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BACTERIA ASSOCIATED TO *RHYNCHOPHORUS FERRUGINEUS* (OLIVIER) (COLEOPTERA DRYOPHTHORIDAE) IN ITALY

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Raio A., Roversi P.F., Francardi V. – Bacteria associated to *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthoridae) in Italy.

Thirty-five bacterial strains were selected on the basis of colony morphology from larvae and pupae of *Rhynchophorous* (Olivier) *ferrugineus* collected from infested *Phoenix canariensis* Chabaud trees growing in different Italian locations. 16S rDNA sequencing showed that bacteria isolated from larvae were mostly Gram positive and belonged to *Bacillus*, *Brevibacillus* and *Paenibacillus* genera. Bacteria isolated from pupae were affiliated to *Bacillus* and five different Gram negative genera (*Alcaligenes*, *Morganella*, *Myroides*, *Providencia* and *Serratia*). Some strains produced lytic enzymes like chitinase, lecithinase, lipase and protease in vitro. The enthomopathogenic strains belonging to *Bacillus thuringiensis, Providencia rettgeri and Serratia marcescens* species may be considered of interest as potential biocontrol agents of *R. ferrugineus*.

KEY WORDS: *Phoenix canariensis,* Red Palm Weevil, entomopathogenic bacteria, *Providencia rettgeri*, biocontrol.

INTRODUCTION

Rhynchophorous. ferrugineus (Olivier) is an exotic invasive insect pest that in a very short time, spread rapidly in Italy with devastating effects on ornamental palms. In the last 16 years it caused the death of thousands *Phoenix canariensis* Chabaud plants mainly in the Central-Southern Italian regions and in Sicily and Sardinia islands. Notwithstanding the species has been subjected to all the authorized control measures to remove dead plants and to treat promptly the attacked ones with chemical insecticides in order to save the palms and to prevent insect pest spread, the insect is still being in expansion in the Country. Palm trees are ubicated mainly in urban parks and boulevards within residential areas, where the use of chemicals elicits serious concerns related to environmental pollution and human health, consequently, eco-friendly biological control methods has now attracted high interests (MAZZA *et al.,* 2014). Previous studies have demonstrated that spore-forming bacteria belonging to *Bacillus* genus (SALAMA *et al.,* 2004) and the gram negative bacterium *Pseudomonas aeruginosa* caused the mortality of the larvae under standard biassay conditions (BANERJEE and DANGAR, 1995). As part of research carried out to develop low environmental impact control methods, several natural enemies and entomopathogenic microorganisms of *R. ferrugineus* have been evaluated (FRANCARDI *et al.,* 2012; 2013a; 2013b; 2014; MAZZA *et al.,* 2013, 2014; CITO *et al.,* 2014). but at present, no effective biocontrol agent is utilized currently.

In Italy, during a two year study (2012-2013) on the natural antagonist complex of *R. ferrugineus*, two important groups of entomopathogens belonging to fungi (*Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metschnikoff) Sorokin and bacteria, which caused the death of 15.74% and 11.88% of larvae and adults respectively (FRANCARDI *et al.,* 2014), were identified. In the present paper the Authors focused the attention on the characterization of bacterial microflora associated to larvae and pupae of *R. ferrugineus* with the aim to evaluate potential biocontrol strains.

MATERIAL AND METHODS

ISOLATION OF BACTERIA

Cocoons and dead medium-large larvae (from 2 cm to 4-5 cm long) of *R. ferrugineus* were obtained from *P. canariensis* infested palms grown as ornamental in different urban areas of Sicily (Provinces of Catania, Ragusa, Agrigento and Enna) and Tuscany (Province of Lucca) regions. Samples were collected during felling operations of heavily attacked plants carried out by the local Plant Protection Services in order to contain the spread of the pest. *R. ferrugineus* larvae and cocoons with pupae inside were transferred in CREA-ABP laboratory in a quarantine room and examined; specimens with suspected symptoms of bacterial attacks were separated and treated to isolate the pathogen/s.

Larvae and pupae were surface sterilized in 70% ethanol for 5 minutes and then washed thoroughly with sterile distilled water three times. Each sample was cut in small pieces with a sterile scalpel and put in flasks containing 50 ml of Nutrient Broth (Oxoid) amended with 0,25% glucose (NGB). Flasks were incubated at 30°C for 48 hours on a rotary shaker. Samples were then serially diluted and aliquots of 100 µl were spread on plates containing the medium previously indicated amended with agar (NGA) and Tryptic Soy Agar medium (Fluka). Twenty microliters of the undiluted samples were also

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streaked on the same agar media. Plates were incubated at 30°C for 48 hours. Bacterial isolates were selected on the basis of colony morphology and purified on NGA medium. For long term storage, suspensions of each isolate were prepared in 30% glycerol solution and frozen at -20°C.

