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REVIEW ARTICLE

Biotechnological Approaches for Enhanced Secondary Metabolite Production using Hairy Root Cultures

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Abstract

Secondary metabolites have great application such as nutraceuticals, pharmaceuticals and additives. A substantial amount of secondary metabolites can be produced by hairy root cultures. There are a list of novel compounds produced by hairy root cultures of different plants such as Agroastrolagoside I, Vulgaxanthin III, IV, 1,8-Di-*O*-methylchrysophanol, Indole alkaloids: anthraserpine, N-methyl pyrrolidinyl-cuscohygrine, 5-Hydroxy-and 6-hydroxy-tetrahydronorharman, 12- hydroxyajmaline two anthraquinones etc. Various biotechnological approaches can be used to improve the secondary metabolites production by hairy root cultures such as selection of efficient producing hairy root clones, optimization and selection of suitable culture media, use of plant growth regulators with elicitors.

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Introduction

In addition to essential primary metabolites (e.g. carbohydrates, lipids and amino acids), higher plants are also able to synthesize a wide variety of low molecular weight compounds – the secondary metabolite which can be used as food additives, nutraceuticals, and pharmaceuticals. The production of these compounds is often low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant. Plant cell cultures represent a potential source of valuable secondary metabolites. In vitro cultures are being considered as an alternative to agricultural processes for producing valuable secondary metabolites. Organized cultures, and especially root cultures, can make a significant contribution in the production of secondary metabolites. *Agrobacterium rhizogenes* causes hairy root disease in plants. The neoplastic (cancerous) roots produced by *A. rhizogenes* infection are characterized by high growth rate, genetic stability and growth in hormone free media. These genetically transformed root cultures can produce levels of secondary metabolites comparable to that of intact plants.

Numerous secondary metabolites have been produced in transformed roots at an advanced level than those in native plants by optimization of cultural forms (Table 1). However, in many previous reports the desired products yields were very low or sometimes not detectable in transformed roots. In order to achieve products at sufficiently high concentrations for commercial manufacture, several efforts have

been made to stimulate or restore biosynthetic activities of hairy root cultures using numerous methods (Sevon and Oksman Calendety, 2002). Hairy root cultures could offer a significant production in controlled fabrication of therapeutic proteins. This process is independent of seasonal variations and enables continuous production. Continuous secretion and recovery of foreign proteins from cell and culture medium can reduce the time and cost of process standardization, develop protein recovery, make the process more easily reproducible and reduce protein degradation during handling. Targeting recombinant proteins with suitable signal peptides for extracellular secretion can mimic the natural route in plants. Most of proteins can be simply recovered from the secretion fluid or culture media. The addition of protein stabilizing agents to the suspension culture standard can enlarge the accumulation of recombinant protein (Magnuson et al., 1996).

The expression of recombinant proteins in suspension and hairy root culture offers promising potential for exploitation as large bioreactors. Two tobacco plant cell lines, Bright Yellow-2(BY-2) and N. tobaccum-1 (NT-1) have been utilized extensively for foreign protein production because of easy transformation and synchronous growth in liquid culture (Hellwig et al., 2004). Nevertheless, improvement in optimizing the conditions of plant cell cultures for stable expression in enduring cultures.

The secreted proteins can be improved simply from the hydroponic medium and used as easy source material for protein enhancement and purification (Komarnytsky et al., 2004). Furthermore, potato hairy roots were employed for

Table 1: Some important secondary metabolites produced by hairy root cultures at levels higher than those found in the parent plants.

Plant species	Product investigated	Ratio of secondary metabolites in transformed culture /control culture	Reference
Aconitum heterophyllum	Aconites	11.7 (R)	
Beta vulgaris	betalains	1.7 (R)	Mukundan, 1993
Calystegia sepium	cuscohygrine	10.0 (R)	Jung and Tepfer, 1987
Catharanthus roseus	ajmalicine	2.7 (R)	Bhadra *et al.,* 1993
(L.) G. Don	serpentine	21.0 (L)	
	catharanthine	7.0 (L)	
Centranthus rubber	Valepotriates	1.0 (R)	Granicher *et al.,*1995
Chaenactis douglasii	thiarubines	3.0 (F)	Constabel and Towers, 1988
(Hook.) H. and A.			Christen *et al.,* 1989
Datura candida	hyoscyamine	1.6 (A)	
	scopolamine	2.6 (R)	Mano *et al.,* 1989
Duboisia leichhardtii	scopolamine	2.0 (L)	
Hyssopus officinalis	rosmaric acid	0.7 (R)	
	litospenic acid	0.33 (R)	Sauerwein *et al.,*1991
Lippia dulcis	camphene	16.2 (R)	
	limonene	18.5 (R)	Tada *et al.,* 1995
*Lobelia chinensis*Lour.	lobetyolin	20.0 (L)	Yonemitsu *et al.,*1990
Lobelia inflata	lobelin	0.03 (R)	Ishimaru *et al.,* 1994
Lobelia sessilifolia	lobetyolin	20.0 (WP)	
Nicotiana rustica	nicotine	0.18 (R)	
	anatabine	0.05 (R)	Yoshikawa & Furuya,1987
Panax ginseng	ginsenosides	2.4 (R)	Lodhi *et al.,*1996
Rubia peregrina	anthraquinones	0.89 (R)	Ogasawara *et al.,* 1993
Sesamum indicum	naphthoquinone	50.0 (R)	Ermayanti *et al.,* 1994
Swainsona galegifolia	swainsonine	7.0 (WP)	Kyo *et al.,* 1990
Tagetes patula L.	thiophenes	12.0 (R)	Granicher *et al.,* 1992
Valeriana officinalis L.	valepotriates	10.0 (R)	
Var. sambucifolia Mikan			

Abbreviations: L=leaf, R= roots, F=floral parts, A=aerial part, WP=whole plant

the expression of HBsAg (Richter et al., 2000; Kumar et al., 2006). The rhizosecretion has also been exploited recently for heterologous expression of human alkaline phosphatase (Gaume et al., 2003) and immunoglobulin G antibodies (Komarnytsky et al., 2006).

The productivity of hairy root cultures has been reported to be enhanced through adopting the following strategies:

Selection of High Producing Hairy Root Clones

It has generally been accepted that a single hairy root that arises from any independent transformation event is a clone (Chilton *et al.,* 1982; Sevon *et al.,* 1998). As the insertion sites of Ri T–DNA varies within each and every transformation event and the site-specific insertion strongly regulates the growth behavior and secondary metabolite productivity, it is therefore highly logical to screen the products of a large number of individual transformation events in order to select the high yielders. The advantage of judicious screening has already been successfully utilized for the selection of superior hairy root clones in several medicinal plant species (De Vries-Uijtewaal *et al.,* 1988; Mano *et al.,* 1989; Oksman-Caldentey *et al.,* 1994; Vanhala *et al.,* 1995; Aoki *et al.,* 1997; Bourgaud *et al.,* 1999; Zehra *et al.,* 1999) (Table-1)

Influence of the Culture Medium

The bio-synthetic potential of the transformed roots is genetically controlled but it has frequently been observed that such transformed roots are sensitive to medium composition with respect to both biomass yield and secondary metabolite productivity (Wyskoinska & Chimel, 1997; Giri and Narasu 2001).A detailed literature survey revealed that the standard media formulations differentially influence the growth as well as production behavior of the hairy root cultures of different medicinal plant species. Several factors seem to be responsible for such differential behavior which differs not only between different plant species and bacterial strains used (Vanhala *et al.,* 1995) but also between different root clones (Oksman-Caldentey *et al.,* 1994; Aoki *et al.,* 1997) of the same plant species with variable root morphology (Hamill *et al.,* 1987; Mano *et al.,* 1989,) and harboring different genetic makeup

Table 2: Novel compounds found in hairy root cultures of some medicinal plants.

