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Antimicrobial activity of the red palm weevil *Rhynchophorus ferrugineus*

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Abstract

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthoridae), is an important pest of palms. Knowledge of both its natural enemies and its defensive mechanisms against predators and microorganisms is important to develop methods for an integrated pest control. Antimicrobial activity of the cuticular surface of adults and larvae, as well as of eggs, of this invasive species was investigated. This activity was tested against the Gram-positive bacteria *Bacillus subtilis* (Ehrenberg) Cohn and *Bacillus thuringiensis* Berliner, the Gram-negative bacterium *Escherichia coli* Escherich, and the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin. A similar analysis was conducted with the hemolymph of *R. ferrugineus*' larvae infected by *Pseudomonas aeruginosa* (Schroter) Migula, *E. coli* and *Staphylococcus aureus* Rosenbach. Polar surface fraction of extracts from adults and large larvae inhibits Gram-positive bacteria and the *B. bassiana*'s growth, but not the growth of *E. coli* and *M. anisopliae*. Similarly, the hemolymph of larvae and the surface extracts of both small larvae and eggs seemed not to show any inhibition. Chemical analyses of the fraction exhibiting antimicrobial activity show the presence of some polar compounds ranging between 1000 and 1500 Dalton. This study improves our knowledge on the biology of *R. ferrugineus* and helps to suggest strategies for the biocontrol of this pest.

Key words: *Rhynchophorus ferrugineus*, palm, pest, biological control, antimicrobial activity, polar substances.

Introduction

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthoridae *sensu* Alonso-Zarazaga and Lyal, 1999), is a large polyphagous insect native to southern Asia and Melanesia and is one of the most important pests of several palm species. Barranco *et al*. (2000) and Decision 2007/365/EC reported that it attacks more than 20 palm species worldwide, including date (Giblin Davis, 2001) and coconuts palms (Faleiro, 2006; Malumphy and Moran, 2007). According to OEPP/EPPO (2005; 2008), the weevil has become naturalised in isolated areas in every country of south-eastern, southern and western Asia. Today, RPW is widely distributed in Europe, Africa, Oceania and Asia (Wattanapongsiri, 1966; Zhang *et al*., 2003; Al-Ayedh, 2008; Yuezhong *et al*., 2009), and it is rapidly spreading in the Mediterranean basin through *Phoenix dactylifera* L. and *P. canariensis* Hort. ex Chabaud. Until recently only the American continent was free from the pest, but since December 2008 the RPW has been found in the Island of Curaçao, Netherlands Antilles (www.redpalmweevil.com), and more recently in Orange County, California (CDFA, 2010). The agro-climatic conditions of the date palm-growing countries, along with monocultures and the intensive modern date palm-farming practices, favoured the establishment of this pest (Faleiro, 2006). In addition, the abundant shipment of planting material from one country to another has contributed to its rapid spread in the Middle East, Africa and Europe (Abraham *et al*., 1998).

The RPW is attracted by kairomones of damaged palms, in the trunk of which larvae develop. As a result, the central tissue of the palm is destroyed and the tree eventually collapses and dies. The RPW's invasive potential is a consequence of the elevated female fecundity (each female lays 58-531 eggs that hatch in 1-6 days; Faleiro, 2006), the ability to complete several generations in a year even in the same tree (Rajamanickam *et al*., 1995; Avand Faghih, 1996), the ability of adults to fly for long distances (Wattanapongsiri, 1966), along with the tolerance to a wide range of climates due to the habit of hiding inside the host palm.

Since the RPW is a concealed tissue borer, it is difficult to detect symptoms of its attack at an early stage of infestation. Preventative measures are thus crucial for the success of any RPW-Integrated Pest Management (IPM) programme (Faleiro, 2006), which should include sanitation, trapping, and chemical and biological control, as well as increase in public awareness (Peter, 1989; Gobinadhan *et al*., 1990; Rajan and Nair, 1997; Murphy and Briscoe, 1999; Hanounik, 1998; Hanounik *et al*., 2000; Abbas *et al*., 2000; 2001).

The development of a biological control component for a successful IPM requires the identification of the natural enemies of the RPW (Murphy and Briscoe,

1999), including its pathogens (Dangar, 1997), and more in-depth information about its immune system and defensive mechanisms against its enemies.

