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Entomopathogenic nematodes as biocontrol against tomato leaf miner *Tuta absolute* compared with the chemical insecticide, Emamectin benzoate.

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Abstract

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is an invasive alien species especially infested tomato crops. It invaded Egypt in 2009 and many plantations. It's difficult to control due to the limitation of insecticides, therefore, the susceptibility of *T. absoluta* to three indigenous isolates of entomopathogenic nematodes in Egypt, i. e. *Heterorhabditis bacteriophora* (EKB20), *Steinernema* sp. (B32) and *Heterorhabiditis* sp. (Kasassien isolate) were determined in the laboratory, greenhouse, and field experimental compared with emamectin benzoate insecticide. Leaf bioassays were carried out to evaluate the affinity of nematode isolates to reach the larvae and affect them at the galleries. The efficacy of the three nematode species after foliar application to potted tomato plants was evaluated under laboratory conditions. High larval mortality (70.6–94%) and low pupae mortality (<25%) were determined. In the leaf bioassay tests, a high level of larval infestation (75.4–88.6%) indicated the nematode's ability to kill the larvae within the galleries compared with 100% mortality of emamectin benzoate with the recommended dose. In the pot experiments treatment plants with nematode caused a reduction in insect infestation by 87–94% compared with 96% in the treatment of emamectin benzoate. The results of the field experiment showed the efficiency of entomopathogenic nematodes for reducing infestation with *T. absoluta* with 70 to 90 % reduction % compared with more than 95% with emamectin benzoate. The results suggested that EPN are considered promising biocontrol agents, if correctly applied and released in integrated control schemes against the tomato leaf miners *T. absoluta.*

1. Introduction

Egypt is one of the most important tomato producers in the world (WP TC, 2011). The invasive pest tomato leaf miner *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a devastating pest of tomato *Lycopersicum esculentum* L., was reported in the first time in Egypt at 2009 [1&2] and it may become a significant problem in greenhouses and open fields. Currently, the most effective method for control of *T. absoluta* in Egypt is the use of commercial synthetic pesticides, However, these pesticides have exhibited low to moderate efficiency due to the cryptic nature of *T. absoluta* [3]. This has considerably increased the spraying frequency per crop cycle, which has accelerated the development of resistance in the pest [4, 5], and has disrupted the natural biological control, as well as serious drawbacks for the environment. Thus, there is an urgent need for finding new eco-friendly tools for the control of *T. absoluta*. Moreover, a chemical approach quickly results in a build-up of insecticide residues on tomato fruits and in the environment and leads to negative ecological effects [6, 7 and 8]. Entomopathogenic nematodes (EPNs) are considered biological control agents for a variety of economically important pests [9, 10, 11, 12]. Most of these EPNs belong to the families; Steirnernematidae and Heterorhabditidae, which are obligate parasites that kill insects with the help of mutualistic bacteria in their intestines [13, 14] They have been used with variable success against insects. Susceptibility of *T. absoluta* to entomopathogenic nematodes has been investigated [15, 16]. Thus, to quickly contain the menace of *T. absoluta* and to avoid the development of resistance, careful management of the infestation that uses a rotational application of insecticides with a different modes of action, together with other control measures, such as biological control with Entomopathogenic nematodes to prevent the development of resistance to insecticides, and decrease insecticide residues in the food chain.

This work aimed to study the susceptibility of different stages of *T. absoluta* to Entomopathogenic nematodes (EPNs), the capacity of EPNs to infect pupa and larvae in the soil and inside galleries in the tomato leaves, and the effectiveness of the foliar application of EPNs in tomato plants under controlled and field conditions controlled with an insecticide.

