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MINI-REVIEW

Application of hairy roots for phytoremediation: what makes them an interesting tool for this purpose?

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Abstract In recent years, hairy roots (HRs) have been successfully used as research tools for screening the potentialities of different plant species to tolerate, accumulate, and/or remove environmental pollutants, such as PCBs, TNT, pharmaceuticals, textile dyes, phenolics, heavy metals, and radionuclides. This is in part due to several advantages of this plant model system and the fact that roots have evolved specific mechanisms to deal with pollutants because they are the first organs to have contact with them. In addition, by using HRs some metabolic pathways and enzymatic catalyzed reactions involved in pollutants detoxification can be elucidated as well as the mechanisms of uptake, transformation, conjugation, and compartmentation of pollutants in vacuoles and/or cell walls, which are important detoxification sites in plants. Plant roots also stimulate the degradation of contaminants by the release of root exudates and oxido-reductive enzymes, such as peroxidases (Px) and laccases, that are associated with the removal of some organic pollutants. HRs are also considered good alternatives as enzyme sources for remediation purposes. Furthermore, application of genetic engineering methods and development of microbe-assisted phytoremediation are feasible strategies to enhance plant capabilities to tolerate, accumulate, and/or metabolize pollutants and, hence, to create or find an appropriate plant system for environmental cleanup. The present review highlights current knowledge, recent progress, areas which need to be explored, and future perspectives related to the application and improvement of the efficiency of HRs for phytoremediation research.

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Introduction

The first studies of HRs and their causative agent date from the early 1900s. Riker (1930) made great advances in HR disease and/or syndrome studies and at first named the implicated bacterium *Phytomonas rhizogenes*. Later, the name changed to *Agrobacterium rhizogenes*. From those findings, the disease mechanism of HRs has been exploited to develop a valuable biotechnological application and great progress in the development of HRs cultures has occurred.

This plant tissue culture is originated by the infection of explants with *A. rhizogenes* strains, a Gram-negative soil bacterium. During this process, *A. rhizogenes* transfers the T-DNA, comprising the loci between the T_R and T_L region of the Ri (*root inducing*) plasmid, into the plant genome (Gelvin 2009; Chandra 2012). Several genes of the pRi such as *vir* and those belonging to T-DNA and chromosomal virulent genes (*chv*) are essential for transformation. In particular, *rol* genes present in T-DNA are responsible for rhizogenic growth with the typical massive adventitious roots and abundant root hairs, which is characteristic of the derived in vitro HRs cultures. The molecular basis of genetic transformation of plant cells by *Agrobacterium* strains has been largely studied and recently detailed by Pacurar et al. (2011) and Chandra (2012). These exhaustive studies have permitted it to be used as a natural genetic engineer for gene transfer experiments since not only *A. tumefaciens* is being used for transgenic plants obtainment but *A. rhizogenes* is also being used as a vehicle to obtain transgenic HRs for the improvement of different processes.

HRs cultures have been obtained from many plant species (to date more than 500), mainly dicotyledonous, which

exhibit susceptibility to *A. rhizogenes* infection (Georgiev et al. 2012). However, HRs from new plant species are continually being induced, even from species recalcitrant to transformation, through different strategies. Induction of *vir* genes by the addition of signal molecules (acetosyringone or related compounds) or by *Agrobacterium* cocultivation with exudates of wounded tissues (Stachel et al. 1985), as well as a new technique, based on transformation mediated by sonication (Georgiev et al. 2011), have been used for obtaining HRs from recalcitrant plant species, such as monocotyledons.

HRs have several advantages such as genotype and phenotype stability, fast and indefinite in vitro growth by subculture in absence of phytohormones under sterile conditions, and high production of secondary metabolites. Due to the last characteristic mentioned, HR cultures have been called "phytochemical factories" since their biosynthetic capacity is similar to the native plant root; moreover, they often accumulate phytochemicals at a higher level than undifferentiated cultures. Furthermore, HRs have also been traditionally used for studying root physiology. In a recent review, we presented a compilation of recent patents related to their use for different purposes, or even the scarce and previously existing patents in some aspects, generated regarding the use of HRs (Talano et al. 2012). In addition, great advances have been made in HRs bioreactors design and optimization for large-scale production (Huang and McDonald 2012). Among other interesting biotechnological applications for HRs that have emerged in recent years, we can mention recombinant proteins production (Georgiev et al. 2007), biotransformation of exogenous substrates (Banerjee et al. 2012), molecular breeding (Huang and McDonald 2012), biosynthetic pathway elucidation (Wevar Oller et al. 2009), organic or inorganic pollutants phytoremediation (Georgiev et al. 2012), and rhizoremediation, when the contribution of an associated microorganism is considered (González et al. 2012b), despite the fact that this area has been less explored.

Phytoremediation, using plants to clean up contaminated environments, is a green and an eco-friendly technology which has gained importance in relation to traditional decontamination methods. Although it was first applied for the removal of inorganic pollutants from soils, phytoremediation has gradually proven to be efficient for the treatment of organic pollutants as well. Since then, the development of genomics, proteomics, and metabolomics has contributed to enhance or manipulate plant metabolism of many pollutants, and considerable efforts are being made by scientists, remediation engineers, and environmental professionals in government and industry to develop practical technologies for phytoremediation (Khaitan et al. 2006; Van Nevel et al. 2007). Based on the type of biological mechanisms adopted by plants to remediate contaminants, phytoremediation is classified either as phytoextraction, phytostabilization, phytotransformation, phytovolatilization, rhyzofiltration, or phytostimulation, which have been extensively described by Pilon-Smits (2005) and Abhilash et al. (2009). Generally, plants use a variety of processes that collectively contribute to the overall level of remediation. However, the ability to exploit the potential of plants for environmental remediation is frequently restricted by the limited understanding of plant metabolic pathways, the full range of enzymes involved, and tolerance mechanisms. In this regard, HRs are frequently applied in phytoremediation research as model plant systems since they permit the examination of the intrinsic metabolic capabilities of plant cells and their capacities for toxicity tolerance (Doran 2009).