Identification of bacteria

Gram reaction of bacterial isolates was determined by the rapid non staining method described by Buck (1982).

Thirty-five isolates selected on the basis of their origin and colony morphology were identified by the partial 16S rRNA gene sequence analysis. Bacterial strains were grown in 5 ml of Luria Broth (Difco) overnight at 27° C. One ml of each culture was used for DNA extraction by using EZNA bacterial DNA kit (Omega bio-tech) following the protocols of the kit manufacturer for Gram positive or Gram negative bacteria.

The amplification was performed in 25 µl volume using 200 ng DNA, 20 pmol of each primer fD1 [(5' – AGAGTTTGATCCTGGCTCAG – 3') and rP1/rP2 (5' – GGYTACCTTGTTACGACTT – 3'; Y=C/T)] (PIOUS and THYVALAPPIL, 2009), 50 µM dNTPs and 0.5 units of Taq DNA polymerase (Invitrogen). Amplification cycle was the same as reported by PIOUS et al., (2008). PCR products were sequenced by Primm s.r.l. (Milano, Italy) by using the primer set involved in PCR reaction. Similarity of partial 16S rDNA nucleotide sequences with known sequences in the NCBI GenBanK database was determined by BLASTn (http://blast.ncbi.nlm.nih.gov/). Strains were identified at species level when they shared more than 97% identity with reference strains. Partial sequence data for the 16S rRNA genes have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence data libraries. Data for bacterial strains identified in this study have been deposited under accession numbers.

Enzyme production by bacterial strains

Strains identified at species level were characterized for production of chitinase, lecithinase lipase and protease. The ability to degrade chitin was checked by streaking the four strains on M9 minimal medium amended with colloidal chitin (1%) (Sambrook *et al.,* 1989). Plates were incubated at 28°C for 96 hours. Strains that determined a clarification of the medium were considered chitinolytic. For the other tests, a 10^8 cfu/ml suspension of each bacterial strain was prepared using fresh cultures grown on NGA for 24 - 48 hours at 28°C. Fifty µl of each bacterial suspension were placed on egg yolk agar (EYA) plates for lecithinase, LB agar plates amended with Tween 40 (1%) for lipase and skim milk agar (SMA) plates for protease tests. Plates were incubated at 28°C and monitored at different times. Each test was repeated three times. An opaque halo around the macrocolonies, easily visible by transmitted light on EYA, indicated lecithinase production. Lipolytic activity was considered positive when the formation of a whitish halo around the macrocolonies was observed on LB agar. The formation of a transparent halo surrounding the macrocolonies on SMA indicated proteolytic activity.

RESULTS

Twenty and fifteen bacterial strains were selected on the basis of colony morphology from *R. ferrugineus* larvae and pupae respectively. Only one strain from larvae was Gram negative and was identified as *Stenotrophomonas* spp. since it shared only 96% identity with the reference strains. Nineteen strains were Gram positive including fifteen *Bacillus* spp., two *Paenibacillus* spp. and one *Brevibacillus* spp. One *Brevibacillus parabrevis* (Takagi *et al.,* 1993) (98% identity) and one *Paenibacillus pabuli* (Nakamura *et al.,* 1984) (98% identity) strains were correctly identified. Among the *Bacillus* spp., one strain was identified as *B. thuringiensis* Berliner, (Skerman *et al.,* 1980) (99% identity). (Fig. I, 1). The remaining *Bacillus* strains were not identified at species level by the analysis of 16S gene sequence because the percentage identity with the sequence of reference strains was lower than 97%.

Bacteria isolated from pupae were mostly Gram negative and belonged to the genera *Alcaligenes*, *Morganella*, *Myroides*, *Providencia* and *Serratia*. At species level one *Providencia rettgeri* (Jackson *et al.,* 1995) (99% identity) and two *Serratia marcescens* Bizio (Skerman *et al.,* 1980) (99% identity) strains were identified. Only three isolates, out of fifteen, were Gram positive and were all affiliated to *Bacillus* genus, the strain E2 was identified as *B. subtilis* subsp. *subtilis*. (Fig. I, 2). EMBL/GenBank/DDBJ accession numbers for the strains identified at species level were: A1, KX002005; E2, KX002006; B4, KX002007; B3, KX002008; D4, KX002009; C3, KX002010; D3, KX002011.

