

Phenylacetic acid-producing *Rhizoctonia solani* represses the biosynthesis of nematocidal compounds *in vitro* and influences biocontrol of *Meloidogyne incognita* in tomato by *Pseudomonas fluorescens* strain CHA0 and its GM derivatives

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ABSTRACT

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Aims: The aim of the present investigation was to determine the influence of *Rhizoctonia solani* and its pathogenicity factor on the production of nematocidal agent(s) by *Pseudomonas fluorescens* strain CHA0 and its GM derivatives *in vitro* and nematode biocontrol potential by bacterial inoculants in tomato.

Methods and Results: One (Rs7) of the nine *R. solani* isolates from infected tomato roots inhibited seedling emergence and caused root rot in tomato. Thin layer chromatography revealed that culture filtrates of two isolates (Rs3 and Rs7) produced brown spots at *Rf*-values closely similar to synthetic phenylacetic acid (PAA), a phytotoxic factor. Filtrates from isolate Rs7, amended with the growth medium of *P. fluorescens*, markedly repressed nematocidal activity and *PhlA*'-*LacZ* reporter gene expression of the bacteria *in vitro*. On the contrary, isolate Rs4 enhanced nematocidal potential of a 2,4-diacetylphloroglucinol overproducing mutant, CHA0/pME3424, of *P. fluorescens* strain CHA0 *in vitro*. Therefore, *R. solani* isolates Rs4 and Rs7 were tested more rigorously for their potential to influence biocontrol effectiveness of the bacterial agents. Methanol extract of the culture filtrates of PAA-producing isolate Rs7 resulting from medium amended with phenylalanine enhanced fungal repression of the production of nematocidal agents by bacteria, while amendments with zinc or molybdenum eliminated such fungal repression, thereby restoring bacterial potential to cause nematode mortality *in vitro*. A pot experiment was carried out, 3-week-old tomato seedlings were infested with *R. solani* isolates Rs4 or Rs7 and/or inoculated with *Meloidogyne incognita*, the root-knot nematode. The infested soil was treated with aqueous cell suspensions (10^8 CFU) of *P. fluorescens* strain CHA0 or its GM derivatives or left untreated (as a control). Observations taken 45 days after nematode inoculation revealed that, irrespective of the bacterial treatments, galling intensity per gram of fresh tomato roots was markedly higher in soil amended with isolate Rs4 than in Rs7-amended soils. Soil amendments with *R. solani* and the bacterial antagonists resulted in substantial reductions of the number of galls per gram of root. These results are contradictory to those obtained under *in vitro* conditions where culture filtrates of PAA-positive Rs7 repressed the production of nematocidal compounds. Plants grown in Rs7-amended soils, with or without bacterial inoculants, had lesser shoot and root weights than plants grown in nonamended or Rs4-amended soils. Moreover, amendments with Rs7 substantially retarded root growth and produced necrotic lesions that reduced the number of entry sites for invasion and subsequent infection by nematodes. Populations of *P. fluorescens* in the tomato rhizosphere were markedly higher in Rs7-amended soils.

Conclusions: PAA-producing virulent *R. solani* drastically affects the potential of *P. fluorescens* to cause death of *M. incognita* juveniles *in vitro* and influences bacterial effectiveness to suppress nematodes in tomato roots.

Significance and Impact of the Study: As most agricultural soils are infested with root-infecting fungi, including *R. solani*, it is likely that some PAA-producing isolates of the fungus may also be isolated from such soils. The inhibitory effect of PAA-producing *R. solani* on the biosynthesis of nematicidal agent(s) critical in biocontrol may reduce or even eliminate the effectiveness of fluorescent pseudomonads against root-knot nematodes, both in nursery beds and in field conditions. Introduction of bacterial inoculants, for the control of any plant pathogen, should be avoided in soils infested with PAA-producing *R. solani*. Alternatively, the agents could be applied together with an appropriate quantity of fungicide or chemicals such as zinc to create an environment more favourable for bacterial biocontrol action.

Keywords: biological control, mycotoxins, plant-parasitic nematodes, root-infecting fungi, soil-borne fungi.

INTRODUCTION

Interest in biological control has increased recently, fuelled by public concern over the use of chemicals in the environment in general and the need to find alternatives to the use of chemicals for disease control. However, micro-organisms as biological control agents typically have a relatively narrow spectrum of activity compared with synthetic pesticides and often exhibit inconsistent performance in practical agriculture, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (Backman *et al.* 1997). The key to achieving successful, reproducible biological control is knowledge of the ecological interactions taking place in soil and root environments that will allow prediction of the conditions under which biocontrol can be achieved (Deacon 1994; Whipps 1997). Recently improvements in this knowledge may be a part of the reason why more biocontrol agents are reaching the market-place (Whipps and Davies 2000).

Plant-associated micro-organisms have been extensively examined for their role in natural and induced suppressiveness of soil-borne diseases. Among the many groups of such organisms are root-associated bacteria, which generally represent a subset of soil bacteria. Although a range of different bacterial genera and species have been tested for their biocontrol potential against plant pathogens, the overwhelming number of studies have involved the use of *Pseudomonas* species and this begs the question as to the features that make this genus so effective and the choice of many researchers. Pseudomonads are characteristically fast growing, easy to culture and manipulate genetically in the laboratory and are able to utilize a range of easily metabolizable organic compounds, making them amenable to experimentation (Whipps 2001). However, commercial application of these bacteria for biocontrol has been hampered in large part because of their inconsistent

performance between field locations (Weller 1988). Such variability has been attributed to different causes, including host plant age and genotype (Siddiqui and Shaukat 2003a), agricultural practices (Schippers *et al.* 1990), mutation of the biocontrol strain (Duffy and D  fago 2000), pathogen resistance to biocontrol mechanisms (Mazzola *et al.* 1995), various soil physical and chemical properties (Ownley *et al.* 2003), and vulnerability of the biocontrol strain to pathogen defense mechanisms (Duffy and D  fago 1997).

