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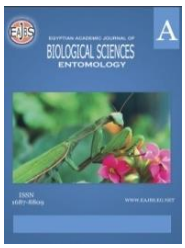

Application of Entomopathogenic Nematodes to Control Larvae of *Temnorhynchus baal* (Reiche and Saulcy) Under Laboratory Conditions and In Strawberry Fields

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ABSTRACT

The white grub, *Temnorhynchus baal*, is a major pest of the strawberry crop in many Asian and African countries. The white grabs cause significant economic losses in the yield. The present study aims to evaluate the effectiveness of entomopathogenic nematodes (EPNs) on the third larval instar of *T. baal* under laboratory conditions. The study extended to compare the efficacy of a combination of EPNs (*Steinernema glaseri+Heterorhabditis bacteriophora*) against a natural infestation of *T. baal* with an organophosphorus insecticide (Diazinon) in an Egyptian strawberry field. In the laboratory, five larvae of *T. baal* were placed in soil and infected with EPNs. At the highest concentration of 4000 IJs/larva, the larvae were more susceptible to infection with *S. glaseri* than *H. bacteriophora*, with mortality percentages of 96 and 88%, respectively. The LC50 of *S. glaseri* and *H. bacteriophora* was 937.44 and 1026.58 IJs/larvae, respectively. The mortality percentage was 96% and 100% when treated for five larvae/cup and one larva/cup, respectively, after being infected with a combination of *S. glaseri+ H. bacteriophora*. The mortality percentage was higher in the mixture than for each species studied individually. In the field, throughout the seasons, the percentage of wilted plants was 17.64, 66.09, and 83.95% in 2020 and 12.56, 67.87, and 75.62% in 2021, for the plots treated with a combination of EPNs, insecticides, and control, respectively. Present findings indicate that entomopathogenic nematodes are good alternatives to control the white grub, *T. baal*, in strawberry fields.

INTRODUCTION

Strawberry, *Fragaria ananassa* Duch, is a widely distributed crop, mainly produced in China, the USA, Mexico, Egypt, Turkey, and Spain (Talavera *et al*., 2019; FAOSTAT, 2019). Egypt is the ninth strawberry-producing country in the world, after China, the USA, Spain, Mexico, Turkey, the Republic of Korea, and Japan. In Egypt, the total harvested area was 11772 ha, yielding approximately 390966 kg/ha in 2019 (FAOSTAT, 2019). Scarabaeoid species are the most serious crop soil pests in the world. Many plants are attacked by white grubs, including sugarcane, maize, millet, and sorghum (Sapkota, 2006; Rahama *et al*., 2014). The white grub (*Temnorhynchus baal* Reiche & Saulcy) (Coleoptera: Scarabaeidae) is one of the main pests that attack the strawberry plant (Shehata *et al*., 2019). The white grub, *T. baal*, is usually found throughout tropical Africa,

Algeria, Libya, Egypt, Israel, Lebanon, Syria, Yemen, Cyprus, and Greece (Endrodi, 1985). It appears polyphagous, attacking roots and/or underground stems (Veeresh, 1988). The first larval instar feeds on organic matter in the soil, while the second and third larval instar grubs feed on roots or underground stems. The economic importance of chafers is primarily due to the feeding activity of the third larval instar grubs (Chandel *et al*., 2015). Strawberry producers have experienced problems with the declining efficacy of the chemical pesticides used to control insects. It has been reported that the development of resistance in the insect is due to prolonged cyclical applications of chemical insecticides (Sato *et al*., 2005). In addition, using chemical pesticides increases farm workers' health problems and causes environmental contamination (Abd El-Salam *et al*., 2013; Parra, 2014; Abd El-Salam, 2019). Several reasons make white grub outbreaks difficult to predict. They are localized and sporadic, and their eggs and young larvae are difficult to sample. Therefore, preventive applications of pesticides are usually made over large areas every year. Consequently, applications of pesticides become expensive, and there is an increase in the chances of resistance development or enhanced microbial degradation. In addition, they increase chemical control independence to the deprivation of endemic natural enemies of host/prey (Koppenhofer and Fuzy, 2008). Entomopathogenic nematodes (EPNs) (Heterorhabditidae and Steinernematidae) have a high potential for curative white grub control and offer an environmentally friendly and IPM-compliant alternative to synthetic insecticides (Grewal *et al.*, 2005). When used under favourable conditions, well-adapted nematode species/strains, such as *Heterorhabditis bacteriophora* Poinar, can be a good alternative biological control of white grubs, often providing suppression comparable to standard insecticides (Georgis and Gaugler,1991; Grewal *et al.*, 2004; Klein, 1993; Cappaert and Koppenhofer, 2003).

