Journal of Invertebrate Pathology 132 (2015) 77–85

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

The fungistatic and fungicidal effects of volatiles from metathoracic glands of soybean-attacking stink bugs (Heteroptera: Pentatomidae) on the entomopathogen *Beauveria bassiana*

Rogério B. Lopes, Raul A. Laumann, Maria C. Blassioli-Moraes, Miguel Borges, Marcos Faria *

EMBRAPA Genetic Resources and Biotechnology, Parque Estação Biológica, W5 Norte (Final), 70770-917 Brasilia, DF, Brazil

ARTICLE INFO

Article history:
Received 29 April 2015
Revised 25 August 2015
Accepted 26 August 2015
Available online 28 August 2015

Keywords:
Entomopathogenic fungi
Biological control
Biopesticides
Mycoinsecticides
Antimicrobial activity
Germination

ABSTRACT

This study was initially designed to evaluate the differential susceptibility of three soybean-attacking pentatomids to the entomopathogenic fungus *Beauveria bassiana* in standardized bioassays. *Euschistus heros* (Eh) was shown to be significantly less susceptible than *Chinavia ubica* (Cu), whereas *Dichelops melacanthus* (Dm) adults were highly susceptible to fungal infections. A deeper look at the mechanisms involved in the possible role of volatiles from metathoracic glands on fungal infections was undertaken, and gland extracts from *Nezara viridula* (Nv), a species known for its resilience to fungal infections, were also included in the assays. Atmospheres with volatiles from pentatomids with very low-susceptibility to *B. bassiana* infections (Eh and Nv) had a significant effect on speed of germination as shown in counts performed up to 22 h post-inoculation, by which time 0.1 (control), 0.6 (Dm), 17.9 (Cu), 32.6 (Eh), and 43.4% (Nv) of conidia had not germinated. The fungistatic (inhibitory) and fungicidal (lethal) effects of Eh and Nv volatile-rich atmospheres were subsequently quantified in Petri dishes with either PDA or PDA medium amended with carbendazim, which allowed germination rates to be determined at 18 and 48 h post-inoculation, respectively. As opposed to control, Eh volatile-rich atmosphere had a clear fungistatic effect, since germination rate was only 27.4% within 18 h, but reached 99.4% at 48 h post-inoculation. For Nv volatile-rich atmospheres, only 15.1% of conidia germinated within 18 h, and by 48 h post-inoculation, approx. 18% of conidia were unviable (neither germ tubes nor intumescence), whereas in the control treatment rates were >99% at both reading times. Therefore, the gaseous phase of defensive secretions from fungus-resilient pentatomids possess a strong inhibitory effect and may display a less pronounced lethal effect on fungal germination, as was the case for Nv.

1. Introduction

Most phytophagous stink bugs (Hemiptera: Pentatomidae) are polyphagous pests, feeding on a wide range of plants, including legumes, cereals and trees (Panizzi and Slansky, 1985; Panizzi, 1997). Although many entomopathogenic fungi have been commercially developed to control hemipterans in the families Cercopidae, Aleyrodidae, and Aphididae, none of these products have pentatomids as primary targets (Faria and Wraight, 2007). Unless very high dosages are used, susceptibility of these insects to fungal infections is usually limited even under laboratory conditions (Ihara et al., 2001; Santos et al., 2002; Patel et al., 2006), and natural epizootics associated with these entomopathogens have not been reported for stink bug populations (Sosa-Gómez and Moscardi, 1998).

Hypocrealean fungi, such as *Metarhizium anisopliae* (Metschn.) Sorokin sensu lato and *Beauveria bassiana* (Bals.-Criv.) Vuill. sensu lato, kill susceptible arthropods by germination of infective propagules on the cuticle, followed by their penetration and propagation in the insect hemocoel (Boucias and Pendland, 1998). Before pathogens are exposed to antimicrobial peptides once they reach the body cavity (Jenssen et al., 2006), compounds present in the cuticle may play important roles against infection (Dettner, 1985; Sosa-Gómez et al., 1997; Boyle and Cutler, 2012). Ants, for example, have a sophisticated chemical defense mechanism to prevent germination of fungal conidia, based on the use of exocrine gland secretions during self-grooming (Nascimento et al., 1996; Fernández-Marín et al., 2006; Schlüns and Crozier, 2009; Graystock and Huges, 2011), and some termites show similar behavior (Yanagawa et al., 2008; Hamilton et al., 2011).

