Chapter 18
Advances in Transformed Root Cultures for Root Biofactory and Phytoremediation Research

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18.1 Hairy Roots Induction: From an Infection Disease to a Biotechnological Resource

Agrobacterium rhizogenes belongs to a group of phytopathogenic bacteria within the order Rhizobiales. The disease, called hairy root disease, is caused by the transformation of the plant cell by plasmid-derived DNA of the bacteria. This process proceeds in analogy to the transformation of plant cells by *Agrobacterium tumefaciens*, the causal agent of crown gall disease (Pitzschke and Hirt 2010). However, for both processes different genes are transferred into the host, which cause on the one hand the tumorous growth of crown gall and on the other hand the extensive root system of hairy roots. The principles of transformation, however, seem to be quite similar. Both species contain plasmids, which are coined Ti-plasmids, for tumor inducing in the case of *A. tumefaciens*, and in analogy Ri-plasmids, for root inducing in the case of *A. rhizogenes*. These contain the T-DNA, for transfer DNA, which is the part of the plasmid transferred into the host plant's nucleus. The T-DNA of *A. tumefaciens* contains the genes for the synthesis of two plant hormones auxin and cytokinin, as well as the genes for the

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synthesis of the opines. The T-DNA of *A. rhizogenes* contains the so-called *rol* genes, of which the function until today is not yet elucidated (Veena and Taylor 2007). Even though four *rol* genes (A, B, C, and D) are transferred to induce hairy roots, the major player seems to be *rolB*, because, first, loss-of-function mutation renders the bacteria avirulent and, second, when *rolB* is introduced into the host plant genome as a single gene, it is capable of hairy root induction (Altamura 2004).

While for genetic engineering the *A. tumefaciens* T-DNA is "disarmed," i.e., the tumor-inducing genes are removed (Tzfira and Citovsky 2006), the complete T-DNA of *A. rhizogenes* is used for genetic transformation to generate organ cultures, the hairy roots. While *A. tumefaciens* transformation is one method of choice to generate transgenic plants with novel traits, generation of hairy roots has been used to increase the production of highly bioactive plant secondary metabolites, potentially beneficial in medicine (Georgiev et al. 2010). Therefore, the focus within the last decades has not so much been on the biology of *A. rhizogenes*, but rather on improving transformation protocols for different plant species with the bacterium.

Biotechnological production of secondary plant metabolites has been of interest for many decades (Georgiev et al. 2007; Verpoorte et al. 2002). Many plant species with metabolites having interesting pharmaceutical properties are on a list of endangered species, and therefore novel approaches have to be found to get hands on these compounds (Gómez-Galera et al. 2007). Organ cultures have been used to produce secondary metabolites under sterile conditions, but these are more expensive in cultivation because they rely on the addition of plant hormones to the culture medium. On the contrary, transformation of plant tissues with *A. rhizogenes* generates organ cultures, which are independent of plant hormones for their growth (Georgiev et al. 2007; Vasilev et al. 2006; Zhang et al. 2009). In addition, a hairy root is a more organized structure, so biosynthetic processes, which need compartmentation in the plant cells, can be much better simulated in a complex organ. Similarly to cell cultures, hairy roots can be cultivated under sterile and controlled conditions depending on the type of cultivation method used. In both cultivation systems, cell and organ cultures, it is possible to produce higher amounts of a given compound in comparison to the mother plant (Srivastava and Srivastava 2007). However, a preselection of plant cultivars before the culture is induced might be advantageous for the production of higher levels of a secondary metabolite. In addition, during the cultivation method the biosynthesis of compounds can be induced by so-called elicitors, either abiotic or chemical factors added during the cultivation procedure (Staniszewska et al. 2003; Zhang et al. 2009). Finally, the generation of hairy root cultures with novel traits is possible by using *A. rhizogenes* with additional information on the T-DNA (Fig. 18.1). These can be genes encoding enzymes for metabolic pathways, which might be rate limiting, but also transcription factors, which could control major steps in a given pathway (Georgiev et al. 2010). Combining these transgenic approaches with elicitation could result in much increased production rates.

One major factor to consider for the genetic alteration of a given pathway is to gain as much knowledge on enzymatic steps, competing reactions and regulatory

