European Journal of Plant Pathology **108:** 187–196, 2002.

Study of bacterial determinants involved in the induction of systemic resistance in bean by *Pseudomonas putida* BTP1

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Accepted 12 November 2001

Key words: biological control, elicitor, induced systemic resistance, plant defence mechanisms, plant growth promoting rhizobacteria

Abstract

The ability of *Pseudomonas putida* BTP1 to induce resistance in bean to *Botrytis cinerea* was demonstrated in soil experiments on plants pre-inoculated at the root level with the bacteria before challenge with the leaf pathogen. As a first step to characterize the molecules from BTP1 responsible for induction of systemic resistance in bean, heat-killed cells and supernatant from culture in an iron-limited medium were tested for their protective effect. Most of the resistance-eliciting activity of the strain was retained in the crude cell-free culture fluid. *In vivo* assays with samples from successive fractionation steps of the BTP1 supernatant led, (i) to the conclusion that salicylic acid, pyochelin and pyoverdin, previously identified as *Pseudomonas* determinants for induced systemic resistance (ISR), were not involved in systemic resistance triggered by BTP1, and (ii) to the isolation of fractions containing one main metabolite that retained most of the resistance-inducing activity in bean. Although this molecule remains to be structurally characterized, its isolation is an addition to the range of determinants from plant growth-promoting rhizobacteria (PGPR) known to stimulate plant defences.

Abbreviations: ACC – 1-aminocyclopropane-1-carboxylate; CAA – Casamino acids medium; ISR – induced systemic resistance; LPS – outer membrane lipopolysaccharides; PGPR – plant growth-promoting rhizobacteria; SA – salicylic acid; SAR – systemic acquired resistance.

Introduction

Strains of the fluorescent species of the genus *Pseudomonas* are potential biocontrol agents able to suppress a variety of plant diseases caused by microbial pathogens (Kloepper et al., 1993). These non-pathogenic strains, also referred to as plant growth-promoting rhizobacteria (PGPR), can exert their biocontrol activity through direct antagonism toward the necrotizing agent. This phenomenon encompasses various mechanisms such as niche exclusion and competition for carbon via effective colonization rate, siderophore-mediated competition for iron and antibiosis related to the production of antibiotics or extracellular lytic enzymes (for reviews see O'Sullivan and O'Gara, 1992; Glick, 1995a). Beside the direct inhibiting effect on pathogen growth, some fluorescent *Pseudomonas* strains activate defence mechanisms in the host plant and thereby indirectly confer some protection against pathogen ingress. As this elevated resistance has been observed in the whole plant and not only at the site colonized by the rhizobacteria, it was termed induced systemic resistance (ISR) (Kloepper et al., 1992).

Although defence mechanisms associated with the pathogen-induced systemic acquired resistance

(SAR) and molecules from pathogens that induce this phenomenon are well characterized (Ryals et al., 1996; Hahn, 1996; Sticher et al., 1997), molecular events and PGPR elicitors involved in ISR are less well understood (Van Loon et al., 1998). Outer membrane lipopolysaccharides (LPS) and siderophores such as pyoverdine, pyochelin and salicylic acid (SA) have been shown to be necessary for ISR induction by pseudomonads. The elicitor activity of LPS or, more specifically of their O-antigenic side chain, was identified for *Pseudomonas fluorescens* strains WCS417r or WCS374 on carnation (Van Peer and Schippers, 1992), radish (Leeman et al., 1995) and tomato (Duijff et al., 1997). The composition of pseudomonad LPSs are strain-specific and their eliciting activity seems to be dependent on the isolate studied. SA is produced by most of the rhizobacteria that induce systemic resistance under iron-limited conditions, and its role in the ISR-elicitation process was demonstrated in the case of *P. aeruginosa* KMPCH (De Meyer et al., 1997; 1999) and for an isolate of *P. fluorescens* P3 which had been converted to an ISR-inducing strain by the insertion of SA biosynthetic genes (Maurhofer et al., 1998). Nevertheless, several reports showed that SA production by other strains was not associated with ISR (Leeman et al., 1996; Press et al., 1997). From experiments involving pyoverdine-non-producing mutants (Maurhofer et al., 1994) or addition of pure pyoverdines (Leeman et al., 1996), these compounds were also suggested as potential ISR elicitors, but this hypothesis is controversial and can probably not be applied to other *Pseudomonas* strains (Van Loon et al., 1998).

