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Special Issue on Hairy Roots (A. Lorence and F. Medina-Bolivar, co-editors)
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Hairy Roots: From High-Value Metabolite Production to Phytoremediation

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ABSTRACT

Environmental pollution is a global concern that is threatening the well-being of all life forms including humans. The cost of cleaning up contaminated sites is high and phytoremediation, the use of plants for removal of environmental pollutants, offers an attractive option due to its low cost and safety of implementation. The hairy roots technology has potential to become an excellent platform for studying numerous aspects encompassing phytoremediation. This is because hairy roots can be grown in large mass in culture media in a controlled environment and can therefore be subjected to various physiological assays. Also, these transformed roots are amenable to genetic manipulation and may facilitate the characterization of genes that influence the phytoremediation capacity of plants. This idea is well supported by the recent success in the development of transgenic plants for use in phytoremediation. Thus, hairy roots offer a good opportunity for the initial assessment of transgene efficacy in phytoremediation. Also, in the near future, hairy roots might be developed into initial screens for plants with enhanced capacity for phytoremediation. This review highlights the recent advances in the use of hairy roots to assess plants for their potential in removing important water and soil pollutants such as metals, explosives, radionuclides, insecticides, and antibiotics.

Environmental pollution is a global concern

Environmental pollution is a global problem that affects both the developing and developed countries (Suresh and Ravishankar, 2004). To a large extent, both human and natural processes contribute to environmental pollution and contaminants are commonly classified as either organic or inorganic. Organic contaminants are a result of human activities including oil spills, military explosives, agriculture, fuel production, and wood treatment (Pilon-Smits, 2005). Common organic pollutants such as trichloroethylene (TCE), herbicides such as atrazine, explosives such as trinitrotoluene, petrochemicals such as benzene, toluene, polycyclic aromatic hydrocarbons, polychlorinated biphenyls (PCBs), and the fuel additive methyl *tert*-butyl ether may contaminate soils and water (Xingmao and Burken, 2003; Pilon-Smits, 2005; Rentz et al., 2005; Suresh et al., 2005; González et al., 2006). In general, inorganic contaminants originate from either natural processes of soil weathering or human activities including agriculture and mining (Pilon-Smits, 2005). Subsequently, both natural and human activities may promote the release of heavy metals e.g. manganese, lead, copper, zinc, molybdenum, mercury, and nickel into soils and water posing a health threat to livestock and human populations (Nedelkoska and Doran, 2000a). For example, mercury is an important health concern to populations that rely heavily on the consumption of fish as a protein source (Hajeb et al., 2008), and to a large extent all global water bodies face the threat of mercury contamination (Harris et al., 2007).

Plants are used to remove environmental contaminants

The health consequences due to environmental pollution are dire and the cost of cleaning up contaminated sites is high (Kuiper et al., 2004; Doty, 2008). Therefore, the use of plants to absorb, stabilize and degrade contaminants, collectively referred to as phytoremediation, is gaining acceptance as a more cost-effective alternative to other cleanup approaches. Phytoremediation is a technology that has been extensively reviewed (for recent reviews see Suresh and Ravishankar, 2004; Pilon-Smits 2005, and Doty, 2008). Our intention here is not to duplicate the efforts of the experts in the field, but instead we will concentrate this review on the potential of hairy roots as a powerful tool to study the phytoremediation capacity of plants.

The process of contaminant extraction by plants and the subsequent fates of the contaminant are described in Figure 1. Plant roots may act as a conduit for the absorption of a contaminant which is then translocated through the vascular system and concentrated in plant harvestable tissues in a process called phytoextraction (Doty, 2008). In addition, roots may provide a haven for microbial growth by secreting exudates that in turn act as a source of nutrition for the microbes and also serve as important cues for enhancing plant-microbe interactions (Bais et al., 2006). The resulting rhizospheric interactions may enhance the biodegradation of organic contaminants in a process referred to as phytostimulation (Pilon-Smits, 2005 and references therein). Prior and after entering the plant via the root system, the contaminant may become target for degradation by either secreted or internal plant enzymes in a process called phytodegradation (Boominathan et al., 2004; Doty, 2008). The phytoremediation of some organic contaminants (e.g. TCE) is influenced by its concentration and the rate of transpiration, and TCE may be released from the plant through volatilization (Xingmao and Burken, 2003). Thus, in phytoremediation plants are used to facilitate optimum conditions for microbial break down of contaminants and to extract contaminants which may be metabolized or sequestered inside the plant (Boominathan et al., 2004; Tamaoki et al., 2005). Even though the rate of detoxification of organic contaminants in plant tissue is slow (Van Aken, 2008), the rising costs of physicochemical cleanup methods of contaminated sites makes phytoremediation a more attractive alternative (Doty, 2008 and references therein).

