Pakistan Journal of Nematology (2019)37(2): 161-170 ISSN 0255-7576 (Print)
ISSN 2313-1942 (Online)
www.pjn.com.pk
http://dx.doi.org/10.18681/pjn.v37.i02.p161-170

Management of *Meloidogyne incognita* on tomato with different biocontrol organisms

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Abstract

The effect of biocontrol agents comprising *Bacillus* sp., and *Streptomyces rubrogriseus* on the management of the root-knot nematode *Meloidogyne icognita* was investigated along with a nematicide Fosthiazate on tomato susceptible cultivar cv. Baiguoqiangfeng. The *S. rubrogriseus* culture filtrates (CF) showed maximum mortality of second stage juvenile (J_2) followed by *Bacillus* sp., at 12 h and 24 h duration of exposure time as compared to the control treatment containing medium (L.B). In the green house experiments Fosthiazate had significant increased the root length, shoot length and reduced the number of root-knot nematode galls on tomato roots followed by biocontrol agent *S. rubrogriseus* and *Bacillus* sp., as compared to the untreated control treatment after 60 DPI and 90 DPI.

Keywords: Root-knot nematode, *M. incognita*, *S. rubrogriseus*, *Bacillus* sp., biocontrol, tomato.

The primary infection in roots becomes initiated by the soil-born root endoparasite nematodes followed by subsequent secondary infection causing complexes of biotic syndrome. Among the phytopathogenic endoparasites the possible known reason is root-knot nematode (*Meloidogyne* spp.) causing damage to vegetable nurseries, organic vegetable farming in tunnels in tropical and subtropical agro-ecologies (Sahebani & Hadavi, 2008; Kiewnick & Sikora, 2006). The vegetable tunnel farming, organic agriculture and protected agricultural practices has been increasing in worldwide, while these methods are boosting with rapid increase from 257.7 million hectare area cultivation in year 2003 to 386.2 million hectare area under progressive cultivation was recorded in year 2014 (Cao *et al*., 2014).

The environmental pollution, climatic change and global warming leads to the worsen condition in agricultural production of vegetable crops around the world. One possible factor is continuous application of hazardous chemical like methyl bromide as a broad spectrum insecticides in the vegetables farming system for the management of soil born pathogenic nematodes as well as other soil born insect pests (Kiewnick & Sikora, 2006; Nico *et al*., 2004). The biological methodologies are being devised as alternatives to avoid the notorious impact of chemicals on vegetable crop production systems and health of living beings in ecological niche. Among those biological methods many antagonistic bacteria and fungi are being investigated and have been proved to become a potential biological control for soil born root pathogenic nematodes (Khan *et al*., 2008). The researchers, bio-chemists, pharmaceuticals and other related disciplines sciences are being investigating the

Published by Pakistan Society of Nematologists
Received:26 Apr, 2018
Accepted:17 May, 2019

microorganism anatagonistic compounds or their secondary metabolites chemistry for the cure and precautionary measures to avoid the primary infection that initiated by root-knot nematode *Meloidogyne* spp., on the roots of vegetable crops (Ashraf & Khan, 2010). Also the pathogen associated microorganism like fungal isolates have been isolated from root-knot nematode itself, root-knot nematode infested soil fauna and root-knot nematode infected roots have ability to produce substances or secondary metabolites compounds inhibit nematode hatch phenomenon or act as nematicide (Nitao *et al*., 1999; Khan & Saxena, 1997). *Paecilomyces lilacinus,* a nematophagous fungus which produces lytic enzymes serine protease and chitinase by penetration inside the nematode eggs and degrades the surface cuticle while the antagonistic microorganisms like bacterial strains *Pseudomonas aeruginosa* and *Pseudomonas* spp. have also proved and being applied as for the biological control of plant-parasitic soil born nematode (Ali *et al*., 2002; Siddiqui *et al*., 2000; Giannakou *et al*., 2004; Khan *et al*., 2003).

Similarly the compounds or secondary metabolites secreted from actinomycetes are being mass cultured by batch fermentation as well as industrial scale fermentation. These are also being used as biological control methods of different soil born nematodes and insect pests in many vegetable crops. The genus *Streptomyces* contains actinomycetes group and about 52 strains of actinomycetes were obtained from root-knot nematode eggs and females that showed noteworthy effect on parstitizing the eggs, inhibition in egg hatch and mortality of second stage (J_2s) *in vitro*. (Luo *et al*., 2006; Sun *et al*., 2006; Moosavi & Zare, 2012).

