Advances in Crop Science and Technology

Aldesuquy et al., Adv Crop Sci Tech 2015, 4:1
DOI: 10.4172/2329-8863.1000201

Research Article Open Access

Evaluation of Some Botanicals and *Trichoderma harzianum* for the Management of Tomato Root-knot Nematode (*Meloidogyne incognita* (Kofoid and White) Chit Wood)

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Abstract

Root-knot nematode disease caused by *Meloidogyne incognita* (Kofoid and White) Chit wood) is one of the major constraints for successful cultivation of tomato (*Lycopersicon esculentum* Mill.) in Ethiopia. Hence, the present study was conducted to evaluate the effect of leaf and seed extracts of four botanicals viz., Rape seed (*Brassica napus* L.), Lantana (*Lantana camara* L.), African marigold (*Tagetes erecta* L.) and Neem (*Azadirachta indica* L.) at 5% and 10% concentrations and *T. harzianum* at 5% plus control were tested on root-knot nematode under *in vitro* and also to evaluate their against root-knot nematode development and their role on plant growth parameters of tomato under *in vivo* condition. Plant extracts were more effective and significantly inhibited egg hatching and immobilizing the J_2 larval mortality of *M. incognita* than *T. harzianum*. Aqueous extract of all the tested plants inhibited egg hatching of nematode and resulted 84.67-100% mortality of the J_2 juveniles of *M. incognita in vitro* at the 10% concentration after 72 h of exposure time. There were no significant differences among the treatments of rape seed leaf (84.7%) at 10% concentration and *Lantana camara* (87%), African marigold (86.3%) and Neem leaf (85%) at 5% concentration after 72 h. Aqueous seed extracts of *A. indica* more significantly inhibited egg hatching and larva mortality of the J_2 of *M. incognita in vitro* at the 10% concentration and immobilized by 89, 93 and 100% after 24, 48 and 72 h of exposures, respectively, while at similar concentration of *T. erecta*, *B. napus and L. camara* leaf extracts exhibited 92, 89 and 93.2% inhibition of egg hatching and 75, 62.1 and 73% larval mortality, respectively. The effect of different botanicals and *T. harzianum* singly and in combination were studied for the management of tomato root-knot nematode under greenhouse condition. There was a significant difference in the reduction of root-knot nematode incidence, root-knot nematode population, nematode reproduction rate (NRR), number of galls and egg masses per plant were recorded. In pot culture condition, the application of leaf extract of individual plant in the presence of the nematode significantly enhanced the growth of tomato seedlings in comparison to the control. A significant increase in plant height, shoot weight and root weight of the seedlings were observed at the 10% concentration of leaf extracts in comparison to control. There was a significant difference in the reduction of root-knot nematode population, nematode reduction rate, number of galls and egg masses per plant of *L. camara* combined with *T. harzianum*. The mean fruit weight and total yield were observed highest in the combination treatment of *L. camara* combined with *T. harzianum*. This study results revealed that the test plants are readily available to farmers at no cost and able to reduce nematode population below economic threshold.

Keywords: Tomato seedlings; Botanical leaf; Seed extracts; *Trichoderma harzianum*; Root-knot nematode; Egg hatching; Larval mortality; Growth

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely grown vegetable in the world and regarded as one of the top priority vegetable which is widely cultivated in tropical, sub-tropical and temperate climates [1]. Tomato fruits contributes to healthy, because it is rich in minerals (potassium, magnesium, calcium, iron and zinc), proteins (essential amino acids), citric acid, sugars, dietary fibers (pectin) and high levels of vitamin C, lycopene, and beta-carotene which are anti-oxidants against oxygen radicals that probably cause cancer, aging and arteriosclerosis [2,3]. In Ethiopia, tomato is also among the most important vegetable crops and its production has shown a marked increase since it became the most profitable crop providing a higher income to small scale farmers compared to other vegetable crops [4]. The total area under production reaches 51,698 hectares and annual production is estimated to be more than 230,000 tons in Ethiopia [5]. However, the national average yield of tomato in the country is very low which is around 7 tons/ha [6] and less than 50% of the current world average yield of about, 27 tons/ha [7]. Its production is hampered by poor soil fertility, unreliable rainfall patterns, poor marketing structures, post-harvest handling problems and most important pests and diseases.

Tomato crops are more susceptible to diseases as compared to other vegetable and cereal crops. Bacterial, fungal and nematode diseases of vegetables are common problem of all agro climatic zones and it is worldwide problem [8]. The root-knot nematode disease (*Meloidogyne incognita*) is the most destructive and widespread diseases of solanaceous vegetables in Ethiopia [9]. Furthermore, the percent incidence of root-knot nematode disease is as high as 65% on tomato and it was recorded in major tomato producing areas of Ethiopia. This

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Received June 09, 2015; **Accepted** November 14, 2015; **Published** November 20, 2015

Citation: Feyisa B, Lencho A, Selvaraj T, Getaneh G (2015) Evaluation of Some Botanicals and *Trichoderma Harzianum* for the Management of Tomato Root-knot Nematode (*Meloidogyne Incognita* (Kofoid and White) Chit Wood). Adv Crop Sci Tech 4: 201. doi:10.4172/2329-8863.1000201

report gives good indication of the losses due to the disease can cause in major tomato producing areas of Ethiopia particularly in Ambo and Toke Kutaye district of West Showa, Ethiopia. *Meloidogyne* spp. is one of the most harmful nematode pests in both tropical and sub-tropical crop production regions and cause extensive economic damage worldwide [10,11]. Many workers have attempted to assess crop losses caused by plant parasitic nematode species in Ethiopia [12,13]. Tomato root-knot nematode species viz., *Meloidogyne incognita, M. javanica* and *M. ethiopica* have been reported to occur in Ethiopia [14,15]. Tadele and Mengistu [15] reported that the occurrence of *M. incognita* on tomato in the Eastern part of the country, particularly in Eastern Hararghe, where many vegetable crops were attacked by root-knot nematodes. Apart from the Eastern parts of the country, root-knot nematode, *M. incognita* is the major problem in tomato cultivation in the Central and Western parts of the country [16].

