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Evaluation of the effects of autochthonous and commercial isolates of Steinernematidae and Heterorhabditidae on *Rhynchophorus ferrugineus*

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Abstract

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier), is a highly prevalent pest worldwide damaging more than 18 different species of palm trees. Developed in Italy in 2004, RPW has begun attacking ornamental palm trees belonging to the species *Phoenix canariensis* (Chabaud) and *Phoenix dactylifera* L., causing serious damage in numerous regions of Italy. Because of the restrictions on pesticide use, numerous alternatives are being investigated employing products such as entomopathogenic nematodes (EPNs). Four *Heterorhabditis* and seven *Steinernema* species and isolates belonging to the collection of EPNs at the section of Entomology and Zoology of the DiBCA, University of Bari, Italy, were assayed for their pathogenicity against the larvae and adults of *R. ferrugineus* and compared with the commercial products NEMATOP [*Heterorhabditis bacteriophora* Poinar] and NEMASTAR [*Steinernema carpocapsae* (Weiser)]. After ten days of testing, the EPNs that yielded the highest larval mortality were *H. bacteriophora* ALG12, CS17 and C3, NEMATOP (93-100%), *Steinernema longicaudum* Shen et Wang (100%), *Steinernema glaseri* (Steiner) (100%), *S. carpocapsae* NEMASTAR (100%) and *Steinernema kraussei* (Steiner) 3D (100%). Compared to the adults, *H. bacteriophora* C3 (100%) and CS17 (80%), *S. longicaudum* (96%), and *S. carpocapsae* MR7 (80%) resulted as being the most effective EPNs. Concerning the ability for *Steinernema* and *Heterorhabditis* species to reproduce in *R. ferrugineus*, one may conclude that commercial product derived from *S. carpocapsae*, as well as the autochthonous isolate, did not produce nematode offspring in the adult weevil and its larvae. On the contrary, *H. bacteriophora* produced adults and new generations but only in *Rhynchophorus* adults. However, *S. glaseri* yielded a slower reproduction rate in both the larvae and adults of *Rhynchophorus*. The same result was obtained for *S. affine* (Bovien), which in some cases was able to produce new generations in adult insects.

Key words: biological control, entomopathogenic nematodes, nematode reproduction.

Introduction

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Curculionoidea Dryophthoridae) is a devastating insect for palm trees. RPW was first detected on *Cocos nucifera* L. in South Asia and is now present worldwide, damaging more than 18 species of palm trees besides *Agave americana* L. and *Saccharum officinarum* L. (RPW, 2006; OEPP/EPPO, 2008). With time this pest has spread to infest *Phoenix dactylifera* L. in many regions of the Middle East, Asia, Egypt (Faleiro, 2006), Curasao Island and South California. Eventually it has spread to Europe through the commercialization of infested palms that were introduced to these continents. RPW is considered the most damaging pest for palm trees in the Mediterranean basin, especially for *Phoenix canariensis* (Chabaud) (OEPP/EPPO, 2008). Damage was first reported in Italy in 2004, since then it has begun attacking palm trees belonging to *P. canariensis* and *P. dactylifera*, causing serious damage in numerous regions of Italy. RPW is in the A2 list of the European and Mediterranean Plant Protection Organization (EPPO) of quarantine pests.

Healthy as well as damaged palm trees can be attacked by the adults of RPW (Murphy and Briscoe, 1999). This insect is difficult to control because of its peculiar biological life cycle and behaviour. Therefore, pesticide use to prevent attacks on both uninfested as well as infested trees must be repeated within the respective growing season (Ferry and Gomez, 2002); however, some specific products (pesticides) may cause environmental pollution. The difficulty in controlling the RPW is also due to inappropriate techniques unable to control insects living in cryptic environments (Deseö, 1982; Triggiani 1983; Georgis and Manweiler, 1994; Triggiani and Tarasco 2002; Curto *et al.*, 2003; Tarasco and Triggiani 2006; Nardi *et al.*, 2009). Furthermore, the restriction on the use of many pesticides places emphasis on biological control with alternative products such as entomopathogenic nematodes (EPNs). These organisms penetrate the bodies of insects through natural ways (mouth, anus and spiracles) or through the cuticle. Once within the host's cavity, EPNs find the hemolymph where they release their symbiotic bacteria, the species of that are linked to the nematode species (Tailliez *et al.*, 2006). These bacteria transform the host tissue into food suitable for nematodes to breed several generations in the host. When the food source is depleted, the new dauer juveniles (DJs) leave the victim to go and find another host to begin a new cycle.