During the last few years, HRs have contributed to our knowledge of the complex biochemical and molecular mechanisms involved in phytoremediation. In the present mini-review, we try to highlight the most relevant studies related to the application of HRs with the aim of identifying the capacity of plant cells to tolerate, assimilate, detoxify, metabolize, and store a wide variety of organic and inorganic pollutants. The areas where HRs offer the greatest potential as devices for the practical study of phytoremediation processes and mechanisms will be described and recent advances are included. In Fig. 1, a schematic summary of the most relevant studies is shown. Some limitations inherent in using HRs instead of whole plants in research programs will also be discussed.

HRs to improve phytoremediation research

Among several plant-based experimental systems available for phytoremediation research, HRs have proved to be a very useful tool and suitable model systems to study xenobiotic detoxification and the activity of central detoxification enzymes, without the interference of soil matrix and microbes. HRs offer important advantages over dedifferentiated cultures and some culture conditions are well controlled in terms of nutrient and phytohormone levels, light and temperature requirements, etc. In addition, they are physiologically closer to real roots than undifferentiated cell cultures, have a short subcultivation period of 2 or 3 weeks, and give good and stable biomass yield over the whole year without dependence on the season, thus providing a more reliable and reproducible experimental system over time, for phytoremediation purposes (Doran 2009). They have a prolific root growth which is a prerequisite to increase the effectiveness of phytoremediation processes. Moreover, HRs develop in an environment that is totally free of microbial contamination and can be used to distinguish the responses and capabilities of plant cells from rhizospheric microbes.

Roots are the main organ to have contact with environmental pollutants and are also the site where the first

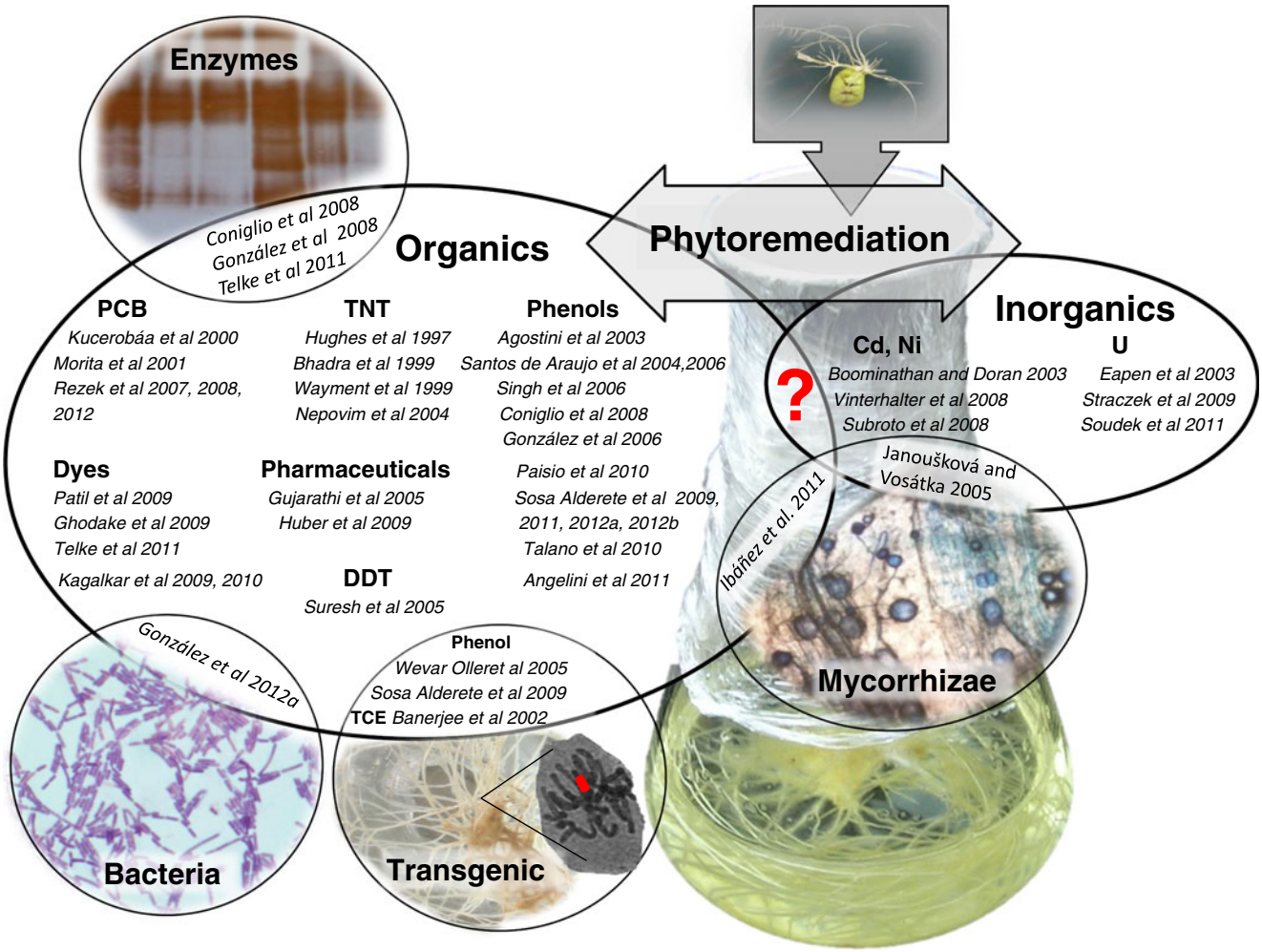

Fig. 1 Schematic summary of the most relevant studies related to phytoremediation processes and mechanisms of organic and/or inorganic compounds removal using HRs. Examples of assisted HR phytoremediation with mycorrhizae and/or bacteria are included, as well as transgenic HR obtained for the improvement of phytoremediation process. The *question mark* (?) indicates an area less/not explored, to our knowledge

reactions against the pollutant take place. Thus, HRs are able to metabolize per se hazardous compounds by common metabolic pathways (Nepovim et al. 2004), which constitute an additional advantage to this plant system. Moreover, the absence of shoots helps in understanding the mechanisms only present in roots, for contaminants remediation, without the effects of translocation.

