

Susceptibility and possible resistance mechanisms in the palm species *Phoenix dactylifera*, *Chamaerops humilis* and *Washingtonia filifera* against *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Curculionidae)

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Abstract

Rhynchophorus ferrugineus, known as the Red Palm Weevil (RPW), is reported as a pest of up to 40 palm species. However, the susceptibility degree and the defense mechanisms of these species against this weevil are still poorly known. In Europe, the RPW is a major pest of *Phoenix canariensis* while other palm species, including the congeneric *Phoenix dactylifera*, seem to be less suitable hosts for this insect. The aim of our study was to compare the defensive response of *P. dactylifera*, *Chamaerops humilis* and *Washingtonia filifera* against *R. ferrugineus* and try to define the mechanisms of resistance that characterize these species. Bioassays were carried out to evaluate the mortality induced on RPW larvae by extracts from the leaf rachis of the studied palm species. Tests at semi-field scale were also conducted, based either on forced palm infestation, with larvae of RPW, or on natural infestation, with adult females. Rachis extracts from *C. humilis* and *W. filifera* caused 100% larval mortality after 2 days of exposure, while extracts of *P. dactylifera* did not impair larval survival. Independently of the effect of the leaf extracts, the weevils were unable to naturally infest the three palm species, although larval survival was high after forced infestation of the plants. We concluded that the observed lack of infestation of *P. dactylifera* by RPW is due to factors other than antibiosis. In *W. filifera* and *C. humilis*, although the presence of antixenosis mechanisms cannot be excluded, resistance to *R. ferrugineus* seems to rely on the presence of antibiosis compounds.

Keywords: antibiosis, antixenosis, bioassays, plant–insect interactions, Red Palm Weevil, semi-field experiments, forced palm infestation

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Introduction

Rhynchophorus ferrugineus (Olivier, 1790) (Coleoptera: Curculionidae), commonly called the Red Palm Weevil (RPW), is native to Southern Asia and Melanesia where it

infests coconut palms (Nirula, 1956a, b). The RPW life cycle starts with the eggs laid in holes made by the female at the base of the host leaves. Oviposition holes contain chemical signals that discourage other females from laying eggs in the same spot, influencing the distribution of eggs on the host plant (Salem *et al.*, 2012). Neonate larvae dig tunnels within the palm core, feeding on its inner tissues. As larvae grow and molt, their food requirements lead them to feed primarily on the soft tissues surrounding the apical meristem, frequently causing the death of the infested plant (Giblin-Davis *et al.*, 1996; Dembilio *et al.*, 2012).

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Following its accidental introduction into North Africa and Europe two decades ago, *R. ferrugineus* has become established in countries of the coastal fringe of the Mediterranean basin and now it represents one of the major pests of ornamental palm species in the urban areas of Mediterranean countries. In 2009, its presence was reported for the first time in America, on the islands of Curaçao, Aruba and the Netherland Antillas; in 2010, a sibling species, *Rhynchophorus vulneratus* (Rugman-Jones *et al.*, 2013), was reported as *R. ferrugineus* in the Laguna Beach area of Orange County, California (EPPO, 2010; Roda *et al.*, 2011). *R. ferrugineus* is included in the EPPO A2 list for quarantine pests in Europe, where it was reported on 19 species of *Arecaceae*, comprehending 15 different genera (EPPO, 2008). Malumphy & Moran (2009) reported it as a pest of 26 palm species belonging to 16 different genera in diverse agro-ecosystems worldwide. Its host range is still expanding and currently, *R. ferrugineus* is considered to infest up to 40 palm species (SAP, 2015). RPW ability to infest host plants belonging to different species is extremely variable: for instance, in Peoples Republic of China and India, *R. ferrugineus* has been reported as a primary pest of coconut palms (*Cocos nucifera*), while in the Arabian Peninsula this weevil mainly attacks date palm (*Phoenix dactylifera*) plantations (Hussain *et al.*, 2013). In the Mediterranean basin, it has become the major pest of the Canary palm (*Phoenix canariensis*), which is extremely sensitive to its attack (Dembilio & Jacas, 2012). In Italy, the RPW has been found recently on the endemic palm *Chamaerops humilis* (Giovino *et al.*, 2012), and also on *Brahea armata*, *Brahea edulis*, *Howea forsteriana* and *Jubaea chilensis* (Longo *et al.*, 2011).