Pseudomonas fluorescens strain CHA0 is a potential biological control agent of plant diseases caused by soil-borne pathogenic fungi (Keel *et al.* 1992) and plant-parasitic nematodes (Siddiqui and Shaukat 2002a). Production of secondary metabolites including 2,4-diacetylphloroglucinol (DAPG) by CHA0, plays a critical role in the suppression of root-knot nematode both *in vitro* and in natural conditions (Siddiqui and Shaukat 2003b). The objectives of the present investigation were to determine the influence of various isolates of *Rhizoctonia solani* from the roots of tomato on nematicidal activity and *phlA*⁺-*lacZ* gene expression by *P. fluorescens* strain CHA0 and its DAPG-overproducing derivative CHA0/pME3424 or DAPG-negative CHA89 *in vitro* and on the biological control potential of the bacterial inoculants against the root-knot nematode, *Meloidogyne incognita* in tomato. *Rhizoctonia solani* was selected here because: (i) the fungus is widespread in most agricultural fields and is considered an important pathogen that causes pre- and postemergence damping-off disease in tomato resulting in serious yield losses; (ii) whereas the influence of other soil-borne root-infecting fungi, including fusaric acid producing *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Notz *et al.* 2002), non-pathogenic *F. solani* (Siddiqui and Shaukat 2003c) and species of *Trichoderma* (Siddiqui and Shaukat 2004a), on the biocontrol potential of *P. fluorescens* strain CHA0 has been reported, the influence of *R. solani* and its patho-

genicity factor on nematode biocontrol by CHA0 is yet to be explored.

MATERIALS AND METHODS

Bacteria and culture conditions

The *P. fluorescens* strain CHA0 used in this study was originally isolated from the rhizosphere of tobacco in a Swiss soil and is considered to be an effective biocontrol agent of soil-borne pathogenic fungi (Keel and Défago 1997). *Pseudomonas fluorescens* CHA0-Rif/pME3424 (a GM derivative of CHA0; Schnider *et al.* 1995), contains *IncP* plasmid (about seven copies per cell) carrying *rpoD* with its natural promoter tetracycline resistant ($125 \mu\text{g ml}^{-1}$) responsible for the overproduction of the antibiotics 2,4-DAPG and pyoluteorin. Strain CHA89 (*gacA::Ω-Km*) is a kanamycin-resistant derivative ($50 \mu\text{g ml}^{-1}$) of CHA0 and is deficient in antibiotic production (Schnider *et al.* 2000). All strains were routinely cultivated at 28°C on King's B agar (King *et al.* 1954). For inoculum production, the strains were grown at 27°C for 24 h with shaking (150 rev min^{-1}) in 250 ml Erlenmeyer flasks containing 100 ml of King's B liquid medium with appropriate amounts of antibiotics. The bacterial culture was centrifuged at $2800 g$ for 20 min, the supernatant discarded and the pellet resuspended in MgSO_4 (0.1 M) prior to use.

Isolation of *R. solani* strains and their disease reaction in tomato seedlings

During a survey of five cultivated fields at Gadap, ca 25 miles west of Karachi city, tomato (*Lycopersicon esculentum*) plants showing yellowing and wilting were randomly collected. Most of the plants growing in the fields were at the flowering stage. From each location, five to 10 plants were taken and brought to the laboratory for the isolation of fungi. Isolations were made from discoloured or necrotic lesions on root and crown tissues. Plant tissues were washed under running tap water, surface sterilized in 0.5% sodium hypochlorite for 2 min and placed on water agar containing 50 mg l^{-1} streptomycin sulphate. After 48–72 h incubation at 28°C , hyphae from the margin of each developing colony were placed on potato dextrose agar (PDA). Isolates of *R. solani* were identified on the basis of characteristics of their vegetative hyphae (Ogoshi 1975; Sneh *et al.* 1991).

The isolates were maintained on PDA at 28°C and tested for their pathogenicity and emergence on tomato cultivar (cv.) SUN 6002 (PVP) seedlings following the procedure described by Yildiz and Döken (2002). The isolates were cultivated separately in 100 ml of Czapek Dox liquid medium at 30°C without shaking for 1 week. The mycelial

mat was removed, placed on a filter paper and dried under laminar flow hood. A 2-g sample was cut into small pieces in 30 ml of sterile distilled water with a flame-sterilized scalpel blade. This suspension was used to infest the soil (Pleban *et al.* 1995). As phenylacetic acid (PAA) is the known pathogenicity factors of *R. solani*, this compound was also tested in pathogenicity bioassays on tomato plants. For synthetic PAA and its derivatives, a 20-ml suspension of the compound ($400 \mu\text{M}$ prepared in sterilized distilled water) was mixed with 350 g of steam-sterilized soil. For each *R. solani* isolate, three replicate pots were prepared and arranged in a randomized pattern on a bench in a glasshouse at 28°C .

Five tomato cv. SUN 6002 (PVP) seeds were placed on the infested soil in each pot and covered with a layer of uninfested soil mix. Seedling emergence was recorded daily from the first day of emergence for 30 days. The percentage of seedling emergence in each treatment was calculated relative to the control. To determine disease severity, tomato seedlings that had been raised in steam-sterilized soil were removed and washed free of soil. To facilitate the penetration of the fungus, the tips of the roots were excised and the seedlings transplanted into 8-cm-diameter plastic pots containing 350 g *R. solani*-infested soil. After 6 weeks, plants were removed, washed and disease severity at the base of the stem and the roots rated on a 0–3-scale (Tezcan and Yildiz 1991) as follows: 0, no visible symptoms; 1, discolouration and wilting of leaves; 2, necrotic lesions on lower stem and 3, plant death.

Production of PAA by *R. solani* strains

The fungi were cultivated for a week in darkness at 30°C in 250 ml capacity Erlenmeyer flasks containing 100 ml of potato dextrose liquid medium. The broth was filtered through two layers of Whatman no. 1 filter paper to obtain the culture filtrate. Initial and final pH of the medium and mycelial dry weight after drying at 80°C for 48 h were determined. Qualitative analysis of PAA in the filtrate was by thin layer chromatography (TLC) using fluorescent Merck silica gel (60F₂₅₄; $5 \text{ cm} \times 12 \text{ cm}$; Merck K GaA, Darmstadt, Germany) after ethyl acetate extraction. A separate TLC plate was used for each *R. solani* isolate. The freshly prepared culture filtrate was spotted at the base of each plate several times and each spot was allowed to dry before the next spot was applied. The TLC plates were developed in toluene : chloroform : acetone (40 : 25 : 35, v/v) using saturation pads. The TLC plates were dried at room temperature and examined under u.v. light (365 nm). The characteristics of all spots, i.e. colour and *R_f*-values, were recorded. Spots were identified by comparing *R_f*-values and colours with those of commercially available

synthetic PAA. The assay was repeated to confirm the production of toxins from the isolates.

