

SHORT COMMUNICATION

PREPARATION AND REGENERATION OF PROTOPLASTS OF *Talaromyces flavus*

T.M.C. Santos¹ and I.S. De Melo²

ABSTRACT

Protoplasts from *Talaromyces flavus* were produced from young mycelia using the lytic enzyme. Novozym 234. The best culture medium for highest mycelia yield was malt extract with 20 hours of incubation. The osmotic stabilizers Glucose, NaCl and KCl in regeneration medium were found to induce high regeneration of protoplasts, such as 65%, 65.75% and 70.75%, respectively. The rate of regeneration with $(\text{NH}_4)_2\text{SO}_4$ was 4.5%.

INTRODUCTION

Talaromyces flavus (Klöcker) Stolk & Samson (Anamorph: *Penicillium vermiculatum* Dangeard) is an effective biocontrol agent of *Verticillium* wilt of eggplant under agronomic production conditions (Marrois *et al.*, 1982). *T. flavus* is also considered to be a potential antagonist for the biocontrol of *Rhizoctonia solani* (Boosalis, 1956) and *Sclerotinia sclerotiorum* (McLaren *et al.*, 1985). The fungus produces an antibiotic that kills microsclerotia of *Verticillium dahliae* (Fravel *et al.*, 1987).

¹ Centro de Energia Nuclear na Agricultura, CENA/USP, 13400 Piracicaba, SP, Brasil.

² Centro Nacional de Pesquisa de Defesa da Agricultura, CNPDA/EMBRAPA, Caixa Postal 69, 13820 Jaguariúna, SP, Brasil. Send correspondence to I.S.M.

Genetic transformation in filamentous fungi can be via the production of protoplasts, in this case facilitating the manipulation of a biocontrol agent.

The present study was undertaken to investigate different types of lytic enzymes, the effects of varying enzyme concentration and osmotic stabilizers to maximize protoplast yield, regeneration and viability of *T. flavus*.

MATERIAL AND METHODS

Wild-type strain of *T. flavus*, obtained from Mycological Institute, Kew, Surrey, UK was maintained on potato - dextrose - agar (PDA). For mycelia production five different culture media were tested: Potato - dextrose broth (PD); complete media (CM) (Pontecorvo *et al.*, 1953); malt extract, dextrose, peptone (ME); yeast extract, dextrose, peptone (YE); glucose, yeast extract, casein (GYEC) in order to verify the best for greatest production of young mycelia in 20 hours of incubation at 28°C. Three agar plugs with *T. flavus* mycelia were put into 50 ml of medium and incubated with orbital shaking at 120 rpm.

The minimal combination of enzyme necessary to produce protoplasts was determined by testing the following enzymes in various combinations: cellulose (Biobras) 2% (w/v); cellulose (Onozuku) 2% (w/v), and Novozym 234 (Novo Enzyme, Windsor, UK). The enzyme mixture consisted of: cellulose (Onozuku) 2% (w/v) and Novozyme 234 1%, cellulose (Onozuku) 1%, Drisilase 1% and Novozym 1% in osmoticum (0.6 M KCl in Mclivaine buffer pH 6.0). Ten mg/ml mycelia (fresh weight) of *T. flavus* were digested for 30, 60, 90 e 120 minutes. Initially, the various enzyme combination were selected as either producing or not producing protoplasts. Protoplasts formed in those times were quantified.

The following osmotic stabilizers were used: 0.5 M glucose, 0.6 M (NH₄)₂SO₄ and 0.6 M NaCl. All-all in Mclivaine buffer, pH 6.0. Mycelia for protoplasts isolation were grown in liquid medium as follows: (g/l) Malt extract, 20; peptone, 1.0.

Protoplasts isolation and regeneration

Young mycelia were filtered through a sintered glass filter (number 3), washed twice with Mclivaine buffer (pH 6.0) and 1.0 g fresh weight was mixed with Novozyme 234 in osmotic stabilizer (0.6 KCl). The mixture was incubated at 28°C with reciprocating shaking for 2 hours. Protoplasts yields were determined in lytic mixtures by counting in a haemocytometer.

