JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2017. Vol. **11**(1), p. 277-284

# Enhancement of Antagonism through Protoplast Fusion in *Trichoderma* spp

**B.P. Chandrappa[1]* and M.P. Basavarajappa[2]**

[1]Department of Plant Pathology, College of Agriculture, Vijayapur - 586 101, India.
University of Agricultural Sciences, Dharwad - 580 005, Karnataka, India.
[2]Associate Professor, University of Horticultural Science- Bagalkot, Karnataka, India.

http://dx.doi.org/10.22207/JPAM.11.1.35

(Received: 10 June 2016; accepted: 03 August 2016)

**The filamentous fungi *Trichoderma* is used asmost important biocontrol agent. Because of the absence of sexual reproduction in this fungus, other methods of genetic improvement have been developed to enhance its bicontrol potential.In the present investigation protoplast fusion of four different *Trichoderma* spp. was evaluated for their antagonistic activity against the soil borne plant pathogen like *Fusarium* spp. which causes wilting diseases in many crops. In protoplast fusion technique, protoplast isolation by lysis enzyme at the concentration of 10 mg/ml, protoplasts were fused intra and inter specifically, with 30% (w/v) PEG solution and those fusants regenerated on osmatic stabilizer amended PDA media. Eight self fusants and eighteen fusants were selected for assessment of antagonastic activity against fusarium spp. in dual culture technique. The results revealed that among eight intra fusants, fusant*Tvdsf*-2 (93.70%) was recorded highest per cent inhibition against *F. o.* f. sp. *udum*and*Tvssf*-1 (86.70%) against the *F. solani* and inter specific fusants, the maximum per cent inhibition mycelia growth was observed in *Tvs*×*Tas*-1 (94.07%) and *Tvd*×*Tas*-1 (94.07%) against *F. o.* f. sp. *udum*and *Tvd*×*Tas*-1 (89.63%) against the *F. solani.* These outcomes indicated that protoplast fusion technique was successful tools to enhance the antagonistic effects of *Trichoderma* species against several fungal plant pathogens.**

**Keywords:** *Trichoderma* spp., protoplast fusion, regeneration and antagonism.

Soil borne pathogens have a broad host range and persist for longer periods in soil by resistant resting structures. Chemical control of soil borne pathogens provides certain degree of control but at the same time have adverse effects on environment affecting the beneficial soil microorganisms. Therefore, biological control of plant pathogens has been considered as a potential control strategy in recent years and search for these biological agents is increasing. *Trichoderma* is the most commonly used fungal biological control agent and have long been known as effective antagonists againstplant pathogenic fungi (Papavizas, 1985).

Soil borne diseases caused *Fusarium* spp.which was causes economical loss in many crops. Pesticide treatment is the mostusual method for controlling these diseases. However, these pesticides are expensive and areharmful on human health. Therefore, using biocontrol agents aremore effective, less expensive and safer for human health. Genus *Trichoderma* is one of the mostimportant filamentous fungi used as a biocontrolagent. Many species under this genus has been usedagainst diseases in a wide variety of economically important crops. Because ofthe

* To whom all correspondence should be addressed.
E-mail: chandrunayakagri@yahoo.com

absence of sexual reproduction, other methods of genetic improvement have beendeveloped in these fungi such as transformation andproptoplast fusion (Balasubramanian and Lalithakumari, 2008).

The present work aimed to apply protoplast fusion of four *Trichoderma* spp. to enhancement their bio-control abilities against some of important plant fungal pathogens (*Fusarium* spp.), which cause wilting diseases that attacking different crops.

## MATERIAL AND METHODS

The mutation was carried out by using four *Trichoderma* species *viz.*, *Trichodermaharzianum, Trichodermavirens* and *Trichodermaviride* isolates were collected from NBAIR (National Bureau of Agricultural Insects Resources) Bengaluru and *Trichodermaasperellum* from IIOR (Indian Institute of Oil Research) Hyderabad and*Fusariumoxysporum* f. sp. *udum* and *Fusariumsolani*, the causal agent of wilt diseases in pigeon pea and acid lime were isolated from diseased plants. Those bio-agent and pathogenic fungi was sub cultured on PDA slants and allowed to grow at 28±1°C for ten days and such slants were preserved in a refrigerator. The procedure or protocol followed for the isolation of protoplast, fusion of protoplasts is given below.

