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RESEARCH ARTICLE

Evaluation of *Pseudomonas chlororaphis* subsp. *aurantiaca* SR1 for growth promotion of soybean and for control of *Macrophomina phaseolina*

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Pseudomonas chlororaphis subsp. *aurantiaca* SR1 was evaluated for control of *Macrophomina phaseolina in vitro* and in soybean plants, for growth promotion of soybean plants and for production of antifungal compounds. Strain SR1 caused a significant inhibition of *M. phaseolina in vitro* and reduced damping-off in the *in vivo* assays. In addition, strain SR1 significantly increased shoot and root length and shoot and root dry weight of soybean plants in *M. phaseolina* infested soil, as compared to control plants in infested soil. Fragments for the phenazine-1-carboxylic acid, pyrrolnitrin and hydrogen cyanide encoding genes were amplified from the DNA of strain SR1 after polymerase chain reaction (PCR) assays with specific primers. Thus, this study establishes that *P. chlororaphis* subsp. *aurantiaca* SR1 provides control of *M. phaseolina in vivo* and suggests that strain SR1 might be applied as an effective biocontrol agent to protect soybean plants from this phytopathogen.

Keywords: *Pseudomonas chlororaphis* subsp. *aurantiaca* SR1; plant growth promotion; soybean seeds inoculation; *Macrophomina phaseolina* biocontrol; detection of antifungal compounds

1. Introduction

Soybean [*Glycine max* (L.) Merrill] is a globally valuable crop. Soybean flour, rich in protein content, and soybean oil, with a high energy value, are essential products for human consumption because they contribute to cover nutritious needs. In Argentina, soybean constitutes the leading legume crop and its expansion restricts the planting of other crops. Indeed, Argentina is one of the main exporters of soybean flour and oil. Soybean plants are susceptible to diseases caused by the fungal genera *Macrophomina* spp., *Fusarium* spp., *Rhizoctonia* spp., *Colletotrichum* spp. and *Phomopsis* spp.

Fungicides are chemicals used in the control of fungal diseases. Nevertheless, chemical fungicides are costly, can produce adverse environmental effects and may cause the pathogens to become resistant (Wang, Yieh, & Shih, 1999). The increasing interest in the sustainability of agriculture is heightening the need for obtaining substitutes to chemical fungicides. Currently, efforts are being made to limit the use

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of chemical fungicides and control diseases more suitably by integrated disease management strategies. For a sustainable agricultural productivity, the constituents implicated in these strategies, such as biological control agents (BCAs), should be friendly to the environment for beneficial organisms to be preserved (Sahni, Sarma, & Singh, 2008).

Biocontrol refers to the use of applied or native organisms, different from the resistant host plants, to disrupt the growth and activity of one or more plant pathogens (Pal & McSpadden Gardener, 2006). Thus, BCAs partake at increasing the currently available practices for the management of plant diseases. The mechanisms on which BCAs are based are well defined and include production of antifungal compounds, induction of systemic resistance and spatial and nutrient competition (Anjaiah, 2004). The fluorescent *Pseudomonas* have emerged as the largest and potentially most promising group of plant growth-promoting rhizobacteria involved in the biological control of plant diseases (Kloepper, Leong, Teintze, & Schroth, 1980; Mercado-Blanco & Bakker, 2007; Suslow & Schroth, 1982). Fluorescent *Pseudomonas* spp. have simple nutritional requirements, which is reflected in the relative abundance of these organisms in nature. In addition, they can be found in soil, foliage, fresh water, sediments and sea water (O'Sullivan & O'Gara, 1992). Fluorescent *Pseudomonas* were proven to control soybean root rotting by *Phytophthora* (Lifshitz et al., 1986), peanut root rotting caused by *Rhizoctonia solani* (Savithiry & Gnanamanickam, 1987) and fusarium wilt of radish (Leeman et al., 1996), among other diseases. Fluorescent *Pseudomonas* yield a broad diversity of secondary metabolites, which are compounds that may act directly or indirectly against microbes.

Soybean plants are sensitive to several phytopathogenic fungal genera such as *Macrophomina*, which causes charcoal rot, dry root rot and damping-off in several agronomically important crops. *Macrophomina* predominates in the tropical and sub-tropical areas and causes diseases in a broad group of hosts (Anjaiah, 2004). Substantial yield decreases of soybean are declared annually due to *M. phaseolina* (Tassi) Goid. *Macrophomina* diseases on soybean are usually controlled with fungicides such as carbendazim and pentachloronitrobenzene (Senthilkumar, Swarnalakshmi, Govindasamy, Lee, & Annapurna, 2009). Also, effective control of *M. phaseolina* has been demonstrated with promising BCAs in groundnut (*Arachis hypogaea;* Arora, Kang, & Maheshwari, 2001), chir-pine (*Pinus roxburghii;* Singh, Pandey, Dubey, & Maheshwari, 2008) and sunflower (*Helianthus annus* L.; Anis, Abbasi, & Zaki, 2010), among others. To our knowledge, the application of a *Pseudomonas chlororaphis* subsp. *aurantiaca*, formerly *P. aurantiaca*, strain to control *M. phaseolina* in soybean has not been tested.

In previous works, we demonstrated that *P. chlororaphis* subsp. *aurantiaca* strain SR1 can promote growth of wheat and maize in field experiments (Carlier, Rovera, Jaume, & Rosas, 2008; Rosas et al., 2009). In addition, strain SR1 was proven to produce indole-3-acetic acid (IAA) and siderophores and suggested to behave as an endophytic bacterium (Rosas et al., 2009; Rovera et al., 2008). This work had two aims. Our first aim was to evaluate the biocontrol efficacy of strain SR1 against *M. phaseolina* (Tassi) Goid. *in vivo*. Our second aim was to detect encoding genes for 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyrrolnitrin (PRN), pyoluteorin (PLT) and hydrogen cyanide (HCN) in the DNA of strain SR1.