The crude protoplasts suspension was filtered, collected by centrifugation at 500 xg for 30 sec., rinsed with a solution containing the stabilizer in McIlvaine buffer. The protoplasts were serially diluted in osmotic stabilizer or distilled water. Aliquots of each dilution were mixed in the regeneration medium supplied with the stabilizers. Regeneration frequency was then calculated for each medium as follows:

$$\text{Regeneration frequency} = \frac{\text{viable count (Dilutions in stabilizer - Dilutions in Water)}}{\text{total Count.}} \times 100$$

RESULTS AND DISCUSSION

Mycelia production

The best medium for highest mycelia yield was Malt-Extract with 1.27 g with 20 hours of incubation. This medium differed from the other four media as follows: BD, 0.137 g; GYEC, 0.276 g; CM, 0.546; and YE, 0.720 g.

Effectiveness of lytic enzyme

Results of the enzyme combination assay are presented in Table I. All combinations containing Novozym 234 resulted in protoplast production. Cellulase (Biobras) from *Trichoderma* sp. was poor in producing protoplasts and cellulase (Onozuku) which contains very little chitinase or 1.3 - B - D - glucanase (Brown *et al.*, 1986), gave low protoplast yield in the times used. Novozym 234, according to Brown *et al.* (1986), possessed the highest specific activities for chitinase and 1.3 - B - D - glucanase. It might be expected that those enzyme preparations, richest in activity to chitin and 1.3 - B - D - glucans, would give the best yields of protoplasts. This enzyme will be used later in studies of protoplasts fusion with different strains.

Effect of the osmotic stabilizers on regenerations frequency

Results of these experiments clearly show that glucose, NaCl and KCl were all suitable for regeneration and reversion of protoplasts to hyphae (Table II). Approximately 65 - 71% of the protoplasts regenerated and reverted to hyphae. The percentage regeneration using $(\text{NH}_4)_2\text{HSO}_4$ into the basal medium was found to be low (4.5%) in all trials tested.

Table I - Enzyme combination assay: number of protoplasts formed/mg mycelium (10^5) of *T. flavus* at 30, 60, 90 and 120 minutes digestion times.

Enzyme combination	Incubation time (min.)			
	30	60	90	120
Celulase (Bibrás) - 2%	0.00 a A	0.00 a A	0.00 a A	0.00 a A
Celulase (Onozuku) - 2%	0.00 a A	0.53 b B	1.08 c B	1.58 d B
Novozym 234, (Novo) - 1%	6.51 a C	11.22 b E	33.08 c D	38.02 d D
Celulase (Onozuku) - 2%				
Novozym 234, 1%	6.07 a B	9.70 b C	36.59 c E	36.59 c C
Celulase (Onozuku) 1% +				
Novozym 234, 1% + Drisilase, 1%	9.77 a D	10.72 b D	30.90 c C	40.12 d E

Means in each column followed by the same capital letters in vertical and small letters in horizontal are not significantly different ($P = 0.05$) according to the Tukey test.

Table II - The effect of stabilizers on regeneration frequency of *Talaromyces flavus* protoplasts.

Stabilizer system	Percentage regeneration ¹
(NH ₄) ₂ HSO ₄	4.50 a
Glucose	65.00 b
NaCl	65.75 b
KCl	70.75 b

Means followed by the same letter are not significantly different ($P = 0.05$) according to the Tukey test.

¹ The regeneration frequency is the percentage of protoplasts regenerating to conidiating mycelial colonies. Average of four replicates in each experiment.

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RESUMO

Protoplastos de *T. flavus* foram obtidos de micélio jovem com a enzima lítica Novozym 234 num tempo de 30 minutos. Altas produções foram obtidas em 90 minutos. Com as misturas Novozym 234 (1%) + Celulose (2%) e Novozym 234 (1%) + Celulose (2%) + Drisilase (1%) não houveram grandes incrementos na produção de protoplastos. O melhor meio de cultura para produção de grandes quantidades de micélio num menor espaço de tempo foi Extrato de Malte. Os estabilizadores osmóticos glucose, NaCl e KCl, no meio de regeneração propiciaram uma alta freqüência de regeneração de protoplastos, da ordem de 65%, 65,75% e 70,75%, respectivamente, ao passo que a regeneração em $(\text{NH}_4)_2\text{SO}_4$ foi da ordem de 4,5%.

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