Enhancement of Lytic Enzymes Activity and Antagonistic Traits of *Trichoderma harzianum* Using γ-Radiation Induced Mutation

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ABSTRACT

Trichoderma species are known as effective agents used for biological control of plant pathogenic fungi. The *Trichoderma harzianum* and its mutant isolates were cultured and their traits including, mycelial growth, antagonistic activity and extracellular proteins and enzymes production (Chitinase and Cellulase) were investigated to select the most effective mutant isolates against plant pathogenic fungus *Rhizoctonia solani*. Also, the purity and composition of enzyme-rich protein samples were evaluated under denaturing gel electrophoresis. This study clearly showed the possibility of improving mycelia growth rate (from 1.18 to 1.33 cm d^{-1}), the antagonistic capability of *Trichoderma* (from 54.9% growth inhibition of *R. solani* to 66%), extracellular proteins and enzymes production for biological control of plant diseases through mutation with γ-radiation. Also, compared to wild type strain, protein production in the mutant isolates increased. Moreover, the highest specific chitinase enzyme activities were observed in mutant isolates T. h M8 (42.48 U mg^{-1}) and T. h M15 (38.25 U mg^{-1}). *Trichoderma* mutant of T. h M8 maintained higher mycelia growth rate and higher ability to inhibit growth of *R. solani*. The SDS-PAGE profiles had several enzyme protein bands such as CelloBioHydrolases (CBHs), EndoGlucanases (EGs), β-Glucosidases (BGLs), endochitinases, and β-(1, 4)-N-acetyl glucoaminidases. SDS-PAGE analysis indicated the presence of different protein bands in the range of 10.5 to 245 KDa. Interestingly, expression of chitinase in 95 percent of mutants was higher than wild type of *T. harzianum*. The results showed that gamma mutation could increase the efficiency and amount of enzymes in *T. harzianum*, while these enzymes are involved in antagonistic properties of *T. harzianum*.

Keywords: Cellulase, Chitinase, Gamma radiation, Mutation.

INTRODUCTION

Trichoderma species are known as an effective biological control agent used for biological control of plant pathogenic fungi. These antagonists protect the plants against soil-borne plant pathogens by colonization of their root system (Etebarian, 2006). Also, some certain strains of *T. harzianum* can increase plant growth and yield (Cumagun, 2012). But, large scale application of these fungal agents in agriculture remains relatively limited, due to the lower levels of control they offer. For this reason, improvement of *Trichoderma* spp. as biocontrol agents is an important goal of researchers (Chet *et al.*, 1997). Mutation of these fungi to produce more effective strains is one of the alternative techniques for isolation and screening them from the soil. The competition of *T. harzianum* isolate mutated by ultraviolet radiation vs. *F. oxysporum* f.sp. *lycopersici* was improved

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via enhancement of the tolerance to growth-inhibitory metabolites produced by *F. oxysporum* (Marzano *et al*., 2013). Mutation and screening of *Trichoderma* species can result in increasing amounts of antagonistic factors such as some lytic enzymes and their antagonistic activities (Jun *et al*., 2009). Many researches have been carried out to explore the role of proteolytic enzymes (Kredics *et al*., 2005; Pozo *et al.,* 2004), β-1, 3-glucanolytic enzymes (Kubicek, 2004) and chitinase (Hoell *et al*., 2005). The presence of chitin in many pathogenic fungal cell walls (for example, *R. solani*) as the structural polymer, indicates the importance of chitinolytic enzymes as a component of the virulence factors in a fungus e.g. *Trichoderma.* Scientist's researches indicate that γ-radiation could affect the morphological characteristics like sporulation of fungus, mycelia colonies shape, color, and its growth rate. The mutants had higher inhibitory capability against *R. solani* (Mohamadi *et al*., 2014). The study clearly indicated that it is possible to improve the antagonistic capability of *Trichoderma* for biological control of plant diseases through mutation with γ-radiation (Mohamadi *et al*., 2014). The mutant of *T. harzianum* produced chitinase and glucanase posed activities between two and four times more than the wild type. In addition, it secretes higher amounts of a yellow pigment and produces about three times more extracellular proteins (Rey *et al*., 2001). During in vitro experiments, this mutant performed better than the wild type, earlier overgrowing and sporulation on *R. solani*, faster killing this pathogen and exerting better protection on grapes against *Botrytis cinerea* (Rey *et al*., 2001). The mutant developed from gamma mutagenesis of *Trichoderma reesei* is the best mutant for endo-glucanase, exoglucanase, and total cellulase activities (Shahbazi *et al*., 2014). Induced mutation is one common measure to change and restrain the genetic construction of microorganisms such as biocontrol species of *Trichoderma* (Rey *et al*., 2001). Many studies indicate that γ-mutagenesis

could improve exo-chitinase production and achieving biological efficiencies of *T. harzianum.*

Therefore, in this study, *T. harzianum* and its mutant isolates were investigated for possible enhancement of lytic enzymes production and biocontrol activity of *T. harzianum* against *R. solani* by induced mutation of gamma radiation.

