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Induction of systemic resistance in rice by *Pseudomonas fluorescens* against rice root knot nematode *Meloidogyne graminicola*

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ABSTRACT

Induction of defense enzymes phenol, peroxidase (PO), polyphenol oxidase (PPO), phenyl ammonia lyase (PAL), super oxide dismutase (SOD) and chitinase by *Pseudomonas fluorescens* isolate Pf1, against challenge inoculation of *Meloidogyne graminicola* in rice was studied *in vitro*. The activity of phenol, PO,PPO, PAL and chitinase was higher in the bacterized rice plants. The analysis of isoform profiles revealed unique PO, PPO and SOD isoforms induced in plants treated with *P. fluorescens*. The present study implies earlier and higher accumulation of phenols and defense enzymes *viz.,* PO, PPO, PAL and chitinase in rice root tissue resulting in significant reduction in nematode infection.

Key words: Control, induced systemic resistance, *Oryza sativa*, rice root-knot nematode

INTRODUCTION

The root-knot nematode, *Meloidogyne graminicola* Golden *et* Birchfield is the most widely distributed serious pest of rice (*Oryza sativa* L.) in the sub-tropics and tropics and is considered economically important in all rice ecosystems (Pankaj *et al*., 2010). It has also been reported from all rice growing regions in India. To reduce the dependence on chemical crop protectants in agriculture, bioagents antagonists to pests are receiving increasing attention. Resistance inducing rhizobacteria offer an excellent alternative in providing a natural, effective, safe, persistent and durable protection. One classical biotic inducer is the plant growth promoting bacterium, *Pseudomonas fluorescens* Migula (Iavicoli *et al*., 2003).

Plants have endogenous defense mechanisms that can be induced in response to attack by plant parasitic nematodes. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy (Ramamoorthy *et al*., 2001). The use of fluorescent pseudomonads for inducing systemic resistance against phytonematodes has been well documented (Siddiqui, 2006; Patricia *et al*., 2009).

In view of the increasing importance of root-knot nematodes in rice based cropping systems in the changing agricultural scenario, the present investigation was undertaken with the objective of assessing the induction of defense enzymes by *P. fluorescens* against *M. graminicola* in rice as a result of induced systemic resistance.

MATERIALS AND METHODS

Pseudomonas treatment and challenge inoculation in glass-house

Pseudomonas fluorescens isolate (Pf1) was used in the defense reaction in rice against root-knot nematode. The treatments included (Table 1): i) *P. fluorescens*– as seed treatment + soil application of 25 mL bacterial suspension containing 9×10^8 cfu mL^{-1} without challenge inoculation; ii) nematode alone at 200 second stage juveniles J2/ plant; iii) *P. fluorescens* (seed treatment + soil application) challenge inoculated with nematode at 200 J2/ plant; and iv) control plants without *P. fluorescens* treatment nor nematode inoculation. The treatments were replicated five times according to a Randomized Block Design (RBD).

The susceptible rice cultivar CO 47 and the root-knot nematode *M. graminicola*, obtained from the culture collection maintained in the Department of Nematology, Tamil Nadu Agricultural University, Coimbatore, were used in this study. Pre-soaked rice seeds (cv. CO 47) were surface sterilized with 0.1 per cent streptomycin sulfate and sown in steam sterilized wetland clay soil (clay 43.2 per cent, silt 8.9 per cent and sand 45.6 per cent) in earthen pots of 5 kg capacity. After 7 days, eggs of *M. graminicola* were inoculated to the rhizosphere and further multiplied.

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The galled rice roots were thoroughly washed, cut into small bits of 1cm and teased in a blender, shaken in 0.5 per cent sodium hypochlorite solution for two minutes. By passing the liquid suspension of eggs through a 200-mesh sieve rested upon a 500-mesh sieve the eggs were collected. The 500-mesh sieve with eggs was quickly placed under a stream of cold water to remove residual sodium hypochlorite (Barker, 1985). The eggs thus collected on the sieve were allowed to hatch. Infective second stage juveniles were inoculated at the rate of 200 per plant before sowing.

