Rev. Brasil. Genet. 17, 1, 15-18 (1994)
(Brazil. J. Genetics)

GENETIC RECOMBINATION BY PROTOPLAST FUSION IN THE DEUTEROMYCETE *Beauveria bassiana*

Luzia Doretto Paccola-Meirelles[1] and João Lúcio Azevedo[2]

ABSTRACT

Protoplasts of auxotrophic mutants of *Beauveria bassiana* were produced by treating germ tubes with lytic enzymes. Regeneration frequency was 33%. The fusogenic agent used was polyethylene glycol and the fusion products were highly unstable. The sectors which emerged from the fusion products were analyzed and recombinants were obtained. The electrophoretic pattern for esterases of parental strains and recombinant sectors was determined. The protoplast fusion technique proved to be a valuable tool for studies of genetics and breeding of *B. bassiana*.

INTRODUCTION

Both the parasexual cycle, first described by Pontecorvo and Roper (1952) for *Aspergillus nidulans*, and protoplast fusion are of considerable interest for the genetic study of Deuteromycete fungi such as the entomopathogenic fungus *Beauveria bassiana*. We have recently described the parasexual cycle of this species (Paccola-Meirelles and Azevedo, 1991). However, it has not always been easy to produce heterokaryons between some strains, probably due to incompatibility factors.

The protoplast isolation and fusion technique can be a potentially valuable tool in such a case since it permits the production of diploid nuclei between different strains, species and even genera (Peberdy, 1971; Peberdy *et al.*, 1976; Anné and Peberdy, 1981; Azevedo, 1986).

Several protoplast isolation techniques have been described for entomophatogenic fungi (Paris, 1977; Pendland and Boucias, 1984; Kawula and Ling, 1984; Kawamoto and Aizawa, 1986; Shimizu, 1986, 1987; Pfeifer and Khachatourians, 1987; Silveira and Azevedo, 1987; Shimizu and Kurisu, 1988 and Vega, 1990). In *B. bassiana*, protoplasts were isolated from blastospores by Kawula and Ling (1984), using the enzyme β-glucuronidase, by Kawamoto and Aizawa (1986), using Zymolyase 5000, and by Pfeifer and Khachatourians (1987), using a mixture of cellulase, chitinase, β-glucuronidase and lysozyme. Shimizu (1986) obtained high levels of protoplast production using young *B. bassiana* mycelium treated with Zymolase 20-T. The protoplast fusion process of *B. bassiana* was described by Kawamoto and Aizawa (1986) and by Shimizu (1987). These investigators reported the frequencies of the fusion products but did not analyze the haploid recombinants originating from these fusion products.

The objective of the present study was to promote protoplast fusion between auxotrophic strains, in an attempt to recover recombinants from such fusion products.

MATERIAL AND METHODS

Microorganisms

Auxotrophic mutants were obtained from two wild-type strains of *B. bassiana* (196-isolated from *Diatraea saccharalis* (Pyralidae, Lepidoptera) and 256-isolated from *Euchistus heros* (Pentatomidae, Hemiptera) after treatment with gamma radiation, following the procedure described by Paccola-Meirelles and Azevedo (1991). The mutants were designated 196/1 (*ade-2; ths-1*) and 256/1 (*nic-1, rib-1*) in which the *ade,*

[1] Centro de Ciências Biológicas, Departamento de Biologia Geral, Universidade Estadual de Londrina, Caixa Postal 6001, 86051-970 Londrina, PR, Brasil. Send correspondence to L.D.P.-M.

[2] Escola Superior de Agricultura "Luiz de Queiroz", Instituto de Genética, Universidade de São Paulo, Piracicaba, SP, Brasil.

ths, nic and *rib* symbols represent nutritional deficiency for adenine, thiosulfate, nicotinic acid and riboflavin, respectively.

Media

Minimal medium was a modified Czapek-Dox medium (Pontecorvo *et al.*, 1953) and complete medium was a complex medium containing yeast extract, hydrolysed casein, peptone, hydrolysed nucleic acids and vitamins (Pontecorvo *et al.*, 1953).

The mycelium were produced in liquid Sabouraud's dextrose medium. The incubation temperature was 28°C.

