Chapter 9
Hairy Root Cultures for Secondary Metabolite Production

Ravi S. Singh, Prakash Singh, Ruchi Kumari, and Sanjeev Kumar

Abstract Hairy root culture (HRC) is a promising biotechnological tool for the production of plant secondary metabolites under in vitro condition. This has been harnessed for the production of several molecules of medicinal and commercial importance. The hairy root phenotype appears at the site of infection of plants by *Rhizobium rhizogenes* which possess characteristics like fast growth even without hormone in the media, high genetic stability, geotropism, profuse lateral branching, differentiated tissue type, and high productivity. The content of secondary metabolites in these hairy roots is usually comparable to that of the fully grown plants. But, the major bottleneck for commercial exploitation of HRC is the developing protocol for scaling up production in bioreactors, as easy handling of interconnected hairy roots that are unevenly distributed in the vessel, is difficult. This chapter is focused on HRC with latest information available on basic method of induction of hairy roots to its uses in production of low volume and up-scaling in bioreactors, for commercial viability of HRC-based technology.

Keywords Secondary metabolites · Hairy roots · *Rhizobium rhizogenes* · *Agrobacterium rhizogenes*

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9.1 Introduction

Hairy root formation is induced by *Rhizobium rhizogenes* (earlier known as *Agrobacterium rhizogenes*) bacteria (Gutierrez-Valdes et al. 2020), at the site of infection by integration of T-DNA of the root-inducing (Ri) plasmid into the host genome. The hairy roots usually grow faster than plant cell cultures and yield more biomass. These hairy roots show rapid growth in hormone-free medium with extensive lateral branching and ageotropic nature, making these roots to be used as an attractive system for producing secondary metabolites. Further, their high genetic stability ensures the uniform productivity for longer period, which also makes these hairy roots as preferred material for HRC-based in vitro production. Several phytochemicals of medicinal and commercial importance, i.e., pharmaceuticals, cosmetics, dye, pesticides, food colorant, and food additives, have been reported. These hairy roots have potential to be utilized for in vitro production of secondary metabolites. Though several in vitro culture methods such as callus culture, cell suspension culture, adventitious root culture, and organ culture are available for secondary metabolites (Singh et al. 2010; Silja and Satheeshkumar 2015; Purwianingsih et al. 2016; Shoja and Shishavani 2021), the genetically transformed hairy roots are very attractive and promising keeping in view their differentiated nature and genetic stability and high production and productivity of secondary metabolites. The hairy root culture (HRC)-based production systems for secondary metabolites were reported for shikonins, camptothecins, azadirachtin, paclitaxel, hyoscyamine, harpagide, ginsenosides, and scopolamine (Sim and Chang 1993; Satdive et al. 2007; Almagro et al. 2015; Zhang et al. 2017; Balasubramanian et al. 2018; Singh et al. 2020; Barba-Espin et al. 2020; Wawrosch and Zotchev 2021; Table 9.1). As the plant-based phytoceuticals are in great demand worldwide, as per the estimate of World Health Organization (WHO), up to 80% of people take traditional herbs as medicines (Khan et al. 2009).

HRCs are usually capable of producing the structurally identical compounds similar to the ones found in roots of the naturally occurring intact parent plant, in contrast to as frequently observed in callus or cell suspension cultures. In comparison to undifferentiated cell culture, higher and stable yield of secondary metabolites are obtained with differentiation hairy roots. HRCs usually surpass the spatial barrier of production or accumulation of metabolites and could be employed also in cases where secondary metabolites accumulate or produced in other aerial tissues (stem, leaf, fruit, flower, seeds). HRC is considered to be more biosynthetically efficient than their mother plants. The hairy roots are also used for the production of those metabolites, which are synthesized and accumulate in aerial part of plants only, for example, lawsone and artemisinin, so irrespective of their origin or site of production, the metabolites could be obtained in hairy roots (Patra and Srivastava 2016; Bakkali et al. 1997). High genetic stability, differentiated tissue type, and high productivity are the features of HRC leading it to be used as valuable alternative method for the production of plant secondary metabolites. High branching, high growth rate, and genetic stability of these roots also suit for commercial up-scaling

Table 9.1 HRCs for secondary metabolite production

Secondary metabolite	Plant	Biological activities	Reference
Shikonin	*Lithospermum erythrorhizon, Arnebia euchroma*	Wound healing	Sim and Chang (1993), Singh et al. (2010)
Flavonoid/ biflavonoid	*Selaginella bryopteris*	Antimicrobial	Singh et al. (2018a, b, c, 2020)
Ginsenosides	*Panax quinquefolium*	Antimicrobial	Kochan et al. (2013)
Terpenoid indole alkaloids	*Catharanthus roseus*	Anticancer drugs (vinblastine, vincristine)	Almagro et al. (2015)
Curcumin	*Atropa belladonna*	Antimicrobial, wound healing	Singh et al. (2021)
Quercetin	*Raphanus sativus*	Antioxidant and anti-inflammatory effects	Balasubramanian et al. (2018)
Tanshinones	*Salvia miltiorrhiza*	Antioxidant, anti-inflammatory, and antitumor activities	Zhang et al. (2017)
Anthocyanin	Black carrots (*Daucus carota*)	Antioxidants	Barba-Espin et al. (2020)
Flavone	*Scutellaria baicalensis*	Antioxidants	Park et al. (2021)
Phenolic compounds	*Momordica dioica*	Antioxidants	Thiruvengadam et al. (2016)

even in bioreactors; hence, in recent years, focus has been on designing appropriate bioreactors suitable to culture the delicate and sensitive hairy roots (Rekha and Thiruvengadam 2017). But, the major bottlenecks for commercial exploitation of HRC are the developing protocol for scaling up production in bioreactors, as easy handling of interconnected hairy roots that are unevenly distributed in the vessel is difficult. In this chapter, we have discussed the HRC with latest information available on basic method of induction of hairy roots to its uses in production low volume and up-scaling in bioreactors for commercial viability of technology.

