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EFFECTIVENESS OF *METARHIZIUM ANISOPLIAE* AGAINST IMMATURE STAGES OF *ANASTREPHA FRATERCULUS* FRUITFLY (DIPTERA : TEPHRITIDAE)

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ABSTRACT

The study evaluated the effectiveness of *Metarhizium anisopliae* var. *anisopliae* (Hyphomycetes : Moniliales) strain E9, isolated from the pasture spittlebug *Deois flavopicta* (Hemiptera : Cercopidae), against larvae, prepupae and pupae stage and emergent adults of *Anastrepha fraterculus*, the South American fruitfly. The bioassay was carried out simulating field conditions, on autoclaved (AS) and non-autoclaved (NAS) soil from typical citrus orchards in the State of São Paulo, Southeastern region of Brazil. Various concentrations of conidia were incorporated into the soil the mortality, calculated based on the percentage of adult emergence, was 86% for the highest conidia concentrations: 2.52×10^{10} for AS and 2.52×10^{10} for NAS. The lethal concentration (LC_{50}), expressed as conidia concentration, was 8.44×10^{9} conidia/g of soil (S) for AS and 12.23×10^{9} conidia/g of soil for NAS.

Key words: microbial control, *Metarhizium anisopliae*, *Anastrepha fraterculus*, fruitfly, bioassay

INTRODUCTION

Tephritids fruitflies of the genus *Anastrepha* Schiner, 1868 (South American fruitflies) and *Ceratitis capitata* Wiedemann, 1848 (Mediterranean fruitflies) represent a group of pests of economic importance for the fruit-growing industry worldwide. Approximately 300 species belonging to 41 botanic families have been reported to be hosts of these tephritids (15). Among the most intensively attacked species are guava, peaches, pears, apples, melons, plums, nectarines, mangoes, oranges, and grapes. Due to their wide geographic distribution in tropical, subtropical and temperate climates, these pests also attack wild fruit varieties.

In Brazil, fruitflies of the genus *Anastrepha*, especially *A. fraterculus*, the South American fly, and *A. obliqua*, have the widest geographic distribution (13). These two species, together with *A. sororcula* and *A. grandis*, are considered quarantine species by protection agencies from various countries (14).

These insects have been controlled in fruits using traditional chemical products although efficient efforts are under way for the development of an Integrated Management Program using biological control agents (11,20,21), in addition to attempts involving the use of bioregulators such as gibberellic acid (10). Entomopathogenic agents such as fungi, bacteria, viruses and nematodes have been reported to infect tephritids (2). However, quantitative studies of the action of predators and of entomopathogenic agents in terms of fruitfly mortality are not as frequent as studies of the action of parasitoids (6). Among the entomopathogenic fungus cited in the literature are *Paecilomyces fumosoroseus* and *Beauveria bassiana* (4). Studies by Garcia *et al.* (8,9) have shown the virulence of *Metarhizium anisopliae* on larvae, pupae and adults of *Ceratitis capitata*.

In the present investigation we evaluated the susceptibility of the species *A. fraterculus* to *Metarhizium anisopliae*, an entomopathogenic hyphomycete, under conditions of bioassay in soil, since under field conditions the larvae, after developing

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in the fruit, fall to the ground and penetrate the soil, later transforming into pupae and emerging as adults (19). The use of *M. anisopliae* is justified by the results already obtained against *C. capitata* and by the fact that it is currently sold as an important biological control agent for various insect species in many countries such as Colombia, Australia and the United States of America. Particularly in Brazil, more than 1,000,000 ha have been treated for the control of the leaf spittlebug *Mahanarva posticata*, and the root spittlebug *M. fimbriolata* (Hemiptera : Cercopidae) in sugar cane and spittlebug in pasture grasses (12). In addition, it should also be mentioned that importers require **"in natura"** fruits, specially those without pesticides residues (5).

