Improvement of Biological Control Activity in *Trichoderma* Against Some Grapevine Pathogens Using Protoplast Fusion

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Abstract: Protoplast was prepared from two mutant isolates of *Trichoderma* characterize with fungicide tolerant, PTk-C and PTv-T. The mutants were selected after EMS/UV treatment of *Trichoderma* isolates that obtained from grapevine farms in Taif Government, KSA. The protoplasts were obtained from 18 h old mycelium digested by Novozyme 234 and then fused by polyethylene glycol (PEG). The frequency of fusion resulting in double fungicide tolerant isolates was about 0.23 % and six fusants were selected for further studies. The phylogeny of the parental strains was carried out using sequence of 5.8S-ITS region. The BLAST of the obtained sequence was identified these isolates as *T. koningii* and *T. viride*. The fused protoplasts of the two mutant strains have been regenerated on carboxymethyl cellulase agar medium supplemented with the two fungicides Carbamate and Topsin-M. Most of the fusants exhibited fast mycelial growth on PDA as compared to parent strains. CMCase activity of fusants indicated the high level extracellular carboxymethyl cellulose than parents. Two-fold increase in enzyme activity of β-glocanase was recorded with the two fusants, Fu.3 and Fu.5 as compared to the parental strains. The protein pattern of extracellular cellulase enzymes in parents and fusants were obtained using SDS-PAGE. The resulted pattern revealed a new recombinant protein that may be indicated the possibility of partial or incomplete genetic recombination during nuclear and cytoplasmic protoplast fusion. Most of the fusants have shown powerful antagonistic activity against the grapevine pathogens *Pythium ultimum* and *Fusarium roseum.* Results of the present study demonstrated the scope and significance of the protoplast fusion technique, which can be used to develop superior hybrid strains of filamentous fungi and enhance biological control activity against some grapevine pathogens.

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Keywords: *Trichoderma*; Protoplast fusion; β-glocanase, CMCase activity, antagonistic activity, grapevine pathogens

1. Introduction

Trichoderma koningii and *T. viride* are known producer of cellulolytic enzymes **Feng *et al.*, (2011)**, that are extensively used for the degradation and other processes of cellulose materials particularly in textile and paper industries besides, it is also used for wastewater treatment **Prabavathy *et al.*,(2006)**. Fungal protoplasts are important tools in physiological and genetic research, as well as genetic manipulation which can be successfully achieved through fusion of protoplasts in filamentous fungi that lack the capacity for sexual reproduction (**Hayat and Christias 2010; Lalithakumari, 2000**). Hence, protoplast fusion is one of the important approaches in the strain improvement programme **Hassan, *et al.*, (2011)**. Isolation, fusion and regeneration of protoplasts have been achieved in the genus *Trichoderma* mainly to enhance its cellulolytic activity (**EL-Bondkly, 2006 and EL-Bondkly *et al.*, 2010**). However, limited attempts were made to improve the strain of *Trichoderma* to enhance enzyme production (**Prabavathy, *et. al.*, 2004 and EL-Bondkly *et al.*, 2010**). Hence, there is ample scope for strain improvement in *Trichoderma* utilizing this technique for enhancing the enzyme production.). However, limited attempts were made to improve the strain of *Trichoderma* to enhance enzyme production (**Prabavathy, *et. al.*, 2006 and EL-Bondkly *et al.*, 2010**).Accordingly; there is ample scope for strain improvement in *Trichoderma* utilizing this technique for enhancing extra-cellular enzymes and antagonistic activity especially against some grapevine pathogens. Several molecular techniques used to characterize fungi species were reported, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and isozymes analysis (**Lieckfeldt *et al.*, 2002; Migheli *et al.*, 2009**). Furthermore, sequence analysis of the nuclear ribosomal internal transcribed spacer of rDNA (ITS-rDNA region) is one of the famous methods among these molecular characterization techniques. The

rRNA genes are universally conserved, while the ITS region and intergenic spacer (IGS) are highly variable. Sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE is an important molecular technique used for the identification at species level of whole cell proteins and extracted enzymes as cellulase, chitinase and Pectinase, it has the advantage of being fairly simple and rapid to perform. But for the identification this technique requires extensive data to cover all known target species (**Sharma and Shanmugam, 2011**), with this background, the present work was aimed to1) isolation and molecular identification of *Trichoderma* spp. isolated from grapevine farmers in Taif Government. 2) Isolate and fuse the protoplasts from *T. koningii* and *T. viride* 3) Enhances the extracellular carboxymethyl cellulase (CMCase) production in the fusant progenies. Evaluate the fusants antagonistic activity against grapevine pathogens *Pythium ultimum* and *Fusarium roseum*.

