Chapter 8

CLONAL MULTIPLICATION OF WOODY PERENNIALS

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Woody perennials comprise a large group of taxonomically diverse species. It includes such valuable crops as fruit and nut trees, plantation species (palms, rubber), forest trees (hardwood and softwood), trees of importance in social forestry, etc. The progress in the improvement of these crops is usually very slow largely because of their long-term life cycle. Vegetative multiplication of adult trees selected for proven performance is certainly a promising method to improve the quality of tree crops. However, owing to the difficulty in rooting the cuttings from elite trees of many of the forest species (Quercus, Fagus, Eucalyptus, most conifers) it has not been possible to harness the goals of clonal forestry. In several cases, such as monocotyledonous palms and some forest species, vegetative propagation is virtually impossible (Jones, 1983). Therefore, traditionally, forests have been regenerated by natural seedlings and, more recently, by collection of seeds from randomly pollinated elite trees (Ahuja, 1986). The exception is Cryptomeria japonica which has been clonally propagated for centuries in Japan. Recently, clonal forestry has been introduced in Sweden and Federal Republic of Germany for establishing Norway Spruce (Picea abies) on a large scale (Ahuja, 1986).

During the past fifteen years considerable efforts have been made to apply tissue culture methods for clonal multiplication of tree species as evinced by the large number of books largely or exclusively devoted to tree tissue culture published during this period (Bajaj, 1986,1988; Bonga and Durzan, 1987a,b,c; Ahuja, 1988; Hanover and Keathley, 1988; Valentine, 1988; Dhawan, 1989). Although the initial progress with tree tissue culture was slow but during the last decade considerable progress has been made. Boulay (1979,1987) developed in vitro methods for mass cloning of 100 years old Sequoia sempervirens. Gupta et al. (1980) reported

micropropagation of 100 years old trees of Tectona grandis trees. Similarly, Ahuja (1984) has demonstrated the feasibility of rapid clonal multiplication of mature trees of Populus tremula, P. tremuloides and their hybrids by bud culture. The most recent success in tree tissue culture is controlled flowering of tissue cultured bamboo (Nadgauda et al., 1990).

This chapter deals with in vitro propagation of some woody perennials. For information on other tree species the books mentioned above may be consulted.

1 BAMBOO

Bamboos are one of the fastest growing and highest yielding perennial grasses belonging to the family Poaceae. They constitute one of the most versatile and useful group of plants ever known to mankind that find use from the cradle to the coffin. Being straight, light, hard and strong, bamboos are extensively used for construction of houses (especially in the flood and earthquack prone areas) and in making a variety of items for domestic and agricultural use including food and cattle feed (for details see Varmah and Bahadur, 1980). Living bamboos are planted as hedges and for landscape gardening. On account of hollow and interwoven root system, they are widely used as a windbreak and for preventing soil erosion. Species like Bambusa vulgaris are grown as ornamentals. However, the most important use of bamboo is in the paper pulp industry for which it serves as the basic raw material.

In order to meet the rapidly growing demand of food and fodder of the ever increasing human and livestock population, vast forest areas are being cleared to bring more land under cultivation. The "slash and burn" practice of agriculture which is widely prevalent in North-East regions of India has caused extensive damage to the natural forest of bamboos, thereby resulting in a substantial decline in bamboo forest cover. Mass utilization of bamboo resources by paper and pulp industry, bad management practices, interference by biotic factors such as grazing and forest fires, over-exploitation by the rural and tribal communities for domestic and commercial use, and lack of state control over natural forest of bamboos, are some other major factors contributing to the scarcity of bamboos. The problem is compounded by the monocarpic nature of bamboo plant. Consequently, there exists a wide gap between the demand and supply of bamboos and it is established that if early and appropriate measures to revamp denuded and degraded bamboo areas are not taken, the current situation would deteriorate further.

1.1 In vivo regeneration

Under normal conditions, bamboos regenerate through seeds with ease. With the onset of monsoon, the fertile seeds of bamboo germinate and give rise to seedlings. However, the seedling growth is adversely affected by the surrounding weeds and shade of other tree species . Forest fires and grazing by the cattle are two important factors that restrict the natural regeneration of bamboos.

Artificial regeneration of bamboo takes place sexually as well as vegetatively. However, both the methods of propagation are beset with many problems that restrict their large-scale propagation. As compared to vegetative methods, propagation through seeds is easier and cheaper, but this method of propagation is unreliable on account of long flowering cycle, short viability of seeds, extremely poor seed-set during sporadic/off-season flowering and large-scale consumption of seeds by wild animals, especially the rodents. With respect to vegetative propagation, the conventionally used propagules (offsets, rhizomes and cuttings in some cases) are available in small number and, being bulky, difficult to transpoprt over long distances. Also, replantation using the vegetative propagules is expensive and season specific. In view of the constant increase in demand, the scarcity of planting material and the problem associated with conventional methods of propagation, development of effective in vitro methods of clonal propagation for different bamboo species is highly desirable.

1.2 Current status of tissue culture

As compared to other dicotyledonous woody species, research on in vitro propagation of bamboos started fairly late. The pioneering report in this regard came from Alexander and Rao (1968). However, extensive in vitro studies on bamboos started only in 1980s and a complete protocol for micropropagation of a bamboo species (Bambusa arundinacea) was published by Mehta et al. (1982). Since then, micropropagation methods for many bamboo species have been successfully worked out.

Micropropagation in bamboos has been attempted both from seed/seedling and mature explants. Although considerable success could be obtained with embryonic tissue, only limited progress has been possible with the adult tissues because of following reasons:

(1) Meristems in bamboos are active only during the rainy season (except for those regions where humidity is high throughout the year; Dekkers et al., 1978) and, consequently, fresh cultures can be initiated

only during this period. Being under the influence of nature, this period is highly variable and, at times, restricted to just a few days.

(2) contamination of cultures especially by the fungi is one of the most serious problems encountered while raising new cultures. This is largely because of the fact that warm and humid conditions that favour meristematic activity are also conducive for the fungal growth.

(3) In many cases, the in vitro raised shoots die immediately after being excised from the mother explant and, therefore, multiplication of such shoots is by and large restricted.