The main objectives of this study were to evaluate the effectiveness of EPNs, *Steinernema glaseri* (NJ strain), and *H. bacteriophora* (HP88 strain), against the third larval instar of the white grub, *T. baal*, under laboratory conditions. Compare the efficacy of a combination of EPNs (*S. glaseri* and *H. bacteriophora*) against a natural infestation of *T. baal* with an organophosphorus insecticide (Diazinon) in an Egyptian strawberry field.

MATERIALS AND METHODS

Target Pest, The White Grub, *Temnorhynchus baal*:

The third larval instar of white grub, *T. baal*, was collected from a farm in Al-Mansourieh district, Giza Governorate, Egypt, between mid-April and early May (2019). This farm had not been treated with insecticides or EPNs during the previous year. White grub larvae were gathered by digging out strawberry roots and soil in 0.30×0.30×0.30 m pits. After that, the collected larvae were placed in a plastic jar (3 kg capacity, 25 cm height, and 12 cm diameter), half filled with moistened sterile sandy soil, covered with a cotton cloth, and fed for a week on the roots of the strawberry plants. Then, the white grubs, *T. baal* larvae, were examined according to Koppenhofer and Fuzy (2008), and the third healthy larval instar was chosen for utilization in laboratory experiments.

Entomopathogenic Nematode Species Sources:

The EPN, *Heterorhabditis bacteriophora* Pionar (Hb88 strain) and *Steinernema glaseri* (NJ strain) were obtained from Randy Gaugler, Rutgers University, New Brunswick, NJ, USA. *Heterorhabditis bacteriophora*, isolated by Poinar (1975) and *S. glaseri*, isolated by Stuart and Gaugler, (1994). Both nematode species and strains were reared *in vivo* on the full-grown larvae of the greater wax moth, *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidadae), following the methods of Dutky *et al.* (1964).

Laboratory Bioassays:

A. First Method:

The efficacy of the EPNs, *S. glaseri,* and *H. bacteriophora* was tested against the third larval instar of *T. baal* (Vashisth *et al.*, 2018; Kajuga *et al.*, 2018). Five larvae of *T. baal* were placed in plastic cups (15-9-7cm) half-filled with sterile sandy soil at a depth of 1 cm from the surface with the roots of the strawberry plant for their feeding. The larvae of *T. baal* have been infested with the tested EPNs, *S. glaseri*, and *H. bacteriophora*. Plastic cups were covered with plastic lids. The nematode suspension was poured into the vials in 2 ml of water and mixed with the sterile sandy soil at five different concentrations: 250, 500, 1000, 2000, and 4000 IJs/larva. For each concentration / 5 larvae / 5 replicates were conducted. In control, therapy was performed utilizing distilled water. The LC_{50} for each species of EPN was estimated.

B. Second Method:

One 3rd larval instar of *T. baal* was placed in a plastic cup (28 cm^2) on filter paper individually following the method by Vashisth *et al.* (2018) with the roots of the strawberry plant for their feeding. The nematode concentrations in vials were put into 1 ml of water and at five different concentrations: 250, 500, 1000, 2000, and 4000 IJs/larva (25 replicates/concentration). In control, therapy was performed utilizing distilled water.

