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Biocontrol of a chickpea root-rot disease complex with *Glomus intraradices, Pseudomonas putida* and *Paenibacillus polymyxa*

M. S. Akhtar[A] *and Z. A. Siddiqui*[A,B]

[A]Department of Botany, Aligarh Muslim University, Aligarh 202 002, India.
[B]Corresponding author. Email: zaki_63@yahoo.co.in

Abstract. The effect of *Glomus intraradices, Pseudomonas putida* and *Paenibacillus polymyxa* on the growth, chlorophyll, nitrogen, phosphorus and potassium contents and on the root-rot disease complex of chickpea (caused by *Meloidogyne incognita* and *Macrophomina phaseolina*) were observed. Inoculation of plants with *G. intraradices*, *P. putida* and *P. polymyxa* alone and in combination significantly increased plant growth, pod number, chlorophyll, nitrogen, phosphorus and potassium contents and reduced galling, nematode multiplication and root-rot index. Inoculation of plants with *P. putida* most effectively reduced galling and nematode multiplication, followed by *G. intraradices* and *P. polymyxa*. Combined inoculation of plants with *G. intraradices*, *P. putida* and *P. polymyxa* caused the greatest reduction in galling, nematode multiplication and root-rot index. Pathogens had adverse effects on root colonisation by *G. intraradices*, while root colonisation by arbuscular mycorrhizal fungus was increased in the presence of *P. putida* and *P. polymyxa*.

Introduction

Chickpea (*Cicer arietinum* L.) is an important pulse crop of India and a chief source of dietary protein in the vegetarian diet. This crop is susceptible to root-knot nematode, *Meloidogyne incognita*, and the root rot fungus, *Macrophomina phaseolina*. The interaction between *M. incognita* and *M. phaseolina* causes a root-rot disease complex that severely damages this important pulse crop (Siddiqui and Husain 1991, 1992).

Rhizosphere organisms provide an initial barrier against pathogens attacking the root (Weller 1988) and microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents. Arbuscular mycorrhizal (AM) fungi colonise the roots of many crop plants (Smith and Read 1997; Ozgonen *et al*. 1999) and are of great value in promoting the uptake of phosphorus, minor elements and water (Allen 1996; Ibijbijen *et al*. 1996; Siddiqui *et al*. 2001). They also influence the severity of several plant pathogens (Dehne 1982; Siddiqui and Mahmood 1995; Linderman 2000; Barea *et al*. 2002; Akkopru and Demir 2005). *Glomus intraradices* is a highly infective species of woody and herbaceous plants over a wide range of conditions and greatly enhanced the growth of plants (Duponnois and Plenchette 2003). However, *Pseudomonas putida* is a metabolically versatile saprophytic soil bacterium. It is known for its diverse metabolism and potential for development of biopesticides and plant growth promoters, due to its ability to colonise the rhizosphere of crop plants. Similarly, *Paenibacillus polymyxa* is known to produce two types of peptide antibiotics (Beatty and Jensen 2002). The species also synthesises an auxin (Lebuhn *et al*. 1997) and a cytokinin (Timmusk *et al*. 1999).

This study examined the effects of *P. polymyxa*, *P. putida* and *G. intraradices* on growth, chlorophyll, nitrogen, phosphorus and potassium contents and the root-rot disease complex of chickpea.

Materials and methods

The root-knot nematode, *M. incognita*, and *M. phaseolina* were the pathogens tested. The potential biopesticides, *P. polymyxa*, *P. putida* and *G. intraradices*, were applied alone and in combination to chickpea (*C. arietinum* cv. Avarodhi). The influence of these treatments on plant growth, number of pods, galling and nematode multiplication and root-rot disease complex was assessed over 90 days in glasshouse experiments.

Preparation and sterilisation of soil mixture

Soil, river sand and organic manure were mixed in a ratio of 3 : 1 : 1 (v/v) respectively and added to jute bags. Water was poured into each bag to wet the soil. The bags were then transferred to an autoclave for sterilisation at 137.9 kPa for 20 min. Sterilised soil was allowed to cool down at room temperature before filling 15-cm diameter clay pots with 1 kg of sterilised soil.

