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ARTICLE

A study of some biocontrol mechanisms of *Beauveria bassiana* against *Rhizoctonia* disease on tomato

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ABSTRACT Tomato damping-off, caused by *Rhizoctonia solani,* is one of the most common diseases worldwide. The use of biological control agents to protect plants against pathogens seems to be an appropriate solution. *Beauveria bassiana* as an endophytic fungus can colonize a wide range of plants in a systemic manner and enhance plant resistance. In this study, the ability of three isolates of *B. bassiana* to control damping-off of tomato seedlings was examined. The effect of *B. bassiana* on the induced systemic resistance enzymes such as peroxidases (POX) and phenylalanine ammonia-lyase (PAL) and also the phenolic compounds content in plant resistance was measured. Our results showed that *B. bassiana* isolates especially TS12 and TS7 resulted in an increase in the activity of POX and PAL enzymes as well as enhanced the contents of phenolic compounds in treated plants. The results of greenhouse studies showed that each of the three isolates were able to successfully control tomato damping-off. However, this ablity was probably due to the stimulation of plant growth by KJ24, but TS12 and TS7 isolates acted through the stimulation of "induced systemic resistance" in plants.
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Introduction

Tomato is one of the most consumed and important vegetables and field crops in the world (Blancard et al. 2012). Apart from being daily consumed, this plant is an important model organism, the complete genome sequence of which has also been determined (The Tomato Genome Consortium 2012). Damping-off and root rot of tomato caused by *Rhizoctonia solani* is one of the most destructive diseases in some of the tomato production areas worldwide. It is also capable of infecting cultivars of tomato in the greenhouse (Montealegre et al. 2010). The disease causes an average decrease of 20% in the annual yield worldwide (Muriungi et al. 2014). Because of the broad host range and high rates of overwintering sclerotia, the control of *R. solani* is very difficult (Grosch et al. 2006). Considering the damages of chemical pesticides and problems associated with other means of control, it seems that biocontrol could be the perfect solution for protecting plants against this pathogen (Ewekeye et al. 2013). Different types of microorganisms as biocontrol agents have been identified, and, among them, the exploitation of biocontrol fungi is developing (Butt et al. 2001). Different bicontrol mechanisms by biocontrol fungi against plant pathogens have been identified. The biocontrol fungi are directly involved in some of these mechanisms, such as competition, antibiosis, and parasitism. Other mechanisms, including induced systemic resistance (ISR), increased growth response, and endophytic colonization in the plant have an indirect role, and, through the activation of plant defense responses and increased expression of genes related to defense they cause induction of plant resistance and disease reduction (Ownley et al. 2010). Induced systemic resistance is activated by mycorrhizae, as well as some biocontrol (Ownley et al. 2010) and endophytic fungi (Gasoni and Gurfinkel 2009). Colonization of plants by endophytic fungi can be local or systemic (Saikkonen et al. 1998). Systemic colonization can be caused by entomopathogenic fungi and act against plant disease (Ownley et al. 2010). Localized colonization by endophytes could also increase production of phenolic compounds and other defence metabolites in the plants (Gasoni and Gurfinkel 2009). The endophytic activity of *Beauveria bassiana* isolates has been reported from several plant species under natural conditions, as well as from plants including tomato, cotton, green beans, soybeans, corn, datura and potatoes inoculated using various methods (Ownley et al. 2008, 2010). The antagonistic abilities of different isolates of *B. bassiana* were investigated against certain plant pathogens including *Fusarium oxysporum*, *Ar-*

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millaria mellea, Rosellinia necatrix, *R. solani*, and *Pythium myriotylum* (Griffin 2007). Application of *B. bassiana* isolate 11-98 on tomato seed resulted in the endophytic and epiphytic colonization of seedlings and protection against damping-off (Ownley et al. 2008). Similarly, in cotton (*Gossypium hirsutum* L.) seed, the application of the mentioned isolate reduced *R. solani* damping-off in seedlings via the activation of induced systemic resistance (Griffin 2007). Several studies have characterized *B. bassiana* as a fungus with the potential to control plant diseases, but this is still being investigated by researchers.

