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ScienceDirect
Journal of Invertebrate Pathology 93 (2006) 210–213
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Short communication

A new bioassay method reveals pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against early stages of *Capnodis tenebrionis* (Coleoptera; Buprestidae)

Pierdomenico Marannino [a,b], Cándido Santiago-Álvarez [a], Enrico de Lillo [b], Enrique Quesada-Moraga [a,*]

[a] *Departamento de Ciencias y Recursos Agrícolas y Forestales, ETSIAM, Universidad de Córdoba, Campus de Rabanales, Edificio C4 Celestino Mutis, Cordoba 14071, Spain*
[b] *Dipartimento di Biologia e Chimica Agro-forestale e ambientale, Università degli studi di Bari, Via Amendola 165/a, Bari 70126, Italy*

Received 1 March 2006; accepted 7 August 2006
Available online 22 September 2006

Abstract

We used a newly developed bioassay method to demonstrate for the first time the potential of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to be used for the control of neonate larvae of *Capnodis tenebrionis*, a major threat to stone-fruit orchards in several countries. Four *B. bassiana* and four *M. anisopliae* isolates were all pathogenic for neonate larvae of *C. tenebrionis*; mortality rates 10 days after inoculation by dipping in a suspension with 10^8 conidia/ml varied from 23.5% to 100%. Three of the four *M. anisopliae* isolates caused 100% mortality. In most cases, postmortem hyphal growth and sporulation of *M. anisopliae* or *B. bassiana* was observed covering the larvae in their galleries. The eight isolates were also evaluated for pathogenicity to *C. tenebrionis* eggs at the same dosage. Only two *B. bassiana* isolates caused significant egg hatching reduction of 84.5% and 94.5%. Our results indicate that *M. anisopliae* and *B. bassiana* may be considered as promising for a new approach to prevent larval infestations by *C. tenebrionis*.

Keywords: *Beauveria bassiana*; *Metarhizum anisopliae*; Pathogenicity; Virulence; Neonate larvae; Eggs; Endophitic; Biological control; *Capnodis tenebrionis*; Buprestidae

The Mediterranean flatheaded peachborer, *Capnodis tenebrionis* (L), is a key pest of stone-fruit crops of the family Rosaceae in Southern Europe and the Mediterranean basin (de la Beffa, 1961; Balachowsky, 1962; Avidov and Harpaz, 1969; Alfaro-Moreno, 2005). Adults cause defoliation by feeding on twigs and young branches (Garrido, 1984). This type of damage is more serious in greenhouse trees and nurseries than in fruit bearing orchards. Females oviposit on the trunk base, on superficial roots or freely on the soil. Neonate larvae move through the soil and penetrate host root tissue (Marannino et al., 2004), where they develop endophytically, forming large, sinuous galleries. Larval feeding causes the principal economic damage; young trees can die within 1 or 2 yr while mature trees are less affected (Balachowsky, 1962). As a result of the severe injury it produces, the EU has included *C. tenebrionis* on the list of noxious organisms prejudicing the quality of stone-fruit propagating material (Comm. dir. 93/48/EEC).

Current *C. tenebrionis* control programs depend almost solely on repeated applications of chemical insecticides against the adults and against neonate larvae on the soil surface. The complexity of *C. tenebrionis* chemical control and its negative environmental impact has encouraged the search for alternative pest management strategies (Sekkat et al., 1997; Ben-Yehuda et al., 2000), especially to protect organic fruit production.

* Corresponding author. Fax: +34 957218440.
E-mail address: cr2qumoe@uco.es (E. Quesada-Moraga).

0022-2011/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2006.08.002

Although there are scarce records of natural enemies affecting *C. tenebrionis* populations (Marannino and de Lillo, 2005), biological control options need to be explored for solutions to this pest problem. Because entomopathogenic nematodes and fungi penetrate the insect host through the integument, they may be able to be used to prevent root colonization by neonates. The main hindrance for evaluating the potential of these agents for control of neonate larvae of *C. tenebrionis* and other wood boring larvae has been the lack of a reliable bioassay protocol simulating the insect's endophytic location for development. The potential of entomopathogenic fungi against *C. tenebrionis* has not been evaluated, and entomopathogenic nematodes (*Steinernema* sp., *Heterorhabditis* sp.) have been ascertained only in laboratory bioassays using neonate larvae under non-endophytic conditions (García del Pino and Morton, 2005). The aim of the present study was to investigate whether or not selected isolates of *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Balsamo) Vuill. could infect *C. tenebrionis* neonate larvae using a bioassay simulating their natural endophytic growing conditions. We also examined the susceptibility of *C. tenebrionis* eggs to isolates of both fungal species.

