LABORATORY AND FIELD PERFORMANCE OF SOME SOIL BACTERIA USED AS SEED TREATMENTS ON *MELOIDOGYNE INCOGNITA* IN CHICKPEA

M.R. Khan*, M.M. Khan, M.A. Anwer and Z. Haque

Department of Plant Protection, Aligarh Muslim University, 202002, India

Received: 26 May 2012; Accepted: 27 September 2012.

Summary. Experiments were conducted under *in vitro* and field conditions to assess the efficacy of the soil bacteria *Bacillus subtilis*, *Pseudomonas fluorescens*, *P. stutzeri* and *Paenibacillus polymyxa* for controlling the root knot nematode, *Meloidogyne incognita*, in chickpea, *Cicer arietinum*, in India. The bacterial strains tested solubilized phosphorous under *in vitro* and soil conditions and produced indole acetic acid, ammonia and hydrogen cyanide *in vitro*. Both pure culture and culture filtrates of the bacteria reduced egg hatching and increased juvenile mortality of the nematode. Under field conditions, seed treatment (at 5 ml/kg seed) with cultures containing 10^{12} colony forming units/ml of *P. fluorescens* and *P. stutzeri*significantly increased yield and root nodulation of chickpea. Inoculation with 2000 juveniles of *M. incognita*/spot (plant) caused severe root galling and decreased the yield of chickpea by 24%. Treatment with *P. fluorescens* suppressed gall formation, and treatment with *P. fluorescens*or *B. subtilis* suppressed reproduction and soil populations of *M. incognita*. However, the suppressive effects of the two bacteria on the nematode were less than that of fenamiphos. In nematodes infested plots, only treatments with *P. fluorescens* increased the yield (14%) compared to fenamiphos, being 31% above the untreated nematode control. *Paenibacillus polymyxa* and *P. stutzeri* increased the yield over that of the control and were on par with fenamiphos treatment. Rhizosphere populations of all bacteria increased over the course of experiment irrespective of the nematode treatment. Seed dressing with *P. fluorescens* appears to be a handy and cost effective treatment to control the nematode and increase yield of chickpea.

Key words: Biological control, *Cicerarietinum*, root-knot nematodes, plant growth promoting rhizobacteria.

Certain soil bacteria when applied as seed or root inoculants improve plant growth and productivity. Such bacteria are commonly known as plant-growth-promoting rhizobacteria (PGPR). The PGPR commonly occurring in India are *Azotobacter chroococcum* Beijerinck, *Beijerinkia indicum* Starkey *et* De, *Paenibacillus polymyxa* (Prazmowski) Mace, *Pseudomonas fluorescens* (Threvesan) Migula, *P. striata* Chester and *P. stutzeri* (Lehmann *et* Neumann) Sijderius (Gaur, 1990). Some strains of these bacteria have also been found to reduce the crop damage caused by nematodes (Siddiqui *et al*., 2001; Oyekanmi *et al*., 2008; Zeinat *et al*., 2010; Anwarul-Haq, 2011). The reduced crop damage due to PGPR application may result through different mechanisms. The bacterium may increase the availability of phosphorus, which may enhance crop tolerance to nematode parasitism by inducing systemic resistance (Leeman*et al*., 1995; Khan, 2007). In addition, phosphorus nutrition is important in plant defence against nematodes (Kirkpatrick *et al*., 1964). Strains of *B. sublitis* produce bulbiformin (Brannen, 1995), whereas strains of *P. fluorescens* may produce hydrogen cyanide, ammonia, phenazine, pyoleutorin and/or pyrrolnintrin (Whistler *et al*., 2000; Schoonbeck *et al*., 2002). These metabolites/toxins may adversely affect egg hatching and nematode parasitism (Siddiqui and Ehtshamul Haque, 2001). Production of phytohormones, such as auxin derivatives and gibberellin-like substances by PGPR (Arshad and Frankenberger Jr., 1991) may modify the plant's own pool of growth regulators, which subsequently stimulates plant growth (Glick, 1995) and may also suppress soil-borne pathogens (Khan *et al*., 2009). It has also been found that PGPR, when present in the rhizosphere, may stimulate host plant defence against pathogens, including nematodes (Kloepper *et al*., 1992). In addition, a change in the microbial community in the rhizosphere may influence nematode movement and host recognition. It has been reported that soil application or seed treatment with *B. subtilis*, *Pseudomonas stutzeri*, *P. aeruginosa*and *P. fluorescens* suppressed the severity of root galling on tomato caused by *Meloidogyne incognita* (Kofoid *et* White) Chitw. or *M. javanica* (Treub) Chitw. (Rao, 1990).

Chickpea, *Cicer arietinum* L., is an important pulse crop in India and an excellent source of high quality protein. Unfortunately, root-knot nematodes are among the most severe pathogens of chickpea that cause significant yield loss to the crop. Most of the previous investigations describing the effects of PGPR on root-knot nematode diseases have been conducted in pots (Siddiqui and Ehteshamu-Haque, 2001). The aim of this study was to examine the effects of *B. subtilis* Cohn *emend*. Prazmowski, *P. polymyxa*, *P. fluorescens* and *P. stutzeri* (Lehmann *et* Neumann) Sijderius on: *i*) phosphate solubilization and indole acetic acid (IAA), ammonia and hydrogen cyanide (HCN) production; *ii*) hatching and mortality of *M. incognita in vitro* by culture filtrates; *iii*) control of *M. incognita* in chickpea, under field conditions, through seed application.

[1] Corresponding author, e-mail: mrkhan777in@yahoo.co.in

MATERIALS AND METHODS

Assay for phosphate solubilization and indole acetic acid (IAA), ammonia and hydrogen cyanide (HCN) production

The amounts of phosphorus solubilized and indole acetic acid produced by the applied bacteria were assayed in culture broths. The phosphate solubilization in culture broth was determined by the colorimetric method (King, 1932) modified by Jackson (1967). The Pikovskaya broth (99.5 ml) was inoculated with 0.5 ml broth of the test organism and incubated at 30 °C for 2-4 days. After incubation, the final volume was made up to 100 ml with distilled water and the suspension was centrifuged at 15,000 rpm (28,117 g) for 30 minutes. Two ml of the suspension were transferred to a 50-ml flask to which 10 ml of chloromolybdic acid were added and mixed thoroughly. Thereafter, 5 drops of chlorostannous acid were added and the final volume was made up to 50 ml with distilled water. The solution produced a blue colour and the absorbance was read at 600 nm in a spectrophotometer (UV-2450, Shimatzu, Japan). The available phosphorus content of soil was also estimated in the bacteria-treated and untreated microplots using Olsen's method (Olsen *et al.,* 1954).

