Mycologia Iranica 6(2): 73–87, 2019 Original Article DOI: 10.22043/MI.2020.121450

Two-dimensional and chitinase activity analysis of a novel mutant of *Trichoderma koningii* for biodegradation of *Macrophomina phaseolina* cell walls

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Abstract: The role of extracellular enzymes in mycoparasitism of *Trichoderma* spp. have been demonstrated in several cases. *Trichoderma* spp. produces several chitinolytic enzymes and this fungus is known as a powerful antagonist against *Macrophomiona phaseolina*, the causal agent of charcoal rot disease of soybean. In this study, two–dimensional protein pattern analysis and chitinaseactivity evaluation were performed for an Iranian *Trichoderma koningii* strain (NAS–K1) and its generated mutants were measured to indicate the potential role of endochitinases (N–acetylglucosam–inidase (NAG–I and NAG–II)) in its mycoparasitism. The results of chitinase activity assay using chitin and cell walls of *M. phaseolina* as substrates showed that the mutant isolates have significantly more enzyme activity compared to the wild type strain. The specific endochitinase enzyme activity in the mutant NAS–K1M25 was increased to approximately 2.5 times more and secreted three times more endochitinase than that of the wild type strain. This superior mutant showed up to 65% growth inhabitation against *M. phaseolina* in dual culture test (five times more than the wild type strain). Moreover, this strain showed sharper spots belong to endochitinase, and N–acetylglucosaminidase (I & II) presented in SDS–PAGE and 2D electrophoresis. Overall, induced mutation by gamma irradiation could be a useful method to develop such superior mutants, and the mutant NAS–K1M25 could be used as a potential biological control agent candidate for plant disease management programs of *M. phaseolina*. However, more detailed fermentation, formulation and field trial studies should be performed to finalize its biocontrol potentials.

Key words: Enzyme activity, γ–irradiation; chitinase, phytopathogen, antagonist

INTRODUCTION

Charcoal rot is one of the most important diseases that limits the cultivation of sesame and soybean. Oilseed crops are widely grown in different parts of the world. *Macrophominaphaseolina* (Tassi) Goidis, which is a soil and seed–borne fungus, is the causative agent of charcoal rot. The fungus can infect the root and lower stem of more than 500 plant species and has a wide geographic distribution (Eladet al. 1986, Etebarian 2006). Charcoal rot is an important soil–borne disease and favors by hot and dry weather and when faces with unfavorable environmental conditions, imposes stress on plants (Eladet al. 1986, Etebarian 2006).

Different strategies including cultural control, physical control, resistant crop varieties, biological control, chemical control, and transgenic plants have been applied to manage this plant disease. (Adekunleet al. 2001).

However, excessive use of these pesticides may contaminate the food or accumulate in the soil and groundwater, and consequently, may be entered into the food chain. Therefore, as a safe and an effective alternative to fungicides application, biological control shows greater potential for disease control (Adekunle et al. 2001). Accordingly, different biocontrol agents (BCAs) can be used for the control of plant diseases.

Species of *Trichoderma* are primarily studied for their ability in controlling plant disease through different mechanisms including antagonism, rhizos–phere competence, enzyme production, induction of defense response in plants, metabolism of germination stimulants, and beneficial growth of the host following

Submitted 4 Sept. 2019, accepted for publication 20 Nov. 2019
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root colonization (Weindling 1932, Weindling 1937, Weindling 1939, Eladet al. 1983, Benhamouet al. 1993, Zimand et al. 1996, Howell 2003, Bailey & Den 1998).

Recently, the role of extracellular enzymes such as proteolytic enzymes (Pozo et al. 2004, Kredicset al. 2005), β–1,3–glucanolytic system (Vázquez–Garcidueñas et al. 1998, De la Cruz & Llobell 1999, Kubicek et al. 2001) and chitinase (Zeilinger et al. 1999, Howell 2003) has been well documented by several researchers. The complex groups of extracellular enzymes have been reported to play key factor in lysing of the pathogen cell wall during mycoparasitism. The presence of chitin, as the structural polymer present in many fungal cell walls (such as *M. phaseolina*), indicates the importance of chitinolytic enzymes as a component of the portfolio of "virulence" factors in fungal species such as *T. koningii* (Gisi et al. 2010).

Induced mutation is one of the common methods to restrain the genetic construction of microorganisms (Whipps & Lumsden 1991). Induced mutagenesis using γ–irradiation was proved to be effective on enhancing exochitinase production and achieving biological efficiencies in *T. koningii* (Baharvand et al. 2016). Radiation treatments of biological materials have been applied in various processes such as sterilization of medical supplies. Sometimes, irradiation cannot completely kill organisms, but it may result in cell lesion (Smith & Pillai 2004), and directly damage the chromosomal DNA of living cell (Barkai–Golan 2001).

The damaging of nuclear DNA may cause the mutagenesis, and some genetic materials of fungi probably can be mutated to higher or lower biological activities through two different mechanisms. First, it splits covalent bonds in target proteins by direct photon energy; and second, via water radiolysis, it indirectly produces reactive oxygen species (ROS) that are responsible for the majority of the protein damages. Therefore, in this study, the wild type of *T. koningii* NAS–K1 (WT) was isolated from the soil samples and then treated with different doses of γ–irradiation for investigating the possible enhancement of bio–control activity in the WT strain against *M. phaseolina* by the induced mutation of γ–irradiation. The objectives of this study were; (1) to select fungal antagonists from WT strain and its γ–irradiation mutants showing abilities to control *M. phaseolina*, (2) show the induced mutation by gamma irradiation could be considered as a useful method to develop some efficient biological control agents for plant disease management programs of *M. Phaseolina* in the polluted soils.

MATERIALS AND METHODS

Trichoderma koningii WT and mutant strains

Trichoderma was isolated from the soil samples collected from different parts of agricultural fields (Khorasan Razavi province, Iran) by serial dilution on *Trichoderma* selective medium (Papavizas & Lumsden 1990). Additionally, identification of fungi was done by Dr. Naser Safaie, Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modarres University, Iran.

After isolation and identification of *Trichoderma* isolates, spore suspensions of the WT strain NAS–K1 were irradiated by a cobalt–60 γ– irradiator at a dose rate of 0.23 Gy/Sec in Nuclear Agriculture Research School, (NSTRI, AEOI). Dosimetry was performed with the Ficke–refrence standard dosimetry system (Gamma–cell Issledovatel, PX–30), and after irradiation process at 250 Gy (in three replicates), the spore suspensions were cultured on PDA medium and then incubated for 72h at 25˚C. The appropriate dose was selected based on approximately 50 % inhibition of spore germination on water agar (WA) medium. Out of 270 irradiated isolates, 26 mutant isolates were selected based on higher growth rate (on PDA during 72h of incubation) and vast number of spores produced per unit area. The mutant isolates were sub–cultured for seven times to ensure the immutability of morphological characteristics on the Mandelś optimized medium (Chand et al. 2005).

In order to investigate the morphological characteristics of the selected mutants, a diameter of 5 mm disk of the colony's mid–area was removed from each mutant samples. One ml of 2 % Tween 80 solution was added to microtubes containing each fungal disk and after two minutes of vortexing, 0.01 ml of spore suspension was poured into a homocytometer (Line–Bright Improved Neubauer, Hamburg, Germany) and the number of spores for each isolate was counted under the microscope. This experiment was performed in thriplicates for 25 spores from each disk of the fungus, and then the mean values were calculated.

