Efficacy of entomopathogenic nematodes against *Tuta absoluta*

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Abstract

BACKGROUND

The South American tomato pinworm, *Tuta absoluta* (Meyrick) is one of the most serious insect pests of tomato plants. Current control strategies for *T. absoluta* primarily involve the use of insecticides, but increasing resistance in field populations has prompted research on alternative control measures. Biological control with entomopathogenic nematodes (EPNs) can be an alternative or one component of an Integrated Pest Management programme. In foliar application, EPNs encounter many factors that affect their survival and efficacy. This paper reports investigations into some of these factors for several EPN species; relative humidity (RH), temperature, time required by EPNs to enter a leaf and number of applications in whole leaf bioassays on tomato leaves.

RESULTS

RH was the most important factor; EPNs efficacy and survival decreased as RH declined. *Steinernema feltiae* was the most effective species followed by *Steinernema carpocapsae* then *Heterorhabditis bacteriophora. Steinernema carpocapsae* survived better at low RH than *S. feltiae.* The two *Steinernema* species induced similar mortality at 25 °C, but *S. feltiae* was more virulent at lower temperatures (15 and 20 °C) while *S. carpocapsae* was more virulent at higher temperatures (30 and 35 °C). First and second instar larvae of *T. absoluta* required more applications compared to third and fourth instar larvae.

CONCLUSION

The species of EPN used to control *T. absoluta* requires careful selection in relation to the temperature and relative humidity environment of the crop to maximise efficacy.

Keywords: South American tomato pinworm; entomopathogenic nematodes; foliar application; relative humidity; temperature.

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1. Introduction

The South American tomato pinworm,*Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is most serious insect pests of tomato (Biondi et al., 2018; Desneux et al., 2010; Mansour et al., 2018; Urbaneja et al., 2013). Since its introduction into Europe in 2006, being first detected in the northern part of Castellón de la Plana in eastern Spain, it has continued to spread rapidly across Europe, Africa, the Middle East and Asia where it immediately reached damaging levels and became a serious pest of tomatoes grown in field and greenhouse (Biondi et al., 2018; Han et al., 2019; Mansour et al., 2018; Urbaneja et al., 2013).

Adults emerge from pupae, mate and females start laying eggs on all above ground parts of plants. After hatching, larvae enter leaves, stems or fruits, where they feed and develop and, as a result, produce visible mines and galleries which expand as larvae develop and increase in size. Yield is reduced as a result of the injuries happening to the leaves and fruits. Fruits can also be invaded by secondary pathogens which enter from the galleries produced by larvae. General development of plants is altered due to the mines formed in the stems. Finally, direct feeding of larvae on the growing tips can result in reducing and stopping plant development, with yield losses can always reach 100% on tomatoes (Biondi et al., 2018; Desneux et al., 2010; Mansour et al., 2018).

The management of *T. absoluta* has commonly relied on the use of chemical insecticides in South America and in newly invaded areas (Biondi et al., 2018; Desneux et al., 2010; Mansour et al., 2018; Urbaneja et al., 2012). Effective chemical control is difficult because of the development of resistant strains and because of the feeding behaviour of larvae in which they are protected from contact with insecticides inside the mines on leaves, fruits and stems (Terzidis et al., 2014). This has prompted frequent and extensive use of insecticides which hastened the development of insecticide resistance in *T. absoluta* in its area of origin and in the newly invaded areas (Guedes et al., 2019; Haddi et al., 2012; Roditakis et al., 2015). There is growing concern about the about the adverse side effects of insecticides that are used in integrated pest management programmes in tomato crops on non-target organisms (Arno and Gabarra, 2011; Biondi et al., 2012; Biondi et al., 2013) as well as the negative impact of insecticides on the environment, users and consumers (Pimentel et al., 1992; Weisenburger, 1993). To reduce reliance on chemical insecticides there has been growing interest in growing interest in biorational alternative management methods for T. absoluta including biological control through releases of parasitoids and/or predators, biopesticide applications, and the potential use of entomopathogenic nematodes (Mansour and Biondi, 2020).

Entomopathogenic nematodes (EPNs) are important biological control agents for several insect pests (Grewal et al., 2005; Lacey and Georgis, 2012) and are potentially promising biological control agents for *T absoluta*. Nevertheless, successful control of *T. absoluta* using EPNs on foliage is not easy because nematode efficacy is reduced by a number of adverse environment

factors such as desiccation, ultra-violet radiation and extreme temperatures (Gaugler et al., 1992; Mason and Wright, 1997). The most important among these factors is desiccation (Baur et al., 1995; Bélair et al., 2003). In addition to the environmental factors, there are other factors that interact with each other and affect nematode efficacy on leaves such as the specific insect pest (e.g., type of feeding, activity) and host plant (e.g., hairy or non-hairy surface) (Mason et al., 1998).

When on foliage, nematodes require a thin water film to survive and move freely to locate and enter the mines where larvae of *T. absoluta* live. The persistence of this water film, before the leaf surface dries, is critical, thus high relative humidity (RH) after application is required for successful nematodes foliar application(Broadbent and Olthof, 1995; Koppenhöfer, 2007).

After application, the period of time that nematodes require to enter a leaf is a factor that affects the efficacy of nematodes against leafminers. When nematodes enter the mines they are protected from environmental constraints and are able to seek and infect larvae. Therefore there are several factors that may alter the efficacy of nematodes used for foliar application including: Nematode search strategy - ambush or cruise foraging or an intermediate strategy (e.g. Jagodič et al., 2017; Williams and MacDonald, 1995); Number of applications - applying nematodes more than once could result in better coverage and deposit more nematodes near the entrances of mines where they can infect larvae; Timing of nematode application - because nematode efficacy is affected by the age of the host larval instar (e.g. Williams and Walters 2000); Temperature – as different EPN species vary in their performance according to temperature (e.g. Grewal et al., 1994).

The aim of this work was to determine which factors affect EPN's efficacy against larvae of *T. absoluta* on tomato leaves and to identify the most effective species. The objectives were to compare (1) the effect of RH on efficacy, lethal concentration and lethal time of EPN species against larvae of *T. absoluta* on tomato leaves, (2) the effect of RH on survival of EPN species, (3) the time required by EPN species to enter a leaf at different RH, (4) the effect of temperature on efficacy of EPN species and (5) the effect of the number of applications on efficacy of EPN species at different RH.

2. Materials and methods

2.1. Source of insects and entomopathogenic nematodes (EPNs)

A *Tuta absoluta* culture was maintained under quarantine conditions at 25 ± 5 °C with an 18:6 h L:D regime using high pressure sodium lamps and at 45-88% RH. Commercial formulations of *S. feltiae* (Nemasys), *S. carpocapsae* (Nemasys C) and *H. bacteriophora* (Nemasys H) were provided as a moist paste by BASF plc. Nematode suspensions were prepared using tap water. Prior to use,

nematode suspensions were acclimatised in an incubator at 25 ± 0.5 °C for 24 h and were examined microscopically to ensure viability. Nematode concentration was adjusted by altering dilution, using the method described by (Glazer and Lewis, 2000).

2.2. Tomato plants

The insects were reared on leaves of tomato plants *Solanum lycopersicum* "Moneymaker" variety (4-6 weeks old) which were grown under greenhouse conditions in individual pots (13 cm diameter) using J Arthur Bower's John Innes growing medium Number 2 loam based compost at 20-25 °C and 18 hours day length. Extra lighting was provided during periods of shorter natural day length using high pressure sodium lamps.

