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Research Article/Araştırma Makalesi

Biocontrol of *Alternaria alternata* causing leaf spot disease on faba bean (*Vicia faba* L.) using some *Trichoderma harzianum* ısolates under i*n vitro* condition

In vitro koşullarda bazı lokal Trichoderma harzianum izolatları kullanılarak bakla yaprak lekesi hastalığı etmeni Alternaria alternata' nın biyolojik kontrolü

Mukaddes KAYIM[1*], Amin Mohammad YONES[1], Ali ENDES[1]

[1]Çukurova Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü, Balcalı 01330 ADANA

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Address for Correspondence:
Mukaddes KAYIM
e-mail:
mkayim@cu.edu.tr

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ABSTRACT

Faba bean is among common legume crops used as a fresh vegetable in Turkey. *Alternaria alternata* is one of the main fungal diseases attacking faba bean in Turkey. Investigation of leaf spot disease of faba bean was carried out to find out suitable management strategies under laboratory condition. Five *A. alternata (*A1, A2, A3, A4, and A5) and five *Trichoderma harzianum* isolates (T1, T2, T3, T4, and Tc) including control were arranged in complete randomized design factorial, forming a total of 25 treatments. Dual culture plate test revealed that the percentage growth inhibition of *A. alternata* by *T. harzianum* was in the range of 53.85 to 82.81 %. In addition, *Trichoderma* isolates (T2 and T3) were found the most effective with the highest antagonistic activity against *A. alternata* isolates fungal pathogen caused minimum length and width diameter of leaves lesions. The species of *Alternaria* and *Trichoderma* isolates were identified according to cultural characteristics and alignment analysis of ITS sequences in GenBank of NCBI. The results point out that T*. harzianum* provides the use of high potential antagonists capable of controlling the leaf spot disease of faba bean plant.

Anahtar Kelimeler: *Alternaria alternata,* Antagonism, Detached leaf, Dual culture, Faba bean (*Vicia faba* L.), ITS, Pathogenicity, *Trichoderma harzianum*

ÖZ

Bakla,Türkiye'de taze olarak tüketilen yaygın baklagiller arasında yer almaktadır. *Alternaria alternata'nın* neden olduğu Bakla yaprak leke hastalığı, Doğu Akdeniz Bölgesi'nde görülen önemli bir fungal hastalığıdır. Bu çalışmada Yaprak leke hastalığına karşı uygun mücadele stratejilerini belirlemek için *in vitro* koşullarda antagonist *Trichoderma harzianum* ile denemeler yürütülmüştür. *Trichoderma harzianum* ile denemeler yürütülmüştür. Deneme, Beşer adet *A. alternata* (A1, A2, A3, A4 ve A5) ve *T. harzianum* izolatları (T1, T2, T3, T4 ve Tc) ile kontrol parseli dahil, tesadüf parselleri deneme desenine göre toplam 25 uygulama olarak kurulmuştur. Petride, İkili kültür şeklinde yapılan testlerde, *T. harzianum izolatlarının A. alternata*'yı engelleme oranın, % 53.85 ile 82.81 aralığında olduğu belirlenmiştir. *A. alternata* fungal patojenlere karşı *T. harzianum*'un T2 ve T3 izolatlarının en yüksek antagonistik etki gösterdiği, yaprak lezyonlarının uzunluk ve genişlik çapının en az olması ile saptanmıştır. *Alternaria* ve*Trichoderma* türlerinin teşhisi, rDNA'sının ITS gen dizisinin NCBI Gen Bankasında nükleotid karşılaştırma analizi (nBLAST) ile belirlenmiştir. Elde edilen sonuçlara göre, *T. harzianum*'un, *A. alternata*'nın gelişiminin engelleyerek, bakla yaprak leke hastalığının kontrolünde bir antagonistik potansiyel olarak kullanılabileceğini göstermiştir.

