INDUCTION OF SYSTEMIC RESISTANCE IN TOMATO AGAINST ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA* BY *PSEUDOMONAS FLUORESCENS*

B. Anita, G. Rajendran and R. Samiyappan*

*Department of Nematology, *Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore - 641 003, India*

Summary. Induction of defence enzymes by *Pseudomonas fluorescens* isolate Pf_1 against challenge inoculation of *Meloidogyne incognita* in tomato was studied *in vitro*. The activities of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), chitinase and catalase were assayed. The isoforms of PO and PPO were examined by discontinuous native polyacrylamide gel electrophoresis. Activities of all the enzymes were significantly higher in bacterized tomato root tissues challenged with the nematode. Isoform analysis revealed unique PO and PPO isoforms induced in *P. fluorescens*-treated plants. There was also a significant reduction in the root-knot nematode population in roots of and soil around bacterized plants. The results suggest that induction of defence enzymes involved in the phenylpropanoid pathway and accumulation of phenolics and pathogenesis related proteins would have contributed to the restriction of invasion of *M. incognita* in tomato roots.

The ability of a plant to survive any pathogen attack depends on pre-formed barriers and on induced active defence mechanisms. Induced defence mechanisms in plants against various pathogens by biotic and abiotic inducers have been reported in many crops (Baker *et al.*, 1997). One classical biotic inducer is the plant growth promoting bacterium, *Pseudomonas fluorescens* Migula (Leeman *et al.*, 1995). It activates defence gene products and enzymes, including peroxidase (PO) and polyphenol oxidase (PPO), that catalyse the formation of lignin and phenylalanine ammonia lyase (PAL), which is involved in synthesis of phytoalexins and phenolics (Van Peer *et al.*, 1991). Other defence enzymes include pathogenesis-related proteins (PRs) such as chitinases, which degrade the cell wall and cause lysis of the cell. Plant growth promoting rhizobacteria also induce systemic resistance against nematode pests (Oostendrop and Sikora, 1990; Sikora, 1988). The use of *P. fluorescens* as a biological control agent has been reported as a successful strategy in management of sugar beet and potato cyst nematodes (Sikora, 1992), rice root nematodes (Swarnakunari *et al.*, 1999) and root-knot nematode, *Meloidogyne incognita* (Kofoid *et* White 1919) Chitw., (Santhi and Sivakumar, 1995).

The present study was carried out to assess the induction of defence enzymes by *P. fluorescens* against *M. incognita* in tomato as a result of induced systemic resistance.

MATERIALS AND METHODS

The susceptible tomato (*Lycopersicon esculentum* Mill.) cultivar PKM-1 (seeds obtained from KVK, Tamil Nadu Agricultural University, Coimbatore, India) and the root-knot nematode, *M. incognita* (obtained from the culture collection of the Department of Plant Nematology, Tamil Nadu Agricultural University) were used in this study. A talc-based formulation of fluorescent *P. fluorescens* (Pf1), isolated from blackgram rhizosphere (TNAU, Coimbatore, India), was prepared as described by Vidhyasekaran and Muthamilan (1995). The population of bacteria in the formulation was 2.8 x 10^8 cfu/g of talc powder at the time of application.

Tomato seeds were treated with *P. fluorescens* at the rate of 10 g/kg seed and sown in earthern pots filled with sterilized potting soil. Seven days after sowing, *P. fluorescens* was applied by adding the talc based formulation to the soil surface at a dose of 1 g/kg. Half of the bacterized plants were challenge inoculated with *M. incognita* (one J_2/g of soil) one day after soil application of *P. fluorescens* and the other half were not inoculated with the nematode. In addition, plants that had not been treated with *P. fluorescens* were inoculated with the nematode, whilst plants that received no treatment at all were kept as control. The experiment was conducted in a randomized block design of four treatments with five replications.

Plants were uprooted at different intervals (7, 14, 21 and 28 days after the nematode inoculation). Young root samples (1 g), randomly collected along the root system, were homogenised in 1 ml of 0.1 M phosphate buffer pH 7.0 at 4 °C in a pre-chilled pestle and mortar. The homogenate was centrifuged at 20,000 rpm at 4 °C for 15 minutes and the supernatant served as enzyme source.