2. Material and Methods

Trichoderma isolation and mutants induced:

The two parental strains *Trichoderma koningii* and *Trichoderma viride* were used in this study isolated from grapevine farms in Taif, KSA according to diluted method. Fungicide tolerant mutants PTk-C (Carbamate tolerant) and PTv-T (Topsin-M tolerant) were induced by EMS/UV treatment, according to **Salama and Tolba (2003)**. Fungicides tolerance was used as marker for protoplast fusion programme

Molecular characterization of parental strains:

Genomic DNA Isolation:

Fungal mycelia of the selected fungus strains were inoculated onto PDA broth for five days. Genomic DNA for each *Trichoderma* strain was extracted using DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.

PCR amplification of ITS region:

ITS1 and ITS2 regions together with 5.8S gene in rRNA from *Trichoderma koningii* and *Trichoderma viride* were amplified as designed by **Hermosa *et al*., (2000).**

Sequence analysis of 5.8S-ITS region:

The nucleotide sequences of 5.8S-ITS region were determined using the sequencer (Gene analyzer 3121). The deduced sequence was aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5.10. The forward and reverse sequences were checked and edited manually when needed. Then, a consensus sequence was generated from each alignment made. The sequencing data were compared against the Gene Bank database (http://www.ncbi.nlm.nih.gov/BLAST/), where a nucleotide blast program was chosen to identify the homology between the PCR fragments and the sequences on the Gene Bank database.

Protoplast preparation:

Protoplasts preparation was carried out using fungicide tolerant isolates of *Trichoderma* according to **Stasz *et al.,* (1988)**.

Protoplast fusion:

Protoplasts were fused using a procedure similar to that described by **Pe′er and Chet (1990)**. 1 ml of the suspension containing 10^6 protoplasts in STC buffer (0.6 M sorbitol; 10 mM CaCl2; 10 mM Tris-HCl at pH 7.5) was prepared and equal number of protoplasts from *T. koningii* and *T. viride* strains was mixed. To this 200 µl of 40% (w/v) polyethylene glycol (PEG, MW 6000; Sigma Chemicals Co., St. Louis, USA), 10 mM CaCl2 and 10 mM Tris-HCl, (pH 7.5) was added and gently mixed by rolling the tube. This step was repeated twice and the mixture was incubated at 28°C for 10 min with 1.1 ml of STC buffer by mixing gently. These dilution steps were repeated two times and 2.2 ml of STC was added. After the fusion and dilution, protoplasts were recovered by centrifugation at 10,000 rpm for 1 min and suspended in 5 ml STC. The interfused protoplasts were serially diluted in STC and plated on selective media.

Protoplast regeneration:

Protoplast regeneration was estimated according to **Prabavathy, *et. al*., (2006)**. Fused protoplasts of parental isolates of *Trichoderma* were collected by centrifugation at 100 rpm for 10 min. These fused protoplasts were suspended in 100 µl of STC buffer and plated on 2 % CMCA medium supplemented with fungicides. The plates were incubated at room temperature and the regenerated colonies were isolated and subcultured on CMCA and PDA. The fusion protoplasts were also incubated with CMC broth containing 0.6 M KCl as osmotic stabilizer for microscopic observation and photographs were taken using digital camera microscope.

Enzymes assay:

The fusant and parental isolates of *T. koningii* and *T. viride* were examined for the production of extracellular CMCase and β-glucosidase according to **Prabavathy *et. al*., (2004)**. The CMCB medium consisting of (g l^{-1} of distilled water) CMC, 5.0; $NaNO_3$, 2.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4$, 0.5 and $FeSO_4$, 0.01, pH 6.5 was used for growing *Trichoderma*. All the isolates were grown in 50 ml of CMCB in 250 ml Erlenmeyer flask. Each flask was inoculated with 1 ml conidial suspension (1×10^7 conidia ml^{-1}) and incubated at room temperature on a rotary shaker at 100 rpm. Triplicate flasks were maintained for each isolate. After 8 days, the cultures were harvested, filtered through Whatman no. 1 filter paper in a glass funnel and the

culture filtrates were centrifuged at 10,000 rpm at 4 °C. The cell free culture filtrates were used as enzyme sources for CMCase assay.