2.3. Experimental set up

In order to obtain the first *T. absoluta* larval instars, eggs laid on leaves by females were used. To obtain second, third and fourth instars larvae were left to feed on tomato leaves until they reached the required life stage. Then they were extracted directly from the mines using a fine needle and brush. The larvae or eggs were placed on the upper side of fresh tomato leaves. To keep these leaves fresh, the petioles were inserted in a 125 ml narrow neck bottle filled with a 20% nutrient solution (Canna Hydro Vega nutrients solution). The leaves containing larvae were left for 24 hours and those containing eggs were left for 48 h to develop well-formed mines before EPN treatment. The leaves were then sprayed on both sides with nematodes (at the desired concentration) suspended in tap water until run off. Control treatments received water only.

The treated leaves were transferred to experiment arenas consisting of 3.25 l clear plastic containers filled either with 400 ml water (to obtain > 95% RH), 400 ml of saturated salt solution of calcium chloride hexahydrate (45% RH) Winston and Bates (1960) or 400 ml of 85% glycerine solution (75% RH) Grover and Nicol (1940). Recorded RH varied 10% above and below the stated values. The containers were closed and kept in an incubator at 25 ± 0.5 °C in darkness in a randomized block design and numbers of dead and live larvae were recorded after 48 h unless otherwise specified. A larva was scored dead if it failed to respond to mechanical stimulation. RH was recorded by hanging a RH and temperature data logger (LASCAR EL-USB-2) from the lid of the container. The number of infective juvenile nematodes (IJs) on the leaf surface area was determined following the method described by Glazer and Navon (1990).

Unless otherwise stated the control treatment received water only. There were four replicate containers for each treatment (n = 4) with a corresponding control treatment (n = 4).

2.4. Effect of RH on efficacy of EPN species

The impact of RH on infectivity of *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* was investigated at > 95%, 75% and 45% RH against 1st, 2nd, 3rd and 4th instar larvae of *T. absoluta*. Ten to twenty-five larvae were placed on each leaf depending on larval instar used. Leaves were sprayed with nematodes suspended in water at a rate of 60 ± 6 IJs cm^{-2}.

2.5. Effect of RH on lethal concentrations (LC) and lethal time (LT) of EPN species

The influence of RH on the concentrations of the three nematode species, which kill 50% of *T. absoluta* larvae (LC50), was tested at > 95%, 75% and 45% RH against 3rd instar larvae. Ten to fifteen insect larvae were placed on each leaf. Nematodes were suspended in tap water at four different rates: 0; 5 ± 0.5; 15 ± 1.5; 30 ± 3 and 60 ± 6 IJs cm^{-2}. Additionally, the effect of RH on the time required by the three nematode species to kill 50% of *T. absoluta* 3rd instar larvae (LT50) was investigated at > 95 %, 75 % and 45 % RH. Ten to fifteen larvae were placed on each leaf. Leaves were then sprayed with water (control) or nematodes suspended in water at a rate of 60 ± 6 IJs cm^{-2} (treatment). Larval mortality was checked every 8 h up to 48 h. Independent samples were used for each time point.

2.6. Effect of RH on survival of IJs of EPN species

The impact of RH on the survival of IJs of the two nematode species *S. feltiae* and *S. carpocapsae* on tomato leaves was investigated at > 90%, 75% and 45% RH. The experiment arena was prepared as before except that leaves were not infested with insect larvae. Leaves were sprayed with nematodes suspended in tap water at a rate of 60 ± 6 IJs cm^{-2}. After incubation for 0, 2, 4, 7, 24 or 48 h, IJs were washed off the leaflet into counting chambers and percentage nematode survival was recorded after 24 h. IJs not moving when mechanically stimulated using a fine needle were recorded as dead.

2.7. Time required by EPN species to enter a leaf

The time required by *S. feltiae* and *S. carpocapsae* to enter a leaf was investigated at > 95%, 75% and 45% RH against 3rd instar larvae. Ten to fifteen larvae were placed on each leaf. Leaves were sprayed with nematodes suspended in tap water at a rate of 60 ± 6 IJs cm^{-2}. At intervals from 1 to 24 h, four leaves were removed from the containers. In order to fix the IJs on the leaf surface, the leaves were immediately dried by blowing cool air across them until no surface moisture was apparent. Each dried leaf was wrapped with moist cotton wool to keep it fresh and placed in a vented plastic Petri dish (9 cm × 1.6 cm) and larval mortality was checked 48 h later.

2.8. Effect of temperature on efficacy of EPN species

The effect of temperature on the efficacy of *S. feltiae* and *S. carpocapsae* was investigated against *T. absoluta* 3rd instar larvae at > 95% RH. Six different temperatures were tested: 10°C, 15°C, 20°C, 25°C, 30°C and 35°C. Ten to fifteen insect larvae were placed on each leaf. Leaves were sprayed with nematodes suspended in tap water at a rate of 60 ± 6 IJs cm^{-2}.

2.9. Effect of number of applications of EPN species

The effect of the number of applications on the efficacy of the most effective nematode species *S. feltiae* against first, second, third and fourth instar larvae of *T. absoluta* was investigated at 45% and 75% RH. Ten to twenty-five insect larvae were placed on each leaf depending on larval instar used. Leaves were sprayed with nematodes suspended in tap water at a rate of 60 ± 6 IJs cm^{-2}. Four different frequencies of application were used: spraying leaves once (at the beginning (0 hours)), twice (0 h and after 24 h), three times (0 h, 24 h and after 48 h) or four times (0 h, 24 h, 48 h and after 72 h).

3. Statistical analysis

Data were analysed using Minitab® 16.1.0 (© 2010 Minitab Inc.). Data that were normally distributed were analysed with a General Linear Model (GLM) and, if there was a significant differences between treatments, Tukey's multiple range test was performed to separate means. Where necessary percentage larval mortality was corrected for mortality in the control using Schneider-Orelli's formula (Püntener, 1981). LC_{50} values and LT_{50} of nematode species at each RH were computed by probit analysis (Finney, 1971).

The assumptions of normality were not met for the effect of temperature on efficacy of *S. feltiae* so these data were analysed using the non-parametric Scheirer-Ray-Hare Test followed by the non-parametric Mann-Whitney-U Test to test significant differences between treatments. The results of the effect of temperature on efficacy of *S. carpocapsae* were transformed (arcsine of the square root) before analysis to meet the assumptions of normality.

To test the effect of number of applications on efficacy of *S. feltiae* and the time required by *S. feltiae* and *S. carpocapsae* to enter a leaf; larval mortalities were corrected for control mortalities and tested for normality. One-way ANOVA was used to reveal significant differences between treatments and, if there was a significant differences between treatments, Tukey's multiple range test was performed to separate means.

4. Results

4.1. Effect of RH on efficacy of EPN species

In general, relative humidity had an impact on efficacy of the tested nematode species (*S. feltiae, S. carpocapsae* and *H. bacteriophora*). The efficacy against all larval instars of *T. absoluta* decreased with the decrease in RH. Mortality of different larval instars induced by the three nematode species was highest in the fourth larval instar followed by third, second and first instar larvae. *Steinernema feltiae* gave the highest mortality against different instar larvae at the three ranges of RH compared to *S. carpocapsae* and *H. bacteriophora* (Fig. 1 a, b and c).

There were significant differences in the efficacy of *S. feltiae* at different RH (GLM: $F_{2, 36} = 1306$; $P < 0.05$) and against different larval stages (GLM: $F_{3, 36} = 74$; $P < 0.05$) (Fig. 1 a); *S. carpocapsae* at different RH (GLM: $F_{2, 36} = 1167$; $P < 0.05$) and against different larval stages (GLM: $F_{3, 36} = 48$; $P < 0.05$) (Fig. 1 b); and *H. bacteriophora* at different RH (GLM: $F_{2, 36} = 746$; $P < 0.05$) and against different larval stages (GLM: $F_{3, 36} = 9$; $P < 0.05$) (Fig. 1 c).

