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Araştırma Makalesi

In vitro Suppression Effects of Rhizobacteria against *Pseudomonas syringae* pv. *tomato* the Agent of Bacterial Speck Disease of Tomato

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Abstract

Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* is significant pathogen of tomato causing economical losses in yield and quality. Bactericides for the control of such plant diseases are insufficient in some cases. Plant Promote Growth Promoting Rhizobacteria is recently considered as a potential alternative approach to the control of plant pathogens. Here, a collection of bacteria (thirty-eight in total) obtained from rhizospheric soil of different plants were examined for *in vitro* antagonistic effects on the *Pseudomonas syringae* pv. *tomato* growth on agar plates through dual culture assay. Seven isolates out of thirty-eight exhibited effects with varying ranges of inhibition zones from 0.1 to 0.867 cm on the growth of pathogen isolate obtained from Çumra District of Konya. Based on MALDI Biotyper classification results, all of the rhizobacterial isolates showing *in vitro* antagonistic actions were identified as *Bacillus* genera, excluding an isolate that was determined as belonged to *Paenibacillus* genera.

Key words: Antagonism, Bacteria, Disease, *In vitro*, Rhizobacteria, Tomato

Domates Bakteriyel Benek Hastalığı Etmeni *Pseudomonas syringae* pv. *tomato*'nun Üzerinde Rhizobakterilerin *In vitro'* da Baskılayıcı Etkileri

Özet

Pseudomonas syringae pv. *tomato'* nun neden olduğu bakteriyel benek domateste verim ve kalitede ekonomik olarak kayıplara neden olan önemli bir patojendir. Bazı durumlarda bu tür bitki hastalıklarının kontrolünde bakterisit uygulamaları yetersiz kalmaktadır. Son zamanlarda bitki patojenlerinin kontrolünde Bitki Büyümesini Destekleyen Rizobakteriler potansiyel bir alternatif yaklaşım olarak görülmektedir. Bu çalışmada, farklı bitkilerin rizosfer toprağından elde edilen bir bakteri koleksiyonunun (otuz sekiz adet), patojen bakterinin besi ortamında büyümesi üzerinde *in vitro* antagonistik etkileri ikili kültür yöntemi ile belirlenmiştir. Çalışmada kullanılan *Pseudomonas syringae* pv. *tomato* izolatı Konya-Çumra'da açık alan üretimi yapılan domates bitkilerinden izole edilmiştir. Otuz sekiz rizobakteriyel izolattan yedi tanesi, besi üzerinde 0.1 ila 0.867 cm arasında değişen inhibisyon zonları oluşturarak *Pseudomonas syringae* pv. *tomato*' ya karşı etkinlik göstermişlerdir. MALDI Biotyper sınıflandırma sonuçlarına göre, *Paenibacillus* cinsine ait olduğu belirlenen bir izolat dışında *in vitro*' da antagonistik etki gösteren rizobakteriyel izolatların tümünün *Bacillus* cinsine olduğu tespit edilmiştir.

Anahtar kelimeler: Antagonizm, Bakteri, Domates, Hastalık, *In vitro*, Rizobakteri

