Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Efficacy of root-associated fungi and PGPR on the growth of *Pisum sativum* (cv. Arkil) and reproduction of the root-knot nematode *Meloidogyne incognita*

Mohd. Sayeed Akhtar[1, 2] and Jitendra Panwar[3]

[1] Department of Biology, College of Natural Sciences, Jimma University, Jimma, Ethiopia,
[2] Department of Botany, Aligarh Muslim University, Aligarh, India
[3] Centre for Biotechnology, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Rajasthan, India

The effects of root-associated fungi (*Aspergillus awamori* and *Glomus mosseae*) and plant growth promoting rhizobacteria (PGPR) (*Pseudomonas putida*, *Pseudomonas alcaligenes* and *Paenibacillus polymyxa*) were studied alone and in combination in glasshouse experiments on the growth of pea, enzyme activity (peroxidase and catalase) and reproduction of root-knot nematode *Meloidogyne incognita*. Application of *A. awamori*, *G. mosseae* and PGPR caused a significant increase in pea growth and enzyme activities of both nematode inoculated and uninoculated plants. *A. awamori* was more effective in reducing galling and improving the growth of nematode inoculated plants than *P. alcaligenes* or *P. polymyxa*. The greatest increase in growth, enzyme activities of nematode-inoculated plants and reduction in galling and nematode multiplication was observed when *A. awamori* was used with *P. putida* or *G. mosseae* as compared to the other combinations tested. Percentage root colonization was higher when AM fungus inoculated plants were treated with *P. putida* both in presence and absence of nematode.

Keywords: Catalase / *Glomus* / *Meloidogyne* / Peroxidase / PGPR

Received: November 30, 2011; accepted: April 30, 2012

DOI 10.1002/jobm.201100610

Introduction

Pea (*Pisum sativum* L.) is one of the most important legume crop and chief source of protein for large vegetarian population in the world. The third largest area in pea cultivation is occupied by India after Canada and Russia [1]. Pea is an annual legume that grows vigorously as vines. The leaves are modified into tendrils used for attachment to support and climbing. As a legume crop, pea contributes to yield and protein content of the succeeding cereal crop in rotation by improving nitrogen status of the soil [2]. The vegetable peas are good source of vitamins, folate, iron and phosphorus. The seeds contain high levels of proteins, digested carbohydrate and low concentration of fat fiber to human diet [3]. In India, the pea cultivated on about 0.81 million hectare, out of which 65% area lies in the state Uttar Pradesh (U.P.) [4]. It was evident from our previous study that pea production has declined drastically particularly in Aligarh and its adjoining districts of U.P., India due to the presence of root-knot nematode *Meloidogyne incognita* in soils [5]. The annual loss due to *M. incognita* infestation is estimated at 40–45%. Pea roots infected with *M. incognita* were found to be severely galled and resulted in decreased plant growth. This nematode has been reported as a serious constraint in the successful cultivation of pea [5].

In soil, the rhizosphere zone is relatively rich in nutrients due to the loss of up to 40% of plant photosynthates from roots. Rhizosphere microorganisms utilize compounds and materials released from the crop roots as nutrition source. Consequently, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral or detrimental effects on plant growth. Among the rhizosphere organisms, plant growth-promoting rhizobacteria (PGPR) have the ability to improve seed germination, root development, mineral nutrition and water utilization and can also suppress plant

Correspondence: Mohd. Sayeed Akhtar. Department of Biology, College of Natural Sciences, Jimma University, Jimma-378, Ethiopia
E-mail: sayeedbot@yahoo.co.in
Phone: +251-923793757
Fax: +251-471110934

diseases. The manipulation of crop rhizosphere by inoculation with PGPR for biocontrol of plant diseases has shown considerable promise [6]. In addition, the association of arbuscular mycorrhizal (AM) fungi can offer multiple benefits to the host plant either by colonizing the roots of crop plants [7] or by increasing the uptake of phosphorus, minor elements and water [7, 8]. They also reduce the severity of several plant diseases [9, 10]. Sikora and Reimann [11] found the significant reduction in galling and egg-mass production of *M. incognita* when *Glomus intraradices* is applied in combination with rhizobacteria. However, Zheng *et al*. [12] reported that the inoculation with *G. intraradices* and P fertilizer confer tolerance of cucumber plants to *M. incognita* by enhancing plant growth and suppressing reproduction and/or galling of nematodes during the early stages of plant growth. Moreover, *Aspergillus* species have been reported to produce a variety of secondary metabolites and are useful in the biocontrol of nematodes [13, 14]. Gaur [15] observed that the *A. awamori* have the ability to increase the grain yield of various crops through the solubilisation of fixed soil phosphorus while, Khan and Khan [16] reported that application of *A. awamori* and *A. niger* resulted in an 80 and 58% increase in the yield of tomato inoculated with test organism. Siddiqui and Akhtar [17] reported that the use of *A. awamori* significantly increases the plant growth, chlorophyll and NPK contents and also reduced the galling and nematode multiplication on chickpea. The results were more pronounced when it was applied in combination with plant growth promoting bacterium. Similarly, Mittal *et al*. [18] also reported that that *A. awamori* increased the shoot height of chickpea (7–12%), and seed number (3 fold) and seed weight (2 fold) compared to control in a green house condition. It has been also reported that inoculation of *A. awamori* produced phenyl ethanol, phenyl acetic acid and phenoxy acetic acid, which may reduced the disease severity in crop plants [19].

