Chapter 7
Bioengineering Hairy Roots: Phytoremediation, Secondary Metabolism, Molecular Pharming, Plant-Plant Interactions and Biofuels

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Abstract Hairy root cultures are an efficient tool to understand plant biology, biotechnology and other applied sciences. In particular such cultures have revealed many cues of plant cells related to growth, development, physiological and biochemical processes. Thus, hairy roots are used to study secondary metabolism and

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production of bioactive compounds such as flavonoids. Transgenic roots are used as biofoactories to produce heterologous recombinant proteins for pharmaceutical purposes. Furthermore, they have shown promising applications in phytoremediation and restoring the environment because of advantages in the reduction of toxic organic and inorganic pollutants from soil, air, wastewater, groundwater and biowaste. This review focuses on the recent progress of bioengineering hairy root culture systems.

Keywords *Agrobacterium rhizogenes* • Biofuels • Plant molecular pharming • Phytoremediation • Recombinant proteins • Secondary metabolites • Transgenic roots

7.1 Introduction

Hairy root cultures are obtained by the transfer of genetic information to a host plant from a gram-negative soil bacterium, *Agrobacterium rhizogenes*. When various parts of plants, such as the leaf, shoot and root are wounded, they excrete simple phenolic substances, such as acetosyringone; these substances induce the virulence (vir) genes which responsible for transferring the T-DNA fragments of Ri-plasmid of *A. rhizogenes* to the plant cells (Doran 2009).This natural process leads to the development of hairy roots at the site of infection. The phenotype of these hairy roots is characterized by the fast and hormone-independent growth, a lack of geotropism, lateral branching and genetic stability (Makhzoum et al. 2013; Shanks and Morgan 1999), so they have been considered as possible biological matrices for producing valuable metabolites (Georgiev et al. 2012). Although the plant cell culture system is extensively used for the production of valuable metabolites due to low cost, ease of management, presence of post-translational modification machinery, but their genetic instability makes them less suitable for stable and long-term cultures (Yesil-Celiktas et al. 2010). In this context, the hairy root cultures presenting added benefits, such as, genetic and biochemical stability, fast growth rates, and growth on hormone free medium. (Habibi et al. 2016; Streatfield 2006).

A remarkable advantage of hairy roots is that their cultures often show approximately the same or greater biosynthetic possibilities for producing secondary metabolites compared to their mother plants (Al-Shalabi et al. 2014; Kim et al. 2002). Over the last 30 years hairy roots have been used for a variety of purposes, ranging from metabolic engineering and recombinant protein production to analyses of phytoremediation, plant–plant interaction and in the near future, they might be considered as potential system for biofuel production (Praveen et al. 2014; Runo et al. 2011; Talano et al. 2010; Wilson and Roberts 2012) (Fig. 7.1). Moreover, hairy root system could allow modifications for the production of phyto-molecules that cannot realistically be generated by chemical semi-synthesis. More recently, hairy root technology has been established as a biotechnological concept in a majority of

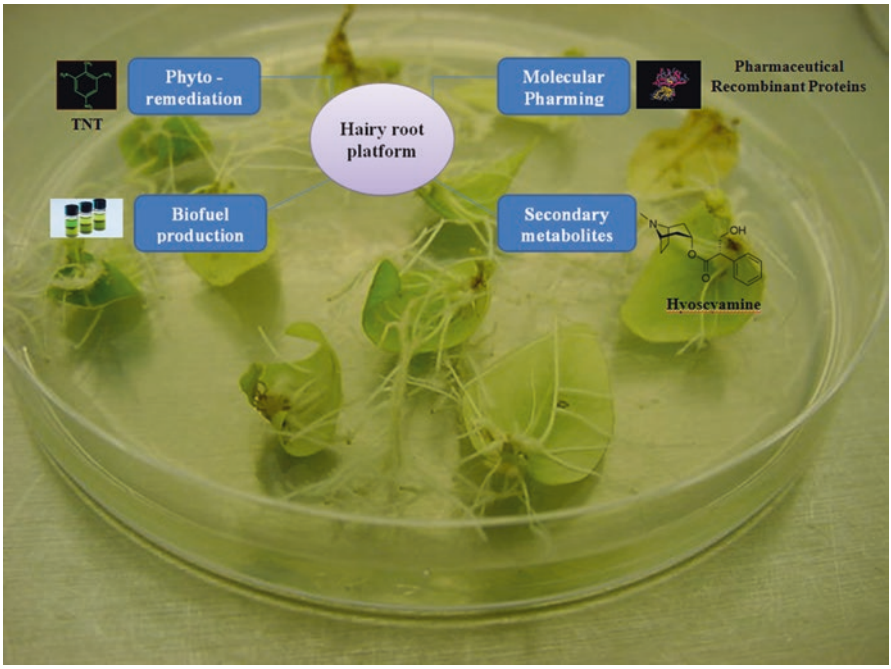

Fig. 7.1 Different applications of hairy root culture system

plant species to exploit novel biological insights. A wide range of chemical compounds have been synthesized using this technology (Giri and Narasu 2000).

Considering the importance of the subject, we highlight the fundamental characteristics of hairy roots that make them amenable to be applied as tools for production of secondary metabolites, molecular farming as well as potential usefulness in phytoremediation and biofuel as well as plant composite production.

7.2 Hairy Roots and Phytoremediation

Phytoremediation is known as an eco-friendly approach for remediation of contaminated soil and water using plant systems. Plants are autotrophic systems, exploiting sunlight and carbon dioxide as a source of energy and carbon. Beside the role of plant roots to take up nitrogen and minerals as well as water from soil and groundwater, they also soak up a diversity of natural and man-made toxic compounds for which they have developed various detoxification mechanisms (Eapen et al. 2007; Van Aken 2008). The process of phytoremediation encompasses a range of detoxification phases. Including; such as phase I, including transformation, including oxidation, reduction, and hydrolysis,and catalyzed catalysis by enzymes such as P450 monooxygenases, peroxidases, reductases, dehydrogenases and esterases.

While Second phase (II) and phase III comprise, initiated with the conjugation of contaminants by with endogenous compounds such as mono-, oligo-, and polysaccharides, lignin, organic acids, peptides, proteins, amino acids), for better solubility, and, to being more soluble. Compartmentation involving transport and storage in the vacuole and then bound to cell wall or being excreted Finally, in the third phase (III) compartmentation will be occurred. In this phase, transportation and accumulation of soluble contaminations will be done into vacuole organelle or they can bound to cell wall and diminished (Abhilash et al. 2009; Schröder et al. 2007).

A successful microbe- based phytoremediation system must meet various conditions including the ability of microbes with the desired metabolic activity to survive in environment containing the bioavailable chemical as well as the presence of inducers to activate the expression of necessary enzymes. Therefore, these requirements make this system inappropriate and costly. In terms of bioremediation, plant cell cultures act as an alternative system to be employed for phytoremediation, to abolish or decrease the concentration of toxic organic as well as inorganic pollutants in soil, air, wastewater, groundwater and biowaste. However, unlike bacteria and mammals, plant-based systems involved in phytoremediation research usually impoverished in catabolic enzymes necessary to accomplish full metabolism of recalcitrant organic compounds which potentially resulting in slow and incomplete treatment performance (Eapen et al. 2007). Therefore, incomplete transformation of contaminants in plants causes the release of toxic compounds from plant tissues which potentially arise the presence of contaminations on the food chain (Yoon et al. 2006). Inherent impedition of plants for the metabolism of recalcitrant xenobiotic compounds emerged the idea of modifying plants genetically by the expression of eukaryotic genes involved in the elimination of toxic chemicals through *Agrobacterium*. In this area, the hairy root technology has been utilized as effective tool to finalized transformation of contaminants by expression of involved genes in treatment of contaminants.

Use of genetically engineered systems with enhanced degradative capabilities such as hairy root cultures, phytoremediation is turning out to be the most promising, cost-effective and resource-conserving approach for environment remediation (Wevar Oller et al. 2005). Hairy root cultures have been considered as a superior model system to investigate phytoremediation processes as rhizofiltration, phytostabilization and phytoextraction of organic and inorganic pollutants because of their biochemical and genetic stability (Majumder and Jha 2012) and easy maintenance (de Araujo et al. 2002; Harms et al. 2004). Physiologically, they follow a prolific root growth template like a real root which is prerequisite to increase the effects of phytoremediation processes, therefore, provide a reliable and stable biomass over the whole year without environmental effects (Doran 2009). Recently, hairy roots have contributed to our knowledge of the complex biochemical and molecular mechanisms involved in the phytoremediation of organic and inorganic pollutants (Agostini et al. 2013). Several reports have been published that HRs derived from different plant species could be used for the treatment of a great variety of organic contaminants. Table 7.1 shows some of the examples of hairy roots used to remove contaminants.

