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Chapter 2
Agrobacterium rhizogenes-Mediated Transformation in Medicinal Plants: Prospects and Challenges

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2.1 Introduction

Root cultures have been studied since the early days of tissue culture research, but have created little interest because of their slow growth rate, although root and shoot organ cultures have been used for studies of alkaloids (Hashimoto et al. 1986; Hirata et al. 1990; Jha et al. 1991; Baíza et al. 1999a; Khanam et al. 2001; Ghosh et al. 2002; Ghosh and Jha; 2005), coumarins (Panichayupakarananta et al. 1998), saponins (Kusakari et al. 2000; Kim et al. 2005a), phenolic acids (Karam et al. 2003), essential oils (Olszowska et al. 1996), terpenes (Pannuri et al. 1993), glycosides (Swanson et al. 1992), steroidal lactones (Ray and Jha 2001), etc.

However, in all but a few species, they are difficult to culture and the auxin concentrations optimal for growth may reduce productivity (Siah and Doran 1991; Bourgaud et al. 2001). Few studies on secondary metabolite production with root and shoot organ cultures in bioreactors have been reported (Kevers et al. 1999; Choi et al. 2000; Bondarev et al. 2002; Piatczak et al. 2005; Kim et al. 2005a).

Although, root and shoot organ cultures are genetically and biosynthetically more stable than cell cultures, interest in root and shoot organ cultures as a source of secondary metabolites has been limited (Flores and Curtis 1992; Flores and Medina-Bolivar 1995).

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2.2 Ri-Transformed Root Cultures

Agrobacterium rhizogenes infects higher plants to produce so-called "hairy roots" with altered phenotype from the wound sites. The transformed roots can be excised to establish axenic root cultures and indefinitely propagated in growth regulator free medium. The root exhibit fast, plagiotropic growth characterized by profuse lateral branching and rapid root tip elongation (Tepfer and Tempé 1981; Chilton et al. 1982; Tepfer 1984). Putatively transformed root lines can be easily screened with these morphological markers. Foreign genes can also be introduced into transformed roots by using binary vectors (Hamill et al. 1988). The rapid biomass accumulation in transformed root cultures is comparable, if not superior, to unorganized cell cultures and the fastest biomass doubling time is 1-day in *Datura stramonium* (Maldonado-Mendoza et al. 1993). The advantage of this transformation approach is that each primary root produced at the infection site is the result of a single transformation event—a clone (Chilton et al. 1982). However, somaclonal variations in transformed root cultures are also known (Sevón et al. 1998; Wilhelmson et al. 2005). Transformed root cultures have been established in several species, including many medicinal plants (Tepfer 1989; Sevón and Oksman-Caldentey 2002).

Transformed clones vary in morphology (Amselem and Tepfer 1992), growth, and metabolite productivity (Aoki et al. 1997; Batra et al. 2004). This is attributed to the nature, site, and number of T-DNA integration into the host genome (Ambros et al. 1986a, b; Jouanin et al. 1987; Amselem and Tepfer 1992). Therefore, clone selection is critical for metabolite productivity through transformed root cultures (Mano et al. 1986). Transformed root clones are genetically and biosynthetically stable for long periods. Growth and alkaloid production were stable over a period of 5 years in transformed roots of *D. stramonium* (Maldonado-Mendoza et al. 1993). Ri-transformed roots also exhibit a high degree of cytogenetic stability (Baíza et al. 1999b). In *Beta vulgaris* and *Nicotiana rustica* (Benson and Hamill 1991), growth, secondary metabolite production, and T-DNA structure in several transformed root lines were unchanged after cryopreservation. However, progressive loss of growth rate under conditions that favor the production of tropane alkaloids was reported in *Duboisia myoporoides* (Yukimune et al. 1994).

Plant roots can synthesize, store, and secrete a vast array of compounds and transformed root cultures have a wide range of biosynthetic capacities (Flores et al. 1999). They can produce the full range of secondary products characteristics of roots of the parent plants (Parr and Hamill 1987; Zárate 1999) as well as novel compounds (Fukui et al. 1998, 1999). The secondary metabolite levels are often comparable to or greater than that of intact plants (Sevón and Oksman-Caldentey 2002). Unlike cell cultures, actively growing transformed roots can continuously produce secondary metabolites (Holmes et al. 1997). Transformed root cultures are reported to synthesize secondary products, including alkaloid, in numerous medicinal plant species (Table 2.1). Many of these have already been reviewed

Table 2.1 Secondary metabolite production in Ri-transformed roots

Plant species	*A. rhizogenes* strain used for transformation	Secondary metabolite	Reference
Aconitum heterophyllum	LBA 9402, LBA 9360, A4	Heteratisine, atisine, hetidine	Giri et al. (1997)
Agastache rugosa	R1000	Rosmarinic acid	Lee et al. (2008)
Ajuga multiflora	A4	20-hydroxyecdysone	Kim et al. (2005b)
Ambrosia maritima	ATCC15834	Thiarubrine A, thiarubrine A epoxide, thiarubrine A diol	Zid and Orihara (2005)
Ammi majus	A4, LBA9402	Umbelliferone	Królicka et al. (2001)
Artemisia annua	–	Artemisinin	Weathers et al. (2005)
	LBA 9402, 8196, A4 1601	–	Mukherjee et al. (1995)
A. dubia, *A. indica*	LBA 9402 and 8196	Artemisinin	Mannan et al. (2008)
Astragalus mongholicus	LBA 9402, ATCC 15834, R 1601 and TR 105	Astragaloside I, astragaloside II, astragaloside III	Ionkova et al. (1997)
Atropa acuminata	LBA 9402	Atropine, scopolamine	Banerjee et al. (2008)
Atropa belladonna	15834	Atropine, scopolamine	Kamada et al. (1986)
Atropa belladonna	MAFF 03-01724, ATCC 15834	Hyoscyamine, 6β-hydroxyhyoscyamine, scopolamine, littorine	Jaziri et al. (1994)
Atropa belladonna	AR15834	Scopolamine, hyoscyamine	Chashmi et al. (2010)
Azadirachta indica	LBA9402	Azadirachtin, nimbin, salannin, 3-acetyl-1-tigloylazadirachtinin, 3-tigloylazadirachtol	Allan et al. (2002)
Brugmansia candida	LBA 9402	Scopolamine, hyoscyamine	Spollansky et al. (2000)
Brugmansia candida	LBA 9402	Cadaverine, putrescine, spermidine, spermine	Carrizo et al. (2001)
Brugmansia suaveolens	15834, TR 105	Tropine, pseudotropine, scopoline, scopine, aposcopolamine, hyoscyamine	Zayed and Wink (2004)
Calystegia sepium	15834	Calystegine	Scholl et al. (2001)
Camptotheca acuminata	ATCC 15834, R-1000	Camptothecin, 10-hydroxycamptothecin	Lorence et al. (2004)

(continued)

Table 2.1 (continued)

Plant species	*A. rhizogenes* strain used for transformation	Secondary metabolite	Reference
Catharanthus roseus	15834, A2, A2-83, A47-83, R1000	Yohimbine, ajmalicine, tetrahydroalstonine, tabersonine, horhammericine, lochnericine, venalstonine, vindolinine, 19-epi-vindolinine, catharanthine, pericalline, O-acetylvallesamine	Toivonen et al. (1989)
	15834	Catharanthusopimaranoside A, catharanthusopimaranoside B	Chung et al. (2008)
	R1000	Vincristin, vinblastin	Zargar et al. (2010)
C. roseus var. Prabal	A4	Serpentine, ajmalicine	Batra et al. (2004)
Centaurium erythraea, *C. pulchellum*	A4M70GUS	Xanthone	Janković et al. (2002)
Cephaelis ipecacuanha	ATCC 15834	Cephaeline, emetine	Yoshimatsu et al. (2003)
Cichorium intybus	LMG 150	Esculin, esculetin	Bais et al. (1999)
Cinchona ledgerina	LBA 9402	Quinine, cinchonidine, quinidine, quinamine	Hamill et al. (1989)
Cinchona officinalis	LBA 9402 harbouring a binary vector	Tryptamine, strictosidine, quinidine,quinine, cinchonine, cinchonidine	Geerlings et al. (1999)
Coleus blumei	A4	Rosmarinic acid	Bauer et al. (2009)
Coleus forskohlii	MAFF 03-01724	Rosmarinic acid	Li et al. (2005)
	–	Forskolin	Sasaki et al. (1998)
Datura stramonium	LBA 9402	Hyoscyamine, apohyoscyamine,	Robins et al. (1991)
	1855, AR-10, TR-105, ATCC15834, A4, A41027, ATCC13333	Hyoscyamine, scopolamine	Maldonado-Mendoza et al. (1993)
	ATCC 15834	Hygrine, tropinone, apoatropine	Berkov et al. (2003)
Duboisia leichhardtii	15834, A4	Scopolamine	Mano et al. (1989)
Glycyrrhiza glabra	LBR56	Glycyrrhizic acid	Tenea et al. (2008)

(continued)

Table 2.1 (continued)