9.2 Molecular Mechanism of Hairy Root Development

Root loci (*rol*) genes present on T-DNA of the root-inducing (Ri) plasmid of *R. rhizogenes* are integrated into the host genome causing hairy root formation. The most studied agropine-type strains, *R. rhizogenes* strains, have two T-DNA regions designated as the TL-DNA and TR-DNA on their Ri plasmid (Nemoto et al. 2009). These regions get independently transferred to the nuclear genome of infected plant cells. The TL-DNA contains about 18 potential genes, of which 4 genes, *rol* A, B, C, and D, are implicated to induce the formation of hairy root in plants (Nemoto et al. 2009). The *rol*A gene suggested as an activator of growth and secondary

metabolism while *rol*B gene as stimulator or growth-suppressor (Bulgakov 2008). The *rol*C has self-activation property and reported to play a significant role in hairy root growth (17-fold increase); however, *rol* A, B, and C together had 75-fold increase in *Atropa belladonna* (Bonhomme et al. 2000), while the TR-DNA possesses the genes for opine synthesis and the genes involved in the auxin biosynthesis, i.e., *aux1* and *aux2*. The synergistic function of *rol*B, *rol*C, *ORF13*, and *ORF14* of TL-DNA of *A. rhizogenes* in hairy root induction in *Nicotiana tabacum* and the effect of these genes on the rolB-mediated rooting were in the order ORF13 > rolC≤ORF14 (Aoki and Syono 1999). The Ri plasmid of *R. rhizogenes* strain has *rolC* and *aux1* genes present on T-DNA designated as TL-DNA and TR-DNA, respectively. The detection of these genes in the hairy roots of host is indicative of T-DNA integration into the host genome. The virulence (*vir*) loci lying outside the T-DNA encode trans-acting products involved in early events in the plant-pathogen interaction. The *vir* loci are composed of six tightly regulated transcriptional units, *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, and *vir*G, in different *Agrobacteria*. The *virD2* gene is localized outside the T-DNA of Ri-plasmid, and this feature serves as diagnostics for the presence of any leftover *Agrobacteria* in the root tissue (Thiruvengadam et al. 2016). These genes play a role in inducing the expression of defense genes, thereby eliciting the production of secondary metabolite in and development of transformed roots.

9.3 Establishment of Hairy Root Cultures

9.3.1 Induction of Hairy Roots

The hairy root induction is affected by the type of strains of *A. rhizogenes*, co-cultivation period, type of explants, media composition, and PGRs. We have investigated these factors in our earlier study (Singh et al. 2020) and discussed here along with updated information available in literature, in brief.

9.3.1.1 *A. rhizogenes* Strains

The virulence of the *A. rhizogenes* strains has been observed to vary strain-wise, and it has effect on the frequency of the hairy root formation. In our experiment (Singh et al. 2020), in *S. bryopteris* the strain LBA 1334 was found to induce hairy root formation, while other two MTCC series strains (MTCC 532 and MTCC 2364) could not. Perhaps, it was believed that the recalcitrance in *S. bryopteris* to genetic transformation and the virulence of these strain affected the hairy root formation. Kim et al. (2015) reported HRC of *Silene vulgaris* by infecting leaf explants with *A. rhizogenes* strains, LBA9402, R1000, A4, 13333, and 15834, and the strain LBA9402 had induced the most hairy roots per plant. In *Withania somnifera*, the hairy root induction by above strains of *A. rhizogenes* (MTCC 2364, MTCC

532, R1000) showed the strain R1000 to be highly virulent in inducing hairy roots (50.6%), in comparison to MTCC 2364 (29.3%) and MTCC 532 (18.6%) (Chandrasekaran et al. 2015). Explants infected by MTCC 2364 and MTCC 532 showed maximum 77.6% and 67.6% of hairy root induction, respectively (Balasubramanian et al. 2018). Among different strains (R1000, 15834, and A4), R1000 was the most promising for hairy root stimulation as it was found to induce the highest growth rate, root number, root length, and transformation efficiency in *Fagopyrum tataricum* (Thwe et al. 2016). Joseph Sahayarayan et al. (2020) investigated effects of *A. rhizogenes* strains such as 15834, 13333, A4, R1200, R1000, LBA9402, R1301, and R1601 hairy root induction in *Cucumis anguria*. Their finding also supported the R1000 strain of *A. rhizogenes* as the most virulent and the best strain for hairy root initiation of *C. anguria* from cotyledon explants. Yousefian et al. (2020) reported hairy root induction in *Mentha spicata* by direct injecting of explants with *A. rhizogenes* strains (A13, R318, ATCC15834 A4, and GMI 9534). They found that of these four strains, the strain A13 exhibited the highest transformation efficiency (~75%).

9.3.1.2 Co-Cultivation Period

The co-cultivation period during transformation of explants with the *R. rhizogenes* strain has also shown to impact the hairy root formation. In *S. bryopteris*, the effect of co-cultivation period was examined for hairy root induction with the strain LBA1334 grown along with explants for 24 and 48 h (Singh et al. 2020). It was observed that the hairy root appeared after 6 days of infection with this strain in case of 48 h co-cultivation only. In *Raphanus sativus*, co-cultivation of explants with *A. rhizogenes* strain in half-MS medium containing acetosyringone (100 μM) for 2 days had the maximum effect on hairy root induction (Balasubramanian et al. 2018).

9.3.1.3 Type of Explants

Hairy roots usually induced in explants such as leaf, cotyledon, hypocotyl, node, stem, and root within 1–4 weeks of culture. Singh et al. (2020) reported hairy root induction after 6 days of infection using *A. rhizogenes* strain LBA1334 co-cultivated with root network as explants for 48 h after transformation. Yousefian et al. (2020) found that the middle and lower internodes of stem were highly susceptible to infection by *A. rhizogenes*, and these showed a higher rate of transformation. In *Cucumis melo,* cotyledon as explants was observed to give higher frequency of hairy root formation (Pak et al. 2009).

9.3.1.4 Media

The media compositions do affect the hairy root formation. Singh et al. (2020) tested different media like MS, SHFR (Stag Horn Fern Rooting), and Knops during transformation for hairy root induction by *A. rhizogenes* and found that SHFR plus TDZ (2 mg/L) and Bavistin (0.1%) showed good response in transformation and hairy root formation. The culture media incorporated with acetosyringone (an amino acid-derived phenolic compound) utilized by the invading *Agrobacterium* for food enhances the transformation rate by inducing the expression of *vir* gene (Veluthambi et al. 1989). Rana et al. (2016) reported the effect of medium supplements (30 g L^{-1} sucrose, 0.1 g L^{-1} l-glutamine and 5 g L^{-1} polyvinylpolypyrrolidone) on *A. rhizogenes*-mediated hairy root induction in *Camellia sinensis* var. sinensis. In HRC of *A. indica*, different media varying in ionic strength such as Ohyama and Nitsch (ON), Gamborg's B5, and MS basal were used that yielded maximum amounts of biomass and azadirachtin in correlation with their ionic strength (Satdive et al. 2007). As ON medium contained the higher ionic concentrations of inorganic salts than the MS and B5, it was found to favor the growth of hairy roots as well as azadirachtin production.

9.3.1.5 PGRs

Though hairy roots have inherent ability to grow even in the absence of PGRs, the addition of PGR elicits the secondary metabolite production. Singh et al. (2020) reported SHFR media modified with PGRs in combination or alone (Kinetin, TDZ and Bavistin). The SHFR media with TDZ (2 mg/L) and Bavistin (0.1%) was found to increase the transformation efficiency and propagation of hairy roots (65%), while with Kinetin (2 mg/L) alone the response was 40% only. The phytohormone treatment, 0.3 mg L^{-1} IBA and 100 μM MeJA, in *M. spicata* was reported to substantially increase in the hairy root growth and phenolic acid accumulation (Yousefian et al. 2020). Joseph Sahayarayan et al. (2020) found that MS medium supplemented with IBA + NAA (2.46 + 1.07) had the maximum accumulation of biomass (0.68 g/L dry wt. and 6.52 ± 0.49 g/L fresh wt.) in 21-day-old transgenic hairy roots of *Cucumis anguria*. Methyl jasmonate elicited the biosynthesis of triterpenoid saponins in the hairy roots of *S. vulgaris* leading to increased level of segetalic acid (fivefold) and gypsogenic acid (twofold) than control hairy root (Kim et al. 2015). In transgenic hairy root line of *Taxus x media* var. Hicksii carrying a *taxadiene synthase* gene, the paclitaxel production and phenyl ammonia lyase activity upon elicitation with nitric oxide and methyl jasmonate yielded the highest paclitaxel content (7.56 mg L^{-1}) (Sykłowska-Baranek et al. 2015).

