Biotechnological Production of Useful Phytochemicals from Adventitious Root Cultures

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Contents

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Abstract

A potent cluster of phytochemicals produced within plants are used as pharmaceuticals, health-promoting substances, biopesticides/agricultural, and industrial chemicals. Various attempts of producing phytochemicals from cell cultures have been undertaken extensively since several decades. However, issues such as low yields and unpredictable behavior of cell lines in the effective production are the barriers in cell cultures. On the other hand, because of their genetic, biochemical stability, and biosynthetic capabilities, transformed roots and adventitious roots have also been tested for in vitro production of useful phytochemicals; however, adventitious root cultures are preferred over hairy root cultures because of their natural origin and noninvolvement of any transgene in their production. In this review, we update the research developments in the area of adventitious root cultures and application of bioreactor technology for the production of adventitious root biomass and useful phytochemicals. In addition, few examples are presented in which adventitious root cultures are used successfully for the production of useful phytochemicals at commercial scale.

Keywords

Adventitious roots · Bioreactors · Ginseng · Purple coneflower · Phytochemicals · St. John's wort

1 Introduction

Plants are rich source of heterogeneous phytochemicals in the form of secondary metabolites which serve as drugs, health-promoting agents/functional food, food additives/coloring agents, flavoring agents, fragrances, agricultural, and industrial chemicals for the benefit of mankind [1]. Production of secondary metabolites in field-cultivated plants is feasible; however, accumulation of secondary metabolites in field-grown plants is influenced by various factors such as genotype, environmental conditions, ecological, and edaphic factors. Moreover, overexploitation of plants from natural habitats for extraction of useful phytochemicals may endanger the species that possesses these compounds [5]. Many of the species belonging to this category (e.g., *Taxus* sp.; *Podophyllum* sp.) are usually slow growing and exist in restricted habitat; moreover, accumulation of the compounds in such plants is in very low quantities [54]. Therefore, in quest of novel techniques to produce these compounds, biotechnological methods like cell and organ cultures have been executed as a source of secondary metabolites. The production of these compounds from cell and organ cultures have several advantages over their traditional extraction from field-grown plants, including constant production of quality compounds, shorter production cycles, reduced contamination, and simpler downstream processing [53]. The possibilities and advances of cell culture system for the production of useful secondary metabolites have been deliberated in several recent reviews [38, 56]. Nevertheless, cell culture systems have serious setback in the production of

secondary metabolites because of unforeseeable behavior of cell lines and low yield of the product. As a result, only a few examples are available in the literature wherein cell cultures have been used successfully for commercial production of secondary metabolites, e.g., production of paclitaxel from *Taxus* sp. and shikonin from *Lithospermum erythrorhizon* [46]. Alternatively, differentiated cells, i.e., organs such as shoots, hairy roots, adventitious roots, and embryos/protocorms, have been evolved as useful systems for sustainable production of secondary metabolites [36, 42, 53]. However, induction of shoots, embryos/protocorms, and further cultivation in large-scale bioreactor cultures are not expedient in majority of plants. Even though induction and cultivation of hairy roots are likely in large number of plants, yet safety and efficacy of hairy root products are the major concerns when the products obtained are used as food ingredients, pharmaceuticals, and nutraceuticals [41, 42]. In contrast, adventitious root cultures are considered as a good system for continuous production of useful phytochemicals of uniform quality and yield as adventitious roots are natural and possess very high genetic stability and biosynthetic capabilities. Therefore, there are many examples available in the literature where induction of adventitious roots, establishment of suspension cultures, and scale-up processes have been effectively achieved [42, 43]. So, this article reviews various applications and perspectives of adventitious root cultures.

2 Establishment of Adventitious Root Cultures: Techniques for Phytochemical Accumulation

2.1 Induction of Adventitious Roots and Selection of Clones

Choice of superior genotype and organ is the first and most important criterion for induction of adventitious roots from desirable plant species (Fig. 1). In selection of superior lines/clones which are efficient in growth, accumulation of biomass is the second criterion to achieve an effective induction of adventitious roots [42]. Application of these methodologies is evident in bacoside-A (saponin) profiling in various accessions collected from different regions of India carried out by Naik et al. [44], and they have reported fourfold variation in bacoside-A content between the accession Bm2 (18.36 mg g^{-1} dry weight) and Bm7 (3.53 mg g^{-1} dry weight). They also estimated the amount of bacoside-A in leaves, nodes, internodes, roots, stolons, and entire plant and reported the accumulation of highest bacoside-A in the stolons compared to other organs. Similarly, Ho et al. [23] reported variation in accumulation of adventitious root biomass: phenolics and flavonoids in different genotypes of *Polygonum multiflorum*. Use of suitable explants for induction of adventitious roots and selection of suitable explants always depends on specific plant species, because the site of biosynthesis and accumulation of specific metabolite/s vary from species to species. For example, site of synthesis and accumulation of terpenoids are in aerial part of *Artemisia annua* and *Mentha piperita* [47, 52]. Site of synthesis and