Plant species	*A. rhizogenes* strain used for transformation	Secondary metabolite	Reference
Glycyrrhiza pallidiflora	pRi 15834	Licoagroisoflavone, licoagroside C, calycosin, erythrinin, isoliquiritigenin, echinatin, maackiain, trifolirhizin, ononin	Li et al. (2001)
Glycyrrhiza uralensis	ACCC 10060 harbouring vector p130/35S-GuSQS1	Glycyrrhizin	Lu et al. (2008)
	pRi A4 harboring the binary vector pCHI	Flavonoids	Zhang et al. (2009)
	pRi A4	Licochalcone A	Zhang et al. (2011)
Gmelina arborea	ATCC 15834	Verbascoside	Dhakulkar et al. (2005)
Gynostemma pentaphyllum	ATCC 15834	Gypenoside	Chang et al. (2005)
Harpagophytum procumbens	ATCC 15834	Harpagoside, gallic acid	Georgiev et al. (2006)
	A4	Harpagoside, verbascoside, isoverbascoside	Grabkowska et al. (2010)
Hyoscyamus albus	LBA9402, A4	Atropine	Zehra et al. (1999)
Hyoscyamus muticus	LBA9402, A4	Scopolamine, hyoscyamine	Zolala et al. (2007)
Linum tauricum ssp. *tauricum*	TR 105, ATCC 15834	4′-demethyl-6-methoxypodophylotoxin, 6-methoxypodophyllotoxin	Ionkova and Fuss (2009)
Nicotiana tabacum cv. Xanthi	LBA9402, LBA9402 pLAL21	Hyoscyamine, scopolamine, nicotine, nornicotine, anabasine, anatabine, anatalline	Häkkinen et al. (2005)
Ocimum basilicum	ATCC 15834, MAFF 03-01724	Rosmarinic acid, lithospermic acid, lithospermic acid B	Tada et al. (1996)
	ATCC 15834	Rosmarinic acid	Bais et al. (2002a)
Ophiorriza pumila	15834	Camptothecin	Saito et al. (2001)
Panax ginseng	A4	Ginsenoside	Bulgakov et al. (1998)

(continued)

Table 2.1 (continued)

Plant species	*A. rhizogenes* strain used for transformation	Secondary metabolite	Reference
Papaver bracteatum	R15834 harbouring the binary vector pBI121	Morphine, noscapine, sanguinarine	Rostampour et al. (2009)
Papaver somniferum var. *album*	LBA 9402	Morphine, codeine, sanguinarine	Le Flem-Bonhomme et al. (2004)
Picrorhiza kurroa	LBA 9402	Kutkoside, picroside I	Verma et al. (2007)
Plantago lanceolata	LBA 9402	Verbascoside, plantamoside	Fons et al. (1999)
Plumbago indica	ATCC 15834	Plumbagin	Gangopadhyay et al. (2011)
Plumbago zeylanica	A4	Plumbagin	Verma et al. (2002)
Przewalskia tangutica	A4	Hyoscyamine, scopolamine	Lan and Quan (2010)
Pueraria phaseoloides	ATCC15834, harboring agropine type plasmid pRiA4b	Puerarin	Shi and Kintzios (2003)
Rauvolfia micrantha	ATCC 15834	Ajmalicine, ajmaline	Sudha et al. (2003)
Ruta graveolens	LBA 9402	Pinnarin, rutacultin, xanthotoxin, bergapten, isopimpinelin, osthole, osthenol, dictamnine, skimmianine, kokusaginine, rybalinine and an isomer of rybalinine	Sidwa-Gorycka et al. (2009)
Salvia broussonetti	ATCC-15834	Brussonol, iguestol	Fraga et al. (2005)
Salvia miltiorrhiza	ATCC 15834	Tanshinones	Chen et al. (2001)
Salvia sclarea	LBA 9402 carrying pRi 1855 plasmid	Salvipisone, aethiopinone, ferruginol	Kuźma et al. (2006)
Saussurea involucrata	LBA 9402, R1000, R1601	Syringin	Fu et al. (2005)
Saussurea medusa	R1601	Jaceosidin	Zhao et al. (2004)
Scopolia parviflora	KCTC 2703 harbouring pBE*pmt* plasmid	Scopolamine, hyoscyamine	Lee et al. (2005)

(continued)

Table 2.1 (continued)

Plant species	*A. rhizogenes* strain used for transformation	Secondary metabolite	Reference
Scutellaria baicalensis	A4	Baicalin, wogonoside, baicalein, wogonin	Kovács et al. (2004)
	ATCC 15834	Baicalin	Hwang (2006)
	A4GUS	Baicalin, wogonin	Tiwari et al. (2008)
Taxus x *media* var. *Hicksii*	LBA 9402	Paclitaxel, 10-deacetylbaccatin III	Furmanowa and Syklowska-Baranek (2000)
Tylophora indica	A4	Tylophorine	Chaudhuri et al. (2005)
Valeriana wallichii	LBA 9402, A4	Isovaleroxyhydroxy didrovaltrate, didrovaltrate, acevaltrate, homodidrovaltrate	Banerjee et al. (1998)
Withania somnifera	LBA 9402, A4	Withasteroids	Ray et al. (1996), Bandyopadhyay et al. (2007)

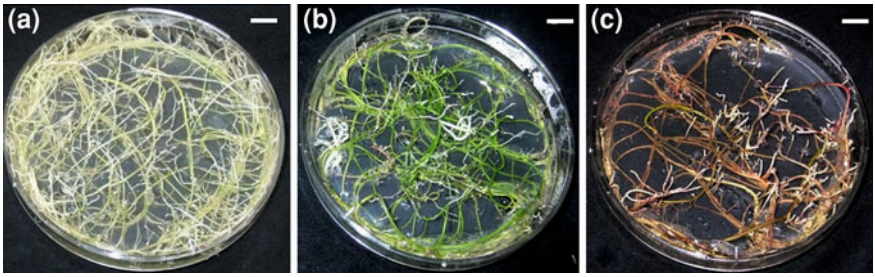

Fig. 2.1 Ri-transformed roots of *Tylophora indica* showing pigmentation **a** white root (bar = 9 mm), **b** green root (bar = 9 mm), and **c** red root (bar = 9 mm)

(Verpoorte et al. 1991; Rhodes et al. 1997; Mukundan et al. 1998; Shanks and Morgan 1999; Sevón and Oksman-Caldentey 2002).

Secretion is a fundamental function of plant cells and it is especially well-developed in plant roots (Roschina and Roschina 1993). Their ability to secrete plethora of compounds into the rhizosphere is a remarkable physiological feature and up to nearly 21 % of all carbon fixed by photosynthesis can be transferred to the rhizosphere in the form of root exudates (Marschner 1995) and contains both low molecular weight (e.g. secondary metabolites) and high molecular weights (e.g. proteins) compounds. Secondary metabolites that are produced in the roots that are transported and stored in other parts of the plants may be released in the culture medium from excised roots (Rhodes et al. 1986). Several secondary products, including a few alkaloids produced in transformed root culture of a number of plant species are released into the culture medium. In transformed root cultures the released secondary metabolites can be adsorbed and removed by a variety of high affinity polymeric resins (Freeman et al. 1993). In addition to a reduction in the production cost, this operation is known to stimulate the productivity of a number of compounds by transformed root cultures (Muranaka et al. 1993a; Holmes et al. 1997; Saito et al. 2001).

Ri-transformed root cultures of *Tylophora indica* shows variation in pigmentation when cultured under light (unpublished data). While, the hairy root cultures of *T. indica* remains white when cultured in dark, on exposure to light, they become green to red depending on the light intensity (Fig. 2.1). Hairy root turning green on exposure to light is also reported in other plants like in *Solanum khasianum* (Jacob and Malpathak 2005), red, and green hairy root lines in addition to white Ri-transformed root cultures are also reported by Yang and Choi (2000) in *Panax ginseng*.

2.3 Ri-Transformed Plants

Regeneration of whole viable plants from hairy root cultures, established from transformation with *A. rhizogenes*, has been reported in a number of plant species. Such transgenic plants frequently show a very characteristic phenotype which differ from their normal counterparts, such as, wrinkled leaf, shortened internodes, decreased apical dominance, altered flower morphology, increase in number of branches, reduced pollen and seed production, and abundant production of highly branched plagiotropic roots. All these altered phenotypic characters the so-called "Hairy Root Syndrome" is due to the combined expression of *rolA*, *rolB*, and *rolC* genes. *rolA* gene is associated with shortening of internodes, wrinkling of leaves, etc., whereas *rolB* gene causes reduced length of stamens, protruding stigmas, and increased adventitious roots on stems. *rolC* gene is responsible for reduced apical dominance, internodes shortening, and increased branching (Nilsson and Olsson 1997; Tepfer 1984). The hairy root phenotype was first described by Ackerman (1977) in Tobacco regenerants. In addition to the above-mentioned changes, biennial species frequently becomes annuals on transformation and regeneration with *A. rhizogenes* (Tepfer 1984; Sun et al. 1991; Kamada et al. 1992). Regeneration of transgenic plants from hairy roots can be either spontaneous or can be induced with the help of plant growth regulators.