Growth and maintenance of test plants

Seeds of chickpea cv. Avarodhi were surface sterilised in 0.1% sodium hypochlorite for 2 min and then washed three times with distilled water. Five seeds were sown in each pot and later thinned to one seedling per pot. Plants were placed in a glasshouse and watered as needed. Two days after thinning, seedlings received the treatments. Uninoculated plants served as a control. The seedlings were inoculated with 2000 freshly hatched second stage juveniles (J_2) of *M. incognita*, *M. phaseolina* (1 g), *P. polymyxa* (10 mL at 1.5×10^7 bacterial cells/mL), *P. putida*

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(10 mL at 1.5×10^7 bacterial cells/mL) and *G. intraradices* (500 infective propagules).

Preparation of nematode inoculum

M. incognita was collected from chickpea field soil and multiplied on egg plant (*Solanum melongena* L.) using a single egg mass. Egg masses were hand picked using sterilised forceps and placed in 9-cm diameter sieves of 1-mm pore size, which were previously mounted with cross layered tissue paper. The sieves were placed for hatching in Petri dishes with distilled water for hatching and incubated at 27°C. Two thousand freshly hatched second stage juveniles (J_2) per plant were used as inoculum.

Preparation of fungal inoculum

M. phaseolina was isolated from chickpea root and maintained on potato dextrose agar (PDA). Fungal inoculum was prepared by culturing the isolates in Richard's medium (Riker and Riker 1936) for 15 days at 25°C. Mycelium was collected on blotting sheets to remove excess water and nutrients. Ten mL of this suspension containing 1 g fungus was inoculated per plant, before macerating 100 g wet mycelium in 1 L distilled water.

Inoculum of microorganisms used as biocontrol agents

The AM fungus, *G. intraradices*, was produced on *Chloris gayana* (Rhodes grass) grown in sandy loam soil mixed with washed river sand and farmyard manure at the ratio of 3 : 1 : 1 (v/v) respectively. The population of *G. intraradices* in the inoculum was assessed by the most probable number method (Porter 1979). Fifty grams of inoculum with soil was added around the seeds to provide 500 infective propagules of *G. intraradices* per pot (1 g inoculum contained ten infective propagules). The crude inoculum consisted of soil, extra matrical spores and reproductive bodies, hyphal fragments and infective Rhodes grass segments. *Pseudomonas putida* (MTCC No. 3604) and *P. polymyxa* (MTCC No. 122) were obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Inocula of both bacterial species were produced from a freshly cultured subplate on nutrient broth incubated at 37 ± 2°C for 72 h. Ten mL of the suspension (1.5×10^7 cells/mL) was used as inoculum.

Inoculation techniques

For inoculation of *M. incognita*, *M. phaseolina*, *G. intraradices*, *P. polymyxa* and *P. putida*, soil around the root was carefully removed without damaging the roots. The inoculum suspensions of these microorganisms were poured around the roots and the soil was replaced. An equal volume of sterile water was added to control treatments.

Experimental design and measurements

The experiment was carried out in a completely randomised block design with four experimental variables: (i) control, (ii) *M. incognita*, (iii) *M. phaseolina* and (iv) *M. incognita* + *M. phaseolina*. Each set was inoculated with the following eight treatments and the experiment was repeated once:

(1) Control
(2) *G. intraradices*
(3) *P. putida*
(4) *P. polymyxa*
(5) *G. intraradices* + *P. putida*
(6) *G. intraradices* + *P. polymyxa*
(7) *P. putida* + *P. polymyxa*
(8) *G. intraradices* + *P. putida* + *P. polymyxa*

The plants were harvested 90 days after inoculation. Data were recorded on dry shoot weight, number of pods, number of nodules, number of galls, percentage root colonisation, root-rot index and estimated nematode population. Chlorophyll, nitrogen, phosphorus and potassium content were estimated per gram of fresh leaf weight. Chlorophyll content of the shoot was estimated by the technique of Arnon (1949) and nitrogen content of the shoot was estimated by the technique of Lindner (1944). Phosphorus and potassium contents were estimated by the methods of Fiske and Subba Row (1925) and flame photometer, respectively. A 250-g subsample of well-mixed soil from each treatment was processed by Cobb's sieving and decanting method followed by Baerman's funnel extraction to determine nematode population (Southey 1986). A root-rot index was determined by scoring on a scale ranging from 0 (no disease) to 5 (severe root-rot). The proportion of root colonised by *G. intraradices* was determined by a grid intersecting method (Giovannetti and Mosse 1980) after clearing the root with KOH in 0.05% trypan blue lactophenol.