MATERIALS AND METHODS

Isolation and Identification of T. harzianum

T. harzianum, isolated from infected soils under cucumber (not published), was obtained from Yazd Agricultural Research Centre, and its mutant isolates were prepared in Nuclear Agriculture School, Alborz, Iran (Moradi, *et al*., 2015). Fungi were maintained on Potato Dextrose Agar (PDA) prepared according to manufacturer's instructions.

Determination of Growth Rates of Fungi

Radial growth rates of *T. harzainum* and their mutant isolates were determined by inoculating centrally three replicate Petri dishes of each test medium with a 5 mm diameter disc of each fungus, after 72 hours incubation at 28°C (Prabavathy *et al*., 2006).

Antagonistic Activity of T. harzianum and Its Mutants against R. solani

The antagonistic activity of *T. harzianum* and its mutant isolates against *R. solani* were determined by a dual culture technique at 28°C for 72 hours on PDA medium (Elad *et al*., 1983). The percentage of growth Inhibition (I) was calculated by the following formula:

$I= (1-C_n/C_o)\times100.$

1036

Where, C_n refers to the average diameter of Colonies of pathogen in the presence of the antagonist and C_o is the average diameter of Colonies of the control.

Chitinase and Cellulase Enzyme Production

Seed Culture Preparation

Spores of *T. harzianum* and its mutants on agar media [MYG agar medium containing (g L^{-1}): Malt extract, 5; yeast extract, 2.5; glucose, 10; agar, 20] were washed with sterilized water and made the spore suspension with concentration at 1×10^7 spores mL^{-1} of the medium. One mL spore suspension was transferred into 250 mL Erlenmeyer flask, which contained 50 mL seed culture medium. Seed culture was produced in Trichoderma Complete Medium (TCM) which contained (g L^{-1}): Bactopeptone, 1.0; urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H2O$, 0.3; $CaCl_2.6H2O$, 0.3; $FeSO_4.7H2O$, 0.005; $MnSO_4$, 0.002; $ZnSO4$, 0.002; $CoSO_4.7H_2O$, 0.002 and 2 Ml L^{-1}, Tween 80. The medium was adjusted to pH 4.8 and supplemented with 0.3% w/v of glucose. Cultures were prepared in 50 mL volumes of TCM in 250 mL Erlenmeyer flasks shaken at 180 rpm at 28˚C for 24 hours (Wen *et al*., 2005; Shahbazi *et al*., 2013).

Extracellular Enzyme Production

To induce production of cellulase or chitinase enzymes, washed mycelium of *Trichoderma* was transferred to 50 mL of *Trichoderma* fermentation medium (TFM) which contained (g L^{-1}): urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; $CaCl_2.6H$ O, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.002; $ZnSO_4$, 0.002; $CoSO_4.7H_2O$, 0.002 and 2 mL L^{-1}, Tween 80. This medium was adjusted to pH 4.8 for cellulase production or pH 5.5 for chitinase and supplemented with 0.5% w/v of Phosphoric

Acid Swollen Cellulose (PASC) or colloidal chitin. Growth conditions were as described previously and triplicate flasks were harvested after fermentation incubation time of 48 hours and incubation temperature 28°C (Wen *et al*., 2005; Shahbazi *et al*., 2013).

Estimation of Extracellular Protein Production and Enzyme Activity

The dye binding method of Bradford was used for estimation of the extracellular protein content in the TFM supernatant (Bradford, 1976). The amount of protein was calculated using Bovine Serum Albumin (BSA) as a standard. Exoglucanase (Avicelase) and Endoglucanase (CarboxyMethyl Cellulase or CMCase), activities were determined by measuring the amount of glucose released from substrates by the DiNitroSalicylic acid (DNS) method with glucose as the standard. The International Unit (IU) of activity is defined as the amount of enzyme that liberates 1 μmol of glucose per hour in a standard assay (Gama and Mota, 1998). The chitinase assay was estimated by the method of Zeilinger *et al*. (1999). One unit chitinase activity was defined as the amount of enzyme that produced 1 μmol of *N*-acetyl-glucosamine from colloidal chitin in the reaction mixture mL^{-1} h^{-1} under standard assay condition.

