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Investigation on the biological control of *Alternaria alternata*

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ABSTRACT

Alternaria alternata (Fr.) Keissl. which has a wide host range is an important fungal pathogen causing losses in yield in agricultural crops. The chemicals used for controlling this disease are directly toxic to beneficial microorganisms in soil. This study was carried out to determine the antifungal activities of a total 13 candidate bioagent bacterial isolates of *Bacillus subtilis* (TV-6F, TV-12H, TV-17C and TV 125 A), *Bacillus megaterium* (TV 87 A and TV 91 C), *Bacillus pumilus* (TV 67 C), *Paenibacillus polymyxa* (TV 12E), *Pantoea agglomerans* (RK 92 and BRT-B), *Pseudomonas fluorescens* Biotip F (FDG 37), *Bacillus thuringiensis* subsp. *kurstakii* (BAB-410) and *Bacillus sphaericus* GC subgroup D (FD 49) and bioagent fungal isolates of *Trichoderma harzianum* (ET 4 and ET 14) against two isolates of *A. alternata* isolated from strawberry and cucumber on petri plate assays. *B. pumilus* TV 67C (87.63%-65.89%), *B. subtilis* TV 6F (77.61%-63.11%) and *B. megaterium* TV 87A (72.93%-68.87%) bacterial isolates were the most effective isolates against pathogenic fungi in *in vitro* and bioagent fungal isolates ET 4 and ET 14 inhibited pathogenic fungi grown in *in vitro* respectively 73.87% -83.33% and 55.85% -74.44%, too. Our results indicated that *B. subtilis, B. pumilus, B. megaterium* and *T. harzianum* should be tested against *A. alternata* in field condition.

Key words: *Alternaria alternata,* Bacteria, Biological control, *Trichoderma harzianum*

Alternaria alternata (Fr. Keissl.) is a fungal pathogen causing leaf spot, rots and blights on many plant parts on over 380 host species of plants. It caused serious losses in the yield. For example, yield losses up to 79% due to early blight damage in tomato were reported from Canada (Abbo *et al*. 2014) and has potential to cause 30% yield loss and postharvest losses of up to 10% (Dube 2014). Disease management strategies including rotation with non-host crops and sanitation are not entirely satisfactory since the fungus is primarily air-borne, has long survival ability in plant debris, and has a wide Solanaceae host range (Chaerani and Voorrips 2006). Fungicide treatments are the most effective way to control the disease to a non-damaging level. Typically, fungicides are applied starting from two weeks after transplanting until two weeks before harvest at two to three week intervals. Such heavy use of chemicals is not economically feasible for the generally resources-limited growers (Abbo *et al.* 2014). However, the widespread use of chemicals in agriculture has been a subject of public concern and scrutiny due to their potential harmful effects on the environment, their undesirable effects on non-target organisms, development of resistant races of pathogens and possible carcinogenicity of some chemicals (Heydari and Pessarakli 2010).

Some researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. For this reason, recent efforts have focused on developing environmentally safe, long lasting and effective biocontrol methods for the management of plant diseases (Cook and Baker 1983). Biocontrol agents may provide a seemingly environmental friendly alternative to potent and toxic fungicides, which cannot be broken down in the environment (Abdalla *et al*. 2014).

There are many studies conducted on the biological control of *A. alternata* by using fungal (Benhamou and Chet 1993, Verma *et al*. 2007, Tozlu *et al.* 2017) and bacterial (Abbo *et al*. 2014, Abdalla *et al*. 2014, Pane and Zaccardelli 2015, Ali *et al*. 2016, Gao *et al*. 2017) biocontrol agents.

In this study, we aimed to determine the antifungal activity of two fungal and 13 bacterial isolates against pathogen causing damage in strawberry and cucumber.

MATERIALS AND METHODS

Pathogenic isolates were isolated from diseased strawberry (ET 57) and cucumber (ET 58) obtained from local market.