In general, a single biocontrol agent is used for biocontrol of plant disease against a single test organism [20]. This may sometimes account for the inconsistent performance by the biocontrol agent because a single agent is not active in all soil environments or against all test organisms that attack the host plant. On the other hand, mixtures of biocontrol agents with different plant colonization patterns may be useful for the biocontrol of different plant diseases via different mechanisms of disease suppression. Moreover, mixtures of biocontrol agents with taxonomically different organisms that require different optimum temperature, pH and moisture conditions may colonize roots more aggressively, improve plant growth and efficacy of biocontrol. The greater suppression and enhanced consistency against multiple cucumber test organisms was observed using strain mixtures of PGPR [21].

Several investigations of enzymes in nematode-infected plants have been conducted. Catalase and peroxidase are the important groups of enzymes which play a very crucial role in the defence mechanism of plants against the test organisms. Catalase is a soluble hemeprotein. It breaks the hydrogen peroxide into hydrogen and water, works as detoxifying agents for enzymes and has important regulatory effect in controlling quantity of indole acetic acid in plants [22], while peroxidase catalyses the polymerization of phenolic compounds and forms cross-links between extensin, lignin and feruloylated polysaccharides [23]. Peroxidases are important in the reinforcement of cell wall and play a significant role in plant resistance against nematodes [24].

In the present study, an attempt was made to examine the effects of root-associated fungi (*Aspergillus awamori* and *Glomus mosseae*) alone and in combination with plant growth promoting rhizobacteria (*Pseudomonas putida* Trevisan, *P. alcaligenes* Monias and *Paenibacillus polymyxa* Prazmowski) on enzyme activities (catalase and peroxidase), pea plant growth and reproduction of root-knot nematode *M. incognita*.

Materials and methods

The root-knot nematode *M. incognita* was the tested organisms. *Glomus mosseae*, *Aspergillus awamori* and PGPR's were applied alone and in combination to pea (*Pisum sativum* cv. Arkil). The influence of these treatments on catalase and peroxidise activity was determined one week after inoculation and the plant growth, galling, percent root colonization and nematode multiplication were assessed 90 d after inoculation in glasshouse experiments.

Preparation and sterilization of soil mixture

Sandy loam soil (pH 7.2, porosity 37%, water holding capacity 41%, electrical conductivity 0.66 (dSm^{-1}), available N 214 kg ha^{-1}, P 26 kg ha^{-1} and K 361 kg ha^{-1}) collected from the field of the Department of Botany, Aligarh Muslim University, Aligarh, India was sieved using a 10 mesh sieve. The soil, river sand and decomposed cow manure were mixed in the ratio of 3:1:1 (v/v) and filled in jute bags. Appropriate amount of water was added into each bag in order to moist the soil before transferring to an autoclave for sterilization at 137.9 kPa for 20 min. After autoclaving, the soil was allowed to cool to room temperature and 1 kg of soil was filled in each clay pots (15 cm diameter).

Raising and maintenance of test plants

Seeds of pea were surface sterilized with 0.1% sodium hypochlorite (NaOCl) for 2 min and then rinsed thrice with distilled water. Five healthy pea seeds of similar sizes were sown in each pot and after germination, thinning was done to maintain one healthy seedling per pot. Two days after thinning, seedlings received the treatments as listed in Table 1. The pea seedlings grown in pot were inoculated with 2,000 freshly hatched second-stage juveniles (J_2) of *M. incognita*, plant growth promoting rhizobacterial isolates (10 ml of 1.5×10^7 cfu/ml), *G. mosseae* (500 infective propagules), and *A. awamori* (10 ml of 1.2×10^5 cfu/ml), while uninoculated plants served as a control. All the pots were kept in a glasshouse at 22 ± 2 °C and watered as needed.

Nematode inoculum

M. incognita was collected from pea field soil and identified with the help of perineal pattern. The pure culture of nematode was multiplied on egg plant (*Solanum melongena* L.) using a single egg mass placed in a glass house. Egg masses were hand-picked using sterilized forceps and placed in 9 cm diameter sieves of 1 mm pore size which were previously mounted with cross layered tissue paper and placed in Petri plates containing water just deep enough to contact the egg masses for hatching and incubated at 25 ± 2 °C. The hatched juveniles were collected from the Petri plates after every 24 h. The concentration of second stage juveniles of *M. incognita* in the water was adjusted so that each milliliter contained 200 ± 5 nematodes. Ten milliliter of this suspension (i.e. 2000 freshly hatched juveniles) was added to each pot containing a pea seedling.

