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Full Length Article

Enhancing the Biocontrol Efficiency of *Trichoderma harzianum* JF419706 through Cell Wall Degrading Enzyme Production

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Abstract

The goal of this study was to enhance the biocontrol efficiency of *Trichoderma harzianum* JF419706 via its mycoparastic affinity as well as inducing the production of cell wall degrading enzymes. Different carbon and nitrogen sources were added to the growing medium of *T. harzianum* JF419706 to induce cell wall degrading enzymes. Production conditions of the target enzymes were optimized. The mycoparasitic affinity was induced by induction of the mutation of *T. harzianum* JF419706 by ethidium bromide and UV radiation. Results indicated that 4 days was appropriate to achieve the maximum production of the enzymes. The slight acidic pH (5.5–6.0) was more favorable for production of enzymes. The results exposed that chitin was responsible for improvement in the productivity of all tested enzymes, except the protease that was improved only by casein. All enzymes exhibited a high level of activity in media supplemented with yeast extract. Ethidium bromide in concentration either 0.5 or 1.0 mg mL^{-1} enhanced the mycoparasitic affinity of *T. harzianum* JF419706 more than UV radiation. However, both treatments enhanced the mycoparasitic affinity of the biocontrol agent compared with the wild type. This observation was confirmed by RAPD-PCR technique using primers chitinase (C15) and R2 that succeeded to differentiate among the mutants and the wild isolate. © 2016 Friends Science Publishers

Keywords: Chitinase; Glucanases; Mutation; Mycoparasitism; Protease; *Trichoderma harzianum*

Introduction

Plant diseases, caused by fungi, are one of the major challenges for agricultural food production sector. Soil-borne pathogens that attack most of the economically important crops resulting in great loss in their values (González *et al.*, 2012). Chemical pesticides are still used to control these phytopathogens. However, these fungicides are very harmful to the environment (Yulianti *et al.*, 2006). Moreover, biocontrol of soil-borne plant pathogens was emerged as a good substituted strategy. This technique is preferred to be used in large scale in plant protection against diseases in the organic farming.

Trichoderma species had been studied as biocontrol agents for many decades; however, a few strains have become available commercially. Different species of *Trichoderma* are used as biocontrol agents against many plant pathogens including soil-borne and foliar phytopathogens, in addition to its application as biofertilizer (Lumsden *et al.*, 1993; Monte, 2001). *Trichoderma* is characterized by its very active mycoparasitic affinity and induction of the system of defense in plants (Küçük and Kıvanç, 2008; Jayalakshmi *et al.*, 2009; Gajera *et al.*, 2011). Competition for space and nutrients, mycoparasitism, production of inhibitory compounds, induction of resistance and secretion of cell wall degrading enzymes are essential means of *Trichoderma* spp. as a bio-agent (Kapulnik and Chet, 2000). The role of degrading enzymes are very important in mycoparasitism process that enable *Trichoderma* to derive the nutrients from the host via haustoria (Kubicek *et al.*, 2001; Verma *et al.*, 2007; Saba *et al.*, 2012). Zeilinger and Omann (2007) reported that *Trichoderma* infect the hyphae of phytopathogenic fungi using prehensile coils and hooks that penetrate their cell walls. This process is assisted by the hydrolytic activity of chitinases, protease and glucanases. The activity and expression of these hydrolytic enzymes are generally vary with the different strains of *Trichoderma*, leading in turn to differences in performance when used as biocontrol agents.

Production of chitinase by *T. harzianum* was reported during mycoparasitism, however its productivity depended on fungal host and parasitic time (Haram *et al.*, 1996; ; Cheng et al., 2015). In all successful stories, *Trichoderma* isolates were used against a number of pathogens, however the case is completely different in nature. The complexity of presence of pathogens is nature could reduce the effectiveness of any biocontrol agent. To develop an efficient biocontrol agent that could suppress a wide range of pathogens or complex

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disease comprises a big challenge facing the researchers and producers.

Therefore, the chief objective of our study was to enhance the biocontrol efficiency of *T. harzianum* JF419706 against many soil-borne plant pathogens by induction its mycoparasitic activity as well as increasing the secretion of cell wall degrading enzymes.

Materials and Methods

Fungal Species

Trichoderma harzianum JF419706, as a biocontrol agent, and the soil-borne fungal pathogens (*Alternaria alternata*, *Exserohilum rostratum*, *Fusarium oxysporum*, *Pythium ultimum*, *Macrophomina phaseolina* and *Rhizoctonia solani*) were obtained during our previous work (Alamri *et al.*, 2012). These phytopathogenic fungi were recovered from naturally infected crops. We identified these fungi based on their macro and microscopic characteristics and the identification was confirmed by amplification of ITS gene using ITS1 and ITS4 primers and the method applied by Handschur *et al.* (2005) was followed.

Enzymes Production and Growth Conditions of *T. harzianum* JF419706

Trichoderma harzianum JF419706 was grown in Czapek salt minimal medium supplemented with 3% glucose. Flask containing 50 mL of the medium was seeded with 4-days old *T. harzianum* JF419706 was. The seeded flasks were incubated for 6 days at 27°C in a shaking incubator (200 rpm). Two mg mL^{-1} of the cell wall of *M. phaseolina* was used as the carbon source. To get the cell wall preparation from *M. phaseolina*, the procedures of Saligkarias *et al.* (2002) was applied. The growth rate of *T. harzianum* JF419706 was determined as a culture dry weight after Moita *et al.* (2005). After 6 days of incubation, culture of *T. harzianum* JF419706, the mycelia were removed by centrifugation for 20 min at 4000 rpm, and the supernatant was considered as the source of the enzymes. Colloidal chitin was obtained from shrimp shell chitin based on the procedures of Roberts and Selitrennikoff (1988).

