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Development of Interspecies and Intergeneric Protoplasm fusant of *Trichoderma* spp. and *Metarhizium anisopliae* and their Efficacy against *Rhizoctonia solani* and *Colletotrichum capsici*

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ABSTRACT: Protoplasts from three fungal antagonists *Trichoderma asperellum* (ITCC-8886.12), *Trichoderma harzianum* (ITCC- 8887.12) and *Metarhizium anisopliae* (ITCC - 8882.12) were obtained following cell-wall digestion by using 20 ml of lysing enzyme (cellulase) @ 8 mg/ml concentration prepared in phosphate buffer. The protoplasts were fused using 40% (w/v) PEG suspended in STC buffer (0.6 M sorbitol, 10mM $CaCl_2$ and 10mM Tris- HCl at pH 7.5). In the present study, the fused protoplasts were regenerated and 9 fusant isolates were used to study their antagonistic activity against *Rhizoctonia solani* and *Colletotrichum capsici.* Among the nine isolates, fusant$_1$ (*T. harzianum* + *T. asperellum*) showed maximum mycelial growth inhibition with inhibition per cent of 68.88 for *R. solani* and 82.58 for *C. capsici*. All the tested fusant isolates exhibited increased antagonistic activity against both the pathogens than the non-fusant isolates of the parent strains except for fusant$_3$ (*T. asperellum* + *M. anisopliae*) against *R. solani*. In addition, frequency of fusion (%) and the radial growth of fusants along with their phenotypic characters were also observed. Protoplast fusion appears to be a useful tool for combining desirable traits from parental strains to produce improved biocontrol strain.

Keywords: *Colletotrichum capsici,* Fusants, *Metarhizium anisopliae,* Protoplast fusion, *Rhizoctonia solani, Trichoderma asperellum, Trichoderma harzianum.*

INTRODUCTION

Fungi in the genus *Trichoderma* has been identified as a potential biocontrol agent against many phytopathogenic fungi. *T. harzianum* is known to produce a wide array of extracellular lytic enzymes that are involved in the process of antagonism against pathogenic mycelia and sclerotia (Benhamou and Chet, 1993, 1996). A number of *Trichoderma* species have been shown to effectively control the soil-borne fungi *Sclerotium rolfsii* (Jinantana, 1995; Henis *et al.*, 1984; Dutta and Das, 2002; Das, *et al*., 2006), *Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., *Aspergillus niger* (Lynch, 1987; Elad *et al*., 1983; Dutta *et al*., 1999), *Sclerotium cepivorum* (De Oliveira *et al.,* 1984), *Sclerotinia sclerotiorum* (Dutta *et al*., 2008), and others.

Metarhizium anisopliae Metsch. is an entomopathogenic fungi which exist as ubiquitous saprophyte in soil and often cause widespread epizootics, wiping out insect pest populations on agricultural crops. Dutta (2012) had isolated a few isolates of *Trichoderma* spp. from different rhizospheres and *M. anisopliae* from the cadavars of insect from Assam. Two isolates of *Trichoderma* (*T. harzianum* and *T. asperellum*) and an isolate of *Metarhizium* were identified to be potential antagonists against plant pathogens (*Rhizoctonia solani*, *Colletotrichum* spp., *Sclerotium rolfsii*) and insect pests (Aphids, Termites) respectively (Pegu *et al*., 2016).

Protoplast fusion is a method which efficiently induces heterokaryosis where it allows the recombination in the progeny of different characteristics from two or more parental strains following the removal of cell wall and exposing the protoplast membrane, processes that are less achievable or impossible with intact cells. Protoplast fusion is an important tool in strain improvement for bringing genetic recombination and developing hybrid strains in filamentous fungi (Lalithakumari, 2000).

