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Using Hairy Roots for Production of Valuable Plant Secondary Metabolites

Li Tian

Abstract Plants synthesize a wide variety of natural products, which are traditionally termed secondary metabolites and, more recently, coined specialized metabolites. While these chemical compounds are employed by plants for interactions with their environment, humans have long since explored and exploited plant secondary metabolites for medicinal and practical uses. Due to the tissue-specific and low-abundance accumulation of these metabolites, alternative means of production in systems other than intact plants are sought after. To this end, hairy root culture presents an excellent platform for producing valuable secondary metabolites. This chapter will focus on several major groups of secondary metabolites that are manufactured by hairy roots established from different plant species. Additionally, the methods for preservations of hairy roots will also be reviewed.

Keywords Hairy roots · Secondary metabolites · Preservation · Terpenoid · Phenolic · Alkaloid

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1 Introduction

Roots play many important roles in plants—they anchor plants to the ground, take up minerals and water from the soil, store nutrients for perennial plants, and produce a diverse array of chemicals for symbiotic interactions or defensive with other plants or microbes in the rhizosphere. These plant-produced chemicals have traditionally been referred to as secondary metabolites and more recently tagged as specialized metabolites. Many secondary metabolites not only protect plants from pathogens, insects, and environmental stresses, but also are invaluable for animal and human health. However, plant cultivation is often time-consuming and metabolite extraction from plant roots is destructive to plant growth. To this end, hairy roots induced from different plant tissues generally grow fast, are genetically stable, and often, but not always, simulate the biochemical profiles of plant roots, which makes hairy roots an attractive system for producing valuable secondary metabolites.

Agrobacterium rhizogenes can infect wounded plants and play a fundamental role in hairy root induction. As tallied in 1991, 463 plant species of 109 families had already been transformed by *A. rhizogenes* [129]. To date, hairy roots established from at least 155 plant species of 41 families, by *A. rhizogenes* strains of diverse host ranges and virulence levels, reportedly produce secondary metabolites (Table 1; Fig. 1). However, a systemic comparative analysis of *A. rhizogenes* strains on effective induction of hairy root growth and secondary metabolite yield has not been performed. It should be noted that, though most of the hairy root cultures resulted from *A. rhizogenes* transformation, high-quality hairy roots were also obtained for *Atropa belladonna*, *Catharanthus roseus*, *Kalanchoe diagremontiana,* and *Nicotiana tabacum* plants infected by *A. tumefaciens* harboring *rolABC* genes [18, 60, 158].

There have been many excellent reviews in the literature on a wide variety of hairy root applications, such as metabolite or therapeutic protein production, biotransformation of core skeletons of secondary metabolites into novel compounds, gene discovery and metabolic pathway characterization, and phytoremediation [13, 20, 39, 45, 47, 54, 52, 53, 124, 150] (and many other reviews). This chapter focuses specifically on production of different classes of secondary metabolites in hairy roots, their bioactivities, and preservation of hairy roots. Modeling and monitoring of hairy root production of valuable compounds will be reviewed and discussed in Chap. “Ramified challenges: Monitoring and Modeling of Hairy Root Growth in Bioprocesses – A review” of this book.

Table 1 Secondary metabolites produced in hairy root cultures

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Amaranthaceae					
Beta vulgaris L.	Red beet	A4	Betalains	Antioxidant, antimicrobial, antifungal, anticancer, anti-inflammatory, neuroprotective, radioprotective	[169]
Apiaceae (*Umbelliferae*)					
Ammi majus L.	Bishops weed	A4, LBA 9402	Coumarins, furanocoumarins	Anti-inflammatory, treatment of leucoderma, psoriasis, and vitiligo	[88]
Ammi visnaga Lam.	Toothpick weed	A4, ATCC 15834, R1601	Furanochromone (visnagin)	Vasodilation, diuretic	[89]
Anethum graveolens L.	Dill	LBA 9402	Essential oils (apiole, falcarinol, dill apiole)	Antiflatulent, antiamenorrhea	[140]
Angelica gigas Nakai	Korean angelica	R1000	Pyranocoumarins (decursin, decursinol angelate)	Neuroprotective, anticancer, antiandrogen-receptor signaling	[194]
Bupleurum falcatum L.	Thorow-wax	A4	Triterpene saponins (saikosaponins)	Anti-inflammatory, antipyretic, antitussive	[2]
Centella asiatica (L.) Urban	Asiatic pennywort	R1000	Triterpenoids (asiaticoside)	Anti-inflammatory, antipyretic	[75]
Daucus carota L.	Carrot	LBA 9402	Phenolic acid (*p*-hydroxybenzoic acid)	Chemical intermediates of synthetic drugs	[156]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Levisticum officinale W.D.J. Koch	Lovage	A4	Essential oils (falcarinol, (Z)-ligustilide, (Z)-3-butylidenephthalide, *trans*-β-farnesene, β-phellandrene, n-octanal, γ-elemene, n-heptanal)	Spasmolytic, diuretic, carminative	[141]
Perezia cuernavacana B.L.Rob. and Greenm.		AR12	Sesquiterpene quinones (perezone)	Natural pigment, laxative, antimicrobial	[5]
Pimpinella anisum L.	Anise	A4	Essential oils (*trans*-epoxypseudoisoeugenyl 2-methylbutyrate, geijerene, pregeijerene, zingiberene, β-bisabolene)	Flavoring and scent agents	[142]
Apocynaceae					
Catharanthus roseous (L.) G. Don	Madagascar periwinkle	ATCC 15834	Indole alkaloids	Anticancer	[149]
Catharanthus trichophyllus (Baker) Pichon		ATCC 15834	Indole alkaloids	Anticancer	[30]
Rauvolfia micrantha Hook. f.	Small-flowered snakeroot	ATCC 15834	Indole alkaloids (ajmalicine, ajmaline)	Treatment of nervous system disorders	[160]
Rauvolfia serpentina (L.) Benth. ex Kurz	Indian snakeroot	ATCC 15834	Indole alkaloids (ajmaline, serpentine)	Treatment of nervous system disorders	[14]
Vinca minor L.	Lesser periwinkle	DC-AR2 (derived from MAFF301724)	Indole alkaloids (vincamine)	Cerebral vasodilator	[167]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Araliaceae					
Panax ginseng C.A. Meyer	Asian ginseng	A4	Triterpene saponins (ginsenosides)	Immunomodulatory, adaptogenic, antiaging	[197]
Panax quinquefolium L.	American ginseng	A4, LBA 9402, ATCC 11325, ATCC 15834	Triterpene saponins (ginsenosides)	Immunomodulatory, adaptogenic, antiaging	[105]
Panax hybrid (*P. ginseng* X *P. quinqifolium*)		ATCC 15834	Triterpene saponins (ginsenosides)	Immunomodulatory, adaptogenic, antiaging	[188]
Asclepiadaceae					
Gymnema sylvestre R.Br.	Gur-mar	KCTC 2703	Triterpene saponins (gymnemic acid)	Antidiabetic, antimicrobial, diuretic, stomachic, anti-hypercholesterolemic, hepatoprotective, antisaccharine	[116]
Asteraceae (*Compositae*)					
Ambrosia artemisiifolia L.	Common ragweed	ATCC 15834, ATCC 11325, TR105	Polyacetylenes, thiophenes	Cytotoxic, antiviral, biocidal	[40]
Artemisia absinthium L.	Wormwood	AR1855, LBA 9402	Essential oils	Antihelminthic, insecticidal, stomachic	[118]
Artemisia annua L.	Sweet wormwood	ATCC 15834	Sesquiterpenes (artemisinin)	Antimalarial	[189]
Bidens sps. [B. sulphurea (Cav.) Sch. Bip, B. pilosa L., B. ferulaefolia (Jacq.) DC]		ATCC 15834, ATCC 11325, TR105	Polyacetylenes, thiophenes	Cytotoxic, antiviral, biocidal	[40]