The production of indole acetic acid was tested by the colorimetric method of Gorden and Weber (1951) as modified by Brik *et al.* (1991). The Luria broth (10 ml) amended with tryptophan (50 mg/100 ml) was inoculated with a loopful of the test organism and incubated at 30 °C for 24 h. The broth was centrifuged at 10,000 rpm (18,785 g) for 15 minutes and 2 ml were transferred to a test tube to which 2-3 drops of orthophosphoric acid and 4 ml of FeCl-$HClO_4$ were added. After 25 minutes, absorbance was read at 530 nm in a UV-spectrophotometer. Concentrations of IAA were determined by reference to a calibration curve prepared using IAA as a standard (10-100 µg/ml in 50% ethanol).

The production of ammonia (Dye, 1962) and hydrogen cyanide (Bakker and Schippers, 1987) by the bacteria in culture broth was tested but no quantification was performed. Change in colour (to yellow) of the culture of the microorganisms in peptone broth upon addition of Nesler's reagent recorded ammonification. Similarly, change in colour of filter paper soaked in alkaline picric acid solution from yellow to orange brown was a positive record for HCN production and this was visually graded on the basis of darkness of the changed brown colour.

Effects of culture filtrates on hatching and mortality of the nematode *in vitro*

Overnight cultures of *P. fluorescens* and *P. stutzeri* in Kings-B broth and *B. subtilis* and *P. polymyxa* in nutrient broth were centrifuged twice at 8,000 rpm (15,028 g) for 20 minutes. The supernatants were collected in beakers. To determine the effect on hatching of eggs, 5 ml of filtrate from pure culture or 5 ml of bacteria culture broth (× $10^{12\text{-}14}$ cfu/ml), separately, were transferred to glass cavity blocks to which ten egg masses of uniform size and surface sterilized (0.5% NaOCl for 2-3 min) were added (Khan *et al.*, 2005b). Cavity blocks containing ten egg masses in distilled water and broth (uninoculated) served as controls. The cavity blocks were placed inside Petri dishes containing distilled water, covered with lids and incubated at 25-27 °C for 7 days. Three cavity blocks were maintained for each treatment. After incubation, nematode second stage juveniles present in the suspension were counted.

To examine the effect of the filtrates on the mortality of the nematode juveniles, 1 ml of pure cultures or culture filtrates were placed in separate glass cavity blocks to which 1 ml aliquots of sterile distilled water suspension containing 40-50 freshly hatched and surface sterilized juveniles of *M. incognita* were added. Juveniles kept in the broth (without the bacterium) or in distilled water served as controls. Each treatment was replicated three times. The blocks were placed in Petri dishes containing distilled water, covered with lids and incubated at 25-27 °C for 2 days. After incubation, the numbers of dead (non-motile) juveniles were counted. The juveniles were transferred to 2 ml fresh water in another cavity block and the mortality (non-motility) was observed again after 24 hr.

Field experiment

Inoculum of root-knot nematode and soil bacteria. A culture of *M. incognita* was prepared from egg masses collected from eggplants. The egg masses were incubated in Baermann funnels at 25 °C for 6-8 days (Southey, 1986). One litre of nematode suspension containing 2000 J_2 was distributed in a small volume of soil (150 mm × 150 mm × 150 mm) in a field, by digging and mixing the soil with a shovel, at 36 positions within each plot (12 spots/row, 3 rows/plot). This population level was chosen because it was found to cause yield loss to chickpea under field conditions (Ali *et al.*, 2010). The inoculation was made (first week of October) one day before sowing seeds of chickpea cv. BG 256 (2-3 seeds/spot). Half of the total plots were inoculated with the nematode and the other half not, so that seeds treated with each bacterium were tested in nematode-inoculated and non-inoculated soil (Table I).Sowing was done in a line passing through the middle of the soil area inoculated with the nematode.

Pure cultures of *P. polymyxa* (MTCC-122)and *P. stutzeri* (MTCC-863) were obtained from the Institute of Microbial Technology, Chandigarh, India, whereas the strains of *B. subtilis* (BS01) and *P. fluorescens* (MB07) were isolated from crop fields (Khan *et al.*, 2005a). The cultures were prepared in nutrient broth and Kings-B supplemented with 75 mg cycloheximide and penicillin (*B. subtilis* and *P. polymyxa*) and Kings-Bsupplemented with 45 mg novobiocin/litre for *P. fluorescens* and *P. stutzeri* (Sands and Rovira, 1970).

The minimum tolerance concentration (MTC) of the bacterial strains to different antibiotics was determined to characterize the strains (Bauer *et al.*, 1966). The 475

and 600 µg tetracycline/20 ml medium were found as the MTC for *P. fluorescens* and *P. stutzeri*, respectively. This MTC of tetracycline was also added in Kings-B throughout the study to make the medium specific for the strain of *P. fluorescens* and *P. stutzeri* used. The MTC could not be determined for *B. subtilis*and *P. polymyxa* as the strains were found sensitive to all antibiotic concentrations tested.

Field plots. An area (24 m × 12 m) of a field consisting of loamy soil at the Agricultural Faculty Farm, Aligarh, was prepared by incorporation of about 500 kg of farmyard manure. The site was divided into 36 plots (3 m × 1.5 m) with 250 mm high bounding (margins) and spaced 50 cm apart to avoid flooding over of water between the plots during irrigation. To determine the population of the indigenous nematodes occurring in the field soil, 25 cores of soil were collected to a depth of 10-20 cm from the site in a stratified manner (Khan, 2008). The cores of soil were mixed together to make a composite sample, from which 1 kg of soil was processed using Cobb's sieving and decanting method followed by the Baermann funnel technique (Southey, 1986). The nematode suspension collected from the funnel was observed under a stereomicroscope in a counting dish to determine the nematode population. There were twelve treatments, as listed in Table I. A treatment with the nematicide fenamiphos at the rate of 2 g active ingredient/kg seed was also included for comparison. Each treatment was replicated in three plots according to a randomized block design. The experiment was repeated the following year in a nearby field under similar agro-climatic conditions within the Faculty Farm. Major climatic factors at the site were 0-0.6 mm and 0-2.8 mm rainfall, 41-93% and 27-93% relative humidity, and 4.0-37.0 °C and 6.5-35 °C air temperature during November, 2007 to March, 2008 and November, 2008 to March, 2009, respectively.

