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Steinernema kraussei and *Moringa oleifera* Extracts Can Suppress *Meloidogyne incognita* Infection on Tomato

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Authors' Contribution
SAK and NJ planned and supervised the study and experiments. AP conducted the study. AM, KJ, HS and AS helped in performing the experiments and data interpretation. AM wrote the manuscript and performed statistical analysis.

Key words
***Lycopersicon esculentum* L, Inhibition, Mortality, Juveniles, Root-knot nematode, Entomopathogenic nematode**

ABSTRACT

Meloidogyne incognita (Kofoid and White) Chitwood (MI) is a potential threat to tomato (*Solanum lycopersicum* L.) production. In this study, the inhibitory effect of *Moringa oleifera* L. (MO) extracts and *Steinernema kraussei* Steiner (SK) as stand-alone treatments and in combination was assessed to suppress the reproduction and development of MI. Highly susceptible variety of tomato cv. 'Money Maker' was tested for the invasion and development of MI. Aqueous and ethanolic MO extracts were tested in combination with SK at three different concentrations and time intervals. After 12-h 'S' concentration of aqueous MO extract + ethanolic MO extract + SK produced 100% juvenile mortality while the treatment MO + SK caused the least juvenile mortality of MI. Juvenile mortality was increased in a time-dependent manner. Aqueous MO extract + ethanolic MO extract + SK produced 100% egg hatching inhibition at standard 'S' concentration. Plant growth was significantly enhanced in plants treated with MO extracts, SK and their combinations compared to MI treated control plants. The total number of infective juveniles/g root and soil, galls, egg masses, females were highest in MI treated plants while lowest in plants treated with a combination of MI + SK + aqueous MO + ethanolic MO after 60 days of inoculation. SK and MO has demonstrated significant potential to suppress MI infection. Therefore, further investigations should focus on their efficacy at field level to prevent the infection of MI.

INTRODUCTION

Plant parasitic nematodes (PPNs) are considered as an unremitting challenge that is responsible for huge production loss annually. Among PPNs root-knot nematodes (RKNs) (*Meloidogyne* spp.) affect major agricultural crops worldwide and are well-documented from Pakistan as a major threat to several vegetable crops with a diverse host range of above 100 (Maqbool, 1986; Zaki, 2000; Abad *et al*., 2008). In Pakistan, 5-20% losses due to PPNs has been reported (Maqbool *et al*., 1988). RKNs has been estimated to cause a 20-33% yield loss in tomato (Sasser, 1979; Sasser and Carter, 1982; Upadhyay and Dwivedi, 1987). In Punjab, Pakistan 75-100% disease incidence has been reported in tomato due to RKNs infection (Shahid *et al*., 2007). Among RKNs, *Meloidogyne incognita* (Kofoid and White) Chitwood (MI) is a major threat to tomato production (Fourie and McDonald, 2000). MI attacks the root system of plants and reduces the uptake of water and nutrients from the soil leading to poor growth of the plants (Walia and Bajaj, 2003).

Currently, the use of synthetic chemicals i.e., nematicides is the most frequent and rapid mean to control RKNs infection. However, synthetic chemicals are a continuous challenge to the environment safety due to their residual effect in soil, ecological imbalance, volatile nature and hazardous effects on human health (Chitwood, 1949). Excessive use of nematicides can lead to pest resistance. In developed countries, growers preferably use relatively safer alternates to synthetic chemicals. Biological control of plant pathogens has achieved huge attention in the past decade. MI has been successfully suppressed by using biological control agents (Murslain *et al*., 2014; Muhae-ud-Din *et al*., 2018). Several studies have reported the environment-friendly nature of antagonistic entomopathogenic nematodes (EPNs) that are safer to the ecosystem and do not affect the free-living nematodes in the soil (Bonning and Hammock, 1996). More than twelve species of entomopathogenic nematodes are commercially available for biological management of RKNs and insect pests persisting in the soil (Klein, 1990; Raichon *et al*., 1994; Georgis and Manweiler, 1994; Bonning and

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Hammock, 1996; Grossman, 1997). Entomopathogenic nematode *Steinernema kraussei* Steiner (SK) has been used as a potential antagonistic agent against nematodes and insect pests (Perez and Lewis, 2002; Chaudhary *et al*., 2017). Botanical extracts with antimicrobial properties provide another environment-friendly option for the management of nematodes (Mukhtar *et al*., 2004). Plants naturally contain toxic antimicrobial compounds (Ngadze, 2014). *Moringa oleifera* L. (MO) extract has been used previously for the suppression of *M. javanica* infection on eggplant in combination with *Trichoderma harzianum* (Murslain *et al*., 2014). Aqueous extracts of *Ocimum gratissimum*, *Veronia amygdalina*, *M. oleifera,* and *Azadirachta indica* were found to possess a suppressive effect on MI race 2 (Claudius-Cole *et al*., 2010).