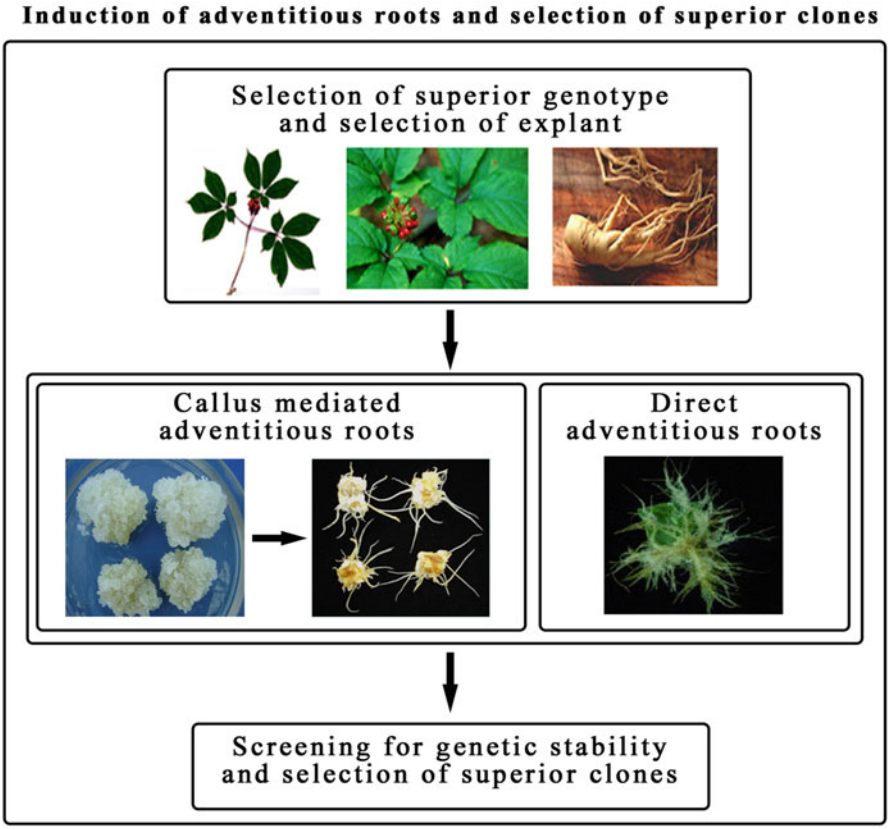


Fig. 1 Flow chart depicting induction of adventitious roots from suitable explants and selection of superior clones for production of phytochemicals

accumulation of quinones are in roots of *Lithospermum erythrorhizon* [51], whereas in *Nicotiana tabacum* and *Atropa belladonna*, the site of synthesis of alkaloids is in the roots, and these alkaloids are accumulated in aerial organs [16]. In *Withania somnifera*, steroidal lactone biosynthesis occurs in roots and aerial parts [50], likewise alkaloid synthesis takes place in both roots and aerial parts, and accumulation takes place in the entire plant in *Papaver somniferum* [15]. Consequently, aerial parts such as leaves, petioles, internodes, and roots should be used as initial explants for induction of adventitious roots. Various endogenous and environmental factors, like hormones, sugars, mineral salts, temperature, and light are responsible for induction of adventitious roots in naturally growing plants [33]. Similarly, nutrient medium, salt strength, the type and amount of growth regulators, and sucrose concentration are the factors which influence induction of adventitious roots from in vitro cultured leaf, root, and stem explants (Fig. 1; [36, 42]). Adventitious root induction with in vitro cultured explants may be direct (without mediation of callus) or indirect (callus mediated) regeneration. For instance, in *Panax*

ginseng adventitious roots were callus regenerated from root explants [28, 65], whereas in *Polygonum multiflorum*, adventitious roots were developed directly from leaf and root explants [23].

