MANAGEMENT OF *MELOIDOGYNE JAVANICA* ON ACID LIME USING *PAECILOMYCES LILACINUS* AND *PSEUDOMONAS FLUORESCENS*[1]

M.S. Rao

Nematology Laboratory, Division of Entomology and Nematology, Indian Institute of Horticultural Research, Hessaraghatta Lake P. O., Bangalore 560 089, India

Summary. Field experiments were conducted to evaluate the efficacy of formulations of *Paecilomyces lilacinus* (10^6 cfu/g) and *Pseudomonas fluorescens* (10^8 cfu/g), applied singly or in combination and with the addition of neem seed cakes, for the management of the root-knot nematode *Meloidogyne javanica* on acid lime (*Citrus aurantifolia*). Application of 10 g *Paecilomyces lilacinus,* 10 g *Pseudomonas fluorescens* and 250 g of neem seed cake per tree once in six months, for a period of two years, reduced the population of the nematode and increased the yield of the crop. Root colonization by both bio-agents increased with the increase in number of applications of their respective formulations, thus resulting in increased percentage of parasitized eggs and egg masses of *M. javanica* by *P. lilacinus*. The combination of the two bio-agents and that of these with neem seed cakes improved root colonization by both bio-agents and the control of the nematode. Combining *P. lilacinus*, *P. fluorescens* and neem seed cakes could form the basis for a sustainable management of root-knot nematodes on acid lime compatible with organic farming practices.

Key words: Antagonistic bacteria, biological control, *Citrus aurantifolia*, fungal egg parasites, root-knot nematode.

Citrus fruits have an important place among popular and exclusively grown tropical and sub-tropical fruits. Acid lime (*Citrus aurantifolia* Christm. *et* Panz.) has a great adaptability to different climatic conditions and is, therefore, grown in tropical and subtropical regions of the world. Mexico and India are the main producers of acid lime. This citrus species is grown in every state of India, but the leading producer states are Andhra Pradesh, Maharashtra, Assam and Karnataka. Acid lime fruits are available throughout the year and are mostly used for flavouring vegetable dishes, fish, meat and salads, and in preparing delicious, refreshing drinks and pickles in addition to being a major source of vitamin C.

The root knot-nematode *Meloidogyne javanica* (Treub) Chitw. is one of the most important factors affecting production of acid lime in India (Mani, 1986; Parvatha Reddy and Rao, 2001), and severe infestations have been observed in Karnataka, Maharashtra, Tamil Nadu and Andhra Pradesh states (Rao *et al*., 2001). Contrary to what is known for the most common species of *Meloidogyne*, the populations of *M. javanica* from India not only enter the roots but also reproduce on acid lime. Since the use of nematicides can be hazardous, it was thought to standardize a method for nematode management by using formulations of *Paecilomyces lilacinus* (Thom.) Samson and *Pseudomonas fluorescens* Migula. The nematophagous fungus *P. lilacinus* is a widespread facultative parasite of the sedentary stages of plant-parasitic nematodes. The fungus colonizes the root surface and parasitizes eggs and egg-masses of root-knot nematodes (Jatala, 1986; Cabanillas and Barker, 1989; Alamgir Khan *et al*., 1997; El-Borai and Duncan, 2005). Similarly, various researchers have reported the bio-control potential of *P. fluorescens* against root-knot and other nematodes (Santhi and Sivakumar, 1995; Parveen *et al.*, 1998; Siddiqui *et al.*, 1999; Rao *et al.*, 2002). This bacterium is known to produce metabolites and lytic enzymes that have nematicidal activity or inhibit egg hatch (Chen and Dickson, 2004). However, there are no reports on the combined use of these two bio-control agents for the management of root-knot nematodes on acid lime. Hence, investigations were carried out to ascertain their compatibility for the management of *M. javanica*, and to test the effects of their combination with applications of neem seed cake.