(4) Browning of the medium during in vitro culturing of mature bamboo shoots has been widely experienced (Zamora et al., 1988).

(5) It has been observed that in those cases where the multiplication of shoots was possible, efficient rooting remained a major bottleneck in the establishment of a complete protocol for micropropagation (Nadgir et al., 1984).

(6) Unlike seeds, which are easily transportable and could be worked upon in any region of the world under controlled conditions, working with mature explants is restricted to those laboratories that are located in the vicinity of bamboo growing area.

The merits and demerits of micropropagation from seed/seedling and adult materials are compared in Table 1.

TABLE 1

Merits and demerits of micropropagation of bamboos from seed/seedling and adult explants.

CHARACTER	EXPLANT	
	SEED/SEEDLING	ADULT
1. Availability of explant	Restricted but available throughout the year	Abundant but restricted to rainy season
2. Contamination problem	Negligible	Serious
3. Multiplication of shoots	Relatively easy	Relatively difficult
4. Rooting of shoots	Fair success	Negligible success
5. Browning of medium	Rare	Common
6. Cloning uniformity	Doubtful	Almost certain
7. Propagation of elites	Impossible	Possible
8. Early flowering	Improbable	Highly probable

All the three approaches of in vitro multiplication, viz., forced axillary branching, somatic embryogenesis, and organogenesis have been tried for the micropropagation of bamboos.

(i) Forced axillary branching. From the point of view of clonal uniformity, this is regarded as the safest mode of shoot multiplication. However, in case of bamboos, for reasons unknown, this approach has not been adopted as widely as it should have been. Nadgir et al. (1984) applied this methodology to multiply the shoots derived from seedlings of Dendrocalamus strictus. They estimated that nearly 10,000 plantlets could be obtained from a single embryo every year. Dekkers and Rao (1989) while working with the same species were able to obtain multiple shoots but very few of them could be rooted. A similar approach has also been employed successfully in the case of B. tulda (Saxena, communicated; Fig. 1A-C).

With regard to adult material, it has been possible to achieve shoot multiplication in B. vulgaris (Saxena and Bhojwani, unpublished), starting from nodal explants of lateral branches. Nodal segments from mature clumps of B. tulda exhibited multiple shoot formation in 100% cultures (Fig. 2; Saxena and Bhojwani, unpublished).

In B. glaucescens excised axillary buds were cultured to obtain multiple shoots on a medium supplemented with 5 mg/l BAP, 1 mg/l NAA and 3 g/l activated charcoal (Banik, 1987).

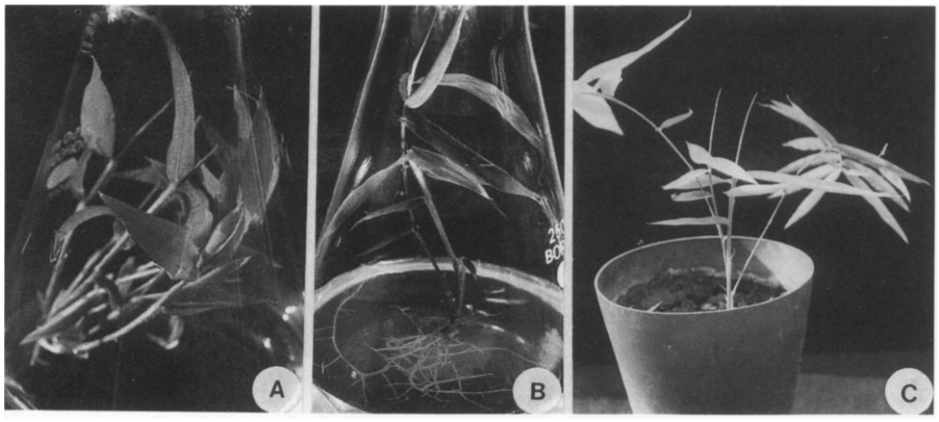

Fig. 1. In vitro propagation of Bambusa tulda from seedlings. A. Three weeks old liquid culture showing shoot proliferation. B. A rooted shoot (4-week-old culture on semisolid medium). C. A micropropagated plant six weeks after transplantation to pot.(Saxena, unpublished)

In vitro propagated shoots derived from seedling material of B. tulda rooted within 3 weeks at the frequency of 92% (Saxena, communicated, Fig. 1B). Similarly, the shoots obtained from seedling explants of D. strictus rooted with ease, but those from mature explants were found to be difficult to root; only 20% of the in vitro multiplied shoots from adult explants rooted within 4-5 weeks when treated with IBA (1 mg/l) for 4 days in dark followed by their transfer to 1/2 strength MS basal medium (Nadgir et al., 1984). However, the same group was unable to root the shoots of B. arundinacea and B. vulgaris by using the same treatment. Similarly, Chaturvedi and Sharma (1988) reported 20% rooting in the adult material of D. strictus when nodal segments bearing in vitro developed shoots were planted upside down in a medium containing phloroglucinol.

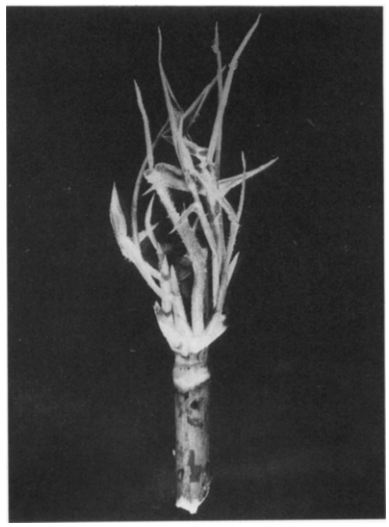

Fig. 2. A nodal segment of adult Bambusa tulda two weeks after culture, showing multiple shoot development. (Saxena and Bhojwani, unpublished)

(ii) Somatic embryogenesis and organogenesis in callus cultures. In bamboos, apart from the mature embryos (Mehta et al., 1982; Rao et al., 1985; Yeh and Chang, 1987), various other explants such as internodal sections containing intercalary meristem (Zamora et al., 1989), shoot-tips (Huang et al., 1989), culm sheath base (Dekkers and Rao, 1989), leaf (Hassan and Debergh, 1987), young inflorescence (Yeh and Chang, 1986a, b) and regenerated adventitious roots (Yeh and Chang, 1986a, b; Dekkers and Rao, 1989) have been used to initiate callus cultures and 2,4-D was found to be the most potent auxin for this purpose.

