

Biocontrol agents induce disease resistance in *Phyllanthus niruri* Linn against damping-off disease caused by *Rhizoctonia solani*

AYYANAR KAMALAKANNAN¹, LINGAIAH MOHAN¹, SANKARASUBRAMANIAN HARISH¹, RAMALINGAM RADJACOMMARE¹, GOPALAN AMUTHA¹, KANDASAMY CHITRA¹, RAMAR KARUPPIAH¹, PETCHIMUTHU MAREESWARI¹, NATARAJ RAJINIMALA¹ and THIRUMALAI ANGAYARKANNI²

¹Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, 641043 Coimbatore, India

²Department of Biochemistry, Avinashilingam Deemed University, 641003 Coimbatore, Tamil Nadu, India

Summary. Five isolates each of *Trichoderma viride* and *Pseudomonas fluorescens* and four isolates of *Bacillus subtilis* were evaluated for their ability to control *Rhizoctonia solani*, the causal agent of damping off of *Phyllanthus niruri*. Among the isolates tested, TVUV10 (*T. viride*), PFMMP (*P. fluorescens*) and BSG3 (*B. subtilis*) showed maximum *in vitro* inhibition of mycelial growth of *R. solani*. Isolate PFMMP was also very effective in reducing disease incidence in greenhouse conditions. The effective isolates were evaluated for their ability to induce defense reactions in *P. niruri* plants. Earlier and increased activity of phenylalanine ammonia lyase, peroxidase, polyphenoloxidase and total phenolics were observed in the biocontrol-agent pretreated *P. niruri* plants challenged with *R. solani*. Isolate PFMMP caused early and increased synthesis of all defense related enzymes and total phenol. The present study showed that isolates TVUV10, PFMMP and BSG3 of *T. viride*, *P. fluorescens* and *B. subtilis* respectively are good candidates for the control of *R. solani* in *P. niruri*.

Key words: *Trichoderma viride*, *Pseudomonas fluorescens*, *Bacillus subtilis*, PAL, PO, PPO, total phenols.

Introduction

Keezhanelli (*Phyllanthus niruri*) is an important medicinal plant, which is cultivated for extracting medicines to cure liver and skin diseases of human beings (Sane *et al.*, 1995; Sastry, 2000). The seedling stage of this plant is highly vulnerable to attack by fungi causing damping-off. Although fungicides have shown promising results in controlling damping off, yet phytotoxic and fun-

gicide residues are major problems posing a human health hazard and contributing to environmental pollution. Sanitation using sterile (or) clean water supplies, application of organic compost and regulation of watering and temperature have managed damping off to some extent. Also, soil solarization has been successful in hot climate (Katan, 1987), though this is impracticable in winter. As a result, existing control measures are on the whole not very effective to control damping-off of *P. niruri*. Biological control is a possible alternative to fungicides, and may be a safe, effective and eco-friendly method for plant disease management. The soil has an enormous untapped potential of antagonistic microbes such as *Trichoderma* spp., *Bacil-*

Corresponding author: A. Kamalakannan
Fax: +91 0422 2431672
E-mail: kamals2k@yahoo.co.in

lus spp., and the fluorescent pseudomonads, which show antagonistic effects against soil borne plant pathogenic organisms. Bio-control agents are becoming increasingly important in plant growth promotion and disease control.

In the last three decades, a lot of research has been carried out on the antagonistic capacity of several species of *Trichoderma* (Chet, 1984; Papavizas, 1985). Soil application of *P. fluorescens* isolate PF1 increased the level of enzymes involved in the phenyl propanoid pathway and pathogenesis related proteins (PR proteins) in response to *Fusarium oxysporum* f. sp. *lycopersici* causing wilt of tomato and *Colletotrichum capsici* causing fruit rot of pepper (Ramamoorthy and Samiyappan, 2001). *Bacillus subtilis* is known to manage diseases of blackgram caused by the fungi *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium solani* (Siddiqui et al., 2001) and root rot of pigeon pea caused by *Fusarium udum* (Vasudeva et al., 1962). In this study it was decided to examine the feasibility to protect *P. niruri* against *R. solani* by using antagonistic fungi and bacteria.