Despite the fact that the ability of *B. bassiana* in improving the protection of plants against a number of soil-borne plant pathogens has been reported, most researches performed on the biological control effect by *B. bassiana* on plant diseases were limited to *in vitro* studies. For this purpose, greenhouse tests along with *in vitro* studies as well as the comparison of the virulence and the operation mode of different strains of this fungus may provide important information about its biocontrol mechanisms reducing the impact of the disease. This study has been undertaken to explore the total phenol content and the activities of peroxidase and phenylalanine ammonia-lyase enzymes in addition to greenhouse and laboratory evaluations.

Materials and Methods

Preparation of pathogen and antagonistic isolates

Three antagonistic isolates of *Beauveria bassiana* (TS12, KJ24, and TS7) were obtained from the culture collection of the Plant Protection Department, Azarbaijan Shahid Madani University. *Rhizoctonia solani* (AG-4) derived from the Plant Protection Department, University of Tehran.

Growth and sporulation pattern of B. bassiana isolates

As the growth and sporulation rates of different *B. bassiana* isolates is variable, the rate of mycelial growth of *B. bassiana* isolates was studied on two media, Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA), based on the methodology proposed by Mostafa et al. (2010). To measure the conidial production of the isolates, the test was carried out in the light of the methodology proposed by Senthamizhlselvan et al. (2010) in two liquid media [Potato dextrose broth (PDB) and Sabouraud Dextrose Broth (SDB)]. After five days, the spore concentration in each Erlenmeyer flask was measured using a hemocytometer and analyzed.

Chitinase activity assay

According to Chan and Tian (2005), cell wall of *R. solani* was prepared and chitinase enzyme was extracted. Chitinase activity was assayed using the method of Kang et al. (1999) at a wavelength of 585 nm.

Toxin production assay

This test was performed by the method described by Parveen and Begum (2010). The experiment was carried out in triplicate based on completely randomized design with four treatments and four replications.

Antagonistic effect of B. bassiana to prevent tomato damping-off under greenhouse conditions

Seed treatment was carried out according to Griffin (2007). After surface sterilization, seeds of tomato (*cv*. mobil) were coated with antagonistic fungal spore suspension of three concentrations (10^5, 10^7, and 10^9 spores/ml), separately. Inocula of the pathogen were prepared on wheat seeds as described by Tseng et al. (2008) and added to the soil at a rate of 3% wt/wt (based on soil fresh weight). Then, seeds were planted in potting soil infested with pathogen inocula (three seeds into each pot). After planting, the pots were transferred to a greenhouse (24-27 °C, 30% relative humidity, and 16 h photoperiod). This experiment was arranged as a completely randomized design with 11 treatments (infected control, healthy control, and nine treatments comprising the three antagonist isolates TS7, KJ24, and TS12 with three spore concentrations) and 10 replicates (pot). Three weeks after the cultivation of plants, healthy plants were evaluated according to the damping-off and wilting symptoms and the percentage of "damping-off" of plants was calculated according to the following equation: $D= [(N–N0) / N] \times 100$, where, N is the number of healthy seedlings in control and $N0$ the number of healthy seedlings in each treatment.

In the fourth week, growth factors such as dry and fresh weight and height of plants were measured.

Preparation of plant extracts and determination of enzyme activities

Enzyme assays were performed with leaf and stem extracts. Tomato leaves were collected in the third and fourth weeks after cultivation of plants. Initially extracts of plants were prepared according to the modified method of Abo-Elyosr et al. (2007) and stored at -80 °C until the determination of the activities of peroxidase, phenylalanine ammonia-lyase, and total phenolic compounds.

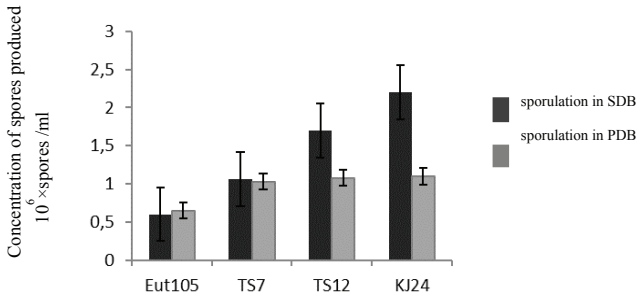

Figure 1. Concentration of spores produced by *B. bassiana* isolates TS12, TS7 and KJ24 in PDB and SDB media at 26±1 °C after 10 days of incubation. The concentration of spores was measured using a hemocytometer. Vertical bars represent ± S.E. of means (n = 3).

