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Combined use of entomopathogenic fungi and their extracts for the control of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

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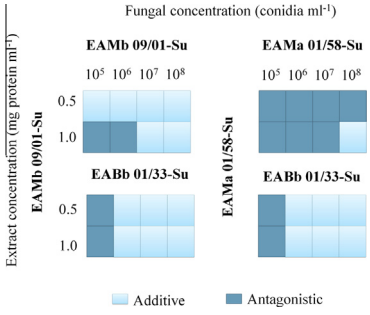
HIGHLIGHTS

- Isolates of *Beauveria* and *Metarhizium* genera were pathogenic against *S. littoralis* larvae.
- The most of their crude extracts were toxic for *S. littoralis* larvae.
- Spray application of crude extracts did not cause mortality on *S. littoralis* larvae.
- Combined application of EF and their extracts obtained additive effect at high doses.

GRAPHICAL ABSTRACT



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ABSTRACT

Both the virulence and insecticidal activities of the crude extracts of 26 isolates of the entomopathogenic mitosporic ascomycetes *Metarhizium* sp. and *Beauveria* sp. (Ascomycota, Hypocreales) were evaluated against the second-instar larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae), a very harmful polyphagous insect pest. Although all isolates caused infection when the second-instar *S. littoralis* larvae were immersed in the conidial suspensions, only four isolates of *Beauveria* (EABb 01/33-Su, EABb 01/88-Su, EABb 01/103-Su, and 3155) and one isolate of *Metarhizium* caused >50% mortality of the larvae, with average survival times (ASTs) of 9.67 and 8.73 days for the isolates EABb 01/33-Su and EABb 01/88-Su, which caused the highest mortality rates of 78.33% and 75.00%, respectively. The LC_{50} and LT_{50} values were 5.69×10^6 conidia ml^{-1} and 6.76 days for EABb 01/33-Su and 1.05×10^7 conidia ml^{-1} and 7.02 days for EABb 01/88-Su. By contrast, the crude extracts obtained from the *Metarhizium brunneum* EAMb 09/01-Su and EAMa 01/58-Su isolates caused the highest mortality rates of 80.00% and 66.66% and the lowest ASTs of 5.13 and 4.43 days, respectively. Topical application of the crude extracts did not cause mortality. Combined treatments with fungal suspensions of the isolates EAMb 09/01-Su and EAMa 01/58-Su and their extracts caused higher mortality rates than the single isolates and extracts, with the increases occurring in a dose-dependent manner, and with mortality rates reaching 100% for the EAMb 09/01-Su isolate and its extract at 1 mg ml^{-1} and 76% for the EAMa 01/58-Su isolate and its extract at 1 mg ml^{-1}. The combination of the maximal concentrations of both fungi and crude extract had an additive effect on larvae, resulting in 100% mortality for the combination of the extract EAMb 09/01-Su with the strains EABb 01/33-Su and EAMb 09/01-Su. These results show the potential of certain entomopathogenic fungal isolates for use in an integrated *S. littoralis* management strategy targeting larvae, as well as the potential of the combined use of entomopathogenic fungi and their extracts.

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1. Introduction

Lepidopteran pests cause billions of dollars in crop loss worldwide each year, making them one of the most dangerous classes of agricultural pests (Fan et al., 2012). The cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae) is one of the most destructive phytophagous pests because it can attack numerous economically important crops in either protected or open fields in the Mediterranean region and the Middle East, particularly in Cyprus, Israel, Malta, Morocco, Italy, Greece, and Spain (Hatem et al., 2009; Azab et al., 2001; Ahmad, 1988; Blackford et al., 1997; Champion et al., 1997; Salama et al., 1970). The life cycle of this insect can be completed in approximately 5 weeks. Females lay egg masses (100–300 eggs each) on the lower surfaces of leaves of host plants. After hatching, larvae feed in groups at night and in the early morning, and they pass through six instars before pupating in soil (Amate et al., 2000; Salama et al., 1970). *S. littoralis* attacks most vegetable crops, including tomato, pepper, eggplant, lettuce, artichoke, strawberry and asparagus, but it also damages ornamental plants and herbs (Lanzoni et al., 2012).

The overuse of and reliance on chemical insecticides for controlling *S. littoralis* has resulted in the emergence of insecticide resistance and cross-resistance (Ghribi et al., 2012). In addition, *Bacillus thuringiensis*-resistant field populations have been detected in several countries (Assaeedi et al., 2011). Therefore, the development of new, environmentally safe and sustainable alternatives has become an urgent need for integrated control programs for the cotton leafworm, with emphasis on biological control through entomophagous predators, parasitoids, and microorganisms.

