Bacterial endophyte-enhanced phytoremediation of the organochlorine herbicide 2,4-dichlorophenoxyacetic acid

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Abstract

2,4-Dichlorophenoxyacetic acid is a selective systemic herbicide for the control of broad-leaved weeds, which is widely used throughout the world. The persistence of its residues and its potential to migrate in the soil make it necessary to reduce its concentrations in contaminated soil and groundwater. The nature of this compound makes it particularly toxic to the broad-leaved plants, such as the poplar (Populus) and willow (Salix), which are often used in phytoremediation projects. We describe the inoculation of a model plant, the pea (Pisum sativum), with a genetically tagged bacterial endophyte that naturally possesses the ability to degrade 2,4-dichlorophenoxyacetic acid. The results showed that this strain actively colonized inoculated plants internally (and in the rhizosphere). Inoculated plants showed a higher capacity for 2,4-dichlorophenoxyacetic acid removal from soil and showed no 2,4-dichlorophenoxyacetic acid accumulation in their aerial tissues. This demonstrates the usefulness of bacterial endophytes to enhance the phytoremediation of herbicide-contaminated substrates and reduce levels of toxic herbicide residues in crop plants.

Introduction

The organochlorine compound 2,4-dichlorophenoxyacetic acid (2,4-D) is used as a selective systemic herbicide for the control of annual and perennial broad-leaved weeds. Its salts are readily absorbed by plant roots, and are translocated to the meristematic tissues of the roots and shoots (Tomlin, 1994). There 2,4-D accumulates and acts as a plant hormone, causing uncontrolled growth in the meristematic tissues. Within these tissues, it inhibits DNA and protein synthesis and thereby prevents normal plant growth and development. It is one of the most commonly used herbicides in the world (Copping, 2002; Watkins, 2002), with 13 000–15 000 tonnes being used domestically and commercially in 2001 in the USA alone (Kiely et al., 2004). Maximum application rates range from 0.335 to 2.0 lb acre⁻¹ (300–1750 mg kg⁻¹ soil), depending on the crop being treated (http://www.24d.org). The mobility of 2,4-D in soil frequently results in the contamination of not only the soil where it is applied but also nontarget surface waters and groundwater. Hence, contamination levels are often exceed the maximum permitted levels (70 p.p.b. in ground water), as set by the US Environmental Protection Agency (EPA) (Kiely et al., 2004). 2,4-D has been detected in groundwater supplies in at least five states in the USA and Canada, and low concentrations have been detected in surface waters throughout the USA (Howard, 1991; US EPA, 1992). With rising public concern regarding the presence of this potentially health-threatening chemical in the environment, there is a particular need to reduce its concentrations in soil and water supplies.

Once applied to the soil, 2,4-D is usually degraded within a few days, through a combination of abiotic and biological mechanisms. However, there are reports of high levels of 2,4-D residues (0.62 mg kg⁻¹ or 2% of the applied 2,4-D) remaining in treated soil for extended periods of time (Kamrin, 1997; Boivin et al., 2005) and observations of phytotoxic effects on crops grown in soil 9 months after 2,4-D application (Crafts & Robbins, 1962). Although 2,4-D is readily biodegradable, it may persist because microorganisms with the required catabolic capacity are not present or because their populations are not large or active enough (Dejonhe et al., 2000).

Phytoremediation is the use of plants to remove toxic chemicals from air, soil or water. It is predicted that by 2010 it will account for 10–15% of all remediation projects (Glick, 2003). The use of plants to remediate recalcitrant chemicals in the soil represents a low-cost alternative when contrasted with expensive, and often destructive, mechanical methods (Sung et al., 2003). However, to realize the true potential of
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this technology, it is necessary for plants to grow to their full capacity in the presence of high levels of contaminants. Plants remove these xenobiocides through one or more biologically active processes, such as phytoaccumulation, enzymatic transformation or degradation, or phytovolatilization. Varying levels of success for the remediation of organic pollutants have been reported (Rubin & Ramaswami, 2001; Susarla et al., 2002; Rasmussen & Olsen, 2004).