Protoplast fusion is a quick and easy method for combining the advantageous properties of distinct promising strains. Protoplast fusion, whether interspecific (Mohamed and Haggag, 2010) or intraspecific (Besoain *et al*., 2007; Fahmi *et al*., 2012), is universally applied as a useful tool for improving fungal strains. Pecchia and Anne (1989) carried out protoplast fusion from *T. harzianum* strains carried out

by treating a mixture of approximately equal numbers of protoplasts from complementary auxotrophs with a 30% (W/V) polyethylene glycol (M.W. 6000) solution in 0.05 M $CaCl_2$, 0.6 M sucrose and 0.05 M glycine, pH 8.0. El-Bondkly *et al*., (2007) showed that there was improvement in antagonistic activity in self-fusants of *T. harzianum* against *R. solani* compared to the parent strain. Complete inhibition (100%) of *R. solani* was recorded with the four fusants ATh1/9, ATh1/12, ATh1/14 and ATh1/17.

The present study aims at development of fusants of intergeneric fusion between *Trichoderma* spp. i.e., *T. harzianum* and *T. asperellum* and interspecific fusion between *Trichoderma* spp. and *M. anisopliae*

MATERIALS AND METHODOLOGY

The various experiments of the present investigation was carried out in the laboratory of Department of Plant Pathology, Assam Agricultural University, Jorhat. The experimental site is located at 26°47/N latitude and 94°12/E longitude at an elevation of 86.60 m above Mean Sea Level.

Source of pathogens: Fresh cultures of two pathogens *viz., Colletotrichum capsici* and *Rhizoctonia solani* were collected from the culture collection of Mycology Research Section, Department of Plant Pathology, AAU, Jorhat, Assam.

The pure cultures of the pathogens *Colletotrichum capsici* (Plate 1a & b) and *Rhizoctonia solani* (Plate 2 a & b) were maintained by periodic sub-culturing in fresh PDA media and stored at 4°C in refrigerator.

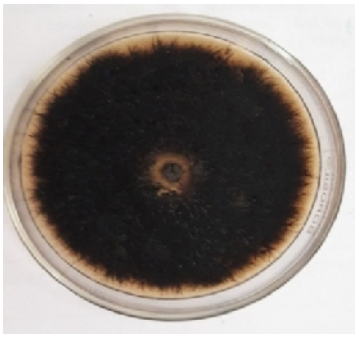

Plate 1 (a): Pure culture of *Colletotrichum capsici*

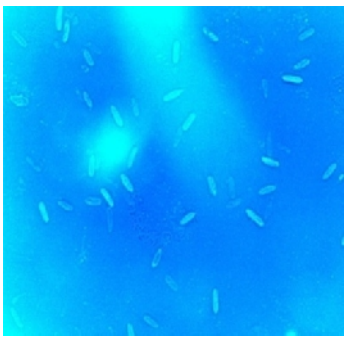

Plate 1 (b): Microscopic observation of *Colletotrichum capsica* (40X)

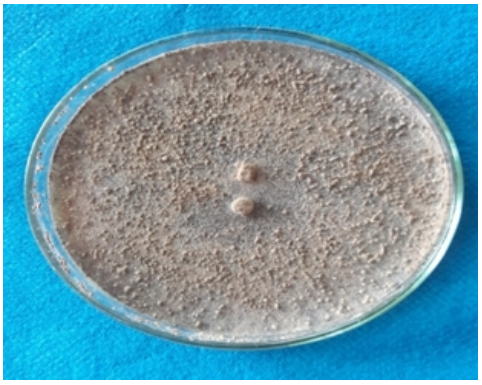

Plate 2 (a): Pure culture of *Rhizoctonia solani*

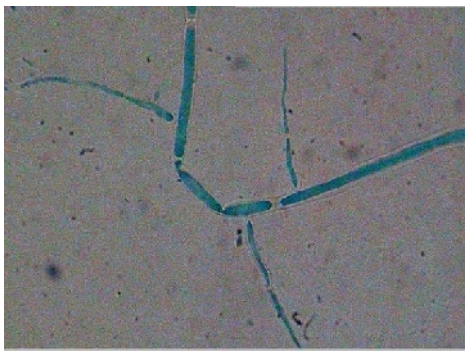

Plate 2 (b): Microscopic observation of *Rhizoctonia solani* (40X).

