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PRODUCTION OF CHITINASES AND Β-1, 3-GLUCANASES BY TRICHODERMA HARZIANUM ISOLATES FOR CONTROL OF VERTICILLIUM DAHLIAE IN PISTACHIO WILT

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ABSTRACT

Twenty isolates of *Trichoderma harzianum* isolated from the rhizosphere soil of healthy pistachio plants from different locations of Kerman Province, Iran. *In vitro* assays demonstrated that the culture filtrate of the fungal antagonist is effective on the growth of *Verticillium dahliae*, the causal agent of pistachio wilt.Also they were screened for chitinases and β-1,3-glucanases activities separately byinoculation into the Minimal Synthetic Medium with colloidal chitin and laminarin, as substrate for spectrophotometric analysis of enzymatic activity. The production of these lytic enzymes by *T. harzianum* isolate was optimized by using different pH, temperature, incubation period and shaking condition. Results showed that all the isolates produced chitinases and β-1,3-glucanases, although there were quantitative differences among isolates in the production of lytic enzymes. The specific activity of chitinases and β-1, 3-glucanases varied from 1.84 to 0.37and 3.14 to 1.18 (U.mg^{-1}protein) respectively. The highest specific activityof chitinases was recorded by Tr4 and Tr12 and the highest specific activity of β-1, 3-glucanases was observed by Tr12 and Tr16. However, the specific activityof chitinases was found least amount in these lytic enzymes. Total activities of the enzymes are greater at when isolates are cultured under the shaking condition at 150 rpm and 72 h after incubation at pH 5. Results also suggested that the maximum specific activity of chitinases at 25°C while the maximum specific activity of β-1, 3-glucanases at 35°C.

Keywords: β-1, 3-glucanases, Chitinases, *Trichoderma harzianum*, *Verticillium dahliae*.

INTRODUCTION

Verticillium dahliae Kleb., is a soilborne plant pathogen worldwide. It causes vascular wilts in more than 300 plant species including pistachio (Agrios, 1997; Inderbitzin and Subbarao, 2014). In some countries, including Iran, *Verticillium* wilt is a serious problem of pistachio (Aminaee and Ershad, 1999). Control of *V. dahliae* is difficult because of the lack of specificity of the host and the extreme variability of *V. dahliae* pathogenicity (Pegg, 2002).

The use of chemical compounds, resistant rootstocks and soil disinfestation methods areparticularly important elements of current management strategies. However, the effectiveness of these management practices is curtailed by *Verticillium* mode of conservation in soil as microsclerotia and the occurrence of new physiological races and chemical control is expensive and may be subject to future governmental restrictions due to environmental and health concerns (Rowe and Powelson, 2002). *Trichoderma* spp. are important biocontrol agents used for management of different diseases (Harman, 2004). They are free living fungi that are common in soil and root ecosystems. *Trichoderma* spp. can directly impact other fungi, after sensing a suitable fungal host, with the production of antibiotic, formation of specialized structures and degradation of the host cell wall, followed by the assimilation of its cellular content (Podile and Kishore, 2002; Chet and Chernin, 2002; Steyaert *et al.*, 2003). They are source of cell wall degrading enzymes which play a great role in biocontrol (Kullnig *et al*., 2000; Kubicek *et al*., 2001; Francesco *et al*., 2008).Mycoparasitism is the most mechanisms of antagonistic activities of *Trichoderma* spp., involving the production of lytic enzymes such as chitinases and glucanases (Elad *et al*., 1982; Chet, 1990; Lorito 1998; Ozbay and Newmann, 2004;

Howell, 2003; Benitez *et al*., 2004; Shoulkamy *et al*., 2006; Verma *etal*., 2007). The lytic enzymes break down cell wall polysaccharides into short oligomers and by this way facilitate the hyperparasite to penetrate into the cytoplasm of the target fungi (De la Cruz *et al*., 1995).

Chitin and glucan are the main polysaccharides and major cell wall constituents of higher fungi, suggest that chitinase and glucanase play an essential role in the lysis of phytopathogenic fungal cell walls during antagonism (Bartnicki-Garcia, 1968; Cherif and Benhamou, 1990; Flach *et al*., 1992; Felse and Panda, 1999; Karasuda *et al*. 2003; Kaur *et al*., 2005). Therefore, chitinases, the hydrolytic enzymes that specifically degrade chitin, are gaining much attention worldwide (Pichyangkura *et al*., 2002; Gkargkas *et al*., 2004; Makino *et al*., 2006; Wang *et al*., 2006). These chitinases are used in various applications such as biological control of fungal pathogens and are an effective tool for complete degradation of mycelia or conidial walls of pathogenic fungi (Chernin *et al*., 1997; Mathivanan *et al*., 1998; Someya *et al*., 2003; De la Vega *et al*., 2006; Chang *et al*., 2007). Microorganisms produce the chitinases primarily for assimilation of chitin as carbon ornitrogen source (Kupiec and Chet, 1998; Wang *et al*., 2006). The role of chitinase in the biological control of various fungal pathogens has already been established (Gunaratna and Balasubramanian, 1994; Chen *et al*., 2004; Huang *et al*., 2005). Several lines of evidence have shown that the production of some lytic enzymes is induced during the parasitic interaction between *Trichoderma* spp. and some pathogenic fungi (Sivan and Chet, 1989; De la Cruz *et al*., 1995; Haran *et al*., 1996a). Recently, the antagonistic properties of purified chitinolytic and glucanolytic enzymes from *T. harzianum* have been described, and evidence provided that these cell walls degrading enzymes may act synergistically with antibiotics. Chitinases are chitin-degrading enzymes that are able to break down polymeric chitin into simple monomers of N-acetyleglucosamine and hydrolyze the β -1, 4-glycosidic bonds between C1 and C4 of the N-acetyl glucosamine residues of chitin (Sahai and Manocha, 1993; Kitamura and Kamei, 2003). The chitinolytic enzymes from *T. harzianum* appeared to be biologically more active than enzymes from other sources and more effective against a wider range of fungi (Lorito *et al*., 1993). De La Cruz *et al*., in 1992 were the first to isolate, purify and characterize chitinases of *T. harzianum*, used as a means of biocontrol. Limon *et al*.,

(2004) detected that *T. harzianum* is a commonly dispersed antagonistic fungus generally correlates with the antifungal enzyme chitinase that degrade the fungal cell walls. The second group of enzymes that is important for the mycoparasitic activities of *Trichoderma* spp. is the glucanases. Glucanases are classified according to the type and location of glycosidic linkages that they cleave (Pitson *et al*., 1993). A glucanase that cleaves β-1,3-glucan-type bonded glucans is β-l,3-glucanase, and a glucanase that cleaves bonds located within the chain are endo-glucanases. The β-1,3-glucanases enzymes break down β-1, 3 glucan and thereby destroy their capacity to act on plant cells. Thrane *et al*., (2000) studied the two antagonistic *Trichoderma* spp. P1 and T3 that produced different kind of lytic enzymes in liquid culture. Inhibition of *Sclerotium rolfsii* correlated activities in the culture filtrate of *T. harzianum* strain T24, suggesting the involvement of these enzymes in the biocontrol process (El-Katatny *et al*., 2001). The production of these lytic enzymes by *T. harzianum* isolatesmay be performed by modifying the growth conditions, such as different pH, temperature, incubation period and shaking condition (Woo and Lorito, 2007). The aim of this study is evaluation of the mechanism in the process of parasitism of *V. dahliae* by twenty isolates of *T. harzianum* isolated from the rhizosphere soil of healthy pistachio plants from different locations of Kerman Province, Iran, involves the production of chitinases and β-1,3-glucanases and determination the physiological conditions which stimulate them *In vitro*. It is important to isolation and selection of *T. harzianum* isolates by potentially higher antagonistic efficiency with high capability of producing lytic enzymes against the test pathogen.

MATERIALS AND METHODS

Isolation of microorganisms

During 2012 – 2013, *Verticillium dahliae* isolate was obtained from pistachio shoots with wilt symptoms onselective media(Christen, 1981) and isoaltion of *Trichoderma harzianum* isolates were done from the rhizosphere soil of healthy pistachio orchards according to Rifai (1969) technique on DAVET selective medium (Davet, 1979), in different areas of Kerman Province, during 2013- 2014. After proper growth, isolates were purified and identified according to their morphology and microscopic characteristics by standard keys (Goud *et al*., 2003; Rifai, 1969; Bissett, 1991a; Bissett, 1991b and Samuels *et al*., 2015.Pathogenicity test of *V. dahliae* isolatewas done. The collected isolates were preserved on potato dextrose agar (PDA) and incubated at 4°C for long time.