Several methods are known to manage the root-knot nematode which includes the use of nematicide, organic amendments, resistant cultivars, soil solarization and biological control and these have been used with different levels of success on tomatoes [17,18]. However, detrimental environmental effects associated with chemical control and the recent losses of methyl bromide as a multipurpose soil fumigant have spurred research into nematode control alternatives [19]. In view of the uneconomical and hazardous effects of chemical nematicide, researchers have focused their attention to adopt biological control of *Meloidogyne* spp. [20]. The persistent pressure on farmers to adopt strategies that do not pollute the environment has increased urgency in the search for alternative sustainable methods [21,22]. Bio-control appears to offer an environmentally safe and ecologically feasible option for plant protection with great potential for promoting sustainable agriculture. The bio control efficiency depends on the nematode species, plant host and their root exudates, and other crops in rotation [23].The beneficial effects of certain types of plants derived materials and microorganisms in soil have been attributed to a decrease in the population densities of plant-parasitic nematodes [24].

Several fungi have been identified and classified according to their nematophagous properties. Fungi that have toxic effects on nematodes include *Aspergillus* spp. and *Trichoderma* spp. *Trichoderma viride* which were reduced egg-hatching [25] and trade formulations have also proven to be efficacious in tropical greenhouse conditions [26]. Some species of *Trichoderma* have been used widely as bio-control agents against soil-borne plant diseases [27] and also they have activity towards root-knot nematode [28,29]. Al Kader [30] reported a high nematicidal effect of the fungus *Paecilomyces lilacinus* culture filtrate on J2 of *M. incognita*, with 99% of J2 immobilized after 2 days of treatment. *Trichoderma* spp. has been reported to produce chitinase into the culture [31], which might help in the inhibition of egg hatching.

Botanicals, plant-based pesticides are favored as alternatives to chemical pesticides in recent times. When French marigold was planted immediately after the termination of a *Meloidogyne* susceptible host, bitter melon (*Momordica charantia* L.) and marigold suppressed approximately 50% of *M. incognita* compared to the bare ground treatment [32]. Several higher plants and their constituents have been successful in plant disease control and have proved to be harmless and non-phytotoxic, unlike chemical fungicides [33]. The fresh leaf extracts of *Azadirachta indica*, *Allium sativum* (Garlic) and *Tagetes erecta* (African marigold) were examined against *M. incognita* on tomato *in vitro* and *in vivo* conditions. All treatments immobilized juveniles (J_2), the highest effect caused by neem leaves extract after 24 and 48 h of exposure. In soil, all treatments significantly reduced the root galling, nematode population, and enhanced the plant growth and yield [34].

So far, little efforts have been made to exploit locally available botanicals and antagonistic fungal organisms for the control of root-knot nematode on crops in Ethiopia. Even if few works were done by botanicals in Ethiopia, their combination with biological and their synergistic effect with antagonistic fungi are not studied. The management of plant parasitic nematodes using plant products and their derivatives are gaining importance in the light of increased awareness of environmental and human health hazards associated with nematicidal chemicals, biodegradability, and selective toxicity to target pests, safety to non target organisms. Therefore, the present study was conducted to find out the effect of different botanicals leaf and seed extracts and *T. harzianum* on egg hatching and juvenile mortality of root-knot nematode under *in vitro* condition and also to compare the effect of different botanicals and *T. harzianum* on individual and in combination for the management of tomato root-knot nematode development and their role on plant growth under greenhouse condition.

Materials and Methods

Description of the study area

Both *in vitro* and *in vivo* experiments were conducted at Ambo Plant Protection Research Center (APPRC), Ambo, Ethiopia. This center is found in Ambo District, West Shewa Zone of Oromia Regional State, Ethiopia, which is away from Addis Ababa 115 km, with an altitudes of 2100 m, latitude 8° 57' 58"N and longitude 37°5'33"E. The minimum and maximum temperature was about 10°C and 28°C, respectively. The average annual rainfall was about 1260 mm with the relative humidity of 70.

Botanicals, antagonistic fungus and tomato cultivar used: Rapeseed, *Lantana* and marigold were collected from Ambo university campus, Ambo. Neem seeds and leaves and the seeds of tomato cv. *Marglobe* were obtained from Melkassa Research Centre, Melkassa, Ethiopia. *Trichoderma harzianum* (Jimma isolate) was obtained from Department of Mycology, *APPRC,* Ambo, Ethiopia.

Sample collection and estimation: Diseased root samples of tomato were collected from pure culture pots grown at APPRC green house. For confirmation roots of tomato infested with root-knot nematode were thoroughly washed, cut into small pieces and stained with Acid Fuchsin in lacto phenol [35]. After cooling to normal temperature, they were keeping in lacto phenol overnight for partial de-staining. Root pieces were dissected under stereomicroscope and adult females were taken out and placed in lacto phenol. The perineal region of females were cut with a sharp razor blade and adhering tissue clear off with a fine pick and the perineal sections were examined under microscope. The ten female patterns of root knot nematode were examined and estimated [36,37].

Multiplication, extraction and counting number of juveniles

Egg masses of *M. incognita* were picked up from pure culture pots of infected roots and placed into the sterilized plastic plates with sterile water and kept on the laboratory benches at room temperature (20-23°C) and allowed to hatch for 3-6 days. Two weeks old transplanted seedlings of tomato cv. Marglobe, raised in autoclaved soil in the wire house were inoculated with the juveniles that emerged out of the egg-mass. Inoculation was done by removing top soil (1-2 cm) around the seedlings two sides, make the hole and nematodes in the water poured on partly exposed root-system with pipette. The removed soils

were again placed on sides of the seedlings. To get regular supply and sufficient culture of root-knot nematode for subsequent experiments, *M. incognita* was sub-cultured by inoculating juveniles to freshly transplanted tomato seedlings raised in sterilized soil in pots.

The pure cultures of *M. incognita* were raised from single egg mass and maintain on tomato roots in wire house. Infected plants then uprooted from soil and the entire root system was dipped in water and washed gently to remove adhering soil particles. Egg masses of nematodes were picked up and kept it in small sieves. Then the sieves were placed in sterilized plastic plates and pour the water up to neck of the sieves and kept in the laboratory at room temperature. After 2 to 7 days, eggs were hatched and active juveniles cross the sieve and settle down in plastic plates. The J_2 juveniles were collected and then counted by using eelworm nematode counting dish for experimental study. Population densities of J_2 were determined from one ml aliquot of an inoculum suspension. 100 J_2 and 10 egg masses of root knot nematode were used for each treatment in *in vitro* experimental study and 2000 J_2 was used for *in vivo* culture study for each treatment.