C. **Combination:**

The efficacy of the combination of the two EPNs was tested by treating cups with a 1:2 ratio of *S. glaseri+H. bacteriophora*, at concentrations of 500+1000 IJs/larva, respectively. The combined impact of nematodes, at one concentration/five larvae/five replicates, was conducted. The same experiment was carried out using one concentration/larva/25 replicates. The control was utilizing distilled water.

In all laboratory experiments, cadavers of larvae were placed individually onto slightly moist filter paper in white traps for another four days. Then, cadavers were dissected under a stereomicroscope to confirm nematode infection and the presence of adults or juveniles of EPNs following Kajuga *et al.* (2018). The mortality percentage of larvae was recorded after one week of treatment. The LC50 of EPNs was measured. All laboratory experiments were carried out at 25°C ± 2 and 55-60% RH. The water content of the soil was always kept constant at 20%. For all experiences, just distilled water was used for the control treatment.

Field Experiments:

A field experiment was conducted on a strawberry farm that was naturally infested with *T. baal*, in the Al-Mansourieh district, Giza Governorate, Egypt, from 1 January to 30 April (2020 and 2021). The soil was sandy and fertilized with organic fertilizer. The strawberry (*Fragaria ananassa* Duchesne) (cv. Festival) seedlings were transplanted onto plots on the 15th and 25th of September in 2019-2020. Each plot (25m in length, 100 cm in width and 50 cm in height) had2 longitudinal tubes to irrigate four strawberry plant rows. According to Koppenhofer and Fuzy (2008), two weeks before the experiment began; natural EPN populations were detected by collecting soil samples and baited with greater wax moth larvae. In soil samples, non-significant natural populations of EPNs were found. In order to make sure that the soil is free from the toxic effects harmful to entomopathogenic nematodes.

Treatments:

The experiment tested the efficacy of the combination of the EPNs (*S. glaseri+H. bacteriophora*) and was compared with an organophosphorus insecticide (Diazinon) against the larvae of *T. baal*. Treatments were arranged in randomized complete block designs with 2 treatments and 14 replicates (470 plants/replicate). The combination of EPNs, *S. glaseri,* and *H. bacteriophora*was applied in aqueous suspension at a 1:2 ratio and

a rate of ($2.5x10^8$ IJs/ha and $5x10^9$ IJs/ha), respectively. The organophosphorus insecticide (Diazinon 60% EC) was applied at 150ml/100L. The control used water only. The net drip irrigation system is employed to apply both organophosphorus insecticide and EPNs to the soil surface. To avoid the effect of the sun's heat on the nematode, the application was one hour before sunset (4:00 PM before dusk, the sunset was around 5:00-5:30 PM during the application period, following the method of Georgis (1990). Both EPNs and insecticides were applied four times a year, on 27 February, March 12th, 26th, and 9 April in 2020, and on 18 February, March 4th, 18th, and 1 April in 2021.

The application's effectiveness in the field was estimated by the weekly counting of wilted plants caused by the feeding of natural infestation by the larvae of *T. baal* on their roots (Tartanus *et al.*,2016; Malusa *et al.*,2020). Moreover, 20 larvae of *T. baal* were collected 7 days after each application to estimate the mortality rate of the larvae. The larvae were collected randomly by digging carefully under the plants at the root area. Larvae were transferred to the laboratory in a 1 kg plastic jar containing sterile sandy soil and strawberry plant roots for larvae feeding. The larvae were examined daily, and dead larvae were dissected to ensure the presence of nematodes, and whether the nematode infection caused the death. The larvae's mortality percentage was estimated.

Statistical Analysis:

Mortality percentages were corrected according to Abbott's formula (Abbott, 1925). Data were analyzed using the Probit analysis (Finney, 1971) and (LC50) values for treated larvae of white grab *T. baal* were estimated. Field data were statistically described using mean ± standard deviation for quantitative variables. One-way analysis of variance (ANOVA) was used to compare the statistical differences between the groups under examination, and then a Duncan post hoc test was used to confirm the results. At a p-value of 0.05, the difference was deemed statistically significant. All statistical computations were performed using SPSS software, version 16.

