Bacterial endophyte-mediated naphthalene phytoprotection and phytoremediation

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PAHs; endophyte; pea; naphthalene; bioremediation; soil.

Abstract
Polyaromatic hydrocarbons (PAHs) are major and recalcitrant pollutants of the environment and their removal presents a significant problem. Phytoremediation has shown much promise in PAH removal from contaminated soil, but may be inhibited because the plant experiences phytotoxic effects from low-molecular-weight PAHs such as naphthalene. This paper describes the construction of a naphthalene-degrading endophytic strain designated Pseudomonas putida VM1441(pNAH7). This strain was found to be an efficient colonizer of plants, colonizing both the rhizosphere and interior root tissues. The inoculation of plants with P. putida VM1441(pNAH7) resulted in the protection of the host plant from the phytotoxic effects of naphthalene. When inoculated plants were exposed to naphthalene, both seed germination and plant transpiration rates were higher than those of the uninoculated controls. The inoculation of plants with this strain also facilitated higher (40%) naphthalene degradation rates compared with uninoculated plants in artificially contaminated soil.

Introduction
Polyaromatic hydrocarbons (PAHs) are byproducts of incomplete combustion of fossil fuels, for example traffic, coal fires, heating, etc., and many PAHs [e.g. benzo(a)pyrene] are considered to be strong carcinogens. In European soils, PAH concentrations range between 0.042 and 11.20 mg kg⁻¹ (Nam et al., 2008). The majority of urban soil concentrations fall in the 0.6–3.0 mg kg⁻¹ range. Areas with heavy transportation networks and industrialization are likely to have much higher levels of PAHs (WHO, 2000). Naphthalene is a low-molecular-weight PAH and is widely used in industry as an intermediate in the production of plasticizers, dyes, pharmaceuticals and insect repellents (USEPA, 2001). In Western Europe, >133000 tonnes of naphthalene were consumed in industrial and domestic applications in 2000 (Lacson, 2000), while in the United States alone, over 500 tonnes of naphthalene were released into the soil environment in 1998, and in Canada, 120 tonnes were released in 2005 (USEPA, 2001; Environment Canada, 2008).

Naphthalene has been identified by the USEPA as one of the most commonly found substances at hazardous waste sites on the ‘National Priorities List’. In contaminated soils and sediments, typical naphthalene concentrations can range from 0.1 to 115 mg kg⁻¹ dry sediment (Simpson et al., 1995). In Germany, naphthalene concentrations of 200 mg kg⁻¹ were found in tar-contaminated sediments (Randow et al., 1996) and about 1 mg kg⁻¹ in soils and sludges in the United Kingdom (Wild & Jones, 1993).

Biodegradation of naphthalene in soil occurs relatively quickly, whereas higher molecular weight PAHs are much more resistant to degradation and can remain in soil for decades. However, naphthalene degradation in soil may be slower if the soil is contaminated with other polycyclic aromatic hydrocarbons. Naphthalene’s evaporation rate is significant at the soil surface, but slowly decreases as the depth and the soil organic matter increases.

Phytoremediation offers an ecologically and economically attractive remediation technique for soils contaminated with PAHs (Olson et al., 2008). Most of the medium to high-molecular-weight PAHs are not toxic to the plants commonly used in phytoremediation projects (Trapp & Karlson, 2001; Smith et al., 2005). Reynoso-Cuevas et al. (2008) showed that germination of four grasses (Bouteloua curtipendula, Cenchrus ciliaris, Echinochloa crusgalli and Rynchelytrum repens) was not affected by a mixture of high-molecular-weight PAHs. However, low-molecular-weight compounds such as naphthalene are toxic to plants...
Materials and methods

