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THE POTENTIAL OF THE NI-RESISTANT TCE-DEGRADING *PSEUDOMONAS PUTIDA* W619-TCE TO REDUCE PHYTOTOXICITY AND IMPROVE PHYTOREMEDIATION EFFICIENCY OF POPLAR CUTTINGS ON A NI-TCE CO-CONTAMINATION

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To examine the potential of* Pseudomonas putida *W619-TCE to improve phytoremediation of Ni-TCE co-contamination, the effects of inoculation of a Ni-resistant, TCE-degrading root endophyte on Ni-TCE phytotoxicity, Ni uptake and trichloroethylene (TCE) degradation of Ni-TCE-exposed poplar cuttings are evaluated.

After inoculation with* P. putida *W619-TCE, root weight of non-exposed poplar cuttings significantly increased. Further, inoculation induced a mitigation of the Ni-TCE phytotoxicity, which was illustrated by a diminished exposure-induced increase in activity of antioxidative enzymes. Considering phytoremediation efficiency, inoculation with* P. putida *W619-TCE resulted in a 45% increased Ni uptake in roots as well as a slightly significant reduction in TCE concentration in leaves and TCE evapotranspiration to the atmosphere.

These results indicate that endophytes equipped with the appropriate characteristics can assist their host plant to deal with co-contamination of toxic metals and organic contaminants during phytoremediation. Furthermore, as poplar is an excellent plant for biomass production as well as for phytoremediation, the obtained results can be exploited to produce biomass for energy and industrial feedstock applications in a highly productive manner on contaminated land that is not suited for normal agriculture. Exploiting this land for biomass production could contribute to diminish the conflict between food and bioenergy production.

KEY WORDS: phytoremediation, endophytes, *Pseudomonas putida*, poplar, co-contamination

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INTRODUCTION

Industrial and agricultural activities together with the enhanced urbanization and military operations resulted in contaminated sites worldwide (Ostroumov 2003). By consequence, the extent of the contamination now imposes a burden on agricultural revenues (Meers *et al*. 2009), regional policy (Flemish government, 2007), public health (Nawrot *et al*. 2006), and the self-cleansing capacity of polluted ecosystems (Dotty, 2008). Conventional remediation strategies are often destructive for soil structure and function and do not provide a reasonable cost-benefit balance to be applied on the imposing scale that is now required (Vangronsveld et al., 2009). Therefore, the need for inventive and efficacious remediation technologies with a low ecological footprint has never been greater. Phytoremediation, an eco-friendly and benign plant-based remediation strategy, has emerged the last two decades as a valuable and sustainable alternative to the commonly used civil-engineering techniques (Vangronsveld *et al*. 2009). Phytoremediation is generally considered to be more cost-effective since it works *in situ*, is largely solar-powered and requires only minimal operational efforts. In addition, during the remediation process, it offers the possibility of economic (non-food) activity and in many cases provides a useful end product with economic value (e.g., biofuel) (Vangronsveld *et al*. 2009, Vassilev *et al*. 2004). Nevertheless, before large-scale application of phytoremediation can be considered, several limitations have to be addressed including: limited bioavailability and translocation of the contaminants, tolerance levels of the plants (phytotoxicity) and insufficient *in planta* degradation (of volatile organic compounds) leading to evapotranspiration to the atmosphere (Newman and Reynolds 2005; Gerhardt *et al*. 2009; Weyens *et al*. 2009a).

In recent years, beneficial plant-bacteria interactions have been exploited to address the limitations of phytoremediation and to improve biomass production (Weyens *et al*. 2009a; 2009b). The high, natural and biotechnological potential of plant-associated bacteria includes (a) several mechanisms by which they can promote plant growth and health as well as (b) an extensive metabolic diversity, which can support their host plants in overcoming phytotoxic effects during phytoremediation applications.

The plant-bacteria interaction entails an intense symbiosis where the plant provides residency and nutrients and in return the bacteria may facilitate plant growth and health either directly or indirectly (Dotty, 2008; Weyens *et al*. 2009b). Direct plant growth-promotion activity may arise through the fixation of atmospheric nitrogen (diazotrophy) (Triplett 1996), the solubilization of minerals such as phosphorus and iron (Rodriquez and Fraga 1999; Braud *et al*. 2006), the production of phytohormones (e.g., cytokinins, indol-3-acetic acid (IAA), acetoin, 2,3-butanediol) (Ryu *et al*. 2003; Garcia de Salamone, Hynes, and Nelson 2005) and the synthesis of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can lower plant ethylene levels during stress (Glick, 2004). Plant-associated bacteria can indirectly benefit plant growth by inhibiting growth or activity of plant pathogens. This inhibition can be attributed to any of a variety of mechanisms including the competition for space and nutrients, the production of biocontrol agents (e.g., antibiotics, antifungal metabolites), the induction of systemic resistance and the depletion of iron from the rhizosphere through the production of siderophores (Glick 2010). These direct and indirect plant growth promoting mechanisms may be of great importance to improve the yields of food, feed, and bioenergy and other feedstock crops on marginal land. This will be needed to meet the rapidly increasing demand for biofuels without infringing upon the food supply (Haberl *et al*. 2010). Furthermore, the production of biofuel and industrial feedstocks on marginal land that is not suited for agriculture can be an approach to moderate the conflict between food and energy crops (Weyens *et al*. 2009b).