Electrophoresis and Molecular Size Determination

Protein samples from TFM supernatants were precipitated with equal volume of acetone and precipitated proteins were resuspended in double distilled water in final volume of 100 μL, frozen and kept at -70˚C until they were used. The molecular weight of the cellulases and chitinases were determined by Sodium Dodecyl Sulfate– Poly-Acryamide Gel Electrophoresis (SDS– PAGE) with a 4% (stacking) and 12.5% (separating) polyacrylamide gel based on

Laemmli (1970). The proteins were separated at constant amperage of 25 mA and were stained with Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (5:1:4, v/v), and decolorized in methanol–acetic acid–water (1:1:8, v/v) (Laemmli, 1970).

Statistical Analysis

All treatments were performed using a completely randomized design and all experiments were carried out at least in triplicate. The experimental data were subjected to Analysis Of Variance (ANOVA) followed by a Duncan's test. Significance was defined at $P < 0.05$. The SPSS (developer 13) program was used for all statistical analysis.

RESULTS AND DISCUSSION

Antagonistic Activity Assay

In this study, *T. harzianum* suppressed the growth of *R. solani* through the overgrowth at all replications. The results showed significant difference in growth inhibition percentage of *R. solani*. However, the isolates T. h M8, T. h M16, and T. h M13 showed excellent antagonistic activity against *R. solani*, respectively (Table 1). It

was observed that T. h M8 reduced the growth of *R. solani* by 66.01%. Chet *et al.* (1997) reported that *Trichoderma* species are common inhabitants of rhizosphere and contribute to control of many soil borne plant diseases caused by fungi. *T. harzianum* and *T. viride* were reported by several workers as the best antagonists for growth inhibition of several soil and seedborne plant pathogens (Dubey, 2002). Most fungi attacked by *T. harzianum* have cell walls that contain chitin as a structural back-bone and laminarin (ß-1, 3-glucan) as a filling material. The fact that *T. harzianum* penetrated the host mycelium and grew extensively within of *R. solani* suggests that it can produce cell wall-degrading enzymes such as chitinases and ß-1, 3 glucanases. Moreover, it could be that other metabolites produced by *T. harzianum* and its mutants might have an important role. Furthermore, Pates *et al.* (1999) found that the strain of *Trichoderma viride* has an important activity to secrete enzymes for end to attack or remove mycotoxines synthesized by the pathogens. The finding that some aggressive *T. harzianum* used as biocontrol agents are capable of producing either antibiotics or extracellular enzymes, or both, has provided crucial information in understanding the events associated with parasitism (Cherif and Benhamou, 1990). Antibiosis mediated by volatile substances has received considerably less attention than antibiosis.

Table 1. Percentage of growth inhibition of *R. solani* by *T. harzianum* mutants after 7 days' incubation in dual culture assay.[a]

Isolate name	Growth inhibition (%)	Isolate name	Growth inhibition (%)
T. h control	54.9 c	T. h M11	49.7 d
T. h M1	53.6 c	T. h M12	40.5 h
T. h M2	24.8 k	T. h M13	60.8 b
T. h M3	47.1 e	T. h M14	43.1 fg
T. h M4	53.6 c	T. h M15	32.7 j
T. h M5	43.1 fg	T. h M16	61.4 b
T. h M6	44.4 f	T. h M17	53.6 c
T. h M7	51 d	T. h M18	46.4 e
T. h M8	66 a	T. h M19	41.8 g
T. h M9	46.4 e	T. h M20	49.7 d
T. h M10	38.6 i	-	-

[a] Different letters in each column, indicate significant differences (P< 0.05, Duncan's multiple range test Duncan's test).

Doi and Mori (1994) showed that *T. harzianum* produced volatiles that had potential to inhibit the hyphal growth of *Lentinus lepidus* and *Coriolus versicolor*. In addition to antibiotic production, is known to be prolific producers of polysaccharide lyases, proteases and lipases, all of which may be involved in host cell degradation (Benhamou and Chet, 1993). Chitinases, cellulases and glucanases, which are produced by *T. harzianum,* are considered important enzymes active against plant pathogens (Chet and Baker, 1981). The involvement of enzymes in biological control complicates the distinction between mycoparasitism and antibiosis. The production of a cell wall degrading enzyme may be involved simultaneously in both parasitism and antibiosis. In this regard, Elad *et al*. (1983) demonstrated hyphal penetration by *Trichoderma* spp., mediated by enzyme activity. In addition, *T. harzianum* was able to parasitize *R. solani* hyphae by producing chitinase (Benhamou and Chet, 1993).

