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Review

Endophyte-assisted phytoremediation

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ABSTRACT

Most plants in their natural state are colonized by endophytes that can form beneficial associations with their hosts. The benefits that some endophytes offer to the plant include but are not limited to: enhancing plant growth through phyto-hormone production, resistance to environmental stresses (heat, cold, drought, salt), supplying biologically fixed nitrogen, and producing important medicinal, agricultural and industrial compounds. Enhancing phytoremediation by using endophytes has been shown to improve uptake and degradation of several toxins. The current review surveys the ongoing research in this field with information on the various pollutants tested, the kinds of plants used, the endophytes studied, and finally, ways in which the plant-endophyte partnership can be exploited for improved phytoremediation.

KEYWORDS: endophytes, phytoremediation, organics, metals

INTRODUCTION

Remediation methods currently applicable to contaminated environments (soil and ground water) are frequently expensive, cause damage to the ecosystem and do not use the existing resources efficiently. It is estimated that traditional global remediation costs are in the range of $US25-50 billion annually [1, 2]. Unfortunately, this high cost of remediation contributes to the abandonment worldwide of a large number of polluted commercial sites or brownfields. The most commonly used traditional methods of treatment are excavation, pump and treat, addition of reactants, incineration, vitrification [3] or transportation of the contaminated material off site resulting in serious environmental degradation and also involving high energy consumption [4]. Therefore, natural remediation techniques have been developed to provide more environment- friendly and cost effective cleanup of contaminated sites. One method is bio-remediation which uses microbes to degrade toxins. Bioremediation can occur on its own or can be spurred on via addition of superior strains of pollutant-degrading microbes and substrates for growth (Bioaugmentation). However, there are multiple challenges to bioremediation. If anaerobic microbes are used, there is potential of producing harmful and persistent by-products. For example, during degradation of trichloroethylene (TCE), more toxic byproducts like vinyl chloride and cis-dichloroethylene are produced that remain in the environment for a long time. Using special (and expensive) microbes like members of dehalococcoides group that can metabolize TCE to ethylene, a harmless end product, can contribute heavily to the overall bioremediation costs. Also to make a site anaerobic, it is a highly energy intensive process requiring lots of carbon inputs, and buffers that can result in fouling of injection tubes. Large volumes of water are used to flush these additives into the contaminated site. Aerobic bioremediation can be too slow, even though there are no toxic intermediates produced. Occasionally, addition of substrates or co-metabolites is required to achieve degradation.

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For example to aid bacterial growth, often thousands of gallons of molasses must be pumped down [5]. Sometimes, harmful co-metabolites are added to induce expression of certain genes to degrade recalcitrant compounds. In spite of these challenges, bioremediation is widely used for several pollutants including xenobiotics, TCE, petroleum, BTEX (benzene, toluene, ethylbenzene, xylene), explosives and heavy metals.

A new emerging cost-effective technology that is gaining tremendous attention is phytoremediation [6, 7, 8]. It is an aesthetically pleasing technology that uses the natural ability of plants to extract chemicals from water, soil and air using energy from sunlight and can be used in several forms including phytoextraction, the absorption and concentration of toxins from soil into the roots and shoots of the plant; rhizofiltration, the use of plant roots to remove pollutants from effluents; phytostabilization, the use of plants to reduce the spread of contaminants in the environment; phytovolatilization, uptake and release of volatiles into the atmosphere; and phytostimulation, the stimulation of microbial degradation in the rhizosphere [9]. Unlike the engineering methods that would remove the fertile soil, phyto-remediation would not reduce the fertility of the site but would enhance it [10]. Phytoremediation has several other advantages over the traditional methods. It is highly cost effective in being approximately 10-fold less expensive than traditional remediation technologies [5]. By planting trees, there is less secondary waste generation [11] and also improvement in wild life habitat [12]. Plants also act as soil stabilizers, minimizing the amount of contaminated dust that could leave the site and enter the neighboring community, hence acting as buffer zones. Other advantages of using phytoremediation over traditional methods include carbon sequestration and generation of a useful product for bioenergy applications (wood, pulp). Also, with phytoremediation, unlike bioremediation by microbes, it is easy to monitor the site. The condition of plants can be visually monitored and samples of plant tissue can be tested for the presence of pollutants over time.