During the cultivation of adventitious roots, the adventitious roots developed from various explants are evaluated for their growth and capability to produce biomass and to accumulate the secondary metabolite/s on semisolid cultures and/or in liquid suspension cultures. Then, lines/strains which exhibit consistency in accumulation of biomass and metabolites are subsequently selected (after four to six subculture cycles) as superior lines. The selected lines are subjected to molecular techniques to verify their genetic stability. Ho et al. [23] evaluated six lines (AR-1, AR-2, AR-3, AR-4, AR-5, and AR-6) developed from leaf explants of *Polygonum multiflorum* and reported that, of the six lines, AR-2 was good in biomass accumulation (60.12 g l^{-1} fresh biomass and 6.36 g l^{-1} dry biomass); AR-6 was excellent in total phenolics and total flavonoids accumulation (50.35 mg g^{-1} dry weight and 22.51 mg g^{-1} dry weight, respectively); and, based on overall performance, they selected line AR-6 for large-scale production of phenolics and flavonoids.

2.2 Optimization of Culture Parameters

Varied factors of culture medium, culture conditions usually affect the biomass and metabolite accumulation. Therefore, chemical factors of medium such as salt strength, sugars, nitrogen, and growth regulators; physical factors, namely, light, temperature, and hydrogen ion concentration (pH); and other factors such as inoculum density and agitation/aeration of cultures are to be standardized for achieving highest biomass and biosynthesis of metabolites in adventitious roots cultures (Fig. 2; [38, 42]). Praveen and Murthy [49] demonstrated that Murashige and Skoog (MS, [34]) medium was superior for the production of biomass and production of withanolides in adventitious root cultures of *Withania somnifera* when compared to Gamborg (B5, [17]), Nitsch and Nitsch (NN, [45]), and Chu [8] media. They also tested the effect of salt strength of MS medium, i.e., 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 salt strength, and reported that 0.5 salt strength MS medium was superior for biomass and metabolite accumulation. Of the various inoculum density tested (2.5, 5.0, 10.0, and 20 g l^{-1} fresh weight), Praveen and Murthy [49] found that 10 g l^{-1} is ideal for both biomass and metabolite accumulation. Murthy and Praveen [35] investigated the effect of carbon source (glucose, fructose, maltose, and sucrose), sucrose levels (1, 2, 3, 4, 5, 6, and 8%), and medium pH (initial medium pH 4.0, 4.5, 5.0, 5.5, 5.8, 6.0, 6.5) on growth and production of withanolides in adventitious root cultures of *Withania somnifera*; their results showed that 2% sucrose was the best for both adventitious root growth and accumulation of withanolides. The biomass of adventitious roots was excellent with initial medium pH of 5.8, whereas withanolide production was highest at the