**Isolation of protoplasts from *Trichoderma* species**

The parental strains of *Trichoderma species* like *Trichoderma harzianum, Trichoderma virens, Trichoderma viride* and *Trichoderma asperellum* were grown separately in 50 ml of PDB in 150 ml Erlenmeyer flasks by inoculating 1 ml conidial suspension of respective culture at H"5´ $10^6$ conidia/ml. The flasks were incubated on a rotary shaker with a speed of 120 rpm at room temperature for 15 hours. Then cultures were harvested and the young mycelia were separated by filtration using Whitman No.1 filter paper. About 100 mg of fresh mycelium was washed with sterile distilled water followed by 0.1 M phosphate buffer (pH 5.8) and osmatic stabilizer (0.7 M KCl) then incubated with Lysing Enzymes (Sigma Chemicals Co.) at 10 mg/ml concentration prepared in phosphate buffer containing 0.7 M KCl as osmotic stabilizer. The mycelia–enzyme mixtures were incubated on a rotatory shaker with a speed of 75 rpm at room temperature and the lysis of fungal cell wall and the release of protoplasts were monitored at 30 min interval under a light microscope. After 4hr, the enzyme-protoplast mixture was filtered through a sterile cotton swab and centrifuged at 3000 rpm for 5 min at $4^0$C. The supernatant was discarded and the protoplast sediment was suspended immediately in buffer and osmotic stabilizer solution. These protoplasts were used for further fusion process.

**Intra and inter-specific protoplast fusion**

Intra and Inter-specific protoplast fusion was carried out by the method given by Prabavathy *et al*. (2006). Intra specific fusion was done within the species, such as, *Trichoderma harzianum (Th×Th), T. virens, (Tvs×Tvs), T. viride (Tvd×Tvd*) and *T. asperellum (Tas×Tas)*. In case of inter specific fusion, on different combination of *Trichoderma* was fused with the another species of *Trichoderma* (*Th×Tvs, Th×Tvd, Th×Tas, Tvs×Tvd, Tvs×Tas* and *Tvd×Tas*).

Polyethylene glycol (PEG), (MW 6000, Himedia company) prepared in STC buffer contained 0.6 M Sorbitol, 10 mM Tris-HCl and 10 mM Calcium chloride (pH 7.5) was used as fusogen. One ml of protoplasts suspension ($1\times10^6$ protoplasts/ml) was mixed with an equal volume of 30% PEG solution and the fusion mixture was incubated at room temperature. After 10 min, the mixture was diluted with 1 ml of STC buffer. The protoplast fusion was observed under microscope (Axiozeiss Company) and microphotographs were taken.

The fusion mixture was serially diluted with the osmotic stabilizer for regenerationand plated on regeneration medium (PDA amended with osmotic stabilizer) and controls were maintained separately for non-fusions. Observations were made every day on regeneration of fusion protoplasts on agar media. After regeneration of protoplasts on media, all the growing fusants were selected based on the fast growth comparing with the parent isolates as check and grown separately on the PDA plates, Selected regenerated intra and inter specific fused protoplasts were enlisted as below.

The antagonistic effects of each intra and inter specific protoplast fusions of different *Trichoderm* spp. and their parental *Trichoderma* spp. isolates were evaluated against *Fusarium oxysporum* f. sp. *udum* and *Fusarium solani* were

evaluated in dual culture plate technique. The percent inhibition of the growth of the pathogen was calculated by using the formula suggested by Vincent (1947)

$$\text{Percent inhibition in mycelial growth/conidial germination} = \frac{C - T \times 100}{C}$$

Where, C = Mycelial growth/conidial germination in control

T = Mycelial growth/conidial germination in treatment

## RESULTS AND DISCUSSION

Protoplast fusion has rapidly become acceptable tools for the fungal genetics with the development of new techniques of gene transfer such as protoplast fusion and enhancement of antagonistic ability of *Trichoderma* spp. byprotoplast fusion. These experiments included protoplast isolation, regeneration andintra and inter specific fusions. In addition, evaluation of obtained fusants by dual culture experiment.

