Hairy Roots and Phytoremediation

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Contents

1 Introduction .................................................................................. 551
2 Hairy Roots ................................................................................ 553
   2.1 Hairy Roots in Phytoremediation: A Rational Approach? ............... 554
3 Remediation of Organic Pollutants by Hairy Roots ................................ 555
   3.1 Phenols and Chlorophenols .......................................................... 555
   3.2 Polychlorinated Biphenyls .............................................................. 558
   3.3 Pharmaceuticals .......................................................................... 559
   3.4 Explosives .................................................................................. 559
   3.5 Insecticides ................................................................................. 560
   3.6 Dyes .......................................................................................... 560
   3.7 Trichloroethylene ....................................................................... 561
   3.8 UV Filter Compounds .................................................................. 561
4 Remediation of Inorganic Pollutants by Hairy Roots ............................... 561
   4.1 Cadmium .................................................................................. 562
   4.2 Nickel ...................................................................................... 563
   4.3 Uranium .................................................................................... 563
   4.4 Copper ..................................................................................... 564
   4.5 Zinc .......................................................................................... 564
5 Phytoremediation by Hairy Root–Microorganism Association .................. 564
6 Conclusions .................................................................................. 565
References ...................................................................................... 566

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Abstract

Contamination of the environment arises either from natural geological processes or due to human activities and has created an alarming situation worldwide. Biological strategies for cleaning up contaminated biosphere have gained much importance in recent years and are preferred over other conventional physical and chemical methods because these are environmentally friendly and cost-effective. Phytoremediation is an ecologically compatible approach using plants to remediate polluted environment. Currently hairy roots have emerged as a notably competent research tool for phytoremediation among the various biological systems investigated for this purpose. Infection of certain plants caused by Agrobacterium rhizogenes is expressed in the form of hairy root disease. The disease is characterized by adventitious roots with copious root hairs developing elaborately from or next to the infection site. The plant genome receives a set of genes from a segment of the large root inducing (Ri) plasmid of A. rhizogenes. Under the effect of these genes, the inherent hormonal balance of the plant is altered resulting in the development of hairy roots. In nature, plant roots are the primary organs having contact with the environmental contaminants. Thus, hairy roots have been used in phytoremediation research as physiologically they resemble the normal roots of the mother plants. Several studies demonstrate the potentiality of hairy roots in removing a vast array of both organic and inorganic pollutants from the environment. In addition, microorganisms colonizing the rhizosphere of hairy roots have also proved to improve the efficacy of hairy roots in eliminating contaminants. The purpose of this review is to summarize the applications of hairy roots in different phytoremediation strategies and provide examples and prospects of the use of hairy roots in the removal of organic and inorganic contaminants from the environment.

Keywords

Agrobacterium rhizogenes · Hairy roots · Inorganic pollutants · Organic pollutants · Phytoremediation

Abbreviations

2,4-DCP 2,4-dichlorophenol
AMF Arbuscular mycorrhizal fungus
Cd Cadmium
cv Cultivar
DDT 1,1,1-trichloro-2,2-bis-(4′-chlorophenyl)ethylene
DNA Deoxyribonucleic acid
FTIR Fourier transform infrared spectroscopy
GC–MS Gas chromatography–mass spectrometry
h Hour
HPLC High-performance liquid chromatography
kb Kilobase
min Minute
NADH–DCIP reductase Nicotinamide adenine dinucleotide reduced–dichlorophenolindophenol reductase
Ni Nickel
OBZ Oxybenzone
PCB Polychlorinated biphenyl
Px Peroxidase
TCE Trichloroethylene
T-DNA Transferred DNA
TNT 2,4,6-trinitrotoluene
U Uranium
UV Ultraviolet

1 Introduction

Contamination of the biosphere is a crisis severely threatening the welfare of all living organisms. Contaminants polluting the environment may be classified as organic and inorganic and are released either naturally through geological processes (viz., erosion, saline seeps) or from anthropogenic activities and extensive industrialization (viz., agriculture, construction, wastewater treatment, mining, melting, military activities, chemical works, electroplating, energy and fuel production, sludge dumping, pharmaceuticals, paper mills, tanneries, textile plants, etc.) [1–5]. These pollutants are ecotoxic substances which are either non-biodegradable or their degradation is very slow, leading to their accumulation in the biosphere, ultimately upsetting the harmony of the ecosystem. Development of remediation technologies to prevent this disruption is of utmost importance. Various techniques (viz., incineration, irradiation, soil washing, pump and treat, surfactant flushing, activated carbon adsorption, or extraction) are available for cleaning up the contaminated environment, but in most cases the methods are expensive and less efficient, require application of huge labor, and cause disorders in the soil or produce by-products which further enhance environmental toxicity [4, 6–8]. Thus, these methods have got limited public acceptance [4]. Consequently research efforts were diverted toward the development of other efficient and reliable technologies which would make the environment cleaner and healthier.

Biological methods score higher over conventional physical and chemical methods in being cost-effective and eco-friendly. Bioremediation of contaminated sites by indigenous microbial flora, a process commonly called natural attenuation, presents certain drawbacks such as prolonged time requirement, production of hazardous by-products, and difficulty in restoration to normal environment [9]. Phytoremediation is a technology where plants are being used to clean up environmental contaminants and is ecologically harmless for restitution and remediation. It is less expensive, does not cause any invasion, is safer than conventional strategies [3, 10], and is thus attracting attention worldwide. Other advantages and limitations of phytoremediation have been discussed in our previous review [11].
The principal mechanism of phytoremediation is based on the solar energy-driven uptake of chemicals from polluted air, water, and soil by plants [10]. The two most vital aspects of phytoremediation are the eradication of pollutants from contaminated sites, also known as **phytodecontamination** and pollutant stabilization, thereby preventing its transport and toxic effects [12].

