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IN VITRO COMPATIBILITY OF AN ACETOGENIN-BASED BIOINSECTICIDE WITH THREE SPECIES OF ENTOMOPATHOGENIC FUNGI

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Abstract

Despite the promising acute and chronic toxicity of commercial acetogenin-based bioinsecticide formulations reported for different arthropod pests and their recent registration, we are unaware of any published study that examines the compatibility of these botanical derivatives with entomopathogenic fungi, which are important natural and applied biological control agents. We investigated the compatibility of *Annona mucosa* Jacq. (Magnoliales: Annonaceae) ethanolic seed extract (ESAM), an effective bioinsecticide previously characterized by its high acetogenin content, with 3 entomopathogenic fungal species, *Beauveria bassiana* (ESALQ-PL63 isolate), *Isaria fumosorosea* (ESALQ-1296 isolate) and *Metarhizium anisopliae* (ESALQ-E9 isolate) at various concentrations in different in vitro assays. The effects of the botanical insecticide varied according to the fungal isolate exposed, the biological variable analyzed, and the concentration used. However, based on the compatibility index adopted, ESAM was classified as compatible with the above 3 fungal species, except at the highest concentration tested (8,000 mg L^{-1}) for *M. anisopliae*, and it was therefore classified as moderately toxic in this case.

Key Words: Botanical insecticides, *Annona mucosa*, *Beauveria bassiana*, *Isaria fumosorosea*, *Metarhizium anisopliae*

Resumo

Apesar da promissora toxicidade (aguda e crônica) reportada sobre diferentes artrópodes-praga e do recente registro de formulações comerciais de bioinseticidas à base de acetogeninas, não é de nosso conhecimento qualquer estudo até então publicado que avaliasse a compatibilidade desses derivados botânicos com fungos entomopatogênicos, importantes agentes de controle biológico natural e aplicado. Assim, este estudo investigou a compatibilidade do extrato etanólico de sementes de *Annona mucosa* Jacq. (Magnoliales: Annonaceae) (ESAM), um eficaz bioinseticida previamente caracterizado pelo seu alto teor de acetogeninas, sobre três espécies de fungos entomopatogênicos: *Beauveria bassiana* (isolado ESALQ-PL63), *Isaria fumosorosea* (isolado ESALQ-1296) e *Metarhizium anisopliae* (isolado ESALQ-E9), em diferentes concentrações e ensaios *in vitro*. Os efeitos do inseticida botânico testado foram variáveis de acordo com o isolado fúngico exposto, a variável biológica analisada e a concentração utilizada. Porém, com base no índice de compatibilidade adotado, ESAM foi classificado como compatível com as três espécies de fungos, exceto com *M. anisopliae* na maior concentração testada (8.000 mg L^{-1}), caso em que foi classificado como moderadamente tóxico.

Palavras-chave: Inseticidas botânicos, *Annona mucosa*, *Beauveria bassiana*, *Isaria fumosorosea*, *Metarhizium anisopliae*

Entomopathogenic fungi are important agents in regulating the populations of arthropod pests of agricultural (Charnley 1977; Shah & Pell 2003; Li et al. 2010), medical (Scholte et al. 2004; Knols et al. 2010; Singh & Prakash 2014), and veterinary importance (Kaaya & Hassan 2000; Fernandes et al. 2012). The fungi interfere with pest populations mainly by naturally occurring as epizootics (conservative biological control), an important factor for ecosystem balance, and via applications of commercial mycoinsecticide formulation (inundative biological control). Mycoinsecticides are increasingly used as an important tool for integrated pest management (IPM) programs of agricultural crops, especially in organic farming systems (Zehnder et al. 2007). However, successful management strategies using entomopathogenic fungi require a deep understanding of how environmental factors affect pathogen/host dynamics and of the possible interactions among

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different agronomic practices used in crop management (Ribeiro et al. 2012).

