Hairy Root Culture: An Alternative Terpenoid Expression Platform

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Jocelyne Trémouillaux-Guiller

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Abstract

A fantastic source of natural products encompassing the vast group of the terpenes, alkaloids, and phenolic compounds is provided by the plants. About 25,000 of these secondary metabolites hold a strong potential in a large domain of industrial and pharmaceutical applications. Plant families, such as *Lamiaceae, Asteraceae, and Taxaceae*, synthesize several terpenoid classes with a great economic value. Hairy roots, resulting from *Agrobacterium rhizogenes*–mediated transformation of plant cells, can be harnessed for terpenoid production on a large scale. *A. rhizogenes* over other transformation systems makes possible an

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easy regeneration of transgenic plants. Based on the deletion of *rol* genes located on the wild-type Ri-T-DNA, disarmed versions are created in order to overcome abnormalities of Ri-plants. Terpenoid accumulation from hairy roots can be optimized by elicitation treatment with jasmonates. Ri-T-DNA activation tagging system and transcriptome analyses are powerful tools to isolate new function genes involved in the terpenoid biosynthesis. Such genes encoding enzymes involved in the secondary metabolism can be inserted between the T-DNA borders. Lastly, some pharmacological properties of these plant terpenes/terpenoids involved in the therapy of more or less severe pathologies are reported.

Keywords

Activation tagging • *Agrobacterium rhizogenes* • Elicitation • Hairy roots • Metabolic engineering • Pharmacological properties • Ri-plants • Ri-T-DNA • Secondary metabolite production • Terpenes • Terpenoids

Abbreviations

2, 4-D	2, 4-Dichlorophenoxyacetic acid
Ac-MVA	Acetate mevalonate pathway
BABA	β-aminobutyric acid
BAP	6-Benzylaminopurine
CPPU	4-*N*-(2-chloro-4 pyridyl) –N′-phenylurea
DMAPP	Dimethylallyl pyrophosphate
DXP	Deoxyxylulose 5-phosphate
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
HMGR	3-Hydroxy-3-methylglutaryl CoA reductase
IAA	Indole 3 acetic acid
IPP	Isopentenyl pyrophosphate
Kin	Kinetin
MeJA	Methyl jasmonate
MEP	2-C-methyl-D-erythritol 4-phosphate pathway

1 Introduction

Plants represent most structurally vast and varied source of natural products, although less than half of the estimated 200,000+ secondary metabolites have been identified at the present time [1]. In nature, a plethora of phytochemicals have specialized functions in ecological interactions and are frequently produced in response to pathogen or abiotic stresses [2]. These natural secondary compounds can play a beneficial or deleterious role in the plant-insect, plant-pathogen, and plant-plant relations [3]. Owing to their large number and great diversity, the secondary metabolites hold an immense potential in industrial and/or medicinal application fields [4], notably as cosmetics, food additives, perfumes, and biopharmaceuticals for the human and

animal healthcare [5]. Based on their biosynthetic origins, these secondary products can be divided into three major groups including the alkaloids (±12,000 types), phenolic compounds (±8,000 types), and terpenes (±25,000 types) which do not seem to be all directly involved in the development and growth of plants [4, 6]. The terpenes that are hydrocarbons exclusively composed of carbon and hydrogen and the terpene-derived terpenoids (or isoprenoids) by oxidation or some alteration of the carbon skeleton encompass more than 40,000 structures and constitute the largest and mostly diverse class of all known natural molecules [7]. Among them, a great chemical diversity is composed of primary metabolites (chlorophylls, carotenoids, cytokinins, gibberellins) and more than 25,000 secondary or specialized metabolites have harmful or beneficial effects on other organisms [3, 6]. Specialized terpenes/terpenoids have a myriad of important industrial applications notably, as flavoring agents, perfumes, insecticides, and biopharmaceuticals [8]. Higher plants biosynthesize commonly terpenes via two IPP (isopentenyl pyrophosphate) sources generated from two independent pathways: acetate-mevalonate (Ac-MEV) (in the cytosol) and 2-C-methyl-D-erythritol 4-phosphate (MEP) or deoxyxylulose 5-phosphate (DXP) (in the plastids) pathways (Fig. 95.1), present in vegetative tissues, flowers, and more occasionally in roots [9]. The early steps of terpene/terpenoid biosynthesis in the plants start by the formation and assembly of C5 isoprene units (1 isoprene unit = a monomer form) that result from two separate pathways. Polymerization of C5 isoprene units leads, for example, to monoterpenes C_{10} (2 isoprene units), sesquiterpenes C_{15} (3 isoprene units), and diterpenes C_{20} (4 isoprene units) [9] (Fig. 95.1). Indeed, terpene biosynthesis occurs, in the cytosol or the plastids, through the condensation of the five-carbon precursor isopentenyl pyrophosphate (IPP) (active isoprene unit) and its allylic isomer dimethylallyl pyrophosphate (DMAPP). The sequential head-to-tail addition of IPP units to DMAPP leads to the prenyl diphosphate geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). These three previous components serve respectively as precursors for sesquiterpenes, triterpenes (in the cytosol) and monoterpenes, diterpenes and tetraterpenes (in the plastids) (Fig. 95.1). Several terpenoids of a great industrial/medicinal interest are accumulated in the plant roots [10].

In the past three decades, hairy root cultures have been extensively investigated in vitro as a fascinating and alternative research tool for producing secondary metabolites at levels comparable to those found in the wild roots [11–20] and/or for revealing novel biomolecules [21, 22]. Hairy roots offer many advantages over cellular suspension cultures, notably their high growth rate, genetic stability, and hormonal independence [23]. Compared with transformed roots, undifferentiated cell cultures show most often a permanent instability responsible for low levels of secondary products and/or a rapid loss of the accumulation potential [24–27].

This chapter focuses on the terpenoid production from plant species, hairy root cultures, and Ri-plants; the terpenoid increase by hairy root elicitation; and recent strategies as T-DNA activation tagging/transcriptome analyses for isolating novel genes and hairy root engineering in order to improve terpene metabolism pathways. Lastly, therapeutic properties of some plant-derived terpenoids are reported.