Like other graminaceous species, bamboos form several types of calli with regard to their morphology, colour and texture. However, generally

compact and rarely gelatinous calli (Mehta et al. 1982) were found to be embryogenic, while the friable callus invariably remained non-embryogenic, although in certain cases such as, B. beecheyana (Yeh and Chang, 1986a) and B. oldhami (Yeh and Chang,1986b), such calli formed adventitious roots. In Phyllostachys viridis, occasional plantlets developed on the non-embryogenic callus and this peculiar phenomenon has been attributed to the possible presence of some embryogenic cells within the non-embryogenic callus (Hassan and Debergh, 1987).

In bamboos, regeneration from calli occurred via somatic embryogenesis (Rao et al., 1985; Hassan and Debergh, 1987) or organogenesis (Huang et al., 1989).

1.3 Future trends of research

Unlike some other woody species, especially the tree legumes, where working with seeds is of virtually no consequence (tree legumes are profuse seed setters), micropropagation of bamboos with seeds, still holds some value as the scarcity of planting material is so acute that even true-to-the seedling type tissue culture raised plants would be useful and welcome by the growers. However, it would be ideal to carry out micropropagation with adult tissues as it would enable one to multiply bamboo plants with identified desirable traits. The elite bamboo clumps are those that produce large number of new culms annually, have a narrow lumen, are straight, do not produce branches in the lower half, possess better paper qualities such as higher cellulose content, increased fibre length etc., and are resistant to diseases and pests and tolerant to drought and frost. Thornlessness, as in the case of Bambusa arundinacea, is also a desirable character. Other elite characteristics will vary from species to species and would depend on the end use for which the plant would be put to.

Till date there is not a single report that refers to multiplication of an elite bamboo. It is envisaged that in future emphasis would be placed in developing protocols for micropropagation with adult explants. Except for two species, viz., Dendrocalamus strictus (Nadgir et al., 1984) and Bambusa tulda (Saxena, communicated), the published micropropagation protocols of all other bamboo species involve an intermediate callus phase which is known to create genetic instability. Until the genetic uniformity of the plants so produced is established, efforts should be directed to multiply shoots through forced axillary branching.

On the basis of personal experience, published reports and the problems encountered with various systems, the task of developing viable methods

of micropropagation of elite bamboos seems stupendous but achievable. Keeping in view the concerted efforts being made by various groups all over the world to attain this goal, an early breakthrough cannot be ruled out.

Conventional breeding in bamboos is extremely difficult because of the long flowering cycle which is highly erratic and unreliable and its basis is still unknown. Tissue culture can provide a vital input to solve this problem. Recently, Nadgauda et al. (1990) have been able to induce in vitro flowering and viable seed production in seedling-derived shoots of B. arundinacea and A. brandisii. Reliable application of this finding to other species of bamboo, particularly elite material, would be highly significant as controlled flowering would not only allow breeding of bamboos to develop improved varieties, but can also provide an everlasting source of bamboo seed production. It is likely to unveil the causative factors that are responsible for induction of flowering in bamboos in nature. The significance of the discovery by Nadgauda et al. would have had enhanced further if it was carried out with an adult tissue. Unfortunately, we do not have the protocols for micropropagation of adult plants of bamboos so far and, consequently, whatever is achieved with the seedling cannot be applied to mature tissues.

It may be concluded that the potential of tissue culture has not yet been fully realized in case of bamboos. But it is hoped that in future, the in vitro studies would not only provide means to multiply elites in large numbers but would also help in developing improved varieties of desirable traits.

2 PALMS

The economic importance of numerous palms, besides the well recognised coconut (Cocos nucifera), oil palm (Elaeis quineensis) and date palm (Phoenix dactylifera), and their recalcitrance to simple horticultural methods of propagation have provided a strong impetus for tissue culture studies. Whilst most studies have been aimed at clonal propagation, the potential of utilising tissue culture methods to produce disease-free palms (e.g., cadang-cadang viroid of coconut which has also been reported in oil palm) or disease resistant palms is well recognised. The inherent difficulties of crop improvement in perennials through breeding and selection create opportunities for utilisation of haploids produced by anther culture and in the long-run perhaps offer scope for genetic engineering through use of protoplast and cell cultures.

Simple methods, such as embryo rescue, have already shown useful application in aseptic transfer of germplasm and rescue of poorly germinating genotypes, e.g. pisifera seeds of oil palm and the makapuno of coconut (De Guzman and Rosario, 1964; Paranjothy et al., 1985). Coupled with cryopreservation, these methods hold realistic promise in long-term germplasm storage and utilization (Engelman et al., 1985; Chin et al., 1989).

In vitro multiplication of several palms (pejibaye, rattan, howei) has been reported but the techniques are better developed for oil and date palm (see Tisserat, 1987). Most methods rely on initiation of callus from various explants (young roots, leaves, inflorescences) followed by somatic embryogenesis and plantlet regeneration. Callus initiation requires an auxin but is strongly inhibited by cytokinins (Paranjothy, 1984). In oil palm, the somatic embryos develop into "calloid" or "polyembryogenic" cultures which consist of a self-perpetuating complex of embryoids and nodules with meristemoids. Shoots develop largely from the latter meristemoids. In rattan (Calamus manan) and in date-palm, a mode of adventive shoot proliferation has also been observed (Aziah, 1989; Tisserat and De Mason, 1985).