The role of plant-associated rhizospheric and endophytic bacteria during phytoremediation applications has become increasingly evident (Weyens *et al*. 2009a). Exploiting the genetic and biochemical potential of plant-associated bacteria can significantly improve phytoremediation of toxic metals or organic compounds.

In case of remediating organics, plants-associated bacteria equipped with the appropriate degradation pathway (naturally or genetically engineered) may enhance rhizo- and *in planta* degradation of the organics thus reducing phytotoxicity and evapotranspiration to the ambient air (Weyens *et al*. 2009a). Proof of this concept was provided by inoculating yellow lupine (Barac *et al*. 2004) and poplar plants (Taghavi *et al*. 2005; Weyens *et al*. 2009c, 2010a) with an endophyte possessing the genetic information required for toluene and trichloroethylene (TCE) degradation to promote *in planta* toluene and/or TCE degradation. After inoculation decreases of both toluene and/or TCE phytotoxicity and evapotranspiration were achieved.

Bioaugmentation of the rhizosphere and/or inoculation with specific bacterial endophytes can also increase metal bioavailability by the excretion of siderophores (Braud *et al*. 2006; Ma *et al*. 2011), and/or of organic acids (Chen *et al*. 2005). After increasing metal uptake, the necessity to reduce metal phytotoxicity is even higher. Increasing metal-tolerance levels of the plants can be accomplished by inoculation with endophytes equipped with a metal resistance/sequestration system. In general, these metal resistance/sequestration systems are leading to bioprecipitation of the metals on the bacterial cell wall, rendering them harmless for the plant and thus reducing metal phytotoxicity (Ma *et al*. 2011; Mastretta *et al*. 2009). Lodewyckx *et al*. (2002) reported the use of endophytes to improve the metal bioavailability, uptake and plant tolerance levels by introducing the ncc-nre nickel resistance system of *Ralstonia metallidurans* into yellow lupine (*Lupinus luteus* L) endophytes. Root inoculation of yellow lupine significantly increased the nickel concentration in the roots by 30%.

Until now, most research concerning improved phytoremediation efficiency mainly focused on single contaminant systems. Consequently, little information is available regarding the effectiveness and processes of phytoremediation of mixed pollutions (e.g., organic compounds and toxic metals). However, numerous sites face contamination with organic pollutants and toxic metals (40% of the hazardous waste sites in the U.S.) (Sandrin and Maier 2003) implicating that plants and their associated bacteria will have to deal with both metals and organics. The remediation of those sites is considerably more complex than single contaminated systems. The presence of metals can inhibit a broad range of microbial processes (*e.g*. growth, dehalogenation) through interaction with specific enzymes responsible for the biodegradation (Sandrin and Maier 2003). As a consequence, phytoremedation of mixed pollutions has been limited to inactivation/stabilisation of the metals (through sequestration/precipitation) and improving the biodegradation of the organic pollutants (Said and Lewis 1991; Burkhardt *et al*. 1993; Qi Lin, 2006). Recently, we demonstrated an innovative approach to cope with mixed pollutions (Weyens *et al*. 2010b). In this approach [33], biodegradation of the organic pollutant (TCE) and at the same time an improved metal phytoextraction (nickel) are intended. The model plant yellow lupine was inoculated with *Burkholderia cepacia* VM1468 possessing (a) the pTOM-Bu61 plasmid constitutively coding for TCE degradation and (b) the *ncc-nre* Ni resistance/sequestration system. Inoculation of *B. cepacia* VM1468 in plants exposed to Ni and TCE resulted in a decreased Ni and TCE phytotoxicity, a decreasing trend in the TCE evapotranspiration and a significant increase in Ni-uptake (Weyens *et al*. 2010b).

Although phytoremediation efficiency can already be improved by applying the appropriate plant-associated bacteria, the duration of the remediation process still ranges

from several years to decades. Considering this, it is essential to select a plant species that can economically be valorized in order to move phytoremediation towards large scale application. For this reason, in this work, the concept of using endophytes to enhance phytoremediation of mixed contaminations is extended from the model plant yellow lupine to hybrid poplar *Populus deltoides x* (*trichocarpa x deltoides*). Poplar is an excellent candidate for phytoremediation applications which also provides a useful end product such as wood, pulp and bio-energy (Dotty 2008; Ruttens *et al*. 2011). For the selection of the appropriate endophyte, we turned to a previously identified *Pseudomonas putida* W619 isolated from the roots of *Populus deltoides x* (*trichocarpa x deltoides*) (Taghavi *et al*. 2005). This strain, naturally resistant to nickel and exhibiting several plant-growth promoting qualities (Weyens *et al*. 2011), was equipped via natural gene transfer with the pTOM–Bu61 plasmid, constitutively coding for TCE degradation (Taghavi *et al*. 2005; Shields *et al*. 1995). Recently, we already reported the capacity of *P. putida* W619-TCE to degrade TCE in a single contaminant system under greenhouse conditions and in a field application. In both cases inoculation with *P. putida* W619-TCE resulted in an improved TCE degradation accompanied by a reduced TCE evapotranspiration (Weyens *et al*. 2009c, 2010a).