Influence of PAA and fungal filtrates on nematicidal activity and *phlA*'-*lacZ* expression by *P. fluorescens* *in vitro*

Autoclaved and semi-cooled potato dextrose liquid medium (20 ml in 100 ml Erlenmeyer flask) was amended with 5 ml of culture filtrate of a *R. solani* isolate. Subsequently, a loopful of the cell suspension of *P. fluorescens* was added to the medium. Treatments were arranged as an 11 × 4-factorial split-plot design, with main plots of the culture filtrates of nine *R. solani* strains, PAA treatment (500 µM) and the control and subplots of the bacterial inoculants (none, CHA0, CHA0/pME3424 and CHA89). In our laboratory experiments, concentrations of 100 and 200 µM of PAA had no significant influence while 400 µM or more markedly repressed bacterial efficacy in causing nematode deaths (I.A. Siddiqui and S.S. Shaukat, unpublished data). Two replicate flasks were kept for each treatment and after 48 h, the bacterial cultures were divided into two equal halves. One of the portions was used to determine the β-galactosidase activity while the other half was used for the assessment of nematicidal activity. In order to compensate for the different amounts of bacterial growth caused by the addition of different *R. solani* culture filtrates, β-galactosidase and nematicidal activities are reported for a certain cell density (O.D.₆₀₀ of 1.0) rather than for incubation time; an O.D.₆₀₀ of 1.0 corresponds to 4.5×10^8 CFU ml⁻¹. β-galactosidase assays were conducted following the method of Miller (1992). Activities were expressed as units per 10⁸ CFU. Unit values were calculated by the method of Miller (1992), following the formula: $1000 \times \text{O.D.}_{420} / [\text{time (min)} \times \text{sample volume (ml)}] = \text{units of } \beta\text{-galactosidase}$.

For the preparation of culture filtrate, the remaining half of the bacterial culture was centrifuged twice at 2800 g for 20 min. The pellet was discarded and the supernatant collected in a beaker. The supernatant was passed through two layers of Whatman no. 1 filter paper and the filtrate was collected in a 250-ml capacity beaker. Nematicidal activity of the culture filtrate was determined as described by Siddiqui (2002) as follows: 1 ml of the culture filtrate was transferred to glass cavity slides to which 1 ml of a freshly hatched juvenile suspension containing 58 ± 8 surface-sterilized juveniles was added. Juveniles kept in 1 ml of potato dextrose liquid medium without the bacterium served as controls. Each treatment was replicated six times and the glass cavity slides were kept at room temperature ($28 \pm 2^\circ\text{C}$). After 48 h of incubation, the number of dead juveniles were counted and the percentage mortality calculated. The nematodes were considered dead if they did not

move on probing with a fine needle. The experiment was repeated.

The effect of a methanol fraction of *R. solani* culture filtrate obtained from medium amended with various substrates on *in vitro* nematicidal activity of *P. fluorescens*

As, in a previous experiment, *R. solani* isolate Rs7 markedly repressed nematicidal and β-galactosidase activities of the bacterial antagonists while Rs4 promoted nematicidal activity, these isolates were tested more rigorously in the subsequent experiments. Potato dextrose liquid medium (250 ml in 500 ml Erlenmeyer flask), either unamended or amended with various substrates, was inoculated with one 7 mm agar plug of *R. solani* taken from 5 to 7-day-old potato dextrose agar cultures. The substrates added separately to the potato dextrose liquid medium before fungal inoculations were: 1 mM ZnSO₄·7H₂O; 4 mM NH₄Mo; 2 g l⁻¹ phenylalanine or asparagine (Aldrich Chemicals, Milwaukee, WI, USA). Minerals, including zinc (Siddiqui and Shaukat 2002b) and molybdenum (Hamid *et al.* 2003) enhance the production of nematicidal agents *in vitro* and improve the biocontrol potential of *P. fluorescens* strain CHA0. Similarly, amino acids are also known to influence nematicidal activity by fluorescent pseudomonads *in vitro* (Siddiqui and Shaukat 2004b). Therefore, phenylalanine and asparagines were included in this experiment. Culture filtrates of the fungal strains were extracted with methanol (1 : 2, v/v) and the fraction concentrated on a rotary vacuum evaporator (Eyela; Tokyo Rikakikai Co. Ltd, Tokyo, Japan) under reduced pressure at 37°C. A 1-mg ml⁻¹ solution of each extract was prepared in methanol and added to KB broth (100 ml in a 250-ml Erlenmeyer flask). Subsequently, a loopful of bacterial culture was mixed with the medium. Treatments were arranged as a 5 × 3 × 4-factorial design with six replicates and were incubated for 48 h at 24°C. The factors comprised five levels of medium amendments (none, zinc, molybdenum, phenylalanine and asparagines), three *R. solani* isolates including control (none, Rs4 and Rs7) and four bacterial strains including nonbacterized control (none, CHA0, CHA0/pME3424 and CHA89). The same procedure was followed for the preparation of bacterial culture filtrate and determination of nematicidal potential as mentioned above. The experiment was repeated twice.

The influence of *R. solani* on nematode biocontrol potential by *P. fluorescens* in tomato

The experimental design was a 4 × 3-factorial. The factors comprised of four bacterial treatments including nonbacterized control (CHA0, CHA0/pME3424, CHA89 and none) and three *R. solani* treatments including a non-*Rhizoctonia*

treatment (Rs4, Rs7 and none). The inoculum of *R. solani* isolates, for the artificial infestation of soil, was prepared as described above. The soil was a sandy loam (sand : silt : clay, 70 : 19 : 11) of pH 8.1 with a moisture-holding capacity of 38%. The soil was pasteurized and infested with a mycelial suspension of *R. solani* at 3 ml kg⁻¹ of soil and 350 g per pot was added to 8-cm-diameter plastic pots. The upper 2 cm of soil was removed and the remainder drenched with a 35-ml cell suspension of CHA0 or its GM derivatives (3×10^8 CFU ml⁻¹). Soils without the bacterium served as controls. After fungal and bacterial inoculations, 3-week-old tomato seedlings were transplanted into each pot, three per pot. Following establishment of the seedlings, they were inoculated with 2000 second stage juveniles of *M. incognita* by making three holes in the soil. Treatments and controls were replicated eight times each and the pots were randomized on a glasshouse bench. The experiment was terminated 45 days after nematode addition and plant growth parameters, including plant height, fresh shoot and root weight, were measured. The number of galls induced by *M. incognita* on each root system was counted using a hand lens. Populations of the bacteria were isolated from the rhizosphere by placing root samples with adhering soil in a 250-ml Erlenmeyer flask containing 10 ml 0.1 mol l⁻¹ MgSO₄ solution (pH 6.5) plus 0.02% Tween 20. Ten-fold serial dilutions of the suspension were prepared and 100 µl aliquots from the appropriate dilutions were plated onto King's medium B supplemented with tetracycline.

