Journal of Invertebrate Pathology 104 (2010) 214–221

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the Red Palm Weevil, *Rhynchophorus ferrugineus*

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ARTICLE INFO

Article history:
Received 22 February 2010
Accepted 10 April 2010
Available online 14 April 2010

Keywords:
Beauveria bassiana
Rhynchophorus ferrugineus
Palmae
Curculionidae
Hypocreales
Pathogenicity
Virulence
Horizontal transmission
Efficacy
Entomopathogenic fungi

ABSTRACT

The potential of a strain of *Beauveria bassiana* (Ascomycota: Clavicipitaceae) obtained from a naturally infected *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) pupa as a biological control agent against this weevil was evaluated both in the laboratory and in semi-field assays. Laboratory results indicate that this strain of *B. bassiana* can infect eggs, larvae and adults of *R. ferrugineus* (LC_{50} from 6.3×10^7 to 3.0×10^9 conidia per ml). However, mortality was not the only indicator of treatment efficacy because adults of either sex inoculated with the fungus efficiently transmitted the disease to untreated adults of the opposite sex, with male-to-female and female-to-male rates of transmission of 55% and 60%, respectively. In addition, treatment with *B. bassiana* significantly reduced fecundity (up to 62.6%) and egg hatching (32.8%) in pairing combinations with fungus-challenged males, females or both sexes. Likewise, 30–35% increase in larval mortality was observed in larvae obtained from eggs from fungus-challenged females or from untreated females coupled with inoculated males, resulting in an overall 78% progeny reduction. Semi-field preventive assays on potted 5-year old *Phoenix canariensis* palms, with efficacies up to 85.7%, confirmed the potential of this strain as a biological control agent against *R. ferrugineus*.

1. Introduction

The invasive red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), has become the major pest of palms in the Mediterranean Basin, where it spread slowly during the mid 1990s and very quickly during the last five years. This pest is at present widely distributed in Oceania, Asia, Africa and Europe (EPPO, 2008) and was found in Curaçao, Netherlands Antilles, in December 2008 (EPPO, 2009). Females lay eggs at the base of the fronds in separate holes made with their rostrum. Neonate larvae bore into the palm core and upon completion of development move back to the base of the fronds to pupate. A new generation emerges and adults may remain within the same host and reproduce until the palm eventually dies. Subsequently, adults move to new hosts. *R. ferrugineus* has been reported on 19 palm species belonging to 15 different genera (EPPO, 2008; Dembilio et al., 2009). Several control methods have been applied against this pest within an Integrated Pest Management strategy. Its main components are phyto-sanitation, which involves cutting down and burning infested palms, use of insecticides and use of pheromone traps for adult monitoring and mass trapping (Murphy and Briscoe, 1999; Faleiro, 2006).

Few studies have been conducted on the natural enemies of *R. ferrugineus* or other *Rhynchophorus* species (Murphy and Briscoe, 1999; Faleiro, 2006). *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae) proved effective against *R. ferrugineus* in semi-field and field trials including both preventive and curative assays (Llácer et al., 2009; Dembilio et al., 2010). Different strains of *Metarhizium anisopliae* (Metschnikoff) Sokorin (Ascomycota: Clavicipitaceae) and *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Clavicipitaceae) were tested against *R. ferrugineus* (Gindin et al., 2006). The former proved more virulent than *B. bassiana* and achieved 100% larval mortality within 6–7 days. However, none of the strains tested was originally obtained from diseased *R. ferrugineus* specimens. In 2005–2006, El-Sufty et al. (2009) obtained a mortality of 12.8–47.1% in adult *R. ferrugineus* population in field assays using a strain of *B. bassiana* isolated in the United Arab

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0022-2011/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2010.04.006

Emirates. More recently, Sewify et al. (2009) successfully reduced the incidence of *R. ferrugineus* under field conditions in Egypt using a native strain of *B. bassiana* isolated from a *R. ferrugineus* cadaver.

In 2007, *R. ferrugineus* pupae presumed to be infected with entomopathogenic fungi were collected in a date palm grove in Spain near the town of Catral (lat.: 38°09′29″N; long.: 00°46′33″W; alt.: 12 m). One of these pupae proved to be infected with the entomopathogenic fungus *B. bassiana*. This species is one of the most widely distributed entomopathogenic fungi in the world and it infects insects in tropical, temperate, humid, and desert areas (Zimmermann, 2007), some of them of agricultural importance, such as the coffee berry borer *Hypothenemus hampei* Ferrari (De la Rosa et al., 1997) and other Curculionidae (Adane et al., 1996). In these studies adults were the most suitable or the only possible stage for treatment. Unlike other insect pathogens, entomopathogenic fungi infect the host by contact, penetrating the insect cuticle. The host can be infected both by direct treatment and by horizontal transmission from infected insects or cadavers to untreated insects or to subsequent developmental stages via the new generation of spores (Lacey et al., 1999; Quesada-Moraga et al., 2004). Passive mechanical transmission of fungi within insect populations has been observed for various entomopathogenic fungi, e.g. *B. bassiana*, *M. anisopliae* and *Isaria fumosorosea* (Lacey et al., 1999; Quesada-Moraga et al., 2004, 2008). These unique characters make entomopathogenic fungi especially important for the control of concealed insects. In the case of *R. ferrugineus*, most of its life cycle occurs within the palm, making the pest inaccessible to direct-contact treatments. Adults are the only exposed stage and can be infected upon emergence. Evidence for successful application of fungi via adults was obtained from experiments on the pinhole borer, *Platypus spp*. (Coleoptera: Curculionidae). Adults that were contaminated with *B. bassiana* or *M. anisopliae* spores transferred the fungal infection to larvae, which resulted in 50–100% larval mortality (Glare et al., 2002).