The aim of this study was to evaluate the efficacy of microorganism like bacteria and actinomycetes against the root-knot nematode infection and development on tomato roots in green house environment conditions.

Materials and Methods

Root-knot nematode *M. incognita* inoculum preparation: The culture of root-knot nematode *M. incognita* was multiplied from the single egg-mass on susceptible Chinese tomato variety cv. Baiguoqiangfeng in green house at Department of Plant Pathology, College of Plant Protection, China Agriculture University, Beijing, China. The egg-masses were collected by heavily infested tomato roots as described by Sun *et al*., 2006, however the second stage infective juvenile (J_2s) were obtained by hatching the egg-masses on 30µm mesh nylon filter in Petri dishes filled with distilled water and kept at temperature 25° C for 5-10 days. The hatched second stage juvenile (J_2s) were collected intermittently and were used in green house bioassay.

***Bacillus* sp., and *S. rubrogriseus* isolate and their culture filtrates:** The *Bacillus* sp., used in this study was previously isolated from the rhizosphere of wheat monoculture soil from local susceptible wheat cultivars "Wenmai 19" at Shang Zhuang Agriculture Research Experimental Area of China Agriculture University, Beijing. The *S. rubrogriseus* isolate was previously known Chinese patent actinomycetes isolate HDZ-9-47 obtained from the Plant Nematology Laboratory, Department of Plant Protection, China Agriculture University, Beijing, China. The *S. rubrogriseus* isolate was requested from Institute of Microbiology, Chinese Academy of Agriculture Sciences (CAAS). It is a potential indigenous isolate in resource culture bank at China General Microbiological Culture Collection Center with identification as CGMCC 2878.

The *Bacillus* sp., was identified by Gram's staining as gram positive. The culture filtrates of *Bacillus* sp., isolates was prepared on L.B culture media at temperature 25° C, while the culture filtrates of *S. rubrogriseus* was prepared on Gauze's medium at temperature 28° C in darkness incubation (Sun *et al*., 2006). The growth of biocontrol agents were enhanced on

gyratory shaker and monitored after 6-12 days under hemocytometer. The biocotrol agent cell suspension was adjusted to a concentration of 10^6 cells/ml in a sterile water suspension. Further for the mass culturing / production of biocontrol agents, 1ml of the mother culture was transferred into 200 ml selected and optimized liquid medium (corn flour 1.65%, bean flour 1.0%, $MgSO_4$ 0.15%, K_2HPO_4 0.1%, $CaCO_3$ 0.1%, $MnSO_4$ 0.0338% w/v, with pH 7.3) in 500 ml quantity Erlenmeyer flasks. These 500 ml quantity Erlenmeyer flasks were incubated in darkness at 28° C on a gyratory shaker at 160 rpm for 6-10 days.

Culture filtrates of the *Bacillus* sp., and *S. rubrogriseus* HDZ-9-47 isolates were prepared by centrifuging the cultures at 10,000 rpm for 25 min to remove the bacterial cell followed by filtering the supernatant through a sterile polyethersulfone filter of 0.22 µm (Whatman, Clifton, NJ, USA) (Sorensen *et al*., 2009).

Effect of culture filtrates on juvenile mortality of root-knot nematode *M. incognita in vitro*: The RKN second stage juvenile (J_2) were collected from hatched eggs after 4-6 days. These juveniles (J_2's) were disinfected by series antibiotics to avoid any contamination. The antibiotics dilutions of 1.0% each such as; (i) Streptomycin sulphate (ii) Ampicillin sodium salt (iii) Amphotericin-B and (iv) Cetyl-trimethyle ammonium bromide for 1-2 min. Each antibiotic was used separately for RKN (J_2's) disinfection by centrifugation at 8000 rpm and followed by three time washing with sterile distilled water (SDW) and centrifugation at 8000 rpm.

