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Transgenic Plants for Enhanced Phytoremediation – Physiological Studies

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1. Introduction

Natural processes such as volcanic eruptions, continental dusts and anthropogenic activities like mining, combustion of fossil fuel phosphate fertilizers, military activities and metal working industries lead to emission of heavy metals and accumulation of these chemicals in ecosystem. So, the metals found in our environment come from natural weathering process of earth's crust, soil erosion, mining, industrial discharge, urban runoff, sewage effluents, air pollution fall out, pest or disease control agents. The concentrations of the contaminants can vary from highly toxic concentrations from an accidental spill to barely detectable concentrations that, after long-term exposure, can be detrimental to human health (Alexander, 1999; Doty, 2008). Particularly, heavy metals are toxic because they cause DNA damage and their carcinogenic effects in animals and humans are probably caused by their mutagenic ability (Knasmuller et al., 1998; Baudouin et al., 2002; Hooda, 2007). Potential threat is that heavy metals are not degradable and without intervention stay in soil for centuries. The cleanup of most of the contaminated sites is mandatory in order to reclaim the area and to minimize the entry of toxic elements into the food chain. Various engineering – based methods such as soil excavation, soil washing or burning or pump and treat systems are already being used to remediate metal contaminated soils ((Hooda, 2007). As a result over recent decades an annual worldwide release of heavy metals reached 22,000 t (metric ton) for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh et al., 2003).

The cost of cleaning up contaminated sites is extremely high. In the USA alone, U$ 6–8 billion is annually spent in remediation efforts, with global costs in the range of U$ 25–50 billion (Glass, 1999; Tsao, 2003). Engineering methods for the remediation of contaminated sites include excavation, transport, soil washing, extraction, pumping and treating of contaminated water, addition of reactants such as hydrogen peroxide or potassium permanganate, and incineration. A serious consequence of the high cost of remediation technologies is that polluted commercial properties are often abandoned rather than cleaned up. There are over 500,000 of these so-called brownfields in the USA.

Elemental pollutants are particularly difficult to remediate from soil, water, and air because, unlike organic pollutants that can be degraded to harmless small molecules, toxic elements

such as mercury, arsenic, cadmium, lead, copper, and zinc, are immutable by all biochemical reactions and hence remain in the ecosystem (Kramer & Chardonnens, 2001). The heavy metals remainings in various ecosystems would seep into surface water, groundwater or even channel into the food chain by crops growing on such a soil (Lin et al., 1998). These heavy metals may adversely affect the soil ecosystem safety, not only agricultural product and water quality, but also the human health (Zhou et al., 2004).

Another popular clean-up method involves augmented bioremediation with the addition of specific microbial strains known to degrade the pollutant. Bacteria and fungi collectively can utilize a vast range of organic molecules. But for bioremediation using microbes at a particular site to be successful, many conditions must be met. These include the ability of the microbes with the desired metabolic activity to survive in that environment, the accessibility or bioavailability of the chemical, and the presence of inducers to activate expression of the necessary enzymes. Many organic pollutants are recalcitrant to degradation and cannot be used as sole carbon source (Doty, 2008). The pollutants are sometimes metabolized by enzymes with other natural substrates; therefore, these substrates sometimes need to be present in order for the genes to be expressed. This requirement is problematic if the inducing chemical is itself a harmful pollutant, such as phenol. Bioremediation also depends on the presence of sufficient carbon and energy sources. Often, thousands of gallons of a food source such as molasses must be pumped down into the site to allow bacterial growth (Doty, 2008).

The use of microorganisms in engineered bioremediation systems has had mixed success. A review of this broad and active field is beyond the scope of this review; a recent book provides an excellent overview of bioremediation of xenobiotics, petroleum, BTEX (benzene, toluene, ethylbenzene, and xylene), explosives, and heavy metals (Fingerman & Nagabhushanum, 2005; Doty, 2008).

Plants are autotrophic organisms capable of using sunlight and carbon dioxide as sources of energy and carbon. However, plants rely on the root system to take up water and other nutrients, such as nitrogen and minerals, from soil and groundwater. As a side effect, plants also absorb a diversity of natural and man-made toxic compounds for which they have developed diverse detoxification mechanisms (Eapen et al., 2007; Van Aken, 2008).

Pollutant-degrading enzymes in plants probably originate from natural defense systems against the variety of allelochemicals released by competing organisms, including microbes, insects and other plants (Singer, 2006; Van Aken, 2008). From this viewpoint, plants can be seen as natural, solar-powered pump-and-treat systems for cleaning up contaminated environments, leading to the concept of phytoremediation (Pilon-Smits, 2005; Van Aken, 2008).

First developed for the removal of heavy metals from soil, the technology has since proven to be efficient for the treatment of organic compounds, including chlorinated solvents, polyaromatic hydrocarbons and explosives (Pilon-Smits, 2005; Salt et al., 1998; Van Aken, 2008). Beyond the removal of contaminants from soil, phytoremediation involves different processes, such as enzymatic degradation, that potentially lead to contaminant detoxification (Pilon-Smits, 2005; Dietz & Schnoor, 2001; Van Aken, 2008). However, despite great promise, rather slow removal rates and potential accumulation of toxic compounds within plants might have limited the application of phytoremediation (Eapen et al., 2007; Van Aken, 2008).

Unlike organic contaminants, metals cannot be degraded. Instead, phytoremediation strategies for metals are based on stabilization, accumulation, and in some cases on

volatilization. The phytostabilization of metals may simply involve the prevention of leaching through the upward water flow created by plant transpiration, reduced runoff due to above-ground vegetation, and reduced soil erosion via the stabilization of soil by plant roots (Vassilev et al., 2004; Martínez et al., 2006).

Phytoremediation, as a cost-effective and environmentally friendly method, is an emerging technology based on the use of plants to remove, transform, clean up or stabilize contaminants including organic pollutants located in water, sediments, or soils (Cunningham et al., 1997; Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008). This method has attracted growing attention because of its distinctive potential and advantages compared with conventional technologies, such as soil replacement, solidification, and washing strategies (Yang et al., 2005; Mello-Farias & Chaves, 2008). The advantages of phytoremediation over usual bioremediation by microorganisms are that plants, as autotrophic systems with large biomass, require only modest nutrient input and they prevent the spreading of contaminants through water and wind erosion (Pulford & Watson, 2003; Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008). Plants also supply nutrients for rhizosphere bacteria, allowing the growth and maintenance of a microbial community for further contaminant detoxification (Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008). Phytoremediation takes advantage of the unique, selective and naturally occurring uptake capabilities of plant root systems, together with the translocation, bioaccumulation and pollutant storage/degradation abilities of the entire plant body. Besides being aesthetically pleasing, phytoremediation is on average tenfold cheaper than other physical, chemical or thermal remediation methods since it is performed *in situ*, is solar driven and can function with minimal maintenance once established (Hooda, 2007).