9.3.2 Molecular Confirmation of Hairy Roots

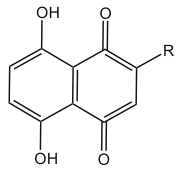
The integration of Ri T-DNA into the genome of host plant cells causes the induction of hairy roots. The Ri T-DNA contains *rol* and *aux* genes, so these genes also integrate into the host genome along with T-DNA. The transgenic nature of hairy roots could be confirmed by simply amplifying these genes by PCR using *rolC* and *aux1* gene primers. While the non-transgenic hairy root line can be detected by amplifying the *virD2* gene present in in Ri-plasmid but not on T-DNA, it could be used as a diagnostic marker for the presence of any remaining *Agrobacteria* in the root tissue (Thiruvengadam et al. 2016). In *S. bryopteris*, *rol*A and *vir*C genes were PCR-amplified from the hairy roots indicating that the hairy roots developed were due to T-DNA integration and not due to any *A. rhizogenes* present (Singh et al. 2020).

9.4 Productions of Secondary Metabolites in Using Hairy Root

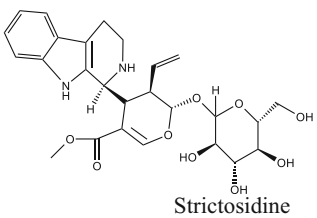
HRC-based production of several plant secondary metabolites with known bioactivities such as shikonins, stilbenes, lignans azadirachtin, camptothecins, paclitaxel, hyoscyamine, harpagide, ginsenosides, and scopolamine was reported (Sim and Chang 1993; Satdive et al. 2007; Almagro et al. 2015; Zhang et al. 2017; Balasubramanian et al. 2018; Singh et al. 2020; Barba-Espin et al. 2020; Wawrosch and Zotchev 2021; Table 9.1; Fig. 9.1).

9.4.1 Shikonin

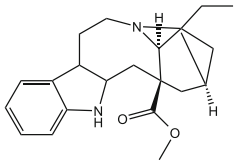
The HRC of *Lithospermum erythrorhizon* was reported long back in 1991 by Shimomura et al. (1991) using in vitro grown shoots for transformation with *A. rhizogenes* strain 15,834. They found that the hairy roots cultured on MS solid medium failed to produce any red pigments; however, in solid or liquid root culture media, a large amount of red pigments was produced. Further, they added adsorbents to the culture medium that stimulated shikonin production by approximately threefold. In *Echium plantagineum*, the hairy root lines developed using *A. rhizogenes* strain ATCC15834 (Fu et al. 2020) showed significant difference in the biomass and shikonin production in the 1/2B5 and M9 media. It was observed that the biomass in the 1/2B5 medium was fivefold than the M9 and the content of acetylshikonin was twofold in the 1/2B5 medium (36.25 mg/L on average) than the M9 medium.



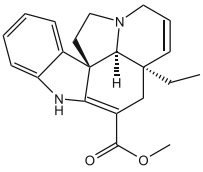
Shikonins



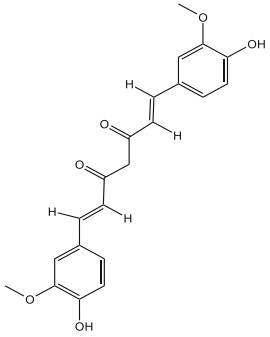
Strictosidine



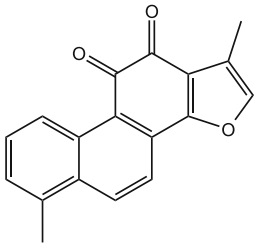
Catharanthine



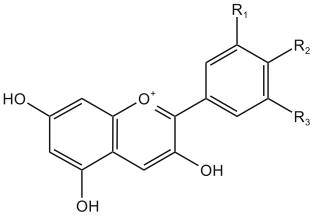
Tabersonine



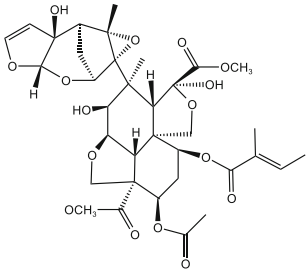
Curcumin



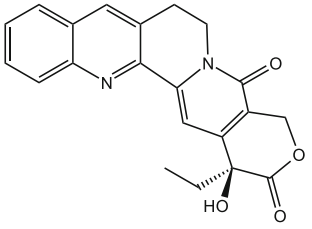
Tanshinones



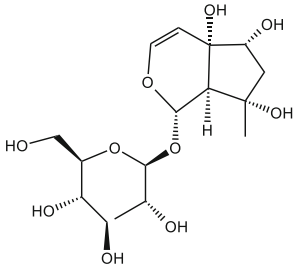
Anthocyanin



Azadirachtin

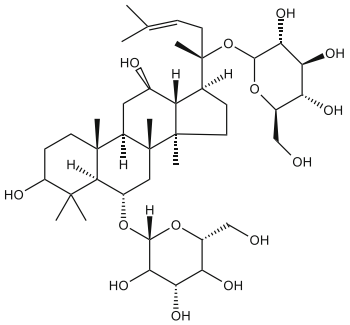


Camptothecins

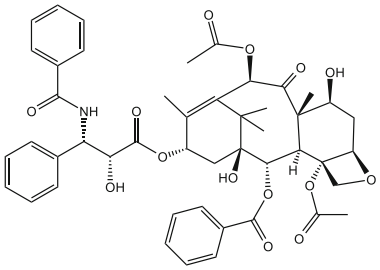


Harpagide

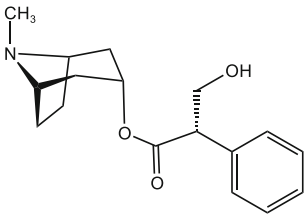
Fig. 9.1 Chemical structures of some of the well-known secondary metabolites produced in HRC



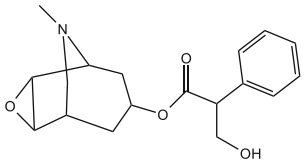
Ginsenosides



Paclitaxel



Hyoscyamine



Scopolamine

Fig. 9.1 (continued)

9.4.2 Phenolic Compounds of HRC

Several phenolic compounds such as rosmarinic acid, caffeic acid, chlorogenic acid, lithospermic acid B, and cinnamic acid were observed in the hairy roots of *M. spicata*, developed by infection with different *A. rhizogenes* strains (ATCC15834, A13, A4, 9534, and R318), and their content was compared (Yousefian et al. 2020). Ho et al. (2018) reported HRCs of *Polygonum multiflorum* from leaf explants infected by *A. rhizogenes* strain KCCM 11879 with more than 60% transformation efficiency after 21 days of co-cultivation. It was found that the line HR-01 yielded the highest biomass (9.7 g L^{-1} of DW) and total phenolic content (26.64 mg g^{-1} DW).