Peroxidase (PO) activity was analysed spectrophotometrically (Hammerschmidt *et al.*, 1982). The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 100 µl of enzyme extract and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2 °C). The change in absorbance at 420 nm was recorded

at 30 sec intervals for 3 minutes. The enzyme activity was expressed as changes in the absorbance min^{-1} g^{-1} of fresh tissue.

PPO activity was determined by the procedure described by Mayer *et al.* (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 100 µl of the enzyme extract. 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} g^{-1} fresh tissue.

PAL activity was determined as the rate of conversion of L-phenyl alanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al.* (1984). A sample containing 100 µl of enzyme extract was incubated with 1.2 ml of 0.1 M borate buffer (pH 8.8) and 1.5 ml of 12 M L-phenyl alanine in the same buffer for 30 minutes at 30 °C. The reaction was stopped by addition of 1 M trichloroacetic acid and, after incubation for 5 min at 37 °C, the absorbance was read at 290 nm. Enzyme activity was expressed as mmol trans-cinnamic acid min^{-1} g^{-1} of fresh tissue.

The calorimetric assay of chitinase was carried out according to Boller and Mauch (1988). Reagents used were colloidal chitin, snail gut enzyme (Sepracor, France), dimethyl amino benzaldehyde (DMAB) (Sigma, USA) and buffer. Colloidal chitin was prepared from crab shell chitin (Sigma, USA) by the method described by Berger and Reynolds (1958).

The reaction mixture consisted of 10 ml of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg). After incubation for 2 h at 37 °C, the reaction was stopped by centrifugation at 1,000 g for three minutes. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 3 ml of 1 M potassium phosphate buffer (pH 7) and incubated with 20 ml of 3% (w/w) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for three minutes and then rapidly cooled in an ice bath. After addition of 2 ml of DMAB, the mixture was incubated for 20 minutes at 37 °C. Immediately thereafter the absorbance was measured at 585 nm. N acteylglucosamine (GluNAC) was used as standard. The enzyme activity was expressed as nmole GluNAC equivalents/min/g fresh tissue.

Catalase activity was measured according to the perborate method of Feinstein (1942). About 200 mg of plant sample was macerated with 10 ml phosphate buffer. It was centrifuged at 3000 g for 10 minutes. One ml of enzyme was added to four 50 ml beakers, except for the blank, and 5 ml of sodium perborate was added to it. After 1, 2, 3 and 4 minutes, 10 ml H_2SO_4 was added to each of the beakers, respectively, to arrest the reaction. With the blank, 10 ml H_2SO_4 was added, then 5 ml sodium perborate and finally 1 ml enzyme extract. The beaker contents were titrated against 0.05 N $KMnO_4$. Activity was expressed in terms of H_2O_2 assuming that 1 ml $KMnO_4$ = 0.85 mg of H_2O_2.

The isoform profiles of PO and PPO were examined by discontinuous native polyacrylamide gel electrophoresis (Laemmli, 1970). Root samples (1 g) were collected 28 days after nematode inoculation, and the protein extract was prepared by homogenizing the samples in 1 ml of 0.1 M phosphate buffer pH 7 followed by centrifugation at 16,000 g for 20 min at 4 °C. Samples (80 µl) were loaded into 8% polyacrylamide gels (Sigma, USA). After electrophoresis, PO isoforms were visualized by soaking the gels in staining solution containing 0.05% benzidine acetate buffer (pH 4.2), for 30 minutes in the dark, after which drops of 30% H_2O_2 were added with constant shaking till the bands appeared. To assess PPO isoforms, the gels were equilibrated for 30 minutes in 0.1% P-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by gentle shaking, which resulted in appearance of dark brown discrete protein bands.

RESULTS AND DISCUSSION

Application of a talc-based formulation of *P. fluorescens* Pf1 as seed treatment, followed by soil treatment, significantly reduced the root-knot nematode population in tomato roots. Root galling in tomato due to infestation by *M. incognita* was also less in bacterized plants compared to untreated plants. This is in agreement with the report that the level of infestation of *M. incognita* on tomato was reduced with fewer galls and egg masses in the root following root dipping with *P. fluorescens* strain (Pf1) (Santhi and Sivakumar, 1995).

