EFFECT OF SEED TREATMENT WITH CERTAIN BIOPESTICIDES ON ROOT-KNOT OF CHICKPEA

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Summary. The efficacy of three biopesticides based on *Pochonia chlamydosporia*, *Trichoderma harzianum* and *Pseudomonas fluorescens* was tested on the root-knot of chickpea caused by *Meloidogyne* spp. under field conditions. The biopesticides were applied to seeds of chickpea cv. BG 256 at the rate of 2 g/kg seed along with the commercial rhizobium. Two thousand J2/kg soil were used as inoculum. Infection by the nematode caused 14 and 15% decline in the dry weight and yield of chickpea, respectively, losses that were avoided when the biopesticides were applied. Dry weight and yield of nematode infected plants were significantly increased by application of *P. fluorescens* (31 and 34%) and *P. chlamydosporia* (28 and 25%), respectively. The dry weight and yield of chickpea not inoculated with the nematode was increased by 39 and 36% when *P. fluorescens* was applied. Seed treatment with fenamiphos resulted in 14 and 18% increase in the dry weight and yield, respectively. Gall formation and egg mass production of root-knot nematodes was decreased by 23 and 18% by the application of *P. chlamydosporia* against 26 and 19% by fenamiphos. Treatment with *P. fluorescens* also significantly decreased the number of galls and egg masses, but less than treatment with *P. chlamydosporia.* Soil populations of the nematodes increased gradually during the growing season in the control plots, but decreased significantly when seeds were treated with *P. chlamydosporia* or *P. fluorescens. Trichoderma harzianum* did not significantly affect nematode pathogenesis. Rhizosphere populations of the bioagents increased both in the presence and absence of root-knot nematode.

India has successfully achieved food security; nutritional security, however, continues to be a cause for concern. For poor people, cereals constitute the staple food, and the major source of energy. However, the addition of pulses, which are the main source of vegetable protein, provides a more nutritionally balanced diet. Chickpea, is an important pulse crop and India ranks first in its production worldwide (Chaturvedi and Ali, 2002). *Meloidogyne* species are important nematode pests of chickpea and are considered among the top five major plant pathogens (Sasser, 1989). A number of control measures have been adopted to manage root-knot disease, including the application of fumigant and non-fumigant nematicides (Johnson, 1985). However, application of nematicides is of concern for the environment. This has resulted in the preference of growers to adopt ecofriendly management practices. Recently, there has been growing interest in the use of biopesticides as a substitute for chemicals in nematode disease management (Zuckerman *et al.,* 1993). Certain microorganisms, such as plant growth promoting organisms (PGPOs), may not only suppress pathogens but also improve the nutritional status of the soil (Van Gundy, 1985). *Pseudomonas fluorescens* (Threvesan) Migula is an effective bioagent; it produces toxins such as phloroglucinol (Howell and Stipanovic, 1980), pyrollnintrin (Leyns *et al.,* 1990), phenazin (Gurusiddaiah *et al.,* 1986), certain siderophores etc., which have deleterious effects on pathogens and also promote plant growth (Kloepper *et al.,* 1980). PGPOs, such as *P. fluorescens*, produce phytohormones (indole-3-acetic acid, gibberellin, etc.), which may enhance plant growth (Glick, 1995). The suppressive effects of *P. fluorescens* and *Paenibacillus polymyxa* (Prazmowski) Mace on *Meloidogyne incognita* (Kofoid *et* White) Chitw. infecting tomato have been reported (Khan and Akram, 2000). Fungi like *Pochonia chlamydosporia* (Goddard) Zare, Gams *et* Nova Hedwigia and *Paecilomyces lilacinus* (Thoms.) Samson may suppress nematode populations through parasitism of eggs or adults (Stirling, 1991). *Trichoderma* spp. are primarily mycoparasites, but they may also parasitise nematodes (Sharon *et al*., 2001).

