Journal of Invertebrate Pathology 127 (2015) 93–100

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Unveiling chemical defense in the rice stalk stink bug against the entomopathogenic fungus *Metarhizium anisopliae*

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ARTICLE INFO

Article history:
Received 4 December 2014
Revised 19 February 2015
Accepted 16 March 2015
Available online 21 March 2015

Keywords:
Chemical ecology
Clavicipitaceae
Virulence
Tibraca limbativentris
Allelochemicals
Oryza sativa

ABSTRACT

Eggs, nymphs (1st–5th instar) and adults of *Tibraca limbativentris* were challenged by conidial suspensions of its major fungal pathogen *Metarhizium anisopliae* in order to assess their susceptibility. The role of chemical defensive compounds from exocrine secretions produced by both nymphs and adults were examined for their participation on *M. anisopliae* infection. Although insect susceptibility to *M. anisopliae* followed a dose-dependent manner, adults followed by older nymphs displayed the highest resistance. Eggs were highly susceptible showing >96% fungal infection. Crude extracts isolated from metathoracic scent gland and dorsal abdominal glands of adults and nymphs, respectively, showed fungistatic effects by impairing spore germination, vegetative growth and sporulation. Gas chromatography–mass spectrometry analysis of these extracts revealed that the major components were short-chain hydrocarbons (C_{10-13}) and unsaturated aldehydes. *In vitro* tests with the corresponding synthetic standards indicated compounds with greater antifungal activity including (*E*)-2-hexenal, (*E*)-2-octenal, and (*E*)-2-decenal, with the latter being the most deleterious to fungal fitness. We demonstrated that differential susceptibility of the rice stalk stink bug to *M. anisopliae* infection is age-specific and partly mediated by fungistatic properties of aldehydes, which are produced by scent glands of both nymphs and adults.

1. Introduction

The rice stalk stink bug, *Tibraca limbativentris* Stal. (Heteroptera: Pentatomidae), is an economic important pest of rice crops (*Oryza sativa* L.) in South America, including Brazil (Pantoja, 1997). Although both nymphs and adults of *T. limbativentris* feed on the developing stalks, the main damage occurs during pre-flowering and panicle formation that consequently leads to rice yield losses by 10–80% (Costa and Link, 1992; Ferreira et al., 1986). In Brazil, the major control method relies primarily on the use of synthetic chemical insecticides (Quintela et al., 2013). However, due to the short-lived effects of synthetic insecticides, the repopulation through migration from non-treated areas, and primarily by the emerging of insecticide resistance in related neotropical stink bugs in Brazilian soybean fields (Sosa-Gómez et al., 1997), there is pressing need to develop alternative control strategies. The use of biological control agents, such as entomopathogenic fungi, represents a practical and ecologically friendly tactic to manage agricultural pests. The entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorok. (Hypocreales: Clavicipitaceae) strain CG168 was first isolated (causing epizootics) from naturally infected *T. limbativentris*; an oil-based formulation of hydrophobic aerial conidia of this isolate is underway to be registered to use in integrated management programs for this pest (Quintela et al., 2013).

Entomopathogenic fungi start the infection process mainly by penetration through the insect cuticle. The outermost surface layer of the cuticle is the epicuticle, mostly composed of lipids. The epicuticle exhibits characteristic water barrier properties and protect insects against predators and pathogens (Borges and Aldrich, 1992; Pareja et al., 2007). Interspersed within the cuticle barrier there are biochemical components such as antimicrobial lipids and phenols, enzyme inhibitors, proteins, and other defensive compounds that entomopathogens must overcome for successful virulence (Pedrini et al., 2013). Thus, fungal virulence is frequently correlated with rapid germination and growth rates on the cuticle, and higher germination rates might help increase the probability of infection

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http://dx.doi.org/10.1016/j.jip.2015.03.009
0022-2011/

before spores are removed from the cuticle (Altre et al., 1999). The epicuticle composition is also associated with the insect susceptibility to entomopathogenic fungi. For example, insects containing a blend of saturated straight and branched chains lipids are more susceptible to fungi that those insects with a predominance of unsaturated chains and/or the presence of some compounds showing an antibiotic role, such as quinone derivatives or short chain lipids (Pedrini et al., 2007). It is known that allomones produced by stink bugs from the family Pentatomidae comprise an assortment of hydrocarbons, saturated and α,β-unsaturated aldehydes and esters (Aldrich, 1988). These compounds are firstly synthesized in specialized exocrine scent glands and then released embedding the cuticle. Nymphs, for instance, produce exocrine secretions of odoriferous compounds from dorsal abdominal glands (DAGs) that are shed along with the exuviae during molting. Thus, extraction of exuviae consists of a suitable method to obtain and identify compounds synthesized in DAGs (Borges and Aldrich, 1992). In adults, defensive secretions constitute allomones produced in the metathoracic scent gland (MTG), which can be easily extracted by dissection (Aldrich, 1988; Fávaro et al., 2011). It is also known that ratio and composition of these compounds vary across nymphal instars and physiological ages in adults (Borges and Aldrich, 1992; Fávaro et al., 2011, 2012). Although some of them are known to constitute the alarm pheromone, their antimicrobial role (Borges and Aldrich, 1992; Sosa-Gómez et al., 1997) is often related with the low susceptibility of pentatomid stink bugs to entomopathogenic fungi observed at field conditions (Sosa-Gómez and Moscardi, 1998; Moraes et al., 2008).