In earlier studies *P. putida* strain BTP1 was shown to enhance the resistance of cucumber to root rot caused by *Pythium aphanidermatum* (Ongena et al., 1999). Results from split root experiments suggested that the protective effect was due to ISR, since systemic accumulation of antifungal compounds was observed in the host plant after treatment with BTP1 or with M3, its siderophore-negative mutant (Ongena et al., 2000). The first objective of the present work was to test the biocontrol potential of BTP1 in another pathosystem where both the bacteria and the infectious agent are inoculated on different plant organs. The bean-*Botrytis cinerea* interaction (De Meyer et al., 1997) was used to compare the efficacy of BTP1 with that of the ISR-inducing *P. aeruginosa* strain KMPCH. The second goal was to characterize the BTP1 determinant(s) involved in the ISR-elicitation process. We tested the protective effect of heat-killed cells and crude or fractionated supernatant samples obtained from bacteria cultured in an iron-limited medium in which iron-regulated genes involved in siderophore biosynthesis are expressed.

Materials and methods

Microbial strains and inocula preparation

Pseudomonas putida isolate BTP1, obtained from barley roots, was originally selected for its specific features regarding pyoverdine-mediated iron transport (Jacques et al., 1995). Strain M3 is a siderophore-negative mutant (sid$^-$) of BTP1 (Ongena et al., 1999). Strain R75.3 is a spontaneous rifampicin resistant isolate of BTP1 which was selected on King's B agar medium (King et al., 1954) supplemented with 100 mg l^{-1} rifampicin. R75.3 does not differ from the wild type based on, (i) the growth rate in Luria Bertani, nutrient broth, Pseudomonas F broth or Casamino acids, (ii) the specificity of the iron-acquisition system, i.e., siderophore (pyoverdin) production rate under iron-limited conditions, electrophoretic profile of iron repressed outer membrane proteins, and (iii) the inability to develop direct antagonism toward plant pathogens (*in vitro*) (unpublished results). The ISR-inducing strain *P. aeruginosa* KMPCH and the fungal pathogen *B. cinerea* were kindly provided by Dr. Monica Höfte (Ghent University) (De Meyer et al., 1997). Bacterial isolates were maintained on King's B medium at 4 °C before experimental use, and stored at −20 °C in glycerol 50% for long-term storage. *B. cinerea* was grown to sporulation on an oat-based medium (oatmeal 25 g l^{-1}; agar 12 g l^{-1}) at room temperature. For the preparation of bacterial inocula used in ISR assays, the *Pseudomonas* isolates were grown overnight on KB medium at 30 °C (*P. putida* strains) or 37 °C (*P. aeruginosa*). Cells were scraped off the plates and suspended in a sterile saline solution (0.85% NaCl) diluted in order to obtain the desired bacterial concentration.

The *B. cinerea* spore suspension was prepared by harvesting spores from 10-day-old cultures in sterile peptone water containing 0.01% Tween 80. After removing mycelial debris, the suspension was centrifuged for 5 min at 5000*g* and the spores were resuspended in an adequate volume of a 0.4% glucose solution supplemented with 0.013 M KH_2PO_4 to a final concentration of 5×10^6 spores ml^{-1}.

Plant material and ISR assays

All ISR assays were performed with bean (*Phaseolus vulgaris*) cultivar Prelude following the procedure described by De Meyer et al. (1997) with minor modifications. In experiments done in pots, plants were grown either in a peat substrate (Brill Substrate GmbH & Co KG, Germany) referred to as 'soil' or in a perlite–vermiculite mix (50/50, v/v). Prior to planting, beans were disinfected in $HgCl_2$ 0.1% for 7 min, rinsed three times in sterile distilled water and soaked for 10 min in a bacterial suspension at a concentration of approximately 4×10^8 CFU ml^{-1} or in NaCl 0.85% in the case of control plants. Bean seeds were sown in 10-cm pots containing compost soil or perlite–vermiculite previously mixed with bacterial inoculum to a final concentration of 3×10^7 CFU g^{-1} or with an equal volume of sterile water for untreated control plants. Beans were germinated at 22 ± 2 °C in the greenhouse with a 16-h photoperiod. Seven and 14 days after sowing, 20 ml of a bacterial suspension at 10^8 CFU ml^{-1} were added as a drench to the roots of each plant except the controls (watered with 20 ml of NaCl 0.85%). After approximately 20 days, bean plants were transferred to a high humidity chamber (19 ± 2 °C) and, 24 h later, they were leaf-infected with *B. cinerea*. To this end, eight wounds were made on both primary leaves by gently touching the epidermis with a red hot pinhead. These wounds were covered with 10 µl of the pathogen spore suspension. After 4–5 days, the disease incidence was expressed in terms of the percentage of *B. cinerea* lesions which clearly grew out of the inoculum drop zone to produce spreading lesions.

For plant growth in nutrient solution, beans sterilized and PGPR-inoculated as described above, were germinated in perlite mixed with a half-strength Hoagland solution (Hoagland and Arnon, 1938). After one week, seedlings were transferred to test tubes filled with 45 ml of the half-strength Hoagland solution. Aerial parts of plants were maintained emerged by fixing the stem base in a styrofoam plug so that only roots were in the nutrient solution. All materials and nutrient solutions used in these experiments were autoclaved at 121 °C for 20 min before use. At the time of transfer, plants were also treated by adding as a drench 1 ml of cell suspensions or supernatant samples (prepared as described below) to each tube containing 40 ml of nutrient solution. The liquid level in the tubes was adjusted daily with sterile distilled water during the experimental time. Thirteen to 15 days after treatment, plants were placed in the high humidity chamber and infected with the pathogen *B. cinerea* as mentioned above except that four infection sites were created on both primary leaves.