A very little progress has been made in Pakistan on the combined use of MO extracts and entomopathogenic nematodes for the management of root-knot nematodes. The project was undertaken to evaluate the antagonistic potential of entomopathogenic nematode SK and aqueous and ethanolic extracts of MO as stand-alone and combined treatments against *M. incognita* infection on tomato.

MATERIALS AND METHODS

Propagation and inoculum preparation of MI

Egg masses were collected from infected roots of eggplant to maintain MI culture. MI culture was reared on tomato seedlings in sterilized pots filled with sterilized medium sand: clay (1:1). Pots were kept in the glasshouse for 60 days to produce nematode inoculum for further studies. A stock culture of the second-stage juveniles (J2's) was obtained from mature egg-masses after immersion in sterilized water for 7–10 days. The extracted nematode juveniles were identified by perineal patterns of the mature MI female. It revealed the presence of a high, square-shaped dorsal arch, consisting of a distinct whorl in the terminal area of the tail and smooth to wavy striations. Distinct lateral lines were absent but breaks and forks in striations were obvious. Eggs of MI were extracted from infected tomato roots using the method described by Hussey and Barker (1973). The number of nematode eggs in the suspension was counted in a counting dish under a stereomicroscope. The number of eggs was adjusted up to 2000/mL by concentrating the suspension.

Culturing and extraction of SK

Cadavers of *G. mellonella* were firstly surface sterilized with 70% methanol for 3 minutes to avoid surface contamination and then transferred to clean Petri plates lined with clean filter paper at the bottom. SK was poured in each Petri plate at the rate of 300 juveniles aseptically. Petri plates were sealed with Nescofilm and incubated at 27ºC for 2-3 days. A modified white trap consisting of a plastic container, filled with distilled water to a depth of 1cm was used for the study. An inverted small Petri plate was placed at the bottom of the container and a filter paper 9-cm-dia was placed on the Petri plate in such a way that the edges of the filter paper barely touch the water. The dead larvae were placed on the filter paper and container was closed with a lid and incubated at 27 ºC till the emergence of new progeny. Water was taken daily and observed under stereoscope for the emergence of SK. Infective juveniles started to leave the cadavers within 8-20 days after infection. Infected juvenile moved through the filter paper into the water and were collected and washed in three changes of distilled water using a sieve.

Preparation of plant extracts

Fresh MO plant sample was collected from the Department of Plant Pathology, Research Farm, University of Agriculture, Faisalabad. The leaves were thoroughly washed, and surface sterilized with sodium hypochlorite 1% NaClO and kept for drying in an incubator at 70 ºC for 2 days. Aqueous extracts were prepared by grinding 10g dried leaves in 100 mL of sterilized distilled water, followed by filtration through a muslin cloth and Whatman No. 1 filter paper to get the clear extract that was used as a standard "S" concentration. S/2 and S/4 concentrations were also maintained by up to 50% dilution of S and S/2 concentrations, respectively.

Plant material and inoculation

The soil was thoroughly mixed and air-dried by spreading in a thin layer on a plastic sheet under the sun. The soil used for the experiment was sandy loam (a mix of 72% sand, 17% silt, and 8% clay) of pH 7.1-7.8, a moisture holding capacity (MHC) of 45% and total organic matter 3.4-3.8%. The soil was sterilized by applying formalin and thorough mixing to equally distribute formalin in the soil. It was left for a week covered with a plastic sheet and later filled in clean earthen pots. Seeds of tomato cv. 'Money Maker' as a susceptible check and test lines were collected from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Tomato nursery was maintained, and 5-week-old seedlings of tomato were transferred to earthen pots (at a rate of 1 seedling per pot).

