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Review Article

IN VITRO HAIRY ROOT CULTURE: A PROMISING APPROACH TO INVESTIGATE MOLECULAR MECHANISM OF PHYTOREMEDIATION

Pradip Hirapure[1], Vijay J. Upadhye[3] and Arti Shanaware[2*]

[1]Department of Biotechnology and Biochemistry, Dr. Ambedkar College, Deekshabhoomi, Nagpur (India)
[2*]Research and Development Cell, Parul Institute of Applied Sciences, Parul University, Vadodara, Gujarat
[3]Rajiv Gandhi Biotechnology Center, Laxminarayan Institute of Technology Campus,
Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur (India)

Abstract

Environmental pollution caused by natural processes or anthropogenic activities is a major global problem. Although several physical and chemical strategies have been used for environmental remediation, these methods are expensive and associated with certain limitations. Phytoremediation is an alternative, biological approach where different plant species are used for the removal of pollutants from the environment or for converting toxic molecules to non toxic forms. Both organic and inorganic compounds of various types are the targets of phytoremediation. Recently Hairy root cultures have been considered as a superior model system to investigate molecular mechanism of phytoremediation processes as rhizofiltration, phytostabilization and phytoextraction of organic and inorganic pollutants because of their biochemical and genetic stability and easy maintenance. This review focuses on the phytoremediation process, induction and establishment of *in vitro* hairy root culture, advantages of hairy root culture, future application of hairy root culture system to understand the molecular mechanism of phytoremediation

Key words: Hairy Root, Phytoremediation, *Agrobacterium rhizogenes*, *In vitro* Culture

Introduction

Environmental contamination has become a concerning issue worldwide due to number of risks it poses to human health and ecosystem functioning (Malik *et al.*, 2017). Contaminants present in soil or water can go up through the trophic chain via microbial or plant incorporation (Chibuike and Obiora, 2014).). Remediation of contaminated sites using conventional practices, such as 'pump-and-treat' and 'dig-and-dump' techniques, is often expensive, has limited potential, and is usually only applicable to small areas. Additionally, these conventional approaches to remediation often make the soil infertile and unsuitable for agriculture and other uses by destroying the microenvironment (Vidali, 2001). Hence now a day's Phytoremediation is an alternative, biological approach where different plant species are used for the removal of pollutants from the environment. 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 (Agostini *et al.,* 2013). It makes the use of various plants to remove, transfer or stabilize pollutants in an environmental friendly manner. Hairy roots of plants are among the several experimental systems which have been employed to improve the efficiency of phytoremediation (Majumder and Jha, 2012). Hairy root disease, characterized by a massive production of adventitious roots with numerous root hairs at the wound site, is caused by *Agrobacterium rhizogenes*, a gram negative soil bacterium. The bacterium transfers a segment (the transferred DNA or T-DNA) of its root inducing (Ri) megaplasmid into the plant genome, modifying the normal hormonal metabolism of the plant; this hormonal imbalance induces the formation of hairy roots at the site of infection (Agostini *et al.,* 2013). This article discusses the phytoremediation process, induction and establishment

***Author for correspondence :** E-mail: artishanware@gmail.com

of hairy root culture, advantages of hairy root culture, future application of hairy root culture system to understand the molecular mechanism of phytoremediation.

Phytoremediation

Phytoremediation ('phyto' means plant) is a generic term for the group of technologies that use plants for remediating soils, sludges, sediments and water contaminated with organic and inorganic contaminants (Champagne, 2007). Plants have evolved a great diversity of genetic adaptations to handle the accumulated pollutants that occur in the environment (Sakina Saadawi *et al.,* 2015). Growing and, in some cases, harvesting plants on a contaminated site as a remediation method is a passive technique that can be used to clean up sites with shallow, low to moderate levels of contamination (Azubuike *et al.,* 2016). Phytoremediation can be used to clean up metals, pesticides, solvents, explosives, crude oil, polyaromatic hydrocarbons, and landfill leachates (Mahendran, 2014). It can also be used for river basin management through the hydraulic control of contaminants (Champagne, 2007). Phytoremediation has been studied extensively in research and small-scale demonstrations, but full-scale applications are currently limited to a small number of projects. Further research and development will lead to wider acceptance and use of phytoremediation (Kokyo *et al.,* 2014).

Phytoremediation is:

A low cost, solar energy driven cleanup technique.

Most useful at sites with shallow, low levels of contamination.

Useful for treating a wide variety of environmental contaminants.

