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Full Length Research Paper

Characteristics of protoplast inter, intra-fusant and regeneration of antagonistic fungi *Trichoderma harzianum* and *Trichoderma viride*

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A standard method for isolation, fusion and regeneration of protoplasts from *Trichoderma harzianum* and *Trichoderma viride* was developed. The protoplasts from *T. harzianum* and *T. viride* were isolated using Novozym 234 as lytic enzyme and potassium chloride as osmotic stabilizer. The maximum number of protoplasts (9.37 × 10^5/ml) was obtained from 16 h old mycelium of *T. harzianum* and 11.1 × 10^5/ml for *T. viride* from 14 h old mycelium at pH 5.5, 28ºC for 3 h. The interspecific and intraspecific fusion frequency was determined using 40% polyethylene glycol (PEG) as fusogen. The intrafusants were selected based on their growth, sporulation, pigmentation on chitin and cellulose amended media, where as the interfusants were selected on fungicide resistance as a marker. The protoplast fusion frequency was found to be 1.92% for interspecific fusion. In the case of intraspecific fusion it was about 6.2 and 7.2%, respectively, for *T. harzianum* and *T. viride.* The protoplast regeneration frequency of intrafusant was 17% for *T. harzianum* on chitin medium and 19.2% for *T. viride* on cellulose medium after two days. The regeneration frequency of 11% for interfusants on fungicide amended medium was observed after three days. The regenerated fusants morphology, growth, sporulation and pigmentation were compared with parental strains.

Keywords: Fusion, isolation, regeneration, *Trichoderma harzianum, Trichoderm viride.*

INTRODUCTION

Many species of *Trichoderma* are potential bio-control agents against a wide range of soil-borne plant pathogenic fungi (Smith et al., 1990; Harman and Hayes, 1993; Elad, 2000). They have a potential to become industrially useful fungi, considering the production of enzymes like cellulase, chitinase, pectinase, protease and lipase. Though the available strains of *Trichoderma* obtained through selection or mutation, possess one or more desirable traits, however, none of them possess all the attributes to realize the full potential of the beneficial fungus (Ahmad and Baker, 1987; Harman and Hayes, 1993; Papavizas, 1985). Therefore, the protoplast fusion technology has stimulated interest in the manipulation of *Trichoderma* as enzyme producers and bio-control agents against diverse plant pathogens (Chet et al., 1993, Balasubramanian, 2003). Furthermore, biosynthesis of new compounds by recombinant strains obtained by intraspecific protoplast fusion as well as by interspecific protoplast fusion was confirmed (Fujimoto et al., 1990).

Most of inter and intraspecific protoplast fusion enhances the activity of parental strains. Interspecific fusants enhanced antibiotic production in *Streptomyces* (Robinson et al., 1981), extracellular glucose oxidase activity in *Aspergillus niger* (Khattab and Bazaraa, 2005) and intraspecific fusants of *Trichoderma koningii* showed better bio-control activity to *Rhizoctonia solani* (Hanson

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and Howell, 2002). Furthermore, intrafusants of *Trichoderma reesei* had increased carboxymethylcellulase activity and *Trichoderma harzianum* increased chitinase and bio-control activity (Prabavathy et al., 2006). In our laboratory, improved *Trichoderma* stains between *T. harzianum* and *Trichoderma lonchibrachiatum* showed effective control over rice diseases (Mrinalini and Lalithakumari, 1998). Using the protoplast fusion technique, improved strains with enhanced antagonistic potential, enzymes and antibiotic production, useful myco-products could effectively be achieved. Moreover, this technique is an important tool for the genetic manipulation of industrially important fungi. The process of protoplast fusion involves cell wall breakdown, release of viable protoplasts, regeneration of protoplasts etc. Different factors are involved in controlling the overall protoplast fusion, the age of mycelium, different types and concentrations of lytic enzymes and osmotic stabilizers are the important parameters to be considered for successful isolation, fusion and regeneration of protoplasts. The protoplast isolation, regeneration have been reported for different fungi, However, not much work has been focused on inter and intraspecific protoplast fusion in filamentous fungi. Many reports are available in protoplast fusion, but there is no report on protoplast fusion between *T. harzianum* and *T. viride.* Since fungal protoplasts fusion is one of the most important approaches in the strain improvement of filamentous fungi, the present investigation was aimed to optimize the conditions for the isolation of viable protoplasts, inter and intraspecific fusion and its maximum regeneration in *T. harzianum* and *T. viride*. A protocol was developed and standardized for the isolation of viable protoplasts, which can readily regenerate and revert to parental strains.