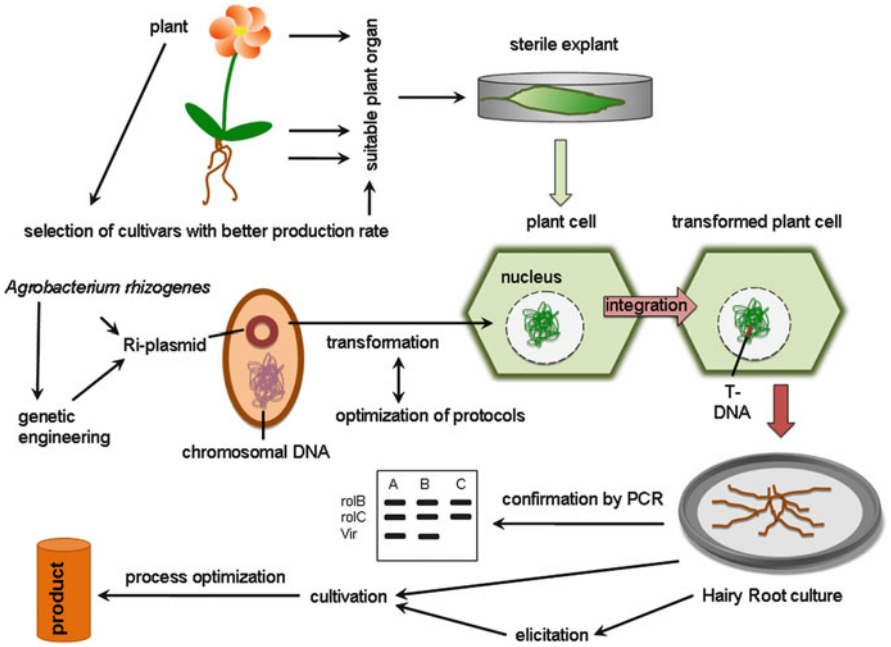

Fig. 18.1 Scheme to demonstrate an example for a common transformation protocol for the generation of hairy root cultures using either wild type or genetically modified *Agrobacterium rhizogenes* strains. In the gel lane A represents bacteria in culture, lane B a contaminated hairy root culture, and lane C a transformed, uncontaminated culture

factors of the native pathway (Georgiev et al. 2010). Alterations can include the increase of a specific bottleneck enzyme (or several), the decrease of an unwanted side pathway to elevate availability of precursors for the main pathway, and the increase in regulatory proteins, i.e., transcription factors. One has to take into account also the transport rates, if several cellular compartments are involved, the availability of precursors and cofactors and possible unwanted effect such as co-suppression of homologous genes, which would actually lead to a decrease in the amount of transcript. Finally, unwanted products could be removed from a beneficial culture by using transgenic approaches to suppress a biosynthetic pathway deliberately (Georgiev et al. 2012). The T-DNA can be transformed with the gene of choice and the plasmid is then brought back into suitable *A. rhizogenes* strains. Suitable strains in this case should be highly virulent, so that transformation events lead to the strong hairy root phenotype.

One of the main obstacles so far is still the transformation protocol. *A. rhizogenes* has a wide host range within monocots and dicots, and in theory, hairy roots can be obtained from any tissue of a plant, i.e., leaves, roots, and flower organs (Ono and Tian 2011; Porter 1991). Mostly parts of leaves are taken, which are then sterilized and incubated with the bacteria. Cutting the leaves induces wounds necessary for penetration of the bacteria. Transformation events are

increased when phenolic compounds, which are signals for the bacteria in nature, are added. For example, acetosyringone and others can enhance the transformation of plant tissues by *A. rhizogenes* as well as *A. tumefaciens* (Giri and Narasu 2000; Joubert et al. 2002). Even though hairy roots can be used themselves to produce antimicrobial compounds (Wang et al. 2012), sometimes the endogenous compounds of a given plant reduce or completely inhibit bacterial growth and thereby transformation. To reduce antimicrobial plant compounds, adsorbing compounds such as charcoal can be added, which often results in increased transformation events. Other protocols use additional sonication to increase the penetration with agrobacteria (Georgiev et al. 2011b). While growth of hairy roots from leaves can be rather easily monitored, the outgrowth of hairy roots, if a root is the origin for transformation, is much more difficult. Here, especially the need for inclusion of selection markers in the *A. rhizogenes* strain might be advantageous. Otherwise, the roots need to be screened by PCR to test for integration events of the T-DNA. This should be in general tested for all hairy root cultures. Here, routine protocols include the amplification of *rol* genes, which are transferred into the plant genome, but could also be present in contaminating bacteria, which have not been removed by antibiotics treatment during initial cultivation periods (Rahman et al. 2004). Therefore, additional PCR reactions are carried out using genes only present in the bacterial chromosome. The expected result for hairy roots would be the amplification of the *rol* genes and not the gene(s) from the chromosome, whereas a control using free bacteria should amplify all bands. This can be done by single PCR reactions, but also multiplex protocols are used for the amplification of several bands in one assay. In these tests the identification of foreign genes could also be included (Georgiev et al. 2011b).