Bacterial strains and culture media

Bacterial strains used in this work include \textit{P. putida} VM1441 [\textit{P. putida} PopHV 9 with a miniTn5 Km\(^\prime\):Ni\(^{2+}\) insert, obtained from Dr Jaco Vangronsveld, VITO (Flemish Institute for Technological Research) Mol, Belgium], \textit{P. putida} VM1453 [\textit{P. putida} PopHV 9 with a miniTn5 Km\(^\prime\):Gfp insert (Germaine et al., 2004)] and \textit{P. putida} G7(pNAH7) (obtained from Dr M.J. Sadowski, University of Minnesota). Bacterial strains were maintained on Luria–Bertani (LB) agar, containing 100 \(\mu\)g mL\(^{-1}\) kanamycin (Km) or minimal media (MM) + naphthalene as a carbon source. The endophytic strain \textit{P. putida} PopHV 9 was isolated from the stem tissue of \textit{Poplar (Populus trichocarpa \times deltoides cv. Hoogvorst)} cuttings (Germaine et al., 2004) and was found to have plant growth promotion abilities. This strain was genetically tagged with either a miniTn5 Km\(^\prime\):Ni\(^{2+}\) (designated strain VM1441) or a miniTn5 Km\(^\prime\):gfp insert (designated strain VM1453).

Introduction of the NAH7 plasmid into \textit{P. putida} VM1441

The naphthalene-degrading endophytic strain \textit{P. putida} VM1441(pNAH7) was constructed by conjugal mating of the NAH7 plasmid through conjugal mating of \textit{P. putida} G7(pNAH7) and \textit{P. putida} VM1441. Briefly, the pNAH7 donor strain, \textit{P. putida} G7, was grown for 72 h in 250 mL of MM broth containing 0.5 g naphthalene crystals as the sole carbon source, and the endophytic recipient strain was grown overnight in LB medium supplemented with 100 \(\mu\)g mL\(^{-1}\) kanamycin. One millilitre of each culture was centrifuged and the pellets were washed in 10 \(^{-2}\)M MgSO\(_4\). Aliquots of 100 \(\mu\)L were added to a sterile filter (0.45 \(\mu\)m) and incubated overnight at 30 \(^\circ\)C on solid LB medium. The mating mixture was plated onto MM agar supplemented with 25 \(\mu\)g mL\(^{-1}\) kanamycin. Naphthalene crystals were placed onto the lid of each Petri dish as the sole carbon source and the plates were incubated at 30 \(^\circ\)C for 4–5 days. Transconjugants were purified by streaking colonies onto new LB plates containing 100 \(\mu\)g mL\(^{-1}\) kanamycin, selecting colonies from these plates and restreaking onto MM with naphthalene as the sole carbon source. Only colonies showing both the Km\(^\prime\) and naphthalene\(^\prime\) phenotype were selected for further study. Presence of the NAH7 plasmid in these strains was confirmed using PCR amplification of the \textit{nah}Y gene using the following primers: \textit{nah}Y F\(^5\)-cgcttggaagcatgcttgg-3\(^\prime\) and \textit{nah}Y R\(^5\)-agagacagccctatcatt-3\(^\prime\). Amplification was performed in 50 \(\mu\)L reaction mixtures, containing 1.25 U Red Taq polymerase (Sigma), 2 \(\mu\)L nucleotide (100 \(\mu\)M), 5 \(\mu\)L 10 \\(\times\) buffer, 3 \(\mu\)L of 25 mM MgCl\(_2\) and 0.5 \(\mu\)M of each of the primers, and 2 \(\mu\)L DNA was subjected to a preliminary denaturation step at 95 \(^\circ\)C for 1 min, followed by 35 cycles of incubation at 95 \(^\circ\)C for 1 min, 55.4 \(^\circ\)C for 1 min and 72 \(^\circ\)C for 1 min, and terminated with one step of 7 min at 72 \(^\circ\)C. Growth rates on naphthalene were assessed by monitoring the OD\(_{600\text{nm}}\) in a 1-L flask containing 250 mL MM broth and 1 g naphthalene.

Soil and plant growth conditions

In all soil-based experiments, the soil used was collected from the grounds at the Institute of Technology, Carlow, Ireland. This soil was classified as a sandy loam (60% sand, 33% clay and 7% silt). Pea plants (\textit{Pisum sativum var Early Onward}, Suttons, UK) were used as the model plant. Plant cultivation was carried out in controlled plant rooms or environmental growth chambers set at 20–25 \(^\circ\)C under a 16-h light/8-h dark regime.