MATERIALS AND METHODS

Strain used and inoculum production

We used strain E_9 of *M. anisopliae* var. *anisopliae*, isolated from *Deois flavopicta*, the pasture spittlebug maintained in the germplasm bank of the Department of Genetics and Evolution, Biology Institute, UNICAMP, and reisolated from *A. fraterculus* larvae. Conidia of the E_9 reisolate of *A. fraterculus* were obtained from a culture grown for 10 days on rice medium containing 100 g rice and 80 mL water in Erlenmeyer flasks autoclaved at 1 atm for 20 minutes. At the end of culture period, the conidia were collected by scraping the surface of the culture medium with the aid of a sterilized spatula and pressed through a sterilized 1.25 µm opening sieve. For the preparation of the desired concentration, the conidia were counted with a hemocytometer. The viability was determined in relation to the percentage of germination in minimal medium (MM) (NaNO3 6.0g, KCl 0.52 g, MgSO4.7 H2O, 0.52 g, KH2PO4 1.52 g, glucose 10.0 g and trace of FeSO4.7H2O and ZnSO4 both at the concentration of 1% and 15 g agar per litre pH 6.9) by counting 500 germinated and non-germinated conidia using a phase contrast microscope 15 hours after inoculation at 28ºC. The doses, expressed as conidia concentration (con) per gram (g) of soil (S), were as follows: (d_1) 2.52×10^8 con/gS, $d_2 = 2.52 \times 10^9$ con/gS, and $d_3 = 2.52 \times 10^{10}$ con/gS.

Collection of larvae of the host insect

The 13 days old larvae of *A. fraterculus* at were obtained from laboratory population using papaya (*Carica papaya* - Caricaceae) as oviposition substrate.

Conidia longevity under soil conditions

Longevity was estimated on the basis of percent of conidia germination calculated as mentioned above in autoclaved (AS) and non-autoclaved (NAS) soil maintained under the same experimental conditions as used in the bioassay and inoculated with 10^7 conidia/g S. Longevity was evaluated over a period of 28 days using weekly 3 samples of 5 g of soil each. The samples were transferred to Erlenmeyer flasks containing 300 mL sterilized distilled water and 5 mL 0.1% Tween 80 and shaken at 150 rpm for 120 minutes. After this time, 0.1 mL aliquots of the supernatant were removed and plated onto dodine medium (3) modified by the addition of 15.0 g rice cream and 1 mg streptomycin per mL of culture medium. The plates were incubated for 96 h at 28ºC for viability evaluation by counting the colony forming units (cfu).

Bioassay preparation

The assay was carried out in 500 mL 7.5 cm deep plastic cups with a mouth diameter of 8.5 cm, containing 400 g of air-dried dirt red latossol typical of the citrus orchards region in the State of São Paulo, in two different preparations, one containing AS and the other NAS and moisturized to 75% humidity, obtained by determination of dry weight and addition of deionized sterile water. Different conidia concentration were incorporated into the soil as cited previously, and 50 larvae in the prepupal phase (13 days old) were then released on the soil surface for each of three replicates in the different treatments. Three replicates without conidia were maintained in each treatment as controls. The preparations were maintained under environmental conditions with humidity of approximately 75% and temperature of 28ºC.

The effect of treatment on the emerging population in relation to the control was evaluated daily after the emergence of the first adult. The infection confirmation by the fungus was done by collecting and carefully washing dead hosts (larvae, pupae and adults) three times in 2.5% (v/v) sodium hypochloride, and 0.1% (v/v) Tween 80 solution for 3 minutes and three times in 0.85% NaCl solution. After being washed, they were transfered to a moisturized chamber and incubated at 28ºC, until development of the propagulus of the fungus on the cadaver. The median lethal concentration (LC_{50}) was calculated by the probit method (7) with correction for the natural population reduction in the control (1).

RESULTS AND DISCUSSION

Under the studied conditions, the developmental cycle of the host insect, consisting of the larvae at the prepupal stages and pupae until adult emergence (considered as the beginning of emergence), lasted approximately 12 to 17 days after larval deposition on the soil surface, when the environmental temperature was about 28ºC and humidity about 75%. The prepupal larval period lasted up to 32 h, with a concentration around 24 h for both AS and NAS. These results are in agreement with data obtained by Orlando and Sampaio (16) who reported a cycle of 10 to 20 days. Thus, no type of antibiosis caused by biotic factor of the microbial population existing in NAS or by other measurable abiotic factor was observed during the experiment. Despite the relatively long developmental cycle of the host, the period of exposure, which is the period when the conidia have the opportunity for adherence and penetration through the integument of the larval stage, was about 24 hours