Antifungal activity of *T. harzianum* isolates metabolites in culture filtrates

The effect of culture filtrate of the fungal antagonist on the growth of *V. dahliae* was studied according to Dennis and Webster (Dennis and Webster, 1971). *Trichoderma* isolates were grown separately in potato dextrose broth at 27^{o}C on rotary shaker (150 rpm) for 10 days. Cultures were filtered through a Whatmann No.1 filter paper and centrifuged at 12,000 rpm for 10 min at 4^{o}C at the end of 10th day. The pellets discarded and supernatants filtered through Sartorius Millipore (0.22 µ) filters. The PDA medium amended with 1000 ppm concentration of cell free metabolites obtained from different isolates of *T. harzianum*. The plates were then inoculated with 6 mm mycelium plug of *V. dahliae* in the center of each plate and incubated at 27^{o}C for five days. The control was maintained without metabolites. There were four replicates of each treatment and percent growth inhibition was calculated by the following formula:

$I= (C-T)/C \times 100$

Where, C is fungal mycelial growth in control plate, T is fungal mycelial growth in *V. dahliae*inoculated plate and I is the percent of inhibition of mycelial growth (El-Naggar *et al*., 2008).

Assay of enzyme activity

For assay of enzyme activity, 20 isolates of *T. harzianum* were separately inoculated into 100 ml Minimal Synthetic Medium (MSM) contained the following components (in grams per liter): $MgSO4. 7H2O$, 0.2g, $K2HPO4$, 0.9g, KCl, 0.2g, $NH4NO3$, 1.0g, $FeSO4. 7H2O$, 0.002g, $MnSO4$, 0.002g and $ZnSO4$, 0.002g with colloidal chitin and laminarin, as substrate for assay of chitinase and β-1,3 glucanase activity in 250 ml Erlenmeyer flasks.

Chitinases assay

Colloidal chitin was used as a substrate with reference to Wen *et al*. (2005). For chitinases assay by Molano *et al*., mehod with minor modifications (Ulhoa and Peberdy, 1992), 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) was taken to that 1 ml of enzyme was added and incubated at 30°C for 30 min. The hydrolysis reaction was terminated by adding 0.6 ml of dinitro salicylic acid (DNS) reagent (Nelson, 1944). The mixture was kept in a boiling water bath for 15 min, chilled and centrifuged to remove the insoluble chitin. The resulting adduct was measured by spectrophotometry (Systronics-2101) at 540 nm (Miller, 1959)and compared with standard graph drawn by following the same procedure but using different concentrations of glucose instead of culture filtrate. The amount of reducing sugar released was calculated from standard curve for glucose. One unit of chitinase activity was defined as the amount of enzyme that catalyzed the release of 1µmol of glucose equivalents per min under the given conditions. Specific activity of chitinases was expressed as Unit.mg^{-1}protein.

β-1,3-glucanases assay

For assay of β-1,3-glucanases enzyme with DNS method (Nelson, 1944), 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) and 1ml culture filtrate was mixed and incubated at 40°C for 60 min (Ramada *et al*., 2010). An equal volume of dinitro salicyclic acid (DNS) reagent was added to the reaction mixture and warmed in boiling water for 15 min. The absorbance of reaction mixture was measured by spectrophotometery (Systronics-2101) at 575 nm (Miller, 1959) and compared with standard graph drawn by following the same procedure but using different concentrations of glucose instead of culture filtrate. The amount of reducing sugar released was calculated from standard curve for glucose. One unit of β-1, 3 glucanases activity was defined as the amount of enzyme that catalyzed the release of 1µmol of glucose equivalents per min under the given conditions. Specific activity of β-1,3 glucanase were expressed as Unit.mg^{-1}protein.

Protein assay

For assays of the total protein concentration in the filtrates by Bradford method (1976) were carried out using Coomassie blue reagent (Coomassie Protein Assay Reagent, Piere) with bovine serum albumin (BSA) as the standard protein. From the protein concentration standard values the specific activity of the enzymes in the total filtrate (recorded as µmol of glucose/ N–acetylglucosomine released/h/µg protein in filtrate) was calculated.

Specific activity of chitinases and β-1,3 glucanases were expressed as Unit.mg^{-1}protein. However, unit activity was defined as the amount of enzyme necessary to produce 1 µM of corresponding reducing sugar per min per ml of culture supernatants.

Non enzymatic controls were also performed using boiled enzymes and were subtracted from the enzymatic values. Standard N-acetylglucosamine was prepared in borate buffer and measured following the above procedure. The amount of N-acetylglucosamine was calculated and expressed as appropriate.

Effect of temperature on chitinases and β-1,3-glucanases activities

100 ml of MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) for chitinases assay and 100 ml of MSM medium with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) for β-1,3-glucanases assay were used. A 6 mm plugs from margins of actively growing colonies of each *T. harzianum* strain and incubated at various temperatures, viz: 15, 20, 25, 30 and 35°C for 72 h with intermittent shaking at 150 rpm. The culture filtrate each isolate was harvested and assayed for chitinases and β-1,3-glucanase enzymes activities immediately.

Effect of pH on chitinases and β-1,3-glucanases activities

100 ml of MSM medium with pH levels of 4, 5, 6, 7 ,8 and 9 in 250 ml Erlenmeyer flasks left for 15 min. Effects of pH were assessed by the following buffers: sodium acetate at pH 4 and 5; potassium phosphate at pH 6 and 7; Tris-HCl at pH 8; and glycine-NaOH at pH 9. A 6 mm plug from the margin of actively growing colony of 20 isolates of *T. harzianum* was separately inoculated at 27°C for 72 h with intermittent shaking at 150 rpm. The culture filtrate each isolate was harvested and assayed for chitinases and β-1,3-glucanases enzyme activities immediately.

Effects of incubation period on chitinases and β-1,3-glucanases activities

The influence of incubation period on chitinases and β-1,3-glucanases enzyme activities was investigated with 100 ml of MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) for chitinases assay and 100 ml of MSM medium with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) for β-1,3-glucanases assay were used. A 6 mm plug from the margin of actively growing colony each of the 20 isolates of *T. harzianum* were separately inoculated at 27°C for different incubation period (24, 48, 72 and 120 hours) with intermittent shaking at 150 rpm. The culture filtrate each isolate was harvested and assayed for chitinases and β-1,3-glucanases enzyme activities, immediately.

Effects shaking condition on chitinases and β-1,3-glucanases activities

The influence of environmental factors such as shaking condition on chitinases and β-1,3-glucanases enzyme activities was investigated with 100 ml of MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) for chitinases assay and 100 ml of MSM medium with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) for β-1,3-glucanases assay were used. A 6 mm plug from the margin of actively growing colony each of the 20 isolates of *T. harzianum* was separately inoculated at 27 °C for 72 h with shaking (150 rpm) or non shaking condition. The culture filtrate each isolate was harvested and assayed for chitinases and β-1,3-glucanases enzyme activities, immediately.

Statistical analysis

Data were analysed on SAS system version 9.1 (SAS institute Inc., 1996). Mean separation was tested using the Duncan's multiple range test at p = 0.05. Test for effect of physical parameters on chitinases and β-1,3-glucanases activities were established under a factorial in complete randomized design with a control and four replications for each test pathogen.

RESULTS AND DISCUSSION

Isolation of microorganisms

One isolate of *Verticillium dahlia* by high pathogenecity isolated and used for further biocontrol investigations. Twenty islolates of *T. harzianum* collected from pistachio orchards in different areas of Kerman Province (Fig. 1), selected and designated as Tr1, Tr2, Tr3, … and Tr20. These 20 isolates showed highest *in vitro* activity.

Effect of metabolites in culture filtrates of *T. harzianum* isolates on mycelial growth of *V. dahliae*

Data of the antagonistic effect of 1000 ppm concentration of metabolites in culture filtrates of *T. harzianum* isolates against the mycelial growth of *V. dahlia In vitro* are shown in Fig. 2. Results revealed that Tr4 and Tr12 isolates were maximum effective for inhibition of mycelial growth of *V. dahliae* by 85.34% and 84.19% inhibition, respectively.

Extracellular enzymatic activity

Chitinases assay

The specific activity of chitinases of 20 isolates of *T. harzianum* varied from 1.84 to 0.37 U.mg^{-1} protein (Fig. 3). The highest specific activity was recorded in case of Tr4 (1.84 U.mg^{-1}protein) and Tr19 significantly produced minimum specific activity of chitinases(0.37U.mg^{-1}protein).