Seed inoculation

Fresh cultures of the inoculum were grown under selective conditions at 30 \(^\circ\)C at 200 r.p.m., to an approximate \(A_{600\text{nm}}\) value of 1.0. Cells were harvested by centrifugation, washed three times in 0.85% sterile saline to remove any selective agents and finally resuspended in 3 \(\times\) volume of saline. Typically, this inoculum contained \(10^{7}--10^{9}\) cells mL\(^{-1}\). A 20-\(\mu\)L aliquot of this inoculum was pipetted onto the surface of each pea seed. The inoculated seeds were then either placed in Petri dishes containing premoistened filter paper and allowed to germinate or placed directly into soil microcosms.

References

(Henner \textit{et al.}, 1999; Kuiper \textit{et al.}, 2001; Trapp \& Karlson, 2001). Phytotoxic symptoms of naphthalene exposure include inhibition of growth, reduced transpiration rates, wilting, chlorosis and even plant death. Henner \textit{et al.} (1999) found that seed germination was strongly inhibited by low-molecular-weight, water-soluble compounds such as toluene, styrene and naphthalene. Many sites are contaminated with mixtures of different PAHs (e.g. creosote), including low-molecular-weight PAHs such as naphthalene; hence, it is important that the phytoremediating plants are protected from the toxicity of lower molecular weight PAHs. The key element for successful phytoremediation is the use of plants that have the ability to proliferate in the presence of high levels of contaminants (Gerhardt \textit{et al.}, 2009). This can be facilitated by inoculation of plants with strains of plant growth-promoting rhizobacteria that increase plant tolerance to contaminants and accelerate plant growth in heavily contaminated soils (Huang \textit{et al.}, 2004).

In this paper, the ability of an endophytic naphthalene-degrading bacterium, \textit{Pseudomonas putida} VM1441(pNAH7), to protect plants from the phytotoxic effects of naphthalene was examined, along with its ability to enhance naphthalene removal from contaminated soil.
Phytoprotection experiments

Effect of naphthalene on seed germination

The aim of these experiments was to assess whether the constructed naphthalene-degrading endophyte, *P. putida VM1441*(pNAH7), conferred phytoprotection properties to *P. putida* constructed naphthalene-degrading endophyte, VM1441(pNAH7), which conferred phytoprotection properties to *P. putida* VM1441. Soil samples were amended with various concentrations of naphthalene (0, 10, 30, 50, 100 and 200 mg kg⁻¹ soil). Pea seeds were inoculated with *P. putida VM1441*(pNAH7) and five seeds were sown per pot. Treatments and experiments were carried out in triplicate. Seeds were allowed to germinate for 2 weeks, after which the number of germinated seeds was determined. Uninoculated seeds were sown under the same conditions and acted as controls.

Hydroponic-based phytoprotection experiments

A hydroponics-based toxicity experiment was used to further assess the protective effect of *P. putida VM1441*(pNAH7) against the phytotoxicity of naphthalene on pea plants. This toxicity experiment was based on the exposure of plants to sublethal doses of xenobiotics. To determine the lethal exposure dose of naphthalene towards pea plants, 4-week-old plants were exposed to 0, 10, 20, 50, 100, 200 and 500 mg L⁻¹ naphthalene in a hydroponic solution [plant nutrient solution (ISO International Organization for Standardization, 1997)]. Transpiration rates of the plants were measured by weighing the whole hydroponic system at regular intervals to determine water loss due to transpiration. Treatments were carried out in triplicate and the experiments were repeated twice. Transpiration rates were monitored over a 4-day period, and were normalized using the following equation (Trapp et al., 2000):

\[
RT = \frac{\sum(T, (t, c))/\sum(T, (0, c))/n}{\sum(T, (0, c))/\sum(T, (0, 0))/m}
\]

where *T* is the transpiration rate, *c* is the concentration, *t* is the time, *n* is the number of replicates and *m* is the number of controls.

To determine whether the naphthalene-degrading endophytic strain *P. putida VM1441*(pNAH7) conveyed protection against naphthalene, 4-week-old, inoculated plants were exposed to sublethal concentrations 0, 10, 20 and 40 mg L⁻¹ naphthalene. For the purpose of comparison, plants inoculated with the parent strain, *P. putida VM1441*, and with the pNAH7 donor strain, *P. putida G7*, were also exposed to these sublethal concentrations of naphthalene. Uninoculated plants received the same treatment and acted as controls. Treatments were carried out in triplicate and the experiments were repeated twice.