In every experiment, 10 plants per treatment were used (160 and 80 infection sites for soil/perlite and nutrient solution assays, respectively). Data for soil assays are from three independent experiments, while perlite and nutrient solution assays were performed twice. The software MINITAB was used for statistical analyses. The homogeneity of variances was tested by ANOVA and data from experiments with the same set-up were pooled for analysis when interaction between experiment and treatment was not significant at $P = 0.05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0.05$).

Plant colonization by PGPR

As *P. aeruginosa* KMPCH is resistant to kanamycin, plant colonization by this bacterium could be evaluated. In the case of *P. putida* BTP1, colonization of bean roots and possible migration through the plant were estimated indirectly by using a spontaneous rifampicin resistant isolate (R75.3) of BTP1. In every soil or perlite experiment, three bean plants were treated in parallel with R75.3 at the same concentrations as the wild type and grown in the same conditions. After 25 days, 1-g samples (fresh weight) of roots (briefly washed with sterile distilled water to remove most of the soil or perlite) or leaves, were randomly collected from three plants per experiment treated either with R75.3 or KMPCH. They were homogenized in 10 ml of sterile peptone water and serial dilutions were plated on King's B medium supplemented with 100 mg l^{-1} rifampicin or kanamycin for R75.3 and KMPCH, respectively. Bacterial counts were made after 24 h incubation at 30 °C (R75.3) or 37 °C (KMPCH).

Treatments with bacterial cell suspensions and fractions from culture supernatant in nutrient solution ISR assays

Bacterial suspensions. BTP1 cell suspensions were adjusted to reach a final concentration of about 5×10^7 CFU ml^{-1}. BTP1 heat-killed cells were obtained by autoclaving. The suspension was used at the same concentration as living cells after checking the absence of growth on King's B medium. In these

experiments, control plants were treated with 1 ml of 0.85% sodium chloride.

Crude supernatant samples. Bacterial strains (BTP1 and M3) were grown in 300 ml of Casamino acids broth (CAA) (Paulitz and Loper, 1991) for 48 h on a rotary shaker (150 rpm) at 30 °C. The flasks were previously washed with 1 N HNO_3 in order to remove most of the traces of contaminating iron and to ensure optimal production of iron-repressed metabolites. Cells were eliminated by centrifugation at 13,500*g* for 15 min. The crude supernatant was harvested, lyophilized and the resulting powder resuspended in 20 ml of distilled water. This solution was adjusted to pH 5.2 with HCl and sterilized by filtration on ACRODISC 0.45 µm. Beans were treated by adding 1 ml per plant to the nutrient solution.

Supernatant samples fractionated on C-18 cartridge. Ten millilitres of a 30-times concentrated crude supernatant were loaded on a ISOLUTE C-18 CE type cartridge (International Sorbent Technology) activated with 40 ml methanol and equilibrated with 80 ml distilled water. The column was rinsed with 60 ml H_2O to remove non-adsorbed material which was collected, lyophilized and resuspended in 10 ml of distilled water. This solution was sterilized by filtration and used in ISR assays as treatment SN-0. Molecules with intermediate hydrophobicity were then eluted from the cartridge with 20 ml of 50% MeOH. The harvested 50% MeOH solution was evaporated to dryness and the residue solubilized in 10 ml of 50% MeOH (SN-50). Finally, hydrophobic compounds were recovered by washing the column with 20 ml of methanol. This methanolic extract (SN-100) was evaporated and the lyophilized material resolubilized in 10 ml MeOH. Plants were treated individually with 1 ml of each treatment and control solution (water, 50% MeOH and MeOH, respectively).

Semi-preparative HPLC. Three-hundred millilitres of culture supernatant was concentrated to 1 ml SN-100 extract. This was further fractionated by semi-preparative reverse phase HPLC (HP 1100 series system from Hewlett-Packard) on a Chromspher 5 C-18 (250 mm × 10 mm, 5 µm packing, Chrompack) column by repeated injections of 100 µl aliquots. The starting mobile phase was 0.1% trifluoroacetic (TFA) acid in milliQ water and elution was performed with a gradient of solvent B (0.1% TFA in 80% acetonitrile in milliQ water) as follows: (time in min/% B/flow rate in ml min^{-1}) 0/0/1, 3/0/4, 6/0/4, 8/5/4, 18/10/4, 48/50/4, 55/100/4, 60/100/4, 61/0/4, 68/0/4. Samples were monitored spectrophotometrically at 280, 214, 254, 320 and 375 nm by means of a diode array detector (DAD). Two-millilitres samples were automatically collected over the entire run time, but three main fractions were constituted by pooling material eluted between 10–30 min (SN-100-1), 30–50 min (SN-100-2) and 50–56 min (SN-100-3). These solutions were evaporated to dryness and the three residues resuspended in 20 ml of methanol. One millilitre aliquots from each of the three fractions were used to treat plants.