as observed by Bechara *et al.* (in preparation). This phase corresponds to adherence and penetration starting with conidia contact with the cuticle, through germination and the presence or absence of differentiation into apressoria, which, according to Roberts *et al.* (17), are initial events in the mechanism of infection by entomopathogenic fungi. The longevity of the conidia in the soil, (germinative capacity of the conidia) ranged from 94.06% for NAS and 94.70% for AS at the beginning of the experiment to 31% on the 28th day (Table 1) for both NAS and AS. Based on the data concerning survival of conidia on AS and NAS (Table 1), normalized by angular transformation α^0 = arc sin $\sqrt{p}$, where p = (%/100), we performed the analysis of variance (Table 2). The ANOVA (NAS and AS) did not show significant differences between AS and NAS. Thus, the conidia exhibited the potential for host infection under the bioassay conditions. The presence of viable conidia throughout the experiment indicates infection not only of larvae, but also of pupae and emerging adults. As observed by soil inspection this appears to have occurred at the end of the bioassay, even though this infection probably occurred during the larval or prepupal or during the pupation stage and was manifested by the fungus growing on cadaver and dead pupae, as well as on dead emergents adults.The mortality confirmation was done by the reisolation of the fungus from the host after being carefully surface washed as mentioned in bioassay preparation. Figs. 1a, b and c, show the development of the fungus in these stages; however, it was not an objective of the present study to evaluate mortality during the different developmental stages but rather to assess the reduction of adult emergence, considered as the additive result of a total lethality action for the various stages. Thus, in the preparation of the bioassays we opted for the incorporation of conidia into the entire soil because of the typical behavior of the larvae, which after falling on the ground, burrow into the soil to different depths, usually ranging from 2 cm to 10.0 cm (18), and more often from 2 to 6 cm. In this movement during the prepupal phase, conidia removal may occur if the conidia were only placed on the surface. Also, the conidia may be exposed to other unfavorable abiotic factors with a consequent reduction in inoculum potential. However, surface application would be much easier and therefore this possibility should be considered when evaluating applicability in the field.

Table 1. Viability of reisolated conidia of *M. anisopliae* strain E9 from soil (NAS and AS) during bioassays expressed as colony forming units (CFUs) on dodine medium.

Days since inoculation	% Viability*	
	NAS	AS
0	94.06	94.70
7	81.90	81.03
14	59.20	57.07
21	49.20	50.33
28	31.00	31.00

* values are means of three replicates

Table 2. The ANOVA for conidia viability of *M. anisopliae* strain E9 on Dodine media expresed as percent of conidia reisolete from NAS and AS based on data from Table 1, normalized by angular transformation α^0 = arc sin $\sqrt{p}$, where p = (%/100).

Sources of variation	GL	F	P	NS
Soil (NAS and AS)	1	0.6667	0.424	NS
Days of inoculation	4	3,920.68	0.000	*
Soil X Days of inoculation	4	2.27	0.098	NS
Residue	20	QMR =	0.4246	

Evaluation of the emerging adult population in relation to the control permitted the determination of the LC_{50} values both in AS and NAS using probit analysis (7) with correction for natural mortality in the control (1).

As can be seen in Fig. 2, the probit lines for AS and NAS did not diverge since their regression coefficients (b = 1.2748 for AS and b = 1.27736 for NAS) were quite close. This was not the case for the coordinates of origin which were 6.1091 and 4.6923, respectively, and which presented a **t** value of 4.2962 < 4.303 for 2. d.f. at the 5% level of probability when tested. However, this could be considered a significant tendency since the **t** probability obtained would be p = 0.052. In both cases, the chi-square values indicated that the curves fitted since there were no significant differences (p = 0.694 for AS and p = 0.627 for NAS).

The LC_{50} values, although different (8.44 x 10^9 con/gS for AS and 12.23 x 10^9 con/gS for NAS), presented different confidence

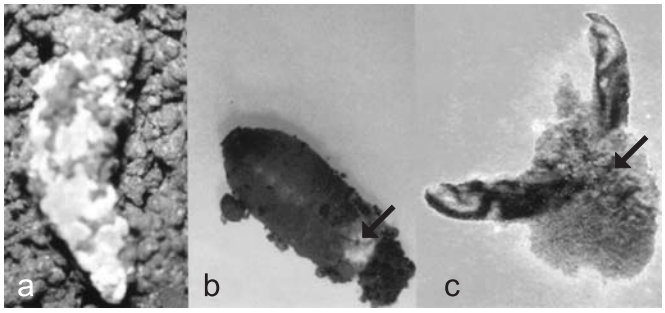
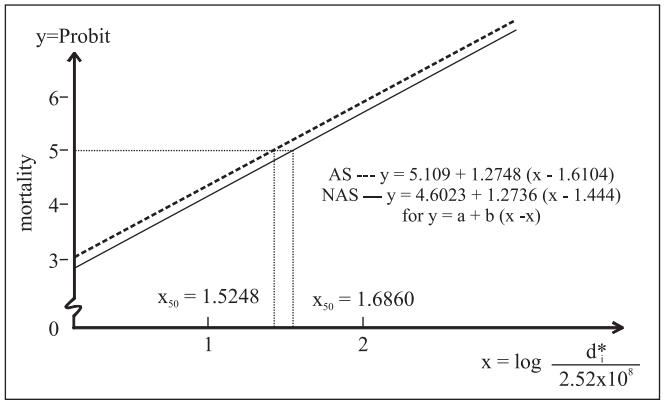


