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DNA POLYMORPHISM AND TOTAL PROTEIN IN MUTANTS OF *METARHIZIUM ANISOPLIAE* VAR. *ANISOPLIAE* (METSCH.) SOROKIN STRAIN E_9

Laurineide Lopes de Carvalho Freire; Ana Bolena Lima da Costa; Larissa Brandão Góes; Neiva Tinti de Oliveira*

Departamento de Micologia, Centro de Ciências Biológicas,Universidade Federal de Pernambuco, Recife, PE, Brasil

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ABSTRACT

Five mutants (MaE_{10}, MaE_{27}, MaE_{24}, MaE_{41} e MaE_{49}) of *Metarhizium anisopliae* wild strain E_9 were analysed for DNA profile through the RAPD technique and for changes in total protein content by spectrophotometry, polyacrylamide gel electrophoresis and densitometry. The pattern of RAPD markers showed genetic polymorphism among the strains: out of twenty primers seven were selected, producing 113 bands. Forty seven bands were present in all strains (41.6% of monomorphic bands) and 66 showed polymorphism (58.4%). The mean coefficient of similarity among all strains was 0.75 (75%). The total protein content varied, staining in the interval of 6.0-8.0 µg/µl. The electrophoresis analysis, through zymogram and protein fraction profiles by densitometry, allowed the observation of seven bands for the wild strain E_9 and five bands for the mutants MaE_{10}, MaE_{27}, MaE_{34}, MaE_{41} and MaE_{49}, evidence of variations in µg% among protein fractions. The RAPD technique was very sensitive to detect genetic differences between the wild type and the mutants obtained through gamma radiation. The total protein analysis also showed changes in quantity and pattern of bands after electrophoresis in the mutants compared to the wild type.

Key words: *Metarhizium anisopliae*, mutants, RAPD, total protein

INTRODUCTION

Among the entomopathogenic fungi, *Metarhizium anisopliae* presents a wide spectrum of pathogenicity and infectivity reaching different species of pest insects. In Brazil this fungus is used with success for the control of *Mahanarva posticata* Stal on sugar-cane and against *Deois flavopicta* Stal. and *Zulia entreriana* Berg on pastures (2,12). In entomopathogenic fungi genetic manipulation allows the alteration of factors such sporulation, dispersion and tolerance to stress in the spores, with the purpose of increasing their efficiency in biological control (29). Knowledge of recombination mechanisms and variability in *M. anisopliae* made it possible to accomplish improved techniques for obtaining strains with expressive characteristics of pathogenicity and infectivity important for biological control (5,17,19,20). Using gamma radiation Oliveira *et al.* (19) obtained mutants of the strain E_9 of *M. anisopliae* var. *anisopliae* with altered germination and suggested the possibility of its ultilization in genetic crossings for incorporation of desirable characteristics.

The RAPD (Random Amplified Polymorphic DNA) technique described by Williams *et al.* (28) and Welsh and Mc Clelland (27) has been used in studies of genetic variability, taxonomy and ecology of several fungi (1,3,6,9,21). The RAPD markers are important tools for evaluation of the variability among different isolates of a species, the degree of genetic relationship among isolates, and may also be useful to help distinguish isolates of nonpathogenic and pathogenic fungi (8,13,16,31,32). Fegan *et al*. (10) observed a great genetic variability in isolates of *M. anisopliae* var. *anisopliae* using this technique. Using RAPD in 13 isolates of *M. anisopliae* var. *anisopliae* Fungaro *et al*. (11) verified a great genetic diversity among them. There was less genetic variability among the isolates from insects, suggesting a certain degree of host specificity. Schlick *et al.* (26) analyzed strains of *Trichoderma harzianum* (Rifai) and mutants of a wild isolate induced by gamma radiation through RAPD, and verified that it was possible to differentiate all mutants by at least one primer. The authors emphasised the great importance of the possibility of discriminating these mutants of the original strain for a single

* Corresponding author. Mailing address: Universidade Federal de Pernambuco, Centro de Ciências Biológicas-CCB, Departamento de Micologia, Av. Prof. Nelson Chaves S/N - Cidade Universitária, 50670-901, Recife, PE, Brasil. Telefax: (+5581) 271-8482 / 271-8483. E-mail: nto@npd.ufpe.br

technique of genomic fingerprint for use in protection of patents of fungal strains.