Raising and maintenance of tomato plants and inoculation with nematode: The seeds of tomato cv. *Marglobe* were axenized by NaOCl method. About 100 seeds were placed in sterilized beaker containing a mixture of 95% ethanol and 5.25% NaOCl in the ratio of 1:1. The mixture was stirred gently and the seeds were allowed to soak for about 10 minutes. The mixture was drained off and the seeds were rinsed thrice with distilled water. Seeds of cultivar *Marglobe* were sown on sterilized soil in plastic pots under greenhouse. Three leaf stage/ one month-old seedlings were transplanted to plastic pots (15 cm dia.) containing 3 kg of sterilized soil with 1:2:3 proportions of sand, compost and clay, respectively. Each pot was planted only one tomato seedling. Fresh roots of tomato were taken from pure culture developed in the wire house and brought to Plant Pathology Laboratory. Egg masses were picked up by using sterile forceps and dissecting needle and placed to Petri dish having sterile water then kept on laboratory benches at room temperature (20-23°C) till hatching was completed. Appropriate suspension of nematode was prepared in a beaker and 3 ml was taken from the total suspension and placed on counting dish, then the number of juveniles of the suspension was determined under stereomicroscope at the magnification of 50 ×. The population of nematode per ml was calculated from one ml aliquot of an inoculum suspension for *in vitro* and *in vivo* experiments. Finally, seedlings of tomato were inoculated with the 2 ml suspension of M. *incognita* at 2000 juveniles/pot after one week of transplanting. For inoculation, 1-2 cm of top soil was separated out and nematode suspension was poured around the plant. Each treatment was replicated three times and the pots were arranged in a randomized complete design. Un-inoculated set of plants were served as control.

Preparation of botanical test plants extracts

The test plants leaves and seeds (Table 1) were shade dried and separately powdered using an electric grinder and 20 g powder of each plant powder was soaked separately in 100 ml of distilled water for 24 h in 500 ml Erlenmeyer flask. After 24 h of soaking, they were filtered through Whatmann No.1 filter papers and then the filtrate was centrifuged at 2000 rpm for 10 min for *in vitro* experiments. Each extract was considered as a standard solution of "S" (100% concentration) and then kept in the refrigerator until use for further studies. Suspensions of the concentrations of 0, 5, and 10% were prepared with distilled water [38]. 5 ml and 10 ml of plant extracts were incorporated in to each pot with different treatments.

Common name of the botanicals	Botanical name	Parts used
Rape seed	*Brassica napus L.*	Leaf
Lantana	*Lantana camara L.*	Leaf
African marigold	*Tagetes erecta L.*	Leaf
Neem	*Azadirachta indica L.*	Leaf and seed

Table 1: List of botanicals used.

Mass multiplication of Trichoderma harzianum: Mass multiplication of T. *harzianum* was performed by the method described by Tiwari and Mukhopadhyay [39]. Pure culture of T. *harzianum* was cultured on Potato Dextrose Agar (PDA) media. 5 mm blocks of the 10 day old pure cultures of T. *harzianum* were placed upside down at the center of each plate and the Petri dishes were kept in the growth chamber at 22°C temperature. After 10 days, an aliquot of 10 ml of sterile water was added to each plate and the mycelium was scraped with a spatula until the culture surface was free from mycelia and the suspension was collected in a 100 ml conical flask. Spores/conidial suspension were separated from mycelia by sieving through cheese cloth and the spore/ conidial suspensions were then adjusted to the desired concentration (10^6spores/ml) after counting spore density using a haemocytometer [40]. The pure cultures of T. *harzianum* was attained by inoculating in one liter jar containing sterile sorghum seeds, sand and water with spore suspensions. Spore suspensions were obtained by adding 20 ml sterile distilled water to three- week old cultures and scraping gently with spatula. The spore suspension of T. *harzianum* was inoculated in to sterilized one litter jar containing sorghum seeds and transferred or inoculated to water medium and preserved at 20°C for 3 days.

In vitro experimental study

Egg bioassay: Test tube bio-assay was carried out to determine the effect of different concentrations of botanical extracts and *T. harzianum on* hatching of *M. incognita* egg masses under *in vitro* [41] condition. Root-knot nematode infected tomato plants from the pure culture pots were up-rooted and washed gently under running tap water. Egg masses of *M. incognita* were picked up from the root using dissecting needle and forceps. Ten uniformly sized egg masses of *M. incognita* were transferred to 5 ml and 10 ml of each concentration of plant extracts and 5 ml of *T. harzianum* alone and combined in sterilized test tubes. Egg-masses in distilled water were only served as control. The experiment was laid out in completely randomized design with three replications. All the test tubes containing the suspensions and the egg masses were kept at room temperature on laboratory bench for seven days to allow eggs hatching.

Juveniles (J2) bioassay: 2 ml suspensions (100 J_2 juveniles) were placed in each test tube containing 5 ml and10 ml of each botanical and 5 ml of *T. harzianum* alone and in combinations. Each treatment was replicated three times. The number of dead J2s were recorded every 24 hours for three days. After 24, 48 and 72 hours, active and inactive J2s were counted in each test tube and sterilized distilled water was served as control [38]. Juveniles were considered dead if they were not move when probed with fine needle and body become straight [42]. Percent J_2 mortality in the test tube was calculated as:

$$\text{Percent J2 mortality} = \frac{\text{No. of inactive (dead) J2s}}{\text{Total J2s in a tube}} \times 100$$

***In vivo* experimental study**: 20 cm wide plastic pots were filled with 3 kg/pot of sterilized mixed soil (sandy clay loam, sand and compost as 2:1:1 (v/v). Seeds of susceptible tomato cultivar were sown

at germination pot and after 21 days, seedlings were transplanted to the green house pots. One seedling per pot was maintained at the center. The experiments were laid out in Complete Randomized Design (CRD) with three replications. Tomato potted plant soils were inoculated with 2 ml suspension of 2000 freshly hatched second stage juveniles (J_2) of *M. incognita* and also infested with 10 ml of each botanical and 20 ml of *T. harzianum* suspension [43]. Then proper watering was provided and the pots were kept at 20°C ± 2°C. Applications of botanicals and *T. harzianum* were also repeated after once in 20 days [38].