Induction Cell Wall Degrading Enzymes Production of by *T. harzianum* JF419706

The productivity of cell wall degrading enzymes by *T. harzianum* JF419706 was enhanced by optimization of the incubation period, pH as well as addition of various carbon and nitrogen sources to the growing medium. *T. harzianum* JF419706 was grown in Czapek salt minimal medium. The growth and enzyme activity were measured daily (1–7 days). To obtain the most appropriate pH, at which the maximum productivity could be attained, the pH of the growing medium was adjusted at 4–9 and at 27°C for 4 days. In other experiment, the best carbon source was detected by growing the fungus in a medium containing 0.1% of either cell wall preparation from *M. phaseolina* (M), glucose, S-glucan, casein, laminarin or colloidal chitin as a carbon source. Also, the best nitrogen source was defined by addition 0.1% of either yeast extract, peptone, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl or KNO_3 to the growing medium of *T. harzianum* JF419706.

Evaluation of Enzymatic Activity

β-1, 3-glucanase activity test: The procedure of Masih and Paul (2002) was followed to determine the activity of β-1, 3-glucanase. The method is based on evaluation of reducing sugars released from laminarin. The culture filtrate of the fungus mixed with 2.5 mg of laminarin in 0.05 M potassium acetate buffer (pH 5.0) was incubated at 40°C for 2 h. Dinitrosalicylic acid reagent (0.5 mL) was added and the mixture was boiling for 5 min. after cooling. The absorbance was measured spectrophotometrically at 595 nm.

α-1,3-glucanase test: Activity of α-1,3-glucanase was determined according to the methods of Hasewaga *et al.* (1969) and Zonneveld (1971). Culture filtrate of the fungus with 0.2 mL of S-glucan (5 mg/mL) in 50 mM potassium acetate buffer (pH 5.5) was incubated at 40°C for 30 min. The mixture was boiled for 5 min and centrifuged 5 min at 500 rpm. The reducing sugars were determined in the supernatant with glucose as standard (Miller, 1959; Ait-Lahsen *et al.*, 2001).

Chitinase activity test: Dinitrosalicylic acid (DNS) method approved by (Miller, 1959) was applied to determine the activity of the enzyme. Culture filtrate of the fungus (0.5 mL) was mixed with 0.5 mL of 0.5% colloidal chitin and 1 mL distilled water in phosphate buffer (pH 5.5). The mixture was incubated for 1 h in a shaker water bath at 40°C. Then, 3 mL of DNS reagent was added to the reaction mixture and was boiling for 5 min. The absorbance was measured at 540 nm using UV spectrophotometer (UV-160 A, Shimadzu, Japan) and the enzyme activity was calculated according to Massimiliano *et al.* (1998).

Protease activity test: The protocol of Takami *et al.* (1989) was applied to determine the protease activity in the culture filtrate of *T. harzianum* JF419706. 1.5 mL of 1.0% casein, dissolved in glycine-NaOH buffer (50 mM, pH 7), was added to 0.5 mL of the culture filtrate of the fungus and incubated in a shaker water bath for 30 min at 40°C. Then, 2.5 mL of TCA solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid) was added to stop the reaction. The produced mixture was filtrated and 0.5 mL of the filtrate was mixed with 2.5 mL of 0.5 M Na_2CO_3 and 0.5 mL of Folin-Ciocalteu reagent. The absorbance was detected at 660 nm.

Induction of Mycoparsitic Affinity of *T. harzianum* JF419706 by Mutation

The random mutation of *T. harzianum* JF419706 was carried out using UV as a physical method and ethidium

Table 1: Primers sequence used in RAPD study primer sequence

Primer name	Sequence 5` to `3	Annealing Temp.
C15	CAG GCC CTT CCA GCA CCCAC	43°C
R2	GAG CCA SGC SGT CCA RTC SGG CCA CCA	45°C

bromide as a chemical method. The mycoparasitic affinity of the mutated *T. harzianum* JF419706 was examined against the pathogenic fungi in dual cultures according to the method of Adelberg *et al.* (1965). *T. harzianum* JF419706 culture was mutated by inoculation Czapek medium containing different concentrations of ethidium bromide (0.25, 0.5 and 1.0 mg/mL) with 0.5 cm hyphal disc from fresh 4 d old culture and incubation the plates at 27°C for 4 d. The mutated stains were cultivated on the solid medium against the pathogenic fungus and the clear zones were measured. *T. harzianum* JF419706 was also mutated physically by using UV with 260 nm. Spore suspension of the fungus was subjected to the UV lamp for different periods (1–10 min). The spore suspension was incubated for 30 min at 30°C in water bath. Then, the cells were harvested by centrifugation at 5000 rpm for 2 min. The produced pellets were washed in phosphate buffer (pH 7.0). The mutated spores were cultivated on the solid medium against the pathogenic fungus and the mycoparasitic potentiality was estimated visually and by aiding the light microscope.

Detection of the Genetic Variability between the Mutated and Wild Isolates

DNA was extracted from *T. harzianum* JF419706 (wild and mutant) isolates following the method of Araujo *et al.* (2004). Four different primers (Table 1) were used to carry out the RAPD-PCR on the extracted DNA using the protocol of Ausbel *et al.* (1995). PCR product was analyzed in agarose gel stained with ethidium bromide. Gel Documentation system (Alpha Imager TM1220, Documentation and Analysis system, Canada) was used to photographed the gels. The phylogeny tree of *T. harzianum* JF419706 isolates was done using the program (Statistica® Version 5).

Statistical Analysis

Statistical program SPSS 16.0 was used to perform the analysis of variance (ANOVA) and means were compared by the least significant difference (LSD) ($P < 0.05$).

