

**Non-chemical based management of *Tylenchulus semipenetrans*, a major threat to the citrus industry**^a Muhammad Wajid*, ^b Adil Mahmood, ^b Haseeb Anwar, ^b Muhammad Imran Hamid, ^a Yannong Xiao, ^c Maleeha Shafqat, ^c Shaista Nasir^a Department of plant pathology, Huazhong Agricultural University, 430070 P.R. China,^b Department of plant pathology, University of Sargodha, 40100 Pakistan,^c Department of plant pathology, University of Agriculture Faisalabad 38000, Pakistan.

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ABSTRACT

There are a vast variety of microbes available in soil performing diverse functions of plants, including root colonization for protection against pathogens and nutrient acquisition. In the present study, the efficacy of rhizospheric microbial strains and plant extracts were evaluated against citrus nematode for their nematicidal activity. Three different concentrations of plant extracts were evaluated *in vitro*, results depicts that 10% solution of *Tagetes erecta* (root) has given 46% the highest percentage mortality at 48hrs followed by *Tagetes erecta* (leaf) and *Datura stramonium* killed 41% and 37% nematodes respectively. Similarly for 20% of plant extract in distilled water, the highest mortality rate was recorded for *T. erecta* (root) 68%, followed by *D. stramonium* 47% and *Acacia nilotica* 44% at 48hrs of application. While using a higher concentration 30% of SS. the percentage mortality elevated multifold i.e. Marigold root has killed maximum 76% nematodes followed by Marigold leaves and Datura with mortality rate 69% and 58% respectively at 48 hrs of the interval. While Eucalyptus killed lowest no of nematodes. Additionally, five bacterial isolates were evaluated for their nematicidal activity. Results indicated that for bacterial cell suspension, *Pseudomonas putida* has shown maximum mortality rate at 84%, followed by *Bacillus subtilis* 73% & *Pseudomonas geniculata* 65% at 48hrs of interval. Similarly, in the 2nd set of experiment bacterial culture filtrates has exhibited promising results, the maximum mortality rate was recorded for *Bacillus* spp. 81%, followed by *B. subtilis* 76%, *Ps putida* 63%, after 48hrs of incubation. Moreover, an insight, investigation of bacterial extracellular metabolites and plant extracts would enable researchers to develop purposeful products and compositions.

Keywords: Citrus; nematode, biological management, plant extracts, rhizobacteria.

INTRODUCTION: Citrus belongs to the family *Rutaceae*, is an economically important crop, majorly grown in tropical and subtropical climatic regions. Citrus grabs the top position in terms of area under cultivation and production among the fruit trees worldwide (Minamikawa *et al.*, 2017). Citrus is a rich source of a variety of nutrients including; vitamins, carbohydrates, amino acids and minerals for human nutrition (Safdar *et al.*, 2010). Towards total citrus production in South Asia, Pakistan is a major contributor (FAO, 2017). Among citrus producers, Pakistan holds 12th position worldwide (Siddique and Garnevska, 2018). Economic survey of the Pakistan revealed that the area under cultivation of Citrus is over 199 thousand hectares in the Pakistan; majorly kinow, (Aatif *et al.*, 2017) with an overall 2.351 million metric tons production during 2018-19 in the Pakistan (Economic survey, 2019). More than 98% of citrus fruit is produced in Punjab province and 70% of which is kinow, its juice content is about half of the total which is the highest of all easy peeler varieties (Memon, 2014; Memon and Kasbit, 2017).

Citrus trees continuously encounter several different types of pathogens throughout their life (Farooq *et al.*, 2018). These interrupts normal physiological and biochemical functioning of host plants (Misaghi, 2012). Nematodes are one of those invisible devastating creatures. These affects plant growth and vigor by interrupting the supply of crucial vitals from roots to aerial parts (Schneider *et al.*, 2003), over 12.6% crop losses in top 20 life-sustaining crops has been reported due to plant-parasitic nematodes worldwide (Abd-Elgawad and Askary,