Table 7.1 Some selected examples of plant hairy roots used to phytodegrade pollutants

Hairy roots obtained from plant species	Compound	References
Alyssum bertoloni	Cadmium	Boominathan and Doran (2003a)
Thlaspi caerulescens	Nickel	Boominathan and Doran (2003b)
Brassica napus	2,4-Dichlorophenol	Agostini et al. (2003)
Brassica juncea	Dichlorodiphenyltrichloroethane (DDT)	Suresh et al. (2005)
Cichorium intybus		
Armoracia rusticana	N-acetyl-4-aminophenol	Huber et al. (2009)
Brassica juncea	Phenol	Singh et al. (2006)
Raphanus sativus		
Azadirachta indica		
Beta vulgaris		
Daucus carota	Guaiacol, catechol, phenol, 2-chlorophenol, and 2,6-dichlorophenol	Araujo et al. (2002) and Araujo et al. (2006)
Ipomoea batatas		
Solanum aviculare		
M. aquaticum	TNT	Hughes et al. (1997)
M. spicatum		
Catharanthus roseus		
Catharanthus roseus	TNT	Bhadra et al. (1999)
Nicotiana tabacum	2,4-Dichlorophenol	Angelini et al. (2014)
Atropa belladonna	Trichloroethylene	Banerjee et al. (2002)
Brassica juncea	Uranium	Eapen et al. (2003)
Chenopodium amaranticolor		
B. juncea	Dichloro-diphenyl-trichloroethane	Suresh et al. (2005)
Cichorium intybus		
Helianthus annuus	Tetracycline and oxytetracycline	Gujarathi et al. (2005)
Lycopersiconesculentum	Phenol	González et al. (2006) and Wevar Olle et al. (2005)
Alyssum murale	Nickel	Vinterhalter et al. (2008)
Rubia tinctorum	Copper	Maitani et al. (1996)
Armoracia lapathifolia	Phenol	Flocco and Giulietti (1998)
Solanum nigrum	Polychlorinated biphenyls	Mackova et al. (1997)

7.2.1 Phytoremediation of Phenolic Compounds

Human activities produce large amounts of chlorinated phenolic chemicals that are often released into soil and water environments during pesticide and insecticide applications, industrial releases and accidental spills. In addition, these chemicals utilized as antiseptics, disinfectants and wood preservatives. In fact, the toxicity of chlorophenols has been shown both *in vitro* and *in vivo*. Phenols as aromatic compounds, are included in the major classes of hazardous pollutants, because of their carcinogenicity, recalcitrance to degradation and high toxicity and presence in industrial wastewaters (Miland et al. 1996). One of the hazardous phenolic compounds, which has been listed in the Agency of Toxic Substances and Disease Registry, is 2,4-dichlorophenol. In particular, 2,4-dichlorophenol has been considered to cause lethargy, tremors and convulsions in mice (Borzelleca et al. 1985). 2,4-dichlorophenol is highly toxic because it is readily captured by the skin, resulted in poisoning (causing hepatic and renal failure, pulmonary edema, hemolytic anemia), or teratogenic and carcinogenic effects (Petroutsos et al. 2008). A new report about whether the hairy root cultures of tobacco are useful and efficient for the removal of halogenated derivative, 2,4-dichlorophenol has been published by (Talano et al. 2010). This study has shown remarkable efficiency of tobacco hairy roots to eliminate high concentrations of 2,4-dichlorophenol by presence of the lignin-type products which formed during 2,4-dichlorophenol transformation and its compartmentalization in hairy root cell walls. Moreover, changes in the deposition pattern of lignin in hairy roots exposed to 2,4-dichlorophenol suggested that these intermediate products would deposit in the cell walls of the xylem and phloem elements, resulting in lignin-type polymers (Talano et al. 2010). Similarly, it has been reported that p-coumaric acid, caffeic acid and ferulic acid, which are natural phenolic compounds from plant cells, were metabolized by the *Brassica napus* hairy roots and converted into lignin/suberin polymers in the xylem cell walls (Sorroche 2006). These findings contribute to the better understanding of metabolic pathway involved in phytoremediation of phenol and lead and efficiency of hairy roots as an inexpensive system for removing contaminants from various environments. Moreover, this capacity and efficiency can be boosted through the incorporation of foreign genes into hairy root genome and consequently the rhizodegradation of highly recalcitrant compounds (Van Aken 2008; Van Aken and Doty 2009).

The search for new insights to enhance the removal process as well as protection of involved enzymes would reduce the costs and improve the process at an industrial scale. In this context, hairy root approaches based on peroxidase overexpression would be useful to improve the removal efficiency of phenolic compounds and reduction of cost. For that reason, a basic peroxidase (TPX1 and TPX2) from tomato was overexpressed in transgenic tomato hairy root to monitor its probability in phenol removal (Angelini et al. 2014; Sosa Alderete et al. 2009). The findings suggested that secreted peroxidases by the hairy root system can polymerize phenolic free radicals in the cell wall. Moreover, investigation of polymerization of phenolic compounds in different hairy root cultures showed that enzymes involved in metabolisim of phenol and chlorophenol free radicals could behave in similar manner as

in plant species which normally do not naturally express isoenzymes (Singh et al. 2006; Sosa Alderete et al. 2009; Wevar Oller et al. 2005). Also, compared with whole transgenic organisms, which have ethic barrier concerns and bioremediation barrier in using of enzymes derived from transgenic organisms, hairy root cultures are characterized by high efficiency and selectivity and also are significantly more environmentally friendly (Abhilash et al. 2009; Sutherland et al. 2004).

The phytoremediation of explosive compounds which led to degradation of great environmental risks is another area of remarkable interest. The large-scale manufacture and exploitation of explosives has brought a high level of environmental pollution by the compounds that comprise explosives (Table 7.1) (Adamia et al. 2006; Pennington and Brannon 2002; Rylott and Bruce 2008). Moreover, the adverse impact of explosives on living organisms, their environmental persistence and low susceptibility to biodegradation has changed into critical concern, hence various methods from physical to chemical have been employed for remediation of these compounds (Pennington and Brannon 2002). Unfortunately, the currently practiced methods are usually expensive, ineffective, time-consuming and cause additional environmental problems, such as NO_2 emissions, recalcitrant and toxic by-products (Rodgers and Bunce 2001). Therefore, in recent decades, the most popular alternative phytoremediation has gained more attention to remove these lethal compounds from soil and water.

7.2.2 Phytoremediation of Explosive Compounds

Two natural model systems including the aquatic species *Myriophyllum aquaticum* (Parrot feather) and hairy root culture of terrestrial species *Catharanthus roseus* (Vinca) have been considered to remove Cyclonite and Octogen (Bhadra et al. 2001). The aqueous levels of Octogen and Cyclonite were dramatically decreased by hairy roots of *C .roseus* as compared to controls. In contrast with *M. aquaticum*, hairy roots of *C. roseus* exhibited greater capacity to eliminate Octogen and Cyclonite from the aqueous medium, which approved the deficiency of explosive compounds from aquatic medium by hairy root cultures. Recently, phytoremediation of Trinitrotoluene by plant systems has gained attention to study the potential of plants to transform this compound. In this regard, knowledge of plant metabolism of nitroaromatic compounds is one of the significant parts for natural attenuation processes and phytoremediation applications. Hence, hairy root cultures have contributed to the understanding of transformation pathways of Trinitrotoluene. Based on the structure of Trinitrotoluene, two initial transformation processes occur; (1) reduction of one or more nitro groups, yielding hydrixylamino or amino groups, and (2) oxidation of either the methyl group or directly the ring. In this context, hairy roots act like a green liver model to provide the complementary information about the conjugation of Trinitrotoluene monoamine derivatives during plant metabolism of Trinitrotoluene and allows us to determine the contribution processes between plants and microbes during Trinitrotoluene disappearance (Bhadra et al. 1999; Wayment et al. 1999).

Hughes et al. (1997) suggested hairy root cultures of different plants when exposed to uniformly labeled ^{14}C-Trinitrotoluene completely transported Trinitrotoluene to an unidentifiable intracellular extractable fraction, which was subsequently converted to an intracellular bound residue. Similar results have been published by (Agostini et al. 2013) and showed the potential of hairy root cultures to metabolize Trinitrotoluene without effects of microbial or symbiotic relationships.

With the advancement in gene (s)/pathway identification, hairy root cultures addressed the Trinitrotoluene transformation pathway and identified specific enzymes which are responsible for oxidative transformations. Cytochrome P-450 is one of the most plausible enzyme candidates for the oxidative metabolism of Trinitrotoluene. (Banerjee et al. 2002) showed that hairy root cultures of *Atropa belladonna* are capable to produce cytochrome P-450 and provide basic mechanisms involved in Trinitrotoluene degradation by this enzyme. These findings not only open up the possibility of new genetic and biochemical approaches to study Trinitrotoluene transformation pathways, they also provide good information in the toxicity concept of the final products and their effects on the ecosystem. In the sense of toxicity, most of the Trinitrotoluene studies with hairy root cultures have been performed on stationary phase cultures (cultures that are metabolically active but not undergoing growth) (Lauritzen 1998). These achievements in hairy root cultures are particularly interesting because Trinitrotoluene oxidation has rarely been observed in microbial studies and little is known about their long-term fate in aquatic environments.

