BIOCONTROL OF ROOT- KNOT NEMATODE (*Meloidogyne* spp.) WITH *Trichoderma harzianum* and *Bacillus subtilis* ON TOMATO (*Lycopersicon esculentum* Mill) PLANTS

M.Sc. THESIS

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July 2012

Jimma University

BIOCONTROL OF ROOT- KNOT NEMATODE (*Meloidogyne* spp.) WITH *Trichoderma harzianum* and *Bacillus subtilis* ON TOMATO (*Lycopersicon esculentum* Mill) AT JIMMA ETHIOPIA

M.Sc. Thesis

Submitted to the School of Graduate Studies Jimma University College of Agriculture and Veterinary Medicine

In Partial Fulfillment of the Requirements for the Degree of Master of Science in Plant Pathology

By

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APPROVAL SHEET

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As thesis research advisor, I hereby certify that I have read and evaluated this thesis prepared under my guidance, by **Abduselam Mahmude**, entitled "Biocontrol of Root knot Nematode *(Meloidogyne* spp*)* with *Trichoderma harzianum* and *Bacillus subtilis* on Tomato ((*Lycopersicon esculentum*) at Jimma Ethiopia**"**. I recommend that it be submitted as fulfilling thesis requirement.

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STATEMENT OF THE AUTHOR

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BIOGRAPHICAL SKETCH

I was born in January 1985 from my father Mahmud Ibrahim and my mother Asma Yassin at Adwa town, Central zone, Tigray regional state. I attended my elementary and junior school education at Maytsadik Elementary and Junior School from 1991-1999. Then I was transferred to Nigiste Saba secondary School for grade 9-10 education from 2000 to 2001 and preparatory school from 2002-2003. I interrupted my education in grade 12 for two years. Then after, I continued my 12 grade education at Nigiste Saba Secondary School in 2004/2005. Finally, I completed senior secondary school education in June, 2005. In December, 2005, I joined Jimma University for tertiary education and graduated with BSc degree in Horticulture in June 2008. Following graduation with BSc degree, in December, 2009, I was employed by the Ministry of Education and served as junior instructor as well as Horticulture Department Head at Samara university Faculty of Dry land agriculture for about two years. Besides my work I studied higher diploma program in pedagogy and graduated as professional teachers educator in January 2010.After two years of service and practical experience in my fields of training, I joined the School of Graduate Studies of the Jimma University in march 2010 to pursue postgraduate (MSc) studies in Plant Pathology at Horticulture and Plant Science Department.

DEDICATION

I dedicated this humble effort to my parents and family whose unprecedented love and affection can never be compensated

Abduselam Mahmude

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I have no words to express my deepest sense of gratitude to Almighty Allah, the Most Merciful, and the Beneficent, Who bestowed upon me the courage and will to complete this project. All respects for the Holy Prophet Muhammad (Peace Be upon Him), who is forever model of guidance and knowledge for humanity.

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(Abduselam Mahmude)

LIST OF ABBREVIATIONS

AMF= Arbuscular Mycorrhizal Fungus

BCAs= biocontrol agents

BC-F= *Burkholderia ambifaria*

CF= culture filtrate

DAPG= Diacetylphloroglucinol

DW= Shoot dry weight

(IPM) = Integrated pest management

(LTSEM) =Low temperature scanning electron microscopy

(PSB)= phosphate solubilizing bacteria

(RKN) = Root knot nematodes

(SLPG)= spore load per gram

(SSF)= Solid-state fermentation

(WT) = Wild-type strain

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ABSTRACTS

Tomato (Lycopersicon esculentum Mill.) Is a crop of great economic importance, having as one of the main problems the plant parasitism by nematodes of the genus Meloidogyne, which are forming galls on the root. To control this disease, chemical method is the most widely used method. However, there are several problems associated with the use of synthetic chemicals. Biological control is favorable alternative for the management of root-knot nematodes, as it is economically sustainable and environmentally friendly. Thus, the objective of this study was to investigate the antagonist effects of Bacillus subtilis and Trichoderma harzianum and their times of application on root knot nematode disease of tomato and yield. Suspensions of T. harzianum, B. subtilis and their combinations at eight levels were used as biological control agents against root-knot nematode. Three incubation periods was arranged for laboratory experiments using completely randomized design. Three times of application (two weeks before, simultaneously and two weeks after nematode application) were arranged for greenhouse experiments using completely randomized block design. The highest egg hatching inhibition (65%) after 4days and J2 mortality (96.2%) after 96 hours were recorded in T. harzianum and B. subtilis combination, followed by T. harzianum and B. subtilis. The galling indices were reduced by all biocontrols with great effect when they were in combination than alone. Galls (25.32) and low egg mass number per root system (47.3) were recorded from the combinations of the biocontrols B. subtilis and T. harzianum. However, only the fungus T. harzianum enhanced plant growth and yield in all the treated pots without nematode. However, all bio-controls showed crop growth enhancement by reducing the disease indices compared to nematode inoculated control. The study revealed that the use of T. harzianium and B. subtilis can help for the management of root knot nematode under controlled conditions. However, further studies should be made to evaluate the effectiveness of the bio-controls and their combinations under field conditions.

Key words: Eggmass, Gall, Hatching, Incubation, Mortality, Suspension

1. INTRODUCTION

Tomato (*Lycopersicon esculentum*Mill*)* is the world's largest vegetable crop after potato and sweet potato, which is widely grown in both tropics and sub- tropics, in home gardens and commercially. Tomatoes are one of the low calorie vegetables containing just 18 calories per 100 g. They are also very low in any fat contents and have zero cholesterol levels. Nonetheless, they are excellent sources of antioxidants, dietary fiber, minerals, and vitamins. Because of their all-round qualities, dieticians and nutritionists often recommend them to be included in cholesterol controlling and weight reduction programs. Tomato is rich in lycopene which is used in the fight against cancer, especially the prostate cancer (Mills *et al.,* 1989; Giovannucci *et al.,* 1995; Giovannucci, 1999). Lycopene Together with carotenoids has the ability to protect cells and other structures in the body from harmful oxygen free radicals. Studies have shown that *lycopene* prevents skin damage from ultra-violet (UV) rays and offers protection from skin cancer.

The global production of tomatoes (fresh and processed) has increased by about 300% in the last four decades (FAS/USDA, 2003). The annual worldwide production of tomatoes in 2003 has been estimated at 110 million ton with a total production area of about 4.2 million hectare (FAS/USDA, 2003).These figures may underestimate the real production area and tonnage, considering that tomatoes are also grown on very small plots and gardens throughout the tropics and subtropics with a large total production that is consumed locally. The global trade of tomatoes and tomato products reached US$4.2 billion, which represents a 33% increase compared to the beginning of the 1990s (FAS/USDA, 2003).

Plant parasitic nematodes cause great economic losses to agricultural crops worldwide by causing severe damage to a wide range of important crops (Sasser and Freckman, 1987). In order to reduce these losses, an estimated amount of US$ 500 million is spent on nematode control globally (Keren-zur *et al.,* 2000). However, the total production and productivity in Ethiopia is far below than the average of major producers in the world and as well in Africa. According to FAOSTAT (2011) in 2009 cropping season the country's

area coverage by this crop was 4, 953 ha and production in tons was 40,426 t with the productivity of 8.8 t/ha, in 2009 (MoARD, 2010) which is very low when compared to the other tomato producer countries. Current productivity under farmers' condition is 9 t/ha, where as yield up to 40 t/ha can be recorded on research plots (Tesfaye, 2008) cited in Eyasu (2010). The national average of tomato fruit yield in Ethiopia is often low (12.5 t/ha) compared even to the neighboring African countries like Kenya (16.4 t/ha) (FAO Production Year Book, 2004). The reason for such yield differences can be attributed to several reasons. The most important among these, is the vulnerability of tomato crop to various diseases including fungal, viral, bacterial and nematode diseases. Unlike other pathogens, nematodes give more problems since they live in the soil and cannot be easily seen by the farmers. They are only noticed when the population is wide spread and yield reduction is high (Mai, 2002).

Root- knot nematodes (*Meloidogyne* spp) are sedentary endoparasites and are considered to be the most economically serious among the plant parasitic nematodes (Maqbool, 1981: Ghaffar and Hashmi, 1983: Qureshi *et al*., 1984) with a host range of more than 2000 species of plants (Barker 1985). They cause high economic losses to tomato crop throughout the world especially in developing countries.

During the 1998-2000 cropping season, surveys for root- knot nematodes were undertaken in the central and western part of Ethiopia. Eight hundred and twenty-eight samples were collected from different vegetable crops during the rainy and off-season periods. The major crops grown in these areas are tomato, pepper and onion. Out of the 192 field samples, 119 (62%) were found to be infested with *Meloidogyne species*. *Meloidogyne incognita* was the dominant species followed by *M. javanica* and *M. ethiopica* (Wondirad *et al*, 2002).Average crop losses due to these nematodes reached above 50% and individual fields may reach to 100% (Wondirad, M. and Tesfamariam, M. (2002). Moreover, association of these nematodes with fungi and bacteria in various disease complexes cause even more economic losses to agricultural crops (Maqbool *et al.,* 1987).

At present, more than 100 species of *Meloidogyne* have been reported from different parts of the world. Four of these are economically important including *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (Eisenback and Triantaphylou, 1991), responsible for 95% of the infestations of the cultivated land (Sasser and Carter, 1982). In order to reduce the population of these nematodes, several control measures are being employed including cultural practices, chemical and biological control methods. Unfortunately there are several problems associated with the use of chemicals because of poor penetration to the nematode egg, rapid leaching and degradation, high cost, environmental pollution including ground water contamination, food safety and worker protection. Therefore, chemical control of root-knot nematodes may no longer be a good option (Ploeg, 2002).

Because of the lack of resistance in plants to most root diseases as well as ecological and economical restrictions on nematicide use for the control of plant-parasitic nematodes, biological control and other ecofriendly disease control measures have gained increasing interest (Siddiqui and Shaukat, 2004). Biological control is favorable alternative for the management of root-knot nematodes, as it is economically sustainable and environment friendly. Therefore, such a strategy is considered to be a major tactic in integrated pest management systems.

Biological control is the reduction of inoculums density or disease producing activity of pathogen or parasite in its active or dormant state by one or more organisms accomplished naturally or through manipulation of the environment, host or antagonist (Baker and Cook, 1974). A large number of biocontrol agents have been tasted so far to control root-knot nematodes with encouraging results. These include bacteria such as *Burkholdera cepacia*, *Pasturia penetrance*, *Bacilluss* spp, and *Pseudomonas flurescens*. (Walia and Dalal, 1994: Meyer *et al.,* 2000; Siddiqui and Shaukat, 2004). Fungi, like *Verticillium chlamydosporium*, *Paecilomyces lilacinus*, *Gliocladium* spp., *Trichoderma* spp., and *Arthrobotrays oligospora*. (Dos Santose *et al*., 1992 ; Duponoris *et al.,* 1998). Similarly, predatory nematodes and enthomopathogenic nematodes have been reported to

control root-knot nematodes including *Steinenema carpocapsea* and *Mononchus aquticus* (Kermarrec *et al*., 1991; Akhtar, 1995).

Trichoderma harzianum has been extensively studied as a biological control organism against a wide range of soil born pathogens including root-knot nematodes and having plant growth promoting capacity (Dos Santose *et al*., 1992: Saifullah and Thomas, 1996; Rao *et al.,* Sharon *et al.,* 2001; Goswami *et al*., 2006). It has been shown that *T. harzianum* stimulated the growth of tomato plants (Chet, 1990; Mc Goven *et al.,* 1992; Dantoff and Pernenzy, 1998).

T. harzianum has been an exceptionally good model to study as biological agent for soil-born plant pathogens (Samuel, 1996). They are easy to isolate and culture, grow rapidly on many substrates, affect a wide range of plant pathogens, are rarely pathogenic on higher plants. They act as mycoparasites, compete well for food and site, produce antibiotics and have an enzyme system capable of attacking a wide range of plant pathogens and are effective biocontrol agents against root-knot nematodes (Parveen *et al.,* 1993, Khan and Saxena, 1997, Hafeez *et al.,* 2000, Meyer *et al.,* 2000, Rangaswamy *et al.,* 2000, Sharon *et al*). Reduction of *M. javanica* infection with several isolates of *T. lignorum* and *T. harzianum* has been reported (Spigel and Chet, 1998). Many species of *Trichoderma* show resistance to various toxic compounds such as herbicides, fungicides, pesticides and nematicides (Chet *et al.*, 1997).

Several mechanisms have been reported to be involved in the biocontrol activity of *Trichoderma* spp. against various fungal diseases such as ; antibiosis, parasitism, competition and enzymatic hydrolysis (Elad, 1995; Sivan and Chet, 1992). All mechanisms except competition can potentially be involved in nematode biocontrol process. Parasitism of *T.harzianum* on potato cyst nematode *Globodera rostochiences* was studied *in vitro* by Saifullah and Thomas (1996). In addition the effect of *T.viride* metabolites on nematodes was demonstrated (Khan and Saxena, 1997).

On the other hand, the effects of *Bacillus subtilis* as a nematocidal biocontrol agent has been investigated for free living nematodes, animal-parasitic nematodes, insect-parasitic and plant-parasitic nematodes (*Li et al.,* 2005). Selected strains of *B. subtilis* have been reported as antagonists of gall-forming nematodes and can be used in the management of cash crops in order to reduce the deleterious effects of the parasite (*Li et al.,* 2005). Sharma & Gomes (1996) reported that the cytotoxin produced by *B. subtilis* in the soil interfere with the reproductive cycle of nematodes, especially in the oviposition and hatching of juveniles. How ever there are only a few studies on the use of *Bacillus* spp as a biocontrol agent of plant parasitic nematodes (Esnard *et al*., 1998; El-Sherif *et al*., 1995; Sharma, 1994).

Root knot nematode (*Meloidogyne* spp.,) is one of the pathogens of tomato which mainly affects its production and yield. Some studies in Ethiopia were undertaken to evaluate the severity of the pathogen. Although the pathogen has been controlled with cultural methods, it causes significance tomato yield loss (Wondirad, M. 2002). Accordingly, alternative method of root knot diseases control is needed to be studied.

Trichoderma harzianum and *Bacillus subtilis* were chosen since these antagonistic micro organism are found in most agricultural fields and are promising antagonist of plant pathogenic fungi, bacteria, and plant- parasitic nematodes (Elad *et al.,* 1982). These biocontrol agents are not harmful to humans, wild life and other beneficial organisms. Biocontrol agents are safe and effective agents in both natural and controlled environments that do not accumulate in the food (Monte and Llobell, 2003). Keeping in view the impotance of *Trichoderma harzianum* and *Bacillus subtilis* as biological control agent the present research was initiated.

1.1. Objectives

General Objectives

- This study was conducted to investigate the antagonistic effects of *B. subtilis* and *T. harzianum* and their times of application on root knot nematode disease and yield of of tomato

Specific Objectives

The specific objectives of the current study were:

- To evaluate the antagonistic effect of *B. subtilis* and *T. harzianum* on root knot nematode disease and yield of tomato
- To evaluate the effect of application times of *B. subtilis* and *T. harzianum* on root knot nematode and yield of tomato
- To evaluate the effect of *B. subtilis* and *T. harzianum* on the growth and yield of tomato

2. REVIEW OF LITRETURE

2.1 A brief description of tomato

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetables worldwide. World tomato production in 2001 was about 105 million tons of fresh fruit from an estimated 3.9 million ha(Youdeowei, 2004). As it is a relatively short duration crop and gives a high yield, it is economically attractive and the area under cultivation is increasing daily(George, 1999). Tomato belongs to the Solanaceae family. This family also includes other well-known species, such as potato, tobacco, peppers and eggplant (aborigine) (Rice, *et al.* 1993).

Tomato has its origin in the South American Andes. The cultivated tomato was brought to Europe by the Spanish conquistadors in the sixteenth century and later introduced from Europe to southern and eastern Asia, Africa and the Middle East(Youdeowei, 2004). More recently, wild tomato has been distributed into other parts of South America and Mexico. Common names for the tomato are: tomate (Spain, France), tomat (Indonesia), faan ke'e (China), tomati (West Africa), tomatl (Nahuatl), jitomate (Mexico), pomodoro (Italy), nyanya (Swahili) (Youdeowei, 2004)

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Tomatoes contribute to a healthy, well-balanced diet. They are rich in minerals, vitamins, essential amino acids, sugars and dietary fibers. Tomato contains much vitamin B and C, iron and phosphorus (Rice, *et al.* 1993). Tomato fruits are consumed fresh in salads or cooked in sauces, soup and meat or fish dishes. They can be processed into purées, juices and ketchup. Canned and dried tomatoes are economically important processed products(George, 1999).

Yellow tomatoes have higher vitamin A content than red tomatoes, but red tomatoes contain lycopene, an anti-oxidant that may contribute to protection against carcinogenic substances. Tomato is an annual plant, which can reach a height of over two metres. In South America, however, the same plants can be harvested for several years in

succession. The first harvest is possible 45-55 days after flowering, or 90-120 days after sowing(Rice, *et al.* 1993). The shape of the fruit differs per cultivar. The color ranges from yellow to red. Three different types of tomato plants can be distinguished: tall or indeterminate type, semi-bush or semi-indeterminate type, bush or determinate type. The tall and bush types are entirely different kinds of crops (George, 1999). The tall varieties are the best choice for a long harvest period. They keep growing after flowering. This feature is called indeterminate. However, under tropical conditions, diseases and insect attacks will stop growth. The plants generally have more foliage. This will keep the temperature lower within the crop and the fruits grow in the shade of the leaves. Because they are covered, the sun does not damage the fruits and they ripen more slowly(George, 1999). Slower ripening and a high leaf/fruit ratio improve the taste of the fruits and in particular the sweetness. The tall types have to be staked, caged or trellised. Short types usually support themselves and need no staking. Under severe weather conditions such as typhoons, however, staking may be advisable. Determinate types stop growing after flowering. They require less labour, so they are popular for commercial cultivation. They have a relatively concentrated fruit set which lasts only two or three weeks and the fruits ripen much faster than those from indeterminate types (Youdeowei, 2004).