Plant species	Compound	Reference
Astragalus membranaceus	Agroastrolagoside I	Hirotani *et al.,*1994
Beta vulgaris	Vulgaxanthin III, IV	Hamill *et al.,*1986
Cassia obtusifolia	1,8-Di-*O*-methylchrysophanol	Ko *et al.,*1989
Catharanthus trichophyllus (Bak.) Pich.	Indole alkaloids: anthraserpine and four of its derivatives	Davioud*et al.,* 1989 a, b
Fagopyrum esculentum	Flavonoids/catechin, pyrocyanidins Licoagrochalcone A, Licoagrocarpine	Trotin *et al.,* 1993
Glycyrrhiza glabra	Piperidone alkaloid: hyalbidone	Saurwein*et al.,* 1991 a
Hyoscyamus albus	Alkaloids: N-methyl pyrrolidinyl-cuscohygrine	Doerk-Schmitz *et al.,*1994 Ishimaru *et al.,*1991;1992
Hyoscyamus albus	5-Hydroxy-and 6-hydroxy-tetrahydronorharman	Folkenhagen*et al.,*1993
Peganum harmala	12- hydroxyajmaline two anthraquinones	Ogasawara *et al.,* 1993 Ishimaru *et al.,* 1990
Rauvolfia serpentina	Xanthone: 8-O-primeverosylbellidifolin	Granicher *et al.,*1995
Sesamum indicum	9-epicatechachol B	
Swertia japonica Makino	Valdiate	
Tanacetum parthenium		
Valeriana officinalis var. sambucifolia Mikan		

(Aoki *et al.,* 1997, Shanks and Bhadra, 1998; Bhadra *et al.,* 1993) resulting from different transformation events (Ermayanti *et al.,* 1994). It is, therefore, all the more important to optimize the yield determining parameters with respect to the specific need of any selected better performer root clone of any specific plant species in order to fully utilize the advantageous characters of such hairy root cultures for the production of important phytochemicals.

Various groups have presents that besides collection of superior hairy root clones with superior than average productivity (Mano *et al.,* 1986) media optimization could increase the biomass and secondary metabolite yield (Table 5).

The growth promoting effect of the Gamborg's B5 medium (Gamborg *et al.,* 1968) has already been recorded with respect to the hairy root cultures of numerous medicinal plant species, such as *Scutellaria baicalensis*; *Lobelia inflata* (Yonemitsu *et al.,* 1990); *Datura quercifolia*; *Panax* hybrid (Washaida *et al.,* 1998); *Fagopyrum esculentum* (Trotin *et al.,* 1993); *Datura stramonium* (Hilton and Rhodes ,1990) *Glycyrhiza urelensis* (Saito *et al.,* 1990) , *Catharanthus roseus* (Parr *et al.,* 1990), *Hyoscyamus albus* (Christen *et al.,* 1992), *Valeriana officinalis* (Granicher *et al.,* 1992); *Psoralea* sp. (Bourgaud *et al.,* 1999); (*Physalis minima*).

The Murashige and Skoog"s (MS) medium (1962) has also widely been used and reported to have positive influence on the growth behaviour of the hairy root cultures of numerous medicinal plant species, such as: *Lobelia inflata* (Yonemitsu *et al.,* 1990), *Lawsonia inermis* , *Wahlenbergia marginata*; *Fragaria X Ananassa* (Motomori *et al.,* 1995), *Hyoscyamus muticus* (Jaziri *et al.,* 1995 ; Zehra et al. 1998), *Hyoscyamus albus* (Saurwein and Shimomura, 1991; Zehra *et al.,* 1998) while it failed to promote growth of the hairy root culture of *Scutellaria baicalensis*.

The positive influence of the WP medium has already been noted in cases of hairy root cultures of *Trigonella foenum graecum* L. (Merkli et al, 1997), *Scutellaria baicalensis* and *Tachelium caeruleum* L. and *Hyoscyamus albus* (Sauerwein and Shimomura, 1991; Sauerwein *et al.,* 1991; Christen *et al.,* 1992).

The Nitsch and Ntisch (NN) medium (1969) proved effective for the hairy root cultures of *Cassia occidantalis Glycyrhiza urelensis* (Ko *et al.,* 1989) and *Swertia chirata* (Keil *et al.,* 2000) but failed to support the growth of the hairy root culture of *Lobelia inflata* (Yonemitsu *et al.,* 1990).

Linsrmaier and Skoog's (LS) medium (1965) has been used in limited number of cases and proved effective for the hairy root cultures of *Duboisia myoporoides* (Deno *et al.,* 1987), *Panex ginseng* (Inomata *et al.,* 1993) and *Rauvolfia serpentina* (Benjamin *et al.,* 1994). Two other media formulations, namely Heller's medium and Monnier medium have successfully been used for the hairy root cultures of *Duboisia leichharditii* (Mano *et al.,* 1989) and *Catharanthus roseus* (Brillanceau *et al.,* 1989) respectively.

A good majority of the earlier studies indicated that the growth behaviors of the hairy root cultures of several medicinal plant species have positively been influenced either by the use of the B5 medium composition (Yonemitsu *et al.,* 1990, Christen *et al.,* 1992, Trotin *et al.,* 1993, Toivonen, 1993; Zehra, 1998; Asada *et al.,* 2001; Azlan *et al.,* 2002) or with the MS formulation (Yonemitsu *et al.,* 1990, Saurwein and Shimomura, 1991; Motomori *et al.,* 1995). Conversely, lesser number of success has so far been reported with respect to the WP (Granicher *et al.,* 1992; Merkli et al, 1997, 2000) or NN media formulations (Ko *et al.,* 1988, 1989).

Nitrogen and phosphate being the most essential elements required for plant tissue cultures, attempts were mostly been made in earlier studies, to find out the correlation, if any, between their source as well as concentrations with both biomass and secondary metabolites productivity of the hairy root cultures (Payne *et al.,* 1987; Weathers *et al.,* 1997).

In certain cases, like *Althaea officinalis* (Ionokova and Alfermann, 1994) and *Salvia miltiorrhiza* (Hu and Alfermann, 1993), presence of ammonium ions in the medium showed a decrease in the quantity of polysaccharide and

Table 3: Influence of bacterial strains and culture media on the secondary metabolites production potential of hairy root cultures.