Bioagent bacteria TV-6F (Erman *et al*. 2010), TV-12H (Mohammadi *et al*. 2017), TV-17C (Erman *et al*. 2010), TV 125 A (Çığ *et al*. 2014) (*Bacillus subtilis*), TV 87 A (Karagöz *et al*. 2016), TV 91 C (Aktaş and Kotan 2016) (*B.*

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megaterium), TV 67 C (Aktaş and Kotan 2016) (*B. pumilus*), TV 12 E (Erman *et al*. 2010) (*Paenibacillus polymyxa*), RK 92 (Kotan 2002) (*Pantoea agglomerans*), FDG 37 (Güneş *et al*. 2015) (*Pseudomonas fluorescens* Biotip F), BRT-B (*P. agglomerans*) (Kotan *et al.* 2005), BAB-410 (Göktürk *et al*. 2018) (*B. thuringiensis* subsp. *kurstakii*) and FD 49 (Dadaşoğlu *et al*. 2013) (*B.sphaericus* GC subgroup D) and fungi ET 4 and ET 14 (Tozlu *et al*. 2017) (*Trichoderma harzianum*) were obtained from the culture collection unit in the Department of PlantProtection, Faculty of Agriculture at Atatürk University, Erzurum, Turkey.The bioagent bacteria had been isolated from the rhizosphere and phyllosphere of wild and traditionally cultivated plants growing in the Eastern Anatolia Region of Turkey.The isolates of bioagent fungi had been isolated from *Aesculus hippocastanum* and *Pinus sylvestris* (Tozlu *et al*. 2017). These bacterial and fungal isolates were selected for their ability to reduce *A. alternata* from apple and tomato; *Sclerotinia sclerotiorum* from red cabbage, cucumber and egg plant; *Fusarium solani* from tomato and cucumber; *Geotrichum candidum* from carrot; *Penicillium digitatum*, *Pseudomonas viridiflava, Erwinia chyrisanthemi* from tomato; *E. amylovora* and *P. syringae* pv. *Syringae* from some pome fruits in previous studies.

Bacteria were identified according to their fatty acid methyl esters (FAME) profiles using Sherlock Microbial Identification System (Miller 1982) and fungi were identified according to their molecular diagnosis (White *et al*. 1990) in previous study. Bacterial cultures were grown on Nutrient agar for routine use, and maintained in Nutrient Broth with 15% glycerol at –80°C for long-term storage. Fungal pathogens and bioagents were grown on Potato Dextrose agar and maintained on PDA slant cultures at 4°C in the refrigerator until use.

The diseased strawberry (ET57) and cucumber (ET58) fruits were brought to the laboratory, and 1 cm pieces obtained from the infected area in the fruits in a laminar cabin were rinsed with tap water for 3 min, and consequently disinfected superficially in 10% ethanol for 1-2 min. Consequently, they were dried for 5 min, and placed in petri plates containing PDA and left to incubation at 20-25°C. Mycelial disks with 4 mm diameter obtained from the edges of colonies that developed within 4-5 days time were transferred to petri plates containing PDA to obtain pure cultures. These isolates were then transferred to the test tubes containing PDA, and were stored at 4°C for later analysis.

Plant fruits were first surface sterilized and inoculated with wounding and than maintained at 25°C and 95% relative humidity conditions in a growth chamber for 10 days. For control treatment, only PDA discs were placed on injured plant. There were symptoms on inoculated plants and no symptoms were observed on control plants. The fungal pathogen was re-isolated from the disease lesions on the inoculated plants; this re-isolated pathogen exhibited the same morphological characteristics as those of the original isolates, so Kochs postulates were completed.

The identification of *Alternaria* isolates was performed by sequencing a fragment of genome. The primers used for the polymerase chain reaction (PCR) were ITS1 (5′TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990). Amplification reactions were carried out in a 50 µl reaction volume under followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 1 min and elongation at 72°C for 1 min, with a final extension step of 72°C for 10 min. PCR products were analyzed in 1% (w/v) agarose gels by horizontal gel electrophoresis. DNAs were visualized by UV excitation after staining with ethidium bromide. The products were purified following the protocols of the PureLink ® PCR Purification Kit. After purification, ITS rDNA gene was sequence in both directions with ITS1 and ITS4 primers. Sequences chromatograms were assembled into one complete sequence using Bioedit Sequence Alignment Editor version 7.2.5 (Hall 1999) and the sequence was compared to all known sequences in the GenBank by use of BLASTN 2.2.26+ program (Zhang *et al*. 2000) and deposited with the GenBank database under the accession number KY774664 and KY774665.