2.3.1 Spontaneous Plant Regeneration from Ri-Transformed Root Cultures

One of the various advantages of using *A. rhizogenes*-mediated transformation system is direct regeneration of transgenic plants from root cultures as it avoids the problems due to somaclonal variations. Spontaneous and direct development of adventitious shoot buds from older regions of transformed hairy roots in hormone-free media without any callus formation is reported in a number of plant species like *Armoracia lapathifolia* (Noda et al. 1987), *Taraxacum platycarpum* (Lee et al. 2004), *Centaurium erythraea* (Subotić et al. 2003), *Hypericum perforatum* (Vinterhalter et al. 2006), *T. indica* (Chaudhuri et al. 2006), *Bacopa monnieri* (Majumdar et al. 2011), *Atropa belladonna* (Jaziri et al. 1994), *Plumbago indica* (Gangopadhyay et al. 2010), *Brassica oleracea* var. Botrytis (David and Tempé 1988), *B. oleracea* var. sabauda, *B. oleracea* var. capitata (Christey et al. 1997; Sretenović-Rajičić et al. 2006), *Populus tremula* (Tzfira et al. 1996), *Lotus corniculatus* (Petit et al. 1987), *Blackstonia perforiata* (Bijelović et al. 2004), *Pelargonium graveolens* cv. Hemanti (Saxena et al. 2007), *Ajuga reptans* var. atropurpurea (Tanaka and Matsumoto 1993), etc. These adventitious shoots when excised and cultured on hormone-free basal media regenerated into whole plants. However, culture conditions and time required for regeneration varied from plant to plant.

Shoot regeneration from transformed roots can be light dependent or independent. In *A. lapathifolia* (Noda et al. 1987), roots maintained in dark showed induction of shoot buds on transfer to light throughout the root except the root tips, but no adventitious shoot bud formation took place in those kept in dark. Non-transformed roots rarely developed adventitious shoot buds on transfer to light. LBA 9402 transformed roots of *P. tremula* (Tzfira et al. 1996) and A4 transformed roots of *B. monnieri* (Majumdar et al. 2011) showed spontaneous shoot bud regeneration when cultured under 16/8 h (light/dark) photoperiod. Interestingly, LBA 9402 transformed roots of *B. monnieri* did not show any regeneration but spontaneously dedifferentiated into callus. In *L. corniculatus* (Petit et al. 1987) and *P. indica* (Gangopadhyay et al. 2010), spontaneous shoot organogenesis is reported, when transformed roots were transferred to continuous light from dark. The hairy roots of *P. indica* did not regenerate in dark even after application of exogenous hormones to the media. Contrastingly, in *T. indica* (Chaudhuri et al. 2006), 17 % of transformed root clones are reported to regenerate shoots directly on hormone-free MS media in light independent manner. Subotić et al. (2003) also reported development of adventitious shoot primordial on older regions of hairy root cultures of *C. erythraea* both under light and in dark. In *Amoracia rusticana*, effect of light on shoot regeneration from transformed root cultures have been studied in details by Saitou et al. (1992). In dark conditions, shoot formation was rarely observed in the hairy roots, but the longer the culture period in the light, higher the frequency of shoot formation, and number of shoots per explant were noted. When cultured under light conditions (16 h light/8 h dark) for the first 4 weeks, no shoot bud regeneration was observed from the hairy roots, but when they were precultured in darkness for 2 weeks and then transferred to light, shoot formation was observed within 1 week of transfer. Shoot regeneration also varied with exposure time of hairy roots to light. A short exposure (1.68 h) to a high light intensity (78 μmol/m^2 s) showed less shoot formation than a long exposure (168 h) to lower light intensity (0.78 μmol/m^2 s). Thus, for adventitious shoot formation from transformed roots of *A. rusticana*, duration of light exposure is more important than the light intensity. The authors also showed that shoot formation in the hairy roots can be induced by white light and red light when precultured for 12 weeks in dark but not by the far red light. Far red light irradiation after red light irradiation partially inhibited shoot formation. Red light is also reported to stimulate shoot formation in *Pseudotsuga menziesi*, Petunia, and Apple (Kadkade and Jopson 1978; Economou and Read 1986; Predieri and Malavasi 1989). Shoot formation in tobacco calli is reported to be stimulated by blue light and inhibited by red light (Seibert et al. 1975; Weis and Jaffe 1969). Hence, effect of light on adventitious shoot organogenesis in hairy roots appeared to be plant specific. Time requirement for regeneration of shoot buds from the Ri-transformed roots also varied from plant to plant. In *A. lapathifolia* (Noda et al. 1987) and *B. monnieri* (Majumdar et al. 2011), shoot bud development was observed within 7 and 10 days of transfer to light, respectively. In others like, *L. corniculatus* (Petit et al. 1987), *P. tremula* (Tzfira et al. 1996), and *P. indica* (Gangopadhyay et al. 2010), shoot bud regeneration took place within 3–4 weeks.

Again, in *T. indica* (Chaudhuri et al. 2006) and *A. belladona* (Jaziri et al. 1994) shoot buds were seen only when transformed roots were kept for more than 8 weeks without subculture.

Regeneration potential of hairy roots also varied with the concentration of sucrose used in the media. When hairy roots of *A. belladona* (Jaziri et al. 1994) were cultured on half strength MS media with 1.5 % sucrose, spontaneous shoot bud regeneration took place. For rooting and further development, these shoot buds were transferred to the same media with 3 % sucrose. Hairy roots of *H. perforatum* (Vinterhalter et al. 2006) is reported to have high potential for spontaneous shoot regeneration which increased from first subculture (40.5 %) to second subculture (62 %) in hormone-free basal media. However, the regeneration frequency varied with the concentration of the sucrose used in the media. Highest shoot bud regeneration took place on hormone-free medium with 1 and 2 % sucrose with 50.3 and 48.8 shoot buds per culture, respectively. Higher concentration of sucrose at and above 4 % have got an adverse effect on both root growth and shoot differentiation, making them necrotic with characteristic brown color. Thus, low level of sucrose seems to favor regeneration of shoot buds from hairy roots in *A. belladona* and *H. perforatum*.

In addition to direct shoot bud regeneration from transformed root cultures, spontaneous callus formation is reported in many plant species like in *T. indica* (Chaudhuri et al. 2006), *Carica papaya* (Cabrera-Ponce et al. 1996), *Catharanthus roseus* (Brillanceau et al. 1989), *Solanum nigrum* (Wei et al. 1986), etc. Shoots regenerated from these calli either in hormone-free medium or with application of hormones. In *T. indica* (Chaudhuri et al. 2006), 44 % of the root clones spontaneously produced yellow friable callus from older regions of root, when kept for more than 8 weeks without subculture. Regeneration occurred via somatic embryogenesis from these calli within 3 weeks on hormone free MS medium (Fig. 2.2). Spontaneous callus development is also reported in many hairy root lines of *C. roseus* (Brillanceau et al. 1989), one of which evolved rapidly on hormone-free media with spontaneous shoot bud formation.

2.3.2 Plant Regeneration from Ri-Transformed Root Cultures Induced by Plant Growth Regulators

Induction of shoot bud directly from the transformed roots in presence of plant growth regulators are reported in many plants like *A. belladonna* (Aoki et al. 1997), *A. reptans* var. *atropurpurea* (Uozumi et al. 1996), *T. platycarpum* (Lee et al. 2004), *Plumbago rosea* L. (Satheeshkumar et al. 2009), *Linum usitatissimum* (Zhan et al. 1988), etc. To induce shoot formation, in most of the cases the cytokinin BA (6-Benzyladenine) is used either alone or in combination with some auxin. Concentration of hormones used for effective regeneration varied from plant to plant along with other conditions like, size of transformed root used as explant, concentration of sucrose used, photoperiod at which cultures were maintained, media used for rooting, etc.

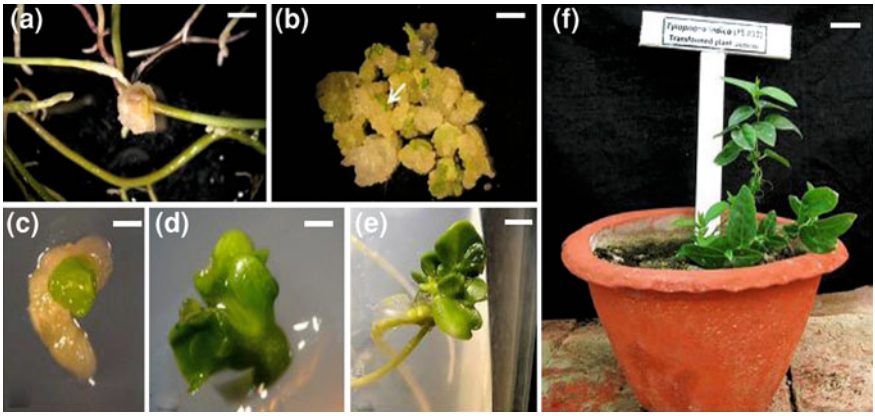

Fig. 2.2 Regeneration of Ri-transformed plant from hairy roots of *Tylophora indica*. **a** Spontaneous induction of callus in transformed roots (bar = 3.3 mm), **b** developing somatic embryos from the embryogenic callus (bar = 5 mm), **c** a single somatic embryo (bar = 1 mm), **d** germinated somatic embryo developing into plantlet (bar = 1.4 mm), **e** Ri-transformed plantlet developed from somatic embryo (bar = 2.5 mm), and **f** Ri-transformed plant transferred to potted soil (bar = 25 mm)

Shoot buds regenerated from hairy roots of *A. reptans* var. *atropurpurea* (Uozumi et al. 1996), *T. platycarpum* (Lee et al. 2004), *Aesculus hippocastanum* (Zdravković-Korać et al. 2004), and *P. rosea* (Satheeshkumar et al. 2009), when cultured on media with BA as the sole hormone supplement. Whereas, in *A. reptans* var. *atropurpurea* (Uozumi et al. 1996) high concentration of BA (10 mg/l) supplementation in media exhibited highest number of plantlet formation in hairy roots. In *T. platycarpum* (Lee et al. 2004) 100 % shoot bud regeneration was noted when the hairy roots were cultured on media supplemented with 1 mg/l BA. In *P. rosea* (Satheeshkumar et al. 2009) when concentration of BA used was within 0.5–2 mg/l shoot formation took place without callusing. But with increase of BA concentration from 2.5 mg/l onwards, callusing increased with less number of shoot bud production. Best shoot bud regeneration frequency was noted on media supplemented with 2 mg/l BA. Root explants cultured in medium containing BA with auxin did not differentiate into shoot buds but proliferated into callus.