Treatments and plant culture. Overnight cultures of the bacteria (× $10^{12\text{-}14}$cfu/ml) were applied on seeds at 5 ml/kg seeds along with the commercial rhizobium (*Mesorhizobium ciceri* chickpea strain). The rhizobium was obtained from the Division of Microbiology, I.A.R.I., New Delhi. Plots sown with seeds treated with rhizobium and no soil bacteria served as controls. Rhizobium treatment was given as a requirement of the crop, not for any other objective, hence the indigenous population of rhizobial bacteria was not assessed. The treated seeds (two-three) were sown at each of the 36 spots in each plot. Two weeks after sowing, the germinated seeds were thinned to one seedling at each spot and the plots were irrigated. Four months after sowing, ten plants from each plot were randomly selected for harvest, and galls, egg masses/root system, fecundity (eggs/egg mass), dry matter production and yield (weight of grain/plant) were determined. The root nodulation was determined by recording the numbers of functional (pink and intact), non-functional (brown and degenerated) and total nodules/root system on a different group of ten two-months-old plants, which were uprooted carefully from a plot to achieve maximum root recovery along with nodules. To determine nodule infection by *M. incognita*, all nodules on the root system were immersed in phloxine B solution (0.15 g/l water) for 20 minutes, which stained in a deep pink colour the gelatinous material secreted by the females. The nodules were examined under a stereomicroscope for galls and/or egg masses.

Gall formation, egg mass production and fecundity of the nematode. The severity of infection and reproduction of *M. incognita* were determined by counting the numbers of galls and egg masses/root system. Galls were counted on the basis of visual observation. To estimate egg masses, the roots were treated with phloxine B solution (0.159 g/l) for 20 min, which stained the egg masses (Hartman, 1983). Fecundity (number of eggs/egg mass) was determined by excising around twenty egg masses carefully from each washed root system. The egg masses from each group of 10 plants per plot (total 100 egg masses) were blended for 4-5 min in an electric blender containing 100 ml NaOCl solution (1%) (Khan, 2008). The egg suspension was rinsed with water on a 500 mesh sieve, collected in a beaker and made up to a standard volume. Eggs in a 1 ml aliquot were counted three times and fecundity calculated.

Soil populations of nematodes and bacteria. A composite sample of soil was collected from the root zone (at 10-15 cm depth and within 15-20 cm of the main root) of ten plants at 0 (planting), 1, 2, 3 and 4 months (harvest) age, from each plot inoculated with the nematode. The nematode juveniles were extracted from 1 kg soil according to Southey (1986). Root zone populations of the applied soil bacteria from the treated, untreated, nematode-inoculated and non-inoculated plots were determined at 1 week and 1, 2, 3, and 4 months age using the dilution plate method. Ten g of soil from the composite sample were mixed and shaken in 100 ml of double distilled water for 5 minutes. One ml of the suspension was transferred to a test tube containing 9 ml of sterile double distilled water (SDDW). This procedure was repeated until a dilution of 10^{-8} was achieved. Petri plates containing solidified Kings-B (supplemented with 475 or 600 µg tetracycline/20 ml for *P. fluorescens* and *P. stutzeri*) or nutrient agar were inoculated with 0.25 ml of suspension of the 10^{-8} dilution. Three plates were maintained for each treatment. After incubation at 35±2 °C for 2 days, the plates were examined under a colony counter. To distinguish the applied strains of *B. subtilis* and *P. polymyxa*, ten colonies were randomly picked and inoculated in nutrient broth in culture tubes. The overnight culture was spread on solidified nutrient agar. Antibiotic discs of amoxicillin (30 µg), cloxacillin (30 µg), chloramphenicol

Table I. Treatments used in the study.

Bacterium applied	Nematode inoculation (2,000 *Meloidogyne incognita* J_2/kg soil)
Control (no bacteria)	No
Control (no bacteria)	Yes
Bacillus subtilis	No
Bacillus subtilis	Yes
Paenibacillus polymyxa	No
Paenibacillus polymyxa	Yes
Pseudomonas fluorescens	No
Pseudomonas fluorescens	Yes
Pseudomonas stutzeri	No
Pseudomonas stutzeri	Yes

col (30 µg), co-trimoxazole (25.75 µg), doxycillin hydrochloride (30 µg), flucanazole (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), methecillin (30 µg), novobiocin (30 µg), penicillin (30 µg) and tetracycline (30 µg) were placed on the medium, and the plates were incubated at 35±2 °C for 48 h. Inhibition zones produced by the above antibiotic discs in the plates were measured. The measurements were used to compare with the measurement of inhibition zones caused by the applied strains of *B. subtilis* and *P. polymyxa*. This technique is not very accurate for the characterization of bacilli, but because of the highly sensitive responses of the micro-organisms to the antibiotics tested, the technique gives some indication about the applied strains (Khan *et al*., 2005a). The native soil populations or background populations of morphologically similar bacteria was also determined in all plots before the experiment,and these were 1.2 - 4.9 × 10^2, 0.76 - 4.1 × 10^2, 0.19 - 1.1 × 10^3 and 1.3-3.5 × 10^2 cfus/g soil of *B. subtilis*, *P. polymyxa*, *P. fluorescens* and *P. stutzeri*, respectively.

Statistical analysis. Observations on plant growth variables, galls, egg masses and root nodules were made from ten plants per plot and these were averaged to represent one replicate. The data for the two years were analysed separately. The data on biomass, yield and nodulation were subjected to a two factor analysis of variance (seed treatment × nematode inoculation). Galls, egg masses, fecundity and soil population were analyzed by one factor analysis of variance and least significant differences (L.S.D.) were calculated at $P \leq 0.05$. The data of the *in vitro* observations on IAA, HCN, NH3, P, etc were also analyzed by single factor ANOVA and Duncan's multiple range test was used to compare mean differences at $P \leq 0.05$. Standard errors were calculated and are presented along with the means in the tables.

RESULTS

Phosphate solubilization and production of IAA, NH_3 and HCN

The bacterial strains used in the study solubilized phosphorus in the culture and in the soil in both years of the study. The greatest solubilization in culture broth was induced by *P. fluorescens* MB-03 ($P \leq 0.05$) followed by *P. stutzeri* MTCC-863, and the least by *B. subtilis* BS-01. Solubilized phosphorus contents of the soil were in-

Table II. Production of hydrogen cyanide (HCN), ammonia (NH3), indol acetic acid (IAA) in culture broth and solubilization of phosphorus in culture broth and in the soil.