The susceptibility degree of each host plant species to *R. ferrugineus* is not well known and, depending on the case, it could be correlated to different mechanisms of plant defense. Painter (1958) defined three mechanisms of plant defense to insects: non-preference, antibiosis and tolerance. According to this classification, non-preference occurred when the plant was not attractive to the insect. Kogan & Ortman (1978) later replaced the term non-preference by antixenosis to indicate a kind of resistance to insect attacks due to mechanical barriers or repellent substances produced by the plant. Antixenosis can affect different kinds of insect behavior such as orientation, colonization, feeding and oviposition. Antibiosis instead displays its effects at the level of insect development, especially during juvenile stages, inducing a decrease in reproductive capacity and premature death. Finally, tolerance occurs when the plant is able to cope with certain levels of infestation and stands insect attacks without suffering important damages. Both antixenosis and antibiosis are currently recognized as mechanisms of plant resistance, i.e. a constitutive or induced plant response against herbivory to avoid or reduce the amount of damage (Núñez-Farfán *et al.*, 2007).

In a previous study, *C. humilis*, one of the two palm species native to Europe, was considered resistant to *R. ferrugineus* (Barranco *et al.*, 2000). Nevertheless, the European Union included this species in the list of *R. ferrugineus*-susceptible plants (EPPO, 2008). The list also included *Washingtonia* spp., but infestation trials conducted in Spain showed that although *Washingtonia robusta* was susceptible to *R. ferrugineus*, the congeneric *W. filifera* was not (Llácer *et al.*, 2012). In a series of semi-field assays (Dembilio *et al.*, 2009), it was shown that both *W. filifera* and *C. humilis* could not be naturally infested by *R. ferrugineus* adult females. Antibiosis was designated as the main mechanism operating in *W. filifera*, since a viscous secretion produced by the plant resulted in complete mortality

of *R. ferrugineus* young instars. In the case of *C. humilis*, antixenosis was proposed as the major mechanism of resistance involved. In fact, it was possible to by-pass this resistance by inserting neonate larvae in holes made with a drill (Dembilio *et al.*, 2009).

Although antibiosis has been proposed as an important defense mechanism against the RPW in some palm species, its underlying nature – chemical, physical or physico-chemical – remains unclear. In this study, we assessed the potential for chemical antibiosis resistance in the palm species *P. dactylifera*, *C. humilis* and *W. filifera* against infestation by the palm weevil *R. ferrugineus*. Since these palm species are known to contain significant amounts of diverse secondary metabolites similar to those involved in the defense response of other plants to biotic and abiotic stresses (Hirai *et al.*, 1986; Asami *et al.*, 1991, El-Sayed *et al.*, 2006), we hypothesized: (1) that palm tissues can show chemical antibiosis activity against *R. ferrugineus*; and (2) that the intensity of the chemical antibiosis activity differs among the studied palm species. Accordingly, palm resistance against the RPW should be the result of multiple factors involving both antibiosis and antixenosis mechanisms. To test these hypotheses we have applied a combined test approach involving toxicity bioassays as well as natural and forced infestation experiments.

Material and methods

Insects

RPW larvae and adult females used all over the experiments were supplied by UTAGRI Institute Eco Enea, Casaccia, Rome, Italy. The larvae originated from eggs laid by females captured through pheromone traps and kept under laboratory conditions (20°C, 16 h photoperiod). Both the females and the larvae were fed with apple slices (*Malus domestica*, cv Golden delicious) and monitored until they were used in the experiments.

Palm species and tissue sampling

The palm species *P. dactylifera*, *W. filifera* and *C. humilis* were used as tissue donors for the chemical bioassays. Tissue samples were obtained from the lower part of the leaf rachis of 15-year-old adult individuals planted in the garden of the Centro Studi e Ricerche per le Palme (CSRP Sanremo, Italy). Leaf rachis tissues between the base of the leaf and the insertion of the first leaflets were chosen because this part of the rachis is known to represent a major oviposition spot for *R. ferrugineus* in *P. canariensis* and several other palm species (Ferry & Gómez, 2002; El-Mergawy & Al-Ajlan, 2011). The plants were taxonomically identified at the CSRP and tissue samples were deposited in the center herbarium.