2. Materials and methods

2.1. Micro-organisms and media

P. chlororaphis subsp. *aurantiaca* SR1 (GenBank accession number GU734089) is a native strain isolated from a rhizosphere soil sample of soybean growing in a field in the area of Río Cuarto, Córdoba, Argentina (Rosas, Altamirano, Schröder, & Correa, 2001). Strain SR1 and reference *Pseudomonas* strains were routinely cultured at 28°C on 25% Tryptic Soy Agar (TSA) and stored at –20°C in Tryptic Soy Broth (TSB; Britania®) amended with 20% (v/v) glycerol. *M. phaseolina* (Tassi) Goid. was cultivated in potato dextrose agar (PDA; Britania®) medium at 28°C, maintained at 4°C and replicated monthly.

2.2. Antagonistic activity in vitro

For this purpose, dual culture antagonism assays were carried out on 25% TSA by using the method previously described by Pastor, Carlier, Andrés, Rosas, and Rovera (2012). *M. phaseolina* was equidistantly placed on the opposite side of petri plates 48 h after inoculation of strain SR1. The plates with *M. phaseolina* and not inoculated with SR1 served as controls. For fungal colonies, two diameters, registered at right angles to one another, were averaged to obtain the average value of the diameter. The average diameter of *M. phaseolina* growth in the presence of SR1 was compared with that of control cultures to determine the percentage of inhibition. The diameters of the fungal colonies were recorded using three replicates.

2.3. Biocontrol activity of P. chlororaphis subsp. aurantiaca SR1 against M. phaseolina

Wheat seeds (200 g) were moisturised with distilled water for 12 h, drained and blotted dry with filter paper. Seeds were then placed in glass flasks and autoclaved at 1 atm for 20 min. Flasks containing sterilised seeds were inoculated with eight mycelial plugs of *M. phaseolina* grown on PDA, and incubated at 28°C for four weeks until complete fungal growth on seeds. Finally, flasks were incubated at 34°C for one week to dry fungal biomass and the infested seeds were milled and stored at room temperature in the dark.

Soybean seeds were surface sterilised by soaking in 1% NaClO for 3 min. Then, seeds were thoroughly washed 10 times with sterile distilled water and inoculated with a peat-based formulation of strain SR1 (2.4×10^9 CFU g^{-1}) prepared and packed by Laboratorios Biagro S.A. Plastic pots (20 cm diameter; 25 cm height) were filled with 800 g of soil, previously sterilised by heating at 180°C for 2 h on four consecutive days. Then, four inoculated seeds were placed into the soil surface in each pot. Pathogen inoculum level was 30 g of fungus–wheat mixture kg^{-1} of soil. The eight treatments were: (1) non-bacterised, non-infested healthy control; (2) non-bacterised, infested with *M. phaseolina* control; (3) bacterised with SR1 at 10^7 CFU g^{-1}; (4) bacterised with SR1 at 10^8 CFU g^{-1}; (5) bacterised with SR1 at 10^9 CFU g^{-1}; (6) bacterised with SR1 at 10^7 CFU g^{-1} and infested with *M. phaseolina;* (7) bacterised with SR1 at 10^8 CFU g^{-1} and infested with *M. phaseolina* and (8) bacterised with SR1 at 10^9 CFU g^{-1} and infested with *M. phaseolina*. The inoculant containing 10^8 and 10^7 CFU g^{-1} was obtained by adding sterile peat to the original formulation. Pots were incubated in a greenhouse. Percentages of pre- and post-emergence damping-off were recorded 10 and 25 days after planting,

respectively. Pre-emergence damping-off was based on the number of non-germinated seeds in relation to the number of sown seeds, whereas post-emergence damping-off was based on the number of seedlings showing disease symptoms in relation to the number of emerged seedlings. In addition, shoot and root length as well as shoot and root fresh and dry (120 h at 60°C) weights were recorded from each treatment after 25 days. Pots were arranged in a completely randomised design with five replicates per treatment. The experiment was repeated twice. On the other hand, the persistence of SR1 in the rhizosphere of soybean plants was determined at 7, 15 and 25 days after planting. One gram of rhizosphere soil was collected on the seventh day from around a seedling from each treatment and placed into 9 ml of sterile phosphate buffer. Additionally, a seedling from each treatment was carefully removed from a pot at 15 and 25 days and the roots were gently shaken to remove all but the firmly adhering soil. One gram of the adhering rhizosphere soil was taken and placed into 9 ml of sterile phosphate buffer. Serial dilutions of the suspension were vortexed and inoculated onto 25% TSA supplemented with ampicillin (100 µg ml^{-1}) and cycloheximide (50 µg ml^{-1}). *P. chlororaphis* subsp. *aurantiaca* SR1 is resistant to the mentioned antibiotics concentrations (data not shown). Plates were incubated for 48 h at 28°C. Colonies showing a characteristic orange pigment were counted and the number of CFU g^{-1} of soil was calculated.

2.4. Detection of antibiotic and HCN encoding genes in P. chlororaphis subsp. aurantiaca SR1

Total DNA was isolated from SR1 cells by a standard protocol (Sambrook & Russell, 2001). Then, polymerase chain reaction (PCR) assays were carried out to detect *phlD, phz*, *prnD* and *pltC* genes, according to the protocols described by Raaijmakers, Weller, and Thomashow (1997) and Souza and Raaijmakers (2003). *Pseudomonas* sp. Phz24 and *P. protegens* CHA0 were used as positive controls. Additionally, the detection of *hcnAB* genes was performed as previously described (Rosas et al., 2012).