Phytoremediation microcosm experiments

Soil was amended with a known concentration of naphthalene (250 mg kg⁻¹ soil). One hundred and fifty grams of this soil was planted with 3-week-old *P. putida VM1441*(pNAH7)-colonized plants (P–I). Three controls were set up: one set of pots with 150 g amended soil planted, but with no inoculum (P–NI), one set of pots with 150 g amended soil with no plants but inoculated with *P. putida VM1441*(pNAH7) at 10⁷ CFU g⁻¹ soil (NP–I) and one set of pots with neither plants nor inoculum (NP–NI). Each of these treatments was carried out in triplicate and the experiment was repeated twice. The pots were left for 2 weeks, and then the soil samples were analysed by HPLC to determine the level of naphthalene remaining. Soil samples were thoroughly mixed before sampling. Three soil samples were taken per pot and analysed individually. Five-millilitre HPLC-grade acetonitrile was added to 2 g soil samples and vortexed vigorously for 5 min. The samples were then centrifuged for 5 min at 20 000 g the supernatant was carefully removed and centrifuged once more. The supernatant was then filtered through a 0.22-μm filter before HPLC analysis. Extractions were carried out in triplicate for each soil treatment. Concentrations of naphthalene were quantitatively analysed by HPLC using the EPA method 610 with modification. This modification involved running the HPLC with isocratic solvent instead of a gradient. The column consisted of a Synergi 4-μm CI2 reverse-phase RP-Max column (250 × 4.6 mm ID: Phenomenex, UK). Mobile phase was acetonitrile–water–phosphate buffer (20 mM, pH 7.1) (80 : 20 : 0.1), the flow rate was 1 mL min⁻¹ and detection was by UV absorbance at 254 nm. A retention time of 5.85 ± 0.03 min was observed under these conditions for naphthalene. A calibration curve of peak area vs. analyte standard concentrations was prepared.

Colonization dynamics of *P. putida POPHV9* in pea plants

To examine the plant colonization patterns of this endophytic strain, the gfp-labelled version *P. putida VM1453* (*P. putida* PopHV 9 with a miniTn5 Km²/gfp insert) was inoculated onto pea seeds. After 4 weeks, plants were harvested, sectioned and examined under an epifluorescent microscope (Germaine et al., 2006).

Results

Construction of an endophyte with the potential to metabolize naphthalene

*Pseudomonas putida* VM1441, a genetically tagged version of the endophytic strain *P. putida* POPHV9, was mated with *P. putida* G7 carrying the pNAH7 plasmid. Successful
transfer of the NAH7 plasmid to VM1441 occurred at a rate of $1.0 \times 10^{-5}$ per recipient cell. The presence of the NAH7 plasmid in these transconjugants was confirmed by PCR (data not shown). The ability of these transconjugants to utilize naphthalene as the sole carbon source was assessed and compared with both the original pNAH7 donor ($P. \text{putida } G7$), which can utilize naphthalene as the sole carbon, and the parental strain $P. \text{putida } VM1441$, which cannot. In growth curve experiments, the generation time ($g$) was 48 min for G7 and 60 min for the VM1441(pNAH7) transconjugant.

Inoculation of peas with the naphthalene endophyte degrader shows phytoprotective effects

Under clean soil conditions (no added naphthalene), both uninoculated and $P. \text{putida } VM1441$(pNAH7)-inoculated seeds displayed identical germination capacities (Table 1). There was also an identical reduction in the germination capacity of both inoculated and uninoculated seeds in soil containing 10 mg kg$^{-1}$ naphthalene. However, at concentrations of 30–100 mg kg$^{-1}$ soil, there was a significant difference in the germination of uninoculated and $P. \text{putida } VM1441$(pNAH7)-inoculated seeds. A higher percentage (60% and 30%, respectively) of inoculated seeds germinated in soil containing 30 and 50 mg kg$^{-1}$ than did uninoculated seeds, and these differences proved to be significant ($P = 0.002$ and 0.024, respectively). In soil containing 100 mg kg$^{-1}$ naphthalene, no uninoculated seeds germinated, while 20% of $P. \text{putida } VM1441$(pNAH7)-inoculated seeds germinated at this concentration, and this also proved to be significant ($P = 0.04$). At 200 mg kg$^{-1}$, no seeds germinated regardless of whether they were inoculated with $P. \text{putida } VM1441$(pNAH7) or not. These results demonstrated that the presence of $P. \text{putida } VM1441$(pNAH7) protected plants from the phytotoxic effects of naphthalene.