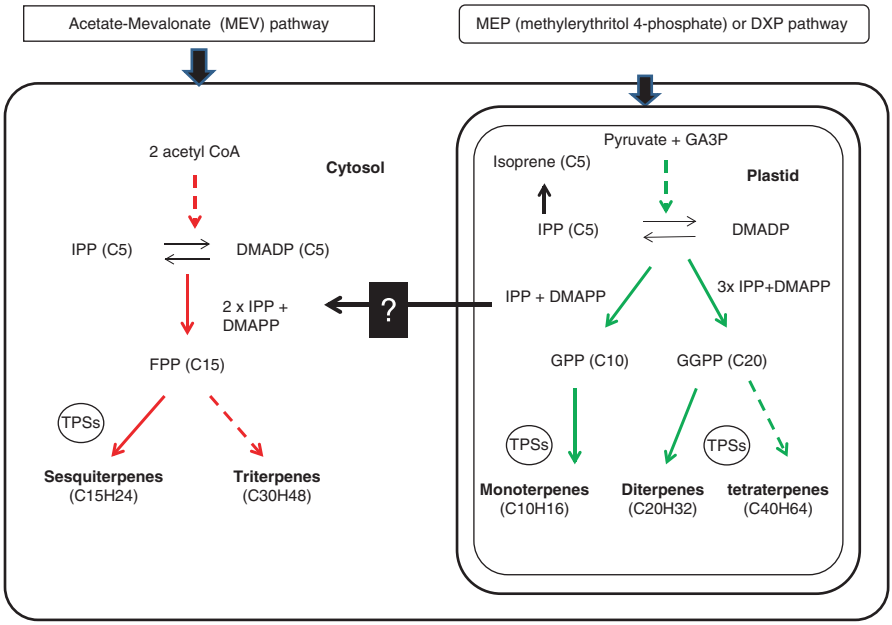

Fig. 95.1 *Terpene biosynthesis pathways and their subcellular localization in the plants*. Different classes of terpenes are respectively formed in the cytosol or the plastid by two independent pathways in the plants, that is, acetate-mevalonate pathway (MEV) (cytosol) and methylerythritol 4-phosphate (MEP) or deoxyxylulose 5-phosphate pathway (DXP) (plastid). Monoterpenes, diterpenes, and tetraterpenes are derived from IPP and DMAPP from the plastidial MEP or DXP pathway. Sesquiterpenes and triterpenes are biosynthesized from IPP and DMAPP from the cytosol pathway. *Black square* with a white question mark suggests a possible transport of IPP (isopentenylpyrophosphate) from the plastid to the cytosol. Other metabolites involved in the different steps are *DMAPP* dimethylallylpyrophosphate, *FPP* farnesylpyrophosphate, *GA3P* D- glyceraldehyde- 3-phosphate, *GPP* geranylpyrophosphate, *GGPP* geranylgeranylpyrophosphate. TPSs in the *circle* correspond to terpene synthases. *Broken arrows* show several enzymatic steps (Adapted from Aharoni et al. [8] and Sallaud et al. [154])

2 Terpenoid Biosynthesis in Plants

Plant species are capable of manufacturing different types of secondary products which can be harnessed by humans for their beneficial properties in a large domain of industrial or medicinal applications [4]. World Health Organization (WHO) estimates that up to 80% of people rely mainly on traditional herbs as remedies for their medicines [28, 29]. Extracted from entire plants, secondary products are used by food and pharmaceutical industries, although most often numerous natural plant-derived molecules remain undiscovered or unexplored for their pharmacological properties [1]. Nowadays, the distribution of terpenes/terpenoids in the nature is extensively studding [30] and numerous terpenoids, presenting tremendous industrial and therapeutic properties, have already been identified in perennial herbs or woody plant species (Table 95.1). A large number of highly efficient terpenes, including

Table 95.1 Different terpene/terpenoid classes synthesized via the Ac Mevalonate/MEV pathway + (in the cytosol) and via the MEP or DXP pathway ° (in the plastid) in some plant species that are known for their therapeutic properties

Terpene class	Plant species	References
Monoterpenes (C10H16)/monoterpenoids		
Menthol	*Mentha piperita* L.	[9]
Menthol-geraniol	*Anethum graveolens* L.	[124]
Monoterpene alcohols (Linalool, geraniol, nerol. . .)	*Vitis vinifera* L.	[32]
Essential oils (E.O)	*Centaurium erythraea* Rafn	[99]
Oxygened monoterpenoids (E.O.)	*Pelargonium graveolens* L'Her Ex Ait	[153]
Volatile terpenoids (Cavacrol, p-cymene)	*Ocimum basiculum* L.	[36]
Thymol; para-cymene; α, γ terpinene	*Ocimum gratissimum* L.	[35]
Volatile oils	*Panax ginseng* CA Meyer, *Prunus japonica*	[47]
Monoterpenoids	*Salvia sclarea* L.	[40]
Sesquiterpenes (C15H24)/Sesquiterpenoids +		
Sesquiterpenoids	*Solanum tuberosum* L.	[97]
	Solanum truncatula L.	[93]
	Solanum habrochaites	[154]
Sesquiterpene lactone/bilobalide	*Ginkgo biloba* L.	[155]
	Salvia sclarea L.	[140]
Sesquiterpene volatiles	*Vitis vinifera* L.	[31, 32]
Sesquiterpene lactone, with an endoperoxide bridge)	*Artemisia annua* L.	[129]
(Artemisinin)	*Artemisia indica* Willd	[42, 54]
Terpene aldehyde – sesquiterpenoid (gossypol)	*Gossypium barbadense*	[135]
Diterpenes (C20 H32)/diterpenoids °		
Ginkgolides/diterpene trilactones	*Ginkgo biloba* L.	[73, 155]
Abietane diterpenes – Diterpenoid: tanshinones	*Salvia scalrea* L.	[40]
	Salvia miltiorrhiza Bunge	[41, 119]
Complex diterpenoid: diterpene-alkaloid	*Taxus cuspidata* Sieb. &Zucc.	[57]
	Taxus media Hicksii	[58]
	Taxus baccata L.	[59]
	Taxus brevifolia Nutt Sieb	[56]
Diterpenoids:	*Panax ginseng* CA Meyer	[44]
Triterpenes (C30 H48)/Triterpenoids +		
Ginseng triterpenoid saponins – Ginsenosides	*Panax ginseng* CA Meyer	[49, 120, 158]
Saponins	*Salvia sclarea* L.	[40]
Saponins-glycyrrhizin	*Glycyrrhiza glabra* L.	[157, 161]
	Glycyrrhiza radix L.	
Dammarane-type saponin glycosides	*Gynostemma pentaphyllum* (Thunb.)	[50, 51]
Aralia saponins V	*Aralia elata* Miq	[103]

(*continued*)

Table 95.1 (continued)

Terpene class	Plant species	References
C-13-norisoprenoid – Lancemaside A	*Codonopsis lanceolata* Trautv.	[104, 160]
Triterpenoid glycosides (saponins) Escin	*Aesculus hippocastanum* L.	[43]
	Aesculus indica L.	[151]
Tetraterpenes (C40 H64)/Tetraterpenoids °		
Carotenoids	*Aesculus hippocastanum* L.	[42, 43]

monoterpenes and sesquiterpenes, play a significant role in the interactions of plants with other environmental organisms [8]; notably terpene volatiles, carrying out important information, permit the pollinators to locate flowers and protect reproductive tissues against diverse attacks [31]. Monoterpenoids, sesquiterpenes, and norisoprenoids of low molecular weights, which have commonly been identified as volatile compounds or volatile oils emitted from flowers, fruits, and leaves of numerous plants, possess important values, such as flavor, fragrance, and aroma compounds. Likewise, other terpenoid volatiles, mainly monoterpene alcohols, for example, linalool, geraniol, nerol, and terpineol, have been considered as flavor and aroma products of grapevine berries and vine [32]. The composition of floral volatiles, for example, pollen volatiles from several grapevine varieties serve as attractants for pollinators, but also may function to deter herbivore attacks and defend the flowers against the pathogen, desiccation, or UV-light damages. Likewise, sesquiterpene volatiles at the highest levels were detected in this floral organ of *Vitis vinifera* L. cv. Cabernet Sauvignon immediately before bloom and at bloom [31]. Aromatic herbs, such as *Ocimum basilicum* L. (sweet basil), *Thymus vulgaris* L., and *Ocimum gratissimum* L., of the family Lamiaceae are largely utilized because of multiple properties as distinctive aroma additives to food or as medicinal herbs [9, 33–35]. These aromatic oils, stored in the leaves, are composed of aroma monoterpenes (eugenol, thymol, carvacrol, and linalool) and exhibit potent antioxidant properties [36]. Essential oils, that is, green pesticides are an excellent alternative to synthetic pesticides in order to reduce negative impacts to human health and environment [37].