This investigation is based on the experiments of late-instar larvae and adults of *R. ferrugineus* infected with strains and species of the two genera *Heterorhabditis* Poinar and *Steinernema* Travassos. The aim of the study was to determine which *R. ferrugineus* stages are sensitive or resistant to EPN + bacteria complexes, which species or strain of nematodes is the most suitable to further control strategies, and to determine the ability of the tested EPN to produce nematode offspring (DJs) emerging from *R. ferrugineus* cadavers.

Materials and methods

In the laboratory, adult pairs of *R. ferrugineus* were allowed to lay eggs on banana and apple. The eggs were then transferred with a brush onto small spheres of sodium polyacrylate, which had been hydrated in distilled water for 24 hours and sterilized in autoclave. The spheres were set in Petri dishes and completely covered the bottom so as not to roll during manipulation.

The young larvae were transferred daily onto slices of pumpkin or apple to feed and develop. An additional breeding method was implemented whereby adult insect pairs were liberated into wooden cages with thick plastic net as walls, each one containing a palm plant of about 2 m high. After the death of the plant the larvae were withdrawn and given apples as food to allow them to reach the last stage of development and be used for testing. Other material was collected from RPW infested plants which had just been cut down.

Four of the nematodes used in the test belonged to the *Heterorhabditis* species and seven to the *Steinernema* species and two strains from the collection of EPNs at the section of Entomology and Zoology of the DiBCA, University of Bari, Italy. The EPNs selected were those with, preferably, different species of symbiotic bacteria (Tailliez *et al.*, 2006) (table 1). All EPNs were assayed for pathogenicity against the larvae and adults of *R. ferrugineus* and compared with commercial products – NEMATOP [*Heterorhabditis bacteriophora* Poinar] and NEMASTAR [*Steinernema carpocapsae* (Weiser)] – supplied by E-nema (Germany). All the EPNs of the collection were reproduced on larvae of late-stage *Galleria mellonella* (L.); early-stage DJ nematodes were taken from water traps, as described by White (1927), and stored for a maximum of 10 days at 10 °C before use. Ten Petri dishes of 9 × 2 cm each were filled with 10 g of sieving peat and sterilized in autoclave. Sterilized tap water was added to the peat in a 1:1 proportion (10 g peat per 10 g water). Three hundred DJs in 0.5 ml sterilized tap water were distributed on the surface of the peat layer in each Petri dish; after 12 hours, one larva of *R. ferrugineus* in the last stage was added. The control received only 0.5 ml of water. A small piece of apple, as food for the larvae or the adults, was placed in each Petri dish and replaced every second day. Each treatment was replicated three times with 10 larvae or 10 adults. The Petri dishes were placed in groups of 10 in a plastic bag with a wet wad and incubated at 25 ± 2 °C in the dark and 16:8 photoperiod. The same tests were repeated with the adults of *R. ferrugineus* excluding *Heterorhabditis megidis* Poinar, Jackson et Klein.

Mortality was recorded every 2 d for 10 d, and half of the dead samples were placed on modified White traps to recover DJs emerging from cadavers. The second half of the dead samples was dissected to assess the presence of EPNs. All the species and strains of *Heterorhabditis* and *Steinernema* were tested in drops of *Rinchophorus* hemolymph to observe their growth (Poinar, 1975). Before being assessed in water traps, the weevil samples were disinfested on the surface with 1% sodium hypochlorite for a few seconds, washed three times with sterile water and left to dry on sterile filter paper in sterile Petri dishes.