RESULTS

Laboratory Bioassays:

A. First Method:

After one week of treatment, the mortality percentage of *T. baal* larvae indicated that the EPN *S. glaseri* was more effective than *H. bacteriophora,* with mortality percentages of 96 and 88% at a concentration of 4000 IJs/larva. The LC_{50} values were 937.4373 and 1026.5813 IJs/larvae when using *S. glaseri* and *H. bacteriophora,* respectively **(**Table 1).

B. Second Method:

After treatment with the EPNs, *S. glaseri,* and *H. bacteriophora* at a concentration of 500 IJs/larva, the mortality percentage was 20 and 16%, respectively. The mortality percentage of the third larval instar of *T. baal* was 100% after treatment with *S. glaseri* at a concentration of 4000 IJs/ larva **(**Table 1**)**. The LC50 values were 682.2645 and 844.3445 IJs/larvae by *S. glaseri* and *H. bacteriophora*, respectively.

C.Combination:

In this experiment, the combination of the EPNs showed an increase in mortality percentage compared to using each species individually. The mortality percentage was 48% at 1000 IJs/larva concentration after the larvae were treated with *H. bacteriophora,* while the mortality percentage was 28% at 500 IJs/larva treated with *S. glaseri* (Table 1). In contrast, the mortality percentage increased to 96% after treatment at a concentration of 500+1000 IJs/larva when used five larvae/cup of the third instar with a combination of *S. glaseri* and *H. bacteriophora,* respectively.

The mortality percentage was 52% at a concentration of 1000 IJs/larva after treating larvae individually with *H. bacteriophora*, while the mortality percentage was 40% at a concentration of 500 IJs/larva after treating larvae individually with *S. glaseri* (Table 1). The mortality percentage increased to 100% after treatment at a concentration of 500+1000 IJs/larva when using larva/cup of the third instar with a combination of *S. glaseri* and *H. bacteriophora*, respectively.

Table 1: The average mortality percentage of the third larval instar of *Temnorhynchus baal* after one week of treatment with *Steinernema glaseri* and *Heterorhabditis bacteriophora* separately.

Concentration IJs/larva	% Mortality of *Temnorhynchus baal* larvae			
	S. glaseri		*H. bacteriophora*	
	One larva/cup	5 larvae/cup	One larva/cup	5 larvae/cup
250	20	12	16	12
500	40	28	36	32
1000	64	56	52	48
2000	80	72	76	68
4000	100	96	100	88
Average	60.8	52.8	56	49.6
Slope	1.8831	1.9355	1.7583	1.5526
LC_{50}	682.2645	937.4373	844.3445	1026.5813
LC_{90}	3269.934	4306.3114	4522.7271	6867.8757

Field Experiments:

According to the laboratory study, combining EPNs, *S. glaseri+H. bacteriophora* caused the highest mortality percentage for white grub larvae; EPNs were 96% when treated with five larvae/cup and 100% when treated with one larva/cup. Thus, the combination of (*S. glaseri+ H. bacteriophora*) will utilize in the strawberries farm. In the two experiment years, applying a combination of EPNs, (*S. glaseri+H. bacteriophora*) successfully reduced the percentage of wilted plants due to being infested by white grubs *T. baal*. In 2020, the mean number of wilted plants before applying the combination of EPNs and insecticides was 12.9±2.62, 17.9±3.83, and 13.9±2.96 wilted plants in the plots treated with a combination of EPNs, insecticides, and control, respectively. The mean number of wilted plants increased gradually in the plots treated with insecticides and control, while it decreased in the plots treated with a combination of EPNs (Fig. 1 a). After the fourth application, no wilted plants were observed in the plots treated with a combination of EPNs, while in insecticides and control, reaching 36.92±8.73 and 57.92±15.52 wilted plants, respectively (Fig.1 a). A significant difference among the treatments was recorded ($F = 11.1$; $df = 2$; p-value =0.00021 at $P < 0.05$). There was a statistically significant difference between the combination of EPNs treatment and the insecticides (P=0.00699). There was a statistically significant difference between the combination of EPNs treatment and control (P=0.00019). There was no statistically significant difference between the insecticide treatment and the control (P=0.40086). In 2021, the mean number of wilted plants before applying the combination of EPNs and insecticides was 6±1.47, 6.21±1.32, and 7.9±1.31 wilted plants in the plots treated with a combination of EPNs, insecticides, and control, respectively. The mean number of wilted plants increased gradually in the plots treated with insecticides and control, while it decreased in the plots treated with a combination of EPNs (Fig. 1 b). After the fourth