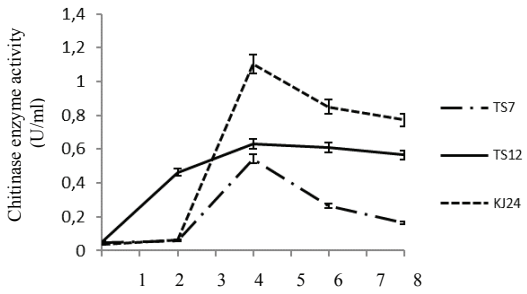

Figure 2. Chitinase enzyme activities of *B. bassiana* isolates TS12, TS7 and KJ24 during eight days of growth at 26±1 °C in Lilly-Barnett (Minimal Salt) medium containing *R. solani* cell wall (2 mg/l) as sole carbon source. Vertical bars represent ± S.E. of means (n = 3).

Peroxidase (POX) activity was determined according to the method of Urbanek et al. (1991) with a little modification. The reaction solution contained 0.5 ml of the enzyme extract, 350 µl phosphate buffer (100 mM), 350 µl pyrogallol (10 mM), and 1 ml of H_2O_2 (70 mM), respectively. Absorption at 470 nm was recorded (extinction coefficient 26.6 mM^{-1} cm^{-1}). POX enzyme activities were calculated as the µMol H_2O_2 decomposed in mg protein per minute.

Phenylalanine ammonia-lyase (PAL) was measured according to the modified method of Pascholati et al. (1986) with cinnamic acid as standard. The reaction solution was obtained by combining the 15 µl of the enzyme extract and 1500 µl phenylalanine (2%). Then the absorption at 290 nm was recorded immediately. Phenylalanine ammonia-lyase enzyme activities were expressed as mg per liter (mg/l) cinnamic acid in mg protein per minute.

The content of phenolic compounds was measured as described in Javanmardi et al. (2003) with a little modification, and based on the color change of phenolic extracts by the folin reagent and sodium carbonate. Absorption was recorded at 765 nm. The contents of total phenolic compounds were calculated using a standard curve obtained with different concentrations of gallic acid expressed as the mg gallic acid per g of tissue fresh weight.

Statistical analysis

The data were analyzed by SAS statistical package version 9.2 and the mean comparisons were made following Duncan's Multiple Range Test at P = 0.01 by MSTATC (version 2.10, Inc, Michigan State University) . The entire experiment was performed three times.

Results

Growth pattern in B. bassiana isolates

On the second, fourth, sixth, eighth, and tenth days after inoculation, the radial growth rate of the antagonistic fungal isolates was measured and compared on PDA and SDA media. Differences could be observed between the examined isolates ($p \leq 0.01$). At both SDA and PDA culture medium, minimum and maximum growth rates were observed in isolates of TS7 and KJ24, respectively (data not shown). Mean comparison of growth rates of isolates on both PDA and SDA showed that isolates TS12, TS7, and KJ24, had the highest growth rates, respectively.

The pattern of sporulation of B. bassiana isolates

In order to compare the pattern of sporulation of the examined antagonistic fungal isolates, amount of spores produced by the isolates was measured on the fifth day after inoculation into SDB and PDB cultures. Mean comparison showed that the amount of spores produced by KJ24 and TS12 isolates in SDB was higher than in PDB, but there was no significant difference in the case of the isolate TS7 between the two media (Fig. 1).

Chitinase enzyme activity and toxin production assay

By using the data obtained from 0, 2, 4, 6, and 8 days, chitinase activity curves were plotted for each isolate of antagonists. The results showed that all isolates of *B. bassiana* produced the maximum enzyme activities 96 hours after inoculation. Maximum and minimum levels of chitinase activities were produced by isolates KJ 24 and TS7, respectively (Fig. 2).

In the toxin production assay, a hyaline zone around the colonies of strains TS7 and TS12 strain was observed after 7

Table 1. Mean comparison of percentage damping-off and plant growth parameters of tomato plants treated with *B. bassiana* strains in the greenhouse.