One of the major problems with 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 (Glick, 2003). This toxicity can be reduced using microorganism-assisted phytoremediation, through the use of rhizosphere-colonizing, pollutant-degrading bacteria or plant growth-promoting rhizobacteria. Shaw & Burns (2004) showed that 2,4-D pollutant-degrading bacteria or plant growth-promoting to remediation, through the use of rhizosphere-colonizing, pollutant-degrading bacteria or plant growth-promoting rhizobacteria. Shaw & Burns (2004) showed that 2,4-D mineralization was enhanced in the rhizosphere of Trifolium, due to the increased population sizes of 2,4-D degraders within the rhizosphere. The absence of a population of degrader microorganisms can be overcome by the inoculation of the plant rhizosphere with pollutant-degrading strains. This approach has had much success in reducing the levels of benzene, ethylene, toluene xylene, polyaromatic hydrocarbons, polychlorinated biphenyls and pesticides in polluted environments (Collins et al., 2002; Chekol et al., 2004; Johnson et al., 2004; Kulper et al., 2004; Sun et al., 2004). However, a reduction in plant biomass and the presence of phytotoxic effects have been reported even with these inocula (Pfender, 1996; Barac et al., 2004).

Another, more novel, plant–microorganism interaction, which has shown much potential in enhancing phytoremediation, is that of plants and their bacterial endophytes. These bacteria are defined as those that colonize the internal tissues of plants without causing disease (Sessitsch et al., 2002). Siciliano et al. (2001) showed that plants grown in soil contaminated with xenobiocides naturally recruited endophytes with the necessary contaminant-degrading genes. Lodewyckx et al. (2001) showed that endophytes of yellow lupine were able to increase the nickel accumulation and tolerance of inoculated plants. More recently, Barac et al. (2004) inoculated lupin with a bacterial endophyte that carried the pTOM plasmid encoding for toluene degradation. They showed that inoculated plants had the ability to significantly reduce the phytovolatilization of toluene (by 50–70%) and reduced the phytotoxic effect of toluene on inoculated plants.

This study examined an endophytic bacterium from poplar trees, which naturally possess the ability to degrade 2,4-D. A genetically tagged version of this strain was inoculated into pea, before exposing these plants to various levels of 2,4-D, and examining them for signs of 2,4-D toxicity and 2,4-D translocation to the plants’ aerial tissues.

Materials and methods

Materials

2,4-Dichlorophenoxyacetic acid (98%) was purchased from Sigma-Aldrich (St. Louis, MO). Strain VM1450 is a chromosomally labelled (mini-Tn5 insertion of gfp::Km<sup>R</sup>) derivative of Pseudomonas putida strain POPHV6, an endophytic isolate from the stem sap of poplar trees (Populus trichocarpa × deltoides cv. Hoogvorst) (Germaine et al., 2004). POPHV6 was found to degrade 27 µM<sup>-1</sup> 2,4-D in culture flasks supplemented with 1 mM 2,4-D. This strain was maintained on Luria–Bertani (LB) agar (Merck, Darmstadt, Germany) (Miller, 1972) supplemented with 100 µg mL<sup>-1</sup> kanamycin.

Pea seeds (Pisum sativum var. Early Onward, Suttons Seeds, Torbay, UK) were selected because of the high growth rate and xenobiotic-sensitive nature of this plant.

Plant inoculation and application of 2,4-D

P. putida VM1450 was grown in LB broth containing 100 µg mL<sup>-1</sup> kanamycin, at 30 °C, and shaken at 200 r.p.m., to an approximate absorbance (A<sub>600 nm</sub>) value of 1.0. Cells from 3 mL of culture were harvested by centrifugation, washed twice in 0.85% sterile saline and resuspended in a final volume of 3 mL of saline. This inoculum contained between 10<sup>9</sup> and 10<sup>6</sup> cells mL<sup>-1</sup> as determined by standard plate counts.

