Journal of Phytology 2009, 1(5): 285–298
ISSN: 2075-6240

Available Online: www.journal-phytology.com

REGULAR ARTICLE

INTER-SPECIFIC PROTOPLAST FUSION IN TRICHODERMA SPP. FOR ENHANCING ENZYME PRODUCTION AND BIOCONTROL ACTIVITY

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SUMMARY

The enzymes, particularly cellulase, have got tremendous industrial application. These enzymes are employed for the conversion of wood and its products, cellulosic agricultural by-products to fermentable substances and in the conversion of lignocellulosic materials into biofuels like ethanol and single cell proteins. In Plant disease management, biological control plays a vital role. The cell-wall degrading enzymes chitinase have been suggested to be essential for the mycoparasitic action of *Trichoderma* species against plant fungal pathogens. In order to develop an unique effective strain as a single source of both these vital enzymes, it was intended to integrate the characterstics of high cellulase producing *Trichoderma reesei* and high chitinase producing *Trichoderma harzianum* by fusing their protoplasts. Protoplasts were isolated from 16 h old cultures of *T. harzianum* and *T. reesei* using Lysing Enzyme. The fusion of protoplasts was achieved by using Polyethylene glycol (PEG) as fusing agent. Among the 20 fast growing fusants, six were selected based on their enhanced growth on selective media and antagonistic activity. All the six selected fusants exhibited morphological variations such as mycelial growth, pigmentation, sporulation and spore coloration among themselves further; they also possessed some common morphological characteristics of the parent strains. The extent of fusion in the fusant strains was confirmed by PCR-RFLP method, which exhibited the presence of DNA from both the fusing parents. The fusant strains were growing 60%-70% faster than the parents, upto 3rd generation. The fusant strains displayed 40-50% increased cellulase production and 10-20% increase in chitinase production than the parent strains, and hence 7%-8% higher antagonistic activity than the parents.

Keywords: Trichoderma, Fusant strains, Chitinase production, Sporulation.

T. R. Srinivasan et al. Inter-Specific Protoplast Fusion in Trichoderma spp. For Enhancing Enzyme Production and Biocontrol Activity. J Phytol 1 (2009) 285-298
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1. Introduction

Trichoderma spp. is genus of asexually reproducing, free-living fungi that are common in soil and root ecosystems. They are one of the most exploited fungal biocontrol agents in the

field of agriculture for the management of crop diseases caused by a wide range of fungal phytopathogens (Elad, 2000; Mathivanan et al., 2000). Seed treatment with *T. harzianum* decreased disease incidence comparable to the imidazole fungicide, prochloraz. (Coskuntuna, 2007). Depending upon the strain, the use of Trichoderma in agriculture can provide numerous advantages: (i) control of pathogenic and competitive/deleterious microflora by using a variety of mechanisms; (ii) improvement of the plant health and (iii) stimulation of root growth (Harman et al., 2004).

Trichoderma, being a saprophyte adapted to thrive in diverse situations, produces a wide array of enzymes. By selecting strains that produce a particular kind of enzyme, and culturing these in suspension, industrial quantities of enzyme can be produced. (Azin et al., 2007). The filamentous fungus *Trichoderma reesei* is the predominant industrial producer of cellulolytic enzymes by secreting an enzyme system capable of degrading crystalline cellulose, which consists of several cellobiohydrolases, endoglucanases and ß-glucosidases. These enzymes are employed for the conversion of wood, cellulosic agricultural by-products to fermentable substances and in the conversion of lignocellulosic materials into biofuels like ethanol and single cell proteins. Production of extracellular hydrolytic enzymes, as chitinase, is one of the biocontrol mechanisms exerted by *Trichoderma* towards fungal pathogens besides antibiosis and competition, which degrades the chitin polymers of fungal cell wall (Mathivanan et al., 1998).

Protoplasts are widely used for DNA transformation (for making genetically modified organisms), since the cell wall would otherwise block the passage of DNA into the cell (Poinssot., 2003). In order to develop an unique effective strain as a single source of both these vital enzymes, it was intended to integrate the characterstics of high cellulase producing *Trichoderma reesei* and high chitinase producing *Trichoderma harzianum* by fusing their protoplasts. Protoplast fusion facilitates the transfer of mitochondrial genomes between taxonomically related but quite distinct species (Lalithakumari, 2000). It can be viewed as one of the recombinant DNA technology that provides the tools for increasing the gene dosage and gene expression from strong promoters, deletion of unwanted genes from the fungal genome, manipulation of metabolic pathways and developing fungal strains for the production of heterologous proteins.