Statistical analysis

Depending upon the experimental design, a one-way analysis of variance (ANOVA) or factorial analysis of variance (FANOVA) was performed using Statistica ver. 5.0 (Statsoft Inc., Tulsa, OK, USA). As a follow-up of FANOVA, the treatment mean values were separated using Fisher's least significant difference (LSD) and Duncan's multiple range tests. Data from repeated trials were pooled after confirming the homogeneity of variance and/or after confirming that the treatment × trial interactions were not significant. Bacterial rhizosphere colonization data were normalized with a log₁₀ plus 1 transformation prior to analysis. Correlation coefficient (*r*) was computed between nematode mortality and β-galactosidase activity.

RESULTS

Rhizoctonia solani isolates and their pathogenicity in tomato seedlings

A total of nine isolates of *R. solani* (listed in Table 1) were isolated from the surface-sterilized roots of tomato plants

Table 1 Growth, influence on medium pH and PAA production (determined in terms of similarity of *Rf*-values and spot colour observation with synthetic PAA) by various *Rhizoctonia solani* isolates in culture medium

Isolates	Initial pH*	Final pH*	Mycelial dry wt. (mg)*	Rf†	Spot colour†
PAA	—	—	—	0.54	Dark brown
Rs1	5.7	6.3 ± 0.7	644 ± 13	0.68	—
Rs2	5.7	6.8 ± 0.9	563 ± 11	0.33	—
Rs3	5.7	6.4 ± 1.0	572 ± 18	0.52	Light brown
Rs4	5.7	6.4 ± 0.6	782 ± 27	0.66	—
Rs5	5.7	6.7 ± 0.8	579 ± 22	0.77	—
Rs6	5.7	6.4 ± 0.8	633 ± 26	0.63	—
Rs7	5.7	6.6 ± 0.5	521 ± 15	0.55	Dark brown
Rs8	5.7	6.7 ± 0.9	622 ± 40	0.71	—
Rs9	5.7	6.5 ± 1.1	588 ± 23	0.49	—

*Each value is the mean (±1 S.D.) of six replicates obtained from two individual experiments each with three replicates.

†Spot colour was visualized under u.v. 365 nm and *Rf*-values determined for each spot from the point of inoculation of culture filtrate.

collected randomly from the agricultural fields of Gadap (Southern Sindh, Pakistan). The isolates were tested for their pathogenicity in an assay on tomato seedlings. PAA, a known phytotoxicity factor, was used as a comparison. Whereas only one isolate (Rs7) reduced seedling emergence (to 40%) and induced damping-off disease symptoms (causing rotting of the roots and discolouration of roots and lower stem, disease score = 1.9), other isolates failed to produce such a response in tomato plants. Synthetic phenylacetic acid induced a disease reaction (seedling emergence = 35.6%; disease score = 2.1) similar to that incited by *R. solani* isolate Rs7. Interestingly, one isolate (Rs4) enhanced growth of tomato plants (data not presented).

Production of PAA by *R. solani* isolates

Qualitatively, TLC analysis of the culture supernatant extracts of all the *R. solani* isolates revealed the production of different compounds at various *Rf*-values (Table 1). Of the nine isolates tested, only two (Rs3 and Rs7) produced light brown and dark brown spots, at *Rf*-values of 0.52 and 0.55, respectively, that are close enough to that of synthetic PAA (dark brown spot at an *Rf*-value of 0.54) and fluoresced under u.v. (365 nm) to suggest that they produce PAA. Interestingly, isolate Rs3, which produced a PAA-like compound *in vitro*, failed to produce disease symptoms in tomato seedlings. Further analytical methods need to be used to confirm the production of PAA from isolate Rs3. Growing *R. solani* isolates in potato dextrose liquid medium

increased the final pH of the growth medium after an incubation period of 1 week. The *R. solani* isolates differed markedly with respect to mycelial dry weights. Isolate Rs7 produced the lowest mycelial dry weight, while Rs4 produced the greatest.

Influence of PAA and fungal filtrates on nematicidal activity and *phl*⁺-*lacZ* expression by *P. fluorescens* *in vitro*

Culture filtrates of *P. fluorescens* strains CHA0 and its GM derivatives CHA0/pME3424 or CHA89, resulting from potato dextrose liquid medium culture, caused significant ($P < 0.05$) mortality of *M. incognita* juveniles *in vitro* compared with the controls (Table 2). Culture filtrate of CHA0 and CHA0/pME3424 obtained from medium amended with culture filtrate of various isolates of *R. solani* induced mortality of *M. incognita* juveniles to a varying degree but the activity of strain CHA89 was not substantially altered by any of the fungal isolates. Bacterial growth medium amended with filtrates from *R. solani* isolate Rs3 or Rs7 repressed ($P < 0.05$) nematicidal activity by *P. fluorescens* strain CHA0 while fungal isolate Rs7 and Rs8 reduced nematicidal activity of the bacterial strain CHA0/pME3424. Amending the growth medium with synthetic PAA markedly reduced nematicidal potential of both CHA0 and CHA0/pME3424. When used

alone, the culture filtrate of *R. solani* isolate Rs7 caused significant ($P < 0.05$) nematode mortality, comparable with that produced by the synthetic PAA. On the contrary, isolate Rs3, which synthesized PAA-like compound *in vitro*, failed to cause juvenile deaths. Interestingly, the hypovirulent isolate Rs4 enhanced ($P < 0.05$) nematicidal activity of the bacterial strains CHA0 and CHA0/pME3424.

Reporter gene expression, determined in terms of units of β -galactosidase activity, revealed that the *R. solani* isolates exerted a differential influence on the expression of the *Phl*⁺-*lacZ* reporter gene by *P. fluorescens* strain CHA0 and its DAPG overproducing derivative CHA0/pME3424 *in vitro* (Fig. 1). β -galactosidase activity of both the bacterial inoculants was reduced ($P < 0.05$) by *R. solani* isolate Rs7 and synthetic PAA but Rs3, which produces a PAA-like compound, failed to inhibit enzymatic activity in bacteria. Regardless of the fungal isolates tested, the DAPG-negative strain of *P. fluorescens* CHA89 failed to express the reporter gene. A strong positive correlation ($r = 0.833$; $P < 0.001$) was obtained between β -galactosidase units and nematicidal activity of *P. fluorescens* strains CHA0 and CHA0/pME3424. When this relationship was tested separately for the two bacterial inoculants, a positive correlation was recorded for both CHA0 ($r = 0.931$; $P < 0.001$) and CHA0/pME3424 ($r = 0.703$; $P < 0.05$). Strain CHA89 was not included in the statistical test as this strain failed to express reporter gene.