The electrophoresis profile of proteins in fungi and techniques for direct observation of DNA have been of great usefulness in taxonomical, phylogenetical and ecological studies (14,18,22,30).

We report the differentiation of five mutants originated from the wild strain E_9 of *M. anisopliae* var. *anisopliae* obtained by gamma radiation, using the RAPD technique and protein electrophoresis.

MATERIALS AND METHODS

Strains

E_9 strain of *M. anisopliae* var. *anisopliae* and five mutants (MaE_{34}, MaE_{49}, MaE_{41}, MaE_{10} and MaE_{27}) obtained by gamma radiation (19) were used.

Growth Conditions

Erlenmeyer flasks with 100 ml of Complete Media (4,23) were inoculated, separately, with 10^8 conidia/ml of the wild strain E9 and mutants MaE_{27}, MaE_{34}, MaE_{41}, MaE_{49} and MaE_{10}, and incubated under agitation at 28ºC for 96 hs. The mycelium was recovered by vacuum filtration, washed with sterilized water, and stored at –12ºC.

Extraction and amplification of DNA

The total genomic DNA of the samples was extracted from mycelium as described by Raeder and Broda (24). Twenty primers of the kit OPW were tested and selected for amplification with the DNA of the wild strain E_9. Kit OPW, Taq DNA polymerase and the dNTPs for RAPD were supplied by Operon Technologies, CA, USA. Amplification reactions were carried out in a total volume of 25 µl containing approximately 20-25 ng of template DNA, primer 0.4 µmol l^{-1} and $MgCl_2$ 3.0 mmol l^{-1}. The reaction control consisted of all components, except the genomic DNA. The amplification process was accomplished in a thermal cycler (MJ Research), programmed to accomplish an initial denaturation of 5 minutes at 92ºC, 40 cycles of 1 minute at 92ºC (denaturation), 1 minute and 30 seconds at 39ºC (annealing), 2 minutes at 72ºC (extension) and finally 5 minutes in 72ºC for a final extension, during 40 cycles. Samples of 22 µl of the amplification products were assayed by electrophoresis in 1.4% agarose gels at 3 V/cm of distance among the electrodes, running with Tris-Borate-EDTA (TBE) buffer. Gels were stained with ethidium bromide, and photographed under U.V. light.

The NTSYS.PC (Numerical Taxonomy System Applied Biostatistics, Setauket, New York) computer program was used for data analysis. The data (band presence or absence) were introduced in the form of a binary matrix and a pairwise similarity matrix was constructed using the JACCARD coefficient (25). The values were obtained by the double number of shared bands between two patterns divided by the sum of all the bands in the same pattern (value 1 indicates identical patterns for two individuals and value 0 indicates completely different patterns). The UPGMA (Unweighted pair-group method with arithmetical averages) grouping of the values was generated using the NTSYS program.

Analysis of the total protein extracts

Humid masses of mycelium were triturated in a mortar with protein extraction buffer (15), centrifuged at 10,000 rpm for 5 minutes and the supernatants stored at –12ºC. The amount of protein present in the extracts was measured spectrophotometrically at 595 nm according to Bradford (7). All reactions were accomplished in triplicate. Aliquots of the extracts were submitted to polyacrylamide gel electrophoresis using a vertical run and a discontinuous system of buffers (22). Each sample was prepared by mixing 100 µl of the sample with 10 µl of bromophenol blue solution (0.025 g bromophenol blue + 10 ml Tris-HCl buffer, pH 6.7). The run buffer was Tris-glycine, pH 8.3. The running was started at 15 mA, corrected to 20 mA after the bromophenol blue front passed through the stacking gel. A bovine serum albumin solution was used as standard (0.001g of bovine serum albumin in 5ml NaCl 0.15M solution). After running, the gel was fixed in 20% glacial acetic acid + methanol (1:1, v/v) for 30 minutes and stained with a fresh solution of Coomassie Brilliant Blue R 250 + 10% glacial acetic acid. An aqueous mixture of 40% glacial acetic acid and 2.5% glycerol was added at a proportion of 1:20. Minimum staining time was of 8 hours. The gel was destained in a 20% methanol + 5% acetic acid + 2.5% glycerol solution. After drying the gel was photographed and submitted to the densitometer (DS-35 of CELM).

