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REGULAR ARTICLE

Simultaneous assessment of the effects of an herbicide on the triad: rhizobacterial community, an herbicide degrading soil bacterium and their plant host

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Abstract
Aims This work addresses the relevant effects that one single compound, used as model herbicide, provokes on the activity/survival of a suitable herbicide degrading model bacterium and on a plant that hosts this bacterium and its bacterial rhizospheric community.
Methods The effects of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), on *Acacia caven* hosting the 2,4-D degrading bacterium *Cupriavidus pinatubonensis* JMP134, and its rhizospheric microbiota, were simultaneously addressed in plant soil microcosms, and followed by culture dependent and independent procedures, herbicide removal tests, bioprotection assays and use of encapsulated bacterial cells.
Results The herbicide provokes deleterious effects on the plant, which are significantly diminished by the presence of the plant associated *C. pinatubonensis*, especially with encapsulated cells. This improvement correlated with increased 2,4-D degradation rates. The herbicide significantly changes the structure of the *A. caven* bacterial rhizospheric community; and it also diminishes the preference of *C. pinatubonensis* for the *A. caven* rhizosphere compared with the surrounding bulk soil.
Conclusions The addition of an herbicide to soil triggers a complex, although more or less predictable, suite of effects on rhizobacterial communities, herbicide degrading bacteria and their plant hosts that should be taken into account in fundamental studies and design of bio(phyto)remediation procedures.

Keywords 2,4-D herbicide · *Acacia caven* · Bioremediation · *Cupriavidus pinatubonensis* JMP134 · Plant soil microcosms · Rhizosphere

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Introduction

The rhizosphere, the layer of soil influenced by plant roots represents a highly dynamic space for interactions between plants and soil microorganisms (Bais et al. 2006, 2008). Abiotic factors (pH, temperature, nutrient levels, water availability, among others) and biotic (plant pathogens, microbial competence,

meiofauna, among others) exert different effects on plants and their associated microbiota (Boyer 1982; Kloepper and Schroth MN 1988; Glick 1995; Lazarovits and Nowak 1997; Bertin et al. 2003; Lindow and Brandl 2003; Dong and Kahmann 2009). One of the relevant abiotic factors affecting plant soil ecosystems is the use of man-made herbicides. For obvious reasons, studies have mainly focused on the effect of herbicides on target and non-target plants (see for reviews Duke 1990; Sterling and Hall 1997; Grossmann 2000; Zheng and Hall 2001; Ninomiya et al. 2004; Grossmann 2010). A lot of attention has been also directed to microorganisms, mainly bacteria, that are able to degrade/mineralize herbicides, especially aromatic compounds (Jacobsen 1997; Aislabie et al. 2005; Pérez-Pantoja et al. 2008; Sørensen et al. 2009; Pérez-Pantoja et al. 2010; Zhang et al. 2011), and studies addressing both the fate of herbicides in plant soil ecosystems and potential for bioremediation (Newby et al. 2000; Germaine et al. 2006; Mastretta et al. 2006). A few studies addressing effects in bulk soil and rhizospheric microbiota are also available (Jofré et al. 1996; Smit et al. 1996; Top et al. 1998; Shaw and Burns 2004; 2005; Macur et al. 2007). The analysis of the relevant literature indicates that specific effects for individual herbicides are observed, however, some general conclusions can be drawn. As examples, organophosphate herbicides are more easily degraded than organochlorinated ones; aerobic bacteria are more versatile and efficient than the anaerobic ones, and that ideal target specificity, i.e. the herbicide only affects the target plants leaving untouched the non target ones, is rarely attained (Sterling and Hall 1997; Grossmann 2000; Zheng and Hall 2001). Despite the extensive literature available, several relevant issues still remain relatively ignored. Among them, do microbial communities in the rhizosphere respond to herbicides in a different way than in bulk soil? Does an exogenously added herbicide degrader behave the same in these two microenvironments? One rarely utilized approach is the simultaneous study of the effects of an herbicide in the triad occurring in planted soils exposed to a man-made compound, i.e. the plant, the associated rhizospheric bacterial community and the herbicide degrading bacteria. Therefore, this work addressed the effects that one single model herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), provokes on an appropriate herbicide degrading model bacterium (*Cupriavidus pinatubonensis*, ex. *C. necator* JMP134), and on a plant that hosts this bacterium and its bacterial rhizospheric community. To get a better knowledge of the effects of the herbicide on the association between colonizing bacteria and the plant, they were studied simultaneously in the same plant soil microcosm system. Briefly, results indicate that the herbicide provokes deleterious effects on the plant, but these negative effects are significantly diminished by the presence of the plant inhabiting *C. pinatubonensis*; the herbicide also produces significant changes in the structure of the bacterial rhizospheric community; and it also profoundly affects the preference of *C. pinatubonensis* for the *A. caven* rhizosphere compared with the surrounding bulk soil.