Plant species, belonging to different genera and synthesizing several under classes or types of terpenes/terpenoids, are succinctly described below (Table 95.1). The first genus *Salvia*, the largest genus of the family Lamiaceae (or Labiatae), distributed throughout the world, offers a great and cosmopolitan group of about 236 genera and 900–1,000 species [38] and is phytochemically characterized by their diterpene contents [39, 40]. *Salvia* species that are of widespread interest owing to their large spectrum of medicinal activities can be cultivated in several countries of Europe [41]. Turkey is one of the major centers of diversity for genus *Salvia*, with 93 species of which 54 % are endemic [38]. Two subgenera *Salvia* and *Sclarea* possess abietane diterpenes, whereas *Calosphace* has clerodane diterpenes and *Leonia*, abietane and clerodane diterpenes. Considered as compounds specific to this species the diterpenoid tanshinones of *Salvia miltiorrhiza* (Chinese sage), contained in the rhizomes, have received more attention [41]. The aerial parts of *Salvia sclarea* contain mostly monoterpenoids and

sesquiterpenoids, whereas twelve diterpenoids and two triterpenoids have been identified in the roots of this species. Most of the diterpenoids, abietane or arranged abietane-type, show various biological activities [40]. Genus *Aesculus* belonging to Hippocastanaceae or Horse-chesnut family regroups 100 species or varieties. *Aesculus* sp. are deciduous trees and shrubs among which several species, such as *Aesculus hippocastanum, Aesculus chinensis, Abronia turbinata, and Acalypha indica*, have been cultured as pharmaceutical crops for providing Standardized Therapeutic Extracts (STEs) [42]. More than 210 biomolecules have been isolated from the genus *Aesculus* and identified as belonging to different terpenoid classes, triterpenoids, triterpenoid glycosides (saponins), and tetraterpenoids (carotenoids) [42, 43] (Table 95.1). Two Eurasian species, of which aescin and individual compounds have been identified and other *Aesculus* sp. exhibit potent antitumor, antiviral, antioxidative, anti-inflammatory effects. The root of *Panax ginseng* CA Meyer is largely used as traditional medicine since ancient time due to its stimulating and tonic properties [44]. Genus *Panax* (Araliaceae family), regrouping about 11 slow-growing species, that is, perennial plants with strong roots, has been used for therapeutic aspects for centuries. Among them *Panax japonicus* (Japanese ginseng), *Panax quinquefolius* (American ginseng), and *Panax ginseng* (Korea or Asia ginseng) [45, 46] are cultured in Japan, China, Korea, but also in the United States and Canada [47]. *P. ginseng* is the most required species for its restorative properties [48], and its roots contain active principles detected as ginseng triterpenoid saponins and ginsenosides (Rx), that is, saponin glycosides, the most prominent constituents of ginseng [49]. The chemical compounds of different *Panax* species are relatively similar and approximately 38 types of ginsenosides have been identified with variable levels according to the *Panax* species [46]. *Panax ginseng* is close to *Gynostemma pentaphyllum* because of the secondary metabolite production and pharmacological properties that its chemical entities confer on this plant species. *Gynostemma pentaphyllum* Thunb. or jiaogulan (Chinese name), a member of the family Cucurbitaceae and genus *Gynostemma*, is an oriental medicinal herb biosynthesizing numerous Dammarane-type saponin glycosides, and about 90 compounds known as gypenosides that have been isolated and considered as responsible for the pharmacological properties recently attributed to this species [50, 51] (Table 95.1).

Lastly, two other important genera regroup precious species biosynthesizing phytochemicals involved in the treatment of mortal pathologies. In first, the genus *Artemisia* is one of the largest of the family Asteraceae with more than 500 species and includes a large number of aromatic plants. Genus *Artemisia* is mostly composed of perennial plants and distributed worldwide, mainly across the temperate zones of the Northern Hemisphere, but a few species can also be observed on the Southern Hemisphere [52]. Overall *Artemisia* species are considered as herbs (*Artemisia annua* L., *Artemisia vulgaris* L.), subshrubs (*A. changaica* Krasch, *A. crithmifolia* L.), or shrubs (*A. tridentate* Nutt.) with strong aroma [52, 53]. Artemisinin, a sesquiterpene lactone with an endoperoxide bridge [42, 54], has been isolated from species *Artemisia annua* L., a model plant owing to its small genome, which is widely used to treat the malaria disorders [42] (Table 95.1).

Another important genus *Taxus*, composed of small coniferous trees and shrubs, belongs to the family Taxaceae including many species, such as *Taxus baccata*, *Taxus brevifolia*, *Taxus chinensis*, *Taxus Canadensis* or *Taxus cuspidata* or still *Taxus media*. These coniferous trees, relatively slow-growing, can reach between 1 and 40 m in length and produce red berry-like fruits. Known commonly as yews, these species are poisonous because most of the trees produce taxol. Taxol or Paclitaxel (its generic name) that was isolated in 1971 [55] from the bark of the yew tree (*Taxus brevifolia* Nutt) is a complex diterpenoid (diterpene alkaloid) possessing very strong antineoplastic efficiency due to its unique mode of action on the cell microtubules [56–58]. Considered as an alternative product to taxol, taxotere or its generic name "docetaxel" is a chemical entity, semi-synthetically via the conversion of non-cytotoxic molecules: baccatin III and 10-deacetylbaccatin III precursors of taxol extracted from the needles of *Taxus baccata* L., the European yew [59] which has provided an interesting and renewable source of taxanes with the similar action mode for treating the same variety of cancer pathologies.

In the last decades, numerous biotechnological investigations have made possible the establishment of hairy root cultures from the plant species, previously described, in order to produce terpene/terpenoids of pharmaceutically great interest.

3 *Agrobacterium rhizogenes:* A Natural Engineer of Genetic Transformation

Phytopathogen gram-negative soil bacterium, *Agrobacterium rhizogenes*, is able to infect wounded plant cells [60] for inducing a tumorous pathology, referred to as "hairy root syndrome" [61, 62] and revealed by the emergence of adventitious roots at or near to the inoculation site of the agrobacteria [63] (Fig. 95.2). Pathogenicity of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* depends respectively on a large and extrachromosomal Ti- (for tumor inducing) or Ri- (for root-inducing) plasmid carrying a DNA region termed Ti-T-DNA or Ri-T-DNA respectively, [64] that can be horizontally transferred and stably integrated into the nuclear genome of higher plants [62], as dicotyledon, gymnosperm, and some monocotyledon host species [65]. Ri-T-DNA harbors between its specific border sequences ($T_{R\ (R=right\ border)}$ and $T_{L\ (L=left\ border)}$) of 25-bp repeats, notably the T_L-DNA region carrying the root loci (*rol*) genes responsible for the hairy root phenotype. Ri plasmids, such as *pRi*A4 or *pRi*1855, belonging to the hypervirulent agropine-type *Agrobacterium rhizogenes* strains, possess a T-DNA formed of two independent regions [66] termed T_R-DNA and TL-DNA. The T_R-DNA region carries the aux1-2 genes that encode enzymes involved in auxin synthesis and play an ancillary role in the transgenic root formation [67]. Eighteen open reading frames (*Orfs*) have been identified from the TL-DNA region, among them the 10, 11, 12, and 15 *Orfs* correspond respectively to the *A, B, C*, and *D rol* genes [68]. Other genes, termed *ops* genes, are equally located in the T_R-DNA and code for opine synthesis, that is, particular amino acid derivatives serving for specific food to the bacteria [69–72]. Genetic transformation of recalcitrant species, as