Purification of SN-100-3 fractions by analytical HPLC. One litre of CAA culture supernatant was processed to a 1 ml concentrated SN-100-3 extract. The three main compounds detected at 280 nm in the SN-100-3 fraction were isolated by repeated HPLC runs using an analytical procedure. Fifty microlitres were injected on a Chromspher C-18 (250 mm × 4.6 mm, 5 µm packing, Chrompack) column using the same solvent system as described for the semi-preparative HPLC method. The flow rate was fixed at 1 ml min^{-1} and the gradient was (time in min/% B) 0/20, 3/20, 30/80, 35/80, 36/100, 40/100, 41/20, 45/20. Eluted material was manually collected on the basis of DAD signals at 214 and 280 nm. Individual fractions from the different runs were pooled and the solvent was evaporated in the Speed Vac concentrator. The residue corresponding to each peak was solubilized in 20 ml of pure methanol and 1-ml aliquots were used for plant treatment.

In vitro *salicylic acid production*

Salicylic acid production by *P. putida* BTP1 in the iron-poor Casamino acids medium was evaluated and compared to that of *P. aeruginosa* KMPCH by HPLC analysis of 15 times concentrated crude supernatants with fluorimetry detection (HP 1100 series system, Hewlett-Packard). Samples of 20 µl were automatically injected onto a INERTSIL 5 ODS-2 (250 mm × 3 mm) column and eluted at a flow rate of 0.55 ml min^{-1} at room temperature. All the other parameters, i.e., mobile phases, gradient, excitation and emission wavelenghts, were as described by Meuwly and Métraux (1993). Under such conditions, SA had a retention time of 16.3 min. It was quantified by reference to the standard curves established after HPLC analysis of commercial SA at various concentrations.

Results

Resistance induced by PGPR in bean

The protective effects of *P. putida* BTP1 and *P. aeruginosa* KMPCH were evaluated on three-week-old bean plants inoculated at the root level with the PGPR strains. Pathogen infection was assessed on the basis of lesion formation (De Meyer et al., 1997). Three independent experiments showed a statistically significant disease reduction in plants grown in compost soil and treated with BTP1 (42%) or KMPCH (35%), as compared to the infected controls. However, pooled data from two separate assays on perlite-grown beans revealed a three-fold reduction in the efficacy of the strains. In these experiments, the 9%-reduction of spreading lesions observed after treatment with KMPCH was not statistically different from controls. Similarly, treatment with BTP1 provided a 15% disease reduction compared to the 42% protection observed in soil-grown plants.

In order to evaluate both local and systemic colonization of plants by the PGPR strains, root and leaf samples were randomly collected at the end of two independent experiments. The colonization rate of BTP1 was estimated by the use of a spontaneous rifampicin resistant isolate of this strain named R75.3. Populations of $1.43 \pm 1.1 \times 10^6$ and $2.42 \pm 0.35 \times 10^6$ CFU g^{-1} for KMPCH and R75.3, respectively were counted on bean roots from soil. Concentrations of $1.05 \pm 0.53 \times 10^6$ and $1.81 \pm 0.42 \times 10^6$ CFU g^{-1} for KMPCH and R75.3, respectively were measured on roots from bean plants grown in perlite. R75.3 and KPMCH were never detected in leaf samples with a detection limit about 100 CFU g^{-1} of leaf, suggesting that bacteria did not migrate through the plants.

Resistance induced by P. putida *BTP1, heat-killed cells and crude CAA supernatants*

Ten-day-old bean plants were transferred from perlite to liquid nutrient solutions and treated accordingly. Plants were inoculated with *B. cinerea* spores two weeks later. The percentage of spreading lesions on leaves from pathogen-challenged, but untreated bean plants (infected controls) varied from 73% to 91% depending on the experiment. Plants inoculated with *P. putida* BTP1 showed a significant reduction of about 15% in the number of spreading lesions, as compared to the infected control plants while heat-killed cells did not provide any protection (Figure 1).

