
Secondary Metabolite Production in Transgenic Hairy Root Cultures of Cucurbits

11

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Abstract

Cucurbits are important group of vegetables due to their nutritional significance and are also used for valuable traditional medicine. The infection of plants by *Agrobacterium rhizogenes* results in a hairy root (HR) phenotype characterized by rapid growth in hormone-free medium, an unusual ageotropism and extensive lateral branching. These genetically transformed root cultures (hairy roots) can produce levels of secondary metabolites comparable to that of intact plants. Hairy root cultures offer promise for high production and productivity of valuable secondary metabolites in many plants. High stability and productivity features allow the exploitation of HRs as valuable biotechnological tool for the production of plant secondary 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. The main constraint for commercial exploitation of hairy root cultivations is the development and scaling up of appropriate reactor vessels (bioreactors) that permit the growth of interconnected tissues normally unevenly distributed throughout the vessel. Emphasis has focused on designing appropriate bioreactors suitable to culture the delicate and sensitive plant hairy roots. To this end, hairy root culture presents an excellent platform for producing valuable secondary metabolites. For these reasons, this chapter describes the establishment of hairy roots and production of secondary metabolites from hairy roots of cucurbits and also phytochemicals uses for biological activity.

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Keywords

Agrobacterium rhizogenes • Hairy roots • Secondary metabolites • Phenolic compounds • Triterpenoids • Charantin • Biological activity

Abbreviations

CaMV	Cauliflower mosaic virus
ELISA	Enzyme-linked immunosorbent assay
GFP	Green fluorescent protein
Gyp	Gypenosides
HIV	Human immunodeficiency virus
HRs	Hairy roots
HSV	Herpes simplex virus
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
RIPs	Ribosome-inactivating protein
RT PCR	Reverse transcription polymerase chain reaction
TLC	Thin layer chromatography
UHPLC	Ultra-high performance liquid chromatography
WHO	World Health Organization

Contents

1	Introduction	269
2	Establishment of Hairy Root Cultures in Cucurbits	271
2.1	Hairy Root Induction of Cucurbits	271
2.2	Molecular Confirmation of Hairy Roots in Cucurbits	271
2.3	Growth Kinetics of Hairy Root Culture in Cucurbits	275
2.4	Effects of Sucrose Concentration and Different Media on Biomass Accumulation of Hairy Root Cultures of Cucurbits	275
3	Production of Valuable Secondary Metabolites from Hairy Root Cultures of Cucurbits	276
3.1	Phenolic Compounds of Hairy Root Cultures in Cucurbits	276
3.2	Triterpenoids of Hairy Root Cultures in Cucurbits	280
3.3	Ribosome-Inactivating Protein of Hairy Root Cultures in Cucurbits	281
3.4	Charantin of Hairy Root Cultures in Cucurbits	282
3.5	Aroma Essential Oils of Hairy Root Cultures in Cucurbits	282
4	Cucurbit Hairy Root Cultures of Secondary Metabolites Using for Biological Activities	283
4.1	Antioxidant Activity	283
4.2	Antimicrobial Activity	284
4.3	Anticancer Activity	284
4.4	Antidiabetic Activity	285
5	Large Scale Productions of Secondary Metabolites in Bioreactors Using Hairy Root Cultures	285
6	Elicitors Increase the Secondary Metabolites Production in Hairy Root Cultures	286
7	Conclusions	287
	References	288

1 Introduction

Cucurbits are the popular name to the plants of family Cucurbitaceae, which include over 130 genera and 800 species [1], among the economically most important plant families [2, 3]. It is a large group of plants which are medicinally valuable. Cucurbitaceae members are primarily established in the tropical regions of the world. Global production of cucumbers, including gherkins, was among the top ten vegetables produced globally (<http://faostat.fao.org>). Cucurbits are a prominent source of secondary metabolites, and many genera of this family received a great level of scientific interest because of the extensive range of pharmacological and nutraceutical properties [4]. It is reported that the bitter flavor of cucurbits is due to tetracyclic triterpenoids [5]. Various phytochemicals such as alkaloids and saponins are extracted from *Momordica*, *Citrullus*, *Cucurbita*, and *Lagenaria* [6]. The family proved itself as a strong source of food and medicine. Major species of importance include: *Citrullus lanatus* (watermelon), *Cucumis sativus* (cucumber), *Cucumis melo* (musk melon), *Cucumis anguria* (bur gherkin), *Cucurbita pepo* (pumpkin), *Momordica charantia* (bitter melon), *Momordica dioica* (spine gourd), *Coccinia grandis* (ivy gourd), and *Praecitrullus fistulosus* (tinda). In recent years, consumption of cucurbits in the average diet has been highlighted for its contribution towards lowering the risks of several life-threatening diseases such as coronary heart disease, stroke, pulmonary disease, and different types of cancer.

Plant species are capable of producing different types of secondary products which can be harnessed by humans for their beneficial properties in a large domain of industrial or medicinal applications [7]. World Health Organization (WHO) estimates that up to 80% of people rely mainly on traditional herbs as remedies for their medicines [8]. Extracted from entire plants, secondary products are used by food and pharmaceutical industries, although most often numerous natural plant-derived molecules remain undiscovered or unexplored for their pharmacological properties [9]. Roots play most important roles in plants and 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. Bioactive compounds are extra nutritional constituents that naturally occur in small quantities in plant and food products [10]. Most common bioactive compounds include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds [10, 11]. Many secondary metabolites not only protect plants from pathogens, insects, and environmental stresses but also are valuable for human health. Many plant species, including crop plants, are capable of producing and releasing biologically active compounds (allelochemicals). Allelochemicals (e.g., phenolics, terpenoids, alkaloids, coumarins, tannins, steroids, and quinines) are released by the plant into the environment by root exudation, volatile emissions, leaching from the leaves and other aerial parts, and the decomposition of plant material [12, 13]. Plant roots

release a range of compounds that are not directly involved in the growth and development of the plant but are very much important for plants during stress conditions (biotic/abiotic). These compounds include aliphatic acids, aromatic acids, fatty acids, sterols, phenolics, enzymes, and other secondary metabolites, including flavonoids [14, 15]. Many plant secondary metabolites of interest are accumulated in roots. However, plant cultivation is often time consuming and metabolite extraction from plant roots is destructive to plant growth.