7.2.3 Phytoremediation of Xenobiotics Compounds

Dichlorodiphenyltrichloroethane (1,1,1-trichloro-2,2-bis-(4′-chlorophenyl ethane), is one of the most persistent xenobiotics because of its cost effectiveness, broad-spectrum, high residual biological activity, and ease of formulation (Twyman et al. 2003). Nearly 14 countries have used dichlorodiphenyltrichloroethane for disease control while several others are reintroducing this compound (Van den Berg 2011). Unfortunately, systematic uses of dichlorodiphenyltrichloroethane have increased the risk of cancer and endocrine disruption; hence phytoremediation of this xenobiotic has gained attention to environmental clean-up of dichlorodiphenyltrichloroethane. Since bioremediation of dichlorodiphenyltrichloroethane by bacteria, fungi and whole plants was not shown to be cost-effective, hairy root cultures have been used for the biotransformation of this xenobiotic because of their fast growth, high metabolic activity, and genetic and biochemical stability. In this context, hairy root cultures of *Cichorium intybus* and *Brassica juncea* have been reported for uptake and degradation of dichlorodiphenyltrichloroethane (Suresh et al. 2005). The general degradative pathway of dichlorodiphenyltrichloroethane involves reductive dechlorination to dichlorodiphenyltrichloroethane and dihydrochlorination to dichlorodiphenyltrichloroethane. Dichlorodiphenyltrichloroethane was shown to be

further degraded to 1-chloro-4-[2-chloro-1-(4-chlorophenyl)ethenyl]benzene by a dechlorination reaction. The high metabolic activity of hairy root cultures during the initial growth phase leads to a higher uptake during the exponential phase. The results suggested that adsorption of dichlorodiphenyltrichloroethane by hairy roots can be assigned to a physical process rather than a biological process. Similar observation of rapid uptake of dichlorodiphenyltrichloroethane has been reported in case of cell suspension cultures of parsley and soybean (Scheel and Sandermann 1977) and in axenic cultures of aquatic plants such as *duckweed* and *Elodea* (Gao et al. 2000).

7.2.4 Phytoremediation of Dyes

Almost 10,000 different textile dyes such as reactive, disperse, basic, etc. are used for coloring and printing purposes by processing industries. As the fixation rate of textile dyes is not 100 % thus, 10–15 % of dyes used in the textile processing industry are released into the environment as wastewater and lead to carcinogenic and mutagenic effects to living organisms(Mathur et al. 2005; Sharma et al. 2009). Use of various methods including; reverse osmosis, chemical oxidation, coagulation-flocculation, filtration, adsorption, photodegradation and advanced oxidation have been reported for dye decolorization and degradation but high cost, generation of hazardous by products and high energy requirement, have limited the use of these methods (Bizani et al. 2006; Crini 2006; Joo et al. 2007; Yun et al. 2006). In this context, the use of biological methods specially hairy root cultures has been more interested, since they are considered to be sustainable, eco-friendly and provide valuable documents for enzymatic reactions and the metabolism products involved in dyes (Jadhav et al. 2009). In this area, laccases (laccase, lignin peroxidase, tyrosinase, and NADH–DCIP reductase) as enzymes of multicopper oxidase family (Ghodake et al. 2009; Kagalkar et al. 2010; Patil et al. 2009) are founded by hairy root culture of *B. juncea* and are able to decolorize textile dyes through oxidation of substituted phenolic and non-phenolic compounds in the presence of oxygen as an electron acceptor (Telke et al. 2011). It has been reported that *B. juncea* hairy root cultures increase the decoloization of Methyl orange to 92 % within 4 days of incubation period. The enzyme assay of HRC obtained after the decolorization of Methyl orange indicated considerable intracellular laccase activity. Similar work by (Patil et al. 2009) showed that the hairy roots of *Tagetes patula* L. decolorized 71 % of Methyl orange (30 mg L-1) within 10 days of incubation period, which confirm the capacity of hairy root culture to produce intracellular laccase and degraded Methyl orange. Since phenolic compounds interferes with laccase enzymes purification and causes high cost of the purification process, hairy root cultures provide hassle-free sources for laccases purification and have shown to be cost-effective for purification process. However, a very few reports on down streaming of these enzymes using hairy roots are available and further work on the purification of the final products and their effects on the pollutants is needed.

7.2.5 *Phytoremediation of Heavy Metals*

Heavy metals are particularly serious environmental contaminants, as the majority of Superfund sites are highly polluted with heavy metals (Peters 1999). Standard remediation methods such as soil excavation followed by coagulation-filtration or ion exchange are too expensive and disruptive methods to the environment. Bioremediation techniques as catabolic reactions which mediated by microbes and its enzymes are the most economical and ecofriendly application for organic and inorganic decontamination. In this context, rhizoremediation is an excellent form of bioremediation that pursuit to harness light energy through plants to biostimulate pollutant degradation by the indigenous soil microbial community (Glick 2010). In rhizoremediation, plant growth-promoting bacteria increase the growth induction of host plants through different mechanisms, including phytohormone production and mineral solubilization. For successful bioremediation of metal contaminated soils, understanding of mechanisms involved in metal uptake, accumulation, and tolerance associated with phytoextraction and metal hyperaccumulation held a great importance. Hairy root cultures provided a model system to monitor the physiology and biochemistry of metal accumulation in plants (Doran 2011). In this sense, hairy root cultures of *B.napus* were studied for uptake, distribution, and hyperaccumulation mechanisms of phenol and chromium (Cr) (Gonzalez et al. 2013; Ontanon et al. 2014). It has been shown that the association between *B. napus* hairy roots and phenol-degrading rhizospheric microorganisms improve the removal process of heavy metals. Probably, these microorganisms obtain nutrients (such as amino acids, enzymes, organic acids, and complex carbohydrates) excreted from roots. In return, the microorganisms convert nutrients into available forms of minerals for plants. Furthermore, the root tips provide a steady-state redox condition and a structural surface for bacterial colonization. Research on different plant species have been carried out in order to study the physiological mechanisms of exogenous metal on the toxicity of cadmium to hairy roots. In all these reports, hairy roots showed a good ability to absorb heavy metal cadmium and remove the toxicity of this compound (Shi et al. 2012; Subroto et al. 2007; Vinterhalter et al. 2008). Investigation on cytogenetic toxicity of cadmium to hairy roots is a prerequisite for using hairy roots with rapid growth or their regenerated plants with well-developed root system to bioremediate cadmium -contaminated environment as well to evaluate and monitor its cytotoxicity, and bioremediate cadmium -contaminated environments. Therefore, hairy root cultures were investigated as a perfect tool for monitoring and evaluation of cadmium pollution in the environment. Additionally, these hairy root cultures can be used as a model system for generating a metal-enriched product from the harvested plant biomass. This procedure might be useful for processing metal-enriched plant material harvested from phytomining operations (Boominathan et al. 2004).