Antagonistic activities

The antagonistic activities of the wild type (WT) strain and the mutantsagainst *M. phaseolina* were determined by dual culture test. Mycelia discs (5 mm diameter) were cut out from actively growing pure cultures of both strains (26 mutants or parental culture of the WT strain and*M. phaseolina*) on PDA for 3 days at 28 ˚C and placed at the opposite sides, 25 mm apart, of 100 mm petri dish plates containing PDA. The plates were then incubated at 28 ˚C in darkness and mycelia growth of the WT strainand its mutant isolates (antagonists) and *M. phaseolina* (pathogen) were measured after 3 days at intervals of 24 h, and finally, percent inhibition of mycelia growth of the pathogen was calculated. The valuation of inhibition by the mutants or the WT strain was estimated by calculating the percentage inhibition of mycelia growth by $I\% = (1–C_n/C_o) \times 100$, where, C_n is the average diameter of colonies of pathogen in the presence of the antagonist and C_o is the average diameter of control colonies.

Preparation of *Macrophomina phaseolina* mycelia

Macrophomina phaseolina strain was obtained from plant protection laboratory of Nuclear

Agriculture Research School in Atomic Energy Organization of IRAN (AEOI). The potato dextrose broth (PDB) culture media was used to grow the mycelia of *M. phaseolina*. About 100 mL of this medium in 500 mL Erlenmeyer flasks were autoclaved for 15 minutes at 121˚C prior to inoculation. After cooling at room temperature, the flasks were inoculated with the stock culture of *M. phaseolina*. The mycelia were collected by centrifugation (4500×g, 7 min) and then washed with water to remove any residual sugar, frozen at –70 ˚C for 24 h, freeze dried for 48 h, and milled to mesh size 53–125 µm.

Production of chitinase and degradation enzymes

Seed culture preparation

Fungal spores of the wild type and mutants on agar media (MYG agar medium containing (g/L): 5, malt extract; 2.5, yeast extract; 10 glucose; 20, agar) were washed with sterilized water and made the spore suspension with concentration at 1×10^7 spores per mL of the medium. One mL spore suspension was transferred into the 250 mL Erlenmeyer flask, which contained 50 mL of seed culture medium. The seed culture was produced in *Trichoderma* complete medium (TCM) which contained (g/L): 1, bactopeptone; 0.3, urea; 2, KH_2PO_4; 1.4, $(NH_4)_2SO_4$; 0.3 $MgSO_4.7H_2O$; 0.3, $CaCl_2.6H_2O$; 0.005, $FeSO_4.7H_2O$; 0.002, $MnSO_4$; 0.002, ZnSO4; 0.002, $CoSO_4.7H_2O$, and 2 mL, Tween 80. The medium was adjusted to pH 4.8 and then supplemented with 0.3 % w/v of glucose. These cultures were prepared in 50 mL volumes of TCM in 250 mL Erlenmeyer flasks were shacked at 180 rpm for 24 h at 28 ˚C (Wen et al. 2005).

Extracellular enzyme production

To induce production of extracellular enzymes, the washed mycelium of *Trichoderma* isolates were transferred to 50 mL of *Trichoderma* fermentation medium (TFM) which contained (g/L): 0.3, urea; 2.0, KH_2PO_4; 1.4, $(NH_4)_2SO_4$; 0.3, $MgSO_4.7H_2O$; 0.3, $CaCl_2.6H_2O$; 0.005, $FeSO_4.7H_2O$; 0.002, $MnSO_4$; 0.002, $ZnSO_4$; 0.002, $CoSO_4.7H_2O$, and 2 mL, Tween 80. This medium was adjusted to pH 5.5 and supplemented with 0.5 % w/v of colloidal chitin. The triplicate flasks were harvested after fermentation incubation for 72h at 28 ˚C (Wen et al. 2005). Moreover, *M. phaseolina* mycelium powder (0.5 % w/v) was used as a substrate for production of cell wall degradation enzymes under the same conditions.

Estimation of extracellular enzyme activities

Inoculum of all mutants and the WT strain were produced in *Trichoderma* complete medium (TCM) (Shahbazi et al. 2016). To induce the production of chitinase enzymes, the washed mycelium of all the isolates were transferred to *Trichoderma* fermentation medium (TFM), and supplemented with cell wall extraction of *M. phaseolina* as a substrate (Wen et al. 2005).

Estimation of protein and extracellular chitinase activity was performed in TFM, after centrifugation at 4500 g for 7 min at 4 °C. The protein content in the TFM supernatants was assayed under different conditions of fermentation using the dye binding method of Bradford and Bovine Serum Albumin (BSA) as a standard (Bradford, 1976).

The protein contents in the TFM supernatant after 72 h fermentation were estimated using the dye binding method of Bradford. The amount of protein was calculated using Bovine Serum Albumin (BSA) as a standard. A standard curve was prepared using 0, 2, 8, 12, 20, and 25 µg protein per ml in distilled water. The test was performed using 150 µl of supernatant of TFM or standard and 3 ml of Bradford reagent. The standard and test samples were replicated three times. The absorbance of the samples was read at 595 nm by a spectrophotometer (Jenway, USA).

The chitinase assay mixture containing 0.2 ml of supernatants of TFM and 0.2 ml of colloidal chitin suspension (0.1 % in 50 mM sodium acetate buffer, pH 5.5) was incubated at 37 ˚C in a water bath with constant shaking controls, without enzyme, substrate, and were then maintained with heat killed enzyme. After one hour of incubation, the released N–acetylglucosamine in reaction mixture was character–rized using the method of Zeilinger (Zeilinger et al. 1999). One unit of chitinase activity was defined as the amount of enzyme, which produced 1 µmol of N–acetylglucosamine from colloidal chitin in the reaction mixture/ml/h under the standard assay conditions. The specific activity of chitinase enzyme is a common unit that gives the measurement of the rate of reaction multiplied by the volume of reaction divided by the mass of total protein.

Electrophoresis and molecular size determination

Protein samples from TFM supernatants (250 µg) were precipitated with equal volume of acetone and precipitated proteins were resuspended in double distilled water in a final volume of 100 µl, and were then frozen and kept at –70 ˚C until use. The molecular weight of the cellulase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with a 5 % (stacking) and 12.5 % (separating) polyacrylamide gel as it was described earlier by Laemmli (1970). Before electrophoresis, equal volumes of sample buffer (100 µl) that contained 65 mM Tris–HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) 2–mercaptoethanol and 0.2 % (w/v) bromophenol blue were added to the protein sample (100 µl), boiled for 5 min, and then applied to loading on the gels. The proteins were separated at constant amperage of 25 mA using the running buffer contained 25 mM Tris, 192 mM glycine, and 0.1 % (w/v) SDS, pH 8.3. The gels 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).