Results

Results clearly revealed that the maximum growth and productivity of glucanases by *T. harzianum* JF419706, in Czapek medium + 3% glucose, were achieved after 4 days of incubation (Fig. 1), whereas the maximum chitinase production was achieved after 3 days of incubation. However, protease enzyme was not detected. The enzyme productivity declined after the fourth day of incubation.

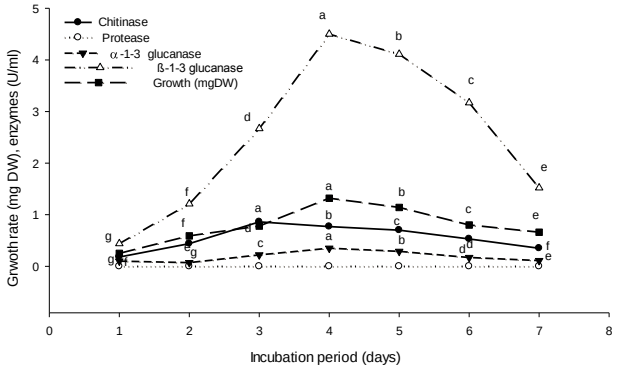

Fig. 1: Effect of incubation periods (in days) on *T. harzianum* JF419706 grown on cell wall preparation and glucose containing medium at 27°C and pH 5. Symbols on the same line followed by different letters are significant different at $P < 0.05$

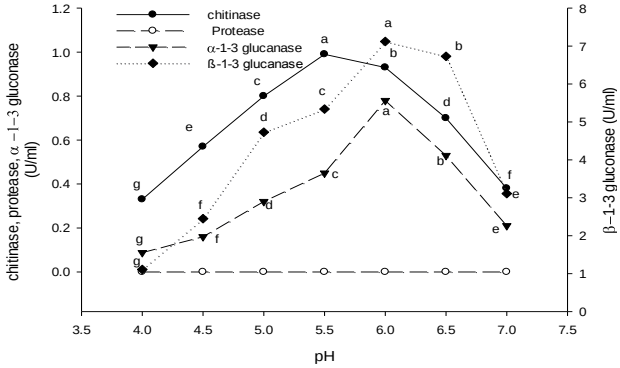

Fig. 2: Effect of pH on *T. harzianum* JF419706 grown on cell wall preparation and glucose containing medium at 27°C after 4 days of incubation. Symbols on the same line followed by different letters are significant different at $P<0.05$

The optimum pH for chitinase production by *T. harzianum* JF419706 was 5.5. At this pH, the fungus produced 0.99 U/mL of chitinase (Fig. 2). Both α-1,3-glucanase and β-1,3-glucanase exposed their maximum activity at pH 6, however, protease was not detected at any pH vlaue.

To induce the enzyme productivity, *T. harzianum* JF419706 grown in the Czapek medium was amended with different carbon sources as mentioned above. The results revealed that *T. harzianum* JF419701 produced both glucanases and chitinase on all carbon sources, but in different concentrations (Fig. 3). β-1,3-glucanase was significantly enhanced by the addition of S-glucan or colloidal chitin. The peak of production of α-1,3-glucanase

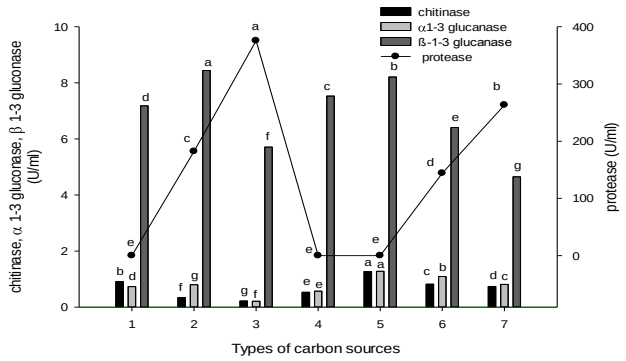

Fig. 3: Effect of carbon sources on enzyme production by *T. harzianum* JF419706 at 27°C, pH 6 after 4 days of incubation. 1= M + Glucose, 2= M + S-glucan, 3= M + Casein, 4= M + Laminarin, 5= M + Colloidal chitin, 6= M + S-glucan + Casein + Laminarin + Colloidal chitin, 7= M + S-glucan + Casein + Laminarin + Colloidal chitin + Glucose. M= cell wall preparation from *Macrophomina phaseolina*. Columns in the same color and symbols on the line followed by different letters are significant different at $P < 0.05$

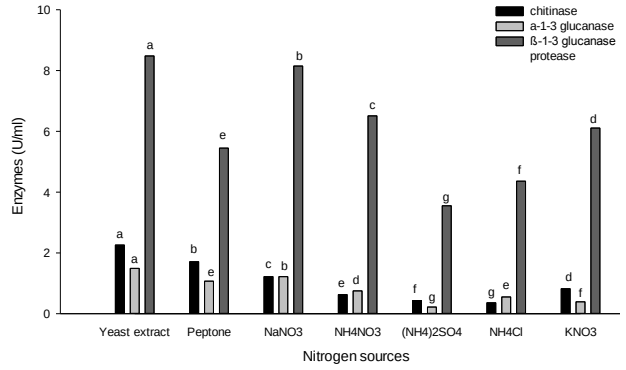

Fig. 4: Effect of different nitrogen sources on production of enzymes by *T. harzianum* JF419706 at 27°C, pH 6 after 4 days of incubation. Columns in the same color followed by different letters are significant different at $P < 0.05$

and chitinase was achieved when colloidal chitin was applied. Both S-glucan and casein, when added singly or combined with other carbon sources, stimulated the production of protease; however, casein was the most appropriate source.

T. harzianum JF419706 was able to utilize all nitrogen sources and produce all enzymes in considerable concentrations, except for protease. In the absence of casein, the fungus did not produce any detectable concentration of protease (Fig. 4). Yeast extract was responsible for the maximum production of both glucanases and chitinase.