Materials and methods

Collection and maintenance of micro-organisms

Rhizoctonia solani was isolated from infected *P. niruri* plants collected from the medicinal plant garden, Department of Horticulture, Tamil Nadu Agricultural University, Coimbatore, India. Isolates of *T. viride* (TVMGLT1, TVMG5, TV21GL, TVUV10 and TV15), *P. fluorescens* (PF1, PFMMP, PFCHAO, PF8 and PFOOTY1) and *B. subtilis* (BSM2, BSG1, BSG3 and BSCBE4) were obtained from the Department of Plant Pathology in Tamil Nadu Agricultural University. The isolates were maintained on slants of potato-dextrose agar (PDA) (*T. viride* and *R. solani*), King's B medium (*P. fluorescens*) and nutrient agar (*B. subtilis*) at 5°C.

In vitro antagonism of *T. viride*

Antagonism of *T. viride* against *R. solani* was assayed with the dual-culture method of Rajeev and Mukhopadhyay (2001). Discs (8 mm) of the pathogen and the antagonist were cut from the edge of a 3-days-old culture and placed on opposite side of Petri dishes containing PDA, 1 cm away from the edge. Petri dishes were incubated at 28±2°C and after 4 and 7 days mycelial growth of

R. solani, the inhibition zone and the overgrowth of the antagonist over the pathogen were measured and expressed in mm and/or as a percentage (Ananthakumar, 1994).

In vitro antagonism of bacterial isolates

Individual bacterial isolates were streaked on one side of Petri dish containing PDA (1 cm away from the edge) and a mycelial disc (8 mm diameter) of a 3-days-old culture of *R. solani* was placed on the opposite side of the dish, perpendicular to the bacterial streak (Vidhyasekaran et al., 1997). The dishes were incubated at room temperature (28±2°C) for 4 days and the mycelial growth of *R. solani* and any inhibition zones were measured in mm.

Effect of biocontrol agents on *R. solani* under greenhouse conditions

Potting soil (red soil:cow dung:manure at 1:1:1 w:w:w) was autoclaved for 1 h per day for two consecutive days and mixed thoroughly with talc based formulations (carboxy methyl cellulose, 10g; talc powder, 1 kg; 400 ml of bacterial inoculum containing 9×10^9 cfu ml⁻¹ and pH adjusted to 7 with calcium carbonate) of each biocontrol agent at the rate of 5 g kg⁻¹ of soil. Treated soil was placed in pots (Vidhyasekaran and Muthamilan, 1995) and *P. niruri* seedlings were planted in this soil. After 15 days, the virulent strain of *R. solani*, mass multiplied in a sand maize/medium (sand and maize powder at the ratio of 19:1 following Riker and Riker, 1936), was incorporated in the root zone at the rate of 10 g of sand/maize + pathogen per kg of soil. Pots inoculated only with *R. solani* and pots inoculated with *R. solani* but treated with carbendazim at 1 g l⁻¹ of water were used as control. Damping-off incidence was evaluated 27 days after planting and expressed as a percentage. Tests were carried out in three replications (3 pots/replication) and pots were randomly distributed.

Induction of defense reaction

Pseudomonas fluorescens isolates PF1 and PFMMP, *T. viride* isolates TVUV10 and TV21GL and *B. subtilis* isolate BSG3 were used to induce a defense reaction in *P. niruri*. The following 7 treatments were included in the experiment: 1, soil application of PF1; 2, PFMMP; 3, TVUV10;

4, TV21GL; 5, BSG3; 6, inoculated control, and 7, un-inoculated control. Talc-based formulations of the five biocontrol agents were applied at the rate of 5 g kg⁻¹ of soil and challenge inoculated with *R. solani* (10 g of sand/maize multiplied pathogen kg⁻¹ of soil) 15 days after planting *P. niruri*. Plant samples were collected 0, 3, 6, 9 and 12 days after pathogen inoculation. Four plants were sampled from each replication of each treatment and maintained separately for biochemical analysis. The experiments were conducted in a randomized block design on a greenhouse bench. Fresh plant samples were homogenized with liquid nitrogen in a pre-chilled pestle and mortar. The homogenized plant samples were stored at -70°C.

