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Larvicidal and Pupicidal Activity of Indigenous Entomopathogenic Nematodes Against Soil-dwelling Stages of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) Under Laboratory and Greenhouse Conditions in Saudi Arabia

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Abstract

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a devastating pest worldwide affecting tomato production. The efficacy of soil treatments of the entomopathogenic nematode (EPN) *Heterorhabditis indica* strains NOAC.N1 and NOAC.N2 were determined against the fourth instar larvae (L4 larvae) and pupae at one, three, and five days old of *T. absoluta*. In the laboratory, each EPN strain was applied at a dose of 0.00, 50,100, and 150 infective juveniles (IJs) cm^2. Both strains reduced the adult emergence to <50%, even at the lowest doses of 50 IJs cm^2 when applied against L4 larvae. No adults of *T. absoluta* emerged from their pupae at one day old after treatment with both strains of EPNs at doses of 50, 100, and 150 IJs cm^2, whereas more than adults emerged from 93% of the pupae of the control treatment (0.00 IJs cm^2). Strain NOAC.N2 performed better than Strain NOAC.N1 and reduced adult emergence to <50% at a dose of 150 IJs cm^2 against pupae at three and five days old. In the greenhouse, both strains were applied at a dose of 150 IJs cm^2 against L4 larvae and pupae at different ages. Strain NOAC.N2 consistently provided a greater reduction in adult emergence than Strain NOAC.N1 and the control treatment. Therefore, the indigenous EPN strain NOAC.N2 could be used as a biological agent to control the soil-dwelling stages of *T. absoluta* in greenhouse conditions if it can be registered and commercialized for use by farmers.

Keywords: Biocontrol; *Tuta absoluta*; *Heterorhabditis indica*; EPN; adult emergence; pupae; L4 Larvae; Tomato

Introduction

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) has invaded more than 90 countries outside of South America (EPPO 2021), thus becoming a serious threat to tomato (*Solanum lycopersicum* L.) production worldwide and, to a lesser extent, a pest of other economically important solanaceous crops, including potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum annum* L.), and tobacco (*Nicotiana tabacum* L.) [1-4]. Tomato crops grown in greenhouses in the Middle East are under constant threat due to widespread infestations of *T. absoluta* [5].

The life cycle of this insect comprises four development stages: egg, larva, pupa, and adult. Females usually lay eggs on

the underside of leaves or stems. Neonate larvae penetrate leaves, stems, or fruits, on which they feed and develop. Last instar larvae usually drop to the ground on a silk thread and pupate in the soil. Pupae complete their development in the soil and after a few days, adults emerge from the soil [6]. The impact of *T. absoluta* on tomato plants is as a result of direct feeding of larvae on photosynthetic areas of leaves and damage to fruit. In addition, feeding on the growing tips of plants shoots results in reducing and stopping plant development, with yield losses reaching 100% on tomatoes [3,4,7].

The economic importance of *T. absoluta* has led to management strategies being focused on pesticides [8]. However, some integrated pest management programs in tomato crops against *T. absoluta* are also based on biological control. *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) and *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) have already been tested for their suitability as predators of *T. absoluta* [9-11]. These studies showed that such predators might be able to regulate *T. absoluta* populations under field conditions when a certain number is present in the crop.

Many entomopathogens such as *Bacillus thuringiensis* (Berliner) Sandeep Kumar et al. [12], *Beauveria bassiana* (Vuill.) and *Metarhizium robertsii* (Sorokin) Abdel-Baky et al. [13] have been tested against *T. absoluta* in laboratory, greenhouse and open staked-tomato field experiments. However, these biocontrol agents only target the stages of *T. absoluta* on the aerial parts of the plant (leaves, stems or fruits). Thus far, few biocontrol strategies have been developed to control the soilborne stages of *T. absoluta*, including the last instar larvae, pupae, and emerged adults.