Induction of mutation of *T. harzianum* JF419706 was performed chemically by ethidium bromide and physically by UV radiation. Results showed that exposure of *T. harzianum* JF419706 to UV for 3 or 5 min enhanced its

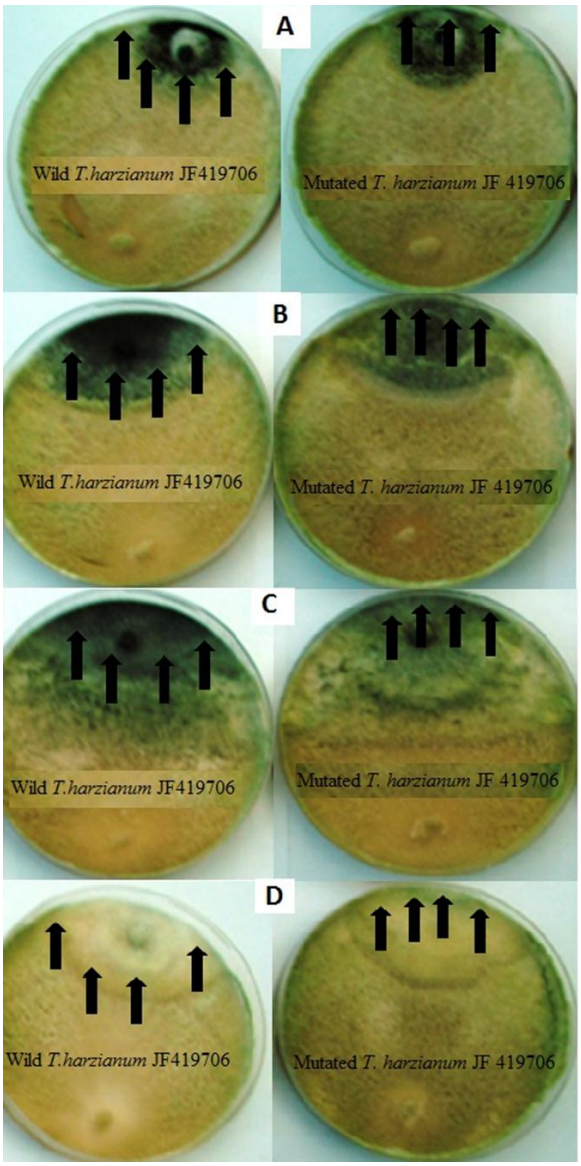

Fig. 5: Comparison between the mycoparasitic affinity of wild *T. harzianum* JF419706 and its mutated strain (using ethidium bromide) against phytopathogenic fungi: *Alternaria alternate* (A), *Exserohilum rostratum* (B), *M. phaseolina* (C) and *Pythium ultimum* (D). The black arrows point to the maximum reach of *T. harzianum* JF419706 over the target pathogen. In wild strain, a wide clear zone of un-colonized surface of each pathogens is shown, however, in case of the mutated stain all surfaces of the pathogens are completely overgrown by the hyphae of *T. harzianum* JF419706 after 4 days of incubation

mycoparasitic activity against the tested phytopathogenic fungi. In dual culture, the mutated *T. harzianum* JF419706 covered the whole colonies of phytopathogenic fungi faster and more competent than the wild type, and reduced the diameter of the target fungal colony (Table 2). It seemed that ethidium bromide either in the

Table 2: Effect of random mutation using ultra violet and ethidium bromide on the effectiveness of *T. harzianum* JF419706 against phytopathogens

Treatment		Phytopathogens											
		A. alternata		*E. rostratum*		*F. oxysporum*		*M. phaseolina*		*P. ultimum*		*R. solani*	
		R	M	R	M	R	M	R	M	R	M	R	M
Control		1.90	++	2.25	++	2.00	+	3.85	++	2.50	++	4.00	++
Ultra violet	1 min	1.85	++	2.00	++	1.90	+	3.75	++	2.60	++	4.00	++
(exposure rate)	3 min	1.85	+++	1.85	+++	1.75	++	3.75	+++	3.00	+++	3.75	+++
	5 min	2.00	+++	2.00	+++	1.70	++	3.00	+++	3.00	+++	4.00	+++
Ethidium bromide	0.25	1.75	+++	1.85	+++	1.75	++	3.25	+++	2.25	+++	4.00	+++
(mg/ml)	0.50	1.75	+++	2.15	+++	1.65	++	2.85	+++	2.25	+++	4.00	+++
	1.00	1.65	+++	2.05	+++	1.65	++	2.70	+++	1.90	+++	3.75	+++
LSD ($P < 0.05$)		0.063		0.17		0.065		0.058		0.154		0.20	

R= mean radius of the colony, M= mycoparasitism (+; up to one-third of colony covered by hyphae of *T. harzianum*, ++; up to two-third of colony covered hyphae of *T. harzianum*, +++; colony is completely covered with hyphae of *T. harzianum*)

concentration of 0.5 or 1.0 mg mL^{-1} enhanced the mycoparasitic activity of *T. harzianum* JF419706 more than UV induction (Fig. 5).

Primers chitinase (C15) and R2 succeeded to differentiate between the wild *T. harzianum* JF419706 isolate (un-mutated) and its mutants, which was initiated by ethidium bromide and UV exposure (Fig. 6, 7). About 72 bands were obtained with different molecular weight. The molecular size of the obtained band ranged from 800 bp to 50 bp. Five monomorphic bands were observed out of the 72 bands. The genetic variability was noticed in the case of R2 primer when 0.5 and 1.0 mg mL^{-1} of ethidium bromide were used, however only 0.5 mg mL^{-1} of ethidium bromide gave the same behavior in case of C15. When UV was used as a mutation source, the exposure time 3 and 5 min involved the production of differentiable monomorphic bands with primer C15 and R2. Analysis of genetic variability obtained from RAPD-PCR reflected the same picture of phenotypic variation, where, all mutants that exhibited stimulatory effect against the pathogens had genetic variation compared with the wild type.