Recently, the removal of synthetic active ingredients from the market, the increased demand by consumer markets, and the expansion of alternative "low-input" farming systems have caused a marked increase in botanical insecticide use for crop protection in many countries. In Brazil, this scenario, together with the recent enactment of specific legislation for registering products used in organic farming [Decree no. 6,913/2009 (Silva 2009)], has catalyzed bioprospecting studies aimed at detecting new sources of raw material for botanical pesticide development, a potential market that is still underexploited in Brazil, in light of the country's vast plant biodiversity.

Among the classes of bioactive secondary metabolites (allelochemicals) synthesized by Neotropical plant species, annonaceous acetogenins, natural compounds (C-35/C-37) derived from long-chain fatty acids (C-32/C-34) combined with a 2-propanol unit, stand out because of their diverse bioactivities. Notable among these activities is their potent insecticidal/acaricidal action, which results from the ability of annonaceous acetogenins to inhibit complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial electron transport systems and plasma membrane enzyme NADH:oxidase (Alali et al. 1999; González-Coloma et al. 2002).

Given this potential, our current screening program (Ribeiro 2014), aimed at obtaining bioactive allelochemicals from Neotropical Annonaceae, found promising insecticidal/insectistatic effects of *Annona mucosa* Jacq. (Magnoliales: Annonaceae) ethanolic seed extract (ESAM) on pest species of stored grains (Ribeiro et al. 2013; Ribeiro et al. 2014a) and other pest species important for tropical fruit crops, horticultural species, and other commodities (cereals and oilseeds) in laboratory tests with crude extracts and in protected and field crops with formulated extracts (Ribeiro et al. 2014 b,c). Subsequently, biomonitored fractionations indicated that the acute and chronic toxicity of this botanical derivative is due to the synergistic interaction of structurally diverse acetogenins (unpublished data).

Despite the promising acute and chronic effects on pest species of agricultural importance already reported in studies available in the scientific literature (Leatemia & Isman 2004; Cólom et al. 2008; Blessing et al. 2010; Ribeiro et al. 2013; Ribeiro et al. 2014 a,b,c) and the recent availability of acetogenin-based formulations on the market in eastern countries (Anosom®, Bio Rakshak®, AnonaCin®), we are unaware of any published study that sought to evaluate the compatibility of Annonaceae derivatives with entomopathogenic fungi, a key issue to be considered within IPM programs. Therefore, this study investigated the in vitro compatibility of ESAM with 3 entomopathogenic fungal species, *Beauveria bassiana* (ESALQ-PL63 isolate), *Metharizium anisopliae* (ESALQ-E9 isolate), and *Isaria fumosorosea* (ESALQ-1296 isolate), which are important microbial control agents under Brazilian conditions. Currently, the first 2 isolates are being used in the production of commercial mycoinsecticides registered in Brazil (Agrofit 2014). In turn, the *I. fumosorosea* isolate in our studies has been showing potential for use in citrus pest management (Conceschi et al. 2013), and it could potentially be used in the development of a new formulation for the Brazilian market.

Materials and Methods

Bioinsecticide Source and Preparation Method

The *A. mucosa* seeds used in preparing the crude extract were obtained from mature fruit collected on 17 Mar 2011 from specimens grown on the "Luiz de Queiroz" College of Agriculture/University of São Paulo campus, in Piracicaba, São Paulo, Brazil (S 22° 42' 28.5" -W 47° 37' 59.6"; altitude: 534 m). One voucher specimen, previously identified by Dr. Heimo Rainer (Department of Systematics and Evolution of Higher Plants, University of Vienna), is deposited in the ESA herbarium of the Department of Biological Sciences at ESALQ/USP, in Piracicaba, SP, under registration number 120985.

