Efficacy of different bacterial strains against *Meloidogyne incognita*

I. Jafri, A.A. Shahid, A. Ibrahim† and M. Atif

Institute of Agricultural Sciences, *Plant Pathology Section*, *University of the Punjab*, *Lahore*, *Pakistan*

†Corresponding author email: asmaibrahim12@gmail.com

Abstract

Study was conducted to determine the effect of *Meloidogyne incognita* on plant health and production of tomato in field. Various strains of bacteria were examined for their influence on shoot, root length, plant height, egg-mass, number of juveniles and number of knots per root system. Outcomes revealed that Controlled (C_1) *Meloidogyne* sp., *Bacillus* sp., (T_1), *Meloidogyne* sp., and *Bacillus thuringiensis* (T_2) remarkably suppressed nematode infestations because they act as growth inhabiting agents for juveniles; reduce gall masses, diminish knot formation on root system, toghether with increasing shoot and root length as compared to other treatments.

Keywords: *Meloidogyne*, *Bacillus thuringiensis*, tomato, developmental stages.

Tomato *Lycopersicon esculentum* Mill., is an important and major vegetable crop growing in all over the world. A tropical perennial plant belonging to family Solanacae is a native to Central and South America. In GDP of Pakistan, agriculture contributed 22%. Value addition of major crop in agriculture by crop production sector was 33.4% (Anonymuos, 2012). Tomato was planted on the 46.2 thousand hectares and production 468.1 thousand tones with 10.1 tons per hectare (Tariq, 2008). Yield and quality of the tomato crop was severely affected by various pathogens. It has been reported that *Pseudomonas solanacearum*, *Verticillium albo-atrum*, *Alternaria solani* and *Fusarium oxysporum* were main bacterial pathogens *(*El-Abyad *et al*., 1993). Root-knot nematodes (RKN) were more effective to tomato and affect more than 2000 species of plants. *Meloidogyne incognita* consists of 51% population in the world than other species (Perry *et al*., 2009). High nematodes population present in soil which was continuously used for the cropping that would be susceptible tomato. The resistant tomato plants were not completely resistant to RKN and a few galls on tomato roots were still found (Blancard, 2012). Stunting, premature wilting, leaf chlorosis or yellowing was the common symptoms on the leaf. Infected roots showed galls and reduced root mass which cause less water uptake and dead roots. The galls developed on the roots were usually ranging from 1-10 mm in diam., and juveniles were about 0.5 mm long.

Most of the chemicals to be used for root-knot nematodes were toxic to plants, hazardous to the human being and accordingly not good to use. Safe management strategy was biological control (Khan *et al*., 2011). It has been reported that the extract of garlic had the maximum potential of reducing the infection of root-knot nematodes on tomato (Agbenin *et al*., 2005). *Bacillus* spores worked as best nematicides (Giannakou *et al*., 2007). It has been reported that the RKN susceptible varieties of tomato were treated with mychorrizhal fungi (Cooper & Grandisons, 1986). This fungus improved the phosphorus uptake and made the plant resistant. *Trichoderma harzianum* reduced the nematodes egg hatching by penetrating into the egg-mass (Sahebani & Hadavi, 2008). Entomophagous nematode *Steinernema glaseri* larvae was used to control the root-knot nematodes (Bird & Bird, 1986).

Materials and Methods

Field survey for soil sample collection: For isolation, purification and identification of root-knot nematodes (*Meloidogyne* spp.), field survey was conducted in the vicinity of Lahore district for the collection of RKN infested soil. Approximately 1 kg of soil was collected with shovel up to 10 cm depth from vegetable fields, packed in polythene bags and wrapped to ensure moisture for the nematodes. The soil samples were transported to Plant Nematology Laboratory, Institute of Agricultural Sciences, University of the Punjab and stored at 4 °C in refrigerator until processed. Data regarding crop history, locality, date of collection, soil type were recorded (Table 1).

Table 1. Collection of root-knot nematode infested soil samples for analysis from Lahore district.

