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In vitro sensitivity of the tomato early blight disease agent *Alternaria alternata* to some fungicides

Domateste Erken yaprak yanıklığı hastalığı etmeni *Alternaria alternata*'nın bazı fungisitlere karşı *in vitro* duyarlılığı

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ABSTRACT

Early leaf blight is a common fungal disease caused by *Alternaria alternata* (Fr.) Keissler. Various fungicides are used for the chemical control of this disease. As a result of the frequent use of fungicides, decreased susceptibility to the pathogen may be observed. In this study, the susceptibility of early leaf blight (*A. alternata*), a problem in tomato plantations, to azoxystrobin, tebuconazole and mancozeb was determined. Sixty *A. alternata* isolates were obtained from 121 infected plant samples collected from the Antalya, Ankara, Bartın, and Zonguldak provinces in 2013 and 2014. Pathogenicity tests revealed that the disease severity of the isolates varied between 50% and 85% on average. The susceptibility of the isolates to azoxystrobin, tebuconazole, and mancozeb was determined by radial growth tests, and ED_{50} values against azoxystrobin, tebuconazole and mancozeb were determined to be 0.4 ppm, 0.6 ppm and 0.6 ppm, respectively. Polatlı was the most susceptible isolate to the three active substances. Alanya isolate to azoxystrobin (ED50= 452 ppm), Derbent isolate to mancozeb (ED50= 14.45 ppm), Serik and Kayaburnu isolates to tebuconazole (ED50= 33.61 ppm) were determined as the highest resistance isolates. As a result of the study, it was determined that some of the isolates developed resistance to these fungicides

INTRODUCTION

Early leaf blight disease on tomatoes is caused by *Alternaria solani* (Ell. and Mart) Jones and Grout. and *Alternaria alternata* (Fr.) Keissler (Akhtar et al. 2004, Bessadat et al. 2014, Loganathan 2014, Ozan and Maden 2005, Yadav et al. 2020). This disease can cause significant crop losses, especially in greenhouse tomato cultivation and in some potato production areas (Anonymous 2008). As a result of the widespread and excessive use of fungicides and misuse applications to control of disease, the sensitivity of pathogens decreases, and fungicide resistance occurs. When the intended results cannot be obtained from the applications, producers increase both the dose of the fungicide and the

number of applications to achieve the desired success. In this case, the resistance problem deepens, more pesticides are consumed, the cost of control increases, and most importantly, the problems in terms of human health and environmental pollution continue to increase.

Fungus-resistance is the most important problem in the application of site-specific fungicides, now known as modern fungicides, and the market life of members of this class is largely determined by resistance risks (Delen 2008). Resistance can be defined as an irreversible decrease in the susceptibility of an organism to plant protection products used to control that organism as a result of a mutation in its genetic structure. One of the factors that causes resistance is the risk of resistance to the fungicide used. The more specialized the site of action of the fungicide in the fungal cell is, the greater the risk of resistance (Yılmaz et al. 2018).

Fungicide resistance in *Alternaria* spp. has been studied in different plants (cauliflower, cabbage, cultivated rocket, basil, potato etc.) (Ding et al. 2019, Matić et al. 2019). Some Alternaria species have been reported to exhibit resistance to certain fungicides (Farrar et al. 2004). *Alternaria dauci* was found to be resistant to azoxystrobin (Amistar) and trifloxystrobin (Zato) (Surviliene and Dambrauskiene 2006), whereas *A. solani* was reported to be resistant to azoxystrobin (Nuwamanya et al. 2022). In addition, *A. alternata* has been shown to be resistant to pyraclostrobin and boscalid (Avenot et al. 2008).

In a study on the susceptibility of *A. solani* to dithiocarbamate, phthalamide, sulfamide, chlorinated hydrocarbon, dicarboximide, and imidazole fungicides, a decrease in susceptibility to some fungicides was reported, and it was stated that this situation should be checked peryodically (Benlioğlu and Delen 1991).

This study was conducted to determine the susceptibility of *A. alternata* isolates obtained from Ankara, Antalya, Bartın, and Zonguldak provinces, where tomato cultivation was intensively carried out between 2013 and 2016, to azoxystrobin, tebuconazole, and mancozeb under *in vitro* conditions.