Isoelectric focusing and 2D SDS–PAGE

Regardless of the initial protein concentration in the TFM supernatant of optimum fermentation conditions, the same amount of protein (1200 µg) was used for 2–D gel. Moreover, proteins were precipitated by incubating the supernatant with 13.3 % (w/v) trichloroacetic acid (TCA) in cold acetone containing 0.2 % (w/v) dithiothreitol (DTT) for 1 h at 4 ˚C. Following centrifugation at 6000 g for 15 min at 4°C, the pellet was resuspended in 20 ml cold acetone and then incubated on ice for 30 min. After that, centrifugation was repeated at 6000 g for 15 min at 4 ˚C, the supernatant was removed, and the pellet was air dried prior to re–suspension in 360 µL of a sample solution containing 8 M urea, 2 % (w/v) 3–[(3–cholamidopropyl)–dimethylam–monio]–1–propanesulfonate (CHAPS), 0.002 % (w/v) bromophenol blue, 40 mM DTT, 0.5 % Bio–Lyte® 3/10 ampholyte in double distilled water. The sample was directly applied to passively rehydrate 3–10, 17 cm immobilized pH gradient (IPG) strips (Bio–Rad, CA, USA) by applying 360 µL of the sample. IPG was equilibrated for 16 h and then placed on top of the PROTEAN IEF Cell (Bio–Rad, USA) and then IPG was focused on a total of 52,500 Volt hours (Vh) at 50 mA constant using a three–step focusing program. The focusing program included a rapid ramp to 500 V for 1 h, a linear ramp to 1,000 V over 1 h, and a 8,000 V step until reaching 52,500 Vh. The second-dimension separation of proteins was performed on 1.5–mm thick continuous 12.5 % (w/v) acrylamide gel using the same gel buffer as at one–dimensional separation stage. The second dimensional electrophoresis, SDS–PAGE, was performed with PROTEAN II xi 2–D Electrophoresis Cell (Bio–Rad) under the conditions of constant current. For the first 30 min, the current was set at 20 mA per gel. The current condition was 40 mA per gel for the rest of electrophoresis time. The SDS–PAGE was developed until the bromophenol blue dye marker had reached to 0.5 cm from the bottom of the gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R–250 in methanol–acetic acid–water (5:1:4, v/v), and then decolorized in methanol–acetic acid–water (1:1:8, v/v) (Sun et al. 2008).

Statistical analyses

All treatments were performed using a completely randomized design, and all experiments were conducted at least in triplicate. The experimental data were subjected to analysis of variance (ANOVA) followed by a Duncan's test. Importance was defined at $p<0.05$. The SPSS (developer 13) program was used for all the statistical analyses.

RESULTS

Morphological characteristics

First, unirradiated colonies (WT strain) were colorless, but after sporulation, they gradually became green. The growth of mutant colonies in 84 % of the mutated isolates was significantly higher than the parental (unirradiated) isolate. Sedimentation sporad–ically occured throughout the entire culture medium or on the sidelines of the second day. Airborne airships were produced in all parental and mutant isolates and began to sporulate. The bottom of the colonies (parental and mutants) were colorless for up to a week; however, in the mutant isolates that produced higher spores, it became yellow by passing a week. Chlamydospores were rarely formed in some mutant isolates after two weeks, often in the middle and rarely in the edges of culture medium. In some isolates, dithiophore had many branches, and branches were usually divided into two to three sub-branches with angles, which were relatively open from the main axis. Primary branches usually produce secondary branches, which may also branch out again. Generally, the branches were produced in pairs and reciprocally. In some of the isolates, the podiophors had long tips leading to a cylindrical phialide, and short and swollen phialides that appeared in the elongation region. Phialides were ampoule shaped and were usually paired in opposite directions. In the mutants with weak phialide production capacity, a large number of phialides were irregularly deposited, but in stronger mutant isolates, phialides produced with three to five layers and they were ellipsoidal; cylindrical; and with a smooth and perimeter wall. Conidia were cylindrical to rectangular and green. In addition, some of the morphological characteristics of parental and mutant isolates are presented in Table 1.

Table 1. The colonial color, mycelial growth rate and conidial size of WT and mutant strain of *Trichoderma koningii*.

Strain	Colony		Conidial size (µm)	
	Color	Growth rate*	Width	Length
WT strain	Green	1.79 ± 0.01	2.44	5.32
NAS–K1M1	Dark green	2.21 ± 0.04	2.70	5.60
NAS–K1M2	Green	1.36 ± 0.04	2.12	4.34
NAS–K1M3	Green	1.59 ± 0.00	2.31	5.19
NAS–K1M4	Green	2.24 ±0.05	2.18	4.69
NAS–K1M5	Green	2.11 ± 0.03	2.29	5.5
NAS–K1M6	Green	2.43 ± 0.04	2.26	5.48
NAS–K1M7	Green	1.72 ± 0.05	2.55	499
NAS–K1M8	Green	1.91 ± 0.03	2.24	5.71
NAS–K1M9	Green	2.24 ± 0.01	2.30	4.87
NAS–K1M10	Green	2.07 ± 0.03	2.07	5.60
NAS–K1M11	Dark green	2.24 ± 0.05	2.44	5.09
NAS–K1M12	Green	2.11 ± 0.05	2.76	5
NAS–K1M13	Green	2.39 ± 0.06	2.17	4.66
NAS–K1M14	Green	2.02 ± 0.02	2.31	4.95
NAS–K1M15	Green	2.15 ± 0.04	2.59	4.87
NAS–K1M16	Green	2.11 ± 0.04	2.39	4.66
NAS–K1M17	Green	2.25 ± 0.01	2.26	5.19
NAS–K1M18	Green	2.14 ± 0.04	2.45	5.12
NAS–K1M19	Green	2.24 ± 0.01	2.81	4.99
NAS–K1M20	Green	2.11 ± 0.08	2.35	5.64
NAS–K1M21	Green	1.45 ± 0.03	2.70	5.60
NAS–K1M22	Green	2.13 ± 0.03	2.12	4.34
NAS–K1M23	Green	2.03 ± 0.02	2.31	5.19
NAS–K1M24	Green	2.11 ± 0.04	2.18	4.69
NAS–K1M25	Dark green	2.11 ± 0.03	2.29	5.5
NAS–K1M26	Green	2.25 ± 0.04	2.26	5.48

*. Cm/day

Antagonistic activity

The dual culture tests results represented some differences between antagonistic activity of the WT strain and the mutantsagainst *M. phaseolina* (Fig. 1). The WT strain suppressed the growth of *M. phaseolina* through mycoparasitism mechanisms, which were previously reported by other *Trichoderma*

species. The first contact between the WT strainand *M. phaseolina* was observed after 48 h (Fig. 2).

Estimation of protein and chitinase activity

The production rate of the extracellular protein and the chitinaseactivity of two mutant isolates NAS–K1M25 (with high antagonistic activity against *M. Phaseolina*) and NAS–K1M22 (with low antagonistic activity against *M. Phaseolina*) were compared to the WT strain.

The amount of extracellular protein production is presented in Table 2.

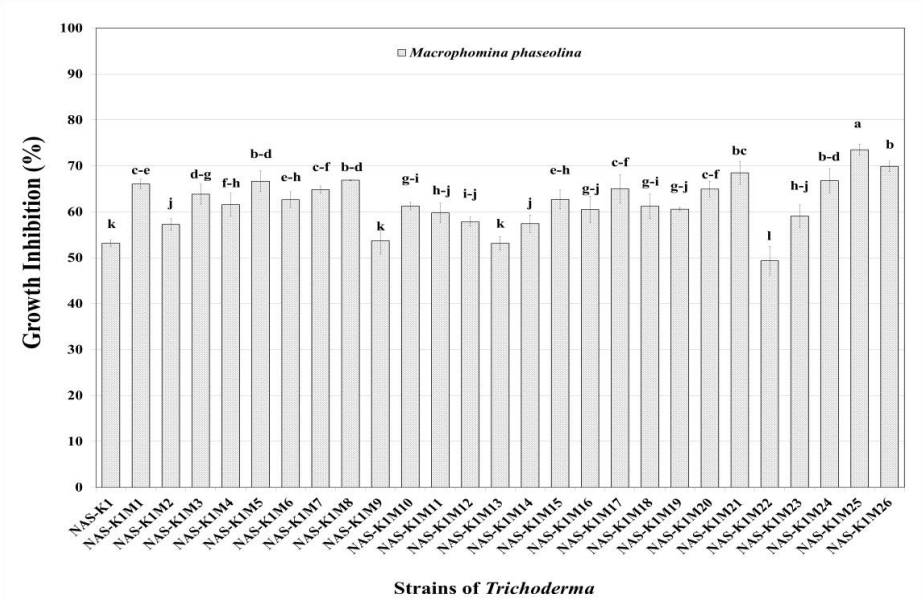

Fig. 1. Dual culture test results of the WT strain (NAS–K1) of *Trichoderma koningii*and its mutants against *Macrophomina phaseolina*. Means with the same letters are not significantly different at $p<0.05$.