Preparation of bacterial inoculum

The bacterial strain P. *fluorescens* strain Pf1 was obtained from the culture collections of the Department of Plant Pathology, TNAU, Coimbatore. It was grown in King's medium B broth under constant shaking at 150 rpm for 48 hrs at room temperature (25 ± 2°C). The culture at its stationary phase of growth was centrifuged at 6000 rpm for 10 minutes and bacterial cells were re-suspended in 10 mM phosphate buffer (pH 7.0). The concentration was adjusted to 9 X 10^8 cfu mL^{-1}. Then 2 per cent carboxy methyl cellosolve was mixed with the bacterial suspension as a sticking agent and used as bacterial inoculum (Thompson, 1996)

Pseudomonas treatment in glass house

For seed treatment, the rice seeds were surface sterilized with 2 per cent sodium hypochlorite solution and soaked in a double volume of bacterial suspension containing 9x10^8 cfu mL^{-1}. After 24 hrs the bacterial suspension was drained and the seeds were shade dried for 30 minutes. The seeds were allowed to sprout for another 24 hours before sowing (Vidhyasekaran *et al.*, 1997). For soil application 25 ml of bacterial suspension (9 x 10^8 cfu mL^{-1}) was poured per pot before sowing.

Biochemical analysis

Plants were carefully uprooted, without causing any damage to root tissues, at different time intervals (7, 14 and 21 days after inoculation of the nematode). Four plants were sampled from each replicate of each treatment and kept separetely. Fresh roots were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized root tissues were stored in a freezer (-70 °C) until used for biochemical analysis.

Determination of phenol. Root samples (1 g) stored at -70°C were homogenized in 10 ml of 80% methanol and agitated 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-Ciocalteau reagent (1/V) 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 mg^{-1} protein.

Determination of peroxidase (PO). Root samples (1 g) maintained at -70 °C were homogenized in 2 ml of 0.1 M phosphate buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 16,000 g at 4 °C for 15 min and the supernatant was used as 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 per cent H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2 °C). The changes in absorbance at 420 nm were recorded at 30s intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min^{-1} mg^{-1} protein (Hammerschmidt *et al.,* 1982).

Determination of polyphenol oxidase (PPO). PPO activity was determined as per the procedure given by Mayer *et al.* (1965). The freeze dried root 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 the activity was expressed as changes in absorbance at 495 nm min^{-1} mg^{-1} protein.

Determination of phenylalanine ammonia lyase (PAL) activity. Root samples (1g) stored at -70 °C were homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinyl pyrrolidine. The extract was filtered through cheesecloth and the filtrate was centrifuged at 16,000 g for 15 min. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nM as described by Dickerson *et al*. (1984). A sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 m M L-phenylalanine in the same buffer for 30 min at 30 °C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 m^{-1} (Dickerson *et al.,* 1984). Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} protein.

Determination of chitinase. The freeze dried root samples (1 g) were homogenized in 2 mL of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 16,000 g for 15 min at 4 °C and the supernatant was used in the enzyme assay. The colorimetric assay of chitinase was conducted according to the method described by Boller and Mauch (1988). Colloidal chitin was prepared according to Berger and Reynolds (1958) from crab shell chitin (Sigma, USA). The reaction mixture consisted of 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 mL of colloidal chitin (10 mg).

After 2 h incubation at 37°C, the reaction was stopped by centrifugation at 8,000 g for 3 min. An aliquot of the supernatant (0.3 mL) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µL desalted snail gut enzyme (Helicase). Finally, the mixture was incubated with 2 ml of dimethyl amino benzaldehyde (DMAB) for 20 min at 37°C and the absorbance was measured at 585 nm. The enzyme activity was expressed as nmol GlcNAc min^{-1} mg^{-1} protein.