Agrobacterium rhizogenes is a Gram negative soil-borne bacterium of the family Rhizobiaceae, which causes the hairy roots disease by infecting wounded higher plants. The transformed roots can be excised to establish axenic root cultures and indefinitely propagated in growth regulator free medium. The root exhibit fast, plagiotropic growth characterized by profuse lateral branching and rapid root tip elongation [16, 17]. Root loci (*rol*) genes harbored by the root-inducing (Ri) plasmid of this bacterium are incorporated into the host plant genome, causing hairy root. *Rol* genes are thought to affect growth and development of transformed roots and induce secondary metabolite synthesis by turning on the transcription defense genes [18, 19]. The *rolB* and *rolC* genes are absolutely essential for induction of hairy roots [20]. Fast growing and genetically stable hairy roots can be efficiently cultured in large scale bioreactors [21]. Besides, hairy root cultures are usually capable of producing the same compound(s) of identical chemistry found in wild-type roots of the naturally occurring parent plant without loss of structural integrity and/or quantity or concentration of the product, which is frequently observed in callus or cell suspension cultures [22]. *A. rhizogenes* to regulate the genes that were involved in the plant secondary metabolite production [23]. 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. Plant roots can synthesize, store, and secrete a vast array of compounds, and transformed root cultures have a wide range of biosynthetic capacities [24]. Various advantages of hairy root culture over cell suspension culture include genotypic and biochemical stability, cytodifferentiation, and growth in hormone free medium. These factors play a vital role during secondary metabolite production. Fast growth, low doubling time, ease of maintenance of hairy roots, and their ability to synthesize a large range of chemical compounds offer an additional advantage as a continuous source for the production of valuable secondary metabolites [25]. A number of secondary metabolites have been reported to be produced from hairy root cultures [26]. 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. There have been few reviews in the literature on a wide variety of hairy root applications in secondary metabolites of medicinal plants. Previously, very few studies of secondary metabolite production in hairy root cultures of cucurbits have been reported. First time, we focus this chapter on establishment of hairy roots and production of secondary metabolites in cucurbits.

2 Establishment of Hairy Root Cultures in Cucurbits

2.1 Hairy Root Induction of Cucurbits

Hairy roots were induced from various explants (leaf, cotyledon, hypocotyl, node, and root) after 3–4 weeks of culture. Control explants failed to induce hairy root formation. High induction of hairy roots was observed in leaves compared to other explants in *Gynostemma pentaphyllum*, *Cucumis anguria*, *Momordica charantia*, and *M. dioica* [27–30]. Cotyledon explants produced higher frequency of hairy root induction in *Cucumis melo* [31–33] and *C. sativus* [34, 35]. Transgenic frequency (80%) of the infected stems of *Luffa cylindrica* formed vigorous hairy roots within 4 weeks from the inoculation of the bacteria [36]. Table 1 shows the different strains of *Agrobacterium rhizogenes* (MAFF 03–01724, K599, R1000, C58C1, A4, 1855, MTCC 532, ATCC15834, R1601, KCTC 2703, and KCTC 2704) that were examined for their ability to induce hairy roots of various cucurbits such as melon, pumpkin, cucumber, sponge gourd, Chinese cucumber, southern ginseng, bitter melon, spine gourd, and bur gherkin [28–45]. Two strains of *A. rhizogenes* differed in their ability to induce hairy roots, with strain KCTC 2703 being more effective than KCTC 2704 [28–30]. Monocyclic phenolic compound, acetosyringone incorporated into the nutrient medium showed enhanced transformation frequency than the medium without it. Acetosyringone was used for co-cultivation in *C. sativus* [34] and *M. charantia* [41]. Acetosyringone is an amino acid derivative which served as a nutrient source for the invading *Agrobacterium* and enhanced the transformation rate. It was reported that acetosyringone would induce the *vir* gene of *Agrobacterium* cultures. The established hairy roots show typical morphological characteristics with rapid growth on phytohormone-free medium, lack of geotropism, and extensive lateral branching [27–30].

2.2 Molecular Confirmation of Hairy Roots in Cucurbits

The transformed root was confirmed by PCR to determine the presence of a T-DNA sequence in their genomes in *Gynostemma pentaphyllum* [27]. The PCR products from the hairy roots for *rolB* regions but not from untransformed roots of *G. pentaphyllum*. This finding indicated that the *rolB* genes from the Ri plasmid of *A. rhizogenes* were integrated into the genome of *G. pentaphyllum* hairy roots. The negative results of PCR amplification for the *virC* gene demonstrated that no bacterial DNA was involved in *rolB* amplification leading to false positives [27]. The transgenic nature of hairy roots was confirmed by PCR using *rolC* and *aux1* gene specific primers, and transgenicity was also confirmed by polymerase chain reaction (PCR), reverse-transcriptase PCR (RT-PCR), and sequencing in *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. The integration of Ri T-DNA into the genome of plant cells caused the formation of hairy roots, in which *rol* and *aux* genes were harbored in *M. dioica* [30]. PCR analysis targeted the *A. rhizogenes* *rolC*, *aux1*, and *virD2* genes in *M. dioica*. The *rolC* and *aux1* genes, located on

Table 1 Secondary metabolites produced in hairy root cultures of cucurbits

Botanical name of plant	Common name	Agrobacterium strains and plasmids	Phytochemicals	Biological activities	References
<i>Cucumis melo</i>	Melon	MAFF 03-01724 (<i>pRi1724</i>)	Aroma essential oils (Z)-3-hexenol, (E)-2-hexenal, 1-nonanol, and (Z)-6-nonenol	Antimicrobial	[32]
<i>Cucumis melo</i>	Oriental melon	K599 (<i>t-PA</i>)			[33]
<i>Cucumis melo</i>	Oriental melon	K599 or DCAR2 (<i>pKGF7S7.0-35SP</i>)			[37]
<i>Cucurbita pepo</i>	Pumpkin	R1000 (<i>pRiA4b</i> and <i>MSU440 pRiA4</i>)			[44]
<i>Cucurbita pepo</i>	Pumpkin	C58C1 (<i>pArA4abc</i>) and C58C1 (<i>pArA4b</i>)			[45]
<i>Cucumis sativus</i>	Cucumber	A4 (<i>pCAMBIA 1301</i>)			[34]
<i>Cucumis sativus</i>	Cucumber	A4 (<i>pARC8</i>)			[42]
<i>Cucumis sativus</i>	Cucumber	A4T C58 with <i>pTic58 pRiA4</i>			[35]
<i>Cucumis sativus</i>	Cucumber	A4rol/ABC			[43]
<i>Luffa cylindrica</i>	Sponge gourd	1855	Ribosome-inactivating protein (RIPs)	Antiviral	[36]
<i>Luffa cylindrica</i>	Sponge gourd	1855	Ribosome-inactivating protein	Cytotoxic	[79]
<i>Trichosanthes kirilowii</i> var. <i>japonica</i>	Chinese cucumber	ATCC15834	Karasunins, ribosome-inactivating proteins (RIPs)	Antiviral	[39]
<i>Trichosanthes kirilowii</i> var. <i>japonica</i>	Chinese cucumber	R1601	Triterpenoids (bryonolic acid, chondrillasterol)	Cytotoxic, growth inhibition of B-16 melanoma cells	[40]
<i>Trichosanthes kirilowii</i> var. <i>japonicum</i>	Chinese cucumber	ATCC 15834	Defense related proteins		[38]