2.5. Production of antibiotics and HCN

The production of PCA and PRN was revealed by thin-layer chromatography (TLC) according to Pliego et al. (2007). Extractions of supernatants from four-day KB cultures were performed with chloroform/methanol (2:1 v/v). The extracted material was fractionated on TLC plates with chloroform/acetone (9:1 v/v). PRN spots were detected after spraying the plates with diazotized sulfanilic acid. Visualisation of PCA spots was carried out under UV light (254 nm). *P. protegens* CHA0 and *Pseudomonas* sp. Phz24 were used as references.

To detect the production of HCN, *P. chlororaphis* subsp. *aurantiaca* SR1 was grown at 28°C in TSB, in a rotatory shaker. Filter paper (Whatman No. 1) was cut into strips of 10 cm long and 0.5 cm wide that were saturated with alkaline picrate solution and placed inside Erlenmeyer flasks, in a hanging position. After 72 h of incubation, the sodium picrate developed a reddish colour in proportion to the amount of hydrocyanic acid released by the strain. The colour was eluted by placing the filter paper into a clean tube containing 10 ml of distilled water and the absorbance was measured at 625 nm (Sadasivam & Manickam, 1992). *P. protegens* CHA0 was used as positive control. Three replicates were performed for each strain.

2.6. Statistical analyses

The pot experiments were conducted two times under the same conditions using the same treatments to ensure reproducibility of results. Five pots per treatment were used. In the experiments for determining plant growth parameters, each pot was considered as a replicate. Since these results were homogenous, they were pooled together and analysed by using analysis of variance (ANOVA). When ANOVA showed treatment effect, the Scheffé test was applied to compare means at $P < 0.05$. All data were subjected to statistical analysis using Statgraphics plus software for Windows V 4.1 (Statistical Graphics, USA).

3. Results

3.1. *Antagonistic activity* in vitro

Strain SR1 was tested for its antagonistic activity against *M. phaseolina* in dual culture *in vitro*. We observed a complete inhibition of *M. phaseolina* mycelial growth after seven days of incubation at 28°C (Figure 1).

3.2. *Biocontrol activity of* P. chlororaphis *subsp.* aurantiaca *SR1 against* M. phaseolina

Results in Table 1 demonstrate that the pathogenic effect of *M. phaseolina* was considerably greater in the non-bacterised, infested control. Indeed, we observed the highest percentage of pre-emergence damping-off (35%) in this treatment. On the other hand, results indicate that *P. chlororaphis* subsp. *aurantiaca* SR1 significantly reduced pre-emergence damping-off of soybean. Results also show that control plants and plants inoculated with strain SR1 alone, at different concentrations, grew normally. With respect to post-emergence damping-off, the non-bacterised, infested control also had the highest percentage (15%), whereas strain SR1 effectively protected soybean plants. No significant differences were observed among the three inoculation doses in the bacterised, infested treatments.
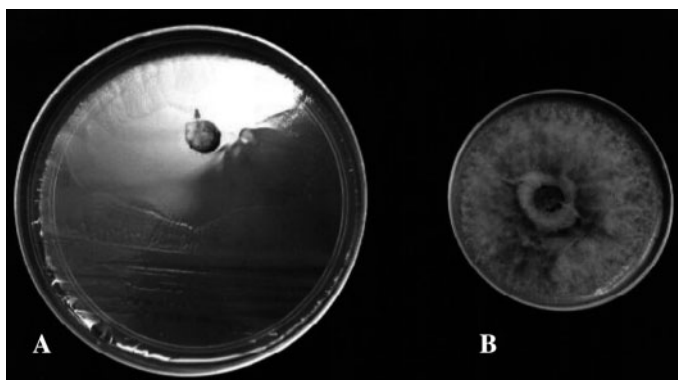
Figure 1. Antagonistic activity of strain SR1 against *M. phaseolina* in 25% TSA. A mycelial disc (9 mm diameter) of a 10-day-old-culture of *M. phaseolina* was equidistantly placed on the opposite side of petri plates 48 h after inoculation of the bacterial strain. Plates were incubated for seven days at 28°C. (A) growth of *M. phaseolina* in medium previously inoculated with strain SR1; (B) *M. phaseolina* placed on the centre of petri plates (fungal control).

Table 1. Percentages of pre- and post-emergence damping-off in the biocontrol assays.

Treatments	Pre-emergence damping-off %	Post-emergence damping-off %
Non-bacterised, non-infested healthy control	0	0
Non-bacterised, infested with *M. phaseolina* control	35a	15a
Bacterised with SR1 at 10^7 CFU g^{-1}	0	0
Bacterised with SR1 at 10^8 CFU g^{-1}	0	0
Bacterised with SR1 at 10^9 CFU g^{-1}	0	0
Bacterised with SR1 at 10^7 CFU g^{-1} and infested with *M. phaseolina*	15b	6b
Bacterised with SR1 at 10^8 CFU g^{-1} and infested with *M. phaseolina*	20b	6b
Bacterised with SR1 at 10^9 CFU g^{-1} and infested with *M. phaseolina*	25b	7b

Percentages of pre- and post-emergence damping-off were recorded for 10 and 25 days after planting, respectively. Data represent the average of two experiments ($n = 20$ seeds per treatment, five pots with four seeds each). Percentages from each column with different letters are significantly different according to the Scheffé test ($P < 0.05$).

The effect of inoculating seeds with strain SR1 on soybean growth parameters is shown in Figures 2 and 3. SR1 inoculated alone at 10^9 CFU g^{-1} enhanced shoot length in 31%, as compared to the uninoculated control treatment. Also, inoculation with SR1 alone at 10^7 and 10^8 CFU g^{-1} significantly increased root fresh weight, as compared to the uninoculated control treatment.