application, no wilted plants were observed in the plots treated with a combination of EPNs, but in insecticides and control, reaching 47.14 and 62.92 wilted plants, respectively (Fig.1 b). A significant difference among the treatments was recorded (F = 11.57; df = 2; p-value =0.000113 at P < 0.05). There was a statistically significant difference between the combination of EPNs treatment and the insecticides (P=0.00082). There was a statistically significant difference between the combination of EPNs treatment and control (P=0.0003). There was no statistically significant difference between the insecticide treatment and the control (P=0.9388). At the end of the seasons, the percentage of all wilted plants throughout the season was 17.64, 66.09, and 83.95% in 2020, and 12.56, 67.87, and 75.62% in 2021, in the plots treated with a combination of EPNs, insecticides, and control, respectively.

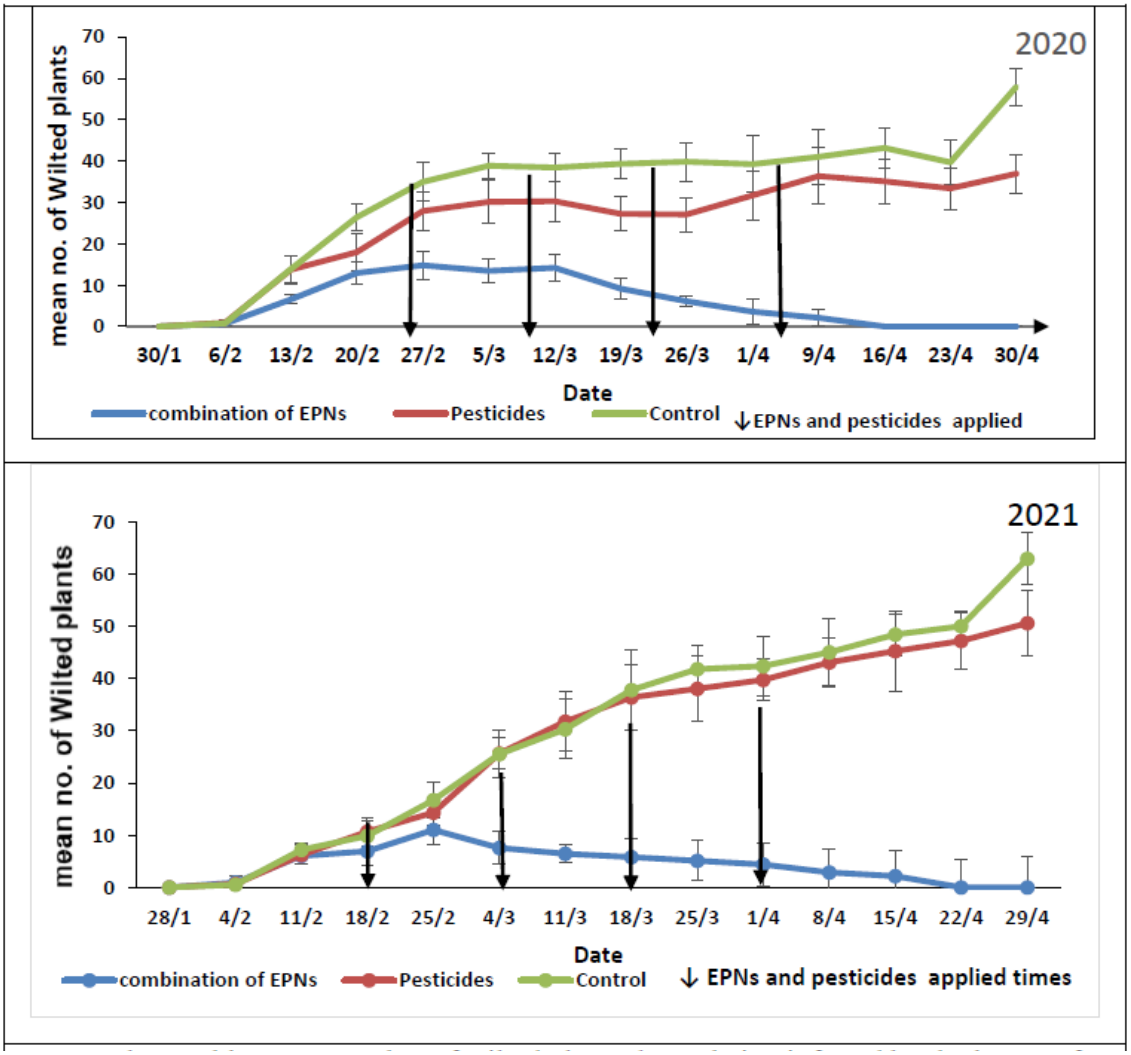

Fig. 1: The weekly mean number of wilted plants due to being infested by the larvae of *T. baal* and the application time of a combination of EPNs and insecticides in strawberry fields a) 2020 and b) 2021.

In addition, in 2020 and 2021, after all, four applications, the mean number of wilted plants in the plots treated with the combination of EPNs was much lower when compared to insecticides and control (Fig. 2 aand b).

In 2020, a significant difference among the treatments was recorded (F = 100.74; df = 2; p-value <0.0001 at P < 0.05). There was a statistically significant difference between the combination of EPNs treatment and the insecticides (P=0.000). There was a statistically