In order to mitigate the downward-migration of contaminants to the below-ground water reservoirs and lateral movement of contaminants via runoff and wind erosion, fast-transpiring trees e.g. poplar (*Populus* sp.) are grown together with grasses resulting in phytostabilization of contaminants (Pilon-Smits, 2005). Therefore, in phytoremediation, plants provide dual benefits; they play the role of providing optimum conditions for root colonizing bacteria and also provide a simple and cost-effective way of extracting contaminants (Suresh and Ravishankar, 2004). Since roots are the primary contact between plant tissues and contaminants in the soil or water they provide a key point for assessment of the phytoremediation potential of a particular plant species. The underground portion of a plant system where roots are in contact with the micro biota is referred to as the rhizosphere (Walker et al., 2003) and the interaction among plant, microbes and mycorrhizal colonies is regulated to a large extent by root exudates (Walker et al., 2003; Bais et al., 2006). To that regard, root exudates are an essential component for pollutant degradation by microbes in the rhizosphere, and rhizosphere processes are thought to be essential for facilitating the uptake of contaminants by plants (Rentz et al., 2005). Therefore, the root environment and interactions among roots and microorganisms are key aspects to consider in phytoremediation (Barea et al., 2005).

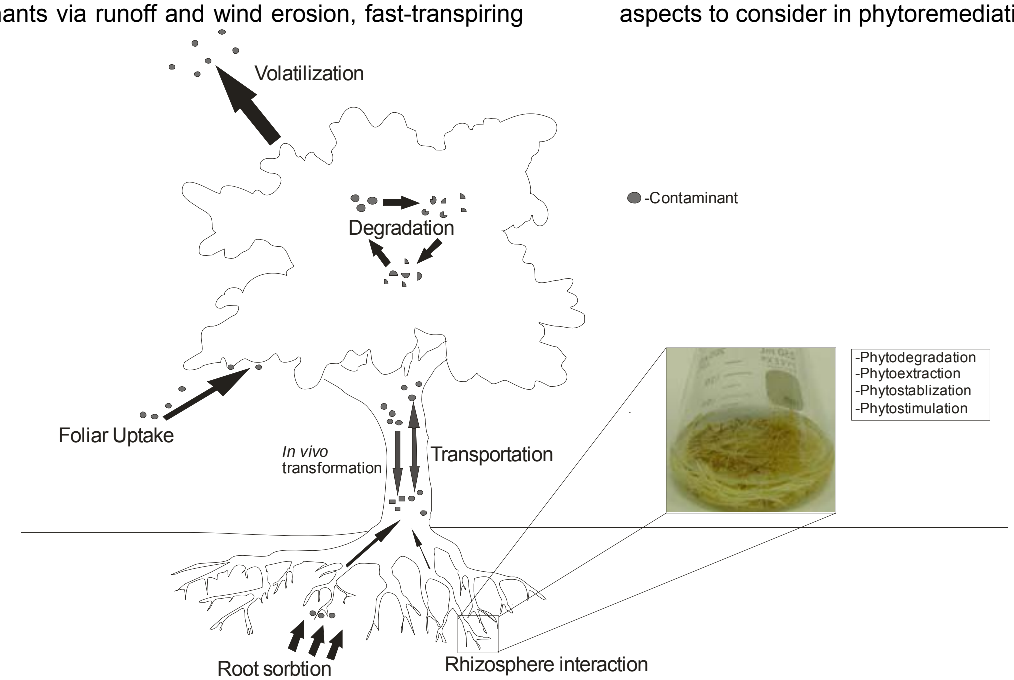

Figure 1. Uptake and metabolism of environmental contaminants by plants: Contaminants can be absorbed by roots and foliage, transformed and degraded *in planta*, or volatilized into the atmosphere; rhizosphere interactions may also contribute to extraction and degradation of contaminants during phytoremediation. Hairy roots are a powerful tool to study various key processes that impact the overall phytoremediation capacity of plants, i.e. the rate of pollutant degradation, extraction, or stabilization. Hairy roots can also be used to study how root exudates may stimulate the degradation of particular contaminants.