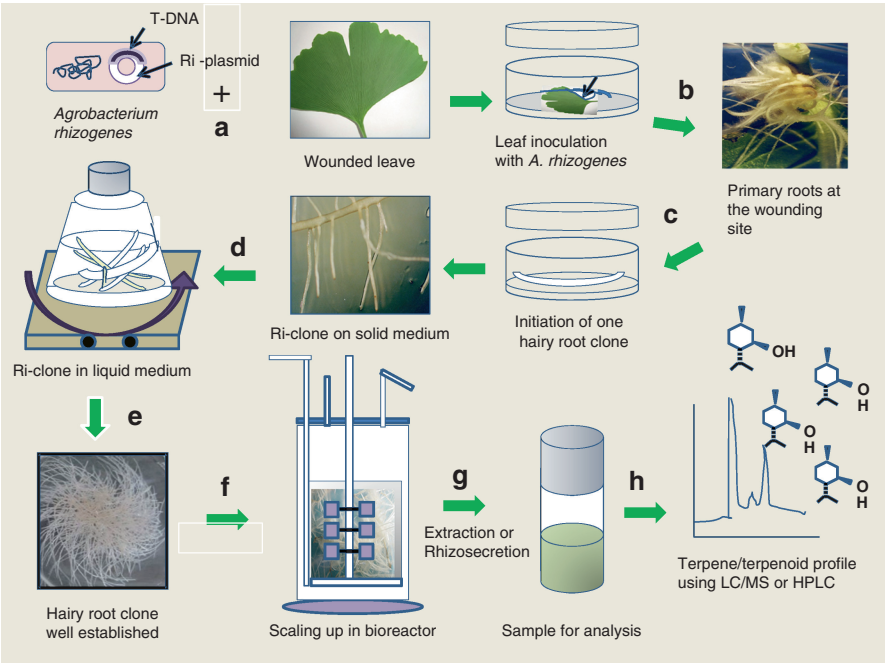

Fig. 95.2 *Establishment, culture on a large scale and terpene/terpenoid production from hairy roots.* (**a**) Both partners involved in the genetic transformation: *Agrobacterium rhizogenes* (a pathogen gram-negative soil bacterium) harbors a large Ri-plasmid carrying the wild-type T-DNA (*black arrows*) or an engineered T-DNA that can be transferred into the plant genome (*black arrow*) and one wounded leave. (**b**) After 48–72 h of co-culture with bacterium/plant cells, emergence of adventitious primary roots at or near the infected site. (**c**) Each primary root excised on the leaf explant and placed on solid medium grows vigorously for generating a true clone of hairy root (HR). (**d**) A crucial step corresponds to the transfer of the root tip segments of HR into the agitated liquid medium. (**e**) One well-established HR clone grows rapidly and shows the characteristics of the HR phenotype, that is, plagiotropic growth, high branching in hormone-free medium. (**f**) Scale-up of hairy root culture in specific bioreactor for commercial exploitation of valuable terpenoids. (**g**) Separation and identification of terpene/terpenoids by liquid chromatography/mass spectrometry (LC/MS) or by high performance liquid chromatography (HPLC)

Ginkgo biloba, required the use of hypervirulent agropine-type strains [73]. On the contrary some Ri plasmids, as *Ri*8196 or *pRi*2659, have a simple T_L-DNA capable of initiating hairy roots and producing different opine types (mannopine, mikimopine, or cucumopine) according to the *A. rhizogenes* strain [74]. Fundamental studies have improved our understanding of the different molecular events resulting in the T-DNA transfer through the host cytoplasm up to nuclear plant genome. Nevertheless, the final steps of T-DNA insertion into the plant genome, involving host factors, are still unknown. The Ti/Ri plasmids possess *vir* genes (*vir* for virulence) that encode Vir proteins responsible for the plant-bacterium attachment, formation, and protection of the simple and mobile copy of the T-DNA for its translocation from bacterium to the host nucleus [75–77]. Most *vir*

genes, carried by the Ti/Ri plasmids and involved in the T-DNA secretion system, are similar. However, the Ri plasmid possesses GALL genes coding for two full-length GALLS (-FL and -CT) proteins, instead of the *vir*E2/*vir*E1 genes present in *A. tumefaciens* [78, 79].

4 Emergence and Development of Hairy Roots

In the previous decades, the strong power of *A. rhizogenes* to transform various plants and induce hairy roots has been largely demonstrated [71]. About 200 species of higher plants (at least 30 families), mostly dicotyledonous species, could be genetically transformed with *A. rhizogenes* and led to the development of adventitious roots [80]. This efficient mode of bacterial transformation is depending on several parameters, including the strain type, bacterial concentration, co-culture time of the bacterium/explant, temperature, light/darkness, as well as the plant species and explant type [81]. Different surface-sterilized explant types (i.e., leaf discs, leaves, stem segments, petioles, roots, embryos, or plantlets) are wounded and then inoculated with *Agrobacteria*. After 2–4 weeks primary adventitious roots, coming from the differentiation of one single plant cell transformed by one bacterium [62], appear at, or near, wound site of plant cells infected by *A. rhizogenes* [82] (Fig. 95.2). Roots, reaching about 1–1.5 cm in length, are excised and placed separately on solid or semi-solid hormone-free medium for initiating a true clone. Hairy root phenotype is characterized by a fast hormone-free growth, an unusual ageotropism, a profusion of root hairs with abundant lateral branching [83–85] (Fig. 95.2). Hairy root cultures can present more and less than root thickness, root length, number of root hairs and root branching, according to plant species and *A. rhizogenes* strain used for hairy root induction [86]. Sometimes, Ri-roots can become brown and stop their growth in liquid medium, because the most crucial step results from the transit of the hairy root clones into agitated liquid medium [87] (Fig. 95.2). But the morphology of *A. rhizogenes*–induced hairy roots is similar in structure to wild-type roots with a few notable exceptions [66]. A recent paper underlined that the A. *rhizogenes*–mediated transformation has made possible the establishment of hairy root cultures from 116 plant species [88].

5 Ri-Plants Taken as Bioreactors

A. rhizogenes–mediated transformation can be exploited for producing terpenoids using plants as bioreactors. Advantages of transformation with *A. rhizogenes* over other methods encompass adventitious organs/roots and rapid shoot regeneration reducing the risk of somaclonal variation [89]. Moreover, this transformation is that it enables the remarkable development of Ri-plants via a marker-free selection through the observation of the hairy root morphology, as the primary indicator of

genetic transfer [90]. Lastly, another fundamental advantage from the wild-type *A. rhizogenes strains*, compared with the *A. tumefaciens* ones, is that the hairy root creation can naturally lead to the plantlets. Thereby such plants are free from the legal controls of GMOs in Japan [91]. One pioneered plant regeneration, performed successfully from *Brassica napus* hairy roots, displayed that fertile Ri-plants could sexually transmit the hairy root phenotype to their progenies [83]. Such a heredity of Mendelian type has recently been confirmed by Crane et al. [92] from Ri-plants of *Medicago truncatula*, an interesting legume, that emits a large spectrum of volatile terpenoids, including monoterpenes, sesquiterpenes, and tetranor-terpenoids, induced by herbivore attacks [93]. Studies reported that transgenic plants were regenerated from hairy roots of 62 different taxa, representing 53 plant species from 24 families [89] or still up to 79 plant species [94].

Besides, Ri-plants potentially can equally offer promising horticultural/floricultural applications. Classical breeding in floriculture serves to create new plant varieties, although the available gene pool for introducing novel agronomic or aesthetic features is genetically limited to the parental background [95]. Appeared after the *rol* gene insertion into the plant genome, cytological abnormalities make possible the creation of the novel floricultural varieties with exciting characteristics. For instance, Ri-regenerants of *Kalanchoe blossfeldiana*, an economically important plant in Europe, exhibited a dwarf and compact growth habit compared to an elongated growth habit, commercially disadvantageous, of the control plants [90].

