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Eco-Friendly Management of Root-Knot Nematode, *Meloidogyne incognita* Infecting Pomegranate at Taif Governorate, KSA.

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ABSTRACT

Pomegranate is one of the most popular fruit trees cultivated in Taif, where special variety called Taify pomegranate is well known. Root-knot nematodes are the most damaging plant parasitic nematodes in pomegranate. The effect of five medicinal plant extracts i.e. colocynth (*Citrullus colocynthis*), marigold (*Tagetes erecta*), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*) and ashwagandha (*Withania somnifera*) as well as entomopathogenic nematodes i.e. *Steinernema* spp. and *Heterorhabditis* spp. and their symbiotic bacteria i.e. *Xenorhabdus* sp. and *Photorhabdus* sp. against *Meloidogyne incognita* infecting pomegranate under greenhouse condition was evaluated. Results indicated that colocynth extract significantly surpassed all other treatments in improving total plant fresh and shoot dry weights with increase percentage values of 126.6 and 140 %, respectively comparing to nematode alone, whereas, marigold extract was the best treatment in suppressing nematode development. *Steinernema* spp. improved plant growth parameters; however, its symbiotic bacteria *Xenorhabdus* sp. showed the highest nematicidal properties with reduction percentages of galls and egg-masses numbers averaged 97.9 and 95.8 %, respectively.

Keywords: Root-knot nematode, Eco-friendly management, and Taify Pomegranate.

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INTRODUCTION

Pomegranate, *Punica granatum* is one of the oldest known fruits, found in writings and artifacts of many cultures and religions, and is an original native of Persia. Pomegranates are widely cultivated in India and are one of the most in demand fruits in the Middle east. In Saudi Arabia, Al-Baha and Taif are both well-known for farming this fruit, but Taify pomegranate supply remains the most popular. Many other good cultivars are also cultivated in Taif where the weather and soil are suitable for its growth. This nutrient dense, antioxidant rich fruit has been revered as a symbol of health, fertility and eternal life. The fruit has beneficial ingredients. It contains high levels of flavonoids, polyphenols, vitamin C and contains vitamins B5, A and E. In addition, it is full of minerals such as calcium, potassium and iron, which add to its many health benefits and offering protection against heart disease and cancer. A glass of pomegranate juice has more antioxidants than green tea, blueberries, and cranberries. Pomegranate seeds are also a great source of fiber.

Plant parasitic nematodes are worldwide an economically important agric-pest, reducing the yield and quality of crops. Several plant parasitic nematodes were recorded as pathogens of pomegranates in many parts of the world [1]. Pomegranates are suspected to infect with a great number of plant parasitic nematodes. The root-knot nematodes, *Meloidogyne* spp. and *Xiphinema* spp. are the economically important parasites of pomegranate cultivars. Two of the root-knot nematodes, *Meloidogyne incognita* and *M. javanica*, are a serious pest of pomegranates which primarily create galls of root system that eventually are seriously hampered in their main functions of uptake and transport of water and nutrients, then reduced the growth and productivity of the pomegranates [1, 2].

Today, chemical nematicides are losing their popularity among farmers for protecting their crops from nematode infestations. Some safe procedures for nematode control have been developed passed on biological control agents and organic amendments; however, there is still a need for alternative, friendly methods or compounds for effective nematode control to be developed [3]. One way of searching for such nematicidal compounds is to screen naturally occurring components in certain plants. Many compounds with nematicidal activity have been found in plants including alkaloids, diterpenes, fatty acids, glucosinolates, isothiocyanates, phenols, polyacetylenes, sesquiterpenes and thienyls [4-6]. Extracts of certain plants and/or their components have been tested for nematicidal activity *in vitro* and *in vivo* by several workers [7-9].