MATERIALS AND METHODS

Sample collection and isolation of Alternaria spp.

In 2013 and 2014, 121 symptomatic leaf samples were collected from tomato cultivation areas, including open fields and undercovers, where chemical uses are intense in Ankara, Bartın, Zonguldak, and Antalya. Of the 60 isolates isolated from diseased leaf samples, 15 were obtained from Bartın, 10 from Zonguldak, 17 from Antalya, and 18 from Ankara. For comparison, isolations were also made from samples taken from two tomato fields in the Polatlı and Sincan counties of Ankara, where no plant protection product was applied. The samples were cleaned with tap water in the laboratory. Then, 4-5 mm pieces, including symptomatic and healthy tissue, were cut with a sterile scalpel, kept in 1.5% sodium hypochlorite (NaOCl) for 2 min, and washed in three series of sterile distilled water. After the plant parts were dried on sterile filter papers, they were placed in 9 cm Petri dishes containing 10 ml of potato dextrose agar (PDA; Merck, Darmstadt, Germany) with four plant parts in each petri dish. Petri dishes were incubated in a dark cabinet at 23±1 °C for 7-10 days. After incubation, mycelia from colonies showing similar development as *Alternaria* spp. were transferred to tomato juice agar (Benlioğlu and Delen 1991). Fungal isolates on tomato juice agar for 10-14 days were transferred to 2% water agar (Agar-Agar; Merck, Darmstadt, Germany) to obtain single spore isolates.

Pathogenicity and morphological identification of isolates

Pathogenicity tests were carried out by modifying the method of Ozan and Maden (2005) with 60 isolates obtained in the study. Stalks of tomato leaves were wrapped with cotton soaked in sterile water and placed in blotter medium. A 10 mm diam. agarose discs excised from 7-14-day-old cultures on tomato juice agar were placed on tomato leafstalks and incubated for 1 week at 25–26 °C with three replicates. One week later, the isolates that caused symptoms on the leaves were considered pathogenic. The evaluation was performed on a rating scale 0 to 4 (0= no signs of disease, 1= local and mild yellowing of the leaf, 2= severe yellowing covering the entire leaf surface, 3= lesion formation on the leaf, 4= completely dried leaf) (Ozan and Maden 2005). Disease severity was calculated by using formula given below (Townsend and Heuberger 1943).

Disease Severity (%) = □ (n * V) *100 / x * N

n: number of leaves entering the scale value; V: scale value; x: highest scale value; N: total number of leaves.

As a result of the pathogenicity test, the morphological features of 60 *A. alternata* isolates confirmed to be pathogens were examined under a compound microscope (Leica DM750, Wetzlar, Germany) with a 40x objective, and identification performed according to Elliot (1917), Ellis (1971), Joly (1964) and Gilman (1959).

In vitro susceptibility testing to fungicides

Susceptibility testing for azoxystrobin, tebuconazole, and mancozeb was conducted *in vitro*. For this purpose, fungicides (azoxystrobin, tebuconazole, and mancozeb) were studied at 0 (control), 0.1 ppm, 0.3 ppm, 1 ppm, 3 ppm, 10 ppm, 30 ppm, 100 ppm, 300 ppm, and 1000 ppm doses, as in preliminary studies (Anonymous 2021). PDAs with fungicide added separately for each dose were poured into 9 cm Petri dishes (14 ml per Petri dish). No fungicide was added to the PDA in the Petri dishes used as a control. After the Petri dishes were prepared in four replications for each dose in a laminar cabinet, 5 mm diameter discs from the pathogen culture grown for 7 days on PDA were placed in the middle. The Petri dishes were incubated at 24±1 °C for 7 days in the dark.

The colony diameters were measured in two directions for each individual culture and averaged. Inhibition values were calculated for each replication of the isolates using the following formula: % inhibition value = [(control growth diameter–application growth diameter)/control growth diameter × 100] (Benlioğlu and Delen 1991).