Although the method was originally developed for removing heavy metals from the soil [13], phytoremediation now relies on the ability of certain plant species to uptake, tolerate, assimilate, detoxify, and store a diverse range of pollutants present in the environment and render them harmless [8]. The recent era of “-omics” has made metabolism of diverse pollutants by plants more effective, and practical technologies are being developed for improving the applicability of phytoremediation [8, 14].

Based on the type of pollutant present and the decontamination strategy applied by the plants, phytoremediation treatments can be of various types, viz., phytorextraction, phytodegradation, phytostabilization or phytosequestration, phytovolatilization, rhizoremediation, rhizofiltration, or phytofiltration [10, 15, 16]. The process by which contaminants are absorbed from the soil and translocated to aerial plant parts is called **phytoextraction** and has been successfully applied for the removal of edaphic contaminants [10, 17]. “Hyperaccumulating” plants, i.e., unusual plants capable of absorbing, storing, and tolerating huge amounts of heavy metals in the foliage, play a crucial role in phytoextraction. Compared to normal plant species growing under identical conditions, these hyperaccumulator species have the capability to accumulate about 100-folds more concentration of heavy metals [17, 18], without affecting the plants in an adverse manner. About <0.2% of angiosperms [19, 20] can hyperaccumulate heavy metal ions, and the phenomenon is reported from members of 45 angiosperm families [4, 21]. **Phytomining**, on the other hand, involves commercial recovery of accumulated metals from plants by ashing, smelting, or liquid extraction [22]. In **phytodegradation**, also known as **phytotransformation** [23], certain organic pollutants such as chlorinated hydrocarbons, polycyclic aromatic hydrocarbons, herbicides, trichloroethylene, and explosives are degraded either through endogenous plant enzymes or enzymes which are secreted [10, 24]. The three phases of metabolism include transformation, conjugation, and compartmentation, leading to the detoxification and breakdown, ultimately accumulating the contaminants [23, 25]. The tropical leguminous tree *Leucaena leucocephala* and *Populus* hybrids [26–28] have been frequently used for phytodegradation. **Phytostabilization** or **phytosequestration** is a strategy, where instead of removing the contaminant from the soil, the polluted soil is stabilized by plants to check the movement of the contaminant to the surrounding neighborhoods [12, 17]. Weathering of contaminated soil by natural elements is reduced by planting of vegetation at the polluted site. Another approach of phytostabilization is to prevent leaching by the addition of various chemicals and organic matter, which prevent solubilization of the metals [17]. After uptake by the roots, contaminants can be transported to the aerial parts from where they can be volatized through **phytovolatilization** [15]. **Phytoimmobilization** denotes the immobilization of pollutants taken up by plants in a soil containing mineral or a geomat (mineral-
containing mat) [3]. Certain pollutants cannot be completely degraded by plants only [29]. In such cases, their alliance with bacteria, colonizing the rhizosphere, has been proposed to enhance phytoremediation potential, a process known as rhizoremediation [29, 30]. Plant root exudates provide the microbial population with nutrients and energy; the microbes in turn degrade contaminants, assisting plants in pollutant remediation. Phytofiltration or rhizofiltration is the plant-aided elimination of pollutants from aquatic bodies [3]. Usually, a variety of these processes utilizing plants are associated with remediation. However, in-depth analysis of plant metabolic pathways, the enzymes involved therein, and understanding the mechanism of tolerance are needed for greater applicability of phytoremediation strategies.

Currently hairy roots have emerged as an important tool for phytoremediation research among the different biological systems investigated for this purpose.

2 Hairy Roots

The history of hairy roots and their causative agent dates back to the early 1900s. The bacterium causing the hairy root syndrome was initially named Phytomonas rhizogenes [31]. P. rhizogenes later came to be known as Agrobacterium rhizogenes, which is a Gram-negative bacterium present in the soil. It induces hairy root syndrome in higher plants, which can be distinctly identified by adventitious roots with copious root hairs developing elaborately at or next to the infection site [32, 33]. The Ri (root inducing) megaplasmid (>200 kb) [32, 34, 35] determines the infectivity of A. rhizogenes. Distinct segments of DNA, the “transferred DNA” or “T-DNA,” carried by the plasmid are transferred to the plant genome [32]. Hairy roots originate from plant wound sites following infection with A. rhizogenes resulting in transfer of T-DNA genes from bacteria to the plant followed by their stable integration and expression in the plant genome. Pacurar et al. [36] and Chandra [37] recently studied in details the molecular mechanism lying behind the genetic transformation of plants by different strains of Agrobacterium. A number of genes of the Ri plasmid, viz., the vir genes (located in the virulence region), which are not delivered into the plant and a group of genes residing on the bacterial chromosome (chv) [14, 38] are essential for T-DNA transfer from the bacteria to the plant cell. Another set of genes, the rol genes, located in the T-DNA, affect the development and phenotype typical to the hairy root disease. Ri-transformed root cultures have been reported for more than 500 plant species, most of them being dicotyledons [23]. However, with improvements in strategies, new species of plant or species which are recalcitrant to transformation are being utilized to produce hairy roots.