Preparation of leaf tissue extracts

Leaf rachis tissues of *P. dactylifera*, *W. filifera* and *C. humilis* (about 80 g of fresh tissue for each species) were chopped and extracted with MeOH in a Soxhlet apparatus for 45 min. Methanolic extracts (about 250 ml for each species) were evaporated to dryness through a rotary evaporator (Buchi, Rotovapor L-200) and stored at –20°C until needed. All the residues were re-suspended in distilled water (70 ml) before use in bioassays with RPW.

Bioassays with RPW larvae

The obtained extracts were screened for their toxic effects on four groups of 10 two-week old larvae of *R. ferrugineus*. The larvae were individually placed in 50 ml plastic jars with perforated caps. They were fed for one week with apple pieces 1.5 cm diameter and 1 cm thick that were dipped for 60 s in one of the different leaf extracts. A control group was established by dipping apple slices in distilled water for the same time, since this was the solvent used to resuspend the dry extracts from the leaves. Extracts from *P. canariensis*, the most preferred host of RPW in the Northern Mediterranean basin, were not tested on the basis of the reported lack of toxicity of date palm tissues to *R. ferrugineus* larvae (Sacco *et al.*, 2011). The apple pieces were replaced every 2 days to prevent microbial contamination. Larval mortality was monitored daily; the weight of each individual was recorded before the start and at the end of the experiment. During the experimental period, the larvae were maintained in a growth chamber at 26°C with a 16 h daily photoperiod. The test was repeated three times over a 10-month period.

Forced infestation trials

Six-year old palm trees, 1.5 m diameter, 2.5 m tall, belonging to the three abovementioned species and grown in 1 m diameter clay pot, were used to check for palm resistance to forced infestation at semi-field scale. The age of the plants was within the reported age range for maximum RPW infestation rates in *P. dactylifera* (Sallam *et al.*, 2012). In order to contrast the results from the selected palm species with the expected results from a RPW susceptible species, an additional set of plants of *P. canariensis* was included in the experimental trials. These plants were of the same age and size as those from the other species. Three potted plants per palm species were individually isolated in cylindrical cages 1.5 m width × 2.5 m height made of an insect-proof steel net of 0.5 cm mesh size. The trials were performed in early spring; plants were regularly watered and kept in a greenhouse at 25 ± 2°C temperature, 14 h photoperiod and 3000 lux illumination. Pot substrate was commercial soil (Flortis universal soil, Orvital S.p.A, Settimo Milanese, Italy). In each palm trunk, six holes 35 mm diameter and 5 cm depth were symmetrically drilled all around the leaf crown in the insertion area of the leaves on the stem. A RPW two-month old larva was introduced in each hole and the hole rim was tightly closed with a loose-texture cotton stopper to ensure air exchange. After 4 weeks, the palms were thoroughly sliced with a saw in order to determine the percentage of infested plants and the percentage of larval survival.

Natural infestation trials

The ability of *R. ferrugineus* to naturally colonize the considered palm species was investigated by means of additional no-choice infestation trials. Each trial involved two individual plants per palm species. The age and size of the plants, as well as the growing conditions and type of cage, were the same as those used for the forced infestation trials. No holes were drilled, but three adult RPW females were freed within each palm-containing cage to allow them to lay eggs on the host plant. Most of these females were assumed to be inseminated because previous field monitoring studies have shown that more than 80% of the females captured in pheromone traps had sperm in their spermathecae and contained fecundated