Treatment	Percentage damping-off	Root length (cm)	Shoot length (cm)	Dry weight (g)	Fresh weight (g)
TS7 (10^5 cell/ml)	82.65 a*	4.50 bc	9.27 bcd	0.419 de	0.036 c
TS7 (10^7 cell/ml)	34.74 g	4.33 bc	12.67 abc	0.404 de	0.038 c
TS7 (10^9 cell/ml)	56.51 e	2.50 c	7.83 de	0.258 e	0.033 c
TS12 (10^5 cell/ml)	78.23 b	4.10 bc	11.07 abcd	0.505 cde	0.046 bc
TS12 (10^7 cell/ml)	34.74 g	5.66 b	11.43 abcd	0.638 bcd	0.068 bc
TS12 (10^9 cell/ml)	69.53 c	4.17 bc	9.50 bcd	0.390 de	0.038 c
KJ24 (10^5 cell/ml)	60.83 d	4.17 bc	9.00 cd	0.353 de	0.032 c
KJ24 (10^7 cell/ml)	52.17 f	11.83 a	13.83 ab	0.966 b	0.093 b
KJ24 (10^9 cell/ml)	21.71 h	12.00 a	15.00 a	1.466 a	0.157 a
Non-infected control	0.00 i	5.67 b	7.50 de	0.796 bc	0.090 b
Infected control	82.62 a	4.43 bc	4.50 e	0.379 de	0.039 c

*Means followed by the same letters in a column are not significantly different.

days of growth on methylene blue agar at 26 °C.

Antagonistic effect of B. bassiana to prevent tomato damping-off in greenhouse

Three weeks after the cultivation of plants, with emergence of the first signs of damping-off, damping-off percentage was calculated (Table 1). The results proved that there was a difference in the effect of the studied isolates on damping-off percentage, root length, fresh and dry weight, as well as shoot length. Mean comparison showed that isolate KJ24 (10^9 spores/ml) and isolates TS12 and TS7 (10^7 spores/ml) had the maximum controlling effect, with 21.73 and 34.74% damping-off, respectively. Isolate TS7 (10^5 spores/ml) with 82.65% damping-off had the minimum control effect (Table 1). Plants treated with isolate KJ24 with a concentration of 10^9 spores/ml had the maximum shoot and root lengths, as well as dry and fresh weight indicating that this isolate controlled tomato damping-off at this concentration. Although there was no difference between the treatments on stem length, all treatments increased the shoot length when compared to the infected and non-infected controls (Table 1).

Preparation of plant extracts for enzyme activity measurements

Plants from treatments with less than 70 percent damping-off were used to determine the activities of peroxidase enzyme, phenylalanine ammonia-lyase, and total phenolic compounds.

Peroxidase enzyme activity

After three and four weeks of tomato cultivation, the peroxidase activities were significantly affected by the treatments. The enzyme activities in all treatments were higher than in case of the infected control in the third week (Table 2). Maximum and minimum activities were observed in plants treated with isolates KJ24 at a concentration of 10^9 spores/ml and TS12 at a concentration of 10^7 spores/ml, respectively. In the fourth week, the enzyme activities were higher than the infected and non-infected control in all treatments, but in some treatments they were increased or decreased in comparison to the values at the third week. Additionally, in the fourth week, the maximum and minimum activities were observed in isolates KJ24 at a concentration of 10^7 spores/ml and TS7 at a concentration of 10^7 spores/ml, respectively (Table 2).

Phenylalanine ammonia-lyase (PAL) enzyme activities

The results showed that PAL activity was different in all treatments in the third and fourth weeks after cultivation of plants. Maximum activity in the third and fourth weeks could be

Table 2. Mean comparison of peroxidase enzyme activities in tomato plants treated with *B. bassiana* strains.

Treatment	Peroxidase enzyme activity (µmol H_2O_2 decomposed per mg protein per minute	
	Third week*	Fourth week*
TS7 (10^7 cell/ml)	0.346 abcd**	0.193 b
TS7 (10^9 cell/ml)	0.186 bcd	Not measured
TS12 (10^7 cell/ml)	0.109 cd	0.566 b
TS12 (10^9 cell/ml)	0.352 abcd	0.257 b
KJ24 (10^5 cell/ml)	0.492 ab	1.524 a
KJ24 (10^7 cell/ml)	0.435 abc	2.274 a
KJ24 (10^9 cell/ml)	0.602 a	0.506 b
Non-infected control	0.296 abcd	0.082 b
Rhizoctonia-infected control	0.080 d	0.113 b

* After cultivation of plants **Means followed by the same letters in a column are not significantly different.