18.2 Bioproduction of High-Value Plant-Derived Molecules by Hairy Roots

Early years of transformed root culture research were mainly focused on underlying the hairy root syndrome (e.g., the host range of plant species susceptible to infection, the mechanism of the infection and genetic transfer from bacteria to host cell). Thus, the immense biosynthetic potential of hairy root cultures was largely neglected for years. Since the 1990s, however, hairy root cultures received increasing attention as biotechnological matrices for the mass production of valuable molecules, because of their several attractive features, as high genetic and biochemical stability (compared to dedifferentiated plant in vitro systems) and relatively fast growth rates (compared to adventitious roots) in hormone-free media (Georgiev et al. 2007, 2012). To date, hairy roots are induced from over 500 plant species, including dozens of endangered and threatened medicinal plant species (Georgiev et al. 2012). The mainstreams of hairy roots application include production of value-added plant-derived metabolites and therapeutic proteins,

phytoremediation, biotransformation, and assistance in molecular breeding (Georgiev et al. 2010; Guillon et al. 2006).

Plants are used since time immemorial to feed people and in the production of beverages but also provide essential materials for clothing and shelter, for writing and coloring materials, for hunting and murdering, and even for ritualistic purposes (as hallucinogens). Moreover plants accumulate a wide spectrum of metabolites (mainly secondary ones), used for centuries as vital source of drugs for treatment of numerous diseases. Nowadays over a quarter of all prescribed current drugs are derived either directly or indirectly from plants (Georgiev 2012). This is especially the case in oncology, immunosuppression, and metabolic disorder therapeutic areas where natural products have played a central role in lead discovery (Butler 2005). The continuously increasing demands for drug leads, produced by ever-greener processes, along with significant reductions in biodiversity, are driving efforts to find alternatives to produce high-value plant-derived metabolites (Georgiev et al. 2007).

Some remarkable examples of high-value metabolites produced by hairy root cultures, including anticancer (paclitaxel, camptothecin, and justicidine B), antimalarial (artemisinin), and anti-inflammatory (harpagoside and verbascoside) substances, are emphasized in Fig. 18.2 and discussed further.

Paclitaxel (Fig. 18.2), a complex diterpenoid that is the active ingredient of Taxol®, has been isolated for the first time in the early 1970s by Wani and his coauthors from the stem bark of the pacific yew *Taxus brevifolia* (Wani et al. 1971). Paclitaxel and related taxanes are microtubule-stabilizing drugs widely used in the treatment of various kinds of cancer, inter alia breast cancer, ovarian cancer, AIDS-related Kaposi's sarcoma, and non-small cell lung cancer (Onrubia et al. 2013). The market of paclitaxel and related taxanes is still expanding, as currently annual global sales are worth over five billion US dollars (Malic et al. 2011). Commercial production of paclitaxel from natural sources is not economically feasible as *Taxus* plants grow very slowly, and their paclitaxel contents are very low (<0.02 % of the dry weight of the bark, where levels are highest). Hairy root cultures of *Taxus* x *media* var. *Hicksii* have been found to produce paclitaxel at levels of 40 μg/g dry roots. Syklowska-Baranek et al. (2009) recently found that the content of paclitaxel in transformed roots can be further enhanced through combinatorial feeding with L-phenylalanine and elicitation with methyl jasmonate, reaching 568 μg/g dry roots or about 14 times higher compared to the control non-stimulated roots.

Camptothecin (Fig. 18.2), a pentacyclic quinoline alkaloid, exhibits anticancer properties due to its inhibition of DNA topoisomerase (Yamazaki et al. 2010). Hairy root cultures of *Ophiorrhiza pumila* were found to produce up to 0.1 % camptothecin per unit dry weight (Yamazaki et al. 2010), which clearly outlines the perspectives for its bioproduction by green root factories. Justicidine B, an arylnaphthalene lignan with cytotoxic, antiviral, fungicidal, antiprotozoal, and antiplatelet properties, was produced by transformed root cultures of *Linum leonii* (Vasilev et al. 2006) in amounts (10.8 mg/g dry weight per unit dry weight) significantly exceeding those obtained from callus cultures (five times less compared to the respective hairy roots) of the same species.