9.4.3 Triterpenoid Saponins

Phytochemical studies of *Gynostemma pentaphyllum* revealed nearly 90 dammarane-type saponin glycosides, known as gypenosides, that show pharmacological activities (Yin et al. 2004). HRC of *G. pentaphyllum* was established by infecting leaf discs with *A. rhizogenes* (Chang et al. 2005); it yielded 7.3 g L(−1) the dry wt. of biomass in MS medium for a period of 49 days with a gypenoside content

of 38 mg g(−1) dry wt. Kim et al. (2015) also reported the in vitro production of triterpenoid sapogenins in HRC of *S. vulgaris* by infecting leaf explants with five strains of *A. rhizogenes*.

9.4.4 Artemisinin

Mass cultivation of hairy roots in *Artemisia annua* was reported in a modified 3-L stirred tank bioreactor using optimized culture conditions (Patra and Srivastava 2014). In this bioreactor, it was possible to produce biomass, 18 g L(−1) (dry wt.), and artemisinin content, 4.63 mg L(−1), in a period of 28 days, which further increased to 10.33 mg L(−1) in response to methyl jasmonate. Elicitation of artemisinin production with 150 mg chitosan l(−1) in hairy roots of *Artemisia annua* was reported to be increased sixfold to 1.8 microg mg(−1) dry weight in 6 days (Putalun et al. 2007). Wang et al. (2006) reported use of oligosaccharide elicitor (MW < 2500) from an endophytic fungus, *Colletotrichum gloeosporioides*, for the stimulation of artemisinin production in hairy roots of *A. annua*. They observed that the 23-day-old hairy roots on exposure to the elicitor at 0.4 mg/mL for 4 days yielded the maximum artemisinin content, 13.51 mg/L (51.63% increase over the control). Further, it was also reported that the nitric oxide generated by an oligosaccharide elicitor from *Fusarium oxysporum* mycelium potentiates its role in induction of artemisinin production in *A. annua* hairy roots (Zheng et al. 2008). The combination of sodium nitroprusside (NO donor) with OE increased artemisinin content from 1.2 to 2.2 mg/g dry wt., whereas the content of artemisinin in HRC was 28.5 mg/L, a twofold increase over the OE treatment alone. Tetraploid clones of *Artemisia annua* hairy roots produce more artemisinin (six times) than diploid parent (De Jesus-Gonzalez and Weathers 2003). The physical and chemical factors such as light, pH value 5.4 of the medium, and 3% sucrose in the medium along with gibberellin (4.8 mg/L) had effect on the growth of the hairy roots and production of artemisinin (Cai et al. 1995).

9.4.5 Paclitaxel

Paclitaxel (trade name: Taxol®) is a potent anticancer agent extracted from the yew plants (*Taxus* spp.). Due to its low accumulation levels in the plants of *Taxus* spp., the yield of paclitaxel is limited and could not meet the demand for the pharmaceutical use. Therefore, alternative methods including HRC have been attempted for in vitro production of paclitaxel from *Taxus* spp. (Kim et al. 2009; Sykłowska-Baranek et al. 2015; He et al. 2022). HRCs have been reported in different *Taxus* spp. such as of *T. cuspidate* (Korean yew) (Kim et al. 2009), *Taxus x media* var. Hicksii (Sykłowska-Baranek et al. 2015); and *T. baccata* (He et al. 2022) using *A. rhizogenes*. Kim et al. (2009) reported that methyl jasmonate treatment of the

hairy root line (RC11106) led to accumulation of 52.5 mg/L of taxol over 2 weeks of incubation. Paclitaxel production in HRC of *Taxus x media* var. Hicksii with a *taxadiene synthase* transgene upon elicitation by nitric oxide and methyl jasmonate enhanced the paclitaxel content to 7.56 mg L^{-1} after 2 weeks of treatment (Sykłowska-Baranek et al. 2015).

9.4.6 Azadirachtin

Azadirachtin is a limonoid ($C_{35}H_{44}O_{16}$, tetranortriterpenoid) and the major component of widely used neem (*Azadirachta indica)*-based biopesticides. In HRC of *A. indica*, the effect of ON, Gamborg's B5, and MS basal media was investigated on yield of biomass and azadirachtin content (Satdive et al. 2007). Out of the three media, ON medium, that contained higher ionic concentrations of inorganic salts than the MS and B5 media, favored the growth and azadirachtin production (0.0166% dry weight, DW). Further, addition of biotic elicitor increased the production of azadirachtin by nearly fivefold (0.074% DW), while jasmonic acid and salicylic acid showed an approximately six- (0.095% DW) and ninefold (0.14% DW) increase, respectively, on ON medium (Satdive et al. 2007). The mass culture of hairy roots of *A. indica* in gas-phase reactors (nutrient spray and nutrient mist bioreactor) yielded the biomass 9.8 g/L dry wt. and azadirachtin accumulation of 2.8 mg/g (Srivastava and Srivastava 2012).

9.4.7 Camptothecin

Camptothecin, an important anticancer drug, is believed to be a potent topoisomerase inhibitor that interferes with the topoisomerase in DNA replication HRC of *Ophiorrhiza alata* which was developed by infection of nodal explants of in vitro-grown plant with *A. rhizogenes* TISTR 1450 for camptothecin (CPT) production (Ya-ut et al. 2011). The content of CPT in various parts of *O. alata* was analyzed by HPLC and found to be double in transformed hairy roots (785 ± 52 μg/g dry wt) than in the soil-grown plants (388 ± 32 μg/g dry wt). Kamble et al. (2011) reported hairy root induction in *O. rugosa*, another source of CPT, using *A. rhizogenes* strain LBA9402. The CPT contents in the hairy roots and in vitro-grown transformed shoots were 0.009% d.w. and 0.012% d.w., respectively.

9.4.8 Ginsenoside

Ginseng (*Panax ginseng*) roots contain ginsenosides (saponins), utilized for its bioactive properties such as immunomodulatory, hepato- and cardioprotective,

stamina booster, antifatigue, and physiological and pharmacological effects. Yu et al. (2005) investigated the impact of temperature and light quality on biomass and ginsenoside content of HRC in the bioreactor. They observed that biomass of hairy roots was highest under dark or red light, while ginsenoside content was optimum under fluorescent light. Kochan et al. (2013) reported three independently generated hairy root (A, G, and B) of *P. quinquefolium* upon infection by *A. rhizogenes*. It was found that the line A had the highest increase of dry biomass (above eightfold) followed by line G (sevenfold) and line B (fivefold) for the period of 28 days culture. In the developed hairy roots of *P. quinquefolium*, the total ginsenoside level in hairy root lines A, G, and B were recorded to be about 10, 8, and 6 mg/g dw, respectively (Kochan et al. 2013).