Table 1. Percentage pea seed germination under various soil naphthalene concentrations

<table>
<thead>
<tr>
<th>Naphthalene treatment (mg kg$^{-1}$ soil)</th>
<th>Uninoculated seeds</th>
<th>$P. \text{putida } VM1441$(pNAH7)-inoculated seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>0</td>
<td>94</td>
<td>0.50</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>0.57</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>0.81</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>0.57</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Statistically significant ($\alpha = 0.05$).

Hydroponics-based naphthalene phytoprotection by inoculated pea plants

To assess the toxicity of naphthalene on pea plants, 4-week-old plants were exposed to 0, 10, 20, 50, 100, 200 and 500 mg L$^{-1}$ naphthalene in a hydroponic solution. Exposure to naphthalene concentrations $> 200$ mg L$^{-1}$ were lethal to plants, with death occurring just after 1 week from the initial exposure time. At naphthalene concentrations of 100 mg L$^{-1}$, plants displayed signs of distress, such as wilting, chlorosis and leaf loss. At concentrations between 20% and 50 mg L$^{-1}$, plants did not display any visible signs of distress.

The effect of naphthalene concentration on the transpiration of pea plants can be seen in Fig. 1a. Naphthalene proved to have a negative effect on the transpiration of pea plants at all the concentrations tested, with increasing reductions in transpiration coinciding with increasing concentrations of naphthalene. At naphthalene concentrations between 10 and 100 mg L$^{-1}$, transpiration rates were moderately affected, dropping to between 12 and 26% of that of the control plant, which did not receive naphthalene.

Figure 1b shows the normalized transpiration rates of inoculated plants exposed to naphthalene after 120 h of

![Hydroponics-based naphthalene phytoprotection by inoculated pea plants](image-url)
expression. Lower transpiration rates correlated to higher phytotoxicity. ANOVA analysis of the data between the inoculated plants and controls showed that there was a significant difference in plant transpiration rates at naphthalene levels of 10, 20 and 40 mg L\(^{-1}\) (\(P = 0.04, 0.03\) and 0.03, respectively). When plants that were inoculated with the endophytic naphthalene degrader strain \(P.\ putida\) VM1441(pNAH7) were exposed to various naphthalene levels, the normalized transpiration rates were 22%, 34% and 35% higher than those of the un inoculated control plants for exposure levels of 10, 20 and 40 mg L\(^{-1}\) naphthalene, respectively. These normalized transpiration rates were significantly higher (\(\alpha = 0.05\)) than those observed with uninoculated, \(P.\ putida\) VM1441- or with \(P.\ putida\) G7-inoculated plants. There was no significant difference between the normalized transpiration rates between plants inoculated with \(P.\ putida\) VM1441 and \(P.\ putida\) G7. The inoculation of pea plants with the endophytic strain possessing the naphthalene degradation genes [\(P.\ putida\) VM1441(pNAH7)] significantly protected these plants from the toxic effects of naphthalene exposure. This protective ability probably stems from the fact that this strain can efficiently utilize naphthalene as a growth substrate and from its inherent ability to maintain large population sizes in the rhizosphere and root tissues (\(10^5–10^6\) and \(10^3\) CFU g\(^{-1}\), respectively), thus leading to a high naphthalene-degrading potential, whereas for \(P.\ putida\) G7, low cell counts were recovered from the rhizosphere and root tissues of inoculated plants (typically of the order of \(10^2–10^4\) and \(10^3\) CFU g\(^{-1}\), respectively), which explains why this strain did not offer a significant phytoprotective effect.

