

**BIOASSAY ASSESSMENT OF *METARHIZIUM ANISOPLIAE* (METCHNIKOFF) SOROKIN  
(DEUTEROMYCOTA: HYPHOMYCETES) AGAINST *ONCOMETOPIA FACIALIS* (SIGNORET)  
(HEMIPTERA: CICADELLIDAE)**

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**ABSTRACT**

Citrus Variegated Chlorosis (CVC) is an economically important, destructive disease in Brazil and is caused by *Xylella fastidiosa* and transmitted by sharpshooter insects. In this study, the efficacy of the fungus *Metarhizium anisopliae* in controlling the sharpshooter *Oncometopia facialis* was studied by bioassay conditions. In the bioassay, insects were sprayed with a suspension containing  $5 \times 10^7$  conidia mL<sup>-1</sup>. Adults captured in the field were treated in groups of 10 in a total of 11 replications per treatment. Significant differences between the natural mortality and the mortality of insects treated with the fungus were observed 6 days after inoculations ( $P < 0.05$ ). These significant differences increased until 10 days after treatment. The fungus caused 87.1% mortality, with the  $LT_{50}$  varying from 5 to 6 days. The  $LC_{50}$  was  $1.2 \times 10^6$  conidia mL<sup>-1</sup>, varying from  $7.7 \times 10^5$  to  $2 \times 10^6$  conidia mL<sup>-1</sup>. The results showed that the sharpshooter *O. facialis* was susceptible to the entomopathogenic action of *M. anisopliae* in controlled condition during bioassay.

**Key words:** microbial control, entomopathogen, Citrus Variegated Chlorosis (CVC), bioassay

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**INTRODUCTION**

The entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin has been isolated from many insect species including the orders Lepidoptera, Coleoptera, Orthoptera and Hemiptera (6,34). *M. anisopliae* is used commercially in Brazil to control the sugar cane spittlebug, *Mahanarva posticata* (Homoptera: Cercopidae), and it is also used in other countries, including Colombia, Australia and the U.S.A. to control a variety of pests. The wide insect host range of the genus *Metarhizium* makes it commercially attractive as a biocontrol agent (24). Excessive use of conventional chemical insecticides has resulted in pest resistance, elimination of economically beneficial insects, residue persistence in the environment, toxicity to humans and wildlife, and a higher cost of crop production (22).

*Xylella fastidiosa* is a fastidious Gram-negative, xylem-limited bacterium (5, 30). Specific strains cause diseases in many crops of economic importance, such as grape, almond, peach, coffee, plum and citrus (12,15-17,25,30). In Brazil, the most economically important disease caused by *X. fastidiosa* is Citrus Variegated Chlorosis (CVC) (15, 31). In Brazil, over 70 million sweet orange trees (38%) are affected annually. CVC is responsible for losses of US\$ 100 million per year to the Brazilian citrus industry and affects all commercial sweet orange varieties (7,8). *X. fastidiosa* is disseminated rapidly from the use of infected nursery trees and transmission of *X. fastidiosa* by several xylem-feeding sharpshooter insect vectors (4).

The insect vectors that transmit *X. fastidiosa* to plants are leafhoppers of the subfamily Cicadellinae (Hemiptera: Cicadellidae), known as sharpshooters. In citrus plants, *Oncometopia facialis* (Hemiptera: Cicadellidae: Cicadellinae)

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is the principal vector of the bacterium *X. fastidiosa* (28). The principal means of controlling this vector has been the use of chemical insecticides (34,35) with consequent ecological problems.

In developing effective replacements for toxic chemicals, entomopathogenic fungi have been considered as an alternative (29). Adoption of a biological control agent, such as *Metarhizium*, in integrated pest management (IPM) often results in overall reduction in the total amount of pesticide applied (26).

The aim of this study was to investigate the efficacy of the entomopathogenic fungus *M. anisopliae* to control the sharpshooter *O. facialis* under conditions of bioassay in citrus plants.

## MATERIALS AND METHODS

### Strain of *M. anisopliae*

We used strain E<sub>9</sub> of *M. anisopliae* var. *anisopliae*, isolated from *Deois flavopicta* (Hemiptera:Cercopidae), obtained from a stock collection of the Department of Genetics and Evolution, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil. This strain is largely used in bioassays and genetic studies (9,11,20,23,32). The conidia used in these studies were produced on complete culture medium (CM) consisting of yeast extract, glucose, minerals, agar, and water, according to Alves *et al.*, 1998 (3). The E<sub>9</sub> strain was plated on Petri dishes containing CM and incubated in a B.O.D. chamber at 27°C ± 1°C for 6 to 7 days.