Table 2 The influence of various *Rhizoctonia solani* isolates on mortality of *Meloidogyne incognita* juveniles by *Pseudomonas fluorescens* strain CHA0 and its derivatives CHA0/pME3424 and CHA89 *in vitro*

Treatments	Mortality (%) [*]			
	None	CHA0	CHA0/pME3424	CHA89
Control	3 ± 0.7	53 ± 2.1	62 ± 2.5	34 ± 1.4
PAA	17 ± 2	29 ± 1.7	33 ± 2.2	28 ± 1.7
Rs1	9 ± 1.2	57 ± 2.5	59 ± 2.8	30 ± 1.3
Rs2	14 ± 1.0	52 ± 1.9	64 ± 1.6	33 ± 1.5
Rs3	12 ± 0.8	41 ± 2.1	56 ± 2.2	33 ± 1.9
Rs4	8 ± 0.7	66 ± 2.4	77 ± 1.8	38 ± 1.4
Rs5	11 ± 0.9	53 ± 2.2	59 ± 2.2	37 ± 1.7
Rs6	14 ± 0.5	48 ± 1.8	66 ± 2.5	28 ± 1.7
Rs7	19 ± 0.8	22 ± 1.5	35 ± 1.8	31 ± 1.4
Rs8	9 ± 0.8	50 ± 1.9	49 ± 1.9	33 ± 1.7
Rs9	9 ± 1.0	47 ± 2.0	58 ± 2.1	36 ± 1.5
LSD _{0.05}				
Treatments (T)	12			
Bacteria (B)	9			
T × B	24			

^{*}Data represent mean values (±1 S.D.) of two experiments each with six replications (a total of 12 replications). Activity measured after 48-h exposure period.

The effect of methanol extracts of Rs4 and Rs7 culture filtrate obtained from medium amended with various substrates on *in vitro* nematicidal activity by *P. fluorescens*

Culture filtrates of the bacterial strains caused significant ($P < 0.05$) mortality of *M. incognita* juveniles compared with the controls, regardless of whether or not the culture medium was amended with methanol extracts of *R. solani* and/or substrates (Table 3). With respect to the fungal isolates, the PAA-producing isolate Rs7 repressed ($P < 0.05$) the potential of CHA0 or CHA0/pME3424 to cause juvenile deaths when the growth medium was or was not amended with molybdenum, phenylalanine or asparagines. However, bacterial efficacy in causing nematode mortality was restored when the bacterial growth medium was amended with Rs7 and zinc. Interestingly, when phenylalanine or asparagines were added to bacterial growth medium amended with extracts of non-PAA-producing isolate Rs4 ($P < 0.05$) the nematicidal potential of the strains CHA0 and CHA0/pME3424 was repressed. Isolate Rs4 alone did not induce juvenile deaths but did enhance ($P < 0.05$) the nematicidal potential of the strain CHA0/

Fig. 1 The influence of various *Rhizoctonia solani* isolates on β -galactosidase activity by *Pseudomonas fluorescens* strain CHA0 and CHA0/pME3424 *in vitro*. Bars with each column represent standard error

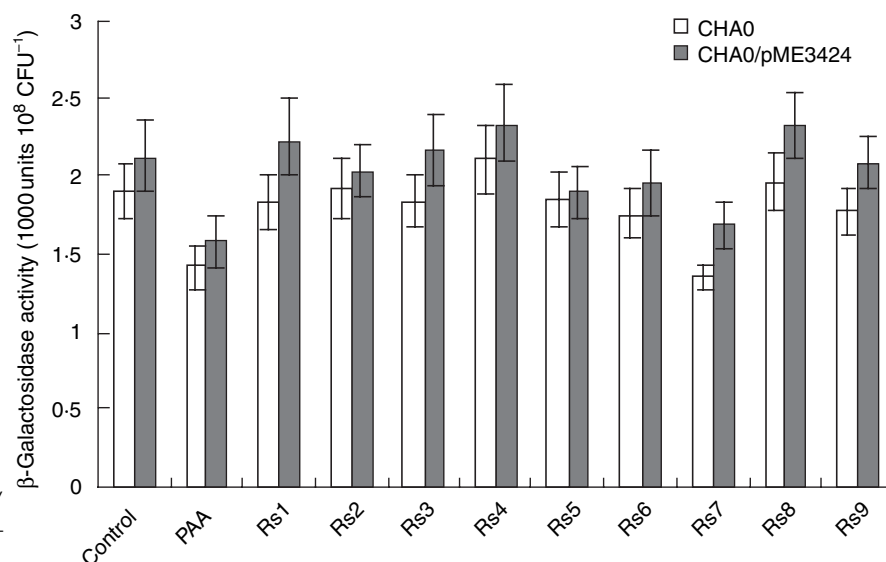


Table 3 The influence of methanol extracts of *R. solani* isolates Rs4 and Rs7 with or without substrate amendment on *in vitro* nematocidal activity (determined in terms of mortality %) by the bacterial agents

Bacterial strains	Mortality (%)*														
	None			Zinc			Molybdenum			Phenylalanine			Asparagine		
	None	Rs4	Rs7	None	Rs4	Rs7	None	Rs4	Rs7	None	Rs4	Rs7	None	Rs4	Rs7
None	2c	7d	16b	2c	10c	9d	3d	9d	12c	5c	15c	18b	3c	13c	16b
CHA0	50a	55b	29a	62a	66a	52b	61b	62b	47a	59a	43a	17b	55a	47a	21a
CHA0/pME3424	59a	67a	37a	71a	72a	68a	74a	73a	52a	63a	46a	20a	61a	51a	29a
CHA89	28b	32c	29a	29b	35b	32c	33c	31c	33b	35b	25b	28a	27b	28b	29a
LSD _{0.05}															
Bacterial strains (B)	10														
Amendments (A)	12														
<i>R. solani</i> strains (Rs)	7														
B × A	24														
B × Rs	21														
A × Rs	16														
B × A × Rs	36														

Mean values followed by the same letters in each column are not significantly different at $P < 0.05$ in accordance with Duncan's multiple range test.