MATERIALS AND METHODS

The local isolates of *P. fluorescens* (IIHR Pf – 2) and *P. lilacinus* (IIHR Pl – 2) (maintained in the collection of the Indian Institute of Horticultural Research, Bangalore, India) were separately mass produced through a liquid and solid fermentation process (the details of the process are not revealed now for patent considerations). In these investigations, formulated products of *P. fluorescens* (10^8 cfu/g) and *P. lilacinus* (10^6 cfu/g) were used.

The study was conducted from September, 2004 to September, 2006 in a field with sandy loam soil at the Indian Institute of Horticultural Research, Bangalore, India. Three-month-old acid lime seedlings, free from any nematode or other diseases, were transplanted in the field in March 1998, when the population density of

[1] IIHR contribution No. 16 - 2008.

Corresponding author e-mail: msrao@iihr.ernet.in

M. javanica was of 52 ± 12 second-stage juveniles (J_2) per 100 cm^3 soil. At the beginning of this investigation in September 2004, the population density of the nematode was of 124 ± 17 J_2/100 cm^3 soil. The nematode species was identified on the basis of observations of perineal patterns of females.

There were six treatments: i) *P. fluorescens* alone applied at 20 g/tree; ii) *P. lilacinus* alone at 20 g/tree; iii) *P. fluorescens* + *P. lilacinus* at 20 g /tree each; iv) neem seed cake alone at 250 g/tree; v) neem seed cake 250 g + *P. fluorescens* at 20 g + *P. lilacinus* at 20 g/tree; vi) control without any treatment. All the treatments were replicated ten times in a randomized block design. Each replicate consisted of four adjacent trees spaced 2.5 m × 2.5 m.

The application of the bio-agents and neem seed cake was repeated at intervals of 6 months. The first application was made on 1st of September, 2004, the second on 1st of March, 2005, the third on 1st of September, 2005, and the last one on 1st of March, 2006. The treatments were applied in a basin (30 cm width × 15 cm depth) made 80 cm away from the trunk of each acid lime tree. After treatment application, the basin was covered with soil. Nematode populations were estimated six months after each application. Standard crop maintenance procedures, such as weeding, pesticide application to the canopy and fertilization, were followed. Irrigation was by flooding in a basin of 1 m radius made around each tree.

Observations were made on nematode population densities in root and soil, root colonization by the bio-agents, effect of *P. fluorescens* on nematode egg hatch, egg parasitism by *P. lilacinus* and yield of the crop.

At each sampling time, soil samples were collected from a tree at 30 cm depth, using a soil auger, from five places around each tree. From each spot, about 50 g of soil were taken to form a composite sample of 250 g per plant. As there were four trees per replicate we collected four samples per replicate, which were mixed to obtain a composite soil sample. Then, *M. javanica* J_2 were extracted from a sub-sample of 100 cm^3 soil per replicate by Cobb's sieving and decanting method (Cobb, 1918) and counted. The nematode population in the roots was estimated by collecting 10 g of root per tree and four root samples per replicate. The roots were washed free of adhering soil, stained using acid fuchsin following the method of Bridge *et al.* (1982), homogenized in a blender (Bazaz make) for 2 minutes, sieved to separate larger root pieces that were discarded, and nematodes in the water suspension collected and counted under a stereo-microscope.

To evaluate root colonisation by *P. fluorescens* and *P. lilacinus,* five months after application of the bio-agents, 10 g of roots per tree were collected at random at the depth of 15-30 cm in the basin surrounding the tree.

Table I. Effects of multiple applications of *Paecilomyces lilacinus* and *Pseudomonas fluorescens* formulations at the rate of 20 g per tree, and neem seed cake at 250 g per tree, alone and in combination, on the dynamics of *Meloidogyne javanica* on acid lime under field conditions.