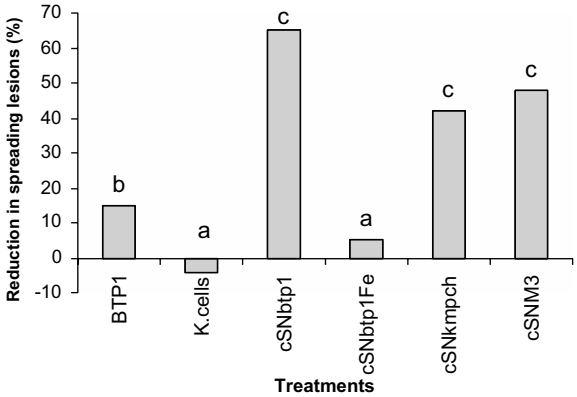

Figure 1. Resistance induced in bean plants grown in nutrient solution treated at the root level with living cells of *P. putida* BTP1 (BTP1), heat-killed BTP1 cells (K. cells) or crude supernatants obtained after growth of strain BTP1 (cSNbtp1, in iron-limited conditions; cSNbtp1Fe, in the presence of 100 μM $FeCl_3$), *P. aeruginosa* KMPCH (cSNkmpch) and strain M3, pyoverdin-deficient mutant of BTP1 (cSNM3), in CAA medium. Protection is expressed as a reduction in *B. cinerea* spreading lesions relative to the number of spreading lesions in control plants. Data are means of two experiments each with 10 plants inoculated on both first leaves (four infection sites per leaf). The homogeneity of variances was tested by ANOVA and data from experiments with the same set-up were pooled for analysis as interactions between experiment and treatment were not significant at $P = 0.05$. Means from the different treatments were compared by the method of Newman and Keuls (least significant difference at $\alpha = 0.05$) and bars marked with 'a' are not statistically different from the control.

Metabolites produced by *P. putida* BTP1, its siderophore-negative mutant M3 and *P. aeruginosa* KMPCH grown in iron-poor liquid medium (CAA) were tested for ISR by treating roots with an equal volume of the corresponding concentrated crude supernatants. In CAA, BTP1 produces 9.1×10^{-14} g pyoverdin per cell while no detectable quantities were found in M3 and KMPCH cultures. The final pyoverdin concentration in nutrient solutions of plants treated with BTP1 crude supernatant was 12.5 μM. A significant reduction in the incidence of spreading lesions was observed for plants grown in the presence of all three supernatants (Figure 1).

Detection of active compounds in the BTP1 supernatant

Since most of the ISR-eliciting activity of BTP1 was retained in the crude CAA supernatant, this solution was further processed and a first fractionation

step was performed on a C-18 Isolute-type cartridge. Compounds eluted from the silica gel with water (SN-0), 50% MeOH (SN-50) and 100% MeOH (SN-100) were separately collected and assayed for ISR (Figure 2). Most of the activity was observed in the SN-100 fraction. Reductions in the number of spreading *B. cinerea* lesions after treatment with SN-0 and SN-50 fractions were not statistically different from infected control plants.

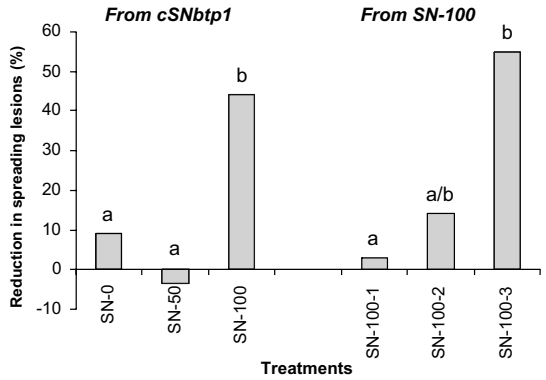

Figure 2. Resistance induced in bean plants grown in nutrient solution and treated at the root level with various supernatant fractions obtained after growth of strain BTP1 in CAA medium. SN-0, SN-50 and SN-100 derived from a first fractionation of crude supernatant (cSNbtp1, see Figure 1) on ISOLUTE C-18 CE cartridge. SN-100-1, SN-100-2 and SN-100-3 were obtained from semi-preparative HPLC separation of material contained in SN-100. Protection is expressed as a reduction in *B. cinerea* spreading lesions relative to the number of spreading lesions in control plants. Data are means of two experiments each with 10 plants inculated on both first leaves (four infection sites per leaf). Statistical analyses were performed as described in Figure 1 and bars marked with 'a' are not statistically different from the control.

The active SN-100 solution was further fractionated by semi-preparative HPLC and the eluted material was divided in three groups on the basis of retention times (Figure 3): peaks eluted between 10–30 min (SN-100-1), 30–50 min (SN-100-2) and 50–60 min (SN-100-3). When tested for ISR on bean plants, only the SN-100-3 fraction showed activity (Figure 2) similar to that expressed by SN-100 or crude supernatant. Three main compounds were present in the SN-100-3 fraction. They correspond to peaks eluted after 51.4, 53.3 and 54.3 min (Figure 3). These molecules were isolated by repetitive analytical HPLC injections and assayed *in vivo*. Bean plants were significantly protected (51% reduction of spreading lesions) upon treatment with fractions corresponding to compounds eluted after 51.4 min and added to the nutrient solution at a concentration of 75 $\mu g\, l^{-1}$. The main molecule present in these fractions (91% purity on the basis of the absorbance at 280 nm) will be subsequently referred to as Cx. Its specific UV-visible spectrum recorded on-line (diode array detector) is presented in Figure 4. A lower 30%-disease incidence reduction was obtained by testing fractions corresponding to the molecule eluted after 53.3 min while material eluted in the 54.3-min peak failed to induce any significant resistance. These percentages are mean values from