2.1 Oil palm

The more successful applications of in vitro propagation has been to oil palm and date-palm which date back to several independent pioneering investigations (Jones, 1974; Rabechault and Martin, 1976; Tisserat, 1979; Paranjothy and Rohani, 1978). Whilst the in vitro technology for large-scale propagation of oil palm is now well established in several commercial and institutional laboratories (for reviews see Wooi, 1984; Blake, 1983; Paranjothy, 1984; Tisserat, 1987), the occurrence of developmental aberrations in the earlier oil palm clonal trials (Corley et al., 1986) has been a serious impediment to large-scale commercialisation of oil palm clones produced through tissue culture. The developmental aberrations essentially reflect a process of feminization (Paranjothy et al., 1989) and consist of: (a) in the flowers of the female inflorescence, parthenocarpic development of the gynoecium and of the development of the vestigial stamens into a mantle of fleshy carpels surrounding the fruit, (b) in the flowers of the male inflorescence, development of the stamens into carpel-like structures, and (c) parthenocarpic fruits and "androgynous" male flowers in the early bunches and male inflorescences, respectively. These features of feminization are also found in seedlings, but do not persist beyond the first few inflorescences. The severity of their expression and persistance of the features in some clones are

the additional source of abnormality. From the practical viewpoint, these developmental aberrations, when severe, result in: (a) bunch failure due to an inadequate number of fertilised fruits to sustain development of the bunch to ripeness, and (b) absence of pollen in abnormal male inflorescences.

Considerable attention is now being paid to elucidate the plausible causal mechanisms of these abnormalities. There is evidence to suggest that the abnormalities are transmitted through meiosis to some seedlings (derived from open-pollinated abnormal palms) with much reduced expression (Rao and Donough, unpublished) and a total absence of expression in some (Paranjothy et al., 1989). These observations taken together with their high frequency in some clones, increase in their frequency and severity with every subculture (Corley et al., 1986) and reversion of abnormal palms to normal (Durand-Gasselin et al., 1989), do not suggest a simple Mendelian mechanism. Possibilities include DNA amplification or cytosine methylation with gradual erosion of the change with progressive cell division and meiosis. Cytological studies have not revealed any changes in chromosome number or karyotype. Differences in restriction fragment length polymorphisms have also not been detected between normal and abnormal palms. Various test crosses involving abnormal palms have been carried out in the field and results from these trials may provide further clues. Cultures maintained on cytokinin containing media generally show evidence of feminization in vitro (Paranjothy et al., 1989). As an early marker, in vitro floral morphology is being used to distinguish between normal and abnormal cultures (Corley, personal communication).

Clonal populations, unaffected by the fruit and floral abnormalities, show a lower variability than seedling population for highly heritable characters, besides yield (Corley et al., 1981). More recent trials have confirmed this even in populations where the fruit abnormalities were present at a commercially acceptable level of about 3-4%. These trials have also indicated potential yield gains of about 15% over seedling populations (Durrand-Gasselin et al., 1989).

2.2 Date palm

Clonal propagation of date palm has also received a great deal of attention. Since the first report of in vitro clonal multiplication by Tisserat (1979), frantic efforts have been made to micropropagate date palm. Methods for in vitro propagation of date-palm are well documented (Tisserat, 1981,1987) and several laboratories are involved in commercial exploitation of these methods. However, nothing is known about the clonal

nature of these plants. Since the propagation protocols involve somatic embryogenesis, as in oil palm, it is important that the micropropagated plants are carefully evaluated up to the fruiting stage before large-scale plantation is undertaken.

2.3 Coconut palm

In vitro studies in coconut started with the pioneering report of De Guzman and Rosario (1964) who cultured the excised embryos of "Makapuno" (a variety of coconut widely appreciated for its delicate endosperm) and obtained complete plantlets. Iyer (1982) raised full plantlets by culturing excised embryos of West Coast Tall variety.

Clonal propagation of coconut palm has been attempted both through somatic embryogenesis and adventitious bud formation. A variety of explants such as fragments of stem, leaf, root, inflorescence, endosperm and shoot apices have been employed for the production of callus along with neoformation of buds or somatic embryos. Although callus was induced in all cases, it was often difficult to maintain it through subculturing (Eeuwens, 1976, 1978; Apavatjrut and Blake, 1977). Attempts to induce shoot buds or somatic embryos on these calli did not yield much success. Pannetier and Buffard-Morel (1982a, b) reported development of embryoids from explants derived from young leaves of seedlings and adult trees. Similarly, Bhaskaran (1985) reported the formation of somatic embryos from induced callus and directly from mid-rib region of seedling leaf explants. However, despite extensive research, success in clonal propagation of coconut remains limited to one or a few plantlets produced in several research centres (Branton and Blake, 1983; Raju et al., 1984; Bhaskaran, 1985; Pannetier and Bufford-Morel, 1986). Routine and large-scale vegetative propagation of coconut still remains elusive.

3 HEVEA

Hevea brasiliensis, a member of the family Euphorbiaceae, is virtually the sole source of natural rubber which constitutes one of the major raw materials for automobiles. Although Hevea can be easily propagated by bud-grafting, the use of clonal root-stocks is restricted due to the lack of taproot formation by the cuttings obtained from mature plants and the difficulty encountered in rooting of some clones. The techniques of micropropagation can play a vital role in rooting of some clones with taproot system. Besides this, there is lot of variation between the yields of modern clones obtained as a consequence of breeding (1637.5 kg/ha/yr) and the unselected seedlings (512.5 kg/ha/yr; Simmonds, 1979). It has

also been observed that rubber trees with greater growth vigour mature faster and, therefore, can be harvested earlier than the ordinary trees, thereby resulting in an overall increase in the yields. In vitro methods of propagation can be very useful in multiplying the high yielding clones and those trees that exhibit greater growth vigour on a large scale.

The initial work on tissue culture of Hevea involved embryo culture to raise seedlings (Muzik, 1956; Paranjothy and Ghandimathi, 1976; Torvan and Suryatma, 1977). In a recent review, Chen (1984) claimed to have obtained complete plantlets from seedling stem segments with pre-existing buds as well as from decotylated embryos followed by successful transfer of some of these plants to the field (details unpublished). However, there is no other published report on micropropagation of Hevea using seed/ seedling explants. The utility of culturing the entire seed in vitro has been restricted to raising seedlings which in turn are used in international transfer of Hevea germplasm.