Our studies of induced defence mechanisms revealed significant accumulation of peroxidase and polyphenol oxidase in bacterized tomato plants challenge inoculated with *M. incognita*. Accumulation of these enzymes began seven days after challenge inoculation with the nematode and gradually increased up to 28 days. Plants inoculated with the nematode alone also showed increased PO and PPO levels, but the increases were less than in *P. fluorescens*-treated plants. In bacterized plants not challenged by nematodes the activities of PO and PPO were similar to those in plants challenged by nematodes alone, but activity hardly challenged in the control plants (Table I).

Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989). Remarkable increases were observed in the peroxidase activity of all the cellular components, viz. soluble fraction, mitochondria and microsomes in roots of resistant pea lines attached by *Heterodera goettingiana* (Arrigoni *et al.*, 1981). Chen *et al.* (2000) reported greater activities of PO and PPO in cucumber root tissues treated with *Pseudomonas corrugata* challenged with *P. aphanidermatum*. Native PAGE revealed unique isoforms of PO and PPO in *P. fluorescens* isolate Pf1 treated roots challenged with the ne-

matode and the expression of the isoforms was more prominent in treated plants (Figs 1 and 2).

PAL is the first enzyme in phenyl propanoid metabolism and in the production of phenolics and phytoalexins that prevent establishment of pathogens (Daayf *et al.,* 1997). In the present study, increased activity of PAL was recorded in *P. fluorescens*-treated tomato roots challenged with the nematode, presumably due to prevention of nematode invasion. In plants inoculated with the nematode alone the activity was, perhaps, due to invasion of root tissues by the nematode. Increased activity of PAL has been reported in resistant but not susceptible soybean cultivars after infection by *M. incognita* or *Heterodera glycines* (Edens *et al.,* 1995).

In the present study, the activity of chitinase was more in tomato root tissues treated with *P. fluorescens* challenged with the nematode, which may have been due to induction of systemic resistance in tomato against the nematode by the bacterium (Table I). Maurohofer *et al.* (1994) reported that induction of systemic resistance by