Materials and methods

Bacterial strains and culture

Cupriavidus pinatubonensis JMP134 (pJP4) (DSM 4058) was obtained from the DSMZ (Braunschweig, Germany), and maintained in a minimal medium containing 2 mM 2,4-D as the sole source of carbon and energy. Double crossover deletion mutant derivative strains of *C. pinatubonensis*, *cheY* and *tfdA* were routinely maintained in minimal medium supplemented with 10 mM fructose as a carbon source plus 50 $\mu g ml^{-1}$ kanamycin. *Escherichia coli* DH5α (pJP4) was obtained after biparental mating with *C. pinatubonensis* JMP134 as donor (Manzano et al. 2007) and maintained in Luria Bertani (LB) medium plus 15 $\mu g ml^{-1}$ nalidixic acid and 0.5 $mg ml^{-1}$ of mercury dibromofluorescein disodium salt.

Green fluorescence protein labelling

C. pinatubonensis JMP134 cells were tagged with the green fluorescence protein (gfp) marker gene by using the mini-Tn*5* system, which forms stable genomic insertions (Mathysse et al. 1996). Wild-type strain JMP134 was conjugated with *E. coli* strain PRK2073, as helper, and strain S17 containing the plasmid with mini-Tn5GFP construct carrying the tetracycline resistance. Transconjugants carrying the gfp marker were selected on 2 mM 2,4-D minimal medium containing 10 $\mu g ml^{-1}$ of tetracycline. Colonies of the gfp tagged strain were examined by optical fluorescence microscopy with a Nikon Labophot 2 model microscope, equipped with a UV light source (100 W HBO Fluorescence lamp housing) and a B-2A fluorescent filter.

Generation of *C. pinatubonensis cheY* and *tfdA* mutants

The *C. pinatubonensis tfdA* gene encoded in plasmid pJP4 was inactivated by double recombination allelic exchange transferring the plasmid to *E. coli* BW25113, as described previously (Trefault et al. 2009). Briefly, PCR primer pairs TFDA-for and TFDA-rev, which contain 37- and 39-bp homology extensions of the *tfdA* gene sequence, respectively, and 20-bp priming sequences for plasmid pKD4 (Datsenko and Wanner 2000), were synthesized. These primer pairs were used with pKD4 as the template to amplify the kanamycin-resistance gene flanked by the corresponding homology extensions of the *tfdA* gene sequence. The following PCR program was used: 95 °C for 5 min, 28 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, and 72 °C for 10 min. The resulting PCR product was used to inactivate the *tfdA* gene in an *E. coli* BW25113 (pJP4) strain harbouring the RecBCD recombinase, according to a previously described procedure (Trefault et al. 2009). pJP4 derivatives containing the inactivated *tfdA* gene were transferred to strain JMP134 by biparental mating. The primer pair TFDA-for and TFDA-rev was used to verify the correct recombinational insertion of the kanamycin resistance cassette in place of the *tfdA* gene. This was confirmed by direct sequencing of the region using the same primer pairs. The *C. pinatubonensis cheY* gene was inactivated by the procedure described by Louie et al. (2002). A 219-bp internal fragment of the *cheY* gene was amplified from JMP134 DNA by using primer pair Chey2For 5'-CTCAAGGAACTGGGCTTCAA-3' and CheY2Rev 5'-CGCAATGATGTTTTCCTTG-3'. The PCR product was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA). Final plasmid DNA (5 to 10 μg) was electroporated into electrocompetent JMP134 cells prepared as described by Louie et al. (2002). To verify interruption of the *cheY* gene sequence, PCR amplification and direct sequencing were carried out using primer pairs CheY1For 5'-GTGGACAAGAACATCAAGATCC-3' / M13Rev and M13For / CheY1Rev 5'-TCAGCCGCC CAGTTTCTC-3'.

Plant-soil microcosms

Plant-soil microcosms were prepared with two soils. A loam soil (EDI) 60.5 % sand, 7.5 % clay and 32 % silt, and a silt loam soil (UAI) 8 % sand, 18 % clay and 74 % silt). Plant soil microcosms were set with germinated *A. caven* seedlings. In order to remove the external seed layer, germination was performed by subjecting *A. caven* seeds to chemical scarification by soaking them in concentrated sulphuric acid for 2 h. Then, after five vigorous washings with sterile distilled water, treated seeds were placed in Petri dishes with wet filter paper during 3 days to allow the early stages of germination. Two types of plant soil microcosms were assembled for each experimental condition: the first contained one germinated seed sown in a plastic flowerpot holder containing 180 g of EDI soil per pot, and the second contained one germinated seed sown in a plastic flowerpot holder containing 500 g of UAI soil per pot. Different amounts of soil were used because the UAI soil microcosms were incubated for longer times, although these conditions were not informative and thus are not reported here. In any case the soil directly exposed to the surface was similar. Control soil microcosms without *A. caven* were prepared in each case. They were set exactly the same way as planted soil microcosms except no germinated seed were sown on them. At least four microcosms were prepared for each condition and inoculum type, and were placed in a plant chamber at 23-25 °C with a light:dark period of 12:12 h. One to two hundred and fifty ml of sterile water were added once a week to keep the moisture of each soil to water holding capacity based on a described procedure (Kalra 1998).