*Nematicidal activity was determined after 48-h exposure period.

pME3424. In growth medium amended with methanol extract of Rs4, addition of zinc or molybdenum did not further enhance the nematocidal activities of bacterial antagonists. In the absence of *R. solani* extracts, addition of zinc to the bacterial growth medium increased the efficacy of both CHA0 and CHA0/pME3424, while addition of molybdenum accentuated the nematocidal activity of only CHA0/pME3424. Neither the addition of *R. solani* extract nor the substrate amendment substantially influenced the activity of DAPG-negative strain CHA89.

Methanol extract of the culture filtrates of *R. solani* Rs4 and Rs7 exhibited the same nematocidal activity (Table 3) as did the culture filtrates (Table 2), implying that active nematocidal compounds produced by *R. solani* are soluble in water and hence polar in nature, but that concentrating the active compound(s) with methanol extraction does not further enhance nematocidal activity of these agents. When compared with the culture filtrates (Table 2), methanol extracts of the culture filtrate (Table 3) from Rs7 did not influence nematocidal activity of the bacterial inoculants while methanol

extraction and subsequent concentration of culture filtrate from isolate Rs4 markedly enhanced ($P < 0.05$) nematocidal activity of CHA0 and CHA0/pME3424. Extraction of the culture filtrates of *R. solani* with solvents having different polarities and subsequent determination of nematocidal activities may yield interesting results.

The influence of *R. solani* on nematode biocontrol potential of *P. fluorescens* in tomato

Application of *P. fluorescens* wild-type strain CHA0 or its DAPG-negative mutant CHA89 to sandy loam soil significantly ($P < 0.05$) inhibited the intensity of galling caused by *M. incognita* per gram of root of tomato, compared with the nonbacterized controls (Table 4). A significant ($P < 0.05$) reduction of root knot disease over the nonbacterized controls was achieved by all the bacterial inoculants tested in soil amended with Rs4 or Rs7. With respect to the fungal strains, galling intensity was markedly higher in soil amended with Rs4 but lower in Rs7-amended soils. Shoot weight was significantly ($P < 0.05$) lower following amendment with *R. solani* Rs7 compared with Rs4 in untreated or in CHA89-treated soils. Inoculation with *P. fluorescens* strain CHA89 increased shoot weight in soil amended with Rs4 over the untreated controls. Treatment with the bacterial strain CHA89 resulted in greater root weight in unamended and Rs4-amended soils relative to the controls. Regardless of bacterial treatment, soil amended with Rs7 produced lesser root weights than unamended or Rs4-amended soils. Furthermore, amendment of soil with isolate Rs7 caused a substantial reduction in root length and most of the root system had brown necrotic lesions or was rotten (data not collected). Bacterial populations were comparatively higher ($P < 0.05$) in soils amended with

Rs7. Among the bacterial strains, wild type strain CHA0 exhibited greater colonization potential in both unamended and *R. solani*-amended soils.

DISCUSSION

Pathogenicity of *R. solani* isolates in tomato

Due to its great ability to adapt to environmental conditions and a wide host range, *R. solani* has a worldwide distribution and is capable of causing severe crop losses in tomato (Ogoshi 1987). In the current study, a total of nine isolates of *R. solani* were isolated from surface-sterilized tomato roots but only one isolate significantly inhibited seedling emergence and caused damping-off disease that resulted in considerable seedling mortality. Similar to our study, one of the most commonly isolated species from the roots of tomato was *Rhizoctonia* sp., with a frequency of 13.51% and producing typical symptoms of chlorosis and wilt in tomato (Yildiz and Döken 2002). Furthermore, Yalcin (1978) found *R. solani* to be one of the commonest fungi in wilted tomato plants with a frequency of 16.8%. The anastomosis group of the *R. solani* isolates that we isolated from tomato roots was not determined, but the knowledge of the anastomosis group of the isolate(s) involved in a particular disease is very useful in determining the reaction and behaviour of the cultivars. Analysis of anastomosis reactions between the tester isolates and *R. solani* isolates from tomato revealed that all the isolates belonged to the AG-4 group (Yildiz and Döken 2002). In our study, only one isolate Rs7 caused typical root rot symptoms in tomato seedlings, while other isolates failed to inhibit seedling emergence or to elicit disease reaction. *Rhizoctonia solani* is a cosmopolitan soil saprotrophic fungus and is highly host specific in pathogenicity. In our case,

Table 4 Effect of virulent and hypovirulent isolates of *Rhizoctonia solani* on *M. incognita* biocontrol by *Pseudomonas fluorescens* strain CHA0 and its derivatives in tomato

Treatments	Number of galls per gram root			Root weight (g)			Shoot weight (g)			Bacterial rhizosphere population [(log CFU g ⁻¹ fr. Root wt.) +1]		
	None	Rs4	Rs7	None	Rs4	Rs7	None	Rs4	Rs7	None	Rs4	Rs7
Control	86	83	74	1.4	1.8	0.8	1.9	2.1	1.1	nd	nd	nd
CHA0	54	61	23	1.8	1.9	1.2	1.9	2.6	1.6	5.30	5.08	5.44
CHA0/pME3424	80	65	55	1.1	1.6	0.9	1.5	1.7	1.5	5.15	4.89	5.12
CHA89	58	55	46	2.0	2.4	1.0	2.2	3.3	1.5	5.09	5.04	5.18
LSD _{0.05}												
Treatments (T)	18			0.31			0.62			0.18		
<i>R. solani</i> strains (Rs)	15			0.26			0.49			0.14		
T × Rs	29			0.55			1.24			0.25		

nd, not detected.

either the isolates are nonpathogenic or host genotype recognition failed and infection did not take place. It is also possible that the isolates, which are nonpathogenic to tomato, could be pathogenic to other crop plants.

PAA production by *R. solani* isolates

In the current study, only two *R. solani* isolates (Rs3 and Rs7) produced PAA-like compounds as detected on the analytical TLC plates. Isolate Rs7 produced *Rf*-values (0.55) and spots (dark brown) closely similar to that produced by synthetic PAA (*Rf* 0.54; dark brown spots) while isolate Rs3 produced *Rf*-values of 0.52 and light brown spots which fluoresced under u.v. 365 nm. However, isolate Rs3 failed to produce phytotoxic symptoms in tomato seedlings, did not cause nematode deaths and did not interfere with the metabolic pathway to biosynthesize nematicidal compounds by *P. fluorescens* *in vitro*, suggesting that the compounds synthesized by this isolate is not PAA but could be one or more of its nonphytotoxic derivative(s) or a compound of a different nature. To ascertain the identity of PAA, other standard techniques such as mass, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, etc. should be employed. The presence of PAA and its *p*- and *m*-hydroxyl derivatives in *R. solani* culture filtrates has been reported. For instance, Frank and Francis (1976) found that a strain of *R. solani* isolated from infected potato stems produced a nonenzymatic, low molecular weight phytotoxin in liquid culture. Purification of the toxic fraction identified it as phenylacetic acid and its *m*-hydroxylated derivatives. Similarly, Mandava *et al.* (1980) used chromatographic and spectroscopic methods to identify the phytotoxins *m*-hydroxy and *m*-methoxyphenylacetic acids from the culture filtrates of *R. solani*.