Statistical analysis

The data were analysed statistically using two factorial analysis (Dospekhov 1984). Least significant differences (l.s.d.) were calculated at $P = 0.05$. Duncan's multiple range test was employed to denote the differences between treatments.

Results

Inoculation of plants with *G. intraradices*, *P. putida* and *P. polymyxa* alone and in combination without pathogens significantly increased shoot dry weight compared with uninoculated controls (Table 1). *P. putida* increased shoot dry weight more than *P. polymyxa*. However, increase in shoot dry weight with *G. intraradices* was similar to that caused by *P. polymyxa*. Combined inoculation of plants with *G. intraradices* + *P. putida* + *P. polymyxa* increased shoot dry weight more than inoculated with *G. intraradices* + *P. polymyxa* or *P. putida* + *P. polymyxa*. However, inoculation of plants without pathogens with *G. intraradices* + *P. putida* increased shoot dry weight by a similar amount to combined inoculations of *G. intraradices* + *P. putida* + *P. polymyxa* (Table 1).

Inoculation of plants with *M. incognita* and *M. phaseolina* alone and in combination caused a significant reduction of shoot dry weight compared to untreated control (Table 1). Inoculation with *M. incognita* and *M. phaseolina* together caused a greater reduction in shoot dry weight than inoculation with either of them alone. *M. incognita* caused a similar reduction in shoot dry weight to that caused by *M. phaseolina*. Inoculation of plants with *G. intraradices*, *P. putida* and *P. polymyxa* alone and in combination significantly increased shoot dry weight of pathogen inoculated plants. Inoculation of plants with *P. putida* increased shoot dry weight of

Table 1. Effect of *Glomus intraradices*, *Pseudomonas putida* and *Paenibacillus polymyxa* on the growth and root-rot disease complex of chickpea
Values within each column followed by the same letter are not significantly different ($P = 0.05$). C, Control; Gl, *Glomus intraradices*; Pp, *Pseudomonas putida*; Pb, *Paenibacillus polymyxa*

Treatments		Shoot dry weight (g)	No. of pods per plant	No. of nodules per plant	Percent root colonisation by AM fungus	No. of galls per root system	Nematode population	Root-rot index
Control	C	6.04l	30ghij	6abc	–	–	–	–
	Gl	6.51hij	35e	7abc	63cde	–	–	–
	Pp	6.92ef	40d	8abc	–	–	–	–
	Pb	6.42ijk	34ef	7abc	–	–	–	–
	Gl + Pp	7.88ab	47ab	10abc	70ab	–	–	–
	Gl + Pb	7.31cd	43cd	9abc	68bc	–	–	–
	Pp + Pb	7.58bc	44bc	9abc	–	–	–	–
	Gl + Pp + Pb	8.12a	49a	11a	74a	–	–	–
M. incognita	C	4.90op	19pq	5bc	–	128a	16 800a	–
	Gl	5.36mn	25lmn	6abc	57fg	96b	12 460b	–
	Pp	6.02l	26klmn	7abc	–	73d	9340e	–
	Pb	5.24mn	24mno	6abc	–	84c	10 740c	–
	Gl + Pp	6.58ghij	30ghij	8abc	62def	47gh	5940h	–
	Gl + Pb	6.12kl	27jklm	7abc	59efg	64e	8140f	–
	Pp + Pb	6.42ijk	29hijk	8abc	–	57ef	7160i	–
	Gl + Pp + Pb	6.78efgh	32efgh	9abc	65bcd	42hi	5360j	–
M. phaseolina	C	5.10no	21op	6abc	–	–	–	3
	Gl	5.56m	26klmn	7abc	59efg	–	–	2
	Pp	6.16kl	28ijkl	8abc	–	–	–	2
	Pb	5.44m	25lmn	7abc	–	–	–	2
	Gl + Pp	6.86efg	33efg	9abc	65bcd	–	–	1
	Gl + Pb	6.26jkl	29hijk	8abc	61def	–	–	1
	Pp + Pb	6.64fghi	31fghi	9abc	–	–	–	1
	Gl + Pp + Pb	6.98de	35e	10abc	68bc	–	–	1
M. incognita + *M. phaseolina*	C	3.51t	11s	4c	–	94b	12 200b	5
	Gl	3.96s	17qr	5bc	51h	77cd	9980d	3
	Pp	4.30r	19pq	6abc	–	58ef	7680g	3
	Pb	3.86s	15r	5bc	–	78cd	10 560c	3
	Gl + Pp	4.88op	24mno	7abc	57fg	44hi	5620h	2
	Gl + Pb	4.42qr	21op	6abc	54gh	56f	8180f	2
	Pp + Pb	4.70pq	23no	7abc	–	52fg	6930ij	2
	Gl + Pp + Pb	5.03nop	26klmn	8abc	62def	39i	4960k	1
l.s.d. ($P = 0.05$)		0.33	3	5	5	7	340	–