S. marcescens was the only chitinolytic species and was able to produce proteases, lecithinase (only strain D3) and lipases too. *B. thuringiensis* produced protease, lecithinase and lipase while *B. subtilis* subsp. *subtilis* showed proteolytic activity only. *B. parabrevis* produced lecithinase and lipase while *P. pabuli* was only lipolytic. *P. rettgerii* did not show any of the tested enzymatic activity (Table 1).

DISCUSSION

The search for natural enemies is a fundamental step to develop an IPM strategy against *R. ferrugineus*, a destructive parasite of palm trees, given the lack of an effective control method environmental friendly and safe for human health. The potential of entompathogenic bacteria has been investigated since from the last century when the efficacy of some *Bacillus* species in controlling dangerous pests was discovered (Sanchis and Bourguet, 2008; Ruiu *et al.,* 2013).

In the present study, bacterial strains isolated from larvae and pupae of *R. ferrugineus* were identified and characterized for their enzymatic activity. Four strains may be considered putative antagonists of *R. ferrugineus* while the remaining strains represent a part of the natural microflora normally associated to the insect.

Bacteria associated with larvae were mostly Gram positive spore-forming bacteria, with the majority of strains belonging to *Bacillus* genus. Strain A1 was identified as *B. thuringiensis*, one of the most effective entomopathogenic bacterial species. This species is able to produce parasporal bodies (Crystals) containing specific endotoxins (Cry proteins) acting by ingestion, that are the active molecules of several commercial insecticides. *B. thuringiensis* strain A1 was able to produce lecithinase, lipase and protease in vitro, three lytic enzymes that could be involved in degradative process of host cells. The presence of *Bacillus* species in the dead specimens of

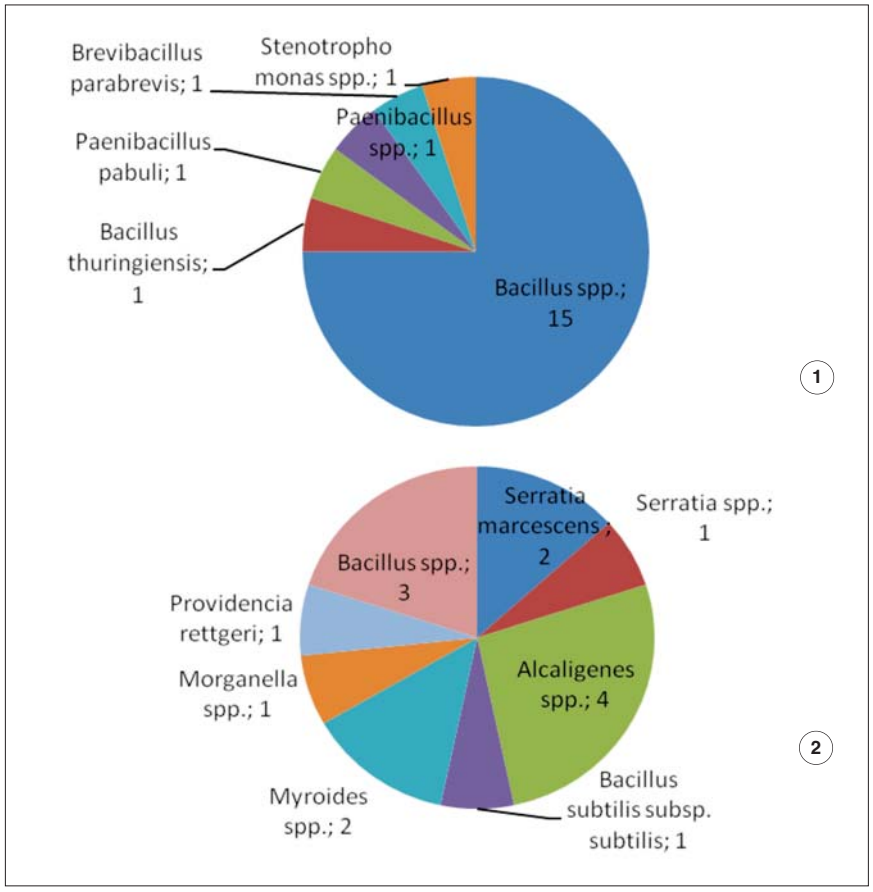

Fig. I – Distribution of bacteria isolated from larvae (1) and pupae (2) of *R. ferrugineus* on the basis of their genera or species affiliation.

