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Advances in Plant Breeding Strategies: Vegetable Crops

Volume 9: Fruits and Young Shoots

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Jameel M. Al-Khayri • S. Mohan Jain
Dennis V. Johnson
Editors

Advances in Plant Breeding Strategies: Vegetable Crops

Volume 9: Fruits and Young Shoots



Springer

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ISBN 978-3-030-66960-7

ISBN 978-3-030-66961-4 (eBook)

<https://doi.org/10.1007/978-3-030-66961-4>

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Preface

Contemporary plant breeders no longer need to rely solely on traditional methodologies in their work of assuring a sustainable and elastic level of world food production. However, the human population is increasing at an alarming rate in developing countries and food availability could gradually become a serious problem. Agriculture production is severely affected because of environmental pollution, rapid industrialization, water scarcity and quality, erosion of fertile topsoil, limited remaining arable land to expand production area, lack of improvement of local plant types, erosion of genetic diversity, and dependence on only few crop species for food supply worldwide. According to FAO, 70 % more food must be produced over the next four decades to feed a projected population of 9 billion people by the year 2050. Currently, only 30 plant species are used to meet 95 % of the world's food requirements, which are considered as the *major crops*. The breeding programs of these crops have been very much dependent on the ready availability of genetic variation, either spontaneous or induced. Plant breeders and geneticists are under constant pressure to sustain and increase food production by using innovative breeding strategies and introducing minor crops that are well adapted to marginal lands and can provide source of nutrition through tolerance of abiotic and biotic stresses. In traditional breeding, introgression of one or a few genes into a cultivar is carried out via backcrossing over several plant life cycles.

With the development of new molecular tools, molecular marker-assisted backcrossing has facilitated rapid introgression of a transgene into a plant and reduced linkage drag. Continued development and adaptation of plant biotechnology, molecular markers, and genomics have established ingenious new tools for the creation, analysis, and manipulation of genetic variation for the development of improved cultivars. For example, molecular breeding has great potential to become standard practice in the improvement of several fruit crops. Adopting a multidisciplinary approach comprised of traditional plant breeding, mutation breeding, plant biotechnology, and molecular biology would be strategically ideal for developing

new improved crop varieties. This book highlights the recent progress in the development of plant biotechnology, associated molecular tools, and their usage in plant breeding.

The basic concept of this book is to examine the best use of both innovative and traditional methods of plant breeding to develop new crop varieties suited to different environmental conditions to achieve sustainable food production and enhanced food security in a changing global climate, in addition to the development of crops for enhanced production of pharmaceuticals and innovative industrial uses. Three volumes of this book series were published in 2015, 2016, and 2018, respectively: Volume 1. *Breeding, Biotechnology and Molecular Tools*; Volume 2. *Agronomic, Abiotic and Biotic Stress Traits*; and Volume 3. *Fruits*. In 2019, the following four volumes were published: Volume 4. *Nut and Beverage Crops*; Volume 5. *Cereals*; Volume 6. *Industrial and Food Crops*; and Volume 7. *Legumes*. In 2021, three volumes are being concurrently published: Volume 8. *Vegetable Crops: Bulbs, Roots and Tubers*; Volume 9. *Vegetable Crops: Fruits and Young Shoots*; and Volume 10. *Vegetable Crops: Leaves, Flowerheads, Green Pods, Mushrooms and Truffles*.

This Volume 9, entitled *Vegetable Crops: Fruits and Young Shoots*, consists of 12 chapters focusing on advances in breeding strategies using both traditional and modern approaches for the improvement of individual vegetable crops. Chapters are arranged in 2 parts according to the edible vegetable parts. Part I: Fruits – Bell Pepper (*Capsicum annuum* L. var. *grossum* Sendl.), Chili Pepper (*Capsicum frutescens* L.), Bitter Gourd (*Momordica charantia* L.), Bottle Gourd (*Lagenaria siceraria* (Molina) Standl.), Eggplant (*Solanum* spp.), Okra (*Abelmoschus esculentus* L.), Plantain (*Musa paradisiaca* L.), Sweet Gourd (*Cucurbita moschata* Duch. ex Poir.), Melon (*Cucumis melo* L. Groups Dudaim and Flexuosus), Tomato (*Solanum lycopersicum* L.), and Zucchini (*Cucurbita pepo* L.); Part II: Young Shoots – Asparagus (*Asparagus officinalis* L.).

Chapters are written by internationally reputable scientists and subjected to a review process to assure quality presentation and scientific accuracy. Each chapter begins with an introduction covering related backgrounds and provides in-depth discussion of the subject supported with high-quality color photos, illustrations, and relevant data. This volume contains a total of 95 figures and 56 tables to illustrate presented concepts. Each chapter concludes with an overview of the current status of breeding and recommendations for future research directions as well as appendixes listing research institutes and genetic resources relevant to the topic crop. A comprehensive list of pertinent references is provided to facilitate further reading.

The book is an excellent reference source for plant breeders and geneticists engaged in breeding programs involving biotechnology and molecular tools together with traditional breeding. It is useful for both advanced undergraduate and post-graduate students specializing in agriculture, biotechnology, and molecular breeding as well as for seed companies and policy makers.

We are greatly appreciative of all chapter authors for their contributions towards the success and quality of this book. We are proud of this diverse collaborative

undertaking, especially since this volume represents the efforts of 43 scientists from 11 countries. We are also grateful to Springer for giving us an opportunity to compile this book.

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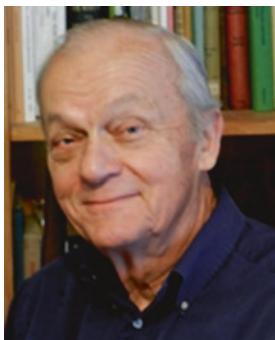


Jameel M. Al-Khayri is a professor of plant biotechnology affiliated with the Department of Agricultural Biotechnology, King Faisal University, Saudi Arabia. He received his B.S. in biology in 1984 from the University of Toledo, M.S. in agronomy in 1988, and Ph.D. in plant science in 1991 from the University of Arkansas. He is a member the International Society for Horticultural Science and Society for In Vitro Biology as well as the national correspondent of the International Association of Plant Tissue Culture and Biotechnology. For the last three decades, he dedicated his research efforts to date palm biotechnology. Dr. Al-Khayri has authored over 70 research articles in refereed international journals and 30 chapters, and has edited several journal special issues. In addition, he has edited 18 reference books on date palm biotechnology, genetic resources, and advances in plant breeding strategies. He has been involved in organizing international scientific conferences and contributed numerous research presentations. In addition to teaching, students advising, and research, he held administrative responsibilities as the assistant director of Date Palm Research Center, head of Department of Plant Biotechnology, and vice dean for Development and Quality Assurance. Dr. Al-Khayri served as a member of Majlis Ash Shura (Saudi Legislative Council) for the 2009–2012 term. Currently he is

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Dennis V. Johnson is a consultant and former university professor. He is a graduate of the University of California Los Angeles where he completed his B.A. (1966), M.A. (1970), and Ph.D. (1972) degrees in geography, with specialization in agriculture and biogeography. He has taught at several colleges and universities, including the University of Houston, and was a visiting professor for 2 years at the University of Ceará, Fortaleza, Brazil. Dr. Johnson also has worked extensively with international development agencies providing technical assistance to agriculture and forestry on projects and programs in Africa, Asia, Europe, and Latin America. He has published numerous articles on palm utilization and conservation and has edited or written books for FAO, IUCN and UNEP. He has also translated into English plant science books from Portuguese and Spanish. A decade ago, Dr. Johnson began to focus his research on date palm, in particular its introduction to non-traditional areas such as Spain, North and South America, and Australia. He co-authored a book on date growing in the USA and has made presentations at five international date palm conferences, and co-edited books on date palm, sago palm, and plant breeding.

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Part I

Fruits

Chapter 1

Advances in Breeding Strategies of Bell Pepper (*Capsicum annuum* L. var. *grossum* Sendt.)



Jyoti Devi, Vidya Sagar, Vineet Kaswan, J. K. Ranjan, Rajesh Kumar, Gyan P. Mishra, R. K. Dubey, and Ravindra K. Verma

Abstract Bell pepper belongs to the Solanaceae family and is in high demand as a vegetable in India, Middle East, USA, Europe and Southeast Asian countries. It has gained the attention of progressive farmers, consumers and international market traders because of its rich nutritional profile and ever-increasing export potential (USD 4.9 billion in 2017). Thirty-eight *Capsicum* taxa are currently documented in the USDA Genetic Resources Information Network (GRIN), including the five under commercial cultivation. In the sixteenth century, bell pepper was introduced to the Asian continent. Currently, China, India, Pakistan, Bangladesh and Indonesia shares over 70% of the world's bell pepper production. The World Vegetable Center in Taiwan holds the world's largest *Capsicum* collection of 8165 accessions, and covers 11% of global diversity. Besides traditionally-important traits like earliness and higher yield, bell pepper breeding is now challenged by the emergence of new pests and diseases. New varieties are needed with desirable fruit color, pungency, shape and nutritional quality, along with resistance to phytophthora, anthracnose, bacteria, viruses, powdery mildews, root-knot nematodes, heat, cold, drought and salinity tolerance; all of these characteristics represent major breeding goals along with higher yields. Conventional breeding methods like introduction, pure line selection, pedigree selection, mutational and heterosis breeding, and backcross breeding are now being assisted by new breeding approaches like rootstock breeding along with modern genomic tools to break down existing barriers, and to speed up traditional breeding programs. Development and implementation of hybrid cultivars are key aspects of bell pepper production, for which the genetic male sterility

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system is being exploited commercially in both the public and private sectors. However, in comparison of chili pepper, utilization of the cytoplasmic male sterility (CMS) system in bell pepper is restricted, owing to high instability of male sterility expression at low temperature, and poor fertility restoration (*Rf*) ability. This chapter describes recent advances in genetic improvement of bell pepper by using various cultivated and wild species as sources of important traits.

Keywords Abiotic stress · Bell pepper · Biotic stress · Breeding · *Capsicum annuum* · Genetic improvement · Molecular markers

1.1 Introduction

The group of pepper species with their enormous genetic and phenotypic diversity of fruit shape, size and other horticultural traits, are the second most important vegetables in the Solanaceae family, following the potato. There are over 200 common names in use for whole pepper groups, creating confusion about its various species and cultivars. Bell pepper is a cultivar group of the species *Capsicum annuum* L. var. *grossum* Sendt. ($2n=2x=24$). Some popular names in various countries include bell pepper (USA, Canada), sweet pepper (UK, Ireland, Malaysia), *Shimla mirch* (India), capsicum (Australia, India, New Zealand, Pakistan), vegetable paprika, or sometimes the term *paprika* is simply added with fruit color like green pepper or red pepper, yellow pepper, etc. Bell pepper is cultivated across the globe for its mild taste, pleasant flavor, versatile range of colors and a rich profile of secondary metabolites. It is also popular among consumers for its crispy, watery and crunchy texture, and a unique taste of slight bitterness tempered by sweetness and hints of saltiness.

Bell pepper has emerged as a leading cash crop under protected structures, attaining a status of high value, low volume crop with immediate economic benefits for their stakeholders across the world. Unlike other members of *Capsicum*, bell peppers are unique in fact that, they produce almost nil quantity of capsaicin ($C_{18}H_{27}NO_3$; 0.003–0.010%), the alkaloid that is responsible for pungency in the genus *Capsicum*. Bell peppers are nonpungent having 0 Scoville heat units (SHU), chili may have 100–500, and their other pungent forms 200,000 to 1,641,183 SHU (Carolina Reaper, the world's most pungent pepper) in dry weight of fruit tissue. Bell peppers are usually blocky with a glossy appearance of bright skin colors of red, yellow, orange, green, purple, brown and black, with a tendency to remain plump, thick fleshed and having three to four lobes. Green peppers are fully developed and unripe, while the other variations are ripe with colors, depending on the type of cultivar grown. The red color of pepper fruits is due to the presence of carotenoid pigments capsanthin, capsorubin, cryptoxanthin and zeaxanthin; among them capsanthin (30–60%) and its isomer capsorubin (6–18%) are more prominent and their balance determines the intensity of red color (Nadeem et al. 2011). The typical aroma of the pepper fruit is because of volatile oils (a mixture of methoxypyrazines,

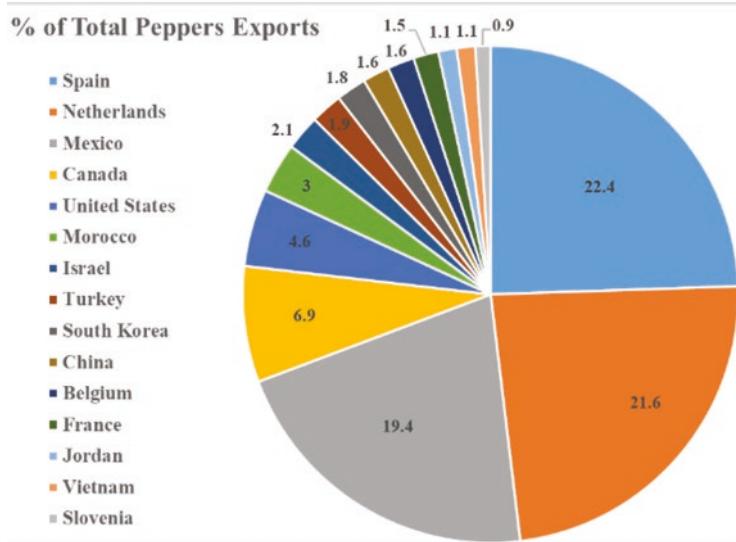


Fig. 1.1 Top 15 countries of world sharing 91.4% of global total peppers export during 2017. (Source:<http://www.worldstopexports.com>)

aliphatic alcohols and esters), present in the mesocarp cells which increases in quantity during fruit ripening. The flavor of capsicum also varies with the color and as the pepper matures, the sugar content increases, thus brighter-colored peppers are sweetest.

Globally, peppers occupy an area of 1.98 million ha with production of 36.09 million mt, including chili peppers; over 70% of the world's bell peppers are produced in Asia (FAO 2017). International exports of sweet peppers, including chili peppers, totaled USD 4.9 billion in 2017. Figure 1.1 presents the leading 15 nations with highest pepper exports during 2017. China, Morocco, Vietnam and Jordan have been the top growing and exporting countries since 2013.

1.2 Origin and Botanical Description

The genus of bell pepper is *Capsicum*, which belongs to the large tropical family Solanaceae, originated from tropical South America. The primary center of diversity of *C. annuum* is in Mexico, with other secondary centers in Central America, South America, India, the Mediterranean Region and Southeast Asia (Andrews 1984). Making their presence with other food fossils, peppers were found as early as 6000 years ago and considered to be the first spice domesticated and used by humans. Christopher Columbus during his first voyage (1492–1493) to the New World, observed that people of the Caribbean Region used a colorful red fruit called *aji* or *axi* in most of their prepared foods. The fruit was introduced to Europe and

later, in the sixteenth century, Portuguese and Spanish explorers introduced capsicum to Asia via trade routes from South America, where new, variable cultivated forms were developed which significantly enhanced the genetic wealth in these countries in addition to the primary center of origin. It is presumed that bell pepper was developed from an ancestral form in the area of Bolivia/Peru. Somos (1984) defined the phylogenetic taxonomy of bell pepper as follows:

Division:	Spermatophyta
Sub-division:	Angiospermae
Class:	Dicotyledones
Tribe:	Solanales
Family:	Solanaceae
Genus:	<i>Capsicum</i>
Species:	<i>Annuum</i>

Many members of the Solanaceae family have the same chromosome number $2n = 2x = 24$, (domesticated and wild species), although genome size varies significantly from one species to another. Some cultivars of *C. annuum* collected from South Sikkim, India, have been reported with $2n=48$ chromosomes (Jha et al. 2012). The size of the *C. annuum* genome (3.48 GB) is around three to four times larger than tomato (*Solanum lycopersicum*; 0.76 GB) and potato (*S. tuberosum*; 0.73 GB). The floral formula of the family is: calyx, K (5–3); corolla, C (5–8); androecium, A (5–8); gynoecium, G (2–4). The plants are herbaceous, short bushes with a woody stem and bright fruits of red, green, orange, chocolate or yellow color. The leaves are alternate and elliptical. The genus is characterized by having perfect and complete flowers comprised of a calyx, corolla, male and female reproductive organs. Flowers are solitary, sometimes paired or occasionally in clusters at the apex of main shoot and axils of lateral branches. The calyx is of cup-shaped, persistent, usually with 5 conspicuous teeth. The corolla is campanulate to rotate, having 5–7 lobes, 8–15cm in diameter and normally white. The flower has 5–7 stamens with pale blue to purplish anthers. The fruit is a non-pulpy berry that varies tremendously in color, shape and size. The seeds have a straw color. The flowers have been observed to open at 05.00 to 06.00 h and anther dehiscence occurs at 08.00 to 11.00 h due to genotypic response to climate.

1.3 Importance, Health Benefits and Factors Affecting Nutritional Quality

Peppers have become a common food ingredient, used by almost one-fourth of the world's population in European, Asian, African and American countries. From being grown as a home garden crop, it has now attained the status of a multibillion dollar industry. They are consumed as salads, stuffed, baked, in soups and as stew

ingredient, dried, pickled or as culinary seasoning. USDA (1984) nutrient database has summarized the nutritional profile of fresh bell pepper per 100 g as comprising 20 kcal of energy, 4.6 g carbohydrate, 1.7 g fiber, 0.2 g fat, 0.9 g protein, 370 IU vitamin A, 80.4 mg vitamin C, 0.4 mg vitamin E, 7.4 mg vitamin K, 0.1 mg thiamin, 0.5 mg niacin, 0.2 mg vitamin B6, 10 mg folate, 0.3 mg iron, 10 mg magnesium, 20 mg phosphorous, 175 mg potassium, 0.1 mg copper and 0.1 mg manganese. Peppers are excellent sources of vitamins C and A. According to Wahyuni et al. (2013), some pepper genotypes can supply between 50% to over 100% of the recommended daily intake (RDI) of vitamin C. Peppers are also a good source of vitamin E and also, contains vitamins B6 and B9 (Tavani et al. 1996). These two 'B' vitamins are crucial for reducing high levels of homocysteine, which damages the blood vessels, thus increasing the risk of heart stroke (Bazzano et al. 2002). They also contain fiber that can help to lower cholesterol levels and also lycopene, a carotenoid which reduces the risk of prostate, bladder and pancreatic cancers (Lu et al. 2001). Green peppers are a rich source of vitamin K and minerals like molybdenum, manganese, potassium and copper. In addition, they contain the beneficial phytonutrients, lutein and zeaxanthin. Recently, Nagasukeerthi et al. (2017) studied the effect of bell pepper juice, integrated with yoga therapy, on blood glucose levels and cardiovascular variables in patients suffering from type 2 diabetes mellitus and reported a significant reduction in post prandial blood glucose, systolic blood pressure, pulse pressure, rate pressure product and double product. Paprika oleoresin is another important commercial product where color, flavor and texture of the ground powder is the same as in the case of chili powder, but lacks in pungency.

Several factors can affect nutritional content of peppers that includes agronomic practices, harvesting time, storage, food preparation technique and genotypes (Kantar et al. 2016). According to one report among the different colored peppers, red pepper contained a higher level of beta-carotene, capsanthin, quercetin and luteolin than the yellow and green pepper (Sun et al. 2007). Likewise, the free radical scavenging abilities of peppers were also low for the green pepper (Sun et al. 2007). Similarly, Nerdy (2018) observed a significant difference in vitamin C content because of variation in fruit color.

1.4 Genetic Resources

All cultivated and related wild species of capsicum constitute the pepper genetic resources, including bell peppers. The species within the *Capsicum* genus that spread globally are still debatable; however, currently, 38 species of *Capsicum* are documented (Hill et al. 2013) and of them 5 species are prominent, including *Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens*. These are distinguished primarily on the basis of flower, seed coat color, calyx shape, flowers/node and their orientation. Among them, *C. annuum* is the most widely grown and includes both, pungent fruits used as spice (hot pepper) and nonpungent including pimiento, Cuban, squash and bell pepper that are consumed as vegetables,



followed by *C. frutescens* (Bosland and Votava 2000, 2003). *Capsicum frutescens* along with other species such as *C. chinense* are commercially cultivated in limited areas of Central America, West and Central Africa, and Oceania, whereas commercial cultivation of *C. baccatum* and *C. pubescens* is limited to Central America, South America and the Caribbean (Kenyon et al. 2014) as shown in Fig. 1.2.

The largest collections of capsicum are held as ex-situ accessions in the various gene banks around the world; most of the exotic genetic resources existing in the genus have not yet been efficiently exploited. The World Vegetable Center, Taiwan, has the world's largest collection of capsicum (8165 accessions with 11% of world diversity) including 11 species. The highest diversity is available for *C. annuum* followed by *C. frutescens*, *C. chinense* and *C. baccatum*. The Center acts as the leading agency for distribution of pepper genetic resources across the globe. India, South Korea, Thailand, China, USA, Vietnam, Taiwan, Indonesia, Netherlands and Tanzania were the top ten recipient countries of *Capsicum* germplasm (Shih-wen et al. 2013) during the last few years.

The next largest collection is the USDA-ARS GRIN seed collection center that alone holds 6200 *Capsicum* accessions, including 4000 *C. annuum* accessions. The USDA germplasm repository includes species *C. chinense*, *C. baccatum*, *C. frutescens*, *C. pubescens*, *C. cardenasii*, *C. chacoense*, *C. flexuosum*, *C. eximium*, *C. rhomboideum*, *C. galapagoense* and *C. tovarii*. Genetic Resources, Wageningen, the Netherlands holds 1011 accessions of capsicum and is the fourth largest in Europe. The Peruvian *Capsicum* collection holds 712 accessions and the collection in Bolivia contains 487 accessions (Van-Zonneveld et al. 2015). The National Bureau of Plant Genetic Resources (NBPGR), New Delhi and the Indian Institute of Vegetable Research, Varanasi, with around 350 accessions, facilitate collection, regeneration, characterization, conservation and distribution of chili germplasm to researchers in India (Figs. 1.3 and 1.4).

1.4.1 Conservation of Genetic Resources

1.4.1.1 In-Situ Conservation

In-situ conservation is on-site conservation of plant genetic resources that includes a geographic area of species diversity in its natural state. Such areas are generally protected from human interference, such as natural parks, biosphere reserves and

Fig. 1.2 Origins and distribution of *Capsicum* species in South and North America; *C. annuum* (USA, Mexico, Caribbean, Belize, Costa Rica, Guatemala, Honduras, Nicaragua, Panama, Colombia); *C. frutescens* (Mexico, French Guiana, Guyana, Suriname, Venezuela, Brazil); *C. baccatum* (Brazil, Bolivia, Colombia, Peru, Argentina, Paraguay); *C. pubescens* (Mexico, Andean Region); *C. chacoense* (Bolivia, Argentina, Paraguay); *C. annuum* L. var. *glabriusculum* (USA, Mexico, Caribbean, Belize, Costa Rica, Guatemala, Honduras, Nicaragua, Panama, Colombia); *C. baccatum* L. var. *pendulum* (South America); *C. galapagoense* (Ecuador); *C. eschbaughii* (Bolivia). (Source:npgsweb.ars-grin.gov)



Fig. 1.3 Species diversity in peppers: (a) *Capsicum annuum* accession LCA-235-A, (b) *C. baccatum*, (c) *C. frutescens*, (d) Bhut Jolokia (one of the most pungent chilis reported with 1,041,427 SHUs from Northeast India, an interspecific cross between *C. frutescens* × *C. chinense*, (e) An interspecific cross between *C. annuum* × *C. frutescens*, (f) Diversity in chili peppers in Indian germplasm. (Source: ICAR-IIVR Annual Report 2015–2016)



Fig. 1.4 Bell peppers diversity available in India, (a, b) Fruit shape, fruit length and color variations in bell peppers, (c) Rich harvest of bell peppers under North Indian Hills, (d, e) Upright bearing and downward bearing in bell peppers

gene sanctuaries. The primary and secondary centers of origin of capsicum have served themselves as major in-situ conservation sites where wild species of capsicum are still growing, guarded by other crop species such as *Helietta parvifolia*, *Diospyros palmeri*, *Acacia rigidula*, *Cordia boissieri*, *Leucophyllum texanum* and *Pithecellobium pallens*, known as *nurse plants* in northeastern Mexico (Murillo-Amador et al. 2015).

1.4.1.2 Ex-Situ Conservation

Ex-situ conservation means protection of genetic diversity outside of its natural habitat; this is done in many gene banks across the globe through various methods. The concept of gene bank conservation arose in the middle of the twentieth century to preserve the biodiversity of crop species, and to avoid further genetic erosion caused by cultivation of modern varieties. With the prime objective to conserve the capsicum genetic resources for long-term use by plant breeders, researchers and users, gene banks have been established throughout the world with certain basic principles established by FAO (2014):

- a) Identity of accessions
- b) Maintenance of viability
- c) Maintenance of genetic integrity
- d) Maintenance of germplasm health
- e) Physical security of collections
- f) Availability and use of germplasm
- g) Availability of information
- h) Proactive management of gene banks

With a worldwide collection of 73,600 accessions, the genus *Capsicum* is well represented in ex-situ collections worldwide, but according to Ebert (2013), wild species such as *C. chinense* and *C. pubescens* have very poor representation in international ex situ conservation. Ex-situ conservation can be done in the various forms like seed banks, tissue banks, DNA banks and cryopreservation of various plant parts, each having their own merits and demerits. The important methods followed worldwide include the following.

1.4.1.3 Seed Banks

Germplasm is stored as seed of various accessions as active, base or working collections. Based on their tolerance to desiccation, capsicum seed has been classified as orthodox and tolerates drying to very low moisture contents ($\leq 3\text{--}7\%$ fresh weight) to short-, medium- or long-term conservation. Many ex-situ collections are mainly kept in seed gene banks across the world due to their ease of maintenance and lower cost.

1.4.1.4 Cryopreservation

Cryopreservation is the process of freezing biological material at extreme temperatures, mostly at -196°C/-321°F in liquid nitrogen. Kuo and Sung (2014) studied the effect of different temperature on pollen storage of chili and found that its ability to germinate started declining after week 12 when kept at -20°C, whereas no decrease was observed under cryopreservation treatment. They further reported a water bath temperature of 40°C was suitable for thawing after pollen cryopreservation. Similarly, Pipithsangchan et al. (2019) recently standardized the protocol for cryopreservation of chili seeds.

Appendix I lists some of the organizations dedicated to *Capsicum* germplasm conservation, distribution and improvement. Appendix II highlights main cultivars being grown by Indian farmers. The species along with available accessions at the US National Plant Germplasm System Center are detailed in the Table 1.1.

1.4.2 Gene Pool

For crop improvement, it is essential to study various species to understand their evolution and the complex relationship among species. Very recently, Pereira-Dias et al. (2019) summarized the cultivated *Capsicum* species as follows:

- a) *C. annuum* most economic, relevant, diverse and rigorously studied that includes bell, jalapenos, numex and ancho types
- b) *C. chinense* that contains pungent peppers such as the habanero
- c) *C. frutescens* in which the well-known form is tabasco
- d) *C. baccatum* or ají, that contains the lemon drop and ají escabeche
- e) *C. pubescens* that covers rocoto and manzano types

A further three complexes holding the cultivated peppers are identified based upon their capability to cross-pollinate (Fig. 1.5, adapted and modified from Van-Zonneveld et al. 2015) are: (i) *Capsicum annuum* complex, (ii) *C. baccatum* complex and (iii) *C. pubescens* complex. In spite of the fact that there exist strong incompatibility crossing barriers among these complexes, viable hybrids have been developed between *C. annuum* and *C. baccatum* by utilization of bridge species (Manzur et al. 2015) and auxin treatment followed by embryo rescue (Phan et al. 2010) to cross *C. pubescens* and *C. annuum*. The crossability rate among species of the *C. annuum* and *C. baccatum* complex varied from 2.2 to 3.7%, and was 14.6% among species of the *C. annuum* complex (Martins et al. 2015).

Table 1.1 List of available *Capsicum* species at US National Plant Germplasm System

Taxon	Accessions	Economic Uses	Native
<i>Capsicum annuum</i>	4131 (3588 active, 3403 available)	Food additives, medicines	S. America
Other related taxa	<i>Capsicum annuum</i> var. <i>annuum</i> : 1(0 active, 0 available)	Ornamental; food additives, human food, medicines, vertebrate poisons	N. America, S. America
	<i>Capsicum annuum</i> var. <i>glabriusculum</i> 71 (70 active, 69 available)	Food additives, medicines	N. America, S. America
<i>Capsicum frutescens</i>	590 (202 active, 197 available)	Food additives, medicines, vertebrate poisons	N. America, S. America
<i>Capsicum baccatum</i> L.	48 (47 active, 45 available)		S. America
Other relative taxa	<i>Capsicum baccatum</i> L. var. <i>pendulum</i> : 359 (315 active, 315 available)	Food additives, medicines	S. America
	<i>Capsicum baccatum</i> L. var. <i>praetermissum</i> : 5 (3 active, 2 available)		S. America
	<i>Capsicum baccatum</i> L. var. <i>umbilicatum</i> 2 (2 active, 2 available)	Ornamental	N. America, S. America
	<i>Capsicum baccatum</i> L. var. <i>pendulum</i> : 359 (315 active, 315 available)	-	S. America
<i>Capsicum chinense</i>	520 (488 active, 479 available) in National Plant Germplasm System	Food additives, medicines	S. America
<i>Capsicum caballeroi</i>	0(0 active, 0 available)	-	S. America
<i>Capsicum pubescens</i>	133 (80 active, 40 available)	Food additives, vegetable	N. America, S. America
<i>Capsicum chacoense</i>	22 (21 active, 19 available)	Food additives	S. America
<i>Capsicum galapagoense</i>	2 (1 active, 1 available)		S. America
<i>Capsicum eximium</i>	9 (3 active, 1 available)	Food additives	S. America
<i>Capsicum cardenasii</i>	4 (1 active, 0 available)	Food additives	S. America
<i>Capsicum eshbaughii</i>	-	-	S. America

Source: npgsweb.ars-grin.gov

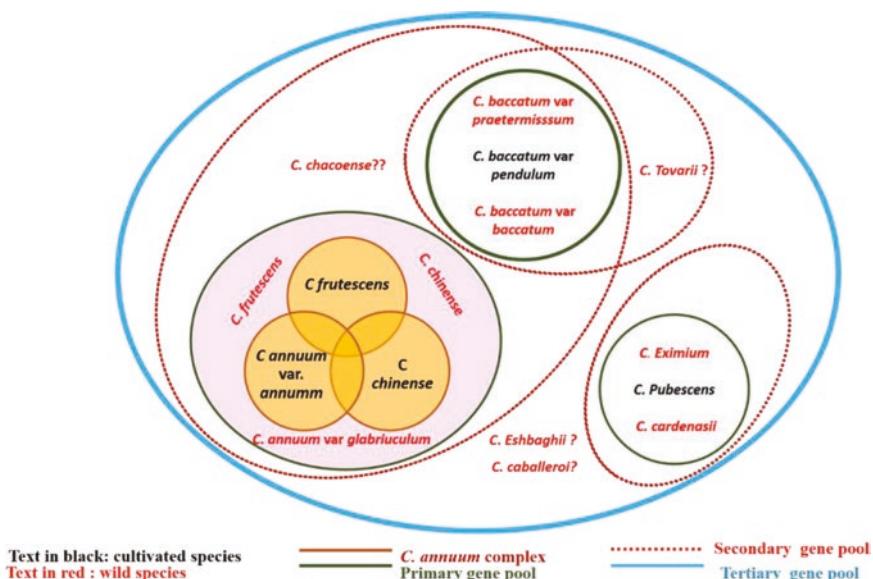


Fig. 1.5 Thirty-eight *Capsicum* species including five cultivated species are currently recognized in the USDA Genetic Resources Information Network (GRIN). Based on cytogenetics and cross-fertility, cultivated species are further divided into three complexes as (I) *C. annuum* complex, (II) *baccatum* complex, (III) *pubescens* complex; *C. annuum*, *C. chinense* and *C. frutescens* along with their wild species, *C. annuum* var. *glabriuscum* constitute the *C. annuum* primary gene pool; *C. baccatum* var. *pendulum*, *C. baccatum* var. *baccatum* and *C. baccatum* var. *praetermissum* constitute the primary gene pool of *Capsicum baccatum* complex. *Capsicum chacoense* has been placed in the *C. annuum* complex (Pickersgill 1971; Zijlstra et al. 1991) although in the *C. baccatum* complex (Van-Zonneveld et al. 2015). Another wild species *C. tovarii* is still debatable (Ince et al. 2010); however, Tong and Bosland (1999) has placed this species in the *C. baccatum* complex. In the *C. pubescens* group, *C. pubescens*, *C. cardenasii* and *C. eximium* constitute the primary gene pool. The other species such as *C. eshbaughii* and *C. caballeroi* are newly-discovered and need further study of their possible relation to these complexes. (Source: Van-Zonneveld et al. 2015)

1.5 Breeding Objectives

In addition to traditionally-important traits like earliness and higher yield, pepper breeding is now challenged by preference for specific horticultural traits, current markets trends and the emergence of new pest and diseases as effects of climate change. Selection and improvement in the domesticated types have resulted in diversity within and among species of capsicum for fruit color, shape, disease resistance and secondary metabolites (Kantar et al. 2016). The most relevant traits in bell pepper breeding are detailed below:

- Earliness
- Indeterminate growth habit
- Higher fruit yield
- Desirable fruit color (green, red, yellow), shape and size

- e) Preferred fruit quality (acceptable flavor, high sugar/acid ratio, high pigment content and vitamin C)
- f) Resistance/tolerance to biotic stress (major diseases – viruses, bacteria, fungi and pests – thrips and mites, fruit borers, nematodes)
- g) Resistance/tolerance to abiotic stress (heat, frost, water, salinity)

Days to flowering, fruiting and picking or earliness are highly desirable attributes in all vegetables including bell peppers, as the price for the produce is consistently higher early in the season. Similarly, high fruit yield is a very relevant trait from the farmers' point of view. Fruit color is another valuable character as it attracts the consumers directly; there is high demand for dark green/red/yellow/orange fruits in bell pepper. Fruit having blocky appearance, lobate/cordate fruit shape at pedicel attachment along with sunken blossom end fruit shape and pendent/erect fruit position on plants are some of the desirable horticultural attributes.

Integration of biofortified peppers with improved nutrients such as vitamins A, B and C into daily diets could help to address nutrient deficiencies by adding a substantial serving of recommended daily nutrients. To unravel the biochemical properties and beneficial effects of secondary metabolites, many studies have been conducted (Nadeem et al. 2011; Sun et al. 2007). Recently, Kantar et al. (2016) examined diverse genotypes of pepper for their nutrient content and reported that some genotypes were high in vitamins A and C, highlighting the opportunities of nutrient supplementation and food fortification through pepper breeding programs to combat vitamin deficiency. Similarly, the *ready to eat* and *ready to cook* life-style has raised demand for processed products like bell peppers, instant chutney powder (Sharma and Joshi 2014) and bell pepper juice (Mohamed et al. 2017) in preparing processed cheese spread (PCS). These are new frontiers for breeders to incorporate the processing traits into the existing cultivars. To support ongoing breeding programs, more comprehensive understanding is required, as how to strike a balance among genotype, environment and cultural practices to obtain maximum metabolites.

Among the various biotic stresses, new strains of bacteria, viruses and fungi have increased the demand for multiple disease-resistant varieties/hybrids, and breeders are looking for sources of resistance for major pathogens of different categories like fungi: anthracnose (*Colletotrichum* spp.), *Cercospora* leaf spot or frogeye leaf spot (*Cercospora capsici*), damping-off (*Pythium* spp., *Rhizoctonia solani*), *Fusarium* wilt (*Fusarium oxysporum*), gray leaf spot (*Stemphylium* spp.), powdery mildew (*Leveillula taurica*), southern blight (*Sclerotium rolfsii*), *Verticillium* wilt (*Verticillium* spp.); bacteria: bacterial canker (*Clavibacter michiganensis*), bacterial spot (*Xanthomonas campestris*, *X. euvesicatoria*, *X. perforans*, *X. axonopodis* (syn. *campestris*) pv. *Vesicatoria*, *X. vesicatoria*, *X. gardneri*), bacterial wilt (*Ralstonia solanacearum*); viruses: cucumber mosaic virus (CMV), potato virus Y (PVY), tomato spotted wilt virus (TSWV) and oomycetes: *Phytophthora* blight (*Phytophthora capsici*). Similarly, insect pests like aphids (*Myzus persicae*), leaf miners (*Lyriomyza* spp.), leaf roller (*Platynota stultana*), pepper weevil (*Anthonomus eugenii*), thrips (*Frankliniella occidentalis*, *Thrips tabaci*), tomato fruit worm (*Helicoverpa zea*), spider mites (*Tetranychus urticae*) and nematodes (*Meloidogyne*

spp.). These all represent challenges to growers, especially under protected structures.

For optimal growth, flowering, and fruit set, the optimal air temperature range for sweet pepper growth is 20–25°C (Rubatzky and Yamaguchi 1999) and root zone temperature (RZT) 21–27°C (Ityel et al. 2014). The plant is very vulnerable to frost and high temperature, as well other biotic stress, limiting its cultivation to specific locations. Also, consumer demand for sweet pepper grown under protected structures has risen since large, high-quality pepper is produced under such an environment along with their availability throughout the year. Keeping these prospects in view, there is urgent need to breed cultivars with wider adaptability and suitability to new niches, especially for protected cultivation.

1.6 Genetics of Important Traits

The inheritance of earliness and total fruit yield are reported to be governed by both additive and nonadditive components (Khalil et al. 2004; Sood and Kumar 2011). Further, additive components were found important for plant height, fruit length, fruit width, pericarp thickness, lobes/fruit, fruits/plant and dry fruit yield/plant (Kamboj et al. 2007; Sood and Kumar 2011), in comparison to traits like branches/plant and fruit yield/plant that were found to be governed by dominant components (Kamboj et al. 2007). The significance of additive components for traits like yield, plant height, branches/plant, fruits/plant, fruit length and width (Anandhi and Khader 2011), and digenic epistasis for days to flowering, fruit length, fruit width, fruit weight, fruits/plant, plant height and yield per plant were also reported. However, it is a well-known fact that most of these traits are complex in nature and with the advancement of molecular-marker technologies, it is now possible to locate the position of various quantitative trait loci. Ben-Chaim et al. (2001) published the first fruit related QTL study in pepper from a cross between the bell pepper cv. Maor and a small-fruited hot pepper cv. Perennial. Since then many yield, quality and disease-related QTL studies have been published on peppers (Barchi et al. 2009; Ben-Chaim et al. 2001; Dwivedi et al. 2013; Han et al. 2016; Rao et al. 2003; Yarnes et al. 2013; Zygier et al. 2005). Table 1.2 summarizes some important QTL studies in bell peppers, including hot peppers, for various fruit and quality traits.

1.6.1 Breeding for Higher Yield

Achieving higher fruit yield is the key objective of bell pepper improvement programs as well as emphasizing other traits like branches/plant, fruit length, width, number of fruits/plant, fruit weight, plant height and harvest duration. The newly-developed cultivar/hybrid must have potential yield equal to or greater than the existing commercial cultivars, otherwise, it will not achieve any success although

Table 1.2 QTLs identified for economic important traits in various peppers mapping population

Traits	Mapping population	Generation	QTL identified	References
Fruit diameter, weight, pericarp thickness and pedicel diameter	180 families of a cross Maor × Perennial	F ₃	QTLs responsible for fruit shape were reported on chromosome 3 and accounts for ≥ 60% of the phenotypic variation	Ben-Chaim et al. (2001)
Fruit, flowering and seed traits	248 plants derived from cross Maor × BG 2816	BC ₂	76 QTLs were deducted for the 07 traits, 10 QTLs were consider to be possibly orthologous including <i>fw2.1</i> , <i>fw3.1</i> and <i>fw4.1</i> for fruit weight; <i>fl2.1</i> for fruit length; <i>fd2.1</i> and <i>fd3.1</i> for fruit diameter; <i>fs3.1</i> for fruit shape; <i>perwd3.1</i> and <i>perwd4.1</i> for pericarp width; <i>flw2.1</i> and <i>flw3.1</i> for flowering and <i>swt2.1</i> for seed weight	Rao et al. (2003)
Fruit and plant traits	297 RIL from a cross Yolo Wonder × Criollo de Morelos 334	F ₆	76 QTLs governing 13 fruit and plant traits were detected, grouped on 28 chromosome, explaining 7–91% of the phenotypic variation	Barchi et al. (2009)
<i>Phytophthora capsici</i>	126-RIL derived from a cross CM334×Yolo Wonder	F ₆	LG5 was detected as a main resistance locus for <i>Phytophthora capsici</i> that explain up to 98.25% of the phenotype variations	Lu et al. (2012)
Capsaicinoids, fruit quality, and plant architecture-related traits	105 RILs derived a cross <i>C. frutescens</i> var. 2814-6 × <i>C. annuum</i> var. NuMexRANAKY	Not Mentioned	96 QTLs were observed for 38 traits, including 12 QTLs for capsaicinoid levels	Yarnes et al. (2013)
Fruit, leaf, and horticultural traits	120 lines from a cross Maor × Criollo de Morelos-334	F ₆	49 QTLs were observed, fruit trait QTLs were distributed onto chromosomes 1, 2, 3, 6, 8, 9, 10, 11 and 12 explaining 14–70% of phenotypic variance	Chunthawodtiporn et al. (2018)

(continued)

Table 1.2 (continued)

Traits	Mapping population	Generation	QTL identified	References
Post-harvest fruit water loss	213 plants of a cross 1154 × USDA 162	BC ₂	2 linked QTLs on chromosome 10 with 35% of the trait variation	Popovsky-Sarid et al. (2016)
Plant architecture, leaf, flower and fruit traits	120 RILs derived from a cross Perennial × Dempsey	F ₇ –F ₁₀	86 significant QTLs controlling 17 traits were observed over the environments. For each morphological trait, 2–10 QTLs were detected explaining 5.6–63.9% of phenotypic variation	Han et al. (2016)
Vitamin E	Population of 104 individuals derived from cross AC2212 × Long Sweet	F ₂	A major QTL on chromosome 10 was deduced, 06 candidate genes were identified in this region which were Gloden-2-Like (GLK), the I subunit of magnesium-chelatase (CHLI) and four homologs of fatty acyl-CoA oxidase (ACX) genes. QTL for phylloquinone (vitamin K) was highly co-localized with the QTL for α-tocopherol (Vitamin E)	Wu (2017)
Capsanthin content	Doubled-haploid (DH) lines derived from a cross of S3586 × KyotoManganji No. 2	-	1 QTL on linkage group (LG) 13 at 90 days after flowering (DAF) and 01 on LG 15 at 45 DAF; were designated as <i>Cst13.1</i> (17.0% of variation) and <i>Cst15</i> (16.1% of variation)	Konishi et al. (2019)
First flower node (FFN)	146 RIL from cross PM702 × FS871	F ₁₀	2 major QTLs, named <i>Ffn2.1</i> and <i>Ffn2.2</i> , identified on LG02, with a phenotypic variance of 28.62 and 19.56%, respectively	Zhang et al. (2019)

having excellent fruit quality and resistance to diseases. Traits/crosses that exhibit relatively higher dominance components in a desirable direction than the additive components, show a possibility of hybrid development, whereas in the traits/crosses with positive and significant additive gene effects and additive × additive gene interactions could be improved by pedigree selection. However, traits/crosses showing a

duplicate type of gene action, recurrent selection and a biparental approach to select desirable segregants could be suitable (Devi and Sood 2018). Further, there are many diseases and insect pests that make pepper production a challenging task for growers. Breeders are continuously trying to find resistance sources both in cultivated and wild relative species to enhance possible breeding strategies.

1.6.2 Breeding for Fruit Color

Fruit color of bell pepper is one of the important commercial traits that arises mainly due to the chlorophyll, anthocyanin and carotenoid pigments. Fruit color in peppers is controlled by three independent loci: *y*, *c1* and *c2*. The dominant *Y* locus encodes for capsanthin-capsorubin synthase (*CCS*), accounting for red carotenoids production (Lefebvre et al. 1998) and could also impart orange color to fruit by changing the carotenoid composition (Kumar et al. 2009b). The inability of expression of the *CCS* gene may result in the genotype producing orange fruit. The yellow fruit phenotype is recessive to red, and is regulated by the *y* locus. The candidate gene of the *y* locus is also a mutation in the *Ccs* gene that includes a partial or complete deletion of a premature stop codon or as a result of frameshift mutation (Li et al. 2013). The *c2* locus is related to phytoene synthase (*PSY*), an enzyme accounting for phytoene production. Mutation at this locus is related to production of an orange fruit in the crosses between red- (*Capsicum annuum*) and orange-fruited (*C. chinense*) genotypes (Huh et al. 2001). So far, the molecular mechanism and function of the *Ccs* gene has been given more attention, lacking the available information on its relation with other genes that are involved in the fruit color production. Further, Hornero-Mendez et al. (2002) reported that to develop carotenoid rich varieties of bell peppers, together with the total carotenoid content, red-to-yellow isochromic fractions ratio and the capsanthin-to-zeaxanthin ratio are the two most useful indexes. Although there are numerous variants for pigments in pepper germplasm, few studies have also been reported on quantitative variations in carotenoid content (Ben-Chaim et al. 2001; Brand et al. 2012). Recently, Rao and Reddy (2016) revealed that the capsanthin capsorubin synthase(*Ccs*) gene specific marker exhibited a polymorphism among all red fruited lines (Arka Mohini, Arka Basant, IIHR-4096, IIHR-4103, IIHR-3338, IIHR-3341, IIHR-3342, IIHR-4104, IIHR-4105, IIHR-4106, IIHR-4034, IIHR-4107, Indira) and yellow-fruited lines (Arka Gaurav, IIHR-4033). The dominant marker PAL ID (*CCS*) is highly useful for selection of parental lines for hybridization programs as well as foreground selection in marker-assisted backcrossing programs.

1.6.3 Breeding for Low Pungency

The primary source of pungency in peppers is capsaicin, which together with its analogues are termed *capsaicinoids*. These compounds are synthesized in epidermal cells of the placental tissues, accumulation of which occurs in the pungent

cultivars between 20–30 days after flowering (Stewart et al. 2007). The presence of pungency is determined by the single dominant gene *C* that was mapped on chromosome 2, and then renamed as *Pun1* (Stewart et al. 2005). Later, another independent gene *pAMT* was discovered on chromosome 3 (Ben-Chaim et al. 2006). It is believed that most bell peppers contain the identical mutated *Pun1* allele, *pun1*. Whereas the *pAMT* mutated nonpungent pepper has been found only in non-bell-type pepper. The *Pun1* locus controls the pungency in peppers, qualitatively encoding a putative acyltransferase enzyme and mutation in this gene could results in a loss of pungency. So far, several *Pun1* alleles (*pun1^{1–4}*) have been discovered. The gene *pun1¹* has characterized with 2.5-kb deletion (Stewart et al. 2005), *pun1²* has a 4-bp deletion (Stewart et al. 2007), *pun1³* has a large deletion of 70 amino acids in the *Pun1* protein (Stellari et al. 2010) and *pun1⁴* has single adenine nucleotide insertion in the second exon region (Kirii et al. 2017). In a very recent study by Tsurumaki and Sasanuma (2019), a novel mutated *pAMT* allele, *pamt10*, with a nonsense substitution at the 11th exon, was discovered.

1.6.4 Breeding for Biotic Stresses

1.6.4.1 Breeding for *Phytophthora* Resistance

Phytophthora capsici is the most destructive pathogen affecting pepper production across the globe, resulting in more than USD100 million in losses yearly (Bosland 2008); to date, 45 physiological races have been described. The pathogens attack root, stem, leaf and fruit, and the disease symptoms may vary with host species, infection point, environmental conditions and stage of plant growth (Barchenger et al. 2018). Besides, the cultural and chemical control, planting of resistant hosts is the most effective management practice. Many inheritance studies have reported that the single dominant gene (Kim and Hur 1990; Lee et al. 2012b), single dominant gene with modifiers (Barksdale et al. 1984), multiple genes (Sy et al. 2005); polygenic inheritance and higher orders have epistasis effects (Lu et al. 2012). The host resistance was first reported in the 1960s in the resistant accessions PI 123469, 187331, 201232 and 201234 (Kimble and Grogan 1960). Paul Bosland developed the genotype CM334 (Criollo de Morales 334) as a primary source of resistance to *Phytophthora*, which is still in use by breeders worldwide. Further, correct screening protocols and precise identification of species is crucial for disease management. Table 1.3 highlights some of the resistant accessions reported across the world.

1.6.4.2 Breeding for Anthracnose Resistance

Anthracnose, a seed-borne disease, is caused by the *Colletotrichum* species complex including *C. acutatum*, *C. gloeosporioides*, *C. capsici* and *C. coccodes*; it is another major disease of the pepper group that affects chilis, bell peppers and

Table 1.3 Resistant sources against the tested isolates of *Phytophthora capsici*

Resistance sources	References
Criollo de Morelos 334	Bosland and Lindsey (1991)
SGM 334	Ortega et al. (1991)
P1188478, PI 189550, PI 201232, PI 201238, PI201234, Criollo de Morelos 331, PM 702, PM 217	Hartman and Wang (1992)
PI201237-4, PI201237-3, PI566811-2, PI640532-1, PI566811-1, PI593573-1 and PI593573-3, KC00807-1, KC01744, KC00937, KC01322	Mo et al. (2014)
41-1, 41-2, 42-6, 55-2	Gomez-Rodriguez et al. (2017)
PI 201237, PI 640532	Candole and Conner (2010)

specialty peppers, such as cubanelle and jalapeno (Park and Kim 1992). *Colletotrichum acutatum* and *C. gloeosporioides* are the two main species which have spread to Asian countries causing up to 50% yield losses (Pakdeevaraporn et al. 2005). Symptoms differ from the tomato fruit, where they are only present in ripe fruit; pepper anthracnose can infect fruit at any growth stage. However, it is even more problematic in the postharvest stage causing deterioration in the quality of fruits by reducing fruit dry weight, capsaicin and oleoresin (Ridzuan et al. 2018). The development of resistant variety/hybrid is a main prerequisite to overcome the pesticide load. Both monogenic and polygenic disease resistances have been reported and numerous studies have been dedicated to work out the inheritance (Kim et al. 2010; Lin et al. 2002; Mahasuk et al. 2013) and to identify resistant sources in various related species of pepper (Table 1.4). For the introgression of a resistant gene, the backcross method of breeding has been widely utilized by using *Capsicum baccatum* and *C. chinense*. To date, *C. baccatum* PBC80 has exhibited the widest resistance to all three *Colletotrichum* species, resistance first identified by the World Vegetable Center, Taiwan (Mahasuk et al. 2013). The genotype PBC932 possesses recessive genes (*co1*, *co3*) on linkage group (LG) P5, while PBC80 possesses recessive (*co4*) or dominant (*Co5*) genes on LG12 and LG9 (Sun et al. 2015). Many other QTL studies have been carried out (Kim et al. 2010; Lee et al. 2010b).

1.6.4.3 Breeding for Bacterial Wilt Resistance

This disease is caused by *Ralstonia solanacearum* which has become a major limitation to commercial bell pepper production in some specific growing areas, especially under hot and humid weather conditions. Yield losses up to 90.62% have been reported (Dharmatti et al. 2009) and the disease has become a major production constraint in South Asian countries. The pathogen has been comprehensively studied in tomato, eggplant and potato; however, limited attention has been given to

Table 1.4 Resistant sources for anthracnose in peppers accessions along with pathogen description

Pathogen species	Pepper Resistant Species	Breeding line/Cultivars	References
<i>Colletotrichum truncatum</i> , <i>C. gloeosporioides</i>	<i>Capsicum annuum</i>	Acchar lanka, CA-4, Pant C-1, Punjab Lal, Bhut Jolokia, BS-35	Mishra et al. (2018)
Isolate not mentioned	<i>C. baccatum</i>	PBC80/VI046804, PBC81/ VI046805, PI594137, PI497985-1, PI260550	Yoon and Park (2005), Montri et al. (2009) and Park et al. (2009)
<i>C. acutatum</i>	<i>C. chinense</i>	0038-9155	Lin et al. (2007)
	<i>C. baccatum</i>	PI594137	Kim et al. (2008)
<i>C. acutatum</i> , <i>C. capsici</i> , <i>C. gloeosporioides</i>	<i>C. chinense</i>	PBC932/VI047018	Park et al. (2009)
<i>C. capsici</i>	<i>C. annuum</i>	83–168.Chungryong	Park et al.(1990a,b) and Lin et al. (2002)
<i>C. capsici</i>	<i>C. annuum</i>	LCA-301, LCA-324, K-1, Byadige Kaddi	Hegde and Anahosur (2001)
<i>C. capsici</i>	<i>C. annuum</i> , <i>C. frutescens</i> and <i>C. baccatum</i> *	BS-35, BS-20, BS28, Punjab Lal, Bhut Jolokia, Taiwan-2, IC-383072, Pant C1, Lankamura Collection	Garg et al. (2012)
<i>C. capsici</i>	<i>C. annuum</i>	LLS, PBC932 (VI047018), Breck-2, PBC80 (VI046804), Breck-1, Jaun, PBC81 (VI046805)	Kaur et al. (2011)

*Individual identification of genotype regarding the species group is not mentioned

peppers. Inheritance studies have revealed resistance to be digenic recessive in nature (Thakur 1990); single dominant gene as well as two genes with dominant and recessive epistasis (Devi et al. 2015). Depending upon the host, five races, whereas based upon biochemical properties, six biovars has been identified (Prior and Steva 1990). Despite its origin in South America, Southeast Asia may be an important region as a source of pepper germplasm resistant to bacterial wilt, as also shown in Table 1.5.

1.6.4.4 Breeding for Powdery Mildew Resistance

This disease is caused by the obligate fungal pathogen *Leveillula taurica*. Conventional breeding is quite time-consuming for the selection of powdery mildew resistant plants. Hence, the molecular marker-assisted breeding of pepper became more reliable after the availability of the *Capsicum* genome sequence information (Manivannan et al. 2018). In recent days, DNA-based single nucleotide polymorphisms (SNPs) markers have revolutionized marker-assisted breeding in various crops due to their high density occurrence in the plant genome. Ahn et al.

Table 1.5 Resistant Pepper accessions reported for bacterial wilt

Accession/ cultivar	Origin	Fruit shape	Pungent/non pungent	References
LS2341	Malaysia	Small New-mex	Pungent	Mimura et al. (2000)
Manganji	Japan	New-mex	Nonpungent	Tsuro et al. (2007)
Fushimi- amanaga	Japan	Cayenne	Nonpungent	Hashimoto et al. (2001)
Mie-midori	Japan	Smallbell	Nonpungent	Matsunaga and Monma (1999)
Ishii-midori	Japan	Smallbell	Nonpungent	Matsunaga and Monma (1999)
Akashi	Japan	Smallbell	Nonpungent	Matsunaga and Monma (1999)
MC4	Malaysia	Small	Pungent	Quezado-Soares and Lopes(1995)
MC5	Malaysia	Small	Pungent	Matos et al. (1990)
EC-464107	AVRDC, Taiwan	Long bell	Pungent	Devi et al. (2015)
EC-464115	AVRDC, Taiwan	Long bell	Pungent	Devi et al. (2015)

(2018) resequenced pepper varieties *C. baccatum* (PRH1- a PM resistant line) and *C. annuum* (Saengryeg- a PM susceptible line) to develop SNP markers related to powdery mildew (PM) resistance. A total of 4,887,031 polymorphic SNP loci were identified leading to design of 306,871 high resolution melting (HRM) markers. In addition, 6281 SNPs associated with 46 resistance genes were identified.

1.6.4.5 Breeding for Virus Resistance

Green and Kim (1990) reported 35 virus species infecting peppers, a number increased to 68 species by Pernezny et al. (2003). However, to the contrary, Moury and Verdin (2012) reported some 20 virus species causing damage to pepper crops. Expansion and intensification of pepper cultivation in tropical and subtropical regions have opened new horizons of global markets, and changing climatic conditions has expanded the geographical range of pathogens, including viruses. According to the latest review by Kenyon et al. (2014), around 80 different viruses of peppers from various groups like potyviruses, cucumoviruses, poleroviruses, begomoviruses and tospoviruses, as well as virus transmitted by seed and invertebrate vectors, are distributed throughout different regions of the world (Fig. 1.6). Potyviruses and begomoviruses are the two important groups of viruses in pepper species. Tolerance to *cucumber mosaic virus* (CMV) was reported in the Indian chili accession, Perennial, having very small fruits into several bell-pepper genotypes. The genetics of resistance in this line was reported as monogenic recessive, partially dominant or polygenic recessive inheritance by (Yao et al. 2013). Inheritance of

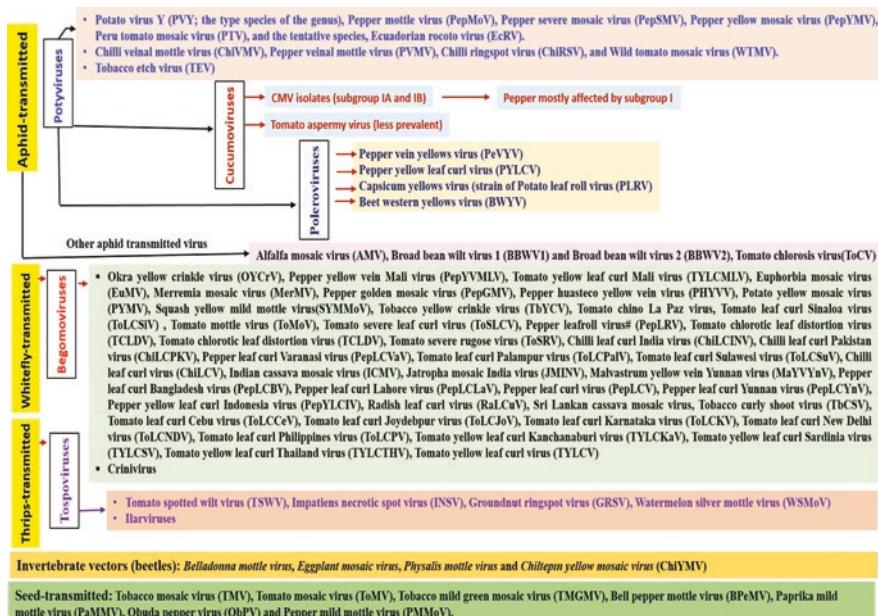


Fig. 1.6 Simplified version of virus species affecting the pepper group along with their vectors

pepper mottle potyvirus as two unlinked, recessive loci, (Murphy et al. 1998); resistance to TSWV in several *Capsicum chinense* accessions, including PI152225 and PI159236 inherited as single dominant gene (Boiteux 1995). Similarly, inheritance of *pepper yellow mosaic virus* in *C. baccatum* var. *pendulum* was found to be polygenic and complex (Bento et al. 2013). Table 1.6 summarizes the resistant accessions reported for various viruses in related species of *Capsicum* currently in use by breeders.

1.6.4.6 Breeding for Root-Nematode Resistance (RKN)

RKN has emerged as one of the major pests of bell pepper, and four contributory *Meloidogyne* species (*M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*) have been reported as occurring separately or together. Resistance to this pest was first documented 50 years ago in the genotype PI 128657 of wild tomato (*Lycopersicum peruvianum*); subsequently, a number of RKN-resistance genes were discovered in the Solanaceae, such as tobacco, tomato, potato, eggplant and its wild species such as *Solanum sisymbriifolium*, *S. wercewiczii* and *S. torvum* (Djian-Caporalino et al. 1999). Unlike other vegetable crops such as tomato or cucurbits, the galls on pepper roots are generally not as obvious and large. Still, an infected pepper root system may carry several million root-knot nematode eggs. Resistance to this parasite has been reported in many pepper accessions including chili. In one of the earlier

Table 1.6 Resistant sources reported for various viruses in pepper species

Viral diseases	<i>Capsicum</i> species	Genotype	References
<i>Pepper yellow mosaic virus</i>	<i>C. annuum</i>	Criollo de Morellos 334	Boiteux et al. (1996)
	<i>C. chinense</i>	PI 159236	Boiteux et al. (1996)
	<i>C. chinense</i>	PI159236, PI152225	Cunha et al. (2004)
	<i>C. chinense</i>	UENF1703, UENF1730, UENF1732, UENF1751, UENF1755, UENF1764 and UENF1803	Bento et al. (2009)
	<i>C. baccatum</i> var. <i>pendulum</i>	UENF1624, UENF1732	Bento et al. (2009)
<i>Pepper golden mosaic virus</i>	<i>C. chinense</i>	BG-3821	Garcia-Neria and Rivera-Bustamante (2011)
<i>Pepper huasteco yellow vein virus</i>	<i>C. chinense</i>	BG-3821	Garcia-Neria and Rivera-Bustamante (2011)
<i>Cucumber mosaic virus</i>	OP variety developed through interspecific cross followed by pedigree breeding	Peacework	Mazourek et al. (2009)
	<i>C. frutescens</i>	BG2814-6	Grube et al. (2000)
	<i>C. annuum</i>	Perennial	Grube et al. (2000)
	<i>C. baccatum</i>	PI 439381-1-3	Suzuki et al. (2003)
	<i>C. annuum</i>	Bukang	Kang et al. (2010)
	<i>C. annuum</i>	BJ0747-1-3-1-1	Yao et al. (2013)
	<i>C. annuum</i>	CA23 (Noakhali), CA12	Rahman et al. (2016)
<i>Pepper mottle potyvirus</i>	<i>C. chinense</i>	PI 159236, PI 152225	Murphy et al. (1998)
	<i>C. annuum</i>	Avelar	Murphy et al. (1998)
<i>Tomato necrotic ringspot virus</i>	<i>C. chinense</i>	PI290972	Persley et al. (2010)
	<i>C. annuum</i>	PY-4300, PY4301, PY-4302	Puangmalai et al. (2013)
	<i>C. baccatum</i>	CA1316, CA1998, CA2000, CA2008, CA2009	Puangmalai et al. (2013)
	<i>C. chinense</i>	PI 152225, PI 159236, cv. Panca, cv. 7204	Jahn et al. (2000) and Hoang et al. (2014)

Table 1.7 Genetic resources reported in peppers for various nematode species

Tested species	Resistant sources	References
<i>Meloidogyne incognita</i>	White Khandari	Peter et al. (1984)
<i>M. incognita</i>	PM687, PM217, Criollo de Morelos 334, Yolo NR	Djian-Caporalino et al. (1999)
<i>M. incognita</i>	Carolina Wonder, Charleston Belle	Thies et al. (2008) and Aguiar et al. (2014)
<i>M. javanica</i>	EC378632, EC378688, EC391083, EC391087, EC402105, EC402113, EC405253, IC214965, IC214985, IC215012, NIC19969	Pandravada et al. (2010)
<i>M. incognita</i> , <i>M. arenaria</i> , <i>M. javanica</i>	PA-566	Fery and Thi (2011)
<i>M. incognita</i>	41-1, 41-2, 42-6, 49-5, 55-2, 55-3, 56-2, 56-3	Gomez-Rodriguez et al. (2017)
<i>M. aberrans</i>	41-1, 41-2, 42-2, 35-3, 35-5	Gomez-Rodriguez et al. (2017)

reports, Peter et al. (1984) found that a local cultivar of chili from India, White Khandari, possessed multiple resistance for diseases including RKN. Thies et al. (2008) and Aguiar et al. (2014) found that cvs. Carolina Wonder (Reimer Seeds, Saint Leonard, MD) and Charleston Belle (Reimer Seeds, Saint Leonard, MD) were found resistant to *M. incognita* in southern California. However, in another later study in India, most of the commercially-grown private varieties like Chocolate Wonder, Indra and Indum Super Gold were found susceptible to *M. incognita* including California Wonder (Bommalinga et al. 2013). Pandravada et al. (2010) found 11 Indian chili accessions free from the incidence of root-knot. Fery and Thi (2011) had developed a variety, PA-566, by transferring nematode resistant gene *N* from the genotype Mississippi Nemaheart into a Pimiento L-type genetic background through backcross breeding programs. The dominant *N* gene has a high level of resistance to the southern RKN *M. incognita*, *M. arenaria* and *M. javanica*. Table 1.7 summarizes the resistant accessions for various species of nematodes.

1.6.5 Breeding for Abiotic Stress

Significant losses are reported due to various abiotic stresses in peppers that make them issues of global concern, such as heat, drought, cold and salinity. Apart from the conventional screening methodologies, transgenic protocols (engineering of genes of important metabolic and defensive pathways) and tissue-culture based screening methods (in vitro screening in NaCl for salt tolerant and PEG or mannitol for drought tolerance) (Rai et al. 2011) have been used. Further, identification of stress responsive genes and transfer of identified genes against these stresses could

be accelerated through marker-assisted breeding. However, the recent concept that a single gene of multiple origin could impact many stress tolerance mechanisms has again broadened the horizons for determining the limit of genetic improvement of crop species. Recently, the potential of a pea DNA *Helicase 45* (*PDH45*) was demonstrated, in combating multiple abiotic stresses in chili (Shivakumara et al. 2017). Breeding for important abiotic stresses are detailed in the following sections.

1.6.5.1 Breeding for High Temperature Tolerance

Just as in other agricultural crops, global warming has emerged as a major production constraint to pepper cultivation. Serious pollination problems resulting in blossom and fruit drop have been observed when temperatures exceed 32°C. As the temperature increases, a reduction was reported in days to first flowering, fruit weight, fruit length, fruit diameter and number of seeds/fruit (Thuy and Kenji 2015). Heat tolerance is a complex trait involving various biochemical and metabolic interactions. It includes maintenance of membrane stability, scavenging of reactive oxygen species (ROS), antioxidant production, gene expression and translation, protein stability, accumulation and adjustment of compatible solutes (Kaya et al. 2001). Drastic changes in cell membrane stability have been reported among heat tolerance and heat susceptible cultivars. Under heat stress, significant increase in the *HSP70* gene has also been observed (Usman et al. 2015). Shieh et al. (2015) concluded that 36°C is the critical temperature to differentiate heat-tolerant and heat-susceptible lines. Several pepper varieties/genotypes having high heat tolerance have been bred through selection and hybridization. Some of the resistant/tolerant genotypes are given in Table 1.8.

1.6.5.2 Breeding for Cold tolerance

Plants have limits of tolerance to chilling (0–15°C) and freezing (< 0°C) temperatures, and may exhibit various symptoms of chilling injury such as chlorosis, necrosis or growth retardation. De-Swart (2007) stated that within the sweet-pepper

Table 1.8 List of heat tolerant accession reported in peppers

Resistant sources	References
CCA-3331, CCA-336B, CCA-984A, Mr. Lee No. 3 selex, PBC142, Maor, CCA-119A, CCA-3288, Susan's Joy, CO-5678	Dahal et al. (2006)
Chilly Chili, Medusa, Thai Hot, Explosive Ember, Treasures Red	Gajanayake et al. (2011)
AVPP0702, AVPP9905, AVPP0116	Usman et al. (2015)
R597*	Li et al. (2015)
AVPP0408(PP0437-7031)*	AVRDC**

*Heat tolerant, **information available at <https://avrdc.org/seed/improved-lines/sweet-pepper>

group, the variation for lower temperature tolerance was limited, emphasizing the need to explore wild relatives in order to breed for this trait. He further reported that relative growth rate (RGR) could be a useful criterion to screen low-temperature tolerant accessions for which morphological plant traits such as leaf, stem and total fresh- and dry-mass, plant height, leaf area and number of leaves could be used to simplify the selection of RGR. Shabetya et al. (2017) described two methods of screening for cold tolerance in sweet peppers: (i) a direct evaluation method by taking into account the plants that survived exposure to low temperature and (ii) plant ability to germinate at low temperature. Gajanayake et al. (2011) screened ornamental pepper species and reported that the genotypes Black Pearl, Calico, Sangria, Red Missile and Salsa Yellow (CTRI =10.08 to >10.86) were tolerant to low temperature.

1.6.5.3 Breeding for Drought Tolerance

As a consequence of global warming, it has been estimated that the frequency and intensity of drought may increase by 1–30% (Fischlin et al. 2007). Drought adaption strategies of plant species has led to the identification of specific traits associated with water mining, water use efficiency (WUE) and water conservation traits. Relative water content, chlorophyll content, activities of superoxide dismutase and catalase enzymes were found to play significant roles in screening methodologies. Sahitya et al. (2018) reported a strong correlation between seed antioxidants and water stress tolerant traits in chili seedlings. It has also been reported that pepper plants are sensitive to water deficit due to large leaf areas and higher stomata conductance (Campos et al. 2014). Initial establishment of transplants and flower initiation are the critical stages of crop growth to moisture stress (Bosland and Votava 2003). Table 1.9 lists some of the resistant accession reported for drought tolerance in peppers.

1.6.5.4 Breeding for Salinity Tolerance

Among the major abiotic stresses, high salinity is significant and affects 930 million ha area of the world, including the Mediterranean Region (Qadir 2016). Peppers are sensitive to moderately-sensitive to salinity during different crop growth stages. An excess of soluble salts, particularly NaCl, may trigger three types of stresses in plants: osmotic, ionic and oxidative. Salinity stress may lead to a reduction in crop

Table 1.9 *Capsicum* accessions tolerant to drought stress

Drought tolerant genotypes	References
<i>C. chinense</i> , <i>C. baccatum</i> var. <i>pendulum</i> , <i>C. eximium</i> , Arka Lohit, IIHR - Sel-132	Singh (2010)
Kca-5, Kca-7, KCa-10	Sahitya et al. (2018)
<i>C. chinense</i> (IHR4502)	Naresh et al. (2017)

Table 1.10 *Capsicum* accession tolerant for salinity stress

Genotypes	References
Demre, Ilica 250, 11-B-14, Bagci Carliston, Mini Aci Sivri, Yalova Carliston, Yaglik 28	Yildirim and Guvenc (2006)
Early Jalapeno, AZ-20	Niu et al. (2010)
Zard, Tasty, Super Shimla, Aristotle	Tehseen et al. (2016)
CO1, K1, Jayanthi, Arka Supha, IC119546	Balasankar et al. (2017)

growth traits such as plant height, number of branches, days to first flowering, leaf area, fruit length, girth weight and number of fruit/plant (Balasankar et al. 2017). In contrast, increased concentration of proline has been reported with tolerance to salt stress in many crop species. Further, Mukhtar (2016) reported that the foliar application of calcium at 20 or 35 mg L⁻¹ and sulfur at 5 or 10g L⁻¹ improved the morphological and physiological traits in chili plants subjected to salinity stress. Table 1.10 lists some of resistant/tolerant accessions of peppers to salt tolerance.

1.7 Breeding Methods

Capsicums are diploid and predominantly self-pollinated; hence, breeding methods adapted to self-pollinated crops can be employed for varietal development. The methods generally used for the development of high-yielding cultivars are introduction, pure line selection, pedigree breeding, single seed descent method (SSD), hybridization, backcrossing, mutation and polyplodiy breeding. However, modern breeding approaches were also successfully incorporated, along with the traditional methods to keep the pace of breeding programs fast, as demanded by the current scenario of production challenges being faced by the breeders/growers due to emerging pests and diseases as result of climate change. Choice of the best method, or a combination of them, depends mainly on the type of inheritance (monogenic, oligogenic, polygenic) of the traits to be improved. Wang and Bosland (2006) summarized a list of 292 genes for morphological and physiological traits, sterility and resistance genes for various biotic and abiotic stresses in peppers. Some commercial varieties grown in India include: California Wonder, Yolo Wonder (ICAR-IARI, New Delhi); Arka Gourav, Arka Mohini, Arka Basant (ICAR-IIHR, Bengaluru); Kt-1 Pusa Deepi (ICAR-IARI, Regional Center, Katrain) and Bharat (Indo American Hybrid Seeds (IAHS), Bengaluru. Some lesser grown varieties in India include Nishat 1 (A selection from Capsicum Sel-2, SKUAS&T, Srinagar), Solan Bharpur (from Department of Seed Science and Technology, UHF, Nauni, Solan, HP), varieties from the private sector that include World Beater, Chinese Giant (introduced by IAHS, Bangalore) and Lario, Indra, Bomby, Orobelle, Picador (Syngenta). The pungency level, bright attractive colors, highly concentrated extracts and few seeds are the main characters that determine bell pepper quality and price. Table 1.11 summarizes the utilization of various breeding methods for improvement of various traits in pepper breeding programs across the world; the

Table 1.11 Breeding approaches to improve the various traits in pepper breeding programs

	Breeding methods	Treatment/Utilization	References
	Mutation	• EMS concentration of 0.6% (v/v) for 12 h	Arisha et al. (2015)
Traditional breeding	Mutation	• Gamma rays treatment with a dose of 0.5, 1.0, 3.0, 5.0, 8.0, 11, 13, 16, 19 and 22kR by using ^{60}CO • EMS at different concentrations of 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 2.0 and 3.0 % for 5.3 h	Sood et al. (2017)
	Mutation	• EMS concentrations of 0.01, 0.1 and 0.5% (V/V) for 3 and 6 h	Jabeen and Mirza (2004)
	Mutation	• Gamma rays treatment at 30kR, 40kR and 50kR and EMS concentration of 20mM, 30mM, 40mM	Aruldoss and Mullainathan (2015)
	Wide Hybridization	• Introgression of <i>Capsicum baccatum</i> genes into <i>C. annuum</i> ; (i) by using <i>C. chinense</i> and <i>C. frutescens</i> as bridge species, (ii) by direct crossing combined with in vitro embryo rescue (ER)	Manzur et al. (2015)
	Back cross breeding	• The introgression of S-type cytoplasm and the Rf allele was conducted from tropical pungent donors CA1 and CC1 to the recurrent parent GC3, a nonpungent bell pepper	Mulyantoro et al. (2014)
Biotechnological interventions	Polyplody breeding	• Possibility to explore high and stable pepper production along with uniform fruit size	Takizawa et al. (2008), Kazi (2015)
	Micropropagation	• Preservation and maintaining of elite plants, sterile and transgenic plants and F ₁ hybrid plants displaying heterosis	Grozeva et al. (2009) and Otroshay et al. (2011)
	Protoplast culture	• Recovering either somaclonal variants or somatic hybrids in cases of sexual interspecific incompatibility	Sura et al. (2015)
	Double haploids via anther culture	• Obtaining homozygous pure lines in the shortest period of time	Keles et al. (2015)
	Marker-assisted breeding	• Marker-assisted introgression of 4 <i>Phytophthora capsici</i> resistance QTL alleles into bell pepper • Marker-assisted breeding of a new fresh pepper cultivar (<i>Capsicum annuum</i>) containing capsinoids and low-pungent capsaicinoid analogs • Marker-assisted selection for resistance to <i>Potyvirus</i> in sweet pepper	Thabuis et al. (2004) Tanaka et al. (2014) Nogueira et al. (2012)

approaches in pepper breeding that are currently being practiced are detailed below in the following sections.

1.7.1 *Plant Introduction*

This is an ancient method of crop improvement: introducing crop plants into new areas where they were never before grown. Plant introduction helps to enrich the diversity of flora in the new areas. Introduction of new genotypes can be done within the country, within the continent and across the continent. Some of most popular, widely grown varieties of the bell peppers like California Wonder and Yolo Wonder were early introductions across many counties of world.

1.7.2 *Pureline Selection*

This method is suitable to handle the landraces/local cultivars and relies on the availability of genetic variation in the existing cultivar/variety. The initial genetic stock is space-planted and selection is done for genetically-superior plants. The following year, individual plant progenies are grown and lines showing superior performance with no further genetic variation within selected plants, are bulk harvested. They are further evaluated with check cultivar(s) in replicated yield trials. Several pepper varieties in India have been developed by this method like Arka Gaurav, Arka Basant and Arka Mohini.

1.7.3 *Pedigree Method*

This method relies on the selection of superior plants in the segregating generations followed by hybridization between superior cultivars along with their pedigree record. In bell pepper, California Wonder is still a dominant and preferred variety and has been used rather frequently in new crosses. Therefore, in the pedigree method, as far as possible, both the parental cultivars should be promising and superior types. The process of generation advancement and selection continues for six to eight generations when the desired level of homozygosity is reached. After attaining homozygosity, the individual lines are evaluated for their performances. The best performing lines are evaluated in replicated trials along with commercial as well as local checks; lines showing superiority over the checks are released as commercial cultivars.

1.7.4 Single Seed Descent

This represents a rapid method for generation advancement in self-pollinated crops. Initially the crossing is made between desirable parents and advanced to the next generation through selfing. This method is highly suitable where more than one generation of crop advancement is possible. Selection is avoided during the early generation of improvement and close planting can be done in a limited space. A single fruit seed is harvested from each plant in a segregating generation and those seeds are advanced to the next generation. This technique provides a scope of selection for seed viability, seedling vigor, virus resistance or other single-gene resistances amenable to controlled inoculation. In pepper, SSD has been employed to fix recessive *Potyvirus* genes into the inbred lines prior to evaluating them in the field for other economically-important traits. This technique is widely employed to generate large numbers of inbred and mapping populations.

1.7.5 Backcross Breeding

Backcrossing is particularly adapted for traits controlled by one or a few genes, involving selection of individual plants and successive crosses to a recurrent parent (Bosland and Votava 2012). Repeated backcrossing with one parent (recurrent parent) is done to recover most of its genotype. The resulting plants are similar in the genetic constitution with the recurrent parent except having a few of the genes from the donor side, the resulting lines are also called *isogenic lines* or *near isogenic lines*. Backcross breeding complemented with molecular-marker systems has been a method of choice for improvement of one or a few gene-governed traits. In pepper this method was used for transfer of CMS into a different background. Also, marker-assisted backcross breeding was used for transfer of *Phytophthora capsici* resistance QTL alleles (Thabuis et al. 2004), breeding of a new fresh pepper cultivar containing capsinoids, low-pungent capsaicinoid analogs (Tanaka et al. 2014) and incorporation of resistance to *Potyvirus* (Nogueira et al. 2012) in bell pepper.

1.7.6 Mutation Breeding

Mutation breeding is generally practiced for those traits where natural variation is insignificant and there is the need to create variation. Naturally the germplasm, breeding lines and wild relatives are sources of improvement. The frequency of mutation per gene varies from crop to crop; in the case of pepper the mutation frequency is 50 Kb/mutation. The first report of mutagenesis traces back to 1940s when X-rays were used for the generation of mutant plants (Raghvan and Venkatasubban 1940). The plants generated varied in plant type, branching habit,

size of leaves and fruits. Several useful mutants were generated through treatment of gamma rays, X-rays and EMS (Daskalov 1968, 1971, 1972, 1973, 1974, 1976, 1977, 1981); for example, male sterile mutants, anthocyanin less mutants, gene markers, mutants with changed fruit form and color, dwarfs, etc., which were used directly, in crossbreeding programs and in heterosis breeding. In the era of chemical mutagenesis, several novel quantitative traits were identified by Videnin (1968) for yield and early fruit maturity. Skripnikova (1976) identified a mutant with increased dry matter content, and a mutant with enlarged fruits was reported by Rubzov and Solomatin (1974) and Skripnikova (1976). Karasz (1974) described induction of a CMV resistant mutant which was released as a cultivar under the name Horgoska Slatka—X—3. Karasz (1974), during another mutation experiment, identified a *Verticillium dahiae* resistant plant. In another mutational study on *Capsicum annuum*, Alcantara et al. (1996) found dwarf, albino and chlorotic seedlings in EMS-treated M1 plants. Dwarf plants and fruit color mutants were also reported by (Honda et al. 2006). Mutation affecting branching pattern was also identified by Mao et al. (2000). Other mutants for chlorophyll, dwarf plants and leaf architecture mutants were also described by Arisha et al. (2015). Sood et al. (2017) also reported isolation of chlorophyll mutants and found that gamma rays were more effective in inducing mutation than EMS. Different varieties were also released and directly utilized in plant breeding (Table 1.12).

1.7.7 *Heterosis Breeding*

The heterosis phenomenon was first understood in maize and thereafter it was utilized across other crop species. It exploits the heterozygosity available in the genome of contrasting parents. It is the quick method of breeding given the condition that the heterotic parents are identified previously. The success of heterosis breeding relies primarily on three factors: (i) extent of heterosis (ii) pollination control and (iii) efficient seed production system. Several cultivars in bell pepper were developed in the past using this method. Low seed rate ~400–600 g/ha and high number of seeds per fruits paved the way for the success of heterosis breeding in pepper. Lack of economic hybrid seed production technology, however, is the major limitation of using these hybrids owing to high seed cost, because hand emasculation is the widely practiced method for their production. The available genetic mechanisms of male sterility were also exploited to some extent.

1.7.7.1 *Manual Hybrid Seed Production*

Currently this is the most successful method of breeding. Bell pepper flowers are hermaphroditic; however, they can cross-pollinate because of their protogynous nature. A day before pollination, buds of appropriate size are selected for emasculation. All the anthers generally, 5–7, are carefully removed from the flower without

Table 1.12 Mutant varieties developed till date in bell pepper across the globe

S. No.	Variety name	Latin name	Common name	Country	Year
1	Krichimski ran	<i>Capsicum annuum</i>	Green pepper	Bulgaria	1972
2	Horgoska slatka-X-3	<i>C. annuum</i>	Pepper	Serbia	1974
3	MDU 1	<i>C. annuum</i>	Chili	India	1976
4	Albena	<i>C. annuum</i>	Green pepper	Bulgaria	1976
5	Lyulin	<i>C. annuum</i>	Green pepper	Bulgaria	1982
6	Friari KS80	<i>C. annuum</i>	Green pepper	Italy	1985
7	Nuzh-51	<i>C. annuum</i>	Sweet pepper	Russian Federation	1991
8	Orangeva Kapija	<i>C. annuum</i>	Sweet pepper	Bulgaria	1991
9	Pirin	<i>C. annuum</i>	Sweet pepper	Bulgaria	1991
10	Gornooriahovska kapia	<i>C. annuum</i>	Pepper	Bulgaria	1997
11	Yujiao 1	<i>C. annuum</i>	Pepper	China	2002
12	Longjiao 9	<i>C. annuum</i>	Pepper	China	2005
13	Yujiao 2	<i>C. annuum</i>	Pepper	China	2006
14	Yujiao 3	<i>C. annuum</i>	Pepper	China	2007
15	Yujiao 4	<i>C. annuum</i>	Pepper	China	2007
16	F ₁ Orange Beauty	<i>C. annuum</i>	Vegetable pepper	Russian Federation	2011

Source: <http://mvgs.iaea.org/AboutMutantVarities.aspx/>. Accessed 11 July 2019

disturbing the stigma, and covered with a bag or cotton. The following morning mature pollen is collected and dusted on the stigma and covered. For every four rows of female plants, one row of male plant is planted.

1.7.7.2 Male Sterility in Hybrid Seed Production

Research on male sterility began in the 1950s. Since then, it has advanced significantly with respect to its utilization (Dhaliwal and Jindal 2014). Male sterility use has reduced the time and labor required for hybrid seed production. Both nuclear/genetic and cytoplasmic male sterility are available in bell pepper. However, the use of this system is limited because of high instability of the male sterility system at low temperatures and poor fertility restoration (Naresh et al. 2018).

1.7.7.3 Genic Male Sterility (GMS) or Nuclear Male Sterility (NMS)

The first description of genetic male sterility in pepper (*Capsicum frutescens*) was provided by Martin and Crawford (1951), who reported it was governed by a single recessive gene. So far, approximately 20 known genes controlling genetic male sterility have been reported; the major ones are listed in Table 1.13. Successful utilization of this system requires diverse parents for crossing with each other to generate

Table 1.13 Discovery and development of the genetic/nuclear male sterility system

Male sterility gene	Inheritance of NMS gene/cultivars	References
<i>ms-1</i>	Natural mutant in cv. All Big	Shiffriss and Frankel (1969)
<i>ms-2</i>	Natural mutant incv. California Wonder	Shiffriss and Rylsky (1972)
<i>ms-3, ms-4, ms-6, ms-7, ms-8</i>	Irradiation induced mutant, <i>ms-3, ms-4</i> from cv. Kalinkov 800/7 and <i>ms-6, ms-7, ms-8</i> from cv. Zlaten Medal	Daskalov (1968, 1971, 1973)
<i>ms-9, ms-10, ms-11</i>	Irradiation and EMS induced mutant, <i>mc-9, mc-509, mc-705</i>	Pochard (1970)
<i>ms-12</i>	Spontaneous mutant of <i>Capsicum annuum</i> incv. Gambo	Shiffriss (1973)
<i>ms-13</i>	A mutant of <i>C. annuum</i> from the genotype Ca452-1	Meshram and Narkhede (1982)
<i>ms-14</i>	Natural mutant from cv. Kalyanpur Selection	Pathak et al. (1983)
<i>msc-1, msc2</i>	Mutation of <i>ms-5</i>	Yang et al. (1994)
<i>Dms</i>	Dominant <i>NMS</i> gene	Daskalov and Poulos (1994)
<i>ACMS-2</i>	Recessive gene governed the inheritance of <i>NMS</i>	Patel et al. (1998)
<i>ms</i>	Unknown origin	Lee et al. (2012a)
<i>Msw</i>	Developed from pepper hybrid Forever	Naresh et al. (2018)

a heterotic combination. The complete fertile plant (*MsMs*) is used as male and male sterile plants (*msms*); as female plants, the resulting progenies will be fully fertile (*Msms*) hybrids. The only major disadvantage of this system is that during hybrid seed production it is necessary to rogue out the 50 % of fertile plants from female rows.

1.7.7.4 Cytoplasmic Male Sterility (CMS)

This form of male sterility originates from dysfunction of mitochondrial genes associated with normal pollen development and the lack of restorer allele in the nucleus. CMS is a three-line system of hybrid seed production which include an A line (male sterile line), B line (maintainer line) and R line (fertility restorer line). The A and B lines are genetically isonuclear only differing for cytoplasmic fertility, while the R line is diverse with nuclear fertility restoration capacity. The A and R lines are crossed with each other to produce hybrid seeds. Generally, the fertility restoration is governed by a single dominant gene (Gulyas et al. 2006). However, the unavailability of complete restoration of fertility is the major lacuna associated with the utilization of CMS system in pepper (Mulyantoro et al. 2014). Alternatively, introduction of strong fertility restoration genes from hot pepper could improve the fertility restoration potential of sweet pepper. Also, development of genic markers could increase the efficiency of transfer of fertility restoration as compared to the currently available systems (Table 1.14). In hot pepper, Fig. 1.7 shows some of

Table 1.14 Molecular markers for male sterility currently being utilized in pepper breeding programs

Marker	Type	Distance	Gene	References
PmsM1-CAPS	CAPS	2 – 3 cM	<i>ms</i>	Lee et al. (2010a)
E-AGC/M-GTG	AFLP	3 cM	<i>ms1</i>	Lee et al. (2010c)
SCAR_P2	SCAR	4.6 cM	<i>ms8</i>	Bartoszewski et al. (2012)
SCAR_V17	SCAR	6.8	<i>ms8</i>	Bartoszewski et al. (2012)
Z05-760	RAPD	4.6 cM	<i>ms8</i>	Bartoszewski et al. (2012)
RAPD W06-520	RAPD	7.4 cM	<i>ms8</i>	Bartoszewski et al. (2012)
AVRDC-PP12	SSR	7.2 cM	<i>ms10</i>	Aulakh et al. (2016)
AVRDC_MD997	SSR	20.8 cM	<i>ms10</i>	Aulakh et al. (2016)
HPGMS3	dCAPS	3.83 cM	<i>ms3</i>	Naresh et al. (2018)
HPGMS2	CAPS	3.83 cM	<i>ms3</i>	Naresh et al. (2018)
SPGMS1	dCAPS	-	<i>msw</i>	Naresh et al. (2018)
OP131400	RAPD	0.37	<i>Rf</i>	Zhang et al. (2000)
OW19800	RAPD	8.12	<i>Rf</i>	Zhang et al. (2000)
OP13CAPS	CAPS	1.1	<i>Rf</i>	Kim et al. (2006) and Min et al. (2008)
CRF3S1S	SCAR	4.8 – 5.3*	<i>Rf</i>	Gulyas et al. (2006)
AFRF8CAPS	CAPS	1.8	<i>Rf</i>	Kim et al. (2006)
CaRf-FL-M2	STS	0.5	<i>Rf</i>	Min et al. (2008)
AFRF4	AFLP	0.1	<i>Rf</i>	Min et al. (2008)
AFRF1	AFLP	1.1	<i>Rf</i>	Min et al. (2008)
AFRF3	AFLP	18.3	<i>Rf</i>	Min et al. (2008)
CRF-SCAR	SCAR	1.4	<i>Rf</i>	Jo et al. (2010)
CRF-S870	SCAR	-	<i>Rf</i>	Lin et al. (2015)
SCAR130/140	SCAR	-	<i>Rf</i>	Yeh et al. (2016)

*5.3 cM in the F₃ generation and 4.8 cM in the F₄ generation

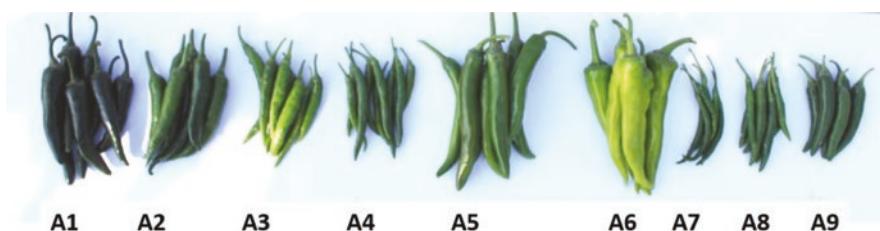


Fig. 1.7 Male sterile lines maintained at ICAR-Indian Institute of Vegetable Research, Varanasi through CMS System. Source: ICAR-IIVR Annual Report (2015–2016)

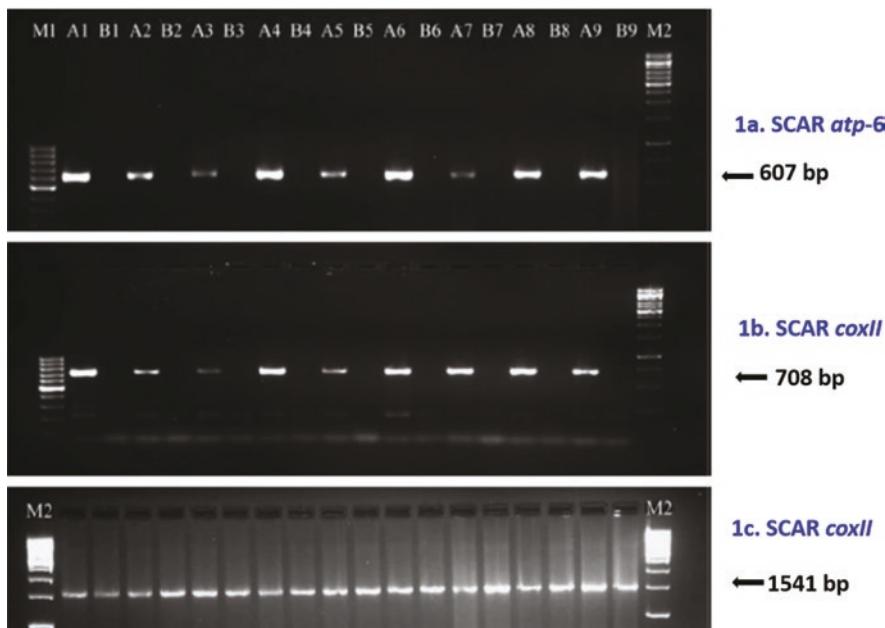


Fig. 1.8 PCR amplification results of 09 CMS Alines having S-cytoplasm and maintainer B lines having N-cytoplasm **1a** SCAR primer *atp6*, **1b** SCAR *coxII* primer, **1c** SCAR *cox II* control; M1 and M2 are IKB markers, respectively. (Source: Kumar et al. 2009c)

CMS-based male sterile lines developed at ICAR-Indian Institute of Vegetable Research, Varanasi. Kumar et al. (2009c) utilized two SCAR markers *atp6*₆₀₇ and *coxII*₇₀₈ to distinguish male sterile (S-) and normal/male fertile (N-) cytoplasms to explore their feasibility in CMS-based pepper breeding. These primers are specific to sterile cytoplasm and no amplification was observed in genotypes carrying normal cytoplasm. This hot pepper based cytoplasm can be utilized through molecular-assisted plant breeding (marker-assisted backcross breeding) to develop novel CMS in sweet pepper (Fig 1.8).

1.7.8 Micropropagation

Seed-based nursery plant production is the predominant conventional system of pepper production and multiplication. It has certain limitations like the short span of seed viability, poor rate of germination, high vulnerability to various types of diseases and very high seed prices. Additionally, climatic conditions, especially temperature extremes, also affect pepper production (Zemene and Worku 2018).

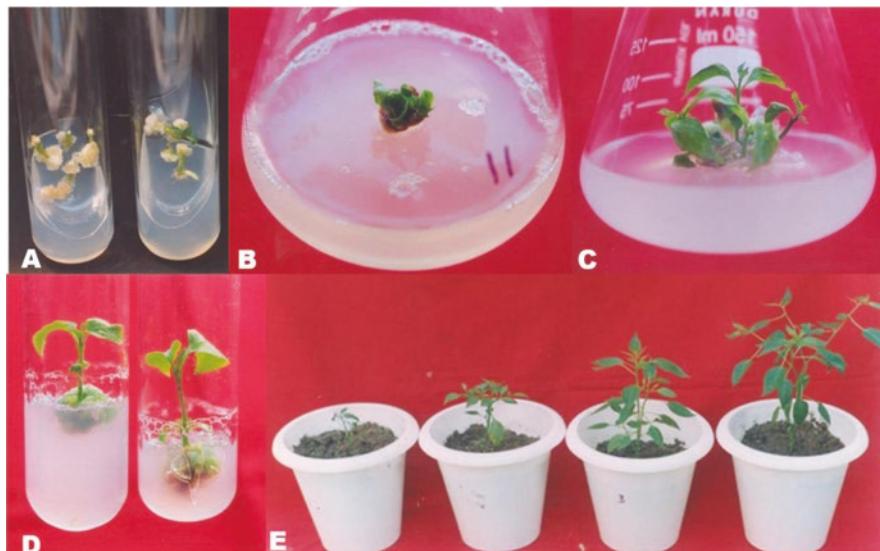


Fig. 1.9 (A) Callusing on MS medium supplemented with 5.0 mg/l 2,4-D + 0.5 mg/l kinetin in hypocotyl and cotyledonary leaf explants, (B) Shoot bud induction on hypocotyl derived callus mass on MS medium supplemented with 1.5 mg/l TDZ, (C) Shoot multiplication on MS medium supplemented with 6.0 mg/l BAP + 1.0 mg/l kinetin + 0.5 mg/l GA₃, (D) Rooting of in vitro regenerated microshoots on 1/2 strength MS medium + 1.0 mg/l IBA, (E) Growth of the plantlets after 30 days of transfer to the glasshouse. (Source: Ranjan et al. 2018)

Tissue-culture based micropropagation is a more reliable approach to overcome the limiting factors of pepper production and multiplication. However, many of the in vitro culture protocols for a specific pepper cultivar have proved inappropriate for proper micropropagation of other cultivars. So, it is necessary to establish reliable micropopagation systems for peppers, especially for commercially-used genotypes. Different explants like, stems, leaves (Swamy et al. 2014) shoot tips, roots, hypocotyls and cotyledons (Zemene and Worku 2018) and induced somatic embryogenesis (Arous et al. 2001), have been used for in vitro multiplication in pepper. Efficient and successful mass propagation in pepper has been reported with Murashige and Skoog medium supplemented with various growth hormones such as 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA) and indole-3-butryic acid (IBA) to provide uniform young plants (Swamy et al. 2014; Zemene and Worku 2018). The micropopagation approach provides additional opportunities for genetic improvement of in-vitro-raised plants by somaclonal variation. These variations occur due to tissue culture cycles and may be of value in pepper improvement in two ways: (1) to create novel variation which may not be present in the existing natural gene pool and (2) to improve performance of an existing cultivar by creating change in one or more its characters (Fig. 1.9).

1.7.9 Marker Assisted Breeding (MAB)

Marker-assisted breeding (MAB) or marker-assisted selection has been extensively used in many crop species, including peppers, to optimize/increase the selection efficiency especially when traits are governed by the oligogene. There are several studies showing the crucial role of molecular markers supporting breeding in *Capsicum*, such as diversity analysis (Cardoso et al. 2018), fingerprinting of genotypes (Kumar et al. 2001), taxa identification and phylogenetic studies (Patwardhan et al. 2014), mapping of important traits (Table 1.2), quality improvement (Rao and Reddy 2016), breeding for biotic stresses (Lu et al. 2012), breeding for abiotic stresses (Usman et al. 2017) and hybrid seed production (Table 1.14). There are numerous examples of marker-assisted breeding in peppers as highlighted in this chapter: Table 1.2, sections 1.11 and 1.14 and Fig. 1.8. In the very latest study by Naresh et al. (2018), two markers (HPGMS2, HPGMS3) were located 3.83 cm away from the *ms3* gene, and were found to be helpful in identification and development of genic male sterile lines in peppers. Similarly, Kim et al. (2019) identified three markers (CaNB5480, CaRP-5130, CaNB-5330) closely linked to the *Phytophthora*-resistance locus that can be used to characterize the genotypes against *P. capsici* in pepper.

1.7.10 Genetic Engineering

Genetic engineering provides a direct method of genetic improvement that selectively targets one or a few genes for introduction into the pepper plant, such as insect, nematode, virus or fungal resistance. The success of transgenic plant creation depends on an efficient and reliable transformation and a plant regeneration protocol. In bell pepper, the plant's recalcitrant nature is a major bottleneck in adoption of transgenic breeding (Kumar et al. 2009a, b, c). However, transgenic pepper has been created by regeneration from various explants, but with low regeneration and transformation efficiencies (Delis et al. 2005; Kim et al. 2006; Ko and Soh 2007; Lee et al. 2004). Hence there is an urgent need for simple, reliable and efficient transformation protocols to create commercial bell-pepper cultivars. Heidmann et al. (2011) reported ectopic expression of the *Brassica napus* BABY BOOM AP2/ERF transcription factor to efficiently regenerate transgenic bell pepper plants.

It would be worthwhile to establish protocols that minimize or bypass tissue culture steps for bell pepper transgenic development. In particular, *Agrobacterium tumefaciens*-mediated in planta transformation methods like the vacuum infiltration and floral dip methods (Zhang et al. 2006) are preferred over other methods because they do not involve tissue culture steps and avoid somaclonal variation. In the vacuum infiltration method, adult plants at the reproduction stage are infiltrated with

Agrobacterium cell suspension (Bechtold et al. 1993). This method creates stable transgenic plants with low transformation frequency. The vacuum infiltration method was further modified as the flower dip method by Clough and Bent (1998). In this method, the floral parts of flower buds are dipped in *Agrobacterium* culture with strong optical density to produce transgenic plants. Although this method is quick, reliable and free from microbial contamination, random integration of a transgene in a host genome with low transformation frequency limits its use in transgenic plant production. Kumar et al. (2009a) successfully transformed two varieties of bell pepper (Arka Gaurav, Arka Mohini) by the *Agrobacterium tumefaciens*-mediated in planta transformation method. They used *Agrobacterium* strain EHA105 harboring the binary vector pCAMBIA1301 that carries the genes for β-glucuronidase (*uidA*) and hygromycin phosphotransferase II (*hptII*) for transforming the meristem of the seedlings with just-emerging plumules. In 2014, a non-expresser of pathogenesis-related gene (*NPRI*) for powdery mildew pathogen (*Leveillula taurica* (Lev.) Arn.) was introduced in the differentiating cells of the apical meristem by using the *Agrobacterium tumefaciens*-mediated in planta transformation method to develop disease-resistant bell pepper transgenic plants (Arthikala et al. 2014).

1.7.11 Genome Editing

Genome editing refers to the strategies and techniques developed for the precise, targeted changes to the genetic information of living cells. Due to complex genomic architecture, it is challenging to edit all of the genes/genomes using a particular genome editing tool. Therefore, to overcome this challenging task, several genome editing tools have been developed to facilitate efficient genome editing. Recent approaches to targeted genome editing, zinc-finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs), enable researchers to generate mutations by introducing double-stranded breaks to activate repair pathways (Gai et al. 2013). These approaches are costly and time-consuming to engineer, limiting their widespread use, particularly for large-scale, high-throughput studies. Recently, methods based on a bacterial CRISPR (clustered regularly interspaced short palindromic repeat)- associated protein-9 nuclease (cas9) have generated considerable excitement (Joseph et al. 2016). The simplicity of the CRISPR system, with only three components (Cas9, crRNA, tracrRNA) makes it attractive for genome editing and studying plants at the molecular level.

The whole genomic sequencing of *Capsicum annuum* CM334 (Kim et al. 2014) opens new avenues for precise pepper molecular breeding programs. To apply the CRISPR genome editing tool to a pepper genome, an in vitro regeneration protocol is indispensable. Kim and Lim (2019) successfully established the conditions for leaf-derived callus formation for two different pepper cultivars, namely, hot pepper (CM334) and bell pepper (Dempsey). This is the basis for regeneration of whole plants to obtain stable protoplasts.

1.8 Grafting: A New Approach to Deal with Biotic and Abiotic Stresses in *Capsicum*

A large number of biotic and abiotic stresses are responsible for the low productivity and quality of vegetables grown worldwide. To overcome these problems, particularly biotic stresses, growers indiscriminately use pesticides, which are a major health concern. An alternative is to combat stresses by developing resistant or tolerant varieties for which we need a genetic resistance source, along with their cross-compatibility within a backcross breeding program. The time needed to develop resistant varieties backcross breeding is several years. The technique of vegetable grafting may bypass some of these barriers to make superior genetic stock available to farmers. Grafting creates a new plant by combining two plants, each with a different genetic background, with one (scion) providing the shoots and the other the roots (rootstock), thereby combining the desirable traits of both plants. Grafting desirable fruiting vegetable varieties to vigorous, disease-resistant rootstocks has become a cost-effective method for growers to overcome many disease and production-related issues. Vegetable grafting can improve production, overall crop health, reduce or eliminate the need for pesticide use, lengthen the harvest period, which in turn significantly increases the net income.

The genus *Capsicum* is affected by numerous biotic and abiotic stresses, and grafting may help overcome these problems. Initially begun in Japan and Korea, *Capsicum* rootstocks are used mainly for pepper (*C. annuum*) production. A review by Lee et al. (2010d) revealed that pepper is currently the least grafted among all the solanaceous vegetables. The reason being the poor availability of suitable rootstocks. Consequently, there is great interest in developing new *Capsicum* rootstocks effective against biotic and abiotic stresses. The primary purpose of grafting peppers worldwide has been to manage losses from soil-borne pathogens of major economic importance such as *Phytophthora capsici*, pests including nematodes, and viruses such as *tobacco mosaic virus* or *potato Y virus* (Pico et al. 2017). Increased nutritional fruit quality with respect to beta-carotene, vitamin C and total antioxidant capacity when grafted on *C. annuum* rootstock has also been reported by Chavez-Mendoza et al. (2013). There are various techniques to graft the scion onto the rootstock, and the proper selection of a technique depends upon the crop, size, growth stage and compatibility of the two plants. A large number of rootstocks have been reported for different purposes. Hence, the selection of suitable rootstock plays an important role in the success of the vegetable grafting industry. The properties of different *Capsicum* rootstock for different purpose are listed in Table 1.15.

Although grafting is costly, pepper's sensitivity to different diseases like bacterial wilt and *Phytophthora* blight, and waterlogging provides an opportunity to utilize grafting in those areas where there is a high risk of these problems frequently occurring. AVRDC successfully utilized tomato and chili rootstock for grafting and recommended chili accessions PP0237-7502, 0242-62 for resistant to flooding, bacterial wilt and *Phytophthora* blight. The apical wedge graft with success rates of 100 % and tube graft with success rates of 49–66% have been reported in the case of chili-tomato grafts (Rodriquez and Bosland 2010). However, tube grafting has been reported to be quicker and less complicated than wedge grafting, with 800–1000 grafts per day/person possible (Lee and Oda 2003).

Table 1.15 Rootstock–scion combinations for *Capsicum* for various biotic and abiotic stresses

Rootstock	Purpose	References
<i>Capsicum annuum</i>	Tolerance to mild salinity	Penella et al. (2015)
	Tolerance to <i>Phytophthora nicotianae</i>	Saadoun and Allagui (2013)
	Changes in fruit shape	Tsaballa et al. (2013)
	<i>Phytophthora</i> blight,	Jang et al. (2012)
	Bacterial wilt and root-knot nematode tolerance	Ros-Ibanez et al. (2014)
	Tolerance to soil borne pathogens	Morra and Bilotto (2006)
	<i>P. nicotianae</i> tolerance	Saadoun and Allagui (2013)
	<i>M. incognita</i> tolerance	Kokalis-Burelle et al. (2009)
	Yield and/or fruit quality	Attia et al. (2003) and Donas-Ucles et al. (2014)
<i>C. annuum</i> F ₁	<i>Fusarium oxysporum</i> and <i>Meloidogyne incognita</i> tolerance and <i>Verticillium</i> wilt tolerance	Geboglu et al. (2011)
	High radiation, temperature tolerance	Lopez-Marin et al. (2013)
	Control of <i>Phytophthora</i> blight	Gilardi et al. (2014)
<i>Capsicum chinense</i>	Tolerance to mild salinity	Penella et al. (2015)
<i>Capsicum frutescens</i>	Increased production under hot wet or hot dry conditions	Palada and Wu (2008)
<i>Capsicum baccatum</i>	Tolerance to mild salinity	Penella et al. (2015)
<i>C. annuum</i> × <i>C. chinense</i>	Superior growth and yield; agronomic performance and fruit quality	Lee et al. (2010d) and Gisbert et al. (2010)
<i>Capsicum chacoense</i>	Root-knot nematode resistance	Oka et al. (2004)
The wild serrano-type pepper SCM334	Root rot and wilt (<i>Phytophthora nicotianae</i>)	Saadoun and Allagui (2013)
Charleston Hot, Carolina Wonder, Charleston Belle, Mississippi Nemaheart and Carolina Cayenne of bell pepper	Resistant to galling by <i>M. incognita</i>	Kokalis-Burelle et al. (2009)

1.8.1 Grafting Growing Facilities

The essential facility for successful grafting includes a screening chamber that excludes insect vectors, and a grafting chamber to maintain high humidity coupled with lower light intensity.

1.8.2 Identification of Suitable Rootstock and Scions

The first step for the successful grafting is the identification of suitable rootstock exhibiting resistant to biotic and abiotic stresses, and having a vigorous root system for better nutrient uptake. Similarly, scions should have high fruiting capacity and graft compatibility with the rootstocks.

1.8.3 Sowing Time of Seedlings and Growth Media

The stock and scion should have equal height and similar stem diameter. To synchronize the stem diameters, chili seeds to be used as rootstock should be sown 5–6 days earlier than the sweet peppers to be used as a scion source, as the chili stem diameter grows at a slower rate than the sweet pepper. The nursery stock can be raised in growth media of various ratios. For example, AVRDC has standardized mixture consisting of field soil, well-decomposed compost, rice husk and river sand in a 2:3:1:1 ratio. Similarly, Camposeco-Montejo (2018) utilized a peat moss/perlite mix in the ratio 70:30.

1.8.4 Grafting Steps

- a) Seedlings may be grafted after developing 2–3 true leaves having stem diameters of 1.6–1.8 mm. This seedling stage can be achieved 35–40 days after sowing.
- b) To make a successful graft union, the vascular bundles of scion and rootstock should have maximum contact. A cut of about 30–45° angle is made about 1.5 cm above the first pair of true leaves. A similar cut is made in the scion at a point having same diameter.
- c) The rootstock bottom and the top of the fruit plant variety are then grafted by using special grafting clips such as latex or silicon tubes or simple plastic clips to make certain the two cut ends are joined together exactly making a perfect match.
- d) Healing and acclimatization are very important at the union for grafted plants to survive. The grafted plants are placed in protected areas away from direct sunlight (grafting chambers). The plants should receive proper moisture under reduced light intensity for which the mist chamber can be covered with a shade cloth (50%) for 7–14 days.
- e) The hardening of plants can be done with air temperature of 24–32°C over the 24-hour cycle.

1.9 Conclusions and Prospects

Depending on market needs and opportunities, bell pepper breeding has vast horizons. Like other crops, yield is an important breeding target in peppers and most of current breeding efforts are mainly devoted toward the improvement of yield and quality attributes. However, the demand for new colors, shapes and tastes has arisen, along with higher digestibility, broadening the scope for future diversification. Further, the type of growing system (protected or open fields), varieties for local adaptation and market segment are important factors to be taken into consideration. For example, for long distance transportation and extended shelf-life, varieties with a thick pericarp are needed, on the other hand, for easy cooking (especially baking), varieties with a thin pericarp are preferred. Resistance or tolerance to any kind of stress, biotic or abiotic, will remain primary objectives of breeding that is essential for stable performance, and again these differ with region. In addition to traditional breeding approaches, rootstock breeding is another frontier area to explore to manage various biotic and abiotic stress as it is playing crucial role in tomato breeding. Likewise, exploitation of male sterility and chemical hybridizing agents in developing new hybrids and the introduction of heat and drought tolerance germplasm as a strategy for climate change is essential. Lastly, to achieve the above targets, the role of biotechnological tools like CRISPR/Cas9 (a precision gene editing tool) is crucial, along with traditional breeding, to accelerate ongoing pepper breeding programs.

Appendices

Appendix I: Some of the Research Institutes Working in Capsicum Germplasm Management and Genetic Improvement

Institution	Specialization and research activities	Contact information and website
World Vegetable Center (AVRDC), Taiwan	<i>Capsicum</i> gene pool management, genetic improvement, production technology	World Vegetable Center, P.O. Box 42, Shanhua, Tainan, Taiwan 74151 Phone: +886-6-583-7801 Email: info@worldveg.org Web: avrdc.org
United States Department of Agriculture-ARS GRIN, USA	Germplasm documentation, conservation, utilization of <i>Capsicum</i> genetic resources	Jamie L. Whitten Building 1400 Independence Ave., S.W Washington DC, 20250 Web: npgsweb.ars-grin.gov Email:Karen.Williams@ars.usda.gov

(continued)

Institution	Specialization and research activities	Contact information and website
Centre for Genetic Resources, Wageningen, Netherlands	To identify the genomic organization of economically important traits including drought and salt stress tolerance, and pathogen resistances within cultivated and related wild species of <i>Solanum</i> and <i>Capsicum</i> crops, mechanical harvesting, database management	Centre for Genetic Resources, the Netherlands (CGN) of Wageningen University & Research, PO Box 338, 6700AH Wageningen Email: cgn@wur.nl Web: www.wur.nl
Central Institute of Genetics and Germplasm, Gatersleben, Germany	Genomics of genetic resources, documentation, conservation	Head, OT Gatersleben Corrensstrabe 3, 06466 Seeland Phone: 039482/5-220 Web: www.ipk-gatersleben.de
Centro Agronomico Tropical de Investigacion y Ensenanza, Turrialba, Costa Rica	Classification, characterization, maintenance, genetic improvement of capsicum	Cartago, Costa Rica Turrialba 30501 Phone:+506 2558 2000 Web: www.catie.ac.cr
Ethiopian Biodiversity Institute (EBI), Addis Ababa, Ethiopia	Genetic diversity, improvement, biodiversity conservation	Addis Ababa, Ethiopia Phone: +251 11 661 2244 Web: http://www.ebi.gov.et/
The Chile Pepper Institute, Las Cruces, USA	Conservation of genetic resources, cultivar development	Gerald Thomas Hall, 945 College Dr, Las Cruces, NM 88003, USA Web: cpi.nmsu.edu
National Bureau of Plant Genetic Resources, New Delhi, India	Augmentation through import and exploration visits, characterization, evaluation, distribution of <i>Capsicum</i> accessions to India and abroad, plant quarantine	Pusa Campus, New Delhi, Delhi 110012 Telephone: + 91-11 -25843697 Email: director.nbprgr(AT) icar.gov.in Web: http://www.nbprgr.erne.in
ICAR-Indian Institute of Vegetable Research, Varanasi, India	Augmentation, evaluation of germplasm, development of multiple disease resistance varieties, standardization of production technology, rootstock breeding, maintenance, documentation, distribution of germplasm	ICAR-Indian Institute of Vegetable Research, Varanasi-221 305, Uttar Pradesh, India Email: directoriivr@gmail.com Web: www.iivr.org.in
ICAR-Indian Institute of Horticulture Research, Bengaluru, India	Augmentation, evaluation, development of multiple disease resistant varieties, hybrids development etc.	ICAR-IIHR, Hessaraghatta Lake Post, Bengaluru-560 089. E-mail: director.iihr@icar.gov.in Web: https://www.iihr.res.in

*A number of institutes are mentioned by Kim et al. (2014) as carrying out advanced molecular work in pepper, including genome sequencing

Appendix II: List of Bell Pepper Cultivars Grown in India

Cultivar	Important traits	Cultivation location
California Wonder	Plants vigorous, upright, prolific, fruits 3–4 lobed smooth with medium thick sweet flesh, perfect for roasting, grilling, stuffing	India (all parts of country)
Yolo Wonder	Large fruit, 3–4 lobed, medium thick flesh mild and sweet. Plants with good leaf foliage, thus reduce sunscald	India (all parts of country)
Arka Gaurav	Indeterminate plant habit with green foliage, thick fleshed, 3–4 lobed, green, blocky fruits, average fruit weight 130–150g, fruits erect which turn orange yellow on ripening, yields 16 mt/ha in 150 days	India (Southern India)
Arka Mohini	Indeterminate plant habit with green foliage, thick fleshed, 3–4 lobed, green, blocky fruits, average fruit weight 130–150g, fruits erect which turn orange yellow on ripening, yields 16 mt/ha in 150 days	India (Southern India)
Arka Basant	Indeterminate plant habit with yellowish green foliage, thick fleshed, 2–3 lobed, conical fruits, average fruit weight 50–80g, fruits erect, cream colored, which turn orange red on maturity, yields 15 mt/ha in 180 days. Excellent keeping and cooking qualities	India (Southern India)
Pusa Deepi (KT-1)	High yielding F ₁ hybrid, resistant to anthracnose and fruit rot disease	India (Northern India)
Bharat	F ₁ hybrid, plants are vigorous, fruit dark green, 4 lobed yields 20–25 mt/ha. Resistant to TMV	India (throughout India)
Solan Hybrid-2	Released by YSPUHF, Solan. Resistant to fruit rot and virus	Northern India
Chinese Giant	Plants are vigorous and prolific bearer, fruits are 3–4 lobed, sweet, flesh and skin is dark green in color	India (all parts of country)
World Beater	Plants are upright, productive, having 3–4 lobed, flesh thick, mild and sweet fruits	India (all parts of country)

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Chapter 2

Genetics, Genomics and Breeding of Chili Pepper *Capsicum frutescens* L. and Other *Capsicum* Species



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Abstract The genus *Capsicum* belongs to the Solanaceae family which contains more than 30 species, of which only five, namely, *C. annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L. and *C. pubescens* L. are cultivated. Capsicums are very important crop plants and widely consumed worldwide as they are important source of several nutritional and dietary compounds which includes capsaicinoids, vitamins A and C, pigments, minerals and essential oils. Among the five cultivated spp., *C. annuum* has gained the maximum attention of researchers. Research on other *Capsicum* spp. is limited despite they are having wide diversity, and are potential sources of desirable genes for several economically-important traits such as disease resistance, fruit shape, size and color. The improvement of *C. annuum* by hybridization with *C. chinense*, *C. frutescens*, *C. baccatum* and *C. pubescence* indicates that these four species may play a significant role as important genetic resources for the transfer of genes of economically-important traits to *C. annuum*. In this chapter, we have compiled the development and advances made in genetics, breeding and genomics with main emphasis on *C. chinense* (world's hottest chili), *C. frutescens*, *C. baccatum* and *C. pubescens*. Highlighted are recent developments in the application of biotechnology especially tissue culture and genetic transformation in *Capsicum* species.