The bioagent bacterial isolates were tested on tobacco plants (*Nicotina tabacum* L. var. Samsun) for hypersensitivity as described by Klement *et al.* (1964). The bacterial suspension (10^8 cfu/ml) prepared in sterile distilled water and infiltrated into the inter costal area of the leaves of tobacco plants by using a 3-cc syringe without needle. The inoculated plants were incubated in a completely randomized design on the greenhouse bench for 24-48 h at 20-28°C. The presence of rapid tissue necrosis at the inoculation site was recorded within 24-48 h after infiltration. This test was repeated at least three times for each strain. Sterilized distilled water (sdH_2O) was used as a negative control. The tobacco plants symptoms were examined. The bacterial isolates which weren't developed any symptoms were evaluated as nonpathogenic, developed were pathogenic.

Petri dishes (9 cm diameter) containing 20 ml PDA were used *in vitro* assay. The bacterial isolates were grown on NA at 26°C for 24 h to obtain fresh culture for Petri plate assay. The pathogenic fungi isolates and bioagent fungi isolates were also cultivated on PDA at 30°C for 3 days.

The discs measuring 6 mm obtained from pathogenic fungal isolates and the discs measuring 6 mm obtained from fungal isolates tested for efficiency as bioagent were placed as opposite to one another on the part close to edge on petri dishes; and bacteria were placed around petri dishes with PDA, where pathogenic fungi were located. As control, only mycelial disks of pathogenic fungi were placed on petri dishes with PDA, and they were incubated at 25ºC in the dark, until (define the days) mycelium of fungal isolates cover the surface of agar surface. For the study, 3 petri dishes were utilized for every fungal and bacterial isolate, and study was carried out in 3 replicated. Fungal growth was determined by measuring the diameter of colony radial growth in mm.

Chitinase enzyme activities were investigated by petri plate assays and clear zones formed around the colonies indicated enzymatic activities. The colloidal chitin was prepared as described by Örtücü (2012). For the chitinase activity of fungi, medium-5 as described by Abd-Aziz *et al.* (2008) with added agar was used. Six mm agar disk taken from seven days old fungal culture was placed in the center of these agar plates. Then, inoculated plates were incubated at 28ºC for 10 days. For the chitinase activity of bacterial isolates, chitinase production medium as described by Woo *et al*. (1996) was used. Similarly, a loop full of each of tested bacterium inoculated in plate containing 3 per centcolloidal chitin and plates were incubated at 30ºC for 2 days. Clear zones that are formed around the colonies indicated degradation of the substrate due to enzymatic activity.

The percentage inhibition of pathogen fungi by bioagent bacterial isolates was calculated by using the formula (Mari *et al.* 1996):

$$\text{Inhibition (\%)} = (C-T) \times 100 / (C-6)$$

where C is the diameter of the pathogen colony of control group, 6 is the diameter of pathogen disk, T is the diameter of pathogen colony after treatments.

The hyperparasitic effect of *T. harzianum* isolates was determined against *A. alternata* was expressed as percentage interference rate (%) and calculated according to (Skidmore and Dickinson 1976) formula:

$$\text{PIRG (\%)} = \frac{R_1 - R_2 \times 100}{R_1}$$

where, PIRG, Percentage interference rate (%); R_1, Semi-diameter of the pathogen mycelium in the control petri plate; R_2, Semi-diameter of the pathogen mycelium in the double culture petri (ET 4 or ET 14 and pathogen fungus). PIRG ≤ 50% : Low, 50 < PIRG % ≤ 60 : Medium, 60% < PIRG ≤ 75% : High, PIRG > 75% : Very high, -: Ineffective.

RESULTS AND DISCUSSION

Pathogenicity of fungal isolates obtained from strawberry and cucumber was performed and the result was positive (Fig 1, Table 1).