Inoculum production of microorganisms

The AM fungus, *G. mosseae* Nicolson and Gerdmann was isolated from the soil of pea field of Aligarh, India. The species was identified using the synoptic keys [25] and the identification was further confirmed from Curator, INVAM, USA (http://invam.caf.wvu.edu/). For inoculum production, the fungus was cultured on *Chloris gayana* Kunth (Rhodes grass) grown in sandy loam soil. The population of *G. mosseae* isolate AI13 (Aligarh, India) in the inoculum was assessed by the most probable number method [26]. Fifty grams of inoculum with soil was added around the seed to provide 500 infective propagules of *G. mosseae* per pot (1 g inoculums contains 10 infective propagules and consisted of soil, extra matrical spores and sporocarps, hyphal fragments and infected Rhodes grass fragments). Plant growth promoting rhizobacteria, *Paenibacillus polymyxa* (MTCC No. 122), *Pseudomonas putida* (MTCC No. 3604), and *Pseudomonas alcaligenes* (MTCC No. 493) were obtained from Microbial Type of Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. These isolates were sub cultured on nutrient broth (Hi-Media Laboratories, Mumbai, India). One ml of nutrient broth suspension contained about 1.5×10^7 cfu/ml. Ten ml of this suspension was used to inoculate the respective pots around the pea seedling. *Aspergillus awamori* Nakazawa (ITCC 4680) was obtained from Indian Agricultural Research Institute, New Delhi and was cultured in Richard's medium at 25 ± 1 °C for 15 d. Ten ml (equivalent to 1 g) suspension was used to inoculate the respective pots around the pea seedling.

Inoculation procedure

For inoculation of *M. incognita*, root-associated fungi (*A. awamori* and *G. mosseae*) and PGPR (*P. putida*/*P. alcaligenes*/*P. polymyxa*), soil around the root was carefully removed without damaging the roots. The inoculum suspensions were poured or placed around the roots and the soil was replaced. An equal volume of sterile water was added to control treatments.

Experimental design

The experiment was carried out in a completely randomized blocked design with three experimental variables: (a) Control; (b) *A. awamori*; (c) *G. mosseae*. Each

Table 1. Effect of root-associated fungi and PGPR on the dry shoot weight (g) in *M. incognita* inoculated and uninoculated pea plants.

		PGPR			
Nematode inoculation	**Root-associated fungi**	**C**	**Pp**	**Pa**	**Py**
Without	C	4.02 ± 0.34^{mn}	5.26 ± 0.26^{ef}	5.08 ± 0.35^{fg}	4.82 ± 0.36^{ghi}
	Gm	5.36 ± 0.29^{e}	6.06 ± 0.39^{abc}	5.80 ± 0.32^{cd}	5.72 ± 0.35^{d}
	Aw	5.42 ± 0.22^{e}	6.22 ± 0.30^{a}	6.10 ± 0.33^{ab}	5.88 ± 0.43^{bcd}
With	C	3.26 ± 0.27^{o}	4.14 ± 0.25^{lm}	3.98 ± 0.35^{mn}	3.78 ± 0.31^{n}
	Gm	4.06 ± 0.27^{mn}	4.68 ± 0.26^{hij}	4.54 ± 0.18^{ijk}	4.38 ± 0.16^{kl}
	Aw	4.20 ± 0.24^{lm}	4.84 ± 0.26^{gh}	4.72 ± 0.25^{hij}	4.52 ± 0.25^{jk}

Values followed by same letter do not differ significantly ($P < 0.05$). $\pm$ = Standard deviation. C = Control; Gm = *G. mosseae*; Aw = *Aspergillus awamori*; Pp = *Pseudomonas putida*; Pa = *P. alcaligenes*; Py = *Paenibacillus polymyxa*.

set was inoculated with the following four treatments: (1) Control; (2) *P. putida*; (3) *P. alcaligenes*; (4) *Paenibacillus polymyxa* ($3 \times 4 = 12$ treatments). These 12 treatments were tested both in presence and absence of *M. incognita* ($12 \times 2 = 24$ treatments). Each treatment was replicated five times ($24 \times 5 = 120$ pots) and the experiment was repeated once. The data in this paper represents the pooled data of both the experiments.

Parameter assessment

The plants were harvested 90 d after inoculation. Data were recorded on dry shoot weight, percentage root colonization by AM fungus, number of galls, and nematode multiplication. Catalase and peroxidase activities in leaves were determined by Chance and Maehly method [27]. Plants were kept in envelops at 60 °C for 2–3 d before dry weight measurements. A 250 g sub-sample of well-mixed soil from each treatment was processed by Cobb's sieving and decanting method followed by Baerman's funnel extraction in order to determine the nematode population [28]. To estimate the number of juveniles, eggs and females inside the roots, 1 g sub-sample of roots was macerated in a Waring blender and counts were made on the suspension thus obtained. The numbers of nematodes present in roots were calculated by multiplying the number of nematodes present in 1 g of root by the total weight of root. The proportion of root colonized by AM fungi were determined by the gridline intersect method [29] after clearing the root with KOH in 0.05% trypan blue lactophenol.

Statistical analysis

The data were analyzed statistically using multifactorial analysis (*M. incognita* × root-associated fungi × plant growth promoting rhizobacteria) of variance $p = 0.05$. Effects of *M. incognita*, root-associated fungi, plant growth promoting rhizobacteria were analyzed individually and also using the interactions of two and three factors. Duncan's multiple range test (DMRT) was employed to distinguish differences between treatments.

