Protoplast fusion enhances antagonistic activity in *Trichoderma sp* .

A. I. Fahmi[1,4], A. D. Al-Talhi[2] and M. M. Hassan[3,4]

[1]Biotechnology Department, Faculty of Science, Taif University, KSA
[2]Biology Department, Faculty of Science, Taif University, KSA
[3]Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, KSA
[4]Genetics Department, Faculty of Agriculture, Minufiya University, Egypt
Khyate_99@yahoo.com

Abstract: Genus *Trichoderma* is one of the most important filamentous fungi used as a biocontrol agent. Because of the absence of sexual reproduction in this fungus, other methods of genetic improvement have been developed such as proptoplast fusion to enhance its bicontrol potential. Therefore the objectives of this study were; 1) protoplast fusion and regeneration of two fungicide tolerant mutants of *Trichoderma viride.* and *T. harzianum* 2) using ISSR for fingerprinting of inter-specific protoplast fusants and 3) assessment of antagonistic ability of *Trichoderma* fusants against three soil born diseases. Protoplast was isolated from two fungicide tolerant mutants PTv-V and PTz-F of *Trichoderma*,. The frequency of fusion tolerant to both pesticides was about 0.3 % and eight fusants were selected for further studies. In fusant stability experiments, only five of these fusants were the result of nuclear fusion of parental cells**.** Molecular characterization of two stable fusants using ISSR indicated the presence of novel fragments which may be due to recombination events between parents. In dual culture biocontrol experiments, five selected fusants showed growth inhibition against three pathogens namely; *Rhizoctonia solani*, *Fusarium oxysporum* and *Pythium ultimum*.. However, Fus 7 indicated the best ability of inhibition the growth of the three pathogens. In greenhouse experiments Fus 7 demonstrated a great ability to reduce tomato damping off of the three pathogens in the presence of the two fungicides understudy. It was concluded that, the protoplast fusion is a powerful tool to enhance the biocontrol ability of *Trichoderma sp.* against diseases.
[A. I. Fahmi, A. D. Al-Talhi and M. M. Hassan. **Protoplast fusion enhances antagonistic activity in *Trichoderma sp.*** *Nat Sci* 2012;10(5): 120-126]. (ISSN: 1545-0740). http://www.sciencepub.net. 14

Key words: Protoplast fusion, PCR-ISSR technique, *Trichoderma* and antagonistic ability

1. Introduction

Soil bore diseases *R. solani, P. ultimum* and *F. oxysporium* causing economical loss in many crops such as tomato [1]. Pesticide treatment is the most usual method for controlling these diseases. However, these pesticides are expensive and are harmful on human health. Also, complete suppression of these diseases with pesticides is difficult [2]. Therefore, using biocontrol agents are more effective, less expensive and safer for human health [3]. Genus *Trichoderma* is one of the most important filamentous fungi used as a biocontrol agent. Many species under this genus such as *T. harzianum*, *T virens*, and *T. hamatum* has been used against diseases in a wide variety of economically important crops [1, 4, 5]. Genetic variation in this fungi occurred by various asexual processes such as mutation and parasexual recombination because of the absence of sexual reproduction [6]. Therefore, other methods of genetic improvement have been developed in these fungi such as transformation and proptoplast fusion [7, 8, 9]. However, protoplast fusion in this fungi more economical than other methods of improvements [10].

Isolation, fusion and regeneration of protoplasts have been achieved in the genus *Trichoderma* mainly to enhance its bicontrol potential [11]. This approach has been found to be useful tool for combining desirable traits in *T. harzianum* [12, 13]. Molecular fingerprinting has been made to obtain satisfactory differentiation in fungus. Many modern techniques offer valuable means for more direct approaches for improving molecular fingerprinting [14]. One of these techniques is ISSR which has been used for assessment of genetic variation in *Trichoderma* species [15, 16, and 17].