A summary of the main advantages and disadvantages of using HR for phytoremediation compared with other plant systems are listed in Table 1. Many of these aspects are also extensively reviewed by Doran (2009) and some of them will be described throughout the present mini-review.

Organic pollutants uptake and metabolism

The first step to be addressed in studies of pollutants metabolism is generally the measurement of the rate and/or the capability of uptake of these compounds by plant roots. However, sorption or binding to the root surface and cell walls is frequently observed and it can be estimated in control experiments using dead or inactivated biomass (Dec and Bollag 1994; Santos de Araujo et al. 2006; Coniglio et al. 2008; Sosa Alderete et al. 2009). Uptake is different for organics and inorganics. Organic pollutants are usually xenobiotic to plant roots; as a consequence, there are no transporters for these compounds in plant membranes and they tend to move into and within plant tissues driven by simple diffusion. Some of the factors affecting uptake and distribution of a contaminant within plant cells include (1) physical and chemical properties of the compound [e.g., water solubility, vapor pressure, molecular mass, and octanol–water partition coefficient (K_{ow}), and especially log K_{ow}, where an optimal uptake is reached by compounds with log K_{ow} in the range between 1 and 3.5 (Dietz and Schnoor 2001; Pascal-Lorber et al. 2008)]; (2) environmental characteristics (temperature, pH, organic matter, and soil moisture component);

Table 1 Advantages and disadvantages of using hairy root cultures for phytoremediation research

Advantages	Disadvantages
Independent of site and weather conditions Indefinite propagation and on demand availability	Many aspects of whole plant cultivation cannot be simulated
Permits easily controlled experimental variables, substantially reduced time required to carry out studies and highly reproducible results Large amounts of biomass generated in a controlled setting	Large-scale application could be complicated in some cases
Amenable to incorporate large amounts of contaminants to recover metabolites and intermediates for quantitative analysis and to study their chemical nature and transformation sequence	Requirement of sterile culture conditions to avoid loss of plant cell viability
Permits easy medium manipulation and availability of reaction products, requiring less purification	Requirement of sugars in the culture medium
Ability to grow rapidly in microbe free conditions providing a great surface area of contact between contaminants and roots Clonal selection of species with suitable phytoremediation traits for plant regeneration	Maintenance could be slightly more costly than in the field for some plant species
Greater degree of authenticity than undifferentiated plant tissue cultures with regard to their biological behavior and properties due to their organized nature Amenable to genetic transformation for improving phytoremediation	Requirement of a judicious interpretation of results in order to avoid some experimental artifacts
Ability to produce large amounts of exudates which may contribute to detoxify or sequester harmful pollutants	

and (3) plant characteristics, specific inherent properties of the root itself, and the transport tissues involved (e.g., type of root systems, presence of different types of enzymes, etc.) (Schröder et al. 2008). Thus, uptake and translocation of various organic pollutants can differ among plant species and, thereby, conclusions concerning any contaminant uptake by a particular plant species cannot be applied to others, even to those belonging to the same genus.

Transformation of organic pollutants into smaller, simpler, and, frequently, less toxic compounds is often referred to phytotransformation or phytodegradation. It has been accepted that several enzymes, not necessarily physiologically connected, form a metabolic cascade for the detoxification, breakdown, and final storage of organic xenobiotics (Schröder et al. 2008). Detoxification mechanisms resemble more the reactions in the animal liver than the bacterial metabolism, following the "green liver" model proposed by Sandermann (1994). This network of reactions can be subdivided into three distinct phases: transformation (phase I), conjugation (phase II), and compartmentation (phase III). The last phase is generally categorized into two independent phases, one confined to transport and storage in the vacuole and a second one involving final reactions, such as cell wall binding or excretion (Schröder et al. 2007; Abhilash et al. 2009). The transformation of the initial substrate includes several enzymatically catalyzed reactions (oxidation, reduction, and hydrolysis), which usually take place simultaneously, involving enzymes such as P450 monooxygenases, peroxidases (Px), reductases, dehydrogenases, and esterases. Pollutant transformation increases its solubility and provides an opportunity for conjugation (phase II), with endogenous compounds (mono-, oligo-, and polysaccharides, proteins, peptides, amino acids, organic acids, lignin, etc.). Then, conjugates can be sequestered or compartmentalized, which is known as phase III of pollutant metabolism. Soluble conjugates are accumulated in vacuoles, with the participation of ATP-binding cassette (ABC) transporters (Schröder et al. 2007). Metabolites stored in the vacuoles could be further processed before exportation to the cell wall. However, very little is known about these processes (Pascal-Lorber et al. 2008). Insoluble conjugates (coupled with protein, lignin, starch, pectin, cellulose, xylan, and other polysaccharides) are moved out from cells via exocytosis and are accumulated in the apoplast or cell wall. This may lead to the formation of so-called bound residues because of their inability to be extracted by chemical methods. Hence, the main objective of compartmentation is essentially to remove toxic compounds from metabolic tissues.

It is important to note that organic compounds are rarely mineralized in plants because only a few plant enzymes are able to catalyze ring opening reactions, in contrast to the degradative metabolism of microorganisms (Sandermann 1992; Schnoor et al. 1995; Schröder and Collins 2002). Unlike total breakdown, conjugation does not lead to complete detoxification of the xenobiotic, which preserves its basic molecular structure and hence reduces only partially its toxicity.

Application of HRs to study uptake, metabolism, and removal of organic pollutants

Several reports have demonstrated that HRs derived from different plant species could be used for the treatment of a great variety of organic contaminants (Fig. 1) as will be described in the following paragraphs.