In an attempt to develop in vitro methods of propagation, Paranjothy and Ghandimathi (1976) cultured shoot apices from aseptic seedlings. The shoots grew into rooted plantlets but could not be multiplied. Mascarenhas et al. (1982) reported the development of about three shoots per explant from terminal buds of 10-12 years old trees. These in vitro raised shoots failed to multiply but were rooted with 60% success. Carron and Enjalric (1982) observed sprouting of nodes obtained from young greenhouse-grown plants by pre-treating the explants in a solution containing IBA (5 mg/l) and BAP (10 mg/l) for two hours. As in the case of shoot apices, these shoots could not be multiplied.

Although plants have been obtained both from somatic (Paranjothy, 1974; Paranjothy and Rohani, 1978; Carron and Enjalric, 1982, 1985; Wilson and Street, 1975) and gametic (Chen et al., 1977, Gymogenic-Guo et al., 1982) cells through somatic embryogenesis, their frequency has remained very low and often the embryoids, particularly somatic embryos, exhibited recalcitrance to develop into plantlets.

Thus, concerted efforts are required to develop effective micropropagation methods which would permit the exploitation of untapped potential of elite clones by making them available in large numbers.

4 TROPICAL AND SUB-TROPICAL FRUIT TREES

Production of tropical and subtropical fruits has been increasing significantly faster than temperate fruits in recent years (FAO Production Yearbook, 1987). Although efficient vegetative propagation methods, i.e., grafting, air layering and removal of suckers, already exist for most

of the important tropical and subtropical fruit crops, the rapid expansion of plantings has been impeded by serious shortages of clonal material, particularly of newly released, superior cultivars, e.g. 'Arkin' carambola (Averrhoa carambola). Among the underexploited tropical and subtropical fruits, whose production is often very important regionally, conventional vegetative propagation may be either quite inefficient (mamey sapote - Calcocarpum sapota; longan - Euphorbia longan) or virtually impossible (mangosteen - Garcinia mangostana). Therefore, many interesting tropical and subtropical fruits have not been exploited for lack of suitable clonal planting material. In this context, in vitro clonal propagation methods hold potential.

The planting of extensive new orchards of vegetatively propagated clones of some tropical fruits has sometimes been limited by pathogens that can severely reduce production. Thus, most Citrus producing countries have established programmes for release of clonal material that has been indexed for freedom from citrus tristeza virus after micro-grafting of meristems onto seedling rootstocks in vitro (Bitters et al., 1970). Other diseases of tropical and subtropical fruits that have seriously limited production, e.g., Phytophthora cinnamomi, which causes root rot of avocado (Persea americana), could be controlled if clonally propagated disease resistant rootstocks were readily available.

4.1 Current status of tissue culture

Unlike temperate fruit trees, where tissue culture has proved fairly succcessful and could be commercialised for some crops (Hammerschlag, 1986; Zimmerman, 1986), the progress in the application of tissue culture for clonal multiplication of tropical fruit trees has been rather slow. The limited literature available on tissue culture of latter group of plants suggests that all the three approaches to in vitro propagation, viz. proliferation from pre-existing buds, differentiation of adventitious shoot buds and somatic embryogenesis have been tried.

There have been several reports of micropropagation from shoot tips of tropical fruit trees in the juvenile phase of development. This underlines the potential for using rejuvenated material as a source of explants. Shoot tips from seedlings of Citrus (Barlass and Skene, 1982), jackfruit (Rahman and Blake, 1988) and Black plum (Yadav et al., 1990) and from immature embryos of avocado (Skene and Barlass, 1983) have responded readily in culture. Nucellar seedlings are naturally rejuvenated clones, and have been utilized as stock plants for micropropagating several polyembryonic Citrus cultivars (Barlass and Skene, 1986) and mangosteen

(Goh et al., 1988).

Besides the composition of medium, the nature of explant, its orientation on the medium and genotype of the material are reported to affect the response of adult materials in vitro. For propagation from shoot tips and nodal segments from adult trees, new vegetative growth which occurs from the base of the main stem during the onset of flowering in jackfruit (Amin, 1987) and during periods of vigorous vegetative growth in guava (Amin and Jaiswal, 1987) have been reliable sources of explants. In avocado, hard pruning of the main stem to stimulate growth of dormant lateral buds has been effective for providing culturable shoot tips (Pliego-Alfaro et al., 1987). The conditioning of the stock plants has been very important for the establishment of shoot tips and nodal explants of some species; guava is most responsive during the warm and humid months (Jaiswal and Amin, 1987).

Horizontal placement of guava shoot tip explants on the medium was superior to the vertical orientation (Jaiswal and Amin, 1987). However, Goh et al. (1988) did not observe substantial influence of explant orientation in mangosteen.

Genotypic effect on in vitro response of shoot tips has been recorded in pineapple (DeWald et al., 1988) and Citrus. Barlass and Skene (1982) have reported that shoot tips from zygotic and nucellar seedlings of different selections of Citrus showed different rates of axillary bud proliferation.

In vitro regeneration through adventitious shoot formation in trtopical and subtropical fruit cultivars, either directly from explanted tissues or via the callus phase, has been reported for several species. However, there have been relatively few reports of organogenesis from adult explants in this heterogeneous group of plants. Citrus spp. (Barlass and Skene, 1986), carambola (Litz and Conover, 1980) , custard apple (Annona squamosa; Nair et al., 1984) and Annona cherimola (Jordan, 1988) have been regenerated from stems, petioles or leaves of young seedlings. Of these, only carambola has also been regenerated from explants of mature origin. Several Citrus spp. clones (Barlass and Skene, 1986) and mangosteen (Goh et al., 1988) have been regenerated from somatic tissues of young nucellar seedlings.

Many of the most important tropical and subtropical fruit crops, including Citrus and mango have been regenerated by somatic embryogenesis from explants derived from mature trees. Nucellus has been the appropriate explant for embryogenesis in the cultures of Citrus spp., mango, jaboticaba, Syzygium spp. and loquat. Most of these species are polyembryonic. However, monoembryonic species of Citrus, and varieties of mango and loquat have

also been regenerated via somatic embryogenesis from nucellar explants (Litz et al., 1986), although the efficiency of regeneration is usually considerably lower than polyembryonate species. It is possible that nucellus of all plants has morphogenetic potential which remains inhibited chemically within monoembryonate species (Tisserat and Murashige, 1973a, b).