2018). These soil inhabitants are hard to manage, and multiple species of citrus nematodes have been reported so far (Khan and Hasan, 2010; Elhady *et al.*, 2018). Among all, *T. semipenetrans* (Cobb) is the major culprit that limits the yield and plant health of citrus groves (Khan and Hasan, 2010). The infestation of nematode up to 64.93% was observed in the Pakistan (Aatif *et al.*, 2017). The characteristic symptoms associated with highly infested trees includes the chlorosis, die back, poor root development showing discoloration, shedding of fruits and leaves of reduced size (Mahmood *et al.*, 2010). Newly emerging health issues associated with chemical pesticides (Nicolopoulou-Stamati *et al.*, 2016) has forced the recent world towards non-conventional control methods. Many alternate integrated practices are being developed and few are in practice (Montasser *et al.*, 2012). Biological control agents and extracts of different botanicals are sustainable, financially affordable and ecofriendly alternative ways to manage plant-parasitic nematodes. Biocontrol efficiency of different plant extracts and bacteria have reportedly shown remarkable results against different nematodes species but their performance in the local harsh environment is questionable (Ahmad *et al.*, 2010; Haque *et al.*, 2018).

OBJECTIVES: The present research therefore, was aimed to study the effects of bacterial cell suspension and culture filtrates of locally isolated *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus* spp, *Pseudomonas putida*, *Pseudomonas geniculata* and extracts of different botanicals like; *Tagetes erecta* (leaves and roots used separately), *Datura stramonium*,

Acacia nilotica and *Eucalyptus globulus* were evaluated for their nematocidal activity against *T. semipenetrans* causing the citrus slow decline.

MATERIALS AND METHODS: Sampling and nematode extraction: Rhizospheric soil samples with root fibers of citrus were collected from Sargodha for nematode extraction. Samples were transferred to lab in properly labeled zipped bags. White Head and hemming Tray method were used to extract healthy nematodes. For this purpose, 50g of soil along with chopped roots were placed on a perforated tray lined with paper towel placed over non-perforated one containing water, so that the healthy nematodes could migrate towards it. Assembly were allowed to stay for 48 h., then water was collected in a beaker and nematodes were allowed to settle down for 2hrs to reduce total volume. Juveniles were stored in the falcon tubes at room temperature.

Preparation of plant extracts: Fresh plant parts of Marigold root (*Tagetes erecta*), Marigold leaf (*Tagetes erecta*), Datura (*Datura stramonium*), Acacia (*Acacia nilotica*) and Eucalyptus (*Eucalyptus globulus*) were collected in zipped bags, washed under tap water and air dried for further processing. Plant materials in a 1:1 w/v ratio were crushed in distilled water and resulted in slurry. The Slurry was filtered using Whatman filter paper #42 in a conical flask and used as a stock solution (Haroon, 1989). Three different dilutions for each treatment of 10%, 20% and 30% strength were made i.e. 1:10, 2:10, & 3:10 respectively in distilled water.

Preparation of bacterium cell Inoculum: The pure cell cultures of the bacterial isolates were prepared by taking a smear from stored tubes. The isolates were further multiplied in 250mL by using nutrient broth with shaking at 150rpm for 3 days at 25. Medium were centrifuged at 7500rpm for 10min. to palletized the bacterial cells, pallet was washed three times using deionized water to remove medium and metabolite contaminants (El-Bendary, 2006), and dissolved in the distilled water of 1×10^8 cells/mL recommended dilution. The supernatant was collected (bacterial filtrate) containing medium along with bacterial metabolites and passed through 40 μ m filters to ensure presence of no bacterial cells in the filtrate.

Application of treatments: To evaluate the nematocidal activity of bioactive botanicals, 0.5mL of nematode suspension was added to 0.5mL said concentrations of plant extracts. Each treatment had 3 replications with one set of control for evaluation. Data was recorded at regular intervals of 12, 24 and 48 h. after the incubation and motile and dead juveniles were observed and calculated over an inverted microscope at 20X magnification lens. Sterile 12 well tissue culture plates were used to evaluate the efficiency of nematocidal bacteria for both cell suspension and bacterial culture filtrates. A dilution was prepared keeping 100 \pm 5 nematodes per ml of distilled water. An equal volume (500 μ L) of both nematode suspension and bacterial treatments were poured in each well, keeping the final volume not more than 1mL. Each bacterium contributes two treatments with three replicates of each and one triplicate of distilled water added to the nematode suspension to serve as control treatment.

Statistical analysis: Data were subjected to Software (Statistix 8.1) for statistical analysis, one-way ANOVA tool was used to calculate analysis of variance for percentage mortality data, the significant difference between treatments applied using Tukey's

test keeping the level of significance $P \leq 0.05$.