As mentioned above, in some species, induction of shoot buds from transformed root cultures was also noted when auxin was used in combination with BA. In *A. belladonna* (Aoki et al. 1997), *Cichorium intybus* cv. Lucknow local (Bais et al. 2001), and Mexican lime (Pérez-Molphe-Balch and Ochoa-Alejo 1998), shoot buds regenerated when root segments were cultured on medium supplemented with low concentration of NAA along with BA. Whereas, in apple (Lambert and Tepfer 1992), low concentration of IBA is reported to be used along with high concentration of BA for development of adventitious shoot buds from the transformed roots. Transformed roots of *L. usitatissimum* (Zhan et al. 1988)

were cultured on MS medium containing NAA, BA, and adenine, for successful shoot bud regeneration. Hence, for efficient regeneration of shoot buds from hairy roots, type, combination, and concentration of hormones to be used varies from plant to plant.

Hormone supplementation in media increases the regeneration frequency in some plants when compared to that on hormone-free media. For example, in hairy roots of *T. platycarpum* (Lee et al. 2004), the regeneration frequency increased from 88.5 ± 9.8 % on hormone-free media to 100 % on BA supplemented media. Similarly, in hairy roots of *A. hippocastanum* (Zdravković-Korać et al. 2004), regeneration frequency was significantly increased on media supplemented with BA compared to that in phytohormone-free media. Contrastingly, in some other plants, like in *B. oleracea* var. sabauda and *B. oleracea* var. capitata (Sretenović-Rajičić et al. 2006), average number of shoots regenerated from transformed roots were lower on hormone supplemented media compared to hormone-free media. Thus, use of phytohormone does not always increase the regeneration frequency.

Hairy roots of different species were maintained under different photoperiod for efficient regeneration of shoot buds from the roots. Ri-transformed roots of *A. belladonna* (Aoki et al. 1997), *A. hippocastanum* (Zdravkovic-Korac et al. 2004), and *L. usitatissimum* (Zhan et al. 1988) were maintained under 16/8 h photoperiod, while hairy root explants of *A. reptans* var. *atropurpurea* (Uozumi et al. 1996) and *P. rosea* (Satheeshkumar et al. 2009) were cultured under 14/10 h (light/dark) and 12 h photoperiod, respectively, for shoot bud induction. Continuous exposure to light was given to transformed root explants of Mexican lime (Pérez-Molphe-Balch and Ochoa-Alejo 1998). Thus, like in case of spontaneous regeneration, effect of light on shoot bud regeneration in presence of phytohormones also varies from species to species.

Apart from use of plant growth regulators to induce shoot from transformed roots, some other agents are also reported to induce shoot regeneration either alone or in addition to exogenous hormones. In *Antirrhinum majus*, Hoshino and Mii (1998) studied effect of phosphinothricin-based herbicide, Bialaphos (a tripeptide antibiotic produced by *Streptomyces hygroscopicus*), and plant growth regulators on regeneration from transformed roots. The various concentrations of phytohormones, NAA in combination with BA or TDZ, tried by them did not result in any improvement in shoot bud induction compared to that in hormone-free media. But bialaphos, although toxic at and above 0.9 mg/l, when added at or below 0.5 mg/l, enhanced shoot regeneration. Fifty-six percent of hairy roots regenerated shoots when cultured on half strength MS with 0.5 mg/l bialaphos after 3 months.

Shoot organogenesis from transformed root cultures induced by phytohormones via callus formation is also reported in many plant species like, *Stylosanthes humilis* cv. Paterson (Manners and Way 1989), *Medicago arborea* L. (Damiani and Arcioni 1991), *Glycine argyrea* (Kumar et al. 1991), *Lotus japonicus* (Stiller et al. 1997), *Alhagi pseudoalhagi* (Wang et al. 2001), *C. roseus* (Choi et al. 2004), *Crotalaria juncea* (Ohara et al. 2000), cultivars of *B. oleracea*, and *Brassica campestris* (Christey et al. 1997), etc. Concentration and combinations of plant

growth regulators used to induce callus formation and for regeneration of shoot buds from callus varied from plant to plant.

In *S. humilis* cv. Paterson (Manners and Way 1989), *G. argyrea* (Kumar et al. 1991), *C. roseus* (Choi et al. 2004), *C. juncea* (Ohara et al. 2000), *L. japonicus* (Stiller et al. 1997) and different cultivars of *B. oleracea* and *B. campestris* (Christey et al. 1997), callus induction from Ri-transformed roots and shoot organogenesis from those calli took place in the same hormone supplemented media. In some of these plants, the development and elongation of the regenerated shoot buds required transferring the regenerating callus or regenerated shoot buds to a different medium. When hairy root segments of *S. humilis* cv. Paterson (Manners and Way 1989) were cultured on MS with 2 mg/l BA, callus induction took place and within 2–3 weeks shoot regeneration occurred on the same media. When less than 2 mg/l BA was used, callus formation took place without any shoot bud regeneration, while shoot regeneration frequency from callus was very low when 4 mg/l BA was used. In *G. argyrea* (Kumar et al. 1991), when Ri-transformed root segments were cultured on B5 medium with BA and IBA (Indole-3-butyric acid), green nodular callus was induced within 20 days. These nodular calli produced shoots within 40–50 days. Shoot elongation was noted when regenerating callus was transferred to B5 medium with IBA and reduced level of BA. When hairy root segments of *C. roseus* (Choi et al. 2004) were cultured on MS supplemented with BA and NAA (α-Naphthaleneacetic acid), callus induction was noted after 2 weeks, from which shoot buds regenerated with 80 % frequency on the same medium. Transformed root fragments of *C. juncea* (Ohara et al. 2000) when cultured on solid B5 medium supplemented with 3 mg/l BA, callus induction followed by shoot bud regeneration was observed. Regeneration frequency which was 30 % in 3 mg/l BA supplemented media reduced to 14 % when concentration of BA increased to 5 mg/l. The induced shoot buds showed severe hyperhydricity, thus mostly failed to develop further on 0.8 % agar and 0.2 % gelrite solidified media. Elevated concentrations of gelling agent (1.2 % agar) was found to be effective in lowering the hyperhydricity and in promoting further development of the shoot buds. In *L. japonicus* (Stiller et al. 1997), when hairy root segments were cultured on B5 medium supplemented with BA and NAA, callogenesis followed by development of shoot primordial took place. Shoot elongation of these regenerated shoot buds was achieved when concentration of NAA was reduced to half.

Contrastingly, in *M. arborea* (Damiani and Arcioni 1991), the Ri-transformed callus only proliferated on the callus induction media (B5 media containing 2,4-D and kinetin). Shoot regeneration needed transfer of these calli to a different media, i.e. on hormone-free solid MS media. Similarly, Wang et al. (2001) reported use of two different media, one for callus induction from the hairy root of *A. pseudoalhagi* and other for regeneration from those calli. Hence, for induction of callus from Ri-transformed roots and subsequent shoot bud regeneration, choice and concentration of exogenous hormone supplementation varies with species.

Induction of embryogenic callus from hairy roots using phytohormones and regeneration of transgenic plants from such calli is reported in many plant species

like in *Cucumis sativus* L. (Trulson et al. 1986), *P. ginseng* (Yang and Choi 2000), *Prunus avium* x *P. pseudocerasus* (Gutièrrez-Pesce et al. 1998), etc.

Yang and Choi (2000) cultured green, white, and red root lines of *P. ginseng* on MS medium supplemented with 1 mg/l 2,4-D. Low frequency of embryogenic callus formation was noted until 1 month which increased with duration of the culture. Among the three root lines, best induction of embryogenic callus was noted in the red line. Efficient production of somatic embryos from this embryogenic callus was achieved by transferring them to MS medium containing 0.5 mg/l 2,4-D, which matured into cotyledonary stage after 2 months. Germination of these cotyledonary somatic embryos were only found when transferred to MS medium containing 10 mg/l GA_3 within 15 days, which continued to grow on half strength MS medium without any growth regulators.

2.4 Production of Secondary Metabolites in Ri-Transformed Plants

While extensive study have been done on secondary metabolite production in hairy root cultures obtained through transformation with *A. rhizogenes*, only a few reports are available regarding analysis of secondary metabolites in roots and shoots of Ri-transformed plants (Table 2.2). Secondary metabolite productions in Ri-transformed plants are at levels, comparable to or even greater than that in non-transformed plant in many cases, whereas in some plants reduction of specific secondary metabolite is also reported. In A4 transformed shoots of *T. indica*, tylophorine content was 20–60 % higher than that in the control (Chaudhuri et al. 2006). Similarly, in A4 transformed plants of *B. monnieri*, the content of four bacopa saponins (bacopasaponin D, bacopasaponin F, bacopaside II, and bacopaside V) were up to five times higher than non-transformed plants of same age (Majumdar et al. 2011). Ri-transformed plants of *P. indica* are also reported to have an increased plumbagin content compared to non-transformed plants (Gangopadhyay et al. 2010). On the contrary, Ri-transformed plants of *Hyoscyamus muticus* showed reduced alkaloid production (Sevón et al. 1997) and same was in case of transgenic plants of *D. myoporoides* × *D. leichhardtii* for scopolamine and hyoscyamine (Celma et al. 2001).