The RKN second stage juvenile (J_2's) mortality assay was carried out in 32 well cell culture plates (Costar Cat# 3524, Corning Inc., Corning, NY 14831, USA). *M. incognita* juveniles (J_2s) population was adjusted as 100 juveniles approximately with the ratio of 900µl of *Bacillus* spp., and *S. rubrogriseus* HDZ-9-47 of culture filtrates (cf) in each well. The control treatment (ck) wells containing 900µl sterile culture medium (L.B). The cell culture plates were incubated in the dark at 25° C. The first observation for mortality was observed after 12 h followed by second observation after 24 h simultaneously under stereoscopic microscope. The mortality was estimated as if nematode bodies were prolonged and immobile even after nudging with a fine needle and considered as dead (Cayrol *et al*., 1989). Each treatment of biocontrol agents were replicated four times and the experiment was repeated three times. The data were means of three separate experiments. The actual mortality and corrected J_2s mortality was calculated according to formula (1) and (2) simultaneously.

$$J2s\ mortality\,(\%) = \left(\frac{Number\ of\ dead\ J2s}{Total\ number\ of\ J2s}\right)\times 100 \qquad (1)$$

$$Correced\ J2s\ mortality\,(\%) = \left(\frac{J2s\ mortality\ in\ the\ treatment - J2s\ mortality\ in\ the\ control}{100\% - J2s\ mortality\ in\ the\ control}\right)\times 100 \qquad (2)$$

Effect of culture filtrates on *M. incognita* infection and development on tomato in green house: Chinese tomato variety cv. Baiguoqiangfeng six weeks old seedlings were transferred into autoclaved potting mix pots. After one week of transplanting the inoculums of second stage injective (J_2) of *M. incognita* about 2000/plant was carefully poured by making holes around the root zone of each plant. Culture filtrates (cfs) of *Bacillus* sp., and *S. rubrogriseus* containing 10^{10} cells at the rate of 100 ml/plant were applied soon after inoculation of *M. incognita* J_2s. The treatment of a chemical nematicide Fosthiazate were used as comparative treatment as described by manufacturer while the untreated control (ck) treatment was served as control. These four treatments were replicated ten times under

glass house environmental conditions at temperature 25° C. The data was collected for the observation on root-knot initiation and knot development after 30 DPI and 60 DPI, respectively from the five plants roots in each treatment. Root gall index were observed using the 0-10 galling index as described by Bridge & Page (1980). The data for fresh shoot length and root length was calculated.

Data analysis

The green house experiments were arranged in completely randomized design with 10 replications. The significance of difference between means values was determined. One way analysis of variance (ANOVA) was carried out and the significance of difference among the treatments was determined according to Least Significant Difference (LSD) (P = 0.05).

Results

Effect of culture filtrates on juvenile mortality of root-knot nematode *M. incognita in vitro*: The sustainable potential of inhibition of *M. incognita* juveniles increased by *S. rubrogriseus* followed by *Bacillus* sp., culture filtrates (cf) as compared to control treatment (Fig. 1) after 12 h subsequently 24 h in which the mean of *M. incognita* J_2 cadavers were of 14.6% and 23.2% were recorded simultaneously. The *S. rubrogriseus* isolate showed 51.0% juveniles inhibition mortality followed by *Bacillus* sp., (42.25%) in first 12 h exposure duration time and followed the mortality about 64.75% with *S. rubrogriseus* and 71.5% by *Bacillus* sp., after 24 h exposure duration time (Fig. 1). The corrected mortality percentage showed that *S. rubrogriseus* isolate significantly kills the maximum number of *M. incognita* J_2 as 42.6% and 62.8%, followed by *Bacillus* sp., as 32.3% and 54.0% in first 12 h and 24 h duration of exposure time to the culture filtrates (Fig. 2).

Effect of culture filtrates (cfs) on *M. incognita* infection and development on tomato in green house: The results of pot experiment in green house indicated that application of *Bacillus* sp., *S. rubrogriseus* and Fosthiazate singly to soil significantly reduced the *M. incognita* second stage juvenile (J_2) penetration in roots and development of root galls as compare to the untreated control treatment on tomato plants (Fig. 6). Shoot length was significantly increased with the application *S. rubrogriseus* followed by nematicide Fosthiazate, *Bacillus* sp., as compared to the control treatment after 60 DPI and 90 DPI (Figs. 2 and 4). However, the root length was significantly observed healthy with the treatment of the nematicide Fosthiazate followed by biocntrol agents *S. rubrogriseus* and *Bacillus* sp., as compared to the control treatment after 60 DPI and 90 DPI (Figs. 3 and 5).