Phytoremediation Processes

Different techniques have been introduced to exploit the potential of plants for the removal of hazardous compounds from contaminated water and soil. Schwitzguebel has explained different technological subsets of phytoremediation (Razzaq, 2017).

Phytoextraction (Phytoaccumulation): Removal of pollutants using the plants having the ability to accumulate pollutants from the soil and store them in their shoots so that they can be harvested.

Phytotransformation: It is the phenomenon in which the complex organic molecules are converted into the simpler one by degrading them and the simpler one can then be incorporated in the tissues of plants.

Phytostimulation: This process includes the stimulation of enzymes present in the rhizosphere which can lead to the bioremediation using microbes or fungal degradation by releasing exudates.

Phytovolatilization: In this, the plants take up the pollutants and then they can volatile from the surface of the leaves.

Phytodegradation: In this technique, there is the use of enzymes for the breakdown of harmful organic pollutants like herbicides or trichloroethylene. This can happen both inside or outside the plants as the plants can also secrete the enzymes outside.

Phytorhizofiltration: It is the inhibition of organic pollutants from mixing into the water streams or groundwater using roots for filtration purpose as they can absorb or adsorb the pollutants.

Phytostabilization: This technique involves the prevention of mobility of organic pollutants into the soil thus reducing its bioavailability and stops them from entering into the food chain. Some types of phytoremediation process and removal of respective pollutants mentioned in table 1.

Does Phytoremediation Work at Every Site?

Phytoremediation technologies have been used to clean up metals, pesticides, solvents, explosives, crude oil, polyaromatic hydro-carbons, and landfill leachates

Table 1: Outline of Phytoremediated Chemicals (Nwoko, 2010)

Type of phytoremediation	Chemicals Treated
Phytoaccumulation/extraction	Cd, Cr, Pb, Ni, Zn, radionuclides, BTEX*, penachlorophenol, short chained aliphatic compounds
Phytodegradation/transformation	Nitrobenzene, nitroethane, nitrotoluene, atrazine, chlorinated solvents (chloroform, carbon tetrachloride, etc)
Phytostabilization	Heavy metals in ponds, phenols and chlorinated solvents
Phytostimulation	Polycyclicaromatic hydrocarbon, BTEX, PCB#, tetrachloroethane
Phytovolatilization	Chlorinated solvents, Hg, Se.
Phytofiltration	Heavy metals, organics and radionucleides

*BTEX = benzene, toluene, ethyl benzene, xylenes; #PCB = Polychlorinated biphenyl

(Mahendran, 2014). Phyto-remediation can be used in combination with other cleanup approaches as a ‘finishing’ or ‘polishing’ step. Some phytoremediation applications are slower than mechanical and chemical methods and are limited to the depths that are within the reach of the plant roots (Singh, 2005). Generally, the use of phytoremediation is limited to sites with low to medium contaminant concentrations and contamination in shallow soils where phytotoxicity does not occur and the roots of plants can easily access the contaminant (Bieby *et al.,* 2011). Plants can also be used to clean up contaminants in streams and groundwater. Researchers are finding that the use of trees (rather than smaller plants) allows for the treatment of contamination at greater depths, as tree roots penetrate more deeply into the ground. Very deep contaminated ground-water may be treated by first pumping the water out of the ground and then using plants to treat the contamination (Pierret *et al.,* 2016).

Further research is needed to study the effects of bioaccumulation and biomagnification in the food chain that could occur if insects and small rodents eat the plants that are collecting contaminants and are then eaten by larger mammals. In addition, scientists need to establish whether contaminants can collect in the leaves and wood of trees used for phytoremediation and be released when the leaves fall in the autumn or when firewood or mulch from the trees is used.