MATERIALS AND METHODS

Culture maintenance and inoculum preparation

T. harzianum and *T. viride* were used from our laboratory culture collection and these strains were maintained on PDA (Potato 200 g; Dextrose 20 g; Agar 20 g; distilled water 1000 ml, pH 6.5) slants. The inoculum was prepared by adding 5 ml of sterile distilled water to 5 days old slant culture and scrapped with an inoculation needle. The suspension was transferred into 100 ml molten PDA, mixed thoroughly and poured into sterile Petri plates and incubated at room temperature (28°C). Mycelial discs of 9 mm were cut randomly from 3 days old cultures with sterile cork borer and used throughout the investigation.

Enzymes and osmotic stabilizers

The lytic enzyme Novozym 234 was purchased from Sigma Chem. Co (St. Louis, USA) and tested with 2, 4 and 5 mg/ml concentration for both strains. Sucrose, mannitol, potassium chloride, sodium chloride, ammonium sulphate, magnesium sulphate were used as osmotic stabilizers at a fixed concentration 0.6 M and pH 5.5 was used.

Preparation of mycelium

Conidia were collected from 5 days old cultures of *T. harzianum* and *T. viride*. The inoculum of *T. harzianum* and *T. viride* were prepared by adding 5 ml of sterile distilled water to 5 days old culture under aseptic conditions and centrifuged at 5000 *g* for 10 min. The conidial suspension of 1×10^6 conidia/ml was transferred aseptically into 100 ml potato dextrose broth. The flasks were incubated for 12-24 h on a rotary shaker at 150 rpm. The young germlings was harvested by filtration using sterilized cheese cloth and aseptically washed once with sterile distilled water followed by two washes with sterile osmotic stabilizer.

Isolation and purification of protoplasts

Novozym 234 at different concentrations was used in the presence of 0.6 M KCl as osmotic stabilizer to release viable protoplasts. Flasks containing mycelium and enzyme solution were kept on an orbital shaker at 100 rpm for 3 h. At every 30 min intervals, the release of protoplasts was observed under phase-contrast microscope (Carl Leitz photomicroscope). After 3 h of incubation, culture was filtered through six layers of cheese cloth to remove mycelial fragments. The resultant filtrates containing protoplasts were washed with osmotic stabilizer to remove the enzyme remnants by centrifugation at 500 *g* for 5 min. The sediment protoplasts were re-suspended in known amount of osmotica, their purity was checked under microscope and the number of protoplasts was counted using haemocytometer (Fein-ofpik Blankenbarc, Germany).

Interspecific protoplast fusion

Protoplasts were fused according to the method of Stasz et al. (1988) with modifications. 1 ml of the suspension containing 10^6 protoplasts in STC buffer (0.6 M sorbitol; 10 mM $CaCl_2$; 10 mM Tris-HCl at pH 7.5) was prepared and equal number of protoplasts from *T. harzianum* and *T. viride* strains was mixed. To this 200 µl of 40% (w/v) polyethylene glycol (PEG, MW 3350; Sigma Chemicals Co., St. Louis, USA), 10 mM $CaCl_2$ and 10 mM Tris-HCl, (pH 7.5) was added and gently mixed by rolling the tube. To the fusion mixture again an aliquot of 500 µl PEG solution was added and mixed gently. This step was repeated twice and the mixture was incubated at 28°C for 10 min with 1.1 ml of STC buffer by mixing gently. These dilution steps were repeated two times and 2.2 ml of STC was added. After the fusion and dilution, protoplasts were recovered by centrifugation at 100 *g* for 1 min and suspended in 5 ml STC. The interfused protoplasts were serially diluted in STC and plated on selective (10 µM carbendazim, 75 mM copper sulphate and 0.6 M KCl supplemented in PDA) and non-selective media (PDA with 0.6 M KCl).