In this work, the potential of the Ni-resistant, TCE-degrading *P. putida* W619-TCE to enhance phytoremediation efficiency of *Populus deltoides x* (*trichocarpa x deltoides*) upon Ni-TCE exposure is investigated. Poplar cuttings are inoculated with *P. putida* W619-TCE and exposed to Ni and TCE. Ten weeks after inoculation, plant health is evaluated based on biomass measurements and the activity of several enzymes involved in the anti-oxidative defence. Phytoremediation efficiency is assessed by measuring (a) TCE concentrations in the leaves and TCE evapotranspiration to the atmosphere and (b) nickel concentrations in the leaves and roots.

MATERIALS AND METHODS

Inoculation of Poplar Cuttings

In order to obtain a successful inoculation, a good root development of the poplar cuttings is desirable. For this reason, poplar cuttings [*Populus deltoides x (trichocarpa x deltoides)* cv. Grimminge] were first putted in aerated tap water for 4 weeks after which roots were inoculated (as described in Weyens *et al*. (2010a)) for 4 days with *Pseudomonas putida* W619-TCE (final CFU $ml^{-1} = 1 \times 10^8$). *Pseudomonas putida* W619-TCE is a TCE-degrading natural root endophyte of poplar, which acquired (via natural gene transfer) the pTOM plasmid containing the constitutively expressed genes for toluene and TCE degradation (Taghavi *et al*. 2005). Control plants were treated in the same way, with the exception of the addition of bacteria.

Re-isolation of *Pseudomonas putida* W619-TCE

To verify a successful inoculation, bacteria were isolated from the root, stem, twig and leaves from 20 cuttings (10 non-inoculated and 10 inoculated) immediately after the 4-day inoculation period. Root, stem, twig and leaf samples were surface sterilized as described in Weyens *et al*. (2010a). After surface sterilization, samples were macerated and serial dilutions were plated on selective 284 medium (Weyens *et al*. 2009d) supplemented with C-mix (per liter medium: 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose, and 0.81 g succinate) with addition of 1mM $NiCl_2$ as a selective agent for *P. putida*

W619(-TCE). The plates were incubated for 7 days at 30°C before the CFU were counted and calculated per gram fresh plant weight (Weyens *et al*. 2010a). To confirm that the isolated strains were identical to the inoculated strains, the BOX1 primer was used for BOX-PCR DNA fingerprinting which was carried out as described earlier (Barac *et al*. 2004).

Growth and Ni-TCE Exposure

After the 4-day inoculation period, poplar cuttings were transferred from the inoculum solution into 2-liter beakers (1 cutting per beaker) containing a 2 cm thick layer of gravel (1–2 mm size) for preventing anaerobic conditions at the bottom. Above this layer, the beakers were almost filled up with calibrated sand with on top of the sand, a 2 cm thick layer of potting soil. Sand, a rather inert material, was chosen as the main substance of the matrix to reduce binding of Ni and TCE to organic matter, which would otherwise affect the effective Ni and TCE exposure. The top layer of potting soil was added to minimize TCE volatilization to the atmosphere. A schematic representation of this experimental set up is shown in Figure 1. During planting, the sand was saturated with 300 ml half strength Hoagland nutrient solution (Weyens *et al*. 2010b) with the addition of 0 and 120 mg l^{-1} $NiSO_4$ combined with 0 and 600 mg l^{-1} TCE respectively for non-exposed and Ni-TCE-exposed cuttings. The beakers were equipped with a 30 cm long glass tube reaching the bottom for watering and additional application of Ni and TCE. To protect the roots from light and to avoid any photodegradation of TCE, aluminium foil was wrapped around the

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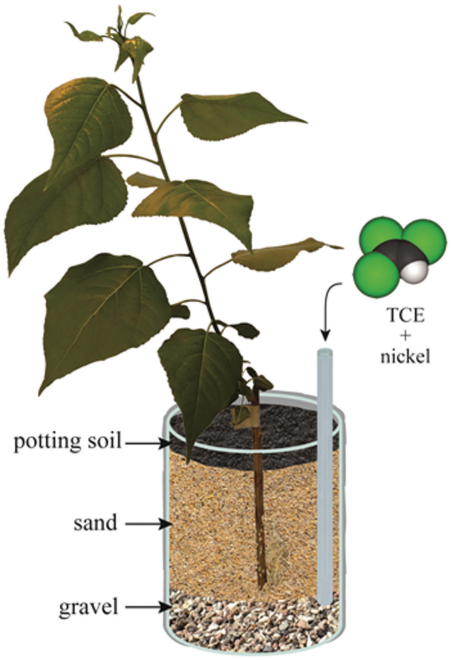