If the ED_{50} value of an isolate ranged between 0 and 1 ppm, it was considered susceptible (S); if it was between 1 and 15 ppm, it was considered reduced susceptibility (RS); if it was between 15 and 100 ppm, it was considered intermediate resistance (IR); and if it exceeded 100 ppm, it was considered resistant (R) (Avenot et al. 2008).

Statistical analysis

After the calculated % inhibition values were converted to arcsine transformation in the MINITAB statistical program, the logarithm of the doses and angle values were written in the MINITAB statistical program, and the regression equation for each isolate for each plant protection product was created separately. The ED50 (50% inhibition of mycelial growth) values of the isolates were determined using regression equations.

RESULTS

A total of 60 isolates of *Alternaria alternata* were obtained from 121 diseased plant samples collected from the Ankara, Bartın, Zonguldak, and Antalya provinces in 2013-2014. The isolates formed colonies ranging from dark green to blackish-brown on PDA media. The conidiophores of the pathogen are single, small, simple or branched, yellowish-brown in colour, and bear one or more conidial scars. The conidia were oval, obpyriform, or elliptical, had 3-5 transverse and several longitudinal septa, with short beak (2-4 µm) and 9-11 x 20-32 µm in size, and were composed of chains of 5-16 (Figure 1).

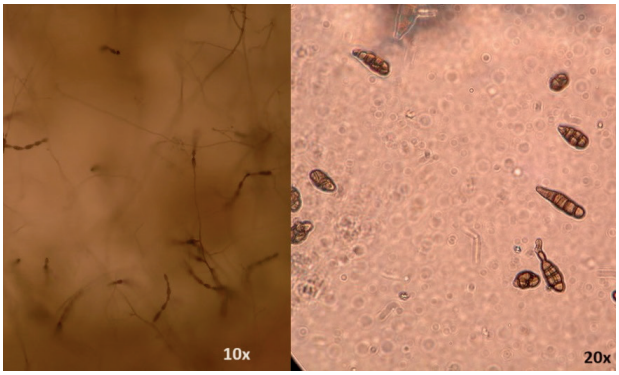

Figure 1. Microscopy images of *Alternaria alternata* conidia

The mean disease severity ranged from 52- 66% for the Bartın isolates, 50–55% for the Zonguldak isolates, 55–75% for the Ankara isolates, and 80–85% for the Antalya isolates. No correlation was found between the susceptibility of *A. alternata* to azoxystrobin, mancozeb, or tebuconazole and disease severity in tomatoes (Table 1).

Reduced sensitivity to azoxystrobin was observed; in all isolates from Bartın, in all except one isolate with moderate resistance from Zonguldak, in all but two sensitive isolates from Polatlı and Malıköy in Ankara, and in six isolates from Antalya. In addition, eight intermediate resistance isolates from Antalya and three high-level resistance isolates obtained from Serik, Kayaburnu and Alanya were identified (Table 1).

All isolates from Beypazarı and Polatlı showed decreased susceptibility to mancozeb, except for two isolates from Ankara, which were sensitive to mancozeb, while no moderate or high-level resistant isolates were obtained. Decreased susceptibility to tebuconazole was detected in all the Bartın and Zonguldak isolates. In Ankara, two isolates from Polatlı and Malıköy were found to be susceptible, whereas the others showed decreased susceptibility. Eight isolates from Antalya developed intermediate resistance, whereas the others showed decreased susceptibility. Isolate number 59 obtained from the Polatlı district of Ankara was susceptible to all three plant protection products, while an isolate obtained from Malıköy was susceptible to azoxystrobin and tebuconazole and showed decreased susceptibility to mancozeb (Table 1).

