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Short communication

Isolation and regeneration of protoplasts from the mycelium of *Fusarium pallidoroseum* – a potential biocontrol agent of water hyacinth [*Eichhornia crassipes* (Mart.) Solms]

A. Naseema*, B. Dhanya, I.P. Anjanadevi, K.G. Sheena, and Swapna Alex

Department of Plant Pathology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695522, Kerala.

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Abstract

A protocol for isolation of protoplast from the mycelium of *Fusarium pallidoroseum*, a potential biocontrol agent of water hyacinth, was standardized. Stable and more medium sized protoplasts were obtained from 18 h old mycelium at 3 h after incubation, using 80 mg of lytic enzyme from *Trichoderma harzianum*, in 4 ml of 0.01 M citrate buffer (pH 5.85) containing 0.6 M KCl and 5 mM DTT. The protoplasts were regenerated in the regeneration medium.

Keywords: Protoplast, isolation, regeneration.

Fusarium pallidoroseum (Cooke) Sacc. is a pathogen of water hyacinth [*Eichhornia crassipes* (Mart.) Solms] with potential biological control applications (Naseema et. al., 2001). For genetic manipulation and strain improvement of this fungus, protoplast fusion is considered an effective strategy (Lalithakumari, 1996). Although several methods are available for the release and regeneration of protoplasts from the mycelium of fungi (Balasubramanian et. al., 2003), in view of the variations in the cell wall composition, no single method is universally applicable. Therefore, an attempt was made to standardize the methods for isolating and purifying the mycelial protoplasts of *F. pallidoroseum* with particular reference to the role of incubation time and concentration of the lytic enzymes in the release of protoplasts.

Mycelial discs of 3 mm size from 7 day-old cultures of *F. pallidoroseum* grown on potato dextrose agar (PDA) were inoculated into 50 ml potato dextrose broth in 250 ml conical flask and incubated at 30° C. Since the age of mycelium plays an important role in the release of protoplast, an experiment was carried out to determine the optimum age of mycelium for the release of protoplast. The mycelia were separated from the broth culture at different time intervals (16, 18, 20, 22, and 24 h) and washed in 3-4 changes of sterile distilled water for removing the broth. The mycelia were then incubated with constant stirring at 100 rpm with 3 ml mixture of EDTA (100 mM), MES (100 mM), and DTT (5 mM) at pH 6 for 60 min at 30°C. The mycelia were separated from the incubation mixture by centrifugation for 10 min. at 10 000 g and then mixed with different concentrations of lytic enzyme from *Trichoderma harzianum* (Sigma-Aldrich) *viz.*, 50, 60, 70, 80, and 90 mg respectively, in 4 ml of 0.01 M citrate buffer (pH 5.85) containing 0.6 M KCl and 5 mM DTT. This mixture was incubated for 5 h at 30°C and stirred slowly at 30 rpm in a 125 ml conical flask. At every 30 min., the contents were examined under phase contrast microscope to determine the time required for the release of protoplast after lysis of the mycelia. The viability of released protoplast was checked by staining with Evan's blue.

*Author for correspondence: Phone +91 471 2740810; Email< naseenmadr@yahoo.com>.

After the release of maximum number of protoplasts, the contents were filtered through sintered glass filter (Jena, porosity D_2). The filtrate was washed free of enzymes with osmoticant, (0.6M KCl) by centrifugation at 100 g for 6 min. and the pelleted protoplasts were resuspended in known amounts of the osmoticant. Purity of the protoplast was checked under the phase contrast microscope to ensure that they were free of mycelia/debris. The number of protoplasts per millilitre sample was counted using a haemocytometer.

The purified protoplast were regenerated (medium: potato extract from 200 g of fresh potato boiled in 800 ml water, D glucose 20 g, KCl 0.6 M, agar agar 20 g). Fifteen millilitre of cooled media was added to sterile petriplates seeded with 1 ml of protoplast suspension and rotated for uniform mixing. Three replications were maintained. The plates were incubated for 24 to 48 h at 25°C in dark. Individual colonies at random were aseptically transferred to PDA medium. The pathogenicity of the regenerated protoplast was tested by inoculating on water hyacinth plants *in vitro*.

Maximum release of protoplast occurred from 18 h old mycelium at 3 h of incubation (Fig. 1). With further increase in age (e.g., 24 h), the mycelia released less number of protoplasts. This is consistent with the findings of Balasubramanian et al. (2003) for *Trichothecium roseum*. Implicit in this is that the effective growth stage for the formation of *F. pallidoroseum* protoplasts is the early mycelial growth phase. That is, at the physiologically active phase (log phase) the mycelia are more sensitive to lytic enzymes than at 22 and 24 h. After 4 h of incubation, complete lysis of the mycelium was observed. Variations in the size of protoplast are the result of the release of protoplast from different regions of the hyphae.

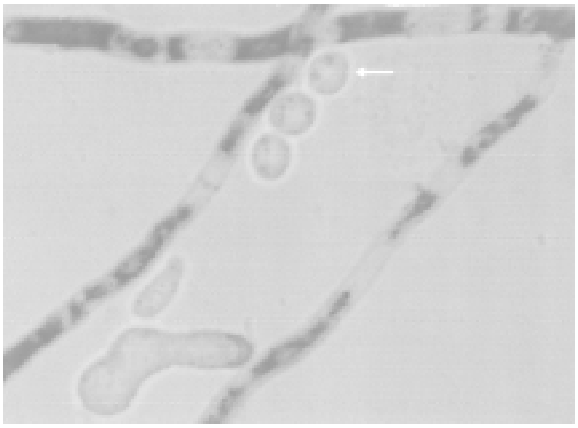

Figure 1. Protoplasts of *F. pallidoroseum* being released (arrow indicates protoplasts).

The experiment to find out optimum concentration of enzyme required for maximum release of protoplast showed that maximum release occurred when 80 mg of lytic enzyme in 4 ml of 0.01 M citrate was used. As the concentration of mycelium increased, the ability to release the protoplast decreased. With 90 mg of enzyme, the protoplasts were found to burst immediately after release. Overall, the number of protoplasts released increased with increasing concentrations of the lytic enzyme, but very high concentrations were harmful, resulting in the lysis of protoplast soon after their appearance, signifying toxic levels. The optimal duration of lytic enzyme treatment may, however, differ among fungal species and also within the strain.

The protoplast of *F. pallidoroseum* incubated at room temperature showed rapid growth, and individual colonies were observed after 24 h. The frequency of regeneration of protoplast to mycelium colonies differed when transferred to PDA medium with KCl as the osmotic stabilizer. According to Santos and De Melo (1991), osmotic stabilizers like KCl in protoplast regeneration medium induce high regeneration (70–75%). The protoplast culture of *F. pallidoroseum* was found to be more pathogenic and took less time for symptom development in water hyacinth. The present protocol for isolation and regeneration of protoplast from the mycelium of *F. pallidoroseum* indicates the possibility of evolving a fungus with enhanced biocontrol activity through inter- specific or inter-generic protoplast fusion.

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