With this background, the present work aims to; 1) protoplast fusion and regeneration of two fungicide tolerant mutants of *T. harzianum*, 2) using ISSR for fingerprinting of intraspecific protoplast fusants and 3) assessment of antagonistic ability of *Trichoderma* fusants against three soil born diseases.

2. Material and Methods

a) Strains:

Two mutants of *T. viride* and *T. harzianum,* namely; PTv-V and PTz-F, tolerated to Vitavax (5, 6-dihydro-2-methyl-1,4-oxathiin-3-carboxamide) and Fungo (thiophanate-methyl) respectively, were obtained by EMS/UV treatment [18].

b) Media:

Potato dextrose agar medium (PDA) (Defico)

was used as growth medium for fungal cultures. 100 µg ampiciline /ml was added to the basal medium (BM) described [19], which was used as growth medium for protoplast isolation. Also, the same medium was used as selected media for fungicides tolerant by adding fungicide, Vitavax and/or Fungo. Protoplast regeneration minimal medium (PRMM) [12] was used for protoplast regeneration.

c) Protoplast isolation and regeneration:

Protoplasts isolation was carried out using fungicide tolerant isolates of *Trichoderma harzianum* [12]. The isolated protoplasts were diluted at concentration of 10^6 protoplast / ml in STC osmotic buffer (0.6 M Sorbitol, 10 mM Tris-HCl and 10 mM $CaCl_2$). 1 ml of diluted protoplast was overlaid on PRMM or PRMM plates supplemented with none or one of the fungicides. They were incubated for 5-7 days at 25°C and developed colonies were counted.

e) Protoplast fusion:

Protoplasts were fused using a procedure described [20]. Fusion frequency was calculated as follows:

Fusion % = fused colonies tolerant to both fungicides / Fused colonies tolerant to first fungicide + Fused colonies tolerant to second fungicide.

f) Fusant stability:

Also, fusant stability of the produced spores from fusant protoplast was evaluated as described [19]. by sub-culturing of selected seven strains on PDA media without fungicide for three generations. Finally, 10^6 spores from each seven fusant strains were subcultured on media supplemented with either or both fungicides. The number of produced colonies from each seven fusant strain was counted.

g) Fusant characterization:

DNA was extracted from fusants and their parents [21]. PCR-ISSR reactions were carried out as demonstrated [22]. ISSR primers P1, 5´-CACACACACACACAGG-3´ and P2, 5´-CACACACACACAAC-3´ were used [23].

h) Antagonistic ability of fusants in dual culture:

The antagonistic ability of both fusants and parental isolates were assessed against soilborne pathogens; *Rhizoctonia solani, Fusarium oxysporum* and *Pithym ultimun* in dual culture without any fungicides [24]. Percent inhibition of radial growth of pathogen was calculated as follows:

Percentage of inhibition = [(R1 - R2)/ R1] × 100

Where, R1 = radius of the pathogen away from the antagonist

R2 = radius of the pathogen

i) **Biocontrol ability of fusants:** The biocontrol efficiency of fungicide tolerant fusant 7 and its parents to damping off tomato disease caused by *R. solani, P. ultimum* or *F. oxysporium* were evaluated in greenhouse experiments [25]. Disease reduction was calculated as follows:

% Disease reduction (% D. R.) = [(Number of control plants – number of diseased plants) / number of control plants] X 100.

Damping–off data were recorded 30 days after sowing. Three replicates were carried out. The percentage of the seedling emergence was taken as index of biocontrol efficiency. Where, control plants are plants without fungicide or pathogen treatment.

Data analysis:

The experimental data were analyzed statistically by one-way ANOVA using Costat Statistical Software, Version 3.01.

3. Results

For enhancing antagonistic ability of *T. Viride* and *T. harzianum* using protoplast fusion, many experiments were conducted. These experiments included protoplast isolation, regeneration and fusion of two fungicides tolerant mutants. Furthermore, assessment experiments of these fusants were carried out which included dual culture and greenhouse experiments.