There have been several attempts to isolate pathogens of the RPW (Gindin *et al*., 2006; El-Sufty *et al*., 2007; Güerri-Agulló *et al*., 2008; Salama *et al*., 2001; Salama *et al*., 2004). These studies led to the discovery of a cytoplasmatic polyhedrosis virus (Gopinadhan *et al*., 1990), a facultative bacterial pathogen, *Pseudomonas aeruginosa* (Schroter) Migula (Banerjee and Dangar, 1995), and a yeast isolated from the RPW's haemolymph (Dangar, 1997). However, none of these can be classified as potential biocontrol agents, mostly because their application in natural conditions is limited (Banerjee and Dangar, 1995; Salama *et al*., 2004). The susceptibility to microorganisms by Italian populations of the RPW, detected through observations and bioassays, shows a different efficacy of microbial infections (Clausi *et al*., 2009; Manachini *et al*., 2008, 2009; Vitale *et al*., 2009). However, the success of biological agents is often insufficient and any control of the RPW seems to be very difficult. Generally, insects use three primary lines of defence to avoid infection. The first is a physical barrier between the internal and the external environment, which includes the exterior cuticle and the lining of the gut. The second and third lines, humoral and cellular, are activated once a foreign invader infects the hemocoel. Studies on the immune system of the RPW were carried out mainly on hemocyte reactions (Manachini *et al*., 2011), but no data are available for humoral defence of this coleopteran.

Humoral and cuticular defences in insects include inducible antimicrobial peptides (Meister *et al*., 2000; Schmid-Hempel, 2005). These are produced mainly in the circulating hemocytes, in the cells of the fat body and to a lesser extent cuticular cells, midgut, salivary glands and reproductive organs (Schmid-Hempel, 2005). A variety of antimicrobial peptides have been described to date from insects (Saido-Sakanaka *et al*., 2005; Turillazzi *et al*., 2004; 2006).

Previous observations by one of us (S. Longo, unpublished data) suggest the ability by the larvae of this pest to inhibit microbial growth on the rearing substratum. The aim of this research was to analyse the outdoor and indoor defence systems of the RPW. Using microbiological techniques, we also attempted to understand whether different development stages of the RPW can inhibit the growth of different microorganisms, including entomopathogens. We used two approaches: the first was aimed at analysing the external surface of the RPW, whereas the second investigated the activity of humoral part of larval hemolymph. We also conducted a preliminary chemical analysis of the fraction with antimicrobial activity by MALDI-TOF mass spectrometry.

Materials and methods

Insect samples

Adults (body length of females and males averaging 3.42 ± 0.04 cm and 3.16 ± 0.03 cm, respectively) and large larvae (body length ≥ 3 cm) were collected in 2008 from infested palms in Sicily and in Latium (Italy) and maintained in the laboratory at the Department of Evolutionary Biology in Florence until used for the microbiological assay. Eggs and small larvae (body length < 3 cm) were obtained after reproduction of the adults in the laboratory. The large larvae and the adults were kept in plastic cages (15 x 15 x 15 cm) with apple slices as food and oviposition substratum for females. Small larvae were kept in plastic cups (diameter: 6 cm) and fed on mashed apple.

Bleeding procedures

To distinguish the humoral antimicrobial activity from other components we performed a protocol to extract clean and pure hemolymph. To this end, older and large larvae of about 4 g were chosen (n = 15). The larvae were sterilised in 70% ethanol for a few seconds and rinsed with sterile water and anaesthetised at -20 °C for 5 to 7 minutes. The hemolymph was obtained from the dorsal blood vessel of larvae. About 1 ml of hemolymph from each larva was collected and placed in sterilised Eppendorf tubes containing an anticoagulant solution (98 mM NaOH, 186mM NaCl, 17mM Na_2 EDTA, 41 mM citric acid and 10 mM phenylthiourea pH 4.5). The hemolymph was centrifuged at 1000 x for 10 min. at 4 °C to collect the hemocytes and were suspended in an anticoagulant solution at a concentration of 1 10^{-6} ml^{-1}.

Microbial strains

We used the following microbial strains: *Escherichia coli* Escherich JM109, *Bacillus subtilis* (Ehrenberg) Cohn ATCC 6633 (American Type Culture Collection), *Bacillus thuringiensis* Berliner subspecies *kurstaki* EG 2348 from a commercial product (*Rapax*, registered against Lepidoptera, Intrachem Bio Italia), *Beauveria bassiana* (Balsamo) Vuillemin JW-1, ATCC 74040 from a commercial product (*Naturalis*, Intrachem Bio Italia), and *Metarhizium anisopliae* (Metchnikoff) Sorokin isolated as described below. These entomopathogenic fungi and *B. thuringiensis* have been chosen because they are currently used in biological control (Alfazariy, 2004; Gindin *et al*., 2006).