Inoculation of *M. phaseolina* caused significant reductions in shoot and root length and shoot and root fresh and dry weights, as compared to non-bacterised, non-infested healthy controls (Figures 2 and 3). In *M. phaseolina* infested soil, there were differences among the SR1 doses for the measured parameters. Although there were no significant differences among 10^7 and 10^8 CFU g^{-1} doses for shoot fresh and dry weight and root dry weight, the optimum inoculation dose was 10^7 CFU g^{-1}. Compared to non-bacterised pathogen controls, this dose increased shoot and root length by 277% and 290%, shoot and root fresh weight by 263% and 351%, and shoot and root dry weight by 275% and 375%, respectively (Figures 2 and 3). On the other hand, we did not observe differences in shoot length between plants infested/bacterised with SR1 at 10^7 and healthy control plants. When soybean seeds were inoculated with the formulation containing strain SR1 at 10^9, colony counts from rhizosphere soil at 25 days were ~10^5 CFU g^{-1} in the non-infested/bacterised and infested/bacterised treatments. When seeds were treated with SR1 at 10^7 and 10^8, colony counts at 25 days were ~10^4 CFU g^{-1} rhizosphere soil in the non-infested/bacterised and infested/bacterised treatments.

3.3. Detection of antibiotic and HCN encoding genes in P. chlororaphis *subsp.* aurantiaca *SR1*

We tested strain SR1 for the presence of operons for the biosynthesis of DAPG, PCA, PRN, PLT and HCN by PCR, using specific primers. A fragment of the predicted size for each one of these compounds was observed from the DNA of the
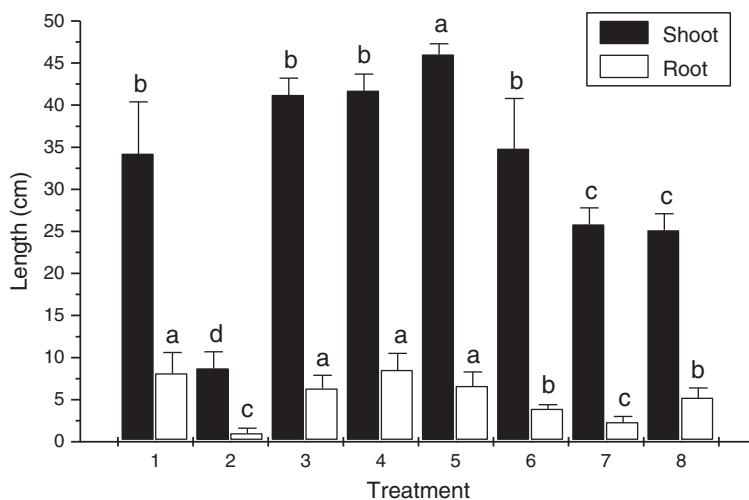
Figure 2. Soybean shoot and root length in the biocontrol assay. Soybean seeds were surface sterilised, inoculated with strain SR1 and sown. Measurements were performed from each treatment after 25 days: (1) non-bacterised, non-infested healthy control; (2) non-bacterised, infested with *M. phaseolina* control; (3) bacterised with SR1 at 10^7 CFU g^{-1}; (4) bacterised with SR1 at 10^8 CFU g^{-1}; (5) bacterised with SR1 at 10^9 CFU g^{-1}; (6) bacterised with SR1 at 10^7 CFU g^{-1} and infested with *M. phaseolina*; (7) bacterised with SR1 at 10^8 CFU g^{-1} and infested with *M. phaseolina*; (8) bacterised with SR1 at 10^9 CFU g^{-1} and infested with *M. phaseolina*. Data represent the average of two experiments ± standard deviation. Bars for each plant fraction with different letters are significantly different according to the Scheffé test ($P < 0.05$).

reference *Pseudomonas* strains that produce these compounds. On the other hand, fragments of the predicted size for PCA (~1, 100 bp), PRN (~780 bp; Figure 4) and HCN (~570 bp; Figure 5) were amplified from the DNA of *P. chlororaphis* subsp. *aurantiaca* SR1.

3.4. Production of antibiotics and HCN

P. chlororaphis subsp. *aurantiaca* SR1 produced spots corresponding to PCA and PRN. The control strain *P. protegens* CHA0 produced a spot corresponding to PRN and *Pseudomonas* sp. Phz24 produced PCA and PRN.

HCN forms a brownish red compound with sodium picrate and the intensity of the colour increases with the amount of HCN. In this study, *P. chlororaphis* subsp. *aurantiaca* SR1 produced HCN, which turned yellow-coloured filter strips to reddish colour. The assay revealed that strain SR1 liberated 0.15 µg/ml HCN after 72 h whereas *P. protegens* CHA0 produced 0.16 µg/ml HCN.