Biotechnological research has advanced greatly based on the immense potential of hairy roots for commercial exploitation. Highly branched, plagiotropic hairy roots grow fast, indefinitely on medium without phytohormones under aseptic conditions and are characterized by genetic and biochemical stability over extended periods [14, 32]. They are often called “phytochemical factories” as these roots can biosynthesize
compounds naturally produced in the native plant roots, and the amounts are often analogous to or more than that of the roots and shoots of mother plants [14, 39, 40]. The laboratory maintenance of hairy root cultures is a low-cost method and requires simple tissue culture procedures. Furthermore, for proper functioning, these roots need not be associated with aerial plant organs and remain free of microbes. Currently, hairy roots are being used for the production of valuable ‘secondary metabolites’ of plant origin, for expression of genes for the production of foreign proteins to be used for therapeutic purpose (viz. antibodies, vaccines, cytokines), enzyme production, molecular farming, elucidation of biosynthetic pathways, bio-transformation of exogenous substrates and environmental decontamination by phytoremediation [41–49]. Great advances have also been made in culturing hairy roots in bioreactors and optimizing conditions for large-scale production [49, 50].

The present review deals with the instances of the use of hairy roots for phytoremediation; relevant studies demonstrating the tolerance, metabolization, and storage of a vast array of organic and inorganic contaminants by plant cells using hairy roots of different plant species are discussed.

2.1 Hairy Roots in Phytoremediation: A Rational Approach?

Among the different experimental systems currently utilized for research on phytoremediation, hairy root cultures of various plant species have proved to be promising experimental tools and suitable isolated model systems to understand not only the mechanisms associated with the elimination or decomposition of contaminants but the activity of pivotal enzymes involved in detoxification processes as well. Hairy roots can be transformed genetically [14] and because of their genetic stability, foreign proteins can be produced by them for a long term [51]. The resultant functional proteins might be involved in metabolization of environmental pollutants. Moreover, introduction of foreign genes from plants, animals, or microbes along with A. rhizogene-mediated transformation of suitable plants results in the production of proteins by the hairy roots which are capable of metabolizing chemical compounds. These genes can also be overexpressed via genetic transformation of hairy roots to improve these metabolic traits further. Hairy root cultures serve as model experimental system for standardization of variables prior to large-scale field application of a particular remediation technique. Keeping this in mind, Flocco and Giulietti [52] designed protocols concerned with Armoracia lapathifolia hairy root production and utilization of the same in detoxification of phenol, a model organic compound.

The initial reactions against the environmental pollutants take place in the roots which are the primary organs having contact with the contaminants [14]. In terms of physiology, hairy roots are more similar to normal roots than undifferentiated cell cultures. Another advantage of hairy roots is that they use common metabolic pathways to metabolize harmful compounds [53]. They can be propagated indefinitely and their prolific growth rates shorten their subculture period, thus yielding stable and large amounts of biomass over the whole year, independent of the season.
This is a necessary prerequisite for phytoremediation research. This is also helpful as large surface area of the hairy root mass comes in contact with the contaminants. Moreover, hairy roots grow in microbe-free environment, and thus it is possible to discriminate the exact role of plant root cells against those of microorganisms residing in the rhizosphere in removal of soil contaminants. Hairy roots are organs without shoots, a characteristic aiding in elucidating the mechanisms of root remediation only, bypassing the event of translocation. Easy regeneration of whole plants from hairy roots is another attribute which can be exploited for clonal selection of plants with appropriate phytoremediation potentialities. Their organized nature makes them suitable candidates for cultivation in bioreactors for studying remediation processes on a large scale and can be reused consecutively in several cycles [54–56]. They also allow medium to be manipulated easily and make the availability of end products easier, thus reducing the procedures of purification [14]. Hairy roots are also advantageous in the sense that they can produce root exudates which cause detoxification and sequestration of harmful pollutants by the action of secretory enzymes or heterologous enzymes which influence the secretory pathway. However, these areas of research are still not completely elucidated. Hairy roots have also contributed to our understanding regarding the compartmentalization and nature of a few of the end products of detoxification processes [55] and the effects of contaminants on certain physiological and biochemical processes, viz., antioxidative stress responses and lipid peroxidation [57]. In addition, hairy root cultures act as indicators of the plants capable of phytoremediation as these roots carry the genetic capacity of its parent plant to transform a particular compound [8].

However, certain disadvantages are also associated with the use of hairy root cultures for phytoremediation research, viz., application on a large scale might become complex in some cases, aseptic culture conditions are required, sugars are required in the culture medium, and maintenance of such cultures could be a bit expensive for some species [14].

Nevertheless, the use of hairy root in phytoremediation research is well documented (as will be described in the following sections) and reviewed widely [5, 11, 14, 58, 59]. Figure 1 demonstrates the application of hairy roots in phytoremediation research.

3 Remediation of Organic Pollutants by Hairy Roots

Several reports demonstrate the phytoremediation of different organic pollutants by hairy roots of different plant species as follows:

3.1 Phenols and Chlorophenols

Aromatic compounds are present in industrial effluents resulting from coal processing, coke ovens, petroleum refineries, as well as manufacture of phenolic resins, fiberglass, herbicides, pesticides, disinfectants, and other activities including paper,
wood, metal, and plastic industries [60–64]. The fractional degradation of certain aromatic organic pollutants (viz., polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and some surfactants) is also responsible for the release of phenolic compounds [14, 65]. Phenols are categorized as one of the major classes of hazardous pollutants as they are toxic and recalcitrant to degradation and have low volatility [5, 66]. Phenol exposure may cause liver damage, hemolytic anemia, blindness, and nervous disorder and is also alleged to cause paralysis [62, 67]. Decontamination processes for eradication of phenol by conventional and commonly used techniques applied to contaminated areas are less efficient and expensive, and the metabolic by-products often have higher toxicity than phenols [6, 54, 61, 62]. In recent years, hairy roots derived from different plant species have not only been successfully applied for the removal of phenol from aqueous solutions but also to test the tolerance capacity of plants to high concentrations of this pollutant. The effect of 2-week-old hairy roots of different species obtained through transformation using A. rhizogenes LBA 9402, on the detoxification of phenol from the culture medium, was studied by Singh et al. [61] demonstrating that the remediation of phenol by hairy roots was species dependent. They observed that Brassica juncea was the most prospective species for phenol removal, removing 97% phenol, followed by Beta vulgaris, Raphanus sativus, and Azadirachta indica in the absence of H2O2 supplementation to the medium. Plant peroxidases (Px), mainly those restricted to the cell walls, are crucial for the removal of toxic compounds. Chemical assays related to contaminant removal potential and kinetics of root extracts as well as purified Px suggested the formation of “inextricably bound residues” resulting due to covalent bond formation between plant cell wall and hydroxylated pollutants and metabolites [68]. They are categorized as the chief enzymes behind the removal of phenolic compounds [14]. In their study, Singh et al. [61] also demonstrated that exposure to phenol increased Px activity in the roots. H2O2 was synthesized in situ and its level was enhanced in the presence of phenol thereby eliminating the necessity of addition of H2O2 to the culture medium.

Fig. 1 Schematic representation of the application of hairy roots in phytoremediation research
The susceptibility of hairy roots to phenols and chlorophenols was investigated by Araujo et al. [62]. They demonstrated that hairy roots of *Solanum aviculare* were most efficient for phenol remediation, removing 98.6% from the medium within 72 h followed by *Ipomoea batatas* and *Daucus carota*. *D. carota*-transformed roots were most effective in removing 2,6-dichlorophenol (2,6-DCP). However, inherent Px activity was highest in *I. batatas*, in the presence of phenol, demonstrating that transforming efficiencies and Px activity in the root cultures were not directly correlated.

*Lycopteron esculentum* cv Pera hairy roots derived by *A. rhizogenes* LBA 9402-mediated transformation of sterile leaf explants were also found to be suitable for removing phenol from water [63]. Conditions (pH, temperature, etc.) for efficient removal of phenol with minimum inactivation of the enzymes possibly involved in the removal process were optimized. Doubly transgenic tomato hairy roots engineered for overexpression of *tpx1* (basic tomato peroxidase 1, pI 9.6) have also been tested for enhanced removal of phenol from aqueous solutions; Px activity was higher in the transgenic hairy roots overexpressing *tpx1* and *Nicotiana tabacum* hairy roots which were double transgenic for *tpx1* and *tpx2* (tomato peroxidase genes) as compared to the hairy roots developed following infection with wild-type strain [57, 68].

In another study, Coniglio et al. [64] observed that up to 500 mg l\(^{-1}\) phenol could be removed by hairy roots of *Brassica napus* developed following transformation with wild-type *A. rhizogenes* LBA 9402 in the presence of exogenously added H\(_2\)O\(_2\). The authors reported that removal efficiency was maximum within 1 h of treatment and the hairy roots when reused for consecutive cycles, the efficiency for removing phenol gradually decreased, together with a decrease of Px activity. They suggested that this decline in Px activity could be due to its inactivation caused by H\(_2\)O\(_2\) or irreversible bonding between Px and phenyl or phenoxy radicals formed during oxidation of phenolic compounds. Competitive inhibition by the end product for the Px active site or the inaccessibility of root biomass due to adsorption of the polymer might be the other causes of reduction in Px activity. But in contrast to studies by González et al. [63], the authors demonstrated that acidic Px might play the crucial role in phenol removal, rather than the basic and neutral Px.

Hairy roots of another plant species *Helianthus annuus* could effectively metabolize phenol at concentrations ranging from 100 to 400 mg l\(^{-1}\) [69]; however, a decline in phenol removal efficiency was noted with increasing phenol concentrations. When l-proline was added to the reaction mixture, more than 90% phenol (100 mg l\(^{-1}\)) was removed after 24 h. Px activity was also induced in the roots in the presence of phenol, and catechol was detected as a major metabolite in the process of biodegradation. Treatment with hairy roots also reduced toxicity of phenol solutions in comparison to untreated solutions.

Mazaheri and Piri [70] reported the phenol-metabolizing potential of *Atropa belladonna* hairy roots up to a concentration of 500 mg l\(^{-1}\) from wastewater in the presence of H\(_2\)O\(_2\) within pH ranging from 4.0 to 9.0. Reuse of the hairy roots in the fifth cycle showed decrease of phenol removal efficiency from 98% to 62%. Toxicity tests using *Lactuca sativa* seeds revealed that the treated solution had less toxic effect than the initial solution.
Chlorophenols are another group of hazardous compounds which are recalcitrant to degradation, extremely toxic, and widely distributed in the biosphere [71]. Several chemicals utilized in agriculture and industries such as herbicides (viz., 2,4-dichlorophenoxyacetic acid), pesticides, germicides, resins, and antiseptics are manufactured using 2,4-dichlorophenol (2,4-DCP), a substituted phenol [54], which is released to the environment through industrial effluents. Exposure to this toxic compound for extensive periods is harmful to both aquatic organisms and human beings [5, 72]. Thus, efforts have been made toward the remediation of 2,4-DCP using eco-friendly phytotechniques. Agostini et al. [54] demonstrated that hairy root cultures developed by \textit{A. rhizogenes} LBA 9402 inoculation of \textit{B. napus} leaves could remove 2,4-DCP from solutions in concentrations ranging from 100 to 1,000 mg l\(^{-1}\) in the presence of external H\(_2\)O\(_2\) (5–10 mM), within an incubation span of 15 min to 1 h, probably due to the effect of Px. Reuse of the roots for six successive removal cycles (with a high efficiency of ~90% after six cycles) distinguished the results from those of Singh et al. [61] and Coniglio et al. [64] and is thus suitable for continuous decontamination purposes in a large scale. 2,4-DCP was also efficiently (98%, 88%, and 83%) removed by tobacco hairy root cultures in a short time for solutions containing 250, 500, and 1,000 mg l\(^{-1}\), respectively [55]. When 10 mM H\(_2\)O\(_2\) was used, 500 mg l\(^{-1}\) 2,4-DCP was removed in 60 min. The hairy roots could also be reused for almost three consecutive cycles. The role of Px in 2,4-DCP dehalogenation was suggested.