Native-PAGE analysis**.** The isoform profile of PO, PPO and SOD were examined by discontinuous native polyacrylamide gel electrophoresis (native-PAGE) (Laemmli, 1970). The protein extract was prepared by homogenizing 1 g of freeze dried root samples in 2 mL of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 16,000 g for 20 min at 4°C. The protein content of the sample was determined by the Bradford method (Bradford, 1976). Samples (50 µg protein) were loaded onto 8% polyacrylamide gels (Sigma, USA). After electrophoresis, PO isoforms were visualized by soaking the gels in a staining solution containing 0.05% benzidine (Sigma, USA) and 0.03% H_2O_2 in acetate buffer (20 mM, pH 4.2) (Nadlony and Sequira, 1980). For assessing PPO isoforms profile, the gels were equilibrated for 30 min in 0.1 per cent p-phenylene diamine followed by addition of 10 mM catechol in the same buffer (Jayaraman *et al*., 1987). SOD isoforms were observed by placing the gel in the stain solution (TRIS 0.61 g; Water 100 ml; pH adjusted to 8.2 with HCl; Sodium EDTA 7.5 mg; Riboflavin 4 mg; NBT 10 mg) and incubated at 37°C for 20 minutes in the dark (Ravindranath and Fridovich, 1975).

Statistical analysis

The data generated were subjected to statistical analysis following the standard statistical procedures (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The bacterized plants expressed higher amounts of defense enzymes in rice plants challenge inoculated with *M. graminicola* when compared to plants without bacterization (Table I).

Phenols. Studies on induction of defense mechanisms revealed higher accumulation of phenolics in bacterized rice roots challenge inoculated with *M. graminicola*. Accumulation of phenolics was observed seven days after challenge inoculation with nematode. The highest accumulation (2.23 µg catechol mg^{-1} protein) was observed in bacterized plants on the 14th day after nematode inoculation**.** Phenolic compounds are known to play a major role in the defense mechanism of plants against various external infectious agents. *Pseudomonas fluorescens* releases antimicrobial factors including lytic enzymes which leads to the accumulation of phenolics (Meena *et al*., 2000) by secretion of indole acetic acid that induced phenol metabolism in plants (Shabaev *et al*., 1999). Accumulation of phenolics by prior application of *P. fluorescens* has been reported in rice (Meena *et al*., 2000).

Peroxidase (PO). Rice roots treated with *P. fluorescens* isolate Pf1 upon challenge inoculation with *M. graminicola* expressed higher activity of peroxidase compared to either *P.*

Table 1. Activity of phenols and defense enzymes in rice roots treated with *Pseudomonas fluorescens* challenge inoculated with *Meloidogyne graminicola* (n = 5)

Treatment	Phenols (µg catechol mg^{-1} protein)			Peroxidase (change in absorbance m^{-1} mg^{-1} protein)			Polyphenoloxidase (change in absorbance m^{-1} mg^{-1} protein)			Phenylalannine ammonialyase (nmol transcinnamic acid $m^{-1}mg^{-1}$ protein)			Chitinase (nmol GlcNAc m^{-1} mg^{-1} protein)		
	Days after treatment														
	7	14	21	7	14	21	7	14	21	7	14	21	7	14	21
Pseudomonas fluorescens	1.59	1.66	1.51	4.32	4.61	3.78	2.31	1.4	1.29	21.1	20.72	20.23	18.27	14.64	14.79
Meloidogyne graminicola	1.79	1.77	1.52	3.82	2.95	2.56	1.86	1.69	1.68	25.48	24.49	21.32	20.53	19.14	13.82
P. fluorescens + *M. graminicola*	1.81	**2.23**	2.17	5.26	5.81	**5.85**	2.85	3.06	**3.28**	32.9	33.96	33.41	28.65	34.4	34.8
Untreated uninoculated control	1.03	0.44	0.09	2.40	2.79	2.01	1.25	1.19	1.17	19.46	20.56	14.99	17.24	13.32	12.22
CD (0.05) Treatment		0.049			0.153			0.129			1.161			0.770	
Days		0.043			0.132			0.112			1.001			0.670	
Treatment × Days		0.086			0.264			0.225			2.01			1.341	

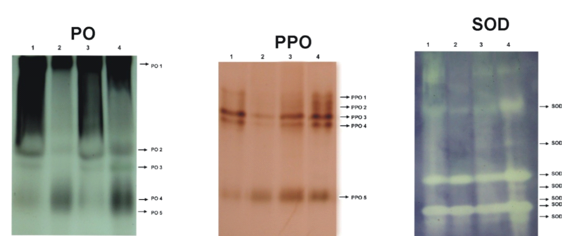
fluorescens alone or nematode alone. The highest activity of (5.85) expressed as change in absorbance min^{-1} mg^{-1} protein was observed 21 days after nematode inoculation.Oxidative enzymes play a decisive role in plant resistance to biotic stress. Various studies reported that *P. fluorescens* induced peroxidase in response to pathogen attack (Chen *et al*., 2000). The present study also indicated that *P. fluorescens* induced higher accumulation of peroxidase upon invasion by *M. graminicola*.