RESULTS AND DISCUSSION

Analysis of the RAPD profiles

The RAPD profiles obtained with the selected primers (OPW01, OPW02, OPW03, OPW04, OPW09, OPW12, OPW13) are shown in Figs. 1 and 2. The seven primers provided a total of 113 bands, with an average of 16 bands per primer. 41.6% were monomorphic, because they were present in all samples, while 58.4% were polymorphic, because at least one sample did not present the band. A similarity matrix and a dendrogram (Fig. 2) using the UPGMA method were constructed. All the mutants of the wild strain E_9 presented a ramification point around 0.75, equivalent to 75% similarity, which corresponds to a moderate variation degree. The MaE_{34} and MaE_{49} mutants presented the highest similarity index (about 87.5%). These mutants presented the same physiological characteristics such as mycelial growth in complete media and minimum media, percentage of germination, beginning of conidia germination, and colony morphology, as reported by Oliveira *et al.* (19). The MaE_{10} mutant presented a ramification point around 0.81 (81.2% of similarity); the wild strain 0.78 (78% of similarity) and the MaE_{27} and MaE_{41} mutants 0.71 and 0.62, corresponding to 71% and 62% of similarity, respectively.

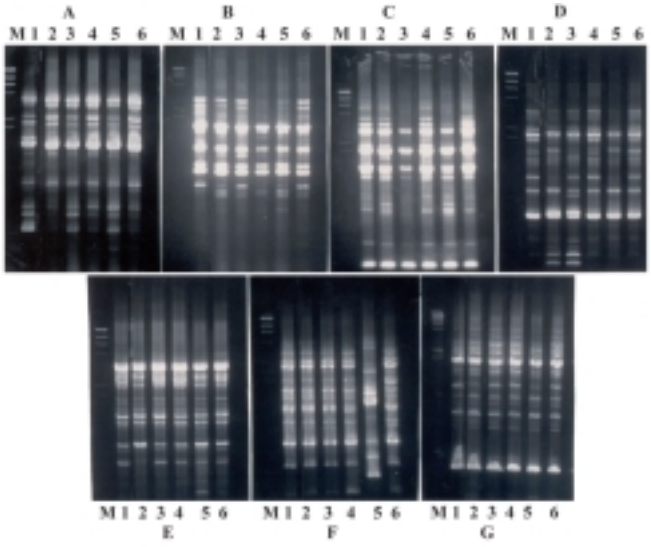

Figure 1. *Metarhizium anisopliae var. anisopliae* wild strain E_9 and five mutants MaE RAPD profiles, obtained with the primers: **A-** OPW01 **B-** OPW02 **C-**OPW03 **D-** OPW04 **E-** OPW09 **F-**OPW12 **G-** OPW13. M- Molecular weight size markers (Hind III cut λ DNA) in base pairs. **M-** Molecular weight size markers (Hind III cut λ DNA) in base pairs. Positions **1-6** corresponding to wild strain E_9, MaE_{10}, MaE_{27}, MaE_{34}, MaE_{41}, MaE_{49} respectively.

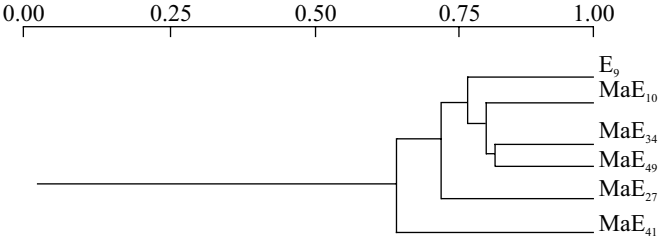

Figure 2. Dendrogram obtained by UPGMA agroupment, with Jaccard coefficient starting from RAPD profiles of *Metarhizium anisopliae* var. *anisopliae* wild strain E_9 and five mutants MaE.