An overview of the literature reveals that plant regeneration from hairy root segments can take, spontaneously, directly or indirectly (after a callusing phase before shoot regeneration) several developmental ways. Thereby, buds were initiated from adventitious Ri-root parts with or without exogenous growth regulators [66], for example in *Aesculus hippocastanum*, a plant species producing triterpenic glycosides [43], buds emerged from hairy roots placed on the solid medium devoid of exogenous hormones [96]. Nevertheless, the number of *A. hippocastanum* buds was found superior when the Ri-roots were placed on the medium supplemented with BAP. Rooting of these shoots was initiated more rapidly with IBA (in 80% of explants) than IAA (in 40% of explants) [96]. Another regeneration model was reported from genus *Solanum* which is one of the largest *genus* of family Solanaceae comprising about 84 genera and 3,000 species and producing numerous flavonoids and terpenoids, such as sesquiterpenoids in *Solanum tuberosum* [97] (Table 95.1). *Solanum nigrum* hairy roots, maintained on a hormone-free medium, developed spontaneously plantlets capable of producing in vitro flowers and fruits, whereas control plantlets remained in the vegetative state [89]. *Pelargonium graveolens* hairy roots, coming from Ri-T-DNA-mediated transformation of leaves and internodal segments, expressed their shoot regenerative capacity from both explant types [98]. *Centaurium erythraea* Rafn (*Gentianaceae*) is a traditional medicinal plant of Croatia that accumulates monoterpenoids in the aerial parts [99] (Table 95.1). *C. erythraea* hairy roots, placed in the medium added with IAA and BAP, formed shoots which were rooted at 68–97% without any hormonal

supplement [100]. The Ri-plants regeneration could be associated to the formation of embryogenic calli [100]. *Panax ginseng* CA Meyer possesses numerous pharmacological activities, mostly attributed to one type of its constituents, namely, ginsenosides belonging to triterpenoid saponins [46] (Table 95.1). Placed on the medium without any growth regulator and in the dark, shoots and yellow calli in *Panax ginseng* appeared simultaneously from hairy root clones. Embryogenic calli, transferred to light, gave rise to somatic embryos leading to shoots/plantlets [101]. Transformed callus-derived embryogenic cell lines of *Panax ginseng* have been able to develop embryos and plantlets arising from only *A. rhizogenes rol* C-gene expression [102]. Root segment-derived hairy root lines of *Aralia elata*, a wood shrub producing pharmaceutically important metabolites as triterpenoids (*Aralia* saponins V) [103], and cultured on nutritive medium supplemented with 2, 4-D could generate embryogenic calli able to develop somatic embryos up to the cotyledonary stage without any phytohormone. Embryo germination led to plantlets and 100% of them were successfully acclimatized under greenhouse conditions [103]. Embryogenic calli, cotyledonary embryos and plantlets were developed from hairy root segments of *Codonopsis lanceolata* (Campanulaceae family) cultured in medium supplemented with 2, 4-D and accumulated, in the stems, higher contents of three major triterpenoids (lancemaside A, foetidissimoside A, and aster saponin Hb) than wild-type plants [104].

The third organogenesis type requires an intermediate callus phase before the formation of shoots and plantlets. Thereby, hairy root–derived calluses of *Mentha piperita* proliferated abundantly in the presence of 1 μM NAA, 10 μM CPPU and 10% coconut powder in the culture medium. Placed onto solid hormone-free medium *M. piperita* calli gave rise to shoots and roots [105].

6 Transgenic Plant Devoid of Hairy-Root Phenotype

Ri-Regenerants, arising from genetic transformation by the T-DNA insertion, exhibit frequently typical abnormalities due to the *rol* gene expression as wrinkled leaves, short internodes, dwarfness, numerous lateral shoots, flowers, and plagiotropic roots with an extensive branching [94, 106, 107]. Nevertheless, normal morphology of the leaves, a good stem elongation, and an extensive root system could be observed from Ri-regenerants of *Alhagi pseudoalhagi*, and similarly of *Hypericum perforatum* [108]. In contrast, transgenic somatic embryo-derived plantlets of *Aralia elata* exhibited an aberrant hairy-root phenotype compared to the mother plants [103]. This particular aspect of Ri-plants can be discouraging for initiating certain programs of applied research, notably, in the agronomy fields. Easy protocols have been established to overcome undesirable abnormalities of the Ri-plants by creating disarmed versions, as it has been achieved with *A. tumefaciens* [66]. The abnormal hairy-root phenotype can be suppressed after deletion of the oncogenes, that is, *rol* genes, located on

the wild-type Ri-T-DNA and responsible for these morphological singularities. This deletion is performed without any consequence for the T-DNA insertion, since with exception of the right and left borders none of the other T-DNA regions are indispensable to the horizontal transfer of foreign genes into the host genome [109]. Therefore, the *Rol* genes carried by the wild-type Ri-T-DNA may be replaced by additional genes overexpressing secondary metabolites/recombinant proteins or encoding the resistance to herbicides, insects, and various pathogens [110]. Two vector types, co-integrative and binary vectors, can be used as genetic transformation systems [111]. A binary vector system contains, for example, the Ti plasmid carrying only virulence genes, whereas its T-DNA has been removed [112] and the second plasmid carries the foreign gene inserted between the border sequences of the Ri-T-DNA with or without deleting the *rol* genes [113]. Plants coming from leaf explants of *Centaurium erythraea* Raf co-cultured with the *A. rhizogenes* strain LBA 9402, carrying a binary vector, were reported by Piatczak et al. [100]. Using binary vectors, deleted oncogenes, the problem of the surprising hairy root phenotype may be bypassed.

7 Novel Genes Revealed by Ri-T-DNA Activation Tagging and Transcriptome Analysis

Hairy roots, coming from the genetic horizontal transfer of the wild or recombined Ri-T-DNA vectors in the host plant genome, represent an ideal engineering platform for exploiting new biotechnology tools (Fig. 95.2). DNA activation tagging [114] and RNA silencing processes followed by transcriptome analyses are recent powerful tools capable of revealing novel genes, notably those involved in the secondary metabolism, but also, of identifying gene function [114]. Activation tagging process permits to generate gain-of-function mutations, whereas gene silencing leads to loss-of-function approaches [115]. RNA silencing corresponds to a natural mechanism of genetic control taking place in virus resistance, genome maintenance, and developmental control in plants [116]. The classical loss-of-function process can be limiting because of the absence of visible phenotypes which restrict the identification of mutants [16]. Thus, one of the most frequently applied approaches is DNA-activation tagging insertional mutagenesis [117] which can circumvent many disadvantages of loss of function [44]. The *Agrobacterium* spp. T-DNA vector, carrier of multimerized transcriptional enhancers of the cauliflower mosaic virus (CaMV) 35 S promoter, is considered as a powerful mutagen [44] capable of discovering novel dominant or semi-dominant mutations observed in primary transformants [117]. Activation tagging system leads to a random genomic insertion, in the plant genome, of Ri-T-DNA constructs harboring constitutive enhancer sequences positioned next to the right- or left-hand T-DNA border, which are able to cause transcriptional activation of flanking genes [22, 117, 118]. For example, Choi et al. [44] generated activation tagged *P. ginseng* hairy root lines in order to dissect, isolate genes responsible for ginsenoside and triterpene lactone biosynthesis and evaluate the ginsenoside profile of these hairy root lines [119].

Another recent approach based upon the methyl jasmonate inducible system that was applied to hairy root cultures of *Panax ginseng* made possible the identification of new genes through the analysis of 3,134 expressed sequence tags (ESTs) [120]. From the isolated transcripts several would correspond to the genes encoding enzymes as Cytochrome P450, glycosyltransferases, and oxidosqualene cyclase (OSC) involved in the ginsenoside (i.e., triterpene glycosides) biosynthesis [120]. G*inseng* (or other plant species) ESTs data set can provide important information on genes involved in the regulation and biosynthesis of secondary metabolites, only, when their accumulation can be induced by MeJA treatment [120]. Besides, Jian et al. [121] emphasized the fundamental role of the *A. rhizogenes*–mediated transformation system coupling, at once, genetic transformation and regeneration for gene function testing in *Lotus corniculatus*.