Plant species	Plasmid strain	Culture Media	Secondary products	Yield	References
Tropane alkaloids					
Atropa belladonna L.	15834	Normal MS medium	scopolamine atropine	0.024% DW 0.371% DW [S]	Kamada *et al.*, 1986
Atropa belladonna L.	8196	MS medium minus NH_4NO_3	scopolamine atropine	0.090% DW 0.950% DW [B]	Jung & Tepfer.,1987
Datura candida hybrid	15834	Normal MS medium	scopolamine hyoscyamine	0.570% DW 0.110% DW [S]	Christen *et al.*, 1989
Datura innoxia Mill.	15834	Normal MS medium	scopolamine hyoscyamine	0.035% DW 0.172% DW [S]	Shimomura *et al.*, 1991a
Duboisia hybrid M-II-8-6	15834	(nd)	scopolamine hyoscyamine	0.250% DW 0.140% DW [S]	Shimomura *et al.*, 1991a
Duboisia leichhardtii	A4	Double strength Heller's medium with Fe-Na-EDTA	scopolamine	1.800% DW [S]	Mano *et al.*, 1989
Duboisia myoporoides R. Br.	HRI & 8196	Normas LS medium	scopolamine hyoscyamine	0.150% DW 0.860% DW [S]	Deno *et al.*, 1987
Datura quercifolia Kunth	9402	4/3 strength of B5 salts	hyoscyamine	1.240% DW [S]	Dupraz *et al.*, 1994
Datura stramonium	9402	(nd)	hyoscyamine	0.300% DW [S]	Payne *et al.*, 1987
Datura stramonium	15834	Normal MS medium	scopolamine	0.560% DW [S]	Jaziri *et al.*, 1988
Datura stramonium	15834 TR 105	Normal B5 medium	scopolamine hyoscyamine	0.005 to 0.077% DW [S] 0.110 to 0.230%DW [SB]	Wilson *et al.*, 1988 ; Maldonado-Mendoza *et al.*, 1993 ; Hilton & Rhodes, 1990
Datura metel	A4	Normal B5 medium	scopolamine	(nd) [S]	
Datura wrightii	9402	Normal B5 medium	scopolamine hyoscyamine	(nd) [S]	
Hyoscyamus albus	15834	Normal MS medium	scopolamine hyoscyamine	0.460% DW 0.340% DW [S]	Shimomura *et al.*, 1991a
Hyoscyamus albus	MAFF 03-01724	Normal WP medium with 15 mM NO_3^- Conc.	scopolamine hyoscyamine	0.040% DW 0.540% DW [S]	Sauerwein *et al.*, 1991
Hyoscyamus albus	A4	Normal WP medium	hyoscyamine littorine	0.580% DW 0.067% DW [S]	Christen *et al.*, 1992
Hyoscyamus niger	15834	Normal MS medium	scopolamine hyoscyamine	0.018 to 0.086% DW [S] 0.460 to 1.250% DW [S]	Jaziri *et al.*, 1988
Hyoscyamus nuticus	15834	Normal B5 medium	scopolamine hyoscyamine	(nd) [S]	

Scopolia japonica Maxim	15834	(nd)	scopolamine hyoscyamine	0.500% DW 1.300% DW [S]	Mano *et al.*, 1986
Scopolia tangutica	15834	(nd)	scopolamine hyoscyamine	0.013 to 0.019% DW [S] 0.041 to 0.052% DW [S]	Shimomura *et al.*, 1991a Knopp *et al.*, 1988
Scopolia carniolica	A4 & 8196	Normal LS medium	scopolamine hyoscyamine	(nd) [S]	Parr, 1992
Nicandra phsaloides	(nd)	(nd)	hygrinne cuscohygrinne	(nd) [S]	
Indole alkaloid					Sauerwein *et al.*, 1991(a) Bhadra *et al.*, 1993
Amsonia elliptical	A4	Normal B5 medium	pliocarpamine	(nd) [S]	
Catharanthus roseus (L.) Don	15834	Normal B5 medium	ajmalicine sepentine catharanthine vindoline	0.400% DW 0.210% DW 0.210% DW 0.030% DW [S]	Toivonen *et al.*, 1989
Catharanthus roseus (Cr)	15834	1/2 B5 medium	-do-	(nd) [S]	Brillanceau *et al.*, 1989 Toivonen *et al.* 1992
Catharanthus roseus	15834	Monnier medium	-d0-	(nd) [S]	
Catharanthus roseus	15834	1/4th B5 medium	-d0-	(nd) [S]	
Catharanthus roseus	9402	½ B5 medium	-do-	(nd) [S]	Davioud *et al.*, 1989
Catharanthus trichophyllus	15834	MO medium	-d0-	(nd) [S]	
Rauwolfia serpentina	15834	Modified LS medium	ajmalicine serpentine	0.045% DW 0.007% DW [S]	Benjamin *et al.*, 1994 Folkenhagen *et al.*, 1993
Rauwolfia serpentina	A4	Normal B5 medium	vomilenine vinorine	280 mg/l 200 mg/l [S]	Tanaka *et al.*, 1993
Vinca minor	AR2	DC medium	vincamine	(nd) [S]	
Other alkaloids					Hamill *et al.*, 1989
Cinchona ledgeriana	15834	(nd)	quinoline alkaloids	50.00 µg/g FW [S]	
Nicotiana hesperis	(nd)	(nd)	nicotine	(nd) [S]	
Nicotiana rustica	(nd)	(nd)	nicotine,	(nd) [S]	Knopp *et al.*, 1988
Nicotiana tabacum	(nd)	(nd)	nicotine, anabasine nornicotine	(nd) [S]	Berlin *et al.*, 1993
Peganum harmala	A4	Normal B5 medium	harmine, harmalol	(nd) [S]	Williams *et al.*, 1993
Papaver somniferum	MAFF 03-01724	Normal B5 medium	codien	(nd) [S]	
Solanum aviculare					Subroto & Doran,

