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1 Evaluation of the efficacy of *Steinernema carpocapsae* against the Red Palm Weevil,
2 *Rhynchophorus ferrugineus* in *Phoenix canariensis*.

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1 Evaluation of the efficacy of *Steinernema carpocapsae* against the Red Palm Weevil,
2 *Rhynchophorus ferrugineus* in *Phoenix canariensis*.

3

4 **Abstract**

5 The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera, Curculionidae),
6 is an important pest of palms. It has recently colonized the Mediterranean Basin where
7 it is a serious problem on ornamental *Phoenix canariensis* (Chabaud) palms. The
8 efficacy of *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae) against
9 this weevil in a semi-field trial including both preventative and curative assays has been
10 studied. Our results prove the potential of this nematode to control *R. ferrugineus*.
11 Efficacies around 80 % were obtained in the curative assay, and up to 98 % in the
12 preventative treatment. Applications repeated every 2-3 weeks during the flight critical
13 periods could prove effective to protect palms from this weevil in the Mediterranean
14 Basin.

15

16 **Keywords:** Curculionidae, Steinernematidae, Palms, EPNs

1 **Introduction**

2 The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera, Curculionidae),
3 is a phytophagous insect that feeds in soft succulent tissues of many palm species
4 (Murphy and Briscoe, 1999). Females deposit their eggs in separate holes or injuries at
5 the base of the fronds. Eggs hatch into legless larvae which bore into the interior of the
6 palms. On completion of their development, larvae move back to the base of the fronds
7 where they pupate in elongate oval, cylindrical cocoons made out of fibrous strands.

8 The red palm weevil is a concealed tissue borer and can spend all of its life stages inside
9 the palm. Adults often remain and reproduce within the same host until the apical
10 growing area of the palm has been destroyed by the larvae causing the palm to die. The
11 complete life cycle of the weevil, from egg to adult emergence, takes an average of 82
12 days (OEPP/EPPO 2008).

13 Nowadays *R. ferrugineus* is considered the main pest of palms in the Mediterranean
14 Basin (OEPP/EPPO 2008). This pest, which is native of South and Southeast Asia
15 (Faleiro 2006) was first detected in Egypt in date palms, *Phoenix dactylifera* L., in
16 1992 (Cox 1993). In Spain *R. ferrugineus* was detected in 1995 (Barranco *et al.* 1995)
17 but remained confined in a small area in Southern Spain until 2004, when the pest
18 appeared in different foci along the Spanish Mediterranean coast. In 2006 it was
19 reported in the Canary Islands and today it can be found in almost all Mediterranean
20 countries. In all these areas, *R. ferrugineus* constitutes a severe pest of the Canary palm,
21 *Phoenix canariensis* (Chabaud), which is an important ornamental plant in both public
22 and private gardens. Furthermore, the original wild populations of this species located
23 in the palm forests existing in the Canary Islands are presently at risk.

24 The methods currently used to control *R. ferrugineus* are mainly based on the
25 application of large quantities of synthetic chemical insecticides although there are

1 deep concerns about the environmental pollution caused by these treatments (Faleiro
2 2006). Insecticides are applied in a range of preventative and curative procedures
3 designed to limit and contain the spread of an infestation. These procedures have been
4 developed and refined since commencing in India in the 1970s (Murphy and Briscoe
5 1999). Methods range from general dusting of the leaf axils after pruning, or spraying of
6 the tree trunk, to localized direct injections of chemicals into the trunk (Faleiro 2006).
7 Researchers have concluded that because of the cryptic habitat of the boring stages of
8 this weevil, chemical insecticides have to be applied frequently and over a long period
9 of time for effective management of established populations (Murphy and Briscoe 1999;
10 Ferry and Gómez 2002).

11 The use of entomopathogenic nematodes (EPNs) could offer an interesting alternative to
12 the chemical control of *R. ferrugineus* (Abbas *et al.* 2001a,b; Elawad *et al.* 2007; Saleh
13 and Alheji 2003). EPNs are safe for non-target vertebrates and to the environment and
14 since they are mass produced in liquid media, production costs have been significantly
15 reduced in recent times (Ehlers 2003). Steinernematids are soil-inhabiting EPNs, and
16 have free-living, parasitic and saprophytic stages (Mráček 2003). The infective third
17 juvenile stages (Dauer Juvenile, DJ) survive outside the insect and can either actively
18 search for hosts (cruisers) or wait for host to pass by (ambushers). DJs enter the insect
19 host through any opening (mouth, anus, spiracles) and grow into the parasitic stage. The
20 death of the insect due to nematode parasitism is caused by Gram-negative bacteria
21 which are carried within the gut of the DJs (Forst and Clarke 2002). *Steinernema*
22 *carpocapsae* (Weiser) (Nematoda: Steinernematidae), which is mutualistically
23 associated with the bacterium *Xenorhabdus nematophila* (Enterobacteraceae), is the
24 most studied, available, and versatile of all EPN. This species is a typical ambusher,
25 standing on its tail in an upright position near the soil surface (nictating) and attaching