Collection of fungal antagonists *Trichoderma harzianum, Trichoderma asperellum, Metarhizium anisopliae*: The fungal antagonist strains *Trichoderma harzianum* (ITCC- 8887.12) (Plate 3), *Trichoderma asperellum* (ITCC-8886.12) (Plate 4), *Metarhizium anisopliae* (ITCC - 8882.12) (Plate 5), were collected in pure culture from Mycology Research Section, Department of Plant Pathology, AAU, Jorhat. The pure culture of the antagonists were maintained by periodic sub-culturing in fresh PDA media and stored at 4°C in refrigerator throughout the period of investigation.

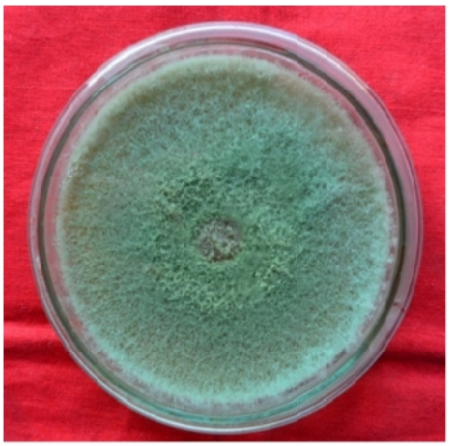

Plate 3: Pure culture of *T. harzianum* after 144 hours

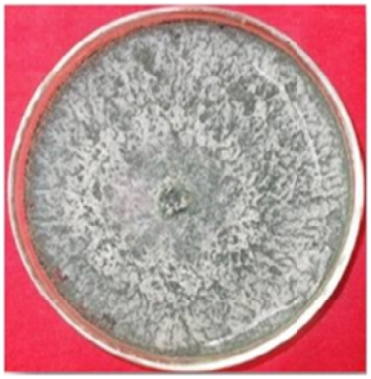

Plate 4: Pure culture of *T. asperellum* after 120 hours

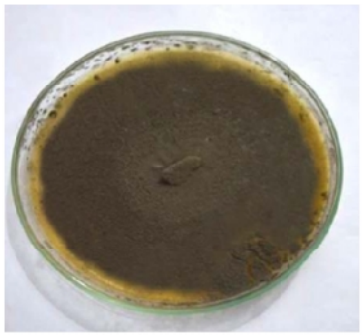

Plate 5: Pure culture of *M. anisopliae* after 240 hours.

Isolation and purification of protoplasts: Protoplasts were isolated and purified according to the method of Ahmed *et al*., (2007), with modifications. About 1ml of conidial suspensions (1×10^7 conidia/ml) of *T. harzianum*, *T. asperellum* and *M. anisopliae* were inoculated into 100ml of potato dextrose broth (PDB) and incubated on a rotary shaker at 100 rpm at room temperature. After 120 hours of incubation, the cultures were harvested and mycelia were separated by filtration. About 50 mg wet mycelia were washed with sterile distilled water followed by 0.1M phosphate buffer at pH 6.0 and incubated with 20 ml of lysing enzyme (Cellulase) 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 24 hours, the protoplasts preparation was filtered through sterile cotton pad and centrifuged at 4000 rpm for 20 minutes. The supernatant was discarded and the sedimented protoplasts were suspended immediately in buffered-osmotic stabilizer solution.

Intergeneric 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*, *T. asperellum* and *M. anisopliae* strains were mixed. To this 200 µl of 40% (w/v) polyethylene glycol (PEG, MW 3350), 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 minutes 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 Czapekdox agar media.

Interspecific protoplast fusion: Similar to intergeneric fusion methods, interspecific protoplast fusion in *T. harzianum* and *T. asperellum* was also carried out using PEG solution. The fused protoplasts were serially diluted with STC buffer. The intrafused protoplasts were then plated on Czapekdox agar media with 0.6 M KCl.

Regeneration of interfusants: The interfused protoplasts were suspended in 100 ml of STC buffer and plated on PDA media at pH 6.5. The intergeneric and interspecific fused protoplasts isolated from *M. anisopliae*, *T. harzianum*, *T. asperellum* were plated on PDA media 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.

