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# Amylase and protease secretion in recombinant strains of *Metarhizium anisopliae* var. *anisopliae* following parasexual crosses

*M.C. Valadares-Inglis*[1] *and J.L. Azevedo*[2]

## ABSTRACT

Parasexual crosses were made between auxotrophic mutants of *Metarhizium anisopliae* var. *anisopliae*. Analysis of recombinants for secretion of amylases and proteases demonstrated that stable prototrophic recombinants have similar levels of enzyme secretion. Unstable prototrophic recombinants varied in amylase production, and segregants originating from these varied in protease secretion. Auxotrophic recombinants showed intense variation in secretion of amylases and proteases although there was no correlation between auxotrophic markers and enzyme production. There were significant differences in enzyme secretion between recombinants and parentals.

## INTRODUCTION

Mutagenesis of entomopathogenic fungi is a tool employed in investigations aimed at production of "marker" genes, to enhance or remove specific activities, or increase virulence by selection of target hosts (Heale *et al*., 1989). Mutants with altered morphology, nutritional requirements, enzyme production, fungicide resistance and virulence have been isolated from *Metarhizium anisopliae* using UV light and chemical agents (Messias and Azevedo, 1980; Al-Aidross and Seifert, 1980; Silveira and Azevedo, 1984; Robert and Messing-Al-Aidross, 1985; Huxlam *et al*., 1989; Bagagli *et al*., 1991). Auxotrophic and conidial color mutants from *Metarhizium* are used mainly for genetic analysis (Messias and Azevedo, 1980; Al-Aidross, 1980; Silveira and Azevedo, 1984). Other mutants have been used to correlate enzyme production with virulence (Al-Aidross and Seifert, 1980; Robert and Messing-Al-Aidross, 1985) in order to study regulation of enzyme production (St. Leger *et al*., 1991) and to determine the involvement of enzymes in the hemocoelic immune response of insects (Huxlam *et al*., 1989).

Mutants showing alteration in metabolism help to elucidate factors related to virulence and species-specificity between host and pathogen. However, pleiotropic effects caused by induced mutation are recognized as deleterious, and crossing of mutants may be necessary to restore or combine advantageous characteristics.

Deuteromycetes have no conventional sexual cycle, however new genotypes can be produced by parasexual crosses. This involves fusion of hyphae or protoplasts, to generate heterokaryons and ultimately, heterozygous diploids. Mitotic recombination in the diploid nuclei and random assortment of chromosomes during haploidization, lead to the production of novel genotypes. The parasexual cycle has been described in *M. anisopliae* by Messias (1979) and Messias and Azevedo (1980), and confirmed later by Al-Aidross

[1] CENARGEN/EMBRAPA, Caixa Postal 02372, 70770-900 Brasília, DF, Brasil. Send correspondence to M.C.V-I.
[2] Escola Superior de Agricultura "Luiz de Queiroz", Departamento de Genética, Caixa Postal 83, 13400-970 Piracicaba, SP, Brasil.

(1980). Diploids obtained from parasexual crosses are generally unstable and following recombination new genotypes are produced. Also some prototrophic stable recombinants as well as auxotrophic recombinants are obtained directly from heterokaryons (Bagagli *et al.*, 1991) which may not always be restored to full virulence (Riba *et al.*, 1985). Some stable diploids of entomopathogenic fungi, however, seem to be more effective in causing mortality in insects than the respective parental mutants (Garcia *et al.*, 1985; Riba *et al.*, 1985).

# MATERIAL AND METHODS

## Strains.

Strains $E_{6-7}$ (*vio met⁻ bio⁻*) and $E_{6-8}$ (*ylo pyr⁻lys⁻*) were described by Silveira and Azevedo (1984, 1987), and strain $E_{9-19}$ (*ylo leu⁻ rib⁻*) by Bagagli (1986). The designations *vio* and *ylo* refer to pale vinaceous and yellow as opposed to the wild type green color of the conidia, and the notations *bio, leu, lys, met, pyr* and *rib* are used to designate nutritional requirements for biotin, leucine, lysine, methionine, pyridoxine and riboflavin, respectively.