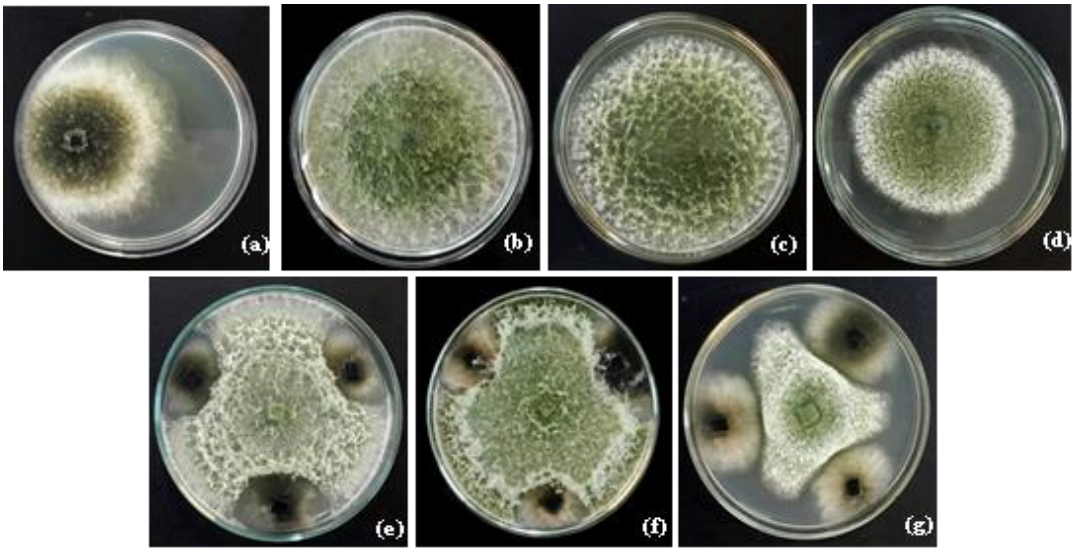

Fig. 2. Above panel: Colony of a. *Macrophomina phaseolina*, b. *Trichoderma koningii* WT strain NAS–K1, c. Mutant strain NAS–K1M25, d. Mutant strain NAS–K1M22. Down panel: *M. phaseolina* growth inhibition on PDA medium for 48 h incubation, at 28 ˚C. e. By WT strain, f. by mutant strain NAS–K1M25, g. By mutant strain NAS–K1M22 strain.

Table 2. The extracellular protein content (µg/ml), specific chitinase activity (U/mg) of the WT strain of *Trichoderma koningii* and its mutants (NAS–K1M25 and NAS–K1M22) in TFM supernatant after 72 h incubation at 180 rpm and 28 ˚C.

Protein	Strain		
	WT strain	**NAS–K1 M22**	**NAS–K1 M25**
Protein (µg/ml)	$280.58^{n.s.} \pm 7.61$	$285.33^{n.s.} \pm 8.04$	$268.88^{n.s.} \pm 1.88$
Specific chitinase activity (U/mg)	$4.69^{b} \pm 0.29$	$3.00^{c} \pm 0.08$	$17.01^{a} \pm 0.88$

In each column, means with same letters are not significantly different at $p\leq0.05$.
n.s.: non–significant.

Moreover, protein concentration ranged from 268 to 285 µg/ml in supernatant of TFM medium from the studied fungi. The highest protein content was 285.33 µg/ml in the supernatant of the mutant NAS–K1M22 isolate, whereas the lowest protein content (268.88 µg/ml) was measured in the culture supernatant of NAS–K1M25. Finally, the results showed that, protein content production in TFM (µg/ml) for all the studied isolates have no significant difference at the level of $p<0.05$.

The results of chitinase activity assay of NAS–K1, NAS–K1M25, and NAS–K1M22 in TFM supernatant after 72 h of incubation are shown in Table 2. These results indicated significant variations ($p<0.05$) in the enzyme activity values of the mutant strains. The activities of the enzymes are shown as international unites (U), in which one unit of activity is defined as the amount of enzyme required to liberate 1 µmol of product per hour. The values of specific chitinase activity in all the isolates have a significant difference at the level of $p<0.05$. The specific activities between 3.00 and 17.00 U/mg were found among these isolates. The maximum and minimum enzyme activity values were observed for NAS–K1M25 (17.01 U/mg) and NAS–K1M22 (3.00 U/mg), respectively. Table 3, shows the production of protein and hydrolytic extracellular enzymes of the cell walls of the *M. phaseolina* by the WT strain and two mutant isolates NAS–K1M25 and NAS–K1M22. All protein and enzyme activity data had significant statistical differences at 0.05 %.

The maximum amount of extracellular protein production was observed in the unirradiated sample (WT). The amount of protein production in the NAS–K1M25 with a high antagonistic activity showed a lower value compared to the unirradiated sample. However, this amount of protein was not significantly different from the NAS–K1M22 with a low antagonistic activity.

The special enzyme activity of total chitinase in the NAS–K1M25 was higher than the WT strain and the NAS–K1M22. In addition, the total chitinase activity in the NAS–K1M22 with a low antagonistic activity, was higher than total chitinase activity in the WT strain. The NAG activity of the NAS–K1M22 isolate was higher than that of the WT strain and the NAS–K1M25 isolate. There was also no significant difference between the NAS–K1M25 and its un–irradiated in terms of the NAG activity. The highest activity of the exoglucanase enzyme, was observed in the NAS–K1M25 and the highest activity of the endoglucanase enzyme, β–1,4–glucosidase enzyme and β–1,3–glucanase were observed in the NAS–K1M22.Theendoglucanase activity in the NAS–K1M22 increased over 4 folds of the NAS–K1M25 and more than 5 folds of its unirradiated. In spite of observing the exoglucanase, endoglucanase, and β–1,4–glucosidase enzyme activities; total cellulase enzyme activity was not observed in all the studied fungi.

Electrophoresis and molecular size determination

The composition and purity of the chitinase–rich protein samples were evaluated under denaturing conditions by SDS–PAGE. The electrophoresis patterns obtained by SDS–polyacrylamide gel electrophoresis (PAGE) analysis of precipitated cell free TFM supernatants is shown in Fig. 3. Accordingly, the SDS–PAGE analysis of the crud proteins on a 12.5 % polyacrylamide gel (Fig. 3) indicated the presence of different protein bands ranging from 11 to 245 KDa.

In the protein profile of the WT strain, a sharp band at the molecular weight of 66 KDa and a relatively weaker band at the molecular weight of 113 KDa, which are related to the presence of the 1, 4–β–N–acetylglucosaminidase, were observed. The 1, 4–β–N–acetylglucosaminidase with the molecular weight of 66 KDa exhibited the highest band intensity in the unirradiated and NAS–K1M22, and the lowest band intensity was observed in the NAS–K1M25.

Moreover, the SDS–PAGE analysis showed additional protein bands for NAS–K1M25 and its wild type strain, which could be attributed to the mutagenic effect. The increased protein secretion was also confirmed by quantitative assay, and SDS–PAGE analysis was done to compare identity among the wild and mutant strains loaded by the same amount of protein. Similarly, SDS–PAGE showed an additional protein band, and the increased extracellular enzymes by the application of five rounds of mutation were reported in *Ophiostoma floccosum* (Wu et al. 2006).