Entomopathogenic nematodes (EPNs) are important biological control agents for a variety of economically important pests [14,15]. These nematodes, belonging to the families Steinernematidae and Heterorhabditidae, are obligate parasites that kill insects with the help of mutualistic bacteria that inhabit the intestine of the infective juveniles (IJs) [16]. They have been used with variable success against insects occupying different habitats. Most success has been achieved against soil dwelling pests or pests in cryptic habitats such as inside galleries in plants where IJs find protection from hostile environmental factors [17,18]. Although EPNs have been used against a number of soil-inhabiting coleopterous insects, less information is available about their use against lepidopterous insects in soil. This is the first study we are aware of to evaluate the activity of indigenous strains of entomopathogenic nematodes of *Heterorhabditis. indica* to control the soil-dwelling stages of *T. absoluta* in laboratory and greenhouse trials under Saudi Arabia conditions.

Material and methods

Isolation and maintenance of nematodes

The nematodes strains NOAC.N1 and NOAC.N2 used in this study were isolated from soil samples from the National Organic Agriculture Center (NOAC) and Algosamy organic farm, Algassium region, Saudi Arabia, using *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) as a live insect bait [19]. The nematodes were identified using PCR by Macrogen Inc., Korea (https://www.macrogen.com/en/main, accessed on 27 January 2023). IT5 primer (5'-GGAAGTAAAAGTCGTAACAAGG-3') and the reverse ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the entire ITS region [20]. The accession number with Macrogen Inc., are (OQ427656) and (OQ427655) for NOAC.N1 and NOAC.N2, respectively. Both H. indica strains NOAC.N1 and NOAC.N2 were cultured in *G. mellonella*, which were reared on artificial media according to [21]. The IJs were recovered in tap water according to Kaya & Stock [22], upon emergence, stored at 12-14 °C, and used within two weeks of harvest.

Laboratory experiments

Effects of entomopathogenic nematode against *T. absoluta* L4 larvae

H. indica strains NOAC.N1 and NOAC.N2 were tested for their virulence against *T. absoluta* L4 larvae. For each strain, a dose of 0.00, 50,100, 150 IJs cm^2 were used. 1 kg of sun sterilized soil with an initial soil moisture content of 0.97%, was added in plastic boxes (15x 10 x 5 cm^3), EPNs were pipetted onto the soil surface in each plastic box in a 50 ml of sterile tap water. The final moisture level was at field capacity (23.2% v/w). Twenty-four hours later, fifteen *T. absoluta* L4 larvae, collected from a naturally infested tomato plantation (cultivar Newton, cultivated at the NOAC under greenhouse conditions) were released on the soil surface of each box. All boxes were incubated at 25 ± °C. Three replicates were used for each dose. The Control (0.00 IJs) was treated with sterile tap water only. To evaluate the larval mortality, nine days post-inoculation, the number of emerging adults was calculated every day for four days. Treatments were arranged in a 2 x 4 factorial experiment, arranged in a randomized complete block design (RCBD).

Effect of entomopathogenic nematode against different pupae ages of *T. absoluta*

Both *H. indica* strains were tested against pupae at one, three, and five days old (time after the larvae were placed on the soil). To ensure the exact pupae age, fifteen *T. absoluta* L4 larvae were released on plastic boxes (15x 10 x 5 cm^3) containing 1 kg of soil, and then each group of pupae were challenged at one, three or five days later. For each pupal age, a separate experiment was conducted using both strains at a dose of 0.00, 50,100, 150 IJs cm^2. The EPNs were pipetted onto the soil surface in each plastic box in 50 ml of sterile tap water. Each dose was replicated three times. The Control was with water only. The experiments were a 2x4 factorial experiment arranged in a randomized complete block design (RCBD). To evaluate pupae mortality, nine, seven- and

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five-days post-inoculations, the number of emerged adults was counted every day for four days from pupae at one, three and five days old, respectively.