M. anisopliae strain was isolated from a dead RPW adult from Sicily in the laboratory of CRA-ABP. Fungal conidia emerging from the insect body after incubation in a moist chamber were collected and then plated on SDA+Y (Sabouraud Dextrose Agar + Yeast extract) to obtain a pure culture for tests. It was identified by both morphological and phylogenetic analysis based on ribosomal DNA (data not shown).

This isolate belongs to the "Entomopathogenic Microorganisms Collection" of CRA – ABP (Florence, Italy) (www.collezionedimicrorganismi.com), coded as Man08/I05.

To test the antimicrobial activity of the hemolymph we used different microbial strains *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027 and *Staphylococcus aureus* Rosenbach ATCC 29213. *E. coli* and *P. aeruginosa* were selected being a Gram-negative strain and a facultative pathogen of the RPW, respectively (Banerjee and Dangar, 1995). Besides, insects were recently found to be a good model for the infection of the human patho-

gen *S. aureus* and a potential source of antimicrobial compounds (Kaito *et al*., 2002; Schmid-Hempel, 2005; Fleming *et al*., 2006).

Microbiological assays

Determination of minimum inhibitory concentrations

Minimum Inhibitory Concentrations (MICs) against bacterial strains were determined as previously described by a broth dilution micro-method (Schillaci *et al*., 2005). A series of solutions with a range of concentration from 500-15 mg/ml were used for the assay. The total protein contained in the plasma was determined using the method of Bradford (1976) and was obtained by twofold serial dilution, made in Mueller Hinton broth (Sigma) in a 96-wells plates. To each well, 10 µl of a bacterial suspension, obtained from a 24 hrs culture and containing ~10^6 cfu ml^{-1}, was added. The plate was incubated at 37 °C for 24 hrs and the MICs were determined by a micro-plate reader (ELX 800, Bio-Tek Instruments). The lowest concentration of compound with optical density (OD) read at 570 nm was compared with the controls (broth without inoculum).

Extracts preparation for microbiological tests

Thirteen small larvae, 26 large larvae and 34 adults (17 males and 17 females) were first inserted into individual glass tubes and frozen at -22 °C for 30 min. The adults, the larvae and the eggs were washed in solvents to extract surface compounds, i.e. the adults in 500 µl of methanol (n = 26) or 500 µl of pentane (n = 8); the large larvae in 1 ml of methanol (n = 13) or 1 ml of pentane (n = 13); the small larvae in 500 µl of methanol (n = 13) and the eggs in 500 µl of methanol (n = 34) and in 500 µl of pentane (n = 20). Methanol and pentane extracted polar and apolar surface compounds, respectively. Since our first results (see below) did not show any antimicrobial activity by pentane extracts, small larvae were only washed with methanol. To test the possible effects of the concentration of polar surface extracts of the small larvae, two pools composed of 10 small larvae each were washed in 1 ml of methanol. Adults, larvae and eggs were shaken for inversion, vortexed for 30 seconds and then removed. The extracts were dried overnight and re-suspended the following day in 30 µl of solvent (pentane or methanol); Tris-HCl 25 mM pH 7.5 was used to re-suspend the methanol extracts because methanol resulted toxic for *B. bassiana*. Aliquots (5 µl) of the extract of each sample were spotted on the plates (see below).

To obtain information about the localisation on the body of the active fraction, six additional adults were frozen as above and their dorsal and ventral sides were separately washed with pads dipped in 500 µl of methanol.

Agar diffusion test

We assayed microbial growth inhibition with the agar diffusion method as previously described (Turillazzi *et al*., 2004). The Gram-positive strains, *B. subtilis* and *B. thuringiensis*, and the Gram-negative strain, *E. coli*, were used as bacterial indicators. Petri dishes with Luria Bertani (LB) solid medium were plated with 10^6 *E. coli* from an overnight culture in LB medium. Petri dishes with Nutrient Agar (NA, Oxoid) medium were overlaid with 7 ml of NA inoculated with 10^6 *B. subtilis* and *B. thuringiensis* spores, respectively. Methanol and pentane extracts of adults, larvae and eggs were spotted (5 µl) onto the plates (2-5 extract spots for each plate) as well as respective solvents as control (one spot for each plate). Penicillin G was used as standard for *B. subtilis*, while Kanamycin was the standard for *B. thuringiensis* and *E. coli*. Standards were spotted onto the plates as 1 µl of solution 50 µg ml^{-1}. Plates were incubated for 24 h at 37 °C.

