ISOLATION OF RECOMBINANT STRAINS WITH ENHANCED PECTINASE PRODUCTION BY PROTOPLAST FUSION BETWEEN PENICILLIUM EXPANSUM AND PENICILLIUM GRISEOROSEUM

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ABSTRACT

Protoplast fusion between complementary auxotrophic and morphological mutant strains of Penicillium griseoroseum and P. expansum was induced by polyethylene glycol and calcium ions (Ca²⁺). Fusant strains were obtained in minimal medium and a prototrophic strain, possibly diploid, was chosen for haplodization with the fungicide benomyl. Different recombinant strains were isolated and characterized for occurrence of auxotrophic mutations and pectinolytic enzyme production. The fusant prototrophic did not present higherpectinase production than the parental strains, but among 29 recombinants analyzed, four presented enhanced enzyme activities. The recombinant RGE27, which possesses the same auxotrophic and morphologic mutations as the P. griseoroseum parental strain, presented a considerable increase in polygalacturonase (3-fold) and pectin lyase production (1.2-fold).

Key words: Protoplast fusion, pectinases, Penicillium

INTRODUCTION

Since the parasexual cycle was first discovered by Pontecorvo and Roper (31), it has been described in important fungal species (6). From a biotechnological point of view, the parasexual cycle is of great importance for the improvement of fungi of industrial interest since most of these fungi do not have a sexual cycle. The parasexual cycle has successfully been applied in enzyme producing fungi, antibiotics and has been used for improvement of biocontrol efficiency in the genus Beauveria (9,21,32,40,44).

The parasexual cycle initiates with heterokaryose, occurring through anastomosis of vegetative cells; followed by nuclear fusion, which gives rise to heterozygous diploids putatives and terminates with recombinant production by mitotic recombination and haplodization (6,10,18,19,44). Spontaneous anastosis between different species can be hindered by the vegetative incompatibility among different strains. The incompatibility can be overcome by use of the protoplast fusion technique, which allows for production of haploid recombinants with the desired characteristics of the parental species. Interspecific protoplast fusion has already been described for several fungi, including Aspergillus nidulans with A. fumigatus (16), A. nidulans with A. rugulosus (22), A. oryzae with A. sojae (42), Aspergillus sp. with A. flavipes (38), Volvariella volvacea with V. bombycina (46) and Beauveria bassiana with B. sulfurescens (13,44).

The spontaneous parasexual cycle in the Penicillium genus has been described for the species P. chrysogenum (30), P. expansum (8), P. italicum and P. digitatum (39), and P. roqueforti (15), while the induced parasexual cycle in P. griseoroseum was described by Santos (37). Fusions of interspecific protoplasts were obtained between P. chrysogenum and P. notatum (3) and between P. chrysogenum and P. roqueforti (4).

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Pectinolytic enzymes are of great commercial interest in the food industry for clarification of fruit juices and wines, for ripening cacao and coffee, for processing of conserved fruit (12,25). These enzymes are also important in the textile industry, since pectic substances contained in the middle lamella and primary cell wall can be softened with enzymes in order to liberate the cellulose fibers from the plant cell wall (7,41,45).

Several genetic studies have been carried out in our laboratories in order to sequence the structural and regulatory genes of the pectinolytic complex, and to isolate strains with enhanced pectinolytic enzyme activities. Different methodologies which have been used to achieve these goals include protoplast production and regeneration, mutant isolation and characterization, protoplast transformation, construction of a genomic library and isolation and characterization of genes coding polygalacturonases in *P. griseoroseum* and *P. expansum* (14,17,33,34,35).

The fusion of interspecific protoplasts to genetically enhance pectinase production has only been described for the *Aspergillus* genus (38), in which fusion of *A. flavipes* with *Aspergillus* sp. protoplasts, resulted in diploids putatives with increased pectinase production.

The objective of this study was to carry out protoplast fusion between *P. expansum* and *P. griseoroseum* to obtain recombinant strains with increased pectinolytic enzyme activity.