<i>Gynostemma pentaphyllum</i>	Poor man's ginseng or Southern ginseng	ATCC 15834	Triterpene saponins (gypenosides)	Antitumor, cholesterol lowering, immunopotentiating, antioxidant, hypoglycemic, antidiabetic	[27]
<i>Gynostemma pentaphyllum</i>	Poor man's ginseng or Southern ginseng	ATCC 15834	Triterpene saponins (gypenosides)	Antitumor, cholesterol lowering, immunopotentiating, antioxidant, hypoglycemic, antidiabetic	[66]
<i>Momordica charantia</i>	Bitter melon	KCTC 2703 and KCTC 2704	Hydroxybenzoic acids, hydroxycinnamic acids, flavonols	Antioxidant, antibacterial, antifungal	[29]
<i>Momordica charantia</i>	Bitter melon	ATCC 15834	Charantin	Antidiabetic	[41]
<i>Momordica dioica</i>	Spine gourd	KCTC 2703	Hydroxybenzoic acids, hydroxycinnamic acids, flavonols	antioxidant, antibacterial, antifungal, and antiviral	[30]
<i>Cucumis anguria</i>	Bur gherkin	KCTC 2703	Hydroxybenzoic acids, hydroxycinnamic acids, flavonols	antioxidant, antibacterial, antifungal	[28]

independent T-DNAs (TL-DNA and TR-DNA, respectively) of the Ri plasmid of *A. rhizogenes* strain, are diagnostic for T-DNA integration into the host genome. The *virD2* gene, located outside the T-DNA, is diagnostic for the presence of any remaining *Agrobacterium* in the root tissue [30]. The *rol* and *aux* genes are essential for the induction of hairy roots, and they act as a potential activator of secondary metabolites in cucurbits [28–30]. The *virD2* gene was used to verify the complete absence of *A. rhizogenes* in the hairy roots lines of *C. anguria* and *M. dioica*. This result indicates that pRi T-DNA fragments of *A. rhizogenes* were successfully integrated into the genome of *C. anguria* and *M. dioica* without bacterial residues [28, 30]. The obtained full length coding sequence of *rolC* gene of *M. charantia* and *M. dioica* [29, 30]. The use of PCR combined with DNA sequencing instead of Southern blotting for the characterization of transgenic plants has the advantage that the newly inserted genes can be detected at an earlier stage with less DNA and less plant material [29, 30]. The presence of pRi T-DNA in pumpkin long-term hairy root cultures was determined by Southern hybridization [31]. Integration of the T-DNA region of Ri-plasmids into the plant genome was confirmed by both opine assay on paper electrophoresis and PCR-based detection of *rol* genes in *Trichosanthes kirilowii* var. *japonica* [39].

Successful integration of the T-DNA into chromosomal DNA of the KMH-009 was first examined by PCR amplifying the *rolC* gene located on the integrated T-DNA in *Cucumis melo* [32]. An immunoblot analysis of the Oriental melon transgenic hairy root extract revealed 97 kDa single bands coincident with the molecular weight of the GFP-GUS fusion proteins. ELISA demonstrated that the highest level of GFP-GUS fusion protein expression was 0.47% of the total soluble protein in a transgenic hairy root of Oriental melon [37]. The integration of T-DNA containing a *gus* reporter gene in hairy root lines was confirmed at low copy numbers ranging from 1 to 4 copies using quantitative real-time PCR, and histochemical staining of cucumber hairy roots showed overexpression of the *gus* gene when driven with the CaMV 35S promoter in *C. sativus* [34]. The presence of GUS activity and its localization were observed in all of the tissues of the root, especially in transgenic cucumber hairy root lines with the CaMV 35S and CaMV 35ST/AMV promoters. The transgenic cucumber hairy roots lines with the CaMV 35S promoter or the CaMV 35ST promoter showed localized GUS activity only in the vascular bundles in *C. sativus* [34]. Quantification of the copy number of the *gus* gene using absolute quantification in real-time PCR revealed a low copy number of the GUS gene per genome [34]. The transgenic plants looked normal and were positive for the neomycin phosphotransferase II. Southern blot analysis of the transgenic plants revealed that all plants contained vector DNA, but only some of them contained DNA from the Ri plasmid [42]. Enzyme-linked immunosorbent assay (ELISA) revealed the highest levels of the recombinant t-PA accumulation in transgenic hairy roots carrying the t-PA transgene under the control of single and dual *rolD* promoters as compared to triple and quadruple *rolD* promoters [33].

Previously, it was reported that changes in secondary metabolite production in hairy roots and Ri plants correlate with changes in the phenotype induced by the insertion of *rol* genes and with the quantity of the polypeptide encoded by the *rolC*

gene [29, 30]. Interestingly, both the capacity to grow and produce nicotine in hairy roots and Ri plants of *Nicotiana tabacum* cv. Xanthi were higher after integration of the three *rol* genes (*A*, *B*, *C*) together than with *rolC* alone. In addition, the level of nicotine accumulation was positively correlated with the levels of the polypeptide encoded by the *rolC* gene, as detected by immunoassays [28–30]. The *rolA* gene appears to be an activator of growth and secondary metabolism. Although the *rolB* gene has emerged as the most powerful stimulator, its use is presently disputed owing to its growth-suppressing effect. More positively, the self-activation of *rolC* gene seems to be promising [28–30].

2.3 Growth Kinetics of Hairy Root Culture in Cucurbits

The time profile of the growth of hairy roots in liquid culture was reported in *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. The sixth day was the lag period of hairy root growth; then it began to increase gradually during the eleventh day. The exponential growth stage during the 21 days was followed by the stationary phase during the 15–25 days. The higher fresh mass (FM) and dry mass (DM) was observed at 20, 21, and 22 days of culture of *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. The culture duration of 49 days of hairy roots increased about 120-fold compared to inoculum and the gypenoside content in *G. pentaphyllum* [27]. Hairy root cultures showed a sigmoidal growth curve, and crude extracts showed a progressively increasing translational inhibitory activity that reached the maximum value during the early stationary phase of *L. cylindrica* [36].

2.4 Effects of Sucrose Concentration and Different Media on Biomass Accumulation of Hairy Root Cultures of Cucurbits

Sucrose is the most significant carbon source for plant tissue cultures and helps as the chief energy source and an important constituent in secondary metabolite biosynthesis in cucurbits [29]. The amount of sucrose usually affects the accumulation of secondary metabolites in cultures. About 3% of sucrose produced the higher amount of biomass accumulation and metabolite production in *C. anguria*, *M. charantia*, and *M. dioica* [28–30, 41]. About 2% of sucrose induced hairy root induction in *Trichosanthes kirilowii* var. *japonica* [39]. Many previous reports focus on the composition of medium nutrients to achieve maximum accumulation of metabolites in cultured cells [46]. The different media, full and half strength MS, B5, NN, and LS were employed in hairy root culture and the results shown that MS medium was superior for biomass accumulation in *G. pentaphyllum*, *C. anguria*, *M. charantia*, *M. dioica*, *Cucumis melo*, *C. sativus*, and *T. kirilowii* [27–34, 38, 41, 42]. However, other media like B5 was also used to induce hairy roots in *L. cylindrica* [36]. Hairy root induction of cucurbits using carbohydrate source is by sucrose and nutrients media is by MS or B5.