	A4	Normal B5 medium	solasodine	32.00 mg/g DW [S]	1994
				29.00 mg/g DW [B]	
Swainsonia galegifolia					Ermayanti *et al.*, 1994
	A4	Normal MS medium	Swainsonine	62.3µg/g DW [S]	
	9402				
Terpenoids and Steroids					
Ajuga reptans					
	MAFF 03-01724	Normal MS medium	Phytoecdysteroids	(nd) [S]	
Atemisia absinthium					Kennedy *et al.*, 1993; Nin *et al.*, 1997
	1855 & 9402	Normal B5 medium	Linalyl-3-methylbutanoate, nerol	(nd) [S]	
					Hirotani *et al.*, 1994
Astragalus membranceus					
	15834	Normal B5 medium	astragalosides	nd [S]	Ionkova *et al.*, 1997
Astragalus mongholicus					
	9402, 15834, R1601 TR105	Normal MS medium Minus $(NH_4NO_3)^+$	astragalosides	7.13% DW [S]	Sasaki *et al.*, 1998
Coleus forskohli					
	MAFF 03-01724	Normal WP medium	forskolin	(nd) [S]	Furze *et al.*,1991
Datura stramonium					
	nd	nd	3-hydro-xylubimine	4.70% DW [S]	Ko *et al.*, 1989
Glycyrrhiza uralensis L.					
	15834	Normal MS medium	glycyrrhizin	(nd) [S]	Singh *et al.*,1994
Hyoscyamus muticus					
	nd	nd	solavetivone	0.25 mg/g DW [S]	Hook,1994
Leontopodium alpinum					
	9402	MS medium plus 0.4 mg/l THCl	sesquiterpene	(nd) [S]	Sauerwein *et al.*, 1991
Lippia dulcis					
	A4	nd	hernandulicin	(nd) [S]	
Perezia cuernavacana					
	AR12	Normal MS medium	perezone	(nd) [S]	
Panax ginseng C.A.Meyer					Inomata *et al.*, 1993
	15834	Normal LS medium	ginsenosides	16.70 mg/g DW [S]	
				16.90 mg/g DW [B]	Hu & Alfermann, 1993
Salvia miltiorrhiza Bge.					
	9402 15834 R1601	Normal MS medium	tanshinones	0.50 to 1.90% d wt. [S]	
	TR105		ferruginol	0.02 to 0.07% d wt. [S]	Ikenaga *et al.*, 1995
Solanum aculeatissimum					
	15834	Normal B5 medium	aculeatiside A	6.71 mg/l [S]	
			aculeatiside B	6.39 mg/l [S]	Subroto & Doran, 1994
Solanum aviculare					
	A4 15834 43057 1132	Normal B5 and MS medium	Solasodine	32.00 mg/g DW [S]	
				29.00 mg/g DW [B]	
Solanum mauritianum					
	(nd)	(nd)	Solasodine	(nd) [S]	

Stevia rebaudiana	15834	Normal MS medium	Stevioside Rebaudioside A	(nd) [S]	
Tanacetum parthenium	9402	nd	Parthenolide	(nd) [S]	
Taxus brevifolia	A4	nd	Taxol	0.24 mg/g DW [B]	Rodriquez-Mendiola *et al.*, 1991; merkli *et al.*, 1997
Trigonella foenum-graecum	A4	Normal WP medium	diosgenin	(nd) [S]	Ray *et al.*, 1996 ; Banerjee *et al.*, 1994
Withania somnifera	9402	Normal MS medium	Withanolides	0.181 mg/l/d	
Flavonoids					Trotin *et al.*, 1993
Fagopyrum esculentum			flavonols		Motomori *et al.*, 1995
Fragaria X ananassa	15834	Normal B5 medium	flavonols	3.00% DW [S]	
Glycyrhiza glabra	15834	Normal MS medium	glabrol,	0.59% DW [S]	Hook, 1994
Leontopodium alpinum	R1601	Normal WP medium	abssinone	(nd) [S]	Robbins *et al.*, 1991
Lotus corniculatus	15834	Normal MS medium plus 0.4 mg/l THCl	anthocyanin Vestitol, satavan	(nd) [S] (nd) [S]	Nishikawa & Ishimaru, 1997; Nishikawa *et al.*, 1999
Scutellaria baicalensis Georgi.	15834 C58C1	Normal B5 medium	baccalin, wogonin		
Aromatic compounds	9402	Normal B5 medium	oroxylin A	(nd) [S]	Reichling & Thorn 1990
Coriopsis tinctoria			phenyl propanoids		Ishimaru & Shimomura, 1990
Geranium thumbergii	1855	(nd)	tannins	2.00 mg/g FW [S]	
Lithospermum erythrorhizon	A4	(nd)	amarogentin shikonin	0.67% DW [S]	Shimomura *et al.*, 1991b Santos *et al.*, 1998
Pimpinella anisum	15834	Normal MS medium	essential oil	5.90 mg/day [B]	Ishimaru *et al.*, 1990
Swertia japonica	A4	Normal SH medium	amaroswerin and xanthones	(nd) [S]	Kisiel & Stojakowska, 1997
Tanacetum parthenium	15834	(nd)	isofraxidine	(nd) [S]	
Linum flavum L.	(nd)	(nd)	5-methoxy-podo-phyllotoxin	(nd) [S]	
Miscellaneous unsaponifiable lipids	LBA 9402	(nd)		1.50 to 3.50% DW [S]	Ko *et al.*, 1988

Cassia obtusifolia	(nd)	(nd)	chrysophanol, emodin	(nd) [S]	Ko *et al.*, 1989
Cassia occidentalis	(nd)	(nd)	germichrysone, pinselin	(nd) [S]	Ko *et al.*, 1989
Cassia torosa	(nd)	(nd)	germichrysone	(nd) [S]	Tada *et al.*, 1995a
Lobelia chinensis	15834	Normal WP medium	lobetyol lobetyolin, lobetyolinin	3.4 mg/flask 10.6 mg/flask	
Lobelia inflata	15834	Half strength MS medium	Lobeline	2.8 mg/flask 18.0-54.0 µg/g DW [S]	Isimaru *et al.*, 1994
Lobelia sessilifolia	15834	(nd)	polyacetylenes (lobetyolin)	4.36% DW [S]	
Platycodon grandiflorum	03-01724	Normal B5 medium	lobetyol lobetyolin, lobetyolinin	0.21% DW [S] 0.41% DW 0.02% DW	Tada *et al.*, 1995b
Miscellaneous nitrogen compounds	MAFF 03-01724				
Beta vulgaris		Normal B5 medium	betacyanin betaxanthin	0.70 mg / l/culture [SB] 1.30 mg/l/culture [S]	Hamill *et al.*, 1986
Miscellaneous sulfur compounds	(nd)				
Chaenactis douglasii (Hook) H. and A.			thiarubrines		Constabel &Towers, 1988
Tagetes laxa (Cabrera)	LBA 9402	Normal B5 medium	thiophenes	0.50% DW [S]	Rodriguez-Talou & Giulietti, 1995
Tagetes patula L.	TR 7	Normal MS medium with RT vitamin complex	thiophenes	277.00 to 1,773.00 µg/g FW [S]	Kyo *et al.*, 1990
Other compounds		Normal SH medium		15.00 to 1,268.00 µg/g DW [S]	Jaziri *et al.*, 1995
Artemisia annua L.	LBA 9402		artemisinin		
Centranthus ruber DC		Normal B5 medium	valepotriates	0.01 to 0.40 x 10^{-3} % DW [S]	Granicher *et al.*, 1995
Valeriana officinalis L.	43057	Normal B5 medium	valepotriates	3.00% DW [S]	Granicher *et al.*, 1992 Banerjee *et al.*, 1998
Valeriana wallichii		1/2 MS medium	valepotriates	10.30 % DW [S]	Kittpongpatana et al.2002
Valeriana locusta	R1601	Normal B5 medium	valepotriates	(nd) [S]	George *et al.*, 1999
Cichorium intybus L.	NCIB 8196	Normal MS medium	Esculin	(nd) [S]	Trypsteen *et al.*, 1991
Echinacea purpurea	R 1601	1/2 MS medium	alkamides	(nd) [S]	Toivonen & Rosen-qvist, 1995
Glycyrrhiza glabra		1/2 B5 medium	liquiritigenin	(nd) [S]	

