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Review

A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures

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Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots, meristems etc for both the ways for multiplication and extraction of secondary metabolites. Strain improvement, methods for the selection of high-producing cell lines, and medium optimizations can lead to an enhancement in secondary metabolite production. However, most often trials with plant cell cultures fail to produce the desired products. In such cases, strategies to improve the production of secondary metabolites must be considered. One of the main problems encountered is the lack of basic knowledge of the biosynthetic routes, and mechanisms responsible for the production of plant metabolites. Where the productivity of the desired metabolites is limited by the lack of particular precursors, biotransformation using an exogenous supply of biosynthetic precursors, genetic manipulation and metabolic engineering may improve the accumulation of compounds. Feedback inhibition of metabolic enzymes as well as inhibition of membrane transport can be eliminated by the accumulation of synthesized products in a second phase introduced into the aqueous medium. Organ cultures and *in vitro* biomass production often have sites of synthesis and storage of secondary metabolites in separate compartments. Elicitors, compounds triggering the formation of secondary metabolites, can be abiotic or biotic. Natural elicitors include polysaccharides such as pectin and chitosan, which are also used in the immobilization and permeabilization of plant cells. Immobilization with suitable bioreactor system provides several advantages, such as continuous process operation, but for the development of an immobilized plant cell culture process, natural or artifically induced secretion of the accumulated product into the surrounding medium is necessary. The present review highlights the nature, applications, perspective and scale up methods for the production of valuable secondary metabolites *in vitro*.

Key words: Plant cell cultures, secondary metabolites, biotransformation, biosynthetic pathways, elicitation, immobilization, bioreactors.

INTRODUCTION

Since the early days of mankind, plants with secondary metabolites have been used by humans to treat infections, health disorders and illness (Wyk and Wink, 2004). Only during the last 100 years have natural products been partly replaced by synthetic drugs, for which plant structures were a lead in many instances, for example salicylic acid and aspirin. Many higher plants are major sources of useful secondary metabolites which are used in pharmaceutical, agrochemical, flavor and aroma industries. The search for new plant-derived chemicals should thus be a priority in current and future efforts towards sustainable conservation and rational utilization of biodiversity (Philipson, 1990). Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants (Rao and Ravishankar, 2002). On a global scale, medicinal plants are mainly used as crude drugs and extracts. Several of the more potent and active substances are employed as isolated compounds, including many alkaloids such as morphine (pain killer), codeine (antitussive), papaverine (phosphordiesterase inhibitor), ephedrine (stimulant), ajmaline (antirrhythmic), quinine (antimalarial), reserpine (antihypertensive), galanthamine (acetylecholine esterase inhi-

bitor), scopolamine (travel sickness), berberine (psoriasis), caffeine (stimulant), capsaicin (rheumatic pains), colchicines (gout), yohimbine (aphrodisiac), pilocarpine (glaucoma), and various types of cardiac glycosides (heart insufficiency) (Wink et al., 2005).

The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, it is not only commercial significance that drives the research initiatives. The deliberate stimulation of defined chemical products within carefully regulated *in vitro* cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled microenvironmental regimes.

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, biobased fuels and plastics, enzymes, preservatives, cosmetics (cosmeceuticals), natural pigments, and bioactive compounds. There is a series of distinct advantages to producing a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant. These include: a) Production can be more reliable, simpler, and more predictable, b) Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants, c) Compounds produced *in vitro* can directly parallel compounds in the whole plant, d) Interfering compounds that occur in the field-grown plant can be avoided in cell cultures, e) Tissue and cell cultures can yield a source of defined standard phytochemicals in large volumes, f) Tissue and cell cultures are a potential model to test elicitation, g) Cell cultures can be radio labeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically.

Secondary products in plant cell culture can be generated on a continuous year-round basis; there are no seasonal constraints. Production is reliable, predictable, and independent of ambient weather. At least in some cases, the yield per gram fresh weight may exceed that which is found in nature. Disagreeable odours or flavors associated with the crop plant can be modified or eliminated *in vitro.* Plant cell culture eliminates potential political boundaries or geographic barriers to the production of a crop, such as the restriction of natural rubber production to the tropics or anthocyanin pigment production to climates with high light intensity. When a valuable product is found in a wild or scarce plant species, intensive cell culture is a practical alternative to wild collection of fruits or other plant materials. Extraction from the *in vitro* tissues is much simpler than extraction from organized, complex tissues of a plant. Plant tissue culture techniques offer the rare opportunity to tailor the chemical profile of a phytochemical product, by manipulation of the chemical or physical microenvironment, to produce a compound of potentially more value for human use.

While research to date has succeeded in producing a wide range of valuable secondary phytochemicals in unorganized callus or suspension cultures, in other cases production requires more differentiated microplant or organ cultures (Dörnenberg and Knorr, 1997). This situation often occurs when the metabolite of interest is only produced in specialized plant tissues or glands in the parent plant. A prime example is ginseng (*Panax ginseng*). Since saponin and other valuable metabolites are specifically produced in ginseng roots, root culture is required *in vitro.* Similarly, herbal plants such as *Hypericum perforatum* (St. John's wort), which accumulates the anti-depressant hypericins and hyperforins in foliar glands, have not demonstrated the ability to accumulate phytochemicals in undifferentiated cells (Smith et al., 2002). As another example, biosynthesis of lysine to anabasine occurs in tobacco (*Nicotiana tabacum*) roots, followed by the conversion of anabasine to nicotine in leaves. Callus and shoot cultures of tobacco can produce only trace amounts of nicotine because they lack the organ-specific compound anabasine. In other cases, at least some degree of differentiation in a cell culture must occur before a product can be synthesized (e.g., vincristine or vinblastine from *Catharanthus roseus*). Reliance of a plant on a specialized structure for production of a secondary metabolite, in some cases, is a mechanism for keeping a potentially toxic compound sequestered. The three long-standing, classic examples of commercially viable production of a secondary metabolite *in vitro* — ginseng saponines, shikonin, and berberine — each feature products that have diversified uses, including medicinal applications. Ginseng is produced in large-scale root cultures, whereas the other two products are produced in highly colored cell cultures. A tremendous research and development effort has advanced a number of other *in vitro* -derived secondary products to semicommercial status, including vanillin and taxol production in cell cultures. In a myriad of other cases, the *in vitro* processes for secondary metabolite production have fallen far short of expectations and have never approached commercial status. Still, the arena of secondary product formation in cell cultures remains as an industrial pursuit. Engineers and biologists are currently joining forces on a global scale to develop new strategies for streamlining the critical bioprocesses.

Research efforts on a broad range of plant cell culture-derived extracts can be cited in each of these major pro-

duct categories: flavors (onion and garlic, peppermint and spearmint, fruit flavors, chocolate aroma, seaweed flavors, vanilla, celery, coffee, spice, sweeteners, and so on); edible colors for foods and medicines (mainly betalains and anthocyanins); non-food pigments for cosmetics and textiles (shikonin, berberine, and various other products); several examples of fragrances and essential oils; and bioactive natural insecticides and phytoalexins useful in current integrated pest management programs. Of course, intensive activity has centered on production of natural drugs or chemoprotective compounds from plant cell culture. Some of the most prominent pharmaceutical products in this latter category include ajmalicine (a drug for circulatory problems) from *C. roseus* and taxol (a phytochemical effective in treatment of ovarian cancer) from *Taxus* species. *In vitro* production of secondary metabolites from higher plants is surveyed and summarized in Table 1.