When efficacy of the three nematode species against the third instar larvae at the three ranges of RH was compared, results showed that there were significant differences in efficacy between the nematode species (GLM: $F_{2, 27} = 128$; $P < 0.05$) (Fig 2). *Steinernema feltiae* caused most mortality and *H. bacteriophora* least.

4.2. Effect of RH on lethal concentrations (LC) and lethal time (LT) of EPN species

LC_{50} and LT_{50} of *S. feltiae, S. carpocapsae* and *H. bacteriophora* were affected by the decrease in RH (Table 1). LC_{50} values for all species of nematode increased as RH decreased. *Steinernema feltiae* at >90% RH had the lowest LC_{50} at 4.3 IJs/cm^2 whilst for *H. bacteriophora* at 45% RH LC_{50} values were not calculated because larval mortality was estimated to less than 10%.

Similarly, *S. feltiae* had the lowest LT_{50} at 7.5 h at > 95% RH whilst that for *H. bacteriophora* at 45 ± 10 % RH was not calculated because larval mortality was estimated to less than 10%.

4.3. Effect of RH on survival of IJs of EPN species

In general *S. carpocapsae* survived better than *S. feltiae*, but survival of both nematode species decreased as RH decreased and as time passed (Fig. 3).

When survival of *S. feltiae* and *S. carpocapsae* was compared after 7, 24 and 48 h at the three RH tested, significant differences were observed in survival between the two nematode species (GLM: $F_{1, 54} = 53$; $P < 0.05$), the three ranges of RH (GLM: $F_{2, 54} = 750$; $P < 0.05$) and the three tested periods (GLM: $F_{2, 54} = 64$; $P < 0.05$) (Fig. 4). Mean survival of *S. carpocapsae* (49%) was significantly ($P < 0.05$) higher than survival of S*. feltiae* (36%) in all humidity conditions and across all times.

4.4. Time required by EPN species to enter a leaf

Using *S. feltiae,* there were significant differences between the mortality achieved with different fixing times at > 95% RH (One-Way ANOVA: $F_{4, 15} = 32$; $P < 0.05$), at 75% RH (One-Way ANOVA: $F_{4, 15} = 20$; $P < 0.05$) and at 45% RH (One-Way ANOVA: $F_{4, 15} = 7$; $P < 0.05$) (Fig. 5). *Steinernema feltiae* required 3 h to enter the leaves at > 95% RH and 6 h at 75% and 45% RH.

With *S. carpocapsae*, there were also significant differences between the mortality achieved with different fixing times at > 95% RH (One-Way ANOVA: $F_{4, 15} = 40$; $P < 0.05$), at 75% RH (One-Way ANOVA: $F_{4, 15} = 20.5$; $P < 0.05$) and at 45% RH (One-Way ANOVA: $F = 4.5$; $P < 0.05$) (Fig. 6). *Steinernema carpocapsae* required 6 h to enter the leaves at > 95% RH and 12 h at 75% and 45% RH.

4.5. Effect of temperature on efficacy of EPN *species*

There were significant differences in the efficacy of *S. feltiae* (Sheirer-Ray-Hair Test; $\chi^2_5 = 74.5$; $P < 0.05$) and in the efficacy of *S. carpocapsae* (GLM: $F_{5, 72} = 198$; $P < 0.05$) at the tested temperatures (Fig. 7 a, b). *Steinernema feltiae* caused the highest larval mortality (above 95%) at 15, 20 and 25 °C. Larval mortality at 10 °C increased sharply as the time passed, reaching 94% after 96 h, whilst mortality at 35 °C was very low (13%). On the other hand, *S. carpocapsae* induced the highest larval mortality (above 91%) at 20, 25, 30 and 35 °C. Larval mortality at 10 °C was very low (12%).

When the two nematode species were compared in the same analysis with larval mortality recorded after 72 h, the results showed that there were no significant differences in overall mean efficacy between *S. feltiae* and *S. carpocapsae* (GLM: $F_{1, 36} = 0.02$; $P > 0.05$), whereas there were significant differences in the efficacy of *S. feltiae* and *S. carpocapsae* due to temperature (GLM: $F_{5, 36} = 263$; $P < 0.05$) (Fig. 8).

4.6. Effect of number of applications of EPN species

There were significant differences in mortality between numbers of applications of *S. feltiae* at 75% RH against the first larval instar (One-Way ANOVA: $F_{3, 12} = 31$; $P < 0.05$), the second larval instar (One-Way ANOVA: $F_{3, 12} = 84$; $P < 0.05$), the third larval instar (One-Way ANOVA: $F_{3, 12} = 18$; $P < 0.05$) and the fourth larval instar (One-Way ANOVA: $F_{3, 12} = 17$; $P < 0.05$) (Fig. 9). The most effective treatments with *S. feltiae* at this RH were three and four applications to the first and second instar larvae and two, three and four applications to the third and fourth instar larvae.

There were also significant differences in mortality between numbers of applications of *S. feltiae* at 45% RH against the first larval instar (One-Way ANOVA: $F_{3, 12} = P < 0.05$), the second larval instar (One-Way ANOVA: $F_{3, 12} = P < 0.05$), the third larval instar (One-Way ANOVA: $F_{3, 12} = 32$; $P < 0.05$) and the fourth larval instar (One-Way ANOVA: $F_{3, 12} = 35$; $P < 0.05$) (Fig. 10). The most effective

267 treatment with *S. feltiae* at this RH was four applications in which mean corrected larval mortality
268 was 92%, 91%, 74% and 50% for the fourth, third, second and first instar larvae respectively.

269 **5. Discussion**

270 Relative humidity had a direct effect on efficacy of the EPNs, the efficacy decreasing with a
271 decrease in RH. These findings are in accordance with those of Jacobson and Martin (2011) who
272 reported high efficacy (> 90% mortality) using an aqueous suspension of the same commercial
273 nematode species used in the current study (*S. feltiae* (Nemasys®)) against larvae of *T. absoluta* in
274 favourable conditions (). However, they observed lower nematode efficacy in two further trials of
275 the same system undertaken in commercial organic tomato crops in Portugal (50% and 43%
276 overall mean mortality in first and second trial respectively). The low efficacy in the first trial was
277 explained as due to inadequate application in one of the plots due to some spray nozzles being
278 temporarily blocked and the authors suggested that the overall mortality would rise to 56% if the
279 data from that plot were excluded. The result of the second trial was attributed to the unfavourable
280 environmental conditions for nematode activity (65-74% RH). Similarly, Baur et al. (1998) reported
281 larval mortality of diamondback moth *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) of 62-
282 87% using *S. carpocapsae* "All" strain suspended in water in Petri dish bioassays lined with filter
283 paper. However, only 41% mean mortality was achieved in two experiments conducted at farms in
284 Hawaii with more challenging environmental conditions using a foliar application of the same
285 nematode species. Comparable results were obtained by Glazer and Navon (1990) who found that
286 an aqueous suspension of *S. feltiae* "All" strain gave a mean mortality of 77% with different larval
287 instars of the cotton bollworm *Heliothis armigera* (Hübner) (Lepidoptera: Noctuidae) in Petri dish
288 bioassays, whereas in harsher environmental conditions in a greenhouse (50-70% RH and 24-28
289 °C), *S. feltiae* "Pay" strain and *S. feltiae* "All" strain induced only 15% and 0% larval mortality
290 respectively on leaves of bean seedlings.