Estimation of phenylalanine ammonia lyase (PAL) activity

Plant samples (1 g) were homogenized in 3 ml of iced 0.1 M sodium borate buffer, pH 7, containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone. The extract was filtered through cheesecloth and centrifuged at 16,000 g for 15 min. The supernatant was used as the enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at 290 nm (Dickerson *et al.*, 1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8, and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of transcinnamic acid synthesized was calculated (Dickerson *et al.*, 1984). Enzyme activity was expressed as nmol transcinnamic acid min⁻¹ g⁻¹ tissue.

Assay of peroxidase (PO)

Plant samples (1 g) were homogenized in 2 ml of 0.1 M phosphate buffer, pH 7, at 4°C. The homogenate was centrifuged at 16,000 g at 4°C for 15 min and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H₂O₂. The reaction mixture was incubated at room temperature (28±2°C). Changes in absorbance at 420 nm were recorded at 30-second intervals for 3 min. Enzyme activity was expressed as changes in the absorbance min⁻¹ g⁻¹ tissue (Hammerschmidt *et al.*, 1982).

Assay of polyphenol oxidase (PPO)

Plant samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µl of 0.01 M catechol was added, and activity was expressed as changes in absorbance at 495 nm min⁻¹ g⁻¹ tissue (Mayer *et al.*, 1965).

Estimation of phenol

Root samples (1 g) were homogenized in 10 ml of 80% methanol and shaken for 15 min at 70°C (Zieslin and Ben-Zaken, 1993). One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteu reagent (1 N) and the solution was kept at 25°C. The absorbance of the developed blue colour was measured using a Spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol g⁻¹ tissue.

Statistical analysis

All the experiments were repeated once with similar results. The data were statistically analyzed (Gomez and Gomez, 1984) and treatment means compared with Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute, Biometrics Unit, Manila, The Philippines.

Results

Screening of *T. viride* isolates

All the *T. viride* isolates tested inhibited growth of *R. solani* (Table 1). TVUV10 achieved the highest mycelial inhibition (61.7% over control), followed by TV21GL (56.9%), TVMG5 (53.9%), TV15 (53.9%) and TVMGLT1 (51.2%). The widest inhibition zone (13 mm) and the most extensive highest overgrowth of *R. solani* (49.3 mm) were also recorded with TVUV10. All other isolates showed less but still considerable inhibition of *R. solani* (Table 1).

Screening of bacterial antagonists

Varying degrees of mycelial growth inhibition

Table 1. Effect of *Trichoderma viride* isolates TVMGLT1, TVMG5, TV21GL, TVUV10 and TV15 on the growth of *Rhizoctonia solani*.

Treatment	Mycelial growth of <i>R. solani</i>		Inhibition zone (mm) ^a	Mycelial overgrowth of <i>R. solani</i> by <i>T. viride</i> (mm) ^a
	(mm) ^a	(% reduction over control)		
TVMGLT1	43.7 f ^b	51.3 f	10.0 c	44.7 b
TVMG5	41.3 d	53.9 d	8.0 e	43.7 c
TV21GL	38.7 c	56.9 c	12.0 b	43.7 c
TVUV10	34.3 b	61.7 b	13.0 a	49.3 a
TV15	41.3 e	53.9 d	8.7 d	41.0 d
Carbendazim	8.0 a	91.1 a	-	-
Control (<i>R. solani</i>)	89.7 g	-	-	-

^a Mean of three replications.