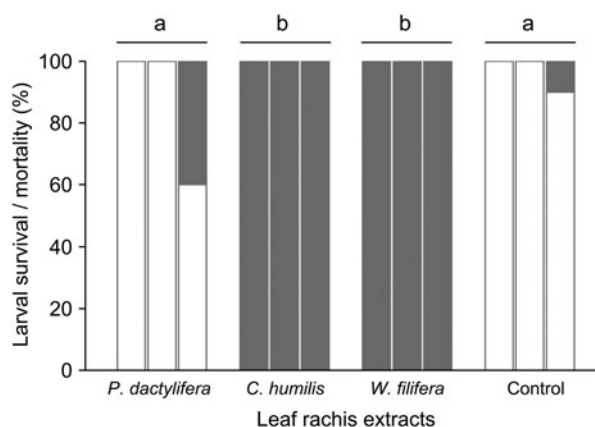


Fig. 1. Effect of leaf rachis extracts from *Phoenix dactylifera*, *Chamaerops humilis* and *Washingtonia filifera* on the survival of neonate larvae of Red Palm Weevil (RPW). For each palm species, bars indicate the percentage of either survival (white background) or mortality (grey background) of the weevil larvae ($n = 10$) after 1 week in three different tests. Mortality induced by *P. dactylifera* extracts was not significantly different from that observed in controls with distilled water according to χ^2 tests at $P \leq 0.05$.

eggs (Lo Bue *et al.*, 2010). Six weeks later, the plants were thoroughly dissected to determine the percentage of infestation and the average number of larvae per plant.

Statistical analysis

Data were analyzed using the R environment (R Core Team, 2015). In the laboratory bioassays, the effect of palm rachis extracts on larval survival was assessed by means of χ^2 tests using the function `chisq.test()`. The effect of palm rachis extract on larval growth was estimated through a meta-analysis of the replicated tests using the functions `es-calc()` and `rma()` from the *metafor* package (Viechtbauer, 2010). Differences in initial weight between the weevil larvae used in the different growth tests were checked through analysis of variance (ANOVA) and Tukey HSD tests with the functions `aov()` and `TukeyHSD()`, respectively. In the semi-field experiments, differences in larval survival after forced infestation of different palm species were tested by means of the Kruskal–Wallis test using the `kruskaltest()` function. Data presented through the text are means ± SE.

Results*Bioassays*

The effect of rachis extracts on the survival of *R. ferrugineus* larvae depended on the palm species considered. In the three tests performed, rachis extracts from *C. humilis* and *W. filifera* caused 100% larval mortality after 2 days of exposure, while extracts of *P. dactylifera* did not impair larval survival. The 40% mortality observed in the third test with *P. dactylifera* extracts did not differ significantly from that observed in the related control ($\chi^2 = 1.07$, $df = 1$, $P = 0.30$; $n = 10$; fig. 1). The larvae used in the three growth tests differed in their initial weight (ANOVA $F_{2,57} = 58.07$, $P < 0.001$; $n = 10$; fig. 2), suggesting an association between the smallest size of the larvae in the

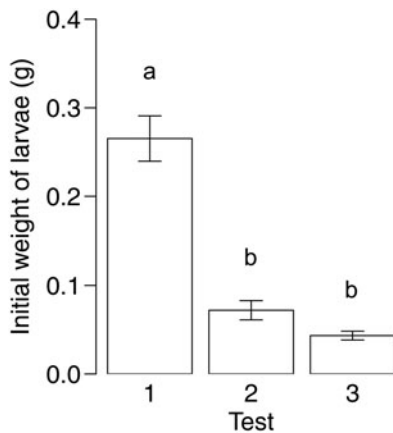


Fig. 2. Differences in initial weight of RPW larvae used in the bioassay tests with leaf rachis extracts from *Phoenix dactylifera*. Vertical bars and whiskers represent mean larval weight \pm SE in the three tests performed ($n=10$). Different letters indicate significant differences in body weight by the Tukey HSD test at $P \leq 0.05$.