By using primers defining pathogenic fungal isolates and PCR, molecular diagnosis was obtained from pure isolates, and pathogenic fungal isolates were specified as *A. alternata* (ET 57, ET 58). Molecular diagnosis results of pathogen isolates used in this study are shown in Table 1.

The 13 bacterial isolates were determined according to fatty acid methyl esters in previous study. Of the bacterial isolates applicated in this study: 4 of bacterial isolates were *Bacillus subtilis* (TV 6F, TV 12H, TV 17C, TV 125A), 2 were *B. megaterium* (TV 87A, TV 91C), 2 were *P. agglomerans* (RK 92, BRT-B), 1 was *B. pumilus* (TV 67C), 1 was *P. polymyxa* (TV 12E), 1 was *P. fluorescens* Biotip F (FDG-37), 1 was *B. thrungiensis* subsp. *kurstaki* (BAB 410) and 1 was *B. sphaericus* GC subgroup D. (FD 49). The hypersensitivity test results of these isolates were found to be negative.

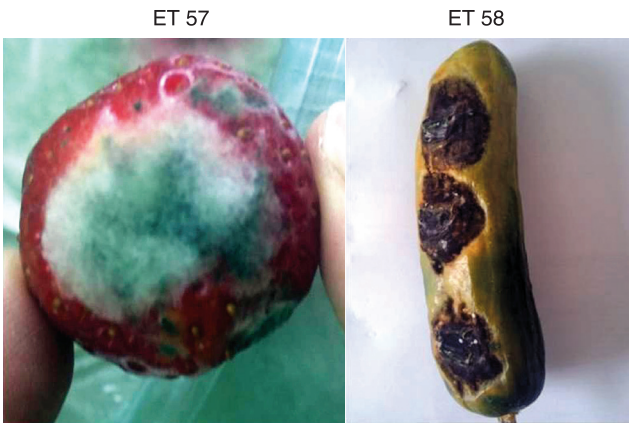

Fig 1 *Alternaria alternata* ET 57 and ET 58 isolates isolated from strawberry and cucumber

During the study, efficiency of both isolates of *Trichoderma* was tested in *in vitro* on petri plate containing PDA, and the hyperparasitic effect of *T. harzianum* isolates were shown (in 6 days period) in Table 2. It was found as 73.87% and 83.33% for ET 4 and 55.85% and 74.44% for ET 14 (Table 2). Isolate ET 4 showed better effect against pathogen isolate ET 57 (83.33%), than pathogen isolate ET 58 (73.87%). The hyperparasitic effects of *T. harzianum* ET 4 and ET 14 isolates *in vitro* conditions are given on Fig 2 and SEM **(***Scanning Electron Microscope)* images on Fig 3.

Table 2 Hyperparasitic effects of *Trichoderma harzianum* isolates tested against the pathogens in *in vitro* conditions

Pathogen	ET 4			ET 14	
	Isolates	PIRG (%)	HL	PIRG (%)	HL
Alternaria alternata	ET 57	83.33	++++	74.44	+++
	ET 58	73.87	+++	55.85	++
Average		78.60	++++	65.15	+++

PIRG, Percentage interference rate (%); HL, Hyperparasitic level; +, Low; ++, Medium; +++, High; ++++, Very high, -: Ineffective

Table 1 The molecular identification and pathogenicity test results of the pathogen isolates

Isolate	Isolated from	ITS identification results	Identify (%)	ITS Sequence*	P	Reference
Pathogens						
ET 57	*Fragaria ananassa*	*Alternaria alternata*	95	KY774664	+	In this study
ET 58	*Cucumis sativus*	*Alternaria alternata*	99	KY774665	+	In this study