Key Words: *Alternaria alternate,* Antagonism, Bakla (*Vicia faba* L.), İkili Kültür, ITS, Koparılmış yaprak, Patojenisite, *Trichoderma harzianum*

Introduction

Pulse crops are kind of legume mainly used as dried seed and they are very important in food security nowadays in the world. Faba bean ranks fourth in the world as an important food legume after garden pea, chickpea and lentil (Torres et al., 2006). The leading faba bean producing countries are China, Ethiopia and Egypt (Tekalign, 2014; FAOSTAT, 2017). In Turkey, faba bean is consumed as a fresh vegetable, canned and freeze foods. Faba beans are mainly grown in Aegean Region, followed by Marmara, Central North, Black Sea, Mediterranean (Adana, Antalya, Hatay) and Central East Region; annual faba bean production in Turkey is 41,929 tons (Balkaya and Karaağaç, 2013). Several infectious and non-infectious disease causal agents are reducing faba bean production and productivity. Among these, *Alternaria alternata,* causing faba bean leaf spot, belongs to the anamorphic fungi and the group of Dothideomycetes (Eriksson and Winka, 1997). This disease causes a symptom of brown-gray foliar spots surrounded by a darker border and showing concentric circles inside. Humid weather and temperature near 20 °C are favorable to *A. alternata* leaf spot of faba bean (Nasraoui, 2008). *Alternaria* disease on different crops is usually controlled by spraying with chemical fungicides (Batta, 2000; El-Gali, 2015).

However, fungicides are hazardous to human health (Leroux et al., 2002). The use of antagonist for plant diseases management is very important and promising method against foliar disease like *A. alternata*, causing diseases on several crops. The rapidly growing human population needs an increase in agricultural production. However, the emergence of plant diseases raised the difficulty of this challenge (Hui, 2013). Fungicidal plant diseases control causes many problems in the environment for living organisms including human health. For instance; plant pathogens can develop resistance against fungicides, the phytotoxic effect on crops, and environmental pollution. Subsequently, to reduce these problems, it is better to find out other safe and effective methods for plant diseases like *Alternaria* leaf spot of faba bean.

So far, several bio-control agents have been used for management of different plant diseases including *Alternaria* leaf spot of faba bean (Suprapta, 2012). Several studies have investigated the role of *Trichoderma as* bio-control agents against diseases of leaves spots (Bigirimana, 1997; Seaman, 2003; Prasad et al., 2013). *Trichoderma* spp. have the ability to inhibit plant pathogens by several mechanisms, either by competition for different nutrient, parasitic or by the production of antibiotic compounds, which can inhibit the growth and reproduction of pathogens. So far, no researches have been done on evaluation of antagonistic bio-agents against *Alternaria* leaf spot on faba bean in Turkey.

Hence, this work was initiated to evaluate the antagonistic capacity of local isolates *T. harzianum* against *A. alternata* leaf spot disease agent of faba bean plant *in vitro* conditions.

Material and Methods

Isolation of A. alternata and T. harzianum isolates

The most aggressive pathogenic fungi of *A. alternata* isolates were collected from a farmer's field in Adana province, Turkey. More than 25 infected faba bean plant samples were collected from different fields. Two of them were from faba bean leaves and three were from stems of faba bean, forming a total of 5 samples. The stems and leaves of faba bean samples were collected, placed in plastic bags and labeled with information of collection sites and origin of samples. All samples were taken randomly.

Infected parts of the leaves and stems were excised with a sterile scalpel. Collected samples from diseased plants were thoroughly washed with tap water; roots were removed and, surface sterilized with 70 % alcohol for 3 seconds and subsequently washed three times in 1 % (w/w) NaOCl for 2 minutes. Sterilized pieces were washed twice with sterile water for 60 seconds. Four-millimeter square of faba bean leaves placed on tetracycline amended potato dextrose agar

(PDA) media and incubated for 3 days at 25 °C. Mycelium from growing edge was subcultured on new PDA medium plates. After incubation for 5 days, single spore technique was adopted to obtain a pure culture.

T. harzianum on faba bean leaf and stems were isolated as they grown in the same Petri dish. All *T. harzianum* isolates were purified by hyphal tip technique or single conidia following methods developed by Brown (1924) and Hansen (1926), respectively. All *T. harzianum* isolates were observed under a microscope and identified based on morphological features (Barnett and Hunter, 1987; Bissett, 1991).