Isolation of pure enzymes is expensive and the enzymes are more readily subjected to inactivation during reaction. The studies discussed so far indicate that enzyme isolation might not be an obligatory prerequisite for decontamination processes, and hairy root biomass or extracts from the roots might be used as economical enzymatic systems for eradication of phenol from contaminated waters. Tissues of the roots might act as protective and stabilizing agents, thereby avoiding enzyme inactivation.

The removal of 2,4-DCP by hairy roots in the presence of H\(_2\)O\(_2\) was applied by Angelini et al. [56] in a novel study related to eradication of the contaminant in a large scale using a discontinuous stirred tank reactor. The authors achieved 98% removal of 2,4-DCP using \textit{B. napus} hairy roots in 30 min with reduction to 86% after six consecutive cycles.

### 3.2 Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) add to the list of recalcitrant chlorinated aromatic pollutants frequently encountered in the ecosystem. These are carcinogenic, mutagenic, teratogenic, and immunotoxic [73]. PCBs are used in the production of electrofluids, hydraulic lubricants, gas turbines, paints, plasticwares, pesticide extenders, adhesives, dedusting agents, cutting oils, flame retardants, heat transfer fluids, etc. PCBs affect human beings after entering the food chain and accumulating in the adipose tissue [74–77]. Thus, removal of PCBs from the environment is important, and considerable scope of PCB remediation has been shown by
phytoremediation. The ability of hairy roots of different species to degrade PCB has been studied. A patent had been obtained by Morita et al. [78] who described that *A. belladonna* hairy roots could absorb and decompose significantly large amounts of PCBs and dioxins in comparison to the natural roots of the plant, thereby providing a cheaper treatment. Kučerová et al. [79] and Rezek et al. [73] demonstrated that hairy root cultures of black nightshade (*Solanum nigrum*) could successfully metabolize an array of PCB congeners. Rezek et al. [77] also demonstrated that *S. nigrum* hairy root-mediated transformation of PCBs produced known hydroxy-PCBs as well as novel metabolites such as methoxy-PCBs and hydroxymethoxy-PCBs [80]. Lack of knowledge on the effect of these compounds has opened up interesting challenges in the field of metabolism of these compounds by plants.

### 3.3 Pharmaceuticals

Bioremediation by hairy roots has also proved to be an inexpensive and green technology that has been used for the removal of other organic pollutants as well. Aquatic bodies and drinking water often contain pharmaceuticals and the metabolites produced from them. Of these compounds, some [viz., antibiotics and N-acetyl-4-aminophenol/acetaminophen (paracetamol), a broadly used analgesic, antipyretic, and anti-inflammatory agent] are detrimental to aquatic organisms. Hairy root cultures of *H. annuus* developed by *A. rhizogenes* 15834 were effective in removing tetracycline and oxytetracycline from liquid media. The cell-free exudates of the hairy roots also showed similar activity. The rate of modification was found to decrease with the increase in oxytetracycline concentration and increase with the increase in age of culture [81]. The authors also suggested that reactive oxygen species (ROS) and not enzymatic catalysis was responsible for the modification of antibiotics. Huber et al. [82] demonstrated that *Armoracia rusticana* (horseradish) hairy roots could absorb and detoxify the xenobiotic compound paracetamol. It was suggested that paracetamol-glucoside, a predominant metabolite, is stable and nondegradable as glucoside serves as a precursor to cell wall lignin-associated insoluble residues. Such studies paved way for remediation of pharmaceuticals from wastewater using plants.

### 3.4 Explosives

Another area of considerable concern is the phytoremediation of explosives, which are hazardous. The most prevalent and persistent among the explosives is 2,4,6-trinitrotoluene (TNT) and its metabolization is extremely difficult. Research has been largely directed to analyze the ability of plants to alter TNT. Uptake and transformation of TNT have been reported by periwinkle (*Catharanthus roseus*) hairy root cultures; products of transformation and their chemical characteristics were identified and the transformation events leading to removal processes were studied [83, 84]. Using *A. rusticana* hairy roots, Nepovim et al. [53] analyzed the effect of explosives, viz., 2,4-dinitrotoluene (DNT), TNT, aminodinitrotoluenes
(ADNTs), and dianmonitrotoluenes (DANTs), on the activity of certain enzymes, viz., glutathione S-transferase (GST) and Px, involved in the metabolism of pollutants upon exposure to the pollutants for different time intervals.