**MATERIAL AND METHODS**

**Micromonogroups**

The wild type strains of *Penicillium griseoroseum* (PG) and *P. expansum* (PE) used in this study were isolated from forest seeds by Dr. James J. Muchovej, Departamento de Fitopatologia, Universidade Federal de Viçosa. The *P. griseoroseum* mutant PGLBF (*leu, bio, fwn*) is auxotrophic for leucine and biotin and produces brown conidia. This mutant was isolated using nitrosoguanidine (N-methyl-N′-nitro-N-nitrosoguanidine), as mutagenic agent (11). The mutant *P. expansum* PELW (*lis, whi*), obtained by irradiation of conidia with ultraviolet light, is a lysine auxotroph and produces white conidia (24). The reversion frequency was < 1 x 10⁻⁶ in all mutants tested.

**Culture media**

The following media were used in this study: 1) Minimal Medium (MM) (31): 6.0 g/L NaNO₃, 1.5 g/L KH₂PO₄, 0.5 g/L KCl, 0.5 g/L MgSO₄·7H₂O, 0.01 g/L FeSO₄, 0.01 g/L ZnSO₄, 10.0 g/L glucose, 15.0 g/L agar (SIGMA); 2) Complete Medium (CM) (5, 31): minimal medium supplemented with 2.0 g/L peptone, 1.5 g/L hydrolyzed casein, 2.0 g/L yeast extract and 1.0 mL of a vitamin solution (0.2 mg biotin, 10.0 mg p-aminobenzoic acid, 50.0 mg pyridoxine, 50.0 mg thiamin, 100.0 mg nicotinic acid and 100.0 mg riboflavin in 100 mL distilled water); 3) enriched oatmeal agar (EOA): 40.0 g/L oatmeal, 1.5 g/L hydrolyzed casein, 2.0 g/L yeast extract, 2.0 g/L peptone, 15.0 g/L agar and 1.0 mL of the vitamin solution; 4) buffered mineral medium (BMM): 13.6 g/L K₂HPO₄, 7.6 g/L KH₂PO₄, 2.0 g/L (NH₄)₂SO₄, 3.0 g/L citric pectin, 0.6 g/L yeast extract and 1.1 g/L MgSO₄·7H₂O; and, 5) non-buffered minimal medium (NBMM): 4.0 g/L K₂HPO₄, 1.28 g/L KH₂PO₄, 2.0 g/L (NH₄)₂SO₄, 3.0 g/L citric pectin, 0.6 g/L yeast extract and 1.1 g/L MgSO₄·7H₂O.

**Spontaneous heterokaryons formation**

*P. griseoroseum* PGLBF conidia and *P. expansum* PELW conidia were inoculated in sterile test tubes containing 3 mL of MM enriched with 2% (v/v) CM and incubated at 25°C for 5 days.

**Protoplast isolation and fusion**

About 10⁶ conidia of the *P. griseoroseum* (PGLBF) and *P. expansum* (PE) mutant strains were inoculated into 50 mL CM in 250 mL Erlenmeyer flasks and incubated for 18 hours at 150 rpm and 25°C. Cultures were filtered in gauze and the retained mycelia washed twice in distilled water. Approximately 300 mg of fresh mycelia were incubated in 5.0 mL of osmotic stabilizer (0.6 M KCl in 10 mM sodium and potassium phosphate buffer, pH 5.8) containing 5.0 mg/mL of *Trichoderma harzianum* lytic enzyme (SIGMA) for 3 hours at 80 rpm and 30°C to produce protoplasts. The protoplasts were filtered through gauze, washed twice in osmotic stabilizer and centrifuged for 10 minutes at 3000 g and 4°C. Protoplast fusion was carried out as follows: protoplast suspensions (10⁶ protoplasts/mL) of each strain were centrifuged at 3000 g for 10 minutes at 4°C, protoplasts were resuspended in 1.0 mL of a solution containing 30% (w/v) polyethylene glycol (PEG 6000 – SYNTH) and 0.05 mM CaCl₂ in 0.05 mM glycine-NaOH buffer, pH 8.0. After 10 minutes of incubation at 30°C the suspension was washed twice with 10 mL of osmotic stabilizer and centrifuged at 3000 g for 10 minutes at 4°C. The sedimented protoplasts were resuspended in the same buffer and serial dilutions were prepared and plated for regeneration in MM and CM containing 1.0 M sucrose as osmotic stabilizer, for the calculation of fusion frequency and incubated at 25°C for 5 days. Diploids putatives were identified by growth in MM and by the green conidial color.