2.5 Large-Scale Production of Secondary Metabolites by *A. rhizogenes* Mediated Transformed Roots in Bioreactors

Hairy roots appear to be potential systems for culture in bioreactors in large scale because of their organized nature, fast growth rate, and stability in metabolite production. As hairy roots grow continuously (Jeong et al. 2002) bioreactors used

Table 2.2 Secondary metabolites from transgenic plants obtained through transformation with *Agrobacterium rhizogenes*

Plant species	Family	*A. rhizogenes* strain used for transformation	Secondary metabolite	Reference
Ajuga reptans var. *atropurpurea*	Lamiaceae	*A. rhizogenes* MAFF03-01724	20-hydroxyecdysone (20-HE)	Tanaka and Matsumoto (1993)
Armoracia lapathifolia	Brassicaceae	–	Isoperoxidase	Saitou et al. (1991)
Atropa belladonna	Solanaceae	*A. rhizogenes* strain 15834	Hyoscyamine and other tropane alkaloids	Aoki et al. (1997)
Bacopa monnieri	Scrophulariaceae	*A. rhizogenes* strain A4	Bacopasaponin D, Bacopasaponin F, Bacopaside II, Bacopaside V, Bacoside A3 and Bacopasaponin C	Majumdar et al. (2011)
Centaurium erythraea	Gentianaceae	*A. rhizogenes* strain LBA 9402	Secoiridoids glucosides	Piatczak et al. (2006)
Convolvulus arvensis	Convolvulaceae	*A. rhizogenes* with synthetic *crypt* gene	Calystegine	Chaudhuri et al. (2009)
Duboisia myoporoides X *D. leichhardtii*	Solanaceae	*A. rhizogenes* strain A4	Scopolamine	Celma et al. (2001)
Hyoscyamus muticus	Solanaceae	*A. rhizogenes* stain LBA9402	Hyoscyamine, scopolamine, Calystegins	Sevón et al. (1997)
Papaver somniferum	Papaveraceae	*A. rhizogenes* strain MAFF 03-01724	Morphinan alkaloid	Yoshimatsu and Shimomura (1992)
Pelargonium graveolens cv. Hemanti	Geraniaceae	*A. rhizogenes* strains A4 and LBA9402	Essential Oil, Linalool, Rose oxides(cis + trans), Isomenthone, Citronellol, Geraniol, 6-Caryophyllene, Guaia-6,9-diene, 10-epi-Y-Eudesmol, Citronellyl esters, Geranyl esters	Saxena et al. (2007)
Pimpinella anisum	Umbelliferae	–	Total phenolics	Andarwulan and Shetty (1999)
Plumbago indica	Plumbaginaceae	*A. rhizogenes* strain ATCC 15834	Plumbagin	Gangopadhyay et al. (2010)
Scoparia dulcis	Scrophulariaceae	*A. rhizogenes* strain 15834 with *bar* gene	Scopadulcic acid B.	Yamazaki et al. (1996)
Solanum khasianum	Solanaceae	*A. rhizogeness* strains A4 and LBA 9402	Solasodine	Jacob and Malpathak (2005)
Tylophora indica	Asclepiadaceae	*A. rhizogenes* strain A4	Tylophorine	Chaudhuri et al. (2006)
Vinca minor	Apocynaceae	*A. rhizogenes* strain DC-AR2	Vincamine	Tanaka et al. (1995)

for the cultivation of hairy roots are complex and quite different from the conventional ones utilized for the culture of plant cell suspensions (Mishra and Ranjan 2008). Reactors with unique configurations are required due to the organized and entangled nature of these roots. The structured as well as delicate hairy roots distribute unevenly throughout the bioreactor (Jeong et al. 2002) and form continuous root clumps composed of interconnected primary and lateral roots which hinder the percolation of oxygen into the roots (Kino-Oka et al. 1999; Bordonaro and Curtis 2000). Root hairs also limit fluid flow and the availability of oxygen (Bordonaro and Curtis 2000). Limitations of nutrient and oxygen mass transfer, which increase with increase in root biomass, are the main drawbacks associated with culture of hairy roots in bioreactors, leading to cell death and necrosis at the core of the biomass (Jeong et al. 2002; Suresh et al. 2005). This in turn causes reduction in growth and metabolite production (Suresh et al. 2005). Transfer of oxygen from the air bubbles to the hairy roots via the medium and from one cell to another within the hairy roots is complicated (Neelwarne and Thimmaraju 2009). Mass transfer can be enhanced by vigorous mixing, but this might lead to reduction in root viability due to increase in hydrodynamic shear stress (Hitaka et al. 1997). Large-scale culture of hairy roots is difficult as nutrients need to be provided simultaneously from the gas and liquid phases (Jeong et al. 2002). Several factors such as growth characteristics, morphological changes of hairy roots during proliferation, nutrient requirements, availability and utilization rates, medium composition, mass transfer, mechanical properties, methods for providing a support matrix, protection from shear damage, inoculum density and even distribution of the inoculum, and chances of flow restriction caused by the highly tangled root masses should be considered while designing a bioreactor for hairy roots (Taya et al. 1989; Yu and Doran 1994; Mishra and Ranjan 2008). Because of continuous proliferation and repeated branching, the rheological properties of the hairy roots change continuously (Neelwarne and Thimmaraju 2009). Thus, it is generally difficult to select a single type of bioreactor for the cultivation of hairy roots as the rheological properties of the roots vary not only from species to species but also within clones of a single species (Mishra and Ranjan 2008). Productivity in bioreactors also depends on certain other parameters like temperature, pH, composition of gases, selection of hairy root clone, reactor operation, removal of toxic byproducts, etc. (Kim et al. 2002a; Mishra and Ranjan 2008). To a large extent, bioreactor design also depends on the location of the product which may be either intracellular or extracellular (Mishra and Ranjan 2008).

2.5.1 Types of Bioreactors Used for the Cultivation of Hairy Roots

Reactors used for the cultivation of hairy roots can be of different types based on the continuous phase viz. liquid phase, gas phase, or the hybrid ones which are a combination of both.

2.5.1.1 Liquid Phase Bioreactors

In liquid phase reactors, also known as submerged reactors, roots remain submerged in the medium and air is bubbled through the culture medium to supply oxygen (Kim et al. 2002a, b). Stirred tank, bubble column, air lift, liquid impelled loop, and submerged connective flow reactors (Wilson et al. 1987; Taya et al. 1989; Buitelaar et al. 1991; Tescione et al. 1997; Carvalho and Curtis 1998) are examples of liquid phase reactors.

Stirred Tank Bioreactors

Among the liquid phase reactors, stirred tank reactor is mechanically agitated (Mishra and Ranjan 2008); aeration and medium currency are regulated by mortar-derived impeller or turbine blades (Choi et al. 2006). Compressed air is sparged into the reactor in the form of bubbles to supply oxygen for the aerobic processes (Mishra and Ranjan 2008). The impeller region has an aeration device which produces a well dispersed gas phase in the continuous liquid phase (Mishra and Ranjan 2008). However, the impeller rotation damaged the roots by shearing, which resulted in callus formation and poor biomass production (Hilton et al. 1988; Wilson 1997). The problem was alleviated by isolating roots from the impeller by using a steel cage or nylon mesh to fix the roots (Kondo et al. 1989; Hilton and Rhodes 1990) or by developing modified stirred tank reactors having flat blade turbines (Mishra and Ranjan 2008). Cardillo et al. (2010) used a 1.5 l modified stirred tank reactor equipped with a plastic mesh to culture hairy roots of *Brugmansia candida*. These cultures produced an increased biomass (49.33 g FW, GI of 3.93), corresponding to a 36 % higher yield than that obtained in Erlenmeyer flasks; alkaloid yields in the reactor were 2.6- to 3-folds higher than the cultures in Erlenmeyer flasks with a fivefold increase in scopolamine yield. The specific productivities for both the systems were similar except a 31 % increase in anisodamine production in the bioreactor. 2.8–4 times higher volumetric productivities were obtained for the processes carried out in the bioreactor when compared with the Erlenmeyer processes. Choi et al. (2006) reported that compared to other types of reactors, temperature, pH, amount of dissolved oxygen, and nutrient concentration can be controlled in a better way within a stirred tank bioreactor. Growth and hyoscyamine production in transformed root cultures of *D. stramonium* was studied in a modified 14 l stirred tank reactor (Hilton and Rhodes 1990). The effects of batch and continuous modes of operation, three different temperatures and half and full strength Gamborg's B5 salts were studied. When cultured at half strength B5 medium, the dry matter content and hyoscyamine levels of the roots were higher than those grown on full strength B5 salts. The amounts of hyoscyamine were similar at both 25 and 30 °C but about 40 % lower at 35 °C. However, highest production rate of 8.2 mg/l/day hyoscyamine was obtained when the roots were grown at 30 °C. Hyoscyamine released into the

culture medium was low in continuous fermentation at 25 °C but up to sevenfolds higher when the fermentors were operated at 30 or 35 °C.

