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Evidence for the involvement of ACC deaminase from *Pseudomonas putida* UW4 in the biocontrol of pine wilt disease caused by *Bursaphelenchus xylophilus*

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Abstract Pine wilt disease, caused by the nematode *Bursaphelenchus xylophilus*, is responsible for devastation of pine forests worldwide. Until now, there are no effective ways of dealing with this serious threat. The use of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (encoded by the *acdS* gene)-producing plant growth-promoting bacteria has been shown to be a useful strategy to reduce the damage due to biotic and abiotic stresses. *Pinus pinaster* seedlings inoculated with the ACC deaminase-producing bacterium *Pseudomonas putida* strain UW4 showed an increased root and shoot development and reduction of *B. xylophilus* induced symptoms. In contrast, a *P. putida* UW4 *acdS* mutant was unable to promote pine seedling growth or to decrease *B. xylophilus* induced symptoms. This is the first report on the use of ACC deaminase-producing bacteria as a potential biological control agent for a tree disease, thus suggesting that the inoculation of pine seedlings grown in a tree nursery might constitute a novel strategy to obtain *B. xylophilus* resistant pine trees.

Keywords ACC deaminase · Biocontrol · *Bursaphelenchus xylophilus* · Pine wilt disease · *Pseudomonas putida* UW4 · Plant growth promoting bacteria

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Introduction

Pine wilt disease (PWD) is considered to be one of the major threats affecting conifer forests and forestry economics throughout the world (Mota and Vieira 2008). This complex disease is caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, through a complex web of interactions with other biological components including PWN-associated bacteria and an insect vector, *Monochamus* spp., which is responsible for the PWN tree-to-tree transportation and dissemination (Vicente et al. 2012a, b). The development of PWD is also affected by abiotic factors such as temperature and water availability, which can lead to increased pine tree susceptibility to the PWN (Suzuki and Kiyohara 1978; Miki et al. 2001).

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The first report of PWD dates to 1905 in Japan (Yano 1913). Later, in the 1980s, PWD was reported in China, South Korea and Taiwan (Cheng et al. 1983; Tzean and Jan 1985a, b; Yi et al. 1989; Shin and Han 2006). In Europe, PWD was first reported in 1999 in Portugal (Mota et al. 1999). Since then, the disease has been observed throughout the Portuguese mainland and, more recently, PWD foci were found in the island of Madeira (Fonseca et al. 2012) and also in Spain (Abelleira et al. 2011). Considering the potential climate change scenarios and possible introduction points in Eastern and Northern Europe (Robinet et al. 2011), PWD may become one of the most common coniferous diseases in European forests in the near future. Although efforts to understand and control PWD have been made, to this point in time there are no effective solutions, thus, leading to huge ecological and economical losses (Dwinell 1997).

Under stressful conditions, such as a pathogen attack, plants produce the phytohormone ethylene (Hyodo 1991). If the pathogen action is intense, the autocatalytic ethylene synthesis and the consequent high levels of ethylene can be responsible for plant damage and ultimately its death (van Loon 1984). Interestingly, several authors reported an increase of ethylene levels in pine upon PWN invasion, and suggest that ethylene can play a role in PWD development (Mori and Inoue 1986; Fukuda et al. 1994; Fukuda 1997).

One potential way of limiting the damage caused by the PWN might include the inoculation of pine trees with plant growth-promoting bacteria (PGPB), which have been used for biocontrol of plant diseases (Compant et al. 2005). In this regard, one of the bacterial traits responsible for plant growth promotion, especially under stressful conditions (Glick 2005), is the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (encoded by the *acdS* gene). This enzyme cleaves the ethylene precursor, ACC, into ammonia and α-ketobutyrate (Honma and Shimomura 1978) and thereby prevents ethylene levels in plants from rising to deleterious levels (Glick et al. 1998, 2007). The ACC deaminase-producing bacterium *Pseudomonas putida* UW4 has been shown to be an effective PGPB protecting several different plant hosts from a variety of stress conditions including flooding, salt, pathogens and metals (Grichko et al. 2000; Wang et al. 2000; Grichko and Glick 2001; Cheng et al. 2007; Hao et al. 2007; Gamalero et al. 2010; Toklikishvili et al. 2010).