Two-week-old seedlings of tomato cultivars were inoculated with 5,000 eggs of MI. The soil was inoculated by slowly dispensing 2.5 mL of previously prepared nematode suspension around the plant near the root zone. A week after inoculation, soil in each pot was drenched with 250 mL of MO leaf extracts (ethanolic or aqueous) and

EPN's SK. The treatment combinations were as follows; 1) MI + MO aqueous (plants inoculated with MI and treated with MO aqueous extract), 2) MI + MO ethanolic (MI inoculated and MO ethanolic extract treated plants), 3) MI + SK (MI inoculated and SK treated plants), 4) MI + SK + MO aqueous (MI inoculated, SK and MO aqueous extract treated plants), 5) MI + SK + MO ethanolic (MI inoculated, SK and MO ethanolic extract treated plants) and 6) MI + SK + MO aqueous + MO ethanolic (MI inoculated, SK, MO aqueous and MO ethanolic extract treated plants), 7) Healthy Control (Healthy untreated plants). Nematode reproductive parameters *viz,* Total no. of egg masses, females, galls, no. of juveniles per gram of soil and no. of juveniles per gram of root were assessed at the end of the experiment.

Assessment of plant growth and nematode reproduction parameters

Total shoot length, root length, shoot weight and root weight of tomato plants were taken at the end of the experiment. Gall indexing was carried out using the method described by Coyne *et al*. (2007). Egg-masses were stained by dipping the roots in 0.015% Phloxine B solution for 20 minutes as described by Daykin and Hussey (1985). Stained roots were then washed under running water to remove the residual stain. The number of eggs per egg-mass were determined by selecting 10 egg-masses randomly from each root system and shaking in 1% NaClO solution for 3 minutes, the suspension was then sieved through 200 and 500 mesh size sieves (75 and 26µm) gently with tap water to remove soil debris during first sieving and eggs were collected after second sieving (Hussey and Barker, 1973). Eggs were collected in 50 mL water suspension and the number of eggs was counted in 1 mL suspension under a light microscope (10X). An average number of eggs/egg-mass was calculated. Roots were stained by lactophenol acid fuchsin method to count the adult females/root system under a stereoscope (6X) (Goodey and Franklin, 1959).

Statistical analysis

Data were statistically analyzed using statistical analysis software M-Stat (Ver. 2.3) Faisalabad, Pakistan. Treatment means were separated using the LSD test at 5% significance level after analysis of variance (ANOVA).

RESULTS

Effect of MO and SK on juvenile mortality and egg hatching inhibition of MI

Aqueous and ethanolic MO leaf extracts alone and in combination with SK tested against MI mortality proved to be significantly effective. The standard concentration of (SK + MO aqueous + MO ethanolic + MI) produced 100% egg inhibition and highest juvenile mortality (Figs. 1, 2). Significantly low mortality was observed at the standard concentration of MO aqueous extract treatment. Least mortality and no egg hatching inhibition was recorded in control treatment containing distilled water only. Larval mortality was significantly increased with the passage of time. The concentration S/4 was least effective as compared to other concentrations to cause juvenile mortality and egg hatching inhibition (Figs. 1, 2). Mortality of root-knot nematode MI was considerably affected by the concentration and time of exposure.

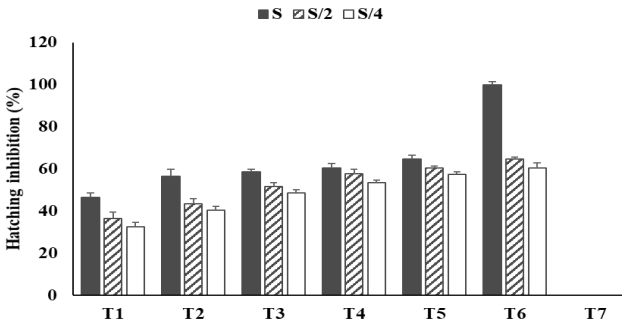

Fig. 1. Effect of *M. oleifera* extracts and *S. kraussei* on egg hatching inhibition of *M. incognita*; The treatments were as follows: T1, *M. oleifera* aqueous; T2, *M. oleifera* ethanolic; T3, *S. kraussei*; T4, *S. kraussei*+ *M. oleifera* aqueous, T5, *S. kraussei*+ *M. oleifera* ethanolic; T6, *S. kraussei*+ *M. oleifera* aqueous +*M. oleifera* + *M. oleifera* ethanolic; T7, Control; S, standard concentration (10%); S/2, S concentration diluted up to 50% (5%); S/4, S/2 concentration diluted up to 50% (2.5%).