Discussion

In this study we observed the potential biological control through secondary metabolites of *Bacillus* spp., and *Streptomyces* spp., against root-knot nematode. During the preliminary *in vitro* observation in *M. incognita* second stage (J_2s) mortality assay the secondary metabolites of used microorganism has showed significant difference in (J_2s) mortality as compare to the untreated control treatment (ck). The secondary metabolites secreted from *S. rubrogriseus* showed noteworthy mortality of J_2s. Similar finding were observed by various scientists that reflects that the secondary metabolites obtained from different bio-control agents are lethal to soil nematodes fauna including *M. incognita* and insect pests (Khan *et al*., 2005; Kiewnick & Sikora, 2006; Siddiqui *et al*., 2009).

The *Streptomyces* species are generally recognized to produce secondary metabolites or compounds with high nematicidal activity such as *S. avermitilis* has metabolites of avermectins that cause J_2s mortality while *S. albogriseolus* contains metabolite A22-1(S1) of *M. incognita* (Faske & Starr, 2006; Sun *et al*., 2006; Zeng *et al*., 2013). Furthermore, the *Streptomyces* sp., CMU-MH021 was studied and found that the secondary metabolites compounds obtained from this isolate had 82.0% J_2s mortality of *M. incognita in vitro* (Ruanpanun *et al*., 2011).

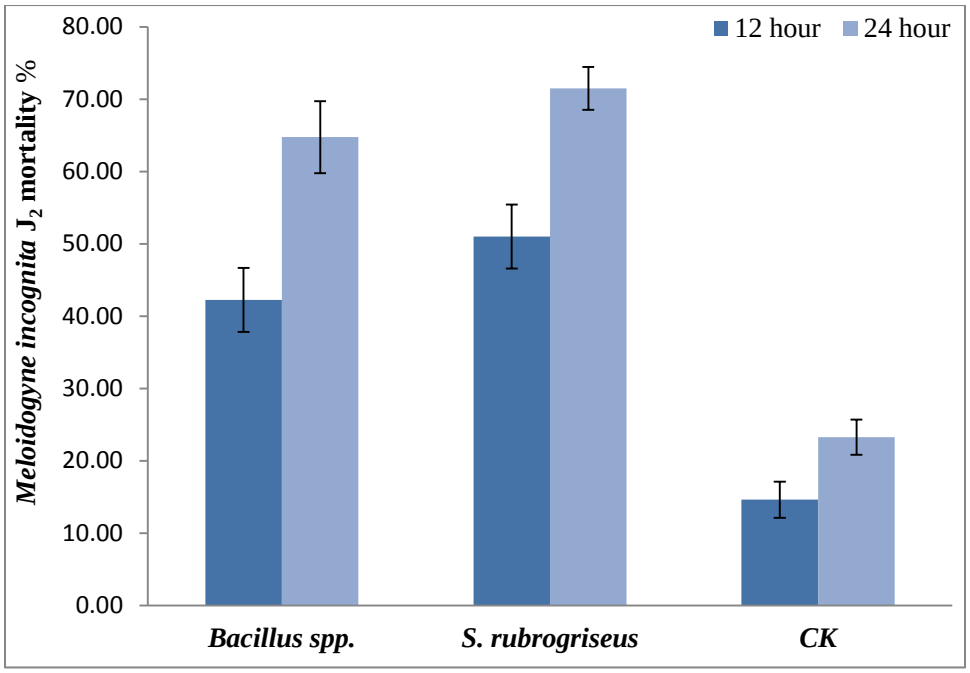

Fig. 1. *In vitro* inhibition rates of mortality by bacterial isolates culture filtrates (cf) relative to the negative control against second stage juveniles (J2s) of *M. incognita* on 12h and 24h duration exposure time.