3.5 Insecticides

Hairy root cultures have emerged as a vital tool in research related to the remediation of insecticides. One of the most commonly used pesticides, DDT [1,1,1-trichloro-2,2-bis-(4′-chlorophenyl)ethylene], has gained popularity over decades owing to its broad-spectrum activity, easy and cheap formulation, and high residual biological activity. However, inclusion in the food chain has resulted in carcinogenesis and endocrinological abnormalities in human beings [85]. *Cichorium intybus* and *B. juncea* hairy root cultures (obtained by inoculation of germinated seedlings with *A. rhizogenes* 15834) were used for the uptake and breakdown of DDT [86]. Their work also suggested that endogenous enzymes from the roots assisted in the degradation of this persistent insecticide.

3.6 Dyes

Dyes are another class of xenobiotic chemicals which are produced industrially. The largest group of dyes, azo dyes, is recalcitrant to biodegradation [87]. Various industries (viz., textiles, leather, plastics, cosmetics, food processing) are presently using a huge number of dyes including approximately 2,000 types of azo dyes, as coloring agents [14, 87]. Certain azo, xanthene, and anthraquinone dyes are toxic and mutagenic agents which may lead to severe health issues and affect aquatic life when discharged into water bodies [88–90]. Although categorized as harmful compounds, till date there are very few reports on remediation of these pollutants utilizing hairy roots. Hairy root cultures of marigold (*Tagetes patula*) could remove reactive red 198 dye up to concentrations of 110 mg l⁻¹ and could be consecutively used for five successive cycles of decolorization. GC–MS analysis revealed that the dye was transformed into nonhazardous metabolites [91]. These roots could also decolorize other dyes, viz., golden yellow HER, methyl orange, orange M2RL, navy blue HE2R, and reactive red M5B after 10 days. However, in another study, 92% decolorization of methyl orange was achieved within 4 days using hairy root cultures of *B. juncea*, suggesting that these hairy roots are extremely potential for degrading textile dyes [92].

Studies demonstrate the association of certain intracellular enzymes, viz., laccase, lignin Px, tyrosinase, NADH–DCIP reductase, azoreductase, and riboflavin reductase with the decolorization of textile dyes by plants or hairy roots [91, 93–95]. These observations led to the purification and characterization of an intracellular laccase from *B. juncea* hairy roots which was applied for removing textile dyes [92]. Among the various redox mediators studied, 2, 2′-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) appeared to be the most appropriate in step-up of the decolorization rate of laccase, suggesting that the combination of
laccase and ABTS could be efficiently used for treating textile dyes. These outcomes demonstrate the feasibility of using enzymes and/or other compounds from hairy root exudates for dye detoxification/sequestration purposes.

Recently, hairy root cultures of the facultative halophyte *Sesuvium portulacastrum* were induced by inoculating pre-cultured leaf and stem explants with *A. rhizogenes* NCIM 5140 [96]. The roots could degrade a wide variety of textile dyes (viz., reactive orange 14, reactive pink MB, reactive red 2M5B, reactive green 19A-HE4BD, Remazol yellow 3GL, Remazol navy blue RGB, Remazol blue RGB, Remazol yellow RGB, and red brown H4R) after 5 days of incubation. Maximum decolorization (98%) was noted in the case of reactive green 19A-HE4BD used at concentration of 30 mg l$^{-1}$. However, decolorization efficiency was reduced when higher concentration (150 mg l$^{-1}$) of the dye was used. Degraded nature of the dye was confirmed by HPLC and FTIR analyses. Also, nontoxic nature of the products of degradation was demonstrated by germination assay using seeds of *Phaseolus mungo*.

### 3.7 Trichloroethylene

Industrial effluent and a xenobiotic compound, trichloroethylene (TCE), is a key pollutant. P450 2E1, an enzyme from mammalian liver, metabolizes TCE. Hairy roots of *A. belladonna* expressing a P450 2E1 enzyme from rabbit were able to metabolize TCE [51].

### 3.8 UV Filter Compounds

In recent times, another compound which is gaining much attention as a contaminant is oxybenzone (OBZ) or benzophenone-3, frequently used in cosmetics which serve as UV filter in sun tans and skin protectants. It is detected in water from swimming pools and surface water samples, and treatment of wastewaters is not adequate to get rid of this contaminant completely. Aquatic organisms tend to accumulate OBZ and related metabolites which induce abnormalities of the endocrine and reproductive system [97–100]. Richardson’s water analysis [101] has categorized OBZ as an emerging pollutant since 2005. A very recent study by Chen et al. [102] demonstrates that hairy root cultures of *A. rusticana* could remove more than 20% of the initial amount of OBZ from the medium after 3 h of exposure, with oxybenzone-glucoside and oxybenzone-(6-O-malonyl)-glucoside being identified as novel metabolites. These findings paved the way for future applications of hairy roots in remediation of UV filter compounds.

### 4 Remediation of Inorganic Pollutants by Hairy Roots

Anthropogenic activities and extensive industrialization have resulted in the release of various types of inorganic pollutants, viz., heavy metals, metalloids, and radionuclides into the environment. These accumulate largely in agricultural and
industrial areas, affecting crop yields, soil biomass, and fertility, finally entering into the food chain [14, 103, 104]. A few of the heavy metals, viz., manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), and nickel (Ni), are essential micronutrients for the normal development of plants and animals, playing key roles in the induction and reaction of enzymes, membrane function, activity of isozymes, etc. [105]; these metals might become toxic at higher concentrations. Heavy metals cannot be destroyed by any chemical or biological means [5] and thus significantly contribute to environmental pollution. Currently, phytoremediation techniques are gaining much commercial importance for the elimination of hazardous inorganics from the environment, and hairy roots, in particular, have proven to be competent tools for investigating the underlying mechanisms involved in metal uptake, accumulation, and tolerance. Extraction and sequestration of metals by plants as well as the physiological and biochemical processes lying behind metal accumulation have also been elucidated utilizing hairy roots as model systems. Remediation of different inorganic pollutants is being mentioned in the following sections.

