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Diatomaceous earth and oil enhance effectiveness of *Metarhizium anisopliae* against *Triatoma infestans*

Christian Luz*, Juscelino Rodrigues, Luiz F.N. Rocha

Instituto de Patologia Tropical e Saúde Pública (IPTSP), Universidade Federal de Goiás, CP 131, 74001-970 Goiânia, GO, Brazil

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ABSTRACT

Entomopathogenic fungi, especially *Metarhizium anisopliae*, have potential for integrated control of peridomestic triatomine bugs. However, the high susceptibility of these vectors to fungal infection at elevated ambient humidities decreases in the comparatively dry conditions that often prevail in their microhabitats. A formulation adapted to this target pest that induces high and quick mortality can help to overcome these drawbacks. In the present study diatomaceous earth, which is used against pests of stored grains or as an additive to mycoinsecticides, delayed but did not reduce *in vitro* germination of *M. anisopliae* s.l. IP 46 conidia after >24 h agitation without affecting viability, and did not hamper the survival of *Triatoma infestans* nymphs exposed to treated surfaces. The settling behavior of nymphs on a treated surface in choice tests depended on the concentration of diatomaceous earth and ambient light level. Conidia formulated with diatomaceous earth and a vegetable oil synergized the insecticidal effect of the fungus in nymphs, and quickly killed all treated insects, even at 75% relative humidity (LT_{90} 8.3 days) where unformulated conidia caused only 25% mortality after a 25 days exposure. The improved performance of a combined oil and desiccant dust formulation of this *Metarhizium* isolate raises the likelihood for its successful mycoinsecticidal use for triatomine control and, apparently, against other domestic insect pests.

1. Introduction

Until some 10 or more years ago, *Triatoma infestans* was the primary vector of Chagas disease in the southernmost countries of Latin America. While the populations of this host have been reduced by various means, this insect still remains prominent, and its vectorial transmission of this disease has not been permanently interrupted (Dias, 2009). Some populations of *T. infestans* in Argentina and Bolivia are reported to be resistant to commonly used pyrethroids (Picollo et al., 2005; Orihuela et al., 2008; Toloza et al., 2008; Cardozo et al., 2010; Juárez et al., 2010). Further, unsprayed wild populations of *T. infestans* still persist in Andean valleys and the Gran Chaco in Bolivia which is thought to be the center of distribution for this vector (Noireau, 2009). Constant entomological surveillance in high risk areas and effective, sustainable vector control are crucial to prevent this and other vector species from invading peridomestic and domestic areas (Dias, 2009; Guhl et al., 2009; Gűrtler, 2009).

T. infestans and related triatomines are highly susceptible to entomopathogenic fungi under laboratory conditions (Luz et al., 1998a,b, 2004a; Lecuona et al., 2001; Pedrini et al., 2009; Rocha et al., 2011; Rocha and Luz, 2011). However, low ambient humidity has been clearly shown to be a limiting factor for fungal infection of these vectors or for sporulation when recycling on fungus-killed individuals (Fargues and Luz, 1998, 2000; Luz and Fargues, 1998, 1999; Lazzarini et al., 2006). Several fungal species have been isolated from field-collected triatomines or were found in their habitats (Parameswaran and Sankaran, 1979; Luz et al., 2003, 2004b; Marti et al., 2005, 2006). These pathogens probably act as natural antagonists of Chagas disease vectors, especially in the rainy season. Promising results were reported in a field test with *Beauveria bassiana* against *T. sordida* in Central Brazil (Luz et al., 2004c), and with the same fungal species against pyrethroid-resistant *T. infestans* in Northern Argentina (Pedrini et al., 2009); these results underline the potential of entomopathogenic fungi for triatomine control, especially in peridomestic areas where the efficacy of synthetic insecticides is often reduced. However, to our best knowledge, there is still no registered, commercialized mycoinsecticide available for use against triatomine bugs.

