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Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects

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ABSTRACT

The current work reports how spray application of entomopathogenic fungi on alfalfa, tomato and melon plants may cause an additional *Spodoptera littoralis* larvae mortality due to a temporal colonization of the leaves and subsequent ingestion of those leaves by the larvae. Most entomopathogenic fungi (EF) (Ascomycota: Hypocreales) endophytes seem to colonize their host plants in a non-systemic pattern, in which case at least a transient endophytic establishment of the fungus should be expected in treated areas after spray application. In this work, all strains were able to endophytically colonize roots, stems and leaves during the first 96 h after inoculation. Whilst the treatment of *S. littoralis* larvae with a 10^8 ml^{-1} conidial suspension resulted in moderate to high mortality rates for the *Metarhizium brunneum* EAMb 09/01-Su (41.7–50.0%) and *Beauveria bassiana* EABb 01/33-Su (66.7–76.6%) strains, respectively, an additive effect was detected when these larvae were also fed endophytically colonized alfalfa, tomato, and melon leaves, with mortality rates varying from 25.0% to 46.7% as a function of the host plant and total mortality rates in the combined treatment of 75–80% and 33–60% for *B. bassiana* and *M. brunneum*, respectively. Fungal outgrowth was not detected in any of the dead larvae feeding on colonized leaves, whereas traces of destruxin A were detected in 11% of the insects fed tomato discs endophytically colonized by *M. brunneum*. The combined effects of the fungal spray with the mortality caused by the feeding of insects on transient EF-colonized leaves have to be considered to estimate the real acute impact of field sprays with entomopathogenic fungi on chewing insects.

1. Introduction

In the last century, entomopathogenic fungi (EF) have been viewed as arthropod pathogens, and research has been focused on all aspects related to their possible development as alternatives to chemicals. Indeed, several hypocrealean ascomycete-based mycoinsecticide products are commercially available (de Faria and Wraight, 2007; Nollet and Rathore, 2015).

Recent studies have revealed that entomopathogenic Hypocreales may play additional unusual roles in the ecosystem such as plant endophytes, rhizosphere-competent fungi, or antagonists of plant pathogens (Vega, 2008; Vega et al., 2009; Quesada-Moraga et al., 2014a). Research on these new lifestyles, particularly plant endophytes, has come of age within the last decade, and the newly recognized importance of these roles is a consequence of the increasing recognition of the potential agronomic benefits that these fungal species may have and their enormous potential in the development of novel integrated crop protection tools (Quesada-Moraga et al., 2014a).

The pioneer studies of Bing and Lewis (1992) in corn first revealed that the establishment of *Beauveria* sp. *in planta* can confer systemic protection from herbivorous pests. Late in the XXI century, some fungal entomopathogens were identified as naturally occurring endophytes, whereas others have been artificially inoculated into plants using various techniques (Quesada-Moraga et al., 2014b). Such studies aimed at using these fungi as biological control agents against specific pests whose life cycles (feeding internally and tunnelling extensively in stems, pseudostems, rhizomes, roots, and seeds) seriously limit the effectiveness of chemical insecticides and other control methods.

The techniques used for the artificial inoculation of EF into different crops include leaf spraying, injection into stems, soil drenching, and seed dressing with conidial suspensions, although differences have been noted in the rates of success, the extension of colonization and the occurrences of systemic colonization (Bing and Lewis, 1992; Tefera and Vidal, 2009; Quesada-Moraga

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et al., 2006; Posada et al., 2007). Despite the occurrence of natural or artificial EF endophytic colonization, much remains unknown about endophyte penetration and colonization processes. Important issues central to the development of this fungus-plant association include the primary portals of fungal entry into plant tissues, the extent of fungal dissemination (especially ascending migration) within the plant host after primary root infection, and fungal population dynamics *in planta*.

The first clue suggesting the ascending migration of an endophytic strain of *Beauveria bassiana* within the opium poppy came from the studies of Landa et al. (2013), who used seed dressing with a conidial suspension to demonstrate, for the first time, the vertical transmission of an entomopathogenic fungus from endophytically colonized maternal plants (Quesada-Moraga et al., 2014a). Interestingly, when the inoculation technique was not seed dressing but leaf spray with the same gfp-tagged *B. bassiana* strain, only a temporal endophytic establishment was detected in the treated area, which gradually decreased until disappearing completely (Landa et al., 2013); these findings were also detected in several crops by different researchers (Bing and Lewis, 1992; Posada et al., 2007; Gurulingappa et al., 2010; Herrero et al., 2012; Biswas et al., 2012; Batta, 2013).

One current drawback to the use of EF endophytes as biocontrol agents is the variability observed in the endophytic persistence of fungi after inoculation. The inoculation method, the fungal strain used, and the host plant genotype are key factors determining the persistence of EF endophytes and the compatibility of plant-endophyte associations (Quesada-Moraga et al., 2014a). As a result, most known fungal endophytes seem to colonize their host plants in a non-systemic pattern (Rodriguez et al., 2009; Marquez et al., 2012), probably due to a "balanced antagonism" in which the host plant can restrain the growth of the fungus, and the fungus can modulate the effectiveness of plant defence mechanisms (Schulz and Boyle, 2005). As a result, it could be expected that spraying plant tissues with an EF fungal suspension could produce the temporary endophytic establishment of the EF fungus in the treated area in many cases, both in systemic and non-systemic colonization patterns.

