

BIO-MANAGEMENT OF *MELOIDOGYNE INCOGNITA* AND *ERWINIA CAROTOVORA* IN CARROT (*DAUCUS CAROTA* L.) USING *PSEUDOMONAS PUTIDA* AND *PAECILOMYCES LILACINUS*

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Summary. An experiment was conducted to test the effects of two biocontrol agents, *Pseudomonas putida* and *Paecilomyces lilacinus*, on the control of *Meloidogyne incognita* and *Erwinia carotovora* in carrot (*Daucus carota*). *Pseudomonas putida* and *P. lilacinus* formulations were enriched in neem cake and evaluated under field conditions, individually and in combination, as seed treatment or as substrate treatment. Twenty grams of this formulation was used for the seed (one kg) treatment and five kg for the enrichment of neem cake (200 kg), which was applied to the beds at the rate of 20 g/m² as a substrate treatment before sowing. Seed treatment with both bio-agents and application of neem cake enriched with *P. putida* and *P. lilacinus* proved to be the best of all the treatments, leading to a reduction in the *M. incognita* (J₂) population (in roots by 69% and soil by 47.6%) and *E. carotovora* by 66%, with a significant increase (27.8%) in the yield of carrot. *Pseudomonas putida* and *P. lilacinus* co-existed without affecting root colonization by either.

Key words: Biological control, neem cake, root-knot nematode, soft rot.

Carrot (*Daucus carota* L.) is an all time favourite fresh vegetable in India and in the world. The crop suffers severely (> 50% loss) in India from soft rot, *Erwinia carotovora* Jones et Waldee, one of the most serious of bacterial diseases with world-wide occurrence. The bacterium causes soft rot disease in carrot by entering into the aerial plant part or tap root through natural openings or wounds. It is soil-borne and attacks a wide range of fruit and vegetable crops (Kokoskova and Pavela, 2005).

During a survey, we observed the association of soft rot with the root-knot nematode, *Meloidogyne incognita* (Kofoid et White) Chitw. The infection by the nematode creates wounds that facilitate entry of the bacteria and we reported the disease complex of carrot caused by *M. incognita* and *E. carotovora* (Sowmya and Rao, 2011). This root-knot nematode is one of the major pathogens of carrot throughout the world, affecting both quality and quantity of the marketable carrot yield (Sasser and Carter, 1985; Gugino et al., 2006).

Excessive use of chemicals to control soil-borne pathogens is affecting soil bio-diversity adversely and has proved to be hazardous to the environment and expensive (Gugino et al., 2006). The nematophagous fungus *P. lilacinus* Tom. et Samson is a widespread facultative parasite of the sedentary stages of plant parasitic nematodes.

It is considered to have potential as a biological control agent for application in nematode infested soils (Jatala, 1986; Rao and Reddy, 1994; Rao, 2008). Similarly, previous research has reported the bio-control potential of *Pseudomonas putida* Trevisan against the bacterium *Erwinia carotovora* (Colyer, 1984; Xu and Gross, 1986).

So far, there are no reports on the combined use of these two promising bio-agents for the management of the disease complex caused by *M. incognita* and *E. carotovora* in carrot. Hence, it was thought to develop a suitable bio-management strategy for the disease complex in carrot by using bio-pesticide formulations of *P. lilacinus* and *P. putida*.

MATERIALS AND METHODS

The local isolates of *P. putida* (code IIHR - PP 2 - NAIMCC - B-01212), maintained in the collection of NAIMCC (National Agriculturally Important Microbial Culture Collection, Mau, Uttar Pradesh), and *P. lilacinus* (code IIHR - PI 2 - ITCC NO. 6887), maintained in the collection of ITCC (Indian Type Culture Collection, IARI, New Delhi), were mass produced through liquid fermentation processes and prepared as talc-based formulations. These talc based bio-pesticide formulations were used to enrich de-oiled neem cake procured from Agro Extracts Limited, Bangalore, India. The enrichment was done by mixing 5 kg of either of the *P. lilacinus* or *P. putida* formulations, or 2.5 kg each of the *P. putida* and *P. lilacinus* formulations together in 200 kg of neem cake and keeping it under shade while maintaining optimum moisture of 25-28% for a period of 15 days.