The aim of this work was to evaluate in the laboratory both lethal and sublethal effects of an indigenous strain of *B. bassiana* against different stages of *R. ferrugineus* and to determine the efficacy of a formulation of this strain in a semi-field trial as a first step to reveal the biocontrol potential of this strain.

2. Materials and methods

2.1. Entomopathogenic fungus

The *B. bassiana* strain used in the experiment was isolated from an infected pupa originally collected in a date palm grove near the town of Catral, Spain, and belongs to the fungal collection of the Departamento de Ciencias y Recursos Agrícolas y Forestales of the University of Córdoba (Spain) with the reference code EABb 07/06-Rf. This strain was deposited with accession No. CECT-20752 on May 13, 2009, following the Budapest Treaty, in the Spanish Collection of Culture Types (CECT) at the University of Valencia (Burjassot, Spain). Fungal cultures were grown on malt agar (12.75 g/l malt extract, 2.75 g/l dextrine, 2.35 g/l glycerol, 0.78 g/l gelatine peptone and 15.0 g/l agar) at 25 °C in the dark. Conidial suspensions for experiments were prepared by scraping conidia from 15-day old, well sporulated cultures into an aqueous solution of 0.2% Tween 80. Suspensions were then filtered through several layers of cheesecloth to remove mycelium. To homogenize the inoculum, they were sonicated for 10 min (P-selecta ultrasounds, Barcelona, Spain). Suspensions were prepared independently for each assay and therefore conidial concentrations used were not exactly the same. Conidial concentrations were determined using a hemocytometer. Viable germinating conidia were counted after 36 h of incubation at 25 °C in malt agar. Germination of conidia was over 95%. Suspensions were kept in 4 °C dark storage before use (Goettel and Inglis, 1997).

2.2. Stock colonies

Adult weevils collected in the province of Valencia in traps baited with ferrugineol (the male *R. ferrugineus* aggregation pheromone) and plant kairomones (ethyl acetate and pieces of palm fronds) were used to start the stock colonies. These colonies were established in 2007 and have been periodically supplemented with the introduction of additional wild specimens. Adult weevils were reared in a controlled environment cabinet at 25 ± 1 °C, 75 ± 5% R.H. and a 16-h light photoperiod in perspex cages (30 × 30 × 45 cm depth) with a density of 120–150 weevils per cage. These cages had a round hole (8 cm in diameter) on the upper side covered by a mesh used for manipulation of the specimens and its bottom side consisted of a 2 mm metal mesh used by females for oviposition. Cages were set on top of a tray containing a folded piece of moistened filter paper containing thin apple slices used by female weevils as oviposition substrate and by both males and females as food. Apple slices were replaced three times per week (Dembilio et al., 2009).

2.3. Experimental insects

Eggs, less than 24-h old, were obtained from the stock colonies and used in our assays. When necessary, eggs were further kept on apple slices until hatching or reared up until the fourth instar or to adulthood (assays with neonate larvae, fourth instars and laboratory-reared adults, respectively). Upon hatching, neonate larvae were individually transferred to 125 ml vials containing 45–50 ml of the weevil's artificial diet (Martín and Cabello, 2006). Larvae were moved to a new vial fortnightly until ready for pupation (45-day old larvae). At that moment, larvae were moved to another vial containing strands of dry esparto grass (*Stipa tenacissima* L.) used by the larvae to build a cocoon. About one month later, laboratory-reared adults were obtained. Adults collected in field-traps as described were also used in our laboratory assays.

2.4. Plant material

Semi-field assays were performed on 5-year old potted *Phoenix canariensis* palms obtained from an officially inspected nursery (EU, 2007) and therefore were presumed to be free of *R. ferrugineus*. The stipe of these palms was 0.35–0.55 m high and 0.30–0.40 m wide. Plants were watered twice a week and kept inside a double mesh security enclosure containing 24 independent cages (4 × 3 × 3 m). Palms were kept under natural light and temperature conditions during summer-fall 2009. Mean temperature during the assays was 24.5 °C (max: 42.2 °C; min: 6.3 °C). A plastic roof protected the enclosure from the rain.

2.5. Bioassays

R. ferrugineus is quite a difficult and expensive insect to rear in the laboratory. Therefore, the number of insects used in our experiments had to be considered very carefully. Preliminary pathogenicity tests were conducted on both adult and immature stages of *R. ferrugienus* firstly to perform the Koch postulates with the indigenous strain against all instars and secondly to define the range of dosages to be used in the biological activity assays. Therefore, each stage was tested at least twice before undertaking the laboratory assays described below. Laboratory assays took place in a controlled environment cabinet at 25 ± 1 °C, 75 ± 5% R.H. and a 16-h light photoperiod, whereas semi-field trials took place at