Introduction

Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (Pst), is a significant disease of tomato influencing economically the yield and quality worldwide including Turkey (Çınar, 1977; Wilson et al., 2002). The pathogen can maintain saprophytically in plant waste, soil and as well as on leaf surfaces. In spring, when the weather is cool and humid which favors the bacteria reproduce, the pathogen attack and causes bacterial speck disease on tomato plants. The disease symptoms can be defined with brownish-black spots surrounded by a yellow margin on leaf and ripe fruit as well as dark specks on green fruit (Preston, 2000). Particularly, in early infections, the yield could be significantly decreased due to a decline in the photosynthetic capacity of infected plant leaves. The speckled symptoms on fruit lead to reducing the market value of tomato fruits. For control bacterial speck caused by Pst, copper-based bactericides have been used, however, prolonged use of such compounds has led to copper-resistance development and hereby loses in compound effectiveness have been reported in several studies (Silva and Lopes, 1995a; Alexander et al., 1999). Also, the antibiotic resistance in the pathogen populations also has been reported by Silva and Lopes (1995b). The resistant varieties in some cases are insufficient for controlling the pathogen since the Pst has different races (Oldroyd and Staskawicz, 1998). Genetically modified tomato plants that possessive Pto/Prf genes expressions might be resistant to the races, however, it is a paradox whether these transgenic lines would be commercialized (Oldroyd and Staskawicz, 1998). The rhizosphere, the soil around the plant roots, hosts numerous of microorganisms including beneficial bacteria and has been focusing attention in recent years (Nautiyal and DasGupta, 2007). Rhizobacteria that promote plant development were first entitled as “Plant Growth-Promoting Rhizobacteria” by Kloepper and Schroth (1978). Plant growth-promoting rhizobacteria (PGPRs) can survive in the rhizosphere, the phyllosphere, or in plants. The PGPRs can boost plant growth by direct and indirect mechanisms based on the way of their impact. Indirect mechanisms involve combating the pathogen through the production of antibiotics and enzymes and the action of siderophores, Inducing Systemic Resistance (ISR), and exo-polysaccharides production. Direct mechanisms are as follows; making the natural nutrition source ready to use for plants through nitrogen fixation, solubilization of phosphorus, potassium, and iron; production of siderophores; producing phytohormones namely, auxins, cytokinins and gibberellins (Ahemad and Kibret, 2014) or by producing an enzyme, 1-aminocyclopropane-1-carboxylate (ACC)-deaminase that hinders the over-secretion of ethylene in plant which is called as “plant stress ethylene” when the plant is exposed to biotic and abiotic stresses such as flooding, high temperature, organic and inorganic residual pollutants, phytopathogens, drought or salinity (Glick, 2014). Bacteria from the *Bacillus* group are microorganisms are highly adaptable to different habitats. The isolates of the genera primarily are known with the ability to produce enzymes such as proteases glucanases, cellulases, chitinases, and lipases that have a role to block the pathogen infection in the plant by demolishing the fungal and bacterial cell wall (Stein, 2005). Among the recent studies relevant with PGPRs, *Paenibacillus polymyxa* (synonym, *Bacillus polymyxa*) has been getting attention as a bio-control agent (Raza et al., 2008). *Paenibacillus* which separated from *Bacillus* in 1993 can enhance the plant growth by direct (e.g. production of antibiotic, hydrolytic enzymes, some other metabolites which kill directly pathogens) and indirect mechanisms (stimulating the protein secretions related to disease resistance and producing plant hormone and converting the nutrients available in soil to uptake by plants (Çakmakçı et al., 2006; Weselowski et al., 2016). The broad and prolonged application of chemical compounds in agriculture has been lead to danger for living organisms including humans and disruption of natural balance as well as ineffectiveness control based on resistant formation. Although biological control studies have been carried out for long years, more studies are still needed to find out novel eligible bio-agent in pathogen control. This study was mainly focused on determining the antagonistic properties of territorial rhizobacterial isolates on the growth of Pst *in vitro* which is the beginning step in the screening of a novel biocontrol agent.

Material and Method

Isolation and identification of the pathogen

The study was conducted in 2019. Tomato plant samples showing typical disease symptoms were collected from the open tomato production area in Çumra, Turkey. A small piece of infected tomato leaf containing infected and healthy parts (shown in Figure 1) was suspended by macerating with a small amount of sterilized distilled water (3-5 ml) in a mortar.

Figure 1. Tomato leaves showing bacterial speck symptoms collected from the open production field in Çumra.

Loopfulls of the suspension was inoculated by streaking on King's B agar medium (Merck, Germany) which allows separating the bacteria produce fluorescent pigment. The petri dishes were kept in an incubator adjusted to 25 ºC for 48 h. The colonies grown intensively were purified on King's B medium. Tomato seedlings (*Lycopersicon esculentum* cv. H-2274) were spray inoculated with suspensions prepared with purified bacterial isolates at the density of ~1x10^8 CFU ml^{-1}. Negative control plants were treated just with sterile distilled water by spraying. For each isolate, three tomato plants were used. Inoculated plants were kept in a climate room at 25 ºC and 70% humidity for 15 days to enhance the disease symptom formation. Bacteria were re-isolated from the leaf displaying bacterial speck symptom. Re-isolated bacterium was identified by a series of biochemical tests as gram reaction using the KOH method, fluorescent pigmentation, formation of levan colonies, reaction of oxidase, pectinase activity as inducing soft rot formation on potato slices, activity of arginine dihydrolase, and HR (Hypersentsivity) activity on tobacco leaves (Lelliot and Stead, 1987). The re-isolate identification was additionally confirmed by the MALDI Biotyper classification system in Plant Clinic Service at Mustafa Kemal University through service purchase.