8 Engineering of Terpene Metabolism Pathways

The fortification of numerous steps by overexpression of multiple biosynthesis genes or transcription factors for controlling the expression of genes is a fascinating strategy to alter the accumulation of secondary metabolites [122]. Metabolic engineering of terpenoids biosynthesized in plants can be directed toward two major objectives: to increase the plant resistance to predators and diseases or still to increase the production of medicinal or industrial compounds and to make possible a better knowledge of genes encoding enzymes involved in the terpenoid metabolism pathways or its regulation [8]. Understanding the function of genes involved in terpene production can lead to discovery of novel compounds or metabolic pathways, which might reveal favorable properties for human applications [4]. Thereby *Nicotiana tabacum* plants displayed an increase of terpene emission from leaves after insertion of monoterpene synthase genes [4]. The red pigment present in *Salvia militiorrhiza* (*Lamiaceae*) roots is composed of many diterpenoid tanshinones. In this plant, diterpenes are synthesized through the MEP pathway in the plastids (Fig. 95.1) and DXR (DXP (1-deoxy-D-xylulose 5-phosphate) reductoisomerase) is an enzyme catalyzing the first step of this pathway. Wu et al. have cloned and sequenced the gene coding for the DXR protein [41]. Functional expression of the *dxr* gene was controlled by genetic complementation with the lethal *dxr*-deficient *E. coli* mutant strain. Besides, the RNA-blot analysis, from hairy root cultures of *S. militiorrhiza*, elicited with a hyperosmotic stress and/or yeast extract, showed a strong induction of the *dxr* gene transcription that was correlated with the diterpenoid tanshinone increase in these cultures. The same authors concluded that the *dxr* gene involved in the MEP pathway may be a target for the metabolic engineering of diterpenoid tanshinone biosynthesis [41]. The regulation of terpenoid metabolism and, particularly, the regulation of factors controlling genes encoding enzymes of the secondary metabolism require subsequent investigations [123]. *S. militiorrhiza*, taken as a model plant owing to its small genome, made possible the identification of 40 terpenoid biosynthesis-related genes, of which 27 are novel. It was reported that 20 of the 40 genes could be involved in the tanshinone biosynthesis [124].

9 Hairy Roots: Fascinating Routes to Produce Terpenoids

Secondary metabolites of low molecular weights are widely biosynthesized throughout the plant Kingdom [122] and a large biological diversity of them is accumulated in the roots [85]. In the past few years, a great interest for the natural products led private companies to consider the progresses of the biotechnologies as an alternative strategy for producing biomolecules of high added value. Numerous plant species, genetically transformed with the natural vector *Agrobacterium rhizogenes*, are good candidates to accumulate terpene/terpenoids in organ culture systems [23, 40]. Important research investigations that use differentiated cultures, instead of cell suspension cultures, have been focused on transformed (hairy) roots [23] which offer a flexible and versatile system for a large-scale production of phytochemicals [125]. Hairy roots, because of their high differentiation, allow a stable and extensive production of secondary compounds [126]. Moreover, owing to their culture mode in a sterile, confined, and controlled environment avoiding the transgene dissemination, hairy roots present serious advantages compared with entire plants cultured in the open field [18, 22]. The transformed root cultures can be propagated indefinitely in liquid medium and conserve, at once, a morphological integrity and biochemical stability [86, 115]. Besides, the *rol* genes are now known to possess a stimulating effect on the secondary metabolism with a durable stability, over long-term culture [127]. After its insertion in the plant genome, the T-DNA appears to be a remarkable element in the gene regulation [128]. As described below, some hairy root cultures have shown their capacity to accumulate terpenes/terpenoids in vitro. Thereby, chemical analysis displayed that the contents of three major triterpenoids in hairy roots of *Codonopsis lanceolata* Trautv were similar to those found in wild roots harvested from soil [104]. Sterile 4-week-old shoots of *Salvia sclarea* transformed with the *A. rhizogenes* strain, LBA 9402, led to hairy root cultures from which four diterpenoids (ferruginol, salvipisone, aethiopinone, and 1-oxoaethiopinone) and two ursine-type triterpenoids were isolated [40]. The light enhanced significantly the abietane diterpenoid levels and affected slightly the triterpenoid spectrum in transgenic cultures. Kuzma et al. [40] concluded that *S. sclarea* hairy roots, accumulating terpenoids at higher levels than the roots of field-grown plants, could significantly provide a commercial source of diterpenoids. Hairy roots of *Artemisia dubia* and *Artemisia indica* arising from sterile plantlet-derived stems were transformed with both *A. rhizogenes* strains, LBA 9402 and 8196. Artemisinin levels and fresh root biomass were significantly enhanced in the Ri-roots compared with the control roots and according to the *A. rhizogenes* strain and *Artemisia* species used. Higher levels (0.603 % and 0.753 %) of this natural sesquiterpene lactone were detected in *A. dubia* hairy roots when the stems have been respectively inoculated either with LBA 9402 or 8196 *A. rhizogenes* strains [129].

Despite numerous positive results, some transformed root systems accumulated less secondary metabolites than plant tissues in vivo. The *A. hippocastanum* zygotic embryos are known to be the major site of aescin, a group of chemically related triterpenic glycosides (saponins) involved in the treatment of peripheral vascular

disorders as venous insufficiency [43, 96]. Androgenic embryos of *Aesculus hippocastanum* (horse-chestnut), induced from anthers and inoculated with the *A. rhizogenes* strain A4GUS, led to hairy root clones [43]. The substantial percentages (3.50 % and 4.1 %) of aescin, detected in two hairy root clones, remained inferior to those found in the cotyledonary embryos placed on the hormone-free medium [43]. These authors concluded that the androgenic embryos of *A. hippocastanum* cultured with 2, 4-D is a better strategy for the aescin production on a large scale. Besides, Zdravkovic et al. [96] reported that the hairy root clones of horse chestnut were subsequently able to regenerate transformed plants which may be useful in horticulture for the bonsai establishment. Another tremendous advantage of hairy roots results from the feasibility of regenerating transgenic plants, whereas the regeneration of tissues transformed with *A. tumefaciens* is proved to be often more problematic. Overall secondary metabolites are biosynthesized and accumulated in the roots/hairy roots; nevertheless, some of them can be produced and/or stored in the aerial parts of the plant. Thereby, the total spectrum of secoiridoids that were detected in the non-transformed plants could be provided in vitro at eightfold superior levels in the roots and aerial parts of *Centaurium erythraea* Ri-plants [100]. Likewise, essential oils were mostly detected in the leaves and stems of *Mentha*, but the highest oil levels of *M. piperita* are found in the leaves of Ri-plants compared with those of the wild-type plants [130]. Moreover, the dwarf regenerants of *Mentha* species displayed the hairy root phenotype abnormalities, which does not present any negative impact on the essential oil production [105]. Most hairy root–derived rose-scented geranium plants (*Pelargonium* species) provided a large panel of monoterpenoids (essential oils) similar to those of the wild-mother plant, at the exception of two Ri-plants that biosynthesized two volatile monoterpenoids possessing better olfactory value offering an improvement from a commercial point of view [98]. Green hairy roots can accumulate some secondary products that are normally synthesized in green aerial parts of the plant [130].

10 Elicitation Treatment: A Booster System of Secondary Products

Plants are capable of producing and releasing active allelochemicals targeted in defense against pathogen attacks and stress conditions [2, 131]. Allelochemicals are accumulated under the impact of stress molecules, called elicitors, and then released by the plants into the environment by root exudation, volatile emissions from leaves and other aerial parts [132]. Several natural elicitors have been observed as efficient inducers for boosting the production and secretion of secondary metabolites in cultures in vitro, notably, when they are added to the hairy root cultures [115]. Thereby, the secondary plant messenger methyl jasmonate (MeJA), and its relatives, from the oxylipin family, is a stress hormone able to induce the improvement or optimization of secondary product contents [10, 18, 97]. In 1962, MeJA was discovered as a sweet-smelling biomolecule present in *Jasminum*

grandiflorum L. flower extracts [133, 134] and described as "hormone" because of the cellular responses of elicitation, at low levels, distant from its site of biosynthesis. The jasmonate elicitors are synthesized via the octadecanoid pathways, beginning at linolenic acid and ending at (+) – 7- epi-JA and its conjugates and isomers [134]. There are some examples of elicitation processes for stimulating the secondary metabolite accumulation. Gossypol (sesquiterpenoid) is a toxic phytoalexin biosynthesized in *Gossypium barbadense* (a cotton plant) that possesses anticancer, antiviral, antimicrobial, and antiparasitic properties and is routinely isolated from cottonseed or from cotton roots. This terpene aldehyde interacts with various anticancer agents for increasing the efficiency of their pro-apoptic proteins, detoxification enzymes, and signaling kinases. Only MeJA was found to able to stimulate the contents of gossypol and methylated forms in cotton hairy root cultures. Moreover, the distribution of enantiomers was not found to be similar in elicited or control transformed roots of *G. barbadense* [135].

