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Endophytic bacteria improve phytoremediation of Ni and TCE co-contamination

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Engineered endophytes can improve phytoremediation of mixed contaminations via enhanced degradation of organic contaminants and improved metal uptake and translocation.

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ABSTRACT

The aim of this work was to investigate if engineered endophytes can improve phytoremediation of co-contaminations by organic pollutants and toxic metals. As a model system, yellow lupine was inoculated with the endophyte *Burkholderia cepacia* VM1468 possessing (a) the pTOM-Bu61 plasmid, coding for constitutive trichloroethylene (TCE) degradation, and (b) the *ncc–nre* Ni resistance/sequestration system. Plants were exposed to Ni and TCE and (a) Ni and TCE phytotoxicity, (b) TCE degradation and evapotranspiration, and (c) Ni concentrations in the roots and shoots were determined. Inoculation with *B. cepacia* VM1468 resulted in decreased Ni and TCE phytotoxicity, as measured by 30% increased root biomass and up to 50% decreased activities of enzymes involved in anti-oxidative defence in the roots. In addition, TCE evapotranspiration showed a decreasing trend and a 5 times higher Ni uptake was observed after inoculation.

1. Introduction

Established methods to remediate contaminated soils and groundwater are frequently expensive, environmentally invasive, labour intensive, and do not make cost-effective use of existing resources. Especially in case of large-scale contaminated areas, phytoremediation is considered to be a cost-effective and sustainable remediation alternative, as it works *in situ*, is solar powered and demands minimal site disturbance and maintenance. However, phytoremediation still has to deal with some important shortcomings such as phytotoxicity, a limited contaminant uptake, and evapotranspiration of volatile organic contaminants.

Plant-associated bacteria can be exploited to overcome these constraints (Weyens et al., 2009a,b). In case of phytoremediation of organic contaminants, endophytes equipped with the appropriate degradation pathway can diminish phytotoxicity and evapotranspiration (Barac et al., 2004; Taghavi et al., 2005). To increase plant availability of metals plant-associated bacteria that are capable of producing siderophores and/or organic acids can be used (Weyens et al., 2009a). To reduce internal metal bioavailability and by consequence metal phytotoxicity, endophytes equipped with a metal resistance/sequestration system (*e.g.* ncc–nre) leading to bioprecipitation of metals on the bacterial cell wall can be inoculated (Weyens et al., 2009a). Combining increased plant availability and reduced internal bioavailability of metals will allow plants to accumulate higher amounts of metals without increasing phytotoxicity. Proof of concept has been provided under controlled laboratory conditions for toluene (Barac et al., 2004; Taghavi et al., 2005) or nickel (Lodewyckx et al., 2001) and for mixed contamination by toluene and nickel (Weyens et al., in press).

At most contaminated sites, the application of phytoremediation is limited because plants and their associated microorganisms are faced with mixed pollutions of organics and toxic metals. The presence of toxic metals can inhibit the biodegradation of a variety of organic pollutants (Said and Lewis, 1991; Burkhardt et al., 1993; Sandrin and Maier, 2003; Lin et al., 2006). Consequently, until now, studies on remediation of co-contaminated sites have mainly focused on metal sequestration and precipitation to improve the biodegradation of the organic contaminants. In this way, metal remediation on these co-contaminated sites is restricted to metal inactivation.

In previous work, we have demonstrated that engineered endophytes that (a) are capable of degrading organic contaminants,

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and (b) are resistant to metals can be used to improve the remediation of both organic contaminants and metals in a mixed waste situation (Weyens et al., in press). Inoculation of yellow lupine exposed to Ni and toluene with a Ni-resistant and toluene-degrading endophytic bacterium results in decreased Ni and toluene phytotoxicity, and reduced evapotranspiration of toluene. In this work, we extended this concept to mixed contamination by Ni and trichloroethylene (TCE), one of the most prevalent groundwater contaminants (Halsey et al., 2005). For this purpose, yellow lupine (*Lupinus luteus*) was inoculated with the endophytic strain *Burkholderia cepacia* VM1468, which possesses the pTOM-Bu61 plasmid coding for TCE degradation and the *ncc–nre* Ni resistance/sequestration system, (Ni^R, Tol^+, TCE) and were exposed to Ni and TCE. To examine if the inoculated endophyte could improve efficiency of phytoremediation, Ni and TCE phytotoxicity, Ni uptake and TCE evapotranspiration were investigated.