Statistical Analysis

The mortality data of adults and larvae were assessed using analysis of variance (ANOVA), and Tukey's (HSD) test was used to compare means. Before conducting ANOVA, all percentages were transformed using the arcsine square root transformation. The cumulative mortality response across the assessment period was analyzed by means of Kaplan-Meier survival analysis. All data were processed utilizing Statistic 9.0. A p value of 0.05 was used in all analyses.

Results and discussion

Larvae mortality

The data from the laboratory test (figures 1 and 2) and survival (tables 2 and 3) demonstrated variable susceptibility of *R. ferrugineus* larvae and adults toward EPNs, with higher percentages of mortality and, on average, lower survival rates of the larvae. After 10 d, the EPNs

Table 1. EPNs + their symbiotic bacteria tested in the experiments.

Nematode	Bacterium
Heterorhabditis bacteriophora *ItH-C3	*Photorhabdus luminescens* subsp. *laumondii*
Heterorhabditis bacteriophora ItH-CS17	*Photorhabdus luminescens*
Heterorhabditis megidis NW European type	*Photorhabdus luminescens*
Heterorhabditis bacteriophora **ALG 12	*Photorhabdus luminescens*
Steinernema carpocapsae ItS-MR7	*Xenorhabdus nematophila*
Steinernema feltiae ItS-CO1	*Xenorhabdus bovieni*
Steinernema glaseri USA	*Xenorhabdus poinarii*
Steinernema affine ItS-FO1	*Xenorhabdus bovieni*
Steinernema feltiae ItS-2D	*Xenorhabdus bovieni*
Steinernema longicaudum USA	*Xenorhabdus ehlersii*
Steinernema apuliae ItS-C5	*Xenorhabdus kozodoii*
Heterorhabditis bacteriophora NEMATOP	*Photorhabdus luminescens*
Steinernema carpocapsae NEMASTAR	*Xenorhabdus nematophila*

*The acronyms ItH and ItS stand for *Heterorhabditis* and *Steinernema* collected in Italy.
**EPN collected in Algeria at Tamanrasset oasis, southern part of the Sahara desert.

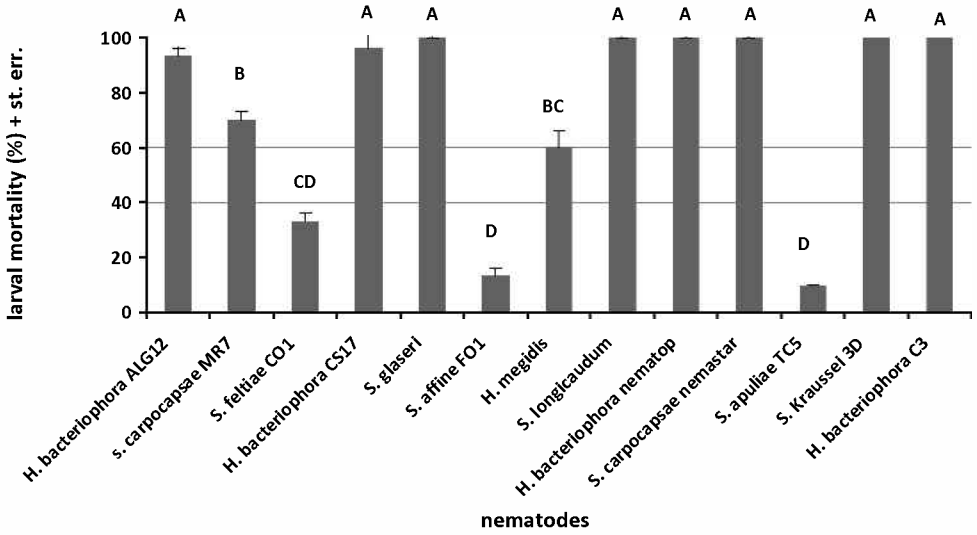

Figure 1. Mortality of last instar larvae of *R. ferrugineus* 10 days after the treatment with 13 species and strains of EPNs. Columns marked with the same letter are not statistically different at $p < 0.05$, according to the Turkey's HSD test.