9.4.9 Hyoscyamine

In *Datura stramonium*, HRC developed with hairy roots of different ploidy (diploid and tetraploid) levels was investigated by Pavlov et al. (2009) for hyoscyamine biosynthesis. The ploidy level difference was observed in content of hyoscyamine and the influence of nutrients growth of hairy root. The HRC of tetraploid plants had the maximum yield of hyoscyamine (177 mg/L) in MS medium with 6% sucrose.

9.4.10 Scopolamine

Scopolamine is a tropane alkaloid that shows anticholinergic property. The overexpression of genes of scopolamine biosynthesis *putrescine N-methyltransferase* (*PMT*) and *hyoscyamine 6 β-hydroxylase* (*H6H*) in *Hyoscyamus niger*'s transgenic HRC produced significantly higher ($P < 0.05$) levels of scopolamine in comparison to the wild-type and transgenic lines harboring *pmt* or *h6h* gene alone (Zhang et al. 2004). The line (T3) overexpressing the genes, *pmt* and *h6h*, was observed to produce the highest content of scopolamine (411 mg/L) followed by single-gene (*h6h*) transgenic line H11 (184 mg/L) and wild type (43 mg/L). Dehghan et al. (2017) found that the tetraploidy improved the overexpression of *h6h* and scopolamine production of *H. muticus*.

9.4.11 Harpagide

Harpagide is an *iridoid* glycosides that show anti-inflammatory properties. Only a few literature are available on the hairy root line development for harpagide production. Piątczak et al. (2019) reported HRC in *Rehmannia elata* (Orobanchaceae) from shoot tips and leaves by the infection of *A. rhizogenes* strain (A4). They

observed the different levels of iridoid glycoside content, in the hairy root line (S1 line) were aucubin (0.2 mg/g DW) and harpagide (1.57 mg/g DW), and in L14 line, harpagoside (0.09 mg/g DW) were the highest.

9.5 Upscaling the Productions of Secondary Metabolites in Bioreactors Using HRCs

For scaling up the production of secondary metabolites and biomass of hairy roots, a large vessel (bioreactor) is needed that can provide the best conditions for optimum growth and secondary metabolite production. But the delicate and interconnected form of the hairy roots makes the growth measurement and designing of a large-scale culture system a difficult task. Stiles and Liu (2013) have discussed the major factors related to large-scale bioreactor cultures such as process intensification technologies and the mathematical models and computer-aided methods of bioreactor design and development. Wyslouzil et al. (2000) suggested that the bioreactor design for HRC is a balancing act between the biological needs of the tissues without inducing an additional and undesirable biological response. The bioreactors used in HRC are usually (1) liquid-phase, (2) gas-phase, and (3) hybrid reactors.

Bioreactor designs for HRC are challenging and should meet the criteria such as least mechanical agitation (may cause wounding and shearing of delicate hairy roots leading to callus formation), uninterrupted nutrient flow (hampers due to branching and interconnected forms hairy roots), regular oxygen supply, nutrient uptake, etc. in the medium. An air-lift fermenter system along with a XAD-2 column was used for the shikonin production from HRC of *L. erythrorhizon* (Shimomura et al. 1991), which continuously produced ~5 mg/day of shikonin during a period of over 220 days. Srivastava and Srivastava (2012) reported azadirachtin production by hairy roots of *A. indica* using HRC in gas-phase reactors (nutrient spray and nutrient mist bioreactor). They found the biomass production of 9.8 g/L dry weight with azadirachtin accumulation of 2.8 mg/g biomass (27.4 mg/L) during 25 days of batch cultivation period.

The study by Zhang et al. (2004) provided an effective approach for large-scale commercial production of scopolamine by using transgenic hairy root culture systems as bioreactors. Homova et al. (2010) investigated the capacity of HRC of *Harpagophytum procumbens* to accumulate four phenylethanoids, viz., glycosides beta-OH-verbascoside, verbascoside, leucosceptoside A, and martynoside in shake flasks and a stirred tank reactor. They observed equally highest contents of verbascoside in both kinds of culture (1.12 mg/g dry weight), while leucosceptoside A content was 1.6 times higher in bioreactor than in shake flask. Several reviews are available discussing bioreactor-based scale-up using HRCs (Valdiani et al. 2019).

9.6 Elicitors Increase the Secondary Metabolite Production in HRCs

Various biotic and abiotic elicitors are used in HRCs to elicit the biosynthesis and the accumulation of secondary metabolites (Yousefian et al. 2020; Singh et al. 2020; Joseph Sahayarayan et al. 2020). These elicitors are known to activate different genes such as secondary metabolic pathway, defense-related or signaling pathway, and transcription factors. In general, biotic elicitors used in HRC include fungal cell extracts, fungal or plant polysaccharides, and heavy metals, while the abiotic elicitors include PGRs (auxins, TDZ, cytokinins), signaling molecules (nitric oxide, hydrogen peroxide, salicylic acid, methyl jasmonate), UV radiation, ultrasound, shaking, and temperature. For the best result on elicitation of in vitro production of metabolites in the medium, the selection of suitable elicitor, its level to be added, and treatment duration need to be standardized. The elicitor treatments like other tissue culture methods also stimulate the production and release of intracellular products in the hairy roots.

9.7 Prospect and Challenges

HRC-based in vitro culture is a promising approach for production of a wide range of secondary metabolites as compared to other in vitro methods such as cell culture and adventitious root culture (Table 9.2). Several factors critical to increase in yield of HRC have been standardized such as light, oxygen, temperature, phytohormones, heavy metals, elicitors, external stimuli, etc. The metabolic pathway engineering for modulation of expression of key genes either by overexpression of genes and transcription factors or by downregulation of competing pathway genes has also

Table 9.2 Comparison of hairy root culture with other tissue culture methods

Description	Hairy root culture	Cell culture	Adventitious root culture
Production of secondary metabolite	Comparable to roots without elicitation	Elicitation may require	Elicitation may require
Tissue type	Differentiated	Undifferentiated	Differentiated
Phytohormone required	No	Yes	Yes
Growth rate	Higher	Slower but high in liquid cell suspension	Higher
Genetic and biochemical stability	Stable for several subculture	Unstable	Stable
Genetic engineering possible	Yes	Yes	Yes
Scale-up	Yes	Yes	Yes
Cost	Expensive	Expensive	Expensive

led to increased yield of end-products. With advances in genomics and molecular biology, today we have a better understanding of the biosynthetic pathway and their regulation, thus realizing that the biosynthetic potential of hairy roots is more feasible. Commercial production of secondary metabolites that have phytoceutical uses, from field-grown plants, can't meet the market demand, for example, the extraction of catharanthine and vindoline from field plants and then chemical semi-synthesis of vinblastine and vincristine, two chemicals usually found in the FDA drug shortage list due to supply chain problems (Kaiser 2011). Due to complex chemical structures, most of secondary metabolite economic feasibility for the industrial mass production through chemical synthesis is not possible and thus not popular as method of choice.