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Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Carthamus tinctorius L. (var. Biggs, var. N-10)	Safflower	ATCC 15834, ATCC 11325, TR105	Polyacetylenes, thiophenes	Cytotoxic, antiviral, biocidal	[40]
Chaenactis douglasii (Hook.) Hook. and Arn.	Douglas' dustymaiden	TR7	Polyines (thiarubrines)	Antifungal	[29]
Cichorium intybus L.	Common chicory	LBA 9402	Sesquiterpene lactones	Anti-inflammatory, hepatoprotective	[102]
Coreopsis tinctoria Nutt.	Golden tickseed	ATCC 15834	Phenolics	Antidiabetic	Thron et al. [172]
Echinacea sps. [*E. angustifolia* DC; *E. pallida* (Nutt.) Nutt.; *E. purpurea* (L.) Moench]	Purple coneflower	A4	Alkamides	Anti-inflammatory, immune-stimulatory	[133]
Lactuca virosa L.	Wild lettuce	LBA 9402	Sesquiterpene lactones	Analgesic, antitussive, sedative	[82]
Leontopodium alpinum Cass.	Edelweiss	LBA 9402	Anthocyanins, hydroxycinnamic acid esters, essential oils	Chemoprotective against toxins	[61]
Rudbeckia hirta L. var. *plucherrima*	Black-eyed susan	ATCC 15834, ATCC 11325, TR105	Polyacetylenes, thiophenes	Cytotoxic, antiviral, biocidal	[40]
Saussurea involucrate Karel. and Kir.	Snow lotus	R1601, R1000, LBA 9402	Syringin	Anti-inflammatory, anti-hypersensitive	[42]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Saussurea medusa Maxim.	Snow lotus	R1601	Flavones (jaceosidin)	Antitumor, anti-inflammatory	[202]
Serratula tinctoria L.	Saw-wort	A4	Triterpenoids (phytoecdysteroids)	Potential use in chemotherapy	[31]
Silybum marianum (L.) Gaertn.	Milk thistle	ATCC 15834	Flavolignans (isosilybin, silychristin, silydianin)	Antihepatotoxic	[3]
Solidago altissima L.	Late goldenrod	A4	Polyacetylenes (*cis*-dehydromatricaria ester)	Cytotoxic, antiviral, biocidal	[65]
Solidago nemoralis Aiton	Gray goldenrod	R1000	Complex alkaloid metabolites	Targeting the insect and human nicotinic receptor for acetylcholine	[55]
Tanacetum parthenium (L.) Sch. Bip. [syn. *Chrysanthemum parthenium* (L.) Bernh.]	Feverfew	LBA 9402	Coumarins (isofraxidin, isofraxidin drimenyl ether)	Antitumor	[81]
Tagetes erecta L.	Mexican marigold	ATCC 15834, ATCC 11325, TR105	Polyacetylenes, thiophenes	Cytotoxic, antiviral, biocidal	[40]
Tagetes patula L.	French marigold	ATCC 43057	Thiophenes (α-terthienyl)	Cytotoxic, antiviral, biocidal	[92]
Bignoniaceae					
Paulownia tomentosa Steud.	Empress tree	LBA 9402	Phenolic glycosides (verbascoside)	Antiviral, antibacterial, antiproliferative, analgesic, anti-hypertensive	[193]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Bixaceae					
Bixa orellana L.	Achiote	Not described	Bixin, tocopherol, ellagic acid, etc.	Antimalarial	[200]
Boraginaceae					
Echium acanthocarpum Svent.	Gomeranneidonkieli	LBA 1334	Polyunsaturated fatty acids	Nutrients, membrane structural components	[25]
Lithospermum erythrorhizon Sieb. et Zucc.	Purple gromwell	ATCC 15834	Naphthoquinones (shikonin)	Anti-inflammatory, antitumor, antiviral	[152]
Brassicaceae					
Arabis caucasica L.	Mountain rock cress	LBA 9402	Glucosinolates (gluconasturtiin, glucoiberverin)	Anticancer, antifungal, antibacterial, antinematode, anti-insect	[190]
Armoracia rusticana P. Gaert., B. Meyer et Scherb.	Horseradish	Not specified	Diterpenoids (fucicoccin)	Unknown	[8]
Barbarea verna (Mill.) Asch.	Land cress	LBA 9402	Glucosinolates (gluconasturtiin, glucoiberverin)	Anticancer, antifungal, antibacterial, antinematode, anti-insect	[190]
Crambe abyssinica R.E. Fr.	Abyssinian mustard	A4, ATCC 15834	Lipids, fatty acids	Nutrients	[56]
Nasturtium officinale (L.) R.Br.	Watercress	LBA 9402	Glucosinolates (gluconasturtiin, glucotropaeolin)	Anticancer, antifungal, antibacterial, antinematode, anti-insect	[190]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Tropaeolum majus L.	Garden nasturtium	LBA 9402	Glucosinolates (glucotropaeolin)	Anticancer, antifungal, antibacterial, antinematode, anti-insect	[191]
Campanulaceae					
Campanula glomerata L. var. *dahurica* Fish.	Bellflower	MAFF 03-01724	Polyacetylenes (lobetyol, lobetyolin, lobetyolinin) Phenolics (anthocyanins, rutin, coniferin)	Cytotoxic, antiviral, biocidal	[166]
Campanula medium L.	Canterbury-bells	A13	Polyacetylenes	Cytotoxic, antiviral, biocidal	[163]
Lobelia cardinalis L.	Cardinal flower	ATCC 15834, MAFF 03-01724	Polyacetylene glucosides (lobetyolin, lobetyolinin)	Cytotoxic, antiviral, biocidal	[195]
Lobelia chinensis Lour.	Chinese lobelia	ATCC 15834	Polyacetylenes	Cytotoxic, antiviral, biocidal	[164]
Lobelia inflata L.	Indian tobacco	ATCC 15834	Piperidine alkaloids (lobeline)	Respiratory stimulant	[196]
Platycodon grandiflorum (Jacq.) A.DC.	Broad bell	ATCC 15834	Polyacetylenes	Cytotoxic, antiviral, biocidal	[71]
Trachelium caeruleum L.	Blue throatwort	ATCC 15834	Polyacetylenes (lobetyol, lobetyolin, and lobetyolinin)	Cytotoxic, antiviral, biocidal	[114]
Caprifoliaceae					
Centranthus ruber (L.) DC.	Red valerian	R1601	Iridoids (valepotriate)	Sedative, spasmolytic	[50]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Cannabaceae					
Cannabis sativa L.	Hemp	Not specified	Choline, atropine	Parasympatolytic, anticholinergic, spasmolytic, antiemetic, precursor of membrane lipids and signaling molecules	[184]
Convolvulaceae					
Calystegia sepium (L.) R.Br.	Hedge bindweed	A4, 8196	Tropane alkaloids	Anticholinergic	[72]
Cornaceae (*Nyssaceae*)					
Camptotheca acuminate Decne.	Happy tree	ATCC 15834, R1000	Quinoline alkaloids (camptothecin)	Antitumor, anticancer, antiviral	[99]
Cucurbitaceae					
Gynostemma pentaphyllum (Thunb.) Makino	Southern ginseng; Jiaogulan	ATCC 15834	Triterpene saponins (gypenosides)	Antitumor, cholesterol lowering, immunopotentiating, antioxidant, hypoglycemic, antidiabetic	[26]
Trichosanthes kirilowii Maxim var *japonicum*	Chinese cucumber	R1601	Triterpenoids (bryonolic acid, chondrillasterol)	Growth inhibition of B-16 melanoma cells	[165]
Euphorbiaceae					
Phyllanthus amarus Schum. and Thonn.	Carry me seed	ATCC 15834	Crude hairy extracts were used for bioactivity assays and were not analyzed for chemical constituents	Anticancer, antiviral	[1, 17]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Fabaceae (*Leguminosae*)					
Abrus precatorius L.	Indian licorice	MTCC 532, MTCCC 2364, NCIM 5140	Triterpene saponins (glycyrrhizin)	Emetic, diuretic, anthelmintic, alexeteric	[73]
Arachis hypogaea L.	Peanut	ATCC 15834, R1000, EHA105	Resveratrol, pterostilbene, and derivatives	Cardioprotective, cancer chemopreventive, antiatherosclerosis, antiaging	[108]
Astragalus mongholicus Bunge	Huangqi	LBA 9402, ATCC 15834, R1601, TR105	Triterpene saponins	Immunostimulant	[66]
Cassia sps. (*C. obtusifolia* L.; C. *occidentalis* L.; C. *torosa* Cav.)	Chinese senna	ATCC 15834, A4	Anthraquinones, polypeptide pigments	Antioxidant, laxative, treatment of fungal skin diseases	[87]
Clitoria ternatea L.	Butterfly pea	A4T, 8196	Triterpenoids (taraxerol)	Laxative, diuretic, anthelmintic, anti-inflammatory	[161]
Glycyrrhiza glabra L.	Licorice	ATCC 15834	Flavonoids, isoprenylated flavonoids	Antioxidant, antimicrobial	[6]
Glycyrrhiza uralensis Fisch.	Chinese licorice	A4	Flavonoids	Antimutagenic, antiulcer, antitumor, antimicrobial protective action against hepatotoxicity	[201]
Lotus corniculatus L.	Bird's-foot trefoil	LBA 9402	Condensed tannins (i.e., proanthocyanidins)	Anthelmintic	[23]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Lupinus mutabilis Sweet	Andean lupin	R1601	Isoflavones (genistein, 2′-hydroxygenistein, wighteone)	Antioxidant, antiproliferative	[9]
Psoralea corylifolia Linn.	Indian bread root	LBA 9402	Isoflavones (daidzein, genistein)	Anticancer, anti-inflammatory, antiallergic, antioxidant, antiangiogenic, inhibition of DNA polymerase, topoisomerase II activities	[153]
Psoralea sps. (*P. canescens* Michx.; *P. cinerea* Lindl.; *P. lachnostachys* F. Muell.; *P. leucantha* F. Muell.; *P. plumosa* F. Muell; P. *pustulata* F. Muell.; *P. obtusifolia* D.C.)		LBA 9402	Phenolics (daidzein, genistein, coumestrol)	Anticancer, anti-inflammatory, antiallergic, antioxidant, antiangiogenic, inhibition of DNA polymerase, topoisomerase II activities	[19]
Pueraria candollei Wall. ex Benth.	Thai kudzu	ATCC 15834	Isoflavonoids (puerarin, daidzin, genistin, daidzein, genistein)	Anticancer, anti-inflammatory, antiallergic, antioxidant, antiangiogenic, inhibition of DNA polymerase, topoisomerase II activities	[177]
Pueraria lobata (Willd.) Ohwi	Kudzu	KCTC 2744	Isoflavones (puerarin, daidzin)	Hypothermic, spasmolytic, hypotensive, antiarrhythmic	[76]
Pueraria phaseoloides (Roxb.) Benth	Tropical kudzu	ATCC 15834	Isoflavones (puerarin)	Hypothermic, spasmolytic, hypotensive, antiarrhythmic	[151]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Swainsona galegifolia (Andrews) R.Br.	Darling pea	LBA 9402	Indolizidine alkaloid (swainsonine)	α-mannosidase inhibitor; antimetastatic, antiproliferative, immunomodulatory	[113]
Trigonella foenum-graecum L.	Fenugreek	A4	Steroidal sapogenin (diosgenin)	Precursor for synthesis of steroidal hormones	[110]
Gentianaceae					
Gentiana macrophylla Pall.	Large leaf gentian	R1000	Secoiridoid glycosides (gentiopicroside)	Anti-inflammatory, analgesic, antirheumatic, antipyretic, diuretic, hypoglycemic	[173]
Gentiana punctate L.	Spotted gentian	A4M70GUS	Secoiridoid glycosides (gentiopicrin)	Anti-inflammatory, analgesic, antirheumatic, antipyretic, diuretic, hypoglycemic	[109]
Gentiana scabra Bunge	Scabrous gentian	ATCC 15834	Iridoids and secoiridoids (loganic acid, gentiopicrosides, sertiamarin)	Anti-inflammatory, analgesic, antirheumatic, antipyretic, diuretic, hypoglycemic	[63]
Swertia japonica (Roem. and Schult.) Makino	Semburi	ATCC 15834	Xanthones (bellidifolin, methylbellidifolin, swertianolin, 8-*O*-primeverosylbellidifolin), iridoidal glycosides (amarogentin, amaroswerin)	Bitter stomachic, treatment of hepatitis and digestive disorders	[68]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Geraniaceae					
Geranium thunbergii Siebold ex Lindl. and Paxt.	Cranesbill	A4	Hydrolyzable tannins	Antiviral, antimicrobial, antitumor	[67]
Ginkgoaceae					
Gingko biloba L.	Ginkgo	A4	Diterpenes (ginkgolide)	Treatment of aging-related disorders	[7]
Hypericaceae					
Hypericum perforatum L.	St. John's wort	A4	Flavonoids, xanthones	Antioxidant, antimicrobial, cytotoxic, hepatoprotective	[176]
Lamiaceae (*Labiatae*)					