Results

Effects of *M. incognita*, root-associated fungi (*A. awamori* and *G. mosseae*) and plant growth promoting rhizobacteria (PGPR) individually and their interactions were found to be significant on dry shoot weight except for the interaction of nematode and PGPR and interaction of all the three factors together. Inoculation of *A. awamori* and *G. mosseae* and PGPR (*P. putida*/*P. alcaligenes*/*P. polymyxa*) alone caused a significant increase in dry shoot weight of plants without nematodes over inoculated ones (Table 1). *A. awamori* alone caused a greater increase in the dry shoot weight of the plants without nematode followed by *P. alcaligenes*/*P. polymyxa*. However, increase in dry shoot weight caused by *G. mosseae*/*P. putida* was similar to that caused by *A. awamori*. Use of *A. awamori* with *P. putida* resulted in greater increase in shoot dry weight of plants without nematodes compared to any other tested combination. However, inoculation of *A. awamori* plus *P. alcaligenes* caused a similar increase in dry shoot weight of plants without nematodes to that caused by *A. awamori* with *P. putida* (Table 1).

Inoculation of *M. incognita* caused a significant reduction in dry shoot weight of plants over uninoculated ones. Inoculation of *A. awamori, G. mosseae* and PGPR (*P. putida*/*P. alcaligenes*/*P. polymyxa*) alone resulted in significant increase in dry shoot weight of nematode inoculated plants. *A. awamori* alone caused a greater increase in nematode inoculated plants than caused by *P. polymyxa* (Table 1).

Effects of *M. incognita*, root-associated fungi (*A. awamori* and *G. mosseae*), and PGPR individually and their interactions were significant on root colonization by AM fungus except for the interaction of nematodes and PGPR and the interaction of all the three factors together on colonization of root. Root colonization by AM fungus was generally reduced in presence of *M. incognita* (Table 2). Root colonization by *G. mosseae* was greater when inoculated together with *P. putida* while the least when inoculated alone (Table 2).

Table 2. Effect of root-associated fungi and PGPR on the percent root colonization by AM fungus in *M. incognita* inoculated and uninoculated pea plants.

		PGPR			
Nematode inoculation	**Root-associated fungi**	**C**	**Pp**	**Pa**	**Py**
Without	C	–	–	–	–
	Gm	53 ± 6.75^{d}	66 ± 6.86^{a}	62 ± 4.52^{ab}	59 ± 9.72^{c}
With	C	–	–	–	–
	Gm	46 ± 4.74^{e}	60 ± 3.80^{bc}	57 ± 4.37^{cd}	53 ± 7.71^{d}

Values followed by same letter do not differ significantly ($P < 0.05$). ± = Standard deviation. C = Control; Gm = *G. mosseae*; Pp = *Pseudomonas putida*; Pa = *P. alcaligenes*; Py = *Paenibacillus polymyxa*.

Table 3. Effect of root-associated fungi and PGPR on the number of galls per root system of pea plants inoculated with *M. incognita*.

	PGPR			
Root-associated fungi	**C**	**Pp**	**Pa**	**Py**
C	73 ± 6.99^{a}	54 ± 6.12^{c}	59 ± 3.77^{b}	62 ± 4.88^{b}
Gm	51 ± 4.00^{cd}	37 ± 4.59^{f}	40 ± 3.19^{f}	44 ± 2.94^{e}
Aw	48 ± 2.98^{d}	32 ± 4.02^{g}	36 ± 3.65^{f}	39 ± 4.54^{f}

Values followed by same letter do not differ significantly ($P < 0.05$). ± = Standard deviation. C = Control; Gm = *G. mosseae*; Aw = *Aspergillus awamori*; Pp = *Pseudomonas putida*; Pa = *P. alcaligenes*; Py = *Paenibacillus polymyxa*.

The root galling and nematode multiplication was highest when *M. incognita* was inoculated alone (Tables 3 and 4). Inoculation of *A. awamori* caused a greater reduction in galling and nematode multiplication followed by *G. mosseae*, *P. putida*, *P. alcaligenes* and *P. polymyxa*. Inoculation of *A. awamori* with *P. putida* caused a highest reduction in root galling and nematode multiplication while inoculation of *G. mosseae* with *P. polymyxa* was less effective in reducing galling and nematode multiplication among combined treatments (Tables 3 and 4).

Effects of *M. incognita*, root-associated fungi (*A. awamori* and *G. mosseae*), and PGPR were significant on catalase and peroxidase activity while the interaction of two factors and the interaction of all the three factors together were non-significant except the interaction of *M. incognita* and root-associated fungi on peroxidase activity. Inoculation of *A. awamori* and *G. mosseae* and PGPR (*P. putida*/*P. alcaligenes*/*P. polymyxa*) caused a significant increase in catalase and peroxidase activity of plants without nematodes over uninoculated ones (Tables 5 and 6). Inoculation of *A. awamori* alone caused a significant increase in catalase activity than caused by *P. putida*/*P. alcaligenes*/*P. polymyxa* or *G. mosseae* (Tables 5 and 6). Inoculation of *A. awamori* alone caused a greater increase in peroxidase activity of the plants without nematodes than caused by *P. putida*/*P. alcaligenes*/*P. polymyxa* (Tables 5 and 6). However, inoculation of *G. mosseae* and *P. putida* caused almost similar increase in peroxidase activity of the plants without nematodes than caused by *A. awamori*

Table 4. Effect of root-associated fungi and PGPR on the nematode multiplication (rhizospheric soils and roots) in pea plants inoculated with *M. incognita*.