Intraspecific protoplast fusion

Similar to interspecific fusion methods, intraspecific protoplast fusion in *T. harzianum* and *T. viride* was also carried out using PEG solution. Intraspecific fusants fuse within the species; it was served as a control for interspecific protoplast fusion. The fused protoplasts were serially diluted with STC buffer. It was then plated on selective medium for *T. harzianum* on 2% chitin with 0.6 M KCl and for *T. viride* on 2% cellulose with 0.6 M KCl and non selective medium (PDA with 0.6 M KCl).

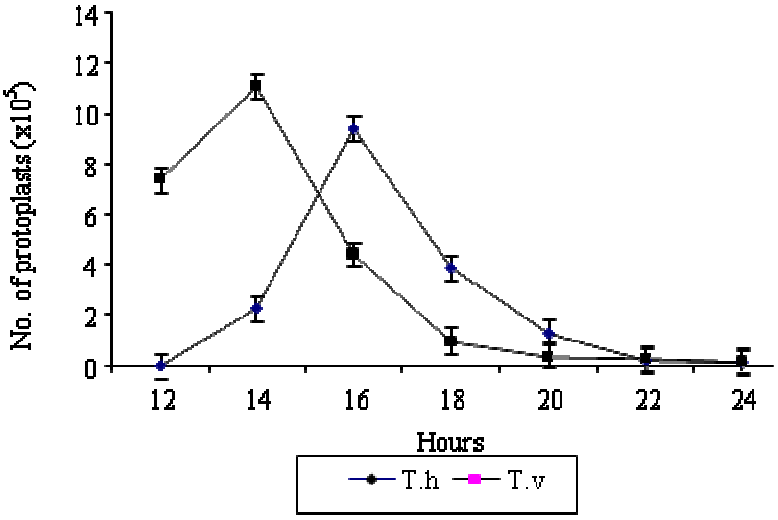

Figure 1. Effect of age of the mycelium on release of protoplasts from *T. harzianum* and *T. viride*.

Regeneration of inter and intrafusants on selective medium at different days

The interfused protoplasts were suspended in 100 µl of STC buffer and plated on selective medium (10 µM carbendazim and 75 mM copper sulphate with 0.6 M KCl supplemented in PDA at pH 6.5). The intraspecific fused protoplasts isolated from *T. harzianum*, *T. viride* were plated on the media containing chitin with 0.6 M KCl at pH 6.5, cellulose with 0.6 M KCl at pH 6.5 and the non-selective media PDA with 0.6 M KCl at pH 6.5. All the plates were incubated at 28°C. The selected colonies were designated using numbers based on growth ability. The regenerated colonies were photographed using a phase-contrast condenser in a Carl Leitz photomicroscope.

Statistical analysis

All the data were subjected to statistical analysis by one way ANOVA procedure of SPSS 10.0 Software. The values $P < 0.05$ was considered as significant and $P < 0.01$ was considered as highly significant.

RESULTS

Age of the culture

Culture age which is suitable for the isolation of maximum protoplasts was standardized by growing the test organisms, *T. harzianum* and *T. viride* for 24 h. With *T. harzianum,* the number of protoplasts increased in 14 h old culture and reached a high count at 16 h, indicating the optimum age of the culture. After 16 h, it decreased drastically and only a few numbers of protoplasts were released at around the 20th h. The optimum age of the culture suitable for the isolation of protoplasts was 16 h for *T. harzianum* and 14 h for *T. viride*. The yield of protoplasts declined with increasing age of the cultures (Figures 1 and 5 a-d).

Enzyme concentration

Protoplasts were released from the fungal mycelium by enzymatic digestion of the cell wall or parts of it. The lytic enzyme (Novozym 234) in osmotic stabilizer (0.6 M) tested at 5 mg/ml was the best concentration for the release of increased number of protoplasts from *T. harzianum* and *T. viride* (Figure 2).

Osmotic stabilizers

Osmotic stabilizers are one of the important parameters for high yield of protoplasts. In order to identify the best osmotic stabilizers, we used various inorganic and organic stabilizers at 0.6 M concentration. Among them, 0.6 M KCl was the best osmotic stabilizer for maximum isolation of protoplasts in both the *Trichoderma* species. This was followed by sodium chloride (Figure 3).

Effect of pH, temperature and incubation period

Different pH (3.5, 4.5, 5.5 and 6.5) of the osmotic stabilizers were tested for *T. harzianum* and *T. viride*. The osmotic stabilizers at pH 5.5 gave maximum release of protoplasts.