Two weeks after the initial naphthalene exposure, there was a pronounced effect on the biomass of uninoculated plants. The biomass of uninoculated plants exposed to 10, 20 and 40 mg L\(^{-1}\) naphthalene were 4, 20 and 27%, respectively, less than that of the control plants (0 mg L\(^{-1}\) naphthalene). In the case of \(P.\ putida\) VM1441(pNAH7)-inoculated plants, plants receiving 10, 20 and 40 mg L\(^{-1}\) naphthalene had 7%, 6% and 3%, respectively, more biomass than that of the control plants (Table 2). It appears that this plant growth promotion ability is maintained even in the presence of naphthalene and may even be stimulated by low levels of naphthalene. ANOVA analysis of these results showed that the biomass of inoculated plants was significantly greater than those of the uninoculated plants receiving 10, 20 and 40 mg L\(^{-1}\) naphthalene treatments.

**Pea plants inoculated with \(P.\ putida\) VM1441 (pNAH7) show an improved capacity to remove naphthalene from soil microcosms**

Two weeks after planting, triplicate soil samples were taken from each of the pots and analysed individually. Figure 2 shows the level of naphthalene remaining in the pots of the four treatments. Analysis of soil samples taken from the original amended soil, kept at 4 °C and analysed 2 weeks later, showed naphthalene levels between 220 and 280 mg kg\(^{-1}\) soil. Statistical analysis of the data using ANOVA showed that there was a significant difference between the treatments (\(\alpha = 0.05\)). In the control experiment with no treatment [no plants–no inoculum (NP–NI)] and held under the same conditions as the planted soil, the naphthalene levels had reduced to 59–83 mg kg\(^{-1}\) soil. This constituted a 70% reduction in the level of naphthalene present in the soil, and this loss is thought to be due to volatilization of the naphthalene from the soil.

In soil that had been planted with uninoculated plants (P–NI), there had been a further reduction in naphthalene levels by up to 19%. However, there was not always a significant difference between this treatment and that of the control (NP–NI) (\(P = 0.06\)). This reduction may have been brought about by sorption of the naphthalene to the roots, enhanced degradation through the promotion of indigenous naphthalene degraders or perhaps even by plant metabolism. There was a significant difference in the naphthalene levels remaining in the pots containing the inoculum alone (NP–I) compared with the control pots (\(P = 0.0007\)). On average, there was 50% less naphthalene remaining in the

![Fig. 2. Naphthalene concentrations remaining in the soil 2 weeks after planting. P–I, soil with inoculated plants; P–NI, soil with uninoculated plants; NP–I, soil with inoculum only; and NP–NI, soil with neither plants nor inoculum.](https://example.com/fec230.png)
pots containing the inoculum alone (NP–I) compared with the control pots. *Pseudomonas putida* VM1441(pNAH7) cell counts were found of the order of $10^5$–$10^6$ CFU g$^{-1}$ soil in the inoculated soil. This high survival rate in soil probably explains why it was capable of removing significant amounts of naphthalene.

Soil containing plants that were inoculated with *P. putida* VM1441(pNAH7) underwent the most dramatic decrease in naphthalene levels. There was a statistically significant difference between this treatment and each of the other three treatments ($\alpha = 0.05$). Compared with the control soil (NP–NI), 68% more naphthalene was removed from the soil ($P = 0.0003$); compared with plants alone, there was a 40% increase in naphthalene removal ($P = 0.0003$); and compared with inoculation alone, 37% more naphthalene was removed ($P = 0.00005$).

Within inoculated plants, the population size of *P. putida* VM1441(pNAH7) in the rhizoplane was of the order of $10^6$ CFU g$^{-1}$, and inside the root tissues, the populations were of the order of $10^4$ CFU g$^{-1}$ (Table 3). However, in the bulk soil VM1441(pNAH7) cell counts were of the order of $10^2$–$10^3$ CFU g$^{-1}$. Total numbers of naphthalene degrader cells were slightly higher than these figures, indicating that there were some indigenous strains present that could also utilize naphthalene or that gene transfer of the pNAH7 plasmid occurred between the inoculated strain and the indigenous microbial population. No *P. putida* VM1441(pNAH7) cells were recovered from uninoculated plants (rhizoplane or root). However, once again, there were low numbers of indigenous strains isolated that could also utilize naphthalene.