Protoplast isolation and regeneration

Table (1) showed the efficient production of protoplasts from 18 h old mycelium of *Trichoderma* wild type, PTv-V and PTz-F on PRMM or PRMM supplemented with either fungicides. Percentage of regenerated colonies from isolated protoplasts was 1.92, 2.23 and 1.76 for wild type, PTv-V and PTz-F strains on PRMM without any fungicide; respectively. The protoplast of wild type parent strain did not grow on media supplemented with either fungicide. However, the protoplast of strains PTv-V and PTz-F were grown on fungicides Vitavax and Fungo; respectively. The percentage of regenerated protoplast of PTv-V decreased when grown on PRMM supplemented with Vitavax. Also, the percentage of regenerated protoplast of PTz-F decreased when grown on PRMM supplemented with Fungo.

Protoplast fusion:

Data of Fig. (1) Showed that colonies regenerated from fused protoplasts of the two mutants were tolerant to both fungicides. However, the frequency of produced regenerated colonies on each fungicide was higher than on both of them. Hence, the calculated fusion percentage was 0.3%. Fig. (2) Showed different stages of protoplast fusion process

Fusants stability:

Genetic stability of the double fungicide tolerant (nuclear fusion) isolates was represented by their ability to maintain tolerance to fungicide after three generations. Table (2) showed that six fusants Fus 3, 4, 5, 6, 7 and 8, were retains the double fungicide

tolerance, whereas, both Fus 1 and Fus 2 lost their double fungicide tolerance after the third generation.

Molecular characterization of stable fusants:

In the present study two DNA primers were used to detect the occurrence of fusion using inter-simple sequence repeat (ISSR) technique (Fig. 3). The first primer showed 21 polymorphic fragments in the two parental strains tested and their corresponding fusants. The molecular weight of fragments ranged from 95 bp to 1124 bp. Also, the second primer showed 23 polymorphic fragments with the same parents, fusants with molecular weights ranging from 138 bp to 593 bp. Comparison of the DNA patterns indicated that many fragments were present in Fus 4, Fus 5, and Fus 6 and they were missing in both parents. For example, Fus 5 showed additional fragment of molecular weight 412 bp with primer P2. Also, additional fragments with molecular weights 408, 400, and 367 bp in the Fus 6 and Fus 7 were noticed with primer P2.

Antagonistic ability of fusants in dual culture:

The antagonistic activity of the parental isolates and stable fusants (Fus 3, Fus 4, Fus 5, Fus 6, Fus 7 and Fus 8) was assessed against soil borne pathogenic fungi in dual culture. The pathogen fungi included; *R. solani*, *F. oxysporum* and *P. ultimum*. Generally, results in Table (3) showed that the three pathogens radius growth decreases significantly with fusants than their parents. However, some of fusants exhibited higher significant inhibition percentage of radial growth than others such as Fus 7. Therefore, it was used for further assessments.

Biocontrol ability of fusants:

The effect of fusant 7 on integrated control of tomato damping-off caused by *R. solani, F. oxysporium* and *P. ultimum* was presented in Table (4) along with their parental strains in presence and absence of the fungicides (Vitavax and Fungo). Results demonstrated that, in presence of *R. solani* as soil born pathogen, the damping-off ranged from 22.5 to 79.3 %, while the disease reduction ranged from 9.1 to 70.9 %. The disease reduction of fusant 7 treatment by itself and fusant with the mixture of the two fungicides (at low and high concentrations) showed lower incidence than the mixture of two fungicides at low concentration. In presence of *F. oxysporium* as pathogen the damping-off ranged from 24 to 79.3%. The fusant by itself and the fusant with the Fungo + Vitavax at high concentration showed higher effectiveness than mixture (Fungo + Vitavax mixture at high concentrations). In presence of *P. ultimum* as pathogen the damping-off ranged from 17.1 to 70.4%. While the disease reduction ranged from 21.4 to 64.3%. The fusant by itself and in combination with (Fungo + Vitavax at high concentrations) caused more reduction than the combination of fungicides. In general the presence of the fusant alone or in combination with the fungicides reduced significantly the disease in most cases.