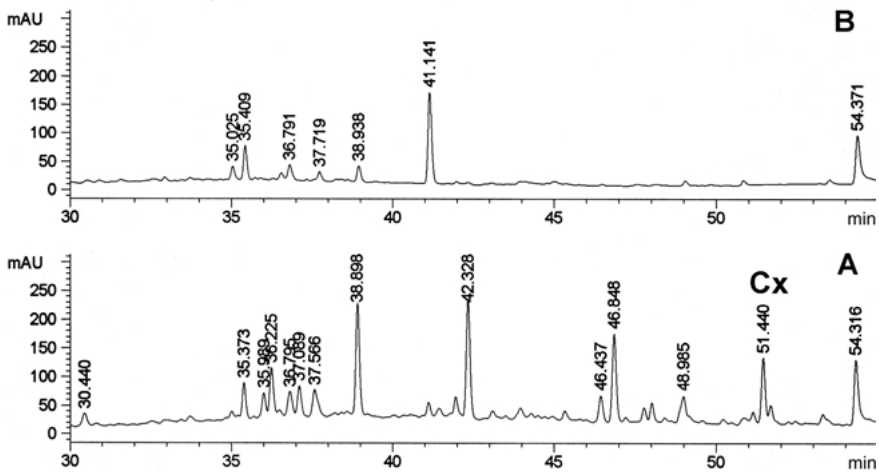

Figure 3. Chromatograms obtained for analytical HPLC analyses of SN-100 (A) and SN-50 (B) extracts (see Figure 2). Samples were injected on a RP C_{18} Chromspher-type column and eluted with a gradient of acetonitrile in acidified milliQ water. Elution was monitored at various wavelengths by photodiode array detection, but only traces recorded at 280 nm are shown for visualization of the active compound Cx. Pyoverdines eluted mainly in peak with retention time of 41.14 min.

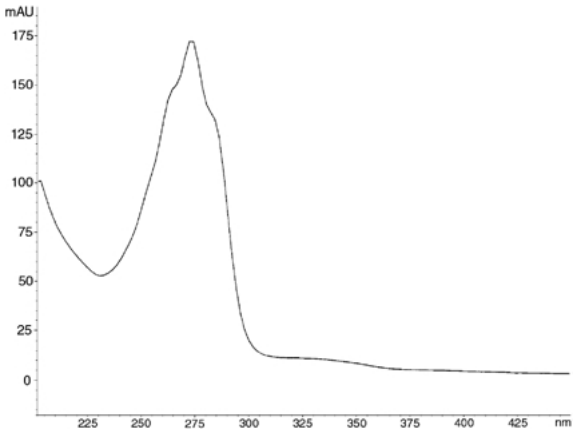

Figure 4. UV-visible spectrum recorded on line at the apex of the peak corresponding to the compound Cx (Figure 3) by means of a photo diode array detector.

two independent experiments and were statistically different from controls.

In the presence of added iron, Cx synthesis was inhibited: only 4.7% of the amounts produced under iron-limited conditions were measured. This mean percentage value was calculated on the basis of peak area at A_{280nm} and from three independent cultures upon addition of 100 μM $FeCl_3$. Similar results were observed by adding $FeSO_4$ at the same concentration. As expected, pyoverdine production was also drastically reduced in these iron-supplemented cultures (12.5 and 0.24 μg 10^8 CFU^{-1} in CAA and in CAA+Fe, respectively). Interestingly, inhibition of Cx synthesis in the presence of added iron was concomitant with the loss of ISR-eliciting activity of the corresponding culture supernatant (cSNbtp1Fe) which failed to induce any significant reduction in disease incidence as shown in Figure 1.

Production of salicylic acid

Production of SA by *P. putida* strain BTP1 was evaluated by reverse phase HPLC analyses of concentrated supernatant from CAA cultures. Crude supernatants from BTP1 (cSN*btp1*) and KMPCH (cSN*kmpch*) were analysed using a specific and sensitive method based on fluorimetric detection of the molecules eluted (Meuwly and Métraux, 1993). Concentrations (mean of values obtained from analyses of four different samples) of 19.1 ± 3.2 μg ml^{-1} culture supernatant and of 0.012 ± 0.009 μg ml^{-1} culture supernatant, were measured for KMPCH and BTP1, respectively. While the production rate observed for KMPCH is similar to values mentioned in the literature (De Meyer et al., 1999), formation of SA by *P. putida* BTP1 is very low with values just above the detection limit of 0.22 ng. No traces of SA were detected in fraction SN-100.