291 The results of the current investigation showed that the rate of nematode survival is related to RH
292 with nematode survival decreasing as RH decreased. In addition, *S. carpocapsae* tolerated
293 desiccation better than *S. feltiae* (Fig. 3 and 4). These results are consistent with those of Bélair et
294 al. (2003) who found that survival rate of *S. carpocapsae* "All" strain on cabbage leaves was
295 higher than survival of *S. feltiae* "UK" strain at 70% RH in growth chambers. They are also in line
296 with those of Glazer and Navon (1990) who recorded extreme reduction in *S. feltiae* "All" strain
297 survival (80% after 4 h and 0% after 8 h exposure) on foliage of bean plants at 50-70% RH under
298 greenhouse conditions. Furthermore, similar results were obtained by Glazer (1992) who reported
299 a reduction in survival of *S. carpocapsae* "Mexican" strain by 40% and 70% at 80% and 60% RH
300 respectively after 6 hours exposure on filter papers. The author recorded a reduction in survival of

301 the same nematode species by 39% at 65% RH after 4 h exposure on tomato leaves under
302 greenhouse conditions.

303 When comparing the efficacy of the three nematode species against third instar larvae at the three
304 ranges of RH, the results showed significant differences in efficacy with *S. feltiae* being the most
305 effective species (68% mean mortality at the three RH levels) followed by *S. carpocapsae* (49%)
306 then *H. bacteriophora* (36%) (Fig. 2). These findings were supported by the results of the effect of
307 RH, in which the LC_{50} and LT_{50} of *S. feltiae* were the lowest followed by *S. carpocapsae* then *H.*
308 *bacteriophora* (Table 1). Despite the fact that *S. carpocapsae* tolerates desiccation better than *S.*
309 *feltiae*, the latter species induced higher mortality. The reason behind this might be the difference
310 in foraging strategies of the two nematode species. *Steinernema carpocapsae* is an ambush
311 forager (exploiting a 'sit and wait' strategy), whereas *S. feltiae* is an intermediate forager that
312 employs both an ambush and a cruise foraging strategy (Campbell and Gaugler, 1997). Therefore,
313 *S. feltiae* is more effective at finding the larvae of *T. absoluta* inside the mines than *S.*
314 *carpocapsae*. *Steinernema feltiae* required less time to locate and enter the mines than *S.*
315 *carpocapsae* and consequently gained protection from desiccation inside the leaf tissue. The high
316 efficacy of the intermediate forager *S. feltiae* compared to the ambush forager *S. carpocapsae*
317 against a sedentary insect pest, the leafminer *Liriomyza huidobrensis* (Blanchard) was also
318 reported by Williams and Walters (2000). The low mortality caused by of *H. bacteriophora*
319 compared to *S. feltiae* and *S. carpocapsae* in this study may be explained by the fact that
320 *Heterorhabditis* spp. have reduced survival on foliage compared to *Steinernema* spp. (Hara et al.,
321 1994). Thus, *H. bacteriophora* was excluded from further investigations.

322 The amount of time that nematodes need to enter a leaf is an important factor, which determines
323 their efficacy after foliar application, because once the nematodes enter the mines, they are
324 protected from detrimental environment conditions and are able to seek and infect larvae (Williams
325 and MacDonald, 1995; Williams and Walters, 1994). The time taken by nematodes to enter a leaf
326 in the present investigation (Fig. 6 and 7) was shorter than that observed by Williams and Walters
327 (1994) who found that *S. feltiae* required 10 hours to successfully enter the leaf tissue and
328 consequently cause high mortality of the late second/early third instar larvae of *L. huidobrensis* at
329 temperatures of 15-25 °C and at high relative humidity. These results may be explained by the fact
330 that the behaviour of the two pests is different. With *Liriomyza*, nematodes enter the mines through
331 the holes made by the female on leaves during oviposition. Larvae are unable to move between
332 leaves and all larval instars occur inside the mines (Ameixa et al., 2007). In contrast, in *T. absoluta,*
333 nematodes enter the mines through the larger holes produced by larvae when they penetrate the
334 leaves, which can be easily used by nematodes to enter the mines and thus avoid desiccation and
335 infect the larvae more rapidly.

The findings of the effect of temperature on nematode efficacy revealed that both nematode species induced similar mortality at 25 °C and that *S. feltiae* was more virulent at lower temperatures (15 and 20 °C), while *S. carpocapsae* was more virulent at higher temperatures (30 and 35 °C) (Fig. 7 and 8). Several other investigations have revealed that infectivity of nematodes was affected by temperature (Grewal et al., 1994; Mason and Wright, 1997). Molyneux (1984) reported that the survival of *S. feltiae* at high temperature was reduced. Bélair et al. (2003) found that *S. feltiae* was more effective than *S. carpocapsae* at 15 and 20 °C against second instar larvae of the imported cabbageworm *Artogeia rapae* L. and that at 30 and 35 °C, infectivity of *S. feltiae* strains declined and infectivity of *S. carpocapsae* remained unaffected compared to 25 °C. Similarly, Ratnasinghe and Hague (1998) reported that infectivity of *S. carpocapsae* against the diamondback moth (*Plutella xylostella* (L.)) was optimum at a temperature between 20 and 30 °C. A possible explanation for the high efficacy of *S. carpocapsae* (ambush forager) at high temperature might be because it resides near the soil surface to ambush passing hosts and thus is adapted to tolerate high temperature and desiccation (Bélair et al., 2003; Campbell and Gaugler, 1993), whereas the reason behind the low survival of *S. feltiae* (intermediate forager) is that food reserves are quickly depleted because of high activity level and respiration (Bélair et al., 2003). Therefore, since the results of the present study revealed that *S. feltiae* is significantly more effective than *S. carpocapsae,* it seems that its use as a biological agent against this pest will be limited to countries that have a temperate climate (Jacobson and Martin, 2011). In countries where temperature is at times high (night time minimum > 20 °C), *S. feltiae* will not be effective and accordingly it is important to find and test other nematode species (indigenous or commercially available species) that are tolerant to high temperature for their potential as biological agents against *T. absoluta*.

The observations of the effect of number of applications on nematode efficacy in the current study showed that four applications gave significantly higher larval mortality than other frequencies of application at 45% RH with some exceptions at 75% RH (Fig. 9, 10). The results also revealed that first and second larval instars required additional applications to achieve significant mortality compared to third and fourth larval instars. These results may be explained by the fact that first and second larval instars are less susceptible to nematode infections. The possible reasons behind this could be that the small size of larvae may have hindered nematodes entering through the normal infection routes (mouth, spiracle, or anus) or that younger larvae may produce smaller amounts of attractants such as CO_2 or other kairomones which makes it more difficult for nematodes to locate them inside the mines (Kaya, 1985). If the nematodes are deposited away from the entrance of the mines, it might be more difficult for them to locate these attractants. Thus, it is important to target the third and fourth larval instar when controlling this pest using EPNs. Williams and Walters (2000) reported that a 24 h repeat application of a dose one tenth (1000 *S. feltiae*/ml) of the

original concentration of the single dose (10,000 *S. feltiae*/ml) at < 90% RH induced higher leafminer (*Chromatomyia syngenesiae* Hardy) mortality than repeat applications at 48 h and 72 h. The authors attributed this to the differences in susceptibility of larval instars present at the time of application. Therefore, the increase in nematode efficacy in the current study can be attributed to the increase in nematode density and coverage on leaf surfaces after each application, which enabled them to locate larvae inside the mines by depositing them near the entrance of these mines, so that they parasitised the remaining live larvae.