**Protoplast isolation fusion and regeneration**

Isolation of protoplast, first completely lysed the young mycelium and release of protoplasts were observed after 3 hour (Fig.1 to 4). Initially released protoplasts were smaller in size, but later enlarged and formed spherical structures. The protoplasts yield significantly affected by the concentrations of lysing enzymes and specific conditions including mycelium age, temperature, medium, etc.. The conditions for releasing the protoplasts were similar as reported by EL-Bondkly (2006).

The lysis of fungal mycelium was confined only to the tip portion, 10 mg/ml concentrations of lysing enzymes with 0.7 M KCl as osmotic stabilizer was optimal for the release of protoplasts. Peåer and Chet (1990) obtained highest protoplasts yield from *T. harzianum* using Novozyme 234 at 10 mg/ml with 0.6 M KCl. The isolated protoplasts were subjected to intra and inter specific protoplast fusion with the use of 30% PEG 6000. The protoplasts were attracted to each other's and pairs of protoplasts were observed when PEG solution was added to the protoplasts. Later, the plasma membrane at the place of contact dissolved and protoplasmic contents fused together that may half union and fully fusion (fig 9 and 10), then followed by nuclear fusion in most cases.

The fused protoplasts became single, larger and round or oval-shaped structure representing in fig 10. As similar concentration of PEG was reported as optimum for inter-specific fusion of protoplasts between *T. harzianum* and *T.*

List of regenerated Intra-specific fused protoplasts

| Sl. No. | *Trichoderma* spp. | Name of regenerated isolates |
|---|---|---|
| 1 | *Trichoderma harzianum* | *Thsf*-1 & *Thsf*-2 |
| 2 | *Trichoderma viride* | *Tvssf*-1 & *Tvssf*-2 |
| 3 | *Trichoderma virens* | *Tvdsf*-1 & *Tvdsf-2* |
| 4 | *Trichoderma asperellum* | *Tassf*-1 & *Tassf*-2 |

Note: *Thsf- Trichoderma harzianum* self fusant *Tvssf- Trichoderma virens* self fusant *Tvdsf- Trichoderma viride* self fusant*Tassf-Trichoderma asperellum* self fusant

List of regenerated Inter-specific fused protoplasts

| Sl. No. | *Trichoderma* spp. | Name of regenerated isolates |
|---|---|---|
| 1 | *Trichoderma harzianum* x *Trichoderma viride* | *Th*×*Tvs*-1, *Th*×*Tvs*-2, *Th*×*Tvs*-3 |
| 2 | *Trichoderma harzianum* x *Trichoderma virens* | *Th*×*Tvd*-1, *Th*×*Tvd*-2, *Th*×*Tvd*-3 |
| 3 | *Trichoderma harzianum* x *Trichoderma asperellum* | *Th*×*Tas*-1, *Th*×*Tas*-2, *Th*×*Tas*-3 |
| 4 | *Trichoderma virens* x *Trichoderma viride* | *Tvs*×*Tvd*-1, *Tvs*×*Tvd*-2, *Tvs*×*Tvd*-3 |
| 5 | *Trichoderma virens* x *Trichoderma asperellum* | *Tvs*×*Tas*-1, *Tvs*×*Tas*-2, *Tvs*×*Tas*-3 |
| 6 | *Trichodermaviride*x*Trichodermaasperellum* | *Tvd*×*Tas*-1, *Tvd*×*Tas*-2, *Tvd*×*Tas*-3 |