Twenty microliters of the inocula were pipetted onto the surface of rehydrated pea seeds. The seeds were allowed to dry for 30 min before planting. Five pots containing 150 g of sterile soil were set up in duplicate. The soil was collected from the campus grounds at Carlow, classified as a sandy loam soil (60% sand, 33% clay and 7% silt), and autoclaved twice for 2 h at 121 °C before use in experiments. Five inoculated seeds were then planted in each pot. The plants were allowed to develop for 4 weeks at 20–25 °C under a 16 h light/8 h dark regime and watered twice a week with 60 mL of modified Plant Nutrient Solution (PNS) ISO 8692 (ISO, 1997). In week 5, duplicate pots received 60 mL of PNS containing 0, 1, 2, 4 or 8 mg L<sup>-1</sup> 2,4-D. In week 6, the respective pots received 60 mL of PNS containing 0, 10, 20, 40 or 80 mg L<sup>-1</sup> 2,4-D. Finally, in week 7, the pots received 60 mL of PNS containing 0, 100, 200, 400 or 800 mg L<sup>-1</sup> 2,4-D. These 2,4-D concentrations corresponded to approximate totals of 0, 7, 13, 27 and 54 mg of 2,4-D (this was equivalent to 0, 47, 87, 180 and 360 mg kg<sup>-1</sup> soil) applied to respective pots over the course of the three weeks. Noninoculated seeds received identical treatment and served as controls. Four days after the final application of 2,4-D, the plants were harvested and analysed for 2,4-D accumulation and signs of phytotoxic effects of 2,4-D exposure.
Determination of biomass and chlorophyll levels

Four days after plants received the final 2,4-D dose, they were removed from their pots and the soil was removed from the roots. The cumulative weight of the five plants from each pot was determined by weighing. Chlorophyll analysis was performed by the method of Huang et al. (2004). Briefly, 1 g samples of the leaves were removed from each plant, and the chlorophyll was extracted by grinding each sample in a pestle and mortar containing 100 mL of 80% acetone solution. The mixture was then filtered, transferred to a 100 mL volumetric flask and made up to the mark with 80% acetone solution. The solution was mixed well before reading the absorbance at both 645 and 663 nm. Chlorophyll levels were calculated (based on fresh weight) using the following equations (Huang et al., 2004):

\[
\text{Chlorophyll } a \ (\text{mg g}^{-1} \text{ tissue}) = (12.7 \times A_{663} \text{ nm}) - (2.69 \times A_{645} \text{ nm}) \\
\text{Chlorophyll } b \ (\text{mg g}^{-1} \text{ tissue}) = (22.9 \times A_{645} \text{ nm}) - (4.68 \times A_{663} \text{ nm})
\]

Analysis of leaves from each treatment was carried out in triplicate.

2.4-D extraction and high-performance liquid chromatography analysis

2.4-D extraction from soil

Two millilitres of high-performance liquid chromatography (HPLC)-grade methanol were added to 1 g soil samples and vortexed vigorously for 5 min. The samples were then centrifuged for 5 min at 23,428 g, and the supernatant was carefully removed and centrifuged once more. The supernatant was then filtered through a 0.22 μm filter prior to HPLC analysis. Spiked samples of soil were kept refrigerated for 7 days prior to analysis, to determine the recovery rate. Analysis of soils from each pot was determined by weighing. Chlorophyll levels were calculated (based on fresh weight) using the following equations (Huang et al., 2004):

\[
\text{Chlorophyll } a \ (\text{mg g}^{-1} \text{ tissue}) = (12.7 \times A_{663} \text{ nm}) - (2.69 \times A_{645} \text{ nm}) \\
\text{Chlorophyll } b \ (\text{mg g}^{-1} \text{ tissue}) = (22.9 \times A_{645} \text{ nm}) - (4.68 \times A_{663} \text{ nm})
\]

Analysis of leaves from each treatment was carried out in triplicate.