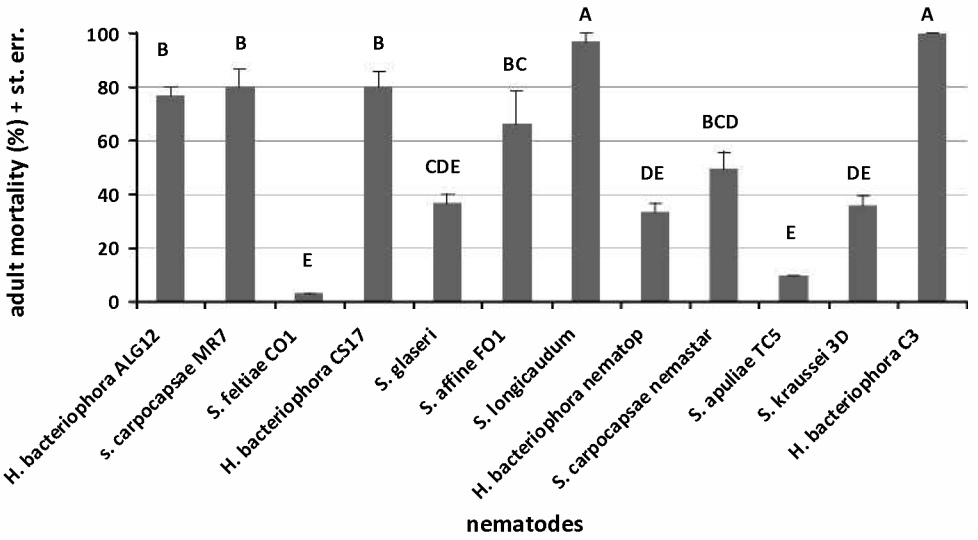

Figure 2. Mortality of adults of *R. ferrugineus* 10 days after the treatment with 12 species and strains of EPNs. Columns marked with the same letter are not statistically different at $p < 0.05$, according to the Turkey's HSD test.

Table 2. Average survival time (AST) (Kaplan-Meier) of last instar of *R. ferrugineus* larvae exposed to 13 EPNs for 10 days. Means with the same letter are not significantly different ($p < 0.05$) according to the Long-rank test.

EPNs / *R. ferrugineus* larvae	[a]AST d + SE	Confidence interval	
H. bacteriophora **ALG12	4.667 ± 0.288	4.102 - 5.231	B
S. carpocapsae ItS-MR7	6.933 ± 0.429	6.092 - 7.775	D
S. feltiae * ItS-CO1	9.200 ± 0.274	8.663 - 9.737	E
H. bacteriophora ItS-CS17	5.067 ± 0.245	4.587 - 5.546	BC
S. glaseri USA	5.067 ± 0.185	4.704 - 5.430	BC
S. affine ItS-FO1	9.467 ± 0.248	8.980 - 9.953	EF
H. megidis NW European type	9.733 ± 0.187	9.366 - 10.101	E
S. longicaudum USA	5.200 ± 0.182	4.843 - 5.557	C
H. bacteriophora NEMATOP	4.267 ± 0.230	3.817 - 4.717	AB
S. carpocapsae NEMASTAR	3.867 ± 0.093	3.685 - 4.048	A
S. apuliae ItSC5	9.600 ± 0.219	9.171 - 10.029	F
S. kraussei ItS3D	5.030 ± 0.192	4.633 - 5.358	B
H. bacteriophora ItHC3	4.5 ± 0.880	4.102 - 4.842	AB

*The acronyms headed by ItH and ItS means: *Heterorhabditis* and *Steinernema* collected in Italy.
**EPN collected in Algeria at Tamanrasset oasis, southern part of Sahara desert.

Table 3. Average survival time (AST) (Kaplan-Meier) of adults of *R. ferrugineus* adults exposed to 12 EPNs for 10 days. Means with the same letter are not significantly different ($p < 0.05$) according to the Long-rank test.