Figure 1 Schematic representation of the set up to grow the poplar cuttings and expose them to Ni and TCE. (Color figure available online).

beakers. After a stabilization period of 2 weeks, Ni and TCE were supplied through the glass tube 2 times a week by adding 50 ml half strength Hoagland containing 120 mg l^{-1} $NiSO_4$ and 600 mg l^{-1} TCE. At the same time, non-exposed plants were provided with 50 ml half strength Hoagland. Besides the application of half strength Hoagland, plants were watered every other day depending on the water consumption of the individual plant. For the non-exposed and for the Ni-TCE-exposed condition, 30 cuttings were planted, 15 inoculated and 15 non-inoculated cuttings. Plants were grown for a total of 10 weeks (2 weeks of stabilization + 8 weeks of additional Ni-TCE exposure) in the beakers before harvesting.

Phytotoxic Effects of Exposure to Nickel and TCE

To investigate Ni and TCE phytotoxicity, changes in biomass production and activity of some stress-related enzymes (involved in anti-oxidative defense) were analyzed.

Effect on Biomass. At harvest, the sand was thoroughly rinsed from the roots and biomasses of root and shoot (twig + leaves) were determined for all cuttings.

Effect on Enzyme Activity. To investigate the effect of Ni-TCE-exposure on enzyme activities, at harvest, root samples (6 replicates from each condition) were taken and snap frozen in liquid nitrogen. These frozen samples were used for an extraction as previously described by Weyens *et al*. (2010b). Ascorbate peroxidase (APX, EC 1.11.1.11), syringaldazine peroxidase (SPX, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1) activities as markers for oxidative stress were measured spectrophotometrically in the extract at 25°C. Ascorbate peroxidase activity was measured at 298 nm following the method of Gerbling (Gerbling *et al*. 1984). Syringaldazine peroxidase activity was measured at 530 nm according to Bergmeyer (Bergmeyer, Gawenn, and Grassl 1974) and analysis of superoxide dismutase activity was based on the inhibition of cytochrome c (McCord and Fridovich 1969).

Ni Content in Roots and Leaves

During harvest, fresh root and leaf samples (6 replicates for each condition) were thoroughly washed with $Pb(NO_3)_2$ to remove Ni present on the surface. Plant samples were oven dried at 65°C after which they were crushed to a fine powder with mortar and pestle. Crushed samples were wet digested in a heating block (as described in Weyens *et al*. (2010b)) and Ni concentrations were determined using flame atomic absorption spectrometry (AAS).

TCE Degradation and Evapotranspiration

To determine TCE concentrations in the leaves, 6 leaf samples of each condition were snap frozen during harvest and stored at –80°C until TCE extraction. TCE was extracted from the leaves and the concentration was determined following the method described in Weyens *et al*. (2010a).

Next to the TCE concentration in the leaves, the TCE evapotranspiration from the leaves to the atmosphere was determined. This analysis was performed on 3 replicates for each condition using the set up previously described by Weyens *et al*. (2009d).

Statistical Analysis

All datasets were statistically compared using one-way or two-way ANOVA and post hoc multiple comparison testing (Tukey Kramer). Transformations were applied when necessary to approximate normality and/or homoscedasticity. The statistical analyses were performed in SAS 9.1.3. Significance levels are indicated as follows: *: $p < 0.5$; **: $p < 0.05$; ***: $p < 0.01$; and ****: $p < 0.001$.

RESULTS AND DISCUSSION

Re-isolation of *Pseudomonas putida* W619-TCE

After the 4-day inoculation period, a re-isolation was performed from non-inoculated as well as from inoculated cuttings.

As expected, *P. putida* W619-TCE could be re-isolated from the roots, the stem and the twigs of all inoculated poplar cuttings. Furthermore, BOX-PCR fingerprints confirmed that the strains isolated on the selective medium were identical to the inoculated strains (data not shown). However, *P. putida* W619-TCE could not be detected in the leaves of the inoculated cuttings. This colonization pattern is very similar to the ones we observed in previous inoculation experiments (Weyens *et al*. 2010a). Obviously, this successful colonization of poplar cuttings under laboratory conditions does not guarantee that the inoculated *P. putida* W619(-TCE) will sustainably establish after inoculation under related Ni-TCE-contaminated field conditions. However, the successful establishment and enrichment of *P. putida* W619-TCE after *in situ* inoculation on a TCE-contaminated site (Weyens *et al*. 2009c) suggest that the chances for successful and stable colonization are high. Consequently, extrapolation of the inoculation results obtained in these laboratory experiments to similar field conditions is realistic. Furthermore, in case long term stability of the inoculated strain would still be a problem, additional inoculations in time can be applied.