2.1.2 Nutritional and medicinal value of tomato

Tomatoes are one of the low calorie vegetables containing just 18 calories per 100 g. They are also very low in any fat contents and have zero cholesterol levels. Nonetheless, they are excellent sources of antioxidants, dietary fiber, minerals, and vitamins (George, 1999). Because of their all-round qualities, dieticians and nutritionists often recommend them to be included in cholesterol controlling and weight reduction programs. The antioxidants present in tomatoes are scientifically found to be protective against cancers including colon, prostate, breast, endometrial, lung, and pancreatic tumors (Rice, *et al.* 1993).

Lycopene, a flavonoid antioxidant, is the unique phytochemical present in the tomatoes. Red varieties are especially concentrated in this antioxidant. Together with carotenoids, it has the ability to protect cells and other structures in the body from harmful oxygen free

radicals. Studies have shown that *lycopene* prevents skin damage from ultra-violet (UV) rays and offers protection from skin cancer(Varela, 2003).

Zeaxanthin is another flavonoid compound present abundantly in this vegetable. Zeaxanthin helps protect eyes from "age related macular disease" (ARMD) in the elderly persons by filtering harmful ultra-violet rays. The vegetable contains very good levels of vitamin A, and flvonoid anti-oxidants such as α and ß-carotenes, xanthins and lutein (Varela, 2003). Altogether, these pigment compounds are found to have antioxidant properties and are take part in vision, maintain healthy mucus membranes and skin, and bone health. Consumption of natural vegetables and fruits rich in flavonoids is known to help protect from lung and oral cavity cancers (Youdeowei, 2004)

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2.1.3 Tomato world production stastics

Tomato is one of the most popular and widely grown vegetables in the world. It is grown throughout the world, either outdoors or indoors, because of its wide adaptability and versatility. The estimated world production of tomato is about 89.8 million Mg from an area of about 3,170,000 ha; the leading producers are China (with 25.3% of the total production), USA, Mexico and Egypt (Bose et al., 2002; Basheer, 2006). Tomato production is widely distributed in Asia, Europe, North and South America, and in North Africa. Demand for tomato products has, in recent years, risen on the international market. In 2003 the main importers were United States of America (26.5%), Germany (19.3%), United Kingdom (12.4%) and France (8.7%), which accounted together for more than 66% of the total world imports. In 2003 the top ten exporters of tomato in the world were Netherland (23.9%), Spain (20.5%), Mexico (20.5%), Belgium (6.5%) and Canada (5.5%), and all accounting together for 77% of the world exports(Varela, 2003).

2.1.4 Tomato production in Ethiopia

Tomato and tomato products are important part of human diets. Currently, tomato has a higher consumption rate in developed countries and is often referred to as a luxury crop. In developing countries, tomato has become important part of the food basket as well. It is also the most widely consumed vegetable in Ethiopia. In Ethiopia, tomato is grown almost all over the country, mostly under irrigation (furrow, drip or spate irrigation) and

sometimes under rain fed conditions. It is both produced by small-scale farmers and commercial growers. The bulk of tomato production is concentrated in river valleys and lakes especially in the Awash Valley and around Lake Zwai for their favorable growing conditions, good access to market outlets and better infrastructure and other facilities. The current productivity of tomato farms is 105 quintals per hectare. However, it is 250 and 400 quintals per hectare at the demonstration and research plots, respectively[1].

In 2001/02, approximately 3,300.55 hectares of private holdings were under tomato cultivation and the total volume of fresh tomato harvested was 347,277.48 quintals, according to the figures of the Central Agricultural Census Commission, Statistical Report on Farm Management Practices, Livestock and Farm Implements Part I(2003) of Ethiopia. On the other hand, production of tomato on state farms in the past ten years is shown in table 1 below.

Table 1: Production of Tomatoes (2003/04 – 2007/08)

Year (E.C/G.C)	**Area Cultivated (ha)**	**Total production**
2003/04	3761	752200
2004/05	2919	583800
2005/06	4788	957600
2006/07		
2007/08	36382	7729141

Source: MoRAD

2.2 Distribution and impact of *Meloidogyne* spp in tomato

The majority of nematodes in the rhizosphere are bacterial feeding taxa that make up typically more than 60% of the nematode community (Griffiths, 1989). However, most studies on the interactions of nematodes and their natural enemies have concerned plant parasitic species. Because plant parasitic nematodes affect crop yields directly through

the alteration of the morphology of the root system that results from their feeding on, and/or invasion of root tissues.

Root knot nematodes (RKN) are the species of nematode genus *Meloidogyne* producing galls on the roots of wide varieties of cultivated plants that provide food, fiber and timber. Most apomictic root knot nematodes (*Meloidogyne* spp.) have host ranges that encompass the majority of flowering plants and *M. incognita* is possibly the world's most damaging crop pathogen (Trudgill and Blok, 2001).

RKN are key nematodes widely distributed throughout the world causing discernible yield losses, quantitatively as well as qualitatively in important agricultural and horticultural crops (Swarup and Dasgupta, 1986). Unlike most other plant pathogens they are density dependent and may vary from little to total crop loss. The difficulties lie mainly in the design of suitable experiments because of the many overlapping interactions involved (Browning, 1998). The association of plant parasitic nematodes with a wilt fungus is reported to produce greater loss than caused by a pathogen alone.

M. incognita is widely prevalent inflicting serious yield loss of tomato crop (Sasser, 1980) and ranged from 24 per cent to 38 per cent in the tropics. In India, it was 46.7 per cent (Bhatti and Jam, 1977), 39.7 per cent (Reddy, 1985), ranged from 42.05 per cent to 54.42 per cent (Subramaniyan *et al*, 1990). Jam *et al*. (1994) reported that crop yield losses due to root knot nematodes exceed 71 .9 per cent.

Therefore, control of nematodes is important to maintain economic and social stability. With the economical globalization, the competition in agriculture production has intensified. These scenarios have provided the great economical, environmental, and social impetus for development of biological method of control of nematodes.

Many commercially important plants such asbetelvine, ginger and tomato suffer severe damage from *M. incognita* infections (Bhatt et al., 2002a, b; Vadhera *et al.,* 1998). Chemical methods have been mostly used tocontrol nematodes. Chemical agents such as

halogennated aliphatic hydrocarbons (e.g., 1,3-dichloropropene), methyl isothiocynate mixtures, oxamyl, Thionazin and carbofuran are effective in the management of nematodes but are not ecofriendly and in the course of time may cause serious threat to the ecological balance. In soil these agents increase the probability of mutagenesis in microbes. Chemical pesticides have been tested and evaluated for their ill effects such as reproductive toxicity and carcinogenesis in mammals. High doses of these agents have been proved to be fatal to animals. These facts have been reported under 'Food and Environment Protection Act, 1985, Part III. Control of Pesticide regulations 1986' by Pesticide Safety Directorate (Kings Pool,York Y01 7PX) in 1992.

Therefore, biological control agents are gaining importance in the field of nematode management. Another importance of these agents is their role as plant growth promoting microorganism (Sharon et al., 2001). *Trichoderma* spp. found in close association with roots contributes as plant growth stimulators (Ousley et al, 1994). Many fungal and bacterial agents have been examined over a period of time for their potential as biocontrol agents. Li et al (2008) evaluated expression of Cry5B protein from Bacillus thuringiensis as environment friendly nematicidal proteins. In research performed on fungi, it has been shown that fungi possess appropriate characteristics for biological control of nematodes; for example, fungal enzymes such as chitinases are capable of rupturing nematode egg shells contributing to parasitism of fungi on nematodes (Gortari and Hours, 2008). Also, mutualistic endophytic fungi such as non-pathogenic strains of Fusarium oxysporum and species of *Trichoderma* have been evaluated for their activity against plant parasitic nematodes (Sikora et al., 2008).

2.2 Fungi and bacteria as biological Control of Root knot nematodes

Culture filtrates of *Rhizoctonia solani* and *Trichoderma viride* adversely influenced hatching *M. incognita* larvae with highest inhibition of hatching occurring in the standard concentration of the filtrate (Sharma and Saxena (1992). When culture filtrates of the two fungi were mixed together, the relative toxicity of the separate filtrates was unaffected. Ahmad *et al*. (1994) reported that *Pasteuria penetrans* applied at 2.5cm soil depth was

the most effective method for the control of *M. incognita* on tomato, followed by soil surface application and application at 5cm depth.

The efficacy of *P. lilacinus* in controlling *M. incognita* on four vegetable crops and soybeans under field conditions was evaluated by Noe and Sasser (1995). Experiments with Marglobe tomato, Clemson Spineless okra, Black Beauty eggplant, and Yolo Won den pepper were carried out in two different sites in Wake County, North Carolina. An experiment with Lee 68 soybeans was carried out in a field in Johnston County, North Carolina. The yield of vegetable crops in plots treated with *P. lilacinus* was higher than untreated plots in both experimental sites. Cumulative yield data for okra and tomato showed a widening difference between control plots and plots treated with fungus as the season progressed and differences between treatments increased with each harvest date. Similarly, *M. incognita* juvenile counts were lower in treated plots versus that in control plots. There were more *M. incognita* juveniles in control plots treated with either *P. lilacinus* or fenamiphos. The fungus provided the same level of nematode suppression as the nematicide.

According to Khan and Saxena (1997) root-dip treatment with culture filtrates of *Aspergillus niger*, *P. lilacinus* and *T. viride* was particularly beneficial in reducing *M. javanica* damage on tomato in pot experiments. Of the other eight fungal species tested, *Alternaria alternata*, *Curvularia clavata*, *pencillium spp*., arid *Rhizoctonia solani* had no effect on the nematode population or on plant growth and fruit yield. Khan and Saxena (1997) studied the effect of fungal filtrates of *A. alternata, A. niger*, *Chaetomium cochliodes*, *Curvularia clavata*, *P. lilacinus*, *Penicillium sp*., *Rhizoctonia solani* and *T. viride* on plant growth, root galling, penetration and development of nematodes, with variable effects. Exposure of roots in the filtrates of different soil fungi not only reduced the penetration of *M.javanica* J2, but also delayed it. The culture filtrates of *A. alternata*, *C. clavata* and *Penicillium sp*. did not significantly affect either the plant growth or the development and multiplication of nematodes. However, filtrates of *A. niger, T. viride, P. lilacinus, C. cochliodes* and *R. solani* significantly improved plant growth by decreasing

the root galling as well as development and fecundity of nematodes. Filtrates of *A. niger*, *P. lilacinus* and *T. viride* also delayed egg laying as compared to the control.

Some bacteria associated with *A. oligospora* ORS 18692 S7 isolated from a vegetable-producing area in Senegal for their potential effects on fungus development (saprophytic growth and trapping activity), multiplication of *M. mayaguensis* on tomato plants and growth of the host-plantwas tested by Duponnois *et al.* (1998) . Three bacterial strains enhanced in vitro fungal activity against the nematode, which resulted in better control of the nematode and improved plant growth. These bacteria were called Nematophagous fungus Helper Bacteria (NHB). No criteria could be found for predicting the helper effect of these bacteria.

The root knot nematode *M. incognita* was controlled more effectively when *P. lilacinus* and *G. mosseae* were applied together in a pot experiment than when either was applied alone (Bhat *et al.*, 2000). Inoculation of tomato plants with *G. mosseae* did not markedly increase the growth of plants infected with *M. incognita.* Inoculation of plant with *G. mosseae* and *P. lilacinus* together or alone resulted in a similar shoot and plant height. The highest root development was achieved when mycorrhizal plants were inoculated with *P. lilacinus* to combat root knot nematode. Inoculation of tomato plants with *P. lilacinus* suppressed galls/root system and eggs/egg masses, compared to seedlings inoculated with *M. incognita* alone. Mycorrhizal colonization was not affected by inoculation of *P. lilacinus,*

The efficacy of *V. chlamydosporium* cultured on different substrates such as sorghum grain, rice grain, broken wheat, maize grain and wheat bran, against *M. incognita* with tomato cv. Pusa Ruby under potted conditionwas tested by Sankaranarayanan *et al*. (1999). All the substrates favored the multiplication of *V. chlamydosporium* and enabled the fungus to suppress the galls, egg masses and nematode population. The degree of suppression of nematode by *V. chlamydosporium* varied with the substrates used and the percentage parasitism of egg masses and eggs of *M. incognita* ranged from 39 to 70 %and 51 to 89%, respectively. *Verticillium chlamydosporium* cultured on sorghum grain

applied at 10 g/plant as well as 5 g/plant was superior to other substrates in terms of parasitism of egg masses (70 and 89.3%, respectively) and eggs (63 and 69%, respectively). A significant increase in growth of tomato plants was observed with *V. chlamydosporium* treated plants.

In Karachi, Pakistan the efficacy of *V. chlamydosporium* as a biological control agent of *M. javanica*, under laboratory and field conditions was tested by Siddiqui and Haque (2000). An ethyl acetate and hexane extract of *V. chlamydosporium* was lethal to *M. javanica* juveniles. *V. chlamydosporium* parasitized *M. javanica* eggs whereas the female was unaffected. Neither egg nor *M. javanica* female were infected with *Pseudomonas aeruginosa*. *V. chlamydosporium* did not colonize the inner root tissue of tomato whereas *P. aeruginosa* did. *V. chlamydosporium* and *P. aeruginosa* applied together had a better biocontrol effect, and promoted plant growth, compared with either antagonists alone or the untreated controls.

Root-knot disease caused by *M. incognita* is a matter of grave concern because it affects several economically important crop plants (Debora *et al*. (2005). The use of solid-state fermentation (SSF) may help to elaborate efficient formulations with fungi to be employed in the biologic control of nematodes. Attempts were made to select low-cost substrates for spore production of a strain of *P. lilacinus* with known nematicide capacity. Coffee husks, cassava bagasse, and defatted soybean cake were utilized as substitutes, and sugarcane bagasse was used as support. Fermentation were were carried out in flasks covered with filter paper at 28°C for 10 d. The products obtained by SSF were evaluated tor their nematicide activity in pot experiments containing one seedling of the plant Coleus inoculated with the nematode *M. incognita*. The plants were evaluated 2 months after inoculation. Fermented products showed a reduction in the number of nematodes. The best results were obtained with defatted soybean cake, which showed almost 100% reductions in the number of nematodes; the reduction with coffee husk was 80% and with cassava bagasse was about 60%.

A collection of bacterial isolates and isolates GL3 and GL 21 of *T. virens* for suppression of diseases caused by *R. solani*, *Pythium ultimuin*, and *M. incognita*, *Trichoderma virens* isolates GL3 and GL21 provided the most effective suppression of damping-off caused by *R. solani* in greenhouse bioassays. *Burkholderia ambifaria* BC-F, *B. cepacia* BC-1, and *Serratia marcescens* Nl-14 also provided significant suppression of *R. solani* relative to the pathogen check in some experiments was tested by Roberts *et al*. (2005). No microbial treatment containing individual or combined microbes significantly suppressed populations of *M. incognita* on cucumber or improved plant vigor in greenhouse bioassays. *Trichoderma virens* GL21 applied as a granular formulation, in combination with *B. cepacia* BC-1 or *B. ambifaria* BC-F applied as a seed treatment, significantly improved suppression of damping-off caused by *R. solanium* over individual applications of these microbes in at least one experiment. *Burkholderia ambifaria* BC-F combined with *T. virens* GL2 in seed treatments resulted in significantly improved suppression of damping- off caused by *P. ultimum* in two of three experiments.

The fungal biocontrol agent, *P. lilacinus* strain 25l (PL251) for its potential to control the root-knot nematode *M. incognita* on tomato was evaluated by Kiewnick and Sikora (2006). In growth chamber experiments, a pre-planting soil treatment reduced root galling by 66%, number of egg masses by 74% and the final nematode population in the roots by 71% compared to the inoculated control. Significant dose-response relationships were established when conidia were applied to soil either with or without the glucose-based formulation. The effective concentration 50 (EC50) values for the commercially formulated product ranged between 0.097g and 0.08 g/500 cm^3 soil, equivalent to an EC5O of $1.29x10^6$ and $9.88x10^5$ colony forming units (cfu)/g soil for the parameters gall index and final population per root, respectively. For the number of egg masses per root the EC5O was 0.007g product or2.64x105 cfu/g soil. Similarly, EC50 values for conidia applied without formulation were 0.O68g or 0.103 g/500 cm^3 soil (EC50 of $8.10x10^5$-$1.40x10^6$ cfu/g soil) for gall index and final population per root. In contrast, the ec5o was 0.096g (EC50 of 1 .28x 10^6 cfu/g soil) for the number of egg masses per root.

2.3. Mycoparasitism and growth promotion ability of *Trichoderma harzianum*

The mycoparasite *Trichoderma harzianum* may prove to be an effective control agent for many phytopathogenic fungi. It has been proposed that its mycoparasitic interaction proceeds in three major steps (Goldman *et al*., 1994a, b; Haran *et al.*, 1996). Initially, the mycoparasite hyphae grow toward the host hyphae (Chet *et al*., 1981). Then the parasite attaches to the target hyphae, presumably mediated by a host lectin, and appressoria-like structures coil around the attacked cells (Elad *et al.*, 1983a,b; Barak *et al*., 1985). Concurrently, degradation of □(1,3)-glucans and chitin from the host cell wall has been observed (Elad *et al*., 1983b). Probably both mechanical pressure and cell wall degradation by hydrolytic enzymes are involved. Finally, the mycoparasite penetrates and/or lyses the host hyphae (Chet *et al*., 1981) and releases cellular contents, which provides nutrients to sustain growth. Extracellular enzymes corresponding to the main chemical constituents of the fungal cell wall, i.e., chitin, glucans, and proteins, have been detected when *T. harzianum* is grown on *Rhizoctonia solani* mycelia or cell walls as the sole carbon source (Ridout *et al*., 1988; Geremia *et al.*, 1993). The enzymes appeared sequentially; an alkaline proteinase was produced first, followed by glucanases and chitinases (Geremia *et al.*, 1991, 1993). Recently, we purified and cloned this alkaline protease gene (*pbr1*) specifically induced by *Rhizoctonia solani* cell walls and chitin (Geremia *et al.*, 1993). Although the results support the hypothesis that this gene is specifically involved in mycoparasitism, there is no direct evidence of its role in this interaction.

