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Suppression of chickpea (*Cicer arietinum* L.) *Fusarium* wilt by *Bacillus subtillis* and *Trichoderma harzianum*

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Abstract

We studied the effect of *Bacillus subtillis* and *Trichoderma harzianum* Rifai, in commercial formulations, alone or in mixture, on glucanassoluble protein content, β-1, 3-glucanase enzyme activity and suppression of *Fusarium* wilt disease caused by *Fusarium oxysporum* f.sp *ciceris* in Hashem and Pirooz chickpea cultivars. Experiment was conducted in a factorial experiment based on randomized complete design with three replications under controlled greenhouse condition with aggressive isolate of *F. oxysporum* f.sp *ciceris* and *B. subtillis, T. harzianum* treatments in liquid and seed coating inoculation methods. Disease severity was significantly reduced by *B. subtillis, T. harzianum* and their mixtures (about 40%). Although the combination of these bio-control agents was effective in controlling *Fusarium* wilt disease but did not differ significantly from bio-control treatments individually. Hashem cultivar exhibited significantly higher level of resistance compared to Pirooz cultivar after inoculation with bio-control agents. Significantly higher levels of soluble protein content and β-1, 3-glucanas activity was observed in chickpea cultivars after inoculation with *B. subtillis, T. harzianum* compared to control. Hashem cultivar exhibited significantly higher levels of soluble protein content and β-1, 3-glucanas activity in compared to Pirooz cultivar, which apparently associated with establishment higher level of resistance to *Fusarium* wilt. Results of this study indicated that *B. subtillis and T. harzianum* effectively suppress the *Fusarium* wilt and increasing the protein content and β-1, 3-glucanas enzyme activity might have contributed to inducing systemic resistance after treatment with bio-control agents. Application of *B. subtillis* and *T. harzianum* either singly or in combination in both seed and liquid inoculation methods protect chickpea from *F. oxysporum* f.sp ciceris infection indicating that the importance of application of biocontrol agents.

Keywords: Bio-control agents; chickpea; *Fusarium oxysporum* f.sp *ciceris*; protein content; systemic resistance; β-1, 3-glucanas.
Abbreviations: SAR: systemic acquired resistance; ISR: induce systemic resistance; PGPR: plant growth promoting rhizobacteria; PR: pathogenesis-related proteins; PPO: poly phenol oxidase.

Introduction

Chickpea (*Cicer arietinum* L.) is an annual legume and the only cultivated specie within genus *Cicer* (Atta and Shah, 2009). Chickpea is valued for its nutritive seed composition which is high in protein content and used increasingly as a substitute for animal protein (Hossain et al., 2010). It is difficult to manage the disease either through crop rotation or application of chemicals because of soil nature persistence and its capacity to survive for long time even in the absence of host (Haware et al., 1996). Efficacy of wilt management was improved when bio-control agents were combined with cultural practices such as sowing date (Landa et al., 2004). Biological control provides an alternative to the use of synthetic pesticides with the advantages of greater public acceptance and reduced environmental impact (Reino et al., 2008). *Trichoderma* spp. have gained wide acceptance as effective bio-control agents against several phytopathogens (Whipps and Lumsden, 2001). Strains of *T. harzianum* are well known for their efficiency to control wide range of disease such as *Sclerotium rolfsii* (Benhamou et al., 1996), *Sclerotium cepivorum* (Kay and stewart, 1994), *Botrytis cinerea* (Bélager et al., 1995), *Fusarium solani* (Chakraborty and Chatterjee, 2008) and *Fusarium oxysporum* (Hervàs et al., 1998; Dubey et al., 2007; Shanmugum et al., 2008). Biological control of plant pathogens using antagonistic bacteria is a promising strategy for plant protection (Kloepper et al., 1999). Several reports have described *Bacillus* strains worthy to be used as bio-control agents for plant disease (Shoda, 2000). *B. subtillis* has many characteristics as an excellent bio-control agent, including the production of structurally diverse antibiotics (Liu et al., 2006). *B. subtillis* have been studied as bio-control agent in several diseases such as black rot of brassicas (*Xanthomonas campestris* pv. *campestris*) (Wullf et al., 2002), Southern Blight of Peanut (*Sclerotium rolfsii*) (Abd-Allah, 2005). Many investigations have indicated that *B.subtillis* is one of the most effective agents in controlling *Fusarium oxysporum* (Hervàs et al., 1998; Baysal et al., 2008; Cazorla et al., 2007; Zhang et al., 2008; Gajbhiye et al., 2010; Chen et al., 2010). In interactions with invading pathogens, plants frequently activate defense-related genes that lead to expression of pathogenesis-related (PR) proteins (Liu et al., 2009). PR proteins are one of the important non-specific defense