	R 1601		isoliquiritigenin		
		(nd)		32.00 mg/g	Ogasawara *et al.*,
Sesamum indicum	A4		naphtho-quinone	DW [S]	1993
	A4	Normal B5 medium		645.00 to	
Lawsonia inermis			lawsone	1100.00 µg/g FW [S]	
	LMG-150				
Paulownia tomentosa	15834	Normal MS medium	Verbacoside	(nd) [S]	Wysokinsa &Rozga, 1997
		Normal MS medium		(nd) [S]	
Psoralea sp.	C58C18		Daidzein		Nguyen *et al.*, 1992
		Normal B5 medium		(nd) [S]	
Taxus x media var. Hicksii Rehd	15834		Paclitaxel & 10-deacetyl-baccatin III		Furmanova & Syklowska-Baranek,
	NCIB 8196	DCR medium		(nd) [S]	2000
Trichosanthes kirilowii Maxim			trichosanthin		
	9402	Normal MS medium		(nd) [S]	
Azadirachta indica			azadirachtin		Allan *et al.*, 2002
Physalis minima	15834, TR 105	Normal MS medium	physalins	(nd) [S]	
Trachelium caeruleum L.	9402	Normal B5 medium	polyacetylenes	(nd) [S]	
Stizolobium hassjoo	nd	Normal MS medium	L-DOPA	(nd) [S]	Sung & Huang 2000
Fragaria x Ananassa		Normal B5 medium	polyphenol	(nd) [S]	
	9402				
Ginkgo biloba		Normal MS medium	ginkgolide bilobalide	(nd) [S]	
	9402	Normal MT medium		(nd) [S]	Laurain *et al.*, 1997
Wahlenbergia marignata	15834		polyacetylenes		
	(nd)	Normal MS medium		(nd) [S]	Ando *et al.*, 1997
Aconitum heterophyllum					
	15834		aconites		
Plumbago zeylanica					
	A4,	Normal MS medium	plumbagin	7.9 mg/g DW [S]	Giri *et al.*, 1997
Digitalis lantana	CFBP 2409	1/2 MS medium	digoxin	0.042 % FW [S]	
Atropa belladonna	15834	1/2 MS medium		(nd) [S]	
	MAFF		littorine		
Brugmansia candida	03-01724	Normal MS medium	cadaverine	(nd) [S]	
Armoracia rusticana					Nakanishi *et al.*, 1998
	A4	1/2 B5 medium	Phytochelatins	(nd) [S]	
			Peroxidase		Carrizo et al, 2001
Gentiana sp.	A4	Normal MS medium		(nd) [S]	
			Gentiopicrine, swertiamarine,		Sakamoto *et al.*, 1992 Kubota *et al.*, 2000
	03-01724	Normal MS medium	gentianine	(nd) [S]	
Polygonum tinctorium			indigo		
	9402				
Ophiorhiza pumila		Normal SH medium		152/µ/DW [S]	
	(nd)		camptothecin		Young-Am *et al.*, 2000
Swertia chirata					

	A4 A4 (nd) 15834 9402 TR105	Normal B5 medium Normal NN medium	amarogentin	(nd) [S,B] (nd) [S]	Sudo *et al.,* (2002)

nd – no data available;S-hairy roots grown in shake flasks; B-hairy roots grown in bioreactor

diterpene respectively, while the root cultures of *Astragalus membranaceus* were unaffected by the presence of these ions (Ionkowa, 1995). Supplementation of heavy metals such as Cu^{2+} has been shown to stimulate alkaloid production (Sevon *et al.,* 1992; Christen *et al.,* 1992).

The supply of carbon as the energy source to such non-photosynthetic hairy root cultures is normally met with the most widely used carbohydrate, sucrose, the optimum supply level of which widely differs according to the specific need of the root clone under consideration (Yamazaki and Flores, 1991; Weathers *et al.,* 1997; Banerjee *et al.,* 1998). The earlier reported observations, accentuated the fact that variable concentrations of sucrose exert notable effects on the growth and secondary metabolite productivity of hairy root cultures (Nguyen *et al.,* Toivonen *et al.,* 1992; Jung *et al.,* 1992; Oksman-Caldentey *et al.,* 1994; Vasquez-Flota *et al.,* 1994; Keil *et al.,* 2000), but in contrast to these, Christen et al. (1992) noticed that the development of the *Hyoscyamus albus* hairy roots are not effected by several concentrations of sucrose.

The ratio of FW/DW of *Datura stramonium* is greatly changed by the level of sucrose supplied compare to the whole ion content of the medium. Sucrose is preferred carbon source for plant tissue cultures. But several workers also studied the effect of few other carbon sources like Glucose, fructose, and maltose on biomass and secondary metabolite production. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. Hairy roots of *Valeriana officinalis* var. *sambucifolia* (Granicher *et al.,* 1992) showed valepotriate content in cultures in ten different media containing varying concentrations of sucrose (2-7%) and were compared with 9-month-old non-transformed plants. The content varied with respect to sucrose concentration but showed maximum quantity (4 times higher) at lower levels (2%) of sucrose. Similarly, hairy roots of *Catharanthus roseus* produced double (41 mg / l) the amount of catharanthine by use of fructose as a carbon source instead of sucrose (Jung *et al.,* 1992), and in some cases the total alkaloid content of *C. roseus* was increased by over 50% as compared to the non-transformed roots (Toivonen *et al.,* 1991). Transformed roots of *Atropa bactica* Willk when cultured on ½ MS medium exhibited maximum accumulation of the alkaloid (Zarate, 1999). Similarly, a variation of media composition changed the metabolite accumulation pattern in a large number of plants including *Atropa belladona* (Aoki *et al.,* 1997), *Linum flavum* (Oostdam *et al.,* 1993).

Effect of Plant Growth Regulators

The effect of growth regulators such as auxins and cytokinin on root growth and morphology has been studied extensively, especially, the influence of these substances on induction and development of hairy roots (Bercetche *et al.,* 1987). Cardarelli et al. (1987) investigated the relative role of auxin and of *A. rhizogenes* T-DNA in the iduction of carrot hairy roots. The physiological role of auxin in the development of hairy root tumors was examined with the use of auxin antagonist in

transformed and normal roots of potato culture. However, there is a little information about the effect of exogenously supplied gibberelins on hairy root growth. Concentrations of gibberelic acid between 10 ng/l and 1mg/l have been found to accelerate the fresh weight increase of Datura innoxia hairy roots and enhance elongation and lateral branching (Ohkawa *et al.,* 1989); however, the effect on secondary product synthesis was not recorded.