To prepare the extract, the seeds (200 g) were dried in an oven at 40 °C for 48 to 72 h, and subsequently the seeds were ground in a knife mill (Model 090, Marconi Equipamentos para Laboratórios Ltda., Piracicaba, SP, Brazil). The plant powder was stored in hermetically sealed glass containers and refrigerated until use. The organic extract was obtained using an ethanol solvent soaking technique (in a 1:5 powder:ethanol ratio, w/v). For this procedure, the plant powder was maintained in the solvent for 3 days and was then immediately filtered through filter paper. The resulting solid material was re-extracted with ethanol, and the entire process was repeated 4 times. The solvent remaining in the filtered solution was eliminated in a rotary evaporator (Model 550, Fisatom Equipamentos Científicos Ltda., São Paulo, SP, Brazil) at 50 °C and -600 mm Hg pressure. The extract yield (18.79%, w/w) was determined after complete evaporation of the solvent under a laminar air flow chamber.

Obtaining and Propagating the Entomopathogenic Fungi

The isolates used in this study (ESALQ-PL63—*Beauveria bassiana*, ESALQ-E9—*Metarhizium anisopliae*, and ESALQ-1296—*Isaria fumosorosea*) were obtained from the Pathogen Collection of the Laboratory of Insect Pathology and

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Microbial Control at ESALQ/USP, in Piracicaba, SP, Brazil. Additional information on the isolate origin and use in commercial mycoinsecticide formulations available on the Brazilian market is detailed in Table 1.

Before using the isolates in the in vitro assays, they were propagated on potato dextrose agar culture medium [PDA Difco® (Becton-Dickinson Company, Franklin Lakes, New Jersey, USA)] with 5 g L^{-1} of the antibiotic Pentabiotico® [benzylpenicillin benzathine, benzylpenicillin procaine, potassium benzylpenicillin, dihydrostreptomycin sulfate, streptomycin sulphate (Fort Dodge Saúde Animal Ltda., Campinas, SP, Brazil)] and incubated in a growth chamber at 25 ± 1 °C and a 12 h photoperiod.

In vitro assays

All in vitro assays were conducted in a climate chamber (at 25 ± 1 °C and a 12 h photoperiod) under a completely randomized design. The concentrations used in the in vitro assays [0, 250, 500, 1,000, 2,000, 4,000, and 8,000 mg L^{-1} (mg of extract per L of medium or solution)] were defined based on the range of the lethal concentrations (LC_{90}) estimated in our previous studies to control different arthropod pest species of agricultural importance with different feeding habits (Ribeiro et al. 2013; Ribeiro et al. 2014 a,b,c). Subconcentrations and superconcentrations were used to determine the thresholds compatible with the aforementioned fungal species.

Effect on Conidia Germination

The conidia produced on PDA culture medium in the absence of ESAM were disaggregated from the Petri dishes by adding 20 mL of Tween 80® adhesive spreader solution (0.01%). The fungal suspensions were quantified in a Neubauer chamber, and the concentration was standardized to 10^6 conidia mL^{-1} in a 50 mL volume, to which different product concentrations were added. The suspensions were then homogenized with a Vortex® apparatus and incubated for 120 min at 25 °C.

After exposing the conidia to ESAM, one 150 µL aliquot was placed in the centers of Rodac® Petri dishes containing 5 mL of Difco® PDA culture medium plus 5 g L^{-1} of Pentabiotico® and 10 µL L^{-1} of Derosal® (Bayer S.A., São Paulo, SP, Brazil). Next, the Petri dishes with the fungal isolates were incubated in a growth chamber (at 25 ± 1 °C and a 12 hour photoperiod) for 24 hours. After this period, the proportions of germinated and ungerminated conidia were counted under an optical microscope (400 x). Seven Petri dishes were used per treatment considered as replicates and the experiment was repeated once (n = 14), per treatment. The controls included an acetone:water solution [1:3 (v v^{-1})] used in solubilizing the extract and sterile distilled water alone (negative control).

Estimating Vegetative Growth

The different ESAM concentrations (0, 250, 500, 1,000, 2,000, 4,000, and 8,000 mg of extract per L of medium) were solubilized in 5 mL of acetone:water [1:3 (v v^{-1})] and were incorporated into Difco® PDA culture medium with Pentabiotico® (5 g L^{-1}), which was added while the medium was still liquid (at approximately 45 °C). For this procedure, 10 mL of culture medium was transferred into Petri dishes (90 mm diam), with 10 Petri dishes per treatment level and the experiment was repeated once (n = 20). The controls included an acetone:water solution [1:3 (v v^{-1})] used to solubilize the extract and one negative control comprised solely of sterile distilled water.