Induction and Establishment of Axenic Hairy Root Culture

Hairy root disease is caused by a naturally occurring gram negative soil bacterium *Agrobacterium rhizogenes* that contains root inducing (Ri) plasmid (more than 200 kb). T-DNA on Ri-plasmid is transferred and integrated into the nuclear genome of the host plant upon infection with *A. rhizogenes* (Nilsson and Olsson, 1997; Riva *et al.,* 1998; Sevón and Oksman-Caldentey, 2002). Opines are produced as a result of infection, and based on the opines produced, *A. rhizogenes* strains are classified into octopine, agropine, nopaline, mannopine, and cucumopine type (Zhou *et al.,* 1998). Choice of proper explant, an appropriate *A. rhizogenes* strain, culture medium, as well as selection of antibiotic to remove the bacteria after cocultivation are important factors for establishing hairy root culture (Hu and Du, 2006). Different explants like root, stem, leaf, hypocotyl, cotyledons, nodal segments, and embryo axis can be used for initiation of hairy roots. Out of all the strains, agropine is the most commonly used due to its strong induction ability. The protocol for successful establishment of axenic hairy root culture (HRC) involves three steps *viz*. root induction, decontamination of roots and establishment in liquid medium. Seedling grown under sterile conditions is inoculated with an appropriate strain of *Agrobacterium rhizogene.* A commonly used technique is to wound the axenic seedling or suitable plant material with an overnight culture of *A. rhizogene*. Hairy root emerge from the site of infection within 2-4 weeks. Putative hairy roots are established on MS medium. The hairy roots are decontaminated by subculturing on MS solid medium containing 250 mg/l antibiotic. The bacteria free hairy roots can be successfully grown in a phytoharmone free MS liquid medium. The cultures are incubated on a rotary shaker at 100 RPM, with 25^{o} C temperature (Uozumi, 2004; Guillon *et al.,* 2006). Hairy roots are characterized by a large number of highly branched fast growing adventitious roots at the wound site which are capable of growing on hormone free medium. These are plagiotrophic in nature and are found to be genetically as well as biochemically stable over a long period of time (Hu and Du, 2006; Uozumi, 2004; Guillon *et al.,* 2006; Chilton *et al.,* 1982).

Table 2 : Pollutants and Hairy Root culture of plant species (Sonia Malik *et al.,* 2017)

Pollutant	Hairy root culture of plant species
Cadmium	*Beta vulgaris, Nicotiana tabacum, Solanum nigrum, Thlaspi caerulescens, Adenophora lobophylla, A. potaninii*
Copper	*Rubia tinctorum, Hyptis capitata*
Uranium	*Chenopodium amaranticolor, Brassica juncea*
Nickel	*Alyssum sp., Alyssum murale*
Phenol	*Brassica juncea, B. napus, Lycopersicon esculentum, Armoracia lapathifolia, Daucus carota*
RDX and HMX	*Catharanthus roseus*
Tetracycline and Oxytetracycline	*Helianthus annuus*
PCBs	*Solanum nigrum*

Abbreviations: (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)

Advantages of Hairy Root Culture for Phytoremediation

Plant roots are in direct contact with pollutants in contaminated soil or water, their responses to toxic substances are of particular importance in phytoremediation and phytomining research. Genetically transformed hairy roots offer many practical advantages compared with untransformed roots or dedifferentiated plant cells in experimental studies, such as ease of initiation, culture, and maintenance, indefinite propagation of material derived from the same parent plant, genotype and phenotype stability, biochemical stability, autotrophy in plant hormones. Hairy roots have been applied mainly in metabolic studies of xenobiotic biotransformation and degradation in plants, and for determining the responses of plant tissues to toxic heavy metal (Arora's, 2010; Doran, 2013). Hairy roots provide a large surface area due to fast growth and highly branched nature and hence contact between the contaminants and tissue in comparison to naturally growing roots thus providing reliable and reproducible experimental system to study the pollutants and their response to toxic substances (Suza *et al.,* 2008; Eapen *et al.,* 2003). Introduction of foreign genes and their resultant proteins to metabolize environmental pollution in transformed hairy roots can be expressed for a long term due to genetic stability (Bernejee *et al.,* 2002). Another advantage associated with the hairy roots is absence of shoots that help in understanding the mechanisms present only in roots for removal of contaminants without the translocation effects (Majumder and Jha, 2012). These roots can also be used to understand the enzymatic processes involved in bioconversion of toxic pollutants to nontoxic compounds (Macek *et al.,* 2000). Literature studies showed the number of reports wherein hairy roots have been successfully used to study phytoremediation. Some of the examples of hairy root cultures from different plant species employed to uptake and degrade the various pollutants are presented in table 2.