Antagonistic test against targeted pathogens: The antagonistic effects of each *Trichoderma* spp. and *M. anisopliae* as well as their fusants against *C. capsici* and *R. solani* were tested by dual culture method. Mycelial disc were cut out from the actively growing culture of fusant and parent culture and placed at the opposite pole on PDA media. The plates were incubated at room temperature and the mycelial growth of pathogens were measured when full radial growth of pathogen in control was observed. The percentage of inhibition (I%) on the mycelial growth of pathogens were calculated using this formula:

$I\% = [(R_1 - R_2)/ R_1] \times 100$

Where, R_1= radial growth of the pathogen in control

R_2= radial growth of the pathogen away from the antagonist

Following treatment combinations were used for antagonistic test-

- T_1 = Pathogen 1 (P_1)
- T_2 = Pathogen 2 (P_2)
- $T_3 = P_1 + M_1$
- $T_4 = P_1 + M_2$
- $T_5 = P_1 + M_3$
- $T_6 = P_2 + M_1$
- $T_7 = P_2 + M_2$
- $T_8 = P_2 + M_3$
- $T_9 = P_1 + Fusant_1$
- $T_{10} = P_1 + Fusant_2$
- $T_{11} = P_1 + Fusant_3$
- $T_{12} = P_2 + Fusant_1$
- $T_{13} = P_2 + Fusant_2$
- $T_{14} = P_2 + Fusant_3$

- T_{15} = P_1 + P_2 + $Fusant_1$
- T_{16} = P_1 + P_2 + $Fusant_2$
- T_{17} = P_1 + P_2 + $Fusant_3$

Where, T = Treatment, P_1 = *Rhizoctonia solani*, P_2 = *Colletotrichum capsici*,
M = Microbe or Biocontrol agent, M_1 = *Trichoderma harzianum*,
M_2 = *Trichoderma asperellum*, M_3 = *Metarhizium anisopliae*
Three (3) replications were maintained for each treatment and each treatment was arranged in Completely Randomized Block design.

RESULTS AND DISCUSSION:

Isolation and purification of protoplasts

Complete lysis of mycelium and release of protoplasts of *T. harzianum* and *T. asperellum* were observed after 24 hours of mixing of lysing enzyme. The enzyme effectively acted on mycelial mass and produced protoplasts and it released soon after the lytic activity from the mycelial structure. They were smaller in size and later gradually enlarged to a hyaline spherical structure. At high enzyme concentrations, the mycelium lysed effectively yielding large number of protoplasts, but they bursted immediately after release and disintegrated as shown in Plate 6 and 7.

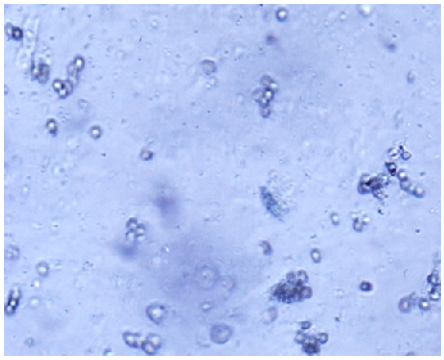

Plate 6: Protoplasts of *T. harzianum*

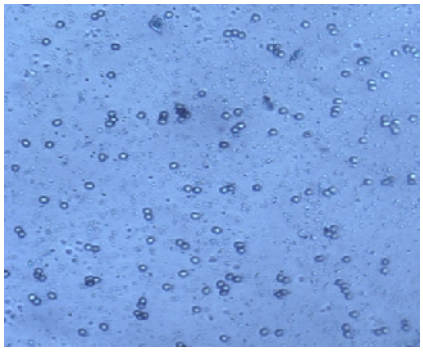

Plate 7: Protoplasts of *T. asperellum*

Incubation of *M. anisopliae* mycelia with lysing enzyme resulted in lysis of cell wall and release of protoplasts. Swelling and rounding up of cell contents were observed initially and subsequently the mycelium started lysing after 24 hours as shown in Plate 8.