In vivo experiment consisted of the following thirteen treatments:

T1- Application of Rape seed leaf extract alone,

T2- Application of *Lantana* leaf extract alone,

T3- Application of African marigold leaf extract alone,

T4- Application of neem leaf extract alone,

T5- Application of neem seed extract alone,

T6- *T. harzianum* alone,

T7- Rapeseed+*T. harzianum,*

T8- Lantana+*T. harzianum,*

T9- Marigold+*T. harzianum*,

T10- Neem leaf+*T. harzianum*,

T11-Neemseed+*T.harzianum*

T12- Un inoculated control and

T13- Nematode only inoculated control.

After 90 days of the growth, the plants were uprooted, thoroughly washed and then the plant height, fresh and dry weight of shoot and roots, root-knot nematode population, nematode reproduction rate (NRR), number of galls/plant and egg masses per plant were recorded. The number of fruits per pot was counted. The galling index and the number of egg masses (gall) per plant in each pot were determined using a scale following the rating scale described by Taylor and Sasser [44] and Colyer et al. [45]. Scale 0=0, 1=1-2; 2=3-10; 3=11-30; 4=31-100; 5=>100. Galling index: 0=no galls, 1=slight infection, 2=moderate infection, 3=moderately severe, 4=severe, 5=very severe. The numbers of egg masses per plant on infected roots were counted after staining with Phloxin B [46]. The nematode population was recorded in soils of each treatment separately, after 90 days. The final population density of nematode was determined based on Cobb's sieving and decanting method [47]. The number of nematodes per pot was counted using counting dish.

The reproduction factor (R_F) was calculated by the formula [48]: $R_F = P_F/P_I$

Where P_f is the final population and P_i is the initial population.

Data analysis: Data on plant height, fresh shoot weight, fresh root weight and dry shoot weight, number of galls, egg mass/root, and final nematode population / pot were statistically analyzed as described by Gomez and Gomez [49]. The data were subjected to an Analysis of Variance (ANOVA) procedures using Statistical Analysis system [50] (version.9.1.3, SAS Institute Inc., Cary, NC, USA). All data were subjected to analysis of variance and Duncan's New Multiple Range Test used to separate means at 5% level of probability.

Results and Discussion

In vitro effect of botanicals and *Trichoderma harzianum* against juvenile mortality

The results of the treatments with plant extracts and *T. harzianum* individually and in combination immobilized with *M. incognita* J_2 after 24, 48 and 72 h of exposures are given in (Table 2). The percentage mortality of the second stage juveniles of *M. incognita* under *in vitro* tests as affected by aqueous plant extracts and *T. harzianum* at 24 h showed that there was a significant difference between the treatments. Plant extracts were more effective in immobilizing J_2 than *T. harzianum*. The neem seed extracts were effective in causing J_2 mortality with 10% concentration being more efficacious. Neem seed extract at 10% concentration caused significant mortality of *M. incognita* J_2 24 h after treatment application when compared to all the other treatments. Neem seed extracts which applied at 10% concentration immobilized J2 by 89, 93 and 100% after 24, 48 and 72 h of exposure, respectively. Similar results were reported by Agbenin, [51] after 24 h of exposure of all the treatment levels of dry leaf neem extract caused 100% mortality of larvae except in the control where 85% of larvae remained alive. Parmar, [52] also reported that aqueous extracts of leaf, flower, fruit, bark, root and gum of neem were reported to be highly toxic to nematodes with fruit extract showing the most lethal activity followed by leaf extract. In the present study, at 5% concentration of botanicals, the highest juvenile mortality within 24 h was shown in neem seed and followed by *L. camara*, African marigold and neem leaf respectively. After 48 h of application both at 5 and 10% concentrations, the highest mortality was shown in neem seed and the lowest mortality was shown in rape seed+*T. harzianum,* respectively. After 72 h treatment application the highest and the lowest percent mortality was found in neem seed and rape seed+*T. harzianum,* respectively. On the other hand, all botanicals which combined with *T. harzianum* and applied at both 5% and 10%

Percent mortality of J2 of *M. incogita*				
Treatments	Con. %	24h	48h	72h
Rape seed leaf extract alone	5	72.33e	76.33e	78.67d
	10	77.67d	81.33d	84.67c
Lantana leaf extract alone	5	82.67bc	86.33bc	87.33c
	10	82.60bc	85.33bcd	96.00b
African marigold leaf extract alone	5	80.33cd	83.33cd	86.33c
	10	84.00b	88.00b	95.00b
Neem leaf extract alone	5	79.00d	82.00d	85.33c
	10	83.33bc	86.67bc	94.67b
Neem seed extract alone	5	84.33b	88.67b	94.00b
	10	89.00a	93.00a	100.00a
***T. harzianum* suspension alone**	5	64.33f	70.00f	80.67d
Rape seed + *T.harzianum*	5	50.33i	53.00e	57.33g
Lantana + *T. harzianum*	5	58.67g	61.67g	65.67f
African marigold + *T. harzianum*	5	55.667gh	58.67gh	64.00f
Neem Leaf + *T. harzianum*	5	54.00h	56.00hi	59.00g
Neem Seed + *T. harzianum*	5	62.30f	67.00f	73.67e
Distilled Water (Control)	5	0.00j	0.00j	0.00h
LSD **CV (%)**		3 2.68	3.69 3	3.54 2.78

Note: Means in each column followed by the same letter were not significantly different at (P<0.0001), according to Duncan's Multiple Range Test (DMRT)

Table 2: Percentage mortality of the J2 of *M. incogita* under *in vitro* test using botanicals and *T. harzianum.*

concentrations showed less mortality of juveniles than individually applied within 24, 48 and 72 h. There were no significant differences among treatments of rape seed leaf at 10% concentration, *L. camara*, African marigold and neem leaf at 5% concentration within 72 h. Effects of all treatments and *T. harzianum* on J2 mobility continued as exposure time increased, although the differences were not significant as such after 24 h (Table 2). Generally, the mortality rates of juveniles increased with an increase in exposure time. A similar result was reported by Elbadri et al. [53].