The use of PGPB has been shown to be a useful strategy in protecting plants against root plant parasitic nematodes (Sikora 1992; Santhi and Sivakumar 1995; Khan et al. 2008). Although PGPB molecular mechanisms responsible for enhanced plant protection are not completely understood, they often reside in nematicidal effects (antagonists). There are no reports on the use of PGPB as biological control agents against tree pathogenic nematodes, such as *B. xylophilus*.

This study aims to evaluate the effectiveness of the ACC deaminase-producing bacterium *P. putida* UW4 as a potential biological control agent for PWD.

Methods

Bacterial growth conditions

The bacterial strains *P. putida* UW4 (Glick et al. 1995) and *P. putida* UW4 (*acdS*$^{-}$), an *acdS* minus mutant of the wild-type strain (Li et al. 2000), were used. The *acdS*$^{-}$ mutant of *P. putida* UW4 was obtained by replacing the functional *acdS* gene with a modified *acdS* gene containing a tetracycline resistance gene inserted within the coding region, through homologous recombination (Li et al. 2000).

The strains were maintained in tryptic soy broth (TSB) medium (Merck, Germany) and supplemented with tetracycline (15 μg ml^{-1}) when necessary. For the inoculation assay, strains were grown in 250 ml Erlenmeyer flasks with TSB medium at 30 °C during 24 h. After reaching the late exponential phase, cells were collected and the optical density was measured at 540 nm. The cell suspensions were diluted in TSB medium so that the cell concentration was $\sim 10^8$ CFU ml^{-1}.

PWN growth conditions

The *B. xylophilus* virulent isolate "HF", collected from a symptomatic pine tree in the Setúbal region, SW Portugal, and maintained in the Nematology Lab (University of Évora, Évora, Portugal), was used in the pathogenicity test. Erlenmeyer flasks containing *Botrytis cinerea* growing in autoclaved barley/water

medium (Evans 1970) for a week at 25 °C, were inoculated with the PWN. Seven days after PWN feeding and reproduction, nematodes were collected by separation over a 24 h period in a plastic tray (Whitehead and Hemming 1965) followed by 6 h in a Baermann funnel (Christie and Perry 1951). A suspension of about 500 nematodes (mixed stages) was prepared for the inoculation assay.

Inoculation assay in *Pinus pinaster*

Three-month-old *P. pinaster* (maritime pine) seedlings, growing on non-sterilized soil, obtained from a nursery (Alfredo Moreira da Silva e Filhos, Lda., Porto, Portugal), were inoculated with wild-type *P. putida* UW4 or *P. putida* UW4 (*acdS*$^{-}$) strains. Each seedling was inoculated with 5 ml of a bacterial suspension applied to the root system. One week after bacterial inoculation, pine seedlings were inoculated with the Portuguese PWN virulent isolate "HF". One milliliter of a nematode suspension (approximately 500 nematodes, mixed stages, in distilled water) was inoculated in the stem of the pine seedlings following the method of Futai and Furuno (1979). Briefly, a small wound (3–5 mm) was made on the pine stem using a sterilized scalpel, and sterilized cotton was placed over the wound and fixed with Parafilm^{TM}, the cotton was subsequently drenched with the PWN suspension.

In addition to the bacterial treatments, two controls were used: a negative control (no bacteria inoculation, stem inoculation with sterile water) and a positive control (no bacteria inoculation, stem inoculation with PWN suspension). The experimental trial was conducted in a growth chamber under controlled conditions (average temperature of 24° ± 2 °C, 80 % humidity, 14 h photoperiod). The seedlings were watered once per week with 100 ml sterilized water. A total of 15 pine seedlings were used per each treatment. The assay was conducted as a randomized block design.