2.Materials and methods

2.1. Source of nematodes: -

Three indigenous species of nematodes were tested in the present study: *Heterorhabditis bacteriophora* strain EKB20 and B32 isolates collected from soils cultivated with clover [10], meanwhile (EL-Kasassien) isolated from the soil cultivated with Date palm trees [9]. All entomopathogenic nematodes were reared on last instar larvae of *Gallaria*

mellonella L. (Lepidoptera: Pyralidade) [17]. Larvae of *G. mellonella* were reared on old bee wax at 28±2 ºC and relative humidity of 65±5 % in the insect-rearing laboratory. The emerging infective juveniles (IJs) were harvested from nematode traps and stored in sterilized water at 10ºC [18]. Bioassay of different local nematode strains against different stages of *Tuta absoluta* was carried out. The virulence of selected nematode isolates was evaluated using 20 larvae from 3rd and 4th larval stages of *T.absoluta* (2 insects in ten replicates) for each concentration from the 3 tested nematode isolates (B32, EKB20 and Kasassien). Two concentrations were used from each isolate 100 and 200 IJs/insect. Two mL from each concentration of the tested nematode were dispensed on moistened filter paper to keep suitable moisture for the nematode activity. Two insects were covered with the wet filter paper used in each replicate. Ten replicates were used for each treatment. Check treatment was treated with 2 ml. of distilled water. Numbers of dead insects were recorded after 72 hours post treatments. Virulence of all the tested nematode isolates in the laboratory was assessed and corrected mortality % was evaluated for each concentration against 3rd, and last instar larvae of *T.absoluta*. The % mortality in each treatment was corrected for control mortality according to the Abbott formula [19].

$$\text{Corrected mortality} = \frac{\%\ \textbf{Responded in treatment} - \%\ \textbf{Responded in control}}{\textbf{100} - \%\ \textbf{Responded in control}} X\ 100$$

A second experiment was carried out to confirm if age is one factor contributing to variability in EPN efficacy. We hypothesized that older IJs would be less successful in penetrating and killing insect larvae. The penetration behavior of "young" (<1 wk. old) and "old" (2-4 wk. old) were evaluated over 4 “exposure periods” using larvae of *T. absoluta*. Groups of *T. absoluta* larvae were exposed to nematode-infested filter paper for exposure periods of 8, 16, 32 and 64 h. Cadavers were dissected after 72 h and the IJs that penetrated the larvae were counted. Larval mortality for all treatments was calculated after 72 h and 144 h "incubation periods”. For each age and species at each time of exposure effects were noted in nematode penetration over time. The experiment was repeated twice. And the % of Penetration, or successful infection, and the average was calculated.

The third experiment was carried out to confirm if 1 h and 3 h of exposure to EPNs were enough to cause larval mortality. In this experiment, the nematodes were applied at a dose of 50 IJs cm−2 (4000 IJs dish−1). A single *T. absoluta* larva was placed in each Petri dish for 1 h or 3 h, and then larvae were moved individually to another Petri dish without nematodes. For each nematode species and exposure time, there were 10 replicates. After 72 h larval mortality was recorded. All dead larvae were dissected to confirm nematode parasitism. An untreated control was identical to the other treatments but no IJs were added. The experiment was repeated twice.

2.2. Methods

2.2. Leaf bioassay

Infected leaves were collected from a naturally infected tomato plantation in Minia University, Faculty of Agriculture farm, Minia, Egypt and transported to laboratory, the number of larvae per leaf was determined from one larva per leaf up to seven. Each infected leaf was sprayed with 5 ml (2.5 ml top and 2.5 ml bottom) of a 1,000 IJs ml−1 concentration for each EPN species tested. This concentration is equivalent to a 60 IJs cm−2 dose. A single treated leaf was placed in Petri dishes (10 cm diameter), sealed with parafilm, and maintained in a climate chamber at 23 ± 2°C in the dark, and insect mortality was checked after 72 h. Nematode’s presence within dead larvae was recorded to ensure nematode infection. Larval relative situation (inside or outside of the tomato leaf) was also determined. The experiment was carried out with 10 replicates per nematode strain and repeated twice.

2.3. Pot experiment under greenhouse conditions

The bioassay was conducted using tomato plants (*L. esculentum* (L.)) and transferred 3 plants /pots. The plants were nurtured in a greenhouse with a range of temperatures from 25°C to 30°C, and with relative humidity (RH) range of 66–80%. Plants were severely infested with this pest. Nine tomato plants for each nematode species (three pots) were sprayed with 1,000 IJs ml−1 and 0.05% of the oil adjuvant Tween 80 ®. All treatments consisted of two applications with 15 ml nematode suspension per plant, using a manual sprayer, with 24 h time intervals between the treatments. The untreated control consisted of nine plants sprayed with water containing 0.05% of Tween 80 adjuvant. After four days larval mortality was assessed. The experiment was repeated twice.