After the culture media solidified, they were inoculated with the fungi by monosporic isolation. For this, the conidia were disaggregated from the Petri dishes by adding 20 mL of 0.01% Tween 80® adhesive spreader solution, and after serial dilution, the conidia were inoculated on Petri dishes (90 mm diam) to obtain approximately 50 conidia per Petri dish. Next, the Petri dishes were incubated in a growth chamber (14:10 h L:D at 25 ± 1 °C) for 24 h for conidia germination. The germinated conidia were observed under a stereoscopic microscope, and with the aid of a sterile needle, one spore was individually transferred to the center of each Petri dish from the different treatment levels.

Colony diam were measured after 2, 4, 6, 8, and 10 days of incubation for *B. bassiana* and *I. fumosorosea* and after 3, 5, 7, and 10 days of incubation for *M. anisopliae* (which had slower initial

TABLE 1. ENTOMOPATHOGENIC FUNGI USED IN THE IN VITRO ASSAYS OF COMPATIBILITY WITH *ANNONA MUCOSA* ETHANOLIC SEED EXTRACT (ESAM).

Species	Isolate	Original host	Sampling location	Commercial brands[1]
Beauveria bassiana	ESALQ-PL 63	*Atta* sp.	Piracicaba, SP	Boveril®
Isaria fumosorosea	ESALQ-1296	*Bemisia tabaci* biotype B	Jaboticabal, SP	—
Metarhizium anisopliae	ESALQ-E9	*Mahanarva posticata*	Boca da Mata, AL	Metarril®

[1]Commercial formulations registered in Brazil (Agrofit 2014) based on the respective isolates.

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growth). The diam comprised the mean of the 2 colony measurements taken along 2 axes perpendicular to one another. Subsequently, colony areas were calculated on the tenth incubation day.

Estimating Conidial Production and Viability

Conidial production (conidiogenesis) was estimated from 5 randomly selected colonies (n = 2 replicates × 5 repetitions) grown in PDA culture medium under the different treatment levels after ten days of incubation. To estimate this parameter, the selected colonies were cut out and individually transferred into test tubes containing 10 mL of sterile distilled water and 0.01% Tween 80®. The conidia were removed from the discs by individually mixing the test tubes with a vortex mixer and an ultrasound apparatus for one minute. Next, the samples were counted in a Neubauer chamber after serial dilutions of the conidia suspensions were performed, which allowed counting under an optical microscope (400 x).

Conidial germination of the fungal species grown on culture medium containing the different botanical insecticide concentrations was evaluated. The suspensions employed to estimate conidial production were adjusted to a concentration of 10^6 conidia mL^{-1}, and the viability was determined following the same procedures described in the in vitro assay about effect on conidia germination above.

Determination of Compatibility between Botanical Insecticide and Entomopathogenic Fungi

The in vitro compatibilities of the botanical insecticide with the 3 entomopathogenic fungal species isolates at different concentrations were calculated using the biological index formula proposed by Rossi-Zalaf et al. (2008), according to the following equation:

$$BI{:}[47*(VG) + 43*(SPO) + 10*Germ]/100$$

where:

BI: biological index;
VG: percentage of vegetative growth compared to the control;
SPO: percentage of sporulation compared to the control;
GERM: percentage of conidia germination compared to the control.

The effect of the botanical insecticide on the fungi was classified based on the following classifications (Rossi-Zalaf et al. 2008): toxic (BI between 0 and 41), moderately toxic (BI between 42 and 66), and compatible (BI greater than 66).