Polyphenol oxidase (PPO). Upon root-knot nematode, *M. graminicola* challenge in bacterized paddy root tissues, the activity of PPO gradually increased and was the highest 21 days after inoculation. Similar to PO, enhanced activity of PPO was observed in the root tissues treated with *P. fluorescens* upon challenge with the nematode. Enhanced activity of peroxidase and polyphenol oxidase was observed in tomato roots treated with *P. fluorescens* and *Fusarium oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al*., 2001), in rice against sheath blight (Nandakumar *et al*., 2001) and in cotton against boll worm (Rajendran *et al*., 2007).

Phenylalanine ammonialyase (PAL). The highest activity of PAL was observed in bacterized root tissues challenged with the nematode on the 14th day after inoculation. PAL is the first enzyme in phenylpropanoid metabolism involved in the production of phenolics and phytoalexins that prevent establishment of the pathogen (Mariutto *et al.,* 2011). The present investigation revealed increased activity of PAL due to *P. fluorescens* treatment, which might have prevented the establishment of the nematode within the roots. Enhanced activity of PAL by *P. fluorescens* has been reported in tomato (Kandan *et al*., 2003) and pearl millet (Niranjan Raj *et al*., 2003) against pathogens.

Chitinase. The activity of chitinase was maximum in bacterized root tissues challenged with the nematode and the highest activity was recorded 21 days after inoculation. The root-knot nematode *M. graminicola* also increased the activity of chitinase in rice roots up to 7 days after inoculation. In rice, seed treatment with PGPR strains increased the chitinase enzyme activity and phenolic content, which was correlated with reduced nematode infestation (Swarnakumari, 1996). Results of the present study revealed that *P. fluorescens* enhanced the accumulation of phenols in rice roots in response to attack by *M. graminicola.*Production and secretion of chitinase by non-pathogenic microorganisms may be important in the biological control of plant parasitic nematodes as it degrades the chitin which is the major component of the egg shell. Chitinases may also play a secondary function as signal molecules, which elicit the induction of other pathogenesis related proteins or metabolites, which are involved in plant-defense reactions (Rahimi *et al.,* 1998). The present study demonstrated increased expression of chitinase in *P. fluorescens* upon challenge inoculation with the nematode. Enhanced activity of chitinase has been suggested to have direct or indirect role in the induction of systemic resistance against pathogens (Dalisay and Kuc, 1995). Many of the *P. fluorescens* strains are known to produce chitinases (Velazhahan *et al*., 1999). *Pseudomonas* treatment of rice cv. IR 50 led to the induction of systemic resistance against *Rhizoctonia solani*, as a result of increase in chitinase activity which directly or indirectly involved in the reduction of sheath blight disease development (Nandakumar *et al.,* 2001).

Figure 1. Native–PAGE analysis for isoform profiles induced by *P.fluorescens* isolate Pf1 in rice challenged with *M. graminicola*



Native PAGE analysis revealed that peroxidase isoforms designated as PO1 to PO5 were observed in *P. fluorescens* treated root tissues challenged with the root-knot nematode *M. graminicola* and the expression of isoforms PO2, PO3, PO4 and PO5 were more prominent in bacterized plants challenge inoculated with the nematode compared to other treatments (Fig. 1). The expression of PO4 and PO5 isoforms was more prominent in healthy plants and plants treated with *P. fluorescens* challenged with the nematode.

Five PPO isoforms, PPO1, PPO2, PPO3, PPO4 and PPO5 were observed in bacterized rice root tissues, inoculated with root-knot nematode. The induction of isoforms PPO1 and PPO2 were observed in all the treatments except in healthy plants. Isoforms, PPO3 and PPO4 were detected both in bacterized root tissues and root knot infected roots but it was more prominent in root tissues treated with *P. fluorescens* challenge inoculated with the nematode.