Phytoremediation has been used to treat a wide variety of chemicals including metals, organics, excess nutrients, and radionuclides [13]. Some of the major constraints this technology has is the phytotoxicity, slow degradation, limited contaminant uptake and evotranspiration of volatile contaminants [14]. To overcome this, plant associated bacteria have been exploited for improving phyto-remediation of several pollutants. The plant roots provide an ideal environment for degradation of organic compounds as a result of several mechanisms. The plant root system offers several benefits including production of exudates, promoting movement of water and gases through the soil, and promoting microbial activity. All these factors influence the contaminant availability for uptake and degradation of pollutants. This is an advantage over bioremediation, where the microbes may not be able to survive at a site or degrade a particular pollutant because it may not be bioavailable. Hence using plants with microbes is a promising green technology as it combines the environmental and cost benefits of plants and microbes for phytoremediation.

Application of endophytes to enhance phytoremediation

One of the major limitations of phytoremediation is that even plants that are tolerant to the presence of these contaminants often remain relatively small, due to the toxicity of the pollutants that they are accumulating or the toxic end products of their degradation [9]. Recently, attention has focused on the role of endophytic bacteria in phytoremediation (reviewed in [15, 16]). Endophytes are a group of bacteria or fungi that reside inside the plant and live asymptomatically. These have been found in many plant species with the highest densities observed in roots, and decrease from stem to the leaves [17, 18, 19]. In general Pseudomonaceae, Burkholderiaceae, and Entero-bacteriaceae are among the most common genera of cultivable endophytic species found. In general, beneficial endophytes promote plant growth, increase plant nutrient uptake, inhibit plant pathogen growth, reduce disease severity and enhance tolerance to environmental stresses (drought, salt, heat, contaminant) [20, 21]. Some endophytes are also diazotrophic and provide fixed nitrogen to the host plant [22]. In the recent years, research has focused on exploiting the benefits of endophytes to overcome the constraints of phytoremediation [23, 24, 25]. Bioaugmentation with endophytic bacteria has shown to offer several benefits over traditional bioaugmentation. In the former case, the bacteria have less competition from the surrounding microbes and the plant provides nutrients to the bacteria, thus supporting growth and establishment. Other advantages associated with the use of endophytic bacteria in phytoremediation include (i) quantitative gene expression of bacterial pollutant catabolic genes can be used to assess the efficiency of the remediation process, (ii) genetic engineering of a bacterial catabolic pathway is easier to manipulate than a plant catabolic pathway, and (iii) toxic pollutants taken up by the plant may be degraded in planta by endophytic degraders reducing the toxic effects of contaminants in environmental soil on flora and fauna. However, there are certain disadvantages including (i) the choice of plant can mean that it is only seasonally effective, (ii) it is associated with phytotoxic effects of contaminants, and (iii) there is potential for the environmental contaminants or their metabolites to enter the food chain if contaminants are not completely detoxified and if the plants are consumed by local fauna [1]. But careful selection of pollutant degrading microbes and inoculating them into native plants can avoid some of the problems associated with phytotoxicity and the ability of plants to survive at non-native locations. Several studies have been done to address these issues and are described below.