CMCase and β-glucosidase activities

The CMCase and β-glucosidase activities were determined according to **EL-Bondkly *et al*., (2010)** as the following, 1 ml of culture supernatant was added to 1 ml of 1 % carboxymethyl cellulose (1 ml of 1 % salicin in the case of β-glucosidase) and 1 ml of 0.1 M citrate buffer (pH 4.8) incubated for 30 min. at 50 °C. The reaction was terminated by adding 3 ml of 3, 5-dinitrosalicilic acid (DNS) reagent and boiling for 15 min then 1 ml of 40 % sodium potassium tartarate was added, cooling and then absorbance was measured at 540 nm. One unit enzyme activity was defined as the amount of enzyme that produced 1 μM of reducing sugars ml^{-1} min^{-1} from CMC under standard assay conditions using glucose as standard.

Antagonistic test against some grapevine pathogens:

The antagonistic effects of each *Trichoderma* sp. and their fusants against *Pythium ultimum* and *Fusarium roseum* were tested according to the method described by **Fahmi *et al*., (2012)**. The percentage of inhibition (I %) on the mycelial growth of Pathogens were calculated using this formula: Percentage of inhibition (I %) = [(R1 - R2)/ R1] × 100 Where, R1 = radius of the pathogen away from the antagonist and R2 = radius of the pathogen.

Protein estimation:

The protein content in the culture filtrates was estimated by the dye-binding method of **Bradford (1976)**. The amount of protein was calculated using Bovine Serum Albumin as standard.

SDS-PAGE gel electrophoresis:

Cellulase protein pattern was done according to **Salama and Tolba (2003)**. Extracellular cellulases enzyme was recovered from culture supernatant after addition an equal volume of methanol and tenth volume of chloroform. Centrifugation at 15000 rpm for 10 min at 4°C was made. The protein was precipitated by addition 1 ml of methanol to the lower phase. Then protein was pelleted and resuspended, after air drying, in Laemmli sample buffer (64 % of 0.15 M Tris-HCl buffer, pH 6.8. 20 % glycerol, 6 % sodium dodecyl sulfate (SDS), 10 % 2-6-mercaptoethanol and 0.1% bromophenol blue). Twenty-microliter samples were subjected to electrophoresis.

3. Results

Phylogeny of the *Trichoderma* isolates based on 5.8S-ITS region:

Data from 5.8S-ITS region analysis is useful in establishing the coarse scale phylogeny of ascomycetes and for sorting them into statistically supported family-level groupings. We therefore used 5.8S-ITS sequence analysis to establish the origin of *Trichoderma* isolates within the ascomycetes. An approximately 600 bp of 5.8S-ITS rDNA fragment was successfully amplified and sequenced from both parental *Trichoderma* isolates Figure (1). Next, we performed a BLAST search with the *Trichoderma* spp. 5.8S-ITS rDNA gene to find most similar sequences in GenBank. The BLAST of the obtained sequence was identified these isolates as *T. koningii* and *T. viride*, respectively.

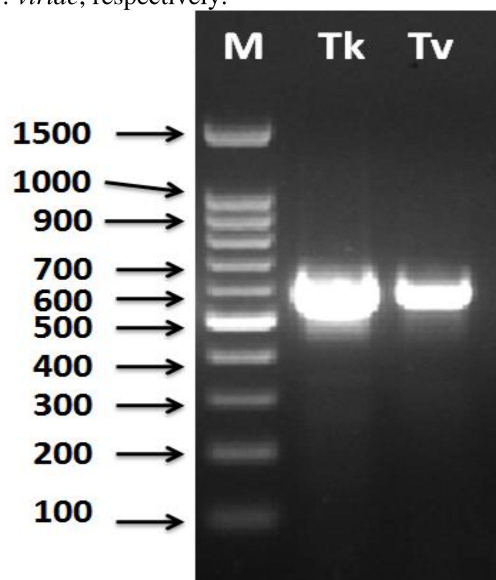

Figure (1): PCR products of 5.8S-ITS rDNA region from genus *Trichoderma koningii* (lane 1) and *Trichoderma viride* (lane 2). Positions and sizes of 100 bp DNA ladder are shown on the left side of the panel.