Table 3. Mean comparison of phenylalanine ammonia-lyase activities in tomato plants treated with *B. bassiana* strains.

Treatment	Enzyme activity (mg per liter cinnamic acid in mg protein in minutes)	
	Third week*	Fourth week*
TS7 (10^7 cell/ml)	3.003 b**	1.776 d
TS7 (10^9 cell/ml)	1.42 e	Not measured
TS12 (10^7 cell/ml)	2.57 c	1.896 d
TS12 (10^9 cell/ml)	1.23 f	1.070 f
KJ24 (10^5 cell/ml)	1.82 d	1.540 e
KJ24 (10^7 cell/ml)	3.56 a	1.563 e
KJ24 (10^9 cell/ml)	2.86 b	2.943 a
Non-infected control	2.60 c	2.113 c
Rhizoctonia-infected control	1.45 e	2.343 b

*After cultivation of plants ** Means followed by the same letters in a column are not significantly different.

Table 4. Mean comparison of amount of total phenolic compounds in tomato plants treated with *B. bassiana* strains.

Treatment	Amount of total phenolic compounds (mg gallic acid per gr fresh weight of the plant)	
	Third week*	Fourth week*
TS7 (10^7 cell/ml)	16.27 d**	8.12 de
TS7 (10^9 cell/ml)	8.82 h	Not measured
TS12 (10^7 cell/ml)	22.82 c	8.80 d
TS12 (10^9 cell/ml)	33.49 a	7.69 e
KJ24 (10^5 cell/ml)	27.13 b	19.10 a
KJ24 (10^7 cell/ml)	13.02 f	10.43 c
KJ24 (10^9 cell/ml)	14.76 e	18.39 a
Non-infected control	9.59 h	18.94 a
Rhizoctonia-infected control	11.12 g	12.71 b

*After cultivation of plants ** Means followed by the same letters in a column are not significantly different.

measured in plants treated with isolate KJ24 at concentrations of 10^9 and 10^7 spores/ml, respectively, but plants treated with TS12 at the concentration of 10^9 spores/ml had the minimum activity of this enzyme at both time points. In the fourth week, the level of PAL activity in all plants – except from those treated with isolate KJ24 at the concentration of 10^9 spores/ml – was significantly reduced, compared with the infected and non-infected control plants (Table 3).

Total phenolic compounds

At the third and fourth weeks after the cultivation of plants, the amount of total phenolic compounds was significant in all of the treated plants. Maximum and minimum amounts of phenolic compounds were produced at the third week by the isolates TS12 and TS7, respectively at the concentration of 10^9 spores/ml. Also, maximum and minimum amounts of phenolic compounds at the fourth week were observed in the plants treated with isolates KJ24 (10^9 spores/ml) and TS12 (10^5 spores/ml), respectively (Table 4).

Discussion

It is likely that more than one antagonistic mechanism is operative in suppression of plant diseases by *B. bassiana* which include the production of antibacterial and antifungal secondary metabolites (Ownley et al. 2010; Azadi et al. 2015a). According to Azadi et al. (2015b), mycelial growth of *R. solani* was inhibited by volatile compounds and culture filtrates containing secreted metabolites of isolates TS12, TS7 and KJ24 of *B. bassiana*. Also in this study, isolates TS12 and TS7 were able to produce toxins in methylene blue agar medium. Certain toxins as well as antifungal and antibacterial compounds produced by microorganisms can be involved in the biocontrol activities as important factors (Harman 2000). The occurrence of the phenomenon of antibiosis by *B. bassiana* against plant pathogens including *Gaeumannomyces graminis* var. *tritici*, *Armillaria mellea, Rosellinia necatrix*, *Fusarium oxysporum*, *Botrytis cinerea*, *Pythium ultimum* and *R. solani* (Ownley et al. 2010) have been reported. Despite the production of toxins and secondary metabolites by isolates of *B. bassiana* they are not able to cease the growth of pathogens and create halos of inhibition in dual culture (Azadi et al. 2015a; Griffing 2007). Evaluation of chitinase enzyme activities showed that all isolates were able to produce chitinase enzymes in the presence of *Rhizoctonia* cell wall as the only carbon source and it is noted that the secretion of chitinase enzymes increased significantly on the fourth day, which is consistent with previous reports (Mustafa and Kaur 2010). It was also found that the amount of chitinases increased in various isolates of *B. bassiana* on the fourth and sixth days after inoculation. In other studies, chitinase production increased in various isolates of *B. bassiana* on the third day after inoculation. Although the amounts of chitinases produced by various isolates of *B. bassiana* are different and influenced by the carbon source and the test situation, the maximum of chitinase enzyme activities can be seen at 72 to 120 hours after inoculation (Petlamul and Prasertsan 2012).