**Table 1.** *In vitro* evaluation of intra specific fusions against *Fusarium oxysporum* f. sp. *udum*

| Sl. No. | Parents and Fusants | Mean per cent inhibition |
|---|---|---|
| 1 | *PTh* | 85.56*(67.65) |
| *2* | *PTvs* | 85.55(67.64) |
| *3* | *PTvd* | 91.11(72.79) |
| *4* | *PTas* | 86.67(68.57) |
| 5 | *Thsf*-1 | 88.52(70.20) |
| 6 | *Thsf*-2 | 91.85(73.50) |
| 7 | *Tvssf*-1 | 90.00(71.55) |
| 8 | *Tvssf*-2 | 90.74(72.35) |
| 9 | *Tvdsf*-1 | 89.63(71.29) |
| 10 | *Tvdsf*-2 | 93.70(75.48) |
| 11 | *Tassf*-1 | 88.15(69.87) |
| 12 | *Tassf*-2 | 89.26(70.85) |
| S. Em± | 1.04 | |
| C.D. @1% | 4.12 | |

*Arc sine values

**Table 2.** *In vitro* evaluation of intra specific fusions against *Fusarium solani*

| Sl. No. | Parents and Fusants | Mean per cent inhibition |
|---|---|---|
| 1 | *PTh* | 78.5*(62.38) |
| *2* | *PTvs* | 82.2(65.04) |
| *3* | *PTvd* | 85.2(67.36) |
| *4* | *PTas* | 78.9(62.63) |
| 5 | *Thsf*-1 | 85.9(67.98) |
| 6 | *Thsf*-2 | 85.2(67.36) |
| 7 | *Tvssf*-1 | 86.7(68.57) |
| 8 | *Tvssf*-2 | 85.6(67.71) |
| 9 | *Tvdsf*-1 | 82.2(65.04) |
| 10 | *Tvdsf*-2 | 84.4(66.75) |
| 11 | *Tassf*-1 | 82.6(65.34) |
| 12 | *Tassf*-2 | 82.2(65.04) |
| S. Em± | 0.78 | |
| C.D. @1% | 3.09 | |

*Arc sine values

**Table 3.** *In vitro* evaluation of inter specific fusions against *F. o.* f. sp. *udum*

| Sl. No. | Parents and Fusants | Mean per cent inhibition |
|---|---|---|
| 1 | *PTh* | 85.56*(67.65) |
| *2* | *PTvs* | 85.55(67.64) |
| *3* | *PTvd* | 91.11(72.79) |
| *4* | *PTas* | 86.67(68.57) |
| 5 | *Th*×*Tvs*-1 | 90.00(71.55) |
| 6 | *Th*×*Tvs*-2 | 90.00(71.67) |
| 7 | *Th*×*Tvs*-3 | 87.41(69.21) |
| 8 | *Th*×*Tvd*-1 | 87.78(69.52) |
| 9 | *Th*×*Tvd*-2 | 87.04(69.02) |
| 10 | *Th*×*Tvd*-3 | 88.52(70.23) |
| 11 | *Th*×*Tas*-1 | 92.59(74.37) |
| 12 | *Th*×*Tas*-2 | 90.74(72.35) |
| 13 | *Th*×*Tas*-3 | 91.11(72.71) |
| 14 | *Tvs*×*Tvd*-1 | 92.59(74.37) |
| 15 | *Tvs*×*Tvd*-2 | 93.70(75.48) |
| 16 | *Tvs*×*Tvd*-3 | 90.00(71.75) |
| 17 | *Tvs*×*Tas*-1 | 94.07(75.97) |
| 18 | *Tvs*×*Tas*-2 | 87.78(69.52) |
| 19 | *Tvs*×*Tas*-3 | 88.52(70.25) |
| 20 | *Tvd*×*Tas*-1 | 94.07(76.34) |
| 21 | *Tvd*×*Tas*-2 | 91.48(73.05) |
| 22 | *Tvd*×*Tas*-3 | 91.85(73.50) |
| S. Em± | 1.39 | |
| C.D. @1% | 5.31 | |