Treatment	Culture broth							Phosphorus in the soil (kg/ha)			
	HCN		NH_3		µg IAA/ml	Phosphorus (µg /ml)		Uninoculated*		Inoculated*	
	Iyr[1]	IIyr	Iyr	IIyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr
Control	-	-	-	-	-	-	-	18.1[a]	17.7[a]	16.9[a]	17.2[a]
								±2.3	±2.0	± 1.9	±2.0
Bacillus subtilis	-	-	++	++	17.3[d]	5.8[b]	5.6[b]	20.5[b]	20.3[b]	19.5[a]	19.9[b]
					±1.9	±0.5	±0.4	±2.0	±1.8	±1.3	±1.8
Paenibacillus polymyxa	-	-	++	++	14.9[a]	4.9[a]	5.1[a]	19.4[a]	19.8[a]	18.4[a]	18.9[a]
					±2.1	±0.4	±0.3	±2.2	±1.7	±0.6	±2.1
Pseudomonas fluorescens	+++	+++	+++	+++	25.2[b]	6.4[c]	6.2[c]	26.8[c]	27.9[b]	27.2[a]	28.5[b]
					±3.1	±0.3	±0.5	±3.6	±3.5	±2.8	±2.7
Pseudomonas stutzeri	+	+	++	++	20.2[c]	5.5[b]	5.8[b]	19.2[a]	19.1[a]	18.4[a]	18.7[a]
					±2.7	±0.6	±0.6	±2.2	±2.5	±2.3	±2.1
LSD					1.52	0.71	0.6	1.51	1.48	1.58	1.61

Each value is mean of three replicates. + production, - no production; + less, ++ medium, +++ high hydrogen cyanide or ammonia

Production; yr year. Values followed by different letters in a column are significantly different at $P \leq 0.05$.

* Inoculated with 2000 $J_2$of *Meloidogyne incognita* or not inoculated. ± Standard error

[1]Iyr = First year; IIyr = Second year.

Table III. *In vitro* effects of pure culture and culture filtrate of some soil bacteria on hatching and mortality of *Meloidogyne incognita*.

Treatment	Number of juveniles hatched / egg mass								Percentage of dead juveniles							
	Distilled water		Broth		Cultured broth		Culture filtrate		Distilled water		Broth		Cultured broth		Culture filtrate	
	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr
Bacillus subtilis	314	319	308	309	230*	227*	254*	252*	00	00	2.6	2.5	24.2*	24.1*	20.6*	20.3*
	±29	±27	±24	±31	±29	±30	±27	±31			±16	±21	±3.1	±2.9	±5.1	±2.9
Paenibacillus polymyxa	314	319	308	309	237*	238*	245*	247*	00	00	2.6	2.5	25.9*	26.3*	21.5*	21.6*
	±32	±24	±25	±27	±31	±31	±31	±35			±21	±17	±5.0	±3.9	±4.4	±3.1
Pseudomonas fluorescens	314	319	304	304	143*	141*	169*	174*	00	00	3	3	55.7*	57.8*	44.1*	42.2*
	±27	±31	±27	±30	±19	±20	±19	±21			±18	±20	±4.3	±7.3	±7.9	±5.4
Pseudomonas stutzeri	314	319	304	304	271*	269*	260*	272*	00	00	3	3	18.3*	18.2*	16.3*	16.5*
	±25	±28	±29	±31	±29	±27	±21	±27			±18	±20	±308	±2.2	±2.0	±2.0

Each value is the mean of three replicates; yr year.*Significantly different from the control (broth) at P< 0.05 ± Standard error.

creased by 61-66% and 50-61% in plots treated with *P. fluorescens*, and inoculated or not inoculated with the nematode, compared to the control in the two years, respectively (Table II). Treatment with the rest of the soil bacteria also resulted in a significant increase ($P \leq 0.05$) in solubilized phosphorus content of the soil but it was significantly less than that of the *P. fluorescens* treatment. The soil bacteria also solubilized phosphorus *in vitro* and the extent was significantly greater by *P. fluorescens* ($P \leq 0.05$) than by the other bacteria. All four strains of the bacteria tested produced IAA in culture broth; the amount, however, varied significantly among the strains ($P \leq 0.05$) and the order was *P. fluorescens*> *P. stutzeri*> *B. subtilis*> *P. polymyxa* (Table I). The applied bacteria were also found positive for the production of NH_3, with *P. fluorescens* the most productive (Table I). The tested strain of *P. fluorescens* was scored +++ for HCN production (Table I), while the rest of the bacteria, except for *P. stutzeri*, produced no HCN (Table II).

Nematode hatching and mortality

The hatching of eggs in egg masses incubated in broth alone (without bacteria) was almost identical to that in distilled water (Table III). However, hatching was suppressed in pure culture and culture filtrates of the bacteria. The hatching decreased ($P \leq 0.05$) by 54 and 43% in the pure culture broth and culture filtrate of *P. fluorescens* compared with the control (broth alone). Treatments with *B. subtilis* (18-26%), *P. polymyxa* (20-23%) and *P. stutzeri* (10-12%) also suppressed egg hatching ($P \leq 0.05$). Bacterial treatments killed the juveniles of *M. incognita* (Table III), with mortality of 58% in pure culture of *P. fluorescens* compared to the broth alone ($P \leq 0.001$). The other bacterial treatments induced 17-26% juvenile mortality ($P \leq 0.05$). The effect of *P. stutzeri* was also significant ($P \leq 0.05$). The culture filtrates of the bacteria caused mortality 13-16% less than those of the pure cultures.

Field experiment

Galling and reproduction of root-knot nematode. The mean soil population of indigenous nematodes in the field plot was 71±28 specimens/kg soil, which included specimens of the genera *Meloidogyne*, *Hoplolaimus*, *Tylenchorhynchus*, *Rotylenchus*, *Hirschmanniella*, *Ditylenchus*, *Trichodorus*, free living nematodes (fast moving), etc. These nematodes were not considered important as the individual populations were too low to cause damage. However, in control plots, occasionally 3-7 galls were recorded on a few plants, which was an average of less than one gall or egg mass/root system. Hence, data on control plots have not been presented. In nematode infested plots, numerous galls and egg masses were recorded on the roots of chickpea. Treatments with *P. fluorescens* suppressed gall formation by 14.8% ($P \leq 0.05$, Table IV), while fenamiphos reduced galls by 29.8% and egg masses by 27.0%/root system over the inoculated control ($P \leq 0.05$). The bacterial

treatments also suppressed egg mass production, especially *P. fluorescens* (20.7%) and *B. subtilis* (12.1%). Fecundity of the nematode (number of eggs/egg mass) was decreased by 31.2 and 13.4% due to the nematicide and *P. fluorescens* treatments, respectively ($P \leq 0.05$, Table IV).