Temperature is one of the key factors for the isolation of protoplasts. Among the various temperatures tested for the effective protoplast release, 28ºC was found to be the

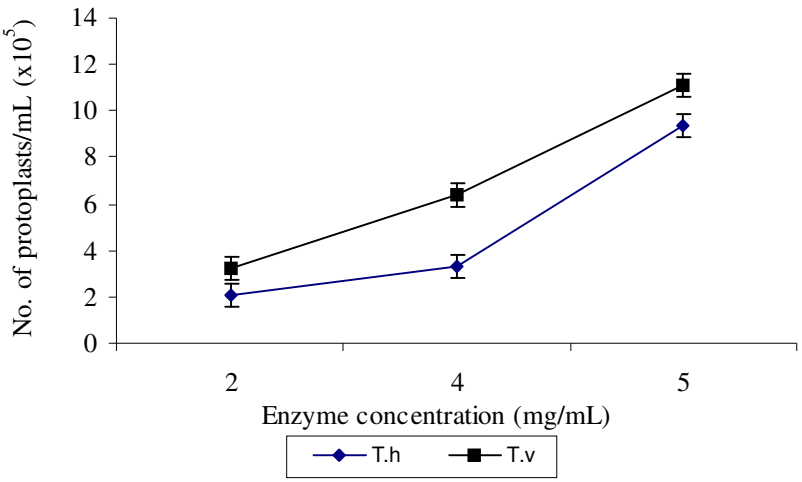

Figure 2. Effect of enzyme concentrations on the release of protoplasts from *T. harzianum* and *T. viride*.

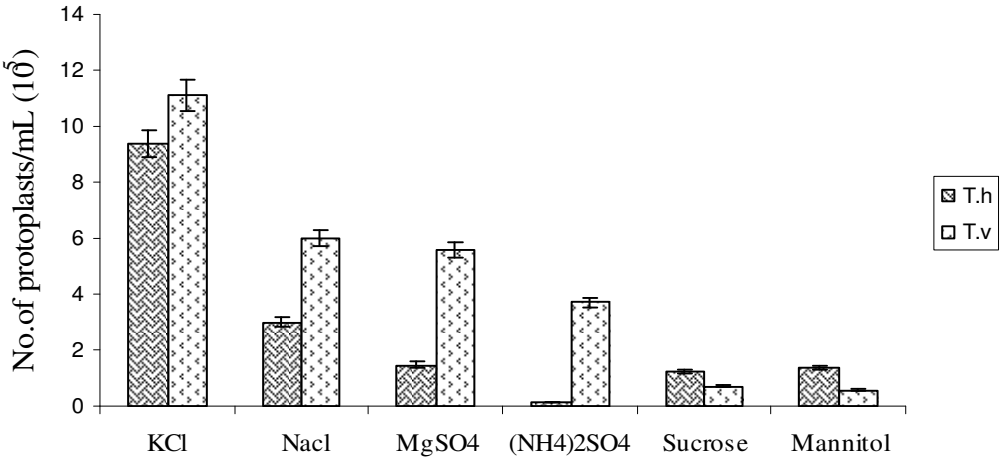

Figure 3. Effect of osmotic stabilizers on the release of protoplasts from *T. harzianum* and *T. viride*.

suitable for the isolation of maximum protoplasts. Any increase or decrease in temperature reduced the release of protoplasts.

The maximum numbers of protoplasts were released after 3 h of incubation at 100 rpm from the mycelium of both *T. harzianum* and *T. viride*. Protoplasts released in the early hours (until 60 min) of incubation were smaller in size and moderately uniform and large size protoplasts after 120 min incubation (Data not shown).

Effect of PEG concentration and temperature on the interspecific fusion

Lower concentrations of PEG (< 30%) did not stabilize the protoplasts and the protoplasts got busted, whereas 40% PEG was observed to be an optimal concentration, further increase of PEG concentration resulted in shrinkage of the protoplasts. Temperature plays a distinct role in protoplast fusion where 28°C was found to be the optimum with a maximum fusion frequency. We clearly observed that higher temperatures of 35 and 42°C resulted in poor fusion frequency in both the fungi (Table 1, Figure 5 g).