Polychlorinated biphenyls (PCBs) are xenobiotic chlorinated aromatic compounds that have been used in a wide range of applications (electric fluids in transformers and capacitors, pesticide extenders, adhesives, dedusting agents, cutting oils, flame retardants, heat-transfer fluids, hydraulic lubricants, sealants, etc.). Local manufacture, usage, spill, and improper disposal of PCBs have led to extensive environmental contamination. Thus, they have been detected in virtually every compartment of the ecosystem (water, soil, air, and sediments) and living organisms, which can accumulate these pollutants in fat tissues (Van Aken et al. 2010). Therefore, removal of PCBs is of great environmental concern and phytoremediation shows considerable promise for PCBs remediation. In this context, Morita et al. (2001) proposed and patented a remediation process for a medium contaminated with PCBs. They found that *Atropa belladonna* HRs were able to absorb and efficiently decompose a considerably large amount of PCBs, as well as large amounts of dioxins compared to the roots of natural *A. belladonna*, constituting a low-cost treatment.

In order to improve phytoremediation, it is crucial to understand plant metabolism of these compounds. Thus, metabolism of the PCBs was studied in approximately 40 in vitro tissue cultures of different plant species, using the commercial mixture Delor 103, consisting of 59 PCB congeners with an average of three chlorine atoms per biphenyl, as the model pollutant (Kucerova et al. 1999; 2001; Mackova et al. 1997a, b). HRs exhibited a higher potential to degrade PCBs as compared to callus cultures and the HR culture of black nightshade (*Solanum nigrum*), SNC-9O, was shown to have the highest ability to metabolize PCBs. Using these HRs, Kucerova et al. (2000) identified mono- and dihydroxychlorobiphenyls as metabolites of three mono- and six dichlorobiphenyls, which would be the activated PCBs metabolites of phase I transformation, according to the green liver concept previously mentioned.

Similarly, Rezek et al. (2007), using the same HR culture, studied the first-step detoxification products of a wide range of PCB congeners. Monohydroxylated metabolites as well as free non-conjugated metabolites were identified in plant cell biomass. The number of metabolites decreases with an increasing number of chlorine atoms per molecule of PCB. The authors concluded that each plant species, even plant tissue, can have a different potential for metabolizing various PCB congeners and can produce different metabolites. More recently, Rezek et al. (2012) demonstrated that the transformation of PCBs in these HRs resulted not only in known hydroxy-PCBs but also in newly discovered plant metabolites of PCBs: methoxy-PCBs and hydroxyl-methoxy-PCBs (Rezek et al. 2008, 2012). It is also interesting to note that methylation of some hydroxy compounds is known to be a detoxification or deactivation reaction in some organisms. Hydroxy–methoxy-PCBs were present preferentially as conjugates; only minor amounts as free metabolites and surprisingly neither free nor conjugated dihydroxy-PCBs were detected. Thus, the methoxy-PCBs should be recognized as another group of metabolites formed from PCBs in plant tissues, although cellular localization, biochemical pathways, and potential toxicity remain unexplained. This is an important issue to be addressed because little knowledge exists on the impact of these compounds and the lack of knowledge regarding these impacts presents new challenges in the study of plant metabolism of these pollutants.

The phytoremediation of explosive compounds is another area of great interest to reduce the levels of these contaminants, which have serious environmental risks. Among them, 2,4,6-trinitrotoluene (TNT) is the most widespread and persistent compound, and most research was concentrated on elucidating the capability of plants to transform this compound. In particular, HRs have contributed to the understanding of the transformation pathways of nitroderivatives. For instance, an important basic research project focused on the identification of the transformation products, their chemical nature, and the sequence of transformation that governs removal, which was performed using periwinkle (*Catharantus roseous*) HRs, an in vitro culture able to take up and transform TNT into dinitroamino-derivatives (Hughes et al. 1997; Bhadra et al. 1999). HRs also provided information about the conjugation of TNTs monoamine-derivatives as a significant pathway during plant metabolism of TNT, which was consistent with the green liver model (Bhadra et al. 1999; Wayment et al. 1999). Using periwinkle HRs, Bahdra et al. (1999) confirmed the formation of soluble, i.e., extractable, conjugates, which appears to be the gateway to the formation of bound residues of the TNT-derived conjugates. They showed that bound residues increased as the conjugates turn over. The distribution of conjugates formed via monoamine derivatives of TNT may be a function of several factors, including the starting xenobiotic type and/or level. As HRs are axenic cultures, all results obtained are indicative of the potential of plant roots to metabolize TNT and do not include any significant microbial or symbiotic relationships. These results can also be extended to whole plants because it is generally known that the spectrum of resulting metabolites in plants and plant cell cultures is in principle identical, although quantitative differences may occur. However, for practical application, further in vivo experiments under real conditions are advisable.

Through comparison of different plant species, a great variation was observed in the fate of TNT and other derivatives, and different concentrations of degradation products could be found, which would be an indication that the metabolism of TNT is controlled by different enzymatic systems, which may have variable substrate specificity, in different plant species. These findings reinforce the importance of

screening and studying different plant species for TNT degradation in order to select the most suitable candidates for phytoremediation. In this context, HRs were shown to be excellent candidates for these purposes because if a compound is metabolized by in vitro HRs, this is a clear indication that the plant has the genetic capacity to biotransform this compound (Doran 2009).

In addition, Nepovím et al. (2004) used horseradish (*Armoracia rusticana*) HRs as a model to explore not only plant metabolism of explosives viz. 2,4-dinitrotoluene (DNT), 2,4,6-trinitrotoluene (TNT), aminodinitrotoluenes (ADNTs), and diaminonitrotoluenes (DANTs) but also to analyze the effect of these pollutants on the activity of enzymes related to pollutants metabolism such as glutathione *S*-transferase (GST) and Px. The presence of some nitroderivatives in the medium caused induction of Px activity whereas the GST activity was significantly affected either by the concentration and/or short- or long-term treatment of roots by DNT, TNT, and by their degradation products ADNT and DANT. Thus, they concluded that nitroamino-derivatives would cause severe stress to plants leading to highly effective defense reactions.

All these studies, related to the elucidation of TNT transformation pathways, helps in developing quantitative models for removal of explosives and aids in understanding the kinetics of uptake and removal. Moreover, these findings open up the possibility of new genetic and biochemical approaches to the study of TNT transformation pathways. However, further work on the toxicity of the final products and the effect on the ecosystem are needed.