Endophyte assisted phytoremediation of organics

Emerging phytoremediation technologies have been applied at various scales to treat several organic pollutants, such as chlorinated solvents, BTEX compounds, explosives, petroleum compounds, polycyclic aromatic compounds and excess nutrients. The first study demonstrating the ability of a poplar endophyte to degrade nitro aromatic pollutants was done by van Aken and colleagues [26]. The authors described an endophyte, Methylobacteriumpopulum sp.nov., strain BJ001, which was isolated from a poplar tree, was capable of degrading explosives such as TNT (2,4,6-trinitrotoluene) and RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine) [26]. It mineralized approximately 60% of RDX to carbon dioxide in 2 months. It is possible that it could assist in phytoremediation of explosive contaminated areas. Endophytes may also assist in phytoremediation of petroleum compounds. Plants grown in soil contaminated with xenobiotics naturally recruited endophytes with the pollutant degrading genes [27]. In the field sites contaminated with petroleum compounds, genes encoding for petroleum compound degradation (alkane mono-oxygenase and naphthalene dioxygenase) were more prevalent in endophytic strains than in the rhizospheric strains. Also the pollutant degrading genes increased proportionally with the concentration and that the increase was dependent on several factors including the type of pollutant, the gene being analyzed, the plant tested and the soils that the plants were grown in. In other words, some plant species have the ability to recruit, or selectively expand, the necessary bacteria to remove pollutants or to help cope with the contaminant stress, than other plants in the same area. This is an important study showing how the microbial community can be altered according to the environmental conditions, but in order to understand how some plant species are able to recruit the necessary bacteria at a given site, further investigation is needed. Khan and Doty (unpublished) isolated endophytes from several willow (*Salix* spp.) and poplar (*Populus* spp.) clones and tested for tolerance to PAHs including naphthalene, phenanthrene and pyrene. Six endophytes were able to tolerate the PAHs and use them as sole carbon source for growth. Studies are underway to test if these endophytes can help the plant in removal and degradation of this important class of pollutants.

Because endophytes can colonize almost any plant, it is interesting to study their diversity at contaminated sites to identify their potential role in phytoremediation. In one such study [28], the authors described the diversity of endophytes found in poplar trees growing at a phyto-remediation field trial site with toluene as the major groundwater contaminant, with the aim of identifying potential candidates for enhancing phytoremediation of toluene and other BTEX compounds. Endophytic bacteria were isolated and characterized from two varieties of poplar.

These bacteria were found to be cultivable, non-pathogenic, plant-specific and located within the plant compartment most suitable for the biodegradation of the target compound. Some of the isolates demonstrated tolerance to TCE, BTEX and heavy metals and 34 were identified as having characteristics that might make them useful to enhance phytoremediation. The focus of most researchers has been to study endophytes within poplar and willow trees because of their fast growth and large transpiration potential. They are the "trees of choice" for phytoremediation purposes [29, 30]. To expand the applicability of endophyte assisted phytoremediation from poplars and willows, Weyens *et al*. [31] studied the potential of English Oak and Common Ash (native to Europe) and their associated microbes for the phytoremediation of a TCE-contaminated site. They found that both the trees had their own specific and non-specific microbes, some also found in association with hybrid poplar [28]. Both the tree species were dominated by TCE and Toluene tolerant strains showing that there is recruitment of pollutant degrading bacteria in response to the TCE contamination similar to the observations of Sicilano *et al*. [27]. This study suggests a possible active role of the trees and bacteria in the remediation process.

From simply looking at the diversity of endophytes, research has turned to adding a specific endophyte for a specific pollutant. The first reported study on endophytic re-colonization of a hardwood deciduous tree was by Germaine *et al*. [32]. Three Pseudomonas endophytes isolated from the xylem sap of hybrid cottonwood (*P. trichocarpa* X *P. deltoides* cv *Hoogvorst*) growing on a phytoremediation site in Belgium, were identified to degrade 2,4-dichlorophenoxyacetic acid (2,4 D), toluene, naphthalene. The authors demonstrated successful re-colonization of poplar trees when inoculated with these strains. However, no work was shown to test if the pollutant degrading strains enhanced phytoremediation ability due to the plant-microbe combination. In another study by the same group, the authors inoculated pea plants with a genetically tagged bacterial endophyte that naturally possesses the ability to degrade the herbicide, 2,4D [33]. The result showed that this strain actively colonized inoculated plants and these inoculated plants showed a higher capacity for 2,4D removal from the soil and showed no 2,4 D accumulation in their aerial tissues compared to uninoculated plants. Also, the inoculated plants had a higher biomass than the controls and this difference became more pronounced with increasing concentrations of 2,4D. The inoculated plants seemed to degrade 2,4D as there was no 2,4D accumulation in the tissues. More recently, the same authors reported that a genetically enhanced endophytic strain of the poplar endophyte *Pseudomonas putida* VM1441, i.e., *Pseudomonas putida* VM1441 (pNAH7), could protect inoculated pea plants from the toxic effects of naphthalene (a probable human carcinogen). They also showed that inoculation of plants with this strain facilitated higher (40%) naphthalene degradation rates compared with uninoculated plants in artificially contaminated soil [34]. All these studies clearly demonstrate that endophytes can be exploited to improve phytoremediation of a variety of pollutants.