Agastache rugosa Kuntze.	Korean mint	R1000	Phenolics (rosmarinic acid)	Antitumor, antimicrobial, anti-inflammatory, antiviral, antimutagenic	[95]
Ajuga reptans L. var. *atropurpurea*	Bugle	MAFF 03-01724	Phytoecdysteroids (20-hydroxyecdysone, norcyasterone B, cyasterone, isocyasterone)	Potential use in chemotherapy	[106]
Coleus sps. (*C. blumei* Benth.; *C. forskohlii* Briq.)		MAFF 03-01724	Diterpenoids (forskolin)	Positive inotropic, positive chronotropic, hypotensive, inhibition of thrombocyte aggregation, reduction of intraocular pressure	[143]
Gmelina arborea Roxb. (formerly Verbenaceae)	Beechwood	ATCC 15834	Phenylpropanoid glycoside (verbascoside)	Anti-inflammatory, wound healing, inhibition of platelet aggregation	[33]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Ocimum basilicum L.	Sweet basil	ATCC 15834	Phenolics (rosmarinic acid)	Antioxidant	[10]
Salvia austriaca Jacq.	Austrian sage	A4	Diterpenoids (royleanone, 15-deoxyfuerstione, taxodione)	Antioxidant, anti-inflammatory	[90]
Salvia broussonetii Benth	Stiff Canary Island sage	ATCC 15834	Diterpenoids (brussonol, iguestol, 7-oxodehydroabietane, 11-hydroxy-12-methoxyabietatriene, taxodione, inuroyleanol, ferruginol, deoxocarnosol 12-methyl ether, cryptojaponol, pisiferal, sugiol, isomanool, 14-deoxycoleon U, 6R-hydroxydemethylcryptojaponol, demethylsalvicanol, demethylcryptojaponol)	Antioxidant, anti-inflammatory	[41]
Salvia cinnabarina M.Martens and Galeotti	Cinnabar sage	ATCC 15834	Diterpenoids	Antimutagenic	[148]
Salvia involucrata Cav.	Roseleaf sage	R1601	Flavonoids (apigenin)	Anticancer	[96]
Salvia miltiorrhiza Bunge	Red sage or Chinese sage	LBA 9402, ATCC 15834, TR 105, R 1601, A 4 1027	Diterpenoid naphthoquinones (tanshinone), phenolic acids	Antioxidant, promotion of blood circulation	[204]
Salvia officinalis L.	Garden sage or Common sage	ATCC 15834, A4	Phenolics (rosmarinic acid)	Neuroprotective, antioxidant, anti-inflammatory, immunomodulatory, photoprotection, melanogenic	[48]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Salvia sclarea L.	Clary sage	LBA 9402	Diterpenes	Antioxidant, antibacterial, anti-inflammatory	[91]
Scutellaria baicalensis Georgi.	Baikal skullcap	ATCC 15834	Flavonoids, phenylethanoids (baicalin, wogonin)	Antiviral, antitumor	[205]
Linaceae					
Linum flavum L.	Golden flax	LBA 9402, TR105	Lignans	Anticancer	[97]
Lythraceae					
Lawsonia inermis L.	Henna	NCIB 8196	Naphthoquinone (lawsone)	Coloring agent, UV sunscreen	[11]
Punica granatum L. (formerly Punicaceae)	Pomegranate	MSU440	Hydrolyzable tannins, other phenolics	Anticancer	[123]
Malvaceae					
Gossypium sps. (*G. barbadense* L.; *G. hirsutum* L.)	Cotton	ATCC 15834	Disesquiterpene (gossypol)	Anticancer, antiviral, antiparasitic, antifungal, antimicrobial	[175]
Meliaceae					
Azadirachta indica A. Juss.	Neem	LBA 920	Triterpenes (azadirachtin)	Biopesticide	[159]
Papaveraceae					
Papaver bracteatum Lindl.	Persian poppy	ATCC 15834	Benzylisoquinoline alkaloids	Antitumor, antimicrobial, sedative	[134]
Papaver somniferum L. var. *album*	Opium poppy	ATCC 15834, LBA 9402	Benzylisoquinoline alkaloids	Antitumor, antimicrobial, sedative	[38]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Pedaliaceae					
Harpagophytum Procumbens (Burch.) DC. ex Meisn.	Devil's claw	ATCC 15834, A4	Iridoid glycosides	Treatment of degenerative rheumatoid arthritis, osteoarthritis, tendonitis, kidney inflammation and heart disease	[51]
Sesamum indicum L.	Sesame	ATCC 15834	Naphthoquinone (2-isopropenylnaphthazarin-2,3-epoxide), anthraquinones [2(4-methyl-1,3pentadienyl) anthraquinone and 2(4-methyl-3pentenyl) anthraquinone]	Antimicrobial	[121]
Plantaginaceae					
Bacopa monnieri (L.) Pennell	Indian pennywort	A4, LBA9402	Triterpene saponins	Anti-inflammatory, analgesic, antipyretic, anticancer	[101]
Digitalis lanata Ehrh.	Woolly foxglove	A4, ATCC 15834, TR105	Anthraquinones, flavonoids	Positive inotropic	[130]
Digitalis purpurea L.	Foxglove	ATCC 15834	Cardiac glycosides	Treatment for heart failure	[137]
Picrorhiza kurroa Royle ex Benth.	Kutki	LBA 9402, A4	Iridoid glycosides (kutkoside, picroside)	Hepatoprotective, anticholestatic, antiulcerogenic, antiasthematic, antidiabetic, anti-inflammatory, immunoregulatory	[182]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Plumbaginaceae					
Plumbago indica L.	Scarlet leadwort	ATCC 15834	Naphthoquinones (plumbagin)	Antimicrobial, abortifacient, vesicant diuretic	[44]
Plumbago zeylanica L.	Ceylon leadwort	A4	Naphthoquinones (plumbagin)	Antimicrobial, abortifacient, vesicant diuretic	[181]
Polygonaceae					
Fagopyrum esculentum Moench.	Buckwheat	ATCC 15834	Flavonoids (rutin)	Antioxidant, anti-inflammatory, anticancer, antithrombotic, cytoprotective, vasoprotective	[79]
Fagopyrum tataricum (L.) Gaertn	Tartary buckwheat	Ri1601	Flavonoids (Rutin, quercetin)	Antioxidant, hypocholesterolemic, antidiabetic, antimicrobial, antitumor	[203]
Polygonum multiflorum Thunb.	Chinese knotweed	KCTC2703	Hydroxybenzoic acids, hydroxycinnamic acids, flavonols	Antitumor, anti-HIV, antibacterial, hemostatic, spasmolytic, analgesics, antioxidant	[171]
Polygonum tinctorium Lour.	Chinese indigo	A4	Indigo	Treatment of leukemia	[199]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Ranunculaceae					
Aconitum heterophyllum Wall.	Atis	LBA 9402, LBA 9360, A4	Diterpene alkaloids (heteratisine, atisine, hetidine)	Bitter tonic; treatment of diarrhea, dysentery, cough, dyspepsia and chronic enteritis	[46]
Rosaceae					
Fragaria x *ananassa* cv. Reikou	Strawberry	ATCC 15834	Polyphenols (proanthocyanidins, flavonoids, hydrolyzable tannin)	Antioxidant, anticancer	[112]
Rubiaceae					
Cinchona ledgeriana Moens ex Trimen	Quinine bark	LBA 9402	Quinoline and indole alkaloids	Antiarrhythmic	[57]
Ophiorrhiza pumila L.	Dwarf Ophiorrhiza	ATCC 15834	Quinoline alkaloids (camptothecin)	Antitumor, antineoplastic	[136]
Rubia akane Nakai	Asian madder	R1000	Anthraquinones (alizarin, purpurin)	Natural dyes; anticancer, antimalarial, antimicrobial, antifungal, antioxidant	[126]
Rubia peregrina L.	Wild madder	LBA 9402	Anthraquinones (alizarin)	Natural dyes; anticancer, antimalarial, antimicrobial, antifungal, antioxidant	[98]
Rubia tinctorum L.	Common madder	ATCC 15834	Anthraquinones	Natural dyes; anticancer, antimalarial, antimicrobial, antifungal, antioxidant	[144]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Rutaceae					
Fagara zanthoxyloides Lam. [syn. Zanthoxylum zanthoxyloides (Lam.) Zepernick and Timle]	Senegal prickly-ash	LBA 9402	Benzophenanthridine alkaloids	Antileukemic, inhibitor of DNA topoisomerases I and II, and protein kinase C, sedative and hypothermic	[37]
Ruta graveolens L.	Common rue	LBA 9402, A4	Coumarins, furanocoumarins, alkaloids	Antimicrobial, treatment of skin diseases	[155]
Solanaceae					
Anisodus luridus Link and Otto	Himalayan scopolia	C58C1	Tropane alkaloids	Anticholinergic	[132]
Atropa belladonna L.	Deadly nightshade	ATCC 15834	Tropane alkaloids	Anticholinergic	[18]
Atropa caucasica Kreyer	Caucasian belladonna	A4	Tropane alkaloids	Anticholinergic	[86]
Brugmansia candida Pers.	Angel's trumpet	LBA 9402	Tropane alkaloids (scopolamine, hyoscyamine) polyamines (cadaverine, putrescine, spermidine, spermine)	Anticholinergic	[22]
Datura candida (Pers.) Safford	Hybrid variety between two cultivars of the same species	ATCC 15834	Tropane alkaloids	Anticholinergic	[28]
Datura candida x Datura aurea	Hybrid variety	Not described	Tropane alkaloids (hyoscyamine, scopolamine)	Anticholinergic	[120]
Datura stramonium L.	Jimson weed	TR105	Tropane alkaloids (hyoscyamine)	Anticholinergic	[162]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Datura sps. (*D. chlorantha* Hook; *D. fastuosa* L. var. *violacea*; *D. ferox* L.; *D. innoxia* Mill.; *D. metel* L.; *D. meteloides* DC. ex Dun.; *D. quercifolia* H.B.K.; *D. rosei* Saff.; *D. sanguinea* Ruiz. et Pav.; *D. stramonium* L.; *D. stramonium* var. *inermis* Timm.; *D. stramonium* var. *stramonium* Dun.; *D. stramonium* var. *tatula* Torr. *Duboisia*-hybrid)		A4	Tropane alkaloids	Anticholinergic	[86]
Duboisia leichhardtii F. Muell.	Corkwood	ATCC 15834, A4	Tropane alkaloids (scopolamine, hyoscyamine)	Anticholinergic	[104]
Duboisia myoporoides R.Br.	Corkwood	HRI	Tropane alkaloids	Anticholinergic	[32]
Hyoscyamus albus L.	White henbane	MAFF 03-01724	Tropane alkaloids (7β-hydroxyhyoscyamine, littorine, scopolamine, 6β-hydroxyhyoscyamine, hyoscyamine)	Anticholinergic	[146]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Hyoscyamus niger L.	Black henbane	ATCC 15834	Tropane alkaloids	Anticholinergic	[69]
Hyoscyamus sps. (*H. albus* L.; *H.aureus* L.; *H. bohemicus* F.W. Schmidt; *H. muticus* L.; H. niger L.; *H. niger* var. *pallidus* Rchb.)		A4	Tropane alkaloids	Anticholinergic	[86]
Nicotiana sps. (*N. tabacum* L.; N. *rustica* L.; *N. occidentalis* subsp. *hesperis* (N.T.Burb.) P.Horton; *N. Africana* Merxm; *N. umbratica* N.T. Burb; *N. velutina* H.-M. Wheeler; *N. cavicola* N.T. Burb.)		LBA 9402	Pyridine alkaloids (nornicotine, nicotine, anatabine, anabasine)	Insecticidal	[127]
Scopolia sps. (*S. carniolica* Jacq.; *S. straminifolia* Shrestha)		A4	Tropane alkaloids	Anticholinergic	[86]
Scopolia japonica Maxim.	Japanese belladonna	ATCC 15834	Tropane alkaloids (scopolamine, hyoscyamine)	Anticholinergic	[103]
Solanum aculeatissimum Jacq.	Indian nightshade	ATCC 15834	Steroidal saponins (aculeatiside A, aculeatiside B)	Hemolytic	[64]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Solanum aviculare Forst.	Poroporo	A4, ATCC 11325, ATCC 15834, ATCC 43057	Steroidal glycoalkaloids (solasodine)	Precursor of steroidal drugs and hormones	[84]
Solanum chrysotrichum Schltdl.	Devil's fig	Not described	Triterpene saponins	Antifungal	[24]
Solanum laciniatum Aiton	Kangaroo apple	ATCC 15834	Steroidal glycoalkaloids (solasodine)	Precursor of steroidal drugs and hormones	[122]
Withania somnifera (L.) Dunal	Indian ginseng	R1601	Steroidal lactones (withanolide A)	Anticancer	[115]
Taxaceae					
Taxus x media var. Hicksii Rehd.	Hicks yew	ATCC 15834, TR 105, LBA 9402	Diterpenoid taxanes (paclitaxel, 10-deacetybaccatin III)	Anticancer	[43]
Valerianaceae					
Valeriana officinalis L. var. sambucifolia Mikan	Valerian	R1601	Iridoids (valepotriates)	Sedative, spasmolytic	[49]
Valeriana wallichii DC	Indian valerian	A4, LBA 9402	Iridoids (valepotriates)	Sedative, spasmolytic	[12]