Pathogenicity test

The experiment was conducted to determine the pathogenicity of the five *A. alternata* isolates on local faba bean. Mycelia disks were used for pathogenicity test of *A. alternata* on faba bean leaves. Faba bean seeds, susceptible to different diseases including *Alternaria* spp., were sown in pots at Plant Protection Research Station of Cukurova University, on October 20, 2016. Leaves were collected from 50 days old seedlings as stated by Zhang et al. (2010). Moistened paper towels with steriled water and leaflets were placed face up on paper towels in a Petri plate. Seven days old agar plugs containing *A. alternata* (4 mm diameter) were placed on the leaflets after removed using cork borer. On control plates, solid PDA free of A. *alternata* was used. Then, all cultures were kept for 6 days at 25 °C in an incubator adjusted to 12 h dark and 12 h light. The length and width diameters of the leaves lesion formed around each agar plugs were measured.

A. alternata growth inhibition by T. harzianum in dual culture

The biocontrol ability of *T. harzianum* against *A. alternata* isolates was carried out under *in vitro* condition using the method described by Rahman et al. (2009). Petri plates of 9 cm diameter, containing tetracycline amended PDA medium were used for dual culture tests. Seven and five days-old culture *of A. alternata* and *T. harzianum* isolates were used, respectively. On dual culture tests, *T. harzianum* and *A. alternate* were placed on the opposite, where both were 2 cm away from the edge of the Petri dish. All treatments were repeated twice. In control plates, only *A. alternata* isolate was inoculated.

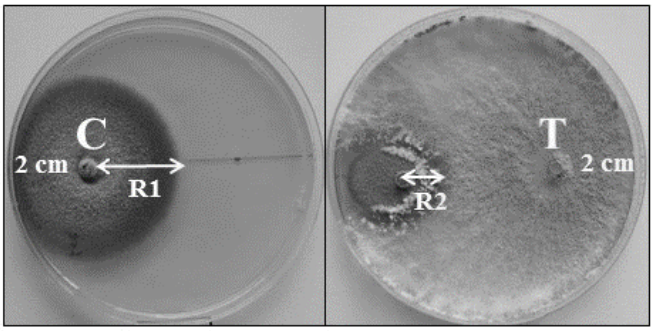

Figure 1. Measurement of radial growth of *A. alternata* mycelia where culture plug placement was 2 cm away from the margin. Note: R1, Radius of *A. alternata* colony in control plate; R2, the radius of *A. alternata* colony in dual culture plate; C, *A. alternata* isolate; T, *T. harzianum* isolate.

Şekil 1. Petrinin karşılıklı kenarlarından 2 cm uzaklığa yerleştirilen A. alternata ve T. harzianum misel disklerini içeren ikili kültür ortamında A. alternata'nın radial misel gelişiminin ölçülmesi. R1, Kontrol petride A. alternata kolonisinin yarıçapı; R2, İkili kültür ortamında A. alternata kolonisinin yarıçapı; C, A. alternata izolatı; T, T. harzianum izolatı

Percentage of inhibition ability was calculated following percentage inhibition of radial growth (PIRG) equation described by Skidmore & Dickinson (1976). Overgrowth ability was calculated as the mycelial growth of *Trichoderma* over the pathogen. The antagonistic effect was assessed based on the scale described by Soytong (1988).

$$\text{(Growth Inhibition (\%)} = \frac{R1-R2}{R1} \text{x100)}$$

R1, the radius of *Alternaria* isolate in the control plate; R2; the radius of *Alternaria* in the antagonistic colony.

Detached leaf experiment

Determination of biocontrol activity of *Trichoderma* isolates by using detached leaf method. The same faba bean variety was used, and the above procedure (pathogenicity test) was

followed so as to get faba bean leaflets. For this study, seventy days old faba bean seedlings were used for leaflets collection. The detached leaflet was placed on paper towels moistened with sterile water after placed in a Petri plate. Three days old PDA plugs containing *T. harzianum* (4 mm diameter) were used for this study. The plugs were placed on the faba bean leaflets, one plug per leaflet. After 36 hours, the equal size of agar plugs containing 7 days old *Alternaria* isolate was placed using a sterile scalpel on the *T. harzianum* isolates. *A. alternata* isolates which placed on paper towels moistened with sterile water in a Petri plate was served as a control. Then, they were kept in an incubator for 6 days at 25 °C and fluorescent light adjusted to 12 h dark and 12 h. The study was repeated two times. The length and width diameters of the leaf lesions formed around each agar plugs were measured.