* Corresponding author.
E-mail address: marcos.faria@embrapa.br (M. Faria).

http://dx.doi.org/10.1016/j.jip.2015.08.011
0022-2011/

Heteropterans, mainly the Pentatomidae, are well known for their repulsive stink smell that is released when they are disturbed. These volatile compounds are produced and stored in the metathoracic (adults) and abdominal glands (nymphs) (Aldrich, 1988; Krall et al., 1999; McBrien and Millar, 1999; Moraes et al., 2008). These compounds are small molecules, short chain (*E*)-2-alkenals, 4-oxo-(*E*)-2-alkenals (C_6, C_8, C_{10}) and linear hydrocarbons, mainly C_{11}–C_{13} (Borges and Aldrich, 1992). In the adults, metathoracic gland compounds are composed basically by the same components identified in the later nymphal stages, with *n*-tridecane being the major compound (Borges and Aldrich, 1992; Pareja et al., 2007). However, the adults also present short-chain alcohols (C_6–C_8) and their esters, and other minor compounds, including monoterpenes, long-chain saturated and unsaturated aldehydes, diols and pyrazines. These compounds serve the dual purpose of alarm pheromones and allelochemicals, in this case acting as weapons against natural enemies (Pareja et al., 2007; Moraes et al., 2008; Blassioli-Moraes et al., 2013). For instance, it has been demonstrated that specific fractions of the multicomponent secretions of the green stink bug, *Nezara viridula* (Linnaeus), display antimicrobial properties when in direct contact with the entomopathogenic fungus *M. anisopliae* (Borges et al., 1993). The detrimental effect exerted by the gaseous phase (volatiles) of glandular secretions on fungi has been reported in orders such as Hemiptera (families Cydnidae and Cimicidae), Isoptera, and Coleoptera (Timonin, 1961; Rosengaus et al., 2000; Gross et al., 2002, 2008; Ulrich et al., 2015), but knowledge about the role of volatile compounds of pentatomids on fungal infections remains fragmentary.

In our institution, we have noticed that insects with fungal diseases have not been seen in colonies of *N. viridula*, *Chinavia ubica* (Rolston) and *Euschistus heros* (Fabricius) kept for biocontrol and behavior studies, despite frequent introduction of field-collected insects to avoid genetic degeneration and room conditions favorable to fungal development. However, in specimens of the green belly stink bug *Dichelops melacanthus* (Dallas) collected in western Brazil (Dourados, Mato Grosso State), we observed some *B. bassiana*-infected adults. These contrasting observations motivated us to undertake this study, designed to investigate whether different stink bugs species show differential susceptibility to *B. bassiana*, and to evaluate whether the gaseous phase of metathoracic gland secretions might be involved in pathogen germination via fungistasis (inhibitory effect) and/or fungitoxicity (lethal effect).

2. Materials and methods

2.1. Insect rearing

Nezara viridula, *Chinavia ubica*, and *Euschistus heros* were kept in colonies at Embrapa Genetic Resources and Biotechnology (Brasilia-DF, Brazil) for over five years. A *Dichelops melacanthus* colony was established with insects originally collected in soybean fields near Embrapa Western Region Agriculture (Dourados-MS). All species were reared on green bean pods (*Phaseolus vulgaris* (L.)) plus a mixed-seed diet composed of sunflower (*Helianthus annuus* (L.)), soybean (*Glycine max* (L.) Merrill), and raw peanuts (*Arachis hypogaea* (L.)), replaced twice a week. Insects were reared in 8-L plastic containers (200 nymphs or 100 adult couples/container) in a room set to a constant temperature of 26 ± 1 °C and 14 h photophase.