Data Analysis

Generalized linear models (GLMs) belonging to the exponential family of distributions (Nelder & Wedderburn 1972) were used to analyze the data. Fit quality was assessed using half-normal probability plots with envelope simulation (Hinde & Demétrio 1998). Whenever there was a significant difference between the treatments ($\alpha = 0.05$), linear and non-linear regression analyses were used to determine the concentration-response relationships for the variables studied, and the fit quality was subsequently assessed. All analyses were performed using the "R" statistical software (R Development Core Team, 2012), version 2.13.1.

Results

The acetone:water [1:3 (v v^{-1})] solution had no significant effect ($P > 0.05$) on any of the biological variables when the values obtained were compared with those of the negative control (sterile distilled water), independent of the entomopathogenic fungal species. Thus, the acetone:water solution appeared adequate for use in solubilization, as it did not interfere with the variables analyzed.

Conidial Germination. The ESAM did not affect conidial viability of the 3 entomopathogenic fungal species [GLM with quasibinomial distribution: $F = 1.78$, $P = 0.1257$ (*B. bassiana*); $F = 0.34$, $P = 0.9095$ (*I. fumorosea*); $F = 0.61$, $P = 0.7198$ (*M. anisopliae*)], when exposed to solutions containing the extract at concentrations of up to 8,000 mg L^{-1} for 2 h. The mean percentages of germination for all three fungi exposed to all ESAM concentrations were higher than 90% (data not presented).

Vegetative Growth. Incorporating ESAM into the culture medium led to a significant decrease in the mean colony area of the isolates (Fig. 1) [GLM with Gaussian distribution: $F = 61.44$, $P < 0.0001$ (*B. bassiana*); $F = 9.99$, $P < 0.0001$ (*I. fumosorosea*); $F = 10.02$, $P < 0.0001$ (*M. anisopliae*)] after 10 days of incubation for the 3 species studied. An exponential model was used to describe the effect of incorporating the extract at different concentrations into the culture medium (Fig. 1).

Conidiogenesis and Viability. When estimating the number of conidia per unit area (cm^2) produced by the colonies grown in culture medium at the different treatment levels, the different isolates behaved differently (Fig. 2). For all concentrations used, ESAM did not inhibit sporulation in the *B. bassiana* isolate (ESALQ-PL 63), (GLM with quasi-Poisson distribution: $F = 2.00$, $P = 0.0986$). However, the significantly reduced sporulation observed in the *M. anisopliae* isolate was concentration-dependent (GLM with quasi-Poisson distribution: $F = 4.13$, $P = 00043$). In contrast, ESAM stimulated sporulation in the *I. fumosorosea* isolate (GLM with quasi-Poisson

