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Hydrophobins from aerial conidia of *Beauveria bassiana* interfere with *Ceratitis capitata* oviposition behavior

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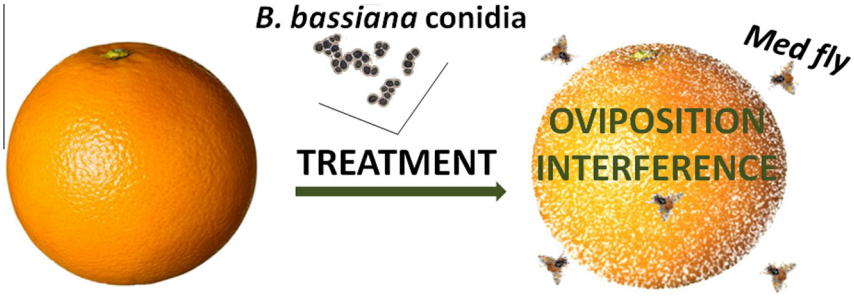
HIGHLIGHTS

- *Beauveria bassiana* interferes with *C. capitata* oviposition behavior.
- Conidia treated fruits are less susceptible to *C. capitata* visits and oviposition.
- Inhibitory effects are concentration-dependent.
- Conidial hydrophobins are important to reduce fruit susceptibility to *C. capitata*.

GRAPHICAL ABSTRACT



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ABSTRACT

A reduction in *Ceratitis capitata* Wiedemann oviposition rate was observed on fruits treated with commercial formulations containing conidia of *Beauveria bassiana*. In an attempt to identify the main sources of disturbance for ovipositing flies, this study investigated the possible role of different fungal fractions in relation to *C. capitata* oviposition behavior on treated fruits.

Orange fruit treatments with different *B. bassiana* (Balsamo) Vuillemin preparations caused an oviposition deterrent effect in terms of reduction in the number of female visits/fruit and of oviposition punctures/fruit, in comparison to the untreated control. The most evident effects were observed for conidia-based preparations, while for the other fungal fractions assayed (mycelium and culture supernatants) these effects were moderate or negligible. These effects were concentration-dependent, and a maximum was achieved at the highest tested concentration of conidia (10^8/ml, 10 ml per fruit).

According to our results, we assumed that the physical and biochemical properties of conidia, in particular the rodlet layers of hydrophobins covering conidia surface, may impair the ability of *C. capitata* to detect orange-derived stimuli, such as orange odors and fruit humidity content. This hypothesis was supported by the significant decrease in the oviposition deterrent effect observed when fruits were treated with conidia deprived of the rodlet layer in comparison to equivalent suspensions of intact conidia. In addition, a suspension containing rodlet proteins alone at a concentration of 5 mg/ml also determined a significant reduction in the number of fruit visits and of oviposition punctures compared to an untreated control.

1. Introduction

The entomopathogenic filamentous fungus, *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is worldwide used as a biological control agent in different agro-ecosystems (Meyling and Eilenberg, 2007). Since its first discovery, the isolation of new strains and the improvement in formulations and application techniques have led to the development of different biopesticidal products (Wraight et al., 2001).

Although many aspects still need to be clarified, the insecticidal action generally involves conidia adhesion to the insect body,

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followed by the production of germ tubes and penetrant hyphae (Boucias and Pendland, 1991; Kumar et al., 1999). The penetration inside the insect body is a molecular-mediated process that involves the fungal production of various proteins such as chitinases, proteases, and other extracellular enzymes (Ortiz-Urquiza et al., 2010). After invasion, the proliferation within the insect hemolymph is associated with the release of fungal toxins, which leads to the death of the host. A wide variety of insecticidal toxins are expressed by different fungal strains, which results in variable degrees of virulence against specific targets (Castrillo et al., 2008). Many of these toxins are secondary metabolites, small peptides, and antibiotics, whose specific role is not always understood (Vey et al., 2001). Besides, new toxins are progressively being discovered (Quesada-Moraga and Vey, 2004; Quesada-Moraga et al., 2006a). Recent investigations based on the whole genome shotgun sequencing of *B. bassiana* strain ARSEF 2860, also revealed the presence of genes encoding for bacterial-like toxins, some of which showing similarities to *Bacillus thuringiensis* Cry toxins (Xiao et al., 2012).

Ceratitis capitata Wiedemann (Diptera: Tephritidae), also known as the Mediterranean fruit fly, is a multivoltine and polyphagous pest species affecting numerous host fruits (White and Elson-Harris, 1992). Since the management of this pest is still mainly based on repeated applications of synthetic chemical insecticides, the integration of these conventional methods with biological control tools would be highly desirable. Previous studies highlighted the pathogenic potential of different *B. bassiana* strains against diverse stages of *C. capitata* (Quesada-Moraga et al., 2006b; Beris et al., 2013; Lozano-Tovar et al., 2013). The use of entomopathogenic fungi in tephritid fruit fly IPM (Integrated Pest Management) includes aerial applications targeting adults and soil inoculation targeting pupariating larvae and puparia (Ekesi et al., 2007). According to a modern approach, entomopathogenic fungi should be applied in localized spots and their dissemination would rely on the ability of conidia to be transmitted from infected to uninfected hosts (Dimbi et al., 2013). The effectiveness of such methods requires successful interactions between fungal conidia and the insect body. Studies in this direction are needed to improve knowledge on the molecular mechanisms regulating pathogen-host interactions (Ortiz-Urquiza and Keyhani, 2013). Among the molecules and genes that are involved in the interaction with the host, fungal hydrophobins are important components of the conidial cell envelope, and are involved in surface interactions, development, and virulence of the fungus (Zhang et al., 2011).