### Insects

Adults of *O. facialis* were collected from orchards of sweet-orange (*Citrus sinensis* [L.] Osbeck cv. pera) containing CVC-symptomatic orange trees in Araraquara, São Paulo, Brazil. The insects were carefully transported from the field to cages. Each cage (20cm x 50cm) contained one potted sweet-orange seedling. The potted plants were maintained in the greenhouse at a temperature of 27°C ± 1°C, 63% ± 5% relative humidity.

### Lethal Concentration Estimation (LC<sub>50</sub>)

Six suspensions (10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup> conidia mL<sup>-1</sup>) of the *M. anisopliae* strain E<sub>9</sub> were tested against field-collected *O. facialis* to determine the LC<sub>50</sub>. The LC<sub>50</sub> values were analysed using POLO-PC, a computer programme for probit analysis (14) based on the method of Finney (10).

### Conidial Viability

Conidial concentrations were determined with an improved hemocytometer adjusted to 10<sup>7</sup> conidia per milliliter by dilution with 0.1% Tween 20. One milliliter of each suspension was removed and diluted to 10<sup>6</sup> conidia mL<sup>-1</sup> using 0.1% Tween 20. Spore viability was determined by plating 100 µl of the conidial suspensions on CM and counting colonies after 48h. Spore

germination of *M. anisopliae* strain E<sub>9</sub> exceeded 94% in all cases. All samples were adjusted to a final concentration of 10<sup>7</sup> conidia mL<sup>-1</sup>.

### Bioassays

Experiments were conducted with field-captured adult sharpshooters. Plots consisted of 10 sharpshooters, totaling 110 sharpshooters per treatment in a completely randomized design with 11 replicates. Insects were sprayed with a suspension of conidia at a 5 X 10<sup>7</sup> concentration conidia mL<sup>-1</sup> with a viability of 94.9%. Evaluations of the entomopathogenic action were done daily and after a period of 10 days. This experiment was conducted in a greenhouse with the same temperature and relative humidity cited above.

Dead sharpshooters were collected daily from the fungal treatment groups and the control groups and were tested to determine if mortality was due to infection. To confirm the identification of the primary pathogen, the sharpshooters were surface-sterilized by dipping them successively in 70% ethanol (10 min), 2% sodium hypochlorite solution (2 min), and sterile water (40 seconds) and transferred with a camel-hair brush to Petri dishes containing CM media (19). These plates were sealed with parafilm and incubated at 27°C ± 1°C for 7-14 days. The dead sharpshooters were observed daily for the presence of external fungal structure such as hyphae and conidia. Dead sharpshooters with external hyphae and conidia were counted and only sharpshooters that showed fungal growth were considered to have died of infection, and only these counts were used to compute the pathogenicity of the fungal pathogens.