Pharmaceuticals and their metabolites are detected in the aquatic environment and in drinking water. Based on ecotoxicological studies, some of these compounds like *N*-acetyl-4-aminophenol (paracetamol) and antibiotics are classified as harmful to aquatic organisms. However, there is little information available on the fate and detoxification of pharmaceuticals in plants. In this sense, Huber et al. (2009) investigated the fate and metabolism of paracetamol, a widely used antipyretic agent, in plant tissues, using HRs of *A. rusticana* L. as a model system. Using this culture seemed favorable because transport, storage, and distribution phenomena in a whole plant would be far more complex and require special discussion. They found that HRs were able to take up and detoxify the model substrate paracetamol. In addition, a paracetamol–glucoside was described as the dominant metabolite, which would be a precursor to insoluble, bound residues, probably associated to the lignin fraction of the cell wall, leaving the xenobiotic compound in a stable and indigestible form. Thus, these results marked the beginning of understanding the fate of acetaminophen in plants and provided new insights to give recommendations for the removal of the pharmaceutical from waste water by phytotechniques (Huber et al. 2009). Further, sunflower (*Helliantus annus*) HRs as well as their root exudates catalyzed the rapid disappearance of tetracycline and oxytetracycline from aqueous media, demonstrating the potential of these HRs for phytoremediation of these two antibiotics in vivo. The chemical mechanism of degradation was studied and the involvement of reactive oxygen species (ROS) in the antibiotic modification process was suggested (Gujarathi et al. 2005).

Currently, more than 2,000 different azo dyes are used to dye various materials such as textiles, leather, plastics, cosmetics, and food. These industrially produced chemicals are all xenobiotic compounds that are very recalcitrant to biodegradative processes (Stolz 2001). Some of the azo, xanthene, and anthraquinone dyes are known to be very toxic and mutagenic to living organisms and their discharge into water bodies may lead to serious health problems and also produce acute and chronic effects on aquatic life (Khandare et al. 2011). The removal of textile dyes mediated by plants has been one of the most neglected areas of phytoremediation research, and the knowledge of the basic mechanisms and pathways involved in the decolorization of dyes is limited (Govindwar and Kagalkar 2010). Thus, the use of in vitro cultures for phytoremediation studies can help to explore the enzymatic status and the metabolism products of dyes. In this context, very few reports on phytoremediation of these pollutants using HRs are available. For instance, Marigold (*Tagetes patula* L.) HRs were selected among few HR cultures from other plants tested for decolorization of Reactive Red 198, and they were able to remove dye concentrations up to 110 mgL^{-1}. These HRs could be successively used for at least five consecutive decolorization cycles. A possible pathway for the biodegradation of Reactive Red 198 was also proposed and some metabolites such as 2-aminonaphthol, *p*-aminovinylsulfone ethyl disulfate, and 1-aminotriazine, 3-pyridine sulfonic acid were identified after treatment. Furthermore, a phytotoxicity study demonstrated the non-toxic nature of the extracted metabolites (Patil et al. 2009). These HRs were also able to decolorize other dyes, such as Methyl orange. However, Telke et al. (2011) have recently demonstrated that *Brassica juncea* L. HRs pose high potentiality towards the degradation of textile dyes, and it would be more efficient than *T. patula* HRs for decolorization of Methyl orange, showing an efficiency of 92 % after 4 days. The decolorization of textile dyes using plants or HRs is associated with the involvement of intracellular enzymes, such as laccase, lignin peroxidase, tyrosinase, and NADH–DCIP reductase (Ghodake et al. 2009; Patil et al. 2009; Kagalkar et al. 2009, 2010). Therefore, Telke et al. (2011) reported the purification and characterization of an intracellular laccase from *B. juncea* HRs and its application for the removal of textile dyes. The dye decolorization rate of this laccase was significantly enhanced in the presence of various redox mediators and

2, 2′-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was found to be the most efficient one. Thus, the laccase–ABTS system could be considered useful for the treatment of textile dyestuffs. Such results demonstrated another advantage of HRs for phytoremediation since enzymes and/or other compounds from root exudates can be used to detoxify or sequester harmful pollutants.

Phenols are among the major organic contaminants found in effluents of coal conversion processes, coke ovens, petroleum refineries, manufacturing of phenolic resins, herbicides, fiberglass, and petrochemicals. Phenolic contaminants can also be introduced into the environment via pesticide applications and as a result of partial degradation of aromatic organic contaminants. They represent a threat to human health and can also produce lethal and teratogenic effects on several species (Agostini et al. 2011). In recent years, phytoremediation technologies have emerged as potential alternatives for detoxification of these harmful pollutants. In this context, HRs derived from different plant species were successfully applied for removing phenol and/or chlorophenols from aqueous solutions and, in some cases, for testing the ability of plants to tolerate high levels of these pollutants. For instance, Singh et al. (2006) screened some plant species viz. *B. juncea*, *Beta vulgaris*, *Raphanus sativus*, and *Azadirachta indica*, finding that *B. juncea* showed the highest potential (97 %) for phenol removal. Similarly, Santos de Araujo et al. (2006) investigated phenol and chlorophenols susceptibility in HRs of various plant species, demonstrating that HRs derived from the ornamental plant *Solanum aviculare* would be the best for remediation of these pollutants. Furthermore, HRs from *Brassica napus*, *Solanum lycopersicon*, and *Nicotiana tabacum* were successfully used to remove phenol and/or 2,4-DCP from aqueous solutions, and the optimal conditions (pH, temperature, concentration of co-substrate, and time of treatment) for the removal process were established (Coniglio et al. 2008; González et al. 2006, 2008, 2012a; Sosa Alderete et al. 2009, 2012b). Moreover, HRs could be re-used for the treatment of solutions containing phenolics, in several consecutive cycles, with high efficiency (Agostini et al. 2003; Talano et al. 2010). Even though HRs derived from different plant species are capable of degrading the same phenolic compound, the products formed after degradation are likely to be different, indicating that the pattern of transformation of the xenobiotic molecule is dependent upon the plant species. Thus, toxicity of final degradation products is another important feature to be addressed because transformation into non-toxic products is preferable for phytoremediation and HRs can help the researchers in such studies. For instance, after phenol treatment using HRs, the toxicity of some post-removal solutions was evaluated by means of AMPHITOX and *Allium cepa* tests, and significant reduction of toxicities were observed. Moreover, the addition of PEG-3350 was efficient to reduce toxicity of post-removal solutions treated with rapeseed HRs (Paisio et al. 2010; González et al. 2012a).