As can be seen from the Phylogenetic tree in Figure (2), the two parental isolates were almost 100% similarity with *Trichoderma koningii* NG-14 (accession No. HQ115672.1) and 99% similarity with *Trichoderma viride* T-9 (accession No. HQ259986.1).The tree is based on the results of distance matrix analyses of all available 5.8S-ITS region primary structures for *Trichoderma* species. The topology of the tree was evaluated by performing maximum parsimony and maximum Close-Neighbor-Interchange analyses of the full data set and subsets, respectively. Only sequences that were at least 98 % similarity were used for treeing (Figure 2). The phylogenetic positions of *Trichoderma* isolates presented by partial sequences were roughly reconstructed by applying the parsimony criteria without changing the overall tree topology. Multifurcations indicate that a common branching order was not significantly supported by applying different treeing methods.

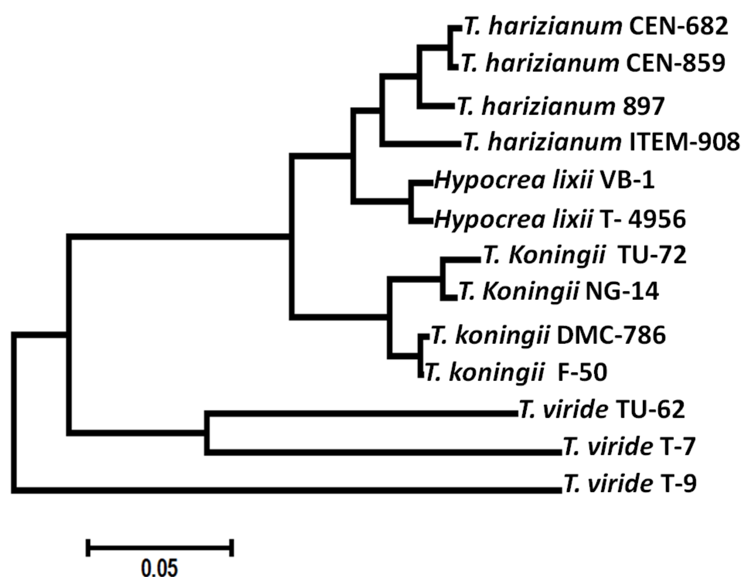

Figure (2): Phylogenetic tree and the diversity of 5.8S-ITS region sequences of two *Trichoderma* strains comparing with some *Trichoderma* strains. Phylogenetic tree was generated using parsimony neighbor-joining and maximum likelihood analysis

Isolation of protoplasts:

Incubation of *T. koningii* mycelium with Novozym 234 resulted in lysis of cell wall and release of protoplasts. Swelling and rounding up of cell content were observed initially and subsequently the *T. koningii* mycelium started lysing after 2 h. Almost complete digestion of mycelia and release of protoplasts occurred prominently after 3 h of incubation Figure (3). The protoplasts just released out of mycelium were smaller in size but later they slowly enlarged to a spherical structure.

Table (1): Number of protoplast fusant regeneration and the frequency of fusion.

Isolates	No. of colonies appearing on PRMM supplemented with				
	None	Car	Top	Car +Top	%
PTk-C	3.6×10^4	1.77×10^2	-	-	0.66
PTv-T	4.3×10^4		1.63×10^2	-	0.34
PTk-C X PTv-T	4.9×10^4	-	-	14	0.29

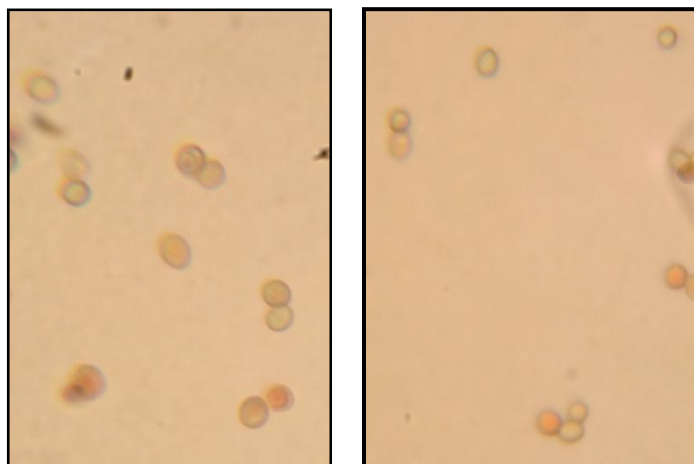

Figure (3): Released protoplasts from parent isolates observed after 3 h of incubation with lysis enzyme