Greenhouse experiments

Effects of entomopathogenic nematode against *T. absoluta* L4 larvae and different pupae ages

For both strains, the best dose of 150 IJs cm^2 from the previous experiments was tested against *T. absoluta* L4 larvae in greenhouse conditions. Plastic boxes of (20x 15 x 10 cm^3) were used in this experiment. Each box was opened from the lower side using a sharp knife. The upper side of the boxes was covered with insect net material to facilitate air circulation. The boxes were then dug into the soil surface under growing tomato plants. EPNs were added on the soil surface in each plastic box in a 100 ml of tap water, using 10 ml medical syringe. Twenty-four hours later, twenty *T. absoluta* L4 larvae were released in each plastic box. Treatments were replicated three times. The control treatment received tap water only. For pupae experiment, the same two strains of EPNs were tested at doses of 0.00 and 150 IJs cm^2. Different pupae ages were prepared as mentioned previously, with using twenty L4 larvae. Nematode application was as discussed previously, and pupae mortality data was collected as mentioned previously. The experiment was a 2x2 factorial experiment arranged in a randomized complete block design (RCBD). Treatments were replicated three times. The control treatment received tap water only.

Statistical analysis

Mortalities data of *T. absoluta* L4 larvae and pupae at different ages were subjected to analysis of variance (ANOVA). Means were compared using Duncan multiple range test at a 5.0% level of significance. The analyses were conducted using GenStat for Windows, 21th edition.

Results

Laboratory trial

Effect of EPNs against *T. absoluta* L4 larvae

Highly significant differences were observed between the tested doses (F= 286.39; P<0.001; df = 3). However, there were no significant differences between the two EPN strains (F= 2.57; P= 0.131; df = 1), and there was no interaction effect between doses and strains (F= 2.10; P= 0.147; df = 3) for their efficacy against *T. absoluta* L4 larvae.

A polynomial response to dose was observed with $R^2 > 0.9$. Adult emergence reduced as a function of dose increase (Figure 1). H. indica strains NOAC.N1 and NOAC.N2 were pathogenic against *T. absoluta* L4 larvae and reduced adult emergence at all doses compared to the control treatment. Strain NOAC.N2 at the highest dose of 150 IJs cm^2 reduced the adult emergence to 22.6% compared to 25% by Strain NOAC.N1. Both strains caused reductions in adult emergence <50% even at the lowest doses of 50 IJs cm^2 (Figure 2). Observation by stereo microscope (Euromex, Netherland) showed the emergence of IJs from the dead cadaver (Figure 3).