One month after nematode inoculation, pine seedlings were harvested and the disease symptomatology was assessed and categorized as described by Li (2007). Disease incidence was calculated according to Fang (1998). Pine seedling shoots were separated from the roots and shoot fresh weight was immediately measured. Posteriorly, the shoot was divided in smaller parts for the extraction of living nematodes following the method described by Whitehead and Hemming (1965) and Penas et al. (2002). Pine seedling roots were washed and dried for 24 h at 60 °C and the root dry weight was recorded.

Nematicidal activity assay

The *P. putida* UW4 strain was tested for nematicidal activity against *B. xylophilus* by the methods described by Samaliev et al. (2000) and Ali et al. (2002). *Bursaphelenchus xylophilus* "HF" culture was used for the nematicidal assays. Nematodes were previously surface sterilized following the method described by Han et al. (2003).

Pseudomonas putida UW4 was grown in TSB medium (overnight at 28 °C and 120 rpm) and centrifuged at 1,000×*g* for 20 min. The supernatant was collected and filtered using a 0.22 µm filter in order to obtain a cell-free culture filtrate (Ali et al. 2002).

The pellet obtained with *P. putida* UW4 was used following the procedure developed by Samaliev et al. (2000). The pellet was re-suspended in 570 µl of 10 mM Tris pH 8.0, 1 mM EDTA and cells lysed by addition of 30 µl of 10 % SDS (sodium dodecyl sulfate). After incubation at 37 °C for 1 h, the suspension was centrifuged at 15,000×*g* for 20 min, and the supernatant collected for further use.

Three treatments were prepared to test the nematicidal activity of *P. putida* UW4: (1) nematodes in the presence of *P. putida* UW4 cell-free culture filtrate, (2) nematodes in the presence of *P. putida* UW4 supernatant of lysed cells, and (3) control (TE and 10 % SDS). Each treatment was repeated three times using 15 nematodes for each test. An observation on the number of dead nematodes was conducted during the following 24 and 48 h.

Statistical analysis

The data obtained from the inoculation assay was examined by ANOVA, and means were compared by Tukey's HSD test. Statistical analysis was carried out using SPSS statistics V.17 (SPSS Inc., IBM Company).

Results

Pinus pinaster seedlings inoculated with nematodes only showed the expected symptoms of PWD (data not shown). The previous inoculation of *P. pinaster* seedlings with wild-type *P. putida* UW4 led to a significant reduction of PWD symptoms development (Table 1). Interestingly, pine seedlings inoculated with *P. putida* UW4 (*acdS*$^-$) were affected by the disease to the same extent as seedlings inoculated with only PWN (Table 1). That is, the *acdS*$^-$ mutant did not protect pine seedlings against PWD. No disease symptoms were observed in the negative control seedlings.

The shoot fresh weight of pine seedlings inoculated with *P. putida* UW4 was significantly higher when compared to the positive control or *P. putida* UW4 (*acdS*$^-$) inoculated plants ($F_{(3,\ 56)} = 33.852, p < 0.001$) (Fig. 1). Regardless of the nematode inoculation, *P. putida* UW4 inoculated seedlings showed approximately the same shoot fresh weight as the negative control seedlings. No significant differences were found between seedlings inoculated with *P. putida* UW4 (*acdS*$^-$) and seedlings inoculated only with the PWN (Fig. 1).

The inoculation of pine seedlings with *P. putida* UW4 lead to an increased level of seedling root development. The root dry weight of wild-type *P. putida* UW4 inoculated seedlings was also significantly higher when compared to all other treatments ($F_{(3,\ 56)} = 22.198$, $p < 0.001$) (Fig. 2). There were no significant differences between the root dry weight of the positive control or *P. putida* UW4 (*acdS*$^-$) inoculated seedlings. As expected, the negative control seedlings had a higher dry weight than the positive control seedlings.