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Keywords Genome sequencing · Molecular breeding · Molecular markers · QTL mapping · Tissue culture · Transgenic

2.1 Introduction

The genus *Capsicum* (chilis or peppers) belongs to the Solanaceae family, one of the largest plant families. *Capsicum* fruits are mostly utilized as a spice and vegetable such as green fresh vegetable, dried whole fruit, fresh/dried powder or in a sauce. Besides these, *Capsicum* also has several medicinal properties and health beneficial compounds (Jaiswal et al. 2020). *Capsicum* fruits are rich in several vitamins including pro-vitamin A, vitamin C and vitamin E. Among the secondary metabolites, capsaicin which is responsible for the pungency (hotness) of *Capsicum* fruits, is the most important and is utilized in medicinal formulations, chemotherapy and radiation therapy to reduce pain (Clark and Lee 2016). *Capsicum* fruits also contain anti-microbial and anti-inflammatory properties. Recently, it was reported that *Capsicum* may have anti-cancerous properties by reducing the chance of organelle cancer (Sarpras et al. unpub). *Capsicum* fruits are also utilized to make natural food colors.

The *Capsicum* genus contains mostly diploid species (with $2n = 2x = 12$) and the genome size of *Capsicum annuum* L. is approx. 3.5 GB (Qin et al. 2014). The chromosome 03 (270 MB) is the largest while chromosome 02 is the smallest (154 MB; Hulse-Kemp et al. 2018). It is believed that *Capsicum* originated in Central and South America and then spread worldwide. In 2017, world annual production of green capsicum was 53.9 million mt, was harvested from 2.7 million ha, while 4.9 million mt of dry capsicum was harvested from 1.9 million ha of land (FAOSTAT 2017). Across the globe, India ranks first in area of harvest (0.84 million ha) as well as for production for dry *Capsicum* crop (2.1 million mt; more than 40% of world production); however, China is at the top for area of harvest (0.76 million ha) and production (17.8 million mt; more than 30% of world production) of green *Capsicum* (FAOSTAT 2017). In developing countries like Nigeria, India, China, Ethiopia, Bhutan, Thailand, *Capsicum* is a valuable cash crop for smallholder farmers (Lin et al. 2013). Lin et al. (2013) categorized *Capsicum* marketing in five major categories including (a) whole fruit (fresh market), (b) sauces and pickling (fresh processing), (c) whole fruit/power (dried spice), (d) capsaicinoid (industrial extracts) and (e) ornamental.

The genus *Capsicum* comprises more than 30 described species worldwide of which 5 are cultivated spp. viz. *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum* and *C. pubescens* (Bosland and Votava 2000; Ibiza et al. 2012). Among them, *C. annuum* has been extensively explored for molecular, genetic, genomic and breeding studies. Several review papers and book chapters are also available, mostly highlighting the progress of research work on *C. annuum* (Sanati et al. 2018; Srinivasan 2016). This chapter reports the research progress made on other *Capsicum* spp. such as *C. chinense*, *C. frutescens*, *C. baccatum* and *C. pubescens*. These species are important genetic resources containing favorable genes/alleles for economically-important traits which can be used in *Capsicum* breeding.

2.2 Morphological Features and Cultivation of *Capsicum* Spp.

In ancient times, capsicums were grown to protect primary crops from birds and insects. Subsequently, it was popularized in America as a flavoring agent. In the sixteenth century, *Capsicum* was introduced into Asian countries. At present, the naturally occurring highest pungency containing *Capsicum*, commonly known as Bhut jolokia/Ghost chili, is found in Northeast India (Sarpras et al. 2016). Bosland and Baral (2007) grouped these chilies into *C. chinense* by RAPD marker analysis. However, Purkayastha et al. (2012a, b) through molecular analysis using internal transcribed spacer DNA sequences (ITS1 and ITS2) and ribosomal RNA(5.8 s) sequence analysis indicated that Bhut jolokia/Ghost chili is distinct from all five cultivated *Capsicum* species mentioned above and named it *C. assamicum* (Purkayastha et al. 2012a, b). However, the species name *C. assamicum* is not widely accepted. Among the five cultivated *Capsicum* spp., *C. annuum* is mainly cultivated in Mexico (as famous as Mexican chili); *C. frutescens* and *C. chinense* are cultivated in African and Asian countries; and *C. baccatum* and *C. pubescens* are largely cultivated in Latin America (Picksgill 1997).

There are more than 400 varieties of *Capsicum* (Bargavi and Elumalai 2010), and cultivation practices differ from variety to variety, representing a challenge to *Capsicum* breeders. In general, *Capsicum* is cultivated in tropical and subtropical climates (up to 2000 m elevation). *Capsicum* cultivation requires 850–120 mm rainfall and 20–25 °C temperature in the growing season. *Capsicum* plants should be grown in soils with adequate moisture and organic matter. Loamy soil is most appropriate for *Capsicum* growth. In India, *Capsicum* is a rainfed crop; sown in August and harvested in December. In India, the major chili growing regions are in the Northeast and South (Bargavi and Elumalai 2010).

Capsicum chinense, including Bonnet pepper or Habanero pepper, and Bhut jolokia/ghost chilis, are known for their extraordinary hot properties. They are herbaceous and bushy plant reaching a height of 70–80 cm. Leaves of *C. chinense* are wrinkled and compound, and consist of three leaflets. Solitary or a pair of small white flowers with five petals is found in the leaf axil. The shape, size and color of *C. chinense* fruits varies; shape may be round, elongated, bonnet, bell- or lantern-shaped. Fruit color varies depending on the different varieties e.g. yellow, orange, red, purple, dark brown, black or chocolate. The seeds are small, round and flattened.

Capsicum frutescens is commonly called Tabasco pepper or Cayenne pepper; the plants are perennial shrubs. Leaves are smooth and simple. Flower color may vary from white to yellowish white. Fruits are smaller and less pungent than *C. chinense* and are mostly erect and with slightly pointed tips.

Capsicum chinense has a different number of pedicels and different calyx constriction than *C. annuum* and *C. frutescens*; it exhibits a calyx constriction, which is absent in *C. frutescens* (Eshbaugh 1976). Similarly, *C. annuum* contains one pedicel, however, in *C. chinense* the number of pedicels ranges from three to five (Jarret and Berke 2008). Besides, *C. chinense* has rugose leaves, the flowers are dull white and the seeds have wavy margins (Smith and Heiser 1957).

2.3 Germplasm Diversity and Conservation

The conservation of genetic diversity is very important for germplasm improvement and utilization in genetic studies or breeding programs (Pereira-Dias et al. 2019). However, it has been observed that wild *Capsicum* spp. are vigorously harvested by local people for sale in markets. For example, due to its great taste, *C. baccatum* and *C. annuum* var. *glabriusculum* are in high demand in Bolivia and Mexico, respectively. Due to lack of conservation assessment, five taxa of *Capsicum* have just been listed as endangered by IUCN (Khoury et al. 2020). Recently, 37 taxa of the genus *Capsicum* were assessed for their in situ and ex situ conservation, and divided into groups consisting of 18, 17 and 2 taxa, designated as having high, medium or low priority, respectively, for conservation purpose. Further, these taxa fall into different conservation threat categories: 6 are critically endangered, 3 endangered, 10 vulnerable, 6 threatened and 12 of least concern, respectively (Khoury et al. 2020).

Diversity analysis facilitates the development and execution of suitable conservation plans through molecular fingerprinting, and development of core collection germplasm sets. It also helps in understanding the relationship among the germplasm accessions for their appropriate utilization. Germplasm conservation is also a part of diversity, dealing with the proper registration and preservation of the material in authentic gene banks. The conservation of genetic diversity (natural accessions) counterbalances the loss occurring due to genetic erosion. The genus *Capsicum* contains broad diversity which is the result of combination of factors such as different center of diversity, domestication and selections processes (both artificial and natural) and evolution. *Capsicum* spp. can be divided into three complexes: (a) CA *annuum* complex (including *C. annuum*, *C. chinense*, *C. frutescens*, *C. galapagoensis* and wild relatives), (b) CB *baccatum* complex (including *C. baccatum*, *C. praetermissum* and *C. tovarii*) and (c) CP pubescence complex (including *C. pubescens*, *C. carenassii* and *C. eximium*). Although it has been suggested that there is strong incompatibility among the three complexes, successful hybrids have been developed by crossing between individuals of these complexes (Manzur et al. 2015; Yoon et al. 2006). Although among the five domesticated *Capsicum* spp., the maximum number of studies have focused on the diversity analysis of *C. annuum*, few genetic diversity studies have been conducted to understand the genetic relationship in other *Capsicum* spp. such as *C. chinense*, *C. frutescens* and *C. baccatum* (Albrecht et al. 2012a, b; Cardoso et al. 2018; Carvalho et al. 2017; Jarret and Berke 2008; Sarpras et al. 2016; Table 2.1). Significant diversity was observed in fruit size, shape and color in the collection of 330 USDA/ARS *C. chinense* accessions (Jarret and Berke 2008). In the case of *C. baccatum*, the presence of substantial genetic diversity was reported between the wild type (*C. baccatum* var. *baccatum*) and the cultivated type (*C. baccatum* var. *pendulum*) and it was suggested that *C. baccatum* originated from independent lineage which is totally different from the *C. annuum* lineage (Albrecht et al. 2012a; Cardoso et al. 2018). Similarly, Brazilian *C. frutescens* germplasm also has good diversity for various morphological features like stem length, stem width, fruit weight, fruit length and days to flowering and fruiting

Table 2.1 Summary of genetic diversity studies conducted in *Capsicum* spp.

Germplasm lines	<i>C. annuum</i> L.	<i>C. chinense</i> Jacq.	<i>C. frutescens</i> L.	<i>C. baccatum</i> L.	<i>C. pubescens</i> L.	Others	Parameter ^a	References
330	330						Fruit traits	Jarret and Berke (2008)
237	2	5	4	226			DNA marker (AFLPs)	Albrecht et al. (2012a)
220				220			DNA marker (AFLPs)	Albrecht et al. (2012b)
48	38	1	3	1	0	5	DNA marker (SSR/RAMPO)	Rai et al. (2013)
10	5	4		1			α/β -esterase polymorphism	Monteiro et al. (2013)
102		102					DNA marker (SSRs)	Moses et al. (2014)
4652	4163	122	152	163	11	41	DNA marker (SNPs)	Lee et al. (2016)
372	369	2	1				DNA marker (SSR)	Zhang et al. (2016)
377	94			283			DNA marker (SNPs)	Nimmakayala et al. (2016)
32	10	4	3	5	1	9	DNA marker (SSR)	Meng et al. (2017)
123	3	15	103	1	1	1	Morphology and SSR	Carvalho et al. (2017)
30	2	25	2	1	0		DNA marker (SSR)	Nanayakkara et al. (2018)
47		47					Morphological features	Luitel et al. (2018)
116				116			Fruit traits + DNA marker (AFLP)	Cardoso et al. (2018)
307	182	57	12	38	10	8	Morphological features	Tripodi and Greco (2018)
9	7	1	1				DNA marker (ISSR)	López Castilla et al. (2019)
190	137	14	2	28	2	7	DNA marker (SNP)	Pereira-Dias et al. (2019)
373	220	62	14	39	13	25	DNA marker (SNP)	Colonna et al. (2019)

^aAFLP amplified fragment length polymorphism, SSR simple sequence repeat, RAMPO random amplified microsatellite, SNP single nucleotide polymorphism, ISSR interSSR

(Carvalho et al. 2017). *Capsicum frutescens* produces the smallest size fruit among all the *Capsicum* spp., perhaps due to the reduced selection made on this species; however, a large number of small-sized, erect and colorful fruits enhance its utilization for ornamental purposes. Substantial molecular diversity was also observed in a set of 96 *Capsicum* accessions using non-coding RNA based and gene based simple sequence repeat (SSR) markers (Dubey et al. 2019; Jaiswal et al. 2020; Sarpras et al. 2016). Of 96 accessions studied, 53 were from *C. annuum*, 26 from *C. chinense*, 8 *C. frutescens*, 1 each from *C. baccatum* and *C. pubescens*; 7 accessions were of unknown species (for complete list of accessions, see Jaiswal et al. 2020). It was observed that 75% of markers showed polymorphism among accessions with high polymorphic information content (PIC up to 0.70) and genetic diversity (up to 0.74) (Jaiswal et al. 2020).

2.4 Traditional Breeding

The availability of wide genetic variability in *Capsicum* spp. opens the door for their successful utilization in breeding programs for further improvement. However, before beginning a breeding program, one first has to understand the phenotype for which breeding has to be done. In the case of *Capsicum*, breeding is focused on several traits such as productivity, fruit traits including fruit metabolites, and resistance/tolerance to biotic and abiotic stresses (Fig. 2.1). After establishing the breeding objectives, a second focus should address the breeding approach to be followed, which again depends on the breeding objectives. Several conventional breeding techniques have been deployed for *Capsicum* improvement. These breeding techniques include:

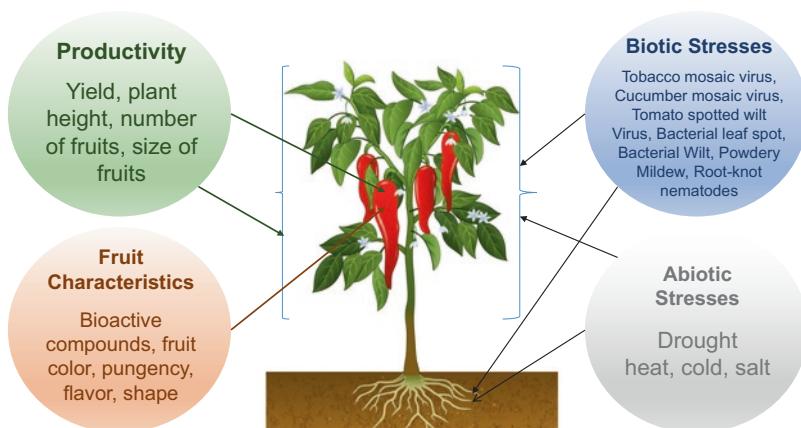


Fig. 2.1 Major breeding objectives in *Capsicum* spp. (Figure constructed by Vandana Jaiswal)

- (a) mass selection: mainly utilized for high heritability traits in the indigenous crop. Best plants are selected for raising next generation (Nsabyera et al. 2013);
- (b) pedigree selection: during pedigree selection record is always maintained for parental mating and derived progenies;
- (c) single seed descent (SSD) method: in this method, single seed of each plant is selected in order to raise the subsequent generations. This method of breeding is mostly utilized for the development of recombinant inbred lines (RIL) mapping population. In this method, maximum diversity can be captured;
- (d) recurrent selection; this selection method involves selection of superior plants and their intercrossing in each generation. This method finds potential when traits need to be combined;
- (e) backcross breeding: in this method selected plants with desirable characters (control by one or few genes) are intercrossed with recurrent parent in subsequent generations.

The above breeding techniques have been utilized by breeders for the development of improved varieties in *Capsicum* crops (Nsabyera et al. 2013; Oliveira et al. 2015; Singh et al. 2014; Ulhoa et al. 2014).

2.5 Hybridization and Heterosis

Interspecific hybridization breeding involves crossing between two individuals belonging to different species of the same genus followed by selection. In *Capsicum*, hybridization breeding is very successful in improving traits, although different cross combinations have shown different responses in terms of seed setting, germination, sterility and viability (Visalakshi and Pandiyan 2018). *Capsicum frutescens* is a good source of disease-resistant genes, and has played a significant role in enhancing disease resistance against viruses in *C. annuum* through interspecific hybridization breeding. Resistance against tobacco mosaic virus and tobacco etch virus has been developed in *C. annuum* by transferring the *L* gene from *C. frutescens* (Visalakshi and Pandiyan et al. 2018).

Similarly, *Capsicum baccatum* is a good source of variation for a range of economically-important and disease-resistant traits. However, there is negligible cross compatibility between *C. baccatum* and *C. annuum*. For interspecific crosses between these two species, a duo of approaches has been suggested (Manzur et al. 2015). The first utilizes the genetic bridge (through *C. chinense* and *C. frutescens*) between *C. annuum* and *C. baccatum*. It has been observed that *C. chinense* acts as an effective genetic bridge for interspecific indirect hybridization between *C. baccatum* and *C. annuum*; however, the potential prezygotic and postzygotic barriers hinder the success of hybridization when *C. frutescens* is used as the genetic bridge between *C. baccatum* and *C. annuum*. The second approach is based on the embryo rescue technique to eliminate the postzygotic hybridization barrier in crosses between *C. baccatum* and *C. annuum*. It has been observed that when crosses

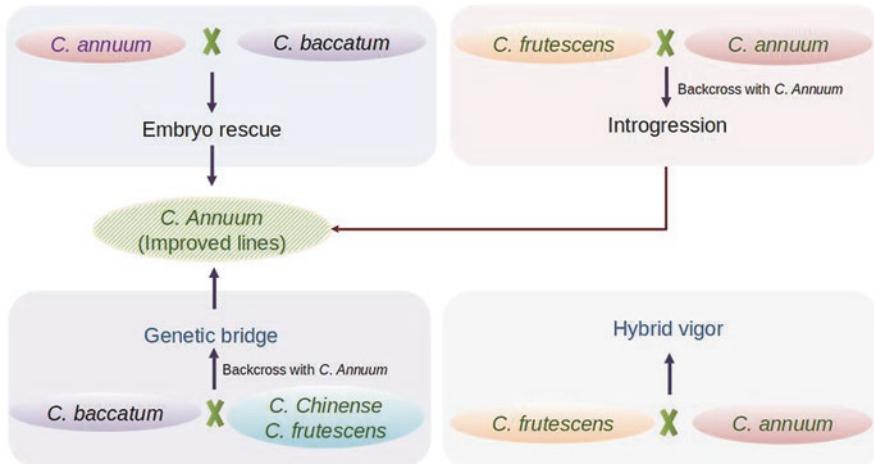


Fig. 2.2 A flow diagram of utilization of other *Capsicum* spp. for the improvement of *C. annuum* through hybridization breeding (Figure constructed by Vandana Jaiswal)

between *C. baccatum* and *C. annuum* are attempted, embryo abortion takes place at a very early stage. Under such condition, embryo rescue is recommended before abortion occurs. Although a higher success rate can be achieved when *C. annuum* and *C. baccatum* are utilized as female and male parent, respectively, success may also vary from genotype to genotype. The only limitation to this method is that it is very tedious and complicated (Manzur et al. 2015).

Besides the introgression of genes from one species to another, another advantage of interspecific hybridization is the superiority of derived progenies over parents, so-called heterosis. Interestingly, heterosis has also been observed in interspecific crosses between *C. frutescens* and *C. annuum* (Ilodibia et al. 2015). F_1 hybrids derived from a cross between *C. frutescens* and *C. annuum* showed significant hybrid vigor for protein, fat, carbohydrate, ash, moisture, and fiber contents (Ilodibia et al. 2015). A flow diagram of the utilization of other *Capsicum* spp. for the improvement of *C. annuum* through hybridization breeding is given in Fig. 2.2.

2.6 Molecular Breeding

With the advancement in high throughput genomic technologies and statistical tools, molecular breeding has become much easier for breeders in development of new and improvement of existing varieties. Molecular breeding has been successfully deployed on several crops including wheat, rice, tomato, and *Capsicum*. A flow diagram of molecular breeding is given in Fig. 2.3. The development of molecular markers, construction of genetic linkage maps and identification of quantitative trait loci (QTLs)/gene(s) are prerequisites of molecular breeding. Several QTLs/

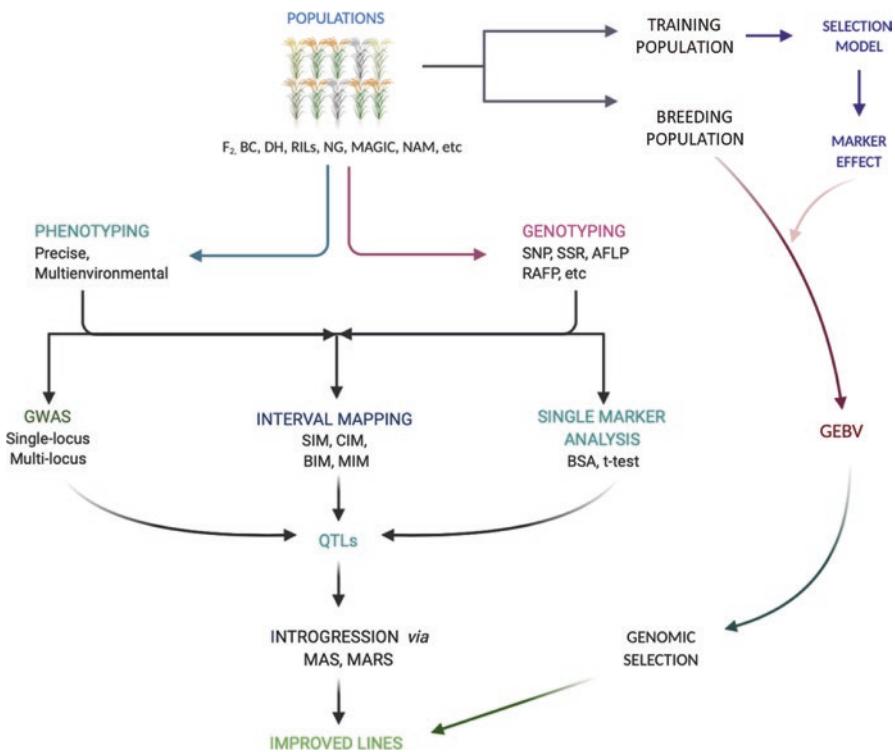


Fig. 2.3 Flow diagram showing different methods/techniques/approach used in molecular breeding; *BC* backcross, *DH* double haploid, *RIL* recombinant inbred line, *NG* natural germplasm, *MAGIC* multi-parental advanced generation intercross, *NAM* nested association mapping, *SSR* simple sequence repeats, *SNP* single nucleotide polymorphism, *AFLP* amplified fragment length polymorphism, *RFLP* restriction fragment length polymorphism, *GWAS* genome-wide association studies, *SMA* single marker analysis, *BSA* bulk segregant analysis, *SIM* simple interval mapping, *CIM* composite interval mapping, *BIM* Bayesian interval mapping, *MIM* multiple interval mapping, *MAS* marker-assisted selection, *MARS* marker-assisted recurrent selection, *GEBV* genome-wide estimated breeding value, *GS* genomic selection. (Figure constructed by Vandana Jaiswal)

genes have been identified for many economically-important traits in different crops using molecular markers. In *Capsicum* spp. (other than *C. annuum*) also, a number of marker systems have been developed and successfully utilized to construct linkage maps and identification of QTLs for important traits. Starting from first generation marker RAPD to RFLP, AFLP, SSRs, to present day markers like SNPs and InDel are available in other spp. of *Capsicum* along with *C. annuum* (Guo et al. 2017; Kang et al. 2001; Lee et al. 2010; Livingstone et al. 1999; Rao et al. 2003; Wu et al. 2009; Zhu et al. 2019). These markers have also been successfully utilized in the identification of QTLs for important traits (Table 2.2). High density linkage maps have also been constructed in interspecific mapping populations derived from crossing *C. chinense*, *C. frutescens* and *C. baccatum* (Hulse-Kemp et al. 2016; Kang et al. 2001; Livingstone et al. 1999; Yi et al. 2006; Zhu et al. 2019). The interspecific

Table 2.2 List of linkage map constructions and QTL mapping studies involving *Capsicum chinense*, *C. frutescens* and *C. baccatum*

Cultivar crosses	Population ^a	Markers ^b	Map length (cM)	Trait	QTL mapping	References
NuMexRNaky (<i>C. annuum</i>) x PI159234 (<i>C. chinense</i>)	F ₂	RFLP, RAPD, AFLP, isozyme	1246	–	Linkage map construction	Livingstone et al. (1999)
TF68 (<i>C. annuum</i>) x Habanero (<i>C. chinense</i>)	F ₂	RFLP, AFLP	1320	–	Linkage map construction	Kang et al. (2001)
Maor (<i>C. annuum</i>) x BG2816 (<i>C. frutescens</i>)	BC ₂	RFLP	1100	Morphological and yield related traits	Interval mapping	Rao et al. (2003)
5226 (<i>C. annuum</i>) x PI159234 (<i>C. chinense</i>)	F ₂	RFLP	–	Anthocyanin and fruit shape	Interval mapping and SMA	Chaim et al. (2003)
Jatilaba (<i>C. annuum</i>) x PRI95030 (<i>C. chinense</i>)	F ₂	AFLP	1060	Anthracnose resistance	Interval mapping	Voorrips et al. (2004)
PI 152225 (<i>C. chinense</i>) x 100/6 (<i>C. annuum</i>); Maor (<i>C. annuum</i>) x BG2816 (<i>C. frutescens</i>)	BC ₂ S ₃ , BC ₂ S ₁	RFLP	–	Fruit shape and size	Interval mapping	Zygier et al. (2005)
TF68 (<i>C. annuum</i>) x habanero (<i>C. chinense</i>)	F ₂	SSR, RFLP	2201	–	Linkage map construction	Yi et al. (2006)
<i>C. annuum</i> x <i>C. frutescens</i>	F ₃ , BC ₂ S ₁	RFLP	–	Fruit shape and size	–	Chaim et al. (2006)
SP26 (<i>C. annuum</i>) x PBC81 (<i>C. baccatum</i>)	BC ₁ F ₂	AFLP, SSR	325	Anthracnose resistance	CIM	Lee et al. (2010)
BG2816 (<i>C. frutescens</i>) x NuMexRNaky (<i>C. annuum</i>)	F ₂	COSII	1613	–	Linkage map construction	Wu et al. (2009)
F68 (<i>C. annuum</i>) x Habanero (<i>C. chinense</i>)	F ₂	rRAMP, gene based, AFLP, SSR	3377	Fruit length	CIM	Lee et al. (2011)
BG2814-6 3 (<i>C. frutescens</i>) x NuMexRNaky (<i>C. annuum</i>)	RIL	SPP	1380	–	Linkage map construction	Hill et al. (2015)

(continued)

Table 2.2 (continued)

Cultivar crosses	Population ^a	Markers ^b	Map length (cM)	Trait	QTL mapping	References
UENF1616 (<i>C. baccatum</i>) x UENF1732 (<i>C. baccatum</i>)	F ₂	SSR, ISSR, RAPD	2547	11 agronomic traits	Interval mapping	Moulin et al. (2015)
BA3 (<i>C. annuum</i>) x YNXML (<i>C. frutescens</i>)	F ₂	InDel, SSR	1250	Flower primordia	CIM and SMA	Tan et al. (2015)
Bangchang (<i>C. annuum</i>) x PBC932 (<i>C. chinense</i>); PBC80 (<i>C. baccatum</i>) x CA1316 (<i>C. baccatum</i>)	F ₂	SNP	824, 1270	Anthracnose resistance	Interval mapping	Mahasuk et al. (2016)
Tabasco (<i>C. frutescens</i>) x P4 (<i>C. annuum</i>)	F ₂	SNP	1392	–	Linkage map construction	Hulse-Kemp et al. (2016)
BA3 (<i>C. annuum</i>) x YNXML (<i>C. frutescens</i>)	F ₂	SNP	1629	Fruit orientation	Interval mapping	Cheng et al. (2016)
PBC688 (<i>C. frutescens</i>) x G29 (<i>C. annuum</i>)	F ₂ , BC ₁ , F _{2:3}	SNP, InDel	–	Cucumber mosaic virus resistance	MQM mapping	Guo et al. (2017)
TF68 (<i>C. annuum</i>) x Habanero (<i>C. chinense</i>)	RIL and germplasm	SNP	–	Capsaicinoid content	Interval mapping and GWAS	Han et al. (2018)
FL201 (<i>C. annuum</i>) x TC07245 (<i>C. galapagoense</i>)	F ₂	SSR	399	Fruit length	CIM	Arjun et al. (2018)
Habanero (<i>C. chinense</i>) x Jolokia (<i>C. chinense</i>); SNU11-001 (<i>C. annuum</i>) x Jolokia (<i>C. chinense</i>)	F ₂ , F ₃	SNP	1150, 1641	Capsaicinoid biosynthesis	CIM	Park et al. (2019)
PB2013071 (<i>C. baccatum</i>) x PB2013046 (<i>C. baccatum</i>)	F ₂	SNP	1319	Aphid resistance	Interval mapping and MQM	Sun et al. (2020)
IBL740 (<i>C. chinense</i>) x CA1 (<i>C. annuum</i>)	F ₂	SLAF	1587	Flowering time	CIM	Zhu et al. (2019)

^aBC backcross, RIL recombinant inbred line^bRFLP restriction fragment length polymorphism, RAPD random amplified polymorphic DNA, AFLP amplified fragment length polymorphism, SSR simple sequence repeat, COS conserved ortholog set, rRAMP reverse random amplification microsatellite polymorphism, SPP single position polymorphism, ISSR inter simple sequence repeat, InDel insertion deletion, SNP single nucleotide polymorphism, SLAF specific length amplified fragments

mapping populations such as F₂, backcrosses, and recombinant inbred lines (RILs), were used to map a number of QTLs for several traits like disease resistance (Lee et al. 2010; Voorrips et al. 2004), fruit shape and size (Chaim et al. 2006; Lee et al. 2011), agronomic traits (Moulin et al. 2015), morphological traits (Rao et al. 2003); capsaicinoid content (Han et al. 2018; Park et al. 2019), flowering time and other traits (Zhu et al. 2019; Table 2.2).

Besides, the other *Capsicum* spp. (other than *C. annuum*) are also utilized to transfer important genes for trait improvement in *C. annuum*. Jeong et al. (2015) transferred *pAMT* gene from *C. chinense* to *C. annuum* through marker-assisted backcross breeding and developed a pungent *C. annuum* variety. For this purpose, cv. SNU11-001 (*C. chinense*) was used as a donor parent and cv. Shinhong (*C. annuum*) was used as a recipient parent. Using SCAR markers for identification of the *C. chinense* allele of *pAMT* gene (foreground selection) and 204 polymorphic SNP markers which are distributed throughout the genome (background selection); the *pAMT* gene was transferred from *C. chinense* and pungent a *C. annuum* variety was developed (Jeong et al. 2015).

Genomics selection (GS) is the most advanced breeding strategy of crop improvement with several advantages like: (a) no need of QTL identification, (b) capture minor QTLs as well, (c) reduces the cost due to limited phenotyping, and (d) faster due to higher genetic gain per cycle. GS has been successfully done in several crops (Varshney et al. 2017); however, no reports are yet available on any species of *Capsicum*.

2.7 Use of Tissue Culture Techniques

It is widely known that plant cells are totipotent and the entire plant can be developed from a single cell. Totipotency of the plant cell allows the application of plant tissue culture technique for the production of plant secondary metabolites. Tissue culture techniques have several advantages over other breeding techniques. For example, they are free from environmental and seasonal changes. Continuous and uniform products are recovered in terms of both quality and quantity (Kehie et al. 2016). In *Capsicum*, tissue culture techniques are well developed and adapted, particularly for the production of secondary metabolite capsaicin and hydrocapsaicin (Gulierrez-Carbajal et al. 2010; Haque and Ghosh 2017; Kehie et al. 2012, 2014, 2016; Sudha et al. 2002). Different types of treatments are being given to induce the production of capsaicin in cell culture. Capsaicin yield has been enhanced through induction of osmotic stress (Kehie et al. 2012) and pH stress (Kehie et al. 2014) in culture medium. Several chemicals such as salicylic acid, jasmonic acid, calcium channel modulator, can also enhance the production of capsaicin in the medium (Gulierrez-Carbajal et al. 2010; Kehie et al. 2016; Sudha et al. 2002).

Micropropagation of genotype Umorok (*Capsicum chinense*) is well established (Santombi and Sharma 2008a, b). For this purpose, the shoot tip is used as an explant and successfully grown in modified MS (Murashige and Skoog) medium supplemented with indole acetic acid. Haque and Ghosh (2017) developed a protocol for micropropagation using germinated seedlings of ten *Capsicum* cultivars from India and Mexico. They used a combination of phyto-hormones benzylaminopurine, indole acetic acid and spermidine for multiple shoot induction. However, cultivars responded differently against different plant growth regulators. Survival rate also differed across the ten cultivars taken for study under particular treatment. Like *C. chinense*, tissue culture is also well established in the case of *C. frutescens*. In the case of *C. frutescens*, different plant parts can be utilized as explants such as the hypocotyl (Guay and Rao 1978), shoot tip (Sanatombi and Sharma 2007), leaf and cotyledons (Sanatombi and Sharma 2008a, b). It has also been suggested that shoot tip regeneration can be substantially enhanced by the addition of activated charcoal to the medium in *C. frutescens* (Gururaj et al. 2004).

2.8 Genetic Transformation

Capsicum annuum has frequently been used as a model gene donor for various bacterial, viral and fungal disease resistances and also resistance against abiotic stresses. Many genes from *C. chinense* germplasm have also been characterized as well as expressed in transgenic plants. *Germin-Like Protein* gene (*CchGLP*) was silenced to reflect its role in gemini virus resistance, in *C. chinense* cv. BG-3821, gemini virus resistant. Silencing of this gene multifolded the propensity of single and mixed infection, thus establishing its critical role as a candidate gene for developing gemini virus resistant transgenic plants (Teniente et al. 2015). *Capsicum chinense pvr1* locus in pepper encodes the eukaryotic translation initiation factor *eIF4E*, and natural point mutation of this gene provides monogenic broad spectrum resistance against wide range of potyvirus infections. Transgenic progenies of tomato overexpressing the *pvr1* allele showed resistance to tobacco etch virus and many potyvirus strains (Kang et al. 2007). The transcription factor CBF (C-repeat binding factor) family regulates chilling stress response in many plants. *CfCBF3* isolate from *C. frutescens* was overexpressed in tobacco plants to mitigate chilling stress and high levels of proline, soluble sugars and reduced content of reactive oxygen species (ROS) was observed; transgenics reflected less susceptibility to chilling stress (Yang et al. 2011). The underexplored *C. pubescens* genes have also been used to confer bacterial blight (*Xanthomonas campestris* pv. *vesicatoria*) resistance to rice, *Oryza sativa*. Wang et al. (2018) created an synthetic gene using *CpBs4C-R* and two other *Bs4C*-like genes, the susceptible allele in the genotype PI585270 of *C. pubescens* (*CpBs4C-S*) and the *CaBs4C-R* homologue gene in the cv.CM334 of *C. annuum* (*CaBs4C*) and the transgenic rice showed broad spectrum resistance confined only to monocots. There are many potentially novel gene in less explored capsicum germplasm, which can be utilized for important resistance traits.

2.9 Whole Genome Sequencing

Among the cultivated *Capsicum* species, whole genome sequences of *C. annuum* (vars. CM334, Zunla1, glabriusculum, UCD-10x-F1), *C. baccatum* (var. PBC81) and *C. chinense* (var. PI159236) are available in public databases. Since the first report, many resequencing projects have submitted refinement over previous ones in terms of gap filling, more coverage, lesser contig counts, and more gene models were refined and repeats and variants were characterized. The current status of the *C. baccatum* genome is 83% (3.9 GB estimated size vs. 3.2 GB assembled size and 2.82 GB anchored to genetic maps) complete, while *C. chinense* is around 94% (3.2 GB estimated vs. 3.0 GB assembled and 2.81GB anchored to genetic maps); respective gene counts are 35,874 and 35,009 for *C. baccatum* and *C. chinense* (Hulse-Kemp et al. 2018; Kim et al. 2014, 2017; Qin et al. 2014).

Transposable elements are an integral part of almost every higher organism's genome but plants carry more remnants in the forms of repeat regions, which ultimately leads to formation of several pseudogenes and genome expansion events. LTR-Rs of Ty3-gypsy superfamily accounts for about half the genome. The gypsy superfamily can further be categorized as *del* elements which are 41.5%, 4.9% and 41.7% for *Capsicum annuum*, *C. baccatum* and *C. chinense* genomes, respectively. In *C. baccatum*, overabundant *athila* element (>2 fold more than the other two) indicates a species-specific genome expansion for Cb lineage (Kim et al. 2017). Genome-wide SSR markers have also been developed interspecifically between *C. annuum* and *C. chinense*. Uncu (2019) developed a total of 53,749 PCR based markers by mining the *C. chinense* genome, utilizing the high degree of compatibility between *C. annuum* and *C. chinense*, and he further developed 17,992 transferable markers. After redundancy removal, a total of 4994 SSRs showing interspecific polymorphism were developed. Zhu et al. (2019) applied the simple length amplified fragment sequencing (SLAF-seq) technique to create a molecular genetic linkage map using 150 F2 interspecific mapping population (derived from *C. annuum* × *C. chinense*). The resulting linkage map contained a total of 9038 markers spanning 1586.78 CM length, and a total of 6 QTLs for flowering time, and flower number per node were identified based on this map. Among the genome-wide explorations, Ahn et al. (2018) resequenced *C. baccatum* powdery mildew resistant line (cv. PRH1) and *C. annuum* powdery mildew susceptible line (cv. Saengryeg) and identified a total of 6281 SNP markers associated with 46 resistance genes. A total of 6,213,009 and 6,840,889 SNPs were identified for PRH1 and Saengryeg, respectively, and a total of 4,887,031 polymorphic SNPs were characterized between the two lines to developed 306,871 high resolution melting temperature marker primer pair sets. These data are being used for further improvement of economically important traits in *Capsicum* species.

2.10 Chloroplast Genome Sequencing

Complete chloroplast genome sequences also serve as important resources for breeding. The whole chloroplast genome sequences with Genbank acc. MH559326 and KR078312 with a total size of 156836 bp and 156817 bp, respectively, are available for *Capsicum frutescens*. The complete chloroplast genome sequences for *C. chinense* are available under acc. MH559321 (156858 bp), KX913217 (156936 bp) and KU041709 (156807 bp). Likewise the complete chloroplast genome sequences are also available for *C. baccatum* (with acc. MH559330 with 157056 bp for cv. Praetermissum, MH559324 with 157053 bp for cv. Baccatum; KR078314 with 157145 bp for Baccatum; MH559320 with 157144 bp for cv. Pendulum) and *C. pubescens* (acc. MH559325 with a cpGenome size of 157390 bp). Agostino et al. (2018) also developed 96 SSR markers from the chloroplast genome.

2.11 Transcriptome Resources

Transcriptomes produced by sequencing of reverse transcribed total mRNA from tissue play an important role in the study of gene expression, including estimation of amount/level of gene(s) expression and splice boundaries. This information has been the basis of overall gene models and annotation refinement for almost every genome assembly project. Several genome sequencing projects and independent submissions to GEO and other expression databases across the globe have generated an enormous number of datasets which are curated with detailed sample and experimental background. Accession SRP166668 comprises 24 samples of high quality triplicate samples of leaf, flower, root, stem tissues and fruit developmental stages, including placenta and pericarp isolated samples (Kim et al. 2017). Accession SRP174493 is hosting 23 *Capsicum chinense* samples in triplicates, representing TMWV (tomato mosaic spotted wilt virus) transfected samples at various dpi (days post inoculation), against TMWV resistant cultivar PI152225 of *C. chinense* at 25 °C and 30 °C. The unreplicated fruit tissue samples of *C. chinense* cvs. Such as Pimenta da neyde and Naga morich, are each available at acc. SRP187959. Transcriptome of placenta isolates of *C. chinense* fruits at 16–18 DPA (days post anthesis) were generated to understand the gene expression involved in capsaicinoid biosynthesis. The unreplicated sequenced dataset of four related cvs. (PI257145, PI257129, PI238048, PI224448) are available under acc. SRP219366. Accession SRP019256 also hosts five tissue stages, i.e. a total of 5 *C. chinense* tissue samples (33 total) are available for further exploration. Primary objective of the study was functional annotation of the pepper genome. The transcriptome data of cv. Bhut jolokia (*C. chinense*) from Northeast India are available at NCBI under acc. SRP116983. This accession contains transcriptome data developed from tissue at early, breaker and mature stages of fruit development along with flower and leaf samples (Table 2.3). There are also transcriptomes available for *C. frutescens*,

among them is whole seedling transcriptome of cv. Honglong 23 for samples under salt stress, a total of 12 samples from 4 conditions in triplicate are available with acc. SRP173426. Transcriptome data from different fruit developmental stages such as early, breaker and mature, along with flower and leaf samples are available under acc. SRP116985. Furthermore, *C. frutescens* transcriptomes were developed in two separate projects and deposited in public database under acc. SRP009477 and SRP009538. These transcriptomes were developed originally to study the expression of genes involved in pungency (capsaicinoids) biosynthesis pathways. Transcriptome sequencing of different fruit developmental stages such as early, breaker and mature were also done in *C. baccatum* cv. PBC81. The pericarp and placenta tissues were also taken for transcriptome sequencing along with the whole genome sequencing for the purpose of studying transposons and disease-resistance genes (Kim et al. 2017; Table 2.3).

2.12 Databases

The *Capsicum baccatum* (acc. MLFT00000000) and *C. chinense* (acc. MCIT00000000) genomes are available at NCBI genomes database. Another dedicated resource for *Capsicum* is the Sol Genomics Network (<https://solgenomics.net>); datasets are browsable in a genome viewer and users can also perform blast query over various *Capsicum* genomes, in addition to that, genetics maps are also available (Mueller et al. 2005). The Pepper Genome Platform is another resource hosting the latest *Capsicum* genomes; data can be locally downloaded and blast based queries can be performed (<http://peppergenome.snu.ac.kr/>). Additional RNA-seq datasets are available at NCBI Sequence Read Archive. The complete proteomes for *Capsicum* are available for *C. chinense* (acc. UP000224522) and *C. baccatum* (acc. UP000224567) at UniProt. The current BUSCO orthology status for *C. baccatum* is 84.4% complete, out of which 81.6% are unique orthologs while 2.8% match with multiple orthologies (Seppey et al. 2019). *Capsicum chinense* is 84.8% complete, out of which 81.9% are unique orthologs while 2.8% are redundant matches; 11.5% and 9.9% fail the test for orthology, hence are reported as missing.

2.13 Conclusions and Future Prospects

Capsicum spp. are a very important spice crop and have several medicinal properties and health benefits. *Capsicum* fruits are rich in several vitamins including pro-vitamin A, vitamins C and E and metabolites. There are five cultivated spp. of *Capsicum*, but a significant knowledge gap is found when comparing (*C. chinense*, *C. frutescens*, *C. baccatum*, *C. pubescens*) with *C. annuum*. Although, these species have wide genetic and morphological diversity, and which would serve as importance genetic resources with potential for carrying biotic and abiotic resistant genes; they are relatively unexplored for genetic improvement. There is an urgent need to

Table 2.3 Transcriptome resources available for *Capsicum chinense*, *Capsicum frutescens* and *Capsicum baccatum* in NCBI sequence read archive database

Study accession	Tissue/stage/condition [^a Accession number]	Isolate/cultivar	Protocol & instrument	Total size Mb ^b /Total bases ^b
A. C. chinense				
SRP019256	Fruit breaker [248815]; fruit mature green [248808]; leaf [248804]; root [248827]; stem [248828]	HYL	CDNA; HiSeq 2000	3211.302/4933556280
SRP116983	fruit early [3165829]; fruit breaker [3165830]; fruit mature [3165831]; flower [3165832]	Bhut jolokia	PCR; HiSeq 1000	1633.81/2772223881.2
SRP166668	Leaf [4926851, 4926853, 4926854]; Stem [4926860, 4926859, 4926871]; Root [4926852, 4926857, 4926858]; Pericarp breaker plus 10 days [4926855, 4926856, 4926869]; pericarp mature green [4926863, 4926864, 4926870]; placenta breaker plus 10 days [4926861, 4926862, 4926867]; placenta mature green [4926865, 4926866, 4926868]; flower [4926872, 4926874]	PI159236	Size fractionation; HiSeq 2500	865.98/1796284317.17
SRP174493	1 dpi mock inoculated [5180527, 5180528, 5180538, 5180539, 5180540, 5180529]; 5 dpi mock inoculated [5180529-5180531, 5180541-5180543]; 1 dpi TSWM inoculated [5180544-5180546, 5180532-5180534]; 5 dpi TSWM inoculated [5180535-5180537, 5180547-5180549]	PI152225	CDNA; HiSeq 2500	2206.94/4349756267.65
SRP187959	Green fruit 16DPA [5495382, 5495383]	Naga morich; Pimenta da neyde	PCR; NextSeq 500	760.58/2169583158
SRP219366	Fruit [6761113-6761116]	PI224448, PI238048, PI257129, PI257145	PCR; NextSeq 500	1319.44/3507233359.25
B. C. frutescens				
SRP009477	Pooled placenta and pericarp [109549]	Xiaomila	PCR; HiSeq 2000	662.08/1238999940
SRP009538	Pooled various tissues [110131]	Xiaomila	PCR; HiSeq 2000	1310.75/2477999880
(continued)				

Table 2.3 (continued)

Study accession	Tissue/stage/condition [^a Accession number]	Isolate/cultivar	Protocol & instrument	Total size Mb ^{bw} /Total bases ^{bw}
SRP116985	Fruit mature [3165834]; flower [3165835]; leaf [3165836]; fruit breaker [3165837]; fruit early [3165838]	Kon jolokia	PCR; HiSeq 1000	1464.67/2482451245.2
SRP173426	WU9 [5129464-5129466]; WU9 salt [5129467, 5129474, 5129475]; Con [5129470, 5129472, 5129473]; Con salt [5129468, 5129469]	Honglong 23	PCR; HiSeq 4000	2450.23/7980974755.64
<i>C. C. baccatum</i>				
SRP166669	Leaf [4926895-4926897]; root [4926892-4926894]; stem [4926876, 4926888, 4926889]; pericarp breaker [4926879, 4926890, 4926891]; pericarp mature green [4926878, 4926884, 4926885]; placenta breaker [4926881, 4926886, 4926887]; placenta mature green [4926880, 4926882, 4926883]; flower [4926875, 4926877]	^b PBCI83, 82, 81; 86, 85, 84; 102, 90, 89; 99, 88, 87; 100, 94, 93; 97, 92, 91; 98, 96, 95; 103, 101]	Size fractionation; HiSeq 2500	694.33/1839732977.65

^a corresponds to NCBI SRA experiment identifier, common prefix SRX has been omitted for brevity, e.g. SRX248815^bPBC is common prefix for all cultivars used in this studyDPA stands for days post anthesis, significant physiological and gene expression changes takes place in different DPA of *Capicum* fruit

focus on and explore these species on a level comparable to *C. annuum*. The application of advanced genetic, genomic and biotechnological tools would enhance their utilization in breeding programs, even for interspecific hybridization breeding and genomic selection. Furthermore, genome editing tools like CRISPR/Cas may also be utilized to understand the function of genes and to develop improved *Capsicum* crops with desirable traits. The availability of whole nuclear and organelle genomes, and transcriptome sequences also help significantly to identify and isolate important genes governing economically-important traits and their application in breeding. The sequencing of whole genome of *C. frutescens*, of moderately pungent species, will highlight the conservation and evolution of *Capsicum* genomes among different species. Furthermore, more genetic and genomic study projects involving a large number of genotypes of the four underutilized *Capsicum* species would provide more insight into their genome organization, evolution and potential for use in future *Capsicum* breeding.

Acknowledgements Thanks are due to the Council of Scientific and Industrial Research for providing funds (MLP-201) and Department of Biotechnology, Government of India, in the form of Ramalingaswami Re-Entry Research Grant to NR., VJ and VG thanks to Department of Science and technology for INSPIRE faculty award. VJ also thanks to Science and Engineering Research Board for Early Career Research Award. CSIR-IHBT publication no-4658.

Appendices

Appendix I: Research Institutes Relevant to Capsicum

Institution name	Specialization and research activities	Address / Country	Contact information and website
ICAR-Indian Institute of Horticultural Research	<i>Capsicum</i> breeding	ICAR-IIHR, Hesaraghatta Lake Post, Bengaluru-560 089, India	Director, ICAR-IIHR, email: director.ihr.res.in ; website: https://ihr.res.in/node?
Chile Pepper Institute	<i>Capsicum</i> breeding and germplasm collection	New Mexico State University, Las Cruces, Mexico	Website: https://cpi.nmsu.edu
The National Germplasm Resources Laboratory	Germplasm resources	0300 Baltimore Avenue BLDG. 003, RM. 102, BARC-WEST Beltsville, MD 20705	https://www.ars.usda.gov/northeast-area/beltsville-md-barc/beltsville-agricultural-research-center/national-germplasm-resources-laboratory/
Instituto Nacional de Innovación Agraria	Germplasm resources	Av. La Molina, N° 1981, La Molina Lima – Perú Central	Telefónica: +(51 1) 240-2100/240-2350 informes@inia.gob.pe https://www.inia.gob.pe/

(continued)

Institution name	Specialization and research activities	Address / Country	Contact information and website
Pairumani Phytoecogenetic Research Center	Germplasm resources	Bolivia Phytoecogenetic Research Center Bolivia	Tel.: + 591-4-4010470 Fax: + 591-4-4263329 Email: fito@fundacionpatino.org http://www.fitogen.fundacionpatino.org/
Instituto Nacional de Innovación Agropecuaria y Forestal	Germplasm resources	Calle Cañada Strongest N ° 1573 Zona San Pedro. La Paz, Bolivia	Telephones (591) 2 2124404 – 2 2124420 contacto@iniaf.gob.bo
International Plant Genetic Resources Institute	Germplasm resources, germplasm characterization, curation	Maccarese, Italy	http://www.ipgri.org/
Universidade Estadual do Norte Fluminense Darcy Ribeiro	Germplasm collection	Av. Alberto Lamego, 2000 – Parque Califórnia Campos dos Goytacazes – RJ CEP: 28013-602 Brazil	(22) 2739-7119 uenf@uenf.br https://uenf.br/
The Asian vegetable research and development centre	Germplasm collection	60 Yi- Min Liao; PO Box 42, 741 Shanhua, Tainan – China	Website: http://www.avrdc.org.tw Institutional email: avrdcbox@netra.avrdc.org.tw
World vegetable center	Germplasm, seedbank, guidelines on characterization	P.O. Box 42 Shanhua, Tainan, Taiwan 74151	Phone: +886-6-583-7801 Email: info@worldveg.org Web: avrdc.org
National Bureau of Plant Genetic Resources	Germplasm curation	ICAR-National Bureau of Plant Genetic Resources, Pusa Campus- New Delhi –110012, India	www.nbprgernet.in

Appendix II: Genetic Resources of Capsicum

Species	Cultivar	Important traits ^a	Cultivation location
<i>C. chinense</i>	Datil	Elongated pods 8–9 cm; extremely hot but also very sweet; green to yellow color transitions	St. Augustine, Florida
<i>C. chinense</i>	Cajamarca	Small pods; color transitions from purple to rich red; intense spicy-citrus fragrance; very high heat level.	Peru

(continued)

Species	Cultivar	Important traits ^a	Cultivation location
<i>C. chinense</i>	Bhut jolokia	Over 1 million SHU, among hottest chili	North – East India
<i>C. chinense</i>	Habanero chocolate / Congo black	Pendant pods (4.0–6.0 cm long × 2.5–5.0 cm wide); medium thick flesh; mature green to chocolate brown; 60–76 cm tall plant; extremely hot	Trinidad
<i>C. chinense</i>	7 Pot Dougla	60–76 cm tall plant; Pendant habanero type, 5.08 cm long × 3.5 cm wide fruit; medium thick flesh; matures from green to red; 1,854,000 SHU	Trinidad
<i>C. chinense</i>	Trinidad Moruga Scorpion	2 million SHU, among hottest chili	Trinidad
<i>C. chinense</i>	Orange Habanero	Large bright orange pods; very high heat levels	
<i>C. chinense</i>	Red Caribbean Habanero	Large bright red pods; fruity aroma; very high heat levels	Caribbean
<i>C. baccatum</i>	Christmas bell (Uba Tuba)	Pod shape resembles bell; mild heat; pickling applications	Brazil
<i>C. baccatum</i>	Aji Limon	Small upright canopy; lemony undertone pods; very high heat levels	Peru
<i>C. baccatum</i>	Omni color	Small upright pods; multicolored; very high heat levels	Peru
<i>C. baccatum</i>	Aji Amarillo	40,000–50,000 SHU, medium heat; 15.24–17.78 cm fruit length; 1.5–1.8 m tall plant in a single season	Peru
<i>C. baccatum</i>	Aji Andean	Medium hot pepper; distinct aji type flowers greenish yellow marks around corolla	Peru
<i>C. baccatum</i>	Aji Ayucullo	Small, thick flashed, oval shaped	Peru
<i>C. baccatum</i>	Aji Bolivian long	Medium heat, 10.16 cm long × 1.27 cm wide thin walled fruit; wide color transitions from green to yellowish orange and finally red	Bolivia
<i>C. baccatum</i>	Aji Brazilian Bonanza	White flowers, 1.21 m high upright plant; colors transitions green orange to finally red. Very hot variety	Brazil
<i>C. baccatum</i>	Aji Brazilian Starfish	Unique starfish shaped fruit; very pungent; color green to red; distinctive baccatum greenish yellow markings on flower petals	Brazil
<i>C. baccatum</i>	Aji Caballero	0.9–1.21 m tall plant; high yields, 2.54 cm long fiery hot peppers deep red color on maturity	Puerto Rico
<i>C. pubescens</i>	AJI MONGOL	Apple shaped red fruits; small plant 0.5 × 0.5 m canopy; purple flowers; brownish seeds; mild heat	Venezuela
<i>C. pubescens</i>	Caballo	Globose to oblate fruit shape, 7.62 cm long × 5.72 cm wide fruit; thin, soft flesh, fruity flavors; very hot variety	Guatemala
<i>C. pubescens</i>	Ecuadorian red pepper from hell	Extremely pungent variety; small oval and fleshy fruit; characteristic black seeds; purple flowers	Ecuador (continued)

Species	Cultivar	Important traits ^a	Cultivation location
<i>C. pubescens</i>	Manzano Amarillo	Fruit appearance like small Manzano (apple); very hard and black seeds very hot	Mexico
<i>C. pubescens</i>	Manzano Canario	Apple-like fruits; hard black seeds; yellow fruit coloration while breaker stage; very hot	Mexico
<i>C. pubescens</i>	PI590503	1.2 m tall woody and erect shrub; purple flowers; fruit color transition green, yellow and finally red; 5 cm long ovate shaped fruits with thick flesh, black seeds and pungent flavor.	Bolivia
<i>C. pubescens</i>	Rocoto canario	Apple shaped yellow fruits (5.08 cm long × 3.81 cm wide); fruit color transitions from greenish to yellow; black seeds; highly pungent	Peru
<i>C. pubescens</i>	Rocoto largo San Isidro	Large fruits; color changes from dark green to red during ripening; black seeds; flower color white unlike most other pubescense, while purple also appear sometimes; 50,000–100,000 SHU	Canary islands
<i>C. frutescens</i>	African devil/ Monbassa / Zanzibar Chile / pili-pili	0.30–1.22 m tall bushy plant; 2.54–3.81 cm long pods, blunt taper at ends; green to red transition at maturity; 50,000–100,000 SHU	Sudan
<i>C. frutescens</i>	Aji Pequante	Around 80 cm high bushy plant; small green flowers with purple stamens; fruit transition green to red	Ecuador
<i>C. frutescens</i>	Angkor sunrise	A tall (1.06 – 1.22 m) bushy variety; 5.08 cm long × 1.27 cm wide erect fruits; color transition from pale yellow to sunrise orange to red; very hot	Cambodia
<i>C. frutescens</i>	Assam	Thin walled cayenne type pods (3.81–5.0 cm long × 0.95–1.27 cm wide); color transition green to red	India
<i>C. frutescens</i>	Bangalore Torpedo	Cayenne type pods (12.7 cm long × 0.6 cm wide) curly at ends; mature to crimson red from lime green	India
<i>C. frutescens</i>	Eleuthera Pepper	0.60 m tall × 0.91 m wide bush; orange pods (2.5–5.0 cm long × 0.63–0.95 cm wide); very hot	Bahamas
<i>C. frutescens</i>	Cabai Burong	Medium sized plant; very seedy pods (3.81–5.0 cm long × 0.95 cm wide); citrus aftertaste	Malaysia
<i>C. frutescens</i>	Dieng Plateau	Erect red fruits (2.54 cm long × 0.85 cm wide); 60.96 × 60.96 cm plant canopy; hot	Indonesia
<i>C. frutescens</i>	Isla Colon Mild	30.5 cm tall plant; 3.81 cm long × 1.9 cm wide, erect fruits; long dark green leaves; mildly hot	Panama
<i>C. frutescens</i>	Japones	Thin long cayenne like red fruits grow in large clusters of 5 cm long; 60–75 cm tall plant; medium heat	China
<i>C. frutescens</i>	Tabasco	1.2–1.5 m tall plant; 3.8 cm long, upright pods; mature from light green to orange and finally red; 50,000–80,000 SHU	Costa Rica

(continued)

Species	Cultivar	Important traits ^a	Cultivation location
<i>C. frutescens</i>	Thai hot	30.5 cm tall plant; 1.27–2.54 cm long red upright fruit; very hot	Thailand

^aSHU = Scoville heat units

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Chapter 3

Recent Advances in Breeding of Bitter Gourd (*Momordica charantia* L.)



Pulipati Gangadhara Rao

Abstract Bitter gourd is valuable for its nutrients and medicinal properties, but neglected in terms of genetic inheritance and molecular breeding. Single plant selection, mass selection, pedigree selection and bulk population improvement methods are commonly used in bitter gourd. Heterosis breeding (F_1 hybrids) is a new approach by using gynoecious and precautious gynoecious lines. Rootstock breeding is another area to address biotic and abiotic stresses in bitter gourd. Scanty information is available on the use of molecular markers including random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and inter-simple sequence repeat (ISSR). Reduced representation sequencing (RRS) methodologies like restriction-associated DNA tag sequencing (RAD-seq) analysis and genotyping-by-sequencing (GBS) technology used to map gynoecy and other economic traits. The SNP marker TP_54890 found at a distance of 3.04 cM to gynoecious (*gy-1*) locus on linkage group 12. This marker will be extremely useful in MAS for the rapid development of new varieties and hybrids in bitter gourd. The draft genome sequence (285.5 Mb ~84%) of bitter gourd will act as a reference genome for future genomic studies. The availability of advanced molecular tools will complement conventional breeding used to accelerate genetic gain in bitter gourd. This chapter discusses conventional breeding, distant hybridization, genetic inheritance and QTLs mapping of horticultural traits along with tissue culture aspects in bitter gourd.

Keywords Bitter gourd · Breeding · Genotyping · Mapping · Molecular marker · QTL · Sequencing

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3.1 Introduction

Bitter gourd, also commonly known as bitter melon, balsam pear, bitter cucumber or African cucumber (*Momordica charantia* L.), is an economically-important vegetable crop. It belongs to the tribe Joliffieae, subfamily Cucurbitoideae and family Cucurbitaceae (De Wilde and Duyfjes 2002; Jeffrey 1980). The generic name *Momordica* is derived from the Latin *mordeo*, meaning *to bite* (Drury 1866; Krishnendu and Nandini 2016) and the species name *charantia* is from ancient Greek, which means *beautiful flower*. It is widely cultivated in India, China, Malaysia, Africa and South America (Raj et al. 1993; Singh 1990). Both fruits and seeds of bitter gourd are consumed together at an immature stage and possess purported medicinal properties such as the following: diabetes in India, China and Central America (Chen et al. 2003), hypoglycaemic compounds (Jayasooriya et al. 2000); anticarcinogenic and hypercholesterolemic (Ahmed et al. 2001; Ganguly et al. 2000); charantin (Yeh et al. 2003); momorcharin (Leung et al. 1997); anti-HIV activity (Lee-Huang et al. 1995) and momordicoside A and B (Okabe et al. 1980). The fruits of bitter gourd also possess antimicrobial (Yeşilada et al. 1999), antifertility (Basch et al. 2003), antiviral (Nerurkar et al. 2006), antiulcerogenic (Gürbüz et al. 2000), steroid (Grover and Yadav 2004) and antitumor (Fang and Ng 2011) properties. The seeds contain pyrimidine nucleoside vicine (Yuk et al. 2015). Bitter gourd seed oil contains high value potential nutraceuticals such as lipids, mainly α -eleostearic acid and considerable levels of phytosterols (Yoshime et al. 2016). It is known that seed lipid of bitter gourd contains more than 50% conjugated linoleinic acids (CLN), reported to remarkably inhibit the development of AOM-induced colonic aberrant crypt foci (ACF) (Kohno et al. 2002), small but distinct amount of CLN are also found in the flesh of bitter gourd (Suzuki et al. 2001). The seed coat is considered diacritical in the taxonomy of the *Momordica* genus (Aguoru and Okoli 2009). The fruits of bitter gourd are rich in β -carotene, vitamin C, folic acid (vitamin B₉), magnesium, phosphorus and potassium (Dhillon et al. 2017; Yuwai et al. 1991).

Consumer preferences vary for fruit color, shape, ridges and size among and within countries. Fruit color varies from white to dark-green, and shapes from longitudinal to round, ridges may be continuous or discontinuous depending upon the variety. Based on the variability of fruit traits, nearly 20 market types (Thailand, Chinese, Taiwan, Okinawa, Vietnam, Philippine, Indonesia, South Asian and other types (Fig. 3.1) of bitter gourd exist in Asia, and nearly half are cultivated in India, China, Nepal, Bangladesh and Sri Lanka (Dhillon et al. 2016b). Fruit color governs marketability; green-fruited types are in high demand in southern China, while white-fruited types are preferred in central China, similarly dark green to glossy green fruits are favored in northern India, white fruits are preferred in southern India and in the eastern parts of the country small and dark green fruited types are preferred (Behera et al. 2010). Long-fruited types are preferred in northern India, while medium-long fruited types are favored in southern India, whereas, short-fruited types are in high demand in the eastern states of India (Behera et al. 2010).

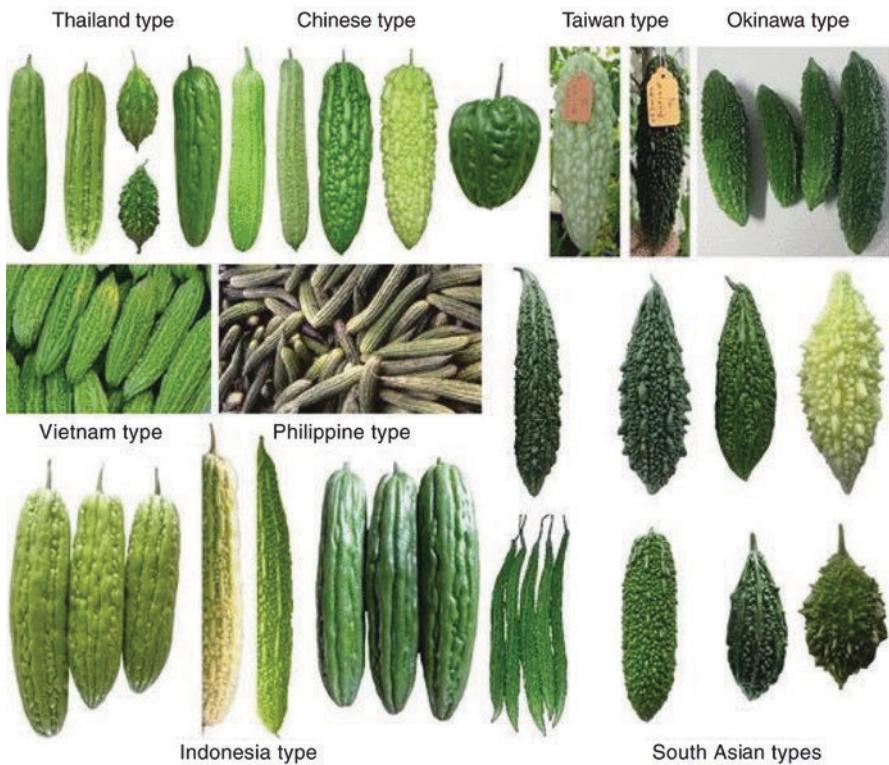


Fig. 3.1 Variability of bitter gourd fruit traits for major market types. (Source: Dhillon et al. 2016b)

Landraces still popular among farmers include Faizabadi Karela, Green Long, White Long, Jaipur Long, Katai and Jhalari in India; Guangdong, Guangxi and Hainan in southern China; Dading (cone-shaped) in the Pearl River Delta of China; Zhenzhu, in southwest to central China; Ranipukur, rampali and Gazkarala in Bangladesh and Kee Nok (small fruit) in Thailand (Dhillon et al. 2016b). Also, local cultivars like Faizabadi, Jhamada (up to 60 cm and less bitter), Barsati, Jaunpuri and Deshi Hra from Bihar. A rare landrace Rudrakshahajli (JMP/10-41) with typical round spindle-shaped fruits from Karnataka was collected and maintained at NBPGR and may be useful for future breeding (Pandey et al. 2019).

Even though India is a major world producer of bitter gourd, average production is very low and has remained static for decades. Major constraints to bitter gourd production are the low genetic potential of improved varieties, lack of early- and high-yielding hybrids and unavailability of quality seeds, besides poor crop management and biotic and abiotic stresses. Thus, in the Indian context production of bitter gourd should be further enhanced by manipulating the sex expression, plant architecture and identifying the best combiners. Despite steady efforts towards the improvement of productivity in bitter gourd using conventional breeding approaches, there is yet no substantial improvement in yield. Moreover, its cross-pollinated

nature, difficulty in accurate phenotyping and linkage drag are problems faced in conventional breeding. Thus, the use of advanced molecular tools like marker-assisted selection (MAS) can be utilized for bitter gourd improvement. Bitter gourd has been a rather neglected crop concerning genomic research and work on molecular markers is in an initial stage.

3.1.1 Taxonomy of Bitter Gourd

The genus *Momordica* consists of 59 species (Schaefer and Renner 2010a); there are 5 other cultivated *Momordica* species in addition to *M. charantia* (Robinson and Decker-Walters 1997). *Momordica* is a monophyletic genus and divided into 11 clades. The Asiatic species consists of 3 sects, namely Sectopm 1, Cochinchinensis (*M. cochinchinensis*, *M. dioica*, *M. sahyadrica*, *M. denticulata*, *M. denudata*, *M. clarkeana* and *M. subangulata*; all are dioecious spp.), sect. 2, *Momordica* (*M. charantia* and *M. balsamina*; monoecious spp.), and sect. 3, *Raphanocarpus* (*M. cymbalaria*) (Schaefer and Renner 2010a; Behera et al. 2011). The taxonomy of *Momordica charantia* is given below, and a taxonomic key of related species is in Table 3.1.

Table 3.1 Taxonomic key of *Momordica* spp.

Group	Species	Characteristics
I		Epigeal, annual, non-tuberous tap root, monoecious, nectary male flowers not closed with corolla scales, fruits are muricated
	<i>M. charantia</i>	(a) Male flower bracts are at the middle of the peduncle
	<i>M. balsamina</i>	(b) Male flower bracts are at the apex of the peduncle
II		Hypogea, perennial, tuberous tap root, dioecious, nectary male flowers closed with prominent corolla scales, fruits are echinate
		(a) Petals with black purple blotch, saucer-shaped male calyx hypanthium
	<i>M. subangulata</i> ssp. <i>renigera</i>	(i) Cordate leaf, un-lobed with dentate margins, glandular petiole
	<i>M. cochinchinensis</i>	(ii) Un-lobed/deeply lobed leaf with undulated margins, 6–12 bead-like glands on petiole and leaf lamina
		(b) Petals without purple blotch, cup-shaped male calyx hypanthium
	<i>M. sahyadrica</i>	(i) The flower opens in the early morning, large, not scented, blackish-purple male calyx
	<i>M. dioica</i>	(ii) The flower opens in the evening, small, with musky scented, whitish-yellow male calyx
III	<i>M. cymbalaria</i>	Hypogea, perennial, tuberous tap root, monoecious, male flowers borne in a short raceme

Source: This table is based on information from Bharathi and John (2013)

Taxonomic Tree of *Momordicacharantia* (NRCS, USDA)

Domain: Eukaryota
 Kingdom: Plantae
 Phylum: Spermatophyta
 Subphylum: Angiospermae
 Class: Dicotyledonae
 Order: Violales
 Family: Cucurbitaceae
 Genus: *Momordica*
 Species: *Momordica charantia*

3.1.2 Origin, Evolution and Domestication

The center of origin of bitter gourd is Africa (Schaefer and Renner 2010a) and the center of domestication is eastern Asia, possibly eastern India or southern China (Raj et al. 1993; Walters and Decker-Walters 1988). The distribution of major *Momordica* species in Southeast Asia is shown in Fig. 3.2. The evolution of bitter gourd has been very slow because of the preference for wild traits like bitterness and fruit size (Matsumura et al. 2019). The South Asian bitter gourd cultivars diverged from wild spp. about 6000 years ago and the further extreme trait divergence occurred about 800 years ago with selection for fruit traits within South Asian cultivars (Matsumura et al. 2020). Long-read genome analysis of bitter gourd revealed that balancing selection within the cultivar groups and divergent selection among the cultivar

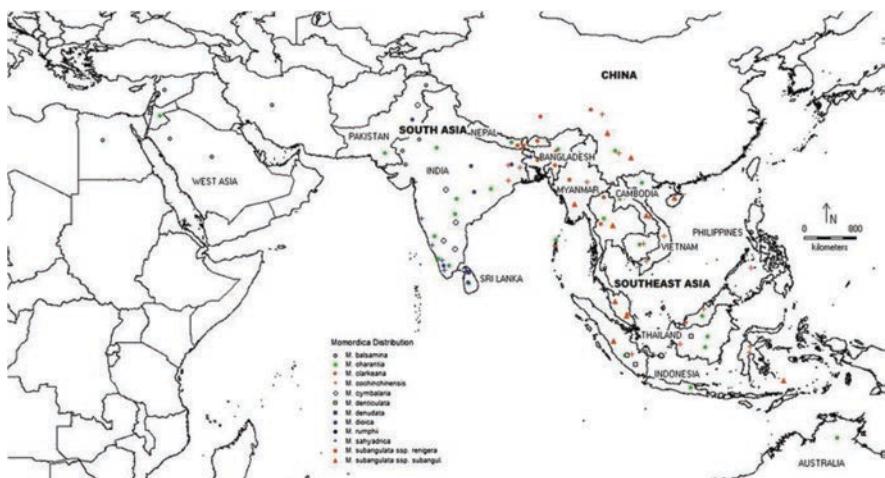


Fig. 3.2 Distribution of *Momordica* spp. in Southeast Asia. (Source: Bharathi and John 2013)

groups are major reasons for the crop diversity (Matsumura et al. 2020). Whole-genome sequencing of wild and cultivated bitter gourd germplasm encountered the candidate domestication genes in South Asia (710), Southeast Asia (412) and China (290) populations (Cui et al. 2020). The wild *M. charantia* var. *muricata* is considered as the progenitor of cultivated *M. charantia* var. *charantia* (Walters and Decker-Walters 1988) and *M. angolensis* is considered as a sister species to *M. charantia* (Schaefer and Renner 2010b).

3.1.3 Botanical Description

Momordica charantia is an annual to a perennial monoecious vine with smooth or slightly hairy stems and can reach up to 2–3 m long. The stems are branched, slightly five angled, or ridged and carry unbranched tendrils in the leaf axils. The leaves are carried singly along the stems on 3–5 cm long stalks, and each leaf is 4–10 cm long, rounded in outline, and deeply 5–9 lobed. Leaf stomata of *Momordica* species are hypostomatic except *M. balsamina* (amphistomatic) and leaf epidermis is smooth except *M. multiflora* (glabrous) and *M. angustisepala* (pubescent) (Kadiri and Olowokudejo 2016). Flowers occur singly in the upper leaf axils on 2–10 cm long stalks with a small leaf-like bract towards the base. Male flowers have a slender basal swelling which is continuous with the base of the sepal tube, which ends in 5 blunt sepals. There are 5 oval yellow petals 10–20 cm long, and 5 central stamens. Female flowers are looks like to the male flowers but have a distinct warty swelling ovary well below the base of the sepal tube and 3 stigmas. The pendulous cylindrical fruits are egg-shaped and 2–10 cm long (up to 20 cm in cultivated varieties) and covered with longitudinal ridges and warts. At maturity, they turn orange to yellow, and the tips split into three and turn back to reveal the yellow pulp and the bright red arils that enclose the seeds which adhere to the inside of the fruit. Each of the flattened woody seeds is 5–9 mm long with finely pitted surfaces.

3.1.4 Cytology

The majority of cytological studies report that the *Momordica charantia* diploid chromosome number is $2n = 22$, with a basic chromosome number $x = 11$ (Shibata 1962; Trivedi and Roy 1972). The chromosome length may vary from 1.32 to 3.24 μm in *M. charantia* var. *charantia* and 1.27–3.07 μm in *M. charantia* var. *muricata* (Ghosh et al. 2018). Among 11 pairs of chromosomes, 6 are median, 3 are submedian and 2 are subterminal chromosomes. Karyotype and ribosomal DNA distribution reveal that chromosome pairs 4 and 11 have 45S rDNA sites in the

terminal position of the small arm and chromosome pair 5 has 5S rDNA sites in the proximal position (Waminal and Kim 2012). Giemsa staining of metaphase found 2 pairs of nucleolar constriction in all *M. charantia* var. *charantia* and 3 such pairs in *M. charantia* var. *muricata* (Ghosh et al. 2018).

3.1.5 Reproductive Biology

Bitter gourd is monoecious; male and female flowers are borne on separate nodes. Generally, flowering begins 35–55 days after sowing, even earlier depending on genotype (gynoecy/precocious gynoecy) growing conditions. Anthesis time typically occurs from 0330 to 0730, when flowers are completely open (Raj et al. 1993). The stigma is usually receptive 1 day before or after anthesis (Rasco and Castillo 1990). Pollen viability is lost relatively rapidly (Desai and Musmade 1998). Bitter gourd pollen can be stored at -25°C under N_2 gas or vacuum for 1 year (Akutsu 2016). The typical sex ratio of male to female flowers in monoecious lines in the complete flowering period is 50:1 (Rasco and Castillo 1990), but it also varies significantly from 9:1 to 48:1 (Dey et al. 2005).

3.1.6 Breeding Objectives of Bitter Gourd

- (a) Yield: high yearly and total yield,
- (b) Earliness: first pistillate flower to appear at the lower node,
- (c) High femininity: high female to male ratio of flowers, resulting in the high number of fruits per plant,
- (d) Fruit quality traits: fruit quality should be as per consumer preference,
- (e) Size: generally the preference for medium-sized fruits, 10–15 cm long,
- (f) Shape: fruit with uniformly medium thickness is preferred,
- (g) Surface: smooth surface and continuous smooth ribs or ridges are preferred in many places and tough spines for long-distance transportation,
- (h) Color: preference varies in different regions,
- (i) Less mature seeds,
- (j) Bitterness: moderately bitter,
- (k) Disease resistance: resistance to important diseases like powdery mildew, downy mildew, mosaic,
- (l) Insect resistance: resistance to important insect pests like red pumpkin beetle and fruit fly.

3.2 Genetic Inheritance of Economic Traits

Bitter gourd hybrids are not very prevalent, thus development of hybrids using stable gynoecious or predominately gynoecious lines would be highly useful. This gynoecious trait is directly related to fruit yield and earliness. The major form of sex in the bitter gourd is monoecious, withal, gynoecious sex also has been reported from India, Japan and China (Behera et al. 2006; Iwamoto and Ishida 2006; Ram et al. 2002). Gynoecious lines originating in India are DBGy-201 and DBGy-202 (Behera et al. 2006), IIHRBTGy-491 and IIHRBTGy-492 (Varalakshmi et al. 2014) and Gy263B (Ram et al. 2002). Some other gynoecious lines popularly used as parental material in the breeding program are K44 (Cui et al. 2018), OHB61–5 in Japan (Matsumura et al. 2014) and Z-1-4 (Wang and Xiang 2013), X Hei-d-d (Wang et al. 2010a) and Yuqiang-2 (Li et al. 2011) in China. Predominately the gynoecious habit is also reported in Indian bitter gourd line PreGy-1 (IC-0591254; INGR12014) (Behera et al. 2012). In bitter gourd, the gynoecium is governed by a single recessive gene (Behera et al. 2009; Matsumura et al. 2014; Ram et al. 2006), partially dominant (Iwamoto and Ishida 2006) and two pairs of genes as reported by Cui et al. (2018).

Consumer choice varies with fruit surface traits such as ridges, fruit color, and fruit tubercles. The inheritance pattern of fruit surface (ridges) was studied in bitter gourd (Dalamu et al. 2012; Srivastava and Premnath 1972) and it was reported that a discontinuous ridge was dominant over the continuous ridge and controlled by a single gene. Fruit (epicarp) color governs marketability; however, color preference varies across regions, dark green to glossy green fruits are preferred in northern India while white fruits are favored in southern India (Behera et al. 2010). Green fruit color appears to be completely dominant over white color and single dominant gene (*FrCol*) (Dalamu et al. 2012; Kole et al. 2012; Srivastava and Premnath. 1972; Suribabu et al. 1986; Vahab 1989) also reported white epicarp to be recessive to green. (Liu et al. 2005) reported high heritability of fruit color controlled by one pair of nuclear genes where green is dominant to white. The fruit tubercle is controlled by a single gene and the presence of tubercles is dominant over the absence of tubercles (Kumari et al. 2015; Vahab 1989). Curved fruit shape is controlled by a single recessive gene and straight fruits are dominant over curved fruits (Kumari et al. 2015). Short fruit length is partially dominant over long fruit (Kim et al. 1990; Kumari et al. 2015). Besides, more than four genes govern fruit length (Kumari et al. 2015). Fruit length was found to be incompletely dominant and governed by a minimum of five genes (Zhang et al. 2006). Seed color loci (*SdCol*) are governed by a single dominant gene (Srivastava and Premnath 1972); dark brown seed dominant over light brown seed color with a digenic (9:7) mode of inheritance (Kole et al. 2012). Further, large seed size (*ls*) is recessive to small seed size (Srivastava and Premnath 1972). Fruit luster (*FRLsr*) and stigma (*StCol*) color to be monogenic and dull fruits are dominant over glossy fruits, and yellow stigma dominant over green stigma color (Kole et al. 2012). The gynoecy (*gy*), fruit wart (*fwa*) and white fruit color (*w*) are qualitative characters (Cui et al. 2018). The first female flower node, female flower number, width of ridge and immature fruit color (lightness variable, L; hue angle, H°) are determined to be quantitative characters. The first female

flower node is more affected by the environment in which it is grown (Cui et al. 2018). Two pairs of major genes (overdominant, incompletely dominant) govern the internode length and have high heritability (Yang 2019). The presence of high fruit bitterness is dominant over low bitterness (Suribabu et al. 1986).

3.3 Conventional Breeding

The traditional breeding methods like single plant selection, mass selection, pedigree selection and bulk population improvement are frequently used for bitter gourd enhancement (Sirohi 1997). MDU 1, a mutant variety with improved yield performance over the parent MC 103, was developed by gamma irradiation of seeds (Rajasekharan and Shanmugavelu 1984). Nowadays heterosis breeding has become more popular due to the availability of good combiner lines with earliness and high yield like the gynoecious lines. The range of heterosis in bitter gourd is as high as 86% to as low as 27% for yield per vine (Behera 2004). Researchers have tried mutation breeding and polyploidy breeding (Cho et al. 2006; Saito 1957) methods, but they are at a preliminary stage. Interspecific hybridization among *Momordica* spp. presents a great opportunity for the development of pest and disease resistance, and abiotic stress tolerance varieties and hybrids, although success is a challenge.

3.3.1 Interspecific Hybridization

A cross between *Momordica charantia* and *M. dioica* failed to set seed (Trivedi and Roy 1972). Crosses among *M. charantia* (monoecious), *M. dioica* (dioecious) and *M. balsamina* (monoecious) completely failed (Sinha et al. 1983). Fluorescence microscopic studies revealed the failure of embryo formation in *M. dioica* × *M. charantia* due to the heavy deposition of callose at the tips of the pollen tube and embryo sac (Dutt and Pandey 1983; Roy 1985). Hybrid seeds are more difficult to produce in crosses between *M. charantia* × *M. balsamina* (Bharathi et al. 2012b; Singh 1990) and complete failure was also reported in the same cross (Trivedi and Roy 1972). Embryo rescue techniques need to be standardized in bitter gourd for transfer of resistance genes from wild *Momordica* spp.

3.3.1.1 Classification of the Gene Pool

Gene pool classification serves as an important aid to choosing a specific population to cross with other species (Harlan and de Wet 1971). Based on the available reports on interspecific hybridization and evaluation of their progeny, Bharathi et al. (2012b) proposed the following gene pool classification (Table 3.2) and crossing behavior of *Momordica* spp. shown in Fig. 3.3 (Bharathi et al. 2012a) (Table 3.3).

Table 3.2 Gene pool classification of *Momordica* spp.

species	Gene pool I	Gene pool II	Gene pool III
<i>M. charantia</i>	<i>M. charantia</i> . var. <i>muricata</i>	<i>M. balsamina</i> (only <i>M. charantia</i> as female parent crossable)	Dioecious spp.
<i>M. dioica</i>	<i>M. sahyadrica</i>	<i>M. cochinchinensis</i> and <i>M. subangulata</i> ssp. <i>renigera</i>	Monoeious spp.
<i>M. subangulata</i> ssp. <i>renigera</i>	Only intraspecific types (hybrid progeny are triploid and sterile)	Dioecious species	Monoeious spp.

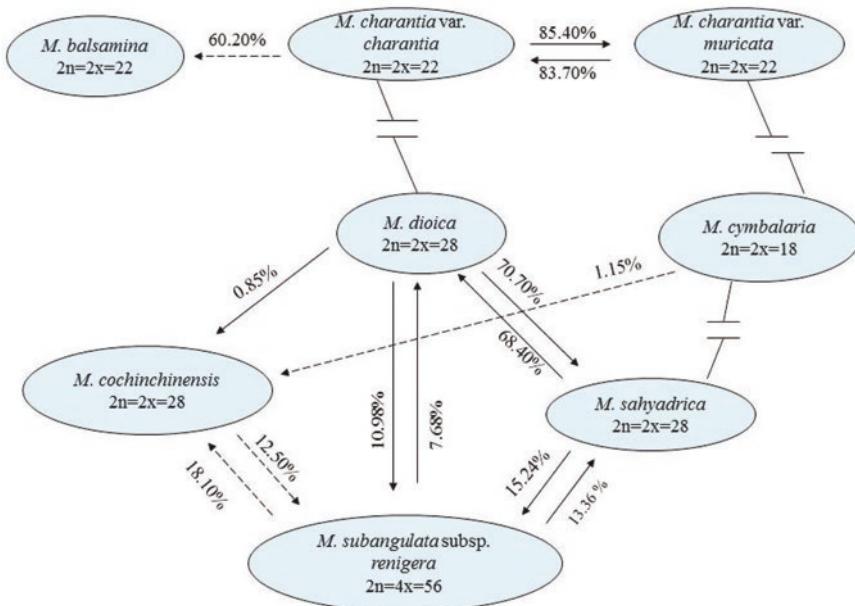


Fig. 3.3 Crossing relationships among *Momordica* species. The cross direction from female to male (→); fruit set/fruits set with viable seeds were not obtained (—||—); partially cross-compatible (< 50% fruits with viable seeds set) (---); completely cross-compatible (> 50% fruits with viable seeds set) (→). The numbers above the arrows represent pollen fertility percentage. (Source: Bharathi et al. 2012a)

Table 3.3 Crossability behavior of five major the *Momordica* spp.

Crossing behavior	<i>Momordica</i> species
Cross-compatible with fertile hybrids	Between two varieties of <i>M. charantia</i> (var. <i>charantia</i> and var. <i>muricata</i>) and <i>M. dioica</i> × <i>M. sahyadrica</i>
Partial cross-compatible with fertile hybrids	<i>M. charantia</i> × <i>M. balsamina</i>
Cross-compatible with sterile hybrid	<i>M. subangulata</i> ssp. <i>renigera</i> × <i>M. dioica</i> , <i>M. subangulata</i> ssp. <i>renigera</i> × <i>M. sahyadrica</i> and <i>M. subangulata</i> ssp. <i>renigera</i> × <i>M. cochinchinensis</i>
Partial cross-compatible with sterile hybrids	<i>M. cochinchinensis</i> × <i>M. dioica</i> , <i>M. cochinchinensis</i> × <i>M. sahyadrica</i>
Cross-incompatible	Monoeious × dioecious species

3.3.2 Intergeneric Hybridization

Patrudu and Murthi (1934) made an intergeneric cross between *Momordica charantia* (female) and *Trichosanthes anguina* (male). About 50% of the pollinated flowers developed into fruit. The F₁ plants were more vigorous than the female parent and were fully fertile. The F₂ segregation showed interesting combinations with gradations in fruit form, size and flavor.

3.3.3 Rootstock Breeding

The main problem with bitter gourd production is *Fusarium* wilt and low temperatures during winter production (Lin et al. 1998). Luffa (*Luffa* spp.), figleaf gourd and pumpkin are common rootstocks for bitter gourd (Lin 2004). Cleft grafting to a loofah (*Luffa aegyptiaca*) rootstock has been widely used during summer production since it is resistant to *Fusarium* wilt and has good tolerance to heat and also resistant to flooding (Liao and Lin 1996). Figleaf gourd and pumpkin are used as rootstocks for winter production since figleaf gourd does not have good heat tolerance. To overcome this problem, figleaf gourd and luffa, are often used together. Figleaf gourd roots will support the scion early and as temperatures rise, luffa roots support the plant (Davis et al. 2008). In Taiwan, bitter gourd yield is increased by grafting with luffa (*Luffa* spp.), which allows bitter gourd to survive in the presence of *Fusarium* wilt and in waterlogged soils (Palada and Chang 2003). Grafting of bitter gourd scions on specific rootstock significantly influences the growth and yield without diminished fruit quality (Tamilselvi and Pugalendhi 2017b). Bitter gourd scion Palee F₁ side grafted onto pumpkin has the highest survival (71.70%) (Tamilselvi and Pugalendhi 2017a). Breeding of rootstocks resistance to soil-borne diseases and abiotic stresses greatly enhances bitter gourd fruit yield and quality.

3.4 Advanced Breeding Strategies

Many economic traits in bitter gourd are governed by several genes and the most important trait, gynoecy, by a single recessive gene. It will take several to 8–9 seasons to transfer even single recessive genes into a good combiner line or any other elite lines. In the case of Pusa Aushadhi and long-fruited gynoecious line (DBGY-202), it took 8 seasons (BC₂S₆) to develop. The improvement of quantitative traits using traditional strategies is time consuming. Breeding for pest and disease resistance is often challenging in bitter gourd. Molecular markers have the potential to overcome the limitations of traditional breeding, since they are nondestructive, can eliminate the environmental variables associated with a trait and can be evaluated for multiple traits simultaneously.

3.4.1 Molecular Markers

Scanty information is available on the use of molecular markers in bitter gourd, including random amplified polymorphic DNA (RAPD) (Behera et al. 2008a,b; Dey et al. 2006b), amplified fragment length polymorphism (AFLP) (Behera et al. 2008a; Kole et al. 2009), simple sequence repeat (SSR) (Dhillon et al. 2016a; Kole et al. 2009) and inter-simple sequence repeat (ISSR) (Behera et al. 2008a), for the assessment of genetic diversity and population stratification. There is an urgent need for high density genetic maps to identify the closely-linked or functional markers for marker assisted selection (MAS) and map-based cloning. Polymorphic microsatellite markers in *Momordica charantia* are the most useful tool to study the genetic diversity, population genetic structure and mapping of horticultural traits in *M. charantia* (Guo et al. 2012; Wang et al. 2010b). In addition, SSR markers between wild and cultivars populations of *M. charantia* also provide polymorphic information in bitter gourd genotypes (Liao et al. 2012). De novo transcriptome sequencing of bitter gourd gynoecious line (Gy323) and monoecious line (DRAR1) enables the detection of candidate genes and the development of functional markers (Shukla et al. 2015).

Various multilocus dominant DNA markers such as RAPD markers (Dey et al. 2006b), ISSR makers (Singh et al. 2007) and AFLP markers (Gaikwad et al. 2008) have been reported so far for genetic diversity analyses of bitter gourd. SSRs are more informative than dominant DNA markers due to their high heterozygosity values (Powell et al. 1996). Although the initial cost of SSR marker development is high, once developed, it is highly repeatable and, consequently, easily transferred across laboratories (Maughan et al. 1995). Based on the suitability in various genetic analyses, SSR markers provide accurate results with a minimum number of loci. However, very scanty information is available on SSR markers in bitter gourd. Out of 70 SSR markers developed, 16 have been reported using the FIASCO technique (Guo et al. 2012; Ji et al. 2012), 11 SSR markers developed through genomic library enrichment (Xu et al. 2011) and 43 SSR markers constructed through cross-species transferability from other cucurbits (Chiba et al. 2003; Watcharawongpaiboon and Chunwongse 2008; Xu et al. 2011). A greater number of markers are necessary for the development of a high-density or saturated genetic map and marker-assisted selection (Tang et al. 2007). A unique set of 160 microsatellite markers has been reported in *Momordica* species through sequencing of small insert genomic libraries (Saxena et al. 2015); however, they showed polymorphisms among the *Momordica* species and less variation within *M. charantia*. A draft bitter gourd whole genome sequence represent a great opportunity to develop new molecular markers in bitter gourd germplasm (Urasaki et al. 2017).

3.4.2 Mapping Population Development

More than seven seasons are required to construct a RIL population and, for some species, the DH population cannot be constructed for lack of standardized tissue culture techniques. It is often simple and rapid to develop a F_2 mapping population for the majority of crop species, therefore F_2 mapping population was widely preferred for early linkage mapping and QTL analysis (Clarke et al. 1995; Gardiner et al. 1993; Harushima et al. 1998), especially in species with limited information on molecular markers (Feng et al. 2012; Levi et al. 2003); moreover, codominant markers provide more information than dominant markers in the analysis of the F_2 population.

3.4.3 Mapping of the Gynoecy Trait

Based on RAD-Seq (restriction-associated DNA tag sequencing) analysis SNP marker GTFL-1 was linked to the gynoecious locus (*gy*) at a distance of 5.46 cM at the distal end of the linkage group aligned at the site of scaffold 44_7277273 (Matsumura et al. 2014). Gynoecious loci (*gy1.1*, *gy1.2*) mapped on LG-1 between scaffold 44_3793313 – scaffold 44_7318231 (Cui et al. 2018). SNP Marker TP_54890 developed through genotyping-by sequencing (GBS) technology at a distance of 3.04 CM to gynoecious (*gy-1*) locus, flanked by markers TP_54865 and TP_54890 on LG12 (Gangadhara Rao et al. 2018), as shown in Fig. 3.4. This marker will be highly useful in MAS for the rapid advancement of various gynoecious lines with the different genetic backgrounds of high combing ability for the development of early and high yielding F_1 hybrids in bitter gourd.

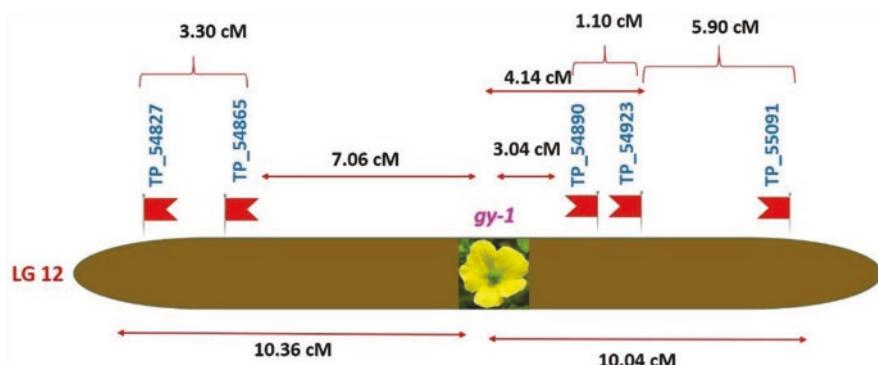


Fig. 3.4 Genetic map of putative gynoecious locus (*gy-1*) of bitter gourd. (Source: Gangadhara Rao et al. (2018). Creative Commons Attribution License (CC BY). <https://creativecommons.org/licenses/by/4.0/>)

Table 3.4 QTL mapping of major quantitative traits in bitter gourd (*Momordica charantia*)

Trait	QTLs	R ² (%)	Parents	Markers	Population	References
Gynoecy	2	11.2–59.6	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)
Stem diameter	2	23.6–25.3	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Internode length	2	16.7–18.1	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Female flower ratios	3	6.7–16.1	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
	9	6.35–20.95	DBGy-201 (gynoecios) × Pusa Do Mousami (monoecious)	2013 SNPs	90 F ₂ and 65 F _{2,3}	Gangadhara Rao et al. (2018)
First female flower node	3	12.2–21.4	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
	2	12.0–32.0	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)
	5	2.09–13.94	DBGy-201 (gynoecios) × Pusa Do Mousami (monoecious)	2013 SNPs	90 F ₂ and 65 F _{2,3}	Gangadhara Rao et al. (2018)
Days to first female flower	8	0.05–58.75	DBGy-201 (gynoecios) × Pusa Do Mousami (monoecious)	2013 SNPs	90 F ₂ and 65 F _{2,3}	Gangadhara Rao et al. (2018)
Female flower number	2	21.2–52.8	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)
Fruit length	2	13.4	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	108 AFLP markers; 11 linkage groups spanning a total of 3060.7 cM	146 F ₂	Kole et al. (2012)
	4	11.2–16.2	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)

(continued)

Table 3.4 (continued)

Trait	QTLs	R ² (%)	Parents	Markers	Population	References
Fruit diameter	1	12.9	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	108 AFLP markers; 11 linkage groups spanning a total of 3060.7 cM	146 F ₂	Kole et al. (2012)
	5	13.0–18.2	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Flesh thickness	2	8.9–12.1	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Fruit shape	5	12.9–23.3	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Fruit pedicel length	3	10.5–27.0	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Fruit pedicel length ratios	5	5.1–33.1	Z-1-4 (gynoecios) × 189–41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Fruit wart	1	52.5–56.7	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)
Width of fruit ridge	1	17.7–30.9	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)
White fruit color (W)	1	70.5–86.1	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)
Fruit color, Hue angle (H°)	1	66.5–75.9	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)
Fruit color, lightness variable (L)	1	65.2–73.4	K44 (gynoecios) × Dali-11 (monoecious)	1009 SNPs	423 F ₂	Cui et al. (2018)

(continued)

Table 3.4 (continued)

Trait	QTLs	R ² (%)	Parents	Markers	Population	References
Fruit weight	1	11.1	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	108 AFLP markers; 11 linkage groups spanning a total of 3060.7 cM	146 F ₂	Kole et al. (2012)
	4	13.1–25.4	Z-1-4 (gynoecios) × 189-41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)
Fruit number	4	39.7	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	108 AFLP markers; 11 linkage groups spanning a total of 3060.7 cM	146 F ₂	Kole et al. (2012)
	3	18.3–20.1	Z-1-4 (gynoecios) × 189-41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang, 2013
Fruit yield	4	38.1	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	108 AFLP markers; 11 linkage groups spanning a total of 3060.7 cM	146 F ₂	Kole et al. (2012)
	2	7.4–15.9	Z-1-4 (gynoecios) × 189-41 (monoecious)	Total: 194 (26 EST-SSR, 28 SSR, 124 AFLP and 16 SRAP)	144 F _{2,3}	Wang and Xiang (2013)

3.4.4 QTL Mapping of Horticultural Traits

Unlike other major cucurbits such as melon, watermelon and cucumber, there is no high-density genetic linkage map for bitter gourd and limited information on precise map positions of horticulturally-important traits, especially for those related to gynoecium sex, fruit-related traits and yield. The genetic linkage map of bitter gourd with 108 AFLP markers in the F₂ population (146 individuals) was developed from a cross between Taiwan White (*Momordica charantia* var. *charantia*) and CBM12 (*M. charantia* var. *muricata*) (Kole et al. 2012). A total of 11 linkage groups were found spanning a distance of 3060.7 cM. The qualitative traits like fruit surface structure, fruit luster and stigma color were mapped on LG1 whereas fruit color and seed color mapped on LG7 and LG3, respectively (Table 3.4). A total of 12 QTLs for 5 fruit traits like length (2 QTLs), diameter (1 QTL), weight (1 QTL), number (4 QTLs) and yield (4 QTLs) were detected on 5 linkage groups that individually explained 11.1–39.7% of the corresponding total phenotypic variance (Table 3.5).

Table 3.5 Mapping of some qualitative traits in bitter gourd (*Momordica charantia*)

Trait	Gene locus	Parents	Population	Linkage group	Linked markers	Type	References
Gynoecy	<i>gy-J</i>	DBGy-201 (gynoecios) × Pusa Do Mousami (monoecious)	90 F ₂	LG12	3.04 cM to TP_54890	SNP	Gangadhara Rao et al. (2018)
	<i>Mgy</i>	OHB61-5 (gynoecios) × OHB95-1A (monoecious)	48 F ₂	LG1	5.46 cM to Gfl-1 distal end	SNP	Matsumura et al. (2014)
Fruit surface structure	<i>FrSrf</i>	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	146 F ₂	LG1	E12M51a distal end 52.9 cM	AFLP	Kole et al. (2012)
Luster	<i>FrLsr</i>	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	146 F ₂	LG1	E12M51a distal end 93.5 cM	AFLP	Kole et al. (2012)
Stigma color	<i>SICol</i>	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	146 F ₂	LG1	E19M57h and E18M53b at 47.6 and 40.4 cM apart, respectively	AFLP	Kole et al. (2012)
Fruit color	<i>FrCol</i>	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	146 F ₂	LG7	E12M47c and E12M52c at distance of 61.0 cM and 42.6 cM, respectively	AFLP	Kole et al. (2012)
Seed color	<i>SdCol</i>	Taiwan White (<i>M. charantia</i> var. <i>charantia</i>) × CBM12 (<i>M. charantia</i> var. <i>muricata</i>)	146 F ₂	LG3	E12M47a and E11M48a at 26.5 and 26.0 cM apart, respectively	AFLP	Kole et al. (2012)

A robust linkage map was constructed for bitter gourd using an F_2 population derived from a cross between gynoecia Z-1-4 \times 189-4-1 in three environments. The linkage map developed with the help of 26 EST-SSRs, 28 SSRs, 124 AFLP markers and 16 SRAP markers spanned over 1005.9 cM with 12 LGs. A total of 43 QTLs, with a single QTL associated with 5.1–33.1% phenotypic variance, were identified on 9 chromosomes for 13 horticulture traits by analyzing F_{2-3} families mapping population and the genetic linkage map. One QTL cluster region was detected on LG-5, which contained the most important QTLs for yield per plant, fruit numbers per plant, first female flower node, female flower ratios and fruit weight with high contributions to phenotypic variance (5.8–25.4%) (Wang and Xiang 2013).

A linkage map using 552 codominant markers derived from RAD-seq (restriction-associated DNA tag sequencing) analysis in the F_2 population (48 individuals) was developed from a cross between OHB61-5 (gynoecious) and OHB95-1A (monoecious). A total of 15 linkage groups were found encompassing 1821 cM distance. SNP locus, GTFL-1 (5.46 cM) was the closest to the putative gynoecious locus at the site of scaffold44_7277273 and transformed to a traditional DNA marker using invader assay technology, which applies to the MAS of gynoecy in bitter gourd breeding (Matsumura et al. 2014).

Construction of a restriction site associated DNA (RAD) based genetic map with 1009 SNP markers spanned over 2203.95 cM with 11 linkage groups for bitter gourd using F_2 mapping population (423 individuals) derived from K44 (gynoecious) and Dali-11 (monoecious) and assembled 113 scaffolds that covered about genome size 251.32 Mb (85.48%) of the 294.01 Mb assembled genome (Cui et al. 2018).

A high-density, high-resolution genetic map was constructed for bitter gourd (*Momordica charantia*). A total of 2013 SNP markers were found, spanning over 2329.2 cM on 20 LGs and each LG ranging from 185.2 cM (LG-12) to 46.2 cM (LG-17). Economically- and horticulturally-important traits like gynoecy, sex ratio, node and days at first female flower appearance were analyzed with 22 QTLS. (Gangadhara Rao et al. 2018).

3.4.5 Draft Genome Sequence

The draft genome sequence (285.5 Mb ~ 84%) of a bitter gourd (monoecious line, OHB3-1) analyzed through Illumina sequencing and de novo assembly (Urasaki et al. 2017) and the estimated genome size of bitter gourd was 339 Mb (Urasaki et al. 2015). Syntonic mapping and phylogenetic studies revealed bitter gourd was more related to watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) than to cucumber (*Cucumis sativus* L.) or melon (*Cucumis melo* L.) (Urasaki et al. 2017). Further, the bitter gourd draft genome sequence revealed that the protein MOMC3-649 in bitter gourd was orthologous to *CmAcs11* (female flower regulation in melon) and MOMC46_189, MOMC518_1 proteins were found in bitter gourd orthologous to *CmAcs-7* (unisexual flower development in melon). This helped as a bitter gourd reference (OHB3-1 reference) and anchored 255 scaffolds to the genetic map (Urasaki et al. 2017). Whole-genome

sequencing of wild and cultivated bitter gourd germplasm revealed the estimated genome size of cv. Dali-11 (*M. charantia*) was 300 Mb, while the wild small-fruited line TR (*Momordica* sp.) was 301 Mb and OHB3-1 (*M. charantia*) was 339 Mb and comparative genome analysis among cucurbit genomes found that *Bi* major gene clusters (cucurbitane triterpenoid biosynthesis) were highly conserved in cucumber, melon and watermelon but absent in bitter gourd (Cui et al. 2020).

Reduced representation of genome sequencing technologies like RAD (restriction site associated DNA markers) (Baird et al. 2008) combined with NGS (next-generation sequencing) and RAD-seq allows an inexpensive, high density SNP marker discovery and the genotyping of a massive population of orphan crops like bitter gourd (Davey et al. 2011).

3.4.6 Transcriptome Analysis

Whole transcriptome sequencing of female and hermaphrodite flower buds of gynoecious bitter gourd (inbreed line; DBGY-201) was done using Roche 454 parallel pyrosequencing technology (Behera et al. 2016). A total of 1,994,113 expressed sequence tags (ESTs) were generated, of which 834,476 were obtained from female and 1,159,637 from hermaphrodite flower buds. Differentially expressed ESTs between the two different flower types and putative simple sequence repeat markers (total of 51 SSRs: 29 gynoecious, 22 hermaphrodite flowers) were also reported (Behera et al. 2016). However, none of these markers exhibited polymorphism. More information is needed on ESTs in bitter gourd, which will provide valuable information to understand the molecular mechanisms of sex determination, marker development for bitter gourd breeding.

3.5 Breeding for Pest and Disease Resistance and Nutrition

Pest and disease resistance breeding in bitter gourd is still at the initial screening level. Very few reports are available regarding genetic inheritance and screening. Improvement of nutritional qualities like vitamins, macro- and micronutrients, and minerals, along with medicinal properties in bitter gourd, as future targeted areas of research, are still at an infantile stage.

3.5.1 Insect Pest Resistance Breeding

The infestation level of melon fruit fly (*Dacus cucurbitae*) was significantly lower in the genotype US-6214 and Meghnad-2 (14.35 and 16.33%) (Sen et al. 2019); also, wild bitter gourd ecotypes and botanical varieties (e.g., *Mormordica*

charantia var. *muricata*). Accessions, such as IC 256185, IC 248256, IC 213311, IC 248282, IC 256110 and IC 248281 have shown resistant to fruit fly (Dhillon et al. 2005). The strain CD Green Rough was consistently the best for resistance to fruit fly and for high yield (Fernando and Udurawana 1941) and wild bitter gourds (var. *muricata*) collected from southwestern India have high fruit fly tolerance (John and Antony 2008). These may be utilized in resistance breeding programs against melon fruit fly. *Mormordica charantia* accession THMC 281 has been found resistant to melon fly (Dhillon et al. 2016c). BG 5–98 and BG 102 had the fewest pumpkin beetles (*Raphidopalpa foveicollis*), per plant and the least damage to leaves (Khaire et al. 1987). Col-II and FSD-Long were found to be resistant to fruit fly (Gogi et al. 2009). Reciprocal recurrent selection is desirable to develop fruit fly resistant varieties after studying the inheritance of fruit fly resistance (Tewatia and Dhankhar 1996).

Line IC 582449 was resistant to bitter gourd gall midge (*Lasioptera bryoniae*) resistance line (Muthukumar et al. 2019). Biochemical parameters total phenols and total sugars are the resistance-conferring factors against the gall midge in bitter gourd. *Mormordica balsamina* (MB-3, MB-5, MB-6, MB-7, Local-1) were resistant to root-knot nematode and *M. charantia* accessions PAUBG-1 and PAUBG-13 were moderately resistant (Sharma et al. 2019).

Mormordica dioica is tolerant of pumpkin caterpillar, gall fly and root-knot nematode, whereas *M. sahyadrica* is highly tolerant of pumpkin caterpillar and root-knot nematode and fruit fly. *Mormordica balsamina* is highly tolerant of most of the typical cucurbit diseases and pests like ladybird beetle, red pumpkin beetle, pumpkin caterpillar, root-knot nematode, cucurbit yellow mosaic and little leaf disease (John and Antony 2008). *Mormordica subangulata* ssp. *renigera* is highly susceptible to a majority of the pests and diseases, especially root-knot nematode, but resistant to cucurbit yellow mosaic virus and little leaf diseases (John 2005). *Mormordica foetida* and *M. rostrata*, are famous for their insect repellent properties (Bosch 2004) and have great scope in resistance breeding of *M. charantia*.

Higher trichome length and density inhibits the feeding of foliage by insect pests (Mandal et al. 2012). The presence of Momordicin I in *Mormordica charantia* leaves deterred the ovipositing of female leaf miners (Mekuria et al. 2005) and Momordicin II was deterrent to feeding of red pumpkin beetle (Chandravadana 1987). Screening of bitter gourd for high trichome density and high Momordicin I & II can reduce the pest incidence and is useful for resistance breeding programs.

3.5.2 Disease Resistance Breeding

Powdery mildew (*Podosphaera xanthii*) is a devastating fungal disease in bitter gourd, causing more than 70% yield losses. Lines IIHR-80-1-2 and IIHR-80-1-3 (*Mormordica balsamina*) were free from powdery mildew and IIHR144-1

(*M. charantia* var. *muricata*) showed mild symptoms of powdery mildew (Prasanth et al. 2019). Five bitter gourd breeding lines at the World Vegetable Centre were screened for resistance to a local isolate of *Px* In Kamphaeng Saen, Thailand (Dhillon et al. 2018); furthermore, they were screened at 12 locations in 5 countries (Thailand, Vietnam, Philippines, India, China). Bitter gourd breeding lines THMC 153 (AVBG1333) and THMC 167 (AVBG1334) showed resistance to the local race of *Px* in all locations, whereas THMC 143 (AVBG1330) was shown resistance in all locations except in China. THMC 113 (AVBG1329) showed resistance to each location except in India; THMC 170 (AVBG1335) was shown susceptible at 3 locations in India; Lines THMC 143 (AVBG1330) and THMC 167 (AVBG1334) originating from India, exhibited good yield potential in trials conducted in Thailand, Myanmar, Vietnam and Bangladesh. Dhillon et al. (2019) studied the inheritance of powdery mildew resistance (BG-CPM race Mc-1); the six generations mean analysis revealed the resistance govern by minimum two independent recessive genes and intercrosses revealed that resistance was controlled by allelic and nonallelic genes.

Downy mildew (*Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev) disease is a major constraint of bitter gourd production during the summer and rainy seasons. Even for crops grown using the bower system, downy mildew is very common in the kharif (summer) season. Lines NIC-12285 and VRBT-39 were moderately resistant with a high degree of tolerance to downy mildew (Pandey and Singh 2001). Accessions 83-006, 83-003 and 9-32 were resistant to bacterial wilt (*Pseudomonas solanacearum* Smith) (Alcazar and Gulick 1983). Lines IC 68296, IC 68335, IC 68263B, IC 68275, IC 68250A, IC 85620, IC 68285, IC 68312 and IC 68272 are resistant to bitter gourd distortion mosaic virus (BDMV). The genotype IC 68275 was the best general combiner for BDMV resistance and fruit yield per plant (Arunachalam et al. 2002).

3.5.3 Breeding for Nutritional and Medicinal Quality

Five lines (K169998, K042800, K170002, Hwanock, Nockwoo) recorded the highest content of charantin at 143.7, 104.6, 103.5, 161.4, 101.1 $\mu\text{g.g}^{-1}$, respectively (Kim et al. 2013). Breeding for nutritional/medicinal quality typically emphasizes accessions with relatively high vitamin C content. Bitter gourd lines DBTG-3, DBTG-8, DBTG-6 and IGBTG-9 recorded high (1000 mg/kg) vitamin C in edible plant parts, as compared to standard cultivated types (500 mg/kg) (Dey et al. 2006a). These high vitamin-C lines are aggressively used in breeding programs whose focus is on the development of cultivars with high nutritional value. High quality is also found in Indian bitter gourd cultivars possessing high TSS (>3.1°Brix; MC-84, Preethi, RHRBG-5) and elevated vitamin C (>950 mg/kg; Konkan Tara, Hirkani) and fruit protein (>1.5%; DVBTG-1, Preethi, Hirkani, Konkan Tara) content (Kore et al. 2003). Association analysis results indicated that breeding for increased TSS would improve the content of vitamin C while

maintaining a reasonably high protein content, but that improving the iron content would require a separate breeding effort (Ramachandran and Gopalakrishnan 1980).

For preserving by canning, cv. BG 14 was found to be the best, followed by C 96, mainly due to their superior color, texture and flavor. For dehydration, BG 14 was recommended, followed by BG 13 (Kalra et al. 1983) because they reconstituted to their original shape reasonably well. Considering physical and biochemical constituents, the most suitable varieties for processing were Coimbatore Long, Ripe Gang Gorakhpur and Karela Jhalardar (Awasthi and Jaiswal 1986). In bitter gourd, several genetic studies have shown that an association exists between morphological traits and insect resistance, and that these associations may be useful for indirect selection during resistance breeding (Dhillon et al. 2005). Lines BG 598 and BG 102 have shown resistant to pumpkin beetle. Biophysical traits of the plants play a major role in deterring foliage-feeding insect pests (Khaire et al. 1987).