Fusion of protoplasts:

When the protoplasts were mixed with PEG solution, they stuck together and pairs of protoplasts were observed. Later the plasma membranes in the place of contact of both the protoplasts dissolved and fusion of protoplasmic contents took place Figure (4). Subsequently the nuclei of the pairing protoplasts fused together (karyogamy) in many cases and in some cases, dikaryotic stage without nuclear fusion was observed. Finally, the fused protoplasts became single, larger and round or oval shaped structures.

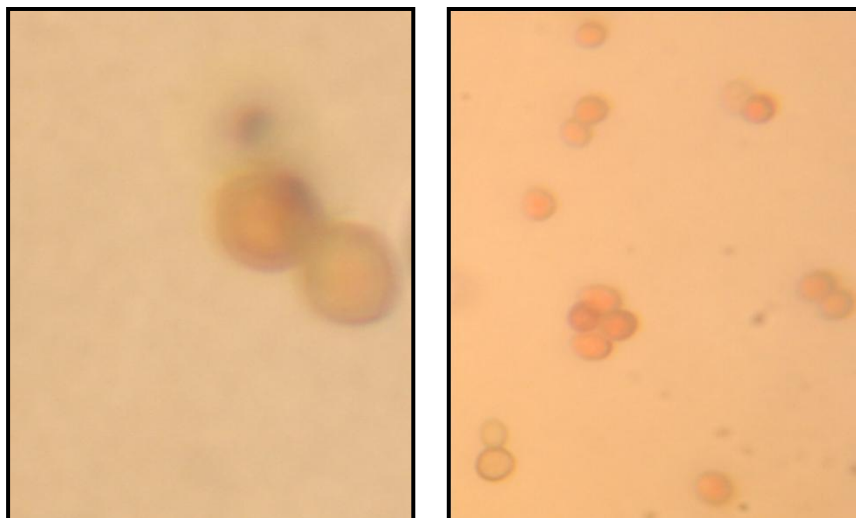

Figure (4): Fusion of protoplasts of *T. koningii* and *T. viride* after treatment with polyethylene glycol

Regeneration of fused protoplasts and selection of fusants:

The fused protoplasts of *T. koningii* and *T. viride* started regenerating after 2 days Figure (5), and developed mycelium after 3 days on CMC medium supplemented with fungicides. The colony development was observed after 4 days on 2 % CMCA. Based on the mycelial growth, six strong growing colonies of twelve fusants were selected and designated as Fus. 1 to Fus. 6. However, the non-fusion protoplasts could not germinate into colonies even after 3 days on selective medium. Two fusant regenerated colonies that appeared on 3th day after plating on selective medium were selected based on fast growth as compared to other colonies, which normally required more than 3 days. These were designated as Fus.3 and Fus.5.

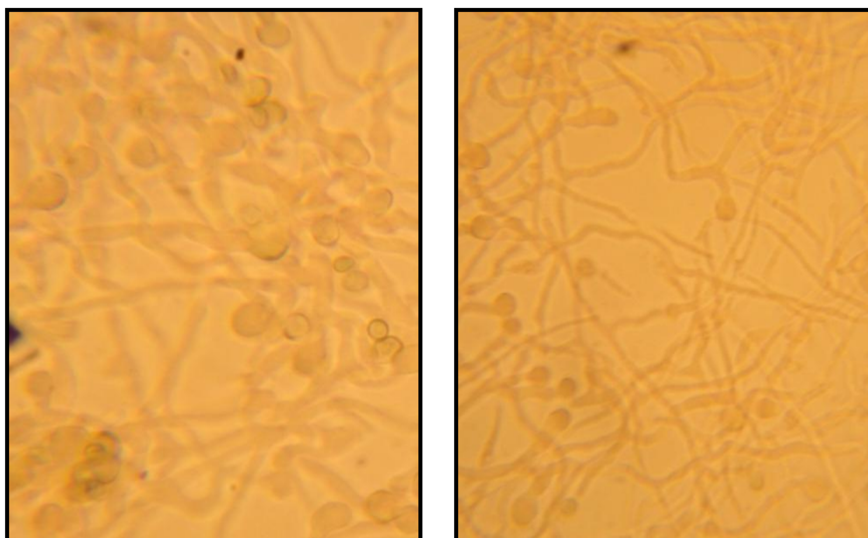

Figure (5): Regeneration of fused protoplasts of *T. koningii and T. viride*

Table (2): Protein content and CMCase activity in culture filtrates of the parents fusants.