Rhizosphere colonization

To establish the distribution of inoculated *C. pinatubonensis* JMP134 in rhizospheric and non-rhizospheric soil, fresh cultures of *C. pinatubonensis* JMP134 gfp were grown in 10 mM fructose at 30 °C and 180 rpm to an approximate optical density (OD_{600nm}) value of 0.8. Cells were harvested by centrifugation, washed twice in 0.85 % sterile saline minimal medium to remove any selective agents and finally resuspended in 100 ml of irrigation water. EDI soil microcosms incubated by 30 days were inoculated once with 50 ml of a solution containing 10^4 or 10^8 CFU ml^{-1}, to give 2.8×10^3 or 2.8×10^7 CFU g^{-1} of soil. The same protocol was used to establish the distribution of *C. pinatubonensis* JMP134 *cheY*. These experiments were carried out in six replicates. Samples of 0.2 g of rhizospheric soil (taken from soil

adhered to roots in planted soil microcosms) and non-rhizospheric soil (taken from unplanted soil microcosms) were taken with a spatula 3-4 weeks post-inoculation, then soil samples were resuspended in 1 ml of 0.85 % sterile saline and subjected to ultrasonic homogenization for 3 min. Supernatants were obtained by centrifugation at 800 rpm for 5 min, and 1/50 supernatants dilutions were plated in minimal medium containing 2 mM 2,4-D and 10 $\mu g ml^{-1}$ of tetracycline. $CFU g^{-1}$ of soil were determined after 24 h of incubation at 30 °C, and the gfp tag was followed by epifluorescence microscopy. To calculate the amount of $CFU g^{-1}$ of dry soil, the soil moisture was determined using a drying oven at 85 °C.

Microscopy analyses

Epifluorescence images were taken using Nikon Eclipse 50i microscope (Nikon, Japan) equipped with GFP HYQ and G-2E/C filters, and photographs were taken with a digital camera DS Fi1. Confocal microscope images were obtained using Olympus FluoView 1000 confocal laser scanning (Olympus, Japan) equipped with high performance sputtered filters.

2,4-D phytotoxicity tests

Plant soil (EDI and UAI) microcosms were used to assess the phytotoxicity of 2,4-D on *A. caven* and the ability of *C. pinatubonensis* JMP134 to prevent it. Thirty days old plant EDI soil microcosms were treated with 0 or 50 mg 2,4-D kg^{-1} soil added on the irrigation water, inoculated or not with 2.8×10^7 CFU g^{-1} of soil, of *C. pinatubonensis* JMP134 (pJP4), *E. coli* DH5α (pJP4) or *C. pinatubonensis* JMP134 (pJP4) *tfdA*. The percentages of surviving plants were measured 14 days after the herbicide/strain addition. Seventy days old plant UAI soil microcosms were treated with 0 or 200 mg 2,4-D kg^{-1} soil of 2,4-D added on the irrigation water, and inoculated or not with $1.1 \times 10^7 CFU g^{-1}$ of soil of *C. pinatubonensis* JMP134 as a liquid suspension (free cells), or in alginate beads. Alginate beads were prepared as reported by Young et al. (2006). The numbers of surviving plants were measured every 7 days through the following 35 days of incubation. All treatments were carried out in four replicates.

Determination of 2,4-D in soil

Residual 2,4-D in soil was determined by high-performance liquid chromatography (HPLC). Soils were extracted using one part by weight and ten parts of an 80 % methanol, 3 % acetic acid aqueous solution in a bead-beater Fast-Prep 24 (MP Biomedicals, OH, USA), during 60 s at minimal power, then centrifuged for 20 min at 16300*xg*, followed by filtration with Millex 0.22 um (Millipore, MA, USA). Samples (20 uL) from the filtrates were injected directly into a Hitachi LaChrom liquid chromatograph system equipped with a Kromasil 100–3.5 C18 (Bohus, Sweden). A methanol-H_2O (65:35) mixture containing 0.1 % (vol/vol) *ortho*-phosphoric acid was used as the solvent, at a flow rate of 1 $ml min^{-1}$. The operation temperature was 35 °C. The column effluent was monitored at 230 nm with a L-2455 diode array detector. Retention volume for 2,4-D was 5.4 ml. HPLC data were transformed to soil residual 2,4-D values, i.e., the percentage of remaining 2,4-D with respect to the initial dose added to soil.

Terminal restriction fragment length polymorphism analysis

Rhizospheric and non-rhizospheric EDI soil/microcosm samples were analysed by the terminal restriction fragment length polymorphism (T-RFLP) technique. UltraClean® soil DNA isolation kit (Mo Bio Laboratories Inc.) was used to extract the total community DNA from 1 g of rhizospheric and non-rhizospheric samples, according to the manufacturer´s instructions. DNA integrity and concentration was estimated by electrophoresis on 1 % agarose gels stained with SYBR safe (Invitrogen). Soil community DNA was used as template for PCR with the primers 8 F: 5'-AGA GTT TGA TCC TGG CTC AG- 3', labelled with the fluorochrome NED, and 1392R: 5'- ACG GGC GGT GTG TAC-3', designed for bacterial 16S rRNA gene sequences (Lane et al. 1985; Lane 1991). For each DNA sample, three 50 uL reactions were performed, containing 5 uL of 10x PCR buffer (200 $mmol L^{-1}$ Tris–HCl pH 8, 500 $mmol L^{-1}$ KCl), 3 $mmol L^{-1}$ $MgCl_2$, 0.2 umol L^{-1} of each primer, 0.2 $mmol L^{-1}$ dNTP, 0.2 $mg ml^{-1}$ bovine seroalbumine, c. 10–50 ng of soil DNA and 1 U of Taq polymerase. Reaction conditions were as follows: 94 °C for 5 min, 25 cycles at 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 2 min, and finally, 7 min at 72 °C. PCR products were