B. bassiana and *M. anisopliae* were used as fungal indicators. Petri dishes with Malt Extract Agar (MEA, Oxoid) were plated with 10^6 spores of each fungus. Extracts were spotted (5 µl) on the plates (table 1). Nystatin was used as standard, spotted as 2 µl of 4 mg ml^{-1} solution for both fungi. Plates were incubated for 2-3 days at 27 °C.

Antimicrobial activity for both bacteria and fungi was indicated by the clear zones of the growth inhibition ring on the plates. Results were considered positive when the diameter of the ring was ≥ 0.5 cm.

The pads were gently put and removed on Petri dishes overlaid with NA medium inoculated with *B. subtilis* spores.

Mass spectrometry analyses

Samples preparation

Ten small larvae, 10 large larvae and 10 adults were used for the analyses. The insects were killed by freezing and were kept at -20 °C until extraction. Each larva was washed in 300 µl of methanol for 1 min to extract the overall surface polar compounds. Then, the crude extracts, dried and re-suspended in 20 µl of methanol, were analysed by MALDI-TOF mass spectrometry.

In order to obtain reference spectra of both the ventral part of the thorax and the abdomen and the dorsal part of the thorax and elytra of the adults, we rinsed these two different parts of the cuticle using the method described by Turillazzi *et al*. (2006) ("*in situ* micro-extraction"): we directly poured twice 10 µl drop of methanol on the cuticle by means of a Gilson Pipette sucking and back it repeatedly and then depositing 1 µl of the extract solution on the MALDI plate.

To reduce salt concentration and enhance the peak signal intensity, we performed three pool methanol extractions of respectively 10 small larvae, five large larvae and five adults (ventral side micro-extraction). Then the samples were filtered using a Zip-Tip unit for salts (C18, for small peptides) and 1 µl of the filtrate was applied on the MALDI plate as described below.

Chemicals

Chromatography grade methanol and acetonitrile were purchased from Riedel de Haen (Sigma Aldrich, Milan, Italy). Purified and deionized water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA). Formic acid and trifluoroacetic acid (TFA) were purchased from Fluka (Sigma Aldrich, Milan, Italy); α-Cyano-4-hydroxycinnamic acid (HCCA) was obtained from Sigma (Sigma Aldrich, Milan, Italy).

Table 1. Results of the agar diffusion tests, with extracts from *R. ferrugineus* adults, larvae (small and large) and eggs. Each test corresponds to a single individual, except the pool.

Stage	Extract	Microorganism	No. tests performed	No. positive tests
Adult	pentane	*B. subtilis*	8	0
"	pentane	*B. thuringiensis*	8	0
"	pentane	*B. bassiana*	8	0
"	methanol	*B. subtilis*	16	16
"	methanol	*B. thuringiensis*	16	16
"	methanol	*E. coli*	10	0
"	methanol	*B. bassiana*	16	16
"	methanol	*M. anisopliae*	10	0
Adult (dorsal part)	methanol	*B. subtilis*	6	0
Adult (ventral part)	methanol	*B. subtilis*	6	6
Large larva	pentane	*B. subtilis*	13	0
"	pentane	*B. thuringiensis*	13	0
"	pentane	*B. bassiana*	13	0
"	methanol	*B. subtilis*	13	10
"	methanol	*B. thuringiensis*	13	10
"	methanol	*E. coli*	13	0
"	methanol	*B. bassiana*	13	12
"	methanol	*M. anisopliae*	13	0
Small larva	methanol	*B. subtilis*	13	0
"	methanol	*B. thuringiensis*	13	0
"	methanol	*E. coli*	13	0
"	methanol	*B. bassiana*	13	0
"	methanol	*M. anisopliae*	13	0
Pool (10 small larvae)	methanol	*B. subtilis*	2	0
"	methanol	*B. thuringiensis*	2	0
"	methanol	*B. bassiana*	2	0
Egg	methanol	*B. subtilis*	10	0
"	methanol	*B. thuringiensis*	10	0
"	methanol	*E. coli*	7	0
"	methanol	*B. bassiana*	7	0
"	pentane	*B. subtilis*	5	0
"	pentane	*B. thuringiensis*	5	0
"	pentane	*E. coli*	5	0
"	pentane	*B. bassiana*	5	0