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Table I. Effects of single and combined applications of *Pseudomonas putida* and *Paecilomyces lilacinus* on plant growth components of carrots in a field infested with *Meloidogyne incognita* and *Erwinia carotovora*.

Treatment	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)
T1 – PPSD	51.8 e	16.5 c	38.7 c	78.0 d
T2 – PPSB	52.8 e	17.1 d	40.1 d	79.7 e
T3 – PP(SD+SB)	47.5 c	14.5 b	35.0 b	73.2 c
T4 – PL SD	45.8 b	13.7 b	32.4 a	70.0 b
T5 – PL SB	49.5 c	15.4 b	36.2 b	75.4 c
T6 – PL (SD+SB)	50.0 d	15.9 b	37.5 b	76.4 d
T7 – (PP+PL) SD	53.8 f	17.4 d	42.6 e	82.9 f
T8 – (PP+PL)SB	54.7 g	18.2 e	45.3 f	86.8 g
T9 –(PP+PL) SD+SB	56.0 h	19.4 f	49.1 g	88.4 g
T10 – Neem	42.2 a	11.2 a	31.9 a	68.5 b
T11 – Control	39.5 a	9.5 a	31.6 a	64.2 a

SD = Seed treatment; SB = Substrate treatment, PP = *Pseudomonas putida*; PL = *Paecilomyces lilacinus*.

All figures are the average of five replicates. Means in the same column followed by a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

These neem formulations enriched with *P. putida* and *P. lilacinus* were used for the experiments conducted at the Indian Institute of Horticultural Research farm in a field infested with *M. incognita* and *E. carotovora*. To assess the nematode soil population densities of the selected areas of the field (50 m × 50 m), a 200-cm³ soil sample, composed of ten cores, was collected and a 100 cm³ sub-sample was processed following Cobb's sieving and decanting method (Cobb, 1918) followed by a modified Baermann's funnel (Petri dish) method (Schindler, 1961). The level of water in the Petri dishes was maintained to keep the tissue paper wet and the dishes were kept undisturbed for 48 hours. The nematode suspensions in the Petri dishes were poured into beakers, adjusted to a suitable volume and root knot nematode specimens were counted, with the help of Fenwick's multi-chamber counting slides and a binocular microscope, as the average number present in five different one ml aliquots of the nematode suspension. The nematode population was 124 ± 15/100 cm³ of soil in the first season and 118 ± 9/100 cm³ of soil in the second season. The population density of *E. carotovora* was determined by making a serial dilution from the soil sample and counting CFUs on nutrient agar plates; it was 2.6 × 10³/g of soil in the first season and 4.3 × 10³/g of soil in the second season.

The soil texture in the experimental plot was sandy-clay-loam, with a pH of 6.6-6.8 and organic matter content of 0.22-0.24%. During the experimental periods, total rainfall was 48 mm (first season) and 44 mm (second season) and soil temperatures were in the range 21-32 °C. The enriched formulations were applied during the first week of September, 2010 and the first week of October, 2011 at the rate of 20 g/m² to the field, which was divided into plots of 4 m² (2 m × 2 m). At the same

time, carrot seeds (cv. Kuroda) were mixed with the bio-pesticide formulations at the rate of 20 g/kg before sowing.