It is well known that the effectiveness of fungi against target pests can be increased by appropriate methods of formulation and application (Faria and Wraight, 2007). This has also been shown for *T. infestans* and *T. sordida* with oil-in-water formulated *B. bassiana* in laboratory and field conditions (Luz et al., 1999, 2004c; Luz and Batagin, 2005). In spite of the promising results it seems that

* Corresponding author at: IPTSP, DMIPP, UFG, CP 131, 74001-970 Goiânia, GO, Brazil. Tel.: +55 62 3209 6154; fax: +55 62 3209 6363.
E-mail address: wchrisluz@hotmail.com (C. Luz).

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oil-in-water conidial formulations do not really overcome unfavorable conditions of low ambient humidity and do not induce a quick and high mortality of treated triatomines comparable to synthetic insecticides. There is a need to strengthen the efforts and to test the suitability of other additives to improve fungal formulations.

Diatomaceous earth (DE) consists of amorphous silica originating from fossilized microscopic diatoms and has both abrasive and absorptive properties on the insect cuticle (Korunic, 1998). As a dust it scratches or abrades the cuticle and kills the insect by desiccation. This natural product is often used to protect stored grains against pest insects, and there are several reports on a combined effect of DE and entomopathogenic fungi (Lord, 2001, 2005, 2007; Akbar et al., 2004; Oliveira and Alves, 2007; Dal Bello et al., 2006; Batta, 2008; Vassilakos et al., 2006; Athanassiou and Steenberg, 2007). Actually, however, we know almost nothing about the activity of DE against triatomine bugs. In field tests in Northern Argentina Pedrini et al. (2009) obtained 52.4% mortality with *B. bassiana* formulated with DE. We report here on the effect of a commercialized product combining DE with *Metarhizium anisopliae* for use against *T. infestans* and on the effectiveness of this fungus when formulated in oil and/or DE against nymphs of this vector species.

2. Materials and methods

2.1. Origin and preparation of triatomines

Experiments were carried out with laboratory-reared *T. infestans*. The colony originated from individuals collected in the State of Paraná, Brazil. Insects were fed on chickens, and maintained in the Institute of Tropical Pathology and Public Health at 25 ± 0.5 °C, 75 ± 10% relative humidity (RH) and natural photophase (Silva, 1985). Unfed third-instar nymphs (N3) were used for all tests. Insects had molted 3 up to 5 days before initiating tests. Nymphs were not fed during the assays.

2.2. Origin and preparation of the fungus

M. anisopliae s.l. IP 46 originated from a soil sample collected in 2001 in the central Brazilian Cerrado. This isolate is stored at the Collection of Entomopathogenic Fungi, IPTSP, UFG, Goiânia, Brazil. Before the reported tests, IP 46 was host-passaged once on N3 of *T. infestans* to stimulate its activity. Aerial conidia were obtained from 15-days cultures grown in Petri dishes (100 mm × 20 mm) at 25 ± 1 °C, 75 ± 10% RH and 12 h photophase on PDA (potato dextrose agar) medium (Bills and Foster, 2004). Conidia were harvested directly by scraping the culture surface with a spatula, transferred to a Petri dish, and dried over silica gel suspended in 10 ml of sterile 0.1% Tween 80 (polyoxyethylene sorbitan monoleate). The slurry was vortexed for 3 min with glass beads, and the number defined was based on hemacytometer counts. Viability of conidia (>95% germination) was assessed routinely at the beginning of each test by inoculating 100 μl of conidial suspension (10^6 conidia/ml) onto SDAY (Sabouraud dextrose agar + 0.2% yeast extract; Bills and Foster, 2004); germination of 100 conidia was quantified from four separate areas on the medium, 6–24 h after incubation at 25 ± 1 °C and 12 h photophase. Progress of germination was monitored by scoring conidia as ungerminated, swollen, germination-initiated or germinated. Conidia were considered germinated when the elongating germ tube was longer than the conidial diameter (Luz and Fargues, 1997). All tests were repeated four times with single fungal cultures for each repetition.

2.3. In vitro tests with diatomaceous earth

KeepDry® (Irrigação Dias Cruz Ltda., Santo André, São Paulo, Brazil) an amorphous DE (86%) was used in the following tests.