7.2.6 *Phytoremediation of Polychorinated Biphenyls*

Polychlorinated biphenyls comprise a large family of 209 possible congeners that differ in their chlorination level, chemical and physical properties. Therefore, these compounds have been used in various industrial applications such as coolants in electric transformers, hydraulic fluids, fire retardants or components in plastic production processes. They are produced as commercial mixtures that differ in congener composition (e.g., Aroclor, Clophen, Delor and Kanechlor). Polychlorinated biphenyls have been banned because of their toxicity, carcinogenicity, mutagenicity, teratogenicity. Regardless, these chemicals are still causing a great environmental concern because of their properties, which make them recalcitrant compounds accumulating in the environment. Although polychlorinated biphenyls can enter the food chains and accumulate in fat tissues, they mostly affect the final consumers, including humans (Derocher et al. 2003; Petrik et al. 2006; Skaare et al. 2002). Therefore, a great environmental concern is to reduce the effects of polychlorinated biphenyls. Bioremediation techniques are becoming promising system for the removal of polychlorinated biphenyls from contaminated soils because of high cost and public opposition of classical physical methods. Developing phytoremediation techniques requires an understanding of where polychlorinated biphenyls accumulate in plants. In this context, phytoremediation using hairy root cultures hold an important attention to polychlorinated biphenyl remediation. Although cellular localization, biochemical pathways and potential toxicity of this derived compound have not been explained but they can be figured out as an another group of metabolites formed from polychlorinated biphenyls in plant tissues. In this context, hairy roots can provide a profound knowledge about metabolism of these compounds and help to improve phytoremediation of these pollutants. The metabolism of polychlorinated biphenyls was investigated in *in vitro* tissue cultures of approximately 40 different plant species, using the commercial mixture Delor 103, consisting of 59 polychlorinated biphenyl congeners with an average of three chlorine atoms per biphenyl, as the model pollutant (Aken et al. 2009; Mackova et al. 1997). Hairy root cultures of *Solanum nigrum* were capable of metabolize almost all of the 22 individual polychlorinated biphenyl congeners examined and provided a good knowledge about transformation pathway of enzymes involved in polychlorinated biphenyls remediation. In similar works, hairy root cultures of the same species (*Solanum nigrum*) were exposed to several di, tri, tetra, and penta-chlorinated polychlorinated biphenyl congeners which show metabolization of all metabolites by hairy roots (Harms et al. 2004; Kucerova et al. 2000; Rezek et al. 2007), suggesting that, plant metabolism of polychlorinated biphenyls differs according to the plant species, substitution pattern as well as degree of chlorination. In fact, oxidation of the biphenyl core represents one of the first steps in the polychlorinated biphenyls plant metabolism although this step can be slowed down by the presence of electron-withdrawing chlorine atoms. More recently, Rezek et al. (2012) recommended that the transformation of polychlorinated biphenyls in these hairy roots resulted not only in production of hydroxy- polychlorinated biphenyls, but also in methoxy and

hydroxylmethoxy- polychlorinated biphenyls. Thus, the methoxy- polychlorinated biphenyls should be clarified as another group of metabolites synthesized from polychlorinated biphenyls in plant tissues. However, cellular localization, biochemical pathways and potential toxicity are important issues to be addressed because little knowledge exists on the impact of these compounds which brings new challenges in the investigation of plant metabolism of these pollutants.

7.3 Hairy Roots: Secondary Metabolites Production

Secondary metabolites have an important role in adaptation of plants to their environment and also exhibit an important source of pharmaceuticals (Malik et al. 2014b; Malik et al. 2014a; Malik et al. 2011; Rao and Ravishankar 2002). Plant cell culture system has emerged as a possible tool for valuable secondary metabolites production, however, in most of the cases, production of phytochemicals is under strict metabolic regulation and require tissue specific localization (Makhzoum et al. 2015; Makhzoum et al. 2011). So the instability and no uniformity associated with the undifferentiated cell suspensions cultures have not brought tremendous achievement in successful commercialization of bioactive compounds from plants except only a few examples e.g. shikonin (Malik et al. 2014b). On the other hand, differentiated cultures have received a great deal of attention due to stable and remarkably higher production of secondary compounds as compared to cell suspension cultures or mother plant. In Table 7.2 there are some illustrated data on successful efforts to produce some of these valuable pharmaceuticals in relatively large quantities using hairy root cultures.

Although the progress in optimization of upstream and downstream processes have tentatively led to the high production of secondary metabolites, substantial gaps still present in our understanding of how these secondary metabolites are produced in plants at both the gene and the pathway levels, because many plants that produce secondary metabolites of interest are either recalcitrant to transformation or have long life and reproductive cycles (Ono and Tian 2011). Hence, with the advent of hairy root platform from many plant species, it has become possible to functionally characterize and manipulate the genes for biochemical and secondary metabolism related studies. Moreover, hairy roots can be used as a promising tool to envisage new metabolic engineering strategies for the improvement of secondary metabolites production and to design new metabolic pathways to induce pharmaceutically important molecules (Wilson and Roberts 2012). Also *A. rhizogenes*-mediated transformation system has enhanced our knowledge in understanding the biosynthetic pathways and key genes and enzymes involved. Moreover, overexpression of single or multiple genes, RNA interference and stable isotope approaches can be employed on hairy root to explicate specific reactions within a biosynthetic pathway. For instance, the explication of an enzyme that involve in pyridine alkaloid biosynthesis in *Nicotiana glauca* hairy root (Deboer et al. 2009; Kajikawa et al. 2009) and the biosynthesis of camptothecin from the 2C-methyl-d-erythritol

Table 7.2 Secondary metabolites produced using hairy root culture

Plant	Secondary metabolites	Content	References
Abrus precatoroius	Glycyrrhizin	71.35 mg/d d.w.	Dixit and Vaidya (2010)
Aconitum heterophyllum	Aconite	29.6 mg/d d.w.	Giri et al. (1997)
Artemisia annua	Artemisinin	13.3 mg/l	Zhang et al. (2013)
Atropa belladonna	Atropine	3.7 mg/d d.w.	Kamada et al. (1986)
	Cuscohygrine	2.8 mg/d d.w.	Jung and Tepfer (1987)
	l-Hyosyamine	9.5 mg/d d.w.	Hashimoto et al. (1993)
	Scopolamine	3.0 mg/d d.w.	Jung and Tepfer (1987)
	Atropine	7.6 mg/d d.w.	Lee et al. (1999)
	Scopolamine	0.3 mg/d d.w.	Lee et al. (1999)
	Littorine	0.9 mg/d d.w.	Nakanishi et al. (1998)
Catharanthus roseus	Ajmalicine	4 mg/d d.w.	Bhadra et al. (1993)
	Catharanthine	2 mg/d d.w.	Bhadra et al. (1993)
	Serpentine	2 mg/d d.w.	Bhadra et al. (1993)
	Vindoline	4 mg/d d.w.	Geerlings et al. (1999)
	Vinblastine	0.003 mg/d d.w.	
Centranthus ruber DC	Valepotriates	31 mg/g d.w	Gränicher et al. (1995)
Cichorium intybus	Hydroxycinnamate	7.00 ± 0.65 % d.w	(Malarz et al. 2013)
	Sesquiterpene lactone (3 8-deoxylactucin glucoside)	1.371± 0.321 %d.w	
Cinchona ledgerino	Cinchonine	1.6 µg/g f.w.	Hamill et al. (1989)
	Cinchonidine	18 µg/g f.w.	
	Quinidine	15 µg/g f.w.	
	Quinine	24 µg/g f.w.	
Glycyrrhiza uralensis	Glycyrrhizin	–	Zhou et al. (2008)
Helicteres isora	Diosgenin	1034 µg g^{-1} FW	Kumar et al. (2014)
	Prolin	28.4 µmol g^{-1} FW	
Liunm mucronatum	Podophyllotoxin	5.78 mg/g d.w.	Samadi et al. (2014)
	6-methoxy podophyllotoxin	49.19 mg/g d.w	
Mitragyna speciosa	Mitragynine	14.25 mg/g d.w	Phongprueksapattana et al. (2008)
	Ursolic Acid	3.47 mg/g d.w	
Nicotiana rustic	Nicotine	0.9 mg/g f.w.	Robins et al. (1987)
	Anatabine	0.4 mg/g f.w.	
Panax ginseng	Ginsenoside	145.6 mg/l^{-1}	Palazón et al. (2003)
Physalis minima	Solasodine glycoside	900 mg/ d.w.	Putalun et al. (2004)
Platycodon grandiflorum	Chlorogenic acid	21.31 µg/100 mg d.w	Tuan et al. (2014)
Polygonum multiflorum	Emodin	211.32 µg/g d.w	Thiruvengadam et al. (2014)
	Physcion	353.23 µg/g d.w	

(continued)

Table 7.2 (continued)

Plant	Secondary metabolites	Content	References
Portulaca oleracea	Dopamin	1.2 mg/g d.w.	Ahmadi Moghadam et al. (2013)
Salvia officinalis	Rosmarinic acid	45 mg/ g d.w.	Grzegorczyk et al. (2006)
Scutellaria baicalensis	baicalin, baicalein, wogonin		Park et al. (2011)
Silybum marianum	Silymarin	0.18 mg/g d.w.	Hasanloo et al. (2013)
Solanum lycopersicon	Vitamin C		Wevar Oller et al. (2005)
Solanum Khasianum	Solanoside		Putalun et al. (2004) Putalun et al. (2003)
Withania somnifera	Withanolide A.	57.9 µg/g d.w.	Murthy et al. (2008)
Rhodiola crenulata	Salidroside	8.58 mg g^{-1} d.w	Lan et al. (2013)

4-phosphate and shikimate pathways using hairy roots of *Ophiorrhiza pumila* (Yamazaki et al. 2004) are some of the interesting proofs of this concept. Several recent reports highlight the important contribution of hairy root cultures to identify biosynthetic and regulatory genes as well as transporters such as tyrosine decarboxylase catalyzes decarboxylation (TYDC) of tyrosine to generate tyramine as the precursor for salidroside (Gyorgy et al. 2009). (Lan et al. 2013) have studied the metabolic characterization of the TYDC gene to engineer the salidroside pathway from hairy roots of *Rhodiola crenulata*. Their results showed that RcTYDC-overexpressed hairy root lines operate at an interface between primary and secondary metabolism, with suggestive role as key factors in the control of end-product biosynthesis. A number of studies have also determined the functions and targets of candidate transcription factors using hairy root culture as a heterologous system. For instance, octadecanoid-responsive Catharanthus AP2/ERF-domain (ORCA) is transcription factor that regulates the coordinate expression of the terpene indole alkaloids biosynthesis genes (Memelink et al. 2001) MYB1 is another transcription factor which is responsible to control the six genes involved in anthocyanin biosynthesis in transformed root of *purple-fleshed sweet potato* (Mano et al. 2007). Overexpression of VvMYBA1-2 gene in grapevine hairy roots suggested its role in the regulation of genes of later biosynthesis steps and transporters of anthocyanins (Cutanda-Perez et al. 2009). Gene activation by VvMybPA1 and VvMybPA2 in grapevine hairy roots demonstrated both expected and novel genes as key players in the proanthocyanidin biosynthetic pathway (Terrier et al. 2009).