Treatment	Nematodes per 10 g root				Nematodes per 100 cm^3 soil			
	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application
P. lilacinus 20 g/tree	23	20	16	11	114	90	88	65
P. fluorescens 20 g/tree	25	22	18	12	121	96	91	74
P. fluorescens 20 g + *P. lilacinus* 20 g/tree	22	15	13	8	110	87	80	62
Neem cake 250 g/tree	20	18	16	15	103	90	86	80
Neem cake 250 g + *P. fluorescens* 20 g + *P. lilacinus* 20 g/tree	18	14	11	6	87	77	62	44
Control	29	31	31	34	129	135	139	142
C. D. (P = 0.05)	3.14	3.56	3.79	2.12	11.81	11.82	12.69	10.25
SEM	1.26	1.22	1.43	0.94	4.03	2.09	2.38	1.66
CV%	12.28	13.68	18.32	14.64	8.12	4.89	5.85	4.76

Values are means of five replicates.
SEM = Standard error of the mean; CV% = Coefficient of variation.

The roots were washed to remove soil particles, dried, weighed, cut in pieces of about 1 cm long and homogenized in a blender, before plating them on semi-selective media. Root colonization by *P. fluorescens* and *P. lilacinus* was assessed using the semi-selective media developed by King *et al.* (1954) and Mitchel *et al.* (1987), respectively.

To estimate the effect of *P. fluorescens* on egg hatching, five egg masses of the nematode (averaging 344 eggs/egg mass) were randomly selected from 20 g of root sample per tree, treated with 0.01% sodium hypochlorite in a Petri plate for 30 seconds for surface disinfection, placed in a Petri plate (5 cm diam.) containing 5 ml of sterile distilled water and incubated at 25 °C. Numbers of emerging J_2 were recorded at 24-hour intervals. After 5 days, per cent suppression of egg hatching was computed on the basis of the number of juveniles hatched in the treatments and in the control.

To assess egg parasitism by *P. lilacinus*, five egg masses of the nematode were randomly selected from 20 g of root sample per tree and treated with 0.05% sodium hypochlorite. Then, the average number of eggs infected was counted under a microscope at ×100. *Paecilomyces lilacinus* was also isolated from 10 egg masses of *M. javanica* per replicate by using the semi-selective medium mentioned above. Data on yield were recorded by harvesting acid limes from each tree and expressed as number of fruits/tree and their weight (Table IV).

Data were subjected to analysis of the variance and compared by CD (critical difference).

RESULTS AND DISCUSSION

Applications of *P. fluorescens, P. lilacinus* and neem cake, alone and in the combination mentioned above, were effective in the management of root-knot nematodes on acid lime (Table I). However, applications of the two bio-agents separately were not as effective as the combination (Table I). The neem cake treatment was at par with the bio-agents in decreasing the root and soil population densities of the nematode until 6 months after the third application, but it did not decrease significantly the nematode populations thereafter when compared with the bio-agents (Table I). Application of the bio-agents over a period of 18 months increased root colonization and they were more effective than neem seed cake alone in lowering the populations of nematodes in roots and soil of acid lime (Tables I and II).

To be effective, *P. lilacinus* has to colonize the root system and then it can parasitize the egg masses of the root-knot nematodes (Jatala, 1986; Cabanillas and Barker, 1989). The degree of root colonization depends on the strain of the fungus and also on rhizosphere conditions. In general, the higher the dosage of the bio-agent applied, the greater will be the extent of colonization of

Table II. Numbers of colony forming units of *P. lilacinus* and *P. fluorescens* on the roots of acid lime under field conditions.