Effect of pH and exposure time with PEG on interspecific fusion

Different pH values were tested to study interspecific fusion frequency. At pH 7.5 with 40% PEG, a maximum fusion frequency of 1.92% was observed between *T. har-*

Table 1. Influence of PEG concentration and temperature on interspecific protoplast fusion.

PEG (%)	Fusion frequency (%)	Temperature	Fusion frequency (%)
30	1.26±0.04	20°C	0.51±1.00
40	1.92±0.01	28°C	1.92±0.08
50	0.91±0.70	35°C	0.90±0.6
60	0.68±1.00	42°C	0.42±0.7

Values are mean ± standard error of five determinations.
*P<0.05; **P<0.01.

Table 2. Influence of pH and exposure time on interspecific protoplast fusion.

pH	Fusion frequency (%)	Exposure time with PEG (min)	Fusion frequency (%)
6.0	0.45±0.01	5	0.91±0.09
6.5	0.91±0.04	10	1.72±0.05
7.0	1.14±0.07	15	1.37±1.00
7.5	1.92±1.00	20	0.91±0.01
8.0	1.26±0.06	25	0.57±0.72

Values are mean ± standard error of five determinations.
*P<0.05; **P<0.01.

zianum and *T. viride.* PEG treatment for 10 min favoured maximum fusion frequency while, increasing time of PEG exposure caused loss of viability of protoplasts due to dehydration associated with rupture of them (Table 2, Figure 5 g).

Effect of PEG concentration on intraspecific fusion

Based on the interspecific protoplast fusion results, the intraspecific protoplast fusion was carried out using 40% PEG with pH 7.5, at 28°C, and 10 min incubation. The fusion frequency for *T. harzianum* and *T. viride* was 6.2 and 7.2%, respectively. In the present study, we used intraspecific fusants to compare and characterize with the interfusant and parental strains (Table 3, Figures 5 e, f).

Regeneration in inter and intraspecific fusants

Interspecific fused protoplasts regenerated after 3 days of incubation at 28°C on selective medium and the recorded regeneration was 11%. The resistance fusant colonies were grown on fungicide amended medium. Once the regeneration started, the development of mycelium was observed after 4 days on fungicides amended medium. The non fused protoplasts did not regenerate even after 5 days on selective medium (Figures 4 and 5 h).

Intraspecific fused protoplasts regenerated after 2 days of incubation at 28°C on selective medium and were found to be 17 and 19.2% for *T. harzianum* and *T. viride,* respectively. *T. harzianum* colonies were regenerated on chitin medium and in *T. viride* on cellulose medium. Here, the mycelia were developed after 3 days at 28°C. The non fusant colonies did not regenerate even after 4 days of incubation. However, they slowly started regenerating after 5 days and their growth was very slow (Figures 4 and 5i). Both fusant colonies were selected based on the fast growth, sporulation and pigmentation, designated as number, transferred to fungicides with PDA medium for interfusants, 2% chitin and 2% cellulose for intrafusants of *T. harzianum* and *T. viride*, respectively.

Table 3. Effect of PEG concentration on intraspecific fusion.

PEG concentration (%)	Fusion frequency (%)	
	T. harzianum	*T. viride*
30	4.8±0.23	5.1±0.32
40	6.2±0.50	7.2±0.10
50	5.0±1.02	6.1±0.44
60	4.2±0.36	4.8±0.62

Values are mean ± standard error of five determinations.
*P<0.05; **P<0.01.

DISCUSSION

Different biochemical and biophysical parameters were studied for protoplast isolation, fusion and regeneration. Since protoplasts are isolated for the strain improvement purpose, care should be taken to isolate maximum number of viable protoplasts. In the present study, 9.37 ×