The effects of plant growth regulators on secondary metabolites in hairy root cultures have been studied extensively. The role of an appropriate concentration of auxin and a cytokinin in a culture medium to induce proliferative growth and callus formation is the well-known effect of plant growth regulators for the *in vitro* growing plants. It is therefore not surprising that the concentration and balance between growth regulators in a culture medium would influence secondary metabolism, a facet of differentiation. Generally, treatments which encourage structural differentiation also influence the biochemical profile. For example, the regeneration of roots from callus of several species tends to be accompanied by a sharp rise in alkaloid content (Hashimoto and Yamada, 1983). Bais *et al.* studied the effect of exogenously fed plant growth regulators on growth and coumarine content of hairy root cultures of *Cichorium intybus* and found that the total caumarine content was corelated with growth and was controlled by the auxin: cytokinin ratio. In contrast, exogenously supplied gibberelic acid at the o.5 mg/l level enhanced growth, coumarin content, and branching patterns over the control and other treatments on the 28th day of culture. In case of *Datura quercifolia*, increasing concentrations of GA3, slightly inhibited the growth in the 35 days of culture and the hyoscyamine content significantly decreased when the GA3 concentration was 1 mg/l. It has also been demonstrated that the concentrations of added growth regulators which support high rates of growth do not necessarily induce higher accumulation of desired secondary metabolites. Rodriguez Talou *et al*. (1995) studied their effect of plant growth regulators on growth and thiophene content of hairy root cultures of *Tagetes laxa*. In the case of IAA, none of the concentrations tested affected growth. This observation is in correlation with the results reported by Croes *et al*. (1989), who found higher concentrations of IAA (10 mM) significantly reduce the secondary metabolite in *Tagestes patula* hairy root culture. Amongst the different gibberellins, GA7 rendered the most pronounced effect on growth of tagetes patula hairy root culture, while it reduced the thiophene content to 1/3 of that noted with control medium (Croes *et al.,* 1989).

Effect of Elicitors

It is widely accepted that microbial invasions of intact plants activate plant defense mechanisms and thereby often elicit the synthesis of specific secondary metabolites increasing their productivity (Yoshikawa *et al.,* 1993). Molecules that stimulate secondary metabolism are called "elicitors". Depending on their origin, "elicitors" are classified as biotic or abiotic. The primary reaction upon elicitation with a biotic elicitor is thought to be composed of recognition of the elicitor and its binding to specific high-affinity receptor which resides in the plasma membrane. The next step in elicitation is thought to be inhibition of plasma membrane ATPase, which reduces the protein electrochemical gradient across this membrane. The elicitor binding to the receptor in the plasma membrane is essential to induce a defensive response, and also to generate a second messenger that transduces intracellularly. The interaction of the elicitor molecule with the plant cell surface ultimately results in the higher accumulation of secondary compounds.

Recently developments in phytochemical elicitation have presented that simple inorganic and organic molecules can generate product accumulation. Even though, the method by which elicitors enhance the productivity of secondary plant metabolites has not been explained, their stimulating action is fairly important if an suitable elicitor is chosen to stimulate synthesis of a particular product. Stimulation of secondary

metabolism by elicitation is the result of a complex interaction between the elicitor and the concerned tissue. The response of cultured plant tissues is affected by a number of factors as described below, some of which are linked to the elicitors, other to the tissue that has been cultured (Singh *et al.,* 1994; Rijhwani and Shanks 1998):

1) Elicitor specificity;
2) Elicitor concentration Time course of elicitation;
3) Growth stage of the culture to be feeded with the elicitor.

The biotic and abiotic elicitors have been extensively used to increase the production or to induce the *de novo* synthesis of secondary metabolites in plant cell cultures.(Eilert, 1987; Di Cosmo and Misawa, 1985; Threlfell and Whitehead, 1988). More recently, elicitation of secondary metabolites has received increasing attention in hairy root culture. Hairy roots are found susceptible to elicitation with variations in the kinetics of induction and extent of release of the desired metabolite, thereby also exerting differential effects on the secondary metabolite profile. Elicitation of hairy root cultures by applying period in several cases (Table 6). In response to elicitors certain compounds, known as phytoalexins which normally defend the plants against micro-organism, are often easily formed but the accumulation of the secondary metabolites has not usually been induced. Although the use of elicitors does not directly increase the secondary metabolite contents of hairy roots, the cell permeability increases which often has a positive effect on the formation of the secondary metabolites. Increase in cell permeability may enhance the formation of secondary products because feedback inhibition and intracellular degradation of products decreased. An added biotechnological benefit to the use of elicitors is the fact that, frequently, they also promote liberation of the metabolites into the medium. Some attempts have already been made to increase the permeability of hairy root cultures through elicitation with biotic and abiotic elicitors including solvents and detergents which have led to release of the products into the medium without any loss of viability and/or productivity. (Pitta-Alvarez *et al.,* 2000 a, b) (Table-6)

Examples of biotic elicitors are enzymes (cellulase, pectinase, etc.) that can liberate endogenous elicitors from the plant cell walls, molecules that act as endogenous signals in the defense mechanisms of plants (salicylic acid, jasmonic acid, etc.) and extracts of diverse microorganisms (glucan, glycoprotein, various fungal cell wall components, fungal culture filtrates or fungal toxins) (Eilert , 1987; Nishi, 1994; Benhamou, 1996). Abiotic elicitors or stress agents, on the other hand, include UV irradiation, heavy metal salts, detergents and other chemical compounds that disturb membrane intigrity or work through the diverse mechanism of action (Eilert, 1987).

Flores and co-workers (1988) reported that hairy roots of *Bidens sulphureus* responded to elicitation with fungal culture filtrates by dramatically increasing the production of specific polyacetylenes (Flores *et al.,* 1988).

Hyoscyamus mutics hairy root cultures have extensively been used for elicitation studies and successfully demonstrated the positive influence of different elicitors on secondary metabolite yield as well as denovo production of sequiterpenes (Signs and Flores, 1989; Sevon *et al.,* 1992; Flores and Curtis, 1992; Singh *et al.,* 1994; 1998; Biondi *et al.,* 2000; Carvalho and Curtis, 2002). Dunlop and Curtis (1991) reported that a combination of phosphate limitation and fungal elicitation with *Rhizoctonia solanii* crude extract synergistically increased the production of solavetivone by *Agrobacterium rhizogenes*-transformed hairy root cultures of *Hyoscyamus muticus* to a significant extent which was considerably greater than that obtained with either method alone. The effect of phosphate limitation combined with fungal elicitation (Rhizoctonia solani) was examined on the production of solavetivone by hairy root cultures of *H. muticus* (Pannuri *et al.,* 1993).

Fungal elicitors obtained from mycelial extracts of *Fusarium conglutinans* and *Aspergilus niger* respectively enhanced the thiophene

Table 6. Enhancement of secondary metabolite production by different biotic and abiotic elicitation of hairy root cultures.