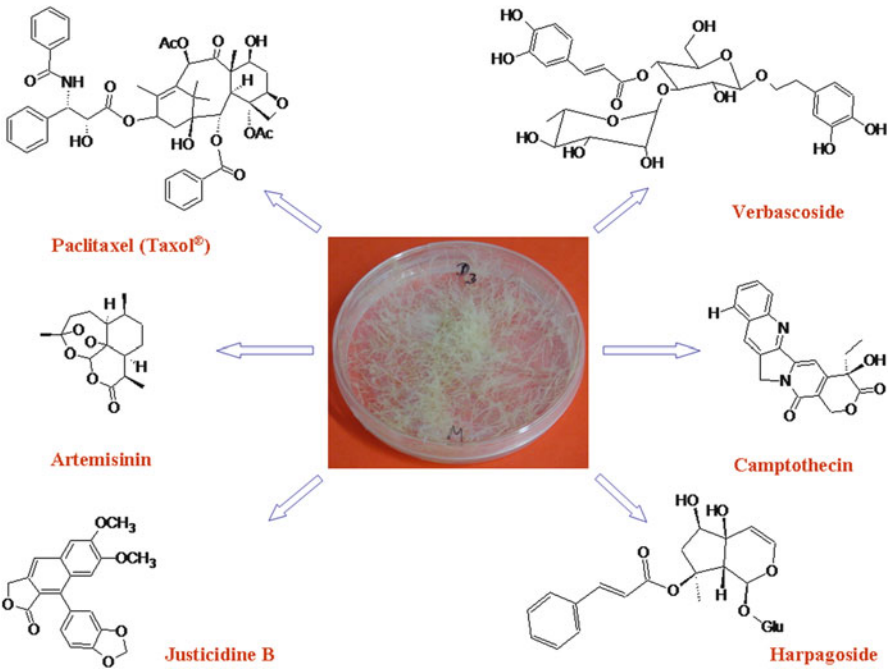

Fig. 18.2 Chemical structures of high-value plant-derived secondary metabolites produced by hairy root culture systems

Artemisinin (Fig. 18.2), a sesquiterpene lactone produced by *Artemisia annua*, is a highly active compound against the parasitic protozoa of the genus *Plasmodium*, the causative agent of malaria. Malaria is a very common and virulent tropical disease, which causes about two million deaths per year (mainly of infants and children; Potterat and Hamburger 2008). Large-scale artemisinin extraction from the natural sources is laborious and costly, because of the low yields in the intact plants (0.01–0.8 %; Potterat and Hamburger 2008). In searching of alternative sources for artemisinin supply, hairy root cultures of *A. annua* were induced in Weathers' laboratory in Worcester Polytechnic Institute (USA). Moreover, the artemisinin biosynthetic process was upscaled in several bioreactors, as the most promising appeared to be mist bioreactor configurations (Kim et al. 2001).

Hairy root cultures were also found to be attractive producers of anti-inflammatory substances. Harpagoside (Fig. 18.2) is an iridoid glycoside (cinnamic ester) with remarkable biological properties, such as antiarthritis, anti-inflammatory, and analgesic effects. It is the major constituent of the iridoid pool in devil's claw plants (*Harpagophytum procumbens*, Pedaliaceae). By transforming shoot tip explants of devil's claw with *A. rhizogenes*, Grabkowska et al. (2010) successfully induced hairy roots capable of growing under submerged conditions and producing ca. 0.32 mg harpagoside/g dry root mass. Verbascoside (Fig. 18.2) is a water-soluble naturally occurring phenylethanoid glycoside, isolated from several

medicinal plants. Several pharmacological studies have shown verbascoside to have a wide spectrum of biological activities (in vitro and in vivo) including antimicrobial, immunomodulatory, anti-inflammatory, and cholinesterase inhibitory properties (Georgiev et al. 2011a; Gyurkovska et al. 2011). Hairy root culture of *Verbascum xanthophoeniceum* was found to produce significant amounts of bioactive verbascoside (over six times more than in mother plant leaves) during their submerged shake-flask cultivation (Georgiev et al. 2011b).

Although no commercial processes based on hairy root culture have been yet established, there have been many proof-of-concept studies (e.g., the production of anticancer, anti-inflammatory, and antimalarial compounds), which allow hairy root-based processes to be upscaled while keeping their immense biosynthetic potential.

18.3 Hairy Root Culture: Green Factories for Biopharmaceuticals

Biopharmaceuticals are the next generation of high-value therapeutic proteins that offer great importance in the treatment of various diseases like cancers, heart attacks, strokes, diabetes, anemia, and hemophilia (Walsh 2005). Commercial production of biopharmaceutical proteins has traditionally relied on bacterial fermentation and mammalian cell cultures. However, the inherent disadvantages of these expression platforms in terms of cost, scalability, safety, and authenticity of proteins produced have prompted research into alternatives. Plants are emerging as one of the most powerful alternative bioproduction platform because of their economic and safety advantages over the traditional systems and the presence of posttranslational modification machinery, such as glycosylation that enables the production of complex human proteins (Hood et al. 2012; Ma et al. 2003; Ono and Tian 2011; Xu et al. 2012). Hairy roots that can be grown in vitro and in bioreactors, just as plant suspension cells, represent an attractive contained plant production system preferred to plants grown in open field. The contained in vitro culture systems combine the merits of whole-plant system with microbial and mammalian cell culture benefits (Georgiev et al. 2012; Xu et al. 2011). They show intrinsic benefits like fast-growing, batch-to-batch consistency, production in compliance with good manufacturing practice (GMP), less concerns over regulatory compliance and product safety, and simpler procedures for downstream processing and protein purification, especially when proteins are secreted into the culture medium (rhizosecretion; Hellwig et al. 2004; Huang and McDonald 2009). Yet as a more organized tissue, hairy root presents additional benefits over suspension cells, including genotype and phenotype stability and being able to grow on plant hormone-free media (Guillon et al. 2006).