2,4-D extraction from plant material

Five grams of plant stem material were ground in 20 mL of 20 mM phosphate salt buffer (PBS) (0.55 g L\(^{-1}\) NaH\(_2\)PO\(_4\), 2.85 g L\(^{-1}\) Na\(_2\)HPO\(_4\), 9.0 g L\(^{-1}\) NaCl, pH 4.8) using a pestle and mortar. The samples were then vortexed vigorously for 5 min before centrifuging at 20,000 g for 15 min. The supernatant was carefully removed and passed through a C\(_{18}\) Sep-Pak\(^{TM}\) cartridge (Waters, Dublin, Ireland) that had been previously activated by passing through 4 mL of methanol. The cartridge was washed with 5 mL of 20 mM PBS and the 2,4-D was eluted from the cartridge with 6 mL of methanol at a flow rate of 1 mL min\(^{-1}\). The methanol was evaporated to dryness, and the residue was resuspended in 2 mL of methanol and filtered through a 0.22 μm filter prior to HPLC analysis. To determine the rate of 2,4-D recovery, 5 g of plant material was spiked with 10 mg of 2,4-D, refrigerated for 7 days and treated as described above.

HPLC

This was carried out on a C\(_{18}\) polar end-capped column (250 × 4.6 mm: Phenomenex, Macclesfield UK), with a mobile phase consisting of methanol–water–acetic acid (60:40:1), at a flow rate of 1 mL min\(^{-1}\). Detection was by UV at 280 nm. A calibration curve was constructed using the integrator values obtained from the quantification of standard solutions. Twenty microlitres of standards or prepared samples were injected onto the column using a Rheodine injector system.

Enumeration of Pseudomonas putida VM1450 within plant tissues

Plants were destructively sampled 4 days after the final addition of 2,4-D. Samples of leaf/stem, root and rhizosphere soil were taken from each plant. For surface sterilization of the root and stem/leaf tissue, a sodium hypochloride solution (1% active chloride + 200 μL L\(^{-1}\) Tween 20) was used. Leaves and stems were surface sterilized by submerging them in the sterilizing solution for 5 min. They were then rinsed three times in sterile water. Roots were surface sterilized by placing them into the sterilizing solution for 5 min, and then rinsed three times in sterile water. This procedure was carried out twice on each root sample. To check for sterility, surface-sterilized tissues were pressed against a plate count agar (PCA) plate (Merck), and samples of the third rinsings were plated onto PCA. One gram of the surface-sterilized tissues was homogenized using a sterile pestle and mortar, and serially diluted in 0.85% sterile saline, and 100-μL samples were spread plated onto PCA containing 100 μg mL\(^{-1}\) kanamycin or 2,4-D plates (solid minimal media + 1 mM 2,4-D). Rhizosphere samples were serially diluted and plated in the same manner. Plates were incubated at 30 °C and examined for growth after 72 h. The number of CFUs g\(^{-1}\) of fresh tissue was calculated. Sampling from each compartment and each treatment was carried out in triplicate.

In planta visualization of Pseudomonas putida VM1450 using epifluorescence microscopy

Hand-cut sections of the root or surface-sterilized stem and root tissues were stained with a 0.1% acridine orange solution for 1 min. The sections were then examined under blue light (395 nm) using a Nikon E400 epifluorescence microscope equipped with a 100-W mercury short-arc photo-optic lamp and a Fluorescein isothiocyanate (FITC)
filter. Lucia® imaging software (version 4.6) was used to capture and process microscopic images.

**Results**

**Biomass and chlorophyll levels**

After 3 weeks of exposure to 2,4-D, the biomass of plants was measured (Fig. 1). Noninoculated plants exposed to 2,4-D appeared smaller than the unexposed control plants. Reductions in biomass appeared to correspond with increasing concentrations of applied 2,4-D. However, ANOVA analysis of the results showed that the applied 2,4-D did not result in an overall significant reduction in biomass in either noninoculated or inoculated plants (Table 1). Plants inoculated with *Pseudomonas putida* VM1450 showed little or no reduction in size at any of the levels of 2,4-D applied. Moreover, ANOVA results showed that the presence of the inoculum did have an overall positive effect on plant biomass that was statistically significant.