RESULTS: Effect of bioactive plant extracts: Table 1 shows the nematocidal activity of bioactive plant extracts viz *Tagetes erecta*; leaves and roots separately (Marigold), *Datura stramonium* (Datura), *Acacia nilotica* (kikar) and *Eucalyptus globulus* (Eucalyptus). Different strengths of botanicals i.e. 10, 20 & 30% were evaluated *In vitro* against citrus nematode *T. semipenetrans* at the regular intervals of 12, 24 and 48 hours, with p-value less than 0.05 for all treatments confirms high significance of all treatments. Plant extracts of *Tagetes erecta* (leaf and root), *Datura stramonium*, *Acacia nilotica*, and *Eucalyptus globulus* were found highly effective against *T. semipenetrans* (table 1). The mortality rate of nematodes was directly proportional to the percentage of plant extract. An increase in nematode mortality was recorded in proportion with an increase in strength of percent solution of plant extracts. At a concentration of 10% after 48h. of application. The root extract of *T. erecta* has recorded the maximum 46% of mortality followed by *Tagetes erecta* (leaf) 41%, *Datura stramonium* 37%, *Eucalyptus globulus* 36% and 30% *Acacia nilotica*. Percentage mortality was elevated accordingly as the concentration of sol. was doubled such as 20% of plant extract stock solution in distilled water. The nematode mortality trend was quiet similar to that of above, where the highest mortality rate was recorded for *T. erecta* (root) 68%, followed by *D. stramonium* 47%, *A. nilotica* 44%, *T. erecta* (leaf) 42% and 40% *E. globulus*. Similarly, percentage mortality increases with an increase in strength of solution. While using a higher concentration 30% of stock solution, the percentage mortality raised multifold i.e. Marigold root ha killed maximum 76% nematodes followed by Marigold leaves and Datura with mortality rate 69% and 59% respectively (figure 1). The Eucalyptus killed a minimum n of nematodes and is presented in table 1.

Bacterial bio-control agents: Nematophagous potential of total five bacterial isolates were evaluated against juveniles of citrus nematode *T. semipenetrans*, bacterial cell suspension and culture filtrates showed nematocidal activity against *T. semipenetrans* at the regular intervals of 12, 24 and 48 h. Results presented in table 2 are with p-value less than 0.05 shows that all the treatments were highly significant; mortality percentage of juveniles due to bacterial cell culture recorded between 42 to 84% in comparison to control treatment was 6% that was only distilled water added in nematode suspension at 48hrs of interval. Results indicated that when nematodes were exposed to bacterial cell culture, *Pseudomonas putida* has shown maximum mortality rate at 84% followed by *Bacillus subtilis* 73%, *Pseudomonas geniculata* 65%, *Bacillus licheniformis* 53% and *Bacillus spp.* 42% at 48 hours of exposure. Similarly, in the 2nd set of experiment bacterial culture filtrate showed promising results, percentage mortality ranged between 44 to 76% in comparison to that of control (distilled water) was 6%, at 48 hours of interval (table 2). In the case of culture filtrates, maximum mortality rate was recorded for *Bacillus spp.* 81% followed by *Bacillus subtilis* 76%, *Pseudomonas putida* 63%, *Bacillus licheniformis* 50% and *Pseudomonas geniculata* 44% after 48h. (figure 2).

Discussion: *Tylenchulus semipenetrans* has been reported from almost all major citrus producing countries as a perilous threat causing a huge cut short in perilous

Table 1: Percentage mortality of *T. semipenetrans* caused by plant extracts.