Rhizobacterial isolates

Thirty-eight isolates were selected randomly from Rhizobacterial Culture Collection (Gul IMRIZ, Dicle University). The isolates were obtained from rhizosphere of different plants in Konya districts in previous study (Imriz et al., 2020). The properties belonging to the isolates including the codes, origin of plants, locations are given in Table 1.

Table 1. Codes, locations and origin plants of rhizobacterial isolates involved in the antagonistic activity assay.

Isolate code	Location / district	Origin plant	Isolate code	Location / district	Origin plant
GP23*	Selçuklu	Wheat	GP412	Ilgın	Wheat
GP28*	Çumra	Nut Grass	GP4	Güneysınır	Wheat
GP110*	Selçuklu	Wheat	GP2	Güneysınır	Wheat
GP211*	Selçuklu	Wheat	GP416	Çumra	Wheat
GP10*	Ilgın	Wheat	GP15	Ilgın	Wheat
GP9*	Seydişehir	Wheat	GP39	Kadınhanı	Wheat
GP7*	Selçuklu	Barley	GP1	Güneysınır	Wheat
GP11	Kadınhanı	Barley	GP11a	Kadınhanı	Barley
GP196	Cihanbeyli	Barley	GP29	Ilgın	Wheat
GP122	Kadınhanı	Wheat	GP16	Güneysınır	Wheat
GP328	Çumra	Nut Grass	GP25	Selçuklu	Wheat
GP129	Sarayönü	Wheat	GP13	Güneysınır	Wheat
GP8	Güneysınır	Wheat	GP20	Selçuklu	Wheat
GP350	Karapınar	Wheat	GP27	Selçuklu	Wheat
GP3	Yalıhöyük	Wheat	GP124	Kadınhanı	Wheat
GP128	Sarayönü	Wheat	GP127	Sarayönü	Wheat
GP19	Selçuklu	Wheat	GP228	Yalıhöyük	Wheat
GP14	Akşehir	Wheat	GP130	Selçuklu	Wheat
GP6	Seydişehir	Wheat	GP125	Selçuklu	Wheat

Rhizobacterial isolates and antagonistic activities

In vitro antibacterial activity of all isolates (Table 1) against Pst was screened by using the dual-culture technique (Weselowski et al., 2016) on Nutrient Agar (NA) (Merck, Germany). A hundred µl of suspension of each pathogen isolate at ~1x10^8 CFU ml^{-1} was spread on the NA plates by using a glass spatula. The treated plates were kept for an hour at room temperature allowed bacteria to be settled on agar medium, and those were inoculated with pure rhizobacterial isolates, which were grown for 48 hours, in three points with an equal distance to each other on agar medium by a loopful. Experiment was settled up according to randomized plot design. Three replicates were applied for each bacterial isolate. Only sterile distilled water was used instead of bacterial culture for the control plates. The experiment was conducted in sterile conditions entirely. The treated petri dishes were incubated at 25 ± 2°C for 7 days, when the control petri dishes were completely covered by pathogen. The inhibition zones were measured in cm. The isolates showed *in vitro* inhibition activity were identified with MALDI Biotyper classification system.

Statistical data analysis

One-way analysis of variance (ANOVA) was applied to statistically analyze the inhibition zone dimensions resulting from interactions between rhizobacterial isolates and pathogen by using software SPSS. The means were separated by the Tukey HSD test ($p < 0.05$).

Results and Discussion

Isolation, diagnosis and re-isolation of the pathogen

As a result of isolations on King's B medium, fluorescent bacteria were obtained from tomato plant leaves showing typical bacterial speck symptoms. The isolates coded as ÇD displayed similar symptoms as bacterial specks on artificially inoculated tomato leaves. Isolations were made from the leaves and re-isolate was identified as *Pseudomonas syringae* pv. *tomato* by traditional bacteriological test methods as well as the MALDI Biotyper classification system. The results of traditional bacteriological tests and MALDI-Biotyper characterization for pathogen isolate are shown in Table2.