The strains used in the experiment belong to the fungal collection of Agricultural and Forestry Sciences and Resources (AFSR), Department of the University of Cordoba (Spain). There were eight fungal isolates, four of *B. bassiana* [EABb 01/33-Su and EABb 01/103-Su isolated from an olive orchard and a forest soil, respectively and EABb 04/01-Tip and 1333 isolated from *Timaspis papaveris* (Hymenoptera; Cynipidae) and *Bactrocera oleae* (Diptera; Tephrytidae), respectively], and four strains of *M. anisopliae* (EAMa 01/121-Su and EAMa 01/152-Su from cotton fields, EAMa 01/58-Su from a wheat crop and EAMa 01/44-Su from a non-cultivated soil, respectively). Fungal isolation from soil samples was performed using the *Galleria* bait method (Zimmermann, 1986).

Conidial suspensions for experiments were obtained by scraping conidia from 15-day-old cultures 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 darkness into an aqueous solution of 0.002% Tween 80. Then they were filtered through several layers of cheesecloth to remove mycelium and the concentrations of viable conidia were estimated as colony forming units using a dilution plate count method.

Adults of *C. tenebrionis* captured in autumn 2005 from heavily infested organic Apulian and Sicilian apricot orchards (Italy) were reared on fresh apricot twigs (de Lillo, 1998) under laboratory conditions (28–30 °C, 40–50% R.H., 16:8 L:D photoperiod). Adults oviposited in dishes filled with sand, previously sifted to 0.8 mm, according to the method described by Garrido et al. (1987). Neonate larvae,