The inoculation of the gfp-expressing derivative strain *P. putida* VM1453 showed that this strain resided in extensive biofilms and colonies along the rhizoplane (Fig. 3a–c). Cells were also visualized in the root cortex and near the vascular tissues (Fig. 3d).

<table>
<thead>
<tr>
<th>Table 3. Populations sizes of inoculated strains within pea plant tissues</th>
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<tbody>
<tr>
<td><strong>P. putida VM1441(pNAH7)</strong> Uninoculated plants <strong>P. putida VM1453</strong></td>
</tr>
<tr>
<td>Rhizoplane</td>
</tr>
<tr>
<td>Km' (CFU g$^{-1}$)</td>
</tr>
<tr>
<td>5.4 x 10^6</td>
</tr>
<tr>
<td>2.2 x 10^4</td>
</tr>
<tr>
<td>Root</td>
</tr>
<tr>
<td>Km' (CFU g$^{-1}$)</td>
</tr>
<tr>
<td>1.4 x 10^5</td>
</tr>
<tr>
<td>1.2 x 10^2</td>
</tr>
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</table>

ND, not detectable.

**Fig. 3.** *Pseudomonas putida* VM1453 residing on the rhizoplane of inoculated pea plants. (a, b) Biofilms and colonies of *P. putida* VM1453 residing on the rhizoplane (x 1000). (c) Epidermal intercellular colonization pattern by *P. putida* VM1453 (x 1000). (d) *Pseudomonas putida* VM1453 cells within the root cortex of inoculated pea plants (x 1000).
Discussion

Germination of pea seeds proved to be sensitive to the presence of naphthalene. This is in agreement with Henner et al. (1999), who studied the effects of PAHs on plant germination. They found that seed germination was strongly inhibited by low-molecular-weight, water-soluble compounds such as toluene, styrene and naphthalene. High-molecular-weight compounds (> 3 rings) did not affect germination. This was also reported by Smith et al. (2005), who found that aged PAH-contaminated soil did not have any adverse effects on plant germination, because aged PAHs are unlikely to contain any significant amounts of volatile low-molecular-weight PAHs such as naphthalene. The presence of the plant-colonizing, naphthalene degrader strain P. putida VM1441(pNAH7) protected the seeds from the phytotoxic effects of naphthalene. Kuiper et al. (2001) reported a very similar effect when they inoculated grass seeds (Lolium multiflorum) with the rhizosphere-colonizing, naphthalene-degrading Pseudomonas strain PCL1444 and planted the seeds in soil containing 3 mg naphthalene per 15 g soil (equivalent to 200 mg kg\(^{-1}\) soil). None of the uninoculated seeds produced above ground growth, while Pseudomonas strain PCL1444-inoculated plants produced above ground growth averaging 6 cm. The presence of plant-colonizing, naphthalene-degrading bacteria had the ability to protect their host plant against the toxic effects of naphthalene exposure.

Fully developed pea plants were tolerant to higher concentrations of naphthalene, as seen in the hydroponic toxicity experiments. Naphthalene concentrations \(> 200 \text{ mg L}^{-1}\) were lethal to plants. At concentrations \(< 50 \text{ mg L}^{-1}\), plants showed a reduction in transpiration rates but no visible signs of toxicity. The reduction in transpiration rates were of the order of 10–40% and were dose dependent. Thygesen & Trapp (2002) carried out similar experiments using willow (Salix alba). They subjected hydroponically grown willow treelets to three different PAHs, naphthalene, phenanthrene and benzo(a)pyrene. The results showed that neither phenanthrene nor benzo(a)pyrene had any significant effect on the growth or transpiration rates of the exposed plants. However, they found that exposure to high doses of naphthalene (325 mg L\(^{-1}\)) were fatal to willow trees. Hulzebos et al. (1993) reported naphthalene lethal dose 50% values of 100 mg L\(^{-1}\) for Lactuca sativa. Thygesen & Trapp (2002) also found that exposure to lower doses of naphthalene had significant effects both on growth and on transpiration rates of willow. Interestingly, when they combined naphthalene with phenanthrene or benzo(a)pyrene, plant sensitivity increased to a level greater than that of phenanthrene, benzo(a)pyrene or naphthalene alone. They also found that naphthalene doses of between 10 and 25 mg L\(^{-1}\) led to reductions of up to 10% in growth and 30–50% reduction in transpiration rates in willow trees. In the present study, naphthalene doses of 10–40 mg L\(^{-1}\) led to a reduction in growth of up to 17%, and a 10–40% reduction in transpiration rates on pea plants.