significant difference between the combination of EPNs treatment and control (P=0.000). Also, there was a statistically significant difference between the insecticide treatment and the control (P=0.01282). Also, in 2021, a significant difference among the treatments was recorded (F = 21.66; df = 2; p-value =0.000104 at P < 0.05). There was a statistically significant difference between the combination of EPNs treatment and the insecticides (P=0.00074). There was a statistically significant difference between the combination of EPNs treatment and control (P=0.00013). There was no statistically significant difference between the insecticide treatment and the control (P=0.5329). In 2020, after the first application of the combination of EPNs, the percentage of mortality of collected larvae was 55%. It increased to 80% after the second application and reached 100% after the third and fourth applications. After the application of insecticides, the collected larvae mortality percentage was 25, 30, and 40, 40 % after the first, second, third, and fourth applications, respectively. In 2021, the collected larvae mortality percentage was 85, 90, and 100% after the first, second, third, and fourth applications. After the application of insecticides, the collected larvae mortality percentage was 10, 15, and 20, 40 % after the first, second, third, and fourth applications, respectively. The collected larvae mortality percentage was zero in the control in the two studied seasons (all the larvae were alive). The percentage of 100% mortality in larvae means that the combination of EPNs succeeds in controlling the white grub, *T. baal*, in the strawberry field. There was no significant difference between the two years at p < .05.