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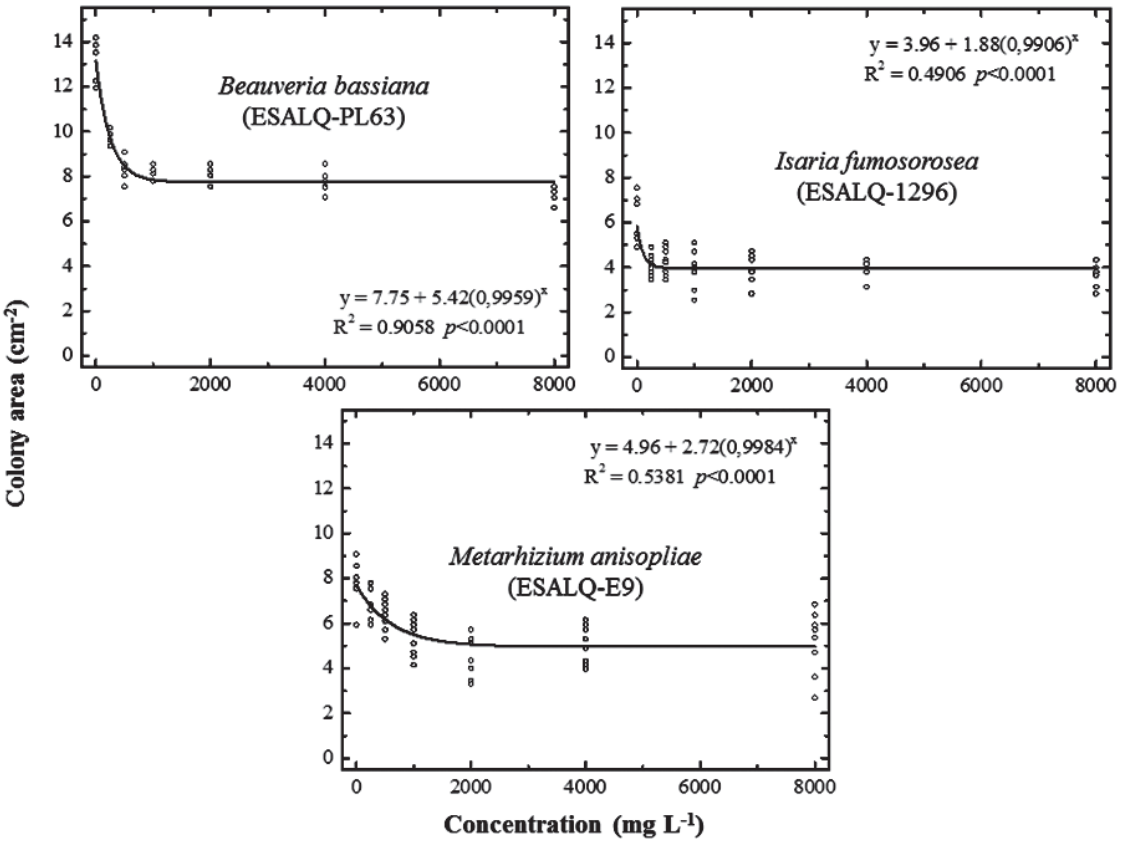


Fig. 1. Colony areas (cm^2) of isolates of 3 entomopathogenic fungal species in culture medium containing different concentrations (mg of extract per liter of culture medium) of *Annona mucosa* ethanolic seed extract (ESAM) after 10 days of incubation.

distribution: $F = 4.31$, $P = 0.0033$), with conidiogenesis tending to increase with increased product concentration in the culture medium up to 4,000 mg L^{-1}, but showing a reduced increase at the highest concentration (8,000 mg L^{-1}). For both species (*M. anisopliae* and *I. fumosorosea*), a third-degree polynomial model was fitted to describe conidiogenesis behavior at different treatment levels (Fig. 2).

Conidia from the 3 species grown in culture medium at the different treatment levels exhibited similar viabilities compared to the control [GLM with quasibinomial distribution: $F = 0.737$, $P = 0.6242$ (*B. bassiana*); $F = 1.5519$, $P = 0.1982$ (*I. fumorosea*); $F = 0.4063$, $P = 0.8685$ (*M. anisopliae*)]. The mean percentages of germination for all three fungi exposed to all ESAM concentrations were higher than 95% (data not presented).

Based on the compatibility index (Rossi-Zalaf et al. 2008), ESAM was compatible with the 3 entomopathogenic fungal species isolates, except for *M. anisopliae* at the highest concentration tested (8,000 ppm), and it was thus classified as moderately toxic in this case (Table 2). In general, and considering all of the variables analyzed, the *M. anisopliae* isolate (ESALQ-E9) was more sensitive to the extract's components, especially to the effect on sporulation.

Discussion

Our results, obtained in vitro assays, indicate that ESAM is compatible, at the concentrations recommended for managing the target arthropod pest species (< 4,000 ppm), with the 3 fungal species studied, which are important microbial control agents in Brazil. In contrast to our results, some studies conducted with the aim of developing new biofungicides reported the toxicity of derivatives of *A. squamosa* or of acetogenins isolated from extracts prepared from its different structures to different fungal species that cause diseases in humans and crops, as well as mycotoxin-producing species in stored food (Gopalakrishnan et al. 2010; Dang et al. 2011; Vidyasagar et al. 2012). However, the following factors may explain the differences observed: variations in the chemical profiles of the acetogenins (with