Although cloning and characterization of biosynthetic enzymes involved in secondary metabolic pathways have been performed, regulation of their gene expression is still poorly understood. Therefore, monitoring the expression of genes in response to different stimuli in a given hairy root culture can boost up our knowledge of gene function and its regulation mechanisms. For instance, gene regulation

of fructan synthesis independent of source tissues in response to external cues, including nutritional status and cold exposure using hairy root cultures induced from *chicory* (*Cichorium intybus*) as a model system (Kusch et al. 2009) and identification of dadienyl/copalyl diphosphate synthase (SmCPS) and miltiradiene synthase (SmKSL) as functional genes involved in consecutive tanshinone biosynthesis in respond to biotic and abiotic factors using hairy root culture of *Salvia miltiorrhiza Bunge* (Gao et al. 2009) have been developed. Therefore, understanding of the correlation between the molecular -cellular state of hairy roots and its potential towards growth and production of the biomolecule of interest is a significant key for improving secondary metabolites reproducibility. Such a structured model is then able to improve our understanding of molecular and cellular behavior, in identifying possible regulatory roles (Wiechert 2002), as well as being a tool for determining adequate controlled culture conditions.

Applications for activation tagging in hairy root cultures for dissecting plant secondary metabolism are another interesting aspect of this technology. The ability to identify and generate novel genes involved in a variety of biological pathways through random insertion of T-DNA containing constitutive enhancer elements to the proximity of affected gene is called activation tagging. Since activation tagging creates gain-of-function mutations, it is not necessary to generate a large collection of fertile transgenic plant lines. Instead, transformed explants or cultured cells can be used for desired phenotypes (Ono and Tian 2011; Seki et al. 2005). This new biotechnological method of mutagenesis has boosted understanding of development in various plants by increasing the range of gene expression and secondary metabolite accumulation. This technique allows Agrobacterium to introduce a viral CaMV35S enhancer into the genome. This enhancer dominantly "activates" and/or intensify the pattern of gene expression near to enhancer (Hsing et al. 2007). For example, the CaMV35S enhancer increases the expression of genes situated upstream or downstream of T-DNA in *Arabidopsis* and rice (Ichikawa et al. 2003; Jeong et al. 2006). An enhancer sequence inserted in the vicinity of an endogenous gene can modify the transcriptional pattern of the gene, and lead to the formation of a mutant phenotype. Activation tagging approach has been extensively undertaken in a number of dicot and monocot plants (Michael and Anthony 2011). Identification of CKI1 as histidine kinase (involved in cytokinin biosynthesis in shoot regeneration of *Arabidopsis*), and ORCA3, an AP2/ERF class of transcription factor that regulates indole alkaloid production from *C. roseus* are specific examples of this system (Seki et al. 2005). More recently, RNA-guided genome editing (cluster regularly interspaced short palindromic repeats [CRISPR]-associated nuclease9 [Cas9]) has been established as a method for targeted mutation of specific genes in plants. The CRISPR/Cas system exploits targeted cleavage of genomic DNA handled by a customizable small noncoding RNA, causing gene transformation by both nonhomologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms. Testing this technology as means for genome editing in HR will enable rapid functional genomics studies in plant to determine gene function at the cell/tissue-specific level. Recently CRISPR/Cas9-based genome editing has been demonstrated in *Arabidopsis*, *N. tabacum* (tobacco), *T. aestivum* (wheat), rice, and *sorghum*.

bicolor (Belhaj et al. 2013). Ron and his co-workers have for the first time evaluated this system in hairy roots of tomato. They performed a series of experiments to evaluate the capacity of the Cas9 system to increase gene knockouts using hairy root modification. Their data demonstrated that *A. rhizogenes* and the CRISPR/Cas9 system facilitate gene function analysis during root development, successfully (Ron et al 2014).

Since *A. rhizogenes* is capable of transferring its T-DNA from the root-inducing (Ri) plasmid to the host genome, and also to their desirable features including (1) rapid and efficient induction of hairy roots from explants of a wide variety of plant species (2) simplicity to identify and separate individual transgenic clones; and (3) ability to synthesize the same compounds as the roots of intact plants (Shanks and Morgan 1999; Uozumi 2004), provide attractive materials for screening of gain-of-function mutations. In this context, the binary vector pHRAT (Hairy Root-Activation Tagging) has been emerged to facilitate of activation-tagging approach to those of plant species that are intractable to the regeneration of transgenic plants. Molecular analyses of the pHRAT-GFP-transformed *Arabidopsis* lines showed activation-tagged root culture lines with high-throughput metabolic profiling which provide opportunities for recognizing regulatory or biosynthetic genes for valuable secondary metabolites production (Seki et al. 2005). Recently, activation tagged transgenic Salvia plant (SH41) has been identified by (Ho et al. 2013). This transgenic plant (SH41) with different leaf morphology increased amount of tanshinones as diterpene in root which definitely intensify the application of activation tagged transgenics for genetic manipulation of other medicinal plants and subsequent improved metabolite content. However, such examples suggest hairy root culture as diverse technique with a wide breadth of applications that continue to multiply with the development of novel gene manipulation techniques and biotechnologies and show the broad potential of hairy roots for the production of pharmaceutically active biomolecules in a confined and secure area but a better understanding of biosynthetic genes and biochemical pathways can in turn boost valuable phytochemical production through metabolic engineering of hairy root cultures.

7.4 Hairy Root Culture and Molecular Pharming

Plants have been emerged as an interesting system for production of recombinant proteins (Klimyuk et al. 2005), including biopharmaceuticals (Fischer et al. 2004; Goldstein and Thomas 2004; Ma et al. 2003; Schmidt 2004), such as monoclonal antibodies (Ma and Hein 1995; Valdes et al. 2003), vaccines (Ma and Vine 1999; Makhzoum et al. 2013; Walmsley and Arntzen 2003) and industrial proteins (Somerville and Bonetta 2001). The production of recombinant therapeutic proteins has been traditionally reported in non-plant organisms, such as bacteria, fungi, cultured insect or mammalian cells (Benatti et al. 1991). In comparison with non-expensive technologies of bacterial and fungal expression systems, they are sometimes not able to deliver the correct product and also do not perform post-translational modifications or perform modifications differently in contrast to cells of higher

organisms, hence are not considered suitable for the production of a wide range of pharmaceutical proteins (Gils et al. 2005; Schmidt 2004). Moreover, recombinant proteins of human origin are usually correctly synthesized and processed in mammalian expression systems (Denman et al. 1991), but the supply of mammalian-based recombinant production systems are restricted with respect to limited scalability, high cost and risk for human pathogen contamination (Mor et al. 2001). In comparison with these systems, plantibodies have received considerable interest as the second generation of plant-made pharmaceutical proteins, because of the low-cost production, post-translational modifications, the ease and speed of increasing the scale, and the lack of risks of potentially harboring animal pathogens (Goldstein and Thomas 2004; Ma et al. 2003; Xu et al. 2012). Although transgenic plants are efficient protein producers, free of human pathogens, and capable of post-translational modifications, the transgenic plant production time (Twyman 2004) along with regulatory uncertainty (Doran 2000) and purification challenges have been cited as disadvantages. Moreover, critical parameters which affect the economics of protein production is product accumulation level, (as much lower than 1 % total soluble protein (TSP) as maximum product accumulation of 0.01–0.1 % TSP or less are commonly suggested) and resulted in limitation of the commercial exploitation of recombinant plant systems (Kusnadi et al. 1997; Makhzoum et al. 2014a, b).