DISCUSSION

Alternaria solani and *A. alternata* cause leaf blight in tomatoes (Akhtar et al. 2004, Loganathan 2014, Ozan and Maden 2005). Ozan and Maden (2005) reported that *A. alternata* was the causative agent of tomato leaf blight in the Nallıhan, Ayaş and Beypazarı districts of Ankara province

Table 1. Disease severity and sensitivity* to azoxystrobin, mancozeb and tebuconazole of Alternaria alternata isolates obtained different locations

Isolate number	Location	% Disease severity Mean ± SE (Min-Max)	Azoxystrobin ED_{50} (ppm)				Mancozeb ED_{50} (ppm)				Tebuconazole ED_{50} (ppm)			
			S	RS	IR	HR	S	RS	IR	R	S	RS	IR	HR
1	Derbent/BARTIN	60,67±2,6 (58-66)		8,81				12,02				8,92		
2	Derbent/BARTIN	52,67±2,67 (50-58)		8,93				8,07				8,15		
3	Derbent/BARTIN	52,67±2,67 (50-58)		8,52				9,07				9,03		
4	Derbent/BARTIN	63,33±2,67 (58-66)		7,58				4,13				3,44		
5	Derbent/BARTIN	66,00±0,00 (66-66)		8,29				12,69				9,27		
6	Derbent/BARTIN	63,33±2,67 (58-66)		9,90				4,40				4,98		
7	Derbent/BARTIN	60,67±2,67 (58-66)		3,99				3,63				3,43		
8	Derbent/BARTIN	63,33±2,67 (58-66)		4,80				9,70				12,88		
9	Derbent/BARTIN	60,67±2,67 (58-66)		10,42				9,03				9,26		
10	Derbent/BARTIN	58,00±0,00 (58-58)		4,70				7,53				8,15		
11	Derbent/BARTIN	63,33±2,67 (58-66)		5,15				14,45				8,92		
12	Derbent/BARTIN	63,33±2,67 (58-66)		6,51				12,02				3,44		
13	Derbent/BARTIN	58,00±0,00 (58-58)		8,03				9,70				9,03		
14	Derbent/BARTIN	60,67±2,67 (58-66)		6,52				8,07				8,15		
15	Derbent/BARTIN	60,67±2,67 (58-66)		4,46				12,69				8,81		
16	Kayıkçılar/ZONGULDAK	50,00±0,00 (50-50)			17,72			8,50				4,34		
17	Kayıkçılar/ZONGULDAK	55,33±2,67 (50-58)		11,57				7,22				8,15		
18	Kayıkçılar/ZONGULDAK	52,67±2,67 (50-58)		10,17				7,57				8,92		
19	Kayıkçılar/ZONGULDAK	52,67±2,67 (50-58)		14,50				9,03				9,15		
20	Bakacakkadı/ZONGULDAK	52,67±2,67 (50-58)		3,49				10,05				3,44		
21	Bakacakkadı/ZONGULDAK	50,00±0,00 (50-50)		3,42				7,22				8,15		
22	Bakacakkadı/ZONGULDAK	52,67±2,67 (50-58)		9,57				8,68				3,43		
23	Kayıkçılar/ZONGULDAK	50,00±0,00 (50-50)		10,30				7,57				9,03		
24	Bakacakkadı/ZONGULDAK	52,67±2,67 (50-58)		6,84				3,63				8,92		