For over ten years, entomopathogenic nematodes (EPNs) belonging to the families Steinernematidae and Heterorhabditidae (Rhabditida) have been used as effective biological control agents against a wide spectrum of insect pests. Steinernematids are symbiotically associated with entomopathogenic bacteria from the genus *Xenorhabdus*, and heterorhabditid nematodes are symbiotically associated with the genus *Photorhabdu*s. Suppression of plant parasitic nematodes has been recorded earlier by using entomopathogenic nematodes [10-12], tested the filtrates of *Xenorhabdus* spp. and *Photorhabdus* spp. cultivated in culture medium and in *Galleria mellonella* (L.) infected with entomopathogenic nematodes on the mortality of *M. incognita* second stage juveniles (J2s) after 24 h. They found that after dilution of the hemolymph from infected *G. mellonella,* the filtrates obtained from the *in vivo* culture and *in vitro* culture produced an 80% and 20% mortality rate, respectively. In 2014, [13] evaluated the nematotoxic activities of the cell-free conditioned media (CFCM) obtained from three strains of entomopathogenic bacterium species i.e., TT01, EMA and EMC, isolated from entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema bicornutum* and *S. rarum*, respectively in-vitro against *Meloidogyne incognita* (J_2s). Data revealed that the highest dose of TT01 CFCM resulted in 100% mortality rate after 48 hrs of exposure time.

The information on the role of alternative friendly methods for nematode management on pomegranate is lacking in Saudi Arabia. Therefore, the present study aimed to evaluate the eco-friendly compounds i.e. plant extracts as well as entomopathogenic nematodes and their symbiotic bacteria to control root-knot nematodes, *M. incognita* infecting Taify pomegranate.

MATERIALS AND METHODS

This study was carried out in the laboratory of Biology department, Faculty of Science, Taif University, Saudi Arabia to assay the efficacy of aqueous extracts of certain medicinal plants and entomopathogenic nematodes as well as their symbiotic bacteria against *Meloidogyne incognita* infecting pomegranate seedlings c.v. Taify under greenhouse conditions 30±5°C.

Plant Materials

The parts of five medicinal plants i.e. colocynth fruits (*Citrullus colocynthis*), marigold flowers (*Tagetes erecta*), rosemary leaves (*Rosmarinus officinalis*), thyme leaves (*Thymus vulgaris*) and ashwagandha leaves (*Withania somnifera*) were used in this study. Plant materials were collected randomly from plants grown in the university experimental farm and gardens.

Plant Extracts Preparation

Plant parts were washed thoroughly under running tap water, cut into small pieces, shade dried and used for extraction. Dried plant materials were homogenized to a fine powder and stored in airtight bottles. 20 g of each plant powder were extracted with 100 ml of distilled water for 24 h. The suspension was filtered with Whatman No. 1 filter paper, the residue re-extracted for an additional 24 hours, and similarly filtered. The two filtrates were combined, and a concentration of 4 % was prepared for each plant extract.

Nematode Inoculum Preparation

The root-knot nematode *M. incognita* eggs were extracted from infected coleus (*Coleus blumei*) roots as described by [14]. These eggs were obtained from a pure culture established from single egg mass of *M. incognita* that previously identified according to the characteristics of its perineal pattern [15] and reared on tomato plants.

Preparing of entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) used in this study were isolated from soil of pomegranate trees grown in Taif University gardens using the *Galleria mellonella* L. baiting method described by [16]. White trap technique was used for re-culturing of EPN [17]. The isolated nematodes were morphologically identified as *Steinernema* spp. and *Heterorhabditis* spp. by examining morphometrics for IJs and first-generation males [18].

Preparing of entomopathogenic bacteria

Infective dauer juveniles (IJ) freshly harvested from *Galleria* white traps were collected and washed by centrifugation with sterilized tap water three times. Some were then put to a drop of physiological saline (M9) solution in a sterile petri plates and then were transferred to 5% of chlorox. After 2 minutes long incubation animals were transferred to a series of sterilized M9 drop and finally were fragmented by using a sterile platinum wire. The drop of M9 were diluted and plated into NBTA indicator plate (5 g of Bacto Peptone, 3 g of beef extract, 15 g of Bacto agar [Difco], 25 mg of bromthymol blue, 40 mg of 2,3,5-triphenyltetrazolium chloride, 1,000 ml of distilled water [pH 6.8]). Colonies of presumptive entomopathogenic bacteria on NBTA were selected and classified based on colony morphology as *Xenorhabdus* sp. and *Photorhabdus* sp. according to [19]. Single colonies of each bacterium were removed from indicator plates and transferred to 5-ml of LB medium (10 g trypton [Difco], 10 g yeast extract [Difco], 10 g NaCl, 1,000 ml of distilled water [pH 6.8]) as an inoculum for 100 ml culture. The cultures were shaken for maximum aeration at room temperature overnight, and then transferred to 1000-ml Erlenmeyer flasks containing 400 ml of LB media, shaking (200 rpm, 25°C for 48 h). The bacterial suspension was centrifuged at 4800 rpm for 10 min. in 250 ml tubes, filtered through Millipore filter of 0.22 µm pore-size to remove the bacterial cells, resulting in a cell-free preparation of bacterial filtrates.