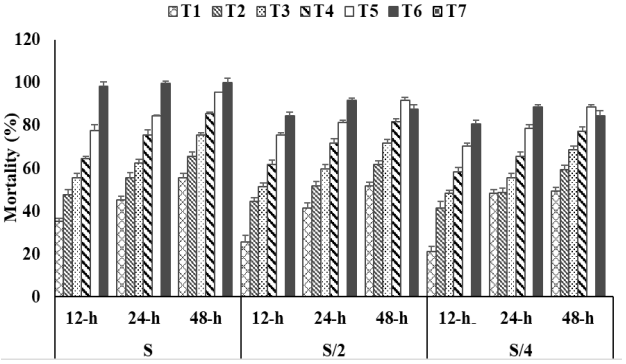

Fig. 2. Effect of *M. oleifera* extract and *S. kraussei* on juvenile mortality of *M. incognita*.
For abbreviations and other statistical details see Fig. 1.

Table I. Effect of *M. oleifera* extracts and *S. kraussei* on the growth parameters of treated plants.

Treatment	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)	Shoot lateral branches	Root hair	No. of leaves
MI (infected control)	30.0e[a]	10.4d	34.364f	12.18d	11.6e	34.4e	84.0d
MI + MO aqueous	31.3e	11.3cd	37.2e	11.8c	13.2d	34.8de	85.8d
MI + MO ethanolic	32.8d	11.5c	40.3d	11.6c	13.2d	35.0de	92.4c
MI + SK	33.0d	12.3c	39.2de	10.1c	16.4c	37.8cd	93.2c
MI + SK + MO aqueous	35.9c	13.9b	40.3d	8.1b	16.2c	40.0bc	96.4b
MI + SK + MO ethanolic	35.5c	13.4b	47.4b	8.0a	18.0b	40.4bc	97.6b
MI + SK + MO aqueous + Mo ethanolic	38.5b	14.1b	49.4b	7.6a	19.2a	41.0b	98.2b
Healthy Control	50.9a	22.7a	61.0a	5.9a	19.6a	44.4a	104a

[a]Means sharing same lettering are not significantly different from each other at $p = 0.05$ analyzed by least significant difference test; MI: *Meloidogyne incognita*, SK: *Steinernema kraussei*, MO: *Moringa oleifera.*

Table II. Effect of *M. oleifera* extracts and *S. kraussei* on reproductive parameters of *M. incognita* in tomato plants.

Treatment	No. of egg masses	No. of juveniles /g soil	No. of juveniles/g root	No. of galls	No. of females
MI (infected control)	549a[a]	2939.8a	7033.6a	465.8a	515.8a
MI + MO aqueous	529.6a	2780.4b	5041b	439.8b	489.8b
MI + MO ethanolic	473b	2623.4c	4434.2c	431.8bc	481.8bc
MI + SK	440.6c	2550.6d	3045.2d	422.2cd	472.2cd
MI + SK + MO aqueous	423c	2489.8e	3022.8e	407.4e	457.6d
MI + SK + MO ethanolic	364d	2391.6f	3007.8f	377f	427e
MI + SK + MO aqueous + Mo ethanolic	323e	2299g	1736.6g	347.8g	397.8f
Healthy Control	0f	0h	0h	0h	0g

For abbreviations, see Table II.

Effect of SK and extracts of MO on growth parameters of plants

Plant height did not vary significantly in all treatments. Plant height was comparatively less in control treatment. Fresh shoot weight was highest in (MI + MO aqueous + MO ethanolic) treatment. Minimum shoot weight was recorded in control. Maximum fresh root weight was recorded in control treatment followed by MO aqueous + MI, and MO ethanolic + MI treatments (Table I).

Effect of MO extracts and SK on reproduction of MI in tomato plants

The number of galls and the number of egg masses varied significantly in each treatment. Highest number of nematodes, number of galls, egg masses, juveniles per gram root and juveniles per gram soil were recorded in MI treated control plants while, minimum in (SK + MO ethanolic + MO aqueous) treatment (Table II). MO aqueous extracts were less effective on reproduction parameters than MO ethanolic extracts. The efficacy of MO aqueous and MO ethanolic extracts was significantly increased in combination with SK.