Antagonistic activities of rhizobacterial isolates

This study exhibited that dual culture assay as a suitable method for preliminary screening of antagonistic activity and made possible the initial screen of potential bio-control rhizobacterial isolates against pathogenic bacteria Pst in tomato crop. Among thirty-eight, seven rhizobacterial isolates (namely; GP23, GP28, GP110, GP211, GP10, GP9, and GP7) displayed an ability to suppress the colonial growth of Pst on NA medium, with inhibition zones ranged from 0.1 to 0.867 cm (Table 2).

The highest inhibiting zone was formed by GP23 coded isolate with inhibition zone in size of 0.867 cm (Figure 2) whereas the minimum was obtained from GP7 (0.1 cm). The MALDI Biotyper results belonging to all isolates which showed *in vitro* suppression on Pst are summarised in Table 4.

According to the results, the isolates coded GP23, GP28, GP110, GP211, GP10, GP9, and GP7 were determined as *Bacillus safensis, Paenibacillus polymyxa, Bacillus subtilis, Bacillus atrophaeus, Bacillus mojavensis, Bacillus pumilus,* and *Bacillus safensis*, respectively (Table 4). The remaining thirty-one rhizobacterial isolates showed no effect against Pst (Table 3). There were statistically significant differences among the inhibition zone sizes of the isolates (F = 61,276, df = 39, P = 0.001).

Table 2. Results of Bio-chemical tests and MALDI Biotyper of pathogen isolated from tomato leaf.

Pathogen isolate code	**Bio-chemical characters**							**MALDI-Biotyper characterization**
	Levan type colony	Oxidase reaction	Potato rot	Arginin dehydrolase	Tobacco hyper sensitive reaction	Gram Reaction	Growth on King's B	Score value
ÇD	+	-	-	-	+	-	Fluorescent	2.598

Table3. *In vitro* antagonistic properties of the rhizobacterial isolates.

Isolate code	Means of inhibition zones (cm)	Std. deviation	% Efficiency	Isolate code	Means of inhibition zones (cm)	Std. deviation	% Efficiency
GP23*	**0,867** [f]	**±0.115**	**86,70**	GP412	0,000 [a]	±0.000	0,000
GP28*	**0,367** [e]	**±0.155**	**36,70**	GP4	0,000 [a]	±0.000	0,000
GP211*	**0,333** [de]	**±0.152**	**33,33**	GP2	0,000 [a]	±0.000	0,000
GP110*	**0,300** [cd]	**±0.000**	**30,00**	GP416	0,000 [a]	±0.000	0,000
GP10*	**0,200** [bc]	**±0.000**	**20,00**	GP15	0,000 [a]	±0.000	0,000
GP9*	**0,200** [bc]	**±0.000**	**20,00**	GP39	0,000 [a]	±0.000	0,000
GP7*	**0,100** [ab]	**±0.000**	**10,00**	GP1	0,000 [a]	±0.000	0,000
Control (Pst)	**0,000** [a]	**±0.000**	**0,000**	GP11a	0,000 [a]	±0.000	0,000
GP11	0,000 [a]	±0.000	0,000	GP29	0,000 [a]	±0.000	0,000
GP196	0,000 [a]	±0.000	0,000	GP16	0,000 [a]	±0.000	0,000
GP122	0,000 [a]	±0.000	0,000	GP25	0,000 [a]	±0.000	0,000
GP328	0,000 [a]	±0.000	0,000	GP13	0,000 [a]	±0.000	0,000
GP129	0,000 [a]	±0.000	0,000	GP20	0,000 [a]	±0.000	0,000
GP8	0,000 [a]	±0.000	0,000	GP27	0,000 [a]	±0.000	0,000
GP350	0,000 [a]	±0.000	0,000	GP124	0,000 [a]	±0.000	0,000
GP3	0,000 [a]	±0.000	0,000	GP127	0,000 [a]	±0.000	0,000
GP128	0,000 [a]	±0.000	0,000	GP228	0,000 [a]	±0.000	0,000
GP19	0,000 [a]	±0.000	0,000	GP130	0,000 [a]	±0.000	0,000
GP14	0,000 [a]	±0.000	0,000	GP125	0,000 [a]	±0.000	0,000
GP6	0,000 [a]	±0.000	0,000				

*The isolates have shown suppression on Pst growth on NA medium
Mean values followed by different superscript in a column are significantly different ($p<0.05$**).**