The sharp bands of endochitinase enzymes were observed in molecular weight of 30, 33, 36, 42, 44 and 46KDa for NAS–K1M25, weak bands with these similar molecular weights were observed in the WT strain and the NAS–K1M22 SDS–PAGE profiles (Fig. 3).

Table 3. Comparison of extracellular protein production, exoglucanase, endoglucanase, β–glucosidase, chitinase, N–acetylglucosaminidase and β–glucanase enzyme activity (U/mg) of NAS–K1M25 and NAS–K1M22 with its unirradiated strain (the WT strain) in TFM supernatant supplemented with *Macrophomina phaseolina* hyphal wall powder after 72 h incubation at 180×g and 28 ˚C.

Acticity	Enzyem	Trichoderma koningii Strain		
		WT strain	NAS–K1M22	NAS–K1M25
Extracellular protein (µg/ml)		$183.91^{a} \pm 1.26$	$155.02^{b} \pm 2.14$	$156.36^{b} \pm 2.50$
Chitinase enzymes activity (U/mg)	Total Chitinase	$27.29^{c} \pm 0.34$	$30.54^{b} \pm 1.84$	$57.40^{a} \pm 0.61$
	N–acetylglucosaminidase (NAG)	$0.41^{b} \pm 0.02$	$0.72^{a} \pm 0.05$	$0.34^{b} \pm 0.01$
Glucanase enzyme activity (U/mg)	β– (1,3)–glucanase	$25.05^{c} \pm 0.17$	$29.14^{a} \pm 0.40$	$26.15^{b} \pm 0.26$

In each column, means with the same letters are not significantly different at $p \leq 0.05$.

The results showed that the superior NAS–K1M25 isolate has a high ability in the production of chitinase enzymes due to growth on colloidal chitin as a fermentation substrate, which has led to a specific activity of the chitinase enzyme in this mutant that can be 3.5 folds more than the unirradiated and 5.5 folds more than the weak antagonist of the NAS–K1M22 isolate. The results of the protein profiles analysis also showed that because of mutation, this isolate produced an extreme variety of chitinase–rich protein bands.

Meanwhile, the intensity of the expression of endochitinase enzymes with molecular weights 30, 33, 35, 42, 44 and 46 KDa, exoglucanase enzyme with molecular weight 40 KDa and NAG enzymes with molecular weight 74 KDa were noted. These results confirmed the high activity of the total chitinase enzyme in this mutant. The clearest chitinase enzyme band of this mutant was found in a molecular weight of 40 KDa, which is probably an exochitinase.

Fig. 3 shows the densitometry and protein profiles from supernatant of the fermentation medium containing *T. koningii* and its mutants (NAS–K1M25 with high antagonistic activity and NAS–K1M22 with low antagonistic activity) and cell wall powder substrate of *M. phaseolina* after 72 hours of incubation at 28 ˚C and 180 rpm. All the studied samples had different molecular weight bands in the range of 20–120 KDa. The amount of protein in each well was equally loaded, and the difference in band strength indicated the presence of the same protein. The intensity of the bands in well associated with the superior antagonist NAS–K1M25 was higher than the unirradiated. Comparison of the molecular weight of the produced bands showed that, the NAS–K1M22 with low antagonistic activity had a generally lower band strength compared to the superior antagonist NAS–K1M25 and unirradiated in 74, 58, 54, 42, 40, 35, 33 and 30 KDa bands. Most of these molecular weights were related to chitinase enzymes, which were also visible in the protein profile of the studied fungi in the fermentation on the colloidal chitin substrate. Reducing bands' intensity indicated a reduction in the expression of these enzymes in the NAS–K1M22, which weakened the antagonistic power of this fungus by inducing mutation. On the other hand, an increasing in the severity of these bands in the NAS–K1M25 superior antagonist isolate resulted in the increased antagonistic activity of this fungus against the pathogen fungus of *M. phaseolina*. This difference in the band intensity was completely observed in the molecular weights of 30, 33, and 35 KDa. Moreover, the lowest band severity was observed in the molecular weight of 35 KDa, and the highest band severity was observed in the molecular weight of 20 KDa in the NAS–K1M22. In addition to the change in the intensity of the bands, mutation induction led to the creation of other new bands as a result of the growth in the vicinity of the cell wall of the pathogen fungus in the mutant isolates (e.g., 48 KDa band at NAS–K1M22), which were not visible in the unirradiated.

Isoelectric focusing and 2D SDS–PAGE

The Fig. 4, shows the 2D–PAGE gel analysis of the proteins produced by the wild type strain (NAS–K1) (Fig.4 left) and the NAS–K1M25 isolate (Fig. 4 right) in the fermentation medium containing the cell walls of *M. phaseolina*, as a fermentation medium substrate. In addition, the highest distribution of stains was observed in the range of isoelectric point 7–4 and the highest accumulation of molecular weight was observed in the range of 20–75 KDa.

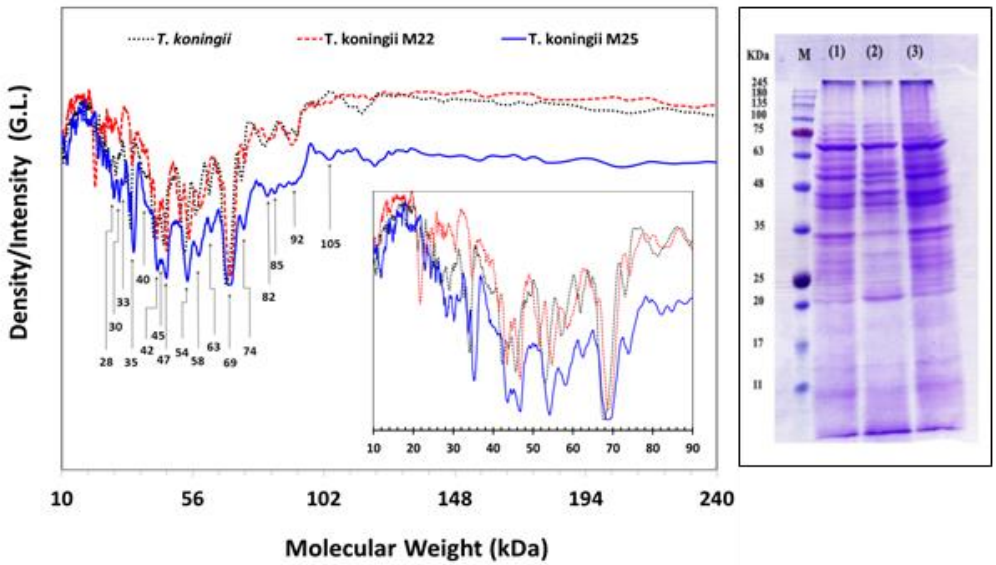

Fig. 3. Densitometry analyses of the proteins present in the SDS–PAGE profiles protein of the 1) WT strain; 2) NAS–K1M22; 3) NAS–K1M25; and M) standard protein molecular weight markers, in TFM supernatant supplemented with *Macrophomina phaseolina* hyphal wall powder after 72 h incubation at 180×g and 28 ˚C. Proteins were visualized on 12.5 % SDS–PAGE gel and stained with Coomassie Brilliant Blue R250.