Although *P. putida* W619 is a natural, Ni-resistant root endophyte of poplar [*Populus deltoides x (trichocarpa x deltoides)* cv. Grimminge], this strain could not be re-isolated from non-inoculated cuttings. As the cuttings were growing for only 4.5 weeks at the moment of isolation, the number of natural abundant *P. putida* W619 strains probably was too low for a successful isolation. Also the TCE-degrading *P. putida* W619-TCE could not be re-isolated from non-inoculated cuttings. This strain was originally isolated from the same poplar clone exposed to toluene and inoculated with a donor of the pTOM plasmid coding for toluene/TCE degradation (Taghavi *et al*. 2005). Taghavi *et al*. (2005) demonstrated that after toluene exposure and inoculation with the pTOM plasmid donor *Burkholderia cepacia* VM1468, the natural poplar root endophyte *P. putida* W619 got equipped with the pTOM plasmid through *in planta* horizontal gene transfer. It is not surprising that *P. putida* W619-TCE could not be re-isolated from non-inoculated cuttings in this experiment, as the cuttings were not (yet) exposed to toluene/TCE and no potential donor of the pTOM plasmid was present.

Effect of Inoculation on Phytoremediation Efficiency

In earlier studies the phytoremediation efficiency of co-contaminated sites was mostly improved by inactivating the toxic metals by means of stabilization in order to achieve an improved degradation of organic contaminants (Said and Lewis 1991; Burkhardt *et al*.

1993; Qi Lin 2006). This means that the tackling of the metal contamination is limited to stabilization and metal phytoextraction is not even considered. This study aims to improve the phytoremediation efficiency on a Ni-TCE co-contamination by not only promoting TCE degradation but at the same time attempting to enhance Ni uptake by poplar. To accomplish these features, the Ni-resistant TCE-degrading poplar endophyte *P. putida* W619 was inoculated in Ni-TCE exposed poplar cuttings. On the one hand, these endophytic bacteria, colonizing the transport tissues of the plant (Weyens *et al*. 2011), are hypothesized to decrease Ni phytotoxicity leading to an increased Ni concentration in the poplar cuttings. On the other hand, these endophytes are capable of degrading TCE while it is transported through the xylem. The combination of both features could result in (a) a decreased Ni and TCE phytotoxicity, (b) a lowered TCE concentration in the leaves and increased Ni uptake in roots/leaves and (c) a reduced TCE evapotranspiration to the atmosphere. To affirm this hypothesis, the phytotoxic effects of Ni and TCE, the Ni uptake in roots and leaves and the TCE degradation and evapotranspiration were tested.

Phytotoxic Effects of Exposure to Ni and TCE. To investigate the phytotoxic effects caused by Ni and TCE exposure on non-inoculated cuttings and cuttings inoculated with *P. putida* W619-TCE, the effects on root and shoot weight and on the activities of some oxidative stress-related enzymes in the roots were determined.

Exposure to Ni and TCE strongly decreased ($p < 0.001$) the weight of the roots and the shoots of non-inoculated as well as of W619-inoculated cuttings (Figure 2). In the roots, a positive effect of the inoculation was observed which was strongly significant ($p < 0.001$) in non-exposed plants and only slightly significant ($p < 0.5$) in Ni-TCE-exposed plants (Figure 2B). Inoculation with *P. putida* W619-TCE did not affect the shoot weight (Figure 2A).

Studying the effects on oxidative stress-related enzyme activities in roots after inoculation with *P. putida* W619-TCE and after Ni and TCE exposure, following observations can be reported. Inoculation of non-exposed cuttings resulted in slightly increased activities of APOD and SOD while inoculation of Ni-TCE-exposed cuttings lead to a slight decrease ($p < 0.5$) of APOD, SPOD and SOD activities (Figure 3). The slightly increased activities of APOD and SOD in non-exposed plants might suggest a minor stress at the root level due to the inoculation of *P. putida* W619-TCE. However, since root weight strongly increased after inoculation, this enhanced anti-oxidative capacity obviously did not have a negative effect on plant growth. However, the decreases in enzyme activities after inoculation of Ni-TCE-exposed cuttings indicate that *P. putida* W619-TCE can reduce the level of oxidative stress in the roots of cuttings that are exposed to Ni and TCE.

To study the effect of inoculation with *P. putida* W619-TCE on Ni and TCE phytotoxicity, the toxic effect caused by Ni and TCE exposure on non-inoculated cuttings is compared with the effect of exposure on W619-inoculated cuttings (Figure 3). In case non-inoculated cuttings are exposed to Ni and TCE, the activity of APOD strongly ($p < 0.001$) and of SOD slightly ($p < 0.5$) increased while the SPOD activity remained unaffected (Figure 3). The increased activities of defence related enzymes like APOD and SOD indicate oxidative stress induced due to exposure to Ni and TCE. In contrast, exposure of W619-inoculated cuttings to Ni and TCE did not affect APOD and SOD activity and even resulted in a slightly decreased ($p < 0.5$) SPOD activity (Figure 3). This suggests that inoculation with *P. putida* W619-TCE results in a mitigation of the phytotoxic effect of Ni and TCE on poplar cuttings.