Molecular identification of A. alternata and T. harzianum

DNA extraction from A. *alternata* and *T. harzianum* isolates was conducted following mini procedures developed by Cennis (1992) with minor modifications. Mycelium was used for this study. Pair primers of ITS4 and ITS5 were used for amplification of internal transcribed spacer (ITS) region of both *A. alternata* and *T. harzianum* isolates as described by White et al. (1990). These primers were synthesized by IDT (Integrated DNA Technology, USA). The polymerase chain reaction (PCR) was carried out with 5 U μL^{-1} *Taq* polymerase, 2.5 mM nucleotide mix, 2.5 mM $MgCl_2$ and 10x buffer supplied by Fermantas Company. Amplification was conducted in a thermal cycle (ProFlex PCR System, AppliedBiosystems, Life Technology) with the following cycle parameters: 94°C for 3 min, 35 cycles of 94 °C for 45 s, 56°C for 45 s, 72°C for 1 min, and a final extension for 7 min at 72°C. Agarose gel electrophoresis was used for the amplified product verification. Based on manufacturer instructions, PCR products were purified with a PCR prep kit (Qiagen, USA). The purified two PCR samples from each *A. alternata* and *T. harzianum* isolates were sequenced by Genoks (Ankara, Turkey) using Sanger Dideoxy Sequencing. The sequences of ITS of rDNA were edited and assembled using BioEdit 6 (free software). The sequences were compared with those deposited in the NCBI GenBank database using the BLAST program (version 2.0; National Center for Biotechnology Information, United States National Institutes of Health). The sequences of the ITS gene location were also submitted to the NCBI GenBank and accession numbers were obtained.

Data analysis

Two-way ANOVA statistical analysis was performed using SigmaStat procedure version 4, software for growth inhibition of pathogens in dual culture and antagonistic assay on a detached leaf, and SAS version 9.1 was used for single factor analysis of variance. Means separation were conducted at LSD=5 %. Square root transformations were done for pathogenicity test and antagonistic assay on a detached leaf. Normality test (Shapiro-Wilk) and equal variance test (Brown-Forsythe) were performed both for all data.

Results and Discussion

Pathogenicity test

The results indicated that maximum lesion length and width were obtained with isolates of A1 and A3 and the minimum was observed leaves inoculated with the isolate of A5. No leaf spot symptom developed on control leaves (Figure 2 and Table 1). This might be due to the fact that there was virulence variability among isolates. To confirm that these isolates were the causal agent of faba bean *Alternaria* leaf spot, *A. alternata* isolates were reisolated from faba bean leaves and grown on PDA and the characteristics of the *A. alternata* pathogen was confirmed by morphological features and PCR of ITS region.

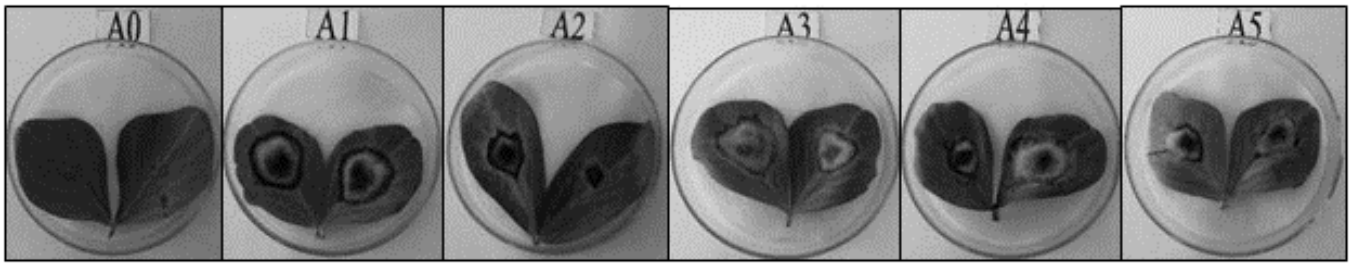

Figure 2. Pathogenicity of *A. alternata* on detached faba bean leaves
Şekil 2. In vitro koşullarda koparılmış bakla yapraklarında A. alternata'nın patojenisitesi

Table 1. Pathogenicity test of faba bean leaf spot caused by *A. alternata* on detached leaf test
Çizelge 1. Koparılmış yaprak testi metodu kullanarak A. alternata'nın neden olduğu bakla yaprak lekesinin patojenisite testi