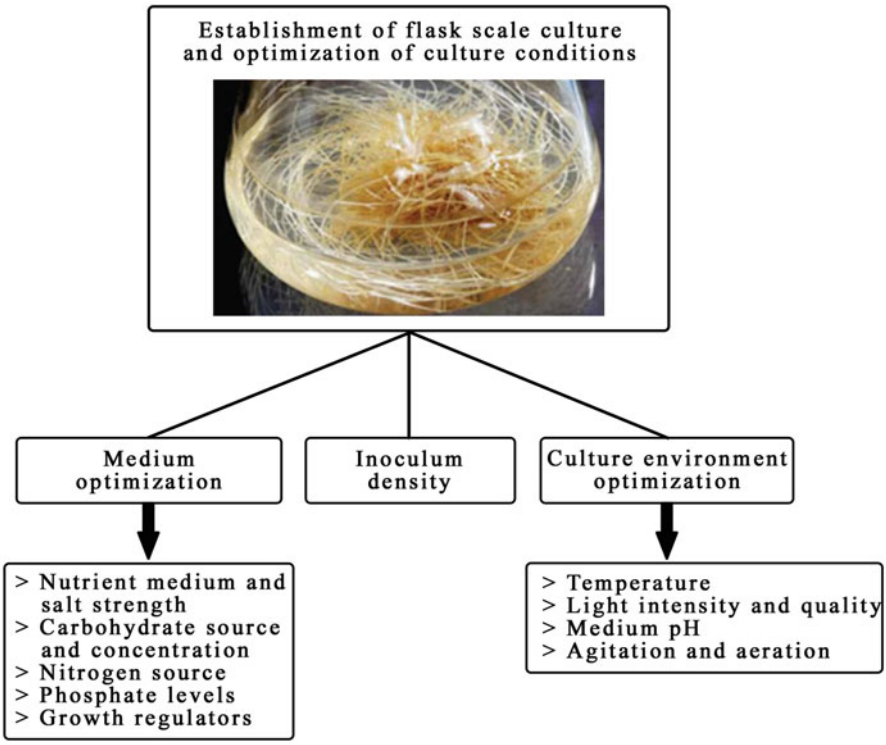


Fig. 2 Flow chart depicting establishment of small-scale adventitious root cultures in conical flasks and optimization of parameters for production of phytochemicals

medium pH level of 5.5. Wu et al. [57] explored the effect of auxins [indole-acetic acid (IAA), indole-butyric acid (IBA), and naphthaleneacetic acid (NAA)] on growth and accumulation of phenolics and flavonoids in adventitious root cultures of *Echinacea purpurea* and recorded that IBA at lower concentration (2 mg l^{-1}) was excellent for adventitious root proliferation, biomass, and metabolites accumulation compared to IAA and NAA. Lee and Paek [31] tested the effects of ammonium to nitrate ratios (0:30, 5:25, 10:20, 15:15, 20:10, 25:5, and 30:0 mM) of MS medium to determine the optimal ammonium and nitrate concentrations on adventitious root biomass and metabolites in *Eleutherococcus koreanum* and are of the opinion that 5:25 mM ammonium and nitrate ratio was congenial for growth of adventitious roots and metabolite accumulation. Influence of temperature and light and dark regimes was studied on growth and production of caffeic acid derivatives in *Echinacea purpurea* adventitious root cultures by Wu et al. [58]; accumulation of biomass and metabolites was optimal under incubation temperature of 20 °C among the different incubation temperatures tested (10, 15, 20, 25, and 30 °C). Adventitious root growth and biomass accumulation was highest in cultures grown under continuous dark, while accumulation of caffeic acid derivatives was optimum in cultures grown under 3/21 light and dark culture conditions.

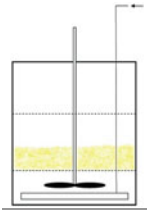
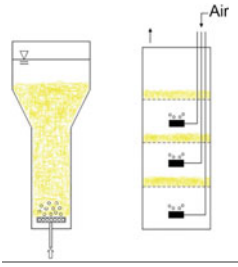
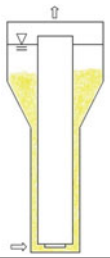
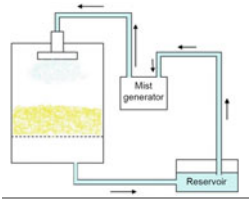
2.3 Elicitation

Biotic and abiotic elicitors are useful to activate the secondary metabolic pathway and to enhance the production of target compounds in cell and organ cultures [20, 38]. Polysaccharides and oligosaccharides such as alginate, pectin, chitosan, mannan, and galacturonides could be used as elicitors and they enhance secondary metabolite synthesis [20]. Chemical elicitors such as jasmonic acid (JA), methyl jasmonate (MJ), salicylic acid (SA), and nitric oxide (NO) are also frequently used for elicitation of cell and organ cultures [20]. Adventitious root cultures of *Panax ginseng* were treated with MJ (10, 30, 50, 100, and 150 μM), and MJ-treated cultures inhibited the adventitious root growth and increased ginsenoside accumulation [29]. Therefore, they have adopted two-stage strategy, i.e., culturing adventitious roots for 40 days without elicitor and then treatment of adventitious root cultures for another 14 days with 100 μM of MJ, and with such treatments, 11-fold increment Rb group ginsenosides were reported. Lee et al. [32] examined the production of bioactive compounds in adventitious root cultures of *Eleutherococcus koreanum* by elicitation with MJ and SA, and they achieved 37% higher production of eleutherosides, chlorogenic acid, phenolics, and flavonoids with the treatment of cultures with 50 μM MJ. These studies demonstrate the usefulness of elicitation strategy in improving accumulation of secondary metabolites in adventitious root cultures.

