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Production and Regeneration of Protoplasts from Orchid Mycorrhizal Fungi *Epulorhiza repens* and *Ceratorhiza* sp.

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ABSTRACT

The aim of this work was to study the standardization of conditions to obtain and regenerate Epulorhiza repens *and* Ceratorhiza *sp. protoplasts. For* E. repens*, the largest number of protoplasts (8.0 × 10^6 protoplasts/mL) was obtained in 0.6 M KCl, using 15 mg/mL of Lysing Enzymes, and 2-day-old fungal mycelium. When 0.5 M sucrose was used as osmotic stabilizer, the highest frequency of regeneration was achieved (8.5 %); 80.0 % of protoplasts were nucleated, and 20.0 % anucleated. For* Ceratorhiza *sp., the largest number of protoplasts (4.0 × 10^7 protoplasts/mL) was achieved in 0.6 M NaCl, when 15 mg/mL of Lysing Enzymes and 15mg/mL of Glucanex, with 2-day-old fungal mycelium were used. The highest frequency of regeneration was 6.7 % using 0.5 M sucrose as osmotic stabilizer; 88.8 % of protoplasts were nucleated, and 11.2 % anucleated.*

Key words: *Ceratorhiza*, *Epulorhiza*, molecular biology, protoplast, regeneration

INTRODUCTION

In nature, orchids are associated with mycorrhizal fungi for seed germination and plant nutrition to ensure to complete plant life cycle (Peterson et al., 1998). Orchids make use of intracellularly formed masses of hyphae (pelotons), present mainly in root cortex cells, as a carbon source during the heterotrophic stage of their lives (Peterson et al., 1998). Orchid mycorrhizal fungi have been isolated from their natural hosts and used to improve *in vitro* seed germination and produce seedlings of endangered species to be reintroduce in their natural habitat (Zettler, 1997; Zettler et al., 2000; Pereira et al., 2003), or to multiply the species of ornamental (Zettler et al., 1999) and medicinal (Chang and Chou, 2001) interest.

Although these fungi present a biotechnological potential, little is known about their physiology and genetic. Establishing an effective protocol to obtain and regenerate protoplasts of orchid mycorrhizal fungi is important for determining their electrophoretic karyotype and for works involving transformation and functional analyses of genes related to fungus-plant interaction.

Knowing which factors affect the protoplast production is essential to optimize the conditions for protoplasts release and regeneration for each fungal species (Kumari and Panda, 1992). Factors such as age and quantity of mycelium, osmotic stabilizer, temperature, pH, digestion time, and lytic enzyme concentration are mentioned as determinants of success (Peberdy et al., 1976; Chadegani et al., 1989). It is also important to

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ensure protoplast regeneration, which consists of cell wall reconstitution and return to mycelial growth. Fungal growth tests at different concentrations of osmotic stabilizers are necessary since high solute concentrations often inhibit the fungal growth. Regeneration frequency obtained for different fungi ranges from 0.1 to 50.0 %, depending, mainly, on fungal species, cell ploidy, and regeneration medium (Peberdy, 1991).

Some protocols for obtaining and regenerating the protoplasts have already been established for some Basidiomycetes species, such as *Rhizoctonia solani* (Hashiba and Yamada, 1982), *Agaricus bisporus* (Royer et al., 1992), *Suillus granulatus* (Dias et al., 1996), *Pseudozyma flocculosa* (Cheng and Bélanger, 2000), *Lentinus lepideus* (Kim et al., 2000), *Crinipellis perniciosa* (Lima et al., 2003) and *Stropharia rugoso-anulata* (Yan et al., 2004).

This work aimed at establishing the conditions for production and regeneration of protoplasts of *Epulorhiza repens* and *Ceratorhiza* sp.