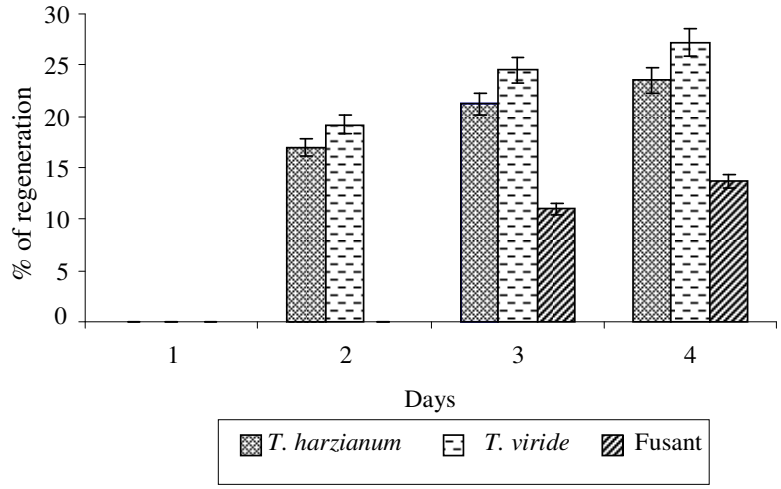

Figure 4. Regeneration frequency (%) of inter, intra fusion on selective medium at different days.

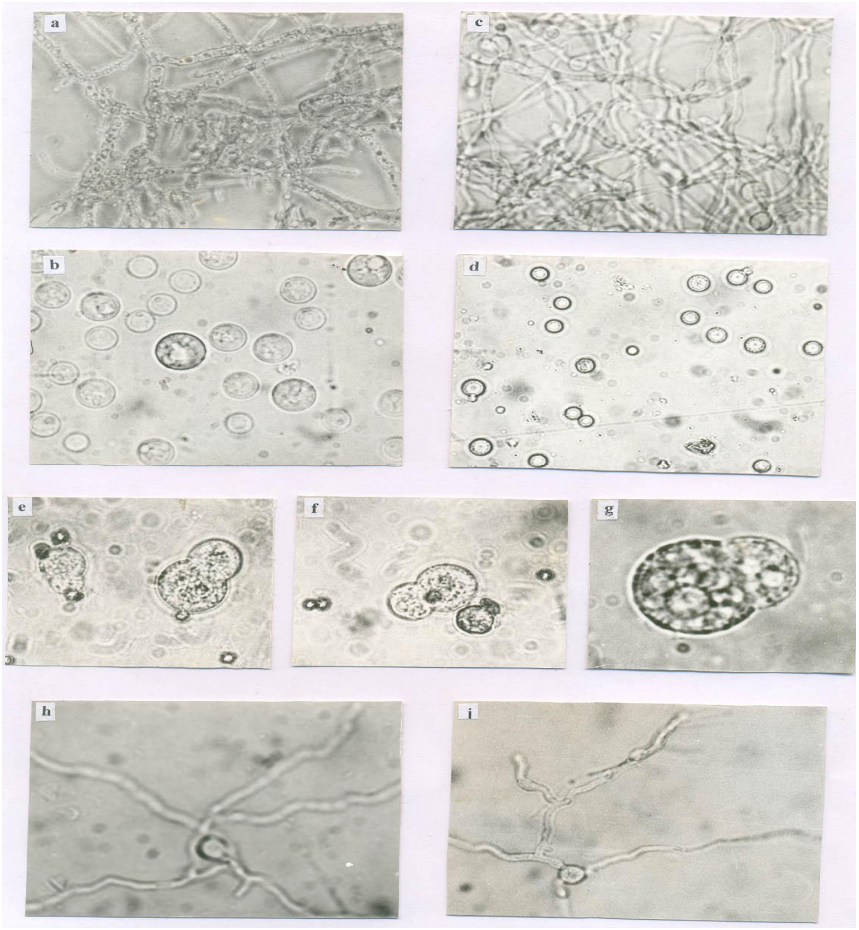

Figure 5. Protoplast isolation, fusion and regeneration in *T. harzianum* and *T. viride.* **a.** Partial lysis of *T. harzianum* mycelium. **b.** Purified protoplasts *T. harzianum*. **c.** Partial lysis of *T. viride* mycelium. **d.** Purified protoplasts in *T. viride*. **e.** Intra fusion of *T. harzianum.* **f.** Intrafusion of *T. viride.* **g.** Interfusion (T.h x T.v). **h.** Regeneration of interfused protoplast. **i.** Regeneration of intrafused protoplasts (Mag nification is × 400).

10^5/ml protoplasts for *T. harzianum* and 11.1 × 10^5/ml for *T. viride* were released from 16 and 14 h old cultures, respectively. However, release of protoplasts from different culture age was reported and it was 20 h in *T. reesei* (Kolar et al., 1985) and 15 - 18 h in *T. viride* (Tomova et al., 1993). On the other hand, in *Trichothecium roseum* (Balasubramanian et al., 2003), *T. harzianum* and *T. longibrachiatum*, 24 h old culture was ideal for maximum release of protoplasts (Mrinalini and Lalithakumari, 1998).