Since fungal cell walls are composed mainly of chitin and β-1, 3-glucans embedded in a matrix of amorphous material (Cherif and Benhamou, 1990), successful wall degradation requires the activity of more than one enzyme. Sivan and Chet (1989) have speculated that a co-ordinated action of polysaccharides, lipases and proteases is important in antibiosis. The present investigation has demonstrated that *T. harzianum* mutants (M1-M20) produce a spectrum of extracellular enzymes. These results may confirm the macro- and microscopical observations according to the aggressive behaviour of *T. harzianum* and its selected mutants towards *R. solani*. In summary, the observations of this study provide evidence that antibiosis by elaboration of volatile compounds and/or extracellular enzymes and the possible production of antibiotic metabolites may contribute to the observed inhibitory effects on *R. solani* when co-cultured with *T. harzianum* or its selected mutants. The results indicated that mutants had the highest antagonistic activity against *R. solani* compared with the wild type. Mutants T. h M8, T. h M16, and T. h M13 maintained highest ability to restrict the growth of *R. solani*. The data revealed that γ-radiation possibly altered the genetic makeup of *T. harzianum* and greatly increased its biocontrol capability as reflected by increasing the inhibition zone and decreasing *R. solani* growth after treatment with T. h M8, T. h M16, and T. h M13 compared to the parental strain *T. harzianum*.

Mycelia Growth Rate of T. harzianum and Its Mutants

Radial growth rate of selected *T. harzianum* and its mutants were estimated after subjecting the spores of the wild type (*T. harzianum*) to the γ-radiation (Table 2). A significant (P< 0.05) difference in mycelia growth was observed. The γ-radiation mutants showed higher growth than its wild type strain (*T. harzianum*), where, γ-induced mutants T. h M8 and T. h M14 showed the highest growth, respectively, compared with its wild type. Also, T. h M15 indicated the lowest growth. Abbasi *et al.* (2016) showed the gamma radiation caused differences in morphological properties of *T. harzianum* such as color, colony appearance, sporulation and growth rate of mycelia at different irradiation (Abbasi *et al*., 2016).

Extracellular Protein and Enzyme Activity Assay

The concentration of extracellular protein produced by *T. harzianum* and its mutant's enzyme complexes was determined by the dye binding method of Bradford and results were shown in Table 2. The highest extracellular protein production was observed in mutant isolate of T. h M11 (0.097 mg mL^{-1}) for chitinase production and in mutant isolate of T. h M9 (0.119 mg mL^{-1}) for cellulase production. Finally, the

Table 2. Extracellular protein production assay and the rate of mycelial growth of *T. harzianum* wild type (T. h control) and its mutant isolates.[a]

Isolate name	Growth rate (cm d^{-1})	Protein concentration (mg mL^{-1})	
		Chitinase production	Cellulase production
T. h control	1.18 e	0.031 j	0.07 ef
T. h M1	1.22 d	0.049 cd	0.072 ef
T. h M2	1.12 g	0.03 hi	0.062 g
T. h M3	0.77 o	0.031 h	0.062 g
T. h M4	0.95 k	0.063 b	0.076 ef
T. h M5	0.85 m	0.039 fg	0.05 h
T. h M6	1.17 f	0.054 cd	0.076 ef
T. h M7	1.2 d	0.042 ef	0.071 ef
T. h M8	1.28 c	0.038 ef	0.085 d
T. h M9	0.75 o	0.044 e	0.119 a
T. h M10	1 j	0.055 c	0.108 b
T. h M11	0.77o	0.097 a	0.101 c
T. h M12	1.02 i	0.055 c	0.078 ef
T. h M13	0.83 mn	0.039 ef	0.081 de
T. h M14	1.33 a	0.042 ef	0.081 de
T. h M15	0.52 p	0.098 a	0.091 cd
T. h M16	1.32b	0.04 ef	0.034 i
T. h M17	0.78n	0.048 cd	0.062 g
T. h M18	1.05h	0.037 fg	0.091 cd
T. h M19	0.88 l	0.027 hi	0.064 g
T. h M20	0.88 l	0.034 h	0.052 h

[a] Different letters indicate significant differences (P< 0.05, Duncan's multiple range test Duncan's test) in each column.

results showed that protein content production in TFM (mg/mL) for all mutant isolates had a significant (P< 0.05) difference in comparison with wild type of *T. harzianum*. Determining the extracellular protein concentration is not always a simple task, since various factors may interfere with the final result (Zaia *et al*., 1998). Specifically, three main factors affect these measurements: (a) Each protein dosage method is based on a different identification and quantification principle; (b) The presence of non-protein components in the enzymatic solution and/or reaction medium can be a source of error if they interfere with the results of the quantitative method; and (c) Other non-chitinase proteins present in the enzyme preparation may compromise the interpretation of the specific activity data. Such differences are also due to the fact that different enzyme isolates have different primary structures, besides different degrees of glycosilation. Therefore, these factors are

reflected in the response of the proteins from wild type of *T. harzianum* and its mutant isolates (Adney *et al*., 1995).