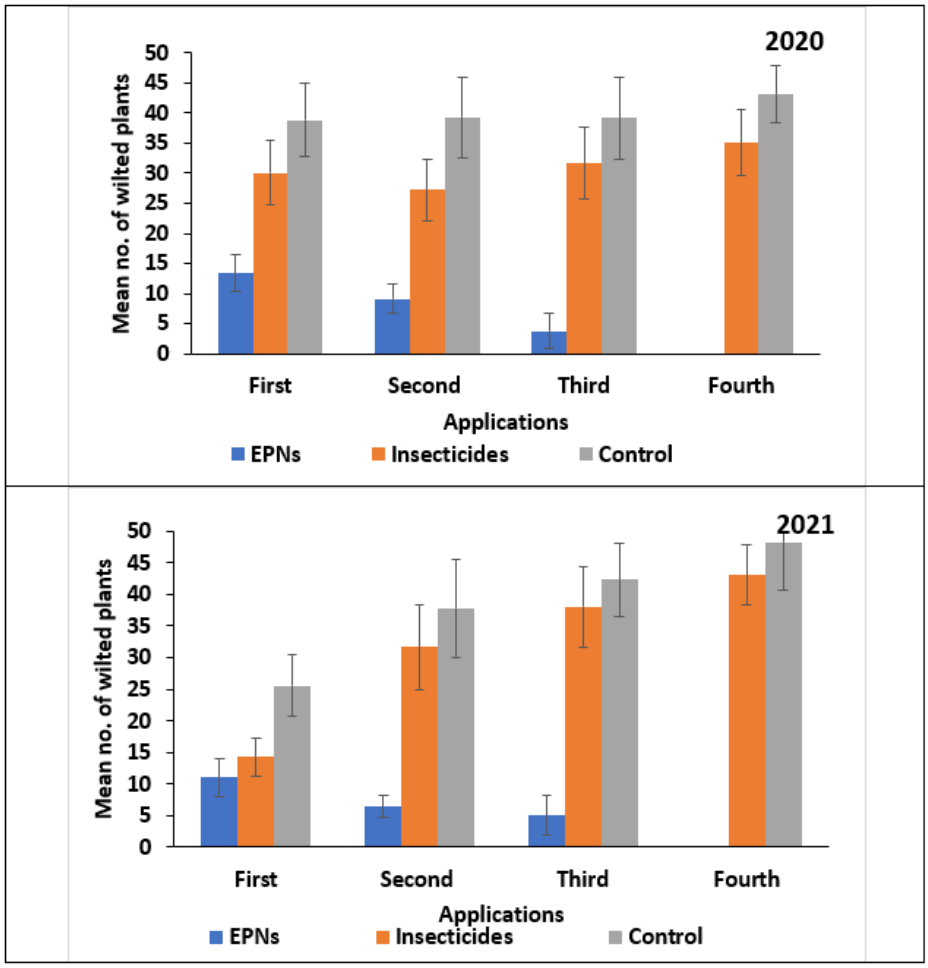

Fig. 2: Mean ± SE number of wilted plants, after first, second, third and fourth applications of a combination of EPNs and insecticides for controlling larvae of the white grub, *T. baal* in strawberry fields in (a2020 and b 2021).

DISCUSSION

Laboratory Experiments:

In this study, the third larval instar of *T. baal* was more susceptible to *S. glaseri,* than the *H. bacteriophora*. Moreover, the highest mortality rate of the third larval instar of *T. Baal* larvae was 96% when infected with the combination of the EPNs. Several studies agree with these results.

Atwa **(**2009), studied laboratory bioassay against *T. baal* using *S. glaseri* (NJ strain) (Steiner). The mortality rate was 100, 100, 94, and 96% for the first, second, and third larval instar and adult stage, respectively, of the white grub, *T. baal*, after three days of treatment with *S. glaseri.* The mortality rate was 68, 62, 60, and 58% for first, second, and third larval instars and adult stages, respectively, after treatment with *H. bacteriophora* (HP 88) (Poinar) strains. Atwa and Hassan (2014) reported that *S. glaseri* was more effective against the third larval instar of *T. baal* than *H. bacteriophora*.

In this study, the mortality rate caused by *S. glaseri* was 56%, which was higher than the 48% caused by *H. bacteriophora* at a concentration of 1000 IJs/larvae. These results agree with those of Kajuga *et al.* (2018), who concluded that white grubs are causing large problems in many crops in Africa. Therefore, EPNs are used to control white grubs. The laboratory bioassays revealed that all EPNs could infect white grubs (Coleoptera: Scarabaeidae), but comparatively high concentrations of infective juveniles were needed. At a concentration of 1000 infective juveniles/larva, the Rwandan nematodes species of *H. bacteriophora* and *S. carpocapsae* caused a mortality rate in white grubs of 34 -58%.