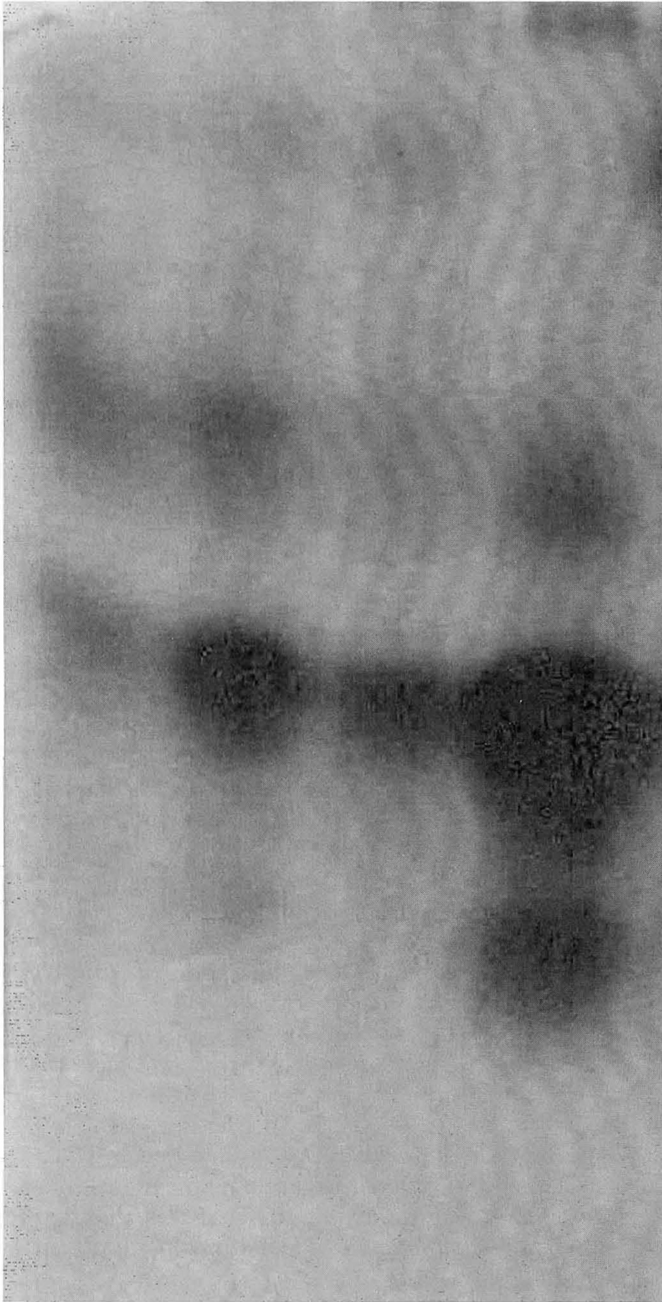

Fig. 1. Native PAGE analysis for PO isoform profile induced by *Pseudomonas fluorescens* in tomato (from left: first lane, control plants; second lane, plants treated with *P. fluorescens* alone; third lane, plants inoculated with *Meloidogyne incognita* alone; fourth lane, plants treated with *P. fluorescens* challenged with *M. incognita*).

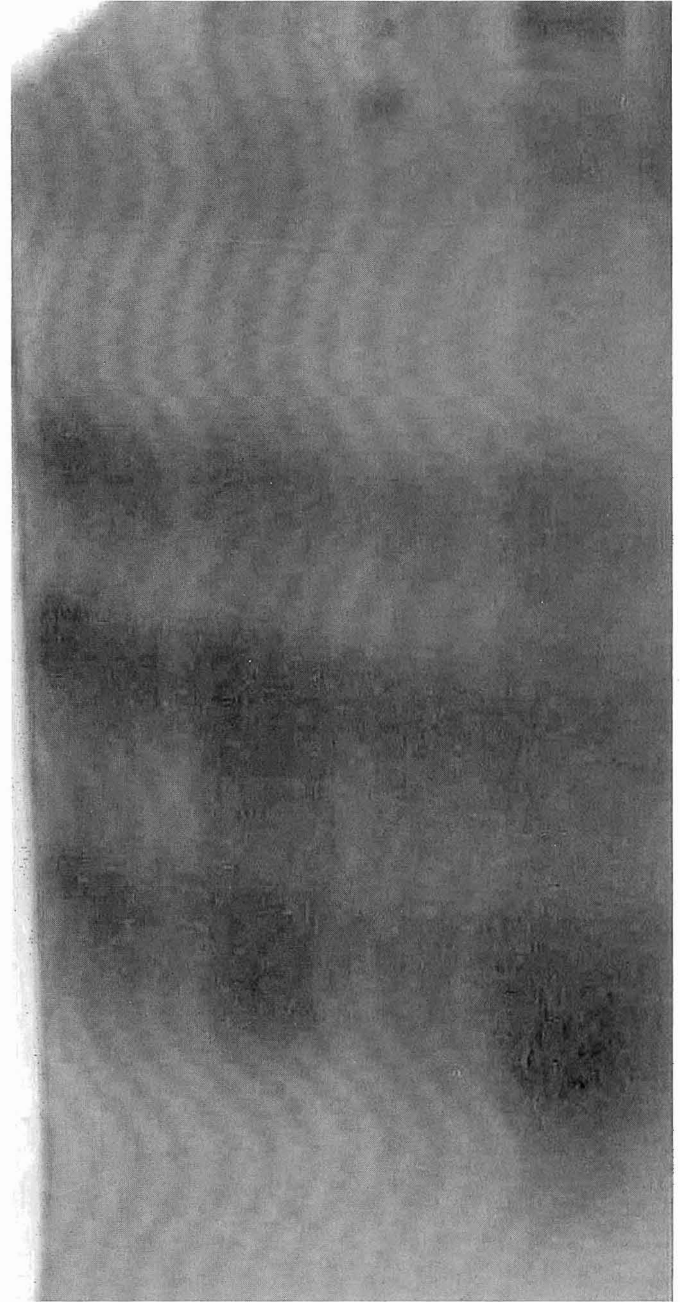

Fig. 2. Native PAGE analysis for PPO isoform profile induced by *P. fluorescens* in tomato (from left: first lane, control plants; second lane, plants treated with *P. fluorescens* alone; third lane, plants inoculated with *M. incognita* alone; fourth lane, plants treated with *P. fluorescens* challenged with *M. incognita*).