Mass-scale multiplication was done on cheaper substrates for application of bioagents in the management of plant-parasitic nematodes and fungal pathogens (Sharma and Trivedi (2005). The bioagents were isolated from the local field soils. Out of the 13 isolated fungi, most of the isolates of *Trichoderma spp*. that were found antagonistic to *F. oxysponim f. sp*. cumini in dual cutlure technique, were mass multiplied on cheaper agro wastes. Suitability of 6 substrates was screened and tea waste was found to be best followed by wheat bran and sorghum straw. *T. harzianum* (T5) had the maximum spore load per gram (SLPG) value on tea waste followed by *T. hatnatum* (T1 6) on wheat bran.

Three isolates of bacteria viz. *Bacillus subtulis*, *P.fluorescens* and *Rhizobium spp*. were multiplied on nutrient broth; King's B broth and yeast extract mannitol broth, respectively.

Trichoderma mycoparasite activity depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall. We have analyzed the extracellular proteome secreted by *T. harzianum* cect 2413 in the presence of different fungal cell walls (Suarez *et al*. (2005). Significant differences were detected in 2DB maps, depending on the use of specific cell walls or chitin. A combination of maldi-tof and liquid chromatography mass spectrometry allowed the identification of a novel aspartie protease (P628 1: mw 33 and pI 4.3) highly induced by antifungal cell walls. A broad EST library from *T. harzianum* cect 2413 was used to obtain the full-length sequence. The protein showed 44% identity with the polyporopepsin (EC 3.4.23.29) from the basidiomycete Irpex lacteus. Lower identity percentages were found with other pepsin-like proteases from filamentous fungi (<3 1%) and animals (<29%), Northern blot and promoter sequence analyses support the implication of the protease P6281 in mycoparasitism.

2.4. *Trichoderma* spp as nematophagous fungus

Neem cake (*Azadirachta indica*) and a biocontrol fungus, *T. harzianum* either singly or in combination for the management of *M. incognita* on tomato was evaluated by Rao *et al*. (1997). Significant increase in plant growth and reduction in root galling and final population of *M. incognita* were observed in tomato seedlings transplanted in neem cake-amended soil incorporated with *T. harzianum*. Increase in colonization of *T. harzianum* on roots of tomato was also observed in the above treatments, which indicated favorable effects of neem cake amendment on the growth of *T. harzianum*.

Reknaa *et al*. (1998) reported that both *T. roseum* (1 g/kg soil) and *T. viride* (5 g/kg soil) reduced the harmful effects of *M. incognita* and improved germination of tomato in pot experiments.

According to Reddy *et al*. (1998) the fungal biocontrol agent *T. harzianum*, *Glomus fasciculatum* and neem cake either singly or in combination (at half the doses) for the management of *M. incognita* infecting tomato under nursery and main field conditions. In the nursery, integration of neem cake with *G. fasciculatum* gave maximum increase in plant growth parameters and the least root-knot index. Root colonization was maximam when neem cake was integrated witn *G. fasciculatum*, while egg parasitism was highest when neem cake was integrated with *T. harzianum.* Under field conditions, planting of tomato seedlings (raised in nursery beds treated with neem cake + *G.fasciculatum)* in pots incorporated with *T. harzianum* grown on sorghum seeds at 0.5 g per plant was effective in increasing tomato fruit yield and for the management of root-knot nematodes.

Sankaranarayanan *et al*. (1999) conducted a pot experiment to find out the nematicidal effect of the antagonistic fungi *T. harzianum* (3 isolates), *T. viride* (2 isolates), *Gliocladium virens* and *G. deliquescens* against *M. incognita* on sunflower. The maximum plant height (78.87 cm) was recorded in *T harzianum* ITCC treated pots. Among the nematophagous fungi tested, *G. virens* was found most effective with respect to treated plants having the least number of galls and egg masses on root systems and nematode populations in soil. Root galling was significantly reduced in treatments where the roots were coated with the fungus.

The use of mixtures of bacteria and some antagonistic fungi in controlling root-knot nematode, *M. incognita*, in lettuce. NK-05 bacteria and *B. megateriurn* which were able to inhibit the egg hatching of root-knot nematode and two antagonistic fungi was studied by Songsak (1999). *P. lilacinus* and *T. harzianum* were used in the study. The experiment was conducted in a screen house and the use of bacteria and fungi were both in single and mixture forms. The results showed that the use of a mixture of *P. lilacinus* and *T. harzianum* and a mixture of *P. lilacinus* and NK-05 bacteria along with chitosan had the tendency to decrease the gall number of lettuce more effectively than other treatments and were significantly different from the control which the number of gall were found 65, 66 and 104 galls/plant, respectively. The treatment using the mixture of *P. lilacinus* and

NK-05 bacteria along with chitosan gave the highest stem fresh weight, which was significantly different from the control: 86.90 and 52.10 g, respectively.

Khan and Goswami (2000) reported that twenty-day-old tomato cv. Pusa Ruby seedlings were transplanted in pots containing different levels (2, 4, 6, 8 and 10 g/kg soil) of *P. lilacinus* (isolate 6) culture on pounded rice grain. Second stage juveniles of *M. incognita* were inoculated in the soil one week after transplanting at about 2000 J2/kg soil. The pots were kept under greenhouse conditions and observations were recorded 60 days after transplanting. All treatments receiving *P. lilacinus* exhibited higher plant growth parameter values compared to those treated with the nematode alone. Increasing the dose of *P. lilacinus* was accompanied by an increase in plant height and root length. The percentage egg infection increased from 30.4% at 2g *P. lilacinus*/kg soil with higher concentrations and the gall index, number of eggs per egg mass and the final soil population of nematodes decreased with increasing inoculum concentrations. The results indicate that 8g *P. lilacinus*/kg soil is the optimum dose for the suppression of *M. incognita*.

Sharon *et al*. (2001) evaluated *T. harzianum* for its potential to control the root-knot nematode *M javanica*. In greenhouse experiments, root galling was reduced and top fresh weight increased in nematode infected tomatoes following soil pretreatment with *Trichoderma* peat-bran preparations. The use of a proteinase PrB1-transformed line (P-2) that contains multiple copies of this gene improved biocontrol activity in the greenhouse experiments compared with the nontransformed wild-type strain (WT). All the *Trichoderma* strains showed the ability to colonize *M. javanica* separated eggs and second-stage juveniles (J2) in sterile in vitro assays, whereas P-2 also penetrated the egg masses. This protease transformed line presented the same nematicidal and overall proteolytic activity as the WT in vitro tests in which concentrated soil extracts from *Trichoderma* treated soils immobilized the infective J2. However, the J2 immobilization and proteolytic activities of both P-2 and the WT were higher than those obtained with strain T-203. Characterization of the activity of all *Trichoderma* strains soil extracts on J2 showed that it was heat resistant and restricted to the low-molecular weight fraction (less

than 3 kDa). They suggested that improved proteolytic activity of the antagonist might be important for the biological control of the nematodes.

The effects of the combination of the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* and the biological control fungus *T viride* on the control of the root-knot nematode (RKN), *M. hapla*, in greenhouse experiments on the tomato cultivars 'Hildares' and 'Tiptop' (less suitable as host for RKN, showing retarded development of the giant cell system, retarded growth of the nematode, and consequently reduced production of egg-sacs) was investigated by Masadeh *et al*, (2004). Neither of the beneficial fungi inoculated singly or together, changed general susceptibility of the cultivars. In 'Hildares' application of the beneficials reduced the number of galls and egg-sacs. However, a combination of *G. intraradices* and *T. viride* did not result in synergism. In 'Tiptop', biocontrol of root-knot was not achieved.

Siddiqui and Shaukat (2004) determined the influence of soil-borne fungus *T. harzianum* on the biocontrol performance of *P. fluorescens* strain CHAO and its 2,4-diacetylphloroglucinol (DAPG) overproducing derivative CHAO/ pME3424 against *M.javanica*. Amendment of the culture filtrate (CF) or methanol extract of the CF of a *T. harzianum* strain Th6 to *P. fluorescens* growth medium enhanced the production of nematicidal compound(s) by bacterial inoculants in vitro. In addition, bacteria overwhelmingly expressed phl-lacz reporter gene when the medium was amended with CF of *T. harzianum*. *P.fluorescens* and *T. harzianum* applied together in unsterilized sandy loam soil caused greater reduction in nematode population densities in tomato roots. The synergistic effect of *T. harzianum* on the production of nematicidal compound(s) critical in biocontrol may improve the efficacy of biocontrol bacteria against plant-parasitic nematodes. Considering the inconsistent performance of the biocontrol agents under field conditions, application of a mixture of compatible *T. harzianum* and *P.fluorescens* would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity with enhanced efficacy and reliability of control.

Sukumar *et al*. (2005) reported that *T. harzianum*-Thn1 parasitizing the egg masses of root knot nematode M. incognita was isolated from galled mulberry roots and evaluated for its potential to control root knot disease. In pot experiments root galling was reduced and leaf yield increased significantly following soil treatment with *T. harzianum*-Thn1. The extracts obtained from the soils inoculated with *T. harzianum*-Thn1 drastically inhibited the hatching of nematode eggs and the effect was irreversible even after the eggs were transferred to fresh water.

Goswami *et al*. (2006) studied the effect of two fungal bioagents along with mustard oil cake and furadan against root knot nematode *M. incognita* infecting tomato under greenhouse condition. Bioagents viz., *P.lilacinus* and *T. viride* alone or in combination with mustard cake and furadan promoted plant growth, reduced number of galls/plant, egg masses/root system and eggs/egg mass. The fungal bioagents along with mustard cake and nematicide showed least nematodes reproduction factor as compared to untreated infested soil.

Saifullah *et al*. (2007) reported that root-knot nematode (*Meloidogyne* spp.) is one of the major nematode problems in the vegetable crops especially tomato growing areas. In these studies, efforts were made to find out the efficacy of *T. harzianum* in suppressing the population of root- knot nematodes. Eleven different localities viz. Sakhakot, Dargai, Jabban, Thana, Zarakhella, Parrai, Goratey, Matta, Barikot, Charbagh and Tindodag were surveyed and *T. harzianum* was isolated from Zarakhella, Swat. Culture filtrates of *T. harzianum* showed strong nematicidal effect against *Melodogyne sp.* The percent inhibition of egg hatching was directly proportional to the concentration of the filtrate, 90% in standard solution of culture filtrate, 79% in 1:1 dilution, 31% in 1:10 and 9% in 1:100 dilutions. Only 5% larval mortality was observed in the sterile distilled water used as control.

Sharon *et al*. (2007) examined parasitism of *Trichoderma* on *M. javanica* life stages *in vitro*. Egg masses, their derived eggs and second-stage juveniles (J_2) were parasitized by *T. asperellum*-203, 44, and *T. atroviride* following conidium attachment. Trichoderma

aspereiiurn-Gh11 attached to the nematodes but exhibited reduced penetration, whereas growth of *T. harzianum* 248 attached to egg masses was inhibited. Only a few conidia of the different fungi were attached to eggs and J_2s without gelatinous matrix; the eggs were penetrated and parasitized by few hyphae, while J_2s were rarely parasitized by the fungi. The gelatinous matrix specifically induced J_2 immobilization by *T. asperellum*-203, 44 and *T. atroviride* metabolites that immobilized the J_2s. Scanning electron microscopy revealed the formation of coiling and *appressorium*-likr structures upon attachment and parasitism by *T. asperellum* 203 and *T. atroviride*. All but *T. harzianum* could grow on the gelatinous matrix, which enhanced conidium germination. A biomimetic system based on gelatinous-matrix-coated nylon fibers demonstrated the role of the matrix in parasitism. All Trichoderma isolates exhibited nematode biocontrol activity in pot experiments with tomato plants.

2.5. *Bacillus* spp.

2.5.1 Characterization of *Bacillus* spp.

Bacteria of the genus *Bacillus* Cohn are widely dispersed in nature, easy to multiply, have a long shelf life when sporulated. Alexander, (1961 reported that The numbers of *Bacillus* are vary from 10^6 in cooler regions to or more per gram in warmer latitudes. *B. subtilis, B. mycoides, B. pumilus, B. megaterium, B. thuringiensis and B. firmus* are wide range of *Bacillus* present in rhizosphere soil (Wipat and Harwood, 1999; Garbeva *et al*., 2003) which produced cytotoxin or/and putative emetic toxin (From *et al*., 2005).

The genus *Bacillus* consists of a heterogenic group of gram positive rods, able to form endospores that allow them to survive for extended periods under adverse environ mental conditions, better than nonsporulating bacterial enteropathogens. Endospore formation is the dominant feature in the characterization of *Bacillus:* There is a boundary that separates this genus from other genera in which endospores are produced. The genus Clostridium is distinguished from *Bacillus* by inability to grow on the surface of agar media in air or, if growth does occur under these conditions, it is slight and does not lead to sporulation. There is also little or no catalase activity. *Sporosarcina* is sharply separated from *Bacillus* by the coccal form of its vegetative cells. Sporolactobacillus has

the physiological properties of the genus *Lactobacillus* including the inability to form catalase (Gibson and Gordon, 1974).

2.5.2 *Bacillus* spp. as potential biocontrol agent

Cook, 1996; Cook *et al*., (1996) reported that Many bacteria including *Pseudomonas* spp*., Bacillus* spp., *Burkholderia* spp., *Enterobacter* spp., etc. have been identified as biocontrol agents. Mazzola (2004) said that identification of biological properties contributing to the function of suppressive soils is a necessary first step to the management of such systems for use in the control of soil borne diseases. Multiple *Bacillus* spp. (McSpadden Gardener, 2004) and number of *B. subtilis* strains (Jacobsen *et al*., 2004) can promote crop health and integrated successfully into several pest management programs.

2.5.2.1 Bio-control efficacy of *Bacillus* spp. on nematode.

From 244 PGPR isolates, only 125 bacterial isolates imparted positive effect on *M. incognita* on tomato and cucumber (Zavaleta-Mejia and VanGundy (1982). And *Bacillus* spp. is a large group of bacteria that have shown diversified effects on plant-parasitic nematodes. *Bacillus* species that demonstrated nematicidal effects include: *B. subtilis* and *B. pumilis* which gave 100% larval mortality at 2.0 O.D. concentration (Gokte and Swarup, 1988), *B. cereus* (Gokte and Swarup, 1988; Kempster et al., 2001) and *B. licheniformis* (Siddiqui and Mahmood, 1992) against *M. incognita*, *Heterodera cajani*, *H. zeae,* and *H. avenae*. The non-cellular extract exhibited high larvicidal properties. The first line of evidence of broad- spectrum activity of antibiotics by PGPR was derived from culture filtrates or purified antibiotics (Nakayama *et al*., 1999).

Bacillus thuringiensis (Bt) is a potential biopesticide of large number of insect pests. Its toxic effects to eggs and juveniles of *M. javanica* under in vitro conditions were first reported by Prasad *et al*., (1972) and under natural conditions (Prasad and Tilak, 1972). They have nematicidal effects against plant-parasitic nematodes (Zuckerman et al, 1993) including *Meloidogyne spp*. (Rai and Rana, 1979), *Meloidogyne hapla* (Chen et al, 2000) and lethality to eggs and second-stage juveniles of root knot nematode under laboratory

conditions (Al Banna and Khyami-Horani, 2004). With cell free filtrates, Dhawan *et al*. (2004) reported that J2 of *M. incognita* completely died after 24h exposure in standard filtrates (s) and s/10 dilution in all four native strains of B. thuringiensis.

According to Jonathan and Umamaheswari (2006) a pot culture study was conducted to assess the biocontrol potential of endophytic bacterial isolates of *Bacillus subtilis* (Epb 5, 22, 31 and Epc 16) prepared in talc-based formulation against the nematodes of banana, viz. *M. incognita, Pratylenchus coffeae, Radopholus similis* and *Helicotylenchus multicinctus* on tissue cultured banana cv. Robusta (*Musa* AAA). Significant increase in shoot height and weight, root length and weight, pseudostem girth and number of leaves coupled with reduction in nematode population was observed in the combined treatment of EPB 5 + 31.

Regina *et al* (1998) tested 21 strains of *Bacillus* spp. against second stage juveniles (J2) of *M. javanica* in vitro and in greenhouse bioassays. Bacterial supernatant and whole culture of *B. thuringiensis brasiliensis* and *B. laterosporus* killed freshly hatched J2 within 24-48 hours, whereas treatments with *B. thuringiensis aizawai*, *B. thuringiensis* morrisoni and *B. circulans* caused only immobilization. Greenhouse bioassays confirmed the nematicidal effect of the in vitro tests. Although the chemical nature of the supernants was not determined, the results suggested the presence of extra cellular toxins with strong nematicidal activity.Field experiments showed *B. thuringiensis* var. kurstaki h3 was highly effective against high population of *R. similis* in banana (Mena et al, 1997) and reduction in tomato root galling by 51-59% when M. javanica was inoculated (Khyami-Horani and Al Banna, 2006).

P. fluorescens and *B. thuringiensis* showed nematidal activity against juveniles and adults of *M. incognita* - infecting tomato plants. The mortality levels of M. incognita increased with increase in the concentration of bacterial cells (5 x 10^8 cfu/ml) (Hanna *et al*, 1999). Jonathan *et al* (2000) reported rhizobacteria, viz. P. fluorescens and *Bacillus spp*. induced profuse root development in banana, tomato and betel vine and reduced *M. incognita* population. *Brevibaccillus brevis* or *Bacillus subtilis* exhibited strong nematicidal activity

by killing the second stage larvae of *Meloidogyne javanica* to varying degrees in the greenhouse (Li Bin *et al*, 2005).

However, *P. fluorescens* and *Bacillus* spp. were most effective against *M. incognita*: Clover plants treated with these bacteria had fewer galls and large root (Becker *et al*., 1988). Bacillus licheniformis caused greater reduction in nematode multiplication than Alcaligenes faecalis on chickpea (Siddiqui and Mahmood, 1992). Rajendran *et al* (2001) reported that *P. fluorescens*, *Bacillus* spp. and VAM could be used as successful biocontrol agents for the management of *M. incognita* and *Tylenchulus semipenetrans* in horticultural crops such as citrus, tomato, potato and chilli. Application of *P. fluorescens* or *B. subtilis* increased the growth and yield of chickpea and reduced the infestation of *M. incognita* by minimizing the number of galls per root system, egg mass production and soil population (Khan *et al*, 2001).