The final pH of the culture medium probably became more alkaline because of the fungal growth. Although the pH of the medium could not be correlated with PAA production by *R. solani* isolates, mycelial dry weight was to some extent correlated with toxin production by the fungi. The *R. solani* isolates differed in their final mycelial dry weights; in general, PAA-producing isolates had a relatively low mycelial dry weight. The mechanism whereby PAA production limits the dry weight of *R. solani* is not known but certain hypothesis can be advanced. Accumulation of PAA in the fungal growth medium might have an inhibitory effect on biosynthesis of certain structural proteins and, as a consequence lead to lesser mycelial dry weight.

Influence of *R. solani* filtrates on nematicidal activity of *P. fluorescens* *in vitro*

Culture filtrates of the *P. fluorescens* strain CHA0 and its GM derivatives caused substantial mortality of *M. incognita*

juveniles *in vitro*. DAPG-overproducing derivative CHA0/pME3424 caused significantly greater mortality of the nematodes while the DAPG-negative CHA89 exhibited the least nematicidal activity. These results indicate the importance of secondary metabolite DAPG produced by *P. fluorescens* in the induction of nematode mortality *in vitro*. In a previous study, exposure of root-knot nematode to culture filtrate of *P. fluorescens* CHA0 or CHA0/pME3424 under *in vitro* conditions reduced egg hatch and caused considerable mortality of *M. javanica* juveniles (Siddiqui and Shaukat 2003b). Bacterial growth medium amended with the culture filtrate of virulent PAA-producing *R. solani* isolate Rs7 substantially lessened the ability of *P. fluorescens* strains CHA0 and CHA0/pME3424 to cause *in vitro* nematode mortality while the activity of DAPG-negative CHA89 remained unaltered. Similarly, addition of 500 μM synthetic PAA to bacterial culture medium also depressed bacterial potential to cause nematode deaths. To our knowledge, this is the first study to demonstrate the *in vitro* repression of the nematicidal activity by *P. fluorescens* following culture amendment with synthetic PAA or culture filtrate of PAA-producing *R. solani*. The phytotoxic pathogenicity factor fusaric acid, produced by *F. oxysporum* strains, represses the production of DAPG, a key factor in the antimicrobial activity of *P. fluorescens* strain CHA0 (Notz *et al.* 2002). In another study, bacterial growth medium amended with the culture filtrate of nonpathogenic strains of *F. solani* repressed the nematicidal activity of the *P. fluorescens* strain CHA0 and its derivative CHA0/pME3424 (Siddiqui and Shaukat 2003c).

In the current study, addition of synthetic PAA also resulted in significant mortality of *M. incognita* juveniles *in vitro*. PAA, an important phytotoxicity factor, is known to suppress a variety of plant pathogens. For instance, Kawazu *et al.* (1996a,b) demonstrated that PAA produced by *Bacillus subtilis* strain HY-16, *Bacillus cereus* strain HY-3 and *Bacillus megaterium* strain HY-17 has a toxic effect *in vitro* against the pine wood nematode *Bursaphelenchus xylophilus*. Further, PAA was found to be more effective than metalaxyl in reducing zoospore germination of *Phytophthora capsici* *in vitro* and suppressed *Phytophthora* blight at the concentration of 1000 $\mu\text{g ml}^{-1}$ in pepper plants (Hwang *et al.* 2001). Details of the mode of action of PAA against root-knot nematode in tomato plants remain to be elucidated.

A hypovirulent non-PAA-producing isolate Rs4 significantly enhanced bacterial ability to cause nematode mortality. The possible role(s) of the promoter compound(s) produced by isolate Rs4 in the enhancement of nematicidal activity by *P. fluorescens* needs further investigation. Enhanced nematicidal activity by *P. fluorescens* strain CHA0 following the addition of the culture filtrate of *Trichoderma harzianum* to the bacterial culture medium has been reported previously (Siddiqui and Shaukat 2004a).

Influence of *R. solani* filtrates on *phlA*'-*lacZ* expression by *P. fluorescens* *in vitro*

The detection of secondary metabolites from the crop rhizosphere is an arduous task but assaying expression of the reporter genes that regulate the biosynthesis of secondary metabolites in bacterial inoculants is relatively straightforward, with high sensitivity and few technical constraints. Reporter gene expression, determined in terms of units of β -galactosidase activity, revealed that enzyme activity of both the bacterial strains, CHA0 and CHA0/pME3424, was reduced by *R. solani* isolate Rs7 and synthetic PAA. The phytotoxic pathogenicity factor fusaric acid produced by *F. oxysporum* strains suppressed DAPG production and *PhlA*'-*lacZ* reporter gene expression in CHA0 under *in vitro* conditions (Notz *et al.* 2002). To our knowledge, ours is the first report to show a repression in the *Phl* reporter gene expression in CHA0 by PAA-producing *R. solani* and synthetic PAA *in vitro*. In this study, reporter gene expression was enhanced by *R. solani* strain Rs4. Notz *et al.* (2001) demonstrated that *Pythium ultimum* can stimulate *phlA* gene expression in CHA0 on crops such as cucumber and maize. Similarly, in our recent study (Siddiqui and Shaukat 2004a), addition of culture filtrate of *T. harzianum* strain Th6 to bacterial growth medium markedly stimulated *Phl*'-*lacZ* reporter gene expression by *P. fluorescens* strain CHA0 and CHA0/pME3424 *in vitro*. Furthermore, *Aspergillus niger*, a cosmopolitan soil fungus, enhanced *in vitro* β -galactosidase activity while *A. quadrilineatus* repressed such activity (Siddiqui *et al.* 2004c).