2.4. Field evaluation of EPN against Tuta absoluta:

Field experiments were carried out at a farm located at the Faculty of Agric., Minia University, and heavily infested with *T absoluta*. The experimental area was divided into smal1 plots (15 plots each 1/100 fed). A randomized complete block design was followed in the whole experimentation area. Two sprays were applied during two successive seasons i.e. 1 st spray on 5th May (2021) and the 2nd spray on 25 May (2021). Samples of 30 leaves were collected at random from each plot to assess *T. absoluta* infestation % pre and 3, 5, and 9 days post-treatment. The percentage of reduction in larval infestation was calculated according to the following formula [20].

$$\textbf{Reduction}\ \% = \left(\mathbf{1} - \left(\frac{\mathbf{Ta} * \mathbf{Cb}}{\mathbf{Tb} * \mathbf{Ca}}\right) \boldsymbol{X}\mathbf{100}\right)$$

Where:

T_b is the number of living larvae (active tunnel) before treatment.

T_a is the number of living (active tunnel) larvae after treatment.

C_b is the number of living (active tunnel) larvae before treatment for the control.

C_a is the number of living larvae (active tunnel) after treatment for the control.

The averages of the two applications were subjected to analysis of variance and means were compared with LSD test, the 5 % level of probability was used in all statistical tests. The software program "Costat" was used for all analyses.

3. Results and Discussion

3.1. Petri dishes assays

As shown in Table (1) results revealed that the late larvae instars of the tomato leaf miner were highly susceptible to all three tested nematode species. When a dose of 30 IJs cm^{-2} has applied mortality of larvae reached 81.6% with *Heterorhabditis bacteriophora* (EKB20), and with *Steinernema* sp. (B32) 70.6% and with *Heterorhabiditis* sp. (Kasassien isolate showed 84% compared with 100% with emamectin benzoate with dose 0.03 ml/cm^{-2}. At the dose of 50 IJs cm^{-2} mortality reached 94% with *Heterorhabiditis* sp. (Kasassien isolate and *Heterorhabditis bacteriophora* (EKB20) (85.86%). The differences between applied doses and nematode species were not significant ($F = 2.288$; 48; $P > 0.05$).

In contrast pupae were hardly infected by nematodes. There were no significant differences between untreated control and treatments ($P > 0.05$). Percentage of pupae infected by nematodes varied from 7.65% caused by *Steinernema* sp. (B32),15.5% by *H.bacteriophora* (EKB20) and 18.65 % by *Heterorhabiditis* sp. (Kasassien isolate when a 30 IJs cm^{-2} dose was applied and 17.66,22.33 and 25.00% respectively at 50 IJs cm^{-2} (Table 2). During the experiment, some of the adults emerged from surviving pupae and were infected by nematodes (7.66% infected by *Steinernema* sp. (B32) and 23.6 and 34% by *H.bacteriophora* (EKB20) and *Heterorhabiditis* sp. (Kasassien isolate), confirming that adults of *T. absoluta* are also susceptible to EPNs.

When larvae were exposed for 3h to EPNs, mortality ranged from 26.17% to 47.26% and in the 6h exposure experiment from 38.49% to 78%. There were no significant differences between EPN species ($F = 1.758$); $P > 0.05$ for 3 h exposure and $F = 2.581$; $P > 0.05$ for 6h exposure). Significant differences were observed between the time of exposure for *Steinernema* sp. (B32) and *H.bacteriophora* (EKB20) ($t = 4.031$; $P < 0.05$) but not for *Heterorhabiditis* sp. (Kasassien isolate) ($t = 5.17$; $P > 0.05$) (Table. 3).

3.2. Pot experiment under greenhouse conditions

The foliar application of nematodes on tomato plants resulted in efficacy between 89% for *H.bacteriophora* (EKB20) and 94 %for *Heterorhabiditis* sp. (Kasassien isolate) and *Steinernema* sp. (B32) with significant differences (Table. 5).

Table (1): Average of corrected percentage of infected larvae (third to the fourth instar) of *T. absoluta* when exposed to *Heterorhabditis bacteriophora* (EKB20), *Steinernema* sp. (B32) and *Heterorhabiditis* sp. (Kasassien isolate) at two doses (30 and 50 IJs cm^{-2}) 72 h after nematode application.