(continued)

Table 1 (continued)

Species	Common names	Agrobacterium strains	Major secondary metabolites produced	Biological activities	References
Verbenaceae					
Lippia dulcis Trevir. [*Phyla dulcis* (Trevir.) Moldenke]	Sweet Aztec herb	A4	Sesquiterpene (hernandulcin)	Natural sweetener	[147]
Vinceae					
Amsonia elliptica (Thunb. ex Murray) Roem. and Schult.	Japanese bluestar	A4	Indole alkaloids	Anticancer	[145]

When different varieties of the same species were reported by multiple groups or with different stimulation methods, only a representative report is listed in the references. Common names of the plant species are indicated when available

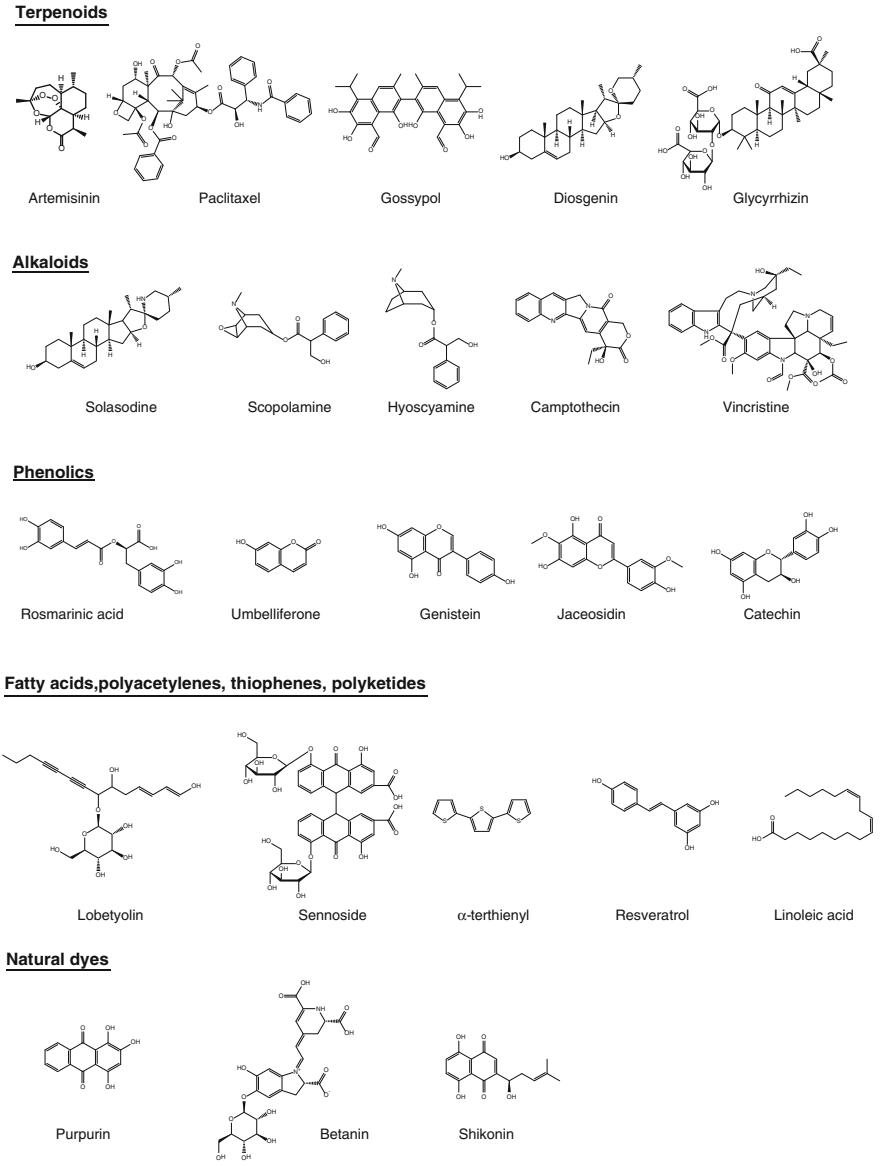

Fig. 1 Chemical structures of selected plant secondary metabolites produced in hairy root cultures

2 Production of Valuable Secondary Metabolites

2.1 Terpenoid Production in Hairy Roots

Terpenoids (also known as isoprenoids) are synthesized from the dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) precursors, and constitute the largest group of secondary metabolites in plants. Based on the number of C_5 isoprene units in their structures, terpenoids can be divided into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), sesquarterpenes (C_{35}), and tetraterpenes (C_{40}). Polymers of isoprenes are also found in plants, such as natural rubber. In addition, terpenoids can conjugate with molecular structures derived from other biochemical pathways to form additional groups of secondary metabolites; for instance, isoprene units are transferred to phenolic compounds to obtain prenylated phenolics.