3.6 Tissue Culture

Micropropagation and genetic transformation are tissue culture techniques which will play a major role as supplementary approaches to conventional breeding in bitter gourd crop improvement. Traditional breeding approaches are time consuming and laborious for the development of improved cultivars. For mass multiplication, regeneration of virus-free plants, production of secondary metabolites, maintenance of gynoecious line and to speed up the breeding program, tissue culture techniques are highly requisite. The breeding process can be accelerated by the production of double haploids (DH) (Ren et al. 2017). Gynoecious lines of bitter gourd micro-propagated through the nodal segment have a higher survival rate (81.3%) on MS basal medium containing 6 BAP (2 mg/l) + α -NAA (0.2 mg/l) than apical bud (Saha et al. 2016). Among BAP, IAA, 2,4-D in MS medium, the combination of BAP/2,4-D is best for callus induction in *Mormordica charantia* cv. Jaunpuri (Ali and Tariq 2013). The 100% callus formation was found on MS medium supplemented with 2, 6 and 8 mg/l 2,4-D in Silifke cv. of bitter gourd (Saglam 2017). The highest rate of callus formation ($93.75 \pm 2.55\%$) through anther culture was found on MS medium supplemented with 2,4-D (1.0 mg/l) and BAP (1.5 mg/l) (Nguyen et al. 2019). Recently five new cucurbitacins, kuguacins II-VI (1–5), along with five known analogs (6–10), were derived from bitter gourd fruit (Chen et al. 2015). There is a need to develop standard protocols for tissue culture morphogenetic response of explants, choice of genotypes and culture medium in bitter gourd. Conventional breeding and application of advanced biotechnological tools will pave the way for bitter gourd crop improvement.

3.7 Conclusion and Prospects

Bitter gourd is a nutritious and medicinal vegetable traditionally consumed in Asian and African countries. Genetic and molecular studies in this crop are still at a very early stage and have wide opportunities for future research. All current varieties and hybrids in bitter gourd were developed through selection from landraces and conventional breeding methods like pedigree selection, mutation and heterosis breeding. However, traditional breeding methods have low efficiency and are time-consuming. Over the last decade researchers have begun using molecular markers in bitter gourd to study the genetic diversity and to construct linkage maps. Transcriptomic resources like ESTs, multi-locus amolecular markers, construction of genetic maps, QTL analysis, next-generation sequencing (NGS) platforms, RAD-Seq, GBS technology and whole genome sequencing studies provide greater opportunities for improvement of bitter gourd.

The efficiency of developing transgenics in cucurbits is still low and there is the need to develop standard protocols for genetic transformation. CRISPR/Cas based technologies are necessary in bitter gourd to further study targeted functional genes. Emerging technologies such as optical mapping will be helpful in developing a physical map of the whole genome. There is a need to develop tissue culture protocols in bitter gourd for production of unique metabolites or functional ingredients.

Appendices

Appendix I: List of Bitter Gourd Varieties Developed in India

Variety	Breeding method	Salient features	Recommended states
Pusa Do Mausmi	Selection from local collection	The fruits are reach edible stage in about 55 days from sowing, fruits are dark green, long, medium thick, club shaped with 7–8 continuous ridges, 18 cm long at edible stage, 8–10 fruits weigh 1 kg	Delhi, Punjab, Haryana, Uttar Pradesh and Bihar
Pusa Vishesh	Selection from local collection	It is a dwarf vine variety suitable for planting in high plant density. Fruits are glossy green, medium long and thick, suitable as vegetable for pickling and dehydration	Delhi, Punjab, Haryana, Uttar Pradesh and Bihar

(continued)

Variety	Breeding method	Salient features	Recommended states
Pusa Hybrid 1	F ₁ hybrid	Fruits are medium long, medium thick, glossy-green, suitable for pickling and dehydration. Higher yielder than Pusa Vishesh and Pusa Do Mausami, first picking in 55–60 days. Suitable for growing in spring summer season. Yield 200 q/ha	Delhi
Pusa Hybrid 2	F ₁ hybrid	Fruits dark green medium long and medium thick, suitable for vegetable pickle, dehydration and export. Maturity 52 days. Average yield 180 q/ha, an increase of 40% over check (Pusa Do Mousami)	Punjab, Delhi, Haryana, Rajasthan, Gujarat, Uttranchal, Bihar, Chattisgarh, Orissa, Andhra Pradesh
Pusa Aushadhi	Back crossing and selection	Fruits of this bitter gourd variety are light green, with 7–8 continuous ridges. Average fruit length 16.5 cm and its fruits mature in 48–52 days. Average fruit weight 85 gm and yield 15–19 mt/ha. Sex ratio 3:1	Rajasthan, Gujarat, Haryana and Delhi states
Pusa Rasdar	Selection from exotic collection	It is first extra early. Average fruit weight is 110 g with an average yield of 0.5 mt/100 m ² in an insect proof net house	Suitable for cultivation in protected conditions.
Pusa Purvi	Selection	Small size (4–5 cm long and 3–4 diameter) and crispy flesh with high dry matter. Average yield is 8.78 mt/ha. It is good source of minerals (calcium, manganese, zinc, iron) and antioxidant activity	Delhi
Arka Harit	Pure line selection from IIHR-4	Fruits are spindle shaped, glossy green skin color without tubercles. Medium-sized fruits, thick flesh, moderate bitterness, with fewer seeds. Yield 12 mt/ha	Karnataka, Tamil Nadu and Kerala
Kashi Urvasi	Pedigree	This variety has been derived from the cross IC-85650B x IC44435A, having dark green and long fruits, mild projection, length 16–18 cm, fruit weight 90–110 g and yield 200–220 q/ha	Uttar Pradesh, Punjab and Jharkhand
Kalyanpur Baramasi	Open pollinated	Fruits are 20–25 cm long, thin, dark green with 8–10 seeds per fruit. Average yield is about 10–12.5 mt/ha. It is tolerant to mosaic and fruit fly	Uttar Pradesh
Co 1.	Local collection	Fruits are green, 30–35 cm long, 100–200 g each. Average yield 14 mt/ha	Tamil Nadu

(continued)

Variety	Breeding method	Salient features	Recommended states
MDU 1	Mutation γ -irradiation (MC-103)	Fruits are green and each weighs 410 g. Average yield 32 mt/ha	Tamil Nadu
COBgoH1	F_1 (MC 84 9 \times MDU 1)	It recorded an average yield of 44.4 mt/ha. crop duration of 115–120 days. Rich in momordicine (2.99 mg/g)	Tamil Nadu.
Coimbatore Green	Selection from local cultivar	fruits are 60 cm long and dark green. Individual fruit weighs of 300–400 g. It gives a yield of 15–18 mt/ha	Tamil Nadu
Coimbatore Long White	Selection from local cultivar	Fruits are long, tender, white and suitable as a rainy season crop with an average yield of 25–30 mt/ha	Tamil Nadu
VK 1 (Priya)	Selection from local cultivar	The fruits are green with white tinge at stylar end and 35–40 cm long, heavy bearing variety with first picking in 60 days. Yield potential is up to 30 mt/ha	Kerala
MC 4 (Preethi)	Selection from local cultivar	Fruits are medium, white, 30 cm long and a single fruit weighs around 310 g. The yield ranged from 15 to 34 mt/ha	Kerala
Priyanka	Selection from local cultivar	Fruits are uniformly white, spindle shaped with spiny ridges, medium long, average fruit weight is 300 g with yield potential of 28 mt/ha	Kerala
Phule Green Gold	Selection	Fruits are dark green, 25–30 cm long with prickles. Average yield 230 q/ha suitable for export market. Tolerant to downy mildew	Maharashtra
Phule Priyanka	Hybrid variety	Dark green color fruits. Fruits are 20–25 cm long with tubercles. Average yield is 35–40 mt/ha	Maharashtra
Phule Ujwala	Open pollinated	Fruits are 18–20 cm long, dark green with tubercles and produce about 30–35 mt/ha	Maharashtra
RHRBGH-1	F_1 hybrid	It is suitable for cultivation in both summer and rainy season. Fruits are tubercled with 20 cm length and dark green. The average yield of this variety is about 20 mt/ha. Tolerant to downy mildew	Maharashtra.
Hirkani	Open pollinated	Fruits are dark green, 15–20 cm long. Average yield is 13.8 mt/ha in a crop duration of 160 days	Maharashtra.

(continued)

Variety	Breeding method	Salient features	Recommended states
Pant Karela 1	Selection	The vine length is about 2 m. Fruits are thick, about 15 cm long with tapering ends. It takes about 55 days to first harvest. It is suitable for planting in the hills. The yield potential is 150 q/ha. It is highly resistant to red pumpkin beetle	Uttar Pradesh and Uttarakhand
Pant Karela 3	Selection	Early and high yielding variety, its fruits are cylindrical 24 cm long of dark-green color, and are suitable for plain and hilly areas of north India. Yield potential of this variety is 16 mt/ha	Uttar Pradesh and Uttarakhand
Konkan Tara	Selection	Fruits are green, prickly medium long (15 cm) and spindle shaped. Yield potential is 24 mt/ha	Maharashtra
Pride of Gujarat	Selection	Fruits are small, round, whitish green in color with a few white Dabson the skin. Each fruit weighs 8–10 g	Gujarat
Punjab 14	Selection and extant variety	Plants bushy and bear light green fruits with an average weight of 35 g. Yield 14 mt/ha	Punjab

Appendix II: Total Bitter Gourd Germplasm Accessions Available in Different Countries

Centre	Total Accessions	Targeted area of research	Address	Website
NBPG (National Bureau of Plant Genetic Resources)	1326	DNA fingerprinting and conservation	Pusa Campus, New Delhi 110,012, India	http://www.nbgr.ernet.in/
AVRDC (Asian Vegetable Research and Development Centre)	434	Fruit fly, powdery mildew, downy mildew, nutrients and yield traits	World Vegetable Center, P.O. Box 42, Shanhua, Tainan, Taiwan 74,151	http://avrdc.org/seed/seeds/
GRIN (Germplasm Resources Information Network)	103	Yield traits	National Genetic Resources Program, USDA, Washington, D.C. 20,250–0110	http://www.ars-grin.gov/

(continued)

Centre	Total Accessions	Targeted area of research	Address	Website
IARI (Indian Agricultural Research Institute)	-	Gynoecy, nutrients and yield traits	Pusa Campus, New Delhi 110,012, India	https://www.iari.res.in/
IIHR (Indian Institute of Horticultural Research)	48	Gynoecy, powdery mildew and yield traits	ICAR-IIHR, Hessaraghatta lake post, Bengaluru-560,089, India	https://iihr.res.in/
IIVR (Indian Institute of Vegetable Research)	320	Gynoecy and yield components	Post Bag No. 01; P. O. Jakhini (Shahanshapur) Varanasi - 221,305, Uttar Pradesh, India	https://www.iivr.org.in/
PCPGR (Pantnagar Centre for Plant Genetic Resources)	154	Yield traits	G. B. Pant University of Agriculture and Technology, Pantnagar Udhampur Singh Nagar, Uttarakhand - 263,145, India	http://www.gbpuat.ac.in/
MPKV (Mahatma Phule Krishi Vidyapeeth)	-	Yield traits	Mahatma Phule Krishi Vidyapeeth, Rahuri Ahmednagar, Maharashtra 413,722, India	http://mpkv.ac.in/
KAU (Kerala Agricultural University)	-	Yield traits	KAU Main Campus KAU P.O., Vellanikkara Thrissur, Kerala 680,656, India	http://www.kau.in/
TNAU (Tamil Nadu Agricultural University)	-	Yield traits	Lawley Road, Coimbatore, Tamil Nadu - 641,003, India	http://www.tnau.ac.in/

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Chapter 4

Breeding of Bottle Gourd (*Lagenaria siceraria* (Molina) Standl.)



A. K. M. Aminul Islam, Sumi Sarkar, and Farzana M. Era

Abstract Bottle gourd (*Lagenaria siceraria* (Molina) Standl.) is an important vegetable crop bearing health benefits and medicinal properties. Continuous increase in bottle gourd consumption, especially in the tropics and subtropics, is attributed to its purported health benefits. Successful cultivation of bottle gourd in these regions requires proper breeding strategies to develop superior genotypes with desired traits aimed at market-driven quality and high yield. For effective bottle gourd breeding, genetic diversity of this crop needs to be studied and documented. The monoecious and open-pollinated nature of bottle gourd supports conventional breeding by pure line selection, pedigree selection, recurrent selection and heterosis breeding. Besides traditional breeding, transgenic breeding, gene editing and marker-assisted selection are also needed to fully exploit the genetic resources of this crop to achieve desirable yield and quality. The main objective of this chapter is to provide an overall assessment of the importance of bottle gourd and its breeding strategies to develop high-yielding and nutritionally-rich varieties using conventional and advanced breeding tools.

Keywords Calabash · Variety · Germplasm · Hybridization · Heterosis · Nutrition · Yield

4.1 Introduction

Bottle gourd (*Lagenaria siceraria* (Molina) Standl.) is a widely cultivated perennial climbing vegetable of the Cucurbitaceae family, usually grown and used in the winter season, but now also cultivated during the summer and rainy seasons (Roopan et al. 2016). The gourd family is a medium-sized and economically-important family of around 118 genera and 825 domesticated species distributed widely in the

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warmer regions of the world. It is an excellent warm season vegetable containing all of the essential nutrients beneficial to human health (Nath et al. 2017). Fruits of bottle gourd are consumed as vegetables and the dried shells used to make bowls, containers, and musical instruments or fishing floats; whereas the tendrils, leaves, immature fruits and seeds are employed in traditional medicine (Kandasamy et al. 2019). Consumption of bottle gourd leaves, seeds and flowers are suggested to have medicinal applications to control diabetes mellitus, hypertension, and liver diseases promote weight loss and other associated benefits (Barot et al. 2018).

In Bangladesh, bottle gourd is used as a popular winter vegetable cultivated throughout the country. For optimum growth and fruiting, bottle gourd needs 20–27 °C of day and 18–23 °C of night temperatures (Quamruzzaman et al. 2019a, b). The winter season of Bangladesh is favorable for the better growth and yield of bottle gourd, although it is also being cultivated now in the summer and rainy season throughout the country, both commercially and in home gardens for family consumption. The production of bottle gourd per year in Bangladesh is around 77,352 mt from an area of 7217 ha; the average yield is 10.71 mt per ha which is very low compared to other countries (Anonymous 2017). Bottle gourd is naturally cross-pollinated and most of the local varieties and cultivars that are being used by a large number of farmers have lost their production potential (Quamruzzaman et al. 2019a, b). For that reason, a well-planned and dynamic breeding program is needed to develop high yielding varieties to meet the production demand of bottle gourd. Scientist are now recommending this underutilized crop species for integration into agricultural development policies to support cash crops and offer opportunities for diversifying food, agriculture and income for rural people (Aya et al. 2019). The main objective of this study is to explore the economic, nutritional importance and different breeding approaches of bottle gourd that may help to develop new higher-yielding bottle gourd cultivars.

4.1.1 Botany

4.1.1.1 Vegetative Phase

Bottle gourd can be grown either on a fence or bower, or on the ground, depending on the variety. There are many forms of bottle gourd cultivated for specific purposes and are named based on fruit shape such as club, dolphin, dipper, kettle and trough. Great variation is found in the size of leaves, vines, flowers and shape of fruits. Bottle gourd is an annual plant with a fast growing vines some 1–3 cm thick, deeply grooved longitudinal ridged, softly pubescent, gland-tipped hairy stems (Doloi et al. 2018). The stem is slender at a young stage but becomes angular at maturity, keeping 5–6 main lateral branches on the main branch (Ajuru and Nmom 2017). Leaves are simple, alternate, variable, large, rounded and kidney-shaped, non-lobed, hairy, bearing long forked tendrils on the leaf axils. This plant has a widely spreading root system and the roots are smooth and circular in cross section. The bulk of the root

system spreads out to a depth of 15–30 cm of the topsoil (depending on soil depth) with a taproot extending down to 80 cm. Flowers are solitary, white in color and appear on leaf axils. Calyx is lobed with 5 sepals and corolla united with 5 petals. The fruit size of some bottle gourd varieties may be more than 1 m. It is a seed propagated plant and the main vegetative growth takes place 12–15 days after seed germination.

4.1.1.2 Reproductive Phase

Bottle gourd is diploid, monoecious, highly cross-pollinated in nature and the cross-pollination ranges from 60–80% (Doloi et al. 2018). In such typical monoecious plant, male and female flowers are borne individually (unisexual) in the same plant (Fig. 4.1) and the proportion of producing male flowers is higher than female flower. The male and female sex ratio range is 5:1–15:1. Male or staminate flowers are produced on a longer pedicel than female flowers and extend beyond the foliage. Staminate flowers contain 3 stamens attached to calyx tube of which two are compound and another remains single (Desai et al. 2011). The female or pistillate flowers are produced singly on a short peduncle and contain ovate, long or cylindrical, hairy and inferior ovary with 3 carpels. Anthesis begins from around 0900 and is finished by 1900 the same day, whereas dehiscence of the anthers starts at around 1100 and is completed by 1400 h. Stigmas become receptive 24 h before anthesis and continue up to 24 h after anthesis. Flowers stay open only for a few hours and after that, the petals wither. After 24 h of anthesis, the stigma losses its shine and receptivity (Adjakidje 2006) becomes brownish and increases to about 3 mm long showing the beginning of fruit formation. The flowering and fruiting starts about 38 days after seed germination and may continue for up to 90 days. The fruit contains many seeds per fruit, called a *berry*, because of its hard and tough rind after maturity.

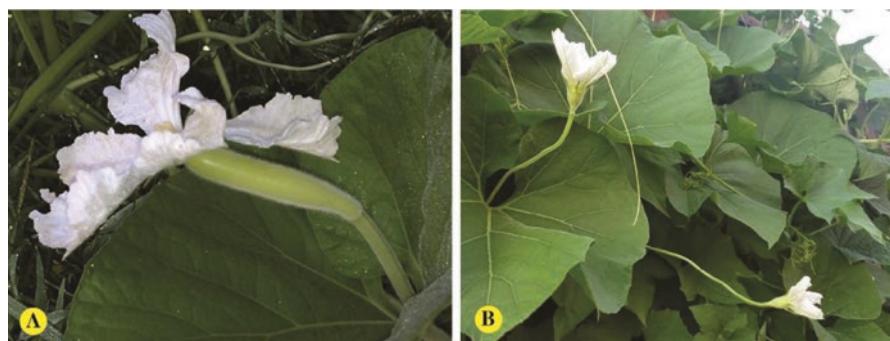


Fig. 4.1 Bottle gourd flower structures. (a) Female flower, (b) Male flower. (Photos by A. K. M. Aminul Islam)

4.1.2 Economic Importance

Bottle gourd is commonly grown as a vegetable crop in tropical and subtropical countries for human consumption. India, Sri Lanka, South Africa, Indonesia and Malaysia are the major producing countries. The total cultivated area is about 405,000 ha worldwide (Barot et al. 2018) with 7217 ha in Bangladesh. The total production in Bangladesh was around 77,352 mt in 2017–2018 (Anonymous 2017). Fruits of bottle gourd can be used as a vegetable and also for making sweets and pickles. It is easily digested as a vegetable even by recovering patients. Young fruits are cooked as a vegetable, young shoots and leaves can be cooked, seeds can be used in soups and edible seed oil production; flesh of immature fruits can also be used in making icing for cakes, and the hard skin is sometimes sliced into thin, dry strips for cooking (McCreight 2016). The dry hard shells of the fruits are used to make a wide range of articles of common use including bottles, bowls, containers, ladles, floats for fishing nets, pipes and musical instruments. Besides its food use, bottle gourd is also utilized in the production of biodiesel (Sokoto et al. 2013). Bottle gourd has great export potential as well. For export, the fruits should be, straight-shaped, light green to dark green colored with a length of 30–100 cm and have good packing qualities in cardboard boxes (Ajuru and Nmom 2017).

4.1.3 Nutritional Importance

4.1.3.1 Health Benefits

Bottle gourd is a valuable source of proteins, carbohydrates, vitamins and minerals (Table 4.1), and acts as one of the most nutritive menu items said to tone up the energy and vigor of humans. Minerals like calcium, phosphorus, iron, potassium,

Table 4.1 Nutritional composition of bottle gourd

Nutritional value	Value per 100 g	Nutritional value	Value per 100 g
Water	95.54 g	Fiber	0.5 g
Carbohydrate	3.39 g	Total fat (Lipid)	0.02 g
Protein	0.62 g	Iron	0.20 mg
Calcium	26 g	Phosphorus	13 mg
Potassium	150 mg	Sodium	2 mg
Magnesium	11 mg	Thiamin	0.029 mg
Zinc	0.70 mg	Riboflavin	0.022 mg
Foliate	6 µg	Vitamin A	16 IU
Niacin	320 mg	Vitamin B6	0.040 mg
Energy	14 Kcal	Vitamin C	10.1 mg

Source: Adapted from Mishra et al. (2019); Singh and Singh (2019a, b)

sodium and iodine are reported to be present in fair amounts in bottle gourd fruit. They are also a good source of essential amino acids such as leucine, cysteine, valine, phenylalanine, aspartic acid, proline and threonine, along with a good source of vitamin B (particularly thiamine, riboflavin, niacin, ascorbic acid) and finally pectin which shows good prospects for preparing jelly (Mishra et al. 2019). The seed extract of bottle gourd contains antibiotics properties and the fruit juice is reported to be helpful against premature graying hair, constipation, urinary disorders and insomnia (Janaranjani et al. 2016). Bottle gourd consumption is associated with a number of traditional health benefits such as antihyperlipidemic activity (Gorasiya et al. 2011); analgesic and anti-inflammatory activity (Shah and Seth 2010); diuretic activity (Parle and Kaur 2011); antioxidant activity (Saha et al. 2011); immunomodulatory activity; hepatoprotective activity; antidiabetic activity; cardioprotective activity; central nervous system (CNS) activity; anti-cancer activity; hypertensive activity and CNS depressant activity (Akter et al. 2019; Bhat et al. 2017). Bottle gourd extract contains a high content of flavonoids and flavanols which may increase its antioxidant potential (Agrawal and Katare 2015).

4.1.3.2 Pharmacological Properties

The bottle gourd leaf is used to make a decoction that purportedly acts as a medicine against jaundice. The fruit of bottle gourd has a cooling, cardiotonic and diuretic effect which is good for people suffering from indigestion, biliousness and in convalescence (Maqsood et al. 2017). The fruit pulp may help to overcome constipation, cough and night blindness and act as an antidote against certain poisons (Tyagi et al. 2017). The plant extract is used as a cathartic; seeds and seed oil are used in dropsy (Thamburaj and Singh 2005). This crop contains high levels of choline which has been reported to heal mental disorders (Singh and Singh 2019a, b). The ethanolic fruit extract of bottle gourd is reported to play a prominent role to prevent almost all the enzymes of isoproterenol-induced heart failure significantly, cure mitochondrial damage and improve myocardial injury (Fig. 4.2) (Roopan et al. 2016).

Bottle gourd juice has an alleged therapeutic role in the treatment of diabetes and dyslipidaemia (Charu et al. 2013). The fruit has also anti-atherosclerotic and anti-thrombotic activities (Rajput et al. 2011). It contains ethnomedicine which provides cardiotonic, anti-hyperlipidemic hepatotonic, anti-hyperglycemic and fat-lowering properties and possesses fibrinolytic qualities (Katare et al. 2014). It is also used in the treatment of pain, ulcers, pectoral cough, asthma, fever and other bronchial disorders. Tender fruits are used to prepare a syrup said to have protective effects in myocardial infarction (Ajuru and Nmom 2017).

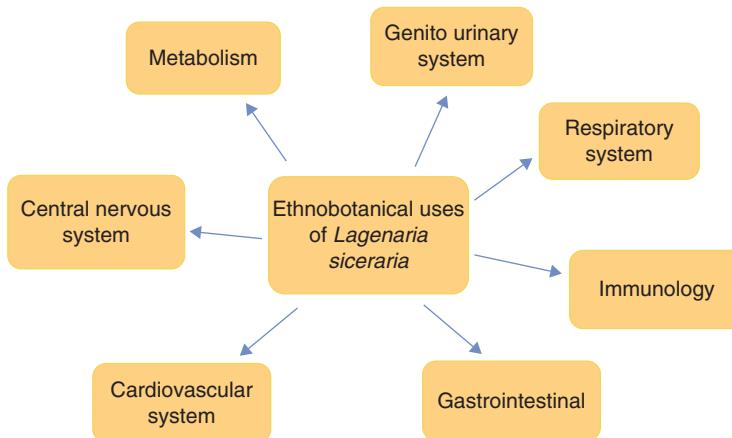


Fig. 4.2 Ethnobotanical application of bottle gourd. (Source: adapted from Roopan et al. (2016); <https://link.springer.com/article/10.1007/s00253-015-7190-0/figures/5>)

4.2 Genetic Diversity and Conservation

4.2.1 Genetic Diversity

Bottle gourd is indigenous to Africa, dating back as much as 10,000 years. From its center of origin it has been dispersed to the temperate and tropical areas of Asia and the Americas by human agency or by flotation of wild species across the seas (Fig. 4.3) (Chomicki et al. 2020; Clarke et al. 2006). Fruit of wild species are known to float in seawater for several months without loss of seed viability. The genus *Lagenaria* includes six species that are distributed in Africa, Madagascar, Indo-Malaysia and the Neotropics. Among these, only one species *Lagenaria siceraria* (Mol.) Standl (Syn. *L. vulgaris* Ser. and *L. leucantha* Duch) is cultivated, whereas the other five *Lagenaria abyssinica* (Hook. fil.) C. Jeffrey, *Lagenaria breviflora* (Benth.) Roberty, *Lagenaria sphaerica* (Sond.) Naudin, *Lagenaria rufa* (Gilg) C. Jeffrey and *Lagenaria guineensis* (G. Don) C. Jeffrey are wild, dioecious and distributed in East Africa and Madagascar (Dhatt and Khosa 2015). There are two subspecies of *Lagenaria siceraria*, ssp. *siceraria* known as African and American landraces, and ssp. *asiatica* known as an Asian landrace (Kistler et al. 2014). These are morphologically distinct from each other. It was believed that bottle gourd arrived in the Americas from Asia rather than from Africa; the Asian type of bottle gourd has been present in Europe for a long time (Emina et al. 2012; Erickson et al. 2005). Fruit size, seed size, fruit shape and seed shape are the major morphological traits which explain most of the variation in bottle gourd through principal component analysis (Emina et al. 2012). *Lagenaria siceraria* germplasm exhibits more diversity than its wild relatives (*L. abyssinica*, *L. sphaerica*, *L. breviflora*) for qualitative and quantitative traits (Dhatt and Khosa 2015).

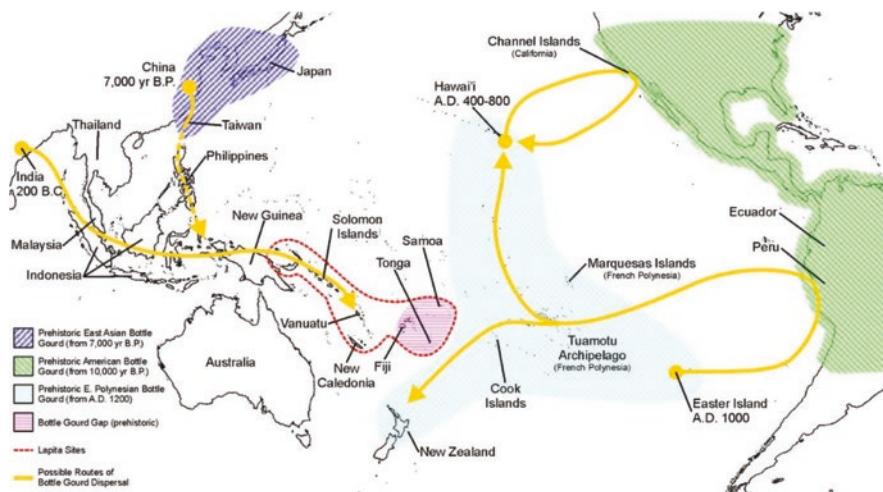


Fig. 4.3 Prehistoric distribution and dispersal of the bottle gourd (*Lagenaria siceraria*) in Asia, the Americas and Oceania. Adapted from Clarke et al. (2006)

In Bangladesh, farmers grow various types of bottle gourd with different local names such as *Lau*, *Panilau*, *Khetlau* and *Kadu*. Broad information of the nature of genetic divergence of bottle gourd genotypes found and introduced to the country is a prerequisite for a variety development program (Quamruzzaman 2020). It is necessary to select suitable parental lines from available indigenous germplasms for a successful breeding program (Fuller et al. 2010; Kistler et al. 2014).

Considerable variation is exhibited among bottle gourd genotypes due to morphological characters such as number of branches per plant, length of main shoot, fruit bearing capacity, fruit length, number of seeds per fruit, seed length, seed weight, seed shape and seed color (Achigan-Dako et al. 2008).

4.2.2 Germplasm Conservation

For effective breeding and conservation of bottle gourd germplasm it is necessary to have knowledge of the genetic diversity which exist among available bottle gourd lines (Mashilo et al. 2017). The major objectives of germplasm conservation of bottle gourd is to collect, characterize and conserve genotypes to study the diversity, identify the most useful variables for discrimination among genotypes, the genetic variations among and within collected samples using progeny test, and correlation among the traits (Mladenovic et al. 2012). Local African farmers maintain a large number of bottle gourd cultivars. Hundreds of Kenyan bottle gourd landraces were collected and are maintained in The International Plant Genetic Resources Institute

(IPGRI) and the Kenya Resource Centre for Indigenous Knowledge (KENRIK), National Museums of Kenya (Morimoto and Mvere 2004). Accessions of bottle gourd are available as well in gene banks in Benin, Cameroon, Ethiopia, Ghana, Kenya, Nigeria, Senegal, South Africa, Sudan, Tanzania, Zambia and Zimbabwe. A bottle gourd germplasm collection in Turkey has great morphological diversity exhibited among their accessions (Yetisir et al. 2012). In Serbia, diversity in the collected bottle gourd germplasm is a result of its adaptation to diverse ecological conditions and farmers' selection according to their preference and ethnobotanical utilization (Mladenovic et al. 2012). The Bangladesh Agricultural Research Institute has an active ex situ conservation collection of bottle gourd germplasm and a base collection in gene bank storage and in a field gene bank; The Bangladesh Agricultural Development Corporation (BADC) provides seed processing and short-term storing and also includes field gene bank.

Many other institution such as the National Bureau of Plant Genetic Resources (NBPGR), India (https://www.bioversityinternational.org/fileadmin/bioversity/publications/Web_version/174/ch18.htm); the Southern African Developing Countries Plant Genetic Resources Centre (SPGRC); the National Plant Genetic Resources Laboratory (NPGRL), Philippines (https://www.genesys-pgr.org/partners/aaa02346-762a_4798-a306f77553479df1#:~:text=The%20National%20Plant%20Genetic%20Resources,the%20wild%20and%20weedy%20relatives) and the International Plant Genetic Resources Institute (IPGRI), Rome, Italy (<http://www.isaaa.org/Kc/cropbiotechupdate/features/ipgri.htm#:~:text=Its%20headquarters%20are%20in%20Maccarese,productive%2C%20resilient%20and%20sustainable%20harvests.>), have ex situ collections of bottle gourd germplasm. The National Bureau of Plant Genetic Resources (NBPGR) holds a significant number of accessions (around 739) of bottle gourd collected from different regions of the world; 329 accessions are held by the World Vegetable Center (WorldVeg), Taiwan, (<https://avrdc.org/about-avrdc/about-us/>) whereas only 9 are available and the majority (97%) found inactive. A total of 500 accessions were listed by GRIN of *Lagenaria siceraria* from 23 countries in 2016. Among them, 185 (37%) were available for distribution; 51% were not available. Africa accounted for 233 of the GRIN accessions. In the Institute for Field Crops, Serbia, ex situ conservation of *L. siceraria* germplasm, African, New World and Asian landraces was developed to lead future studies and breeding of bottle gourd (Mladenovic et al. 2011). The National Gene Bank of Turkey (AARI) maintains an ex situ collection of 2223 accessions of Cucurbitaceae species and among them around 7.7% are of bottle gourd (Sari et al. 2008).

In vitro conservation is a kind of ex situ conservation through which plant parts can be conserved and used for regeneration purpose. Although in vitro conservation of bottle gourd is difficult because it is a seed propagated plant, cotyledons and other plant parts can be regenerated and preserved as in vitro collections (Han et al. 2005).

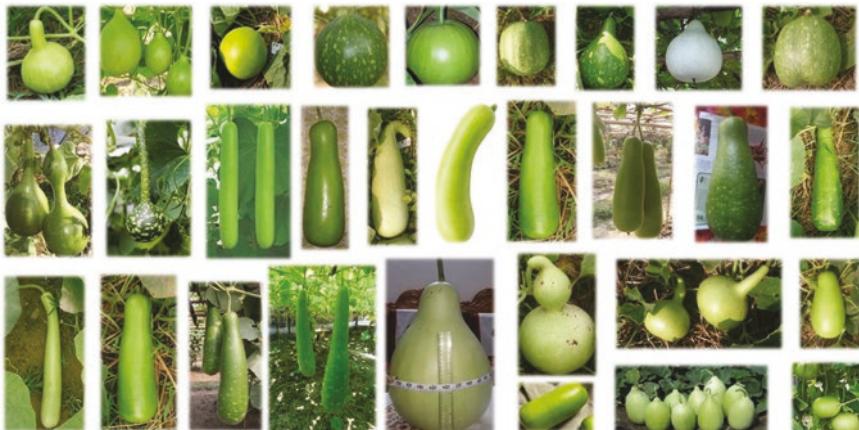


Fig. 4.4 Various fruit shape and size of bottle gourd landraces, varieties and cultivars. (Photo credit: A. K. M. Aminul Islam)

4.3 Varieties and Cultivars

The species *Lagenaria siceraria* of bottle gourd provides the largest variation in plant fruit shape, resulting from thousands of years of selection in isolated areas of the world (Yetişir et al. 2008). Bottle gourds have several different fruit shapes; for example, elongate, long and cylindrical, curved, crooked necked, pyriform and globular (Fig. 4.4).

The fruit shape has also undergone change with domestication. There are several varieties and cultivars of bottle gourd having diverse fruit shape and size (Table 4.2).

4.4 Cultivation Practices

4.4.1 Land Preparation and Sowing

Land is prepared by ploughing to a fine tilth and making furrows at 2–3 m distance. Seeds are sown in furrows at a distance apart of 1–1.5 m after incorporating farm-yard manure in the soil. Seed can either be sown directly or germinated in raised nursery beds and then transplanted, depending on weather conditions. Bottle gourd is usually planted on mounds during the rainy seasons, and in depressions during the dry season. Transplanting of seedlings are done with inter-row spacing of 1.5–2 m and intra-row spacing of 1–2 m. A spacing of 3 x 1 m is followed when bottle gourds are trained on a bower (www.eagri.org). On sloping land, seeds are sown in pits with 2–3 plants per pit. Before sowing, seeds should be soaked in water for 12 h to improve germination. The recommended seeding rate is 3–6 kg per ha.

Table 4.2 Different varieties and cultivars of bottle gourd *Lagenaria siceraria*

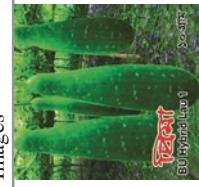
Images	Cultivar name	Botanical name	Characteristics	Source
Bottle gourd varieties developed by Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh				
	BARI Lau-1	<i>L. siceraria</i>	<ul style="list-style-type: none"> Fruits are long, slender, bottle shaped Each fruit is about 1.5 kg Cultivate all year 	http://baritechnology.org/en/home/commodity_detail_pop/670
	BARI Lau-2	<i>L. siceraria</i>	<ul style="list-style-type: none"> Fruit shape is round with square end Average fruit weight 1.5 kg High yielding than other varieties Exportable due to attractive shape 	http://baritechnology.org/en/home/commodity_detail_pop/670
	BARI Lau-3	<i>L. siceraria</i>	<ul style="list-style-type: none"> Variety for both early and main season Attractive green color with white spots Average fruit weight 2.7 kg 	Http://baritechnology.org/en/home/commodity_detail_pop/670

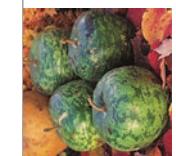
	BARI Lau-4	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Temperature tolerant variety • Cultivated in summer season • Average fruit weight 2.5 kg 	Http://baritechnology.org/en/home/commodity_detail_pop/670
	BARI Lau-5	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Deep green, bottle shape, long (42–44 cm) fruit with slight narrow neck • Average fruit weight 1.9–2 kg 	Http://baritechnology.org/en/home/commodity_detail_pop/670
	BARI Shita Lau-1	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Perennial in nature, root bulb and 4-winged variety • Leaf is parabola shaped • Average fruit weight 750 g • This variety become edible 30 days after seeding 	http://baritechnology.org/en/home/commodity_detail_pop/670
	BU Lau 1	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Fruit is pale green colored with prominent bottleneck • Early fruiting variety with less vegetative growth • Average weight of fruit 1 kg 	https://bsmrau.edu.bd/dres/varieties-released

Bottle gourd varieties developed by Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh

(continued)

Table 4.2 (continued)

Images	Cultivar name	Botanical name	Characteristics	Source
	BU hybrid Lau 1	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Long bottle shape fruit • Less vegetative growth • Low male female ratio • Average fruit weight 3 kg 	https://bsmrau.edu.bd/dres/varieties-released
Varieties in India				
	Arka Bahar	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Fruits medium long, straight without a crookneck • Light green shining fruit skin • Tolerant to blossom end rot 	https://iibr.res.in/bottle-gourd-arka-bahar-1
	Pusa Naveen	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Cylindrical and straight fruits • Free from crooked neck. • Average weight 550 g 	https://www.plantmojo.com/seeds-bulbs/bottle-gourd-pusa-naveen http://eagri.org/eagri50/HORT281/pdf/lec10.pdf
	Punjab Komal	<i>L. siceraria</i>	<ul style="list-style-type: none"> • Early maturing variety • Medium sized light green colored fruit • Tolerant to CMV (cucumber mosaic virus) 	https://www.apnkhett.com/en/pn/agriculture/horticulture/vegetable-crops/bottle-gourd#TYPESOFVARIETIES https://www.indiamart.com/proddetail/bottlegourd-punjab-komal-seeds-21031349233.html

	Bottle gourd round	<i>L. siceraria</i>	<ul style="list-style-type: none"> Plant is a creeper type Fruits become ready to harvest within 75 days Plant needs trellis to grow 	https://www.ankurnursery.com/store/S-VS-024/bottle-gourd-round-seeds
	Calabash	<i>L. siceraria</i>	<ul style="list-style-type: none"> Fruits can be huge and rounded, small and bottle shaped, or slim and serpentine, They have a crooked neck Can grow to be over 1 m long 	https://artsandculture.google.com/exhibit/african-calabash-collection/bwIC1-GIC08IJw
	Ornamental types			
	Apple	<i>L. siceraria</i>	<ul style="list-style-type: none"> Not an edible variety This cultivar is ideal for arts and crafts 	File:///G:/ALL%20PAPERS/other%20Papers%20For%20Publish/breeding%20bottle%20gourd/varieties/1.Html
	Extra long handle dipper	<i>L. siceraria</i>	<ul style="list-style-type: none"> Produces elegant curved handles Handles grow straight downwards Useful for making attractive decorations Not edible 	File:///G:/ALL%20PAPERS/other%20Papers%20For%20Publish/breeding%20bottle%20gourd/varieties/1.Html

(continued)

Table 4.2 (continued)

Images	Cultivar name	Botanical name	Characteristics	Source
	Speckled swan	<i>L. siceraria</i>	<ul style="list-style-type: none"> Grassy green fruits with creamy white specks The large fruit grows usually with a flat base It got its name from the sweeping 30–40 cm neck that curves elegantly downwards Used for crafts and decorations, not edible, retains its color when dried 	File:///G:/ALL%20PAPERS/other%20Papers%20For%20Publish/breeding%20bottle%20gourd/varieties1.Html
	Narendra Shivani	<i>L. siceraria</i>	<ul style="list-style-type: none"> Average weight of each fruit 10–15 kg Length of fruit 150–200 cm whereas normal bottle gourd fruit height is 30–60 cm It is edible when fruit length become 90 cm Also used to make musical instruments 	https://www.thebetterindia.com/132392/narendra-shivani-bottle-gourd-developed-by-prof-sheo-pujan-singh/

4.4.2 Fertilizer Management and Irrigation

It is difficult to suggest specific recommendations of fertilizer application per hectare to obtain maximum yield and reduce cost associated with production of bottle gourd. However, the Institute of Vegetable Research of India Council of Agricultural Research recommended fertilizer dose at a rate of 50–100 kg N, 40–60 kg P₂O₅, 30–60 kg K₂O /ha for bottle gourd, but amounts may vary from soil to soil and can be affected by climate conditions. Bottle gourd requires frequent irrigation because high humidity is needed for prolific fruit bearing. Little attention is required when growing calabash gourd under normal rainy season conditions because watering is necessary every 3–4 weeks during hot and humid weather (Sivaraj and Pandravada 2005).

4.4.3 Intercultural Operations

4.4.3.1 Training and Pruning

Proper training and pruning are advantageous for bottle gourd for it to attain good vegetative growth. Training of plants on a bower is an efficient practice to trap sunlight more effectively which helps to obtain high yield (Teppner 2004). Axillary buds of growing vines should be removed until vines reach the height of the bower. Apical buds are removed when vines reach 10–15 cm below the bower to allow 2 or 3 branches to spread on the bower. Vines are again pruned allowing 2–3 axillary buds to grow on primary vines after formation of 4–5 fruits. All the yellow and pale colored older leaves near bottom portion should also be removed.

4.4.3.2 Cutting

A new cutting technology is being practiced in bottle gourd to increase the number of female flower and yield. This technique is called the 3G technique (www.krishijagran.com). In general, from one vine of bottle gourd around 50–150 gourds are produced, whereas it is possible to produce up to 800 gourds in one vine using the 3G technique. This is a very simple and effective technique to increase the number of female flower in bottle gourd plant. In this technique the tip of second vine growing from main vine is pinched to enhance the emergence of a third vine as all the flowers produced by the third vine are female.

Table 4.3 Cultural and chemical control against diseases of bottle gourd

Disease name	Symptoms	Chemical control	Cultural control
Powdery mildew	Powdery whitish superficial growth on growing parts	Spraying 1 ml L ⁻¹ Dinocap or 0.5 g L ⁻¹ Carbendazim	Proper air circulation and aeration of soil before sowing, humidity level should be kept in check, removal of diseased plant and weeds, using clean tools, use of virus free seed, use of resistant varieties
Downy mildew	Owing to presence of moisture, the corresponding lower surface of affected plant leaves have purplish growth	2 g L ⁻¹ Mancozeb or Chlorothalonil twice at 10 day intervals	
Cucumber mosaic virus	Foliage covered in distinctive yellow mosaic leaves curl downward	Vector control by systematic insecticides like 0.1% Metasystox	
Cucumber green mottle mosaic virus	Mottling and mosaic on leaves, mottling and distortion of fruit	Vector control by systematic insecticides	

Source: Modified from Sultana and Ghaffar (2010); <https://gardeningtips.in/>

4.4.4 Disease Management

Common diseases in bottle gourd are anthracnose, powdery mildew, damping off, *Cercospora* leaf spot and downy mildew, and viral diseases such as cucumber mosaic virus, cucumber green mottle mosaic virus, papaya ringspot virus, watermelon mosaic virus, clover yellow vein virus and chlorotic curly stunt. Anthracnose mainly occurs during the wet season and powdery mildew during the dry season. Both cultural and chemical controls can help to overcome these diseases (Table 4.3).

4.4.5 Pest Management

Insects infecting bottle gourd are fruit fly, fruit and shoot borer, leaf folder, yellow beetle, spider mites and aphids, beetles and caterpillars (Dhatt and Khosa 2015). Aphids suck juice from bottle gourd plants. Releasing ladybird beetle in the bottle gourd field is a control measure from aphid infestation. Use of insecticidal soap or neem oil can also control aphids. To avoid infestations of fruit fly the fruits can be covered with polythene or paper bags and infected plants removed from the field. Integrated pest management (IPM) strategies can also be utilized to control insect infestation in bottle gourd. Some pesticides have been recommended against bottle gourd insect pests (Table 4.4).

Table 4.4 Recommended dose of pesticides by CIB and RC (Central Insecticidal Board and Registration Committee) against insect pests of bottle gourd

Pests/Pesticides	Dosage/ha			Waiting period (days)
	Active ingredient (g)	Formulation (g)	Dilution (Liter)	
Fruit fly				
Cyantraniliprole 10.26% OD	90	900	500	5
Red pumpkin beetles				
Cyantraniliprole 10.26% OD (oil dispersion)	90	900	500	5
Dichlorovos 76% EC (emulsifiable concentrate)	500	627	500–1000	–
Trichlorphon 5% GR (granules)	500	–	–	–
Trichlorphon 5% dust	500	–	–	–
Trichlorphon 5% EC (emulsifiable concentrate)	500	–	–	–
Red spider mite				
Dicofol 18.5% EC (emulsifiable concentrate)	250–500	1350–2700	500–1000	15–20

Source: Adapted from Dhatt and Khosa (2015); Kousik et al. (2008); <http://ppqs.gov.in/sites/default/files/bottle-gourd-ipm-for-export.pdf>

4.5 Genetics of Yield and Quality Traits

In the plant kingdom, bottle gourd is one of the most interesting species due to its diversity of fruit shapes, sizes and ways of use. The inheritance of bottle gourd fruit color is monogenic. A single pair of genes (Bi, Bt) govern the bitterness of fruits. The shape is controlled by two major genes. The cultivars having a long fruit contain AA genotypes whereas round-fruited cultivars have aa with partial dominance (<https://www.slideshare.net/firdosvani/breeding-of-cucurbits>). In a monogenic recessive character andromonoecious sex form is reported (Prakash et al. 2017).

Number of branches per plant, length of main shoot, time to maturity (earliness), number of seed per fruit, seed size, 100 seed weight, days to opening of first female flower and yield are polygenic traits. Fruit yield is controlled by mainly dominant alleles and most of the economic traits exposed equal importance of additive and nonadditive gene effects. Mladenovic et al. (2013) investigated the pattern of inheritance and genes responsible for fruit skin color and warty phenotype segregation. Genetic analysis of two parental lines, LAG 70 (with warty fruit of light green color) and LAG 71 (variegated and smooth fruit), F₁, F₂ and backcrosses populations, indicated that monogenic inheritance is responsible for warty fruit type, whereas the trait warty fruit type is dominant (*Wt*) over the non-warty fruit type (*wt*) and recessive epistasis controlled the mode of inheritance of fruit color (Mladenovic et al. 2012). The inheritance of fruit weight of bottle gourd is not only due to direct nuclear gene effects but also to maternal effects caused by cytoplasmic factors and their interactions (Yao et al. 2015). Amangouav et al. (2018) used calabash type

bottle gourd as a female parent showing a maternal inheritance in traits like seed yield and its components through the expression of heterosis and the degree of dominance. In order to improve seed yield and yield components of *Lagenaria siceraria* breeders can use a calabash type like female parent (Singh et al. 2012).

4.6 Breeding Objectives

The breeding objectives of bottle gourd depend mainly on key production problems and consumer preferences. The main breeding objective is to develop high-yielding and early varieties. Several breeding approaches are used to develop high yielding varieties of bottle gourd as follows.

The major objective of bottle gourd breeding is to get higher yield. Other objectives may include earliness of first pistillate flower at an early node number, a low male and female sex ratio, well branched and vigorous plants, higher fruit number, fruit qualities such as: a) uniformly long, cylindrical, medium sized and without a neck fruit or spherical fruit, b) immature fruits with pubescent hairs and glossy green surface at market level, c) fruits should not become fibrous and have bitterness at edible stage, d) the fruits should have a lesser number of immature soft seeds at edible stage and e) fruits which are attractive, retain green color and have resistance to diseases and insect pests.

In the tropical zones, the major threats for vegetable production are the changing patterns of climatic parameters such as atmospheric temperature rise, changes in precipitation patterns and the higher incidence of extreme weather events like droughts and floods. On the other hand, low temperature can also be a threat to bottle gourd production since it is generally grown in the tropical areas as a summer vegetable. Therefore, other objectives of breeding bottle gourd are adaptation to all temperatures, day length and tolerance to drought and flood.

4.7 Conventional Breeding

Conventional breeding methods such as pure line selection, pedigree selection, recurrent selection or progeny testing and reciprocal recurrent selection methods can be followed to improve or develop new varieties of bottle gourd. As bottle gourd is a monoecious and open-pollinated (OP) crop, there is a considerable scope to develop hybrid varieties, OP varieties, hybrid seed production and exploitation of heterosis (Singh and Singh 2019a, b).

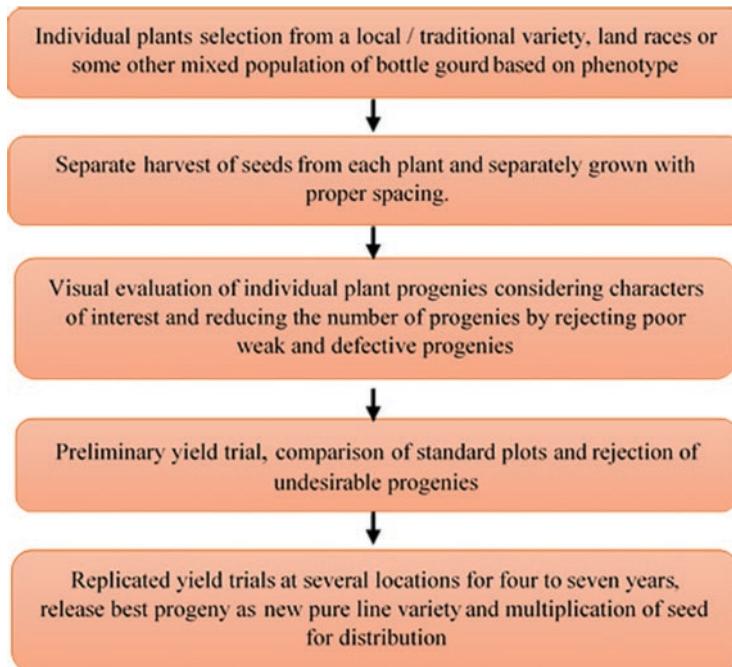


Fig. 4.5 Pure line selection of bottle gourd

4.7.1 *Pure Line Selection*

Pure line selection is a simple and rapid selection method of crop improvement (Fig. 4.5). In case of bottle gourd, inbreeding in landraces followed by pure line selection or individual plant selection can be used to develop improved cultivars from heterogeneous local types (www.biologydiscussion.com). In pure line selection, lines of crop can be fixed genetically and yield trials can be conducted immediately. Plants of those varieties developed through pure line selection perform uniformly in different environmental conditions and maximum improvement of the existing variety is possible through this method (Koundinya 2017).

4.7.2 *Pedigree Selection*

Two parental lines of bottle gourd can be crossed and the pedigree method then used to breed bottle gourd (Singh 2005) (Fig. 4.6). Pedigree selection is still the main conventional method of bottle gourd breeding; hybrid and population improvement methods are also used. Inbred lines developed in bottle gourd are nearly homozygous due to long inbreeding through self-pollination or sib-mating and these lines can be used in genetic research such as mapping genes and QTLs (quantitative trait

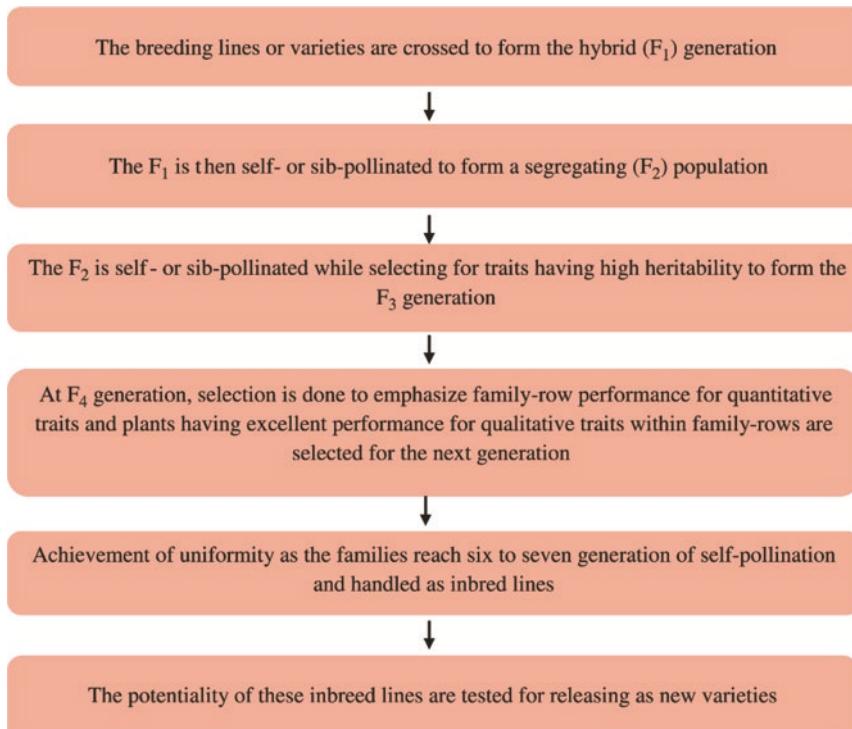


Fig. 4.6 Pedigree selection of bottle gourd

loci), allele discovery or as parents of hybrids and synthetic cultivars (Dias and Ortiz 2019). In this method two or more parental lines are crossed which complement each other, such as one parent is generally good (yield, earliness, type) excluding disease resistance and the other is generally good (yield, earliness, type) except for fruit quality.

4.7.3 *Heterosis Breeding*

Heterosis breeding has been utilized commercially in both public and private sector research and a number of popular hybrids of different crops have been developed (Singh and Nath 2011). One of the main achievements in vegetable breeding is the development of hybrid cultivars based on the exploitation of heterosis which is much easier in bottle gourd as a cross-pollinated crop (Dias and Ortiz 2019). For exploitation of heterosis, it is a prerequisite to have a significant amount of nonadditive gene action. Quamruzzaman et al. (2019a, b) analyzed the genetic components of variation and suggested that additive components are more important in the inheritance of fruit yield of bottle gourd, and this character was controlled by two to three pairs of genes or group of genes.

If heterosis is high for an economic trait like fruit yield in a specific cross of a crop, it ensures that the pollination system of that crop permits commercial hybrid seed production or there exists a male sterility-fertility system (Singh and Singh 2019a, b). There is an opportunity to apply heterosis breeding in bottle gourd as it has monoecious flowers, high seed number per fruit and requires a lesser number of plants per unit area. The objective of breeding is not only to obtain high heterosis but also to attain a high level of production within the shortest possible time, which can be exploited for early maturity, uniform size and shape of fruit, longer harvesting span and resistance to biotic and abiotic stresses (Yadav et al. 2010). Heterotic effects have been recorded in several studies for substantial increase in yield and earliness (Kumar et al. 2007; Ojha et al. 2009). Singh and Singh (2019a, b) did diallel cross analysis to determine the genetic architecture and heterosis of yield and yield-contributing traits. Maximum heterotic response for fruit yield per plant was detected in the cross between two diverse Indian bottle gourd parents/varieties-Pusa Naveen x NDBG-140 and its reciprocal cross, NDBG-140 x Pusa Naveen, provided the maximum heterosis over the standard variety. In another study, half-diallel crossed 8 parents conducted by (Malviya et al. 2017) to develop 28 F₁ hybrids where desirable standard heterosis for fruit yield and yield contributing characters found for the crosses of diverse Indian lines Pusa Naveen × JBOGL01–6, Coimbatore-3 × JBOGL-01-6 and JBOGL-01-6 × JBOGL-01-2.

4.7.4 Hybridization and Hybrid Seed Production

Hybridization is mainly done to create a variable population from which to select plants with desired characters, combining them into a single individual in order to exploit and utilize the hybrid varieties and hybrid vigor (Singh et al. 2006). For the exploitation of hybrid vigor in bottle gourd, the considerable existing genetic diversity can be utilized. Hybridization becomes easy and convenient in bottle gourd due to the size and monoecious character of the flowers (Doloi et al. 2018). To satisfy the interest and demand of producers and consumers, hybrid varieties may play a vital role. In order to achieve commercial cultivation of hybrids, it is very important to identify and utilize the most heterotic and useful crosses of the parental lines (Janaranjani et al. 2015). F₁ hybrid development in bottle gourd hybrid seed production can be simplified by manipulating the sex mechanism and sex expressions through different techniques such as use of gynoecious line, hand pollination, use of male sterile lines and use of plant growth regulators or other chemicals to suppress male flowers and open pollination (Kumar et al. 2014). Special consideration is given to standardize the techniques of suppressing male flowers of bottle gourd for hybrid seed production. Application of auxin like 1-naphthaleneacetic acid suppresses early flowering and accelerates the bearing of female flowers and increasing their over-all proportion (Heslop-Harrison 2008).

- (a) **Hand pollination:** Hand emasculation and natural or a hand-pollination mechanism are used in hybrid seed production of bottle gourd. In this method all the male flowers are pinched and the selected male parent undergoes either natural cross pollination or manual pollination. The male and female parents are grown in alternate rows and the planting ratio varies for different crops such as 4:1 in bottle gourd and muskmelon whereas 3:1 in cucumber summer squash (Heslop-Harrison 2008). Fruit set on female lines are hybrid and are harvested for the extraction of F₁ seeds. But hand pollination is a laborious, skill-requiring technique and can be applied only for a limited scale of production.
- (b) **Use of male sterile lines:** Many types of male sterility have been identified in cucurbits. Genetic male sterility (GMS) is used mainly in muskmelon and other cucurbits; most genetic male-sterile mutants are monogenic recessive meaning they are controlled by a single recessive gene (msms). The male sterile line is used as a female parent in hybrid seed production. Genetic male sterile lines are maintained in heterozygous form, which is why it is necessary to remove 50% fertile plants at flowering stage and the remaining 50% carrying non-dehiscent empty anther retained in female rows. The seed parent or female parent should be sown at double the normal seed rate in order to maintain a good plant population in female rows (Heslop-Harrison 2008). Male sterile lines can be used for hybrid seed production of bottle gourd, but commercial exploitation of male sterile lines is still lacking because this type of male sterile line has not yet been developed for bottle gourd (Brar et al. 2019).
- (c) **Chemical sex expression:** Chemicals can also be used for hybrid seed production in bottle gourd. To suppress the staminate flowers and initiate the pistillate flowers in the first few nodes of the female parent, 200–300 ppm 2-chloroethyl phosphonic acid (ethrel) is applied at the 2 and 4 leaf stage of the bottle gourd plant (Heslop-Harrison 2008). The male parent row is grown by the side of female parent and allows natural pollination. At the initial nodes, 4 to 5 fruit are sufficient for hybrid seed set. Higher concentration (400–500 ppm) of ethrel can be applied twice for the suppression of male flowers completely, making hybrid seed production comparatively easier (Brar et al. 2019).

4.8 Mutation Breeding

Mutation breeding in combination with conventional breeding can result in mutant varieties and improved phenotype isolation of vegetable crops with new and desirable variations of agronomical traits. The major purpose of mutation breeding is to cause maximum genomic variation with a minimum reduction in viability (Gupta 2019). For the improvement of a vegetable crop like bottle gourd, induced mutation can help to increase the crop genetic diversity along with the maintenance of its biodiversity. The advancement and incorporation of molecular biology tools and techniques, micropropagation and other in vitro culture methods in modern crop breeding have made induced mutagenesis easy to apply in vegetable breeding

programs (Gupta 2019). Mutation breeding deals with the utilization of induced mutations for crop improvement and it has been used to obtain direct mutants or use of these mutants in hybridization program of vegetable crops that may help to improve yield and generate desirable traits (Ahloowalia et al. 2004). In different vegetable crops, mutation breeding has already been practiced such as in tomato (Minoia et al. 2010), cucumber (Wang et al. 2014), pumpkin (Kurtar et al. 2017), okra (Solanki et al. 2011) and cauliflower (Hadi and Fuller 2013). The availability of genomic sequences of many crop species has enhanced the ability to detect variation at specific genetic loci (reverse genetics) that may expand the capacity for both probing gene function and genetic engineering in vegetable crops, although a high-quality sequence of the modern bottle gourd genome is still not quite available and their genetic relationships remains largely unknown (Wu et al. 2017a, b). Effective and efficient mutagen selection is essential in any mutation breeding program to produce a high frequency of desirable mutations. A chemical mutagen like ethyl methylsulfonate (EMS) was used by Shah et al. (2015) and Wang et al. (2014) at different doses to create cucumber mutants and this type of mutation can also be used in obtain mutants in bottle gourd (Fig. 4.7). The mutant population developed can also serve as a resource for high throughput reverse genetic studies in order to screen point mutations in specific regions of the targeted genes (Reddaiah et al. 2014). The combined effect of both physical and chemical mutagens is another effective tool to improve vegetable crops through the creation of variability in the crops. A huge number of mutant varieties of vegetable crops have been developed and widely cultivated in developing countries to improve food security. Integrating mutation techniques with other molecular technologies, such as high-throughput mutation-screening techniques and molecular marker is becoming more powerful and effective in breeding vegetable varieties. The general applicability of the rapid

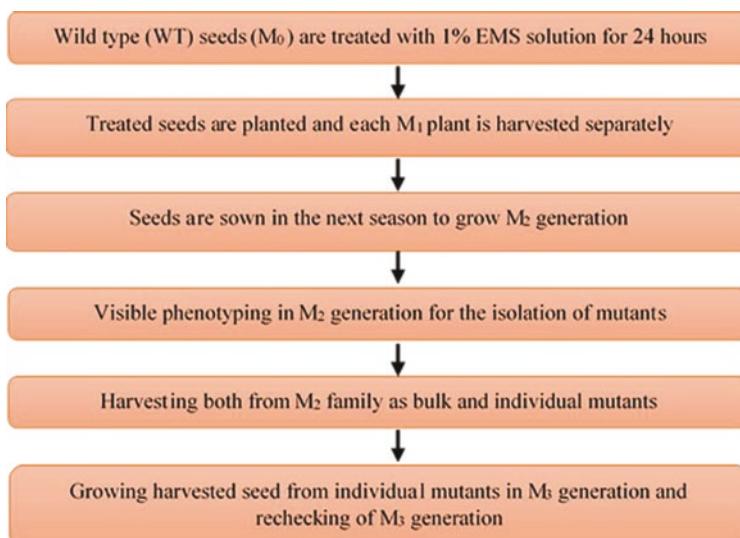


Fig. 4.7 Chemical mutagenesis in vegetable crops. Source: adapted from Wang et al. (2014)

mutational screening method called TILLING (targeting induced local lesions in genomes) that targets lesions to specific genes, may speed up the gene function analysis process and the efficiency of mutation breeding for better improvement of vegetable crop in terms of yield and quality (Minoia et al. 2010).

4.9 Biotechnological Methods

4.9.1 Tissue Culture

Tissue culture techniques are being widely used for mass propagation and elite plant preservation. In plant tissue culture, the most important characteristic is the aptitude of the cultured cells and tissue to grow into complete plants. Many plants, both temperate and tropical, can successfully be propagated through tissue culture. Conventional plant breeding programs can be improved through a rapid and efficiently reproducible in vitro regeneration system. Through genetic transformation done in vitro, specific traits can be added with the least alteration of the target plant genome. Direct shoot development from explants is more desirable compared to the intermediate callus phase. In the present work, propagation of *Lagenaria siceraria* through tissue culture techniques was achieved and the factors influencing the growth of this plant were studied by Hasbullah et al. (2017).

4.9.1.1 In Vitro Plant Regeneration

Indirect organogenesis through callus formation is possible from multiple shoots of bottle gourd. For callus formation in bottle gourd explant, BAP was used by Hasbullah et al. (2017). Callus with a good, compact structure can be obtained when the explant is cultured on MS medium supplemented with BAP and NAA. Hairy roots form callus from leaf explants when they are placed on MS medium supplemented with 1 mg L^{-1} BAP and 1 mg L^{-1} NAA (Fig. 4.8a). Callus can also be obtained from stem explants after placing them on MS medium added with 1 mg L^{-1} BAP and 2 mg L^{-1} NAA (Fig. 4.8b) and regeneration of shoots can be attained from petiole explants by culturing on MS medium with the addition of 2 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (Fig. 4.8c) (Lood et al. 2014).



Fig. 4.8 Indirect organogenesis. (a) Root formation from leaf explant, (b) Callus formation from stem explant, (c) Shoot formation from petiole explant of bottle gourd. (Source: Hasbullah et al. 2017)

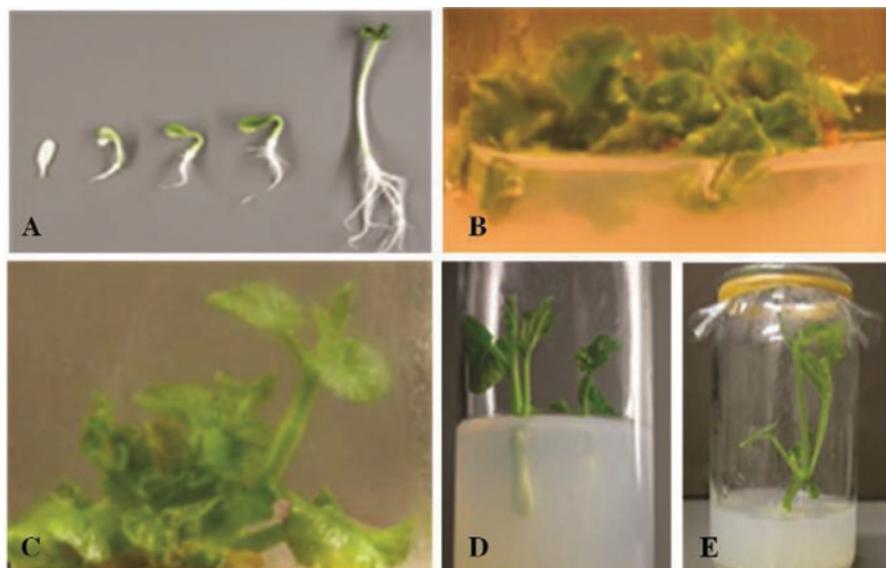


Fig. 4.9 Plant regeneration from the cotyledonary explants of bottle gourd. (a) Seedling germination to obtain cotyledonary explants, (b) Bud proliferation, c Shoot initiation, (d) Shoot elongation, (e) Root induction. (Source: Saha and Kazumi 2007)

4.9.1.2 Plant Regeneration from Cotyledons

In plant regeneration for breeding purposes of bottle gourd, cotyledon explants can also be used. In a study by Saha and Kazumi (2007), kinetin (1 mg L^{-1}) and benzyl adenine (BA) (2 mg L^{-1}) provided the highest regeneration efficiency in cotyledon explants of bottle gourd (Fig. 4.9). Root formation can be done by placing the regenerated shoots in half strength MS media containing 0.1 mg L^{-1} indole acetic acid (IAA).

4.9.1.3 Anther Culture

The main objective of anther culture is to accelerate plant breeding through the development of homozygous double haploid lines (DHL) that facilitate desired selection of genotypes to produce improved varieties and highly homogeneous and vigorous F1 hybrid seed. Homozygous parental line development using traditional self-pollination method takes around 6–8 years to develop a variety with desirable traits (Asadi et al. 2018). Therefore, the development of doubled haploids through anther culture (Fig. 4.10) can reduce the time required for cultivar development of bottle gourd. In the Cucurbitaceae, haploid plants have recently been obtained using different methods such as in situ induction of haploid embryos via irradiated pollen in winter squash (*Cucurbita maxima* Duchesne ex. Lam.) (Kurtar and Balkaya

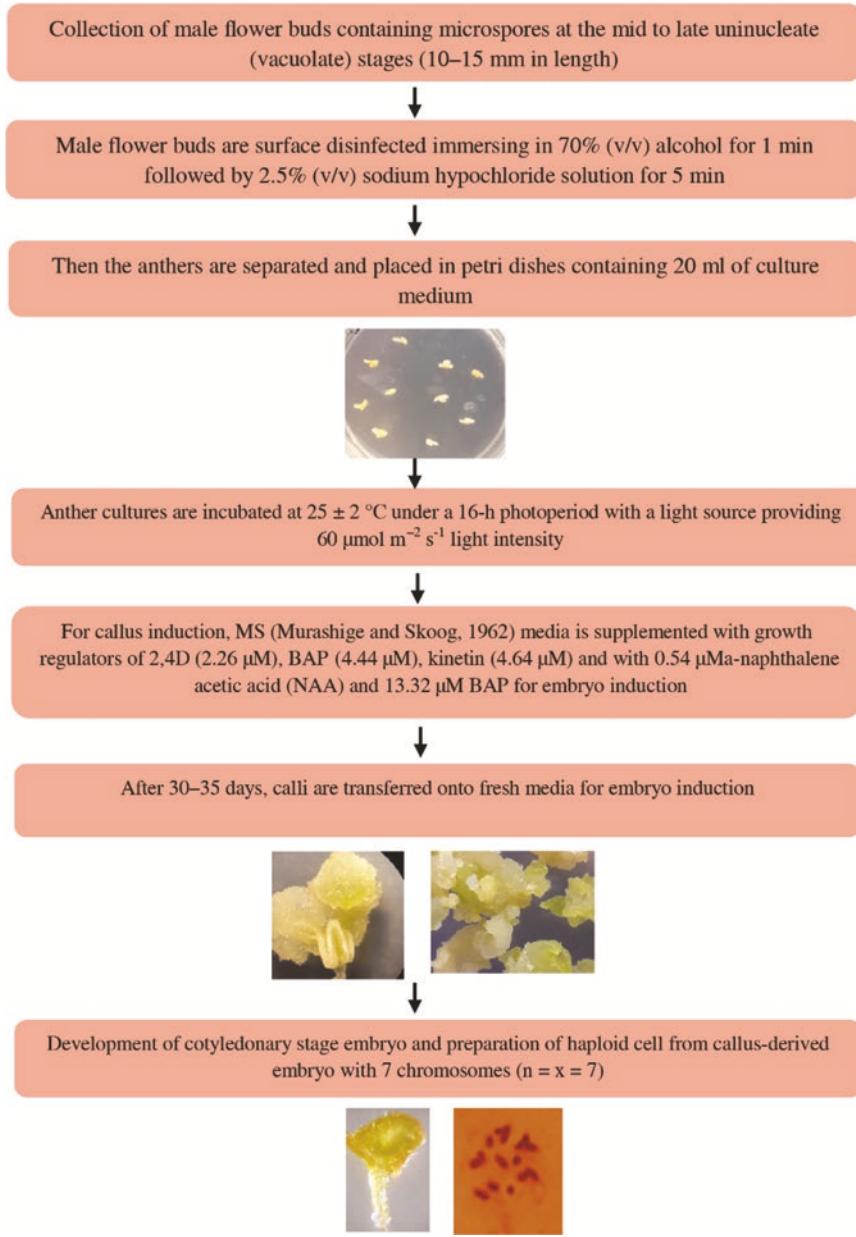


Fig. 4.10 Callus and embryo induction through anther culture. Source: adapted from Abdollahi et al. (2016); Asadi et al. (2018)

2010), in vitro gynogenesis in summer squash (*Cucurbita pepo* L.) (Shalaby 2006) and anther culture method in watermelon (*Citrullus lanatus* L.) (Abdollahi et al. 2016). Most of the studies found anther culture more effective for double haploid production as compared to total irradiation of pollen (Gałazka and Niemirowicz-Szczytt 2013). Doubled haploid (DH) technology through anther culture is a faster and cheaper way of producing pure lines in vegetable crops compared to conventional breeding approaches (Seguí-Simarro 2016). Although, DH technology has not yet been explored successfully in bottle gourd, a very few studies to produce DH via androgenesis in cucumber have been done (Abdollahi et al. 2016; Hamid et al. 2013; Zhan et al. 2009).

4.9.2 Transgenics

Tissue culture genetic transformation has become a routine approach of crop plant improvement due to rapid advancements in biotechnology, but in bottle gourd it is in a nascent stage. The advances in genetic transformation methods have unlocked new ways for crop improvement. In most of the transformation experiments involving *Agrobacterium* inoculation, wounded tissue has been used as explants (Han et al. 2005). In bottle gourd an efficient shoot regeneration system was developed via organogenesis using cotyledon and adventitious shoot explants to establish an efficient *Agrobacterium*-mediated transformation method (Han et al. 2005; Mendi et al. 2010). The cotyledon explants of bottle gourd cultured by Han et al. (2005) on MS media supplemented with 3 mg l⁻¹ of BA (6-Benzylaminopurine) and 0.5 mg l⁻¹ of AgNO₃ at 16-h photoperiod and highest shoot regeneration (80.6%) efficiency showed in Kinetin (1 mg l⁻¹) and BA (2 mg l⁻¹) without adding AgNO₃ or Polyamines, rather than BA or Kinetin alone (Shyamali and Hattori 2007). Hirschi (2001) developed transgenic bottle gourd lines expressing an *Arabidopsis* H+/Ca + transporter gene to test its efficiency in conferring biotic and abiotic stress tolerance and to investigate the effect of ethylene action and biosynthesis inhibitors on the infection of *Agrobacterium*. In bottle gourd, genetic engineering protocols are available for transgenic breeding and it can be utilized if target traits are unavailable in breeding populations or gene banks. Some limiting factors in production, such as pests and pathogens, can be overcome through developing transgenic cultivars of bottle gourd (Dias and Ortiz 2019).

4.9.3 Gene Editing

Gene editing is a new type of genetic engineering technology based on editing or deleting a genetic sequence and contributes to revolutionizing vegetable breeding as it precisely alters DNA sequences. Currently CRISPR-Cas9 system has become the

most popular gene editing system due to its ability to insert and turn off desired traits more accurately and efficiently (Tian et al. 2016). Gene editing is hence comparable to conventional breeding, but can be used for faster, cheaper and more precise introduction of genetic variation.

Genome wide studies in bottle gourd may help to edit the genome to achieve the desired genetic outcome related to high yield and nutritional importance. Wu et al. (2017a, b) conducted a genome-wide association study (GWAS) to identify the relationship between umami (savory) chemical ingredients of bottle gourd fruits (bottle gourd fruits have a peculiar umami taste that is important for their market value), but the free amino acid (FAA) as the genetic determinants of this chemical remain unidentified and free glutamate (Glu) was found to have the most significant association with umami taste, suggesting that free Glu is the main umami-conferring ingredient of bottle gourd. This kind of study may help to facilitate molecular breeding of bottle gourd cultivars with improved taste and flavor.

4.9.4 Marker-Assisted Selection (MAS)

Although having a relatively small genome size, very few molecular genomic resources such as DNA sequences are publically available in the DNA database for bottle gourd. In bottle gourd, molecular markers that have been used to assess genetic variability are chloroplast markers, simple sequence repeat (SSRs) or microsatellite markers, sequence related amplified polymorphisms (SRAP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), amplified fragment length polymorphisms (AFLP), single nucleotide polymorphisms (SNPs) and allozyme markers (Table 4.5) (Koffi et al. 2009; Saxena et al. 2015; Xu et al. 2011). A limited number of anonymous random amplified polymorphic DNA (RAPD) molecular markers have been used for various studies in bottle gourd (Morimoto et al. 2005). Decker-Walters et al. (2004) initially used RAPD (random amplified polymorphic DNA) markers to study genetic resources in bottle gourd. The potential of RAPD markers for testing the purity of bottle gourd hybrid seed was indicated by Singh et al. (2010). Next-generation sequencing is another recent emerging technique that provides a powerful alternative for creating a tremendous number of DNA sequences for marker development and genomics study of bottle gourd. The bottle gourd genome was partially sequenced using this technology and tens of thousands of genes with a broad range of functional types were recognized through assembling and annotating the sequence reads (Xu et al. 2011). For studying the transferability in bottle gourd and for better understanding of bottle gourd genomics, SSR markers can be used that are developed in cucumber and other cucurbits. Several polymorphism among *Lagenaria siceraria* accessions collected by Levi et al. (2008) and they elucidated the phylogenetic relationship of bottle gourd with other cucurbit species using watermelon derived EST (expressed

Table 4.5 Markers used for genetic diversity analysis in bottle gourd (*Lagenaria* spp.)

Country	Marker type	No. of landraces evaluated	No. of cultivars evaluated	Bottle gourd species	References
USA	RAPD	31	–	<i>L. siceraria</i> , <i>L. sphaerica</i>	Decker-Walters et al. (2001)
Kenya	RAPD	95	–	<i>L. siceraria</i> , <i>L. sphaerica</i> , <i>L. abyssinica</i> , <i>L. breviflora</i>	Morimoto et al. (2005)
India	SSR	–	40	<i>L. siceraria</i>	Bhawna et al. (2015)
India	SSR	–	20	<i>L. siceraria</i>	Sarao et al. (2013)
India	SSR	–	44	<i>L. siceraria</i>	Bhawna et al. (2015)
India	ISSR	–	42	<i>L. siceraria</i>	Bhawna et al. (2014)
China	SSR	39	5	<i>L. siceraria</i>	Xu et al. (2011)
Turkey	SSR, SRAP	–	30	<i>L. siceraria</i>	Yildiz et al. (2015)
Turkey	SSR	60	31	<i>L. siceraria</i>	Gurcan et al. (2015)

sequence tags) based SSR markers. Marker-assisted breeding began with the development of SSR markers that help to provide sequence information of bottle gourd which will allow comparative genomics studies across different cucurbit species. Simple sequence repeat markers are used in diversity analysis because of their high degree of polymorphism and random distribution across the genome (Ji et al. 2012). Some 400 SSR loci were designed and a subset of 14 SSR markers for bottle gourd genotyping was selected by Xu et al. (2011). A total of 40 SSRs were developed by Bhawna et al. (2015) that are useful for identification of cultivars and breeding in bottle gourd. In order to identify breeding lines retaining desirable traits, SSR markers developed for bottle gourd can be employed to screen segregating populations in a marker-assisted breeding program of bottle gourd (Mashilo 2016).

4.9.5 Resistant Breeding

Controlling insect pests and diseases in bottle gourd by resistance breeding is an economical and environmentally-friendly approach. Breeding procedures for developing resistance vary with the source of resistance, nature of the pathogen, inheritance of resistance, quality and horticultural traits required in a resistant variety. Different diseases attack different growth stages of the bottle gourd plant and

different plant parts are inoculated for screening, such as first or second true leaf stage are taken for powdery mildew and viruses, cotyledon leaves are inoculated for screening downy mildew and bacterial wilt. In the case of resistance breeding, resistant genes from an available genetic resource are transferred to a commercial variety already having superior horticultural traits. Resistance controlled by one or two genes can be transferred through the backcross method (Fig. 4.11) and polygenic controlled resistance is generally transferred through recurrent selection (Dhatt and Khosa 2015). Artificial screening is done for every cross generation and selfing is continued in resistant plants by repeatedly growing them until the development of stable resistance, along with superior horticultural traits. Comparing to vertical resistance, horizontal resistance along with general adaptability and broad genetic base is more stable. Two bottle gourd breeding lines (USVL#1–8, USVL#5–5) derived from Indian accessions, exhibited resistant to zucchini yellow mosaic virus (ZYMV), papaya ringspot virus strain watermelon (PRSV-W), watermelon mosaic virus (WMV), and squash vein yellowing virus (SqVYV) (Ling et al. 2013). Resistant to cucurbit powdery mildew caused by *Podosphaera xanthii*, resistant to ZYMV, tomato ringspot virus (TmRSV), SqMV, watermelon mosaic virus-1 (WMV-1) and watermelon mosaic virus-2 (WMV-2), found in another Indian bottle gourd accession PI 271353 (Kousik et al. 2008). In bottle gourd, dominant inheritance was found for resistance against zucchini yellow mosaic virus (Ling and Levi 2007). Careful selection and screening of moderately-resistant germplasm may provide novel sources of disease resistance in bottle gourd and the breeding lines exhibiting resistant to certain pathogens or insects can be utilized in breeding programs of bottle gourd improvement. Markers associated with diseases can be found through mapping populations of bottle gourd. Bottle gourd can also be used to

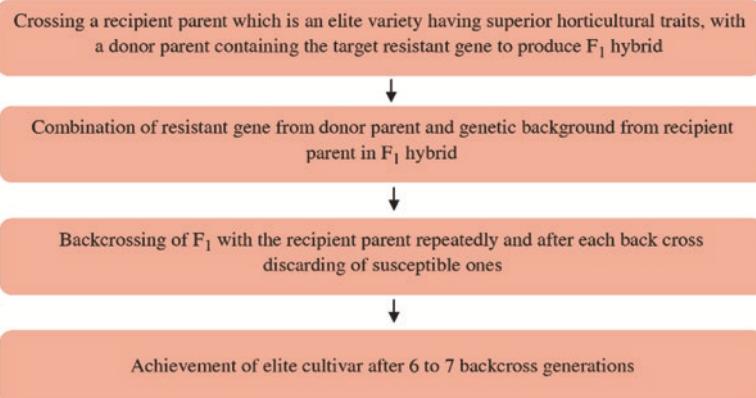


Fig. 4.11 Transfer of resistance gene through backcross method

develop resistant rootstock for resistance against soil borne pathogens and shorten the prolonged breeding program (Karaca et al. 2012).

4.10 Conclusions and Prospects

Bottle gourd is widely distributed and cultivated in the tropical and subtropical countries of the world. It is highly variable due to its mode of pollination which makes it highly adaptable to changing environments. Nevertheless, little is known about genetics of different qualitative and quantitative traits of bottle gourd such as plant habit, sex expression, fruit abortion, fruit shape, fruit size, fruit yield and so on. The major objectives in breeding bottle gourd are the development of early and high-yielding varieties, fruit set at high humidity and low temperatures, small and uniform size of fruit, and resistance to common diseases and pests. Information on genetics and breeding of yield related and nutritional traits will help to improve farming strategies of bottle gourd. The major breeding techniques practiced for bottle gourd improvement are pure line and pedigree selection. Progeny testing and recurrent selection are also used to develop new varieties of bottle gourd. Pure line and pedigree selection are also used to develop inbred lines from available germplasm and later these inbred lines are exploited to develop hybrid varieties. Hybrids have shown advantageous yield compared to open pollinated plants. Open pollinated (BARI Lau 1–5, BARI Shita Lau 1, BU Lau 1–2) and hybrid (BU Hybrid Lau 1, Barsha, Moyna, Diana, Martina, High Green, Merina) varieties are being commercially grown in Bangladesh. Interspecific crosses may help to develop hybrids or to transfer desirable genes from other species. Climate change and global warming are serious threats to food security. It is an unavoidable phenomenon of natural origin against which mitigation and adaptation are required to reduce the magnitude of impact and vulnerability, especially to avoid risk in vegetable farming, because vegetables are highly sensitive to climate change. Climate resilient varieties and hybrids have to be developed to preserve sustainable yield of bottle gourd. Genetic improvement of bottle gourd is an important strategy to overcome climate change hardships. Studies can provide a clear understanding of the environmental effect on the transformation of a genotype into phenotype. Modern breeding tools such as marker assisted selection genomics, phenomics and gene editing are also needed to develop varieties with multiple resistances against biotic and abiotic stress.

Appendices

Appendix I: Research Institutes Relevant to Bottle Gourd

Institution name	Specialization and research activities	Address and Country	Contact information and website
Bangladesh Agricultural Research Institute (BARI)	Agricultural research Specially vegetables	Bangladesh	www.bari.gov.bd
Bangladesh agricultural development corporation (BADC)	Agricultural research	Bangladesh	www.badc.gov.bd
Bangladesh Agricultural Research Council (BARC)	Maintaining national agricultural research system	Bangladesh	www.barc.gov.bd
Sher-e-Bangla Agricultural University (SAU)	Agricultural research	Bangladesh	www.sau.edu.bd
Bangladesh Agricultural University (BAU)	Agricultural research	Bangladesh	www.bau.edu.bd
Bangabandhu sheikh Mujibur Rahman Agricultural University (BSMRAU)	Agricultural research	Bangladesh	Bsmrau.edu.bd
Bangladesh Rural Advancement Committee (BRAC)	Agricultural research	Bangladesh	www.brac.net
Lal Teer Seed Limited	Agricultural research and quality seed development	Bangladesh	https://www.lalteer.com/ Contact/#
World Vegetable Center (WorldVeg)	Vegetable research	Taiwan	https://avrdc.org/about-avrdc/ about-us
Indian Agricultural Research Institute (IARI)	Agricultural research, education and extension	India	www.iari.res.in
Department of Vegetable Science	Research related with vegetables	India	http://cohvka.kau.in/cohvka/ department-olericulture
Indian Institute of Vegetable Research (IIVR)	Research related to vegetables	India	www.iivr.org.in
Aegean Agricultural Research Institute (AARI)	Agricultural research	Turkey	https://arastirma.tarimorman. gov.tr/etae/Sayfalar/EN/ AnaSayfa.aspx

(continued)

Institution name	Specialization and research activities	Address and Country	Contact information and website
U.S. National Plant Germplasm System (NPGS)	Agricultural research and germplasm conservation	USA	www.ars-grin.gov
United States Department of Agriculture (USDA)	Executing federal laws related to farming	United States	www.usda.gov
Institute of Agrifood Research and Technology	Food technology	Spain, Catalonia	www.irta.cats
Institute for Vegetable Crops	Research institute attributed for scientific work	Serbia	www.institut-palanka.co.rs

Appendix II: Genetic Resources of Bottle Gourd

Cultivar	Important traits	Cultivation location
BARI Lau-1	Cultivated whole year	Bangladesh
BARI Lau-2	Exportable for having attractive shape	Bangladesh
BARI Lau-3	Can be grown both in early and main season	Bangladesh
BARI Lau-4	Temperature tolerant	Bangladesh
BARI Lau-5	Narrow neck and dark colored	Bangladesh
BARI Shita Lau-1	Perennial in nature	Bangladesh
BU Lau 1	Early fruiting and less vegetative growth	Bangladesh
BU hybrid Lau 1	Less vegetative growth and low male female ratio	Bangladesh
Arka Bahar	Tolerant to blossom end rot	India
Pusa Naveen	Free from crooked neck	India
Punjab Komal	Tolerant to CMV (cucumber mosaic virus)	India
Bottle gourd round	Plant is creeper type	India
Calabash	Crooked neck	India
Apple	Used for arts and crafts	Africa
Extra long handle dipper	For making attractive decorations	USA
Speckled swan	Used for decorations and retains its color when dried	USA
Narendra Shivani	Both used for food and making musical instrument	India
PI 271353	Resistance to cucumber mosaic virus (CMV), squash mosaic virus, tobacco ringspot virus (TRSV), tomato ring spot virus (TmRSV), watermelon mosaic virus 1 (WMV1)	India

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Chapter 5

Eggplant (*Solanum melongena*, *S. aethiopicum* and *S. macrocarpon*) Breeding



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Abstract Brinjal eggplant (*Solanum melongena*) is an annual vegetable cultivated for its edible fruits, which are variable in shape, size and color. Scarlet eggplant (*S. aethiopicum*) and gboma eggplant (*S. macrocarpon*) are also cultivated but more locally in Africa. Domestication of brinjal eggplant took place in India and China, but today the plant is cultivated globally and with tremendous economic importance, especially in Asia and the Mediterranean. It is low in calories but rich in antioxidants and phenolic compounds. A warm climate favors the plant as it has a long growing season. Current eggplant breeding aims to develop F₁ hybrids and traditional methods include pure-line selection, pedigree methods, and backcrossing for breeding for higher fruit yield, quality and resistance to diseases. In this chapter, we review recent developments including doubled haploids, marker-assisted breeding and tissue culture technologies, but we also focus on pre-breeding, trait discovery and hybridization with crop wild relatives. Broadening the genetic base of cultivated eggplant is a key to develop robust varieties. The primary gene pool includes only *S. insanum*, which can be easily crossed with cultivated eggplant

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to give highly fertile hybrids. The secondary and tertiary gene pools include several species of interest (*S. dasypodium*, *S. incanum*, *S. linnaeanum*, *S. tomentosum*, *S. torvum*, *S. sisymbriifolium*), which are more distant. Crosses of cultivated eggplant with tertiary gene pool species result in sterile or low fertility hybrids after embryo rescue or somatic hybridization. Still, these wild species are of interest as they are genetically very diverse and could provide tolerance to abiotic stresses, as well as resistance to pests and diseases.

Keywords Crop wild relatives · Genetic resources · Genomics · Hybridization · Resistance breeding · QTLs · Tissue culture

5.1 Introduction

5.1.1 Background

Eggplant (*Solanum melongena* L.), also called brinjal eggplant in Asian and South African English or aubergine in French, is an important vegetable cultivated for its edible fruits. A range of fruit shapes, colors and sizes, and accordingly market diversity, exist in addition to two additional cultivated eggplant species, the scarlet eggplant (*S. aethiopicum* L.) and the gboma eggplant (*S. macrocarpon* L.), both of local importance in Africa (Daunay and Hazra 2012). When not specified, the term *eggplant* we will refer to brinjal eggplant/aubergine. In cooking, eggplant fruits can absorb large amounts of fats and spicy sauces and they are popular both as fried, roasted, steamed or stewed ingredients. Regarding cultivation, the plants thrive under warm conditions and prefer tropical or subtropical climates, but they are also cultivated in temperate areas. In cooler climates, plastic tunnels or glasshouses can be used to extend the growing season.

5.1.2 Botanical Classification and Distribution

Eggplant belongs to a non-tuberiferous group of species of the Solanaceae family (Daunay et al. 1991), which includes more than 3000 species distributed in some 90 genera and many species of economic use such as tomatoes, potatoes, peppers and eggplants (Knapp and Peralta 2016; Vorontsova and Knapp 2012). *Solanum* is one of the largest genera of the Solanaceae occurring on all continents except Antarctica, and comprises around 1500 species divided into 13 clades. Most taxa of *Solanum* have a basic chromosome number of $n = 12$ (Chiarini et al. 2010).

Eggplant and its relatives are members of the largest clade of the genus, the *Leptostemonum* clade (the *spiny solanums*), which contains around 450 currently recognized species (Aubriot et al. 2016; Särkinen et al. 2013; Weese and Bohs

2010). The spiny solanums are most diverse in Central and South America and are less common in the Old World (Knapp et al. 2013; Vorontsova and Knapp 2012). The Old World, including Africa, Asia and Australia, is home to more than 300 *Solanum* species (Levin et al. 2006; Vorontsova and Knapp 2016). All three cultivated eggplants species have an Old World origin, unlike other solanaceous crops, which are native to the New World. *Solanum melongena* and *S. macrocarpon* belong to the section *Melongena* (Lester and Daunay 2003; Lester et al. 2011) and *S. aethiopicum* to the section *Oliganthes* (Lester 1986).

Solanum melongena is the most commonly cultivated eggplant and represents large morphological variation (Fig. 5.1). Lester and Hasan (1991) divided eggplants into four taxonomic/geographical groups, labeled E to H. The groups represent two species: the cultivated eggplant *S. melongena* and its wild ancestor *S. insanum* L. (Knapp et al. 2013). Group E and F represent extremely prickly plants, which grow wild or weedy in India and Southeast Asia and are *S. insanum* (Ranil et al. 2017). Group G represent primitive cultivars with small fruits. Group H consist of large-fruited landraces and modern cultivars. These plants are also less prickly than the ones found in the other groups (Daunay et al. 2001; Weese and Bohs 2010). Both groups G and H are *S. melongena* (Knapp et al. 2013). Genetic and



Fig. 5.1 Cultivation of an eggplant variety (a Taiwan local variety) with very elongated fruits favored by Asian countries people (a) and some other example of the diversity in fruit shape and color: purple oval (b), a clustering pink elongated (c), red oval (d) and white circular (e). (Photos Courtesy of World Vegetable Center)

morphological differentiation has been observed between eggplants from the Mediterranean area, North Africa and the Middle East (Occidental) and eggplants from southeast and eastern Asia (Oriental) (Cericola et al. 2013; Hurtado et al. 2012; Vilanova et al. 2012).

Like *Solanum melongena*, *S. aethiopicum* is also hypervariable. However, this species can be easily distinguished from *S. melongena* by its small white corollas and bright scarlet fruits that often resemble *Capsicum* peppers. Based on morphological characteristics and uses, *S. aethiopicum* is grouped into Gilo, Shum, Kumba and Aculeatum (Lester 1986). The Gilo group is the most important and their fruits are edible. The Shum group has plants with glabrous and small leaves that are eaten as a green vegetable, but with fruits that are inedible. The Kumba group also has plants with glabrous leaves but the fruits are flattened and large, and they are edible. The Aculeatum group has plants that are very prickly and with flattened fruit, often grown as ornamentals (Gramazio et al. 2016; Plazas et al. 2014b; Taher et al. 2019).

Solanum macrocarpon is grown for its leaves and fruits (Maundu et al. 2009; Nyadanu and Lowor 2015). It can be distinguished from *S. melongena* by its usually deeply lobed leaves and very large calyces. Although it has less morphologically diverse than *S. melongena* and *S. aethiopicum* (Plazas et al. 2014b) it has local importance in several parts of Eastern and Central Africa (Nyadanu and Lowor 2015; Schippers 2000). All three domesticated eggplant species can be intercrossed giving hybrids with intermediate fertility (Khan et al. 2013; Prohens et al. 2012; San José et al. 2016).

Unlike cultivated eggplant, which have lost much genetic diversity through domestication, crop wild relatives (CWR) are a rich sources of variation and may house traits related to pest and disease resistance, and could be useful for breeding for climate change adaptation (Rotino et al. 2014; Syfert et al. 2016; Vorontsova et al. 2013). Eggplant wild relatives are distributed from Africa throughout the Middle East into India and Asia (Weese and Bohs 2010). Wild eggplants are generally very prickly and have small and bitter fruits. Some species are very high in bioactive compounds, but inedible (Meyer et al. 2015). The wild relatives of eggplant are one of the most variable and intricate groups regarding their taxonomic and phylogenetic relationships (Vorontsova et al. 2013). Harlan and de Wet (1971) reported classification of wild species into three gene pools, based on crossability relationships and biological concept of species. Recently, Syfert et al. (2016) suggested the inclusion of one species (*S. insanum*) in the primary gene pool (GP1) of brinjal eggplant, which can be crossed easily and produce normal fertile hybrids (Plazas et al. 2016). More than 40 African and Southeast Asian species belong to the secondary gene pool (GP2). These can be crossed and are phylogenetically relatively close to brinjal eggplant. However, the success of the crosses and the viability and fertility of the hybrids may vary. Hybrids with *S. incanum* L., which is in GP2, have good fertility levels, while some interspecific hybrids derived from other GP2 species such as *S. dasypetalum* Schumach. & Thonn., *S. linnaeanum* Hepper & P.-M.L.Jaeger or *S. tomentosum* L. are partly sterile or weak due to reproductive barriers (Kouassi et al. 2016; Rotino et al. 2014). Finally, the tertiary gene pool (GP3) includes more distantly related species that are harder to use in breeding, e.g.

Solanum torvum Sw., *S. elaeagnifolium* Cav. and *S. sisymbriifolium* Lam. GP3 species might be of interest due to their resistance features, but crossing needs specific breeding techniques (Kouassi et al. 2016; Plazas et al. 2016).

5.1.3 Economic and Health Importance

5.1.3.1 Economic Importance

On a global scale, the economic value of eggplant, calculated as farm-gate value and global numbers, exceeds USD 10 billion a year, ranking fifth in the very important Solanaceae family, just after potato, tomato, pepper and tobacco (FAO 2017). Eggplant is especially popular in Asia and the Mediterranean where the plant ranks fifth among all vegetables (Frary et al. 2007). Exact numbers of produced quantities are difficult to obtain, especially for subsistence production. According to FAO statistics, eggplant occupies more than 1.8 million ha with a total production of more than 52 million mt (FAO 2017). Asia has the highest production share and accounts for more than 90% of the global production. China alone has the more than half of the production (32.8 million mt) and India has another 30% (12.5 million mt). Number 3 is Egypt (1.3 million mt), followed by Turkey (820,000) and Iran (750,000 mt). Among the Asian countries, Indonesia (530,000), Japan (308,000), and the Philippines (241,000 mt) are among the countries with the highest production. In Africa, Egypt with its production of 1.3 million mt is the most important eggplant producing country, followed by Algeria (156,000), Ivory Coast (99,000) and Sudan (90,000 mt). In Europe, Italy (286,000) and Spain (226,000 mt) are the most important eggplant producing countries, followed by Romania, Ukraine, Greece, Netherlands and France. In the Americas, Mexico has the highest production (185,000) followed by the USA (103,000 mt).

5.1.3.2 Health Value

The importance of fruits and vegetables as sources of bioactive substances is well recognized in human nutrition. Eggplant is a low-calorie vegetable rich in some vitamins but perhaps more important is its content of antioxidants and phenolic compounds (Braga et al. 2016; Cao et al. 1996; Docimo et al. 2016; Plazas et al. 2013, 2014a; Raigón et al. 2008). According to the USDA National Nutrition Database (USDA 2019), every edible 100 g gives only 25 Kcal and 2.2 mg vitamin C (Table 5.1). The pro-vitamin A content is only around 1 µg/100 g. Anthocyanin is one of many groups of compounds with antioxidant capacity. In eggplant, anthocyanin is found in the skin (Niño-Medina et al. 2017), where purple-colored fruits have a higher content than non-colored fruits (Mennella et al. 2012). The phenolic compounds include various phenolic acids and flavonoids, but in eggplant the first group is of significant value (Plazas et al. 2013; Stommel et al. 2015). Total phenolic

Table 5.1 Chemical composition of eggplant fresh weight (FW)

Chemical constituents	USDA Nutrition database ^a
Water (g/100 g)	92.3
Energy (kcal/100 g)	25
Carbohydrates (g/100 g)	5.88
Protein (g/100 g)	0.98
Total lipids (g/100 g)	0.18
Fatty acids, total saturated (g/100 g)	0.034
Fiber (g/100 g)	3.0
Calcium (mg/100 g)	9
Potassium (mg/100 g)	229
Magnesium (mg/100 g)	14
Sodium (mg/100 g)	2
Phosphorus (mg/100 g)	24
Iron (mg/100 g)	0.23
Zink (mg/100 g)	0.16
Vitamin C, total ascorbic acid (mg/100 g)	2.2
Thiamine (mg/100 g)	0.039
Riboflavin (mg/100 g)	0.037
Niacin (mg/100 g)	0.649
Vitamin B6 (mg/100 g)	0.084
Vitamin B12 (μ g/100 g)	0.0
Vitamin D (IU/100 g)	0
Vitamin K (μ g/100 g)	3.5
Folate, DFE (μ g/100 g)	22
Vitamin A (μ g/100 g)	1
Vitamin A (alpha.tocopherol, mg/100 g)	0.30

^a USDA (2019) National nutrition database for standard reference legacy release <http://ndb.nal.usda.gov>

Source: USDA 2019

content in eggplant is 40-fold higher than vitamin C content (San José et al. 2013). Phenolic compounds of importance in eggplant include, in particular, delphinidin found in the skin and chlorogenic acid found in the flesh (Niño-Medina et al. 2017).

As in other vegetables, variation in content among genotypes can be found. Hanson et al. (2006) examined 35 accessions from the World Vegetable Center's (Taiwan) collection and found highly significant differences among accessions in antioxidants measured as superoxide scavenging activities (SOS) in both water and methanol. No association was found between such activity and fruit skin color and but with content of phenolic compounds and with dry matter. Also, small fruits had higher antioxidant activity than large fruits. A second study to be highlighted is a study of different Turkish eggplant cultivars that showed a three-fold difference in total water-soluble antioxidant activity between the accessions, with a range of 2664–8247 μ mol Trolox/kg fresh weight (Okmen et al. 2009). Furthermore, they showed a two-fold range in total phenolic contents between the same accessions, of

615–1376 mg/kg and that antioxidant activity and phenolic contents were significantly correlated. The abovementioned studies indicate that breeders can develop eggplant cultivars with high antioxidant activity.

5.1.4 Domestication, Selection and Early Improvement

The origin of a plant species is generally determined based on archeological evidence as well as the existence of wild species or ancestors of domesticated forms. The natural forces including mutation, migration, selection and recombination through hybridization modified the wild species resulting in the evolution of cultivated species (Pratap and Kumar 2011). The evolutionary history and biogeography of eggplant and its wild relatives are incompletely understood (Aubriot et al. 2018). Vavilov (1951) considered *Solanum melongena* as being native to the *Indo-Chinese center of origin*. However, the putative progenitor of eggplant, *S. insanum* is widespread in tropical Asia (Knapp et al. 2013; Syfert et al. 2016). Cultivation of eggplant is believed to have started in India and China but archeological studies point at an earlier domestication in India than in China, with subsequent additional and independent centers of domestication in the Philippines (Meyer et al. 2012). Around the eighth century, cultivation spread to Japan and later along the Silk Road into Western Asia, Europe and Africa. Here Arab traders during the fourteenth century may have played an important role. The cultivation of eggplant was introduced into the Americas in 1492 (Prohens et al. 2005; Taher et al. 2017) and has expanded further into other parts of world. The domestication of eggplant primarily involved dramatic expansion of fruit shape, size, color diversity and a decrease in plant prickliness (Weese and Bohs 2010).

Little is known about the domestication of the scarlet and gboma eggplants. Certainly, both were domesticated in Africa from their wild ancestors. These are *Solanum anguivi* Lam. in the case of *S. aethiopicum* (Lester and Niakan 1986) and *S. dasypodium* in the case of *S. macrocarpon* (Bukenya and Carasco 1994). Hybrids between the cultivated species and their respective wild ancestors are fully fertile (Bukenya and Carasco 1994; Lester and Thitai 1989; Plazas et al. 2014a).

5.2 Current Cultivation Practices and Challenges

5.2.1 Current Cultivation Practices

Brinjal eggplant is produced all around the world and for different purposes, thus current practices are very diverse. A warm climate favors plant growth and development. The plants prefer a deep, nutrient-rich soil with good drainage and access to irrigation. Production normally starts from transplants, 6–8 weeks old, planted at a

distance in the 40–65 cm range. Plants benefit from being staked and plant protection and a proper weed management is required throughout the growing season. Plants are very frost-sensitive and warm-season cultivars can be damaged even at temperatures around 10 °C (Abe et al. 1974). Cultivation under poly-tunnels or other protected environments will reduce such risks. Heavy rains and thunderstorms may also cause damage. A crop rotation system of a minimum of 4–6 years is preferred due to the accumulation of damaging soil-borne diseases and nematodes.

5.2.2 Current Agricultural Challenges

As eggplant requires a relatively long growth period of warm and often humid conditions, it is vulnerable to a range of plant diseases, pests, nematodes and weed competition. Brinjal eggplant is among the crops that is sprayed the most often with the risks this causes. Important diseases are bacterial wilt, *Verticillium* wilt and *Fusarium* wilt but a range of other diseases including also viruses as leaf spot, little leaf of brinjal and mosaic virus can damage the plants (Abe et al. 1974). Among insect pests, we find eggplant fruit and shoot borer, mites, whiteflies, aphids and others (Medakker and Vijayaraghavan 2007; Rotino et al. 1997). Furthermore, climate and climate change are a challenge and many farmers are faced with extreme temperatures, drought or flooding. Developing resistant or tolerant cultivars is therefore of great importance.

5.2.3 Genetic Improvement Strategies

Yield and quality reduction of eggplant results from a number of factors that will be intensified by breeding efforts of eggplant under climate change, including tolerance to adverse weather conditions, diseases, insects, nematodes and weeds. Little effort has been devoted to eggplant breeding and selection compared to other major vegetable crops (Daunay and Hazra 2012), mostly because production is overwhelmingly concentrated in developing countries where investments in breeding are often reduced. Current eggplant breeding programs aim to develop F₁ hybrids with high yield and fruit quality, nutritive value such as high dry matter, sugars, anthocyanins and total phenol contents, low level of polyphenol oxidase activity to avoid browning of cut fruits, and tolerance or resistance to abiotic and biotic stresses. Breeding for abiotic stress includes high temperature, salinity and drought, and biotic stress focuses on resistance to nematodes, wilt diseases such as *Ralstonia solanacearum*, *Fusarium oxysporum* f. sp. *melongenae*, *Verticillium dahliae*, and resistance to insect pests such as eggplant fruit and shoot borer (*Leucinodes orbonalis* Guenée), whitefly (*Bemisia tabaci* (Genn.)), two-spotted spider mites (*Tetranychus urticae* Koch), leafhopper (*Amrasca devastans* Distant), and aphids (*Aphis gossypii* Glover) (Taher et al. 2017). The most common breeding methods

for combining high production traits including resistance to biotic and abiotic stress are pure-line selection, pedigree method, bulk method, single-seed descent, a combination of bulk and pedigree methods, backcrossing and development of hybrids to exploit heterosis.

Exploitation of eggplant genetic resources and pre-breeding activities in using promising landraces, open-pollinated cultivars and wild relatives are high priority for eggplant breeders to develop introgression lines from wild species from the primary, secondary and tertiary gene pools for adaptation to climate change, and to broaden the genetic base (Daunay and Hazra 2012; Rotino et al. 2014). Eggplant is related to many crop wild relatives growing in a wide range of environmental conditions and other highly stressful environments, and found to be resistant or tolerant to some prevailing eggplant pests and diseases (Daunay and Hazra 2012; Syfert et al. 2016). At WorldVeg, considerable efforts have been done to evaluate WorldVeg's germplasm collection of eggplant, with more than 3200 accessions collected from 90 countries, for traits of economic importance, as well as resistance to biotic stress for identifying desired genotypes for use in eggplant breeding programs. Accessions with important traits such as early maturity, high yield, and resistance to bacterial wilt, two-spotted spider mites, eggplant fruit and shoot borer and aphids have been identified in the WorldVeg germplasm collection (Taher et al. 2017, 2019).

5.3 Germplasm Biodiversity and Conservation

5.3.1 *Germplasm Biodiversity*

Similar to other crops, the introduction of new high-yielding hybrid cultivars has led to the risk of losing agrobiodiversity. However, much has been safeguarded in gene banks (ex situ) around the world. Some collection holders also include crop wild relatives of cultivated eggplants. Tolerance to pest and diseases and to unfavorable growth conditions is most likely to be found in crop wild relatives. Wild eggplants are greatly underrepresented in ex situ conservation (gene banks and botanic gardens), as demonstrated by Castañeda-Álvarez et al. (2016). They showed that priority areas for future collecting missions should be in Africa and that 14 wild relative species of eggplant are threatened or near threatened. Many of these are concentrated in eastern Africa. While cultivated eggplants are represented by more than 6600 accessions in the global holdings, the sum of all wild eggplant species is 1300 accessions, and some species have only a few accessions (GENESYS 2019).

It is quite common among collection holders to carry out morphological descriptions of the accessions (characterization). Such data are about plant growth habit, stem characters, plant height, branching, leaf characters, inflorescence characters, and fruit characters such as fruit length, shape and color at various stages, maturity date, fruit flesh density, number of fruits per plant and seed data. Wide variation is



Fig. 5.2 Illustration photo of the diversity in the germplasm collection at the World Vegetable Center, Taiwan. (Photo Courtesy of World Vegetable Center)

present, for example at the World Vegetable Center collection (Fig. 5.2). Large variations in taste, fruit dry matter content, total sugar content, fiber content and antioxidant activity have been determined, but such studies have only been made on a limited number of accessions (AVRDC 1996; Hanson et al. 2006). Information on other important properties, such as yield stability and resistance traits is less common.

5.3.2 *Cultivar Characteristics and Phylogeny*

In brinjal eggplant, cultivar differences are characterized by fruit color, size, shape and height of the fruits, but also by differences in chemical composition of the fruits and agricultural properties such as yield and earliness. Geographical patterns, which reflect regional preferences, are common in this crop (Martin and Rhodes 1979; Sekara et al. 2007). Examples of different cultivars and their geographical areas is provided in Appendix 2. Occidental eggplant types (Western eggplants) are most common in Europe, the Americas and the Middle East but are also grown in Asia. Fruits are often oval or elongated and relatively large (200–600 g), most often dark purple. Oriental eggplants (Asian eggplants) are early and vigorous, fruits are in different colors, from purple, violet green, pink, white, and yellow, with or without stripes, often round or slender in shape. They are often sweet in taste, and tender and with a thin skin. In China, the preferred cultivars are often long, in various color but

sweet and tender. In Japan people prefer large and firm, sweet in taste, violet to purple and long or egg-shaped cultivars. In Thailand, cultivars usually have small fruits (40–100 g), and they are either round or very long. In India, a range of sizes, shapes and colors are preferred. Miniature eggplant includes cherry or finger types that are small, narrow or rounded, sweet in taste and tender in consistency with a thin skin but can vary in color, from dark purple to green and white, or striped.

5.3.3 Genetic Resources Conservation

According to the Global Gateway to Genetic Resources (GENESYS 2019) there are 5665 accessions of *Solanum melongena*, 798 of *S. aethiopicum* and 196 accessions of *S. macrocarpon*, conserved in different gene banks around the world. However, not all of the national collection holders report to this global gateway and the total number of accession is higher. Among the large collections not included in Genesys are those in India, like the National Bureau of Plant Genetic Resources, and in China, the Institute of Vegetables and Flowers. Among those reporting to Genesys, the largest collections are found at the World Vegetable Center, with 2209 accessions of *S. melongena*, 481 of *S. aethiopicum* and 63 accessions of *S. macrocarpon*. Other important eggplant collections are found at USDA-ARS and Centre for Genetic Resources, the Netherlands (Table 5.2).

Table 5.2 Major gene banks with significant brinjal eggplant collections

Holding institute and country	Number of accessions
World Vegetable Center, Taiwan http://www.avrdc.org/	2209
University of Georgia, USDA-ARS (USA) http://www.ars-grin.gov/ars/SoAtlantic/Griffin/pgrcu/	813
Centre for Genetic Resources, Netherlands http://www.cgn.wur.nl	373
Institute of Vegetable and Melon Growing, Ukraine	366
Embrapa Hortaliças, Brasil	346
Embrapa Recursos Genéticos e Biotecnologia, Brasil	265
Institute of Botany, Armenia	224
Institute for Plant Genetic Resources, Bulgaria	178
Universidad Politécnica de Valencia, Spain	169
Centro de Investigación y Tecnología Agroalimentaria, Spain	146

Source: GENESYS 2019

5.4 Traditional Breeding

Traditional breeding through sexual hybridization has been used in eggplant to improve agronomical traits by the production of F_1 hybrids including the exploitation of heterosis, and to introduce useful characteristics from its wild related species into the cultivated species by interspecific crosses (Blestos et al. 1998). More than 90% of eggplant cultivars produced by breeding companies are hybrids in the developed countries (Daunay and Hazra 2012). Considerable progress has been made in recent years by eggplant breeders in obtaining hybrids with increased yield, earliness, reduced prickliness and bitterness, and improved fruit quality. However, manual emasculation and pollination of the inbred parents is time-consuming and expensive. Therefore, incorporation of cytoplasmic male sterility (CMS) into breeding lines of eggplant would save time, effort and cost required for hybrid seed production. Male sterile lines have been developed by crosses between cultivated eggplant as male parent with wild species such as *Solanum violaceum* Ortega, *S. virginianum* L., *S. grandifolium* C.V. Morton and *S. anguivi* (Isshiki and Kawajiri 2002), followed by several generations of backcrossing to eggplant. Disease and pest resistance have been reported in many wild relatives belonging to primary gene pool species (*S. insanum*), secondary gene pool species such as *S. campylacanthum* Hochst., *S. incanum*, *S. lichtensteinii* Willd., *S. linnaeanum*, *S. anguivi* and *S. dasypodium*, *S. lidii* Sunding, *S. vespertilio* Aiton, *S. violaceum*, *S. tomentosum*, or *S. pyracanthos* Lam., and tertiary gene pool species such as *S. torvum*, *S. sisymbriifolium*, and *S. elaeagnifolium* (Daunay and Hazra 2012; Plazas et al. 2016; Rotino et al. 2014). However, breeding progress for disease and insect resistance is still limited except for some disease resistance such as bacterial wilt and *Fusarium* wilt.