The results of chitinase activity of *T. harzianum* and its mutants in TFM supernatant are shown in Table 3. These results indicate variations in the enzyme activity between mutants. The values of chitinase activity in different mutants have a significant (P< 0.05) difference in comparison with wild type of *T. harzianum*. Some of mutants showed chitinase activity higher than that detected for the wild strain, whereas the others revealed lower activity. Generally, the chitinase enzyme activities in all tested isolates were affected by induced gamma radiation. Specific chitinase enzyme activities between 1.995 to 42.479 U mg^{-1} were found among these isolates. Two of the 20 mutant strains (T. h M8 and T. h M15; respectively) secreted significantly more chitinase than the wild type strain of *T. harzianum*. The cellulase enzymes activity

Table 3. Specific Cellulase (Endo- and Exo-glucanase) and Chitinase enzyme assay of *T. harzianum* and its mutant isolates in TFM supernatant after 48 h incubation at 180 rpm and 28 °C.

Isolate name	Specific enzyme activity (U mg^{-1}) [a]		
	Chitinase	Exoglucanase	Endoglucanase
T. h control	9.21 fg*	48.56 c-f	51.93 d-h
T. h M1	16.73 de	48.92 c-e	48.06 c-e
T. h M2	31.51 b	55.74 bc	55.05 bc
T. h M3	8.63 f-h	47.92 d-f	63.59 c-e
T. h M4	11.96 e-g	47.47 d-f	54.22 c-e
T. h M5	19.57 d	59.18 b	73.00 ab
T. h M6	29.41 b	40.91 f-h	48.11 d-h
T. h M7	2.00 h	45.11 e-g	59.43 cd
T. h M8	42.48 a	62.21 b	47.67 e-h
T. h M9	5.34 gh	41.53 e-h	37.73 h
T. h M10	19.70 d	46.64 ef	42.40 f-h
T. h M11	26.71 bc	34.40 hi	38.27 gh
T. h M12	13.93 d-f	38.04 gh	49.80 d-g
T. h M13	20.52 cd	36.64 hi	41.72 f-h
T. h M14	20.42 cd	30.67 i	45.98 e-h
T. h M15	38.25 a	41.52 e-h	38.88 gh
T. h M16	8.41 f-h	76.89 a	81.85 a
T. h M17	16.23 de	60.49 b	54.53 c-e
T. h M18	11.99 e-g	55.65 bc	39.33 gh
T. h M19	9.35 fg	54.99 bc	48.84 d-h
T. h M20	19.30 d	62.74 b	55.74 cde

[a] Different letters in each column, indicate significant differences (P< 0.05, Duncan's multiple range test Duncan's test) for specific enzyme activities.

(Endo- and Exo-glucanase) of mutant and wildtype isolates of *T. harzianum* in TFM that was supplemented with colloidal cellulose were investigated and results are shown in Table 3. In the case of endo-glucanase (U mL^{-1}), T. h M7, T. h M9 and T. h M10 were the best mutated isolates, although T. h M8, T. h M9, T. h M10, and T. h M18 showed the best exo-glucanase (U mL^{-1}) enzyme activity. The isolates of T. h M9 and T. h M10 not only had Endo- and Exo-glucanase activity [CelloBioHydrolase (CBH) I and II, Figure 2] but were also able to produce more protein. On the other hand, T. h M16 had more specific glucanase enzyme activity (Endo- and Exo-glucanase) than the wild type. These results occurred because of low amount of protein that was produced and contained only CBH I and II in its SDS-PAGE profile of protein (Figure 2, lack of endo-glucanases) (Shahbazi *et al*., 2013; Baharvand *et al*., 2015; Naseripour *et al*., 2015). Exoglucanases cut the accessible

ends of cellulose molecules to liberate glucose and cellobiose. The *T. harzianum* CBH I and II act on the reducing and non-reducing cellulose chain ends, respectively (Zhang and Lynd, 2004). CBH (exoglucanases) is classified as exo-acting based on the assumption that they all cleave β-1, 4-glycosidic bonds from chain ends releasing cellobiose and some glucose molecules. Commercial Avicel (also called microcrystalline cellulose or hydrocellulose) is used for measuring exoglucanase activity because it has a low degree of polymerization of cellulose and it is relatively inaccessible to attack by exoglucanases despite some amorphous regions. Exoglucanases show relatively high activity on Avicel and little activity on CMC (Maki *et al*., 2009). Exoglucanase enzyme activities or Avicelase activities were analyzed using pure Avicel, and results are given in Table 3. The highest specific Avicelase activity was 76.89 U mg^{-1} in T. h

M16. Enoglucanase enzyme activities or CMCase activities were analyzed using CMC, and results are given in Table 3. The highest specific CMCase activity was 81.85 U/mg in T. h M16. EGs (CMCase) can randomly hydrolyze internal glycosidic bonds in cellulose chains. *Trichoderma* spp. are known to degrade the plant cellulosic biomass by producing hydrolytic enzymes that are collectively called cellulases (Seiboth *et al.*, 2002; Gusakov, 2011).