In the present study, the efficacy of seed treatments with three biopesticides, based on *T. harzianum* Rifai*, P. chlamydosporia* (= *Verticillium chlamydosporium*) and *P. fluorescens,* was evaluated against a mixed population of root-knot nematodes, *M. incognita* and *M. javanica* (Treub) Chitw. on chickpea under field conditions.

MATERIALS AND METHODS

A field site of 18 × 14 m was selected and divided in 30 plots (4 × 2 m), distributed according to a completely randomised block design. The soil was loam with pH 7.7 and 2.28% organic matter. The binding consisting of soil (25 cm wide and high) between the plots prevented lateral movement of microorganisms and nematodes. Ten treatments (Table I), each replicated three times, were used.

Table I. Effect of seed treatments with three biopesticides on dry weight and yield of chickpea, *Rhizobium* nodulation and gall and egg mass formation of *Meloidogyne* spp., in soil inoculated or not with the nematodes.

Treatment	Plant dry weight (g)	Yield per plant (g)	Nodules/ root system		Per root system	
			Functional	Non functional	Galls	Egg mass
Control	21.9	7.2	35	14	-	-
Trichoderma harzianum (Th)	25.9[a]	8.1[a]	46.7[a]	13.4	-	-
Pochonia chlamydosporia (Pc)	23.2	7.6	42.5[a]	11.4[a]	-	-
Pseudomonas fluorescens (Pf)	30.5[a]	9.8[a]	49.9[a]	14.5	-	-
Fenamiphos	22.2	7.5	30.2[a]	14.5	-	-
Nematode control (N)	18.9[a]	6.1[a]	24.1[a]	11.9[a]	49.0	43.0
N + Th	20.1	6.8	25.2	16.9[a]	45.5	40.0
N + Pc	24.2[a]	7.6[a]	27.5[a]	14.7[a]	37.8[a]	35.2[a]
N + Pf	24.8[a]	8.2[a]	28.7[a]	15.1[a]	40.2[a]	36.2[a]
N + Fenamiphos	21.5	7.2[a]	26.6	15.6[a]	36.2[a]	34.7[a]
C. D. (P = 0.05)	2.4	0.7	3.2	1.3	4.3	3.8
F. value						
Nematode (df = 1)	44.5[b]	40.3[b]	50.5[b]	60.4[b]	-	-
Biopesticides (df = 4)	35.1[b]	39.6[b]	26.7[b]	10.4[b]	-	-
Interaction (df = 4)	9.9[b]	4.9[b]	10.6[b]	4.8[b]	-	-
Treatment (df = 4)	-	-	-	-	12.5[b]	7.9[b]

Each value is a mean of three replicates
[a] Significantly different from the 'Respective control' at P = 0.05
[b] Significant at P = 0.05

The nematodes were maintained on eggplants in culture beds. To obtain fresh nematodes, infected plants were uprooted, washed gently and egg masses were excised and placed on wire gauze in a Baermann funnel. The hatched second stage juveniles (J2) were collected from the funnel after 3-4 days. Ten litres of water containing $7x10^5$ J2 of *M. incognita* and *M. javanica* were added to a plot to obtain a uniform population of the nematode (2000 J2/kg soil). The inoculation was done one day prior to sowing.