There were significant differences between treatments in number of infective juveniles/egg mass of *M. incognita* (Table 3). Different botanicals applied at different concentrations and *T. harzianum* individually and in combination adversely inhibit egg/juvenile hatching. Among botanicals applied, neem seed at concentration of 10% can inhibit egg mass hatching to juveniles, because this concentration had least number of infective juveniles per 10 egg masses, in comparison to 301 juveniles in control. There were no significance difference between the treatment of Rape seed, *L. camara,* African marigold because there were 30-36 number of juveniles per ten egg masses at the concentration of 10%, respectively, but *L. camara* were more effective next to neem seed and neem leaf than other treatment at 10% concentrations. On the other hand there were no significance difference statistically between *T. harzianum* which applied at 5% concentration individually and combination with other botanicals but *T. harzianum* with combination of neem seed at both 5% concentration each were inhibit juvenile hatching. Rape seed leaf applied at both concentrations was less effective because 56 juveniles were hatched per 10 egg masses when compared to control. In general botanicals applied at concentration of 10% was more effective than botanicals applied at 5% concentration on egg mass hatching than *T. harzianum* applied at 5% concentration. Neem seed, neem leaf and *L. camara* at both concentrations, African marigold at 10% concentrations reduce the hatching maximum (>90%) over the control. Both at 10 and 5% concentrations, the greatest percentage of hatching inhibition (96%) and (92%) was achieved by neem seed, neem leaf followed by *L. camara* and African marigold (90%). Among the botanicals the least egg mass inhibition was obtained by rape seed leaf at both concentrations individually (88%) and combination with *T. harzianum*. Susan and Noweer, Susan AH and Noweer EMA [54] reported that the plant extracts of basil, marigold, pyrethrum, neem and china berry proved to be effective against *M. incognita*. Also, the inhibitory effect of the extracts might be due to the chemicals present in the extracts that possess ovicidal and larvicidal properties [55]. These chemicals either affected the embryonic development or killed the eggs or even dissolved the egg masses. Similar results were reported that the extracts contained alkaloids, flavonoids, saponins, amides including benzamide and ketones that singly and in combination inhibit egg mass hatching [56,57]. Also, Salawu EO [58] reported that the neem seed extracts to inhibit egg hatch, and juvenile activity. In the present study, the neem seed was acted as the highest in juvenile mortality and egg mass hatch inhibition by *in vitro*. Meira et al. [59] reported that the soluble plant extracts were very effective in inhibiting egg-hatch and larval motility of nematodes. The active principles of neem viz. nimbidin and thionimone were reported to be highly active against nematodes. Fatema and Ahmad [60] have been reported that the extracts of neem leaf and garlic bulb completely inhibited hatching of egg masses of *M. incognita* and were lethal to larvae. In this study, the neem leaf extracts can inhibit 90% of egg hatching. The inability of the egg mass to hatch is as a result of ingress/entrance of plant extracts into the egg mass. Larvae in the egg mass were exposed to the toxic effect of the extract resulting first in reduced mobility and finally death or moribund state. Once this state is reached the larva cannot pierce through the wall with its stylet hence hatching ceases. The egg mass which is a part of the perineal region of the female in root-knot is permeable to the active ingredient in the extracts [61]. These compounds act by various mechanisms like blocking molting of larvae, disrupting mating and sexual communication of nematodes, reducing the motility of gut and by inhibiting the formation of chitin [62]. Sharon et al. [63] showed that eggs adhered with *Trichoderma* conidia became non-viable, thus decreasing the eclosion rate. In this study, the botanicals used only they were effective but when they were used in combination they show less effective so it is evident that as extract was diluted, toxicity was decreased resulting in correspondent decrease in inhibition and any inhibition was observed in distilled water.

Treatments	Con.	No. eggs hatched to J_2 after 7days	Z**
Rape Seed Leaf extract alone	5	36.00cdef	88
	10	33.60cdef	89
Lantana leaf extract alone	5	30.67cdef	90
	10	32.60cdef	91
African marigold leaf extract alone	5	33.00cdef	89
	10	27.00ef	91
Neem Leaf extract alone	5	33.33cdef	89
	10	29.00def	90
Neem Seed extract alone	5	25.00f	92
	10	10.67g	96
T.harzianum suspension alone	5	38.67cde	87
Rape seed + *T.harzianum*	5,5	56.33b	81
Lantana + *T. harzianum*	5,5	40.30cd	87
African marigold + *T. harzianum*	5,5	43.00c	86
Neem Leaf + *T. harzianum*	5,5	54.67b	82
Neem Seed + *T. harzianum*	5,5	39.33cde	87
Distilled Water (Control)	5	301.33a	
LSD		11	
(CV)%		13	

Note: means in column with the same letter are not significantly different (P<0.0001) by DMRT.
Z** Hatching inhibition over the control in percent

Table 3: Egg mass hatching and hatching inhibition of *Meloidogyne incogita* by *in vitro* test using botanical aqueous plant extracts and *Trichoderma harzianum*.

In vivo effect of botanicals and *Trichoderma harzianum* against *M. incognita*

Plant height: The treatments did not showed any negative effects on plant growth. There were significant differences in the height of tomato plants treated with aqueous plant extracts and *T. harzianum* over inoculated control plants (Table 4). The highest plant height was observed in pots treated with combination of *L. camara* and *T. harzianum* followed by neem seed and neem leaf with *T. harzianum* over inoculated control. The lowest height of plants was recorded in pots treated with rape seed leaf. The highest plant height was 160% increased in pots treated with combination of *L. camara* and *T. harzianum* over inoculated control. Pots treated with combination of botanicals and *T. harzianum* showed more height than botanicals applied alone or without fungus. The addition of botanicals to soil leads to a better environment for the growth of the roots. This enhances the utilization of soil nutrients, as a consequence of which the nematode damage might have been markedly reduced [64]. These botanicals may be act as substrate for the growth and multiplication of *T. harzianum*. Some *Trichoderma* isolates were reported to do both enhanced plant growth and reduced root-knot nematode damage [65]. It has been reported that *Trichoderma* has not only been proved to parasitize

nematodes and inactive pathogen enzymes but also help in tolerance to stress condition by enhanced root development. It participates in solubilization of inorganic nutrients [66]. The shortness' of the plant height might be due to the stunting action of *M. incognita*. Jinfa et al. [67] also reported that this kind of height reduction caused by root-knot nematode. In inoculated control, the lowest growth performances by the control plants could be as result of the combined effect of nematodes and availability of nutrients [68]. The galls on the root system might disturb important root functions like uptake and transport of water and nutrients [13].