MATERIALS AND METHODS

Microorganisms and growth conditions

Orchid mycorrhizal fungi isolates from Brazilian native orchids, *Epulorhiza repens* (VIC 27806) and *Ceratorhiza* sp. (VIC 27808) (Pereira et al., 2005) belonging to "Laboratório de Associações Micorrízicas, Departamento de Microbiologia/BIOAGRO, Universidade Federal de Viçosa/MG/Brazil" were used. Fungal cultures were maintained at 25 ºC in solid modified Melin Norkrans (MMN) medium (Marx, 1969).

Protoplasts production

Three hundred milligrams of mycelium, slightly dried in gauze and toilet paper, were transferred to 50-mL Erlenmeyer flasks containing 5 mL of osmotic stabilizer and different amounts of hydrolytic enzymes. This hydrolytic preparation was incubated at 30 °C and 80 rpm from 1 to 24 h. Protoplast countings were carried out using a Neubauer chamber in a microscope.

Both fungi mycelium were grown by inoculating 4 agar discs, cut off from the edge of colonies cultivated for 10 days at 25 ^{o}C in solid MNM into Petri dishes containing 20 mL of liquid MNM medium, and then incubated at 25 ^{o}C for 2 and 4 days, respectively for *Ceratorhiza* sp. and *E. repens*. For protoplast production, *Ceratorhiza* sp. mycelium was directly used while *E. repens* mycelium was fragmented in a blender by applying two pulses of 2 seconds each, and aliquots of mycelial suspension were inoculated into 100 mL of MMN medium and incubated at 25 ^{o}C for 2 days at 150 rpm.

The osmotic stabilizers mannitol, KCl, NaCl and $MgSO_4$ were tested at the following concentrations 0.6; 0.8; 1.0 and 1.2 M. The lytic enzymes, Glucanex (Novo Nordish Ferment Ltd., CH4243), Lysing Enzymes (Sigma Chemical Co., L1412), and Cellulase (Sigma Chemical Co., C8546), were also tested individually or in combination. Cellulase was used at 20 mg/mL and Lyzing Enzymes and Glucanex at 15 mg/mL.

Protoplast Regeneration

After digestion, the hydrolytic preparations were filtered with gauze to retain the hyphal fragments. The filtrate, containing the hydrolytic preparation and protoplasts, was centrifuged at 710 *g* for 10 minutes. The sediment was resuspended in 5 mL of osmotic stabilizer, centrifuged again and resuspended in 1 mL of the same medium. Protoplasts were counted in a Neubauer chamber. Protoplasts were transferred by the "pour plate" technique to MMN medium containing osmotic stabilizers (sucrose, mannitol, KCl and NaCl, at 0.5 or 1.0 M). A control was done in MMN medium, with no osmotic stabilizer, to calculate the regeneration frequency. Plates were incubated at 25 ºC until colonies formation on the surface of the medium.

All experiments were done twice and the results represent the mean of two repetitions.

Protoplast nuclei staining

To determine the percentage of nucleated protoplasts, a droplet of the protoplast suspension was spread on a slide glass and dried at room temperature, then placed in methanol for 5 minutes and washed three times with 100 mM KH_2PO_4, pH 7.0. After drying, 15 µL of a solution containing 1 µL/mL of SYBR Green I 10.000 X (Sigma Sigma Chemical Co., S9430) was used for nuclei staining (Meinhardt et al., 2001). After 5 minutes of contact with the dye solution, the protoplasts were observed in a Nikon E600 microscope with a Y-FL epi-fluorescence attachment, excitation wavelength range of 450-520 nm. Images were captured using a Fuji HC-300Z digital camera and processed with Photograb and Eztouch (Fujifilm). For both the fungal species, 150 protoplasts were observed.