Discussion

Secretion of the cell wall degrading enzymes by *Trichoderma* species is the key factor in the success of its biological control activity against the phytopathogens, because these enzymes (chitinase, glucanases and protease) lysis the cell wall of the pathogen and facilitate the mycoparsitism by *Trichoderma* spp. (El-Katatny *et al.*, 2003; Gajera *et al.*, 2011). Our results approved that the productivity of the cell wall degrading enzymes by *T. harzianum* JF419706 could be induced by growing *T. harzianum* JF419706 on specific carbon or nitrogen source. Also, the pH and incubation period have a considerable effect on the enzymes productivity. The activity of β-1,3-glucanase was significantly enhanced by the addition of S-glucan or colloidal chitin. The maximum activity of α-1,3-glucanase and chitinase was achieved when colloidal chitin was added. Casein was the most appropriate source that induced the production of protease. El-Katatny *et al.* (2003), reported that the addition of chitin or dried fungal mycelium as adjuvant enhanced the enzyme production up to 2 and 5 fold for β-1,3-glucanase and chitinase, respectively. San-Lang *et al.* (2002) reported that chitin is a vital source for induction of antifungal activity. Šimkovič *et al.* (2008) found that the secretion of proteolytic activity by *T. viride* is induced in the presence of 1% pure protein. They also reported that the secretion of proteases by *T. viride* is not only a phenomenon triggered by nutrition status of fungus and/or starvation, but it is a specific reaction of the fungus induced by recognition of some protein(s) in the environment. The differences in their nutritive value (i.e. composition of proteins) might explain the quantitative differences in the induction of extracellular proteolytic activity (Šimkovič *et al.*, 2008). Szekeres *et al.* (2004) and Kredics *et al.* (2005) indicated that proteases is an important element that enhances the mycoparasitism of different strains of *T. harzianum*. *T. harzianum* JF419706 was not able to produce any detectable concentration of protease unless casein as a nitrogen source was added. This observation could support the assumption that a nitrogen source is needed to stimulate the production of protease by this fungus (El-Katatny *et al.*, 2003). Other enzymes were produced in the presence of any other nitrogen source, however, yeast extract was responsible for the maximum production of both glucanases and chitinase. Šimkovič *et al.* (2008) mentioned that nitrogen is very important element for either microbial growth or enzyme productivity, especially yeast extract that functions as a source of vitamins for fungus and it does not have a crucial effect on protease secretion.

We detected the maximum productivity of both glucanases after 4 days, and chitinase after 3 days of incubation. After the fourth day of incubation, the enzymes productivity was greatly declined. These findings are in partial agreement with those of other researches, who dealt with optimization process for the production of extracellular metabolites (Prapagdee *et al.*, 2008). In some exceptional cases, El-Katatny *et al.* (2003) found that 12 d was the appropriate time for chitinase production, and 10 d was optimum incubation time for production of β-1,3-glucanase. El-Katatny *et al.* (2001) reported that glucanases and chitinases inhibited the growth and spore germination of

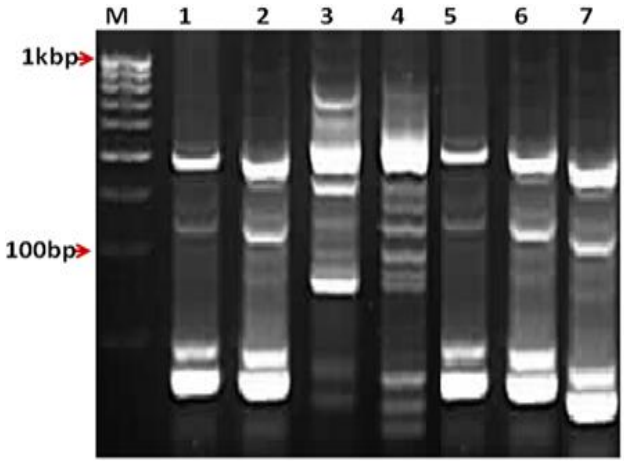

Fig. 6: RAPD-PCR for the mutated and the wild *T. harzianum* JF419706 isolate using R2 primer as arbitrary primer. Lanes; M: 1 kb ladder, lane 1: wild fungus, lane 2: Eth 0.25 mg mL^{-1}, lane 3: Eth 0. 5 mg mL^{-1} lane 4: Eth 1 mg mL^{-1}, lane 5: UV exposure for 1 min, lane 6: UV exposure for 3 min, lane 7: UV exposure for 5 min

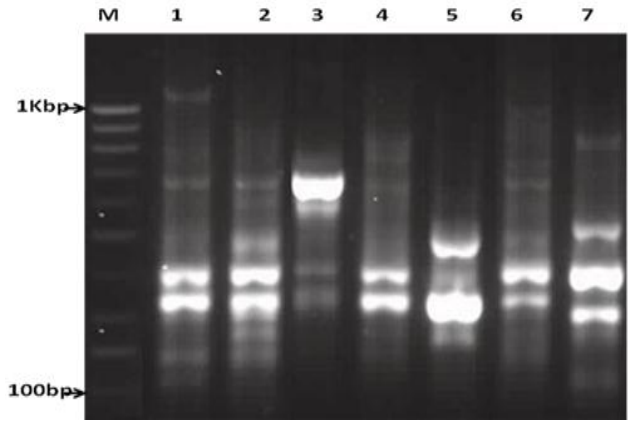

Fig. 7: RAPD-PCR for the mutated and the wild *T. harzianum* JF419706 isolate using C15 primer as arbitrary primer. Lanes; M: 1 kb ladder, lane 1: wild fungus, lane 2: Eth 0.25 mg mL^{-1}, lane 3: Eth 0. 5 mg mL^{-1} lane 4: Eth 1 mg ml^{-1}, lane 5: UV exposure for 1 min, lane 6: UV exposure for 3 min, lane 7: UV exposure for 5 min

different pathogens.