Table (1): Percentage of regenerated colonies derived from *T. harzianum* and their fungicide tolerante mutants.

Strains-derived protoplast	% of regenerated colonies on PRMM supplemented with		
	None	Vitavax	Fungo
Wild type	1.92 %	0.0 %	0.0 %
PTz-V	2.23 %	0.24 %	0.0 %
PTz-F	1.76 %	0.0 %	0.11 %

* PTz-V = *T. harzianum*, Vitavax tolerant: PTz-F = *T. harzianum*, Fungo tolerant mutant.

Table (2): Number of colonies on PRMM supplemented with either/both fungicides after three generations.

Protoplast fusants	No. of colonies on PRMM supplemented with			
	no fungicides	no fungicides	Vit + Fun	Vit + Fun
	0 generation	3 generation	0 generation	3 generation
Fus 1	82.5^{f}	80.2^{f}	64^{h}	0^{n}
Fus 2	91.3^{c}	91.1^{c}	83^{ef}	0^{n}
Fus 3	94^{b}	93.3^{bc}	91.3^{c}	13.2^{m}
Fus 4	84.1^{e}	82.2^{f}	70.3^{g}	23^{k}
Fus 5	95.7^{ab}	94^{b}	87.3^{de}	18.3^{l}
Fus 6	91.3^{c}	90^{cd}	88.4^{d}	42.8^{i}
Fus 7	96.4^{a}	94.8^{b}	89.2^{d}	33^{j}
Fus 8	96.4^{a}	90^{cd}	83^{ef}	13.2^{m}

Table (3) Radial growth of pathogens; *R. solani*, *F. oxysporum* and *P. ultimum* against fusants or their parents.

Parents and fusants	% Radial Growth		
	R. solani	*F. oxysporum*	*P. ultimum*
P 1	83.3^{bc}	78.6^{d}	68.9^{f}
P 2	83.3^{bc}	85^{b}	78.9^{d}
Fus 3	87.8^{b}	82.9^{c}	70.1^{ef}
Fus 4	87.4^{b}	78.3^{d}	72.2^{e}
Fus 5	94.4^{a}	82.9^{c}	72.2^{e}
Fus 6	91.1^{ab}	78.6^{d}	71.1^{e}
Fus 7	86.7^{b}	81.3^{c}	77.8^{de}
Fus 8	83.3^{bc}	82.9^{c}	70.1^{ef}

P1, PTz-V of *T. harzianum* and P2, PTz-F of *T. harzianum*

Table (4): Effect of fusant 7 on percent of tomato damping-off and disease reduction caused by *Rhizoctonia solani, Fiusarium oxysporium* and *Pythium ultimum.*

Treatmants	Dose of fungicide	Infested soil					
		R. solani		*F. oxysporium*		*P. ultimum*	
		D. O	D. R	D. O	D. R	D. O	D. R
PTz-F		40.7f	16.6f	34.8f	8.30 f	52.6f	42.8f
PTz-F + Fungo	0.15 g	58.5d	41.6d	58.5c	41.6c	55.6e	46.0e
PTz-V		79.3a	70.9a	49.6e	29.1e	67.4b	60.7b
PTz-V + Fungo	0.15 g	73.3b	62.5b	79.3a	70.9a	34.8g	21.4g
Fus 7		73.3b	62.5b	64.4b	49.9b	70.4a	64.3a
Fus 7 + Fungicides	0.15 g + 0.15 g	73.3b	62.5b	52.6d	33.3d	70.4a	64.3a
Fus 7 + Fungicides	0.10 g + 0.10 g	64.4c	49.9c	49.6e	29.1e	61.5d	53.6d
Fungo + Vitavax	0.15 g + 0.15 g	22.4h	9.1h	24.1h	6.8h	64.4c	57.1c
Fungo + Vitavax	0.10 g + 0.10 g	46.7e	25.0e	52.6d	33.3d	64.4c	57.1c
Control (untreated)		28.9g	00.0g	28.9g	00.0g	17.0h	00.0h

Values within a column followed by the same letter(s) are not significantly different at the P= 0.05 level according to the least significant difference test.