S.No	Plant System	Secondary metabolite	*Elicitor*		Reference
			Biotic	**Abiotic**	
1.	Artemisia annua	Artemisnin	*Colletotrichum* sp.		Wang *et al.*,2001
2.	Armoracia lapathifolia	Peroxidases	*Verticillum sp.* *Monodyctis cataneae* *Aspergillus niger*	$AgNO_3$ $CuSO_4$	Flocco *et al.*,1998
3.	*Atropa belladonna*	Tropane alkaloids	Rhizoctonia solani MeJA Phytosulfokine-α Chitosan Chitin Yeast extract	$CuCl_2$ $CdCl_2$ H_2O_2 Glutathione	Rothe *et al.*,2001 Spollansky et al. ;2000
4.	*Brugmansia candida*	Tropane alkaloids	Hemicellulase Salicylic acid Yeast extract	$AlCl_3$ $CaCl_2$ $CdCl_2$ $AgNO_3$	Pitta-Alvarez et al.; 2000a;b
5.	*Catharanthus roseus*	Indole alkaloids	*A.niger* *Trichoderma viride* *T. r*eseii *Rhodotorula marina* *Penicillium sp.* MeJA Pectinase Chitinase Macerozyme Cellulase		Sim etal., 1994; Vasquez-Flota *et al.,* 1994; Rhizwani & Shank,1998
6.	*Datura stramonium*	Tropane alkaloids Lubimin & 3-hydroxylubimin	Cellulase MeJA Yeast *Fusarium solani* *T. viride* *Gongrenella sp.* *A.alternata* *A. niger* *Botrytis cinerea* *C. bakenseae*	$CdCl_2$ $CuSO_4$ $Pb(NO_3)_2$ $HauCl_4$	Furze *et al.*,1991; Whitehead & Threlfall,1992
7.	Datura metel			Tween-20	
8.	*Hyoscyamus muticus*	Tropane alkaloids Sesquiterpene (Lubimin&	Rhizoctona solani Salicylic acid Ethane MeJA JA chitosan	H_2O_2	Sevon *et al.*,1992; Pannuri i1993; Singh *et al.*,1994; 1998; Biondi, 2000; Carvalho & Curtis, 2002.
9.	Jimson weed	Solavetivone		$CuSO_4$	
10.	*Lipia dulcis*		chitosan		Whitehead & Threlfall,1992
11.	*Lotus corniculatus*	Rhistin		Glutathione	Sauverwein*et al.*,1991

12.	*Nicotiana tabacum*	hernandulcin	Yeast extract		Robbins *et al.*,1991
13.	*Panax ginseng*	Phytoalexin	JA		Wibberely*et al.*,1994
		Sesquiterpene	Phenylalanine Caffeic acid		Yu *et al.*,2000
		Ginsenoside	Catechin Chitin Gum Karaya Fucoidan Peptone		
14.	*Pimpinella anisum*		MeJA		
15.	*Polygonum tinctorium*		Chitosan Pectinase		Santos *et al.*,1998
16.	*Psoralea* spp.	Essential oil	Chitosan		Young-Am *et al.*,2000
		Indigo			Bourgaud *et al.*,1999
17.	*Rauvolfia serpentina*	Coumestrol	Salicylic acid		
18.	*Salvia miltiorhiza*	Daidzein Genistein	Yeast extract Methyl viologen	H_2O_2	Shelirdko *et al.*,1996
19.	Stizolobium hassjoo	Indole alkaloids		$CoCl_2$ $AgNO_3$	Sung &Huang, 2000
		Tanshinone			
20.	*Swainsona galegifolia*			$CuSO_4$	Chen and Chen 2000
21.	*Swertia chirata*	L-DOPA		$VdSO_4$	
			Salicylic acid MeJA		Ermayanti *et al.*,1994
		Swainosonine	Cinnamic acid m-coumaric acid		
		Amarogentin	Chitosan		
22.	*Tagetes laxa*		Scleretoniasclerotiio rum		
23.	Tagetes patula		*A. niger*		Rodriguez- Talau & Giulietti, 1995
		Thiophene			
24.	*Taxus* X *media* Var..Hicksii Rehd.	Thiophene	MeJA		Buitelaar *et al.*,1992; Mukundan *et al.*,1990
25.	*Trigonella foenum-graceum*		Chitosan		Furmanova & Syklowska-Baranek, 2000
26.	Valeriana locusta	Paclitaxel, 10-deacetylbaccatin	MeJA Salicylic acid	$CuSO_4$ $HgCl_2$	Merkli *et al.*,1996
		Ciosgenin	Yeast extract	$CaCl_2$	Kittipongpatana *et al.*,2002
		Valepotraits			

production in hairy root culture of *Tagetes patula* (Mukundan and Hjortso, 1990; Buitelaar *et al.,* 1993). The artemisnin content in the hairy roots of *Artemisia annua* was inreased by using elicitor treatment of mycelial extracts from the endophytic fungus *Collectotrichum* sp.and the increase of artemisnin was dependent on the growth stage of hairy roots as well as on the dose of the elicitor applied (Wang *et al.,* 2001). Flocco et al. 1998 reported a transient increase of approx. 100% in peroxidase production from the hairy root cultures of *Armoracia lapathifolia* after 24 h elicitation

with *Verticillum* sp., while other biotic elicitors (*Monodyctis cataneae* and *A. niger*) and abiotic elicitors ($AgN0_3$ and $CuSO_4$) exerted little effect on peroxidase activity.

Fungal elicitation of *Catharunthus roseus* hairy root cultures by *Penicillium* sp. enhanced the production as well as secretion of specific indole alkaloids and the combination of treatments with permeabilizing agent along with fungal elicitation and in situ adsorption proved optimum for both production and secretion of such specific indole alkaloids (Sim *et al.,* 1994). According to an earlier report, elicitation of *C. roseus* hairy root cultures with *Aspergillus* homogenates and macerozyme selectively enhanced the yield and accumulation of ajmalicine whereas elicitation with *Trichoderma viride, T. reseii* and a yeast-*Rhodotorula marina* failed to exert any effect (Vazquez-Flota *et al.,* 1994). Furthermore, treatment with increasing concentrations of macerozyme induced an increase in indole alkaloid and coumarine accumulation in *C.roseus* hairy root cultures (Moreno-Valenzuela *et al.,* 1999).

Jasmonic acid was reported to be a unique elicitor leading to an enhancement in flux to several branches in the indole alkaloid pathway of *C.roseus* hairy root culture and increased the specific yields of ajmalicine, serpentine, lochnericine and horhammericine (Shanks *et al.,* 1998; Rijhwani and Shanks, 1998) or only ajamalicine and catharanthine (Vazquez-Flota *et al.,* 1994). Jasmonic acid or its methylester are known to function in the signal transduction pathway and appeared to have a posite effect on secondary metabolite production in over 36 plant species (Gundlach *et al.,*1992). They have already been used for elicitation of hairy root cultures of several medicinal plant species. It has considerably improved the production of secondary metabolites in *Hyocyamus muticus* (Singh *et al.,* 1998; Biondi *et al.,* 2000); *Panax ginseng* (Yu *et al.,* 2000) *Valeriana locusta* (Kittipongpatana *et al.,* 2002) and *Taxus x media* var.Hicksii Rehd (Furmanova and Syklowska-Baranek, 2000).