Soleimani *et al*. [35] recently studied the effects of two grass species, sterile or colonized by endophytic fungi, on the degradation of petroleum hydrocarbons in an aged petroleum contaminated soil. The endophyte (+) plants contained more root and shoot biomass, higher levels of water-soluble phenols and dehydrogenase activity in the soil than the uninoculated plants. Although there was no difference in the PAH (polycyclic hydrocarbons) and TPH(total petroleum hydrocarbons) removal between both the E(+) and E(-) plants, there was more degradation of TPHs in the rhizosphere soil of the E(+) plants when compared to the E(-) plants. Overall the presence of the fungus seemed to improve the plant growth, released more phenols and could have a potential to degrade TPHs. However the authors need to further investigate with more controls to confirm if the degradation is due to the endophytes or the enhancement of microbial degradation that has resulted from more root biomass and increased phenol levels in the E(+) plants.

Not every microbe possesses the ability to degrade every toxin, and not every pollutant degrading bacterium has the ability to thrive in plants on contaminated sites. Because of this, attempts have been made to genetically modify endophytes. This is done by using the natural ability of microbes to conjugate with each other, by transferring mobile DNA elements (plasmids) amongst a microbial population. Van der Lelie's group [36] developed a method for genetically endowing a plant endophyte with the ability to degrade toluene. Toluene is a component of BTEX that are major contaminants of environmental soil and groundwater and are usually found near petroleum and natural gas production sites. In their work, the pTOM plasmid from *Burkholderia cepacia* G4 was naturally inserted into the lupine endophyte *B. cepacia* BU 0072 via bacterial conjugation. When yellow lupine plants were inoculated with this altered endophyte, the plants had higher tolerance of toluene. The plants grown in the presence of toluene had two characteristics imparted by the endophyte- the toxicity of toluene to the plants decreased significantly and the plants grew at high concentrations of toluene (1000mg/l) without any toxic effects, whereas the uninoculated controls showed growth reduction with as little as 100mg/l of toluene. Also the inoculated plants showed 50-70% reduction in toluene release from the aerial portions of the plant through phyto-transpiration. Another important observation was that the original host of the plasmid, *B. cepacia* G4 did not confer toluene tolerance, perhaps because it was not able to establish a relationship with the plant, In fact, it had a negative effect on plant development.

Although application of engineered endophytic bacteria to improve phytoremediation of volatile organic contaminants has several obvious advantages, some of the concerns include the persistence and stability of the engineered organisms and their degradation capabilities in field-grown plants. As long as there is a selection pressure, there will be a selective advantage for those microbes possessing the appropriate degradation genes [37]. To overcome issues concerning the stability of the engineered organisms, horizontal gene transfer has been illustrated where the plant's endogenous endo-phytic populations can be adapted to deal with the environmental stress. Taghavi *et al.* [38] have demonstrated that horizontal gene transfer could be used to change natural endophytic bacterial communities in order to improve phytoremediation. When poplar (*Populus trichocarpa* X *deltoides* cv *Hoogvorst*) was inoculated with the endophytic strain *Burkholderia cepacia* VM1468 (containing pTOM-Bu61 that constitutively expresses toluene degradation), it had a positive effect on plant growth in the presence of toluene and reduced the amount of toluene released. Remarkably there was in planta horizontal transfer of the toluene degrading gene into the different members of the endogenous community and the inoculated poplars had increased tolerance to toluene and evotranspired less toluene than the uninoculated controls. This study shows that no long-term establishment of the original inoculants is required, which could have practical implications.