6. Conclusion

In conclusion, all factors studied in the current investigation affected nematode efficacy to a certain extent. RH was the most important and reduced nematode survival and efficacy significantly as it decreased. It will be important to enhance nematode survival and consequently efficacy when RH is low. This can be achieved by artificially increasing RH if tomato plants are grown in the greenhouse or by adding adjuvants to nematode suspensions to increase their survival and efficacy (Bélair et al., 2003; Schroer et al., 2005).

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536 *Table 1* ***Effect of different levels of RH on LC_{50} values (IJ/cm^2) and LT_{50} (hours) of EPNs***
537 ***applied in water on 3rd larval instar of* T. absoluta *on tomato leaves***

Nematode species	RH	LC_{50} (IJs/cm^2)	Fiducial limits (95%)		LT_{50} (hours)	Fiducial limits (95%)	
			lower	upper		lower	upper
S. feltiae	> 90%	4.3	3.2	5.5	7.5	6.2	8.9
	75 ± 10%	17	13.5	21.8	29	26.9	32.3
	45 ± 10%	100*	59.8	285	53*	46.1	65.3
S. carpocapsae	> 90%	6.4	4.8	8.2	10	7.2	12.4
	75 ± 10%	35	26.5	50.1	49*	43.3	56.9
	45 ± 10%	160*	85.9	1000	72*	57.5	113
H. bacteriophora	> 90%	11.2	7.9	15.3	14	9.9	17.3
	75 ± 10%	153*	85.5	496	63*	53.2	85.2
	45 ± 10%	×	×	×	×	×	×

(×) LC_{50} and LT_{50} were not calculated because larval mortality was less than 18%.
(*) LC_{50} and LT_{50} were calculated when mortality was below 50% and above 18%.

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Figure legends

Figure 1 *Mean corrected mortality at different RH values and 25 ± 0.5 °C of different larval instars of* T. absoluta *48 h after application of (a)* S. feltiae*, (b)* S. carpocapsae *and (c)* H. bacteriophora *in water at 60 IJs ± 6 cm^{-2}. Bars (mean ± SE) with the same letter do not differ significantly according to Tukey's multiple range test at P <0.05 (n = 4)*

Figure 2 *Mean corrected mortality of 3rdlarval instar of* T. absoluta *48 h after application of three EPNs (60 ± 6 IJs cm^{-2}) in water at three different RH and 25 ± 0.5 °C. Bars (mean ± SE) with the same letter do not differ significantly according to Tukey's multiple range test at P <0.05 (n = 4). Data used in the analysis here were from the experiment shown in Figure 1*

Figure 3 *Mean survival of IJs (60 ± 6 IJs cm^{-2}) of (a):* S. feltiae *and (b):* S. carpocapsae *applied in water over 48 h on tomato leaves at various RH and 25 ± 0.5 °C. Error bars indicate standard error (n = 4)*

Figure 4 *Mean survival of IJs (60 ± 6 IJs cm^{-2}) of* S. feltiae *and* S. carpocapsae *applied in water after 7, 24 and 48 h on tomato leaves at various RH and 25 ± 0.5 °C. Bars (mean ± SE) with the same letter do not differ significantly according to Tukey's multiple range test at P < 0.05 (n = 4). Data used in the analysis here were from the experiment shown in Figure 3*

Figure 5 *Mean corrected mortality of 3^{r} larval instar of* T. absoluta *after 48 h of fixing* S. feltiae *(60 ± 6 IJs cm^{-2}) applied in water after various times on tomato leaves at (a): > 95%; (b): 75 ± 10%; (c): 45 ± 10% RH and 25 ± 0.5 °C. Bars (mean ± SE) with the same letter do not differ significantly according to Tukey's multiple range test at P < 0.05 (n = 4)*

Figure 6 *Mean corrected mortality of 3^{r} larval instar of* T. absoluta *after 48 h of fixing* S. carpocapsae *(60 ± 6 IJs cm^{-2}) applied in water after various times on tomato leaves at (a): > 95%; (b): 75 ± 10%; (c): 45 ± 10% RH and 25 ± 0.5 °C. Bars indicated with the same letter (mean ± SE) do not differ significantly according to Tukey's multiple range test at P < 0.05 (n = 4)*

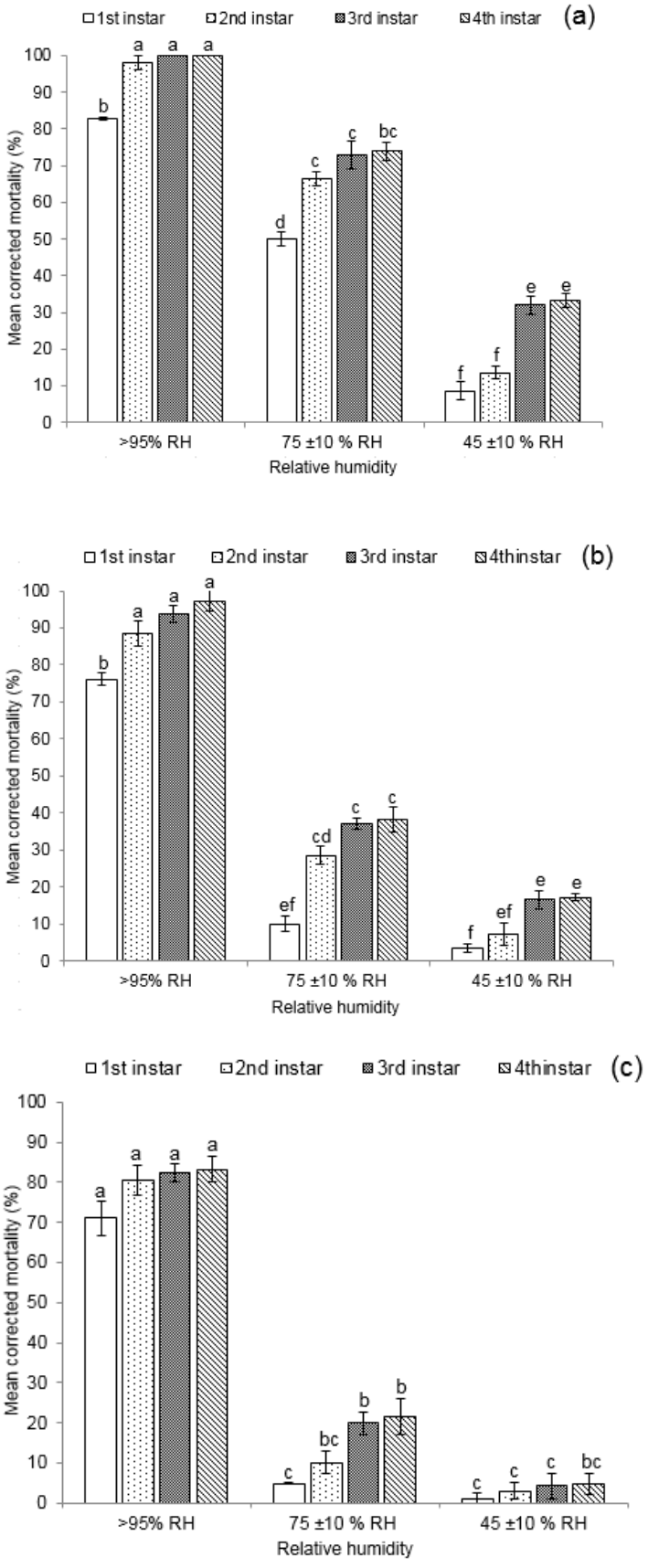
Figure 7 *Mean (± SE) corrected mortality of 3rd larval instar of* T. absoluta *at different temperatures after application of (a)* S. feltiae *and (b)* S. carpocapsae *applied in water at 60 ± 6 IJs cm^{-2} and > 95% RH (n = 4)*