Fresh and dry shoot weight: The higher fresh shoot weight was significantly obtained in seedlings treated with aqueous plant extracts and *T. harzianum* over inoculated control (Table 4). The highest and the lowest shoot fresh biomass was observed in plants treated with the combination of *T. harzianum* with *L. camara* and rape seed leaf, 146 and 80 g, respectively, when compared with inoculated control. The results of the present experimental study was not agree with Agbenin et al. [51], Neem seed powder increased root and shoot weights and heights and decreased root galling index and presence of mycelium on root. Generally, *T. harzianum* individually and combination with botanicals showed more effective on plant fresh shoot weight than botanicals. Dry shoot weight of plants after 90 days were significantly lower in inoculated control plants than inoculated treated plants (Table 4). There were no significance difference between pots treated with all botanicals applied individually, rape seed and neem leaf with combination of *T. harzianum* and un inoculated control but they were significant difference when compared with inoculated control. The maximum total plant shoot dry weights were recorded in pots treated with combination of *L. camara* and *T. harzianum* followed by *T. harzianum* with combination of neem seed and neem leaf over inoculated control. The lowest dry shoot weight was observed in pants treated with rape seed than other treatments (Table 4).

Fresh root weight: There were highly significance differences among recorded fresh root weight between pots treated with aqueous plant extracts and *T. harzianum* when compared with inoculated control (Table 4). The highest fresh root weight was recorded by plants grown on pots with inoculated control followed by neem leaf with *T. harzianum* and African marigold jointly with *T.harzianum*. The lowest weight was recorded in pots with un inoculated control or negative control when compared with inoculated control. *Trichoderma* spp. found in close association with roots contributes as plant growth stimulators [69]. In the present study, the root weight of inoculated control was greater than that of un inoculated weight. Wong and Mai [70] reported that differences in root weight may be explained by gall development, gall mass being heavier than an equivalent linear length of similar non-galled roots. Perry et al. [71] reported similar results that root weight increased in untreated infected plants compared with those amended with herbal powder due to the formation of galls and giant cells.

Number of galls per root system: The number of galls per root system was observed significantly reduced between the pots treated with aqueous plant extracts and *T. harzianum* over inoculated control (Table 5). Maximum inhibition of gall formation was observed in pots treated with combination of lantana and *T. harzianum* and followed by neem seed with *T. harzianum* .The highest and lowest reduction of number of galls per root system was observed in pots treated with combination of *L. camara* with *T. harzianum* and rape seed leaf because they reduced number of galls by 88 and 37% over inoculated control, respectively. Combination of *T. harzianum* with neem seed, neem leaf and the fungus only also showed gall reduction next to combination of neem seed with *T. harzianum* that shows gall reduction 83, 79 and 75%, respectively. Generally the highest reductions in number of galls per root were observed in pots treated with combination of botanicals and *T. harzianum* than botanicals treated individually. The lowest growth rate, high galling due to nematode activity at root zone resulting in giant cell formation, high population of nematodes because the nematodes larvae were able to penetrate roots freely and reproduce without any inhibition. A reduction in root knot development could be attributed to poor penetration of the second stage juveniles and later retardation in their activities, for example feeding and /or reproduction

Treatment	Cons.	Plant height (cm)	Z**	Fresh shoot weight (g)	z**	Dry shoot weight(g)	Z**	Fresh root weight(g)
Rape seed leaf extract alone	10	68.00d	50	80.00d	46	23.17e	71	41.00cd
Lantana leaf extract alone	10	80.0bcd	66	109.00c	88	26.00e	86	29.33efg
African marigold leaf extract alone	10	80.bcd	66	108.00c	86	25.50e	82	27.00fg
Neem leaf extract alone	10	73.33cd	52	86.00d	48	25.00e	78	24.00gh
Neem seed leaf extract alone	10	72.33cd	41	85.00d	38	24.00e	64	20.00h
T.harzianm **suspension only**	20	83.0bcd	73	110.00c	89	27.00e	93	30.00efg
Rape seed + *T.harzianum*	10+10	88.0bcd	83	120.00bc	106	30.00de	114	35.00de
Lantana + *T. harzianum*	10+10	125.00a	160	146.47a	151	62.00a	342	10.00i
African marigold + *T. harzianum*	10+10	89.00bc	85	121.00bc	108	38.00cd	171	45.00bc
Neem Leaf + *T. harzianum*	10+10	91.67bc	90	128.00b	120	40.00bc	185	48.00b
Neem Seed + *T. harzianum*	10+10	95.00b	98	130.00b	124	48.00b	242	32.00ef
UC	-	84.67bcd		111.00c		28.00e		19.00h
IC CV (%)	- 12.82	48.00e 8.2	16	58.00e	12.7	14.00f		55.00a
LSD	17.84	14.76	8.5		6.8			

Note: means in column with the same letter are not significantly different (P<0.0001) DMRT.
Z** increase over inoculated the control in percent.
Values are averages of three replicates.
Significance is given compared to positive controls (inoculated control).
Control positive = control in conjunction with inoculation of *M. incognita* juveniles.
Control negative = control without inoculation of *M. incognita* juveniles

Table 4: Effect of aqueous plant extracts and Trichoderma harzianum Table 4 Effect of aqueous plant extracts and Trichoderma harzianum on growth of tomato plants against root-knot nematode infested soil under green house condition on growth of tomato plants against root-knot nematode infested soil under green house condition.