4.2 Limitations

So far the only tropical fruit species which have been commercially propagated in vitro are banana, papaya and pineapple (Litz et al., 1986), None of the tree species of this group of crops has qualified to enter the list.

Nucellus appears to be the explant of choice for in vitro regeneration of tropical fruit trees. However, acceptability of this approach for commercial propagation would depend on several considerations (Litz et al., 1986). So far it has been very difficult to achieve efficient maturation and germination of somatic embryos except in the case of Citrus. Germination of these embryos has been spontaneous. Exceptionally large size of the somatic embryos (4-6 cm in mango) of these plants often causes bruising of the embryos in vitro leading to necrosis and eventual death of some embryos (Litz, 1986). In addition, the plants regenerated via nucellar embryogenesis exhibit juvenile traits and, therefore, would require 7-20 years to reach the fruiting stage. Moreover, Citrus plants regenerated from nucellar callus were found to exhibit considerable variation with respect to morphological and biochemical features (Moore, 1985, cited in Litz, 1986).

5 GYMNOSPERMS

Gymnosperms account for much of the world produce of timber. Their long life-span and 'open-pollination' make clonal afforestation programmes desirable. Clonal multiplication can capitalize on the genetic traits of a superior clone while avoiding the segregation and the resulting variability associated with propagation by seed. However, vegetative propagation by cuttings has its drawbacks; cuttings often lack the vigour of seedlings and their growth rate declines with increasing age of the ortet (Sweet and Wells, 1974; Shelbourne and Thulin, 1974). In vitro techniques are expected to help overcome these problems and regenerate plants at faster rates.

5.1 Current status of tissue culture

Gymnosperms were, in fact, among the first species to be cultured in vitro. Gautheret (1934) cultured cambial tissue of Pinus pinaster and Abies alba. As early as 1936, La Rue cultured embryos of several Gymnosperm species into normal looking seedlings. Buds were obtained from callus cultures of Sequoia sempervirens (Ball, 1950). However, despite the early start Gymnosperm tissue culture lagged behind similar studies in Angiosperms.

In the 1960s medium for callus growth was defined (Risser and White, 1964). Although embryo-like structures from cotyledon cultures of Thuja orientalis was reported in 1965 by Konar and Oberoi, the first report of complete plantlet regeneration from tissue cultures of a Gymnosperm (Pinus palustris) was published in 1975 (Sommer et al., 1975). Plantlets via organogenesis have since been regenerated in about 25 diverse species (Dunstan, 1988). Another decade lapsed before somatic embryogenesis (SE) was convincingly demonstrated in conifer tissue cultures (Hakman et al., 1985). Further progress has been rapid and somatic embryogenesis has to date been reported in ca 10 species.

(i) Organogenesis. Clonal multiplication via organogenesis requires 3 in vitro steps, viz. shoot multiplication, elongation and rooting. So far, maximum success has been achieved in inducing multiple shoot formation in primary cultures of embryos or embryonal explants, especially cotyledons (Dunstan, 1988; Mehra-Palta and Thompson, 1988). Generally, excised cotyledons are more organogenic than intact embryos. In radiata pine excised cotyledons produced twice as many shoot buds as whole embryos (Aitken et al., 1981). Age of the cotyledon, which is critical for optimum shoot bud formation, may vary with the species. While in radiata pine cotyledons loose the potentiality to form shoot buds shortly after embryo germination, in Douglas fir cotyledons from 2 to 4-week-old seedlings are still able to form buds (Wochok and Abo El-Nil, 1977).

In conifer tissue cultures, a cytokinin alone can bring about non-callus mediated differentiation of shoot buds (Pinus radiata - Horgan, 1987; P. contorta - Patel and Thorpe, 1984; P. resinosa - Noh et al., 1989; Picea abies - Von Arnold, 1982; P. glauca - Toivonen and Kartha, 1988). Addition of an auxin often decreases the response as in Pinus elliottii (Perez-Bermudez and Sommer, 1987), P. gerardiana (Banerjee and Bhojwani, unpublished) and Calocedrus decurrens (Jelaska and Libby, 1988). Of the various cytokinins tested, BAP has been found to be most effective (Flinn et al., 1986).

Under optimal conditions distinct shoot buds appear after 8-12 weeks, and after 16 weeks the whole cotyledon may get covered with the buds (Fig. 3). The differentiation of buds is asynchronous, and the number of buds per cotyledon or embryo may vary with the species. Wochok and Abo El-Nil (1977) and Aitken et al. (1981) reported the formation of 264 buds per embryo in Douglas fir and 180 buds per embryo in radiata pine, respectively. In Pinus gerardiana, an excised cotyledon developed on an average, 20 buds (Banerjee and Bhojwani, unpublished).

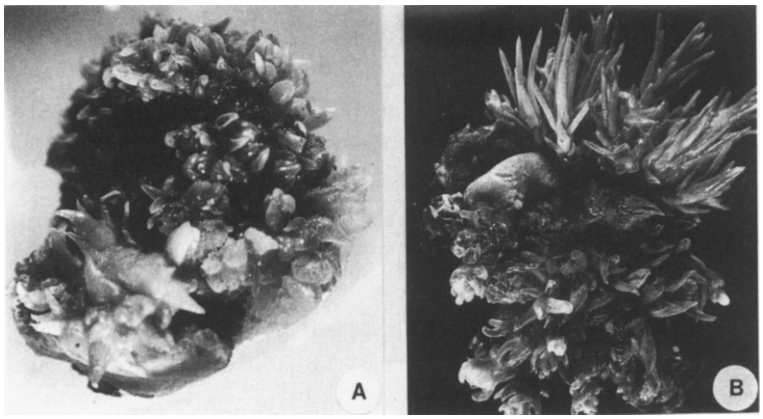

Fig. 3. Shoot bud differentiation from excised cotyledons of Pinus gerardiana. A. A cotyledon completely covered with adventitious buds, 12 weeks after culture. B. Asynchronous elongation of shoot buds, after 8 weeks on elongation medium.