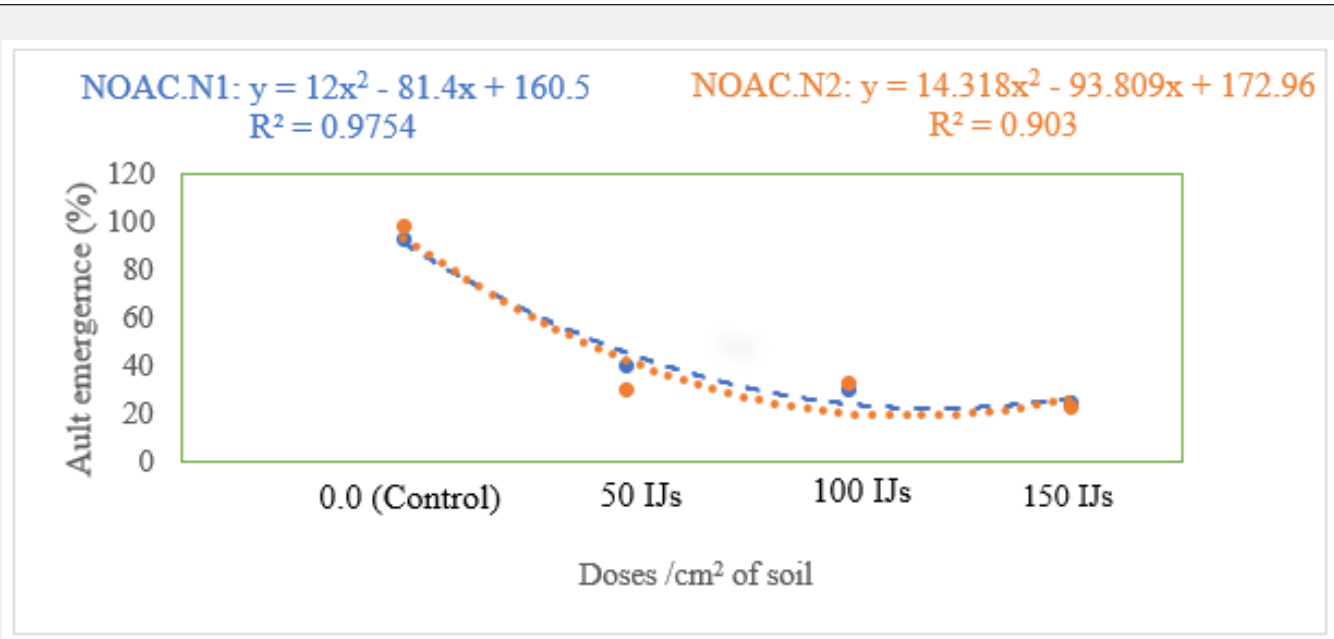

Figure 1: Polynomial regression analysis showed the relationship between reduction of adult emergence and *Heterorhabditis indica* strains NOAC.N1 and NOAC.N2 applied at different doses against *Tuta absoluta* L4 larvae.

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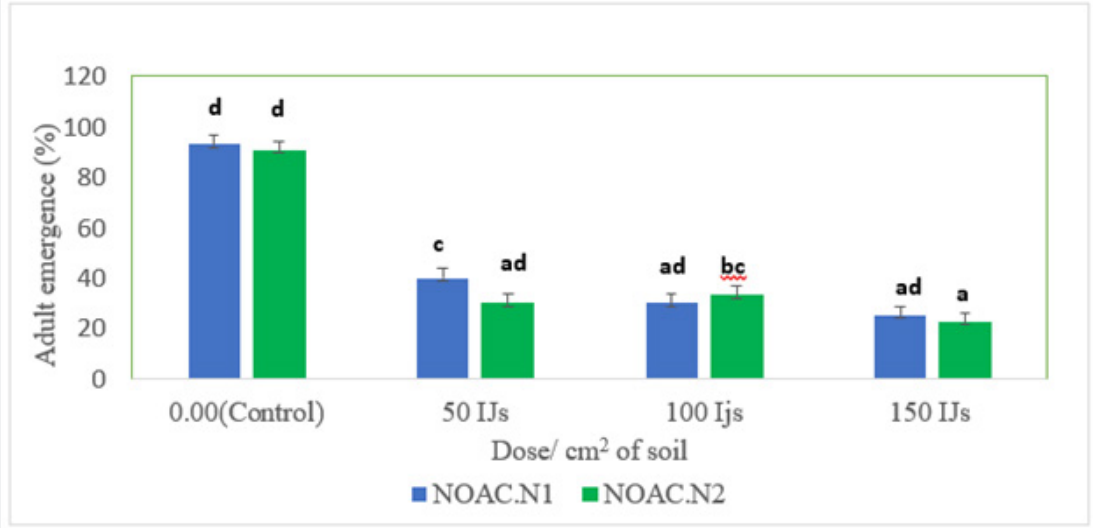

Figure 2: Adult emergence (%) following exposure of *Tuta absoluta* L4 larvae to *Heterorhabditis indica* Strain (NOAC.N1 and NOAC.N2 applied as soil treatments at a dose 0.00, 50, 100 and 150 IJs cm^2. The error bars represent the SE.