5.4.1 Improvement Strategies

Breeding strategies to improve eggplant have been focused on higher yield, fruit shape, fruit size and color, and earliness. Other consumers' preference traits, such as low or high number of seeds (depending upon use), soft flesh, low solanine content, and fruit color retention in summer is also of importance. The resistance traits are also of importance, such as resistance to bacterial wilt, *Phomopsis* blight, little leaf, root knot nematodes, tolerance to eggplant fruit and shoot borer, whitefly and spider mites (Daunay and Hazra 2012). Although high levels of resistance to disease and insect pests are available in wild species, the linkage drag and various incompatibility barriers between cultivated and wild species are a major limitation for using wild species in eggplant breeding programs (Frary et al. 2007). Therefore, pre-breeding provides a solution and opportunity to broaden the genetic base of cultivated eggplant by exploiting genetic variability present in wild species and cultivated germplasm, and using it in breeding pipelines to develop new cultivars having high levels of resistance. Major pre-breeding activities in eggplant are (1) identification of desirable traits and crosses for development of mapping

populations for identification of genes from unadapted germplasm such as exotic landraces/wild species; (2) transferring these traits into well-adapted genetic backgrounds and development of pre-breeding materials; (3) characterization of pre-breeding materials for relevant traits of commercial interest and for resistance to biotic and abiotic stresses and (4) introduction of materials in eggplant breeding pipelines. Overall, these pre-breeding activities enable breeders to broadening the genetic base of cultivated eggplant exploiting the high diversity in wild relatives for further use in breeding pipelines.

In addition to pre-breeding, heterosis breeding and doubled haploid technology are used for eggplant improvement to develop cultivars/hybrids with preferred traits of commercial interest (Collonnier et al. 2001; Frary et al. 2007; Kashyap et al. 2003). Pal and Singh (1949) were early to report that hybrids in eggplant showed around 50% increased yields compared to the better parent. Mishra (1961) saw increased yields in hybrids due to the higher number of branches, increased fruit size, fruit number and fruit weight. Raina and Iyer (1973) found that the haplo-method has been successfully applied to many varieties or genotypes of eggplant since the first reports on androgenesis in eggplant. Salas et al. (2011) studied the androgenic response of 12 eggplant genotypes. The results revealed that the anthers of 11 of the 12 accessions produced somatic calli, and only 5 genotypes (ANS3, ANS26, Bandera, Ecavi, IVIA371) produced microspore-derived embryos with highly variable rates of 0.7–60.9 embryos/100 anthers. Embryos of responding accessions were initially haploid, and then reached doubled haploid status.

5.4.2 Traditional Breeding Methodologies

The most important breeding methods for eggplant improvement in the private and public sectors are pure line selection, pedigree method of selection, single seed decent, the backcross method and hybrid development for heterosis exploitation. Considerable heterosis for fruit yield, quality and resistance to diseases has long been reported in eggplant (Mishra 1961; Pal and Singh 1949). More recent works, such as those of Rodríguez-Burrueto et al. (2008) and Kaushik et al. (2018) reveal that hybrids between different eggplant varieties are generally heterotic for traits of vigor and yield, with high values for the ratio between general combining ability (GCA) and specific combining ability (SCA) and high values for broad-sense heritability. Because of these productive advantages of hybrids, the cultivation of F₁ hybrids is becoming more and more popular, especially where manual emasculation and pollination of eggplant are efficient in terms of number of seed obtained per hybridization performed.

In pure-line selection, individual plants are characterized at full fruiting stage for earliness, fruit shape, size, color, yield (visual estimate) plant type; resistance to diseases/pests are selected and harvested separately. This method is applicable in population varieties (landraces). In this method, individual plants are conserved within each landrace are evaluated and uniform progenies obtained by the selfing of each individual selected plant. Each of these progenies may constitute a pure line.

In pedigree selection, individuals from segregating generations, including F_2 or backcross generations obtained after hybridization, are selected and families are obtained by self-pollination of each selected plant. In subsequent cycles first families are selected and then seed obtained by selfing of the best individuals within each of these selected families is conserved and used for the next generation. In this system, a register of the pedigree of each individual plant is conserved and in new selection cycles, selection is performed based not only on traits of the individual plants, but also on pedigree records.

In the single seed descent method, starting from a segregating highly-heterozygous population, such as an F_2 , no selection is performed and a single seed is obtained from each individual and used to obtain the next generation. By repeating this scheme for several generations, a highly variable and homozygous population is obtained on which selection can be performed. In eggplant, it is generally considered that 6–7 generations of selfing are appropriate for this method. One advantage of this method is that no selection is performed until the population is fixed.

In the backcross method, one or a few genes are transferred via sexual hybridization to a recipient parent and selection of the plants carrying the desired trait are performed in each backcross generation. When the gene(s) to be transferred are dominant, selection can be performed directly in the backcross generations; if the gene(s) to be introduced are recessive, then progeny testing is required. This method is commonly used to transfer genes for resistance to diseases and insect pests into a recipient genotype with good productive and quality characteristics. In eggplant, in general 4–6 backcross generations are required for a successful introgression of the desired gene/s and a recovery of all the characteristics of interest of the recipient parent.

5.4.3 Traditional Breeding Limitations

Classical eggplant breeding has been hampered by several factors such as sexual incompatibilities for the crosses between cultivated eggplant and wild relatives, and for the fact that most agronomic traits are under polygenic control, the labor-intensive aspects of hybrid seed production, and association of resistance with horticulturally detrimental traits associated with the wild species (linkage drag) (Frary et al. 2007). In addition, there is limited information about genetic control of the most important traits, particularly in resistance to diseases and insect pests. Biotechnology methods such as protoplast culture and genetic engineering can be used to overcome sexual incompatibilities for the crosses between cultivated eggplant and wild relatives as described below. Screening eggplant germplasm for resistance to diseases and insect pests is laborious and time consuming, particularly for genetic studies of traits that require a relatively large plant population.

5.4.4 Role of Biotechnology

Biotechnology protocols including embryo culture, protoplast culture, organogenesis, somatic embryogenesis, somaclonal variation, haploidization and genetic transformation, have been used successfully to introduce resistance traits into the cultivated eggplant (Collonnier et al. 2001; Kashyap et al. 2003). Eggplant is attacked by many diseases and parasites, nematodes and insects and sources of resistance to biotic stresses have been identified mostly in wild relatives of eggplant including *Solanum sisymbriifolium*, *S. aethiopicum*, *S. macrocarpon*, *S. aculeatissimum* Jacq. and *S. torvum* (Daunay et al. 1991). Crosses between cultivated eggplant and wild relatives have resulted in limited success due to sexual incompatibilities. Both embryo culture and somatic hybridization through chemical and electrical fusion procedures have been used to overcome potential sexual barriers in order to facilitate the introduction of resistance traits from wild relatives into cultivated eggplant. Resistance to bacterial and fungal wilts has successfully been introduced into the cultivated eggplant through somatic hybridization. However, breeding efforts have been hampered by sterility or low fertility in the interspecific hybrids developed (Collonnier et al. 2001; Gleddie et al. 1986; Kashyap et al. 2003; Rattan et al. 2015). Anther and microspore cultures are common techniques to obtain doubled haploid (DH) plants in vegetable crops including eggplant. Production of DH lines through androgenesis and microspore culture is a powerful approach to overcome the limitations of classical breeding techniques for pure line generation for producing uniform F₁ hybrid seed of eggplant commercial cultivars. In eggplant, in vitro androgenesis has been commonly employed for the last 40 years. However, few studies have been conducted on anther culture on interspecific hybrids developed from the crosses between cultivated eggplant and wild relatives. Furthermore, tolerance to salinity, resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say), eggplant fruit and shoot borer (*Leucinodes orbonalis*), and root knot nematode *Meloidogyne incognita* have been engineered in eggplant (Collonnier et al. 2001) and successfully released in some countries such as Bangladesh (Shelton et al. 2018).

5.5 Molecular Breeding

5.5.1 Marker-Assisted Breeding

Utilization of molecular markers for marker-assisted breeding (MAS) has depended on the availability of markers linked to traits. Although markers that do not require previous knowledge on genomic information, markers such as RFLPs, RAPDs or AFLPs, have been used in developing markers for MAS, the advent of next-generation-sequencing (NGS) techniques has facilitated the availability of molecular markers, mostly SSRs and SNPs, for MAS in eggplant (Gramazio et al. 2018).

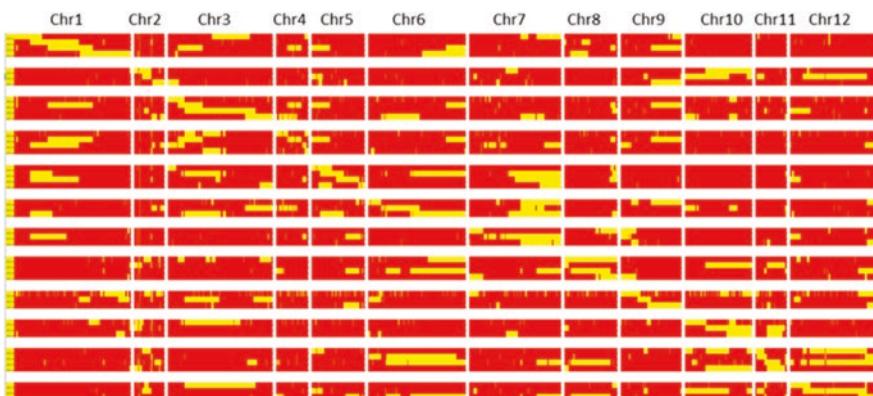
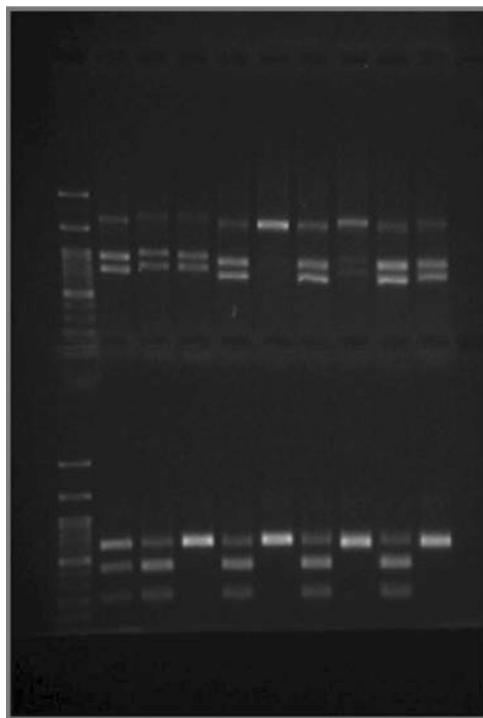


Fig. 5.3 Graphic representation of the genotypes obtained with SPET genotyping of plants of a BC3 generation between eggplant (recurrent parent) and *Solanum insanum* (donor parent) used for a development of introgression lines program. Red cells represent the recurrent allele, while yellow cells are heterozygotes containing introgressions from the *S. insanum* donor parent. Each of the 12 vertical blocks represents one chromosome. (Photo by Jaime Prohens)

Dominant and codominant markers have been developed for MAS in eggplant for different traits and using different methodologies, such as bulked segregant analysis, conventional genetic mapping based on segregating populations, or through GWAS, for targeting both major genes and QTLs (Barchi et al. 2012; Cericola et al. 2014; Daunay and Hazra 2012; Doganlar et al. 2002; Frary et al. 2003, 2014; Gramazio et al. 2018; Portis et al. 2014, 2015; Toppino et al. 2016). Below, we report, according to the type of trait, some relevant cases of marker development for MAS in eggplant. Also, in addition to being used for selection of plants carrying specific genes and/or QTLs, MAS has also been used in eggplant to develop introgression lines (Fig. 5.3), including the first set of introgression lines in eggplant, covering a relevant part of the genome of *S. incanum* (Gramazio et al. 2017), as well as to identify the cytoplasm of wild species in alloplasmic materials of eggplant (Fig. 5.4).

Multiple QTL studies, based on segregating populations in intraspecific and interspecific crosses, as well as on association mapping in germplasm collections, have been used to detect over 200 QTLs associated to many other traits of morphological and agronomic interest, such as plant, leaf, flower and inflorescence, and fruit characteristics. In this way, Barchi et al. (2012), Portis et al. (2014) and Toppino et al. (2016) have used an intraspecific F₂ population grown at two locations to detect multiple QTLs for traits related to morphology, yield, and fruit color, with different percentages of variation explained. In many cases, QTLs for related traits (prickliness or anthocyanin presence in different parts of the plants, yield traits) clustered together, revealing a likely pleiotropic effect of some QTLs. In this way, several QTLs with a major effect clustered together in the same genomic region in chromosome 2 (Portis et al. 2014). A similar situation occurred for several QTLs for anthocyanin content, which mapped in the same region of chromosome 10 (Barchi

Fig. 5.4 Agarose gel (2%) with the digestion with enzymes BamHI (above) and AflIII (below) of a fragment of chloroplastic DNA from F_1 individuals obtained from reciprocal backcrosses between *Solanum melongena* and a wild relative. The sheared fragments correspond to the wild species chloroplastic DNA, identifying the individuals with wild species cytoplasm. (Photo by Jaime Prohens)



et al. 2012). In an interspecific F_2 population between *Solanum linnaeanum* and *S. melongena*, Doganlar et al. (2002) and Frary et al. (2003, 2014) also found multiple QTLs for morphological traits with a wide range of percentages of variation explained and again many of the QTLs clustered together. Other studies, such as those of Ge et al. (2013), Cericola et al. (2014) and Portis et al. (2015) used a GWAS approach in eggplant germplasm collections and also discovered an important number of QTLs for morphological traits, which in many cases validated and confirmed the QTLs already discovered in mapping populations, but also led to the discovery of new QTLs.

Like in many other crops, a major effort for eggplant is underway to develop markers associated to resistance to diseases. Three of the most devastating diseases for eggplant are bacterial wilt (*Ralstonia solanacearum* species complex), *Fusarium* wilt (*Fusarium oxysporum*), and *Verticillium* wilt (*Verticillium dahliae*). In this way, Salgon et al. (2017, 2018), used GBS for genotyping an intraspecific doubled haploid eggplant population, which was phenotyped for resistance to two strains of *R. pseudosolanacearum*, and confirmed a previously major QTL detected in a RIL population of the same cross (Lebeau et al. 2013) and detected several new QTLs, spread out in several chromosomes, some of which were stable. *Fusarium* wilt resistance was introduced to brinjal eggplant from the scarlet eggplant *Solanum aethiopicum* through somatic hybridization (Toppino et al. 2008) followed by back-cross breeding schemes involving anther culture at a certain stage to recover the

diploid state ($2n = 2x = 24$) of eggplant. Segregation results were compatible with a major resistance gene (*Rfo-sa1*) and a BSA approach was used to detect several RAPDs markers that were transformed into CAPS markers linked to *Rfo-sa1*. Subsequently, Barchi et al. (2018) developed a genetic map in an F_2 intraspecific population where one of the parents was one line carrying *Rfo-sa1* and detected a major QTL (almost 70% of the variance explained) co-locating with *Rfo-sa1*. In addition, these authors found another minor QTL in chromosome 11. Using different materials, Mutlu et al. (2008) screened several segregating generations and found a codominant SRAP, a dominant SRAP-RGA and a RAPD marker linked to a major gene used for resistance to *Fusarium*. These same authors converted the SRAP marker in two SCAR markers. Miyatake et al. (2016) using interspecific crosses of eggplant, which among two other sources of resistance included as parent the same resistance source used by Mutlu et al. (2008), found two alleles for a major QTL *Fm1^L* and *Fm1^E* that mapped in the same genomic region than *Rfo-sa1*. An additional QTL coming from a different source of resistance was found in chromosome 4 (Miyatake et al. 2016). Regarding *Verticillium* wilt, Liu et al. (2015) introgressed resistance from *S. linnaeanum* in the genetic background of eggplant through a backcross breeding program with phenotypic selection for tolerance to *Verticillium* wilt and developed a marker for the tomato *Ve* gene homolog in eggplant, which was found to be linked to the resistance to *Verticillium* wilt. More recently, Barchi et al. (2018) used an F_2 intraspecific population found three QTLs with moderate effect for *Verticillium* wilt resistance at different times after inoculation (one in chromosome 8 for early response and two in chromosomes 5 and 9 for late response).

Markers have also been developed for other important traits for which the evaluation requires considerable effort. In this way, parthenocarpy is an important agro-nomic trait in eggplant as it allows fruit set without the need of pollination and fertilization (Daunay and Hazra 2012). Using F_2 populations derived from crosses between two non-parthenocarpic lines and a parthenocarpic line, were able to detect two QTLs in chromosomes 8 and 3. Also, for fruit composition traits, Toppino et al. (2016) found several QTLs for anthocyanins, glycoalkaloids, sugars and organic acids, although in general with the exception of anthocyanins, where two major QTLs were found for nasunin and D3R in the same genomic region of chromosome 5, the percentage of variation explained was low to medium.

5.5.2 Genomics

Despite its economic importance, eggplant has lagged behind other major *Solanum* crops such as tomato and potato in the development of genomics. A first eggplant genome draft of cv. Nakate-Shinkuro was published in 2014 (Hirakawa et al. 2014) and a new genome assembly, in this case of line cv. 67/3 of much better quality was recently developed by Barchi et al. (2018). Apart from the de novo sequencing of these two common eggplant varieties, very recently, a genome draft of the scarlet

eggplant (*S. aethiopicum*) has become available (Song et al. 2019). These genome sequences are of great utility for multiple plant breeding applications (Gramazio et al. 2018).

Regarding resequencing projects of common eggplant, to our knowledge, there is just a single study involving the resequencing of seven eggplant cultivars, which together with a *Solanum incanum* accession, are the parents of a MAGIC population (Gramazio et al. 2019). This resequencing project allowed the discovery of over nine million SNPs in the eight parents. Within the framework of the sequencing project of the scarlet eggplant *S. aethiopicum*, 65 accessions of this species have been resequenced, which has revealed an expansion of resistance genes in the last millions of years and the selection of genes for drought tolerance in some cultivar groups (Song et al. 2019).

Transcriptomic and RNA sequencing studies have also been performed in the eggplant gene pool, and again, the number of transcriptomes from the cultivated brinjal eggplant *Solanum melongena* is relatively low (Gramazio et al. 2018). In this way, a de novo transcriptome of one eggplant accession (SRR1104129) was obtained by Yang et al. (2017). These authors found a number of unigenes similar to the one obtained from the genome annotation. An additional *S. melongena* transcriptome was released by Ramesh et al. (2016) to identify putative allergens in the eggplant fruit. Regarding other cultivated species, the transcriptome of *S. aethiopicum* was sequenced by Gramazio et al. (2016) and used to develop a large number of markers. Apart from *S. melongena*, other transcriptomes of wild eggplant relatives are available. In this way, the transcriptomes of four wild species of interest for eggplant breeding for their resistance to diseases (*S. aculeatissimum*, *S. sisymbriifolium*, *S. torvum*) and tolerance to drought (*S. incanum*) have been sequenced (Gramazio et al. 2016; Wixom et al. 2018; Yang et al. 2017; Zhou et al. 2016).

5.6 Tissue Culture Application

The application of tissue culture (Fig. 5.5) such as organogenesis, somatic embryogenesis, anther culture and protoplast culture, is of great interest to overcome constraints of conventional breeding and development of disease free plant in eggplant (Kashyap et al. 2003; Khatun et al. 2006; Magioli et al. 2001).

5.6.1 Organogenesis

The induction of plant regeneration via in vitro organogenesis from various explants such as leaf, stem, cotyledon, hypocotyl (Fig. 5.6), epicotyl and root has been developed in cultivated eggplant and wild varieties as well as their hybrids (Calvo-Asensio et al. 2014; Franklin et al. 2004; Sharma and Rajam 1995; Zayova et al. 2012). Shoot regeneration was obtained from hypocotyl segments of *Solanum*

Fig. 5.5 In vitro microppropagation of an interspecific hybrid between eggplant and *Solanum elaeagnifolium*. (Photo Courtesy of Jaime Prohens)

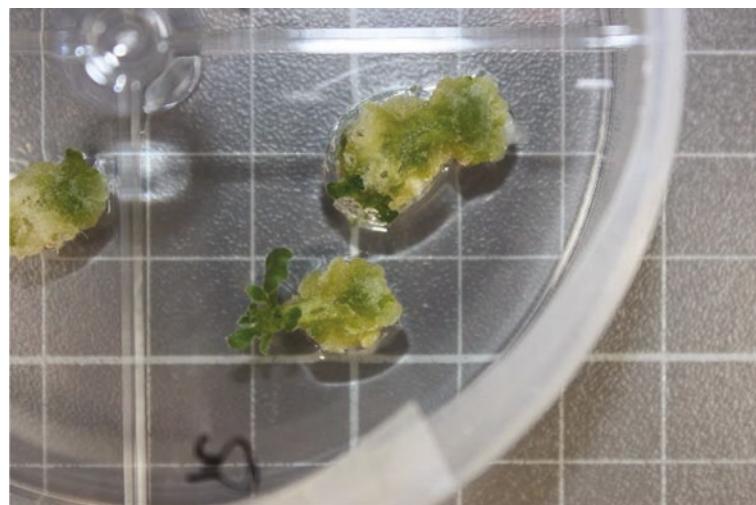


Fig. 5.6 Organogenesis in a hypocotyl explant of eggplant. (Photo Courtesy of Jaime Prohens)

melongena and the F₁ hybrids in the presence of cytokinins, kinetin and zeatin (Kamat and Rao 1978); zeatin riboside and indole-3-acetic acid (Muktadir et al. 2016). Organogenesis has been also achieved in eggplant wild relatives, such as *S. aviculare* G. Forst., *S. acuminatissimum* Merr. & L.M. Perry, *S. sisymbriifolium*, *S. indicum* and *S. torvum* (Fassuliotis 1975; Kashyap et al. 1999; Kowalozyk et al. 1983; Rao and Narayanaswami 1968).

5.6.2 Somatic Embryogenesis

Somatic embryogenesis involves obtaining embryos from somatic plant tissues. Eggplant somatic embryogenesis studies using different growth regulators and the various explants including leaves, cotyledons, zygotic and hypocotyls have been reported (Kaur et al. 2013; Magioli et al. 2001; Yadav and Rajam 1998). Yamada et al. (1967) were the first to report somatic embryogenesis in eggplant. Zygotic embryos were cultured on MS supplemented with indole-3-acetic acid (IAA). Somatic embryogenesis of eggplant also was initiated from intact seedlings when seeds were cultured on medium containing 6-benzylaminopurine (BAP) or thidiazuron (TDZ) (Kaparakis and Alderson 2002). Somatic embryogenesis is a method commonly used in large-scale production of synthetic seeds (Stuart et al. 1987). In eggplant, synthetic seeds have also been developed by encapsulating somatic embryos in sodium alginate and calcium chloride (Huda et al. 2007; Kashyap et al. 2003; Mariani 1992). In this way, by using sucrose at 1% as a carbon source, germination rates of 90% or higher were obtained for the synthetic seeds (Huda et al. 2007).

5.6.3 Anther Culture

Anther culture technique (Fig. 5.7) is applied in eggplant as an effective tool to obtain androgenic haploid and double-haploid (DH) plants (Gemesne et al. 1998). Haploid and diploid plant production have been of great value to breeders to overcome the limitations of classical breeding when it comes to producing new pure lines from a varied gene pool (Dunwell 2010; Germana 2011). The first in vitro haploid eggplant production via anther culture was obtained by Raina and Iyer (1973). Subsequently, researchers have used various media and treatments to produce DH plants (Alpsoy and Seniz 2007; Rotino 1996; Rotino et al. 1991; Salas et al. 2011, 2012; Seguí-Simarro et al. 2011; Tuberosa et al. 1987). Microspore embryogenesis is also a powerful tool to produce doubled haploids (Rivas-Sendra et al. 2015). Isolated microspore culture (IMC) in eggplants was first reported by Gu (1979). Miyoshi (1996), and Corral-Martínez and Seguí-Simarro (2012) carried out

Fig. 5.7 Haploid being regenerated from anther culture of eggplant. (Photo Courtesy of Jaime Prohens)



extensive studies and developed protocols of microspore culture in eggplant. Although isolated microspore cultures have several advantages over anther culture, such as that no regeneration can take place from somatic tissues, avoiding the negative effects of the tapetal layer secretions or of the barrier of the thick eggplant anther to the access of media components reaching the anther locule; this technique is still poorly explored in eggplant (Corral-Martínez and Seguí-Simarro 2014).

5.6.4 Embryo Rescue

Embryo rescue is one of the oldest and most successful in vitro tissue culture techniques used to promote the development of an immature or weak embryo into a viable plant and is commonly used for producing plants from hybridizations that produce unviable/aborted seeds (Reed 2004). The successful production of plants from the cultured embryos largely depends upon the maturation stage and the composition of the medium. Sharma et al. (1980) successfully rescued hybrid embryos from crosses of *Solanum melongena* and *S. aculeatissimum*; the hybrids were obtained when embryos (25 days old) were cultured on Nitsch and Nitsch (1969) medium. In another study, hybrids were developed with *S. torvum* and *S. sisymbriifolium* through embryo rescue (Blestos et al. 1998). The hybrids were obtained when immature ovules were cultured on MS medium for 50 days. Numerous other researchers attempted embryo culture technique to produce interspecific hybrids between cultivated eggplant and its wild relatives (Kumchai et al. 2013; Plazas et al. 2016; Rattan et al. 2015). The chances of successful embryo rescue become higher when *S. melongena* is used as a female parent.

5.6.5 Protoplast Culture and Somatic Hybridization

Protoplast culture is an excellent system for studying the structure and function of cell organelles, cytoplasmic membrane transport in plants and cell wall formation (Fournier et al. 1995). Sihachakr and Ducreux (1987) were able to isolate protoplasts from eggplant mesophyll cells using both cytokinin and auxin. Plant regeneration from isolated protoplasts of *Solanum aculeatissimum*, *S. aviculare*, *S. torvum* and *S. sisymbriifolium* has been achieved (Gleddie et al. 1985; Guri et al. 1987). Somatic hybridization by protoplast fusion has become an important tool to overcome the limitation of conventional sexual hybridization. The first successful somatic hybrid of *S. melongena* with *S. sisymbriifolium* was produced by Gleddie et al. (1986). The fusion was assured by the use of polyethylene glycol. Since then, tremendous progress has been made in protoplast fusion. Somatic hybrids were also developed by fusing protoplasts of *S. melongena* with *S. aculeatissimum* (Sihachakr et al. 1988), *S. aethiopicum* (Daunay et al. 1991; Rotino et al. 2001), *S. torvum* (Guri and Sink 1988a; Jarl et al. 1999) and *S. nigrum* (Guri and Sink 1988b). Protoplast fusion between interspecific tomato hybrid (*Solanum lycopersicum* and *S. pennellii* Corr.) and eggplant was developed, but the somatic hybrids obtained exhibited flower abscission. Somatic hybridization was also successfully used to transfer resistance traits into the cultivated eggplant. Somatic hybridization was used between *S. melongena* and *S. sanitwongsei* for production of fertile hybrids that showed resistance to *Pseudomonas solanacearum* (Asao et al. 1994). Another example was provided by Collonnier et al. (2001) who succeeded in producing somatic hybrids between *S. melongena* and *S. aethiopicum* groups *aculeatum* and *gilo*, and found that all hybrids were fertile. Of these, 16 were tested tolerant against bacterial wilt. Furthermore, Collonnier et al. (2003) found that interspecific somatic hybrids of eggplants and *S. sisymbriifolium* were more resistant than eggplant parents to *Ralstonia solanacearum* bacteria. Since somatic hybrids between eggplant and its wild relatives are tetraploid, anther culture is used for the development of dihaploids (Rizza et al. 2002; Rotino et al. 2005; Toppino et al. 2008).

5.7 Genetic Engineering and Gene Editing

Eggplant is prominent among vegetable crops in the commercial use of transgenic materials. In this way, an eggplant expressing the *Bacillus thuringiensis* (Bt) *cry1Ac* gene developed by the Maharashtra Hybrid Seed Company is currently used with great success in Bangladesh, mostly to control the eggplant fruit and shoot borer (*Leucinodes orbonalis*), which is a devastating pest of eggplant in southeast Asia (Shelton et al. 2018). This transgenic eggplant has had a rapid adoption by farmers, as it allows an effective control of eggplant fruit and shoot borer without the need to spray pesticides.

Since the first report of the development of transgenic eggplant plants in the late 1980s, there have been a large number of reports dealing with genetic transformation of eggplant for several traits of agronomic interest (Rotino et al. 2014), demonstrating that eggplant is amenable to genetic transformation. Apart from initial works on developing methodologies for the genetic transformation of eggplant (Guri and Sink 1988c; Rotino and Gleddie 1990), many works have been devoted to the development of transgenic plants tolerant to pests such as the eggplant fruit and shoot borer and the Colorado potato beetle (*Leptinotarsa decemlineata*) using different Bt *cry* genes (Acciarri et al. 2000; Arpaia et al. 1997; Pal et al. 2009). Partial resistance to other pests, such as the aphids *Myzus persicae* and *Macrosiphum euphorbiae* has been obtained using oryzacystatin, which is a protein inhibitor of rice (Ribeiro et al. 2006). Resistance to nematodes has been obtained in eggplant transformed with the tomato *Mi-1.2* gene (Goggin et al. 2006) and a modified rice cystatin (Papolu et al. 2016).

Regarding disease resistance, *Verticillium* wilt resistance has been observed by overexpressing a yeast Δ -9 desaturase gene (Xing and Chin 2000), while resistance to *Botrytis cinerea* by the expression of the gene *Dm-AMP1*. Enhanced resistance to *Alternaria solani* was obtained by the expression of the *Wasabi defensin* gene (Darwish et al. 2014). Also, the expression of rice chitinase or alfalfa glucanase genes conferred resistance of eggplant to *Fusarium oxysporum* and *Verticillium* wilt (Singh et al. 2014, 2015). Interestingly, as pointed out by Rotino et al. (2014), transgenic eggplants expressing the *mtlD* gene, which confers tolerance to some abiotic stresses, such as salinity drought and chilling (Prabhavanthi et al. 2002), resulted in increased resistance to several fungi, such as *Fusarium oxysporum*, *Rhizoctonia solani* and *Verticillium dahliae*. Regarding resistance to viruses, the expression in eggplant of the *Sw-5* gene, which confers resistance to tomato spotted wilt virus in tomato, resulted in resistance to the related tomato chlorotic spot virus (Picoli et al. 2006), while the expression of the coat protein of cucumber mosaic virus resulted in resistance to this virus (Pratap et al. 2011).

Apart from the use of gene *mtlD* indicated previously (Prabhavanthi et al. 2002), tolerance to abiotic stresses has been obtained by using the expression of other genes such as the yeast gene *HAL1* (Kumar et al. 2014). Tolerance to drought and cold stress of eggplant was improved by the expression of the *Arabidopsis AtCBF3* and *AtCOR15A* genes (Wan et al. 2014), as well as by the overexpression of the *IPT* gene (Xiao et al. 2017). Also, the development of parthenocarpic eggplants able to set fruits without pollination and at low temperatures has been obtained by the expression of the *iaaM* gene under the control of an ovule specific promoter (Donzella et al. 2000). In addition, these parthenocarpic fruits, due to the lack of seeds, have an increased quality (Rotino et al. 2014). Parthenocarpic fruits have also been obtained in eggplant transgenic materials harboring RNA interference of the transcription factor *SmARF8* (Du et al. 2016).

Development of reversible male sterile plants has also been obtained using different approaches. In this way, Cao et al. (2010) used a *Barnase* gene under a tapetum-specific promoter to induce pollen sterility, which could be reversed when crossing with *Cre* expressing transgenic eggplants. Another transgenic approach to

obtain reversible male sterility was developed by Toppino using microRNA-mediated silencing of endogenous TBP-associated factors genes in the anthers. Use of an ethanol-inducible alternative form of the TBP-associated factors allowed fertility recovering after an application of short treatments with ethanol (Toppino et al. 2011).

Gene editing has not yet been used extensively in eggplant. However, some works are being performed to knock-out four PPO genes expressed in the fruit flesh in order to develop eggplant materials with reduced browning (Gianoglio et al. 2018).

5.8 Mutation Breeding

Induced mutation in crop improvement is called *mutation breeding*. Mutation breeding has been used since the 1930s. This breeding method is not considered to produce genetically modified organisms, which might accelerate the process of developing new eggplant cultivars with valuable agronomic traits, disease resistance, and tolerance to biotic stress, and broadening biodiversity in vegetable crops including eggplant. Mutation breeding is a cost effective method, ubiquitously applicable for both oligogenic and polygenic traits, non-hazardous and environmentally friendly. However, breeders have to evaluate a large plant population to select desirable traits, the frequency of desirable traits are very low and might be associated with undesirable side effects, and mutations are often recessive and produce pleiotropic traits. Globally, more than 3200 mutant varieties have been released for commercial use in more than 210 plant species across the world, including five eggplant cvs. (Daijiro, Floralba, Macla, Picentia, PKM 1) obtained by different mutagenesis methods from three countries (India, Italy, Japan), as referenced in the Mutant Varieties Database (<https://nucleus.iaea.org/Pages/mvd.aspx>).

Breeders can induce mutations in seeds, cuttings, or the shredded leaf of plant (tissue) through chemical or physical mutagens, followed by selection for targeted traits in the resulting mutants. Chemical mutations are generally induced by compounds such as ethyl methane sulfonate (EMS), diethyl sulfate (DES), methyl methane sulfonate (MMS), hydroxyl amine and nitrous acid. Physical mutagens such as gamma ray irradiation, x-rays and others (Ahloowalia and Maluszynski 2001) can also induce genetic diversity that is useful in plant breeding. Eggplant has a narrow genetic base, and breeders can use mutation induction to broaden the genetic base of germplasm. In addition, mutant lines can be used directly as new cultivars or as sources of new variation in breeding programs. Gamma irradiation has been used for improving both qualitative and quantitative traits of many crops, but there have few breeding efforts using mutations for eggplant improvement worldwide. Srivastava and Roy (1981) tested 5–40 krad gamma radiation effects on eggplant and found that cv. KT3 was affected with 100% lethal under 40 krad gamma radiation, but it was 73% for cv. BG. In African eggplant (*Solanum aethiopicum*), 40 Gy and 60 Gy doses of gamma irradiation were appropriate for creating beneficial traits (Titus et al. 2018).

5.9 Hybridization

Cultivated eggplants are self-pollinated, but outcross pollination may occur especially during high activities of pollinator insects such as bumblebees (*Bombus* spp.) and honeybees (*Apis* spp.). Under these conditions of high pollinator activity, cross-pollination may reach 60–70%. Eggplant is a day neutral plant, and the optimum temperatures for fruit setting are 21–27 °C. Anthesis in the eggplant flower starts in the early morning and continues for a couple of hours. Pollen dehiscence starts a little later. The stigma is most receptive at the time of flower opening. Anthesis and dehiscence are influenced by factors such as temperature, humidity and light. Concerning interspecific hybridization, emasculation and hybridizations should be made in the early morning (7–9 am) to avoid the hours of higher temperatures. The anthers can be removed by forceps, and the flowers should be covered immediately with a paper bag to avoid the contamination of foreign pollen (Fig. 5.8). Plazas et al.

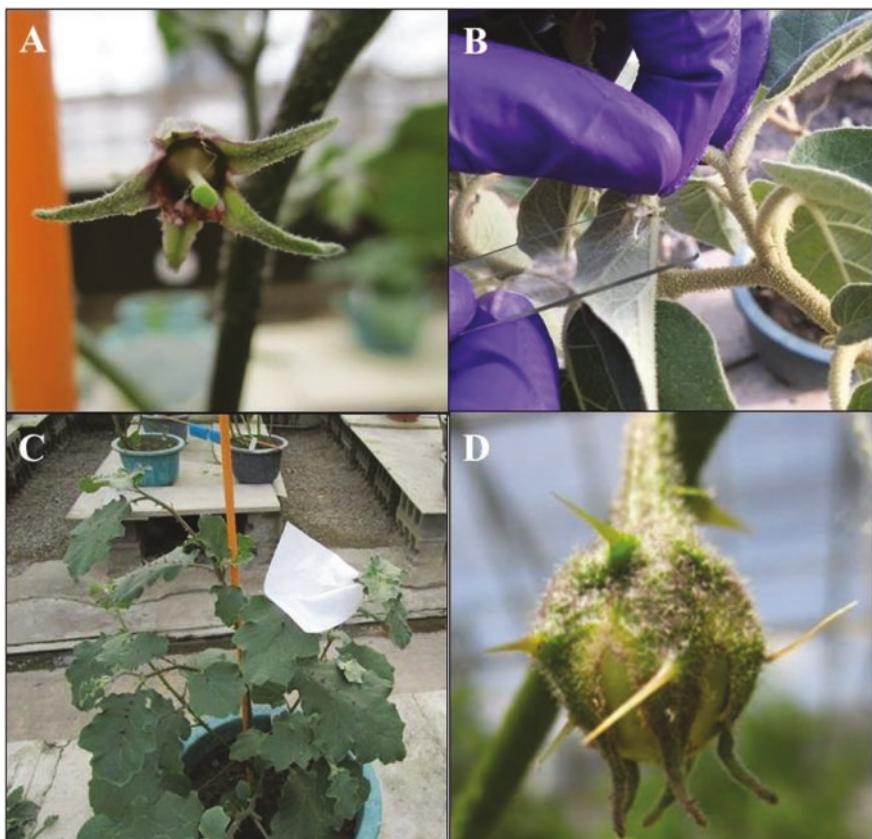


Fig. 5.8 Interspecific hybridization between eggplant and wild relatives: (a) Emasculated female bud 1 day before anthesis; (b) Artificial pollination; (c) Pollinated female bud covered by paper bag to avoid the contamination; (d) Early stage fruit after successful pollination. (Photos Courtesy of Ahmed Namisy, a master's student at Kafrelsheikh University, Egypt)

(2016) confirmed that cultivated eggplant is amenable to interspecific hybridization with a large number of wild species, including wild species from the primary, secondary and tertiary gene pools. Crosses between eggplant and primary gene pool species *Solanum insanum*, provides fertile hybrids with eggplant (Knapp et al. 2013). Crosses with secondary gene pool species give interspecific hybrids with different degrees of fertility when they are hybridized with eggplant (Daunay and Hazra 2012; Rotino et al. 2014). Finally, crosses between eggplant and the tertiary gene pool species such as *S. torvum* and *S. sisymbriifolium* may be possible to obtain sterile or low fertility hybrids after embryo rescue or somatic hybridization (Daunay and Hazra 2012; Rotino et al. 2014). These wild species are of interest for eggplant breeding as they are genetically very diverse and could provide tolerance to abiotic stresses, as well as resistance to pests and diseases (Daunay and Hazra 2012; Plazas et al. 2016).

5.10 Conclusion and Prospects

Brinjal eggplant has large economic importance, especially in Asian and Mediterranean countries. In this chapter, we provided an overview of both traditional breeding methods and new developments in biotechnology and marker-assisted techniques in eggplant. There are many activities going on in various parts of the world and at a range of institutions (Appendix 1). Current breeding research is often aimed at producing F₁ hybrids and to identify markers and QTLs associated with important traits to speed up breeding programs. Germplasm evaluation and phenotyping are ongoing; however, a large proportion of the global gene bank holdings remains uncharacterized. Little effort has been made to evaluate crop wild relatives for resistance and tolerance traits. Crop wild relatives are also highly underrepresented in gene banks, especially secondary and tertiary gene pool species. Hybridization is possible from such species but there is a need for more knowledge to secure fertile hybrids after embryo rescue or somatic hybridization. We foresee a need for more joint pre-breeding projects on a regional or global scale, which could include trait discovery but also development of pre-breeding materials and markers to introgress key traits into high-yielding genetic backgrounds. We also expect that genomics studies in eggplant will be of great utility in eggplant breeding.

Acknowledgements This work was undertaken as part of the initiative Adapting Agriculture to Climate Change: Collecting, Protecting and Preparing Crop Wild Relatives, which is supported by the Government of Norway. The project is managed by the Global Crop Diversity Trust with the Millennium Seed Bank of the Royal Botanic Gardens, Kew. It is implemented in partnership with national and international gene banks and plant breeding institutes around the world. For further information, see <http://www.cwrdiversity.org/>. Funding was also received from the European Union's Horizon 2020 Research and Innovation Programme (grant agreement No. 677379, G2P-SOL project, Linking genetic resources, genomes and phenotypes of Solanaceous crops). We are also thankful for grants from Spanish Ministerio de Ciencia, Innovación y Universidades, Agencia Estatal de Investigación and Fondo Europeo de Desarrollo Regional/European Regional

Development Fund (grant RTI-2018-094592-B-100 from MCIU/AEI/FEDER, UE). Long-term strategic donors to the World Vegetable Center are Republic of China (Taiwan), UK aid (the UK government), United States Agency for International Development (USAID), Australian Centre for International Agricultural Research (ACIAR), and Germany, Thailand, Philippines, Korea and Japan.

Appendices

Appendix I: Important Institutions for Eggplant Research

Institutions	Research area	Location and website
Asia		
World Vegetable Center (AVRDC)	Germplasm evaluation, crop wild relatives	Tainan, Taiwan https://avrdc.org/
National Pingtung University of Science and Technology, Department of Tropical Agriculture and International Cooperation	Breeding research	Pingtung, Taiwan http://dtaic.npu.edu.tw/
Nanjing Agriculture University	Resistance research	Nanjing, China http://english.njau.edu.cn/
Wuhan Vegetable Research Institute, Wuhan Academy of Agricultural Science and Technology	Biotechnology	Wuhan, Hubei, China http://www.chinaavf.com/
Shanghai Jiao Tong University, School of Agriculture and Biology	Biotechnology	Shanghai, China http://www.agri.sjtu.edu.cn/
National Institute of Vegetable and Tea Science NARO	Biotechnology	Tsu, Japan http://www.naro.affrc.go.jp/
International Center for Tropical Agriculture (CIAT)	Crop wild relatives	Cali, Colombia https://ciat.cgiar.org/
Indian Council of Agricultural Research (ICAR)	Breeding research, biotechnology	New Delhi, India https://icar.org.in/
Indian Institute of Science, Department of Microbiology and Cell Biology	Biotechnology, gene technology	Bangalore, India http://mcbl.iisc.ac.in/
Chaudhary Charan Singh University, Department of Genetics and Plant Breeding	Breeding research	Meerut, India http://ccsuniversity.ac.in/
Pondicherry University, Department of Ecology and Environmental Sciences	Crop diversity, crop wild relatives	Kalapet, Pondicherry, India http://www.pondiuni.edu.in/

(continued)

Institutions	Research area	Location and website
University of the Philippines Los Baños, College of Agriculture and Food Science	Breeding research	Laguna, Philippines http://uplb.edu.ph/
Bati Akdeniz Agricultural Research Institute (BATEM), Department of Vegetable Crops and Ornamentals	Breeding research, genetic resources, diseases	Antalya, Turkey
Izmir Institute of Technology, Department of Molecular Biology and Genetics.	Molecular markers, breeding research, bioactive compounds	Izmir, Turkey https://en.iyte.edu.tr/
Horticultural Crop Research and Development Institute	Breeding research	Peradeniya, Sri Lanka http://doa.gov.lk/ HORDI/en/
Americas		
The Boyce Thompson Institute for Plant Research	Biotechnology, gene transformation	Ithaca, NY, USA
Genetic Improvement for Fruits & Vegetables Laboratory, Beltsville Agricultural Research Center	Breeding, phenolics, diversity	Beltsville, Maryland, USA https://btiscience.org/
Cornell/NYSAES, Department of Entomology	Resistance research	Geneva, New York, USA https://entomology. cals.cornell.edu/
Department of Plant Pathology, Physiology, and Weed Sciences, Virginia Tech.	Resistance research	Blacksburg, Virginia, USA https://www.ppws.vt. edu/
Department of Biological Sciences, Boise State University	Crop wild relatives, cultivation	Boise, Idaho, USA https://www.boisestate. edu/biology/
Department of Evolution, Ecology, and Organismal Biology, Ohio State University	Crop diversity, crop wild relatives	Columbus, Ohio, USA https://eeob.osu.edu/
Europe		
Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València	Crop wild relatives, pre-breeding, biotechnology, molecular markers, phenotyping	Valencia, Spain https://www.upv.es/ entidades/COMAV/
Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universitat Politècnica de València	Biotechnology, breeding research	Valencia, Spain http://www.ibmcpcsic. es/en
CREA- Consiglio per la Ricerca in Agricoltura e l'Analisi dell'economia Agraria, Unità di Ricerca per l'Orticoltura	Abiotic stress, biotechnology, association mapping	Lodi, Italy http://www.crea.gov.it/ home
University of Turin-DISAFA-Plant Genetics and Breeding, University of Turin	Biotechnology, association mapping, abiotic stress	Torino, Italy https://en.unito.it/

(continued)

Institutions	Research area	Location and website
Section of Genetics and Plant Breeding, Department of Plant, Soil and Food Science, University of Bari	Resistance breeding	Bari, Italy https://www.uniba.it/
Consiglio per la Ricerca e Sperimentazione in Agricoltura, Genomic Research Centre	Biotechnology	Piacenza, Italy http://centrodigenomica.entecria.it/
INRA, Unité de Génétique & Amélioration des Fruits et Légumes	Genetic resources, crop wild relatives, breeding, disease resistance	Montfavet Cedex, France http://institut.inra.fr/en
School of Biosciences, University of Birmingham	Crop wild relatives, conservation	Birmingham, UK https://www.birmingham.ac.uk/
Natural History Museum, Department of Life Sciences	Taxonomy	London, UK https://www.nhm.ac.uk/
Wageningen University Research	Resistance breeding, genetic resources	Wageningen, Netherlands https://www.wur.nl/
Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University	Biotechnology	Gent, Belgium https://www.ugent.be/bw/en
Biotechnical Faculty, Department of Agronomy, Chair for Fruit, Wine and Vegetable Growing, University of Ljubljana	Grafting research	Ljubljana, Slovenia https://www.uni-lj.si/
Dipartimento Agraria, Università Mediterranea di Reggio Calabria	Nutrient use efficiency	Reggio Calabria, Italy http://www.agraria.unirc.it/
Technological Institute of Western Greece, Department of Agricultural Technology	Local varieties, breeding research	Amaliada, Greece http://www.tewest.gr/
Africa		
Horticulture Department, Faculty of Agriculture, University of Kafrelsheikh	Disease resistance and breeding research	Kafr El-Sheikh, Egypt http://www.kfs.edu.eg/
Vegetable Crops Research Department, Horticulture Research Institute, Agriculture, Research Center	Breeding research, diversity in crop wild relatives	Giza, Egypt http://www.arc.sci.eg/
Laboratory of Genetics, Félix Houphouët-Boigny University	Breeding research, local varieties, diversity	Abidjan, Côte d'Ivoire http://univ-fhb.edu.ci/
Uganda Christian University	Diversity, abiotic stresses	Mukono, Uganda https://ucu.ac.ug/
African Orphan Crops Consortium, World Agroforestry Centre (ICRAF)	Diversity in minor crops, breeding research, genomics	Nairobi, Kenya http://worldagroforestry.org/

(continued)

Appendix II: Examples of Commercial Cultivars of Eggplant Worldwide

Cultivar	Important traits	Cultivation location
Little Fingers F ₁	Long, purple	China
Long White Angel F ₁	Long, white	China
Ma Zu Purple	Long, purple	China
Megadok F ₁	Long, purple	China
Ping Tung Long	Long, pale purple	China
Lucky Green	Long, green	China
Asian Bride	Long, white-purple	China
Machiaw	Long, pink	China
Fengyuan Purple	Long, purple	China
Round Mauve	Round, reddish purple	China
Kurume Long Purple	Long, dark purple	Japan
Kyoto Egg F ₁	Round, dark purple	Japan
Milionaire F ₁	Long, dark purple	Japan
Senryu Ni Gou F ₁	Long, dark purple	Japan
Shoya Long F ₁	Long, dark purple	Japan
Green Doll F ₁	Round, small, white with green stripes	Thailand
Kermit F ₁	Round, small, green/white	Thailand
Thai Round Green	Round, small, green	Thailand
Thai White Ribbed	Round, ribbed, white	Thailand
Violet Prince F ₁	Round, small, violet	Thailand
White Ball F ₁	Round, small, white	Thailand
Puangyok Thai Pea	Round, small, green	Thailand
Thai Yellow Egg	Round, small, yellow	Thailand
Thai White Egg	Round, small, pinkish	Thailand
Rolex F ₁	Long, green	Thailand
Tai Long Green F ₁	Long, thin, green	Thailand
White-Purple	Long, light purple with white streaks	Thailand
Apsara F ₁	Oval, small, purple with white stripes	India
Red Chu F ₁	Round, small, purpura	India
Black Chu F ₁	Round, small, blackish purple	India
Ratna	Oval, small, dark purple	India
Rhim Jhim	Oval, small, purple with white streaks	India
Hari F ₁	Elongated, green	India
Bali F ₁	Elongated, small, purple	India
Bharata Star F ₁	Round, dark purple	India
Suphal	Oval, dark purple	India
Supriya	Round, violet	India
Orissa	Oval, small, yellow	India
Harihar	Elongated, green with white stripes	India

(continued)

Cultivar	Important traits	Cultivation location
Tarini	Elongated, large, green and white	India
Harabegan F ₁	Long, green	India
Black Beauty	Oval, blackish purple	Western countries
Udumalapet	Oval, green with purple streaks	Western countries
Bonica F ₁	Oval, dark purple	Western countries
Classic F ₁	Oval, dark purple	Western countries
Epic F ₁	Oval, dark purple	Western countries
Galine F ₁	Oval, dark purple	Western countries
Sonata F ₁	Oval, dark purple	Western countries
Nadia F ₁	Oval, dark purple	Western countries
Tudela F ₁	Oval, dark purple	Western countries
Velia F ₁	Oval, dark purple	Western countries
Santana F ₁	Oval, blackish purple	Western countries
Megal F ₁	Oval, purple	Western countries
Bartok F ₁	Elongated, dark purple	Western countries
Lemmy F ₁	Elongated, dark purple	Western countries
Gostbuster F ₁	Oval, white	Western countries
Listada de Grandia	Oval, purpura stripped with white	Western countries
Neon F ₁	Oval, pink purpura	Western countries
Rosita	Oval, pink lavender	Western countries
Zebra F ₁	Oval, violet stripped with white	Western countries
Baluroi F ₁	Elongated, dark purple	Western countries
Black King	Elongated, dark purple	Western countries
Fabina F ₁	Elongated, dark purple	Western countries
Ichiban F ₁	Elongated, dark purple	Western countries
Long Purple	Long, dark purple	Western countries
New Purple	Elongated, purple	Western countries
Nite Lady F ₁	Elongated, dark purple	Western countries
Lavender Tough F ₁	Elongated, white	Western countries
Cloud Nine F ₁	Elongated, white	Western countries
Antigua	Elongated, white stripped with lavender	Western countries
Fairy Tale F ₁	Elongated, lavender with white strikes	Western countries
Baby Bell	Oval, small, blackish purple	Italy
Bianca Rosa	Oval, small, white with lavender streaks	Italy
Prosperosa	Round, ribbed, violet	Italy
Violetta di Firenze	Round, dark purple, ribbed	Italy
Casper	Elongated, white	France

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Chapter 6

Breeding Strategies for Yield Gains in Okra (*Abelmoschus esculentus* L.)



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Abstract In okra, we follow the breeding methodologies of autogamous plants. This includes crossing or controlled hybridization of parents selected based on their better-combining abilities. This is followed by the pedigree selection of the segregating population for the traits of interest like biotic and abiotic stress tolerance and yield. The major breeding objectives are nearly the same across all the okra-growing countries: high yield, tolerance to various pests and diseases, better organoleptic qualities, appealing color and size of the harvestable fruits. Hand pollination is the most commonly used method of hybrid seed production in okra and improving yield and ensuring its sustainability under adverse conditions through resistant hybrids is the major objective of heterosis breeding. Sufficient genetic diversity has been reported among the parents and crosses for selection to be effective for okra hybrid production. Although both additive and dominant gene actions were found regulating the phenotypic expression of various characters, dominant gene action is considered more important. Through intensive research efforts, a large number of varieties and hybrids have been released around the world. Some of these varieties have already made a significant impact in revolutionizing the production of okra

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worldwide. This chapter summarizes the current status of okra crop production and its future improvement strategies.

Keywords Hybrid seed production · Improvement · Ladyfinger · Okra breeding

6.1 Introduction

Okra (*Abelmoschus esculentus* (L.) Moench), is considered one of the important vegetable crops grown in several countries of tropical, subtropical and Mediterranean regions (Duzyaman and Vural 2003). The genus *Abelmoschus* probably originates from the Arabian word *abul-l-mosk*, meaning *the source of musk or father of musk* (Quattrocchi 2000). World over, cultivated okra is known by different names such as lady's finger (English), gombo (French), quimngombo (Spanish), bhindi (Hindi), quiabeiro (Portuguese) and bamiah (Arabic). Fruits are picked at an immature stage before the seeds are fully developed and eaten as a vegetable. Nearly 99% of okra cultivation is practiced exclusively in developing countries in Asia and Africa. When compared with the world average productivity (7.7 mt ha^{-1}), the average productivity of most of these countries is quite low, and the poorest producers are found in Africa (2.7 mt ha^{-1}). Globally, okra occupies a total area of 1.08 m ha , resulting in an annual yield of 8.36 million mt, with an average yield of 7.7 mt ha^{-1} . With the production of 6 million mt, nearly 72% of the total world production, India ranks first. This production was obtained from over 0.5 million ha area and with the average productivity of 12 mt ha^{-1} (FAOSTAT 2018).

Okra belongs to the Malvaceae or mallow family; formerly under the genus *Hibiscus*, it was later classified as the distinct genus *Abelmoschus* (Dhankar 2012). This genus contains two cultivated species namely, *A. esculentus* and *A. caillei* (Patil et al. 2015). Okra is considered to be native to South Africa (Akanbi et al. 2010), but Vavilov very strongly indicated its Ethiopian ancestral connection (Lamont 1999). However, *A. tuberculatus* ($2n = 58$) which is considered as a probable ancestor species from Uttar Pradesh, indicates an Indian origin. However, *A. ficulneus*, ($2n = 72$) which is native to East Africa, indicates Northern Egypt and Ethiopia as the geographical region for the origin of *A. esculentus* ($2n = 130$) (Purseglove 1984).

Okra is an allopolyploid that has $2n = 56$ as the lowest known chromosome number as reported in *Abelmoschus angulosus* (Ford 1938). Furthermore, the maximum number of chromosomes reported is nearly 200 in *A. caillei* (Siemonsma 1982), which is an allotetraploid derived from the cross between *A. esculentus* ($2n = 130\text{--}140$) \times *A. manihot* ($2n = 60\text{--}68$). Also, within the *A. esculentus* species a perfect series of polyploids having $2n$ chromosome numbers 72, 108, 120, 132 and 144 are known. These are considered to be the derivatives having basal chromosome number $n = 12$ (Datta and Naug 1968). Until now, out of 50 identified and well-defined okra species, only 9 species are widely cultivated (IBPGR 1990).

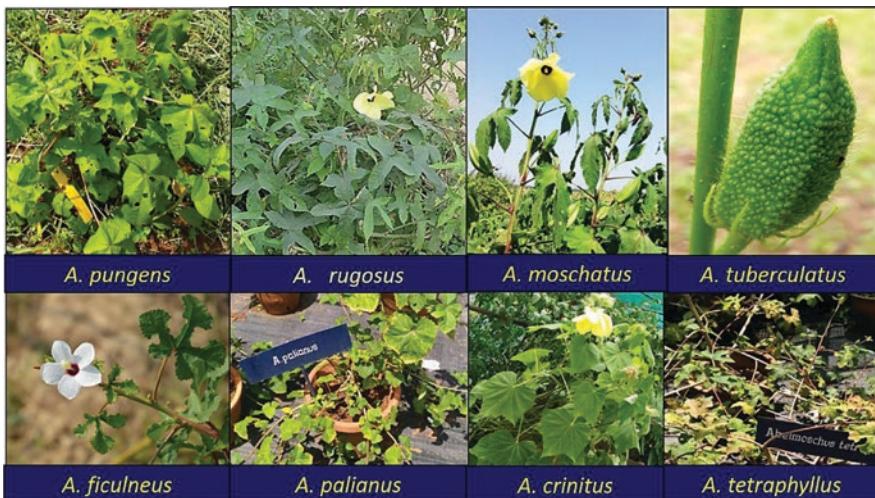


Fig. 6.1 A few wild *Abelmoschus* species. (Photos by: Gyan P. Mishra and Tania Seth)

Although *Abelmoschus moschatus* is cultivated as an ornamental plant, it is also found in the wild. Whereas, other wild species like *A. tetraphyllus*, *A. tuberculatus*, *A. ficulneus*, *A. crinitus*, *A. enbeepegearensis*, *A. palianus*, and *A. angulosus* are considered as true wild species (Patil et al. 2015). A few wild species of *Abelmoschus* are presented in Fig. 6.1. If we exclude potato, onion, and garlic; then okra is one of the major forex earners among the vegetable crops, accounting for nearly 60% of the total fresh vegetable export from India to mostly the Middle East and European countries (Singh et al. 2014).

Earlier okra was placed in the genus *Hibiscus* but later, based on the caducity of the calyx, and adnations of the calyx to the petals and the staminal column, it was placed in the genus *Abelmoschus*. In 1990, during the international workshop on okra genetic resources, New Delhi, India, a new classification was adopted based on cytogenetic evidences. It was concluded that Guinean type okra and *A. manihot* var. *caillei* (A. Chev.) are the same and named *A. caillei* (A. Chev.) Stevols. The plant is mostly robust, erect (occasionally branched), annual herb with a well-developed tap root system. The calyx is generally nonlobed, tubular; corolla with 5 large yellow petals, purplish-red pigmentation on one or both sides of the claw. Flowers are solitary, usually axillary with a lobed epicalyx. The first flower generally appears at the 5–7th nodes. A tubular staminal column with numerous stamens attached to the corolla base; stigma 5–10 lobed, velvety red/purplish. Okra is an often-cross-pollinated crop, as several insects like honeybees, bumblebees and many other small insects can frequently lead to the cross-pollination (Lamont 1999).

Plants generally flower 30–60 days after sowing (DAS). Flowering starts from the 5th to 7th node from the base and continues along with the growing stems/branches for 40–80 days. The time of anthesis varies with the cultivar, temperature, and humidity, but normally occurs in the morning.

It was observed that the stigma of okra crop becomes receptive only 2 h before anthesis (protogyny), which persists for 3–4 h post-anthesis with variable receptivity. This crop requires generally 45–50 days from sowing until flowering and final fruit setting (tender). The tender fruits attain marketable maturity in 5–6 days, while it requires 30–32 days to reach the seed harvest stage post-anthesis. Dark green fruit color and small size (8–10 cm) fruits are desirable characters for export because the percentage share of okra in total vegetable export is second after onion. On the biotechnology front, okra crop is lagging especially in areas like genome sequencing, use of robust molecular markers in marker-assisted breeding, SNPs identification and even identification of differentially expressed genes using transcriptomic approaches. This crop still lacks a large number of reliable polymorphic molecular markers and also there is no serious effort to create molecular genetic maps or linkage groups. The condition becomes worse due to the presence of a very large number of chromosomes ($2n = 56\text{--}196$) in association with its complex polyploid genome (Mishra et al. 2017; Sastry and Zitter 2014).

6.1.1 Economic and Uses

Okra is mostly cultivated for its tender fruits, which are harvested and consumed as a cooked vegetable in a variety of ways like salads, soups, and stews, or fried or boiled as a vegetable dish (Salameh 2014). Furthermore, due to the presence of dietary fiber, and unique amino acid composition which is rich in lysine and tryptophan, it is regarded as a vital constituent for a balanced diet (Hughes 2009). It is also a very important source of several vitamins (A, B, C, K, and folates), and minerals including iron, calcium, potassium, manganese, and magnesium (IBPGR 1991; USDA National Nutrient Database 2018). The roots and stems are used for cleaning sugarcane juice for brown sugar preparation (Shetty et al. 2013). Dried okra seeds can be used to prepare vegetable curd or roasted and ground into a powder as a coffee additive or substitute (Moekchantuk and Kumar 2004). Seeds have also gained much attention as a new oil (30–40%) and protein (15–20%) source (Duzyaman 1997). Okra leaves are considered good cattle feed, but rarely considered as their primary use (Benchasri 2011). Okra fruits contain approximately 86% water, 2% protein, 0.2% fat, 10% carbohydrate, 1.0% fiber and 0.8% ash (Saifullah and Rabbani 2009). The fruits have recently been used to study their ethnopharmacological and medicinal (Tseng et al. 2004) properties against cancer, high-cholesterol (Jenkins et al. 2005) and diabetes mellitus (Amin 2011). Moreover, okra mucilage is used to replace blood plasma to expand the blood volume (Akinyele and Temikotan 2007).

Among the various *Abelmoschus* species, the most widely cultivated is *A. esculentus*. Others include *A. caillei* which is mostly cultivated for both leaves and fruits in the Western and Central African regions (Siemonsma 1982). However, *A. manihot* is widely grown only for its leaves in the South Pacific islands. *Abelmoschus*

moschatus is cultivated not only for its aromatic seeds but also as an ornamental plant.

6.2 Breeding Objectives

The primary breeding objectives to enhance okra as a crop plant are as follows:

- (a) To develop high yielding cultivars having wide adaptabilities.
- (b) To develop cultivars of different maturity groups suited to specific climatic conditions.
- (c) To develop multiple diseases and pest-resistant cultivars, with special emphasis on yellow vein mosaic virus (YVMV), okra leaf curl virus (OELCV), shoot and fruit borer, jassids, leafhoppers, and root-knot nematodes.
- (d) To create cultivars tolerant to abiotic stresses, especially tolerance to low temperature, drought, excessive rain, and saline and alkaline soils.
- (e) To develop cultivars having dark green, tender and smooth fruits suitable for both domestic and export purposes.
- (f) To create cultivars with enhanced nutritive attributes which are also suitable for dehydration, canning, and freezing.

6.2.1 Breeding for High Yield

Important characteristics in any high-yielding cultivar should be medium plant height, upright branching, a high number of fruit-bearing nodes, lower position of first fruiting node, deeply-lobed leaves, short internodes, dark-green colored fruit, earliness, and tolerance to important pests and diseases. Details of some major world institutions working on the improvement of okra are given in [Appendix I](#).

6.2.2 Breeding for Resistance Attributes

Okra is prone to be affected by several biotic stresses like yellow vein mosaic virus (YVMV), okra enation leaf curl virus (OELCV), *Cercospora* leaf spot and pests - jassids, whitefly, and shoot and fruit borers. Among these YVMV and OELCV are very serious and cause losses of up to 50–90%. Resistance against these diseases is not stable in cultivated species and resistant cultivars become susceptible generally after 5–8 years of development. Okra crop wild relatives like *Abelmoschus caillei* and *A. tetraphyllus* have been used in breeding programs for the transfer of resistance to various biotic and abiotic stresses. However, their use is generally restricted

due to sterility problems. A few viable seeds could be produced in F_1 hybrid and using backcross approach it could be possible to transfer YVMV tolerance from *A. caillei* and *A. tetraphyllus* to the conventional okra cvs. Parbhani Kranti and Arka Anamika, respectively. Crossing between promising parents combined with pedigree selection or backcrossing are the most common breeding procedures. Okra lines resistant to root-knot nematode, shoot and fruit borer and jassids have been identified. Remarkable native diversity in cultivated and wild types occurs in the Indian subcontinent, besides the variability observed among introduced cultivars (Mishra et al. 2017).

The genotype IC-1542 from West Bengal contributed genes for YVMV resistance to Pusa Sawani; the most widely cultivated symptomless carrier cultivar. In this cultivar, two recessive alleles at two loci were found conferring the resistance (Singh et al. 1962). Later, a virus-resistant accession from Ghana was identified as *Abelmoschus manihot* (L.) Medikus ssp. *manihot* which has contributed to the development of resistant cultivars, Punjab Padmini, Punjab-7 and Parbhani Kranti, in India. Two complementary dominant genes are reported responsible for the YVMV resistance (Sharma and Dhillon 1983; Sharma and Sharma 1984; Thakur 1976). Additive genes are also reported responsible for the plant attributes associated with virus resistance. However, resistant genes are reported to be sensitive to environmental changes, particularly low temperature, thus the possibility of polygenic resistance is also inferred. However, Jambhale and Nerkar (1981) reported a single dominant gene for resistance in this species.

6.3 Utilization of Crop Wild Relative for the Transfer of Desirable Traits

A rich diversity of *Abelmoschus ficulneus* along with *A. tuberculatus* observed in India may be the focal point of the disagreement for *A. esculentus* to be considered as having Asian origin (Vredenburg 1990). The second genome of cultivated okra is considered to have come from *A. ficulneus*, whereas, the other genome is supposedly from *A. tuberculatus* (Bisht and Bhat 2007). Among the various *Abelmoschus* species, nine are reported to occur in India; these include *A. angulosus*, *A. crinitus*, *A. esculentus*, *A. ficulneus*, *A. manihot*, *A. moschatus*, *A. tetraphyllus* var. *pungens*, *A. tetraphyllus* var. *tetraphyllus* and *A. tuberculatus*. A YVMV-resistant, wild species of okra (*A. angulosus* Wall. ex Wight & Arn.) was crossed with the susceptible species *A. angulosus* and susceptibility was found dominant over resistance. Furthermore, the resistance was found to be regulated by two recessive genes in an additive manner (Wasala et al. 2019). The detailed gene pool of okra is presented in Table 6.1.

Variation in agro-morphological characters is present in the cultivated, semi-cultivated forms and crop wild relatives (Table 6.2). After laborious screening and evaluation of large number of germplasm accessions, several promising

Table 6.1 Gene pool of okra, *Abelmoschus* spp.

Species	Ploidy Level ^a	Gene pool
<i>A. esculentus</i>	2	GP-1
<i>A. manihot</i> ssp. <i>manihot</i>	1	GP-3
<i>A. manihot</i> ssp. <i>tetraphyllus</i> var. <i>tetraphyllus</i>	2	GP-3
<i>A. manihot</i> ssp. <i>tetraphyllus</i> var. <i>pungense</i>	2	GP-3
<i>A. moschatus</i>	1	GP-3
<i>A. ficulneus</i>	1	GP-2
<i>A. angulosus</i>	1	GP-3
<i>A. tuberculatus</i>	1	GP-2
<i>A. tuberculatus</i>	3	GP-3

Source: Singh (2007)

^aPloidy level 1: 2n = 56–72; Ploidy level 2: 2n = 108–144; Ploidy level 3: 2n = 185–199**Table 6.2** Identified source in crop wild relatives for various traits in okra

Traits	Germplasm accessions
Resistance to <i>Fusarium</i> wilt (<i>Fusarium oxysporum</i> f. <i>vasinfectum</i>)	<i>Abelmoschus manihot</i> , P.I. 379,584
Resistance to powdery mildew (<i>Erysiphe cichoracearum</i>)	<i>A. tetraphyllus</i> , <i>A. manihot</i> ssp. <i>manihot</i> , <i>A. moschatus</i> , <i>A. angulosus</i>
<i>Cercospora</i> and <i>Alternaria</i>	<i>A. crinitus</i> , <i>A. moschatus</i> cv. 7-1, <i>A. angulosus</i>
Yellow vein mosaic virus	<i>A. angulosus</i> , <i>A. manihot</i> ssp. <i>manihot</i> , <i>A. manihot</i> ssp. <i>manihot</i> var. <i>ghana</i> , <i>A. tetraphyllus</i> , <i>A. crinitus</i> , <i>A. pungens</i>
Jassids (<i>Amrasca biguttula</i>)	<i>A. manihot</i> ssp. <i>manihot</i> var. <i>Ghana</i> , <i>A. moschatus</i> , <i>A. crinitus</i>
Shoot and fruit Borer (<i>Earias</i> spp.)	<i>A. tuberculatus</i> , <i>A. caillei</i> cv. <i>Narnaul Special</i>
Low temperature and frost	<i>A. angulosus</i>
Fruits per plant	<i>A. tetraphyllus</i> , <i>A. manihot</i> ssp. <i>manihot</i> , <i>A. caillei</i>
Number of branches per plant	<i>A. tetraphyllus</i>
Dark green fruit and extended fruiting period	<i>A. manihot</i> ssp. <i>manihot</i>

Source: Singh (2007)

accessions have been identified by several researchers. These accessions do possess resistance or tolerance to several biotic and abiotic stresses and various economically-important horticultural traits. Furthermore, resistance to several biotic stresses is also identified in some wild okra species. However, the transfer of these traits to cultivated species using conventional breeding tools is very difficult due to sterility issues. However, modern biotechnological methods like embryo rescue have been found quite successful (Bisht and Bhat 2007).

6.4 Genetics of Different Traits of Okra

6.4.1 Plant Architecture Traits

Plant architecture traits are very important for any crop, as they not only determine yield ability but also market preference. Several studies have been carried out to determine the genetics of various okra traits like plant color, pigmentation and fruit color. Venkitaramani (1952) reported that the dark-green color of a plant was dominant over light-green color whereas, greenish-red was dominant over the green. The yellow color of the corolla of *Abelmoschus manihot* was dominant over the cream color of *A. esculentus*. Kolhe and D'Cruz (1966) reported that pigmentation of calyx, corolla, and fruit was under monogenic control. Jasim and Fontenot (1967) opined that cut leaves were dominant over lobed leaves and the white-fruit color was dominant over green color. Leaf shape, fruit-color, and fruit-spininess were under the control of single genes (Jasim and Fontenot 1967) while fruit hairiness and leaf lobing were governed by single incompletely dominant genes (Nath and Dutta 1970). Additive gene action for the conditioning of days to first flowering (Arora et al. 2008; Kishor et al. 2013) and days to 50% flowering (Khanpara et al. 2009; Seth et al. 2016a; Sood and Kalia 2001) are reported. Node at first flowering had a predominance of both additive and non-additive genetic effect (Jindal et al. 2009; Seth et al. 2016a); whereas, the response of non-additive gene actions for internodal length, plant height, number of branches per plant, fruit-length, number of fruits per plant and fruit-yield per plant has been observed (Das et al. 2013; Reddy et al. 2013b; Seth et al. 2016a). Thus, the genetics of plant architectural traits has helped in the targeted transfer of these traits in a precise way to develop a cultivar suitable for different market segments. However, more studies are required to understand such traits by using more diverse okra germplasm.

6.4.2 Genetics of Resistance to Yellow Vein Mosaic Virus (YVMV)

Okra can be infected by not less than 27 begomoviruses (family: Geminiviridae), which have circular ssDNA and are transmitted by *Bemisia tabaci* (Seal et al. 2006) and yellow vein mosaic disease (YVMD) and okra enation leaf curl disease (OELCD) are the most severe (Mishra et al. 2017; Sanwal et al. 2014). Various types of responses to YVMV have been reported to occur in cultivated and wild species. Several reports showed that the YVMD resistance is controlled by two dominant complementary genes (Bharathkumar et al. 2019; Dhankhar et al. 2005; Sharma et al. 1981); on the contrary, others have shown that there is a single dominant gene (Bharathkumar et al. 2019; Jambhale and Nerker 1981) responsible for governing the resistance against YVMD. Additive genes were responsible for plant attributes associated with virus resistance. Das et al. (2013) reported that the YVMV

disease was controlled by non-additive gene effects. Seth et al. (2016a) reported that YVMV disease was controlled by both additive and non-additive gene effects. Similarly, in interspecific crosses between *Abelmoschus manihot* and *A. tetraphyllus*, a single dominant gene controlled the resistance (Jambhale and Nerker 1981). Arora et al. (2008) indicated duplicate gene action for the resistance. YVMV resistance was found regulated by both major and minor gene(s) which means that the plant resistance mechanism for YVMV is very complex. Thus, the resistance to the YVMD should be studied in detail using comprehensive and integrated approaches like transcriptomics, proteomics and metabolomics (Mishra et al. 2017).

Resistance mechanisms of okra against YVMV disease are complex, highly variable and associated with biochemical parameters and defense-related enzymes (Prabu and Warade 2009; Seth et al. 2017). The absence of absolute resistance to begomoviruses in okra requires various alternative means of control. Also, there is a need to do large scale germplasm screening under artificial epiphytic conditions for the identification of resistance source to the begomoviruses. Furthermore, the development of transgenic okra using a coat protein (CP) gene should also be kept as an option (Mishra et al. 2017). The reduction of white-fly populations in the field from the beginning of the cropping season has also shown positive results in the effective management of *Begomovirus*-caused diseases like YVMV and OELCV. Among several strategies, the disease can be controlled by various means including (i) planting the border crop with maize (20 days before sowing of okra); (ii) seed treatment with Imidaclorpid (5–10 g/kg of seeds) followed by spraying with Imidaclorpid (0.5 ml/L water) at 15-day intervals.

6.4.3 Genetics and Selection of Cultivated Okra

As an often-cross-pollinated crop, okra's improvement depends upon the magnitude of genetic variability present in existing populations (Purewal and Randhawa 1947). Many characters have a simple genetic base such as green or purple coloration of the stem at its base and veins, presence or absence of soft or hard hairs on the leaves and fruit, leaf-lobes (Erickson and Couto 1963; Kalia and Padda 1962; Nath and Dutta 1970). Fruit-yield is positively correlated with fruit-weight, number of fruits per plant, plant height, number of nodes, days to 50% flowering, fruit-length and fruit-width (Dhankar and Dhankar 2002; Singh et al. 2006, 2007). Selection for traits such as per plant fruit number, branch number and medium plant height are considered most important for the improvement of okra yield (Dhankar and Dhankar 2002). Nwangburuka et al. (2012) recorded positive and significant phenotypic and genotypic correlation between plant-height at maturity with various fruit characters and overall yield.

Characters like plant-height, internodal length, days to flowering, fruit-shape, length and diameter, number of fruits per plant, weight of the fruit, yield, plant-height and vitamin C content show high heritability (Bali et al. 2004; Seth et al. 2016b; Singh et al. 2006, 2007). Yield is influenced directly or indirectly by

fruit-weight and fruit-length suggesting they are the most useful parameter/character for selection in breeding for yield improvement (Duzyaman and Vural 2003). Nwangburuka et al. (2012) reported the importance of additive gene effects over non-additive gene effects for the genetic control of fruit weight in okra. Kishor et al. (2013) and Adiger et al. (2013) suggested a predominance of non-additive action. In addition to these, both additive and dominance gene effects were reported for this trait by Kumar et al. (2005). For primary branches per plant, the additive gene effect was reported by Khanpara et al. (2009). Plant height showed high heritability estimates and high genetic advance (Abdelmageed 2010). The additive gene effect was observed for fruit yield per plant by Khanpara et al. (2009) and Kumar et al. (2013).

6.5 Breeding Methods

Although okra is often cross-pollinated, breeding methods adapted to self-pollinated crops can be employed for varietal development. The methods generally used for the development of high yielding cultivars are through introduction, pure-line selection, hybridization (heterosis breeding), backcrossing, mutation and polyploidy breeding. Several cultivars released as genetic resources in India are presented in [Appendix II](#).

6.5.1 Plant Introduction and Pureline Selection

In India, inter-varietal hybridization and subsequent pedigree selection have resulted in the development of a high-yielding and YVMV-tolerant cv. Pusa Sawani. However, in due course of time, this cultivar has become susceptible to YVMV in Northern and Southern parts of India, but it is doing well at high altitude regions of Jammu and Kashmir, Himachal Pradesh and Uttarakhand where YVMV does not appear. Similarly, Selection-2 is a derivative of multiple inter-varietal crossing. Interspecific hybridization has been followed in the development of cultivars like Punjab Padmini, Prabhani Kranti, P-7, Arka Anamika and Arka Abhay. Indigenous introductions have played a significant role in improving the okra germplasm resources of many countries. *Abelmoschus manihot* ssp. *manihot*, now known as *A. caillei*, a semi-wild species introduced from Ghana, has served as a source of resistance to YVMV. In India, the first improved cultivar of okra, Pusa Makhmali, was developed through pureline selection from material collected from West Bengal. Using the single plant selection approach viz. pureline selection method, the high-yielding cultivar Co-1 was developed. This was derived from the cross between Red Wonder and Gujarat Bhindi-1 (Singh et al. 2017).

6.5.2 *Heterosis Breeding*

Researchers have reported yield improvement in okra from 50 to 70% through heterosis breeding (Jagan et al. 2013; Reddy et al. 2013a). Among various factors contributing to yield, the number of fruits per plant was found most effective, followed by the height of the plant, weight of the fruit and earliness. Heterosis breeding in okra is aimed not only at improving the yield but also safeguarding its sustainability under various biotic and abiotic stress conditions through the incorporation of resistance and tolerance to these stresses (Kerure et al. 2019). Combining resistance to one or more pests (jassids, aphids, and borers), diseases (YVMV, OELCD) through morphological or biochemical (sugars, phenols, alkaloids, and phytoalexins) processes may be an important objective.

Sufficient genetic diversity has been reported among the parents and crosses for selection to be effective for okra hybrid production. Although both additive and dominant gene actions were found regulating the phenotypic expression of various characters, dominant gene action is more important. Furthermore, the okra hybrids were also found responding to the environmental conditions for various traits (Rajesh et al. 2019). Due to its additive gene action, the traits like plant height, days to 50% flowering, number of branches per plant, fruit-weight and ridge number per fruit are suggested for selection. Hundred seed weight and the number of leaves per plant can also be considered as important traits for capturing the dominance gene action for hybrid production (More et al. 2017; Oyetunde and Ariyo 2014; Singh et al. 2017).

6.5.2.1 Methodology of Hybrid Seed Production

Lately, inter-varietal hybrids in okra have been found useful and researchers do have reported yield improvement to the tune of 50–70% through heterosis breeding (Jagan et al. 2013; Reddy et al. 2013a). Yield improvement through the resistant-breeding approach seems most effective in any okra breeding program including hybrid development. During emasculation, first, the stamens are removed very carefully using forceps from the unopened flower buds, mostly in the evening. These emasculated buds are pollinated in the morning of the next day using selected parents as pollinator or male parent. The emasculation is carried out daily from 15:00–17:00 h. Both emasculation and pollination are performed by hand. After emasculation, the buds are covered with the cotton bud to avoid the possibility of any cross-pollination (Fig. 6.2). While selecting the male flowers as the pollinator, the just-opened flowers which are fully loaded with the yellow color pollen grains are selected. One male flower is used to pollinate 4–10 female emasculated buds and after crossing the pollinated flower is covered with a paper bag. Pollination is carried out in the morning between 08:00 to 11:00 h.

The crossing can be continued for approximately 8 weeks from the date of first flowering. Care must be taken that the remaining flowers and buds if any should be

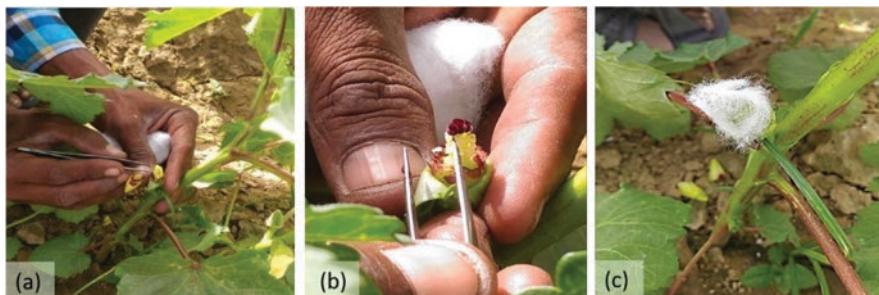


Fig. 6.2 Emasculation of the okra flower bud. (a) Opening of the flower bud using forceps; (b) Removal of anthers; (c) Covering of emasculated flower buds using cotton for next day pollination. (Photos by: Gyan P. Mishra and Tania Seth)

removed manually once the crossing program comes to an end. This helps in better development of crossed fruits and simultaneously it also prevents the mixing of selfed seeds with that of hybrid seeds.

The best quality hybrid seed in terms of its productivity can be obtained if the stigma receptivity of the female parent coincides perfectly with that of the pollen viability of the male parent. In okra hybrid seed production, generally, the pollination success is 30–50%. It was observed that if the viable pollen grains are deposited early on the stigma, it may lead to poor seed development because of the poor or non-receptivity of the stigma. Similar results are also obtained if pollen grains are deposited on the sigma very late, as there is drying of the stigma and also the loss of pollen viability. The quantity of the male pollen depositing on the receptive stigma of the female parent also determines the amount of F₁ hybrid seed. Thus, pollination timing and female-to-male flower ratio for the crossing should be optimized to achieve maximum hybrid seed production from the female okra plant (Singh et al. 2017).

Male-to-female row ratio is generally kept as 1:8 for hybrid seed production in okra. Generally, the minimum number of rouging for variety and hybrid seed production in okra is 3 and 4, respectively. These inspections are performed at vegetative (01 No), flowering (02) and fruit maturity (01) stages. Furthermore, various plant traits like hairiness, fruit-color, ridge numbers, and fruit-length are used for the rouging. Also, any off-types and YVMV infected plants should be positively rogued out from the seed production field.

Furthermore, any wild species of okra must be removed from the seed production plots before flowering. Harvesting of dried fruits generally should be done 30–35 days after crossing and these should be threshed, winnowed and sun-dried until nearly 10% seed moisture content is achieved.

The dried seeds are then placed in a container of water to remove the floaters (poor seeds) while good seeds, or sinkers, are again dried under shade followed by sun-drying. The cleaning of the seed is then performed and the cleaned seeds are treated using Captan or Thiram and processed through BSS-7 wire mesh sieve. In general, nearly 1000–1200 kg per ha seed yield can be obtained (Seth et al. 2018; Singh et al. 2017).

6.5.2.2 Exploiting Genetic Male Sterility for Hybrid Seed Production in Okra

In the Indian subcontinent commercial hybrid seed production of okra is done by hand-emasculation and hand-pollination and hence a labor-intensive proposition which ultimately increases the expense of hybrid seed production and the price of hybrid seeds in okra. To reduce the cost of hybrid seed production we can utilize the genetic pollination control mechanism like male sterility which is being used in other crops worldwide for the production of hybrid seed (Mishra et al. 2001, 2003). In okra one such mechanism, genetic male sterility, can be exploited for the production of F₁ hybrid seed with the inherent disadvantage of identification of sterile plants and roughing out of 50% male fertile plant during hybrid seed production. Although male sterility in okra is not common in nature, it can be induced by gamma radiation. In okra, the male sterility is reportedly regulated by a single recessive gene (*ms1*). Thus, it gets expressed only if the gene is present in homozygous (*ms1ms1*) condition and expression was found stable and not much influenced by the environmental factors (Dutta 1980; Mishra et al. 2003).

At ICAR-IIVR, Varanasi, India, a single plant showing male sterility in the genotype HRB55 was identified, which was confirmed by pollen staining using Acetocarmine (Fig. 6.3). Besides selfing of a few flower buds, crosses were also attempted on the putative male-sterile plant with fertile genotypes like VRO-22 and VRO-109. The F₁ plants [HRB55 (ms) x VRO22] were sown to get F₂ progenies which were found male fertile. This indicated that the putative male-sterile plant identified in the genotype HRB55 could be a case of genetic male sterility (GMS) (Anonymous 2017). Once we get a stable GMS line, it will help in the large-scale hybrid seed production in okra.

Dutta (1980) characterized the genetic male sterile line and observed that anthesis was normal but anther dehiscence was partial and microsporogenesis was normal up to the tetrad stage. Therefore, this GMS line has great potential for its utilization in F₁ hybrid development, to extract the heterotic effect for yield and its contributing traits. For maximizing the seed yield in GMS-based hybrids, a planting

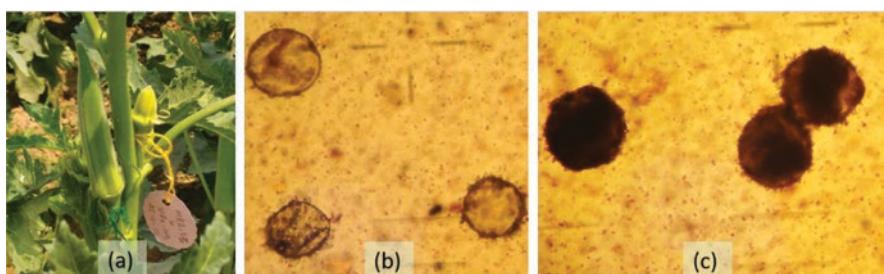


Fig. 6.3 (a) Putative male-sterile plant identified from the genotype HRB55, (b) Pollen staining using Acetocarmine of a putative male-sterile plant (HRB55), (c) Pollen staining using Acetocarmine of a male fertile plant (HRB55). (Source: Anonymous 2017)

system/ratio of male sterile line and its pollinizer have been standardized. Further, the male parents or pollinizers were identified based on the specific combining ability with genetic male sterile lines (Pitchaimuthu and Dutta 2002). Under field conditions, 2:1 planting ratio of sterile and fertile parents i.e. alternate planting of 2 rows of male-sterile plants and 1 row of fertile plants can accommodate about 4200 male-sterile plants per hectare. From this one can harvest nearly 566 kg of hybrid seed per ha, which is almost 52.56% higher seed yield in comparison with the fertile control (Dutta 1980). In male sterile line one person can pollinate 92 flowers per hour and 1 kg of hybrid seed can be produced in 3 h from 274 hybrid fruit which is quite low as compared to hand emasculation and hand pollination. Hand emasculation and hand pollination of 274 perfect flowers may require over 9 h to produce 1 kg of seed (Dutta 1980). Therefore, a considerable amount of labor cost is minimized in hybrid seed production in okra by utilizing genetic male sterility.

6.5.3 *Mutation Breeding*

Variability can be created in okra through induced mutagenesis mostly by the use of either some physical agent (gamma rays) or chemical agent (EMS, ethyl methane-sulfonate). By intensive selection, a novel genotype or a variety can be developed. For induced mutagenesis, we need genetically pure seeds (dry seeds) of any variety or a potential genotype, which need to be improved for a specific trait. These seeds can be treated with either different doses of gamma rays or different concentrations of EMS. The treated seeds are sown in the field as M₁ generation and progenies are harvested in bulk to get the M₂ generation. A large M₂ generation should be screened and the suitable mutant line(s) selected for detailed evaluation. A mutant EMS-8 (Punjab-8) carrying resistance to YVMV and tolerance to fruit borer has been evolved through induced mutation of cv. Pusa Sawani treated with 1% EMS (Singh et al. 2017). There is great potential for the development of high-yielding and YVMV-resistant varieties using the mutagenesis approach. At the international level, the International Atomic Energy Agency (IAEA) Vienna, Austria and the Bhabha Atomic Research Centre (BARC), located at Mumbai, India, are supporting the development of high yielding varieties of many crop species through project funding involving induced mutagenesis (Gupta and Sood 2019).

6.6 Method of Seed Production: Seed to Seed

The various stages of seed production include (i) breeder seed, (ii) foundation seed (iii) certified seed and (iv) truthfully labeled seed. The field isolation distance of 500 m in the case of foundation seed and 250 m in the case of certified seed is maintained for the production of genetically pure seeds. Okra seed production fields must be isolated from contaminants by providing proper distances as specified in Table 6.3.

Table 6.3 Various parameters of okra (*Abelmoschus* spp.) seed production

	Minimum distance (m)	
	Foundation	Certified
Contaminants		
Other varieties field	500	250
Same variety field not maintaining desired varietal purity for certification & wild species ^a	500	250
Factors (Maximum permitted % ^b)		
Off-types	0.10	0.20
^a Objectionable weed plants	None	None
Standards for each class		
Pure seed (minimum)	99.0%	99.0%
Inert matter (maximum)	1.0%	1.0%
Other crop seeds (maximum)	None	None
Total weed seeds (maximum)	None	None
Other distinguishable varieties (maximum)	10 per kg	20 per kg
Germination (hard seeds included) (minimum)	65.0%	65.0%
Moisture (maximum)	10.0%	10.0%
For vapor-proof container (maximum)	8.0%	8.0%

^aObjectionable weeds such as wild okras [*A. ficulnus* *A. manihot* and *A. moschatus*

^bMaximum permitted at and after flowering

6.7 Ridge Number Variation in Okra Fruits

Okra consumers worldwide have a wide choice of smooth fruits to ridged-fruits. In the Indian subcontinent, the preference is for the five-ridged and dark green colored slender okra cultivars with slow fiber development. Furthermore, in the southern parts of India, fibrous okra cultivars are mostly preferred for the making of *sambar* (a type of curry). However, in African countries and also in America, the preference is for the okra with small and thick fruits with more than five ridges on the fruit surface (Fig. 6.4). Several germplasm lines are known to possess varying numbers of fruit ridges, but typically cultivated okra genotypes have five; but Kashi Satdhari is a seven-ridged cultivar which was developed by ICAR-IIVR, Varanasi, India and was released in 2006. Interestingly, cv. Kashi Satdhari was named based on the number of ridges it possesses; as in the Hindi language the word ‘Satdhari’ means ‘seven ridges’.

The Indian Institute of Vegetable Research described an accession (IC-117090) having 9 ridges on its fruit (Fig. 6.5). However, some of its fruits were also found having up to 11 ridges. Besides, three more accessions, IC-117088, IC-117245, and IC-117333, were also identified as having 7-ridged fruits. Therefore, there are all possibilities of developing a high yielding line having viral disease resistance and 9 ridges using this accession as a parent, which may be released as a 9-ridged cultivar. At ICAR-IIVR, we are using the IC-117090 genotype in various combinations in our breeding program to achieve the goal of creating a genotype having 9 ridges along with high yields and viral disease resistance (Seth et al. 2016c).



Fig. 6.4 Variation in okra fruits for its shape, size, color, and ridge numbers. (Photo by: Gyan P. Mishra and Tania Seth)

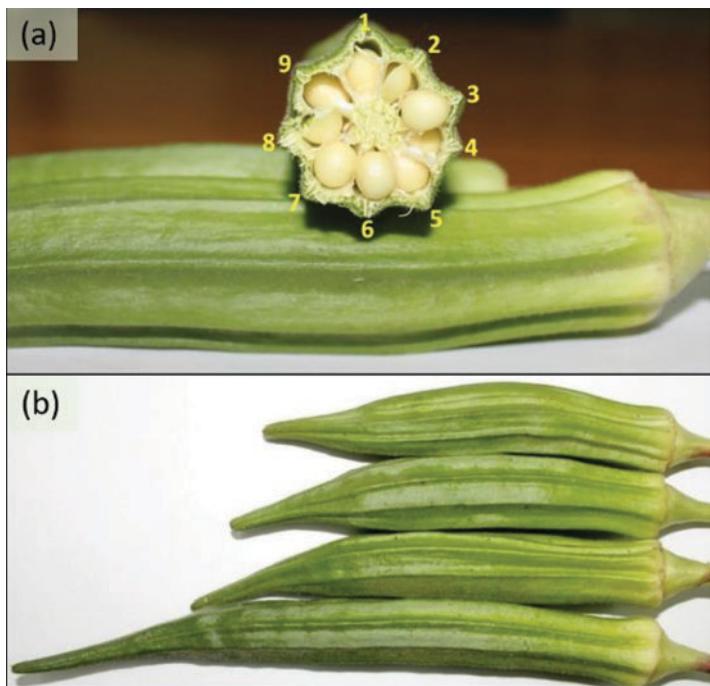


Fig. 6.5 Okra accession IC-117090, identified having nine ridges on its fruit (a) Cross-Section of fruit, (b) Fruits of IC-117090. (Source: Seth et al. 2016c)

6.8 Ideal Plant Type in Okra

A model or ideal plant type is described in detail and studied in several crops such as wheat, rice, maize, etc. As per the literature, the concept of an ideotype frequently denotes the breeding process, but it can also be extended in quest of the best phenotype in a crop plant which can be grown under a particular growing condition with distinct cropping systems and for specific uses. At the commercial level, cultivars distant from the typical definition of the crop ideotype are developed using some very limited traits of immediate importance. Thus, the ideotype definition for crops like okra should be widened to combine both morphological and physiological characters giving the okra crop a substantial adaptation under certain specific biophysical environment, crop management, and end uses (Martre et al. 2015). The ideal plant type of okra should possess the following characters:

Breeder/Farmer Traits Preference

- (a) Plants should be medium-tall 100–120 cm to facilitate easy manual harvesting.
- (b) Intermodal length should be short as there is a positive correlation between the number of nodes and yield per plant.
- (c) Plants should have 2–3 primary branches at acute angles (25–30°) which may supplement the number of fruits per plant without decreasing plant population.
- (d) The leaves should be deeply-serrate or cut-leaf or papaya-leaf type and the vector load will be less in such type of plants, thus less viral disease infection.
- (e) Fruit should be free from dense hair or trichomes at harvestable maturity.
- (f) The plant must have resistance against YVMV and ELCV.

Consumer Traits Preference

- (g) Fruit-length should be 10–12 cm with 1.5–2.0 cm diameter for culinary purposes; 6–7 cm long fruits are preferred for canning.
- (h) Fruit-color should be uniform dark-green from base to tip without any discoloration at the base.
- (i) Fruits should have 5–6 ridges which are most preferred by consumers; multi-ridges (7–10) are common in the USA.

6.9 Molecular Analysis

In okra, only a few molecular or biotechnological studies have been conducted in this genetically very complex genus (Mishra et al. 2017). The presence of a large amount of mucilage in the okra prevents easy and quality DNA isolation from its leaves. Therefore, for the isolation of quality DNA, a range of plant material like dark-grown seedlings (Kochko and Hamon 1990; Seth et al. 2018), fresh leaves (Adiger and Sridevi 2014; Ahmed et al. 2013; Khanuja et al. 1999; Narendran et al. 2013; Seth et al. 2018; Singh and Kumar 2012) and dried leaves (Meena et al. 2014) are used by different researchers. Success has been achieved for the isolation of

good quality DNA using a suitable sample and DNA isolation protocol. Molecular analysis for YVMV resistance has been done in okra genotypes using gene-specific markers (Patil et al. 2018). The differentially expressed proteins (DEPs) associated with photosynthesis antenna proteins and RNA degradation were found playing roles in salt stress tolerance in okra (Yu et al. 2019). Also, the comparative proteomic analysis of okra at seedling stage under salinity stress has identified response to stress, protein processing in the endoplasmic reticulum and heat shock proteins, as the most strongly associated DEPs (Zhan et al. 2019).

6.9.1 Marker-Assisted Germplasm Characterization

Several genetic diversity studies have been conducted using various molecular markers (Martinello et al. 2001, 2003). RAPD markers were used by Bisht et al. (1995) for the genetic differentiation of *Abelmoschus esculentus*, *A. ficulneus*, *A. manihot*, *A. moschatus*, and *A. tuberculatus*. RAPD markers were also used extensively by a number of researchers for the differentiation of Jordanian landraces (Rawashdeh 1999), West African (*A. callei*) and Asian (*A. esculentus*) genotypes (Aladele et al. 2008), Nigerian accessions (Nwabngburuka et al. 2011), Indian and Brazilian genotypes (Kaur et al. 2013, Prakash et al. 2011) and genotypes grown in Pakistan (Haq et al. 2013). Furthermore, SSR markers were also used for the differentiation of Burkina Faso accessions (Sawadogo et al. 2009), Indian accessions (Fougat et al. 2015) and also for the differentiation of three okra species (Schafleitner et al. 2013). Additionally, the diversity of okra accessions from Burkina Faso which were studied using SSR markers revealed 42.10% polymorphism (Ouedraogo et al. 2018). Recently, 69 Indian okra genotypes were evaluated using microsatellite markers to identify the diverse groups of genotypes for further crossing and conservation purposes (Kumar et al. 2017). Figure 6.6 shows the polymorphism in 43 okra genotypes amplified using SSR marker (unpublished data). Also, other DNA based marker system like SRAP (Gulsen et al. 2007), AFPL (Akash et al. 2013; Kyriakopoulou et al. 2014; Salameh 2014; Younis et al. 2015), ISSR (Younis et al.

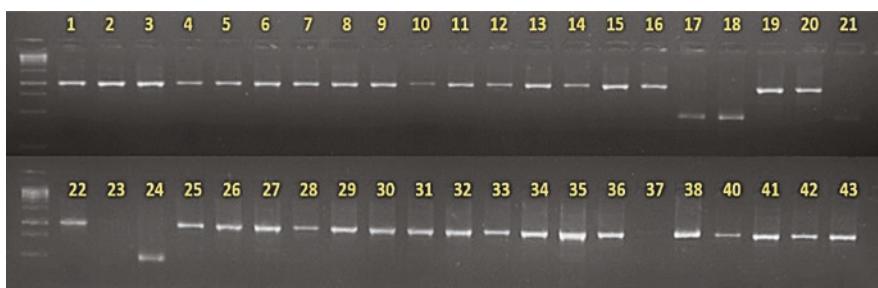


Fig. 6.6 PCR amplification polymorphism in 43 okra genotypes using SSR marker Where, 1–43 are okra genotypes, First lane: Markers lane. (Photo by: Gyan P. Mishra and Tania Seth)

2015; Yuan et al. 2014) and iPBS-retrotransposons (Yildiz et al. 2015) are being used to characterize okra germplasm. Moreover, a detailed genetic diversity analysis of global okra germplasm is needed to provide precise and more oriented use in okra breeding programs.

6.9.2 RNA-Seq Analysis, Genetic Transformation, and Transgenic Development

The RNA-Seq analysis helps in the identification of differentially expressed genes (DEGs) and also in the development of EST-SSR markers (Bosamia et al. 2015). Leaf and fruit RNA-Seq analysis have identified 935 SSR motifs of which, 199 when tested in a germplasm set resulted in 161 polymorphic SSR markers (Schafleitner et al. 2013). In a genetically complex crop like okra, there is a need to do deep sequencing so that the correct de-novo assembly can be generated (Mishra et al. 2017). Transcriptomic studies using infected and uninfected samples of resistant and susceptible genotypes will result in the identification of DEGs associated with *Begomovirus* resistance in okra.

Optimization of the transformation protocol is continuously performed on several okra genotypes by various research groups around the world. For this, different explants such as nodes and shoot tips (Mangat and Roy 1986), cotyledonary axils (Roy and Mangat 1989), hypocotyls (Anisuzzaman et al. 2008; Ganesan et al. 2007; Haider et al. 1993), shoot tips (Anisuzzaman et al. 2008) and zygotic embryos (Narendran et al. 2013) have been successfully used. The improved and reproducible transformation protocol will help to accelerate the progress of transgenic research in okra. Not only this, the much-discussed reverse genetics technology, CRISPER/cas9 or gene-editing technology, will only be successful once we have better transformation protocols, along with the detailed information about its genome sequence. Furthermore, Narendran et al. (2013) developed transgenic Bt okra having resistance to *Earias vittella* (shoot and fruit borer) by optimizing the *Agrobacterium*-mediated transformation protocol. Although a humble beginning has been made for molecular analysis in okra, much is still to be done, especially in the areas of whole genome sequencing and marker-assisted breeding for economically-important traits.

6.10 Conclusions and Prospects

Climate change has caused significant deviation from the mean of the different climatic parameters such as temperature, precipitation, relative humidity, etc. when estimated over a long-time period and also for expanded geographic areas. These changes may be due to natural forces and also due to human activities such as the

burning of fossil fuels. Like any other crop, climate change is also affecting overall okra productivity. The constant increase in global temperature along with occurrences of drought, heat stress, flooding, and increasing soil-salinity are the major outcomes of climate change which the current century is experiencing. Other negative impacts of climate change are observed in terms of rising biotic stresses which crops are now facing. This ultimately makes overall vegetable production less productive and hence less profitable (Abewoy 2018).

In spite of okra as one of the very important vegetable crops, limited attention has been given to its genetic enhancement using recent biotechnological tools. Okra is an agronomically less explored/neglected crop and researchers are still trying to optimize the quality of DNA isolation protocols for their efficient use in contemporary genomic studies. Recently, Seth et al. (2018) reported a fast and reproducible genomic DNA isolation protocol for cultivated and wild *Abelmoschus* species as well as related species of *Hibiscus*. The use of molecular markers in okra improvement is still in its infancy. This is limited because of the lack of availability of large numbers of robust and validated markers, along with the absence of genetic maps. Linkage groups are not yet being developed by any research group around the world; also, the development of transgenics is very limited and mostly grappling with the optimization of various protocols.

Furthermore, high-end biotechnological tools such as chromosome engineering, RNAi, MARS, GBS, targeted gene replacement, NGS and nanobiotechnology can provide rapid ways for okra improvement (Mishra et al. 2017). Hybrid breeding programs will have to proceed in reverse from the market or consumer feedback to product design and value addition by R&D teams. This is taking place in the private sector but perhaps not to the desired level in the public sector. The deployment of dominant genes conditioning resistance to disease and pests needs to be implemented urgently to reduce the cost and extensive use of fungicides and insecticides, and ultimately to reduce cultivations costs and to minimize generating toxic residues. The future objective is to harness heterosis which is stable, along with economic hybrid seed production and the incorporation of biotic- and abiotic-stress tolerances and enhanced nutraceutical properties. Future research efforts should include the following:

- (a) Identification of stable YVMV resistant sources in the cultivated okra species.
- (b) The immediate need to develop okra varieties having combined resistance to YVMV, ELCV, and nematodes.
- (c) Develop okra lines having absolute resistance to fusarium wilt and powdery mildew diseases.
- (d) Develop okra lines suitable for baby-okra types (less than 5 cm size) for export purposes.
- (e) Develop cultivars tolerant to low temperature for the processing industry.

Appendixes

Appendix I: Research Institutes Involved in Okra Research

Institution	Specialization and research activities	Contact information and website
ICAR-Indian Institute of Vegetable Research	Research for the development of improved okra varieties	Director, ICAR-Indian Institute of Vegetable Research, Post Bag No. 01; P. O. Jakhini (Shahanshapur), Varanasi-221,305, Uttar Pradesh, India Telephone: +91-542-2,635,247; 2,635,236 + 91-5443-229,007 Website: https://www.iivr.org.in/
ICAR-Indian Institute of Horticultural Research (IIHR)	Research for the development of improved okra varieties	ICAR- ICAR-Indian Institute of Horticultural Research (IIHR), Hessaraghatta Lake Post, Bengaluru-560,089, India E-mail: director.iihr@icar.gov.in website: https://www.iihr.res.in
ICAR-Indian Agricultural Research Institute	Research for the development of improved okra varieties	Director, ICAR-Indian Agricultural Research Institute Ph: +91-11-25842367; Fax: +91-11-25846420 E-mail: director@iari.res.in http://www.iari.res.in
Botanic Garden Meise	Maintenance of plant herbarium	Botanic Garden Meise, Bouchout Domain, Nieuwelaan 38, 1860 Meise Telephone: +32 2 260 09 20 Fax: ++32 2 260 09 45 https://www.plantentuinmeise.be/en/home/
Institute of Vegetables and Flowers	Research for the development of improved okra varieties	Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS), 12 Zhongguancun Nandajie, 100,081 Beijing - China email: ivfcaas@public3.bta.net.cn Website: http://www.ivfcaas.net.cn
ICAR-National Bureau of Plant Genetic Resources	Okra germplasm conservation	ICAR-National Bureau of Plant Genetic Resources, PUSA, New Delhi-110,012, India Telephone: + 91-11-25843697, Fax:+ 91-11-25842495 Email: director.nbpgr(AT)icar.gov.in Website: http://www.nbpgr.ernet.in/
The New York Botanical Garden	Maintenance of plant herbarium	The New York Botanical Garden,2900 Southern Boulevard, Bronx, NY 10458-5126 Telephone: +1 (718) 817-8622 email: bthiers@nybg.org Website: http://sweetgum.nybg.org/science/
World Vegetable Center	Okra germplasm collection maintenance and breeding	World Vegetable Center, P.O. Box 42, Shanhua, Tainan, Taiwan 74151 Phone: +886-6-583-7801 Email: info@worldveg.org Web: avrdc.org

Appendix II: Genetic Resources of Okra

Cultivar	Important traits	Cultivation location
Kashi Vibhuti (VRO-5)	Dwarf growth habit/bushy type, plant height 60–70 cm during rainy and 45–50 cm during the summer seasons. It bears 2–3 branches with short inter-nodal length. Flowering starts on 4–5th nodes after 38–40 days after sowing. A plant bears 18–22 fruits with 8–10 cm length at marketable stages; yield 170–180 q/ha	India: Rajasthan, Gujarat, Haryana, and Delhi
Kashi Mohini (VRO-3)	Plants are tall, height 110–140 cm, flowers at 4–5th nodes during summer and 5–7th nodes during rainy season after 39–41 days of sowing, fruits 5 ridges, 11.3–12.6 cm long at marketable stage, suitable for summer and rainy season cultivation; gives yield of 130–150 q/ha	Different parts of India
Kashi Mangali (VRO-4)	Plants are tall, height 120–125 cm, flowers at 4–5th nodes after 40–42 days after sowing, fruits 5 ridges, light green; yield 130–150 q/ha	India: Punjab, Uttar Pradesh, Bihar, Jharkhand, Chhattisgarh, Orissa, and Andhra Pradesh
Kashi Pragati (VRO-6)	Plants are tall, height 130–175 cm, with 1–2 effective branches. Fruits are 8–10 cm in length at the marketable stage, 23–25 per plant and yield 180–190 q/ha during rainy and yields 130–140 q/ha during the summer season	India: Chhattisgarh, Orissa and Andhra Pradesh
Kashi Satdhari (IIVR-10)	Plant height is 130–150 cm with 2–3 effective branches, flowering at 42 days after sowing at 3–4th nodes. A plant bears 18–25 fruits with 7 ridges, length 13–15 cm at the marketable stage and yields 110–140 q/ha	India: Uttar Pradesh, Bihar, and Jharkhand
Kashi Lila (IIVR-11)	Plants are of medium height (110–130 cm), flowering starts 30–34 days after sowing. This is suitable for cultivation during rainy and summer season as early crop due to low-temperature tolerance. Fruits with 5 ridges, green and 13–15 cm long. This is resistant to YVMV and gives yields of 150–170 q/ha	India: Chhattisgarh, Orissa, Andhra Pradesh, Rajasthan, Gujarat, Haryana, and Delhi
Shitla Upkar (DVR-1)	Hybrid variety. Plants are medium tall, height 110–130 cm, flowering starts at 38–40 days after sowing at 4–5th nodes. Fruits are green, 11–13 cm long at the marketable stage and yields 150–170 q/ha. This is resistant to yellow vein mosaic virus and OLCV	India: Punjab, Uttar Pradesh, Bihar, Madhya Pradesh, and Maharashtra
Shitla Jyoti (DVR-2)	Hybrid variety. This hybrid is suitable for the warm humid climate with relatively long day length. Plants are medium tall, height 110–150 cm, flowering starts on 30–40 days after sowing at 4–5th nodes. The fruit is green, 12–14 cm long at the marketable stage, yields 180–200 q/ha. This is resistant to YVMV and OLCV	India: Rajasthan, Gujarat, Haryana, Delhi and Chhattisgarh, Orissa and Andhra Pradesh

(continued)

Cultivar	Important traits	Cultivation location
Kashi Bhairav (DVR-3)	Hybrid variety. Plants are medium tall with 2–3 branches; fruits are dark green with 10–12 cm length at the marketable stage; yield 200–220 q/ha. This is resistant to YVMV and OLCV under field conditions	All the okra growing region of India
Kashi Mahima (DVR-4)	Hybrid variety. Plants are tall, height 130–170 cm, flowering starts at 36–40 days after sowing at 4–5th nodes, fruits are green with 12–14 cm of length at the marketable stage and yield 200–220 q/ha. This has shown field resistance against YVMV and OLCV	India: Punjab, Uttar Pradesh, Bihar, Jharkhand, Chhattisgarh, Orissa, Andhra Pradesh, Madhya Pradesh, and Maharashtra
Kashi Kranti (VRO-22)	It is an early, medium-tall (100–115 cm) variety with short internodes. Resistant to yellow vein mosaic virus and leaf curl virus under field conditions. It takes 38–42 days for first flowering and each plant bears 18–20 fruits of dark green color. The fruits are available from 45 to 95 days after sowing and total yield is 140–150 q/ha	India: Uttar Pradesh, Bihar, Jharkhand, and Punjab
Kashi Shristi (VROH-12)	High yielding (180–190 q/ha) okra hybrid variety having short internodal length, and resistant to YVMV diseases. It is suitable for both summer and rainy season cultivation	India: Uttar Pradesh
Kashi Chaman (VRO-109)	High yielding (150–160 q/ha) variety having short inter-nodal length and resistant to YVMV and OLCV diseases. It is suitable for both summer and Kharif season cultivation	India: Uttar Pradesh
Kashi Lalima (VROR-157)	Early maturing, high-yielding (140–150 q/ha) reddish-purple fruited okra variety, rich in anthocyanin and also having resistance to YVMV and OLCV. It is suitable for both summer and Kharif season cultivation	India: Uttar Pradesh
Kashi Vardan (VRO-25)	High yielding (140–150 q/ha) okra variety having a short inter-nodal length along with 2–3 branches and having resistance to both YVMV and OLCV, while moderately tolerant to major pests under field conditions. It is suitable for both summer and rainy season cultivation	India: Uttar Pradesh, Bihar, Jharkhand, and Punjab

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Chapter 7

Plantain (*Musa paradisiaca* L.) Genetic Improvement and Germplasm Management with Emphasis on Cross River State in Nigeria



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Abstract In recent years, there has been an increase in demand for plantain as a major staple with enormous potential for domestic and global trade. It provides a vital source of income for many developing countries and increased awareness exists about its nutritional value and nutraceutical properties. However, knowledge regarding the scope of genetic and phenotypic diversity among most commercial plantain cultivars has received less attention. Achievements have been made in the genetic improvements of plantain through in vitro culture techniques, cryopreservation, induced mutation breeding, haploid production and production of virus-free plantain. In addition, estimation of cultivar variability based on molecular markers revealed wide genetic diversity useful for selecting elite genetic resources. Clustering based on scores of standard phenotypic traits delineate plantains into distinct groups, with one of these presenting a dichotomization event that results in both a double and triple bunching phenotype at fruiting. The best agronomic practices adopted for high yield of plantain are also evaluated. Findings suggest the presence of significant variability that symbolizes an excellent opportunity to bring about genetic improvement, management and conservation of plantain germplasm through selection of high-yielding cultivars exhibiting unique traits. This chapter highlights research progress relevant to these aspects.

Keywords Genetic diversity · Inflorescence · Molecular breeding · Micropagation · Microsatellite markers · Phenotypic plasticity · Plantain

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7.1 Introduction

Plantain (*Musa* spp. AAB genome) is one of the perennial giant herbaceous plants in the genus *Musa*. Plantain is one of the most useful food crops in Africa, where it has been widely utilized and is very prominent ingredient in local delicacies and as a major driver of local agrarian economies (Crouch et al. 1998). The crop remains one of the most important dietary energy providers in the wet humid and subhumid ecological climates of the globe, where it is farmed and provides essential nutrition for more than half a billion people (Ogazi 1996). The fruits (fingers) are of extraordinary significance, providing an important and cheap source of carbohydrates, vitamins, and several essential minerals including potassium, sodium, phosphorus and iron. Presently, Sub-Saharan Africa produces more plantain than the rest of the world combined (FAOSTAT 2018). Presently, in many parts of the African continent, plantain and certain other crops such as rice and manioc constitute all year and/or seasonal staples crops. Plantain also serves as an ingredient in many community cottage industries for the manufacture of beverages and local brewed products (Ssebuliba et al. 2000), chemicals and fermented liquor, which are very nutritious due to their high content of B-complex vitamins and high population of fungal yeast in the brew. Plantain also finds wide utilization in the area of excellent flour production in terms of quality by using the peeled unripe fruit and dried pulp. The flour is a very good source of carbohydrate and is thus prescribed for use by diabetics as it has proven to have higher biological value than dietary energy sources obtained from other food crops (Vuylsteke et al. 1996).

Thus far, progress in plantain production in Africa has depended to a large extent on the ability to select high-yielding cultivars from a segregating population (IITA 2007). And given that the estimation of genetic diversity in a crop species is a prerequisite for its improvement, there is an encouraging possibility that different plantain cultivars can be improved once genetic variability has been ascertained using appropriate selection indices. However, the expected genetic response to selection is determined by heritability and the variability of the traits for which the crop is selected for cultivation are to be made relative to the farming systems prevalent in a given socioeconomic environment. There is no doubt that an accurate knowledge of genetic diversity and relationships among plantain collections in any preserved germplasm is essential and important for the establishment, management and guarantee of long-term success of plantain improvement programs through breeding (Dzomeku et al. 2007).

Plantain cultivars show considerable variations for many horticultural traits such that different criteria including pedigree records, morphological traits and DNA marker technology (Weising et al. 2005) have been used in the past to estimate genetic diversity prevalent within the species.

Like with banana, different plantain-producing areas currently suffer from newly emerging pests and diseases and rapidly changing environmental conditions. In the absence of locally-adapted resistant varieties and a general lack of characterized

germplasm that could be used as potential parents for breeding purposes, farmers need to extensively use pesticides, which threaten the sustainability of not only the crop but also the environment (Pennisi 2010).

There is therefore an urgent need for the selection of cultivars with significant variability of genetically-improved characters with more improved disease resistance, enhanced yield and superior potential to adapt to a robust area with optimum growing conditions. In consideration of the fact that an understanding of the variability among different plantain varieties is desirable for setting up of an efficient strategy for breeding improved cultivars and support the choice of parents that can be used for regeneration, a very robust appreciation of the genetic and phenotypic diversity of available resources is of paramount importance (Jarret and Gawel 1998). Consequently, this chapter is designed to provide details on the diversity available, based on information generated through molecular fingerprinting and variations in morphological and yield-related traits within a large plantain germplasm collection, as a foundation for selection and conservation of genetically-superior cultivars that can be used for further research into the breeding and improvement of this important staple crop (Ogazi 1996; Swennen 1997).