Dates	Locality	Soil type	Field samples		Cropping pattern	
			Total	RKN infested	History	Duration
August 11, 2012	I	Sandy loam	3	2	Brinjal	1 year
	I	Sandy loam	3	2	Brinjal	2 years
	I	Sandy loam	3	1	Cucumber and tomato	> 5 years
	I	Sandy loam	3	-	Cucumber	> 5 years
	I	Sandy loam	3	2	Cucumber and brinjal	> 5 years
August 23, 2012	K	Sandy loam	3	-	Cucumber and pumpkin	> 5 years
October 1, 2012	P	Sandy loam	3	1	Cucumber, pumpkin and brinjal	> 5 years
	P	Sandy loam	4	2	Cucumber, pumpkin and tomato	> 5 years
	P	Sandy loam	5	-	Cucumber	4 years
	P	Sandy loam	3	-	Cucumber and bitter gourd	4 years
	P	Sandy loam	3	-	Cucumber and bitter gourd	4 years
October 17, 2012	P	Sandy loam	4	1	Carrot	>15 years
	P	Sandy loam	4	-	Carrot	>10 years
	P	Sandy loam	3	-	Carrot	> 5 years
	P	Sandy loam	3	1	Carrot	> 9 years
October 18, 2012	A	Clayey loam	4	5	Tomato and chillies	> 7 years
October 28, 2012	A	Clayey loam	3	4	Tomato and chillies	> 7 years

I = Iqbal Town sabzimandi, Lahore; K = Khudpur, Multan road, Lahore; P = Punjab University vegetable field sample and A = Institute of Agricultural Sciences, University of the Punjab, Lahore.

Methods of nematodes extraction: Collected soil samples were processed to extract the RKN for further study. For extracting the nematodes; modified funnel technique (Baermann, 1917), tray method (Whitehead & Hemming, 1965), decanting and sieving method (Cobb, 1918) were used. For comparison, in addition to water, hydrogen peroxide and *p*–hydroxybenzoic acid were used to check the efficiency of nematodes isolation from the soil sample.

Mass culturing of *Meloidogyne*: As root-knot nematodes are obligate parasite and need some host for reproduction; therefore, initial culture was multiplied on eggplant (purple round). The nursery was purchased from Iqbal Town market and was transplanted to 12″ earthen pots. Nematodes were extracted through above mentioned procedures to inoculate the eggplant rhizospheric soil in the pots. Pots were watered regularly. Multiplication of culture was carried out on tomato (Rheo grand).

Counting of nematodes: Stereoscopic examination of extracted and stained root-knot nematodes adult ♂ and ♀ were observed at 4x, juveniles at 10x and eggs at 40x magnification of stereoscope by self-designed scale1sq = 1 cm^2.

Staining of roots: Acid fuchsin (Goodey, 1937; Vos *et al*., 2012; Windham & Williams, 1994) and Phloxine B (Luc *et al*., 2005; Thies *et al*., 2002) methods were used for the staining of RKN egg-masses. Both procedures were optimized for RKN estimation.

Phloxine B stained RKN egg-masses were separated out from root system under stereoscope and were given vigorous stirring on magnetic stirrer by dipping in 1.0% NaOCl solution for 10 minutes to liberate RKN eggs from egg-mass jelly like material (Stanton & O'Donnell, 1994; Sudirman & Webster, 1995). RKN eggs were collected from sieve that were rinsed under running tap water and were incubated at 30 °C for 16 hours for hatching and were studied under stereoscope.

Isolation and identification of bacteria: Bacteria were isolated from soil collected samples and identification was received from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences (IAGS), University of the Punjab.

***In vitro* biochemical tests:** Total 20 bacterial strains were tested for their protease and chitinase production potential by *in vitro* study. Spectrophotometric analysis was used to calculate protease and chitinase production standardizing the equipment (Spectrophotometer UV-1900) through control taken as blank at 560 nm and 540 nm, respectively.

***In vitro* tests to check effect of bacteria on nematodes:** Chitinase producing bacteria were screened to test freshly hatched RKN juvenile mortality rate in the Petri plates experiment. Twenty five freshly hatched (60 minutes of age) were dispensed in the Petri plate having 5 ml of dH_2O and 100 µl of each strain having 10^8cfu/ml (Sondi & Salopek-Sondi, 2004) inoculated in the dish and left for solubilizing the J_2 chitin molts. Data regarding mortality rate were taken after 12 hrs.

RKN disease management in field: Experimental design for *in vivo* test of chitinase producing five bacterial strains used as biocontrol agent each with three replicates along with controls (+ve/-ve). Treatment description was as below:

C_1 = -ve control/ no inoculum.
C_2 = +ve control/*Meloidogyne* juveniles.
T_1 = *Meloidogyne* and *Bacillus* sp.
T_2 = *Meloidogyne* and *Bacillus thurengesis*.
T_3 = *Meloidogyne* and *Bordetella* sp.
T_4 = *Meloidogyne* and *Bacillus subtilis.*
T_5 = *Meloidogyne* and *Bacillus farraginis.*

Estimation of RKN disease management: Tomato (*Lycopersicon esculentum* Mill.) was used as host plant for evaluation of RKN disease management after applying bacterial strains as biocontrol agents. The studied parameters were root length, total number of RKN knots in whole root system, counting of RKN egg-masses per RKN knot, RKN ♀ per RKN knot, average number of RKN eggs per egg-mass, live adult ♂ and J_2s of RKN were counted under stereoscope. All parameters data were taken for estimation of RKN disease management and analyzed statistically by using DASTAAT software for biostatistics and self-designed MS Excel Macros.