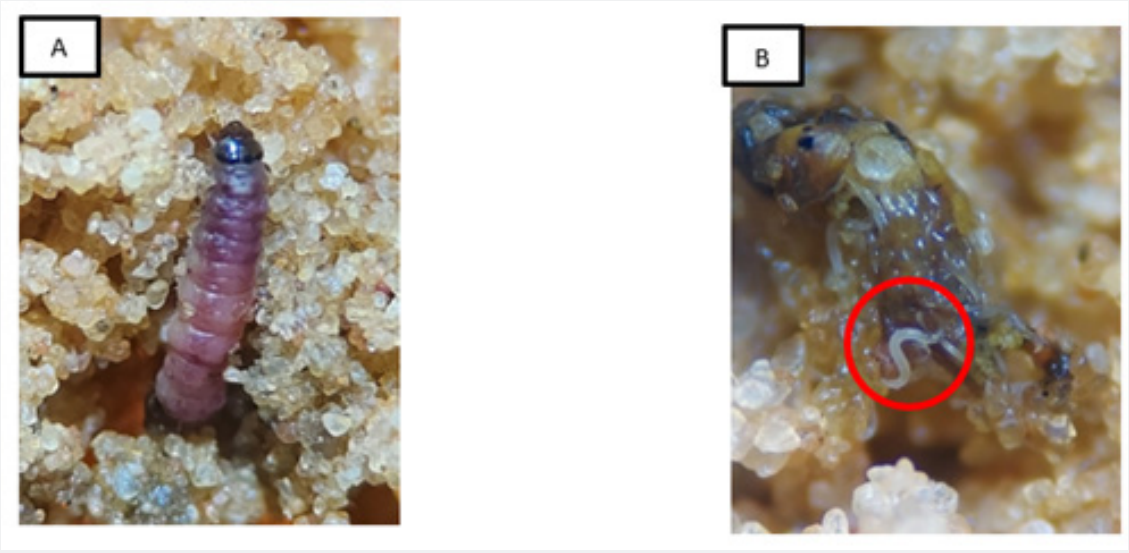

Figure 3: *Tuta absoluta* L4 larvae A) dead larva on the soil surface B) Infective juveniles IJs emerging from dead larva following exposure to *Heterorhabditis indica* strain NOAC.N2 at a dose of 150 IJs cm^2.

Effects of entomopathogenic nematode against *T. absoluta* L4 larvae and different pupae ages

For all tested pupal ages, no significant differences were observed between the EPN strains. However, highly significant differences were observed between doses. No significant interactions effects were found between strains and doses (Table 1). Generally, pupae at one day old were more susceptible to the nematode infection, followed by pupae at three days old and five days old. No adult emergence was observed from pupae at one day old after treatment with *H. indica* strains NOAC.N1 and NOAC.N2 at a dose of 50, 100 and 150 IJs cm^2. In contrast, >93% of adults emerged in the control treatment (0.00 IJs) (Table 1) Increased levels of adult emergence were observed on pupae at three and five days old following exposure to the same strains at the same doses. *H. indica* Strain NOAC.N2 at a high dose of 150 IJs cm^2 reduced the adult emergence to <50% against pupae at three and

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five day old (Table 1). A linear response to dose was observed with all tested pupal ages and strains with $R^2 > 0.6$. Adult emergence reduced as the result of EPN dose increases (Figure 4).