Although large number of adventitious buds are induced on embryonal explants the number of plants finally established in soil is extremely low because of losses at successive stages of shoot elongation, rooting and transplantation. For example, in Picea abies only one rooted propagule per cotyledon could be obtained (Bornman, 1983).

Most of the buds differentiated from cotyledons remain stunted and are unsuitable for rooting. For example, minimum size of shoot required for rooting is ca 1 cm in radiata pine and over 2 cm in Pseudotsuga menziesii (Mohammed et al., 1989). Therefore, an intermediary step of shoot elongation is necessary. Some of the treatments found favourable for shoot elongation include the transfer of explant pieces bearing adventitious shoot buds to a hormone-free medium with reduced salt concentration (Patel and Thorpe, 1984) or to a basal medium containing activated charcoal, which possibly adsorbs the excess hormones and other inhibitory substances from the tissues. In black and white spruce the charcoal source was important, as only conifer-derived charcoal was effective for shoot elongation (Rumary and Thorpe,

1984). Far red light, vitamin D and coconut milk stimulated shoot elongation in Pinus species (Bornman, 1983; Konar and Singh, 1980).

Spontaneous rooting in Gymnosperms is rare and occurs at low frequency. Therefore, the regenerated shoots must receive a "rooting" treatment. Generally an auxin has been used for both in vitro and in vivo rooting (Horgan, 1987). However, rooting of shoots continues to be a bottleneck.

Large-scale planting out of embryo/cotyledon-derived plants has been done in some species (Table 2). The plantlets transferred to the field often show a higher mortality rate and slower growth than seedlings. Plagiotropic growth is seen in many species (Pinus taeda - Leach, 1979; P. radiata - Horgan, 1987; Pseudotsuga menziesii - Mapes et al., 1981, Boulay, 1987; Cunninghamia lanceolata - Boulay, 1987). The field survival of plagiotropic plantlets is less than orthotropic plantlets. In Douglas-fir plagiotropic plantlets recovered and became orthotropic sooner than cuttings (Abo El-Nil, 1987). After 3 years, field growth of plantlets was comparable to seedlings (Abo El-Nil, 1987; Amerson et al., 1988).

Amerson et al. (1988) regenerated plantlets from cotyledons of Pinus taeda and established a field trial in which tissue culture-derived plants and seedlings from half-sib families were compared. There was a lag in the growth of tissue cultured material in the first season but it showed greater percentage increase in growth in years 2-4. Growth increments in 4-6 year trials were similar, indicating that the initial lag in growth of the tissue-cultured plants would cause no significant difference at rotation age. In contrast, P. radiata plantlets were shorter and had less stem volume than seedlings after 4 years in the field (Horgan, 1987). Tissue culture plants appeared more mature in character in their 2nd year than the equivalent seedlings.

Table 2. Large-scale transplantation of plantlets regenerated in vitro.

Species	No. of propagules planted by 1985
Pinus taeda	6,000
Pinus pinaster	1,000
Pseudotsuga menziesii	5,000
Sequoia sempervirens	50,000
Pinus radiata	8,400

*After Mehra-Palta and Thompson (1988).

The real importance of in vitro techniques lies in the clonal multiplication of superior trees. However, so far only limited success has been achieved with in vitro regeneration of plants from adult tissues. Shoot tips of Douglas fir trees up to 25 years old (Thompson and Zaerr, 1981) and Norway spruce trees up to 120 years old (Von Arnold and Eriksson, 1979, 1986) have been induced to form adventitious buds. The possibility of clonally multiplying older trees and establishing field trials with in vitro derived plants has been demonstrated in species such as Sequoia sempervirens (Ball, 1987), Pinus pinaster (Franclet et al., 1980) and Pinus radiata (Horgan,. 1987).

(ii) Somatic embryogenesis. A major breakthrough in tissue culture of Gymnosperms occurred when somatic embryogenesis was reported from immature embryos of Picea abies (Hakman et al., 1985) and female gametophyte of Larix decidua (Nagmani and Bonga, 1985). Hakman et al. (1985) noted that 50% of the immature zygotic embryos of P. abies, excised from developing seeds and cultured on LP medium (Von Arnold and Eriksson, 1981) in the presence of 10 uM 2,4-D and 5 uM BAP produced a translucent and friable callus from suspensor cells. After about a month the callus contained somatic embryos at various stages of development, which resembled zygotic embryogenesis.

Following the report of Hakman et al. (1985) somatic embryogenesis has been reported in immature embryo cultures of Pseudotsuga menziesii (Durzan and Gupta, 1987), Larix sp. (Klimaszewska, 1989), Pinus taeda (Gupta and Durzan, 1987), and P. strobus (Finer et al., 1989). In all these cases somatic embryogenesis occurred when the culture were raised from pre-cotyledonary stage embryos (2-3 weeks after fertilization); older embryos yielded non-embryogenic calli.

Although immature embryos continue to be the best material for raising embryogenic cultures, in some cases it has been possible to obtain somatic embryos from mature zygotic embryos. Von Arnold and Hakman (1986) showed, for the first time, that 11% of mature zygotic embryo explants of Norway spruce (Picea abies) could produce somatic embryos. Subsequently mature embryos from stored seeds of Pinus lambertiana (Gupta and Durzan, 1986) have also shown the potential to form somatic embryos. It has been possible to preserve embryogenic cells of Picea abies (Gupta et al., 1987) and P. glauca (Kartha et al., 1988) without affecting their embryogenic potential. Well developed somatic embryos have also been obtained from protoplasts derived from embryogenic calli of Pinus taeda (Gupta and Durzan, 1987) and Picea glauca (Attree et al., 1987).

Similar to organogenic differentiation, somatic embryogenesis in conifers has so far been restricted to the culture of zygotic embryos. However, while organogenic differentiation occurs directly from the explants in the presence of high cytokinin to auxin ratio, somatic embryogenesis involves a callus phase and is usually induced in the presence of high auxin to cytokinin ratio.