Strains	Protein content (µg ml^{-1})	β-glocanase activity (U/ml)	CMCase activity (U/ml)
PTk-C	97^d	0.80^d	0.86^c
PTv-T	78^e	0.73^e	0.81^d
Fus. 1	102^c	0.42^f	0.55^f
Fus. 2	105^c	1.09^b	0.31^g
Fus. 3	162^a	1.66^a	1.87^a
Fus. 4	108^c	0.86^c	0.61^e
Fus. 5	137^b	1.61^a	1.07^b
Fus. 6	109^c	0.23^g	0.86^c

Values are mean of three replicates. Means within a column followed by the same letter(s) are not significantly different at the P= 0.05 level.

CMCase and β-glocanase activity in culture filtrates of the parents and fusants:

The CMCase activity remarkably increased in most (80%) of the fusants except fus. 1, fus. 2 and fus. 4. parents PTk-C and PTv-T. The maximum enzyme activity of 1.87 unit was estimated in culture filtrate of the fus. 3 and the minimum (0.31 unit) was recorded in fus.2. More than 1.5-fold increase in CMCase activity was recorded in one out of six selective fusants as compared to the parents. Interestingly the fus.3 registered two-fold increase in CMCase activity than the parents. Moreover, β-glocanase activty was estimated with 50 % of the fusants and more than two-fold increase in enzyme activity was recorded with two fusants, fu.3 and fu.5 as compared to the parental isolates Table (2).

Antagonistic activity against some grapevine pathogens:

Antagonistic effects of all *Trichoderma* parental and fusant strains were tested against *Pythium ultimum* and *Fusarium roseum* on PDA at 28°C for 7 days. In all the dual culture plates tested, the contact zone was a curve, with concavity oriented towards the pathogenic fungi. The averaged inhibition percentage (I %) of mycelial growth for grapevine pathogens were presented in Figure (6). All fused strains showed the ability to inhibit the mycelial growth of grapevine pathogens *P. ultimum* and *F. roseum.* However, Fus. 5 and Fus. 6 exhibited the lowest inhibition to the mycelial growth of *P. ultimum* with inhibition percentage of 86%, Fus. 5 exhibited the highest percentage of inhibition, 85%. Moreover, among all *Trichoderma* fused strains, Fus. 8 exhibited the lowest inhibition to the mycelial growth of *Fusarium roseum* with inhibition percentage of 34% while Fus. 5 exhibited the highest percentage of inhibition 86%.

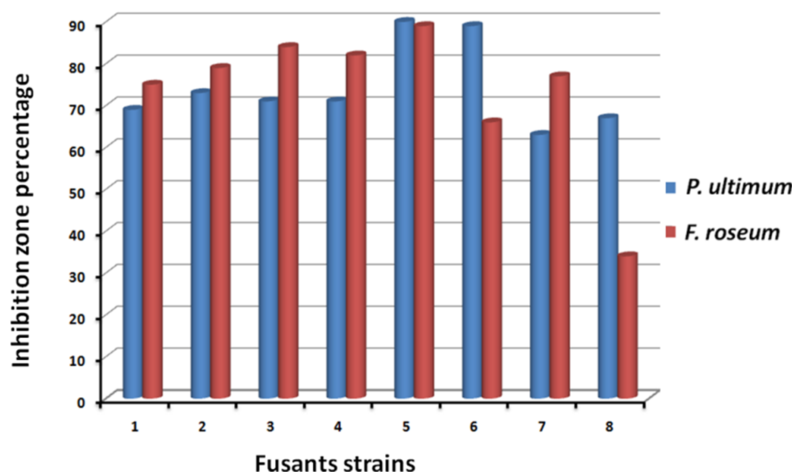

Figure (6): Antagonistic activity of *Trichoderma* parents and fusion against *P. ultimum* and *F. roseum,* 1and 2 = parental strains, 3-8 = fusants.