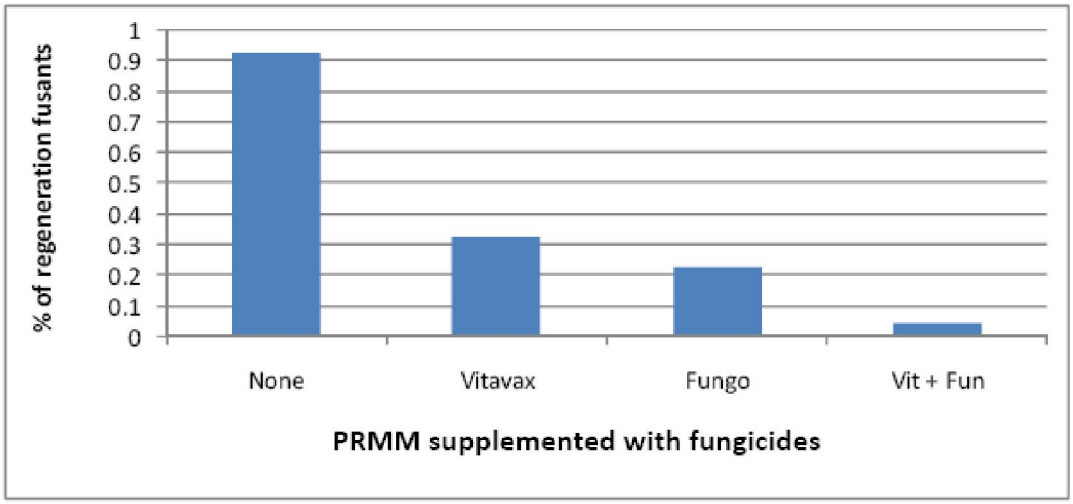

Fig. (1): Percentage of regenerated fusants developed on PRMM supplemented with either/both fungicides

Fig. (2): Stages of protoplast fusion: a) mycelium of *T. harzianum* before lysis, b) isolated protoplast and c) two fused protoplast.

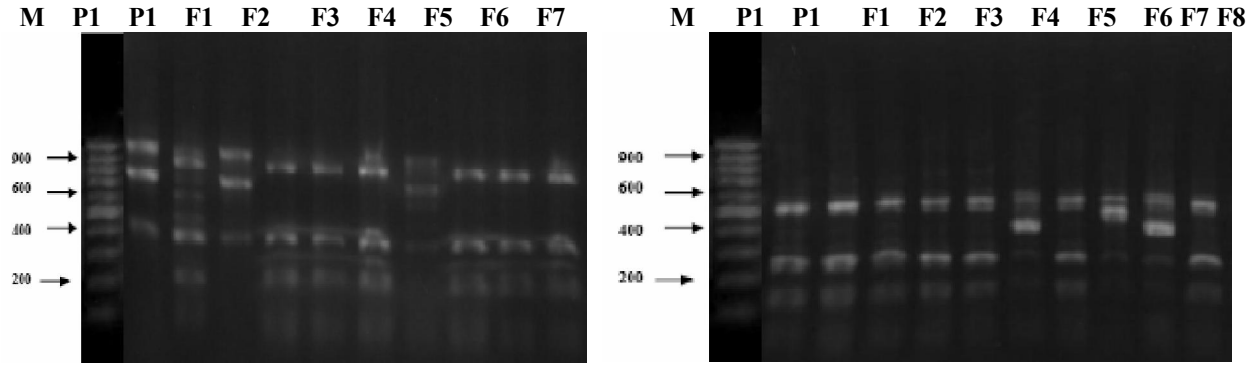

Fig. (3): Inter simple sequence repeats (ISSR) patterns obtained with two primers yes-1(a) and yes-2 (b) of parental and some fusants, M = 100 bp DNA ladder,1 = PTz-V, 2 = PTz-F of *T. harzianum*, 3 and 4 = two fusants