Treatments	10% Solution			20% Solution			30% Solution		
	12h	24h	48h	12h	24h	48h	12h	24h	48h
Marigold (Leaf)	13±4.4 ^c	39±7.6 ^d	41±8.4 ^d	20±9.8 ^a	38±5.5 ^b	42±5.5 ^b	21±4.5 ^{ab}	62±7.1 ^a	69±6 ^a
Marigold (Root)	30±4.6 ^a	41±4 ^b	43±2 ^b	29±4.6 ^a	58±6.1 ^a	68±7.1 ^a	52±2.6 ^a	70±5.5 ^a	76±3.1 ^a
Datura	21±4 ^{ab}	33±7 ^b	37±6 ^{bc}	29±0.6 ^a	45±5 ^b	47±6 ^b	34±1.2 ^b	56±2.1 ^b	59±2.6 ^b
Acacia	18±6.6 ^b	28±5.5 ^b	30±5 ^c	26±4 ^a	43±3.1 ^b	44±2.5 ^b	36±5 ^b	52±2.3 ^{bc}	55±2.1 ^{bc}
Eucalyptus	21±1.2 ^{ab}	33±1.5 ^b	36±3 ^{bc}	21±2.6 ^a	38±0.6 ^b	40±1.2 ^b	28±1 ^b	42±0.6 ^{cd}	44±2.1 ^{cd}
Control	6±0.6 ^c	7±0.6 ^c	7±1.2 ^d	4±3.5 ^b	7±2.6 ^c	7±2 ^c	1±1.7 ^d	6±0.6 ^e	9±1.5 ^e
F-value	10.3	38	65	10.6	46.5	48.2	10.3	85	94
P-value	<0.0005	<0.00	<0.00	<0.0005	<0.00	<0.00	<0.00	<0.00	<0.00

Note: Treatments; Plants extracts that were evaluated to manage the nematodes.

Table 2: The percentage mortality of *T. semipenetrans* by bacterial cell culture and bacterial culture filtrates.

Treatments	Bacterial cell culture			Bacterial culture filtrates		
	12h	24h	48h	12h	24h	48h
<i>Bacillus licheniformis</i>	20±1.3 ^{bc}	39±2.3 ^{bc}	53±3.5 ^c	16±3.1 ^b	26±2.5 ^c	50±2.5 ^c
<i>Pseudomonas putida</i>	41±1.3 ^a	69±3.6 ^a	84±2.5 ^a	19±3.5 ^{ab}	33±3.1 ^{bc}	63±4.9 ^b
<i>Bacillus subtilis</i>	12±1 ^d	24±2.1 ^d	73±5.6 ^b	21±4.4 ^{ab}	46±6.1 ^{ab}	76±4.7 ^a
<i>Bacillus spp.</i>	14±1.2 ^{cd}	30±4 ^{cd}	42±4.2 ^d	25±4.5 ^{ab}	47±9.6 ^a	75±3.5 ^a
<i>P. geniculata</i>	21±4.9 ^b	42±4.6 ^b	65±5 ^b	29±3.8 ^a	40±3.6 ^{abc}	44±0.6 ^c
Control	3±0.6 ^e	6±2.1 ^e	6±1.4 ^e	3±0.6 ^c	6±2.1 ^d	6±1.4 ^d
F-value	10.5	19.6	12.2	25.8	14.6	19.7
P-value	<0.004	<0.00	<0.001	<0.00	<0.0009	<0.00

Note: Treatments; Bacteria that were evaluated to manage the nematodes.