The biopesticides based on *P. chlamydosporia* (VC-02), *T. harzianum* (TO-14) and *P. fluorescens* (MB-07) (Khan, 2003) were applied on seeds (2 g/kg seed) along with commercial rhizobium of the gram strain. The strains of each organism have been deposited in the NBAIM, New Delhi. Colony forming unit counts (CFU) of *P. chlamydosporia, T. harzianum* and *P. fluorescens* were 22×10^6, 11×10^8 and 80×10^{12}/g formulation, respectively. The effectiveness of these biopesticides was compared with that of a seed treatment with fenamiphos at the rate of 2 g/kg seed. The treated seeds of chickpea, *Cicer arietinum* L. cv. BG-256 were sown in rows (57 seeds/row, 3 rows/plot). Rows were 4 m long and 50 cm apart. The field was irrigated one week after sowing. Plants were grown for four months. The field was irrigated by flood irrigation 2 and 10 weeks after planting and weeds were removed manually 1 and 3 months after planting. Plants were observed regularly for any disease symptoms. At maturity, in April, four months after sowing, ten plants from each plot were chosen at random and uprooted, and the dry weight, grain yield, number of galls and egg masses were determined. The whole root system was examined visually to count galls. The roots were treated with 0.15% Phloxin B to stain egg masses for ease of counting. The ingredients of the biopesticides are not described here to protect application for a patent. Nodulation, in terms of functional (pink) and non-functional (brown) nodules, was assessed on ten plants per plot two and a half months after planting. Soil populations of root-knot nematodes were extracted using Cobb's decanting and sieving method followed by the Baermann funnel technique (Southey, 1986), and the rhizosphere populations of bioagents were estimated, using dilution plate method (Seeley Jr. and Van Demark, 1981), monthly from December (planting) to April (harvest). Antibiotic and fungicide sensitivity tests were performed using various concentrations of antibiotics (amoxicillin, chloramphenicol, cloxacillin, co-trimoxazole, doxycillin hydrochloride, fluconazole, methicillin, nalidixic acid, nitrofurantoin, novobiocin, penicillin and chlorotetracycline) (Bauer *et al.*, 1966) and fungicides (PCNB, carbendazim, captan, metalaxyl and mancozeb) (Sharma *et al.,* 2001) to determine the maximum tolerance concen-

trations (MTC) for the bioagents. Specific antibiotics and/or fungicides at MTC were added to the respective growth medium to make it strain selective. The media used were potato carrot agar supplemented with 50 mg each of streptomycin, chloramphenicol and chlortetracycline/l for *P. chlamydosporia*, potato dextrose agar supplemented with 1.25 g metalaxyl/l for *T. harzianum*, and Kings B agar supplemented with 75 mg cycloheximide, 45 mg penicillin and 45 mg novobiocin/l for *P. fluorescens*. Despite making the growth media selective, some contaminants also developed in the Petri plates, but *T. harzianum* and *P. fluorescens* were easily distinguished from colony characters. Identification and counting of *P. chlamydosporia* colonies was, however, done by randomly picking five morphologically similar colonies from each Petri plate for microscopic examination and comparison with the standard strain. Chlamydospores of *P. chlamydosporia* have a unique shape and are readily identified under a microscope (Crump and Kerry, 1981). The growth media supplemented with antibiotics/fungicides as described above have given satisfactory results in previous studies (Khan, 2005; Khan *et al.*, 2004). Natural populations of the bioagents in the experimental plots were not detected.

The data on plant growth parameters and nodules were subjected to a two factor analysis of variance (ANOVA), and on galls, egg masses and soil populations were analyzed by a single factor analysis of variance. Critical Difference (CD) was calculated for each variable at the probability level of P = 0.05 (Dospekhov, 1984). Analysis of variance on soil/rhizosphere populations was done in two ways i.e., pre-plant population versus monthly populations, and control versus treatment, (CDs or *F*-values are not given in Table II).

RESULTS AND DISCUSSION

Infection by *Meloidogyne* spp. caused a 14% decrease (P = 0.05) in the dry weight of chickpea compared to the uninoculated controls (Table I). Seed treatment with *P. fluorescens* significantly increased the dry weight of healthy chickpea plants (39%) not inoculated with the nematode. Treatment of inoculated plants with *P. chlamydosporia* or *P. fluorescens* partially suppressed gall formation and nematode reproduction, thus helping to increase plant dry weight by 28 and 31%, respectively.