Nematicidal Activity Test

Two-months-old pomegranate seedlings c.v. Taify were grown in 25- cm-d. plastic pots (one seedling/pot) filled with 2500 g steam-sterilized sandy loam soil (1:1). Twenty seven pots were inoculated with 2000 *M. incognita* eggs per pot by pipeting around the base of each plant. Two days after nematode inoculation, 20 ml extract of each previous medicinal plants at the concentration of 4 % was applied to the soil surface. Entomopathogenic nematodes, *Steinernema* spp. and *Heterorhabditis* spp. were added at the rate of 5 ml of water containing about 4000 infective juveniles of each as well as bacterial filtrates of *Xenorhabdus* sp. and *Photorhabdus* sp. were applied at 5 ml. Three untreated and uninoculated as well as another three untreated with any of such extract and inoculated seedlings were left to serve as control. Each treatment was

replicated three times. All plastic pots were randomly arranged on a greenhouse bench at 30±5°C and watered regularly as needed.

After 45 days from nematode inoculation, plants were removed from pots and roots were washed free of soil. Data dealing with fresh weights of shoot and root as well as shoot dry weight were measured. The total number of galls and egg-masses per root system were recorded. Infected pomegranate roots were stained in 0.01 lactic acid fuchsin and examined for the numbers of galls and egg-masses [20]. The root gall index (RGI) and egg-mass index (EI) were rated according to the scale of 0 to 5, where 0= no galling or egg-masses, 1= 1-2 galls or egg-masses, 2= 3-10 galls or egg-masses, 3= 11-30 galls or egg-masse, 4= 31-100 galls or egg-masses and 5= more than 100 galls or egg-masses per root system [15]. Statistically, the obtained data were subjected to analysis of variance (ANOVA) [21] followed by Duncan's multiple range to compare means [22].

RESULTS AND DISCUSSION

Data in Table (1) represent the influence of aqueous extracts of five medicinal plants on the growth of pomegranate infected with *M. incognita*. Results indicated that all treatments obviously improved pomegranate plant growth parameters in terms of fresh and dry weight of shoot with various degrees as compared to control. Moreover, among tested treatments, colocynth extract significantly surpassed all other treatments in improving total plant fresh and shoot dry weights with increase percentage values of 126.6 and 140 %, respectively comparing to nematode alone. However, pots receiving marigold ranked second to colocynth extract regarding the same previous growth characters that were amounted to 93.5 and 100 %, whereas rosemary extract treatment gave moderate enhancement, since their values averaged 52.5 and 54.3 %, respectively. On the other hand, ashwagandha treatment gave the lowest values which were 23 and 37.1 %, respectively.

Table 1: Influence of plant extracts on the growth of pomegranate infected with *Meloidogyne incognita* under greenhouse conditions.

Treatment	*Plant Growth Response					
	Fresh weight (g)		Total F.W. (g)	Inc. %	Shoot D.W. (g)	Inc. %
	Shoot	Root				
Citrullus colocynthis	21.8 a	9.7 a	31.5 a	126.6	8.4 a	140
Tagetes erecta	19.3 b	7.6 b	26.9 b	93.5	7.0 b	100
Rosmarinus officinalis	14.3 c	6.9 c	21.2 c	52.5	5.4 c	54.3
Thymus vulgaris	13.7 cd	5.5 cd	19.2 cd	38.1	5.0 c	42.9
Withania somnifera	12.9 d	4.2 d	17.1 d	23.0	4.8 cd	37.1
Healthy Plants	14.1 c	5.9 c	20.0 c	43.9	5.2 c	48.6
N alone	11.3 de	2.6 e	13.9 e	--	3.5 d	--

*Each value is the mean of three replicates. Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple- rage test. N= 2000 eggs of M. incognita.