Cellular and Molecular Mechanisms of Phytoremediation

Exposure to pollutants may cause a series of symptoms in plants. Pollutant action can result in inhibition of cellular activity or rupture of cell structure, due to possible damages of essential components (Cherian and Oliveira, 2005). Plants show some potential cellular and molecular mechanisms and strategies, which can be involved in detoxification of organic and inorganic pollutants such as herbicides, explosives and heavy metals. These mechanisms can be related to the cell wall composition and root environment, plasma membrane properties and integrity, enzymatic transformation, complexation with ligands and vacuolar compartmentalization (Hall, 2002; Mello-Farias and Chaves, 2008). Depending on the nature of pollutant (organic or inorganic) plant cells can use one or some of these systems of remediation (Cherian and Oliveira, 2005); Hall, 2002; Mello-Farias and Chaves, 2008). Hairy root cultures provide an ideal model system to identify the role of plants in phytoremediation and have been used frequently for this purpose (Doran, 2009). They allow researchers to monitor and quantify the uptake of pollutants and follow the detoxification process in detail. Thus, hairy roots will help in gaining a greater understanding of the way plants deal with pollutants. In addition, hairy roots can be subjected to various physiological assays. This information will be valuable in choosing the best plant species for use in bioremediation (Suza *et al.,*2008).

Much work on metal transport in plants is done with the hyperaccumulator T. calerulescens. Molecular genetics comparison of different ecotypes has resulted in the identification of genes involved in transmembrane metal transport and hyperaccumulation (Plaza *et al.,* 2007; Plessl *et al.,* 2010; Visioli *et al.,* 2010), a key role is played by genes belonging to the ZIP, HMA, MATE, YSL, and MTP families (Rascio and Navari-Izzo, 2011). High-throughput technologies, in particular microarray, have allowed the complexity of plant stress response to be tackled. Much work has been reported recently in these filed. Here, we reviewed the progresses since 2009. For more information, readers can refer to the reviews by Verbruggen *et al.,* (2009), Thapa *et al.,* (2012), Claire-Lise and Nathalie (2012) and other related papers.

Transgenic Hairy Roots for the Improvement of Phytoremediation

The understanding of the physiological and biochemical processes, types of enzymes and genes involved in the metabolism of a particular compound, allows the obtainment of genetically modified plants with the purpose of improving the efficiency of pollutants removal or transformation. Furthermore, encouraging results have been obtained using transgenic HRs. For inorganic compounds, subcellular targeting of metal-binding proteins to cytoplasmic membrane or to desired cell organelles could enhance metal accumulation (Bizily *et al.,* 2003; Hussein *et al.,* 2007). As it was mentioned before, Cu hyperaccumulating *A. thaliana* plants were generated by expressing Cu-binding periplasmic protein CopC (Rodrý´guez-Llorente *et al.,* 2012). This novel strategy has been also applied to obtain transgenic HRs, which accumulated high Cu concentrations by expressing

CopC protein either in the cytoplasm of cells or by targeting it to the vacuole (Pe´rez-Palacios, 2015). HRs by a multi-transgene strategy, could contribute with an increased phytoremediation of mixed polluted environments.

Hairy Roots Versus Plants for Phytoremediation Studies

HRs have proved to be useful for phytoremediation researches providing basic information related on the capability of plant cells to tolerate, detoxify, metabolize, and store a wide variety of organic and inorganic pollutants. Particularly, many aspects of the primary interaction roots-pollutants have been elucidated taking advantage of the physiologic similarity between HRs and real roots (Nedelkoska and Doran, 2000). Recently, omic technologies have allowed deepening our knowledge about the HRs biotechnological potential and they have revealed the main similarities and differences between HRs and their mother plants (Sharma *et al.,* 2013; Gao *et al.,* 2014; Georgiev *et al.,* 2015). Thus, it is expected that these technologies would be useful tools to clarify some aspects related with phytotransformation of recalcitrant xenobio-tics, which still remain unclear. In addition, transgenic HRs give the possibility to study genes functionality and the role of some key proteins and enzymes involved in several metabolic pathways used by plant cells to tolerate and detoxify environmental pollutants. As it was pointed out, HRs have an undeniable potential regarding to the use of plants for *in vitro* phytoremediation studies. However, some limitations, mostly associated with their application in a real scenario should be considered

Conclusion and Perspectives

The environment has become more and more concerned in the fields of economics, politics, social and cultural affairs, science and technology. To enhance the potential for environmental protection and conservation, a considerable amount of researches have been done for phytoremediation. However, those studies in the field of biological applications do not provide much attention to uncertainty in the molecular principles. HRs have contributed to the knowledge of the complex biochemical and molecular mechanisms involved in phytoremediation. In this sense, a better understanding of the intrinsic roots metabolism is allowed since there is not translocation of the pollutant or its intermediates to aerial parts. HRs have proved to be effective tools for studying the mechanisms of metal uptake, accumulation and tolerance. The phytoremediation is still under investigation as well in methodological aspects as in concrete applications. In order to move phytoremediation forward, it is important to look for and investigate the molecular mechanism of new plant species with the ability to remove contaminants from our environment.

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