2.5.2.2 Bio-control efficacy of *Bacillus* spp. on other pathogens

Treatment of cucumber seeds with strain mixtures comprising *B. pumilus* - INR7, *B. subtilis* –Gb03 and *Curtobacterium flaccumfaciens* - Mel with a mean bacterial density of 5 x 10^6cfu/seed reduced intensity of angular leaf spot and anthracnose (Raupach and Kloepper, 1998).

Among *Bacillus subtilis*, *B. amyloliquefaciens* and *B. megaterium*, *B. amylolequefaciens* proved to be the most effective antagonist against Phytophthora palmivora, the causal agent of bud rot of coconut (Jayasuja and Iyer, 2003). Bacillus sp. isolated from naturally occurring compost was found to be antagonistic to Fusarium solani, the causal agent of black rot of chickpea (Sriveni *et al*., 2004).

In vitro, *Bacillus sp*. Tr1 6, *B. pumilus* Tr24, *Serratia marcescens* Tr10 and Ochrobactrum anthropi Tr9 from rhizosphere of tea plants were antagonistic against three fungal pathogens, viz. Fomes lamaoensis, Poria hypobrumea and Sphaerostilbe repens (Chakraborty *et al.,* 2005).

2.5.2.3 Plant growth promotion by *Bacillus* spp.

PGPR enhance soil fertility by increasing the amount of available nitrogen and phosphorus and other plant nutrient and synthesize several different phytohormones that can act to enhance various stages of plant growth. Haque and Dave (2005) studied ecology and diversity of phosphate solubilizing microorganisms in soil under organic and non organic farming, virgin and barren soils of Gujarat. They found *Pseudomonas* spp., *Bacillus* spp., Saccharomyces spp. and *Aspergillus niger* to be most prevalent among the isolates. Plant growth responses were variable and dependent upon the inoculant strain, soil organic matter content, growing stage, and harvest date and growth parameters evaluated (Cakmakci *et al*., 2006).

Bacillus species have been reported to promote the growth of a wide range of plants (De Freitas *et al,* 1997; Kokalis-Burelle *et al*., 2002). Trials with rhizosphere associated plant growth promoting N2-fixing and phosphate solubilising *Bacillus* species indicated yield increase in sorghum (Broadbent *et al*., 1977), maize (Pal, 1998), rice (Sudha *et al*., 1999), sugarbeet (Cakmakci et al., 1999), barley (Sahin *et al*, 2004) and apples (Aslantas *et al.,* 2007).

Podile and Dube (1988) reported enhanced plant growth and yield for five crops through seed inoculation with an antibiotic producing strain of *B. subtilis*. In growth chamber, *B. subtilis* b2 significantly increased in shoot (12-94%) and root (13-100%) dry weight and shoot height (12-40%) over control in onion (Reddy and Rahe, 1989). *B. subtilis* A-13 increased yield of peanut up to 37% and reduced plant disease in field conditions (Turner and Backman, 1991). *B. subtilis* increased the dry and fresh weight of cucumber plants by 29%, fruit yield by 14% and fruit number by 50% in greenhouse (Uthede *et al*., 1999).

De Freitas *et al*. (1997) assessed the potential use of phosphate solubilizing bacilli and other rhizobacteria as biofertilizers for canola and reported that *Bacillus thuringiensis* isolate was that most effective inoculant which significantly increased the number and weight of pods and seed yield without rock phosphate.

Rajarathnam *et al*. (1995) found *B. megaterium*, *B. polymyxa* to be the most efficient phosphate solubilizing bacteria (PSB) in Tamil Nadu (India). Chanway (1995) studied *B. polymyxa* strain L6-16R and obtained significant increase in seedling emergence, height and biomass accumulation of western hemlock. The influence of inoculation of *Pseudomonas striata* and *B. polymyxa* on sorghum significantly increased in yield and nutrient uptake of sorghum as well as available phosphate content in soil (Jisha and Alagawadi, 1996). Increase in growth and yield of black gram under rice fallow conditions was reported by Sundari and Sureshkumar (2004) due to inoculation of *B. megaterium* var. phosphaticum.

The application of some *Bacillus spp*. has shown increased grain yield and plant biomass accumulation (Pal and Jalali, 1998). Afzal *et al*., (2005) reported increased yield and P uptake of wheat plants due to inoculation of mixture of *Pseudomonas* and *Bacillus* spp. Bacillus strains increased total bacteria and the PSB population, root and shoot weight as well as total N and P uptake by plants (Canbolat *et al*., 2006

3. MATERIALS AND METHODS

3.1. Description of the Experimental Site

The experiment was conducted at Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) under greenhouse and laboratory conditions in the year 2011/12. Jimma University College of Agriculture and Veterinary Medicine is geographically located at 346 km southwest of Addis Ababa at about 7^0, 33'N latitude and 36^0, 57' E longitude at an altitude of 1710 meter above sea level (m.a.s.l). The mean maximum and minimum temperature are 26.8^0C and 11.4^0C, respectively and the mean maximum and minimum relative humidities are 91.4% and 39.92% respectively. The mean annual rainfall of the area is 1500mm (BPEDORS, 2000).

3.2. Experimental Material

3.2.1. Plant material

Tomato cv. Marglobe was used for greenhouse experiments. Seeds were sterilized with 1% sodium hypoclorite solution for 3 minutes and were sown in germination tray which is filled by snad, clay and compost composition at 2:1:1 v/v.

3.2 .2 Preparation of soil

Soil used for the pot experiments was obtained from JUCAVM horticultural field. The soil was thoroughly mixed and air-dried by spreading in a thin layer on plastic sheet in sun. After drying the large stones and plant debris was removed by sieving through 3.5 mm pore size sieve. After this, mechanical analysis of the soil was performed. Soil used for the experiments was sandy and clay composition at 2:1 (v/v) with pH ranging from 7.2 with moisture holding capacity (MHC) of 45%. Total organic matter was 3.4-3.8%.

3.2.3. Transplanting of seedlings

Tomato plants grown in germination tray were shifted to 15×20 cm earthen pots. One day before transplanting the nursery pots (germination tray) were not watered for the hardening of seedlings. Seedlings were removed from germination tray carefully and then adhering soil was removed gently by shaking and then three seedlings planted in each pot.

3.2.4. Management of plant material in green house

During the course of greenhouse experiments, plants were always watered carefully by avoiding the risk of leaching nematodes out of the soil or drying out, especially for the first few days after nematode inoculation. Before conducting any experiment, plants were chosen for their uniformity in terms of height and size of root system. Seedlings were kept under greenhouse conditions with ambient temperatures ranging from 25 to 34 °C. Plants were not watered the day before harvest, facilitating washing out root systems the following day. At harvest, lose soil was first shaken off before soaking the roots in water for a few minutes. Having washed off the bulk of the soil, the roots were then transferred to a tray of shallow water and the remaining soil particles were washed out using a jet of water or picked out by hand. Once washed, clean roots were kept in a beaker of water until further processing.

3.2.5 Experimental design

The experiment was arranged in a Randomized Complete Block Design using three blocks. Two bio-controls (*Bacillus subtils* and *Trichoderma harzianum* were applied either alone or in combinations with nematode to check the nematode incidence and without nematode to check the growth promotion capacity of the bio-controls (table 2).

Table 2. Treatment arrangement in green houseexperiment

Treatment number	Treatments		Times of application
	Type	*Meloidogyne* spp	
1	Plant (control)	Un-inoculated	Two weeks before nematode application
2	Plant (control)	Inoculated	
3	*T. harzianum*	Un-inoculated	
4	*B. subtilis*	Un-inoculated	Simultaneously with nematode application
5	*T. harzianum* + *B. subtilis*	Un-inoculated	
6	*T. harzianum*	Inoculated	
7	*B. subtilis*	Inoculated	Two weeks after nematode application
8	*T. harzianum* + *B. subtilis*	Inoculated	

3.3. *Trichoderma harzianum* and *Bacillus subtilis* culture

Pure culture of *T. harzianum* was obtained from Ambo Agricultural Research Center (AARC) and pure culture of *B. subtilis* was obtained from Haramaya University. *T. harzianum* was grown using PDA (potato dextrose agar), but, *B. subtilis* was grown using Nutrient agar.

3.3.1 Preparation of *Trichoderma harzianum* suspension

Fungal suspensions was prepared by direct washing of 10 days old *T.harzianum* using sterile distilled water then filtered and counted using hemocytometer the spores were adjusted to $1x10^6$ spores / ml. two ml of this suspension was used for *in vitro* experiment. However, twenty ml of this suspension was inoculated per pot.

3.3.2 Preparation of *Bacillus subtilis* suspension

Three loopful of *B. subtilis* per 15 ml of nutrient broth was inoculated into 250 ml Erlenmeyer flasks of the conical flasks and grown for 72 hours on rotary shaker (120 rpm).The bacterial cells were harvested and counted following serial dilution, One ml contained $1.5x10^7$ CFU/ml. two ml and 20 ml of this suspension was inoculated in to each cuveets for *in vitro* experiments and into each pot around the tomato seedlings for *in vivo* experiments.

3.4. Nematode culture

Large numbers of *Meloidogyne* spp eggmasses were handpicked using sterilized forceps from heavily infected tomato roots on which a pure culture of the nematode was maintained. These egg masses were washed in distilled water and then poured in 10 cm diameter 15 mesh course sieves containing crosses layered tissue paper and placed in Petri plates containing water just deep enough to contact the egg masses. The hatched juveniles were collected from the Petri plates every 24 hours and fresh water was added to the Petri plates. The concentration of second stage juveniles of *Meloidogyne* spp in the water was adjusted so that each milliliter contained 100 ± 5 nematodes. Twenty ml of this suspension (i.e. 2000 freshly hatched juveniles) was added to each pot containing tomato seedlings.

3.5. Extraction of Root- knot Eggs

Egg masses of approximately the same size were hand picked from the roots of 8- week-old tomato plants. Roots infested with root-knot nematode were cutted it to small pices and placed in a wide mouth bottle in which 1% sodium hypochlorite solution was placed and the mouth was tightly closed. The bottle was shaken vigorously by hand for 3 min and contents were poured onto a 100-mesh sieve, fitted over a 400-mesh sieve. After washing under running tap water for 1 min, the residue collected on 400-mesh sieve was transferred into 250-ml beaker. Number of eggs per ml of suspension was determined with the help of a counting chamber (Hussey and Barker, 1973).

3.6 *In vitro* bioassays

Cuveets were carried out to determine activity of suspensions of *Trichoderma harzianum* and *Bacillus subtilis* against *Melodogyne* spp.

3.6.1 Egg bioassay

The eggs of *Meloidogyne* spp were surface disinfested with sadium hypchlorite solution (NaOCl) by agitation in a sterile vial for 2-3 min. surface sterilized eggs pipetted onto an autoclaved 25- p.m aperture sieve and rinsed them with sterile water (Meyer *et al*., 2000). The eggs were then placed into *Trichoderma harzianum,* and *Bacillus subtilis,* suspensions in 60- Cuveets. The cuveets were arranged in completely randomized design (CRD). Eggs placed in sterile distilled water served as control. There were five replications per treatment and approximately 100 eggs were placed into each well. Total number of unhatched eggs in each vial were counted at three different days interval (i.e. after four days, after six days and after eight days) after they were placed in the filtrates. The unhatched eggs were then transferred into sterile distilled water for 48 hours to see whether the egg hatching was inhibited partially or permanently. Percentage of unhatched eggs in a well was calculated as;

$$\text{Percent of egg hatching inhibition} = \frac{\text{Total unhatched eggs}}{\text{Total eggs per well}} \times 100\%$$

3.6.2 Juveniles (J_2) bioassay

Surface sterilized eggs were placed in sterile chamber to collect active juveniles (J2s) which hatch in 3-4 days. About 50 J_2s were placed in each micro well containing 2 ml of the suspensions. Each treatment was replicated five times. The wells were arranged in completely randomized design (CRD). After 48 hours, 72 hours and 96 hours incubation active and inactive J2s were counted in suspensions and sterilized distilled water (SDW) control. The treatments were replaced with water the following day, and motile J2s were counted again the next day. Juveniles were considered dead if they did not move when probed with fine needle (Siddiqui and Shaukat, 2004). Percent J_2 mortality in a well was calculated as:

$$\text{Percent J2 mortality} = \frac{\text{No. of inactive J2s}}{\text{Total J2s in a well}} \times 100\%$$

3.7. *In vivo* Studies

3.7.1 Experimental Design and Layout

The experiment was laid down as a factorial experiment with completely randomized block design where the factors were antagonistic microorganisms (TN, BN, TBN, T, B, TB, C and D) and three different times of application with three replications. Treatments were assigned to the experimental pots randomly.

3.8. Collection of greenhouse data

For collection of nematode data, all plants in each treatment were carefully uprooted 90 days after pot transplantation, (at the time of termination of the experiment or at last fruit picking). Roots were washed gently with running tap water and data were recorded on different parameters as follows:

3.8.1 Number of galls per plant root system

Galls on entire root system of all tomato plants were counted with low power stereo microscope (6X) and their means were calculated.

3.8.2 Galling, Index (GI)

The galling index for each root system was determined using a 0 to 5 scale ((0=no galls, 1= 1-2 galls, 2= 3-10 galls, 3=11-30 galls, 4= 31-100 and 5= >100 galls), following Bridges and Page (1980). The Disease severity index (DSI) was calculated based on the following formula:

Disease severity index (DSI) = $\frac{\Sigma(A \times B) \times 100}{\Sigma B \times 5}$

Where; A – Disease class (0, 1, 2, 3, 4 or 5)

B – Number of plants showing that disease class per treatment

3.8.3 Number of egg masses per root system.

Tomato roots were stained for 5-20 minutes in an aqueous solution of phloxine B (15 mg/l tap water as reported by Southey (1986). The number of egg masses was counted with a stereoscope.

3.8.4 Number of eggs per egg mass

Eggs were released from the gelatinous matrix with 1 .0% NaOCl (Hussey and Barker, 1973). The assessment of the number of eggs per egg mass was carried out in 10 randomly selected egg masses and their means were recorded.

3.8.5 Root and shoot fresh weight (g)

Tomato roots were carefully rinsed in tap water, separated from the shoot, blotted dry and weighed. Roots and shoots were weighed separately, and their weights (in grams) were recorded.

3.8.6 Root and shoot dry weights (g)

Roots and shoots of tomato plants were placed separately into paper bags and dried in an oven at 43°C for four days (McGovern *et al.,* 1992). Weights of the dry roots and shoots were determined and their means were calculated.

3.8.7 Root and shoot lengths (cm)

Root and shoot lengths were measured from the soil line to root and shoot apices. Data were taken on root and shoot lengths (cm) of all tomato plants from each pot and their means were calculated.

3.8.8 Number of flowers per plant

Number of flowers was counted separately from each plant.

3.8.9 Fruit set per plant

Number of fruits per plants was recorded by counting the fruits from all plants in each treatment.

3.8.10 Days to maturity

Days to maturity were recorded by counting number of days from sowing to flower initiation.

3.8.11 Fruit weight (g)

Tomato fruits from each plant and each pot were picked and weighed and averaged to calculate fruit weight per fruit.

3.9. Statistical analysis

Data of all the parameters were analyzed using analysis of variance (ANOVA) techniques and means were separated by Tukey's test, using SAS virsion 9.2 software (SAS Institute Inc 2002).

4. RESULTS AND DISCUSSIONS

4.1. In vitro bio-assay

Several bio-controls have been tested so far in controlling root-knot nematodes, *Meloidogyne* spp., with varying results (Kerry *et al*, 1992; Parveen *et al*., 1993; Hafeez *et al*., 2000, Meyer *et al*,, 2000, Rangaswamy *et al*, 2000, Sharon *et al.,* 2001; Sharon *et al*., 2007). There are many nematophagous fungi, and bacteria but not all of them are used as bio-control agents under field conditions due to their adverse effect on environment. However, some fungi and bacteria play a vital role in the natural decline of root-knot nematodes in heavily infested soils (Khan and Saxena, 1997; Eapen *et al*., 2005). In the present study, bio-control agents (*Trichoderma harzianum* and *Bacillus subtilis*) were evaluated for their biocontrol activity against root-knot nematode *Meloidogyne* spp., *in vitro* bioassays and under greenhouse conditions.

4.1.1 Egg hatching and Juveniles (J_2) mortality

Suspensions obtained from *T. harzianum*, *B. subtilis* and their combinations at different times of incubation significantly ($P \leq 0.05$) inhibited egg hatching of *Meloidogyne* spp., in the range from 40.80% inhibition after 8 days of incubation in *Bacillus subtilis* to 65% after 4 days of incubation period in *T. harzianum* and *B. subtilis* combination (TB). The minimum 3.40 % - 6.40% egg hatching inhibition was recorded in the sterile water control (C) (table 3). Inhibition of egg hatching was in opposite direction to the time of applications of the bio-control suspensions. This study was in agreeing with Sharma and Saxena (1992) report, which indicates the highest inhibition of *Meloidogyne* spp., eggs incubated with *T. harzianum*. This was also similar with (Imran, 2000) in which B. *subtilis* were found to release some compounds in culture media that reduced egg hatching of *M. javanica.*

Eggs play a key role in nematode perpetuation and survival under adverse environmental conditions. Egg shell protects the nematode larvae from environmental hazards. Most plant-parasitic nematode eggs have a second layer as a thick chitin layer of egg shell, and the biocontrols *Trichoderma harzianum* and *Bacillus subtilis* Produce chitinases, a-

gluconase and Cellulase, which helps in parasitizing the eggs of *M.javanica* (Chet and Baker, 2001).

Table 3. Effect of *Trichoderma harzianum*., *Bacillus subtilis*., and their combinations on the egg hatching inhibition (%) of *Meloidogyne* spp., *in vitro*.

	Time of application		
Treatment	1	2	3
C	6.4000 h	4.6000 hi	3.4000 i
T	56.0000 bc	47.2000 e	45.2000 ef
B	52.4000 d	43.6000 f	40.8000 g
TB	65.00 a	56.4000 b	54.0000 cd
Pvalue	<0.0001		
Cv%	2.61		
MSD	2.379		

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey test

C= sterile water control, T= *Trichoderma harzianum* , B= *Bacillus subtilis* , TB= combination of *Trichoderma harzianum* and *Bacillus subtilis* , 1= un hatching eggs after 4 days, 2= un hatching eggs, after6 days, 3 = un hatching eggs after 8 days, MSD = minimum significance difference.