purified with Wizard PCR Preps DNA Purification System (Promega) and digested with *Hha*I or *Msp*I endonucleases (Promega). After desalting, the DNA fragments were separated by capillary electrophoresis using a Perkin- Elmer ABI Prism 310 (Foster City, CA, USA).

Statistical analysis

Two ways ANOVA was used to establish differences in CFU enumeration of rhizospheric soil versus non-rhizospheric soil for each plant soil microcosms treatment. Values of 2,4-D HPLC determinations were analysed by Kruskal-Wallis non-parametric test to establish differences between values of residual 2,4-D in soil microcosms. The T-RFLP profiles were compared by non-metric multidimensional scaling (NMDS) and analyses of similarity (ANOSIM) to examine the statistical significance of grouping as described elsewhere (Pavissich et al. 2010). The T-RF areas were analysed with the multivariate statistical software Primer 5 8 (Primer-E Ltd, Plymouth, UK).

Results

Effect of the herbicide 2,4-D on the bacterial component of plant-soil microcosms

To study the effect of the herbicide 2,4-D on the bacterial component of *A. caven* - EDI soil microcosms, we selected the culture independent molecular technique, T-RFLP, to compare the structures of the rhizospheric and non-rhizospheric bacterial community in the presence or absence of the herbicide. NMDS analyses of 4 weeks planted soil microcosms showed that the composition of the bacterial community of rhizospheric soil and non-rhizospheric soil not amended with 50 mgkg-1 2,4-D (triangles in Fig. 1), were highly different (R: 0.572; P: 0.01), but when 2,4-D was present the composition of bacterial communities of rhizospheric soil and non-rhizospheric soil (circles in Fig. 1), were not distinguishable (R: 0.038; P: 0.01). The comparison of the bacterial community structure of non-rhizospheric soils, amended or non-amended with 2,4-D, (open symbols in Fig. 1) indicated a similar structure (R: 0.149, P: 0.01), which was in contrast with the comparison of rhizospheric soil bacterial community structures, amended and non-amended with 2,4-D, (closed symbols) that showed a moderate difference (R: 0.437; P: 0.01). The comparison of the four groups (2,4-D amended and non amended rhizospheric and non-rhizospheric soils) indicated that bacterial community of rhizospheric soil not exposed to 2,4-D (closed triangles) was quite distinguishable from those of the other three soils (R: 0.633; P: 0.01). Therefore, the presence of 2,4-D changes the bacterial community structure of the rhizospheric soil in a way that shifted to a bacterial structure such as those found in non-rhizospheric soils irrespective if are in contact or not with 2,4-D.

Effect of the herbicide 2,4-D on the survival and distribution of the herbicide degrader in plant-soil microcosms

To test if the addition of 2,4-D provokes changes in the distribution of *C. pinatubonensis* JMP134, an

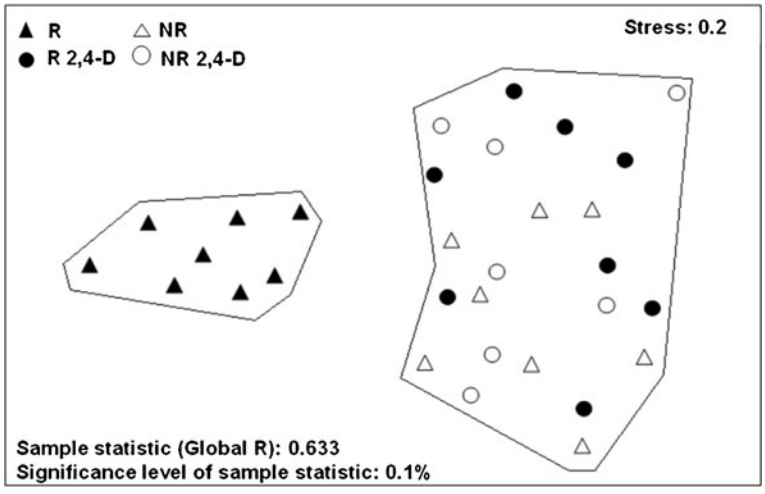

Fig. 1 Non-metric multidimensional scaling plot (NMDS) showing the grouping of non treated rhizospheric soil (**R**, *closed triangles*), 2,4-D treated rhizospheric soil (**R 2,4-D**, *closed circles*), non treated non-rhizospheric soil (**NR**, *open triangle*), and 2,4-D treated non-rhizospheric soil (**NR 2,4-D**, *open circles*) *Hha*I-T-RFLP profiles. R and P values correspond to analysis of similarity by 2,4-D and soil factor