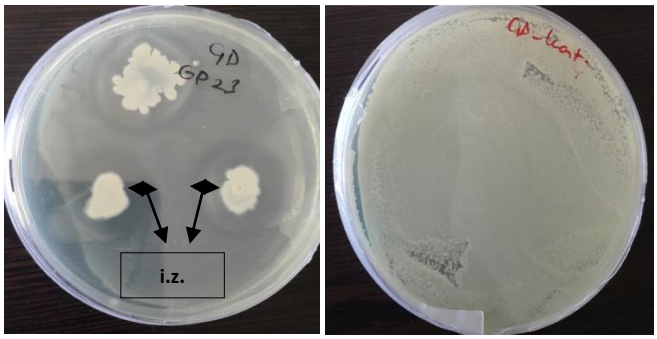

Figure 2. The finest inhibition zones (i.z.) with 0,867 in cm provided by isolate GP23 which identified as *Bacillus safensis* on the left and control petri completely covered by Pst on the right.

Table 4. MALDI Results for the rhizobacterial isolates which showed *in vitro* antagonistic activity.

Isolate Code	MALDI Scores	MALDI Biotyper Results
GP23	1.726	*Bacillus safensis*
GP28	2.101	*Paenibacillus polymyxa*
GP110	1.762	*Bacillus subtilis*
GP211	2.119	*Bacillus atrophaeus*
GP10	1.987	*Bacillus mojavensis*
GP9	1.712	*Bacillus pumilus*
GP7	1.853	*Bacillus safensis*

The results of this study are consistent with several studies that reported the bacteria in the *Bacillus* and *Paenibacillus* groups to have multifaceted properties, including antagonistic effects (Glick 2014; Weselowski et al., 2016). In this study, the presence of *Paenibacillus* and *Bacillus* was proved in the area that they had been collected from the agricultural production area in Konya-Karaman provinces (as seen in Table 1-4) where the drought is subjected. These bacteria are known as gram-positive and have the ability to produce endospores that endure even under unfavorable environmental conditions (Logan et al., 2007). The data in this study displayed isolates belong to *Paenibacillus* and *Bacillus* genera, that are gram-positive, have antagonistic potential for Pst and these gram-positive rhizobacterial isolates have ability to survive despite arid conditions. In the present study, the majority of successful isolates found as *in vitro* suppressor of Pst were identified as *Bacillus* (6 isolates). The bio-control potential of *Bacillus* isolates has been shown by numerous studies (Milijašević-Marčić and Todorović, 2017). Milijašević-Marčić et al., (2018) reported a study on the *in vitro* actions of *Bacillus* spp. against bacterial pathogens of tomato and pepper plants. In their study, two *B. subtilis* strains coded as B-338 and B-348 provided the highest inhibition zone on *C. michiganensis* subsp. *michiganensis* whereas the best inhibition was obtained by the treatment of *B. subtilis* strain B-319 for *X. vesicatoria*. Mirik et al., (2008) determined the *in vitro* antagonistic activities of *Bacillus* strains. The researchers observed the antagonistic suppression activity with 3 *Bacillus* strains namely; M1-3, M3-1, and against *X. axonopodis* pv. *vesicatoria*. Of the tested isolates, an isolate coded as GP28 was identified as *Paenibacillus polymxa* (Table 4). This bacterial species has been demonstrated in previous studies for antagonism to bacterial pathogens *Xanthomonas campestris* causing agent for bacterial blight, cankers and leaf spots (Weselowski et al., 2016). On the other hand, Kim et al., (2009) indicated not all *P. polymyxa* strains have antagonistic features to pathogenic organisms, therewithal, the diversity was observed among strains in their gene clusters that encode antimicrobial peptides (Xie et al., 2016; Eastman et al., 2014). This study is the beginning of identifying potential biocontrol agents for control of Pst in tomato. Results obtained in the study are giving promise for biological control possibilities of the disease with potential rhizobacterial isolates as bio-control agents in tomato. Nevertheless, control of seed-borne pathogens, such as Pst would be initiated when the seed first being placed in soil. So that, the protection of plants against such pathogen attacks would be achieved by either seed coating or soil application with a bio-control agent which is proven for success in suppressing the pathogen. The next action has to be surely determining the efficiencies of isolates on the plants *in vivo* conditions as well as natural conditions with field experiments. Additionally, the survival of isolates in/on seed and in the soil should be revealed by detailed studies to achieve significant milestones for developing an effective bio-control agent.

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