T. harzianum produces different lytic enzymes such as polysaccharide lyases, proteases and lipases (Sherif and Benhamou, 1990 and Harman *et al*. 1993) and *B. subtilis* produced cytotoxin or/and putative emetic toxin (From *et al*., 2005). These enzymes play an important role in penetration of nematode egg surface, resulting in death of juveniles inside the hard and protective egg barriers.

In the present study both biocontrol treatments alone and in combination were found more effective in suppressing egg hatching, thereby increasing J_2 mortality. The maximum (96.20 %) and (93.80 %) mortality of juvenile two (J2) was shown in the combination of *T. harzianum* and *B. subtilis* (TB) and *T. harzianum* (T) alone respectively after 96 hours period of incubation. This was followed by *B. subtilis* in which 91.60% of the juveniles were killed after incubated for 96 hours. J_2 mortality

percentage was positively correlated with the time of incubation period in opposite to egg hatching inhibition. Minimum J_2 mortality was recorded from sterile water control (C) that is 6.6% after 48 hours of incubation to 14.0% at 96 hours of incubation periods (table 4). The differences in antagonistic activity of Bio-controls might be due to variations at the molecular level or the production of different toxic compounds and mechanisms (Hallmann and Sikora, 1996). The J_2 cuticle is composed mainly of proteins (Blaxter and Robertson, 1998) and the proteolytic enzymes produced by *T. harzianum* and *B. subtilis* (Harman *et al.,* 1993), decreased egg hatching and J_2 activity (Abd *et al.,* 1993; Pathak and Kumar 1995) which had similar result with the current study.

Table 4. Effect of *Trichoderma harzianum, Bacillus subtilis,* and their combination on the J_2 mortility of *Meloidogyne* spp., *in vitro*.

Treatments	Times of application		
	1	2	3
C	6.60 h	9.80 gh	14.00 g
T	61.20 f	81.40 d	93.80 ab
B	57.00 f	81.40 d	91.60 bc
TB	71.20 e	86.40 c	96.20 a
Pvalue	<0.0001		
Cv%	3.11		
MSD	4.2979		

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey test.

C= sterile water control, T= *Trichoderma harzianum,* B= *Bacillus subtilis* TB= combination of *Trichoderma harzianum* and *Bacillus subtilis,* 1= J2 mortility after 48 hours 2= J2 mortility after 72 hours 3= after 96 hrs the percentage of J2 mortality

T. harzianum has been reported as an egg parasite of *M. incognita* race 3, killing 53% eggs under *in vitro* (Dos Santos *et al*., 1992). And the use of this fungus also reported its ability to grow on the egg surface of potato cyst nematodes (Saifullah and Thomas, (1996), which were similar process with the current findings.

4.2 *In vivo* bio-assay

4.2.1 Number of galls and index per plant

The aim of this study was to investigate the antagonistic potentials of these biocontrol agents for the management of RKN that suit best to the local agro-ecological conditions. Results obtained are very encouraging showing drastic reduction in galling index and

increased plant growth. *T. harzianum* and *B. subtilis* when they become alone and in combination in all times of applications was greatly reduced the galling indices of tomato roots in root knot nematode-infested pots. Significant variations of the bio-control agents and time of applications depicted that these bio-agents had effective bio-control potential in times when they are applying before nematode inoculation.

Maximum reduction (25.32) in galls per plant was caused in combination of *T. harzianum* and *B. subtilis* (TBN), followed by (TN) from which 28.52 galls per plant was found, and this was statically similar with *B. subtilis* (30.18) (fig.1). Regarding the time of applications, significant reduction in number of galls per plant resulted at first (two weeks ago before nematode) than at simultaneously and two weeks after, that is (27.45), (32.09), and (36.02) galls respectively (fig 1).

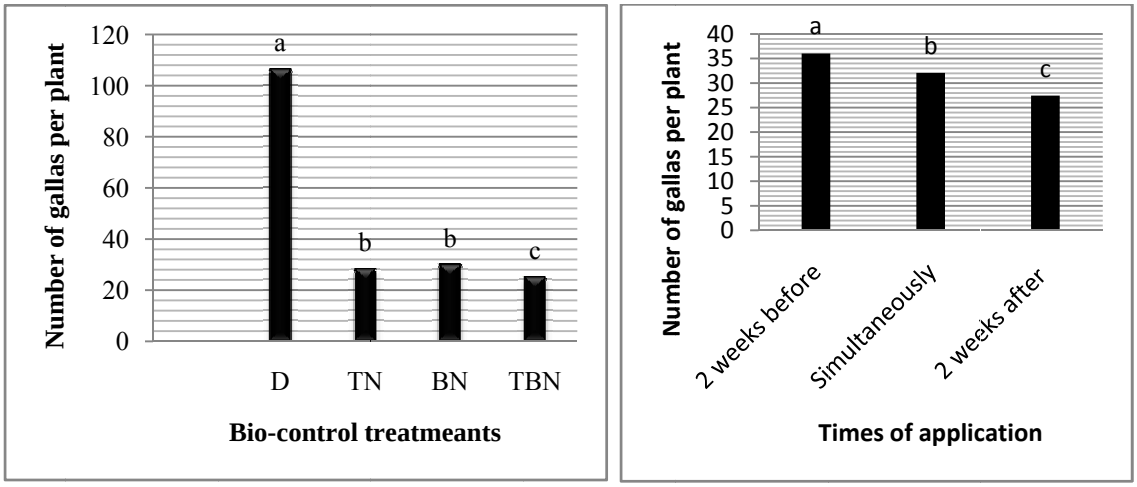


Fig.1 Effect of *Trichoderma harzianum*, *Bacillus subtilis,* and their combinations at different time of applications on the number of galls per plant caused by *Meloidogyne* spp., on tomato in greenhouse

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey's test.

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trychoderma harzianum,* BN= *Bacillus subtilis*+nematode TBN= combination of *Trychoderma* and *Bacillus* + nematode, before 2w = two weeks before nematode inoculation, simultaneously = simultaneously with nematode, after 2w= two weeks after nematode inoculation

The galling index was highly reduced (32.3 %) in combinations of *T. harzianum* and *B. subtilis* (TB), followed by *T. harzianum* (45%) which was stastically similar with *B. subtilis* (49%). The un-treated but infected control D caused maximum galling index (100

%). The data pertaining to application times depicts that the lower (33 %) galling index was recorded in first application (two weeks before nematode application time) followed by (43 %) at simultaneous application period. The maximum galling (64 %) index was shown when plants were inoculated by bio-controls after two weeks of nematode application (fig. 2). This result was in agree with the report by Hafeez *et al*. (2001), which observed 55.95% reduction in the number of galls in *T. harzianum* treated tomato plants. Rao *et al*. (1997) which reported significant reduction in root galling in tomato crop incorporated with *T. harzianum* under field conditions is also in agree with the result obtained. Concerning to the result found by *B. subtilis* was also similarly discussed by (Khan *et al*, 2001) in which application of *P. fluorescens* or *B. subtilis* were minimized the number of galls per root system.

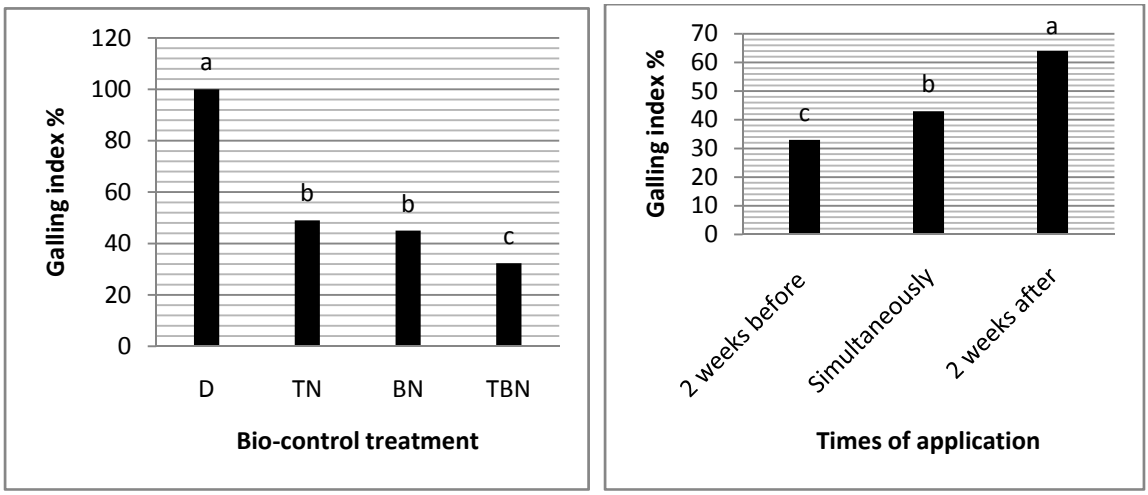


Fig.2 Effect of *Trichoderma harzianum*, *Bacillus subtilis*, and their combinations at different time of applications on the galling index of greenhouse grown tomato in *Meloidogyne* spp.,– inoculated plants

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey test.

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trichoderma harzianum,* BN= *Bacillus subtilis*+nematode TBN= combination of *Trichoderma* and *Bacillus* + nematode, before 2w = two weeks before nematode inoculation, simultaneously = simultaneously with nematode, after 2w= two weeks after nematode inoculation

In all most all parameters the combining suspensions of *T. harzianum*, and *B. subtilis* exhibited great reduction in galling index, the exact reason is not known, however, it might be due to different chemicals created and the mechanisms used by both bio-agents

4.2.2 Eggs and egg masses per root system

Meloidogyne spp. egg masses per root system were significantly reduced by TN and BN alone and with great effect when they were used in combinations. There was a significant effect of bio-control and time of applications on number of eggs per egg mass. The bio-controls reduced egg masses in the treated tomato plants to minimum (47.30) egg masses per root system and statistically similar (47.42) in both *T. harzianum* and *B. subtilis*, followed by (65.76) in the combinations of *T. harzianum* and *B. subtilis*. And the maximum egg masses per root system were recorded in the positive control (D). As for time of applications are concerned, the egg masses per root system were lower (46.14) in the first application time followed by the second (53.4) and third (58.59) respectively (Fig 3).

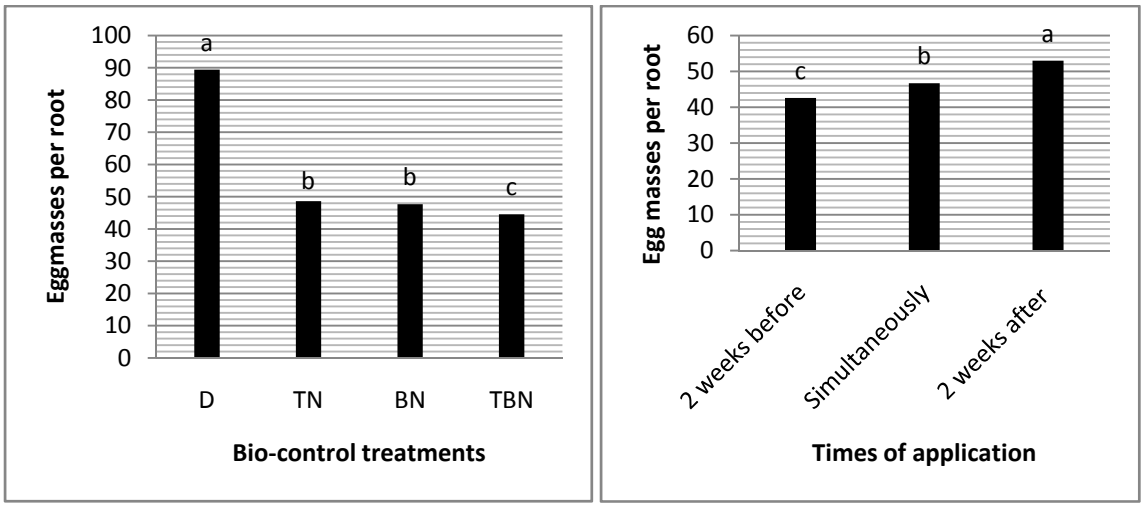


Fig.3 Effect of *Trichoderma harzianum*, *Bacillus subtilis*, and their combinations at different time of applications on egg masses per root system of greenhouse grown tomato in *Meloidogyne* spp., – inoculated plants

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey test.

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trichoderma harzianum,* BN= *Bacillus subtilis*+nematode TBN= combination of *Trichoderma* and *Bacillus* + nematode, before 2w = two weeks before nematode inoculation, simultaneously = simultaneously with nematode, after 2w= two weeks after nematode inoculation

The highest (297.77) number of eggs per eggmass was caused by nematode inoculated positive control D. This was followed by inoculated *T. harzianum* (276.08) and *B. subtilis* (273.28) both at third time of application with stastically similar to inoculated *T. harzianum and B. subtilis* alone and in combination. The minimum (210.00) with stastically similar (218.32) eggs per egg mass was noted in *T. harzianum* and *B. subtilis combination* and *T. harzianum* alone, both in the first time of applications. However all treatments show significant reduction of eggs per eggmass effect as comparing to the positive control (D) (Table 5.).

Table 5. Effect of different time applications of *Trichoderma harzianum, Bacillus subtilis*, and their combination on number of eggs per egg mass: *in vivo*

	Time of application		
TREATMENT	1	2	3
C	0.000 e	0.00 e	0.000 e
D	297.77 a	297.77 a	297.77 a
TN	218.32 d	255.72 b c	276.08 a b
BN	233.76 c d	249.70 b c	273.28 a b
TBN	210.00 d	249.66 b c	267.33 b
MSD	6.0354		
Cv%	2.99		
Pvalue	<0.04		

*Means of the same category followed by different letters are significantly different at 5% level of probability using Duncan's range test.

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trichoderma harzianum,* BN= *Bacillus subtilis* +nematode TBN= combination of *Trichoderma* and *Bacillus* + nematode, 1= two weeks before nematode inoculation, 2= simultaneously with nematode, 3= two weeks after nematode inoculation

Eggs of RKN are protected in a tough covering of gelatinous matrix; outside the nematode body which could have protected the eggs from toxic metabolites. The earlier study by Sankaranarayanan *et al*. (1999) was agreeing with the present studies in which three isolates of *T. harzianum* was reduced egg masses on root system of tomato plants.

However Sharon *et al*. (2001) disagrees as he reported that certain strains of *T. harzianum* could not grow on egg masses or penetrate the eggs inside gelatinous matrix. Goswami *et al*. (2006) found significant reduction in the egg masses of *M. incognita* on tomato treated with certain fungi alone and in different combinations including *B. subtilis*. And this was similar process with the current finding.

4.2.3. Growth promotion

The most typical effect of root knot nematode infection is a general reduction in plant growth. Results of the present studies clearly indicated that an encouraging result of growth parameters recorded of tomato plants. From *T. harzianum* alone and its combination with *B. subtilis* treated pots was significantly increased when it becomes alone without nematode and with nematode infested pots .but in *Bacillus subtilis* had less effect for the growth promotion parameters when inoculated without nematode as compared to the control and *T. harzianum* although it was antagonistic for the disease incidence.

4.2.3.1.Root and shoot length

The maximum increase (22.19cm) in root length of un-inoculated plants was caused by combination of *T. harzianum* and *B. subtilis* (TB) at first time of application. This was followed by un-inoculated *B. subtilis* (B) and *T. harzianum* at first time of application where root length was recorded (20.02cm) and (19.78cm) respectively. However the rest treatments shows non-significant effect as comparing to the negative control (C) from which 18.42 cm root length was recorded. Regarding inoculated treatments maximum root length was also recorded from the combination (TB) two weeks before nematode application. This was stastically similar with T, and B at first and second time of applications. The minimum root length was recorded from inoculated control D (15.55 cm) this was significantly varied from all bio-controls applied at different times (Table 6).

Maximum (81.27cm) tomato shoot length was noted in Un-inoculated combination of *T. harzianum* and *B. subtilis* (TB) at first time of application with stastically similar (21.43cm) to TB at simultaneous application time. This was followed by un-inoculated *T. harzianum* (T) at first time of application where shoot length was recorded (76.43cm) with stastically similar to Un-inoculated *T. harzianum* (T) from which (76.13cm) shoot length was obtained. However the rest treatments shows non-significant effect as comparing to the negative control (C) from which 74.75 cm shoot length was recorded. On the subject of inoculated treatments maximum shoot length was also confirmed from the combination (TB) two weeks before nematode application (73.28cm). This was stastically similar with T, from which 71.0 cm was obtained. The minimum shoot length was recorded from inoculated control D (55.77cm) which was significantly varied from all bio-controls applied at different times (Table 6).

In general regarding the root and shoot length the present study indicates that, *T. harzianum* alone and in combination with *B. subtilis* showed significant increase in tomato root and shoot length. Although *B. subtilis* alone not shown significant variations when it become without nematode it was shown a significance variation as comparing to positive control D. differences in interactions among the bio-controls with time of applications infer that both these bio-controls have different RKN antagonistic but *T. harzianum* had better plant growth promoting capability in each time of applications. Molecules produced by *T. harzianum* have potential for promoting plant growth and nematode suppression that lead to enhanced root and shoot length of tomato plants. This finding have been agreed with the report said different strains of *T. harzianum* causes the increment of root and shoot length (Bjorkman *et al*. 1994; Inbar *et al*. 1994; Altomare *et al.* 1999; Harman, 2000; Yang *et al*. 2000).