mechanisms of plants against pathogen (Van loon and Van strien, 1999). PR proteins such as β-1, 3-glucanase (PR-2 family) preferentially hydrolyze 1,3-β-D-glycosidic linkages in (1→3)-β-D- and (1→3),(1→6)-β-D- glucanase in the cell walls of many pathogenic fungi (Jin et al., 2007). β-1, 3-glucanase appears to be a part of the inducible defense response of higher plants (Göhl et al., 1998). Raju et al. (2008) assessed induction of β-1, 3-glucanase (PR-2) in roots and shoots of two different genotypes of chickpea cultivars treated with salicylic acid and spermine against *Fusarium oxysporum* f.sp *ciceris*. In this study we (1) evaluated *B. subtillis, T. harzianum,* alone or in mixture as antagonistic agents against *Fusarium* wilt of chickpea in greenhouse condition (2) assessed protein content and β-1, 3-glucanase (PR-2) activity as systemic response of plants against *Fusarium* wilt of chickpea after inoculation with bio-control organisms including *B. subtillis* and *T. harzianum.*

Results and discussion

Biocontrol of Fusarium wilt

Analysis of variance obtained for disease severity in bio-control treatments against *F. oxysporum* f.sp *ciceris* are given in Table 2. Data indicated that there were significant differences between cultivars (p <0.01) and bio-control agents (p <0.05). All the treatments of bio-control agents in commercial formulations including trichodermin (*T. harzianum*) and subtillin (*B. subtillis*) alone and their mixture were significantly different from untreated control (without bio-control treatment) (p <0.05). *Bacillus* sp. has been used as bio control organism and successfully reduces the disease severity of *F. oxysporum* of banana (Nel et al., 2006), cucumber (Hammad and El-Mohandes, 1999) and chickpea (Hervàs et al., 1998). *T. harzianum* were effective in control of *F.solani* (Chakraborty and Chatterjee, 2008), *F. oxysporum* f.sp *cubense* (Thangavelu et al. 2003), *F. oxysporum* f.sp *dianthi* (Shanmugum et al., 2008) and *F. oxysporum* f.sp *ciceris* (Hervàs et al., 1998). The mixture of two bio-control agents not significantly differed from treatments with either of bio-control agents alone. The application of biocontrol agents mixture has not yielded better results than the agents applied alone. Nel et al. (2006) indicated the commercial formulation; Patostop (*Bacillus* sp., *Pseudomonas* sp. and *Gliocladium* sp.) reduced the severity of *F. oxysporum* f.sp *cubense* but was not better than *Pseudomonas* isolates alone. Hervàs et al. (1998) studied the efficiency of *B. subtillis, T. harzianum* and nonpathogenic *F. oxysporum,* applied alone or in combination to suppress *Fusarium* wilt of chickpea caused by *F. oxysporum* f.sp *ciceris.* Their result showed the combination of *B. subtillis* and *T. harzianum* was effective in suppressing *Fusarium* wilt development but it did not differ significantly from treatments with either of these antagonists alone. Therefore *B. subtillis* and *T. harzianum* effectively suppress the *Fusarium* wilt disease of chickpea alone, but the mix of them did not differ from *B. subtillis* and *T. harzianum* alone in suppressing this disease.