The pool of genetic variation within a parthenocarpic population of plantain is the basis for selection and improvement of plantain germplasm. In recent years, there has been increasing awareness of a holistic approach in agricultural biodiversity conservation for sustainable utilization and development for food security and income generation (Simmonds and Shepherd 1995).

Hence, in the conservation of plantain (*Musa paradisiaca* L.) genetic diversity (germplasm) is indispensable for sustainable agriculture and for the well-being of present and future generations. Thus, understanding the distribution and scope of biodiversity in plantain germplasm availability in Cross River State, Nigeria, would help in the proper conservation and sustainable use, since changes in genetic variability among plantain populations largely depend on time and space. Moreover, the scope and distribution of genetic diversity in the plantain germplasm relies greatly on its evolutionary pathways, breeding systems, ecological and geographical factors, past bottlenecks as well as biotic and other abiotic factors (Brisibe and Ekanem 2019).

This chapter presents an overall view of the historical development, origin and distribution, taxonomic classification, germplasm diversity, genetic, species and ecological diversities of plantain, sustainable agronomic practices, characterization and conservation, traditional and modern cultivation practices for elite plantains cultivated in Cross River state, Nigeria (Fig. 7.1). It also presents recent developments on biotechnology and molecular phylogeny and their application for the crop improvement in association with conventional plantain breeding methods along with utilization.

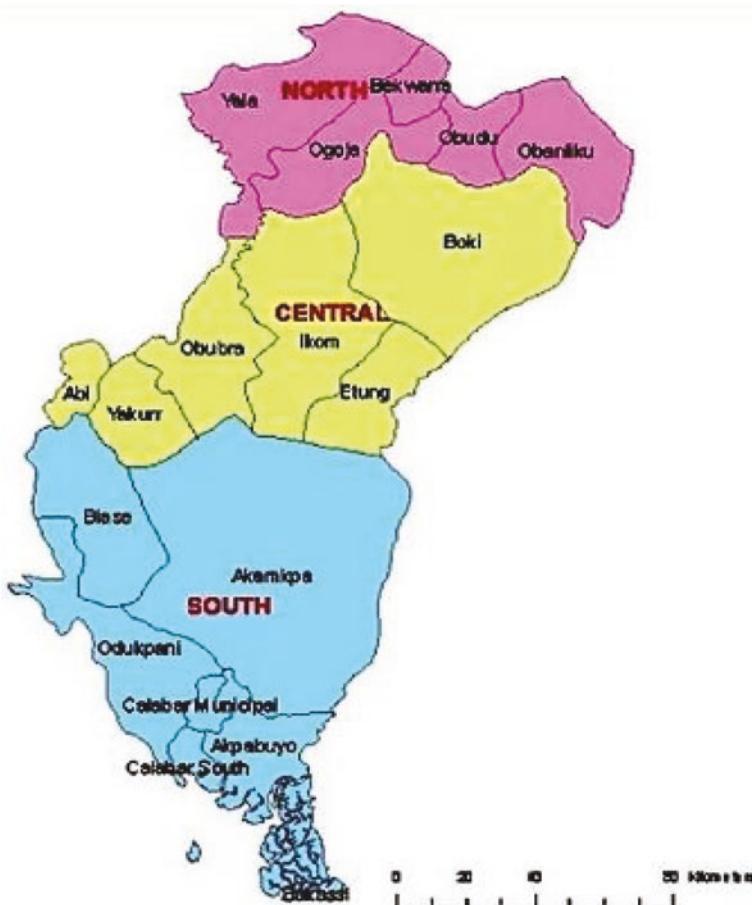


Fig. 7.1 Map of Cross River State, Nigeria, showing major areas of plantain production. Pink (low production area), Yellow (high production area), Sky blue (average production area). (Source: Fadama III project, Cross River State, Nigeria, 2015)

7.2 Multiplication Techniques Adopted in Cross River State

7.2.1 Split Corm

This is normally obtained from a ball-earth corm that is usually split into several units weighing about 50 g each. The unit should have eyes or buds to facilitate sprouting. Each unit should be treated with fungicides such as Captan or Furadan against infections before spreading in the sun to harden the cut surface. The units are then planted in a nursery for 7–8 weeks for sprouting. The sprouted seedlings are the peepers which are then transplanted at a spacing of 15 × 15 cm into the field (Fogain et al. 1998; Speijer 1959; Wilson 1983).

7.2.2 *Bakers Technique*

This is also called the stripping technique and involves the stripping of the outer leaves to the base rhizome, which stimulates the growth of auxiliary buds to form suckers. The suckers are removed as soon as they sprout (peepers) and cultured in the nursery (Gerda 1990).

7.2.3 *Hamilton Technique*

This is carried out on a mature plantain of about 6–12 months of age that has not produced fruits. The pseudostem is cut above the corm and the apical meristem is destroyed, which stimulates and promotes the growth of adventitious buds. Peepers are then removed and cultured in the nursery (Dzomeku et al. 2007).

7.2.4 *Use of Peep and Sword Ratoon/Follower Crops Suckers*

Most traditional farmers use healthy corms obtained from ratoons and follower crops as planting materials or parent stock. This is mostly practiced by rural communities on small and large plantation farms. This practice has the advantage of sporadic spreading and development of plantains from ratoons and follower peepers and sword suckers which develop within a very short time from the corm. In most cases, the farmers remove the peep and sword suckers to another planting hole using appropriate spacing distance (Fogain et al. 1998).

7.2.5 *Traditional Breeding Limitations*

The traditional breeding method has several limitations including the inability to check and improve the genetic makeup of the cultivars. The genetic characteristics of the cultivars are not ascertained by farmers before planting, leading to inbreeding depression. Associated diseases such as black sigatoka disease and bunchy top diseases are simply transmitted from one generation to another. There is continuous declining yield and outputs due to low genetic makeup and poor breeding/management practices (Gill 1998).

7.3 Biotechnological Improvement Efforts for Plantain Germplasm

Conventional efforts to propagate, conserve and breed cultivated *Musa* are fraught with obstacles such as low reproductive fertility, slow rate of vegetative propagation and long growth cycle. Interestingly, tissue culture and molecular genetic methods are increasingly being used as enabling techniques for handling and improvement of *Musa* germplasm worldwide. In some research institutes such as the International institute for Tropical Agriculture, Ibadan, Nigeria, shoot-tip culture is routinely used for rapid propagation, safe international exchange and germplasm conservation (Vuylsteke et al. 1996).

7.3.1 *Microppropagation*

This technique has been crucial for the rapid supply of large numbers of female and male plantain plants for crossing and for the continual supply of promising new hybrids for field evaluation trials. Over 300 new *Musa* accessions have been introduced through shoot-tip culture during the last decade (Vuylsteke 2000).

Plant tissue culture gives the opportunity to grow plant material in artificial conditions with advantages such as: possibility for sanitation (culture in sterile conditions, pathogen cleaning); rescue and conservation (continuous availability, space saving, cost-effectiveness, cryopreservation); micrografting and micropagation (mass multiplication: bioreactor, cell suspension, polyshoot development); germplasm exchange; open access for more research (genetic transformation, somatic embryogenesis, haploid production, embryo rescue, molecular, pathological/virological investigations) in the production of plantain and other *Musa* species (IITA 2018).

Micropropagation using shoot tip culture reveals that plantain is very responsive to in vitro plant tissue culture meristem regrowth in 4–6 weeks, followed by shoot development proliferation to subculture of fully developed plantlets. Micropropagation of plantain multiplication using semi-solid culture medium reveals that plantain proliferation medium shows an average multiplication rate of 4:1 in semi-solid culture medium components using 4:1 MS basal medium (Murashige and Skoog 1962) supplemented with 4.43 mg/L myo-inositol, 100 mg/L sugar, 30 mL/L indolacetic acid (IAA), 0.18 mg/L benzylamino purine (BAP) and 4.5 mg/L ascorbic acid in 10 mg/L agar (IITA 2018).

7.3.2 *Micropropagation of Plantain Using Temporary Immersion System (TIS) Bioreactor*

Micropropagation and multiplication of plantain using the temporary immersion system (TIS) bioreactor Rocket kit shaker and Southern sun bioreactor air-lift are instruments used for the micropropagation of plantain plantlets. Micropropagation

of plantain multiplication using the TIS bioreactor showed that the average multiplication rate using TIS was very high. The hardening and acclimatization process revealed more than 95% success of micropropagation using this protocol (IITA 2018).

7.3.3 Somatic Embryogenesis in *Musa*

In plantain, like any angiosperm, embryo production by using somatic cells shows special attributes of plasticity development in the plant cells. Somatic embryogenesis (SE) induction with somatic embryos using embryogenic cell suspension (ECS) cultures is an authentic achievement protocol for enhancing mass-propagation and production of plantain because of the high percentage of regeneration, and remains a tool to reckon with in cellular proliferation in traditional breeding of the crop. Mechanisms and laboratory approaches used for somatic embryogenesis are basic for many *Musa* species like those having AA and/or BB genomes, wild dessert (AA, AB, AAA), cooking banana (ABB) and cultivated plantain (AAB) using various forms of the explants. Although, in most instances, the procedures are confined to low embryo sprouting and plant establishment rates. Hence, the need for a conscious attempt to understand the biochemical, physiological and genetic procedures underlying plantain zygotic and somatic embryo development, so as to develop a reliable somatic embryogenesis methodology with a high percentage of embryo sprouting, establishment and plant development (Vuylsteke 2000).

An efficient and simple procedure has been developed for explant development using an in vitro somatic embryo protocol in *Musa* spp. In vitro propagation, using the somatic embryogenesis approach, has been exploited to obtain identical planting stock in *Musa* spp. In a current study, somatic embryogenic plants were achieved in selected *Musa* genotypes with immature male flower bud explants. Evaluation carried out using two stages of somatic embryogenesis, including induction and maturation of somatic embryos, revealed that embryogenic calli desiccated for up to 2 h at 25 ± 1 °C resulted in higher frequencies of embryonic induction and maturation, compared to nondesiccated embryos. Regenerated explants are hardened and the genetic fidelity of the plantlets confirmed using sequence-related amplified polymorphism (SRAP) molecular markers. Furthermore, it was observed that plantain plants derived from somatic embryogenesis showed normal morphotypes, the same as phenotypes derivable from a parent plant. The establishment of follower plants from male flower bud was achieved in 6–7 months, which is a relatively good period of time for plantain genotypes when compared to related studies involving different accessions of same plant. The procedure adopted can be very useful for large-scale micropropagation investigations for important commercial species of plantain (Jain 2007).

Somatic embryogenesis signifies a process in which bipolar appendages, similar to a zygotic embryo, develop from a nonzygotic cell without vascular connections to the original tissue. SE is an important system where the multiplication of plantain

plantlets can be carried out speedily. During the development process, somatic embryogenesis undergoes four consecutive stages: (a) somatic embryogenesis induction in the plantlets, (b) formation of somatic embryo, (c) somatic embryo maturation and (d) germination of somatic embryos and their conversion into viable explants (López et al. 2013). Somatic embryogenesis thus remains an ideal approach for the development of procedures to achieve rapid multiplication of plantlets, new genotypes and synthetic seed production. The selection protocol using an in vitro approach utilizes different living and non-living components and their effects to study genetic manipulation. The best established explants are obtained from the proliferating meristems and male bud inflorescences. This allows for the development of embryonic cell suspension (ECS), outright formation of somatic embryos, and the subsequent development into plantlets that are further exposed for field establishment and evaluation. The procedure is based on the use of explant shoot apices derived from the multiplication of the axillary buds in an ancymidol solution (0.2–0.4 mg/L) and depends on the plant genotype used. It also depends on the somatic embryos potential to achieve maximum development in semisolid and liquid culture media. The use of shoot apices of axillary buds to induce embryonic cell suspensions in AAB group plantain cultivars prompted the need to scale up propagation using somatic embryogenesis and temporary immersion system (biofactory) that are being used as an alternative for propagation of good clones (IITA 2018) and its application in the plantain genetic improvement program by mutations (Jain 2010).

7.3.4 *Embryo Culture and Rescue Techniques*

These techniques are applied to increase the germination rate of true seeds from crosses, with more than 10,000 seeds cultured at once in the laboratory each year.

Unlike suckers, the adoption of explants developed through tissue culture (TC plantlets) show several merits. Tissue culture developed plants tend to be cheaper and show easily transported and propagated. The TC plantlets have a high establishment rate in the field. Plantlets are resistant and help reduce the cost of controlling foliar diseases by at least 50%. The homogeneity of their development avails the farmer the opportunity to be in charge of routine developments in the plants such as flowering and harvesting time, resulting in a significantly improved yield output and quality (Hwang et al. 1984). At the preliminary stage of development, tissue culture plantlets are more susceptible to herbicide tolerance than sword suckers. Because labor costs are high, weed control in large farmlands by hand hoeing is impractical. Thus the indiscriminate use of herbicides will result in serious plant damage. Mature plants developed from tissue culture plantlets will produce *high mat* or exposed roots thus making the plants vulnerable to lodging and wind action after shoot development and possible topple over.

7.3.5 Use of Somaclonal Variation

Somaclonal variation as a tool for genetic improvement of plantain germplasm derived from shoot-tip has also been extensively explored, but with limited success (Vuylsteke et al. 1996). Plantain suckers were subjected to in vitro shoot-tip culture to produce true-to-type somaclones. Field performance of the somaclonal variants were evaluated for development of multiple bunching horticultural traits. Somaclones from the first, second and third ratoon crops produced the same number of multiple bunches, similar to the parent stock, were regenerated, multiplied and distributed (Vuylsteke 2000).

7.3.6 Cryopreservation of *Musa* Germplasm

Cryopreservation refers to the storage of biological samples at an ultra-low temperature (-196°C) in liquid nitrogen. Cryopreservation techniques may also serve as a pathogen eradication tool. Meristem clumps of in vitro cultures regenerated from viral-infected plantain plants were exposed to the ultra-low temperature in liquid nitrogen to reduce viral infections. The procedure for subculturing is strenuous and provides an opportunity for plants to become infected by any pathogenic microbial contaminants. In addition, plants established in vitro, especially under reduced growth conditions, are prone to exhibit somaclonal variation. Overcoming these limitations and achieving long-term conservation of *Musa* germplasm resources and biodiversity is supported by cryopreservation research and storage. This cryopreservation method ensures cost-effective, safe and long-term storage of genetic resources of species which are not seed-bearing and are vegetatively propagated, such as *Musa*. This research was earlier developed and carried out at the Katholieke Universiteit Leuven, Belgium (KULeuven) and the techniques developed are now being utilized for routine cryopreservation of accessions held in different plant biodiversity collections. About one-half of the plant germplasm is at the moment preserved safely in liquid nitrogen for long-term storage. Cryopreserved collections are considered complementary to in vitro collections and serve as safe gene banks in case genotypes are lost due to impurities, somaclonal variation or laboratory mistakes arising from subculture procedures (Roux et al. 2003).

Approaches in cryopreservation are routinely applied to any type of plant tissue with high potential for regeneration. Model protocols have now been developed for over 200 diverse plant species, nurtured in different forms such as cell suspensions, calli, apices, somatic and zygotic embryos. Two types of meristematic and regenerative in vitro tissue can be obtained from plantain: (i) individual meristems obtained from shoot-tip cultures and (ii) highly proliferating meristem cultures containing *cauliflower-like* meristem clusters. Cryopreservation methods exist for both tissue types. In addition, embryogenic cell suspensions of different cultivars belonging to distinct genomic groups are also now being stored in liquid nitrogen (López et al.

2005b). The primary purpose of preserving embryogenic cell suspensions of plantain for the long term is not the conservation of *Musa* diversity. Some plantain accessions are recalcitrant toward the establishment of embryogenic cell suspensions and moreover this process is extremely time consuming (usually 15 months); cryopreservation should be considered in this case as an aid for biotechnological applications such as genetic engineering (López et al. 2005a). The merits and demerits of each protocol are explained and areas not properly addressed are further explored with a view to further optimize all the methods identified. The availability and the nature of the recipe used, the accessions undergoing cryopreservation and resource availability will have to be taken into consideration so as to determine which of these methods are most suitable for use in other laboratories (López et al. 2005b; Roux et al. 2003).

7.3.7 Production of Virus Free Plantain

Virus-infected planting materials are a major problem in plantain cultivation, exchange and storage of plantain germplasms. Most *Musa* gene banks are severely affected by viral diseases. Viral resistance can confer by the transgene in plantain; genetic manipulation in plantain, including the introduction of viral resistance, has been reviewed several times. Therefore, viral elimination therapies like meristem tip culture, chemotherapy and cryotherapy to produce virus-free *Musa* planting material are pertinent. Some of the efficiency of in vitro approaches used for viral eradication in other crops are discussed at the end of this section. In vitro culture of apical meristems or shoot tips are the best choice for explant initiation of in vitro clonal propagated selected *Musa* genotypes. The meristem-tip culture technique is generally considered a tool for virus elimination. However, the meristem culture technique is not able to remove CMV (cucumber mosaic virus) from plantain plants. In another report, few virus-free plantlets could regenerate via in vitro meristem culture technique from BSV (banana streak virus) infected plantain plants. Further, this technique was unable to recover even a single BBTV-free (banana bunchy top virus) *Musa* cultivar. Several other researchers also reported the inefficiency of the tissue culture technique to eradicate viruses in *Musa*. Therefore, other therapies in combination with the in vitro meristem-tip culture technique were screened to regenerate virus-free planting material. Furthermore, the plant tissue culture process itself may induce excision of BSV integrated sequences to cause BSV infection (Jain et al. 2007).

Furthermore, antiviral molecules adefovir, tenofovir or 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine (PMEDAP) containing culture medium was used to target viral reverse transcriptase for a duration of 6 months. The treated ones were cultured to regenerate plantlets. Six-month-old plantlets in the greenhouse exhibited 69, 88 and 90% BSV eradication after PMEDAP, adefovir or tenofovir treatments, respectively. Thus, chemotherapy of the infected in vitro cultures was found quite efficient for the elimination of BSV. Chemotherapy may prove

effective for the other viruses as well in future viral disease eradication programs (Roux et al. 2003).

7.3.8 *Induced Mutation in Musa Species*

This section looks at a procedure which involves the use of embryogenic cell suspensions (ECS) in plantain (*Musa paradisiaca*) using both the in vitro gamma irradiation and plant regeneration approaches in order to attain maximum genetic improvement. The procedure involves an array of activities to properly select embryonic cell suspension for irradiation and the handling of posttreatment plant regeneration in addition to mutant selection through the acclimatization process and under field conditions (Xu et al. 2012). Mutation-induced plantlets will unmask a recessive phenotype by either using mutation processes, inhibition or deletion of the homozygous dominant allele (Jain and Swennen 2004). The success of in vitro mutation breeding depends on the development of reliable and viable in vitro plant regeneration protocols, optimization and types of mutagens used as well as the efficient screening of the mutation inbred lines for the desired variations (Xu et al. 2012). Thus, somatic embryogenesis offers an efficient protocol for clonal propagation and mutation induction in plantain. Somatic embryos originate from a single cell, preventing somaclonal variations in regenerated plantlets and makes them an excellent tool for mutation breeding. Research has also revealed that somatic embryos emerging from plantain embryonic cell suspensions in most cases possess a single-cell origin. Thus, the merit of utilizing embryonic cell suspension for mutagenesis would best be obtained either in nonchimeric populations or the quick dissociation of the chimeric sectors on emergence (Roux et al. 2001).

7.3.9 *Haploid Production in Plantain*

The regeneration of haploid plants by the classical method of anther culture (androgenesis) is employed in various crops. It requires the evaluation of several generations, which is difficult in false fruit crops like plantain. Research reveals that the most effective means of haploid plants production using anther culture of plantain is by adopting flow cytometric procedures for polyploid determination (Jain et al. 2007).

Plantains with haploid genotypes were developed from the anther callus of plantain *Musa paradisiaca*. The highest rate of callus induction of about 90% was obtained at 2,4-D concentration of 3 mg/L. After 3 weeks of incubation, embryonic cells were developed from the callus mass. A combination BAP at 4 mg/L and indole acetic acid at 0.4 mg/L induced shoot growth of the embryonic cells and well developed roots system at a concentration of 0.6 mg/L NAA with an augmented media of 0.2% activated charcoal. The results of the investigation showed that the

protocol was efficient in the production of haploid plants from anther culture (Roux et al. 2003).

Analyses of flow cytometry was carried out by determining the intensity of leaf DNA of the in vitro regenerated plants. Jain et al. (2007) showed that identification of plantlets with lower nuclear DNA intensity value was as a result of the prevalence of aneuploid plants. This result was based on the assumption that many of the chromosomal changes were identified by the flow cytometric method were associated with variations in the number of chromosomes. These variations were capable of inducing total or partial loss of chromosomes during the doubling process (Roux et al. 2003). Flow cytometric studies thus provide the basis for other investigations to detect plants with values of nearly twice the nuclear DNA content, suggesting possible in vitro polyploidization.

7.4 Plantain Diversity in Cross River State

The biological diversity of plantain (*Musa paradisiaca*) can be viewed from three perspectives:

- (a) Genetic diversity—which has to do with the heritable variations in genes and genotypes of the plantain germplasm availability in Cross River state;
- (b) Plantain (*Musa paradisiaca*) species diversity—which has to do with the plantain species richness in Cross River state (elite cultivars that are mostly cultivated);
- (c) Ecosystem diversity of plantain which takes into consideration the ecosystem (ecology), that best suits and favors the cultivation and growth of plantain (elite cultivars) germplasm in Cross River state.

The importance of biodiversity to mankind cannot be overemphasized. It has paved the way for the sustenance of the socioeconomic systems in ways that allow the poorest of the poor to obtain their food and nutritional needs while retaining their cultural diversity as a people (Tingey et al. 1994).

Plantain (*Musa paradisiaca*) is an important food crop with the most diverse uses in the tropics; it is seen as a very important aspect of food security and provides a reliable source of income to rural agrarian localities due to its excellent morphological attributes (Table 7.1). Plantain is undoubtedly one of the most important staple plant based foods and one of the oldest cultivated fruit crops in Sub-Saharan Africa, Central Africa, South America and Asia (Swennen et al. 1995). Plantain cultivation is undoubtedly an activity of important from a socioeconomic standpoint in the rainforest zone of Nigeria by ensuring food availability, job creation and sustainability, and it dominates the farming communities and remains a very important source of rural income and is an economic mainstay (Ortiz and Vuylsteke 2002).

Plantain production remains a promising agricultural investment with enviable economic returns in Central and West Africa region and South America, which remain the major core plantain growing regions globally. It is one of the few most

Table 7.1 Morphological attributes of elite plantain accessions cultivated in Cross River State

Elite plantain accessions	Length of cycle (months)	Shape	Bunch weight (kg)	No. of hands/bunch	No. of fingers/hand	Finger skin color	Pulp color	Harvest interval (days)	Fingers cross section	Pseudostem color	Bunch pheno-type	Hardness (kg/cm ³)	Weight ratio	
Ogoni red French	15	Medium /curve	13.23	6	4	24	Red	Brown	69	Triangular	Purple	French type	1.9–1.7	1.3–1.5
Kigwa brown false horn	14	Medium/ curve	12.01	4	4	16	Brown	Milky white	62	Quadrilateral	Gray	False horn	1.8–1.6	1.3–1.5
Enugu black false horn	15	Big /curve	23.54	8	8	64	Dark green	Creamy	71	Pentagonal	Green	False horn	1.4–1.1	1.2–1.3
Ebi egome false horn	14	Big /flat	22.13	9	9	81	Pale green	Creamy	74	Quadrilateral	Green	False horn	1.4–1.1	1.2–1.3
Owomoh true horn	13	Medium /flat	15.79	6	5	30	Pale green	Milky white	65	Triangular	Gray	True horn	1.7–1.5	1.3–1.3
Kenkwa false horn	14	Medium /curve	16.23	7	6	42	Pale green	Creamy	66	Pentagonal	Brown	False horn	1.3–1.0	1.02–1.15
Kainjen false horn	15	Big /flat	19.88	8	6	48	Olive green	Whitish green	62	Quadrilateral	Brown	False horn	1.7–1.5	1.3–1.4
Uhom false horn	13	Medium /curve	14.23	7	5	35	Pale green	Creamy	67	Quadrilateral	Green	False horn	1.7–1.5	1.3–1.4
Ekamkwam French	14	Medium /flat	16.22	9	8	72	Dark green	Creamy	63	Triangular	Green	French type	1.1–0.9	1.14–1.3
Ikpobata French cooking bananas)	13	Small /curve	7.21	4	4	16	Dark green	Milky white	69	Triangular	Brown	French type	1.9–1.7	1.5–1.7
Mgbeghe false horn	14	Big /flat	24.97	6	7	42	Pale green	Creamy	73	Quadrilateral	Green	False horn	1.9–1.7	1.4–1.6

(continued)

Table 7.1 (continued)

Elite plantain accessions	Length of cycle (months)	Shape	Bunch weight (kg)	No. of hands/bunch	No. of fingers/hand	No. of fingers/bunch	Finger skin color	Pulp color	Harvest interval (days)	Fingers cross section	Pseudostem color	Bunch pheno-type	Hardness (kg/cm ³)	Weight ratio
Ingwam French	17	Medium /curve	15.64	6	5	30	Pale green	Creamy	65	Quadrilateral	Brown	French type	1.7–1.5	1.2–1.4
Bakpri French (dwarf mutant)	16	Small / curve	5.11	3	3	9	Dark green	Milky white	61	Triangular	Green	French type	1.8–1.6	1.4–1.6
Ejorgom True horn	15	Medium /curve	13.80	6	5	30	Pale green	Creamy	68	Pentagonal	Brown	True horn	1.7–1.5	1.3–1.5

Source: Ubi et al. (2016)

important sources of energy diet in most areas where it is cultivated and used as a main staple food crop by more than a billion people globally (Ray et al. 2006).

Plantain is a tropical crop which originated in Southeast Asia with a wide variety found in the area of Myanmar. It is a monocot plant with parallel venation. It is an herbaceous crop which matures within a year or two, but is naturally a perennial crop, because the suckers continue to produce. It does not have a woody stem. The true stem is found underground and is known as the rhizome. It has many eyes or buds, which grow into suckers.

Nigeria is one of the major plantain producing countries of the world, which is partly fueled by the higher rate of utilization of plantain products in the country due to the ever-increasing population and their need for sustenance (Racharak and Eiadthong 2007). Nigeria is the largest producer of plantain in West Africa, producing more than 64% of the total plantain in the sub region. Cross River state in Nigeria is the highest producer of plantain with almost all the local government areas of the state serving as hubs for plantain trade and distribution.

Plantain genetic resource conservation merits far greater attention than it is presently receiving due its wide economic advantage over most other indigenous/local food tree crops, arising from its non-seasonality, adaptability to the wide range of ecological habitats prevalent in Cross River state, availability of high yielding elite cultivars, ease of cultivation, availability of markets, high demand, availability of good postharvest processing techniques, better shelf life of products and good farm gate prices.

These factors have endeared the elite plantain (*Musa paradisiaca*) cultivars to Cross Riverians and thus enhanced the realization of the productive potentials of plantain by farmers. This has also created a heightened need for this discussion which intends to highlight the ecological biodiversity of elite plantain (*M. paradisiaca*) cultivars available in Cross River state in terms of genetic diversity, plantain species diversity and spread as well as ecological system diversity of the elite plantain germplasms in the state.

7.5 Molecular Characterization of Elite Plantain Germplasm in Cross River State

Plant genetic resources are among the most essential of the world's natural resources. Variability in genomic DNA sequences of plant cultivars have played a major role in varietal characterization and improvement of many crops species, including plantain, and have contributed to the capacity to assess biodiversity, evaluate phylogenetic relationship and estimate potential yield capacity (Alvarez 1997; Pillay et al. 2000).

Genetic diversity is the genetic variability existing among individuals of a species of the same genus (Nei 1978). Genetic diversity derives from the various

genetic differences or polymorphisms between and among individual species and may unfold in variations in DNA nucleotide sequences, in biological and physiochemical attributes (as seen in protein structure or isoenzyme properties), in chemical properties (temperature, nutrient deficiency, and other abiotic stress resistance or growth rate) and in phenological attributes such as plant height or color of unripe fruit (Ruangsuttapha et al. 2007).

Genetic diversity in plantain can be evaluated in terms of the different forms (alleles) as may be found in the different populations, their distribution and the overall distinctiveness between different populations. The variation that underscores genetic diversity in the elite plantain cultivars in Cross River state arises from mutations and recombination (Agoreyo et al. 2008). Selection, genetic drift and gene flow are among the indices of the alleles present in different populations which induce variation. It is generally believed that genetic variability in a plant population is structured in space and time (Lakshmanam et al. 2007).

Presently, conservation efforts for plantain germplasm include in situ and ex situ measures, which have proceeded with little or no information on genetic diversity. Gene banks have also been established for storage of plantain germplasm in the form DNA sequences in most of the biological databases like the National Centre for Biotechnology Information and the European Molecular Biology (IITA 2018).

Thus genetic diversity remains one of the sole bases for speciation, adaptation and survival, thereby preempting the possibility to advance the foundations upon which evolutionary relatedness and the survival of human depend. Hence, the knowledge of the genetic diversity of plantain biodiversity in Cross River state will help in the preservation of the rich and available plantain germplasms in gene banks and other in situ conservation media to prevent the loss of plantain biodiversity occasioned by biotic and abiotic stresses, competition, predation, parasitism, pests and diseases, isolation, habitat alteration, urbanization, climate change, natural disasters and human advancement. Thus it has become imperative, given this threat, to fully understand and utilize the knowledge of genetic diversity in the conservation of plantain genetic resources for food security, income generation and agricultural developmental sustainability.

Studies by Ubi et al. (2016) revealed the existence of sufficient amounts of genetic diversity among the elite plantain cultivars in Cross River state. Fourteen elite plantain cultivars sampled from the state and subjected to molecular analysis using microsatellite markers or simple sequence repeat (SSR) are given in Table 7.2; the markers in Table 7.3:

- (a) 55 mean total number of amplified bands
- (b) 65.47 mean % polymorphism
- (c) 0.67 mean polymorphic information content (PIC)
- (d) 3.54 mean marker option index
- (e) 0.832 mean gene diversity
- (f) 0.11–0.91 genetic distance range

Table 7.2 Selected microsatellite primers used for the 14 elite plantain (*Musa paradisiaca*) genotyping

S/N	Primer	Motif	References	Annealing temp. (°C)
1	Ma-1-32	(GA) ₁₇ AA(GA) ₈ AA(GA) ₂	Crouch et al. (1998)	58
2	Ma-3-90	(CT) ₁₁	Crouch et al. (1998)	53
3	mMaCIR 307	(CA) ₆	Hippolyte et al. (2010)	54
4	mMaCIR 264	(CT) ₁₇	Hippolyte et al. (2010)	53
5	mMaCIR 260	(TA) ₈	Hippolyte et al. (2010)	55
6	mMaCIR 39	(CA) ₅ GATA(GA) ₅	Lagoda et al. (1998)	52
7	mMaCIR 196	(TA) ₄ (TC) ₁₇ (TC) ₃	Hippolyte et al. (2010)	55
8	mMaCIR 214	(AC) ₇	Hippolyte et al. (2010)	53
9	mMaCIR 01	(GA) ₂₀	Lagoda et al. (1998)	55
10	mMaCIR 03	(GA) ₁₃	Lagoda et al. (1998)	53

Source: Ubi et al. (2017a, b)

The reports also showed that the 14 elite plantain cultivars in Cross River state are genetically grouped into 4 distinct clusters as shown in Fig. 7.2.

Microsatellite markers like simple sequence repeats (SSR) are more informative in the identification and discrimination of closely related genotypes and are considered a great asset in fingerprinting, mapping, and genotyping of plants, and can be used for distinguishing within and between the groups of plantains evaluated in this study. Hence, simple sequence repeat markers have been fully utilized for the purposes of genetic analysis, including genetic diversity (Fig. 7.3 and Table 7.3) of plants. It also used for the determination of the relationships between plant accessions, elucidation of evolutionary relationships (Fig. 7.4), as an instrument in the taxonomic classification of many plants including tropical pasture grasses, as well as in the molecular genotyping of important crops such as rice, orphan legume species, banana and plantain. Also, their use in the evaluation of dichotomous bunching plantain cultivars is highly valuable and highly recommended as this will help to pinpoint the degree of divergence within them on the one hand and between them and the single bunching cultivars of False Horn plantains on the other. In addition, utilizing molecular markers which can help to identify many genetic polymorphisms would equally make it feasible to address the relationship, if any, between morphological and genotypic variations among multiple (dichotomous) bunching plantain varieties that consequently will provide substantial insights in directing introgression and molecular breeding strategies in these varieties of plantain.

Table 7.3 Genotyping of the 14 Elite Plantain accessions using the selected microsatellite markers

S/N	Marker	Major allele frequency	Number of allele	Heterozygosity	Mean allele frequency	Coefficient of gene differentiation	Gene diversity	Polymorphic information content
1	mMaCR 01	0.105	18.00	0.543	0.405	0.364	0.886	0.942
2	mMaCR 03	0.357	10.00	0.400	0.634	0.433	0.765	0.694
3	Ma-3.90	0.167	15.00	0.474	0.543	0.436	0.852	0.913
4	Ma-1-32	0.215	12.00	0.493	0.665	0.555	0.831	0.876
5	mMaCR 0476	0.476	8.00	0.321	0.772	0.421	0.635	0.533
307								
6	mMaCR 0.239	18.00	0.522	0.475	0.678	0.836	0.874	
264								
7	mMaCR 0.329	14.00	0.357	0.609	0.628	0.762	0.685	
260								
8	mMaCR 0.250	13.00	0.453	0.584	0.546	0.829	0.855	
196								
9	mMaCR 39 0.200	18.00	0.531	0.665	0.455	0.860	0.893	
214								
10	mMaCR 0.383	9.00	0.365	0.576	0.626	0.772	0.670	

Source: Ubi et al. (2017a, b)

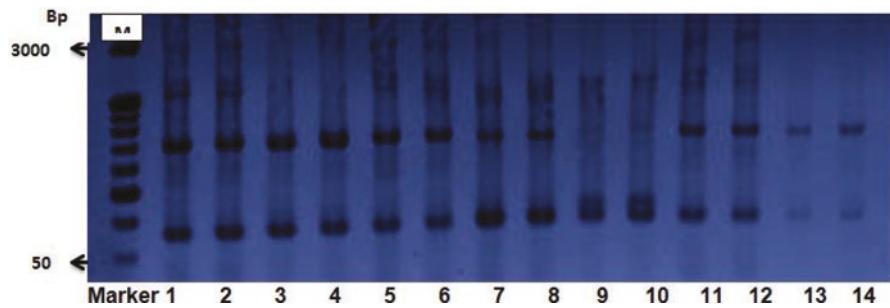


Fig. 7.2 Electropherogram showing amplified fragment bands of 14 elite cultivars of plantain amplicons in agarose gel using microsatellite SSR markers. Arrows in gel photo indicates the range (50–3000 bps) of size of the molecular marker (DNA ladder). Numbers 1–14 represents the different 14 plantain cultivars evaluated as presented in Table 7.4. (Source: Ubi et al. 2017a, b)

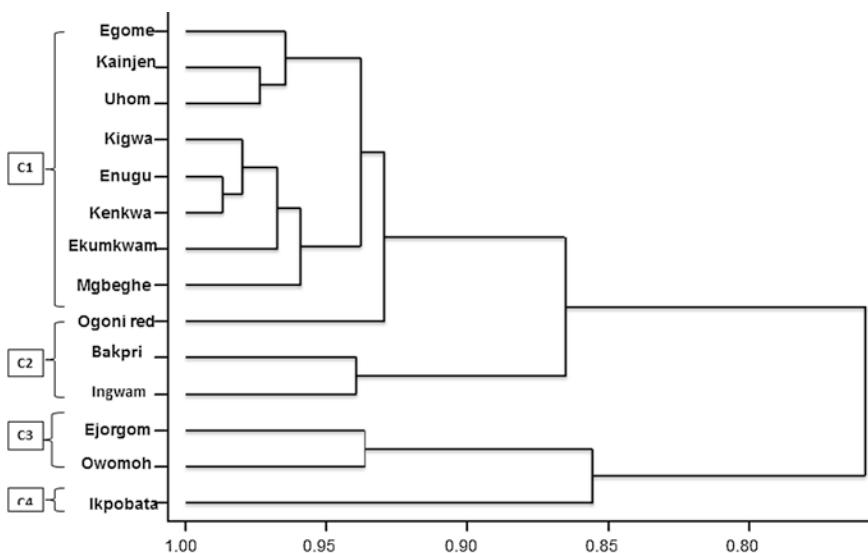


Fig. 7.3 Dendrogram showing genetic diversity showing among 14 elite plantain (*Musa paradisiaca*) cultivars in Cross River state. (Source: Ubi et al. 2017a, b)

7.6 Molecular Phylogeny of Species Diversity of Plantain in Cross River State

The phylogenetic tree above represents the evolutionary tree and pathway followed by the elite cultivars of plantain (*Musa paradisiaca*) in the study area. The phylogenetic tree is composed of branches, also known as edges, which connect and terminate at nodes.

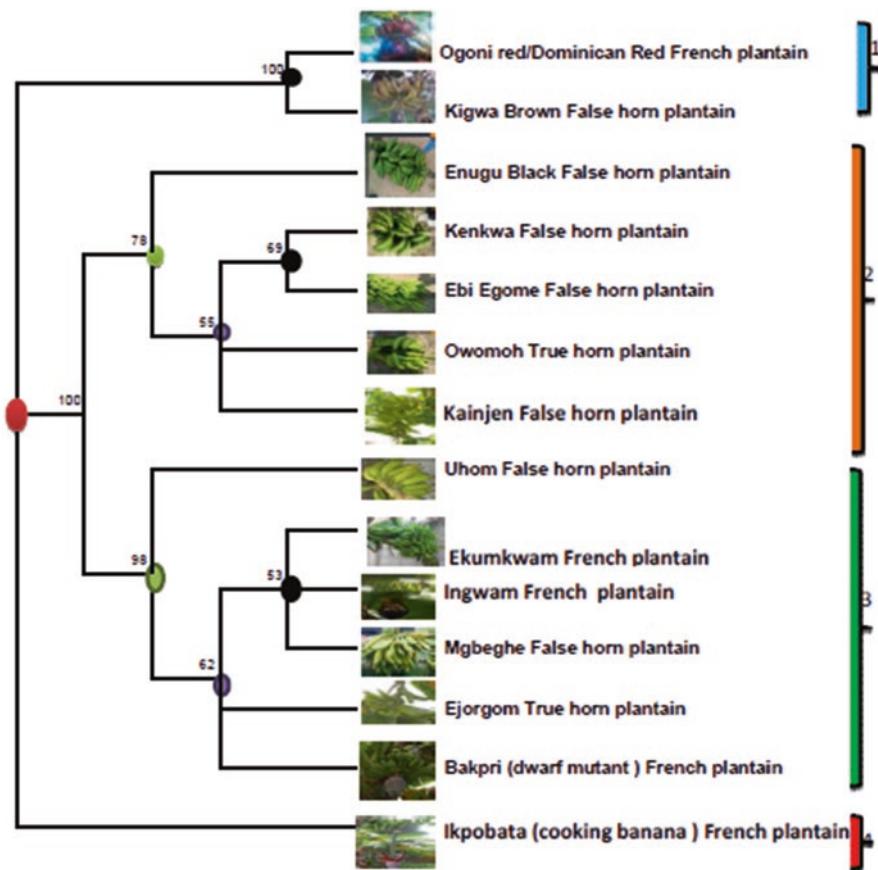


Fig. 7.4 Phylogenetic tree inferring evolutionary relationship on elite plantain cultivars grown in Cross River state. (Source: Ubi et al. 2017a, b)

- This color indicates the branches and nodes which can be internal or external (terminal) as observed from the phylogenetic tree (Fig. 7.4). The branches length varies and represents the evolutionary pathway and time of evolution. Longer branches indicates longer period of evolutionary development and mutation while shorter branches indicates shorter period of evolutionary development between and among the plantain species.
- This color indicates the terminal node at the tips of the tree and represents the operational taxonomic units (OTUs). OTUs corresponds to the molecular sequences or taxa (species) from which the tree was inferred.
- This color indicates the internal nodes and represents the last common ancestor (LCA) to all nodes that arise from that point.
- The red color indicates the tree root which emanated from a single gene from many taxa (*Musa* spp.) and through evolutionary sequences and mutation changes developed into multigene families (gene tree).

The edge length represents mutation events which are supposed to have occurred on the evolutionary path. Variations in edge dimensions in the phylogenetic tree shows the rate at which mutations accumulate in the sequences varies among the cultivars or the taxa.

7.6.1 *The Operational Taxonomic Units (OTUs) and the Branches*

Plantain cultivars used here represent the leaves and are referred to as taxa or OTUs. The phylogenetic tree was reconstructed for this set of taxa, using their respective genes or protein sequences (Fig. 7.4). The OTUs species in the first cluster have a 100 G consensus with only two relatives, the species in the second cluster also have two relatives extending to develop into branches and have a 69 G consensus while the third cluster group has three relatives extending to the branches with 53 G consensus. OTUs can be used to build an unrooted phylogenetic tree that clearly depicts a path of evolutionary changes (Ubi et al. 2017a, b).

7.6.2 *The Clades*

These are a group of OTUs that include several sequences and their common ancestor nodes. In the above phylogeny tree, 2 clades are represented in the tree comprising 5 cultivars of plantain as seen in cluster 2 and 6 cultivars of plantain as seen in cluster 3 above. In the first clade which is represented as cluster 2, there are 2 branches (cultivars) with a first internal node or last common ancestor and a second descendant of its neighboring close relative linked to the clade (Fig. 7.4).

The second clade which is represented as cluster 3 has 3 branches (cultivars) with first internal node or last common ancestor and a descendant of its neighboring relatives linked to the clade (Ubi et al. 2017a, b).

7.6.3 *The OTU Group*

At times it is possible to gather information on the evolutionary relationships of a particular ingroup from a more distant taxon, unrelated to the other taxa. This type of taxon is called an outgroup. Hence, the addition of a root node to the edge and then to the outgroup, then allows interpretation of bifurcations with regard to the time of divergence. An OTU group therefore is for the purpose of finding the root of the tree. In the phylogenetic tree above, the cooking banana cultivar with an ABB genome is distantly related to all other cultivars in the study with AAB genomes. This cultivar therefore was basically used to obtain the common root or ancestry for the 14 elite cultivars of plantain used for the study (Fig. 7.4).

A tree is said to be rooted if there is a single node or outgroup that is an external point of reference point from which all OTUs in the tree arise. The root is the oldest point in the tree and the common ancestor of all taxa in the analysis. In the absence of a known outgroup, the root can be placed in the middle of the tree or a rootless tree may be generated.

In molecular phylogeny, trees are drawn so that branch length corresponds to the amount of evolution that is the percent difference in molecular sequences between nodes. Once a gene has been duplicated, all other subsequent species in the phylogeny will inherit both copies of the gene to create orthologs as shown in clusters 1, 2 and 3 above from the phylogenetic tree.

The monophyletic groups consist of an internal last common ancestor (LCA) node and all OTUs arising from it. All members within the elite plantain cultivar groups here presented were derived from a common ancestor and have inherited a set of unique common traits. On the other hand, polyphyletic groups are an assemblage of distantly-related OTUs that possess similar characteristic or phenotype, but are directly not descendants from a common ancestral parent.

Almost all phylogenetic tree reconstruction methods reconstruct an unrooted binary tree which cannot be interpreted with respect to a time scale. In an unrooted tree one may not know if an internal node is the LCA or the descendant of its neighboring adjacent node.

Interestingly, evolutionary divergence species may result in many variations of protein, all with similar structures and functions, but with very different sequences. Bioinformatics and phylogenetic studies have been widely used to trace the origin of protein sequences to an ancestral protein family or gene line. Molecular sequence may evolve over time as a result of multiple sequence or gene mutations that result in small, but evolutionarily-important changes in the nucleotide sequences. At the protein level, these may not initially affect protein structure or function, but over time, may eventually shape a new purpose for a protein within different species.

This does not apply in all findings, as all elite cultivars have descended from a common ancestor. An evolutionary event is shaped by homology, which describes any similarity arising from the possession of common ancestry. Furthermore, phylogenetic trees are defined in terms of their homologous relationships. Paralogs are homologous sequences that are separated by a gene duplication event while orthologs are homologous sequences are those that are separated by a speciation event when one species separates or diverges into two. Paralogs are created by the duplication of gene events.

7.7 Plantain Species Diversity and Cultivation Type

Studies by Ude et al. (2003) revealed that in Africa, 116 cultivars of plantain (*Musa paradisiaca*) exist and these vary greatly from one country to another as well as from one geographical locality to the other.

Until recently, identification of these wide varieties of plantain has traditionally been based on morphological criteria (Reddy et al. 2002). However, this does not reveal the close genetic relationship and molecular characteristics that exist among the identified cultivars occasioned by frequent somatic mutations and morphological changes due to environment, thus posing a major obstacle to the proper identification of existing cultivars (Delaporta et al. 1983). This triggers the call for genetic diversity studies using molecular markers to complement the use of morphological characteristics in the identification of existing cultivars in Cross River state.

A wide range of plantain cultivars are grown in Cross River state. Local cultivars with names such as Ukom, Uhom, Egome, Ejorgom, Owomoh, Ingwam, Kenkwa, Kainjen, Mgheghe, are some of the most prominent (elite) cultivars of plantain cultivated in the state (Ubi et al. 2016). Botanically, plantain cultivars in Cross River state exhibit a triploid cytogenetic structure with chromosome number $2n = 22$ with an AAB Genome. These cultivars belong to the following types.

7.7.1 *True Horn Plantain*

True Horn (Fig. 7.5a) bears a bunch with fewer hands and fruits, but individual fingers are very large. It possesses an incomplete inflorescence. There is a complete absence of hermaphrodite flowers and a male floral bud. The horned plantain (Table 7.4) possess incomplete bunches at maturity, that is, the fingers and hands do not fill up or complete all the available buds in the inflorescence (Simmonds 1959).

7.7.2 *False Horn Plantain*

According to Stover and Simmonds (1987), False Horn (Fig. 7.5b) bears a bunch with increased number of hands and fruits, but individual fruits or fingers are small compared to the True Horn type. The False Horn plantain (Table 7.4) also possesses an incomplete inflorescence. However, this type of plantain contains only a few hermaphrodite flowers and the remains of the male floral buds. False Horn and True Horn are found extensively in Ivory Coast where they represent 91–96% of the country's plantain production.

7.7.3 *French Plantain*

French Plantain (Fig. 7.5c) bears a bunch containing many hands with an increased number of fruits per hand, but the fruits are smaller than the horn types. It bears a complete inflorescence with the presence of both hermaphrodite flowers and a

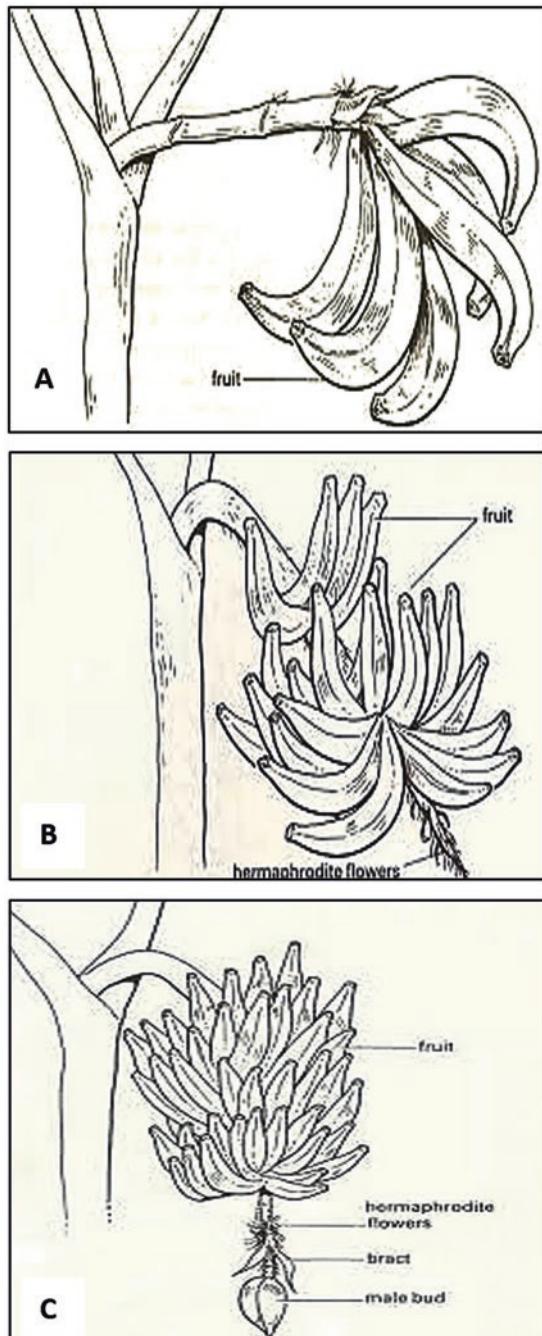


Fig. 7.5 Species diversity showing the True Horn (**a**) False Horn (**b**) and French Plantain (**c**) types in Cross River state. (Source: Ogazi 1996)

Table 7.4 Elite plantain showing species diversity in Cross River State

S/N	Elite plantain cultivar (local name)	Plantain species types	Genome
1	Ogoni red plantain	French	AAB
2	Kigwa brown plantain	False horn	AAB
3	Enugu black plantain	False horn	AAB
4	Ebi egome plantain	False horn	AAB
5	Owomoh plantain	True horn	AAB
6	Kenkwa plantain	False horn	AAB
7	Kainjen plantain	False horn	AAB
8	Uhom/Ukom plantain	False horn	AAB
9	Ekumkwam plantain	French	AAB
10	Ikpotabata (cooking banana)	French	ABB
11	Mgbeghe plantain	False horn	AAB
12	Ingwam plantain	French	AAB
13	Bakpri plantain	True horn	AAB
14	Ejorgom plantain	True horn	AAB

Source: Ubi et al. (2016)

persistent male floral bud. The French Plantain (Fig. 7.6 and Table 7.4) is extensively found in the coastal regions of West Africa, Cameroun, Rwanda and Burundi (Tezanas 1987).

According to Swennen and Vuylsteke (1987), bunch phenotype determines yield and fruit quality and is considered the most striking morphological trait for differentiation of clones. In the French Plantain, the male flowers persist until the bunch matures. The bunch is complete at maturity.

Studies by Ubi et al. (2017a, b) reveal that False Horn plantains are the dominant cultivar in Cross River state constituting up to 54%, followed by the French Plantain with 26.0%, while the True Horn plantain is the least cultivated with only 20% of production.

7.8 Plantain Ecosystem Diversity in Cross River State

Ecological and geographical differences in the distribution of plantain biodiversity (genetic, species, ecological diversity) are extremely common. In fact, differences in geographical distribution are nearly almost impossible to separate from ecologically-induced variations. Different geographical localities will always differ with respect to some potentially significant ecological characteristics such as latitude, longitude, temperature and moisture availability. Several studies have clearly demonstrated that there is a strong association between plantain population characteristics and the environments in which they grow (Ubi et al. 2017a).

Ecological factors tend to play a very significant role in determining the extent and distribution of biodiversity in plantain and their wild relatives (Venkatachalam



Fig. 7.6 Fourteen elite plantain cultivars. Names are listed in Table 7.4. (Source: Ubi et al. 2016)

et al. 2008). Ecological differences in plantain affects many traits in plantain such as the relative rate of development, resistance to biotic and abiotic stresses, edaphic responses to soil fertility and adaptation to cultivation, irrigation, quality differences, as well as methods of harvesting and utilization (Venkatachalam et al. 2007). Thus, the resultant traits or attributes of the plantain crop are a combination of climatic, soil or edaphic factors and breeding system. This explains why different communities and local government areas maintain a greater or lesser number of different types of plantain (Fig. 7.5 and Table 7.5) (Ubi et al. 2017b).

Table 7.5 Cultivars of plantain showing ecosystem diversity in Cross River state with coordinates readings showing areas of high population/ecological diversity

S/N	Plantain cultivar	Latitude (N)	Longitude (E)	Elevation (m)	Area of least diversity (LGA)
1	Ogoni red	06° 54. 58'	09° 17. 79'	178	Bendi II (Obanliku)
2	Kigwa brown	06° 48. 62'	09° 15. 72'	183	Baterico (Boki)
3	Enugu black	06° 02. 84'	08° 41. 11'	49	Nde (Ikom)
4	Ebi egome	05° 56. 54'	08° 50. 46'	131.4	Etomi (Etung)
5	Owomoh	05° 55. 88'	08° 26. 01'	175	Ochon (Obubra)
6	Kenkwa	06° 04. 45'	08° 54. 77'	129	Bashua (Boki)
7	Kainjen	05° 58. 20'	08° 63. 52'	181	Mkpani (Yakurr)
8	Uhom	05° 40. 19'	08° 03. 52'	56	Akpet 1 (Biase)
9	Ekumkwam	06° 38. 46'	08° 52. 29'	110	Mbube (Ogoja)
10	Ikpotoba	06° 28. 43'	09° 08. 84'	97	Agoi Ekpo (Yakurr)
11	Mgbeghe	05° 38. 71'	08° 48. 02'	119	Eku (Akamkpa)
12	Ingwam	06° 39. 99'	08° 51. 60'	92	Abuochichie (Bekwarra)
13	Bakpri	04° 97. 78'	08° 36. 01'	54	Awi (Akamkpa)
14	Ejorgom	06° 30. 72'	09° 10. 69'	118	Ukelle (Yala)

Source: Ubi et al. (2016)

7.9 Inflorescence Developmental Polymorphism in Plantain

This section discusses the occurrence and persistence of inflorescence developmental polymorphism (multiple bunching) in plantain. It also highlights the molecular and genetic characteristics of the different forms of inflorescence developmental polymorphism in plantain using DNA-based molecular markers such as the simple sequence repeat (SSR or microsatellites) and single nucleotide polymorphism (SNP).

Inflorescence developmental polymorphism or multiple bunching in plantain is a form of variation which can contribute to crop improvement, if valuable and genetically stable clones are selected and regenerated (Vuylsteke 2000). Plantains which bear several bunches have occasionally been observed and reported. Stover and Simmonds (1987) observed an unusual plant of Grande Naine with a double inflorescence of the pseudostem, with an inflorescence borne on each pseudostem.

The first detailed account of inflorescence developmental polymorphism in plantain in Africa was provided by Pospisil (1966). It is, therefore, certain that this character is invariably propagated through the suckers. The occurrence of twin fingers is a common feature of the first bunch (Karikari et al. 1971), which is also an indication that subsequent bunches will produce the inflorescence variants.

7.9.1 *Advantages of Inflorescence Developmental Polymorphic Plantain*

Multiple bunching plantain has several and innumerable advantages over the single bunching plantain in that the former produces many fingers per hand, many hands per bunch and many bunches per plant giving a higher yield per hectare compared to the single bunches. This multiple bunching plantain ensures increase income to plantain farmers and food security for local citizenry (Tenkouano 2000).

7.9.2 *Phenotypes of Multiple Bunching in Plantain*

Robinson (1996) viewed the inflorescence as a single bract spike, with a stout peduncle which bears most of the biserrate nodal clusters of flowers. Each nodal cluster is anchored by an inflorescence bract that guards the tender developing flowers. Inflorescence developmental polymorphism in plantain is a dichotomization event which can contribute to crop improvement if valuable and genetically stable clones are selected and regenerated (Vuylsteke 2000). Mutants of inflorescence polymorphic plantain types are rare. In fact, in most areas, they are cut away as soon as they are found for superstitious reasons, and this may be the cause of their scarcity. There are therefore, various identified forms of inflorescence polymorphism in plantain discussed as follows.

7.9.2.1 Double Bunching in One Peduncle (DB1P)

According to Baiyeri (1994), these are two bunches of plantain that are borne on one peduncle on a single plant (Fig. 7.7a). The two bunches may vary significantly in their hand and finger numbers per bunch as well as in their finger size. However, the two bunches are of the same bunch phenotype, False Horn, and may have the same qualitative attributes such as pulp color, finger skin color and finger orientation.

7.9.2.2 Double Bunching Borne in Two Peduncles (DB2P)

These are two bunches of plantain that are borne on two separate peduncles (one on each peduncle) on a single plant (Fig. 7.7b). The two bunches may vary significantly in their hand and finger numbers per bunch as well as in their finger sizes. However, the two bunches are of the same bunch phenotype, that is, False Horn, and may have the same qualitative attributes such as pulp color, finger skin color and finger orientation (Baiyeri 1994).



Fig. 7.7 Inflorescence polymorphic plantain types. (a) Double bunching plantain on one peduncle (DB1P), (b) double bunching plantain on two peduncles (DB2P), (c) triple bunching plantain on one peduncle (TB1P), (d) triple bunching plantain on two peduncles (TB2P), (e) triple bunching plantain on three peduncles (TB3P). (Source: Brisibe and Ekanem 2019)

7.9.2.3 Triple Bunching in One Peduncle (TB1P)

These are three bunches of plantain that are borne on one peduncle on a single plant (Fig. 7.7c). The three bunches may vary significantly in their hand and finger numbers per bunch as well as in their finger sizes. However, the three bunches are of the same bunch phenotype, False Horn, and may have the same qualitative attributes such as pulp color, finger skin color and finger orientation (Baiyeri 1994).

7.9.2.4 Triple Bunching in Two Peduncles (TB2P)

These are three bunches of plantain that are borne on two peduncles (two on one peduncle and one on the other) on a single plant (Fig. 7.7d). The three bunches may vary significantly in their hand and finger numbers per bunch as well as in their finger sizes. However, the three bunches are of the same bunch phenotype, False Horn, and may have the same qualitative attributes such as pulp color, finger skin color and finger orientation (Baiyeri 1994).

7.9.2.5 Triple Bunching in Three Peduncles (TB3P)

These are three bunches of plantain that are borne on three separate peduncles (one on each) on a single plant (Fig. 7.7e). The three bunches may vary significantly in their hand and finger numbers per bunch as well as in their finger sizes. However, the three bunches are of the same bunch phenotype, False Horn, and may have same qualitative attributes such as pulp color, finger skin color and finger orientation (Boumah-Mensah 1970).

7.10 Breeding Constraints in Inflorescence Developmental Polymorphic Plantain

At present, breeding efforts in *Musa* species (AAB and ABB genomes) is faced with serious challenges, especially due to the low level of fertility and seed production in several genotypes, structural incompatibility, absence of well-defined germplasm as potential stocks for breeding and the general lack of knowledge of the genetic factors responsible for important agro-morphological attributes. Expectedly, this has also affected investigations on inflorescence dichotomous *Musa* varieties. An accurately monitored and controlled scientific experiment to identify the remote causes and nature of inflorescence developmental polymorphism in plantain can go a very long way in providing and identifying the pathway underlying the cytological and molecular mechanisms responsible for controlling its expression, which will obviously be crucial for understanding this phenomenon.

Studies by Ubi and Brisibe (2017) have shown that inflorescence developmental polymorphism is multidimensional; to understand it, there are three clear goals: (a) unravel the cytological profile of these plants in terms of number, structure and behavior of mitotic chromosomes, (b) precisely determine the DNA ploidy status of a set of multiple bunching *Musa* cultivars over several crop production cycles based on chromosome counting and (c) characterize single nucleotide polymorphisms from the genomic sequences of the different inflorescence dichotomous accessions studied. These are important because such details will not only increase the knowledge of the diversity but will equally assist agronomist and plant breeders in understanding the pattern and extent of existing genetic resources available within the *Musa* genus, which can be used for exploitation in future varietal improvement programs for the crop.

7.11 Inflorescence Developmental Polymorphism (Multiple Bunching) in False Horn Plantain

7.11.1 Use of Concordance Coefficient Analysis

The concordance coefficient estimate for natural occurrence and persistence of inflorescence developmental polymorphism in plantain revealed that a unit concordance coefficient equals 1, which is an indication of normal occurrence and persistence, is observed in the single-bunching plantain only. A concordance coefficient of 0.11, far less than 1, was obtained for the plants with triple bunch borne on one peduncle, equally indicative of the random nature of occurrence and nonpersistence of the phenomenon in the accession (Ubi and Brisibe 2018).

Concordance coefficient values of zero were obtained for plants with three bunches borne on two and three peduncles, respectively, which is also an indication of the random nature of occurrence and nonpersistence of the phenomenon in the accessions. Thus, the double-bunching accessions occur more frequently than the triple-bunching accessions even though the occurrence is not persistent (Ubi and Brisibe 2018).

7.11.2 Use of Poisson Distribution Analysis

To further confirm that inflorescence developmental polymorphism phenomenon in plantain is a random event, the Poisson distribution was also used. It was gathered from the statistical inferences that Tabulated X^2 of 11.070 is greater than the Calculated X^2 of 3.013, showing that there is no significant differences between the observed and expected polymorphism. Consequently, the occurrence of

inflorescence developmental polymorphism in plantain is a random event or random occurrence which is not persistent (Ubi and Brisibe [2018](#)).

7.12 Factors Influencing Inflorescence Developmental Polymorphism in Plantain

7.12.1 Environmental Factors

7.12.1.1 Acid Rain and Mist

The effect and influence of acid rain on the persistence of multiple bunching at 7 months of age of a first ratoon/follower crop was investigated in Calabar for three cropping cycles from 2014 to 2016. Results showed that plots treated with dilute sulfuric acid (acid rain) produced 100% persistence of all the morphotypes evaluated for the first ratoon/follower crops at 7 months of growth (Ubi and Brisibe [2017](#)).

7.12.1.2 Crude Oil Pollution in Soils

The effect and influence of crude oil soil pollution on the persistence of multiple bunching in 7-month-old first ratoon/follower crop was investigated in Calabar for three cropping cycles from 2014 to 2016. Plots treated with crude oil as a soil pollutant produced 67% of all the morphotypes evaluated for the first ratoon/follower crops at 7 months of growth (Ubi and Brisibe [2017](#)).

7.12.1.3 Chemical Mutagens from Industrial Effluents

The effect and influence of chemical mutagen (sodium azide, NaZ) on the persistence of multiple bunching in 7 months old first ratoon/follower crop was investigated in Calabar for three cropping cycles from 2014 to 2016. Plots treated with the chemical mutagen (sodium azide, NaZ) produced 100% of all the morphotypes evaluated for the first ratoon/follower crops at 7 months of growth (Ubi and Brisibe [2017](#)).

7.12.1.4 High Organic Residue in Soil

The effect and influence of high volume of organic residue on the persistence of multiple bunching in 7-month-old first ratoon/follower crop was investigated in Calabar for three cropping cycles from 2014 to 2016. The plot with a high volume of organic residue treatment produced 33% of all the morphotypes evaluated for the first ratoon/follower crops at 7 months of growth. (Ubi and Brisibe [2017](#)). These

results substantiate our earlier position that this phenomenon of multiple bunching is triggered by some environmental factors and not necessarily nutritional factors.

7.12.2 *Genetic Factors*

7.12.2.1 DNA Methylation

DNA methylation is a process by which methyl groups are introduced into a DNA molecule leading or giving rise to methylation. Methylation of the DNA can alter the expression or functionality of the associated genes of a DNA segment without altering the sequence orientation.

DNA methylation can also be seen as the introduction of a methyl (CH₃) group to the DNA molecule, basically to the fifth carbon atom of a cytosine ring, thereby modifying the function of the *GTPase* protein binding genes thus affecting gene expression. The findings in the sequence analysis and mutation assay in the polymorphic accessions revealed that the changes in amino acids composition of the *GTPase* protein binding genes in the polymorphic accessions mostly accounted for the phenotypic plasticity observed. DNA methylation cannot be ruled out as being partly or totally responsible for the epigenetic signaling showing the phenotypic plasticity (Ubi et al. 2017a, b).

7.12.2.2 Epigenetic Control

Epigenetics is the study of the heritable phenotypic changes that do not involve alterations in the DNA sequence. A single genotype controls different phenotypes. The genotypes of the chromosomes for the polymorphic accessions are similar, especially in their chromosome numbers, but revealing different phenotypic expressions (Ubi and Brisibe 2018).

7.12.2.3 Pleiotropy

This is the phenomenon that involves the influence of more than a single phenotype by a single gene. The *GTPase* protein binding gene that is a part of the *leaf tissue* complex gene that controls many of the biochemical processes in the plant can induce this attribute in the genome of the plantain, thus controlling multiple phenotypes like the phenotypic variants by the single *GTPase* single gene. Pleiotropy is a condition where a single genic mutation causes more than one phenotypic effect as observed in the multiple bunching phenomenon in plantain. In pleiotropy, one gene affects multiple characteristics (Ubi and Brisibe 2018).

7.12.2.4 Incomplete Penetrance

Penetrance is the proportion of individuals of a species with specific genotypes that show or manifest variable characteristics or phenotypes. It is a factor that influences the effects of particular genetic changes. Here, the genetic traits have reduced with incomplete penetrance, thus being expressed in only a part of the population. The inconsistency in multiple bunching phenomena in inflorescence polymorphic plantain can be attributed to incomplete penetrance of the genetic traits in the follower crop generations (Ubi and Brisibe 2018).

7.12.2.5 Transversional or Nonsynonymous Mutations

These types of mutations result in the transformation and conversion of amino acids from one form to another, thus significantly influencing protein expressivity. The changes in the study involve the conversion of purines to pyrimidines and pyrimidines to purines leading to the changes in genetic expressions. The changes in amino acid compositions in the genome of the plantain during the reproductive stage accounts for the changes from multiple bunch to single bunches in subsequent follower or ratoon crop generations. This is informed by the fact that in the single bunching phenotype, transition mutations in which there are no changes in amino acids compositions in the genome after mutation and hence no change in bunch phenotype (Ubi and Brisibe 2018).

7.12.2.6 Missense Mutations

These are changes in amino acids that results in phenotypic changes in gene expression. These have already been explained and are similar to transverse and non-synonymous mutations (Ubi and Brisibe 2018).

7.13 Cytogenetics of Inflorescence Developmental Polymorphic Plantains

7.13.1 Cytology of Plantain Chromosome (AAB Genome)

Most cultivated *Musa* species are triploids ($2n = 3x = 33$). Being almost completely sterile, they develop fruits by parthenocarpy. The genome of cultivated varieties is derived from the diploid related to *Musa acuminata* and *M. balbisiana* with A and B genomes, respectively (Fig. 7.8).

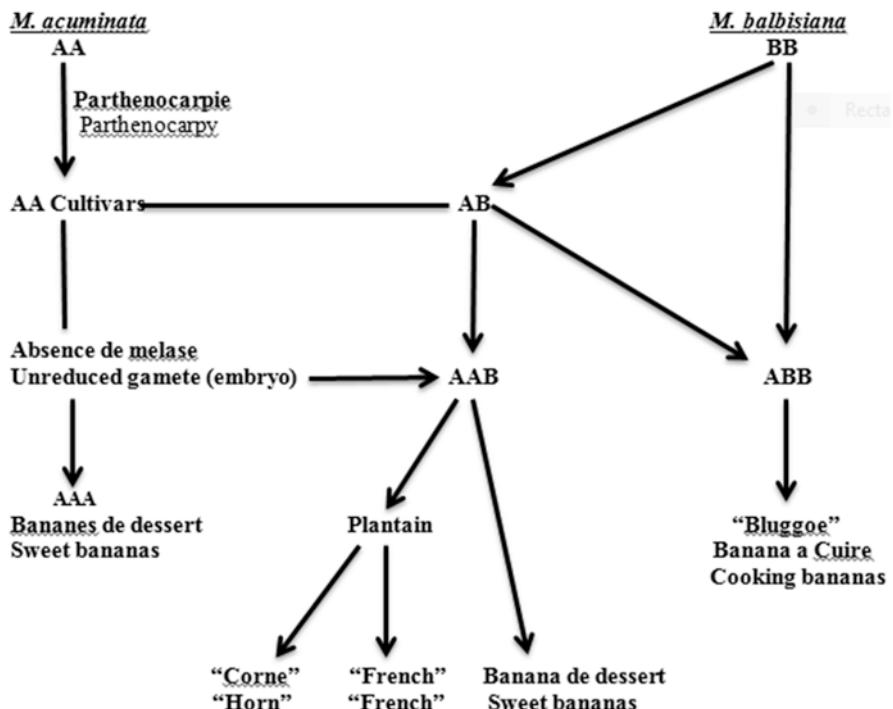


Fig. 7.8 Cytological development of AAB Genome of *Musa paradisiaca*. (Source: Gerda 1990)

The most important cultivars have characteristic genomic constitutions like dessert banana (AAA), East Africa highland banana (AAA), plantain (AAB) and cooking banana (ABB). (Simmonds 1959).

7.13.2 Fluorescence In Situ Hybridization in Plantain

The following conclusions can be made from the results of fluorescence in situ hybridization in multiple bunching plantain (*Musa paradisiaca*) accessions:

- Fluorescence in situ hybridization in the single-bunching plantain accession that served as the control in the study was observed to commence on a single chromosome 6 after dual multicolored fluorochrome (DAPI/DA) staining and rearrangements (Fig. 7.9a);
- The number of peduncles formed per inflorescence polymorphic plantain was found to be associated with the number of translocated chromosome(s) which hybridized to the DNA probe;

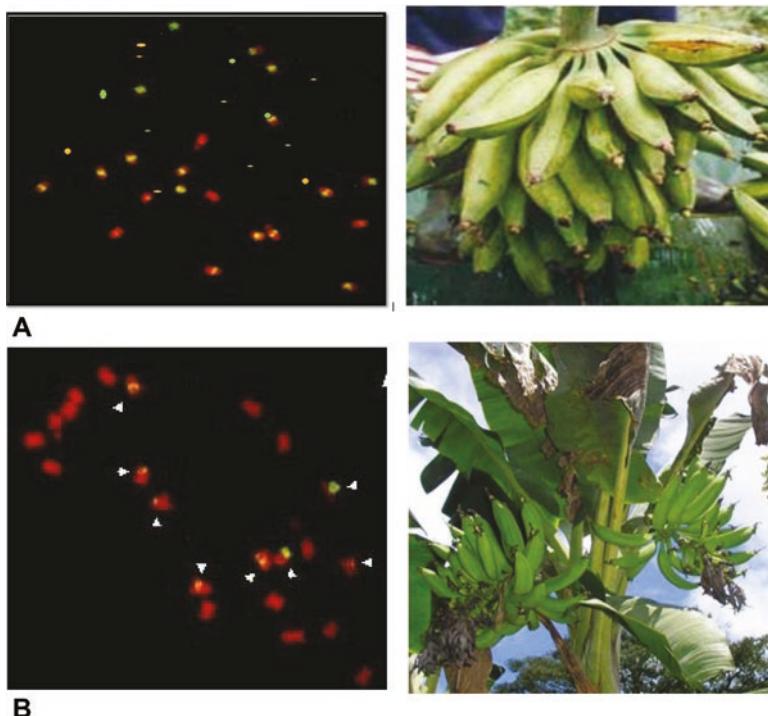


Fig. 7.9 (a) Fluorescence in situ hybridization of single bunching plantain showing triploid chromosomes with stable genome at $2n = 2x = 33$, (b) fluorescence in situ hybridization of multiple bunching plantain showing diploid chromosomes with unstable genome at $2x = 2n = 22$. (Source: Ubi and Brisibe 2018)

- (c) The number of bunches per inflorescence polymorphic plantain was found to be associated with the maximum number of chromatids of chromosome pairs hybridizing with the DNA probe;
- (d) Two pairs of hybridizing chromatids with the DNA probe were found to be associated with the double bunches;
- (e) Three chromatids hybridizing with the DNA probe were found to be associated with triple bunches;
- (f) Univalent chromosomes were found to be associated with the number of peduncles (Fig. 7.9b);
- (g) Bivalent hybridized chromosomes were found to be associated with double bunching in plantain;
- (h) Trivalent chromosomes were found to be associated with triple bunching in plantain.

7.13.3 Genomic In Situ Hybridization (GISH) Study in *Musa* Species

Racharak and Eiadthong (2007) in their studies with GISH on *Musa*, reported that it is feasible to detect the rate of development of univalent, bivalent and multivalent hybrids in a plantain population by comparison with ancestral genomes, and also the chiasma formation or recombination frequencies between homologous chromosomes. The great advantage is that, with this approach, it is possible to observe and identify the factors that induce irregular meiotic divisions, and how they affect plantain fertility.

The analysis of plantain plant behavior at meiosis was ascertained by use of genomic in situ hybridization of interspecific hybrids of *Musa* cultivated banana species (Ji and Chetelat 2003; Jin et al. 2006). These hybrids plantains were obtained from the cross between *M. acuminata* ($2n = 2x = 22$, AA) and *M. balbisiana* ($2n = 2x = 22$, BB). The interspecific triploid hybrids earlier referred to as Figure Pomme ($2n = 3x = 33$, AAB) and Praha ($2n = 3x = 33$, ABB) developed as multivalent, bivalent and univalent hybrids in both cultivars, with homologous bivalents seen in all chromosomes analyzed; and with all the multivalent hybrids (trivalent, tetravalent) showing homologous chromosomes. Research has shown that it is possible to re-engineer the recombination between the two genomes, A and B, which is very relevant in the domestication and improvement of interspecific plantain cultivars (Jeridi et al. 2011; Ji and Chetelat 2007).

The results of genomic in situ hybridization showed the following:

- (a) No hybridization was observed between genomic and DNA segment probes in the single-bunching plantain accession, which consequently may be the reason for the persistence of the single bunching expression at every generation (Fig. 7.10a);
- (b) The number of peduncles formed per inflorescence polymorphic plantain depended on the number of sites of hybridization between the target chromosomes and the genomic probe;
- (c) The number of bunches per inflorescence polymorphic plantain accession depended on the number of chromatids of chromosomes hybridizing with the genomic probe (Fig. 7.10b);
- (d) Two univalent chromosomes hybridizing in a T-shape manner was found to be associated with double bunches;
- (e) Multiple bivalent chromosomes hybridizing in a random manner was found to be associated with the formation of triple bunches.

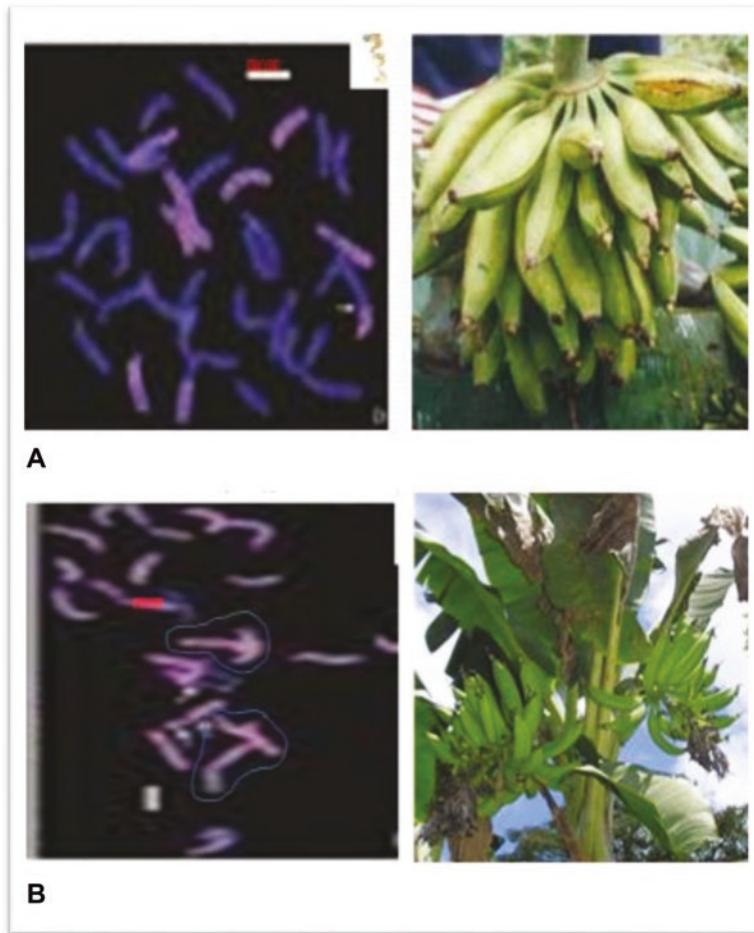


Fig. 7.10 Genomic *in situ* hybridization in a single bunching plantain (a) and in multiple bunching plantain (b). (Source: Ubi and Brisibe 2017)

7.14 Chromosomal Number and Architecture of Inflorescence Polymorphic Plantains

Considering the possibilities for ploidy variations in some plantain morphotypes, the ploidy level of all six cultivars were carefully re-evaluated in a second series of experiments in the plant, with first and second follower crops using DNA flow cytometric analysis to verify the mechanism of the crop-cycle dynamics in the inflorescence polymorphic plants. A total of 100 plantain plants (10 each for all 5 polymorphic variants during 2 crop cycles) were evaluated in the first and second ratoon crops, in which 95 were diploid and 5 triploid (Fig. 7.11), suggesting that the multiple-bunching plantains were mainly diploid and genetically unstable resulting



Fig. 7.11 (a) Stable triploid in single bunching plantain accession showing $2x = 2n = 33$, (b) unstable diploid in multiple bunching plantain accessions showing $2x = 2n = 22$, (c) unstable triple in multiple bunching plantain accessions showing $2x = 2n = 22$. (Source: Brisibe and Ekanem 2019)

from mutations in some or all of the chromosomes. Concomitant with these variations in ploidy status, there were changes in the chromosome numbers from diploid ($2n = 2x = 22$) (Fig. 7.11a) to the more stable triploid ($2n = 3x = 33$) (Fig. 7.11b), usually seen as a natural attribute of all plantain landraces. Thus, it can be speculated that the ploidy changes identified may result from different amounts of genetic changes that may have taken place as the number of both bunches and crop cycles increased. Collectively, these observations tend to suggest the presence of a high

incidence of genetic instability in the inflorescence dichotomous plantains early in the crop cycle. However, these genetic changes are favorably more disposed towards achieving the more stable triploid status in later cropping cycles of the follower crops, especially as the number of bunches obtained on a single plant showed reduced bunches instead of an increasing number of bunches. Based on this phenomena and observations, it therefore created a heightened need of major horticultural significance, for the additional studies to be designed which would help to examine and unveil the genetic and environmental basis of these ploidy level changes since cultivars with a higher number of fruit bunches appeared to be more unstable than those with the conventional single bunch.

7.15 Bioinformatics Studies in Multiple Bunching Plantain Accessions

The *GTPase* gene of the complex *leaf tissue* gene in multiple bunching plantain accessions were sequenced after amplification of the gene. The chromatograms containing the sequences were further analyzed using Chromas software to obtain the individual sequences. The sequences obtained were further analyzed using Bioedit software, molecular evolutionary and genetic analysis (MEGA X) software, and some online programs like the Expasy, Genscan, Nsopma and Phyre and Phyre to obtain the properties of the gene, SNPs, mutations types, phylogenetic tree relationship and their tertiary protein structures.

7.15.1 Time of Divergence and Evolutionary Relationship Among Inflorescence Polymorphic and the Single Bunching Plantain Accessions

As shown in the phylogenetic tree (Fig. 7.12), the plantain accession with double bunches borne on one peduncle was the nearest neighbor to the single-bunching accession in terms of evolutionary sequence. The time of genetic divergence for the single-bunching plantain was zero years (0 MYA, million years ago).

The accessions with double bunches borne on one peduncle and double bunches on two peduncles were very close in terms of their evolutionary sequence and mutation with 99% similarity that showed a genetic divergence time of 10 MYA.

The percentage similarity in evolutionary changes between the plantain accession with triple bunches on one peduncle and the double bunches in two peduncles is 97% with a relative genetic divergence time of 12 MYA.

The percentage similarity in evolutionary changes between the triple bunches on one peduncle and triple bunches on two peduncles is 91% showing a slight variation in mutation. Time of genetic divergence of the gene in this inflorescence polymorphic plantain accession is 20 MYA.

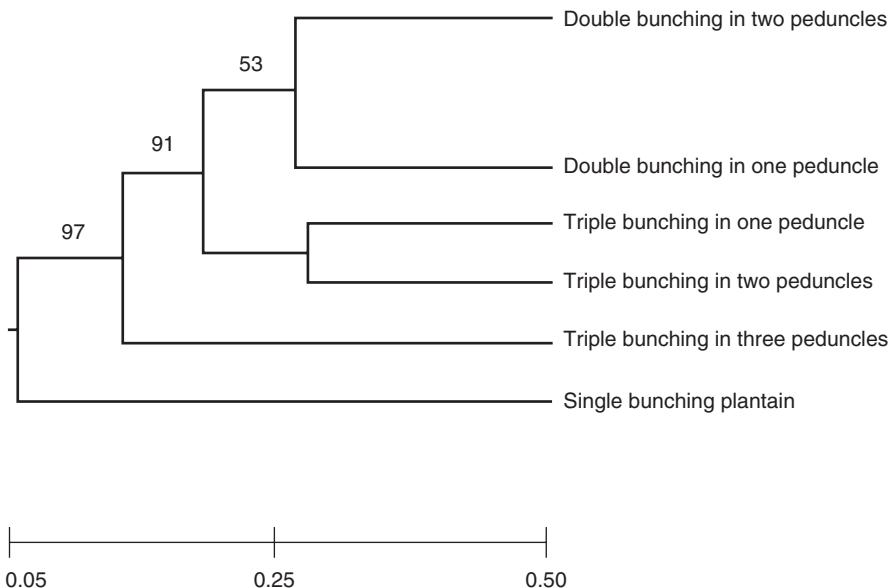


Fig. 7.12 Phylogenetic tree showing evolutionary relationships and time of divergence of the *GTPase* protein binding genes among the single and multiple-bunching plantain accessions. (Source: Ubi et al. 2017a, b)

The percentage similarity in evolutionary changes between the triple bunches on three peduncles and the other triple-bunching polymorphic types is 53% showing that significant changes in evolutionary sequence and mutation have taken place. The average time of genetic divergence for this inflorescence polymorphic plantain accession is 15 MYA.

Multiple sequence alignment data are indicative of the differences in chromosome numbers and ploidy levels among the different cultivars evaluated and provided some insights into the polymorphism phenomena detected. However, the cascade of reactions in the genome which triggered these phenotypic variations in plantain still remains unclear. Thus, to have a better understanding of the molecular mechanisms underlying this highly unstable genetic attribute, a possible strategic study approach should involve nucleotide diversity studies and single nucleotide polymorphisms (SNPs) exploration and identification in inflorescence dichotomous plantains. This initiative could find/identify diverged and conserve regions in the gene sequences associated with floral development and which are utilized in the design of primers that are further used in the polymerase chain reaction (PCR), thus annealing the amplicons to only a single DNA target (Zwierzykowski et al. 2008).

Multiple sequence alignments created from pairwise alignments used to select the highly conserved variation-enriched regions are presented in Fig. 7.13. Two distinct attributes were identified. First, there was a major nucleotide deletion in the inflorescence dichotomous cultivars (Fig. 7.13). Second, there was a high level of nucleotide diversity in the genome of the inflorescence dichotomous cultivars,

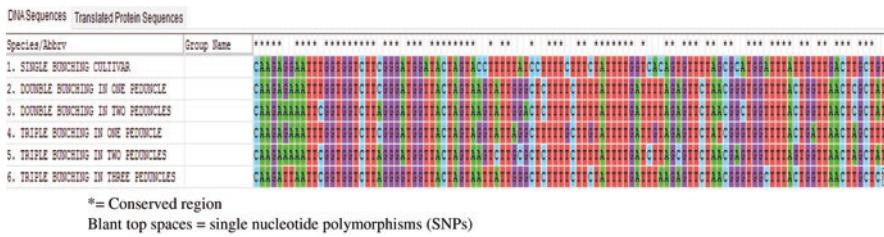


Fig. 7.13 Multiple sequence alignment of inflorescence polymorphic plantain showing single nucleotide polymorphisms and conserved regions. (Source: Ubi and Brisibe 2018)

* = Conserved region

Blant top spaces = single nucleotide polymorphisms (SNPs)

Table 7.6 Base pairs of nucleotide sequences and number of amino acids of *GTPase* gene of the leaf tissue complex gene of inflorescence polymorphic and single-bunching plantain accessions

S/N	Accessions	Number of nucleotide (bp)	Number of amino acid	Genome
1	Single bunch control	1185	221	AAB
2	Double bunches in one peduncle	3478	83	AAB
3	Double bunches in two peduncles	3486	86	AAB
4	Triple bunches in one peduncle	3890	105	AAB
5	Tripe bunches in two peduncles	3864	105	AAB
6	Triple bunches in three peduncles	3862	105	AAB

Source: Ubi and Brisibe (2018)

which are represented as nucleotide swaps that may have resulted from duplicated loci or allele separation from the ancestors at the time of divergence from the single-bunching cultivar (Fig. 7.12). Descriptive details for the single nucleotide polymorphic loci, nucleotide substitutions types and amino acid swaps in the genomic sequences of the different cultivars are shown in Table 7.6. As expected, the single-bunching plantain cultivar was genetically stable as it possessed the least number of nucleotide changes with no associated nucleotide substitutions. Hence, all the variations in the base pair in the aligned sequences involved only a change in position of the last base nucleotide (for a three base codon) resulting in a swap of amino acids from guanine to adenine and adenine to guanine, without the prediction of any concomitant changes in the expressed proteins (Table 7.6).

Of particular interest and significance is that this aspect of observations has revealed that transitional and synonymous point mutations in single nucleotide polymorphisms (that involving a change from purine to purine and pyrimidine to pyrimidine or leucine to leucine and lysine to lysine and vice versa) were only observed and peculiar to the single-bunching plantain cultivar. This obviously, is an indication that silent mutation (with no changes on amino acid sequence),

Table 7.7 Transitions, transversions, synonymous and non-synonymous mutations in the *GTPase* gene of the *leaf tissue* gene complex of inflorescence polymorphic accessions of plantain

Plantain accession	Number of SNP sites	Nucleotide substitution	Transitions/transversions	Mutation type
SBC	55	Pu-Pu	Transition	Synonymous
DB1P	82	Py-Py	Transversion	Nonsynonymous
DB2P	77	Pu-Py	Transversion	Nonsynonymous
TB1P	81	Py-Pu	Transversion	Nonsynonymous
TB2P	79	Pu-Py	Transversion	Nonsynonymous
TB3P	86	Pu-Py	Transversion	Nonsynonymous

Source: Ubi and Brisibe (2018)

SNP single nucleotide polymorphism, *Pu* purines, *Py* pyrimidines

consequently does not lead to an alteration in the expression and function of the *GTPase* complex *leaf tissue* gene. This provides experimental evidence and the possibility that the absence of any changes in amino acid sequence may have accounted for the genetic stability that was observed in all replicates of the single-bunching plantain cultivar. On the contrary, a number of transversions and nonsynonymous single nucleotide changes were detected in the coding regions of the *GTPase* complex *leaf tissue* gene (Table 7.7), depicting that the amino acid substitutions resulted in the alternative receptor isoforms. These changes in amino acid types can be speculated to have contributed to and be responsible for the bunch variability that was detected in the inflorescence dichotomous cultivars.

However, unlike in the single-bunching cultivar, the basic nucleotide changes detected in the inflorescence polymorphic phenotypes were precisely from purine to pyrimidine and pyrimidine to purine while some of the typical amino acid changes in the different accessions included glycine to valine in DB1P, leucine to proline in DB2P, valine to lysine in TB1P, leucine to glycine in TB2P and glycine to valine in TB3P, respectively (Table 7.7). These different change manifestations resulting in nucleotide substitutions and changes in the types and positions of amino acids equally suggest highly complex processes that can be associated with alterations in both gene function and expression. It is speculative from these details, therefore, that the high mutation rates characteristic of both nucleotide base pairs and amino acids could have led to the high genetic instability detected, which may have developed as a consequence of incomplete penetrance or pleiotropy and may equally have contributed to the presence of multiple genetic variants within the *Musa* population evaluated.

Table 7.7 shows that transitional and synonymous point mutations in single nucleotide polymorphisms (SNPs) were only peculiar to the single-bunching plantain accession (that is change of purine to purine and pyrimidine to pyrimidine/leucine to leucine and lysine to lysine) indicating only a change in position of the same amino acid, which does not alter the gene expression and function.

All forms of inflorescence polymorphic plantain accessions were found to undergo transversional and nonsynonymous point mutations in their single nucleotide polymorphisms (SNPs). Changes observed showed purine to pyrimidine and

pyrimidine to purine/glycine to valine and lysine to proline. These changes in types and position of amino acids are responsible for the change and alterations in gene functions and expressions leading to genetic instability.

This was thus probably seen to be responsible for the high genetic instability in the inflorescence polymorphic plantain accessions and instability of the gene expression for the yield traits and thus responsible for the nonpersistence of inflorescence polymorphism in the False Horn plantain accessions.

7.16 Tertiary Protein Structures of Inflorescence Polymorphic Plantain

The tertiary protein structure characteristics for the inflorescence polymorphic plantain variants were determined using the protein homology Y recognition engine (PHYRE) online interactive tool and the protein sequences. The tertiary protein structure reflected the bunch phenotypes corresponding to the extended strand components (Ubi and Brisibe 2017). These are discussed relative to the Fig. 7.14a–c shown below.

The protein structures exhibited by the different inflorescence polymorphic plantains and single-bunching plantain accessions can be summarized as follows:

- (a) Only 1 extended strand was observed in the single-bunching accession that corresponded to the single peduncle (Fig. 7.14a);
- (b) In DB1P, 2 extended strands were joined at one end to produce one peduncle with 2 bunches (Fig. 7.14b);
- (c) In DB2P, 2 separate extended strands each corresponding to a peduncle with 1 bunch;
- (d) In TB1P, 3 extended strands that were joined at one end corresponding to 1 peduncle with 3 bunches (Fig. 7.14c);
- (e) In TB2P, 2 extended strands joined at one end to form a peduncle with 2 bunches and another separate extended strand corresponding to a peduncle with a single bunch;
- (f) In TB3P, 3 separate extended strands each corresponding to a peduncle with a single bunch.

7.17 Conclusions and Prospects

Diversity studies of elite plantain cultivars in Nigeria have provided baseline information and created awareness on the genetic, species and ecological diversity of elite plantain (*Musa paradisiaca*) cultivars in Cross River state. This represents a significant step in the right direction toward the collective struggle to eradicate poverty, hunger and unemployment of the citizenry. This chapter provides baseline information needed for urgent intervention in the preservation, conservation,

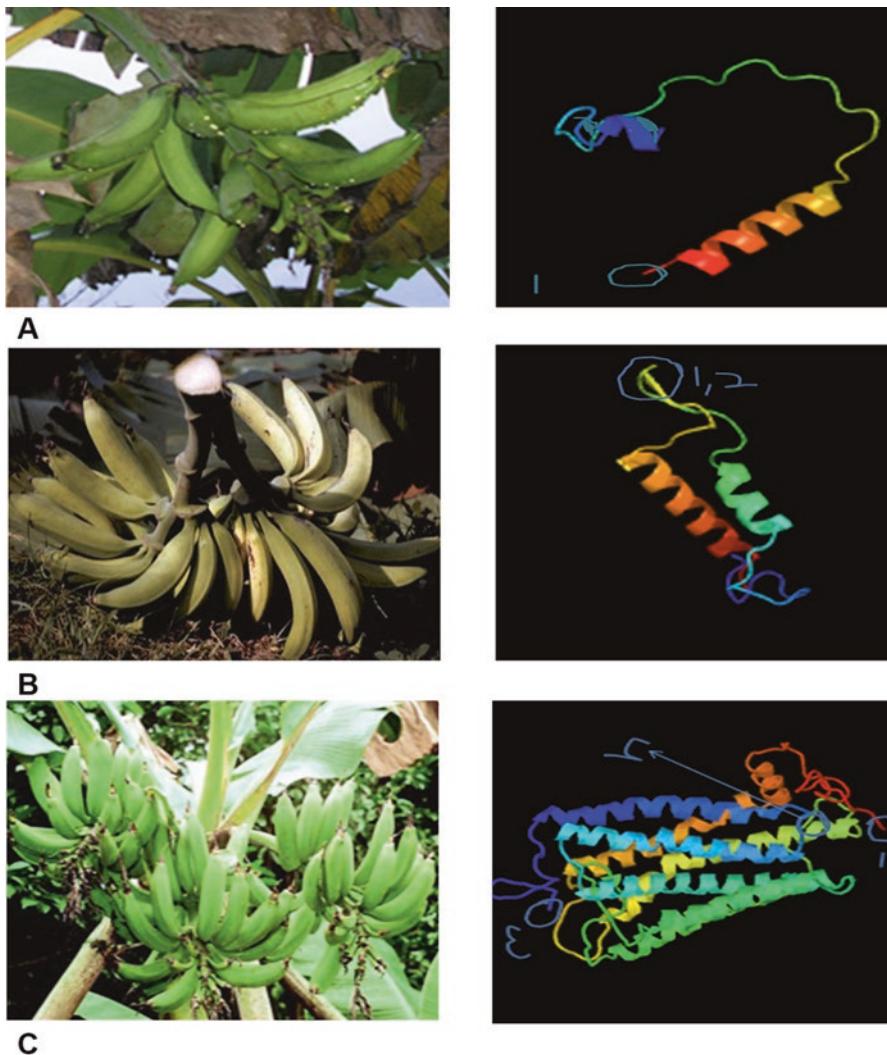


Fig. 7.14 (a) Tertiary protein structure for single bunching plantain showing 1 extended strand corresponding to single bunching, (b) tertiary protein structure for double bunching plantain showing 2 extended strands corresponding to double bunching, (c) tertiary protein structures for triple bunching plantain showing 3 extended strands corresponding to triple bunching. (Source: Ubi and Brisibe 2017)

improvement and management of plantain genetic resources. Plantain is an important cash crop in Nigeria and forms the mainstay of most rural economy in the state and country at large.

With the teeming population and the upsurge in rural-to-urban migration, youth restiveness, high unemployment rate, high rate of poverty and increased crime rate among Cross Riverians, it is imperative for a holistic and collective approach to be

adopted for the improvement, conservation, production, management and utilization of the elite plantain cultivars for the attainment of sustainable development drive for contemporary Cross River state.

It has also been revealed that current breeding techniques such as micropropagation through somatic embryogenesis, in vitro propagation, temporary immersion systems (bioreactors), haploid production, induced mutation breeding, cryopreservation and the use of virus free suckers can be widely utilized and applied in the development and increased production of plantain for food security. This will probably offer significant relief to the resource-poor farmers who are struggling against these traditional obstacles and barriers to plantain production, especially in developing country like Nigeria.

The study on the molecular, cytogenetic and phylogenetic characteristics of inflorescence developmental polymorphism in plantain is an important approach toward unveiling the probable causes and reason(s) for the nonpersistence of this yield attribute in plantain, a phenomenon that has generated so much interest among plant breeders and farmers. This study revealed that the phenomenon of inflorescence developmental polymorphism in plantain accessions occurs mostly among the False Horn varieties. The variability in the phenotypic expression of this trait was highly contributed to by the first principal component.

From the molecular, cytogenetic and phylogenetic characteristics of inflorescence polymorphic plantain accession unveiled by this study, it could be concluded that transversional, non-synonymous point mutations of the highly unstable *GTPase* gene of the *leaf tissue* gene complex are responsible for the nonpersistence of inflorescence developmental polymorphism in False Horn plantain (AAB group). This review chapter has revealed the following thrusts:

Some of the molecular, cytogenetic and phylogenetic basis underlying the dichotomous phenomenon in plantain revealed in this chapter shows that molecular and genetic data and information are available on multiple-bunching plantain accessions. The basic types of mutations associated with nonpersistence of inflorescence polymorphism among variant accessions in the field are discussed.

Chromosomal translocations also occur in inflorescence polymorphic plantain accessions during in situ hybridization. The meiotic behavior of plantain polyploids was associated with unstable genetic composition. Fluorescence and genomic in situ hybridization techniques as cytogenetic tools can be used to reveal the cytogenetic mechanisms in inflorescence polymorphic plantain accessions. Molecular and phylogenetic characterization studies can be useful in promoting and sustaining biodiversity in plantain species.

Important prospects of future research and utilizations include the production of multiple bunching plantain contributes positively to food security, especially in the current global pandemic era. Conservation efforts geared towards the preservation of multiple bunching plantain germplasm in gene banks can be very promising towards the crop future production, management and utilization. In addition, more research is needed to reveal the underlying factors necessitating the inconsistencies associated with inflorescence developmental polymorphism in plantains. Biotechnological approaches should be adopted in the production, management and

conservation of plantain germplasm. Governments should encourage multiple bunching plantain farming by establishing mandated institutions with a view to ensuring food security, creating employment and means of livelihood for her teeming populace as the world look forward to developing post COVID-19 economy recovery strategies.

Appendix I: Research Institutes Relevant to Plantain

Institution name and location	Website
International Institute of Tropical Agriculture IITA, Moore Plantation, Ibadan, Nigeria	www.cgiar.iita.org
Farm Focus International FFI, Calabar South, Cross River State, Nigeria	www.farmfocusinternational.org
University of Calabar, Department of Genetics and Biotechnology, University of Calabar, Calabar, Nigeria	www.unical.edu.org
Australian Plant DNA Bank (APDB), Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW, Australia	http://www.dnabank.com.au
Botanic Garden and Botanic Museum (BGBM) DNA Bank, Berlin, Germany	http://www.bgbm.org/bgbm/research/dna/
DNA Bank Brazilian Flora Species, Rio de Janeiro Botanic Garden, Brazil	http://www.jbrj.gov.br/pesquisa/div_molecular/bancodna/index.htm
DNA Bank at Kirstenbosch, South African National Biodiversity Institute, Kirstenbosch, South Africa	http://www.nbi.ac.za/research/dnabank.htm
International Rice Research Institute (IRRI), DNA Bank, Philippines	http://www.irri.org/GRC/GRChome/Home.htm
Missouri Botanic Garden DNA Bank, (MBGDB) St Louis, MO, USA	http://www.mobot.org/MOBOT/research/diversity/dna_banking.htm
National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India	http://www.nbpgr.ernet.in/
National Herbarium Netherlands DNA Bank (NHNDB), Netherlands	http://www.nationalherbarioum.nl/taskforcemolecular/dna_bank.htm
National Institute of Agrobiological Science (NIAS) DNA Bank, Tsukuba, Ibaraki, Japan	http://www.dna.affre.go.jp/
Plant DNA Bank Korea (PDBK), Graduate School of Biotechnology, Korea University, Seoul, Korea	http://www.pdbk.korea.ac.kr/index.asp
Royal Botanic Garden Edinburgh DNA Bank, Edinburgh, Scotland	http://www.rbge.org.uk/rbge/web/science/research/
Royal Botanic Garden Kew DNA bank, Richmond, England	http://www.rbgkew.org.uk/data/dnaBank/
TCD DNA Bank, Department of Botany, School of Natural Sciences, Trinity College, Ireland	http://www.dnabank.bot.ted.ie
Tropical Plant DNA Bank, Fairchild Tropical Botanical Garden and Florida International University, FL, USA	http://www.ftg.org/research
International Institute for Tropical Agriculture, Nairobi, Kenya	www.cgiar.iita.org

Appendix II: Some Genetic Resources of Plantain, Cultivation Area Genome Group and Main Characteristics

Cultivar	Cultivation location			Main characteristics					
	Latitude (N)	Longitude (E)	Elevation (m)	Genomic group	Finger size/ shape	Bunch phenotype	Finger cross section	Unripe Finger skin color	Unripe Pulp color
Elite plantain cultivars	06° 02.84'	08° 41.10'	210	Genome/ type	Big/curve	False horn	Triangular	Dark green	Creamy
Enugu black	06° 02.84'	08° 41.10'	210	False horn/ AAB	Big/curve	False horn	Quadrilateral	Pale green	Milky white
Ebi Egome	05° 56.54'	08° 50.45'	132	False horn/ AAB	Medium/ curve	French type	Pentagonal	Red wine	Milky white
Ogoni red	06° 54.58'	09° 17.79'	178	French/AAB	Medium/flat	False horn	Quadrilateral	Brown	Creamy
Kigwa Brown	06° 48.62'	09° 15.30'	183	False horn/ AAB	Medium/flat	True horn	Triangular	Pale green	Creamy
Ejorgom	06° 30.72'	09° 10.68'	119	True horn/ AAB	Small/curve	French type	Pentagonal	Pale green	Milky white
Bakpri (dwarf mutant)	04° 97.78'	08° 36.01'	54	French/AAB	Medium/ curve	False horn	Quadrilateral	Pale green	Creamy
Owomoh	05° 55.88'	08° 26.39'	175	True horn/ AAB	Medium/flat	False horn	Quadrilateral	Dark green	Creamy
Kainjen	05° 58.20'	08° 63.52'	181	False horn/ AAB	Small/curve	French type	Triangular	Dark green	Milky white

Ikpobata (cooking banana)	06° 28.43'	09° 08. 85'	97	French/ABB	Big/flat	False horn	Triangular	Pale green	Milky white
Mgbeghe	05° 38.71'	08° 46. 02'	119	False horn/ AAB	Big/flat	False horn	Quadrilateral	Pale green	Milky white
Kenkwa	06° 04.45'	08° 54. 77'	130	False horn/ AAB	Medium/ curve	French type	Quadrilateral	Dark green	Creamy
Uhom	05° 42.19'	08° 03. 23'	56	False horn/ AAB	Medium/ curve	French type	Triangular	Pale green	Creamy
Ekunkwam	06° 33.46'	08° 52. 29'	110	French/AAB	Medium/ curve	True horn	Pentagonal	Pale green	Milky white
Ingwam	06° 39.99'	08° 51. 61'	92	French/AAB					

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Chapter 8

Breeding of Sweet Gourd (*Cucurbita moschata* Duch. ex Poir.)



A. K. M. Aminul Islam, Sumi Sarkar, Kamrun N. Ruma, Marium Khatun, Farzana M. Era, and Mohammad S. Raihan

Abstract Sweet gourd (*Cucurbita moschata* Duch. ex Poir) is an annual crop with an open-pollinated sexual reproductive system via cross-pollination and can easily be grown year-round. The reproductive system, mode of pollination and large monoecious flowers are extensively used for conventional breeding in sweet gourd and involves pedigree selection and hybrid breeding. Distant crossing between domesticated and wild species gives some success to overcome hybrid sterility and transfer of resistance genes by using different breeding tools. The advanced lines from distant hybridization may be useful in diverse cucurbit breeding programs. Backcrossing using non elite and wild germplasm donors is an effective strategy for a single trait transfer such as specific disease resistance or bush growth habit into a cultivar. It can also be bred into commercial lines via transgenesis. Application of biotechnological tools and molecular breeding may help sweet gourd breeders to improve yield, disease and pest resistance as well as nutritional quality. This chapter provides an overview of the importance of sweet gourd and its breeding strategies for the development of high yielding and nutritionally rich varieties using conventional and frontier breeding tools.

Keywords Cultivar · Germplasm · Hybridization · Pedigree · Sweet gourd · Yield

8.1 Introduction

Sweet gourd (*Cucurbita moschata* Duch. ex Poir.) belongs to the family Cucurbitaceae and has a chromosome number of $2n = 40$. Sweet gourd originates from Central and South America (Fig. 8.1) (Khoury et al. 2019). The wild ancestor of *C. moschata* is still unidentified. Recent investigations (Montero-Pau et al. 2018) based on DNA data indicate the phylogenetic relationships among wild and

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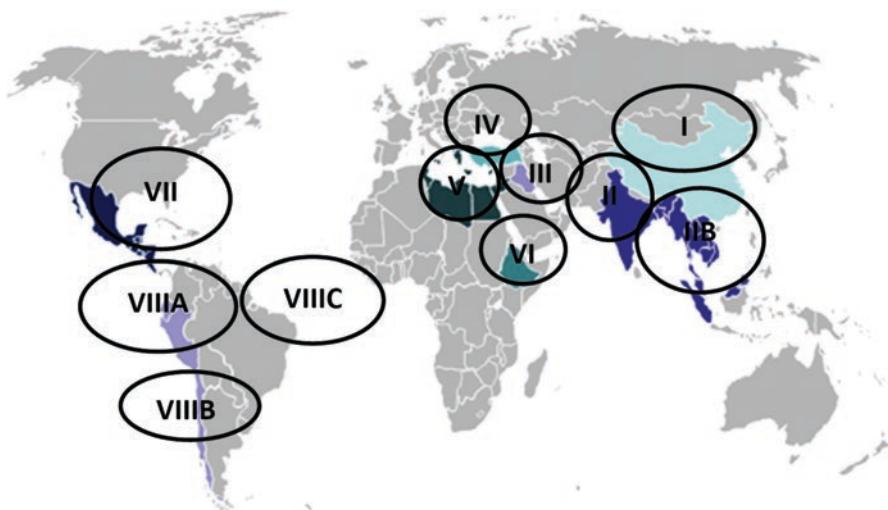


Fig. 8.1 The seven centers of origin proposed by NI Vavilov (I-Chinese Center, IIA-Indian Center, IIB-Indo-Malayan Center, III-Central Asiatic Center, IV-Near-Eastern Center, V-Mediterranean Center, VI-Abyssinian Center, VII-South Mexican and Central American Central, VIII A- Peruvian, Ecuadorean, Bolivian Center, VIII B- Chile Center, VIII C-Brazilian-Paraguayan Center) whereas VII and VIII A are the major center of origin for *Cucurbita* especially *C. moschata* and *C. maxima*, respectively. (Map courtesy from: <http://en.softonic.com>)

domesticated *Cucurbita* taxa suggest their probable existence in lowland northern South America up to 5000 years BC (Castellanos-Morales et al. 2018). After the European arrival in the New World, the cultivated cucurbits were introduced into the Old World and spread all over the tropics and subtropics after the seventeenth century. Now it is cultivated all over the world (Chomicki et al. 2019).

8.1.1 Botany

8.1.1.1 Vegetative Phase

Sweet gourd is an annual and scandent herb having a well-developed root system of 40-cm root depth and 5 m length. It is characterized by a thick climbing or creeping stem up to 10 m long covered with soft white pubescence and often produces adventitious roots at the nodes. Leaves are alternate, simple, without stipules; petiole 10–20 cm long; blade usually reniform, not lobed to shallowly 5–7 cm lobed, margins finely toothed, softly hairy, occasionally with white blotches 7–25 mm in diameter, deeply cordate at base, 3-veined from the base stipulate and densely pubescent. The plant bears tendrils with light pubescence, coiled and positioned at 90° to the leaf axil (Hazra et al. 2007).

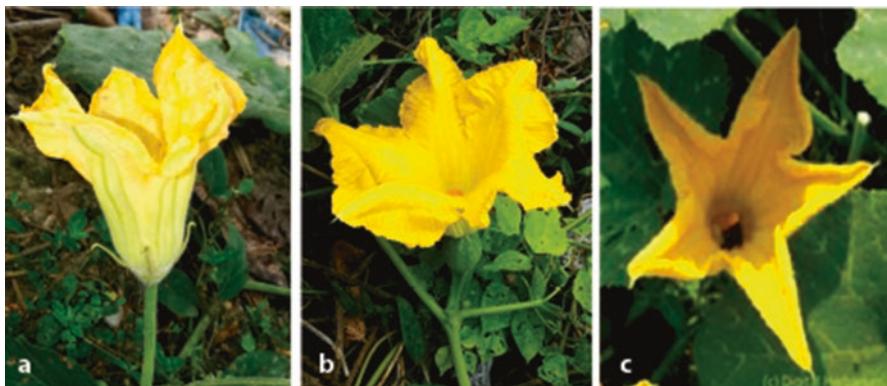


Fig. 8.2 Sweet gourd flower. (a) Male flower, (b) Female flower, (c) Pollination by a bee. Photo credit: Sumi Sarkar

8.1.1.2 Reproductive Phase

Sweet gourd is a monoecious plant bearing both male and female flowers on the same plant (Fig. 8.2). Anthesis begins 30–60 days after emergence of the plant and it is more or less continuous. Male flowers appear first and generally outnumber the female flowers. The ratio of female to male flower is around 20:1. The female flower usually appears on the eighth node of the plant. Pollination occurs early in the morning. Insects, mostly thrips and bees, are pollinators; flowers are principally cross-pollinated. A male flower produces around 100,000 pollen grains; about 90% remain viable for up to 9 h. Pollen viability gradually decreases and reaches 8% after 1 day. Stigma remains receptive from 1 day before anthesis to 2 days afterwards (Agbagwa et al. 2007). Flowers are solitary, unisexual, regular, and lemon yellow to orange-yellow; sepals free, subulate to linear 5-merous, corolla campanulate, with widely spreading lobes. Male flowers have peduncles 10–16 cm long, calyx tube club-shaped, 5–10 mm long, lobes 5, ovate-orbicular, 2–3 cm long, apex obtuse, margin rugose, linear, hairy, corolla yellow and tubular. Androecium having stamens 3, filaments 5–8 mm long, anthers 12–16 mm long, connivent and almost glabrous or puberulous at the base. Female flowers have peduncles of 5–7 cm long, solitary, 3 staminodes, calyx and corolla the same as male flower. Gynoecium with ovoid ovary, style short, stigma 2-lipped (Ferriol and Pico 2008).

Sweet gourd fruits are classified as berries and are highly variable in shape, size and color. Fruit shape may be elongated, oval, flattened, globular, heart-shaped or cylindrical. Length varies from 5.8–71.6 cm and width from 11.2–48.6 cm. Skin can be smooth, warty, wrinkled or have shallow-to-deep longitudinal ridges. Flesh is also variable in thickness and white, yellow, or orange in color, and typically 1–6.4 cm thick. Some pumpkins and squashes are over 90 kg (~200 pounds), but usually range from 0.3–50 kg. Each fruit bears a few to many seeds (Mythili and Kavitha 2017). Seed shape is oval. Seed color may be white, cream, orange or brown, and contains 34–54% oil. Seed size (length, width, thickness) and weight

vary from fruit to fruit and ranges between 1.6–2.9 cm long, 0.7–1.6 cm wide, 0.28–0.69 cm thick, and 14–60 g per 100 seeds. Seeds reportedly remain viable for 6–8 years, but bear no endosperm. The embryo consists of leaf-like cotyledons and a short radicle (Ferriol and Pico 2008).

8.1.2 Economic Importance

In Bangladesh, the total annual production of sweet gourd is 191,087 mt from an area of 16,861 ha. Sweet gourd production is high in the Khulna region where 44,656 mt is produced. It is a well-known as a beneficial vegetable. All parts of the sweet gourd plant are important due to their nutritive values (Ahmed et al. 2017). Vegetable exports have become an important part of total agricultural exports of the country. Bangladesh exports 54 different vegetables; 60% of the total quantity to the Middle East and the remaining 40% to European and other countries. Major exported vegetable crops include yard-long bean, cucumber, snake gourd, bitter gourd, tomatoes, eggplant, lady's finger, sweet gourd, amaranth, spinach, Indian spinach and green chili. The demand for vegetables export is increasing steadily. According to FAO, world vegetable production has increased fivefold in the past 40 years. Some 20–25 types of vegetables, including sweet gourd, are produced year-round (www.freshplaza.com). Bangladesh ranks third in global vegetable production, after China and India. Sweet gourd has great economic importance as a vegetable in Bangladesh and excellent export potential due to its nutritional value and storability.

8.1.3 Nutritional and Health Importance

Sweet gourd leaves contain large amounts of iron and other minerals and vitamins; the stems provide dietary fibers. The orange and yellow flowers are laden with carotenoids and phytochemicals that may decrease the risk of some cancers, heart attacks and slow certain aging processes (Muntean et al. 2013). The pulp is an outstanding source of protein and also has pharmacological qualities purported to be antidiabetic and anti-inflammatory. It is an excellent source of calories, proteins, minerals and fiber-regulating cholesterol. The pulp of sweet gourd contains dietary antioxidants which supply bioactive mechanisms to reduce oxidative stress due to free radical produced in the human body. It is said to play a vital role to prevent aging and various diseases associated with oxidative stress, such as cancer and cardiovascular disease (Nagar et al. 2018).

Seeds contain lipids, proteins, carbohydrates and minerals for the human diet which are necessary to maintain proper health (Alfawaz 2004). The proximate compositions evaluation from the seed reveals 33.48% protein, 28.68% carbohydrate, 30.66% lipid, 3.07% fiber, 3.98% ash and 524.58 kcal of available energy (Karaye

Table 8.1 Some medicinal compounds found in sweet gourd

Compound	Derived from	Benefit
Antioxidants (carotenoid, tocopherols, phenolic acids, and flavonols)	Sweet gourd pulp	Antioxidants decrease the risk of several diseases such as cardiovascular diseases (heart attack atherosclerosis, stroke), cancers, neurodegenerative diseases, etc. by inhibiting free radicals
Antidiabetic substances	Sweet gourd pulp	Works against diabetes
Unsaturated fatty acid	Seeds of sweet gourd	Works against many diseases such as diabetes, hypertension and many other diseases
Antibacterial and anti-inflammatory properties	Sweet gourd seed oil	Decreases the risk of high cholesterol level in blood and other health benefits
Anti-carcinogenic substances	Sweet gourd pulp	Works against chronic disease like cancer

Source: Kulczynski et al. (2017), Montesano et al. (2018), Sharma et al. (2015)

et al. 2013; Pevicharova and Velkov 2017). Sweet gourd seeds also contain significant amounts of various essential microelements such as K, Cr and Na (Durante et al. 2014).