The hairy root culture-based process for biopharmaceutical production is shown in Fig. 18.3. Tobacco (*Nicotiana tabacum*) is by far the most largely used plant species for the production of recombinant biopharmaceutical (Daniell et al. 2009). For high-level protein expression and secretion in hairy root system, a strong constitutive promoter, e.g., *cauliflower mosaic virus 35S* (*CaMV35S*), and a signal peptide to direct nascent proteins through the default ER-Golgi secretory pathway are strategically designed. Further enhancement of the transgene expression can be achieved by using a double-enhanced *CaMV35S* promoter (Medina-Bolivar et al. 2003; Woods et al. 2008) or a chimeric super-promoter [*(Aocs)3AmasPmas*] (Nopo et al. 2012; Rukavtsova et al. 2007) as well as introduction of a translational enhancer (5′-untranslated leader sequence), such as that derived from tobacco etch virus (TEV) (Liu et al. 2008; Nopo et al. 2012) or from alfalfa mosaic virus (AMV) (Anuar et al. 2011; Gallie et al. 1987). Except for constitutive expression, controlled and temporal gene expression in the hairy root system by inducible promoters, such as *Arabidopsis small heat shock protein 18.2* promoter (Lee et al. 2007) and *glucocorticoid-inducible* promoter (Peebles et al. 2007), has been achieved. Since a fully functional murine IgG1 monoclonal antibody was produced by tobacco hairy roots in 1997 (Wongsamuth and Doran 1997), nearly 20 therapeutic proteins, including antibodies, vaccines, cytokines, therapeutic enzymes, etc., have been successfully expressed in hairy root systems, which were summarized in a recent review (Georgiev et al. 2012). Protein yields as high as 64 mg/L of a murine IgG1 produced by tobacco hairy root were achieved (Martinez et al. 2005). In addition, the rhizosecretion feature of hairy roots has also been exploited. For example, up to 43 % of the murine IgG1 was found to be secreted into tobacco hairy root culture medium when polyvinylpyrrolidone (PVP) and gelatin were supplemented (Wongsamuth and Doran 1997).

The scaling-up of culture systems is of critical importance for achieving commercial level of biopharmaceutical production. However, the special morphological characteristics of hairy roots including nonhomogenous growth and highly branched phenotypes present major challenges to culture scale-up in bioreactors (Ono and Tian 2011). Compared with the production of phytochemicals by hairy roots, protein offers additional challenges due to the susceptibility of products to proteolytic degradation and their increased sensitivity to shear stresses (Hood et al. 2012). To achieve high production yields, a culture system with low shear stresses and higher oxygen transfer efficiency (thus low levels of protease generation) is especially needed. So far, two types of bioreactors have been successfully used for scaling up hairy root cultures for biopharmaceutical production: The first, based on airlift concept, is a liquid-phase bioreactor where compressed air released from the base of the culture vessel provides aeration and agitation as the air moves up through the root bed (Caspeta et al. 2005). The second type, the mist reactor, is a gas-phase reactor where hairy roots are exposed to humidified air or a gas mixture and nutrients delivered as droplets by spray nozzles or ultrasonic transducers (Eibl and Eibl 2008). Compared to liquid-phase bioreactors, the mist bioreactor offers advantages in that it reduces the volume of culture medium, enhances oxygen transfer efficiency, and totally eliminates hydrodynamic stress imparted on root