Iridoids are monoterpenes that arise from geraniol through cyclization reactions; cleavage of iridoids leads to the formation of secoiridoids. Iridoids and secoiridoids often accumulate in plants in the glycosylated forms. The root extracts of the Gentianaceae family plants have been used for treating many diseases and disorders due to the bioactivities (e.g., hypoglycemic, anti-inflammatory) of secoiridoid glycosides and the active principles of the root tissue [168]. Explants of several Gentiana species, including *Gentiana macrophylla*, *Gentiana punctate*, *Gentiana scabra*, were transformed with *A. rhizogenes,* and in general, the hairy roots grew at a faster pace than the non-transformed control roots [63, 109, 173]. The increase in hairy root biomass also correlated with an average of 2.5-fold higher accumulation of the secoiridoid gentiopicroside in hairy roots than the non-transformed roots [63, 173]. A similar elevated production of iridoids glycosides was also observed for a *Picrorhiza kurroa* hairy root clone, which manufactured 4-fold higher kutkoside and picroside in liquid culture than roots of 3-year-old plants [182]. Besides glycosylation, (seco)iridoids can also conjugate with alkaloids to form (seco)iridoid alkaloids, which are classified as terpene indole alkaloids (TIAs). The TIA valepotriates are valued for their sedative and spasmolytic activities and are mainly found in roots of plants that produce these compounds [36, 183]. It was shown that *A. rhizogenes* transformed *Centranthus ruber*, *Valeriana officinalis*, and *V. wallichii* hairy roots grew rapidly and produced significantly more concentrated valepotriates than the non-transformed roots [12, 49, 50]. In addition, valepotriates were retained in the hairy roots, unlike the cell cultures of these plants that released them into the growth medium. Overall, the above-mentioned hairy root clones provide an alternative means for isolating the valuable TIAs other than the more destructive plant root extraction method, which facilitates the preservation of these endangered medicinal plant species.

Plant essential oils are often used for fragrances, flavoring, and toning. Bioactivities have also been found for various classes of essential oils [34]. While the parent *Anethum graveolens* (dill) plant contains monoterpenes as the major essential oil components in roots and fruits, its hairy roots produced essential oils that had the

signature of phenylpropanoid derivatives [140]. Additional examples can be drawn from *Pimpinella anisum* (anise), *Artemisia absinthium,* and *Levisticum officinale* (lovage) where the hairy roots exhibited different essential oil profiles than that of the parent plants [118, 141, 142]. Although not suitable for synthesizing the characteristic essential oils from the parent plants, these hairy roots could still be employed, in the future, for producing the hairy root-type essential oils.

The tropical disease malaria is caused by parasitic infections. According to the World Health Organization (WHO), there were over 200 million cases of malarial illness reported worldwide and more than 600,000 people died from malaria in 2012 [192]. Artemisinin, a sesquiterpene derivative, is the only Food and Drug Administration (FDA)-approved antimalarial drug on the market, and an artemisinin-based combination therapy (ACT) is currently being used for treating malaria. Artemisinin has traditionally been extracted from leaves and flowers of the annual herb *Artemisia annua* where low concentrations of artemisinin are found. Total chemical synthesis of artemisinin, though possible, poses high costs to patients. Complementary to the whole-plant extraction, semi-chemical synthesis, and synthetic biology approaches, hairy root cultures of *A. annua* were established to provide year-round production of this valuable antimalarial metabolite [70, 131, 189]. Additionally, gas-phase and liquid bioreactors have been designed, implemented, and improved for large-scale, hairy root-based production of artemisinin. A 1-L mist (gas phase) bioreactor was modified with a flexible-wall growth chamber, which allowed a dense inoculum bed at the early stage of culture growth and resulted in more proliferative hairy root growth than shake flasks [157]. When the growing *A. annua* hairy roots were separated from the impeller of a 3-L stir tank liquid bioreactor, damage of the growing roots was avoided. A combination of this improved stir tank bioreactor design and application of the elicitor methyl jasmonate (MeJA) led to an enhanced yield of 10.33 mg L^{-1} artemisinin [128]. However, despite these technological advancements, to date, commercial production of artemisinin from hairy root cultures has not been realized.

Although the ACT has been effective in combating malaria, artemisinin-resistant plasmodium parasites were reported recently [35] and there is an immediate need for the discovery of new antimalarial drugs. The seed extracts of *Bixa orellana* are commonly used in food coloring and cosmetics. Ethnopharmacy records showed that several Bixa species were also among a few indigenous plants adopted for treating malaria by Amazonians [135]. In contrast to a previous report where in vitro antiplasmodial activity assays were negative for crude Bixa extracts [135], a recent study indicated that crude extracts and purified compounds (e.g., stigmasterol) from Bixa hairy roots exhibited EC_{50} values on a micromolar scale against two plasmodial strains [200]. The discrepancy in antiplasmodial activities reported in these two studies could be due to different metabolite profiles, chemical extraction methods, assay procedures, and parasite strains that were used. Further studies are necessary to verify the observations made by Zhai et al. [200] and identify the antiplasmodial compounds from the hairy root extracts. As documented by Kaur et al. [74], there are continuous efforts in the search for additional antimalarial drugs from natural sources; many of the lead compounds have already

been identified from hairy roots induced from various plant species (Table 1), and hairy roots will find great utility in the development of new antimalarial drugs.

The dimeric sesquiterpene compound gossypol is uniquely localized in the specialized glandular tissues of cotton plants. Gossypol could inhibit the growth of several types of cancer cells, while antiviral, antiamoebic, and antiprotozoan effects were also ascribed to this compound [186]. Hairy roots induced from *Gossypium hirsutum* and *Gossypium barbadense* were highly productive, with an average yield of 15 mg g^{-1} and a maximum yield of over 40 mg g^{-1} gossypol on a dry culture mass basis [175]. Gossypol-related compounds that contain additional functional groups were also present in these hairy root clones. Between 60 and 95 % of gossypol and derivatives were retained in the hairy roots, which allows for targeted extraction of these valuable compounds from hairy root tissues.

The renowned diterpene anticancer drug paclitaxel was directly extracted from the bark of pacific yew trees (*Taxus brevifolia*). The lengthy growth cycle of the trees, the destructive extraction procedure, and the low yield (0.01 %) of paclitaxel prompted the use of a cell culture system for paclitaxel production (0.6 % yield). Complementary to the cell culture-based approach, hairy roots of *T. brevifolia* were induced and could synthesize 69–210 μg g^{-1} paclitaxel (i.e., up to 0.02 % yield) upon elicitation by MeJA [43]. Though the hairy roots are twice as productive as the tree bark, there is still much less paclitaxel being made in hairy roots than in cell cultures. Nevertheless, this *T. brevifolia* hairy root culture could be useful for biochemical pathway characterization and possibly biotransformation studies.

The Salvia species contain diterpenes with diverse structures, such as the labdane, abietane, clerodane, and pimarane types of diterpenes, which were reported to possess antioxidant, anti-inflammatory, antimicrobial, antitumor, and anticancer activities [41]. These diterpene compounds have been identified from hairy roots induced from various Salvia plants [41, 90, 91, 148]. Fraga et al. [41] also analyzed the biogenetic relationships of the abietane diterpenoids from *S. broussonetii* hairy roots and determined their antifeedant and toxic properties, thus providing new insights into this group of bioactive compounds. Hypotensive, positive inotropic, and chronotropic activities were shown for a labdane diterpenoid, forskolin, produced by the roots of *Coleus forskohlii* [27]. The hairy root culture of *C. forskohlii* yielded high concentrations of forskolin, and this production appeared to be affected by the *A. rhizogenes* strains and the culture media being used [143]. Diterpene lactones ginkgolides can be extracted from leaves and roots of *Ginkgo biloba*. Previous studies of differentiated *G. biloba* cell cultures produced ginkgolides [94], suggesting that the differentiated hairy roots could possibly be a source of these health-beneficial diterpenoids. Hairy roots of *G. biloba* were established; however, chemical analysis of hairy roots was not described in that study [7].

Triterpene saponins (C_{30}) are amphiphilic compounds with glycosidic chain(s) conjugated to the triterpene aglycone core structures. A group of triterpene saponin compounds, namely saikosaponins, contribute to the anti-inflammatory, antipyretic, and antitussive activities of the root extracts of the traditional Chinese herbal plant *Bupleurum falcatum* (Apiaceae) [2]. In consideration of the slow natural root growth, hairy root cultures of *B. falcatum* were established for speedy production of

saikosaponins [2]. Sucrose and mineral contents in the growth medium showed differential impacts on hairy root growth and saikosaponin yield [2]. Further optimization of the culturing conditions is still needed for commercially viable production of this valuable metabolite. Another plant of the Apiaceae family, *Centella asiatica*, accumulates saponins, such as centellasaponin, asiaticoside, madecassoside, and sceffoleoside, mainly in leaves. These triterpene saponins are recognized for their antipyretic and anti-inflammatory properties [185]. Non-transformed roots and hairy roots of *C. asiatica* did not show significant accumulation of saponins. However, upon elicitation by MeJA, 7.12 mg g^{-1} asiaticoside accumulated in hairy roots [75]. This encouraging result suggested that saponin production could potentially be further enhanced in *C. asiatica* hairy roots using elicitors and additional manipulations.

The roots of *Panax ginseng* are rich in saponin ginsenosides, which are appreciated for their toning, immunomodulatory, adaptogenic, and antiaging activities. The lengthy cultivation time and various problems involved in culturing stimulated the development of Panax hairy root cultures. Interestingly, 2-fold more ginsenosides were achieved in *P. ginseng*-derived hairy roots than the wild-type roots [197]. Another important Panax species, *P. quinquefolium*, was subjected to hairy root induction, and the ginsenoside content reached 0.2 g g^{-1} dry weight at 10 weeks of hairy root growth [105]. The hybrid plant between *P. ginseng* and *P. quinquefolium* was more vigorous in ginsenoside production than either parent [83]. However, since the hybrid plant is sterile, hairy roots of the hybrid were developed to maintain this elevated biosynthetic capacity [188]. The promising finding from this work was that the 8-week-old hairy roots contained comparable amounts of ginsenosides to the roots of field-grown parental plants, suggesting that the biosynthetic potential of the parent plants was maintained in the hairy roots [188]. Structural analogs of ginsenosides, gypenosides, are produced by *Gynostemma pentaphyllum* (Cucurbitaceae) and are noted particularly for their antidiabetic effects [119]. A maximum of 280 mg L^{-1} gypenoside accumulation was reached at day 49 of *G. pentaphyllum* hairy root growth, and kinetic evaluations revealed an intriguing concomitant production of gypenosides with the primary metabolites [26]. It will be interesting to understand how *G. pentaphyllum* hairy roots partition carbons between the primary and secondary metabolism.