1 to passing hosts. Consequently, *S. carpocapsae* tends to be most effective when applied
2 against highly mobile surface-adapted insects (García del Pino 2006; Gaugler 2007).
3 Cryptic habitats are considered as the most favorable, enhancing the infectivity,
4 survival, and persistence of EPNs, because these environments minimize nematode
5 death from ultraviolet radiation and desiccation (Mráček 2003). Till present, field
6 experiments in these habitats have provided mostly consistent and efficient results.
7 *Steinernema carpocapsae* has proved successful in the control of geophilous insects,
8 such as *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) (Martínez de Altube *et al.*
9 2007), *Popilia japonica* Newman (Coleoptera: Scarabaeidae) (Simões *et al.* 1993),
10 *Delia radicum* (L.) (Diptera: Anthomyiidae) (Schroeder *et al.* 1996), as well as species
11 with partially hidden life cycles, such as borers, *Ostrinia nubilalis* (Hübner)
12 (Lepidoptera: Pyralidae) (Ben-Yakir *et al.* 1998), and leafminers, *Liriomyza trifolii*
13 Burgess (Diptera: Agromyzidae) (Hara *et al.* 1993). *Steinernema carpocapsae* has
14 already been used against *R. ferrugineus*. In the laboratory, results were good, but
15 inconsistent in the field when used in date palms (Abbas *et al.*, 2001b). The objective of
16 this study was to test the efficacy of *S. carpocapsae* against *R. ferrugineus* in a semi-
17 field trial including preventative and curative assays.

18

19 **Materials and methods**

20 The assays reported in this study were carried out at the Institut Valencià
21 d'Investigacions Agràries (IVIA) during the months of June, July and August 2007.
22 Trials were performed in a double mesh security enclosure containing 24 independent
23 cages (4 * 3 * 3 m) under natural light and temperature conditions. Mean temperature
24 during the assays was 28.2°C (max: 34.2°C; min: 22.3°C). A plastic roof protected the
25 enclosure from the rain.

1 **Plant material.** Trials were performed on 48 4-year old potted *P. canariensis*. The stipe
2 of these palms was 0.35 to 0.55 m high and 0.3 to 0.5 m wide. Plants were watered
3 twice a week.

4 **Experimental insects.** Adult weevils used to infest the palms in the preventative tests
5 were captured in the province of Valencia in traps baited with ferrugineol (male RPW
6 aggregation pheromone) and plant kairomones (ethyl acetate, pieces of palm leaves).
7 Before release, adults were kept for 3 d in a plastic lunchbox with a perforated lid where
8 thin apple slices were provided as food source.

9 Immature stages used in curative tests were obtained from an artificial rearing
10 maintained at IVIA. Eggs were obtained from wild specimens kept in plastic boxes as
11 above and offered thin apple slices both as food and oviposition substrate as described
12 by Martín and Cabello (2006). Eggs were subsequently transferred to an artificial
13 complex diet (Martín and Cabello 2006) until they reached the desired age.

14 **Nematode application.** The commercial product Biorend R Palmeras[®] consisting of *S.*
15 *carpocapsae* with a chitosan adjuvant was used. Chitosan is an organic biodegradable
16 product with the active ingredient N-acetyl-glucosamine. The use of nematodes with
17 chitosan is patented and has already been used in other systems (Martínez de Altube *et*
18 *al.* 2007). Product was applied with a manually operated backpack compact sprayer at a
19 dose of $1.8 * 10^6$ DJs + 18 ml chitosan per liter of water. Approximately 2 l of this
20 solution were applied on the trunk and the bases of the fronds of each palm until run-
21 off.

22 **Curative tests.** Twenty four palms were infested with nine larvae of *R. ferrugineus*
23 each. A hole 3 cm deep and 1 cm in diameter was drilled on one side of the palm.
24 Subsequently, an open vial containing the insects's artificial diet and four 7-d old larvae
25 was introduced into the hole. Two days later the vial was removed and checked under

1 microscope for evidence of larval exit. Ten days later, a new hole was drilled on the
2 other side of the palm trunk and five 15-d old larvae were similarly introduced in each
3 palm. One month after the first infestation, twelve palms were treated with *S.*
4 *carpocapsae*, and the remaining 12 were used as control. Fourteen days after the
5 treatment 4 control and 4 treated palms were carefully dissected and checked for the
6 presence of *R. ferrugineus*. The remaining 16 plants were similarly dissected two weeks
7 later. All *R. ferrugineus* specimens found, either dead or alive, were counted. To
8 ascertain whether the nematode was the cause of the death, dead immature stages were
9 observed under microscope and streaked on NBTA (Nutrient agar supplemented with
10 bromothymol blue and triphenyl-tetrazolium chloride) plates (Akhurst 1980) to verify
11 the presence of *X. nematophila* phase I which absorbs bromothymol blue, producing
12 dark blue colonies (Chavarría-Hernández *et al.* 2007).