Despite vast potential and prospect of HRCs, very few companies (public or private sector) have initiated the HRC-based commercial production phytochemicals from hairy roots or any in vitro methods unlike a large number of companies worldwide involved in the mass production of tissue culture raised plants. ROOTec bioactives Ltd. (www.rootec.com) is the only company founded in 2005 in Switzerland for the commercial production of plant-derived molecules using bioreactor technology to provide products for the pharmaceutical and cosmetic markets. Besides in vitro production of secondary metabolites, the hairy roots have been useful for the functional validation of genes, plant synthetic biology, genome editing, symbiotic root colonization, antimicrobial excretion, and phytoremediation (Morey and Peebles 2022). Deciphering the competing biosynthetic pathways or identifying feedback or feed-forward reactions in metabolite production and then devising strategies is important for increasing the yield desired product. The major hurdle in commercial exploitation of HRC is the development of suitable protocol for the sustainable growth of hairy roots and designing of appropriate bioreactors for scaling-up without damaging the delicate hairy roots. Metabolic engineering for manipulation of biosynthetic pathways for high yield of secondary metabolites is easier nowadays with the availability of vast genomic and transcriptomic datasets helping better understanding of regulation of pathways.

9.8 Conclusion

Among various tissue culture methods, HRC has the potential to be harnessed for in vitro production of secondary metabolites at commercial level. It could be an economically viable option for sustainable in vitro production of secondary metabolites, as the hairy roots are normally high yielding due to their fast growth rates, possible growth in hormone-free media, and genetic and biosynthetic stability. These are also amenable to genetic manipulation using genetic engineering including recent gene-editing approaches. In future, the HRC efficacy and commercial viability could be enhanced by adopting advanced biochemical processing methods for metabolite extractions, genetic manipulations, and elicitations for enhanced yield and bioreactor-based up-scaling.

References

Almagro L, Fernández-Pérez F, Pedreño MA (2015) Indole alkaloids from Catharanthus roseus: bioproduction and their effect on human health. Molecules 20(2):2973–3000

Aoki S, Syōno K (1999) Synergistic function of rolB, rolC, ORF13 and ORF14 of TL-DNA of Agrobacterium rhizogenes in hairy root induction in Nicotiana tabacum. Plant Cell Physiol 40 (2):252–256

Bakkali AT, Jaziri M, Foriers A et al (1997) Lawsone accumulation in normal and transformed cultures of henna, *Lawsonia inermis*. Plant Cell Tissue Organ Cult 51:83–87. https://doi.org/10.1023/A:1005926228301

Balasubramanian M, Anbumegala M, Surendran R, Arun M, Shanmugam G (2018) Elite hairy roots of *Raphanus sativus* (L) as a source of antioxidants and flavonoids. 3 Biotech 8:128. https://doi.org/10.1007/s13205-018-1153-y

Barba-Espin G, Chen ST, Agnolet S, Hegelund JN, Stanstrup J, Christensen JH et al (2020) Ethephon-induced changes in antioxidants and phenolic compounds in anthocyanin-producing black carrot hairy root cultures. J Exp Bot 71:7030–7045. https://doi.org/10.1093/jxb/eraa376

Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux MA, Jacquin-Dubreuil A (2000) Tropane alkaloid production by hairy roots of Atropa belladonna obtained after transformation with Agrobacteriumrhizogenes 15834 and Agrobacterium tumefaciens containing rolA, B, C genes only. J Biotechnol 81(2–3):151–158

Bulgakov VP (2008) Functions of rol genes in plant secondary metabolism. Biotechnol Adv 26: 318–324

Cai G, Li G, Ye H, Li G (1995) Hairy root culture of *Artemisia annua* L. by Ri plasmid transformation and biosynthesis of artemisinin. Chin J Biotechnol 11(4):227–235

Chandrasekaran T, Soundar RC, Kandhan V, Abubakker A, Appakan S (2015) Improved *Agrobacterium rhizogenes*-mediated hairy root culture system of *Withania somnifera* (L.) Dunal using sonication and heat treatment. 3 Biotech 5:949–956

Chang CK, Chang KS, Lin YC, Liu SY, Chen CY (2005) Hairy root cultures of *Gynostemma pentaphyllum* (Thunb.) Makino: a promising approach for the production of gypenosides as an alternative of ginseng saponins. Biotechnol Lett 27(16):1165–1169. https://doi.org/10.1007/s10529-005-8653-7

De Jesus-Gonzalez L, Weathers PJ (2003) Tetraploid artemisia annua hairy roots produce more artemisinin than diploids. Plant Cell Rep 21(8):809–813. https://doi.org/10.1007/s00299-003-0587-8

Dehghan E, Reed DW, Covello PS, Hasanpour Z, Palazon J, Oksman-Caldentey KM, Ahmadi FS (2017) Genetically engineered hairy root cultures of *Hyoscyamus senecionis* and *H. muticus*: ploidy as a promising parameter in the metabolic engineering of tropane alkaloids. Plant Cell Rep 36(10):1615–1626. https://doi.org/10.1007/s00299-017-2178-0

Fu JY, Zhao H, Bao JX, Wen ZL, Fang RJ, Fazal A, Yang MK, Liu B, Yin TM, Pang YJ, Lu GH, Qi JL, Yang YH (2020) Establishment of the hairy root culture of *Echium plantagineum* L and its shikonin production. 3 Biotech 10(10):429. https://doi.org/10.1007/s13205-020-02419-7

Gutierrez-Valdes N, Häkkinen ST, Lemasson C, Guillet M, Oksman-Caldentey KM, Ritala A, Cardon F (2020) Hairy root cultures-a versatile tool with multiple applications. Front Plant Sci 11:33. https://doi.org/10.3389/fpls.2020.00033. PMID: 32194578; PMCID: PMC7064051

He J, Plácido JPA, Pateraki I, Kampranis S, Favero BT, Lütken H (2022) Hairy root induction of *Taxus baccata* L. by natural transformation with *Rhizobium rhizogenes*. Horticulturae 9(1):4. https://doi.org/10.3390/horticulturae9010004

Ho TT, Lee JD, Ahn MS et al (2018) Enhanced production of phenolic compounds in hairy root cultures of *Polygonum multiflorum* and its metabolite discrimination using HPLC and FT-IR methods. Appl Microbiol Biotechnol 102:9563–9575. https://doi.org/10.1007/s00253-018-9359-9

Homova V, Weber J, Schulze J, Alipieva K, Bley T, Georgiev M (2010) Devil's claw hairy root culture in flasks and in a 3-L bioreactor: bioactive metabolite accumulation and flow cytometry.