4. Discussion

During the last few years, special attention has been paid to the study of novel technologies that facilitate the use of sustainable agricultural practices. Such practices focus on a proper utilisation of natural resources for reducing the application of chemical pesticides. In this regard, the use of natural organisms
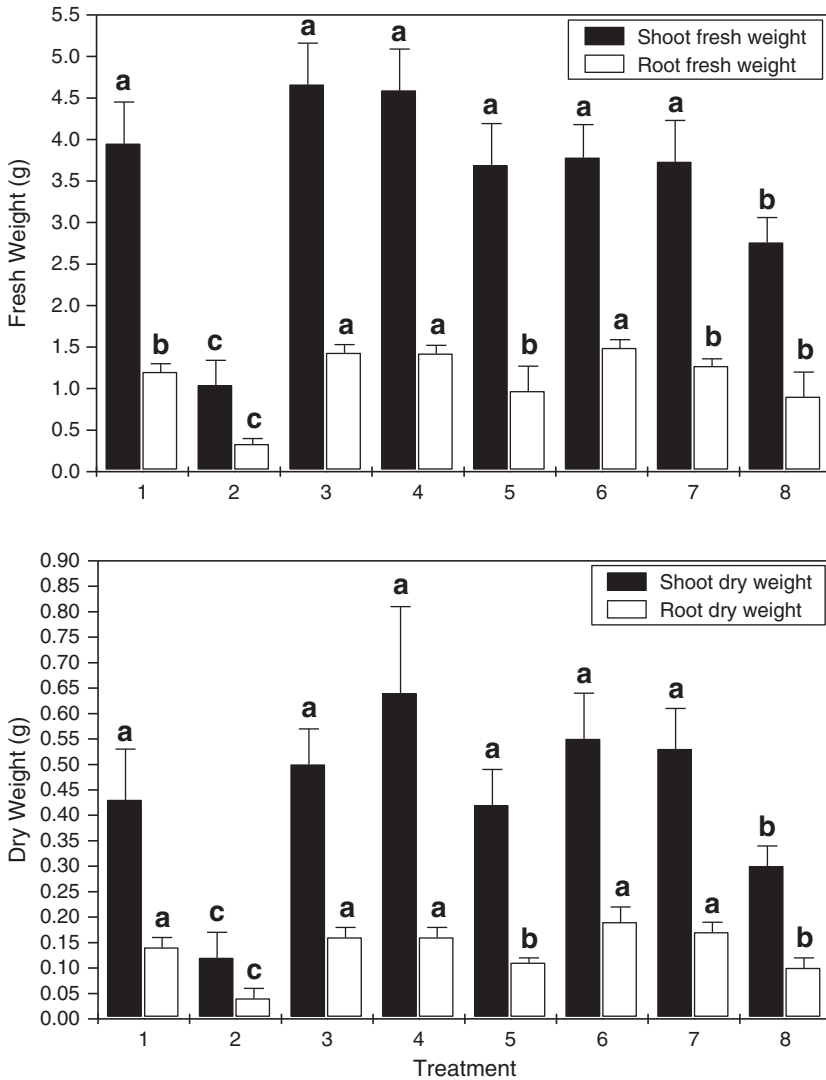
Figure 3. Soybean shoot and root fresh and dry weights in the biocontrol assay. Soybean seeds were surface sterilised, inoculated with strain SR1 and sown. Measurements were performed from each treatment after 25 days: (1) non-bacterised, non-infested healthy control; (2) non-bacterised, infested with *M. phaseolina* control; (3) bacterised with SR1 at 10^7 CFU g^{-1}; (4) bacterised with SR1 at 10^8 CFU g^{-1}; (5) bacterised with SR1 at 10^9 CFU g^{-1}; (6) bacterised with SR1 at 10^7 CFU g^{-1} and infested with *M. phaseolina*; (7) bacterised with SR1 at 10^8 CFU g^{-1} and infested with *M. phaseolina*; (8) bacterised with SR1 at 10^9 CFU g^{-1} and infested with *M. phaseolina*. Data represent the average of two experiments ± standard deviation. Bars for each plant fraction and for each weight with different letters are significantly different according to the Scheffé test ($P < 0.05$).
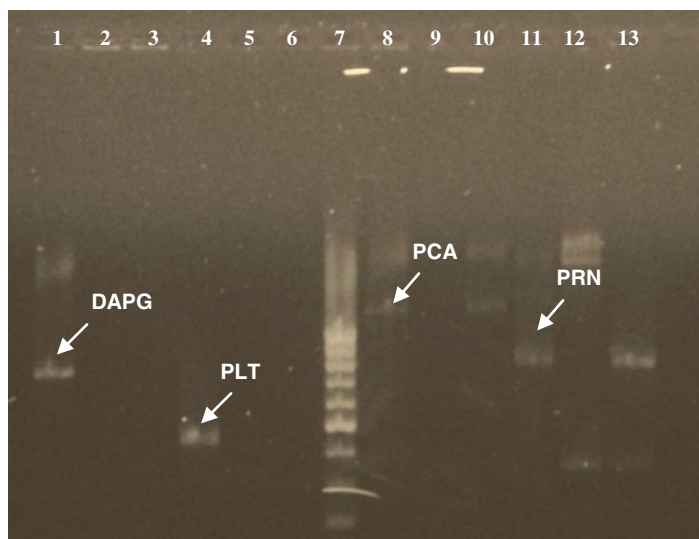
Figure 4. (Colour online) Gel electrophoresis of PCR products amplified from the DNA of *P. protegens* CHA0, strain SR1 and *Pseudomonas* sp. Phz24 with primers that target for genes for the biosynthesis of DAPG, PLT, PCA and PRN. Lane 1: CHA0; Lane 3: SR1; Lane 4: CHA0; Lane 6: SR1; Lane 7: molecular weight marker (mi-100 bp+ DNA Marker Go); Lane 8: Phz24; Lane 10: SR1; Lane 11: CHA0; Lane 13: SR1. Arrows indicate the presence of bands corresponding to DAPG, PLT, PCA and PRN from the DNA of strains CHA0 (DAPG, PLT and PRN) and Phz24 (PCA).

(rhizobacteria) has found a potential role in the development of sustainable systems for crop production. Unlike chemical control, the repercussions of biocontrol on the environment are lower due to the biodegradability of its compounds. The host specificity is also an important attribute for a safe, effective biocontrol.

Isolation of micro-organisms, screening for aspired characteristics, selection of strains and manufacturing of inocula and formulations are all primordial for the development of a sustainable technology. Besides the direct benefits of the use of plant growth-promoting micro-organisms, the ecological and rhizosphere competence and their effect on the native rhizosphere micro-organisms are also important for selecting suitable microbial inoculants (Pandey, Sharma, & Palni, 1998; Trivedi, Pandey, & Palni, 2012). In this context, it is important to consider the ecological specificity of micro-organisms and promote the use of native micro-organisms as microbial inoculants for field applications.