Table 6. Effect of *Trichoderma harzianum*, and *Bacillus subtilis*, and their combinations at different times of application on the growth of greenhouse grown tomato in *Meloidogyne* spp., – inoculated or un-inoculated plants, Jimma

Meloidogyne spp	Treatment	Root length (cm)	Shoot length (cm)	Dry root weight (g)	Fresh shoot weight (g)
Un-inoculated	Control	18.42 defgh	74.75 cdef	7.66 c	62.14 cdefg
	T1	19.78 cd	76.43 bcd	7.98 bc	66.56 a b
	T2	19.6967 cde	76.13 bcde	7.93 bc	65.38 abc
	T3	19.23 cdef	75.30 cdef	7.73 bc	64.45 abcde
	B1	20.0200 bc	74.11 def	7.62 c	63.37 abcde
	B2	18.6400 cdefg	72.46 fgh	7.36 c	62.49 bcdef
	B3	18.28 efgh	71.13 gh	7.32 c	60.96 defgh
	TB1	22.19 a	81.27 a	9.14 a	66.92 a
	TB2	21.4300 ab	78.99 ab	8.54 ab	65.04 abcd
	TB3	19.23 cdef	77.12 bc	8.02 bc	63.23 abcde
Inoculated	Control	15.55 k	55.77 m	9.23 a	45.357 k
	T1	18.20 fgh	71.00 ghi	8.03 bc	60.72 efgh
	T2	17.1133 hij	68.10 ijk	7.82 bc	58.28 ghi
	T3	16.44 ijk	65.80 kl	7.69 bc	53.550 j
	B1	17.77 ghi	69.61 hij	7.68 c	61.003 defgh
	B2	17.0633 hij	66.82 jkl	7.54 c	57.88 hi
	B3	16.23 jk	64.65 l	7.37 c	54.693 ij
	TB1	18.40 defgh	73.28 efg	7.72 bc	61.70 cdefgh
	TB2	17.5833 ghij	70.94 ghi	7.42 c	58.77 fghi
	TB3	16.34 ijk	66.600 kl	7.21 c	55.237 ij
Pvalue		<0.0001	<0.0001	<0.0307	<0.014
Cv %		2.45	1.21	3.55	2.25
MSD		1.4353	2.9078	0.8577	4.1602

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trichoderma spp.,* BN= *Bacillus*+nematode TBN= combination of *Trichoderma* and *Bacillus* + nematode, T= *Trichoderma spp only B= Bacillus only, TB*= combination of *Trichoderma* and *Bacillus only,* 1= two weeks before nematode inoculation, 2= simultaneously with nematode, 3= two weeks after nematode inoculation.

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey's test.

4.2.3.2.Root and shoot weight

Root weight of tomato plants was found to be reduced in the *Trichoderma harzianum* and *Bacillus subtilis* alone treated plants as compared to the infested control (D). This might be due to the accumulation of synthesized food in the galled roots of root knot nematode infected tomato plants. Galls induced by *Meloidogyne* spp., in tomato have decreased concentration of polysaccharides such as cellulose and increased the concentration of nucleic acids and amino acids (Owens and Specht, 1966). Gommers and Dropkin (1977) observed high concentrations of protein and caiioliydndes in giant cell induced by *Meledogyne* spp.and this was in agree with present findings in which maximum root dry weight was recorded in positive control (D) from which (9.23gm) was recorded . This was followed by TB (7.72gm) at two weeks before application period. Regarding to Un-inoculated treatments maximum (9.14gm) tomato dry root weight was noted in combination of *T. harzianum* and *B. subtilis* (TB) at first time of application with stastically similar (8.54) to TB at the second times of application. This was followed by un-inoculated *T. harzianum* (T) at first time of application where dry weight was recorded (7.98 gm). This was not stastically different with *T. harzianum* (T) from which (7.93 gm) was recorded. However the rest treatments shows non-significant effect as comparing to the negative control (C) from which 7.66 gm dry weight was recorded (Table 6).

The minimum fresh root weight (22.09 g) with statically similar to (22.24 g) and (22.36g) was recorded in BN, TBN and B respectively. While maximum (26.26 g) fresh root weight was noted in the infected control plants (D) (plant with nematode but without bio-control agents) .This was followed by TN (23.62 g) with stastically similar to TB (23.53g) (Fig. 4.).The report by Jonathan *et al* (2000) was in agree with the present study in which rhizobacteria, viz. *P. fluorescens* and *Bacillus* spp., were induced profuse root development in banana, tomato and betel vine and reduced *M. incognita* population.

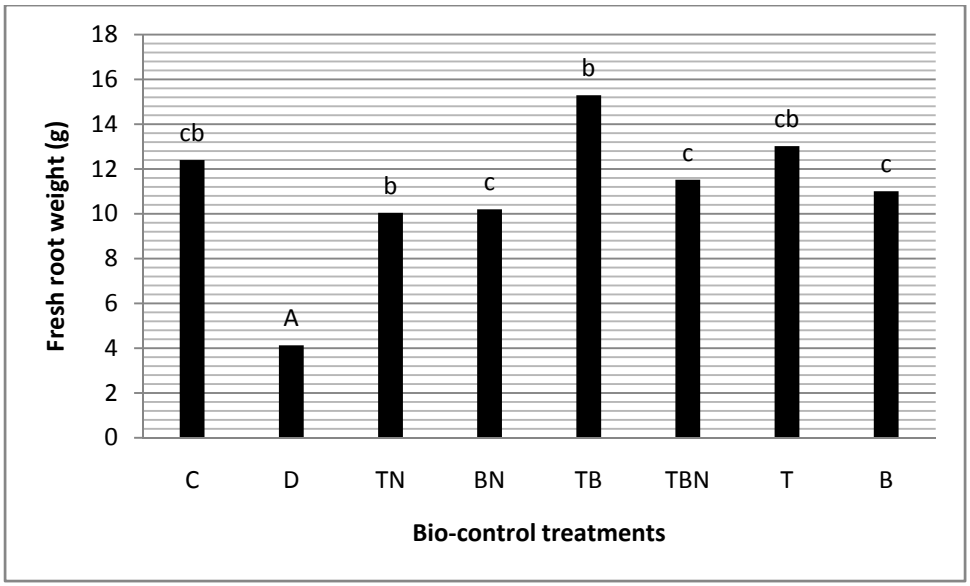


Fig.4. Effect of *Trichoderma harzianum*, *Bacillus subtilis*, and their combination on fresh root weight of greenhouse grown tomato in *Meloidogyne* spp., – inoculated and un-inoculated plants at Jimma

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey test.

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trychoderma harzianum.,* BN= *Bacillus subtilis* +nematode TBN= combination of *Trychoderma harzianum* and *Bacillus subtilis*+ nematode, T= *Trychoderma harzianum only B= Bacillus subtilis only, TB*= combination of *Trychoderma* and *Bacillus,* 1= two weeks before nematode inoculation, 2= simultaneously with nematode, 3= two weeks after nematode inoculation.

The enhanced-growth characteristic contributed by the incorporation of *T. harzianum* into the RKN-infested soil has been reported by Kleifield and Chet (1992). It might also be due to the antagonistic properties of *T. harzianum* against RKN that was exhibited in reduction of root knot galling thereby reducing nematode population in the soil (Sharon *et al*., 2001). Several researchers (Chet, 1990; Datnoff and Pernezny, 1998; McGovern at al. 1998; Rao *et al*. 1997 and Stephan *et al*. 1996) reported that *T. harzianum* significantly enhanced shoot weight of tomato plants. And this report was in agree with the current finding in which Maximum (66.92gm) tomato shoot fresh weight was noted in Un-inoculated combination of *T. harzianum* and *B. subtilis* (TB) at first time of application with stastically similar to T (66.56gm). However the rest treatments shows

non-significant effect as comparing to the negative control (C) from which 62.14 gm fresh shoot weight was recorded. Regarding inoculated treatments maximum fresh shoot weight was recorded from the combination (TB) two weeks before nematode application (61.70gm). This was stastically similar with T, in which 60.72 gm was recorded. The minimum shoot fresh weight was obtained from inoculated control D (45.35g) this was significantly varied from all bio-controls at different times of applications (Table 4).

Maximum (25.62 g) dry shoot weight was documented in un-inoculated *T. harzianum* (T1) followed by (24.76g) that was observed in *T. harzianum* and *Bacillus subtilis* combination (TB1) at first time of application. However the rest treatments shows non-significant effect as comparing to the negative control (C) from which 21.44 g dry shoot weight was recorded. As to inoculated treatments maximum shoot dry weight was recorded in the combination (TB) two weeks before nematode application (24.69g). This was stastically similar with T, from which 23.79g was obtained.The minimum shoot dry weight was caused by inoculated control D (14.80 g) this was significantly varied from all bio-controls at all times of applications (table 7).

4.2.3.3.Number of flowers and fruits

Results of the present studies show that *Trichoderma harzianum* alone and in combination significantly increased flower production and fruit set in *T. harzianum* tomato plants as compared to others bio-controls and both controls. The fastest (52 days) in flower initiation was caused by TB followed by T1 (56.33 days) at first time of application. And the late in maturit was shown (65) days in un-inoculated B3, which was statistically similar to negative control C from which 64.33 days was recorded. Concerning inoculated treatments the fastest days to maturity was recorded from the combination TB (54.33days) two weeks before nematode applications. The lowest speed of flower initiation was recorded from positive control D from which 71.66 days was recorded and this was stastically varied from all treatments at all times of applications (Table 7).

Table 7. Effect of *Trichoderma harzianum*, *Bacillus subtilis*, and their combinations at different times of application on the growth of greenhouse grown tomato in *Meloidogyne* spp., – inoculated or un-inoculated plants, Jimma

Meloidogyne spp	Treatment	Dry Shoot Weight(g)	Fruit Weight(g)	Days to Maturity	Number of flowers per plant
Un-inoculated	Control	21.4433 bcd	54.540 cde	64.3333cde	60.523 fg
	T1	25.623 a	60.323 ab	56.3333 h	67.690 b
	T2	23.810 abcd	54.033 cdef	60.6667 fg	65.130 cd
	T3	23.2667 abcd	52.947 def	62.6667 defg	61.230 ef
	B1	20.7100 bc	54.000 cdef	63.3333 defg	60.717 gf
	B2	20.5200 bc	51.113 ef	63.666D def	60.143 gf
	B3	20.7167 cd	50.520 f	65.0000 bcde	60.073 gf
	TB1	24.7600 ab	61.230 a	52.0000 i	70.540 a
	TB2	23.4100 abc	57.430 abcd	56.0000 h	63.115 ed
	TB3	22.09 bcd	51.230 def	62.0000 efg	56.670 ih
Inoculated	Control	14.80 e	18.853 j	71.6667 a	33.457 k
	T1	23.79 abc	55.070 cd	62.6667 defg	63.107 ed
	T2	22.34 abcd	43.097 gh	65.0000 bcde	58.390 gh
	T3	20.92 cd	39.290 hi	68.0000 b	53.810 j
	B1	22.12 bcd	51.730 def	63.666 def	59.437 gf
	B2	20.9667 cd	42.597 gh	65.3333 bcde	56.810 ih
	B3	19.9567 d	37.200 i	67.6667 bc	53.410 j
	TB1	24.69 ab	57.197 bc	54.3333 hi	66.180 bc
	TB2	21.67 bcd	50.437 f	60.0000 g	60.427 fg
	TB3	20.57 ef	44.787 g	65.6667 bcd	55.023 ij
Pvalue		<0.0026	<0.0001	<0.0001	<0.0001
Cv %		4.84	2.53	1.86	2.27
MSD		3.494	3.8409	3.6161	2.5213

*Means of the same category followed by different letters are significantly different at 5% Level of probability using Tukey test.

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trichoderma harzianum.*, BN= *Bacillus*+nematode TBN= combination of *Trichoderma* and *Bacillus* + nematode, T= *Trichoderma harzianum only B= Bacillus only, TB*= combination of *Trichoderma* and *Bacillus only,* 1= two weeks before nematode inoculation, 2= simultaneously with nematode, 3= two weeks after nematode inoculation

Similarly maximum (70.54) tomato flowers were confirmed in TB, followed by (67.69) flowers per plant in T at first time of applications of the nematode un-inoculated treatments. Similarly combination TB nematode inoculated treatments caused vast flowers (66.18) at two weeks before nematode applications. The minimum flowers were obtained from positive control D from which 33.47 was recorded which was statically varied from all treatments at all times of application (Table 7).

The highest (15.29) fruits per plant was recorded in TB combinations followed by *T. harzianum* (T) un-inoculated treatments from which 13.02 fruits was recorded. Concerning to the inoculated treatments maximum fruit number was recorded in TBN which was 11.52 fruits. And the minimum number of fruits was recorded from the positive control D from which 4.13 fruits was obtained. Application of the bio-controls before two weeks records maximum fruit number (12.45 % in T, and 12.40 % in C followed by (10.82) fruit per plant in BN (Fig. 5).

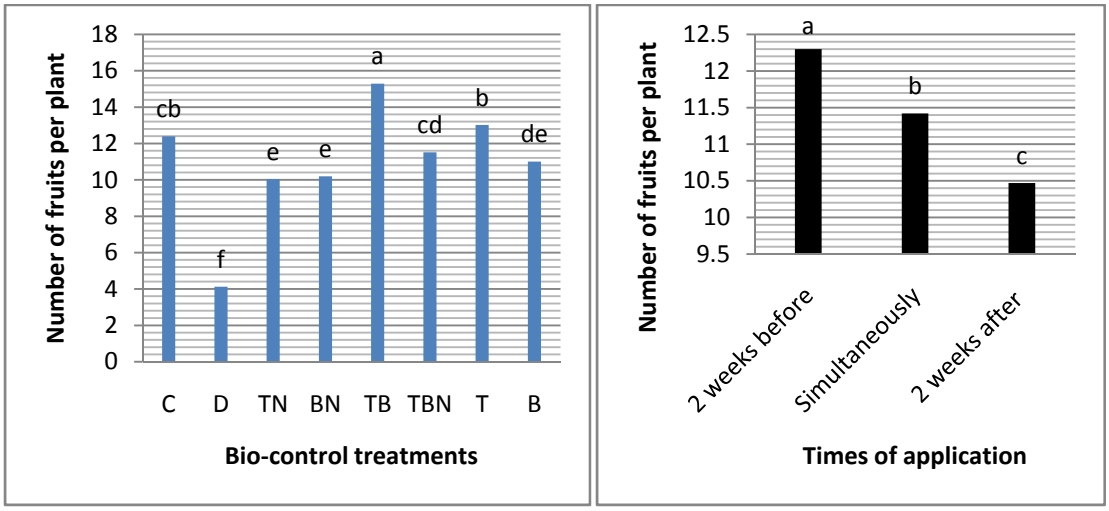


Fig.5. Effect of *Trichoderma harzianum*, *Bacillus subtilis*, and their combinations on the fruit set per plant of greenhouse grown tomato in *Meloidogyne* spp.,– inoculated and un-inoculated plants

*Means of the same category followed by different letters are significantly different at 5% level of probability using Tukey test.

C= Tomato plant without nematode and bio-control D= Tomato plant with nematode but without bio-controls, TN= nematode+ *Trychoderma spp.,* BN= *Bacillus*+nematode TBN= combination of *Trychoderma* and *Bacillus* + nematode, T= *Trychoderma spp only B= Bacillus only, TB*= combination of *Trychoderma* and *Bacillus only,* 1= two weeks before nematode inoculation, 2= simultaneously with nematode, 3= two weeks after nematode inoculation

Vascular system of galled roots is partially or completely blocked and the translocation of water and nutrients to flowers and fruits is reduced (Dropkin and King, 1956) which causes a reduction in flower number and fruit set. Monte and Llobell (2003) concluded

similar to the present study that that *T. harzianum* particularly *T. harzianum* is not only a good biocontrol agent, but also a growth promoter, as it enhances plant growth and increases fruit production. *Bacillus* species have been reported to promote the growth of a wide range of plants (De Freitas *et al,* 1997; Kokalis-Burelle *et al*., 2002), however, this was disagree with the present study.

Although Podile and Dube (1988) reported enhanced plant growth and yield for five crops through seed bacterization with an antibiotic producing strain of *B. subtilis*, however in this result *B. subtillis* in almost all growth promotion parameters were shown non-significant difference to the negative control when inoculated alone. But there was growth enhancement by *B. subtilis* also by reducing the disease incidences in all parameters as comparing to the positive control D. the present study was disgree with reports in growth chamber, by Reddy and Rahe, 1989, that B. *subtilis* b2 significantly increased in shoot (12-94%) and root (13-100%) dry weight and shoot height (12-40%) over control in onion. *B. subtilis* A-13 increased yield of peanut up to 37% and reduced plant disease in field conditions (Turner and Backman, 1991). *B. subtilis* has been also suggested in increment of the dry and fresh weight of cucumber plants by 29%, fruit yield by 14% and fruit number by 50% in greenhouse (Uthede *et al*., 1999). The application of some *Bacillus* spp. has shown increased grain yield and plant biomass accumulation (Pal and Jalali, 1998). Afzal *et al*., (2005) reported increased yield and P uptake of wheat plants due to inoculation of mixture of *Pseudomonas* and *Bacillus* spp. *Bacillus* strains increased total bacteria and the PSB population, root and shoot weight as well as total N and P uptake by plants (Canbolat *et al*., 2006). However all these reports were varied from the current findings as *B. subtilis* was not caused an increment of growth parameters

The present findings confirmed results of previous research conducted in different places of the world and elsewhere on the use of *Trichoderma harzianum* and *Bacillus subtilis* for management of root knot nematodes. *Trichoderma* and *Bacillus* are known as mycoparasites of many organisms causing diseases (Chet and Baker, 1980; Elad *et al*., 1980; Papavizas *et a*l., 1982; Radar *et al*., 1984; Lifshitz *et al*, 1986; and Sivan *et al*., 1987). However, it is not at all pathogenic in the absence of *Meloidogyne spp*. (Windham

et al., 1986). In general, *Trichoderma spp*., and *Bacillus spp* are not plant pathogenic organism, but rather plant growth promoters ((Windham et al, 1989).), No disease symptoms were seen on all bio-control treated roots of tomato plants at the time of data collection and thus our bio-controls were not pathogenic to the plants.

5. CONCULUSION

The tested bio-controls and three times of application, *Trichoderma harzianum* and *Bacillus subtilis,* alone and with great effect when it comes in combination two weeks before nematode applications were found effective antagonists of root knot nematode *Meloidogyne* spp both under laboratory and greenhouse conditions. However, only *T. harzianum* enhanced growth of the tomato plants when inoculated alone without nematode and with nematode. But *B. subtilis* controlled the disease but it had not growth enhancement capacity when inoculated alone with out nematode. Further studies are required to solve problems in relation to, the effectiveness of the bio-controls under different agro-ecological zones of Ethiopia in field conditions; and interactions of the antagonist and nematodes within roots; effect of the bio-agents on giant cell.

6. REFFERENCES

Ahmad, R., Abbas M.K., Khan MA., Inam-uI-Haq M., Javed N and Sahi. S.T. 1994. Evaluation of different methods of application of *Pasteuria penetrans* for the biocontrol of root-knot nematodes (*Meloidogyne incognita*) of tomato. Pak. J. Nematol. 12: 155-160.