efficient degrader of 2,4-D, in the soil compartments of these *A. caven* - EDI soil microcosms, we determined the CFU of this bacterium per g of dry soil. The results observed after 3 weeks and 4 weeks are essentially the same. Plant soil microcosms inoculated with 10^8 cells of *C. pinatubonensis* JMP134 per ml showed a mean abundance of 5.46±0.35 log CFU g^{-1} in rhizospheric soil and 4.15±0.55 log CFU g^{-1} in non-rhizospheric soil. These values indicated that *C. pinatubonensis* JMP134 shows a preference for rhizospheric soil versus non-rhizospheric soil, with a rhizosphere to soil (R/S) averaged ratio of 20.7±3.4. A quite similar distribution was determined when *C. pinatubonensis* JMP134 was inoculated at 10^4 cells per ml: its abundance after 4 weeks of incubation was 5.38±0.85 log CFU g^{-1} in rhizospheric soil and 4.04±0.27 log CFU g^{-1} in non-rhizospheric soil (averaged R/S ratio of 21.5±6.4). To test the effect of chemotaxis towards the herbicide 2,4-D, a gfp tagged *C. pinatubonensis cheY* mutant was obtained. The *C. pinatubonensis* (gfp) *cheY* behaved as the wild type followed by comparison of growth curves with fructose, benzoate, 2,4-D, or pyruvate (for clarity purposes, only the result with fructose is shown in Fig. 2), except that did not give positive for the standard chemotactic test against 2,4-D, carried out as described by de Weert et al. (2002). The distribution of the *C. pinatubonensis cheY* mutant was 5.08±0.44 log CFU g^{-1} in rhizospheric soil and 3.17±0.9 log CFU g^{-1} in non-rhizospheric soil (averaged R/S ratio of 40.0±9.3). In contrast, when the herbicide 2,4-D was added 5.22±0.12 log CFU g^{-1} and 5.16±0.21 log CFU g^{-1} of *C. pinatubonensis* JMP134, in rhizospheric and non-rhizospheric soil were observed, respectively, and the averaged R/S ratio fell down to 10.3±0.4. Essentially the same averaged R/S ratio (13.3±0.8) was observed for the *C. pinatubonensis cheY* mutant, although log CFU g^{-1} values were about one order of magnitude lower compared with the wild type strain. Therefore, in the presence of 2,4-D, *C. pinatubonensis* JMP134 apparently loses its preference for rhizospheric soil. The strong preference of this bacterium for the *A. caven* rhizosphere was supported by the detection of gfp-tagged *C. pinatubonensis* cells in close contact with the external and internal parts of the root surface of *A. caven* (Fig. 3).

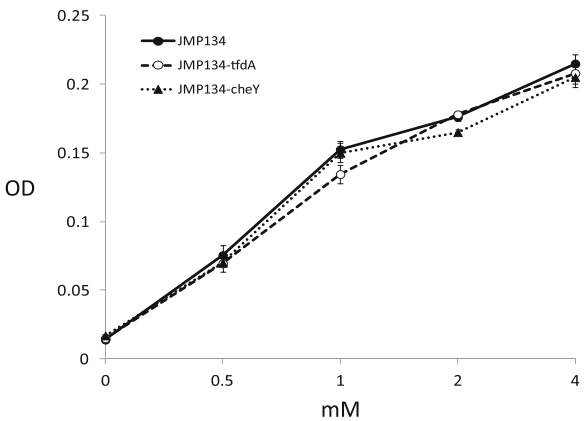

Fig. 2 Growth of *C. pinatubonensis* JMP134 and its mutant derivative strains on fructose as a sole carbon source. Liquid minimal saline medium cultures supplemented with different concentrations of fructose were inoculated with strain JMP134 tagged with gfp (●), or the gfp tagged mutant derivative strains *tfdA* (○) and *cheY* (▲). OD600 was registered at the stationary phase after 48 h cultivation. Values are means from three replicates and are representative for three different experiments

Effect of the herbicide 2,4-D on the plant component of plant-soil microcosms

To explore the effects of 2,4-D herbicide in the plant component of these *A. caven* – soil microcosms we selected two representative soils of foothills areas where *A. caven* is naturally found (EDI and UAI). Among other differences, these two soils have a distinct particle size composition, and the UAI soil allowed slower growth of plants, therefore, different times (30 vs. 70 days after sown) for herbicide addition/bacterial amendment had to be chosen. A 50-mg kg^{-1} dose of the herbicide killed 100 % of the exposed plants after 1 week of incubation in the EDI soil microcosms, whereas a higher dose (200 mgkg^{-1}) killed the totality of *A. caven* plantlets in the UAI soil microcosms (Fig. 4). The inoculation of *A. caven* - soil microcosms exposed to 2,4-D with 1.1-2.8×10^7CFU g^{-1} of soil of *C. pinatubonensis* JMP134 completely prevented plant death in EDI soil microcosms, and delayed but did not fully avoid plant death in UAI soil microcosms (Fig. 4). To study if protective effect depends on 2,4-D degradation carried by strain JMP134, a *C. pinatubonensis* (gfp) *tfdA* mutant was obtained. This mutant grew on fructose, pyruvate or benzoate as the wild type (for clarity purposes only growth on fructose is shown in Fig. 2), except it completely lost the ability to grow on 2,4-D. The protective effect of strain JMP134 is fully dependent of the ability