Noninoculated control plants [receiving no 2,4-D treatment (0 mg)] showed total chlorophyll levels of 18.7 ± 2 mg g⁻¹ leaf tissue. *P. putida* VM1450-inoculated control plants [receiving no 2,4-D treatment (0 mg)] showed total chlorophyll levels of 16.7 ± 0.5 mg g⁻¹ leaf tissue. This reduction in chlorophyll level due to the inoculum alone was noted in all inoculated plants exposed to 0, 7 and 13 mg of 2,4-D, but these reductions did not prove to be significantly lower (*P < 0.05*) than those experienced by noninoculated plants. It can be seen from Figs 2(a) and (b) that levels of both chlorophyll *a* and chlorophyll *b* decreased with corresponding increases in the levels of 2,4-D. ANOVA analysis of the results showed that the addition of 2,4-D led to statistically significant reductions in both chlorophyll *a* and chlorophyll *b* levels in both uninoculated and inoculated plants (Table 1). Plants inoculated with *P. putida* VM1450 that were exposed to 27 and 54 mg of 2,4-D did have higher levels of chlorophyll *a* and chlorophyll *b* when compared to noninoculated plants exposed to the same levels of 2,4-D. However, these differences were only statistically significant for chlorophyll *a* at 27 mg of 2,4-D and for chlorophyll *b* at 54 mg of 2,4-D. ANOVA analysis of the results showed that there was not an overall significant difference in the chlorophyll reductions between inoculated and noninoculated plants (Table 2).

![Fig. 1. Biomass of control and inoculated plants after exposure to 2,4-dichlorophenoxyacetic acid.](image1)

![Fig. 2. Effect of 2,4-dichlorophenoxyacetic acid levels on (a) the chlorophyll *a* levels and (b) the chlorophyll *b* levels of pea plants.](image2)

<table>
<thead>
<tr>
<th></th>
<th>Chlorophyll <em>a</em></th>
<th>Chlorophyll <em>b</em></th>
<th>Plant biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated plants</td>
<td>0.00008*</td>
<td>0.00003*</td>
<td>0.49925</td>
</tr>
<tr>
<td>Inoculated plants</td>
<td>0.00037*</td>
<td>0.0006*</td>
<td>0.54164</td>
</tr>
<tr>
<td>Total</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001*</td>
<td>0.956</td>
</tr>
</tbody>
</table>

*Indicates value is statistically significant.
The phytotoxic effects of 2,4-D on the roots of non-inoculated plants were clearly visible (Figs 3a and b). These 2,4-D-exposed plants developed enlarged root tips and thickening of the secondary roots. This occurred in all the noninoculated plants that received 2,4-D treatment. The severity of the condition corresponded to the level of applied 2,4-D. This condition was not observed in the roots of inoculated plants (Fig. 3c), with the exception of minor calluses that had developed on some of the roots of plants that had received the highest level of 2,4-D (54 mg).

Inoculated plants did not accumulate 2,4-D

The rates of recovery of 2,4-D from spiked soil and plant tissue (after 7 days) were determined to be 100 ± 10% and 98 ± 5%, respectively. The retention time for 2,4-D under the HPLC conditions described was 16.87 min. Analysis of plant stem/leaf tissue showed 2,4-D accumulation only in the noninoculated plants (Fig. 4a). These plants showed accumulation of 2,4-D corresponding to the increased levels of applied 2,4-D and accounted for 24–35% of the total 2,4-D applied to the pots. Plants inoculated with *P. putida* VM1450 did not show any accumulation of 2,4-D within their aerial tissue at any level of applied 2,4-D.

After plants were removed from the pots, soil samples were analysed to determine the quantity of 2,4-D that remained in the soil. The results showed high levels of 2,4-D remaining in soil that held the noninoculated plants (Fig. 4b). These levels increased with the levels of added 2,4-D and accounted for 10–20% of the total 2,4-D applied to the pots. This quantity of 2,4-D, along with the levels recovered from the stem tissues, accounted for 34–55% of the total 2,4-D applied to the pots. The remaining 45–66% is thought to have accumulated in the roots of the noninoculated plants. Although the 2,4-D levels in the roots of plants were not determined, Fig. 3 clearly shows that 2,4-D caused severe damage to the root systems, indicating that 2,4-D did accumulate in the roots. This accumulation is not thought to have occurred in *P. putida* VM1450-inoculated plant roots, as there were few or no visible signs of toxic effects on their root systems. No 2,4-D was detected in the soil of inoculated plants exposed to either 7 or 13 mg of 2,4-D. However, 2,4-D was extracted from the soil where either 27 or 54 mg of 2,4-D had been applied. These levels corresponded to both 1.8% and 7.04%, respectively, of the total amount of 2,4-D applied.