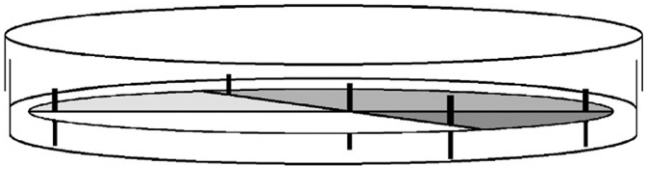

Fig. 1. Device to test the settling behavior of *Triatoma infestans* nymphs: circular filter paper (125 mm diameter) in a Petri dish (140 mm × 20 mm), subdivided in four equal areas of approximately 30 cm^2 each, perforated with 5–15 mm shortened pipette tips adjusted at a 5 mm height from the bottom, permitting free circulation of nymphs on both upper and lower surfaces of the filter paper inside the dish.

Dried conidia (100 mg) were mixed with 600 mg DE at a final concentration of 1.5×10^7 conidia/mg and then agitated on a rotating shaker at 250 rpm, 25 ± 1 °C in darkness for up to 240 h. Samples of 10 mg were taken after 0 h, 0.5 h, 1 h, 2 h, 3 h, 5 h, 12 h, 24 h, 72 h, 120 h and 240 h of agitation and suspended in 1 ml of sterile 0.1% Tween 80; 100 μl of the suspension were inoculated onto SDAY and incubated at 25 ± 1 °C. Germination rates were determined at 24 h and 48 h as mentioned.

2.4. Settling behavior of nymphs and toxicity of DE

The upper and lower surfaces of a circular filter paper (125 mm diameter) were subdivided in four equal areas of approximately 30 cm^2 each. Areas on each surface were treated with the DE or kept untreated according to the test. The filter paper was carefully perforated with 5–15 mm shortened pipette tips (≤200 μl), four in peripheral and opposed and one in central position, and the filter paper adjusted at a 5 mm height from the bottom. Each prepared filter paper set was then arranged in a Petri dish (140 mm × 20 mm) to permit free access of nymphs on both surfaces of the filter paper inside the dish (Fig. 1).

Two different choice tests were run in order to study the settling behavior of nymphs and their reactions to DE at 25 ± 1 °C, 75 ± 10% RH and a 12 h photophase. Behavior was assessed with dual option tests (areas alternately untreated and treated with the DE at a single concentration in the same Petri dish at 0.1, 0.33 or 1 mg/cm^2) or multiple option tests (areas untreated and treated with DE at increasing concentrations on the same filter paper in the same Petri dish at 0.1, 0.33 and 1 mg/cm^2). Survival of nymphs and the number of individuals settled on the different untreated or treated areas were evaluated daily during repose of the nymphs (between 11 and 12 am) for 14 days. All assays were repeated four times with 10 different nymphs in each Petri dish.

2.5. In vivo tests with formulations

M. anisopliae IP 46 was assayed on *T. infestans* N3 by exposing nymphs to unformulated, previously dried conidia (10^7 conidia/cm^2) or the same conidial concentrations formulated in DE (1 mg/cm^2), in soybean oil Graxol® (Agrária Indústria e Comércio Ltda, Jardinópolis, São Paulo, Brazil) (3 μl/cm^2), or in both DE + oil at the same concentrations mentioned. For this each preparation was applied on filter paper in a Petri dish (100 mm × 20 mm), and nymphs continuously exposed to the treated paper. Control insects were exposed to untreated filter paper or paper treated with DE or oil only or DE + oil. All tests were run at 25 ± 1 °C, 12 h photophase and 75 ± 10% RH or humidities close to saturation. Petri dishes were arranged in airtight plastic box (33 cm × 37 cm × 22 cm). Humidity of 75% inside the box was regulated with a saturated solution of NaCl at the bottom with 1000 g salt/500 ml H_2O (Winston and Bates, 1960). Near saturated humidities (>98%) in moist chambers were maintained with moistened filter paper. In each test, four independent repetitions with