25	Bakacakkadı/ZONGULDAK	50,00±0,00 (50-50)	10,16			8,50	8,15	
26	Alanya/ANTALYA	80,33±2,67 (75-83)	10,24			7,77	4,93	
27	Serik/ANTALYA	80,33±2,67 (75-83)			239,10	6,69		15,99
28	Serik/ANTALYA	85,67±2,67 (83-91)		28,18		6,33		17,56
29	Serik/ANTALYA	83,00±0,00 (83-83)		33,61		4,87		24,18
30	Serik/ANTALYA	80,33±2,67 (75-83)	14,81			8,81	8,70	
31	Serik/ANTALYA	85,67±2,67 (83-91)		18,28		6,33	12,95	
32	Kumluca/ANTALYA	80,33±2,67 (75-83)	14,57			10,23	11,05	
33	Serik/ANTALYA	80,33±2,67 (75-83)	7,16			6,42	12,92	
34	Serik/ANTALYA	80,33±2,67 (75-83)		33,50		5,56		33,61
35	Turunçova/ANTALYA	80,33±2,67 (75-83)		17,32		5,66		17,64
36	Serik/ANTALYA	80,33±2,67 (75-83)		18,88		6,61		16,90
37	Kayaburnu/ANTALYA	83,00±0,00 (83-83)	14,42			3,63	12,95	
38	Kayaburnu/ANTALYA	80,33±2,67 (75-83)			204,00	4,93		18,94
39	Kayaburnu/ANTALYA	80,33±2,67 (75-83)		54,08		5,52		33,61
40	Kumluca/ANTALYA	83,00±0,00 (83-83)	9,88			9,15	9,57	
41	Alanya/ANTALYA	85,67±2,67 (83-91)			452,00	5,34	7,03	
42	Alanya/ANTALYA	85,67±2,67 (83-91)		64,65		5,52	7,57	
43	Çubuk/ANKARA	69,00±3,00 (66-75)	12,05			4,46	4,30	
44	Çubuk/ANKARA	60,67±2,67 (58-66)	4,20			4,36	4,20	
45	Çubuk/ANKARA	66,00±0,00 (66-66)	10,91			2,40	2,43	
46	Çubuk/ANKARA	60,67±2,67 (58-66)	4,64			1,89	1,85	
47	Çubuk/ANKARA	58,00±0,00 (58-58)	14,57			4,29	4,23	
48	Çubuk/ANKARA	60,67±2,67 (58-66)	8,90			2,40	2,43	
49	Çubuk/ANKARA	60,67±2,67 (58-66)	8,32			4,17	4,14	
50	Ayaş/ANKARA	58,00±0,00 (58-58)	3,93			2,01	2,00	
51	Haymana/ANKARA	58,00±4,62 (50-66)	2,71			2,63	2,56	
52	Haymana/ANKARA	60,67±2,67 (58-66)	6,86			4,18	4,06	

53	Haymana/ANKARA	55,33±2,67 (50-58)		11,76		1,94		1,89
54	Beypazarı/ANKARA	72,00±3,00 (66-75)		2,61	0,73			2,43
55	Akkaya/ANKARA	75,00±0,00 (75-75)		8,50		2,67		3,84
56	Akçakavak/ANKARA	72,00±3,00 (66-75)		4,96		4,31		4,08
57	Dibecik/ANKARA	60,67±2,67 (58-66)		3,63		1,96		2,10
58	Ayaş/ANKARA	60,67±2,67 (58-66)		1,33		1,92		1,89
59	Polatlı/ANKARA	58,00±0,00 (58-58)	0,38		0,59			
60	Malıköy/ANKARA	55,33±2,67 (50-58)	0,50			1,71		

*S=Sensitive (0-1 ppm), RS=Reduced sensitivity (1-15 ppm), IR= Intermediate-resistant (15-100 ppm), HR=Highly resistant (100< ppm)

and had an average prevalence of 12%. The finding that *A. alternata* is a pathogen that causes early leaf blight in tomatoes is consistent with the results of Ozan and Maden (2004, 2005), Gazozcuzade (2010), Mutlu and Üstüner (2017).

Although there is no record of resistance of *A. alternata* to azoxystrobin in tomato, it has been reported in pistachio (Avenot and Michallides 2007, Ma et al. 2003), apple (Ishii 2008), citrus fruit (Mondal et al. 2009), potato (Fairchild et al. 2013) and leafy vegetable plants (Matić et al. 2019).

The susceptibility to mancozeb was reduced in 96.6% of the isolates, with ED_{50} values between 1 and 15 ppm. There was no intermediate or high-level resistance to mancozeb, and the decrease in susceptibility was less than that of azoxystrobin. Benlioğlu and Delen (1991) reported that the ED_{50} values of mancozeb for 60 *A. solani* isolates ranged from 3-300 µg/l. In that study, *A. alternata* appeared to be more sensitive to mancozeb than *A. solani*. He et al. (2017) reported no resistance of *A. alternata* to mancozeb in China, as in this study.

The ED_{50} values of tebuconazole for 86.6% of the isolates were between 1 and 15 ppm. The decrease in sensitivity to tebuconazole was lower than that to azoxystrobin, as was the case for mancozeb. Moreover, resistance to tebuconazole, a fungicide of the azole group with demethylation inhibiting activity, has been reported to be moderate in many fungi of the ascomycete group, such as black spot (*Venturia ineaqualis*), gray mold (*Botrytis cinerea*), monilia (*Monilinia fructicola*), powdery mildew (*Erysiphe graminis*) (Anonymous 2022). A total of 13.3% of the isolates in the study were found to be moderately resistant to tebuconazole. Malandrakis et al. (2015) reported that 42 *A. alternata* isolates obtained from tomato fields and greenhouses in southern Greece, were resistant to mancozeb (ED_{50} = 2.34 - >100 µg/l), and the ED_{50} values of tebuconazole ranged from 0.43 - 20 µg/l.