Organ cultures for secondary metabolite production

The technique of *in vitro* organ culture of *Fritillaria unibracteata* has been established and chemical composition of the metabolites has been confirmed. *F. unibracteata* can be rapidly propagated, directly from small cuttings of the bulb by the technique of organ culture. The cultured bulb can be harvested after a 50-day culture period in MS media supplemented with 4.44 µM BA and 5.71 µM IAA. The growth rate was about 30–50 times higher than that under natural wild growth conditions. The content of alkaloid and beneficial micro-elements in the cultured bulbs were higher than found in the wild bulb. It is therefore possible to establish a novel process to produce this natural plant drug in future studies and achieve scale-up for industrial production (Gao et al., 2004).

In vitro shoot multiplication of *Frangula alnus* was obtained on woody plant medium (WPM) with indole-3-acetic acid (IAA) and 6-benzylaminapurine (BAP), the highest metabolite production (1731 mg/100 g of total anthraquinone was in the shoots grown on the MS medium with addition of 1-naphthilaceneacetic (NAA) (0.1 mg l^{-1}) and thidiazuron (TDZ) (0.1 mg l^{-1}). Similarly, shoot multiplication of *Frangula rupestris* was obtained on MS medium supplemented with BAP (0.5 mg l^{-1}). The highest metabolite production of total anthraquinone was in the shoots grown on medium with 2,4-D (0.1 mg l^{-1}) and BAP (0.5 mg l^{-1}) (Kovacevic et al., 2005).

Shoot cultures of *Gentianella austriaca* (A. and J. Kerner) Dostal established from seedling epicotyls were maintained on MS medium supplemented with 2.22 µM BA and 0.54 µM NAA. Shoot cultures contained the same types of secondary metabolites as plants from nature. Xanthones were the major constituents, with DMB (demethylbellidifolin), DGL (demethylbellidifolin-8-*O*-glucoside) and BGL (bellidifolin-8-*O*-glucoside) present at roughly two times lower concentrations than in samples from nature. Secondary metabolite production was strongly affected by the presence of BA in the medium (Vinterhalter et al., 2008).

Precursor addition for improvement of secondary metabolite production

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses (Zhao et al., 2005). Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures. The most frequently used elicitors in previous studies were fungal carbohydrates, yeast extract, MJ and chitosan. MJ, a proven signal compound, is the most effective elicitor of taxol production in *Taxus chinensis* Roxb. (Wu and Lin, 2003) and ginsenoside production in *Panax ginseng* C.A. Meyer (Yu et al., 2000; Yu et al., 2002; Kim et al., 2004; Thanh et al., 2005) cell/organ culture. In the present study, the effect of different concentrations of MJ on embryogenic cell growth and eleutheroside accumulation was tested and results reveled that addition of 200 µM MJ was suitable for optimum accumulation of eleutheroside B, E, E1 and chlorogenic acid. However, addition of MJ at higher concentration (above 100 µM) was detrimental for biomass accumulation. Similar to the present results, MJ inhibited the cell growth and promoted the secondary metabolite production with cell/adventitious root cultures of *Bupleurum falcatum* L. (Aoyagi et al., 2001), *Taxus* spp. (Yukimune et al., 1996; Ketchum et al., 1999) and *Panax ginseng* C.A. Meyer (Kim et al., 2004; Thanh et al., 2005). Differential accumulation of eleutherosides was observed during elicitation experiments.

In the bioreactor cultures, eleutheroside content increased significantly by elicitation of methyl jasmonate (MJ) when the *Eleutherococcus senticosus* embryo was cultured in Liquid MS with suspension. However, the fresh weight, dry weight and growth ratio of embryos was strongly inhibited by increasing MJ concentrations. The highest total eleutheroside (7.3 fold increment) and chlorogenic acid (3.9 fold increment) yield was obtained with 200 µM methyl jasmonate treatment (Shohael et al., 2007).

The involvement of amino acids in the biosynthesis of hyperforin and adhyperforin was reported in *H. perforatum* shoot cultures. Valine and isoleucine, upon administration to the shoot cultures, were incorporated into acyl side chain of hyperforin and adhyperforin, respectively. Feeding the shoot cultures with unlabelled l-isoleucine at a concentration of 2 mM induced a 3-7-fold increase in the production of adhyperforin. The addition of 3 mM threonine, a precursor of isoleucine, stimulated a

Table 1. *In vitro* secondary metabolites from plant cell, tissue and organs cultures.

Plant name	Active ingredient	Culture medium and plant growth regulator(s)	Culture type	Reference (s)
Aconitum heterophyllum	Aconites	MS + 2,4-D + Kin	Hairy root	Giri et al., 1997
Adhatoda vasica	Vasine	MS + BAP + IAA	Shoot culture	Shalaka and Sandhya, 2009
Agastache rugosa	Rosmarinic acid	MS + 2,4-D + Kin + 3% sucrose	Hairy root	Lee et al., 2007
Agave amaniensis	Saponins	MS + Kinetin	Callus	Andrijany et al., 1999
Ailanthus altissima	Alkaloids	MS + 2,4-D + Kinetin	Suspension	Anderson et al., 1987
Ajuga reptans	Phytoecdysteroids		Hairy root	Matsumoto and Tanaka, 1991
Allium sativum	Allin	MS + IAA + Kinetin	Callus	Malpathak and David, 1986
Aloe saponaria	Glucosides	MS + 2,4-D + Kinetin	Suspension	Yagi et al., 1983
Ambrosia tenuifolia	Altamisine	MS + Kinetin	Callus	Goleniowski and Trippi, 1999
Ammi majus	Umbelliferone	MS + BAP	Shootlet	Krolicka et al., 2006
Ammi visnaga	Furanocoumarin	MS + IAA + GA3	Suspension	Kaul and Staba, 1967
Amsonia elliptica	Indole alkaloids		Hairy root	Sauerwein et al., 1991
Anchusa officinalis	Rosmarinic acid	B_5 + 2,4-D	Suspension	De-Eknamkul and Ellis, 1985
Angelica gigas	Deoursin	MS (Liq.) + 2,4-D + GA3	Hairy root	Xu et al., 2008
Anisodus luridus	Tropane alkaloids	MS + 2,4-D + BA	Hairy root	Jobanovic et al., 1991
Ammi majus	Triterpenoid	MS + 2,4-D + BA	Suspension	Staniszewska et al., 2003
Arachys hypogaea	Resveratol	G5 + 2,4-D + Kin.	Hairy root	Kim et al., 2008
Armoracia laphthifolia	Fisicoccin	MS + IAA	Hairy root	Babakov et al., 1995
Artemisia absinthum	Essential oil	MS + NAA + BAP	Hairy root	Nin et al., 1997
Artemisia annua	Artemisinin	MS + IAA + Kinetin	Hairy root	Rao et al., 1998
Artemisia annua	Artemisinin	MS + NAA + Kinetin	Callus	Baldi and Dixit, 2008
Aspidosperma ramiflorum	Ramiflorin	MS + 2,4-D + BAP	Callus	Olivira et al., 2001
Aspidosperma ramiflorum	Ramiflorin alkaloid	MS + 2-4,D + BAP + 30 g/l Sucrose	Callus	Olivira et al., 2001
Astragalus mongholicus	Cycloartane saponin	MS + 2,4-D + Kin	Hairy root	Ionkova et al., 1997
Astragalus mongholicus	Cycloartane	MS + IAA + NAA	Hairy root	Ionkova et al., 1997
Azadirachta indica	Azadirachtin	MS + 2,4-D	Suspension	Sujanya et al., 2008
Azadirachta indica	Azadirachtin	MS + 2,4-D + Cyanobacterial elicitor	Suspension	Poornasri Devi et al., 2008
Beeta vulgaris	Betalain pigments	MS + IAA	Hairy root	Taya et al., 1992
Brucea javanica	Alkaloids	MS + 2,4-D + Kinetin	Suspension	Lie et al., 1990
Brucea javanica	Cathin	MS + IAA + GA3	Suspension	Wagiah et al., 2008
Brugmansia candida	Tropane	MS + 2,4-D + IAA	Hairy root	Marconi et al., 2008
Brugmansia candida	Tropane alkaloid	MS + BA + NAA	Hairy root	Giulietti et al., 1993
Bupleurum falcatum	Saikosaponins	B_5 + IBA	Root	Kusakari et al., 2000
Bupleurum falcatum	Saikosaponins	LS + 2,4-D	Callus	Wang and Huang, 1982
Calystegia sepium	Cuscohygrine	MS + 2,4-D + BA	Hairy root	Jung and Tepfer, 1987
Camellia chinensis	Flavones	MS + 2,4-D + NAA	Callus	Nikolaeva et al., 2009
Camellia sinensis	Theamine	MS + IBA + Kinetin	Suspension	Orihara and Furuya, 1990
Campanula medium	Polyacetylenes	MS + IAA + BA	Hairy root	Tada et al., 1996
Canavalia ensiformis	Canavanine	LS + NAA + Picloram	Callus	Ramirez et al., 1992
Capsicum annum	Capsiacin	MS + 2,4-D+ GA3	Callus	Varindra et al., 2000
Capsicum annum	Capsiacin	MS + 2,4-D + Kin.	Callus	Umamaheswari and Lalitha, 2007
Capsicum annuum	Capsaicin	MS + 2,4-D + Kinetin	Suspension	Johnson et al., 1990
Cassia acutifolia	Anthraquinones	MS + 2,4-D + kinetin	Suspension	Nazif et al., 2000