The leaves and infusions of *Gymnema sylvestre* are valued in traditional Indian medicine for their antidiabetic properties. The active principles of *G. sylvestre*, triterpene saponins (e.g., gymnemic acid), were also reported to have additional health-promoting benefits. As with many other medicinal plants, overexploration of this herb caused concerns for a sustainable supply of these antidiabetic compounds. The *G. sylvestre* hairy roots generated almost 5-fold more gymnemic acid than the non-transformed roots [116]. The highly productive *G. sylvestre* hairy roots hold great potential for producing gymnemic acid. However, it remains to be determined whether the hairy root extracts exhibit comparable efficacy to that shown for the leaf and whole-plant extracts.

A special group of triterpene compounds are phytoecdysteroids, which are synthesized from the mevalonic acid pathway with cholesterol as a biosynthetic

intermediate. These plant-produced ecdysteroid analogs are purposed to mimic the action of ecdysteroids (steroidal hormones) from insects and other anthropods to interfere with their molting processes (ecdysis). On the other hand, immunostimulatory effects of phytoecdysteroids have been described in humans [21]. High levels of phytoecdysteroids accumulated in the hairy roots of *Serratula tinctoria*, to an extent of 0.1–0.2 % of dry weight [31]. Biosynthesis of phytoecdysteroids in *S. tinctoria* hairy roots was supported by feeding of radiolabeled cholesterol and mevalonic acid and incorporation of the isotope into phytoecdysteroids [31]. The hydrolysis product of saponins, sapogenins (i.e., the aglycone, e.g., diosgenin), can be used for commercial synthesis of the human steroidal hormones. Fenugreek (*Trigonella foenum-graecum*) hairy roots were shown to produce diosgenin [110]. Cholesterol supplementation (i.e., precursor feeding) largely decreased the biomass of *T. foenum-graecum* hairy roots and only led to a moderately increased diosgenin content (5 %). However, addition of chitosan to the growth medium induced diosgenin production by 5-fold [110]. Further optimization of biomass growth and diosgenin yield could improve the prospect of using fenugreek hairy roots as human steroidal hormone "feedstocks".

2.2 Alkaloid and Glucosinolate Production in Hairy Roots

Alkaloids are nitrogen-containing (*N*-containing) compounds found in plants, microorganisms, and animals. The current classification of alkaloids is based on the *N*-containing structures, such as indole or quinoline alkaloids. The nitrogen atom in alkaloids is derived from an amino acid either directly or indirectly through a transamination reaction. Tropane alkaloids are likely synthesized from the monomethylated polyamine putrescine. Many solanaceous plants produce tropane alkaloids, particularly hyoscyamine and scopolamine, specifically in the root tissue. Tropane alkaloids can counteract the function of the neurotransmitter acetylcholine in the brain (i.e., anticholinergic) [100]. The pharmaceutical properties and the root-enriched accumulation of tropane alkaloids inspired hairy root studies in solanaceous plants (Table 1). A survey of Datura and Hyoscyamus species revealed large variations in the quantity of tropane alkaloids produced by hairy roots [86]. Most of the tropane alkaloids accumulated in *Datura candida* hairy roots, while only a small portion were released to the growth medium [28]. However, Sáenz-Carbonell and Loyola-Vargas [162] noticed that the use of ammonium as the nitrogen source for hairy root growth stimulated secretion of tropane alkaloids. It was found that UV B irradiation and acetylsalicylic acid elicitation could enhance tropane alkaloid production in *Anisodus luridus* hairy roots [132]. Interestingly, in addition to hyoscyamine and scopolamine, a novel polyamine cadaverine was detected in the hairy roots, but not the whole plant, of *Brugmansia candida* [22].

Indole alkaloids originate from the aromatic amino acid tryptophan. The terpene and indole alkaloid conjugates (TIAs) constitute one of the largest groups of alkaloids in plants. TIA biosynthesis presents an excellent example of highly complex and

coordinated secondary metabolic pathways that encompass multiple distinct biosynthetic routes, diverse cell types, and different subcellular compartments. The rhizomes and roots of Rauvolfia species are rich sources of TIAs that have been used for treating nervous system disorders due to their anxiolytic activities. Hairy roots established from *Rauvolfia serpentine* produced ajmaline and serpentine in both solid and liquid cultures [14], while targeted analysis of ajamaline and ajamalicine in *Rauvolfia micrantha* hairy roots indicated that this hairy root system could synthesize both TIAs [160]. Development of hairy roots as sources of Rauvolfia TIAs will help alleviate overharvest of these endangered species.

C. roseus (Madagascar periwinkle) reportedly produces over 130 TIAs and is valued for its many health-beneficial activities. Two major alkaloids from Catharanthus, vinblastine and vincristine, are structurally very similar—each is composed of two alkaloid monomers, with vinblastine possessing an *N*-methyl group and vincristine possessing an *N*-formyl group at the corresponding position. In principle, both vinca alkaloids bind to tubulins, disrupt microtube functions, and terminate cell divisions. Yet, these dimeric TIAs exhibit distinct bioactivities and are components of cancer therapies. Since the yield of vincristine and vinblastine from whole plants is extremely low (0.0002 %), these chemicals are currently obtained from semisynthesis using intermediates isolated from *C. roseus*. Alternative methods are desirable for economic production of these valuable vinca alkaloids. Hairy root cultures of *C. roseus* were established, and various environmental, chemical, and biotic factors were applied to the hairy roots for yield increase [149]. In addition, biosynthetic and regulatory genes were also employed for manipulation of alkaloid content in hairy roots. To this end, the transcription factor octadecanoid-derivative-responsive catharanthus AP2 domain (*ORCA3*) and one of the biosynthetic genes geraniol 10-hydroxylase (*G10H*) were overexpressed in *C. roseus* hairy roots and resulted in enhanced accumulation of catharanthine, but not vindoline, vinblastine, and vincristine [187]. It remains to be explored how the enzyme organization (e.g., whether metabolons are involved and delineation of the regulons) and the subcellular localization of the biosynthetic enzymes affect the production of vinca alkaloids.

The TIA camptothecin (CPT) contains a quinoline-type alkaloid and is obtained from the Chinese happy tree *Camptotheca acuminata* [99]. CPT can bind to DNA and DNA topoisomerase I; the formation of such a complex blocks the activity of DNA topoisomerase I, which contributes to the antitumor, anticancer, and antiviral activities of CPT [85]. Synthetic analogs of CPT have already been approved by the FDA for treating cancers. Both *C. acuminata* and *Ophiorrhiza pumila* hairy roots produced quantities of CPT that were similar to the wild-type roots [99, 136]. The CPT produced in hairy roots was released to the liquid growth medium. It was shown that addition of polystyrene resins to the growth medium of *O. pumila* hairy roots absorbed CPT and further stimulated its secretion. These promising results bode well for hairy root production of this bioactive chemical. One may envision that high levels of CPT could be achieved by a combined elicitation, absorbent addition, and metabolic engineering of hairy roots.

Additional groups of alkaloids with piperidine or indolizidine structures were also identified from hairy root cultures. Piperidine alkaloids from *Lobelia inflata*

(e.g., lobeline) have been used as antiasthmatic agents. Hairy roots of *L. inflata* produced similar or higher levels of lobeline when cultured in different growth media as compared to the soil-grown non-transformed roots [196]. Swainsonine, an indolizidine alkaloid derived from lysine, is produced from the hairy roots of the legume plant *Swainsona canescens* [113]. Its concentration in hairy roots was further increased by feeding biosynthetic precursors, lowering the pH of the growth medium, and supplementation of copper sulfate. These various treatments also stimulated the secretion of swainsonine to the growth medium [113]. Though release in the medium may facilitate downstream recovery of swainsonine, it is unclear whether swainsonine remains stable in the growth medium. One possible solution is adding absorbents to the growth medium to prevent degradation, assist in extraction, and stimulate further release of swainsonine.

Steroidal alkaloids are conjugates of a steroidal saponin and an alkaloid, such as solasodine found in solanaceous plants, and are often glycosylated to form steroidal glycoalkaloids. Steroidal alkaloids can impart toxicity to humans, particularly when accumulated at high concentrations in food crops. On the other hand, these steroidal alkaloids share similar structures to steroidal saponins and can serve as the biosynthetic precursors of steroid drugs and synthetic human hormones. Though these compounds are generally more concentrated in the photosynthetic and reproductive tissues of the plants, low concentrations of steroidal alkaloids were also detected in roots. By manipulating the composition of the growth medium, 6.2 mg g^{-1} solasodine was obtained in *Solanum aviculare* hairy roots and the solasodine-containing glycoalkaloids accounted for 0.3–1 % of the dry weight of *Solanum laciniatum* hairy roots [84, 122]. The yield of steroidal glycoalkaloids could be further improved in hairy roots by treatment with elicitors and feeding of biosynthetic precursors.

Besides alkaloids, another group of *N*-containing compounds in plants is known as glucosinolates, which are amino acid-derived thioglycosides. Hydrolysis of glucosinolates by myrosinase releases isothiocyanate that could be toxic to humans when ingested in large quantities. Conversely, cancer chemopreventive activities have been found in several glucosinolate compounds [58]. Hairy roots of *Arabis caucasica, Barbarea verna*, *Nasturtium officinale*, and *Tropaeolum majus* were obtained and produced gluconasturtiin, glucotropaeolin, and glucoiberverin [190, 191]. When amino acid precursors were added to the growth medium of the hairy roots, the yield of the corresponding glucosinolates was enhanced [190]. While the glucosinolate profiles varied in different plant species, these studies indicated that hairy roots can be used as sources of glucosinolates in addition to the parent plants.