Table 1 – Production of lytic enzyme by the bacterial strains isolated from larvae and pupae of *R. ferrugineus* (size of halo surrounding the colony: + : < 1 cm; ++: > 1 cm).
B = *Bacillus*; Br = *Brevibacillus*; P = *Paenibacillus*; Pr = *Providencia*; S = *Serratia*.

	Origin	Gram	Chitinase	Lecithinase	Lipase	Protease
B. thuringiensis A1	larvae	+	-	++	+	++
P. pabuli B3	larvae	+	-	-	+	-
Br. parabrevis B4	larvae	+	-	+	+	-
S. marcescens C3	pupae	-	+	-	+	+
S. marcescens D3	pupae	-	++	+	++	++
Pr. rettgeri D4	pupae	-	-	-	-	-
B. subtilis. subsp. subtilis E2	pupae	+	-	-	-	+

larvae showed that in some cases the antimicrobial defense compounds detected in the cuticola of *R. ferrugineus* larvae that inhibit *Bacillus* spp. growth, may be overcome (Mazza *et al.,* 2011). Evidences derived from literature reported that *B. thuringiensis* activity is low against *R. ferrugineus* immature stages notwithstanding midgut damages and feeding inhibition (Dembilio and Jacas, 2013) while *B. thuringiensis* var. *kurstaki* activity against *R. ferrugineus* larvae is high (Mahmoud *et al.,* 2011). Strain B4 of *B. parabrevis* isolated from larvae was able to produce lecithinase and lipase in vitro. This species has never been reported as an enthompathogen, but it was previously isolated from adults of the Coleoptera *Lasioderma serricorne*, (Fabricius) (Yaman *et al.,* 2008) where probably it is able to establish a symbiotic interaction with the host. The species *Brevibacillus laterosporus* is able to develop mutualistic (with *Apis mellifera*) or pathogenic (with *Musca domestica*) interactions with diverse insect species, probably as the result of a coevolutionary process (Marche *et al.,* 2016). In the light of this findings, the kind of interaction occurring between *R. ferrugineus* larvae and *B. parabrevis* may deserve further analysis.

Bacterial microflora of *R. ferrugineus* pupae showed a strong diversity. Five different Gram negative and one Gram positive genera of bacteria were identified. Strain E2 was identified as *B. subtilis* subsp. *subtilis* (Skerman *et al.,* 1980) and was able to produce protease. For some

spore-forming isolates belonging to the *Bacillaceae* family, the 16S sequence similarity was less than 97% so that they could not be affiliated to any species. Given the complexity and variability of this group of bacteria, a multiphasic approach is often needed to precisely identify *Bacillus* strains (Schmidt *et al.,* 2011). Strains D4 of *P. rettgeri* and C3 and D3 of *S. marcescens* are the most interesting bacteria isolated from pupae. *P. rettgerii* is an insect pathogenic bacterium that has been found associated to *Galleria mellonella* L. larvae and *Drosophila melanogaster* Meigen, 1830; the bacterium may be highly lethal to these hosts (Jackson *et al.,* 1995; Galac and Lazzaro, 2011). *S. marcescens* is the most frequently reported entomopathogen for a wide of invertebrate hosts (included Coleoptera) where it is able to colonize the gut and later pass to the hemocoel causing the destruction of hemocites and internal organs (Sanchez-Contreras and Vlisidou, 2008). This mechanism seems to be very common among the entomopathogenic non spore-forming bacteria (Mahar *et al*., 2005) that do not elicit the systemic immune response of the host. Bacteria belonging to *Bacillus* and *Serratia* genera have been already reported in association to *R. ferrugineus* larvae or pupae (BUTERA *et al.,* 2012; Mahmoud *et al.,* 2001; Salama *et al.,* 2004). To our knowledge this is the first time that the entomopathogenic bacterial species *Providencia rettgeri* was found as component of the microflora associated to this insect.

Many bacteria pathogens to insects are active producers of secondary metabolites toxic to insects or other kind of organisms, that can be used as novel molecules for controlling both plant pathogens and pests (Bode, 2009). The extraction and identification of secondary metabolites produced by the entomopathogenic bacteria isolated in this study as well as the in vivo activity of bacterial cells against *R. ferrugineus* needs to be evaluated.

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