According to Sarma (2011), there are different strategies of phytoremediation, each having a different mechanism of action for remediating metal-polluted soil, sediment or water, like: 1) Phytoextraction: plants absorb metals from soil through the root system and translocate them to harvestable shoots where they accumulate. Hyperaccumulators mostly used this process to extract metals from contaminated site. The recoveries of extracted metals are also possible through harvesting the plants appropriately; 2) Phytovolatilization: plants used to extract certain metals from soil and then release them into the atmosphere by volatilization; 3) Phytostabilization: plant root microbial interaction can immobilize organic and some inorganic contaminants by binding them to soil particles and as a result reduce migration contaminants to grown water; 4) Phytofiltration: plant roots (rhizofiltration) or seedlings (blastofiltration) absorb or adsorb pollutants, mainly metals from water and aqueous waste streams (Prasad & Freitas, 2003)

Many genes are involved in metal uptake, translocation and sequestration; the transfer of any of these genes into candidate plants is a possible strategy for genetic engineering of plants to improve phytoremediation traits. Depending on the strategy, transgenic plants can be engineered to accumulate high concentrations of metals in harvestable parts. Transfer or overexpression of genes will lead to enhanced metal uptake, translocation, sequestration or intracellular targeting. Genetic engineering of plants for synthesis of metal chelators will improve the capability of plant for metal uptake (Karenlampi et al., 2000; Pilon-Smits & Pilon, 2002; Clemens et al., 2002; Eapen & D´Souza, 2005).

The application of powerful genetic and molecular techniques may surely identify a range of gene families that are likely to be involved in transition metal transport. Considerable progress has been made recently in identifying plant genes encoding metal ion transporters

and their homologous in hyperaccumulator plants. Therefore, it is hoped that genetic engineering may offer a powerful new means by which to improve the capacity of plants to remediate environmental pollutants (Yang et al., 2005, Mello-Farias & Chaves, 2008).

2. Types of pollutants

There is a variety of different pollutants, originated, in most cases, by human action. To facilitate the development of studies on decontamination techniques and also according the different physical and chemical characteristics they present, the different types of contaminants were divided into two major classes: organic and inorganic. These two groups are further subdivided. Organic pollutants include various compounds such as polychlorinatedbiphenyls (PCB's), polycyclicaromatichydrocarbons (PAH's), nitroaromatic (explosives), halogenated hydrocarbons, chlorinated solvents. When compared to inorganic, the organic pollutants are relatively less toxic to plants because they are less reactive and do not accumulate readily. Many of these compounds are not only toxic or teratogenic, but also carcinogenic.

The inorganic contaminants include heavy metals, such as mercury, lead, cadmium, among others; and non-metallic compounds like arsenic and radionuclides like uranium, cesium, chromium, strontium, technetium, tritium, etc. Many metals are essential to growth and development of living forms. However, when in high concentrations, they become extremely toxic, leading the organism to oxidative stress with great production of harmful free radicals, highly dangerous to cells and tissues. Some particularly reactive metals interfere in the structure and function of proteins, and also cause the substitution of other essential nutrients (Garbisuet al. 2002; Pulfort; Watson, 2003; Taiz & Zeiger, 2002).

Many elemental pollutants penetrate the plant through regular systems of nutrient absorption. The plants protect themselves from these xenobiotics through degradation of endogenous toxic organic or sequestering them in the vacuoles (Meagher, 2000).

Different technologies of phytoremediation are compatible with a great number of pollutants. Constructed wetlands have been applied for many inorganics, including metals, nitrates, phosphates, cyanides, as well as organics such as explosives and herbicides (Horne 2000; Schnoor et al., 1995; Jacobson et al, 2003).

There is a special category of plants called hyperaccumulators (described later), for they accumulate a considerable amount of toxic metals and radionucleides in their tissues (phytoextraction), keeping these compounds above the ground surface. This is the main goal of phytoremediation.

2.1 Inorganics

The absorption of any metal by plants depends on the metal relative bioavailability in the contaminated array. Changes in the soil chemistry, such as decreased pH, may increase the availability of many metals for the absorption by the roots. Many plants can absorb significant levels of metals in some soil conditions. Changes in rhizosphere microbial status (e.g.: presence of mycorrhizae) can also have profound effects (positive or negative) on the uptake of metals by the roots (Smith, 1996). The general consensus of researchers in this area, however, is that phytoremediation, especially for heavy metals, will only be economically viable through the use of hyperaccumulators. The research in the past two decades has shown that certain specialized plants have the ability to accumulate more than 3% (dry weight) of heavy metals and over 25% (dry weight) in sap / latex with no apparent

damage to the plant (Baker & Brooks, 1989, Baker et al. 1994; Huang & Cunningham, 1996). The mechanisms that govern this tolerance and absorption of excessive concentrations of metals in leaves were the subject of active research and vary according to the element (Cunningham & Lee, 1995; Huang & Cunningham, 1996). The mechanisms of tolerance include the accumulation of Zn in cell walls; Ni associated with the pectin in large cells; Ni, Co and Zn being chelated by malic acid; phytochelatin associated to Zn, Ni chelation by citrate; and Co associated with oxalate crystals calcium in plant tissues. Knowledge of the mechanisms of tolerance will aid in identifying the genetic characteristics necessary for the transfer of metal tolerance of plants capable of producing greater biomass with deeper rooting. It was suggested that in some cases, the resulting biomass rich in metals (biominery) could be incinerated and have metals economically recycled. This "biomineration" of metals can also be applied as a mining technique for metals with significant economic value (Robinson et al., 1998).