## Media

Minimal medium (MM): 6 g/l $NaNO_3$; 1.5 g/l $KH_2PO_4$; 0.5 g/l KCl; 0.5 g/l $MgSO_4.7H_2O$; 0.001 g/l $FeSO_4$; 0.001 g/l $ZnSO_4$; 10 g/l glucose; 15 g/l agar; pH 6.8. Complete medium (CM): minimal medium supplemented with 0.5 g/l yeast extract; 2 g/l peptone; 1.5 g/l hydrolyzed casein; 1 ml/l vitamin solution (100 mg/l nicotinic acid; 10 mg/l p-aminobenzoic acid; 50 mg/l tiamine; 50 mg/l pyridoxine; 0.02 mg/l biotin; 100 mg/l riboflavin, distilled water to 100 ml/l), pH 6.8. Medium for amylolytic activity: minimal medium without glucose containing 0.2% (w/v) soluble starch (Reagen), pH 6.0. Medium for proteolytic activity: minimal medium without $NaNO_3$, plus 1% (w/v) solution of skimmed milk, pH 6.0.

Minimal medium supplements were prepared in sterile distilled water at concentrations: 10 mg/ml leucine, 10 mg/ml lysine, 10 mg/ml methionine, 1 mg/ml riboflavine, 0.02 mg/ml biotin, 0.5 mg/ml pyridoxine, 7.5 mg/ml adenine. Supplements were added at 0.1 ml per 20 ml minimal medium.

## Exoenzyme activity

Spores of the experimental strains were collected in a solution of Tween 80 (0.1% v/v) and plated at appropriate dilutions on CM. After seven-days incubation at 28°C the colonies were transferred to the activity media described above, supplemented as appropriate for the auxotrophic mutant or recombinant under investigation. Amylolytic and proteolytic activities were estimated after five days incubation at 28°C. Iodine (1% v/v in ethanol) was added to detect amylolytic activity. Proteolytic activity was detected by fixing and staining plates with a 0.01% (w/v) solution of Comassie brilliant blue R250 in 50% (v/v) methanol/ 5% (v/v) acetic acid. Enzymatic index was calculated based on diameter of the halos + colonies, divided by colony diameter.

## Genetic techniques

Heterokaryons were obtained as described by Bagagli *et al.* (1991) between strains $E_{6-7}$ + $E_{6-8}$ and $E_{6-7}$ + $E_{9-19}$. Spores collected from the heterokaryon were plated on complete, minimal and minimal medium supplemented with nutritional supplements for the auxotrophic recombinants recovered, according to Bagagli *et al.* (1991). Colonies were inoculated on CM (26 colonies per plate in a 5 x 5 x 1 disposition) and transferred to MM + nutritional requirements in order to classify them according to color and nutritional requirements. Stability of prototrophic recombinants was tested, using benomyl (Upshall *et al.*, 1976).

# RESULTS AND DISCUSSION

Higher frequencies of recombinants were obtained by crossing mutants $E_{6-7}$ + $E_{6-8}$ (Table I). No colonies were obtained on minimal medium by plating spores from the heterokaryon $E_{6-7}$ + $E_{9-19}$; however, recombinants were isolated on media supplemented with the combined nutritional requirements of the parentals. The high frequencies of recombinants from one of the heterokaryons of $E_{6-7}$ + $E_{6-8}$ in relation to the other $E_{6-7}$ + $E_{9-19}$ can be explained by the origin of these strains. Both $E_{6-7}$ and $E_{6-8}$ were derived from the same strains ($E_6$). The other heterokatyon had two origins ($E_6$ and $E_9$) which could result in incompatibility. Genetic analysis of data from selective media growth tests showed a high level of recombination, as previously described by Bagagli *et al.* (1991).

Prototrophic colonies obtained from one of the crosses ($E_{6-7}$ + $E_{6-8}$) on minimal medium were analyzed for stability using benomyl. Two types of prototrophs could be distinguished: unstable ones, which were derived from probable diploids, and stable prototrophic colonies. Unstable prototrophic recombinants were

assayed for amylase and protease. Amylase secretion varied significantly; however, this difference is likely to be due to the low level of activity of the recombinant R12 alone (Table II). Similar results were obtained for protease (data not shown). Segregants isolated from the unstable recombinants had similar levels of amylase secretion, whereas protease secretion was found to vary greatly (Table III). No significant differences in amylase and protease secretion were observed among the stable prototrophic recombinants, isolated on minimal media.