The yield of chickpea was significantly (P = 0.05) improved by application of the bioagents, except *P. chlamydosporia*, in comparison to the uninoculated control (Table I). The greatest increase in yield was obtained with *P. fluorescens* (36%), followed by *T. harzianum* (12%). The increase in plant growth and yield of chickpea not inoculated with nematode, due to application of *P. fluorescens*, might have been due to the production of phytohormones such as indole-3-acetic acid and gibberellin (Glick, 1995). *Trichoderma* spp. are reported to mineralise phosphorus in soil (Sharma, 2003), which may have been responsible for the significant increase in the yield when *T. harzianum* was applied. Suppression of some unknown soil-borne fungal pathogens by *T. harzianum* may also have helped enhance yield. Root-knot infection caused a 15% decrease in yield of chickpea compared to the uninoculated control (P = 0.05). Application of all three bioagents significantly (P = 0.05) increased the yield of nematode infected plants, the increase being greatest with *P. fluorescens* (34%), followed by *P. chlamydosporia* (25%) and *T. harzianum* (11%) in comparison to the inoculated control. Sharon *et al*. (2001) reported a significant increase in the yield of root-knot nematode infected tomato plants due to application of *T. harzianum*.

Nodule formation on the roots of chickpea was quite good and, on average, 49 nodules were formed per plant, of which 71% were functional and 29% were non-functional (Table I). Root nodulation was promoted by the treatments with the bioagents. Rhizobacteria (*Pseudomonas* spp.) and mycoparasites (*Trichoderma* spp.) are known to synergise *Bradyrhizobium* spp. (Khan *et al*., 1998; Prabakaran, 1998). The number of functional nodules was increased by 21, 33 and 43% following seed treatment with *P. chlamydosporia, T. harzianum* and *P. fluorescens,* respectively. Another reason for increased nodulation may be the suppression of nematode pathogenesis caused by the biopesticides. Infection by root-knot nematodes resulted in a 31% decrease in the number of functional nodules/root system. Several reports of decreased root-nodulation due to infection by root-knot nematodes have been made (Taha, 1993).

Characteristic galls developed on the roots of plants grown in the plots inoculated with *Meloidogyne* spp. On average, 49 galls per root system were formed in plots inoculated with the nematode only (Table I). Application of the biopesticides partially suppressed gall formation. Treatment with *P. chlamydosporia* resulted in a 23% decrease in the number of galls, followed by *P. fluorescens* (18%), in comparison to the control (P = 0.05). Egg mass production was inhibited significantly following application of *P. chlamydosporia* (18%) and *P. fluorescens* (16%) (Table I), but a decrease of only 7% was caused by *T. harzianum*. Earlier studies also report significant decreases in gall formation and egg mass production by root-knot nematodes due to application of *P. chlamydosporia* (Khan *et al*., 2002) and *P. fluorescens* (Khan and Akram, 2000). Fenamiphos treatment resulted in 26 and 19% decreases in the numbers of galls and egg masses per root system compared to the inoculated control (Table I).

Soil populations of *Meloidogyne* spp., in plots not treated with biopesticides or fenamiphos, increased during January to April in comparison to the pre-plant population (December) (Table II). The increase was significant on all dates except January. Treatment with *P. chlamydosporia* or *P. fluorescens* significantly (P = 0.05)

Table II. Effect of seed treatment with *Trichoderma harzianum, Pochonia chlamydosporia* or *Pseudomonas fluorescens* on the dynamics of the soil population of *Meloidogyne* spp. and of the three bioagents.