Treatment	Con_{st}	Gall/root	X**	Eggmass/ root	X**	Final Nematodepopulation./ pot	X**	Reproduc- tion factor(R=PF/P)
Rape Seed leaf extract alone	10	203.00b	37	175.00b	39	650.00b	79	0.325b
Lantana leaf extract alone	10	115.00ef	64	91.00de	68	513.00c	83	0.26de
A. marigold leaf extract alone	10	138.00d	57	128.00c	55	548.00c	82	0.27c
Neem leaf extract alone	10	158.00c	51	129.00c	55	560.00c	81	0.275c
Neem seed extract alone	10	120.00de	63	96.00d	66	521.00c	83	0.261cde
***T.harzianum* suspension alone**	20	79.00hi	75	62.00g	78	532.00c	82	0.266f
Rape seed+*T.harzianum*	10+10	99.00fg	69	83.00ef	71	509.00c	83	0.25de
Lantana+*T.harzianum*	10+10	39.00k	88	35.00i	87	303.00d	90	0.15g
African margold+*T.harzianum*	10+10	90.00gh	72	78.00f	73	490.00c	84	0.245ef
Neem leaf+*T.harzianum*	10+10	68.00ij	79	53.00h	81	463.33c	85	0.23cd
Neem seed+*T.harzianum*	10+10	55.00jk	83	42.00i	85	341.00d	89	0.17h
UC	-	0.00l		0.00j		0.00e		0.00e
IC	-	325.00a		290.00a		3080.00a		1.54a
CV (%)		9.81		5.44		8.2		
LSD		18.85		8.86		89.8		

Note: means in column with the same letter are not significantly different (<0.0001) PDMRT
Each pot contains 3000 cc sterilized soil.
X**: Reduction over inoculated control in percent

Table 5: Effect of aqueous plant extracts and *Trichoderma harzianum* on nematode population, gall and egg mass on tomato plants (cv. Marglobe) in root-knot nematode infested soil under green house.

as suggested by Abdi M [72].

Number of egg masses per root system: There were significant differences between treatments on egg mass reduction over inoculated control (Table 5). Similarly all the treatments were found to be highly effective in their ability to reduce egg mass per root system when compared with inoculated control/untreated plants. The highest and the lowest egg mass reduction was observed in pots treated with *L. camara* combined with *T. harzianum* and botanical rape seed leaf over inoculated control. The highest percentage of egg mass reduction was observed with pots treated with combination of *L. camara* and *T. harzianum* (87%) and followed by combination of neem seed with *T. harzianum* (85%) and *T. harzianum alone* (80%). Concerning the effect of rape seed on nematodes it is true with results reported by Johnson et al. [73].

Final nematode population and reproduction factor: The suppressive effect of aqueous plant extracts and *T. harzianum* was recorded as the nematode population in the soil at the end of the experiment 90 days after nematode inoculation. Significantly, the less number of parasitic nematodes was observed in the soil samples obtained from pots treated with *L. camara* with *T. harzianum* as compared to the control. Among treatments, pots treated with *L. camara* and *T. harzianum* showed more effective in reducing the final nematode population over inoculated control. For this reason it is suggested that the use of plants residue too would be more efficient against nematodes when used in combination with other management practices that are currently available. Except rape seed there were no significant difference between pots treated with botanicals each other. The maximum and minimum final nematode population was recorded from combination of rape seed leaves and *T. harzianum* and *Lantana* leaves with *T. harzianum,* respectively, (Table 5). The highest percentage (90%) of final nematode population reduction was shown in pots treated with combination of *L. camara* combination with *T. harzianum* followed by combination of neem seed with *T. harzianum* (89%) over inoculated control. The lowest nematode population reduction was observed in pots treated with rape seed leaf when compared with other treatments (Table 5). A reduction in root-knot development could be attributed to poor penetration of the second stage juveniles and later retardation in their activities, for example feeding and/or reproduction as suggested by Abdi [72]. Nematotoxic compounds especially the Azadirachtin released through gradual decomposition of the neem seeds [29] and suppress nematode populations throughout the whole period of the nursery stage. Nematode population in nematode + fungus treatment was 532 but *L. camara* has decreased population to 303. Except rape seed leaf extract, there were no significant differences between the pots treated with all botanicals including *T. harzianum* which applied individually. In this study, African marigold and neem seed treated pots were reduced nematode population in the soil 82 and 83%, respectively. Similarly, Hasabo and Noweer [74] found that the soil treatment with aqueous extracts of marigold leaves and neem seeds significantly reduced *M. incognita* J_2 in soil and roots of egg plants. The J2 population in roots was reduced by 90% and 75% respectively, 4 months after treatments, applied at 50 ml/plant as soil drench. Begum et al. [75] and Qamar et al. [76] observed that isolated chemical constituents such as lantanoside, lantanone, camaric acid and oleanolic acid from aerial parts of *L. camara*, possessing nematicidal activity against *M. incognita*. Ahmad et al. [77] also noted that various concentrations of leaf extract of *L. camara* were deleterious to *M. incognita*.

Reproduction factor: The reproduction rate of *M. incognita* was significantly suppressed by all the treatments as compared to untreated inoculated plants (Table 6). Reproduction rate of *M. incognita* was 0.23 in nematode+ fungus but its decrease to 0.15 by *L. camara*. Nematode reproduction factor was reduced in pots treated with combination of lantana and *T. harzianum* followed by pots treated with neem seed and *T .harzianum* when compared with inoculated control than other treatments. The highest nematode reproduction factor was observed in pots treated with rape seed leaf aqueous extracts than other treatments. This is because we suggest that *L. camara* act as substrate for the growth and multiplication of *T. harzianum*. Decomposed leaves have been found to support greater sporulation and multiplication of *T. harzianum* and *P. chlamydosporia* [78]. Several authors have been shown the potential of using plant extracts in the control of plant parasitic nematodes [79,80,81]. The reduction in population of *M. incognita* in this investigation may be due to the accumulation of nematicidal components and/or to increase host resistance. This significant reduction on the final nematode population density in

the soil could be due to the chemicals present in the extracts that possess ovicidal or larvicidal properties resulting in inhibition of its multiplication. *T. harzianum* and *L. camara* not only could decrease nematode population but also increase growth parameters of tomato.