Treatment	*P. lilacinus* (CFU/g root)				*P. fluorescens* (CFU/g root)			
	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application
P. lilacinus 20 g/tree	6,443	7,345	10,548	14,562	0.0	0.0	0.0	0.0
P. fluorescens 20 g/tree	0.0	0.0	0.0	0.0	9,457	12,542	15, 674	17,547
P. fluorescens 20 g + *P. lilacinus* 20 g/tree	5,848	7,129	10,964	13,679	9, 138	11,864	14,879	18,345
Neem cake 250 g/tree	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neem cake 250 g + *P. fluorescens* 20 g + *P. lilacinus* 20 g/tree	7,548	8,987	13,658	17,542	10,547	13,683	18,547	20,458
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C. D. (P = 0.05)	785.4	674.3	1,234.8	1,267.4	876.8	976.7	1,363.5	1,465.7
SEM	93.45	93.38	325.40	245.18	198.65	205.20	425.78	268.9
CV%	6.31	5.32	12.41	7.18	9.15	7.22	11.63	6.40

Values are means of five replicates.

CFU = Colony Forming Units; SEM = Standard error of the mean; CV% = Coefficient of variation.

Table III. Effects of applications of *P. lilacinus* and *P. fluorescens* on the percentage of eggs parasitized or percentage of egg hatching suppression of *M. javanica* on acid lime under field conditions.

Treatment	% eggs parasitised by *P. lilacinus*				% egg hatching suppressed by *P. fluorescens*			
	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application
P. lilacinus 20 g/tree	5	12	18	21	0.0	0.0	0.0	0.0
P. fluorescens 20 g/tree	0.0	0.0	0.0	0.0	7	13	17	29
P. fluorescens 20 g + *P. lilacinus* 20 g/tree	6	10	16	23	5	12	18	27
Neem cake 250 g/tree	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neem cake 250 g + *P. fluorescens* 20 g + *P. lilacinus* 20 g/tree	8	14	22	32	11	17	26	35
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C. D. (P = 0.05)	1.48	2.14	2.68	3.49	1.78	2.24	3.28	3.86
SEM	0.57	0.51	0.66	0.83	0.70	0.79	0.68	1.18
CV%	16.15	19.36	15.71	14.59	14.11	15.22	14.92	17.34

Values are means of five replicates.

SEM = Standard error of the mean; CV% = Coefficient of variation.

Table IV. Effects of *P. lilacinus*, *P. fluorescens* and neem seed cake on the yield of acid lime in a field infested with *M. javanica*.

Treatment	Fruits in 6 months (No. per tree)				Fruit weight (kg per tree)			
	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application	6 months after 1st application	6 months after 2nd application	6 months after 3rd application	6 months after 4th application
P. lilacinus 20 g/tree	1109	1157	1212	1274	22.18	23.14	24.24	25.48
P. fluorescens 20 g/tree	1031	1145	1236	1266	20.62	22.90	2472	25.32
P. fluorescens 20 g + *P. lilacinus* 20 g/tree	1181	1186	1243	1302	23.62	23.72	24.86	26.04
Neem cake 250 g/tree	1042	1239	1217	1254	20.84	24.78	24.34	25.08
Neem cake 250 g + *P. fluorescens* 20 g + *P. lilacinus* 20 g/tree	1354	1404	1376	1512	27.08	28.08	27.52	30.24
Control	943	907	879	860	18.86	18.14	17.58	17.20
C. D. (P = 0.05)	145.29	176.94	165.2	153.85	2.58	3.16	3.59	3.12
SEM	33.81	36.26	29.08	32.67	1.26	1.04	0.72	0.57
CV%	6.81	6.91	5.44	5.86	12.7	9.96	6.77	5.20

Values are means of five replicates.

SEM = Standard error of the mean; CV% = Coefficient of variation.

the roots by a bio-agent. However, it is not feasible to apply higher dosages of the bio-pesticides than we used as it would be too expensive. It is possible to change rhizosphere conditions by applying organic amendments like neem cake. In this investigation, when *P. fluorescens* and *P. lilacinus* were applied in combination with neem seed cake, their efficacy in controlling the nematode was greater than when applied alone (Table I). The neem seed cake may provide favourable conditions for significant root colonization by these bio-agents (Table II), thus increasing their efficacy by allowing parasitization of a larger proportion of nematode eggs by *P. lilacinus* and greater suppression of egg hatching by *P. fluorescens* (Table III). This treatment also increased significantly the yield of acid lime (Table IV). However, only a small proportion of the yield increase could be attributed to the reduction in nematode population in the soil and roots as the nematode infestation was low throughout the course of the experiment, even in the untreated control. Therefore, the observed yield increases could also be due to other effects, such as growth promotion by *P. fluorescens*.