3 Cultivation of Adventitious Roots in Bioreactors

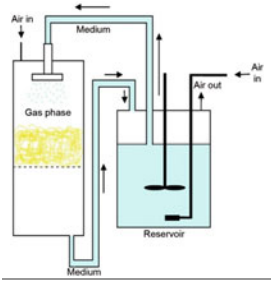
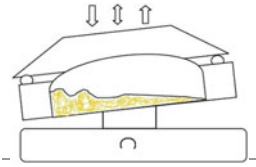
Bioreactors used for cultivation of plant cells and organs are glass or steel containers with different configurations (such as vertical/horizontal cylinders, columns, circular, or balloon shaped) and sizes that are developed in the line of fermenters which are used for culturing microbial cells. Bioreactors are provided with sterile environment, and they contain liquid nutrient medium, and the medium is aerated with sterile air. The bioreactors are designed in such a way that culture conditions such as aeration, temperature, gases, hydrogen ion concentration (pH), and other essential parameters are optimized and monitored in them [19, 55]. Bioreactors used for culturing plant cells and organs are of two types, namely, liquid-phase bioreactors and gas-phase bioreactors (Table 1). Conventional liquid-phase bioreactors are stirred tank bioreactors, in which cells/organs are submerged in the liquid medium; mixing is achieved by mechanically driven rotors/impellers for agitation and aeration. Agitation of the medium prevents aggregation and settling of biomass; however, agitation of the medium with impellers is responsible for shear stress on the cultured cells and organs. Further, stirred tank bioreactors consume high energy, and they are not useful for organ cultures such as hairy and adventitious roots as these bioreactors are responsible for mechanical damage of the organs [19, 55]. Pneumatically driven bioreactors were developed subsequently in which sterilized air or mixture of gases passed though the spargers kept at the bottom of the bioreactors for aeration and agitation of the medium (Table 1). Bubble column bioreactors are the simplest pneumatic type of bioreactors in which shear stress is

Table 1 Configuration of varied bioreactors used for cultivation of plant cell and organ cultures

Type of bioreactor	Advantages	Disadvantages
Liquid phase bioreactors		
Mechanically driven		
Stirred tank 	Agitation prevents aggregation and settling of biomass	High shear force
		High-energy consumption
	Enhanced aeration	Difficult to optimize multiple parameters
Pneumatically driven		
Bubble column 	Simple design and construction	Foaming
	Low shear stress	Irregular growth of biomass due to undefined flow pattern
	Low capital and operational costs	
Airlift 	Reduced shear stress	Not suitable for high-density cultures
	Low-energy requirement	Inadequate mixing and oxygen mass transfer
Gas-phase bioreactors		
Nutrient mist 	Improved oxygen and nutrient availability	Consumption of high energy
	Reduced shear stress	Labor-intensive setup

(*continued*)

Table 1 (continued)

Type of bioreactor	Advantages	Disadvantages
Trickle bed 	Better oxygen supply	Creates mass transfer barrier by forming viscous liquid layer on roots
	Low-energy requirement	Labor-intensive setup
Novel bioreactors		
Wave mixed (disposable) 	Flexible, user-friendly	Limitations in large-scale production
	Cost- and laborsaving	Multiple manual procedures may lead to the risk of leakage

reduced with cultured cells and organs; these bioreactors require low capital and operation costs [7]. Nevertheless, disadvantages of bubble column reactors are nonuniform growth of biomass due to undefined flow pattern due to coalescing of air bubbles and foaming during accumulation of biomass [7]. Airlift bioreactors are modified bubble column bioreactors which contain a draft tubes (internal or external) which facilitate the circulation of the medium and enhance oxygen mass transfer. However, these bioreactors were not suitable for high-density cultures because of insufficient mixing and nutrient transfer [55]. Various gas-phase bioreactors were designed and used for hairy root cultures including nutrient mist bioreactors, trickle-bed bioreactors, and wave-mixed bioreactors (disposable bioreactors) as they provide better oxygen and nutrient supply, good gas exchange, and reduced shear stress [27, 55]. However, these bioreactors have limitations of scale-up process due to high-energy consumption and are not cost-effective [55].