Table 1. Contd.

Cassia obtusifolia	Anthraquinone	MS + TDZ + IAA	Hairy root	Ko et al., 1995
Cassia senna	Sennosides	MS + NAA + Kin	Callus	Shrivastava et al., 2006
Catharanthus roseus	Indole alkaloids	MS + IAA	Suspension	Moreno et al., 1993
		MS + NAA + Kinetin	Suspension	Zhao et al., 2001
Catharanthus roseus	Vincristine	MS + 2,4-D + GA3	Suspension	Lee-Parsone and Rogce, 2006
Catharanthus roseus	Indole alkaloid	MS + 2,4-D + GA3 + Vanadium	Suspension	Tallevi and Dicosmo, 1988
Catharanthus roseus	Catharathine	MS + 2,4-D + UV-B radiation	Suspension	Ramani and Jayabaskaran, 2008
Catharanthus trichophyllus	Indole alkaloids	MS + IAA + GA3	Hairy root	Davioud et al., 1989
Cayratia trifoliata	Stilbenes	MS + IAA + GA3	Suspension	Roat and Ramawat, 2009
Centella asiatica	Asiaticoside	MS + 2,4-D	Hairy root	Kim et al., 2007
Centella asiatica	Asiaticoside	Ms + 2,4-D + Kin	Callus	Kiong et al., 2005
Centella asitica	Asiaticoside	MS + BAP + IAA	Shoot	Kim et al., 2004
Centella asitica	Asiaticoside	MS + 2,4-D	Hairy root	Paek et al., 1996
Centranthes ruber	Valepotriates	MS + IAA + Kin	Hairy root	Granicher et al., 1995
Cephaelis ipecacuanha	Alkaloids	MS + IAA	Root	Teshima et al., 1988
Chaenatis douglasei	Thiarbrins	MS + NAA	Hairy root	Constabel and Towers, 1988
Chrysanthemum cinerariaefolium	Pyrithrins	MS + 2.4-D + Kinetin	Callus	Rajasekaran et al., 1991
		MS + Kinetin	Suspension	Kuch et al., 1985
Cinchona ledgeriana	Quinine	MS + 2,4-D	Hairy root	Hamill et al., 1989
		B_5 + 2,4-D	Suspension	Schripsema et al., 1999
		B_5 + 2,4-D + Kinetin	Suspension	Wijnsma et al., 1985
Cinchona succirubra	Anthraquinone	MS + IAA + GA3	Suspension	Khouri et al., 1986
Citrus sp.	Limonin	MS + 2,4-D + Kinetin	Callus	Barthe et al., 1987
Coffea arabica	Caffeine	MS + 2,4-D + Kinetin	Callus	Waller et al., 1983
Coleus forskohlii	Forskolin	MS + IAA + Kin	Hairy root	Sasaki et al., 1998
Corydalis ambigua	Corydaline	MS + IAA + 3% sucrose	Embryo	Hiraoka et al., 2004
Corydalis cava	Corydaline	MS + IAA + GA3	Shoot	Rueffer et al., 1994
Corydalis ophiocarpa	Alkaloids	MS + 2,4-D + Kinetin	Callus	Iwasa and Takao, 1982
Corydylis terminalis	Corydalin	MS + 2,4-D + BAP	Callus	Taha et al., 2008
Coscinium fenustratum	Berberin	MS + 2,4-D + BAP	Callus	Khan et al., 2008
Coscinium fenustratum	Berberine	MS + IAA +BAP	Callus	Nair et al., 1992
Coscinium fenustratum	Berberine	MS + 2,4-D + GA3	Suspension	Narasimhan and Nair, 2004
Crataegus sinaica	Flavonoid	MS + 2,4-D + NAA + BAP	Callus	Maharik et al., 2009
Croton sublyratus	Plaunotol	MS + NAA + BA	Callus	Morimoto and Murai, 1989
Cruciata glabra	Anthraquinones	LS + NAA + Kinetin	Suspension	Dornenburg and Knorr, 1996
Cryptolepis buchanani	Cryptosin	B_5 + 2,4-D + Kinetin	Callus	Venkateswara et al., 1987
Cymbopogon citratus	Essential oil	MS + IAA + GA3	Shoot	Quiala et al., 2006
Datura stramonium	Hyocyamine	MS + IAA	Hairy root	Hilton and Rhodes, 1993
Digitalis purpurea	Cardenolides	MS + BA	Suspension	Hagimori et al., 1982

Table 1. Contd.