Reduced productivities, often recorded during scale-up cultures, might also result from handling problems during inoculation and uneven distribution of the roots in the bioreactor (Hilton and Rhodes 1990; Kwok and Doran 1995; Woo et al. 1996). Lee et al. (1999) cultivated randomly cut hairy roots of *A. belladonna* in 3 l and 30 l modified stirred reactors. For a good distribution, roots were inoculated and immobilized on a stainless steel mesh in the reactors that separated the roots from the stirrer. After a period of 1 month 1490 mg tropane alkaloids were produced by the roots cultured in the 30 l reactor; 5.4 mg/gDW atropine could be detected in the roots which was equivalent to the amount found in plants grown in the field for 12 months and contained considerable amounts of other alkaloids like 1.6 mg/gDW 6-β-hydroxyhyoscyamine, 0.9 mg/gDW scopolamine, and 2.0 mg/gDW littorine. The study demonstrated that the use of this type of modified stirred bioreactor would provide sufficient supply of oxygen and nutrition for the growth of roots and alkaloid production. In another study, *A. belladonna* hairy roots were cultured in a bioreactor using porous polypropylene membrane tubing as a supplementary aeration device and an emulsion of Fluorinert^{TM} FC-43 perfluorocarbon was added to the medium (Kanokwaree and Doran 1998). The treatments were applied to improve oxygen supply to the roots. Combination of air sparging and membrane tubing aeration in a gas driven bioreactor supported 32–65 % higher biomass levels than sparging only of oxygen-enriched air at the same total gas flow rate. However, growth was not improved by the addition of perfluorocarbon to the medium in the sparged stirred tank reactor. The study demonstrated the need for site directed aeration of hairy root cultures for supplying oxygen into the zones of highest root biomass. Large-scale culture of *Ophiorrhiza pumila* in a 3 l modified stirred tank reactor fitted with a stainless steel net (to minimize physiological stress of the hairy roots by agitation) decreased the biomass yield compared to shake flasks; while a biomass of 162 ± 20 gFW/l was achieved in a 100 ml shake flask, only 87.2 ± 12.4 gFW/l was achieved in the aerated 3 l reactor (Hiroshi et al. 2002). Camptothecin yields in the reactor (8.7 ± 1.3 mg/l) were, however, almost equivalent to that of the roots cultured in shake flasks (8.9 ± 1.4 mg/l).

Nuutila et al. (1994) demonstrated that growth and alkaloid content in hairy roots of *C. roseus* was affected by different shear levels. For culturing the roots a stirred tank reactor with a metal mesh used to isolate the roots from the impeller system was used. Although the impeller was used to avoid shear stress, root growth was severely inhibited. Best growth and alkaloid production was obtained in an air sprayed bioreactor with no other mixing.

Mehrotra et al. (2008) cultured a fast growing hairy root clone G6 of *Glycyrrhiza glabra* in a 5 l bench top air sparged and mechanically agitated bioreactor, provided with a nylon mesh septum containing 4 l modified NB (Nitsch and Nitsch 1969) medium. After 30 days of incubation, the root biomass harvested (310 g) represented 20 times increase over initial inoculum (16 g).

Bubble Column Bioreactors

In bubble column reactors roots are submerged in the medium and an air distributor situated at the bottom of the column generates an upflow of air bubbles leading to liquid mixing (Mishra and Ranjan 2008). Compared to other stirred types of reactors this reactor is advantageous for the culture of organized structures like hairy roots as the bubbles create less shearing stress. Kwok and Doran (1995) improved supply of oxygen to the roots in a 2.5 l bubble column reactor by introducing gas at multiple points of the reactor, divided into three segments with wire mesh, each segment containing a sparger. After 43 days of culture 9.9 gDW/l of *A. belladonna* hairy roots were harvested. However, bulk mixing was very poor in the reactor. McKelvey et al. (1993) reported that in hairy root cultures of *H. muticus*, the yields of tissue mass in submerged air sparged reactors was 31 % of that accumulated in shake flask controls. The authors suggested that liquid phase channeling and stagnation leading to impaired oxygen transfer were the probable causes resulting in poor growth of the roots. When *Lobelia inflata* hairy roots were cultivated in a cylindrical 10 l bubble column bioreactor, a 3.5 times increase in biomass was noted at the end of the culture period (Bálványos et al. 2004). High levels of polyacetylene (36.5 mg/g lobetyolin and 15.9 mg/g lobetyolinin) were detected by HPLC.

Jeong et al. (2002) used two types of bioreactors viz. an air bubble four stage column bioreactor and a modified stirred bioreactor, both fitted with stainless steel meshes, for the culture of *P. ginseng* hairy roots. After 32 days of culture in the four stage bubble column reactor, each stage of the column was closely packed with hairy roots and a 36.3-fold increase in biomass was noted. The crude saponin content was 20 % on dry weight basis, which was similar to flask cultures. A 52-fold increase from the initial inoculum was observed in the modified stirred bioreactor after 42 days. In both the types of reactors growth was about three times as high as in flask cultures.

A prototype basket bubble bioreactor was used for the coculture of hairy roots and shoots of *Genista tinctoria* (Shinde et al. 2008). Large amounts of isoliquiritigenin were produced by the hairy roots.

Connective Flow Bioreactor

Carvalho and Curtis (1998) developed a connective flow reactor which consisted of a 14 l stirred tank, equipped with agitation and temperature control, a peristaltic pump and a tubular reactor. Sparging and agitation in the fermentor transferred oxygen into the medium and a peristaltic pump recirculated the medium between the stirred tank and the tubular reactor. 556 $\pm$ 4 gFW/l of *H. muticus* hairy roots, were produced after a period of 30 days whereas a bubble column reactor produced only 328 $\pm$ 5 gFW/l hairy roots. Although better than a bubble column reactor, it was suggested that a connective flow reactor may not be suitable from a realistic point of view as a pressure is required to circulate the culture medium at a

high velocity to overcome the flow resistance of the root bed (Carvalho and Curtis 1998). However, reactors similar to connective flow reactors have been proved to be effective as research tools. Williams and Doran (1999) minimized the liquid solid hydrodynamic boundary layer at the root surface and determined the critical oxygen level of hairy roots of *A. belladonna* by using a packed bed recirculation reactor.

Air Lift Bioreactors

Air lift reactors are basically bubble column reactors containing a draught tube (Mishra and Ranjan 2008). In these reactors air is supplied through a sparger ring into the bottom of a central draught tube that controls the circulation of air and the medium (Mishra and Ranjan 2008). The draught tube also prevents bubble coalescence by causing the bubbles to move in one direction and the shear stress is distributed equally throughout the reactor. Thus cells can grow in a more stable physical environment. Shear stress generated in air lift reactors is lower than that generated in bubble column reactors, giving the air lift reactors an advantage over the bubble column reactors (Al-Masry 1999). Although reports indicate the use of conventional air lift reactors for the scale-up culture of hairy roots (Buitelaar et al. 1991; Oka et al. 1992; Uozumi et al. 1995), optimum biomass could not be yielded mainly because of two factors—uneven distribution of roots at certain regions of the reactor and excessive channelling of gas phase, blocking liquid flow due to clumping of roots (Kim and Yoo 1993). However, immobilization of roots has been reported to increase biomass of hairy roots in air lift reactors (Taya et al. 1989). In another study, after 39 days of culture in a 5 l airlift bioreactor, growth of hairy roots of *P. ginseng* was about 55-fold of the initial inoculum whereas a 38-fold increase in biomass was noted after 40 days of culture in a 19 l airlift bioreactor (Jeong et al. 2003). When cultured in a 30 l airlift bioreactor for 20 days, the dry weight of hairy roots (11.5 g/l dry weight) and astragaloside IV yields (1.4 mg/g) from hairy root cultures of *Astragalus membranaceus* were higher than a 10 l bioreactor (dry weight 9.4 g/l and astragaloside IV 0.9 mg/g) (Du et al. 2003). Yields from the 30 l bioreactor were almost similar to cultures from 250 ml and 1 l flasks. Growth of hairy roots and the production of scopolamine in hairy root cultures of *Datura metel* were improved following treatment with permeabilizing agent Tween 20 in a 4 l airlift bioreactor with root anchorage (Cusido et al. 1999). After 4 weeks of culture, biomass yield was 2.3 and 0.84 mg/l/day scopolamine was produced. 30 g FW of *Duboisia leichhardtii* hairy root clone DL47-1 was inoculated into 3 l airlift bioreactor packed with Amberlite XAD-2 (Muranaka et al. 1993b). Biomass of the roots increased eightfolds during the culture period and 0.5 g/l scopolamine was obtained after 11 weeks. When a polyurethane foam was used in the vessel, the root tissue spread uniformly and grew well, showing a 14-fold increase in biomass after 12 weeks, with a 0.8 g/l yield of scopolamine. In the same study, a two-stage culture system, the first stage for the growth of hairy roots, and the second one for scopolamine release was

carried out in a 1 l turbine blade reactor fitted with a stainless steel mesh as a support for the uniform growth of the roots and also packed with Amberlite XAD-2 column. The root biomass increased 12-folds during 4 weeks of culture in the growth medium; after culturing in the medium for scopolamine release for 11 weeks 1.3 g/l scopolamine was recovered.