The individual treatments (Tables I-III) were as follows: T1, seeds of carrot treated with *P. putida* (CFU 2 × 10⁸/g) at the rate of 20 g/kg and sown in untreated plots (PP SD); T2, untreated seeds sown in plots mixed with neem cake enriched with *P. putida* at the rate of 20 g/m² (PP SB); T3, seeds treated with *P. putida* sown in plots containing neem cake enriched with *P. putida* (PP SD+SB); T4, seeds treated with *P. lilacinus* (CFU 2 × 10⁶/g) at the rate of 20 g/kg and sown in untreated plots (PL SD); T5, untreated seeds sown in plots mixed with neem cake enriched with *P. lilacinus* at the rate of 20 g/m² (PL SB); T6, seeds treated with *P. lilacinus* sown in plots mixed with neem cake enriched with *P. lilacinus* (PL SD+SB); T7, seeds treated with both formulations of *P. putida* and *P. lilacinus*, each at the rate of 10 g/kg, and sown in untreated plots (PP+PL SD); T8, untreated seeds sown in plots mixed with neem cake enriched with both formulations of *P. putida* and *P. lilacinus* at the rate of 20 g/m² (PP+PL SB); T9, seeds treated with both formulations of *P. putida* and *P. lilacinus*, sown in plots mixed with neem cake also enriched with both the formulations of *P. putida* and *P. lilacinus* (PP+PL SD+SB); T10, untreated seeds sown in plots mixed with neem cake at the rate of 20 g/m²; T11, untreated seeds sown in plots without any treatment. T10 and T11 served as controls. All treatments were replicated five times in a randomized block design. The crop was maintained by applying recommended dosages of fertilizers (N-P-K at the rate of 75-62.5-50 kg/ha, respectively) and plant protection chemical (sulphur spray at the concentration of 3 g/l two months after sowing, to control powdery mildew). The plots were drip irrigated and spaced 1 m apart to

Table II. Rhizospheric density in soil and root colonization by *P. putida* and *P. lilacinus* in a field infested with *M. incognita* and *E. carotovora*.

Treatment	Soil density of <i>P. putida</i> (CFU/g) at harvest ($\times 10^6$)	Root colonization (CFU/g) of <i>P. putida</i> at harvest ($\times 10^6$)	Soil density of <i>P. lilacinus</i> (CFU/g) at harvest ($\times 10^5$)	Root colonization (CFU/g) of <i>P. lilacinus</i> at harvest ($\times 10^5$)
T1 - PPSD	3.6 ^a	1.2 ^b	0 ^a	0 ^a
T2 - PPSB	6.2 ^c	4.3 ^c	0 ^a	0 ^a
T3 - PP(SD+SB)	7.3 ^c	5.6 ^d	0 ^a	0 ^a
T4 - PL SD	0 ^a	0 ^a	2.6 ^a	2.2 ^b
T5 - PL SB	0 ^a	0 ^a	5.1 ^c	4.7 ^c
T6 - PL (SD+SB)	0 ^a	0 ^a	5.8 ^c	5.3 ^c
T7 - (PP+PL) SD	3.8 ^b	1.4 ^b	2.8 ^b	2.5 ^b
T8 - (PP+PL)SB	6.7 ^c	5.8 ^d	5.6 ^c	5.8 ^d
T9 -(PP+PL) SD+SB	7.8 ^d	6.7 ^c	6.9 ^d	5.5 ^d
T10 - Neem	0 ^a	0 ^a	0 ^a	0 ^a
T11 - Control	0 ^a	0 ^a	0 ^a	0 ^a

SD = Seed treatment; SB = Substrate treatment, PP = *Pseudomonas putida*; PL = *Paecilomyces lilacinus*.

All figures are the average of five replicates. Means in the same column followed by a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

avoid cross contamination.

At harvest (90 days after sowing), five plants were uprooted at random from each plot and observations on plant growth components shoot length (from the tip of the top leaf to the base of the root) and root length (from the lowest root tip to the start of the shoot part), and fresh shoot and root weights. Rhizosphere density of both bio-agents in the soil, root colonization by *P. putida* and *P. lilacinus*, and root and soil populations of *M. incognita* (J_2) were recorded following standard procedures. Percentage of disease incidence by *E. carotovora*, percentage reduction of *M. incognita* and *E. carotovora* populations, yield per plot and percentage increase in the yield of carrot at harvest were also recorded.

To estimate the root populations of *M. incognita* (J_2), five plants per plot were uprooted at random and 1 g of root was collected from each of the five plants. The root samples were stained using an acid fuchsin solution following the method of Bridge *et al.* (1982), homogenised in a blender and the number of nematodes (J_2) were counted under a stereo-microscope. The soil population density of *M. incognita* was estimated collecting at random ten soil cores per replicate, for a total of 200 cm³, and processing 100 cm³ soil using Cobb's sieving and decanting method (Cobb, 1918). The data on eggs were not recorded. The percentage reductions in the nematodes densities in root and soil due to the applica-

tion of *P. lilacinus* or *P. putida* or both were calculated by comparison with the nematodes densities in the respective controls.