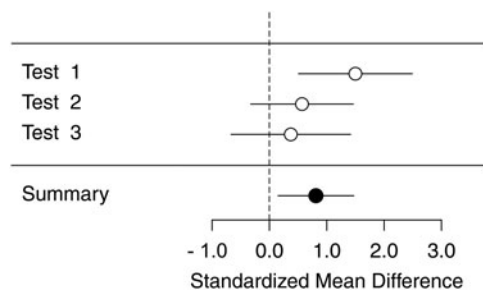


Fig. 3. Forest plot showing the effect of leaf rachis extract from *Phoenix dactylifera* on the growth of neonate larvae of RPW after 1 week. White symbols indicate larval average growth relative to controls in three different tests ($n=10$). The black symbol represents the summary estimate of the effect of the extract on larval growth, following a meta-analysis based on a random effects model of the data. Horizontal lines through the symbols represent 95% confidence intervals. The dashed vertical line indicates the line of no effect, i.e., the lack of differences between treatment and control.

third test and the recorded mortality. In the growth tests, the addition of rachis extracts from *P. dactylifera* to the food provided to RPW larvae had an overall positive effect in their growth (Random-effects model, $Z = 2.41$, $P = 0.016$; fig. 3), increasing larval weight by $34.2 \pm 8.3\%$ after 7 days.

Infestation trials

In the case of forced infestation, when the larvae were directly introduced into the stipe of the palm trees, 100% of infestation was scored on all the tested species. The boring activity operated by the larvae was well evident in all the plants, with the presence of deep feeding galleries in the inspected plant tissues. In *W. filifera* and in *C. humilis*, in correspondence with the galleries dug by the larvae, the production of viscous secretions was observed. This response was more intense in

W. filifera than in *C. humilis*, forming tubular-like structures, several centimeter long, that protruded from the holes initially drilled on the stipe of the palms. These tubular-like structures were made of sawdust produced by the larvae mixed with viscous substances produced by the plants. Larval survival exceeded 75% of the inserted larvae and did not significantly differ between palm species ($\chi^2 = 2.42$, $df = 3$, $P = 0.49$; $n = 3$; table 1).

In the natural infestation trials, neither larvae of *R. ferrugineus* nor larval trails were retrieved from the main study species after 6 weeks. The presence of larvae was only recorded in *P. canariensis* individuals, where infestation took place in both the tested plants (table 1).

Discussion

Our results show that toxic substances can play a significant role in palm resistance against RPW in some species (hypothesis 1). Antibiosis-like responses of palms against natural and forced infestation by *R. ferrugineus* have been previously reported, but such responses were based on the excretion of large quantities of viscous substances resulting in the obstruction of the RPW larval galleries (Barranco *et al.*, 2000). The composition of these excretions is poorly known and, as far as we know, there is no information about their toxic or repellent effect on the larvae of *R. ferrugineus*. In the present case, however, the observed effect of the leaf extracts of *W. filifera* and *C. humilis* on the RPW larvae indicates the presence of a true chemical antibiosis resistance in these species. This chemical effect was absent in the extracts of *P. dactylifera*. Not only extracts of *P. dactylifera* did not impair larval survival, but they had a positive effect on larval growth, excluding any potential toxicity due to the extraction procedure. These results indicate that leaf extracts are not necessarily toxic to *R. ferrugineus* larvae, which is in partial agreement with our hypothesis 2 regarding the species-specificity of the palm resistance mechanisms against the RPW.

The diversity and specificity of the chemical compounds responsible for the observed effects of the rachis extracts from *W. filifera* and *C. humilis* on the larvae of *R. ferrugineus* are not known, but a recent study regarding *W. filifera* (Cangelosi *et al.*, 2015) points to the existence of previously undescribed molecules with specific toxic effects for insects. Despite the presence of specific molecules, the chemical resistance of palms to the RPW does not necessarily rely on single compounds. In *C. humilis*, leaves show a significant concentration of secondary metabolites such as tannins, flavonoids and saponins (Benmehdi *et al.*, 2012), which are generally involved in both the constitutive and induced resistance of most plants species to the attack of parasites and pathogens (Yamane *et al.*, 2010). The ability of RPW to infest a certain palm species may be influenced by the identity of these metabolites and their leaf concentration in the palm. Our results suggest that *P. dactylifera* leaf extracts contain either a lower concentration or a different type of defense compounds as compared with the extracts from the other two test species. The latter possibility is in agreement with the reported differences in flavonoid composition between *C. humilis* and *P. dactylifera* (Hirai *et al.*, 1986; Asami *et al.*, 1991).