2.2. Production of Beauveria bassiana conidia for bioassays

Strain CG1105 of *Beauveria bassiana* s.l. was originally isolated from infected adults of *D. melacanthus* collected in a soybean field in Mato Grosso do Sul State, Brazil. Conidia preserved in liquid nitrogen in the Invertebrate Fungal Collection at Embrapa Genetic Resources and Biotechnology were inoculated on potato dextrose agar medium (PDA – Difco Laboratories, Detroit-MI, USA) and maintained at 25 ± 0.5 °C and 12 h photophase. Conidia were scraped from 10–12 day-old cultures with a spatula and immediately used for preparation of suspensions.

2.3. Susceptibility of stink bug species to Beauveria bassiana infection

Suspensions containing 0.5 g conidia of *B. bassiana* CG1105 per 10 mL of 0.05% v/v Tween 80® were prepared in 50-mL polypropylene centrifuge tubes, and then vortexed and ultrasonicated for 2 min. After agitation, suspensions were filtered through cheesecloth to remove mycelial fragments and adjusted to a final concentration of 2×10^9 conidia mL^{-1} using a Neubauer hemocytometer. Viability testing showed that germination of fresh conidia was ⩾98%. Unsexed *E. heros*, *C. ubica*, and *D. melacanthus* adults were anesthetized with gaseous CO_2 for 30 s, and each insect was then inoculated on the thorax (sterna region) with 1 μL of the conidial suspension (2×10^6 conidia/insect) using a glass micro-syringe coupled to a micro-applicator. For each pentatomid species, 30 adults divided into three groups (replicates) were treated with the pathogen. Insects treated with 0.05% Tween 80® were considered as the control treatment. Following inoculation, insects were transferred to plastic cups (15 cm diameter × 12 cm high) containing moistened cotton and fresh green beans and kept at 26 ± 1 °C and 12 h photophase. Evaluations were performed daily between the 5th and 10th days post-inoculation. Cadavers were transferred to Petri dishes with moistened filter paper for confirmation of mortality by the fungus. The experiment was performed three times on different dates. Due to limited availability of *N. viridula* in the colony by the time the bioassays were conducted, this species was not included in these experiments.

2.4. Extraction of metathoracic glands from pentatomid adults

To test the influence of the volatile compounds present on the metathoracic glands on *B. bassiana* germination, natural blends of these glands were obtained by dissecting male and female adults of all four pentatomid species followed by extraction of compounds, according to procedures described by Laumann et al. (2009) and Pareja et al. (2007). Both methodologies are described in the literature as being able to extract all defensive compounds stored in the metathoracic glands of stink bugs (Aldrich et al., 1993; Pavis et al., 1994; Pareja et al., 2007; Fávaro et al., 2012). In the first approach, considered to be simpler and faster, insects were anesthetized in CO_2 flux for 30 s and then dissected under stereoscopic microscope using microdissecting forceps; the abdomen was opened and viscera removed revealing the metathoracic scent gland. For bioassay purposes, each extract was obtained from 4 adult (2 from each sex) metathoracic glands, by submerging them directly in *n*-hexane for 2 h. Then, the tissues were removed filtering the solution with a Celite column in a Pasteur pipette, and the volume was adjusted with hexane to four glands per milliliter. Therefore, each extract used in the bioassays had one gland equivalent/250 μL. For each pentatomid species, a total of five gland extracts were obtained. In the second approach, known to yield clean extracts (not contaminated by fatty acid traces and other non-volatile compounds) appropriate for chemical analyses, metathoracic glands of CO_2-anesthetized insects were pierced with a flame-stretched glass capillary, causing the contents to rise through the capillary. The contents were then emptied into 200 μL of *n*-hexane by immersing the tip of the capillary into the solvent and briefly passing charcoal-filtered air through the capillary. Each sample was composed of the content of one gland. For