Trichoderma cellulase complexes trigger the Induced Systemic Resistance (ISR) in plants such as tobacco, lima bean, and corn, by increasing the up-regulation of Ethylene (ET) or Jasmonate (JA) pathways (Piel *et al.*, 1997; Hermosa *et al.*, 2013). A concentration-dependent pattern resulted in the response. This response occurs based on the *Trichoderma* concentration in the roots

and the interaction (Segarra *et al.*, 2007). However, the mechanism by which the cellulase-like macromolecules produced by *Trichoderma* induces the ISR in plants is still unclear. Interestingly, ISR is initiated by both pathogens and Plant Growth-Promoting Microbes (PGPR) like *Trichoderma* (Djonovic *et al.*, 2006).

SDS-PAGE Analysis and Molecular Size Determination

The electrophoresis patterns obtained by SDS-PolyAcrylamide Gel Electrophoresis (PAGE) analysis of precipitated cell free TFM supernatants are shown in Figures 1 and 2. The SDS-PAGE analysis of the crude proteins on a 12.5% polyacrylamide gel (Figures 1 and 2) indicated the presence of
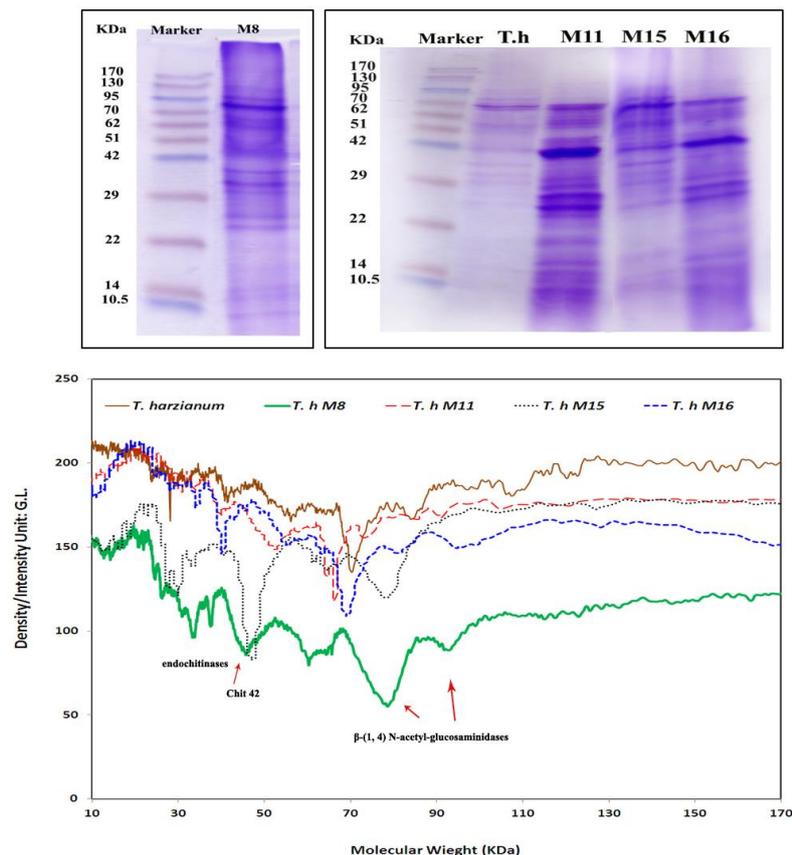
Figure 1. The profile of chitinase enzyme protein and optical density measurement of protein bands in the *T. harzianum* wild type and its mutant isolates by Gel –Pro (Ver. 6). Marker: Prestain Protein Ladder (Cinna Gen[TM] PR901641); M8 (T. h M8); T. h (wild type *T. harzianum*), M11 (T. h M11); M15 (T. h M15), M16 (T. h M16).
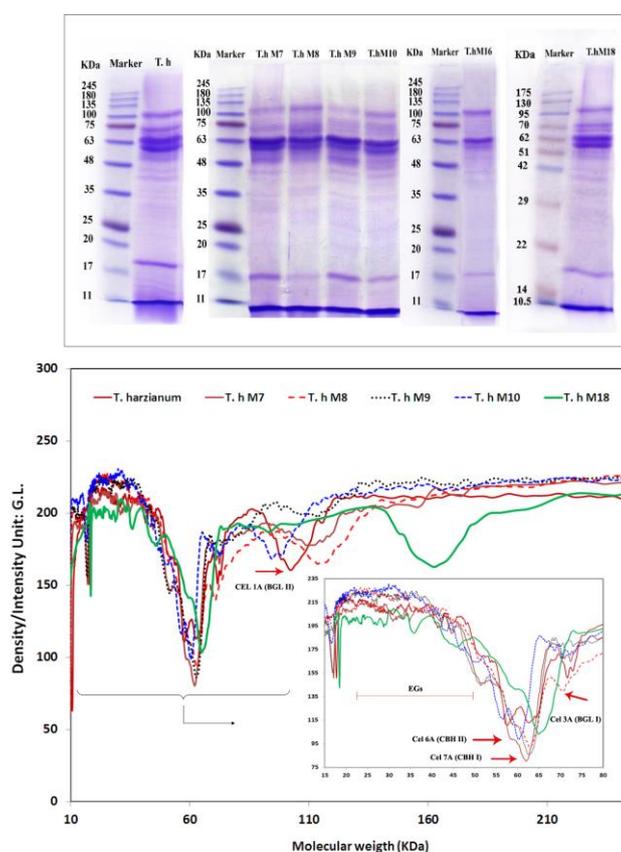
Figure 2. The profile of cellulase enzyme protein and optical density measurement of protein bands in the *T. harzianum* wild type and its mutant isolates by Gel –Pro (Ver.6). Marker: Prestain Protein Ladder (Cinna Gen[TM] PR901641); T. h (wild type *T. harzianum*), T. h M7; T. h M8; T. h M9; T. h M10); T. h M16, T. h M18.