Improving the foreign protein accumulation in plant systems remains a major difficulty in development of this technology. Also, concern has been increased among regulatory agencies and the public for use of transgenic plants (grown in open fields or in greenhouses) to produce pharmaceutical proteins due to significant limitations including environmental containment (in terms of both transgenic escape and the inadvertent contamination of non-transgenic plant material). The lack of strictly controlled growth conditions that is typical of plant cultivation in open fields or greenhouses is an additional perceived difficulty. Therefore, increasing of the protein expression level in plant tissue culture and clearing the regulatory barriers for plant-based heterologous production systems are keys issues for improvement of recombinant protein production based on plant systems. In comparison with the whole plant, plant cell cultures in particular offers several persuasive benefits for recombinant protein production, but the low level of protein accumulation in plant cells and the instability of secreted proteins in plant culture medium which contains a number of hydrolyses and proteases, create new challenges in the plant production platform, therefore, increasing protein stability and enhancing protein expression levels in plant tissue culture are key issues for improvement of recombinant protein production. The nondestructive secretion process offer high yields of recombinant proteins by facilitating the downstream purification process over the longevity of a plant.

Hairy roots are potential biopharming factories because they are capable of producing wide range of recombinant proteins safely and inexpensively. Many therapeutic proteins and functional recombinant proteins have been produced using hairy root technology as listed in Table 7.3. Of particular relevance for foreign protein production, hairy roots are determined as non-photosynthetic organs and therefore capable of bio-contained easily and can be scaled up to increase large content of biomass in industrial scale bio-reactors (Boehm 2007; Rigano et al. 2013; Shih and Doran 2009). In this context, recombinant proteins such as green fluorescent protein

Table 7.3 Summary of recombinant proteins produced by hairy root culture (HRC)

Hairy root from plant species	Recombinant protein	Activity	Product concenteration	Refrence(s)
Solanum tuberosum	Hepatitis B surface antigen (rHBsAg)	Anti hepatit B	97.1 ng/g vs. 19.11 ng/g FW	Sunil Kumar et al. (2006)
Nicotiana tabacum	Surface-protective antigen A (SpaA)	Anti swine erysipelas	N/A	(Ko et al. 2006)
Nicotiana tabacum *Lysopersicon esculentum*	B-subunit heat-labile toxin antigen (LTB)	Antigene	65 µg/g	Guzman et al. (2011)
Petunia parodii			10 µg/g	
			70 µg/g	
Nicotiana tabacum	Ricin B chain(RTB:RTB:F1:V) antigen	Anti pneumonic and bubonic	0.25 ng/ µg TS	Woffenden et al. (2008)
Nicotina benthamiana and Petunia hybrida	LicKM-HPV-16 E7 antigene	Anti-cancer	N/A	Massa et al. (2009)
Nicotiana tabacum	Recombinant G1/G2 (Gc/Gn) glycoprotein	Anti Crimean-Congo hemorrahagic fever	1.8 µg/g FW	Ghiasi et al. (2011)
Petunia hybrida	LicKM–PAD4 antigen	Anti anthrax	38 mg/kg	Skarjinskaia et al. (2013)
Thaumatococcus daniellii	Thaumatin I	flavour modifier	2.63 mg/L	Pham et al. (2012)
Sesamum indicum	Recombinant phytase	Feed-supplement	196 units/mL	Jin et al. (2005)
Nicotiana tabacum	Antibody M12	Monoclonal antibody	1600 µg/L	Häkkinen et al. (2014)
Brassica raparapa	Green fluorescent protein (GFP)	Reporter protein	>120 mg/l	Huet et al. (2014)
Brassica oleracea	Human growth hormone (hGH1)	Growth hormone	7.8 ± 0.3 µg/g DW	López et al. (2014)
Nicotiana tabacum	Interleukin 12	T cell-stimulating factor	434.8 µg/g DW	Liu et al. (2009)
Nicotiana tabacum	Immunoglobulin G1(IgG1)	Monoclonal antibody	36.4 µg/g	Komarnytsky et al. (2006)

Nicotiana tabacum	14D9 antibody	Murine antibody IgG1 type useful for organic synthesis	5.95 mg/l^{-1}	Martinez et al. (2005)
Nicotiana tabacum	Human secreted alkaline phosphatase (SEAP)	Human enzyme	280 mg/g DW	Sharp and Doran (2001)
Nicotiana plumbaginifolia	Cholera toxin B-surface protective antigen (CTB-spaA)	Antigen	N/A	Ko et al. (2006)
Nicotiana tabacum	Human epidermal growth factor (hEGF)	Growth factor	2 μg/g FW	Parsons et al. (2010)
N. benthamiana	Human acetylcholinesterase	Bioscavenger of organophosphate toxins	3.3 % TSP	Woods et al. (2008)
Atropa belladonna	Rabbit cytochrome P450 2E1	Mammalian liver detoxifying enzyme	N/A	Banerjee et al. (2002)
Nicotiana tabacum	b-Glucuronidase (GUS)	Reporter protein	N/A	Lee et al. (2007)
N. tabacum cv Xanthi	Ricin-B	Mucosal vaccine for intranasal immunization	N/A	Nopo et al. (2012)
Oriental Melon(Cucumismelo)	Human tissue plasminogen activator (t-PA	Thrombolytic protein that converts plasminogen into plasmin	798 ng mg^{-1}	Kim et al. (2012)
Atropa belladonna	Carrot ADP-ribosylation factor gene	Endoplasmic reticulum (ER) targeting signal sequence	N/A	Asakura et al. (2008)

and human secreted alkaline phosphatase, are examples of proteins which can be secreted into the surrounding media and intensify the concept of low-cost downstream purification process for valuable therapeutic proteins production by hairy roots (Gaume et al. 2003). (Guzman et al. 2011) investigated the capacity of hairy roots from three species in the Solanaceous family for production and secretion of lymphotoxin beta protein. They observed that *Petunia parodii* hairy root cultures have been capable of expressing high levels of lymphotoxin beta protein. Lymphotoxin beta protein production using hairy roots has also been reported in other transgenic plant systems (Mason et al. 1998; Rosales-Mendoza et al. 2008; Wagner et al. 2004; Walmsley et al. 2003). In all of these works, the stability of hairy root cultures provides consecutive production of foreign proteins for long duration. Moreover, the maintenance of secreted protein in transgenic plants derived from hairy root culture regeneration is possible for long time (Christey 2001), and provide a cheap as well as simple technique for germplasm conservation. Also, growth and productivity of roots from these transgenic plants showed similar to first generation of hairy root cultures. For example, (Guzman et al. 2011) investigated the ability of re-generation of hairy roots from transgenic plants produced by hairy root system and found that regeneration of plants from lymphotoxin beta -containing tobacco hairy root culture could be readily achieved and hairy root culture growth and lymphotoxin beta production showed similar pattern as that of original hairy root culture. Therefore, in comparison with more expensive techniques, such as, cryopreservation, which is used for long-term perpetuation of cell lines, this system provides a low cost technology with repeated sub-culturing.

Secretion of recombinant pharmaceutical proteins expressed in hairy root into medium as well as product homogeneity and purification facility are advantages to develop optimized induction protocols for the cultivation of hairy roots secreting monoclonal antibody.

In this context, engineering of immunoglobulin complexes secretion from hairy root into hydroponic medium (rhizosecretion) was accomplished to increase the productivity and stability of immunoglobulin complex (Borisjuk et al. 1999; Gaume et al. 2003; Komarnytsky et al. 2004). Moreover, extensive branching, being meristematic tissues (characterized by large central nuclei), uncondensed nuclear chromatin, ribosome-rich cytoplasm and small vacuoles of hairy root culture, have shown to be other advantages of this system which can significantly produce high contents of single-chain and full-size immunoglobulins (Kolek and Kozinka 1991)

In comparison with these advantages, there are important barriers and concerns in development of recombinant protein production by hairy root platform which can be influenced the structural integrity and activity of recombinant proteins (Benchabane et al. 2008). For instance, protein degradation by protease activity into intercellular spaces or during the transit between the ER and Golgi apparatus can influence harvesting, extraction and downstream protein purification (Ma et al. 1994; Sharp and Doran 2001). In this context, RNA interference, organelle specific-cell targeting, protein-stabilizing protease inhibitors and recovering the recombinant protein continuously in small amounts are some of the effective strategies to simplify recombinant protein purification and minimize proteolysis of intact recombinant proteins produced in hairy root cultures (Ono and Tian 2011).

To fully exploit the bio-production potential of transgenic hairy roots, gene constructs also have been strategically engineered to have a strong promoter for high-level gene expression and a scaffold attachment region for regulation of gene expression (Häkkinen et al. 2014). The strong constitutive promoter cauliflower mosaic virus 35S (CaMV35S) has been most commonly established to drive transgene expression in hairy roots. Strategies recently developed to enhance transgene expression include the use of a double-enhanced CaMV35S promoter (de35S) (Woffenden et al. 2008; Woods et al. 2008) a chimeric super-promoter ((Aocs)3AmasPmas) and a 5′-untranslated leader sequence (translational enhancer), such as that from tobacco etch virus (TEV) (Liu et al. 2008; Nopo et al. 2012) or alfalfa mosaic virus (AMV) (Anuar et al. 2013; Shirokikh et al. 2010). In addition, inducible promoters, induced for instance by glucocorticoids (Hughes et al. 2002) or heat (Lee et al. 2007) have been used to drive controlled gene expression at desired times in hairy root systems. Therefore, discovering and installation of new promoters with or without of enhancer for expression of proteins in hairy roots may be allow the continuous or inducible production and recovery, while maintaining the absolute containment of the recombinant product.