The enzymatic method (lytic enzymes) is almost invariably used now for the isolation of protoplasts, since it gives large quantities of protoplasts, where cells are not broken and osmotic shrinkage is minimum. It indirectly helps strain improvement and development of new strains which are economically viable for industrial use. (Kanth, et al, 2000). Polyethylene glycol (PEG)[28-50% (MW 1,500-6,000) for 15-30 min], are polyethers, that can induce protoplast fusion, in the fungi cells, it induces reproducible high frequency fusion accompanied with low toxicity to most cell types (Scharzorevic et al, 2004).

RFLP may be very important in detecting the genes of the fusant progenies that are to be obtained and to co-relate them to the parents and to display the variation.. The frequency and position of the bands would indicate the distance of genetic relatedness of the fusant progenies from the parents. Also it will be instrumental to determine that whether the fusion is taking place within the same species (intra-specific) or between the two parent strains, that is, intra – specific (Chatterjee; 2005).

2. Materials and Mehtods

Media used

Potato Dextrose Agar (PDA)

Czapek Dox Agar (CDA)

Minimal medium (Toyama et al., 1984) Culture conditions of the fungi and maintenance. Fungal stock cultures were maintained on PDA slants at 28°C Using a sterile cork borer, mycelial discs (9 mm dia) were cut and used for further experiment throughout the investigation.

Isolation of protoplasts

The parent strains of *T. harzianum* and *T. reesei* were grown Erlenmeyer flasks at 1×107 conidia ml-1. The Erlenmeyer flasks were incubated on a rotary shaker with a speed of 100 rpm at room temperature for 16 h. The cultures were harvested and the young mycelia were separated by filtration. About 100 mg fresh mycelium was washed with sterile distilled water followed by 0.1 M phosphate buffer (pH 6.0) and incubated with Lysing Enzymes (Sigma Chemicals Co., USA) at 8 mg ml -1 concentration prepared in phosphate buffer containing 0.6 M KCl as osmotic stabilizer. The mycelia-enzyme mixtures were incubated on a shaker with a speed of 75 rpm at room temperature and the lysis of cell wall and the release of protoplasts were monitored at 30 min intervals under a light microscope. The viability of the isolated protoplasts of *T. harzianum* and *T. viride* was assessed by the method of Wiebe et al., (1997) using fluorescein diacetate reagent prepared in acetone (5 mg/ml).

Fusion of protoplasts

Inter-specific protoplast fusion between *T. harzianum* and *T. viride* was carried out by the method of Prabavathy et al. (2006). Polyethylene glycol (PEG) (MW 3500, Sigma Chemicals Co., USA) prepared in Sorbitol tris-HCl calcium chloride buffer (STC) was used as fusogen. One milliliter of protoplasts suspension (1×106 protoplasts ml^{-1}) was mixed with an equal volume of 80% PEG solution and the fusion mixture was incubated at room temperature. The protoplasts fusion was observed under phase contrast microscope.

Regeneration of fused protoplasts

The fusion mixture was serially diluted with the osmotic stabilizer and plated on regeneration (PDA amended with osmotic stabilizer) and selective media (media amended with fungicide and colloidal chitin) and checked for regeneration. Suitable controls were maintained separately for non-fusion .

Growth of parents and fusants

The growth of both the non-fusion parents and 20 selected fusants was assessed on PDA, by marking the end points of the mycelia in every 24 hours intervals from the time of inoculation, continuously for three days. The growth (in mm) was recorded.

Morphological characterization

The non-fusion parents and fusants were grown on PDA and their colony morphology, pigmentation and sporulation were observed. The growth pattern morphology and sporulation were studied by cover slips technique. Sterilized cover slips were placed at an angle of 45°C into solidified PDA medium in a Petriplate so that half of the cover slip was in the medium. A loopful of spore was spread along the line of the upper surface of the cover slip where it meets the agar and incubated at room temperature. The organisms grew both on the medium and in a line across the upper surface of the cover slip. After 2 days of incubation, the cover slip was removed and examined under microscope. The parent culture of *T. harzianum* has greenish yellow mycelium, green pigmentation and greenish white spores whereas *T. reesei* showed white mycelium with greenish yellow spores and produce dark yellow pigmentation. Among six fusants, three (Th+Tr1, Th+Tr2 and Th+Tr5) have light yellow to off-white and pale

pigmentation as that of *T. reesei* and rest of the three (Th+Tr6, Th+Tr12 and Th+Tr13) produced green pigmentation as that of *T. harzianum*. All the six fusants produced greenish spores similar to that of parents, and possess common morphological characteristics of the parent strains.