A diverse set of bioreactor configurations were experimented for cultivation of adventitious roots of *Panax ginseng*, and it was a modified airlift bioreactor, namely, balloon-type bubble bioreactor (BTBB) (Fig. 3) which was found more suitable for biomass and metabolite production [48]. This bioreactor was good enough for mixing of biomass with the medium, aeration, and oxygen mass transfer and was responsible for low shear stress and foaming [48]. Balloon-type bubble bioreactors were also suitable for scale-up process from small scale (5, 20 l) to pilot scale (100 l, 500 l) and even to commercial/industrial scale (1000 l and 10,000 l) without hampering the productivity [6, 39, 48]. Balloon-type bubble reactors were

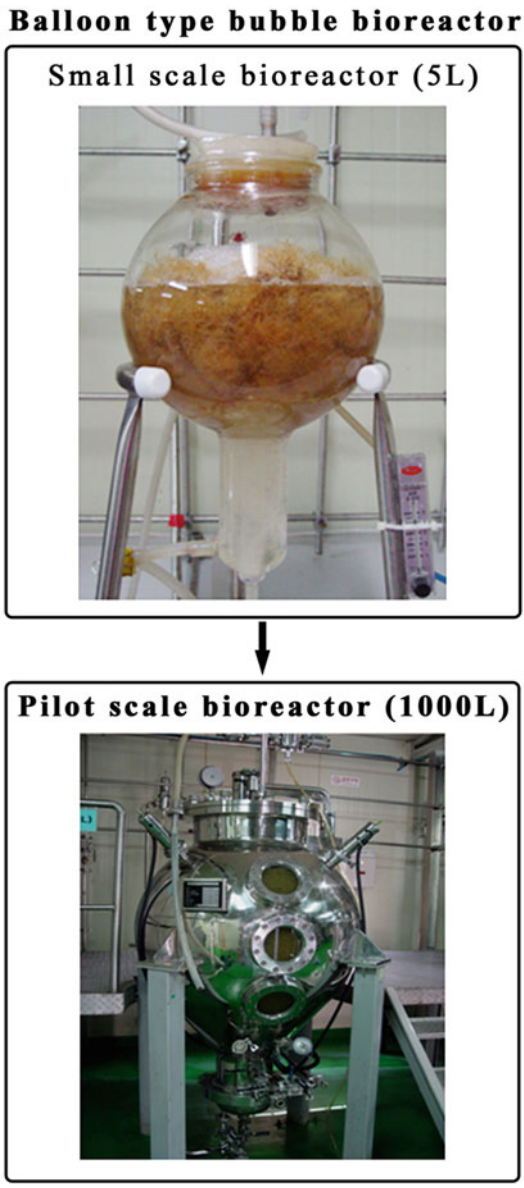


Fig. 3 Balloon-type bubble bioreactors used for adventitious root cultures

successfully used for culturing adventitious roots of *Echinacea purpurea*, *E. angustifolia* [37, 58–60], *Hypericum perforatum* [13, 14, 40], and *Polygonum multiflorum* [22] for the production of secondary metabolites at pilot scale (500 l and 1000 l). BTBBs were also used for cultivation of adventitious roots of *Astragalus membranaceus* [62], *Echinacea pallida* [18], *Eleutherococcus koreanum* [32], *Morinda citrifolia* [2], and *Oplopanax elatus* [21, 25]. Wu et al. [61] experimented

interspecific adventitious root co-culture of ginseng and *Echinacea* for simultaneous production of ginsenosides and caffeic acid derivatives using BTBBs. Similarly, BTBBs are also quite useful for co-culturing of *Echinacea pallida*, *E. angustifolia*, and *E. purpurea* adventitious roots [63, 64].

4 Successful Examples of In Vitro Production of Phytochemicals by Adventitious Root Cultures

4.1 Ginsenosides

Ginsenosides are triterpenoid compounds (saponins) that are produced in *Panax* species especially *Panax ginseng* (Asian ginseng or Korean ginseng). Ginsenosides have been shown to possess major pharmacological activities, and it is the major ingredient in herbal preparations and functional foods [39]. Ginsenosides are classified into three categories based on their structure, namely, Rb group (protopanaxadiols), the Rg groups (protopanaxatriols), and Ro group (oleanolic acid) (Fig. 4A).