RESULTS AND DISCUSSION

KCl at 0.6 M provided the highest protoplast release for *E. repens* (2.0×10^4 protoplasts/mL) (Table 1). Low protoplast yields, in the order of 10^4 protoplasts/mL, obtained for the different stabilizer suggested that Glucanex was not appropriate for *E. repens*. For *Ceratorhiza* sp., 0.6 M NaCl was selected since 4.1×10^5 protoplasts/mL were released and greater protoplast stability was observed when this stabilizer was used (Table 1). Choosing the osmotic stabilizer and its concentration is an important feature to be considered when isolating protoplasts. The best osmotic stabilizer varies for each type of microorganism and its ideal concentration is related to differences in internal osmotic pressure in the cells (Villanueva and Acha, 1971). For *Suillus granulatus*, the stabilizer which allowed the largest protoplast release was 0.7 M $MgSO_4$, for *Agaricus bisporus*, 0.8 M $MgSO_4$, and for *C. perniciosa*, 0.6 M KCl (Royer et al., 1992; Dias et al., 1996; Lima et al., 2003).

Table 1 - *E. repens* and *Ceratorhiza* sp. protoplast production in media with different osmotic stabilizers, after a 5-h treatment with hydrolytic preparation.

Osmotic stabilizers	*E. repens*	*Ceratorhiza* sp.
	Protoplasts/mL	
Mannitol 0.6 M	0	1.5×10^5
Mannitol 0.8 M	0	2.0×10^5
Mannitol 1.0 M	0	5.0×10^4
Mannitol 1.2 M	0	3.5×10^4
KCl 0.6 M	2.0×10^4	1.3×10^5
KCl 0.8 M	1.5×10^4	9.0×10^4
KCl 1.0 M	0	3.0×10^4
KCl 1.2 M	0	1.5×10^4
NaCl 0.6 M	0	4.1×10^5
NaCl 0.8 M	1.0×10^4	1.2×10^5
NaCl 1.0 M	0	3.5×10^4
NaCl 1.2 M	0	1.5×10^4
$MgSO_4$ 0.6 M	1.0×10^4	6.5×10^4
$MgSO_4$ 0.8 M	0	5.0×10^4
$MgSO_4$ 1.0 M	0	5.0×10^4
MgSO4 1.2 M	0	1.5×10^4

In all tests, 300 mg of mycelium was treated with 15 mg/mL de Glucanex.

Cell wall composition varies among organisms, and it is assumed that enzyme preparations should be optimized for each species. For *E. repens*, when Lysing Enzymes were used, 1.0×10^6 protoplasts/mL were obtained after a 24-h digestion, while for *Ceratorhiza* sp., the best result (1.6×10^6 protoplasts/mL), with a 5-h digestion, occurred when a combination of Glucanex and Lysing Enzymes was used (Table 2).

Different lytic enzymes have been used alone or in combinations and in different amounts for cell wall digestion. An important characteristic of these commercial preparations is that they are complex compounds of several hydrolytic enzymes. For instance, enzymes obtained from *Trichoderma harzianum* have α-glucanase, β-glucanase, and chitinase activities (Kelkar et al., 1990; Kavanagh and Whittaker, 1996). Larger amounts of protoplasts were obtained from *Rhizoctonia solani* when an enzyme combination (Cellulase Onozuka R-10, Macerozyme R-10 from *Rhizopus* sp., and β-glucuronidase from *Helix pomatia* digestive juice) was used (Hashiba and Yamada, 1982). For *Suillus granulatus* and *Agaricus bisporus*, the best enzyme was Novozym 234 (Royer et al. 1992; Dias et al., 1996). Glucanex was the most appropriate enzyme for *Crinipellis perniciosa* (Lima et al., 2003).

Mycelium age is regarded as one of the main factors which influence the protoplast release (Gold et al., 1983; Peberdy et al., 1976). Although mycelium cultivation time that allows best protoplast release vary for each species, optimal cultivation time for *Ceratorhiza* sp. and *E. repens* protoplasts was two days (Table 3).

Table 2 - *E. repens* and *Ceratorhiza* sp. protoplast production in media with different enzyme preparations after different times of treatment with hydrolytic preparations.