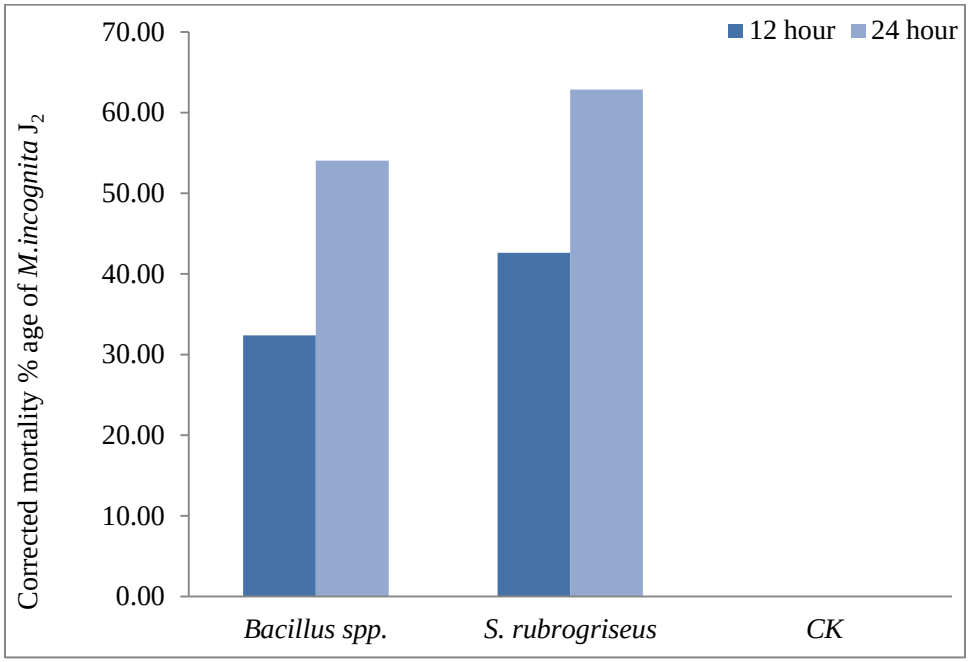

Fig. 2. Corrected mortality percentage (%) by bacterial isolates culture filtrates (cf) relative to the negative control against second stage juveniles (J2s) of *M. incognita* on 12h and 24h duration exposure time.

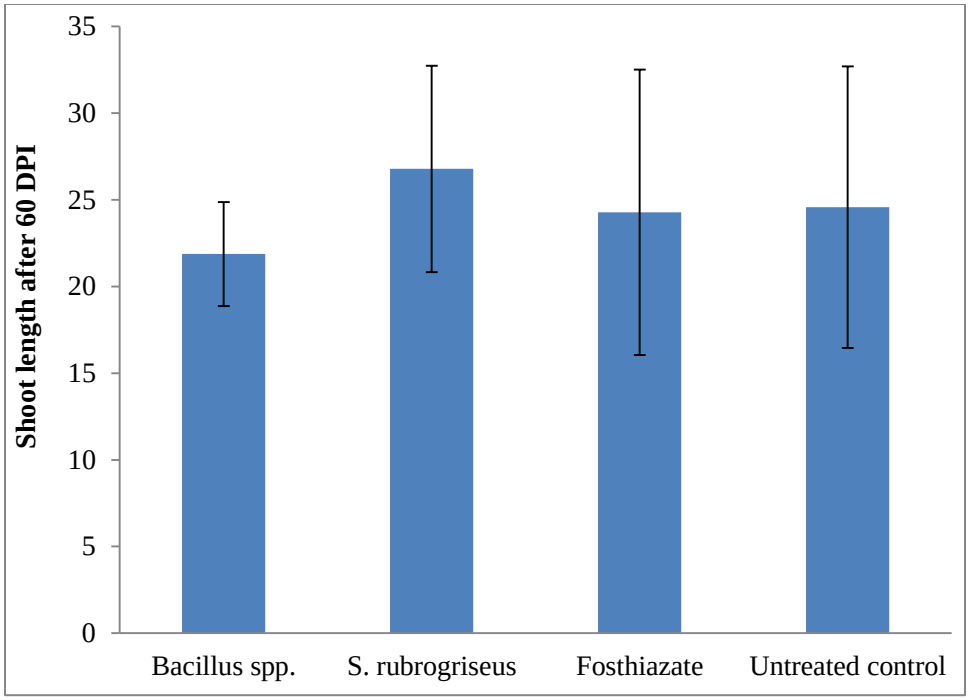

Fig. 3. Effect of different biocontrol agents and avermectine on the on tomato plant shoot length after 60 DPI.