Table I. Defence enzymes in tomato treated with *Pseudomonas fluorescens* challenge inoculated with *Meloidogyne incognita*.

Treatment	Peroxidase change in absorbance $m^{-1} g^{-1}$				Polyphenoloxidase change in absorbance $m^{-1} g^{-1}$				Phenyl Alanine Ammonia Lyase nmol trans-cinnamic acid $m^{-1} g^{-1}$				Chitinase nmol GluNac $m^{-1} g^{-1}$				Catalase $\mu g\ H_2O_2\ g^{-1}$			
	Days after inoculation																			
	7	14	21	28	7	14	21	28	7	14	21	28	7	14	21	28	7	14	21	28
P. fluorescens + *M. incognita*	0.66	1.38	1.53	2.04	0.13	1.52	2.38	3.71	5.32	4.43	4.81	2.74	1897	2133	2442	2386	12.26	11.10	10.21	10.04
M. incognita alone	0.15	0.54	0.38	0.29	0.11	0.15	0.99	0.58	1.95	2.05	2.36	1.62	615	633	583	538	22.63	23.45	22.36	21.98
P. fluorescens alone	0.05	0.25	0.53	0.62	0.39	0.53	0.56	0.60	1.67	1.57	1.58	1.42	1085	1362	1412	1428	20.10	19.28	18.85	18.60
Control	0.06	0.04	0.11	0.20	0.01	0.02	0.03	0.07	0.06	0.01	0.04	0.02	589	507	468	403	18.40	18.76	18.46	18.13
CD (0.05) : t		0.058				0.061				0.0423				33.56				0.169		
d		0.058				0.061				0.0423				33.56				0.166		
td		0.115				0.122				0.085				67.12				0.338		

P. fluorescens was correlated with the accumulation of chitinase. Several studies have shown that various preparations of chitinases can be used successfully to control plant parasitic nematodes both *in vitro* and in soil (Miller and Sands, 1977; Mercer *et al.*, 1992; Swarnakumari and Lakshmanan, 1999). Chitinases play a secondary function as signal molecules, which elicit the induction of other PR proteins or metabolites that are involved in plant defence reactions. After invasion of potato plants by *Globodera pallida* and the appearance of chitinase in roots, large amounts of new proteins accumulated in leaves (Rahmi *et al.,* 1996).

Catalase is an enzyme related to stress tolerance as it converts cytotoxic hydrogen peroxide to water and oxygen, which is lethal towards pathogenic microorganisms. In tomato plants treated with *P. fluorescens* there were decreased concentrations of H_2O_2 indicating increased catalase activity, which might have imparted tolerance to the plant against root knot nematode stress (Table I). However, Rajasekhar *et al.,* (1997) reported that the activity of catalase was of lower magnitude in a resistant tomato cultivar.

The present study has shown an accumulation of defence enzymes, viz. peroxidase, polyphenol oxidase, chitinase, phenylalanine ammonia lyase and catalase, in tomato root tissue treated with *P. fluorescens* isolate Pf1 in response to invasion by the root knot nematode *M. incognita*.

LITERATURE CITED

Arrigoni O., Zacheo G., Bleve Zacheo T., Arrigoni Liso R. and Lamberti F., 1981. Changes in superoxide dismutase and peroxidase activities in pea roots infested by *Heterodera goettingiana*. *Nematologia Mediterranea, 9:* 189-195.

Baker B., Lambryski P., Staskaawiczad B. and Dineshkumar S.P., 1997. Signalling in plant microbe interactions. *Science, 276:* 726-733.

Berger L.R. and Reynolds D.M., 1958. The chitinase system of a strain of *Streptomyces griseus. Biochemica et Biophysica Acta,* 29: 522-534.

Boller T. and Mauch F., 1988. Colorimetric assay for chitinase. *Methods in Enzymology, 161:* 430-435.

Bruce R.J. and West C.A., 1989. Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiology, 91*: 889-897.

Chen C., Belanger R.R., Benhamou N., and Paulitz T., 2000. Defence enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum. Physiological and Molecular Plant Pathology, 56*: 13-23.