Hairy roots biotechnology for valuable metabolite production

Hairy roots are fine fibrous structures that are formed on plant tissues infected by *Agrobacterium rhizogenes*, a soil bacterium responsible for the root mat disease (Georgiev et al., 2007; Veena and Taylor, 2007). After infecting the cells, *A. rhizogenes* stably transfers several of its genes to the plant genome resulting in physiologic changes in the host cell leading to enhanced growth in hormone-free media (Srivastava and Srivastava, 2007). The observed changes in root physiology and morphology are associated with the transfer of a cluster of genes from the *A. rhizogenes* large Ri (root-inducing) plasmid into the plant genome. The symptoms

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observed with *A. rhizogenes* infection may suggest that the transformed cells have been rendered more sensitive to auxin without altering the production of these plant hormones (McAfee et al., 1993; Srivastava and Srivastava, 2007).

Humankind has tapped into the plant natural products reservoir not only for nutritional needs, but also for medicinal and aesthetic purposes (Srivastava and Srivastava, 2007). However, to a high degree most valuable plant natural products are produced in small amounts from specialized metabolic pathways that fluctuate with respect to environmental conditions. The versatility of the hairy roots system has allowed the development of platforms for the production of high-value natural products, at times in scaled up bioreactors (Georgiev et al., 2007; Cuello and Yue, 2008; Villarreal et al., 2008; Weathers et al., 2008). In addition, the inherent characteristics of hairy roots including their fast growth, genetic stability, short doubling time, and ability to produce a broad range of metabolites similar to wild type make this system a powerful tool for metabolic engineering (Veena and Taylor, 2007). In combination with transgenic approaches, the capacity of hairy roots metabolism can be manipulated for the enhancement of *de novo* synthesis of high value phytochemicals (Guillon et al., 2006).

Hairy roots technology offers important advantages for phytoremediation studies

Hairy roots offer several advantages for use in phytoremediation studies, these include: their ability to grow rapidly in microbe-free conditions, providing a greater surface area of contact between contaminant and tissue, and they are genetically and metabolically more stable in comparison to wild type (Gujarathi et al., 2005; Georgiev et al., 2007). Hairy roots are also amenable to genetic transformation, making gene transfer and characterization possible in a system that may pose minimum health or environmental concerns. Another advantage of using hairy roots for studying phytoremediation is their ability to produce large quantities of exudates which are composed of enzymes and some metal chelating compounds that may detoxify or sequester harmful organic and inorganic contaminants (Gujarathi et al., 2005; Bais et al., 2006; Doty, 2008). As shown in Table 1, hairy roots have been used to assess the potential of several plant species to remove contaminants from the environment. For example, the hairy root cultures of black nightshade (*Solanum nigrum*) may metabolize and remove PCBs from solutions spiked with PCB congeners (Macková et al., 1997a,b; Kučerova et al., 2000; Rezek et al., 2007). Also, by studying the rates of removal and the fate of contaminants such as the explosives hexahydro-1,3-5-trinitro-1,3-5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), Badhra et al. (2001) discovered that periwinkle (*Catharanthus roseus)* hairy roots have an “intrinsic ability” to remove these molecules from the medium. RDX and HMX are the two most common pollutants found in military sites where explosives are commonly tested (Pilon-Smits, 2005).