	PGPR			
Root-associated fungi	**C**	**Pp**	**Pa**	**Py**
C	9680 ± 411.50^{a}	7060 ± 361.11^{c}	7640 ± 275.04^{b}	7860 ± 339.54^{b}
Gm	6560 ± 251.39^{d}	4810 ± 308.29^{hi}	5180 ± 392.51^{g}	5680 ± 334.13^{f}
Aw	6120 ± 389.53^{e}	4180 ± 339.02^{j}	4660 ± 390.36^{i}	5120 ± 370.11^{gh}

Values followed by same letter do not differ significantly ($P < 0.05$). ± = Standard deviation. C = Control; Gm = *G. mosseae*; Aw = *Aspergillus awamori*; Pp = *Pseudomonas putida*; Pa = *P. alcaligenes*; Py = *Paenibacillus polymyxa*.

Table 5. Effect of root-associated fungi and PGPR on the catalase activity (H_2O_2 per minute) in *M. incognita* inoculated and uninoculated pea plants.

		PGPR			
Nematode inoculation	**Root-associated fungi**	**C**	**Pp**	**Pa**	**Py**
Without	C	6.10 ± 0.38^{ij}	7.03 ± 0.26^{ef}	6.80 ± 0.39^{g}	6.46 ± 0.16^{h}
	Gm	7.10 ± 0.16^{e}	8.13 ± 0.26^{b}	7.86 ± 0.22^{c}	7.43 ± 0.29^{d}
	Aw	7.36 ± 0.26^{d}	8.53 ± 0.27^{a}	8.23 ± 0.22^{b}	7.70 ± 0.30^{c}
With	C	5.26 ± 0.21^{l}	6.16 ± 0.24^{i}	5.90 ± 0.21^{j}	5.65 ± 0.17^{k}
	Gm	6.13 ± 0.19^{i}	7.13 ± 0.22^{e}	6.86 ± 0.20^{fg}	6.43 ± 0.29^{h}
	Aw	6.50 ± 0.30^{h}	7.43 ± 0.15^{d}	7.20 ± 0.21^{de}	6.73 ± 0.15^{g}

Values followed by same letter do not differ significantly ($P < 0.05$). ± = Standard deviation. C = Control; Gm = *G. mosseae*; Aw = *Aspergillus awamori*; Pp = *Pseudomonas putida*; Pa = *P. alcaligenes*; Py = *Paenibacillus polymyxa*.

Table 6. Effect of root-associated fungi and PGPR on the peroxidase activity (Purpurogallin per mg per minute) in *M. incognita* inoculated and uninoculated pea plants.

Nematode inoculation	Root-associated fungi	PGPR			
		C	Pp	Pa	Py
Without	C	0.226 ± 0.010^{mn}	0.268 ± 0.005^{ghi}	0.259 ± 0.007^{ij}	0.246 ± 0.009^{kl}
	Gm	0.276 ± 0.011^{fg}	0.308 ± 0.005^{bc}	0.302 ± 0.007^{cd}	0.288 ± 0.014^{e}
	Aw	0.282 ± 0.011^{ef}	0.322 ± 0.006^{a}	0.312 ± 0.005^{b}	0.298 ± 0.016^{d}
With	C	0.204 ± 0.009^{o}	0.239 ± 0.007^{l}	0.228 ± 0.004^{m}	0.218 ± 0.005^{n}
	Gm	0.242 ± 0.008^{l}	0.269 ± 0.012^{gh}	0.262 ± 0.013^{hij}	0.254 ± 0.006^{jk}
	Aw	0.246 ± 0.010^{kl}	0.282 ± 0.005^{ef}	0.272 ± 0.013^{g}	0.262 ± 0.006^{hij}

Values followed by same letter do not differ significantly ($P < 0.05$). $\pm$ = Standard deviation. C = Control; Gm = *G. mosseae*; Aw = *Aspergillus awamori*; Pp = *Pseudomonas putida*; Pa = *P. alcaligenes*; Py = *Paenibacillus polymyxa*.

plus *P. alcaligenes*. Inoculation of *A. awamori* with *P. putida* caused a greater increase in peroxidase and catalase activity of plants without nematodes compared to caused by other treatments (Tables 5 and 6).

Inoculation of *M. incognita* caused a significant reduction in catalase and peroxidase activity of plants over uninoculated ones (Tables 5 and 6). Inoculation of root-associated fungi (*A. awamori* and *G. mosseae*) and PGPR (*P. putida*/*P. alcaligenes*/*P. polymyxa*) caused a significant increase in peroxiase and catalase activity of nematode inoculated plants. *A. awamori* alone caused a greater increase in peroxidase activity in nematode inoculated plants than caused by *P. alcaligenes*/*P. polymyxa* while the inoculation of *A. awamori* alone caused a significant increase in catalase activity of nematode infected plants than caused by *P. putida*/*P. alcaligenes*/*P. polymyxa* or *G. mosseae* (Tables 5 and 6). However, inoculation of *A. awamori* with *P. putida* caused a greater increase in catalase and peroxidase activity of plants infected with nematodes than other treatments except to that caused by *A. awamori* plus *P. alcaligenes* for catalase activity (Tables 5 and 6).