EPNs/*R. ferrugineus* adults	[a]AST d + SE	Confidence interval	
H. bacteriophora ALG12	6.933 ± 0.309	6.328 - 7.539	B
S. carpocapsae ItSMR7	5.533 ± 0.429	4.693 - 6.374	A
S. feltiae ItSCO1	9.933 ± 0.066	9.805 - 10.062	D
H. bacteriophora ItHCS17	7.667 ± 0.268	7.141 - 8.192	B
S. glaseri USA	8.800 ± 0.378	8.059 - 9.541	C
S. affine ItSFO1	6.933 ± 0,419	6.112 - 7.754	B
S. longicaudum USA	5.867 ± 0.187	5.500 - 6.233	A
H. bacteriophora NEMATOP	7.733 ± 0.594	6.569 - 8.898	BC
S. carpocapsae NEMASTAR	6.533 ± 0.653	5.254 - 7.813	ABC
S. apuliae ItSTC5	9.800 ± 0.110	9.585 - 10.015	D
S. kraussei ItS3D	7.533 ± 0.394	6.544 - 8.398	BC
H. bacteriophora ItHC3	5.366 ± 0.287	4.826 - 6.133	A

that yielded the greatest efficacy were: all *H. bacteriophora* strains killing 93-100% of the larvae; the American strains of *Steinernema glaseri* (Steiner) and *Steinernema longicaudum* Shen et Wang; *S. carpocapsae* NEMASTAR and *Steinernema kraussei* (Steiner) controlling 100% of RPW larvae (figure 1). The same species recorded inferior survival rates of the larvae (table 2); *S. carpocapsae* NEMASTAR provided the best results (AST = 3.867), followed by *H. bacteriophora* NEMATOP (AST = 4.267) and *H. bacteriophora* ItHC3 (AST = 4.5). Whereas, the high susceptibility of the weevil larvae to *S. carpocapsae* was verified in our test and confirmed by the trials of Gomez Vives *et al.* (2008).

These data seem to be in contrast with what has been asserted by Shamseldean (2000), where the higher concentration tested (500 infective juveniles IJs/larva) *S. carpocapsae* controlled only 40% of late-stage RPW larvae. The same author (Shamseldean *et al.*, 1994) also stated that six isolates of *Heterorhabditis* collected from different places in Egypt infected the larvae of *R. ferrugineus* to a high degree and were more effective against the larvae than to the pupae and adults of the insect. Shamseldean maintains that the nematode isolates could infect and reproduce on both *G. mellonella* and *R. ferrugineus.* The high sensibility of *R. ferrugineus* toward *H. bacteriophora* noted in our laboratory test was also confirmed by Atakan *et al.* (1979), who reported that in Turkey, where trunks had been cut down, a natural infestation of *H. bacteriophora* killed 69% of *R. ferrugineus* larvae. Our laboratory tests demonstrated that *H. megidis* controlled at least 60% of *R. ferrugineus* larvae, whereas Martin-Molina reported 100% larval mortality (Martin-Molina *et al.*, 2001). Among the EPNs tested by us, the poorest results were recorded by *Steinernema apuliae* Triggiani, Mracek et Reid (10%), *Steinernema affine* (Bovien) (13%) and *S. feltiae* (33%). This low incidence of *Steinernema feltiae* (Filipjev) mortality on the larvae of *R. ferrugineus* in laboratory testing was also pointed out by Abbas *et al.* (1991).