MALDI-TOF spectra profiling

Methanol extracts of the small and the large larvae and of the adults were analysed with a MALDI-TOF/TOF Ultraflex III (Bruker Daltonics, Bremen, Germany). The instrument was operated in positive ion reflector mode. The accelerating voltage and the Ion Source 2 were set to 25.0 and 21.9 kV, respectively, and the delay time was 20 ns. The matrix for MALDI-TOF experiments was a solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) (10 mg ml^{-1}) dissolved in 70/30 Acetonitrile/TFA 0.1%. One µl of the sample was mixed with MALDI matrix (1:1, vol:vol) and the mixture was spotted in a stainless steel target; the droplet was allowed to evaporate before introducing the target into the mass spectrometer. All the samples were analysed using an automatic procedure available on the MALDI mass spectrometer in order to standardize the results. 800 shots were automatically accumulated for each spectrum. External calibration was done with the Bruker Standard Peptide Calibration kit. The peptidic fraction of the samples was acquired in the range *m/z* 800-3000 and was calibrated using the Bruker peptide calibrant kit (*m/z* 1000-3500).

Results

Microbiological results of outdoor components

The results are given in table 1. No control spots (solvent only) showed inhibitory activity whilst all standards inhibited the growth of the respective microorganisms. On one hand, methanol extracts of the adults and the large larvae showed inhibitory activity against the growth of *B. subtilis*, *B. thuringiensis* and *B. bassiana* but not against the growth of *E. coli* and *M. anisopliae*, whereas methanol extracts of the small larvae, including the pool, did not inhibit the growth of any microorganism. On the other hand, pentane extracts of the adults and the large larvae did not show any effect. No growth inhibition was found in any microbiological test carried out with eggs extracts. Methanol extracts of the ventral side of the adults using pads showed inhibitory activity against *B. subtilis*, in contrast to the extracts of the dorsal side.

Antimicrobial results of hemolymph

The MICs' determination showed that the hemolymph extracted from all RPW larvae dorsal vessel was inac-

tive against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027 and the human pathogen *S. aureus* ATCC 29213 at all the tested concentrations ranging from 500 to 15 mg/ml of the total protein contained in the plasma.

Chemical results

The MALDI-TOF analyses of samples of both the larvae and the adults provide good reference spectra but not enough for the specific identification of the substances. This was probably due to the high concentration of salts dissolved in the samples which breeding Na^+ and K^+ adducts. However, it is evident that the extracts of both the larvae and the ventral side of the adults contain some polar compounds ranging between 1000 and 1500 Dalton. On the contrary, the dorsal side of the adults lacks polar compounds in 9 out of the 10 analysed samples.

The MALDI-TOF analyses of the purified pools of small larvae (n=10), large larvae (n=5) and the adults (n=5, ventral side) also provided good reference spectra (figure 1) but did not allow to identify the substances. The peak at 1060 Da corresponds to the principal medium weight polar compound present on the extracts of the large larvae and the adults (the peaks at 1082 Da and 1098 Da correspond to 1060 + Na^+ and 1060 + K^+, respectively) and the peak at 1294 Da is the second principal peak in the large larvae and in the adults and the principal peak in the small larvae. The peaks at 1424 Da and at 1476 Da are well represented in the adult extracts only (table 2).

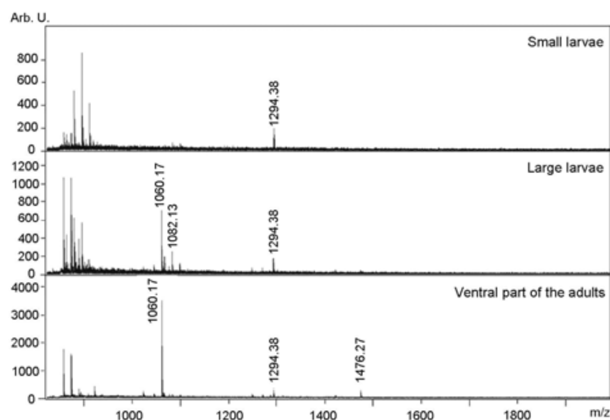

Figure 1. Reference MALDI-TOF spectra in the range 850–2000 *m/z* of methanol filtered extracts from the pools of: small larvae (n = 10), large larvae (n = 5) and ventral part of the adults (n = 5) of *R. ferrugineus*. As z = 1 (in the x-axis), values of *m/z* are in equivalent Daltons.