Another type of inorganic compounds that may be susceptible to phytoremediation are radionucleides. The presence of radionucleides in soil and water poses serious risk to human health. These contaminants come from the explosion of atomic bombs or nuclear power plant accidents such as Chernobyl, Ukraine and, more recently in Fukushima, Japan. The selection of an appropriate cleaning technology of these contaminated areas is based on the environmental chemistry of each element, character of deposition and rate of radioactive decay. A variety of physicochemical methods are available, like soil washing, ion exchange, leaching with chelating agents, flocculation and osmosis-ultrafiltration. Recently there has been increasing interest in the use of biological methods to remove radionucleides (Duschenkov, 2003). Negri and Hinchman (2000) reported data in the use of plants for the treatment of ^{3}H, U, Pu, ^{137}Cs and ^{90}Sr.

2.2 Organics

More recently, with the development of the pesticide industry, the metabolic capacity of the plant system began to be assessed. The most modern herbicides are based on the selectivity of crops due to metabolic differences between species of plants. This capability, often created by man, is the cornerstone of the highly profitable market of herbicides. "Desirable" plants rapidly metabolize the herbicide compound in a nontoxic one, while "undesirable" herbs do not, and are therefore dead. This mechanism developed by the natural selection of plants, proves to be potentially exploitable in the remediation of contaminated soils.

This ability of plants to detoxify xenobiotics is widely recognized and with current utility. Besides, plants generally have a metabolic system with differences in the efficiency of degradation of toxic compounds when compared to microorganisms, what makes the union of these two distinct systems in the rhizosphere, an ideal situation for a more efficient phytoremediation. Recent research includes plant selection, alternative patterns of rooting, the composition of exudates produced by the plant and its effect on microbial communities, exudation of specific compounds inducing specific metabolic pathways and, inoculation with rhizosphere microorganisms capable of degrading xenobiotics efficiently (Langenbach, 1994). The plants and their roots can create an environment in the soil which is rich in microbial activity, able to change the availability of organic contaminants or increase the degradation of certain organic compounds, such as hydrocarbons derived from Petroleum.

Siciliano et al. (2003) evaluated the impact of microbial remediation on soil mass and the capacity of microbial community to degrade hydrocarbons in order to determine whether phytoremediation treatments increase the metabolic potential of microbial community by

altering its taxonomic structure. It was found that the best remediation system to reduce hydrocarbons in the soil was obtained by increasing the population of bacteria containing genes for the catabolism of hydrocarbons in the rhizosphere community, thus demonstrating the importance of using microorganisms in phytoremediation. However, it is necessary to identify the species of suitable plants that can beneficially alter microbial diversity for soil remediation. According to Pires et al. (2003), the absorption of herbicides by plants is affected by the compound's chemical properties, environmental conditions and the characteristics of plant species. Actually, the probability of a plant being phytoremediator depends on the type of pollutant; plants should be tested to detect that one with the greater resistance to a specific pollutant. Esteve-Nunez et al. (2001) evaluated trinitrotoluene (TNT), and found that its chemical structure influences its biodegradability. According to these authors, the oxygenated metabolism for aromatic compounds by bacteria does not occur in TNT because of its chemical properties generating compounds not metabolized by microorganisms. However, anaerobic processes have advantages because of the absence of oxygen. Therefore the use of fungi for the bioremediation of TNT has generated considerable interest. Esteve-Nunez et al. (2001) concluded that the remediation of TNT by these organisms is a very valid process; and the rhizoremediation by microbes able to colonize the rhizosphere of plants, will provide a fast and efficient mechanism for the removal of this pollutant. Figure 1 shows some types of organic pollutants.

There are other types of contaminants called Persistent Organic Pollutants (POPs) that resist long in the soil. Some examples are Dichlorodiphenyltrichloroethane (DDT), Polychlorinated biphenyls (PCB), Dioxins, etc. Research has shown that a variety of plants can remove persistent compounds, transporting them to aerial plant tissues (Coutinho & Barbosa, 2007). It is important to highlight that, due to variety of contaminants, the study of pollutant is very important to generate an effective phytoremediation.

Most current methods of cleaning metals and volatile organic compounds not on the soil surface are coarse, expensive and physically destructive (Baker et al., 1994). The remediation by conventional methods of engineering often costs 50 to 500 dollars per ton of soil, and certain specialized techniques can cost up to US$ 1000 (Cunningham & Ow, 1996). Phytoremediation associated with biotechnology is an emerging technology that promises a viable remediation when pollutants: a) are close to the surface, b) are relatively non-leachable, and c) have little immediate risk to the environment (Cunningham & Lee, 1995). The results are more effective in slightly or moderately polluted areas. For heavy contamination, the decontamination time is too long (Robinson et al., 1998). The combination of metal hyperaccumulation and degradation or, increased sequestration of organic compounds with greater biomass and deeper rooting systems can result in a powerful technology of phytoremediation that will provide cheaper, permanent and intrusive remediation. Table 1 shows a summary of the techniques applied to the different types of phytoremediated compounds.