Enzymes	*E. repens* (5 hs)	*E. repens* (24 hs)	*Ceratorhiza* sp. (5 hs)
	Protoplasts/mL		
Glucanex	6.0×10^5	5.3×10^5	1.5×10^5
Cellulase	2.0×10^4	8.0×10^4	0
Lysing Enzymes	8.4×10^5	1.0×10^6	2.0×10^5
Glucanex + Cellulase	5.0×10^4	1.7×10^5	2.7×10^5
Glucanex + Lysing Enzymes	5.8×10^5	3.2×10^5	1.6×10^6
Cellulase + Lysing Enzymes	5.0×10^4	1.0×10^4	5.8×10^5
Glucanex + Cellulase + Lysing Enzymes	1.8×10^5	3.0×10^5	NT

In all tests, 300 mg of mycelium was used. For *Epulorhiza repens* 0.6 M KCl and for *Ceratorhiza* sp. 0.6 M NaCl were used as osmotic estabilizers. NT not tested.

Table 3 - *E. repens* and *Ceratorhiza* sp. protoplast production from mycelia cultivated during different periods of time, after a 5-h treatment with hydrolytic preparation.

Cultivation time (Days)	*E. repens*	*Ceratorhiza* sp.
	Protoplasts/mL	
1	0	3.3×10^5
2	1.9×10^6	1.7×10^6
3	4.0×10^5	9.0×10^5

In all tests, 300 mg of mycelium was used. For *Epulorhiza repens* 0.6 M KCl and Lysing Enzymes and for *Ceratorhiza* sp. 0.6 M NaCl and the combination of Lysing Enzymes and Glucanex were used.

Fungi with very high growth rates present a mycelium that is more susceptible to degradation than that of those with slower growth rate, allowing a greater protoplast release within less than 24 h of cultivation (Hashiba and Yamada, 1982). Although *Ceratorrhiza* used to have faster growth than *Epulorrhiza*, this characteristic didn't affect the mycelial degradation, but the number of protoplasts was higher to *Ceratorhiza* sp. (Fig. 1).

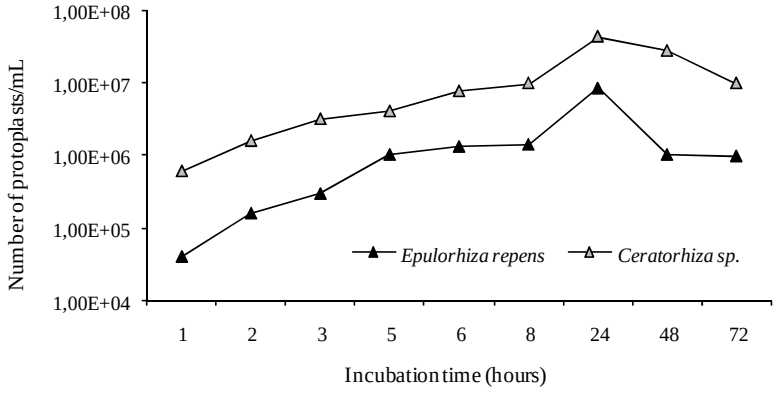

Figure 1 - Number of protoplasts of *E. repens* and *Ceratorhiza* sp. produced during incubation of 500 mg of 2-day-old fungal mycelium in hydrolytic preparations for 72 h. For *E. repens* 0.6 M KCl containg Lysing Enzymes and for *Ceratorhiza* sp., 0.6 M NaCl and a combination of Glucanex and Lysing Enzymes were used.

Incubation time must be considered during protoplast release, since the length of contact with lytic enzymes may lead to disrupture of the first protoplasts formed (Kim et al., 2000). For *E. repens* and *Ceratorhiza* sp., the largest protoplasts number was obtained after a 24-h incubation (Fig. 1). Under this condition protoplast yields for *E. repens* and *Ceratorhiza* sp. were 8.0×10^6 and 4.0×10^7 protoplasts/mL, respectively (Fig. 1). Longer treatments, exceeding 24 h, did not improve yields. Long incubation of digestion mixtures has been related to lyse protoplasts (Chadegani et al., 1989), while other researchers have suggested that a longer time of incubation is more favorable (Toyama et al., 1984; Kolar et al., 1985; Laurila et al., 1985).