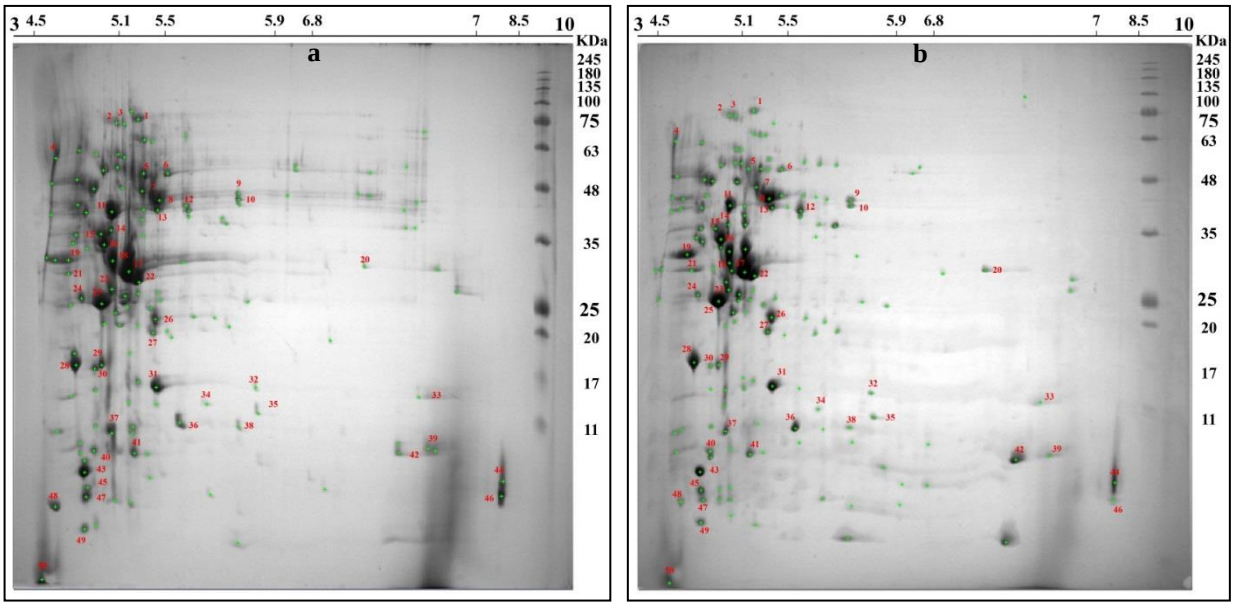

Fig. 4. Coomassie blue–stained 2DE gel of secreted proteins fromthe WT (a) and NAS–K1M25 strain (b) of *Trichoderma koningii* cultivated on *Macrophomina phaseolina* hyphal cell wall powder in Trichoderma Fermentation Medium (TFM).

Totally, 137 spots in the WT strain and 157 spots in the NAS–K1M25 isolate were identified and numbered. Moreover, molecular weight, isoelectric point (Ip), and percentage of the stains in the gels were determined and compared using the Melanie software. About 50 spots in each gel had the same molecular weight and isoelectric point. Moreover, 20 spots in the NAS–K1M25 isolate showed a higher percentage of stain intensity than the unirradiated, which indicates more protein expression in those spots.

The spots with higher protein expression were shown in Fig 5.b, by the filled symbol points, and molecular weight and the isoelectric point were individually determined.

Most of the more intense spots in the NAS–K1M25 were related to chitinase enzymes including spots number 37 (5.54–40.7), number 48 (4.99–37.17), number 53 (4.9–36.37), and number 63 (5–33).

DISCUSSION

The antagonistic specifications of *Trichoderma* spp. and their capability in reducing the growth of other soil borne pathogenic fungi have been described by several authors like Ellil et al. (1998). This antagonistic capability in attacking pathogenic fungi at different stages of their development has led to this belief that they could be strong biocontrol agents.

The role of enzymes in biological control of plant disease often can be allocated to mechanisms of parasitism and antibiosis. In particular, cell wall degrading enzymes, such as chitinases, cellulases, β–1,3–glucanases and protease, not only are important features of mycoparasites for colonization of their host

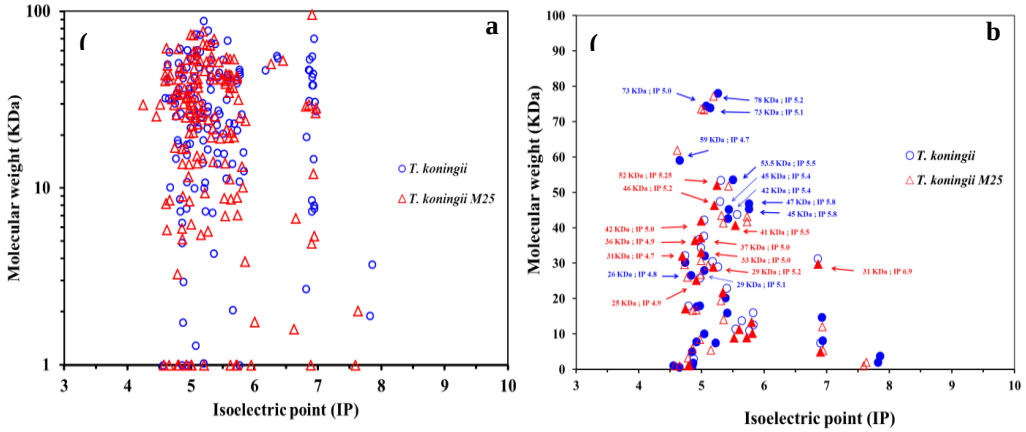

Fig. 5. a. Distribute protein spots at the 2DE gel of secreted proteins; b. The identified protein spots are labeled by the protein molecular weight (Mw) and isoelectric point (Ip) and the closed and open symbols (●, ○; the WT strain and ▲, Δ; NAS–K1M25) show the high and low protein secreted in media, respectively.

fungi, but also exhibit considerable antifungal activity on their own. One of the mechanisms through which enzymes act relies on the recognition, binding, and enzymatic disorder in the host–fungus cell wall (Elad 2000; Hermosa et al. 2000).

The production increase of chitinase (Limon et al. 1999) and cellulase (Gadgil et al. 1995; Wadhwa & Srivastava 1997) could be achieved by mutants of *Trichoderma* and important levels of toxic metabolites were also identified in *Trichoderma* mutants, which may be associated with the increased rhizosphere competence with other microflora and pathogens (Cotes et al. 1996, Migheli et al. 1998).

The tested samples of gamma irradiation induced mutants showed higher chitinase enzymes production compared to their parental unirradiated strain (NAS–K1). A major part of *Trichoderma* antifungal system contains a number of genes encoding for an astonishing variety of the 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 (Hermosa et al. 2000). Particularly, chitinolytic and glucanolytic enzymes are useful for biocontrol applications due to their ability in efficiently degrading the cell wall of plant pathogenic fungi. Each of these two classes of enzymes contains a number of proteins with different enzyme activities, and some of the enzymes have been purified and characterized and their genes were cloned. The cell–wall–degrading enzymes (CWDEs) from *Trichoderma* strains have great potential in agriculture as active components in new fungicidal formulations (Hermosa et al. 2000). According to the findings, purified CWDEs from different strains of *T. harzianum* are highly effective on the inhibition spore germination and mycelial growth in a broad range of pathogens such as *Rhizoctonia*, *Fusarium*, *Alternaria*, *Ustilago*, *Venturia*, *Pythium*, *Phytophthora*, *Colletotrichum* and especially *Botrytis* (Baek et al. 1999, Elad 2000, Benitez et al. 2000, Haggag & Mohamed 2002). Haran et al. (1996) reported an N–acetylglucosaminidase of 102 KDa (CHIT102), which was expressed by *T. harzianum* strain TM when grown on chitin as the sole carbon source. Accordingly, they assumed this could be the same enzyme described by Ulhoa & Peberdy (1991) and moreover, different estimates of its molecular mass were suggested to be occurred as a result of applying different procedures. The other 1, 4–β–N–acetylglucosaminidase, which was purified from *T. harzianum* strain Pl, was estimated to be 72 KDa and had a pI of 4.6 (Lorito et al. 1994). Haran et al. (1996) reported a 73 KD a glucosaminidase (CHIT73) that was expressed and excreted when *T. harzianum* strain TM was grown on chitin as the sole carbon source; however, it was not detected when the fungus was grown on glucose. The activity of CHIT73 was found to be heat–stable. N–acetylglucosaminidase enzymes were observed in molecular weight of 74 KDa and 94 KDa only for NAS–K1M25 SDS–PAGE profile.