3 Production of Valuable Secondary Metabolites from Hairy Root Cultures of Cucurbits

Increased secondary metabolite production in hairy roots cultured in vitro, over their wild-type counterparts, may be seen as one of the most exciting spin-offs of biotechnology. Due to their great richness in secondary products, such as triterpenoids and phenolic compounds, plants represent an immense source of therapeutic and/or industrial compounds. For example, plant-derived biomolecules, such as saponins (*G. pentaphyllum*), triterpenoids of bryonolic acid, and chondrilasterol (*Trichosanthes kirilowii* var. *japonica*), ribosome-inactivating protein (*Luffa cylindrica*), charantin (*Momordica charantia*), hydroxybenzoic acids, hydroxycinnamic acids, and flavonols (*C. anguria*, *M. charantia* and *M. dioica*), are efficient in the treatment of different pathology types relating to cancer, cardiovascular and metabolic disorders, and/or other infectious diseases (Table 1 and Fig. 1). Many plant metabolites are commercially available as drugs, flavors, food additives, cosmetics, fragrances, and insecticides. Here, several important phytochemicals from hairy roots of cucurbits are discussed (Fig. 1).

3.1 Phenolic Compounds of Hairy Root Cultures in Cucurbits

Phenolic compounds are secondary metabolites, ubiquitous in plants and plant derived foods. They show a large diversity of structures, including rather simple molecules (e.g., vanillin, gallic acid, caffeic acid) and polyphenols such as stilbenes, flavonoids, and polymers derived from these various groups [47]. Phenolic compounds are classified into three major groups based on the number and binding position of exchangeable hydroxyl groups on aromatic compounds: simple phenol and phenolic acid group, hydroxycinnamic acid derivative group, and flavonoid group. 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. Flavonoids are phenylpropanoid metabolites, most of which are synthesized from *p*-coumaroyl-CoA and malonyl-CoA, and share their precursors with the biosynthetic pathway for lignin biosynthesis [48]. Flavonoids are low-molecular-weight compounds having approximately 15 atoms of carbon, which are organized in a C6–C3–C6 configuration [49]. More than 9000 flavonoids have thus far been identified in plants [50]. Phenolic acids are considered as simple phenolics, and they are categorized into two groups, i.e., the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids have C6–C1 arrangement, such as protocatechuic, vanillic, gallic, syringic, and *p*-hydroxybenzoic acids, while hydroxycinnamic acids have C6–C3 arrangement, such as *p*-coumaric, sinapic, caffeic, and ferulic acids. Flavonoids and phenolic acids are considered as potentially health-promoting substances and have a number of physiological properties, such as antithrombotic,

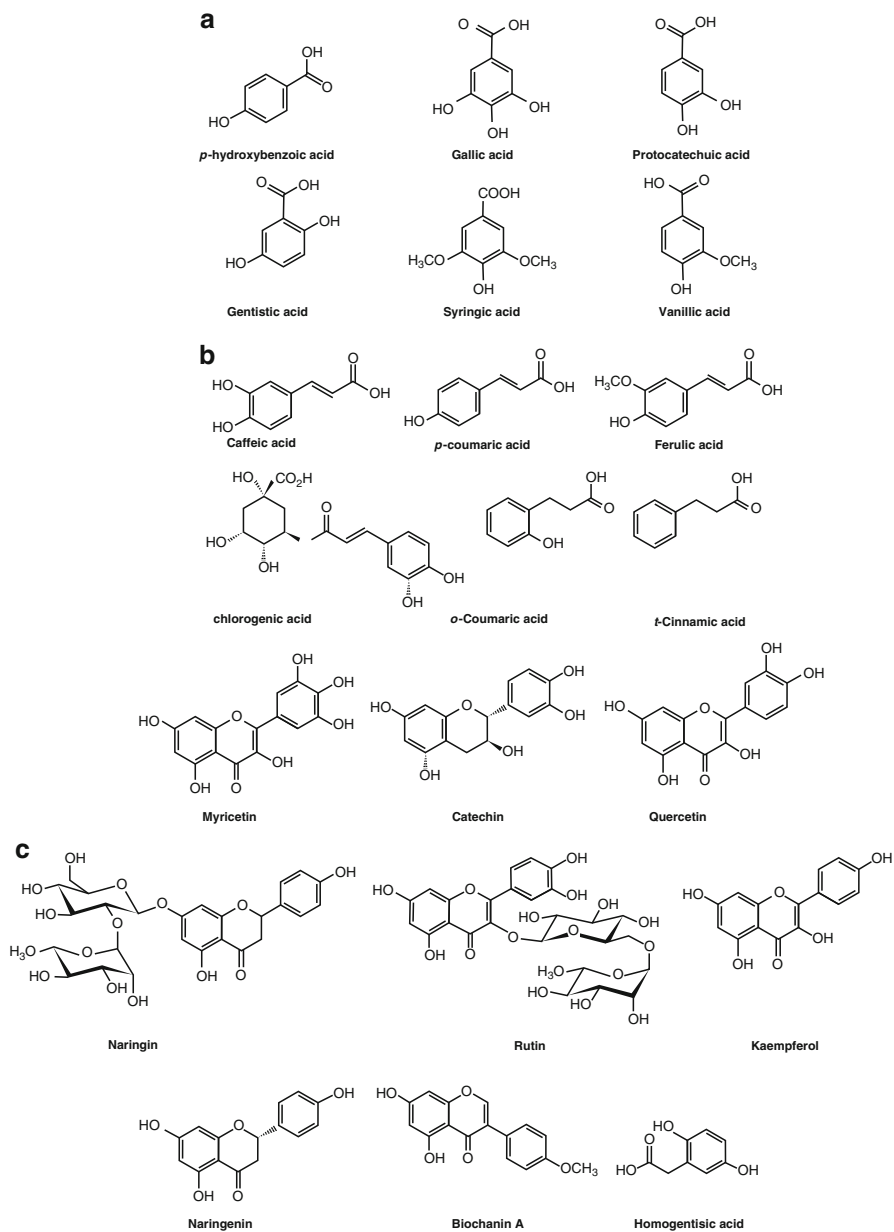


Fig. 1 (continued)