Almost all parts of sweet gourd plants are edible and healthful. Moreover, sweet gourd has certain claimed medicinal properties which are shown in Table 8.1.

The flesh of the ripe fruit is used to prepare sweets and soft or slightly alcoholic drinks, and the unripe fruit is consumed as a boiled vegetable. The fruits have numerous cooking uses including bread, biscuits, pie, desserts, soups, beverages and puddings. Sweet gourd fruits are used in festivals for decoration purposes.

The flowers can be consumed raw in salads or cooked. The leaves are used as vegetables and smaller new leaves are tasty ingredients in salads, while stems can be chopped and boiled with other vegetables. The seeds can be dehydrated and eaten raw or baked. Edible oil is also expressed from the seed of sweet gourd which is rich in oleic acid. Sweet gourd seeds are an important food resource and thus their consumption should be promoted (Muntean et al. 2013).

The dried flesh of sweet gourd can be processed into flour which has a lengthy shelf life and can be utilized for its flavor, sweetness, deep yellow orange color and substantial amount of dietary fiber (Fig. 8.3). This flour can be used to enhance the quality of bakery products, sauces, instant noodles, and soups and used as a coloring ingredient for food.

In North America, peoples use sweet gourd seed oil for skin problems e.g. sores and ulcers (Martinez et al. 2018). American Indians utilize the seeds for treating intestinal infections. They also use the flowers for curing minor injuries. In India, the paste of fruit stalks is utilized for curing earache and boils. In Nigeria, Cameroon and other West African countries, seeds are also popularly used (Aziah and Komathi 2009; Aziah et al. 2011).



Fig. 8.3 Different food uses of sweet gourd. (a) Sweet gourd juice. (Source: <https://www.food-power.info/>), (b) Powder. (Source: <https://kang-med.com/>), (c) Cake. (Source: <https://www.twosisterscrafting.com/>), (d) Vegetable. (Source: <https://www.jagsfresh.com/>)

8.2 Genetic Diversity and Conservation

8.2.1 Genetic Diversity

Pumpkin (*Cucurbita maxima*) is cultivated as a sweet gourd in several countries. It was domesticated in the Americas (Hazra et al. 2007). Nowadays the genetic variation of this species is very extensive. Some reasons for this variation seed and fruit morphology, fruit color, shape, flesh thickness and durability of the fruit skin. There are differences in duration of life cycle in sweet gourd varieties. Phenotypes with exceptional agronomic characteristics were developed in different parts of the world leading to local varieties. Variations present in sweet gourd (*C. moschata*) cultivars are used as sources for hybridization. A cultivar, native to Nigeria, represents the only source of resistance to certain viral diseases is the best example. Sweet gourd (*C. moschata*) has shown, along with other cultivated species, confirmation that there are good prospects for the perfection of these crops. Numerous commercial cultivars are characterized by using genetic stock of sweet gourd, developed mainly in the USA and to a lesser extent in Brazil (Khoury et al. 2019). Among them butternut squash, golden cushaw, large cheese, Tennessee sweet potato, Kentucky field and menina brasileira are prominent. These commercial cultivars have different levels of resistance and/or susceptibility to certain diseases

and are suggestive of the broad genetic variation of this species (Sanjur et al. 2002). However, the introduction of improved cultivars affects ancient landraces and threatens their survival. Therefore, collecting germplasm of landraces deserves priority for genetic improvement of sweet gourd. Ex situ and in situ conservation are essential to exploit the accessible genetic resources for better phenotypic assessments and improvement of *Cucurbita* crops (Kates 2019).

8.2.2 Germplasm Collections

Germplasm of *Cucurbita* spp. can be collected and managed both in local more informal and formal ways (Fig. 8.4). Plant genetic resources (PGRs) are managed by farmers in a combined and adaptive way. The exchange and adaptation of seed via friends, relatives and merchants are other ways to collect *Cucurbita* germplasm. Plant genetic materials are actually collected from local farmers and stored in gene banks which predominantly serve breeders (Kiramana and Isutsa 2019).

Appropriate characterization of genetic resources and resourceful exploitation of pumpkin (*Cucurbita maxima*) germplasm for crop improvement that provides researchers with accurate information about genetic resources of pumpkin, depend upon the capability of the world's gene banks to conserve the existing genetic diversity in pumpkin and their wild and weedy relatives (Lebeda et al. 2007).

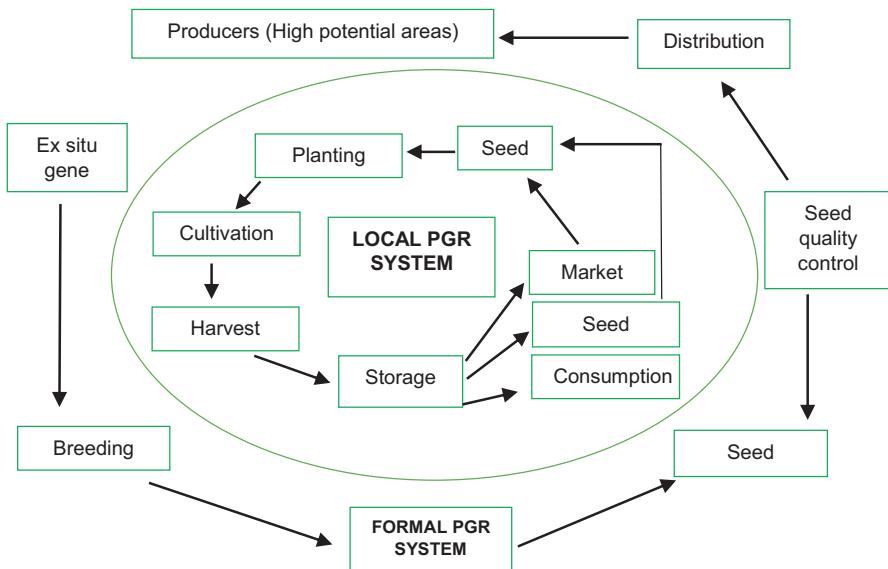


Fig. 8.4 Plant Genetic Resource (PGR) Management of germplasm collection through local and formal systems. Adapted from Kiramana and Isutsa (2019)

The Southern African Developing Countries Plant Genetic Resources Centre (SPGRC) collection recently exceeded 24 *Cucurbita* spp. Accessions (SPGRC Annual report 2018–2019). *Cucurbita moschata* is the best represented germplasm of *Cucurbita* in the gene banks of the Americas where more than 2000 accessions are held. The most important accessions were collected from Mexico and Central America and the USA. In Mexico, the CIFAP (Forestry, Agricultural and Livestock Research Center) collection is the most representative of *C. moschata* variation in that country (Ferriol et al. 2004). However, there are some other base collections, namely, the Vavilov Institute of Plant Industry (VIR), St. Petersburg, Russia and the National Seed Storage Laboratory (NSSL), Fort Collins, Colorado, USA (Grubben and Chigumira 2004).

8.2.3 Conservation Approaches

Existing and primitive *Cucurbita* species containing significant genetic traits should be conserved. Different conservation approaches are used to accumulate, safely preserve and adequately document the genetic resources of pumpkin (*Cucurbita maxima*) in order to make them available to the science for the benefit of present and future generations. The ability of genebanks to conserve cucurbit germplasm depends on the conservation protocols that ensure high-quality seed production (Lebeda et al. 2007). Seeds should be preserved under proper temperature and moisture content in order to prolong their endurance to extend the period between regeneration events and diminish probable alteration to genetic profiles.

8.2.3.1 Ex Situ Conservation

Significant effort has been focused on increasing ex situ conservation since the 1980s through the collection of weedy, wild and landrace populations of *Cucurbita* spp. from their center of diversity and conventional agroecosystem all over the world (Lebeda et al. 2007). Pumpkin seeds are orthodox and can be dried and frozen completely and grown after storage (<https://blog.doublehelix.csiro.au>). That explains why ex situ conservation is the most useful method of pumpkin (*Cucurbita maxima*) germplasm conservation. There are mainly two types of ex situ conservation: in vivo and in vitro.

8.2.3.2 In Vivo Conservation

Among the techniques, in vivo is mainly used where seeds and other vegetative propagules of *Cucurbita maxima* are preserved. As pumpkin is mainly a seed propagated plant, in vivo methods are easy and cost effective for germplasm conservation. There are several types of in vivo conservations as follows:

8.2.3.3 Seed Gene Bank

Seed gene bank is one of the most safe conservation approaches to avoid the risk and expense of *Cucurbita maxima* seed storage (Kiramana and Isutsa 2019). Storing pumpkin seeds under proper temperature and moisture content, high level of seed viability and integrity can be maintained for a long time. To avoid the risk of genetic damage to seed, they can be stored at sub-zero temperature, in a fully imbibed state or with liquid nitrogen. In seed gene bank three types of conservation are done depending on storage duration:

- (a) Base collection: This method is used for long-term conservation of pumpkin seed at about -20°C temperature and 5% moisture content in order to provide for regeneration purposes.
- (b) Active collection: Pumpkin seeds are conserved for medium duration such as 10–15 years at 0°C temperature and 5–8% moisture content for evaluation, multiplication and distribution purposes.
- (c) Working collections: This is a short-term conservation program where pumpkin seeds are preserved at 5–10 $^{\circ}\text{C}$ temperature and about 10% moisture content for only 3–5 years to use frequently in crop improvement programs.

8.2.3.4 Field Gene Bank

Traditional varieties or primitive cultivars cannot be conserved *in situ* as this system needs to preserve the associated farming system which is not possible. But conservation of these species is possible through field gene banks as genetic resources can be collected in the field as representative samples and preserved safely for long time. Genetic resources of pumpkin are also being preserved by many institutions of the world in field gene bank by establishing research farms, botanical gardens and wild farms (Rajasekharan and Ramanatha 2019).

8.2.3.5 InVitro Conservation

Sweet gourd is mainly a seed propagated plant. As a result, in vitro preservation of its genetic resources is difficult as in vitro is preferably matched to germplasm conservation of vegetatively-propagated plants. Nevertheless certain species such as *Cucurbita moschata* and *Cucurbita maxima* are suitable for cryopreservation and in vitro preservation (Sari et al. 2008).

8.2.3.6 In Situ Conservation

The most suitable way of biodiversity conservation is in situ or on farm conservation as this type of conservation allows conservation of plant species in their natural habitat. This type of conservation includes areas of biosphere reserve or gene sanctuaries, natural parks which are kept protected from human interference. Wild relatives of pumpkin are conserved through this method. However, it is a costly method for conserving germplasm. Linkages must be created between ex situ and in situ conservation to avoid genetic erosion different valuable species of pumpkin (Rajasekharan and Ramanatha 2019).

Cucurbita spp. are well represented in the germplasm collections as both ex situ and in situ conservation of many institutions all over the world (Table 8.2).

8.2.4 Varieties and Cultivars

The wild *Cucurbita* are classified into two forms based on life cycle:

- (a) Annual/short-lived perennial/mesophytic forms (*C. ficifolia*, *C. sororia*, *C. lundellina*);

Table 8.2 Approaches of *Cucurbita* germplasm conservation by many institutions

Institutions	Conservation programs/activities
Bangladesh Agricultural Research Institute, Bangladesh	Ex situ conservation of germplasm as active and base collection in gene bank storage and in field gene bank
Bangladesh Agricultural Development Corporation (BADC), Bangladesh	Seed Processing and short-term storing (also includes field gene bank)
National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India	Active germplasm collection
Southern African Developing Countries Plant Genetic Resources Centre (SPGRC), Zambia.	Ex situ Conservation of <i>Cucurbita</i> spp. as base collection
Southern African Developing Countries Plant Genetic Resources Centre (SPGRC), Tanzania	Ex situ Conservation of <i>Cucurbita</i> spp. as base collection
Bioversity International	In situ conservation of crop wild relatives
The National Plant Genetic Resources Laboratory (NPGRL), University of the Philippines Los Baños	Regeneration and documentation of plant genetic resources as seeds, live plants in the field, greenhouse, nursery and in vitro
Svalbard Global Seed Vault, Svalbard	World largest ex situ conservation of plant germplasm through frozen seed
International Plant Genetic Resources Institute (IPGRI)	Conservation of gene pools of useful plants
National ex situ Cucurbitaceae collections of Turkey	Both ex situ and in situ conservation

Source: BARI Annual Report 2017–2018; Razzaque and Hossain (2007); SPGRC Annual Report 2018–2019

- (b) Perennial/xerophytic forms (*C. digitata*, *C. palmata*, *C. cylindrica*, *C. foetidissima*).

Sweet gourd appears to be most likely related to the xerophytic forms, considered as the axis through which the species under xerophytics forms are related to each other. The mesophytic forms are mostly cross compatible and *C. lundelliana* is seen as the key species in this group. *Cucurbita lundelliana* has a wide spectrum of compatibility with the cultivated species particularly *C. moschata*. This suggests that *C. lundelliana* is involved in the ancestry of the cultivated types. Several types of sweet gourd are based primarily on fruit shape and texture (Table 8.3). In Bangladesh, some institutions have also developed sweet gourd varieties (Table 8.4).

8.3 Cultivation Practices

8.3.1 Land Preparation

Land preparation is very important in terms of where a plant is to be grown and where the main roots exist (Whitaker and Davis 1962). Preparation of the soil around the whole area where the sweet gourd vines spread is equally important. A pit is prepared by excavating a $1 \times 1 \times 1$ m hole and filling it with manure and compost. By using a large amount of rich materials, a nutrient rich and soft soil composition will allow the roots to grow fully. The soil should not be compacted as that will impede root spreading. The material should be well composted, otherwise, it can be harmful to the plant, burning the roots or robbing the soil of nitrogen. Manures and compost should be added to the soil in generous portions. These amendments should be thoroughly mixed into the soil (BBS 2015; Chadda 2004).

8.3.2 Sowing

Sweet gourd seeds may be sown on mounds (creating a hill or raised area) or level beds (Chadda 2004; Hazra and Som 1999; Rubatzky and Yamaguchi 1997). Mounding promotes better drainage. Excessive moisture can promote bacterial growth, damping off disease or *drowning* of the roots by depriving them of oxygen. Plant 4–5 seeds per hill at 3 cm depth. Maintain 2 m between hills, and space rows 3–4 m apart. Keep 2–3 healthy seedlings per hill after thinning. Maintain spacing of 1 m between hills and 2.5 m between rows for semi-bush varieties, 0.75 m in a row and 3–4 m between rows for miniature varieties, and 1 plant/m and 1–2 m between rows for bush varieties. The recommended seeding rate is 3.36–4.47 kg/ha (Bose et al. 2009; Hazra and Som 1999).

Table 8.3 Types of sweet gourd *Cucurbita* spp. based on fruit shape and texture

Images	Cultivar name	Botanical name	Characteristics	References, source, photo credit
	Acorn	<i>C. pepo</i> var. <i>turbinate</i>	Deeply ridged and tapered at one end Have a dark green rind Weight 1–4 kg Both bush and vining types are available	OCED (2016). Photo credit: Johnny's Selected Seeds
	Butternut and Waltham butternut	<i>C. moschata</i>	Less symmetrical, cylindrical, odd-shaped fruit that often bulges around the seed cavity Weight around 1 kg They have light tan rinds with orange flesh and are vining in growth habit.	Gourmet Sleuth (2020). Photo credit: George Chernilevsky
	Buttercup and Turk's Turban	<i>C. maxima</i>	Medium sized fruit Tricolor gourd spotted with orange, green, and white shades Weight 1–2 kg	Linda Stradley (2013). Photo credit: https://www.westcoastseeds.com/shop/vegetable-seeds/squash-seeds/specialty-squash-seeds/turks-turban-squash-seeds/
	Spaghetti	<i>C. pepo</i> var. <i>styriaca</i>	Also called vegetable spaghetti, cylindrical in shape (20–23 cm long) Fruit has a yellow flesh that is stringy	Beany et al. (2002). Photo credit: http://steamykitchen.com/11285-baked-spaghetti-squash-with-garlic-and-butter.html

	Hubbard	<i>C. maxima</i>	Round in general but taper to a point at the blossom end Warty gray-green rind	Piperino and Stothert (2003). Photo credit: https://www.westcoastseeds.com/products/baby-blue
	Marrows	<i>C. pepo</i>	Fruits are very large, green mottled, ovoid and non-ribbed They may also be pear shaped and rounded	Lust and Paris (2016). Photo credit: https://stock.adobe.com/ee/search?k=vegetable+marrow
	BARI Mistikuma-1	<i>C. moschata</i>	Fruit round, deep orange flesh color and very sweet Average marketable weight 4.5–5.0 kg	Source: http://dhicrob.bsmrau.net
	Crookneck	<i>C. moschata</i>	Fruit extended with bent neck Yellow skin and flesh	Paris et al. (2003). Photo credit: https://www.adaptiveseeds.com/wp-content/uploads/2014/12/p-8160-winter_squash_canada_crookneck.jpg
	Aehobak or Korean zucchini	<i>C. moschata</i>	Thin, smooth skin and more dedicated flesh Used both in fresh and dried form	Uddain et al. (2019). Photo credit: http://photo.naver.com/view/2015062613372403976

(continued)

Table 8.3 (continued)

Images	Cultivar name	Botanical name	Characteristics	References, source, photo credit
	Calabaza or West Indian pumpkin	<i>C. moschata</i>	Fruit has a round or pear shape Year-round availability	https://specialtyproduce.com/produce/Calabaza_Squash_1198.php
	Dickinson pumpkin	<i>C. moschata</i>	It has tan skin of orange color Fruit has oblong shape and dark orange flesh used for pumpkin pie	https://www.rareseeds.com/store/vegetables/squash/winter-squash/dickinson-pumpkin;https://gardentabs.com/dickinson-pumpkins/
	Giromon	<i>C. maxima</i>	Also known as the sweetness of Caribbean Grainy flesh used to make traditional <i>sopapegiromon</i>	https://azmartinique.com/en/all-to-know/fruit-vegetables/turban-squash-giromon
	Golden cushaw	<i>C. moschata</i>	Golden strip present on fruit skin The neck of fruit is slightly crooked	Kates (2019). https://www.seedway.com/product/9423-cushaw-orange-stripe-treated-seed/
	Lochezapallo squash	<i>C. moschata</i>	High quality landrace of <i>C. moschata</i> Fruits have longitudinal ridges with few or no seeds	https://specialtyproduce.com/produce/Loche_Zapallo_Squash_16996.php
	Long Island cheese pumpkin	<i>C. moschata</i>	Fruits are round to semi flattened shaped Buff colored and smooth fruit skin	https://www.rareseeds.com/store/vegetables/squash/winter-squash/long-island-cheese-pumpkin

	Naples long squash	<i>C. moschata</i>	Fruits have elongated necks like butternut Flesh is thick and dense	https://www.specialtyproduce.com/produce/Lunga-Di_Napoli_Squash_11350.php
	Seminole pumpkin	<i>C. moschata</i>	Flesh is orange, firmer and less fibrous than other pumpkin Their thick skin helps to store them for up to a year	Photo credit: Miranda Castro; https://gardeningsolutions.fas.ufl.edu/plants/edibles/vegetables/seminole-pumpkin.html
	Tromboncino or zucchetta	<i>C. moschata</i>	Fruits have long neck and always free of seed Pale green colored fruit that become beige when mature	https://www.rootsimple.com/2013/11/what-does-tromboncino-squash-taste-like/

Table 8.4 Sweet gourd varieties developed by Bangladesh Agricultural Research Institute (BARI, Bangladesh) and Bangladesh Agricultural University (BAU)

Name of variety	Developed by	Year of release	Growing season	Average yield (mt ha ⁻¹)
BARI Mistikumra-1	Horticulture Research Center (HSC), BARI	2007	Winter (Rabi)	35–40
BARI Mistikumra-2	Horticulture Research Center (HRC), BARI	2007	Winter (Rabi)	25–30
BARI Hybrid Mistikumra-1	Horticulture Research Center (HRC), BARI	2015	Winter (Rabi)	40
FTIP BAU Mistikumra-2	Bangladesh Agricultural University (BAU)	2009	Kharif (Summer)	30–40

8.3.3 Fertilizer Management

Sweet gourd is a very heavy feeder, growing well in rich soil with a lot of compost and manure. It grows even larger when fertilizer is added to the soil. Three basic plant nutrients are required at higher or lower levels depending upon the growth stage of the sweet gourd. These basic nutrients are N (nitrogen), P (phosphorus) and K (potassium). Sweet gourd responds well to ample dressings of organic manure, and artificial fertilizers may be applied at a rate of 672–897 kg/ha of a 5:10:10 NPK mixture (Grubben and Ngwerume 2004; Hazra and Som 1999; Rai and Yadav 2005).

Application of higher concentrations of N at the early growth stage helps for leaf, root and vine growth, but excessive nitrogen can burn the plants and reduce or delay the emergence and number of flowers and fruits. So, avoid direct contact to leaves and vines. As the crop moves towards fruiting stage, higher phosphorus levels should be used (5-10-5 or 5-15-5 are good ratios of N:P:K). Phosphorus promotes fruit set and development. Potassium promotes fruit growth. After fruit set, a high potassium fertilizer should be used. Over application can cause sweet gourd to grow so quickly that it outgrows its skin and splits or explodes. Over applications should be avoided and the other essentials of good soil management and plenty of water should not be overlooked (Bose et al. 2009; Rubatzky and Yamaguchi 1997).

Micronutrients are essential to plant growth. These can be applied in the form of liquid fertilizer. Liquid fertilizer can be applied to secondary roots, and included in the water supply or irrigation channel. Application methods are (a) during the growing season, most fertility needs of sweet gourds can be met by applying water-soluble plant foods once or twice a week over the entire plant area, (b) give fertilizer to the seedlings that stresses phosphorus, such as 15-30-15 (N-P-K). Shift to a more balanced formula, such as 20-20-20, once fruits are set, and (c) once fruit set is evident, use a formula with a high potassium percentage, such as 15-11-29 (Rai and Yadav 2005).

8.3.4 Intercultural Operations

In the first few weeks of growth sweet gourds are not very competitive with weeds because they are planted in widely spaced rows, plants are still short in stature, and require 8–10 weeks to close the canopy. Weeds in the early stages of growth can be controlled by transplanting the seedlings closer. As sweet gourd vines senesce at the later stage of the crop they potentially open the crop canopy and allow weeds to become established and produce seed (Rai and Yadav 2005; Rubatzky and Yamaguchi 1997; Whitaker and Davis 1962).

Weed control can be achieved with herbicides and a good crop-rotation system. Several pre-plant and post-emergence herbicides are available for sweet gourd, depending on the specific weed problem and growth stage. If infestation levels are low, early cultivation (prior to vine running) can help minimize weed problems (BBS 2015; Muimba-Kankolongo 2018).

8.3.5 Irrigation

Good water management is critical to the development of vigorous vines. It helps in the maintenance of the foliage canopy, which supports fruit growth and protects developing fruit from sunburn. Different types of irrigation are used for sweet gourd cultivation. They are sprinkler irrigation (effective), drip and furrow irrigation, trickle irrigation (ideal) and soaker hoses (Bose et al. 2009; Grubben and Ngwerume 2004; Hazra and Som 1999).

Sweet gourd needs to be watered regularly throughout the growing season; they require a lot of indirect water. The foliage should not be watered as wet foliage increases the chance of disease, especially mildew. When it gets warmer it may be necessary to water more than once a day. The best indicator of water need is the plant leaves (Chadda 2004; Whitaker and Davis 1962). If they are green and look healthy, they are probably getting enough water; if they appear wilted, they need more water.

8.3.6 Disease Management

Disease control is essential in the production of high-quality sweet gourd. A preventive program that combines the use of cultural practices, genetic resistance and chemical control as needed, usually provides the best results (Table 8.5). Some common diseases affecting sweet gourd production include: anthracnose, gummy stem blight, powdery mildew, bacterial wilt, *Phytophthora* root rot and downy mildew.

Table 8.5 Disease management with cultural and chemical control

Disease name	Infected parts	Chemical control	Cultural control
Anthracnose <i>Colletotrichum lagenarium</i>	Foliage, fruit	Seed treatment with systematic fungicide	
Gummy stem blight (<i>Mycosphaerella melonis</i>)	Stems, leaves and fruit	Bravo® (chlorothalonil), Maneb® (maneb)	
Powdery mildew (<i>Erysiphe cichoracearum</i>)	Leaves	Mancozeb	
Bacterialwilt (<i>Erwinia tracheiphila</i>)	Leaves	Mancozeb	
Downy mildew (<i>Peronospora cubensis</i>)	Leaves, fruits	Mancozeb	
<i>Phytophthora</i> blight (<i>Phytophthora infestans</i>)	Seedlings, stems, leaves, fruit	Ridomil and Aliette have shown to be effective in managing this disease	
Viral disease	Leaves	Insecticides are used	

Source: Tuttle McGrath (2004)

Table 8.6 Pest management with cultural and chemical control

Name	Infected parts	Chemical control	Cultural control
Squash bug (<i>Anasa tristis</i>)	Young plants and leaves	Insecticides like Carbaryl or Sevin are used	Deep tillage or removal of crop residue; control weeds along drains, banks, roads, and other non-cultivated crops; destroy the debris of field
Squash vine borer (<i>Melitaea cucurbitae</i>)	Vines	Carbaryl or Sevin	
Aphid (<i>Myzus persicae</i>)	Leaves,foliage	Insecticidal soap sprays	
Cucumber beetles (<i>Acalymma vittatum</i>)	Seedlings, leaves, flower, root	Malathion	
Striped Cucurbit Beetle (<i>Acalymma vittata</i>)	Seedlings	Malathion, Sevin, Fastac, Decis and Karate	

Source: Adapted from Painkra et al. (2019)

8.3.7 Pest Management

The main pests of sweet gourd are aphids, pumpkin beetles, cucumber beetles, squash vine borers, squash bugs and the leaf eating ladybird. Dusting or spraying regularly before an infestation, especially during season of egg laying and hatching, crop rotation etc. are recommended actions (Table 8.6).

8.4 Genetics of Yield and Quality Traits

The genes responsible for yield and quality traits of the species *Cucurbita moschata* and *C. maxima* are presented in Table 8.7.

Table 8.7 Gene symbols with characters of two *Cucurbita* species

Gene symbol	Characters	Species	References
<i>B</i>	Bicolor.Precious yellow fruit pigmentation; pleiotropic, affecting fruit and foliage, modified by Ep-1, Ep-2 and Ses-B. Originally from cv. Vaughn's Pear Shaped ornamental gourd.B in <i>C. moschatav</i> . Precocious PI 165561 derived from <i>C. pepo</i> through backcrossing. Complementary to L-2 for intense orange, instead of light yellow, fruit-flesh color	<i>C. moschata</i>	Paris (1988, 2005, 2016), Shiffriss (1981)
<i>B-2</i>	Bicolor. Precious yellow fruit pigmentation, from ssp. <i>andreana</i> PI 165558	<i>C. maxima</i>	Shiffriss (1989)
<i>Bi</i>	Bitter fruit. High cucurbitacin in fruit. Bi FROM <i>C. maxima</i> ssp. <i>andreana</i>	<i>C. maxima</i>	Herrington and Brown (1988)
<i>bl</i>	Blue fruit colour. Incompletely recessive to Bl for green fruit color,in hubbard squash	<i>C. maxima</i>	Hutchins (1935)
<i>Bn</i>	Butternut fruit shape, fromcv. New Hampshire Butternut, dominant to Bn for crookneck fruit shape, as in cv. Canada Crookneck	<i>C. moschata</i>	Mutschler and Pearson (1987)
<i>Bu</i>	Bush habit. Short internodes, dominant to vine habit, Bu, in young plant stage	<i>C. maxima</i>	Denna and Munger (1963), Wu et al. (2007)
<i>Cmv</i>	Cucumber mosaic virus resistance, from Nigerian local. Dominant to Cmv susceptibility, from cv. Waltham Butternut	<i>C. moschata</i>	Brown et al. (2003), Paris (2005, 2016)
<i>de</i>	Determine plant habit;stem lacking tendrils and terminating with female flowers. Recessive to de for interminate plant habit	<i>C. moschata</i>	Kwack (1995)
<i>Fr</i>	Resistance to fruitfly	<i>C. maxima</i>	Nath et al. (1976)
<i>Gr</i>	Green rind.Dominant to buff skin of mature fruit	<i>C. moschata</i> <i>C. maxima</i>	Robinson (1987)
<i>lo</i>	Lobbed leaves, recessive	<i>C. maxima</i>	Dyutin (1980)
<i>M</i>	Mottled leaves,silver grey areas in axils or leaf veins	<i>C. maxima</i> <i>C. moschata</i>	Coyne (1970), Paris (2002, 2005, 2016)
<i>s</i>	Sterile, male flowers small, without pollen, female flower sterile	<i>C. maxima</i>	Hutchins (1944)

8.5 Conventional Breeding Methods

8.5.1 Breeding Objectives and Challenges

The concerns of breeders to improve traits are summarized in the following objectives:

- (a) To increase fruit yield;
- (b) To attain early fruiting and maturity;
- (c) To have first pistillate flower at early node number;
- (d) To gain high ratio of female to male flower;
- (e) To produce yellow or mottled fruit skin;
- (f) To develop non-ridged surface of fruit;
- (g) To get good fruit shape for market demand;
- (h) To get high antioxidants specially carotenoids;
- (i) To achieve small seed cavity and thick fruit flesh;
- (j) To get high β carotene as precursor of vitamin a and orange color of fruit flesh;
- (k) To achieve resistance to diseases and pests;
- (l) To develop tolerance to abiotic stresses.

To achieve the above breeding objectives, sweet gourd breeders must overcome the following conditions:

- (a) Requirements of space and time for the plants limit breeding efficiency;
- (b) Only two cycles can be carried out per year in most breeding programs;
- (c) Development of molecular marker for traits that demonstrate environmental variation is difficult;
- (d) Biotechnological methods are not extensively utilized, possibly because sweet gourd is not a high-priority crop and has attained adequate breeding success with traditional practices.

Several breeding methods have been developed to improve fruit yield and quality of sweet gourd. The goal of conventional breeding is to develop a unique and superior variety of sweet gourd with desirable yield and quality such as thick flesh with high β carotene, high sugar content and disease/pest resistance. Billions of diverse gene combinations in sweet gourd can be generated by crossing, selfing, mutation and other breeding methods.

8.5.2 *Hybrid Seed Production*

Commonly, hybrid seeds of sweet gourd are produced by hand pollination and manual defoliation with open pollination in an isolated field. Hand pollination is done between selected male-fertile parents. These parents are selected based on superiority of fruit yield and quality traits of sweet gourd (Soltani et al. 2017). Hybrid seeds can also be produced using male sterility systems.

8.5.2.1 Hand Pollination

To produce hybrid seed of sweet gourd by hand pollination female parents are planted in several rows, whereas male parents are planted in single row. The male buds from the female parental plants are removed some days before anthesis. Generally the female flowers open in the morning and are primarily pollinated by bees. To avoid this unwanted pollination the unopened tip of both male and female flowers can be tied up by clip before anthesis when petals start to turn to a yellowish-orange. Then the pollen grains from male flowers can be deposited directly on the stigmas of female flowers the next morning. Petals of the female flowers can again be tied up until the development of fruits. Hand pollination is usually done with fresh pollen.

8.5.2.2 Chemical Suppression of Male Flowers

Commercial hybrid seed production can be done using chemicals such as ethephon. Repeated spraying of 250 ppm ethephon can suppress the production of male flowers temporarily (2–3 weeks) on monoecious plants of sweet gourd. This chemical is usually applied to young plants at the first, third and fifth true-leaf stages. The development of staminate flowers is prevented by ethephon but the pistillate flowers remain unaffected. The effect of ethephon dissipates by the time two-three fertile fruits have developed on each mother plant. Additional spraying of ethephon at this stage is not be effective. At this time, the plant growing points are cut off with a knife to stop the development of lateral male flowers.

8.5.3 Pedigree Selection

Sweet gourd is a cross-pollinated crop and exhibits no significant loss in vigor due to inbreeding (Restrepo-Salazar et al. 2019). So, inbreeding and individual plant selection of sweet gourd can effectively be done through the pedigree method (Figs. 8.3 and 8.5).

Pedigree selection is the most conventional and widespread selection method. It is a popular selection method for sweet gourd as hand-pollination is easy with the flowers and many successful interspecific crosses have been done between domesticated and wild species of sweet gourd (Zhang et al. 2012). Selection for fruit yield should be made at the end of the growing cycle of the sweet gourd plant. Row selection in the field is done based on the average fruit yield performance of the lines. Seeds are extracted from the fruits of the desirable plant which is selected based on fruit yield and quality traits. These seeds are used for the next breeding cycle.

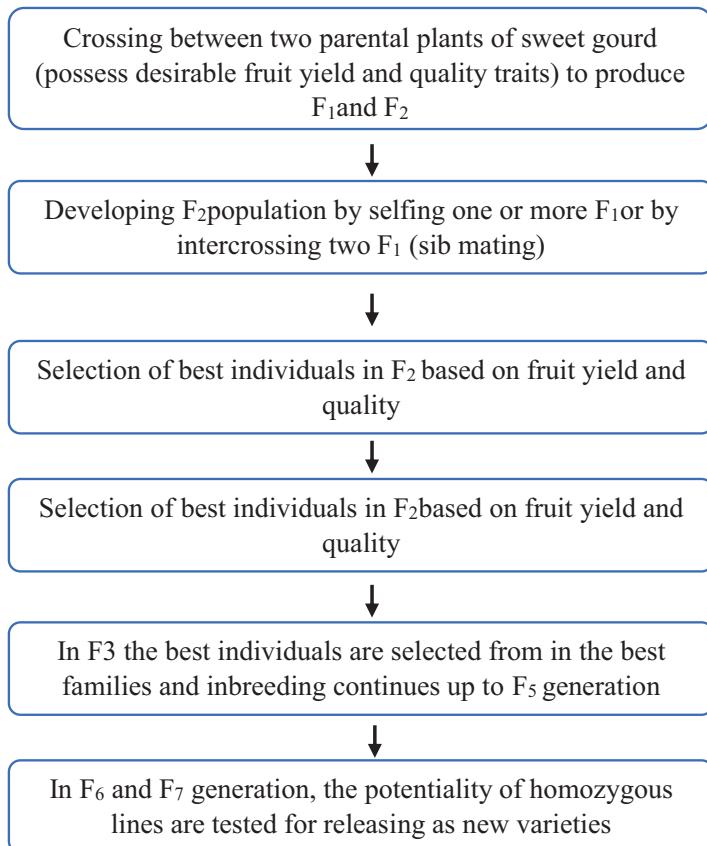


Fig. 8.5 Stepwise schematic diagram of pedigree selection in sweet gourd

Objectives and Limitations

Pedigree breeding is mainly done to attain uniformity in the quality traits of sweet gourd, to increase the fruit yield of individual plants and to recombine suitable inbreed lines (Kumar et al. 2018a, b). This method is laborious and time consuming as breeders need to keep pedigree records for a huge number of plants.

8.5.4 Recurrent Selection

This selection method can be used to improve the population of sweet gourd. Steps involved in recurrent selection for sweet gourd are provided in Fig. 8.6.

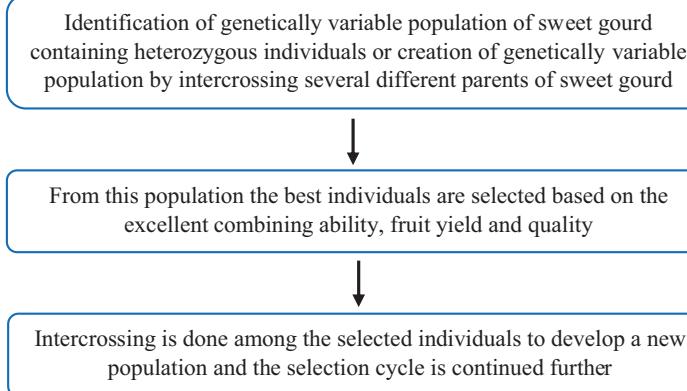


Fig. 8.6 Stepwise schematic diagram recurrent selection in sweet gourd

8.5.5 **Hybrid Breeding**

Selection of sweet gourd varieties, crosses among these varieties and selection of superior hybrid crosses are required for the development of new hybrid lines of sweet gourd. F_1 hybrid variety is mainly developed by crossing two genetically dissimilar homozygous parents which are obtained by inbreeding upto several generations and from a gene pool, which is genetically heterogeneous (Kumar et al. 2018a, b; Mohanty and Prusti 2002). Major phenomenon in hybrid breeding for sweet gourd is described in Fig. 8.7.

Objectives and Limitations

Hybrid breeding enhances yield of sweet gourd through utilizing vigor or heterosis. Hybrids show earliness, uniformity in maturity, fruit size and shape compared to open-pollinated cultivars (Kumar et al. 2017; Mahajan and Sirohi 2002). Hybrid breeding is complicated, laborious and time-consuming because it requires testing of the combining ability of parental lines.

8.5.6 **Mutation Breeding**

Mutation breeding is mainly done in sweet gourd for resistance to diseases and abiotic stresses. It is also a method of initiating new traits into the existing varieties of sweet gourd. But the success of this method depends largely on the appropriate doses of mutagen application. Among several mutagenic agents, gamma rays and ethyl methane sulfonate (EMS) have been found to be widely used in sweet gourd breeding. Gamma rays are commonly used for their easy availability, simple application, good penetration, high reproducibility, large mutation frequency and low disposal problems (Mba et al. 2012).

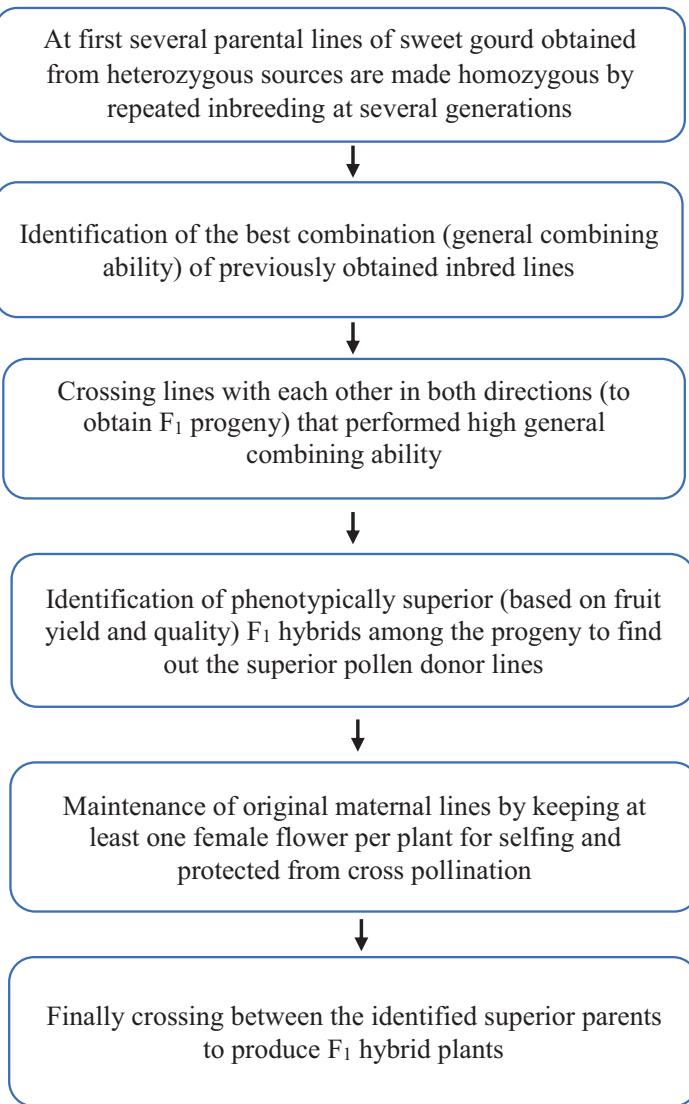


Fig. 8.7 Schematic diagram for production of hybrid sweet gourd

Researchers have developed TILLING (targeting induced local lesions in genomes) populations using EMS mutagenesis to produce a variety of mutant genotypes in sweet gourd (Fraenkel et al. 2014; Vicente-Dolera et al. 2014). Recently Kurtar et al. (2017) used EMS to produce mutant line of sweet gourd for salt tolerance. They screened sweet gourd for NaCl tolerant mutants (Fig. 8.8) to enrich the germplasm source of sweet gourd. Around 1000 seeds of sweet gourd can be mutagenized with 1.5% EMS for 24 h in case of large-scale mutagenesis. Initially

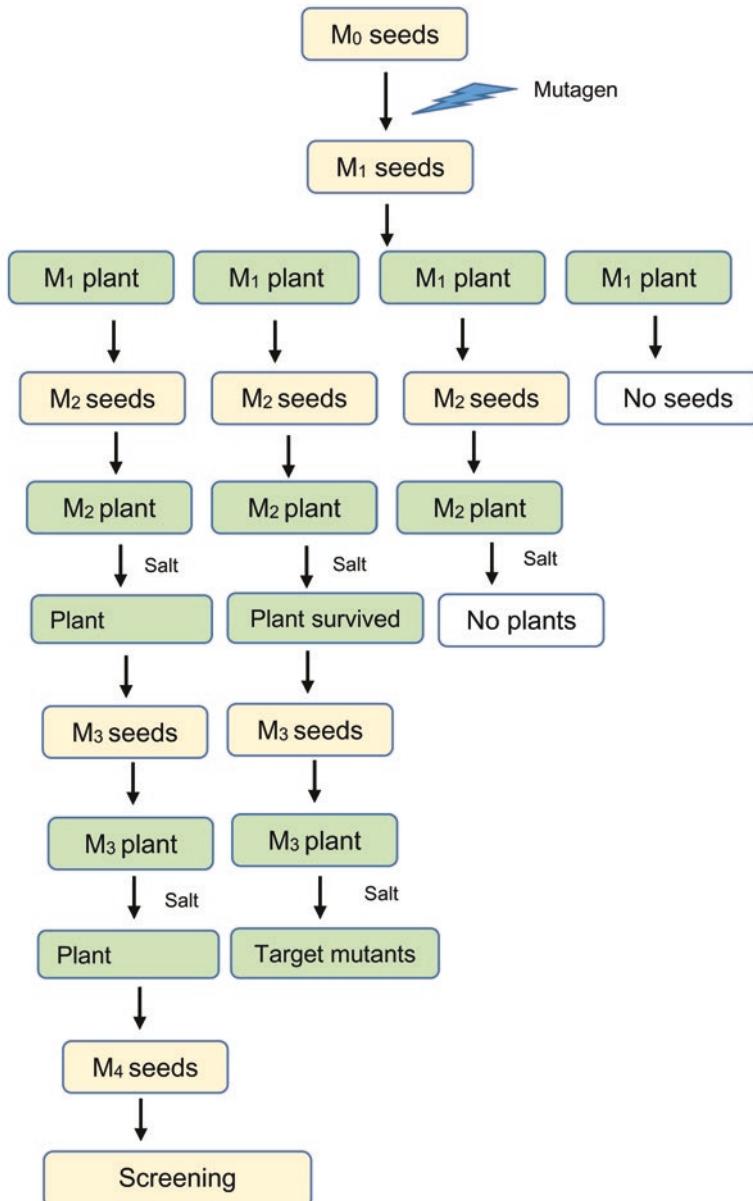


Fig. 8.8 Screening of sweet gourd with EMS for NaCl-tolerant mutants. Source: Kurtar et al. (2017)

young seedlings should be grown under controlled climatic and long day condition (16 h light/8 h dark cycle, at 25 °C) and after treating with EMS the surviving 14-day-old plants should be transplanted to the field. Mutants derived by this method can be used as excellent breeding materials in further research program to identify salt-tolerant genes and understand salt tolerant mechanisms in sweet gourd.

Objectives and Limitations

Mutation breeding may help to develop stress tolerant mutants and to understand the salt tolerant mechanisms in the plant. The main limitations of mutation breeding are the deleterious effect of mutagens and alteration of gene of quality traits.

8.5.7 *Polyplody Breeding*

Induction of haploids, followed by doubling of chromosome is accepted as the most useful method of producing inbred lines of sweet gourd. This method is faster than selfing and completely homozygous plants can be obtained in a single generation (Kazi 2015). Production of inbred lines of sweet gourd can be improved through doubled haploid practices either by regenerating plants from haploid egg cells or derived from microspores, which are known as gynogenesis and androgenesis, respectively (Fig. 8.9). Haploid plants are developed primarily by these techniques and dihaploidization is done by treating these plants with chemical compounds such as colchicine (Budhani et al. 2018).

Colchicine Treatment

Colchicine should be applied in the growing shoot apex at the concentration of 0.1–1% which is more effective compared to seed treatment at the concentration of 0.001–1% and 0.2% (Nikolova and Niemirowicz-Szczytt 1996). Colchicine also can apply as emulsion (0.2–0.4%) or in agar (1% colchicine/2% agar). The objectives of dihaploidization are (a) to fix hybrid vigor, (b) to increase fruit size and (c) to reduce breeding cycle. The success of dihaploidization depends on genotype and the health and legal concerns related to handling the doubling chemical agent.

8.5.8 *Distant Hybridization*

8.5.8.1 Interspecific Crossing

The degree of success in interspecific crossing of sweet gourd depends on the compatibility of the cultivars. Interspecific crosses are mostly difficult to make. The cross-compatible species of *Cucurbita* produce partially fertile hybrid (Fig. 8.10).

From Fig. 8.10 it can be seen that the species produce partially-fertile hybrids those can cross with each other. Cross compatibility of the species is highly dependent on cultivar and very few viable seeds are produced. Partially fertile

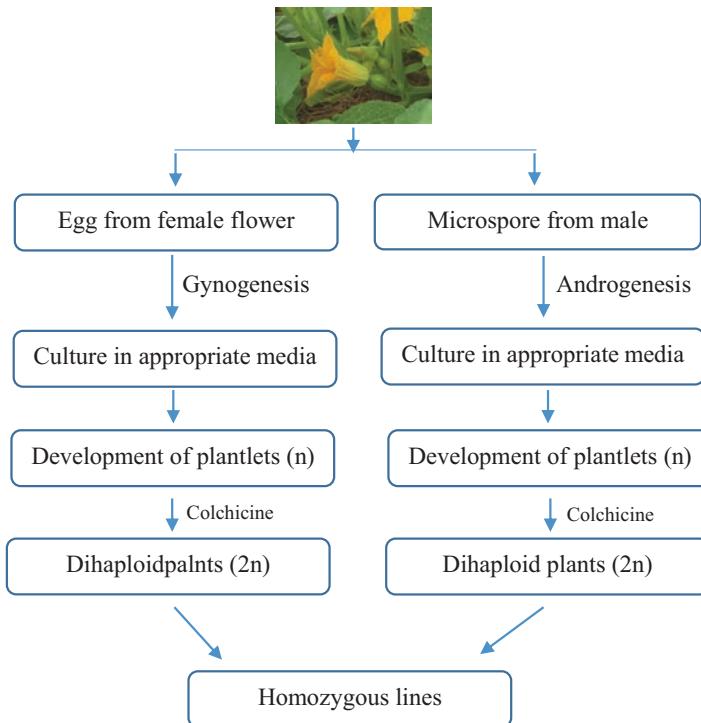


Fig. 8.9 Production of inbred lines of sweet gourd by dihaploidization

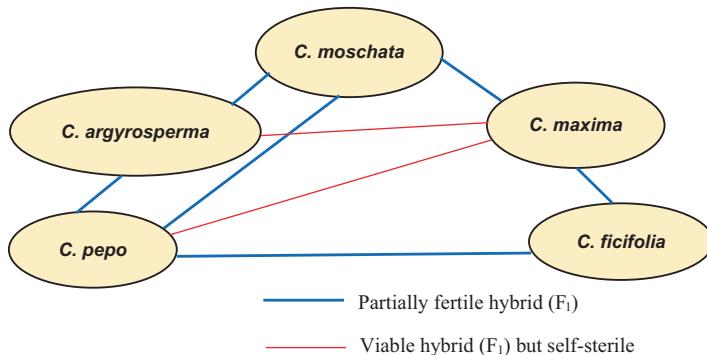


Fig. 8.10 Cross compatibility among different *Cucurbita* spp. of sweet gourd. (Source: Adapted from Yongan et al. 2002)

hybrids may be produced when two species related taxonomically (e.g. *Cucurbita maxima* and *C. moschata*) are planted close together and exposed to high pollinator pressure (mostly hand pollination) under favorable environmental conditions (Fig. 8.11). The highest potentiality of interspecific cross has been found between



Fig. 8.11 Sweet gourds (**a, b**) produced by interspecific cross between *Cucurbita moschata* and *C. maxima* through hand pollination. Photo credits (**a**): Bryan Connolly; (**b**): A.K.M. Aminul Islam

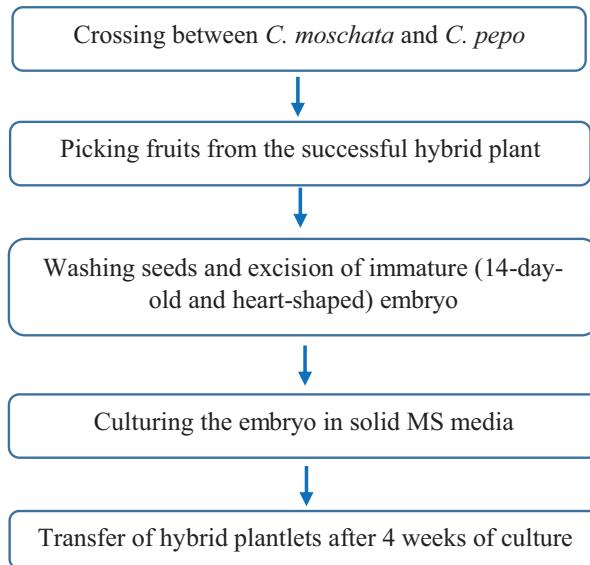


Fig. 8.12 Schematic diagram of interspecific gene transfer between *C. Moschata* and *C. pepo* through embryo rescue

C. maxima and *C. moschata*, particularly when *C. moschata* is used as the maternal parent (Paris 2016).

Interspecific crossing has also been done between *Cucurbita pepo* and *C. moschata* (Fig. 8.12). Although this type of cross is difficult, it is not impossible. *Cucurbita moschata* crossed with several cultivars of *C. pepo* produced successful and unsuccessful crosses (Table 8.8). To develop new generation from these crosses,

Table 8.8 Crosses between *Cucurbita moschata* and *C. pepo* resulted in successful and unsuccessful interspecific hybridization

Sl. No.	Interspecific hybridization
	Successful interspecific hybridization
1	<i>C. moschata</i> × <i>C. pepo</i> Eskandarani
2	<i>C. moschata</i> × <i>C. pepo</i> Queen F ₁
3	<i>C. moschata</i> × <i>C. pepo</i> Jedida F ₁
4	<i>C. moschata</i> × <i>C. pepo</i> MHTC77F ₁
	Unsuccessful interspecific hybridization
5	<i>C. moschata</i> × <i>C. pepo</i> Diamant F ₁
6	<i>C. moschata</i> × <i>C. pepo</i> Hurricane F ₁
7	<i>C. moschata</i> × <i>C. pepo</i> Cavili F ₁
8	<i>C. moschata</i> × <i>C. pepo</i> Arlika F ₁
9	<i>C. moschata</i> × <i>C. pepo</i> Revennue F ₁
10	<i>C. moschata</i> × <i>C. pepo</i> Eskandarani × Arlika

Source: Rakha et al. (2012)

embryos can be rescued from the seeds of the successful interspecific hybrid fruit (Moon and Meru 2018).

Objectives and Limitation

The objectives of interspecific crossing are (a) to develop resistance varieties by transferring desirable genes and (b) to manipulate chromosome for crop improvement. Linkage of undesirable characters, cross-incompatibility, hybrid inviability, hybrid sterility and hybrid breakdown are the major limitations of interspecific crossing.

8.6 Biotechnological Methods

8.6.1 Tissue Culture

Tissue culture can regenerate sweet gourd plants that are mostly true to type of the mother plant (Haque et al. 2010). The regenerable cells of a sweet gourd plant which can be used in tissue culture are the meristem, embryos, callus, pollen anthers, pistils and protoplasts.

8.6.2 Meristem Culture

In sweet gourd breeding, the meristem is widely used to regenerate plants through tissue culture. The meristem is a very efficient portion for the regeneration of virus free plants (Haque et al. 2010; Stipp et al. 2012). Due to the absence of a vascular

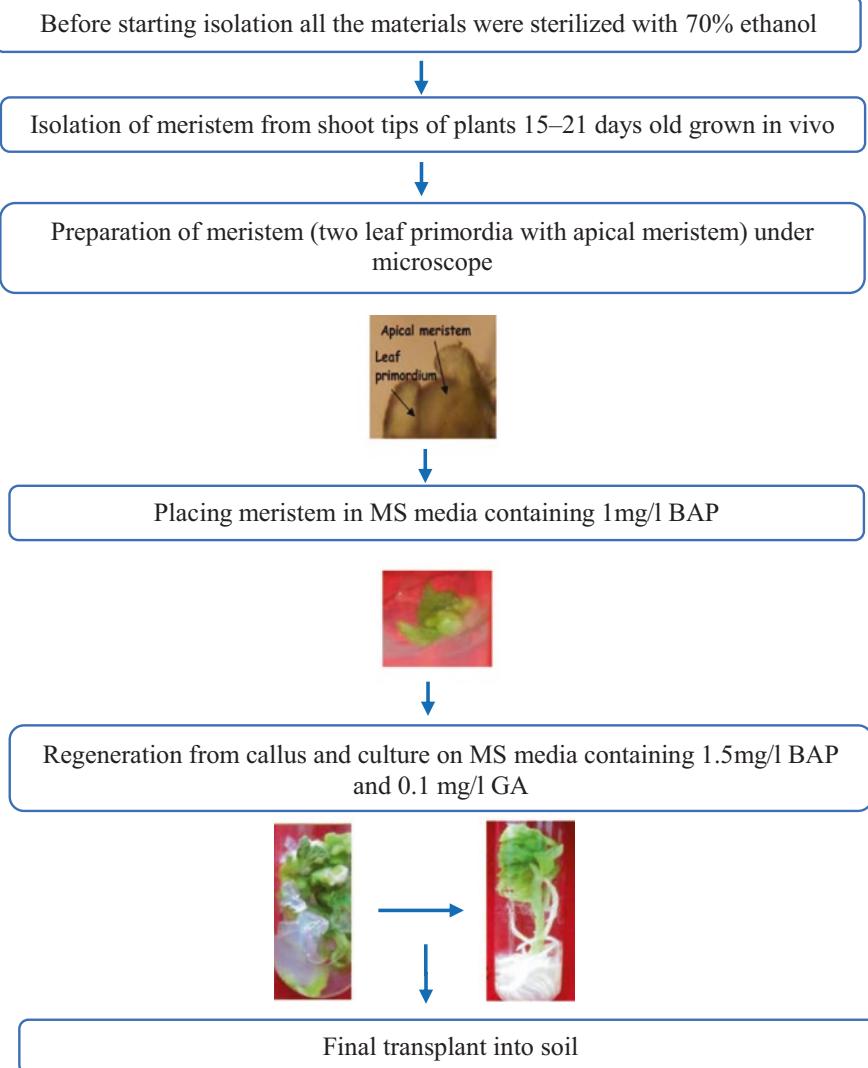


Fig. 8.13 Schematic diagram for production of plantlet through meristem culture. Source: Haque et al. (2010)

system, rapid cell division and lack of plasmodesmata in the meristematic tip, meristems remain free from virus. Meristem culture in sweet gourd can be carried out as shown in Fig. 8.13.

Objectives and Limitations

Meristem culture helps for rapid propagation and production of virus free plant in sweet gourd. The major limitation is the loss of characteristics of the original tissue. It also requires more technical knowledge and skill.

8.6.3 *Embryo Culture*

In sweet gourd, unique uniform lines can be provided by embryo culture. As sweet gourd is an open-pollinated crop, development of F₁ cultivars is highly time consuming and laborious. In vitro embryo culture is mainly done to obtain interspecific F₁ hybrids. Diploid and tetraploid plants from embryo culture were found by Kwack and Fujieda (1988). Successful regeneration of immature embryos from *Cucurbita maxima* × *C. pepo* and *C. pepo* × *C. Moschata* were created by Kwack and Fujieda (1988), Leljak-Levanić et al. (2004) and Mihaljević et al. (2011). Embryo culture of sweet gourd can be conducted as follows (Fig. 8.14).

8.6.4 *Somatic Embryogenesis*

The genetic constitution of the mother plant seems to play a key role in the somatic embryogenesis of sweet gourd. That is why embryogenesis from ovulesis commonly practiced in the plant recovery of sweet gourd. According to Kwack and Fujieda (1988), Leljak-Levanić et al. (2004), Mihaljević et al. (2011) and Kurtar et al. (2018) somatic embryogenesis can be practiced in sweet gourd in the following way (Fig. 8.15).

8.6.5 *Marker-Assisted Selection (MAS)*

Marker-assisted selection has gained a valuable place in the molecular breeding of sweet gourd. Genetic linkage maps establish a suitable arrangement for it. Construction of genetic linkage and consensus maps by phenotypic traits and QTLs has been carried out to develop DNA markers which are widely used for marker-assisted selection in sweet gourd breeding. To analyze genetic variations, molecular markers are convenient tools that effectively help to link phenotypic and genotypic variations (Kong et al. 2020). Brown and Myers (2002) conducted QTL mapping for leaf indentation and fruit shape using RAPD markers. Paris et al. (2003) tested AFLP and SSR in sweet gourd for diversity analysis among various lines and these markers may be combined into a gene mapping program of sweet gourd. Markers that are linked to the desirable fruit yield and quality traits can be used as a powerful selection tool in sweet gourd breeding (Sawazaki et al. 2018). Most sweet gourd traits can be mapped with RFLP, RAPD or AFLP markers (Meru et al. 2019). But before using a marker, it needs to be transformed into PCR-based user-friendly dominant or co-dominant marker. The conversion of markers often involves marker DNA sequencing from both parents, identifying polymorphic segments and designing PCR-primers according to the sequence difference.

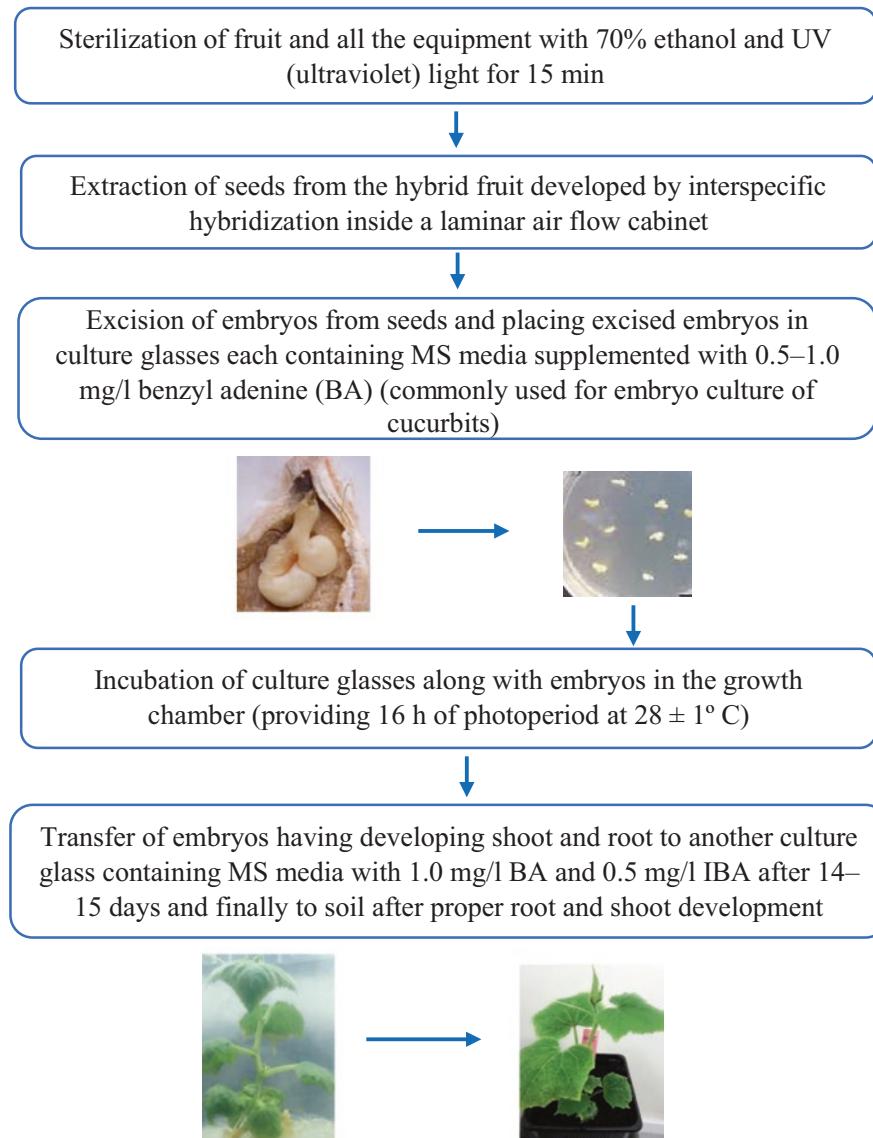


Fig. 8.14 Schematic diagram for production of plantlet through embryo culture. (Photo credit: A. K. M. Aminul Islam)

MAS can also be used as an effective tool to develop disease resistance in sweet gourd. The disease resistance genes act as excellent molecular markers to screen sweet gourd genotypes for disease resistance. Ramos et al. (2020) examined University of Florida breeding line #394-1-27-12 of *Cucurbita moschata*, which was resistant to *Phytophthora* crown rot, using three independent dominant genes

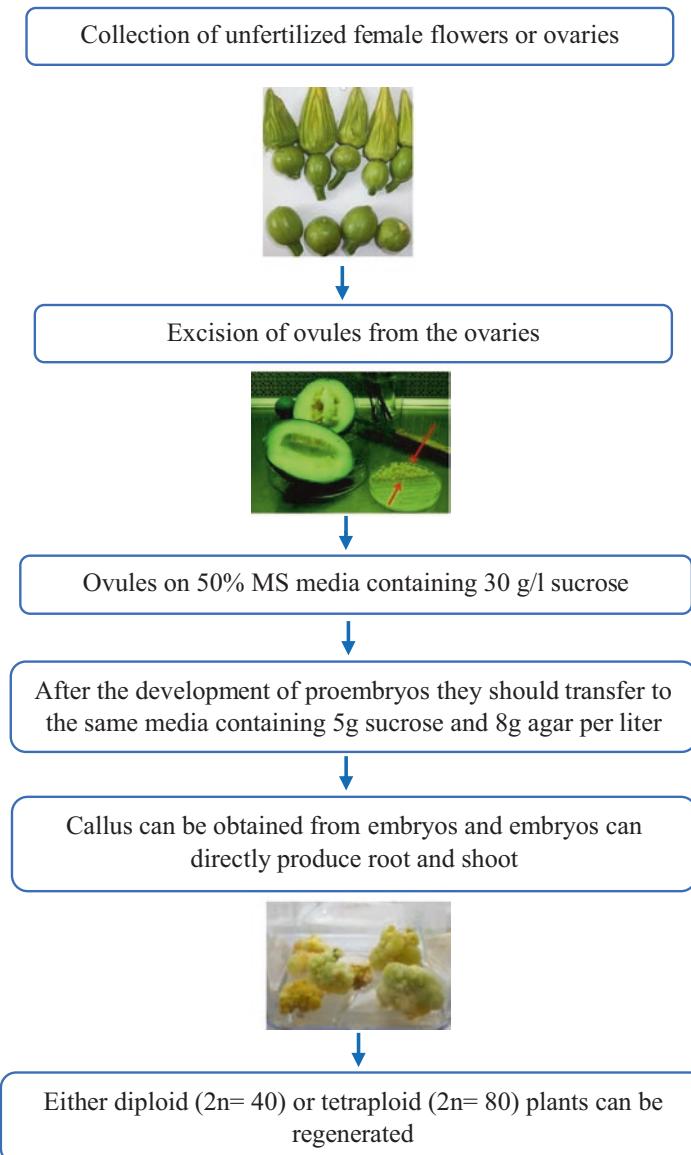


Fig. 8.15 Schematic diagram for production of plantlet through somatic embryogenesis. (Source: Kurtar et al. 2018)

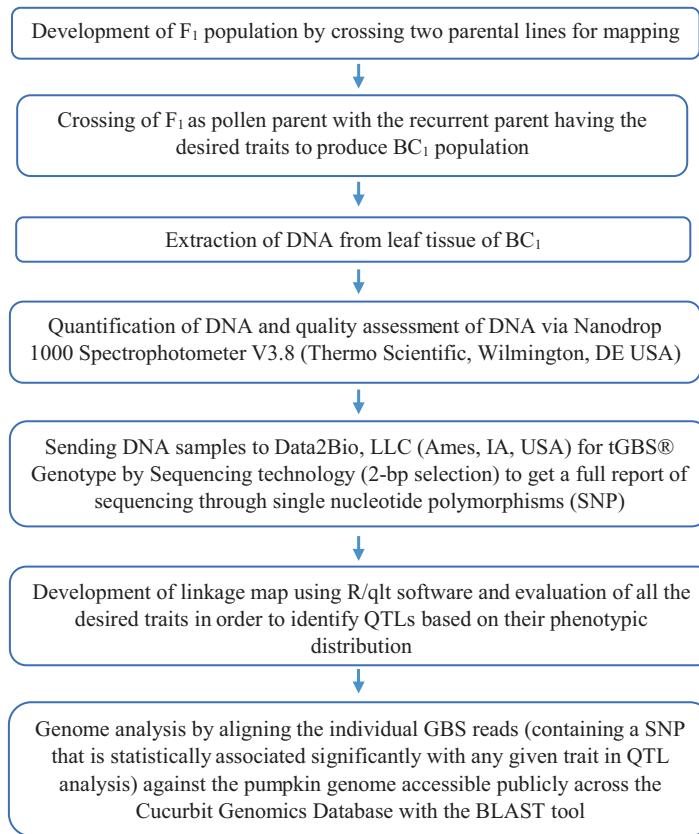


Fig. 8.16 Schematic diagram used to identify QTLs associated with fruit yield and quality traits of pumpkin using genotype-by-sequencing (GBS) method. (Source: Del Valle Echevarria et al. 2020; R Core Team 2020; Zheng et al. 2018)

(R1R2R3) and concluded that efficient breeding for *Phytophthora* crown rot resistance through marker-assisted selection (MAS) is possible through using DNA markers linked to R1R2R3 genes. Del Valle Echevarria et al. (2020) applied the genotype-by-sequencing (GBS) method to identify the QTLs associated with fruit yield and quality traits of *C. moschata* (Fig. 8.16).

QTLs linked with the depth of the indentation between primary leaf veins and shape of sweet gourd was identified by Brown et al. (2003). They also described the technique for extracting *Cucurbita* DNA with modified CTAB (hexadecyl-trimethyl-ammonium bromide).

8.7 Resistance Breeding

8.7.1 Disease Resistance

Attaining disease resistance is one of the major objectives of sweet gourd breeding. Sweet gourd is predominantly susceptible to various fungal, bacterial and viral diseases. Fungal diseases like powdery mildew, bacterial disease like *Phytophthora* blight and viral diseases such as cucurbit latent virus, squash leaf curl virus, watermelon mosaic virus, papaya ring spot virus, zucchini yellow mosaic virus and cucumber mosaic virus are common in sweet gourd. Backcross breeding is the most widely used breeding method to attain resistance to these diseases of sweet gourd. This is the single method of interspecific gene transfer. In this method the resistance gene is transferred to the commercially-cultivated species from the resistant donor species of sweet gourd through conventional approaches (Fig. 8.17). Researchers found different sweet gourd species resistant to different diseases and effective methods for transferring the resistant genes (Table 8.9).

8.7.2 Insect Resistance

It is difficult to breed sweet gourd for insect resistance. Very few breeding studies have been published on insect resistance in sweet gourd. Differences in susceptibility in sweet gourd cultivars and sources of resistance to a number of insects have been discovered by many researchers. Combined resistance to striped cucumber beetle, prickle worm and serpentine leaf miner were found by Hall and Painter (1968); resistance to red pumpkin beetle was observed by Grewal (1981) and resistance to silvery white fly by Wessel-Beaver and Katzir (2000) in several species or accessions of sweet gourd such as *Cucurbita moschata*, *C. pepo* and *C. maxima*.

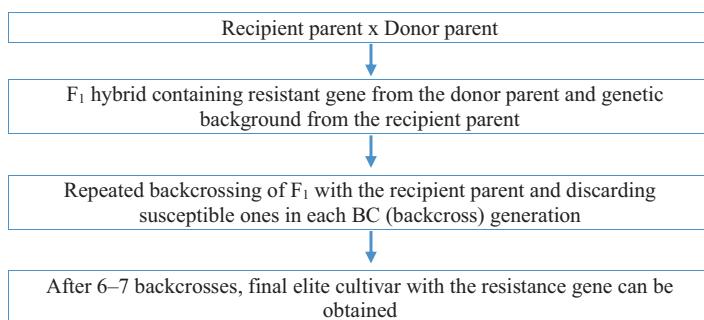


Fig. 8.17 Schematic diagram of gene transfer through backcrossing

Table 8.9 Sweet gourd species resistant to various diseases

Disease	Resistant species	Transferred to (cultivated form)	Breeding methods	References
Powdery mildew	<i>Cucurbita marintezii</i>	<i>C. moschata</i> & <i>C. pepo</i>	Backcross, marker assisted selection (MAS)	Contin and Munger (1977), Guo et al. (2020)
	<i>C. lundellina</i>			Rhodes (1964)
Papaya ring spot virus	<i>C. equadorensis</i>	<i>C. moschata</i> & <i>C. pepo</i>	Backcross	Herrington et al. (1989)
Zucchini yellow mosaic virus	<i>C. moschata</i> (wild form)	<i>C. moschata</i> & <i>C. pepo</i>	Backcross	Brown et al. (2003)
Cucumber mosaic virus	<i>C. pepo</i> (wild form)	<i>C. moschata</i> & <i>C. pepo</i>	Backcross	Pink (1987)
Watermelon mosaic virus	<i>C. maxima</i>	<i>C. moschata</i> & <i>C. pepo</i>	Backcross	Maluf et al. (1985)
<i>Phytophthora</i> blight	<i>C. martinezii</i> & <i>C. moschata</i>	–	Genomics studies	Quirin et al. (2005)

8.8 Conclusions and Prospects

Since Bangladesh gained independence in 1972, the country has made remarkable progress in the export of agricultural products including gourds. Still sweet gourd (*Cucurbita moschata*) is the least studied species among the *Cucurbita* species. It is most widely distributed and cultivated among the tropical and subtropical countries. It is highly polymorphic due to its pollination mechanism which makes it highly adaptable in the changing climate. However, little is known about genetics of different qualitative and quantitative traits such as plant habit, sex expression, fruit set, fruit shape, fruit size and fruit yield. Knowledge on genetics of yield related traits will help farming the breeding strategy of sweet gourd. The major objectives in breeding sweet gourd have been to improve fruit yield and quality of fruit flesh. The primary breeding method of sweet gourd in most tropical countries has been selection of inbred lines from available germplasm and interspecific crosses. Inbreds are used to develop hybrid varieties that have shown more desirable yield compare to open pollinated varieties. Development of gynoecious lines or male sterile lines in sweet gourd may help to improve hybrid development as well as hybrid seed production. Interspecific crosses, tissue culture, embryo culture, somatic embryogenesis techniques may also help to develop hybrid and to transfer desirable genes from other *Cucurbita* species. Modern biotechnological and molecular interventions are also needed to develop cultivars with multiple resistances against biotic and abiotic stress which will help new varieties cope with climate change.

Acknowledgements Authors are thankful to Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh authority for their support.

Appendixes

Appendix I: Research Institutes Relevant to Sweet Gourd

Institution name	Specialization and research activities	Address / Country	Contact information and website
Bangladesh Agricultural Research Institute (BARI)	Agricultural research, especially vegetables	Bangladesh	www.bari.gov.bd
Bangladesh Institute of Nuclear Agriculture (BINA)	Agricultural research with radiation technology	Bangladesh	www.bina.gov.bd
Bangladesh Agricultural Research Council (BARC)	Maintaining national agricultural research system	Bangladesh	www.barc.gov.bd
Bangladesh Agricultural University (BAU)	Agricultural research	Bangladesh	www.bau.edu.bd
Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU)	Agricultural research	Bangladesh	www.bsmrau.edu.bd
Bangladesh Rural Advancement Committee (BRAC)	Agricultural research	Bangladesh	www.brac.net
Advanced Chemical Industries Limited (ACI Limited)	Variable research	Bangladesh	www.aci-bd.com
Lal Teer Seed Limited	Agricultural research and quality seed development	Bangladesh	www.lalteer.com
Indian Agricultural Research Institute (IARI)	Agricultural research, education and extension	India	www.iari.res.in
Indian Institute of Vegetable Research (IIVR)	Research related to vegetables	India	www.iivr.org.in
World Vegetable Center (WVC)	International Institute for Vegetable Research	Taiwan	Avrdc.org
US Department of Agriculture, Agricultural Research Service	Extend scientific knowledge of agriculture and solve problems	USA	www.ars.usda.gov
Institute of Agrifood Research and Technology	Food technology	Catalonia	www.irta.cats
Institute for Vegetable Crops	Research institute attributed for scientific work	Serbia	www.institut-palanka.co.rs

Appendix II: Genetic Resources of Sweet Gourd

Cultivar name	Important traits	Cultivation location
BARI mistikumra-1	Early winter variety, 5–6 months storability of fully mature fruit tolerant to papaya ring spot virus (PRSV)	Bangladesh
BARI mistikumra-2	Year round variety, tolerant to papaya ring spot virus (PRSV), powdery mildew and cucumber mosaic virus	Bangladesh
Baromashi	Early matured and high degree of femaleness	Bangladesh and Uganda
Sweety F ₁	Tolerance to heat, salinity and viral disease	Bangladesh
Maya F ₁	Day neutral, year round	Bangladesh
Atlantic giant	Maximum number seeds are found	Belgium,Germany,South Africa,Spain
Baby blue	Excellent storage and good for roasting, soups	USA
Banana, pink	Excellent storage and good for industrial product	Mexico
Buttercup	Flavor and taste is excellent	Japan
Golden turban	Used as an ornamental plant	Northeastern united states
Hubbard, blue	Taste is excellent	South America
Queensland blue	Sweet flavor lasts months in storage	Australia
Hokkaido, green	Storage capacity is good	Japan
Hubbard, baby	Attractive, flavorful variety for gardeners and commercial growers	Japan
Kuri, blue	Taste is good	America
Boston marrow special	Delicate nutty flavor, good quality	Native America
Sweet keeper	Decorative as well as flavorful	Australia
Alagold	Excellent storage capacity	Africa
Butternut	Taste is sweet	Australia and New Zealand
Seminole	High productive	Central America
Dickinson	Sweet with high flesh	Central America
Butterbush	Aromatic nutty flavor	American
Chirimin	Pretty and decorative	Japan
Kentucky field	The industry standard for canning pumpkin and feeding to animal	Developed by the Asgrow company
Tahitian	Taste is excellent	Mexico
Aehobak	Thin, smooth skin and more delicate flesh	Korea

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Chapter 9

Breeding of Melon (*Cucumis melo* L. Groups Dudaim and Flexuosus)



Forouzandeh Soltani

Abstract Cultivated melons have spread through trade and exploration from centers of origin in Asia to Europe and the Americas and are important in local, regional and world trade. Iran ranks second in world melon production (*Cucumis melo* L.), generating over 1.7 million mt in 2018. Local farmers prefer to cultivate melon groups based on yield, fruit sweetness, firmness and color. However, some groups like Dudaim and Flexuosus have high fragrance and adaptation to warm and dry season; and are distributed in different culture regions. Since standard melon cultivars have evolved and been introduced, most commercial types exhibit variable, late female flower production and, consequently, late fruit ripening which are encountered with unfavorable condition of late season ripening, disease susceptibility and cucurbit fly as a critical problem of field melon production. Melons are cross-pollinated crops without inbreeding depression and are well suited to heterosis breeding. Furthermore, access to monoecious or gynoecious lines in commercial and popular melons with andromonoecious sex expression could reduce time and cost for emasculation and accelerate hybrid production. Priority of melon breeding should be given to produce multiple disease resistance, fruit size and uniformity, fruit quality attributes (color, mild aroma, firmness, thickness) and good shelf life for storage to reduced postharvest loss. This chapter reviews and explores the past, present and future prospect of melon breeding relative to Flexuosus and Dudaim Groups as genetic resources, flower and fruit attributes and also organic volatile compounds.

Keywords Breeding · Diversity · Domestication · Genomic · Hybrid · Melon · Molecular marker

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9.1 Introduction

Melon (*Cucumis melo* L.) belongs to the Cucurbitaceae family which includes many edible and non-edible plants that have long been cultivated in eastern and western Asia. Iran is among the primary melon producing countries and one of the main centers of origin and domestication which contain rich genetic diversity of melon germplasm. Approximately, 73% of Iranian territory consists of arid and semiarid climatic areas with an average annual rainfall is 240 mm and sunny, warm weather from late spring to mid-September. These climatic conditions are suited to melon cultivation and create variations in fruit ripening and harvesting time, skin and flesh volatile compounds, presence or absence of suberized net tissue of the fruit skin, sweetness and color.

Besides the fragrance and aromatic properties of melon, the water, antioxidants, vitamins, polyphenols and minerals as functional food make it a popular fruit throughout the world (Rolin et al. 2019; Vella et al. 2019). Melon fruit contains important bioactive compounds, mainly in the peel which has a high content of antioxidants which are of interest in the food, cosmetic and pharmaceutical industries (Gomez-Garcia et al. 2020). The high consumption of melon accompanied with a large amount of postharvest loss, especially in developing countries, make it necessary, as a main goal of breeding programs, to create varieties with long shelf life and good storability. Furthermore, climate change could modify water deficiency and salt accumulation along with rising disease infections, such as powdery mildew, *Fusarium* wilt and viruses, pushing scientists to consider local landraces and their use to introduce new traits into modern melon cultivars for genetic improvement (Dhillon et al. 2012; Islam et al. 2020).

With respect to traditional breeding methods, biotechnological tools such as RNAseq, which enables the identification of resistance genes to *Phytophthora capsici* L. (Wang et al. 2020), can enhance the efficiency of breeding programs. As cucurbits are model species in different areas of fruit ripening, floral biology, volatile compounds and other fruit quality attributes, these could also be used by the scientific community for melon improvement. This chapter presents a detailed outline of melon's importance, breeding methods, floral biology, genetic resources with respect to Flexuous and Dudaim groups, and genomic and biotechnology tools to improve melon breeding.

9.1.1 Origin and Distribution

The most common name for *Cucumis melo* is melon. Other names include sweet melon, round melon, muskmelon, casaba, cantaloupe and winter melon (Nayar and Singh 1998; Robinson and Decker-Walters 1997). Melon belongs to the gourd family (Cucurbitaceae) which has 120 genera and around 1000 species, including numerous crops, such as cucumber (*Cucumis sativus* L.), bitter gourd (*Momordica*

charantia L.), watermelon (*Citrullus lanatus* Thunb), preserving melon (*Citrullus amarus* Schrad), squash and zucchini (*Cucurbita pepo* L.), and bottle gourd (*Lagenaria siceraria* L.) (Renner and Schaefer 2016; Schaefer and Renner 2011). Melon is divided into two subspecies, *C. melo* ssp. *agrestis* and *C. melo* ssp. *melo*, differentiated by the pubescence on the female hypanthium; ssp. *melo* has spreading hairs, and ssp. *agrestis* appressed hairs (Kirkbride 1993).

In ancient reference books, based on Marco Polo's writings, dried melon is mentioned as a special melon with high sugar content in Iran and the way people sliced them, dried them in the sun and then packed them in baskets just like Malaga figs are treated (Pitrat 2008). Archeological remains indicate that melon was cultivated in Iran as early as 3000 BC (Robinson and Decker-Walters 1997) and over time many cultivars representing high fruit diversity have been created. Some botanists believe that the origin of muskmelon is Persia (Iran) or Central Asia, while others report that the origin is uncertain and is a complicated issue.

Looking back, based on historical records under Darius the Great, the Persian Empire, which stretched from Europe to the Indus River Valley in northwest India and south to Egypt, and known as Persia, it can be concluded that melon originated and was domesticated in Persia. Persian melons are known for their firmness and long shelf life and therefore considered as promising genetic material for breeding programs and crossing with other *Cantalupensis* type melons to create hybrids that produce firmer fruit, good shipping qualities and longer shelf life.

In Iran, curcurbits are considered to be the most important horticultural crops and the amount of total production is about 7.3 million mt (FAO 2018), more than 50% of total vegetable production. Among them melon is the most important, and various groups of landraces and some hybrid cultivars are grown. The most important ones are the sweet type melon of Groups *Cantalupensis* and *Inodorous*, with production at about 2.2 and 1.9 million mt, respectively. They are mainly cultivated in the eastern and southern parts of Iran, where the climate is semiarid and warmer than western and northern parts of the country. In areas with less rainfall, underground water is used to irrigate melon fields (Soltani et al. 2010). The muskmelons, or *Reticulatus* Group, have a reticulate (corky or *netted*) rind and are the most widely grown. Muskmelon is native to Persia (Iran) and adjacent areas to its west and east. *Musk* is a Persian word for a kind of perfume; melon is French, from the Latin *melopepo*, meaning *apple-shaped melon* and is derived from Greek words of similar meaning. The cantaloupes, or *Cantalupensis* Group, are similar but have a smooth or warted rind and are popular in parts of Europe.

Other groups of melon, such as *Flexuosus* and *Dudaim*, are also cultivated in Iran. *Flexuosus* is known as Armenian cucumber, as well as other names, such as snake cucumber and snake melon. The fruit is usually slender, almost 1 m long and 8 cm in diameter, and is typically bent and twisted. The fruit changes to yellow when ripe, at which time it has a strong muskmelon aroma. Actually, their flavor, fruit color and shape is generally closer to cucumber (Soltani et al. 2010). The shape of seeds is more like muskmelon than cucumber, but they are rather slender like cucumber seeds. Growers pick the fruits at a size of 20–25 cm for their best taste and high quality and consumed as raw vegetable for salad and for medicinal use.

Growers formerly planted snake melon in the hot, dry seasons in arid and semiarid regions as a good alternative for cucumber under such conditions. Today it is grown in spring and summer, sown in April to the first week of May and harvested from June to September, according to local climatic conditions. Snake melon is also grown in other countries from Africa to India in Asia (Pitrat et al. 2000) and in Greece (Staub et al. 2004). The Dudaim, or Queen Anne's pocket melon, is a special melon traditionally cultivated in Iran. It is characterized by small reddish yellow fruit with ochre stripes, and a round or slightly oval shape with velvety skin. Dudaim melons are not sweet, but are valued for their high fragrance, ornamental beauty and medicinal properties (Soltani et al. 2010). In some parts of Iran, as a main center of cultivation and diversification, farmers grew almost 789 ha of Dudaim melon, with a total production of 15,000 mt in 2016. The fruit matures 1 month after pollination and detaches from the plant when ripe and also has stripes which change color as the fruit approaches maturity (Hatami et al. 2019). The dark-green stripes become intense orange, maroon or brown and the light-green stripes become intense yellow (Paris 2012).

Muskmelons, or Persian melons, are the most popular in Iran. This species of melons has yellow-white flesh with small yellow seeds. Its taste is sweet, flavorful and semi-juicy. The outer rind is yellow, thin with light-green vertical stripes covering its outer surface. Its shape is long and elongated or circular and its weight exceeds 1 kg. Muskmelons are an excellent source of vitamins A and C, and a good source of potassium. Therefore, reportedly they can help or treat some diseases. Persian melons are best utilized in fresh preparations.

9.1.2 *Cultivation Practices*

Melon and other cucurbits are propagated by seed and need warm temperatures (25–30 °C) to induce germination. Because its domestication occurred in arid and semiarid areas, it is adapted to hot and dry conditions and needs plenty of sunshine. Muskmelons may be planted by direct seeding or from greenhouse transplants after environmental condition are favorable and frost danger is passed (Welbaum 2015). In the past local farmers planted melons in deep furrows to minimize water deficit and to offer some disease control (Fig. 9.1a). Their season runs from April–May through August–September, according to the variety and climate conditions.

Direct seeding in traditional culture of some Iranian melons (Kharbuze) is done by planting 6 seeds per hill (then thinned to 2–3 plants) with hills 1.5–2 m apart, and 1 m between plants to allow the vines to extend. Plastic mulch is also used to conserve soil moisture and reduce weed growth. In Iran, most commercially-grown melons are produced with plastic mulch and drip irrigation (Fig. 9.1b). Plastic mulch may be effective, but has the disadvantage, compared to organic mulch, of not being biodegradable. It must be removed and disposed of in landfills.



Fig. 9.1 (a) Traditional method for melon irrigation, (b) Drip irrigation of melon without plastic mulch, (c) With mulch in a field of the Research Station of Horticultural Science, University of Tehran. (Photo by Forouzandeh Soltani)

9.2 Floral Biology

Cucurbits such as melons produce male, female and bisexual flower with respect to their genotype and environment. Remarkably, different sexual types also coexist within the melon species (Revanasidda and Belavadi 2019). Although the usual common sex type of commercial and most of Iranian melon cultivars is andromonoecious, however, germplasm screening of Iranian genotypes showed that most of the snake melon (*Cucumis melo* Group Flexuosus) expressed male and female

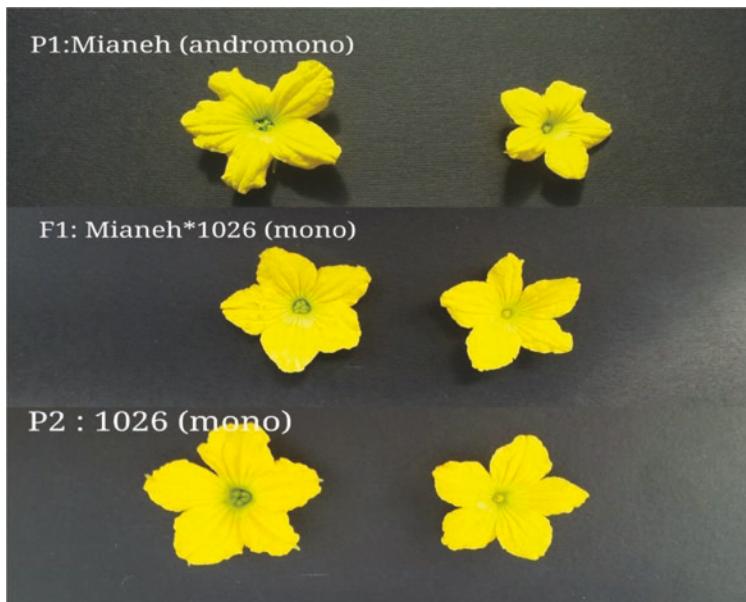


Fig. 9.2 Cross between andromonoecious (P1) and monoecious (1026 as parental (P2) and (F1) with female flower. (Phot courtesy of Moein Shajari, Horticultural Science Department, University of Tehran)

flower separately on a plant (unpublished data). The scientific and economic interest of sex expression, besides the fruit quality and ripening characters, are very important issues. Crossing 6 andromonoecious inbred line with 1 monoecious type, all F1 hybrids exhibited undeveloped stamen and female sex expression (Fig. 9.2). Selected hybrids continued by the backcross method exhibited a relative frequency of femaleness in the evaluated population, as shown in Table 9.1.

Today, andromonoecious types are being replaced by new hybrid varieties that tend to be monoecious because of the agronomic advantages this provides, as parental lines do not require hand emasculation to produce F1 hybrids and thus have gradually become an important goal for melon breeding. At least three genes, *G* (gynoecious) *M* (monoecious) and *A* (andromonoecious) contribute to sex determination in *Cucumis melo*, depending on the combination of different sex phenotypes appearing on the plant (Grumet and Katzir 2007; Grumet et al. 2017).

9.2.1 Pollination

Melon is a cross-pollinated crop with monoecious or andromonoecious flowering habits; honey bees are the chief insect pollinators. However, the andromonoecious sex type can shed a considerable portion of pollen on its own stamen providing the

Table 9.1 Segregating generation of sex expression of (andromonoecious x monoecious) and their BCs populations

Generation name	Andromonoecious	Monoecious
Khatouni (p1) Inodorous Group	100	0
Ut.92.1026 (p2) Cantalupensis	0	100
p1*p2	25.2	74.8
P2*p1	94	6
BC1(P1,F1)	50	50
BC2(P1*BC1)	27	73
F2	49	51
F3	40	60

Courtesy: Franoosh Yosefi, Horticultural Science Department, University of Tehran

probability of selfing. Each flower should be visited by a minimum of 8–10 bees to achieve the same level of pollination efficiency. For hybrid seed production it is better to allow an isolation distance of 1500 m because of the large, yellow flowers which attract bees. Anthesis takes place at around 0530 h and stigma is receptive 12 h before the day of anthesis. Anther dehiscence occurs between 0500 and 0600 h. (Revanasidda and Belavadi 2019); therefore, pollination must follow in the early morning 0600–1100 h to achieve maximum fruit set, considering climatic and regional conditions. To ensure a greater pollen load in hybrid production, 3–5 anthers should be transferred for cross-pollination, even in the greenhouse, for fruit production.

9.2.2 Selfing and Crossing Techniques

Melons can be considered as a self-pollinated or cross-pollinated crop. Inbreeding depression has not been reported in melon and most varieties have been developed by selection and controlled inbreeding (Rosa 1927; Scot 1932). One day prior to anthesis in the andromonoecious type, flowers are emasculated and covered to prevent contamination by insects, then the male flowers, before opening, are placed on emasculated flower for pollination and follow up fertilization (Fig. 9.3). In successful crossing the ovary grows and remains green vs. turning yellow without further growth, an indication of unsuccessful fruit set.

9.3 Genomics of Melon

Melon (*Cucumis melo*) is an eudicot diploid plant species ($2n = 2x = 24$); its genome size is 454 Mbp. The melon genome sequence data (Melonomics) was assembled and annotated in 2012 (Garcia-Mas et al. 2012). The last data from Ruggieri et al.



Fig. 9.3 Crossing in melon. (a) Selecting the proper flower, (b) Emasculation, (c) Pollination, (d) Covering with gelatin capsule. (Photo courtesy of Tahereh Javanmard, Horticultural Science Department, University of Tehran)

(2018) indicated that assembly (v3.6.1) of the melon genome and a new genome annotation have improved and more than 8000 new genes were identified, one third of them being well supported by RNA-Seq data. This work considerably increases the reliability of the melon genome assembly and resolution of the gene models, paving the way for further studies in melon and related species (www.melonomics.net/melonomics).

The chloroplast genome was obtained from the whole-genome sequence reads; it is 156 Kb in size, and includes 132 genes and is 95% similar to the cucumber chloroplast sequence. The melon chloroplast gene coding regions contain 75 protein-coding genes and 6 conserved ORFs, 4 rRNA genes and 30 tRNA genes, which represent 51.6, 2.9 and 5.2%, respectively, of the total sequence. The melon mitochondrial genome is 2.74 Mb in size, one of the largest among the cucurbits, and includes 51 protein-coding genes and a large fraction of repetitive sequences and DNA of nuclear origin. Due to the repetitive nature of the mitochondrial genome, its sequence was obtained after the purification and isolation of mitochondria (Rodriguez-Moreno et al. 2011). Melon mitochondria is different from other crops because of its paternal inheritance and 1.7% of total sequences belong to

coding regions containing 51 protein-coding genes, 4 conserved ORFs, 3 rRNA genes and 24 tRNA genes. There are 1.41% similar regions between mitochondrial and chloroplast genomes and 46.47% with nuclear in melon (Rodriguez-Moreno et al. 2011; Shen et al. 2019).

9.3.1 Traditional Breeding Methodologies

No particular breeding method is best for melon, many have been used, and more than one may be used in pursuing any objective. Conventional plant breeding has been based on fruit characters such as size, firmness, sweetness and skin odorant and they still are characters for elite parent selection. In Iran, with the largest genetic diversity among others, vegetable crops provide opportunities for breeders to develop new, improved varieties (Kalloo and Bergh 1993). Useful new melon seed introduced from other melon-production countries and cultivated across the southwest, central and northeast parts of Iran are used directly or recommended for release after minimum selection for commercial production.

9.3.2 Mass Selection and Pure Line Selection

Mass selection and pure line selection are the most ancient breeding approaches in which desirable plants are selected from genetically variable populations. Local accessions are the target for such a selection procedure (Acquaah 2012). Some melon accessions may be domesticated and adapted as cultivated over time in a certain Eco geographic area such as Khorasan province, for some of melon varieties from the Inodorous Group. Local farmers consciously and unconsciously select similar looking plants (mass selection) to improve the resilient genetic stocks. Selection of melon through seven to nine generations leads to produce interesting inbred lines. Reciprocal full-sib selection and selective diallele mating schemes generate variability for population improvement and inbred development (Andrus and Acquaah 2016). Pedigree selection permits the development of unique genotypes from parents possessing desirable traits (Andrus and Bohn 1967; Kalloo and Bergh 1993).

9.3.3 Hybridization

The mating or crossing of two plants or lines of dissimilar genotypes are known as hybridization, which is used to create genetic variability. Therefore, it is often necessary to combine desirable traits from different parental lines into a single plant by hybridization (Acquaah 2012). Some target traits for hybridization in melon often

advocated in breeding programs relate to enhanced fruit uniform shape and maturity, high yield, small seed cavity, fruit quality traits such as flesh and skin thickness, total soluble solid (TSS), color and texture, firmness, presence or absence of netting on the skin and sugar content. Hybrids should be resistance to the more important biotic stress such as powdery mildew, *Fusarium* wilt mosaic virus and curcurbit fly (Nakazumi and Hirai 2004).

9.3.4 Heterosis Breeding

Heterosis is the case when an F1 hybrid resulting from a cross between two inbred lines exhibits increased performance in comparison to the average performance of both parents mid-parents value. This state is the ultimate goal for F1 seed breeders as it is the complete opposite case to inbreeding. Therefore, average heterosis percentage (compared to mid-parent values) and useful heterosis percentage (compared to the best parent values) are very useful breeding indicators and both are considered in breeding programs, depending on breeding aims.

Newly-created inbred lines should be tested in order to determine which ones will deliver heterosis in the F1 generation. Heterosis is the state of maximal heterozygosity where the maximum number of alleles is obtained by crossing genetically different inbred lines. Since the tested inbred lines could be genetically similar, it is necessary to test the combining ability of the new lines, as the breeding value of the line is estimated based on the heterosis it gives in combination with other lines. Additionally, the final estimation of the value of even more carefully selected inbred lines is based on their results in hybrid combinations. There are general and specific combining abilities where general combining ability (GCA) is the average value of an inbred line based on its behavior in crosses with other lines, while specific combining ability (SCA) is the value of the line in a specific cross where the F1 progeny illustrates superiority over mid-parent or even best parent value. There is general agreement that GCA and associated heterotic effects are important determinates of parental choice during hybrid melon development. Moreover, heterosis, as a function of performance, is often related to the degree of genetic relatedness [i.e. genetic distance (GD)] and is dramatically influenced by environmental conditions (Fasahat et al. 2016; Napolitano et al. 2020).

In melon, monoecious lines used as female parents illustrate interesting heterosis results for earliness, total yield and yield-related traits, especially that the time required for crossing in these types is reduced by more than one-half and fruit set is highly successful in comparison to andromonoecious variety breeding (Kesavan and More 1991). Additionally, many breeding programs illustrate cases of heterosis in many characteristics such as yield, total fruit yield per plant, plant length, number of branches per plant, flowering date, maturity date, fruit number per plant, acceptable yield, average fruit weight, in addition to fruit characteristics such as fruit flesh thickness, fruit shape index, fruit netting degree, fruit skin color, total soluble solids, moisture content, β - carotene, vitamin C and total sugars (Duradundi et al. 2018;

Kamer et al. 2015). Some Iranian melon inbred lines illustrate heterosis in various yield traits such as average fruit weight, total yield and marketable yield (Shajari et al. 2021), although the inbred line Khatouni illustrates superiority in F1 progeny for yield, fruit quality and postharvest-related traits (Alabboud et al. 2020).

As part of research on melon breeding using collected germplasm, 43 different types of melon genotypes were planted to evaluate characteristics of fruits and flowers and then selected genotypes were self-crossed for inbred line production. The 7 selected parents were crossed in a diallel scheme to obtain all possible combinations of 21 hybrids, 21 reciprocals and 7 parents during 2016–2017. The conventional crossing method was carried out by emasculating female flowers and isolating both male and female flowers the day before pollination, and pollinating the following day as described in (Darrudi et al. 2018). After fruit ripening and harvest, seeds were extracted and stored for use the next year. In the second season (spring-summer of 2017), the seeds of both the parents and F1 hybrids were planted in a randomized complete block design (RCBD) with 3 replications and 6 plants per replication (Fig. 9.4).

Whole and cut fruit characters and flower traits (Fig. 9.5) were recorded in addition to the storability of some hybrids and parents, compared under different storage temperatures. The significant GCA for yield and fruit trait refers to the tendency of introducing new melon hybrids with desirable characteristics, taking into consideration the significantly positive SCA observed in many hybrids (Alabboud et al. 2020; Javanmard et al. 2018). Our results showed that the GCA/SCA ratio for yield, fruit number and fruit weight traits were 0.74, 0.92 and 0.79, respectively; thus, corroborating the results reported by Pouyesh et al. (2017). Barros et al. (2011) refer to the importance of additive gene effects in explaining the variation in yield trait for the evaluated melon lines.

Ripening behavior in melon ranges from typical climacteric types with a high ethylene production, such as *Cucumis melo* var. *cantalupensis* with a fast ripening and short shelf and storage life to non-climacteric melon types, such as *Cucumis melo* var. *inodorus*, which are unable to produce autocatalytic ethylene, generally



Fig. 9.4 Dialle cross and selfing of some selected parents for breeding program. (Photo courtesy of Majid Esmaeili, Research Station of Horticultural Science Department, University of Tehran)

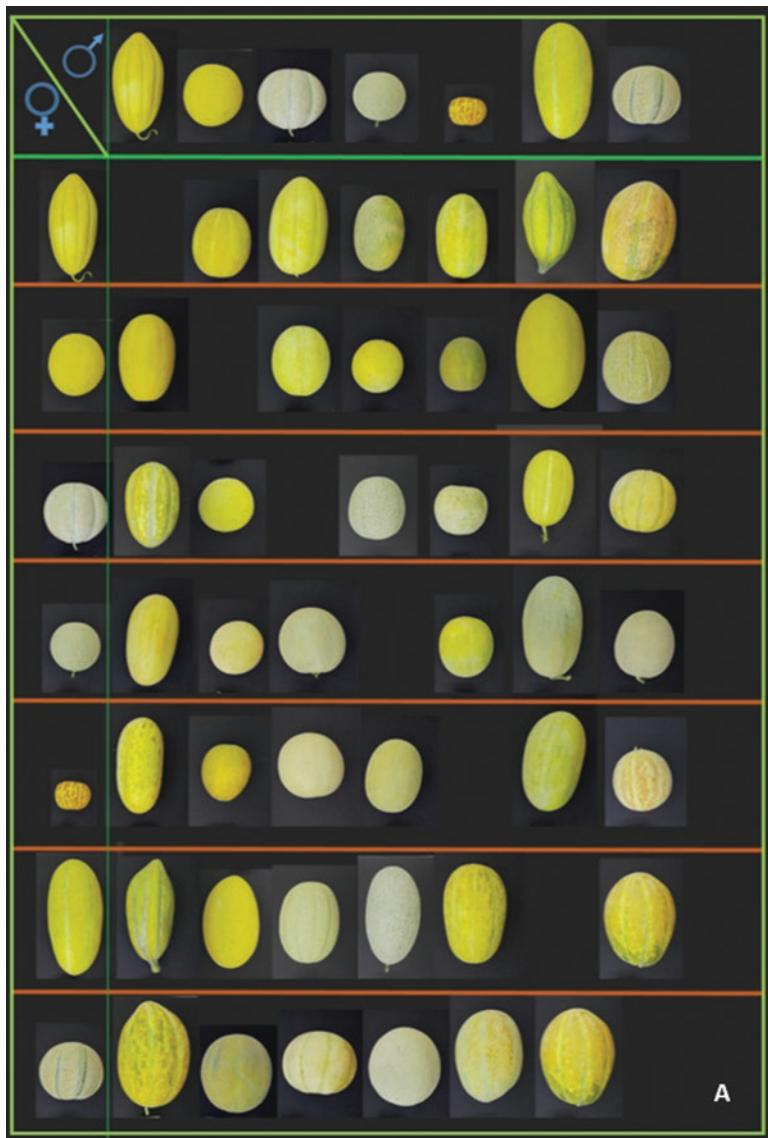


Fig. 9.5 Melon flower shapes resulting from various crosses. (a) Full diallel crossing of selected melon parents, whole fruits view. Source: Alabboud et al. (2020), (b) Full diallel crossing of selected melon parents, shown by male and female flowers. (Photos courtesy of Moein Shahari, Horticultural Science Department, University of Tehran)

have a slow ripening rate and are associated with a long shelf and storage life (Pech et al. 2008).

In Iran and other developing countries postharvest melon losses can reach 70% because of the lack of postharvest infrastructure to store and to retail the

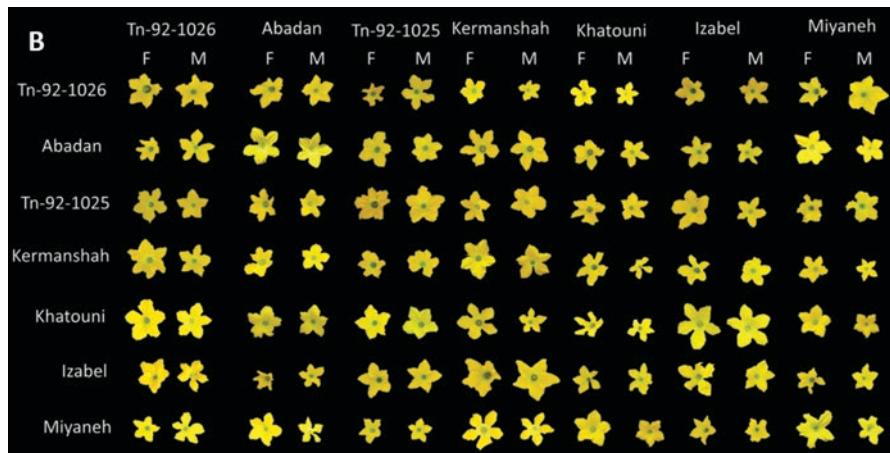


Fig. 9.5 (continued)

commodity; therefore, introducing new melon types with suitable firmness, texture and sugar content for the expanding fresh cut fruit industry and more suitable for long transportation and an extended storability are considered most important in melon breeding programs (Lester et al. 2007; Pitrat 2008). Furthermore, increasing the firmness of cantaloupe types by breeding with Inodorus types would be of interest (Perpiñá et al. 2016). Therefore, in our study the significant GCA with the high GCA/SCA values for TSS in addition to flesh firmness, thickness and flesh-to-cavity ratio presented in the current study showed the capability of introducing new types with such traits.

Similar to Pouyesh et al. (2017) results, GCA/SCA ratio for fruit length to diameter ratio found that this trait is strongly controlled by the additive genetic effect rather than dominant gene effect with a great potential to control this trait using classic hybridization methods with melon types of different fruit shape indices.

9.3.5 Backcross Method

Typically, improvement of an existing variety or type is sought through the addition of one or more specific characteristics, such as disease resistance. Backcrossing is used to introgress a character from a donor parent which is often horticulturally undesirable into an acceptable variety. The superior inbred is referred to as the recurrent parent. Often, six generations of selection and backcrossing to the recurrent parent are used to recover the genotype of the recurrent parent (except for the addition of the new trait) without the other undesirable traits from the non-recurrent (donor) parent. Two versions of the backcross method are used depending on whether the gene of interest is recessive or dominant (Kumar 2006).

Given the current importance of numerous diseases to melon production and the occurrence of disease resistance genes in wild melons, backcrossing may be the most common breeding method to use. The first example of backcrossing in melon is the variety PMR 45 (Chopra 2008; Pryor et al. 1946; Stoskopf et al. 1993). Backcrossing is simple when the trait transferred is conditioned by a single dominant gene, but becomes complex for traits conditioned by one or more recessive genes.

Introgression line (IL) generation is an excellent breeding strategy for incorporating exotic natural variation into modern breeding programs. To investigate the volatile compound inheritance and find the marketable odorant population with high metabolite content, selected parents are used to generate an IL population where the cultivar of the Inodorous Group, Khatouni, is the recurrent parent (odorless) and a genotype of Dudaim Group as donor parent. It is worth mentioning that the Iranian commercial melons such as the inbred line has numerous fruit qualities like crisp texture and high sugar content, and Dudaim types are popular for their flavor and aroma compounds. In F1 and BC1 populations, fruit skin thickness is reduced and organic volatile compounds increase more than in the recurrent parent (unpublished data). Fruits obtained from the BC2 population showed more similarity to the recurrent parent with thicker skin and obvious strips and netting of the skin and more odorant. These results show that backcrossing can improve metabolite and flavor content of odorless Inodorus; however, there is a need for more investigation of subsequent generations (unpublished data).

9.4 Germplasm Biodiversity and Conservation

Melon germplasm serves as a source of valuable genes. Local landraces and accessions are primary sources of genetic variation due to the evolution of crops over time under different climatic conditions. Ex situ conservation of melon germplasm ensures maintenance and accessibility of the materials, documentation, descriptors and properly recorded characterization. In local agricultural institutes within the melon growing areas of Khorasan, Sistan and Baluchestan, Fars and Alborz provinces there are gene banks of local landraces. However, some melon groups, such as Dudaim and Flexuosus, are threatened with genetic extinction because of the shift to hybrid melon production.

Many different classes of DNA markers, including nuclear and plastid, have been used to evaluate melon germplasm. *Cucumis melo* exhibits a high level of polymorphism in simple sequence repeats (SSR) (71%) Katzir et al. (1996) and restriction fragment length polymorphisms (RFLP) (33%) Neuhausen (1992). The development of microsatellites in melons was initiated by Katzir et al. (1996) and since many microsatellite markers have been used to differentiate elite melon germplasm (Garcia et al. 1998; Katzir et al. 1996) and Spanish melon landraces (Danin-Poleg et al. 2001; Lopez-Sese et al. 2002). From genetically-enriched libraries Ritschel

et al. (2004) identified 65 SSR markers that revealed DNA polymorphisms within the melon germplasm.

Feyzian et al. (2007), Kohpayegani and Behbahani (2008) and Raghami et al. (2014) estimated the genetic diversity and relationship among different kinds of melon collected from different parts of Iran. They roughly divide geographical groups of accessions by using cluster analysis, and indicated genetic differences between local landraces and improved cultivars. However, most of the studies focused mainly on the Inodorus Group because of its economic importance and less attention has been paid to other endemic groups. In Iran, a large genetic pool of Flexuosus and Dudaim germplasm is available. A study on four groups of melon including, Inodorous, Cantalupensis, Flexuosus and Dudaim, by morphological and molecular markers, revealed that a closer genetic affinity of Flexuosus, Cantalupensis and the Inodorus groups (Fig. 9.6) which suggests that random crossing between groups has been going on for years in Iran (Soltani et al. 2010). There were clear differences among accessions in relation to leaf and fruit characters; oriental melon is separated from other groups (Fig. 9.7) in addition to fruit yield. Flexuosus fruit harvested when they are immature, with the presence or absence of bitterness, was varied among them. Bitterness is related to triterpene (cucurbitacin) which is also

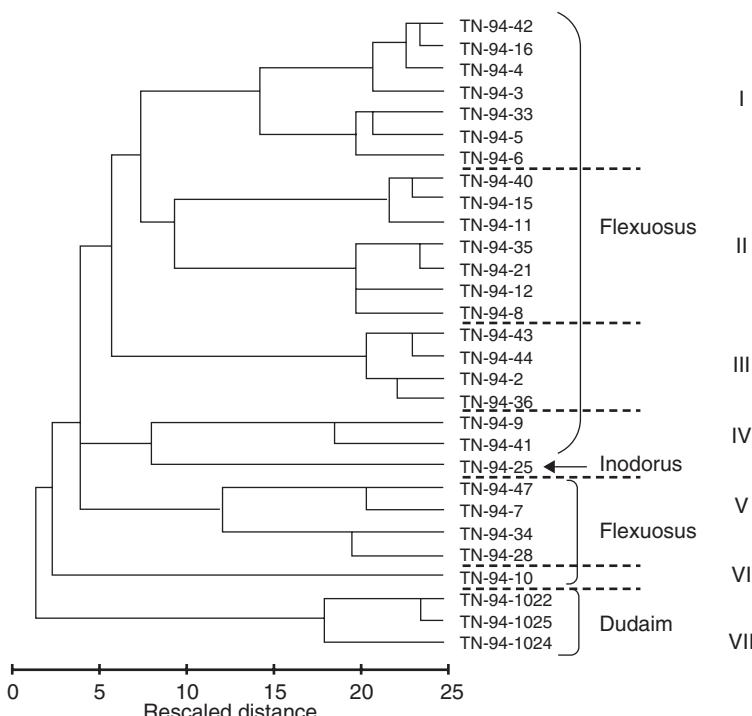


Fig. 9.6 Cluster analysis of morphological and physiological traits of Iranian melon. (Source: Soltani et al. 2010)

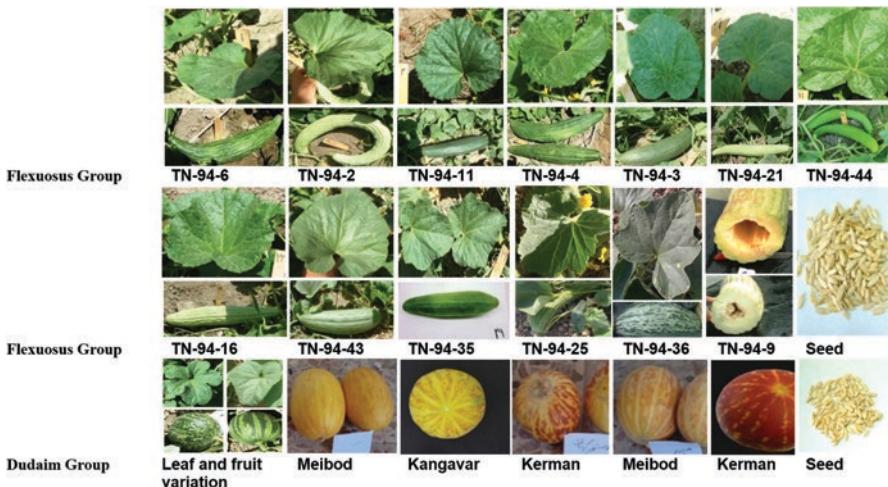


Fig. 9.7 Flexuosus Group variation in fruit and leaf characters and Dudaim Group collected from different parts of Iran (see Appendix II). (Source: Soltani et al. 2010)

detected in other cucurbits such as local cucumber. Dudaim Group fruit harvested after ripening as the fruit skin color changes from yellow to orange, a stage associated with high odorant. Their ovaries have trichomes that are present even on mature fruits. Both Flexuosus and Dudaim can easily cross with other melons and on some farms it happens spontaneously where Inodorus and Cantalupensis varieties are planted near each other resulting in an intermediate type. Some Dudaim types exhibit an elliptic fruit shape and enhanced sweetness. Another intermediate type from the past is Garmak, exhibiting certain characters of both the Inodorus and Reticulatus types. They often bear ovate fruits and ripen earlier than other types, with crispy, sweet fruit and a mild odorant. Genotyping-by-sequencing of melon (GBS) can provide useful information about genetic structure and large-scale SNP discovery. It is also employed to detect polymorphisms in melon accessions with sequence details (Pavan et al. 2017).

9.4.1 *In Vitro and In Situ Conservation*

Although various in vitro melon preservation programs are reported, the regeneration of various melon types has been highly variable and in some cases impossible (Molina and Nuez 1995, 1997; Nuñez-Palenius et al. 2008). In recent years, landraces were replaced with improved varieties, but some farmers still prefer to continue to grow their own landraces for their personal consumption and for local markets, together with commercial varieties. Additionally, preserving these types is of high importance for breeding programs. In situ preservation of the plant genetic resources of some melon landraces has been carried out for generations within communities

without financial support; however, maintaining this tradition is becoming more complicated with presence of new incoming varieties. Action is required to preserve traditional varieties in some manner.