Antagonistic activity of *Trichoderma* against *Rhizoctonia solani* in dual culture

The antagonistic potential of all the isolates against plant pathogen *R. solani* was determined by dual culture technique on PDA. The growth inhibition of the pathogen in terms of radial growth with reference to control pathogen plates was measured as:-

$$I = \frac{C-T}{C} \times 100$$

where, I = percent inhibition C = radial growth in control and T = radial growth in dual plate.

Selection of Fusants

A total of 20 isolates of *Trichoderma* strains were screened for growth kinetics and antagonistic activity against *Rhizoctonia solani*. Among them, the best growing 6 fusant strains were selected for further studies as they effectively inhibited *R. solani* in dual culture, designatedas *Th+Tr1, Th+Tr2, Th+Tr5, Th+Tr6, Th+Tr12 and Th+Tr13*, respectively.

Estimation of protein

The protein content of the culture filtrates was estimated according to dye binding method of Bradford (1976).

Qualitative assay of chitinase and cellulase production

Colloidal chitin (0.5%) agar medium and Carboxylmethyl Cellulose Agar medium were prepared separately in Petriplates. The well of 9 mm was cut out from these plates and loaded by the 100 µl of the culture filtrate from the fusants as well as from the parents. After incubation at room temperature for 24 hours, the plates were flooded with 1.0 % solution of Congo red and shaken at 50 rpm for 15 min on a rotary shaker. Then the plates were again flooded with 1 N NaCl. After 15 min, the clear zone around the mycelial growth was observed.

Quantitative Assay for Chitinase Production (Reissig et al., 1955)

A mixture of 1 ml of enzyme solution and 1 ml of suspension of colloidal chitin (0.1 %in 50 mM Sodium acetate buffer, pH 5.2) was incubated at 37°C in a water bath for 2 h with constant shaking. The release of N-acetyl glucosamine in reaction mixture was estimated by the method of Reissig et al. (1955). Controls without enzymes, substrate and with boiled enzyme were maintained. The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate (pH 9.2) to 0.5 ml of reaction mixture and boiled in a water bath for 3 min. After terminating enzyme reaction 3 ml of diluted DMAB (1 ml DMAB + 9 ml glacial acetic acid) reagent was added and incubated at 37°C for 15 min. The released product was read at 585 nm in Spectrophotometer. Specific activity was calculated as -Specific activity = Total activity/mg of protein

Quantitative Assay for Cellulase Production (Gail Lorenz Miller, 1959; modified)

0.45ml of 1% Carboxylmethyl Cellulose solution in sodium citrate buffer was taken at 55oC. This was added to 0.05ml of enzyme extract from each strain. The mixture is then incubated at 55oC for 15 minutes. Immediately after this, 0.5ml of DNS reagent was added. The mixture is kept in boiling water for 5 minutes. While the tubes are still warm, 1.0ml of 40% sodium potassium tatatrate was added. It is then cooled to room temperature and the volume was made up to 5ml by adding water. The activity of cellulase was determined by spectrophotometric reading at 540 nm. Liberated glucose was determined and specific activity of cellulase was expressed as µg of glucose per mg of protein per hour.

DNA Extraction

The DNA was extracted from both the parents, *T. harzianum* and *T. Reesei* as well as from the six seleted fusants by cell lysis method. (Sambrook, 2000).

DNA Amplification

After the DNA has been extracted from both the parents and all the selected six fuast strains, the Polymerase Chain Reaction was accomplished according to a modified Saiki *et al.* protocol, using the purified DNAs as templates, using following conditions:-

Forward Primer TharzF1:
5'TTGCCTCGGCGGGAT
Reverse Primer TharzR1:
5'ATTTTCGAAACGCCTACGAGA
Probe TharzP1:
5'-CTGCCCCGGGTGCGTCG

1× PCR buffer (Invitrogen), 1.5 mM MgCl2, 1 U of *Taq* DNA polymerase (Invitrogen), and Milli-Q water 25 µl. The reaction was carried out in an MJ Research thermocycler programmed for 30 cycles of 94°C 1 min, 55°C 1 min and 72°C 1.5 min, with 4-min initial and final steps.