different protein bands in the range of 10.5 to 245 KDa. All crude proteins obtained from chitinase TFM supernatants had sharp protein band with a molecular weight 67 KDa, which expresses the enzyme N- acetyl glucosaminidase (Figure 1).

Results showed that mutant isolates T. h M15, T. h M11, T. h M8, and T. h M6 had higher chitinase enzyme expression compared to wild type of *T. harzianum* and other mutants. The sharpest endo-chitinase bands with molecular weight 42, 24.5 and 26 KDa, in addition to β-(1, 4)-N-acetyl glucoaminidase with molecular weight 68 KDa was related to mutant T. h M15. There was another enzyme sharp band with molecular weight 42 KDa, which indicated the presence of enzyme endochitinase (Chit42).

Interestingly, expression of chitinase in 95 percent of mutants was higher than wild type of *T. harzianum*. Haran *et al*. (1995) reported that, depending on the strain, the chitinolytic system of *T. harzianum* may contain five to seven individual enzymes. In the well-characterized strain *T. harzianum* TM, this system comprises two β-(1, 4) N-acetyl-glucosaminidases (102 and 73 kDa), four endochitinases (52, 42, 33, and 31 kDa), and one exochitinase (40 kDa) (Lorito *et al*., 1993). Two 1, 4-β-N-acetylglucoaminidase have been reported to be excreted by *T. harzianum*. Ulhoa and Peberdy (1991) described the purification of these from *T. harzianum*.

Extracellular cellulase protein profiles of mutant isolates in the supernatant of TFM were assessed using SDS-PAGE and the results are shown in Figure 2. Two sharp

1043

bands were observed at molecular weight of 64 and 58KDa in the protein profiles of *T. harzianum* that were related to enzymes Cel 7A (CBH I) and Cel 6A (CBH II), respectively. Also, a 73 KDa enzyme band appeared in gel that showed production of Cel 3A (BGL I) by wild type strain of *T. harzianum*.

CONCLUSIONS

The antagonistic properties of *Trichoderma* spp. and their ability to reduce the growth of other soil borne pathogenic fungi have been described by several authors (Abo-Ellil *et al*., 1998; Marzano *et al*., 2013; Elad *et al*., 1983). The ability of these antagonists to attack the pathogenic fungi at different stages of their development has led us to the concept that they could be powerful biocontrol agents. The role of enzymes in biocontrol can often be attributed to mechanisms such as parasitism and antibiosis, in particular, for cell wall degrading enzymes such as chitinases, β-1, 3-glucanases, proteases and cellulases. These features not only are important for mycoparasites colonization of their host fungi, but also may exhibit considerable antifungal effect on their own activity. *Trichoderma* enzymatic antibiosis relies on disruption of the host-fungus cell wall (Elad, 2000; Hermosa *et al*., 2000). Increased production of chitinase (Limon *et al*., 1999) and cellulase (Gadgil *et al*., 1995; Wadhwa *et al*., 1997) could be achieved by mutants of *Trichoderma*. Significant levels of toxic metabolites were also found in *Trichoderma* mutants, which may be associated with increasing in rhizosphere competence with other microflora and pathogen (Cotes *et al*., 1996; Migheli *et al*., 1998). The tested γ-radiation-induced mutants were higher in their potential of biocontrol activity than their parental wild type strain (*T. harzianum*). A major part of *Trichoderma* antifungal system consists of a number of genes encoding for an astonishing variety of secreted lytic enzymes including; chitinase