9.4.2 Ex Situ Conservation

Due to the high genetic diversity in Iranian melons it is exceedingly important to collect and preserve traditional types from different areas of the country. This will provide a valuable opportunity to compare all these types side-by-side in an experimental scientific manner and will provide better understanding of the potential of each type in every criterion. Ex situ conservation of recovered propagation material can be carried out through the creation and management of catalog fields within experimental farms, long-time seed preservation in seed banks aimed to keep the agamic propagation material in good conditions while reducing conservation costs and space.

9.5 Biotechnology and Molecular Breeding

9.5.1 Molecular Marker-Assisted Selection

Marker-assisted selection (MAS) is a complementary tool for conventional breeding wherein a molecular marker linked to a trait is used for indirect selection. Several reports show how to identify and develop markers for traits such as disease and pest resistance and abiotic stresses (drought, salinity). DNA based molecular markers have been extensively used in melon breeding for the identification of some economic traits, in particular disease resistance. Marker-assisted selection with linked markers to resistance genes of *Fusarium* wilt has been studied for melon (Burger 2003; Oumouloud et al. 2012; Wang et al. 1997, 2000). Our research (unpublished data) on 41 genotypes of different melon and some F1 hybrids employed phenotypic and molecular markers to introduce some resistance types to FOM race 2 which were important for proper genotypes production. As indicated in Table 9.2, genotypes Tabas, Talebi Miyane, Jimabad and Jafa were resistant, based on phenotypic assays as well as molecular markers, making them valuable for use in melon breeding. Khatouni or Mashhadi genotype is more popular and a traditional melon in Iran which has been planted in most areas and represents 70% of total melon production; it revealed a resistance pattern for most of the molecular markers, while an inoculation test showed moderate resistance. A single uncut PCR product identified the homozygous resistance genotypes (703-bp band) (Fig. 9.8).

Table 9.2 Score of primers in melon genotypes with different reactions to *Fusarium oxysporum* f. sp. *melonis* races 2

No.	Genotypes	E07	CAPS-2	SSR ₁₅₄	STS ₁₇₈	S ₂₄₇	S ₃₄₂	R ₄₀₈	R ₅₂₉
1	Tabas	R	R	S	R	R	R	S	S
2	Atashbar	S	S	S	SR	S	S	S	S
3	Khorasan	S	S	S	S	S	S	S	S
4	Eyvanaki	S	S	S	S	R	R	S	S
5	Nikabad	S	R	S	S	R	R	S	S
6	Tashkandi	S	R	S	S	S	R	S	R
7	Kharboze Miyane	S	R	S	S	S	S	S	S
8	Khatouni	R	R	S	R	R	R	R	R
9	French	S	S	SR	SR	S	S	S	S
10	Talebi Miyane	R	S	S	R	R	R	R	R
11	Ananasi	S	S	S	S	S	S	S	S
12	Jimabad	R	R	S	R	R	R	R	S
13	Esfahan	S	R	S	S	S	S	S	R
14	Mahvelat	S	S	S	S	S	S	S	S
15	Majdi	S	S	S	S	S	S	R	S
16	Dastan	S	S	S	S	S	S	S	S
17	Ardakan	S	R	S	S	S	S	S	S
18	Atrak	S	S	NT	S	S	S	S	S
19	Chaghirje	S	S	S	S	S	S	S	S
20	Zamcheh1	S	S	S	S	S	S	S	S
21	Jarjue	S	S	S	S	S	S	S	S
22	Turkmenistan	S	R	S	S	R	R	S	R
23	Chahpaliz	S	S	S	S	R	R	S	S
24	Neishabour	S	S	S	S	S	S	S	R
25	Bojnourd	S	S	S	S	S	S	S	S
26	Jafa	S	S	R	R	R	R	R	S
27	Alien	S	S	S	S	R	R	S	S
28	Arkanga	S	S	S	S	R	R	S	S
29	Ana	S	S	S	SR	S	S	S	S
30	Charentaise	S	R	S	S	S	S	S	S
31	Cavaillon	S	S	S	S	S	S	S	R
32	Ceistel	S	R	S	S	S	S	S	S
33	Visio	R	S	R	SR	S	S	R	S
34	F ₁ : Alien*Tashkandi	S	S	SR	SR	R	R	S	S
35	F ₁ :(Arkanga*Tashkandi)	S	S	S	S	R	R	S	S
36	F ₁ :(Khatouni*Alien)	S	S	S	S	R	R	S	R
37	F ₁ :(Khatouni *Arkanga)	S	S	S	S	R	R	S	S
38	Tabriz	S	S	S	S	S	S	R	S
39	Isabelle	R	R	R	R	R	R	R	S
40	Bola de Oro	S	S	S	S	S	S	NT	S
41	PII61375	R	R	R	R	R	R	NT	R

Source: Courtesy of Tahereh Javanmard, Horticultural Science Department, University of Tehran
 R Resistant, S Susceptible, SR Semi resistant, NT not tested

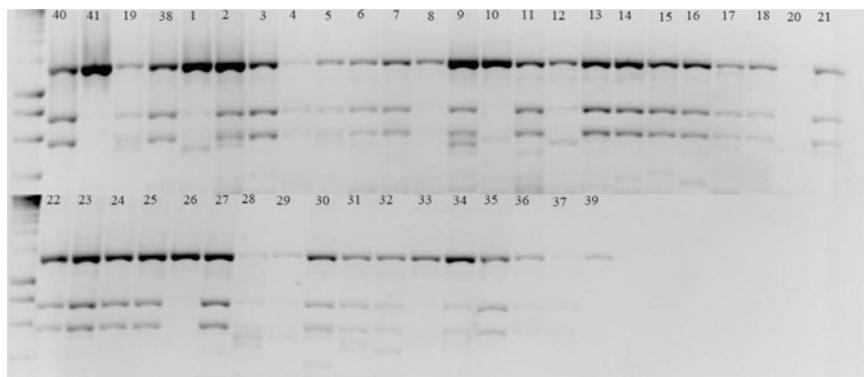


Fig. 9.8 E07 CAPS marker- PCR products were obtained by using E07 specific primer digested with Bcl1 restriction enzyme and separated on agarose gel. A single uncut PCR product identified the homozygous-resistance genotypes (i.e. 41: PI 161375), while digested products identified either the heterozygous-resistance or the susceptible (i.e. 40: Bola de Oro). The numbers of each column are related to the genotypes in Table 9.2. (Photo Courtesy of Tahereh Javanmard, Horticultural Science Department, University of Tehran)

9.5.2 Mutation Breeding

Mutation induction has been used for crop improvement to create new varieties and supplant current germplasm. Many successful experiments have shown that mutation breeding is an effective way for vegetable crop improvement (Hadi and Fuller 2013; Reddaiah et al. 2014). Besides the conventional mutation breeding, mutation induction by in vitro techniques speeds up breeding for disease resistance, salt and drought tolerance and other characters. Altered fruit pigment composition was reported in a mutant family of Charentais type melon which is the novel variety with pro-lycopene accumulation in the fruit as a major pigment (Galpaz et al. 2013).

Despite the time consuming and many efforts to manage the high number of plants in mutant family screening, this method provides a crop library as an important source for new traits. Furthermore, mutation libraries afford essential infrastructure to find important genes, for the annotation of unknown sequences, and for phenotypic and genetic comparison with mutation libraries of other plant species.

9.5.3 Tissue Culture

To complement conventional breeding, in vitro techniques including embryo rescue, somatic embryogenesis and somaclonal variation lead to improved plant varieties and to overcoming the genetic barriers for inter- and intraspecific crossing. One

possible method of introducing disease resistance is by interspecific hybridization using various methods of in vitro culture to rescue the embryo.

Successful regeneration of mature embryos has been achieved from interspecific crosses of cucumber and melon in different culture media by Lebeda et al. (1996) and Skalova et al. (2008). Regeneration of the whole plant of snake melon cultivars with an established protocol and culture media created the right platform for improvement of the crop to resist infection (Ben Ghnaya et al. 2009).

Another benefit of in vitro culture is production of doubled haploid (DH) plants to accelerate producing homozygote pure lines and to advance breeding objectives. However, some common methods to obtain haploid embryos have been tested on melon crops such as *inspecting the seeds one by one* and irradiation of pollen (Lotfi et al. 2003; Sauton and de Vaulx 1987), but it is still necessary to set up an experiment to explore the method that can reduce the cost and time of double haploid plant production.

9.5.4 Genetic Engineering and Transformation

Advances in biotechnology can also accelerate genetic improvement in melon by gene manipulation. Development of novel genetic and genomics tools has enabled the identification and study of genes with agronomic interest in melon. Oligo based microarray allows identification of gene expression profile of melon fruit ripening and also cotyledon and root response to viral and pathogen infection (Mascarell-Creus et al. 2009). Over the last decade bacterial artificial chromosome (BAC) libraries and EST collections have been providing valuable information about genetic content that can be used for melon breeding and the development of genetic markers for disease resistance screening and other characters (Gonzalez-Ibeas et al. 2007; Gonzalez et al. 2010a, b; Luo et al. 2001).

Ethylene as a key regulator plant hormone which has a critical role in melon ripening and postharvest attributes. Manipulation of ethylene biosynthesis by genetic engineering can lead to delayed ripening and extended postharvest life in climacteric fruit, which in turn reduces postharvest loss resulting from produce that is overripe and senescent. Recent whole genomic sequencing experiments have shown that melon can be considered a proper model for ripening studies (Yano and Ezura 2016). Ripening behavior in melon ranges from typical climacteric types with a high ethylene production, such as *Cucumis melo* var. *cantalupensis* with a fast ripening and short shelf and storage life, to non-climacteric melon types, such as *Cucumis melo* var. *inodorus*, which are unable to produce autocatalytic ethylene, generally have a slow ripening rate and are associated with a long shelf and storage life (Pech et al. 2008). Among these genes, *CMe-ACS1* and *CMe-ACO1* are important in ripening and climacteric behavior. Transgenic melon with targeting *etr1-1*

resulted in increased and earlier carpel-bearing buds and flowers followed by more fruit set and earlier ripening fruit than wild type (Switzenberg et al. 2014).

In this genomic era, targeting induced local lesions in genomes (TILLING) is a method that allows direct identification of mutations in a specific gene and has been used as reverse genetic approach in some crops. It has also become an obligate technology to dissect gene function as well as to engineer alleles of agronomic importance in crops (Triques et al. 2007). A TILLING platform has been set up in a monoecious and climacteric melon (Cantaloupe type) line; cloned genes that control ethylene production and a list of candidate genes that enhance melon fruit quality were *tilled* (Dahmani-Mardas et al. 2010). Another experiment on andromonoecious from Inodorus Group as non-climacteric melon has been reported that created new populations with a higher mutation rate. This new resource will facilitate reverse genetics studies in the Inodorus Group, and contribute materially to future genomic and breeding studies (Gonzalez et al. 2011).

An alternative method, alongside the use of natural genetic germplasm of melon, is transgenic plant production to improve disease resistance. Coat protein in transgenic plants increases resistance to virus-mediated disease. Multiviral resistance generated by engineering the plants with viral coat protein (CP) genes from more than one virus have been reported for some transgenic cucurbits include transgenic squash resistant to Cucumber mosaic virus (CMV), WMV, and ZYMV (Fuchs and Gonsalves 1995; Fuchs et al. 1998; Tricoli et al. 1995) and transgenic cantaloupe (*Cucumis melo* L. var. *cantalupensis*) resistant to CMV, ZYMV and WMV (Fuchs et al. 1997).

In recent studies, for the first time, Hooghvorst et al. (2019) performed gene editing by CRISPR/Cas9 in melon for the PDS gene, a key enzyme in the carotenoid biosynthesis pathway involved in at least 20 metabolic pathways, including the inhibition of many genes in carotenoid, chlorophyll and 21 GA biosynthesis pathways. Efficient gene editing in melon presents the possibility to study new gene functions for basic research, and new opportunities for melon productivity by improving metabolite content, resistance to biotic stress, melon production and postharvest utilization.

9.6 Conclusion and Prospects

Given seven commercial and horticulturally important melon varieties (Cantaloupensis, Reticulatus, Saccharinus, Inodorus, Flexuosus, Conomon, Dudaim) and hundreds of muskmelon cultivars, more research should be undertaken to introduce new improved cultivars with good performance and resistance to climate change. Breeding programs are labor intensive, time consuming and require proper planning, expertise and continuity, as well as biological, human and financial resources. Currently Asia produces more than 70% of the world melon production.

Iran, with different climatic conditions and a rich gene pool of melon groups, requires greater attention to develop more breeding projects. Some local genotypes with high fruit quality and production quantity need improvement for their resistance to abiotic stresses, such as drought and salinity, as well as biotic stresses, such as powdery mildew and *Fusarium* wilt. Uniformity, color change, fruit shape, metabolite enhancement and biotic and abiotic resistance leading to achieving high productivity are hybrid breeding goals. Development of elite breeding lines with superior combining ability is one of the prime breeding objectives in melon.

Attaining the monoecy line can reduce the time and cost for emasculation up to 50% and improve fruit set and fruit number per plant. Most commercial melons have perfect flowers and need to be emasculated for hybrid seed production. Since the snake melon is monoecious, it can be used in breeding programs to change sex expression. There is linkage between sex expression and fruit shape and research should be considering the round type as more popular and marketable, so there is a need to use backcrossing to gain monoecious parents with desirable fruit shapes. Considering the relatively small genome size of melon makes it possible to have a draft genome sequence to address the biological questions and for their application in breeding projects. Identification of candidate genes/QTLs underlying tolerance to drought, salinity, disease, fruit ripening and quality attributes and storability using multiparent advanced generation intercrossing populations could be a possible way to adjust against improper environmental condition.

A variety of targeted gene editing methods such as RNA interference, transcription activators like repeat/Cas9 (CRISPER/Cas9) and a TILLING platform developed for melon can contribute to fruit ripening and sex determination studies. The most popular and current precise gene edition technique is the CRISPER/Cas9 system, where gRNA sequences direct the Cas 9 protein to induce the site-specific double-strand break in the genomic DNA. Gynoecious inbred lines for melon and also cucumber populations can be created by selection from crosses of monoecious inbreds, or can arise spontaneously from natural variation. Besides, the time and cost breeders sometimes encounter with undesirable traits, and spontaneous evolution of gynoecious varieties, may not occur in lines of interest for breeders. In melon, modification in *CmWIP1* by gene editing tools such as CRISPR/Cas9 produced a gynoecious phenotype (Martin et al. 2009) and improved the development of gynoecious inbred lines. To date, CRISPR/Cas9 gene editing has been reported in cucumber and watermelon (Chandrasekaran et al. 2016; Hu et al. 2017; Zhang et al. 2019). It can be used widely in the future to alter the sex expression in melon and achieve disease resistance to accelerate breeding programs.

Appendices

Appendix I: Research Institutes Relevant to *Cucumis melo* L. Groups Flexuosus and Dudaim

Institution name	Specialization	Address	Contact information and website
Department of Horticultural Science, College of Agriculture and Natural Resources, University of Tehran	Collection and conservation of germplasm, genetic diversity evaluation, traditional and molecular breeding	Daneshkadeh Street, College of Agriculture and Natural Resources, Horticultural Science Department, University of Tehran, Tehran, Iran	Tel: +982632248721 utcan.ut.ac.ir
Karaj Seed and Plant Improvement Institute	Collection and conservation of melon germplasm	Shahid Fahmideh Blvd., Seed and Plant Improvement Institute, Karaj, Iran	Tel: +982632702859 www.spii.ir
Instituto de Investigaciones Agropecuarias (INIA)	Collection and conservation of melon germplasm	Andes 1365, Piso 12 CP. 11100 Montevideo, Uruguay	Tel: +56-42 206 800 http://www.inia.uy
National Agriculture Research Organization (NARO)	Collection and conservation of germplasm, genetic diversity evaluation, traditional and molecular breeding	Department of Regional Strategy, Central Region Agricultural Research Center, 2118 Kannondai, Tsukuba, Ibaraki 305-8666, Japan	Tel: +81298388481 www.naro.go.ug
Institut National de la Recherche Agronomique (INRA)	Introduce, maintain and characterize the germplasm and breeding program	Domaine St Maurice, 84143, Avingon, France	Tel: +331 42 75 90 00 www.paca.inra.fr
Institute for Conservation and Improvement of Valencian Agrobiodiversity (COMAV)	Germplasm conservation, introduction and breeding program	Genebank of the Instituto de Conservación Mejora de la Agrobiodiversidad Valenciana (COMAV). Universitat Politècnica de València. Building 8E, access J, floor 0. Camino de Vera s/n, 46022 Valencia, Spain	Tel: 0034-963879421 www.comov.upv.es

**Appendix II: Genetic Resources of *Cucumis melo L.* Groups
Flexuosus and Dudaim in Iran**

Cultivar	Important traits	Cultivation location
Kermanshah	Dudaim Group, round flattened fruit shape, dark yellow with orange areas in skin, without netting, light yellow flesh color, more aromatic, andromonoecious	Western parts, Kermanshah, Ilam
TN-94-33	Flexuosus Group, elongated fruit shape, green skin color, monoecious	Markazi
TN-94-34	Flexuosus Group, ovate fruit shape, dark green skin color	Qom
TN-94-42	Flexuosus Group, elongated fruit shape, dark green with mottled in green texture, monoecious	Isfahan
TN-94-44	Flexuosus Group, elongated fruit shape, green skin color, monoecious	Isfahan
TN-94-9	Flexuosus Group, elbowed elongated fruit shape, cream skin color, monoecious	Khuzestan
TN-94-2	Flexuosus Group, elongated fruit shape, green skin color, monoecious	Khuzestan
TN-94-28	Flexuosus Group, elongated elliptical fruit shape, dark green with mottled in green texture, monoecious	Khuzestan
TN-94-12	Flexuosus Group, elongated fruit shape, dark green skin color, monoecious	Fras
TN-94-8	Flexuosus Group, elongated elliptical fruit shape, dark green with mottled in green texture, monoecious	Fars
TN-94-4	Flexuosus Group, elongated elliptical fruit shape, dark green skin color, monoecious	Fars
TN-94-16	Flexuosus Group, elongated fruit shape, light green skin color, monoecious	Fars
TN-94-5	Flexuosus Group, elongated fruit shape, light green skin color, monoecious	Fars
TN-94-6	Flexuosus Group, elbowed elongated fruit shape, dark green with mottled in green texture, monoecious	Fars
TN-94-41	Flexuosus Group, elliptical fruit shape, light cream skin color, monoecious	Fars
TN-94-11	Flexuosus Group, elongated fruit shape, green skin color, monoecious	Fars
TN-94-40	Flexuosus Group, elliptical fruit shape, dark green skin color, monoecious	Fars
TN-94-15	Flexuosus Group, elongated fruit shape, green skin color, monoecious	Fars
TN-94-21	Flexuosus Group, elongated fruit shape, green skin color, monoecious	Fars
TN-94-3	Flexuosus Group, elongated fruit shape, dark green skin color, monoecious	Fars
TN-94-7	Flexuosus Group, elongated elliptical fruit shape, dark green skin color, monoecious	Fars

(continued)

Cultivar	Important traits	Cultivation location
TN-94-36	Flexuosus Group, elongated fruit shape, dark green with mottled in green texture, monoecious	Fars
TN-94-43	Flexuosus Group, elliptical fruit shape, green skin color, monoecious	Kerman
TN-94-10	Flexuosus Group, elliptical fruit shape, dark green spots in green texture, monoecious	Kerman
TN-94-47	Flexuosus Group, elongated fruit shape, dark green skin color, monoecious	Bushehr
TN-94-35	Flexuosus Group, elongated fruit shape, green skin color, monoecious	Bushehr
TN-92-1024	Dudaim Group, globular fruit shape, dark orange spots in orange texture, monoecious	West Azarbayan
TN-92-1022	Dudaim Group, globular fruit shape, dark orange spots in orange texture, monoecious	Khuzestan
TN-92-1025	Dudaim Group, globular fruit shape, dark orange spots in orange texture, monoecious	Kerman
TN-92-1017	Dudaim Group, globular fruit shape, dark orange spots in orange texture, monoecious	Mahabad
TN-92-1014	Dudaim Group, globular fruit shape, dark orange spots in orange texture, monoecious	Markazi
TN-92-97	Dudaim Group, small elliptical fruit shape, dark orange spots in orange texture, monoecious	Yazd-Meibod
TN-92-98	Dudaim Group, flattened round fruit shape, dark orange spots in orange texture, monoecious	Ilam
TN-93-491	Inodus Group, elliptic fruit shape, yellow skin color whiteout net, mild odorant, yellow flesh color, andromonoecious	Fars
TN-93-25	Inodus Group, ovate fruit shape, yellow fruit skin color, dark yellow flesh color, mild aroma, crispy flesh andromonoecious	Kerman
TN-93-525	Inodus Group, green skin color, elliptical fruit shape, light green flesh color, low aroma andromonoecious	Kerman

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Chapter 10

Tomato (*Solanum lycopersicum* L.)

Breeding Strategies for Biotic and Abiotic Stresses



Adel Rezk, Mohammad Abhary, and Abdullah Akhkha

Abstract The cultivated tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family. The origin of tomato plant traces back to the Andean Region in South America, where wild types are still growing in their natural habitat. Tomatoes went through stages of domestication before it was discovered by the European expeditions in the sixteenth century and introduced to the Old World as an ornamental plant. Today, tomatoes are cultivated and consumed worldwide, either fresh or processed. The total world production of tomatoes in 2018 reached more than 182 million mt, steadily rising annually in consumption. The genomic characteristics and growth habits of tomato have made it a model in plant research with an increasing interest in breeding and development of molecular tools for crop improvement. The main breeding objectives of tomato are fruit yield, quality and resistance to biotic stress and tolerance to abiotic environmental factors. Traditional breeding of tomato is based on gene transfer from wild tomato relatives into modern cultivars. This chapter describes the origin and domestication of tomatoes. Traditional breeding strategies, such as mass selection, pedigree and hybridization methods and use of molecular markers are used to construct linkage maps. Biotic resistance and abiotic tolerance strategies are reviewed and the contributing role of biotechnology in generating new tomato cultivars through mutagenesis and the role of biotechnology and transgenic approaches are discussed.

Keywords Biotechnology · Breeding · Domestication · Molecular markers · Mutagenesis · Quality traits · Wild tomato

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10.1 Introduction

At present, tomato is the essential ingredient of many cuisines, such as in sauces and salads. At its European discovery in the early sixteenth century, tomatoes were first considered as ornamental plants (Colvine and Branthôme 2016; Vergani 2002) but within 200 years it became a world crop with great social and economic values. Tomatoes are now annually celebrated on April 8 in the USA as National Tomato Day (Saavedra et al. 2017).

The tomato crop generates thousands of direct and indirect jobs in farming, marketing, processing factories and it is one of the major traded crops in the world (Eboigbe and Edemevughe 2019). According to FAOSTAT (2018), among vegetables, tomato products rank fourth in the world, in terms of value. The total world tomato production in 2018 reached more than 182 million mt (FAOSTAT 2018). On a regional scale, Asia produces more than 61% of the total world production, followed by the Americas, Europe and Africa as illustrated in Fig. 10.1. Country-wise, China produced 61.6 million mt in 2018, 33.8% of the total word production, followed by India (10.6%), USA (6.9%), Turkey (6.6%) and Egypt (3.6%) (FAOSTAT 2018).

The modern tomato processing industry officially began in 1869, when Henry John Heinz established his ketchup company in Pittsburgh, USA (Colvine and Branthôme 2016). Since then, tomato has been the leading processed vegetable in the world in diverse product forms, such as soups, ketchup, pastes, and canned, peeled, diced, chopped, frozen, dry or in ready-to-eat meals.

In the twentieth century, the tomato market was further revolutionized with the aid of breeding programs (Bai and Lindhout 2007; Bauchet et al. 2017; Panthee and Gardner 2011) to provide high yielding varieties with desirable traits for the consumer as fresh or processed tomatoes. Modern techniques and tools of conventional breeding, molecular breeding and genetic studies have used wild tomato species to generate new hybrid tomatoes (Bai and Lindhout 2007) with larger fruit size (Frary

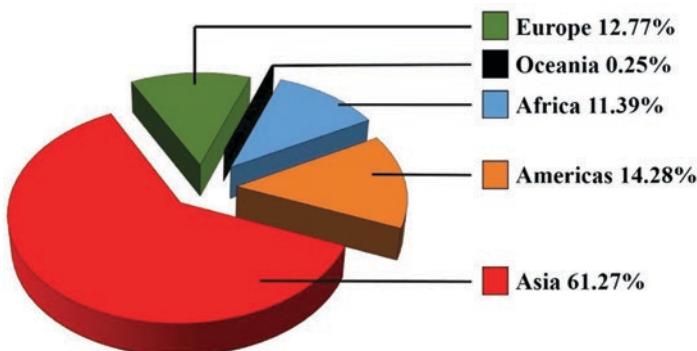


Fig. 10.1 World tomato production, regional percentage of tomato production, according to FAOSTAT (2018). (Figure constructed by M. Abhary)

et al. 2000), fruit shape (Van der Knapp et al. 2014), fruit ripening (DellaPenna et al. 1986), color (Andrade et al. 2015), sugar content and flavor (Jones and Scott 1983), antioxidants (Pal et al. 2018), disease resistance (Acharya et al. 2018; Caro et al. 2015), stress tolerance (Venema et al. 2005; Wang et al. 2003) and many other traits. Moreover, cherry tomatoes (Mukherjee et al. 2020; Rodriguez et al. 2011), black tomatoes (Blando et al. 2019) and customized hybrids for organic culture (Campanelli et al. 2015) were developed to enrich the tomato market with new competing commercial choices.

In this chapter, the historical journey of tomato from the Andes to the rest of the world is detailed as introductory data for the nomenclature and taxonomy of this plant. The classification of cultivated tomato highlights the relationship with its wild relative counterparts and clarifies the importance of its genomic resources for breeding. Tomato breeding methodologies to improve varieties and cultivars are discussed from traditional to technological perspectives, where mass selection, pedigree and cross hybridization are aided with biotechnological tools to accelerate the breeding process in the subjects of biotic resistance and abiotic tolerance. Several approaches are detailed and included; mutagenesis, gene editing and in vitro techniques that explore and expand the known genetic pool of tomato and jump over the barriers that stand in the face of traditional breeding processes, with a perspective on the current status of research capabilities to improve the tomato crop for the interest of the consumer.

10.2 Tomato Classification and Taxonomy

The origins of cultivated tomato (*Solanum lycopersicum* L.) goes back to the Andes Mountains of South America (Bai and Lindhout 2007), where wild ancestors of tomato such as (*S. cheesmaniae*, *S. chilense*, *S. pimpinellifolium*, *S. peruvianum*) still occur naturally (Peralta and Spooner 2000; Rick 1988; Rick and Holle 1990;). In the sixteenth century, the first domesticated tomatoes found their way to Europe and were subjected to further domestication processes (Sims 1980). Tomatoes was assigned the name *Solanum lycopersicum* by Linnaeus in 1753 and *Lycopersicon lycopersicum* by Miller in 1768 (Valdes and Gray 1998); the two given names created a scientific disagreement, which lasted until the twentieth century when morphological and molecular sequencing data placed the tomato plant in the Solanaceae with the name *S. lycopersicum* (Knapp 2002; Olmstead and Palmer 1997; Peralta and Spooner 2001; Peralta et al. 2006; Spooner et al. 1993, 2005).

The Solanaceae, also known as nightshades, is a diverse family containing 98 genera and more than 3000 recognized species (Olmstead and Bohs 2007) occurring in diverse geographical areas of the Old and New worlds with diverse uses (Knapp and Peralta 2016). The Solanaceae includes other important economic crops, such as, potato (Solanum tuberosum L.), eggplant (Solanum melongena L.), pepper (*Capsicum annuum* L.), tobacco (*Nicotiana tabacum* L.), medicinal plants such as *Mandragora* spp., *Datura* spp., the deadly nightshade (*Atropa belladonna* L.) and ornamentals such as those in the genera *Browallia*, *Lycianthes* and

Petunia. The Solanaceae family is part of the Asterid Clade of flowering plants, which originated 59 million years ago (Bell et al. 2010).

The genus *Solanum* has the largest number of species among the Solanaceae with approximately 1500 species (Bohs 2005; Olmstead et al. 1999). Cultivated and wild tomatoes are all placed under the section *Lycopersicon* within 4 groups (Arcanum, Eriopersicon, Lycopersicon and Neolycopersicon), consisting of 13 species, as indicated in Table 10.1. Although the species within these groups are easily distinguished by their morphological characteristics, such as its bright yellow flowers and pinnate non-spiny leaves (Peralta et al. 2006), recent taxonomic considerations of the tomato groups have depended on morphological, biochemical and genetic similarity studies (Bauchet et al. 2017; Baek et al. 2015; Knapp and Peralta 2016; Mata-Nicolas et al. 2020; Peralta and Spooner 2000), providing solid evidence for their placement within the genus *Solanum*.

10.2.1 Domestication

The historical journey of tomato domestication (*Solanum lycopersicum*) began well before the European expeditions to the New World in the sixteenth century (Colvine and Branthôme 2016). Although the exact time and place of the first tomato domestication is still unknown (Knapp and Peralta 2016; Peralta and Spooner 2007), morphological and molecular evolution studies have shown that the process of domestication went through several stages at different locations (Razifard et al. 2020).

Similar to other Solanaceae crops, the domestication of tomatoes included obvious morphological features, such as the selection for fruit size, shape, color and other characters (Bai and Lindhout 2007). Such quality traits are present in the genomes of wild tomatoes; molecular studies have demonstrated that a mutation in the *fruit weight 2.2* (*fw2.2*) gene, found in *S. pennellii*, is responsible for fruit size and has secondary effects on fruit number (Frary et al. 2000; Nesbitt and Tanksley 2001). Moreover, four genes were identified in *S. pimpinellifolium* controlling fruit shape (*SUN*, *OVATE*, *LC* and *FAS*) (Van der Knapp et al. 2014). Other quantitative and qualitative genes that are involved in domesticating, such as growth habit (plant height, self-pruning and earliness), were also identified in wild tomato (Doganlar et al. 2000; Frary and Doganlar 2003; Grandillo and Tanksley 1996; Tanksley 2004).

Genetic studies have shown that cultivated tomato *Solanum lycopersicum* is considerably low in genetic diversity when compared to wild types (Ranc et al. 2008; Razifard et al. 2020). Molecular marker analyses of *S. lycopersicum* var. *cerasiforme* available accessions showed that this variety is structured in two groups; the first is very close to the cultivated tomato (*S. lycopersicum*) and the second is close to the admixture of *S. lycopersicum* and *S. pimpinellifolium* (Ranc et al. 2008), providing evidence for the previous suggestion that the *cerasiforme* variety is the link between the wild type and cultivated tomato (Cox 2000; Nesbitt and Tanksley 2001). Furthermore, population genetics of *S. lycopersicum* var. *cerasiforme* alleles,

Table 10.1 Wild species of tomatoes (*Solanum* spp.) and their distribution in the Andes

Species	Distribution	Group; according to Peralta et al. (2008)	Fruit color	Breeding compatibility	No. of available accessions in TGRC ^a	Reference
<i>S. arcanum</i> Peralta	North Peru	Arcanum	Green with a dark green strips	Self-incompatible	45	Dung-Dinh et al. (2019)
<i>S. cheesmaniae</i> (L. Riley) Fosberg	Galápagos Islands	Lycopersicon	Yellow to orange fruits	Self-compatible	41	Peralta and Spooner (2000)
<i>S. chilense</i> (Dunal) Reiche	Coastal Chile and Southern Peru	Eriopersicon	Green with purple strips	Self-incompatible	111	Peralta and Spooner (2000)
<i>S. chinense</i> (C.M. Rick, Kesicki, Fobles & M. Holle)	South-central Peru	Arcanum	Green	Self-compatible	16	Peralta and Spooner (2000)
<i>S. corneliiomulleri</i> J.F. Macbr.	South Peru	Eriopersicon	Light green	Self-incompatible	53	Baeck et al. (2015)
<i>S. galapagense</i> S.C. Darwin & Peralta	Galápagos Islands	Lycopersicon	Yellow to Orange	Self-compatible	28-	Andrade et al. (2015)
<i>S. habrochaites</i> S. Knapp & D.M. Spooner	Southwestern Ecuador to South-central Peru	Eriopersicon	Green	Self-incompatible	115	Peralta and Spooner (2000)
<i>S. huaylasense</i> Peralta	North-central Peru	Eriopersicon	Pale green	Self-incompatible	16	Peralta et al. (2005)

(continued)

Table 10.1 (continued)

Species	Distribution	Group; according to Peralta et al. (2008)	Fruit color	Breeding compatibility	No. of available accessions in TGRC ^a	Reference
<i>S. neorickii</i> D.M. Spooner, G.J. Anderson & R.K. Jansen	South Ecuador South-central Peru	Arcanum	Green	Self-compatible	47	Peralta and Spooner (2000)
<i>S. pennelli</i> Correll	North-central to South-central Peru	Neolyopersicon	Yellow to green	Self-incompatible and self-compatible in some accessions	45	Peralta and Spooner (2000)
<i>S. peruvianum</i> L.	North-central Peru to North Chile	Eriopersicon	Pale green	Self-incompatible	70	Peralta and Spooner (2000)
<i>S. pimpinellifolium</i> L.	Southern Ecuador and Peru	Lycopersicon	Red	Self-compatible	262	Peralta and Spooner (2000)
<i>S. lycopersicum</i> L.	Ecuador and Peru	Lycopersicon	Red	Self-compatible	266	Peralta and Spooner (2000)

^aSource: Available accession in the Tomato Genetic Resources Center at University of California, Davis <https://tgrc.ucdavis.edu/Wild%20species%20stock%20list-2013-v2.pdf>

in Peru, have demonstrated that several domestication traits, in central and southern *cerasiforme* accessions, are diminished in the northern *cerasiforme* accessions (Razifard et al. 2020). The later finding suggests that cultivated tomato began with the South American *cerasiforme* variety.

The early tomato varieties, such as the *cerasiforme*, found their way from the Peruvian Andes to Mesoamerica through trade between pre-Hispanic cultures (Blanca et al. 2012), where it was further domesticated through diverse agricultural systems (Blanca et al. 2015; Saavedra et al. 2017).

The tomato's dispersal journey to the Old World, along with other Solanaceae members, began with the European navigators, who carried New World plants to Europe in the sixteenth century. The new exotic fruit was first introduced to Andalusia, welcomed by the Spanish and Italians as an appetizer (Vergani 2002) and as an ornamental plant in the rest of Europe (Colvine and Branthôme 2016). The name *tomato* comes from the Aztec word *tomatl* (Saavedra et al. 2017).

In Europe, tomato was cultivated as an ornamental plant in English and French gardens until the eighteenth century (Fournier 1948). During that time, Italian breeders developed the ornamental small-fruited tomato into a smooth red juicy form (Colvine and Branthôme 2016). Concurrently, tomato returned to the New World, to the USA, as an ornamental plant (Rick 1978). In the nineteenth century, awareness of the tomato's value as a nutritional food increased and the first tomato processing factory was established in the USA (Colvine and Branthôme 2016). Since then, the USA has improved and cultivated tomatoes on a large scale (Atherton and Rudich 1986) bringing new varieties to market which became internationalized. The journey of tomato domestication is illustrated in Fig. 10.2.

10.2.2 Germplasm Diversity

The exploration of wild tomato genome continues to reveal tremendous knowledge about the applicable variations that can be used in breeding and genetic engineering (Blanca et al. 2012; Chetelat and Ji 2006; Miller and Tanksley 1990). Tomato has a genome size of 950 Mb, distributed in 12 chromosomes ($2n = 24$) (Barton 1950) with more than 30% of its DNA being in repetitive forms in heterochromatin regions (Van der Hoeven et al. 2002). The simplicity of tomatoes' diploid genetic system, short generation time and simple transformation procedures has made it a model for the Solanaceae family in plant research (Ranjan et al. 2012).

Cultivated tomato (*Solanum lycopersicum*) is estimated to have only 5% of the genetic diversity found in its wild types (Miller and Tanksley 1990); this latter genetic variance was extensively exploited using genetic tools for breeding programs (Bai and Lindhout 2007). Effective crossing experiments between cultivated and wild tomatoes have enriched outcome varieties with numerous traits, such as disease resistance (Acharya et al. 2018; Caro et al. 2015), abiotic tolerance (Venema et al. 2005; Wang et al. 2003) and fruit quality traits (Jones and Scott 1983; Pal et al.

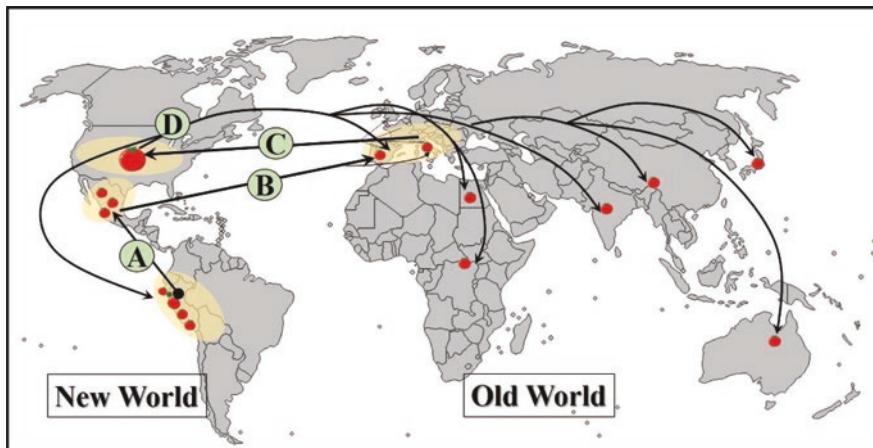


Fig. 10.2 The domestication journey of cultivated tomato. (a) Origin of tomatoes and early domestication in the Andes of South America, transfer to Mesoamerica, Mexico, second domestication, (b) Transfer and distribution of tomato from Mesoamerica to Europe by the Spanish navigators, third domestication by Italian breeders, (c) Transfer from Europe to the United States, fourth domestication, (d) Improved cultivated tomato and distribution from North America to the rest of the world. (Figure constructed by M. Abhay)

2018). Such breeding experiments were officially started after collecting different accessions of wild-type tomatoes from the Andes (Wang et al. 2017).

10.2.3 Conservation of Genetic Resources

Peru has the largest collection of wild tomato species (Jenkins 1948; Peralta and Spooner 2007; Rick 1978; Vilchez et al. 2019), including ten species (*S. pimpinellifolium*, *S. peruvianum*, *S. pennellii*, *S. neorickii*, *S. huaylasense*, *S. habrochaites*, *S. chmielewski*, *S. corneliomulleri*, *S. Arcanum*, *S. chilense*) distributed along the western coast of the country and one variety (*S. lycopersicum* var. *cerasiforme*) found everywhere in Peru (Vilchez et al. 2019). On the other hand, Mexico houses the largest collection of tomato varieties cultivated in different climatic areas, utilizing diverse agricultural systems (Jenkins 1948; Rick 1978; Saavedra et al. 2017) and considered the a center of diversity and domestication of tomatoes (Bai and Lindhout 2007; Jenkins 1948; Knapp and Peralta 2016). The germplasm of wild tomato species is well conserved in the Tomato Genetic Resources Center (TGRC, UC Davis, <http://tgrc.ucdavis.edu/>), as indicated in Table 10.1. The Andes also harbors a large collection of landraces, which are highly diverse, preserved in the Argentinean Vegetable Crop Germplasm Bank System (Clausen et al. 2008; <http://inta.gob.ar/documentos/red-de-bancos-y-colecciones-de-germoplasma>).

10.3 Tomato Breeding

Crop breeding is a normal practice to improve crop yield and other traits related to crop quality. As market demand for tomato increases along with a burgeoning population, much effort has been directed toward the quality and production of the tomato crop using classical breeding alone or in association with the modern gene technologies and bioinformatics. Such breeding programs will definitely help in improving tomato crop traits and producing new varieties with qualities associated with yield improvement, nutritional value, and biotic and abiotic stress tolerances.

10.3.1 Traditional Breeding

The main tomato breeding objectives are fruit yield and quality, resistance to biotic stress and tolerance to abiotic environmental factors. Traditional breeding of tomato is based on gene transfers from wild relatives of tomato species into the modern cultivated cultivars (Fentik 2017). Such breeding aims to produce hybrids from inbred lines with desirable traits such as high productivity and quality, and resistance/tolerance to biotic/abiotic stress factors. A number of methods are used in the traditional breeding including mass selection, pedigree, and hybridization (Iqbal et al. 2019).

Fruit yield is a complex character measured by fruits number per plant, size and weight of fruits. To increase fruit size breeders, have to tackle the problem of lines sensitive to biotic and abiotic stress to stop the yield loss due to such factors. This involves intensive breeding programs to screen for tolerant cultivars and high yield but sensitive cultivars in order to produce hybrids with desirable yield and fruit quality, such as total solid content, shelf life, sweetness, antioxidants and color.

10.3.2 Traditional Tomato Breeding

Mass Selection Mass selection, or phenotypic selection, is based on the appearance of each individual plant within a population. Phenotypically attractive plants are selected, seeds are harvested to obtain a stock of mixed seeds which are then used to produce the next generation. The procedure is repeated until the desirable character is obtained (Iqbal et al. 2019). Mass selection procedures are used to improve existing varieties of tomato, as well as other crops. It is considered to be the easiest and least expensive method of plant breeding.

Pedigree The pedigree breeding method of tomato generates progeny of selected plants from a single cross, starting in F_2 generation and continuing through successive generations until F_6 . The main objective of this method is to finally obtain a new

variety with the preferred traits and it is very fast in developing new cultivars, compared to mass selection (Iqbal et al 2019).

Hybridization Although the hybridization method is usually used for the cross-pollinated crops, it was also proven successful in the case of tomato as a self-pollinated crop. The goal of hybridization is to combine desirable traits from two or more different varieties in order to obtain pure-breeding progeny with more improved traits compared to that of the parent plants. This technique is applied for tomato using both intraspecific (between different tomato varieties) and interspecific (between cultivated tomato varieties and tomato wild relative lines) (Ghani et al. 2019).

Grafting Tomato grafting methods are based on selecting scion stock with biotic resistance to soil-borne pathogens or abiotic tolerance to drought and salinity which has quantitative high yield and desirable fruit qualities. Grafting tomatoes is a widely used horticultural practice to reduce the amount of pesticides applied over the crop (Grieneisen et al. 2018).

10.4 Molecular Breeding Tools

Interest in tomato breeding has increased during the last 50 years concurrently with the development of new molecular tools to facilitate the breeding process. Molecular markers have enabled scientist to locate mutations and genes of interest in the genome to construct reference linkage maps in tomato (Grandillo and Tanksley 1996; Tanksley et al. 1992) and simplified genotyping for breeders. Currently, genome maps of tomato and other Solanaceae members are available on websites, such as the SGN (<https://solgenomics.net>) (Causse and Grandillo 2016).

With the advancement of DNA marker technology, isozyme and morphological markers were strengthened and gene mapping provided a significant plant-breeding tool (Foolad 2007). Molecular breeding involves identifying regions of the genome, such as quantitative trait loci (QTLs) which are related to favorable traits, then pooling genes to develop new hybrids or varieties (Van Alfen 2014). It also refers to the molecular technique of using DNA markers associated with phenotypic traits to assist in a particular breeding program. However, some of these DNA techniques are more laborious, expensive and time consuming, motivating molecular breeders to develop and adopt PCR-based markers which are easier to use (Osei et al. 2018).

There are a number of PCR based marker techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP), all of which are routinely being used in genetic studies of different tomato plant traits in order to associate such markers with a particular trait.

Recent intensified research in the field of genomics has speeded up the development of new varieties with desirable traits related to yield, quality and/or challenges of different environmental factors. Unlike morphological or biochemical markers, molecular markers, which are based on DNA polymorphism, have dramatically increased successful genetic diversity characterization in crop species germplasm proving it to be more advantageous over other markers (Ghani et al. 2019). Several molecular markers were identified in tomato species, which are related to yield, quality, resistance to biotic and tolerance to abiotic stresses.

10.5 Tomato Breeding for Biotic Stress

The genetic diversity in wild tomato types, especially in the case of self-incompatible species like *Solanum peruvianum* and *S. chilense*, is enormous. Tomatoes are extensively grown all over the world in tropical and subtropical regions. The demand for tomatoes is increasing daily, but there are many factors responsible for the limitation of the development and growth of tomatoes, such as drought, high or low temperatures, salinity and attacks by insects and pests. Developing disease-resistant and stress-tolerant varieties are major plant breeding priorities. Genetic engineering methods can also be influential in enhancing and growing disease-resistant cultivars. It is becoming obvious that developing this crop is an essential activity to resolve the constraints of tomato production (Fentik 2017). Currently breeding for plant diseases and pests is one of the most important issues. There are more than 200 species of pathogens and pests that can cause damage to and significant economic loss of tomato plants all over the world (Bai and Lindhout 2007). One of the main reasons for breeding to produce plants resistant to diseases and pests is to lessen the use of chemical pesticides. Chemical compounds such as pesticides and fungicides are usually used to control the pests and pathogens. Use of these chemical compounds may lead to many health and environmental risks in addition to raising production costs. The continuous use of chemical compounds to control diseases can lead to the emergence of new strains of pests and diseases that are resistant to these chemical compounds and thus chemical control will be ineffectual. Wild tomato plant species are rich in many genes that are resistant to many viral, fungal and bacterial diseases in addition to nematodes. There are many examples of successfully inherited resistance gene through breeding programs to produce plants that are less affected by pests and diseases. The first example of producing plants resistant to biotic stress through plant breeding was in 1934, using wild plants of *S. pimpinellifolium* as a source to produce tomato cultivars resistant to the fungal pathogen *Cladosporium fulvum* (Link) which causes tomato leaf mold (Bai and Lindhout 2007).

10.5.1 Breeding for Viral Diseases

Most tomato cultivars (*Solanum lycopersicum*) are susceptible to several viruses like tomato yellow leaf curl virus (TYLCV), tomato spotted wilt virus (TSWV), tomato mottle virus (ToMoV), tomato brown rugose fruit virus (ToBRFV) tomato mosaic virus (ToMV), yomato leaf curl virus (ToLCV), tomato chlorotic viruses (ToCV), pepino mosaic virus (PepMV), cucumber mosaic virus (CMV), tomato mosaic viruses (ToMV), tomato leaf curl New Delhi virus (ToLCNDV), tomato yellow leaf curl Thailand virus (TYLCTHV), and many others. The begomoviruses are conceded to be the most important viruses that infect cultivated tomato (*S. lycopersicum*) in tropical and subtropical regions. Begomoviruses consist of monopartite and bipartite viruses such as TYLCV, ToLCV, TYLCTHV, and two others newly-identified viruses, tomato leaf curl purple vein virus and (ToLCPVV) tomato leaf deformation virus (ToLDeV) (Gill et al. 2019). The symptoms of most of these viruses cause mosaic patterns on leaves, stunted leaf growth and distortions, and bronzing or marbling patterns on the fruit. The strategies of tomato breeding have mainly focused on introgression of the resistance alleles from wild germplasm. Numerous resistance genes have been introgressed into genetically-recognized cultivars of tomato (Table 10.1). Three major resistance genes to ToMV have been reported in the wild tomato *S. peruvianum*, namely *Tm1*, *Tm2* and *Tm2a*. Among these three genes, *Tm2a* confers resistance to most strains of the ToMV so it is widely deployed in most breeding programs (Panthee et al. 2013). In the last 20 years, a breeding program has been carried out at Miguel Hernández University (Spain) to integrate the resistance to ToMV, TSWV and TYLCV into several tomato landraces (Carbonell et al. 2018). Breeding line UMH1400 that is homozygous for *Ty-1*, *Tm-2a* and *Sw-5* genes (resistance genes for TYLCV, ToMV and TSWV, respectively) and line UMH1401 that is homozygous for *Tm-2a* and *Sw-5* genes are the first statements obtained within the cherry tomato type in the EPSO-UMH tomato-breeding program (García-Martínez et al. 2020). Those two lines were obtained by a cross between the cherry cultivar (selected for fruit morphological characteristics and high quality fruits) (Ruiz and García-Martínez 2009) with an F₁ of commercial cultivar Anastasia as the donor parent of the *Ty-1*, *Tm- 2a*, and *Sw-5*, genes (Perez de Castro et al. 2007). UMH1400 and UMH1401 lines can be used to develop F₁ hybrids through crossing with other landraces of tomato to increase the production by using the genetic resistance to those three viruses (TYLCV, TSWV, ToMV) in heterozygous case (García-Martínez et al. 2020).

Previous studies indicated that TYLCV is one of the most critical plant diseases and it is transmitted by sweet potato white fly (*Bemisia tabaci* Gennadius). In addition, few studies indicated the possibility transmit by seeds (Kil et al. 2016). Recently, breeders have been focused on the transfer of resistant alleles from wild tomato species to cultivated tomato plants, as there are many genes with resistance to some begomovirus such as TYLCV have been found in wild types of tomato (Al-Saikhan et al. 2020). The resistance to TYLCV has been found in many wild tomato species (Table 10.1), including *S. peruvianum*, *S. chilense*, *S. pimpinellifolium*, *S.*

cheesmaniae and *S. habrochaites* (Hutton et al. 2012; Ji et al. 2007; Pico et al. 1996; Scott 2006). To date, there are six resistance loci (*Ty-1*, *Ty-2*, *Ty-3*, *Ty-4*, *Ty-5*, *Ty-6*) were recorded as resistance genes for some begomoviruses (Gill et al. 2019). *Ty-1* resistant gene was derived from *S. chilense* and located on chromosome 6 (Zamir et al. 1994). *Ty-2* resistant gene was derived from *S. habrochaites* and mapped to chromosome 11 (Hanson et al. 2000, 2006). *Ty-3* resistant gene was derived *S. chilense* and mapped to chromosome 6 near to the *Ty-1* locus (Ji et al. 2007). *Ty-4* resistant gene was introgressed from *S. chilense* and was mapped to chromosome 3 (Ji et al. 2009). *Ty-5* resistant gene was derived from *S. peruvianum* and was mapped to chromosome 4 (Hutton et al. 2012). Finally, *Ty-6* resistant gene was introgressed from *S. chilense* and mapped on chromosome 10 (Dhaliwal et al. 2012; Gill et al. 2019). Many genes resistant to begomovirus were introgressed from species related to *Solanum* and they are available for breeding purposes. Scott et al. 1996 demonstrated that *Ty-1* and *Ty-3* genes, derived from *S. chilense*, introgressed into tomato lines, displayed multigenic control of TYLCV resistance, and indicated the presence of additional resistance loci in the studied introgressed tomato lines. In addition, the resistance genes *Ty-1* and *Ty-3* were determined as allelic and coding for RNA-dependent RNA polymerase (RdrRP), that provides the resistance through increasing the cytosine methylation of the viral genomes (Butterbach et al. 2014). Recently, *Ty-2* gene was determined as a nucleotide binding domain and leucine-rich repeat containing (NB-LRR) gene (Yamaguchi et al. 2018).

The hybrid cultivar Tyking, developed by the Royal Sluis Company, Enkhuizen, Netherlands, has been used as a donor for resistance to many of begomoviruses in some breeding programs all over the world. The Tyking cv. is derived from *S. peruvianum* and is useful against numerous of monopartite and bipartite begomoviruses, such as TYLCV, ToMoV Tomato chlorotic mottle virus (TCMV) and ToLCV (Bian et al. 2007; Giordano et al. 2005; Scott 2006). The breeding lines Fla. 8753 and Fla. 344 were developed by (Hutton et al. 2012) and both were derived from cv. Tyking (source of resistance derived from *S. peruvianum*) and LA 1938 (derived from *S. chilense*, but it was clear from molecular markers analysis that this resistance is not based on *Ty-1*, *Ty-2*, *Ty-3* or *Ty-4*. Hutton et al. (2012) reported a recessive allele from cv. Tyking that provides resistance to TYLCV and maps to the *Ty-5* loci. However, Gill et al. (2019) demonstrated that *ty-5* is ineffective against ToMoV. Dhaliwal et al. (2012) developed the tomato line IC613995 resistant to ToLCV and its resistance was confirmed though inoculation with white flies and under natural infection in the field for 3 years. The appearance symptoms of the ToLCV was mild and delayed 45 days after inoculation. The average production yield was 22% more than the control (commercial hybrid NS 524) and the fruits were round and firm with weight average 90 g each. Gill et al. (2019) reported that *Ty-6* as a major resistance gene against monopartite (TYLCV) and bipartite (ToMoV) of begomoviruses and located on chromosome 10 of tomato. They found that the gene action of *Ty-6* gene has incomplete dominance and with intermediate response to resistance when *Ty-6* gene is heterozygous. The populations segregating analysis of *Ty-6* with *Ty-3* and/or *Ty-5* demonstrated that the maximum resistance level against TYLCV is accomplished when *Ty-6* is combined with another

additional resistance gene. Sometimes the use of a single gene for resistance is insufficient and it is better to combine more than one gene. It was found in some cases that the use of one gene was effective to resist some strains of the virus but not effective to resist other strains. Al-Saikhan et al. (2020) evaluated the resistance degree in F_1 , F_2 and BC_1F_1 tomato plants derived from the crossing of CLN2498E (source of resistance gene *Ty-2*) and Castle Rock (TYLCV susceptible parent) against three TYLCV strains. They found that plants (parents, F_1 , F_2 and BC_1F_1) which have the resistance gene (*Ty-2*) even heterozygous (*Ty-2/ty-2*) or homozygous (*Ty-2/Ty-2*) were resistance to some strains (TYLCV-IL and (TYLCV-Has) and susceptible to other (TYLCV-Pep) even if they were carrying the resistance gene. Therefore, these genes are considered to confer partial or complete resistance against TYLCV according to the type of strain. Multiple strains of viruses should be taken in into consideration when establishing breeding programs to control viral diseases, especially begomoviruses viruses, which are the most dangerous viruses that threaten the cultivation of tomatoes all over the world, are known for their multiple strains.

10.5.2 Breeding for Fungal Diseases

More than 20 fungal diseases commonly infect tomato (*Solanum lycopersicum*) plants all over the world. The most important fungal diseases tomatoes are early blight caused by *Alternaria solani* (Ellis & G. Martin), late blight, caused by *Phytophthora infestans*, gray mold caused by *Botrytis cinerea*, *Fusarium* wilt caused by *Fusarium oxysporum* fsp. *oxysporum*, leaf spot caused by *Septoria lycopersici*, *Verticillium* wilt caused by *Verticillium dahliae* and *V. albo-atrum*, leaf mold caused by *Fulvia fulva*, buckeye rot caused by *Phytophthora* spp. and anthracnose disease caused by *Colletotrichum coccodes*. Some of these fungi are causing foliar diseases such as *Alternaria*, *Phytophthora*, *Botrytis*, *Septoria* and *Colletotrichum*, while some are causing vascular diseases such as *Fusarium* and *Verticillium*. Late blight disease caused by *P. infestans* is one of the most important and affective disease in tomato and potato plants everywhere. Five resistance genes for late blight disease have been reported and used in tomato breeding programs (Table 10.2). Those three genes identified in wild tomato species *S. pimpinellifolium*. *Ph-1* gene was the first identified gene as late blight dominant resistance gene and mapped in long arm of the chromosome 7 of tomato wild species *S. pimpinellifolium* (Foolad et al. 2008; Peirce 1971). *Ph-1* gene was not effective against the new races of the pathogen *P. infestans* such as T-1 race that (Foolad et al. 2008; Panthee and Chen 2010). *Ph-2* gene was the second resistance gene for late blight and located near to the bottom chromosome 10 of *S. pimpinellifolium* (Moreau et al. 1998). *Ph-2* gene is incomplete dominant and was affective against T-1 race of *P. infestans*. TP105 and TG233 are two molecular markers were reported as to be closely associated with *Ph-2* gene (Panthee and Chen 2010). Recently, *Ph-3* gene was reported as the strongest source of resistance to late blight disease in tomato breeding and it is an

Table 10.2 Resistance genes sources in wild type tomato species (*Solanum* spp.)

Resistance Gene	Resistance source	Pathogen	Tomato chromosome location	Inheritance	Reference
Viruses					
<i>Ty-1</i>	<i>Solanum chilense</i>	TYLCV	6	Incomplete dominance	Zamir et al. (1994)
<i>Ty-2</i>	<i>S. habrochaites</i>	TYLCV	11	Dominant	Hanson et al. (2000, 2006)
<i>Ty-3</i>	<i>S. chilense</i>	TYLCV, ToMoV	6	Incomplete dominance	Ji et al. (2007)
<i>Ty-4</i>	<i>S. chilense</i>		3	Incomplete dominance	Ji et al. (2009)
<i>Ty-5</i>	<i>S. peruvianum</i>		4	Recessive	Hutton et al. (2012)
<i>Ty-6</i>	<i>S. chilense</i>	TYLCV, ToMoV	10	Recessive	Gill et al. 2019; Dhaliwal et al. (2012)
<i>Tm-1</i>	<i>S. hirsutum</i> Dunal	ToMV	2	Dominant	Young et al. (1988)
<i>Tm-2</i>	<i>S. peruvianum</i>	ToMV	9	Dominant	Young et al. (1988)
<i>Tm-2a</i>	<i>S. peruvianum</i>	ToMV	9	Dominant	Panthee et al. 2013
<i>Sw-5</i>	<i>S. peruvianum</i>	Tospovirus TSWV	9	Dominant incomplete	Brommonschenkel et al. (2000)
<i>Pot-1</i>	<i>S. hirsutum</i>	Potyviruses	3	Recessive	Ruffel et al. (2005)
<i>Cmr</i>	<i>S. chilense</i>	CMV	12	Dominant	Stamova and Chetelat (2000)
Fungi					
<i>Ph-1</i>	<i>S. pimpinellifolium</i>	Late blight <i>P. infestans</i> Mont.	7	dominant	Peirce (1971); Foolad et al. (2008)
<i>Ph-2</i>	<i>S. pimpinellifolium</i>	Late blight <i>P. infestans</i>	10	Incomplete dominant	Moreau et al. (1998); Kumar et al. (2019)
<i>Ph-3</i>	<i>S. pimpinellifolium</i>	Late blight <i>P. infestans</i>	9	Incomplete Dominant	Chunwongse et al. (2002); Shah et al. (2015a, b)
<i>Ph-4</i>	<i>S. pimpinellifolium</i>	Late blight <i>P. infestans</i>	2	Unknown	Chen et al. (2014)
<i>Ph-5</i>	<i>S. pimpinellifolium</i>	Late blight <i>P. infestans</i>	1	Unknown	Merk et al. (2012)
<i>I-1</i>	<i>S. pimpinellifolium</i>	<i>Fusarium</i> wilt <i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> W.C. Snyder & H.N. Hansen	7	Dominant	Sarfatti et al. (1991)

(continued)

Table 10.2 (continued)

Resistance Gene	Resistance source	Pathogen	Tomato chromosome location	Inheritance	Reference
<i>I-2</i>	<i>S. pimpinellifolium</i>	<i>Fusarium</i> wilt <i>F. oxysporum</i> f. sp. <i>lycopersici</i>	11	Dominant	Sarfatti et al. (1989)
<i>I-3</i>	<i>S. pennellii</i>	<i>Fusarium</i> wilt <i>F. oxysporum</i> f. sp. <i>lycopersici</i>	7	Dominant	Hemming et al. (2004); Lim et al. (2006)
<i>Ve-1</i>	<i>S. lycopersicum</i>	<i>Verticillium</i> wilt <i>V. alboatratum</i> and <i>V. dahliae</i> Kleb.	9	Dominant	Fradin et al. 2009; Inderbitzin et al. 2019
<i>Ve-2</i>	<i>S. lycopersicum</i>	<i>Verticillium</i> wilt <i>V. alboatratum</i> and <i>V. dahliae</i>	9	Dominant	Fradin et al. (2009); Inderbitzin et al. (2019)
Bacteria					
<i>Bwr-3,</i>	<i>S. pimpinellifolium</i>	<i>Bacterial</i> wilt <i>Ralstonia solanacearum</i> Smith	3	Incomplete Dominant	Yang and Francis (2007)
<i>Bwr-4,</i>			4	Incomplete Dominant	Yang and Francis (2007)
<i>Bwr-6,</i>			6	Incomplete Dominant	Yang and Francis (2007)
<i>Bwr-8,</i>			8	Incomplete Dominant	Yang and Francis (2007)
<i>Bwr-10,</i>			10	Incomplete Dominant	Yang and Francis (2007)
<i>Bwr-12,</i>			12	Incomplete Dominant	Yang and Francis (2007); Shin et al. (2020)
<i>Bwr-6,</i>	<i>S. lycopersicum</i> var. <i>cerasiforme</i>	<i>Bacterial</i> wilt <i>R. solanacearum</i> ,	6	Incomplete Dominant	Yang and Francis (2007); Shin et al. (2020)
<i>Bwr-7</i>			7	Incomplete Dominant	Yang and Francis (2007)
<i>Bwr-10</i>			10	Incomplete Dominant	Yang and Francis (2007)
<i>Pto-1</i>	<i>S. pimpinellifolium</i>	<i>Bacterial</i> speck <i>Pseudomonas syringae</i> pv. <i>tomato</i> Van Hall, Race 0	5	Dominant	Yang and Francis (2007)

(continued)

Table 10.2 (continued)

Resistance Gene	Resistance source	Pathogen	Tomato chromosome location	Inheritance	Reference
<i>Pto-2</i>	<i>S. pimpinellifolium</i>	Bacterial speck <i>P. syringae</i> pv. <i>tomato</i> Race 0	5	Dominant	Yang and Francis (2007)
<i>Pto-3</i>	S. habrochaites	Bacterial speck <i>P. syringae</i> pv. <i>tomato</i> Race 0	5	Dominant	Yang and Francis (2007)
<i>Pto-4</i>	S. habrochaites	Bacterial speck <i>P. syringae</i> pv. <i>tomato</i> Race 1	5	Dominant	Yang and Francis (2007)
Rx-3	<i>S. lycopersicum</i>	Bacterial spot <i>Xanthomonas euvesicatoria</i> Jones Race T1	5	Dominant	Yang et al. (2005)
Rx-4	<i>S. pimpinellifolium</i>	Bacterial spot <i>X. perforans</i> Race T3	11	Dominant	Pei et al. (2012)
<i>RXopJ4</i>	<i>S. pennellii</i>	Bacterial spot <i>X. perforans</i> Race T4	6	Dominant	Sharlach et al. (2013)
Nematode					
<i>Mi-1</i> , <i>Mi-HT</i> and <i>Mi-9</i>	<i>S. peruvianum</i>	Nematode <i>Meloidogyne</i> spp.	6	Dominant	El-Sappah et al. (2019)
<i>Mi-3</i> and <i>Mi-5</i>	<i>S. peruvianum</i>	Nematode <i>Meloidogyne</i> spp.	12	Dominant	El-Sappah et al. (2019)

incomplete dominant gene that provides resistance against a wide range of *P. infestans* races (Ohlson et al. 2018). The *Ph-3* gene is located in the long arm of chromosome 9 of *S. pimpinellifolium* (Chunwongse et al. 2002). TG591A is a molecular marker reported as closely and associated with *Ph-3* gene. A combination of genes *Ph-2* and *Ph-3* was used in tomato some breeding program (Panthee and Chen 2010). Another late blight resistance gene *Ph-4* was identified in Asia and reported in the line L3708 derived from *S. pimpinellifolium* and mapped in the short arm of chromosome 2 as described by Chen et al. 2014. Additional late blight resistance gene *Ph-5* was characterized in an accession line PI 270,443 derived from *S. pimpinellifolium* (Merk et al. 2012). *Ph-5* resistant gene was mapped and located in the long arm of chromosome 1. *Ph-5* gene is considered highly resistant to late blight disease and provide level of resistance as the line which has combination of *Ph-2* and *Ph-3* genes (Foolad et al. 2014).

Panthee et al. 2017 studied the genes map and QTLs of the genes associated with late blight resistance in a population of tomato plants derived from the crossing between NC 1CELBR and Fla. 7775 lines. They evaluated 250 plant individuals of F₁, F₂ families and two major of QTLs (probably caused by the *Ph-2* and *Ph-3* genes) associated with the late blight disease resistance which were reported and located on chromosomes 9 and 10. Additionally, there was a minor QTL mapped on chromosomes 12, not previously reported, that may be novel and worth investigating. *Fusarium* wilt is a tomato disease caused by *F. oxysporum* f. sp. *lycopersici* and three races of the pathogen are reported to cause the disease in tomato (Panthee and Chen 2010). *I-1*, *I-2* and *I-3* are three loci characterized as resistance genes for these three races of *F. oxysporum* f. sp. *lycopersici* (Table 1). *I-1* locus is reported as a single dominant resistant gene for *Fusarium* wilt in tomato, the *I-1* gene was identified in *S. pimpinellifolium* and mapped to chromosome 7 (Sarfatti et al. 1991). The *I-2* gene was mapped to chromosome 11 and identified in the PI126915 line derived from *S. pimpinellifolium* (Sarfatti et al. 1989). *I-3* locus is reported as a resistant gene against the *Fusarium* wilt of tomato race-3 and identified in lines LA716 and PI414773 derived from the wild type *S. pennellii* and located on chromosome 7 (Hemming et al. 2004; Lim et al. 2006). *Verticillium* wilt is considered one of the most important damaging disease in tomato and causes a vascular disease in the plants. The disease is caused by *Verticillium dahliae* and *V. alboatratum* fungi that are soil borne and have a wide host range worldwide; there are two races of the *Verticillium* wilt disease (*Ve-1*, *Ve-2*) (Carrer Filho et al. 2016). There are two single dominant resistant genes (*Ve-1*, *Ve-2*) against *Verticillium* wilt disease recognized to provide resistance to *V. dahlia* and other species in different hosts (Fradin et al. 2009). The *Ve-1* gene was identified in *S. lycopersicum* var. *cerasifore* and mapped on the short arm of chromosome 9 near marker GP39 (Diwan et al. 1999). Fradin et al. (2009) showed that the *Ve-1* gene provided resistance against race 1 of *V. alboatratum* and *V. dahliae* in tomato, but did not provide any resistance to race 2.

10.5.3 Breeding for Bacterial Diseases

Viral and bacterial diseases, as well as nematodes, may cause severe damage and reduce the yield and quality of tomatoes; they more difficult to control than those caused by fungal pathogens. To manage these diseases, integrated pest management has been followed, such as sanitation, crop rotation, using resistant varieties and disease elimination. Devising resistant varieties through breeding is considered one of the most important means to control these types of diseases. In the past three decades great progresses has been achieved to develop numerous cultivars with resistance to viral diseases, fungal diseases and nematodes, while less work has been directed in breeding programs to provide lines resistant to bacterial disease (Yuqing et al. 2018). Bacterial wilt is caused by *Ralstonia solanacearum* and bacterial spot by *Xanthomonas campestris* pv. *vesicatoria*; bacterial speck produced by

Pseudomonas syringae pv. *tomato* and bacterial canker, caused by *Clavibacter michiganensis* ssp. *michiganensis* are four common bacterial diseases in tomato fields. Bacterial wilt is one of the major serious disease affecting tomato and other solanaceous crops in tropical and subtropical regions. There are many sources of resistance to the bacterial wilt disease that were identified in some tomato cultivars and species, including *S. lycopersicum* var. *cerasiforme* and *S. pimpinellifolium* (Aslam et al. 2017; Yang and Francis 2007; Yin et al. 2005).

Resistance to bacterial wilt disease can be dominant, partially dominant or recessive, depending on the resistance sources and bacterial strains used (Yuqing et al. 2018). Seven QTLs located on chromosomes 3, 4, 6, 8, 10 and 12 from the resistant line Hawaii7996, derived from *Solanum pimpinellifolium*, and 3 QTLs located on chromosomes 6, 7 and 10 from resistant accession L285, derived from *S. lycopersicum* var. *cerasiforme* (Table 10.1) have been reported (Yang and Francis 2007). In the past 5 decades, at least 20 cultivars with partial resistance to bacterial wilt were developed (Yuqing et al. 2018). On the other hand, there is an effort to use the wild type *S. nigrum* L. as a source of resistance to produce tomato plants resistant to bacterial canker. Moderate to high levels of resistance were obtained through back-crossing of the susceptible line of tomato 98-1 with a wild type of *S. nigrum* as a source of resistance, but they produced plants short in stature and bore small fruits. Therefore, it is suggested that *S. nigrum* might not be a suitable source to produce tomato cultivars resistant to bacterial canker (Li et al. 2012). Other sources of resistance to bacterial canker have been reported in many other wild types of tomatoes and have been incorporated into cultivated tomatoes. There are some molecular markers linked to QTLs that provide resistance to bacterial canker disease identified in the tomato wild type *S. habrochaites* accession LA407 and *S. peruvianum* accession LA2157 (Sen et al. 2015). The locus *Rcm2* and *Rcm5.1* were identified in *S. habrochaites* and were mapped to chromosomes 2 and 5, respectively (Sen et al. 2015; Yang and Francis 2007). Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* is an important disease causing economic losses in tomato fields in cool and moist environments (Yuqing et al. 2018); two races (0, 1) are reported by Thapa et al. (2015). Several resistance genes to bacterial speck disease have been identified in several wild types of tomato (Table 10.1) and the most important study suggests that resistance to this disease is simply inherited (Yuqing et al. 2018). *Pto-1* gene derived from *S. pimpinellifolium*, *Pto-2* gene derived from *S. pimpinellifolium* and *Pto-3* gene derived from *S. habrochaites*, are reported as resistant to race 0, while *Pto-4* gene derived from *S. habrochaites* reportedly is a resistance gene to race 1 (Yang and Francis 2007). *Pto* genes provide effective resistance against bacterial speck disease race 0, so it has been widely used in many breeding programs (Pedley and Martin 2003; Yang and Francis 2007).

Bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* is an important disease in tomato fields, in partially wet and humid environments of the world (Jones et al. 2014). Four races (T1, T2, T3, T4) have been reported for the pathogen *X. campestris* based on their virulence ability (Jones et al. 2005). Resistance for those races (T1, T2, T3, T4) is quantitatively inherited and several QTLs have been characterized and confirmed in both wild and cultivated types of tomato germplasm

(Hawaii7998, Hawaii7981, PI114490) derived from *S. lycopersicum* var. *cerasi-forme*, PI128216 derived from *S. pimpinellifolium* and LA716 derived from *S. pennelli* (Liabeuf et al. 2015).

10.5.4 Breeding for Nematodes

Nematodes are one of the most important pathogens affecting plant health, growth and production everywhere. Tomatoes are considered one of the main hosts of root-knot nematode (*Meloidogyne* spp.) There are more than 90 species of this genus; in addition, some have several races. *Meloidogyne javanica*, *M. incognita*, *M. arenaria* and *M. hapla*, are the most economically- destructive species worldwide (El-Sappah et al. 2019). To reduce the pesticide use as a chemical control against nematodes, many modern tomato varieties that carry the *M. incognita* (*Mi*) gene as a single dominant resistant gene were produced through plant breeding. The *Mi* gene can provide resistance to three species of root knot nematodes (*Meloidogyne* spp.). The *Mi* nematode resistant gene is derived from the tomato wild type *S. peruvianum* and was introduced into the cultivated tomato (*S. lycopersicum*) in 1940 (Milligan et al. 1998). The resistance of *Mi* genes is induced 2 weeks after tomato seed germination (Martinez de Ilarduya et al. 2001). Rex-1 is reported as a molecular DNA marker associated with the *Mi* gene. Ten genes (*Mi-1*, *Mi-2* *Mi-3*, *Mi-4*, *Mi-5*, *Mi-6*, *Mi-7*, *Mi-8*, *Mi-9*, *Mi-HT*) are identified and reported as resistant to root knot nematode (*Meloidogyne* spp.), but only five have been mapped (El-Sappah et al. 2019). *Mi-1*, *Mi-9* and *Mi-HT* genes are mapped to the short arm of chromosome 6, while *Mi-3* and *Mi-5* are located on chromosome 12 and located between the Rex-1 and C&B DNA markers (Jablonska et al. 2007). The *Mi-1* gene is considered the most successful *Mi* gene used commercially in breeding to produce tomato cultivars with high resistant to root knot nematode. The *Mi-1* gene provides resistance against three species of root knot nematode (*M. incognita*, *M. arenaria*, *M. javanica*), while it does not provide any resistance to other species such as *M. hapla*, while *Mi-2* gene was reported previously as a resistant gene of four species of nematode (El-Sappah et al. 2019). The main problem facing breeders is that the resistance to root knot nematode breaks down at high temperatures, especially *Mi-1* gene while some genes (*Mi-2*, *Mi-3*, *Mi-4*, *Mi-5*, *Mi-6*, *Mi-9*, *Mi-HT*) are able to provide resistance at high temperature levels (Wu et al. 2009).

10.6 Breeding for Abiotic Stress Tolerance

Tomato production is affected by several environmental factors, such as temperature, salinity and drought (Lin et al. 2006). The evaluation of the genetic basis in plant tolerance to abiotic stress began by using genetic model plants, such as thale

cress (*Arabidopsis thaliana*). However, recent advances in DNA technology and molecular marker methodologies, have opened up a whole era of evaluating inheritance of different abiotic stresses such as drought, salinity, heat and chilling. Molecular markers of abiotic stresses became more attractive since specific QTLs could be identified and the association of each QTL to a certain trait became possible (Blumwald et al. 2004). The impact of climate change on the production yield of tomato cultivars can be directly evaluated by molecular markers (Lin et al. 2006).

10.6.1 Salinity Stress Tolerance

Tomato became an important model plant for the study of genetic basis of tolerance to salinity stress due to the possibility of obtaining hybrids from intraspecific and interspecific crosses with the wild relatives of tomato (*Lycopersicon pimpinellifolium*, *L. cheesmanii*, *L. peruvianum*, *L. pennellii*). Successful identification of quantitative trait loci (QTLs) by marker analysis has revealed the genomic locations of salt tolerance genes in tomato (Foolad and Jones 1993). RAPD markers were used to identify salinity tolerance genes in parental DNA and bulks, two RAPD markers (OPX-17, MRTOMR-022) were found in the resistant parent (LA1606) as well as the resistant F₂ bulk (Ezin et al. 2018). Foolad et al. (1999, 2001) suggested that salinity tolerance trait could be improved by MAS using interspecific variation during the vegetative stage. A number of molecular markers were identified in wild tomato relatives (*Solanum pennellii*, *S. pimpinellifolium*) and associated with salt tolerance by promoting rapid seed germination (Table 10.3) (Foolad et al. 1997, 1998).

10.6.2 Heat Stress Tolerance

Increased temperature causing heat stress to plants is recognized as an important global problem. Short- or long-term high temperatures can lead to alterations in morphological, anatomical, physiological, biochemical and on molecular levels. Such alterations are manifested as reduction in growth, yield and quality. The use of advanced genetic tools in breeding for heat stress tolerance aims at creating varieties that have improved thermo-tolerance. Heat tolerance is considered a genetically-complex trait due to significant genotype x environment interaction (Palta et al. 1979). Recently, heat tolerance molecular markers were screened and marker-assisted selection (MAS) used to develop and improve heat tolerance in tomato (Faruq et al. 2012). Most QTL mapping was carried out on chilling and salinity stress; less work was assigned to mapping in heat-stressed plants (Foolad 2007).

Table 10.3 Markers used for locating quantitative traits in tomato (*Solanum*) species and cultivars

Tomato species and crosses	Molecular Marker ^a	Quantitative trait locus (QTLs) on chromosome number	Traits governed	References
<i>S. pennellii</i>	RFLP	1, 2, 3, 7, 8 9,12	Contribute to rapid germination under salt stress	Foolad et al. (1997)
	RAD-seq	2, 7, 8, 9	Salinity stress seedling survival, leaf/plant damage	Bolger et al. (2014)
<i>S. pimpinellifolium</i>	RFLP	1, 2, 5, 7, 9,12	Contribute to rapid germination under salt stress	Foolad et al. (1998)
	SNP analysis	Salinity tolerant related QTLs	Inositol phosphate pathway	Razali et al. (2018)
Cross between: <i>S. lycopersicum</i> x <i>S. pimpinellifolium</i>	RFLP	1, 3, 5, 9	Affect ST during vegetative stage	Foolad and Chen (1998)
	SNP analysis	1, 7, 12	Affect heat tolerance during reproductive stage	Gonzalo et al. (2020)
<i>Cross between: S. lycopersicum</i> x <i>S. pimpinellifolium</i>	RFLP	1, 3, 5, 6 , 11 (salt Tolerant related QTLs)	Affect ST during vegetative stage	Foolad et al. (2001)
<i>Lycopersicon pimpinellifolium</i>	RFLP	Several salt tolerant related QTLs	Affect ST during reproductive stage	Monforte et al. (1996)
<i>S. lycopersicum</i> cv. MAGIC.	SNP analysis	1, 2, 3, 10, 11	Affect ST during vegetative stage	Diouf et al. (2018)
cv. Nagcarlang	RNA-seq	1, 2	Heat tolerance during vegetative stage	Wen et al. (2019)
	SNP analysis	1, 2, 3, 7, 8, 11	Heat tolerance during reproductive stage	Xu et al. (2017)

^aRAD-seq = Restriction site-associated DNA sequencing; RFLP = restriction fragment length polymorphism; SNP = Single-nucleotide polymorphism

10.7 Tomato Breeding Biotechnology

The genomic characteristics and growth habits of tomato have made it a model in plant research (Ranjan et al. 2012). Nevertheless, the high diversity and the genetic variability of tomato landraces is not yet fully explored and the scarcity of genetic background information and performance in diverse climates hinders the use of such varieties in breeding (Alvarenga 2004; Carelli et al. 2006). Successful traditional breeding and improvement methods of qualitative and quantitative tomato traits are time-consuming (Carbonell et al. 2018; Panthee and Gardner 2011), with an average time for market commercialization of 5 years (Bai and Lindhout 2007). On the other hand, recent advances in tomato tissue culture (Wolters et al. 1994),

mutagenesis (Chaudhary et al. 2019), recombinant DNA technologies (Gosal et al. 2009), transformation protocols and transient expression assays (Fernandez et al. 2009), provide additional options for the improvement of tomato cultivars. Obstacles in breeding, such as incompatibility stands as a barrier in crossing wild and cultivated tomatoes, can be overcome with tissue culture technologies through embryo rescue, in vitro cultivation or protoplast fusion and somatic hybridization (Wolters et al. 1994).

10.7.1 Mutagenesis

Mutations play an important role in the development of plant varieties and biological diversity, in addition mutations lead to the emergence of new varieties that contribute to sustainable production. Since the rate of occurrence of natural mutations is very low, the creation of mutations is very important to enhance genetic diversity. Inducing mutations helps breeders to produce plant varieties with characteristics consistent with climatic and environmental conditions. Through breeding, it is possible to create the desired induced mutations in order to keep them from being lost due to climatic fluctuations. Mutagenesis is an effective method for the creation of influencing modifications. Several methods have been developed to create genetic mutations, including physical and chemical methods. Mutagenesis is one of the effective ways to improve the chances of obtaining genetic variation linked to desirable phenotypes and it can help to understand gene functions. Tomato is considered a good model for successful use of mutations affecting important genes for plant breeding. There are several studies on mutagenesis in tomato to discover gene function related to economic characteristics. In addition, many studies have used chemical mutagenic agents such as methane sulfonate and physical agents, like gamma rays, as catalysts to produce mutations in tomatoes. (Chaudhary et al. 2019). Physical and chemical mutagenic agents that lead to random mutations produce many unwanted result (Chaudhary et al. 2019).

10.7.1.1 Chemical Mutagenesis

Several chemical agents have a mutagenic effect in plants such as nitric oxide, ethyl methanesulfonate (EMS), sodium azide, nitrous acid and hydrazine hydrate. Ethyl methanesulfonate is considered the most widely used chemical mutagen due to its high proficiency to create mutations. Ethyl methanesulfonate, sodium azide and hydrazine hydrate have been used as chemical mutagenesis in some tomato cultivars, such as many maker M82 and Red, to produce phenotypic differences and to improve some desirable traits such as crop and fruit quality, and plant disease resistance (Chaudhary et al. 2019). Ethyl methanesulfonate and hydrazine hydrate have been used to induce mutant tomato populations of genotype Arka Vikas. It was found that there was a gradual decrease in the percentage of germination and the

height of seedlings with an increase in the concentration of mutagens, while the maximum biological inhibition for the tomato plants occurred in the combination of both ethyl methanesulfonate and hydrazine hydrate treatments (Laskar et al. 2016). Ethyl methanesulfonate has been used as an effective mutagen to develop tomato varieties resistance against some potyviruses, such as tobacco etch virus (TEV) and potato virus Y (PVY) (Piron et al. 2010). Chemical mutagenesis has been found to be more efficient to control some widespread parasitic weeds such as broomrape (*Orobanche ramosa* L.) which causes significant economic losses in tomato fields. Tomato seeds treated with ethyl methanesulfonate and mutant tomato plants were screened for resistance to broomrape. Six tomato lines with significantly an increased level of resistance to broomrape were selected (Kostov et al. 2007).

10.7.1.2 Physical Mutagenesis

Physical mutagenesis plays an important role in producing various genetic mutations that can be used for many purposes in plant genetic studies. The use of physical mutagens requires special requirements such as highly skilled technicians and special laboratory precautions due to the use of radiation gamma rays, X-rays and fast and thermal neutrons which are typically employed as physical mutagenesis to produce mutations. Radiation, using gamma rays and ionization with neutrons, are commonly used for mutagenesis in tomatoes. Fast neutron and certain other physical mutagens induce promising variabilities in tomato plants for enhanced yield and quality. Several phenotypic mutations can be produced using gamma rays, as they cause large-scale deletions and chromosomes reconstruction. Matsukura et al. (2007) found that low and medium doses of gamma rays might cause a high amount of valuable mutants with regular yielding properties. They produced about 6000 tomato mutant lines by using gamma rays and 24 morphological mutant lines and in addition 8 brix mutant lines were screened. QTL analysis demonstrated the presence of 2 loci in brix mutations that could be used to determine the brix regulation genes in tomatoes. Physiological effects of gamma radiation and LD50 were determined on in vitro growth of Ramsi tomato seedlings in Saudi Arabia by (Alwael et al. 2020). The seeds were treated by different doses of gamma radiation from 0 to 500 Gy using ^{60}Co Nordion Gammacell 220. They found gradual inhibition of germination percentage. The inhibition of the germination was more than 50% of seeds when they use the doses greater than 300 Gy during the first 3 days after treatments. In addition, gradual inhibition of root length, Shoot height and cotyledon length were observed with an LD50 of 43.3, 24, and 57.5 Gy, respectively.

Reverse genetics is a new method developed to identify the deletion mutations of targeted genes. Recent studies have shown to be effective in the use of fast neutron mutagenicity in reverse genetics. The Deletagene method is the major fast neutron technique used in plants. Through this method fast neutrons are used to produce mutations in seeds and caused deletions which were analyzed using specific primers flanking target genes in a polymerase chain reaction (Gilchrist and Haughn 2010; Li

et al. 2001). This generated a mutant library may add value to genetic resources for plant breeding and gene function in tomatoes (Matsukura et al. 2007).

10.7.2 Gene Editing

The protracted domestication processes, over time, of cultivated tomato have led to a massive erosion of genetic diversity. The consequences of breeding for higher yield and larger fruit size were some loss of flavor and nutritive value (Meyer and Purugganan 2013), along with the loss of biotic resistance and abiotic tolerance traits.

Recent advances in genome editing strategies present new options for breeders (Fig. 10.3) to regain the lost desirable traits through targeting genes in a sequence-specific manner to insert sequences, change amino acids or to modulate the expression (Zsogon et al. 2018). The first two strategies are based on protein-DNA interactions like zinc finger (Urnov et al. 2010) and transcription activator-like nucleases (Joung and Sander 2013). The third strategy depends on the RNA-guided DNA endonuclease system associated with clustered regularly interspaced short palindromic repeats (CRISPR-Cas9: Chaudhary et al. 2019). The application of CRISPR-Cas9 technology has flourished in the past decade on tomato, revealing lost traits of cultivated varieties in the genome of wild-type tomatoes (Fig. 10.4).

Domestication of wild tomatoes, as an alternative option for modifying cultivated tomato, was greatly accelerated by gene editing technologies. Although gene editing is still being developed, results have demonstrated the strength of its tools in modifying and improving cultivated and wild tomatoes. The improvement of wild tomato species, such as *Solanum pimpinellifolium*, was successfully achieved by

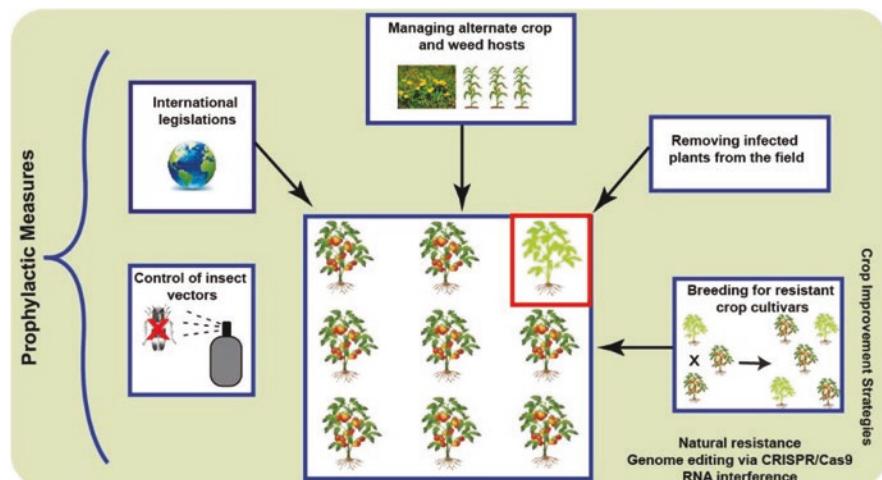


Fig. 10.3 Various control strategies against insect/pests and viruses in tomato. Figure produced using version CS5 of Adobe Illustrator software. (Figure constructed by A. Rezk, and M.N.Sattar)

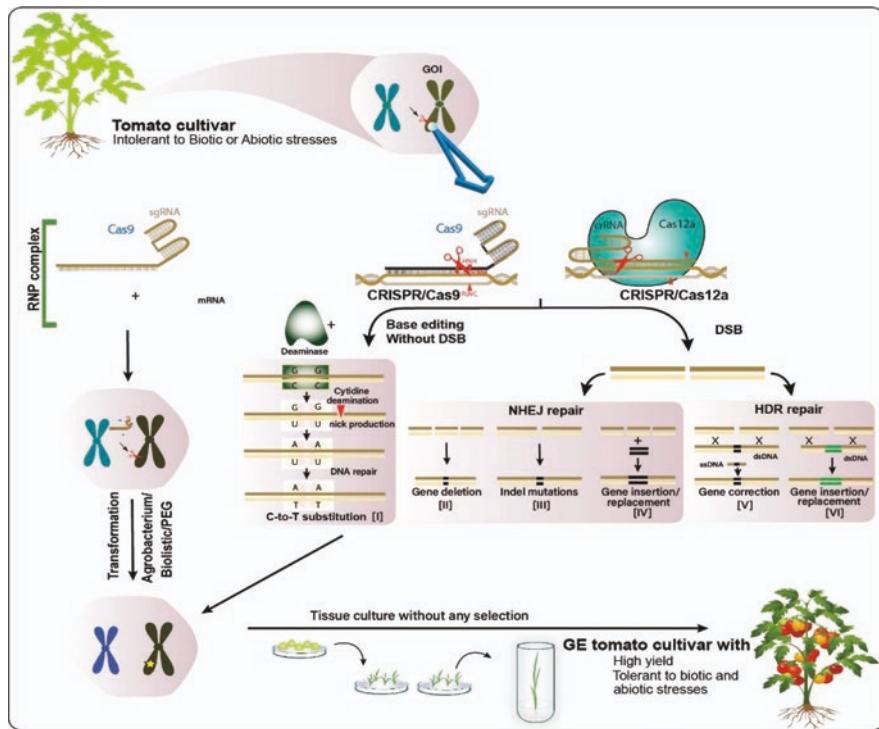


Fig. 10.4 CRISPR-Cas mediated genome editing, base editing/substitution and production of transgene-free/DNA-free GE tomato plants. Two Cas proteins (Cas9, Cas12a) with higher GE potential in tomatoes are depicted. CRISPR-mediated GE can be executed either through DSB dependent pathways (either through homology arms [HDR] or randomly [NHEJ]) or DSB independent pathway (Base editing/substitution), which can be accomplished using a deaminase (C-to-T BE is shown). In addition, transgene-free/DNA-free GE tomato plants can be achieved via RNP-complex by transiently expressing CRISPR-Cas system in tomatoes. Figure produced using version CS5 of Adobe Illustrator software. (Figure constructed by A. Rezk, and M.N.Sattar)

targeting key genes for fruit shape, size, weight, number and nutritive value via CRISPR-Cas9 technology, where the resulting *S. pimpinellifolium* showed a three-fold increase in fruit size and tenfold in fruit number, with a 500% increase in lycopene (Zsogon et al. 2018). Moreover, editing genes related to growth habits, such as day-length, shoot development and flowering (Li et al. 2018) resulted in a domesticated form of wild tomatoes with its original qualities of biotic resistance and abiotic tolerance (Bouzrour et al. 2020). Using CRISPR-Cas9 on tomato has several advantages over other gene editing methods, including simplicity and cost effectiveness (Zsogon et al. 2018). The absence of exogenous additives in gene editing approaches mark the finalized product as a mutant, but not as a genetically-modified organism GMO (Chaudhary et al. 2019), an extra positive feature for gene editing by CRISPR-Cas9 technology.

10.7.3 In Vitro Techniques

The art and science of plant propagation by tissue culture has been widely used for multiplication, disease elimination, improvement and production of secondary metabolites from plants, where a small explant source can generate several thousand plants under controlled growth conditions. The amenability of tomato species to in vitro techniques has enabled scientists to mass propagate (Al-Khayri et al. 2017; Fari et al. 1992), develop cell lines for biotic and abiotic stress (Rahman and Kaul 1989), produce interspecific hybrids (Piosik et al. 2019), generate somaclones (Van den Bulk et al. 1990) and generate anther culture to produce haploids (Farooq et al. 2010).

In vitro culture employs growth regulators to grow and develop tissues of the propagated plants; the propagation process includes several steps starting with the selection of the proper explant source and sterilization. Callus is initiated by the proper balance of auxin and cytokinin growth regulators, where callus can be divided into small pieces and each one will develop into a complete plant. The process of micropropagation of cv. Ramsi tomato is illustrated in Fig. 10.5 (Al-Khayri et al. 2017).

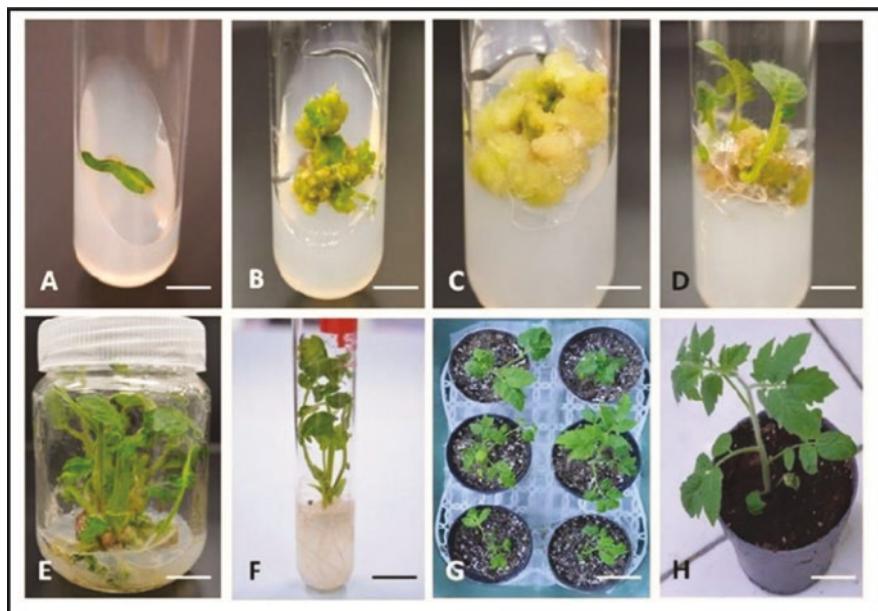


Fig. 10.5 Tissue culture micropropagation of Ramsi cv. tomato cotyledons. (a) The source explant (bar = 9.6 mm), (b) Initiation of shoots from callus (bar = 9.2 mm), (c) Tomato callus (bar = 6.8 mm), (d) Formation of tomato shoots (bar = 8.5 mm), (e) Growth of shoots (bar = 15.3 mm), (f) Single plantlet (bar = 18.4 mm), (g) Acclimatization of plantlets (bar = 64.5 mm), (h) Full acclimatized tomato plant in soil (bar = 48.4 mm). (Source: Al-Khayri et al. 2017)

In tomato, caulogenesis protocols have been optimized for different cultivars and wild tomatoes to generate plant organs or develop somatic embryos (Rzepka-Plevneš et al. 2007; Shah et al. 2015a). Surprisingly, wild tomatoes have shown more satisfactory results than the cultivated *Solanum lycopersicum* in response to in vitro culture; *S. pimpinellifolium*, *S. peruvianum* and *S. glandulosum* have shown better potential for regeneration and morphogenesis (Lech et al. 1996).

The transfer of quality traits from wild types or related species by crossing with cultivated tomatoes can be restricted for many reasons, especially incompatibility between species and low viability of progeny seeds. Hybridizing *Solanum lycopersicum* with *S. sisymbriifolium* produced undeveloped embryos at the globular stage, but this cross was rescued at the early embryo stage and regenerated a hybrid tomato with biotic resistance traits, transferred from the latter species (Piosik et al. 2019). Another example is the attempt to cross *S. lycopersicum* with *S. peruvianum*, resulting in embryo or flower abortion, this cross was also rescued by in vitro techniques (Sacks et al. 1997).

Repeated cycles of tissue culture can result in changes, observed in tissue culture plants, designated as somaclonal variation. Such variants present a new genetic resource to tomato breeders (Van der Bulk et al. 1990). Tomato somaclones are evaluated and screened for new traits, such as bacterial resistance (Van den Bulk et al. 1991), fungal resistance (Popoola et al. 2015), viruses (Smith and Murakishi 1987) and abiotic stress tolerance (Rzepka-Plevneš et al. 2007).

10.7.4 Transgenesis

Recombinant DNA and transformation technologies allow the insertion of a specific gene into the genome by *Agrobacterium* mediation or particle bombardment transformation protocols, avoiding the transfer of undesirable traits and overcoming the incompatibility barrier. It is a powerful approach for detailed characterization of specific gene function and interaction with other genes. Release of the first transgenic food crop was the tomato cultivar Flavr Savr™ in 1994, followed by the long shelf-life tomatoes in 1996 (Bai and Lindhout 2007), delayed ripening (PK-TM8805R) and insect resistant (5345) cultivars (Gerszberg et al. 2014). All these transgenic cultivars had undesired field traits (Van Heusden and Lindhout 2018).

Transgenic tomatoes were also developed for various traits, such as abiotic tolerance (Khare et al. 2010; Wang et al. 2014), disease resistance (Dutta et al. 2015), metabolic engineering (Gonzali et al. 2009; Lu et al. 2013) and the production of pharmaceuticals, such as the human interleukin-12 protein (Elías-López et al. 2008), human beta-amyloid (Youm et al. 2008) and edible vaccines (Sohrab et al. 2017). Several genes were identified and transferred into the tomato genome for abiotic tolerance, such as soil heavy metals, drought, salinity and temperature (Gerszberg et al. 2014). Strategies for biotic resistance to viruses, bacteria and fungal pathogens differ according to the pathogenic agent, from the expression of resistance R genes

of other plant species in tomato (Lin et al. 2004) to gene silencing methodologies (Abhary and Rezk 2015).

The development of new tomato cultivars, through either breeding or transgenic approaches, requires extensive field trials under natural conditions (Witcombe et al. 2008), an important test to understand the biological process of the generated cultivars and a mandatory requisite for stakeholders. The high cost of filing patent applications is an obstacle, as well as the current ban on the commercial sale of transgenic tomatoes. At present tomatoes are genetically transformed only for research purposes (Bai and Lindhout 2007). A major drawback of transgenic tomatoes is public acceptance and the confusion created by different regulations in the USA and the EU.

10.8 Conclusions and Prospects

Tomato (*Solanum lycopersicum*) belongs to the diverse genus *Solanum* of the Solanaceae family (Bai and Lindhout 2007). The Solanaceae family is part of the Asterid Clade in flowering plants, which emerged 59 million years ago (Bell et al. 2010) and contains important economic crops, such as potato, eggplant, pepper and tobacco. Wild tomatoes thrive in their center of origin, the West Coast of South America (Peralta and Spooner 2007), along with diverse varieties and landraces in Mesoamerica (Blanca et al. 2015). Morphological and genomic studies have shown that domestication of tomatoes began in the Andes with *S. lycopersicum* var. *cerasiforme* (Razifard et al. 2020). Later, the *cerasiforme* variety was dispersed to Mesoamerica by pre-Hispanic trading (Blanca et al. 2012), where it underwent further domestication. In the sixteenth century, Spanish navigators introduced tomato seeds, along with other plants, to the Old World through Europe and named the plant *tomato*, derived from the Aztec word *tomatl* (Saavedra et al. 2017). In the nineteenth century, tomato was extensively domesticated in Europe (Colvine and Branthôme 2016) and the USA (Atherton and Rudich 1986), to the form of a smooth, juicy, red-fruited plant. Today, tomatoes are cultivated and consumed worldwide, fresh or processed, with a total world production reaching more than 182 million mt in 2018 (FAOSTAT 2018).

Cultivated tomato (*Solanum lycopersicum*) is estimated to contain only 5% of the genetic diversity found in its wild relatives. A worldwide collaborative network has been informally established to improve tomato varieties and genetic resources were explored and collected in gene banks (Appendices I, II), gene maps were constructed by molecular markers, biotechnological approaches were applied to mutagenize and edit genes, and transgenic approaches clarified the functional properties of genes and tissue culture technologies facilitated the transfer of traits between incompatible species.

There are more than 10,000 varieties of tomato available in gene banks and markets (Gerszberg et al. 2014), a huge number that requires high analytical research power, experimentation and evaluation processes to exploit. Traditional breeding

methodologies to obtain biotic and abiotic stress tolerance qualities have been accelerated by new biotechnological tools, starting from gene mapping with the aid of molecular markers to mutagenesis, gene editing, transgenic approaches and in vitro techniques. Although tomato varieties and cultivars are being developed to resist many pathogens, the high number of disease agents exceeds current research capabilities. Catastrophic tomato crop losses are reported in many countries due to various tomato viral, bacterial and fungal diseases. Therefore, tomato targeted funding and research power is a necessity to follow-up the arising disease problems facing tomato cultivation. Although transgenic approaches to improve tomato quality are developed with standard operating protocols, unknown field outcomes and consumer acceptance stand as a barrier to commercialization. Currently, transgenic tomatoes are generated for research purposes only. Mutagenesis and gene editing approaches are promising technologies for the future of tomato breeding with the ability of targeting more than one gene at the same time; such emerging technologies require extensive background studies on the genomes of tomato wild species and cultivars, including gene mapping and gene function in addition to metabolomics and transcriptomic studies, beyond the current available capabilities. In future, the advancement of tomato basic research in –omics will enrich the core database of tomato informatics, and breeders will have to devise a way to plan and work on precise tomato improvement in accord with consumer demand.

Acknowledgements We thank the editors for inviting us to contribute a chapter on tomato biotic and abiotic breeding in this book. We thank Dr. M. N Sattar, King Faisal University for helping to design Figs. 10.3 and 10.4.

Appendices

Appendix I: Research Institutes Relevant to Tomato

Institution name	Specialization	Address	Contact information and website
California Tomato Research Institute (CTRI)	Processing tomato crop production	Vegetable Crops Research & Information Center - University of California, Davis, California, USA	zach@tomatonet.org https://tomatonet.org/ContactUs
Tomato Genetic Cooperative	Tomato genetic resources	University of Florida, Gainesville, FL 32611, USA	https://tgc.ifas.ufl.edu/

(continued)

Institution name	Specialization	Address	Contact information and website
Department of Tomato and Self Pollinated Vegetable Crops Research	Tomato breeding research	Agriculture research center (ARC), Giza, Egypt	abdo@clae.sci.eg hortinst@yahoo.com http://www.hortinstitute.com/
Asian Vegetable Research and Development Center (AVRDC)	Crops and vegetable research and development	60 Yi- Min Liao; PO Box 42 , 741 Shanhua, Tainan, China	avrdcbox@netra.avrdc.org.tw http://www.avrdc.org.tw/
ICAR-Indian Institute of Horticultural Research	Production and pest management of vegetables and tomato	Indian Institute of Horticultural Research, Hessaraghatta Lake, Bangalore 560 089, India	https://iihr.res.in/tomato-varieties atic.iihr@icar.gov.in
Faculty of Agronomy at University of San Carlos of Guatemala	Breeding of tomato, potato and medicinal plants. Crossing and evaluation of segregating populations and tomato breeding lines are carried out annually	Faculty of Agronomy at University of San Carlos, Guatemala	http://www.usac.edu.gt/
TOMATECH	Focusing exclusively on the development of innovative, superior quality, hybrid tomatoes	Calle Zurbano, 23 - PISO 1 DR, Madrid, 28010 , Madrid, S.L. Spain	https://www.tomatech.com/team-2-4-2/ maury@tomatech.es

Appendix II: Genetic Resources of Tomato

Cultivar	Important traits	Reference	Image	Image source
Plum tomato	Deep red color, oval or cylindrical in shape, fewer locules than round tomatoes semi-determinate its market varieties are Roma VF and San Marzano. Grown for sauce and packing purposes. Resistant to <i>Fusarium</i> and <i>Verticillium</i> wilt, early blight	https://www.deruitseeds.com/en-no/tomato/plum.html		http://www.gradinamea.ro/Tomato_3262_574_1.html
Better Boy	Deep red color, round, medium or large in size, maturity from 70–80 days, grown for sauce purposes, indeterminate, resistant to <i>Fusarium</i> and <i>Verticillium</i> wilt and root knot nematode	Tomato - Vegetable Directory - Watch Your Garden Grow - University of Illinois Extension. urbanext.illinois.edu . Retrieved 22 June 2018		CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=99259
Celebrity	Deep red color, large firm fruit, round, determinate, maturity 70 days, hybrid, grown for sauce purposes, crack resistant, resistant to <i>Fusarium</i> and <i>Verticillium</i> wilt, root knot nematode and TMV	https://njaes.rutgers.edu/tomato-varieties/variety.php?Celebrity		By © 2005 User:FoeNyx - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=115428
Moneymaker	Red color, firm fruit, round tomato, greenhouse tomato, indeterminate, maturity 75–80 days, grown for sauce purposes, popular cash crop for farmers, and it is a favorite of home gardeners, highly heat tolerant, 113–170 g fruits	T&M Seeds online shop entry for Tomato Moneymaker. Retrieved 2011-03-07		http://www.reimerseeds.com/tomato_1162.aspx
Mountain Pride	Deep red color, round tomato, determinant hybrid, grown for sauce purposes, juicy flavor perfect for serving fresh or cooking, crack resistant, grown for sauce, <i>Fusarium</i> and <i>Verticillium</i> tolerant, maturity days 77 days, 227–283 g fruits	https://cloversgarden.com/products/mountain-pride-tomato-plants		https://cloversgarden.com/products/mountain-pride-tomato-plants

Big Beef	Deep red color, round, large fruits, indeterminate hybrid, maturity days 70–80 days. Grown for sauce purposes, resistant to <i>Fusarium</i> wilt races 1 and 2, <i>Verticillium</i> wilt, <i>Alternaria</i> stem canker, nematodes, gray leaf spot and TMV, outstanding taste fruits	Selecting tomatoes for the Home Garden. University of Nebraska-Lincoln, Institute of Agriculture and Natural Resources. Retrieved 4 September 2012		https://bonnieplants.com/product/big-beef-tomato/
Adoration	Red color, small fruits 28–57 g, round, cocktail tomato, indeterminate, maturity days 70–80 days, resistant to <i>Fusarium</i> and <i>Verticillium</i> wilt and TMV	Enza Zaden - Adoration. Archived from the original on 2012-04-15. Retrieved 2011-11-15		By Roseridge1 at English Wikipedia - Own work, CC0, https://commons.wikimedia.org/w/index.php?curid=26733320
Enchantment	Red color, small fruit 3 oz, firm flesh, indeterminate hybrid, slicing shape, maturity days 70–80 days, resistant to <i>Fusarium</i> wilt races 1 and 2, <i>Verticillium</i> wilt and nematodes	Enchantment. Agricultural Experiment Station. Rutgers University. Retrieved 4 September 2012		https://njaea.rutgers.edu/tomatovarieties/vanety.php?Enchantment
Giulietta F1	Red color, large fruit and plum shape, extremely juicy and delicious with a high yield, ideal for greenhouses and outdoors. maturity days 70–80 days, resistant to <i>Fusarium</i> wilt, <i>Verticillium</i> wilt, <i>Alternaria</i> stem canker, nematodes and TMV	https://www.dobbies.co.uk/Garden/Vegetables/Vegetable-Seeds/All-Vegetable-Seeds/Tomato-Seeds%2D%2D-Giulietta-F1_439074.htm		https://www.dobbies.co.uk/Garden/Vegetables/Vegetable-Seeds/All-Vegetable-Seeds/Tomato-Seeds%2D%2D-Giulietta-F1_439074.htm

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Chapter 11

Disease Resistance Breeding with Genomic Tools in Zucchini (*Cucurbita pepo* L.)



Giuseppe Andolfo, Ciro Gianmaria Amoroso, and Maria Raffaella Ercolano

Abstract Zucchini (*Cucurbita pepo* L.) is a valuable vegetable crop with a wide assortment of cultivars that differ in fruit shape, color, flavor and culinary uses. This species is highly challenged from several pathogens including fungi, bacteria and viruses that can cause severe yield losses. To counteract invading pathogens, the plants have developed a complex defense system able to recognize enemy molecules, carry out signal transduction and respond promptly through many gene products. The plant's innate immunity is triggered by resistance (*R*) genes that can detect a variety of changes, both through non-self and modified-self recognition. The cultivation of pathogen-resistant zucchini varieties, or rather with *R*-genes, is one of the most straightforward strategies to assure high quality and quantity fruit yield. The increasing availability of high-throughput sequencing technology has the potential to develop innovative genome-based strategies for the identification of loci involved in disease resistance. Improved knowledge of plant defense mechanisms and advancements in genomics can provide new opportunities to accelerate classical breeding programs. Zucchini breeding for disease resistance needs suitable gene candidates, which can be discovered through the understanding of the genetic basis of resistance mechanisms in cucurbit resources. Indeed, the dissection of pathogen recognition mechanisms through non-self-pathogen molecules or cellular components that have been disrupted upon infection will facilitate the identification of new functional *R*-genes. In addition, the annotation of genes involved in disease resistance could be combined with phenotypic and molecular analyses to better dissect the genetic control of resistance. The information generated from genomic scanning will help breeders integrate genetic and genomic data to obtain a more durable resistance to cucurbit pathogens. In this chapter, we illustrate how innovative strategies can enhance the discovery of *R*-gene candidates, highlighting some genomic applications for improving zucchini resistance against biotic stress.

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Keywords Cucurbitaceae · Genomic resource · Marrow squashes · Next-generation sequencing (NGS) · *R*-genes identification

11.1 Introduction

The Cucurbitaceae family is the second largest horticultural family in terms of economic importance after Solanaceae. It includes several important crops, such as melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* Thunberg), cucumber (*Cucumis sativus* L.) and many *Cucurbita* species with edible fruits (Jeffrey 1980). The genus *Cucurbita* ($2x = 2n = 40$), which originated in the Americas, encompasses several economically-important crops, cultivated throughout temperate, subtropical and tropical regions (Paris 1986, 2010). The cultivated *Cucurbita* species are: *C. argyrosperma* (Huber), which includes the cushaw type (long curved neck) of squash; *C. maxima* (Duchesne), which includes pumpkins, hubbard, turban and buttercup squash; *C. moschata* (Duchesne), which includes the winter squashes and *C. pepo*, which includes both summer squash (zucchini, scallop, scallopini, crookneck, cocozelle squash) and winter squash (common or *true* pumpkin, delicata, acorn, spaghetti squash) as well as ornamental gourds. *Cucurbita ficifolia* (Bouché) includes the fig leaf gourd and lacayote. The most important of these species in terms of world agricultural production are *C. maxima*, *C. moschata* and *C. pepo*. The *C. pepo*, *C. maxima* and *C. moschata* species present the highest commercial value with more than 2 million ha cultivated in the world and over 25 million mt of annual production.

Cucurbita pepo domestication began in Central America around 8000 BC (Smith 1997) while in Europe and in the rest of the world it has quickly spread after its introduction in the sixteenth century (Grubben and Denton 2004). *Cucurbita pepo* cultivation extends from cold to temperate climates, and the cultivars grown in the various areas differ in shape and color according to the varieties. All *C. pepo* subspecies can successfully hybridize with each other; they were once part of an extended contiguous population reaching from Mexico through the eastern United States (Gong et al. 2012; Newsom et al. 1993; Smith 2006). Systematic, ethnobotanical and morphometric research, together with archaeological information, constitute the main sources of information concerning its origin and domestication.

11.2 Genetic Diversity and Pathogen Susceptibility of *Cucurbita pepo* ssp.

The identification of *Cucurbita pepo* subspecies and cultivars is a rather daunting task given the morphological differences within the species. The taxa conventions proposed by Decher-Walters et al. (2002) were: *C. pepo* ssp. *pepo*, *C. pepo* ssp. *ovifera* and *C. pepo* ssp. *fraterna*. *Cucurbita pepo* ssp. *pepo* includes pumpkin, zucchini and other marrow squashes, Mexican landraces and a few ornamental gourds

(Decker et al. 1993; Decker-Walters et al. 2002; Gong et al. 2012; Smith 2006); *C. pepo* ssp. *pepo* and *C. pepo* ssp. *ovifera* arose from two separate domestication events. *Cucurbita pepo* ssp. *ovifera* comprises both domesticated and wild populations; it is further divided into three varieties: *ovifera*, *texana* and *ozarkana* (Decker et al. 1993; Decker-Walters et al. 2002; Smith 2006). *Cucurbita pepo* ssp. *fraterna* (also known as *C. fraterna*), occurs only in a few localities in northeastern Mexico (Nee 1990).

Based on basic fruit shape, Paris (1986) proposed a taxonomy of *Cucurbita pepo* consisting of eight edible groups (acorn, cocozelle, crookneck, pumpkin, scallop, straightneck, marrow, zucchini) and one inedible cultivated variety (ornamental gourds) (Fig. 11.1). Recently, to improve the knowledge of independent evolutionary processes and domestication events within *C. pepo*, a whole-genome resequencing of seven (out of eight) morphotypes was realized (Xanthopoulou et al. 2019). A total of about 3.8 million high confidence single-nucleotide polymorphisms (SNPs) confirmed the genetic divergence between subspecies *pepo* and *ovifera* (Fig. 11.2).

The *Cucurbit* cultivars are notoriously sensitive to several fungal diseases (powdery mildew, downy mildew, leaf sport *Septoria*, gummy stem blight, black rot, *Choanephora* fruit rot, *Fusarium* spp., *Phytophthora* blight, *Plectosporium* blight, *Anthracnose*) and most serious viral diseases (zucchini mosaic virus, squash mosaic virus, tomato yellow leaf curl, new delhi virus). Pesticide use is not only detrimental to the environment and human health, but it also increases selection pressure on pathogen populations to adapt and acquire increasing levels of pesticides resistance (Jones et al. 2014). The cultivation of pathogen-resistant cucurbit varieties is one of the most straightforward strategies to reduce chemical use while assuring the quantity and quality of yield.