DISCUSSION

M. incognita has a broad host range and infects several field crops, fruits and vegetables. The present study aimed to control the infection of MI on tomato through MO extracts and entomopathogenic nematode SK to overcome the environmental hazards caused by synthetic nematicides. MO leaf extracts (MO aqueous and

MO ethanolic extracts) and entomopathogenic nematode SK alone and in combinations showed nematicidal properties against MI and caused juvenile mortality and egg hatching inhibition of MI. Previously, Murslain *et al*. (2014) reported that MO extracts in combination with *Trichoderma harzianum* caused highest juvenile mortality and egg hatching inhibition of *M. javanica* compared to control. The stand-alone treatment of MO extracts was also quite effective to cause juvenile mortality and egg hatching inhibition of MI. El-Ansary *et al*. (2018) reported that crude protein extracts from MO exhibited nematicidal effect against MI and can be proposed as effective and environment-friendly means to control MI infection. The mortality of root-knot nematodes might be due to toxic compounds in plant extracts that inhibit nematodes by penetrating directly (Chopra *et al*., 1963). Some of them like fatty acids (Tarjan and Cheo, 1956; Loos, 1958), phenolics (Hasan and Saxena, 1974) and alkaloids (Khan *et al*., 2009) were found to be lethal against nematodes. Phytochemical profiling of MO revealed that it contains tannins, saponin, alkaloids, steroids and reducing sugars (Izuogu et al., 2013). The nematicidal effect of MO might be due to the high content of certain oxygenated compounds that act on the cytoplasmic membrane of nematode and causes its disruption (Knobloch *et al*., 1989). The efficacy of MO extracts was significantly enhanced in combination with SK. The application of treatments in combination enhances disease suppression than a stand-alone application.

SK was tested to suppress MI on tomato as a stand-alone treatment and in combination with MO aqueous and ethanolic extracts at the rate of 1000 IJs per pot. The application of SK with MO aqueous + MO ethanolic + SK caused the highest reduction in MI egg production as compared to the application of SK alone. The antagonistic activity of SK is due to *Xenorhabdus* a symbiotic bacterium associated with this nematode (Ferreira and Malan, 2014). Our results are in conformation with the previous studies stating that EPNs can be used for the management of MI (Bird and Bird, 1986; Ishibashi and Kondo, 1986; Ishibashi and Choi, 1991; Kermarrec *et al*., 1991). However the suppressive effect of entomopathogenic nematodes on RKNs depends upon application time and species (Perez and Lewis, 2002). *Steinernema* spp. increased the inhibition of RKNs in combination with MO aqueous and ethanolic extracts due to their ability to enter the roots and inhibitory action of bacteria residing inside their body. The bacteria discharge allelochemicals in root tissues that are poisonous and inhibitory to root-knot nematodes (Fallon *et al*., 2002). The application of MO extracts and SK reduced the reproductive potential of MI and improved the plant growth. Previously, Murslain *et al*. (2014) reported the effect of MO extract and *Trichoderma harzianum* as stand-alone treatments and in combinations on reproductive parameters of *M. javanica* and growth of eggplant. They found that the highest reproduction of nematodes was observed in control treatments infected with nematode only. Similarly, Onyeke and Akueshi (2012) reported that treatment with MO reduced the reproductive potential of MI on African yam bean. Our results revealed that all treatments used in this study significantly ameliorated tomato plant growth. In support of our findings, El-Sherif *et al*. (2014) reported that MO powder caused a highest increment in the growth of eggplant viz., length of the plant, number of branches, number flowers, fresh and dry weight of the plant compared to control treatment. Previously, it has been reported by Foidl *et al*. (2001) that MO leaf extracts stimulated plant growth and enhanced yield up to 30% of different vegetables when applied as a seed treatment (Foidl *et al*., 2001). MO leaf powder improved plant growth and vigor by reducing nematode population density without any phytotoxicity (Makkar and Becker, 1996). It has been also reported that MO ethanolic extract possesses growth enhancing compounds belonging to cytokinin group that is effective in reducing nematode population with a subsequent increase in plant growth and development (Guzman, 1984). Therefore, the increment in the growth of tomato plant can also be attributed to growth enhancing compounds in MO extracts. It can also be concluded from the present study that MO extracts and SK improved the growth of the plant by hindering the growth and reproduction of MI. Therefore, the use of indigenous botanical extracts should be considered as a component of an integrated disease management strategy. The efficacy of these treatments should be further investigated at field level to include them as a component of an integrated disease management strategy of MI infection on tomato.

CONCLUSION

MO extracts and SK alone and in combination caused *in vitro* juvenile mortality and egg hatching inhibition of MI and suppressed the reproduction of MI on treated tomato plants. MO extracts and SK also improved the growth of plants by suppressing the development of MI. Hence, MO extracts and SK can be used as potential alternates to chemicals to control MI infection on tomato.

Statement of conflict of interest

The authors declare there is no conflict of interest.

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