*Arc sine values

**Table 4.** *In vitro* evaluation of inter specific fusions against *Fusarium solani*

| Sl. No. | Parents and Fusants | Mean per cent inhibition |
|---|---|---|
| 1 | *PTh* | 78.52*(62.38) |
| *2* | *PTvs* | 82.22(65.04) |
| *3* | *PTvd* | 85.18(67.36) |
| *4* | *PTas* | 78.89(62.63) |
| 5 | *Th*×*Tvs*-1 | 80.74(63.95) |
| 6 | *Th*×*Tvs*-2 | 83.33(65.95) |
| 7 | *Th*×*Tvs*-3 | 82.96(65.64) |
| 8 | *Th*×*Tvd*-1 | 85.18(67.36) |
| 9 | *Th*×*Tvd*-2 | 86.30(68.27) |
| 10 | *Th*×*Tvd*-3 | 84.44(66.75) |
| 11 | *Th*×*Tas*-1 | 86.30(68.27) |
| 12 | *Th*×*Tas*-2 | 83.33(65.88) |
| 13 | *Th*×*Tas*-3 | 81.11(64.22) |
| 14 | *Tvs*×*Tvd*-1 | 87.41(69.19) |
| 15 | *Tvs*×*Tvd*-2 | 89.26(71.16) |
| 16 | *Tvs*×*Tvd*-3 | 87.78(69.56) |
| 17 | *Tvs*×*Tas*-1 | 87.04(68.90) |
| 18 | *Tvs*×*Tas*-2 | 82.22(65.04) |
| 19 | *Tvs*×*Tas*-3 | 82.96(65.61) |
| 20 | *Tvd*×*Tas*-1 | 89.63(71.21) |
| 21 | *Tvd*×*Tas*-2 | 85.55(67.64) |
| 22 | *Tvd*×*Tas*-3 | 82.59(65.33) |
| S. Em± | 0.94 | |
| C.D. @1% | 3.57 | |

*Arc sine values

*reesei*, as well as intergeneric protoplast fusion between *T. harzianum* and *A. niger* (ELBondkly, 2002; EL-Bondkly, 2006). However, Peåer and Chet (1990) used 33 % PEG for Protoplast fusion in *T. harzianum*.

Finally regeneration the fused protoplasts on PDA medium supplemented with osmatic stabilizer was done. Out of number of regenerated colony forming units on PDA media, based on fast growth, eight intra specific and 18 inter specific protoplast fusions were selected. They were subjected to their antagonistic activity evaluation along with the parent isolates against the soil borne pathogens like *F. oxysporum* f. sp.*udum* and *F. solani*.

**Antagonistic activity of intra and inter fusants in dual culture**

Evaluaton of antagonistic activity in intra specific fusions was observed against *F. oxysporum* f. sp. *udum* and *F. solani*, as compared to their parental isolates in below following tables. Among eight fusants *Tvdsf*-2 (93.70%) recorded the highest per cent inhibition (fig 6), followed by *Thsf*-2 (91.85%), *Tvssf*-2 (90.74%), *Tvssf*-1(90.00%) and *Tvdsf*-1 (89.63 %) against *F. oxysporum* f. sp. *udum*. The least inhibition was noticed in *Tassf*-1 (88.15 %) and *Thsf*-1 (88.52 %). *Tvdsf*-2 and *Thsf*-2 recorded more per cent inhibition against *F. oxysporum* f. sp. *udum* than the parent isolates (Table.1). Highest per cent inhibition against *Fusarium solani* observed in fusant*Tvssf*-1 recorded 86.70 per cent (fig 5), followed by *Thsf*-1 (85.9%), *Tvssf*-2 (85.6%), *Thsf*-2 (85.2%) and *Tvdsf*-2 (84.4%) against *Fusarium solani*. The least inhibition (82.2 %) was observed in *Tvdsf*-1 and *Tassf*-2 against *Fusarium solani* (Table.2).These results are in agreement with findings of Prabavathy *et al*. (2006) noticed the improvement of antagonistic activity in self-fusions. Maximum mycelial growth of *Rhizoctonia solani* inhibited