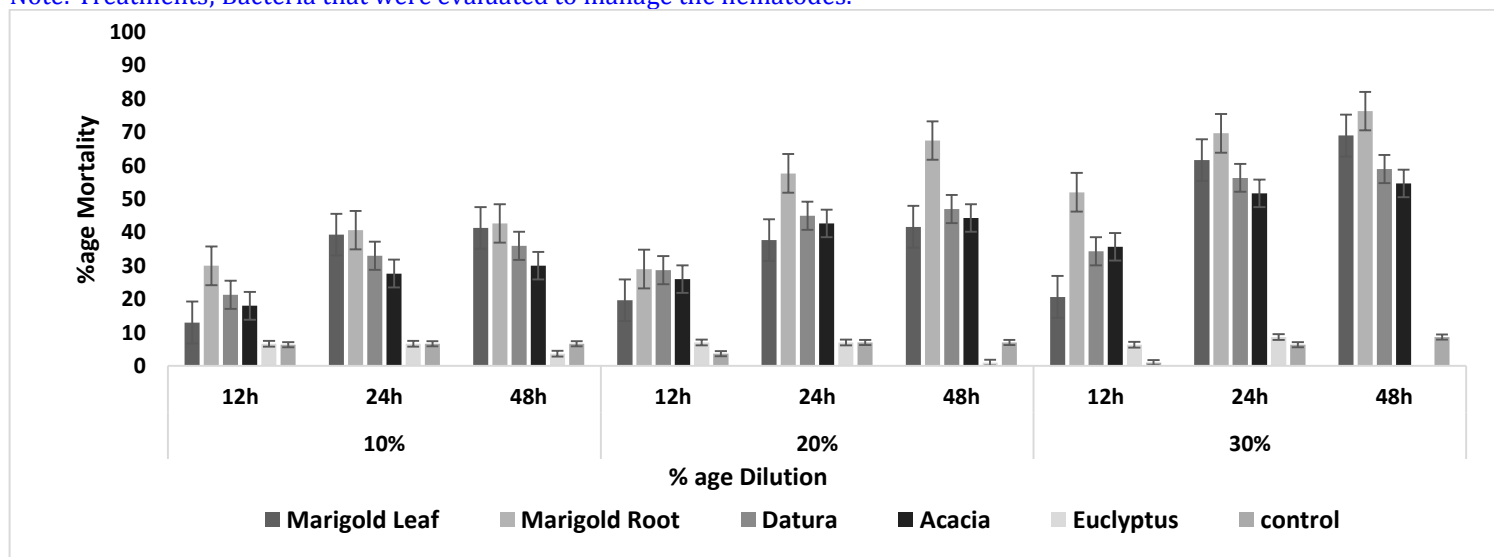


Figure 1: The Effect of three different dilutions of biologically active plant extracts showing percentage mortality at intervals. citrus yield and overall vigor of citrus groves (Abd-Elgawad, 2020). This nematode gives distinguishing aerial symptoms of dieback and is a major cause of citrus slow decline syndrome (Etebu and Nwauzoma, 2014). Somehow a conducive environment has established to facilitate their exponential growth in soil ecosystem. This might be due to excessive use of chemical pesticides those facilitate the imposition of the theory suggested "survival of fittest" (developed tolerance against conventional combinations of chemicals in practice) (Aktar et al., 2009) several previous studies depicted that citrus root diseases caused by the nematodes lead to significant losses (Ibrahim et al., 2010; Hallmann, 2013). Moreover, several different reports are available providing the evidence on nematicidal effect of plant extracts multiple microbes have also been reported worldwide but their efficacy in the local harsh environment is questionable (Kepenekçi et al., 2016). There are two ways either to acclimatize already available products or to evaluate and commercialize the native rhizospheric microbes and other integrated non chemical methods to encounter aforesaid potential threats to citrus industry. To manage these problems different control strategies other than chemical nematicides have to be integrated. Previously, several plant extracts (Aires et al., 2009), essential oil (Naem et al., 2018), beneficial microorganism (Oyekanmi et al., 2007) and cultural practices such as intercropping (Chang et al., 2020) were used to minimize the crop losses. In the current study, the efficacy of beneficial rhizobacteria and plants extracts were evaluated to manage the *T. semipenetrans* associated with citrus. Our results suggested that the cell culture of *Pseudomonas putida* have shown the stronger inhibitory effects followed by the *Bacillus subtilis*, *Pseudomonas geniculata* and *B. licheniformis* at 48h after the exposure. Similarly, the culture