2.3 Phenolic Production in Hairy Roots

Phenolic compounds contain at least one hydroxylated benzene ring in their chemical structures, including simple phenolics, such as coumarins, flavonoids, isoflavonoids, and anthocyanins, and more complex polymers, such as lignins and tannins. A majority of the plant phenolic metabolites are derived from the aromatic

amino acids that are synthesized from the shikimate pathway. Phenolics are collectively valued for their wide variety of health-promoting activities. Some of the phenolic compounds, such as flavone glycosides from citrus, isoflavones from soy, and flavonoids and tannins from tea, were formerly referred to as vitamin P which are linked to antioxidant, cardioprotective, and cancer chemopreventive activities.

Coumarins originate from the orthohydroxylation of the cinnamic acid precursor and the subsequent lactone formation. Substitutions of the simple coumarin ring lead to the formation of furanocoumarins (a five-membered furan ring attached to the coumarin backbone) and pyranocoumarins (a six-membered pyran ring attached to the coumarin backbone). A plethora of pharmacological properties have been ascribed to coumarins, such as anti-inflammatory, antiviral, anticancer, and antihypertensive activities [180]. Coumarins are commonly found in the families of Umbelliferae and Rutaceae. Fruits of *Ammi majus* are rich in coumarins and furanocoumarins. Umbelliferone and furanocoumarins were identified from methanol extracts of hairy roots induced from *A. majus* stalk and leaf explants [88]. *Ruta graveolens* is also an abundant source of coumarins and furanocoumarins [155]. Unexpectedly, the hairy roots of *R. graveolens* synthesized novel coumarins, osthole and osthenol, which do not normally accumulate in the parent plants [155]. Another interesting observation was that the dark-grown hairy roots accumulated more coumarins than those grown under a light–dark regime. It was unclear why depletion of light may enhance the coumarin levels, which could result from an increase in coumarin biosynthesis, a decrease in its turnover, or a combination of these two processes.

The efficacy of flavonolignans in treating liver disorders is presumably due to their abilities to scavenge free radicals produced from the detoxification reactions in the liver. The flavonolignan silybins found in *Silybum marianum* are the oxidative coupling products of dihydroquercetin and coniferol alcohol. Hairy roots were induced from seeds of *S. marianum* in an attempt to produce silybins [3]. Although silybins and other variants of flavonolignans were identified from the non-transformed plant roots, isosilybin was the predominant flavonolignan in the hairy roots, where only trace amount of silychristin and silydianin and no silybin were identified. Though producing a different flavonolignan profile than the non-transformed roots, this hairy root system could be used for investigation of the flavonolignan biosynthetic pathway and for producing different types of flavonolignans.

The trihydroxyflavone jaceosidin acts as an inhibitor of neuroinflammation [117]. Jaceosidin has been isolated from several herbal plants. Hairy root clones of *Saussurea medusa* were obtained and able to form jaceosidin with a 37-fold increase in production within a 27-day growth period [202]. It was also found that the root tips were more active in biomass growth and jaecosidin production than the other sections of the hairy roots. Besides synthesizing flavonoids, the phenylalanine/tyrosine-derived ring structure in the common flavanonone precursor could rearrange to a different position (i.e., ring migration) and form isoflavonoids. In contrast to flavonoids that distribute ubiquitously in plants, isoflavonoids have more limited taxonomic distributions and are mostly found in Fabaceae. Isoflavonoids exhibit phytoestrogenic, anticancer, and anti-inflammatory activities [107]. With the

exception of genistin (glycoside of genistein), isoflavonoids in *Lupinus mutabilis* hairy roots accumulated at a much higher level than the non-transformed roots [9]. The drop in genistin concentration could possibly be due to reduced glycosylation of genistein to genistin in *L. mutabilis* hairy roots. To improve isoflavonoid production, elicitors were applied to the hairy root culture and showed various effects. Elicitor treatments (chitosan, salicylic acid, yeast extract, polyamines) were more effective in enhancing daidzein and genistein accumulation than feeding the *Psoralea corylifolia* hairy roots with the phenylalanine precursor [153]. The biotic and abiotic elicitors stimulated isoflavonoid production, but did not affect the growth of *Pueraria candollei* hairy roots [177]. Of the five elicitors used (MeJA/MJ, chitosan, salicylic acid, yeast extract, lysate of *A. rhizogenes*), MJ was most effective and led to a 4.5-fold increase in total isoflavonoids than the non-elicited control roots [177]. These results suggest that, for optimal production of isoflavonoid metabolites, multiple elicitation strategies should be tested and evaluated on different hairy root cultures. It may be necessary to combine several elicitors to bring about the maximum metabolic potential of the hairy roots.

Anthocyanins are colorful pigments and excellent antioxidants produced largely in flowers, fruits, and tissues under stress. Anthocyanins and hydroxycinnamic acid esters were detected in the hairy roots of *Leontopodium alpinum* [61]. Anthocyanin production in both *L. alpinum* and *Campanula glomerata* hairy roots was increased upon addition of benzylaminopurine (i.e., benzyladenine), presumably due to the stimulated root growth [61, 166]. Light inhibited *C. glomerata* hairy root growth, but promoted anthocyanin accumulation [166]. The accumulation of polyacetylenes was not affected by light or benzylaminopurine, suggesting that these hormonal and environmental factors play distinct roles in producing different classes of secondary metabolites.

In addition to phenolic compounds with simple structures, phenolic polymers, such as condensed tannins (CTs) and hydrolyzable tannins (HTs), are also synthesized in hairy roots [67, 111, 123]. Though sharing the common name tannin, HTs and CTs have different biosynthetic origins; HTs are synthesized from an intermediate of the shikimate pathway, and CTs are derived from the end products of the shikimate pathway. Antioxidant and anticancer activities have been reported for both groups of tannins [62]. CTs are enriched in the *Lotus corniculatus* leaf, stem, root, flower, and seed tissues. Both *L. corniculatus* non-transformed and hairy roots produced CTs [111]. Interestingly, electron microscopy studies revealed that CTs distributed specifically in "tannin cells" of the hairy roots. Exogenously supplied auxin enhanced biomass growth, did not affect flavonoid accumulation, and inhibited CT production in *L. corniculatus* hairy roots. The underlying mechanism for a differential impact of auxin on flavonoid and CT accumulation is not known. Pomegranate fruit peel is highly abundant in HTs, particularly punicalagins. Hairy roots developed from radicle, cotyledon, and leaf explants also showed punicalagin production [123]. The pomegranate hairy root culture constitutes an excellent system for genetic characterization of HT biosynthetic genes. While HTs were the major tannins identified from hairy roots of *Geranium thunbergii*, catechin (precursor of CTs) also accumulated in this hairy root system [67], suggesting that *G.*

thunbergii hairy roots could potentially be used as a platform for understanding the biosynthetic relationships between the two groups of tannins.

2.4 Fatty Acid, Polyacetylene, Thiophene, and Polyketide Production in Hairy Roots

Fatty acids, polyacetylenes, thiophenes, and polyketides share the common biosynthetic scheme of condensing multiple acetate units and forming poly-β-keto chains and differ in the chain extension mechanisms. These various groups of secondary metabolites show diverse taxonomic distributions and exhibit distinct activities in plants and humans. Polyunsaturated fatty acids (PUFAs) are considered "good fats" as consumption of PUFAs has been linked to reduced levels of low-density lipoproteins (LDLs) and improvement on heart health [16]. Since extraction of PUFAs from fish contributes to a decline of fish stocks, plant oils have emerged as an alternative source of PUFAs. In addition to plant oils, hairy roots have also been explored for production of PUFAs and lipids. For example, hairy roots established from a PUFA-producing plant, *Echium acanthocarpum*, accumulated 18:2n-6 (linoleic acid) and 18:3n-6 (γ-linolenic acid) that accounted for 55 % of total fatty acids in the hairy roots [25]. Unlike oil seeds that are capable of amassing PUFAs to large quantities, hairy root production of PUFAs is limited by the competition of carbon precursors for formation of PUFAs or biomass. Nevertheless, hairy roots of PUFA-producing plants present an opportunity for gene discovery and functional characterization that could play a role in improving PUFA production in other plant organs.

Alkamides are amides of unsaturated fatty acids. Many plants that manufacture alkamides have been used for medicinal purposes. Herbal preparations of dried Echinacea roots or plant extracts are still being used nowadays as immunostimulants. Alkamides are active principles of Echinacea roots and comprise PUFAs as amides of 2-methylpropanamine or 2-methylbutanamine. Hairy root cultures of three Echinacea species were established and showed enhanced production of alkamides compared to the wild-type roots [133]. JA stimulated alkamide production in these hairy roots though hairy root growth was thwarted at high JA concentrations (10 μM or greater). The auxin indolebutyric acid (IBA) promoted hairy root branching and growth rate of biomass while having no impacts on alkamide production. This study suggested that a balanced growth and elicitation regime should be designed and implemented for optimal yield of alkamides in Echinacea hairy roots.

Polyacetylenes and thiophenes (heterocyclic sulfur-containing aromatic rings conjugated to acetylenes) share common biosynthetic pathways and are mainly found in roots of plants that belong to Asteraceae, Umbelliferae, and Campanulaceae. Antiviral, antimicrobial, and antitumor properties have been reported for these compounds [154]. Hairy root cultures have been established in multiple species of

these families, and one interesting observation was that the hairy roots grew at least 2-fold faster than the corresponding non-transformed roots, while both root systems produced polyacetylenes and thiophenes [40]. The incorporation of radiolabeled oleic acid precursors to polyacetylenes and thiophenes, stable production of polyacetylenes and thiophenes over a 12-month period with multiple passages, and increased metabolite production upon elicitation by fungal culture filtrates collectively suggested that these compounds are synthesized locally in the root tissue, rather than remanents of the parent metabolites transferred to the roots [40]. One surprising observation was that the hairy roots turned green upon light exposure and the "green hairy roots" contain chloroplasts and perform photosynthesis. It is unclear whether carbon allocation to photosynthetic activities affects synthesis of secondary metabolites in these "green hairy roots".