Plant dry weight and yield. Seed treatments with *P. stutzeri* or *P. fluorescens* significantly ($P \leq 0.05$) promoted the dry weight of plants not inoculated with nematodes (Table IV). Yield of chickpea was significantly greater in the plots that received *P. fluorescens* compared to the uninoculated control. The other bacterial treatments did not improve yield. Nematode infection alone significantly suppressed dry plant weight (15.3%) and yield (23.8%) compared to the non-inoculated control. Bacterial treatments, except *B. subtilis*, significantly ($P \leq 0.05$) increased plant dry weight and yield of chickpea in infested plots over the inoculated control (Table IV). In nematode infected plots, the greatest increases in dry plant weight and yield occurred in the treatments with *P. fluorescens* (14.6 and 30.9%), followed by *P. polymyxa* (15.6 and 13.1%), *P. stutzeri* (14.6 and 15.6%) and fenamiphos (12.6 and 16.7%), respectively.

Root nodulation. Seed treatments with *P. fluorescens* and *P. stutzeri* promoted ($P \leq 0.05$) root nodulation by *Mesorhizobium ciceri*, leading to 18.8 and 14.3%, and 12.5 and 9.5% increases in the numbers of functional and total nodules/root system of non-inoculated plants, respectively (Table III). Nematode infection decreased functional (31.2%) and total (14.3%) nodules/root system. Some 5-11% nodules/root system were invaded by the nematode (Table IV). Application of the soil bacteria, however, increased the number of functional nodules/root system by 9-18% on nematode-infected plants; the increase was greatest with *P. fluorescens* in comparison to the nematode inoculated control.

Table IV. Effect of seed treatment with some soil bacteria on the dry plant weight, yeld and modulation of chickpea and galling, egg mass production and fecundity of *Meloidogyne incognita*.

Biocontrol agent	Nematode Inoculation	Dry plant weight (g)		Weight of seed per plant (g)		Number of nodules/root system						Number root system				Fecundity	
						Functional		Non-Functional		Total		Galls		Egg mass		Egg/eggmass	
		Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr	Iyr	IIyr
Control (non-inoculated)	0	33.0 ±2.2	35.4 ±4.3	70.4 ±8.2	69.0 ±5.3	16 ±1.3	16 ±1.0	5 ±0.4	5 ±0.8	21 ±1.8	21 ±1.9						
Bacillus subtilis	0	34.5 ±3.1	36.5 ±4.1	68.7 ±4.2	67.9 ±5.9	16 ±1.5	16 ±1.2	5 ±0.3	5 ±0.7	21 ±1.7	21 ±2.0						
Paenibacillus polymyxa	0	36.2 ±2.9	37.7 ±3.8	74.8 ±5.6	73.6 ±6.7	18[a] ±1.9	17 ±1.7	5 ±0.6	5 ±0.6	23[a] ±2.1	22 ±2.2						
Pseudomonas fluorescens	0	38.8[a] ±4.1	38.0[a] ±4.2	77.6[a] ±8.1	77.0[a] ±8.1	18[a] ±1.4	19[a] ±1.2	5 ±0.3	5 ±0.9	23[a] ±2.0	24[a] ±2.2						
Pseudomonas stutzeri	0	37.3 ±3.9	38.3[a] ±3.5	72.6 ±6.4	72.3 ±7.0	18[a] ±1.7	18[a] ±1.4	5 ±0.8	5 ±0.4	23[a] ±1.9	23[a] ±2.1						
Fenaniphos	0	35.3 ±2.2	34.5 ±3.9	70.1 ±7.3	70.9 ±6.3	15 ±1.6	15 ±1.7	5 ±0.3	6[a] ±0.5	20 ±2.3	21 ±1.9						
Control (inoculated)	2000	29.2 ±2.5	29.6[a] ±3.1	52.1[a] ±6.2	53.3[a] ±6.1	11[a] ±1.4	11[a] ±1.6	7[a] ±0.8	7[a] ±0.7	18[a](2) ±1.5	18 ±1.6	76 ±6.5	70 ±7.3	67 ±5.5	65 ±7.0	259 ±20.7	247 ±30.1
Bacillus subtilis	2000	30.9[a] ±3.2	30.1 ±2.5	53.1 ±5.9	53.9 ±5.5	12 ±0.9	12 ±1.5	6[a] ±0.7	6[a] ±0.5	18(2) ±1.6	18(2) ±1.6	69 ±7.2	67 ±7.0	59[a] ±5.1	57[a] ±5.1	238 ±21.1	234 ±29.0
Paenibacillus polymyxa	2000	33.5[a] ±2.9	32.5[a] ±3.7	59.9[a] ±6.3	59.3[a] ±7.4	12 ±1.2	12 ±0.9	6[a] ±0.4	6[a] ±0.4	18(2) ±1.5	18(2) ±1.7	70 ±6.5	72 ±6.8	65 ±6.9	69 ±6.2	245 ±19.6	237 ±27.5
Pseudomonas fluorescens	2000	35.7[a] ±3.6	34.4[a] ±3.2	69.4[a] ±7.0	68.8[a] ±7.0	14[a] ±1.5	13[a] ±1.0	6[a] ±0.3	6[a] ±0.5	19(1) ±1.6	19(1) ±1.8	55[a] ±48	61[a] ±6.0	44 ±4.0	48[a] ±3.8	215[a] ±17.9	224 ±29.6
Pseudomonas stutzeri	2000	33.2[a] ±3.5	34.1[a] ±3.5	60.4 ±6.9	61.4[a] ±5.4	12 ±0.8	12 ±1.1	6[a] ±0.4	6[a] ±0.6	18(2) ±1.7	18(2) ±1.5	66 ±5.1	72 ±7.0	69 ±5.7	63 ±7.1	248 ±20.7	244 ±20.0
Fenaniphos	2000	33.5[a] ±2.9	32.7[a] ±2.9	61.1 ±5.2	61.9[a] ±5.9	12 ±1.0	12 ±1.0	6[a] ±0.4	7[a] ±0.5	18(2) ±1.6	19(1) ±1.9	46[a] ±3.8	50[a] ±4.8	43[a] ±3.9	41[a] ±5.0	170[a] ±15.0	178[a] ±15.5
LSD ($P \leq 0.05$)		2.3	2.5	6.9	6.5	1.9	1.7	0.3	0.3	1.8	2	6.5	6.9	4.8	5	32.5	32
F-values:																	
Control agent (df=5)		42.2[b]	46.0[b]	23.0[b]	23.4[b]	9.5[b]	9.0[b]	NS[c]	NS[c]	6.0[b]	6.6[b]	26[b]	34[b]	79[b]	78[b]	22.1	22.5[b]
Nematode (df=1)		113.0[b]	110.6[b]	28.3[b]	28.9[b]	8.4[b]	8.4[b]	NS[c]	NS[c]	NS[c]	NS[c]						