P: Pathogenicity, *GenBank

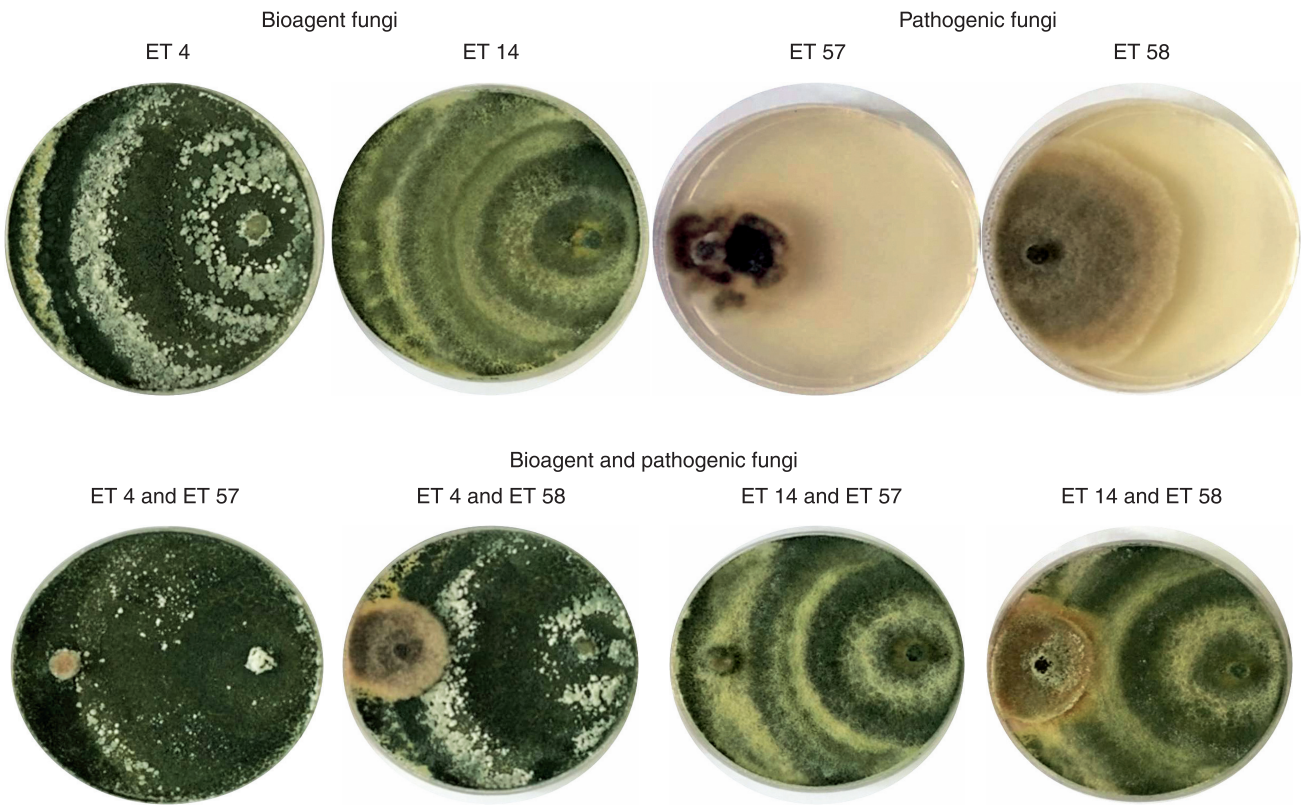

Fig 2 The hyperparasitic effects of *Trichoderma harzianum* ET 4 and ET 14 in *in vitro* conditions

The results of antifungal activities of bacterial isolates tested against *A. alternata* ET 57 and ET 58 isolates in *in vitro* tests are given on Table 3. The inhibition rate of bacterial isolates ranged from 26.87% to 87.63% for ET 57 and from -1.06% to 68.87% for ET 58. The highest inhibition was obtained from TV 67-C (87.63%) and it was followed by TV 6F (77.61%), RK 92 (76.12%) and the lowest inhibition rate was obtained from TV 12 E (26.87%) for ET 57 (Table 3). The bacterial applications more or less effected the growth of ET 57 in *in vitro*. All of bacterial isolates were different group from control application (0.01%) for ET 57. In bacterial isolate applications performed against ET 58 pathogenic isolate; the highest inhibition rate was observed in TV 87A (68.87%), followed by BRT-B (64.18%) and TV 6F (63.11%), the lowest inhibition rate was obtained from control application (-1.06%), TV 12E (-1.06%), BAB 410 (1.07%), TV 91C (5.33%) and TV 125A (5.55%). They were in the same group (Table 3).