Figure 8 *Mean (± SE) corrected mortality of 3rdlarval instar of* T. absoluta *72 h after application of* S. feltiae *and* S. carpocapsae *(60 ± 6 IJs cm^{-2}) in water at different temperatures and > 95% RH. Bars (mean ± SE) with the same letter do not differ significantly according to Tukey's multiple range test at P <0.05(n = 4). Data used in the analysis here were from the experiment shown in Figure 7*

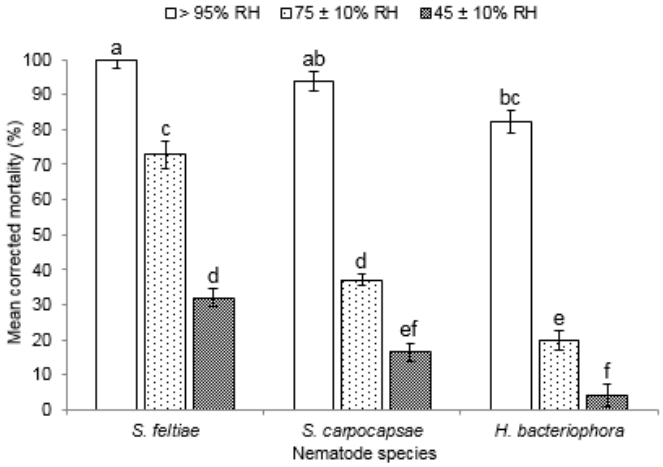
Figure 9 *Mean corrected mortality at 75 ± 10% RH and 25 ± 0.5 °C of (a):1st; (b): 2nd; (c): 3rd and (d): 4th larval instars of* T. absoluta *48 h after different number of applications of* S. feltiae *(60 ± 6 IJs cm^{-2}) applied in water. Bars (mean ± SE) with the same letter do not differ significantly according to Tukey's multiple range test at P < 0.05 (n = 4)*

Figure 10 *Mean corrected mortality at 45 ± 10% RH and 25 ± 0.5 °C of (a):1st; (b): 2nd; (c): 3rd and (d): 4th larval instars of* T. absoluta *48 h after different number of applications of* S. feltiae *(60 ± 6 IJs cm^{-2}) applied in water. Bars (mean ± SE) with the same letter do not differ significantly according to Tukey's multiple range test at P < 0.05 (n = 4)*

Figure 1


1st instar
2nd instar
3rd instar
4th instar
(a)
Mean corrected mortality (%)
>95% RH
75 ±10 % RH
45 ±10 % RH
Relative humidity
1st instar
2nd instar
3rd instar
4thinstar
(b)
Mean corrected mortality (%)
>95% RH
75 ±10 % RH
45 ±10 % RH
Relative humidity
1st instar
2nd instar
3rd instar
4thinstar
(c)
Mean corrected mortality (%)
>95% RH
75 ±10 % RH
45 ±10 % RH
Relative humidity

581 **Figure 2**



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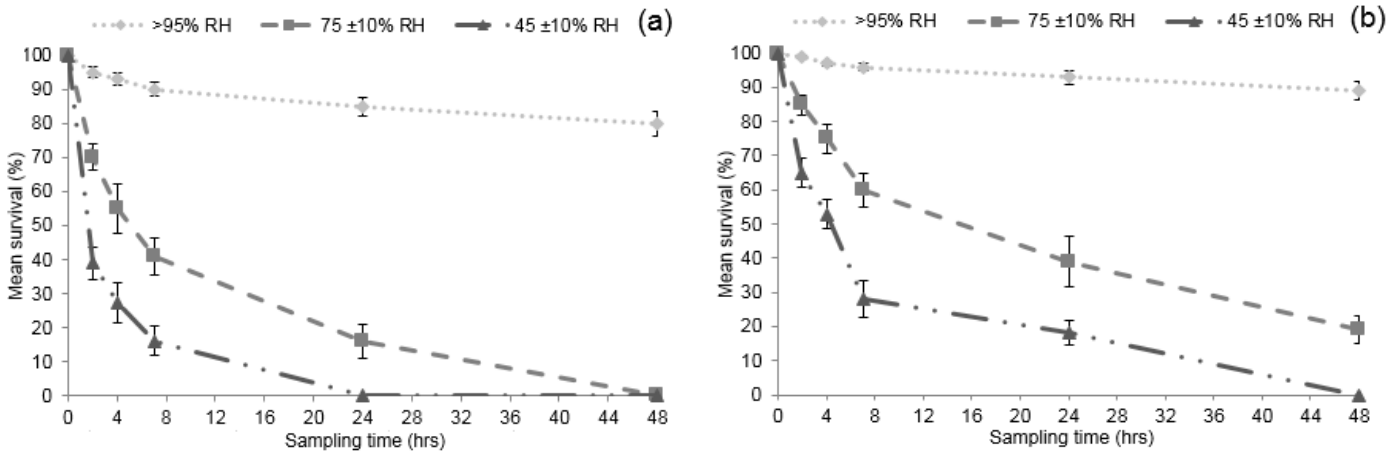
584 **Figure 3**



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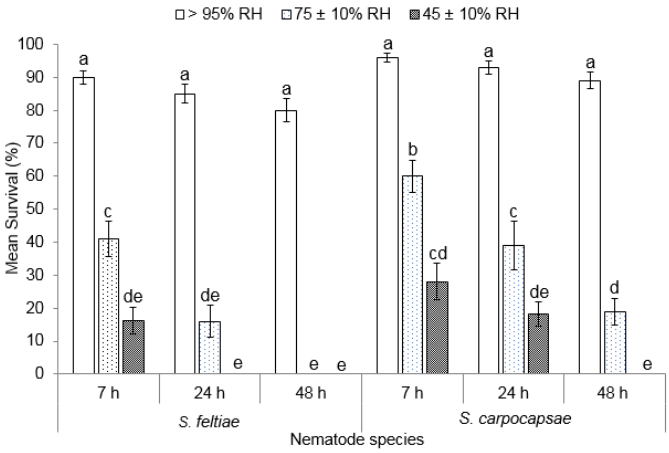
587 **Figure 4**