Ni Uptake in Roots and Leaves. In case of Ni-TCE-exposed cuttings, a strong increase ($p < 0.001$) in Ni concentration in the roots and a slight increase ($p < 0.5$) in

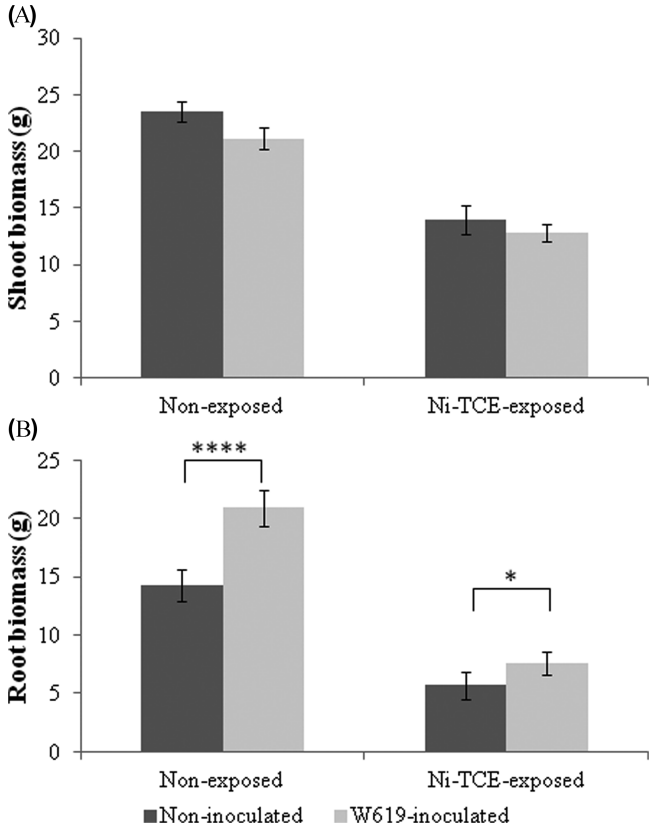

Figure 2 Root (A) and shoot (B) biomass (g) of non-inoculated and W619-inoculated (inoculated with *P. putida* W619-TCE) poplar cuttings exposed to 0 mg l^{-1} $NiSO_4$ and 0 mg l^{-1} TCE (non-exposed) and to 120 mg l^{-1} $NiSO_4$ and 600 mg l^{-1} TCE (Ni-TCE-exposed). Values are mean ± standard error of 15 biological independent replicates (significance level: *: $p < 0.5$; ****: $p < 0.001$).

Ni concentration in the leaves were found after inoculation with *P. putida* W619-TCE (Figure 4). Strong increases in Ni-uptake were previously also observed in roots of yellow lupine plants that were exposed to Ni (Lodewyckx *et al*. 2002) and to a Ni-TCE co-contamination (Weyens *et al*. 2010b). Also the higher Ni concentrations in the roots as compared to those in the leaves (Figure 4) are in accordance with the results of Lodewyckx *et al*. (2002) and Weyens *et al*. (2010b). An essential aspect for phytoremediation in field applications is to obtain a high Ni translocation from the roots to the above ground plant parts since these plants tissues are easier to harvest. Therefore, in future experiments, it is important to investigate if the increase in Ni concentration in the above ground plant parts will become higher when cuttings are grown for a longer time period. In this experiment, only a short term (10 weeks) exposure was applied which could explain why the increase in Ni concentration was mainly found in the roots. Inoculation did not affect the available amount of Ni present in the sand (non-inoculated and inoculated sand contained respectively 1337.14 ± 187,74 and 1143.57 ± 87.36 mg Ni kg^{-1} DW^{-1}). However, in this experiment, Ni was highly available, while this is mostly not the case in the field. It is possible that in case of limited Ni availability, inoculation could contribute to an improved availability