4.1 Cadmium

Cadmium (Cd) is a heavy metal contaminant derived from the use of fertilizers, sewage sludges, compost, and from metallurgical industries [5]. Nedelkoska and Doran [105] developed transformed roots of Cd hyperaccumulator species *Thlaspi caerulescens* with *A. rhizogenes* 15834 and compared with transformed roots of non-hyperaccumulator species *N. tabacum* and *A. belladonna*. They reported that Cd accumulation was 1.5–1.7-fold greater in roots of *T. caerulescens* than in the hairy roots of *N. tabacum* and *A. belladonna*. *T. caerulescens* hairy roots continued to grow till 100 ppm Cd, whereas roots of *N. tabacum* turned brown under similar treatment.

A comparative study on Cd tolerance was carried out by Wu et al. [106] using hairy roots *Adenophora lobophylla*, an endangered species, and *A. potaninii*, spread broadly in the same habitat. They demonstrated that although closely related, the plants might use different metabolic strategies for detoxification of Cd. *A. lobophylla* synthesized a high amount of phytochelatin, whereas *A. potaninii* harbored a Cd removal system and maintained GSH (reduced glutathione) level in the cell via homeostasis, together with phytochelatin synthesis.

Boominathan and Doran [107] also used hairy roots of the Cd hyperaccumulator *T. caerulescens* to study the function of antioxidative metabolism in tolerance of heavy metal. Their work demonstrated that although growth is unaltered by heavy metals, oxidative stress induced by metals occurs in hyperaccumulator tissues. Their results also suggested that enhancement of the antioxidative defenses of plants like *N. tabacum* (which have high biomass and are non-hyperaccumulating) is required in order to genetically improve the metal hyperaccumulating characteristics of these plants. Overexpression of certain enzymes like catalase and/or superoxide dismutase in addition to other molecular approaches could be considered.
A huge literature indicates the involvement of organic acids in heavy metal tolerance, transport, and storage in plants [88, 108–115]. Although high concentrations of citric, malic, and malonic acids are a constitutive property of *T. caerulescens* and *Alyssum bertolonii* hairy roots hyperaccumulating Cd and Ni, respectively, after exposure to 20 ppm Cd and 25 ppm Ni, 13% Cd and 28% Ni taken up by the respective roots were associated with the organic acids [116]. Cd was mostly found in *T. caerulescens* cell walls, similar to the results of Nedelkoska and Doran [105], whereas Ni was located in the symplasm of *A. bertolonii* hairy roots. Treatment with diethylstilbestrol, a plasma membrane H⁺-ATPase inhibitor, resulted in retention of viability of *T. caerulescens* roots, while concentration of Cd in the symplasm was increased by about six times. However, similar treatment caused reduction in Ni transport across the plasma membrane and root viability in *A. bertolonii*. Hence, plasma membrane depolarization does not affect hyperaccumulation of Cd in *T. caerulescens* hairy roots. However, Ni hyperaccumulation is negatively affected in *A. bertolonii* hairy roots under such conditions.

### 4.2 Nickel

Ni hyperaccumulation by hairy roots of *Alyssum tenium, A. bertolonii*, and *A. troodii* was reported by Nedelkoska and Doran [117]. Using long-term hairy root cultures, the authors demonstrated that Ni tolerance and hyperaccumulation are not essentially shoot dependent or dependent on transport from root to shoot. Hairy roots and whole plants also varied noticeably in their capacity to uptake Ni; hairy root-regenerated plants of *A. tenium* were much more tolerant to Ni, accumulating greater amounts compared to the hairy roots of the same species. Shoots of *A. murale* were inoculated with *A. rhizogenes* A4M70GUS [118] producing hairy roots, and the shoots regenerated from the roots could accumulate up to 24,700 μg g⁻¹ dry weight Ni.

### 4.3 Uranium

*Brassica* and *Chenopodium* can uptake heavy metals from aqueous solutions [119]. To exploit such potentiality, hairy root cultures of *B. juncea* and *C. amaranticolor* were induced by *A. rhizogenes* A4 and these were utilized for the removal of uranium (U) from solutions [119]. Up to a concentration of 5,000 μM U, the efficiency of *B. juncea* hairy roots was found to be two- to fourfolds greater than that of *C. amaranticolor* and thus appeared to be more suitable for U removal from contaminated solutions. Also, 97% U uptake was noted in *B. juncea* roots in medium lacking phosphate compared to only 40% uptake in medium containing phosphate. Reduction in the levels of free uranyl cations and uranyl hydroxides due to formation of uranium–phosphate complex in the presence of phosphate was suggested as the cause of greater U uptake in *B. juncea* hairy roots in the absence of phosphate. In contrast to these results, Soudek et al. [120] demonstrated that the presence of phosphates could stimulate accumulation of U.
by hairy roots of *A. rusticana*. Straczek et al. [121] studied U toxicity on *D. carota* hairy roots. Under the experimental conditions, U accumulation was recorded to be 4–563 mg kg\(^{-1}\) fresh weight in the presence of 2.5 and 20 mg l\(^{-1}\) U in 34 days with progressive decrease in the threshold of U toxicity for root length over a period of time. Such accumulation level would be suitable in terms of contaminated soil.