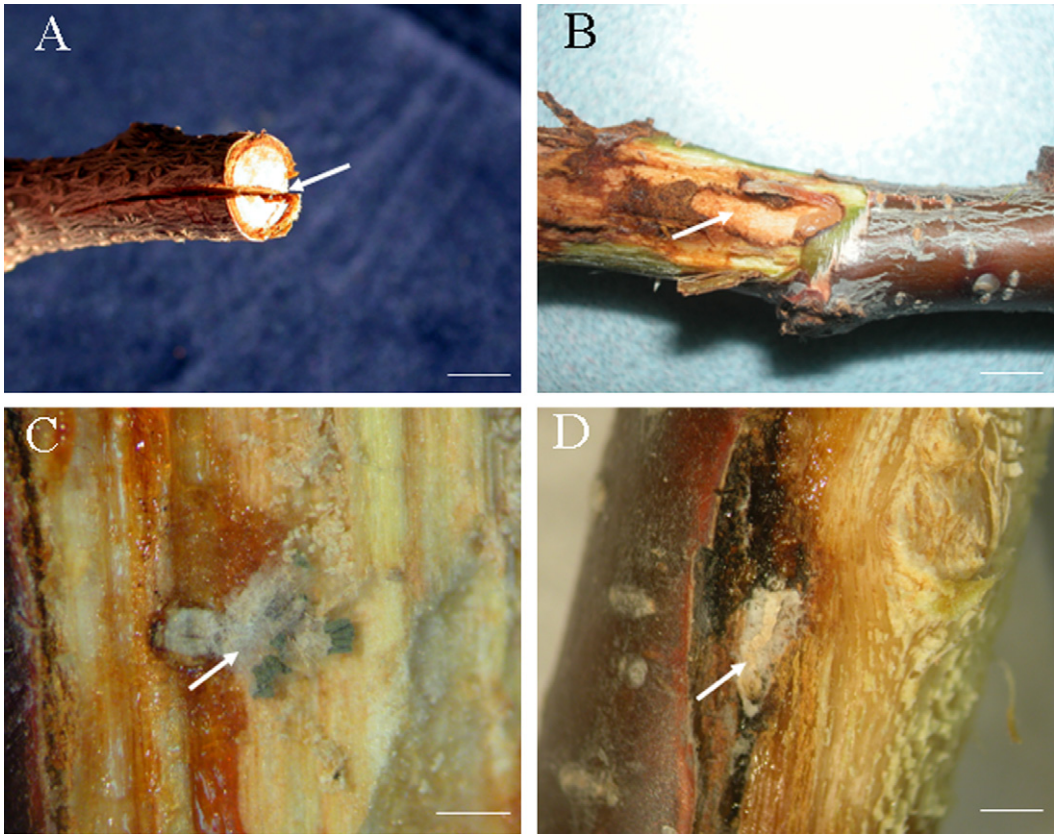

Fig. 1. Apricot branches used in bioassays of fungi against *C. tenebrionis*. (A) Branch piece prepared for larval rearing with a longitudinal cut at one end (arrow). (B) Control larva within a gallery (arrow). (C) Neonate larval cadaver mycosed by *M. anisopliae* within a gallery. (D) Neonate larval cadaver mycosed by *B. bassiana* within a gallery. Bars = 10 mm.

24-h-old, selected from the rearing boxes were immersed individually for 10 s in a spore suspension (1.0×10^8 conidia/ml) or in 0.002% Tween 80 aqueous solution (for controls). Then they were transferred with a soft paintbrush (no. 2) onto the cut surface of a 10 cm by ~1 cm diameter piece of apricot branch (collected from an organic orchard) (Fig. 1A). Infested branches were kept in rearing boxes at 25 °C and 65% R.H. in the dark. After 10 days, each branch piece was stripped of its bark and examined for larvae using a dissecting microscope. Each of four replicates per fungal isolate and control consisted of 10 larvae; the experiment was done twice. When external signs of fungal infection were not directly observed on larvae, cadavers were immediately surface sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water, placed on sterile wet filter paper in sterile Petri dishes, sealed with parafilm and kept at room temperature.

Bioassays on egg masses were conducted using seven-day-old eggs immersed individually in a spore suspension (1.0×10^8 conidia/ml) or in 0.002% Tween 80 aqueous solution (for controls), for 10 s, placed in sterile Petri dishes and kept at 25 °C, in dark, until hatching was completed. Each of four replicates per fungal isolate and control consisted of 10 eggs; the experiment was done twice.

The effect of isolate on mortality was analyzed using a one-way analysis of variance (ANOVA) ($\alpha = 0.05$) using SPSS (Statistical Package for Social Sciences in personal computers) 11.0 for Windows (SPSS, 2002).

Neonate larvae of *C. tenebrionis* were susceptible to entomopathogenic fungi. There was a significant effect of the fungal treatment ($F_{8,35} = 266$, $P < 0.001$), with mortality of larvae ranging from 23.5% to 100% among the isolates (Fig. 2A). The entomopathogenic fungi were recovered from all cadavers appearing typically dried. No mortality was recorded in the control (Fig. 2A). Larvae surviving the fungal treatment had reached the 2nd instar and were found in galleries extending over more than one-third of the total length of the branch piece (Fig. 1B). In general, *M. anisopliae* isolates caused higher mortality rates than those of *B. bassiana*. The isolates can be categorized in four groups: a first group comprised weakly pathogenic isolate 1333 (23.5% mortality), a second group comprised moderately virulent *B. bassiana* isolates 01/33-Su and 01/103-Su (43.5% and 53.5% mortality, respectively), a third group with *M. anisopliae* isolate 01/44-Su and *B. bassiana* isolate 04/01-Tip (68.5% and 73.0% mortality, respectively), and a fourth group including the most virulent *M. anisopliae* isolates 01/58-Su, 01/152-Su and 01/121-Su causing 100% mortality (Fig. 2A). In most cases, postmortem hyphal growth and sporulation of *M. anisopliae* or *B. bassiana* was observed covering the larvae in the galleries (Fig. 1C and D); such a phenomenon could be promoted by the high humidity conditions prevailing in the microclimate surrounding the endophytic larvae. To our knowledge, this is the first investigation showing the susceptibility of *C. tenebrionis* neonate larvae to entomopathogenic fungi. The experimental protocol followed in the present research has