Zeitschrift fur Naturforschung. C. J Biosci 65(7–8):472–478. https://doi.org/10.1515/znc-2010-7-809

Joseph Sahayarayan J, Udayakumar R, Arun M, Ganapathi A, Alwahibi MS, Aldosari NS, Morgan AMA (2020) Effect of different *Agrobacterium rhizogenes* strains for *in-vitro* hairy root induction, total phenolic, flavonoids contents, antibacterial and antioxidant activity of (*Cucumis anguria* L.). Saudi J Biol Sci 27(11):2972–2979. https://doi.org/10.1016/j.sjbs.2020.08.050

Kaiser J (2011) Shortages of cancer drugs put patients, trials at risk. Science 332(6029):523–523. https://doi.org/10.1126/science.332.6029.523

Kamble S, Gopalakrishnan R, Eapen S (2011) Production of camptothecin by hairy roots and regenerated transformed shoots of *Ophiorrhiza rugosa* var. decumbens. Nat Prod Res 25(18): 1762–1765. https://doi.org/10.1080/14786419.2011.559469. Epub 2011 Sep 8. PMID: 21899480

Khan MY, Saleh A, Kumar V, Rajkumar S (2009) Recent advances in medicinal plant biotechnology. Indian J Biotechnol 8:9–22

Kim JA, Baek KH, Son YM et al (2009) Hairy root cultures of *Taxus cuspidate* for enhanced production of paclitaxel. J Korean Soc Appl Biol Chem 52:144–150. https://doi.org/10.3839/jksabc.2009.027

Kim YB, Reed DW, Covello PS (2015) Production of triterpenoid sapogenins in hairy root cultures of *Silene vulgaris*. Nat Prod Commun 10(11):1919–1922

Kochan E, Wasiela M, Sienkiewicz M (2013) The production of ginsenosides in hairy root cultures of American Ginseng, *Panax quinquefolium* L. and their antimicrobial activity. In Vitro Cell Dev Biol Plant 49(1):24–29. https://doi.org/10.1007/s11627-012-9469-5. Epub 2012 Nov 13. PMID: 23459700; PMCID: PMC3580037

Morey KJ, Peebles CAM (2022) Hairy roots: an untapped potential for production of plant products. Front Plant Sci 13:937095. https://doi.org/10.3389/fpls.2022.937095. PMID: 35991443; PMCID: PMC9389236

Nemoto K, Hara M, Suzuki M, Seki H, Oka A, Muranaka T, Mano Y (2009) Function of the aux and rol genes of the Ri plasmid in plant cell division in vitro. Plant Signal Behav 4(12): 1145–1147. https://doi.org/10.4161/psb.4.12.9904

Pak HK, Sim JS, Rhee Y et al (2009) Hairy root induction in oriental melon (*Cucumis melo*) by *Agrobacterium rhizogenes* and reproduction of the root-knot nematode (*Meloidogyne incognita*). Plant Cell Tissue Organ Cult 98:219–228. https://doi.org/10.1007/s11240-009-9556-4

Park CH, Xu H, Yeo HJ, Park YE, Hwang GS, Park NI et al (2021) Enhancement of the flavone contents of *Scutellaria baicalensis* hairy roots via metabolic engineering using maize Lc and Arabidopsis PAP1 transcription factors. Metab Eng 64:64–73. https://doi.org/10.1016/j.ymben.2021.01.003

Patra N, Srivastava AK (2014) Enhanced production of artemisinin by hairy root cultivation of *Artemisia annua* in a modified stirred tank reactor. Appl Biochem Biotechnol 174(6): 2209–2222. https://doi.org/10.1007/s12010-014-1176-8

Patra N, Srivastava AK (2016) Artemisinin production by plant hairy root cultures in gas- and liquid-phase bioreactors. Plant Cell Rep 35(1):143–153. https://doi.org/10.1007/s00299-015-1875-9

Pavlov A, Berkov S, Weber J, Bley T (2009) Hyoscyamine biosynthesis in *Datura stramonium* hairy root *in vitro* systems with different ploidy levels. Appl Biochem Biotechnol 157(2): 210–225. https://doi.org/10.1007/s12010-008-8264-6. Epub 2008 May 29. PMID: 18509605, 157, 210

Piątczak E, Jeleń A, Makowczyńska J, Zielińska S, Kuźma Ł, Balcerczak E (2019) Establishment of hairy root cultures of *Rehmannia elata* N.E. Brown ex Prain and production of iridoid and phenylethanoid glycosides. Ind Crop Prod 137:308–314. https://doi.org/10.1016/j.indcrop.2019.05.022. ISSN 0926-6690

Purwianingsih W, Febri S, Kusdianti (2016) Formation flavonoid secondary metabolites in callus culture of *Chrysanthemum cinerariefolium* as alternative provision medicine. AIP Conf Proc 1708(1):030005. https://doi.org/10.1063/1.4941150

Putalun W, Luealon W, De-Eknamkul W, Tanaka H, Shoyama Y (2007) Improvement of artemisinin production by chitosan in hairy root cultures of Artemisia annua L. Biotechnol Lett 29(7):1143–1146. https://doi.org/10.1007/s10529-007-9368-8

Rana MM, Han ZX, Song DP, Liu GF, Li DX, Wan XC, Karthikeyan A, Wei S (2016) Effect of medium supplements on *Agrobacterium rhizogenes* mediated hairy root induction from the callus tissues of *Camellia sinensis var. sinensis*. Int J Mol Sci 17(7):1132. https://doi.org/10.3390/ijms17071132. PMID: 27428960; PMCID: PMC4964505

Rekha K, Thiruvengadam M (2017) Secondary metabolite production in transgenic hairy root cultures of cucurbits. Trans Sec Metabol 18:267–293. https://doi.org/10.1007/978-3-319-28669-3_6. PMCID: PMC7123301

Satdive RK, Fulzele DP, Eapen S (2007) Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* a. Juss by elicitation and media optimization. J Biotechnol 128(2): 281–289

Shimomura K, Sudo H, Saga H, Kamada H (1991) Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. Plant Cell Rep 10(6–7):282–285. https://doi.org/10.1007/BF00193142

Shoja HM, Shishavani HK (2021) Effects of different hormonal treatments on growth parameters and secondary metabolite production in organ culture of Hyssopus officinalis L. Biotechnologia 102(1):33–41. https://doi.org/10.5114/bta.2021.103760

Silja PK, Satheeshkumar K (2015) Establishment of adventitious root cultures from leaf explants of *Plumbago rosea* and enhanced plumbagin production through elicitation. Ind Crops Prod 76: 479–486. https://doi.org/10.1016/j.indcrop.2015.07.021; ISSN 0926-6690

Sim SJ, Chang HN (1993) Increased shikonin production by hairy roots of *Lithospermum erythrorhizon* in two phase bubble column reactor. Biotechnol Lett 15:145–150. https://doi.org/10.1007/BF00133014