β-1, 3-glucanases assay

The specific activity of β-1, 3-glucanases of the strains varied from 3.73 to 0.33 U.mg^{-1} protein (Fig. 4). The highest specific activity was recorded in case of Tr12 (3.73 U.mg^{-1}protein).The specific activity of β-1, 3-glucanases of Tr18 (0.33 U.mg^{-1}protein) and Tr17 (0.36 U.mg^{-1}protein) were not significantly different (P<0.05). Tr18 significantly produced minimum specific activity of β-1, 3-glucanase (0.33 U.mg^{-1}protein).

Effect of temperature on chitinases and β-1,3-glucanases activities

The specific activities of chitinases and β-1,3-glucanasesenzymes of 20 *T. harzianum* isolates varied in MSM medium at different temperatures (15, 20, 25, 30 and 35°C) for 72 h with intermittent shaking at 150 rpm. The results suggested that maximum specific activity of chitinases at 25°C while the maximum specific activity of β-1, 3-glucanases at 35°C and the specific activity of chitinases and there was a gradual decrease in the specific activity of chitinases enzyme above and below at 25ºC and the specific activity of β-1, 3-glucanases enzyme was decreased below at 35ºC (Fig. 5). At25°C the highest specific activityof chitinases was recorded in Tr4 (2.31 U.mg^{-1}protein). Also the highest specific activityof β-1, 3-glucanasesat 35°C was recorded by Tr12 (4.13 U.mg^{-1}protein).

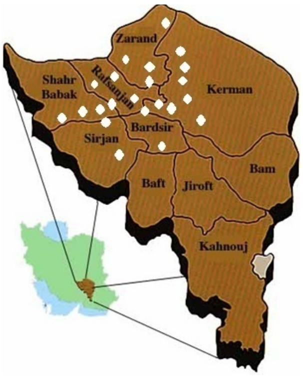

Fig. 1: Sites in Kerman Province which samples were collected and isolates of *Trichodermaharzianum* were obtained.

▨ **Sampling areas**

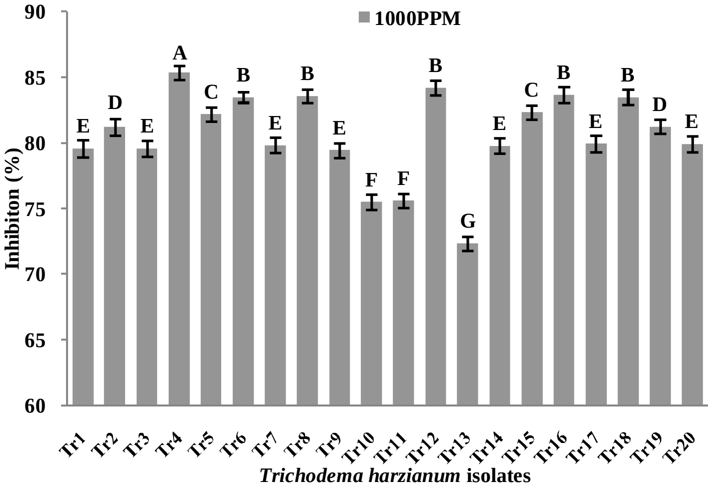

Figure 2: Effect 1000 ppm concentration of metabolites obtained from 20 isolates of *Trichoderma harzianum*. Metabolites mixed with PDA medium and growth of *V. dahliae* at 27°C measured at 5th day.

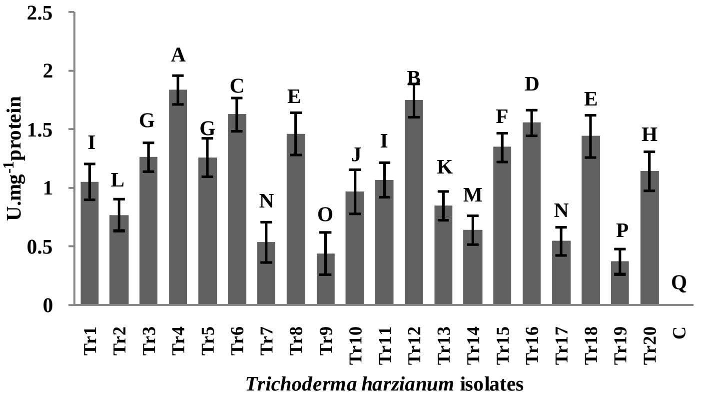

Figure 3: Specific activity of chitinases of 20 isolates of *Trichodema harzianum* incubated in MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) at 27 °C with intermittent shaking at 150 rpm measured at 5th day.

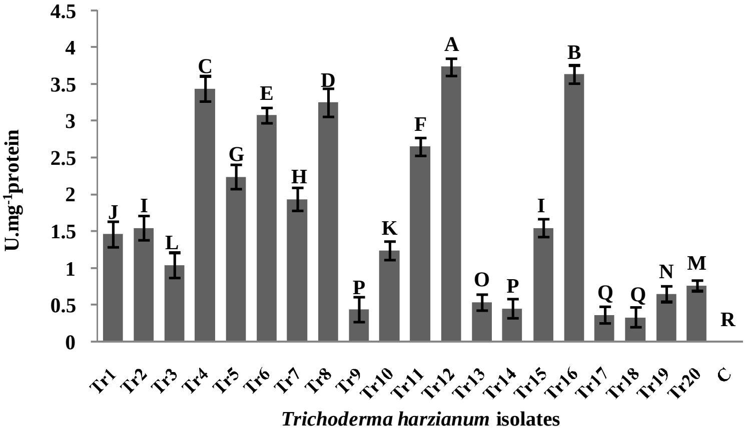

Figure 4: Specific activity of β-1,3 glucanases of 20 isolates of *Trichoderma harzianum* incubated in MSM medium with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) at 27 °C with intermittent shaking at 150 rpm measured at 5th day.

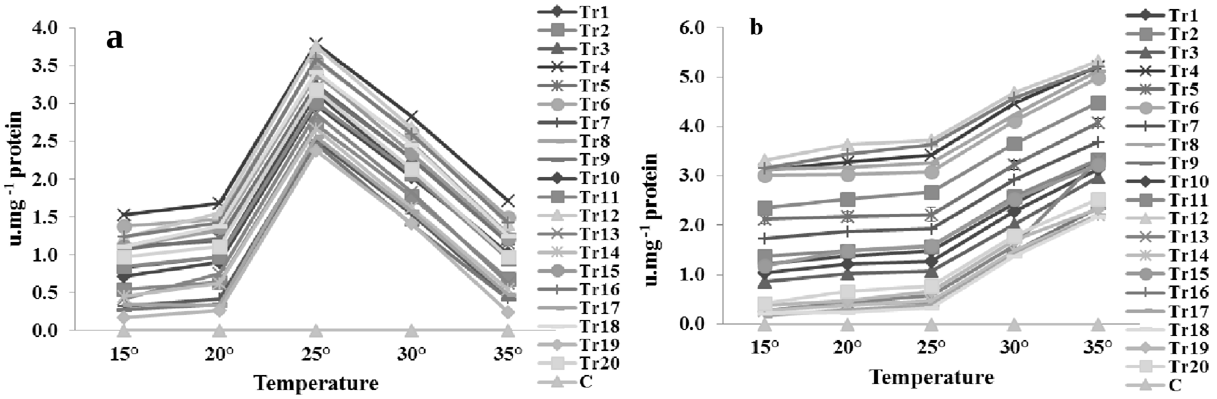

Figure 5: Effect of different temperatures (15, 20, 25, 30 and 35 °C) on specific activities of chitinases (a) β-1,3 glucanases (b) by 20 isolates of *Trichoderm harzianum* incubated in MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) for chitinases and with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) for β-1,3 glucanases at 27 °C for 72 h with intermittent shaking at 150 rpm.

Effect of pH on chitinases and β-1,3-glucanases activities

The results of the effect of different pH (4, 5, 6, 7, 8 and 9) on specific activities of chitinases and β-1,3 glucanases production by 20 isolates of *T. harzianum* indicated a with significant difference value at p=0.05 (Fig. 6). However, It was observed that the maximum Specific activities of chitinases and β-1,3 glucanases productions by all isolates at the 3th day of inoculation was found to be pH 5 (3.08 U.mg^{-1}protein) whereas minimum production was at pH 9 (o.o6 U.mg^{-1}protein) (Fig. 6). Tr4 and Tr12 (2.04 and 1.94 U.mg^{-1}protein) showed a maximum production of chitinases at pH 5. Also Tr12 and Tr16 (3.72 and 3.56 U.mg^{-1}protein) showed a maximum production of β-1, 3-glucanases at pH 5.