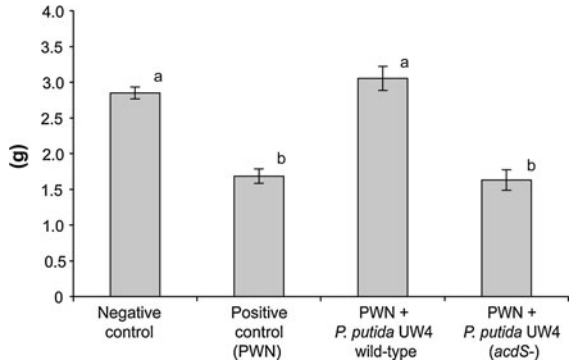

Fig. 1 Shoot fresh weight of *P. pinaster* seedlings, one month after PWN inoculation. Data correspond to the mean and SE values of 15 seedlings per treatment. Different letters (a, b) correspond to statistical significant differences ($p < 0.001$)

Interestingly, the number of living nematodes recovered was lower in seedlings inoculated with wild-type *P. putida* UW4 (Table 1) compared to the high number of living nematodes recovered from the positive control or from *P. putida* UW4 (*acdS*$^-$) inoculated seedlings. Nevertheless, in vitro assays indicated that *P. putida* UW4 has no nematicidal effects towards *B. xylophilus*. Nematode viability was not affected by the presence of either *P. putida* UW4 cell-free culture filtrate or cell lysis supernatant.

Table 1 Disease symptomatology following the inoculation of *P. pinaster* seedlings with the nematode *B. xylophilus* and with either wild-type *P. putida* UW4 or the mutant *P. putida* UW4 (*acdS*$^-$)

Treatment	Average symptom stage	Disease incidence index	Average shoot growth[a] (%)	Average root growth[a] (%)	Estimated number of living nematodes[b]
Negative control	1	0.11	0	0	0
PWN (positive control)	3	0.82	−41 ± 4	−35 ± 6	702
PWN + *P. putida* UW4	2	0.56	7 ± 6	61 ± 13	160
PWN + *P. putida* UW4 (*acdS*$^-$)	3	0.76	−43 ± 5	−17 ± 4	570

Disease stage symptoms (Li 2007): 0, no needle discoloration; 1, only needles around the inoculated place are yellowish, needles in the other part are green; 2, needles in the upper and lower part of the inoculation spot are brown yellowish, and needles in the top of the tree are greyish green; 3, needles in the upper and lower part of the inoculation spot are brown yellowish, and needles in the top are yellowish green; 4, all needles of the plant are yellowish brown; 5, all needles are brown

[a] Compared to the negative control. The values represent the average ± SE

[b] Total number of living nematodes extracted from all pine seedlings belonging to each treatment

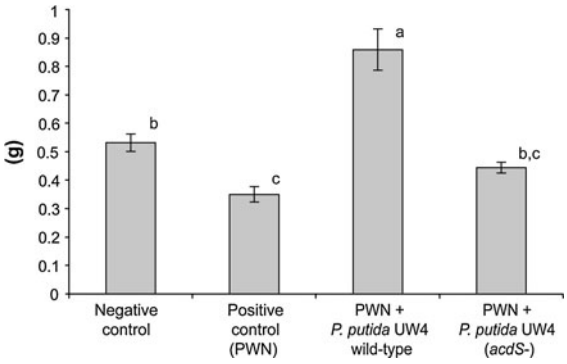

Fig. 2 Root dry weight of *P. pinaster* seedlings, one month after PWN inoculation. Data correspond to the mean and SE values of 15 seedlings per treatment. Different letters (a, b, c) correspond to statistical significant differences ($p < 0.05$)

Discussion

The results presented here indicate that, by producing ACC deaminase, wild-type *P. putida* UW4 acts indirectly as a biological control agent, decreasing PWD development. It has been previously demonstrated that ethylene production increases in pine trees during PWN invasion, suggesting that increased ethylene levels are, at least partially, responsible for the development of PWD symptoms (Mori and Inoue 1986; Fukuda et al. 1994; Fukuda 1997). The data presented here are consistent with the possibility that, through ACC deaminase production, *P. putida* UW4 reduces the deleterious ethylene levels in pine seedlings induced by PWN invasion, thereby decreasing the disease severity and progression. This idea is supported by the fact that *P. putida* UW4 (*acdS*$^{-}$), with its *acdS* gene interrupted, is unable to decrease PWD symptoms or to induce any beneficial effect on the PWN infected seedlings.