Each value is mean of 3 replicates;

[a] Significantly different with in a column from the respective controls at $P \leq 0.05$ (Treatments not inoculated with the nematode are compared with non-inoculated control, where as inoculated-treatments were compared with inoculated control); Values in parenthesis are number of nodules showing infection (galls) by M. incognita;

[b] Significant at $P \leq 0.05$

[c] NS Not significant at $P \leq 0.05$ ± Standard error; yr year

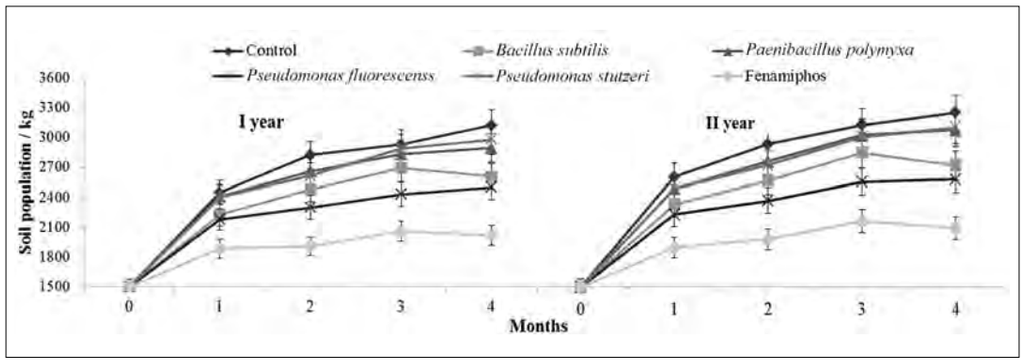

Fig. 1. Effects of seed treatment with some soil bacteria on the soil population of *Meloidogyne incognita.* Vertical bars indicate standard deviation from the mean.

Soil population of the nematode and bacteria. The background soil population of *Meloidogyne* juveniles at the site was 33-59 juveniles (J_2)/kg soil. In the plots inoculated with *M. incognita*, soil populations of the nematode increased gradually and significantly ($P \leq 0.05$) until harvest and were greatest at harvest irrespective of the bacteria applied (Fig. 1). The nematode populations at 2 months, in comparison to the respective control, decreased where applications were made of fenamiphos (28-34%), *P. fluorescens* (12-23%) and *B. subtilis* (7-20%) ($P \leq 0.05$).

The native population of the bacteria varied from 0.4-1.3 × 10^3cfu/g soil. Root zone populations of the applied bacteria were greatly increased over time irrespective of nematode treatment (Fig. 2). At 2 months, the greatest increase was recorded for *P. polymyxa* (734-761%), followed by *P. fluorescens* (600-618%), *B. subtilis* (558-578%) and *P. stutzeri* (473-487%) over their respective initial populations (one week after sowing). At three and four months, the populations were less than the 2 months population and the decrease was much greater at 4 months. The harvest populations of *P. fluorescens, P. stutzeri, P. polymyxa* and *B. subtilis*, in comparison to the respective 2-month populations, decreased by 67-78%, 63-66%, 82-84% and 78-81%, respectively ($P \leq 0.0$); however, these populations were 109-117%, 99-109%, 50-56% and 38-40%, respectively, greater than the populations of the respective bacteria at planting.

DISCUSSION

In vitro, pure culture and culture filtrates of *P. fluorescens* suppressed hatching and killed almost half of the nematode juveniles. This antagonism may have occurred through the effects of HCN and NH_3, as the bacterium produced these nematoxic chemicals in culture broth. Ammonia produced during microbial ammonification and nitrification has been found responsible for decline in plant damage and the soil populations of *Tylenchulus semipenetrans* (Mankau and Minteer, 1962) and *Pratylenchus penetrans* (Walker, 1969). Wilt and Smith (1970) reported that *Chromobacterium* sp. produced ammonia and cyanide, which were toxic to nematodes. The effects that we observed on nematode juvenile mortality were stronger in the pure cultures than in the culture filtrates. This indicates that the nematoxic compounds were released by the bacteria; their concentration would have kept increasing in the pure cultures, whereas in culture filtrate the concentration might have ceased to increase as the bacteria were re-

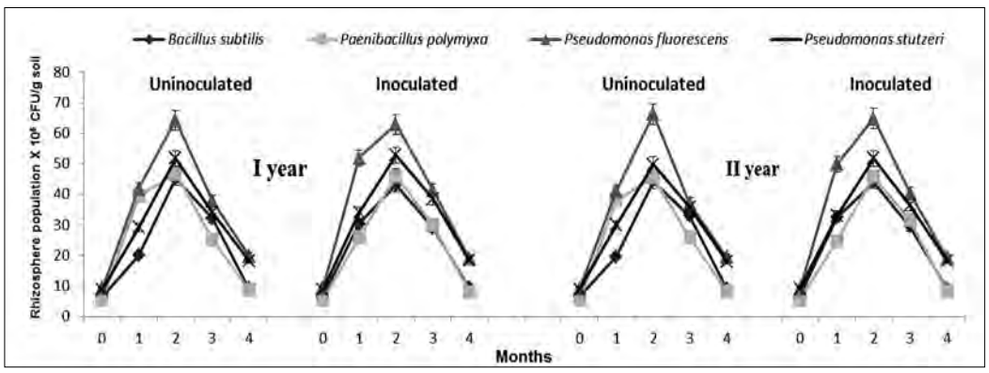

Fig. 2. Populations of some soil bacteria in the rhizosphere of chickpea inoculated or not with *Meloidogyne incognita*. Vertical bars indicate standard deviation from the mean.

moved during filtration. Kapoor and Kar (1989) recorded greater suppression of plant pathogens by pure cultures of a phosphate solubilizing rhizobacteria than their culture filtrates. There is less possibility that the nematoxic compounds were short-lived or rapidly degradable; if this were so, significant mortality in culture filtrates would have not occurred.