Protoplast production

A mutant conidial suspension was inoculated into flasks containing liquid Sabouraud medium and the cultures were incubated at 28°C for 24 hours in a rotary shaker at 150 rpm. The cultures were centrifuged and the pellet was washed three times with an osmotic stabilizer (potassium phosphate buffer, pH 6.0, 5 mM $MgSO_4 7H_2O$, 0.7 M KCl, and 3 mM casein). The material (approximately 50 mg) was resuspended in 10 ml of the osmotic stabilizer, preincubated for 60 minutes at 28°C and treated with the enzyme complex cellulase CP (2.5 mg) and Novozym (2.5 mg) for 2 hours and 30 minutes at 28°C on a rotary shaker at 150 rpm. After this step, the protoplast containing suspension was centrifuged at 2000 rpm for 10 seconds. The supernatant was centrifuged at 3000 rpm for 10 minutes and the pellet resuspended in the osmotic stabilizer. This operation was repeated two more times.

Protoplast fusion

Approximately 10^6 protoplasts ml^{-1} of each mutant strain were mixed and centrifuged for 10 minutes at 3000 rpm in the osmotic stabilizer. The pellet was resuspended and centrifuged twice. Polyethylene glycol 30% (PEG, 1 ml) was added and the mixture was incubated at 28°C for 10 minutes and centrifuged three times in the osmotic stabilizer for total PEG removal. The pellet was resuspended in the osmotic stabilizer and appropriate dilutions were made. Approximately 0.1 ml of the suspension was plated onto minimal medium supplemented with 0.7 M KCl and covered with a 5 ml layer of semisolid minimal medium, also containing 0.7 M KCl. The control consisted of preparations made in minimal medium and complete medium without an osmotic stabilizer and of minimal medium plates containing protoplasts of both strains which were treated with PEG separately. Fusion products were isolated after 10 to 15 days of growth at 28°C.

Analysis of fusion products

The fusion products isolated in minimal medium supplemented with 0.7 M KCl, were transferred to plates containing complete medium. Some products were highly unstable in complete medium and produced sectors that were isolated and characterized for the nutritional requirements involved in the cross.

Gel electrophoresis and enzyme straining procedures

Electrophoresis was carried out according to Paccola-Meirelles *et al.* (1988) in polyacrylamide gels.

RESULTS

The cellulase CP and Novozym 234 mixture was effective for protoplast isolation from *B. bassiana*, with 33% protoplast regeneration frequency. In the minimal media 41 prototrophic colonies were recovered. In contrast, no prototrophic colony was formed on the control plates. This indicates that these colonies are the product of the complementation and fusion of auxotrophic mutants.

The fusion products were transferred to complete medium, where four of these products were found to be highly unstable diploids. The others fusion products were classified as stable prototrophs haploids.

Conidia derived from sectors that emerged from diploid products were plated onto complete medium at suitable dilutions to give isolated colonies. One colony from each sector was then analysed for nutritional requirements. The results are presented in Table I.

Table I - Auxonographic analysis of sectors resulting from the protoplast fusion between strain 196/1 and strain 256/1 of *Beauveria bassiana*.

Class	(1)	Observed number
1	*ade*	3
2	*nic*	1
3	*ths*	1
4	*ade rib*	1
5	*ade ths*	6
6	*ade nic*	2
7	*ade ths rib*	2
8	*ade ths nic*	1
9	*ths nic rib*	1
10	*ade ths nic rib*	17
11	*ade+ ths+ nic+ rib+*	3

(1) The symbos *ade, ths, nic* and *rib* represent nutritional deficiencies for adenine, thiosulfate, nicotinic acid and riboflavin, respectively. The symbol + represents prototrophy.

Class 10 sectors (Table I) presented poor growth on all media, including minimal medium with all nutritional requirements added. However, when transferred to complete medium, these sectors grew normally and sporulated less than the parental strains. These colonies, when incubated in minimal medium for 10 days at 28°C, with all nutritional requirements added, released sectors of normal growth and conidiation.

In addition to the analysis of auxotrophic marker segregation, the electrophoretic pattern of some of these recombinants was determined and is presented in Figure 1. The parental types 196/1 and 256/1 showed different electrophoretic patterns for esterases. The segregants produced by protoplast fusion presented bands similar to those of either 196/1 or 256/1, or similar to both. In some cases, as in sectors A, B, C, D, E, F, nonparental bands were observed.