Chitin determination was performed in 2 fungal and 13 bacterial isolates. thirteen bacterial isolates were tested for qualitative analysis of chitinase enzymes by measuring clear zone. Only one isolates *P. agglomerans* (BRT-B) showed chitinase activity in the plate assays. We did not observe the formation of clear zone in the chitinase activity for

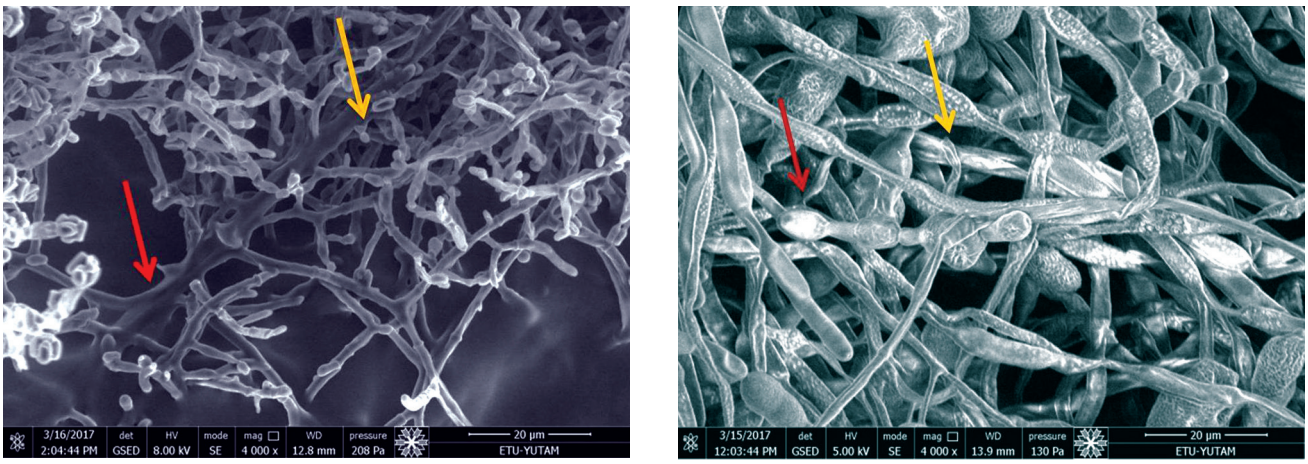

Fig 3 The images of *Alternaria alternata* isolates and *Trichoderma harzianum* in SEM (*Scanning Electron Microscope) (Red arrow: A. alternata, yellow arrow:T. harzianum)*

Table 3 Antifungal activities of the bacterial isolates against *Alternaria alternata* ET 57 and ET 58 pathogens isolates on petri plate assays

Isolate	Petri plate assays*			
	ET 57		ET 58	
	Percentage inhibition rate (%)		Percentage inhibition rate (%)	
TV 67C	87,63	A	65,89	AB
TV 6F	77,61	AB	63,11	AB
RK 92	76,12	AB	42,86	D
TV 87A	72,93	BC	68,87	A
FD 49	72,71	BC	53,10	C
TV 17C	72,07	BC	61,83	B
BRT-B	65,46	BC	64,18	AB
FDG 37	65,03	BC	47,12	CD
TV 12H	59,06	C	60,77	B
TV 91C	36,46	D	5,33	E
BAB 410	29,64	D	1,07	E
TV 125A	27,72	D	5,55	E
TV 12E	26,87	D	-1,06	E
Control	0,01	E	-1,06	E
CV	15.82		10.63	
LSD	14.69		6.97	

*In the same column by the same letter are not significantly different to the test of LS Means Differences Student's (P<0.01)

other 12 isolates. *T. harzianum* (ET 4 and ET 14) showed chitinase activity.