Cell wall regeneration and reversion to normal cells is an important step in genetic manipulation of fungi (Peberdy, 1989). For both studied fungi, the best stabilizer was 0.5 M sucrose with regeneration percentages of 8.5 and 6.7 %, respectively (Fig. 2). The stabilizers varied with the fungi and, for *Rhizoctonia solani*, the highest frequency (90.0 %) was obtained with the use of 1.0 M mannitol (Yang et al., 1993), for *Suillus granulatus*, 8.0 % when used 0.6 M $MgSO_4$ added to MMN medium (Dias et al., 1996), for *C. perniciosa*, was 0.5 M sucrose (Lima et al., 2003). With few exceptions, protoplast regeneration frequency for filamentous fungi varies from 0.1 to 50.0 % (Peberdy, 1991). Low values were found for some Basidiomycetes and could be associated to slower growth in most of these organisms (Peberdy, 1991). Protoplast regeneration for *E. repens* and *Ceratorhiza* sp. was slow, with the first colonies appearing only after 5 to 7 days of incubation at 25 ºC, same time was also required for *C. perniciosa* (Lima et al., 2003), but in *R. solani*, after 48-72 h, small colonies were observed (Hashiba and Yamada, 1982).

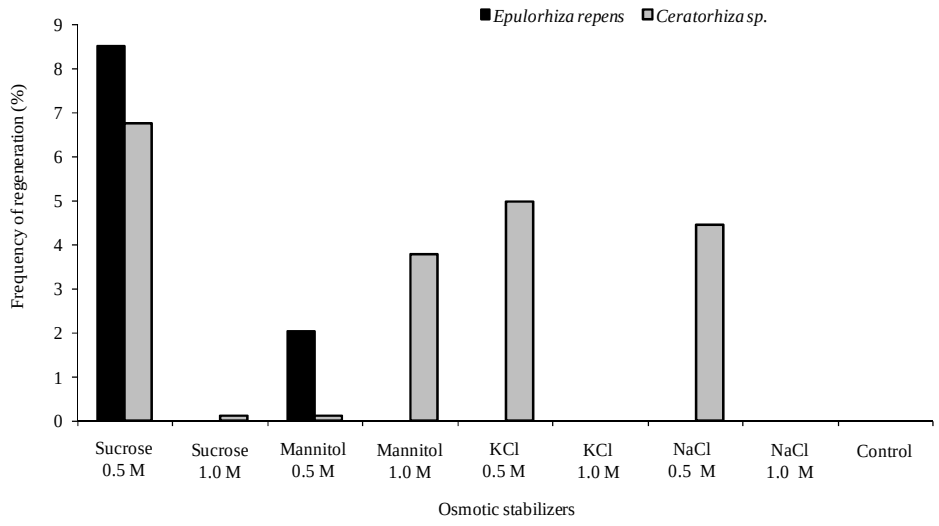

Figure 2 - Frequency of protoplasts regeneration of *E. repens* and *Ceratorhiza* sp. in modified Melin Norkrans (MNM) medium with different osmotic stabilizers.

In *E. repens* protoplast, 58.6 % were uninucleated, 21.4 % binucleate and 20.0 % anucleated, and in *Ceratorhiza* sp., protoplasts, 61.0 % were uninucleated, 24.6 % binucleate, 3.2 % trinucleate and 11.2 % anucleated (Fig. 3). The absence of nuclei in the protoplast may be one of the factors that influence the regeneration (Peberdy, 1991). The percentage of nucleate protoplasts of *E. repens* and *Ceratorhiza* sp. was considered high when compared to other Basidiomycetes. At least one nucleus was present in 80 % of the protoplast of *E. repens* and 88.8 % of *Ceratorhiza* sp. (Fig. 3). So, this was not the main effect in this case. In *C. perniciosa*, for instance, 58.5% of protoplasts were anucleated, 37.5 % uninucleated and 3.55% binucleated (Lima et al., 2003).