Results and Discussion

Comparison of nematodes extraction methods: Minimum population of nematodes was extracted from the Whitehead & Hemming tray and Cobb's decanting and sieving methods. For the quick and more extraction in addition to water, hydrogen peroxide and *p*–hydroxybenzoic acid were used. Addition of Hydrogen peroxide showed more positive results (Fig. 1).

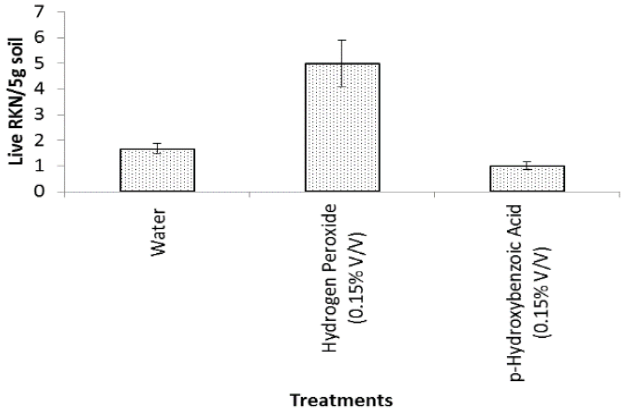

Fig. 1. Different methods to extract nematodes from soil samples

Culturing of root-knot nematodes: Root-knot nematodes are obligate parasites and need hosts for growth and survival. The isolated nematodes from the samples were inoculated into the eggplant and tomato for the mass culturing and multiplication of root-knot nematodes.

RKN disease severity scale: The roots disease severity was determined by RKN disease severity scale 1-4 (number of galls/root system).

Staining of RKN infected roots: Plants treated with RKN were uprooted, root galls were observed showing black arrows for RKN gelatinous egg-masses and green arrows showing knots Phloxin B stained root and acid fuchsin stained root Fig. 2 (A and B).

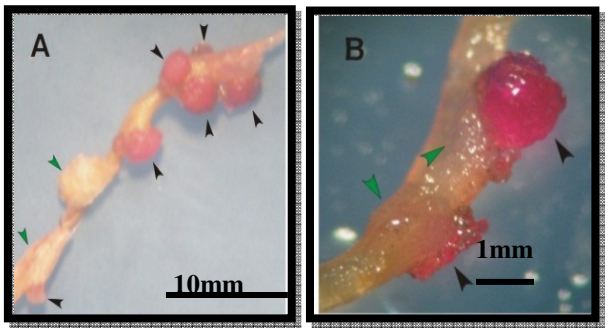

Fig. 2 A. Egg-masses stained by Phloxin B.
B. Root staining through acid fuchsin.

Different stages of RKN: From the egg-masses treated with Acid fuchsin and Phloxin B different life stages of RKN were observed with different incubation time.

Compaction of egg A; RKN egg is developing in to Juvenile 1 ($J_{1)}$ B; Mature J_1 C; Developing juvenile 2 (J_2) D and mature J_2 is ready to hatch E (Fig. 3).

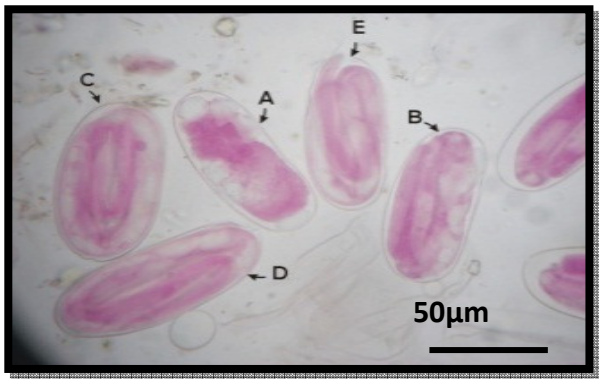

Fig. 3. Different developmental stages of RKN from egg to juveniles.

Micrometry of root-knot nematodes: Micrometry of the different stages of the RKN was made in which length and width of the egg, ♂ and ♀ of RKN were observed under 10x, 40x and 100x and calculations were carried out. The results of root-knot nematodes showed that the length of isolated J_2 was 847.57 µm in length and 16.49 µm in width. Egg was 88.75 µm and 42.50 µm in width.