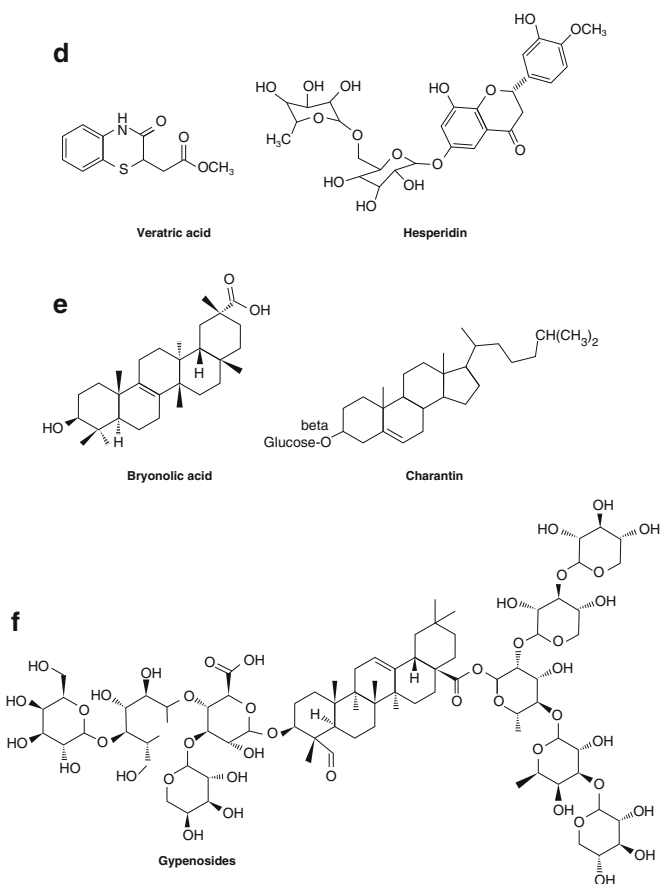


Fig. 1 Chemical structures of selected plant secondary metabolites produced in hairy root cultures of cucurbits

antiallergenic, antimicrobial, cardioprotective, anti-inflammatory, antioxidant, arterogenic, and vasodilatory effects [51–53]. Phenolic compounds are synthesized via the phenylpropanoid pathway that begins with conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase (PAL). In the last few years, great attention has been paid to the bioactive compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of some degenerative diseases like cancer and diabetes [54, 55], reduction in risk factors of cardiovascular diseases [10, 56], antioxidant, antimutagenic, antiallergenic, anti-inflammatory, and antimicrobial effects [49, 57], among others. Due to these countless beneficial characteristics for human health, researches have been intensified aiming to find fruits, vegetables, plants, agricultural, and agro-industrial residues as sources of bioactive phenolic compounds.

3.1.1 Individual Phenolic Compounds of Hairy Root Cultures in Cucurbits

The qualitative and quantitative analysis of phenolic compounds from hairy roots and untransformed (roots from in vitro seedling) root extracts of *C. anguria*, *M. charantia*, and *M. dioica* [28–30] were studied using ultra-HPLC. The phenolic compounds in the *C. anguria*, *M. charantia*, and *M. dioica* extracts were identified by comparisons of the retention time and UV spectra of authentic standards and the quantitative data were calculated from calibration curves [28–30]. Both transgenic and nontransgenic roots contained flavonols, hydroxycinnamic, and hydroxybenzoic acids. Hairy roots contained higher amounts of flavonols compared to nontransgenic roots of *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. Myricetin, quercetin, catechin, kaempferol, and rutin levels were higher in hairy roots compared to nontransgenic roots of *C. anguria* [28]. The contents of naringenin and biochanin A were lower in concentrations in hairy roots than nontransgenic roots of *C. anguria* [28]. Myricetin, quercetin, catechin, kaempferol, rutin, biochanin A, and naringenin levels were higher in hairy roots compared to nontransgenic roots of *M. charantia* [29]. Naringenin was presented in nontransgenic roots, but it was absent in hairy roots of *M. charantia* [29]. Quercetin, kaempferol, catechin, and rutin levels were higher in hairy roots compared to nontransgenic roots of *M. dioica* [30]. Myricetin, naringenin, and biochanin A contents were lower in concentrations in hairy roots than nontransgenic roots of *M. dioica* [30]. Kaempferol, myricetin, naringenin, quercetin, and rutin have antimicrobial activity against human pathogenic microorganisms with some mechanisms of action such as inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolisms [58].

Caffeic acid and chlorogenic acid were major hydroxycinnamic acid derivatives in hairy roots and nontransformed roots compared to *p*-coumaric acid, ferulic acid, *o*-coumaric acid, and *t*-cinnamic acid in *C. anguria* [30]. Caffeic acid, ferulic acid, *o*-coumaric acid, and *t*-cinnamic acid levels decreased in hairy roots compared to nontransformed roots of *C. anguria* [30]. Chlorogenic acid and *p*-coumaric acid contents were higher in hairy roots than nontransformed roots of *C. anguria* [30]. Chlorogenic acid containing plant materials have been shown to have antiviral, antifungal, and strong antibacterial activities [59]. Chlorogenic acid, *p*-coumaric acid, and ferulic acid levels were higher in hairy roots compared to nontransformed roots of *M. dioica* [30]. Caffeic acid, *o*-coumaric acid, and *t*-cinnamic acid contents were lower in hairy roots than nontransformed roots of *M. dioica* [30]. Caffeic acid, *p*-coumaric acid, *o*-coumaric acid, chlorogenic acid, and *m*-coumaric acid levels were higher and ferulic acid content was lower in hairy roots than nontransgenic roots of *M. charantia* [29]. Protocatechuic acid, β -resorcylic acid, syringic acid, gentisic acid, and salicylic acid levels were higher and gallic acid, *p*-hydroxybenzoic acid, and vanillic acid were lower in hairy roots than nontransgenic roots of *C. anguria* [28]. Gallic acid, *p*-hydroxybenzoic acid, gentisic acid, and salicylic acid levels were higher and protocatechuic acid, β -resorcylic acid, and vanillic acid were lower in hairy roots compared to nontransformed roots of *M. dioica* [30]. Gentisic acid has an effective role in the anticarcinogenetic activity [60]. Gallic acid, protocatechuic acid, β -resorcylic acid, vanillic acid, syringic acid, gentisic acid,

and salicylic acid levels were higher and *p*-hydroxybenzoic acid was lower in hairy roots compared to nontransformed roots of *M. charantia* [29]. Veratric acid was higher and vanillin, hesperidin, and homogentisic acid were lower in hairy roots compared to nontransformed roots of *C. anguria* [28]. Vanillin was higher and veratric acid, hesperidin, and homogentisic acid levels were lower in *M. charantia* and *M. dioica* [29, 30].