Soluble protein content

Analysis of variance for soluble protein content in bio-control assay showed that there were significant differences between cultivars (p <0.01), bio-control agents (p <0.01) and inoculation method (p <0.01) (Table 1). Methods of inoculation led to significantly differences in levels of protein content (Fig. 4, Fig. 5). The data regarding the inoculation methods and protein content revealed that the protein content was significantly higher in seed inoculation treatments. Higher levels of protein content in seed inoculation method might relate to superiority this method in effective inoculation. Gajbhiye et al. (2010) showed that treatment of cotton seed with *B. subtillis* strains was more efficient in controlling the *F. oxysporum* infection. Data showed that Hashem cultivar had significantly higher protein content than pirooz cultivar in plant tissues after inoculation with bio-control agents (Fig. 3, Fig. 4 and Fig. 5). Hashem cultivar is more resistant than pirooz. Higher protein content of hashem maybe related to accumulation of more pathogenesis-related proteins (PR-proteins) and induction systemic resistance. Synthesis and accumulation of PR-proteins have been reported to play an important role in plant disease resistance (Van loon, 1997). Raju et al. (2008) claimed that induction of proteins and accumulation of phenolics might have contributed to restrict the invasion of *F.oxysporum* f. sp. *ciceri*, in resistant cultivar ICCV10. The pathogen–induced resistance has been termed systemic acquired resistance (SAR) and plant growth promoting rhizobacteria (PGPR) mediated resistance is known as induced systemic resistance (ISR) (Hammerschmidt, 1999). SAR requires the signal molecule salicylic acid and is associated with accumulation of PR-proteins, which are thought to contribute to resistance (Durrant and Dong, 2004). In some PGPRs, ISR mechanisms related to systemic accumulation of PR-proteins. Maurhofer et al. (1994) indicated that systemic resistance induced by *Pseudomonas fluorescence* was related to accumulation of PR-proteins such as β-1, 3-glucanas and chitinase. Tjamos et al. (2005) showed induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the bio-control bacterium *Paenbacillus alvei* was related to PR-proteins. Therefore, it is possible that higher levels of protein content after inoculation with bio-control agents and difference levels of protein between two cultivars may be related with defense mechanism of SAR or ISR that conform resistance to *Fusarium* wilt disease.

β-1, 3-glucanas activity

Analysis of variance for β-1, 3-glucanas activity assay indicated that there were significant differences between cultivars (p <0.01) and bio-control agents (p <0.01) (Table 1). Hashem cultivar significantly showed higher level of β-1, 3-glucanas activity than Pirooz cultivar (Fig. 6). The higher level of resistance to *Fusarium* wilt in Hashem cultivar after inoculation with bio-control agents (Fig. 1) apparently correlated with induction higher level of protein content (Fig. 3) and β-1, 3-glucanas activity (Fig. 6). Correlation between β-1, 3-glucanas and resistance to many diseases were showed in many studies. Lawrence et al. (1996) showed the correlation between resistance to *Alternaria solani* and β-1, 3-glucanas levels in leaves of tomato. Transgenic Kiwifruit with elevated expression of β-1, 3-glucanas showed higher level of resistance to *Botrytis cinerea* (Nakamura et al., 1999). Raju et al. 2008 in their investigation showed that ICCV10 (resistant cultivar) contained higher levels of β-1, 3-glucanas, poly phenol oxidase (PPO), phenyl alanine ammonia-lyase (PAL) in shoots and roots rather than L550 (susceptible cultivar) after treatment with elicitors and pathogen. Therefore differences between β-1, 3-glucanas activities in cultivars may be led to variation in resistance to *Fusarium* wilt disease. Comparison between control (pathogen free and without bio-control organism treatment) and untreated (inoculated with pathogen only) treatments (Fig. 7) showed that invasion of pathogen can inducing the β-1, 3-glucanas activity therefore β-1, 3-glucanas is involved in defense response mechanism. Results of β-1, 3- glucanas

Table 1. Analysis of variance for soluble protein and β-1, 3-glucanase content in chickpea cultivars after inoculation with bio-control agents in infected pots with *Fusarium oxysporum* f.sp *ciceris*