Greater amounts of solubilized phosphorus in the soil may also have had an inhibitory effect on the nematode (Kirkpatrick *et al*., 1964); *P. fluorescens* might have induced qualitative and/or quantitative alterations in the root exudates of chickpea that may have affected nematode pathogenesis. Alteration in the root exudates of even susceptible plants results in disorientated movement of the nematode juveniles, leading to their failure to locate the root; consequently, juveniles either starve or develop into males (sex reversal), which are non-pathogenic (Van Gundy, 1985). Some antagonism to *M. incognita* may also have resulted from the altered microbial composition in the soil around roots due to the presence of the bacteria (Rao, 1990). The greater number of functional nodules, as in the *P. fluorescens* treatment, may also have contributed to the inhibition of nematode parasitism (Taha, 1993). Rhizobial nodules convert molecular nitrogen into nitrates and make them available to plant roots. This form of nitrogen is readily utilized by plants, leading to better plant growth and yield (Rao, 1990). Hence, the type of nitrogen and its availability to the plant may greatly influence plant growth and host reaction to nematodes (Huang, 1987).

Seed treatment with *P. fluorescens* and *P. stutzeri* (without nematodes) significantly ($P \leq 0.05$) increased nodulation. Bacteria are known to produce indole-3-acetic acid or other phytohormones that contribute significantly to the growth promotion of plants (Gracia do Salamone *et al.*, 2001). *In vitro*, these two bacteria produced IAA in significantly greater amounts than the other tested bacteria. IAA also plays a major role in the development of rhizobial nodules (Glick, 1995). Infection by root-knot nematodes decreased the number of functional nodules either through inhibition in overall nodulation or premature conversion of functional nodules into non-functional ones due to nematode invasion. Nodule invasion by root knot nematodes may affect histopathology and functioning of the nodule (Taha, 1993). Giant cells and nematode females have been found in the vascular tissue and bacteroidal tissue of cowpea nodules (Taha and Kasseab, 1979). Infected nodules degenerated earlier than uninfected nodules and show reduced nitrogen fixation (Taha and Raski, 1969). *Meloidogyne incognita* at 2000 J_2/plant has been reported to significantly reduce nitrogen activity, leg haemoglobin and bacteroid content of nodules (Chahal and Chahal, 1988). The nematode infection also suppresses the formation of root hairs, leading to availability of fewer sites for infection by root-nodule bacteria and a subsequent decrease in the development of nodules on the roots.

The strain of *P. fluorescens* significantly increased the phosphorus content of the soil and produced more IAA than the other bacteria tested. Phosphorus is a major plant nutrient (Gaur, 1990) whereas IAA is a very important growth promoting hormone (Glick, 1995), and both play vital roles in plant growth and development. *Pseudomonas fluorescens* reduced the impact of the nematode, leading to significantly greater yield of chickpea. Siddiqui and Ehteshamul-Haque (2001) reported a significant decrease in root galling and population of *M. javanica* following application of *P. aeruginosa* in tomato. Similar effects of *B. subtilis, P. stutzeri* and *P. fluorescens* on the root-knot caused by *M. incognita* on tomato have also been recorded (Khan and Tarannum, 1999). The greater effect of *P. fluorescens* might have been due to both growth stimulation and nematode suppression, whereas *P. polymyxa* or *P. stutzeri* increased the yield only through growth promotion. The study has demonstrated that seed dressing with *P. fluorescens* may effectively control root-knot nematodes and improve the yield of chickpea.

LITERATURE CITED

Ali S.S., Naimuddin and Ali M., 2010. Nematode infestation in pulse crops. Pp. 288-325. *In:* Nematode Infestations Part I: Food Crops. National Academy of sciences, India.

Anwar-ul-Haq M., Anwar S.A., Shahid M., Javed N., Khan A.S. and Mehamood K., 2011. Management of root knot nematode *Meloidogyne incognita* by plant growth promoting rhizobacteria on Tomato. *Pakistan Journal of Zoology, 43*: 1027-1031.

Arshad M. and FrankenbergerJr W.T., 1991. Microbial production of plant hormones. *Plant Soil*, *133*: 1-8.

Bakker A.W. and Schippers B., 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth stimulation. *Soil Biology and Biochemistry, 19*: 451-457.

Bauer A.W., Kirby W.M.M., Sherris J.C. and Turch M., 1966. Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology, 45*: 494-496.

Brannen R., 1995. Production of antibiotics by *Bacillus subtilis* and their effect on fungal colonists of various crops. *Transaction of the British Mycological Society, 65*: 203.

Brik J.M., Bostock R.M. and Silversone S.E., 1991. Rapid *in situ* assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. *Applied Environmental Microbiology, 57*: 535-538.

Chahal P.P.K. and Nahal V.P.S., 1988. Effect of different population levels of *Meloidogyne incognita* on nitrogenase activity, leg haemoglobin and bacteroid contents of chickpea (*cicer arietinum*) nodule for med by *Rhizobinem* spp. *Zentralblatt für Microbiologia, 143*: 63-65.

Dye D.W., 1962. The inadequacy of the usual determinative tests for identification of *Xanthomonas* spp. *New Zealand Journal of Science, 5*: 69-71.

Gaur A.C., 1990.*Phosphate solubilising microorganisms as biofertilizers.* Omega Scientific Publishers, New Delhi, India, 176 pp.

Glick B.R., 1995. The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology, 41*: 109-117.

Gorden S.A. and Weber R.P., 1951. Colorimetric estimation of indalcacitic acid. *Plant Physiology, 26*: 192-195.

Gracia do Salamone I.E., Hynes R.K.I. and Nelson L.M., 2001. Cytokinin production by plant growth promoting rhizobacteria and selected mutants. *Canadian Journal of Botany, 47*: 401-411.

Hartman K.L., 1983. Enhancement technique for staining egg masses of the root-knot nematode with Phloxine B. Pp. 130. *In*: Proceedings of the Third Research and Planning Conference on Root Knot Nematodes, *Meloidogyne* spp., 22-26 March, 1983. Lima, Peru (Carter C.C. and Sasser J.N., eds). North Carolina State University Graphics, Raleigh, USA.