Tikhomiroff et al. (2002) reported the use of a two-liquid phase bioreactor for the extraction of indole alkaloids from *C. roseus* hairy roots with silicon oil. The roots were inoculated and immobilized in a stainless steel screen mesh box placed 1 cm from the bottom of the reactor in the aqueous phase. The second phase was DC200 silicon oil. Although growth of the hairy roots was not affected by silicon oil, the specific yields of tabersonine and löchnericine were improved by 100–400 and 14–200 %, respectively. When jasmonic acid was used as an elicitor, 10–55 % tabersonine and 20–65 % löchnericine were produced. The alkaloids accumulated in the silicon oil phase and were never detected in the culture medium.

Liu et al. (1998) investigated the effect of four different culture systems—250 ml Erlenmeyer flasks, bubble column reactor, modified bubble column reactor, and modified inner loop air lift reactor, for the production of artemisinin. The modified inner loop air lift bioreactor was designed to improve the transfer of nutrient medium and supply of oxygen and to increase growth homogeneity. When compared it was found that growth rate and artemisinin content (26.8 g/l and 536 mg/l, respectively) in the hairy roots cultivated in the modified air lift reactor was higher than the other types of reactors. The hairy roots grew more homogenously in the modified inner loop air lift reactor, proving that optimization of design of air lift reactor can promote growth and artemisinin production. In another study, *Pueraria phaseoloides* hairy roots were cultured in 2.5 l airlift bioreactors for 3 weeks (Kintzios et al. 2004). 5,570 μg/gDW puerarin, corresponding to 200 times as much as in 250 ml flask cultures was produced after incubation for 3 weeks. Puerarin was also released into the culture medium at concentrations higher than that found in the hairy roots themselves. Sharp and Doran (1990) cultured root tips of *A. belladonna* in hormone-free MS medium containing 3 % sucrose in two 2.5 l airlift bioreactors. A 33-fold increase in biomass yield was noted over a 26 day culture period and concentration of atropine in the dried roots was 0.37 %. In another study, Caspeta et al. (2005a) studied the growth of *Solanum chrysotrichum* hairy roots in shake flasks, a glass-draught internal-loop 2-l basic design air lift reactor (BDR) and a novel modified mesh-draught with wire-helix 2 l reactor (MR). Growth patterns were different in each of them with specific growth rates being 0.08, 0.067, and 0.112 per day for shake flasks, BDR, and MR, respectively. After 42 days of culture, tissue density in the MR was almost the same as that found in the shake flasks and twice as that obtained in the BDR. Cultures were scaled-up into 10 l MR level. Results indicated the overall tissue density reduced slightly compared to that of 2 l reactor. *S. chrysotrichum* hairy roots were also cultivated in 250 ml flasks and 2 l modified draught-tube internal-loop airlift reactors for the production of five antifungal saponins (SC-2 to SC-6) (Caspeta et al. 2005b). In the 2 l reactor, yield of SC-2 was 0.7 % on dry weight basis, which were sixfolds greater than that found in plant leaves. SC-4 was recovered both from the flasks and the reactor while SC-5

and SC-6 were only detected from the culture medium of roots grown in the 2 l reactor.

2.5.1.2 Gas Phase Bioreactors

Trickle bed, droplet phase, liquid dispersed, and nutrient mist reactors are examples of gas phase reactors (Taya et al. 1989; Flores and Curtis 1992; Wilson 1997; Williams and Doran 2000; Woo et al. 1996; Liu et al. 1999). Hairy roots are intermittently exposed to air or other gaseous mixtures and the nutrient liquid in these reactors. Liquid nutrients are either sprayed onto the roots or the roots get nutrient media as droplets, the size of which varies considerably (Kim et al. 2002a, b). The droplets are 0.5–30 μm in mist reactors using ultrasonic transducers (Weathers et al. 1999) whereas they are of much larger sizes for trickle bed or other gas phase reactors using spray nozzles (Wilson 1997). Any oxygen deficiency in the dense root clumps can be eliminated using these reactors; stress caused by shearing is also minimized (Kim et al. 2002b). However, excessive delivery of the medium can cause liquid phase channeling and medium retention. Oxygen can be completely depleted in the submerged regions of the root clumps and nutrient concentrations can be different from that in the bulk medium (Singh and Curtis 1994; Williams and Doran 2000). Since gas is the continuous phase in these reactors, roots need to be immobilized. Horizontal sheets of mesh, vertical structures, packing rings made of nylon mesh, or intalox metal process packing have been used variously for the purpose of immobilization (Woo et al. 1996; Liu et al. 1999; Chatterjee et al. 1997; Williams and Doran 2000).

Nutrient Mist Bioreactors

In nutrient mist reactors roots are dispersed in an air phase by immobilization on a mesh support and a mist phase, consisting of liquid medium is introduced into the reactor (Mishra and Ranjan 2008). The hairy roots are continuously bathed in nutrient mist, thereby replenishing the nutrients rapidly, and removing toxic byproducts (Dilorio et al. 1992). Compared to other reactors, these reactors have certain advantages viz. these are easy to operate and scale up, gas composition can be closely controlled, and oxygen is not a limiting factor and provides an environment of low shearing forces and pressure drops (Weathers et al. 1999; Mishra and Ranjan 2008). As the nutrient mist can be dispersed homogenously within the culture chamber, mechanical agitation is not required, and this reduces root damage caused by shearing (Dilorio et al. 1992). Certain parameters need to be taken into account while designing a nutrient mist reactor. These are composition of the mist, availability of the nutrients, recycling of medium, feeding schedules, and a feed rate sufficient for the growth of the roots (Dilorio et al. 1992).

Whitney (1992) used various types of bioreactors like nutrient mist reactor, trickle bed reactor, stirred tank reactor, and an air lift reactor for the culture of hairy roots of *Nicotiana tabacum* and *D. stramonium* and found that cultivation in nutrient mist reactors resulted in a higher growth rate and yield of tobacco compared to the other reactors used. Kim et al. (2001) found that the amount of artemisinin was significantly higher (2.64 μg/g dry weight) when *Artemisia annua* hairy root clone YUT16 was cultured in a nutrient mist bioreactor, compared to a bubble column reactor (0.98 μg/g dry weight). On the contrary, overall biomass concentration was higher in a bubble column reactor (15.3 gDW/l) than in a nutrient mist reactor (14.4 gDW/l) (Kim et al. 2002b). Also, the average growth rate was higher in the bubble column reactor than in the nutrient mist reactor. *A. annua* hairy roots have also been cultivated in three different types of nutrient mist bioreactors, each of 2.3 l working volume, fitted with three stainless steel meshes, namely, a nutrient mist bioreactor, an inner-loop nutrient mist bioreactor and a modified inner-loop nutrient mist bioreactor (Liu et al. 1999). After a culture period of 25 days, growth indices in the three types of bioreactors were 42, 61, and 68, respectively. 13.6 gDW/l medium biomass yield was achieved in the modified inner-loop nutrient mist bioreactor. The development and growth kinetics of single hairy roots of *A. annua* were characterized in nutrient mist reactor and shake flasks (Wyslouzil et al. 2000). The effects of mist duty cycle, medium formulation, gas composition on growth kinetics, and morphology of the roots were also studied. Dilorio et al. (1992) reconfigured a nutrient mist bioreactor for batch operation to homogenously deliver nutrient mist to the hairy root cultures of *Carthamus tinctorius*. Misting cycle, inoculum size, batch or continuous operation, and sucrose concentration were varied to obtain maximum growth over a period of 1 week. Williams and Doran (2000) scaled-up *A. belladonna* hairy roots in a liquid dispersed bioreactor, where the liquid medium was sprayed onto the roots growing on a stainless steel support.

Bioreactors of various configurations viz. bubble column reactor, nutrient sprinkle reactor, and an acoustic mist bioreactor have been used to study the growth of *C. intybus* hairy roots (Bais et al. 2002b). Among all the types of bioreactors used, roots grown in acoustic mist bioreactor had greater final biomass (295 gFW/l, 29.8 gDW/l); this reactor also showed better specific growth rate of 0.075/day that was very close to that of shake flasks (0.086/day). Also, accumulation of esculin was maximum in the acoustic mist bioreactor (18.5 g/l) which was 1.4 times greater than nutrient sprinkle and bubble column reactors.

In an interesting study, Souret et al. (2003) examined the effect of three different culture systems like shake flasks, a mist reactor and a bubble column reactor on the expression levels of four key terpenoid biosynthetic genes, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), and farnesyl diphosphate synthase (FPS) in hairy root cultures of *A. annua*. All the genes showed temporal regulations when the roots were cultured in shake flasks and the production of artemisinin could only be correlated with the expression of FPS. The expression of the genes in the reactors was equivalent to

or greater than that of the hairy roots grown in the shake flasks. However, the expression level within the six different zones of each reactor could not be correlated with their respective oxygen levels, light and root packing density. The authors also observed that the genes were expressed unusually; the position of the roots in the reactors affected the transcriptional regulation of all the genes under study. It was thus concluded that to characterize gene activity in a whole reactor, analysis of a single reactor sample could be misleading. Also it was found that the terpenoid gene expression in hairy roots of *A. annua* cultured in a bubble column reactor was different when compared with a mist bioreactor.

In another study, Flores and Curtis (1992) showed that when grown in a trickle bed reactor, production of solavetivone by hairy root cultures of *H. muticus* was 3.5 times higher when compared with roots grown in a submerged reactor.