We found that pH 5.5 was optimum for chitinase production by *T. harzianum* JF419706, however the optimum pH for both glucanases was 6. These results are in accordance with previous studies. In this regard, Nampoothiri *et al.* (2004) stated that pH of the medium is an important factor that affects the activity and growth of the microorganisms. In addition, Moreno-Mateos *et al.* (2007) reported that 4.5–5.5 was optimum pH for growth, sporulation of strain *T. harzianum* CECT 2413. El-Katatny *et al.* (2003) found that the optimum pH for

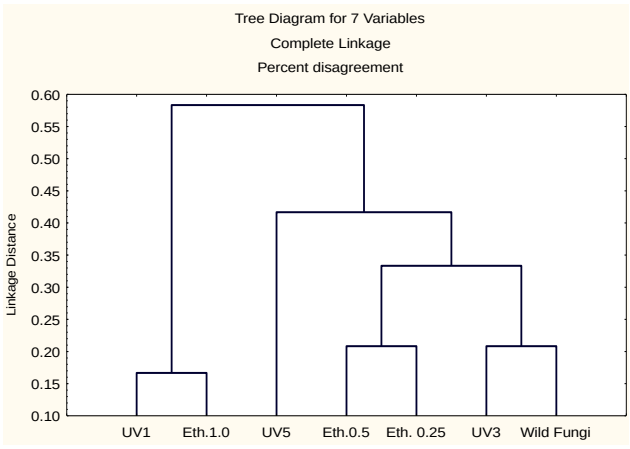

Fig. 8: Demdogram of RAPD analysis for the wild type fungus and its mutated isolates using ethidium bromide and UV

chitinase production was pH 6 and for β-1,3-glucanase production was pH 7. This approves the important role of pH in the production of enzymes by *Trichoderma*, and confirms the moderately acidophilic character of *Trichoderma* species (Kubicek-Pranz, 1998; Pandey *et al.*, 2001; Kredics *et al.*, 2005).

Our results showed that ethidium bromide enhanced the mycoparasitic activity of *T. harzianum* JF419706 more than UV against the tested phytopathogenic fungi. The mutated *T. harzianum* JF419706 covered the whole colonies of phytopathogenic fungi faster and more competent than the wild type, and inhibited the growth of the target pathogens. In results similar with ours, Abbasi *et al.* (2014) compared the mutated strains of *T. harzianum* by gamma radiation and their wild types against *M. phaseolina* in dual culture. They detected the maximum growth inhibition was observed in Th1, Th4, Th15, Th9 and Th22 mutants after three days faster than the wild type. Haggag and Mohamed (2002) noticed that all mutant isolates of *Trichoderma* were fast growing and covered the whole plates in 4 days compared to 6 days than the wild isolates. We assume that the mycoparasitic affinity of the mutated strain was induced through the production of cell-wall degrading enzyme such as chitinase and antifungal metabolites. This could be supported by other authors' findings, who approved that mutagensis of *T. harzianum* and *T. koningii* by γ-ray produced approximately three times more extracellular chitinase than their wild type and exhibit reduction in the growth of *Botrytis cinerea* by culture filtrate (Haggag, 2002). Gadgil *et al.* (1995) reported that mutation may not only effect the cellulase genes directly but also affect the synthesis and secretion of the chitinase and cellulase (Limon *et al.*, 1999). Also, Zaldivar *et al.* (2001) observed an increase in production of endoglucanase, filter paper activity and cellobiase in two to fourfold of mutated *T. aureoviride* 7-121 as compared with

the wild type strain. Induction of mutagenesis by gamma irradiation can also increase the ability of *Trichoderma* species to produce antibiotics and phenolic compounds such as trichodermine, gliotoxin and viridian (Haggag and Mohamed, 2002).

Primers chitinase (C15) and R2 succeeded to differentiate between the wild *T. harzianum* JF419706 isolate (un-mutated) and its mutants. Analysis of genetic variability obtained from RAPD-PCR reflected the same picture of phenotypic variation, where, all mutants that exhibited stimulatory effect against the pathogens had genetic variation compared with the wild type. We could assume that in the case of *T. harzianum* JF419706 the most mutated genes, which could be affected by ethidium bromide are chitinase genes that enhanced the mycoparasitic activity of the fungus. This could be interpreted as; in mutated strains, the chtinase genes were more expressed than the wild type. Prabavathy *et al.* (2006) stated that, the antagonistic activity of *T. reesei* against *Rhizoctonia solani* was enhanced by in self-fusant strains compared with the parent strain. Their finding approved a clear relationship between the chitinase activity and mycoparasitic performance of the bioagent. In accordance with our results, Haggag (2002) reported that fractional culture filtrate of the mutant isolates using gel electrophoresis showed that TH 12 and TK 5 produced high level of isozyme bands of chitinase compared with wild type. Balasubramanian *et al.* (2010) approved that using UV to induce the mutation in *T. harzianum* and the mutant strain H11 showed extra cellular chitinase and protein production, more than the adapted mutant and wild strain. SDS-PAGE analysis demonstrated that UV mutant had 20 major protein bands with up regulation than adapted mutant (15 bands) and wild strains (11 bands). Gajera *et al.* (2013) characterized the overexpressed a 33 kDa chitinase (*chit33*) gene from the transformants *T. harzianum* strain CECT 2413. This strain was cotransformed with the *amdS* gene and its own *chit33* gene under the control of *pki* constitutive promoter from *T. reesei.* Southern blotting indicated that the *chit33* gene was integrated ectopically, mostly in tandem.