Discussion

Results presented in this paper describe the ability of *P. putida* strain BTP1 to protect bean against infection by the leaf pathogen *B. cinerea*. This was demonstrated in soil experiments on plants pre-inoculated at the root level with bacteria before challenge with the pathogen. Disease suppression was due to induction of resistance in the host plant since colonization studies confirmed that PGPR and pathogen remained localized on different plant organs and that bacterial populations established on roots were above the threshold necessary to trigger ISR (Raaijmakers et al., 1995). The protection level provided by BTP1 was not statistically different from the one observed after treatment with *P. aeruginosa* strain KMPCH, an efficient inducer of resistance in bean (De Meyer et al., 1997).

Biocontrol assays carried out with cell-free culture fluids of BTP1 clearly indicated that ISR was mostly induced by one or several metabolite(s) excreted by the strain under iron-limited *in vitro* growth conditions. Indeed, root treatment of beans grown hydroponically with concentrated crude CAA supernatants induced a significant reduction of *B. cinerea* infection (Figure 1). The protection level conferred by such supernatant samples was higher than the one triggered by living BTP1 cells tested under the same conditions. This may indicate that ISR-eliciting molecules were present in CAA supernatant aliquots in more effective quantities than those produced by the bacteria between the time of inoculation and the time of pathogen infection (10 days). Hydroponic conditions and particularly iron concentration in the nutrient solution could be partially inhibitory to the production of the active compound(s) if their synthesis was regulated by this element (see below). The shorter period for root colonization and/or a less favourable medium for elicitor synthesis could also explain the lower efficacy of BTP1 at inducing ISR in hydroponic assays compared to soil experiments. Similarly, disease protection levels induced by PGPR in compost soil were much higher than those observed in perlite (see first section of Results), but root colonization was equivalent in both conditions. The metabolism of BTP1 cells living in the rhizosphere of plants grown

in perlite compared to soil may also be affected by various nutritional factors. A lower availability of some minerals or some unfavourable changes in the composition of root exudates could lead to a reduced production rate of the ISR determinant.

Most of the ISR-eliciting activity of BTP1 was retained in crude CAA supernatant and autoclaved BTP1 cells were fully impaired in providing the protection observed against *B. cinerea* upon treatment with living bacteria (Figure 1). These observations suggest that no heat-stable cell envelop components play a major role in the ISR-elicitation process. Some bacterial isolates such as *P. fluorescens* WCS417 (Leeman et al., 1995; Van Peer et al., 1992) and *Rhizobium etli* (Reitz et al., 2000), mediates ISR through their outer membrane LPS. In these cases, a thermal inactivation does not appear to inhibit the eliciting property of the cells. LPS from BTP1 could be heat labile and their non-involvement should be proved unambiguously by testing purified molecules and/or mutants with modified LPS. However, our data are much in favour of an inefficient structure of the strain-specific O-antigenic side chain as it was suggested in the case of *P. putida* WCS358 on radish plants (Leeman et al., 1995).

Pyoverdines are the main siderophores produced by most of the fluorescent *Pseudomonas* strains to ensure their growth in iron-limited environments (Budzikiewicz, 1993). Their (partial) involvement in the stimulation of resistance was demonstrated in radish against Fusarium wilt (Leeman et al., 1996) and in tobacco against tobacco necrosis virus (Maurhofer et al., 1994). In the case of *P. putida* BTP1, our results showed that culture supernatant fractions (SN-50) containing pyoverdines (at a final concentration of 2.4 μM in the nutrient solution) failed to induce resistance in bean plants (Figure 2). Moreover, cell-free fluids from the culture of M3, a siderophore-deficient mutant of BTP1, were still active (Figure 1) strongly suggesting that pyoverdines are not involved in ISR induced by BTP1. This is in line with previous observations on cucumber with the same strain (Ongena et al., 1999). Studies with pyoverdine-mutants (De Meyer et al., 1997) or with purified compounds (Leeman et al., 1996) from other pseudomonads have led to the same conclusion. The ISR-eliciting activity of pyoverdines could tightly depend on the amino acid sequence of these chromopeptides since the chromophore moiety is highly conserved among the structures described so far in contrast with the usually strain-specific peptide chain composition (Budzikiewicz, 1993). In this respect, (iso)pyoverdines produced by BTP1 are specific since a structural change in the chromophore induces a modification of the peptide fixation site (Jacques et al., 1995). However, this trait does not seem to positively influence the ISR-eliciting activity of the molecule.