Treatments *A. alternata* isolates) *Uygulamalar* *(A. alternata izolatları)*	LD of the leaf lesions (mm) *Yapraktaki lezyon uzunluğu (mm)*	WD of the leaf lesions (mm) *Yapraktaki lezyon genişliği (mm)*	LD / WD leaf lesions *Yapraktaki lezyonun uzunluk / eni*
A1	29.9 (5.5) a	27.8 (5.3) a	1.08
A2	18.2 (4.3) b	16.9 (4.1) b	1.08
A3	28.9 (5.4) a	27.3 (5.2) a	1.06
A4	15.4 (3.9) bc	15.5 (3.9) bc	0.99
A5	12.1 (3.5) c	12.0 (3.5) c	1.01
A (control)	0.0(0.0) d	0.0 (0.0) d	-
LSD at 5%	0.57	0.62	-
CV (%)	8.5	9.6	-

Figures in parenthesis are square root transformed values, A=*A. alternata*, T=*T. harzianum* LSD=Least significant difference, CV=Coefficient of variation, LD = Length diameter, WD =width diameter

Antagonistic assay on detached leaves

This study revealed that all *T. harzianum* isolates showed antagonistic activity against *A. alternata* isolates. But among them, T3 and T2 showed the highest antagonistic activity with the lowest length and width diameter of leaves lesion than others while the maximum was scored on control (Table 2).

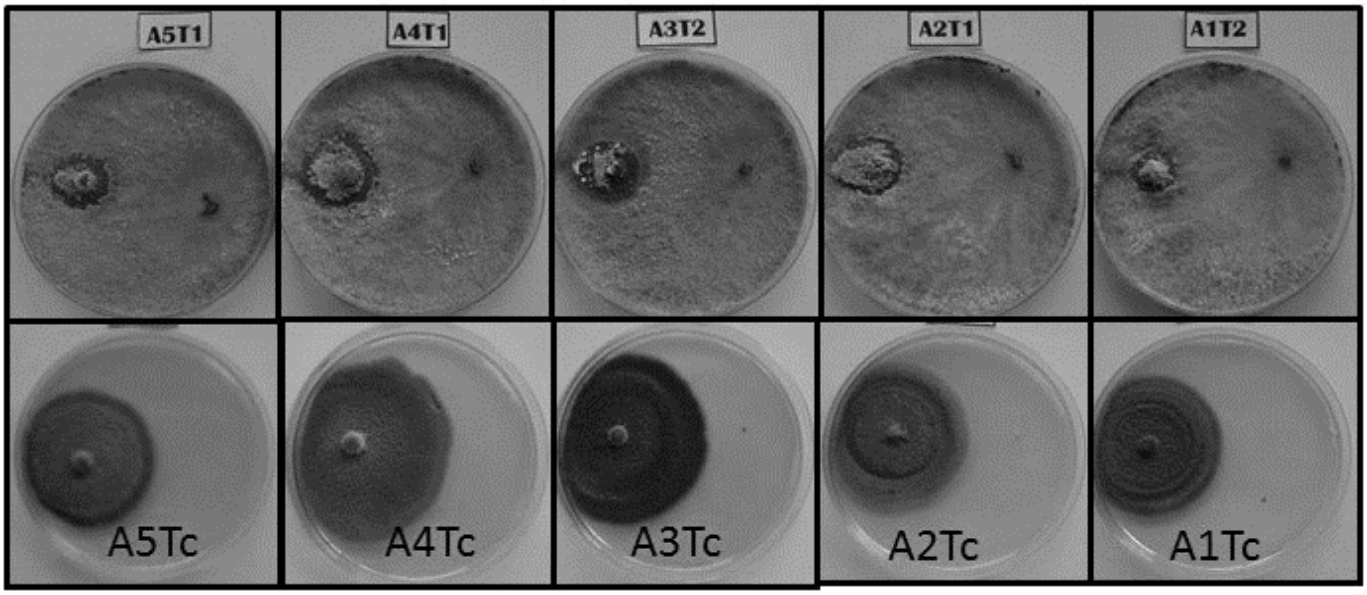

Figure 3. *In vitro* antagonistic activity of *T. harzianum* against the *A. alternata* with control in different replicates, 8 days after incubation in dual culture method. A: *A. alternata*, T: *T. harzianum*, Tc: Without *T. harzianum*
Şekil 3. In vitro koşullarda 8 günlük inkübasyondan sonar ikili kültürde T. harzianum'un A. alternata'ya karşı antagonistic etkisi A: A. alternata, T: T. harzianum, Tc: T. harzianum içermeyen

Table 2. The Antagonistic effect of *T. harzianum* on faba bean leaf spot caused by *A. alternata* isolates on detached leaf test. The LSD=least significant difference, CV=Coefficient of variation, LD=Length diameter, WD=width diameter.