4. Discussion

In this study, a fundamental question concerning the enhancement of biocontrol ability of *T. harazianum* was addressed. To answer this question the protoplast fusion experiments were conducted and the biocontrol ability of the resultant fusants was assessed.

The efficiency of isolated protoplasts from *T. harazianum* and its mutants using PRMM containing either fungicide gave different responses. Regenerated colonies of wild type completely inhibited with either fungicides. However, the regenerated colonies of mutant PTz-F were tolerant to Fungo. These results indicated the ability of mutant protoplasts to regenerate and forming normal colonies in the presence of fungicides with different frequencies. These results were similar with [9, 22, 26] who indicated different frequencies of regeneration ability.

Protoplast fusion of the two mutants PTv-V and PTz-F was carried out and regeneration colonies were evaluated under different fungicides stresses. The results demonstrated that many regenerated fusants were emergent in fungicides treatments. These fusants were tolerated to both fungicides may be due to nuclear fusion or cytoplasmic fusion between the two fused parents. To distinguish between the two events, fusants stability experiments were conducted by subculturing the obtained fusants on PRMM for three generations. Results indicated that some of fusants were unstable and lost through successive cycles of mitotic division during mycelial growth. It was concluded that these unstable fusants (Fus 1 and Fus 2) were produced from temporary cytoplasmic fusion. However, the viable five fusants namely; Fus 3, Fus 4, Fus 5, Fus 6, Fus 7 and Fus 8 should be the result of nuclear fusion of parental cells. Similar findings were observed [9, 12, 13, 27].

Molecular characterization of two stable fusants; Fus 3 and Fus 4 using ISSR indicated the presence of novel fragments which may be due to recombination events between parents [17]. This result was agreed with Savitha et al [9], who found that fusant formation was confirmed by genetic markers such as mycelial protein pattern, restriction digestion pattern and random amplified polymorphic DNA (RAPD)

analysis. Also, this data confirmed the occurrence of nuclear fusion between parental strains PTv-V and PTz-F in the produced stable fusants. Therefore, present result reflects the good occurance of nuclear fusion accompanying protoplast fusion between *Trichoderma* strains and species leading to the production of recombinants. Therefore, we could savely recommend protoplast fusion between different and within species to obtain recombinant strains. This fact could overcome the absence of sexual reproduction to introduce desired characters in *Trichoderma* species. Therefore, the five stable fusants were used further in biocontrol experiments. Many authers indicated that protoplast fusion is an effective tool for inducing genetic recombinations and developing superior hybrid strains in filamentous fungi [12, 20, 28].

In dual culture biocontrol experiments, the five stable fusants were tested for their ability to inhibit radial growth of three pathogens namely; *R. solani*, *F. oxysporum* and *P. ultimum*. They showed different frequencies of growth inhibition against the three pathogens. However, Fus 7 indicated the best ability of inhibition the growth of the three pathogens. Therefore, biocontrol efficiency of Fus 7 was evaluated in greenhouse biocontrol experiments in the presence of fungicides which may help in minimizing their harmful effects on the environment. Results demonstrated the ability of Fus 7 to reduce tomato damping off of the three pathogens in the presence of the two fungicides understudy more than its parental strains. Similarly, EL- Bondkly and Talkhan,and EL-Bondkly et al [6, 28] were able to construct superior *Trichoderma harzianum* isolates for improving β-glucosidase and chitinase productivity through protoplast fusion technique. Also, Prabavathy et al [11] Produced fusants with two-fold increase of chitinase and antagonistic activity against *Rhizocotonia solani* when compared with the parental strains. Moreover Prabavathy et al, [29] used protoplast fusion to enhance carboxymethylcellulase activity and they found that, most of the fusants exhibited fast growth and abundant sporulation compared to non-fusant and parental strains.