3. Hyperaccumulators

Over recent years a special interest has emerged in the phenomenon of heavy-metal hyperaccumulation since this property may be exploited in the remediation of heavy-metal-polluted soils through phytoextraction and phytomining (Robinson et al., 1997; Martínez et al., 2006).
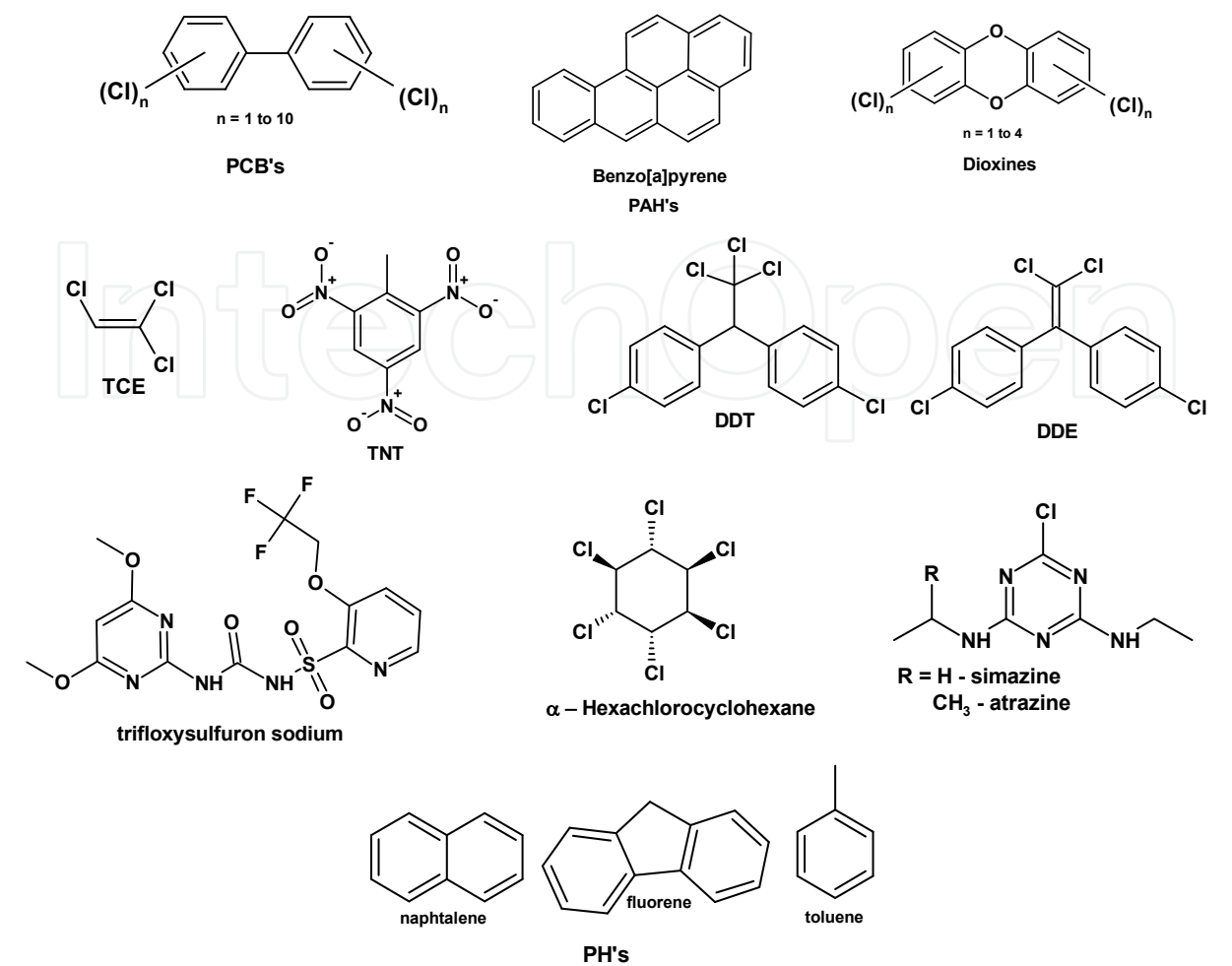
Fig. 1. Different types of organic pollutants

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
Table 1. Outline of phytoremediated chemicals (Nwoko, 2010).

The concept of hyperaccumulation was originally introduced to plants containing more than 0.1% (1000 mg.kg^{-1}) of Ni in dried plant tissues (Jaffré et al., 1976). At present, the criteria used for hyperaccumulation vary per metal, ranging from 100 mg.kg^{-1} dry mass for Cd, to 1000 mg.kg^{-1} for Cu, Co, Cr, and Pb, to 10000 mg.kg^{-1} for Zn and Mn. These values exhibit a shoot-to-soil metal concentration ratio, the so-called bioaccumulation factor that is higher than 1 (Baker et al., 1994).

An ideal plant for environmental cleanup should have a high biomass production, combined with superior capacity for pollutant tolerance, accumulation, and degradation, depending on the type of pollutant and the phytoremediation technology of choice. Hyperaccumulators are good candidates in phytoremediation, particularly for the removal of heavy metals. Phytoremediation efficiency of plants can be substantially improved using genetic engineering technologies (Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008).

Transferring the genes responsible for the hyperaccumulating phenotype to higher shoot-biomass-producing plants has been suggested as a potential avenue for enhancing phytoremediation as a viable commercial technology (Pilon-Smits & Pilon, 2002; Martínez et al., 2006).

Some of the plants belonging to *Brassicaceae* such as *Alyssum* species, *Thlaspi* species and *Brassica juncea*, *Violaceae* such as *Viola calaminaria*, *Leguminosae* such as *Astragalus racemosus* are known to take up high concentrations of heavy metals and radionucleides (Reeves & Baker, 2000; Negri & Hinchman, 2000, Eapen & D´Souza, 2005).

To date, there are approximately 400 known hyperaccumulators metal in the world (Reeves & Baker, 2000; Eapen & D´Souza, 2005) and the number is increasing. However, the remediation potential of many of these plants is limited because of their slow growth and low biomass (Chaney et al., 2000; Lasat, 2002; McGrath et al., 2002; Eapen & D´Souza, 2005).

4. Cellular and molecular mechanisms involved in 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 (Coutinho & Barbosa, 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; Cherian & Oliveira, 2005). Depending on the nature of pollutant (organic or inorganic) plant cells can use one or some of these systems of remediation (Coutinho & Barbosa, 2005; Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008).

4.1 Cell wall composition and rhizosphere

Organic contaminants, when in contact with roots, may be sorbed to the root structure. The hydrophobic or hydrophilic nature of the organic compounds also determines their possible uptake. Hemicellulose in the cell wall and the lipid bilayer of plant membranes can bind hydrophobic organic pollutants effectively (Pilon-Smits, 2005). In addition, the root uptake of chemicals depends on many factors as plant's uptake efficiency, the transpiration rate, and the chemical concentration in soil water. Further, organic pollutants can be degraded or mineralized by plants, either independently or in association with microorganisms. For example, organics like polycyclic aromatic hydrocarbons (PAHs), polychlorinated

biphenyls, and petroleum hydrocarbons are sufficiently degraded by rhizospheric microbial activity (Olson et al., 2003). Plants have significant metabolic activity in both roots and shoots, and some of the enzymes involved in these metabolic processes (e. g. nitroreductases, dehalogenases, laccases, peroxidases, etc.) are useful in the remediation process (Schnoor et al. 1995; Wolfe & Hoehamer, 2003).

Even though mycorrhizas and ectomycorrhizas are not considered in general reviews of plant metal tolerance mechanisms, they can ameliorate the effects of metal toxicity on the host plant. However, the mechanism involved in conferring this increase of tolerance is not yet well explained; they may be quite diverse and show considerable species and metal specificity since large differences in response to metals have been observed, both between fungal species and to different metals (specially Zn, Cu and Cd) within species (Hall, 2002).