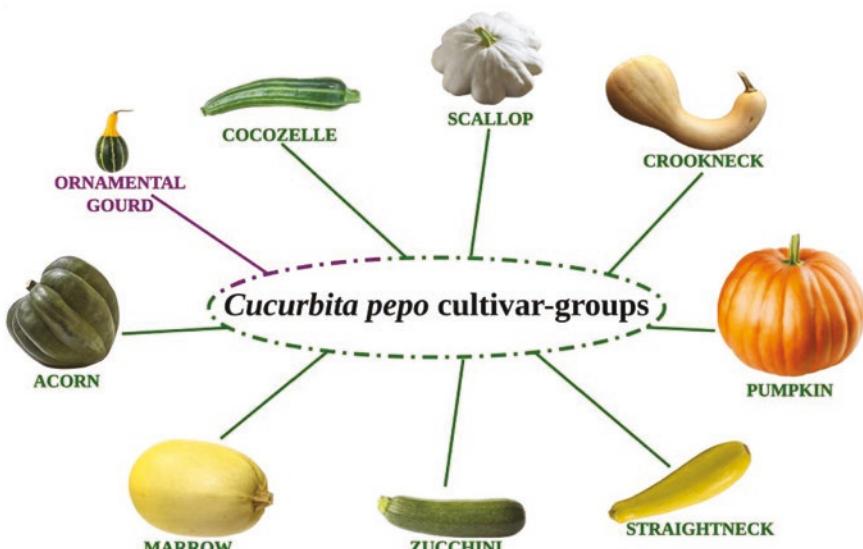


Fig. 11.1 Fruit shape diversification of nine *Cucurbita pepo* cultivar-groups. The eight edible-fruited cultivar-groups and one inedible cultivated variety are marked in green and violet, respectively

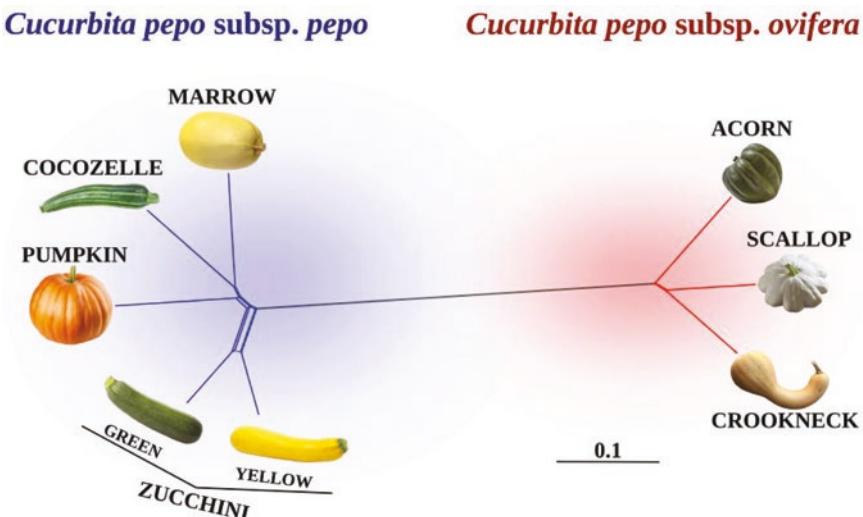


Fig. 11.2 Evolutionary relationship of *Cucurbita pepo* subspecies. Unrooted network based on the genetic distance between seven edible-fruited cultivar-groups (acorn, cocozelle, crookneck, pumpkin, scallop, marrow, zucchini) of *C. pepo* is shown. Fruit morphological diversity is indicated by pictures. (Source: Modified from Xanthopoulou et al. 2019)

The study of plant immunity and a deep understanding of the pathogen recognition layers contributing to the immune system can accelerate classical plant breeding. A suite of plant receptors distinct in specific protein classes able to detect pathogenic molecules have been classified and studied (Andolfo et al. 2013). Moreover, a global picture of pathways and signaling involved in defense response has been provided (Andolfo and Ercolano 2015). Innovative genomic approaches offer the potential for a rapid development of resistant cultivars by the addition or deletion of one or a few genes, while maintaining the specific qualities of individual cultivars. Breeding programs first need suitable genetic candidates, which can be identified through the characterization of the genetic and genomic basis of resistance in cucurbit-resistant accessions.

11.3 *Cucurbita pepo* Genome: Unexploited Breeding Resources

Several Cucurbitaceae crop genomes of great economic importance, including *Cucumis melo* (melon), *Cucumis sativus* (cucumber) *Citrullus lanatus* (watermelon) and *Cucurbita pepo* (zucchini), were recently sequenced. Despite the economic importance and global consumption of the former species, until a few years ago the study of their genomes was limited to molecular markers analysis including amplified fragment length polymorphism (AFLP), random amplification of polymorphic

DNA (RAPD) and simple sequence repeat (SSR) associated with some morphological and pathogen-resistance traits (Montero-Pau et al. 2017). Blanca et al. (2011) obtained the first *Cucurbita pepo* genomic resource from RNA sequencing (RNA-seq). Subsequently, a genetic map based on single nucleotide polymorphism (SNP) markers was built up increasing the *Cucurbita* genome research activity (Esteras et al. 2012). Although the total size of the *C. pepo* genome is similar to that of the other Cucurbitaceae members and the number of genes is not much greater, the genome of the genus *Cucurbita* has 20 chromosomes, unlike the genera *Cucumis* and *Citrullus* which have 12 (*Cucumis melo*), 11 (*Citrullus lanatus*) and 7 chromosomes (*Cucumis sativus*).

Recently, the entire *Cucurbita pepo* ssp. *pepo* genome of accession MU-CU-16 (version 4.1 of the genome) was released by Montero-Pau et al. (2018). Based on a shotgun sequencing approach several DNA fragments were produced and assembled in contigs which in turn were grouped into larger portions (scaffolds). Shotgun sequencing involves randomly breaking up DNA sequences into lots of small pieces and then reassembling the sequence by looking for regions of overlap. The final sequence assembly covered a total of 263 Mb (93% of the genome) in 32,754 contigs and 26,005 scaffolds, with a scaffold N50 of 1.8 Mb. The entire *C. pepo* genome is organized in 20 chromosomes (pseudomolecules) representing approximately 80% of the assembled genome. A total of 34,240 genes of which 27,870 coding for proteins were predicted in the MU-CU-16 genome. Altogether the predicted genes represent 45% of the genome and the coding regions represent 14%. More than the 90% of the genes resulted in integers when compared to the database of 1440 benchmarking universal single-copy orthologs (BUSCO) genes. A functional description based on GO annotation and orthology analysis is available for 77% of the transcripts. Repetitive genome elements make up to 38% of the assembly with a high abundance of Gypsy and Copia elements. The *C. pepo* genome showed a high level of synteny with other cucurbit species and synthetic regions seem to cover most of its chromosomes. It is interesting to note that most of the genes present in *C. pepo* are equally present in other Cucurbitaceae and that many of these have at least one parologue in *C. pepo*, suggesting that the entire genome has been duplicated (Montero-Pau et al. 2018).

Additional assembled transcriptomes generated through the quantitative sequencing-based method called RNA sequencing (RNA-Seq) for mapping transcribed regions, in which complementary DNA fragments are subjected to high-throughput sequencing and mapped to the genome. The transcriptomes can be used as a reference for gene expression analysis in different organs and tissues and under different environmental conditions (Andolfo et al. 2017; Blanca et al. 2011; Wyatt et al. 2015; Vitiello et al. 2016; Xanthopoulou et al. 2016, 2017). Furthermore, 40 assembled transcriptomes belonging to 11 species of *Cucurbita* were made available on the CucurbiGene database (Montero-Pau et al. 2018) allowing exploration of an atlas of 18,446–67,366 genes and 18,902–92,522 transcripts. In the future, genetic and breeding studies will be enhanced by investigation tools, which will use the considerable potential of the available genomic resources (Wang et al. 2009).

11.4 Strategies to Discover Resistance Genes

The vast amount of data generated in *Cucurbita* sequencing projects can be useful to promote the in silico identification of important classes of genes. In recent years, the identification of genome-wide resistance (*R*) gene candidates has become a popular research aim in several species due to the development of prediction tools based on the identification of distinctive structural domains (Andolfo et al. 2013; Osuna-Cruz et al. 2018). To date, more than 150 (*R*-genes) have been cloned and characterized in plants (www.prgdb.org). Most of the resistance encoding genes belong to receptor-like kinases (RLK), receptor-like proteins (RLP) and to nucleotide binding and leucine-rich repeat domains (NB-LRR) protein. The latter class, reported also as NLR, is subdivided into TIR-NB-LRR proteins (TNLs) and non-TNL proteins, depending on whether the Toll/interleukin receptor/domain (TIR) is present or absent. A few other protein classes are able to confer resistance to pathogens such as MLO (Andolfo et al. 2019; Iovieno et al. 2015) and ASC (Brandwagt et al. 2000) have been identified. The plant resistance gene database (PRGdb) is the most important *R*-genes information repository, collecting reference manually-curated as well as novel putative *R*-genes sequences discovered in plant genomes (Sanseverino et al. 2009). The exploration of such resources could be an important starting point to gain access to a large body of information to facilitate the analysis of *Cucurbita* *R*-gene repertoires. The identification of putative R-proteins on the basis of sequence and domain similarity is a challenging task due to the high level of R-proteins diversification promoted from the pathogen selection pressure (Andolfo and Ercolano 2015). Several methodologies such as BLAST search, domain matching, sequence alignment and phylogenetic analysis methods can be employed for R-proteins identification (Andolfo et al. 2013; Esteras et al. 2012; Kushwaha et al. 2016; Sanseverino and Ercolano 2012). A robust prediction tool to search for plant resistance genes in plant genome, named DRAGO 2 (disease resistance analysis and gene orthology), that combines the advantage of hierarchical (HMM) and (BLAST) search, is available for scientists working in this field of research on the PRGdb platform (Osuna-Cruz et al. 2018). Additional learning machine methodologies to facilitate the extraction and classification of putative R-protein were also developed by Kushwaha et al. (2016). Furthermore, to improve the annotation and discovery of pathogen resistance genes, resistance gene enrichment and sequencing (RenSeq) methodologies were implemented. Indeed, RenSeq technology was successfully applied in the analysis of genome resistance gene complements and gene cloning (Andolfo et al. 2014; Jupe et al. 2013).

Recently, an effort to explore *Cucurbita* *R*-genes architecture and diversification was conducted in *C. pepo* True French cultivar proteome (Table 11.1). Interestingly, out of a total of 64 NB-LRR proteins 11 are similar to the *Arabidopsis* RPW8 gene, that confers resistance against *Erysiphe cichoracearum*, one of the causal agent of powdery mildew (PM) (Micali et al. 2008). A comparative analysis conducted with other Cucurbitaceae genomes confirmed that RPW8-NLRs members (11) in *C. pepo* resulted in high duplicated respect in other *Cucurbit* species (3). An expansion in

Table 11.1 Numbers of *Cucurbita pepo* cv. True French genes that encode domains similar to plant R proteins

Protein domain		Number of genes
Full-length	CC-NB-LRR	25
	TIR-NB-LRR	2
Total full-length		27
Partial	CC-NB	11
	TIR-LRR	—
	TIR-NB	6
	NB	6
	TIR	3
	LRR	11
Total partial		37
Total		64

Source: Modified from Andolfo et al. (2017)

RLK and RLP gene families with high degrees of homology and with well-characterized *R*-genes was also found in zucchini (Andolfo et al. 2017). PM disease, caused by another causal agent *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*) has an important economic impact on *C. pepo* varieties and the expansion of RPW8 genes identified in *C. pepo*, could suggest an adaptive diversification induced by species-specific pathogen pressure. In addition, the expansion of cell surface receptors (RLKs, RLPs) may originate from a dose-compensation process to balance the limited number of cytoplasmic receptors (NRLs) identified in the *C. pepo* genome.

Currently, data from many plant sequencing projects are freely available along with several comparative genomic tools to manage, compute, query sequences and display multiple genome alignments can be used (Aversano et al. 2015). The study of plant *R*-genes diversification in different species raises important questions on how complex biological systems evolve and function (Shao et al. 2016). The *R*-genomic architecture in different species has been shaped by large- and small-scale rearrangements occurring in orthologous loci and by loci selection processes (Di Donato et al. 2017). It is well known that selection pressure on a gene in a structurally variant region can significantly affect diversities across entire regions. The reconstruction of evolutionary trajectories that shaped Cucurbitaceae *R*-gene performances can improve our understanding of *Cucurbita* immune system organization and functioning.

Furthermore, a comprehensive effort to integrate genomic tools can facilitate gene localization. High resolution genetic maps can be combined with sequence data to accelerate *R*-gene discovery. Knowing the location of a given *R*-gene locus can be a great advantage for mining its nucleotide sequences using both recombination analysis and protein-function prediction. Once a source of resistance has been discovered, sequencing and genetic analysis can be combined to predict the location and the sequence of a resistance gene (Andolfo et al. 2014). Information on chromosome recombination rates and *R*-gene distribution may be useful to select the most promising candidate genes (Andolfo et al. 2014; Nieri et al. 2017). Collocation of a

predicted R-gene within mapped loci for resistance to a given pathogen, or the detection of syntenic regions among related genomes, can accelerate the selection of a positional or functional candidate gene for the trait. Recently, the integration of prediction data with genetic analysis promoted the discovery of NLR protein-encoding genes located near markers associated to ZYMV resistance in zucchini (Capuozzo et al. 2017).

11.5 Accelerating Resistance Breeding in *Cucurbita pepo*

To increase the resistance of plants to diseases, breeders employ different sources of resistance. Improved plant resistance properties can be achieved through conventional phenotype- and genome-based breeding, or via genetic engineering. In recent decades, genomic and the biotechnological tools have been used successfully to explore the genetic basis of biotic tolerance/resistance and to develop cultivars with enhanced resistance to pathogens and pests.

The massively parallel sequencing techniques enlarged sequencing capabilities and launched the *next generation* in genomic science. Next-generation sequencing (NGS), also known as deep sequencing, is the catch-all term used to describe a number of different modern sequencing technologies which have revolutionized genomic research.

NGS approaches have emerged as dominant genomics technologies, mainly because of their cost-effectiveness and number of wide applications. Genomic strategies based on NGS technology provide a basis for elucidating the genetic structures of complex traits and for marker-assisted molecular breeding. Marker-assisted selection is a method of selecting desirable individuals in a breeding scheme based on DNA molecular marker patterns. So far, whole genome, via whole-genome sequencing (WGS), or parts of it, via whole-exome sequencing (WES), can rapidly provide a large quantity of sequences with great depth and increasing quality. Genotyping by sequencing (GBS) and whole-genome resequencing (WGR) permit genetic relationships studies, creation of detailed genetic mapping of targeted genes and genome-wide association studies to discover genomic variants associated with resistance-valuable traits (He et al. 2019; Kayondo et al. 2018). Genome-wide association studies (GWAS) have been successful in several crops, including maize (Farfan et al. 2015; Xiao et al. 2017); rice (Descalsota et al. 2018; Huang et al. 2010); soybean (Sonah et al. 2015; Zhang et al. 2015) and melon (Gur et al. 2017; Pavan et al. 2017) and can be employed as well in *Cucurbita pepo*.

A cDNA-based GBS technique named restriction site associated RNA sequencing (RAR-seq), enables the discovery of SNPs and alleles mainly from transcribed regions, reducing the representation of the genome and assessing the effect of candidate mutations directly at the expression levels (Alabady et al. 2015).

Plant genotyping can improve the selection of individuals resistant to pathogens causing substantial losses in agriculture. The recent decrypting of the zucchini genome sequence represents a reliable tool to identify new DNA markers linked to

candidate *R*-genes. Marker-assisted selection can speed up the transfer of these genes from donor cultivars into new elite cultivars. Indeed, the modern DNA-marker technology (GBS, RARseq, RNA-Seq) can help to achieve a finer marker-aided selection (MAS) to produce zucchini resistance plants.

A manually curated catalogue of the expressed extracellular (RLP, RLK) and intracellular (NLR) zucchini receptors (<https://figshare.com/s/8a083f60df238acdbc19>) is available in a RNA-Seq dataset (Andolfo et al. 2017). This *R*-gene dataset, in combination with genomic knowledge about zucchini *R* loci, can be integrated with phenotypic and molecular data to develop new resistant varieties in less time (Fig. 11.3).

Future research should focus on filling the gaps in the existing knowledge of biotic resistance resources on zucchini to develop an integrated program for disease-resistance breeding. The information generated from recent genomic projects will help breeders expedite breeding research in zucchini crops by exploring integrated genetic and genomic data.

11.6 Genome Editing

Sequencing techniques are able to provide important details on the position of functional elements of DNA, highlighting differences even of a few bases between genotypes of the same species; at the same time, great progress has been achieved in developing genomic engineering tools. With the creation of the first genetically *edited* plants, it became clear that the enormous opportunities deriving from the use of these technologies could be used to obtain genetically-enhanced plants (Andolfo et al. 2016; Andolfo and Ercolano 2015).

First-generation editing tools used to manipulate desired genes in crop plants are transcription activator-like effector nucleases (TALEN), zinc-finger nucleases (ZFN) and meganucleases (Mns). These approaches are expensive and laborious and involve complex procedures for successful editing. The new CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) technique has totally transformed genetic engineering, due to its versatility, precision and reduced costs (Boch et al. 2009; Moscou and Bogdanove 2009; Shan et al. 2013). Mainly it involves a complex of single-guide RNA (sgRNA) and the Cas9 protein in contrast to TALENs and ZFNs (Jinek et al. 2012). Genome editing, also referred to as *gene editing*, consists of the use of endonucleases that form breaks in genomic DNA. These enzymes are transported to the site of interest leading to the modification of the gene itself and to a no more functional protein. The discovery of programmed sequence-specific nucleases (SSN) has facilitated precise gene editing; although all types of SSNs have unique features, the mechanism for producing double-strand breaks (DSBs) in the target DNA is similar for all. The repairs may be via non-homologous end joining (NHEJ) generating insertions or deletions (InDel) at target sites which could lead to a gene *knock out*. Using these technologies, it is possible to introduce DNA fragments into a genome with a mechanism of

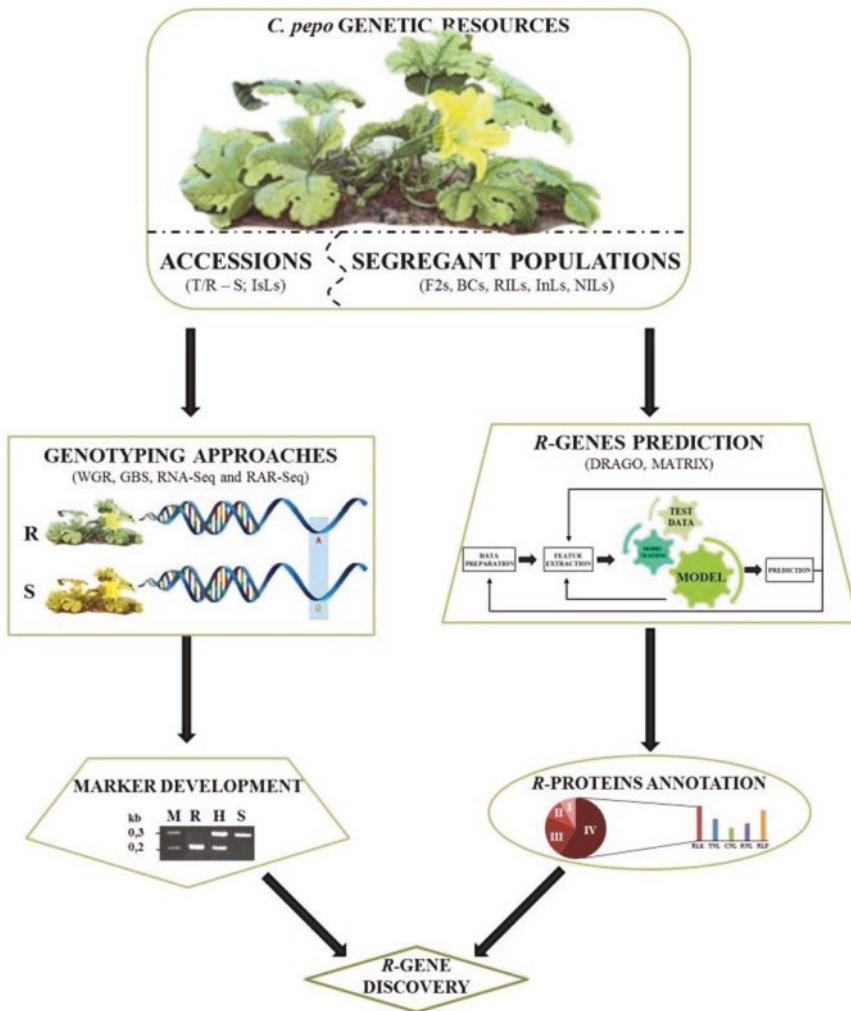


Fig. 11.3 Flowchart for biotic resistance breeding from zucchini genetic resources (*T/R* tolerant or resistant plant, *S* susceptible plant, *IsLs* isogenic-lines, *F2s* *F*2 populations, *BCs* backcross populations; *RILs* recombinant inbred lines, *InLs* introgression lines, *NILs* near isogenic lines). The workflow shows the mapping (*WGR* whole-genome resequencing, *GBS* genotyping by sequencing, *RNA-Seq* RNA sequencing, *RAR-Seq* restriction site associated RNA sequencing) and prediction (machine learning) data processing steps for the selection of the novel markers associated to disease resistance (*R*) traits and the identification of *R*-genes

homology-directed recombination (HDR) that needs a homologous template to mediate repair and can be used to obtain precise changes like gene insertion and gene replacement (Feng et al. 2013; Jinek et al. 2012; Kim et al. 2014). There are also other ways, for example, mutation of a single nucleotide (*CBE* cytosine base editor, *ABE* adenine base editor) and modifying a nitrogen base that could be

responsible for a disease (Lee et al. 2020). Despite the enormous advantages, these technologies are not without drawbacks, indeed much still needs to be done to improve them.

For crop improvement and the development of more productive varieties, a breeding strategy needs to involve the traditional cross between two good varieties to obtain an excellent one (Abdelrahman et al. 2015). Genome editing, in this regard, stands as a strategy for an efficient and precise manipulation of the genome, especially for those crops that are more complex to improve through traditional breeding methods (Andolfo and Ercolano 2015; Cappetta et al. 2020; Feng et al. 2013). Currently, CRISPR/Cas9 system has been widely applied in many plant species to induce genome mutations, to study gene function and to improve crops. Recently fully and chimeric albino plants have been obtained in melon (*Cucumis melo* L.) using the CRISPR/Cas9 system to target the phytoene desaturase gene, a key enzyme for the carotenoids production (Hooghvorst et al. 2019). Also in cucumber (*Cucumis sativus* L.) it has been demonstrated that disrupting the eIF4E (eukaryotic translation initiation factor 4E) gene, that is involved in viral infections (Sanfaçon 2015), the non-transgenic heterozygous plants developed partial resistance to an ipomovirus (*cucumber vein yellowing virus*) and two potyviruses (*zucchini yellow mosaic virus* and *papaya ring spot mosaic virus-W*) (Chandrasekarna et al. 2016). To date, despite several efforts made in zucchini, no edited plants have been obtained. The limiting step seems to be the regeneration process. Using available regeneration protocols (Chee 1990; Stipp et al. 2012), unstable transformation events were achieved in this species. However, gene editing technology has great potential in zucchini. Such a methodology can strongly contribute to making zucchini more resistant to biotic/abiotic stress varieties and improving significantly crop yields.

11.7 Conclusions and Prospects

Modern agriculture must provide an alternative to the application of chemical agents and so researchers are exploring the genetic composition of plants to enhance resistance to pathogenic infections. The decreasing cost of sequencing has led to a rapid rise in the magnitude of crop genomic data, which represents a substantial opportunity for breeders. The zucchini genome sequence provides an important foundation to identify agronomically-relevant variation.

Conventional breeding plays an essential role in zucchini improvement, to generate superior genotypes through genetic recombination. Usually, it entails growing and examining large zucchini populations derived from cycles of phenotypic selection and crossing: a lengthy and labor-intensive process. The availability of zucchini genome sequence will allow identification of all genes and genetic variants contributing to agronomics traits, so that changes made during the breeding processes can be assessed at the genotype level. Indeed, given the ready availability of genomic data for breeders today, genomics plays an increasingly important role in all aspects

of zucchini breeding, such as quantitative trait loci (QTL) mapping and genome-wide association studies (GWAS), where genomic sequencing can allow gene-level resolution of agronomic variation. The advances in genomics-based breeding allow the identification of genetic variation in *Cucurbita* species, which can be applied to produce climate-resilient varieties.

Genetic engineering, based on conventional transgenic approaches or the more recent genome-editing technologies, possesses several advantages compared with conventional breeding. First, it enables the introduction, removal, modification, or fine-tuning of specific genes of interest with minimal undesired changes to the rest of the zucchini cultivar genome. As a result, zucchini varieties exhibiting desired agronomic traits can be obtained in fewer generations as compared with conventional breeding. These features make genetic engineering a powerful tool for enhancing resistance against plant pathogens (Christou 2013). Increased knowledge regarding the molecular mechanism underlying plant-pathogen interactions and advancements in biotechnology will provide new opportunities for disease resistance to pathogens in zucchini.

Appendix I: Research Institutes Relevant to Zucchini

Institution name	Country	Contact person	Website
Università degli Studi di Napoli Federico II	Italy	Maria Raffaella Ercolano	http://www.unina.it/home
Universitat Politècnica de Valencia	Spain	Belen Pico	http://www.upv.es/
University of Çukurova	Turkey	Nebahat Sari	https://www.cu.edu.tr/
The Agricultural Research Organisation of Israel – the Volcani Centre	Israel	Tadmor Yaakov	https://www.agri.gov.il
Asian Vegetable Research and Development Center	Taiwan	Maarten van Zonneveld	https://www.gfar.net
Universidade Federal Rural do Semi-árido	Brazil	Glauber H. de Sousa Nunes	https://ufersa.edu.br/
Leibniz-Institut fuer Pflanzengenetik und Kulturpflanzenforschung	Germany	Ulrike Lohwasser	https://www.ipk-gatersleben.de/
Centro Agronomico Tropical de Investigacion y Ensenanza Catie	Costa Rica	Muhammad Ibrahim	https://www.catie.ac.cr/
Szkola Główna Gospodarstwa Wiejskiego	Poland	Marta Olas-Sochacka	https://www.sggw.pl/
Stichting Wageningen Research, Centre for Genetic Resources	Netherlands	Willem van Dooijeweert	https://www.wur.nl

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Part II
Young Shoots

Chapter 12

Asparagus (*Asparagus officinalis* L.) Breeding



Roberto Moreno-Pinel, Patricia Castro-López, José Vicente Die-Ramón,
and Juan Gil-Ligero

Abstract Garden asparagus (*Asparagus officinalis* L.) is an important horticultural plant worldwide. The proposed Center of Origin of this crop comprises Eastern Europe, Caucasus and Siberia. *Asparagus officinalis* is a monocotyledonous, perennial, diploid, dioecious species with a chromosome number of $2n = 2x = 20$. This species belongs to the *Asparagus* genus which comprises dioecious (*Asparagus* subgenus) and hermaphroditic species (*Myrsiphyllum* and *Protaspasparagus* subgenera). Polyploidization seems to have played an important role in the evolution of this genus due to a wide range of ploidy levels ($2x, 4x, 6x, 8x, 10x, 12x$), found in inter- and intraspecific taxa. Nowadays, the modern cultivars are diploid hybrids with a narrow genetic base due to their common origin, the Violet Dutch population. The genetic resources of asparagus are an excellent source of genes of interest in breeding programs by using different landraces and wild related species tolerant to biotic and abiotic stresses. These genetic resources may also contribute to develop new varieties with higher bioactive compounds that is an increasing consumer demand. In this chapter, we present an overview of the new advances on genealogy, cytogenetics, genetic resources characterization and conservation, biotechnology and molecular markers linked to sex. Garden asparagus male plants are economically more profitable than females and therefore breeders aim to develop all-male hybrids. Recently, the asparagus reference genome was released and the first saturated genetic map has been obtained. These tools can be used to locate genes and/or QTLs controlling agro-morphic traits and to carry out marker-assisted selection (MAS).

Keywords All-male hybrids · Breeding · Marker-assisted selection · Reference genome

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12.1 Introduction

Cultivated asparagus (*Asparagus officinalis* L.) is a monocotyledonous, diploid ($2n = 2x = 20$), perennial and dioecious species. It is the most economically important species in the genus *Asparagus* and an important vegetable crop worldwide. In 2017, the cultivated area was comparable to other vegetable crops such as garlic, carrot and eggplant (Faostat 2019). China is, by far, the most important asparagus producer with a cultivated area that represents 92% of global production. China is followed by Mexico, Peru and Germany in the ranking of countries with largest cultivated area. In these countries, the cultivated area has increased since the 1970s. Conversely, an opposite tendency is observed in the same period in some traditional asparagus-producing countries such as the USA or France but, globally the cultivated area of this vegetable has been increasing over recent decades (Table 12.1).

In the face of increasing global human population amid the threat of climate change, interdisciplinary studies will facilitate the development of new asparagus varieties. These studies may allow achievement of not only the traditional asparagus breeding goals but also the emerging challenges such as varieties suitable for other cropping systems (drought conditions, organic farming) or varieties optimized to meet new consumer demands such as asparagus with high concentration of functional compounds.

A review focusing on asparagus plant breeding may be helpful to breeders or related scientists to design or plan their new research studies. Two reviews on asparagus plant breeding have been published so far. The first one took place in the 1980s (Ellison 1986) and more recently by Anido and Cointry (2008). Also, a review on the genus *Asparagus* as a resource to be used in breeding has been published (Kanno and Yokoyama 2011). Since then, many studies and three international asparagus symposia have been held. The aim of this chapter is to provide an updated review of

Table 12.1 World historical series (1961–2017) of the average harvested area in hectares by the major asparagus producing countries

Country	Average harvested area (ha) during different year ranges					
	1961–1970	1971–1980	1981–1990	1991–2000	2001–2010	2011–2017
China	313,499	240,940	344,371	646,128	1,166,492	1,356,342
Peru	1009	743	4487	17,398	22,730	32,857
Mexico	1388	5443	5569	10,849	14,815	20,621
Germany	6327	6022	6934	9491	16,953	20,536
Spain	5400	12,222	20,619	19,910	12,540	11,271
USA	52,612	40,045	37,119	31,604	19,229	9775
Italy	6040	6322	5458	6034	6315	6095
Japan	4995	6208	8436	7847	6641	5705
France	24,810	18,657	16,117	10,322	6081	4450
Others	21,311	24,354	22,933	33,694	22,465	18,458
Total	437,391	360,956	472,043	793,277	1,294,261	1,486,110

Source: Faostat (2019)

plant breeding with special emphasis on the opportunities that the new knowledge obtained in the last decade may facilitate acceleration of the development of new asparagus varieties.

12.1.1 Center of Origin

According to De Candolle (1885), the origin of asparagus is believed to have been in Europe and western temperate Asia and supposedly cultivation began over 2000 years ago. The first time that asparagus is mentioned in a manuscript was by Theophrastus (372–287 BCE) during the Greek civilization although it is not clear whether he referred to *Asparagus officinalis*. According to Sturtevant (1919), the cultivated asparagus seems to have been unknown to the Greeks at the time of Theophrastus and Disocorides and, supposedly, the term *asparagos* (of Persian origin) seems to have been used for a wild plant of another species. Probably, at the beginning of the domestication of *A. officinalis*, young shots of this species were combined with those of wild relatives such as *A. maritimus* (L.) Mill. collected from their natural habitats for human consumption (Ellison 1986). Roman civilization spread asparagus growing along with their empire throughout Europe (Anido and Cointry 2008; Ellison 1986). The first guide for gardeners was written during the Roman civilization by Cato (234–149 BCE). Later, Columella (4–70 CE) and Pliny the Elder (23–79 CE) pointed out in their writings certain aspects of asparagus growing. After the fall of the Roman Empire, during the Middle Ages in Europe (5–15th centuries), asparagus cultivation was relegated to monasteries as a medicinal plant. However, at that time cultivation continued in Arab Spain as can be deduced from the Al-Awam Book of Agriculture (12–13th centuries) (Cubero 2003). During the Renaissance asparagus was rediscovered as an appreciated vegetable (Lužný 1979). At an elaborate banquet given by the Doge of the Republic of Venice in 1534, the asparagus from Bassano (Veneto, Italy) is cited for the first time (Falavigna 2000). Later, in about the nineteenth century, varieties were developed in different parts of Europe from the landrace Violet Dutch. These varieties were the base of new cultivars in Europe and North America (Knaflowski 1996). Afterward the crop was introduced to other countries, mainly China, Peru, Japan, New Zealand and Mexico (Fig. 12.1).

The proposed Center of Origin of this crop comprises Eastern Europe, Caucasia and Siberia (Sturtevant 1919) (Fig. 12.1). Wild populations of *Asparagus officinalis* from Turkey (Geoffriau et al. 1992), Iran (Mousavizadeh et al. 2015; Sarabi et al. 2010) and Armenia (Melyan et al. 2016) have been reported. However, little is known about the geographical distribution of wild populations in its Center of Origin. *Asparagus officinalis* can also be found as naturalized populations in different parts of Central and Southern Europe, North Africa, and Western and Central Asia (Sturtevant 1919).

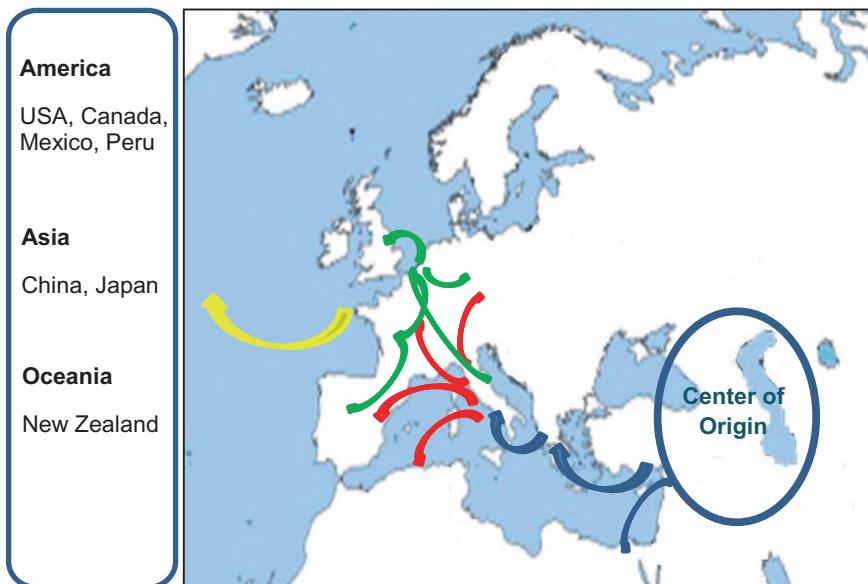


Fig. 12.1 Main dissemination pathways of asparagus as a crop from its Center of Origin over time. Blue arrow, first dispersal to the Mediterranean Basin; red arrow, dispersal by Roman Empire (~ I BCE – ~ IV CE); green arrow, the first varieties bred from Violet Dutch population (~ XVIII – ~ XIX); yellow arrow, asparagus crop introduction to different parts of the world. (Figure constructed by J. Gil)

12.1.2 Genealogy of Asparagus Genus

Asparagus is a large genus with species distributed throughout Asia, Africa, Europe and Australia (Kubota et al. 2012). The genus includes diverse life forms, such as herbaceous perennials, tender woody shrubs and vines (Bailey 1942). The latest infrageneric classification divides the genus *Asparagus* into three subgenera: *Asparagus*, *Myrsiphyllum* and *Protaspargus* (Clifford and Conran 1987; Obermeyer 1983, 1984). The species of the *Asparagus* subgenus are dioecious and distributed in Europe, Asia and North Africa, whereas the other subgenera contain hermaphroditic species mainly distributed in South Africa (*Myrsiphyllum*) and Africa, Oceania, south of Asia along with southwestern regions of Europe (*Protaspargus*) (Komarov et al. 1935; Kubota et al. 2012; Obermeyer 1983, 1984; Tutin et al. 1980; Xinqi and Tamanian 2000).

The genus *Asparagus* is composed by 209 accepted species, among 432 scientific names that have been proposed in this genus so far (The Plant List 2013). The number of species included in this genus has changed since the first classification by Linnaeus (1799) and Baker (1875) who included 20 and 98 species, respectively. Probably, the number may change again in the future as new taxonomic studies are conducted or new species are discovered. Thus, new *Asparagus* spp. have been

recently discovered in Iran (Hamdi 2016; Hamdi and Assadi 2009, 2013, 2017; Hamdi et al. 2017), Spain (Regalado et al. 2017) and Zimbabwe (Demissew 2008).

Asparagus officinalis is the only cultivated species of the genus and belongs to the subgenus *Asparagus*. In addition, young shoots of some wild *Asparagus* spp. are collected for self-consumption and are also sold in local markets in southern Europe (mainly *A. acutifolius* L., but also *A. albus* L., *A. aphyllus* L., *A. horridus* L., *A. verticillatus* L.), Asia (*A. acerosus* Thunb. ex Schult. & Schult. f.) and in Southern Africa (*A. laricinus* Burch.). Other species belonging to *Protaspasragus* or *Myrsiphyllum* subgenera have economic importance because they are cultivated and/or collected from their natural habitats for ornamental use (*A. scandens* Thunb., *A. plumosus* Baker, *A. densiflorus* (Kunth) Jessop, *A. falcatus* L.) or for their medicinal properties (*A. cochinchinensis* (Lour.) Merr., *A. racemosus* Willd.) (Pieroni 2005; Prance and Nesbitt 2012).

Good knowledge of the geographical distribution of *Asparagus* spp. and the phylogenetic relationships among *Asparagus* subgenera may be helpful in plant breeding programs as well as in the development of conservation strategies for endangered species, such as *A. macrorrhizus* Pedrol, Regalado et López-Escina or *A. oligoclonos* Maxim (Red List 2014, 2019; Regalado et al. 2017). Besides, the phylogenetic studies employing species of the three subgenera may provide new information about the evolutionary pathway which occurred in the *Asparagus* genus. In this sense, the most comprehensive studies of the *Asparagus* genus phylogeny have been conducted employing cpDNA (Fukuda et al. 2005; Kubota et al. 2012), rDNA (Ito et al. 2008; Štajner et al. 2002) and both plastid and nuclear DNA (Norup et al. 2015). According to these authors, South Africa could be the ancestral area of the *Asparagus* genus and Eurasian species may have evolved from an African ancestor. In their studies the authors also pointed out that this genus has undergone a recent and rapid radiation.

In order to introgress genes from wild species into the crop, interspecific hybrids between *Asparagus officinalis* and wild species have been solely achieved with some of the *Asparagus* subgenus species evaluated for this purpose. Although a few attempts using the other two subgenera have been tried, results have not been successful so far (see review by Kanno and Yokoyama 2011). This outcome suggests that the crossability of the cultivated species with wild species might be easier with, or perhaps restricted to, those included in the *Asparagus* subgenus. In addition to the studies aforementioned, other phylogenetic studies mainly focused on *Asparagus* subgenus have been performed (Castro et al. 2013; Moreno et al. 2008a; Mousavizadeh et al. 2018; Nothnagel et al. 2017; Plath et al. 2018). These studies highlight the existence of a set of closely-related species to *A. officinalis*, cross compatible with this one.

To date, *Asparagus* genus phylogeny remains incomplete and its evolutionary pathway is not well understood. Good knowledge of the phylogenetic relationship between *Asparagus* spp. could be helpful for plant breeders not only in the choice of the wild species to cross with the cultivated one, but also to select which one/s might be suitable to use as a genetic bridge with those species that in previous

attempts were not possible to cross with *A. officinalis*, or other ones that can be considered distant phylogenetic species but interesting in plant breeding.

12.1.3 Botany

Garden asparagus is a perennial plant whose underground part consists of a rhizome (crown) with downward growing primary roots (storage) harboring other secondary (absorbent) and tender shoots (spears) that emerge from buds located in the upper part of the rhizome. Once the shoots reach commercial length they are cut for human consumption. When the harvest season is completed, the remaining uncut spears form primary and secondary branches becoming the aerial part of the plant (fern), which is responsible for the replenishment and accumulation of carbohydrates until the next harvest season. The color of asparagus spears range between light green to dark purple depending on plant pigment content (Fig. 12.2). White spears are obtained when they are forced to grow without exposure to light.

In garden asparagus male and female flowers are similar in the early stages with both sets of sexual organs present. Later in floral development one set usually aborts, leaving a male flower with an outer and inner whorl of 3 stamens each, or a female flower with a 3-lobed pistil and 3-locule ovary, and the other parts rudimentary (McGregor 1976). In field conditions and under a temperate climate a wide range of the established plants (50–90%) start to flower during the second growing season depending on cultural practices, which greatly affect the time of flowering (Hung 1979). In this sense, a higher percentage of plants blooms the first year if the plant develops enough to promote the first flowering (pers obs.). Male plants tend to flower earlier than females, with a 1:1 expected ratio in populations. In mature plants, flowering mainly occurs during spring although some stems with flowers can be usually found during summer and fall. Pollination is conducted by insects, mainly flies, bees and bumblebees. Fruits are red berries when mature bearing 1–8 seeds. The number of seeds borne by the females usually increases after first flowering to the third or fourth year and then the number of seeds can increase or decrease depending on the number of stalks produced in each growing season.

A single gene (M/m) regulates the sex in this species. Male plants are heterozygous (Mm) and females are homozygous recessive (mm) (Rick and Hanna 1943). Sex is an important trait in garden asparagus because in crop conditions male plants are more profitable than females. Therefore, all-male cultivars are preferred for asparagus cultivations (see Sect. 12.2.2).



Fig. 12.2 Different colors of asparagus spears. (a) Purple, (b) Pale purple, (c) Green-purple, (d) Green with purple bracts, (e) Green with pale purple bracts, (f) Pale green. (Photos by J. Gil)

12.1.4 Functional Compounds

Asparagus spp. have been used for medicinal purposes since ancient times, prior to their use for human food (Ellison 1986). In antiquity, roots, shoots or seeds were used as a sedative, a painkiller and a liniment (Cumo 2013). Nowadays, different pharmacological properties have been documented in garden asparagus, including antidiabetic, anticancer, antioxidant, antiparasitic, to treat urinary tract infections and to lower cholesterol (Al-Snafi 2015; Iqbal et al. 2017; Li 2008; Wang et al. 2010). Edible asparagus is rich in different minerals (phosphorus, potassium, calcium, magnesium), vitamins (C, E, folate), fiber and bioactive compounds, mainly

phenols and saponins (Amaro-López et al. 1995; Fuentes-Alventosa et al. 2008; Guillén et al. 2008; Hedges and Lister 2009; Rodríguez et al. 2005; Vázquez-Castilla et al. 2013; Wang et al. 2003).

The therapeutic activity of garden asparagus has long been well known. Nowadays, this vegetable is becoming increasingly popular as a health-promoting food, probably due to its both antitumor activity (Bousserouel et al. 2013; Jaramillo-Carmona et al. 2018; Jaramillo et al. 2016; Shao et al. 1996) and antioxidant capacity (Chin and Garrison 2008; Chin et al. 2002; Guillén et al. 2008; Maeda et al. 2005; Rodkiewicz 2008). In asparagus, different phenols such as flavonoids, hydroxycinnamic acids and anthocyanins, as well as other compounds like glutathione or vitamins (C, E) contribute to the antioxidant capacity. Thus, garden asparagus normally ranks among vegetables with the highest antioxidant capacity (Chun et al. 2005; Pellegrini et al. 2003; Vinson et al. 1998; Wu et al. 2004). Moreover, Jiménez-Sánchez et al. (2016) detected new compounds belonging to different chemical groups, with both recognized and unknown bioactivity capacity, which may provide better knowledge of garden asparagus as a functional food.

Recent studies have shown that different genetic resources such as the landrace Morado de Huétor or wild *Asparagus* subgenus species have different composition and higher concentrations of bioactive compounds than garden asparagus (Ferrara et al. 2011; Fuentes-Alventosa et al. 2008; Jaramillo-Carmona et al. 2017; Vázquez-Castilla et al. 2013). This fact suggests that the genetic resources of this crop could be a source of genes of interest to develop cultivars with higher profiles in bioactive compounds, as increasingly demanded by consumers.

On the other hand, commercial asparagus production generates large amounts of byproducts from processing that contain a significant content of phytochemicals and fiber. In this sense, the development of new varieties with spears containing higher levels of bioactive compounds could prompt development of bioactive extract production for the functional food market derived from byproducts (Chitrakar et al. 2019; Fuentes-Alventosa et al. 2009a, b; Jaramillo-Carmona et al. 2013; Missaoui 2018; Nindo et al. 2003).

12.1.5 Cytogenetics

The large genus *Asparagus* has a remarkably consistent basic chromosome number of $x = 10$, found in species with different ploidy levels. The basic chromosome number of $x = 10$ was described for the first time in *A. officinalis* ($2n = 2x = 20$) in the late of 1920s and early 1930s by Flory (1932); Kamo (1929) and Shoji and Nakamura (1928). Intraspecific ploidy variations have been also found in some species (Bozzini 1959; Ito et al. 2007; Jessop 1966; Moreno et al. 2006; Mousavizadeh et al. 2016; Rice et al. 2015). Thus, polyploidization seems to be frequent in asparagus, which is favored by its perennial condition which could have played an important role in the evolution of this genus (Castro et al. 2013).

The size of the *Asparagus officinalis* genome has been estimated to be 1300 Mb/1C (Arumuganathan and Earle 1991). Asparagus chromosomes are relatively small (2.3–5.2 µm) but general agreement on the asparagus karyotype has not yet been reached. The most accepted classification of the chromosomes of the species was carried out by Löptien (1976). Based on chromosome length, they can be grouped into long (L1–L5), medium (M1) and short (S1–S4) classes. Chromosome L5 was proposed as the one harboring the sex gene (*M/m*) (Löptien 1979). No sex heteromorphic chromosomes have been detected in the species (Kamo 1929).

Different studies based on fluorescence in situ hybridization (FISH) technique and probes of both rDNA 45S and 5S loci have showed three and one signals that can be used as cytogenetic markers (De Melo and Guerra 2001; Moreno et al. 2005; Mousavizadeh et al. 2016; Reamon-Büttner et al. 1999). In this sense, there is no general agreement concerning either chromosomal localization and signal size obtained in the 45S rDNA loci. The discrepancies may be due to intraspecific chromosome polymorphism, or difficulties in localizing rDNA probes on small chromosomes of asparagus (Moreno et al. 2018).

An alternative methodology for microscopy techniques is the development of a flow karyotype (an histogram of relative fluorescence intensity obtained with DAPI-stained chromosomes) by which it is possible to isolate different chromosomes of the haploid number of a given species, provided they differ enough in size (Doležel et al. 2011). Recently, the technique has been applied in garden asparagus resulting into eight chromosomes groups (peaks I to VIII) (Moreno et al. 2018). Among them, five groups (peaks I, II, IV, VII, VIII) contained one single chromosome, another two tight groups (peaks V and VI) included three other ones and one group (peak II) included two chromosomes (Fig. 12.3). Thus, these eight groups of chromosomes could be classified into three major groups: (i) peaks I–III, (ii) peak IV and (iii) peaks V–VIII which appear to correspond, respectively, to the small, medium and large chromosome groups proposed by Löptien (1976). DNA obtained from flow-sorted chromosomes can be used for a variety of applications which include, among others, the development of molecular markers from those chromosomes that harbor genes of agronomic importance or their sequencing for gene isolation.

In addition, the sensitivity and specificity of FISH analysis can be increased by using flow-sorted chromosomes comparing with the obtained by microscopy techniques (Doležel et al. 2011). This fact enables a better characterization (localization and size) of both rDNA signals and may facilitate long-range mapping of DNA sequences that may be helpful to characterize genetic resources of this crop or to support phylogenetic studies. In asparagus, FISH analysis using flow-sorted chromosomes showed 3 signals for 45 s rDNA and 1 signal for 5S, each one in different chromosomes (Fig. 12.3b) (Moreno et al. 2018). According to these authors, the 5S rDNA locus was located on LG5, which was associated with the sex locus.

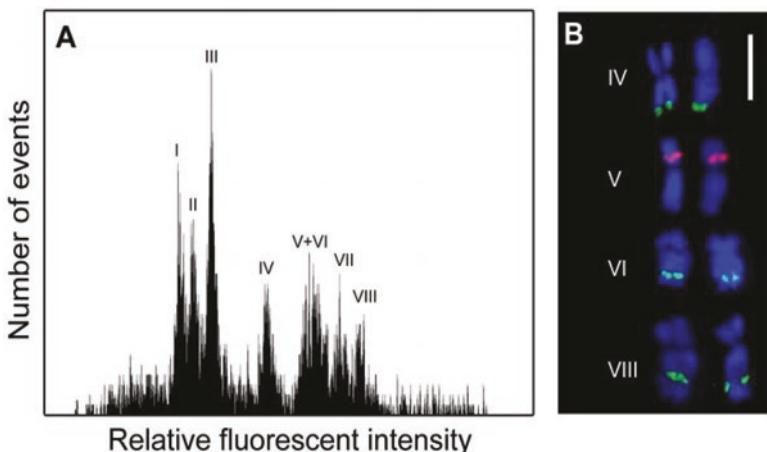


Fig. 12.3 Flow cytometric chromosome analysis and sorting in garden asparagus. **(a)** Histogram of relative fluorescence intensity (flow karyotype) obtained after the analysis of DAPI-stained chromosomes. Eight major peaks (I–VIII) could be resolved. **(b)** Examples of chromosomes sorted from peaks IV, V, VI and VIII. The chromosomes were identified after fluorescence in situ hybridization/FISH with probe for 45S rDNA (green) and 5S rDNA (red) loci. The chromosomes were counterstained with DAPI (blue). Scale bar: 5 mm. (Source: Moreno et al. 2018)

12.2 Breeding

12.2.1 *Cultivars Development*

Asparagus cultivars for green and white spears production are mostly diploid with green-colored spears when exposed to light. The development of asparagus varieties has evolved since improved populations were obtained by mass or recurrent selection toward different types of hybrids, including double, three or two way. As a result, an increase of both homogeneity for the agro-morphological traits of interest in this crop and yield have been achieved (Corriols-Thévenin 1979; Corriols and Doré 1988; Falavigna et al. 1990).

As mentioned in Sect. 12.1.1, the rediscovery of this vegetable for human consumption took place during the Renaissance. During the sixteenth and seventeenth centuries asparagus became popular in different countries such as Germany, France, England and The Netherlands, where different asparagus populations were being developed, identified geographically according to the countries and towns where they were grown (Anido and Cointry 2008). One of them, Violet Dutch, is considered the population from which subsequent improved modern cultivars were obtained. (Geoffriau et al. 1992; Kidner 1947; Knaflowski 1996; Mercati et al. 2015) (Fig. 12.4). This could explain the narrow genetic base of the diploid cultivars described by different authors employing both isozyme (Brettin and Sink 1992; Geoffriau et al. 1992; Lallemand et al. 1994) and DNA molecular markers (Khandka et al. 1996; Mercati et al. 2015; Moreno et al. 2006).

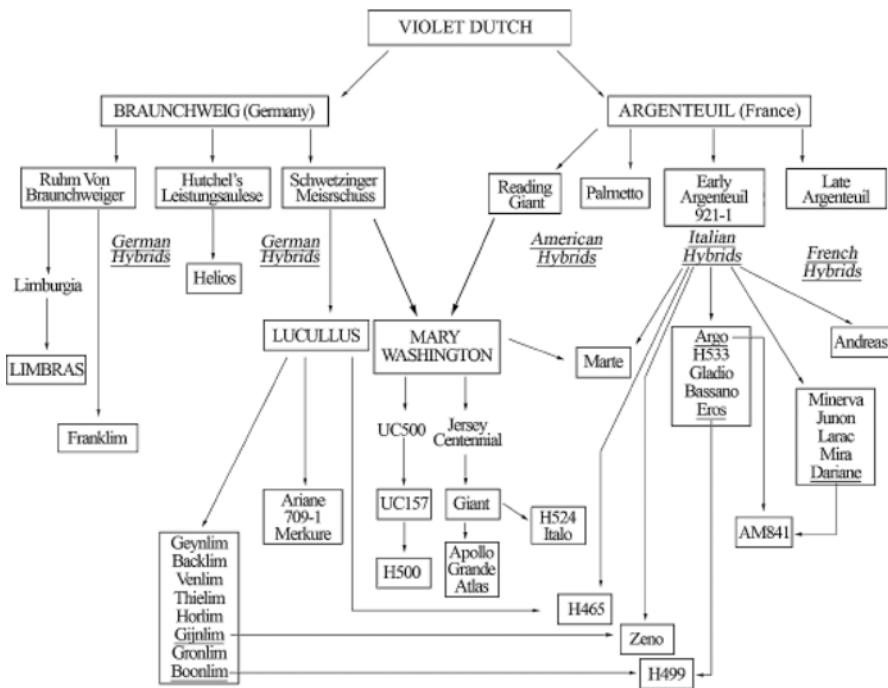


Fig. 12.4 Updated genealogy of the major asparagus cultivars proposed by Knaflawski (1996). (Source: Mercati et al. 2015)

Nowadays, cultivars are mostly diploid hybrids (known as clonal hybrids) derived from crosses between two parents, female and male, with good combining ability. In the development of asparagus cultivars, single genotypes (heterozygous or homozygous) or inbred lines have been employed as parents which have to be propagated or cloned in order to produce a sufficient quantity of commercial seed.

12.2.2 Sex Trait

As mentioned above (Sect. 12.1.3), sex is an important trait in garden asparagus because under crop conditions male plants are more profitable than females. According to the sex trait of progeny of the hybrids, clonal hybrids can be classified in two main groups: all-male hybrids, compounded solely of male plants (Mm), and mixed hybrids (Mm and mm) that contain both sexes in a 1:1 ratio. Nowadays, the tendency in plant breeding is to aim toward the development of all-male hybrids as male plants usually exhibit higher productivity and/or earlier yields during the harvest season than females (Ellison and Scheer 1959; Ellison and Schermerhorn 1958; Falloon and Nikoloff 1986; Franken 1970; Moon 1976; Robbins and Jones 1925;

Thévenin 1967; Yeager and Scott 1938). Moreover, other studies have pointed out that male plants live longer than females (Bannerot et al. 1969; Böttner 1921; Ellison and Scheer 1959; Franken 1970; Yeager and Scott 1938). However, differences in longevity based on sex have been disputed by some other authors (Bouwkamp and McCully 1972; Tiedjens 1924). Nevertheless, all-male cultivars are preferred for asparagus cultivation. Supermale plants (*MM*) are necessary to breed all-male hybrids. Supermale plants can be obtained by in vitro anther culture or through the self-pollination of andromonoecious plants (those bearing male and bisexual flowers) found in asparagus populations at a very low frequency (Fig. 12.5).

The genetic control of the andromonoecy is not yet well understood. Previous studies evaluated progenies of andromonoecious plants, but there is no general agreement on the inheritance of the trait (Franken 1970; Nikoloff and Falloon 1990; Peirce and Currence 1962; Rick and Hanna 1943; Sneep 1953b; Thévenin 1967; Wricke 1973). However, all these studies reported the strong influence of environment conditions on the expression of this trait. The most comprehensive study was developed by Franken (1970) who proposed a genetic model for the control of this trait based on an inhibitor gene (*A/a*) with intermediate inheritance being *AA* male plants that are non-andromonoic. Also, this author proposes a second modifier gene (*G/g*) with genetic effects on the expression of andromonoecy influenced by both environment conditions (photoperiod, temperature) and plant age. The *A* gene is considered responsible for the suppression of pistil development in male plants and, according to Galli et al. (1993), this gene segregates independent of the *M/m* locus. This result suggests that it is possible to create supermale plants nonandromonoic (*MMAA*) to be used in the development of all-male hybrids. Various studies have pointed out that the frequency of the andromonoecious plants in asparagus

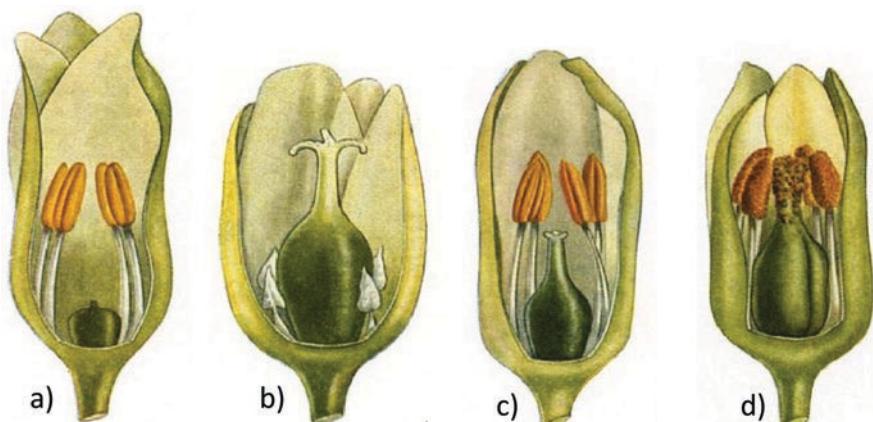


Fig. 12.5 Schematic draw of the different type of flowers in *Asparagus officinalis*. (a) Normal male flower. The rudimentary ovary is clearly visible. The stigmas are lacking. (b) Normal female flower. The rudimentary stamens are very small and produce no pollen. (c) Androgynous flower at a young stage. Style and stigmas not fully grown. (d) Androgynous flower with swollen ovary. Pollination has been effected. (Source: Sneep 1953a)

populations is very low (0.1%) with a range of 0–2% (Franken 1970; Sneep 1953a; Thévenin 1967). This could be related to a high frequency of the dominant allele of the inhibitor gene (*A/a*) in asparagus populations proposed by Franken (1970). Recently, with the sequencing of the asparagus genome, a sex-linked region of about 835 Kb has been reported as the locus of *M/m* (Harkess et al. 2017). This region was present in the male chromosome but absent in the female. Two genes tightly linked and related to male sex expression were found, a suppressor of female function (SOFF) and one implicated in the stamen development (TDF1). Therefore, if the gene *A/a* is independent of locus *M/m* it would be interesting to know the relation of gene *A/a* with the suppressor of female function (SOFF) in the sex-linked region. As mentioned above, another independent *G/g* gene is hypothesized to promote an increase of fructification rates in andromonoecious plants although its effects depend on the (*A/a*) gene genotype. In order to obtain all-male hybrids without the tendency for berry-bearing, it is necessary that the fruitless condition occurs in the supermale parents. Therefore, according to Franken's theory, supermale genotypes *MMAA*gg should be selected to get hybrids with nil or low andromonoecious expression. Our group has obtained different levels of fructification for andromonoecious plants in a progeny ($n = 170$) derived from a cross between two tetraploid plants; a female and an andromonoecious plant, with a low tendency to berry-bearing (unpublished results). This result agrees with the inheritance genetic model proposed by Franken (1970).

12.2.3 Inbred Lines

As mentioned above, heterozygous and homozygous plants, or inbred lines, are employed as breeding parents for asparagus cultivars. Homozygous parents can produce more uniform hybrids. In this sense, different methodologies and techniques have been employed in asparagus breeding in order to obtain homozygous plants or inbred lines. One of them is based on the polyembryony phenomenon that consists of developing extra embryos per seed. Normally in polyembryonic seeds, two embryos are formed. In diploid plants it is assumed that one of the embryos has asexual origin and theoretically proceeds from either a nucelli (diploid) or embryo sac (haploid). Polyembryony has been described in diploid plants of *Asparagus officinalis* and the tetraploid landrace Morado de Huétor as having a low frequency: 0.95% (Randall and Rick 1945), 0.22% (Marks 1973; Thévenin 1968), 0.34% (Uno et al. 2002), 0.31% (Moreno et al. 2010a) and 0.60% (Zenkteler et al. 2012). Thévenin and Doré (1976) developed a methodology based on the backcrossing of the derived homozygous female plants from monoploids as the recurrent parent with heterozygous male plants. That methodology produced different male and female inbred lines (near isogenic for sex trait) facilitating their propagation by seed. One of those inbred lines was employed as the female parent of the first released true F1 all-male hybrid (Andreas) developed by the French breeding program. The male parent of that hybrid was a supermale plant derived from in vitro

anther culture (Corriols et al. 1990). Commercial seeds of cv. Andreas were available in the 1990s. It should be noted that the development of all-male hybrids began around 1965 when cv. Lucullus was released by the German breeding program. Lucullus was developed using a supermale plant derived through self-pollination of an andromonoecious plant (Boonen 1988; Greiner 1990).

Another early method to produce inbred lines was through self-pollination of andromonoecious plants (Sneep 1953a; Thévenin 1967). The development of inbred or homozygous lines through traditional breeding is difficult and time-consuming due to the dioecious nature of the crop (Ellison 1986). However, this methodology provided a set of all-male hybrids that has been widely cultivated such as Venlim, Gynlim, Boonlim, Backlim, Thielim, Jesey Giant, Greenwich, Jersey Knight and Jersey King among others (Boonen 1988; Ellison and Kinelski 1985; Ellison et al. 1990; Scholten and Boonen 1996). Another methodology to obtain homozygous plants in asparagus is in vitro anther culture (Doré 1990; Falavigna et al. 1999; Tsay 1996; Wolyn and Nichols 2003). The induced chromosomes doubling by colchicine treatment, or the spontaneous doubling via endoreduplication that eventually occurs at the callus stage, yields doubled-haploid males (supermales) and females. Anther in vitro culture has allowed the development of doubled-haploid supermale plants that have been employed as parents of different all-male hybrids developed in various plant breeding programs. Two distinct types of hybrids have been developed by this method: two-way all-male hybrid from two homozygous doubled-haploid plants (Hybrid F1), and three-way all-male hybrid, in which female plants derived from a cross (F1) are crossed with a double-haploid supermale. As previously mentioned, cv. Andreas was the first all-male hybrid developed employing a doubled haploid supermale. It was followed by other cultivars developed in Canada (Guelph Millennium) or Italy, where this technique has been widely used to develop female and male doubled haploid plants, giving rise to a set of cultivars such as Eros, Zeno, Marte, Ercole and Vittorio, among others (Falavigna et al. 1999, 2012).

12.2.4 Polyploidy

As mentioned, polyploidy is present in the *Asparagus* genus. Indeed, a wide range of ploidy levels ($2x$, $4x$, $6x$, $8x$, $10x$, $12x$) has been detected in different species in both inter- and intraspecific taxa (Bozzini 1959; Castro et al. 2013; Ito et al. 2007; Jessop 1966; Moreno et al. 2006; Mousavizadeh et al. 2016; Rice et al. 2015). To a lesser degree, polyploidy breeding has been applied in asparagus. It is known in certain tetraploid cultivars such as the clonal hybrid Seto Green (Okimori et al. 1984) or some landraces cultivated in different countries like Spain (Morado de Huétor), Italy (Violetto d'Albenga) and Argentina (Cereseto and Poire) (Falavigna and Fantino 1985; López Anido et al. 2000; Moreno et al. 2006). Violetto d'Albenga has been employed to breed tetraploid cultivars with green spears such as Dulce

Verde (Wehner 2002) or purple spears like Purple Passion (Benson et al. 1996), Purple Pacific (Falloon and Andersen 1999), NJ1016 and Sweet Purple. Besides, an octoploid hybrid (HT801) has been obtained from a cross of two spontaneous octoploid plants (female and male) found in the Morado de Huétor landrace (Moreno et al. 2012).

In addition, triploid cultivars can be obtained from crosses between tetraploid and diploid plants. Asparagus triploid hybrid cultivars would have a similar behavior to male hybrids because of their very low or even nil fertility. With this aim, Ammal and Kaul (1967) obtained triploids by reciprocal crosses between diploid plants with tetraploids from colchicine treatments. These authors also mentioned that triploid lines were in progress, but no results are given in this study or subsequently. The first triploid cultivar (Hiroshima Green) was obtained from a cross between tetraploid and diploid plants both derived from cv. Mary Washington 500 W (Hasegawa et al. 1987). In this sense, tetraploid plants have been obtained in this species by colchicine treatment (Hasegawa et al. 1987; Ozaki et al. 2004; Skiebe et al. 1991). Tetraploids can also arise spontaneously through both in vitro anther culture (Falavigna et al. 1999; Tsay 1996) and the formation of unreduced gametes that have been described in this species (Camadro 1992; Regalado et al. 2015). The unreduced gamete phenomenon might explain the tetraploid plants found in an F2 population obtained from the clonal hybrid UC-157 (Camadro 1994). Also, unreduced gametes could explain the unexpected tetraploid plants observed in crosses aimed to develop triploid plants (Moreno et al. 2010a; Ozaki et al. 2004; Skiebe et al. 1991). However, tetraploid local cultivars such as Morado de Huétor or Violetto d'Albenga show a different origin from current diploid cultivars (Caruso et al. 2008; Geoffriau et al. 1992; Moreno et al. 2008a). Therefore, in crosses between local cultivars and diploids, a higher level of heterosis is expected. The development of triploid hybrids between tetraploid Morado de Huétor and diploid cultivars was explored in our group obtaining a wide variation on fertility (from high to very low) within tetraploid females (Moreno et al. 2010b). In this study, a set of triploid hybrids was evaluated for yield under field conditions. Triploid hybrids had yield values similar to the diploid cultivar (Grande) having the highest yield, suggesting that triploid hybrids could be of value in asparagus breeding. Accordingly, Morado de Huétor could be an interesting genetic resource for individual selection of females with both high fertility and good agronomic behavior, which can be used to obtain triploid hybrids.

Other hybrids with different ploidy levels (5x, 6x, 8x) could also be developed. For instance, taking advantage of the different polyploid levels (6x, 8x) found within the tetraploid landrace Morado de Huétor (Moreno et al. 2006) an octoploid hybrid was recently released (Moreno et al. 2012). The use of polyploidy in cultivated asparagus, employing local cultivars with a different genetic background, should contribute to enlarge the range of asparagus cultivars.

12.2.5 Breeding Objectives

12.2.5.1 Spear Quality and Yield

As mentioned above, most asparagus cultivars are diploid with green-colored spears. In addition to the total number of spears sprouted (yield), there are other agro-morphic traits of interest in asparagus plant breeding, including spear diameter, spear appearance, in which rounded sections and tight close tips are preferred, as well as the plant's ability to exhibit earliness over years (Fig. 12.6a).

For green spear production under warm climates, spears with tight close tips are desirable in order to avoid high percentages of non-marketable spears (Fig. 12.6b). Usually open asparagus heads occur when the temperature increases during the harvest season (spring-summer). Tightly closed spear tips are highly and positively correlated with height of branching of the fern stalks during summer or fall (Ellison 1986). It is well known among breeders that in the parental-selection process of elite plants it is strongly recommended that the potential parents show high branching stalks during summer and fall. Earliness is also an interesting agronomic trait in asparagus varieties because on the one hand, the price of the spears at the beginning of the harvest season is much higher, and on the other hand, this trait is positively correlated with yield (Ellison and Schermerhorn 1958; Ellison et al. 1960; Rameau 1990).

A large spear diameter is a current demand of some asparagus markets, although in others the consumption of large spears is not in high demand. In this sense, in some markets the price for large spears is usually higher compared with thin or medium-size spears. On the other hand, varieties with thick spears are preferred by growers because fewer spears have to be harvested per kilogram, thereby reducing harvest costs (Siomos 2018). A reduction in the size of spears has been observed as



Fig. 12.6 (a) Plant displaying earliness in an experimental trial conducted to evaluate a set of plants selected as potential parents, (b) Plants of an experimental hybrid exhibiting tolerance to low branching. (Photos by R. Moreno)

plants age (Ellison 1986; Krzesiński et al. 2008). Furthermore, some cultural practices have an influence on this trait, i.e. a high plantation density or severe cutting during the harvest season can reduce the size of the spears the following harvest season (Brock 2012; Lloyd and McCollum 1938).

To date, few studies have focused on the genetic control of these three traits. Therefore, their genetic inheritance model remains unresolved, being unknown which major and/or minor genes are involved. Recently, the asparagus reference genome has been released, and the first saturated genetic map has been obtained employing two parents that differ for important agro-morphic traits in this crop (Harkess et al. 2017; Moreno et al. 2018). The results obtained will be helpful in future works to locate genes and/or QTLs controlling agro-morphically-interesting traits in this crop and to carry out marker-assisted selection (MAS).

12.2.5.2 Disease Resistance

The development of varieties resistant to significant asparagus diseases has been one of the main goals in plant breeding since the beginning of the nineteenth century. Among diseases of economic importance, it is known that a group of soil borne diseases involving different phytopathogenic species of the genus *Fusarium* cause serious damages to the vascular and root systems of the asparagus plant (Elmer et al. 1996; Graham 1955; Johnston et al. 1979). In this sense, *Fusarium* spp. is the most important biotic factor of the asparagus decline syndrome. This syndrome is considered as a complex problem and is characterized by a slow decline in the productivity of old asparagus plantings to the point where they become unprofitable to maintain (Grogan and Kimble 1959). Decline in old plantations is generally considered the result of multiple biotic and abiotic stressors, whereas another problem of the asparagus decline, the replant problem, seems to be mainly caused by allelopathy and *Fusarium* pathogens (Elmer 2018).

Different *Fusarium* spp. are associated with this disease, among them, *F. oxysporum* f. sp. *asparagi*, *F. redolens* f. sp. *asparagi* and *F. proliferatum*, are considered as the most frequently occurring species (Elmer 2001). To date, studies focused on the genetic control of this disease have not been reported, although Lassaga et al. (1998) suggested a polygenic control with important environmental effects involved. An integrative approach including both good agronomic management and tolerant cultivars that promote vigorous growth seems to be a good strategy to control asparagus decline (Damicone et al. 1987; Nigh 1990). Up to now, cultivars resistant to *Fusarium* crown and root rot have not been identified. Nevertheless, some all-male hybrids have been described as tolerant (Ellison and Kinelski 1985; Ellison et al. 1990; Elmer 2018; Marcellán et al. 2010; Stephens et al. 1989).

Another important disease is asparagus rust caused by the aerial fungus *Puccinia asparagi* DC, which was first described by De Candolle (1805). This fungus is an obligate parasite which at the uredinal stage can cause serious damages during the vegetative period of the crop when environmental conditions are suitable for the

disease to develop. In this case, premature defoliation occurs and, therefore, the process of storage in the root system is interrupted, compromising yield in the next harvest season. Differences in resistance to rust infection have been observed within *Asparagus officinalis* after evaluation of different genetic stocks such as cultivars, landraces, wild or naturalized populations (Benson 2000; Foster and McDonald 2018; Johnson 2012; Norton 1913). To date, no physiological races of *P. asparagi* have been reported (Ellison 1986; Johnson 2012), although Benson (2000) suggests their existence based on differences to disease reaction to rust of a set of cultivars evaluated in different localities. Some studies have pointed out a quantitative, rather than qualitative, inheritance for resistance to rust (Hepler et al. 1957; Johnson 1986; Norton 1913). In crosses between resistant and susceptible plants, the rust resistance of the progeny showed a continuous distribution, in accord with the hypothesis of quantitative inheritance proposed for the trait (Hepler et al. 1957; Johnson and Peaden 1993). Transgressive segregation for rust was observed in populations derived from crosses between moderately resistant parents (Johnson and Peaden 1993).

Stemphylium vesicarium (Wallr.) Simmons is another aerial fungal disease known as purple spot, first described in Japan (Suzuki 1973). During the harvest season, if the infection is severe, this disease can affect the marketable yield due to reduced spear quality. Once the harvest season is finished, the fungus may infect the ferns (cladodes and branches) causing defoliation, dieback and their premature senescence (Bansal et al. 1986). Thus, the process of storage in the root system is interrupted and, consequently, an important yield loss may occur in the following harvest season. According to Menzies (1983), losses can reach 52%. Differences in disease reaction for purple spot have been observed in this species (Benson 2000; Broadhurst 1996; Foster and McDonald 2018).

Phytophthora spp. are responsible for rot disease, which may cause severe reduction in asparagus stands in the first year after transplantation, affecting also the quality of harvested spears (Falloon 1985). The disease appears to be related to heavy and prolonged rainfall and/or asparagus producing areas with wet or water-logged soils (Elena 2007). To date, resistant varieties have not been reported although differences in tolerance between cultivars have been detected (Woods and Hausbeck 2018).

Other asparagus diseases causing economic losses are *Phomopsis* stem blight, *Cercospora* blight and different viruses.

12.2.6 Genetic Resources

12.2.6.1 Opportunity of Use

As mentioned above, nearly all current cultivars of garden asparagus cultivated worldwide are diploid and derived from the Dutch population Violet Dutch (~ XVIII). For this reason, a narrow genetic base of this crop has been reported (Brettin

and Sink 1992; Geoffriau et al. 1992; Khandka et al. 1996; Lallemand et al. 1994; Mercati et al. 2015; Moreno et al. 2006). Thus, nowadays the development of new germplasm is a major goal in asparagus breeding. In order to broaden the gene pool for breeding, different genetic resources are available such as landraces, old cultivars and wild populations of both cultivated and related species. Some studies have pointed out the wide range of environmental conditions (elevation, climate, soil) that comprise the natural habitat of wild populations of *Asparagus officinalis* and wild *Asparagus* spp. (Kanno and Yokoyama 2011; Mousavizadeh et al. 2015). Some studies employing different wild asparagus populations or landraces have revealed a high genetic variability with a different distribution of current cultivars (Castro et al. 2013, 2014; Moreno et al. 2006; Mousavizadeh et al. 2018). Such results suggest that these genetic resources may be a source of new alleles or genes potentially useful in asparagus breeding for yield, nutritional quality, adaptability and resistance to different biotic and abiotic stresses affecting the crop.

Several studies using wild *Asparagus* spp. have pointed out in some species the existence of tolerance or resistance to different stresses (see Kanno and Yokoyama 2011). Among them, *A. densiflorus* (Kunth) Jessop, which belongs to the *Protaspasparagus* subgenus, has been reported as resistant to *Fusarium* spp. (Lewis and Shoemaker 1964; Marcellán and Camadro 1996; Stephens et al. 1989). Also, resistance to both rust and virus was found in *A. maritimus* (Nothnagel et al. 2014) and resistance to both purple spot and rust disease was reported in *A. acutifolius*, *A. albus*, *A. horridus* and *A. aphyllus* (Alberti et al. 2004; Falavigna et al. 2008). Related to abiotic stress, tolerance to salinity, drought and acidic soil was observed in *A. maritimus*, *A. acutifolius* and *A. tenuifolius* L., respectively (Venezia et al. 1993). *Asparagus breslerianus* Schult. & Schult. f. has been reported growing in dry gypsum hills and dry lands in Iran (Rechbineer 1982) and a population was collected in a region of hot and dry climate with a high saline soil (Mousavizadeh et al. 2015).

Interspecific hybridization is a technique that might be useful to introgress genes from wild *Asparagus* spp. into the cultivated one. The first interspecific hybrid reported between the cultivated asparagus and a wild species (*A. dauricus* Fish. ex Link) was carried out by Norton (1913). Since then, different authors have developed studies aimed at obtaining interspecific hybrids between *A. officinalis* and wild species belonging to the three *Asparagus* subgenera, but thus far, successful crosses have been solely obtained with some related species included in the *Asparagus* subgenus (see Kanno and Yokoyama 2011). In this sense, only a reduced number of wild asparagus species have been successfully crossed with *A. officinalis* including: *A. maritimus* (Castro et al. 2013; Falavigna et al. 2008; Plath et al. 2018), *A. pseudoscaber* Grec. (Castro et al. 2013; Plath et al. 2018), *A. tenuifolius* (Bozzini 1963), *A. prostratus* Dum. (Castro et al. 2013; McCollum 1988a; Plath et al. 2018), *A. brachyphyllus* Tunc. (Castro et al. 2013; Ito and Currence 1965), *A. macrorrhizus* (Amian et al. 2018), *A. acutifolius* (Thévenin 1974), *A. oligoclonos* (McCollum 1988b), *A. schoberioides* Kunth (Ito et al. 2007; Ochiai et al. 2002) and *A. kiusianus* Makino (Ito et al. 2011).

Different factors such as postzygotic barriers or large differences in ploidy level of the parents employed in the crosses could make impossible hybridization between

species. However, the use of a genetic bridge might be helpful to overcome these crossing barriers. Falavigna et al. (2008) obtained one hybrid plant from the cross between a tetraploid plant, derived from *Asparagus officinalis* x *A. maritimus* and *A. acutifolius*. Besides, the tetraploid Morado de Huétor has been employed as a bridge to transfer wild germplasm with different ploidy level (4x, 6x, 12x) into cultivated plants because of its tetraploid condition and the different polyploidy levels (6x, 8x) found in this landrace (Amian et al. 2018; Castro et al. 2013; Moreno et al. 2006). Our group has successfully crossed cultivated asparagus with *A. persicus* Baker and *A. breslerianus*. (unpublished results).

Despite the important role that wild asparagus species could play in the genetic improvement, studies focused on development of new 2x germplasm using wild *Asparagus* spp. have been scarce so far. In this sense, Castro et al. (2014) and Regalado et al. (2016) obtained diploid plants carrying germplasm from Morado de Huétor, a tetraploid landrace for which is proposed a natural interspecific origin (*A. officinalis* x *A. maritimus*) (Moreno et al. 2008a). Moreover, Riccardi et al. (2011) obtained dihaploid plants via in vitro anther culture employing tetraploids plants from backcrosses involving two wild species (*A. acutifolius*, *A. maritimus*) and one landrace as recurrent parent (Violetto d'Albenga). Recently, it has been reported that new 4x and 6x germplasm with introgression of wild relative polyploid species including 4x (*A. prostratus*), 6x (*A. maritimus*, *A. pseudoscaber*, *A. brachyphyllus*) and 12x (*A. macrorrhizus*) from a first backcross using the landrace Morado de Huétor as the recurrent parent (Amian et al. 2018).

12.2.6.2 Conservation Status

According to FAO (2019), a total of 1287 accessions are maintained in different gene banks, representing 57 *Asparagus* spp. The number of different asparagus species may be understated, because 772 of the 1287 accessions lack a complete scientific name. Among the 515 accessions classified by species, 309 belong to *A. officinalis*; these are divided into different groups including advanced cultivars (54), breeders' line (17), traditional cultivar/landraces (26) or wild (73). The remaining 139 accessions lack information related to their genetic stock origin. Additionally, the International Minor Leafy Vegetables Database, a section of the European Search Catalogue for Plant Genetic Resources (EURISCO), lists 334 accessions; 170 belonging to *A. officinalis* (EURISCO 2019). To date, the accessions held by EURISCO come from 21 countries. Not all the accessions data and availability are given (Lohwasser and Börner 2018). According to Van Treuren et al. (2012), cultivated species are well represented, considering the number of accessions catalogued in the different gene banks. However, the number of accessions of wild relative species is very small. In this sense, collecting missions are urgently needed to fill the gap of wild relatives in the gene banks (Lohwasser and Börner 2018).

12.2.7 Marker-Assisted Selection

Traditional breeding has improved asparagus cultivars. However, the selection of new cultivars is laborious and time-consuming. Cultivar quality must be constantly adjusted and optimized to satisfy consumer demands and industry needs in an environment of increasing barriers to productivity. These demands may be effectively addressed through implementation of biotechnology. The integration of genomic technologies in asparagus breeding may increase the efficiency of breeding programs and ultimately facilitate the development of new varieties. Molecular markers have become a valuable tool with different applications that include linkage mapping, genetic diversity evaluation and cultivar identification. Also, molecular markers are useful for identifying and selecting genotypes with favorable traits. Molecular markers linked to traits of interest can be used to select desirable traits in a segregating population and facilitate crop improvement through MAS (Collard and Mackill 2008). Despite markers being a valuable molecular tool, the number of available markers in asparagus remains limited. Most molecular studies in asparagus have focused on identifying markers for sex determination. Initial studies (Biffi et al. 1995; González-Castañón and Carbajal-Carcedo 1996; Jamsari et al. 2004; Jiang and Sink 1997; Maestri et al. 1991; Reamon-Büttner et al. 1998; Reamon-Büttner and Jung 2000; Restivo et al. 1995; Spada et al. 1998) reporting markers linked to the sex determination locus have been reviewed by Anido and Cointry (2008). Here we provide an update on additional studies.

In 2006, Nakayama et al. (2006) used the previously published Asp1-T7 marker (Jamsari et al. 2004) to identify male and female plants from the asparagus cv. Mary Washington 500 W and found out that some male plants and all the female plants could not be detected using that marker. Hence, they used the sequence amplified by Asp1-T7 marker to develop new sex-linked primers (Asp1-T7spf, Asp1-T7spr) that are applicable to various cultivars of garden asparagus. Later, that new marker was successfully used by Regalado et al. (2014) to determine sex in the tetraploid landrace Morado de Huétor. Telgmann-Rauber et al. (2007) used a bulked segregant analysis to develop 12 new amplified fragment length polymorphism (AFLP) markers linked to the *M*-locus. In their study, the authors employed those markers to enrich a genetic map, which included 26 markers and covered a distance of 8.01 cM around the *M*-locus on chromosome L5 of *Asparagus officinalis*. The sex locus was flanked by the closely linked AFLP EM3646 and a cluster of two AFLP (EM4150, EM3156) and two STS markers (STS4150.1, STS3156). Also, in an attempt to identify sex-linked DNA markers, Ii et al. (2012) screened random amplified polymorphic DNA (RAPD) markers in male and female plants of cv. Mary Washington 500 W and reported the identification of a new sex-linked RAPD marker named T35R54-1600. The authors also reported the utility of this marker for sex identification in cv. Zuiyu, but the applicability of this marker in other asparagus cultivars has not been tested. Since the RAPD marker technique is sensitive to changes in

reaction conditions and lacks reproducibility among laboratories, Kanno et al. (2014) converted T35R54-1600 into a stable male-specific STS marker MSSTS710. This marker is suitable for sex identification in *A. officinalis* but not in closely related *Asparagus* species.

Although several genetic maps, with an emphasis on the sex-determining region, and a number of sex-linked markers have been reported, more efforts are still needed. The asparagus research community must keep working on developing a codominant marker useful to distinguish males from supermales and two new sex-linked markers were reported (Mitoma et al. 2018; Stone et al. 2018). Mitoma et al. (2018) developed a new sex-linked marker because the available markers (Asp1-T7sp, MSSTS710) were not suitable to identify males of cv. Pacific Purple, which was bred from an Italian tetraploid landrace Violetto d'Albenga. They used the sequence of sex determination gene *MSE1/AoMYB35/AspTDF1* (Harkess et al. 2017; Murase et al. 2017; Tsugama et al. 2017) to develop a new male-specific marker (AspMSD). The *MSE1/AoMYB35/AspTDF1* gene is responsible for stamen development. The AspMSD marker successfully amplified in males but not in females of cv. Pacific Purple, indicating that it is suitable for sex identification in this cultivar. In their study, the authors also demonstrated the applicability of this marker for sex identification in various cultivars of *Asparagus officinalis* and three related species *A. kiusianus*, *A. schoberioides* and *A. maritimus*. Therefore, this marker is more useful for sex identification than previously-reported markers. However, AspMSD is a dominant marker, which is a disadvantage.

Stone et al. (2018) developed a new sex-linked codominant sequence-tagged site (STS) marker useful for discriminating between normal males (*Mm*), supermales (*MM*) and females (*mm*). These authors employed the asparagus reference genome sequence to design primers in the sex-determining region. They located the putative male promoter gene and the putative female suppressor gene in the asparagus genome. These two genes define the putative sex determination locus and allowed them to narrow the candidate region down to 835 Kb (Harkess et al. 2017). Near the 5' end of that region, they found a 26 bp deletion that was present in the females and absent in the males. So, they made use of that finding to develop a sex-linked marker named RM17.

RAPD, RFLP and restriction fragment length polymorphism (RFLP) are the kind of markers most used in asparagus so far. Microsatellites or simple sequence repeats (SSR) markers are not widely used because they are expensive to obtain. However, with the advent of next-generation sequencing (NGS) technologies, sequence information became publicly available (see Sect. 12.3) and SSR marker development more affordable. In 2008, Caruso et al. (2008) developed a set of SSR primers derived from a public EST collection (Kuhl et al. 2005) and employed them to assess genetic diversity among asparagus cultivars. These markers are worth mentioning because they were the first SSR markers provided to the asparagus research community, and they were used in many of the studies published in subsequent years. Nevertheless, SSR are valuable for marker assisted breeding and positional cloning of genes associated with traits of interest, a new group of SSR markers

would not be reported until five years later when Mercati et al. (2013) developed the first EST-SNP and new EST-SSR markers for asparagus. These markers, together with the previously reported marker Asp1-T7sp, were genotyped in a backcross population, and the data were used to construct a preliminary genetic linkage map. The genetic map covered a total of 773.5 cM and consisted of 81 markers (65 SNP, 15 SSR, Asp1-T7sp) located on 13 linkage groups. The sex locus (M-locus) mapped on their LG10, which included only two markers (Asp1-T7 and one SNP). Therefore, the sex determination region should be saturated with yet more markers. A new collection of SSR markers was developed by Li et al. (2016). These researchers conducted a genome-wide SSR characterization using the genome sequencing data previously obtained by Li et al. (2014). Although the sequencing data only represented partial sequences of the *Asparagus officinalis* genome (Li et al. 2014), they were useful to develop genomic SSR markers. In their study, Li et al. (2016) used some of those SSR markers to study the genetic diversity of *A. officinalis* cultivars. Unfortunately, they did not use these markers in other applications and did not report whether the markers are linked to agronomic traits of interest. The abundance of SNP and SSR markers developed by Caruso et al. (2008), Mercati et al. (2013) and Li et al. (2016) provide a foundation for the development of saturated genetic maps and their utilization in asparagus breeding programs. The first high-density genetic linkage map was reported by Moreno et al. (2018) who employed genotyping-by-sequencing (GBS) to identify SNPs in a F1 population. They used those SNPs and 27 SSR markers previously reported (Caruso et al. 2008; Li et al. 2016; Mercati et al. 2013) for genetic map construction. The resulting parental maps included 907 and 678 markers covering a genetic distance of 1947 and 1814 cM, for female and male, respectively, over 10 linkage groups representing 10 haploid chromosomes of the species. The authors mapped the sex trait on LG5 and suggested that it represents the sex chromosome of asparagus mentioned in Sect. 12.1.5.

12.3 Integrating New Biotechnologies into Breeding Programs

Recent efforts to understand agronomic traits through different genomic approaches have paved the way to explore the relationships between genetic and phenotypic diversity, with a resolution that was impossible even five years ago. The availability of molecular markers and genetic and physical maps, transcript data and recent large-scale genomic resources, such as the asparagus reference genome sequence, combined with cost-effective and high-throughput genotyping technologies and the development of improved bioinformatics tools, open new prospects for the biological understanding of a wide range of traits. Future directions on developing a genomics-assisted breeding strategy by integrating outputs of the breeding program with candidate gene-based trait association mapping, high-throughput sequencing, QTL mapping and differential gene expression profiling will have significant implications for asparagus genetics and breeding.

12.3.1 Genomic Resource Availability

In recent years, a considerable amount of large-scale genomic resources, such as molecular markers, BAC-end sequences, transcript reads and comprehensive genetic and physical maps, have been developed in *Asparagus officinalis*. As of September 1, 2019, a total of 82,195 nucleotide and 65,154 protein sequences were available from the GenBank at the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/). In terms of numbers of curated nucleotide sequences (Reference Sequence database, NCBI), *A. officinalis* ranks 63rd among plants in general and third among plants in the Asparagales order. As sequence technologies have evolved, genomic projects in asparagus have moved away from generating clone libraries and ESTs in favor of next-generation data. Recent advances in next-generation sequencing (NGS) technologies, with increased throughput and reduced sequencing costs, have massively increased the number of asparagus nucleotide sequences deposited in public databases and have opened up many new opportunities to explore the relationship between genotype and phenotype with greater resolution than ever before (Fig. 12.7). In July 2019, the NCBI retired the databases Genome Survey Sequences and Expressed Sequence Tags (GSS and EST), which hosted a number of asparagus records. These data are, however, still available in GenBank through the NCBI Nucleotide database (nucore). In this section, we describe a number of advanced technologies and available genomic resources for garden asparagus which may lead to new strategies for crop improvement.

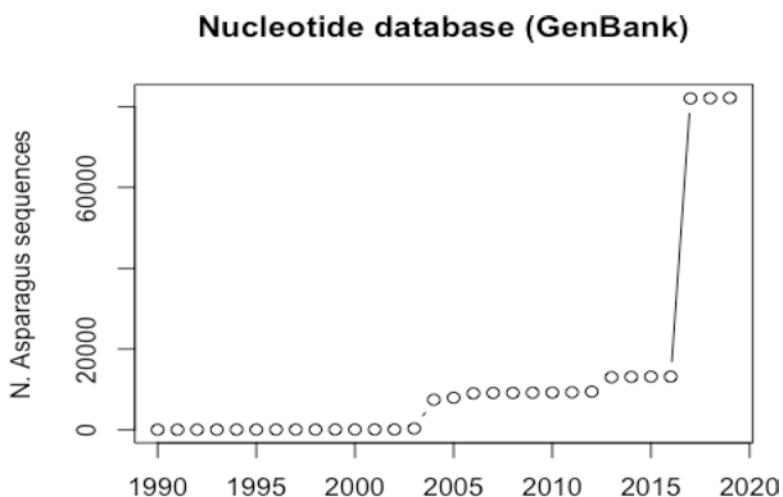


Fig. 12.7 Number of *Asparagus officinalis* nucleotide sequences submitted to the nucleotide division of GenBank. Sequences distribution peak at 2017 with the release of the reference genome sequence. (Figure constructed by J.V. Die)

12.3.2 Genomic Libraries and Expressed Sequences Collections

The first genomic sequences in asparagus were based on large-insert DNA libraries to provide a resource for map-based cloning of the sex gene. Jamsari et al. (2004) constructed the first bacterial artificial chromosome (BAC) library of *Asparagus officinalis*. The library consisted of 86,784 clones. After selection of sex-specific BAC clones, four diagnostic markers were developed to differentiate between male and female plants. Then, the markers were used to construct a high-density map around the sex gene (BioSample: SAMN00184329). The generation of genomic and expressed sequences were also used for comparative analysis between the Asparagales and Poales, the two most economically-important monocot orders. The comparative approach revealed important genomic differences from Poales, suggesting that genomic resources developed for the grasses might not be applicable to Asparagales species (BioSample: SAMN00182338, SAMN00183528; Kuhl et al. 2004, 2005). Another outcome from the analysis was that the smaller genome size of *Asparagus* species could be useful as a genomic model for Asparagales plants with enormous nuclear genomes, such as those in the Agavaceae and Amaryllidaceae families (Jakse et al. 2006; Kuhl et al. 2005). The production of new BAC libraries coupled with survey sequences and comparative genomics have extended our understanding of monocot genome evolution. These BAC libraries were initially made to assess synteny between species and facilitate map-based cloning (Jakse et al. 2006). More recently, annotation and analysis of the BAC libraries have revealed that LTR retrotransposons are major components of the asparagus genome (BioSample: SAMN02264503; Vitte et al. 2013).

Transcriptome data provide insights into the processes underlying gene expression and may facilitate gene discovery. Although EST collections are a valid and reliable source of gene expression data, random sampling of cDNA clones gives preferential access to more highly abundant transcripts. Thus, other strategies are necessary when the aim is to identify differentially-expressed transcripts that may play a key role in the processes under study. To address the underlying molecular differences between male and female flowers of asparagus, Deng et al. (2015) constructed a subtractive hybridization library of male flowers by the suppression subtraction hybridization (SSH) approach (BioSample: SAMN02364165).

12.3.3 Large-Scale Sequencing Projects

Next-generation sequencing technology for large-scale transcriptome analysis has become routine for generating large amounts of expression data in a relatively short time. In 2013, a newly developed dataset of EST (>208,000 sequences) was released for male and female garden asparagus using 454-GS FLX Titanium technology (Mercati et al. 2015). The raw sequences are publicly available in the SRA database of the NCBI (Bioproject PRJNA184373). This represents the first high-throughput

transcriptome of the species using NGS methodologies. These data generated a novel EST-SSR panel together with the first EST-SNP panel in asparagus, which markedly expanded genomic resources for the species available at that time. Li et al. (2014) performed a comprehensive characterization of the transposable elements (TE) fraction of *Asparagus officinalis* by analyzing whole genome sequences via Illumina paired-end technology from both male and female asparagus (Bioproject PRJNA237704). Although the assembly contigs represented only partial sequences (~ 30%) of the genome, the analysis illustrated the transposon-rich complexity of the *A. officinalis* genome. The majority of the identified TE belonged to LTR retrotransposons (~28% of genomic DNA).

As sex determination is such a very important trait for breeders and plant biologists, the topic has received considerable attention from researchers and a number of genomic libraries are publicly available. A common approach is to use RNA-sequencing (RNA-Seq) to identify genes exhibiting gender-biased expression. De novo transcriptome sequencing via Illumina paired-end sequencing revealed more than 26 billion bases of high-quality sequence data from male and female asparagus flower buds (Li et al. 2017). About 5000 genes and 130 TF-related genes belonging to various families were found to be differentially expressed (Bioproject PRJNA357520). This outcome, however, does not mean those genes regulate gender determination. It is expected that many genes exhibit sex-based expression as most of them must be required for the development of male and female flowers, thus functioning downstream of gender specification. An alternative approach to narrow the search is to annotate differential expressed genes (DEG) and list only those that are known to play some role in the anther and pistil developmental pathways. Those few may be better candidates to regulate sex determination. Following this strategy, Harkess et al. (2015) generated a large collection of libraries using Illumina RNA-Seq technology and used sequencing data of female, male and supermale spear-tip tissue to identify DEG by gender (Bioproject PRJNA259909). The study identified some genes involved in pollen microspore and tapetum development that were specifically expressed in males and supermales. Reanalysis of this RNA-Seq data could further identify a male-specific MYB transcription factor, which is expressed in tapetal cells of male flowers (Tsugama et al. 2017). The gene is homolog of *DEFECTIVE IN TAPETUM AND FUNCTION 1* (*TDF1*) in *Arabidopsis*. Using transcriptomic sequences from early developmental buds of male and female plants combined with genome sequencing data, Murase et al. (2017) found the same transcription factor involved in sex determination. Results from their linkage analysis suggest that loss of function of *TDF1* causes the arrest of male organ development in asparagus female flowers (Bioproject PRJDB4559). Another approach to study sex determination and the reproductive developmental process is the analysis of microRNAs (miRNA). Chen et al. (2016) identified 154 conserved miRNA in the asparagus genome, which clustered into 26 families. Comparative profiling revealed significant differential miRNA expression between male and female plants (Bioproject PRJNA294446). Recently, Kakrana et al. (2018) shed some light on another class of small regulatory RNAs. Although the evolutionary origins of plant reproductive small-interfering RNAs (siRNAs) are poorly characterized, they are

known to play a role in male reproductive success. Using Illumina and PacBio sequencing data, the authors found an abundance of phased siRNAs, which displayed spatial localization and temporal dynamics similar to grasses (Bioproject PRJNA449058).

Current investigations using large-sequencing data in asparagus are being conducted to resolve deep phylogenetic relationships or to understand the molecular basis underlying important agronomic traits. Ren et al. (2018) generated RNA-Seq derived from young leaves. Whole-transcriptome Illumina sequencing yielded nearly 16.8 million paired-end reads. After transcripts assembly, they estimated 4788 gene duplications and obtained phylogenomic support for whole genome duplication (WGD) in asparagus (Bioproject PRJNA421868). Understanding the salt tolerance mechanism is of interest because garden asparagus is a moderately salt-sensitive crop. Two recent projects have investigated global gene transcription changes in the leaf tissues of *Asparagus officinalis* using the Illumina sequencing platform. Bioproject PRJNA560341 contains the raw sequence reads from a time-course experiment (1, 24 and 72 h after treatment), whereas Bioproject PRJNA527697 explored the molecular mechanisms underlying salt tolerance in asparagus initiated by arbuscular mycorrhizal fungi (Zhang et al. 2019).

12.3.4 Reference Genome

It is important to mention that advances have not been limited to transcriptome sequences. In 2017, the University of Georgia USA made available the first annotated chromosome-level reference genome (Bioproject PRJNA317340). The asparagus genome assembly (Aspof.V1) spans 1.187 Gb with 93.6% (1.11 Gb) of sequences assigned to 10 chromosomes and 11,792 unassigned scaffolds representing 75.65 Mb.

A fully-assembled high-quality reference genome allows researchers to discover genes related to agronomic traits, determine their location and function as well as develop genome-wide molecular markers. Thus, the release of the asparagus reference genome has laid a solid foundation for downstream molecular research. For example, based on the genome assembly, Harkess et al. (2017) characterized a non-recombinant region of the Y chromosome, which likely contains all genes necessary for sex determination in males. Also, to elucidate the important role of NBS-LRR genes in garden asparagus, our group reported 49 NBS-LRR gene homologs and analyzed their structural diversity. Gene mapping analysis disclosed an important genomic region on chromosome 6 that contained 10% of the NBS genes. Our conclusions regarding the evolution of NBS-LRR in asparagus is that recent duplications are likely to have dominated the NBS expansion, with both tandem genes and duplication events across multiple chromosomes (Die et al. 2018).

Breeding programs will likely benefit from the available current and future genomics resources, as these will facilitate marker-assisted and genomic selection, and it is hoped the development of new high-quality cultivars in shorter breeding cycles.

12.3.5 Databases and Online Resources

One important challenge in using high-throughput sequencing technologies is the downstream computational analysis. Thus, the development of bioinformatic tools is highly important for managing data and generating transcription profiles. Various websites have been established to support such genomic experiments. One of the first that included access to asparagus sequences was The Floral Genome Project (FGP, <http://www.floralgenome.org>). The FGP was a multi-institutional study funded by the US National Science Foundation Plant Genome Research Project with the ultimate goal to understand how floral developmental pathways originated and diversified. FGP was designed to generate large EST datasets, capturing thousands of sequences of genes expressed during early flower development in 15 lineages of flowering plants and gymnosperms. FGP also provided new bioinformatic tools for the comparative analysis of gene history, gene function and molecular evolution. As asparagus plants are dioecious, two libraries were sampled and sequenced from male and female floral tissues. Although the FGP website is no longer maintained (2002–2006), the data are available for retrieval at GenBank (BioSample: SAMN00175615, SAMN00175591).

Another website is the Asparagus Genome Project (<http://asparagus.uga.edu>). The dataset houses the asparagus reference genome assembled for a doubled haploid YY. The website provides public access to CDS and protein sequences, permits BLAST and allows researchers to search data through the genome browser.

The asparagus reference genome is also available at the assembly database from NCBI (assembly Aspof.V1, Bioproject PRJNA317340) and hosted by the Bioconductor Project, an open-source and open-development software for the analysis and comprehension of high-throughput data in genomics and molecular biology (Die 2018).

12.4 Future Goals in Asparagus Breeding

12.4.1 Enlargement of the Genetic Base

For a successful breeding program, the availability of genetic variability is of crucial importance. Thus, outstanding results were obtained in the Dutch breeding program by combining two major germplasm stocks (Argenteuil, Ruhm von Braunschweig). The approach resulted in different varieties that have been largely cultivated for white spear production, such as Venlim, Gynlim, Boonlim and Backlim (Boonen 1988; Knaflowski 1996). Besides, other varieties cultivated worldwide for green spear production in warm climates such as Apollo, Atlas and Grande, were obtained at the end of the last century by crossing plants from different genetic stocks developed at Rutgers University (New Jersey) and University of California (Davis and Riverside) (Benson et al. 1996), respectively. However, the

germplasm stocks employed until now have the old Violet Dutch populations as common origins.

The availability of wide genetic variability in a plant breeding program opens the possibility to incorporate new genes or alleles controlling both currently-demanded agronomic traits in asparagus varieties and new traits that consumers or growers could demand in the future. Also, under the negative effects of climatic change new varieties adapted to new environmental conditions should be developed. In addition, if a highly-variable genetic collection of parents is available, new combining of genetic abilities may be explored in order to maximize favorable heterosis in garden asparagus. As mentioned before, new variability from relative species can be introgressed into the genetic background of the crop. More efforts should be made to explore the potential of germplasm of wild relatives in asparagus breeding programs. Wild related species could be a genetic source for bioactive components and for resistance or tolerance to different biotic or abiotic stresses affecting the crop (Sects. 12.1.5 and 12.2.6). In spite of the importance that the studies focused on the assessment of both heterosis and inbreeding depression might have in this dioecious crop, few studies have been carried out with this aim so far, although heterosis in garden asparagus seems to be very high (Rameau 1990). In this sense, heterosis was obtained by Ito and Currence (1965) and Rameau (1990) respectively in crosses between inbred lines and homozygous plants derived from in vitro anther culture. Moreover, inbreeding depression has been reported in inbred lines (Ito and Currence 1965). Also, a reduction of yield in F₂ progenies derived from the clonal hybrid UC-157 was found by different authors (Farías et al. 2004; González and Del Pozo 2002; Guangyu 2002) that might be explained by inbreeding depression.

According to Anido and Cointry (2008), the general ability (related to additive gene action) and specific ability (related to nonadditive gene actions) effects are of equivalent importance in determining the yield output of hybrids. Thus, in order to secure the highest hybrid performance, breeding programs should devote efforts to maximize both types of gene actions. Recurrent selections could be applied to increase the frequency of alleles with additive gene action. Whereas in order to maximize the potential heterotic effect (related to nonadditive gene actions), the parents for hybrid crosses should be derived from populations with high genetic divergence. Moreover, taking into account the narrow genetic base of the crop, the development of new genetic base populations with introgression of different germplasm such as landraces and wild populations might be also useful in the selection of new parents to be used in the development of new hybrids with higher heterotic potential.

12.4.2 *Polyplody Management*

Polyplody has great potential in asparagus breeding in order to enlarge the genetic base of asparagus cultivars. Tetraploid landraces have different origins than current diploid cultivars and wild related species also show ploidy variation (Caruso et al.

2008; Castro et al. 2013; Moreno et al. 2006, 2008a). On the other hand, polyploid hybrids can exhibit progressive heterosis (Birchler et al. 2010). As a rule, the higher the ploidy level in a genome, the larger the magnitude of heterosis expected. Our group has obtained hybrids with different ploidy levels (3x, 4x, 5x, 6x) that are currently under experimental field trials. Also, a diploid plant collection has been obtained by crosses between tetraploid plants of Morado de Huétor and diploid plants of different cultivars (Mary Washington, UC157, Grande, Atlas). The 3x plants were backcrossed using the diploid as recurrent parents (Castro et al. 2014). Crosses between plants having high differences in ploidy levels were less successful (6x * 2x, 8x * 4x or 12x * 4x). However, this type of crossing could be useful to introgress germplasm from wild related species into the crop.

The development of a wide genetic collection of diploid supermales is to use them as a parent of new experimental hybrids to develop all-male hybrids. In this case, considering the diverse origin of tetraploid varieties, the use of tetraploid male plants as anther donors for in vitro culture may be helpful to obtain a wide genetic collection of supermales. In order to get dihaploid plants *MM*, a set of male plants *MMmm* as anther donors could be employed as well. In this sense, dihaploid female plants (*mm*) have been previously obtained by in vitro culture of anthers derived from a *Mmmm* genotype of Morado de Huétor landrace (Regalado et al. 2016). Thus, these results open the possibility to produce dihaploid (*MM*) supermale plants employing *MMmm* plants in which 1/6, 4/6 and 1/6 are the expected frequencies of the *MM*, *Mm* and *mm* gametes, respectively. Male genotype *MMmm* can be obtained by self-pollination of andromonoecious plants *Mmmm*. This genotype *Mmmm* is practically the only present in tetraploid populations (Moreno et al. 2008b; Regalado et al. 2014).

12.4.3 Molecular Marker-Assisted Breeding

As it has been mentioned, sex is an important trait in asparagus breeding and efforts have been done in order to get a codominant marker to distinguish male and supermale plants. Stone et al. (2018) developed the codominant marker RM17, which is tightly linked to the sex locus. This marker amplifies a 189 bp fragment in plants with the *m* allele and a 215 bp fragment in plants with the *M* allele. To check the applicability of the RM17 marker, the authors tested it in numerous plants from the University of California Riverside germplasm collection. The RM17 marker results agreed with flower morphology for all plants tested, demonstrating its usefulness for diploid plants. The authors also surveyed plants from the UC Riverside Third International Cultivar Trial and found out that at least one possible crossover event between the marker and the sex locus likely occurred in an ancestor of Purple Passion, Sweet Purple, NJ1016 and Pacific Purple. These cultivars are all tetraploid and related to Violetto d'Albenga and, therefore, this marker highlights the divergence between the tetraploid and diploid asparagus varieties. The authors also concluded that difference in the mechanism of sex expression among diploids and

tetraploids could account for the failure of RM17 to correctly predict the sex determining locus in tetraploids. In our lab this marker was tested in two hexaploid populations of *Asparagus maritimus* being monomorphic (unpublished data). The deletion of 26 bp was not present in female of this wild species. It seems that introgression of *A. maritimus* is present in tetraploid cultivars by natural hybridization (Moreno et al. 2008a). This result could explain why RM17 fails to predict correctly the sex determining locus. So, a useful marker to predict the status of the sex locus in tetraploid germplasm is still needed. Regarding other traits of agronomic interest, more efforts should be made to find molecular markers linked to them.

12.5 Conclusions and Prospects

One of the main goals in asparagus breeding is the development of new germplasm. For that, different genetic resources such as landraces and wild populations related to the crop can be useful. Nevertheless, the use of these resources to develop new germplasm has yielded few results. It is known that some tetraploid landraces such as Morado de Huétor and Violetto d'Albenga have different origins than current diploid cultivars. These landraces seem to have emerged from natural introgression of wild asparagus *Asparagus maritimus* into the crop. Today, some tetraploid cultivars have been bred from Violetto d'Alvenga. On the other hand, a diploid population with germplasm introgression from Morado de Huétor has been obtained. Therefore, the use of polyploidy in the development of new cultivars might contribute to enlarge the genetic base of asparagus varieties. Polyploidy is also highly present in wild related species which can be used to introgress new germplasm into asparagus breeding.

The integration of biotechnology in asparagus breeding has lagged behind other important vegetable crops. The main efforts have been focused on the development of molecular markers linked to sex locus. Both dominant and codominant markers are available to be used in current diploid cultivars, the codominant ones being the most interesting. However, in tetraploid cultivars and wild species the codominant markers available do not predict correctly male or female plants. Thinking about the use of these resources in asparagus breeding programs, more efforts should be done to develop a more universal marker. The genetic and molecular base of other important traits like agro-morphic traits, disease resistances or nutraceutical compounds, which could be important in future, has been scarcely studied. Therefore, there are no effective markers for rapid screening of these traits in asparagus germplasm.

The availability of new biotechnological tools, such as the asparagus reference genome sequence, combined with cost-effective and high-throughput genotyping technologies and the development of improved bioinformatics tools, open new prospects for their use in asparagus breeding. In the near term, it will be helpful to identify genes and/or QTLs controlling interesting traits in this crop and develop new markers useful for marker assisted selection.

Appendices

Appendix I: Research Institutes Relevant to Asparagus

Institution name	Specialization and research activities	Address	Contact person	Website
CRA-Research Institute of Vegetable Crops	Plant breeding, genetics and in vitro culture	CRA - Unità di ricerca per l'orticoltura (ORL) 26836 Montanoso Lombardo, Lodi	Agostino Falavigna agostino.falavigna@entecri.it	http://www.entecri.it/
University of California	Plant breeding and genetics	Department of Botany and Plant Sciences, Riverside, CA 92521, USA	Mikeal L. Roose mikeal.roose@ucr.edu Neil Stone neil.stone@ucr.edu	http://www.plantbiology.ucr.edu/
Institute of Vegetables and Flowers, Jiangxi	Physiology, genetics and agronomy	Academy of Agricultural Sciences, 330200, Nanchang, China	Guangyu Chen genebksh@gmail.com	http://www.caas.cn/en/
Julius Kühn-Institut, Poznan University of Life Sciences	Plant breeding and genetics	Institute for Breeding Research on Horticultural Crops Erwin-Baur-Str. 2706484 Quedlinburg, Germany	Thomas Nothnagel thomas.nothnagel@julius-kuehn.de	http://www.julius-kuehn.de/
Rutgers University	Plant breeding and genetics	Department of Vegetable Crops. Wojska Polskiego 28, 60-637 Poznań, Poland	Mikolaj Knafliewski miknaf@au.poznan.pl	http://www.woak.upoznan.pl/en/faculty/
		Agricultural Experiment Station Rutgers, 88 Lipman Drive New Brunswick, NJ 08901-8525 USA	Chee-Kok Chin chin_c@sebs.rutgers.edu	http://www.njaes.rutgers.edu/

Connecticut Agricultural Experiment Station	Phytopathology	Department of Plant Pathology and Ecology, New Haven CT USA	Wade H. Elmer Wade.Elmer@ct.gov	http://www.portal.ct.gov/CAES/
Tohoku University	Plant breeding and genetics	Graduate School of Life Sciences, 2-1-1, Katahira, Aoba-ku, Sendai 980-8577, Japan	Akira Kanno kanno@ige.tohoku.ac.jp	http://www.ige.tohoku.ac.jp/
Universidad Nacional de Rosario	Plant breeding and genetics	Facultad de Ciencias Agrarias, Campo Experimental Villarino CC N° 14 (S2125ZAA) Zavalla Santa Fe, Argentina	Fernando López-Anido flopez@fcagr.unr.edu.ar	http://www.fcagr.unr.edu.ar/
Universidad Nacional Agraria, La Molina	Physiology and agronomy	Av. La Molina s/n - La Molina. Lima, Peru	Andrés V. Casas-Díaz cda@lamolina.edu.pe	http://www.lamolina.edu.pe/
University of Cordoba	Plant breeding and genetics	Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Córdoba, Campus de Rabanales Edificio C-5, 14,071 Córdoba, Spain	Juan Gil-Ligero juan.gil@uco.es	http://www.uco.es
University of Georgia	Biotechnology	Department of Plant Biology, Athens, GA, 30602 USA	James H. Leebens-Mack jleebensmack@uga.edu	http://www.franklin.uga.edu/majors-degrees/plant-biology-bs
University of Guelph	Plant breeding, genetics and in vitro culture	Department of Plant Agriculture, Edmund C. Bovey Building, Guelph, Ontario, Canada	David Wolyne dwolyn@uoguelph.ca	https://www.plant.uoguelph.ca
Utah State University	Physiology, plant environmental stress	Department of Plants, Soils and Climate. Logan, USA	Daniel Thomas Drost dan.drost@usu.edu	http://www.usu.edu/academics/departments/

Appendix II: Cultivar Type and Color Spear Production of some Important/Newly Asparagus Cultivars Developed in Different Breeding Programs Since 1990s (Aprox)

Cultivar name	Type ^{a,b}	Cultivation	Institution /Company (Country)	Additional information
Pacific Challenger	H-Mixed	Green	Aspara Pacific Ltd. (New Zealand)	http://www.asparapacific.co.nz
Pacific Purple	O-Mixed-(4x)	Purple/White		
Pacific 2000	H-Mixed	Green		
Vegalim	H-Male	Green	Asparagus Beheer BV (Holland)	http://www.limgroup.eu
Gijnlim	H-Male	Green/White		
Backlim	H-Male	White		
Frühlim	H-Male	White		
Grolim	H-Male	White		
Herkolim	H-Male	White		
Terralim	H-Male	White		
Vitalim	H-Male	White		
Atticus	H-Male	Green	Bejo Zaden BV (Holland)	http://www.bejo.com
Magnus	H-Male	Green/White		
Cumulus	H-Male	White		
Ercole	H-Male	Green	C.R.A-ORL Unità di Ricerca per l'Orticoltura (Italy)	http://www.vissers.com
Giove	H-Male	Green		
Eros	H-Male	Green/White		
Franco	H-Male	Green/White		
Vittorio	H-Male	White		http://www.globalplantgenetics.com
Apollo	H-Mixed	Green	California Asparagus Seeds (USA)	http://www.walkerseed.com
Atlas	H-Mixed	Green		
Grande	H-Mixed	Green		
UC157	H-Mixed	Green		
Purple Passion	O-Mixed-(4x)	Purple		
Walker Deluxe F1	H-Male	Green	Walker Brothers Inc. (USA)	

(continued)

Cultivar name	Type ^{a,b}	Cultivation	Institution /Company (Country)	Additional information
Ramada	H-Male	White	Südwestdeutsche Saatzaht GmbH & Co. KG (Germany)	http://www.suedwestsaat.de
Ramires	H-Male	White		
Rhapsody	H-Male	White		
Ravel	H-Male	White		
Raffaelo	H-Male	White		
Jersey Giant	H-Male	Green		
Jersey King	H-Male	Green		
Jersey Knight	H-Male	Green		
Greenwich	H-Male	Green		
NJ 1113	H-Male	Green		
NJ 1189	H-Male	Green	Rutgers University (USA)	http://www.breeding.rutgers.edu
NJ 1123	H-Male	Green		
NJ 1192	H-Male	Green		
NJ 977	H-Male	Green		
NJ 1019	H-Male	Green		
NJ 1016	H-Male-(4x)	Purple		
Darbella	H-Mixed	Green	Plantas de Navarra SA – Planasa (Spain)	http://www.planasa.com
Darzilla	H-Mixed	Green/White		
Darlise	H-Mixed	White		
Placosesp	H-Mixed	Green		
DePaoli	H-Mixed	Green	University of California, Riverside (USA)	http://www.eurosemillas.com
Espada	H-Mixed	Green		
HT 801	H-Mixed-(8x)	Green-purple		
Guelph Millennium	H-Male	Green	Centro Sur SCA / University of Córdoba (Spain)	http://www.centro-sures.com
Guelph Eclipse	H-Male	Green		
Guelph Equinox	H-Male	Green		

^aH-Male is clonal hybrid male 100%. H-Mixed is clonal hybrid 50% male +50% female. O-Mixed is open pollination variety with 50% male +50% female

^bIn brackets is detailed the ploidy level of polyploid varieties

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