Colonization of roots by *P. lilacinus* and *P. putida* was recorded from the five plants per plot mentioned earlier. To assess root colonization by *P. putida*, a one gram root sub-sample was taken and washed gently to remove the soil, ground in a mortar and pestle, and the number of CFUs (Colony Forming Units) was estimated by the serial dilution technique (Rao *et al.*, 1997; Tarun *et al.*, 2005). Freshly prepared King's B agar medium (King *et al.*, 1954) was poured into each plate and allowed to spread evenly and solidify. Serial dilutions up to 10⁻⁶ concentration were prepared. One ml from each of 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions were pipetted into the Petri dishes and spread completely over the surface of the plate. Three replicates of each dilution were prepared and incubated at 27±1 °C. After 24 hr, *P. putida* colonies on the King's B medium emitting a pale green fluorescent light under UV at 302 nm were counted. Root colonization by *P. lilacinus* was assessed by the above mentioned serial dilution technique but using a semi-selective medium (Mitchell *et al.*, 1987). Petri plates were incubated at 25-27 °C for 15 days until the colonies turned lilac in colour. These colonies were also observed for their structure under a stereo microscope and counted.

Table III. Effects of single and combined applications of *P. putida* and *P. lilacinus* against disease caused by *M. incognita* and *E. carotovora* in carrot.

Treatment	J ₂ in 100 cm ³ soil	% reduction in <i>M. incognita</i> population	<i>M. incognita</i> J ₂ in 5 g root	% reduction in <i>M. incognita</i> population	Disease incidence by <i>E. carotovora</i> (%)	% reduction in disease incidence by <i>E. carotovora</i>	Yield per 4 m ² (kg)	% increase in yield
T1 – PPSD	116 c	20 c	12.24 e	32 b	31 g	32 d	25.18 a	8.54 c
T2 – PPSB	111 c	23.4 c	10.44 d	42 d	23.25 d	49 g	26.99 b	16.35 e
T3 – PP(SD+SB)	98 b	32.4 d	8.46 b	53 e	19.60 b	57 i	28.41 d	22.45 g
T4 – PLSD	125 d	13.8 b	12.6 f	30 b	33.28 g	27 c	24.35 a	4.98 b
T5 – PLSB	102 b	29.7 d	9.18 c	49 e	25.08 e	45 f	26.02 a	12.15 d
T6 – PL (SD+SB)	97 b	33.1 d	7.56 b	58 f	21.43 c	53 h	27.52 c	18.6 f
T7 – (PP+PL) SD	112 c	22.8 c	11.34 e	37 c	28.27 f	38 e	25.73 a	10.89 d
T8 – (PP+PL)SB	81 a	44.1 e	6.84 a	62 g	17.32 a	62 j	28.84 e	24.30 g
T9 – (PP+PL) SD+SB	76 a	47.6 e	5.58 a	69 h	15.50 a	66 k	29.65 e	27.82 h
T10 – Neem	132 e	8.97 b	12.96 f	28 b	35.11 h	23 b	23.67 a	2.03 a
T11 – Control	145 f	0 a	18 g	0 a	45.6 i	0 a	23.2 a	0 a

SD = Seed treatment; SB = Substrate treatment, PP = *Pseudomonas putida*; PL = *Paecilomyces lilacinus*.

All figures are the average of five replicates. Means in the same column followed by a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

The rhizospheric density of *P. putida* and *P. lilacinus* in soil was also estimated – by collecting a random sample of the rhizospheric soil and following the serial dilution technique, using a one gram sub-sample of soil and the respective selective media for *P. putida* and *P. lilacinus*.

Depending upon experimental design, a one-way analysis of variance (ANOVA) was performed using SPSS ver. 10.0. As a follow-up of ANOVA, the treatment means 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 variances.

RESULTS AND DISCUSSION

There were significant increases in shoot length, shoot weight, root length and root weight in treatment T9 [(*P. putida* + *P. lilacinus*) Seed treatment + Substrate treatment] (Table I) compared to all other treatments. This was probably due to the plant growth promoting activity of *P. putida*, which has been very well documented by many researchers (Xu and Gross, 1986; Pandey *et al.*, 2006; Elbanna *et al.*, 2011). Our finding agrees with those of Goswami *et al.* (2006) and Prakob *et al.* (2009) that *P. lilacinus* enhanced growth of tomato and lettuce infected by *M. incognita* and *Meloidogyne* spp.