Akhtar, M. 995. Biological control of the root-knot nematode *M. incognita* in tomato by the predatory nematode *Mononchus aquaticus*. Internl. Pest Control. 37: 18-19.

Al Banna, L. and Khyami-Horani, H. 2004, Nematicidal activity of two Jordanian strains of *Bacillus thuringiensis* on root-knot nematodes. Nematol. Medit. 32: 41-45.

Alexander, M. 1961. Introduction to Soil Microbiology. Wiley International Edition, Topapn Company Ltd., Japan, p. 19-44.

Aslantas, R., Cakmakci, R. and Sahin, F. 2007, Effect of plant growth promoting rhizobacteria on young apple trees growth and fruit yield under orchard conditions. Sci. Hort. 111:371-377.

Baker, K.F. and Cook.R.I. 1974. Biological control of plant pathogens. W.H. Freeman, San Francisco, CA. pp. 433.

Becker, J. O., Zavaleta-Mejia, E., Colbert, S. F., Schroth, M. N., Weinhold, A. R., Hancock, J. G and VanGundy, S. D. 1988, Effects of rhizobacteria on root-knot nematodes and gall formation. Phytopath. 78: 1466-1469.

Bhat, M.S., Irshad M and Mahmood. I. 2000. Role of *Glomus mosseae* and *P .lilacinus* in the management of root knot-nematode on tomato. Arch. of Phytopath and Plant Prot. 33: 131-140.

Bhatti, D. S. and Jam, R. K., 1977, Estimation of loss in okra, tomato and brinjal due to Meloidogyne incognita. India J. Nematoh, 7: 37-41.

Broadbent, P., Baker, K. F., Franks, N and Holland, J. 1977, Effect of *Bacillus* spp. on increased growth of seedlings in steamed and in non treated soil. Phytopathol. 67:1027-1034.

Browning, J. A., 1998, One phytopathologist's growth through 1PM to holistic plant health: the key to approaching genetic yield potential. Ann. Rev. Phytopathol., 36: 1-24.

Cakmakci, R., Kantar, F and Algur, O. F. 1999. Sugar beet and barley yields in relation to *Bacillus polymyxa* and *Bacillus megaterium* var. phosphaticum inoculation. J. Plant Nutr. Soil Sci. 162: 437-442.

Cakmakci R., Donmez, F., Aydin, A and Sahin, F. 2006. Growth promotion of plants by plant growth promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biol. Biochem. 38: 1482-1487.

Canbolat, M., Bilen, S., Cakmakci, R., Sahin, F and Aydin, A. 2006. Effect of plant growth promoting rhizobacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biol. Fertil. Soil. 42: 350-357.

Chakraborty, U., Chakraborty, B., Basnet, M and Lanjey Bhutia, P. 2005. PGPR mediated induction of resistance in tea plants for management of root rot diseases. In: Proc. Asian Con. on Emerging Trends in P1. Microbe Interactions, Chennai, Dec. 08-10, 2005, (Eds. S. S. Gnanamanickam, R., Balasubramanian and N. Anand), p. 42-50.

Chanway, C. P. 1995. Differential response of western hemlock from low and high elevations to inoculation with plant growth promoting *Bacillus polymyxa*. Soil Bio. Biochem. 27:767-775.

Chanway, C. P and Holl, F. B. 1993. First year field performance of spruce seedlings inoculated with plant growth-promoting rhizobacteria. Can. J. Microbiol. 39: 1084- 1088.

Chanway, C. P., Hynes, R. K and Nelson, L. M. 1989. Plant growth-promoting rhizobacteria: Effects on growth and nitrogen fixation of lentils and pea. Soil Bio. Biochem. 21: 511-517.

Chen, J., Abawi, G. S. and Zuckerman, B. M. 2000. Efficacy of *Bacillus thuringiensis, Paecilomyces marquandii*, and *Streptomyces costaricanus* with and without organic amendments against Meloidogyne hapla infecting lettuce. J. Nematol. 32: 70-77.

Chet, I. 2010. Biological control of soil-borne plant pathogens with fungal antagonists in the presence of biological control agent *Trichoderma harzianum*. Plant Dis. 70: 145-148.

Chet, I and R. Baker. 2000. Induction of suppressiveness to *Rhizoctonia solani* in soil. Phytopathol. 70: 994-998.

Chet, I and R. Baker. 2001. Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive to *Rhizoctonia solani* in the soil. Phytopathol. 71: 286-290.

Cook, R. J. 1996. Assuring the safe use microbial biocontrol agents: a need for policy based on real rather than perceived risks. Can. J. Plant. Pathol. 18: 439-445.

Cook, R. J., Bruckart, W. L., Coulson, J. R., Goettel, M. S., Humber, R. A., Lumsden, R. D and Vaughn, J. L. 1996. Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. Bio. Cont. 7: 333-351.

Dantoff, L.E and Pernenzy, K.L. 1998. Effect of bacterial and fungal microorganism to colonize tomato roots, improve transplant growth and control of *Fusarium* crown and root rot. Florida Tomato Intl. Proceedings. 100: 337-346.

Dasgupta, D. R. and Gaur, H. 5., 1986, The root knot nematodes, Meloidogyne spp. in India. In: Plant parasitic nematodes of India problems and prospects. Eds. Swarup, G. and

Dasgupta, D. R., IARI (Allied Publisher), New Delhi, India, p. 139-1 71.Tomato Intl. Proceedings. 100: 337-346.

Debora, B., R. Sevastianos., Ashok., C.Z. Paulo., P. Jorge. 2005. Development of a bionematicide with *Paecilomyces lilacinus* to Control *Meloidogyne incognita*. World 3. Agric. Sci. 1: 1 8-21.

De Freitas, J. R., Banerjee, M. R and Germida, J. J. 1997. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol. Fert. Soil. 24: 358-364.

Dhawan, S. C., Kaur, S and Singh, A. 2004, Effect of *Bacillus thuringiensis* on the mortality of root knot nematode, *Meloidogyne incognita*. Indian J. Nematol., 34: 98-99.

Dos Santos, M.A., Ferraz. S and Muchovez, J.J. 1992. Evaluation of 20 species of fungi from Brazil for biocontrol of *Meloidogyne incognita* race-3. Nematropica. 22: 183-192.

Eisenback, J.D and H.R. Triantaphyllou. 1991. Root-knot nematodes: *Meloidogyne* spp. and races. Pages.191-274. in: Manual of Agricultural Nematology. W.R. Nickle Marsel Dekker, Inc. New York, USA.

Elad, Y. 1995. Mycoparasitism. Pages. 289-307. In: Pathogenesis and Most specificity in Plant Diseases. Vol.2. K. Kohmoto, U.S. Singh and R.P. Singh, ed. Elsevier Science, Oxford, UK.

Elad, Y., Chet, I and Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can. J. Microbiol. 28: 7 19-725.

From, C., Pukall, R., Schumann, P., Hormazabal, V and Granum, P. E. 2005, Toxin producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. App. Environ. Microbioh, 71:1178-1183.

Garbeva, P., Van Veen, J. A and Van Elsas, J. D. 2003. Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microb. Ecol. 45: 302-31 6.

Gibson, T and Gordon, R. E. 1974, Genus I *Bacillus* Cohn 1872, 174. In: Bergey's Manual of Determinative Bacteriology. Eds. R. E. Buchanan & N. E. Gibbons. Baltimore, Williams and Wilkins, 81h edn., p. 529-550.

Giovannucci, E., Ascherio. A., Rimm. E.B., Stampfer, M.J., Colditz, G.A and Willett, W.C. 1995. Intake of carotenoids and retinol in relation to risk of prostate cancer. 3. Natl. Cancer Inst. 87: 1767-76.

Giovannucci, E. 1999. Tomatoes, Tomato-Based Products, Lycopene, and Cancer: Review of the Epidemiologic Literature. 3. NatI. Cancer Inst. 01: 317-33 1. 122

Ghaffar, A and S. Hashmi. 2003. *Lens esculenta* a new host of *M. incognita*, F.A.O. P1. Prot. Bull. 21-132.

Gokte, N and Swarup, G. 1988. On the potential of some bacterial biocides against root-knot and cyst nematodes. Indian J. Nematol., 18: 152-153.

Goswami, B.K., Pandey, R.K., Rathour, K.S., Bhattacharya, C and Siugh. L. 2006. Integrated application of some compatible biocontrol agents along with mustard oil seed cake and furadan on *Meloidogyne incognita* infecting tomato plants. J. Zhejiang Univ. Sci. 8. 7: 873-875.

Griffiths, B. S. 1989, The role of bacterial feeding nematodes and protozoa in rhizosphere nutrient cycling. Asp. AppL BioL, 22: 141 -145.

Hanna, A. L., Raid, F. W and Tawfik, A. E. 1999. Efficacy of antagonistic rhizobacteria on the control of root knot nematode, *Meloidogyne incognita* in tomato plants. Egyptian J. Agr. Res., 77: 1467-1476.

Hafeez, U.K., Riaz, A., Waqar, A., Khan S.M and Akhtar, S. 2000. Evaluation of chemical vs. biological control treatments against root-knot nematode (*M. incognita*) on tomato, Pak. J. Phytopathol. 12: 118-120.

Haque, N. A and Dave, S. R. 2005, Ecology of phosphate solubilizers in semi-arid agricultural soils. Indian J. Microbiol. 45: 27-32.

Jacobsen, B. J., Zidack, N. K and Larson, B. J. 2004. The role of *Bacillus* based biological control agents in integrated pest management systems: Plant diseases. Phytopathoh. 94: 1271-1275.

Jam, R. K., Dabour, K. R. and Gupta, D. C., 1994, Assessment of avoidable losses in yield due to root knot nematodes (Meloidogyne spp.) in a few vegetable crops. Indian J. Nematol., 24: 181 -184.

Jayasuja, V and Iyer, R. 2003. Multiplication and translocations of introduced endohytic antagonistic bacterium (*Bacillus amyloliquefaciens*) in coconut seedlings. 6' Intl. PGPRworshop, 5-10 October2003, Calicut, India, p.33-37.

Jisha, M. S. and Alagawadi, A. R. 1996. Nutrient uptake and yield of sorghum (*Sorghum bicolor* L.) inoculated with phosphate solubilizing bacteria and cellulolytic fungus in a cotton stalk amended vertisol. Microbioh Res. 151: 213-21 7.

Jonathan, E. I and Umamaheswari, R. 2006, Biomanagement of nematodes infesting banana by bacterial endophytes (*Bacillus subtilis*). Indian J. Nematol. 36: 230-233.

Hussey, R.S. and Barker. K.R. 1973. A comparison of methods of collecting inocula for *Meloidogyne* spp., including a new technique. Plant Dis. Rep. 57: 1025-1028.

Kemiarrec, A., Maulcon M. and Sirusirigh. C. 1991. Action of the entomopathogen in nematodes Stein *Emeina carpocapsae* and *Heterorhabditis bacteriophora* on the reproduction of the root-knot nematode *M. incognita*. Mededelingen van de Faculteit Landbouwweten schappen, Rijksuniversteit Gent. 56: 1293-1296.

Khan, T.A and Saxena. S.K. 1997. Effect of root-dip treatment with culture filtrates of soil fungi on multiplication of *M.javanica* and yield of tomato. Tests of Agrochemic. and Cultivars. 18: 50-5 1.

Khan, M. R., Khan, S. M and Khan, N. 2001. Effects of soil application of certain fungal and bacterial bioagents against *Meloidogyne incognita* infecting chick pea (Abstr.). Pro. Nation. Con. Cent. Nematol.: Appraisal and Future plans. IARI, New Delhi, 5-7 December, p.148.

Khan, M.R and B.K. Goswami. 2000. Effect of different doses of *Paecilomyces lilacinus* isolate 6 on *M. incognita* infecting tomato. Indian J. Nematol. 30: 5-7.

Khyami-Horani, H. and Al Banna, L. 2006, Efficacy of *Bacillus thuringiensis* jordanica against *Meloidogyne javanica* infecting tomato. Phytopathol. Mediter. 45: 153-157.

Kiewnick, S. and Sikora. R.A. 2006. Biological control of the root-knot nematode *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. Biol. Control. 38: 179-187.

Kleifeld, O and Chet. I. 2002. *Trichoderma*-plant interaction and its effect on increased growth response. Plant and Soil. 144: 267-272.

Kokalis-Burelle, N., Vavrina, C. S., Rosskopf, E. N and Shelby, R. A. 2002. Field evaluation of plant growth promoting rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. P1. Soil. 238: 257-266.

Li Bin, Xie Guan-Lin, Soad A., Coosemans J. 2005. Suppression of *Meloidogyne javanica* by antagonistic and plant growth-promoting rhizobacteria. J. Zhejiang Univ. Sci., 6: 496-501.

Mai, W. F. 2002. World-wide distribution of potato-cyst nematodes and their importance in crop production. J. Nematol. 9: 30-34.

Masadeh, B., Von Alten, H., Grunevaldt-Stoecker, G., Sikora. R.A. 2004. Biocontrol of root-knot nematodes using the arbuscular mycorrhizal fungus *Glomus intraradices* and the antagonist *Trichoderma viride* in two tomato cultivars differing in their suitability as hosts for the nematodes. Zeitschrift for Pflanzenkrankheiten and Pflanzenschutz. 11: 322-333.

Maqbool, M.A. 1981. Occurrence of root-knot and cyst nematode in Pakistan. Nematol. Medit. 9: 211-212.

Maqbool, M. A., Hashmi S and Ghaffar, A. 1987. Effect of Latex extract from *Euphorbia caducfo1ia* and *Calolropis procera* on root-knot nematodes *Meloidogyne incognita* infecting tomato and egg plant. Pak. J. Nematol. 5: 43-47.

Mazzola, M. 2004. Assessment and management of soil microbial community structure for disease suppression. Ann. Rev. Phytopathol., 42: 35-59

McGovern, R.T., Datnoff, L.E and L. Tripp. 1992. Effect of mixed infection and irrigation method on colonization of tomato roots by *T. harzianum* and colonies intradix. Proc. Fla. State Hort. Soc. 105: 36 1-363. 127

McSpadden Gardener, B. B. 2004. Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. Phytopathoh, 94: 1252-1258.

Meyer, S.L.F., Massoud, D.J. Chitwood and D.P. Roberts. 2000. Evaluation of *Trichoderma virens* and *Burkholderia cepacia* for antagonistic activity against root-knot nematode, *Meloidogyne incognita*. Nematol. 2: 87 1-879.

Mills, P.K., Beeson, W.L., Phillips, R.L., Fraser. G.E. 1989. Cohort study of diet, lifestyle, and prostate cancer in Adventist men. Cancer. 64:598- 604.

Monte, F and A. Llobell. 2003. *Trichoderma* in organic agriculture. Proceedings in World avacado congress. 725-733.

Nakayama, T., Homma, Y., Hashidoko, Y., Mizutani, J and Tahara, S., 1999, Possible role of xanthobaccins produced by Stenotrophomonas sp. strain SB-K88 in suppression of sugar beet damping off disease. Appl. Environ. Microbioh, 55: 4334-4339.

Noe, J.P and Sasser. J.N.1995. Evaluation of *Paecilomyces lilacinus* as an agent for reducing yield losses due to *Meloidogyne incognita*. Biocontrol. 1: 57-67

O'Bannon, J.H. (1975). Report of nematode survey in Ethiopia. FAO, Rome, 29pp.

Pal, S. S., 1998, Interaction of an acid tolerant strain of phosphate solubilizing bacteria with a few acid tolerant crops. Ph Soi4 198: 169-1 77.

Pal, V. and Jalali, I. 1998. Rhizosphere bacteria for biocontrol of plant diseases. Indian J. Microbiol. 38: 187-204.

Parveen, S., Haque E.S and Ghaffar. A. 1993. Biological control of *M. javanica* on tomato and okra in soil infested with *Fusariurn oxysporum*. Pak.J.Nematol. 11: 151-156.

Ploeg, A.T. 2002. Effect of selected Marigold varieties on root-knot nematodes and tomato and melon yields. Plant Dis. 86:505-508.

Podile, A. R and Dube, H. C., 1988, Plant growth promoting activity of *Bacillus subtilis* AF1. Curr. Sci. 57: 183-186.

Prasad, S. S. V and Tilak, K. V. B. R. 1972, Aerobic spore forming bacteria from a root-knot nematode infested soil. Indian J. Micro. 11: 59-60.

Prasad, S. S. V., Tilak, K. V. B. R. and Gollakota, K. G. 1972. Role of *Bacillus thuringiensis* var. thuringiensis on larval survivability and egg hatching on *Meloidogyne* spp. the causative agent of root knot disease. J. Invt. Pathol., 20: 377.

Qureshi, S.H., Alam S.S and Bashir. M. 1984. Mungbean (*Vigna radiata*), a new host of *Meloidogyne incognita* in Pakistan. Pak. J. Nematol. 2:101- 102.

Rai, G. P. and Rana, R. S., 1979, Effectiveness of beta-exotoxin of *Bacillus thuringiensis* var. thuringiensis on the ability of *Meloidogyne* sp. from brijal (*Solanum melongenea* L.) to survive. J. Biosci., 1: 271-278.

Rajarathnam, K., Balamurugan, T., Kulashera, Pandian, R., Veersamy, S. and Jayabalan, M. 1995. Isolation and screening of phosphate solubilizers from soil of Kamarajar district. J. Ecotoxicoh Environ. Monitoring. 5: 155-157.

Rangaswamy, S.D., Reddy P.P and Nagesh, M. 2000. Evaluation of bio-control agents (*Pasteuria penetrans* and *Trichoderma viride*) and botanicals for the management of root-knot nematode, *Meloidogyne incognita* infecting tomato. Pest Manag. Hort. Ecosys. 6: 135-138.

Rao, M.S., Reddy, P.P. and Nagesh, M. 1997. Management of root-knot nematode (*Meloidogyne incognita*) on tomato by integration of *Trichoderma harzianum* with neem cake. Zeitschrift fur Pflanzenkrankheiten and Pflanzenschutz. 104: 423-425.

Rao, M.S., Reddy, P. and Nagesh, M. 1998. Evaluation of plant based formulations of *Trichoderma harzianum* for the management of *Meloidogyne incognita* on eggplant (*Solanum melongena* L.). Nematologia Mediterranea. 26: 59-62. 130

Raupach, G. S and Kloepper, J. W. 1998. Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathoh. 88: 1158-1164.

