

## SELECTION OF *Metarhizium anisopliae* FOR EXTRACELLULAR ENZYME PRODUCTION AND VIRULENCE TOWARD *Triatoma infestans*

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### ABSTRACT

Recurrent mutation and selection have been used to increase the production of the extracellular enzymes amylase, lipase and protease by the entomopathogenic fungus *Metarhizium anisopliae*. A selection program was initiated from strain E<sub>9</sub> and continued through two cycles of mutation and selection. 8-Methoxyorsoralen plus near ultraviolet light were used as mutagen and enzyme production was estimated semiquantitatively by the enzymatic index. Isolates with high enzymatic indices were tested for virulence toward 3rd-instar nymphs of *Triatoma infestans*. Isolates selected for amylase and lipase showed greater virulence than the original strain, while the virulence of isolates selected for protease was equal to, or lower than that of the original strain.

### INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* is being widely used in Brazil for the control of Cercopidae (Homoptera), especially pasture and sugar cane spittlebugs. The fungus also shows good potential for the control of blood-sucking insects such as triatomines (Moura-Costa, 1978; Sherlock and Guitton, 1982; Silva and Messias, 1985; Messias *et al.*, 1986; Romana and Fargues, 1987).

*M. anisopliae* var. *anisopliae* shows a change in virulence against *Rhodnius prolixus* due to a modification in the production of extracellular enzymes, which was demonstrated through studies on mutants deficient in the production and/or excretion of such enzymes and their revertants (Silva and Messias, 1986). If quantitative genes

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are also involved in the production of these extracellular enzymes, this trait could be genetically improved and recurrent mutation and selection would represent a promising tool for obtaining more efficient strains for the control of insect pests.

Furocoumarins, particularly 8-methoxypsoralen (8MOP), in combination with near UV light (NUV, 365 nm), under conditions of moderate lethality, act primarily as inducers of point mutations without gene specificity, rarely inducing chromosome aberrations. These types of mutagens are of preferred use for microorganisms of economic interest (Townsend *et al.*, 1971; Ball, 1973; Simpson and Caten, 1979a,b).

The objective of the present study was to use the 8MOP-NUV combination as a mutagen for the selection of *M. anisopliae* with increased production of the extracellular enzymes amylase, lipase, and protease, and to test the virulence of the most promising isolates against *Triatoma infestans*.

## MATERIAL AND METHODS

### *Strain and general procedures*

The strain of *M. anisopliae* employed in the present study is denoted E<sub>9</sub> and was isolated from adult *Deois flavopicta* (Homoptera, Cercopidae) collected in the State of Espírito Santo, Brazil. The complete medium (CM) of Pontecorvo *et al.* (1953) was used for routine plating and for storage at 4°C. The conidial suspensions were prepared from 7-day old plates: the conidia were collected, suspended in 0.1% Tween-80, shaken, counted using a hemocytometer and properly diluted in 0.85% saline. Incubation was at 28°C in all experiments.

### *Mutagenesis*

8MOP (Sigma Chem. Co.) dissolved in 40% ethanol (500 µg/ml) was added to a conidial suspension ( $1.0 \times 10^6$ /ml) for a final concentration of 25 µg/ml. After 30 minutes in the dark at room temperature, the suspension was irradiated with NUV (Mineralight UV Lamp, model UVSL 25) in an open Petri dish with gentle shaking in a darkened environment. The dose of NUV was 8.3 KJ/m<sup>2</sup> (Black-Ray UV Intensity Meter, model J-221) which allows 25% survival.

### *Evaluation for extracellular enzyme production*

The production of the extracellular enzymes amylase, lipase, and protease by colonies was evaluated semiquantitatively using solid media with specific substrates in Petri dishes by the method of Hankin and Anagnostakis (1975). Enzyme activity was

determined by incubating conidia for 5 days and then measuring colony and halo diameter. The enzymatic index was estimated by the halo diameter/colony diameter ratio.

### *Selection program*

Following mutagenesis, the conidia were plated onto CM to obtain isolated colonies. Random samples of 20 surviving colonies were picked, tested with two repetitions for extracellular enzyme production, and the isolates with the highest enzymatic index were selected and submitted to a second cycle of mutagenesis and selection.

### *Evaluation of conidial germination*

Proper aliquots of conidial suspension were plated onto CM and incubated for 18 hours. Conidial germination was estimated by observing 500 spores under the microscope 24 hours before utilizing each isolate in the bioassay.

### *Bioassays*

Strain E<sub>9</sub> and the isolates selected for enzyme production were assayed for virulence toward starved third-instar nymphs of *T. infestans*. Groups of five nymphs each were placed in sterilized polypropylene cylindrical flasks (5 x 10 cm) lined with filter paper and closed with a sterilized gauze stopper. Aliquots of 0.2 ml of a conidial suspension ( $8.0 \times 10^6$ /ml) were spread uniformly on the filter paper before introducing the nymphs. The flasks were placed in a cylindrical glass container (15 x 20 cm), relative humidity was maintained near 95% using a saturated solution of potassium sulfate, and the material was incubated at 28°C. Twenty-five insects were employed for each assay and mortality was determined daily. Control insects were treated in the same manner, except that 0.2 ml saline was spread on the filter paper. The results of the bioassay were expressed as lethal time (LT<sub>50</sub>), calculated by the probit method (Finney, 1971).