It is known that RPWs use chemical cues to locate their hosts (Giblin-Davis *et al.*, 1996) and therefore chemical inter-specific variation may play a significant role in the preference of *R. ferrugineus* for certain palm species. This variation may be associated to factors such as plant age, type of cultivar,

Table 1. Percentage of infestation and survival of individuals of *R. ferrugineus* in forced and natural infestation trials. Values followed by the same letter are not significantly different according to the Kruskal–Wallis test at $P \leq 0.05$. *P. canariensis* was included as a positive infestation control.

Species	Forced infestation assay		Natural infestation assay	
	% infestation	% larval survival	% infestation	No. of individuals per palm
<i>Washingtonia filifera</i>	100	83.3 ± 0 ^a	0	0
<i>Chamaerops humilis</i>	100	94.43 ± 5.56 ^a	0	0
<i>Phoenix dactylifera</i>	100	77.76 ± 14.69 ^a	0	0
<i>Phoenix canariensis</i>	100	88.86 ± 5.56 ^a	100	100 ± 64

phenology and health status (Farazmand, 2002; Al-Ayedh, 2008; Faleiro *et al.*, 2014). This is in agreement with the observation that in the Northern Mediterranean basin, *R. ferrugineus* preferentially infests *P. canariensis* over *P. dactylifera* (Dembilio & Jacas, 2012), but does not explain the complete absence of *P. dactylifera* infestation in our natural infestation experiment. Taking into account the statistical limitation posed by our two replicates per palm species, such a result may be due to the presence of resistance factors reducing infestation success to less than 50%. Dembilio *et al.* (2009) found an infestation success of 66.7% in *C. humilis* under similar experimental conditions, revealing a limited resistance of this palm species against RPW. Intraspecific variability in resistance may explain the occurrence of date palm individuals that escape episodic infestation events. In this regard, recent results showed differences in RPW oviposition depending on the *P. dactylifera* cultivar (Faleiro *et al.*, 2014), suggesting that sometimes date palms can avoid infestation by *R. ferrugineus*. We exclude that methodological issues might have prevented RPW infestation of our plants in the natural infestation assay because infestation did take place in all the *P. canariensis* individuals as expected. The average reproductive output we found in *P. canariensis* was 33.3 ± 21.3 ($n = 2$) larvae per RPW female, very similar to values previously reported (33.25 eggs per female) for the same palm species (Dembilio & Jacas, 2012).

The antixenosis resistance of *P. dactylifera* as based on the toughness of its leaves seems unlikely to explain the observed differences between this species and *P. canariensis* in our natural infestation experiment, particularly when considering the morphological similarity between these congeneric species and the fact that *P. dactylifera* constitutes a major host for *R. ferrugineus* in other geographical areas (Abraham *et al.*, 1998). Whatever mechanism was involved in the observed lack of infestation of *P. dactylifera* by *R. ferrugineus*, it was circumvented by the forced inoculation of larvae. This reinforces the hypothesis that antixenosis mechanisms may play an important role in *P. dactylifera* and it is in agreement with the early observed habit of *R. ferrugineus* females of choosing wounds in the cuticle of the palms to lay their eggs (Wattanapongsiri, 1966). Forced infestation also succeeded in the other two tested species, revealing not only that infestation can take place in palm species which produce toxic substances, but also that the observed antibiosis effect may be restricted to the leaves. Accordingly, the relative role of antixenosis and antibiosis may depend on the part of the palm considered. Ju *et al.* (2011) found that trunk tissues of *W. filifera* are a suitable food source for neonate RPW larvae, which agrees with the results of our forced infestation trial. The ability of *R. ferrugineus* to feed on trunk tissues but not on the leaves leads again to the hypothesis that the antibiosis compounds of this palm species accumulate, or are synthesized, at the leaf level.

In conclusion, our experiments suggest that the lower susceptibility of *P. dactylifera* to the RPW is not due to antibiosis, while in *W. filifera* and *C. humilis*, although we cannot exclude the presence of antixenosis mechanisms, chemical antibiosis factors seem to have a significant role in the resistance against *R. ferrugineus*. In this regard, the production by certain palm species of endogenous molecules involved in antibiosis resistance represents a potential new resource for the control of this pest.

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