Other hypotheses to explain low levels of protoplasts regeneration are protoplasts agglomeration after centrifugation (Deed and Seviour, 1989), high proteolytic activity of the

enzyme preparations, long exposure of cell wall to lytic enzymes (Hamlyn et al., 1981), and growth conditions (Keller et al., 1983), such as osmotic stabilizer (Solís et al., 1996; Kim et al., 2000), and high concentrations of sugar or salts may affect cell metabolism.

E. repens protoplasts do not regenerate in high concentrations of mannitol and sucrose. In the work of Dias et al. (1996), *S. granulatus* mycelium was cultivated in medium with different stabilizers. Sucrose, KCl and $MgSO_4$ provided the best mycelial growth, indicating that they could be used for the production and regeneration of *Suillus* protoplasts. These stabilizers, in addition to providing osmotic stability, do not cause other changes in cell metabolism (Dias et al., 1996).

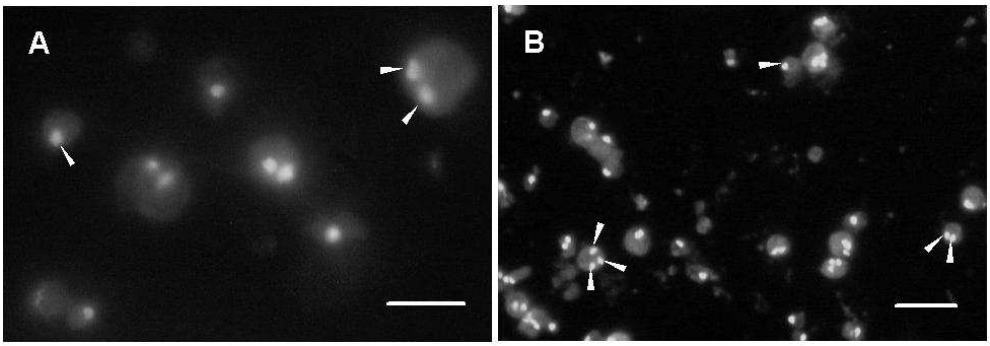

Figure 3 - Nuclei (arrows) in protoplasts of *E. repens* (A) and *Ceratorhiza* sp. (B) stained by SYBR Green I observed in a fluorescence microscope with excitation wavelength of 450-520 nm. Bars: (A) 10 μm, (B) 20 μm.

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RESUMO

O objetivo deste trabalho foi padronizar as condições de obtenção e regeneração de protoplastos de *Epulorhiza repens* e *Ceratorhiza* sp. Para o fungo *E. repens*, a maior produção de protoplastos, 8.0 x 10^6 protoplastos/mL, foi obtida em KCl 0.6 M, na presença de 15 mg/mL de "Lysing Enzymes" e micélio fúngico com 2 dias de idade. A maior freqüência de regeneração obtida foi de 8,5 % quando sacarose 0.5 M foi utilizada como estabilizador osmótico. Do total de protoplastos obtidos, 80 % eram nucleados e 20 % anucleados. Para *Ceratorhiza* sp., a maior produção de protoplastos, 4,0 x 10^7 protoplastos/mL, foi obtida em NaCl 0.6 M, na presença de 15 mg/mL de "Lysing Enzymes" e 15mg/mL de Glucanex, e micélio fúngico com 2 dias de idade. A maior freqüência de regeneração obtida foi de 6.7 % utilizando sacarose 0.5 M como estabilizador osmótico. Do total de protoplastos obtidos, 88.8 % eram nucleados e 1.2 % anucleados. O estabelecimento de protocolo otimizado para obtenção e regeneração de protoplastos dos fungos *E. repens* e *Ceratorhiza* sp. é importante, permitindo o estabelecimento de técnicas de transformação genética, o isolamento de mutantes, a determinação de cariótipo eletroforético e o cruzamento de linhagens.

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