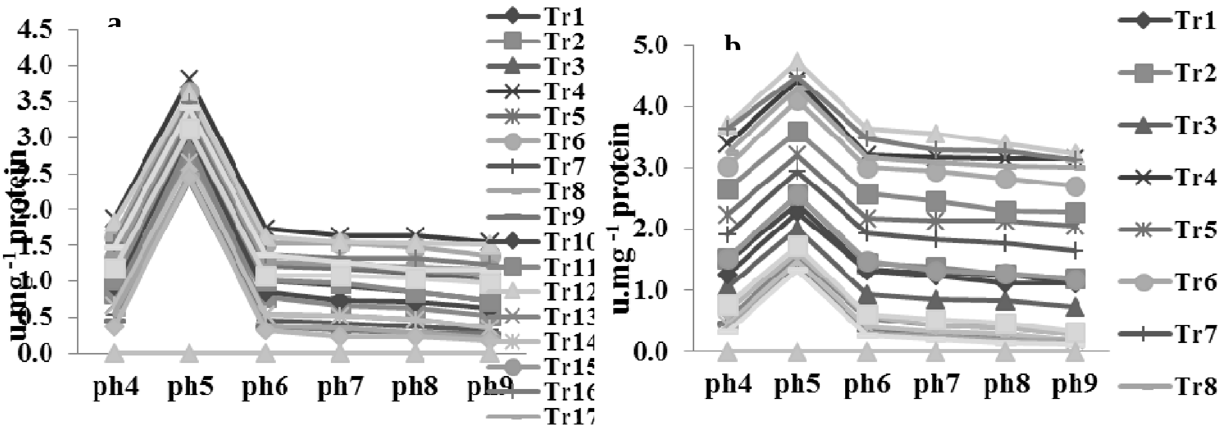

Figure 6. Effect of different pH (4, 5, 6, 7 ,8 and 9) on specific activities of chitinases (a) and β-1,3 glucanases (b) by 20 isolates of *Trichoderma harzianum* incubated in MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) for chitinases and with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) for β-1,3 glucanases at 27°C for 72 h with intermittent shaking at 150 rpm.

Effects of incubation period on chitinases and β-1,3-glucanases activities

From Fig. 7, it is clear that as incubation period is increased, specific activities of chitinases and β-1,3 glucanases enzymes also increased up to 72 hours after incubation. Although this effect was common to all *Trichoderma* isolates therefore, there is moderate for chitinases and β-1,3 glucanases productions. Then, the specific activities of both of chitinase and β-1,3 glucanases enzymesreduced at 120 h after incubation. Maximum specific activity of chitinases at 72 h of incubation period showed by Tr4 and Tr6 isolates (1.54 and 1.52 U.mg^{-1}protein) .Tr16 and Tr12 isolates (3.54 and 3.52 U.mg^{-1}protein) showed maximum β-1,3-glucanases activity as compared to other isolates.

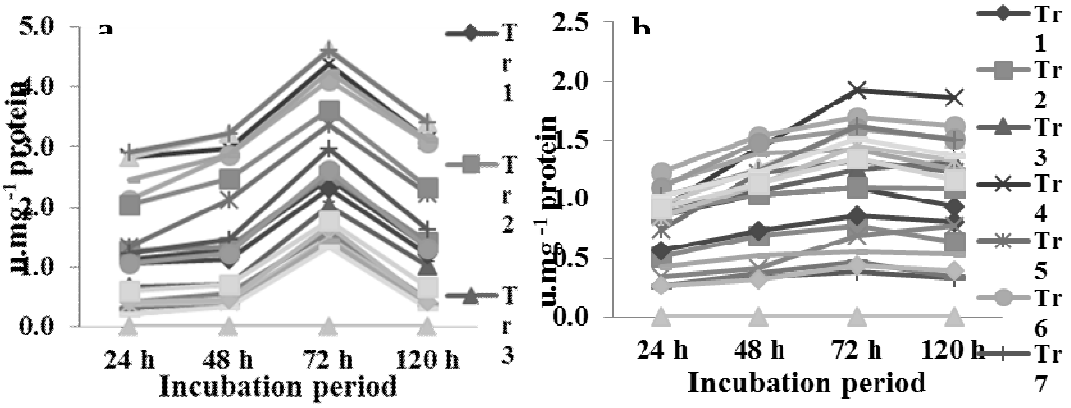

Figure 7. Effect of different incubation periods(24, 48, 72 and 120 hours) on specific activities of chitinases (a) and β-1,3 glucanases (b) by 20 isolates of *Trichoderma harzianum* incubated in MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) for chitinases and with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) for β-1,3 glucanases at 27 °C with intermittent shaking at 150 rpm.

Effects of shaking condition on chitinases and β-1,3-glucanases activities

Continuous shaking condition significantly favors maximum specific activities of chitinases and β-1,3-glucanasesenzymes of *T. harzianum* isolates in MSM medium using an orbital shaker at 150 rpm as compared to non shaking condition. At 150 rpm shaking condition, all isolates showed maximum specific activities of both enzymes (Fig. 8). Significant decrease in specific activity of both enzymes were observed under non shaking condition.

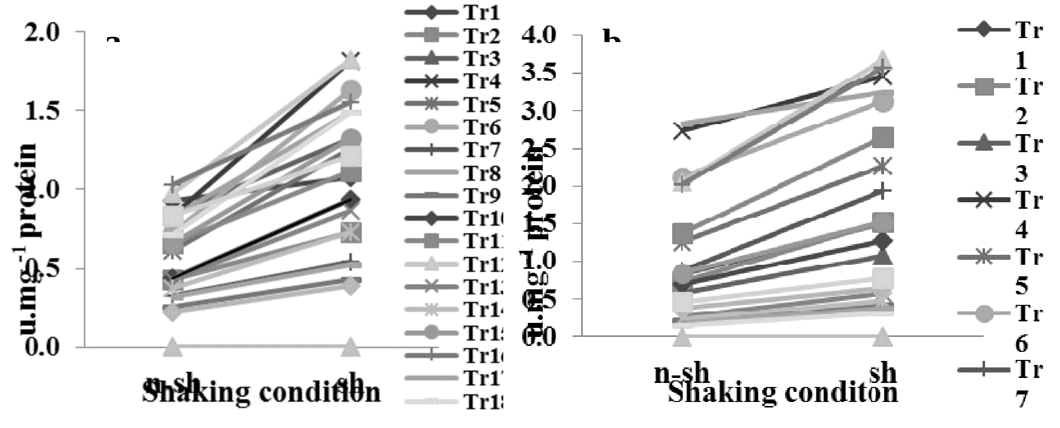

Figure 8. Effect of different shaking conditions (150 rpm (sh) and non shaking (n-sh) on specific activities of chitinases (a) and β-1,3 glucanases (b) by 20 isolates of *Trichodema harzianum* incubated in MSM medium with 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) for chitinases and with 1 ml 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH 4.8) for β-1,3 glucanases at 27 °C for 72 h.