Efficiency of 2 isolates of *T. harzianum*, 4 isolates of *B. subtilis*, 2 isolates of *P. agglomerans*, 2 isolates of *B. megaterium*, 1 isolate of *B. pumilus*, 1 isolate of *P. polymyxa*, 1 isolate of *P. fluorescens* Biotip F, and 1 isolate of *B. thuringiensis* subsp. *kurstakii,* 1 isolate *B. sphaericus* GC subgroup D were tested against 2 isolates of *A. alternata*.

Trichoderma species are most studied fungal biocontrol microorganisms (Singh *et al*. 2008, Altınok and Erdoğan 2015, Saravanakumar *et al*. 2016) and they are among biologic control factors that receive the most encouraging results against many fungal plant pathogen. In this study, the hyperparasitic effects of *T. harzianum* isolates were evaluated according to their inhibition rates were classified as low, medium, high, very high and ineffective. Pursuant to mean values obtained, ET 4 isolate was very highly effective; while ET 14 was highly effective against *A. alternata*. Similar observation was reported by Tozlu *et al*. (2017). *T. harzianum* has several mechanisms of action, including chitinase, production of β-1-3 glucanases and β-1-4 glucanases, antibiotics, antagonism, solubility of inorganic plant nutrients, inactivation of pathogenic enzymes, encouraging stability (Harman 2006) and production of volatile metabolites and nonvolatile antibiotics that suppress soil fungal pathogens, antagonism mechanisms with effects such as competition in terms of location and nutrition (Akrami *et al*. 2012). Furthermore, it was revealed that they induce changes in plant metabolism by colonizing on plant root surfaces (Howel *et al*. 2003).

Many species of *Bacillus* are known to suppress several pathogens. (Abdalla *et al*. 2014). *Bacillus* species as a group offer several advantages over other bacteria for protection against pathogens because of their ability to form endospores, and because of the broad-spectrum activity of their antibiotics (Abdalla *et al.* 2014). There are many studies that *Bacillus* species showed strong antimicrobial activity against *A. alternata* (Abbo *et al*. 2014). *A. alternata* which were inoculated with *B. subtilis* and *B. megaterium* showed significantly less mycelial growth during the incubation period in *in vitro* and this finding agreed with the reports by Abbo *et al.* (2014) who indicated the ability of these bacteria to inhibit *in vitro* mycelial growth of many plant pathogenic fungi. Similar results were reported in these studies. Tozlu *et al.* (2016) reported that *B. subtilis* TV-6F was the most effective bacteria against *Sclerotinia sclerotiorum* and this result is similar to our study result.

B. pumilus is one of the most effective isolate in this study but no significant differences in the inhibition of *A. alternata* were observed between *B. pumilus* and the control treatment in Abbo *et al*. (2014). It is thought that the difference is due to isolate.

The degradation of fungal cell walls with the production of hydrolytic enzymes of bacterial isolates is one of the most important mechanisms for biocontrol of pathogenic fungi (Elshafie *et al*. 2012). These enzymes such as chitinase (Ordentlich *et al*. 1988) protease (Saligkarias *et al*. 2002) and glucanase (Leelasuphakul *et al*. 2006). Chitin is a major structural component of most fungal cell walls (Selitrennikoff 2001). The enzyme could be used directly in biological control of microorganisms (Gomes *et al*. 2000) or indirectly using purified protein (Gomes *et al*. 2001) or through gene manipulation (Tsujibo *et al*. 2000). Senol (2014) have reported that produced chitinase enzyme from *B. subtilis* which has antifungal activity against *Fusarium culmorum* can be used in agriculture as bioagents against fungal pathogens. But, according to Örtücü (2012), there isn't interest between the production of chitin and the antifungal activity. Both of the fungal and only one of the bacterial isolates produced chitinase enzyme in this study.

According to the results, fungal isolates of *T. harzianum* and bacterial isolates of *B. subtilis, B. pumilus* ve *B. megaterium* 10^8 cfu/ml suspension were found to be more effective against *A. alternata* (ET 57 and ET 58) isolates, isolated from strawberry and cucumber in *in vitro* conditions. It is probable that this effect may vary in site conditions with different temperature and moisture values. In the future, conducting studies in conditions that include this fungus and bacteria species being effective against *A. alternata* has crucial importance.

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