Entomopathogenic fungi (EF), in particular entomopathogenic mitosporic ascomycetes, have been shown to successfully control a large variety of lepidopteran pests (Quesada-Moraga et al., 2013; Schulte et al., 2009; Devi et al., 2005; Vänninen and Hokkanen, 1997). Their unique tegumentary mode of action and horizontal transmissibility have been used for the development of alternative control strategies in recent years (Quesada-Moraga and Santiago-Álvarez, 2008; Quesada-Moraga et al., 2006; Wraight et al., 2000). Moreover, EF have been shown to contribute not only as controllers of insect populations but also as producers of novel insecticidal compounds (Isaka et al., 2005). These insecticidal compounds can be either high-molecular-weight proteins or low-molecular-weight secondary metabolites, both showing high mortality and antifeedant properties against lepidopteran insects (Amiri et al., 1999; Quesada-Moraga et al., 2006).

The combined use of EF and synthetic insecticides or natural compounds has been proposed as a strategy to improve their efficacy (Hu et al., 2007) by reducing the lethal times (Sharififard et al., 2011) or enhancing the virulence of the fungi (Zurek et al., 2002). To date, the interactions between EF and chemical insecticides or even fungal compounds have been addressed for lepidopteran pest control (Ribeiro et al., 2012; Cazorla and Morales Moreno, 2010; Hu et al., 2007; Devi et al., 2004). However, the effect of combining treatments of EF and of their own extracts has not previously been tested for lepidopteran pest control.

The aim of this study was to evaluate the biological activities of 26 isolates from different species of EF and the insecticidal activities of their crude extracts and to evaluate combined treatments of EF and their crude extracts against *S. littoralis* larvae, supported by the findings of previous studies of pathogens and toxins demonstrating positive results against Lepidopteran pests (Lastra et al., 1995).

2. Materials and methods

2.1. S. littoralis (Boisduval) (Noctuidae, Lepidoptera) rearing

S. littoralis larvae used in this study were obtained from a stock colony at the Department of Agricultural and Forestry Sciences of the University of Córdoba (Spain). These insects were originally collected in the field from various crops. They were maintained in an environmental chamber programmed at 26 ± 2 °C, 70 ± 5% RH, and photoperiod of 16:8 (L:D) h (Poitout and Bues, 1974). Adults were placed in oviposition chambers consisting of a cylindrical filter paper (150 × 120 mm) closed at both ends with a layer of filter paper. Cotton moistened with a 10% honey solution was placed inside, in a small container. This cotton was replaced every two days to prevent possible contamination and fermentation. Each chamber contained 5 pupae of each sex. The chambers were observed daily to monitor and collect the egg clusters, which were externally disinfected by immersion in 10% formalin for 10 min and then washed three times with sterile water to remove any formalin residues. The disinfected egg clusters were then placed on pieces of filter paper to remove water. Filter papers containing 12 or 15 egg clusters were placed at the bases of plastic containers (6×10^6 mm^3) with perforated covers. Approximately 2400 to 3000 eggs were placed in each container. The larvae were reared until pupation on an artificial diet consisting of alfalfa meal (85 g), brewer's yeast (34 g), wheat germ (32 g), agar–agar (18 g), casein (14 g), ascorbic acid (4.5 g), benzoic acid (1.3 g), nipagin (1.1 g), 10% formaldehyde (5 ml) and distilled water (800 ml) (Santiago-Álvarez, 1977). The pupae were removed and separated by sex using a stereomicroscope (Nikon SMZ800, Japan), after which they were classified by size and vigour and transferred to oviposition chambers under the insectary conditions described above.

2.2. Fungal isolates and preparation

The 26 fungal isolates used in this study were obtained from the culture collection of the Department of Agricultural and Forestry Sciences (AFS) of the University of Córdoba (Table A.1). They were selected based on various criteria, including fungal species (*Beauveria bassiana*, *Beauveria pseudobassiana*, *Metarhizium anisopliae*, *M. brunneum*, *Metarhizium robertsii*) and habitat of isolation (soil, plant, and insect). All of the isolates were obtained from monosporic cultures and maintained lyophilised at −80 °C. To prepare inoculums for the experiments, slant cultures of the lyophilised isolates were sub-cultured on malt agar for 15 d at 25 °C in darkness. The Petri plates were sealed with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL). Conidial suspensions were prepared by scraping conidia from the Petri plates into a sterile aqueous solution of 0.1% Tween 80. These initial suspensions were sonicated for 5 min and then filtered through several layers of cheesecloth to remove mycelial structures. The germination rates were evaluated in Petri plates with water agar as the substrate at 25 °C and 12 h after inoculation, with consistent rates of above 90% (Goettel and Inglis, 1997). The conidial suspensions used for the bioassays were adjusted by diluting the conidia with 0.1% Tween 80 to a final concentration of 1.0×10^8 conidia ml^{-1}. The number of conidia was estimated using a Malassez chamber.

2.3. Initial pathogenicity assay for entomopathogenic fungal isolates against S. littoralis larvae

Second-instar (L_2) *S. littoralis* larvae were treated with the 26 isolates. The larvae were immersed in 5 ml of conidial suspension