Clearly, the ability of plants to metabolize contaminants will depend on the biochemical characteristics of metabolizing enzymes and other protective mechanisms that may prolong tissue survival. In this sense, HR cultures also supply valuable information about the enzymes related to pollutant metabolism, like Px isoenzymes, which are described as the main enzymes involved in the removal of phenolic compounds. Through the use of different HRs, it was suggested that Px isoenzymes may show variation in substrate preference and catalytic efficiency towards phenol (Coniglio et al. 2008; González et al. 2008; Sosa Alderete et al. 2012b). For instance, Agostini et al. (2003) and Coniglio et al. (2008) suggested the major participation of acidic peroxidases from *B. napus* HRs for 2,4-DCP as well as for phenol removal. By contrast, González et al. (2008) found that the group of basic peroxidase isoenzymes from tomato HRs would be the most likely involved in 2,4-DCP and phenol removal, whereas Sosa Alderete et al. (2012b) found similar results using tobacco HRs. As it is well known, in the phenol removal process, Px can be inactivated by three distinct mechanisms (Coniglio et al. 2008). However, the addition of protective compounds like polyethylene glycol (PEG) could decrease the adsorption of polymers onto the enzyme's active site and increase the lifetime of the active enzyme, increasing phenol removal efficiency as well as retaining post-removal Px activity, as was shown using Px isoenzymes from tomato HRs (González et al. 2008). It is noteworthy that the studies in establishing and understanding the enzymatic mechanism of contaminant degradation are important for the selection of candidate enzymes that might be produced in large amounts and used as catalysts for contaminant breakdown (González et al. 2006). In this context, Santos de Araujo et al. (2004) studied the kinetic behavior of Px pools from HR extracts of carrot, sweet potato, and kangaroo apple using phenol, catechol, 2-CP, and 2,6-DCP as model pollutants in order to compare their ability to detoxify phenols. In addition, interesting results have been obtained with HRs as a source of Px isoenzymes demonstrating that some purified or partially purified enzymes may behave as powerful catalysts in the remediation of harmful phenolics (Santos de Araujo et al. 2004; González et al. 2008; Coniglio et al. 2008; Talano et al. 2010; Sosa Alderete et al. 2012a). However, in order to implement an enzyme-based treatment for phenol removal, isolation, purification, and production costs should be considered. Thus, the use of plant materials, such as root tissues as enzyme sources, constitutes a good alternative. In this sense, HRs offer an attractive system for this purpose because they produce and also exude enzymes, like Px, that can remove organic pollutants, such as phenolics. Moreover,

they provide a greater surface area of contact between contaminant and tissue, and could be used for the removal of phenolics on a large scale in bioreactors as was described by Angelini et al. (2011).

In addition, HRs offer the possibility to determine the nature and the compartmentalization of some of the final products of the removal reaction (Talano et al. 2010) and to examine further biochemical and physiological processes such as the antioxidative stress responses and lipid peroxidation after pollutants exposition (Sosa Alderete et al. 2011). Recently, it was shown that phenol treatment may induce changes in the phospholipid turnover and an increase in the phospholipase D (PLD) activity in tobacco HRs. The participation of minor phospholipids, mainly phosphatidic acid, in the activation of intracellular mechanisms that might be important in the response of plant tissues to phenol treatment was suggested (Sosa Alderete et al. 2012b). Thus, such results demonstrated that HRs were able to offer a better understanding about the signaling mechanisms involved in the response to environmental pollutants.

Understanding the physiological and biochemical processes, types of enzymes, and genes involved is important in suggesting not only a mechanism for transformation but perhaps also a way of improving efficiency by generating genetically modified plants with the sole purpose of transforming pollutants. Furthermore, through the application of foreign genes, new routes of degradation may be emphasized resulting in less toxic metabolites than those produced by the existing pathway. Although limited studies were carried out using transgenic HRs in order to improve pollutants removal, encouraging results were obtained. For instance, Banerjee et al. (2002) demonstrated that *Atroppa belladonna* HRs expressing a rabbit P4502E1 enzyme were able to metabolize trichloroethylene whereas transgenic tomato and tobacco HRs expressing basic Px were capable of removing phenol with a higher efficiency than wild-type HRs (Wevar-Oller et al. 2005; Sosa Alderete et al. 2009). Another potential advantage of HRs would be the possibility to produce enzymes in root exudates through the expression (or overexpression) of secretory enzymes involved in pollutants removal or through the expression of heterologous enzymes with signal sequences to drive them to the secretory pathway. However, these aspects have not yet been fully explored.