3.1.2 Total Phenolic Compounds of Hairy Root Cultures in Cucurbits

Previous studies have revealed that polyphenolic compounds are commonly found in both edible and nonedible plants and that they have multiple biological effects, including antioxidant activity [61]. Flavonoids and other phenolic substances may play a preventive role in the development of cancer and heart disease [62]. Biological activities related to antibacterial and antioxidant activities may be correlated with total polyphenol and flavonoid contents [63]. The total phenolic and flavonoid contents were higher in hairy roots compared to untransformed roots of *C. anguria*, *M. charantia*, and *M. dioica* [28–30].

3.2 Triterpenoids of Hairy Root Cultures in Cucurbits

Gypenosides (Gyp) are the major components of *Gynostemma pentaphyllum* Makino, a Chinese medicinal plant. Phytochemical studies of *G. pentaphyllum* have identified approximately 90 dammarane-type saponin glycosides, known as gypenosides, which are responsible for its pharmacological activities [64]. Saponins are a class of chemical compounds found in particular abundance in various plant species. More specifically, they are amphipathic glycosides grouped phenomenologically by the soap-like foaming they produce when shaken in aqueous solutions and structurally by having one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. Triterpenoid saponins are triterpenes which belong to the group of saponin compounds. Triterpenes are a type of terpene containing 30 carbon atoms. Triterpenes are assembled from a five-carbon isoprene unit through the cytosolic mevalonate pathway to make a thirty-carbon compound. Cucurbitacins are triterpenoids that confer a bitter taste in cucurbits such as cucumber, melon, watermelon, squash, and pumpkin. These compounds discourage most pests on the plant and have also been shown to have antitumor properties [65]. Gypenoside content was higher compared to roots of control parent plant of *Gynostemma pentaphyllum* hairy root cultures [27] which was significantly higher than that previously reported for hairy root cultures [66]. Transformed roots can synthesize and store significant quantities of secondary metabolites. Although the hairy roots under these conditions produced approximately 30% to 40% less gypenosides than commercial sources of *G. pentaphyllum*, the growing time was much shorter when compared to field-grown plants. With hairy root cultures, product quality and quantity are easy to control because natural variances in seasonal climates and geographical environments are excluded and culture conditions and process variables are easily optimized [67]. The hairy root cultures have been

considered as a potential alternative for production of gypenosides. Several strategies for the enhancement of biomass and gypenosides have been adopted like the effects of medium compositions, culture conditions, and elicitations [27].

3.3 Ribosome-Inactivating Protein of Hairy Root Cultures in Cucurbits

Ribosome-inactivating proteins (RIPs) are widely distributed plant enzymes that inhibit protein synthesis by virtue of their N-glycosidic activity, selectively cleaving an adenine residue from a highly conserved and surface-exposed stem loop structure in the 28S rRNA [68]. This cleavage prevents the binding of the EF-2/GTP complex, with the subsequent arrest of protein synthesis leading to autonomous cell death [69]. RIPs are either enzymatically active single polypeptides (type I) or heterodimers (type II). A type II RIP consists of an A chain, functionally equivalent to a type I RIP, which is attached to a sugar-binding B chain [70]. Besides RNA N-glycosidase activity, some RIPs have ribonuclease, DNase, DNA glycosylase, and apurinic/apyrimidic lyase activities [71, 72]. In addition, RIPs from *Trichosanthes kirilowii* cell cultures have been demonstrated to possess chitinase activity [73]. Certain type I RIPs display a variety of antimicrobial activities, including antifungal, antibacterial [74], and broad-spectrum antiviral effects against different plant and animal viruses [75], including human immunodeficiency virus [76]. RIPs have been studied as potential tumor cytotoxic agents, both in their native form and after conjugation with monoclonal antibodies. RIP activity of *L. cylindrica* plantlets, grown in vitro on MS medium, was evaluated in crude extracts from different parts and organs and compared to the inhibitory activity shown by extracts from seeds and from transformed roots [36]. The inhibitory activity, as far as normal, nontransformed tissues are concerned, is in agreement with what was already known from previous reports of *L. cylindrica* [77, 78]. RIP-producing hairy roots promise to be much more stable than conventional in vitro grown calluses and cell suspensions [36]. This study tested the sc-RIP extracts from the seeds and hairy root tissue cultures of *Luffa cylindrica* (established by transformation with *Agrobacterium rhizogenes* strain 1855) for inhibitory effects on the growth of in vitro melanotic and amelanotic human melanoma cell lines [79]. The results reported that RIPs can be produced and purified from hairy root cultures, in good agreement with what has been recently reported [38] for hairy root lines of *Trichosanthes kirilowii*. Ribosome-inactivating proteins (RIPs) from plants catalytically damage eukaryotic ribosomes, making them unable to perform the elongation step of protein synthesis. Type 1 RIPs are single-chain proteins, whereas type 2 RIPs consist of two polypeptide chains and possess a galactose-specific binding domain to cell surfaces. Type 1 RIPs are more common and have been identified and purified from more than 30 plants. Interest in type 1 RIPs has been growing due to their widespread physiological activities as abortifacient agents and immunotoxins [39]. The antiviral activity of RIPs has also focused attention on their potential use as anti-HIV agents [39]. There have been few reports on the production of RIPs by plant tissue or cell cultures. A low level of

trichosanthin was reported to accumulate in transformed hairy root cultures of *Trichosanthes kirilowii* var. *japonica* [38]. Trichosanthin was also identified in cell extracts of the transformed callus tissues resulting from infection by *Agrobacterium rhizogenes* but not in the untransformed callus of *T. kirilowii* [39]. The major protein in the basic protein fraction was tentatively identified as a class III chitinase based on the N-terminal amino acid sequence. This is consistent with the report [38], who identified two major extracellular basic proteins and one intracellular basic protein produced by *T. kirilowii* var. *japonica* hairy roots as class III chitinases. However, the N-terminal sequence of HR-PB 1 was very similar to but not identical with the sequence of any of these proteins.

3.4 Charantin of Hairy Root Cultures in Cucurbits

A molecule of charantin consists of aglycone or a steroidal portion, which is highly soluble in relatively nonpolar solvent such as chloroform and dichloromethane. However, the glucosides attached to its molecules make it slightly soluble in polar organic solvents such as ethanol or methanol. Conventionally, isolation of this compound involves extraction with mixtures of these solvents using Soxhlet apparatus. Chloroform is highly toxic and carcinogenic, and its use has now been replaced with its much less toxic relative, dichloromethane, which still carry some health risks. Chronic exposure to dichloromethane has been linked to cancer of lungs, liver, and pancreas in laboratory animals. It is a mutagen and may cause birth defect if women were exposed to it during pregnancy [80]. This compound could be used to treat diabetes and can potentially replace treatment by injection of insulin which has not been successful in stimulating the pancreas of the diabetic patients to lower blood sugar to the desired level [81]. In some cases, the injected patient shows signs of side effects. Plant derived compounds that show antidiabetic property such as charantin and others are now being widely accepted as an alternative medicine for diabetes mellitus, and they are free from side effects [82]. Charantin, a naturally occurring steroidal glycoside, is widely distributed throughout the plant of *Momordica charantia*. The presence of charantin was confirmed by performing thin layer chromatography (TLC) in hairy roots as well as in fruit and leaf [41]. The charantin content was lower in hairy roots compared to leaf and fruit of *M. charantia* [41].

