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Fast Isolation and Regeneration Method for Protoplast Production in *Trichoderma harzianum*

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A B S T R A C T

Trichoderma is one of the most important filamentous fungi used as a biocontrol agent. Because of the absence of sexual reproduction in this fungus, other methods of genetic improvement have been developed such as protoplast fusion to enhance its biocontrol potential. In order to develop an unique effective strain as a single source of vital enzyme, it was intended to integrate two biocontrol species for high chitinase production by fusing their protoplasts. Protoplasts were isolated from *Trichoderma harzianum* fungus using Sigma Lyticase with 0.6 M KClas osmotic stabilizer. Intra-strain *T. harzianum* protoplast fusion has been carried out using polyethylene glycol with STC (sorbitol, Tris – HCl, $CaCl_2$) buffer. The maximum number of protoplasts ($2.0x10^3$/ml) was obtained from 16 h culture at pH 6,28°C for 3h. Five self-fusant strains (*Th3fu1*, *Th3fu2*, *Th3fu3*, *Th3fu4*, *Th3fu5)* were selected. Most of the fusants exhibited fast and vigorous mycelial growth on 2 % colloidal chitin agar. The fused protoplasts were regenerated on chitin agar selective medium. In dual culture biocontrol experiments, five selected fusants showed growth inhibition against three pathogens namely; *Sclerotinia sclerotiorum*, *Fusarium oxysporum* and *Altenaria brassicicola.* However, Fus 5 indicated the best ability of inhibition the growth of the three pathogens. Molecular characterization of five fusants using RAPD profile indicated the presence of novel fragments which may be due to recombination events .Results indicated a fast method to generate protoplast fusants to be used to enhance biocontrol potential intraspecifically as well as interspecifically.

Introduction

The most exploited and widely used fungal biocontrol agents in agriculture for the management of plant diseases caused by a wide spectrum of fungal pathogens (Sharma *et al.*, 2014). Trichoderma is among the most exploited fungal biocontrol agents in agriculture for the management of plant diseases caused by a wide spectrum of fungal pathogens (Mathivanan *et al.*, 2000). Production of extracellular hydrolytic enzymes is one of the biocontrol mechanisms exerted by Trichoderma towards fungal pathogens. Several biocontrol agents alleviate the growth of

pathogenic fungi by producing extracellular Production of extracellular hydrolytic enzymes is one of the biocontrol mechanisms exerted by *Trichoderma* towards fungal pathogens. There are several biocontrol agents hindering the growth of pathogenic fungi by producing extracellular chitinase, which degrades the chitin polymers of fungal cell wall (Mathivanan *et al.*, 1998).Genetic variation in this fungi occurred by various asexual processes such as mutation and parasexual recombination because of the absence of sexual reproduction. Protoplast fusion can be used as a tool in strain improvement for bringing genetic recombination and developing hybrid strains in Filamentous fungi (Lalithakumari, 2000). Isolation, fusion and regeneration of protoplasts have been carried out in *Trichoderma* mainly for improving the biocontrol potential (Prabavathy *et al.,* 2006). The present study was aimed to isolate the protoplasts from *Trichoderma harzianum* and carry out self-fusion of protoplasts with the objective of enhancing the chitinase production. In addition, the antagonistic potential of the fusant strains was also studied in comparison to parent.

Materials and Methods

Collection of isolate for study

The pure cultures of *T. harzianum* (Th3) (ITCC: 5593) was obtained from The Indian Type Culture Collection available in Division of Plant Pathology, IARI, New Delhi. After serial dilution, the samples were plated on PDA media at room temperature (28±2°C). The morphological parameters were studied using light microscopy.

Isolation, fusion and regeneration of protoplasts

About 1ml of conidial suspension of *T. harzianum* (Th3) was inoculated into 100ml of potato dextrose broth (PDB) and incubated on a rotary shaker at 100 rpm at room temperature. After 16 h, the culture was harvested and mycelia were separated through Whatman filter paper.100mg of mycelia was washed with sterile water and incubated in 0.1M phosphate buffer, pH 6.0 and incubated with lysing enzymes (Sigma Chemicals Co., India) at 8mg/ml concentration prepared in phosphate buffer containing 0.6M KCl as osmotic stabilizer.