Rhizospheric density in soil of *P. putida* in treatment T9 [(*P. putida* + *P. lilacinus*) Seed treatment + Substrate treatment], after harvest, was 7.8×10^6 /g of soil, which was at par with that of 7.3×10^6 /g of soil in treatment T3 [*Pseudomonas putida* (Seed treatment + Substrate treatment)]. Similarly, the rhizospheric soil density of *P. lilacinus* in treatment T9 [(*P. putida* + *P. lilacinus*) Seed treatment + Substrate treatment], after harvest, was 6.9×10^5 /g of soil, which was at par with that of 5.8×10^5 /g of soil in treatment T6 [*P. lilacinus* (Seed treatment + Substrate treatment)]. Root colonization of *P. putida* and *P. lilacinus* (enriched in neem cake) was also high in treatment T9 [(*P. putida* + *P. lilacinus*) Seed treatment + Substrate treatment] compared to when they were applied singly. The numbers of CFUs of *P. putida* and *P. lilacinus* per gram of root were 6.7×10^6 and 5.5×10^5 , respectively (Table II).

These results indicated that rhizospheric density and root colonization by *P. putida* and *P. lilacinus* was higher when they were applied together in comparison to individual treatments (Table II). Root colonization by *P. lilacinus* was not affected by the presence of *P. putida* when they were applied together. Similarly, root colonization by *P. putida* also was not affected by the presence of *P. lilacinus*. Thus, the two bio-agents can co-exist without the root colonization by either being affected.

Seed treatment with both bio-agents and application of neem cake enriched with *P. putida* and *P. lilacinus* (T9) proved to be the best treatment for the management of the disease complex caused by *E. carotovora*

and *M. incognita* in carrot under field conditions. There was a reduction of *M. incognita* population in the root by 69% and in the soil by 47.6%, of the disease incidence by *E. carotovora* of 66%, and a significant increase (27.8%) in the yield of carrot (Table III).

Seed treatment followed by substrate treatment using the combination of *P. putida* and *P. lilacinus* in neem was more effective than either of these treatments individually in reducing *M. incognita* population and disease incidence by *E. carotovora* (Table III). Fluorescent pseudomonads produce antibiotics and other metabolites that may play an additional role in suppressing *E. carotovora* (Kloepper, 1983). *Paecilomyces lilacinus* colonizes the root surface and parasitizes eggs, egg-masses, juveniles and females of *Meloidogyne* spp. by direct hyphal penetration (Jatala *et al.*, 1979; Mohd *et al.*, 2009; Prakob *et al.*, 2009; Mucksood and Tabriez, 2010).

Combinations of more than one bio-agent can provide greater protection against disease than that of a single bio-agent (Ehteshamul-Haque *et al.*, 1995; Izhar *et al.*, 1995; Rao *et al.*, 2004). Parveen *et al.* (1998) reported the combined efficacy of *P. aeruginosa* and *P. lilacinus* in reducing disease complex caused by the root-knot nematode *Meloidogyne javanica* (Treub) Chitw. and the fungi *Macrophomina phaseolina* (Tassi) Goid. and *Fusarium oxysporum* (Synder *et* Hansen) on pumpkin and *F. solani* (Mart.) Sacc. on watermelon.

Application of neem cake has proved to be very effective in the management of nematodes as it controls the entry of pathogens through a variety of mechanisms, including the production of antimicrobial compounds (Mankau, 1962; Alam and Khan, 1980; Muller and Gooch, 1982). This resulted in smaller populations of root knot nematodes in our experiment, which in turn reduced the incidence of disease caused by *E. carotovora*. As the entry of the bacteria is mainly through the wounds caused by the nematodes, the lesser the nematode infestation the fewer the wounds created and the lesser the bacterial infection. Hence, the use of a combination formulation of *P. putida* and *P. lilacinus* enriched in neem for seed and substrate treatment would be very useful for the management of the nematode induced disease complex in carrot.

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