Figure 1. Carcasses of imatures stages and adult of *A. fraterculus* infected with *M. anisopliae* showing mycelial development after infection with conidia incorporated into the soil. (a) larvae, the white color is given by the young mycelial growth; (b) pupae: mycelium and conidiation growing out from the extremity (arrow) and (c) emergent adult completely covered with mycelia and young conidia (arrow).

intervals (CI) of 5.15 x 10^9 con/gS to 13.84 x 10^9 con/gS and 7.43 x 10^9 con/gS to 20.14 x 10^9 con/gS, respectively, which crossed and contained the LC_{50} for both treatments at their extremes (Fig. 3a). The LC_{50} value for AS had a lower confidence interval than for NAS, which did not reach 2/3 of the confidence interval for NAS. If the CI for NAS were equal to that for AS, they would not include the LC_{50}. This may be due to a greater heterogeneity possibly occurring in NAS, which would disperse its CI. On the other hand, this is reflected in the calculation of the potency (R) of AS taking NAS as the standard. The potency of AS was R = 1.45 and CI of 0.85 to 2.47 (Fig. 3b) indicates that NAS could be almost identical to AS, perhaps because of its dispersion, with the latter being on average 45% more potent and reaching a maximum value around 147% higher. This amplitude of the data is possibly due to the dispersion produced by the number of doses, a fact that could be improved using intermediate doses.



* were corresponds to different doses based on conidia concentration/gS.

Figure 2. Probit curves corrected for mortality (reduction on the emergent adult population) in relation to control.

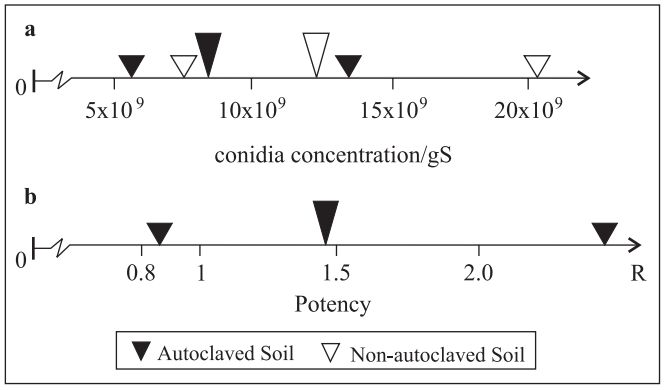


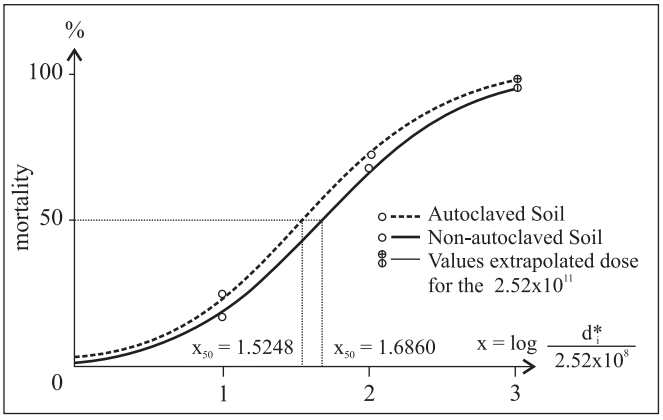
Figure 3. a) LD50 for AS and NAS and its respective confidence intervals; b) Relative Potency = R=1.45 (0.82 - 2.42). (standard = non-autoclaved soil)

Using a graphic extrapolation (Fig. 4), we may state that a concentration of 2.52 x 10^{11} con/gS may produce mortality rates of 97% and 95% for AS and NAS, respectively. However, we obtained respective mortality rates of 70 and 67% with the doses of 2.52 x 10^{10} con/gS which is not negligible as a control and which potentiates the large-scale use of the inoculum. It is important to mention that 50 g of rice media yielded 1 g of conidia having approximately 2.68 x 10^{10} viable conidia.