Digitalis purpurea	Cardioactive glycosides	MS + 2,4-D + BA	Hairy root	Saito et al., 1990
Diocorea doryophora	Diogenin	MS + 2,4-D + BA	Suspension	Huang et al., 1993
Dioscorea deltoidea	Diosgenin	MS + 2,4-D	Suspension	Heble and Staba, 1980
Drosera rotundifolia	7-Methyljuglone	MS + BAP + NAA	Shoot culture	Hohtola et al., 2005
Duboisia leichhardtii	Alkaloids	LS + NAA + BA	Callus	Yamada and Endo, 1984
Duboisia leichhardtti	Scopalamine	MS + 2,4-D + BA	Hairy root	Muranaka et al., 1992
Duboisia myoporoides	Scopalamine	MS + IAA	Hairy root	Deno et al., 1987
Echinacea purpurea	Alkamides	MS + 2,4-D	Hairy root	Trysteen et al., 1991
Eleutherococcus senticosus	Eleuthrosides	MS + 2,4-D	Suspension	Shohael et al., 2007
Ephedra sp.	L-Ephedrine	MS + Kinetin + 2,4-D	Suspension	O'Dowd et al., 1993
Eriobotrya japonica	Triterpenes	LS + NAA + BA	Callus	Taniguchi et al., 2002
Eucalyptus tereticornis	Sterols and phenolic compounds	MS + 2,4-D	Callus	Venkateswara et al., 1986
Fabiana imbricata	Rutin	MS + NAA + 2,4-D	Callus and Suspenson	Schmeda-Hirschmann et al., 2004
Fagopyrum esculentum	Flavonol	MS + IAA + GA3	Hairy root	Trotin et al., 1993
Fagopyrum esculentum	Rutin	MS + NAA	Hairy root	Lee et al., 2007
Frangula alnus	Anthraquinones	WPM + IAA + BAP	Callus	Kovacevic and Grabisic, 2005
Fritillaria unibracteata	Alkaloids	MS + 2,4-D + Kin	Multiple shoot	Gao et al., 2004
Fumaria capreolata	Alkaloids	LS + IAA	Suspension	Tanahashi and Zenk, 1985
Gentiana macrophylla	Glucoside	MS + IAA + Kin	Hairy root	Tiwari et al., 2007
Gentiana sp.	Glucosides	B_5 + Kinetin	Callus	Skrzypczak et al., 1993
Gentianella austriaca	Xanthone	MS + BAP	Multiple shoot	Vinterhalter et al., 2008
Geranium thunbergii	Tannin	MS + 2,4-D + BAP	Hairy root	Ishimaru and Shimomura, 1991
Ginko biloba	Ginkoside-A	MS + NAA + Kinetin	Suspension	Carrier et al., 1991
Glehnia littoralis	Furanocoumarin	LS + 2,4-D + Kinetin	Suspension	Kitamura et al., 1998
Glycyrrhiza echinata	Flavonoids	MS + IAA + Kinetin	Callus	Ayabe et al., 1986
Glycyrrhiza glabra	Triterpenes	MS + IAA + Kinetin + 2,4-D	Callus	Ayabe et al., 1990
Glycyrrhiza glabra	Glycyrrhizin	MS + 2,4-D + GA3	Hairy root	Mehrotra et al., 2008
Glycyrrhiza glabra	Flavonoid	MS + IAA	Hairy root	Asada et al., 1998
Gymnema sylvestre	Gymnemic acid	MS + 2,4-D + IAA	Callus	Gopi and Vatsala, 2006
Gymnema sylvestre	Gymnemic acid	MS + IAA + BA	Callus	Devi et al., 2006
Gynostemma pentaphyllum	Saponin	MS + 2,4-D + BAP	Hairy root	Fei et al., 1993
Gypsophila paniculata	Saponin	MS + IAA + TDZ	Root suspension	Fulcheri et al., 1998
Hemidesmus indicus	Lupeol, Rutin	MS + BAP + NAA	Shoot culture	Misra et al., 2005
Hyocyamus niger	Tropane alkaloids	MS + 2,4-D + BA	Callus	Yamada and Hashimoto, 1982
Hyocyamus niger	Tropane alkaloid	MS + IAA + Kinetin	Hairy root	Jaziri et al., 1988
Hyoscyamus albus	Phytolexins	MS + NAA + GA3	Hairy root	Kuroyanagi et al., 1998
Hyoscyamus muticus	Hyoscyamine	MS + 2,4-D	Hairy root	Halperin and Flores, 1997
Hypericum perforatum	Hypericin	Liquid MS + NAA + GA3	Suspension	Hohtola et al., 2005
Hypericum perforatum	Hypericins	MS + BA + IAA	Multiple shoot	Kornfeld et al., 2007
Hypericum perforatum	Hypericin	MS + BA + TDZ	Multiple shoot	Santarem and Astarita, 2003
Hypericum perforatum	Hyperforin	MS + 2,4-D + Leusine	Multiple shoot	Karppinen et al., 2007
Hyssopus officinalis	Titerpenes	G5 + 2,4-D + IAA	Suspension	Skrzypek and Wysokinsku, 2003
Hyssopus oficinalis	Sterols	MS + 2,4-D + NAA	Suspension	Skrzypek and Wysokinsu, 2003

Table 1. Contd.

Ipomoea cairica	Lignan	MS + IAA + Kin	Callus	Paska et al., 1999
Isoplexis isabelliana	Anthraquinone	MS + 2,4-D + Kinetin	Suspension	Arrebola et al., 1999
Lactuca virosa	Sesquiterpene lactones	MS + 2,4-D	Hairy root	Kisiel et al., 1995
Leontopodium alpinum	Essential oil	MS + IAA + BA	Hairy root	Hook, 1994
Linum flavum	5-Methoxyphyllotaxin	MS salts + B_5 Vitamins	Suspension	Uden et al., 1990
Linum flavum	Lignan	MS + IAA + GA3	Hairy root	Oostdam et al., 1993
Lithospermum erythrorhizon	Shikonin derivatives	LS + IAA + Kinetin	Suspension	Fujita et al., 1981 ; Fukui et al., 1990
Lithospermum erythrorhizon	Shikonin	MS + 2,4-D + Kinetin	Hairy root	Fukui et al., 1998
Lobelia cardinalis	Polyacetylene glucosides	MS + 2,4-D	Hairy root	Yamanaka et al., 1996
Lycium chinense	Cerebroside	MS + 2,4-D, Kinetin	Suspension	Jang et al., 1998
Mentha arvensis	Terpenoid	MS + BA + NAA	Shoot	Phatak and Heble, 2002
Momordica charantia	Flavonoid	MS + BAP + NAA	Callus	Agarwal and Kamal, 2007
Morinda citrifolia	Anthraquinones	B_5 + NAA	Suspension	Zenk et al., 1975
		B_5 + NAA + Kinetin	Suspension	Assetti et al., 1995
Mucuna pruriens	L-Dopa	MS + IAA	Suspension	Wichres et al., 1993
		MS + 2,4-D	Callus	Brain, 1976
Myristica fragrans	Myristin	MS + NAA + TDZ	Shoot	Indira Iyer et al., 2009
Nandina domestica	Alkaloids	MS + 2,4-D + Kinetin	Callus	Ikuta and Itokawa, 1988
Nicotiana hesperis	Anatabine	MS + IAA	Hairy root	Parr and Hamill, 1987
Nicotiana rustica	Alkaloids	LS + 2,4-D + Kinetin	Callus	Tabata ad Hiraoka,1976
Nicotianan tabacum	Nicotine	MS + NAA + Kinetin	Suspension	Mantell et al., 1983
Ophiorrhiza rugosa var. *decumbens*	Camptothecin	MS + BA + Kin.	Shoot	Vineesh et al., 2007
Panax ginseng	Saponin and spogenins	MS + 2,4-D	Callus	Furuya et al., 1973 ; Asaka et al., 1993
Panax ginseng	Glycoside	MS + NAA + Kin.	Hairy root	Jeong and Park, 2007
Panax notoginseng	Gensenosides	MS + 2,4-D + Kinetin	Suspension	Zhong and Zhu, 1995
Papaver bracteatum	Thebaine	MS + Kinetin + 2,4-D	Callus	Day et al., 1986
Papaver somniferum	Alkaloids	MS + Kinein	Callus	Furuya et al., 1972
	Morphine and codeine	MS + 2,4-D + Kinetin	Suspension	Siah and Doran, 1991
Papaver somniferum	Codeine	LS + BA + NAA	Hairy root	Williams and Ellis, 1992
Peganum harmala	Alkaloids	MS + 2,4-D	Suspension	Sasse et al., 1982
Perezia cuernavacana	Sesquterpene quinone	MS + IAA + BA	Hairy root	Arellano et al., 1996
Phiorrhiza pumila	Alkaloids	LS + 2,4-D + NAA	Callus	Kitajima et al., 1998
Phytolacca americana	Betacyanin	MS + 2,4-D	Suspension	Sakuta et al., 1987
Picrasma quassioides	Quassin	B_5 + 2,4-D + Kinetin	Suspension	Scragg and Allan, 1986
Pimpinella anisum	Essential oil	MS + IAA + BAP	Hairy root	Santos et al., 1998
Piper solmsianum	Piperine	MS + 2,4-D + BA	Suspension	Balbuena et al., 2009
Plantago media	Verbascoside	B5 + IAA + Kin.	Callus	Kunvari et al., 1999
Platycodon grandiflorum	Polyacetylene	MS + 2,4-D	Hairy root	Tada et al., 1995
Pluchea lanceolata	Quercetin	MS + NAA + BAP	Callus	Arya et al., 2008