To date, results on the involvement of bacterially produced SA in the induction of plant resistance are conflicting. The capacity to produce SA *in vitro* and the ISR-inducing activity of the bacteria were associated in the case of *P. aeruginosa* KMPCH on bean (De Meyer et al., 1997), tobacco (De Meyer et al., 1999) and tomato (K. Audenaert, Ghent University, Belgium, pers. comm.), *P. fluorescens* CHAO on tobacco (Maurhofer et al., 1998) or *P. fluorescens* WCS374 and WCS417 on radish (Leeman et al., 1996). Direct involvement of SA in the elicitation process was further evidenced in the case of KMPCH by the use of various specific siderophore mutants and through the verification of *in vivo* SA gene expression. In contrast, the work of Press et al. (1997) suggested that plant resistance induction by *S. marcescens* 90-166 was not or only slightly related to salicylate synthesis since SA-deficient mutants were able to trigger ISR to the same level of protection as the wild type. In supernatants from stationary-phase BTP1 cultures, SA was measured at a concentration of 12 ng ml^{-1} corresponding to a production rate of 0.0132 μg 10^9 CFU^{-1} while no traces of its methyl ester form or pyochelin were detected (data not shown). This SA production is much lower than those observed *in vitro* for the above-mentioned PGPR strains considered as efficient SA producers (2–50 μg 10^9 CFU^{-1}). Working with the same pathosystem, De Meyer et al. (1999) showed that nanogram amounts of SA are sufficient to induce systemic resistance in bean plants and that such amounts (11–18 ng) can be readily produced by strain KMPCH colonizing the roots at about 10^5 CFU g^{-1} root. So these authors showed that SA quantities produced in soil correlated well with those synthesised *in vitro* since theoretically 10^5 CFU should produce about 2 ng in 48 h in synthetic media. If we apply such a logical line of reasoning in the case of BTP1, the amounts of SA produced in soil should be of about 15 pg based on the population density of 10^6 CFU g^{-1} root that we determined in this study. This hypothetical value is about 1000-fold lower than the one observed in the case of KMPCH or by feeding 1 nM SA in the nutrient solution used for bean growth. This suggests that induction of resistance by BTP1 is probably not associated with SA production at the root level. However, we cannot obviously conclude that the 15-pg amount putatively produced

by BTP1 in soil is below the threshold limit for resistance induction since the efficiency of SA concentrations lower than 1 nM at triggering ISR in bean plants is not known. In contrast, no traces of SA were detected in the SN-100 supernatant fraction that retained most of the initial activity of the strain. This also indicates that the low amounts of SA that may be produced by strain BTP1 do, probably, not represent the main eliciting activity for ISR in bean.

The perception of ethylene *in planta* is involved in the signaling pathway leading to ISR as shown in studies on *Arabidopsis thaliana* (Pieterse et al., 1998; Knoester et al., 1998). Some *Pseudomonas* strains can synthesize this plant hormone from 1-aminocyclopropane-1-carboxylate (ACC) via the ACC oxidase (Glick et al., 1995b) and may therefore produce amounts sufficient to activate ISR. However, it is probably not the case for BTP1 since this strain is not able to metabolize ACC and since no traces of ethylene were detected in the gas phase after growth in various media (results not shown).

Interestingly, *in vivo* assays with samples from successive fractionation steps led to the isolation of HPLC fractions enriched with unknown compounds that retained the ISR activity of crude CAA supernatant from BTP1. One molecule named Cx represented most of the absorbing material present in these extracts. So it is obvious that Cx is tightly involved in ISR induction. The production of this molecule seems to be iron-regulated since it was practically undetectable in cell-free culture fluids obtained from growth of BTP1 in CAA medium supplemented with ferric chloride (CAAFe) at a concentration inhibitory to siderophore synthesis. In fact, the CAAFe supernatant failed to induce resistance in bean. Moreover, the difference in protection levels conferred by BTP1 in compost soil compared to the one observed in perlite suggests that the synthesis of Cx may also depend on the presence of other minerals such as inorganic phosphate and zinc that have already been reported to influence the production of other bioactive *Pseudomonas* metabolites (Duffy and Défago, 1999; Höfte et al., 1993). Similarly, the excretion of Cx *in planta* might also be modulated by the nature of the carbon sources available for growth of strain BTP1 in the rhizosphere. Such nutrients mainly derive from root exudates that can be qualitatively and quantitatively influenced by the environment of the plant. The structural characterization of Cx is in progress in order to know if it corresponds to any previously identified bacterial metabolite. Although conclusive evidence on its plant resistance elicitor potential can only be gained after testing the compound in a pure form, our results are in support of a wider variety of *Pseudomonas* determinants for ISR than is reflected in the very limited number of such molecules reported to date.

Acknowledgements

Marc Ongena is a Scientific Research Worker of the Fonds National de la Recherche Scientifique (F.N.R.S.) in Belgium. This work also received financial support from the F.N.R.S. (Programme F.R.F.C. no. 2-4557-95). We thank Dr. Monica Höfte for providing the strain KMPCH and for critical reading of the manuscript.

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