Treatment	Nematode juveniles/kg soil					Colony forming units per g soil														
						T. harzianum x 10^6					*P. chlamydosporia* x 10^4					*P. fluorescens* x 10^7				
	Dec.	Jan.	Feb.	Mar.	Apr.	Dec.	Jan.	Feb.	Mar.	Apr.	Dec.	Jan.	Feb.	Mar.	Apr.	Dec.	Jan.	Feb.	Mar.	Apr.
T. harzianum (Th)	-	-	-	-	-	8.3	14.2[a]	14.9[a]	15.9[a]	16.3[a]	-	-	-	-	-	-	-	-	-	-
P. fluorescens (Pf)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	26.0	25.0	38.0[a]	45.0[a]	49.0[a]	
P. chlamydosporia (Pc)	-	-	-	-	-	-	-	-	-	-	1.2	1.3	2.3[a]	1.5[a]	1.5[a]	-	-	-	-	-
Meloidogyne spp.(N)	2000*	2149	2987[a]	3456[a]	3943[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
N + Th	2000	2153	3039[a]	3117[ab]	3278[ab]	8.3	13.5[a]	13.5[a]	16.2[a]	16.4[a]	-	-	-	-	-	-	-	-	-	
N + Pf	2000	1939	1593[ab]	1484[ab]	1237[ab]	-	-	-	-	-	-	-	-	-	26.0	28.0	46.0[ab]	60.8[ab]	64.2[ab]	
N + Pc	2000	1820[ab]	1631[ab]	1298[ab]	1209[ab]	-	-	-	-	-	1.2	2.3[ab]	3.1[ab]	2.6[ab]	3.9[ab]	-	-	-	-	-
N + Fenamiphos	2000	1769[ab]	1563[ab]	1485[ab]	1058[ab]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Each value is a mean of three replicates.
[a] Significantly different from the pre-plant (December) population at P = 0.05.
[b] Significantly different from the respective month control.
* Initial population (Juveniles of *Meloidogyne* spp. added to soil).

decreased the nematode population throughout the observation period, by 15-69% and 10-69%, respectively (Table II). Application of *T. harzianum* also caused a significant decrease in the soil population of *Meloidogyne* spp. in the March and April samples. *Pochonia chlamydosporia* is a parasite of root-knot nematodes (Stirling, 1991) and its application has previously resulted in a significant decrease of galling and reproduction of nematodes (Bharadwaj, 1997; Khan *et al*., 2002). Suppressive effects of *P. fluorescens* on root-knot nematodes have also been recorded in other studies (Khan and Tarannum, 1999; Khan and Akram, 2000). *Trichoderma harzianum*, *T. hamatum* (Beb.) Bain, *T. viride* Pers. ex Gray, etc. are active colonizers in soil and grow preferably on organic material. They might have colonized egg masses, eggs and larvae, thus resulting in significant decreases of the soil population of *Meloidogyne* species (Sharon *et al*., 2001). Fenamiphos decreased (P = 0.05) the soil population of *M. incognita* by 12-47% in comparison to the pre-plant population. This decrease was greater than those of other treatments, except *P. chlamydosporia* in March.

Populations of all three bioagents increased in the rhizosphere of chickpea, indicating that the cultivar used, BG 256, supported their growth and multiplication in the rhizosphere. Populations of *T. harzianum* increased by 71-96% during the course of the experiment (Table II). In the presence of root-knot nematode, the increase in the rhizosphere population was, however, lower than in the absence of the nematode and the difference was frequently significant. In the inoculated plots, the rhizosphere population of *P. chlamydosporia* increased by 8.3 to 92% (in February), whereas the population of *P. fluorescens* increased by 46-89% during the course of experiment (except for a small decrease in January) (Table II). The numbers of CFUs of *P. chlamydosporia*/g soil were relatively greater than those reported in European soils. It is likely that the fungus is favoured by the warm organic soil in our conditions, and so may provide better control of root-knot nematode in subtropical and tropical climates, including India. Kerry (1988) applied a granular formulation of *P. chlamydosporia* and recorded a soil population of the antagonist in the range of 10^4 CFUs/g soil. In nematode-inoculated microplots, the percentage increase of *P. chlamydosporia* was significantly greater.

Our study has demonstrated that seed treatment with a *P. fluorescens* biopesticide increased the yield of nematode-infected and non-infected chickpea plants to an extent acceptable and attractive to commercial growers. The bacterium also established in soil and its rhizosphere population increased over time.

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