Recently, hairy roots have been used to test plants for their ability to tolerate high levels of phenols (de Araujo et al., 2002). Phenols are commonly used in various agricultural applications or released from coal and petroleum refining activities, and they pose a threat to human health (de Araujo et al., 2002; Agostini et al., 2003; Coniglio et al., 2008). In hairy roots of carrot (*Daucus carota*) and other plant species the role of peroxidase enzymes might be the key factor in the removal of phenol and chlorophenols from the culture medium (Agostini et al., 2003; González et al., 2006; de Araujo et al., 2006; Singh et al., 2006; Coniglio et al., 2008). Also, the inherent activity of peroxidases in hairy roots of rapeseed (*Brassica napus)* was associated with the effective removal of 2,4-dichlorophenol and phenol from the medium for several cycles and the removal process was enhanced by exogenously-applied hydrogen peroxide (Agostini et al., 2003; Coniglio et al., 2008). It appears that other plants use additional mechanisms to remove phenol. For instance, cells of carrot, kangaroo apple (*Solanum aviculare)* and sweet potato (*Ipomoea batatas*) hairy roots are able to incorporate and conjugate phenolic compounds with polar cellular materials (possibly sugars and proteins) as well as with insoluble materials such as cell walls and membranes (de Araujo et al., 2006).

To a greater extent, 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. Indeed, results from a comparative study of peroxidase enzymes from hairy roots of carrots, sweet potato and kangaroo apple demonstrated an inter-specific variation in the preference for phenol and chlorophenol among peroxidases (de Araujo et al., 2004). Also, peroxidase isozymes involved in phenol removal within a species may show variation in substrate preference and catalytic efficiency of phenol metabolism (Coniglio et al., 2008). It is noteworthy that, these studies are important in establishing an understanding of the enzymatic mechanisms of contaminant degradation for the selection of candidate enzymes that might be produced in large amounts and used as catalysts for contaminant break down (González et al., 2006).

An inspiring study by Eapen et al. (2003) demonstrated that hairy roots of the Indian mustard (*B. juncea*) and *Chenopodium amaranticolor* could remove uranium from solutions and could withstand high concentrations of this radionuclide for days. It is encouraging to imagine that in the near future it may become possible to use plants to cleanup sites contaminated with radioactive waste and alleviate the devastating environmental problems that may arise through uranium contamination of soils and water (Gavrilkescu et al., 2008).

The uptake of metals and their distribution in plant tissues are both important aspects governing the capacity of plants to remove heavy metals from the soil. Hairy roots have demonstrated that they can be used as a means for screening a wide variety of plant species for their capacity to extract and sequester metals (Nedelkoska and Doran, 2000a). A comparative assessment of nickel tolerance between hairy roots and whole plants revealed that the translocation of nickel to above ground shoots may not be required for nickel tolerance and hyperaccumulation in certain species of *Alyssum* (Nedelkoska and Doran, 2001). This suggests that nickel tolerance may be conferred by a reduced oxidative damage of hairy roots tissue due to enhanced catalase activity (Boominathan and Doran, 2002). Therefore, additional mechanisms to metal translocation and accumulation in shoots of hyperaccumulators may play a significant role in heavy metal tolerance. Indeed, using hairy roots, Boominathan and Doran (2003a) demonstrated that cadmium was extracted by alpine pennygrass *(Thlaspi caerulescens)* and accumulated in high levels in complexes with organic acids inside the cell walls.

Table 1. Phytoremediation of various environmental pollutants by hairy root cultures as tools to study the uptake and degradation of xenobiotics

Plant species	Model pollutant	Reference
Black nightshade (*Solanum nigrum*)	PCBs	Macková et al. (1997a; b)
Alpine pennygrass *(Thlaspi caerulescens)*	Cadmium	Nedelkoska and Doran (2000b)
Alyssum sp.	Nickel	Nedelkoska and Doran (2001)
Periwinkle (*Catharanthus roseus*)	RDX and HMX	Bhadra et al. (2001)
Carrot (*Daucus carota*)	Phenol and chloroderivatives	de Araujo et al. (2002)
Wild mustard (*Alyssum bertolonii*) and alpine pennygrass (*T. caerulescens*)	Nickel, and cadmium	Boominathan and Doran (2002)
Deadly nightshade (*Atropa belladonna*)	TCE	Banerjee et al. (2002)
Rapeseed (Brassica napus)	2,4-Dichlorophenol	Agostini et al. (2003)
Indian mustard (*Brassica juncea*) and *Chenopodium amaranticolor*	Uranium	Eapen et al. (2003)
Indian mustard (*B. juncea)* and chicory *(Cichorium intybus)*	DDT	Suresh et al. (2005)
Sunflower (*Helianthus annuus*)	Tetracycline and oxytetracycline	Gujarathi et al. (2005)
Tomato (*Lycopersicon esculentum*)	Phenols	Oller et al. (2005)
Carrot (*D. carota*), sweet potato (*Ipomoea batatas*), and kangaroo apple (*Solanum aviculare*)	Guaiacol, catechol, phenol, 2-chlorophenol, and 2,6-dichlorophenol	de Araujo et al. (2004; 2006)
Indian mustard (*B. juncea*)	Phenol	Singh et al. (2006)
Tomato (*L. esculentum*)	Phenol	Wevar-Oller et al. (2005); González et al. (2006)
Rapeseed (B. napus)	Phenol	Coniglio et al. (2008)
Yellow tuft *(Alyssum murale)*	Nickel	Vinterhalter et al. (2008)