Nematode strains	Mean of Corrected % infestation of last Larvae instars					
	First treatments		Second treatments		Average	
	Concentrations		Concentrations		Concentrations	
	300IJ /cm^{-2}	500IJ/c m^{-2}	300IJ/c m^{-2}	500IJ/c m^{-2}	300IJ/c m^{-2}	500IJ/c m^{-2}
H.bacteriophora (EKB20)	82.3	84.42	80.90	87.3	81.6	85.86
Steinernema sp. (B32)	69.3	77.5	71.9	80.30	70.6	78.9
Heterorhabiditis sp. (Kasassien isolate	82.6	89.66	85.4	98.33	84.00	94.00
Emamectin benzoate	100	100	100	100	100.00	100.0

Laboratory experiments on tomato leaves revealed that EPNs can find and kill larvae on tomato leaves, despite their relative position (inside or outside the tomato leaf). Mortality caused by nematodes at a rate of 500 IJs /10 ml varied from 79.3% by *H.bacteriophora* (EKB20)to 89.6% and 94% by *Steinernema* sp. (B32) and *Heterorhabiditis* sp. (Kasassien isolate). There were no differences among the three treatments. During the bioassay, most of the larvae (70–80%) were found outside the galleries. However, larval mortalities recorded outside the galleries (94.00% for *H.bacteriophora* (EKB20), 90.1% for *Steinernema* sp. (B32) and 91.9% *Heterorhabiditis* sp. (Kasassien isolate) were slightly superior to percentages observed inside (82.33% for *H.bacteriophora* (EKB20), 74% for *Steinernema* sp. (B32)and 79.33% for *Heterorhabiditis* sp. (Kasassien isolate)), but no statistical differences were recorded.

Table(2): Corrected percentage of infected pupae of *T. absoluta* exposed to *Heterorhabditis bacteriophora* (EKB20), *Steinernema* sp. (B32) and *Heterorhabiditis* sp. (Kasassien isolate) at two doses (300 and 500 IJs cm^{-2}) 72 h after nematode application.

Nematode strains	Mean of Corrected % infestation of Pupae					
	First treatments		Second treatments		Average	
	Concentrations		Concentrations		Concentrations	
	300IJ /cm^{-2}	500IJ/c m^{-2}	300IJ/c m^{-2}	500IJ/cm^{-2}	300IJ/c m^{-2}	500IJ/c m^{-2}
H.bacteriophora (EKB20)	14.99	20.66	15.99	24.00	15.50	22.33
Steinernema sp. (B32)	6.00	19.66	9.3	15.66	7.65	17.66
Heterorhabiditis sp. (Kasassien isolate	17.3	26.33	20.00	23.66	18.65	25.00
Emamectin benzoate (03ml/cm-2	86.3	87.00	84.9	89.00	85.6	88.00

3.3. Field experiments:

Table 6 shows the reduction % of *T. absoluta* larval infestation after treatment with *H.bacteriophora* (EKB20), *Steinernema* sp. (B32), and *Heterorhabiditis* sp. (Kasassien isolate) compared with emamectin benzoate on tomato plants under field conditions with foliar application of nematodes on tomato plants with knapsack sprayer with rate 300L/fed with concentration of 5000 IJs/ml resulted in efficacy between 69.66 % for *Steinernema* sp. (B32) and 81.8% when tomatoes treated with *H.bacteriophora* (EKB20) and 88.33 %for *Heterorhabiditis* sp. (Kasassien isolate) and *Steinernema* sp. (B32) with significant differences between strains and abamectin benzoate (Table. 6).

Table (3): Corrected percentage of infected larvae of *T. absoluta* exposed to *Heterorhabditis bacteriophora* (EKB20), *Steinernema* sp. (B32) and *Heterorhabiditis* sp. (Kasassien isolate) at doses (500 IJs cm^{-2}) for 3 h and 6 h exposure.