Application of in vitro techniques for the production of ginsenosides in *Panax ginseng*, *P. notoginseng*, and *P. japonicus* var. *repens* cell cultures was experimented by various workers of Russia, China, Japan, and Korea, and in chemostatic cultures, daily biomass yield was approximately 1 g/l, and ginsenoside content was varying at 2–5% of dry weight [46]. For the improvement of biomass and ginsenoside content, adventitious root cultures were established in *Panax ginseng* by Korean researchers. They selected hundred-year-old mountain ginseng after ginsenoside fingerprinting and induced adventitious roots and selected superior lines which possessed threefold higher ginsenosides (32.46 mg/g dry weight) compared to cultivated ginseng (11.35 mg/g DW) and wild ginseng (14.19 mg/g DW) [43]. The adventitious root clones selected were also superior in accumulation of higher ginsenosides compared to suspended cells (7.86 mg/g DW) and hairy roots (9.83 mg/g DW) [43]. Korean researchers systematically established various parameters which are responsible for biomass and metabolite production including nutrient medium, levels of various macro-salts, viz., nitrate, phosphate levels; growth regulators, sucrose, inoculums density, hydrogen ion concentration, temperature, light quality, and intensity for adventitious root cultures in small-scale cultures [39, 43, 48]. They also established bioreactor cultures using balloon-type bubble bioreactors and worked out various parameters like aeration; supplementation of various gases such as oxygen, carbon dioxide, and ethylene; elicitation (using chemical elicitors such as methyl jasmonate, salicylic acid); and feeding of precursors (squalene) or fresh medium on biomass and ginsenoside accumulation [39, 43, 48]. Korean researchers were also successful in conducting scale-up process and established pilot scale (500 l) and commercial scale bioreactors (1000 and 10,000 l; Fig. 3). Commercial bioreactors are implemented and operated by CBN Biotech Company, South Korea, and they are producing 35 to 45 t of ginseng adventitious root biomass every year the root biomass used by pharmaceutical, food, and cosmetic industries on regular basis.