Daayf F., Bel-Rhlid R. and Be'langer R.R., 1997. Methyl ester of P. coumaric acid: a phytoalexin like compound from long English cucumber leaves. *Journal of Chemical Ecology, 23*: 1517-1526.

Dickerson D.P., Pascholati S., Hagerman A.E., Butler L.G. and Nicholson R.L. 1984. Phenylalanine ammonia lyase and hydroxy cinnamate: CoA ligase in maize mesocotyls

inoculated with *Helminthosporium maydis* or *H. carbonum* *Physiological Plant Pathology, 25*: 111-123.

Edens R.M., Anand S.C. and Bolla R.I., 1995. Enzymes of the phenyl propanoid pathway in soybean infected with *Meloidogyne incognita* or *Heterodera glycines. Journal of Nematology, 27*: 292-303.

Feinstein R.M., 1949. Perborate as a substrate in a new assay of catalase. *Journal of Biological Chemistry, 180*: 1197-1202.

Hammerschmidt R., Nuckles E.M. and Kuae J., 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium. Physiological Plant Pathology, 20*: 73-82.

Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*: 680-685.

Leeman M., Van Pelt J.A., Den Ouden F.M., Heinsbroek M., Bakker P.A.H.M. and Schippers B., 1995. Induction of systemic resistance against *Fusarium* wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens. Phytopathology, 85*: 1021-1027.

Maurohofer M., Mewly C., Metraux J.P. and Defago G., 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root colonizing *Pseudomonas fluorescens* strain CHAO: influence of the gacA gene and of pyoverdine production. *Phytopathology, 84*: 139-146.

Mayer A.M., Harel E. and Shaul R.B., 1965. Assay of catechol oxidase a critical comparison of methods. *Phytochemistry, 5*: 783-789.

Mercer C.F., Greenwood D.R. and Grant J.L., 1992. Effect of plant and microbial chitinases on the eggs and juveniles of *Meloidogyne hapla. Nematologica, 38*: 227-236.

Miller P.M. and Sands D.C., 1977. Effects of hydrolytic enzymes on plant parasitic nematodes. *Journal of Nematology, 9*: 192-197.

Oostendorp M. and Sikora R.A., 1990. In vitro relationship between rhizosphere bacteria and *Heterodra schachtii. Revue de Nématologie, 13*: 269-274.

Rahimi S., Perry R.N. and Wright D.J., 1996. Identification of pathogenesis related proteins induced in leaves of potato plants infected with potato cyst nematodes, *Globodera* species. *Physiological & Molecular Plant Pathology, 49*: 49-59.

Rajasekhar S.P., Ganguly A.K. and Swain S.C., 1997. Quantitative changes in superoxide dismutase, catalase and peroxidase with reference to resistance in tomato to *Meloidogyne incognita. Indian Journal of Nematology, 27*: 79-85.

Santhi A. and Sivakumar V., 1995. Biocontrol potential of *Pseudomonas fluorescens* (Migula) against root-knot nematode, *Meloidogyne incognita* (Kofoid *et* White, 1919) Chitwood, 1949 on tomato. *Journal of Biological Control, 9*: 113-115.

Sikora R.A., 1988. Interrelationship between plant health promoting rhizobacteria, plant parasitic nematodes and soil microorganisms. *Med. Fac. Landbouww. Rijksuniv. Gent.* 53/2b: 867-878.

Sikora R.A., 1992. Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. *Annual Review of Phytopathology, 30*: 245-270.

Swarnakumari N. and Lakshmanan P.L., 1999. Effect of organic amendments and *Pseudomonas fluorescens* against rice root nematode, *Hirschmanniella oryzae*. International Seminar on Integrated Pest Management, Hyderabad, India, p.101.

Swarnakumari N., Lakshmanan P.L. and Samiyappan R., 1999. Screening of different isolates of *Pseudomonas fluorescens* against rice root nematode, *Hirschmanniella oryzae*. International seminar on Integrated Pest Management, Hydrabad, India, p.102.

Van Peer R., Niemann, G.J., Schippers, B., 1991. Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathology, 81*: 728-734.

Vidhyasekaran P. and Muthamilan M., 1995. Development of formulations of *Pseudomonas fluorescens* for control of chickpea wilt. *Plant Disease, 79*: 782-786.

Accepted for publication on 12 December 2003.