DISCUSSION

Biological control of plant pathogens by microorganisms has been considered as a natural and environmentally acceptable alternative to the existing chemical treatment methods (Baker and Paulitz, 1996). *Trichoderma* spp., are widespread in almost any soil and rhizosphere, have also been reported as biocontrol agents due to its ability to successfully antagonize other fungi including plant pathogenic species particularly many common soil borne pathogens (Spiegel and Chet, 1998; Elad, 2000; Freeman *et al*., 2004; Ashrafizadeh *et al*., 2005; Dubey *et al*., 2007; Woo and Lorito 2007; Vinale *et al*., 2008). *Trichoderma* spp. are known to act through several mechanisms (Weindling, 1932; Hadar *et al*., 1979; Siameto *et al*., 2011; Mokhtar and Aid, 2013). Defense mechanisms of *Trichoderma* comprise both enzymatic and chemical weapons, which make they efficient mycoparasites, antagonists, and biocontrol agents (Vinale *et al*., 2009). Therefore, most of the recent research on *Trichoderma* spp. are understanding the mechanisms involved in the antagonistic effect against plant pathogen and led to the purification of cell wall degrading enzymes. These biocontrol species particularly *Trichoderma harzianum* are known to produce different kinds of cell wall degrading (chitinolytie and glucanoltic) enzymes, hundreds of antibiotic and lot of bioactive compounds hence playing a key role in mycoparasitism (Lorito *et al*., 1996). Lytic enzymes such as chitinases and β-1, 3-glucanases function by breaking down the polysaccharides, chitin and β-glucans that are responsible for the rigidity of fungal cell walls and dissolves them (Henis and Chet, 1975; Elad *et al*., 1982; Elad *et al*., 1983; Haran *et al*., 1996b). These cell fragments in turn induce the production of further enzymes and trigger a cascade of physiological changes, stimulating rapid and directed growth of *Trichoderma* sp. (Zeininger *et al*., 1999). Previous studies have demonstrated that before mycelia of fungi interact, *Trichoderma* sp., produces low quantities of extracellular exochitinase (Kullnig *et al*., 2000; Brunner *et al*., 2003). This could be due to a high degree of mycoparasitism and production of some lytic enzymes by this *Trichoderma* isolate. In this study, we evaluated the ability of *T. harzianum* strains isolated from different locations of Kerman Province, Iran, that they are effective biocontrol agents against *Verticillium dahlia*e, (the causal agent of pistachio wilt) for antifungal activity of metabolites in culture filtrates and chitinases and β-1,3-glucanases activity. Effect of filterate concentrations of *T. harzianum* that collected on mycelial growth of *V. dahlia*e was revealed that the aqueous extracts of *T. harzianum* reduced the mycelial growth of the test pathogen. The present investigation suggests that metabolites released by this *Trichoderma* isolates are toxic and fungistatic to *V. dahliae*. The results were in agreement with Hajieghrari *et al*., (2008) who evaluated six isolated of *Trichoderma* sp. against five isolates of soil borne phytopathogenic fungi by production of non-volatile inhibitors and Barakat *et al*.,(2013) that they evaluated effect of volatile and non-volatile compounds of *Trichoderma* spp. on *Botrytis fabae*. The antibiotics produced by *T. harzianum* are responsible for the inhibitor action against root pathogen, *F. culmorum* (Iqbal *et al*., 1994) and *F. oxysporum* (Michrina *et al*., 1995). Benitez *et al*., (2004) demonstrated that *Trichoderma* strains that over produce chitinases have been shown to be effective biocontrol agents against pathogens such as *B. cinera* and *R. meloni*. Major cause of biocontrol activity of *Trichoderma* is concerned with production of chitinases to disintegrate the cell wall of fungal phytopathogens (Anand and Reddy 2009). As the cell wall of *V. dahlia*e are composed of chitin and β-1, 3 glucan (Adams, 2004), one idea that has been advanced is that enzymes such as chitinases and glucanases produced by by *T. harzianum* might be involved in hydrolysis of *V. dahlia*e cell wall during antagonism. Production of four β-1,3-glucanases by *T. harzianum* has been described by Kitamoto *et al*., (1987). Also, Simmoms, 1994 described that glucanases are among the plant defense responses to pathogen attack. Lorito *et al*., (1994) reported the involvement of glucanase in mycoparasitism. Jones *et al*. (1974) have shown that *T. viride* solubilized hyphae of *Sclerotinia sclerotiorum* by β-1, 3-glucanases activity. Also, results indicate that different isolates of *Trichoderma* have different antagonistic capacity, therefore their enzyme activities may also vary. Elad *et al.* (1982) reported that the isolates of *T. harzianum*, which were found to differ in their ability to attack *Sclerotium rolfsii*, *Rhizoctonia solanii* and *P. aphanidermatum*, also differed in the levels of mycolytic enzymes produced by them. The variation in fungicidal activity among the *T. harzianum* isolates could be attributed to the presence of different types of chemical constituents in different isolates (Wang *et al*., 2003; Zhou *et al*., 2008; Eneyskaya *et al*., 2009; Yang *et al*., 2009). Thrane *et al.* (2000) studied the two antagonistic *Trichoderma* spp. P1 and T3 that produced different kind of lytic enzymes in liquid culture. In the present study was observed that the specific activity of chitinases ranges of 20 isolates of *T. harzianum* to be from 3.14 to 1.18 ($U.mg^{-1}$protein) and specific activity of β-1,3-glucanases ranges from 3.73 to 1.04($U.mg^{-1}$protein) and the highest specific activity of chitinases in Tr4 isolate (1.84 $U.mg^{-1}$protein) and minimum specific activity in Tr19 isolate ($0.37U.mg^{-1}$protein). Also the maximum specific activity of β-1, 3-glucanases was recorded in case of Tr12 (3.73 $U.mg^{-1}$protein) and minimum specific activity in Tr18 isolate (0.33 $U.mg^{-1}$protein). Results also revealed that Tr4 and Tr12 isolates were maximum effective for inhibition of mycelial growth of *V. dahliae* by 85.34% and 84.19% inhibition, respectively. This might be one of the reasons for its biocontrol potentiality. The lytic activity of several strains of *Trichoderma* spp. on cell walls of phytopathogenic fungi was correlated with the degree of biological control of these pathogens *in vivo* (Papavizas, 1985; Vidhyasekaran and Balasubramanian, 1995; Vidhyasekaran *et aI.*, 1996). Matroudi *et al.*, (2009) tested 30 *Trichoderma* isolates and on the basis of maximum level of chitinases and indicated that *T. atroviride* can be employed in the field as biological control agents against *S. sclerotiorum*.

Environment also causes either negative or positive effects on Trichoderma. For the negative effect, Davet (1979) recorded around a 10% loss of conidial viability over roughly a two-year period. Therefore, physical parameters including initial medium pH, incubation temperature, incubation period and aeration play important roles in enhancing enzymeactivity. Therefore, they need to be optimised. For optimisation of initial medium, we used different pH, temperature, incubation period and shaking condition. In the present study temperature showed a significant role in chitinase and β-1, 3-glucanase production by *T. harzianum* isolates. It was observed that maximum specific activity of chitinases at 25°C while the maximum specific activity of β-1, 3-glucanases at 35°C and there was a gradual decrease in the specific activity of chitinases enzyme above and below at 25ºC and the specific activity of β-1, 3-glucanases enzyme was decreased below at 35ºC. Whilemaximum enzyme production of chitinases was observed from 30 to 40°C and the optimum temperature at 40°C was shown a maximal relative chitinases activity of 99% (Skujins *et al.*, 1965; Gupta *et al.*, 1995; Mahadevan and Crawford, 1997; Sayed *et al.*, 2000; Gomes *et al.*, 2001). Ambient pH seems to be a general factor controlling enzyme secretion in fungus – host interactions through a conserved genetic circuit is also, one of the most important factors for any type of enzyme production (Maccheroni *et al.*, 2004).The results of the effect of different pH on specific activity of chitinases and β-1,3 glucanases indicated a with significant difference so that maximum specific activities of chitinases and β-1,3 glucanases productions by all isolates at the 3th day of inoculation was found to be pH 5 (3.08 U.mg^{-1}protein) whereas minimum production was at pH 9 (o.o6 U.mg^{-1}protein). Kim *et al*., (2011) test the pH of the medium plays an important role for the chitinases production from degradation of shrimp shells by *Streptomyces* sp. They reported that it was capable of high amount of chitinases production and chitinases activity at pH 7. Also the optimum pH for the chitinases produced by the strains *Bacillus* sp.13.26 and *Pseudomonasaeruginosa* K-187 was nearly neutral (Wang and Chang, 1997; Purwani *et al*., 2004). Maximum chitinases production by *Paenibacillus* sp. D1 was observed at 7.0 (Anil kumar, 2010). Also, it is clear that as incubation period is increased, specific activity of chitinases and β-1,3 glucanases enzymes increased up to 72 hours after incubation and then, reduced at 120 h after incubation. Maximum chitinases production of *Streptomyces* sp. was recorded on 6 days after incubation and then it decreased (Reynolds, 1954). Also continuous shaking condition significantly favors maximum specific activity of chitinases and β-1,3-glucanasesat 150 rpm as compared to non shaking condition. As similar observation El-Katatny *et al*., (2000), tested twenty four isolates of *T. harzianum* for β -1,3-glucanases and chitinases activity. Enzymes production was significantly influenced under various culture conditions. Maximum activity of both enzymes was at acidic pH from 5.5 to 6.0 at 4th after incubation. Majority of the fungi reported to produce maximum level of chitinases in acidic conditions (Kovacs *et al*., 2004; Sharaf, 2005). Concluding remarks research on the mechanisms responsible for the biocontrol exerted by *Trichoderma* spp. on phytopathogenic fungi have led to a better understanding of such mechanisms, as well as to the isolation of several genes encoding either enzymes and structural or regulatory proteins, or components of signaling pathways that are involved in processes such as the specific recognition of hosts by *Trichoderma* strains. Therefore, *T. harzianum* isolates were selected and its growth conditions were standardized in order to optimize the chitinases and β-1,3-glucanases production. These tools will allow the isolation of improved strains and thus of more efficient formulations to control fungal pathogens in pre and post-harvest periods.