^b In a column, means followed by a common letter(s) are not significantly different at 5% level by DMRT.

were observed with *P. fluorescens* and *B. subtilis* isolates (Table 2). Among the five *P. fluorescens* isolates, PFMMP had a higher inhibitory effect on mycelial growth (50%). The other isolates, PF1, PFCHAO, PF8 and PFOOTY1, caused 37, 22.5, 21.7 and 17.3% inhibition respectively compared with the control. The widest inhibition zone was with in PFMMP (15 mm) followed by PF1 (8 mm), PFCHAO (7 mm), PF8 (3.7 mm) and PFOOTY1 (1.3 mm). Among the four *B. subtilis* isolates tested, BSG3 had lower mycelial growth (40.2%) with a

10 mm inhibition zone. The other isolates, BSM2, BSCBE4 and BSG1, also had lower mycelial growth inhibition, with reductions of 24.8, 23.2 and 20.9% respectively over the control. The inhibition zone was also narrower in all the other isolates than it was with BSG3 (Table 2).

Effect of biocontrol agents in the pot culture experiment

Soil application of the biocontrol agents reduced the disease incidence in the pot culture experiments

Table 2. Effect of different *Pseudomonas fluorescens* and *Bacillus subtilis* isolates on the growth of *Rhizoctonia solani*.

Treatment	Mycelial growth of <i>R. solani</i>		Inhibition zone (mm) ^a
	(mm) ^a	(% reduction over control)	
PF1	53.0 c ^b	37.0 c	8.0 c
PFMMP	42.3 b	50.0 b	15.0 a
PFCHAO	65.7 e	22.5 e	7.0 d
PF8	66.3 f	21.7 f	3.7 e
PF OOTY1	70.0 g	17.3 g	1.3 g
BSM2	56.3 d	24.9 d	8.3 c
BSG3	56.0 d	40.3 d	10.0 b
BSCBE4	65.0 e	23.5 e	1.7 f
BSG1	67.0 f	20.9 f	1.7 f
Carbendazim	8.0 a	90.5 a	-
Control (inoculated)	84.7 h	-	-

^a See Table 1.

^b See Table 1.

(Table 3). *P. fluorescens* isolate PFMMP effectively controlled root rot incidence, which was only 33.3 per cent. For comparison damping off incidence was 16% when the standard chemical fungicide carbendazim was used, and 89% in the inoculated controls.

Induction of defense-related enzymes and phenolic compounds

Soil application of biocontrol agents caused plants to synthesize PAL. An additional increase in the synthesis of PAL was observed in *P. fluorescens* isolate PFMMP pretreated plants challenge inoculated with *R. solani* (Fig. 1). This activity reached maximum level on day 9 after pathogen challenge and thereafter remained at higher levels throughout the experimental period of 12 days. Earlier and increased activity of PO (Fig. 1A) and PPO (Fig. 1B) was observed in plants pretreated with the biocontrol agents. PO and PPO activity was greatest in plants treated with *P. fluorescens* isolate PFMMP, followed by PF1. PO and PPO activity peaked 9 days after challenge inoculation, then declined. In plants treated with the pathogen alone, PAL activity increased for 3 days and then declined drastically (Fig. 1C). The highest phenol content occurred in plants treated with *P. fluorescens* isolate PFMMP, with the highest phenolic content (10.9 mg g⁻¹) 9 days after inoculation, followed by PF1 (max 10.7 mg g⁻¹ phenols), TV21GL (10.5 mg g⁻¹), TVUV10 (10.5 mg g⁻¹) and BSG3 (10.7 mg g⁻¹). In plants inocu-

lated with the pathogen alone phenol content declined to below-initial level 12 days after inoculation (Fig. 1D).

Discussion

Identification and selection of effective isolates of biocontrol agents is the first and foremost step in biological control. In this study at least three antagonistic agents, *T. viride* TVUV10, *P. fluorescens* PFMMP and *B. subtilis* BSG3 were good candidates for control of damping-off in *P. niruri*. All these organisms inhibited *in vitro* growth of *R. solani* (Tables 1 and 2).