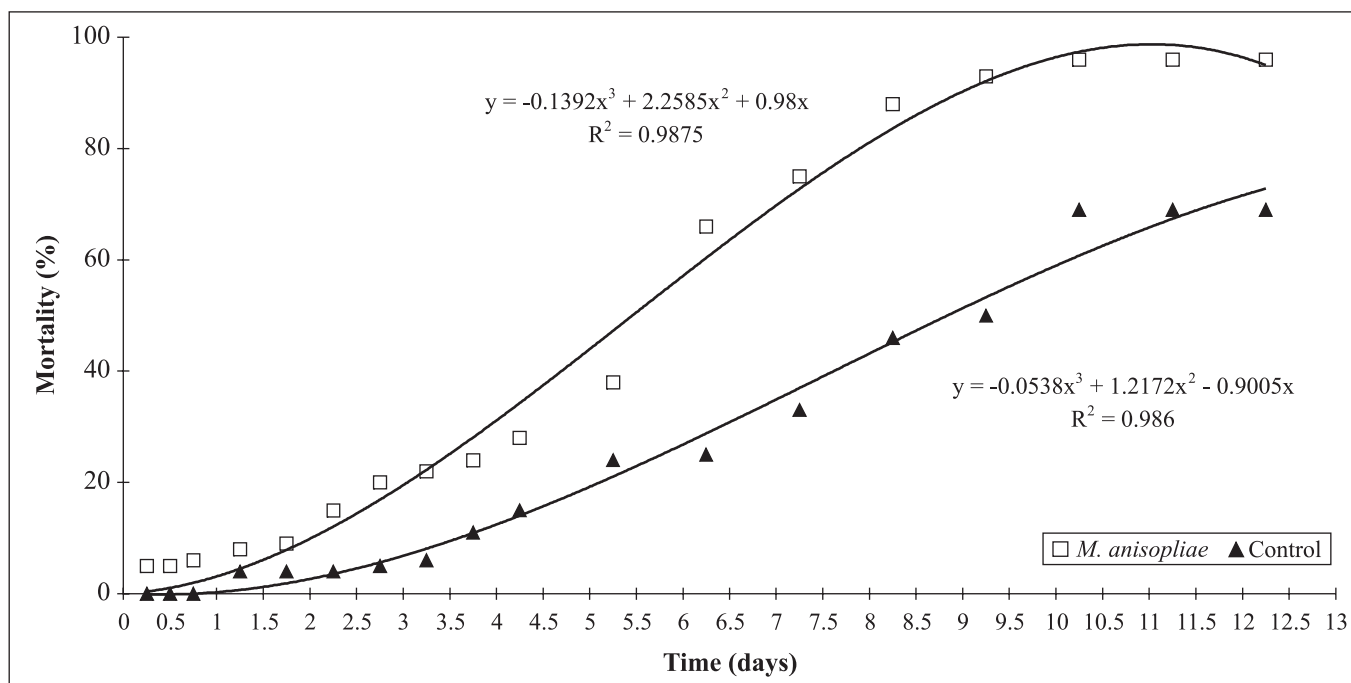
### Statistical Analysis

The daily mortality values were accumulated during the experiments to allow LT<sub>50</sub> (lethal time) calculation by Probit analysis using the Mobae computer program (13). The mite mortality data were submitted to analysis of variance using the F test and the means were compared by Tukey test (*P* < 0.05) using the Sanest (version 3.0) software package.

## RESULTS AND DISCUSSION

The *M. anisopliae* strain E<sub>9</sub> was tested against *O. facialis* sharpshooters at six suspensions of 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup> conidia mL<sup>-1</sup> and compared with controls. Each treatment and control group had 10 insects. The *M. anisopliae* strain E<sub>9</sub> showed an LC<sub>50</sub> of 1.2 X 10<sup>6</sup> conidia mL<sup>-1</sup>, varying from 7.7 X 10<sup>5</sup> to 2 X 10<sup>6</sup> conidia mL<sup>-1</sup>.

After 10 days, 96% mortality was observed for *M. anisopliae*-infected *O. facialis* and 69% mortality was observed for non-infected *O. facialis* (negative control) (Fig. 1). Natural mortality was excluded because it happened in both treated and negative control groups. Mortality percentage was corrected using Abbott's formula (1). Corrected mortality was 87.1%, which



**Figure 1.** Mean cumulative mortality (%) of *Oncometopia facialis* during an observation period of 12 days after application of the *Metarhizium anisopliae* suspension of  $5 \times 10^7$  conidia mL<sup>-1</sup> and the untreated control.

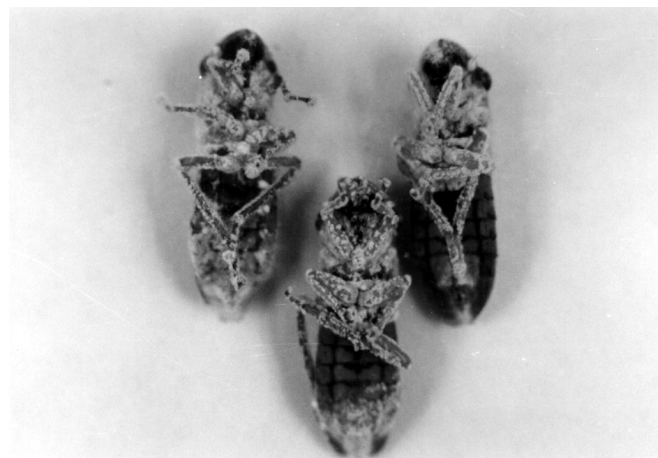
represented the percentage of dead sharpshooters due to action of *M. anisopliae* (Table 1).

Colonization of *O. facialis* by *M. anisopliae* occurred between 24h and 72h. As typical in *M. anisopliae*-infected insects, the external fungal growth initiated on the intersegmental regions before spreading over the entire host. Fungal growths were especially concentrated on the head, thorax, and legs (Fig. 2).

The *M. anisopliae* E<sub>9</sub> strain caused the highest total mortality (87.1%) and the shortest LT<sub>50</sub> (5 to 6 days) (Table 1). These values are similar to those obtained by Kaya and Dara (21) for *Homalodisca coagulata*, a sharpshooter insect vector of *X. fastidiosa* in Pierce's Disease (2), using *M. anisopliae* isolates at a concentration of  $5 \times 10^8$  conidia mL<sup>-1</sup>. In addition, Kanga *et al.* (19), working with *M. anisopliae* at  $10^8$  conidia mL<sup>-1</sup> and *H. coagulata*, verified a total mortality of 75% after 21 days and an LT<sub>50</sub> value of 14 days. These authors also suggested the use of *M. anisopliae* in IPM to control *H. coagulata*. The results of the present study are promising because 87.1% mortality was obtained with a concentration of  $10^7$  conidia mL<sup>-1</sup> and LT<sub>50</sub> of 5 to 6 days.