Reddy, D. D. R., 1985, Analysis of crop losses in tomato due to Meloidogyne incognita. Indian J. Nematol., 15: 55-59.

Reddy, P.P., Nagesh, M. Devappa, V. Reddy P.P., (ed), Kumar, N, K, K. (ed) and Verghese, A. 1998. Management of *Meloidogyne incognita* on tomato by integration of *Trichoderma harzianum*, *Glomus fasciculatum* and neem cake. Advances in IPM for horticultural crops. Proceedings of the First National Symposium on Pest Management in Horticultural Crops: environmental implications and thrusts, India. 349-352.

Reddy, M. S. and Rahe, J. E. 1989. Growth effects associated with seed bacterization not correlated with population of *Bacillus subtilis* inoculant in onion seedling rhizospheres. Soil BioL Biochem. 21: 373-378.

Regina, M. D. G. C., DeSouza, I. S and Belarmino, L. C. 1998. Nematicidal activity of *Bacillus* spp. strains on juveniles of *Meloidogyne javanica*. Nematol. Brasiliera. 22: 12-2 1.

Rekha, A., Saxena, S.K. and Arya. R. 1998. *Trichothecium roseum* together with *Rhizoctonia solani* and *Meloidogyne incognita* on germination of seeds of tomato cv. 'Pusa Ruby'. Ind. J. Nematol. 28: 217-221.

Roberts, D.P., Lohrke, S.M., Meyer, S.L.F., Buyer, J.S., Bowers, J.H., Baker C.J and Chung. S. 2005. Biocontrol agents applied individually and in combination for suppression of soil borne diseases of cucumber. Crop Prot. 24: 141-155.

Saifullah and Thomas B.J.1996. Studies on the parasitism of *Globodera rostochiensis* by *Trichoderma harzianum* using low temperature scanning electron microscopy. Afro-Asian J. Nematol. 6: 117-122.

Saifullah, Stephen, M and Khattak, B. 2007. Isolation of *Trichoderma harzianum* and in vitro screening for its effectiveness against root-knot nematodes (*Meloidogyne* spp.) from Swat, Pakistan. Pak. J. Nematol. 25: 313-322.

Samuel, G.J. 1996. *Trichoderma*: a review of biology and systematics of the genus. Mycol. Res. 100: 923-935.

Sankaranarayanan, C., Hussaini, S.S., Kumar, P.S., Rangeshwaran, R., Dhawan S.C and K.K. Khushal. 1999. Antagonistic effect of *Trichoderma* and *Gliocladium* spp. against the root-knot nematode (*Meloidogyne incognita*) on sunflower. Proc. National symp. Rational Approaches in nematode management for sustainable agriculture, India. 23-25.

Sasser, J. N., 1980, Root knot nematodes: A global menace to crop production. Ph Dis., 64:36-4 1.

Sasser, J.N and Carter, C.C. 1982. Overview of the International *Meloidogyne* project-Rationale, Goals, Implementation and progress to date. Proc. 3rd Res. Plan. Conf. on root-knot nematodes *Meloidogyne* spp. Panama.l-7.

Sasser, J.N and Freckman. D.W. 1987. A world perspective on Nematology. The role of society. Pages 7-14 in Vistas on Nematology, J.A. Veech and D.W. Dickson, eds. SON. Inc., Hyattsville, MD, USA.

Subramaniyan, S., Rajendran, G. and Vadivelu, 5., 1990, Estimation of loss in tomato due to Meloidogyne incignita and Rotylenchulus reniformis. Indian J. Nematol., 20: 239-240.

Sharma, M. and Saxena. S.K. 1992. Effect of culture filtrates of the *Rhizoctonia solani* and *Trichoderma viride* on hatching of larvae of root-knot nematode (*Meloidogyne incognita)*. Current Nematol. 3: 61- 63. 132

Sharma, N and Trivedi, P.C. 2005. Microbial bioagents: Economic multiplication and management of fungal-nematode complex on cumin. Indian J. Biotech. 4:419-421.

Sharma, D.D. 1994. Effect of culture filtrates of biocontrol agents on larval mortality of *Meloidogyne incognita* in comparison with Rugby 10 G. Indian J. Sericulture. 2: 152-154.

Sharon, E., E.M. Bar, I. Chet, E.A. Herrera, O. Kleifeld and Y. Spiegel. 2001. Biological control of the root-knot nematode *M. javanica* by *T. harzianum*. Phytopathol. 91: 687-693.

Sharon, E., Chet, I., Viterbo, A., Meira, B.E., Nagan, H., Samuels, G and Y. Spiegel. 2007. Parasitism of *Trichoderma* on *Meloidogyne javanica* and role of the gelatinous matrix. Europian J. Plant Pathol. 118: 247— 258.

Siddiqui, Z. A and Mahmood, I. 1992, Biological control of root knot disease complex of chickpea caused by *Meloidogyne incognita* race 3 and *Macrophomina phaseolina*. Nematol. Medit., 20: 199-202.

Siddiqui, I.A and Haque, E.S. 2000. Effect of *V. chlamydosporium* and *Pseudomonas aeruginosa* in the control of *M.javanica* on tomato. Nematologia Mediterranea. 28: 193-196.

Siddiqui, I.A, Shaukat. S.S. 2004. *Trichoderma harzianum* enhances the production of nematicidal compounds in vitro and improves biocontrol of *Meloidogyne javanica* by *Pseudomonas fluorescens* in tomato. Letters in App. Microbiol, 38: 169-175.

Siddiqui, I.A and Shaukat. S.S. 2004. Systemic resistance in tomato induced by bio-control bacteria against root knot nematode *Meloidogyne javanica* is independent of salicylic acid production. J. Phytopathol. 154: 48-56. 133

Siddiqui, Z. A and Mahmood, I. 1992, Biological control of root-knot disease complex of chickpea caused by *Meloidogyne incognita* race 3 and *Macrophomina phaseolina*. Nematol. Medit. 20: 199-202.

Sivan, A and Chet, I. 2002. Microbial control of plant diseases. Pages. 335- 354. In: New concepts in environmental microbiology R. Miteehell, ed. Wiley-Liss Inc., New York.

Songsak, C.U. 1999. Use of bacteria and some antagonistic fungi in controlling root-knot nematode (*Meloidogyne incognita*) in lettuce, Rajamangala Inst. Tech., Bangkok (Thailand). 2: 157-165.

Spiegel, Y and Chet, I. 1998. Evaluation of *Trichoderma* spp. as a biocontrol agent against soil borne fungi and plant-parasitic nematodes in Israel. Inter. Pest Mang. Rev. 3: 169- 175.

Sriveni, M., Rupela, O. P., Gopalkrishnan, S and Krajewski, M. 2004. Spore-forming bacteria, a major group among potential antagonists isolated from natural sources such as termitaria soil and composts used by organic farmers. Indian. Microbiol., 44:95-100.

Suarez, M.B., Sanz, L., Chamorro, M.J., Rey, M., Gonzalez, F.J., Liobell A and Monte. F. 2005. Proteomic analysis of secreted proteins from *Trichoderma harzianum*: Identification of a fungal cell wall-induced aspartic protease. Fungal Genetics and Biology. 42: 924-934.

Sudha, S. N., Jayakumar, R. and Sekar, V. 1999. Introduction and expression of the crylAc gene of *Bacillus thuringensis* in a cereal associated bacterium, *Bacilllus polymyxa*. Curr. Microbioh, 38: 163-167.

Sukumar, J., Padma, S.D and Bongale. U.D. 2005. Biological control of mulberry root-knot nematode *Meloidogyne incognita* by *Trichoderma harzianum*. Internl. J. Indust. Entomol, 8: 175-179.

Sundari, A and Sureshkumar, S. M. 2004. Influence of DAP and phosphobacteria on growth and yield of blackgram under rice fallow condition. J. Ecobioh, 16: 75-77.

Swarup, G. and Dasgupta, D. R., 1986, Plant Parasitic Nematodes of India: Problems and Progrress, Director, IARI (Allied Publisher), New Delhi, India. 497p.

Tesfamariam, M., Wondirad, M. and Greco, N. (2003). Relationship between initial population density and damage caused by *Meloidogyne javanica* populations from Ethiopia on pepper and tomato. *Nematologia Mediterranea* (in Press).

Trudgill, D. L. 2005. An assessment of the relevance of thermal time relationships to nematology. Fund and Appl. Nematol. 14: 407-417.

Turner, J. T. and Blackman, P. A. 1991. Factors relating to peanut yield increases following *Bacillus subtilis* seed treatment. P1. Dis. 75: 347-353.

Uthede, R. S., Koch, C. A and Menzies, J. G. 1999. Rhizobacterial growth and yield promotion of cucumber plants inoculated with *Pythium aphanidermatum*. Can. J. Plant Pathol. 21: 265-271.

Walia, R.K and Dalal. M.R. 1994. Efficacy of bacterial parasite *Pasteuria penetrans* application as nursery soil treatment in controlling root-knot nematode *M. javanica* on tomato. Pest Manag. and Economic. Zool. 2: 19-2 1.

Wipat, A and Harwood, C. A. 1999. The *Bacillus subtilis* genome sequence: the molecular blueprint of a soil bacterium. FEMS Microb. Ecol., 28: 1-9.

Wondirad, M. and Tesfamariam, M. (2002). Root-knot nematodes on Vegetable crops in Central and Western Ethiopia. *Pest Management Journal of Ethiopia* 6:37- 44

Yitzhak, S. and Chet. I. 2004. Evaluation of *Trichoderma* spp. as a Biocontrol Agent against Soil borne Fungi and Plant-parasitic Nematodes in Israel.

Young, L.D. 1992. Problems and strategies associated with long-term use of nematode resistance cultivars. J. Nematol. 24. 228-23 3.

Zavaleta-Mejia, E and VanGundy, S. D. 1982, Effects of rhizobacteria on *Meloidogyne* infection. J. Nematol., 14: 475-476.

Zuckerman, B. M., Dicklow, M. B and Acosta, N. 1993. A strain of *Bacillus thuringiensis* for the control of plant-parasitic nematodes. Biocont. Sci. Tech. 3: 41-46.

7. APPENDICES

Appendix 1. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on *Meloidogyne spp.,* eggs hatching inhibition

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	3	25505.11667	8501.70556	7942.15	<.0001
Time	4	10.50000	2.62500	2.45	0.0598
rep	2	908.13333	454.06667	424.18	<.0001
Biocontrol*Time	6	145.73333	24.28889	22.69	<.0001
Total	59	26616.58333			
Mean	39.58333				

Coeff Var =2.613796

Appendix 2. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on *Meloidogyne* spp., J_2 mortality

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	3	55455.78	18485.26	4873.29	<.0001
Time	4	21.10000	5.27500	1.39	0.2528
rep	2	6345.30000	3172.65000	836.41	<.0001
Biocontrol*Time	6	1247.76667	207.96111	54.82	<.0001
Total	59	63236.85000			
Mean	62.55000				J2

Coeff Var = **3.113684**

Appendix 3. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on Root length (cm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	140.90	20.12	98.69	<.0001
Time	2	15.86	15.86	7.93	<.0001
block	2	0.86	0.43	2.11	0.1349
Biocontrol*Time	10	18.6156852	1.86	9.13	<.0001
Total	59	178.46			
Mean	18.38				

Coeff Var = 2.45

Appendix 4. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on shoot length (cm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	1873.08	267.58	355.35	<.0001
Time	2	140.32	70.16	93.18	<.0001
block	2	6.735203	3.367602	4.47	0.0180
Biocontrol*Time	10	47.76	4.77	6.34	<.0001
Total	59	1994.893873			
Mean	71.51567				

Coeff Var = 1.213398

Appendix 5. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on Fresh root weight (gm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	50.21	7.17	8.97	<.0001
Time	2	0.80	0.40	0.50	0.6086
block	2	1.77	0.88	1.11	0.3400
Biocontrol*Time	10	6.48	0.64	0.81	0.6199
Total	59	93.7529			
Mean	23.00350				FRtWt

Coeff Var = 3.886780

Appendix 6. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on Dry root weight (gm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	13.15	1.87	24.07	<.0001
Time	2	1.04	0.52	6.71	0.0032
block	2	0.10856333	0.0542816	0.70	0.5052
Biocontrol*Time	10	1.80629630	0.18	2.31	0.0307
Total	59	20.32497333			
Mean	7.852667				DRtWt

Coeff Var =3.558301

Appendix 7. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on fresh shoot weight (gm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	1420.58	202.94	109.58	<.0001
Time	2	188.872	94.4363	50.99	<.0001
block	2	1.98	0.99315	0.54	0.5893
Biocontrol*Time	10	49.4603	4.946	2.67	0.0141
Total	59	1620.27			
Mean	60.3880				FShWt

Coeff Var =2.253505

Appendix 8. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on dry shoot weight (gm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	271.56	38.79	34.40	<.0001
Time	2	27.44	13.72	12.17	<.0001
block	2	8.18	4.0906017	3.63	0.0362
Biocontrol*Time	10	39.13	3.91	3.47	0.0026
Total	59	363.522			
Mean	21.91033				DShWt

Coeff Var = 4.846911

Appendix 9. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on Fruit set.

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	413.16	59.02	75.24	<.0001
Time	2	95.89	47.94	61.12	<.0001
block	2	5.76	2.88	3.67	0.0348
Biocontrol*Time	10	12.34	1.23	1.57	0.15
Total	59	495.75			
Mean	11.49100				Fs

Coeff Var =7.707713

Appendix 10. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on fruit weight (gm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	4888.91	698.41	446.79	<.0001
Time	2	1037.82	518.91	331.96	<.0001
block	2	2.27592	1.13796	0.73	0.4895
Biocontrol*Time	10	193.15	19.3157	12.36	<.0001
Total	59	5473.07			
Mean	49.3811				

Coeff Var = 2.531874

Appendix 11. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on days to maturity (gm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	1042.68	148.95	109.77	<.0001
Time	2	348.77	174.38	128.51	<.0001
block	2	3.10	1.55	1.14	0.3298
Biocontrol*Time	10	130.55	13.05	9.62	<.0001
Total	59	1391.00			
Mean	62.5				DaMa

Coeff Var = 1.863858

Appendix 12. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on number of floweres per plant

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	7	2560.034894	365.719271	208.02	<.0001
Time	2	85.968044	42.984022	24.45	<.0001
block	2	12.511690	6.255845	3.56	0.0383
Biocontrol*Time	10	149.330600	14.933060	8.49	<.0001
Total	59	2836.994760			
Mean	58.36800				NF/pl

Coeff Var =2.271692

Appendix 13. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on number of galls per plant (cm)

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	4	20171.56087	5042.89022	1342.40	<.0001
Time	2	264.45769	132.22884	35.20	<.0001
block	2	3.72061	1.86030	0.50	0.6167
Biocontrol*Time	4	3.13496	0.78374	0.21	0.9306
Total	32	20670.58162			
Mean	32.61515				Ngs/Pl

Coeff Var =5.942629

Appendix 14. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on galling index

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	4	40.82	10.20567667	219.26	<.0001
Time	2	2.29	1.14654444	24.63	<.0001
block	2	0.16212727	00.08106364	1.74	0.2008
Biocontrol*Time	4	0.24695556	0.06173889	1.33	0.2945
Total	32	48.28596364			
Mean	3.413636				GINX

Coeff Var =6.320165

Appendix 15. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on eggmasses per root

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	4	12127.97746	3031.99437	753.99	<.0001
Time	2	630.68042	315.34021	78.42	<.0001
block	2	13.63617	6.81808	1.70	0.2088
Biocontrol*Time	4	13.13742	3.28436	0.82	0.5295
Total	32	12844.979795			
Mean	46.55394				egma/r

Coeff Var =4.307503

Appendix 16. Analysis of variance for the effect of *Trichoderma harzianum, Bacillus subtilis* and their combinations at different time of applications on eggs per eggmass

S.O.V	DF	S.S	M.S	F.Value	Prob
Biocontrol	4	152052.6243	38013.1561	798.00	<.0001
Time	2	12116.0071	6058.0036	127.17	<.0001
block	2	919.1999	459.5999	9.65	0.0012
Biocontrol*Time	4	576.7266	144.1817	3.03	0.0420
Total	32	196481.5606			
Mean	230.1494				eg/egma

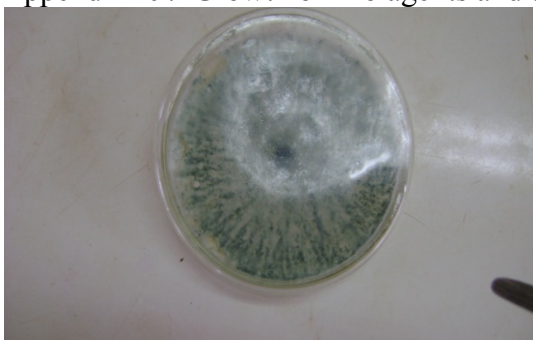
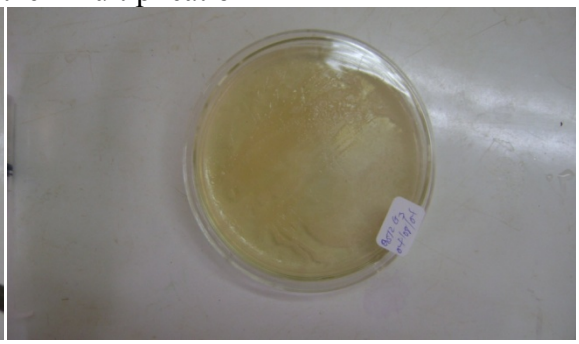
Coeff Var =2.998857

Appendix 17 Nematode extraction methods

Appendix 18. Nematode eggs extraction



Appendix 19: Growth of Bio agents and their multiplication

Appendix 20: In vitro tests


C= counting of un hatching eggs and J2 mortality. D= identifying of active and in active J2

Appendix 21= *in vivo* tests

Appendix 22. Preparation of suspensions and inoculation of bio-controls

Appendix 23: Data collection

Appendix 24: Counting of Root knot galls per root system

A= Root knot galls B= counting of galls with stero microscope

A= counting of egg masses with stero microscope B= egg masses looks red when observed by microscope

Appendix 25: Shoot parts of tomato in green house


BN
C
TB
TBN

Appendix 25: Tomato Root parts