Because of the toxic effects of TCE on human health, it has been a subject of study by many researchers. In a recent study by Weyens *et al.* [39], the authors report in situ inoculation of poplar trees growing on a TCE contaminated site, with TCE-degrading poplar endophyte *Pseudo-monas putida* W619-TCE. Inoculation by the endophyte resulted in 90% reduction of evapo-transpiration under field conditions. The endo-phyte established successfully, dominated as a root endophyte and subsequently transferred the TCE metabolic activity to the natural endophytes of Poplar. Pseudomonas and *Frigoribacterium* spp. were found to receive the pTOM-Bu61 plasmid responsible for TCE degradation, from the inoculated strain. However both lost the degradative ability within 20 generations when cultivated under nonselective conditions thereby demonstrating that once the contaminants' concentration is decreased, the endophytic community is restored to its original situation. In another similar study by the same authors [40] using poplar cuttings inoculated with the same engineered endophyte, a positive effect was noticed in both the type of setups used - hydroponic and soil. The conclusions from their work were that *P. putida* W619-TCE not only protects its host from phytotoxicity, but also reduces the amounts of TCE accumulation in plant tissue and decreased TCE evapotranspiration. The authors suggest that TCE is being degraded; however they could not detect any of the expected TCE metabolites.

To conclude, it is clear that plant-endophyte partnerships are extremely valuable for remediation of a variety of organic pollutants and the results of most work done are of major significance in the development towards large-scale field applications of endophyte-assisted phytoremediation. This technology also offers better public acceptance than either using genetically engineered plants or genetically engineered bacteria in the environment. Modifying endophytes by natural conjugative plasmid transfer for enhanced uptake of organics could help this technology to be easily adapted and put to widespread use.

Endophyte assisted phytoremediation of metals

Among pollutants, heavy metals pose a critical risk to human health and environment due to their high occurrence as a contaminant, low solubility and bioavailability and being carcinogenic and mutagenic [41]. Moreover the metals cannot be degraded to harmless products and hence persist in the environment indefinitely. Several conventional remediation methods have been tried to remove metals from the environment including soil washing with acids/chelators or metal stabilization using soil amendments. These methods are expensive and pose environmental damage. Phytoremediation has been tried successfully for removal of metals from soil. Examples include Thlaspi, Urtica, Chenopodium, Polygonumsachalase and Alyssum that have shown to accumulate unusually high concentrations of heavy metals [42, 43]. However these hyperaccumulators usually have small biomass and metals at elevated levels are generally toxic to most plants. Interactions between endophytes and hyper accumulator plants have been studied by various researchers. Hyperaccumulating plants can be colonized by a high number of different species of metal resistant endophytic bacteria, an overview of which is described by Rajkumar *et al*. [44].

One such study where the phytoremediation of Ni was improved by *Lupinus luteus* L., was demonstrated by Lodewyckx *et al*. [45] where the plants were inoculated with an engineered endophyte that had genes for Ni tolerance. The inoculated plants showed 30% increased Ni concentration in roots whereas the Ni concentration in the shoots was comparable to the controls. When another engineered endophyte having genes for Ni tolerance was inoculated into its host plant *Lolium perenne*, the uninoculated plants accumulated more Ni (roots and shoots) compared to inoculated plants (either with Ni sensitive or Ni resistance) indicating the presence of these strains (both Ni-/Ni+) resulted in some kind of protection resulting in decreased uptake of Ni by the host plant. So both plants responded differently in terms of Ni uptake, showing the presence of Ni genes does not necessarily improve Ni translocation which would have been an important feature to improve the phytoextraction process. Interestingly the modified endophytes by themselves had increased resistance to the toxic effects of nickel.