3.5 Aroma Essential Oils of Hairy Root Cultures in Cucurbits

The typical cucumber flavor results from the enzymatic action of LOX on linolenic and linoleic acids, which introduces molecular oxygen at C13 or C9, forming 13-hydroperoxylinolenic acid (13-HPOT) or 9-hydroperoxylinolenic acid (9-HPOT). HPL cleaves 13-hydroperoxide (13-HPO) and 9-HPO to produce the C6 and C9 aldehydes that are responsible for the cucumber flavor [83]. These aldehydes can then be reduced to the corresponding C6 alcohols by alcohol

dehydrogenase (ADH). Studies have reported that only the oxylipin metabolic pathway contributes to aldehyde and alcohol content and hence flavor [84]. To date, 78 volatile compounds have been identified in cucumber fruits, including aldehydes, alcohols, esters, alkanes, furfurans, and others [85], and (E,Z)-2,6-nonadienal and (E)-2-nonenal are the main aroma compounds [86]. The hairy roots could newly synthesize some essential oils such as (Z)-3-hexenol, (E)-2-hexenal, 1-nonanol, and (Z)-6-nonenol, which were reported to be important aroma volatiles in melon [87]. The stable production of the fruity aroma volatiles by the KMH-009 was assessed by comparing the yields of the compounds from the hairy roots repeatedly subcultured for more than 3 years. The data revealed that the essential oils for aroma scent were constantly synthesized with no relation to the increased number of times for subculture, and the constant production by this clone was successfully maintained in the hairy roots repeatedly subcultured of *Cucumis melo* [32]. The volatile compounds were extracted and identified by GLC-mass spectrometry. Some essential oils such as (Z)-3-hexenol, (E)-2-hexenal, 1-nonanol, and (Z)-6-nonenol were stably synthesized by these hairy roots despite the increased number of subcultures. The productivity of these compounds by the best hairy root line was shown to be considerably higher than naturally ripened melon fruits [32].

4 Cucurbit Hairy Root Cultures of Secondary Metabolites Using for Biological Activities

Phenolic compounds have multiple additional roles in plants, including attracting insects for seed dispersion and pollination. They are also part of the natural defense system against insects, fungi, viruses, and bacteria, and they can act as plant hormone controllers. Moreover, in recent years, phenolic compounds have been intensively investigated because of their potential health-promoting effects [88, 89]. They have been reported to possess many useful properties for human health, including anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, vascular, and cytotoxic antitumor activity, but the most important action of phenolics is their antioxidant activity [58, 89, 90]. It has been demonstrated recently that quercetin and kaempferol synergistically suppress cell proliferation in human gut cancer lines [91]. The translational inhibitory activity found in extracts from our hairy root cultures is the highest that has been found in various tissues of *L. cylindrica*, including seeds [36].

4.1 Antioxidant Activity

The antioxidant potential of hairy roots and nontransformed roots were determined using free radicals scavenging, reducing potential, phosphomolybdenum assays, and chelating effects on ferrous ions. The highest antioxidant activity was exhibited in hairy roots compared to nontransformed roots in *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. Reducing capacity of extracts suggests that hairy roots were more

potential when compared to untransformed roots in *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. The antioxidant capacity shown by phosphomolybdenum method was higher in the hairy root extract than nontransformed root extract of *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. The percentage of metal scavenging capacity of transgenic hairy roots was higher than nontransgenic roots of *M. charantia*, *C. anguria*, and *M. dioica* [28–30]. Hairy roots exhibited higher antioxidant activity in *M. charantia* [29].

4.2 Antimicrobial Activity

The hairy roots and nontransformed roots of *C. anguria*, *M. charantia*, and *M. dioica* revealed varying antibacterial activity, as exposed by the growth inhibition zones [28–30]. The results from the disc diffusion method indicated that both hairy roots and nontransformed root extracts had comparable antibacterial effects against Gram positive and Gram-negative bacteria. Hairy roots exhibited highest activity with both Gram-positive and Gram-negative bacteria compared to nontransformed roots of *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. Gram-positive (*S. aureus*) bacteria exhibited greater inhibition compared to Gram-negative (*P. aeruginosa* and *E. coli*) bacteria in *M. charantia*, *C. anguria*, and *M. dioica* [28–30]. By using the disc diffusion method against the fungal strains, it can be seen that extracts of *M. charantia*, *C. anguria*, and *M. dioica* hairy roots and nontransformed roots exhibited good antifungal activity [28–30]. Hairy roots exhibited greater inhibition of fungus (*F. oxysporum* and *A. niger*) in hairy roots than nontransgenic roots of *C. anguria*, *M. charantia*, and *M. dioica* [28–30]. Hairy roots exhibited higher antibacterial and antifungal activity compared to nontransformed roots [92, 93]. Flavonoid derivatives have also been reported to possess antiviral activity against a wide range of viruses such as HSV, HIV, Coxsackie B virus, corona virus, cytomegalovirus, poliomyelitis virus, rhinovirus, rotavirus, poliovirus, sindbis virus, and rabies virus [94]. Cytotoxicity activity and quantitative assay of virus yields using plaque assay were carried out for hairy roots and nontransgenic roots of *M. dioica* [30]. Hairy roots exhibited higher antiviral activity compared to nontransgenic root extracts of *M. dioica* [30]. *M. charantia* was reported to possess several antiviral activities including hepatitis B virus, dengue virus, human immunodeficiency virus (HIV), and influenza A subtypes including H1N1, H3N2, and H5N1 [95]. Hairy roots have potential antiviral activity compared with nontransgenic roots of various plants like *Phyllanthus amarus* [96] and *Daucus carota* [97].

4.3 Anticancer Activity

Gypenosides (Gyp) are compounds found in the crude extracts from *G. pentaphyllum* and they have been shown to exert various biological effects such as anti-inflammatory and antioxidative [98], antihyperlipidemic, anticardiovascular [99], and anticancer [100–102]. Our previous studies have shown that

gypenosides induced apoptosis in human colon cancer colo 205 cells [103] and human tongue cancer SCC-4 cells through endoplasmic reticulum stress and mitochondria-dependent pathways [104]. Although gypenosides have been shown to induce cell cycle arrest and apoptosis in several human cancer cell lines, there is no available information to address whether gypenosides induce DNA damage or affects DNA repair genes in SAS human oral cancer cells.