13 **Preventative tests.** Twelve uninfested palms were treated with *S. carpocapsae* as
14 described above and 12 additional palms constituted the control treatment. Immediately
15 after the treatment, four control palms and four treated palms were infested with *R.*
16 *ferrugineus* by releasing 4 adult presumably-mated females per plant. To evaluate
17 product persistence the same procedure was repeated 15 and 30 days after the treatment.
18 One week after their release, when found, females were removed from the cage. One
19 month after the release, palms were carefully dissected and checked for the presence of
20 *R. ferrugineus* larvae. All specimens found, either dead or alive, were counted and
21 checked for presence of *X. nematophila* as above.

22 **Data analysis.** Results (percentage mortality and number of immature stages found
23 alive for the curative and preventative tests, respectively) were subjected to a two-way-
24 analysis of variance (ANOVA, $P > 0.05$). The two factors were time and treatment. The
25 efficacy of treatments was evaluated according to Abbott (1925).

1

2 **Results**

3 Both the treatment and the time until palm dissection significantly affected the mortality
4 caused by *S. carpocapsae* in *R. ferrugineus* when used as a curative treatment (Table 1).

5 The longer the time elapsed since EPN application, the higher the mortality observed
6 and hence the efficacy of the treatment. A high percentage (77.1%) of the grubs found
7 dead in treated palms 14 d after treatment proved positive for the presence of either
8 nematodes or the symbiotic bacterium *X. nematophila*. However, this figure dropped to
9 just 30.2 % 14 d later. One difficulty encountered during the execution of this assay was
10 the impossibility of recovering the nine larvae inoculated at the beginning of the assay
11 in the treated palms. Only one to four individuals could be identified 28 d after the
12 treatment. Around 40% of the immature stages found dead in this assay corresponded to
13 pupae and the remaining 60% were larvae.

14 In the case of the preventative treatment, both the application of *S. carpocapsae* and the
15 timing of this application in relation to infestation significantly affected the number of
16 immature stages found alive in the palms. The interaction between these factors was
17 significant and the number of living immature stages per palm remained low and did not
18 significantly change from 0 to 15 d, but increased at 30 d. As a consequence efficacy
19 decreased at the end of the assay (Table 2). Females of *R. ferrugineus* released for
20 oviposition at the beginning of this assay were able to cause infestation in all control
21 palms. On the contrary, the *S. carpocapsae* treatment prevented infestation in 75% of
22 the palms for releases made either 0 or 15 d after nematode application. This result
23 could be explained by the fact that around 50% of ovipositing females released for
24 infestation on treated palms were found dead at the base of the fronds when the palms
25 were dissected at the end of the assay. These fronds showed feeding holes made by

1 these females, but no evidence of larval galleries. However, because of the long time
2 elapsed since their death, it was not possible to prove the involvement of *S.*
3 *carpocapsae*. This was not the case of 60% of the females recovered from treated palms
4 one week after their release which proved infected by *S. carpocapsae* when examined
5 under binocular microscope.

6 **Discussion**

7 Our results prove the potential of *S. carpocapsae* to control *R. ferrugineus* infestations
8 in palms. The dose used in our assays ($3.6 * 10^6$ DJs per palm) is higher than the dose
9 used by Abbas *et al.* (2001b) in a field assay in date palms (approximately $0.9 * 10^6$ DJs
10 per palm) and this may partly explain the lack of effect reported by these authors.

11 Contrarily, our dose is similar to that used by Dillon *et al.* (2006) against *Hylobius*
12 *abietis* (L.) (Coleoptera: Curculionidae) on pine stumps (*Pinus contorta* Dougl. ex Loud
13 and *Pinus sylvestris* L.) ($3.5 * 10^6$ DJs/tree), where efficacies of up to 47 % were
14 reported.

15 Efficacies around 80 % were obtained in the curative assay, and up to 98 % in the
16 preventative treatment. These efficacies are very high, especially when compared to
17 chemical pesticides used against this pest (Azam and Razvi 2001; Hernández-Marante
18 *et al.* 2003; El-Sabaey 2004; Kaakeh 2006). Furthermore, our results demonstrate the
19 ability of *S. carpocapsae* to infect and kill not only larvae but also more robust stages of
20 this weevil, such as the pupa and the adult. Whether infected adults could contribute to
21 the natural spreading of this EPN within palm groves or gardens deserves further
22 investigation.