The mechanism of action of the antagonists against *R. solani* was not investigated, but according to previous reports, it seems likely that the *in vitro* fungal inhibition caused by *T. viride*, *P. fluorescens* and *B. subtilis*, was due to the coiling and disintegration of hyphae of *R. solani* (Weindling, 1932; Elad *et al.*, 1983; Prashanthi *et al.*, 2000); to production of siderophores (Yeole and Dube, 2000), or to the production of antibiotics (Vasudeva *et al.*, 1962; Singh *et al.*, 1965; Podile and Dube, 1985).

The study also showed that soil-applied, talc-based formulations of *P. fluorescens*, *T. viride* and *B. subtilis* isolates reduced damping-off incidence in potted plants (Table 3).

In addition to direct antagonism, the biocontrol agents increased activity of various defense-related enzymes and chemicals in response to pathogen infection. All plants are known to be endowed with defense genes, which are quiescent in nature and require the appropriate stimulation signals to activate them. It has been reported that biocontrol agents trigger/activate latent plant defense mechanisms in response to pathogen infection. Inducing the plant's own defense mechanism by applying biological agents is a novel strategy in plant disease management. In the present study, soil application of biocontrol agents increased the activity of defense related enzymes in various plants, leading to the synthesis of defense chemicals in those plants (Fig. 1A, 1B, 1C and 1D). PAL plays an important role in the biosynthesis of phenolic phytoalexins (Daayf *et al.*, 1997). Similarly PO and PPO catalyse the last step in the biosynthesis of lignin and other oxidative phenols. Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall.

Table 3. Effect of biocontrol agents on the incidence of damping-off of *Phyllanthus niruri* caused by *R. solani*

Biocontrol agent	Damping off incidence in percentage ^a
PF 1	46.0 (42.7) c ^b
PFMMP	33.3 (35.7) b
TVUV10	52.0 (46.1) d
TV21GL	56.0 (48.8) e
BSG3	62.3 (52.1) f
Carbendazim	16.0 (23.6) a
Control (inoculated)	89.0 (70.6) g

^a See Table 1.

^b Figures in parenthesis are arcsine transformed values. In a column, means followed by a common letter(s) are not significantly different at 5% level by DMRT