CONCLUSION

According to the results obtained in this research, we conclude that *Trichoderma* spp. are potent agents to control the devastating phytopathogen *Verticillium.* It is well known that soil inhibitant pathogens are hard to control chemically; however, biological control has the ability to maintain a long lasting effect with least environmental adverse effects. In this path, meticulous research is needed to get better insight into biological status of rhisosphere and attaining bio-tools to manipulate it twards benefit of plants and against the pathogens. We believe that there exists more work to be perfomed for attaining indepth knowledge for such man-made manipulations. As with so many other aspects of science, basic knowledge about the mechanisms involved in the biocontrol process will be of immense value to those scientists intent on developing new methods for utilizing biocontrol agents. In this area, our results and outcome of other researchers are the early steps for a great future goal to attain a sustainable biological environment. We hope that the early steps taken in the present investigation will be taken into account by other scientist to develop strategies in controlling *Verticillium* wilt disease in pistachio trees wordwide.

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REFERENCES

[1] Adams D.J., 2004. Fungal cell wall chitinase and glucanases.Microbiology 150: 2029-2035.

[2] Agrios G. N., 1997. Plant Pathology. 4th Ed. San Diego, Academic Press, 635 pp.

[3] Aminaee M. M. and Ershad D., 1999. Occurrence of *Verticillium* wilt on pistachio trees in Kerman province (Iran). Iran. J. Plant Pathol. 35:59.

[4] Anand S. and Jayarama R., 2009. Biocontrol potential of *Trichoderma* sp. against plant pathogens.Int. J. Agri. Sci., 1(2): 30-39.

[5] Anil Kumar S., 2010. Optimization of culture conditions for thermostable chitinase production by *Paenibacillus* sp. D1 African J of Microbiol Res 4(21): 2291-2298.

[6] Ashrafizadeh, A., Etebarian H.R. and Zamanizadeh H.R. 2005. Evaluation of Trichoderma isolates for biocontrol of Fusarium wilt of melon. Iranian J. Phytopathol., 41: 39-57.

[7] Baker R. and Paulitz T.C., 1996. Theoretical basis for microbial interactions leading to biological control of soilborne plant pathogens In: Principles and Practice of

Managing Soilborne Plant Pathogens. Hall, R. (ed.). The American Phytopathol. Soc. St. Paul, MN. pp. 50-79.

[8] Barakat F.M., Abada K.A., Abou-Zeid N. M. and El-Gammal Y. H. E., 2013. Effect of volatile and non-volatile compounds of *Trichoderma* spp. on *Botrytis fabae* the causative agent of faba bean chocolate spot. Research WebPub. 1(3):42-50.

[9] Bartnicki-García S., 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Annu Rev Microbiol 22:87-108.

[10] Benitez T., Rincon A. M., Limon M. C. and Codon A. C., 2004. Biocontrol mechanisms of Trichoderma strains. International microbiology. 7: 249–260.

[11] Bissett J., 1991a. A revision of the genus Trichoderma. II. Infrageneric classification. Canadian. Journal of Botany, 69: 2357–2372.

[12] Bissett J., 1991b. The revision of the genus Trichoderma III. Section Pachybasidium. Canadian. Journal of Botany, 69: 2357-237.

[13] Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

[14] Brunner K., Peterbauer C.K., Mach R.L., Lorito M., Zeilinger S. and Kubicek R.L., 2003. The N-acetylgucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin of and major relevance to biocontrol. Curr. Gen. 43:289-295.

[15] Chang W.T., Chen Y.C. and Jao C.L., 2007. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. Bioresour. Technol. 98: 1224-1230.

[16] Chen C.Y., Wang Y.H. and Huang C.J., 2004. Enhancement of the antifungal activity of *Bacillus subtilis* F29-3 by the chitinase encoded by *Bacillus circulans* chiA gene. Can. J. Microbiol. 50: 451-454.

[17] Chérif M. and Benhamou N., 1990. Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporum* f.sp. radicis lycopersici. Phytopathology 80: 1406-1414.

[18] Chernin L.S., De La Fuente L., Sobolev V., Haran S. and Vorgias C.E., 1997. Oppenheim AB, Chet I. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. Appl. Environ. Microbiol. 63: 834-839.

[19] Chet I. and Chernin L., 2002. Microbial enzymes in the biocontrol of plant pathogens and pests. In Enzymes in the environment, R.G. Burns and R.P. Dick, Eds (New York: Marcel Dekker, Inc.), pp. 171-226.

[20] Chet I., 1990: Biological control of soil-borne plant pathogens. (Hornby C.A.B., ed.) pp. 15-26.

[21] Christen A. A., 1981. A selective medium for isolating *Verticillium albo-atrum* from soil. Phytopath. 72: 47–49.

[22] Davet P., 1979. Technique pour 1' analyse des population et de Trichoderma et de Gliocladium virens dans le sol. Annual Review of Phytopathology, 11: 529-533.

[23] De la Cruz J., Hidalgo-Gallego A., Lora J.M., Benitez T., Pintor-Toro J.A. and Llobell A., 1992. Isolation and characterization of three chitinases from *Trichoderma harzianum*, European Journal of Biochemistry 206, 859–867.

[24] De-La Cruz, J., Toro-Pintor J. A., Benitez, T. and Llobell A., 1995. Purification and characterization of anendo-beta-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J. Bacteriology 177: 1864–1871.

[25] De la Vega L.M., Barboza-Corona J.E., Aguilar-Uscanga M.G. and Ramirez_lepe M., 2006. Purification and characterization of an exochitinase from *Bacillus thuringiensis* subsp. aizawai and its action against phytopathogenic fungi. Can J. Microbiol. 52: 651-657.

[26] Dennis C. and Webster J., 1971. Antagonistic properties of species-groups of Trichoderma: I. Production of non-volatile antibiotics. Transactions of British Mycological Society, 57: 25-39.

[27] Dubey S. C., Suresh M. and Singh B., 2007. Evaluation of

Trichoderma species against *Fusarium oxysporum* f.sp. ciceris for integrated management of chickpea wilt. Biol. Contr., 40: 118-127.

[28] Elad Y, Chet I. and Henis Y., 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can J Microbiol 28:719–725.

[29] Elad Y., 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Prot., 19: 709-714.

[30] Elad Y., Barker R., Chet I. and Henis Y., 1983. Ultrastructural studies of the interaction between *Trichderma* spp. and plant pathogenic fungi. Phythol. Z., 107: 168-175.

[31] El-Katatny M. H., Somitsch W., Robra K. H., El-Katatny M. S. and Gübitz G. M. 2000. Production of Chitinase and ß -1,3-glucanase by *Trichoderma harzianum* for Control of the Phytopathogenic Fungus *Sclerotium rolfsii*. Food technol. biotechnol. 38 (3) 173–180.

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

[33] El-Naggar M., Kövics G. J., Sándor E. and Irinyi L., 2008. Mycoparasitism and antagonistic efficiency of *Trichoderma reesei* against *Botrytis* spp. Contributii Botanice 43:141-147.

[34] Eneyskaya E.V., Sundqvist G., Golubev A.M., Ibatullin F.M., Ivanen D.R., Shablin K.A., Brumer H. and Kulminsskaya A.A., 2009. Transglycosylating and hydrolytic activics of the ß-mannosidase from *Trichoderma reesei*. Biochimie. 91:632-638.

[35] Felse P.A. and Panda T., 1999. Regulation and cloning of microbial chitinase genes. Appl. Microbiol. Biotechnol. 51: 141-151.

[36] Flach J., Pilet P.E. and Jolles P., 1992. What's new in chitinases research Experientia (Basel) 48: 701-716.

[37] Francesco V, Krishnapallai S, Emiloi L, Ghisalberti R M, Sheridan L, Woo M.L., 2008. Trichoderma-Plant-pathogen interactions, Soil Biol. Biochemist. 40: 1-10.