Table 2. Peaks identified in the MALDI-TOF spectra profiling of the 950-4000 Da fraction of the cuticle of the small larvae, large larvae and ventral part of the adults of *R. ferrugineus*. As z = 1, values are in equivalent Daltons.

Peaks *m/z*	Small larvae	Large larvae	Adults (ventral part)
1060	-	x	x
1082	x (trace)	x	x (trace)
1098	x (trace)	x	x (trace)
1294	x	x	x
1424	-	-	x
1476	-	-	x

Discussion and conclusions

The surface extracts of RPW larvae, but not their hemolymph, and adults showed antimicrobial activity that may allow this species to live in habitats full of microorganisms, such as the interior of the palm tissue (Butera *et al*., 2009).

To face pathogens, insects have a complex immune system including the responses of hemocytes in hemolymph, and physical barriers, such as the integument and gut (Boman, 1995; Gillespie *et al*., 1997). They also synthesize, in both the hemolymph and the cuticle, antimicrobial proteins and peptides against microorganisms. The presence of polar compounds with antimicrobial activity has already been detected on the epicuticular layer of arthropods (Kuhn-Nentwig, 2003), including social insects (Hölldobler, 1990; Turillazzi, 2006). Although fat bodies are the main sources of the inducible anti-pathogenic peptides in insects (Gillespie *et al*., 1997), the epidermis may also produce antibacterial and antifungal peptides in response to local infections (Brey *et al*., 1993; Ferrandon *et al*., 1998).

Our microbiological results show that the growth of some microorganisms, such as *B. thuringiensis* and *B. bassiana* usually used in biological control (Faleiro, 2006; El-Sufty *et al*., 2006; El-Sufty *et al*., 2007), was inhibited by polar compounds, but not apolar ones, which were found in the cuticle of the RPW adults and larvae. On the contrary, the growth of *E. coli* and *M. anisopliae* was not inhibited.

The growth of the Gram-positive bacteria *B. subtilis* and *B. thuringiensis* is inhibited by the polar extracts of RPW adults and large larvae. *B. thuringiensis* is the most important biological agent used in pest control due to its action mode and its specificity (US-EPA, 2008). Alfazariy (2004) reported the successful control against the RPW in Egypt by infection with *B. thuringiensis* subspecies *kurstaki* isolated from the larvae. On the contrary, other authors (Bauce *et al*., 2002; Birda and Akhursta, 2007; Sivasupramaniam *et al*., 2007; Manachini *et al*., 2008; 2009) showed a different susceptibility of the RPW to *B. thuringiensis*. This bacterium has an insecticidal action on the weevil when ingested but not sufficient compared to commercial application (Manachini *et al*., 2009). However, a larval growth inhibition was observed for the RPW and an interaction with hemocytes was primarily described, showing that the bacterium was able to grow in the hemolymph when ingested by the larvae (Manachini *et al*., 2011).

Our microbiological assays also confirm the higher virulence of *M. anisopliae* strains with respect to *B. bassiana* ones, as reported by Gindin *et al*. (2006). These authors showed in the laboratory that *M. ani-*

sopliae strains cause a mortality of up to 80-100% of the larvae and adult weevils. Positive results in the biological control of RPW were also obtained by Vitale *et al*. (2009) using a commercial product of *M. anisopliae* mixed with *B. bassiana*, whereas the use of *B. bassiana* only, isolated from a dead RPW, was not successful in killing the adults. Indeed, we found that polar extracts of the adults inhibit the germination of *B. bassiana*'s spores. However, the use of *B. bassiana* as a biocontrol agent gives ambiguous results: recent studies (Sewifi *et al*., 2009; Dembilio *et al*., 2010; Güerri-Agulló *et al*., 2010) reported that strains of this fungus obtained from naturally infected RPW are promising agents to control the RPW. Thus, we cannot completely discard its use in biological controls.