The enzyme-mycelial mixture was incubated at room temperature with mild shaking and the release of protoplast was monitored regularly under light microscope. After 2 h, the protoplasts preparation was filtered through sterile cotton wad and centrifuged at 100 rpm for 15min. The supernatant was discarded and the sedimented protoplasts were suspended immediately in buffered-osmotic stabilizer solution. Self-fusion of protoplasts of *T. harzianum* was done by the method of Stasz et al. (1988). Polyethylene glycol (PEG) (MW 3500, Sigma Chemicals Co., India) prepared in STC buffer (0.6M Sorbitol; 10mM Tris–HCl; 10mM CaCl2, pH 6.5) was used as fusogen. One ml of protoplast suspension was mixed with equal volume of 80% PEG solution and the fusion mixture was incubated at room temperature. After 10min, the mixture was diluted with 1ml of STC buffer.

The PEG in the fusion mixture was washed away, using STC buffer and the fused protoplasts were collected by centrifugation at 100 rpm for 15min, suspended in STC buffer and plated on 2% colloidal chitin agar (CCA) selective medium containing (g/l) colloidal chitin, 5.0; sucrose, 1.0; NaNO3, 2.0; K2HPO4, 1.0; KCl, 0.5; MgSO4, 0.5; FeSO4, 0.01; agar 15; distilled water 1000ml at pH 6.5. The plates were incubated at room temperature and the protoplast regeneration and development of colonies were observed.

Growth of parent and fusants on CCA media

Eight regenerated self-fusants of *T. harzianum* were selected based on their growth and microscopic examination. Mycelial disc of each self-fusant was inoculated on 2 % CCA and incubated at room temperature. The mycelial growth and morphology were observed after 3–5 days.

Antagonistic activity of parent and self-fusants of Trichoderma harzianum against phytopathogens

The antagonistic activity of *T. harzianum* against *Fusarium, Rhizoctonia solani, Pythium and S.sclerotiorum, Rhizoctonia solani* were determined by dual culture technique. Mycelial discs were cut out from actively growing selffusant and parent cultures of *T. harzianum* and pathogens and placed at the opposite poles on PDA. The plates were incubated at room temperature and the mycelial growth of *T. harzianum* strains (antagonists) and *Fusarium, Rhizoctonia solani, Alternaria brassicicola and S.sclerotiorum* were measured after four days and percent inhibition ofmycelial growth of the pathogen was calculated.

Molecular characterization of fusants

DNA was extracted from fusants and parents PCR-RAPD reactions were carried out using different ng 10-mer oligonucleotide primers (Operon Technologies, USA) to identify polymorphic amplicons. PCR was performed in 15 µl volumes containing 1 µl DNA (50 ng), 1.5 µl primer (10 pmol), 1.5 µl of 10x PCR buffer, 1 µl of 25mM $MgCl_2$, 0.5 µl Taq DNA polymerase (0.5U), 1.5 µl dNTP mix (2mM), 8 µl double distilled water. Amplification were performed on a Bioneer-Mygenie 32 thermocycler programmed for 5 min at 95^0C (denaturation), 40 cycles each of 1 min at 94^0C, 1 min at 32^0C (annealing), 1 min at 72^0C and final extension of 2min at 72^0C. The amplified products were loaded onto 1.5% agarose gels (Agarose T2 Low EEO, Biomatrix) containing 0.2mg l^{-1} Ethidium bromide (Sigma, USA), and electrophoresed in 1x TAE buffer. All the PCRs were repeated thrice to check the reproducibility of the amplicons (Fig.4to Fig.9).