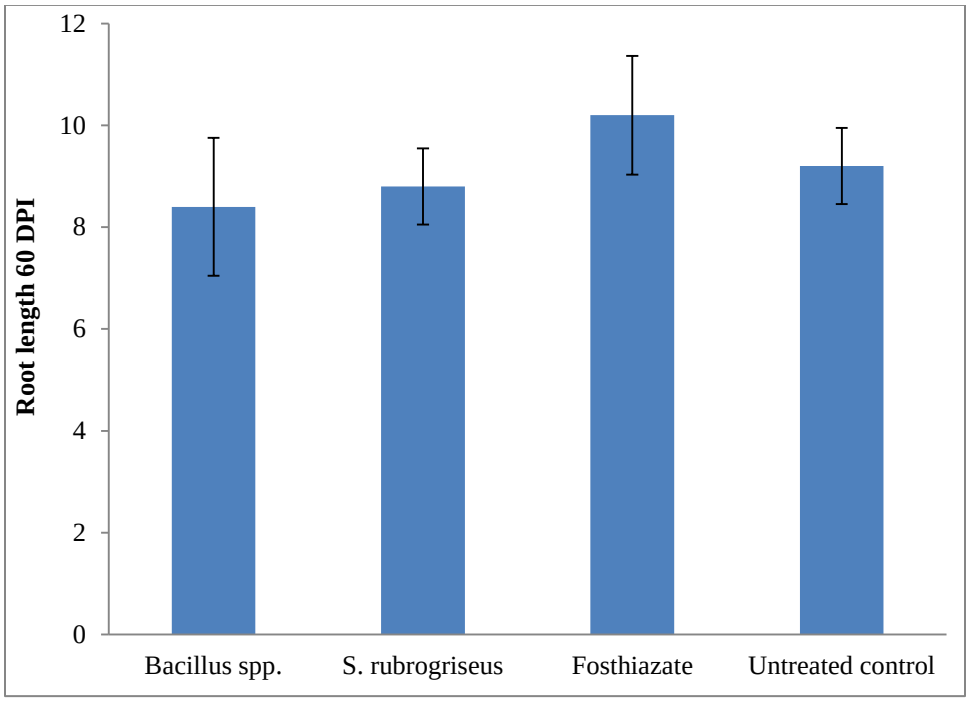

Fig. 4. Effect of different biocontrol agents and avermectine on the on tomato plant root length after 60 DPI.

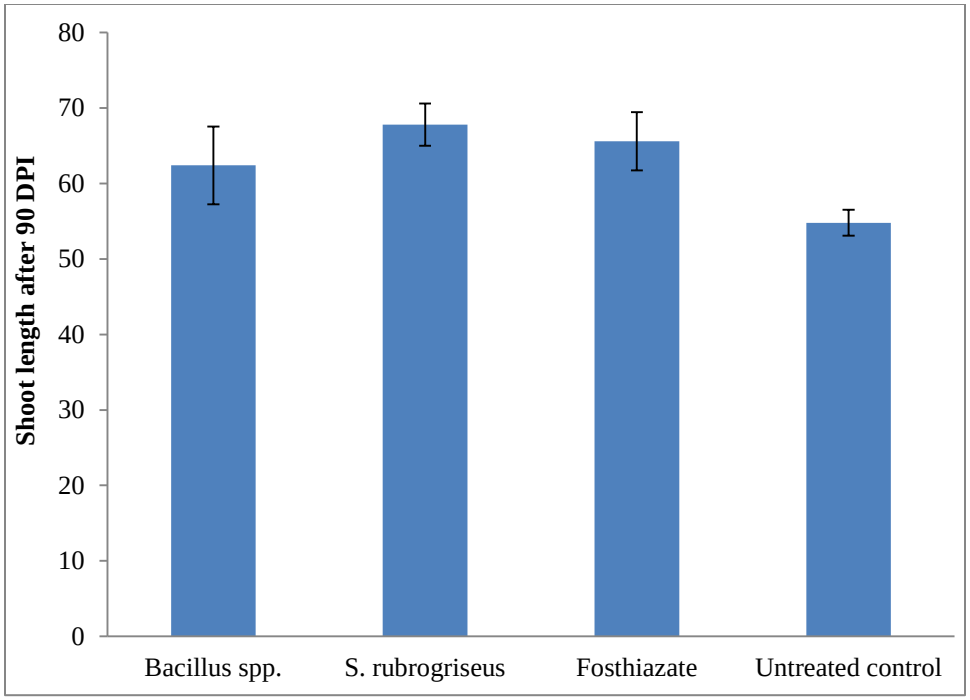

Fig. 5. Effect of different biocontrol agents and avermectine on the on tomato plant shoot length after 90 DPI.