The RAPD molecular markers allowed verification of DNA polymorphism among the wild strain E_9 and the mutants obtained by gamma radiation. This confirms the capability of the RAPD technique to distinguish mutants obtained by gamma radiation, as observed by Schlick *et al.* (26). These authors used the RAPD technique to detect mutants of *Trichoderma harzianum* obtained by gamma radiation, observing an evident DNA polymorphism among the wild strain and the mutants this was interpreted as an indication of the occurrence of deletions and inserts, detected by the molecular markers of RAPD.

Total protein analysis

The concentration of total protein in the mycelium extracts of the wild strain E_9 of *M. anisopliae* var. *anisopliae* and its mutants varied from 6.0 to 8.0 µg/µl (Table 1). The zymogram obtained after electrophoresis of the total proteins is shown in Fig. 3. The wild strain E_9 presented seven bands (considered as 100% for a comparative analysis), and the mutants five bands, resulting therefore in 71.4% of monomorphic bands and 28.6% of polimorphic bands. Quantitative analysis of the fractions of total proteins obtained by densitometry are shown Fig. 4. As shown in Table 1, the wild strain E_9 presented seven fractions of proteins (A, B, C, D, E, F and G) in the electrophoretic profile. Variation in the amount of proteins in fractions was observed. All samples presented the fractions A, C, E, F, G. Fractions B and D were not observed in mutants. The amount of protein in fraction A varied from 1.44 to 2.5 µg%, and the MaE_{10} mutant presented the highest percentage. For fraction C the amount varied from 1.7 to 2.63 µg%. The percentage of protein in fraction E varied

Table 1. Percentage (µg%) of protein total fractions of wild strain E_9 and five mutants MaE of *Metarhizium anisopliae* var. *anisopliae*.

Mutants	Fractions µg%							Total protein µg/µl
	A	B	C	D	E	F	G	
Wild strain E9	1.44	0.42	1.80	0.30	0.42	1.02	0.60	6.0
MaE_{10}	2.50	-	1.85	-	0.90	0.64	0.51	6.4
MaE_{27}	1.94	-	1.73	-	0.72	2.16	0.65	7.2
MaE_{34}	2.22	-	1.70	-	0.37	2.37	0.74	7.4
MaE_{41}	2.20	-	2.63	-	0.71	1.21	0.35	7.1
MaE_{49}	2.16	-	2.40	-	1.12	1.92	0.40	8.0

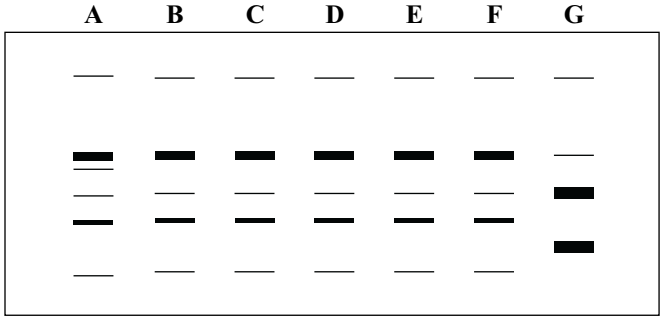

Figure 3. Zymogram obtained after protein total electrophoresis of *Metarhizium anisopliae* var. *anisopliae* wild strain E_9 and five mutants MaE. **A-** wild strain E_9; **B-** MaE_{10}; **C-** MaE_{27}; **D-** MaE_{34}; **E-** MaE_{41}; **F-** MaE_{49}; **G-** bovine serum abumin standard.

from 0.37 to 1.12 µg%; MaE_{49} presented the highest percentage. The MaE_{34} mutant presented the highest percentage of protein for the fraction F and MaE_{10} the lowest. For the fraction G the MaE_{41} mutant presented the lowest while the MaE_{34} showed the highest percentage of protein.

The RAPD technique was sensitive enough to detect genetic polymorphism among mutants of the wild strain E_9 of *M. anisopliae* var. *anisopliae* obtained by gamma radiation. The average similarity index revealed a moderate degree of variation. The zymogram and protein electrophoretical profiles also indicated genetic variation, revealing the presence of seven proteins bands in the wild strain and five in the mutants.