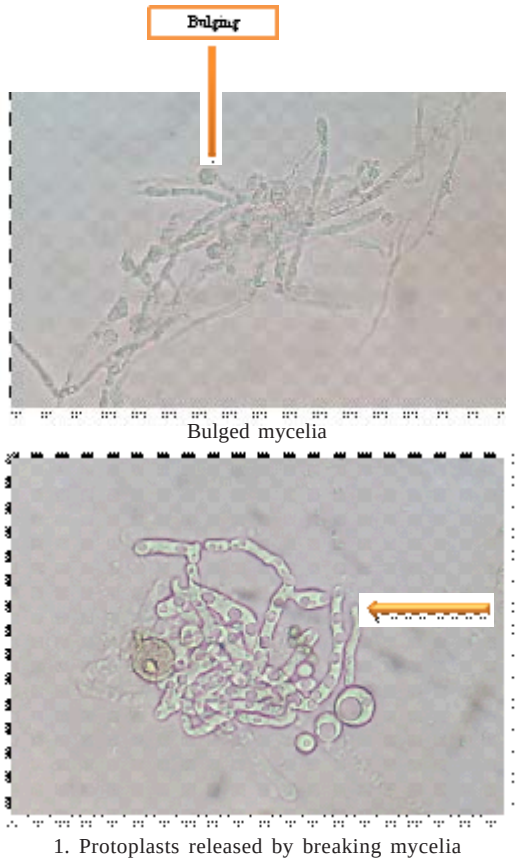



**Fig. 1.** Protoplast releasing from young mycelia

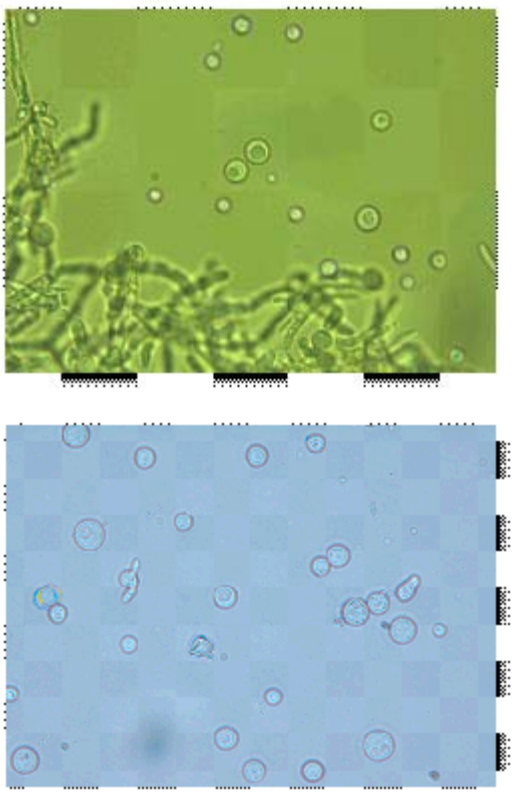


**Fig. 2.** Released protoplast free from mycelia

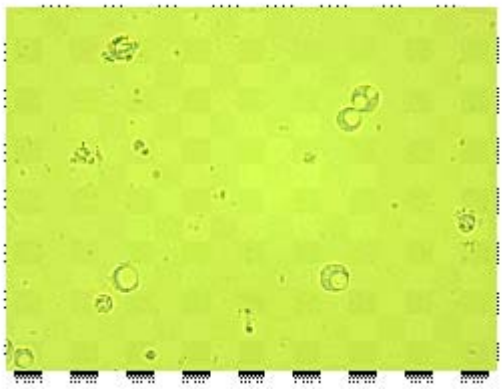
self-fusions (*SFTh*2, *SFTh*8, *SFTh*10, *SFTh*12 and *SFTh*13) as compared to the parent strain (67.6%).