Adult mortality

Adult mortality of RPW, due to the *H. bacteriophora* ItHC3 strain was demonstrated for all adults of the *Rhynchophorus* species (AST = 5.366) (figure 2, table 3), whereas the commercial NEMATOP was less effective (less than 40% adults mortality). *S. longicaudum* confirmed its own excellent activity against the adult of *Rhynchophorus* (96% mortality, AST = 5.867). Of the *S. carpocapsae* strains, the commercial one (NEMASTAR) (very effective against the larvae of the RPW) was not so efficient as the Italian strain (ItS-MR7, collected in Italy) in limiting the vitality of the adult insect. Although *S. kraussei* yielded promising results against RPW larvae (100% larval mortality) even if with slower activity, it failed to control adult insects effectively. The excellent activity of *S. glaseri*, though slower than that of *S. carpocapsae* and *H. bacteriophora*, against the larvae of *Rhynchophorus* was not so effective against the adult RPW; similarly, *S. glaseri* exhibited scarce effectiveness against the adult insect. According to Shamseldean (2000), the adults of *R. ferrugineus* demonstrate higher sensitivity to the Egyptian isolate of *H. bacteriophora* (local isolate-EBR 30), whereas *S. carpocapsae* (European isolate) would yield the lowest, even at elevated concentrations.

Poor capacity to kill the adults of RPW, as well as for the larvae, was highlighted by *S. glaseri* (35%), *S. feltiae* (3%) and *S. apuliae* (10%) with higher survival time of the adults (figure 2, table 3).

The excellent activity of *S. glaseri*, though slower than that of *S. carpocapsae* and *H. bacteriophora*, against the larvae of *Rhynchophorus* was not so effective against the adult RPW; similarly, *S. glaseri* exhibited scarce effectiveness against the adult insect.

Reproduction of EPNs in *R. ferrugineus*

Based on our laboratory tests, NEMASTAR, the commercial *S. carpocapsae*-based product, as well as the autochthonous isolate did not produce nematode offspring in the larvae and adults of RPW. In contrast, 3 d after treatment with *S. carpocapsae* in chitosan, Gómez Vives *et al.* (2008) found a very large number of EPNs in all dead adults of *R. ferrugineus.* Whereas, after 3 weeks no *S. carpocapsae* EPNs were isolated in 25% of the insects. The ability of *S. carpocapsae* to generate offspring in adult RPW is also pointed out by Saleh and Alheji (2003). Furthermore our tests showed that the larvae of *R. ferrugineus* killed by *H. bacteriophora* iso-

Figure 3. DJs of *S. glaseri* emerging from adults of *R. ferrugineus*.
(In colour at www.bulletinofinsectology.org)

lates very rarely harboured live EPNs, despite the highlighted red and green colour variations which are typical of *Photorhabdus* bacterial proliferation where tissues appear "gummy".

The difficulty of the *H. bacteriophora* species and isolates to reach adult stage in the larvae of the weevil host was tested by adding some DJs in drops of *Rhynchophorus* haemolymph, according to the technique illustrated by Poinar (1975). In contrast, *H. bacteriophora* reached the adult stage and produced new generations in *Rhynchophorus* adults. The ability of *H. bacteriophora* to reproduce in adult RPW was confirmed by Shamseldean (2000). We conclude that the majority of the species and/or isolates of *Heterorhabditis* used in the laboratory tests in Egypt were reproduced in the adult RPW, yielding a high number of DJs, whereas a lower number of DJs emerged from larvae and pupae. *S. glaseri* was the only EPN tested in our laboratory that was able to reproduce in *R. ferrugineus* larvae and adults. Despite its slow action in time tests, *S. glaseri* killed all of the larvae reproducing in *Rhynchophorus* species, but was less capable of controlling the adults it reproduced in high numbers (figure 3).

A similar observation can be made for *S. affine*, which resulted as being less effective but was able in some cases to use the tissue of the adult insects to produce new generations. In *R. ferrugineus* larvae and adults, different reactions occurred from the attack of related nematodes and bacteria; it is evident that the bacteria *Photorhabdus luminescens* and *P. luminescens* subsp. *laumondii* (*H. bacteriophora*), *Xenorhabdus poinarii* (*S. glaseri*) and *X. bovieni* (*S. affine*) provide nematodes with tissues of their victim to reproduce in adult insects. The lack of reproduction of some EPNs species in the larvae and adults of *R. ferrugineus* constitutes striking data that warrant further studies.

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