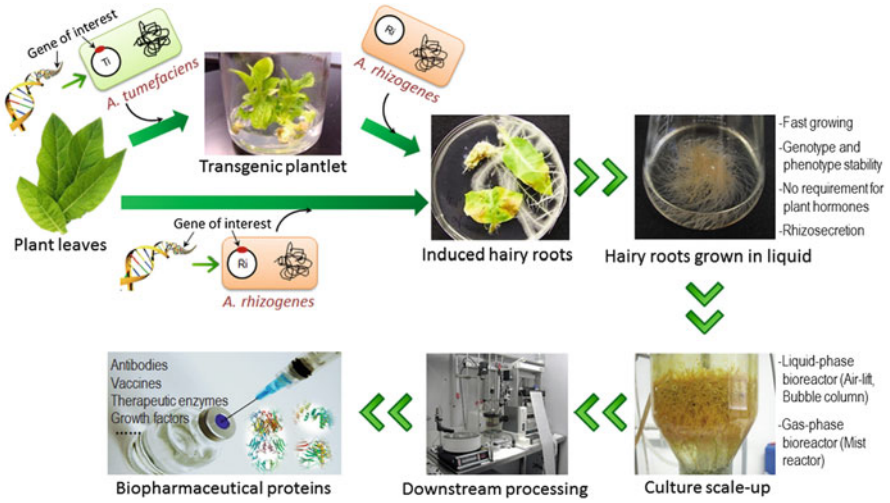

Fig. 18.3 Schematic illustration of the hairy root culture process for producing biopharmaceuticals. Transgenic hairy roots expressing certain therapeutic protein can be established either by infecting stably transformed plants (expressing the target protein) with *Agrobacterium rhizogenes* (*upper pathway*) or by directly infecting wild-type plants with *A. rhizogenes* harboring binary vectors containing gene of interest (*lower pathway*) (Pham et al. 2012). Scale-up production and downstream purification methodologies adapted from other cell-based bioproduction systems can be used in delivering biopharmaceuticals to the market. *Ti* Ti-plasmid, *Ri* Ri-plasmid

biomass (Liu et al. 2009; Towler et al. 2006; Weathers and Giles 1988). A recent study scaling up the tobacco hairy root cultures for production of murine interlukin-12 (mIL-12) showed that a mist reactor made from disposable plastic bag supported better protein production than an airlift reactor (Liu et al. 2009). This is actually the first demonstration of successful production of a pharmaceutical protein in a mist bioreactor. Future research should focus on establishing effective and economical bioreactor culture systems for commercial-scale production.

18.4 Applications of Hairy Roots for Phytoremediation Research: Current Knowledge and Challenges

Hairy roots have been frequently applied in phytoremediation research as model plant systems, for screening the potentialities of different plant species to tolerate, accumulate, remove, and/or degrade environmental pollutants. This is in part due to several advantages of hairy roots and the fact that roots have evolved specific mechanisms to deal with pollutants, because they are the first organs to have contact with them. Therefore, they are the sites where the first reactions against the contaminant take place. Among the main advantages, they are independent of site and weather conditions, and they have a short sub-cultivation period (2 or 3 weeks)

which substantially reduces the time required to carry out studies. The experimental variables are easily controlled, thus providing a more reliable and reproducible experimental system over time, for phytoremediation purposes (Doran 2009; Harms et al. 2003). They also have a prolific root growth, and thus, large amounts of biomass are generated in a controlling setting (Erlenmeyer's or bioreactors). Moreover, hairy roots grow in an environment that is totally free of microbes and can be used to distinguish the responses and capabilities of plant cells from rhizospheric microorganisms, without the interference of soil matrix.

The results derived from these in vitro cultures can be used to predict the responses of plants to environmental contaminants because if a compound is metabolized by in vitro hairy roots, this is a clear indication that the plant has the genetic capacity of biotransforming this compound (Doran 2009). In fact, hairy roots closely reflect the biochemical pathways typical of the parent plant, and although the natural translocation from roots to shoots is obviously blocked, this characteristic frequently constitutes an additional advantage of this plant system. They can also improve the design and reduce the cost of subsequent conventional whole-plant experiments. Furthermore, hairy roots have the ability to produce large amounts of exudates which may contribute not only to remove harmful pollutants but also to sequester them.

As it is well known, the metabolism of xenobiotic organic pollutants is a very complex process and a great variation was observed in the fate of pollutants and their degradation products among different plant species. In this context, hairy roots could provide valuable information regarding plant metabolic pathways. For instance, they have been used to explore metabolism of explosives, phenolics, polychlorinated biphenyls (PCBs), and textile dyes as well as to analyze the effect of these pollutants on the activity of plant pollutant-converting enzymes [glutathione S-transferase (GST); peroxidases (Px); cytochrome P450, laccases, lignin peroxidases, tyrosinases among others] (Agostini et al. 2011; Ghodake et al. 2009; Govindwar and Kagalkar 2010; Patil et al. 2009; van Aken et al. 2010). The chemical mechanism of degradation, the involvement of reactive oxygen species (ROS) in the removal process (Gujarathi et al. 2005), the nature, and the compartmentalization of some final products of the removal reaction (Talano et al. 2010) and several biochemical and physiological processes such as the antioxidative stress responses (Ibáñez et al. 2011), lipid peroxidation, as well as the signaling mechanisms involved in the responses to environmental pollutants (Sosa Alderete et al. 2011, 2012a) were also studied using hairy roots (Table 18.1). All these studies not only help in understanding the pollutant uptake, their metabolism, and removal but also open the possibility of designing new genetic and biochemical approaches to perform a deep study of transformation pathways.

On the other hand, hairy roots could be used as sources of enzymes (Px and laccases) which demonstrated to be powerful catalysts for the removal of harmful pollutants, and they provide valuable information about them, such as variations in substrate preference and catalytic efficiencies toward contaminants (Coniglio et al. 2008; González et al. 2008; Sosa Alderete et al. 2012b; Telke et al. 2011).