Auxotrophic recombinants, originating from the heterokaryon $E_{6-7}$ + $E_{6-8}$, showed significant variation in amylase and protease secretion. The amylase index varied from 1.000 to 3.447, and from 1.000 to 2.150 for proteases. Nutritional deficiency seems to have a pleiotropic effect on the secretion of these enzymes though there is no correlation of their activity with specific markers. Similar data was obtained for recombinants originating from heterokaryon $E_{6-7}$ + $E_{9-19}$.

**Table I** - Frequencies of colonies obtained from conidia of heterokaryons plated on selective media.

| Crosses | Selective media* | Frequency ($x10^{-5}$) |
|---|---|---|
| $E_{6-7}$ + $E_{6-8}$ | MM | 28.82 |
| | MM + *met* + *pyr* | 12.35 |
| | MM + *met* + *lys* | 15.76 |
| | MM + *bio* + *pyr* | 12.94 |
| | MM + *bio* + *lys* | 11.29 |
| $E_{6-7}$ + $E_{9-19}$ | MM | 0.00 |
| | MM + *leu* + *met* | 8.04 |
| | MM + *leu* + *bio* | 64.30 |
| | MM + *rib* + *met* | 0.00 |
| | MM + *rib* + *bio* | 8.04 |

*MM: Minimal medium; MM + *met* + *pyr*: minimal medium supplemented with methionine and pyridoxine; MM + *met* + *lys*: minimal medium supplemented with methionine and lysine; MM + *bio* + *pyr*: minimal medium supplemented with biotin and pyridoxine; MM + *bio* + *lys*: minimal medium supplemented with biotin and lysine; MM + *leu* + *met*: minimal medium supplemented with leucine and methionine; MM + *leu* + *bio*: minimal medium supplemented with leucine and biotin; MM + *rib* + *met*: minimal medium supplemented with riboflavine and methionine; MM + *rib* + *bio*: minimal medium supplemented with riboflavine and biotin.

**Table II** - Amylase secretion by unstable prototrophic recombinants of *Metarhizium anisopliae* obtained by crossing $E_{6-7}$ + $E_{6-8}$. Values are the mean of triplicate tests. Those followed by different letters represent significant differences in the Tukey test at the 5% level (dms = 0.434).

| Recombinant | Amylase index (Mean ± SD) |
|---|---|
| R1 | 1.967 ± 0.115 a |
| R2 | 1.967 ± 0.057 a |
| R3 | 1.940 ± 0.144 a |
| R4 | 1.923 ± 0.225 a |
| R5 | 1.907 ± 0.090 a |
| R6 | 1.900 ± 0.000 a |
| R7 | 1.867 ± 0.058 ab |
| R8 | 1.867 ± 0.115 ab |
| R9 | 1.833 ± 0.289 ab |
| R10 | 1.833 ± 0.058 ab |
| $E_{6-7}$ | 1.795 ± 0.183 ab |
| R11 | 1.770 ± 0.199 ab |
| $E_{6-8}$ | 1.669 ± 0.157 ab |
| R12 | 1.460 ± 0.147 b |

**Table III** - Protease secreted on solid media by segregants obtained from the unstable prototrophic recombinants, originating from crosses of $E_{6-7}$ + $E_{6-8}$. Values are the means of triplicate tests. Those followed by different letters represent statistical differences in the Tukey test at the 5% level (dms = 0.4181).