Source of variation	*Df* *	Mean square **	
		Soluble Protein	β-1,3-glucanase
Cultivar	1	9206.35**	0.0985080**
Inoculation method of bi-control agents	1	663.39 n.s	0.000531 n.s
Bio-control agents	4	3172.16**	0.039806**
Cultivar × inoculation method of bi-control agents	1	0.32 n.s	0.002205 n.s
Cultivar× Bio-control agents	4	396.50 n.s	0.00746 n.s
inoculation method of bi-control agent × bi-control agents	4	1173.47**	0.00526086 n.s
Cultivar × inoculation method of bio-control agent × Bio-control agent	4	445.47 n.s	0.0066951205 n.s
Error	40	297.44	0.0041334105
CV %	-	18.56	2.631694

*Degrees of freedom, ** (**: p <0.01, *: p <0.05, ns: no significant)

Table 2. Analysis of variance of disease severity for chickpea cultivars after inoculation with bio-control agents in infected pots with *Fusarium oxysporum* f.sp *ciceris*

Source of variation	*Df* *	Mean square **
Cultivar	1	22.60**
Application method of biological control agent	1	0.74 n.s
biological control agent	3	0.74*
Cultivar × Application method of biological control agent	1	0.03 n.s
Cultivar× Biological control agent	3	0.30 n.s
Application method of biological control agent × Biological control agent	3	0.23 n.s
Cultivar × Application method of biological control agent × Biological control agent	3	0.26 n.s
Error	32	0.11
CV %	-	22.60

* Degrees of freedom , ** (**: p <0.01, *: p <0.05, ns: no significant)

activity (Fig. 7) indicated that Subtillin (*B. subtillis*) and Trichodermin (*T. harzianum*) treatments significantly increased activity of β-1, 3-glucanas as compared to control treatments. Elicitation of ISR by *Bacillus* spp. is associated with ultra structural changes in plants during pathogen attack and with cytochemical alterations (Kloepper et al., 2004). Eliciting PGPR such as *Bacillus* spp. have a different mechanisms that one of them is systemic accumulation of PR-proteins such as β-1, 3-glucanas (PR-2 family). Induction of ISR by *Bacillus mycoides* (Bargabus et al., 2002) and *Bacillus pumilus* (Bargabus et al., 2004) was correlated with increasing the two isozymes of β-1, 3-glucanas and one chitinase isozyme with enhanced peroxidase activity in sugar beet. Benhamou et al. (1998) studied the effect of *B. pumilus* strain SE34 alone or in combination with chitosan on cytochemical changes of tomato infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Their result showed higher amount of β-1, 3-glucanas accumulated in roots from plants treated with *B. pumilus* strain SE34 with chitosan in compare with untreated plants. In current study increasing of β-1, 3-glucanas activity after inoculation with *B. subtillis* maybe related with eliciting ISR in chickpea against *Fusarium* wilt by this bio-control agent. Recent evidence indicate that many *Trichoderma* spp., including *T. virens*, *T.atroviride* and *T. harzianum*, can induce both localized and systemic resistance in a range of plants to a variety of plant pathogens, and certain strains can also have substantial influence on plant growth and development (Harman et al., 2004). Several *Trichoderma* spp. can activate systemic induced resistance in plants (Brunner et al., 2005). Systemic resistance was induces in cucumber with *Trichoderma hamatum* bio-ocontrol agent against *Phytophthora* root rot (Khan et al., 2004). Inoculation of cucumber (*Cucumis sativus* L.) by the *Trichoderma harzianum* biocontrol agent led to Induction of defense responses in these plants (Yedidia et al., 1999). Expression of defense related genes such as those encoding pathogenesis-related proteins are used as markers for the establishment of SAR (Du and Klessig, 1997). β-1, 3-Glucanase is normally classified as a pathogenesis-related (PR) protein, induced upon pathogen attack (Menu-Bouaouiche et al., 2003) Saksirirat et al. (2009) indicated that the antagonistic fungus *Trichoderma* spp. induced systemic resistance in tomato plants against bacterial and gray leaf spot with increasing activities of chitinase and β-1, 3-glucanase. The result of our study showed *Trichoderma harzianum* biocontrol agent induced systemic resistance in chickpea cultivars that correlated with increasing activity of β-1, 3-glucanase. Results of this study showed that biological control agents *Bacillus subtillis* and *Trichoderma harzianum* effectively improve the resistance to *Fusarium* wilt disease. However, a mix of these two biocontrol agents did not show significant difference individually applied one. β-1, 3-glucanase activity increased following application of both biocontrol agent . This PR protein could be used as a marker for showed a resistance in chickpea. In conclusion, *B. subtillis* and *T. harzianum*