Bioassay for J_2 mortality by chitinase producing bacteria: Bioassay for juvenile (J_2) mortality by chitinase producing bacterial strains was carried out. Results showed that T_1, T_2 and T_3 gave more mortality rate against RKN, as compared to T_4 and T_5. Control (C_2) showed comparatively very low mortality of RKN than all the treatments. Significant difference was observed between controlled and treated plants (Fig. 4).

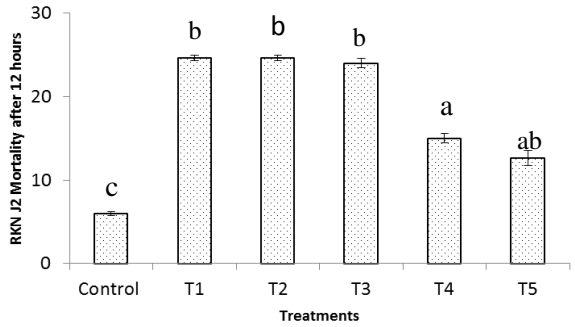

Fig. 4. Bioassay for J_2 mortality by chitinase producing bacterial strains.

RKN disease management in tomato: Experiment showing *in vivo* RKN disease management in tomato by chitinase producing bacterial strains under the following treatments (C_1) -ve control/no inoculum; (C_2) +ve control/*Meloidogyne* juveniles; T_1 = *Meloidogyne* sp., and *Bacillus* sp.; T_2 = *Meloidogyne* sp., and *Bacillus thurengesis*; T_3 = *Meloidogyne* sp., and *Bordetella* sp.; T_4 = *Meloidogyne* sp., and *Bacillus subtilis* and T_5 = *Meloidogyne* sp., and *Bacillus farraginis*. Treatments showed varied results that were assessed by some characteristics e.g., plant height, root length, root-knots per root system, egg-masses per root system, juveniles (J_2) per root system and number of eggs per egg-masses.

Assessment of plant height: The effect of chitinase producing bacteria on the height of tomato plant explained that treatments C_1, T_1, T_2, T_3 and T_5 as compared to C_2 and T_4 showed positive results on plant height. Significant difference was observed between control and treatment (Fig. 5).

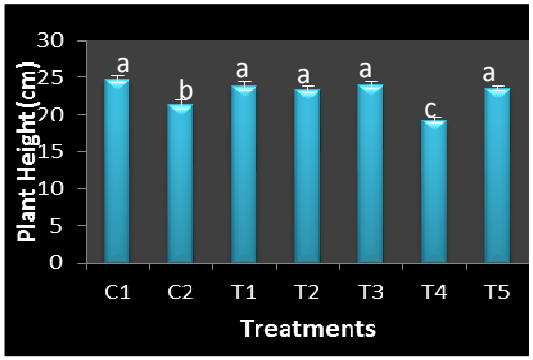

Fig. 5. Average plant height (cm).

Assessment of tomato root length: Experiment demonstrated variable results in case of root length. Different treatments showed diverse effect in the root length of the tomato plant. C_1 and C_2 showed the same root length while bacterial treated plants roots showed slight less growth than controlled. T_4 as compared to T_1, T_2, T_3 and T_5 provided better root length while as T_2 showed slightest root length than all treated plants. Significant difference was observed between controlled and treated plant specifically in T_2 (Fig. 6).

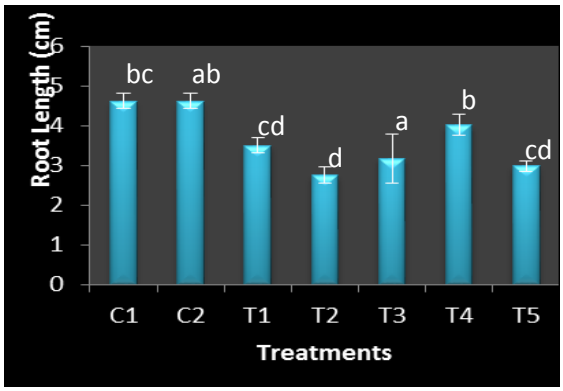

Fig. 6. Average root length (cm).

Assessment of RKN knots per root system: Findings showed that the number of knots per roots system was effective resulted in T_2 as compared to other treatments while in case of C_2 huge numbers of knots were developed. Significant difference was observed between controlled C_2 and treated plants (Fig. 7).