23 The results from the curative assay are indicative that *S. carpocapsae* does not stay on
24 the outside of the palm waiting for its host, but rather penetrates in the palm crown
25 actively looking for and infecting *R. ferrugineus* larvae. These results differ from the

1 general consensus that this species is a classic ambusher (García del Pino 2006; Gaugler
2 2007), but are in agreement with results on similar cryptic systems (Dillon et al. 2006).
3 These authors presented evidence that *S. carpocapsae* did not simply remain on the
4 treated surface and behaved in the same way as other EPN with different foraging
5 strategies. Similarly to our results, these authors also found that the percentage of
6 insects parasitized by *S. carpocapsae* increased between the 2 and 4 weeks and they
7 attributed this fact both to the time taken by the nematodes to find the insects and that
8 taken by the insects to die after EPN infection.

9 The percentage of grubs found dead in treated palms which proved positive for the
10 presence of either nematodes or the symbiotic bacterium decreased from 14 to 28 d.
11 This result is in agreement with the hypothesis that EPNs leave their hosts once they are
12 dead and thus contribute to the natural spread of the disease (Ehlers 2001). The same
13 hypothesis could explain why it was not possible to isolate *S. carpocapsae* from the
14 females found dead when the palms from the preventative treatment were dissected at
15 the end of the assay. Besides, the ability of EPNs to decompose their hosts (Chavarría-
16 Hernández *et al.* 2007) could account for the results obtained in treated palms whereby
17 on completion of its infectious cycle, host larvae could no longer be recognized.

18 *Rhynchophorus ferrugineus* presents in the Mediterranean two main flight periods:
19 around April-May and in September-October. Open field applications of EPN timed
20 slightly before these months could protect the palms by affecting (1) immature stages
21 from the old generation within the palm, (2) adults before oviposition and (3) young
22 larvae from the new generation. Because the results obtained in the preventative assay
23 prove that *S. carpocapsae* can survive in the palm for at least two weeks without
24 losing its efficacy, applications repeated every 2-3 weeks during these critical periods
25 could prove effective to protect palms from this weevil.

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6

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Table 1. Results of the curative assay: mean number of immature stages of *R. ferrugineus* found in *P. canariensis* 14 and 28 days after EPN application (3.6×10^6 DJs + 36 ml chitosan per palm), mortality and efficacy of each treatment. (Initial infestation was 9 larvae introduced 1 month before treatment)

Treatment	Time (d) after treatment application	n	Mean number of immature stages (\pm SE)		Mortality (%)	Efficacy (%)
			Alive	Dead	(%)	(%)
Control	14	4	5.50 \pm 1.11	0	0	
EPN		4	1.25 \pm 0.29	3.25 \pm 0.29	72.9 \pm 2.4	72.9 \pm 2.4
Control	28	8	5.13 \pm 0.62	0.13 \pm 0.13	2.5 \pm 2.6	
EPN		8	0.50 \pm 0.29	2.00 \pm 0.45	81.3 \pm 10.3	80.8 \pm 10.6

ANOVA results*		
Source of variation	F	df
Treatment	163.28	1, 20
Time	5.62	1, 20
Interaction	3.10	1, 20

Data subjected to a two-way-analysis of variance (ANOVA $P > 0.05$).

*Data subjected to the angular transformation prior to analysis.

Table 2. Results of the preventative assay: mean number of immature stages of *R. ferrugineus* found in *P. canariensis* as a function of the time elapsed since EPN application (0, 15 and 30 d; dose 3.6×10^6 DJs + 36 ml chitosan per palm) and efficacy of each treatment. Palms were infested with 4 mated females each and dissection took place 1 month after infestation.

Treatment	Time (d) after treatment application	n	Mean number of immature stages (\pm SE)		Efficacy (%)
			Alive	Dead	
Control	0	4	23.00 \pm 3.50	0	93.5 \pm 7.5
EPN		4	1.50 \pm 1.73	0.75 \pm 0.87	
Control	15	4	14.25 \pm 7.53	0.25 \pm 0.29	98.2 \pm 2.0
EPN		4	0.25 \pm 0.29	0	
Control	30	4	83.50 \pm 11.13	2.00 \pm 1.56	75.7 \pm 8.1
EPN		4	20.25 \pm 6.79	0	
ANOVA results*					
Source of variation	F	df	P		
Treatment	53.72	1, 23	< 0.0001		
Time	39.52	2, 23	< 0.0001		
Interaction	11.64	2, 23	0.0006		

Data subjected to a two-way-analysis of variance (ANOVA $P > 0.005$).

*Data subjected to the $\log(x+1)$ transformation prior to analysis.