Finally, cell walls may play an important role in detoxifying metals in plant cells of the Ni and Zn/Cd hyperaccumulating plant species. About 60–70% of Ni and/or Zn accumulated is distributed in the apoplast cell walls (Krämer et al., 2000; Li et al., 2005; Yang et al., 2005b). However, molecular bases of metal detoxification by cell walls are not well understood (Yang et al., 2005b).

4.2 Plasma membrane properties and integrity

Although there is no direct evidence for a role for plasma membrane efflux transporters in heavy metal tolerance in plants, recent research has revealed that plants possess several classes of metal transporters that must be involved in metal uptake and homeostasis in general and, thus, could play a key role in tolerance (Yang et al., 2005a). Transport proteins and intracellular high-affinity binding sites mediate the uptake of metals across the plasma membrane. A comprehensive understanding of the metal transport processes in plants is essential for formulating effective strategies to develop genetically engineered plants that can accumulate specific metals (Yang et al., 2005b).

Several classes of proteins have been implicated in heavy metal transport in plants. These include the heavy metal (or CPx-type) ATPases that are involved in the overall metal-ion homeostasis and tolerance in plants, the natural resistance-associated macrophage protein (Nramp) family of proteins, and the cation diffusion facilitator (CDF) family proteins (Williams et al., 2000), zinc–iron permease (ZIP) family proteins, etc. (Yang et al., 2005a; 2005b). CPx-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of essential as well as potentially toxic metals like Cu, Zn, Cd, Pb across cell membranes (Williams et al., 2000; Yang et al., 2005a; 2005b). These transporters use ATP to pump a variety of charged substrates across cell membranes and are distinguished by the formation of a charged intermediate during the reaction cycle (Yang et al., 2005a; 2005b).

Transport of metals and alkali cations across plant plasma membrane and organellar membranes is essential for plant growth, development, signal transduction, and toxic metal phytoremediation (Cherian and Oliveira, 2005).

Another factor concerning plasma membrane seems to be the maintenance of its physical integrity in presence of heavy metals, in order to prevent or reduce their entry in the cell, besides the efflux mechanisms described above (Hall, 2002; Coutinho & Barbosa, 2005).

4.3 Enzimatic transformation

Enzimatic transformation in plants concerns mainly organic pollutants and it can be considered a case of phytotransformation. In this process plants uptake organic pollutants and, subsequently, metabolize or transform them into less toxic metabolites. Once taken up

and translocated the organic chemicals generally undergo three transformation stages: (a) chemical modification (oxidations, reductions, hydrolysis); (b) conjugation (with glutathione, sugars, amino acids); and (c) sequestration or compartmentalization (conjugants are converted to other conjugates and deposited in plant vacuoles or bound to the cell wall and lignin) (Ohkawa et al., 1999; Cherian and Oliveira, 2005).

Plant enzymes that typically catalyze the first phase of the reactions are P450 monoxygenases and carboxylesterases (Coleman et al., 1997; Burken, 2003). The second phase involves conjugation to glutathione (GSH), glucose, or amino acids, resulting in soluble, polar compounds (Marrs, 1996). For instance, detoxification of herbicides in plants is attributed to conjugation with glutathione catalyzed by glutathione S-transferase (GST) (Lamoureux et al., 1991). It was also reported that a group of GSTs mediate conjugation of organics to GSH in the cytosol (Kreuz et al., 1996; Neuefeind et al., 1997). Sometimes organic pollutants, such as atrazine and TNT, are partially degraded and stored in vacuoles as bound residues (Burken & Schnoor, 1997). The third phase of plant metabolism is compartmentalization and storage of soluble metabolites either in vacuoles or in the cell wall matrix. The glutathione S-conjugates are actively transported to the vacuole or apoplast by ATP-dependent membrane pumps (Martinoia et al., 1993). Also, an alternate conjugation-sequestration mechanism for organics exists in plants and involves coupling of a glucose or malonyl group to the organic compound, followed by the transport of the conjugate to the vacuole or the apoplast (Coleman et al., 1997).

Mechanisms as complexation whit ligands and vacuolar compartmentalization are described below.

4.4 Complexation with ligands

Complexation with ligands is a process associated to heavy metal pollutants, and it can be an extracellular or an intracellular molecular event. These ligands can be chelators as organic acids or peptides such phytochelatins (PCs), methallothioneins (MTs) or glutathione (GSH) (Mello-Farias & Chaves, 2008).

Plant tolerance to heavy metals depends largely on plant efficiency in the uptake, translocation, and further sequestration of heavy metals in specialized tissues or in trichomes and organelles such as vacuoles. The uptake of metals depends on their bioavailability, and plants have evolved mechanisms to make micronutrients bioavailable (Cherian and Oliveira, 2005). Chelators such as siderophores, organic acids, and phenolics can help release metal cations from soil particles, increasing their bioavailability. For example, organic acids (malate, citrate) excreted by plants act as metal chelators. By lowering the pH around the root, organic acids increase the bioavailability of metal cations (Ross, 1994). However, organic acids may also inhibit metal uptake by forming a complex with the metal outside the root. Citrate inhibition of Al uptake resulting in aluminum tolerance in several plant species is an example of this mechanism (De la Fuente et al., 1997; Pineros & Kochian, 2001; Papernik et al. 2001). Copper tolerance in Arabidopsis is also the result of a similar mechanism (Murphy et al., 1999).

Intracellular complexation involves peptide ligands, such as metallothioneins (MTs) and phytochelatins (PCs) (Yang et al., 2005b). Chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of heavy-metal detoxification and tolerance (Hall, 2002).

Metallothioneins (MTs) are cysteine-rich proteins that have high affinity to cations such as Cd, Cu, and Zn (Cobbet & Goldsbrough, 2002; Singh et al., 2003; Cherian & Oliveira, 2005).

They confer heavy-metal tolerance and accumulation in yeast. Overexpression of genes involved in the synthesis of metal chelators may lead to enhanced or reduced metal uptake and enhanced metal translocation or sequestration, depending on the type of chelator and on its role and location (Cherian & Oliveira, 2005; Pilon-Smits, 2005). MT proteins were originally isolated as Cu, Cd and Zn binding proteins in mammals. There is now good evidence that four categories of these proteins occur in plants, which are encoded by at least seven genes in *Arabidopsis thaliana* (Cobbett & Goldsbrough, 2002; Hall, 2002; Gratão et al., 2005).