2.5.1.3 Hybrid Bioreactors

Hybrid reactors represent the third type of reactor used for the cultivation of hairy roots. In these reactors, roots are grown in a gas phase after being exposed to liquid phase (Mishra and Ranjan 2008). A major drawback of gas phase reactors is that manual loading is required to uniformly distribute roots in the growth chamber (Kim et al. 2002a; Mishra and Ranjan 2008). Ramakrishnan et al. (1994) used a hybrid reactor to solve the problem by initially running the reactor as a bubble column to suspend, distribute, and attach the roots to the packing rings in the reactor. After 2 weeks the reactor was switched to a trickle bed operation, thus exposing the dense root clumps to a gas environment. Roots were also pumped as slurry into the reactor after being chopped in a blender, thereby eliminating the manual labor of cutting and inoculation. Using these processes a 14 l reactor was run for 4 weeks and at harvest 20 gDW/l packing density of *H. muticus* was obtained (Ramakrishnan et al. 1994).

Datura stramonium hairy roots were cultured in a 500 l hybrid reactor that used a droplet phase for 40 days after an initial submerged culture for 21 days (Wilson 1997). At the end, 39.8 kgFW biomass was harvested, yielding a packing density of 79.6 gFW/l. *D. stramonium* hairy roots have also been cultivated in 8.2 l root tube bioreactor; good growth and productivities have been recorded (Greens and Thomas 1996).

2.5.1.4 Rotating Drum and Ebb-and-Flow Bioreactors

Cycles of liquid and gas phases are alternated in rotating drum reactors and ebb-and-flow reactors (Kim et al. 2002a). Rotating drum reactors are also mechanically agitated (Mishra and Ranjan 2008). Kondo et al. (1989) reported that as the roots rotated during drum rotation they did not attach well to the vessel wall; the roots were lifted above the medium and then dropped back. As a result of these repeated drops, cells were damaged resulting in low biomass yield. This effect was

overcome by laying polyurethane foam on the inner wall of the drum, which acted as a support for the hairy roots. The authors also demonstrated that hairy roots of *Daucus carota* grew well in turbine blade reactors (0.63 g/l/day) yielding 10 g/l dry mass after 30 days whereas only 4 gDW/l biomass was obtained when the roots were cultured in shake flasks. They concluded that a stirred tank reactor fitted with a turbine blade or an immobilized rotating drum reactor with a high volumetric mass transfer coefficient is more conducive for the culture of hairy roots. Repeated ebbing and flowing or periodic rising and falling of the liquid medium is the main characteristic feature of an ebb-and-flow bioreactor. *H. muticus* hairy roots were also cultivated in a 2.5 l bench top ebb-and-flow reactor for a period of 18 days (Cuello et al. 2003). A productivity of 0.481 gDW/l/day was noted demonstrating a 50-fold scale up compared to cultures in 50 ml shake flasks.

2.5.1.5 Wave Bioreactors

Wave bioreactors are another type of novel reactors. Stress levels are significantly reduced as these reactors are based on wave-induced agitation (Mishra and Ranjan 2008). Palazon et al. (2003) showed that both the accumulation of biomass and production of ginsenosides by *P. ginseng* were higher in a 2 l wave bioreactor than in shake flasks.

2.6 Use of Ri-Transformed Roots in Research and Practical Application

Apart from the production of secondary metabolites, Ri-transformed root cultures have also been used in other research and practical applications like phytoremediation, biotransformation, and plant environment interactions.

Phytoremediation refers to the ability of plants to uptake chemicals from polluted soil, water or air, and thus clean up environmental pollution. Ri-transformed root cultures are excellent tools for phytoremediation. The roots provide a large surface area of contact between the contaminant and the tissue as they grow fast and are highly branched. As mentioned earlier, the roots can be scaled up in bioreactors and the biomass can be used for clean up of the environment. Also, hairy root exudates contain enzymes and metal chelating compounds which can be used to detoxify or sequester harmful complexes (Gujarathi and Linden 2005; Doty 2008), thereby recycling roots for future use. These roots are also potential tools to better understand the enzymatic machinery involved in the bioconversion of toxic pollutants to non-toxic metabolites (Macek et al. 2000) and the mechanism involved in metal tolerance and hyperaccumulation. As shoots are absent, these root cultures help in assessing the mechanisms present only in the roots for remediation of contaminants without the effects of translocation. Also, foreign genes introduced in the hairy roots can be expressed for a long period of time and

the resultant functional proteins can be used to metabolise environmental contaminants (Banerjee et al. 2002). There are several reports indicating the use of Ri-transformed root cultures in the uptake and degradation of various environmental pollutants like phenols, chlorophenols, polychlorinated biphenyls, heavy metals, etc. (Macková et al. 1997; Agostini et al. 2003; Coniglio et al. 2008; Vinterhalter et al. 2008).

Biotransformation is the process whereby a substance is changed from one chemical to another (transformed) by a chemical reaction within the body of a living organism. Plant cell cultures have the ability to specifically convert exogenously administered organic compounds into useful analogues. The main problem with use of cell suspension culture is the phenomenon of somaclonal variation, which might lead to unstable biochemical behavior. This problem can be overcome with the use of plant root and shoot cultures. The reactions involved in biotransformation include oxidation, reduction, esterification, methylation, isomerization, hydroxylation, and glycosylation. Glycosylation only takes place readily in plant cells but laboriously in microorganisms. Ri-transformed root cultures of many plants are reported to be useful in biotransformation. For example, the hairy roots of *Coleus forskohlii* biotransformed methanol to β-D-glucopyranosides, ethanol to β-D-*ribo*-hex-3-ulopyranosides, and 2-propanol to its β-D-glucopyranoside (Li et al. 2003). *B. candida* hairy roots bioconverted hydroquinone into arbutin (Casas et al. 1998). Yan et al. (2007) reported use of Ri-transformed root cultures of *Polygonum multiflorum* for the biotransformation of 4-hydroxybenzen derivatives (1,4-benzenediol, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, and 4-hydroxybenzoic acid) to their corresponding glucosides. The conversion of 4-hydroxybenzoic acid into its α-D-glucopyranoside was for the first time reported in a plant biotransformation system. In addition, these hairy root cultures were able to reduce the 4-hydroxybenzaldehyde to its corresponding alcohol. Thus, biotransformation using Ri-transformed root cultures has got a good prospective to generate novel products or to produce known products more efficiently.

Ri-transformed root cultures have also been used to study plant environment interactions. Isoflavone reductase (IFR) and (+) 6a-hydroxymaackiain 3-*O*-methyltransferase (HMM) are enzymes apparently involved in the synthesis of pisatin, an isoflavonoid phytoalexin synthesized by *Pisum sativum*. Wu and VanEtten (2004) produced transgenic pea hairy root cultures using sense and antisense-oriented cDNAs of *Ifr* and *Hmm* fused to the 35 s CaMV promoter and *A. rhizogenes* R1000. The virulence of *Nectria haematococca* (a pea pathogenic fungus) on the transgenic roots that produced the minimum amount of pisatin was studied in order to assess the effectiveness of pisatin in disease resistance. It was found that control hairy roots were less susceptible to isolates of *N. haematococca* that are either virulent or nonvirulent on wild-type pea plants than hairy roots expressing antisense *Hmm*. It was demonstrated for the first time that transgenic plant tissue with reduced ability to produce phytoalexin can be generated and such tissues are more susceptible to fungal infection. Interactions between roots and rhizobia (Quandt et al. 1993), mycorrhizal fungi (Mugnier 1997) and nematodes (Narayanan et al. 1999) have also been elucidated using hairy roots.

2.7 Conclusion

Since the first reports of establishment of Ri-transformed hairy root cultures, researchers have attempted to utilize these systems for the production of stable transgenic plants, analysis of genes, production of secondary metabolites and engineering of the biosynthetic pathways, production of therapeutically recombinant proteins, trapping of biomolecules released into the culture medium, for elucidating molecular aspects of biological processes, etc. Compared to plant cell suspension cultures, hairy root cultures appear to be potential systems for continuous production of valuable secondary metabolites because of their fast growth rates, ease of maintenance, genetic and biosynthetic stability, and ability to synthesize a vast array of compounds. Till date hairy root cultures have been established from several plant species, many of which are endangered and pharmaceutically important. Although these cultures have shown tremendous potential for the production of several important phytochemicals, their culture in large scale is still very challenging. As the culture environments in bioreactors and shake flasks are totally different, the results obtained from the studies on improvement in yields from shake flasks may not be directly applicable to bioreactors. Development of more effective and economic scale-up culture systems is required so that bioreactors can be used successfully for the large-scale production of secondary metabolites. Plants regenerated from hairy roots following transformation with *A. rhizogenes* have got a lot of use in different fields. Higher levels of some target metabolite production in transformed plants are very important as a tool for improvement of secondary metabolite production in medicinal plants. Apart from pharmaceutical use, other importance of hairy root regenerants include, use of transgenic plants in micropropagation of plants that are difficult to multiply for example in *A. majus* (Hoshino and Mii 1998), in Mexican lime (Pérez-Molphe-Balch and Ochoa-Alejo 1998), in cherry (Gutièrrez-Pesce et al**.** 1998), etc. Morphological characters such as adventitious shoot formation, reduced apical dominance, altered leaf and flower morphology have ornamental value in some plants while proved effective in plant breeding programmes in some others (Giovanni et al. 1997; Pellegrineschi et al. 1994; Handa et al. 1995). The promising developments and applications of hairy root cultures indicate that in the near future these cultures will provide researchers with powerful tools for further biotechnological research.

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