Restriction Fragment Length Polymorphis Each of the amplified DNA of parents and the six fusant strains were subjected to restriction digestion using EcoR1.The obtained bands agarose gel electrophoresis were analyzed using PCR 100 bp low ladder (DirectLoad)- D3987.

3. Results

Mycelial radial growth of *Trichoderma* spp. on PDA medium

Good mycelial growth in Petri-plates (9 cm) was observed and all the fusant strains showed excellent growth on PDA medium and completed growth on third day itself (Table 1).

Table 1. Mycelial radial growth of *Trichoderma* spp.

Strain	1st day	2st day	3st day
T. harzianum	3.3	7.6	9.0
T. reesei	2.7	6.8	9.0
Th+Tr 1	6.4	9.0	9.0
Th+Tr 2	5.7	9.0	9.0
Th+Tr 3	5.8	9.0	9.0
Th+Tr 4	5.1	9.0	9.0
Th+Tr 5	6.1	9.0	9.0
Th+Tr 6	5.5	9.0	9.0
Th+Tr 7	6.5	9.0	9.0
Th+Tr 8	5.6	9.0	9.0
Th+Tr 9	6.4	9.0	9.0
Th+Tr 10	5.6	9.0	9.0
Th+Tr 11	5.7	9.0	9.0
Th+Tr 12	6.8	9.0	9.0
Th+Tr 13	5.4	9.0	9.0
Th+Tr 14	5.5	9.0	9.0
Th+Tr 15	5.9	9.0	9.0
Th+Tr 16	5.2	9.0	9.0
Th+Tr 17	6.2	9.0	9.0
Th+Tr 18	5.4	9.0	9.0
Th+Tr 19	4.6	9.0	9.0
Th+Tr 20	4.0	9.0	9.0

Table 2. Antagonistic activity of *Trichoderma* spp. against *Rhizoctonia solani*

Strain	Growth (cm)		Percentage inhibition of *R. solani* (%)
	Trichoderma	R. solani	
T. harzianum	5.5	3.5	44.44
T. reesei	4.0	5.0	20.63
Th+Tr 1	8.2	0.8	87.30
Th+Tr 2	6.5	2.5	60.32
Th+Tr 3	8.8	0.2	96.83
Th+Tr 4	7.5	1.5	76.19
Th+Tr 5	7.8	1.2	80.95
Th+Tr 6	8.5	0.5	92.06
Th+Tr 7	6.5	2.5	60.32
Th+Tr 8	6.5	2.5	60.32
Th+Tr 9	7.1	1.9	69.84
Th+Tr 10	7.0	2.0	68.25
Th+Tr 11	6.1	2.9	53.97
Th+Tr 12	8.0	1.0	84.13
Th+Tr 13	6.8	2.2	65.08
Th+Tr 14	7.0	2.0	68.25
Th+Tr 15	6.8	2.2	65.08
Th+Tr 16	7.0	2.0	68.25
Th+Tr 17	8.5	0.5	92.06
Th+Tr 18	8.0	1.0	84.13
Th+Tr 19	6.9	2.1	66.67
Th+Tr 20	5.8	3.2	49.21

Table 3. Viability of protoplast of *T. harzianum* and *T. reesei*

Strain	Protoplast (Nos)		Viability (%)
	Total	Viable	
T. harzianum	15	13	86.66
T. reesei	45	42	93.33

Table 4.Colony morphology, pigmentation and sporulation of fusant and parent strains

Strains	Morphology	Pigmentation	Spore coloration
T. harzianum	Dark green dense mycelium	Light green	Dark Green
T. reesei	Yellowish green compact mycelium	Dark yellow	Light green
Th+Tr 1	Loose mycelium	Fade brown	Light green
Th+Tr 2	Sparse mycelium	Cream white	Light green
Th+Tr 3	Dense whitish green mycelium	Yellow	Whitish green
Th+Tr 4	Thick white mycelium	Yellow	Light green
Th+Tr 5	Green dense mycelium	Pale Yellow	Green
Th+Tr 6	Sparse mycelium	Green	Light green
Th+Tr 7	Thick Whitish mycelium	Greenish yellow	Whitish green
Th+Tr 8	Thick Greenish white mycelium	Yellowish brown	Green
Th+Tr 9	Sparse greenish white mycelium	Off-white	Greenish white