**Enumeration of *Pseudomonas putida* VM1450 within plant tissues**

Enumeration of *P. putida* VM1450 cells in the various tissues of inoculated pea plants showed that it efficiently colonized inoculated plants (Fig. 5). Population sizes in the rhizosphere increased from an order of 10⁵ with no selective pressure, to between 10⁶ and 10⁷ when 13–54 mg of 2,4-D was added. The same pattern was seen in the roots where numbers increased with increasing levels of 2,4-D, with the exception of the highest level applied (54 mg), where numbers fell slightly. The most noticeable change in population size was in the stem, where no detectable levels were obtained in inoculated plants that had received no 2,4-D (0 mg). With the application of 7 mg of 2,4-D, the

<table>
<thead>
<tr>
<th>2,4-D level (mg)</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
<th>Plant biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.504</td>
<td>0.190</td>
<td>0.385</td>
</tr>
<tr>
<td>7</td>
<td>0.849</td>
<td>0.054</td>
<td>0.801</td>
</tr>
<tr>
<td>13</td>
<td>0.394</td>
<td>0.133</td>
<td>0.450</td>
</tr>
<tr>
<td>27</td>
<td>0.032        *</td>
<td>0.088</td>
<td>0.046        *</td>
</tr>
<tr>
<td>54</td>
<td>0.071</td>
<td>0.033        *</td>
<td>0.041        *</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0.356</td>
<td>0.236</td>
<td>0.0320        *</td>
</tr>
</tbody>
</table>

*Indicates value is statistically significant.
2,4-D, 2,4-dichlorophenoxyacetic acid.
The population of *P. putida* VM1450 in the stem grew by two orders of magnitude. Doubling the concentration from 27 to 54 mg led to a doubling of the numbers of *P. putida* VM1450 in the stem and leaf tissue. 2,4-D-degrading bacteria other than *P. putida* VM1450 were detected in the pots, and these represented 0–0.01% of the *P. putida* VM1450 population. The source of these extra 2,4-D-degrading bacteria is thought to be the indigenous plant endophytic microbial populations that are carried within the pea seeds. Also, while the soil was initially sterilized prior to use, it was not maintained under sterile conditions during the experiment, and therefore airborne contamination cannot be ruled out.

No *gfp:KmR* bacteria were recovered from any of the samples from noninoculated plants. However, analysis of these pots showed that there was a small population of 2,4-D degraders present in the rhizosphere of the 2,4-D-exposed plants. These populations ranged from $1.80 \times 10^1$ to $1.27 \times 10^2$ g$^{-1}$ of rhizosphere soil, and were not thought to be responsible for any significant degradation of the applied 2,4-D.

**In planta visualization of *Pseudomonas putida* VM1450**

*Pseudomonas putida* VM1450 cells could be visualized residing on the rhizoplane of all inoculated plants. The number of cells that were visualized increased with the increase in the 2,4-D levels applied. Many microcolonies and biofilms could be seen located on the rhizoplane (Figs 6a and b). *Pseudomonas putida* VM1450 cells could also be seen inside the root and stems, residing as discrete microcolonies (Fig. 6c and d) or inhabiting the intercellular spaces of these tissues.

**Discussion**

Pea seeds were inoculated with a genetically labelled (*gfp:KmR*) version of an endophyte (*Pseudomonas putida* POPHV6) that had been previously isolated from the interior stem tissue of poplar. Inoculated plants were cultured for 4 weeks before being subjected to 0, 7, 13, 27 or 54 mg of 2,4-D over the course of the following 3 weeks. Noninoculated controls were treated identically. Four days after the final application of 2,4-D, plants were sacrificed and analysed for signs of phytotoxicity and accumulation of 2,4-D.