[38] Freeman S., Minz D., Kolesnik I., Barbul O., Zreibil A., Maymon M., Nitzani Y., Kirshner B., Rav-David D., Bilu A., Dag A., Shafir S. and Elad Y., 2004. Trichoderma biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea*, and survival in strawberry. Eur. J. Plant Pathol., 110: 361-370.

[39] Gkargkas K., Mamma D., Nedev G., Topakas E., Christakopoulos P., Kekos D. and Macris B.J., 2004. Studies on N-acetyl-ß-D-glucosaminidase produced by *Fusarium oxysporum* F3 grown in solid-state fermentation. Process Biochem. 39: 1599-1605.

[40] Gomes R.C., Semedo L.T.A., Soares R.M.A., Linhares L.F., Ulhoa C.J., Alviano C.S. and Coelho R.R.R., 2001. Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. J Appl Microbiol 90: 653-661.

[41] Goud J. K., Termorshuizen A. J., and Gams W., 2003. Morphology of *Verticillium dahliae* and *V. tricorpus* on semi-selective media used for the detection of *V. dahliae* in soil. Mycol. Res. 107:822-830.

[42] Gunaratna K.R. and Balasubramanian R., 1994. Partial purification and properties of extracellular chitinase produced by *Acremonium obclavatum*, an antagonist to groundnut rust, *Puccinia arachidis*. World J. Microbiol. Biotechnol. 10: 342-345.

[43] Gupta R., Saxena R.K., Chatuverdi P. and Virdi J.S., 1995. Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. J Appl Bacteriol 78: 378-383.

[44] Hadar Y., Chet I. and Henis Y., 1979. Biological control of *Rizhoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum*. Phytopathology, 69: 64-68.

[45] Hajieghrari B., Torabi-Giglou M., Mohammadi M.R. and Davari M.,

2008. Biological potential of some Iranian Trichoderma isolates in the control of soil borne plant pathogenic fungi. Afr. J. Biotechnol. 7: 967-972.

[46] Haran S., Schickler H. and Chet I., 1996a. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*, Microbiology 142(9), 2321-2331.

[47] Haran S., Schickler H., Oppenheim A. and Chet I., 1996b. Differential expression of *Trichoderma harzianum* chitinases during mycoparasitism. Phytopathology 86:980-985

[48] Harman G. E., 2004. *Trichoderma* species opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2: 43-56.

[49] Henis Y. and Chet I., 1975. Adv Appl Microbiol. 19:85-111.

[50] Howell C. R., 2003. Mechanisms employed by Trichoderma species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis. 87: 4-10.

[51] Huang C.J., Wang T.K., Chung S.C. and Chen C.Y., 2005. Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. J. Biochem. Mol. Biol. Sci. 38: 82-88.

[52] Inderbitzin P. and Subbarao K.V., 2014. *Verticillium* systematics and evolution: how confusion impedes *Verticillium* wilt management and how to resolve it. Phytopathology, 104, 564–574.

[53] Iqbal S. M., 1994. Antagonism to *Colletotrichum falcatum* Went, the cause of sugarcane red rot. Sarhad Journal of Agriculture 10: 575-579.

[54] Jones D., Gordon A.H. and Bacon J.S.D., 1974. Cooperativeaction by endo and exo-β-(1-3) glucanase from parasitic fungi in the degradation of cell wall glucans of *Sclerotinia sclerotium*. Biochem. J., 140: 47-55.

[55] Karasuda S., Tanaka S., Yamamoto Y. and Koga D., 2003: Plant chitinase as a possible biocontrol agent for use instead of chemical fungicides. Bio Sci. Biotech. and Biochem. 67(1): 221-224.

[56] Kaur J., Munshi G.D., Singh R.S. and Koch E., 2005. Effect of carbon source on production of lytic enzymes by the sclerotial parasites *Trichoderma atroviride* and *Coniothyrium minitans*. Journal of Phytopathology 153, 274–279.

[57] Kim C.H., Tzu H., Chung S.L.,Ying T.C. and Chun Y.L., 2011. The Chitinolytic Activities of *Streptomyces* sp. TH-11. Int J Mol Sci 12: 56-65.

[58] Kitamoto Y., Kono R., Shimoyori A., Mori. N. and Ichikawa Y., 1987. Purification and some properties of an exo-ß-glucanase from *Trichoderma harzianum*. Agric. Biol. Chem. 51: 3385-3386.

[59] Kitamura E. and Kamei Y., 2003. Molecular cloning, sequencing and expression of the gene encoding a novel chitinase A from marine bacterium, *Pseudomonas* sp. PE2, and its domain structure. App Microbiol Biotechnol 61:140–149.

[60] Kovacs K., Szakacs G., Pusztahelyi T. and Pandey A., 2004. Production of chitinolytic enzymes with *Trichoderma longibrachiatum* IMI 92027 in solid substrate fermentation. Appl. Biochem. Biotechnol. 118:189-204.

[61] Kubicek C.P., Mach R.L., Peterbauer C.K. and Lorito M., 2001. Trichoderma: from genes to biocon-trol. J Plant Path 83: 11-23.

[62] Kullnig C., Mach R. L., Lorito M. and Kubicek C. P., 2000. Enzyme diffusion from *Trichoderma atroviride* to *Rhizoctonia solani* is a perequisite for triggering of Trichoderma ech42 gene expression before mycoparasitic contact. Appl. Environ. Microbiol. 66:2232-2234.

[63] Kupiec C.R., Chet I., 1998. The molecular biology of chitin digestion Curr. Opin. Biotechnol. 9: 270-277.

[64] Limon M.C., Chacon M.R., Mejias R., Delgado-Jarana J., Rincon A.M., Codoón A.C. and Benitez T., 2004. Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain.Appl Micro and Biotech., 56: 137-143.

[65] Lorito M., 1998. Trichoderma and Gliocladium, Harman G.E., and Kubicek C.E., Eds., London: Taylor and Francis, (2):73–99.

[66] Lorito M., Farkas V., Rebuffat S., Bodo B. and Kubicek C.P., 1996. Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. Journal of Bacteriology 178: 6382–6385.

[67] Lorito M., Hayes C., Broadway K. R. M., Tronsmo A., Woo S. L. and Di-Pietro A., 1993: Chitinolytic enzymes produced by *Trichoderma harzianum* antifungal activity of purified endochitinase and chitobiosidase. Phytopathology. 83 (3): 302-307.

[68] Lorito M., Peterbauer C., Hayes C.K. and Harman G.E., 1994. Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiology 140: 623–629.

[69] Maccheroni J. W., Welington L.A. and Azevedo J.L., 2004. A*mbient pH* regulated enzyme secretion in endophytic and pathogenic isolates of the fungal genus *colletotrichum*. Sci. Agric. 61(3): 298-302.

[70] Mahadevan B. and Crawford D.L., 1997. Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108. Enzyme Microb Technol 20: 489-493.

[71] Makino A., Kurosaki K., Ohmae M. and Kobayashi S., 2006. Chitinase-catalyzed synthesis of alternatingly N-deacetylated chitin: a chitin-chitosan hybrid polysaccharide. Biomacromolecules, 7(3):950-957.

[72] Mathivanan N., Kabilan V. and Murugesan K., 1998. Purification, characterization and antifungal activity from *Fusarium chlamydosporum*, a mycoparasite to groundnut rust, *Puccinia arachidis*. Can. J. Microbiol. 44: 646-651.

[73] Matroudi S., Zamani M.R. and Motallebi M., 2009. Antagonistic effects of three species of *Trichoderma* sp. on *Sclerotinia sclerotiorum*, the causal agent of canola stem rot. Egypt. J. Bio., 11: 37-44.

[74] Michrina J., Michalikova A., Konacik T. and Kulichora R., 1995. Antibiosis a possible mechanism of antagonistic action of *Trichoderma harzianum* against

Fusarium culmorum. Ocharna Rostlin 31: 177-184.

[75] Miller G.L.,1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, (31) 3: 426-428.

[76] Mokhtar H. and Aid D., 2013. Contribution in isolation and identification of some pathogenic fungi from wheat seeds, and evaluation of antagonistic capability of *Trichderma harzianum* against those isolated fungi *in vitro*. Agric. Boil. J. N. Am. 4(2): 145-154.

[77] Nelson N., 1944. A photometric adaption on the Somogyi method for the determination of glucose. Journal of Biological Chemistry. 153: 375-380

[78] Ozbay N. and Newman S.E., 2004.:Biological control with *Trichoderma* spp. With emphasis on *Trichoderma harzianum*. Pakistan Jr. of Bio-logical Sciences.7(4), 478-484.