(endochitinases, 1,4-β-chitobiosidases), β-glucanases (endo- and exo-glucanases, β-1,3-glucosidases, β-1,6-glucosidases, N-acetyl-β-glucosaminidases), proteases, lipases, xylanases, mannanases, pectinases, amylases, phospholipases, RNases, and DNases (Lorito, 1998; Hermosa *et al*., 2000).

In the present study, *T. harzianum* and its isolate mutants exerted an effect synergistic competition and mycoparasitism, over *R. solani*. The study clearly shows the possibility of improving the antagonistic microorganisms for biological control of plant diseases through mutation with γ-radiation. The results showed that gamma mutation could increase the efficiency and amount of enzymes in *T. harzianum*. The enzymes involved in the antagonistic properties of *T. harzianum*. The mutation process, not only can increase lytic enzymes production but also retains the good traits of mutants such as antagonism. The authors finally suggest that *T. harzianum* and T. h M8 could be a good biocontrol agent of *R. solani*.

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افزایش فعالیت آنزیم‌های تجزیه‌کننده و قدرت آنتاگونیستی *Trichoderma harzianum* با استفاده از القای موتاسیون پرتو گاما

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چکیده

گونه‌های قارچ تریکودرما به عنوان عوامل موثر در کنترل بیولوژیک قارچ‌های بیمارگر گیاهی شناخته می‌شوند. در این تحقیق، تغییرات در رشد میسلیومی، فعالیت آنتاگونیستی، تولید آنزیم‌ها (کیتیناز و سلولاز) و پروتئین‌های خارج سلولی گونه *Trichoderma harzianum* و موتانت‌های آن برای انتخاب موثرترین آنها در مقابل قارچ بیمارگر گیاهی *Rhizoctonia solani* مورد بررسی قرار گرفت. همچنین خلوص و محتوی پروتئین‌های آنزیمی آنها با استفاده از ژل الکتروفورز مورد بررسی

قرار گرفت. نتایج نشان داد که افزایش نرخ رشد میسلیوم‌های قارچ (از ۱/۱۸ به ۱/۳۳ سانتیمتر در روز)،
توانایی آنتاگونیستی قارچ (از ۵۴/۹ درصد بازدارنگی از رشد به ۶۶ درصد) و افزایش تولید پروتئین‌های
خارج سلولی و آنزیم‌ها جهت کنترل بیماری‌های گیاهی از طریق موتاسیون با اشعه گاما امکان‌پذیر می‌-
باشد. در این تحقیق، تولید پروتئین‌ها در موتانت‌ها در مقایسه با شاهد افزایش نشان داد. علاوه بر این
بیشترین فعالیت ویژه کیتینازی در موتانت‌های T. h M8 (۴۲/۴۸ U/mg) و T. h M15 (۲۵ U/mg
۳۸/) مشاهده شد. موتانت T. h M8 بیشترین نرخ رشد میسلیومی و بیشترین توان بازدارندگی از رشد
قارچ بیمارگر *R. solani* را نشان داد. در این بررسی، پروفایل ژل الکتروفورز، چندین باند آنزیمی
مانند سلوبیوهیدرولاز (CBHs)، اندوگلوکاناز (EGs) و بتاگلوکوزیداز (BGLs)، اندوکیتیناز و بتا
(۱و۴)- ان- استیل گلوکوآمینیدازها را نشان داد. این تکنیک حضور باندهای پروتئینی مختلفی را در
طیف ۱۰/۵ تا ۲۴۵ کیلودالتون نشان داد. جالب اینکه طبق نتایج بدست آمده مشخص شد، بیان آنزیم
کیتیناز در ۹۵ درصد از موتانت‌ها نسبت به گونه وحشی افزایش یافته است. نتیجه اینکه، موتاسیون
قارچ *T. harzianum* با اشعه گاما می‌تواند کارایی و مقدار تولید آنزیم‌ها را در آن افزایش دهد،
درحالی است که می‌دانیم این آنزیم‌ها در قدرت آنتاگونیستی *T. harzianum* دخالت دارند.