Results and Discussion

Swelling and rounding up of cell content were observed at initial stage and the lysis of mycelium started after 60 min (Fig.1). Complete lysis of mycelium and release of protoplasts were observed after 1h.The protoplasts released initially were smaller in size but later they enlarged to spherical structures. Interestingly, the protoplasts yield was significantly affected by the concentrations of lysing enzymes. At low concentrations, the lysis of fungal mycelium was confined only to a small portion whereas at high enzyme concentrations, the mycelium lysed effectively yielding large numbers of protoplasts, but they bursted immediately after release and disintegrated. Among different concentrations of lysing enzymes tested, 8mg/ml with 0.6M KCl as osmotic stabilizer was optimal for the release of protoplasts from different *Trichoderma* spp. However, Pe'er and Chet (1990) obtained highest protoplasts from *T. harzianum* using Novozyme 234 at 10mg/ml with 0.6M KCl and Tschen and Li (1994) used 15mg/ml of Novozyme 234 with 0.6M sucrose to isolate maximum number of protoplasts from *T. harzianum* and *Trichoderma koningii*. Balasubramanian et al. (2003) obtained maximum protoplasts from *Trichoderma roseum* using Novozyme 234.in combination with chitinase and cellulase, each at 5mg/ml.

Table.1 Radial growth of pathogens; *R. solani, F. oxysporum, A. brassicicola* and *S.sclerotiorum*

Parents and Fusants	% Radial growth			
	R.solani	***F.oxysporum***	***A.brassicicola***	***S.sclerotiorum***
Fusant1	**50**	**25**	**28**	**10**
Fusant2	**53**	**30**	**28**	**12**
Fusant3	**52**	**28**	**29**	**15**
Fusant4	**43**	**30**	**28**	**20**
Fusant5	**70**	**50**	**50**	**40**
Parent 1	**90**	**85**	**80**	**80**

*Values are mean of two replicates.

Fig.1 Digestion of mycelium and release of protoplasts in *T. harzianum*. (A) *T. harzianum* mycelium with lysing enzymes showing swelling and rounding up of cell content (B) Partially digested mycelium with prominent release of protoplasts occurring after 2 h of incubation.