Nematode strains	Mean of Corrected % infestation of larvae					
	First treatments		Second treatments		Average	
	Time of exposure		Time of exposure		Time of exposure	
	3 h	6h	3h	6h	3h	6h
H.bacteriophora (EKB20)	26.66	65.33	30.33	71.33	28.5	68.33
Steinernema sp. (B32)	28.66	36.66	23.67	40.33	26.17	38.49
Heterorhabiditis sp. (Kasassien isolate	44.3	73.33	50.2	82.66	47.26	78.00

This work showed that EPNs can ability to infect larvae and pupae, of *T. absoluta* and cause mortality of the larvae inside the galleries in the leaves of tomato plant. All three EPNs tested showed high efficacy against larvae despite the dose, but limited success against pupae. Differences in susceptibility between larvae and pupae observed in our study are confirmed with [20, 21]. [22,] concluded that pupae of *P. gossypiella* were not susceptible unless injured, explainable by the lack of entry routes (mouth and anus) for nematodes in this stage.

Table (4): Corrected percentage of infected larvae of *T. absoluta* with its relative position on the leaf (inside galleries and outside galleries) exposed to *H.bacteriophora* (EKB20), *Steinernema* sp. (B32)and *Heterorhabiditis* sp. (Kasassien isolate)

Nematode strains	Mean of Corrected % infestation of larvae					
	First treatments position on the leaf		Second treatments position on the leaf		Average position on the leaf	
	inside galleries	outside galleries	inside galleries	outside galleries	inside galleries	outside galleries
H.bacteriophora (EKB20)	79.50	95.33	80.1	93.66	79.8	94
Steinernema sp. (B32)	90.20	90.2	89.00	9.00	89.6	90.1%
Heterorhabiditis sp. (Kasassien isolate	88.2	90.00	87.2	93.8	87.7	91.9

The high mortality obtained on *T. absolute* larvae in the present study (85.6 to 94.00 %) is similar to the mortality observed by [16] in Petri dish trials using the entomopathogenic fungus *Beauveria bassiana* (96% mortality) and superior to results found by [23] using neem seeds extract (52.4–95.4% mortality). The results obtained in leaf bioassay showed that the nematodes applied at a dose of 60 IJs cm−2 were able to penetrate inside the leaf galleries and caused between 79.8 to 89.6 % and 87.7 % mortality of *T. absoluta* larvae. Tomato leaf bioassays carried out by [24] with EPN against larvae of *T. absoluta* recorded only 94% mortality after 144 h. [16, 25] showed in similar experiments that EPNs caused up to 88% mortality of the third instar larvae of the tomato leafminer. The efficacy of EPNs obtained in the pot experiment was like the efficacy recorded by [26] in field experiments with chemical pesticides (triflumuron, chlorfenapyr, and abamectin) on *T. absoluta* larvae (83–100% mortality), but these products also harmed the parasitoid *Trichogrammatoidea backtrace* (Hymenoptera: Trichogrammatidae). In addition, chemical pesticides can induce future resistance in *T. absoluta* populations as assessed by [3] who detected incipient abamectin resistance because of the frequent use of this insecticide.

The present study showed that the larva is the most susceptible stage to the EPNs, therefore the foliar application of these nematodes is necessary to achieve successful control of this insect. EPNs have been already used in foliar applications under field conditions against other insect pests dwelling inside or outside of plant stems or trunks [27]. One of the major obstacles to EPNs efficacy in foliar applications is its limited persistence. Desiccation is the key factor influencing nematode persistence in foliage [28]. [29] showed that, in foliar applications of EPNs, insect habitat in the leaves determines the efficacy of the nematodes, as in boreholes and cryptic foliage the nematodes are more effective than in exposed foliage. The galleries made by the insect in leaves provide nematodes an excellent habitat to avoid harmful environmental factors (desiccation and ultraviolet light) and parasites the insect target. [30] obtained an efficacy of 95% when applied a suspension of 10,000 IJs of *S. feltiae* ml−1 with 0.02% of a wetting agent against the larvae of the leafminer *Liriomyza bryoniae* (Diptera: Agromyzid). In our study, efficacies between 78.9 and 94% against the tomato leafminer larvae were obtained using a 500 IJs /cm2 concentration. The difference between concentrations of EPNs needed to achieve similar efficacies could be due to the different behavior of the two insects. In Liriomyza the only entry to the tunnel is those resulting from punctures made by the female on the leaves during the oviposition. All larval development happens inside the galleries, and larvae are incapable of moving between leaves [31]. However, tomato leafminer larvae produce tunnels generating big entry holes to the galleries that can be effortlessly used by nematodes to penetrate and avoid desiccation and ultraviolet light and finally infect the larvae.