In general, the resistance rates to azoxystrobin, mancozeb, and tebuconazole in isolates from greenhouses in Antalya, Bartın, and Zonguldak provinces were greater than those in isolates from open-field tomato-growing areas in Ankara Province. This is thought to be because disease is more common in greenhouses than in open fields, and because plant protection products are applied more often in greenhouses.

It is important to recognize and monitor resistance related to resistance in *A. alternata* and develop resistance management strategies. In this study, the susceptibility of *A. alternata* to azoxystrobin, tebuconazole and mancozeb was determined in tomatoes from Antalya, Bartın, Zonguldak and Ankara provinces. Fungicide-resistant, less susceptible and sensitive strains of *A. alternata* were detected in our country. The results of this study showed that since *A. alternata* strains vary in their sensitivity to fungicides depending on the region, fungicide recommendations may differ according to the areas where resistance occurs rather than countrywide.

It is important to determine the sensitivity of plant pathogenic fungi to plant protection products, and studies should be conducted regularly to determine the decrease in sensitivity in advance and to establish strategies for resistance management.

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presented as a poster presentation titled "Determination of the susceptibility of Early Leaf Blight in Tomatoes (*Alternaria* spp.) to Azoxystrobin" at VI. The Plant Protection Congress held in Konya on 5-8 September 2016. Another part of the study was presented as a poster at the 3rd Agriculture and Food Congress of the Central Anatolia Region held in Sivas on October 26-28, 2017, titled "Determination of resistance to azoxystrobin and mancozeb in tomato early blight (*Alternaria alternata*)."

Author's Contributions

Authors declare the contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

ÖZET

Domateste erken yaprak yanıklığı, *Alternaria alternata* (Fr.) Keissler)'nın neden olduğu yaygın bir fungal hastalıktır. Hastalığın kimyasal mücadelesinde çeşitli fungisitler kullanılmaktadır. Fungisitlerin çok sık kullanımı sonucunda patojende duyarlılık azalışı görülebilmektedir. Bu çalışmada, domates ekiliş alanlarında sorun olan erken yaprak yanıklığı hastalığının, azoxystrobin, tebuconazole ve mancozeb etkili maddelerine karşı duyarlılığı belirlenmiştir. Antalya, Ankara, Bartın ve Zonguldak illerinden 2013 ve 2014 yıllarında toplanan 121 enfekteli bitki örneğinden yapılan izolasyonlar sonucunda 60 adet *Alternaria alternata* izolatı elde edilmiştir. Patojenisite testleri sonucunda izolatların hastalık şiddetlerinin ortalama %50-85 arasında değişiklik gösterdiği tespit edilmiştir. İzolatların azoxystrobin, tebuconazole ve mancozeb'e duyarlılık düzeyleri radyal gelişme testi ile belirlenmiş ve azoxystrobin, tebuconazole ve mancozeb'e karşı ED_{50} değerleri sırasıyla 0.4 ppm, 0.6 ppm ve 0.6 ppm olarak belirlenmiştir. Polatlı izolatı üç etken maddeye karşı en duyarlı izolat olmuştur. Alanya izolatı azoxystrobine karşı (ED_{50}= 452 ppm), Derbent izolatı mancozeb'e karşı (ED_{50}= 14.45 ppm) , Serik ve Kayaburnu izolatları tebuconazole'e karşı (ED_{50}= 33.61 ppm) en yüksek dirence sahip izolatlar olarak belirlenmiştir. Çalışma sonucunda izolatlardan bazılarının bu fungisitlere karşı direnç geliştirdiği tespit edilmiştir.

Anahtar kelimeler: *Alternaria alternata*, azoxystrobin, tebuconazole, mancozeb, duyarlılık

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