DDT= Dichloro-diphenyl-trichloroethane;, HMX=oxtahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; PCBs = polychlorinated biphenyls; RDX=hexahydro-1,3-5-trinitro-1,3-5-triazine; TCE=Trichloroethylene;

Also, in another study, Boominathan and Doran (2003b) revealed that an inherent high catalase activity may play an important role in cadmium hyperaccumulation in *T. caerulescens* hairy roots. Therefore, the establishment of hairy root cultures for a variety of plant species might be a good strategy in studies of growth and heavy metal tolerance in plants (Nedelkoska and Doran, 2000b). Ultimately, the application of tissue culture technology may prove powerful in the regeneration of shoot cultures from hairy roots of selected species of plants with superior phytoremediation traits (Vinterhalter et al., 2008).

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It is important to monitor and limit the release of pesticides and antibiotics into the environment, and of equal importance is the identification of methods for cleanup in the case of contamination. Hairy roots of sunflower (*Helianthus annuus*) are effective in extracting and metabolizing antibiotics including tetracycline and oxytetracycline through a process that is thought to involve reactive oxygen intermediates (Gujarathi and Linden, 2005). There is controversy regarding the continuous use of the insecticide DDT to combat mosquitoes that spread malaria in developing countries (Sadasivaiah et al., 2007) even though some studies suggest that DDT might have negative health effects on human health (Hatcher et al., 2008). Hairy roots of chicory *(Cichorium intybus)* and Indian mustard (*Brassica juncea)* have been used to study their potential in removing DDT from contaminated sites (Suresh et al., 2005). Interestingly, *C. intybus* and *B. juncea* might produce enzymes that degrade DDT (Suresh et al., 2005), thus offering a promising possibility for the characterization of these enzyme(s) and for similar studies to be done in other plant species.

The expression of heterologous proteins in hairy roots has successfully been done (Banerjee et al., 2002). Such an approach was used to express a mammalian cytochrome P450 enzyme in deadly nightshade *(Atropa belladonna)* and the transgenic plants were able to metabolize the environmental pollutant TCE (Bernejee et al., 2002). Five years later, Doty et al. (2007) were successful in transforming poplar (*Populus tremula* x *Populus alba*) with this mammalian enzyme to generate plants with a superior capacity to remove various organic pollutants from hydroponic solutions and air. Of the several lines transformed with the mammalian enzyme, line 78 metabolized TCE a hundred-fold more than non-transgenic control trees (Doty et al., 2007). Also, others have used transgenic approaches that involved the over-expression of plant genes encoding contaminant metabolizing enzymes in hairy roots. For example, by over-expressing a tomato (*Lycopersicon esculentum*) *tpx1* gene encoding a peroxidase in hairy roots, Wevar-Oller et al. (2005) generated roots with enhanced capacity of removing phenol from the medium. These studies demonstrated that transgenic approaches may be adopted to produce plants with novel and improved phytoremediation capacity (Van Aken, 2008). Therefore, in the near future the use of transgenic hairy root systems may become more common in testing the efficacy of transgenes and the enzymes they encode for the removal of hazardous environmental pollutants.

All these studies demonstrate the power of using hairy roots in screening for candidate genes involved in the metabolism of environmental contaminants. Figure 2 illustrates a model of the mechanism(s) by which wild type or transgenic hairy root cells may metabolize environmental contaminants. It is noteworthy, however, that although the generation of transgenic plants with enhanced phytoremediation capacity might seem as a plausible solution, public skepticism and resistance to transgenic organisms might make this option less favorable for application in the near future. Alternatively, the selection of local plant species with enhanced phytoremediation capacity through hairy root screens may become more favorable and practical in the immediate future.