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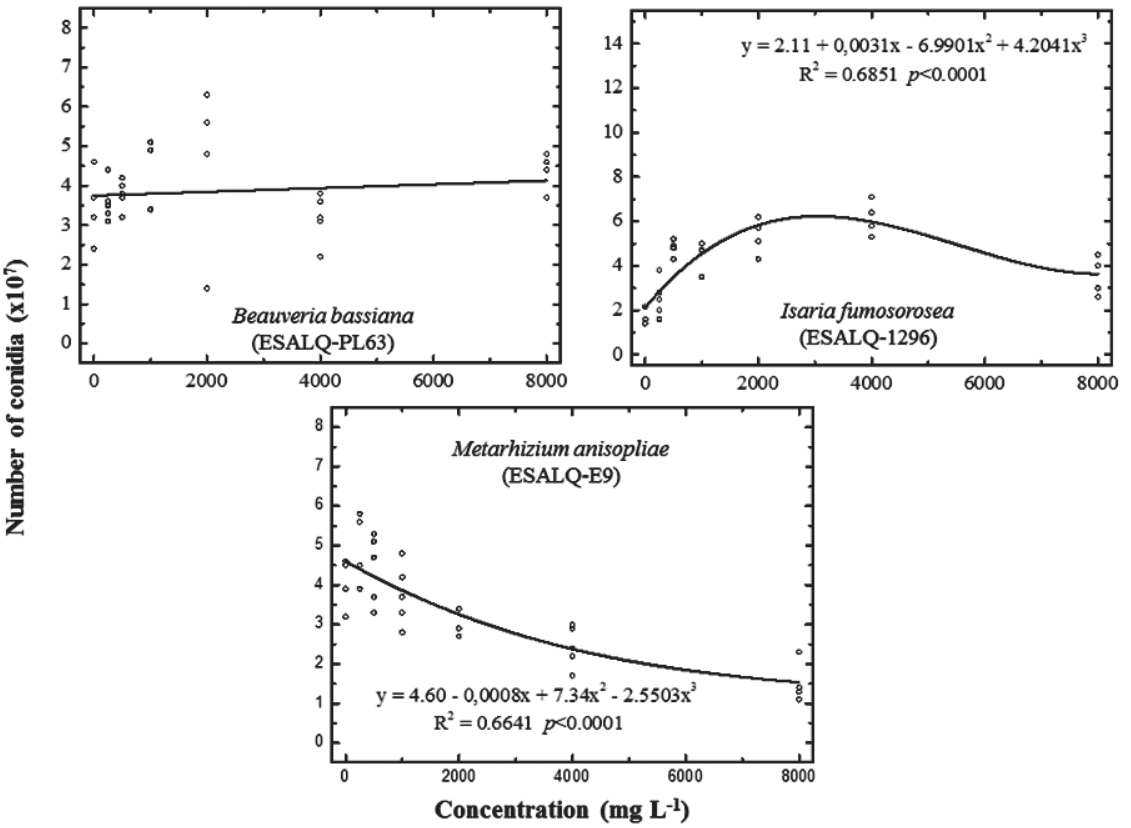


Fig. 2. Number of conidia ($\times 10^7$) produced by each colony of 3 entomopathogenic fungal species in culture medium containing different concentrations (mg of extract per liter of culture medium) of *Annona mucosa* ethanolic seed extract (ESAM) after 10 days of incubation.

their marked structure-activity relationship perculiarities) present in the derivatives of the different species, the use of different solvents and/or extraction methods (with their different selectivity/extraction capacity levels), and the different tolerances of fungal species to the active components. Corroborating the results, we also did not observe significant inhibition of vegetative growth in the CCT7638 *Aspergillus flavus* isolate or an effect on its aflatoxin (AFB_1) production in our previous study (Unpublished data).

According to Alves & Lecuona (1998), the toxicity of pesticides to entomopathogens varies according to the fungal species, the isolate, the active ingredient and its mode of action, the product's formulation, and the recommended dose/concentration. In our study, the effects of the botanical insecticide on the entomopathogenic fungi *B. bassiana* (ESALQ-PL63 isolate), *I. fumosorosea* (ESALQ-1296 isolate), and *M. anisopliae* (ESALQ-E9 isolate) depended on the fungal species exposed, the biological variable analyzed, and the concentration used.