The biosynthesis of MTs is regulated at the transcriptional level and is induced by several factors, such as hormones, cytotoxic agents, and metals, including Cd, Zn, Hg, Cu, Au, Ag, Co, Ni, and Bi (*Yang et al.*, 2005a).

Phytochelatins are a class of post-translationally synthesized (cysteine-rich metal-chelating) peptides that play a pivotal role in heavy-metal tolerance in plants and fungi by chelating these substances and decreasing their free concentrations (Vatamaniuk et al., 1999). PCs have been most widely studied in plants, particularly in relation to Cd tolerance (Cobbett, 2000; Goldsbrough, 2000). PCs consist of only three amino acids, glutamine (Glu), cysteine (Cys), and glycine (Gly). They are structurally related to the tripeptide glutathione (GSH), and are enzymatically synthesized from GSH. PCs form a family of structures with increasing repetitions of the -Glu-Cys dipeptide followed by a terminal Gly, (-Glu-Cys)n-Gly, where n is generally in the range of 2–5, but can be as high as 11 (Cobbett, 2000; Yang et al., 2005b).

Many plants cope with the higher levels of heavy metals by binding them to PCs and sequestering the complexes inside their cells (Yang et al., 2005a). As mentioned above, PCs are synthesized non-translationally, using glutathione as a substrate by PC synthase, an enzyme that is activated in the presence of metal ions (Cobbett, 2000). So, PCs are structurally related to glutathione (GSH; γ-GluCysGly), and numerous physiological, biochemical, and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC biosynthesis (Cobbett, 2000; Cobbett and Goldsbrough, 2002).

Although PCs clearly can have an important role in metal detoxification, alternative primary roles of PCs in plant physiology have also been proposed. These have included roles in essential metal ion homeostasis and in Fe or sulphur metabolism (Sanita di Toppi & Gabbrielli, 1999; Cobbett and Goldsbrough, 2002). However, there is currently no direct evidence that PCs have functions outside of metal detoxification.

Because of MTs and PCs peptidic nature and because they bind metals in thiolate complexes, these peptide molecules demand a greater input of amino acids (especially cysteine), sulfur and nitrogen from the plant as the level of accumulated metals rise. Their synthesis is energy expensive and requires significant amounts of the growth limiting elements sulfur and nitrogen. Increased synthesis might thus at some point affect plant growth and therefore limit their use as phytoremediators (Tong et al., 2004).

4.5 Vacuolar compartmentalization

The vacuole is generally considered to be the main storage site for metals in yeast and plant cells and there is evidence that phytochelatin–metal complexes are pumped into the vacuole in fission yeast (*Schizosaccharomyces pombe*) and in plants (Tong et al., 2004; Yang et al., 2005b). Compartmentalization of metals in the vacuole is also part of the tolerance mechanism of some metal hyperaccumulators. The Ni hyperaccumulator *Thlaspi goesingense*

enhances its Ni tolerance by compartmentalizing most of the intracellular leaf Ni into the vacuole (Krämer et al., 2000; Tong et al., 2004). High-level expression of a vacuolar metal ion transporter TgMTP1 in *T. goesingense* was proposed to account for the enhanced ability to accumulate metal ions within shoot vacuoles (Persans et al., 2001; Tong et al., 2004; Yang et al., 2005b).

5. Genetically engineered plants for phytoremediation

The genetic and biochemical basis is becoming an interesting target for genetic engineering, because the knowledge of molecular genetics model organisms can enhance the understanding of the essencial metal metabolism components in plants.A fundamental understanding of both uptake and translocation processes in normal plants and metal hyperaccumulators, the regulatory control of these activities, and the use of tissue specific promoters offer great promise that the use of molecular biology tools can give scientists the ability to develop effective and economic phytoremediation plants for soil metals (Chaney et al., 1997; Fulekar et al., 2008). Plants such as *Populus angustifolia, Nicotiana tabacum* or *Silene cucubalis* have been genetically engineered to overexpress glutamylcysteine syntlietase, and thereby provide enhanced heavy metal accumulation as compared with a corresponding wild type plant (Fulekar et al., 2008).

Candidate plants for genetic engineering for phytoremediation should be a high biomass plant with either short or long duration (trees), which should have inherent capability for phytoremediation. The candidate plants should be amicable for genetic transformation. Some of high biomass hyperaccumulators for which regeneration protocols are already developed include Indian mustard (*Brassica juncea*), sunflower (*Helianthus annuus*), tomato (*Lycopersicon esculentum*) and yellow poplar (*Liriodendron tulipifera*) (Eapen & D'Souza, 2005; Mello-Farias & Chaves, 2008).

The application of powerful genetic and molecular techniques may surely identify a range of gene families that are likely to be involved in transition metal transport. Considerable progress has been made recently in identifying plant genes encoding metal ion transporters and their homologous in hyperaccumulator plants. Therefore, it is hoped that genetic engineering may offer a powerful new means by which to improve the capacity of plants to remediate environmental pollutants (Yang et al., 2005a; Mello-Farias & Chaves, 2008).

Brassica juncea was genetically engineered to investigate rate-limiting factors for glutathione and phytochelatin production. To achieve this, *Escherichia coli* gshl gene was introduced. The γ-ECS transgenic seedlings showed increased tolerance to cadmium and had higher concentrations of phytochelatins, γ-GluCys, glutathione, and total nonprotein thiols compared to wild type seedlings (Ow, 1996; Fulekar et al., 2008). Study showed that c-glutamylcysteine synthetase inhibitor, L-buthionine-[S,R]-sulphoximine (BSO), dramatically increases As sensitivity, both in non-adapted and As-hypertolerant plants, showing that phytochelatin-based sequestration is essential for both normal constitutive tolerance and adaptative hypertolerance to this metalloid (Schat et al., 2002; Fulekar et al., 2008).