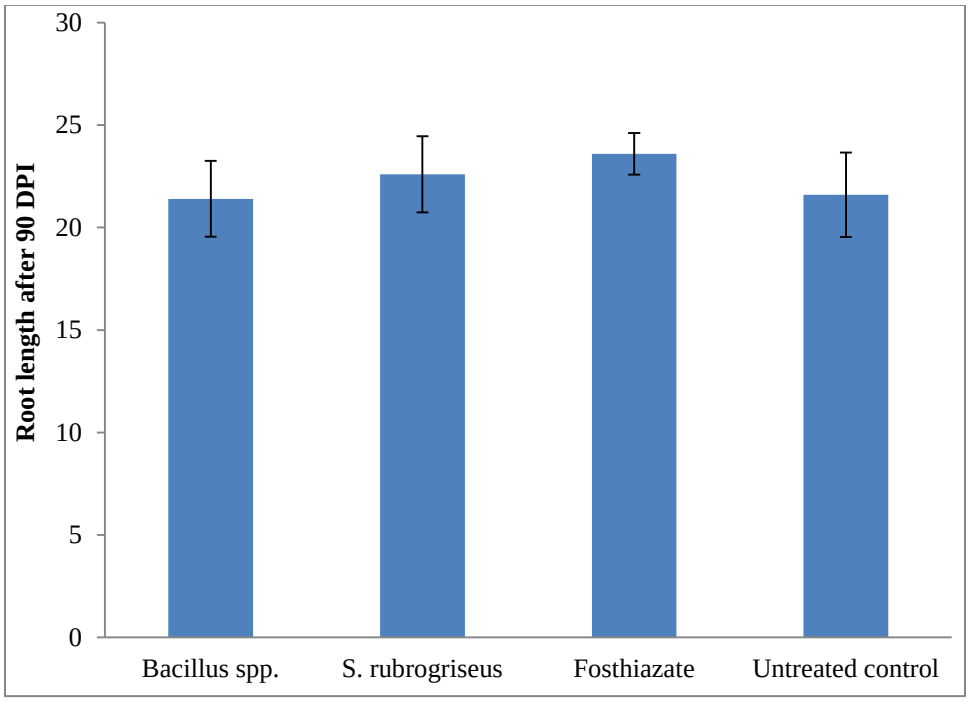

Fig. 6. Effect of different biocontrol agents and avermectine on the on tomato plant root length after 90 DPI.

In green house experiment, the secondary metabolites obtained from *S. rubrogriseus* effects on better shoot length after 30 DPI and 60 DPI followed by Fosthiazate treatment. The *Bacillus* sp., did not show any significant impact on shoot and root length on tomato plants.

It was noticed that majority of biocontrol agents are effective at a specific nematode development stage like against egg hatching inhibition, 1st , 2nd , 3rd stage larvae or adult stage. While the particular inciting the infection on roots by the infective juveniles of the root-knot nematode may decrease the infection but not reduce the nematode population, especially of those endoparasite root-knot nematodes that have more than one generation and simultaneously multiplying in a growing season. Moreover, the control of females and nematodes eggs did not avert the root invasion and damage, while the burgeoning of the nematode becomes condensed. Furthermore, it was observed that the sedentary stage of root-knot nematodes that cannot be parasitized by all the rhizosphere microorganisms because of escape situation from niche. The root-knot nematode *Meloidogyne* spp., life cycle completes when a sedentary female nematode inside the galls produces eggs that extrudes from the root surface. The female of RKN however, stays hidden inside the galls. The high temperature affects the early eggs hatch and the eggs parasitizing microorganism cosmos are unable to obliterate the eggs. The chitin producing bacteria having chitin degrading enzymatic activity with the soil amendments into ammonium can reduces majority of the nematode fauna and insect pests in soil (Kerry, 1992; Hallmann *et al*., 2009).

Because of inherent limitation as low efficacy and inconsistent in active ingredient of biocontrol agents or microorganisms are never playing significant role or could be substitute of chemical management of soil born pathogenic nematode fauna and insect pests. However, their application on long term scale is environmentally safe for protected agriculture as well as organic vegetable food production. The underground water quality will be safeguarded and human health will improved. The application of biological control methods has been accepted at large scale by the farmers in their economic field crops which is a significant stimulus for continued research and development in sustainable agriculture production in developing countries.

Acknowledgement

This research was financially supported by the Special Fund for Agro-scientific Research in the Public Interest in China (No. 201503114) and the National Key Research and Development Program of China (No. 2017YFD0201104). I am also thankful to China Scholarship Council (CSC) for providing the Ph. D. Scholarship funds.

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