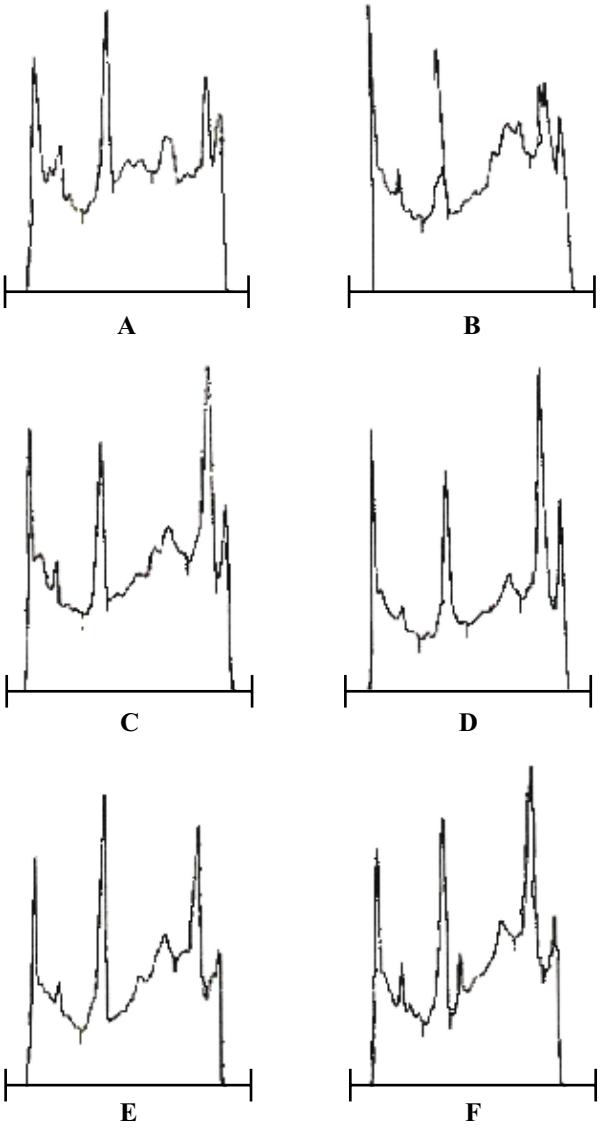

Figure 4. Total protein densitometric analysis of wild strain E_9 and five mutants of *Metarhizium anisopliae* var. *anisopliae*. **A**-wild strain E_9; **B**-MaE_{10}; **C**-MaE_{27}; **D**-MaE_{34}; **E**-MaE_{41}; **F**-MaE_{49}.

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RESUMO

Polimorfismo de DNA e proteína total em mutantes da linhagem E_9 de *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin

Foram analisados cinco mutantes MaE (MaE_{10}, MaE_{27}, MaE_{34}, MaE_{41} e MaE_{49}) da linhagem selvagem E_9 de *Metarhizium anisopliae* var. *anisopliae* quanto ao perfil de DNA pela técnica de RAPD e também quanto ao conteúdo de proteína total por espectrometria e eletroforese em gel de poliacrilamida e densitometria. O padrão de marcadores de RAPD evidenciou polimorfismo nas amostras; dos 20 primers testados foram selecionados 7 que geraram 113 bandas. Deste total, 47 estavam presentes em todas as amostras (41.6% de bandas monomórficas) e 66 mostraram polimorfismo (58.4%). O coeficiente médio de similaridade foi de 75%. O conteúdo de proteína total variou de 6 a 8 µg/µl. O zimograma e perfís das frações de proteínas obtidos por densitometria revelaram 7 bandas para a linhagem selvagem E9 e 5 bandas para os mutantes com variações nos percentuais em µg% entre as frações de proteínas. A técnica de RAPD mostrou-se bastante sensível para detectar diferenças entre a linhagem selvagem e os mutantes obtidos por radiação gama. A análise de proteína total também evidenciou mudanças ocorridas na quantidade e no padrão de bandas nos mutantes em relação à linhagem selvagem.

Palavras-chave: *Metarhizium anisopliae*, mutantes, RAPD, proteína total

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