We can conclude that the maximum production of growth yield and enzymatic activity of *T. harzianum* JF419706 were achieved after 4 days. Slight acidic pH 6 was more favorable for the production of different enzymes. Addition of colloidal chitin improved the cell wall degrading enzymes, however addition of casein was important to stimulate the production of protease. The productivity of most enzymes was positively affected by addition of yeast extract as a nitrogen source. UV and ethidium bromide effectively induced the mutation in *T. harzianum* that were able to antagonize the selected phytopathogens more than the wild types. Ethidium bromide and UV exposure affected the genetic materials of the treated fungus. This genetic variability reflected on the production of the active materials of fungus, which considered as positive results.

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References

Adelberg, E.A., M. Mandel and G.C. Chen, 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichia coli K-12. *Biochem. Biophys. Res. Commun.*, 18: 788–795

Ait-Lahsen, H., A. Soler, M. Rey, J. Cruz, E. Monte and A. Llobell, 2001. An Antifungal Exo-α-1,3-Glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum. Appl. Environm. Microbiol.*, 67: 5833–5839

Alamri, S.A., M. Hashem and Y.S. Moustafa, 2012. *In vitro* and *in vivo* biocontrol of soil-borne phytopathogenic fungi by certain bioagents and possible mode of action. *Biocont. Sci.*, 17: 155–167

Araujo, W.L., D.A. Angellis and J.L. Azevedo, 2004. Direct RAPD evaluation of bacteria without conventional DNA extraction. *Braz. Arch. Biol. Technol.*, 47: 375–380

Ausbel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl, 1995. *Current Protocols in Molecular Biology*. John Wiley and Sons, New York, USA

Balasubramanian, N., V.T. Priya, S. Gomathinayagam, V. Shanmugaiah, J. Jashnie and D. Lalithakumari, 2010. Effect of chitin adapted and ultra violet induced mutant of *Trichoderma harzianum.* Enhancing biocontrol and chitinase activity. *Aust. J. Bas. Appl. Sci.*, 4: 4701–4709

Cheng, P., W. Song, X. Gong, Y. Liu, W. Xie, L. Huang and Y. Hong, 2015. Proteomic approaches of *Trichoderma hamatum* to control *Ralstonia solanacearum* causing pepper bacterial wilt. *Int. J. Agric. Biol.*, 17: 1101–1109

El-Katatny, M.H., A.M. Hetta, G.M. Shaban and H.M. El-Komy, 2003. Improvement of Cell Wall Degrading Enzymes Production by Alginate Encapsulated *Trichoderma* spp. *Food Technol. Biotechnol.*, 41: 219–225

El-Katatny, M.H., M. Gudelj, K.H. Robra, M.A. Elnaghy and G.M. Gubitz, 2001. Characterization of a chitinase and an endo-b-1,3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii. Appl. Microbiol. Biotechnol*., 56: 137–143

Gadgil, N.J., H.F. Daginawala, T. Chokrabarti and P. Khanna, 1995. Enhanced cellulase production by a mutant of *Trichoderma reesei. Enzy. Microb. Technol.*, 17: 942–946

Gajera, H., K. Rakholiya and D. Vakharia, 2011. Bioefficacy of *Trichoderma* isolates against *Aspergillus niger* van Tieghem inciting collar rot in groundnut (*Arachis hypogaea* L). *J. Plant Prot. Res.*, 51: 240–247

González, I., D. Infante, B. Martínez, Y. Arias, N. González, L. Miranda and B. Peteira, 2012. Induction of chitinases and glucanases in *Trichoderma* spp. strains intended for biological control. *Biotecnol. Appl.*, 29: 12–16

Haggag, W.M. and H.A.A. Mohamed, 2002. Enhancement of antifungal metabolites production from gamma-ray induced mutants of some *Trichoderma* species for control onion white rot disease. *Plant Pathol. Bull.*, 11: 45–56

Haggag, W.M., 2002. Induction of hyperproducing chitinase *Trichoderma* mutants for efficient biocontrol of *Botrytis cinerea* on tomato and cucumber plants growing in plastic houses. *Arab J. Biotechnol.*, 5: 151–164

Handschur, M., G. Pinar, B. Gallist, W. Lubitz and A.G. Haslberger, 2005. Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. *Food Chem. Toxicol.*, 43: 1595–1605

Haram, S., H. Schickler, A. Oppenheim and I. Chet, 1996. Differential expression of *Trichoderma harzianum* chitinases during mycoparasitism. *Phytopathology*, 86: 980–985

Hasewaga, S., J.H. Nordin and S. Kirkwood, 1969. Enzymes that hydrolyze fungal cell wall polysaccharides. Purification and properties of an Endo-α-D-(1-3) glucanase from *Trichoderma viride*. *J. Biol. Chem*., 244: 5460–5470

Jayalakshmi, S.K., S. Raju, S. Usha Rani, V.I. Benagi and K. Sreeramulu, 2009. *Trichoderma harzianum* L. as a potential source for lytic enzymes and elicitor of defense responses in chickpea (*Cicer arietinum* L.) against wilt disease caused by *Fusarium oxysporum* f. sp. *ciceri*. *Aust. J. Crop. Sci.,* 3: 44–52

Kapulnik, Y. and I. Chet, 2000. Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *T. harzianum* strain T-203. *Plant Physiol. Biochem.,* 38: 863–873

Kredics, L., Z. Antal, A. Szekeres, L. Hatvani, L. Manczinger, C. Vágvölgyi and E. Nagy, 2005. Extracellular proteases of Trichoderma species. A review. *Acta Microbiol. Immunol. Hung*., 52: 169–184

Kubicek, C.P., R.L. Mach, C.K. Peterbauer and M. Lorito, 2001. *Trichoderma*: From genes to biocontrol. *J. Plant. Pathol*., 83: 11–23