Other reports demonstrated the potential role of endophytes in phytoremediation of heavy metals. In a recent study by Mastreta *et al*. [2] the authors showed that endophytes isolated from *Nicotiana tobacum* reduced cadmium toxicity by increasing uptake of trace elements such as Zinc and iron by plants. Similarly, lead resistant endophytes were isolated from *Brassica napus* that enhanced phytoremediation potential by promoting the plant growth and lead uptake by rape [46]. Inoculation of the plants with the Pb resistant endophytes increased uptake into the shoot from 76% to 131% by *Pseudomonas fluorescens* and from 59% to 80% by *Microbacterium* sp. compared to the uninoculated control plants. Chen *et al*. [47] isolated Cu tolerant bacteria from the rhizosphere of *Elsholtzia splendens*, a copper accumulator plant generally growing on copper mines. Re-inoculation of these strains in the plant rhizosphere resulted in an increased water copper concentration and increased Cu accumulation by the plants. Arbuscular mycorrhizas (AMs) are fungi that intimately associate with plant roots, increasing uptake of nutrients, especially phosphorous. In a study by Trotta *et al*. [48] the authors showed enhancement of growth and leaf area as well as of the arsenic (As) translocation factor in the As hyperaccumulating fern *Pteris vittata* L., when induced by two different species of AM fungi. The arsenic translocation factor (TF) was 730 in Glomus mosseae-inoculated plants compared with a TF factor of 50 in control plants. Thus endophyte-assisted phytoremediation of toxic metals shows a lot of promise and more studies are essential to understand the interactive mechanisms of endophytic bacteria and hyper accumulators to fully exploit the plant-bacterial partnerships for a sustainable future.

Endophyte assisted phytoremediation in mixed pollutant scenarios

At most contaminated sites, phytoremediation is limited because plants and their associated microbes are faced with mixed pollution of organics and toxic metals. Toxic metals can inhibit biodegradation of several organics [49, 50, 51, 52]. Endophytes can be specifically tailored to deal with co-contamination. In a study by Weyens *et al*. [53] the authors used an engineered endophyte that possesses the ability to degrade TCE and sequester nickel (pTOM-Bu61 and ncc-nre Ni plasmids). The engineered endophyte *Burkholderia cepacia* VM1468 when inoculated into Yellow lupine plants resulted in a decreasing trend in evapotranspiration of TCE and a 5 times increased uptake of Ni, thus demonstrating the role of endophytes in improving phytoremediation even in mixed contamination scenarios. Although these studies do not guarantee similar results in the field, more studies using plants like poplar and willows that are excellent candidates for phytoremediation, should be undertaken to prove this concept.

Application of consortia of metal-detoxifying endophytes and endophytes degrading organics have also been proposed to maintain the endophytes offering concurrent metal resistance and organic degradation under field conditions. Use of such consortia have been successfully demonstrated by Roane *et al*. [54] during bioaugmentation of contaminated soil. Their study examined the potential of cadmium-resistant microorganisms to reduce soluble cadmium levels and to enhance degradation of 2,4-D. Although none of the cadmium resistant isolates could degrade 2,4-D, they supported the degradation of the cadmium sensitive 2,4-D degrader *Ralstonia eutropha* JMP134.

CONCLUDING REMARKS

Research on endophytic bacteria is in its early stages of development and there are a lot of challenges to be met both from the fundamental and applied perspectives. From the research done so far, this technology shows great promise for increasing the number of sites where phyto-remediation can be deployed by showing that it is possible to increase plant tolerance to a given toxin and decrease the release of the compound to the atmosphere. With studies demonstrating the beneficial effects of endophytes and in order to improve the applicability of endophyte-assisted phytoremediation in the field level, intensive future research is needed demonstrating successful clean up at contaminated sites. One area that needs to be extensively studied and that has huge practical application, is the mechanism in which the endophytes contribute to the overall degradation of the contaminant. With the availability of complete genome sequences of key endophytic bacteria, the genes governing colonization and establishment of endophytic bacteria in planta can be identified. This information will be vital in improving the phytoremediation potential of a plant by using options like enrichment of pollutant degrading microbes, introducing microbes having enhanced contaminant degrading abilities, or re-introducing natural endophytes with modified properties. Finally, this technology offers great promise in achieving sustainable production of biomass and bioenergy crops in conjunction with phyto-remediation of contaminated environments.

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