Table 18.1 Phytoremediation of organic environmental pollutants using hairy root cultures from different plant species

Plant species	Summary of remediation strategies and/or main aspects studied	References
Brassica juncea, Beta vulgaris, Raphanus sativus, Azadirachta indica	Screening of some plant species to find those with the highest potential for phenol removal	Singh et al. (2006)
Solanum lycopersicon, Brassica napus	Removal of phenol and/or 2,4-DCP with high efficiency. Establishment of optimal conditions (pH, temperature, concentration of co-substrate, time of treatment) for the removal process. Tolerance studies	Agostini et al. (2003), González et al. (2006, 2008), Coniglio et al. (2008)
Nicotiana tabacum	Studies related to physiological responses of plant tissues against phenol (oxidative stress, signal transduction pathways)	Ibáñez et al. (2011), Sosa Alderete et al. (2011, 2012a)
Brassica napus	Removal of 2,4-DCP on a large-scale in bioreactors. Hairy roots could be reused in several consecutive cycles, with high efficiency. The compartmentalization of some of the final products of the 2,4-DCP removal was shown	Angelini et al. (2011), Talano et al. (2010)
Armoracia rusticana	Plant metabolism of various explosives (TNT, DNT, ADNTs, DANTs) was studied as well as the effect of these pollutants on enzymes such as GST and Px	Nepovim et al. (2004)
Solanum nigrum	Detoxification products of PCB metabolism were identified. Newly discovered plant metabolites of PCBs were described: hydroxy-PCBs, methoxy-PCBs, and hydroxy-methoxy-PCBs	Rezek et al. (2007, 2012)
Tagetes patula L.	High concentrations of the textile dye Reactive Red 198 were removed. Hairy roots could be reused for five consecutive decolorization cycles. A possible pathway for the biodegradation was proposed and some metabolites were identified	Patil et al. (2009)
Brassica juncea L.	An intracellular laccase was purified, characterized, and applied for the removal of textile dyes. The dye decolorization rate was significantly enhanced in the presence of various redox mediators	Telke et al. (2011)

(continued)

Table 18.1 (continued)

Plant species	Summary of remediation strategies and/or main aspects studied	References
Armoracia rusticana L.	Hairy roots were able to take up and detoxify *N*-acetyl-4-aminophenol (paracetamol). A paracetamol-glucoside was described as the dominant metabolite, which would be a precursor of insoluble bound residues associated to the cell wall	Huber et al. (2009)

2,4-DCP 2,4-dichlorophenol, *TNT* 2,4,6-trinitrotoluene, *DNT* 2,4-dinitrotoluene, *ADNTs* aminodinitrotoluenes, *DANTs* diaminonitrotoluenes, *PCBs* polychlorinated biphenyls, *GST* glutathione S-transferases, *Px* peroxidases

Toxicity of final degradation products is another important feature to be addressed, because frequently unknown and final transformation products are difficult to identify. Transformation into nontoxic products is preferable for phytoremediation, and hairy roots can help researchers in such studies (González et al. 2012a; Paisio et al. 2010). As could be seen, there are several examples in the literature which illustrate the potentialities of hairy roots to understand the complex biochemical and molecular mechanisms involved in phytoremediation of organic pollutants. Some selected examples are summarized in Table 18.1.

Hairy roots are also suitable for studying the mechanisms of metal uptake, as well as to investigate the physiology and biochemistry of metal accumulation and tolerance in a wide range of plant species (Doran 2011). Although the mechanisms involved are still only partially understood, several sequestration and detoxification strategies are known to occur in plant cells; among them complexation with phytochelatins (PCs) synthesized from glutathione has been identified as an important mechanism for detoxifying metals. In this way, hairy roots seem to be adequate systems for studying PC induction, as was first demonstrated by Maitani et al. (1996) in *Rubia tinctorum* hairy roots. Furthermore, some analogous new families of PC-related peptides as well as PCs were detected using *Armoracia rusticana* hairy roots exposed to Cd (Kubota et al. 2000). These and other works (Sanità di Toppi et al. 1999; Wu et al. 2001) highlight the valuable contribution of hairy roots to improve understanding of the complex mechanisms triggered by inorganic pollutants. Hairy roots are also a convenient laboratory tool for investigating hyperaccumulation mechanisms of heavy metals. Moreover, they contribute to understanding the role of organic acids in heavy metal detoxification as well as their localization in roots (Boominathan and Doran 2003). In addition, hairy roots of *Alyssum bertolonii* were used for generating a metal-enriched product from the harvested plant biomass, which might be useful for phytomining operations (Boominathan et al. 2004).