Th+Tr 10	Dense Green mycelium	Dark Brown	Dark green
Th+Tr 11	Sparse Greenish white mycelium	Faded yellow	Green
Th+Tr 12	White & green mycelium	Yellowish brown	Green
Th+Tr 13	Thick Green & white mycelium	Dark greenish white	Greenish white
Th+Tr 14	Thick dense Green mycelium	Brownish green	Whitish green
Th+Tr 15	Sparse Yellowish brown	Off-white	Light green
Th+Tr 16	Very loose dark green mycelium	Yellow	Green
Th+Tr 17	White mycelium	Yellowish white	Light green
Th+Tr 18	Thick light green mycelium	Dark brownish to yellow	White
Th+Tr 19	Thick green mycelium	Light yellow	Light green
Th+Tr 20	Very thick greenish yellow	Yellow	Yellowish green

Isolation of protoplasts

The lysing enzyme treated mycelium of *T. harzianum* started lysing after 90 min and complete lysis of mycelium and the release of protoplasts were observed after 3 h of incubation (Fig.2). In contrast, *T. reesei*, the mycelial lysis appeared only after 100 minutes and the maximum release of protoplasts was observed after 3 h.

Qualitative assay of selected fusant and parent strains on carboxyl methyl cellulase agar (Table 5) and colloidal chitin agar (Table 6)

No.	Strain	Clear zone (cm)
1	*T. harzianum*	1.3
2	*T. reesei*	1.5
3	Th+Tr 1	2.5
4	Th+Tr 2	2.0
5	Th+Tr 5	1.7
6	Th+Tr 6	1.8
7	Th+Tr 12	1.9
8	Th+Tr 13	1.6

Table 5

Regeneration of fused protoplasts

The fused protoplasts started regenerating after 2 days and developed mycelium after 3 days in liquid medium. The colony development was observed in selective medium the regenerated fusants were observed only after 4 days of incubation (Fig.5).

Growth of parents and fusants

Table 6

S. No.	Strain	Clear zone (cm)
1	*T. harzianum*	1.5
2	*T. reesei*	1.2
3	Th+Tr 1	1.7
4	Th+Tr 2	2.0
5	Th+Tr 5	2.3
6	Th+Tr 6	2.1
7	Th+Tr 12	1.8
8	Th+Tr 13	1.9

Fusion of protoplasts

The protoplasts were attracted each other and pairs of protoplasts were seen under phase contrast microscope, which were subsequently fused together. Although aggregation of more than two protoplasts has occurred, fusion was observed between only two protoplasts. The fused protoplasts became larger in size and spherical in shape (Fig.4).

Among six fusants, six (Th+Tr1, Th+Tr2, Th+Tr5 Th+Tr6, Th+Tvr 12 and Th+Tr 13) exhibited fast mycelial growth than the parents on first day while the growth of other fusants was on par with the non-fusion parent of *T. harzianum* and *T. reesei* (Table 1). However, all the fusant cultures completely covered the Petriplates on 2nd day itself, within 45 hours while the parents have taken nearly 70 hours to cover the full plate growth.

Antagonistic activity of *Trichoderma* spp against *R. solani*

Table 7. Quantitative assay of cellulase production and protein content by selected fusant and parent strains

Strain	Protein content (µg/ml)	Total chitinase activity (Units/ml)	Specific chitinase activity (Units/ml)
T. harzianum	30.45	10.33	0.33
T. reesei	29.96	20.67	0.69
Th+Tr 1	27.44	29.33	1.06
Th+Tr 2	33.88	27.50	0.81
Th+Tr 5	32.34	10.83	0.33
Th+Tr 6	42.98	33.67	0.78
Th+Tr 12	39.55	27.83	0.70
Th+Tr 13	32.34	21.33	0.66

Table 8. Quantitative assay of chitinase production and protein content by selected fusant and parent strains