We observed a higher number of protoplasts from *T. harzianum* (9.37 × 10^5) and *T. viride* (11.1 × 10^5) formation treatments with Novozym 234 at 5 mg/ml. However, 2 mg/ml Novozym 234 was optimum for *T. longibrachiatum* (Vijaya Palani and Lalithakumari, 1999), 15 mg/ml for *T. koningii* and *T. harzianum* (Tschen and Li, 1994), 8 mg/ml optimum for *Trichoderma* sp. (Prabavathy et al., 2006), and 10 mg/ml yielded 20 × 10^6/ml protoplasts in *Lentinus lepideus* (Kim et al., 2000). These results suggest that the cell wall lysis depends on the concentration and combination of lytic enzymes.

The osmotic stabilizers play a crucial role in protecting the nascent protoplasts in different environments. They support the protoplasts from being lysed. Organic stabilizers have been reported to be effective in *R. solani* (Hashiba and Yamada, 1982), *Aspergillus oryzae* (Yabuki et al., 1984) and *Venturia inaequalis* (Revathi and Lalithakumari, 1993). However, inorganic stabilizer are also used including 0.6 M KCl for *T. harzianum* and *T. longibrachiatum* (Mrinalini and Lalithakumari, 1998), and 0.4 M $MgSO_4$ for *A. niger* (Das et al., 1989). In the present investigation, six osmotic stabilizers were tested. Of these, potassium chloride (0.6 M) was found to be the best for isolation of maximum number of protoplasts in *T. harzianum* and *T. viride.* Our findings suggested that KCl followed by NaCl was suitable to isolate more protoplasts, whereas ammonium sulphate was not suitable for the fungal, since the cell wall lysed only after 2 h incubation. In *T. viride*, organic stabilizers like sucrose and mannitol were not suitable since the cell wall started lysis after the end of 3 h and minimum number of protoplasts were released.

The pH of osmotic stabilizers and temperature greatly influence the release of protoplasts from the fungal mycelium. We observed that the pH 5.5 and the temperature of 28ºC were highly favourable for maximum release of protoplast in *T. harzianum* and *T. viride*. However, Tschen and Li (1994) reported that pH 5.6 at 31ºC were optimum for an effective release of protoplasts in *T. harzianum* and *T. koningii.* The optimal pH 5.5 at 31ºC is essential for *Trichoderma* (Mrinalini and Lalithakumari, 1998).

In the present study, it was recorded that 3 h incubation was optimum for maximum release of protoplasts from *T. harzianum* and *T. viride* but the numbers decreased after 3 h incubation due to busting and prolonged incubation caused the protoplasts to lyse (autolysis). However, this effect differs among the species. The protoplasts from *T. longibrachiatum* were released after 1.5 h incubation, 2.3 h for *T. harzianum* (Mrinalini and Lalithakumari, 1998), 1.5 - 2 h for *T. harzianum* (Sivan et al., 1990) and for *T. roseum* maximum number of protoplasts were released at 4 h (Balasubramanian et al., 2003).

Before selecting interspecific fusant both the parental strains need to be specifically identified for their individual resistance level to fungicides and heavy metals (Mrinalini and Lalithakumari, 1998). Resistance to different fungicides in *Trichderma* sp. have already been reported (Elad et al., 1981; Papavizas, 1985; Balasubramanian, 2003). As it is very important to look for a better strategy of selection of heterokaryon more emphasis was given to screeing the parental strains for fungicide and heavy metal resistance. We used 10 µM carbendazim, 75 mM copper sulphate as a marker to select interfusants. The true fusants only could grow on fungicides supplemented medium. Similar results were reported by Mrinalini and Lalithakumari (1998) and Hatvani et al. (2006). To select intrafusants, *T. harzianum* grow fast on chitin, similarly *T. viride* grow on cellulose medium. We used chitin and cellulose medium to select intrafusants with growth rate, sporulation and pigmentation as selection marker. Similar findings were published by Mrinalini and Lalithakumari (1998) and Prabhavathy et al. (2006).