Discussion

Aspergillus species are common in habitats such as soils in warmer climates, compost, decaying plant material and stored grains and many are known to produce a variety of secondary metabolites [30]. Some *Aspergillus* species have also been reported for their biocontrol potential against root-knot nematodes [31]. *Aspergillus* species isolated from the rhizosphere of crop plants produce number of secondary metabolites that are soluble in ethyl acetate and can potentially influence the efficacy of the biocontrol strains of PGPR [13]. Thus, biocontrol by *A. awamori* may be attributed to its production of secondary metabolites. Moreover, *Aspergillus* species can also inhibit egg hatching, indicating the involvement of mechanisms other than parasitism [32]. Neither of these species was isolated from eggs or females of nematodes, so they apparently did not parasitize nematodes; hence their effect is exogenous. Moreover, enzymatic disintegration of the vitelline and chitin layers of the nematode eggshell might have increased the permeability of the eggshell and enhanced mycelial penetration leading to total disintegration of the egg contents [32]. The increase in catalase activity may be responsible for it.

In the present study, AM fungi improved the growth of nematode-infected plants by reducing nematode multiplcation. These results are in conformity with those of Bagyaraj *et al*. [33]. In our study, we presumed that disease inhibition by *G. mosseae* may not be completely related to increase in phosphorus content despite significant increase in phosphorus and dry mass of roots (data not shown). Elson *et al*. [34] have demonstrated that the AM fungi have the ability to induce systemic resistance against plant parasitic nematodes in a root system. In addition, Li *et al*. [35] have demonstrated that AM fungi induce resistance giving a defense response against root-knot nematodes in grapevine roots that involves transcriptional control of VCH3 gene (*Vitis* Class III Chitinase) expression throughout the whole root system. Moreover, treatment with mycorrhizal fungi has also been reported to increase phenylalanine and serine in crop plant and these amino acids have an inhibitory effect on nematodes [36].

Pseudomonas had the ability to improve the plant growth by reducing the nematode galling and multiplication through the production of metabolites such as antibiotics, siderophores and HCN [37] or the conversion of unavailable minerals and organic compounds into forms that are available to plants [38]. *Pseudomonas* sp. can synthesize enzymes that can modulate the plant hormone levels, may limit the available iron via siderophore production and can also kill the test organism

with the synthesis of antibiotics [6]. In addition, many *Pseudomonas* strains can indirectly protect plants by inducing systemic resistance against the various test organisms [39]. Similarly *Bacillus* isolates have been reported to promote the growth of a wide range of plants [40]. *B. pumilus* induces callose and pectin in close association with phenolic compounds in newly formed wall appositions in pea roots in response to attack by *Fusarium oxysporum* [41]. However, treatment with *B. pumilus*, induced a rapid lignification in cucumber plants in response to ingress of *Colletotrichum orbiculare* and total peroxidase and superoxide dismutase activities increased more than those in the buffer control [42]. These responses may be due to the production of siderophores, antibiotics, wall appositions and defense enzymes which adversely affect on the test organisms.

The results of the present study showed that the use of AM fungus, growth promoting fungus (*Aspergillus awamori*) and PGPR's alone and in combination significantly improved the plant growth and reduced the nematode galling multiplication. This study also gave an indication about the use of suitable combination to control root-knot nematodes. Combined inoculation with biocontrol agents having different mechanisms of action is known to provide greater biocontrol against the test organisms on different crops than an inoculation with a single agent [43, 44]. The effectiveness of the PGPR, AM fungus and plant growth promoting fungus was found to be species dependent. In our experiment, the combined application of *P. putida* with either *Aspergillus awamori* or with *G. mosseae* caused a greater decrease in galling and nematode populations as compared to other combinations or individual inoculation. The different modes of action by these organisms probably resulted in synergistic effects in increasing plant growth [44]. Similarly, plant growth promoting fungus with AM fungus also had an additive effect in reducing galling and nematode population, but less than when either was used with PGPR because PGPR was found to be better than either AM fungus or plant growth promoting fungus in controlling galling and nematode population. *Pseudomonas* spp. have been reported to promote and stimulate colonization of AM fungi and are called 'Mycorrhiza helper bacteria' [45]. They stimulate the germination of AM spore and mycelial development, and are also known to synthesize antibiotics that prevent proliferation of phytopathogens [6]. It is also obvious from the earlier reports that when the nematode infected plants were inoculated with beneficial bacteria, various mechanism such as induced systemic resistance, competition for nutrients, siderophore production, predation or parasitism etc. occur which may cause the deleterious effect of the nematode multiplication in the host plant [21]. This may be the reason that when nematode infected plants when inoculated with these beneficial bacteria influenced the above said parameters as compared to only nematode inoculated plants [21]. It was also evident from the results of our study that the enzyme activities of both enzymes (catalase and peroxidase) were less in nematode-infected plants as compared to uninoculated plants. The increase in enzyme activity was observed when AM fungus/*A. awamori*/PGPR was inoculated alone or in combination. Blilou *et al*. [46] observed an increase in the activity of both these enzymes when tobacco roots were inoculated with *G. mosseae*. The rhizobacterium inoculated plants had the higher enzyme activity compared to uninoculated control [47].

The experiments described in the present study were done in pots with sterilized soil. When these microorganisms will be added in the field, they will have to compete with other soil microorganisms and survive under natural environmental conditions that will influence their efficacy as biocontrol agents. Our study suggested that use of biocontrol agents (*A. awamori*, *G. mosseae* and *P. putida*) have the potential to control *M. incognita* on pea plants. However, studies under different field conditions are required to confirm these results. These approaches will increase our scope to control the nematode population for enhaced growth of other crop plants under field conditions.