Polyketides are synthesized in a similar fashion as fatty acids. Stilbenes are one class of polyketides that have the characteristic C6–C2–C6 backbone structures. A particular stilbene compound resveratrol has drawn much attention in recent years due to its cardioprotective, anticancer, antiangiogenic, and immunomodulatory activities [125]. To build a tissue culture-based platform for resveratrol production, hairy root lines of peanut (*Arachis hypogaea*) were established and resveratrol production was induced using five different elicitors that encompass biotic and abiotic stress factors, including copper sulfate, chitosan, cellulose, laminarin, and sodium acetate [108]. Sodium acetate was most effective in stimulating resveratrol formation in the *A. hypogaea* hairy roots, and 99 % of the resveratrol synthesized was released to the growth medium [108]. The mist bioreactor design used for *A. annua* hairy roots was also applied to *A. hypogaea* hairy roots and resulted in a slightly higher biomass gain as compared to the shake flask-grown hairy roots [157]. In addition to yield improvement, optimization of the extraction method could further increase the overall resveratrol production from *A. hypogaea* hairy roots.

2.5 Natural Dye and Biopesticide Production in Hairy Roots

Besides tackling various health-related problems, there are also growing interests from the food industry in using naturally occurring secondary metabolites, specifically pigments, for food coloration. The roots of red beet (*Beta vulgaris*) contain the water-soluble betalains, including the red/violet-colored betacyanins (aromatic indole pigments derived from tyrosine) and the yellow-colored betaxanthins. The betalain pigments mainly accumulated in vacuoles of the *B. vulgaris* hairy roots [169]. It was noted that temporary cessation of oxygen supply led to release of betalain pigments from the vacuolar compartments to the growth medium, thus facilitating downstream pigment extractions. More importantly, the oxygen starvation by cessation of shaking did not affect regeneration of hairy roots, and thus allowed regeneration and reuse of the root biomass for metabolite production. The

betalain profiles in the growth medium resembled those of hairy roots and the parent roots, providing further support to the utility of this hairy root system for the production of natural betalain pigments.

Shikonin is a naphthoquinone (dicyclic quinone molecule) that is derived from the shikimate and methylerythritol phosphate (MEP) pathways. This reddish pigment has shown antimicrobial, anti-inflammatory, and anticancer activities [4] and has been used in food additives and cosmetics. Although shikonin is one of the first plant metabolites produced in cell cultures, it reportedly requires a complex two-step culture system for shikonin production. The cell culture-produced shikonin is secreted to the cell wall after fusion of ER-derived vesicles to the plasma membrane and trapped in between the plasma membrane and the cell wall. Upon optimization of culturing conditions, the hairy roots of *Lithospermum erythrorhizon* secreted shikonin to the growth medium [152]. Microscopic examination revealed that the mature, but not the newly grown, roots accumulated shikonin in aggregated granules [152]. Since about 25 % of shikonin produced was secreted to the growth medium, absorbents were added to the medium in an attempt to sequester shikonin and stimulate further secretion of this metabolite. Indeed, a 3-fold increase in shikonin yield was observed and over 85 % of total shikonin produced was sequestered in the XAD-2 absorbents. Overall, this is a promising example of producing a water-soluble pigment using the hairy root system.

Anthraquinone derivatives (tricyclic quinone molecules), such as alizarin and purpurin, are natural red dyes produced from the roots of the perennial herb *Rubia akane*. Hairy root cultures of various Rubia species have been established, and production of alizarin and purpurin correlated with hairy root growth [80, 126]. Maximum production of these compounds was reached after 20 days of culturing in liquid medium [126]. Upon oxygen starvation treatment, anthraquinones were secreted to the culture medium, similar to what was observed in *B. vulgaris* hairy roots with limited oxygen supply [169]. Like the *B. vulgaris* hairy roots, the usage of absorbents could also be examined for enhancing anthraquinone production and release to the liquid culture.

To protect crop plants from pathogens and insects, pesticides have been applied to agricultural fields in large quantities, causing environmental and human health concerns. To this end, pesticides produced from biosources are degradable and preferred by the general public. The principle insect repelling component of neem (*Azadirachta indica*) is azadirachtin. This tetranortriterpenoid structurally resembles the insect hormone ecdysones and works to disturb the hormone-controlled developmental processes of insect pests. Since neem trees are grown in more restricted climate and soil conditions, a hairy root culture system will ensure more sustainable production and broad utilization of this useful biopesticide. Crude extracts of *A. indica* hairy roots exhibited high antifeedant activities against the desert locust *Schistocerca gregaria*, which was previously demonstrated to be sensitive to azadirachtin [159]. It is worth noting that azadirachtin is photolabile and sensitive to the pH of the environment. Additional work still needs to be carried out

for optimization of additives to this biopesticide to ensure its efficacy and shelf life. In this regard, the *A. indica* hairy roots provide a convenient system for carrying out these studies.

3 Preservation of Hairy Roots

As described in the previous sections, hairy roots produce many valuable secondary metabolites. It is conceivable that the established hairy roots should be maintained for long-term use. However, the constant efforts involved in maintaining hairy root cultures in solid or liquid media are cumbersome and the potential loss of hairy root cultures due to contamination or facility failures could be devastating. In addition, as cultured in solid or liquid media, shipping of hairy root specimens could be challenging. Taking these into consideration, it is highly desirable to develop methods for long-term storage of hairy roots and the capability for regeneration when needed. As with the induction of hairy roots, different plant species may have different optimal conditions for preservation and some may be recalcitrant for the preservation methods.

Cryopreservation is a process where tissues are frozen and preserved at subzero temperatures. Two major challenges encountered by cryopreservation of hairy roots are maintaining the integrity of cellular and subcellular structures and ensuring successful regeneration of the preserved hairy roots. To this end, multiple methods, including vitrification, encapsulation vitrification, and droplet vitrification, have been successfully applied to cryopreservation. Vitrification entails mixing root tips with chemical agents that reduce the cellular water content and suppress the formation of ice crystals. The vitrification method has traditionally been used for saving plant tissues and has been adapted to long-term storage of hairy roots. More recently, two new methods, encapsulation vitrification and droplet vitrification, build upon vitrification and encapsulate the explants in alginate beads or put them in individual droplets prior to freezing in liquid nitrogen for easy handling [138]; these improved methods are more suitable for processing a large quantity of samples. Successful cryopreservation of hairy roots has been reported in a number of plant species using either the traditional or the improved methods (Table 2). Rehydration and recovery of frozen hairy root tissues remain to be the challenging and often bottleneck steps in cryopreservation of hairy roots. In addition to ensuring viability of the cryopreserved tissues, it is also of importance for the recovered hairy roots to maintain the biochemical phenotype of the untreated controls, particularly for those that are used for valuable secondary metabolite production.

The perceivable limitations of cryopreservation inspired development of alternative methods for long-term storage of hairy roots. Previously, hairy roots were reported as a means to generate plantlets in the form of "artificial seeds" [178]. In these studies, the plantlets were induced from fragmented hairy roots, encapsulated and dehydrated prior to regeneration into whole seedlings. This artificial seed procedure was modified by manipulating the composition of the growth media, which was

Table 2 Examples of cryopreservation of hairy roots

Species	Common names	Preservation methods	References
Artemisia annua L.	Sweet wormwood	Slow freezing	[170]
Armoracia rusticana Gaertn. Mey. et Scherb.	Horseradish	Encapsulation dehydration	[59]
Beta vulgaris L.	Red beet	Slow and fast freezing	[15]
Nicotiana rustica L.	Mapacho	Slow and fast freezing	[15]
Panax ginseng C.A. Meyer	Asian ginseng	Vitrification	[198]
Atropa belladonna L.	Deadly nightshade	Vitrification	[174]
Maesa lanceolata Forssk.	False assegai	Encapsulation–dehydration	[93]
Medicago truncatula Gaertn.	Barrel medic	Encapsulation–dehydration	[93]
Rubia akane Nakai	Asian madder	Droplet vitrification	[77, 78, 139]

adapted for the preservation of hairy root clones without the induction of plantlets [179]. However, a caveat was that the rate of hairy root regeneration decreased as time lapsed. The longest period of viable storage for *Scutellaria baicalensis* (Baikal skullcap) hairy roots via the artificial seeds method was 4–5 months, after which the hairy roots needed to be regrown and recapsulated [179]. Though artificial seeds are still at the early stage of development and require further optimizations, they present a promising method for maintaining hairy roots.

4 Future Perspectives

Overall, the major groups of secondary metabolites have already been produced from hairy roots (Table 1). Novel compounds were also identified in hairy roots, and exogenously added metabolites could be biotransformed by the metabolic pathway or grid of hairy roots. Environmental factors, such as light, oxygen, and temperature, as well as abiotic and biotic stress factors, such as heavy metals and fungal elicitors, have all been applied to hairy roots for increased yield of secondary metabolites. In addition to these external stimuli, secondary metabolic pathways have also been modified for enhanced metabolite production, such as overexpression of biosynthetic genes and transcription factors, and suppression of catabolic or competing pathway genes. A better understanding of the biosynthetic pathway and regulation architecture of valuable secondary metabolites is crucial for genetic engineering and fully realizing the biosynthetic potential of hairy roots.

Progress has been made on commercialization of hairy root products. ROOTec Bioactives Ltd., founded in 2005 in Switzerland, currently produces phytochemicals from hairy roots induced from 17 plant species in their proprietary mist bioreactors. In the future, more investigations could be directed toward determining the efficacy of crude hairy root extracts or hairy root-produced chemicals. Plant biologists can work closely with engineers to tackle the challenges with scaling up hairy root cultures, such as optimal biomass growth and adaptation of the extraction methods to industrial-scale metabolite production. Looking forward, establishment of hairy roots guided by bioassays, augmented by elicitations and genetic manipulations, and coupled with efficient metabolite extractions will streamline the process and allow full exploitation of hairy roots as a production platform of valuable secondary metabolites.

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