Inorganic pollutants uptake and metabolism

Contamination of the environment by inorganic pollutants such as heavy metals, metalloids, and radionuclides has become a great problem in areas of intense industry and agriculture, and leads to their bioaccumulation in the food chain (Rajkumar and Freitas 2009). Sources of anthropogenic inorganics contamination include smelting of metalliferous ore, electroplating, gas exhaust, energy and fuel production, application of fertilizers and municipal sludges to land, and industrial manufacturing, among others (Prasad 2007). Roots are usually able to uptake these pollutants by a process known as phytoextraction, which refers to the physical removal of contaminants, and promote long-term cleanup of soil or wastewater (Sas-Nowosielska et al. 2008). The uptake of these inorganic pollutants by plant cells is selective and depends greatly on the redox state and chemical speciation as well as the plant species. Unlike many organic contaminants that can be completely degraded, inorganics are indestructible and, hence, they cannot be degraded chemically or biologically. For this reason, the mechanisms involved in their uptake and metabolism are different (Wu et al. 2010). Inorganics are taken up via membrane transporter proteins. These transporters are present in plant cells because some inorganic pollutants are either nutrients themselves (such as nitrate, phosphate, manganese, and zinc) or are chemically similar to nutrients; thus, they are incorporated inadvertently (e.g., arsenate is taken up by phosphate transporters, selenate by sulfate transporters) (Shibagaki et al. 2002). In the sequestration, transport, and translocation of inorganics, phytochelatins (PC) and metallothioneins (MT) play an important role. Certain metals and metalloids may be complexed by phytochelatins, which are small peptides capable of an efficient sequestration of multiple metal and metalloid ions in metal(loid)–thiolate complexes (Cobbett 2000; Clemens 2006). Their synthesis starts by the activation of the enzyme phytochelatin synthase. MT, another class of metal chelating molecules, are low molecular weight, cysteine-rich proteins that have a high affinity for binding metal cations, such as Cd, Cu, and Zn (Goldsbrough 2000; Cobbett and Goldsbrough 2002). Once inorganic pollutants have been chelated, they may be stored in the vacuole or exported to the shoot via the xylem. The compartmentalization of metal ions in the vacuolar compartment is carried out by means of specialized transporters, and the best-characterized system involves a family of ATP-binding cassette (ABC) transporters (van der Zaal et al. 1999). However, when inorganic pollutants are accumulated in tissues this can often affect cell structure, causing oxidative stress and replacing essential nutrients (Taiz and Zeiger 2002). In this sense, metal hyperaccumulator plant species are of particular interest for phytoremediation since they are capable of taking and storing high concentrations of heavy metals without developing toxicity symptoms. Although great progress has been made, the biochemical and physiological mechanisms of metal uptake, tolerance to high metal concentrations, and the exact roles that high levels of metals play in the survival of hyperaccumulators are still a matter of debate.

Application of HRs for inorganic pollutants remediation

HRs have proven to be effective tools for studying the mechanisms of metal uptake, accumulation, and tolerance

in relation to metal hyperaccumulation and phytoextraction. They have also been used as a model system to investigate the physiology and biochemistry of metal accumulation in plants (Doran 2011). In this sense, Cd and Ni uptake, distribution, and hyperaccumulation mechanisms were investigated in HRs from *Thlaspi caerulescens* and *Alyssum bertolonii*. These HRs remained healthy and grew well with high concentrations of these heavy metals (Boominathan and Doran 2003). In addition, a systematic comparative study on Cd response was carried out using *Adenophora lobophylla* and *Adenophora potaninii* HRs demonstrating that these two closely related species might employ different strategies for Cd detoxification, i.e., the first one was capable of synthesizing high levels of PC while a Cd exclusion system was described in *A. potaninii* (Wu et al. 2001). Vinterhalter et al. (2008) reported high Ni tolerance and accumulation up to 24.7 $\mu g g^{-1}$ dry weight in *Adenophora murale* HRs, whereas Subroto et al. (2007) studied Zn uptake and accumulation by two cultures of *Solanum nigrum* HRs. These authors demonstrated that both cultures were capable of growing with Zn 13.98 $mg l^{-1}$ and accumulated up to 98 % and 90 %, respectively, within 15–18 days of the culture period. Following the demonstration that HRs of *A. bertolonii* can hyperaccumulate Ni, HRs of this species were 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).

In addition to potential practical applications, hyperaccumulating HRs have been useful in physiological studies for understanding the role of roots in heavy metal and radionuclide uptake and accumulation by plants (Soudek et al. 2006). Furthermore, HRs, due to their highly branched nature, have a large surface area in comparison with control roots and can also be used for rhizofiltration purposes. Rhizofiltration can be defined as the use of plant roots to absorb, concentrate, and/or precipitate hazardous compounds, particularly heavy metals or radionuclides, from aqueous solutions (Pilon-Smits 2005). For example, *B. juncea* and *Chenopodium amaranticolor* HRs were applied for removal of U from a solution with a concentration up to 5,000 μM in a short period of incubation. As a result, more than 98 % of U disappeared from the medium in the presence of phosphates compared to 86 % of U removal from the medium without phosphates (Eapen et al. 2003). In addition, Soudek et al. (2011) described the ability of *A. rusticana* HRs to accumulate U and found that the presence of phosphate has a stimulating effect on both the growth of culture and the accumulation of this pollutant. Moreover, Straczek et al. (2009) proposed a test to determine the threshold toxicity of U using carrot HRs. This in vitro device seems to be appropriate to study toxicity and distribution of U in plant roots in optimal conditions, and it was recommended to examine further physiological processes (effect on stress enzymes, genetic material, lipid peroxidation, etc.) and the influence of microorganism interactions because HRs play an important role of providing optimum conditions for root colonizing bacteria.

Understanding plant–microbe interactions for phytoremediation: studies using HRs

Increasing evidence suggests that any phytoremediation system is greatly affected by the interaction of the plant with endophytic or rhizospheric communities of free-living or symbiotic microorganisms, including bacteria and fungi, and the surrounding polluted soil medium. In fact, the success of this microbe-assisted phytoremediation approach requires the interaction of the plant with microbial communities at the molecular and ecological levels. An understanding is needed of these soil–plant–microbe interactions to determine the fate of contaminants in the soil–plant ecosystem. The metabolic capacity of microbes is extremely diverse and greater than that of plants alone; thus, their combination would be a better approach to reach complete remediation of contaminated environments.

Lately, several authors have reviewed the advances in this field and they have even suggested potential applications of some strategies to achieve multiple environmental, economic, and social benefits from degraded environments (Abbilash et al. 2012). Some authors have focused on organic (Karthikeyan and Kulakow 2003), inorganic (Rajkumar et al. 2012; Bhargava et al. 2012; Ma et al. 2011), or both types of pollution (Kuiper et al. 2004). This section presents an overview of some plant–microbe interactions as affected by organic and inorganic contaminants, as well as the role of HRs in the study and elucidation of the biochemical and physiological mechanisms involved.