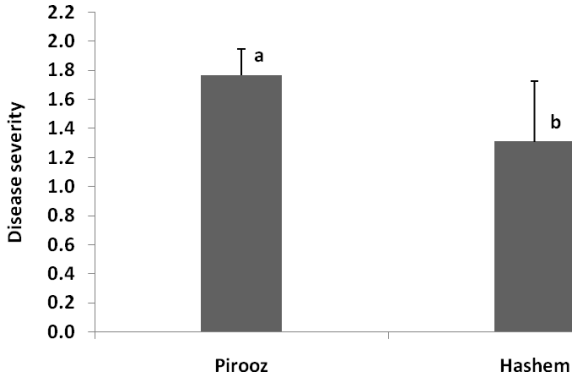

Fig 1. Disease severity of chickpea cultivars after inoculation with bio-control agents in pots that infected with *Fusarium oxysporum* f.sp *ciceris.* Error bars represent the standard deviation for each factor. Data were transformed with logarithmic transformation. Different letters show significant differences.

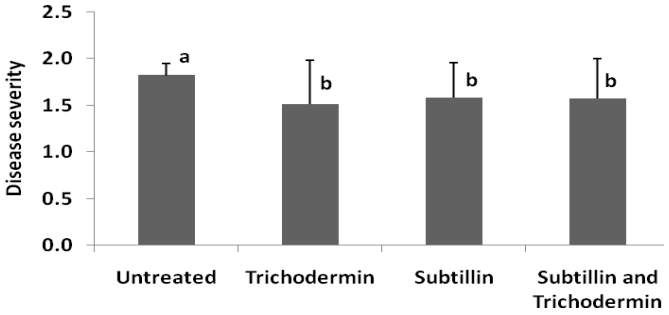

Fig 2. Disease severity of chickpea cultivars after inoculation with Subtillin (*Bacillus subtillis*), Trichodermin (*Trichoderma harzianum* Rifai) and mix of them in sick pots that infected with *Fusarium oxysporum* f.sp *ciceris.* Error bars represent the standard deviation for each factor. Data were transformed with logarithmic transformation. Different letters show significant differences.

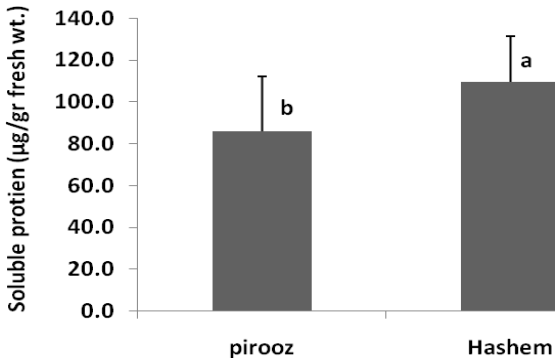

Fig 3. Soluble protein content of chickpea cultivars after inoculation with bio-control agents in pots that infected with *Fusarium oxysporum* f.sp *ciceris.* Error bars represent the standard deviation for each factor. Different letters show significant differences.

have potential, as chickpea inoculants, for inhibiting *F. oxysporum* f.sp *ciceris*.

Materials and method

Plant materials

Two chickpea cultivars, Pirooz and Hashem were obtained from Agricultural and Natural Resources Research Center of Kurdistan, Sanandaj, Iran.