Most studies conducted on the regulation of chitinase formation in *Trichoderma* spp. have identified chitinases only by enzyme assays, and have not addressed the possibility of differential regulation for various isozymes. The N–acetylglucosaminidase (NAGase) produced by Firmino et al. (2002) from *T. harzianum* culture fluid had a molecular mass of 68 KDa. Four endochitinase have been reported to be expressed by *T. harzianum*.

Haran et al. (1995) reported that, depending on the strain, the chitinolytic system of *Trichoderma* may contain five to seven individual enzymes. In the well–characterized strain *T. harzianum* TM, this system comprises two β–(1,4)–N–acetylglucosaminidases (102 and 73 KDa), four endochitinases (52, 42, 33, and 31 KDa), and one exochitinase (40 KDa) (Lorito et al. 1993, Haran et al. 1996). In addition, two 1, 4–β–N–acetylglucsoaminidase have been reported to be excerted by *T. harzianum*. Ulhoa & Peberdy (1991) described the purification of all these from *T. harzianum* 39.1. They estimated its native molecular mass to be 118 KDa by gel filtration, whereas by SDS–PAGE, this value was 66 KDa. Therefore, they suggested that, the active form was a homodimer. Haran et al. (1996) reported an endochitinase of 52 KDa (CHIT52). This enzyme was excreted when *T. harzianum* (strain TM) was grown on chitin as a sole carbon source, and it was highly sensitive to heat treatment. The highest expression of this enzyme was observed in the wild type and the studied mutant samples showed a lower bandwidth compared to the wild type. This enzyme band was shown in a molecular weight of 57 KDa in the NAS–K1M25. An endochitinase of 42 KDa (CHIT42) was reported by De La Cruz et al. (1992), Haran et al. (1996), and many other research studies (Table 4).

Table 4. Comparison of chitinase molecular weight from other *Trichoderma* species.

Organisms	Protein type (Size)	Substrate specificity	Ref.
T. virens UKM–1	42 KDa	Colloidal chitin	(Nadiawati et al. 2009)
T. virens Gv 29–8	42 KDa	ND*	(Baek et al. 1999)
T. harzianum	42 KDa	Colloidal chitin	(De La Cruz et al. 1992)
T. harzianum	44 KDa	$(GlcNAc)_3$	(Draborg et al. 1996)
T. harzianum strain 39.1	40 KDa	Swollen chitin	(Ulhoa&Peberdy, 1992)
T. atroviride	28 KDa	Chitosan 7B	(Omumasaba et al. 2001)
T. reesei PC–3–7	46 KDa	Chitosan (51.3 % deacetylated)	(Ike et al. 2006)
T. atroviride strain P1	30 KDa	GlcNAc oligomers β–chitin	(Hoell et al. 2005)

*ND: not determined

The pI value of the purified endochitinase from *T. harzianum* strain P1 was approximately 3.9, which showed an optimum activity at pH 4 (Harman et al. 1993). According to the preliminary reports, genes encoding endochitinase 42 (CHIT42), endochitinase 33 (CHIT33), and N–acetyl–β–D–glucosaminidase (nag1) were induced by fungal cell walls or colloidal chitin (Carsolio et al. 1994, Garcia et al. 1994; Limon et al. 1995, Peterbauer et al. 1996), as well as carbon starvation (Limon et al. 1995, Margolles et al. 1996). The expression of ech42 associated with light–induced spore germination was inhibited by carbon catabolites (Carsolio et al. 1994, Lorito et al.1996); whereas the transcription of nag1 (exochitinase) was induced by N–acetyl–β–D–glucosamine (Carsolio et al. 1994, Garcia 1994, Limon et al. 1995, Peterbauer et al. 1996). In spite of the chitinase producing micro–organisms that may be effective on the biological control and also despite the possible role of chitinases on the antagonistic process, literatures concerning purification, molecular and kinetic properties, molecular structures, and physiological roles of extracellular chitinases from mycoparasitic fungi are rarely conducted (Sahai & Manocha, 1993). The chitinase purified by De La Cruz et al. (1992) from *T. harzianum* culture fluid had a molecular mass of 40 KDa. Three chitinases purified by De La Cruz et al. (1992) showed molecular masses of 33, 37, and 42 KDa. Accordingly, these enzymes were necessary for maximum efficiency of the biological control of chitin containing plant pathogenic fungi.

Lorito et al. (1994) reported that, CHIT2, CHIT37, and CHIT33 enzymes in *T. harzianum* have 6.2, 6.6, and 7.8 isoelectric points, respectively. Moreover, the CHIT42and CHIT37 enzymes were matched with sharp spots 12 and 15, respectively. On the other hand, another sharp spot of this mutant isolate was found at a molecular weight of 31 KDa and isoelectric point (Ip) 6.9, which was related to the β–1.3 glucanase enzyme according to the results. These results were consistent with the level of enzyme activity of the NAS–K1M25 and increase of the chitinase and β–1,3glucanase enzymes in this mutant isolate relative to the unirradiated. In the unirradiated, spots with a molecular weight of 73 KDa and Ip 5 exhibited more spot intensity compared to the NAS–K1M25. Lorito et al. (1994) showed that the N–acetylglucosaminidase enzyme (NAG) in the *T. harzianum* had a molecular weight of 72 and Ip 4.6. Additionally, further expression of N–acetylglucosaminidase (NAG) in the unirradiated isolate led to a slight increase in the NAG activity in the unirradiated than that of the mutant isolate.

The pI value of CHIT42 purified from *T. harzianum* strain CECT 2413 was 6.2 (De La Cruz et al. 1992). Ulhoa and Peberdy (1992) reported a similar endochitinase of 40 KDa from *T. harzianum* strain 39.1, which exhibited an optimum activity at pH 4. Two endochitinase, estimated at 37 KDa and 33 KDa, were expressed by *T. harzianum* strain CECT 2413 when grown on chitin as the sole carbon source, and their pI values were 4.6 and 7.8, respectively (De La Cruz et al. 1992). Haran et al. (1996) detected two similar endochitinases of 33 KDa (CHIT33) and 31 KDa (CHIT31), which were expressed by *T. harzianum* strain TM.

Depending on available carbon source for *Trichoderma*, it can produce various types of chitinases with different molecular weight; therefore, fermentation of colloidal chitin by this fungus and the high production of chitinase enzymes and the high diversity of endochitinase enzymes; such as the enzymes raised in previous studies. For example, molecular weights of 31, 33, 40, 42 and 73 KDa, cannot be considered as a valid reason for the mechanism of the production of enzymes involved in the good antagonist of *Trichoderma* against the pathogen because the nature of the chitin wall of the pathogen fungus, both in terms of the molecular weight of the chitin polymer in the cell wall and polymer crystallization and the presence of side branches and wall protector compounds to prevent enzyme access, and also the diameter of the polymer filaments are very different compared to the chitin used in the laboratory (shrimp skin, etc.). Therefore, our understanding from the comparison of the results may be far from the truth. Hence, in this study, for the first time, in order to evaluate the enzymes that are effective on antagonistic activity, a pathogen fungus cell wall powder was used as a substrate for fermentation.