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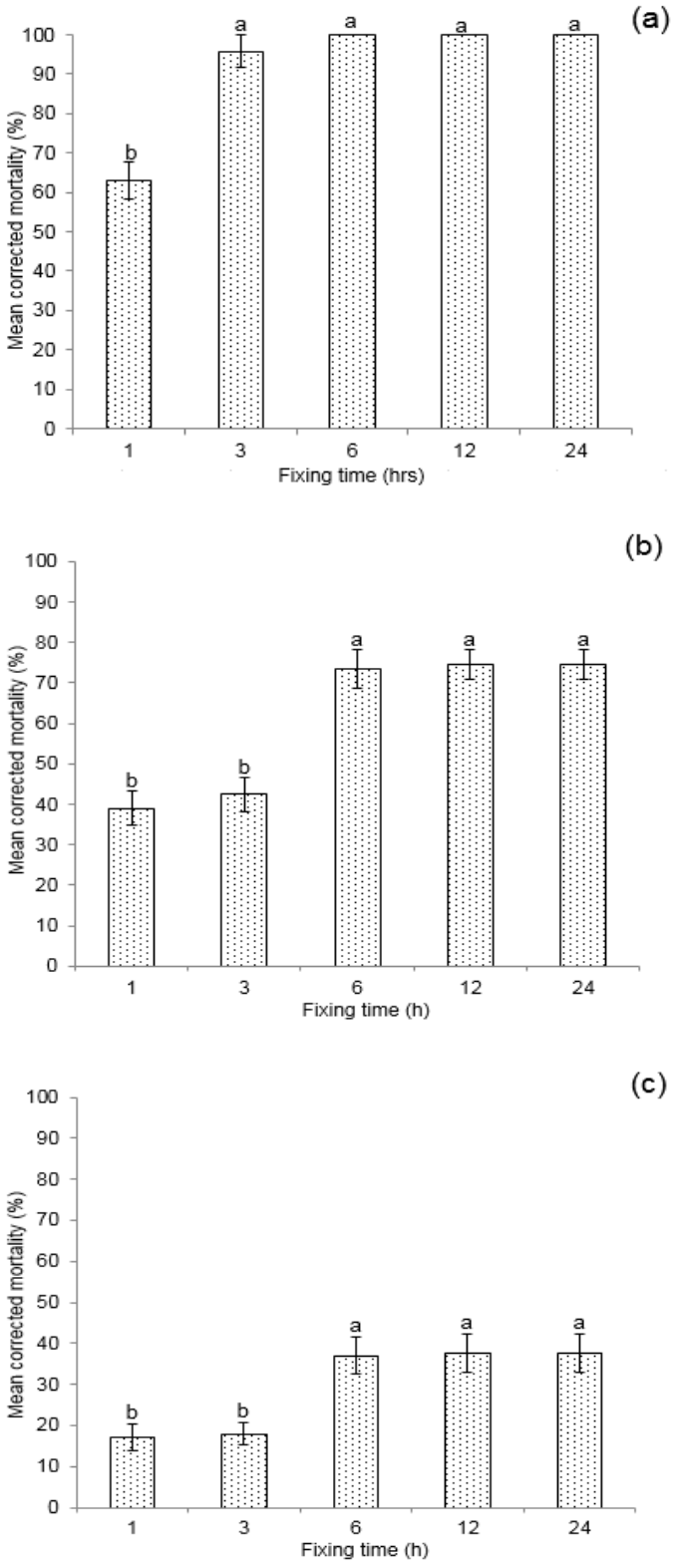
590 **Figure 5**



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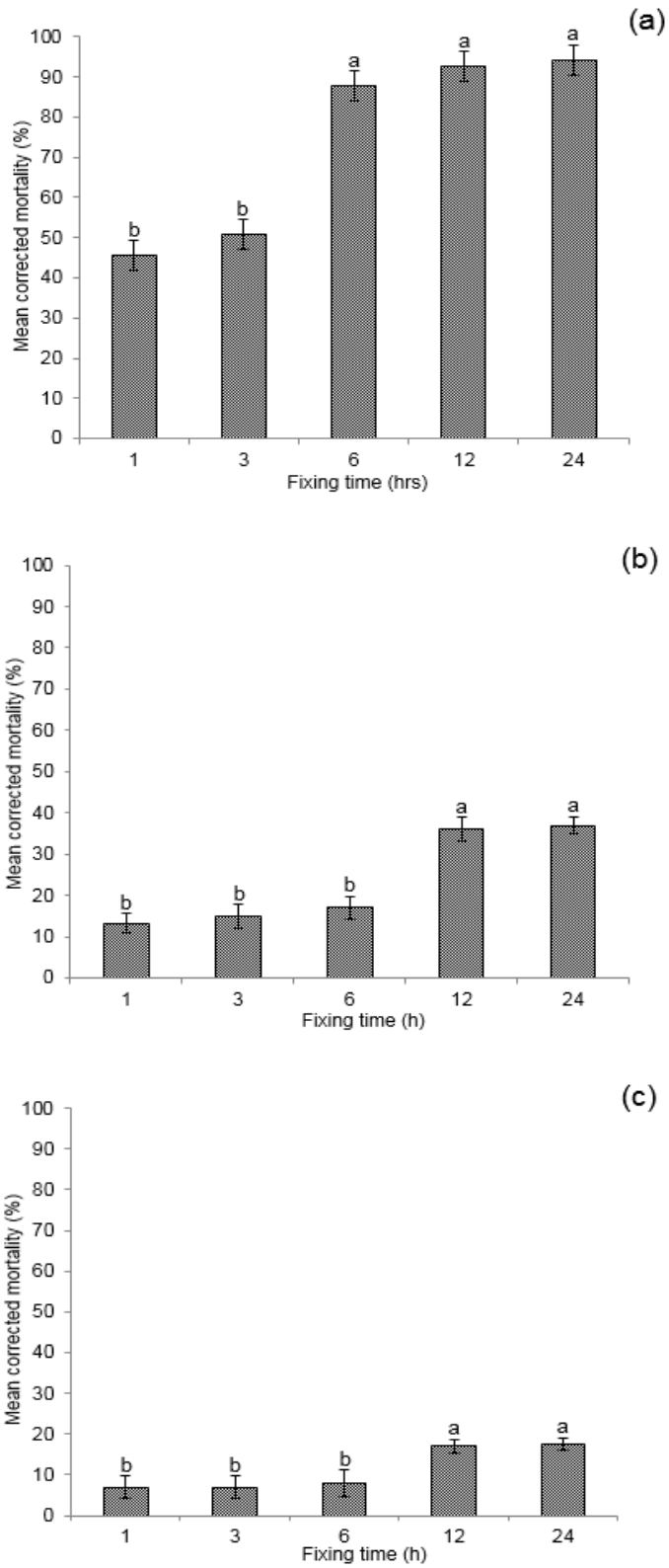
593 **Figure 6**



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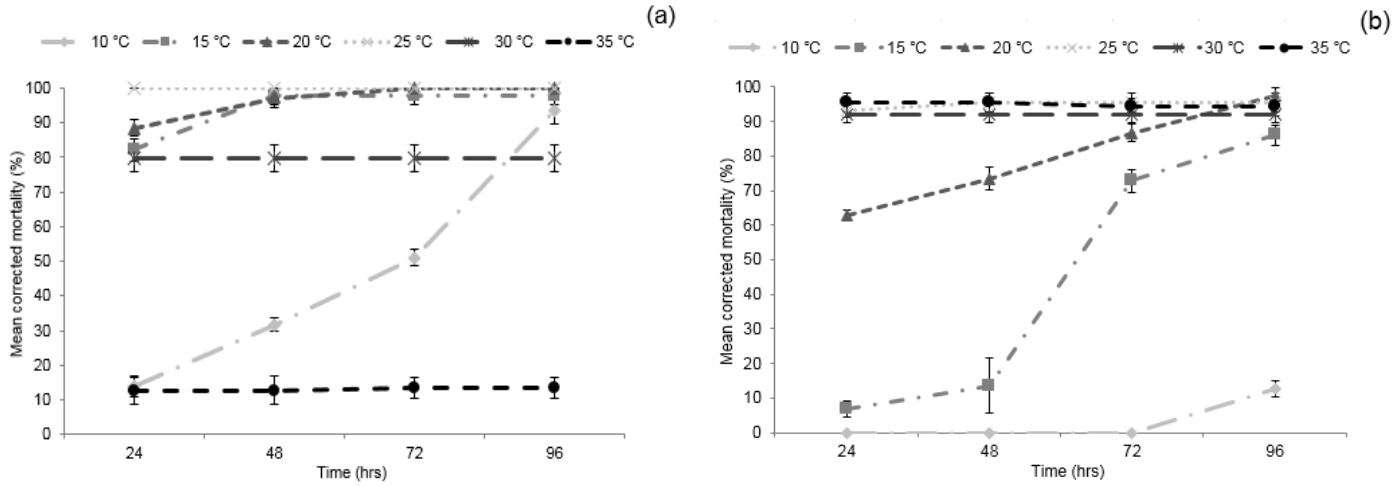
596 **Figure 7**