Paecilomyces lilacinus has been reported to parasitize the eggs and egg masses and can help in the management of root-knot nematodes on various crops (Dube and Smart, 1987; Cabanillas and Barker, 1989; Alamgir Khan *et al*., 1997; Rao *et al.*, 1998; Rao and Reddy, 2001). *Pseudomonas fluorescens* has also been effective in the management of root-knot nematodes (Santhi and Sivakumar, 1995; Parveen *et al.*, 1998; Siddiqui *et al.*, 1999; Rao *et al.*, 2002). In this experiment, parasitism of *M. javanica* eggs or egg masses by *P. fluorescens* was not observed, but this bio-agent suppressed nematode egg hatching (Table III). The efficacy of neem seed cake to control nematodes is well documented (Mankau, 1962; Alam *et al.*, 1980; Muller and Gooch 1982; Rao *et al.*, 1997).

When the two bio-agents were applied simultaneously, *P. lilacinus* did not affect root colonization by *P. fluorescens* and *P. fluorescens* did not affect the ability of *P. lilacinus* to parasitize eggs and egg masses of the nematode (Table II). Also, the application of neem seed cake in combination with these two bio-agents did not affect root colonization by the bio-agents (Table II). Rather, neem seed cake increased the performance of both bio-agents (Table III) and the yield of acid lime (Table IV). Therefore, this combined treatment could form the basis of a sustainable management strategy for root-knot nematodes on acid lime that meets the requirements of organic farming practices. In previous studies, increased efficacy of *P. lilacinus* on *M. incognita* infecting eggplants (Rao and Reddy, 2001) and of *Trichoderma harzianum* on *M. incognita* infecting tomato (Rao *et al.,* 1997) when treatments were combined with neem cake was observed. The effect of root colonization by *P. fluorescens and P. lilacinus* on root physiology and the role of *P. fluorescens* in inducing a systemic resistance response needs to be investigated to thoroughly understand the effects of these bio-agents.

ACKNOWLEDGEMENTS

The author thanks Dr. S.D. Shikhamany, Director, Indian Institute of Horticultural Research, Bangalore, for providing the facilities in the Division of Entomology and Nematology.

LITERATURE CITED

Alam M.M. and Khan A.M., 1980. Effect of organic amendments on the growth and chemical composition of tomato, egg plant, chilli and their susceptibility to attack by *Meloidogyne incognita. Plant and Soil, 57*: 231-236.

Alamgir Khan M.M., Holland R.J. and Williams K.L., 1997. Recent studies on *Paecilomyces lilacinus* as a bionematicide. Suppression of *Heterodera avenae* populations, infection of *Meloidogyne javanica* eggs, females and juveniles in a pot trial and *Radopholus similis* eggs in laboratory studies. *Australasian Nematology Newsletter, 8*: 2.

Bridge J., Page S.L.J. and Jordon S., 1982. An improved method for staining nematodes in roots. *Report of Rothamsted Experimental Station for 1981, Part 1*: 171.

Cabanillas E. and Barker K.R., 1989. Impact of *Paecilomyces lilacinus* inoculum level and application time on control of *Meloidogyne incognita* on tomato. *Journal of Nematolology, 21*: 115-120.

Chen Z.X. and Dickson D.W., 2004. Biological control of nematodes with bacterial antagonists. Pp. 1041-1082. *In*: Nematology – Advances and Perspectives. Vol. 2. Nematode Management and Utilization (Chen Z.X., Chen S.Y. and Dickson D.W., eds). CABI Publishing, Wallinford, UK.