Hairy roots have a large surface area in comparison with nontransformed roots, due to their highly branched nature. Therefore, they can also be used for rhizofiltration purposes, i.e., to absorb, concentrate, and/or precipitate hazardous compounds, particularly heavy metals or radionuclides, from aqueous solutions

Table 18.2 Use of hairy root cultures from different plant species to study several aspects of inorganic compound phytoremediation

Plant species	Summary of remediation strategies and/or main aspects studied	References
Rubia tinctorum	Induction of PCs containing Cd and Cu by adding Cd to the culture medium	Maitani et al. (1996)
Daucus carota	Short-term response to Cd mainly in terms of production of PCs and stress proteins, synthesis of stress ethylene, and involvement of lipid peroxidation	Sanità di Toppi et al. (1999)
Armoracia rusticana	A new group of SH-containing peptides as well as PCs were induced after exposure to Cd	Kubota et al. (2000)
Adenophora lobophylla; A. potaninii	A comparative study on Cd detoxification was performed. PCs have been isolated and characterized from hairy roots. Different strategies for Cd detoxification in both species were suggested	Wu et al. (2001)
Thlaspi caerulescens; Alyssum bertolonii	Uptake and storage of Cd and Ni, respectively. Role of organic acids in Cd detoxification; localization of heavy metals in hyperaccumulator and non-hyperaccumulator roots and the distribution of metals. Investigation of the energy source for transmembrane transport of Cd and Ni	Boominathan and Doran (2003)
Brassica juncea	Rhizofiltration of Cd and Pb with high efficiency within a short period of exposure. The influence of high concentrations of these heavy metals on antioxidative enzymes was studied	Eapen et al. (2007)
B. juncea and Chenopodium amaranticolor	Both hairy roots removed U within a short period of incubation. *B. juncea* hairy roots proved to be a better candidate for U removal, as they could take up two to four times more U from the solutions, at concentrations up to 5,000 μM	Eapen et al. (2003)
Solanum nigrum	High efficiency for Zn uptake and accumulation	Subroto et al. (2007)
Daucus carota	A test to determine the threshold toxicity of U in optimal conditions of both exposure and observations was proposed	Straczek et al. (2009)
A. rusticana	High accumulation of U in the presence of phosphate, which had a stimulating effect on growth of hairy roots and accumulation of the pollutant	Soudek et al. (2011)

PCs phytochelatins

(Eapen et al. 2007). For instance, *Brassica juncea*, *Chenopodium amaranticolor*, *Daucus carota*, and *A. rusticana* hairy roots were applied for the removal of high U concentrations (Eapen et al. 2003; Soudek et al. 2011; Straczek et al. 2009). These authors also demonstrated that hairy roots would be appropriate to study toxicity and distribution of U in plant roots in optimal conditions and to examine further physiological processes triggered by radionuclides. A summary of the main applications of hairy roots for inorganic phytoremediation is provided in Table 18.2.

Until now, limited studies were carried out using transgenic hairy roots in order to improve pollutant removal. Nevertheless, encouraging results were obtained. For instance, Banerjee et al. (2002) demonstrated that *Atropa belladonna* hairy roots expressing a rabbit P4502E1 enzyme were able to metabolize trichloroethylene,

whereas transgenic tomato and tobacco hairy roots were used to remove phenol with higher efficiency than non-transgenic roots (Sosa Alderete et al. 2009, 2012b; Wevar Oller et al. 2005).

A very recent and emerging field of research is the application of hairy roots to investigate microbe-assisted phytoremediation of pollutants, which offers important advantages and challenges. As was demonstrated, the association between hairy roots and microorganisms, such as arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria, could positively contribute to the improvement of the phytoremediation process (González et al. 2012b; Ibáñez et al. 2011). Studying these complex relationships at this appropriate scale would probably be the key to answering several aspects that are unknown about rhizoremediation.

In conclusion, the combinatorial use of different strategies and adequate approaches at the laboratory scale would be very helpful to increase tolerance and removal of pollutants and thus to improve the efficiency of phytoremediation in the field.

18.5 Perspectives

Today, hairy root cultures can be induced from any plant species, including rare and threatened plant species (e.g., some medicinal plants), which have a clear ecological benefit and contribute to the biodiversity preservation. The immense biotechnological potential of hairy root cultures to serve as green factories for mass production of high-value plant-derived metabolites and recombinant therapeutic proteins is clearly recognized and acknowledged. In addition, transformed root cultures show promising potential for other ecologically valuable applications, e.g., their utilization for phytoremediation purposes. Although no commercial processes based on hairy root culture have been yet established, there have been many proof-of-concept studies, which allow hairy root-based (bio)processes to be upscaled while keeping their biosynthetic potential. Finally, recent developments in so-called "omics" approaches help us to understand the roots' biochemical machinery and give us a powerful tool for deliberate adjustment of desired metabolites' biosynthetic pathways as well as to improve pollutant removal. Of course, several fundamental and technological challenges still remain to be addressed. It can be concluded that hairy root culture technology is entering an exciting period and will undoubtfully have a bright future for diverse purposes.

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