**Haplodization**

To induce haplodization, diploids putatives were transferred by punctual inoculation to Petri dishes containing CM supplemented with 1.0 µg/mL of benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate) and incubated at 25°C for 10 days. The sectors presenting color similar to the parental strains were purified in CM and analyzed for genetic markers and pectinase activity.

**Conditions for enzyme production**

The fungi were grown for 9 days on plates containing EOA and 5.10⁷ conidia were harvested and inoculated in 125 mL
protoplast fusion between conidia of 

Table 1. Characterization of recombinant strains obtained by haploidization of the putative diploid strain (DGE), after protoplast fusion between P. expansum PELW and P. griseoroseum PGLB.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>PELW</td>
<td>lys whi</td>
<td>RGE14</td>
<td>leu fwn</td>
</tr>
<tr>
<td>PGLBF</td>
<td>leu bio fwn</td>
<td>RGE15</td>
<td>leu fwn</td>
</tr>
<tr>
<td>DGE</td>
<td>prototrophic</td>
<td>RGE16</td>
<td>prototrophic whi</td>
</tr>
<tr>
<td>RGE01</td>
<td>leu fwn</td>
<td>RGE17</td>
<td>prototrophic whi</td>
</tr>
<tr>
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<td>leu fwn</td>
<td>RGE18</td>
<td>prototrophic whi</td>
</tr>
<tr>
<td>RGE03</td>
<td>leu fwn</td>
<td>RGE19</td>
<td>leu fwn</td>
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<tr>
<td>RGE04</td>
<td>leu fwn</td>
<td>RGE20</td>
<td>leu bio fwn</td>
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<tr>
<td>RGE05</td>
<td>leu bio fwn</td>
<td>RGE21</td>
<td>leu fwn</td>
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<tr>
<td>RGE06</td>
<td>leu bio fwn</td>
<td>RGE22</td>
<td>leu fwn</td>
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<tr>
<td>RGE07</td>
<td>leu bio fwn</td>
<td>RGE23</td>
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<tr>
<td>RGE09</td>
<td>leu fwn</td>
<td>RGE25</td>
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<td>leu bio fwn</td>
<td>RGE26</td>
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<td>RGE13</td>
<td>leu fwn</td>
<td>RGE29</td>
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lys = lysine auxotroph; leu = leucine auxotroph; bio = biotin auxotroph; whi = white conidia; fwn = fawn-colored conidia.
Isolation of pectinase producing recombinants

When the spontaneous parasexual cycle of *P. expansum* was analyzed (24), it was noted that diploids putative were rather unstable and that recombinant sectors could easily be isolated. Nevertheless, Santos (37) observed quite different results with *P. griseoroseum*, in which the parasexual cycle was not obtained spontaneously, and the prototrophic products of fusion were

![Figure 1](image_url)

**Figure 1.** Polygalacturonase (A) and pectin lyase (B) activities of wild type strains of *P. expansum* (PE) and *P. griseoroseum* (PG), of the mutant parental strains (PELW and PGLBF), of the diploid obtained by protoplast fusion (DGE) and of the haploid recombinants (RGE1 to RGE29) grown in minimal medium at pH 6.3 with pectin as sole carbon source. Segments followed by the same letter are not significantly different by the Scott-Knott test at a level of 1%.
stable and produced few discrete sectors even when placed in CM supplemented with benomyl. The diploid putative resulting stable and produced few discrete sectors even when placed in P. expansum, with regard to haploidization, presenting readily observable sectors.