Huang J.S., 1987. Interactions of nematodes with rhizobia. Pp. 301-306. *In*: Vistas on Nematology (Veech J.A. and Dickson D.W., eds). Society of Nematologists, Hyattsville, USA.

Jackson M.L., 1967. *Soil chemical analysis*. Printice Hall of India, New Delhi, India, 318 pp.

Kapoor I.J. and Kar B., 1989. *Azotobacter* and *Bacillus*antagonistic to *Fusasriumoxysporum* f. sp. *lycopersici. Indian Phytopathology, 42*: 400-404.

Khan M.R., 2007. Prospects of microbial control of root-knot nematodes infecting vegetable crops. Pp. 643-665. *In*: Biotechnology: Plant Health Management (Sharma N. and Singh H.B., eds). International Book Distributing Co., Lucknow, India.

Khan M.R., 2008. *Plant nematodes - methodology, morphology, systematics, biology and ecology*. Science Publishers, Enfield, New Hampshire, USA, 563 p.

Khan M.R. and Tarannum Z., 1999. Effect of field application of various microorganisms on the root-knot disease of tomato. *Nematologia Mediterranea, 27*: 33-38.

Khan M.R., Mohiddin F.A. and Khan S.M., 2005a. Effect of seed treatment with certain biopesticides on root-knot of chickpea. *Nematologia Mediterranea, 33*: 107-112.

Khan M.R., Khan S.M. and Mohiddin F.A., 2005b. Root knot problem in some winter ornamental plants and its biomanagement.*Journal of Nematology, 37*: 198-206.

Khan M.R., Altaf S., Mohiddin F.A., Khan U. and Anwer A., 2009. Biological control of plant nematodes with phosphate solubilizing microorganisms. Pp. 395-426. *In*: Phosphate Solubilizing Microbes for Crop Improvement (Khan M.S. and Zaidi A., eds). Nova Science Publishers Inc., New York, USA.

King J.E., 1932. The colonimetric determination of phosphories. *Biochemistry Journal, 26*: 292-294.

Kirkpatrick J.D., Mai W.F., Parker K.G. and Fisher E.G., 1964. Effect of phosphorus and potassium nutrition of sour cherry on the soil population level of five plant parasitic nematodes.*Phytopathology, 54*: 706-712.

Kloepper J.W.M., Tsuzum S. and Kuc J., 1992. Proposed definition related to induced disease resistance. *Biocontrol Science and Technology, 2*: 347-349.

Leeman M., Vanpelt J.A., Hendrickz M.K., Scheffer R.J., Balkker P.A.H.M. and Schippers B., 1995. Biocontrol of *Fusarium* wilt of radish in commercial greenhouse trials by seed treatment with *Pseudomonas fluorescens* WCS 374. *Phytopathology, 85*: 1301-1305.

Mankau R. and Minteer R.J., 1962. Reduction of soil population of the citrus nematode by addition of organic matter. *Plant Disease Reporter, 46*: 375-379.

Olsen S.R., Cole C.V., Watanabe F.S. and Dean L.A., 1954. *Estimation of available phosphorus in soils by extraction with sodium bicarbonate*. USDA Circular 939, Gov. Printing Office Washington D.C., USA, 19 pp.

Oyekanmi Z., Coyne D.L., Fawole L.B., 2008; Utilization of the potential of selected microorganisms as biocontrol and biofertilizer for enhanced crop improvement. *Journal of Biological Science, 8*: 746-752.

Rao A.B., 1990. Role of microorganisms in plant nutrition under acid conditions. Pp. 67-84. *In*: Biofertilizers (Vyas L.L., ed.).Scientific Publ., Jodhpur, India.

Sands D.C. and Rovira A.D., 1970. Isolation of fluorescent pseudomonas with a selective medium. *Applied Microbiology, 20*: 513-514.

Schoonbeek H., Raaijmakers J.M. and Waard M.A. De, 2002. Fungal ABC transporters and microbial interactions in natural environments. *Molecular Plant Microbe Interaction, 15*: 1165-1172.

Siddiqui A.I. and Ehteshamul-Haque S., 2001. Suppression of the root rot-root knot disease complex by *Pseudomonas aeruginosa* in tomato: The influence of inoculum density, nematode populations, moisture and other associated plant bacteria. *Plant and Soil, 237*: 81-89.

Siddiqui A.I., Ehteshamul-Haque S. and Shaukat S.H., 2001. Use of rhizobacteria in the control of root-rot-root-knot disease complex of mungbean. *Journal of Phytopathology, 149*: 337-346.

Southey J.F., 1986. *Plant Nematology*. Her Majesty's Stationery office, London, UK, 202 pp.

Taha A.H.Y., 1993. Nematode interactions with root-nodule bacteria. Pp. 170-202. *In*: Nematode Interactions (Khan M.W., ed.). Chapman and Hall, London, UK.

Taha A.H.Y. and Raski D.J., 1969. Interrelationships between the root nodule bacteria, plant parasitic nematodes and their leguminaus host. *Journal of Nematology, 1*: 201-211.

Taha A.H.Y. and Kassab A.S., 1979. The histopathological reaction of *Vignasinensis* to separate and concomitant parasitism by *Meloidogynejavanica* and *Rotylenchulusreniformis*. *Journal of Nematology, 11*: 117-123.

Van Gundy S.D., 1985. Ecology of *Meloidogyne* spp. - emphasis on environmental factors affecting survival and pathogenecity. Pp. 177-182. *In*: An Advanced Treatise on *Meloidogyne* - Vol. I. (Sasser J.N. and Carter C.C., eds). North Carolina State University Graphics, Raleigh, USA.

Walker J.T., 1969. *Pratylenchuspenetrans* (Cobb) population as influenced by microorganisms and soil amendment. *Journal of Nematology, 1*: 260-263.

Whistler C.A., Stockwell V.O. and Loper J.E., 2000. Lon protease influences antibiotic production and UV tolerance of *Pseudomonas fluorescens* Pf-5. *Applied and Environmental Microbiology, 66*: 2718-2725.

Wilt G.R. and Smith R.E., 1970. *Studies on the interaction of aquatic bacteria and aquatic nematodes*. In Microfiche. Alabama Water Resources Research Institute Bulletin 701, 67 pp.

Zeinat K.M., Nagwa M.A., El-Sayed S.A. and Abd El-Wahab G.S., 2010. Optimization of microbial biomass production as biocontrol agent against root knot nematode on faba plants. *Journal of American Science, 6*: 245-255.