| Segregant number | Protease index (Mean ± SD) |
|---|---|
| S-28 | 1.923 ± 0.132 a |
| S-27 | 1.810 ± 0.035 ab |
| S-26 | 1.770 ± 0.080 abc |
| $E_{6-7}$ | 1.712 ± 0.069 abcd |
| S-12 | 1.710 ± 0.035 abcd |
| S-24 | 1.660 ± 0.154 abcde |
| S-11 | 1.620 ± 0.159 abcde |
| S-10 | 1.610 ± 0.056 abcde |
| S-35 | 1.610 ± 0.270 abcde |
| S-4 | 1.590 ± 0.182 abcde |
| S-18 | 1.567 ± 0.058 abcde |
| S-13 | 1.563 ± 0.035 abcde |
| S-15 | 1.547 ± 0.108 abcde |
| S-39 | 1.543 ± 0.131 abcde |
| S-7 | 1.533 ± 0.095 abcde |
| $E_{6-8}$ | 1.530 ± 0.058 abcde |
| S-37 | 1.527 ± 0.123 abcde |
| S-6 | 1.510 ± 0.046 bcde |
| S-17 | 1.487 ± 0.165 bcde |
| S-21 | 1.473 ± 0.085 bcde |
| S-8 | 1.467 ± 0.152 bcde |
| S-25 | 1.453 ± 0.222 bcde |
| S-5 | 1.450 ± 0.202 bcde |
| S-3 | 1.447 ± 0.221 bcde |
| S-9 | 1.440 ± 0.052 bcde |
| S-40 | 1.423 ± 0.145 bcde |
| S-14 | 1.407 ± 0.114 bcde |
| S-16 | 1.400 ± 0.125 bcde |
| S-2 | 1.387 ± 0.129 cde |
| S-33 | 1.373 ± 0.106 cde |
| S-19 | 1.357 ± 0.031 cde |
| S-36 | 1.353 ± 0.035 cde |
| S-1 | 1.343 ± 0.130 de |
| S-23 | 1.340 ± 0.114 de |
| S-30 | 1.333 ± 0.116 de |
| S-38 | 1.327 ± 0.065 de |
| S-31 | 1.320 ± 0.155 de |
| S-29 | 1.303 ± 0.040 de |
| S-32 | 1.273 ± 0.110 e |
| S-34 | 1.257 ± 0.049 e |

**Table IV** - Amylase and protease enzymes secreted by parentals, prototrophic unstable and stable recombinants, segregants and auxotrophic recombinants.

| Strains | Enzymatic index | |
|---|---|---|
| | Amylases | Proteases |
| Parentals $E_{6-7}$ and $E_{6-8}$ | 1.732 | 1.621 |
| Prot. Rec.[1] | 1.853 | 1.461 |
| Prot. Rec.[2] | 1.952 | 1.498 |
| Segregants[3] | 1.692 | 1.487 |
| Recombinants[4] | 2.047 | 1.741 |
| Parentals $E_{6-7}$ and $E_{9-19}$ | 1.879 | 1.556 |
| Recombinants[5] | 1.718 | 1.346 |

[1]Unstable prototrophic recombinants (Prot. Rec.) from heterokaryon $E_{6-7}$ + $E_{6-8}$; [2]stable prototrophic recombinants from heterokaryon $E_{6-7}$ + $E_{6-8}$; [3]segregants from heterokaryon $E_{6-7}$ + $E_{6-8}$; [4]auxotrophic recombinants originated from heterokaryon $E_{6-7}$ + $E_{6-8}$; [5]auxotrophic recombinants originated from heterokaryon $E_{6-7}$ + $E_{9-19}$. Results are the mean of triplicate tests.

The means of protease and amylase activities were calculated for the different prototrophic recombinants, segregants and parentals. No significant differences were found in the amylase index among the unstable prototrophic recombinants and the parentals $E_{6-7}$ and $E_{6-8}$. However, stable prototrophic and auxotrophic recombinants originating from the same heterokaryon were significantly different from their parentals, with higher values for amylases (Table IV). Auxotrophic recombinants originating from the heterokaryon $E_{6-7}$ + $E_{6-8}$, showed no differences in protease secretion when compared to the parentals, $E_{6-7}$ and $E_{6-8}$. The protease index of the stable and unstable prototrophic recombinants and segregants was found to be lower than the respective parental strains.

Evaluation of enzyme activity of recombinants appears to be a promising method for screening new genotypes with potential for biocontrol, as these exoenzymes have a role in cuticle digestion during penetration of the fungus into the insect. Pleiotropic effects of auxotrophic mutations and recombination of mutants by heterokaryotic crosses produce a wide variation in amylase and protease activity. Recombination mechanisms appear to be efficient in producing new strains with higher activities of enzymes involved in pathogenesis.