Several fungal cell wall components and fungal enzymes also exerted slight positive influence on the secondary metabolite productivities of the hairy root cultures of Lippia dulcis (Sauerwein *et al.,* 1991); Hyoscyamus muticus (Sevon *et al.,* 1992); Trigonella faenum – graecum L.(Merkli et al. 1997), Atropa belladonna (Lee *et al.,* 1998); Psoralea sp.(Bourgaud *et al.,* 1999); and Polygonum tinctorium (Young-Am *et al.,* 2000).

On the other hand, contrasting result has also been noted in case of hairy root cultures of *Atropa belladonna* where the addition of chitosan; MeJA or ABA did not improve the accumulation of calystegines (Rothe *et al.,* 2001). Moreover, the addition of chitosan, chitin, glutathion and yeast extract failed to render any effect either on the yield or realease of the secondary metabolites while H_2O_2, $Cu^{2+,}$ and Cd^{2+} enhanced the release of alkaloids from the transformed roots into the medium (Lee *et al.,* 1998). However, glutathione enhanced the production of isoflavone phytoalexins upon elicitation of *Lotus corniculatus* hairy root cultures (Robins *et al.,* 1991).

Whitehead and Threllfall (1992) reported elicitation of thorn apple and Jimson weed hairy roots cultures with abiotic elicitors. The Thorn apple was elicited with Carnium chloride and resulted in enhanced production of lubimin. Elicitation of Jimson weed on the otherhand, with $CuSO_4$ resuled in the production of traces amounts of Rhishitin.

Treatment of *Swainsona galegifolia* hairy root culture with $CuSO_4$ enhanced the production as well as the release of swainsonine in the medium (Ermayanti *et al.,* 1994). Methyl viologen, a superoxide anion generator, triggered the formation of cryptotanshinone (phytoalexins) in cultures of hairy roots of *Salvia miltiorhiza* (Chen and Chen 2000).

In an attempt to increase productivity in hairy roots of *Brugmansia candida* several biotic and abiotic elicitors were tested (Pitta- Alvarez *et al.,* 2000 a, b). Amongst which, Salycilic acid significantly increased the release of both alkaloids and it also acted positively on specific production without altering the production profile. $AgNO_3$,

on the other hand, significantly enhanced the scopolamine release and accumulation of both the alkaloids in the roots, thus favoring the production of scopolamine. Yeast extract increamented the intracellular content of both alkaloids but particularly increased the release of scopolamine. $CaCl_2$ had little effect on accumulation or release of either alkaloid, while $CdCl_2$ acted positively on the release of both the alkaloids, but both were found to be highly detrimental to the root growth (Pitta- Alvarez et al. 2000).According to another report of *B.candida*, MeJA failed to influence the production or release either of the two alkaloids while $AlCl_3$ significantly increased the hyoscyamine accumulation in the roots (Spollansky *et al.,* 2000).

In an attempt to reduce the accumulated headspace ethylene produced *by Stizolobium hassjoo* hairy roots, treatment with $COCl_2$ led to a significant improvement of root dry weight and L-DOPA production (Sung & Huang, 2000).

The elicitation of *Datura stramonium* hairy root cultures with copper and cadmium salts has been found to induce the rapid accumulation of high levels of sesquiterpenoid defensive compounds, notably lubimin and 3-hydroxylubimin (Furze *et al.,* 1991). According to another later study, the hairy root culture of *Datura stramonium* when elicited with either methyl jasmonate, or a cell wall preparation from baker's yeast or oligogalacturonides respectively, an increased alkaloid accumulation in order Meja> fungal elicitor> oligogalacturonides was observed (Zabetakis *et al.,* 1999).

Elicitation with yeast extract has also stimulated the secondary metabolite productivity of the hairy root clones of several medicinal plant species such as , *Salvia miltiorhiza* (Chen and Chen, 2000), Datura stramonium (Zabetakis *et al.,* 1999), Brugmansia candida (Pitta-Alvarez *et al.,* 2000a); Valerianella locusta (Kittipongpatana *et al.,* 2002). Wibberley et al. (Wibberley *et al.,* 1994) described that a hairy root culture of *Nicotiana tabacum* synthesized and accumulated sesquiterpenoid phytoalexins, capsidiol, and debneyol, a portion of which were also released into the culture medium.

Even although, this mechanism by which elicitors enhance the productivity of secondary plant metabolites has not been explained, their stimulating action is fairly important if an suitable elicitor is chosen to stimulate synthesis of a particular product. However, the employ of microbial elicitors may not be inexpensive since an elicitor-producing micro-organism should be cultivated in a fermentor independently from the cultivation of plant cells using other fermentor. The cost of fermentation for an elicitor-producing micro-organism is not always reasonably priced. In this case, a simple and low cost compound should be used as an elicitor. These processes such as these, employing simple and low cost elicitors have much undertake in industrial scale plant hairy root cultures.

SCALE-UP STUDIES OF HAIRY ROOTS CULTURES IN BIOREACTORS

Large-scale culture of hairy roots in a bioreactor for the production of phytochemicals at commercial scale has gained considerable attention over the last few years (Toivonen, 1993; Singh and Curtis, 1994; Banerjee *et al.,* 1995; Giri and Narasu, 2000). However, one of the most important limitations for the commercial exploitation of hairy roots is the non availability of the technologies for scale-up cultures, specifically the design of novel bioreactor, that permit the optimum growth of hairy roots in the reactor vessel.

The growth of hairy roots and the production of secondary metabolites depend on the composition and availability of liquid media and gases at the root surface. The unique growth patterns and fragile nature of hairy roots have led to many different reactor configurations ranging from simple modifications of the existing one to completely novel designing (Table-7) in-order to avoid injuring the roots while optimizing the supply of nutrients and gases (Kondo *et al.,* 1989).

Nonuniform distribution of biomass in the culture vessel and three-dimensional structures of hairy root cultures lead to the formation of

interconnected, non-homogenous material unevenly distributed throughout the reactor vessel which results in altered rheology and insufficient mass transfer compared to that of cell suspension culture. Besides growth restriction, the densely packed mass of roots also renders nutrient and especially oxygen limitations leading to a reduction in secondary metabolite production or even to cell necrosis and autolysis. The oxygen and substrate diffusion through the root system may be improved to some extent by increasing the aeration and/or agitation rates in the bioreactor. However, there are limitations in the increase of these parameters, since shear stress causes callus formation and disorganization of hairy roots with consequently lower productivity. A wide variety of bioreactors have been designed suitable for growing hairy root cultures under controlled conditions, well equipped with diverse systems for the measurement and regulation of the process parameters (e.g. mixing, oxygenation, foaming, pH, temperature).

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