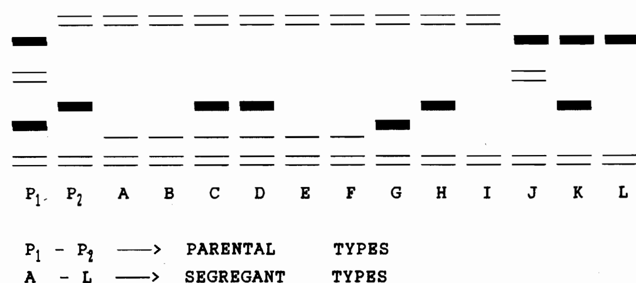

Figure 1 - Electrophoretic pattern for esterases fo parental types and segregants produced by protoplast fusion.

DISCUSSION

The isolation of protoplasts from germ tubes proved to be efficient in *B. bassiana*, in agreement with results reported by other authors (Pfeifer and Khachatourians, 1987).

Several investigators have reported the importance of pre-incubating mycelium to be digested by lytic enzymes. Pfeifer and Khachatourians (1987) tested some thiol compounds on *B. bassiana* during this phase, and Dithiothreitol (DTT) was found to be the most effective. In the present study, the osmotic stabilizer itself proved to be adequate as a preincubating agent, as described by Paris (1977) for *B. tenella*.

Four fusion products were highly unstable and liberated sectors spontaneously (Table I), without the need for haploidization induction with chemical agents.

The occurrence of gene exchange and transfer between strains is confirmed by the results presented in Table I. At least one segregant of each expected type was recovered. Class 10 sectors, which presented poor growth and conidiation on all media, have also been detected during the process of protoplast fusion in other fungi such as *Penicillium* and *Metarhizium* (Peberdy *et al.*, 1977; Vega, 1990). New sectors of normal growth and conidiation arose from these recombinants. Possibly one of the parents carries a chromosome duplication which, through mitotic permutation during the process of haploidization, gives, origin to translocations (resulting in this phenotype), which may be lost during the subsequent divisions, thus giving origin to normal sectors. Another possibility is the involvement of some cytoplasmic incompatibility factors. The loss of these factors would then restore the normal phenotype. And, thirdly the recombinants could be aneuploids and the extra chromosome may be lost during the celular division, originating normal sectors.

New crosses involving these parental lines are currently being performed in our laboratory in an attempt to clarify and confirm one of these hypotheses.

The electrophoretic pattern of some these recombinants was determined (Figure 1). Since the isozymes may be controlled by one or more genes at different loci, and since they can be easily separated and identified, they may be considered as appropriate genetic markers for the identification of hybrid and segregant colonies.

The parental types showed different electrophoretic patterns for esterases. The segregants showed a banding pattern similar to that of either parental type, of a mixture of both types, or a pattern differing from those of both parents, with the appearance of new bands, as seen in sectors, A-F (Figure 1). Banding patterns differing from the parental types may be attributed to the development of proteins with new or modified properties, as reported by Anné and Peberdy (1981), when they compared the isozyme pattern of hybrids obtained between *P. chrisogenum* and *P. roquefortii* by protoplast fusion. They also observed new bands in the recombinants and hybrids.

The recovery of haploid prototroph products in minimal medium could be due to a high instability of diploid nuclei. These diploids difficulty could be maintained even on minimal medium. Others recombinant classes could be recovered by plating fused protoplasts on complete medium or selective media.

The results suggest that diploidization by nuclear fusion in this species is transient and is followed by rapid chromosome segregation.

Paccola-Meirelles and Azevedo (1991) demonstrated the high instability of the *B. bassiana* diploids in the conventional parasexual cycle. In other fungi such as *Cephalosporium acremonium* (Ball and Hamlyn, 1982), *A. niger* (Bonatelli Jr. *et al.*, 1983) and *M. anisopliae* (Silveira and Azevedo, 1987; Vegas, 1990) a similar phenomenon was described and the term

parameiosis was proposed to designate this type of instability (Bonatelli Jr. *et al.*, 1983).

The results showed that the phenomenon of parameiosis is common in the products of the protoplast fusion of *B. bassiana*, as occurs in the normal parasexual cycle of this species.

ACKNOWLEDGMENTS

Publication supported by FAPESP.

RESUMO

Proplastos de mutantes auxotróficos de *Beauveria bassiana* foram produzidos pelo tratamento de tubos germinativos com enzimas líticas. A freqüência de regeneração foi de 33%. O agente fusogênico usado foi o PEG e os produtos de fusão mostraram uma alta instabilidade liberando setores recombinantes. O padrão eletroforético para esterases de linhagens parentais e setores recombinantes foi determinado. A técnica de fusão de protoplastos descrita aqui representa mais uma alternativa para a condução de estudos genéticos e de melhoramento em *B. bassiana*.