The cumulative emergence data were converted to percentages of the total number of larvae used for each treatment (150 larvae) and are reported without the 1st dose since the results of the latter were closely similar to those of the controls (Fig. 5). Cumulative emergence increased in a linear manner in both controls, reaching a maximum of 70% on the 6th day. With the 2nd dose there was no emergence on the first day (in relation to the beginning of emergence) in AS, and the curve soon took on an irregular sigmoidal shape until a maximum of 54% emergence was reached on the sixth day. With respect to NAS, emergence started on the first day at 2%, rapidly increasing to 58% on the fifth day and stabilizing at 60% on the sixth day, in a sigmoidal manner, a value practically parallel to that for AS.

With the 3rd concentration, emergence was the same as obtained with the 2nd concentration on the first day or 0% for AS and 2% for NAS, with the curves following a slightly sigmoidal shape and with little difference between them, both reaching maximum emergence on the fifth day at 14% both at the conidia concentration of 2.52 x 10^{10} for AS and NAS.

These last two results indicate that at these conidia concentrations there was no difference in maximum emergence on the fifth day, a fact possibly due to the death of more sensitive larvae before pupation or to death of adults when emerging from the pupae. Despite these hypotheses, however, it is clear



* where di corresponds to the different doses based on conidia concentation/gS utilized.

Figure 4. Mortality rate corrected in relation to control mortality in autoclaved soil (AS) and non-autoclaved soil (NAS).

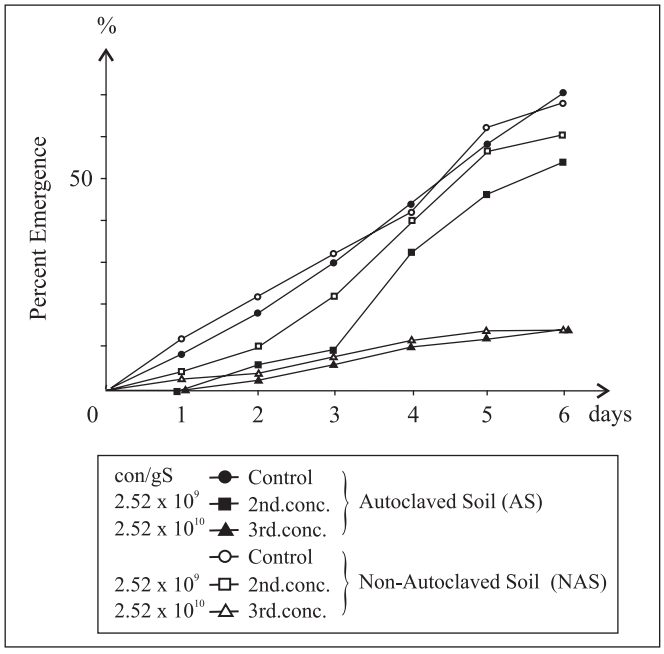


Figure 5. Percent daily adult emergence for the control and 2nd and 3rd conidia concentration (The curve for the 1st conidia concentration is not presented in the figure because it was considerably similar to the control curve) con/gS = conidia per gram of soil; conc.= conidia concentration.

that the effect of the fungus led to an 86% reduction in population emergence, a fact that recommends its use in field tests as a control agent.

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RESUMO

Eficácia de *Metarhizium anisopliae* a estágios imaturos de *Anastrepha fraterculus* a mosca das frutas (Diptera : Tephritidae)

O estudo avaliou a eficácia de *Metarhizium anisopliae var. anisopliae*, (Hyphomycetes : Moniliales) linhagem E9, isolada da cigarrinha das pastagens *Deois flavopicta* (Hemiptera : Cercopidae), contra larvas, prepupas, pupas e adultos emergentes de *Anastreha fraterculus*, a mosca Sul Americana das frutas. Os bioensaios foram conduzidos simulando condições de campo em solo autoclavado (AS) e não autoclavado (NAS) de pomares típicos de citros no Estado de São Paulo, sudeste do Brasil. Várias concentrações de conídios foram incorporadas no solo. A mortalidade calculada sobre a porcentagem de adultos emergentes, foi de 86% para as concentrações mais altas de conídios: 2,52 x 10^{10} para AS e 2,52 x 10^{10} para NAS. A concentração letal (LC_{50}) expressa pela concentração de conídios, foi 8,44 x 10^9 conídios/g de solo para AS e 12,2 x 10^9 conídios/g/S para NAS.

Palavras-chave: controle microbiano, *Metarhizium anisopliae*, *Anastrepha fraterculus*, mosca-das-frutas, bioensaio

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