It can be concluded that, the protoplast fusion is a powerful tool to enhance the biocontrol ability of *Trichoderma sp*. Also, it is a good tool to combine different characters from different strains of filamentous fungi that lack inherent sexual reproduction.

Corresponding author
M. M. Hassan
Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, KSA
Khyate_99@yahoo.com

References

1. Srivastava, R., A. Khalid, U. S. Singh and A. K. Sharma, (2009). Evaluation of arbuscular mycorrhizal fungus, fluorescent Pseudomonas and *Trichoderma harzianum* formulation against *Fusarium oxysporium f. sp. lycopersici* for the management of tomato wilt. Biol. Cont. 53: 24-31.
2. Kerkeni, A., M. Remadi, N. Tarchoun and M. Ben Khedher, (2007). In vitro and in vivo suppression of *Fusarium oxysporum f. sp. radicis lycopersici* the causal agent of *Fusarium* crown and root rot of tomato by some compost fungi. Int. J. Agric. Res. 2: 1022-1029.
3. Riccarda M., I. Pertota, Y. Elada and R. Raffaelli, (2008). Farmers' attitudes toward the use of biocontrol agents in IPM strawberry production in three countries. Biol. Cont. 47: 125-132.
4. Eziashi E. I., I. B. Omamor, E. E. Odigie, (2007). Antagonism of *Trichoderma viridae* and effects of extracted water soluble compounds from *Trichoderma* species and benlate solution on *Ceratocystis paradoxa*. Afr. J. Biotechnol. 6:388-392.
5. Hassan D. G., M. A. Ahanger, A. Nadeem and F. A. Ahangar, (2011). Management of root rot caused by *Rhizoctonia solani* and *Fusarium oxysporum* in blue pine (*Pinus wallichiana*) through use of fungal antagonists. Asian J. Plant Pathol. 5: 62-74.
6. EL- Bondkly A.M., F.N. Talkhan, (2007). Intra-strain crossing in *Trichoderma harzianum* via protoplast fusion to chitinase productivity and biocontrol activity. Arab J. Biotechnol., 10: 233 -240.
7. Hanson E. L. and C. R. Howell, (2002). Biocontrol efficacy and other characteristics of protoplast fusants between *Trichoderma koningii* and *T. virens*. Mycol. Res. 106:321-328.
8. Balasubramanian N. and D. Lalithakumari, (2008). Characteristics of protoplast inter, intra-fusant and regeneration of antagonistic fungi *Trichoderma harzianum* and *Trichoderma viride*. Afric. J. Biotechnol. 7: 3235-3243.
9. Savitha S. : S. Sadhasivam and K. Swaminathan. (2010). Regeneration and molecular characterization of an intergeneric hybrid between *Graphium putredinis* and *Trichoderma harzianum* by protoplasmic fusion. Biotechnol. Adv. 28: 285-292.
10. Hayat S. and C. Christias (2010). Isolation and fusion of protoplasts from the hytopathogeneic fungus *Sclerotium rolfsiiL* (SACC). Brazilian J. Microbiol. 41: 253-263.