The contribution of *P. chlororaphis* subsp. *aurantiaca* for the biocontrol of plant diseases was amply demonstrated (Mandryk, Kolomiets, & Dey, 2007; Mehnaz, Baig, Jamil, Weselowski, & Lazarovits, 2009). *P. chlororaphis* subsp. *aurantiaca* SR1 has a strong antifungal activity *in vitro* against different pathogenic fungi such as *M. phaseolina*, *R. solani*, *Pythium* spp., *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Fusarium* spp. and *Alternaria* spp. (Rosas et al., 2001). In addition, survival of SR1 was demonstrated both in the rhizosphere and in the internal structures of different crops (Rosas et al., 2005). More recently, we reported on the efficiency of SR1 to improve the productivity of several agronomically important crops such as alfalfa, wheat, maize and soybean (Rosas et al., 2012).
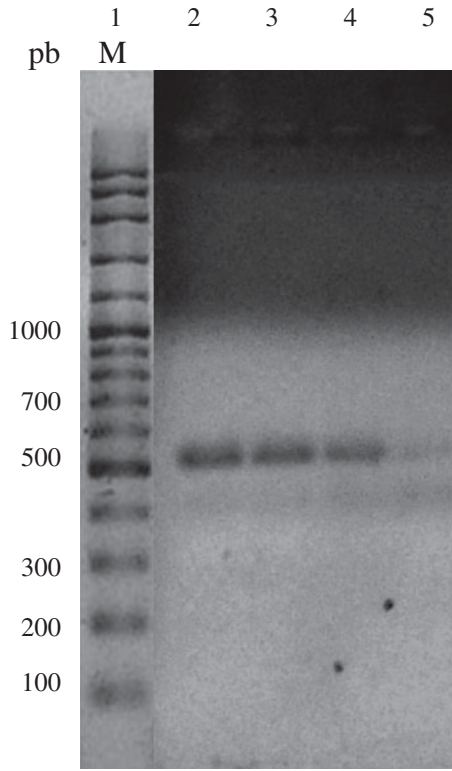
Figure 5. Gel electrophoresis of PCR products amplified from the DNA of *P. protegens* CHA0 and strain SR1 with a primer for HCN. Lane 1: molecular weight marker (Mass Ruler DNA ladder Mix; FERMENTA); Lane 2: CHA0; Lanes 3 and 4: SR1; Lane 5: negative control.

More than 100 fungal species attack soybean worldwide. Of these, only 35 have economic relevance. Seeds carrying *M. phaseolina* do not germinate or yield diseased, malformed seedlings. *M. phaseolina* is present in all of the soybean crop areas around the world and can cause production losses of up to 80%. As an alternative management method, we evaluated SR1 for control of this phytopathogen. Strain SR1 completely inhibited the mycelial growth of *M. phaseolina in vitro*, which indicated secretion of certain antifungal metabolites by this strain.

To our knowledge, this is the first report on the biocontrol of *M. phaseolina* by a *P. chlororaphis* subsp. *aurantiaca* strain in soybean plants. Interestingly, we observed a lesser damage by *M. phaseolina* on seedlings after inoculation of seeds with an inoculant carrying 10^7 CFU of SR1 g^{-1} peat. Singh et al. (2008) showed that inoculation of chir-pine (*P. roxburghii*) seeds with 10^8 CFU g^{-1} seedling of *Bacillus subtilis* BN1 caused 75% and 130% increases in root and shoot dry weights of plants, respectively, in *M. phaseolina* infested soil, as compared to plants from soil infested with *M. phaseolina* alone. In contrast, Arora et al. (2001) reported that bacterisation of groundnut seeds with strains of *Sinorhizobium meliloti* increased seedling biomass between 16.5% and 22% in *M. phaseolina* infested soil. By comparison, we observed increases of over 200% in shoot and root length and in shoot and root dry weights of

plants, as compared to non-bacterised/infested controls. On the other hand, results indicate that SR1, inoculated at 10^9 CFU g^{-1}, reduces root dry weight. Similarly, when we measured soybean shoot length in the presence of the pathogen, we observed that the best results were obtained when SR1 was inoculated at the lowest concentration evaluated. This highlights the importance of standardising the most effective dose of a bioformulation for its potential application. Most biocontrol strains of *Pseudomonas* spp. with a proven effect in plant bioassays produce one or several antibiotic compounds. High concentrations of antibiotics produced by these micro-organisms affect pathogens, but may also result, in some cases, in phytotoxic effects (Barea et al., 1998; Haas & Défago, 2005).

We carried out experiments for detecting biosynthetic genes implicated in production of antifungal compounds and HCN in *P. chlororaphis* subsp. *aurantiaca* SR1. Antibiotics are toxins produced by microbes that can kill other micro-organisms (Pal & McSpadden Gardener, 2006). Antibiosis is one of the most studied mechanisms of biological control and one of the selection traits to consider when screening potential BCAs (Glick, 1995; O'Sullivan & O'Gara 1992). Results from PCR reactions suggest that SR1 contains the genes for the biosynthesis of PCA and PRN. Furthermore, detection of PCA and PRN by TLC indicated that strain SR1 produces these compounds. Raaijmakers et al. (1997) and Souza and Raaijmakers (2003) concluded that the primers used allow the specific detection of bacterial strains that produce PCA and PRN. Nevertheless, further experiments such as sequencing of the amplified fragments could be performed in order to verify the specificity of PCR products. Phenazine type antibiotics, such as PCA, are particularly active against lower fungi (Feklistova & Maksimova, 2008). Park et al. (2011) demonstrated that PRN produced by *P. chlororaphis* O6 is a key constituent in the inhibition of some phytopathogenic fungi such as *Phytophthora infestans*, which causes the tomato late blight disease. Genes for biosynthesis as well as production of HCN were also detected in *P. chlororaphis* subsp. *aurantiaca* SR1. HCN is known to be involved in the biocontrol of root diseases by several fluorescent pseudomonads (Ramette, Frapolli, Défago, & Moënne-Loccoz, 2003). Thus, it seems reasonable to assume that production of compounds such as PCA, PRN and HCN play a role in the inhibition of *M. phaseolina*. These attributes would add up to other already investigated traits of this strain, which are possibly involved in its plant growth improvement effect, such as production of IAA and siderophores. Thus, SR1 would exert its beneficial effect as a potential antagonistic agent and/or plant growth promoter.