Recent laboratory and field observations have shown a reduction in *C. capitata* oviposition on fruits treated with a commercial formulation containing conidia of *B. bassiana* strain ATCC 74040 (Naturalis®, CBC (Europe) Srl) (Ortu et al., 2009). However, the mechanism producing this effect was not investigated.

In an attempt to study the interaction between the fungus and ovipositing *C. capitata* and to identify the main sources of disturbance for ovipositing females, we investigated the possible role of different fungal fractions, including conidial hydrophobins, in relation to fly oviposition behavior on treated fruits.

2. Materials and methods

2.1. Insect rearing

Experiments were conducted with insects from a colony established in the entomology laboratory of the Dipartimento di Agraria of the University of Sassari (Italy). Flies were reared at 25 ± 1 °C on a photoperiod of L14:D10 (Cavalloro and Girolami, 1969). Adults were maintained in plastic cages (60 by 35 by 11 cm) with a lateral face covered with gauze. Water and a yeast powder-sugar mixture (1:1) were provided *ad libitum* to adults. Larvae were reared on a medium made of wheat bran (24,9%), sucrose (16.0%), yeast powder (8.0%), citric acid (0.6%), benzoic acid (0.5%), and water (50.0%) (wt/wt) contained in a plastic tray (36 by 24 by 3 cm).

2.2. Cultivation and recovery of fungal fractions

The microorganism used in this study was *B. bassiana* strain ATCC 74040 which was isolated and purified from the commercial formulation Naturalis® (CBC Europe S.r.l., Nova Milanese, Italy). A blank of the same formulation, containing all co-formulants, was also included in this study.

The microorganism was routinely cultured on Sabouraud dextrose agar (SDA) plates at 28 °C to ensure colony maintenance and production of aerial conidia. Conidia suspensions were collected by scraping conidia from plates into 0.1% Triton solution. Suspension purity was checked under a phase microscope, and conidia quantification was based on Thoma chamber counting.

To generate liquid cultures, the microorganism was grown in Sabouraud broth inside Erlenmeyer flasks. In this case the medium was inoculated with a pure conidia suspension and incubated at 28 °C with shaking at 180 rpm. In these conditions conidia germination normally occurred in approximately 48 h. After 4 days, this primary culture was used to inoculate a secondary culture grown in the same conditions. The mycelium and culture supernatants were harvested after 7 days by centrifugation and filtration (Fuguet et al., 2004).

2.3. Conidia treatments and separation of rodlets

Suspensions of intact conidia (before germination) applied in our bioassays (experiment 2) were identified as the main fungal fraction responsible for the observed effects on fly oviposition behavior. Hence, samples of pure conidia were further treated to evaluate the possible implication of hydrophobic proteins adhering to their surface. In fact, it is known that the surface of *B. bassiana* conidia is covered by layers of class I hydrophobins, Hyd1 and Hyd2, that normally polymerize to form amyloid fibrils known as rodlets (Zhang et al., 2011). Rodlets can be dislodged from conidia surface by sonication (Paris et al., 2003) or dissolved by chemical treatments (Bidochka et al., 1995; Holder and Keyhani, 2005). Hence, conidia were subjected to the following two extraction procedures, in order to collect a fraction of conidia deprived of rodlets and a fraction of rodlets, respectively.

To prepare a void of rodlet conidia fraction, conidia were treated with 98% formic acid in a sonicating water bath (Transsonic Digital, Elma Gmbh & Co KG, Germany) for 1.5 h, followed by oxidation with performic acid as described by Wessels et al. (1991). The fraction of conidia collected by centrifugation at 6000×*g* for 30 min at 4 °C was named ConidiaΔRod.

To prepare a rodlet fraction, rodlet layers were removed from conidia surface following the method described by Paris et al. (2003) with few modifications. In this case conidia suspensions were sonicated at 140 W for 2 × 10 min in an Ultrasonic cell disrupter (Model 150 VT, Biologics Inc., Virginia, USA). Conidia and cell debris were then removed by differential centrifugation including three consecutive cycles at 6000×*g* for 30 min at 4 °C. The resulting supernatant was ultra-centrifuged for 1 h at 50,000×*g* to collect a rodlet fraction (Rod).

At each step of these procedures, the presence of intact or disrupted conidia was checked by phase microscopy.

The protein concentration of the rodlet fraction was determined using the Folin-phenol reagent (Lowry et al., 1951) and bovine serum albumin (Sigma) as a standard.