Cobb N.A., 1918. Estimating the nematode population of the soil. *Agriculture Technical Circular No. 1,* Bureau of Plant industry, United States Department of Agriculture, 48 pp.

Dube B. and Smart G.C. Jr., 1987. Biological control of *Meloidogyne incognita* by *Paecilomyces lilacinus* and *Pasteuria penetrans*. *Journal of Nematolology, 19*: 222-227.

El-Borai F.E. and Duncan L.W., 2005. Nematode parasites of subtropical and tropical fruit tree crops. Pp. 467-492. *In*: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture, Second edition (Luc M., Sikora R.A. and Bridge J., eds). CABI Publishing, Wallingford, UK.

Jatala P., 1986. Biological control of plant parasitic nematodes. *Annual Review of Phytopathology, 24*: 455-489.

King E.O., Ward M.K. and Raney D.E., 1954. Two simple media for demonstration of pyocyanin and fluoresceins. *Journal of Laboratory and Clinical Medicine, 44*: 301-307.

Mankau R., 1962. Effect of organic soil amendments on nematode populations. *Phytopathology, 53*: 881-882.

Mani A., 1986. Occurrence of *Meloidogyne javanica* on citrus in Andhra Pradesh. *International Nematology Network Newsletter, 3*: 11-13.

Mitchell D.J., Mitchell K. and Dickson D.W., 1987. A semi-selective medium for the isolation of *Paecilomyces lilacinus* from soil. *Journal of Nematology, 19*: 255-256.

Muller R. and Gooch P.S., 1982. Organic amendments in nematode control. An examination of literature. *Nematropica, 12*: 319-326.

Parvatha Reddy P. and Rao M.S., 2001. Integrated manage-

ment of nematodes in fruit crops. *National Nematology Congress – Centenary Celebrations*, December 5-7 2001, New Delhi, India, p. 32.

Parveen S., Ehteshamul Haque S. and Ghaffar A., 1998. Efficacy of *Pseudomonas aeruginosa* and *Paecilomyces lilacinus* in the control of root rot-root knot disease complex on some vegetables. *Nematologia Mediterranea, 26*: 209-212.

Rao M.S. and Reddy P.P., 2001. Control of *Meloidogyne incognita* on egg plant using *Glomus mosseae* integrated with *Paeciloymces lilacinus* and neem cake. *Nematologia Mediterranea, 29*: 153-157.

Rao M.S., Parvatha Reddy P. and Nagesh M., 1997. Management of root-knot nematode, *Meloidogyne incognita* on tomato by integration of *Trichoderma harzianum* with neem cake. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz* (Journal of Plant Diseases and Protection), *104*: 423-425.

Rao M.S., Reddy P.P. and Sukhada M., 1998. Biointensive management of *Meloidogyne incognita* on egg plant, by integrating *Paeciloymces lilacinus* and *Glomus mosseae*. *Nematologia Mediterranea, 26*: 217-219.

Rao M.S., Walia R.K. and Reddy P.P., 2001. Biological control of nematodes in horticultural crops. *National Nematology Congress, Centenary Celebrations,* December 5-7, 2001, New Delhi, India, pp. 58-59.

Rao M.S., Dhananjay Naik., Shylaja M. and Parvatha Reddy P., 2002. Prospects for the management of nematode disease complex in vegetable crops using biological control agents. *Proceedings of International conference on Vegetables*. November 11-14, 2002, Bangalore, India, pp. 347-351.

Santhi A. and Sivakumar C.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.

Siddiqui I.A., Ehteshamul S. and Ghaffar A., 1999. Root dip treatment with *Pseudomonas aeruginosa* and *Trichoderma* spp., in the control of root rot-root knot disease complex in chilli (*Capsicum annuum* L.). *Pakistan Journal of Nematology*, *17*: 67-75.

Accepted for publication on 10 March 2008.