HRs have been previously used to study nutrient uptake, elicitation processes in root–microbe interactions such as rhizobial and mycorrhizal symbioses as well as pathogenic interactions. When studying nodulation, however, those cultures are not useful because nodule development requires shoot photosynthates (Ding and Oldroyd 2009; Clemow et al. 2011). In this sense, the development of chimeric "composite" plants that have transformed "hairy" roots but a wild-type shoot have proven to be useful in studies of root nutrient uptake, hormone transport, and rhizobial and mycorrhizal symbioses (Sinharoy et al. 2009; Rodríguez-Llorente et al. 2010; Pavli et al. 2010). However, the application of HRs to investigate microbe-assisted phytoremediation is very recent.

Application of HRs to study microbe-assisted phytoremediation of pollutants, which is indeed a rhizoremediation process, is an emerging field that offers important advantages, such as a simplified environment that allows the study

of a specific microbe–plant association avoiding other positive or negative microbe interactions.

Regarding microbe-assisted phytoremediation, research has focused in the last few years on endophytic bacteria and fungi (Doty 2008). Endophytes are microbes that inhabit the internal plant tissues without causing harm to the host (Kuklinsky-Sobral et al. 2004). Sufficient data confirms their ability to degrade xenobiotic compounds (Germaine et al. 2006; Van Aken et al. 2004) and remove heavy metals from a solution (Lodewyckx et al. 2001). The presence in plant tissues of naturally recruited or genetically engineered endophytic bacteria can result in manifold increases in the capacity to degrade xenobiotic compounds compared with uninoculated plants (Barac et al. 2004; Germaine et al. 2006). However, to our knowledge, to date there are no reports describing its combination with HR cultures to study the phytoremediation process. At this point, it is important to mention the need for particular care in establishing truly axenic plant cultures for the purposes of identifying the intrinsic metabolic or metal accumulation capacity of plant cells because of the difficulty of removing these bacteria using surface sterilization.

On the other hand, arbuscular mycorrhizal fungi (AMF) are beneficial microorganisms intimately associated with plant roots that increase the uptake of nutrients, especially phosphorus. Thus, the plant–AMF partnership has been used to enhance phytoremediation of contaminated substrates and to reduce the concentration of toxic compounds. It has been shown that the interaction of *Pteris vittata*–AMF can increase arsenic uptake in this hyperaccumulating fern (Trotta et al. 2006). It has also been reported that the symbiosis with AMF has been suggested to confer a benefit to plants growing in soils with toxic Cd concentrations, though its contribution to plant resistance and the underlying mechanisms are as yet unclear (Meharg and Cairney 2000). Inoculation of carrot HRs with the AMF *Glomus intraradices* alleviated growth inhibition induced by toxic concentration of Cd compared to the non-inoculated control (Janoušková and Vosátka 2005). In a similar study, colonization of transgenic tobacco HRs with *G. intraradices* efficiently protected roots against phenol-induced oxidative damage (Ibañez et al. 2011). Therefore, the plant–AMF partnership could be used to enhance phytoremediation of contaminated substrates and to reduce the concentration of toxic compounds. These results clearly demonstrate the utility of using inoculations of AMFs to increase phytoremediation potential.

Finally, the potential contribution of plant-growth-promoting rhizobacteria (PGPR) to reduce environmental pollution has been recently reviewed (de-Bashan et al. 2011). Regarding HRs, they have been used for the discovery and acquisition of effective elicitors and growth-promoting substances from PGPR bacteria (Zhao et al. 2010). Very recently, in our laboratory, the association of *B. napus* HRs with *Burkholderia kururiensis* KP 23 and *A. rhizogenes* LBA 9402 has been used to evaluate and compare phenolic compounds tolerance and degradation. The presence of both rhizospheric microorganisms, along with *B. napus* HRs, enhanced phenol degradation compared to *B. napus* HRs alone (González et al. 2012b). Such data suggest that the association between microorganisms and HRs could positively contribute to the improvement of the phytoremediation process. However, more research is needed to fully understand these complex relationships.

Conclusion

HRs should be considered as optimal and effective model systems which may contribute to address some limitations of phytoremediation studies and may help the research community to understand the complex interactions between toxic chemicals, plant cells, and microorganisms to clean up the environment. They have proven to be appropriate for the study of uptake and tolerance, pollutants metabolism, key enzymes involved, toxicity, antioxidative stress responses to toxic compounds, and signal transduction pathways. In vitro, HRs are also very useful to predict the responses of plants to environmental contaminants and to improve the design and reduce the cost of subsequent conventional whole-plant experiments. Furthermore, a better understanding of molecular and ecological mechanisms of plant–microbe interactions can provide the scientific basis for developing new strategies for more effective phytoremediation. Since bacteria and other microbes, such as AMF, help indirectly in better absorption and remediation of organics and inorganics, studies in this direction would also help to develop suitable remediation techniques.

However, emphasis should be placed on evaluating results obtained in simplified experiments, such as those performed with these in vitro cultures, as well as those carried out with hydroponics or pot plants, on applying these findings to heterogeneous and polluted field sites, and also on the functioning of phyto-/rhizoremediation systems under various ecological conditions.

Although an extensive knowledge is now available on genes and enzymes involved in pollutants removal, one of the most important challenges is how to use this basic scientific information to improve the efficiency of phytoremediation in the field. This could imply the obtention of genetically modified plants and/or the use of efficient microorganisms for rhizoremediation. In addition, it is expected that considering the recent advances in genetics, proteomics, and metabolomics, novel detoxifying enzymes could be identified and expressed into plants allowing the host plant to have a wider range of phytoremediation capabilities. Furthermore, genetic

engineering could be useful not only to obtain plants for a better pollutant transformation but also to mineralize some complex compounds, which are difficult to metabolize by plant cells. In this sense, microorganism may also be used to avoid this difficulty because they have enzymes to fully break down organic pollutants. These plant improvements may have great potential for field applications, assuming public acceptance of the use of more genetically modified organisms.

Therefore, studies should be focused on the optimization of pollutants tolerance and metabolism as well as on the enhancement of pollutants uptake and degradation capabilities in order to improve phytoremediation processes which is an important challenge of current research.

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