4.4 Antidiabetic Activity

Diabetes mellitus is an endocrine metabolic disorder in which the body does not produce sufficient insulin or lack of responsiveness to insulin, resulting in hyperglycemia (high blood glucose level). The classical symptoms include polyuria, polydipsia, weight loss, lethargy, polyphagia, visual blurring, frequent or recurring infections, cuts and bruises that are slow to heal, tingling and/or numbness in hands and/or feet, drowsiness, nausea, and decreased endurance during exercise [105]. A number of potential medicinal components from bitter melon, such as α and β momorcharin, momordin, and cucurbitacin B, have been isolated. A number of reported clinical studies have shown that bitter melon extract from fruits, seeds, and leaves contain several bioactive compounds that have hypoglycemic activity in both diabetic animals and humans [106]. Fruits, seeds, and leaves extract of *Momordica charantia* possess hypoglycemic activity in antihyperglycemic activity in alloxan [107] or streptozotocin [108]. The major compounds that have been isolated and identified as hypoglycemic agents include charantin, polypeptide-p, and vicine. Charantin is a steroidal glycoside shown to possess powerful hypoglycemic properties when administered orally and intravenously in diabetic rabbits [109]. Hairy roots produced higher amount of charantin which used for antidiabetics [41].

5 Large Scale Productions of Secondary Metabolites in Bioreactors Using Hairy Root Cultures

The vast potential of hairy root cultures as a stable source of biologically active chemicals has focused the attention of the scientific community for its exploitation. Scaling up of hairy roots in novel bioreactors can provide the best conditions for optimum growth and secondary metabolite production, comparable to or higher than that in native roots. Though the need for developing bioreactors suitable for the hairy root cultivation has long been recognized, root cultures present unique challenges [110]. The complex fibrous structure of the roots makes the growth analysis and development of a large-scale culture system difficult. Hairy root growth is not homogeneous, which affects the reactor performance. Furthermore, the hairy root morphology is quite plastic as the roots respond to the changes in the local environment. Changes in morphology, including changes in the density and length of the root hairs, directly affect the secondary metabolite production from hairy roots

[111]. Thus, bioreactor design for root cultures is a balancing act between the biological needs of the tissues, without inducing an additional, undesirable biological response [112]. Reviews on hairy roots briefly discuss the importance of the use of bioreactors for hairy root cultures [26]. Mechanical agitation causes wounding of hairy roots and leads to callus formation. Due to branching, the roots form an interlocked matrix that exhibits resistance to nutrient flow. Hairy roots are heterotrophic, respiratory organisms that rely on oxygen for energy generation and other metabolic functions. Substantial progress has been made in understanding the mechanisms of oxygen limitation, one of the principle challenges for large-scale growth of hairy root cultures [113]. Because of the solid phase nature of the roots and the development of oxygen gradients within root tissues, relatively small reductions in the dissolved oxygen concentration in the medium can lead to a significant decrease in growth rate and may also affect the synthesis of certain secondary metabolites. In fact, hairy roots can be oxygen limited even in shake flask cultures [114]. Restriction of nutrient oxygen delivery to the central mass of tissue gives rise to a pocket of senescent tissues. Mass transfer resistances near the liquid and solid boundary affect the oxygen delivery to the growing hairy roots. Thus, exploitation of hairy root culture as a source of bioactive chemicals depends on the development of suitable bioreactor system where several physical and chemical parameters (nutrient availability, nutrient uptake, oxygen, and hydrogen depletion in the medium, mixing, and shear sensitivity) must be taken into account. The design of bioreactors for hairy root cultures should also take into consideration factors such as the requirement for a support matrix and the possibility of flow restriction by the root mass in certain parts of the bioreactor. Several bioreactor designs have been reported for hairy root culture taking into consideration the above factors that permit the growth of interconnected tissue unevenly distributed throughout the culture vessel. Reactors used to culture hairy roots can roughly be divided into three types: liquid-phase, gas-phase, or hybrid reactors that are a combination of both [22]. Previously, there are no reports on the large scale production of hairy roots using bioreactor system in cucurbits and the production of phenolic compounds.

6 Elicitors Increase the Secondary Metabolites Production in Hairy Root Cultures

Various biotic and abiotic elicitors applied to hairy root cultures and their stimulating effects on the accumulation of secondary metabolites. According to their origin, elicitors can be divided into different types: (a) biotic and (b) abiotic. Abiotic elicitors can be considered as substances of nonbiological origin, being predominantly inorganic compounds such as salts or physical factors [115, 116]. Inorganic chemicals like salts or metal ions have been used to increase the production of bioactive compounds by their modification of plant secondary metabolism. Among the many elicitors applied to hairy root cultures, the most common and effective elicitors are fungal cell extracts, polysaccharides from fungal and plant cells, and heavy metal salts. With the crude fungal cell extracts, it is essential to observe the

preparation conditions carefully for achieving reproducible effects. In addition to the chemical agents, UV-radiation, hyperosmotic stress, and temperature shift have been shown effective for some plant species/metabolites. Elicitor type, dose, and treatment schedule are major factors determining the effects on the secondary metabolite production. In addition to the accumulation of products in roots, elicitor treatments often stimulate the release of intracellular products. Although elicitation is mainly effective to increase specific product yield on per unit mass of roots, the incorporation of nutrient feeding strategies can be applied to enhance the volumetric product yield. The integration of in situ product recovery from the roots/liquid medium is another synergistic strategy with the elicitor treatment to improve the process. So far, there are no reports on the elicitation of hairy roots and production of phenolic compounds from hairy root cultures of cucurbits. Further, researchers can use the elicitation to improve the contents of secondary metabolites in cucurbits.

7 Conclusions

Hairy root technology has been significantly improved in various fields for past few years. Overall, the major groups of secondary metabolites have already been produced from hairy roots of cucurbits. Compared to plant cell suspension cultures, hairy root cultures appear to be potential systems for continuous production of valuable secondary metabolites because of their fast growth rates, ease of maintenance, genetic and biosynthetic stability, and ability to synthesize a vast array of compounds. Environmental factors, such as light, oxygen, and temperature, as well as abiotic and biotic stress factors, such as phytohormones, heavy metals, and fungal elicitors, have all been applied to hairy roots for increased yield of phytochemicals. 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. The discovery of new genes that participate in the metabolic pathways from hairy root studies increases the tremendous potential of such cultures. It is also predicted that this model of pharmaceutical production is relatively safe. Driven by the demand for productive, robust, and stable hairy root cultures for the production of active agents for the food, cosmetics, and pharmaceutical industry, the development of a direct available measuring method for the biomass concentration of hairy root cultures in liquid medium still does not exist. Transgenic hairy roots grew rapidly than nontransgenic roots in standardized liquid culture conditions and produced greater amount of biomass and phenolic compounds. The higher amount of secondary metabolites possibly contributes to greater biological activity of hairy roots in cucurbits. The genetic and biochemical stability of the hairy roots as well as its high productivity offers an effective platform for further studies on the biosynthetic pathways of phytochemicals. This prediction is strengthened by the observation that emerging

private companies have converted this technology to allow production at a commercial scale. 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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