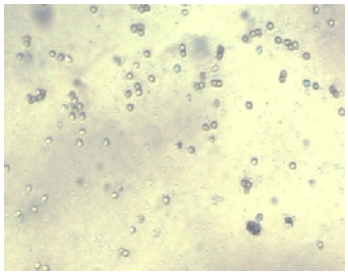

Plate 8: Protoplasts of *M. anisopliae*

Interspecific and intergeneric protoplast fusion: When the protoplasts were mixed with PEG solution, they stuck together and pairs of protoplasts were observed. The plasma membranes in the place of contact of both the protoplasts dissolved and fusion of protoplasmic contents took place. The nuclei of pairing protoplasts fused together (karyogamy) and in some cases dikaryotic stage without nuclear fusion was observed. The fused protoplasts became single, larger and round or oval in shape as shown in Plate 9(a-c).

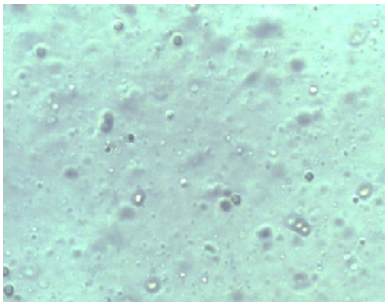

Plate 9 (a): Fused protoplasts of *T. harzianum* and *T. asperellum*

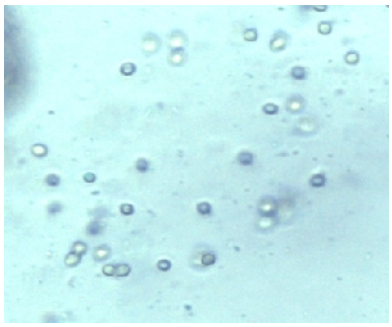

Plate 9(b): Fused protoplasts of *T. harzianum* and *M. anisopliae*

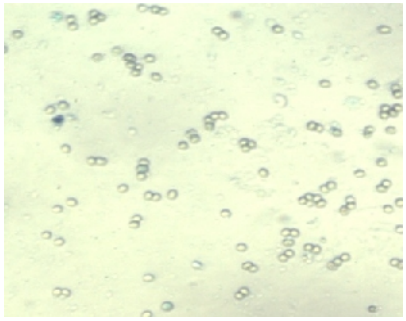

Plate 9(c): Fused protoplasts of *T. asperellum* + *M. anisopliae*

The frequency of fusion was found to be highest in case of fusion between *T. harzianum* and *T. asperellum* (0.51%) followed by the fusion between *T. harzianum* and *M. anisopliae* (0.45%). The least fusion frequency was found in the fusion between *T. asperellum* and *M. anisopliae* as shown in Table 1.

Regeneration of interfusants: Once the interfused protoplasts were sub-cultured in a PDA media a prominent phenotypic characters were observed among the fusants and non-fusants as shown in Table 2.

Table 1: Frequency of fusion (percentage) of interspecific and intragenericfusion.

Isolates	No. of protoplast/ml	No. of fusants	Fusion frequency (%)
T. harzianum +*T. asperellum*	13.50×10^5	7.00×10^5	0.51
T. harzianum + *M. anisopliae*	13.00×10^5	6.025×10^5	0.45
T. asperellum + *M. anisopliae*	12.50×10^5	5.2×10^5	0.43

(The number of fusants by the number of protoplasts per millilitre is calculated as the fusion frequency)

Table 2: Radial growth of fusants (millimeter) of *Trichoderma harzianum*, *Trichoderma asperellum* and *Metarhizium anisopliae*.