Conidial germination is the first step in fungal infection in the insect. Thus, if a chemical product reduce or inhibit conidial germination, it will probably reduce the efficiency of the fungi in controlling pests and establishing epizootics in the field. In the present study, we showed that conidial germination of the 3 fungal species was not affected when they were exposed to concentrations of up to 8,000 mg L^{-1} for 2 h, a scenario that simulates direct contact of the conidia in a liquid spray mixture. Thus, it is possible to infer that ESAM and these entomopathogens can be tank mixed. Also, the natural populations of these pathogens will not be impacted after of ESAM applications in the field. Several studies reported a strong positive correlation between germination speed and virulence in fungal pathogens (Hassan et al. 1989; Altre et al. 1999; Rangel et al. 2008).

Entomopathogenic fungal mycelia grow within the insect, and thus there is small chance that they would be negatively affected by a chemical product at this phase (Jaronski 2010). Vegetative growth precedes the conidiogenesis, an important part of fungal life cycle for the development of secondary infections and for the fungus to persist in the environment (Schumacher & Poehling 2012). In the present study, reduced vegetative growth

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TABLE 2. *IN VITRO* TOXICITY CLASSIFICATION OF THE *ANNONA MUCOSA* ETHANOLIC SEED EXTRACT (ESAM) FOR ISOLATES OF 3 ENTOMOPATHOGENIC FUNGAL SPECIES.

Species	Concentration (ppm)	BI*	Classification**
Beauveria bassiana (ESALQ-PL63)	250	92.72	Compatible
	500	90.99	Compatible
	1,000	95.34	Compatible
	2,000	106.10	Compatible
	4,000	80.52	Compatible
	8,000	96.87	Compatible
Isaria fumosorosea (ESALQ-1296)	250	111.45	Compatible
	500	155.56	Compatible
	1,000	131.56	Compatible
	2,000	112.54	Compatible
	4,000	128.46	Compatible
	8,000	93.43	Compatible
Metarhizium anisopliae (ESALQ-E9)	250	95.99	Compatible
	500	96.86	Compatible
	1,000	84.96	Compatible
	2,000	74.15	Compatible
	4,000	69.19	Compatible
	8,000	64.83	Moderately toxic

*BI: Biological index proposed by Rossi-Zalaf et al. (2008);
**Classification: BI between 0 and 41 = toxic; BI between 42 and 66 = moderately toxic, and BI higher than 66 = compatible.

(fungistatic effect) was observed in the 3 fungal species in the presence of botanical insecticide in the culture medium. However, the fact that a chemical product reduces the mycelial growth of an entomopathogenic fungus is not necessarily indicative of a significant reduction in conidial sporulation and germination (Zimmermann 1975). We observed reduced conidiogenesis (conidia cm^{-2} of colony) of *M. anisopliae*, whereas *B. bassiana* was not affected by the different ESAM concentrations. On the other hand, effects on *I. fumosorosea* depended on the concentration used, either stimulating or reducing conidiogenesis. Fungal stimulation by growth-promoting substances present in plants extract has been reported (Akinbode & Ikotun 2008; Ribeiro et al. 2012). In our study, independent of the fungal species, the conidia produced in culture medium under different bioinsecticide concentrations exhibited high viability (> 95%). In general, the *M. anisopliae* isolate was more sensitive to the bioinsecticide, especially in terms of conidiogenesis, which affected its compatibility classification.

The low toxicity of ESAM to entomopathogenic fungi opens new perspectives for the combined use of these strategies in arthropod pest management, especially in organic and ecologically based food production systems, considering that these systems have few control options. Additionally, the feasibility of on-farm preparation of the ESAM bioinsecticide from plant matter [see preparation method in Ribeiro (2010)] and economic viability of this control strategy will facilitate its adoption in developing countries.

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