In case of inter specific fusions, the maximum mycelial growth was inhibited by fusant *Tvs*×*Tas*-1 (94.07%) and *Tvd*×*Tas*-1 (94.07%) represented in figure 6, followed by fusant*Tvs*×*Tvd*-2 (93.70%) which was on par with other fusants *Th*×*Tas*-1, *Th*×*Tas*-2, *Th*×*Tas*-3, *Tvs*×*Tvd*-1, *Tvs*×*Tvd*-3, *Tvd*×*Tas*-2 and *Tvd*×*Tas*-3. Fusants were found better to their parental strain, in inhibiting the maximum mycelial growth of *F. oxysporum* f. sp. *udum*, except in parent *T. viride-PTvd* (91.11%) recorded maximum growth inhibition more than some of the fusants (Table.3).

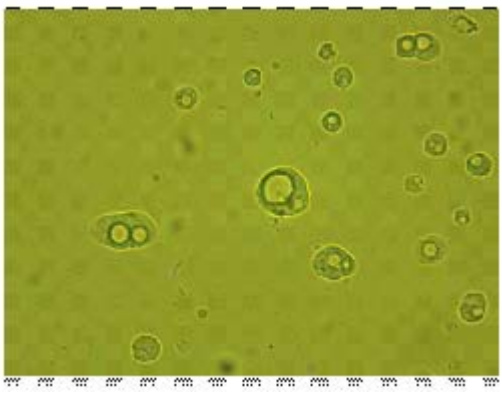
Similarly against *F. solani*, the maximum per cent inhibition of mycelial growth in *F. Solani* was observed in the fusant*Tvd*×*Tas*-1 (89.63%) represented in figure 5, followed by *Tvs*×*Tvd*-2(89.26%), *Tvs*×*Tvd*-3 (87.78%), *Tvs*×*Tvd*-1 (87.41%), *Tvs*×*Tas*-1 (87.04%), *Th*×*Tvd*-2 (86.30%) and *Th*×*Tas*-1 (86.30%) they were on par with fusant*Tvd*×*Tas*-1. But many fusants exhibited better results than that of the parental isolate (Table.4).

These results are in line with the findings of Hassan Abdel-Latif and Haggag (2010) who described that evaluation of inter specific fusion of *Trichoderma koningii* and *Trichoderma reesei*, against the four fungal pathogens like *Fusarium oxysporum*, *Pythium ultimum*, *Sclerotia rolfsii* and *Sclerotinia sclerotiorum* indicated the effectiveness in suppressing the mycelial growth more than the parental isolates. Similar kind of results have also revealed by Mighel*i et al.* (2008) who noticed that the protoplast fusants have higher bio-control potential against *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Pythium ultimum.*

In summary, the intra and inter-fusion of protoplasts in *Trichoderma* spp. resulted in