References

[1] Khan, T.N., Dixit, G.P., 2001. Breeding field peas in India and Australia. Indian J. Pulses Res., **14**, 1–9.

[2] Rowland, I., Mason, M., Pritchard, I., French, R., 1994. Effect of field peas and wheat on the yield and protein of subsequent wheat crops grown at several rates of applied nitrogen. Aust. J. Exp. Agric., **34**, 641–646.

[3] Saharan, K., Khetarpaul, N., 1994. Protein quality traits of vegetables and field peas:varietial differences. Plant Foods Hum. Nutr., **45**, 11–12.

[4] Ramteke, P.W., Joseph, B., Mani, A., Chacko, S., 2012. *Pisum sativum* and associated plant growth promoting rhizobacteria: Effect of normal and sewage irrigation. Int. J. Soil Sci., **7**, 15–27.

[5] Siddiqui, Z.A., Qureshi, A., Akhtar, M.S., 2009. Biocontrol of root-knot nematode *Meloidogyne incognita* by *Pseudomonas* and *Bacillus* isolates on *Pisum sativum*. Arch. Phytopathol. Plant Protec., **12**, 1154–1164.

[6] Siddiqui, Z.A., 2006. PGPR: prospective biocontrol agents of plant pathogens. In: Siddiqui, Z.A. (ed.). PGPR: Biocontrol and Biofertilization, Springer, The Netherlands, pp. 111–142.

[7] Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis, (3rd Eds.), Academic Press, London.

[8] Clark, R.B., Zeto, S.K., 2000. Mineral acquisition by arbuscular mycorrhizal plants. J. Plant Nutr., **23**, 867–902.

[9] Barea, J.M., Azcon, R., Azcon-Anguilar, C., 2002. Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhock, **81**, 343–351.

[10] Akhtar, M.S., Siddiqui, Z.A., 2008. Arbuscular mycorrhizal fungi as potential biprotectants against plant pathogens. In: Siddiqui, Z.A., Akhtar, M.S., Futai, K. (eds.), Mycorrhizae: Sustainable Agriculture and Forestry. Springer: The Netherlands, pp. 61–97.

[11] Sikora R.A., Reimann, S., 2004. Suppressive soils, the edge of chaos and multitrophic strategies for biocontrol of pests and diseases in soil ecosystems. Bulletin OILB/SROP **27**, 251–258.

[12] Zheng L., Zhang, J., Christie, P., Li, X., 2009. Effect of inoculation with the arbuscular mycorrhizal fungus *Glomus intraradices* on the root-knot nematode *Meloidogyne incognita* in cucumber. J. Plant Nutr., **32**, 967–979.

[13] Siddiqui, I.A., Shaukat, S.S., Khan, A., 2004. Differential impact of some *Aspergillus* species on *Meloidogyne javanica* biocontrol by *Pseudomonas fluorescens* strain CHA0. Lett. Appl. Microbiol., **39**, 74–83.

[14] Akhtar, M.S., Siddiqui, Z.A., 2006. Effects of phosphate solubilizing microorganisms on the growth and root-rot disease complex of chickpea. Mycol. Phytopathol., **40**, 246–254.

[15] Gaur, A.C., 1985. Phosphate solubilizing microorganisms and their role in plant growth and crop yield. Proceed. Soil Biol. Symp. Hissar, India, pp. 125–138.

[16] Khan, M.R., Khan, S.M., 2001. Biomanagement of Fusarium wilt of tomato by the soil application of certain phosphate solubilizing microorganisms. Int. J. Pest Manag., **47**, 227–231.

[17] Siddiqui, Z.A., Akhtar, M.S., 2007. Biocontrol of a chickpea root-rot disease complex. J. Plant Pathol., **89**, 67–77.

[18] Mittal, V., Singh, O., Nayyar, H., Kaur, J., Tewari R., 2008. Stimulatory effect of phosphate solubilizing fungal strains (*Aspergillus awamori* and *Penicillium citrinum*) on the yield of chickpea (*Cicer arietinum* L. cv. GPF2). Soil Biol. Biochem., **40**, 718–727.

[19] Nair, M.G., Burke, B.A., 1988. A new fatty acid, methyl ester and other biologically active compounds from *Aspergillus niger*. Phytochem., **27**, 3169–3373.

[20] Wilson, M., Backman, P.A., 1999. Biological control of plant pathogens. In: Ruberson, J.R. (ed.), Handbook of Pest Management, Marcel Dekker, Inc., New York, pp. 309–335.

[21] Raupach, G.S., Kloepper, J.W., 1998. Mixture of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology, **88**, 1158–1164.

[22] Willekens, A., Inze, D., Montago, M.V., van Champ, W., 1995. Catalases in plants. Mol. Breed., **1**, 207–228.

[23] Espelie, K.E., Franceschi, V.R., Kolattukudy, P.E., 1986. Immounocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing of potato tuber tissue. Plant Physiol., **81**, 487–492.

[24] Zacheo, G.C., Orlando, C., Bleve-Zacheo, T., 1993. Characterization of anionic peroxidase in tomato isolines infected by *Meloidogyne incognita*. J. Nematol., **25**, 249–256.