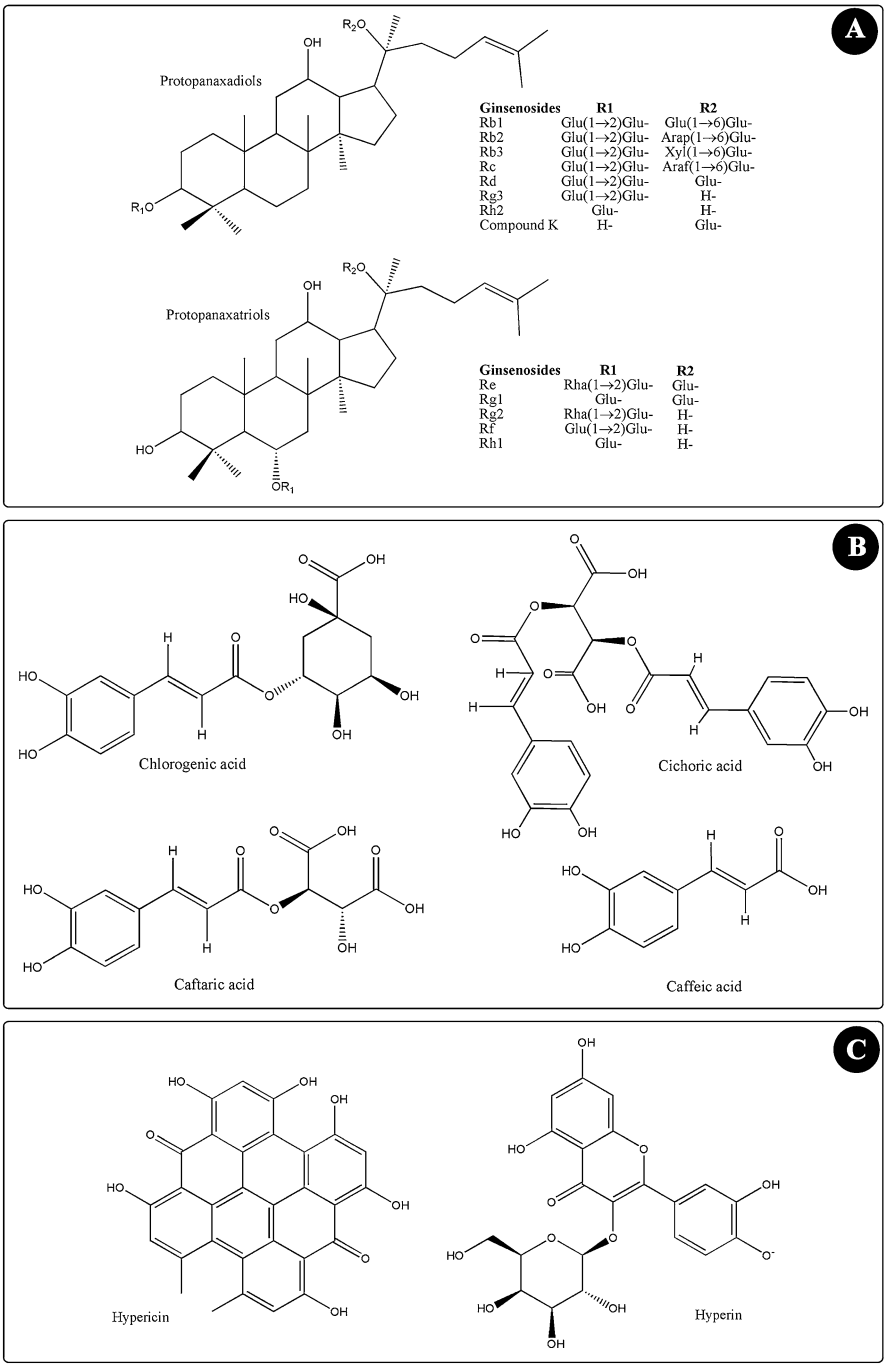


Fig. 4 Structure of phytochemicals produced from adventitious root cultures

4.2 Caffeic Acid Derivatives

Caffeic acid derivatives (CADs) are polypropenoids which are produced by *Echinacea* species, namely, *Echinacea purpurea* (purple coneflower), *E. angustifolia* (Narrow-leaf coneflower), and *E. pallida* (Pale purple coneflower), and are used as herbal medicines and a dietary supplements in North America and Europe [3, 37]. Cichoric acid, chlorogenic acid, caffeic acid, and caftaric acid are major CADs which are reported to possess antioxidant, anti-inflammatory, antiviral, and immunostimulatory activities (Fig. 4B).

Adventitious root culture system has been developed in *E. purpurea*, and factors affecting growth of biomass and CADs production including medium, salt strength of the medium, sucrose levels, growth regulators, pH, light, and temperature have been standardized [24, 37, 58–60]. Similarly, parameters affecting the growth and accumulation of CADs in adventitious root cultures of *E. angustifolia* have been also worked out [13]. Based on standardized data, Wu et al. [60] and Cui et al. [13] established pilot scale cultures (500 l and 1000 l) of *E. purpurea* and *E. angustifolia* by using BTBBs, and they reported accumulation of 25.3 kg and 50.3 kg fresh biomass in 500 l batch cultures and higher accumulation of CADs in adventitious root biomass [37].

4.3 Hypericin

Hypericin is a class of naphthodianthrones (Fig. 4C) and the characteristic constituent of the genus *Hypericum* species especially *Hypericum perforatum* (St. John's wort), and it possesses antitumor, antiviral, and antidepressant properties [26, 30]. In *H. perforatum*, the hypericin content ranges from 0.03 to 0.09%, but it can vary depending on the cultivar, altitude, light conditions, and period of the year [4]. Therefore, various attempts were made for the production of hypericin from in vitro cell and organ cultures of *H. perforatum* [40]. Adventitious roots were induced in *H. perforatum*, and systematic experiments were conducted by Cui et al. [9–12, 14] for the production of biomass and hypericin. Cui et al. [14] established pilot scale adventitious root cultures of *H. perforatum* in 500 l capacity BTBB and horizontal drum type bioreactors and obtained 76.2 kg and 80.4 kg fresh biomass with good content of hypericin.

5 Conclusions

Plant cell and organ cultures have been exploited for commercial production of a wide-ranging phytochemicals having pharmaceutical, nutraceutical, and other industrial values. In vitro culture of adventitious roots is one of useful system for the production of useful phytochemicals because of their high biomass accumulation capability as well as high stability during physical chemical conditions during scale-up process [39, 42, 48]. Selection of superior genotype, induction of adventitious

roots, and choice of superior clone, establishment of adventitious root cultures, and working culture conditions and parameters responsible for biomass and metabolite accumulation are the key steps for successful production of useful phytochemicals in vitro. However, in majority of the cases, researchers are overlooking the key parameters responsible for successful production of phytochemicals through in vitro cultures. Another area yet to be explored for further research is bioreactor cultivation of adventitious roots which involves criteria like selection of bioreactor type, developing optimal process parameters, and standardizing operation methods under defined optimal conditions. Most challenging and critical step is the economic feasibility in the production of useful phytochemicals using adventitious root system. Advancement in research on the development of low-cost and low-maintenance systems and developing low-cost techniques of harvesting biomass, extraction of phytochemicals, and evaluation of toxicity and efficacy of target phytochemicals will be valuable in this venture.

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