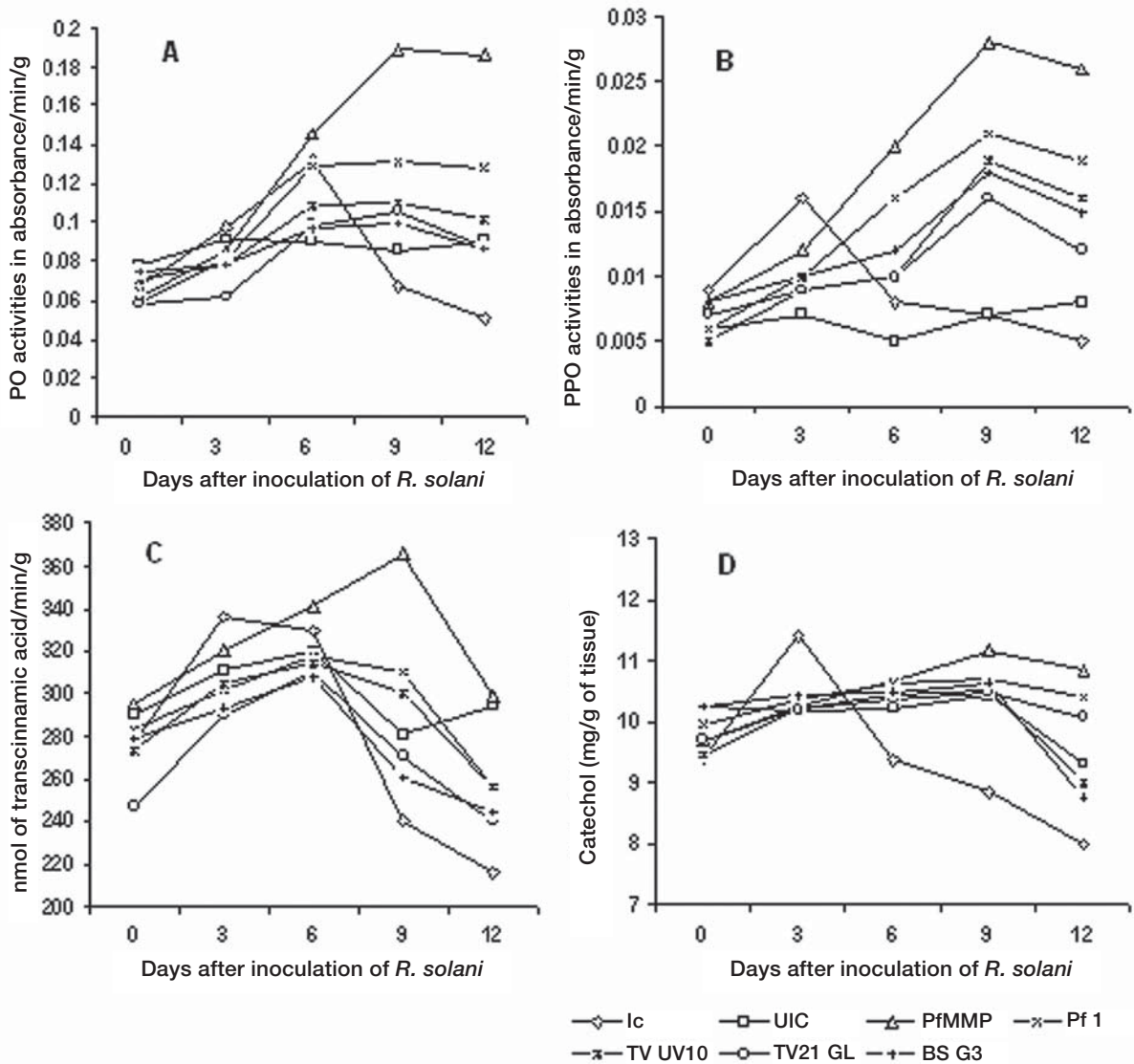


Fig. 1. A, Change in peroxidase activities (PO) by biocontrol agents in *P. niruri* against *R. solani*. B, Changes in polyphenol oxidase activities (PPO) by biocontrol agents in *P. niruri* against *R. solani*. C, Changes in phenylalanine ammonia lyase activities (PAL) in *P. niruri* plants by biocontrol agents against *R. solani*. D, Changes in total phenol content by biocontrol agents in *P. niruri* plants against *R. solani*.

Increased PAL activity was observed with *P. fluorescens* in groundnut against rust (Meena *et al.*, 2000), in cucumber against *Pythium aphanidermatum* (Chen *et al.*, 2001) and in tomato and pepper against *F. oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al.*, 2002). High rates of PAL synthesis were observed in cauliflower and cabbage treated with *T. viride* MNT7 and PF1 along with chitin (Loga-

nathan, 2002), and in pigeon pea treated with *B. subtilis* (Podile and Lakshmi, 1998). Higher levels of PO, PPO and total phenol content in plants pretreated with biocontrol agents and challenge-inoculated with various pathogens were reported by M'Piga *et al.* (1997), Benhamou *et al.* (2000), Chen *et al.*, (2000), Howel *et al.* (2000), Loganathan (2002) and Ramamoorthy *et al.* (2002).

It is concluded that the fungal and bacterial antagonists tested effectively reduced growth of *R. solani* *in vitro*, and that talc-based formulations of these antagonists consistently reduced the incidence of damping off in *P. niruri*. Among the three biocontrol agents used *P. fluorescens* isolate PFMMP gave broad-spectrum protection to *P. niruri* against *R. solani*.

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