Another potential barrier to production of recombinant proteins in plants on a large scale is the requirement for antibiotic-resistance selection markers and the complexity in recognizing transgenes with the highest expression levels. A selectable marker gene for the isolation and propagation of a rare transformation event from non-transformed plant tissue is one of the critical steps for transformation systems. Traditionally, this is achieved using co-expression of gene of interest together with the antibiotic or herbicide resistance marker, which remains in the genome of a transgenic plant after the selection and regeneration processes .While the product of the antibiotic-resistance gene does not interfere with plant development directly, the use of appropriate selective agents may significantly limit the proliferation and regeneration capabilities of the transformed cells. In addition, the potential release of the antibiotic selection markers into the environment causes safety and ethical concerns. Antibiotic resistance genes can be demolish from the genome of the transgenic plant by a number of excision techniques, but each method has limitations that need to be considered (reviewed in Ebinuma et al. 2001). An alternative approach to obtain genetically transformed plants without resistance marker genes, is phenotypic-based selection using regeneration-promoting genes (reviewed in Zuo et al. 2002). This system may improve the overall efficiency of plant transformation by abandoning the need for antibiotic selection (Kunkel et al. 1999). Using this technique, (Endo et al. 2002) have developed a single-step transformation system for rice, where marker-free transgenic rice plants were regenerated directly from the ipt-induced rice embryogenic tissues. A novel, antibiotic-free transformation strategy that results in rapid and efficient expression and secretion of recombinant proteins from the hairy roots only 2 weeks after the initial infection was engineered by (Komarnytsky et al. 2004). They showed that the hairy root phenotype, which is regarded as being undesirable in standard transformation experiments (Cui et al. 2001; Ebinuma et al. 1997) is beneficial if the goal of the transformation is to rapidly produce and analyze significant quantities of the recombinant protein. Marker-free transformation has been successfully demonstrated by

using several plant and non-plant genes that are capable of promoting organ differentiation or explants regeneration (Zuo et al. 2002). As a phenotypic selection marker *rol* genes provide an additional advantage to generate a high copy number of active root tissues with multiple adventitious root growth (Khelifi et al. 2011), therefore result in increasing of protein expression. Since a large number of transformed roots can be induced rapidly and every hairy root emerges as a result of an independent transformation event (Tepfer 1984), the described system provide new means for simultaneous analysis of multiple protein targets and/or cDNA libraries on a large scale. This system also enables the simultaneous sub-cloning and expression of more than one target gene; for example, a heavy and a light chain of an antibody (Al-Shalabi and Doran 2013; Walmsley et al. 2003). Moreover, with improved scale-up technology, hairy roots could therefore provide a promising production platform for the secretion of stable and homogeneous pharmaceutical proteins.

7.5 Hairy Roots and Plant-Plant Interactios

Parasitic plants are considered in 13 angiosperm families and distributed at wide range of places. Striga and Orobanche species of the *Orobanchaceae* are the most economically important parasitic plants, a monophyletic group of root parasites with approximately 90 genera and more than 2000 species (Westwood et al. 2010)**.** Striga is reported to be infecting about 217,000 ha in Kenya, causing annual crop losses of US $53 million (Atera et al. 2013). Strategies for Striga control are limited to modified/improved cultural practices such as different hand weeding and planting techniques, crop rotaion and trap crops intercropping, using chemicals to reduce soil seeds and employing special hosts germplasm (Scholes and Press 2008). Moreover, striga management needs a profound understanding of the biology of the parasite-host interaction for development of appropriate management strategies using both genetic modification and non- genetic modification approaches (Runo et al. 2011; Makhzoum et al. 2015). Since, by the parasitic plant genome project (http://ppgp.huck.psu.edu/) a wide range of genes such as resistance genes can be identified which and used as functional genomics tools for studying host-parasite interactions (Westwood et al. 2012; Makhzoum et al. 2015). Since, roots are the most favorable organelle for expression of genes involved in Striga-host interactions, thus a reliable and successful genetically modification method that rapidly and efficiently accomplished a large number of transgenic host roots would bring to light an excellent system for investigation of genes functions involved in all aspects of Striga-host interactions. In this sense, the various potential applications of range of *A. rhizogenes* mediated transformation provides a key milestone in the development of ‘composite’ plants (Taylor et al. 2006). The term ‘composite’ plant was referred to those of plants with wild type shoot and a transformed root stock. Composite plants were coined to provide an ideal system for investigation of gene function in plants and their association with other organisms. These chimeric plants

can extremely perform precise analyses involving infection of legumes with rhizobia and nitrogen fixation (Boisson-Dernier et al. 2001; Kereszt et al. 2007) as well as host plant associations with mycorrhiza (Boisson-Dernier et al. 2001).

Since, composite plants offer some important advantages from investigation of roots biology of whole plants to maintenance of induced roots outside of tissue culture, the amount of time necessary to produce transgenic plant tissue in transformation is greatly decreased. Successful applications of transgenic hairy root composite system have been emerged in elucidating plant-microbe and host-parasite interactions. In this context (Runo et al. 2012) showed that transgenic roots of composite maize can be infected by the parasitic plant *S. hermonthica* and could be used as a functional genomics tool to study Striga-maize interactions. They established a transformation protocol that allowed production of transformed maize roots at the infection site that led to development of composite plants. This protocol as a tool in functional genomics studies provides three distinct advantages. Firstly, high production of inoculated plants from one transgenic root (Ishida et al. 2007). Secondly, in comparison with standard transformation protocol which usually takes up to 14 months, *A. rhizogenes* mediated method is rapid as well as easy and not laborious that making it efficient for functional assessment and rapid validation of gene expression in the roots. Thirdly, a huge amount of independent transformation events can be achieved and evaluate in a single plant because every transgenic root derives from a single cell (Bercetche et al. 1987; Choi et al. 2004) and figure out an independent transformation mechanism. Agrobacterium rhizogenes-mediated transformation can produce numerous roots and large amount of mutants for those of genes that are expressed in the roots. Then, the system can be used in gene identification from screening of *Striga* defense genes to other genes involved in parasitism (Mann et al. 2011) .

Another application of hairy roots is symbiotic nitrogen fixation studies where they used as a tool for root nodule investments. *A. rhizogenes* can be used to produce composite plants with induced hairy roots on a no transgenic shoot (Beach and Gresshoff 1988; Hansen et al. 1989). In comparison with generated plants through *A. tumefaciens*, this system offers a more rapid analysis of transformed roots. Although, induced hairy root from composite plants with transgenic roots and not transgenic shoots are not able to produce transgenic seeds, but they have a number of significant features that make these composite plants quite useful in plant research. For example, with a broad host range, A. *rhizogenes* is capable to transform many plant species, especially dicots. Hence, it has known as reliable system in genetic manipulation of plants. Moreover, this useful pathogen can infect tissues and explants directly without priority to produce composite plants by transformation of tissue cultures, so this system is very useful for many recalcitrant plant species. The generation of trangenic roots is very fast and can be occurred in a matter of weeks. In this context (Deng et al. 2011) reported transgenic roots induction of *Medicago truncatula* in as short as 3 weeks. Moreover, it was demonstrated how hairy root composite plants exploit to evaluate plant-rhizobium association and nodulation in the difficult-to-transform species. In other reports, the usefulness of *A. rhizogenes* to assessment of nitrogen fixing interactions has been shown in

Casuarina glauca, (family *Casuarinaceae)* (Davioud et al. 1989), *Dactylisglomerata*, (family *Datiscaceae)* (Diouf et al. 1995), and *Discariatrinervis*, (family *Rhamnaceae* (Valverde and Wall 1999). In all these experiments, hairy root composite plants present a valuable and fast technology to regulate or over express symbiotic genes and to gain precise attitude on their role during infection and nodule ontogenesis in these plant with diverse nitrogen-fixing system which result in profound understanding of the wide range of molecular mechanisms involved in the actinorhizal nodulation in variety of plant families.