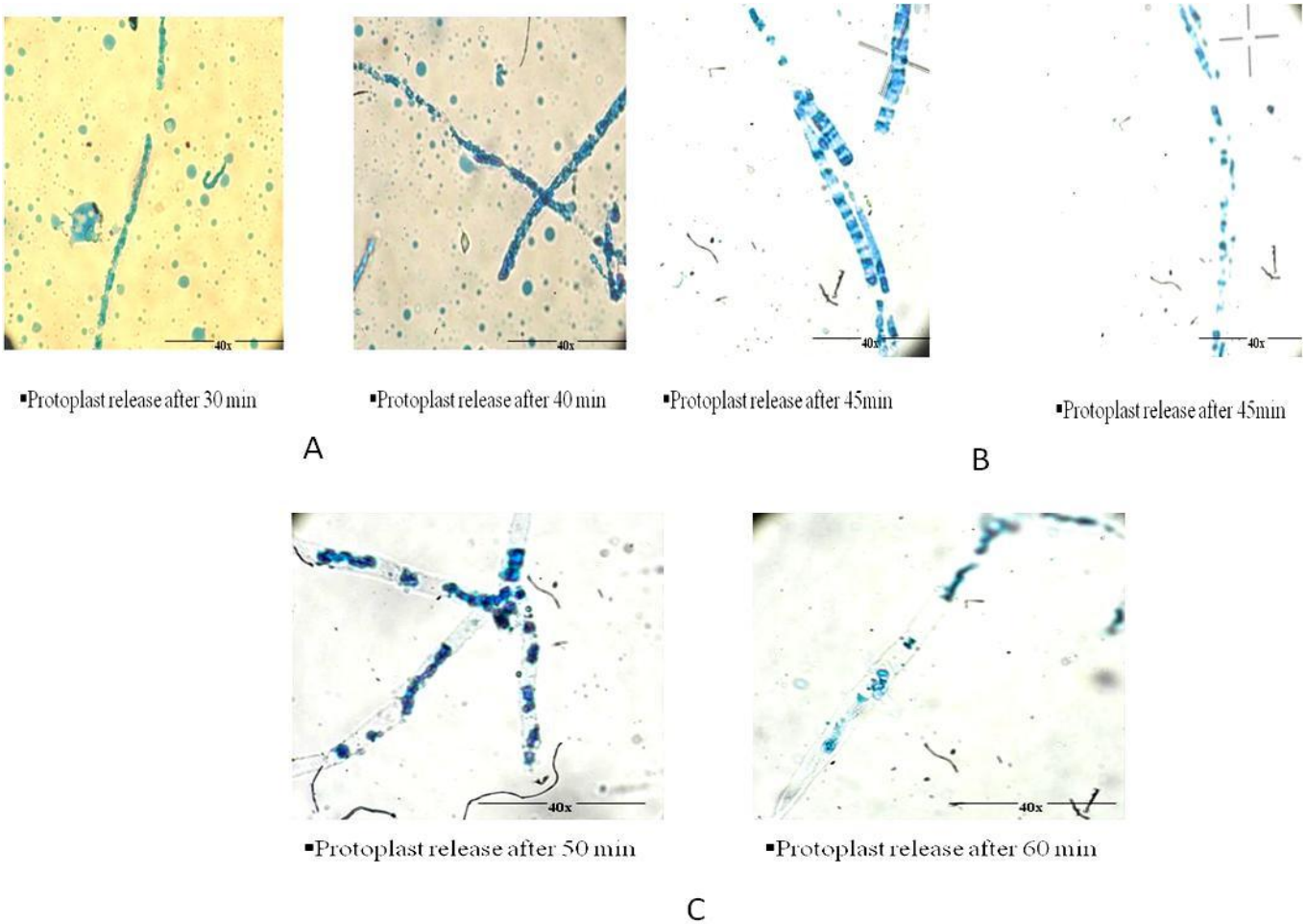


Fig.2 Regeneration of fusion protoplasts of *T. harzianum*

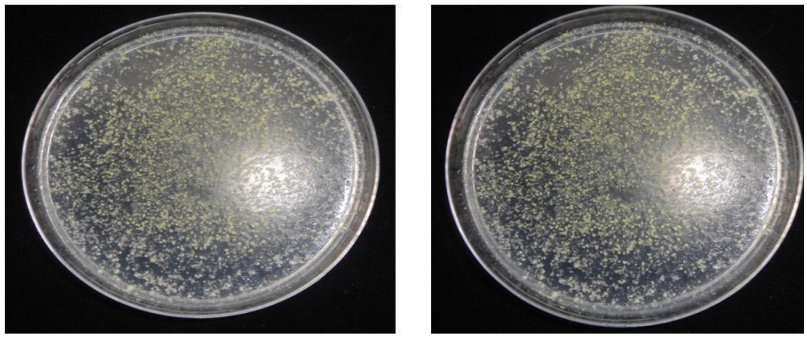


Fig.3 Regenerating protoplast of *T. harzianum* on 2%CCA medium.