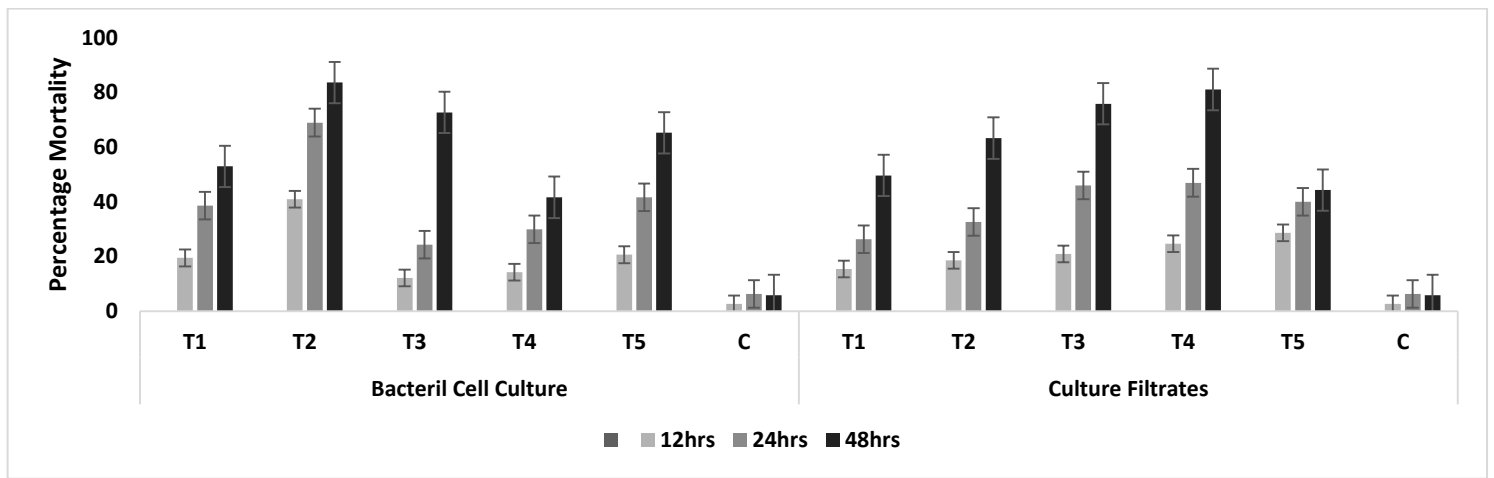


Figure 2: Effect of bacterial biocontrol agents' cell culture and culture filtrates on percentage mortality of second stage juveniles (*T. semipenetrans*). T1: *B. licheniformis*, T2: *P. putida*, T3: *B. subtilis*, T4: *Bacillus* spp, T5: *P. geniculata* & T6: control.

filtrate of *Bacillus* spp. showed that the maximum mortality ranged between 44 to 76% followed by the *B. subtilis*, *P. putida*, *B. licheniformis* and *P. geniculata*. In addition, the plants extracts showed that mortality rate was increases gradually with the increase in concentration when exposed to *T. semipenetrans*. Our results indicated that *T. erecta* (20%) showed the highest mortality rate (68%) followed by the *D. stramonium* (47%), *A. nilotica* (44%), *T. erecta* (42%) and *E. globulus* (40%). Moreover, in consistent with our study many rhizobacteria are reported to suppress the plant parasitic nematodes such as *Pseudomonas* (Spiegel *et al.*, 1991), *Bacillus subtilis* (Oka *et al.*, 1993), *B. sphaericus* (Racke and Sikora, 1992), *Arthrobacter* (Klopper *et al.*, 1989) and *Agrobacterium* (Racke and Sikora, 1992). Except these biocontrol agents, plants extracts are also familiar to combat with phytopathogens. Previously, six plants extracts (cauliflower, Portuguese cabbage, broccoli, collards of kale, collards of *Brassica rapa* and watercress were evaluated against the cyst nematode of potato and showed the inhibitory effects (Aires *et al.*, 2009). Similarly, other plant extracts have significantly controlled the nematodes infecting the pepper crop.

However, the present study was conducted to sort these issues; in the given experiment percentage mortality because of different bioactive plant extracts and local isolates of rhizospheric bacteria is recorded against second stage juveniles of citrus nematode. The effect of cell suspension and bacterial culture filtrates was investigated. The results predicted that, an increase in percentage strength of plant extracts is directly proportional to the juvenile mortality, i.e. 10%, 20%, & 30%. The results of the second experiment indicated a comparatively higher rate of mortality due to bacterial culture filtrates instead of cell suspension. For preparation of a commercial product preliminary investigation of potential microbes has to be done. *Tagetes erecta*, *P. putida* and *B. subtilis* has satisfactorily performed better in the lab, investigative pot and field trials would lead to the development of a purposeful commercial product for sustainable management of citrus nematodes. Thus, it can be concluded that the present investigation will be very help in long term and effective management and breeding resistance of citrus diseases caused by the nematodes.

Conclusion: Among all major plant parasitic nematodes (PPN) associated to citrus species. Citrus nematode *T. semipenetrans* has been reported as predominant

nematode specie of different citrus cultivars grown generally. Out of the major citrus species, even rough lemon was found to be highly infested by *T. semipenetrans* that is an extensively been used as a root stock and previously has been considered as a cultivar resistant to almost all major problems. To our knowledge, this is probably first study that shows to assess the all possible integrated investigative approaches that would serve as an effective alternate strategy to manage nematodes non-chemically.

CONFLICT OF INTEREST: Authors have no conflict of interest in this study.

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