Strain	Protein content (µg/ml)	Total chitinase activity (Units/ml)	Specific chitinase activity (Units/ml)
T. harzianum	1.68	15.8	5.83
T. reesei	6.72	11.3	4.16
Th+Tr 1	8.05	11.7	4.33
Th+Tr 2	10.01	13.9	5.16
Th+Tr 5	6.51	18.0	6.66
Th+Tr 6	10.01	15.3	5.66
Th+Tr 12	10.5	12.6	4.66
Th+Tr 13	11.13	12.6	4.66

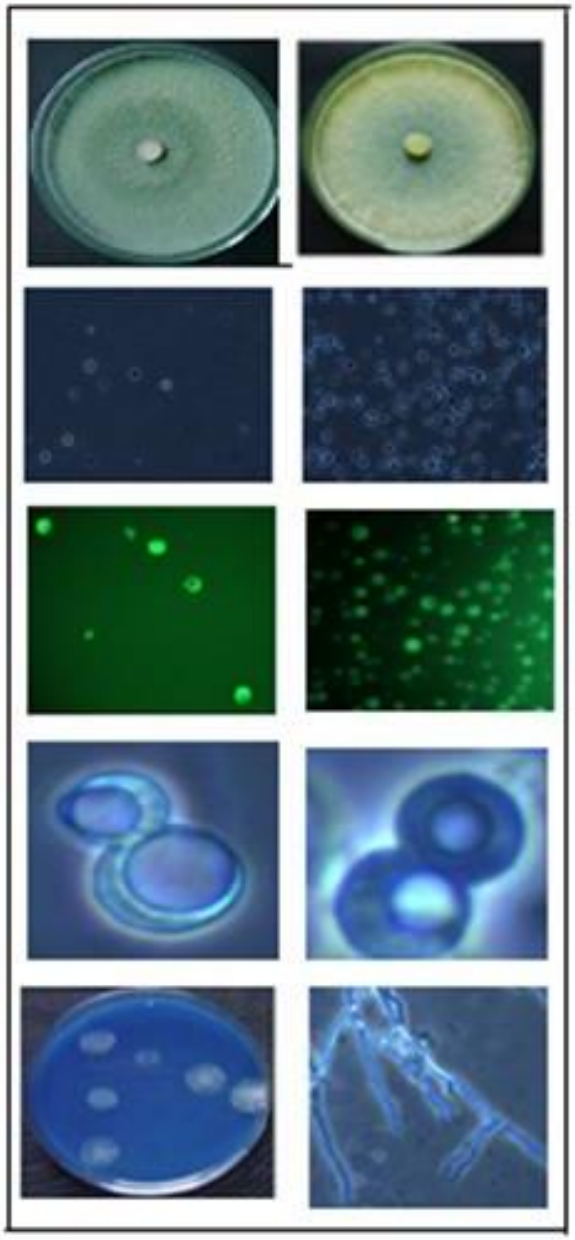
All the fusant strains inhibited *R. solani* ranged from 5.8 to 8.8 cm (Table 2). The average antagonistic activity exhibited by the fusant was better than that of the both the parent, *T. harzianum* and *T. reesei* by 7%-8%zz