Isolates	Phenotypic characteristics	Colony diameter (mm) after 96 hours
Trichoderma harzianum	Bright green	56.00
Trichoderma asperellum	Green patches	62.00
Metarhizium anisopliae	Olive green in mass	48.00
T. harzianum + *M. anisopliae*	Transparent conidia with white conidia scattered around the whole plate	90.00
T. harzianum + *T. asperellum*	Powdery growth with white conidia scattered around the whole plate	90.00
T. asperallum + *M. anisopliae*	Light green with whitish fluffy growth	90.00
S.Ed (±)	—	1.53
$CD_{0.05}$	—	3.52

The non-fusant strain of *T. harzianum* exhibited bright green colour growth in culture plate (Plate 10) while *T. asperellum* shows green patches (Plate 11) and the growth of *M. anisopliae* was olive green in mass (Plate 12). The fusant strains achieved by fusion between *T. harzianum* and *T. asperellum* showed powdery growth with white conidia scattered around the whole plate (Plate 13). The fusion between *T. harzianum* and *M. anisopliae* showed white conidia scattered around the whole plate (Plate 14). The regenerated strain from the fusion between *T. asperellum* and *M. anisopliae* showed light green with whitish fluffy growth (Plate 15).

Plate 10: Growth of *T. harzianum* after 96 hours

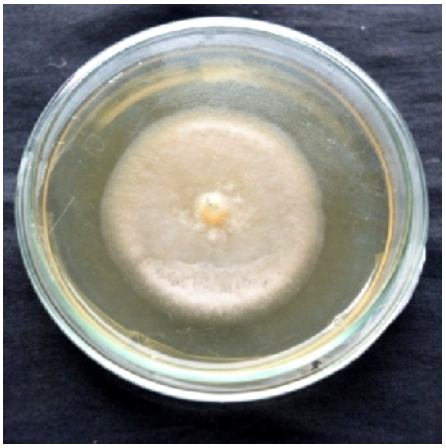

Plate 11: Growth of *T. asperellum* after 96 hours

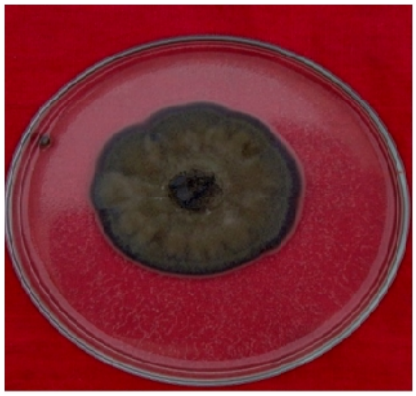

Plate 12: Growth of *M. anisopliae* after 96 hours

Plate 13: Growth of fusant *T. harzianum* + *T. asperellum*

Plate 14: Growth of fusant *T. harzianum* + *M. anisopliae*

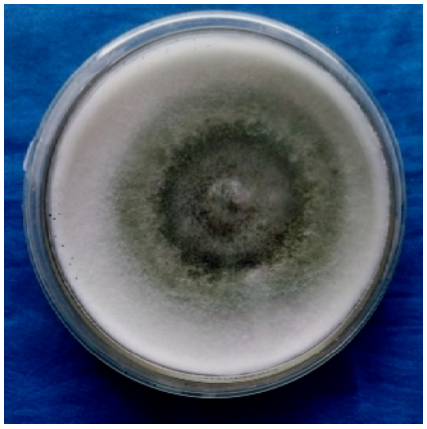

Plate 15: Growth of fusant *T. asperellum* + *M. anisopliae*

The radial growth of the fusants and non-fusant strains was observed after 96 hours of sub-culture and it was seen that the fusant ones exhibited luxuriant growth than the non-fusant ones as shown in Table 2. The interfusants of *T. harzianum*, *T. asperellum* and *M. anisopliae* exhibited full colony diameter growth in a PDA media cultured in a petriplate. The growth of the non- fusant strains of *T. harzianum* (56 mm), *T. asperellum* (62 mm) and *M. anisopliae* (48 mm) was slow as compared to the fusant ones.

Antagonistic tests against *Rhizoctonia solani* and *Colletotrichum capsici*: *In vitro* efficacy of the fusants was tested against *R. solani* and *C. capsici* by dual culture method and the per cent inhibitions observed against both the pathogens are presented in Table 3 and Fig. 1.