pathogen inoculated plants by more than inoculation with *G. intraradices* or *P. polymyxa*. Combined inoculation of plants with *G. intraradices* + *P. putida* + *P. polymyxa*, along with pathogens, caused a greater increase in shoot dry weight than by *G. intraradices* + *P. polymyxa* or *P. putida* + *P. polymyxa*. However, inoculation of plants with *G. intraradices* + *P. putida* increased shoot dry weight by a similar amount to combined inoculation with *G. intraradices* + *P. putida* + *P. polymyxa* (Table 1).

Inoculation of plants with *G. intraradices*, *P. putida* and *P. polymyxa* alone and in combination significantly increased the number of pods per plant, both in pathogen inoculated and uninoculated plants (Table 1). The numbers of pods per plant were reduced when inoculated with *M. incognita* or *M. phaseolina* or with both. Nodulation was very poor in all the plants inoculated with pathogens or with *G. intraradices*, *P. putida* and *P. polymyxa*. Root colonisation by *G. intraradices* was high when inoculated alone. In the presence of *P. putida* and *P. polymyxa*, root colonisation by the AM fungus was found to have increased, while inoculation of pathogens reduced root colonisation by the AM fungus. The number of galls per root system and nematode multiplication was found to be reduced in the presence of *M. phaseolina*. Inoculation of plants with *P. putida* caused greater reduction in galling and nematode multiplication than *P. polymyxa* and *G. intraradices*. Combined inoculation of plants with *G. intraradices* + *P. putida* + *P. polymyxa* caused greater reduction in galling and nematode multiplication than *G. intraradices* + *P. putida*, *P. putida* + *P. polymyxa* or *G. intraradices* + *P. polymyxa*. Pathogens had adverse effects on root colonisation caused by *G. intraradices*. However, root colonisation by the AM fungus was increased when coinoculated with *P. putida* and *P. polymyxa*, both in the presence or absence of pathogens (Table 1).

Root-rot indices were 3 and 5 when *M. phaseolina* was inoculated alone and together with *M. incognita*, respectively

Table 2. Effect of *G. intraradices*, *P. putida* and *P. polymyxa* on total chlorophyll, nitrogen, phosphorus and potassium contents in *M. incognita* and *M. phaseolina* inoculated and uninoculated chickpea plants

Values within each column followed by the same letter are not significantly different ($P = 0.05$). C, Control; Gl, *Glomus intraradices*; Pp, *Pseudomonas putida*; Pb, *Paenibacillus polymyxa*