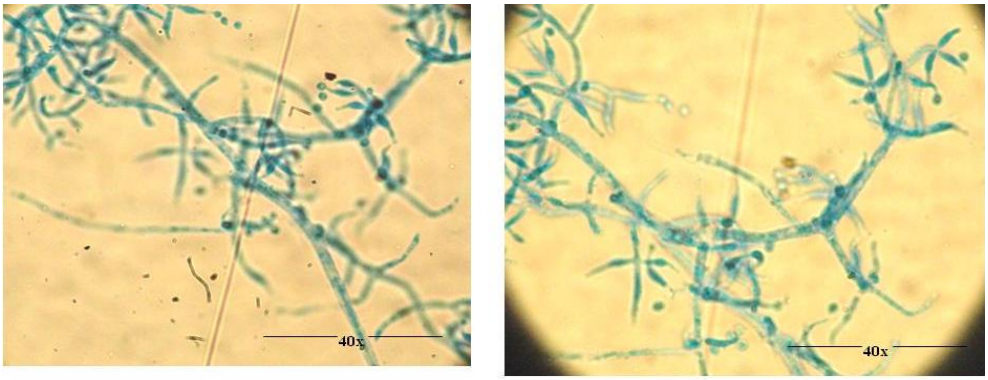


Fig.5

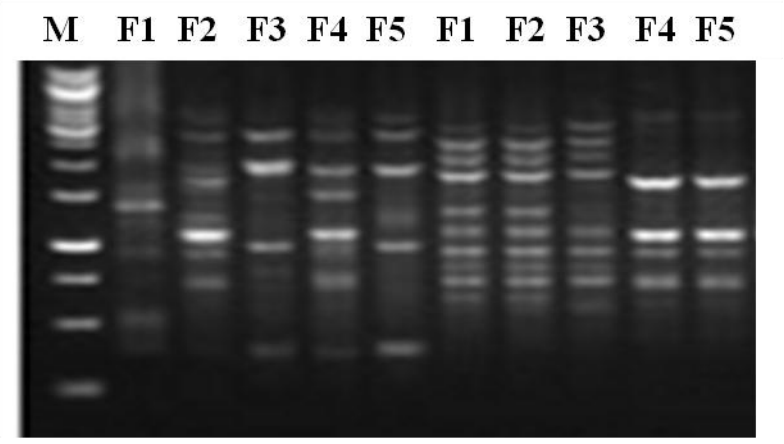
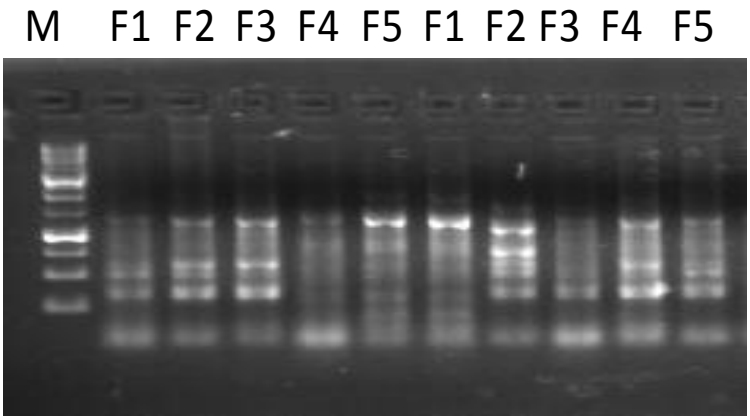


Fig.5 Amplicon generated from OPA-10 and OPA-14 combination for protoplast fusants

Fig.6 Amplicon generated throgh OPA-8 and OPA-11.



When PEG solution was added to the protoplasts, they were attracted to each other and pairs of protoplasts were observed.Self-fusion of protoplasts in *T. harzianum* has been achieved in the present study using 60% PEG which is in accordance with the concentration used by (Hashiba and Yamada, 1984; Mrinalini, 1997). At higher concentrations, PEG caused shrinking and bursting of protoplasts (Lalithakumari, 2000; Lalithakumari and Mathivanan, 2003). The fused protoplasts were plated on high concentration (2%) of CCA for further selection. They started regenerating after two days (Fig. 2) and developed mycelium after three days (Fig.3). Prominent colonies were observed after four days on 2% CCA selective medium. Improvement of antagonistic activity in self-fusant strains was observed against *R. solani* as compared to the parent strain. Inhibition (70%) of mycelia growth of *R. solani* was recorded with (Fus5) (Table 1) which reveals the competence of the fusant generated from *T. harzianum.* The RAPD primers OPA10, OPA13, OPA14, OPA9, OPA8 showed polymorphic fragments in the fusants. The molecular weight of fragments ranged from 100 bp to 600 bp. The fusants showed polymorphic fragments as like parent and are evolutionary similar. Comparison of the DNA patterns indicated that many fragments were present in Fus 3, Fus 5, and Fus 4 showed in Fig.4 to 9.

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