Çizelge 2. Koparılmış yaprak testi ile A. alternata izolatlarının neden olduğu bakla yaprak lekesine T. harzianum'un antagonistic etkisi LSD=En az fark, CV=Varyasyon katsayısı, LD=Lezyon uzunlugu, WD=Lezyonun eni

A.alternata isolates *A. alternata* izolatları	*T. harzianum* isolates *T. harzianum izolatları*	LD of leaves lesion (mm) *Yapraktaki lezyon uzunluğu(mm)*	WD of leaves lesion (mm) *Yapraktaki lezyon genişliği (mm)*	LD/WD of leaves lesion *Yapraktaki Lezyonun uzunluk/ eni*
A1	T1	7.7 (2.7)	6.0 (2.4)	1.28
	T2	5.7 (2.3)	3.3 (1.7)	1.73
	T3	2.7 (1.6)	1.7 (1.3)	1.59
	T4	6.3 (2.5)	5.7 (2.4)	1.11
	Tc (control)	31.7 (5.6)	24.7 (4.9)	1.28
A2	T1	12.7 (3.5)	7.3 (2.7)	1.74
	T2	8.3 (2.7)	8.0 (2.8)	1.04
	T3	7.3 (2.6)	5.7 (2.3)	1.28
	T4	8.0 (2.6)	6.7 (2.5)	1.19
	Tc (control)	22.0 (4.7)	18.0 (4.2)	1.22
A3	T1	19.3 (4.3)	13.0 (3.5)	1.48
	T2	10.0 (3.2)	6.7 (2.6)	1.49
	T3	6.7 (2.0)	7.7 (2.7)	0.87
	T4	19.0 (4.3)	15.0 (3.8)	1.27
	Tc (control)	32.0 (5.6)	25.3 (5.0)	1.26
A4	T1	7.3 (2.2)	7.0 (2.6)	1.04
	T2	5.3 (2.2)	7.7 (2.7)	0.69
	T3	4.3 (2.0)	3.0 (1.7)	1.43
	T4	3.7 (1.8)	3.7 (1.8)	1.00
	Tc (control)	16.0 (4.0)	18.3 (4.3)	0.87
A5	T1	5.3 (2.31)	5.3 (2.3)	1.00
	T2	8.7 (2.9)	6.0 (2.4)	1.45
	T3	5.7 (2.2)	4.7 (2.1)	1.21
	T4	10.7 (3.0)	7.3 (2.6)	1.47
	TC (control)	19.7 (4.4)	18.7 (4.3)	1.05
LSD at 5 %		ns	0.38	-
CV (%)		28.4	18.4	-

Figures in parenthesis are square root transformed values, A=*A. alternata*, T=*T. harzianum*

Dual culture test

Mycelial growth of *A. alternata* isolates colony faced with *T. harzianum* isolates showed a minimum in radial growth (Figure 3). There was significant interaction difference between *A. alternata* and *T. harzianum* isolates (Table 3).

Table 3. ANOVA of radial growth and percent inhibition radial growth of *A. alternata* isolates as inhibited by *T. harzianum* isolates, 8 days after incubation.

Çizelge 3. İkili kültürde 8 günlük inkübasyondan sonra T. harzianum izolatları tarafından A. alternata izolatlarının % radial inhibisyonları ve radial gelişimlerinin varyans analizleri

Source of Variation *Varyasyon kaynağı*	Percentage of inhibition (%) *Engelleme oranı(%)*		Radial growth (mm) *Radyal gelişme (mm)*	
	DF *Serbestlik derecesi*	MS *Kareler ortalaması*	DF *Serbestlik derecesi*	MS *Kareler ortalaması*
A. alternata	4	131.7***	4	52.6***
T. harzianum	3	54.9***	4	1089.9***
A. alternata x *T. harzianum*	12	14.3*	16	10.1***
Residual	40	6.3	50	0.6
Total	59	18.9	74	64.4