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## RESUMO

Cruzamentos via ciclo parassexual entre mutantes auxotróficos de linhagens de *Metarhizium anisopliae* var. *anisopliae* foram conduzidos. Recombinantes foram analisados para secreção de amilases e proteases, demonstrando que recombinantes prototróficos estáveis não apresentam diferenças significativas. Recombinantes prototróficos instáveis variam em termos de amilases e segregantes originados destes apresentam variações para proteases. Recombinantes auxotróficos mostraram intensa variação para amilases e proteases, sendo que nenhuma correlação entre auxotrofia e produção destas enzimas foi detectada. Na comparação entre secreção de amilases e proteases em linhagens parentais, recombinantes prototróficos e segregantes foram encontradas diferenças significantes.

## REFERENCES

**Al-Aidross, K.** (1980). Demonstration of a parasexual cycle in the entomopathogenic fungus *Metarhizium anisopliae*. *Can. J. Genet. Cytol. 22*: 309-314.

**Al-Aidross, K.** and **Seifert, A.M.** (1980). Polysaccharide and protein degradation, germination, and virulence against mosquitoes in the entomopathogenic fungus *Metarhizium anisopliae*. *J. Inv. Pathol. 36*: 29-34.

**Bagagli, E.** (1986). Parameiose em *Metarhizium anisopliae* (Metsch.) Sorokin. Master's thesis, ESALQ/USP, Piracicaba, SP.

**Bagagli, E., Valadares, M.C.C.** and **Azevedo, J.L.** (1991). Parameiosis in the entomopathogenic fungus *Metarhizium anisopliae* (Mestch.) Sorokin. *Rev. Bras. Genet. 14*: 261-271.

**Garcia, A.E., Baracho, I.R., Souza, H.M.** and **Messias, C.L.** (1985). Virulência de linhagens mutantes e diplóides de *M. anisopliae* em *Ceratitis capitata* (Wield.) (Diptera, Tephritidae). *Rev. Bras. Genet. 29*: 267-270.

**Heale, J.B., Isaac, J.E.** and **Chandler, D.** (1989). Prospects for strain improvement in entomopathogenic fungi. *Pest. Sci. 26*: 79-92.

**Huxlam, I.M., Samuels, K.D.Z., Helae, J.B.** and **McCorkindale, N.J.** (1989). *In vivo* and *in vitro* assays for pathogenicity of wild types and mutant strains of *Metarhizium anisopliae* for three insect species. *J. Inv. Pathol. 53*: 143-151.

**Messias, C.L.** (1979). Parassexualidade em *Metarhizium anisopliae* (Metsch.) Sorokin. Doctoral thesis, ESALQ/USP, Piracicaba, SP.

**Messias, C.L.** and **Azevedo, J.L.** (1980). Parasexuality in the Deuteromycete *Metarhizium anisopliae*. *Trans. Brit. Mycol. Soc. 75*: 473-477.

**Riba, G., Azevedo, J.L., Messias, C.L., Silveira, W.D.** and **Tuveson, R.** (1985). Studies of the inheritance of virulence in the entomopathogenic fungus *Metarhizium anisopliae*. *J. Inv. Pathol. 46*: 20-25.

**Robert, A.** and **Messing-Al-Aidross, K.** (1985). Acid production by *Metarhizium anisopliae*, effects on virulence against mosquitoes and on detection of *in vitro* amylase, protease and lipase activity. *J. Inv. Pathol. 45*: 9-15.

**Silveira, W.D.** and **Azevedo, J.L.** (1984). Isolation of auxotrophic mutants of *Metarhizium anisopliae* by filtration enrichment technique. *Rev. Bras. Genet. 7*: 1-8.

**Silveira, W.D.** and **Azevedo, J.L.** (1987). Protoplast fusion and genetic recombination in *Metarhizium anisopliae*. *Enz. Microbiol. Technol. 9*: 149-152.

**St. Leger, R.J., Hajek, A.E., Staples, R.C.** and **Roberts, D.W.** (1991). Fungi for the biocontrol of insects: Tools and trends. In: *Molecular Biology of Filamentous fungi* (Stahl, U. and Tudzynski, P., eds.). *Proceedings of the European Molecular Biology Organization*. VCH, Berlin. 45-63.

**Upshall, A., Giddings, B.** and **Mortimore, I.D.** (1976). The use of benlate for distinguishing between haploid and diploid strains of *Aspergillus nidulans* and *Aspergillus terreus*. *J. Gen. Microbiol. 100*: 413-418.

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