11. Prabavathy V., N. Mathivanan, E. Sagadevan, K. Murugesan, D. Lalithakumari, (2006a). Self fusion of protoplasts enhances chitinase production and biocontrol activity in *Trichoderma harzianum*. Bioresour. Technol., 97: 2330- 2334.
12. Stasz T.E., G.E. Harman and N.F. Weeden, (1988). Protoplast preparation and fusion in two biocontrol of *Trichoderma harzianum.* Mycol., 80: 141 - 150.
13. Watanapokasin R., N. Sawasjirakij, S. Usami and K. Kirimura, (2007). Polyploid formation between *Aspergillus niger* and *Trichoderma viride* for enhanced citric acid production from cellulose. Appl. Biochem. Biotechnol. 143: 176-186.
14. Salama S. A. and A. F. Tolba, (2003). Identification of *Trichoderma reesei* using specific molecular marker and their ability as biocontrol agent. Egypt. J. Genet. Cytol. 32: 137-151.
15. Hermosa M.R., I. Grondona, E.A. Iturriaga, J.M. Diaz Minguez, C. Castro, E. Monte and I. G. Acha, (2001). Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. Appl. Environ. Microbiol., 66: 1890 - 1898.
16. Lee C. F. and T. H. Hseu, (2002). Genetic relatedness of *Trichoderma* sect. *Pachybasium* species based on molecular approaches. Can. J. Microbiol. 48: 831-840.
17. Kumar A. and P. Sharma, (2011). Molecular and morphological characters: An appurtenance for antagonism in *Trichoderma* spp. Afr. J. Biotech. 10: 4532-4543.
18. Papavizase G. C., D. P. Roberts and K. K. Kim, (1990). Development of mutants of *Gliocladium virens* tolerant to benomyl. Can. J. Microbiol. 36: 484 - 489.
19. Toyama H., K. Yamaguchi, A. shinmyo and H.J. Okada, (1984). Protoplast fusion in *Trichoderma reesei* using immature conidia. Appl. Environ. Microbiol., 47: 363 - 368.
20. Pe'er S. and I. Chet, (1990). *Trichoderma* protoplast fusion: a tool for improving biocontrol agents. Can. J. Microbiol., 36: 6 - 9.
21. Al-Samarrai T.H. and J. Schmid, (2000). A simple method for extraction of fungal genomic DNA. Lett. Appl. Microbiol. 30: 53 - 56.
22. Salama S.A., G.A. Amer and SH. M. El-Desouky, (2000). Phylogenetic relationships among *Trichoderma* species using molecular markers to assess their efficiency as biocontrol agents. Egypt. J. Genet. Cytol. 31: 253 – 266.
23. Morgan L. Vis, (1999). Intersimple sequence repeats (ISSR) molecular markers to distinguish gametophytes of *Batrachospermum boryanum* (*Batrachospermales, Rhodophyta*). Phycologia. 38(1): 70-73.
24. Fokkema N.J. (1973). The role of saprophytic fungi in antagonism against *Drechslera sorokiniana (Helminthosporium sativurri)* on agar plates and on rye leaves with pollen. Physiol. Plant Pathol. 3:195-205.
25. Elad Y., I. Chet and J. Katan, (1980). *Trichoderma harzianum*: A Biocontrol Agent effective Against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathol. 70:119-121.
26. Hatvani L., L. Manczinger, L. Kredics, A. Szekers, Z. Antal and C. Vagvolgyi, (2006). Production of *Trichoderma* strains with pesticide-polyresistance by mutagenesis and protoplast fusion. *Antonie Van Leeuwenhoek*. 89: 387-393.
27. Gunashree B. S. and G. Venkateswaran, (2010). Enhanced phytase production through interspecific protoplast fusion of *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 auxotrophic mutants. Enz. Microbiol. Technol. 46: 562-567.
28. EL-Bondkly A. M., A. M. Aboshosha, N. H. Radwan and S. A. Dora, (2011). Application and comparison of two different intraspecific protoplast fusion methods in *Trichoderma harzianum* and their effect on β-glucosidase activity. Afr. J. Biotechnol. 10: 10683-10690.
29. Prabavathy V.R, N. Mathivanam, E. Sagadevani, K. Murugesan, D. Latithakumari, (2006b). Intra-strain protoplast fusion enhances carboxymethyl cellulase activity in *Trichoderma reesei*. Enz. Microbial. Technol., 38: 719- 723.

4/12/2012