Table 1. Contd.

Plumbago rosea	Plumbagin	MS + $CaCl_2$	Callus	Komaraiah et al., 2003
Plumbago zeylanica	Plumbagin	MS + BAP + IBA	Hairy root	Verma et al., 2002
Podophyllum hexandrum	Podophyllotaxin	B_5 + NAA	Suspension	Uden et al., 1989
Podophyllum hexandrum	Podophyllotaxin	MS + BAP + GA32	Shoot	Li et al., 2009
Polygala amarella	Saponin	MS + IAA	Callus	Desbene eet al., 1999
Polygonum hydropiper	Flavonoids	MS + 2,4-D + Kinetin	Suspension	Nakao et al., 1999
Portulaca grandiflora	Betacyanin	MS + 2,4-D + Kinetin	Callus	Schroder and Bohm, 1984
Primula veris	Saponins	MS + BAP + GA3	Shoot	Okrslar et al., 2007
Psoralea cordifolia	Isoflavones	MS + TDZ + BAP	Multiple shoot	Shinde et al., 2009
Psoralea corylifolia	Isoflavones	MS + TDZ + BAP	Multiple shoot	Shinde et al., 2009
Ptelea trifoliata	Alkaloids	MS + 2,4-D +Kinetin	Callus	Petit-Paly et al., 1987
Rauvolfia sellowii	Alkaloids	B_5 + 2,4-D + Kinetin	Callus	Rech et al., 1998
Rauvolfia serpentina	Reserpine	LS + NAA +BA	Suspension	Yamamoto and Yamada, 1996
		LS	Callus	Gerasimenko et al., 2001
Rauvolfia serpentina	Serpentine	MS + BAP + IAA	Callus	Salma et al., 2008
Rauvolfia serpentina	Reserpine	MS + IAA + Cu2+	Callus	Nurchgani et al., 2008
Rauvolfia tetraphylla	Reserpine	MS + 2,4-D + Tryptophan	Callus	Anitha and Kumari, 2006
Rhamnus catharticus	Anthraquinones	WPM + Kin + 2,4-D	Callus	Kovacevic and Grabisic, 2005
Rheum ribes	Catechin	MS + IBA + BA	Callus	Farzami and Ghorbant, 2005
Rhodiola rosea	Rosarin	MS + NAA + IAA	Callus culture	Hohtola et al., 2005
Rhus javanica	Gallotannins	LS + IAA +Kinetin	Root	Tanoguchi et al., 2000
Rubia akane	Anthraquinone	B5 + NAA + Kin	Hairy root	Park and Lee, 2009
Rubia akane	Anthraquinone	MS + 2,4-D + Chitosan	Suspension	Jin et al., 1999
Rubia tinctorum	Anthraquinone	MS + 2,4-D	Hairy root	Sato et al., 1991
Ruta sp.	Alkaloids and coumarins	MS + 2,4-D + Kinetin	Callus	Baumert et al., 1992
Salvia miltiorrhiza	Rosmarinic acid	MS + 2,4-D + BA	Callus	Morimoto et al., 1994
		MS + 2,4-D + Kinetin		Miyasaka et al., 1989
	Cryptotanshinone		Suspension	
Salvia officinalis	Flavonoid	LMS + IAA + BAP	Multiple shoot	Grzegorczyk and Wysokinska, 2008
Salvia officinalis	Terpenoids	MS + 2,4-D + BA	Callus	Santos-Gome et al., 2002
Saponaria officinalis	Saponin	MS + IAA + TDZ	suspension	Fulcheri et al., 1998
Saprosma fragrans	Anthraquinone	MS + 2,4-D + NAA	Callus	Singh et al., 2006
Scoparia dulcis	Scopadulic acid	LMS + Kin + Phenyl urea	Callus	Hayashi et al., 1998
Scopolia parviflora	Alkaloids	LS + 2,4-D + IAA	Callus	Tabata et al., 1972
Scutellaria baicalensis	Flavonoids	MS + IAA	Hairy root	Zhou et al., 1997
Scutellaria columnae	Phenolics	MS + 2,4-D + Kinetin	Callus	Stojakwska and Kisiel, 1999
Serratula tinctoria	Ecdysteroid	MS + 2,4-D + BA	Hairy root	Delbeque et al., 1995
Sesamum indicum	Napthaquinone	MS + NAA + Kinetin	Hairy root	Ogasawara et al., 1993
Silybium marianum	Silymarin	MS + IAA + GA3	Hairy root	Rahnama et al., 2008
Silybium mariyanm	Flavonolignan	LS + TDZ	Root	Alikaridis et al., 2000
Silybum marianum	Silymarin	MS + IAA + Kin	Hairy root	Rahnama et al., 2008
Silybum marianum	Silymarin	MS + IAA + BA	Callus	Tumova et al., 2006
Simmondsia chinensis	Fixed oil	MS + TDZ + GA3	Callus	Aftab et al., 2008
Simmondsia chinensis	Fixed Oil	MS + IAA + 2iP	Callus	Aftab et al., 2008
Solanum aculeatissi	Steroidal saponin	MS + 2,4-D	Hairy root	Ikenaga et al., 1995
Solanum chrysotrichum	Saponin	MS + 2,4-D + Kinetin	Suspension	Villarreal et al., 1997
Solanum laciniatum	Solasodine	MS + 2,4-D + Kinetin	Suspension	Handler and Dodds, 1983

Table 1. Contd.