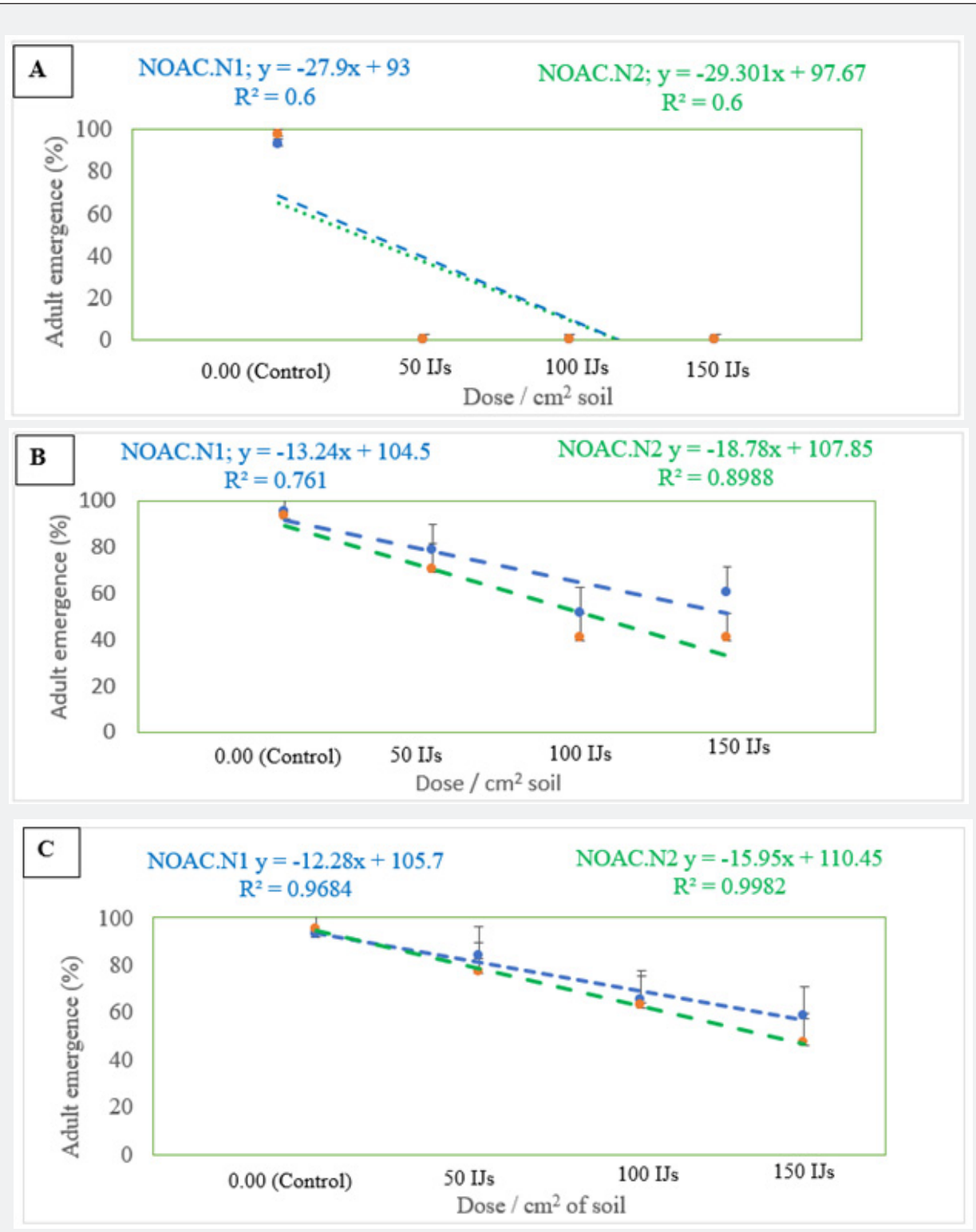

Figure 4: Linear regression analysis showed the relationship between reduction of adult emergence and *Heterorhabditis indica* strains NOAC.N1 and NOAC.N2 applied at different doses against *Tuta absoluta* pupae of, A) one day old, B) three days old and C) five days old. The error bars represent the SE.