[25] Schenck, N.C., Pervez, Y., 1990. A Manual of Vesicular Arbuscular Mycorrhizal Fungi, INVAM (3rd Eds.), University of Florida, Florida, p. 286.

[26] Porter, W.M., 1979. The "most probable number" method for enumerating infective propagules of vesicular-arbuscular mycorrhizal fungi in soil. Aust. J. Soil Res., **17**, 515–519.

[27] Chance, B., Maehly, A.C., 1955. Asssay of catalase and peroxidase. In: Calowick, S.P., Kaplan, N.O. (eds.), Methods in Enzymology II'. Academic Press New York p. 987.

[28] Southey, J.F., 1986. Laboratory Method for Work with Plant and Soil Nematodes. Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationary Office, London.

[29] Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol., **84**, 498–500.

[30] Domsch, K.H., Gams, W., Anderson, T.H., 1980. Compendium of Soil Fungi. Academic Press, London, UK.

[31] Siddiqui, I.A., Ali, N.I., Zaki, M.J., Shaukat, S.S., 2001. Evaluation of *Aspergillus* species for the biocontrol of *Meloidogyne javanica* in mungbean. Nematol. Medit., **29**, 115–121.

[32] Eapen, S.J., Beena, B., Ramana, K.V., 2005. Tropical soil microflora of spice-based cropping systems as potential antagonists of root knot nematodes. J. Invert. Pathol., **88**, 218–225.

[33] Bagyaraj, D.J., Manjunath, A., Reddy, D.D.R., 1979. Interaction of vesicular arbuscular mycorrhizas with root knot nematodes in tomato. Plant Soil, **51**, 397–403.

[34] Elsen, A., Gervacio, D., Swennen, R., De Waele, D., 2008. AMF-induced biocontrol against plant parasitic nematodes in Musa sp.: A systemic effect. Mycorrhiza, **18**, 251–256.

[35] Li, H.Y., Yang, G.D., Shu, H.R., Yang, Y.T., et al., 2006. Colonization by the arbuscular mycorrhizal fungus *Glomus versiforme* induces a defense response against the root-knot nematode *Meloidogyne incognita* in the grapevine (*Vitis amurensis* Rupr.), which includes transcriptional activation of the class III chitinase gene VCH3. Plant Cell Physiol., **47**, 154–163.

[36] Ravichanda, N.G., 2008. Interaction of nematodes with other microorganisms. In: Ravichanda N.G. (ed.), Plant Nematology, I. K. International Publishing House Pvt. Ltd., New Delhi, India, p. 272.

[37] Weller, D.M., Thomashow, L.S., 1993. Microbial metabolites with biological activity against plant pathogens. In: Lumsden, R.D. Vaughn, J.L. (eds.), Pest Management: Biologically Based Strategies, American Chemical Society, Washington, DC., pp. 172–180.

[38] Siddiqui, Z.A., Mahmood, I., 1999. Role of bacteria in the management of plant parasitic nematodes. A review. Biores. Technol., **69**, 167–179.

[39] Ramamoorthy, V., Viswanathan, R., Raguchander, T., Prakasam, V., Samiyappan, R., 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Protec., **20**, 1–11.

[40] Kokalis-Burelle, N., Varvina, C.S., Rosskopf, E.N., Shelby, R. A., 2002. Field evaluation of plant growth-promoting rhizbacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. Plant Soil, **238**, 257–266.

[41] Benhamou, N., Kloepper, J.W., Quadt-Hallman, A., Tuzun, S., 1996. Induction of defence-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. Plant Physiol., **112**, 919–929.

[42] Jetiyanon, K., Tuzun, S., Kloepper, J.W., 1997. Lignification, peroxidase and superoxide dismutases as early plant defense reactions associated with PGPR-mediated induced systemic resistance. In: Ogoshi, A., Kobayashi, K., Homma, Y., Kodama, F., Kondo, N., Akino, S. (eds.), Plant Growth-Promoting Rhizobacteria - Present Status and Future Prospects, Nakanishi printing, Sapporo, pp. 265–268.

[43] Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E., Dinoor, A., 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology. **92**, 976–985.

[44] Siddiqui, Z.A., Akhtar, M.S., 2008. Synergistic effects of antagonistic fungi and a plant growth promoting rhizobacterium, an arbuscular mycorrhizal fungus, or composted cow manure on the populations of *Meloidogyne incognita* and growth of tomato. Biocont. Sci. Technol., **18**, 279–290.

[45] Barea, J.M., Andrade, G., Bianciotto, V., Dowling, D., et al., 1998. Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for the biocontrol of soil-borne plant fungal pathogens. Appl. Environ. Microbiol., **64**, 2304–2307.

[46] Blilou, I., Bueno, P., Ocampo, J.A., Gracia-Garrido, J.M., 2000. Induction of catalase and peroxidase activities in tobacco roots inoculated with the arbuscular mycorrhizal *Glomus mosseae*. Mycol. Res., **104**, 722–725.

[47] Siddiqui, Z.A., Mahmood, I., 1994. Effect of *Heterodera cajani* on growth, chlorophyll content and activity of some enzymes in pigeonpea. Nematropica, **24**, 103–111.