Similar results have been obtained with castor bean and tomato plants infected by *Agrobacterium tumefaciens*. While *P. putida* UW4 was able to decrease crown gall formation and subsequent damage and loss of plant biomass, the *acdS* knockout mutant had no significant impact on protecting tomato plants from the *A. tumefaciens* infection (Hao et al. 2007; Toklikishvili et al. 2010). These results suggest that ACC deaminase activity, and the consequent lowering of deleterious plant ethylene levels, is the mechanism responsible for *P. putida* UW4 plant growth promotion activity as well as its ability to protect plants against biotic as well as abiotic stress (Grichko et al. 2000; Wang et al. 2000; Cheng et al. 2007; Glick et al. 2007; Hao et al. 2007; Gamalero et al. 2010; Toklikishvili et al. 2010). In addition, it has been previously demonstrated that transgenic plants that express a bacterial *acdS* gene under the control of a root specific promoter are more resistant to pathogen induced stress as well as abiotic stress caused by salt, flooding and metals (Robison et al. 2001a, b; Grichko and Glick 2001; Grichko et al. 2000; Stearns et al. 2005; Sergeeva et al. 2006).

In this work, the PGPB *P. putida* UW4 successfully induced pine tree seedling growth, in the presence of the PWN. It also reduced PWD symptoms caused by the PWN. It is probable that by both reducing deleterious ethylene levels and directly promoting plant growth, *P. putida* UW4 can boost plant defense systems, thus helping pine seedlings to overcome some of the negative consequences of PWN infection. This is consistent with the low number of living nematodes recovered from *P. putida* UW4 inoculated plants. Moreover, *P. putida* UW4 showed no nematicidal effects against *B. xylophilus*, suggesting that it has no direct effect on the observed decrease of the nematode population.

Further studies may allow the improvement of the biocontrol effect of *P. putida* UW4 in PWD, including its co-inoculation with other microorganisms namely arbuscular mycorrhiza. Synergistic interactions between *P. putida* UW4 and the arbuscular mycorrhizal fungus *Gigaspora rosea*, that positively affected cucumber plant growth, have been described (Gamalero et al. 2008, 2010).

Altogether, the results obtained suggest that the inoculation of pine seedlings with ACC deaminase-producing PGPB in a nursery system may be used as part of a strategy to obtain PWN resistant pine trees. Moreover, since temperature and water availability stresses also play a role in disease development and expression, the use of these bacteria may prove useful to reduce both abiotic and PWN induced stress, leading to an increased level of plant protection. This is the first report describing the use of an ACC deaminase-producing bacterium as a biological control agent for PWD.

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Author Biographies

Francisco X. Nascimento focuses mainly on beneficial bacteria, including bacteria used in the biocontrol of pine wilt disease caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, as well as rhizobia, together with S. Oliveira and B. Glick.

Cláudia S. L. Vicente is a Postdoctoral Fellow investigating bacteria associated with the pinewood nematode including a possible role of bacteria in pine wilt disease.

Pedro Barbosa research is focused on phytoparasite nematodes, including the use of phytochemicals for controlling PWN.

Margarida Espada is a PhD student investigating the genetics of PWN.

Bernard R. Glick is a full Professor of molecular biotechnology and has expertise in the biochemical and genetic mechanisms used by plant growth-promoting bacteria.

Manuel Mota is the Leader of the Portuguese team that detected the PWN, for the first time, in Portugal and in Europe, and since then has been investigating *B. xylophilus*.

Solange Oliveira has focused mainly on the molecular biology of rhizobia as well as other plant beneficial bacteria.