Little is known about the mechanisms underlying the low susceptibility of *T. limbativentris* to its major fungal pathogen *M. anisopliae*. Here, we have hypothesized that defensive compounds derived from scent glands of nymphs and adults of this stink bug could be involved in this natural resistance to fungal infection. Therefore, our goal in this communication is to describe the susceptibility of eggs, nymphs and adults of *T. limbativentris* to *M. anisopliae* (strain CG168), and at the same time to understand in what extent the chemical compounds isolated from both MTG and DAGs affect infection by this pathogen.

2. Materials and methods

2.1. Insect colony

Rice stink bugs were reared on 5-L potted rice plants (*Oryza sativa* L., cv. BR-IRGA-409) under screenhouse conditions. Insect founders were natural and annually collected from rice fields free of chemical pesticides at the National Rice and Beans Research Center of the Brazilian Agricultural Research Corporation (EMBRAPA Rice and Beans) located in St. Antônio de Goiás, GO, Brazil. Insect density per pot consisted of 100 adults (sex ratio 1♀:1♂) at the same age. This insect colony was free of any chemical phytosanitary treatment. The colony was reared under screenhouse conditions and provided immature stages and adults for experimentation.

2.2. Entomopathogen

For the virulence bioassays, the strain CG168 (=ARSEF1883, USDA, Ithaca, NY) of *M. anisopliae* was used to challenge different life stages of the rice stink bug (Quintela et al., 2013). This fungal strain was originally isolated from *T. limbativentris* adults in the same location of the insects (St. Antônio de Goiás, GO, Brazil). Pure stock cultures were obtained through re-isolation of the fungus on potato dextrose agar (commercial PDA, Acumedia®, Baltimore, MD, USA) plates and then grown for 15 days until full sporulation. Afterwards, aerial conidia were harvested from these plates and dried in silica gel for 4 days at 4 °C, and then finally stored at −20 °C in 2-mL Eppendorf tubes.

To produce inocula for bioassays, the fungus was obtained from stock cultures and cultured on PDA for 10–15 days in a growth chamber at 26 ± 1 °C, 70 ± 10% relative humidity (RH), and 12:12 (L:D) h photoperiod. The fungal inocula were always fresh and subcultured only once on PDA plates using the frozen stock cultures in order to standardize bioassays. Before each trial, conidial viability was checked by counting germinated conidia showing germ tubes of any size on PDA plates after 20 h incubation at same environmental conditions previously indicated, using a phase contrast microscope (Leica® DM 2500) at 400× magnification. Conidial viability scored >90% germination in all cases. All conidial suspensions used in bioassays were prepared with 0.01% (v/v) aqueous solution of Tween 80 (Polysorbate 80, Sigma–Aldrich®).

2.3. Egg susceptibility to M. anisopliae

Eggs aging 5 days of *T. limbativentris* were randomly collected from different rice pots and groups of 10 eggs were placed individually in 6-cm plastic Petri dishes lined with 5.5-cm Whatman No. 1 filter paper and moistened daily with 0.5 mL of sterile distilled water. A fungal suspension of 10 μL containing 5×10^6 conidia mL^{-1} was applied onto the egg masses (10 eggs per mass) using a precision dispensing equipment (microapplicator, Burkard Manufacturing Co., Ltd., UK). Controls were treated with Tween 80 only and egg mortality was indirectly recorded daily by the difference between total number of eggs and number of hatched nymphs. Each treatment had four replicates (10 eggs per repetition) and the entire bioassay was repeated twice.

2.4. Susceptibility of nymphs and adults to M. anisopliae

Bioassays with different life stages of *T. limbativentris*, including immatures and adults, were performed to assess their susceptibility to fungal infection caused by *M. anisopliae* under laboratory conditions. Immature stages from 1st through 5th instar nymphs and adults (1:1 sex ratio) were used in these bioassays. Experimental protocols were adapted to the insect life stage accordingly.

Nymphs from 1st through 5th instars were bioassayed separately as well as adults. Individuals of each life stage less than 2 days old were tested. Five individuals of each instar were placed separately in glass tubes (2.5 cm diameter × 20 cm height) and fed with three fresh rice stems (cv. BR-IRGA 409), previously rinsed three times in distilled water, with their base wrapped by moistened cotton in order to maintain turgor. The tubes were closed with a piece of nylon fabric (30 μm pore size) on the top to prevent insects from escape. Spore suspensions of *M. anisopliae* were prepared by scrapping conidia from PDA plates and diluting in 10 mL of 0.01% (v/v) Tween® 80 aqueous solution. Spore concentrations were then adjusted to 5×10^6, 5×10^7 and 5×10^8 conidia mL^{-1}. Conidial germination in all cases was greater than 90% after 20 h incubation at 26 °C and 12 h photophase. Conidial concentration was enumerated with a hemacytometer under a phase contrast light microscope. Insects were anesthetized for 20 s with CO_2 before fungal inoculation. There were six groups (repetitions) of five specimens of each insect stage per treatment. Each group of insects was treated separately with different conidial suspensions. Insects were individually inoculated on the dorsal region of the thorax (scutellum) with 10 μL of each fungal suspension using a micropipette (Quintela et al., 2013). Insects from the control groups were treated only with 10 μL of 0.01% Tween® 80. All treatments including controls were incubated in a growth chamber set to 26 ± 1 °C, 70 ± 12% RH, and 12:12 (L:D) h photoperiod. Live and dead insects were counted daily and percent survival was determined throughout 8–10 days incubation. Fungal infection in dead insects was confirmed by transferring