2. Materials and methods

2.1. Inoculation of yellow lupine plants

Yellow lupine plants were inoculated with *B. cepacia* VM1468. This strain was constructed as described by Barac et al. (2004): (a) *B. cepacia* L.S.2.4., a natural endophyte of yellow lupine, was equipped with Ni resistance by conjugation between *Escherichia coli* CM2520 carrying the ncc–nre Ni resistance and the natural endophyte resulting in *B. cepacia* BU72; (b) conjugation between this Ni-resistant *B. cepacia* BU72 as a receptor and *Burkholderia vietnamiensis* BU61, a soil isolate possessing the pTOM plasmid as a donor, resulted in the Ni-resistant, toluene-degrading endophyte *B. cepacia* VM1468. Fresh cultures of *B. cepacia* VM1468 were grown in 869 medium (Mergeay et al., 1985) at 30 °C until an approximate absorbance (A_{660}) value of 1 was reached. The cells were collected by centrifugation (15 min at 4630*g*), washed in 10 mM $MgSO_4$ and resuspended in the original volume of 10 mM $MgSO_4$.

Seeds of yellow lupine plants were surface sterilized for 20 min at room temperature in a solution containing 1% active chloride (w/v, added as a NaOCl solution) and supplemented with 1 drop Tween 80 (Merck) per 100 ml solution. After surface sterilization, seeds were rinsed 3 times in sterile tap water and soaked overnight in the last rinsing water. Seeds were planted in 400 ml plastic jars filled with perlite and saturated with half strength Hoagland's nutrient solution to which the bacterial inocula were added at a final concentration of 10^8 CFU ml^{-1}. Non-inoculated plants were used as controls. Only after 2 weeks, plants were transferred to 400 ml pots filled with sand and were exposed to TCE and $NiSO_4$ as described below. For each condition, at least 15 biological independent replicates were tested.

2.2. Recovery of inoculated bacteria

To ensure the inoculation was successful, bacterial endophytes were re-isolated from shoots and roots of lupine plants after 4 weeks of growth. Root and shoot samples were taken from 3 plants and pooled together before the isolation. The endophytes were isolated as described earlier (Barac et al., 2004). Samples were plated on (a) 284 medium with addition of a carbon 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) and 0.24 g l^{-1} $NiCl_2 \cdot 6H_2O$ (284 + Ni) that is selective for *B. cepacia* VM1468. The 284 medium contains per liter distilled water 6.06 g Tris–HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g $NaSO_4$, 0.20 g $MgCl_2 \times 6H_2O$, 0.03 g $CaCl_2 \times 2H_2O$, 40 mg $Na_2HPO_4 \times 2H_2O$, 0.48 mg Fe(III)NH_4 citrate, 1 ml microelements solution, final pH 7. The microelement solution contains per liter distilled water: 1.3 ml 25% HCl, 144 mg $ZnSO_4 \times 7H_2O$, 100 mg $MnCl_4 \times 2H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2 \times 6H_2O$, 17 mg $CuCl_2 \times 2H_2O$, 24 mg $NiCl_2 \times 6H_2O$ and 36 mg $NaMoO_4 \times 2H_2O$.

2.3. Phytotoxic effects of exposure to nickel, toluene and TCE

After 14 days of growth, non-inoculated control and VM1468 inoculated plants were exposed to 0 and 40 mg l^{-1} NiSO4 respectively, and were irrigated every other day with half strength Hoagland's solution to which TCE was added to obtain final TCE concentrations of respectively 0 and 10 mg l^{-1}. The half strength Hoagland's solution contains per liter distilled water 50 ml macroelements, 500 µl microelements and 300 µl Fe–EDTA (macroelements (g l^{-1}): 10.2 HNO_3, 7.08 $Ca(NO_3)_24H_2O$, 2.30 $NH_4H_2PO_4$, 4.9 $MgSO_4 \cdot 7H_2O$; microelements (g l^{-1}): 2.86 H_3BO_3, 1.81 $MnCl_2 \cdot 4H_2O$, 0.08 $CuSO_4 \cdot 5H_2O$, 0.09 $H_2MoO_4 \cdot H_2O$, 0.22 $ZnSO_4 \cdot 7H_2O$; Fe–EDTA (g l^{-1}): 5.00 EDTA–Na, 7.60 $FeSO_4 \cdot 7H_2O$). After two weeks exposure plants were harvested. To investigate TCE and Ni phytotoxicity, changes in growth and activity of some stress-related enzymes (involved in anti-oxidative defence) were analyzed. Plant growth and enzyme activities can be affected by both the microbial inoculation and by exposure to contaminants (Taghavi et al., 2005, 2009). To eliminate the effects caused by inoculation, the phytotoxic effects induced by TCE and/or Ni were determined by calculating biomass and enzyme activities relative to corresponding non-exposed control plants that were inoculated with VM1468.