Solanum paludosum	Solamargine	MS + BA + Kinnetin	Suspension	Badaouti et al., 1996
Stevia rebaudiana	Stevioside	MS + BA + NAA	Callus	Dheeranapattana et al., 2008
Swertia japonica	Amarogenetin	MS + IAA	Hairy root	Ishimaru et al., 1990
Tabernaemontana divariacata	Alkaloids	MS + NAA + BA	Suspension	Sierra et al., 1992
Tagetes patula	Thiophenes	MS + IAA + Kinetin	Hairy root	Arroo et al., 1995
Tanacetum parthenium	Sesquiterpene	MS + 2,4-D + Kinetin	Hairy root	Kisiel and Stojakowska, 1997
Taxus baccata	Taxol baccatin III	B_5 + 2,4-D + Kinetin + GA_3	Suspension	Cusido et al., 1999
Taxus spp.	Taxol	B_5 + 2,4-D + BA	Suspension	Wu et al., 1992
Thalictrum minus	Berberin	LS + NAA + 2,4-D + BA	Suspension	Kobayashi et al., 1987
		LS + NAA + BA	Suspension	Nakagawa et al., 1986
Tinospora cordifolia	Berberin	MS + IAA + GA3	Suspension	Rama Rao et al., 2008
Torreya nucifera	Diterpenoids	MS + 2,5-D	Suspension	Orihara et al., 2002
Trichosanthes kirilowii	Protein	MS + IAA	Hairy root	Savary and Flores, 1994
Trigonella foenu-graecum	Saponins	MS + 2,4-D + Kinetin	Suspension	Brain and Williams, 1983
Vaccinium myrtillus	Flavonoids	MS + BAP + NAA	Callus culture	Hohtola et al., 2005
Vinca major	Vincamine	MS + BAP	Hairy root	Tanaka et al., 2004
Vitis vinifera	Anthocyanin	MS + BAP + NAA	Suspension	Qu et al., 2006
Vitis vinifera	Resveratrol	MS + IAA + GA3 + UV	Callus	Kin and Kunter, 2009
Withania somnifera	Withaferin A	MS + BA	Shoot	Ray and Jha, 2001
Withania somnifera	Withaferin	MS + IAA + Kintin	Hairy root	Banerjee et al., 1994
Withania somnifera	Withanoloid A	MS + IAA + Kin	Hairy root	Murthy et al., 2008
Withania somnifera	Steroidal lactone	MS + 2,4-D + BA	Callus	Mirjalili et al., 2009
Zataria multiflora	Rosmarininc acid	MS + IAA + Kin	Callus	Francoise et al., 2007

2-fold increase in the accumulation of adhyperforin (Karppinen et al., 2007). Production of triterpenes in leaf derived callus and cell suspension cultures of Centella asiatica was enhanced by the feeding of aminoacids. In the callus culture manifold increase of asiaticoside accumulation was reported with the addition of leucien (Kiong et al., 2005).

The effect of coniferyl alcohol as a precursor of flavonolignan biosynthesis on silymarin components production in *Silybum marianum* suspension culture was reported (Tumova et al., 2006). Coniferyl alcohol showed the changes in silymarin complex production. A significant increase of silydianin was observed only after 72 h of the application of 46 µM coniferyl alcohol. The same precursor – coniferyl alcohol in the form of complex with β-cyclodextrin was used as precursor for podophyllotoxin accumulation in Podophyllum hexandrum cell suspension cultures.

Elicitation of *in vitro* products

Pharmaceutically significant secondary metabolites or phytopharmaceuticals include alkaloids, glycosides, flavonoids, volatile oils, tannins, resins etc. Currently, most of these secondary metabolites are isolated from wild or cultivated plants because their chemical synthesis is either extremely difficult or economically infeasible. Biotechnological production in plant cell cultures is an attractive alternative, but to date this has had only limited commercial success because of a lack of understanding of how these metabolites are synthesized. Plants and/or plant cells *in vitro*, show physiological and morphological responses to microbial, physical or chemical factors which are known as 'elicitors'. Elicitation is a process of inducing or enhancing synthesis of secondary
metabolites by the plants to ensure their survival, persistence and competitiveness (Namdeo, 2007).

The production of secondary metabolites in callus, cell suspension and hairy roots of *Ammi majus* L. is by exposing them to elicitors: benzo(1,2,3)-thiadiazole-7-carbothionic acid *S*-methyl ester and autoclaved lysate of cell suspension of bacteria—*Enterobacter sakazaki* (Staniszewska et al., 2003). GC and GC–MS analysis of chloroform and methanol extracts indicated a higher accumulation of umbelliferone in the elicited tissues than in the control ones. Plants generally produce secondary metabolites in nature as a defense mechanism against pathogenic and insect attack. The study was applied in several abiotic elicitors to enhance growth and ginseng saponin biosynthesis in the hairy roots of *Panax ginseng* (Jeong and Park, 2007). Generally, elicitor treatments were found to inhibit the growth of the hairy roots, al-

though simultaneously enhancing ginseng saponin biosynthesis. Tannic acid profoundly inhibited the hairy root growth during growth period. Also, ginseng saponin content was not significantly different from that of the control. The addition of selenium at inoculum time did not significantly affect ginseng saponin biosynthesis. However, when 0.5 m*M* selenium was added as an elicitor after 21 d of culture, ginseng saponin content and productivity increased to about 1.31 and 1.33 times control levels, respectively. Also, the addition of 20 µM $NiSO_4$ resulted in an increase in ginseng saponin content and productivity, to about 1.20 and 1.23 times control levels, respectively, and also did not inhibit the growth of the roots. Sodium chloride treatment inhibited hairy root growth, except at a concentration of 0.3% (w/v). Increases in the amounts of synthesized ginseng saponin were observed at all concentrations of added sodium chloride. At 0.1% (w/v) sodium chloride, ginseng saponin content and productivity were increased to approximately 1.15 and 1.13 times control values, respectively. These results suggest that processing time for the generation of ginseng saponin in a hairy root culture can be reduced via the application of an elicitor.

Chitosan was the biotic elicitor polysaccharide and it is eliciting the manyfole increase of anthraquinone production in *Rubia akane* cell culture (Jin et al., 1999).

Metabolic engineering and production of secondary metabolites

Metabolic engineering involves the targeted and purposeful alteration of metabolic pathways found in an organism to achieve better understanding and use of cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard, 1996). This technique applied to plants will permit endogenous biochemical pathways to be manipulated, resulting in the generation of transgenic crops in which the range, scope, or nature of a plant's existing natural products are modified to provide beneficial commercial, agronomic and/or post-harvest processing characteristics (Kinney, 1998). Over the last decades, plant cell cultures have been intensively investigated as a possible tool for the production of commercial plant secondary metabolites, including fine chemicals such as pharmaceuticals, agrochemicals, flavors, insecticides, fragrances and cosmetics (Whitmer et al., 2002). In spite of the efforts in the field of *in vitro* production of phytochemicals, few industrial processes have been developed, involving only a limited number of secondary products, such as shikonin, berberine, ginsenosides and paclitaxel (Ramachandra and Ravishankar, 2002). As in many cases production is too low for commercialization, metabolic engineering can provide various strategies to improve productivity, such as: increasing the number of producing cells, increasing the carbon flux through a biosynthetic pathway by overexpression of genes codifying for rate-limiting enzymes or blocking the mechanism of feedback inhibition and competitive pathways and decreasing catabolism.

Many of the isolated pure compounds with biological activity are alkaloids, a diverse group of nitrogen-containing chemical ring structure compounds, with alkali-like chemical reactivity and pharmacological activity. Although the pharmacological effects of alkaloids have been studied, the biosynthetic pathways of these compounds are still obscure. Among the most famous are the tropane alkaloids, such as (-)-hyoscyamine, its racemate atropine, and scopolamine (hyoscine), which have an 8-azabicyclo[3.2.1]octane esterified nucleus. These alkaloids are commonly found in plants of different families: Solanaceae, Erythroxylaceae, Convolvulaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae and Cruciferae (Griffing and Lin, 2000). Related to the tropane alkaloids, a new group of nortropane alkaloids, the calystegines, was discovered only 15 years ago. Calystegines bear three to five hydroxyl groups in various positions, making them water-soluble, and they share metabolic steps and enzymes of the formation of tropane alkaloids.