The effect of methanol extracts of *R. solani* on *in vitro* nematocidal activity by *P. fluorescens*

Culture filtrates of *P. fluorescens* caused significant mortality of *M. incognita* juveniles *in vitro* and amendments of the bacterial growth medium with methanol extract of PAA-producing *R. solani* isolate Rs7 reduced the nematocidal activity of the bacteria. However, amendment of the bacterial growth medium with zinc or molybdenum overcame the inhibitory effect of Rs7 and subsequently enhanced nematocidal activity of the bacterial inoculants. These results suggest that addition of zinc to the culture medium influences metabolic pathways of the bacterial agents and thereby restores bacterial nematocidal potential *in vitro*. Slininger and Jackson (1992) demonstrated that zinc stimulated the production of phenazine-1-carboxylate, the primary biocontrol determinant of *P. fluorescens* strain 2-79 (Thomashow and Weller 1996).

Interestingly, addition of phenylalanine enhanced the repression of nematocidal compound(s) production in CHA0 and CHA0/pME3424 by the PAA-producing virulent *R. solani* isolate Rs7 and the non-PAA-producing

hypovirulent isolate Rs4. However, asparagines only enhanced the repressive activity of isolate Rs4. It is clear from this study that PAA is an important determinant that represses the nematocidal activity of *P. fluorescens*. How a non-PAA-producing Rs4 induces repression of bacterial secondary metabolite production and subsequent nematocidal potential, following the addition of phenylalanine to the fungal growth medium, is yet to be elucidated. In fungi, the shikmic acid pathway and the quinic acid pathway are connected by two intermediate products, dehydroshikmic acid and dehydroquinic acid, needed for the completion of both pathways. The shikmic acid pathway leads to the synthesis of the three aromatic amino acids (phenylalanine, tryptophan and tyrosine) and other important metabolites (Bentley 1990; Hawkins *et al.* 1993). It is noteworthy that PAA is a catabolic product of phenylalanine. Liu *et al.* (2003) reported that a 3.6-kb double-stranded RNA, designated M2, is associated with hypovirulence in *R. solani* isolate Rhs 1A1 and proposed that the M2-encoded putative polypeptide A (pA) might interfere with the regulation of the shikimate pathway, with the result that phenylalanine synthesis and consequently PAA production are blocked. Presumably, the addition of phenylalanine, as a precursor, in the fungal growth medium restored the PAA-producing capacity of isolate Rs4 and, hence, turned the hypovirulent strains into a virulent one. Like phenylalanine, addition of asparagine in pathogen nutrition is known to increase the virulence of *R. solani* (Weinhold *et al.* 1969).

Nematode biocontrol by *P. fluorescens* in tomato

Soil application of *P. fluorescens* strain CHA0 or its DAPG-overproducing mutant caused marked reductions of the intensity of galling incited by *M. incognita* per gram of root of tomato. From the transformed data (galls per gram of root) it is evident that the DAPG-negative mutant CHA89 also suppressed galling severity. However, the root systems in CHA89-treated soils had many galls and this obviously increased the weights of such roots. Data from the untransformed values (galls per root system) indicate that, compared with nonbacterized controls or treatment with CHA0 or CHA0/pME3424, strain CHA89 had no significant effect on root-knot nematodes. These results further strengthen our previous findings (Siddiqui and Shaukat 2003b) that production of the secondary metabolite DAPG plays an overriding role in the suppression of root-knot disease in tomato. It is interesting to note that galling intensity by *M. incognita* was lower in soil amended with the PAA-producing virulent isolate Rs7 compared with the non-PAA-producing isolate Rs4 in both bacterized and nonbacterized soils. It is likely that PAA production by Rs7 may reduce nematode survival and infectivity directly by causing nematode mortality in the tomato rhizosphere and indirectly

by inducing systemic resistance in the plants. However, it will be difficult to determine whether PAA can trigger systemic-acquired resistance in tomato plants, because the chemical has direct nematocidal activity *in vitro* against nematodes. Certain plant pathogenic fungi, including *R. solani* are known to produce PAA, a phytotoxicity factor that is capable of inducing the same disease syndrome as the pathogen itself (Sherwood and Linberg 1962; Frank and Francis 1976; Mandava *et al.* 1980; Tavantzis and Lakshman 1995). On the contrary, a positive effect of PAA has been demonstrated on the growth and development of maize (Sarwar and Frankenberger 1995).

Interestingly, culture filtrates of isolate Rs7 repressed nematocidal potential of the bacteria *in vitro* while addition of the fungus in the form of mycelium to the soil promoted bacterial effectiveness to control root-knot disease in tomato plants under glasshouse conditions. At this point it is difficult to explain these contrasting results. In Rs7-amended soil, plants exhibited phytotoxic symptoms, in terms of reduced shoot and root weights of the plants. Furthermore, visual assessment of the root systems growing in such soils showed that they had necrotic lesions and were distorted (data not collected). It is likely that a reduction in root growth depleted the availability of potential entry sites for the nematodes and as a consequence, there was reduced galling on such roots. Bacterial populations were significantly higher in Rs7-amended soils, presumably because leakage from *M. incognita*- and *R. solani*-infected roots provided an ample food source for bacteria in the tomato rhizosphere, leading to increased bacterial population densities. Increased bacterial populations in Rs7-amended soils might also interfere with the penetration of nematodes into the roots leading to a lower intensity of galling. Although isolate Rs7 amendments in the soil enhanced bacterial populations in the rhizosphere, it is not yet fully understood what role it has on DAPG production in the rhizosphere and the subsequent biocontrol potential of the bacterial agents. Determination of the expression of bacterial *PhlA*'-*lacZ* reporter genes, which translate the production of DAPG, in the presence of Rs7 from the tomato rhizosphere may prove this fact. Likewise, Notz *et al.* (2002) demonstrated that fusaric acid-producing *F. oxysporum* strain 798 limited the expression of *phlA* in the wheat rhizosphere. Tomato cv. Sun 6002 (PVP) was used in this study as it is highly susceptible to infection by *R. solani* Rs7. A *R. solani*-resistant cultivar of tomato might grow better and provide a better chance for the nematodes to invade the root system in both bacterized and nonbacterized soils, so allowing study of this complex interaction between rhizosphere organisms of very divergent phylogeny (bacteria, fungi and nematodes) and the impact on plant disease.

In order to achieve successful nematode control with bacterial antagonists such as CHA0, a thorough understand-

ing of the factors that influence bacterial efficacy in the rhizosphere is essential. The present study provides insight into the production of PAA by *R. solani*, which may drastically affect the production of bacterial secondary metabolites that are considered to be important determinants in the biocontrol efficiency of the inoculants against the plant pathogens. If the soil harbours pathogenic *R. solani*, an application of the bacterial antagonist together with zinc is recommended as the latter helps overcome the PAA toxicity in bacteria, thereby providing an environment conducive to bacterial biocontrol potential.

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