To conclude, this work demonstrated the ability of *P. chlororaphis* subsp. *aurantiaca* SR1 to significantly reduce damping-off of soybean caused by *M. phaseolina* and to increase the biomass of soybean plants, under greenhouse conditions. The ability of *P. chlororaphis* subsp. *aurantiaca* SR1 to colonise soybean roots and to synthetize PRN, PCA and HCN suggests the potential use of this bacterium for reducing the incidence of damping-off of soybean and the use of fungicides. Thus, future studies will focus on the use of strain SR1 as a BCA against *M. phaseolina* in field experiments. Finally, the beneficial effects of strain SR1, studied *in vitro* and in different crops, allow us to hypothesise that SR1 could be applied for controlling other necrotrophic pathogens in other plant hosts or that it could be applied in other hosts infected by *M. phaseolina*.

References

Anis, M., Abbasi, M. W., & Zaki, M. J. (2010). Bioefficacy of microbial antagonists against *Macrophomina phaseolina* on sunflower. *Pakistan Journal of Botany*, *42*, 2935–2940.

Anjaiah, V. (2004). Biological control mechanisms of fluorescent Pseudomonas species involved in control of root diseases of vegetables/fruits. In K. G. Mukerji (Ed.), *Disease management of fruits and vegetables* (pp. 453–500). Netherlands: Kluwer Academic.

Arora, N. K., Kang, S. C., & Maheshwari, D. K. (2001). Isolation of siderophore-producing strains of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut. *Current Science*, *81*, 673–677.

Barea, J. M., Andrade, G., Bianciotto, V., Dowling, D., Lohrke, S., Bonfante, P., … Azcon-Aguilar, C. (1998). Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for biocontrol of soil-borne fungal plant pathogens. *Applied and Environmental Microbiology*, *64*, 2304–2307.

Carlier, E., Rovera, M., Jaume, A. R., & Rosas, S. B. (2008). Improvement of growth, under field conditions, of wheat inoculated with *Pseudomonas chlororaphis* subsp. *aurantiaca* SR1. *World Journal of Microbiology and Biotechnology*, *24*, 2653–2658. doi:10.1007/s11274-008-9791-6

Feklistova, I. N., & Maksimova, N. P. (2008). Obtaining *Pseudomonas aurantiaca* strains capable of overproduction of phenazine antibiotics. *Microbiology*, *77*, 176–180. doi:10.1134/S0026261708020094

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology*, *41*, 107–120.

Haas, D., & Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology*, *3*, 307–319. doi:10.1038/nrmicro1129

Kloepper, J. W., Leong, J., Teintze, M., & Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. *Nature*, *286*, 885–886. doi:10.1038/286885a0

Leeman, M., Den Ouden, F. M., Van Pelt, J. A., Cornelissen, C., Matamala-Garros, A., Bakker, P. A. H. M., & Schippers, B. (1996). Suppression of fusarium wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. *European Journal of Plant Pathology*, *102*, 21–31. doi:10.1007/BF01877112

Lifshitz, R., Simonson, C., Scher, F. M., Kloepper, J. W., Rodrick-Semple, C., & Zaleska, I. (1986). Effect of rhizobacteria on the severity of *Phytophthora* root rot of soybean. *Canadian Journal of Plant Pathology*, *8*, 102–106. doi:10.1080/07060668609501850

Mandryk, M. N., Kolomiets, E. I., & Dey, E. S. (2007). Characterization of antimicrobial compounds produced by *Pseudomonas aurantiaca* S-1. *Polish Journal of Microbiology*, *56*, 245–250.

Mehnaz, S., Baig, D. N., Jamil, F., Weselowski, B., & Lazarovits, G. (2009). Characterization of a phenazine and hexanoyl homoserine lactone producing *Pseudomonas aurantiaca* strain PB-St2, isolated from sugarcane stem. *Journal of Microbiology and Biotechnology*, *19*, 1688–1694. doi:10.4014/jmb.0904.04022

Mercado-Blanco, J., & Bakker, P. A. H. M. (2007). Interactions between plants and beneficial *Pseudomonas* spp.: Exploiting bacterial traits for crop protection. *Antonie van Leeuwenhoek*, *92*, 367–389. doi:10.1007/s10482-007-9167-1

O'Sullivan, D. J., & O'Gara, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiological Reviews*, *56*, 662–676.