P < 0.001***, P<0.05*, MS=mean square, DF=degree of freedom

In the present study, *T. harzianum* was reducing the mycelial growth of *A. alternata*. The minimum radial growths were scored on the interaction of T3 x A5 followed by T3 x A1 and T2 x A5. Nevertheless; the maximum radial growths were recorded on interactions of Tc-control x A4 and Tc x A3, followed by Tc-control x A3 and Tc-control x A2, which was without *T. harzianum* isolates application (Figure 4). The inhibition percentage of mycelia growth, overgrowth (days) and scale of antagonistic activity of *T. harzianum* isolates on *A. alternata* isolates were presented in Table 4. It has been seen that *T. harzianum* restricted the growth of pathogenic *A. alternata* isolates.

As *T. harzianum* isolates are fast growing, they overgrew on all *A. alternata* isolates with an increase in the incubation period. The results revealed that on the 8th day of incubation, *T. harzianum* recorded maximum interaction radial growth inhibition on A3 × T2 against *A. alternata* followed by A3 × T3 and A5 × T3. Moreover, the highest antagonistic was observed on this treatment. While the minimum radial growth inhibition was observed in interaction isolates A2 × T1 followed by A2 × T4 and A2 × T3. Other studies have found different results than the current study in that present study percentage growth inhibition of *A. alternata* by *T. harzianum* was between the range of 53.85- 82.81 % but according to Jat and Agalave (2013), percentage growth inhibition of *A. alternata* by *T. harzianum* was 48.33 %, this might be due to antagonistic ability and virulence of *T. harzianum* and *A. alternata* isolates are different, respectively. In addition, host-pathogen interaction might be different. The present study in agreement with El-Gali (2015), *T. harzianum* showed parasitic behavior against *A. alternata. T. harzianum* grew faster than *A. alternata*.

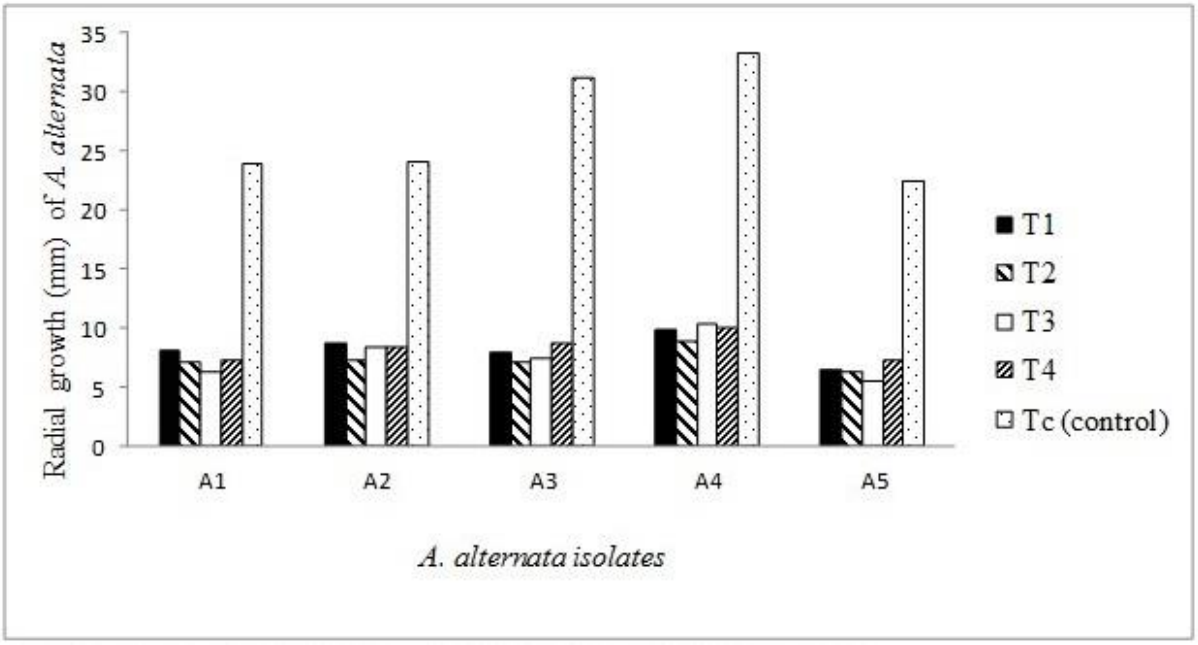

Figure 4. Radial growth of *Alternaria* leaf spot on faba bean as inhibited by *T. harzianum* isolates, 8 days after incubation. LSD (5%) = 1.29, SE ± 0.46 SE= Standard error, LSD=least significant difference