SDS-PAGE:

Electrophoretic banding patterns of SDS-PAGE of extracellular cellulases enzymes of parental and fusant isolates are shown in Figure (7). As expected small numbers of bands were detected. PTv-T and fusant isolates showed a significant band of ~ 67 KDa that was not observed in PTk-C parent isolate. This band previously obtained as endoglucanase I (EGI). However, PTk-C and PTv-T gave a significant band of ~ 40 KDa that identified as endoglucanase V (EGV) that was not observed in some fusants. In conclusion the obtained pattern gave a new recombinant protein due to new recombinant DNA. This indicated that partial or incomplete genetic recombination might have taken place during protoplast fusion.

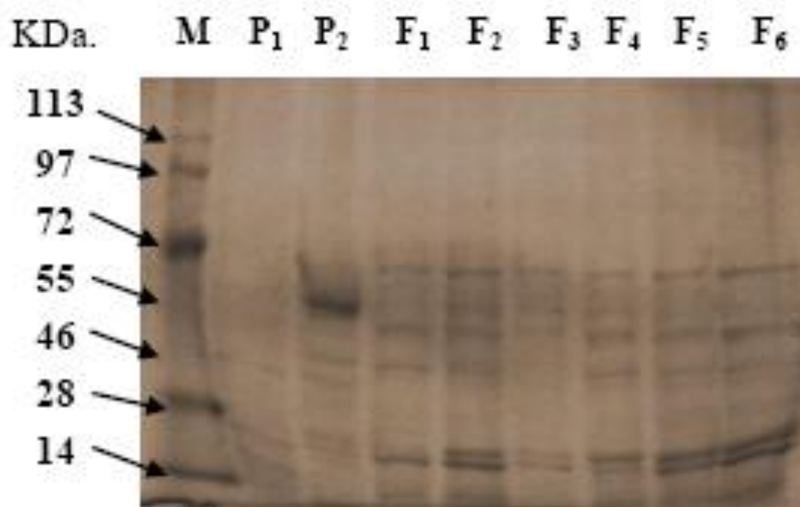

Figure (7): Polyacrylamide-gel electrophoresis of extracellular cellulases enzyme recovered from culture supernatant of *Trichoderma* grown on Mandel medium containing 1% acid-swollen cellulose powder.

4. Discussion

In this study, DNA sequencing of the 5.8S-ITS region of the selected two parental strains of protoplast fusion was carried out. The ITS region is one of the most reliable loci for the identification of a strain at the species level (**Kullnig-Gradinger *et al.,* 2002**). By comparing the sequences of the 5.8S-ITS region to the sequences deposited in GenBank database, all of the *Trichoderma* strains could be identified to species level with homology percentage of at least 99%. However, **Lieckfeldt *et al.*, (2002)** mentioned that GenBank database contain many sequences of *Trichoderma* strains that was recently used by many literatures and resulted in successful identification of *Trichoderma* strains (**Anees *et al.*, 2010 and Migheli *et al.*, 2009**). In addition, results obtained from the *Trich*OKEY were in agreement with the BLAST results. And identification of all strains was confirmed.

Protoplast fusion is an effective tool for bringing genetic recombination and developing superior hybrid strains in filamentous fungi **(Mrinalini and Lalithakumari 1998; Lalithakumari 2000)**. In this present study, a CMCase producing *T. koningii* and *T. viride* strains PTk-C and PTv-T were used for interspecific protoplast fusion programme with the aim of enhancing the extracellular CMCase production. Previously **EL-Bondkly (2006)**, demonstrated the interspecific crossing by protoplast fusion for genetic recombination in *Aspergillus niger* and *Trichoderma* Further, **Hassan *et al.*, (2011)**, achieved intra-specific hybridization in *T. harzianum*. The commercial Lysing enzymes (Sigma Chemicals Co.) at 8 mg ml^{-1} prepared in STC buffer was used to release the protoplasts from PTk-C and PTv-T with 0.6 M KCl as osmotic stabilizer. We have already optimized the conditions for releasing the protoplasts at our laboratory using different permutation combinations in various filamentous fungi including *Trichoderma* **Hassan *et al.*, (2011)**. Interestingly we observed that the release of protoplasts was significantly affected by the concentrations of Lysing enzymes. At low concentrations, the lysis of fungal mycelium took place only at the tip portion resulting in a minimum release of protoplasts whereas at high enzyme concentrations, though the mycelium effectively lysed, the protoplasts bursted immediately after release and disintegrated. Among different concentrations of Lysing enzymes tried, we optimized that 8 mg ml^{-1} with 0.6 M KCl as osmotic stabilizer to release higher number of protoplast from different *Trichoderma* spp. However, **Pe'er and Chet (1990)**, obtained highest protoplasts from *T. harzianum* using Novozym 234 at 10 mg ml^{-1} with 0.6 M KCl and **Tschen and Li (1997)**, used 15 mg ml^{-1} of Novozym with 0.6 M sucrose to isolate maximum protoplasts from *T. harzianum* and *T. koningii*. Further, **Balasubramanian *et al.*, (2003)**, obtained maximum number of protoplasts from *Trichothecium roseum* using Novozym 234 in combination with chitinase and cellulase each at 5 mg ml^{-1}.