[79] Papavizas G.C., 1985. Trichoderma and Gliocladium. Biology, ecology and potential for biocontrol. Ann. Rev. Phytopathol. 23, 23-54.

[80] Pegg G.F. (ed.), 2002. *Verticillium* Wilts. CAB International, Wallingford, UK, 576 pp.

[81] Pichyangkura R., Kudan S., Kultiyawong K., Sukwattanasinitt M. and Aiba S., 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. Carbohydr. Res. 337: 557-559.

[82] Pitson S. M., Seviour, R J. and Mc Dougall B. M., 1993. Non cellulolytic fungal β-glucanases: their physiology and regulation. Enzyme and Microbial Technology. 15: 178-192.

[83] Podile A. R. and Kishore G. K., 2002. Biological control of peanut diseases. Biological control of crop diseases, ed. By S. S. Gnanamanickam. Marcel Dekker, Inc., New York, pp.131-160.

[84] Purwani E.Y., Maggy T.S., Yaya R., Jae K.H. and Yu R.P., 2004. Characteristics of thermostable chitinase enzymes from the indonesian *Bacillus* sp.13.26. Enzyme Microb Technol 35: 147-153.

[85] Ramada M.H.S., Lopes F.A.C., Ulhoa C.J. and Silva R.N., 2010. Optimized microplate b-1,3-glucanase assay system for *Trichoderma* spp. screening. J. Microbiol. Methods 81: 6–10.

[86] Reynolds D.M., 1954. Extracellular chitinase from a *Streptomyces* sp. J Gen Microbiol 11: 150-159.

[87] Rifai M. A., 1969. A revision of the genus Trichoderma. Mycological Papers. 116: 1-56.

[88] Rowe R. C. and Powelson M. L., 2002. Potato early dying: Management challenges in a changing production environment. Plant Dis. 86:1184-1193.

[89] Sahai A. S. and Manocha M. S., 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiology Reviews. 11: 317-338.

[90] Samuels G. J., Chaverri, P., Farr D. F., and Mc Cray, E. B., 2015. *Trichoderma* Online, Systematic Mycology and Microbiology Laboratory, ARS, USDA. http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm, 2015.

[91] SAS Institute Inc. 1996. SAS/STAT Software: Changes and Enhancements through Release 6.11. SAS 20 Institute Inc., Cary, NC.

[92] Sayed S.A., Ezzat S.M., Ghaly M.F., Mansour M. and Bohey M.A., 2000. Purification and characterization of two chitinases from *Streptomyces albovinaceus* S-22. World J Microbiol Biotechnol 16: 87-89.

[93] Sharaf E.F., 2005. A potent chitinolytic activity of *Alternaria alternata* isolated from Egyptian black sand. : Pol J Microbiol.54: 145-51.

[94] Shoulkamy M. A., Guebitz G. M., El-Katatny M. H. A., Shaban M. and Abdelzahel H. M. A. (2006): Antagonism of Trichoderma or Gliocladium species on two phytopathogenic species of Fusarium. J. Natur. Fibers. 3(2/3), 1-17

[95] Siameto E. N., Okoth S., Amugune N. O. and Chege N. C., 2011. Molecular characterization and identification of biocontrol isolates of *Trichoderma harzianum* from Embu district, Kenya. Tropical and Subtropical Agroecosystems, 13 (2011): 81-90.

[96] Simmoms C.R., 1994. The physiology and molecular biology of plant 1,3-ß-D-glucanases and 1,3:1,4-ß-D-glucanases. Crit. Rev. Plant Sci. 13: 325-387.

[97] Sivan A. and Chet I., 1989. Degradation of fungal cell wall by lytic enzymes by *T. harzianum*. J. Gen. Microbiol. 135, 675-682.

[98] Skujins J.J., Pogieter H.J. and Alexander M., 1965. Dissolution of fungal walls by a *Streptomycete*- chitinase and glucannase. Arch Biochem Biophys 111: 358-364.

[99] Someya N., Numata S., Nakajima M., Hasebe A., Hibi T. and Akutsu K., 2003. Biological control of rice blast by the epiphytic bacterium *Erwinia ananas* transformed with a chitinolytic enzyme gene from an antagonistic bacterium, *Serratia marcescens* strain B2. J. Gen Plant Pathol . 69: 276-282.

[100] SpiegelY. and Chet I., 1998. Evaluation of *Trichoderma* spp., as biocontrol agent against soil borne fungi and plant parasitic nematodes in Israel. Integr. Pest Manage. Rev., 3: 169-175.

[101] Steyaert J. M., Ridgway H. J., Elad Y. and Stewart A., 2003. Genetic basis of mycoparasitism: A mechanism of biological control by species of *Trichoderma*. J. Crop. Horticul. Sci. 31: 281-291.

[102] Thrane C., Jensen D.F. and Tronsmo A., 2000. Substrate colonization, strain competition, enzyme production In Vitro, and Biocontrol of *Pythium ultimum* by *Trichoderma* spp. Isolates P1 and T3. Eur. J. Plant Pathol., 106: 215-225.

[103] Ulhoa C.J. and Peberdy J.F., 1992. Production and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. Enzyme Microb. Technol. 14:236-240.

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

[105] Vidhyasekaran P. and Balasubramanian P., 1995. Disease management by exploiting pathogenesis-related proteins with lytic action. In: Verma, J.P., Varma, Anupam, Kumar, Dinesh eds. Detection of Plant Pathogens and their Management. Angkor Publishers, New Delhi, pp. 259-272.

[106] Vidhyasekaran P. and Velazhahan R., 1996. Elicitor induced defense responses in suspension cultured rice cells. In: Chopra V.C., Sharma R.P., Swaminathan, M.S., eds. Agricultural Biotechnology. Oxford and IBH Publishing Co, New Delhi, pp. 249-255

[107] Vinale F., Ghisalberti E.L., Sivasithamparam K., Marra R., Ritieni A., Ferracane R., Woo S. and Lorito M., 2009. Factors affecting the production of *Trichoderma harzianum* secondary metabolites during the interaction with different plant pathogens.Lett. Appl. Microbiol. 48: 705–711.

[108] Vinale F., Sivasithamparam K., Ghisalberti E.L., Marra R., Woo S.L. and Lorito M. 2008a. Trichoderma–plant–pathogen interactions. Soil Biol. & Biochem., 40: 1-10.

[109] Wang L.S., Lin J., Zhang Y.Z., Zhao Y. and Gao P.J., 2003. Comparison of domains functions between cellobiohydrolasel and endoglucanasel from *Trchoderma pseudokoningii* S-38 by limited proteolysis. J. Mol. Cata. B-Enzyme. 24:27- 38.

[110] Wang S.L. and Chang W.T., 1997. Purification and characterization of two bifunctional chitinase/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. Appl Environ Microbiol 63(2): 380-386.

[111] Wang S.L., Lin T.Y., Yen Y.H., Liao H.F. and Chen Y.J., 2006. Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118.

[112] Weindling R., 1932. *Trichoderma lignorum* as a parasite of other soil fungi. Phytopathology, 22: 837-845.

[113] Wen C.M., Tseng C.S., Cheng C.Y. and Li Y.K., 2005. Purification, characterization and cloning of a chitinase from *Bacillus* sp. NCTU2. Biotechnol. Applied Biochem., 35: 213-219.

[114] Woo S.L. and Lorito M., 2007. Exploiting the interactions between fungal antagonists, pathogens and the plant for biocontrol. In: Vurro, M., Gressel, J. (Eds.), Novel Biotechnologies for Biocontrol Agent Enhancement and Management. IOS, Springer Press, Amsterdam, the Netherlands, pp. 107–130.

[115] Yang H.H., Yang S.L., Peng K.C., Lo C.T. and Liu S.Y., 2009. Induced proteome of *Trichoderma harzianum* by *Botrytis cinerea*. Mycol. Res. 113:924-932.

[116] Zeininger S., Galhaup C., Payer K., Woo SL., Mach R.L., Fekete C., Lorito M. and Kubicek C.P., 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal genet. Boil. 26:131-140.

[117] Zhou J., Wang Y.H., Chu J., Zhuang Y.P., Zhang S.L. and Yin P., 2008. Identification and purification of the main components of cellulases from a mutant strain of 474 *Trichoderma viride* T100-14. Bioresour. Technol. 99:6826-6833.