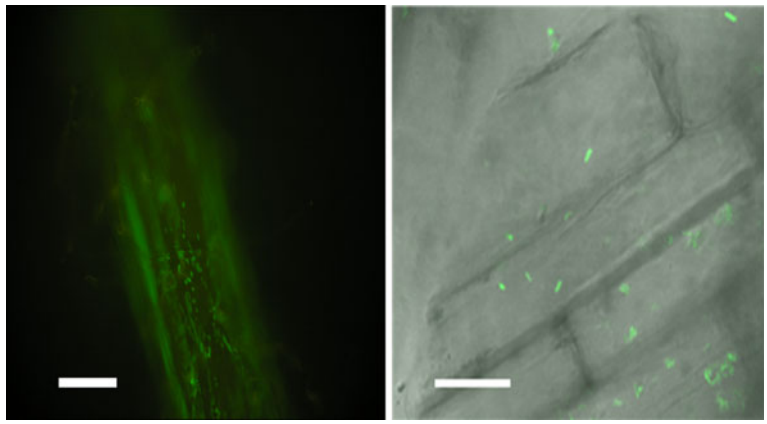

Fig. 3 Epifluorescence (left) and confocal (*right*) microscopy of *A. caven* lateral roots colonized by gfp-tagged *C. pinatubonensis* cells. (*Left*) Individual cells can be seen in the focus plane, directly on the surface of the root. Root hairs are faintly visible at both sides of the root body. The scale bar width represents 30 μM. (*Right*) Strain JMP134::gfp abundantly colonizing *A. caven* rhizodermal cells. Scale bars: 10 μm

of the inoculated strain to mineralize 2,4-D, since this *C. pinatubonensis tfdA* mutant did not protect *A. caven* from the lethal effect of the herbicide. Not only the presence, but also adequate *tfd* genes expression levels are required since an *Escherichia coli* (pJP4), a derivative that cannot efficiently express *tfd* genes (Newby et al. 2000), could not protect *A. caven* from the toxic effect of 2,4-D.

As the efficiency of protection by *C. pinatubonensis* was lower in the UAI soil microcosms, we tested the effect of inoculation with alginate encapsulated JMP134 cells. Trapping of cells in alginate beads

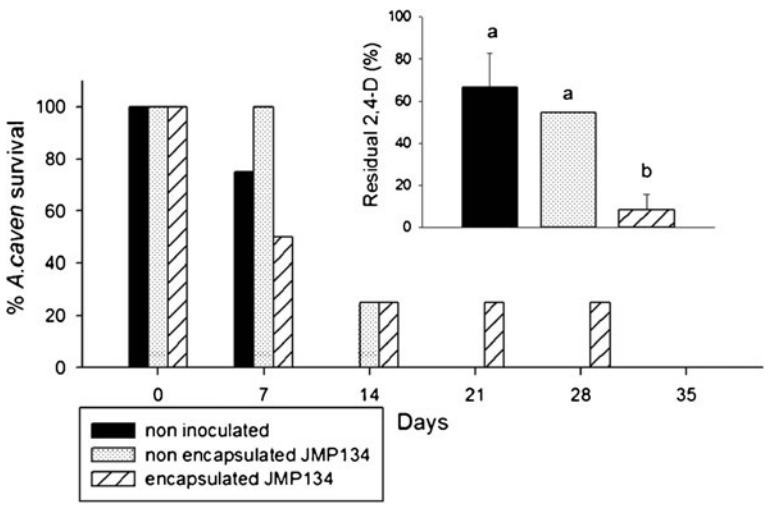

Fig. 4 Survival kinetics of *A. caven* exposed to lethal dose of 2,4-D.UAI plant soil microcosms were amended with a liquid suspension of *C. pinatubonensis* JMP134 at a concentration of 1.1×10^7CFUg^{-1} of soil, and with an encapsulated *C. pinatubonensis* JMP134 at a concentration of 1.1×10^7CFUg^{-1} of soil, or without the addition of the strain. Bars represent the survival of a total of four plant soil microcosms replicates. Standard deviations were lower than 5 %, so cannot be seen in bars. Insert: Percentage of the initial dose of 2,4-D in soil UAI microcosms at day 14 post inoculation (*insert*). 2,4-D contents in UAI plant soil microcosms were measured at day 14 after amendment with a liquid suspension of *C. pinatubonensis* JMP134 at a concentration of 1.1×10^7CFUg^{-1} of soil, with an encapsulated *C. pinatubonensis* JMP134 at a concentration of 1.1×10^7CFUg^{-1} of soil and without the addition of strain. Error bars represent standard deviation values. Kruskal-Wallis non-parametric test and a box and whisker plot were used to establish difference between treatments. Different letters mean treatments are significantly different at $P\leq0.05$

extended part of the protection to *A. caven* for at least 30 days (Fig. 4). The increased protection effect of alginate entrapped *C. pinatubonensis* cells was in agreement with the significantly lower 2,4-D residual levels found after 14 days of incubation in *A. caven* soil microcosms, in contrast with non inoculated and inoculated with non encapsulated cells (Fig. 4, insert). 2,4-D removal levels in soil followed a first order, exponential kinetics ($[2,4\text{-D}]=e^{-kt}$), with k values for non inoculated soils, soils inoculated with non encapsulated cells and encapsulated cells of 0.057 ($R^2=0.75$), 0.074 ($R^2=0.93$), and 0.219 ($R^2=0.84$) day^{-1}, respectively.