Pathogen

We used 11 isolates of *F. oxysporum f.sp ciceris* that were isolated and maintained in research plant pathology laboratory, Agriculture Faculty, Kurdistan University, Sanandaj, Iran for pathogenic assay. Pathogencity of each isolate were determined using disease severity on Kaka cultivar which is highly susceptible to *F. oxysporum* f.sp *ciceris* (Haji-Allahverdipoor et al., 2011). The experiment performed in a completely randomized design for *F. oxysporum* f.sp *ciceris* isolates. The preparation and transformation of inoculums to pots describe in next section. Disease severities were determined based on percentage of affected foliage (Jiménez-Gasco et al., 2001) and wilt incidence (Gowda et al., 2009). Significantly differences were observed among isolates for disease severity. In this study we selected one of the most aggressive isolates for perform other aspects of this study.

Bio-control assay of Fusarium wilt

We used bio-control agents *Bacillus subtillis* and *Trichoderma harzianum* Rifai in commercial formulations, subtillin and Trichodermin-b that provided from Telfig Dane Company, Tehran, Iran. Disease suppression of bio-control organisms was determined in pots at greenhouse of Agriculture Faculty, Kurdistan University, Sanandaj, Iran using factorial experiment based on randomized complete design with three replications. The inoculums were prepared with Corn Meal Sand (CMS) mixture (Trapero-Casas and Jiménez-Díaz, 1985) in conical flasks and incubated for 21 days at room temperature (Gowda et al., 2009). Soil was sterilized by autoclaving at 120 ℃ for 30 min on three consecutive days. The infected CMS mixture was mixed thoroughly with autoclaved soil mixture (clay loam, sand, peat 1:1:1 V/V) at 1:12 (W/W) and transformed to pots according to Brinda and Ravikumar, (2005). Seeds of plants were surface disinfected in 2% $NaOCl_2$, then washed three times in sterile distilled water and dried under laminar airflow cabinet prior to coating with bio-control agents or sowing in treated pots. Plants were inoculated with bio-control organisms in two different manners, seed coating and liquid inoculums. In liquid inoculum method solution was mixed with soil pots following 5 days after infection and seeds were sown next to three days after inoculation. Experiment was conducted in a factorial experiment based on randomized complete design with three replications .The bio-agent treatments used this study were (1) control: uninoculated plants (pathogen free and without bio-control organism treatment), (2) untreated: only pathogen inoculated (*F. oxysporum* f.sp *ciceris*), (3) subtillin: pathogen and bio-control organism inoculated (*B. subtillis*+ *F. oxysporum* f.sp *ciceris*), (4) Trichodermin: pathoen and bio-control organism inoculated (*T. harzianum* + *F. oxysporum* f.sp *ciceris*), (5) subtillin and Trichodermin (1:1 w/w): pathoen and

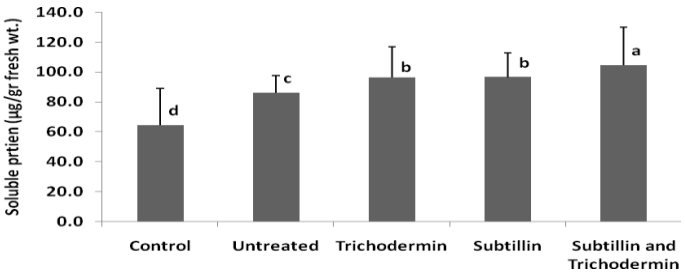

Fig 4. Soluble protein content of chickpea cultivars after seed inoculation method with Subtillin (*Bacillus subtillis*), Trichodermin (*Trichoderma harzianum* Rifai) and mix of them in pots that infected with *Fusarium oxysporum* f.sp *ciceris.* Error bars represent the standard deviation for each factor.
Different letters show significant differences.

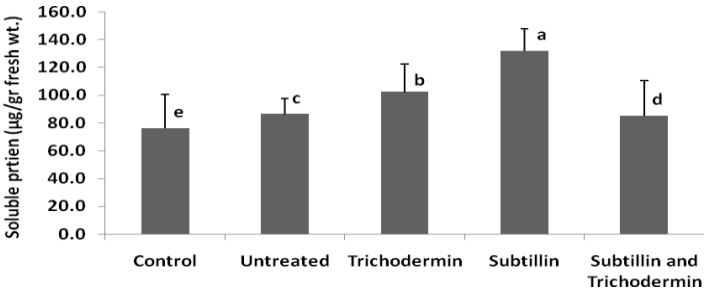

Fig 5. Soluble protein content of chickpea cultivars after liquid inoculation method with Subtillin (*Bacillus subtillis*), Trichodermin (*Trichoderma harzianum* Rifai) and mix of them in pots that infected with *Fusarium oxysporum* f.sp *ciceris.* Error bars represent the standard deviation for each factor.
Different letters show significant differences.