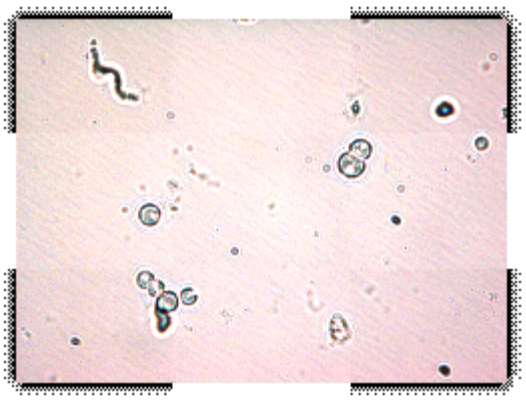


Protoplast attachment

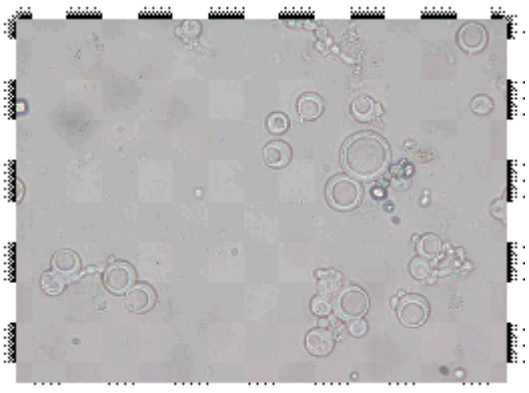


Half union of protoplasts

**Fig. 3.** Protoplast fusion



Fully union protoplasts

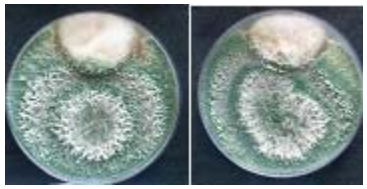


Completely fused protoplasts

**Fig. 4.** Protoplats fusion

Parental isolates against *F. solani*

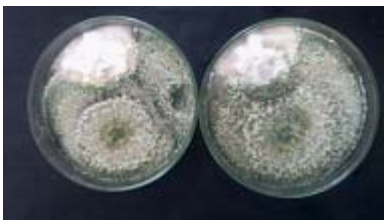
Maximum per cent inhibition in intra fusants*Tvssf*-1



Maximum per cent inhibition in inter fusant*Tvd*×*Tas*-1

**Fig. 5.** Dual culture plates of parents and fusantsagainst *F. solani*

Parental isolates against *F. oxysporum* f. sp. *udum*



Maximum inhibition in intra fusants of*Tvdsf*-2

Maximum per cent inhibition in inter fusant*Tvs*×*Tas*-1

**Fig. 6.** Dual culture plates of parents and fusantsagainst *F. o.* f. sp. *udum*

appreciable increase of bio-control activity in most of the fusants. Results revealed the scope and significance of the protoplast fusion technique, which could successfully be used to develop superior hybrid strains in filamentous fungi that lack sexual reproduction.

## REFERENCES


1. Balasubramanian, N. and Lalithakumari, D., Characteristics of protoplast inter, intra-fusant and regeneration of antagonistic fungi *Trichoderma harzianum* and *Trichoderm aviride. African J. Biotechnol*., 2008; **7**(18):3235-3243.
2. EL-Bondkly, A. M. Genetic transformation in *Trichodermareesei* for the improvement of cellulase production.*Ph.D., Thesis*,Kafr EL-Sheikh, TantaUniversity 2002.
3. EL-Bondkly, A. M. Gene transfer between different *Trichoderma* species and *Aspergillus niger* through intergeneric protoplast fusion to convert ground rice straw to citric acid and cellulases. *Appl. Biochem. Biotechnol.*, 2006; **135**(2): 117-132.
4. El-Bondkly, A. M., Gene transfer between different *Trichoderma* species and *Aspergillus niger* through intergeneric protoplast fusionto convert ground rice strawto citric acid and cellulases.*Appl.Biochem. Biotechnol*., 2006; **135**: 117-132.
5. Hassan Abdel-Latif A. and Haggag, W. M., Mutagenesis and inter-specific protoplast fusion between *Trichoderma koningii* and *Trichoderma reesei* for bio control improvement. *American J. Scientific Indust.Res*, 2010; pp.504-515.
6. Migheli, Q., Whipps, J. M., Budge, S. and Lynch, J., Production of inter and intra hybrids of *Trichoderma* spp. by protoplast fusion and evaluation of their bio control activity against soil-borne and foliar pathogens. *J. Phytopathol.*, 2008; **143**: 91-97.
7. Papavizas, G. C., *Trichoderma* and *Gliocladium* biology, ecology and potential for biocontrol. *Ann. Rev. Physiopathol*., 1985; **23**:23-54.
8. Peer, S. and Chet, I., *Trichoderma* protoplast fusion: a tool for improving bio control agents. *Canadian J. Microbiol*., 1990; **36**:6-9.

9. Prabavathy, V. R., Mathivanan, N., Sagadevan, E., Murugesan, K. and Lalithakumari, D., Intra-strain protoplast fusion enhances carboxy methyl cellulase activity in *Trichoderma reesei*. *Enz.Microb. Technol.*, 2006; **38**: 719–723.
10. Vincent, J. M., Distortion of fungal hyphae in the presence of certain inhibitors. *Nature*, 1947; **159**: 850-851.