7.6 Hairy Root Culture and Biofuel Production

Biodiesels or Biofuels names were used by the pioneer National Soy Diesel Development Board in USA in 1992 as alternative source of petroleum. This new energy resource is of recent growing interest and has been strongly recommended because of its low-hazardous emission (Ju and Chen 2011). Biodiesel is processed from renewable biological sources such as animal fats and vegetable oils. The oils fats are extracted or processed to obtain the crude oil, which usually contains phospholipids, free fatty acids, odorants, sterols, water, and other impurities. The water and free fatty acids contents negatively affect the transesterification process of glycerides with alcohols which cause to consume catalysts, soap formation, reduction of its effectiveness and low conversion (Leao et al. 2011; Yee et al. 2009). Biofuels and biobased chemicals produced from renewable resources have emerged as a reliable technology for degradation of fossil fuel-based resources which address increasing concerns about their global environmental pollution. Energy derived from renewable sources has a remarkable role in the energy matrix worldwide, and its use is expected to increase in near future (Huergaa et al. 2014). A number of studies have been carried out using vegetable oils as a source for diesel fuel viz., palm oil, soybean oil, rapeseed oil, sunflower oil, coconut oil, and so on. Since, animal fats may require expensive processing cost due to their high level of saturated fatty acids in solid forms especially at room temperature, vegetable oils can reduce this expensive processing cost (Gao et al. 2009). Thus, vegetable oils are more favorable and draw greater attention than animal fats because of being renewable and potentially are sustainable source of energy (Leao et al. 2011). Only a small share of biofuel research is focused on developing plant varieties for biofuel production. Despite low patent numbers in biofuel, research into product quality could improve biofuel production efficiencies (Kamis and Joshi 2008).

Vegetable oils from plants can be used as a promising biomass-derived fuel and have a potential for biofuel production. Due to high seed-oil content, and adaptability to wide range of environmental conditions, plants are favorable for biofuel production throughout the world. Hence it can be foreseen that plants have a potential capacity for next-generation of biodiesel fuels. The high oil content in plants means that they have great capacity for inexhaustible biodiesel production; however, this feature strongly depends on the growth conditions and environment. To improve the

economic properties of plants, breeders must develop a superior genotype that can exhibit high yield. Genetic engineering is a powerful technique to develop plants for achieving this purpose. Improvement in *in vitro* regeneration systems and stable genetic transformation systems are necessary for successful genetic modifications. Fortunately, plant genes involved in fatty acid biosynthesis, biotic and abiotic stress and tolerance mechanisms have been identified and also the regulation and production of secondary metabolites and phorbol esters as important targets for biofuel production have been achieved.(Sato et al. 2011).

Among non-edible plants, *Jatropha curcas* has been considered as a prominent biodiesel plant (Annarao et al. 2008) due to its high oil content (50–60 % of total seed biomass), which could easily replace the conventional fossil fuels(Forson et al. 2004). Quality, fuel stability of monounsaturated fatty acids and its low content of nitrogen oxides, unburnt hydrocarbon and sulphur oxides emissions offer *J. curcas* as a potential biodiesel crop (Basili and Fontini 2012). It is to note that *J. curcas* seeds contain many toxic substances, such as curcin, phorbol ester, trypsin inhibitor, lectin, and phytate, which show adverse effects on human health and on nutrient utilization in animals (Khemkladngoen et al. 2011).

Since, demand for feedstock, biodiesel and a medicinal application has been raised, developing of biotic and abiotic stress-resistant, high-yielding, and toxic-free *J. curcas* is necessary. However, it is hampered due to genetic erosion, long time difficulties and the complexity of fertile progenies generation by sexual incompatibilities made conventional breeding inconspicuous. To address, *J. curcas* explants (cotyledons, embryonic axes, and leaf) have been treated by *Agrobacterium* and the transformed plants are recovered by direct (Khemkladngoen et al. 2011; Kumar et al. 2010; Li et al. 2008; Pan et al. 2010) or indirect (Mazumdar et al. 2010; Qu et al. 2012) organogenesis. The occurrence of gene silencing and multiple gene insertions have been limited through *Agrobacterium*-mediated genetic transformation which offer more advantages over particle bombardment. On the other hand, this technique allow to introduce wide range of genes into genome through integration of transgenes into transcriptional active regions of the chromosome (Li et al. 2005).

Initiations and multiplication of transformed shoots by *Agrobacterium*-mediated genetic transformation, are strong process to obtain a large number of transformed plants in a relatively short time. In this context, (Kajikawa et al. 2012) proposed a protocol that allowed the integration of a foreign gene and its expression in *Jatropha* hairy roots within 2 weeks; this new protocol will be useful not only for functional investigation on endogenous genes, but also for assessment of transformation characteristics in *Jatropha* cultivars for commercial purpose. In another work, a high-frequency and efficient plant regeneration protocol from transgenic plants using *Agrobacterium*/biolistic-mediated transformation have been achieved (Daud et al. 2013). These results indicate that hairy roots in *J. curcas* can be considered as a potential feedstock for biodiesel production since the hairy root system is proposed as a rapid and effective way to study gene function in the plant roots. Improvement and optimization of the regeneration and transformation procedure for *Jatropha* is a promising method that can lead to the mass production of this plant as potential feedstock for biodiesel production (Basa and Sujatha 2009).

Crambe abyssinica (Abyssinian mustard) is belong to Brassicaceae family and well known as Mediterranean oilseed crop. It contains a high level of erucic acid (55–60 %) and is, therefore, exploited extremely for industrial usage. Nowadays, coating agents, lubricants, surfactants, corrosion inhibitors and anti-sticking agents are used as by-product of crambe oil. Moreover, in comparison with high erucic acid rape oil, crambe oil is more striking to the chemical industry due to lower contents of polyunsaturated fatty acids (Nath et al. 2007). Although, this plant is also an ideal species for genetic manipulation targeted to improve its industrial suitability (Wang and Peng 1998) but separated only in very limited areas. Moreover, transformation protocols using *A. tumefaciens* for this plant (Engler and Montanez 2010; Li et al. 2010) were shown not to be very efficient. In this context, (Głab et al. 2013) developed an alternative transformation method using *Agrobacterium rhizogenes*. They reported the first analysis of the lipids in hairy roots and non-*Agrobacterium* induced roots of this species. The result suggested that fatty acids contents of the lipids in hairy roots and the non-transformed roots of *C. abyssinica* have not shown significant difference in chemical composition. A-linolenic acid (27 %), palmitic acid (17–20 %) and linoleic acid 12–15 % were the most fatty acids in both types of roots. All other identified fatty acids content ranged from 1 to 4 %, and there were no significant differences between both types of roots transformed and untransformed. Moreover, in similar work, the potential of *Echium acanthocarpum* HRs was studied for polyunsaturated fatty acids production. (Cequier-Sa´nchez et al. 2011). The establishment of novel *Echium acanthocarpum* hairy root cultures represents a powerful tool to study the accumulation and metabolism of fatty acids in a plant particularly rich in gamma-linolenic acid (GLA) and stearidonic acid (SDA).

These novel results showed the utility and high potential of *E. acanthocarpum* hairy roots for biosynthesis and accumulation of wide range of polyunsaturated fatty acids especially the target fatty acids, GLA and SDA, in sufficient quantities. It can be to draw the conclusion that hairy roots could be a very useful tool for investigation of both catabolism and anabolism of root lipids. Therefore, the high capacity of hairy roots to accumulate fatty acids in large quantities can make striking this system as an alternative method for production of second- and third-generation biofuels in the future. Moreover, the optimization of upstream and downstream processes to development of large scale production of fatty acids from hairy root cultures can lead to boost the industrial synthesis of fatty acids in an effective way.

7.7 Conclusion

This review article reports the most recent achievements of using hairy root cultures in the production of plant bioactive compounds, plant molecular pharming as well as other important issues, such as phytoremediation, plant-plant and plant microbe interactions and plant biodiesel research. Due to the ease of using this system as a transgenic platform, and counting on the huge progress in functional genomics and

epigenomic studies, analysis, tools and techniques, it is required to design new hairy roots lines capable to express, produce and metabolize more genes, enzymes and pollutants in a more efficient manner under *in vitro* or field conditions or in environment. Based on the above discussed reports, it can be drawn that over, hairy roots act as a very promising tool to generate invaluable data and information on studying plant-plant and microbes interactions both in *in vivo* and *in vitro* conditions based on employing composite or even whole transgenic plants. Additionally, the exciting potential of these transformed roots can be foreseen in optimizing and increasing the yield of biofuel producing plant species. Interestingly, in plant molecular pharming, using these transgenic roots as bio-factories will accelerate some outstanding studies focusing on repressing proteases or targeting the transgenes into specific transcriptional and open chromatin positioning structure loci to increase the levels of expression and thus the accumulation and production of pharmaceutical proteins of interest. By employing some of the above genomics tools like RNA interference, chromatin acetylation and DNA demethylation the entire genome can be largely shifted in favor of the expression of pharmaceutical heterologous proteins. This plant system platform will continue to advance and help scientists to decipher many interesting phenomena in various fields of plant science and biotechnology and their applications.

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