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Table 1: Effects of *Heterorhabditis indica* Strain (NOAC.N1 and NOAC.N2 applied as soil treatments at a dose of 0.00, 50, 100 and 150 IJs cm^2 against Tuta absoluta pupae at one, three and five days old.

parameter	Dose Infective juvenile (IJs/ cm^2)	Experiment 1		Experiment 2		Experiment 3	
		Strain		Strain		Strain	
		NOAC.N1	NOAC.N2	NOAC.N1	NOAC.N2	NOAC.N1	NOAC.N2
		Pupae (one day old)		Pupae (3days old)		Pupae (5 days old)	
Adult emergence (%)	0.00 (Control)	93 b	97.67 b	95.3 d	93 d	93 d	95.3 d
	50	0.00 a	0.00 a	78.7 cd	70 bcd	83.7 bcd	77.3 bcd
	100	0.00 a	0.00 a	51.3 ab	40.3 a	63 ab	62.7 abc
	150	0.00 a	0.00 a	60.3 abc	40.3 a	58.3 ab	47 a
Strain effects		Ns		Ns		Ns	
Dose effects		***		***		**	
Strain x dose		Ns		Ns		Ns	
LSD		5.3		24.9		26.62	
CV%		12.6		20.8		20.9	

Means followed by the same letter do not differ significantly at P <0.05 according Duncan multiple range test. asterisks indicate significant differences at *** P<0.001 **P<0.01

Greenhouse experiment

Effect of entomopathogenic nematode against *T. absoluta* L4 larvae and different pupae ages

There were significant differences between the two tested Strains (F= 12.48; P<0.012; df = 1). Doses, (F= 97.13; P<0.001; df = 1). and the interaction between Strain and Dose effects were also significant (F= 12.48; P<0.012; df = 1). Application of H. indica strain NOAC.N2 caused a reduction in adult emergence to 35.3% compared to 67% by Strain NOAC.N1 when applied at a dose of 150 IJs cm^2 against L4 larvae in greenhouse conditions. With the Control treatment, 95.3% of adults emerged (Figure 5). *H. indica* Strain NOAC.N2 performed better than Strain NOAC.N1 in reducing the adult emergence following application pupae at three and five days old However, both strains reduced adult emergence to <30% when applied against pupae at one day old (Table 2). Strain NOA. N2 at a dose of 150 IJs cm^2 reduced the adult emergence to the level of 51.67% even against pupae at five days old, compared to a higher level of emergence of 85% after treatment with Strain NOAC.N1. Again, >93% of adults emerged after the control treatment (Table 2).

Table 2: Effects of *Heterorhabditis indica* Strains NOAC.N1 and NOAC.N2 applied as soil treatments at doses of 0.00 and 150 IJs cm^2 against Tuta absoluta pupae aged one, three and five days, under greenhouse conditions.

Parameter	Dose Infective juvenile (IJs/cm^2)	Experiment 1		Experiment 2		Experiment 3	
		Strain		Strain		Strain	
		NOAC.N1	NOAC.N2	NOAC.N1	NOAC.N2	NOAC.N1	NOAC.N2
		Pupae (one day old)		Pupae (3days old)		Pupae (5 days old)	
Adult emergence (%)	0.00 (Control)	95.3 b	95.3 b	95.3 c	93.0 c	95.33 d	95.33 d
	150	21.7 a	28.3 a	78.3 b	60.0 a	85 b	51.67 a
Strain effects		Ns		**		Ns	
Dose effects		***		***		**	
Strain x dose		Ns		**		Ns	
LSD		13.64		8.66		30.39	
CV%		11.3		5.3		18.6	

Means followed by the same letter do not differ significantly at P <0.05 according Duncan multiple range test. asterisks indicate significant differences at ***P<0.001 **P<0.01*P<0.05