REFERENCES

Anné, J. and **Peberdy, J.F.** (1981). Characterization of interspecific hybrids between *Penicillium chrysogenum* and *P. roquefortii* by isoenzyme analysis. *Trans. Br. Mycol. Soc. 77*: 401-408.

Azevedo, J.L. (1986). Melhoramento Genético em Microrganismos. *Ciência e Cultura 38*: 1161-1168.

Ball, C. and **Hamlyn, P.F.** (1982). Genetic recombination studies with *Cephalosporium acremonium* related to the production of the industrially important antibiotic *Cephalosporin C. Rev. Brasil. Genet. 5*: 01-13.

Bonatelli Jr., R., Azevedo, J.L. and **Valent, G.V.** (1983). Parasexuality in a citric acid producing strain of *Aspergillus niger. Rev. Brasil. Genet. 6*: 399-405.

Kawamoto, H. and **Aizawa, K.** (1986). Fusion conditions for protoplasts of an entomogenous fungus *Beauveria bassiana. Appl. Entomol. Zool. 21*: 624-626.

Kawula, T.H. and **Ling, A.J.** (1984). Production of protoplasts from *Beauveria bassiana* blastopores. *J. Invertebr. Pathol. 43*: 282-284.

Paccola-Meirelles, L.D. and **Azevedo, J.L.** (1991). Parasexuality in *Beauveria bassiana. J. Invertebr. Pathol. 57*: 172-176.

Paccola-Meirelles, L.D., Valarini, M.J., Azevedo, J.L. and **Alfenas, A.C.** (1988). *Manual de Técnicas Eletroforéticas em Microrganismos*. Ed. FEALQ, Piracicaba, São Paulo, pp. 54.

Paris, S. (1977). Heterocaryons chez *Beauveria tenella. Mycopathologia 61*: 67-75.

Peberdy, F.F. (1971). Protoplasts from *Mortierella vinacea. Trans. Br. Mycol. Soc. 56*: 67-72.

Peberdy, J.F., Buckley, C.E., Daltrey, D.C. and **Moore, P.M.** (1976). Factors affecting protoplasts release in some filamentous fungi. *Trans. Br. Myco. Soc. 67*: 23-26.

Peberdy, J.F., Eyssen, H. and **Anné, J.** (1977). Interspecific hybridization between *Peniccilium chrysogenum* and *Penicillium cyaneo-fulvum* following protoplast fusion. *Mol. Gen. Genet. 157*: 281-284.

Pendland, J.C. and **Boucias, D.G.** (1984). Production and regeneration of protoplasts in the entomogenous hyphomycete *Nomuraea rileyi. J. Invertebr. Pathol. 43*: 285-287.

Pfeifer, T.A. and **Khachatourians, G.G.** (1987). The formation of protoplasts from *Beauveria bassiana. Appl. Microbiol. Biotechnol. 26*: 248-253.

Pontecorvo, G. and **Roper, J.A.** (1952). Genetic analysis without sexual reproduction by means of polyploidy in *Aspergillus nidulans. J. Gen. Microbiol. 6*: vii.

Pontecorvo, T.A., Roper, J.A., Hemmons, L.M., McDonald, K.D. and **Bufton, A.W.J.** (1953). The genetics of *Aspergillus nidulans. Adv. Genet. 5*: 141-238.

Shimizu, S. (1986). Properties of protoplasts of *Beauveria bassiana. J. Seric. Scien. JPN. 55*: 510-517.

Shimizu, S. (1987). Protoplast fusion of insect pathogenic fungi. In: *Biotechnology in Invertebrate Pathology and Cell Culture* (Maramorosch, K., ed.). Academic Press, London, pp. 401-414.

Shimizu, S. and **Kurisu, K.** (1988). Isolation of protoplasts from several entomopathogenic fungi. *J. Seric. Scien. JPN. 57*: 81-82.

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

Vega, M.S. (1990). Aspectos genéticos da parameiose via fusão de protoplastos em *Metharhizium anisopliae* (METSCH). Sorokin. Master's Thesis, ESALQ-USP, Piracicaba.

(Received May 24, 1993)