CONCLUSIONS AND FUTURE DIRECTIONS

Hairy roots can be generated from many plant species by infecting them with *A. rhizogenes*. This technology has facilitated a more stable production of important medicinal and high-value products at times in scaled up bioreactors. The versatility of hairy roots makes this system more attractive for the assessment of various physiological aspects of plants. The problem of environmental pollution affects both local and global human populations and physicochemical technologies of environmental cleanup are costly. Therefore, the use of plants in phytoremediation is gaining more support. Plants have intrinsic abilities to extract and metabolize contaminants and their cooperation with soil microorganisms and endophytes, microbes that live inside plants, may enhance the removal of contaminants from the environment. However, it is conceivable that not all species will possess superior capacities to extract and metabolize pollutants. These valuable plant traits can be screened for using hairy root cultures. Thus, the initial selection of superior plant species for use in phytoremediation can begin in the laboratory followed by the actual growing and testing plants in the greenhouse and the field. As hairy roots are amenable to genetic transformation, transgenic approaches may be used to study candidate genes that affect pollutant removal.

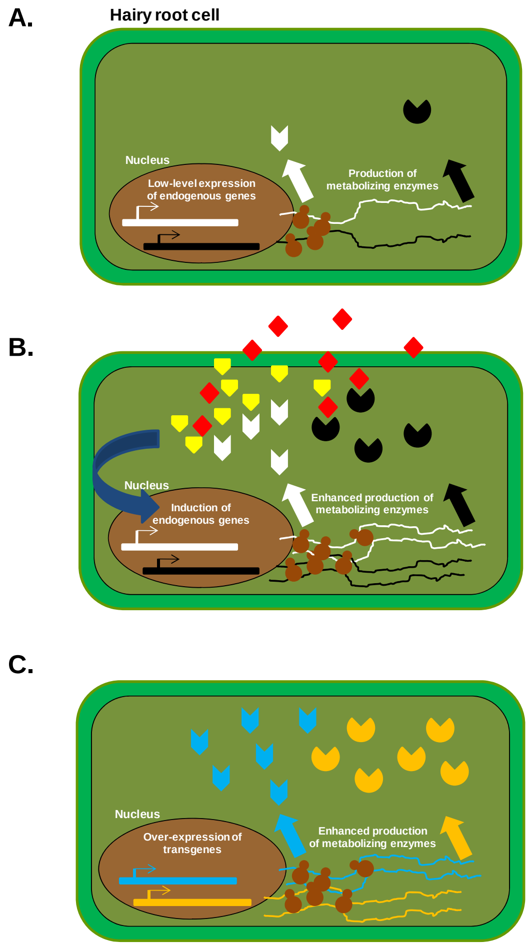

Figure 2. Metabolism of environmental contaminants by hairy root cells: **(A)** a cartoon depiction of a hairy root cell expressing contaminant metabolizing enzymes (white chevron and black pie) at basal levels; **(B)** environmental contaminants (red diamonds) may promote the production of reactive oxygen species (yellow pentagon), the enhanced production of ROS scavenging enzymes and antioxidants (white chevron), and/or contaminant metabolizing enzymes (black pie); **(C)** the expression of transgenes of animal or plant origin may also result in the enhanced production of contaminant metabolizing enzymes (blue chevron and orange pie) and phytoremediation capacity of plants.

Therefore, in the near future the hairy roots technology might be used more commonly in biotechnological efforts ranging from metabolite production to phytoremediation. Despite the large potential of hairy roots in phytoremediation studies, the ongoing challenge will be the actual translation of laboratory results to field applications. The lack of microbes in axenic hairy roots media may prevent our full appreciation of the benefits of the rhizospheric organisms that often enhance the uptake and breakdown of pollutants. Nevertheless, it is encouraging to witness the recent development of transgenic plants, poplar trees in particular, that promise to offer a tremendous impact on phytoremediation. In summary, hairy roots provide a promising tool in the field of phytoremediation but the work of environmental remediation has just begun.

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