Table 3: *In- vitro* efficacy of hybrid against common pathogens like *Rhizoctonia solani* and *Colletotrichum capsici* by dual culture method.

Sr.No.	Treatments	Mean (mm)	Percent inhibition (%)
T_1	*Rhizoctonia solani*	90.00	—
T_2	*Colletotrichum capsici*	90.00	—
T_3	*R. solani + T. harzianum*	28.73	68.07
T_4	*R. solani + T. asperellum*	37.47	58.37
T_5	*R. solani + M. anisopliae*	40.73	54.74
T_6	*C. capsici + T. harzianum*	19.33	78.52
T_7	*C. capsici + T. asperellum*	26.67	70.36
T_8	*C. capsici+ M. anisopliae*	45.83	49.07
T_9	*R. solani + (T. harzianum + T. asperellum)*	28.00	68.88
T_{10}	*R. solani + (T. harzianum + M. anisopliae)*	34.27	61.92
T_{11}	*R. solani + (T. asperellum + M. anisopliae)*	37.40	58.44
T_{12}	*C. capsici + (T. harzianum + T. asperellum)*	15.67	82.58
T_{12}	*C. capsici + (T. harzianum + T. asperellum)*	15.67	82.58
T_{13}	*C. capsici + (T. harzianum + M. anisopliae)*	25.93	71.18
T_{14}	*C. capsici + (T. asperellum + M. anisopliae)*	20.86	76.82
T_{15}	*R. solani + (T. harzianum + T. asperellum)*	45.53	49.41
T_{16}	*R. solani + (T. harzianum + M. anisopliae)*	47.10	47.66
T_{17}	*R. solani + (T. asperellum + M. anisopliae)*	47.23	47.52
	S.Ed(±)	**2.27**	-
	$CD_{0.05}$	**4.56**	-

(Data are mean of three replications)