The growth of *E. coli* is never inhibited by RPW extracts neither in the hemolymph. This result perhaps may explain the efficacy of the nematode *Steinernema carpocapsae* (Weiser) (Nematoda Steinernematidae) as a biocontrol agent. Nematode juveniles of the third stage infect and kill larvae, along with pupae and adults (Faleiro, 2006; Llácer *et al*., 2009), due to the Gram-negative bacterium, *Xenorhabdus nematophila* (Poinar and Thomas) Thomas and Poinar (Enterobacteraceae), carried in its gut. Indeed, the adults and larvae of the RPW have no antimicrobial activities against Gram-negative bacteria. However, a limitation in the use of *S. carpocapsae* against RPW is the inability to complete its life cycle and to reproduce inside the weevil, possibly due to some unknown inhibitory substances produced by the RPW (Clausi *et al*., 2009). The higher susceptibility to the nematodes by the small larvae rather than by the large larvae (the 4th stage larvae reported by Clausi *et al*., 2009) also supports our findings. Confirming Aronson and Shai (2001), methanol extracts of the small larvae do not show any antimicrobial activity, thus suggesting that they are the most susceptible stage to diseases. The lack of antimicrobial activity of the small larvae (single or pooled) could be due to a low quantity of polar substances on the cuticle of this developmental stage.

The eggs extracts seem to be unable to inhibit the growth of the microorganisms. Even if the eggs are laid in the same environment inhabited by the larvae and the adults, their short hatching time (about three days, Murphy and Briscoe, 1999) and the physical barrier provided by the chorion might protect them from the attack of microorganisms.

Moreover, it is known that, in response to microbial infection or septic body injury, insects synthesize *de novo* a battery of antimicrobial peptides (AMPS), which are then rapidly released into the hemolymph (Saido-Sakanaka *et al*., 2005; Schmid-Hempel, 2005). AMPs previously identified in many insects operate synergistically against a wide range of Gram-positive and Gram-negative bacteria (Meister *et al*., 2000; Andrejko *et al*., 2009). The lack of clear *P. aeruginosa* growth inhibition in the case of hemolymph of RPW larvae suggests the absence of inducible specific antimicrobial peptides in the hemolymph against the strain tested. However, different authors found that several strains of *P. aeruginosa* are entomopathogenic (Andrejko *et al*., 2009). Similarly, the hemolymph of larvae of the lepidopteran *Galleria mellonella* L. inhibited *P. aeruginosa* but not *E. coli* (Andrejko *et al*., 2009). Thus, even if *P. aeruginosa* is considered to be a facultative entomopathogen of RPW (Banerjee and Dangar, 1995), our results underline the potential of finding a more infective strain to improve the biological control of the RPW. Recently, it has also been shown that innate immune systems of mammals and insects share a high degree of structural and functional homology. For example, a positive correlation exists between the virulence of *P. aeruginosa* and both several insects, like *G. mellonella* larvae, and mice (Jander *et al*., 2000; Schmid-Hempel, 2005; Andrejko *et al*., 2009). Therefore, analysis of insect responses to pathogens may provide data comparable to those obtained for mammals. Thus, RPW could be a good model for the identification and characterisation of virulence factors of microbial pathogens like *P. aeruginosa* and *S. aereus*, not only for insects but also for mammals, similarly to other insects, such as *Drosophila melanogaster* (Meigen) (Needham *et al*., 2004; Scully and Bidochka, 2006).

Moreover, our microbiological findings suggest that polar substances on the body of both large larvae and adults of RPW provide them with a protective barrier against microorganisms, including those growing inside palms. These substances have only been found in the ventral side of the adults of both sexes, which suggests that they are produced by an unknown secreting structure (e.g. epithelial or gland cells) located in this part of the body.

Interestingly, our preliminary MALDI TOF analysis of the large larvae and the adults shows an important peak (1060 Da) in addition to the peaks found in small larvae. This finding, combined with the revealed antimicrobial activity of both adult and large larvae against *B. subtilis*, *B. thuringiensis* and *B. bassiana*, suggests that the peak at 1060 Da has probably the highest antimicrobial competence. Further studies are needed to investigate the characterization of the polar substances of adults and larvae and to test which peaks are responsible for the microbial inhibitory activity.

In conclusion, our results pinpoint the importance of understanding the antimicrobial abilities of the RPW and open new perspectives for the study of the biological control of this pest. The study may help us to understand the limits in the biological control of the RPW, which is difficult in its field applications for practical reasons (Banerjee and Dangar, 1995; Salama *et al*., 2004; Gindin *et al*., 2006). This knowledge will be useful to improve correct RPW-IPM programmes in order to focus on the identification of more virulent strains of natural pathogens.

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