The recovery of plantlets from somatic embryos is presently very poor and remains a limiting step to the implementation of this technique for propagation of Gymnosperms (Dunstan, 1988). On the induction medium somatic embryos remain suppressed. Embryo maturation requires the removal of phytohormone or application of ABA (Durzan and Gupta, 1987; Von Arnold and Hakman, 1988; Dunstan et al., 1988). The conversion of the somatic embryos into plantlets is generally low. It was 1-2% in the case of Pinus lambertiana (Gupta and Durzan, 1986) and up to 35% in Picea abies (Von Arnold and Hakman, 1988).

5.2 Limitations

Most of the literature on tissue culture of Gymnosperms pertain to embryo/seedling tissues. A lot has been written on conifer tissue culture, and the list of species that have regenerated from seedling explants in vitro is long. However, each step in the process is beset with problems and while the achievements have been justifiably highlighted the problems and particularly the failures have not been adequately projected.

One of the main problems in conifer tissue culture is the non-repeatability of results. Such variability is often due to some inherent character of the explant and is independent of media or hormonal formulations. The number of buds/explant shows much variation: 1 to 200 in Pinus radiata (Reilly and Washer, 1977; Aitken et al., 1981), 3-150 in Picea abies (Von Arnold and Eriksson, 1986), 0-50 in Calcocedrus decurens (Jelaska and Libby, 1988). A positive correlation exists between seed weight and the ability of cotyledons to form buds (Ab El-Nil and Wochok, 1986). Variation in morphogenetic potential is also related to the geographic region (Von Arnold and Eriksson, 1986). By contrast, seeds from 11 open-pollinated trees of Pinus resinosa did not show much variation in their bud forming potential (Noh et al., 1988). In this species the scope of increased shoot regeneration through embryo selection is limited.

When using embryonic explants the genetic gain possible is limited to the quality of the untested genotype of the embryo used to initiate the culture. The process is more useful in multiplying specific crosses.

Success with adult explants is very limited. They exhibit much variation in their in vitro response. This variation is often seasonal; the most responsive explants are available in spring corresponding to a period of active growth in vivo. Shoots from mature trees are more difficult to root than juvenile explants. In vitro culture may sometimes reverse the maturation process (Francelet, 1979) as in Sequoia where, on repeated subculture, shoots from a 100-year-old tree became orthotropic and one shoot rooted spontaneously.

Elongation of regenerated buds is the most recalcitrant step. Elongation is slow, asynchronous and the frequency of bud to shoot conversion is low. A shoot requires 16-25 weeks to reach a rootable size (6 mm-1 cm).

Rooting is another bottle-neck. Rooting has been achieved in a number of species but the rooting percentage has been generally very low: 25% in Picea abies (Von Arnold and Eriksson, 1986), 4% in Pinus resinosa (Noh et al., 1988), and 3% in Pinus monticola (Stiff et al., 1989). In the latter case only 10% shoots had well developed roots.

Somatic embryogenesis has so far been reported from embryonic tissues only. True rejuvenation will be achieved when embryos can be formed from mature explants. Physiological abnormalities of the somatic embryos also need to be overcome before it can be considered as a realistic approach to conifer micropropagation.

The present success with in vitro regeneration of plants does not offer a commercially viable method for micropropagation of conifers.

6 CONCLUDING REMARKS

Among woody perennials, temperate fruit species (see Zimmerman et al., 1986) and some forest trees, such as Populus, Eucalyptus and Sequoia (only some clones of some species) have proved amenable for large scale propagation through tissue culture techniques. Most of the tropical tree species appear comparatively difficult. Tissue culture of nut trees is just beginning.

Despite the voluminous literature on regeneration in tissue cultures of tree species, the success is largely restricted to juvenile materials and often up to the stage of differentiation of shoot buds, embryo and/or embryo-like structures. Commercial exploitation of tissue culture for the clonal multiplication of woody perennials requires considerable more work, especially to apply the findings with juvenile material to adult explants, to find effective treatments to induce efficient rooting of in vitro multiplied shoots, and to improve the quality of somatic embryos

to achieve high frequency conversion to plantlets.

As remarked by Dunstan and Thorpe (1986), commercial application of tissue culture to perennial crops must await adequate quality check and field testing. Violation of this basic rule may cause large scale losses as experienced with oil palm. The temptation to project the possibility of producing large number of plants on the basis of preliminary laboratory experiments should be moderated with words of caution against realistic undesirable possibilities associated with it.

In general, the tree species which exhibit continuous growth throughout the year or nearly so, such as Sequoia, Metasequoia, Thuja, are much easier to stabilize in cultures while those which show highly seasonal growth (episodic) are difficult to culture (McCown, 1986). The latter category of species often maintain their growth dynamics in cultures and, therefore, the resultant cultures are never truly predictable and uniform. Widespread application of microculture for the production of woody perennial plants would require a better understanding of controls of the growth dynamics in these plants.

Total decontamination of tissues is another serious problem faced with the cultures of woody perennials. The contaminants present inside the tissues, such as vascular supply, laticifers, and glands may not show up even on bacteriological media but may affect growth. With some of the bamboo species it was essential to add antibiotics to the culture media at least during the first two passages (Saxena, unpublished).

Production of phenolics by tree tissues, specially when derived from adult material, is of common occurrence. It causes browning of the medium which is toxic to the explant, often resulting in its death prior to its establishment in cultures.

In the cultures of nodal segments of several tropical tree species (Feijoa - Bhojwani et al., 1987; Acacia nilotica - Bhojwani, unpublished; Pterocarpus - Patri et al., 1987) bud break in primary cultures may be good but the in vitro developed shoots excised from the parent cuttings and planted on fresh medium usually do not survive. Understanding the role of the tissue of parent cutting in supporting shoot growth may help evolving suitable culture medium for sustained growth and multiplication of shoots from tree species.

Libby (1988) has expressed concern about the dangers associated with the research of forest biotechnologists working in isolation from foresters. According to him "with some exceptions most good biotechnoligists do not have skills or training in field testing nor the intimate experience with field grown trees to spot an unusual value or uncharacterized variation

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