Seven isozymes of superoxide dismutase could be detected in *P. fluorescens* treated plants, inoculated with *M. graminicola*. Out of the seven isozymes (SOD 1 - SOD 7)

four isozymes *viz*., SOD 1, SOD 3, SOD 6 and SOD 7 were also detected in *M. graminicola* infected root tissues without treatment with *P. fluorescens*. However, the intensity was less, compared to the plants treated with *P. fluorescens*. All the seven isoforms of SOD appeared with a high intensity in bacterized plants challenge inoculated with the nematode and SOD 2 and SOD 4 were two newly induced isoforms expressed in rice root tissues by this treatment.

Enhanced activities of defense enzymes have been suggested to have a direct or indirect role in the induction of systemic resistance in plants against pathogens (Dalisay and Kuc, 1995). In the present study the strong induction of various isoforms of PO, PPO and SOD in bacterized plants challenge inoculated with the root-knot nematode, *M. graminicola* induced systemic resistance, which alternately reduced the development of the nematode. Detection of greater activity but similar banding pattern for peroxidase and polyphenol oxidase in treated and untreated plants suggests that differences in the isoforms associated with induced resistance are quantitative but not qualitative, A similar quantitative type of ISR was observed by Nandakumar *et al*. (2001) in rice against sheath blight. Radjacommare *et al*. (2003) detected unique PO and PPO isoforms in rice treated with *P. fluorescens* against *Rhizoctonia solani*. PO and PPO isoforms were expressed at higher levels in bacterized tomato root tissues challenge inoculated with *M. incognita* and *Fusarium oxysporum* f sp. *lycopersici* (Ramamoorthy *et al*., 2001).

Similar to other enzymes, SOD activity was induced by *P. fluorescens* against the inoculated nematode. Induction of two new SOD isoforms and a higher level of expression of other isoforms of SOD might be implicated in induced defense responses against the nematode invasion. Root colonizing *P. fluorescens* of the saprophytic fluorescent pseudomonads group expressed increased levels of SOD and catalase (Katswon and Anderson, 1990). The presence of numerous copies of genes conferring tolerance to oxidative stress (i.e., 10 peroxidases,six catalases, and two superoxide dismutases) in the genome of Pf-5 supports the proposed importance of oxidative stress tolerance to the fitness of this rhizosphere bacterium (Loper *et al*., 2007) as an effecient biocontraol agent.

Table 2. Nematode population in rice root per plant (n=5)

Treatment	Days after treatment		
	7	14	21
Pseudomonas fluorescens	0[c] (0.61)	0[c] (0.61)	0[c] (0.61)
Meloidogyne graminicola	21.6[a] (4.69)	29.4[a] (5.46)	34.9[a] (5.94)
P. fluorescens + M. graminicola	12.8[b] (3.63)	9.3[b] (3.11)	8.7[b] (3.01)
Untreated uninoculated control	0[c] (0.61)	0[c] (0.61)	0[c] (0.61)
CD (0.05)	0.72	0.91	1.02

Figures followed by the same alphabets are statistically not significant

Nematode population in rice root tissues treated with *P. fluorescens* challenge inoculated with *M. graminicola* (Table 2). The population of the root knot nematode M.graminicola was significantly lower in rice roots treated with P.fluorescens.There was 40.7% reduction in the nematode population 7 days after inoculation in the P.fluorescens treated plants.Similarly there was 68.4% and 75.1% reduction in the nematode population 14 and 21 days after inoculation respectively in the treated plants. Earlier reports have proved the effectiveness of P. fluorescens in suppression of economically important plant diseases (Jeyalakshmi *et al.*, 2010) and plant parasitic nematodes (Muthulakshmi et al., 2010; Deepa *et al.*, 2011). Significant reduction in root and soil population of the rice root nematode, *H. gracilis* was obtained with *P. fluorescens*. Another important species of rice root nematode, *H. oryzae* was also successfully managed by the application of *P. fluorescens*.

In conclusion, the present study implies that earlier and higher accumulation of phenols and defense enzymes *viz*., peroxidase, polyphenoloxidase, phenylalanine ammonia lyase and chitinase in rice root tissue treated with *P. fluorescens* in response to invasion by *M. graminicola* collectively contribute to induced systemic resistance and decrease in nematode infection.

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