Discussion

We report that the addition of 2,4-D to *A. caven* – soil microcosms significantly alters the structure of the rhizospheric bacterial community, strongly decreases the clear preference of *C. pinatubonensis* JMP134 for *A. caven* rhizospheric soil, and completely kills *A. caven* plantlets, unless the microcosms is previously inoculated with strain JMP134. The protective effect requires the presence and adequate expression of *tfd* gene functions and it is enhanced when *C. pinatubonensis* cells are encapsulated.

A. caven is a leguminous shrub belonging to *Mimosaceae* family reported to be a host for beta proteobacteria, including *Burkholderiales* members closely related to *C. pinatubonensis* (Barrett and Parker 2006; Diouf et al. 2007; Hoque et al. 2011). This bacterium shows a strong preference for *A. caven* rhizospheric soil (this work). *A. caven* – *C. pinatubonensis* gnotobiotic agar cultures also show significant numbers ($>10^7$ cells g^{-1}) of strain JMP134 interacting with the *A. caven* rhizosphere, and ($>10^4$ cells g^{-1}) the endorhizospheric environment (Ledger et al. 2012). That work also shows that *C. pinatubonensis* establishes rhizospheric and endorhizospheric interactions with the plant model *Arabidopsis thaliana*.

The strong preference of *C. pinatubonensis* with the root environment may, in part, be explained by provision of carbon sources from root exudates (Uren 2007; Bais et al. 2006). *C. pinatubonensis*, as several other proteobacteria especially of the β-, and γ- classes, uses an impressively wide range of aromatic compounds (Pérez-Pantoja et al. 2008; Ussery et al. 2009; Pérez-Pantoja et al. 2010; 2012), which are quantitatively relevant components of plant exudates (Narasimhan et al. 2003; Bais et al. 2006). Interestingly, analysis of the genome of *C. pinatubonensis* (Lykidis et al. 2010), as well as growth tests indicate that this bacterium is completely unable to grow on common sugars and amino acids as carbon sources, indicating that this bacterium as well as several other close relatives (Pérez-Pantoja et al. 2012), are specialized for catabolism of the aromatic fraction of plant root exudates. In agreement, *C. pinatubonensis* grows on *A. caven* and *A. thaliana* root exudates requiring key aromatic catabolism genes (Ledger et al. 2012).

In the present study, we demonstrate that both proliferation and death of *C. pinatubonensis* is taking place in *A. caven* – soil microcosms. Rhizospheric soil $CFU\,g^{-1}$ levels attained after inoculation with 2.8×10^3 or $10^7\,CFU\,g^{-1}$ of soil are essentially the same, around $10^5\,CFU\,g^{-1}$ of soil, representing the *C. pinatubonensis* population equilibrium in this rhizospheric soil. Chemotaxis is suggested one of the first step for bacterial root colonization (van de Broek et al. 1998). Chemotaxis towards plant roots exudate components may be responsible for a displacement of inoculated cells from the non-rhizospheric soil to the rhizospheric soil. However, in this study we show that chemotaxis is not significantly contributing to the preferential distribution of *C. pinatubonensis* towards the rhizosphere, since a *cheY* mutant behaves essentially equal as the wild type strain. This mutant shows the lowest $CFU\,g^{-1}$ of soil levels in both compartments, indicating some general detriment in survival/proliferation abilities in the plant soil environment.

The presence of 2,4-D in these plant - soil microcosms levels the numbers of *C. pinatubonensis* in both the rhizospheric and non-rhizospheric soil, rising in one order of magnitude the $CFU\,g^{-1}$ of soil of this bacterium in the latter compartment. This strongly suggests that 2,4-D supports proliferation of the bacterium regardless if cells are in the root surrounding soil or in the non-rhizospheric soil. 2,4-D provides an obvious selective growth advantage for *C. pinatubonensis*, but does not rule out other effects in these plant soil microcosms. Among them, competence with other 2,4-D degraders, decrease of competing microorganisms, and alteration of plant root exudates composition after plant intoxication response. Some of these possibilities are discussed below.