### 4.4 Copper

Uptake and tolerance of Cu were analyzed using *Hyptis capitata*, *Polycarpaea longiflora*, and *Euphorbia hirta* hairy root cultures [122]. While similar levels of Cu uptake were noted in *H. capitata* and *P. longiflora* hairy roots, *E. hirta* hairy roots accumulated lower amounts of Cu. The authors also demonstrated the biphasic uptake of Cu by *H. capitata* hairy roots, with faster accumulation during the initial phase as compared to the second phase.

### 4.5 Zinc

Zn is another dominant heavy metal contaminant commonly encountered in water bodies. Subroto et al. [123] reported that up to 98% Zn was taken up from the culture medium and accumulated by *S. nigrum* hairy root cultures, and thus these roots can be used as an efficient means for the remediation of areas polluted with Zn.

### 5 Phytoremediation by Hairy Root–Microorganism Association

An emerging area of interest in the field of phytoremediation is rhizoremediation or phytoremediation assisted by bacteria, which refers to the confluence of phytoremediation and microbial bioremediation [30, 124]. This approach employs microorganisms residing on or near plant roots, called rhizospheric microorganisms. Apart from the removal of contaminants, plants are also benefitted by the presence of these rhizospheric microbial communities as they can provide plants with important nutrients, can protect plants by reducing plant stress hormone levels, or can protect them from plant pathogens [125]. Natural substances exuded by the plants in turn create a nutrient-rich environment for the microorganisms, which boost up their biological activities and help in the degradation of pollutants by induction of enzymes in the microbial populations. This synergism between plants and the rhizospheric microbes can result in either enhanced elimination or breakdown of harmful compounds. As mentioned, hairy roots of different plant species have proved to be ideal model systems to study different aspects of phytoremediation. But to our knowledge, there are very few reports demonstrating association of hairy roots with microbes which would provide useful basis for enhancing the process of phytoremediation. *Burkholderia* and *Agrobacterium* are two genera of microorganisms
which have been shown to degrade phenolic compounds [126, 127]. *Burkholderia* species are a very diverse group of organisms, thriving on rhizosphere surrounding crop plants such as coffee, maize, legumes, tomato, etc. [128]. With the aim to improve phenol phytoremediation techniques, *B. napus* hairy root cultures growing on phenol-supplemented media were inoculated with *B. kururiensis* KP 23 or *A. rhizogenes* LBA 9402 [29]. A promotive effect on phenol eradication from the medium was noted by the existence of both the microorganisms; phenol removal was improved by 34% by co-inoculation of *B. kururiensis*, whereas a 40% improvement was noted in the presence of *A. rhizogenes* compared to roots without the rhizobacteria. The results were encouraging enough to test the rhizoremediation potential of whole plants using these strains.

Another Gram-negative bacterial strain, *Pantoea* sp. FC 1, resistant to phenol and chromium [Cr (VI)] was used in association with *B. napus* hairy roots to test the potentiality of this system in rhizoremediation of phenol and chromium [Cr (VI)] [30]. Significant enhancement in phenol and Cr (VI) removal efficiencies were noted by the hairy roots inoculated with the bacterium than in non-inoculated hairy roots, indicating a positive effect of the co-inoculation process. Their results indicated that *B. napus* hairy roots–*Pantoea* sp. FC 1 association would be an attractive alternative for the rhizoremediation purposes.

The effect of symbiotic association between arbuscular mycorrhizal fungus (AMF) and hairy roots on phytoremediation has also been studied. Ibáñez et al. [129] observed that colonization of transgenic hairy roots of *N. tabacum* with *Glomus intraradices* resulted in higher activity of antioxidative enzymes, viz., Px, superoxide dismutase, and ascorbate peroxidase in the presence of phenol. Furthermore, these roots showed lower oxidative damage when exposed to phenol in comparison to wild-type hairy roots colonized by AMF.

### 6 Conclusions

The importance of phytoremediation lies in its being an effective and optimal model system for the elimination of harmful contaminants from the biosphere. In this aspect, the application of hairy roots has proved to be beneficial. The success of the technique is based on the ability of hairy roots to predict the response of a particular plant species to the pollutant, and thus these roots are important means for screening the plants for phytoremediation. The hairy roots also help in better understanding of plant–soil microbe interaction for rhizoremediation. The challenge lies in applying the knowledge obtained from experimental systems using hairy roots to polluted field sites. Furthermore, the efficiency of phytoremediation ability of a particular species might be enhanced by introduction and expression of genes producing degradation enzymes, proteins capable of binding heavy metals, etc., in hairy roots developed by *A. rhizogenes*-mediated genetic transformation. However, in-depth elucidation of xenobiotic detoxification pathways, enzymes involved, and rate-limiting steps are necessary for optimization of experimental setups using hairy roots. As hairy roots originating from a single plant can be propagated extensively, difficulties arising due to variability among individual specimens can be overcome.
Since hairy roots do not possess identical characteristics to whole plants, they may serve as supporting systems to provide information prior to whole-plant field trials. Rapid rate of hazardous contaminant accumulation in the environment due to either geological or man-made reasons necessitates the utilization of hairy root cultures in phytoremediation, keeping in mind their stable nature, fast growth rate, and easy maintenance.

References

126. Patil SS (2014) Biodegradation study of phenol by Burkholderia sp. PS3 and Bacillus pumilus OS1 isolated from contaminated soil. Thesis, National Institute of Technology, Rourkela