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Discussion

Our study demonstrated the potential of two indigenous EPNs strains when applied as IJs as a soil treatment to control L4 larvae and pupae of *T. absoluta* in both laboratory and greenhouse experiments. Low levels of adult emergence of 22.6% and 25% were observed following soil treatments at a dose of 150 IJs cm^2 by *H. indica* strains NOAC.N1 and NOAC.N2, respectively. Both strains caused a reduction in adult emergence of less than 50%, even at the lowest dose of 50 IJ cm^2. Our results agree with those of Garcia-del-Pino, Alabern, & Morton [23], who studied the ability of EPNs to find and infect L4 larvae of *T. absoluta* in soil. We also observed the emergence of fresh IJs from dead cadavers, which would have the potential to extend the epizootic to other larvae and pupae, reflecting the ability of EPNs as biocontrol agents to multiply in their target hosts and extend the scale and efficacy of control as long as there are target insects in the soil. Although the pupal stage has been considered to be less susceptible to EPNs than other life stages Malan, Knoetze, & Moore [24], Steyn, Malan, & Addison [25], Vicente-Díez et al [26], in our trial, no adults emerged from pupae at one day old after treatment with *H. indica* strains NOAC. N1 and NOAC.N2 at a dose of 50, 100, and 150 IJs cm^2, whereas adults emerged from more than 93% of the pupae of the control treatment. We postulate that the efficacy of the EPNs against pupae was because the pupae are sessile, and at only one day into the pupation process, the silk covering of the pupae would have been incomplete. Combined, these two factors would have made it relatively easy for IJs to find, penetrate and kill the pupae.

Interestingly, both EPNs strains were able to infect and kill three and five day old pupae, with Strain NOAC.N2 killing >50% of the pupae. In doing so, the EPN's demonstrated an ability penetrate the completed silk cocoons of mature pupae, and to infect the pupae. This result contradicts the finding of Garcia-del-Pino et al. [23], who observed no mortality of in the *T. absoluta* pupal stage following exposure to three EPNs (*Steinernema carpocapsae, S. feltiae,* and *Heterorhabditis bacteriophora*). Batalla-Carrera, Morton, & García-del-Pino [27] also observed that pupae were relatively resistant to the IJs of EPNs in laboratory trials conducted in Petri dishes on filter paper. Another observation in this study was that some adults were infected and killed by IJs after they emerged from the soil, which enhanced the overall efficacy of the two strains of EPNS as biocontrol agents.

Overall, the environmental conditions greenhouse tomato production appears to be compatible with the environmental requirements for these EPNs to achieve high levels of infection of the soilborne stages of *T. absoluta*. *H. indica* Strain NOAC.N2 performed better than NOAC.N1 against L4 larvae and three pupal ages of *T. absoluta,* and reduced the adult emergence to 35.3% when applied against L4 larvae. Interestingly, reduced the adult emergence to the level of 51% was also recorded with *H. indica* Strain NOAC.N2 at a dose of 150 IJs cm^2 when applied against pupae at five days old, compare to an 85% adult emergence after treatment with Strain NOAC.N1, and more than 95% adult emergence in the control treatment. This result confirmed that the pupal stage is less susceptible to EPNs than the L4 larvae, and required higher doses of IJs. In similar research Andaló et al. [28] documented a mortality level of 80% against *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) pupa after they were treated with IJs of *Heterorhabditis amazonensis* (Andalo et al.) at doses of 600 and 800 IJs $pupa^{-1}$. Vicente-Díez et al. [26] also found that killing pupae required more IJs than were needed to control larvae of the grapevine moth, *Lobesia botrana* (Denis & Schiffermuller) (Lepidoptera: Tortricidae).

In the present study, *H. indica* strain NOAC.N2 demonstrated its potential to control the soilborne stages of *T. absoluta*. Therefore, it could be incorporated into management programs for greenhouse-grown tomatoes as soil applications, if the EPN is successfully registered and manufactured for farmers' use. Some additional greenhouse studies will be necessary to optimize the efficacy of EPNs for *T. absoluta* with regards to application volumes, use of adjuvants, timing of applications regarding the presence of different pest stages, and importance of foliar versus soil applications, and a cost-benefit analysis of using both foliar and soil applications.

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