Pal, K. K., & McSpadden Gardener, B. (2006). Biological control of plant pathogens. Retrieved from http://www.apsnet.org/edcenter/advanced/topics/Documents/PHI-Biologi calControl.pdf

Pandey, A., Sharma, E., & Palni, L. M. S. (1998). Influence of bacterial inoculation on maize in upland farming systems of the Sikkim Himalaya. *Soil Biology and Biochemistry*, *30*, 379–384. doi:10.1016/S0038-0717(97)00121-1

Park, J. Y., Oh, S. A., Anderson, A. J., Neiswender, J., Kim, J. C., & Kim, Y. C. (2011). Production of the antifungal compounds phenazine and pyrrolnitrin from *Pseudomonas chlororaphis* O6 is differentially regulated by glucose. *Letters in Applied Microbiology*, *52*, 532–537. doi:10.1111/j.1472-765X.2011.03036.x

Pastor, N., Carlier, E., Andrés, J., Rosas, S. B., & Rovera, M. (2012). Characterization of rhizosphere bacteria for control of phytopathogenic fungi of tomato. *Journal of Environmental Management*, *95*, S332–S337. doi:10.1016/j.jenvman.2011.03.037

Pliego, C., Cazorla, F. M., González-Sánchez, M.Á., Pérez-Jiménez, R. M., de Vicente, A., & Ramos, C. (2007). Selection for biocontrol bacteria antagonistic toward *Rosellinia necatrix* by enrichment of competitive avocado root tip colonizers. *Research in Microbiology*, *158*, 463–470. doi:10.1016/j.resmic.2007.02.011

Raaijmakers, J. M., Weller, D. M., & Thomashow, L. S. (1997). Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology*, *63*, 881–887.

Ramette, A., Frapolli, M., Défago, G., & Moënne-Loccoz, Y. (2003). Phylogeny of HCN synthase-encoding *hcnBC* genes in biocontrol fluorescent pseudomonads and its relationship with host plant species and HCN synthesis ability. *Molecular Plant-Microbe Interactions*, 16, 525–535. doi:10.1094/MPMI.2003.16.6.525

Rosas, S. B., Altamirano, F., Schröder, E., & Correa, N. (2001). *In vitro* biocontrol activity of *Pseudomonas aurantiaca*. *Фyton-International Journal of Experimental Botany*, *67*, 203–209.

Rosas, S. B., Avanzini, G., Carlier, E., Pasluosta, C., Pastor, N., & Rovera, M. (2009). Root colonization and growth promotion of wheat and maize by *Pseudomonas aurantiaca* SR1. *Soil Biology and Biochemistry*, *41*, 1802–1806. doi:10.1016/j.soilbio.2008.10.009

Rosas, S. B., Pastor, N. A., Guiñazú, L. B., Andrés, J. A., Carlier, E., Vogt, M. V., … Rovera, M. (2012). Efficacy of *Pseudomonas chlororaphis* subsp. *aurantiaca* SR1 for improving productivity of several crops. In P. Sharma (Ed.), *Crop Production Technologies* (pp. 199–210). Rijeka, Croatia: InTech.

Rosas, S., Rovera, M., Andrés, J. A., Pastor, N. A., Guiñazú, L. B., & Carlier, E. (2005). Characterization of *Pseudomonas aurantiaca* as biocontrol and PGPR agent. Endophytic properties. In S. Sorvari & O. Toldo (Eds.), *Proceeding prospects and applications for plant associated microbes* (pp. 91–99). Lapland, Finland.

Rovera, M., Carlier, E., Pasluosta, C., Avanzini, G., Andres, J., & Rosas, S. (2008). *Pseudomonas aurantiaca*: Plant growth promoting traits, secondary metabolites and inoculation response. In I. Ahmad, J. Pichtel, & S. Haya (Eds.), *Plant–bacteria interactions. Strategies and techniques to promote plant growth* (pp. 155–164). Weinheim: Wiley–VCH.

Sadasivam, S., & Manickam, A. (1992). *Biochemical methods for agricultural sciences*. New Delhi: Wiley Eastern.

Sahni, S., Sarma, B. K., & Singh, K. P. (2008). Management of *Sclerotium rolfsii* with integration of non-conventional chemicals, vermicompost and *Pseudomonas syringae*. *World Journal of Microbiology and Biotechnology*, *24*, 517–522. doi:10.1007/s11274-007-9502-8

Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Savithiry, S., & Gnanamanickam, S. S. (1987). Bacterization of peanut with *Pseudomonas fluorescens* for biological control of *Rhizoctonia solani* and for enhanced yield. *Plant and Soil*, *102*, 11–15. doi:10.1007/BF02370893

Senthilkumar, M., Swarnalakshmi, K., Govindasamy, V., Lee, Y. K., & Annapurna, K. (2009). Biocontrol potential of soybean bacterial endophytes against charcoal rot fungus, *Rhizoctonia bataicola*. *Current Microbiology*, *58*, 288–293. doi:10.1007/s00284-008-9329-z

Singh, N., Pandey, P., Dubey, R. C., & Maheshwari, D. K. (2008). Biological control of root rot fungus *Macrophomina phaseolina* and growth enhancement of *Pinus roxburghii* (Sarg.)

by rhizosphere competent *Bacillus subtilis* BN1. *World Journal of Microbiology and Biotechnology*, *24*, 1669–1679. doi:10.1007/s11274-008-9680-z

Souza, J. T., & Raaijmakers, J. M. (2003). Polymorphisms within the *prnD* and *pltC* genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia* spp. *FEMS Microbiology Ecology*, *43*, 21–34. doi:10.1111/j.1574-6941.2003.tb01042.x

Suslow, T. V., & Schroth, M. N. (1982). Rhizobacteria of sugar beets: Effects of seed application and root colonization on yield. *Phytopathology*, *72*, 199–206. doi:10.1094/Phyto-72-199

Trivedi, P., Pandey, A., & Palni, L. M. S. (2012). Bacterial inoculants for field applications under mountain ecosystem: Present initiatives and future prospects. In D. K. Maheshwari (Ed.), *Bacteria in agrobiology: Plant probiotics* (pp. 15–44). Berlin, Heidelberg: Springer-Verlag.

Wang, S.-L., Yieh, T.-C., & Shih, I.-L. (1999). Production of antifungal compounds by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as a carbon source. *Enzyme and Microbial Technology*, *25*, 142–148. doi:10.1016/S0141-0229(99)00024-1