Protoplasts fusion in *Trichoderma* has been achieved using 40 % PEG that was already reported as optimum concentration for interspecific fusion of protoplasts between *T. harzianum* and *T. longibrachiatum* **Mrinalini and Lalithakumari (1998)**. However, Pe'er and Chet (1990) used 33 % PEG for intra-specific protoplast fusion in *T. harzianum*. The concentration of PEG is highly critical for effective fusion of protoplasts. Higher

concentrations of PEG caused shrinking and bursting of protoplasts (**Lalithakumari, 2000; Lalithakumari and Mathivanan 2003**). The concentration between 40 and 60% was suitable for protoplasts fusion in different fungi (**EL-Bondkly 2006**).

The fusion and parental protoplasts were plated on high concentration 2% of CMCA suplimented with different fungicides for further selection. Though we observed an initial set back in growth of fusants, the colonies exhibited fast mycelial growth after 3 days. However, the parental protoplasts could not germinate on selective medium. Moreover, the protoplasts, which formed clumps, were not viable and failed to germinate into colonies as already reported **Lalithakumari (2000)**. Once subcultured, most of the inter fusants grew very fast on PDA as compared to the parents, which indicated the quicker adaptability of fusion strains in a newer environment. All the fusant strains grew luxuriantly and sporulated profusely than the parents. The intensity of yellow pigmentation in fusant strains was high as compared to parents as observed by **Priyasundari, (2002)**.

Although the growth of parental fusants of *T. koningii* and *T. viride* seems to be apparently similar in CMCA, the clear zone around the mycelium differed distinctly among them. It was prominent and larger in most of the fusants than the parents indicating the enhanced production of CMCase in those fusants and this could be directly related to strain improvement in *Trichoderma*. The increased production of β-glocanase was also confirmed by quantitative assays, in which more than two-fold increase in enzyme activity was recorded with two fusants. Although majority of the fusants had shown enhanced enzyme activity, few fusants exhibited decreased activity as compared to the parents. This indicated that partial or incomplete genetic recombination might have taken place during protoplast fusion, which could have led to negative effects in some fusants. The same results obtained by SDS-PAGE and new recombinant protein bands observed. The antagonistic capacities of all *Trichoderma* fusant isolates and their parents against *P. ultimum* were tested using dual culture method. In all the dual culture plates, the contact zone appeared as a curve, with concavity oriented towards pathogens. The curvature of the contact area between the colony of antagonistic fungi and the colony of pathogenic fungi in the same PDA plate depend on the growth rate of the colonies. If one colony has a faster growth rate than the other, a curve in the contact zone will most probably be observed. However, if the two colonies have the same growth rate, a straight line would be observed when mycelia from both fungi come into contact (**Hayat and Christias 2010; Petrescu *et al.,* 2012 and El-Refai *et al.,* 2013**). Moreover, all *Trichoderma* isolates exhibited inhibition to the mycelial growth of all pathogens. This could be due to the production of diffusible components, such as lytic enzymes or water-soluble metabolites **Anees *et al*., (2010)**.

The outcome of the present study has clearly demonstrated the scope and significance of the protoplast fusion technology for developing superior industrially important fungal strains as already reported by **Hassan *et al*., (2011)**. Furthermore, the inter protoplasts fusion in *T. koningii* and *T. viride* resulted in considerable increase of CMCase activity in most of the fusants and more than two-fold activity increase in two of the fusants has revealed the potential of strain improvement in *T. koningii* and *T. viride*. Hence, this technique can successfully be used to develop superior hybrid strains in filamentous fungi that lack inherent sexual reproduction.

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