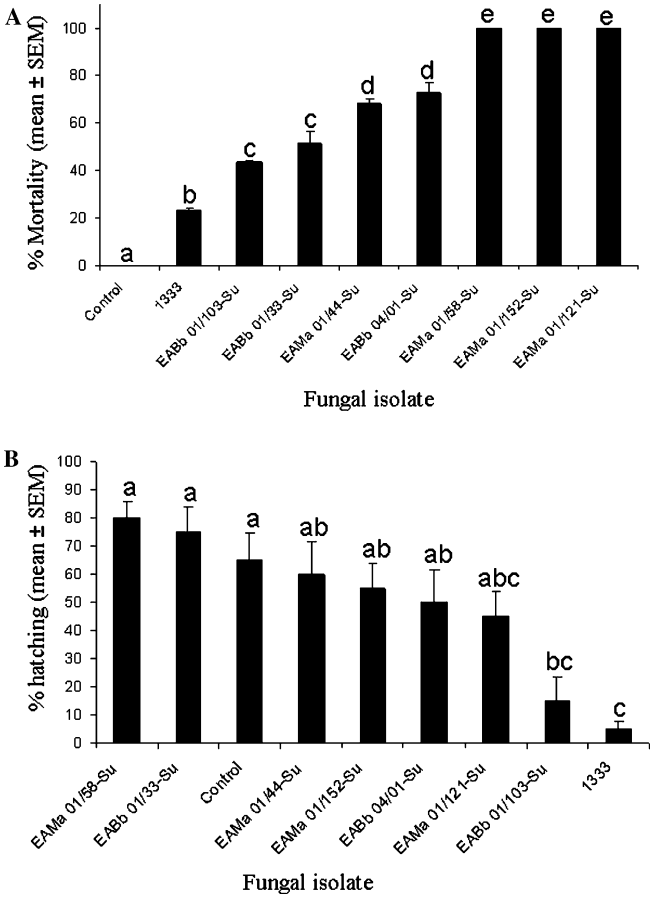

Fig. 2. (A) Pathogenicity of eight fungal isolates on neonate larvae of *C. tenebrionis* at 1.0×10^8 conidia/ml based on mean mortality (±SEM) 10 days after treatment. (B) Percentage hatching (mean ± SEM) of *C. tenebrionis* eggs treated with 1.0×10^8 conidia/ml of eight fungal strains. For each experiment, mortality data with the same letter are not significantly different (one-way ANOVA, $P < 0.05$; Tukey test).

allowed us to provide evidence of fungal infection once the larvae have colonized the host branches and have begun forming their typical galleries. Based on our findings, newly hatched first instar larvae are susceptible to infection by entomopathogenic fungi. This larval stage is the only free-living one, demonstrating a potential practical target of application in the field.

Egg hatch was also significantly reduced by fungal inoculation ($F_{8,35} = 7.9$, $P < 0.001$ for 1×10^8 conidia/ml). However, only *B. bassiana* isolates 01/103-Su and 1333 caused significant reduction of egg hatching, 84.5% and 94.5%, respectively (Fig. 2B). Interestingly, these isolates were weakly pathogenic to neonate larvae. Differential response of life stages to fungal infection was also reported by Gindin et al. (2000), who observed the higher virulence of some *Lecanicillium lecanii* isolates to occur against newly emerged *Bemisia argentifolii* (Hemiptera; Aleyrodidae) nymphs, while some other were more virulent against the eggs. However, our results disagree with those of Samuels et al. (2002), who found *M. anisopliae* isolates to be more virulent to *Blissus antillus* (Hemiptera; Lygaidae) eggs than *B. bassiana* ones.

In conclusion, laboratory bioassays have clearly demonstrated the pathogenicity of some isolates of *M. anisopliae* and *B. bassiana* for larvae and eggs of *C. tenebrionis*. These fungi should be considered as promising for development of a new, environmentally compatible approach to peachborer management aiming at preventing larval infestation. Further studies are needed to determine the biological activity of fungal isolates, appropriate formulation and delivery methods, persistence after field application and agronomic factors that may improve their performance against *C. tenebrionis* throughout its oviposition period.

Acknowledgments

The authors gratefully acknowledge Professor Brian Federici from the Department of Entomology, University of California Riverside and Dr. John D. Vandenberg from the US Plant, Soil and Nutrition Lab of the USDA Agricultural Research Service (Tower Road, Ithaca), for the kind revision of the manuscript. This research was supported by Project no. 36 of the I.F.A.P.A. (expdte 92162/1) from the regional government of Andalusia (Junta de Andalucía) and by a grant from the University of Bari. E. Quesada-Moraga is a contract holder of a 'Ramón y Cajal' grant from Ministerio de Educación y Ciencia of Spain. P. Marannino is a fellowship holder at University of Bari.

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