Kubicek-Pranz, E.M., 1998. Nutrition, cellular structure and basic metabolic pathways in *Trichoderma* and *Gliocadium*. *In*: *Trichoderma & Gliocladium*, pp. 49–72. Harman, G.E. and Kubicek, C.P. (eds.). Taylor & Francis, London

Küçük, Ç. and M. Kıvanç, 2008. Mycoparasitism in the biological control of Gibberella zeae and Aspergillus ustus by *Trichoderma harzianum* strains. *J. Agric. Technol*., 4: 49–55

Limon, M.C., J.A. Pintor-Toro and T. Benitez, 1999. Increased antifungal activity of *Trichoderma harzianum* transformants that overexpress a 33-KDa chitinase. *Phytopathology*, 89: 254–261

Lumsden, R.D., J.A. Lewis and D.R. Fravel, 1993. Formulation and delivery of biocontrol agents for use against soilborne plant pathogens. *In*: *Biorational Pest Control Agents: Formulation and Delivery*, pp: 166-188. Hall, F.R. and J.W. Barry (eds.). American Chemical Society, Washington, USA

Masih, E.I. and B. Paul, 2002. Secretion of β-1,3-glucanase by the yeast *Pichia membranefaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mould of grapevine. *Curr. Microbiol*., 44: 391–395

Massimiliano, F., L. Jean-Louis and F. Federici, 1998. Chitinolytic enzymes activity of *Penicillium janthinellum* P9 in bench top bioreactor. *J. Ferment. Bioeng*., 86: 620–622

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.,* 31: 426–429

Moita, C., S.S. Feio, L. Nunes and J.C. Roseiro, 2005. Optimization of physical factors on the production of active metabolites by *Bacillus subtilis* 355 against wood surface contaminant fungi. *Int. Biodeterior. Biodegrad.,* 5: 261–269

Monte, E., 2001. Editorial Paper: Understanding *Trichoderma*: Between agricultural biotechnology and microbial ecology. *Int. Microbiol.*, 4: 1–4

Moreno-Mateos, M.A., J. Delgado-Jarana, A.C. Codon and T. Benitez, 2007. pH and Pac1 control development and antifungal activity in *Trichoderma harzianum*. *Fung. Genet. Biol*., 44: 1355–1367

Nampoothiri, K.M., T.V. Baiju, C. Sandhya and A. Sabu, 2004. Process optimization for antifungal chitinase productionby *Trichoderma harzianum*. *Process Biochem.,* 39: 1583–1590

Pandey, A., C.R. Soccol, J.A. Rodriguez-Leon and P. Nigam, 2001. *Solid State Fermentation in Biotechnology*, p: 23. Asia Tech Inc., New Delhi, India

Prabavathy, V.R., N. Mathivanan, E. Sagadevan, K. Murugesan and D. Lalithakumari, 2006. Self-fusion of protoplasts enhances chitinase production and biocontrol activity in *Trichoderma harzianum*. *Biores. Technol.,* 97: 2330–2334

Prapagdee, B., C. Kuekulovng and S. Mongkolsuk, 2008. Antifungal potential of extracellular metabolites produced by *Streptomyces hygroscopicus* against phytopathogenic fungi. *Int. J. Biol. Sci.,* 4: 330–333

Roberts, W.K. and C.P. Selitrennikoff, 1988. Plant and bacterial chitinases differ in antifungal activity. *J. Gen. Microbiol*., 134: 169–176

Saba, H., D. Vibhash, M. Manisha, K.S. Prashant, H. Farhan and A. Tauseef, 2012. *Trichoderma* – a promising plant growth stimulator and biocontrol agent. *Mycosphere*, 3: 524–525

Saligkarias, I.D., F.T. Gravanis and H.A. Epton, 2002. Biological control of *Botrytis cinerea* on tomato plants by the use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182. I. *In vivo* studies. *Biol. Cont.,* 25: 143–150

San-Lang, W., I.L. Shihb and C.H. Wang, 2002. Production of antifungal compounds from chitin by *Bacillus subtilis*. *Enz. Microb. Technol*., 31: 321–328

Šimkovič, M., A. Kurucová, M. Hunová and L. Varečka, 2008. Induction of secretion of extracellular proteases from *Trichoderma viride*. *Act. Chim. Slov.,* 1: 250–264

Szekeres, A., L. Kredics, Z. Antal, F. Kevei and L. Manczinger, 2004. Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum*. *FEMS Microbiol. Lett*., 233: 215–222

Takami, H., T. Akiba and K. Horikaoshi, 1989. Production of extremely thermostable alkaline protease from *Bacillus* sp. No. AH-101. *Appl. Microbol. Biotechnol*., 30: 120–124

Verma, M., S.K. Brar, R.D. Tyagi, R.Y. Surampalli and J.R. Valéro, 2007. Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. *Biochem. Eng. J.,* 37: 1–20

Yulianti, Y., K. Sivasithamparam and D.W. Turner, 2006. Saprophytic growth of *Rhizoctonia solani* Kühn in soil amended with fresh green manure affects the severity of damping-off in canola. *Soil Biol. Biochem.,* 38: 923–930

Zaldivar, M., J.C. Velasquez, I. Contreras and L.M. Pérez, 2001. *Trichoderma aureoviride* 7 121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. *Elect. J. Biotechnol*., 4: 1–9

Zeilinger, S. and M. Omann, 2007. *Trichoderma* biocontrol: signal transduction pathways involved in host sensing and mycoparasitism. *Gene Regul. Syst. Biol.,* 1: 227–234

Zonneveld, B.J., 1971. Biochemical analysis of the cell wall of *Aspergillus nidulans*. *Biochim. Biophys. Acta*, 249: 506–514

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