PEG solution with Ca^{2+} is a good osmotic stabilizer for protoplast fusions (Ferenczy and Pesti, 1982). The protoplast fusion between *T. harzianum* and *T. lonchibrachiatum* was brought about by 10 mM $CaCl_2$ with PEG concentration 40 - 60% (Mrinalini and Lalithakumari, 1998); 60% PEG was more suitable for obtaining higher fusion frequency in *T. harzianum* (Stasz et al., 1988; Peer and Chet, 1990). In the present study, we observed that 40% PEG with 10 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5) seems to be optimum osmotic stabilizer (maximum fusion frequency = 1.92%) in interspecific fusion. Intraspecific fusion frequency was 6.2 and 7.2% for *T. harzianum* and *T. viride*, respectively. Similarly, Prabavathy et al. (2006) reported in *T. reesei, T. harzianum* 40% PEG with STC buffer as suitable for intrafusion. When the PEG solution was added to the protoplasts, they were attracted and joined together as pairs and plasma membrane disintegrated into fused protoplasmic contents, and finally the fused protoplasts became single and large sized.

Incubation period is important for the fusion frequency. Short period of PEG treatment is necessary to induce protoplast fusion, and exposure of protoplasts to PEG even up to 60 min did not increase fusion frequency. Hence, our results showed that 10 min exposure time at 28°C was optimum for maximum inter- and intrafusion frequency in *T. harzianum* and *T. viride* and further prolonged incubation exhibited shrinkage of fused protoplasts. However, 40 min at 25°C was optimum for *Saccharomyces cerevisiae* (Yulan et al., 2004), while 20

min incubation at 30°C was optimum for yeast *Candida* sp (Fuji et al., 1988).

The application of protoplasts for molecular studies and fusion experiments depends on the successful reversion and regeneration of protoplasts to normal mycelia (Balasubramanian et al., 2003). When fused protoplasts were inoculated in osmotically stabilized media, a part of population underwent cell wall regeneration, reverting to normal mycelium. When the protoplasts fusants were cultured on solid and liquid growth media for regeneration, the solid medium exhibited greater efficiency than the liquid medium, possibly owing to better aeration and some protoplasts loose their ability to regenerate in the anaerobic conditions. However, the regeneration failure is also attributed to anucleated protoplasts (Mrinalini and Lalithakumari, 1998; Tschen and Li, 1994). In the present investigation, we showed that the optimum pH for the release of protoplasts was 5.5 and regeneration of fused protoplasts was pH 6.5. The viability of protoplasts was checked based on the regeneration and reversion of the protoplasts on osmotically stabilized agar medium. Reversion frequencies for *T. harzianum* and *T. viride* were 17 and 19.2%, respectively, after two days. In interfusants, 11% was recorded after three days of incubation. Furthermore, we also observed that the protoplasts of *Trichoderma* regeneration produced normal germ tube like structure and did not form any bud like structure as described in few other fungi (Peberdy and Gibson, 1971). In addition, it was noted that inter fused protoplasts regenerates after three days on fungicides amended medium, whereas intra fusant protoplasts of *T. harzianum* and *T. viride* regenerated after two days of incubation on chitin and cellulose with 0.6 M KCl, medium, respectively; the colonies developed into mycelium after three days.

We tested fusants stability using carbendazim and copper sulphate for inter fusants, whereas 2% chitin and cellulose medium for intra fusants of *T. harzianum* and *T. viride* up to five generations; the fusants exhibited their stability and resistance. Similarly, Hatvani et al. (2006) reported intra specific fusion between carbendazim and tebuconazole-resistant mutants of *T. atroviride* isolated recombinants resistant to both the fungicides. Mrinalini and Lalithakumari (1998) also reported that, penconazole with carbendazim can be used to check the stability of interfusants of *T. harzianum* and *T. lonchibrachiatum*.

In summary, although many researchers have suggested different protocols for the isolation, fusion and regeneration of protoplasts in the filamentous fungi, the present findings with fine tuned and precise information of protoplasts release, inter, intrafusion and regeneration would immensely be useful in the strain improvement. Interspecific fusion of protoplasts is a means of acquiring desirable strain characteristic from other species and fungicide marker was standardized for interfusant selection. The results demonstrated the objective and significance of the protoplast fusion technique, which could successfully be used to develop superior hybrid strains in filamentous fungi that lack sexual reproduction.

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