Applications of 2,4-D did not result in reductions in plant biomass that were statistically significant. In inoculated plants, biomass actually increased from between 1.5% and 16% compared to that of noninoculated controls that had not been treated with 2,4-D. ANOVA analysis showed that the presence of the inoculum led to statistically significant increases in plant biomass. This increase in biomass correlated with increasing populations of *P. putida* VM1450 within the plant tissues and suggests that this strain may have growth-promotion properties. Another possible explanation for this increased biomass is that trace levels of 2,4-D or its metabolites stimulated plant growth. De Araujo et al. (2002) found that low levels of chlorinated phenols actually stimulated the growth of root tissue cultures.

Maintaining the chlorophyll content of leaves is fundamental for the photosynthetic activity of plants and is often used as an indicator of stress in plants (Huang et al., 2004).
Results from the chlorophyll analysis showed that applications of 2,4-D led to statistically significant reductions in chlorophyll $a$ and $b$ levels. This effect on chlorophyll has been reported for other environmental pollutants. Kummerova & Kmentova (2004) found that increasing levels of fluoranthene decreased the chlorophyll content of tomato plants by up to 40%. Our findings showed that chlorophyll $b$ was particularly sensitive to 2,4-D, with reductions of up to 57%.

Even without the addition of 2,4-D (0 mg), the inoculation of $P$. putida VM1450 into pea plants reduced plant chlorophyll levels by c. 5–18%. These plants showed no other ill-effects from the inoculum. The VM1450 strain has never shown any adverse effects on other plant species (Germaine et al., 2004). This apparent reduction in the chlorophyll levels of inoculated plants may be due to a decrease in the dry weight/wet weight ratio brought about by an increase in water uptake by the plants. This may have had a dilution effect on the chlorophyll levels per gram of tissue (wet weight) and would also account for the increases in the biomass levels that were recorded. Although the presence of $P$. putida VM1450 did significantly help to maintain chlorophyll levels in some cases, the overall effect in all the pots was not statistically significant.

The root systems of noninoculated plants showed severe callus formation and root thickening even at the lowest level of 2,4-D tested. The presence of the inoculum had a profound protection effect on the root system of inoculated plants. These plants did display very minor toxicity symptoms but only at the highest level of 2,4-D tested (54 mg). The ability of plants to maintain biomass is critical for phytoremediation. Maintaining a healthy root system and photosynthetic activity allows an increase in water uptake through the roots and production of root exudates, which in turn results in a greater accumulation/uptake of pollutants both in the rhizosphere (where increased microbial activity can degrade the pollutant) and within the plant (where plant enzymes and endophytic populations can act on it). Our results indicate that $P$. putida VM1450 helped inoculated plants to maintain growth and a healthy root system in the presence of high levels of 2,4-D.

Accumulation of high levels of 2,4-D was observed within the stem/leaf tissue of 2,4-D-treated noninoculated plants.

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Fig. 6. (a) *Pseudomonas putida* VM1450 biofilm within the rhizosphere of inoculated plants exposed to 54 mg of 2,4-dichlorophenoxyacetic acid (2,4-D); (b) *Pseudomonas putida* VM1450 cells in the rhizosphere of inoculated plants exposed to 27 mg of 2,4-D; (c) *Pseudomonas putida* VM1450 microcolony within the root of inoculated plants exposed to 54 mg of 2,4-D; (d) microcolony of *P. putida* VM1450 within the stem of plants exposed to 27 mg of 2,4-D.
This accumulation increased with corresponding increases in the level of applied 2,4-D. Accumulation in the aerial tissue was expected, as 2,4-D is a weak acid with $K_a = 2.83$ (Dubus et al., 2001), allowing it to be readily transported to the leaves via the transpiration stream. Pea plants inoculated with *P. putida* VM1450 did not show any accumulation of 2,4-D in the stem/leaf tissue. The fact that the roots displayed few or no symptoms of 2,4-D toxicity suggests that *P. putida* VM1450 populations in the rhizosphere and/or inside the root were partly responsible for the degradation of the 2,4-D. The pronounced reduction in the chlorophyll levels of the leaf tissues indicates that considerable levels of 2,4-D were translocated to the aerial parts of the plants. The significant increase in the population of *P. putida* VM1450 and the inability to detect any 2,4-D within the stem/leaf tissues suggest that the VM1450 cells residing in these tissues degraded the translocated 2,4-D. The degradation of 2,4-D in the plant tissues by *P. putida* VM1450 is advantageous because it prevents the entry of 2,4-D into the food chain, thereby eliminating any toxic effects on herbivorous fauna residing on or near contaminated sites.