Some genes have been isolated and introduced into plants with increased heavy metal (Cd) resistance and uptake, like *AtNramps* (Thomine *et al.*, 2000), *AtPcrs* (Song et al., 2004), and *CAD1* (Ha et al., 1999) from *Arabidopsis thaliana*, library enriched in Cd-induced cDNAs from *Datura innoxia* (Louie et al., 2003), *gshI, gshII* (Zhu et al., 1999a) and PCS cDNA clone (Heiss et al., 2003) from *Brassica juncea*.

There are some examples of transgenic plants for metal tolerance/phytoremediation, as tobacco with accumulation of Cd, Ca and Mn transformed with gene *CAX-2* (vacuolar

transporters) from *A. thaliana* (Hirschi et al., 2000); *A. thaliana* tolerant to Al, Cu, and Na with gene *Glutathione-S-transferase* from tobacco (Ezaki et al., 2000); tobacco with Ni tolerance and Pb accumulation with gene *Nt CBP4* from tobacco (Arazi et al., 1999); tobacco (Goto et al., 1998) and rice (Goto et al., 1998; 1999) with increased iron accumulation with gene *Ferretin* from soybean; *A. thaliana* and tobacco resistant to Hg with gene *merA* from bacteria (Rugh et al., 2000; Bizily et al., 2000; Eapen & D'Souza, 2005); indian mustard tolerant to Se transformed with a bacterial glutathione reductase in the cytoplasm and also in the chloroplast (D'Souza et al., 2000); transgenic *A. thaliana* plants expressing SRSIp/ArsC and ACT 2p/γ-ECS together showed high tolerance to As, these plants accumulated 4- to 17-fold greater fresh shoot weight and accumulated 2- to 3-fold more arsenic per gram of tissue than wild plants or transgenic plants expressing γ-ECS or ArsC alone (Dhankher et al., 2002; Mello-Farias & Chaves, 2008).

Even though there is a variety of different metal tolerance mechanisms, and there are many reports of transgenic plants with increased metal tolerance and accumulation, most, if not all, transgenic plants created to date rely on overexpressing genes involved in the biosynthesis pathways of metal-binding proteins and peptides (Zhu et al., 1999b; Mejäre & Bülow, 2001; Bennett et al., 2003; Gisbert et al., 2003), genes that can convert a toxic ion into a less toxic or easier to handle form, or a combination of both (Dhankher et al., 2002; Yang et al., 2005b; Mello-Farias & Chaves, 2008).

At least three different engineering approaches to enhanced metal uptake can be envisioned (Clemens et al., 2002), which include enhancing the number of uptake sites, alteration of specificity of uptake system to reduce competition by unwanted cations and increasing intracellular binding sites. Each metal has specific molecular mechanism for uptake, transport and sequestration (Eapen & D'Souza, 2005; Mello-Farias & Chaves, 2008).

New metabolic pathways can be introduced into plants for hyperaccumulation or phytovolatilization as in case of *MerA* and *MerB* genes which were introduced into plants which resulted in plants being several fold tolerant to Hg and volatilized elemental mercury (Bizily et al., 2000; Dhankher et al., 2002; Eapen & D'Souza, 2005) developed transgenic *Arabidopsis* plants which could transport oxyanion arsenate to aboveground, reduce to arsenite and sequester it to thiol peptide complexes by transfer of *Escherichia coli ars C* and *γ-ECS* genes (Eapen & D'Souza, 2005).

Alteration of oxidative stress related enzymes may also result in altered metal tolerance as in the case of enhanced Al tolerance by overexpression of glutathione-S-transferase and peroxidase (Ezaki et al., 2000; Eapen & D'Souza, 2004). Overexpression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase led to an enhanced accumulation of a variety of metals (Grichko et al., 2000; Eapen & D'Souza, 2005).

According to Eapen & D'Souza (2005), it is essential to have plants with highly branched root systems with large surface area for efficient uptake of toxic metals. Experiments had shown that *Agrobacterium rhizogenes* could enhance the root biomass in some hyperaccumulator plants (Eapen, unpublished work). The hairy roots induced in some of the hyperaccumulators were shown to have high efficiency for rhizofiltration of radionuclide (Eapen et al., 2003) and heavy metals (Nedelkoska and Doran, 2000; Eapen et al., unpublished work).

Nowadays there are many different examples of genes that have been used for the development of transgenic plants for metal tolerance and/or phytoremediation, as shown on Table 2.

6. Advantages and disadvantages of phytoremediation

Admittedly, phytoremediation has benefits to restore balance to a stressed environment, but it is important to proceed with caution. Plants enjoy enormous reduction in energy cost and utilization by virtue of deriving energy from solar radiation. The plant tolerates a wide range of environmental conditions.

Gene transferred	Origin	Target plant species	Effect
MT2 gene	Human	Tobacco, oil seed rape	Cd tolerance
MT1 gene	Mouse	Tobacco	Cd tolerance
MTA gene	Pea	*Arabidopsis*	Cu accumulation
CUP-1 gene	Yeast	Cauliflower	Cd accumulation
CUP-1 gene	Yeast	Tobacco	Cu accumulation
γ-Glutamylcysteine synthetase	*E. coli*	Indian mustard	Cd tolerance
Glutathione synthetase	Rice	Indian mustard	Cd tolerance
Cysteine synthetase	Rice	Tobacco	Cd tolerance
CAX-2 (vacuolar transporters)	*Arabidopsis*	Tobacco	Accumulation of Cd, Ca and Mn
At MHX	*Arabidopsis*	Tobacco	Mg and Zn tolerance
Nt CBP4	Tobacco	Tobacco	Ni tolerance and Pb accumulation
FRE-1 and FRE-2	Yeast	Tobacco	More Fe content
Glutathione-s-Transferase	Tobacco	*Arabidopsis*	Al, Cu, Na tolerance
Citrate synthase	Bacteria	*Arabidopsis*	Al tolerance
Nicotinamine amino transferase (NAAT)	Barley	Rice	Grew in iron deficient soils
Ferretin	Soybean	Tobacco	Increased iron accumulation
Ferretin	Soybean	Rice	Increased iron accumulation
Zn transporters ZAT (At MTPI)	*Arabidopsis*	*Arabidopsis*	Zn accumulation
Arsenate reductase γ- glutamylcysteine synthetase	Bacteria	Indian mustard	As tolerance
Znt A-heavy metal transporters	*E. coli*	*Arabidopsis*	Cd and Pb resistance
Selenocysteine methyl transferase	*A. bisculatus*	*A. thaliana*	Resistance to selenite
ATP sulfurylase CAPS		Indian mustard	Se tolerance