Singh RS, Gara RK, Bhardwaj PK et al (2010) Expression of 3-hydroxy-3-methylglutaryl-CoA reductase, p-hydroxybenzoate-m-geranyltransferase and genes of phenylpropanoid pathway exhibits positive correlation with shikonins content in arnebia [*Arnebia euchroma* (Royle) Johnston]. BMC Mol Biol 11:88. https://doi.org/10.1186/1471-2199-11-88

Singh RS, Chattopadhyay T, Thakur D, Kumar N, Kumar T, Singh PK (2018a) Hairy root culture for *in vitro* production of secondary metabolites: a promising biotechnological approach. In: Kumar N (ed) Biotechnological approaches for medicinal and aromatic plants. Springer, Singapore. https://doi.org/10.1007/978-981-13-0535-1_10

Singh RS, Kesari R, Kumar U et al (2018b) Candidate genes of flavonoid biosynthesis in *Selaginella bryopteris* (L.) baker identified by RNA-Seq. Funct Integr Genomics 18:505–517. https://doi.org/10.1007/s10142-018-0603-2

Singh RS, Thakur D, Jha VK, Kumar U, Pal AK, Singh PK, Fulzele DP (2018c) Antibacterial activity of *Selaginella bryopteris* fronds extract on bacteria isolated from Mastitic Milk. Int J Curr Microbiol App Sci 7:4711–4715

Singh RS, Jha VK, Chattopadhyay T, Kumar U, Fulzele DP, Singh PK (2020) First report of *Agrobacterium rhizogenes*-induced hairy root formation in *Selaginella bryopteris*: a pteridophyte recalcitrant to genetic transformation. Braz Arch Biol Technol 63:e20180679. https://doi.org/10.1590/1678-4324-2020180679

Singh S, Pandey P, Akhtar MQ, Negi AS, Banerjee S (2021) A new synthetic biology approach for the production of curcumin and its glucoside in *Atropa belladonna* hairy roots. J Biotechnol 328:23–33. https://doi.org/10.1016/j.jbiotec.2020.12.022

Srivastava S, Srivastava AK (2012) *In vitro* azadirachtin production by hairy root cultivation of *Azadirachta indica* in nutrient mist bioreactor. Appl Biochem Biotechnol 166(2):365–378. https://doi.org/10.1007/s12010-011-9430-9. PMID: 22083394

Stiles AR, Liu CZ (2013) Hairy root culture: bioreactor design and process intensification. Adv Biochem Eng Biotechnol 134:91–114. https://doi.org/10.1007/10_2013_181. PMID: 23604206

Sykłowska-Baranek K, Grech-Baran M, Naliwajski MR et al (2015) Paclitaxel production and PAL activity in hairy root cultures of *Taxus x media var. Hicksii* carrying a taxadiene synthase

transgene elicited with nitric oxide and methyl jasmonate. Acta Physiol Plant 37:218. https://doi.org/10.1007/s11738-015-1949-x

Thiruvengadam M, Rekha K, Chung IM (2016) Induction of hairy roots by *Agrobacterium rhizogenes*-mediated transformation of spine gourd (*Momordica dioica* Roxb. Ex. willd) for the assessment of phenolic compounds and biological activities. Sci Hortic 198:132–141. https://doi.org/10.1016/j.scienta.2015.11.035

Thwe A, Valan Arasu M, Li X, Park CH, Kim SJ, Al-Dhabi NA, Park SU (2016) Effect of different *Agrobacterium rhizogenes* strains on hairy root induction and phenylpropanoid biosynthesis in Tartary buckwheat (*Fagopyrum tataricum* Gaertn). Front Microbiol 7:318. https://doi.org/10.3389/fmicb.2016.00318

Valdiani A, Hansen OK, Nielsen UB, Johannsen VK, Shariat M, Georgiev MI et al (2019) Bioreactor-based advances in plant tissue and cell culture: challenges and prospects. Crit Rev Biotechnol 39:20–34. https://doi.org/10.1080/07388551.2018.1489778

Veluthambi K, Krishnan M, Gould JH, Smith RH, Gelvin SB (1989) Opines stimulate induction of the *vir* genes of the agrobacterium tumefaciens Ti plasmid. J Bacteriol 171(7):3696–3703. https://doi.org/10.1128/jb.171.7.3696-3703.1989

Wang JW, Zheng LP, Tan RX (2006) The preparation of an elicitor from a fungal endophyte to enhance artemisinin production in hairy root cultures of Artemisia annua L. Sheng Wu Gong Cheng Xue Bao Chin J Biotechnol 22(5):829–834

Wawrosch C, Zotchev SB (2021) Production of bioactive plant secondary metabolites through in vitro technologies-status and outlook. Appl Microbiol Biotechnol 105(18):6649–6668. https://doi.org/10.1007/s00253-021-11539-w

Wyslouzil BE, Waterbury RG, Weathers PJ (2000) The growth of single roots of *Artemisia annua* in nutrient mist reactors. Biotechnol Bioeng 70(2):143–150. https://doi.org/10.1002/1097-0290(20001020)70:2<143::AID-BIT3>3.0.CO;2-B

Ya-ut P, Chareonsap P, Sukrong S (2011) Micropropagation and hairy root culture of *Ophiorrhiza alata* Craib for camptothecin production. Biotechnol Lett 33(12):2519–2526. https://doi.org/10.1007/s10529-011-0717-2. Epub 2011 Aug 9. PMID: 21826402

Yin F, Hu LH, Pan RX (2004) Novel dammarane-type glycosides from *Gynostemma pentaphyllum*. Chem Pharm Bull 52:1440–1444. https://doi.org/10.1248/cpb.52.1440

Yousefian S, Lohrasebi T, Farhadpour M, Haghbeen K (2020) Production of phenolic acids in hairy root cultures of medicinal plant *Mentha spicata* L. in response to elicitors. Mol Biol Res Commun 9(1):23–34. https://doi.org/10.22099/mbrc.2020.36031.1475

Yu K-W, Murthy HN, Hahn E-J, Paek K-Y (2005) Ginsenoside production by hairy root cultures of *Panax ginseng*: influence of temperature and light quality. Biochem Eng J 23(1):53–56. https://doi.org/10.1016/j.bej.2004.07.001

Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus Niger* hairy root cultures. Proc Natl Acad Sci U S A 101(17):6786–6791. https://doi.org/10.1073/pnas.0401391101

Zhang J, Zhou L, Zheng X, Zhang J, Yang L, Tan R et al (2017) Overexpression of SmMYB9b enhances tanshinone concentration in *Salvia miltiorrhiza* hairy roots. Plant Cell Rep 36:1297–1309. https://doi.org/10.1007/s00299-017-2154-8

Zheng LP, Guo YT, Wang JW, Tan RX (2008) Nitric oxide potentiates oligosaccharide-induced artemisinin production in *Artemisia annua* hairy roots. J Integr Plant Biol 50(1):49–55. https://doi.org/10.1111/j.1744-7909.2007.00589.x