In such a scenario, it remains unknown whether spray applications with conidial EF suspensions targeting chewing insect pests can be accompanied by such transient endophytic colonizations of plant tissues or whether this temporary establishment may cause any level of pest control mortality, thereby improving the overall efficacy of fungal treatment. Even if one expects that the bio-controlling abilities of endophytic EF are due to the infection of the insect upon feeding on endophytically colonized plants, very few fungal-infected insects have been observed in the aforementioned studies; thus, apart from antibiosis and feeding deterrence, it could be argued that endophytes kill insects during the first stages of development by secreting toxic compounds *in planta*. Likewise, various species of endophytes are known to produce metabolites that deter insect feeding (Daisy et al., 2002); this finding suggests that the production of such compounds *in planta* may inhibit insects from foraging on the plants (Vega et al., 2009). In the current work, we monitored the extent of endophytic colonization of alfalfa, tomato, and melon plants when fungal suspensions of *B. bassiana* and *Metarhizium brunneum* are sprayed onto plant leaves, in order to ascertain whether a spray application of a fungal suspension could produce a temporal colonization of the plants that could improve the overall effect of the treatment. We evaluated whether the feeding of larvae of the beet armyworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) on the leaves of three crops temporally colonized by the fungi caused any level of pest mortality, investigated possible causes of mortality, and assessed whether mortality may improve the efficacy of spray applications with fungal suspensions.

2. Material and methods

2.1. Insects

2.1.1. S. littoralis (Boisduval) (Noctuidae, Lepidoptera) culture

The *S. littoralis* larvae used in this study were obtained from a stock colony from the Department of Agricultural and Forestry Sciences of the University of Córdoba (Spain). These insects were originally collected in the field from different crops. They were maintained in an environmental chamber set at 26 ± 2 °C, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h (Poitout and Bues, 1974).

The adults were placed in oviposition chambers containing cylindrical filter paper (150 × 120 mm) that were closed at both ends with a layer of filter paper. Cotton moistened with a 10% honey solution was placed inside 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, and clusters of collected eggs were externally disinfected by immersion in 10% formalin for 10 min and three washes with sterile water to remove the formalin residues. Finally, the disinfected egg clusters were laid on pieces of filter paper to remove the water using the methodology developed by Santiago-Álvarez (1977).

The larvae were fed on artificial diet consisting of 85 g of alfalfa meal, 34 g of brewer's yeast, 32 g of wheat germ, 18 g of agar-agar (Industrias ROKO, S.A., Spain), 14 g of casein (Merck KGaA, Germany); 4.50 g of ascorbic acid (Scharlab, Spain), 1.30 g of benzoic acid (Scharlab, Spain), 1.10 g of nipagin (Guinama S.L.U., Spain), 5 ml formaline 10% (formaldehyde 37–38% w/w stabilized with methanol) (Panreac, Spain) and 800 ml of distilled water (Santiago-Álvarez, 1977). A 50 mm layer of sterilized peat was added when the larvae reached the last larval instar and kept under controlled conditions of 26 ± 2 °C, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h. Then the pupae were removed and separated by sex using a stereomicroscope (Nikon SMZ800, Japan). Finally the pupae were classified and transferred to the oviposition chambers under control conditions.

2.2. Fungal strains and suspensions

The four fungal strains, which included three *Beauveria* strains and one *Metarhizium* strain, used in this study came from the culture collection in the Department of Agricultural and Forestry Sciences (AFS) of the University of Córdoba (Table 1). They were sub-cultured on malt agar (MA) supplied with 500 mg l^{-1} streptomycin sulphate salt (product of China) for 15 days at 25 °C in darkness. The Petri plates were sealed with Parafilm® (Pechiney Plastic Packaging Co, Chicago, IL). Conidia suspensions were prepared by scraping conidia from the Petri plates into a sterile aqueous solution of 0.1% Tween 80 and filtered through a piece of cheesecloth. The conidia suspensions used for the inoculation bioassays were adjusted by diluting the conidia with 0.1% Tween 80 to a final concentration of 1.0×10^8 conidia ml^{-1} using a Malassez chamber.

2.3. Substrate and preparation of plant material

Alfalfa (*Medicago sativa* L.), tomato (*Lycopersicon esculentum* Mill cv Tres Cantos) and melon var. Galia (*Cucumis melo* L. var. *reticulatus* Naud) seeds were surface disinfected. To accomplish this, the seeds were immersed in 70% (v/v) ethanol for 2 min followed by 2% NaOCl for 5 min and rinsed with sterilized water three times. Lastly, they were again immersed in 70% ethanol (v/v) for 1 min and dried under sterile air flow. The last rinse water was plated out to assess the effectiveness of the surface-disinfecting procedure. The seeds were transferred into plastic pots of 110 wells