Taxol or Paclitaxel (generic name), a complex diterpenoid, is one of today's better known antineoplastic drugs [56] and acts as a strong antitumor agent owing to its unique mode of action on the cellular microtubules. Its extraction from its natural source and costly synthetic process make it one of the products with the highest added value [58]. Taxotere or Docetaxel (generic name) is a chemical entity semi-synthetically performed via the conversion of non-cytotoxic molecules: baccatin III and 10-deacetylbaccatin III precursors of taxol extracted from the needles of *Taxus baccata* L., the European yew. Paclitaxel can equally be semi-synthesized from its natural precursor, 10-deacetyl baccatin III. As a promising alternative, cell suspension cultures of various *Taxus* species have been harnessed for the efficient production of taxol and its related taxane compounds [57]. The impact of explant source and growth regulators was tested in order to optimize the induction and selection of fast growing callus lines of *Taxus baccata* (European yew tree) [136]. In *Taxus baccata* L. (European yew tree), Ashrafi et al. [136] tested different combinations of growth regulators and various explants (stems, needles, and base of needle) for callus production. Taxol and baccatin III were respectively increased by 3.1- and 5-fold in the callus-derived cell suspensions cultured in medium added with 2, 4-D + Kin, at different concentrations according to both expected secondary metabolites [136]. *Taxus media* cell lines, transformed with *A. rhizogenes* and termed "TXS" and "Rol C" (*txs* corresponding to the transgene encoding enzyme taxadiene synthase of *T. baccata*), were treated with MeJA for optimizing taxane production. The highest taxane level was observed in the "TXS" cell line and resulted from the activity of enzyme taxadiene synthase and MeJA [58]. To date, the most favorable proceeding for a sustainable production of taxol and related taxoids at industrial level is provided by plant cell cultures [58]. Although production has been scaled-up, a serious drawback of the cell suspension cultures is their instability and unpredictability of the expected secondary products [137]. Due to their great genetic stability and potentially unlimited propagation in vitro, hairy roots as well-differentiated organs, are able to accumulate of important taxanes. For example, three strains of *A. rhizogenes* (R1000, A4 and 15834) were compared for the initiation of *Taxus cuspidata* hairy root lines. As regards its

growth capacities, a selected line was treated with MeJA and produced a total paclitaxel content of 52, 5 mg/l over 2 weeks of incubation and at 20-L culture scale. These taxol levels produced after MeJA addition were similar to those detected in the cell suspension cultures of *Taxus* [57]. As another example, taxol and 10-deacetylbaccatin III (10-DAB III) were increased in hairy root cultures of *Taxus* x *media* var. Hicksii Rehd in presence of the BABA precursor, used alone or in combination with MeJA [137]. To date, only two reports have been published for the production of paclitaxel and its related compounds by hairy root cultures [137].

Other molecules, biotic or abiotic, can act as elicitors for successfully boosting the accumulation and/or secretion of secondary products. A non-protein amino acid, that is, β-aminobutyric acid (BABA), used against pathogen attacks and added separately or in combination with the yeast elicitor for enhancing the tanshinone production in *Salvia miltiorrhiza* hairy roots, showed that BABA could strongly potentiate elicitor-induced secondary metabolism in plant organ cultures [138]. From another experimental work, Ge and Wu [139] studied the separate and combined action of biotic (yeast=YE) and abiotic (Ag+) elicitors on the secondary metabolism activities of *Salvia miltiorrhiza* hairy roots. These authors showed that the YE-induced tanshinone production seemed to be mainly derived from a non-MVA pathway encompassing DXS (1-deoxy-D-xylulose 5-phosphate synthase) since YE did not stimulate HMGR (3-hydroxy-3-methylglutaryl CoA reductase). In contrast, Ag+-induced tanshinone production seemed to be dependent on both pathways (MVA and non-MVA pathways), because it was suppressed by the inhibitors of both pathways [139]. Elicitation, but, also cell permeabilization and trapping processes have been investigated to increase the secretion of secondary products in the culture media [18].

11 Hairy Root Cultures on a Large Scale: A True Challenge

Hairy root offers a flexible and versatile culture system that is a promising biotechnology for the large-scale production of valuable phytochemicals [125]. The transfer in bioreactors is the key step toward a commercial production of terpenes/terpenoids by hairy root cultures. Bioreactors offer several advantages for the mass culture of plant cells or organs, for example, a constant regulation of conditions at various stages of the culture [28]. To scale up hairy root cultures, several operational factors, including inoculum size, medium, growth rate, secondary product recovery (intracellular/extracellular), and reproducibility of results, must be overpowered before the technology is ready for commercialization [23]. Thereby a perfect sterility, maintenance of the homogenization of roots as well as biomass measurement, agitation, oxygenation, and nutrition availability are the major goals to reach for scaling-up hairy root cultures [23, 130, 140]. In fact, growth of transgenic roots in liquid medium results in dense and packed root biomass, leading to the formation of stagnation which slows down the fluid nutrient circulation and limits oxygen availability [86]. Mechanical agitation generates wounding of hairy

roots and callus formation [130]. To overcome these limiting hurdles, hairy roots can predominantly be grown in spray or droplet bioreactors and mist bioreactors in which the roots are immobilized on mesh support and exposed to humidified air or a gas mixture and nutrients [86]. Recent bioreactors with their different advantages/drawbacks for producing hairy roots, on a large scale, have been detailed and classified by Srivastava and Srivastava [23] as liquid-gas, gas-phase, or hybrid reactors, for example, airlift bioreactors, bubble column reactors, and nutrient mist bioreactors. Conductivity measurements between small- and large-scale reactor cultures of hairy roots were compared [141]. Scaling studies showed that at 1 L *Artemisia annua* and *Arachis hypogaea* hairy roots had better growth in the mist reactor than shake flasks. The mass production of *Panax ginseng* hairy root cultures has been established in bioreactors [142] and the ginsenoside accumulation from transformed root cultures through large-scale reactor system was assessed to 1–10 t [28, 143]. Roots cultured in bioreactors have been shown to release therapeutically active compounds into the liquid medium of the bioreactor, for example, the antineoplastic drug isolated from various *Taxus* species [29].

12 Therapeutic Merits of Plant Terpene/Terpenoids

Numerous terpenoids, such as taxol, ginkgolides, digoxin, gossypol, and artemisinin, have been found efficient in the prevention and therapy of severe pathologies encompassing cancer and other severe diseases. Nevertheless, a vast proportion of higher plants have not still been screened for medicinal activities. Drug discovery from plant species remains an essential strategy in the search for future medicines [144]. From pharmaceutical point of view, terpenoids achieve a rich pool for harnessing new drugs and leading products. Cancer, after cardiovascular pathologies, is the second cause of death [145]. In fact, numerous terpenoids exhibit cytotoxicity against a wide variety of tumor cells as well as anticancer efficacy in preclinical animal models [146]. Terpenoids, a large class of mevalonate-derived phytochemicals, suppress the multiplication of neoplastic cells and the growth of implanted tumors; for example, geraniol, a volatile isoprenoid, is an acyclic dietary monoterpene which exerts an antineoplastic activity against different cancer cells both in vitro and in vivo. This antineoplastic property has successfully been assessed on TC-118 human tumors transplanted into Swiss nu/nu mice [145]. Likewise, it has been exemplified by diterpenoid drug taxol relating to the cancer [123]. Indeed, taxane biomolecules coming from genus Taxus represent one of the most powerful types of compounds among all the chemotherapeutic drugs efficient to treat a variety of cancers (encompassing ovarian, breast, lung, head and neck carcinomas, and the AIDS-related Karposi's carcinoma) [56, 57]. The antitumor property results of the binding of drug to the human β-tubulin, such a chemical interaction leads to inhibit the depolymerization of the microtubules [147]. Docetaxel (taxotere) and Paclitaxel (taxol) offer some different characteristics in their molecular pharmacology, which are potentially responsible for their clinical activity and toxicity profiles [136, 148]. Taxotere displays the greater

affinity to β-tubulin, targeting centrosome organization and acting on cells in three phases of the cellular cycle (S, G2, M), whereas Taxol causes cell damage by altering the mitotic spindle at the beginning of the mitosis [57, 136]. Artemisinin (a sesquiterpene lactone) isolated from *Artemisia* sp. is an antimalarial drug efficient against multidrug-resistant strains of the malaria parasites: *Plasmodium* sp. [42], notably, *Plasmodium falciparum* that is the responsible agent for one of the world's most severe pathologies causing at least 500 million cases globally each year and resulting in more than one million deaths [149].