The presence of the endophytic strain P. putida VM1441 (naphthalene nondegrader) did not display any protective effects, and the inoculated plants experienced similar toxic effects as the uninoculated control plants. Although this strain does possess a plant growth promotion ability, this did not appear to have protected the host plant against the toxic effects of naphthalene. It appears that in order to have a phytoprotection effect the inoculum must possess the ability to degrade naphthalene. Plants inoculated with the naphthalene-degrading strain P. putida VM1441(pNAH7) (a nonendophytic, naphthalene degrader) did experience less toxic effects from naphthalene exposure compared with control plants. This strain did not colonize the rhizosphere of pea plants particularly well nor did it seem to be capable of being a root endophyte (resulting in low cell numbers), which may explain why the strain did not have better phytoprotection ability. Plants that were inoculated with the naphthalene-degrading endophytic strain P. putida VM1441(pNAH7) and were exposed to sublethal concentrations of naphthalene experienced significantly less toxicity compared with uninoculated plants both in terms of plant growth and in terms of water uptake (transpiration). We conclude that in order for a strain to demonstrate significant phytoprotection ability, it must have both the xenobiotic-degrading ability and the capacity to develop large population sizes on and within the host plant.

Uninoculated pea plants grown in naphthalene-amended soil were capable of increasing the rate of naphthalene removal compared with unplanted soil, although this was not always significant. Qiu et al. (1994) assessed the removal of naphthalene in the presence of prairie buffalo grass (Buchloe dactyloides) in a field test. They found that the rate of naphthalene removal was greater in the presence of plants. Olson et al. (2008) also found that planting was the most effective treatment for increased PAH dissipation (with nearly 80% reduction in total PAHs) after 14 months. In the present study, inoculation of naphthalene-amended soil with P. putida VM1441(pNAH7) showed that the inoculum removed more naphthalene than the plants alone. Therefore, in this study, the presence of a naphthalene degrader inoculum was more important than the presence of a plant in the removal of naphthalene. Kuiper et al. (2001) inoculated the naphthalene degrader Pseudomonas strain PCL1444 directly into naphthalene-contaminated soil, but they did not see any significant amount of degradation. Pseudomonas putida VM1441(pNAH7) is capable of surviving in bulk soil in considerably high numbers, which probably accounts for its ability to remove significant amounts of naphthalene. The highest removal of
naphthalene was seen when *P. putida* VM1441(pNAH7)-inoculated plants were grown in the naphthalene-amended soil. Kuiper *et al.* (2001) carried out a similar experiment where plants were inoculated with the naphthalene degrader *Pseudomonas* strain PCLI444 and sown in naphthalene-contaminated soil. After 5 days, they found a 100-fold decrease in the amount of naphthalene present in this soil compared with the control soil. In the present study, *P. putida* VM1441(pNAH7)-inoculated plants were capable of removing 68% more naphthalene than in the untreated soil.

A genetically tagged (gfp, kmR) version of *P. putida* VM1441 designated strain VM1453 was used to visualize and track the colonization patterns of this strain in the phytosphere of inoculated pea plants. Epifluorescent microscopy revealed that *P. putida* VM1453 resided in biofilms on the rhizoplane and, in particular, within the intercellular grooves between the epidermal cells of the root. Within the root tissues, microcolonies were observed near the root cortex. It is concluded that these biofilms and microcolonies were responsible for the degradation of naphthalene when the inoculated plants were grown in the naphthalene-amended soil.

Previously, we have described the use of endophytes to enhance the removal of the herbicide 2,4-dichlorophenoxyacetic acid from contaminated soil (Germaine *et al.*, 2006), and endophytes have been shown to protect plants from the phytotoxic effects of toluene and to reduce toluene volatilization (Barac *et al.*, 2004). This work further demonstrates the potential of endophytic bacteria for improving phytoremediation.

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