## RESULTS

The effects of 8MOP in combination with NUV on the enzymatic indices were determined to be significant by analysis of variance. The means were compared by the Tukey test (Table I). The results indicated progress in the enzymatic indices, and the population derived from the isolates submitted to the second treatment showed a higher mean enzymatic index value for amylase, lipase, and protease.

Table I - Comparisons of mean enzymatic indices of control and treated populations by the Tukey test.

Enzyme	Isolate*	Mean**
Amylase	Control (strain E <sub>9</sub> )	1.31 a
	After first treatment (amy 1)	1.50 b
	After second treatment (amy 2)	1.96 c
Lipase	Control (strain E <sub>9</sub> )	1.47 a
	After first treatment (lip 1)	1.63 b
	After second treatment (lip 2)	1.81 c
Protease	Control (strain E <sub>9</sub> )	1.27 a
	After first treatment (prt 1)	1.44 b
	After second treatment (prt 2)	1.59 c

\*Amy = amylase; lip = lipase; prt = protease.

\*\*Means followed by different letters for each enzyme are significant at the 5% level.

The isolate with the highest enzymatic index for each enzyme in each selection cycle was selected for the bioassays. The conidia of the various isolates and of the original strain had nearly 90% germination.

Virulence is reported as  $LT_{50}$ , which is the time needed to kill 50% of the *T. infestans* nymphs. No mortality was observed in the control (without conidia).  $LT_{50}$  results showed that the isolates with high enzymatic indices for amylase were the most virulent followed by those with the highest indices for lipase. Isolates with high indices for protease showed virulence similar to, or lower than that of the original strain (Table II). All dead nymphs were analyzed and the fungus was reisolated from all of them, indicating that mortality was probably due to *M. anisopliae*.

## DISCUSSION

The 8MOP-NUV combination was efficient in inducing mutations in *M. anisopliae* for increased production of exoenzymes. Recurrent mutation and selection cycles using gamma rays as a mutagen have been applied to *Aspergillus niger* for the production of organic acid (Silva and Azevedo, 1978). Using the 8MOP-NUV combination as a mutagen, this improvement program was applied to *Aspergillus nidulans* for penicillin production (Simpson and Caten, 1979b) and is currently being tested in our laboratory for biomass production.

Table II - LT<sub>50</sub> values and confidence intervals (C.I.) obtained for the different isolates of *M. anisopliae* against *T. infestans*.

Isolates	LT <sub>50</sub> (days)*	C.I.
Strain E <sub>9</sub>	3.63 a	3.28 - 4.01
Amy 1	3.18 b	2.87 - 3.75
Amy 2	2.92 b	2.66 - 3.22
Lip 1	3.23 b	2.90 - 3.85
Lip 2	3.03 b	2.70 - 3.41
Prt 1	5.83 c	5.50 - 6.18
Prt 2	4.00 a	3.63 - 4.45

\*Different letters indicate significantly different levels of virulence (Wilcoxon test).

The data in Tables I and II show that the treatment used caused effect(s) of a genetic nature. The populations derived after treatment showed greater variability than untreated populations in terms of enzymatic indices. The 8MOP-NUV combination was effective in increasing variability, which is indispensable for later selection (Sermonti, 1969).

The observed relationship between production of the extracellular enzymes amylase and lipase (but not protease) by *M. anisopliae* and fungal virulence toward *T. infestans*, supports previous conclusions (Silva and Messias, 1986). Other investigators (Al-Aidroos and Seifert, 1980; Soza-Gomes and Alves, 1983; Sundarababu *et al.*, 1984; Robert and Messing-Al-Aidroos, 1985; Leite, 1987) have reported conflicting results using different pathogens and/or hosts, but the importance of extracellular enzymes in the virulence of entomopathogenic fungi is fully recognized. Finally, we want to emphasize that the extracellular enzyme production trait can be improved in *M. anisopliae*, with the possibility of obtaining more efficient strains for the control of pests. This is a very important fact, especially if applicable also to other entomopathogenic fungi.

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## RESUMO

Mutação recorrente e seleção foram empregados para aumentar a produção das enzimas extracelulares amilase, lipase e protease no fungo entomopatogênico *Metarhizium anisopliae*. Um programa de seleção foi iniciado com a linhagem E<sub>9</sub> e continuou por dois ciclos de mutação e seleção. Como agente mutagênico foi empregada a 8-metoxipsoralaina associada à luz ultravioleta longa e a produção das enzimas foi estimada semiquantitativamente pelo índice enzimático. Isolados com altos valores de índices enzimáticos foram ensaiados para virulência a ninfas de terceiro estágio de *Triatoma infestans*. Os resultados do bioensaio indicaram que os isolados selecionados para amilase e lipase apresentam uma maior virulência que a linhagem original, enquanto que os isolados selecionados para protease apresentam virulência igual ou inferior que a da linhagem original.

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