Several genes in the biosynthetic pathways for scopolamine, nicotine, and berberine have been cloned, making the metabolic engineering of these alkaloids possible. Expression of two branching-point enzymes was engineered: putrescine *N*-methyltransferase (PMT) in transgenic plants of *Atropa belladonna* and *Nicotiana sylvestris* and (*S*)-scoulerine 9-*O*-methyltransferase (SMT) in cultured cells of *Coptis japonica* and *Eschscholzia californica*. Overexpression of PMT increased the nicotine content in *N. sylvestris*, whereas suppression of endogenous PMT activity severely decreased the nicotine content and induced abnormal morphologies. Ectopic expression of SMT caused the accumulation of benzylisoquinoline alkaloids in *E. californica* (Sato et al., 2001).

Hairy root cultures as a source of secondary metabolites

The hairy root system based on inoculation with *Agrobacterium rhizogenes* has become popular in the two last decades as a method of producing secondary metabolites synthesized in plant roots (Palazon et al., 1997). Unorganized plant tissue cultures are frequently unable to produce secondary metabolites at the same levels as the intact plant. This is also the case of scopolamine production in undifferentiated *in vitro* cultures of Solanaceae, probably due to the specific location of some of the key enzymes involved in this biosynthetic pathway (Palazon et al., 2006). Suzuki et al. (1997) have demonstrated that the expression of the *pmt* gene was pericycle-specific, and it has also been shown

that H6H is localized in the root pericycle (Kanegae et al., 1994). In addition, Nakajima and Hashimoto (1999) have observed that TR proteins accumulate in the lateral roots of *Hyoscyamus niger*. Another possible reason for the low production of scopolamine in undifferentiated *in vitro* cultures could be that the auxin added to the callus and cell culture media for normal growth inhibit the activity of some of the key enzymes involved in scopolamine biosynthesis, such as PMT (Rothe et al., 2003).

The hairy root phenotype is characterized by fast hormone-independent growth, lack of geotropism, lateral branching and genetic stability. The secondary metabolites produced by hairy roots arising from the infection of plant material by *A. rhizogenes* are the same as those usually synthesized in intact parent roots, with similar or higher yields (Sevon and Oksman-Caldentey, 2002). This feature, together with genetic stability and generally rapid growth in simple media lacking phytohormones, makes them especially suitable for biochemical studies not easily undertaken with root cultures of an intact plant. During the infection process *A. rhizogenes* transfers a part of the DNA (transferred DNA, T-DNA) located in the root-inducing plasmid Ri to plant cells and the genes contained in this segment are expressed in the same way as the endogenous genes of the plant cells. Some *A. rhizogenes*, such as strain A4, have the T-DNA divided in two sections: the TR-DNA and TL-DNA, each of which can be incorporated separately into the plant genome. Two sets of pRi genes are involved in the root induction process: the *aux* genes located in the TR region of the pRi T-DNA and the *rol* (root loci) genes of the TL region (Jouanin, 1989). The *ags* genes responsible for opine biosynthesis in the transformed tissues are also located in the TR region (Binns and Tomashow, 1988). Opines are synthesized by plant transformed cells and are only used by *Agrobacterium* as a source of nitrogen and carbon. Due to the similarities of the *A. rhizogenes* and *A. tumefaciens* infection processes, and because both microorganisms are very closely related, it has been suggested that the most important *A. rhizogenes* oncogenes encode proteins involved in the regulation of plant hormone metabolism. *Aux* genes provide transformed cells with an additional source of auxin (Chriqui et al., 1996), but they do not seem essential for developing hairy root disease. However, *rol* genes have functions that are most likely other than that of producing mere alterations in plant hormone concentrations (Nilsson and Olsson, 1997). Several authors have investigated the effect of TR and TL regions of *A. rhizogenes* on growth and morphology of transformed roots and plants, but until now there have been few studies on the direct effects of oncogenes on secondary metabolism. As has been previously reported, a correlation exists between the expression of the *rol*C gene and tropane alkaloids (Fumanova and Syklowska, 2000), *Catharanthus roseus* alkaloids (Palazon et al., 1998), and ginsenoside production (Bulkagov et al., 1998). No correlation between *rol*A and *rol*B expression and secondary metabolism was found in any of these studies. Moyano et al. (1999) showed that the inoculation of leaf sections of tobacco, *Duboisia* hybrid and *Datura metel* plants with the A4 strain of *A. rhizogenes* induced transformed roots with the capacity to produce putrescine-derived alkaloids such as nicotine, hyoscyamine and scopolamine. In general, the obtained hairy roots presented two morphologies: typical hairy roots with a high capacity to produce alkaloids, and callus-like roots with faster growth and lower alkaloid production. The *aux*1 gene of *A. rhizogenes* located in the TR-DNA of *A. rhizogenes* was detected in all roots showing callus-like morphology. However, this gene was only detected in 25-60% of the established root cultures showing typical hairy morphology. These results demonstrate a significant role of *aux* genes in the morphology of transformed roots and the importance of typical hairy root morphology in the production of secondary metabolites. The studies with *Panax ginseng* hairy roots also support the effects of the genes located in the TR-DNA on root morphology and secondary metabolism (Mallol et al., 2002). The hairy roots are normally induced on aseptic, wounded parts of plants by inoculating them with *A. rhizogenes*. In scopolamine-producing Solanaceae plants, roots usually emerge at the inoculation sites after 1-4 weeks, but in the case of other plant species such as *Taxus* it can be more than 4 months before the roots emerge. Root tips are cultured separately in a hormone-free medium, the most commonly used being MS (Murashige-Skoog, Gamborg's B5 or SH (Schenk and Hildebrandt, 1972). The next step for establishing hairy root cultures is to select and characterize the root clones according to their capacity for growth and production of the desired compounds. Sometimes these productions were achieved after a laborious process to optimize the growth conditions, such as the selection of the more productive clones, and optimization of the production conditions by testing different ionic concentrations as well as the carbon source and pH of the medium.

Srivastava and Srivastava (2007) have recently summarized the attempts to adapt bioreactor design to hairy root cultures; stirred tank, airlift, bubble columns, connective flow, turbine blade, rotating drum, as well as different gas phase reactors have all been used successfully. In the case of tropane alkaloids, different types of bioreactors are used for scopolamine production. Wilson (1997) describes the only large droplet bioreactor system with a volume of 500 L designed for hairy root cultures of *Datura stramonium*. On a smaller scale, modified airlift and stirred tanks have been used for scopolamine production in hairy root cultures of *D. metel*, connective flow reactors for *H. muticus* (Carvalho and Curtis, 1998) and *Atropa belladonna* (Williams and Doran, 2000) and more recently a bubble column bioreactor has been employed for root cultures of *Scopolia parviflora* (Min et al., 2007). One such advance is the development of dis-

posable wave bioreactor systems, with working principle is based on wave-induced agitation, which significantly reduces stress levels. This type of bioreactor has been successfully used for *H. muticus* and *Panax ginseng* hairy root cultures (Eibl and Eibl, 2004).