Number of galls was significantly reduced in all treatments compared with control. Root gall indices were ranged from 0.7- 4.0. Application of marigold gave the highest reduction percentage in root galling with value of 98.8 %, followed by colocynth extract which averaged 96.4 % without significant differences between them (Table 2). Our results are in line with several reports that claimed that these plants contain several biologically active compounds which are a source of nematotoxic effect. The nematicidal effect of the tested extracts may possibly be attributed to their high contents of certain oxygenated compounds which are characterized by their lipophilic properties that enable them to dissolve the cytoplasmic membrane of nematode cells and their functional groups interfering with the enzyme protein structure [23]. *Tagetes* species contain biocidal compounds of the thiophene group as non-polar products of secondary metabolites [4].

Egg-masses were significantly suppressed with egg-mass index (EI) ranged from 0.0-3.0 as compared with that of the control. The lowest EI was obtained from plants receiving marigold extract (0.0) with reduction percentage value of 100 % (Table 2). These findings agreed with [24] in respect to *Citrullus colocynthis* who mentioned that mentholic extracts of seed had the highest *Meloidogyne* spp. mortality (100%) at highest

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exposure time (72 hrs) while, the fruit extract showed 80% mortality, whereas, aqueous fraction (1 and 0.5) showed 40% mortality after 72 hrs exposure.

Table 2: Impact of certain plant extracts on development of *M. incognita* infecting Taify pomegranate under greenhouse conditions.

Treatment	No. of galls *	RGI	No. of Egg-masses	EI
Citrullus colocynthis	3.0 d	1.3 d	2.3 d	1.7 b
Tagetes erecta	1.0 d	0.7 e	0.0 e	0.0 c
Rosmarinus officinalis	9.3 c	2.0 c	5.3 c	2.0 b
Thymus vulgaris	17.3 b	3.0 b	9.0 b	2.0 b
Withania somnifera	23 b	3.0 b	11.0 b	2.7 a
N alone	82.3 a	4.0 a	24.0 a	3.0 a

*Each value is the mean of three replicates. Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple- rage test.

In the present work, treatment of *Withania somnifera* extract did not act as strong nematicide on nematodes infecting pomegranate, since it was found to be the least effective to reduce root-gall index as well as stimulate the reproduction of *M. incognita*. This findings are disagree with previous report of [25] who concluded that *W. somnifera* extract was the best treatment among tested extracts and callus tissue for both ameliorating eggplant growth criteria and controlling *M. incognita*. Our results support the observation of [26] who determined the inhibitory effects of the water extracts as well as powder of different parts of three plants, oak (*Quercus brantii* L.), true myrtle (*Myrtus communis* L.) and bitter cucumber (*Citrullus colocynthis* (L.) Schrad. on *Meloidogyne incognita* under *in vitro* and greenhouse conditions and recommended to use of whole plant (except root) extract (2%) and or powder (0.02%) of true myrtle in controlling *M. incognita* infecting tomato. On the other hand, this result does not agree with our findings regarding the nematicidal effects of oak and bitter cucumber which were very low especially in in vitro condition.

Data in Table (3) summarize the impact of two entomopathogenic nematodes i.e. *Steinernema* spp. and *Heterorhabditis* as well as their symbiotic bacteria i.e. *Xenorhabdus* sp. and *Photorhabdus* sp. filtrates on Taify pomegranate growth parameters under greenhouse conditions. Of the different treatments, pots received IJ of *Steinernema* accomplished the maximum values in whole plant fresh weight with increment percentage of 60.4 %, as well as shoot dry weight with percentage increase value of 65.7 %. Untreated and un-inoculated plants (Healthy plants) ranked second to *Steinernema* regarding the same characters with values of 43.9 and 48.6 %, followed by *Heterorhabditis* with values of 36.7 and 31.4 %, respectively. Lower fresh and shoot dry weights of pomegranate plants were obtained by *Photorhabdus* treatment with percentage increase value averaged 21.6 and 14.3 %.

Table 3: Impact of entomopathogenic nematodes and their symbiotic bacteria on the growth of pomegranate infected with *Meloidogyne incognita* under greenhouse conditions.

Treatment	Plant Growth Response					
	Fresh weight (g)		Total F.W.	Inc.	Shoot D.W.	Inc.
	Shoot	Root	(g)	%	(g)	%
Steinernema spp.	15.8 a	6.5 a	22.3 a	60.4	5.8 a	65.7
Heterorhabditis spp.	13.9 b	5.1 bc	19.0 b	36.7	4.6 b	31.4
Xenorhabdus sp.	12.8 bc	6.0 a	18.8 b	35.3	4.3 b	22.9
Photorhabdus sp.	12.3 bc	4.6 c	16.9 c	21.6	4.0 c	14.3
Healthy Plants	14.1 c	5.9 b	20.0 ab	43.9	5.2 a	48.6
N alone	11.3 d	2.6 d	13.9 d	--	3.5 d	--

*Each value is the mean of three replicates. Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple- rage test. N= 2000 eggs of M. incognita.