Pharmaceutical properties of several plant species can be due to the association of terpene/terpenoids with other active molecules. That will explain the large spectrum of therapeutic activity of these plant species. *Gynostemma pentaphyllum* (Thunb.) or jiaogulan (Chinese name), a member of the family Cucurbitaceae and genus *Gynostemma*, is an oriental medicinal herb characterized by a large spectrum of pharmacological activities, of which antitumor, antioxidant, cholesterol-lowering, immunopotentiating, and hypoglycemic effects could be observed [50]. Genus *Aesculus* (Horse chesnut) encompasses more than 210 compounds from which different classes have been isolated, in particular triterpenoids, triterpenoid glycosides (saponins), flavonoids, and long fatty chain molecules [42]. Standardized therapeutic extracts (HCSE: Horse chesnut seed extract) and aescin (a saponin mixture) of *Aesculus* sp. are considered as the main source of triterpenic glycosides, a group known as aescin and traditionally used against rheumatism, skin, and peripheral vascular disorders [43]. In Europe, the bark, leaves, HCSE, and aescin have been used in the treatment of chronic venous insufficiency, hemorrhoids, and postoperative edema [42]. Several other medicinal properties have been ascribed to saponins, such as immunostimulant, hypocholesterolaemic, and anticarcinogenic effects [150]. But, one of the most interesting properties is the antitumor action of some novel saponins found recently from the *Aesculus pavia* species [42]. The *Aesculus indica* extract, used against the MCF-7 breast cancer cell line, displayed a significant antiproliferative activity, and dose-dependent effect ranging from 34.2 % (at 10 μ-g/ml) to 94 % (at 500 μ-g/ml) could be observed [151]. Inflammation is a natural response of the mammalian body to a variety of pathologies or hostile agents. Rheumatoid arthritis is one of the challenging diseases associated with inflammatory manifestations. In order to treat similar disorders, various molecules have been isolated as, for example, a potent anti-inflammatory analgesic molecule termed "Aspirin" which was harnessed from bark of *Salix alba* L. Recent findings of several species such as *Achillea millefolium* (Asteraceae) or *Aspilia Africana* C.D. Adams (Asteraceae) and *Bacopa monnieri* (Scrophulariaceae) showed an anti-inflammatory activity due to their chemo-profiles revealing the presence of essential oils/sesquiterpenes, terpenoids (α-pinene, limonene), and triterpene, respectively [152]. Aromatic oils, stored in the leaves, are composed of aroma monoterpenes, such as eugenol, thymol, carvacrol, and linalool, and exhibit potent antioxidant properties which may prevent in vivo oxidative damages such as lipid peroxidation associated

with cancer, premature aging, atherosclerosis, and diabetes [36]. Besides, the essential oils, that is, green pesticides, are excellent alternatives to synthetic pesticides in order to reduce negative impacts to human health and the environment [37], notably the essential oils of *Pelargonium graveolens* showed insecticidal effects with a maximal mortality rate of 100 % [153].

Since ancient times, genus *Salvia* (*Lamiaceae*) has been used throughout the world, owing to its large spectrum of beneficial effects, such as its antineoplastic, antiviral, cardioactive, antidiabetic, and antiplasmodial activities [39, 40]. Among these species, *Salvia miltiorrhiza* (Chinese sage) with a rhizome possessing medicinal powers. Different molecules of *S. miltiorrhiza* (or Danshen in Chinese) are widely recommended in modern and traditional medicine for the treatment of various disorders, for example, blood-circulation diseases. The red pigment of its roots is mainly composed of numerous diterpenoid tanshinones, such as tanshinone IIA, major bioactive molecules of the *Salvia* species offering numerous pharmacological activities notably antineoplastic effects [41]. Tanshinone IIA induces apoptosis and inhibits proliferation, migration, and invasion of a number of neoplastic cell types belonging to hepatoma, osteosarcoma, leukemia, prostate cancer, gastric cancer, breast cancer, colon cancer, and glioma. Besides, the prevention and treatment of cerebral infarction by *S. miltiorrhiza* Bunge involves several action modes, including anti-atherosclerosis, anti-hypertensive, anti-platelet aggregation, anti-inflammatory, and anti-oxidative effects. Plant-derived natural products have provided many bioactive terpenoids, some of which have led to important pharmaceuticals available on the market or promoted in clinical trials by the emerging "biotech" companies [144].

13 Conclusion

Plants hold a strong potential for pharmaceutical and industrial applications, resulting from the great richness in secondary metabolites, notably in terpenes/terpenoids which represent the largest group of natural products. Potentially terpenoids/terpenes remain to be identified in numerous plant species, known or still unexplored. Plant transformation by *Agrobacterium rhizogenes* leads to the formation of hairy roots, that is, of well-differentiated roots presenting at once a high genetic stability, an unlimited propagation, and spontaneous regeneration in media devoid of growth regulators. Hairy roots and *A. rhizogenes*–derived plants are potentially promising bioreactors which can be used for producing biomolecules of high added value. Ri-plants make possible the production of secondary metabolites exclusively accumulated in the aerial parts of the plant. In the *Catharanthus roseus* leaves, the final step of terpenoid indole alkaloid (TIA) biosynthesis leads to vinblastine and vincristine (bisindole alkaloids), whereas their two precursors are produced in the roots. Moreover, Ri-regenerants can allow the creation of new varieties in horticulture/floriculture. Bacterial Ri-T-DNA, responsible for the genetic transformation, is a true secretion system based on the genetic horizontal transfer of foreign genes into the host plant genome. Thereby, hairy

roots open large avenues for engineering terpenoid metabolism pathways or revealing novel genes by recent approaches as T-DNA activation tagging or MeJA-inducible system following transcriptome analyses. Nevertheless, due to the great complexity of these pathways, numerous terpenoid biosynthesis-related genes are always to be isolated and identified in plants. Overall, hairy roots produce terpenoids/terpenes at levels comparable to or higher than those found in the wild roots. The cultures of hairy roots assure serious safety advantages compared to those of plants cultured in the open field. Biotic or abiotic elicitors have been used to enhance terpenoid contents. Most often methyl jasmonate was found to be efficient to improve the secondary metabolite synthesis. Nevertheless, the major challenge for commercial exploitation of the hairy roots is scaling up at industrial level. Based on the cell suspension culture biomass concept, specific reactors have been altered to overcome the hurdles limiting the hairy root culture on a large scale. Nowadays medicinal plants provide therapeutically active terpenoids for treating more and less severe pathologies. A vast pool of higher plants biosynthesizing secondary products remain again to be identified. Certain biomolecules of known plants have not been screened for therapeutic properties. In the near future, drug discovery from plant species is a crucial investigation to treat, notably, severe pathologies.

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