Şekil 4 İkili kültürde 8 günlük inkübasyondan sonar baklada Alternaria yaprak ekesinin T. harzianum izolatları tarafından inhibe edilen radial misel gelişimi. LSD (5%) = 1.29, SE ± 0.46, SE= Standart hata, LSD=En az fark

Table 4. Mean radial growth inhibition of *A. alternata* isolates by *T. harzianum* isolates, 8 days after incubation in dual culture method

Çizelge 4. İkili kültürde 8 günlük inkübasyondan sonraT. harzianum izolatları tarafından A. alternata izolatlarının radyal misel gelişimlerinin engellenme ortalaması

Dual culture *A. alternata x T. harzianum* *İkili kültür* *A. alternata x T. harzianum*		Mean % inhibition of radial growth (PIRG) *Radyal gelişimin % engellenme ortalaması*	No. of days to over growth *A. alternata* colony *A. alternata kolonisini parazitlediği gün*	Antagonistic scale *Antogonistik skala değerleri*
A1	T1	65.94	8	+++
	T2	70.12	8	+++
	T3	73.30	7	+++
	T4	69.19	8	+++
A2	T1	63.23	9	+++
	T2	69.65	8	+++
	T3	64.99	8	+++
	T4	64.80	10	+++
A3	T1	74.41	8	+++
	T2	77.11	7	++++
	T3	76.07	7	++++
	T4	71.91	8	+++
A4	T1	70.34	8	+++
	T2	73.07	8	+++
	T3	68.88	8	+++
	T4	69.50	8	+++
A5	T1	70.91	8	+++
	T2	71.43	7	+++
	T3	75.75	8	++++
	T4	67.30	9	+++
	TC-control	0	-	-
SE ± (A x T)		1.44		
LSD (5%) (A x T)		4.13		

++++ = very high antagonistic activity, +++ = high antagonistic activity, ++ = moderate antagonistic activity, + = low antagonistic activity, IRG= inhibition of radial growth, SE= Square error, LSD=least significant difference, A=*A. alternata,* T=*T. harzianum*

As shown in Table 4 of this study, the interaction of different *T. harzianum* and *A. alternata* isolates exhibited different percentage inhibition of radial growth. As reported by Patale & Mukadam (2011), *Trichoderma* spp. exhibited high antagonistic activities for controlling *Alternaria solani.* Other studies' results by Ambuse et al.(2012) agreeing that *Trichoderma* spp. against leaf spot caused by *Alternaria tenuissima* on faba bean and found 80 % antagonistic activity under *in vitro* condition.

As a reported by Nasraoui (2008), *Alteraria* leaf spot of faba bean may survive in seeds and infected debris as mycelium which, after resuming its activity, can produce conidia able also to start a primary infection. Seed treatment with biocontrol agents like *T. harzianum* could reduce this pathogen. In this study evaluation of different *T. harzianum* isolates against *A. alternaria* showed promising result under *in vitro* condition. Nevertheless, the study should go to field condition where environmental conditions are complex. So that *A. alternaria* pathogen could be managed without affecting the environment.

Molecular identification of A. alternaria and T. harzianum isolates

Both *T. harzianum* and *A. alternata* were identified based on morphological structures as described by Kubicek & Harman (2002) and Simmons (2007), respectively. Sequences obtained from ITS1, 5.8S, and ITS2 region were compared in a BLAST search in GenBank. Results identified as *A. alternata* (identity of 100 % JN618076 and KY365582) and *T. harzianum* (identity of 100 % to KT336515), respectively. The sequences of *A. alternata* isolates (ASF-At1 and ASF-At4) and *T. harzianum* isolates (ASF-T1 and ASF-T4) were deposited in GenBank (accession numbers: KY783936, KY783937, KY783938, and KY783939) for ITS region, respectively and will be released and notified after 3 months.

As a result of this study, *T. harzianum* isolate (T3) showed maximum antagonistic both on dual

culture and antagonistic detached leaf assay. *A. alternata* isolate A1 and A3 were more virulent than the others.

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