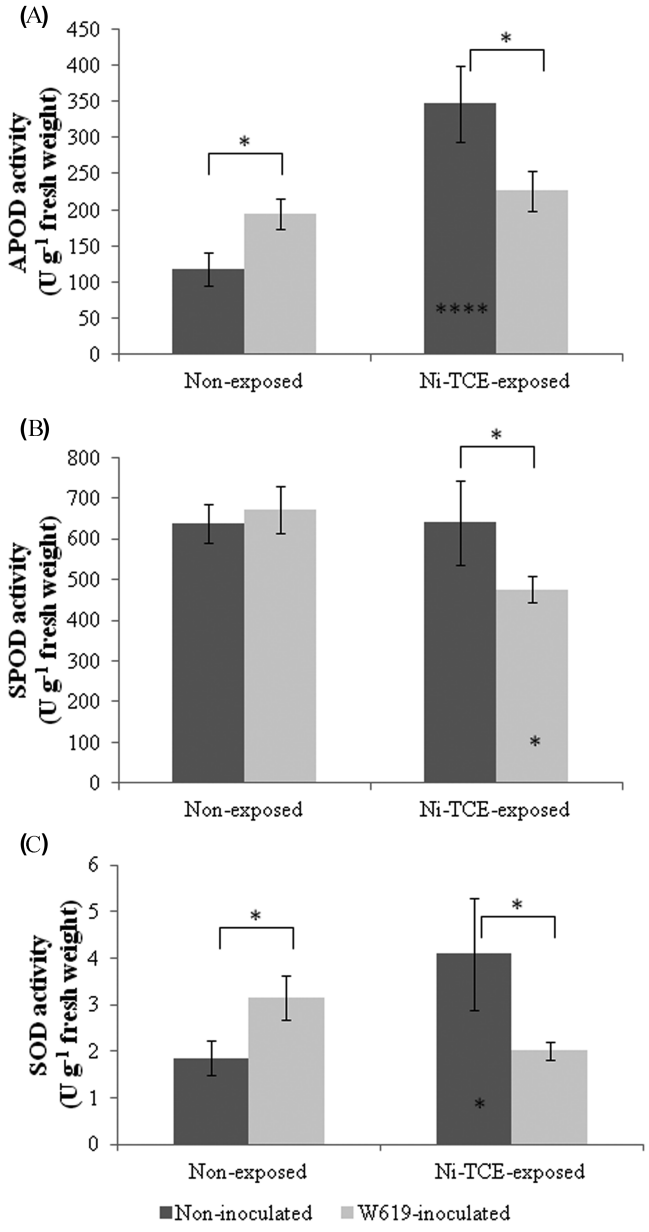

Figure 3 Ascorbate peroxidase (APOD) (A), syringaldazine peroxidase (SPOD) (B) and superoxide dismutase (SOD) (C) activities (U g^{-1} fresh weight) in the roots of non-inoculated and W619-inoculated (inoculated with *P. putida* W619-TCE) poplar cuttings exposed to 0 mg l^{-1} $NiSO_4$ and 0 mg l^{-1} TCE (non-exposed) and to 120 mg l^{-1} $NiSO_4$ and 600 mg l^{-1} TCE (Ni-TCE-exposed). Values are mean ± standard error of 6 biological independent replicates (significance level: *: $p < 0.5$; ****: $p < 0.001$). Significance levels shown inside the bars are referring to comparison (a) between non-inoculated Ni-TCE-exposed cuttings and non-inoculated non-exposed cuttings and (b) between W619-inoculated Ni-TCE-exposed cuttings and W619-inoculated non-exposed cuttings.

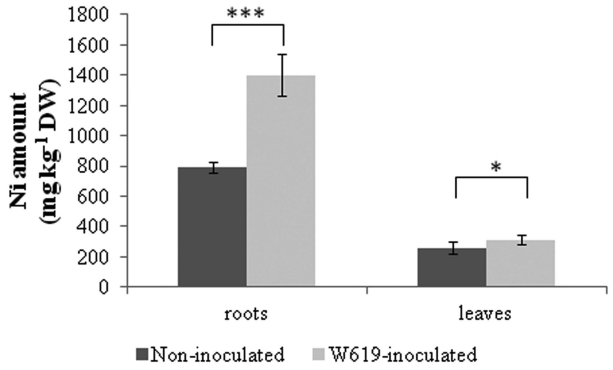

Figure 4 Ni concentration (mg kg^{-1} dry weight) in the roots and the leaves of non-inoculated and W619-inoculated (inoculated with *P. putida* W619-TCE) poplar cuttings exposed to 120 mg l^{-1} $NiSO_4$ and 600 mg l^{-1} TCE. Values are mean ± standard error of 6 biological independent replicates (significance level: *: $p < 0.5$; ***: $p < 0.01$).

due to production of organic acids and siderophores by the bacteria. Further, lower Ni availability under field conditions could be related to a decreased Ni uptake. It would be interesting to verify these assumptions in future experiments using contaminated soil for a field site instead of spiked sand.

Since *Pseudomonas putida* W619-TCE is equipped with a Ni resistance/sequestration system, it probably reduced Ni phytotoxicity by sequestrating Ni on its cell wall thereby possible achieving an increased Ni-uptake without negative effects on the plant growth. This concept can easily be extended to other metals. For example, retrotransfer by broad host range plasmids like RP4 can be used to acquire the desired metal resistance (Top *et al*. 1992).

TCE Degradation and Evapotranspiration. Although plants may often metabolize or sequester organic contaminants, plants are at a significant disadvantage in comparison with bacteria. Since plants are autotrophic, and by consequence do not rely on organic compounds as a source of energy or carbon, they were not under selective pressure to develop the capacity to degrade chemically intransigent materials. Bacteria, on the other hand, are capable to metabolize an extensive set of chemical structures. Moreover, microbial metabolism often ends with the compound being transformed to CO_2, water and cellular biomass. For this reason, we can put forward that plants rely on their associated micro-organisms to achieve a more efficient degradation of organic contaminants (Weyens *et al*. 2009a). In this work, it was hypothesized that the TCE-degrading poplar endophyte *Pseudomonas putida* W619-TCE can assist its host plant in the degradation of TCE, even when the host plant is exposed to a Ni-TCE co-contamination.