Treatments		Chlorophyll (mg/g fresh leaves)	Nitrogen (mg/g fresh leaves)	Phosphorus (mg/g fresh leaves)	Potassium (mg/g fresh leaves)
Control	C	2.402lm	3.40hijk	0.338hij	1.67ij
	Gl	2.518ij	3.58defg	0.366de	1.79def
	Pp	2.614gh	3.72cd	0.356ef	1.85cde
	Pb	2.492jk	3.55efg	0.352fg	1.76fgh
	Gl + Pp	2.892ab	3.88ab	0.388ab	1.95ab
	Gl + Pb	2.776cd	3.75bc	0.374cd	1.86cd
	Pp + Pb	2.856bc	3.83b	0.380bc	1.88bc
	Gl + Pp + Pb	3.974a	3.98a	0.394a	1.98a
M. incognita	C	2.142p	2.88qr	0.276qr	1.41pqr
	Gl	2.298no	3.16nop	0.326jk	1.54lmn
	Pp	2.468jkl	3.34jklm	0.306lmn	1.65ijk
	Pb	2.252o	3.02pq	0.298mno	1.52lmno
	Gl + Pp	2.642fgh	3.54efgh	0.334ij	1.77efg
	Gl + Pb	2.584hi	3.38ijkl	0.328jk	1.68hi
	Pp + Pb	2.616hi	3.52fghi	0.332ij	1.70ghi
	Gl + Pp + Pb	2.724def	3.60defg	0.348fgh	1.79def
M. phaseolina	C	2.218o	3.10op	0.289opq	1.47nopq
	Gl	2.428klm	3.30klmn	0.330ij	1.59jkl
	Pp	2.514ij	3.46ghij	0.316kl	1.66ij
	Pb	2.382mn	3.25lmn	0.308lm	1.57klm
	Gl + Pp	2.682efg	3.68cde	0.348fgh	1.79def
	Gl + Pb	2.598ghi	3.57efg	0.336hij	1.70ghi
	Pp + Pb	2.648fgh	3.63cdef	0.343fghi	1.73fghi
	Gl + Pp + Pb	2.746de	3.72cd	0.352fg	1.83cde
M. incognita + *M. phaseolina*	C	1.608u	2.48t	0.251s	1.21t
	Gl	1.736t	2.68s	0.284pq	1.33r
	Pp	1.824s	2.84r	0.270r	1.39qr
	Pb	1.710t	2.64s	0.267r	1.30s
	Gl + Pp	2.076pq	3.19no	0.294nop	1.52lmno
	Gl + Pb	1.912r	2.86r	0.288opq	1.44opq
	Pp + Pb	1.996qr	3.04p	0.291op	1.49mnop
	Gl + Pp + Pb	2.132p	3.23mno	0.310lm	1.59jkl
l.s.d. ($P = 0.05$)		0.084	0.14	0.013	0.08

(Table 1). This index was reduced to 3 when *M. incognita* and *M. phaseolina* inoculated plants were treated with *P. putida*, *G. intraradices* or *P. polymyxa*. The index was found to be 2 when *M. phaseolina*-inoculated plants were treated with *P. putida*, *G. intraradices* or *P. polymyxa*. The index was also found to be 2 when *M. incognita* + *M. phaseolina* inoculated plants were treated with *G. intraradices* + *P. polymyxa*, *P. putida* + *P. polymyxa* or *G. intraradices* + *P. putida*. In other treatments, the index was reduced to 1 (Table 1).

Inoculation of plants with *P. putida*, *P. polymyxa* and *G. intraradices* alone and in combination in the absence of pathogens caused a significant increase in chlorophyll, nitrogen, phosphorus and potassium contents compared with uninoculated controls (Table 2). Inoculation of plants without pathogens with *P. putida* increased chlorophyll, nitrogen and potassium contents more than inoculation with *P. polymyxa*. However, an increase in phosphorus contents by *P. putida* was similar to that caused by *G. intraradices*. Moreover, increase in chlorophyll, nitrogen and potassium contents caused by *G. intraradices* was similar to *P. polymyxa*. Combined inoculations of *G. intraradices* + *P. putida* + *P. polymyxa* caused increases in chlorophyll, nitrogen, phosphorus and potassium contents that were greater than the increases caused by *P. putida* + *P. polymyxa* or *G. intraradices* + *P. polymyxa*. However, the inoculation of plants without pathogens using *G. intraradices* + *P. putida* caused a similar increase in chlorophyll, nitrogen, phosphorus and potassium contents to that caused by inoculation of *G. intraradices* + *P. putida* + *P. polymyxa* (Table 2).