This study clearly showed the possibility of improving the antagonistic microorganisms for biological control of plant diseases through mutation with δ–irradiation. Induced mutation is one of the usual methods to restrain the genetic construction of microorganisms (Meloet al. 1997, Rey et al. 2000). Induced mutagenesis using δ–irradiation is proved to be effective on enhancing the exochitinase production and achieving biological efficiencies on NAS–K1.

Endochitinases randomly cleave chitin and *Trichoderma* endochitinase tends to be effective on controlling fungi more than chitinases found in plants or other fungi (Lorito et al. 1993b). Extensive investigations have shown that, there are virtually no chitinous pathogens tested in vitro resistant to *Trichoderma*chitinases (Lorito et al. 1993a, b, 1994a, b 1996). The high level and broad spectrum of resistance can be acquired with the endochitinase genes from *Trichoderma* spp. compared to the transgenic expression of plant or bacterial chitinases in plants (Lorito et al.1998). Mycoparasites are fungi that can parasitize other fungi, and this term is generally used to include parasites, which coil around the host hyphae or overgrow other colonies on agar (Paul 1999). Competition may play an important role in fungal interactions, and may occur as an independent phenomenon or in combination with other mechanisms (Whipps & McQuilken 1993).

In the present study, the WT strain and its mutants apply a synergistic, competition, and mycoparasitism effect on *M. phaseolina*. The authors suggest that, the WT strain and NAS–K1M25 have this potential to be as biocontrol agents of *M. phaseolina*. We also confirmed the enhanced biocontrol activity and chitinase and protein production by gamma irradiation mutants over the wild type strain. The results of chitinase enzyme activity assay clearly showed that, one of the reasons for antagonistic activity enhancement can be the high activity of the chitinase enzyme in the NAS–K1M25, and the results of the activity of chitinase enzyme assay confirmed the high antagonistic activity of this fungus compared to its unirradiated sample. In this regard, this case emphasizes that, the chitinase enzyme may not be the only enzyme involved in antagonistic activity and the activity of other enzymes, either alone or as synergistic effects, may be involved in the high antagonistic activity of the NAS–K1M25 mutant.

An additional protein band in gamma irradiation mutants increased the probability of enhanced biocontrol activity, chitinase and protein production in the mutant strains. These results demonstrated the potential of using improved strains as biocontrol agents for plant disease control by gamma irradiated and adapted mutants. This technique can also be used in other fungal strains to develop potential mutants with different applications. Chitinases are divided into 1,4–β–acetylglucosaminidases (GlcNAses), endochitinases, and exochitinases. Endochitinases are regulated by a variety of mechanisms; however, induction by stress has been reported for CHIT33, CHIT36, and CHIT42. Nevertheless, the induction under mycoparasitic conditions is not clear yet. Ech42 is induced prior to any physical contact with *Rhizoctonia solani* (Kullnig et al. 2000). CHIT33 can be expressed only during the contact phase and not before overgrowing *R. solani* (De las Mercedes Danaet. al. 2001), and CHIT36Y need no direct contact of the pathogen to be expressed. In order to test the role of chitinases in mycoparasitism; CHIT33, CHIT42, and CHIT36 have been overexpressed in *Trichoderma* spp. and the 42 KDa chitinase is believed to be a key enzyme. *Trichoderma virens* transformants overexpressing CHIT42 showed a significantly enhanced biocontrol activity compared to the unirradiated when assayed against *R. solani* in the cotton seedling experiments (Howell 2003).

Trichoderma harzianum transformants overex–pressing CHIT33 chitinase constitutively inhibited the growth of *R. solani* under both repressing and derepressing conditions; and the antagonist tests demonstrated that this chitinase plays an important role in mycoparasitism (Limon et al. 1999).

T. harzianum transformants overexpressing CHIT36 chitinase inhibited *F. oxysporum* and *Sclerotium rolfsii* more strongly than the unirradiated. Moreover, culture filtrates almost completely inhibited the germination of *B. cinerea* (Viterboet al. 2001).

ACKNOWLEDGEMENTS

This study was supported by grants from the Radiation Application Research School, Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy Organization of Iran (AEOI).

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ارزیابی الکتروفورز دو بعدی و فعالیت کیتینازی جدایه جهش‌یافته جدید *Trichoderma koningii* برای تجزیه بیولوژیکی دیواره سلولی *Macrophomina phaseolina*

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چکیده: در پژوهش های متعدد، به نقش آنزیم‌های خارج‌سلولی، در فرایند مایکوپارازیتیسم *Trichoderma*، اشاره شده است. گونه‌های این جنس، آنزیم‌های تجزیه کننده کیتین متعددی تولید می‌نمایند و این قارچ‌ها به عنوان آنتاگونیست موثر علیه *Macrophomina phaseolina*، عامل بیماری پوسیدگی ذغالی سویا، شناخته می‌شوند. در این پژوهش، آنالیز دو بعدی الگوی پروتئینی و ارزیابی فعالیت کیتینازی برای سویه ایرانی *Trichoderma koningii* (تیپ وحشی) و جدایه‌های جهش‌یافته آن به‌منظور نمایش نقش احتمالی اندوکیتینازها (N——acetylglucosaminidase I & II) در فعالیت آنتاگونیستی آن، انجام گردید. نتایج ارزیابی فعالیت تجزیه کیتین با استفاده از سوبسترای کیتین و دیواره سلولی میسلیوم *M. phaseolina* نشان داد که جدایه‌های جهش‌یافته به وضوح، فعالیت بیشتری نسبت به سویه والدی دارند. فعالیت ویژه آنزیم اندوکیتیناز در جدایه جهش‌یافته NAS——K1M25 ، تقریبا ۲/۵ برابر و ترشح آن، ۳ برابر سویه والد بود. این جدایه جهش‌یافته برتر، در آزمون کشت دوطرفه در آگار، بیش از ۶۵٪ (۵ برابر سویه والد) از رشد *M. phaseolina* جلوگیری نمود. همچنین، لکه ها و باندهای مرتبط با آنزیم‌های اندوکیتیناز و (N——acetylglucosaminidase I & II) در این جدایه، در آزمون الکتروفورز SDS——PAGE و 2D، از وضوح بیشتری برخوردار بود. به‌طور کلی، جهش القاء شده با پرتوتابی گاما، می‌تواند به عنوان یک روش موثر در توسعه جدایه‌های جهش‌یافته برتر، تلقی گردد و جدایه جهش‌یافته NAS——K1M25، می‌تواند به عنوان یک عامل کنترل بیولوژیکی بالقوه در برنامه‌های مدیریت بیماری های گیاهی ناشی از *M. phaseolina* مورد استفاده قرار گیرد. با این وجود، به منظور نهایی سازی پتانسیل‌های کنترل بیولوژیکی آن، مطالعات مربوط به تولید و تکثیر، فرمولاسیون و ارزیابی می‌بایست در شرایط مزرعه اجرا شود.

کلمات کلیدی: فعالیت آنزیمی، پرتوتابی گاما، کیتیناز، بیمارگر گیاهی، آنتاگونیست

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تاریخ دریافت: ۱۳۹۸/۰۶/۱۳ تاریخ پذیرش: ۱۳۹۸/۰۸/۲۹