The period that nematodes need to infect the insect is a relevant factor that must be considered to determine the nematodes' efficacy after a foliar application. The larval mortality observed in our study after 3h and 6 h of nematode exposure was 26.17–47.26 % and 38.49–78% respectively. [32] Also showed a 12 h survival time of IJs of *S. Carpocap* in the foliar application on Chinese cabbage leaves. Consequently, a foliar application of EPNs against larvae of *T. absolute* would allow the survival of the IJs long enough to find and infect the larvae on the surface of the leaf. Furthermore, this period should be enough to penetrate the galleries where nematodes will be protected from adverse environmental conditions and infect larvae. Results of this study indicated that EPNs can be an efficient bio-control agent of *T. absoluta* and two complementary strategies could be used to control this pest with these nematodes. The foliar application of EPNs could control efficiently feeding larvae of *T. absoluta* in and outside the galleries. Complementary the application of EPNs on soil would control the last instar larval, when they slide down from the leaves to pupate, as well as emerging adults from the buried pupae.

Results of Pot and field study showed that EPNs isolated are good elements in a sequence of pesticide resistance management in *T absoluta* control program. The foliar application of the three isolated species of EPN gave good results in reducing the infestation of plants with *T. absoluta* ranged from 87 to 94% compared with 96% emamectin benzoate in greenhouse experiments and from 69.66 to 88.33 in field trials compared to 90.6% with emamectin benzoate. Emamectin benzoate is highly potent in a broad spectrum of lepidopteron insect pests. It has the potential to penetrate leaf tissues by translaminar movement and it has been recommended for controlling tomato leaf miners in many countries such as Algeria [33] and Greece [5]. Invasive alien species causes a high degree of loss in crop productivity, and it is difficult to control [34], it should be managed carefully from infected land to maximize productivity and to limit the spreading and invasion to other land [35]. Our results trend support the use of *Heterorhabditis bacteriophora* (EKB20), *Steinernema* sp. (B32) or *Heterorhabiditis* sp. (Kasassien isolate) individually or within a rotation with insecticide to control *T. absoluta* and to delay resistance development. The individual use *Heterorhabditis bacteriophora* (EKB20),

Steinernema sp. (B32) and *Heterorhabiditis* sp. (Kasassien isolate) may be used in programs in a sequence with the most effective insecticides to increase efficiency in controlling *T. absoluta* larvae. We believe that we are needed to apply Integrated Crop Management (ICM) for tomato plants to get the best management for *Tuta absoluta*. Also, Integrated Pest Management will be the most sustainable managing tool that counts on different types of control not just pesticides and not just applied at the outbreak, but it will be earlier.

Table (5): Reduction (%) in *T.absoluta* infestation after application of *H.bacteriophora* (EKB20), *Steinernema* sp. (B32), and *Heterorhabiditis* sp. (Kasassien isolate) and emamectin benzoate on potted tomato plants under greenhouse conditions.

Nematode strains	Reduction % of infestation after application								
	First treatments Post-treatment a Time			Second treatments Post-treatment Time			Average Post-treatment Time		
	3-dys	5-days	9-days	3-days	5-days	9-days	3-days	5-days	9-days
H.bacteriophora (EKB20)	97.6	82.6	96.3	89.9	89.0	95.3	93.8	85.8	95.8
Steinernema sp. (B32)	88.3	86.0	87.5	81.9	88.0	90.3	85.1	87.0	88.9
H. sp. (Kasassien isolate	87.8	98.8	94.4	95.4	94.0	93.6	91.6	96.4	94.0
Emamectin benzoate (03ml/cm-2	85.0	100	96.0	95.0	100	100	90	100	98.0

Table (6): Reduction % of *T. absoluta* infestation after treatment with *H.bacteriophora* (EKB20), *Steinernema* sp. (B32), and *Heterorhabiditis* sp. (Kasassien isolate) compared with emamectin benzoate on tomato plants under field conditions.

4. Conclusion

It could concluded that Entomopathogenic nematodes (EPNs) are promising biocontrol agents, if correctly applied and released in an integrated control scheme against the tomato leaf miners *T. absoluta* under both green house and field conditions.

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