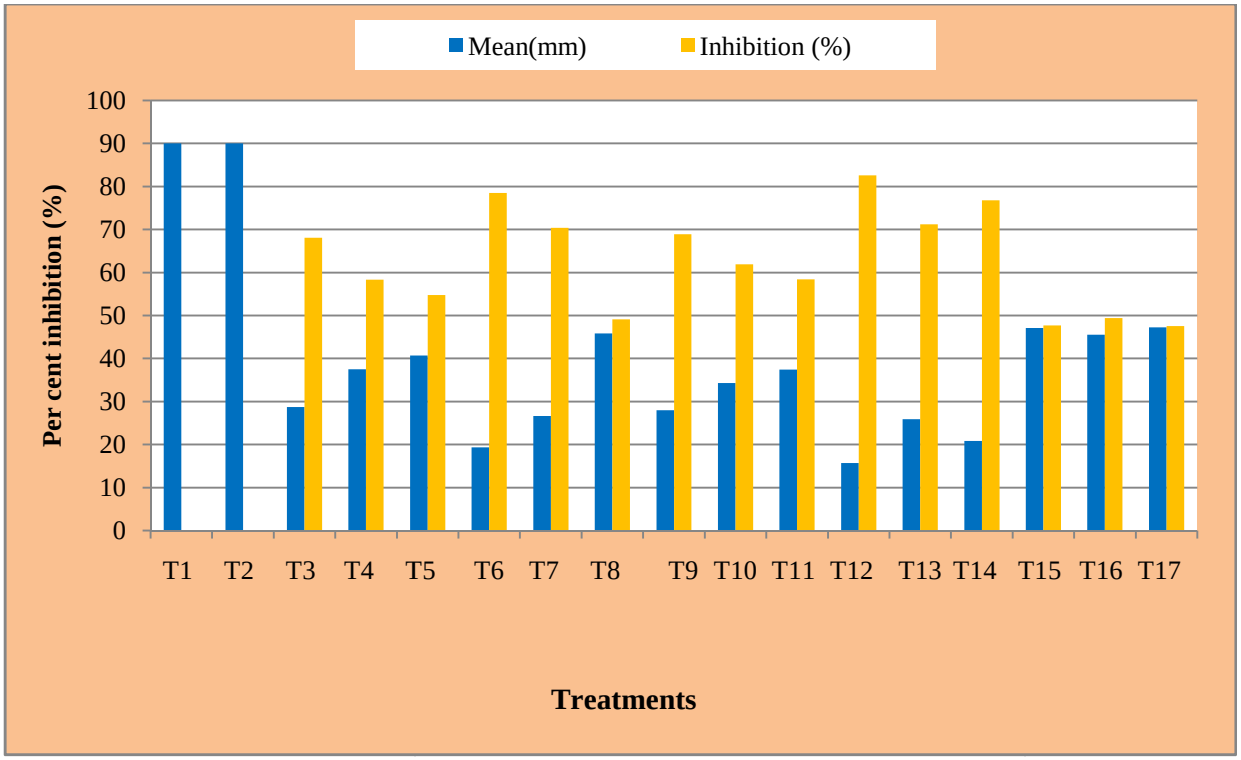

Fig. 1. *In vitro* efficacy of hybrid against common pathogens like *Rhizoctonia solani* and *Colletotrichum capsici* by dual culture method.

Among all the non-fusant strains, *T. harzianum* was found to be most effective against *R. solani* with per cent inhibition of 68.07 (Plate 16) followed by *T. asperellum* with per cent inhibition of 58.37. *T. harzianum* was also found to be most effective microbe against *C. capsici* with inhibition per cent of 78.52 (Plate 17) followed by *T. asperallum* with inhibition per cent of 70.36.

Among all the fusant strains, *T. harzianum* + *T. asperellum* was found to be most effective against *R. solani* with per cent inhibition of 68.88 (Plate 18) followed by *T. harzianum* + *M. anisopliae* with per cent inhibition of 61.92, respectively. *T. harzianum* + *T. asperellum* (Plate 19) was also found to be most effective against *C. capsici* with per cent inhibition of 82.58 followed by *T. asperellum* + *M. anisopliae* with per cent inhibition of 76.82, respectively.

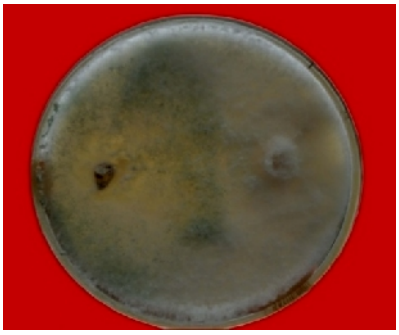

Plate 16: Dual culture plate of *T. harzianum*+ *R. solani*

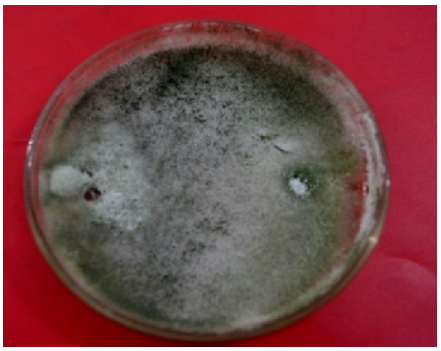

Plate 17: Dual culture plate of *T. harzianum* + *C. capsici*

Plate 18: Dual culture plate of (*T. harzianum* + *T. asperellum*) + *R. solani*

Plate 19: Dual culture plate of (*T. harzianum* + *T. asperellum*) + *C. capsici.*

CONCLUSION AND FUTURE SCOPE

The present study clearly demonstrated that fusant produced from the fusion between *Trichoderma harzianum* and *Trichoderma asperellum* showed maximum mycelial growth inhibition of the pathogens *C. capsici* and *R. solani in-vitro*. The radial growth of fusants of *T. harzianum*, *T. asperallum* and *M. anisopliae* were fast as compared to the non-fusants ones. The inter-generic and inter- specific protoplast fusion resulted in considerable increase in enzyme activity. The protoplast fusion technology can be further applied for developing superior industrially important fungal strains and mycopesticidal strains. This technique can successfully be used to develop superior hybrid strains in filamentous fungi and entomopathogenic fungi that lack inherent sexual reproduction.

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