After inoculation with *Pseudomonas putida* W619-TCE, a significant decrease ($p < 0.05$) in TCE concentration in the leaves and a minor decrease ($p < 0.5$) in TCE evapotranspiration through the leaves were observed (Figure 5). The decrease in TCE evapotranspiration is comparable with the decrease that was obtained after inoculation with a TCE degrading endophyte in yellow lupine plants exposed to a Ni-TCE co-contamination (Weyens *et al*. 2010b). However, when poplar cuttings were exposed to TCE as a single contaminant, a stronger decrease in TCE extracted from the leaves as well as in TCE evapotranspired to the atmosphere was acquired under greenhouse conditions (Weyens *et al*.

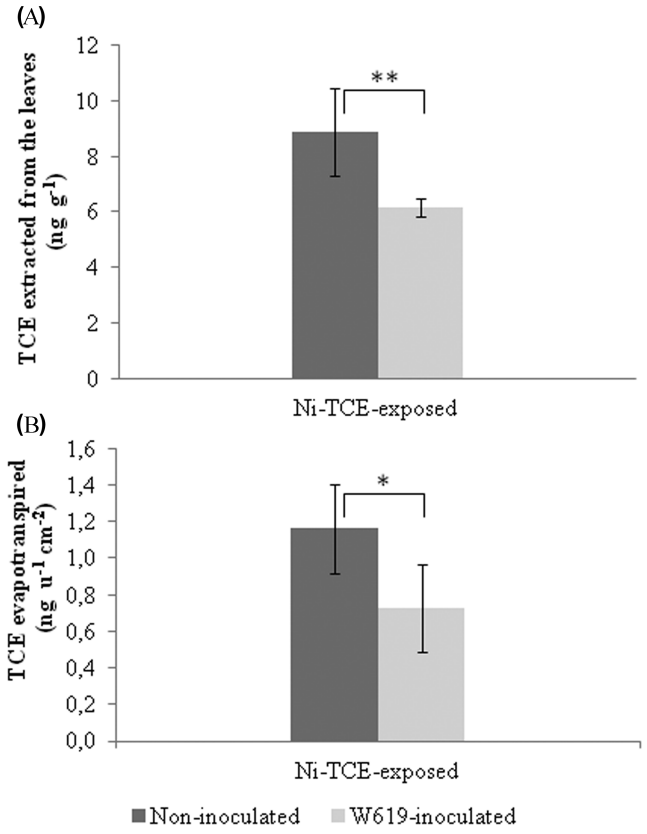

Figure 5 Amount of TCE extracted from the leaves (A) (ng g^{-1}) and evapotranspired through the leaves (B) (ng u^{-1} cm^{-2}) of non-inoculated and W619-inoculated (inoculated with *P. putida* W619-TCE) poplar cuttings exposed to 120 mg l^{-1} $NiSO_4$ and 600 mg l^{-1} TCE (Ni-TCE-exposed). Values are mean ± standard error of 6 (A) or 3 (B) biological independent replicates (significance level: *: $p < 0.5$; **: $p < 0.05$).

2010a), and even in the field (Weyens *et al*. 2009c). However, considering the complexity of a co-contaminated system, the increased TCE degradation observed in this study in combination with the increased Ni uptake in the roots are very promising and could contribute to move phytoremediation of co-contaminations to field applications. Furthermore, also for organic contaminants it will be relatively straightforward to extend the concept to many other contaminants than TCE. As many endophytic bacteria are closely related to environmental strains that are equipped with pathways against a broad spectrum of organics on mobile DNA elements, endophytes can easily be provided with the appropriate degradation pathways by horizontal gene transfer.

CONCLUSION

The results obtained in this work show that after inoculation of poplar cuttings with the TCE-degrading Ni-resistant root endophyte *P. putida* W619-TCE, (a) a slightly reduced Ni-TCE phytotoxicity, (b) a strongly increased Ni uptake in the roots and (c) slightly significant decreases of TCE concentration in the leaves and evapotranspiration through the

leaves can be acquired. In previous work, proof of this concept of using endophytes equipped with the appropriate characteristics to improve phytoremediation of mixed contamination, was already delivered by using yellow lupine as a model plant. However, a crucial step to move this concept towards large scale field applications was realized in this work, namely by replacing the model plant yellow lupine with poplar that is better suited for phytoremediation applications in practice. Moreover, poplar is also an excellent species to realize high biomass production for bioenergy applications on contaminated sites. A next step towards field applications would be to verify if the promising results obtained in this short term experiment can be confirmed in a long term experiment.

In conclusion, if the results obtained in this work can be extrapolated to field applications, this would imply that biomass for bioenergy applications can be optimally produced on marginal land in this way moderating the conflict between food and energy crops and in the meantime, cleaning up contamination.

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