Some of the above mentioned factors were explored through the comparison of the structures of

the soil bacterial communities, using the culture independent, molecular technique T-RFLP. The bacterial community structures of the rhizospheric and non-rhizospheric soil compartments differ significantly (Fig. 1), which reflect the gross influence that plant roots have on surrounding microorganisms (Lugtenberg et al. 1999; Bertin et al. 2003). Notably, 2,4-D provokes a strong effect in the rhizospheric community with a shift towards the structure of the non-rhizospheric soil (Fig. 1). One possible explanation is that 2,4-D increases the relative numbers of 2,4-D degraders in both compartments leading to a reduction of the differences in bacterial community structure. Results reported by Shaw and Burns (2004) indicate that the addition of 2,4-D to soil increases the numbers of 2,4-D degraders that were naturally present in the rhizosphere of *Trifolium pratense*. A 2,4-D doses dependent effect on soil microbial community and 2,4-D degraders has been also described (Macur et al. 2007). In addition, the relatively common presence of *tfdA* gene variants, the initial catabolic key gene for 2,4-D degradation, in β- and γ-*Proteobacteria* (Suwa et al. 1996), and in soil metagenomic DNA (Gazitúa et al. 2010) supports the idea that bacterial community members using 2,4-D as growth source are relatively abundant. In agreement, significant 2,4-D removal by soil endogenous microorganisms has been observed in several cases (this work, insert in Fig. 4, Top et al. 1998; Manzano et al. 2007). Another explanation for changes that 2,4-D provokes in the bacterial community structure would be the response to toxicity of 2,4-D and its catabolic intermediates. Among them, 2,4-dichlorophenol seems to be the more relevant as it is easily formed during 2,4-D degradation (Fukumori and Hausinger 1993; Ledger et al. 2006) and detoxification strategies based on rapid turnover of this and other 2,4-D intermediates are clearly required to avoid intoxication (Pérez-Pantoja et al. 2003; Ledger et al. 2006). Again, 2,4-D degradative abilities would be determinant in changing relative abundances of relevant members of the rhizospheric bacterial community.

An additional explanation is that 2,4-D causes significant effects in the plant. *A. caven* responses to the presence of the herbicide may produce local changes in the roots environment (Ninomiya et al. 2004) that changes the amount and composition of plant root exudates and/or the inhibition of bacterial attachment to the cell surface (Jofré et al. 1996). For example, if herbicide toxicity reduces plant root exudation, this could decrease the advantage for bacteria to be associated with the plant reducing the differences between the rhizospheric and non-rhizospheric bacterial communities.

To explore the effect of 2,4-D on *A. caven*, we selected two different soils (EDI and UAI) and determined the lethal dose of 2,4-D, which turned to be different for each case. These soils were taken from representative places in Central Mediterranean Chile, where this common shrub is normally found (Ovalle et al. 2006). 2,4-D is used as a selective systemic herbicide for the control of annual and perennial broad-leaved weeds, including members of the *Mimosaceae* family as *A. caven*. 2,4-D mimics auxins, and at low concentrations induces cell division and elongation while at higher concentrations has inhibitory effects on growth and development (Sterling and Hall 1997; Grossmann 2000; Zheng and Hall 2001). The phytotoxic effect of 2,4-D includes epinasty, leaf abscission, and abnormal elongation of root and aerial structures leading to senescence (Grossmann 2000). Accordingly, *A. caven* soil microcosms exposed to 50–400 mg kg^{-1} of 2,4-D (levels which are 0.5 - 4 times higher than those used in agronomic activities), clearly show detrimental effect on plants, including death. The effects on *A. caven* are fully or partially prevented by *C. pinatubonensis*, depending of the soil used and the presence and full expression of the key *tfd* genes (see below). Plant protection using 2,4-D degraders has been used previously. *Burkholderia cepacia* DBO1 (pRO101), harbouring a derivative plasmid encoding *tfd* genes, was shown to protect barley plants against phytotoxic levels of 2,4-D (Jacobsen 1997). *Pseudomonas putida* VM1450 has been used to help pea plants to phytoremediate 2,4-D polluted soils avoiding symptoms of 2,4-D toxicity (Germaine et al. 2006).

The *C. pinatubonensis tfdA* mutant does not prevent the death of *A. caven* plants, which is explained because this gene encodes the first step in 2,4-D degradation, and it is not replaced by any other gene function in this mutant (Clement et al. 2001; Pérez-Pantoja et al. 2008; Lykidis et al. 2010). The protective effect relies on the efficient expression of genes, since *tfd* genes are not (or are less efficiently) expressed in hosts such as *E. coli*, or others (Top et al. 1998; Newby et al. 2000; Manzano et al. 2007). Accordingly, *E. coli* (pJP4) does not protect *A. caven* from the toxic effect of 2,4-D. The failure of *E. coli* (pJP4) and the *C. pinatubonensis tfdA* mutant to protect *A. caven* from lethality of 2,4-D, dismiss the

possibility that endogenous 2,4-D degraders may remove 2,4-D levels with the efficiency of the wild type *C. pinatubonensis.*

Survival of introduced bacterial cells is strongly influenced by the soil type (van Veen et al. 1997; Thompson et al. 2005). In the case of *C. pinatubonensis*, predation by protozoa, among other factors, has been shown detrimental for survival in soil (DiGiovanni et al. 1996; Kragelund and Nybroe 1996; Manzano et al. 2007). A clear protective enhancement on *A. caven* was achieved with *C. pinatubonensis* JMP134 encapsulated in alginate beads, reflected in a longer period of protection to the plant and higher 2,4-D removal levels and removal rates in soil microcosms (Fig. 4). The use of encapsulated cells for environmental applications has several advantages over free cell formulations, namely, protection from biotic stresses (Smit et al. 1996) and abiotic stresses (Weir et al. 1995; Cassidy et al. 1997).

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