Analysis of the soil showed that substantial levels of 2,4-D remained in the soil after the removal of noninoculated pea plants. In soil that had *P. putida* VM1450-inoculated plants, no remaining 2,4-D was detected in the pots that had received 7 or 13 mg of 2,4-D. At the two highest levels of applied 2,4-D (27 and 57 mg) there was still 2,4-D remaining in the soil of inoculated plants. This accounted for 1.8% and 7.0%, respectively, of the total 2,4-D level applied to these pots. These levels were significantly less than those in the soil of noninoculated plants. The fact that these high levels of 2,4-D remained in the soil can probably be explained by the plants being sampled 4 days after the final application (and highest dose) of 2,4-D. It is therefore likely that there was simply insufficient time for plant uptake of all of the 2,4-D. Even in the presence of higher 2,4-D-degrading inoculum populations, the uptake of 2,4-D by the plant is still the rate-limiting factor in 2,4-D removal from soil. The inoculated plants were much healthier than the noninoculated plants and would have a greater capacity to completely remove the remaining levels of 2,4-D than noninoculated plants, if the experiment was allowed to run longer.

The population dynamics of *P. putida* VM1450 were monitored within the various tissues of inoculated plants. The results showed that the presence of selective pressure, i.e. the introduction of 2,4-D, had a positive effect on *P. putida* VM1450 populations within the rhizosphere, root and aerial tissues. This selective pressure effect was also reported by Jacobsen (1997), who inoculated barley seeds with a *Burkholderia* strain that could degrade 2,4-D. Without any selective pressure, this strain showed weak rhizosphere colonization ability, but when 10 or 100 mg kg$^{-1}$ 2,4-D was added, the population grew to $10^7$ cm$^{-1}$ root. However, in their study the inoculated plants still developed small stubby root systems when exposed to 2,4-D. Shaw & Burns (2004) also found that the addition of 2,4-D to soil caused an increase in the numbers of 2,4-D degraders that were naturally present in the rhizosphere of *Trifolium pratense*. In our experiment, the most dramatic population change was in the stem and leaves, where initially numbers were undetectable. With the addition of 2,4-D, this population increased with the increasing levels of applied 2,4-D.

Epifluorescence microscopy revealed that *P. putida* VM1450 resided in large biofilms on the rhizoplane and in microcolonies within the root and stem tissues. These cells showed normal cell morphology, suggesting that they did not experience nutrient deficiency as observed in some rhizosphere colonizers (Li et al., 1999). It is proposed that these biofilms and microcolonies were responsible for the degradation of the 2,4-D within the rhizosphere, root and stem/leaf tissues.

The inoculation of pea plants with an endophytic strain possessing the ability to degrade 2,4-D protected this broad-leaved plant from some of the toxic effects of high levels of the herbicide. This allowed inoculated plants to increase their biomass, and hence increased the plant uptake of 2,4-D from the soil. Once within the plant tissues, the 2,4-D was quickly degraded by the root- and stem-colonizing endophytic biofilms and microcolonies of *P. putida* VM1450. In conclusion, this strain allowed plants to maintain their growth and increase xenobiotic removal through a high level of colonization and competence within the plant.

It is envisaged that the phytoremediation of other more toxic or recalcitrant herbicides could be enhanced in a similar fashion as described here, through the introduction of mobile genetic elements carrying the catabolic pathway (e.g. the pADP plasmid encoding for atrazine degradation) similar fashion as described here, through the introduction of mobile genetic elements carrying the catabolic pathway (e.g. the pADP plasmid encoding for atrazine degradation) into this strain. The feasibility of this method has been shown for improving the phytoremediation of nonherbicide contaminants such as toluene (Barac et al., 2004). With phytoremediation playing an ever-increasing role in the clean-up of contaminated land and water, we believe that endophytes will play a central role in enhancing both the range of contaminants that can be phytoremediated and the rate of their degradation.

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