Fruit number per pot: Highest number of tomato fruit was found in pots treated with lantana camara + *T. harzianum* followed by neem seed+ *T. harzianum* over inoculated control (Table 7). Among all treatment the lowest number of fruit was recorded from pots treated with rape seed leaves. There were no significance difference between pots treated with only botanicals but they were significant difference from inoculated control. The inability of the control plants to flower and fruit is probably due to the combined action of the nematode and inadequate availability of nutrients.

Yield of tomato per hectare: Application of aqueous plant extract and *T. harzianum* on root knot nematodes; *Meloidogyne incognita* infested pot shows significant difference ($P \leq 0.0001$) on yield of tomato over control (Table 7). The highest yield of tomato was observed in pots treated with combination of *Lantana camara* with *T. harzianum* and followed by neem seed with *T. harzianum* over inoculated control, respectively. The lowest yield was observed in pots treated with aqueous rape seed leaf extracts. The presence of nematode on tomato plants significantly affected their yield, un inoculated plants had 82% yield higher ($P \leq 0.0001$) than inoculated plants. Root colonization by *Trichoderma* spp. frequently enhances root growth and development, crop productivity, resistance to a biotic stresses and uptake and use of nutrients [82,83]. Cuevas VC [84] showed that the presence of the fungus in the soil in sufficient population resulted in the uptake of more mineral nutrients especially P and Zn available for plant use that increased crop growth and yield in the screen house and farmers' field.

Conclusions

For its management, different plant species (botanicals) and an antagonistic fungus, *T. harzianum* were being tried in different forms as an alternative to nematicide. Water extract of all tested plants significantly inhibited egg hatching of nematode and resulted in 100% mortality of the second juveniles of *M. incognita in vitro* after 72 h of exposure. Results on mortality of infective juveniles (J_2) up to 100% in72 h and egg hatch inhibition up to 96% in seven day duration were observed in test tubes treated with neem seed and *L. camara* individually in laboratory experiment. Egg inhibition and larval mortality decreased with increase in dilution of all the extracts. Juvenile mortality increased corresponding to an increased time of exposure. Similarly a prominent reduction in final nematode population density, egg mass, galls per root system and a significant increase yield per plant and total yields of tomato were observed from plants treated with the combination of *T. harzianum* with *L. camara* and neem seed extracts compared to any other treatments. These results suggest that in laboratory experiment application of aqueous neem seed and *L. camara* in green house combination of *T. harzianum* jointly with *Lantana* leaf and neem seed would be a good alternative to manage root-knot nematode populations in tomato production. These combinations not only reduce nematode infestation and population buildup on tomato but also increase soil fertility. Therefore, bio-control is suggested to be a safer solution. Botanicals are more effective jointly with fungus than applied individually in green house because some botanicals act as a substrate for the growth and multiplication of *T. harzianum*. Non chemicals and eco-friendly management such as bio-control management system by using *T. harzianum* and botanicals mentioned above were gaining importance and also greater attention which are easily available, less cost effective with no pollution hazards.

Treatment	Con_s	Total no. of fruits/pot	Kg/Pot	t/he
Rape Seed leaf extract alone	10	7.50h	0.75ef	14.00f
Lantana leaf extract alone	10	10.00fgh	0.60fg	18.00def
A. marigold leaf extract alone	10	9.00gh	0.90def	16.00ef
Neem leaf extract alone	10	8.00h	0.80ef	15.00ef
Neem seed extract alone	10	11.00e-h	1.10cde	22.00cdef
T.harzianum suspension alone	20	14.00edf	1.15cde	23.00cdef
Rape seed+*T.harzianum*	10+10	15.00cde	1.20cde	24.00cde
Lantana+*T.harzianum*	10+10	34.00a	2.2a	44.00a
African margold+*T.harzianum*	10+10	17.33cd	1.30bcd	26.00bcd
Neem leaf+*T.harzianum*	10+10	19.00bc	1. 5bc	30.00bc
Neem seed+*T.harzianum*	10+10	22.00b	1.70b	34.00b
UC	-	13.00d-g	1.12cde	22.40cdef
IC	-	3.00i	0.20g	4.00g
CV (%)		17.5	22.6	22.1
LSD		4.13	0.4	8.35

Note: means in column with the same letter are not significantly different (P<0.05) by DMRT

Table 6: Effect of aqueous plant extracts and *Trichoderma harzianum* on yield of tomato plants (cv. Marglobe) in root-knot nematode infested soil under green house.

	HT	FSW	DSW	FRW	G.root	E.Mas	N.po	N.Fruit	Kg/pot	Ton/ha
HT	1	0.83	0.81	-0.48	-0.69	-0.70	-0.60	0.85	0.77	0.75
FSW		1	0.80	-0.35	-0.79	-0.79	-0.65	0.81	0.79	0.78
DSW			1	-0.31	-0.62	-0.61	-0.46	-0.90	-0.83	-0.81
FRW				1	0.54	0.55	0.59	-0.38	-0.42	-0.43
G.root					1	0.99	0.86	-0.68	-0.75	-0.73
E.Mas						1	0.88	-0.67	-0.74	-0.73
N.pop							1	-0.47	-0.57	-0.57
N.Frui								1	0.86	0.87
Kg/ pot									1	0.97
Tons/ ha										1

** Correlation is significant at the 0.0001 level
* Correlation is significant at the 0.05 level.
Where
HT=Height; FSW=Fresh dry weight; DSW=Dry shoot weight; FRW=Fresh root weight; G. root=Number of gall per root system; E.mas=number of egg mass per root system,; N.pop=Final nematode population; N. fruit=Number of fruit per plant; Kg/pot=kilogram per pot; Ton/ha=ton per hectare

Table 7: Correlation of plant height, fresh shoot weight, dry shoot weight, fresh root weight, yield/tons/hectare, egg masses, final population, and number of gall Meloidogyne incognita in tomato plant under green house.

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Citation: Feyisa B, Lencho A, Selvaraj T, Getaneh G (2015) Evaluation of Some Botanicals and *Trichoderma Harzianum* for the Management of Tomato Root-knot Nematode (*Meloidogyne Incognita* (Kofoid and White) Chit Wood). Adv Crop Sci Tech 4: 201. doi:10.4172/2329-8863.1000201