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Data presented in table (4) show root number of galls and egg-masses on pomegranate roots as influenced by entomopathogenic nematodes and their symbiotic bacteria. It is evident that a significant reduction in number of galls on pomegranate plant roots was achieved with root gall indices ranged from 1.0 to 4.0. Among all tested materials, *Xenorhabdus* treatment significantly inhibit formation of galls on roots with reduction percentage of 97.9 %, followed by those treated with *Photorhabdus* with value of 96 %. Regarding egg-masses numbers, *M. incognita* was barely reproduced on plants treated with *Xenorhabdus* filtrate with reduction percentage value of 95.8 %, followed by *Photorhabdus* with value of 91.7 %.

Table 4: Effect of entomopathogenic nematodes and their symbiotic bacteria on development of *M. incognita* infecting Taify pomegranate under greenhouse conditions.

Treatment	No. of galls *	RGI	No. of Egg-masses	EI
Steinernema spp.	6.0 bc	2.0 b	3.0 c	1.7 b
Heterorhabditis spp.	10.0 b	2.3 b	6.0 b	2.0 b
Xenorhabdus sp.	1.7 cd	1.0 c	1.0 c	1.0 c
Photorhabdus sp.	3.3 c	2.0 b	2.0 c	1.0 c
N alone	82.3 a	4.0 a	24.0 a	3.0 a

*Each value is the mean of three replicates. Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple- rage test.

Our findings agreed with those of [10] who showed that the same rate of *S. riobrave* suppressed *M. incognita* on tomato in both growth chamber and greenhouse trials. EPNs can affect the number of RNK infecting plants, or the number of eggs produced when they are applied near the root system [27]. Regarding entomopathogenic bacteria, these results are on par with Samaliev et al. (2000) who demonstrated that *X. nematophila* completely inhibited the hatching of *M. javanica* and paralyzed the emergence of J_2 and that of [25] in respect to *X. szentirmaii* filtrate at 80 and 60 % which achieved the highest percentage of *M. incognita* mortality (100%) after 24 hrs of exposure. [29] demonstrated that these bacteria produce metabolites that act as nematicides toward a large number of nematodes, including some phytonematodes. On the other hand, the *Steinernema feltiae-Xenorhabdus bovienii* complex could not suppress the development of *M. javanica* and penetration of the parasite into the roots of the host [30]. Insecticidal toxic proteins from *P. luminescens* strain W-14 different from that of TT01 consists of high molecular weight complexes that include toxin A and toxin B. Several of the active compounds of the symbiotic bacteria have also been identified [31], [32]. The presence of natural nematicidal activity of either *Xenorhabdus* or *Photorhabdus* was confirmed with insufficient larval mortality percentages in this study, a situation which can explain the reasons of applying such components against *M. incognita* J_2s in-vitro as well as in greenhouse. These findings are agreed with [33] who mentioned that *Xenorhabdus* and *Photorhabdus* produce several agents with nematicide and antimicrobial activity.

CONCULOSION

The present study clearly suggested that the percentage increase of fresh weights of whole plant as well as shoot dry weights in the treated plants over those of the untreated ones was resulted due to the exist tolerance of pomegranate plants to nematode infection that expressed by the application of the tested materials within the soil. Regarding the plant extracts, our results showed that aqueous extracts of colocynth and marigold improved pomegranate plant growth parameters and reduced root-knotting severity. Apparently, it is worth to note that no nematode reproduction occurred on pomegranate roots received marigold extract. In the present work, *Steinernema* spp. and its symbiotic bacteria *Xenorhabdus* sp. showed the highest nematicidal properties against the target nematode, *M. incognita* infecting pomegranate and improving plant growth. Undoubtedly, medicinal plant extracts as well as entomopathogenic nematodes and its symbiotic bacteria tested against *M. incognita* on Taify pomegranate under greenhouse conditions in this investigation, clearly assured the possibilities of using such bioagents against any plant parasitic nematode pests. Ongoing research is evaluating methods to use some of these materials as alternative eco-friendly ones for root-knot nematode control in the field in the future.

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