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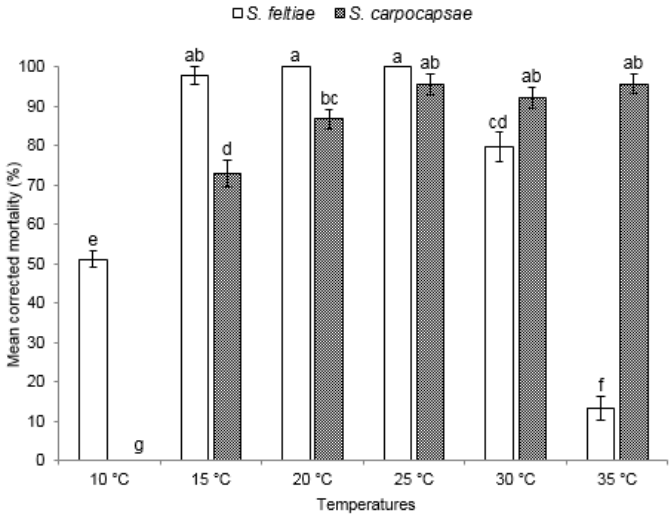
599 **Figure 8**



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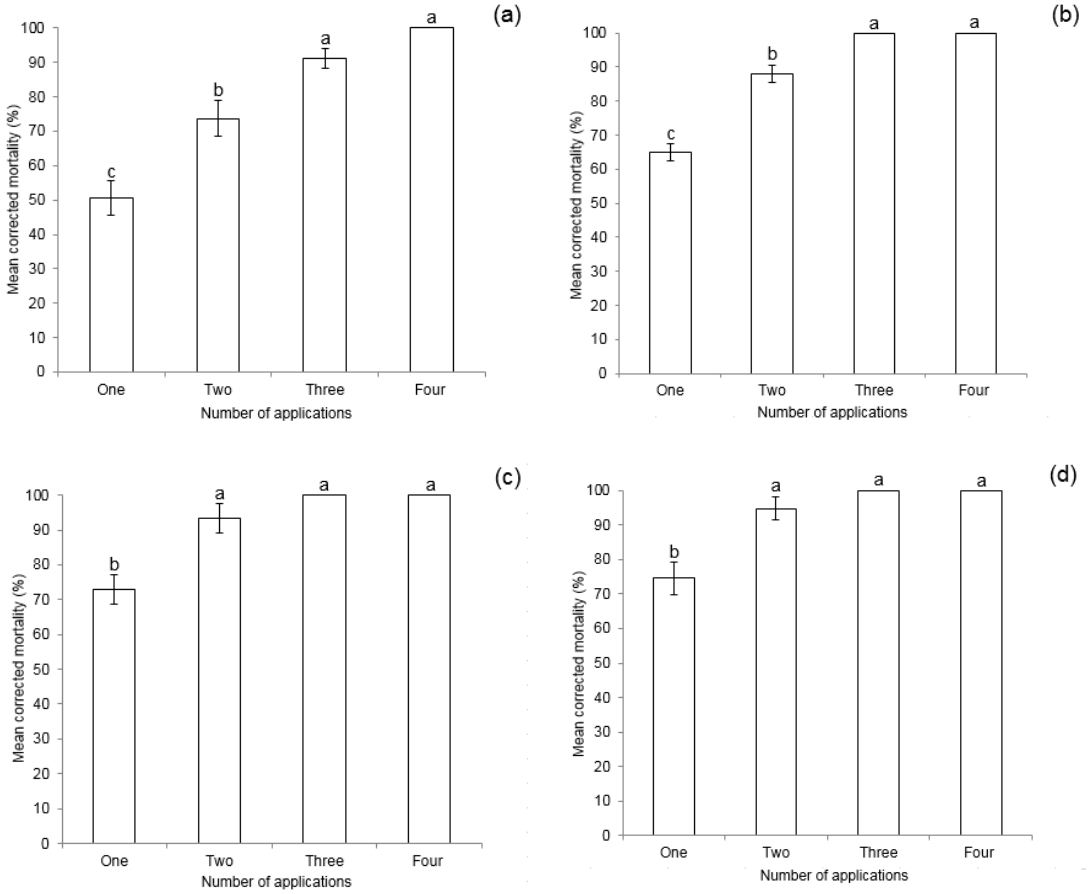
602 **Figure 9**



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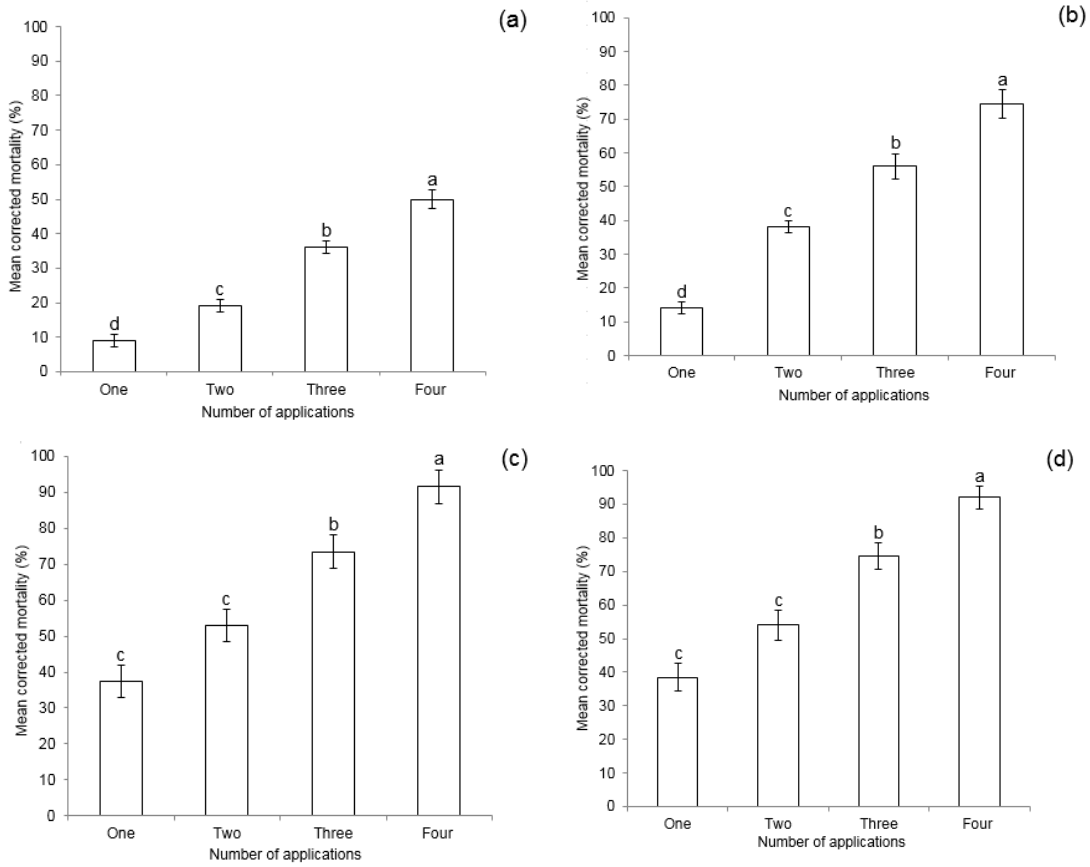
605 **Figure 10**



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