**Enzymatic activity**

Polygalacturonase and pectin lyase activities of the wild type (P. expansum and P. griseoroseum), parental (PGLBF e PELW), putative diploid strain (DGE) and recombinant strains (RGEs) were measured. The DGE strain did not present higher polygalacturonase or pectin lyase activity than the parental strains (Figure 1A e B). This result is similar to that found for diploids of A. niger var. awamori, which did not present a higher quantity of chymosin than the parental strains (9). Hoh et al. (20) obtained five diploids from A. niger, among which only one presented a higher β-glucosidase activity than the parental strains. However, Kirimura et al. (23) obtained one A. niger diploid strain that produced 1.2 times more citric acid than the parental strain in solid culture. Martinková et al. (26) also obtained A. niger diploids with a 15% higher citric acid production than the parental strains.

Several recombinants presented higher polygalacturonase activities than the wild type strains. Recombinants RGE 14, 20 and 27 had higher activities than the parental mutants (Figure 1A). In relation to pectin lyase production, most recombinants presented activity similar to that of the parental mutants, and inferior to the activity of the wild type strains. However, two recombinants, RGE26 and RGE27, presented increased PL activity, corresponding to 163.82% and 146.52%, respectively, of the activity found in the P. griseoroseum wild type strain (Figure 1B). The recombinant RGE27 presented polygalacturonase activity of 121.58% in relation to PELW and of 340.58% in relation to the PG. Recombinant RGE2D did not present higher polygalacturonase activity than the parental mutants, and likewise for its pectin lyase activity. Among 29 recombinants obtained, RGE27, which presented the same auxotrophic and morphologic mutations as the P. griseoroseum parental mutant, was the most outstanding since it presented a considerable increase in both polygalacturonase and pectin lyase activities.

Hoh et al. (20) obtained A. niger recombinants by protoplast fusion which presented glycoamylase activity 2.5 times higher than in the parental strain. A similar result was obtained by Rubinder et al. (36) who was able to obtain a Thermomyces lanuginosus recombinant, which presented five-fold higher α-amylase and a-glycoamylase activities than the wild type strain. A. niger var. awamori recombinants were obtained which presented 15% greater chymosin production than the parental strains (9). Tahoun (40) obtained P. chrysogenum segregants with 290 to 390% greater penicillin production than the parental strains.

Our results confirm that protoplast fusion is a promising technique for obtaining strains with increased enzyme production. Regulatory mechanisms, organization and localization of the genes responsible for the enzymes of the pectinolytic complex of the Penicillium genus are not yet thoroughly understood but, based on the results presented in this paper, we can affirm that these genes were recombined during the parasexual cycle initiated by protoplast fusion. The recombinant RGE27, with enhanced polygalacturonase and pectin lyase activities, demonstrates that our principal objective, obtaining a recombinant with the desired characteristics, was achieved. The production of pectinases by RGE27 strain will be studied in a submerged fermentation system where different carbon sources will be tested with the objective of reducing production costs.

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**RESUMO**

Isolamento de linhagens recombinantes com maior produção de pectinases por meio de fusão de protoplastos entre Penicillium expansum e Penicillium griseoroseum

Fusões de protoplastos entre linhagens mutantes auxotróficas e morfológicas complementares de Penicillium griseoroseum e P. expansum foram induzidas por polietilenoglicol e íons cálcio (Ca²⁺). Fusionantes foram obtidos em meio mínimo e uma linhagem prototrófica, possivelmente diplóide, foi selecionada para a haploidização com o fungicida benomil. Diferentes linhagens recombinantes foram isoladas e caracterizadas quanto à presença de mutações auxotróficas e a produção de enzimas pectinolíticas. O fusionante prototrófico não apresentou maior atividade de pectinases em relação às linhagens parentais, entretanto, entre 29 recombinantes analisados, quatro apresentaram maiores atividades enzimáticas. O recombinante RGE27, o qual possui as mesmas mutações auxotróficas e morfológicas que a linhagem parental de P. griseoroseum, apresentou um aumento considerável na produção de poligalacturonase (3 vezes) e de pectina liase (1,2 vezes).

**Palavras-chave:** Fusão de protoplastos, pectinases, Penicillium

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