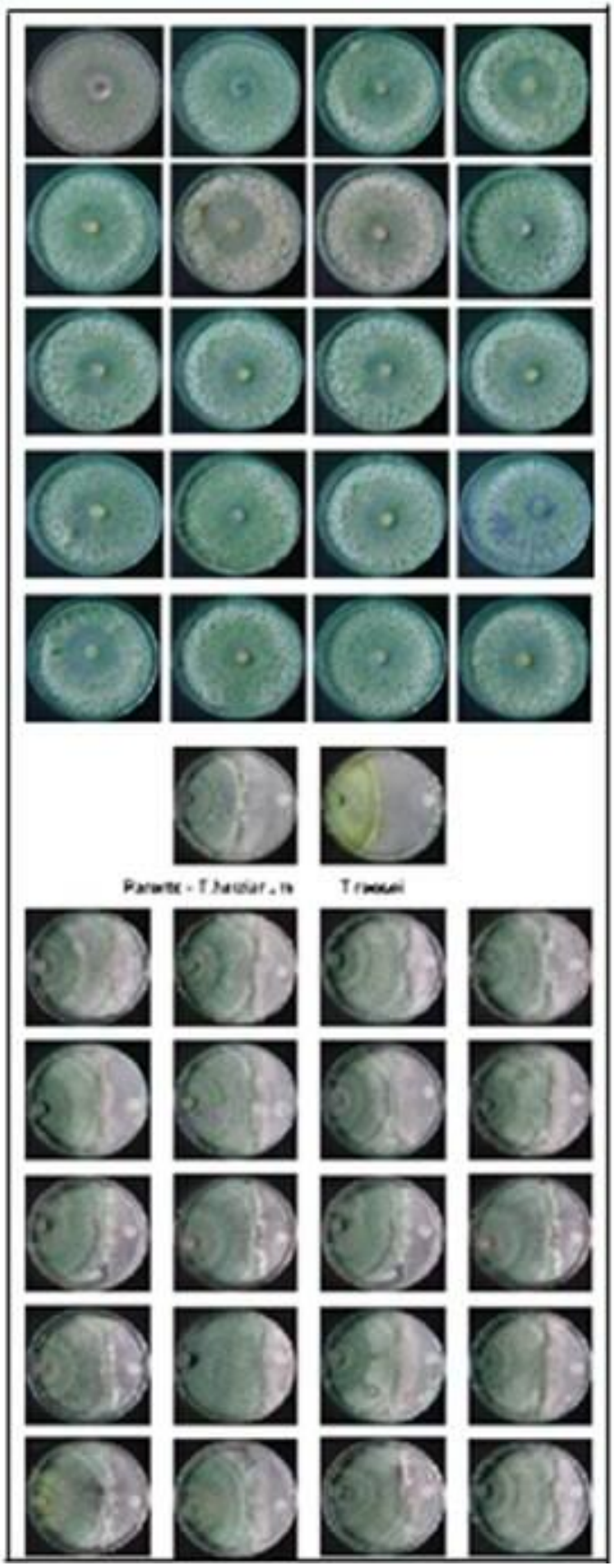
Qualitative assay of chitinase and cellulase production

All the *Trichoderma* strains lysed the colloidal chitin as well as carboxyl methyl cellulase to a very good extent, which was observed as clear zone around the mycelial growth.. However, the levels of clear zone vary among the isolates indicated the differences in enzyme production. The fusant exhibited better performance regarding enzyme activity as compared to both the parents strains. Among the fusant, Th+Tr 1 produced the clear zone of 2.5 cm on carboxylmethyl cellulose media, as against 1.3 – 2.0 cm in rest of the isolates and Th+Tr 5 produced the clear zone of 2.3 cm on colloidal chitin amended media, as against 1.2 – 2.1 cm in rest of the isolates (Table 5 & 6).

Quantitative assay of chitinase and cellulase production

All the selected fusant strains performed better regarding chitinase and cellulase activity than the parent strains. However, Th+Tr 5 produced highest extracellularcellulase with a total activity of, 18.0 U/ml and specific activity 6.66U/ mg of protein (Table 7). Whereas, Th+Tr1 produced highest extracellular chitinase with a total activity of (29.33U/ml and specific activity 1.06U/ mg of protein (Table 8.).





Left panel (from top)
Fig. 1. Morphology of the parents
T.harzianum Treesei
Fig. 2 ISOLATED PROTOPLAST
Fig. 3 Viability Of Isolated Protoplasts
Fig. 4 Fusion of Protoplast
Fig. 5 Regeneration of Fused Protoplast
New Mycelium Developing From The Regenerated Protoplast

Right panel (from top)
Fig. 6 Morphology of the Regenerated Fused Strains
Fig. 7 Antagonistic Activity

RFLP analysis of the parents and the fusants

The DNA was extracted from both the parents and the six selected fusant strains and was subjected to RFLP, with the restriction enzyme EcoR1.The RFLP analysis of both the parents *T. harzianum* and *T. reesei* (Figure 8.0, represented as P1 and P2) and all the six fusants (Figure 8.0, represented as I, II….VI), has revealed that almost all the fusant, except IV (Fusant 6th) contain all the bands from both the parents. This shows and confirms that the fusion of the nuclear genes has taken place between *T. harzianum* and *T. reesei*, that has yielded the mutually fusant strains.