Innate traits of the applied strains, as well as nutritional combinations of the media, carbon sources, and concentration, and carbon:nitrogen ratios are known to influence the growth and conidiogenesis of fungi (Petlamul and Prasertsan 2012). Therefore the growth rate and sporulation of every strain are different. The results showed that the presence of nitrogen in the SDA medium accelerates the growth of isolate TS12; while the growth rate of TS7 and KJ24 isolates was high on

PDA. Pandit and Som (1988) suggested that PDA can be used for the culturing of *B. bassiana* (Senthamizhlselvan et al. 2010), while some others successfully used SDA medium for the mass culturing of *Metarhizium anisopliae* and *B. bassiana* (Petlamul and Prasertsan 2012). In terms of sporulation, all isolates were able to produce more spores in SDB medium than in PDB medium. According to the studies carried out by Sharma et al. (2002), the better growth and sporulation of *Beauveria* spp. in the SDB medium may be due to the presence of peptone as a source of nitrogen (Senthamizhlselvan et al. 2010). Thus the presence of nitrogen in the SDA medium may accelerate the growth of isolate TS12. Similarly as in the natural environment, the amount of carbon and nitrogen in soil can affect the growth and sporulation, colonization, and the use of appropriate biocontrol mechanisms by different isolates.

For the evaluation of the mechanisms involved in the control of damping-off, enzyme activities, phenolic compounds and growth features were investigated. Previous studies have shown that some enzymes are related to ISR, including peroxidase and polyphenol ammonia-lyase. These enzymes cause the release of molecules involved in pathway signaling defense mechanisms of plant, and produce compounds such as phenolics (Alves Silva et al. 2004). Our results showed that the peroxidase activity increased in some treatments in the fourth week in comparison to the third week after cultivation of plants. The rapid increase of this enzyme activity followed by its reduction during the plant's defense responses is related to the generation of reactive oxygen species (ROS), which act as signal molecules to activate defense responses (Yang et al. 2014). Therefore, the amount of peroxidase and ROS increases rapidly under pathogen attack; but the high concentrations of ROS are toxic to the plant itself and cause cell damage. To control the level of ROS and to protect cells under stress conditions, plants produce ROS scavenging enzymes such as peroxidase to reduce ROS (Tsai 2011). Rapid increase of peroxidase enzyme activities in the third week after the treatments (*e.g.*, with TS7 at 10^7 spores/ml) is likely due to the activation of defense mechanisms. The activity of peroxidase enzyme in isolate KJ24 at 10^5 and 10^7 spores/ml increased in the fourth week after cultivation of plants. Although the activity of this enzyme was higher than in case of the infected control plants, because of the delay in the activation of plant defense responses, it was not able to control the disease in proper time and percentage of damping-off was high. Therefore the extent and speed of activation of defense responses for the inducing of resistance against the pathogens is important and vital (Ku and Preisig 1984).