Inoculation of *M. incognita* and *M. phaseolina* alone and in combination caused a significant reduction in chlorophyll, nitrogen, phosphorus and potassium contents compared with the uninoculated controls (Table 2). Reduction in chlorophyll, nitrogen, phosphorus and potassium contents was greater when *M. incognita* and *M. phaseolina* were inoculated together than when they were used individually. *M. incognita* caused a similar reduction in phosphorus and potassium contents to that

caused by *M. phaseolina*. Inoculation of plants with *P. putida* in the presence of pathogens caused a greater increase in chlorophyll, nitrogen and potassium contents than that caused by *G. intraradices* or *P. polymyxa*. However, the increase in phosphorus content caused by *G. intraradices* was greater than that caused by *P. putida*. An increase in chlorophyll, nitrogen and potassium contents caused by *G. intraradices* was similar to that caused by *P. polymyxa*. Combined inoculations of *G. intraradices* with *P. putida* plus *P. polymyxa* caused a greater increase in chlorophyll, nitrogen, phosphorus and potassium contents than that caused by *P. putida* + *P. plymyxa* or *G. intraradices* + *P. plymyxa*. However, inoculations of plants with *G. intraradices* + *P. putida* in the presence of pathogens caused a similar increase in chlorophyll, nitrogen, phosphorus and potassium contents to that caused by inoculation of *G. intraradices* + *P. putida* + *P. polymyxa* (Table 2).

Discussion

G. intraradices has a potential to improve plant growth of nematode-infected plants by reducing their multiplication (Bagyaraj *et al*. 1979). The root-rot index of *M. phaseolina*-inoculated plants was also reduced by *G. intraradices*. Bødker *et al*. (1998) observed a reduction in root-rot of pea caused by *Aphanomyces etueiches*, while Akkopru and Demir (2005) observed reduced Fusarium wilt of tomato by inoculation of plants with *G. intraradices*. Reduced pathogen damage of mycorrhizal plants may be due to physiological and biochemical changes in the host or to an increase in the flow of nutrients, which provides greater mechanical strength (Schonbeck 1979; Augé 2001). In addition, inoculation with mycorrhizal fungi increases phosphorus uptake sufficiently to offset symptoms of the pathogen (Hussey and Roncadori 1982). Treatment with *Glomus* spp. is also reported to increase phenylalanine and serine in tomato roots (Suresh 1980) and these amino acids have an inhibitory effect on nematodes (Reddy 1974).

Pseudomonads may also improve plant growth by suppressing parasitic and non-parasitic root pathogens (Oostendrop and Sikora 1989) by the production of biologically active substances (Gamliel and Katan 1993), or by changing unavailable minerals and organic compounds into forms that are available to plants (Broadbent *et al*. 1977; Siddiqui and Mahmood 1999). In addition, an induced systemic resistance by *Pseudomonas* is also considered a mechanism for the biocontrol of plant pathogens (Wei *et al*. 1996). Siderophore production by *P. putida* was greater than with *P. polymyxa* (Siddiqui *et al*. 2006). This may be a reason why *P. putida* caused greater reduction in galling and nematode multiplication than *P. polymyxa*.

P. polymyxa is also involved in plant growth promotion (Timmusk *et al*. 1999). Indirect promotion of plant growth occurs when *P. polymyxa* antagonises or prevents the effects of phytopathogens or deleterious microorganisms (Glick 1994). *P. polymyxa* is known to produce antibiotic compounds and inoculation with *P. polymyxa* suppresses several plant pathogens (Yuen *et al*. 1991; Oedjijono *et al*. 1993). Induced resistance is also known as a mechanism of disease suppression which may be a result of root colonisation by plant growth promoting rhizobacteria (Alström 1991; Wei *et al*. 1991). Lipopolysaccharides from the bacterial outer membranes (van Peer and Schippers 1992), siderophores (Leeman *et al*. 1996), jasmonic acid and ethylene (Pieterse *et al*. 1998) have all been proposed to be involved in the induction of induced systemic resistance. Siderophore production by *P. polymyxa* was lesser than *P. putida* (Siddiqui *et al*. 2006) and, as a result, *P. polymyxa* caused less reduction in galling and nematode multiplication than *P. putida*.

P. putida, *P. polymyxa* and *G. intraradices* interacted additively in reducing galling and nematode multiplication. As a result, plant growth and root-rot disease index was also reduced greatly.

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