Chemical control with pyrethroid and neonicotinoid insecticides looks promising against immature and adult of sharpshooters (34,35), but it is associated with residual contamination and interferes with biological control strategies (33). Jaramillo *et al.* (18) tested the use of combined applications

of *M. anisopliae* with the neonicotinoid insecticide imidacloprid and did not observe colony growth inhibition for *M. anisopliae* in media containing this insecticide. These data reinforce that the use of insecticides to control *O. facialis* can be reduced when a fungus agent such as *M. anisopliae* is used as part of a biocontrol strategy.



**Figure 2.** *Metarhizium anisopliae*, sprayed at suspension of  $5 \times 10^7$  conidia mL<sup>-1</sup>, emerging from dead glassy sharpshooters collected from the treated samples after 6 days incubation.

**Table 1.** Total mortality and confirmed mortality percentages of *Oncometopia facialis* by *Metarhizium anisopliae* strain E<sub>9</sub> at suspension of 5 X 10<sup>7</sup> conidia mL<sup>-1</sup>

Observations	Time (days)	Mortality (% ± SE)	CV (%)	Calculated $\chi^2$
1	0	0	0	0
2	0.25	5.0 ± 0.02	1.7	8.3*
3	0.5	5.0 ± 0.02	1.7	8.3*
4	0.75	6.0 ± 0.02	1.7	17.5*
5	1.25	4.2 ± 0.03	3.1	2.8 <sup>ns</sup>
6	1.75	5.2 ± 0.03	3.4	3.5 <sup>ns</sup>
7	2.25	11.5 ± 0.04	4.1	9.8*
8	2.75	15.8 ± 0.05	5.4	12.7*
9	3.25	17.0 ± 0.06	6.2	9.9*
10	3.75	14.6 ± 0.07	7.6	4.7*
11	4.25	15.3 ± 0.08	8.7	3.9 <sup>ns</sup>
12	5.25	18.4 ± 0.11	12.9	3.0 <sup>ns</sup>
13	6.25	54.7 ± 0.13	17.5	22.8*
14	7.25	62.7 ± 0.13	19.6	25.8*
15	8.25	77.8 ± 0.12	20.6	40.2*
16	9.25	86.0 ± 0.10	18.6	59.7*
17	10.25	87.1 ± 0.12	25.0	25.0*
18	11.25	87.1 ± 0.12	25.0	25.0*
19	12.25	87.1 ± 0.12	25.0	25.0*

Coefficient of variation (CV); <sup>ns</sup> not significant (p>0.05); \* significant (p<0.05).

Little is known about predators, parasitoids, and pathogens that attack sharpshooter nymphs and adults (18). The results obtained in this study suggest that *M. anisopliae* can be successfully used for the control of *O. facialis*, indicating the importance of entomopathogenic fungi in an IPM strategy.

This is the first report that describes the use of *M. anisopliae* against *O. facialis* and demonstrates the potential to develop IPM strategies for this insect vector. However, additional studies are needed to provide a better understanding of host-pathogen interactions, and identify the factors that enhance or limit pathogenicity in sharpshooter populations under natural conditions.

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#### RESUMO

#### Avaliação do bioensaio de *Metarhizium anisopliae* (metchnikoff) sorokin (deuteromycota: hyphomycetes) contra *Oncometopia facialis* (signoret) (hemiptera: cicadellidae)

A Clorose Variegada dos Citros (CVC) é uma doença economicamente importante e destrutiva no Brasil e é causada pela bactéria *Xylella fastidiosa* e transmitida por insetos vetores tal como *Oncometopia facialis*. Nesse estudo, a eficácia do fungo *Metarhizium anisopliae* em controlar o inseto vetor *O. facialis* foi estudada em condições de bioensaio. Nesse bioensaio, insetos foram pulverizados com uma suspensão de 5 X 10<sup>7</sup> conídios mL<sup>-1</sup>. Insetos-adultos capturados no campo foram tratados em grupos de 10, em um total de 11 replicatas por tratamento. Diferenças significativas entre a mortalidade natural e a mortalidade dos insetos tratados com o fungo foram observadas em 6 dias após a inoculação (P<0.05). Estas diferenças significativas aumentaram antes do décimo dia após o tratamento. O fungo causou uma mortalidade de 87,1%, com uma LT<sub>50</sub> variando entre 5 e 6 dias. ALC<sub>50</sub> foi de 1,2 X 10<sup>6</sup> conídios mL<sup>-1</sup>, variando de 7,7 X 10<sup>5</sup> a 2 X 10<sup>6</sup> conídios mL<sup>-1</sup>. Estes resultados mostraram que o vetor *O. facialis* foi susceptível a ação entomopatogênica de *M. anisopliae* em condições controladas durante o bioensaio.

**Palavras-chave:** controle microbiano, entomopatógeno, Clorose Variegada dos Citros (CVC), bioensaio

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