Based on the results from measurement of plant phenolic compounds, the amount of these compounds increased in the third week and then reduced in the fourth week in all plants treated with fungal isolates (except those treated with KJ24 at 10^9 spores/ml), which represents the activation of plant defense responses by the treatments, particularly by TS12 and TS7 at the concentration of 10^7 spores/ml (34.78% damping-off). Our results are consistent with the findings of Madhavan et al. (2011). Increasing plant resistance is associated with increased phenolic compounds (Velazhahan and Vidhyasekaran 1994). In plants treated with KJ24 at the concentration of 10^9 spores/ml with 21.73% damping-off, the level of these compounds were high in comparison to infected control plants, but defense responses were not activated because these compounds increased gradually. Production of phenolic compounds and their concentrations are among the most important defense reactions of plants against pathogens. Under rapid damage of the cells, phenolic compounds are released quickly, which effectively prevents the penetration of pathogens; otherwise, under slow release of the components, it may let the rapid spread of diseases and quick development of symptoms.

Additionally, results showed that the activity of the PAL enzyme increased in the fourth week in comparison to the third in the case of all isolates except for KJ24 at the concentration of 10^9 spores/ml. Although this rapid increase can be indicative of defense mechanism activation, in some treatments including TS12 and TS7 at the concentration of 10^9 spores/ml, the amount of this enzyme was lower than those of the infected and non-infected control plants. PAL is one of the most important enzymes in the activation of defensive responses (Alves Silva et al. 2004), leading to increased phenolic compound production in response to pathogens (Parr and Bolwell 2000). Based on the above mentioned results, the gradual increase in PAL enzyme and phenolic compounds in KJ24 treatment at 10^9 spores/ml concentration cannot be the reason for the activation of defense responses; therefore the control of damping-off by this treatment was probably not due to the stimulation of induced systemic resistance, and it seems that another mechanism is involved in the reduction of disease by this treatment.

Lignification of the walls and its strengthening were done by H_2O_2 in the early stages of growth and defensive responses (Yang et al. 2014). Considering the role of peroxidase enzymes in the generation of ROS, increased production of H_2O_2 is associated with the increase of peroxidase activities. After passing through the stage of maturity and reducing the amount of H_2O_2, the amount of peroxidase enzymes also decreases. In the uninfected control plants, the amount of peroxidase enzymes increased in the third week after cultivation of plants. However, this increase was less than in the case of the treated plants. Studies carried out by Ippolito and Nigro (2000) also showed that the amount of peroxidase enzymes increased in the uninfected control fruits, however, it was less than that of the fruits treated by antagonist *Aureobasidium pullulans*. So, rapid increase in peroxidase enzyme, as well as the increase in growth factors in KJ24 treatment at 10^9 spores/ml have probably been due to plant growth stimulation for the passage

of the initial phase. Plant growth stimulation by antagonists plays an important role in the protection of plants against root diseases. With the growth stimulation of the plant, the infected roots are replaced quickly by new roots; the result caused the passage of seedlings from the susceptible stage and the reduction of disease damage. Harman et al. (2004) found that the existence of such activity by entomopathogenic fungi is similar to the activity of plant growth promoting rhizobacteria. In TS12 and TS7 at the concentration of 10^7 spores/ml (34.78% damping-off), the activity of PAL, and the amount of phenolic compounds in the third week showed a rapid increase in comparison to the fourth week. Therefore, it seems that an increase in this enzyme under the mentioned treatments may be due to the activation of induced systemic resistance; and, probably, treatments with TS7 and TS12 at the concentration of 10^7 spores/ml are able to reduce damping-off, and their effective control is due to the plant resistance stimulation.

In the case of isolate TS7 the induction of plant resistance could be due also to its rapid and abundant sporulation. The spores could act as a stimulant and cause the activation of the plant defensive responses. Based on *in vitro* studies, isolate TS12 demonstrated the same behavior as KJ24 (Azadi et al. 2015b), but in the greenhouse they were different. This behavior can rise from differences in the growth pattern of two strains. Based on the results, this strain had high growth rate on the SDA medium (nitrogen rich). It is likely that because of the nitrogen shortage in the soil, this strain cannot grow well and colonize the plant, but it can induce plant resistance by producing high number of spores. Induction of resistance along with other biocontrol mechanisms such as competition and antibiosis can be effective in plant protection against plant pathogens. Since the proper functioning of antagonists against soil-borne pathogens is related to environmental conditions, soil, and population of antagonists, it seems that the difference in the behavior of antagonists and the type of mechanisms used to control damping-off by isolates of *B. bassiana* is dependent on its compatibility with the existing conditions.

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