Shamseldean and Atwa (2004) reported that *S. glaseri* caused a 95% mortality of the third larval instar of *T. baal*, after 72h treatment with infective nematode juveniles. In contrast, *Steinernema* sp. (EGG4), *Steinernema* sp. (EBNE), Egyptian isolate of *S. kushidai* (EBN32), Egyptian isolate of *S. carpocapsae* (EGBX), and Egyptian isolate of *H. bacteriophora* (EKB20) caused 50, 50, 52, 28 and 28% insect mortality when applied on the third larval instar, respectively.

Field Experiments:

In this study, the application of the combination of EPNs *S. glaseri* and *H. bacteriophora*, succeeded in reducing the percentage of wilted plants compared to the application of the organophosphorus insecticide (Diazinon) when controlling the white grub, *T. baal* in strawberry fields.

The results showed that the EPNs were more efficient to control of the larvae the white grub, *T. baal*, than the organophosphorus insecticide (Diazinon). This disagrees with Koppenhofer *et al.* (2000), who used *H. bacteriophora* and *S. glaseri* Steiner (Rhabditida: Steinernematidae) strains as control agents against white grub, *Popillia japonica* Newman (Coleoptera: Scarabaeidae: Rutelinae) and showed that the third larval instar of white grub populations was killed at levels like that of an organophosphate insecticide.

The results indicated that repeated applications of the EPNs combination could help keep the white grub and *T. baal* populations low. This agrees with Lola-Luz *et al.* (2005), who reported that three applications of EPN reduced the number of live black vine weevil larvae compared to the control growing bags.

In the field, a combination of both nematode species (*S. glaseri* and *H. bacteriophora* at a 1:2 ratio) was applied according to the in-vivo results, which showed that *S. glaseri* was more effective than *H. bacteriophora* against the third larval instar of *T. baal*. Atwa (2009) reported that *S. glaseri* was more effective when applied on the soil surface than *H. bacteriophora* in both methods of application, inundative and inoculative release. The inoculative release was more effective than the inundative release for controlling *T. baal* in strawberry fields in Egypt. Moreover, the application of *S. glaseri* in

strawberry fields against scarab beetles *T. baal* was applied by Shamseldean and Atwa (2004) and recorded a percentage of population reduction of up to 96.8 and 99.1% after four and eight field applications, respectively.

Furthermore, Yadava and Sharma (1995) have reported that several microorganisms such as *Paenibacillus* (*Bacillus*) *popilliae* (Dutky), *M. anisopliae* (Metchnikoff), Sorokin, *Beauveria bassiana* (Bals.) Vuill, *B. brongniartii* (Saccardo), *H. bacteriophora* Poinar, *S. glaseri* (Steiner) and *S. feltiae* (Filipjev) are pathogenic to white grubs and are effective in suppressing their population under field conditions. In Germany, epizootics have been observed in grub populations infested with *Heterorhabditis*sp., achieving 71% control in a sugarcane field (Akhurst *et al.*, 1992).

A promising future for nematodes in white grub management may lie in developing alternative approaches to their use as bio-pesticides (Ravinder *et al.*, 2019).

Conclusions:

In the laboratory study, the third larval instar of the white grub, *T. baal*, was more susceptible to the EPN, *S. glaseri*, than the *H. bacteriophora*. The third larval instar of *T. baal* larvae had the highest mortality rate when infected with a combination of *S. glaseri* and *H. bacteriophora*. In the field study, applying the combination of the EPNs, *S. glaseri*, and *H. bacteriophora* resulted in a lower percentage of wilted plants than applying an organophosphorus insecticide (Diazinon) when controlling white grub *T. baal* in a strawberry field. Entomopathogenic nematodes as biological control agents are becoming an important component in integrated pest management programs against *T. baal* in strawberry fields.

Abbreviations

(EPNs): Entomopathogenic nematodes; (IJs): Infective juveniles

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