Gene transferred	Origin	Target plant species	Effect
Cystathione-gamma synthase (CGS)		Indian mustard	Se volatilization
Glutathione-S-transferase, peroxidase		*Arabidopsis*	Al tolerance
Glutathione reductase		*B. juncea*	Cd accumulator
ACC-deaminase	Bacteria		Many metal tolerance
YCF1	Yeast	*Arabidopsis*	Cd and Pb tolerance
Se-cys lyase	Mouse	*Arabidopsis*	Se tolerance and accumulation
Phytochelatin synthase (Ta PCS)	Wheat	*Nicotiana glauca*	Pb accumulation

Table 2. Selected examples of transgenic plants for metal tolerance/phytoremediation (from Eapen & D'Souza, 2005)

The molecular composition of plants, mainly related to their enzyme and protein profiles, is of great interest to phytoremediation, because this technology can exploit plant molecular and cellular mechanisms of detoxification, through the use of genetic engineering tools.

The nature of plants is still an advantage because they are able to develop, over time, complex mechanisms to absorb nutrients, detoxify pollutants and control the local geochemical conditions. The plants play an important role in regulation water contant in soil avoiding the penetration of liquids by infiltration, which is the main mechanism of entry of contaminants. Plant roots supplement microbial nutrients and provide aeration to the soil, increasing consequently microbial population compared to non-vegetated area. Above all, phytoremediation gives better aesthetic appeal than other physical means of remediation.

On the other hand, phytoremediation has several limitations that require further intensive research on plants and soil conditions. A major disadvantage is that this method of detoxification is too slow or only seasonally effective. Regulatory agencies often require significant progress in remediation to be made in only a few years, making most phytoremediation unsuitable. In many cases, like trichloroethylene and carbon tetrachloride, the concentration of pollutant is not reduced satisfactorily. Besides, in some contaminated sites, the pollutants can reach phytotoxic concentration, making the plant ineffective. For this reason, recent studies have been conducted with the aim of increasing the phytoremediation potential of plants using genetic engineering (Danh et al. 2009). In phytoremediation technology, multiple metal contaminated soil and water require specific metal hyperaccumulator species and therefore, a wide range of research prior to the application. Other factors are also tied to the success of phytoremediation such as the existence of a pollutant in a bio-available form. If the metal is strongly linked to the organic soil it will not be available to the plant. Moreover, the plants are quite specific to certain pollutants. Hyperaccumulators of Cd and Zn (*Thlaspi caerulescens*) can be sensitive to other metals, such as Cu, not allowing the detoxification of polluted areas with different pollutants (Mijovilovich et al., 2009). Despite the current limitations, present day phytoremediation technology is used worldwide and several researchers are working to

overcome these limitations. Table 3 resumes advantages and limitations of some of the sub-process of phytoremediation.

7. Perspectives on biotechnology - based phytoremediation

The environmental contamination by pollutants, organic or inorganic, has great importance due to its impacts on human and animal health. Thus, the most effective and inexpensive technologies to promote detoxification are necessary in the recovery of affected biomes. Great efforts have been made in identifying plant species and their detoxification mechanisms more efficient on those places. The mechanisms of pollutant uptake, accumulation, exclusion, among others, vary according to each plant species and are very important, for they will determine its specific role in phytoremediation.

Plants can have their detoxification capabilities significantly enhanced through the identification of specific genes in certain promising species and the transmission of these to other species, using genetic engineering tools. This can play a significant role in the more effective detoxification of contaminated sites by improving the cost-benefit.

Advantage	Limitation
Phytoextraction	
The plant must be able to produce abundant biomass in short time. e. g.: in a greenhouse experiment, gold was harvested from plants.	Metal hyperaccumulators are generally slow-growing and bioproductivity is rather small and shallow root systems. Phytomass after process must be disposed off properly
Phytostabilization	
It circumvents the removal of soil, low cost and is less disruptive and enhances ecosystem restoration/re-vegetation	Often requires extensive fertilization or soil modification using amendments; long-term maintenance is needed to prevent leaching.
Phytovolatilization	
Contaminant/Pollutant will be transformed into less-toxic forms. e. g.: elemental mercury and dimethyl selenite gas. Atmospheric processes such as photochemical degradation for rapid decontamination/transformation.	The contaminant or a hazardous metabolite might accumulate in plants and be passed on in later products such as fruit or lumber. Low levels of metabolites have been found in plant tissue.
Phytofiltration/rhizofiltration	
It can be either *in situ* (floating rafts on ponds) or *ex situ* (an engineered tanks system); terrestrial or aquatic.	pH of the medium to be monitored continually for optimizing uptake of metals; chemical speciation and interactions of all species in the influent need be understood; functions like a bioreactor and intensive maintenance is needed.

Table 3. Advantages and limitations of some of the phytoremediation sub-processes (Prasad, 2004; Gratão et al. 2005)

Studies on phytoremediation are developed in order to benefit the environment. Several pollutants are bringing some kind of harm to all habitats. Thus, the use of specific techniques already represents hope. The necessary mechanisms are different, however, the organisms, especially plants, have specific ways for the removal, detention or conversion of specific pollutants. The study and subsequent evaluation of the interaction between the soil and its microorganisms, plant and pollutant is very necessary and guiding.

All things considered, more studies must be carried out in this area to better know the phytoremediation capacity of living organisms and their possible use in combating pollution through plant transformation technology.

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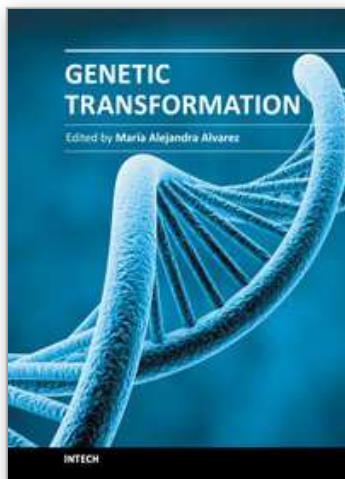
Genetic Transformation

Edited by Prof. María Alvarez

ISBN 978-953-307-364-4

Hard cover, 328 pages

Publisher InTech

Published online 06, September, 2011

Published in print edition September, 2011

Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power fool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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