4. Discussion

The genus *Trichoderma*, particularly *T. harzianum* and *T. reesei,* is an excellent source of enzymes, particularly cellulase and chitinase (Christian P. Kubicek, 2006). In the present project, *Trichoderma* has exhibited a good cellulase production. However, the fusant progenies that were obtained from the inter-specific fusion between *T. harzianum* and *T. reesei*., have proved to have far greater potential than the parent strains, regarding the production of cellulase and have resulted in 40-50% more cellulase production as compared to the parents species, when assayed Carboxy Methyl Cellulose medium. The possible secretion of extracellular cellulase by fusant and parent strains, was observed through the clear zone on CMC agar medium. Industrial application of cellulase include stonewashing denims, household laundry detergents, animal feeds, textile bioploshing, fruit juice and beverages processing, baking and alcohol production (Tolan and Foody, 1999; Vyas et al., 2003) as well as ethanol and single cell proteins.

However, production of chitinase has been shown to be very less as compared to the cellulase activity, from both the parents as well as the fusant strains. Although, some of the fusant strains have shown a slight increase in chitinase production, when assayed in colloidal chitin media, even though none of the fusant strain has shown satisfactory good chitinase production. One of the possible reasons may be the lack of strong chitinase activity in the parent stains, which have been subsequently transferred to the fusant progenies also. Due to the combined activity of *T. harzianum* and *T. reesei*, fusants have shown slight increase in chitinase activity.

Many of the *Trichoderma* species were exhibiting antagonistic activity against several fungal pathogens (Elad et al., 1982; Sivan and Chet, 1989; Baby and Manibhushanrao, 1996; Lalithakumari, 2000b; Mathivanan et al., 2000a) through the inhibition of spore germination, mycelial growth and degradation of host cell wall. In this present study, fusants as comaperd to the parents, exhibited more effective inhibition of the mycelial growth of fungal pathogens in dual culture, against *R. solani*. The antagonism of *Trichoderma* could be attributed to the competition for nutrients, release of toxic metabolites and extracellular lytic enzymes. Generally, *Trichoderma* species grow very faster than other fungi (Mathivanan et al., 2000b) and are reported to produce toxic substances like viridin, trichodermin, etc. (Weindling, 1941; Weindling and Emerson, 1936). The secretion of extracellular enzymes such as chitinase is very well documented and its role on the biological control was established (Sivan and Chet, 1989; Chellappa, 1998; Prabavathy et al., 2003). The antagonistic mechanism of *Trichoderma* is mainly due to the production of lytic enzymes, chitinase that degrade the cell walls of pathogenic fungi (Chet, 1987; Chet and Baker, 1981; Cook and Baker, 1983; Lorito et al., 1993; Sivan and Chet, 1986).

Fungal protoplasts are an important tool in physiological and genetic research (Isaac et al.,

1986; Peberdy, 1989; Lalithakumari, 2000). Genetic manipulation in filamentous fungi can be successfully achieved through fusion of protoplast and it is an effective tool in fungal biotechnology. Interspecific, intraspecfic and intergeneric hybridation could be done by this technique and hence strain improvement of industrial fungal strains is possible (Ohnuki et al., 1982; Quigley et al., 1987; Lalithakumari, 2000).

The isolation of protoplasts from fungi using lytic enzyme is now a well established technique (Hamlyn et al., 1981). Novozyme 234 was reported to be the most effective enzyme for high yields of protoplasts in fungi. Osmotic stabilizer plays an important role in the release and maintenance of protoplasts (Hocart et al., 1987; Mukherjee and Sengupta, 1988).

The aggregation of two or more protoplasts is not enough to start a fusion. Protoplast surfaces bear strong negative charges. Intact protoplast in suspension thus repels each other. They can be very impressively linked and fused by the addition of calcium ions or non-ionic water soluble surfactant polyethylene glycol (PEG/CA2+) (Anne and Peberdy (1976) and Ferenczy et al. (1975).

The fusants progenies exhibited great morphological variations such as mycelial growth, pigmentation and sporulation and spore colouration among themselves and also compared to their wild parents. These variations could be due to the various levels of genetic recombination occurred during the fusion of protoplasts.

The present study reveals the applicability of protoplast fusion technology for the genetic manipulation of industrially important fungi, *T. harzianum* and *T. reesei* which do not possess a sexual stage and therefore it would appear difficult to achieve recombination among themselves. Under such circumstances, protoplast fusion helps in overcoming vegetative incompatibility and results in hybridization. The fusants obtained in this study from inter-specific cross between *T. harzianum* and *T. reesei* have been shown to enhane the level of enzyme production and hence increased biocontrol activity. These could be further exploited for enhanced enzyme prvarious Biotechnological applications.

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