2.3.1. Growth reduction

At harvest, roots and shoots were separated and their biomass was determined for at least 15 biological independent replicates for each condition. The biomass relative to corresponding non-exposed plants was calculated as follows:

$$\text{Biomass}_{\text{relative to non-exposed}} \text{ of plant inoculated with x and exposed to y (\%)} = \left[(\text{biomass of plant inoculated with x and exposed to y}) \times (\text{biomass of non-exposed plant inoculated with x})^{-1}\right] \times 100$$

x, being no bacterial strain or *B. cepacia* VM1468
y, being 40 mg l^{-1} $NiSO_4$ and 10 mg l^{-1} TCE

2.3.2. Activity of enzymes involved in anti-oxidative defence

To determine the activities of stress-related enzymes, root samples (6 replicates for each condition) were harvested and immediately frozen in liquid nitrogen before storage at −80 °C. The frozen root tissues were homogenized in ice-cooled 0.1 M Tris–HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol and 4% insoluble polyvinylpyrrolidone (1 ml buffer per 100 mg fresh weight). This homogenate was squeezed through a nylon mesh and centrifuged for 10 min at 20,000*g* and 4 °C. Catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPOD, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1) activities as markers for oxidative stress (Vangronsveld and Clijsters, 1994) were measured spectrophotometrically in the supernatant at 25 °C. CAT and GPOD activities were determined at 240 nm and 436 nm respectively according to Bergmeyer et al. (1974). Analysis of SOD activity was based on the inhibition of the reduction of cytochrome c measured at 550 nm (McCord and Fridovich, 1969). The enzyme activities relative to corresponding non-exposed plants were calculated as follows:

$$\text{Enzyme activity}_{\text{relative to non-exposed}} \text{ of plant inoculated with x and exposed to y (\%)} = \left[(\text{enzyme activity of plant inoculated with x and exposed to y}) \times (\text{enzyme activity of non-exposed plant inoculated with x})^{-1}\right] \times 100$$

x, being no bacterial strain or *B. cepacia* VM1468
y, being 40 mg l^{-1} $NiSO_4$ and 10 mg l^{-1} TCE

2.4. Ni concentrations in roots and shoots

During harvest, fresh root and shoot samples (at least 3 replicates for each condition) were vigorously washed with distilled water to remove all traces of Ni present on the surface. Root and shoot samples were oven-dried (48 h at 65 °C) and subsequently crushed to a fine powder with a mortar and pestle, and wet digested in Pyrex tubes in a heating block. The digestion consisted of 3 cycles in 1 ml HNO_3 (65%) and 1 cycle in 1 ml HCl (37%) at 120 °C for 4 h. Samples were then dissolved in HCl (37%) and diluted to a final volume of 5 ml (2% HCl). Ni concentrations were determined using flame atomic absorption spectrometry (AAS).

2.5. TCE evapotranspiration

After plants were grown for 3 weeks under conditions as described above, 3 non-inoculated and 3 inoculated plants exposed to 40 mg l^{-1} $NiSO_4$ were used to evaluate TCE degradation and evapotranspiration. The lupine plants were taken out of the jars and their roots were vigorously rinsed in sterile water to remove bacteria from the surface. Subsequently, plants were transferred into a two-compartment glass cuvette system (Fig. 1) (Barac et al., 2004) and grown hydroponically in half strength Hoagland's nutrient solution supplemented with 40 mg l^{-1} $NiSO_4$ and 10 mg l^{-1} TCE. The TCE evapotranspiration was measured by GC–MS as described previously (Barac et al., 2004) and calculated per g shoot.

2.6. Statistical analysis

All datasets were statistically analyzed using one way or two way ANOVA and post hoc multiple comparison testing (Tukey Kramer). When necessary, log-transformations were applied to approximate normality and/or homoscedasticity. The statistical analyses were performed in SAS 9.1.3. Further, all results shown in this work were confirmed in an additional independent experiment.

3. Results

3.1. Recovery of endophytic bacteria

After 4 weeks of growth, yellow lupine plants were harvested. The cultivable endophytic bacteria were isolated from root and