Genetic manipulation in hairy root culture for secondary metabolite production

Transformed roots provide a promising alternative for the biotechnological exploitation of plant cells. *A. rihzogenes* mediated transformation of plants may be used in a manner analogous to the well-known procedure employing *A. tumifaciens*. *A. rihzogenes* mediated transformation has also been used to produce transgenic hairy root cultures and plantlets have been regenerated. None of the other T-DNA sequences are required for the transfer with the exception of the border sequences. The rest of the T-DNA can be replaced with the foreign DNA and introduced into cells from which whole plants can be regenerated. These foreign DNA sequences are stably inherited in a Mendelian manner (Zambryski et al., 1989). The *A. rhizogenes* mediated transformation has the advantage of being able to transfer any foreign gene of interest placed in binary vector to the transformed hairy root clone. It is also possible to selectively alter some plant secondary metabolites or to cause them to be secreted by introducing genes encoding enzymes that catalyze certain hydroxylation, methylation, and glycosylation reactions. An example of a gene of interest with regard to secondary metabolism that was introduced into hairy roots is the 6-β-hydroxylase gene of *Hyoscyamus muticus* which was introduced to hyocyamin rich *Atropa belladonna* by a binary vector system using *A. rhizogenes* (Hashimoto et al., 1993). Engineered roots showed an increased amount of enzyme activity and a five-fold higher concentration of scopolamine. Hairy root cultures of *Nicotiana rustica* with ornithin decarbosylase gene from yeast, and *Peganum harmala* with tryptophane decarboxylase gene from *Catharanthus roseus* (Berlin et al., 1993) have been shown to produce increased amounts of the secondary metabolites nicotine and serotonin when expressing transgenes from yeast. Downs et al. (1994) reported transgenic hairy roots in Brassica napus containing a glutamine synthase gene from soybean showing a three-fold increase in enzyme activity. This approach may be a reality for the commercial production of pharmaceutically important compounds using transgenic hairy root culture system.

Role of endophytes in *in vitro* production of secondary metabolites

There are three schools of thought on the origins of secondary metabolism in plants (Wink, 2008). There is the argument that both plants and endophytic microbes co-evolved with pathways to produce these natural products. Another thought is that an ancient horizontal gene transfer took place between plants and microbes. The third suggests that either plants or endophytic fungi produce these secondary metabolites and transfer them to the other symbiont. Biosynthetic pathway studies using radio-labeled precursor amino acids reveal that plants and endophytic fungi have similar, but distinct metabolic pathways for production of secondary metabolites (Jennewein et al., 2001). Evidence to support the independent production of Taxol by endophytic fungi is the isolation of the gene 10-deacetylbaccatin-III-10-O-acetyl transferase from the endophytic fungus *Clasdosporium cladosporiodes* MD213 isolated from *Taxus media* (yew species). This gene is involved in the biosynthetic pathway of Taxol and shares 99% identity with *T. media* (plant) and 97% identity with *T. wallichiana var. marirei* (plant). These data lead to the hypothesis that plants and endophytic fungi through mutualistic symbiosis produce similar secondary metabolites. Recently, it was reported that plants other than yew species also have endophytic fungi associated with them that make Taxol. This suggests that plants and fungi are independently capable of producing these important secondary metabolites. The question is whether bioactive phytochemicals of plants are produced by the plant itself or as a consequence of a mutualistic relationship with beneficial organisms in their tissue. The fact that a combination of inducing factors from both plants and endophytic fungi increased the accumulation of secondary metabolites in plants and fungi respectively (Zhang et al., 2009; Li et al., 2009) suggest that the fungal endophyte may play important roles in the biosynthesis of secondary metabolites. Therefore, the symbiotic association and effects of plants and endophytes on each other during the production of other important pharmacological bioactive natural products such as camptothecin, vinblastine, and podophyllotoxin need to be explored. This could provide the framework for future natural product production through genetic and metabolic engineering (Engels et al., 2008).

Bioreactors scaling up of production of secondary metabolites

This is the application of bioreactor system for large-scale cultivation of plant cells for the production of valuable bioactive compounds in an active field. Plant cells in liquid suspension offer a unique combination of physical and chemical environments that must be accommodated in large-scale bioreactor process. Some of the well known drawbacks of the cell suspension cultures include the instability of the productive cell lines, the slowness of the cell growth and limited knowledge about the metabolic pathway (Fulzele, 2005). There are indications

that sufficient oxygen supply and proper mixing in airlift bioreactors may not be suitable for high density plant cell suspension cultures. Well known problem shear sensitivity and rapid setting characteristics of plant cell aggregates and cell floating tendencies of the cell cultures have to be solved when bioreactors are designed.

The main constraint for commercial exploitation of *in vitro* cultures is the scaling up at industrial level. Hairy roots, callus and suspension cultures are complicated when it comes to scaling up and pose unique challenges. Mechanical agitation causes wounding of hairy roots and leads to callus formation. With a product of sufficiently high value it is feasible to use batch fermentation, harvest the roots, and extract the product. For less valuable products it may be desirable to establish a packed bed of roots to operate the reactor in a continuous process for extended periods collecting the product from the effluent stream. Scale up becomes difficult in providing nutrients from both liquid and gas phases simultaneously. Meristem dependent growth of root cultures in liquid medium results in a root ball with young growing roots on the periphery and a core of older tissue inside. Restriction of nutrient oxygen delivery to the central mass of tissue gives rise to a pocket of senescent tissue. Due to branching, the roots from an interlocked matrix that exhibits a exploit hairy root culture as a source of bioactive chemicals depends on development of suitable bioreactor system where several physical and chemical parameters must be taken into consideration.

Immobilization scaling up of secondary metabolite accumulation

Advances in scale-up approaches and immobilization techniques contribute to a considerable increase in the number of applications of plant cell cultures for the production of compounds with a high added value. Plant-derived compounds with cancer chemotherapeutic or antioxidant properties use rosmarinic acid (RA) and taxol as representative examples. Critical issues are thoroughly discussed already, including the dependence of *in vitro*, compound-specific production on culture growth and differentiation, elicitation strategies, physiological effects of immobilization, and the current status of scale-up production systems (Ramawat and Merillon, 2008).

Cell cultures of *Plumbago rosea* were immobilized in calcium alginate and cultured in Murashige and Skoog's basal medium containing 10 mM $CaCl_2$ for the production of plumbagin, an important medicinal compound (Komaraiah et al., 2003). Studies were carried to find out the impact of immobilization on the increased accumulation of this secondary metabolite. Immobilization in calcium alginate enhanced the production of plumbagin by three, two and one folds compared to that of control, un-crosslinked alginate and $CaCl_2$ treated cells respectively. Cells subjected to combined treatments of chitosan, immobilization and *in situ* extraction showed a synergistic effect and yielded 92.13 mg g^{-1} of plumbagin which is 21, 5.7, 2.5 times higher than control, immobilized, immobilized and elicited cells, respectively.

Conclusion

Advances in biotechniques, particularly methods for culturing plant cell cultures should provide new means for the commercial processing of even rare plants and the chemicals they provide. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. The major advantage of the cell cultures include synthesis of bioactive secondary metabolites, running in controlled environment, independently from climate and soil conditions. The use of *in vitro* plant cell culture for the production of chemicals and pharmaceuticals has made great strides building on advances in plant science. The increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable levels of product. The increased level of natural products for medicinal puroposes coupled with the low product yields and supply concerns of plant harvest has renewed interest in large-scale plant cell culture technology. Knowledge of biosynthetic pathways of desired phytochemicals in plants as well as in cultures is often still in its infancy, and consequently stratigies needed to develop an information based on a cellular and molecular level. These results show that *in vitro* plant cell cultures have potential for commercial production of secondary metabolites. The introduction of newer techniques of molecular biology, so as to produce transgenic cultures and to effect the expression and regulation of biosynthetic pathways, is also likely to be a significant step towards making cell cultures more generally applicable to the commercial production of secondary metabolites.

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