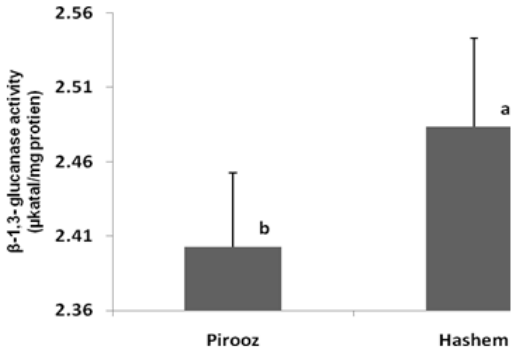

Fig 6. β-1, 3-glucanases activity in chickpea cultivars after inoculation with bio-control agents in pots that infected with *Fusarium oxysporum* f.sp *ciceris.* Error bars represent the standard deviation for each factor.
Data were transformed with logarithmic transformation.
Different letters show significant differences.

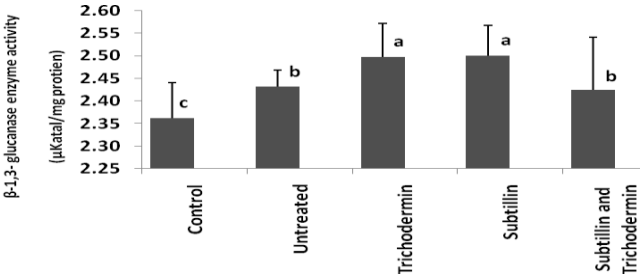

Fig7. β-1, 3-glucanases activity in chickpea cultivars after inoculation with Subtillin (*Bacillus subtillis*), Trichodermin (*Trichoderma harzianum* Rifai) and mix of them in pots that infected with *Fusarium oxysporum* f.sp *ciceris.* Error bars represent the standard deviation for each factor.
Data were transformed with logarithmic transformation
Different letters show significant differences.

bio-control organism inoculated (*B. subtillis*+ *T. harzianum*+ *F. oxysporum* f.sp *ciceris*). These treatments combined with two inoculation methods were checked on two cultivars in a factorial experiment. The symptoms of disease were recorded at daily intervals after exhibition first symptoms in infected plants. Determination of disease severity (0-100%) described in pathogencity assay. To validate the experiments, the pathogen was isolated from stem of infected plants for each isolate to determine the occurrence of vascular infections.

Protein extraction

Proteins were extracted from frozen plant tissues that harvested from plants of all treatments of greenhouse experiments according to Liang et al. (2005). Samples were ground to fine powder with mortar and pestle and homogenized in a cold, 0.1 M sodium acetate buffer (PH 5.1) containing 4 M ascorbic acid, 2 mM 2-mercaptoethanol and 2% poly vinyl pyrrolidone (PVP). The crude extracts were centrifuged twice at 20000 × *g* for 20 min at 4°C. The supernatants were collected and protein concentration was determined by the method of Bradford (1976).

β-1, 3-glucanas activity assay

β-1, 3glucanas activity was measured with laminarin as substrate (Miller, 1959) according to Jin et al. 2007. The reaction was terminated by dinitrosalicylic acid and boiling for 5 minutes. Enzymatic activity of β-1, 3-glucanas measures based on the amount of reducing saccharides released from laminarin with spectrophotometer at 540 nm. One nkatal was defined as the enzymatic activity the formation of 1 nmol of glucose equivalent per second (Liang et al., 2005).

Data analysis

The greenhouse data of disease severity, protein content and β-1, 3-glucanas activity were analyzed by ANOVA and treatment means separated by Duncan Multiple Range Test using SAS program (SAS Institute Inc, 2001). In disease severity and enzyme activity data were transformed with logarithmic transformation.

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