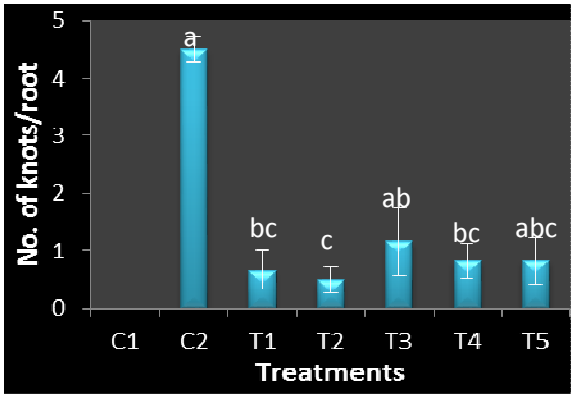

Fig. 7. Average RKN knots per root system.

Assessment of RKN egg-masses per root system: Findings showed that the number of egg-mass per roots system results that T_1 and T_2 were more effective as compared to other treatments for egg-masses formation while in case of C_2 huge number of egg-masses were developed. Significant difference was observed between controlled C_2 and treated plants (Fig. 8).

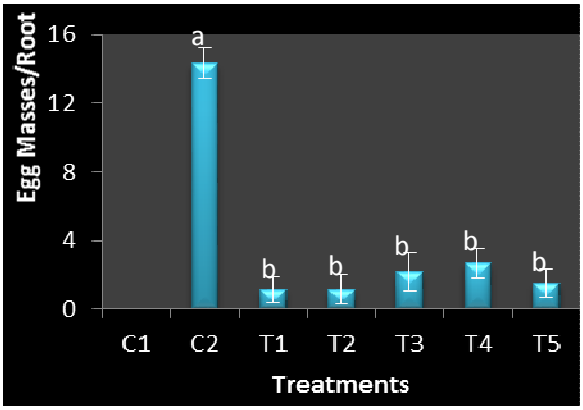

Fig. 8. Average RKN egg-masses per root system.

Assessment of extracted J_2 per root system: Average RKN infectious juveniles (J_2) extracted per root system determined that T_1 and T_2 totally suppress the nematodes growth and cause mortality of the eggs. T_3, T_4 and T_5 were also suppressive for juvenile, while as highest growth rate of J_2 were observed in C_2. Significant difference was observed between controlled C_2 and treated plants (Fig. 9).

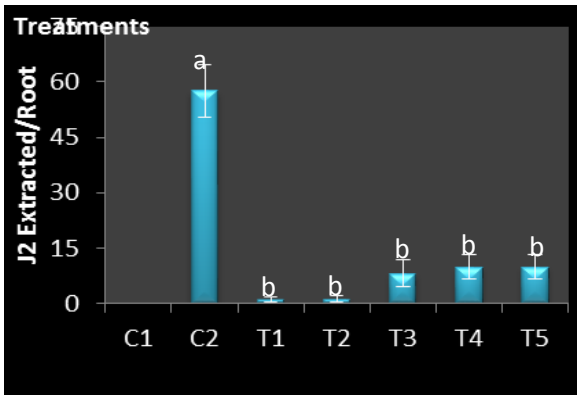

Fig. 9. Average RKN infectious juveniles (J_2) extracted (nos.) per root system.

Assessment of RKN eggs per egg-mass: Findings showed that the number of controlled RKN eggs in the treatments. The C_2 was controlled with only nematodes contained high number of eggs whereas in treated plants with bacteria showed less number of RKN eggs Significant difference was observed between controlled (C_2) and treated plants (Fig. 12).

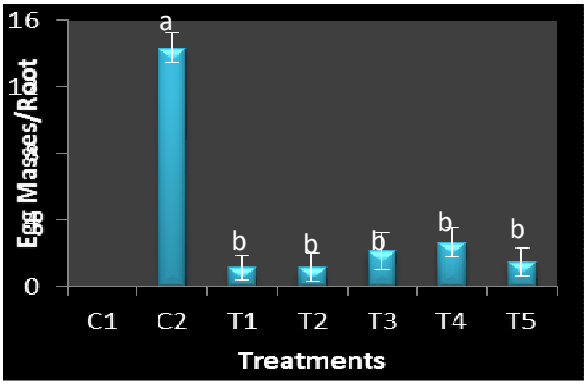

Fig. 12. Average RKN egg numbers/root system.

Root-knot nematodes were parasitic to most of the plants (Zarina & Shahina, 2010). They not only reduce nutrient values but also affect the market value of tomato by damaging the fruit quality. Chemical control of this pathogen is ultimately hazardous to our health, environment and also costly for common farmer (Weller, 1988). Therefore the scientists are using biological approaches to manage this pathogen. Bacterial strain like *Pseudomonas aeruginosa* and *Paecilomyces lilacinus* efficiency was checked against root-knot nematodes (Siddiqui *et al*., 2000).

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