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Biological Control of *Rhizoctonia Solani* Kühn. with Rhizobacteria Isolated from Different Soiland *Calligonum Polygonoides* L. Subsp. *Comosum* (L'hér.)

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ABSTRACT

In this study, the biological control activities of the bacteria strains, which have been isolated from the volcanic, sandy, salty soils and Ebu Cehil plant *Calligonum polygonoides* L. subsp. *comosum* (L'Hér.) were investigated against isolates of *Rhizoctonia solani* causing root rot in beans. As the result of the isolation, 83 strains were obtained and the bacteria were identified by biochemical tests and fatty acid methyl esterase analyses. In the *in vitro* conditions, 20 strains from among 83 bacteria strains were found to be effective and it was determined that these strains inhibited the mycelial growth by forming a prevention zone ranging between 0.1 cm and 2.4 cm. The prevention rate of bacteria strains against pathogen isolates was found to range between 50%-66% maximum. In the *in vivo* conditions, the bacteria strains were found to successfully inhibit the disease development compared to the control group. *Bacillus thuringiensis israelensis* HV43, *Bacillus subtilis* HV34, *Bacillus cereus* GC subgroup A HT21 and *Bacillus subtilis* HT30 strains were determined to prevent the disease incidence with an effectiveness of 100%.

Keywords: Bean, *Rhizoctonia solani*, Biological control, *Calligonum polygonoides* (L.'Hér)

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INTRODUCTION

Dry beans rank first among edible legumes in the world with 29 million ha cultivation area and 27 million tons of production (FAO, 2018). In Turkey, it ranks third after chickpea and lentil with 848,045 da cultivation area and 220,000 tons of production (TUIK, 2019). However, it is reported that many soil-borne fungal disease agents cause significant losses in bean, both in the seedling stage and in other growth stage of plants. Among these pathogens, *Rhizoctonia solani*, *Fusarium* spp., *Macrophomina phaseolina* (Tassi) Goidanich, *Pythium ultimum* Trow, *Sclerotinia sclerotiorum* (Lib.) de Bary and *Sclerotium rolfsii* Sacc. are reported to cause serious problems in the product (Willetts and Wong, 1980; Sippell and Hall, 1982; Dixon, 1984; Bruehl, 1987; Hall, 1991; Vural, 2008).

Among the above-mentioned disease agents, *R. solani* is an important fungal pathogen causing serious losses in yield and quality in many plant groups (Bakalı and Martin, 2006; Aydın, 2008; Lehtonen, 2008; Jehtonen, 2009; Kılıçoğlu and Özkoç, 2010). The agent is a fungus of both soil and seed origin and has a strong pathogenic feature, with its rapid growth on nutrients, colonization on the surface of the roots, and its ability to rapidly invade epidermal cells in the infected area (Carling et al., 2002; Aydın et al., 2011; Çapar, 2012).

Difficulties are experienced in the control of *R. solani,* due to the propagating material and soil origin and easy transmission with seeds (Çapar, 2012). In addition, the very large host environment of the pathogen and its ability to survive in the form of mycelium for a long time makes controlling the disease even more difficult (Aydın et al., 2011). In order to slow down the development of *R. solani* and prevent emergence of the disease the following year, it is recommended to destroy the plant and plant residues with the disease, to use certified production material, solarization, to select resistant varieties, and to employ crop rotation applications with plants such as wheat and corn (Yanar et al., 2005; Larkin and Honeycutt, 2006; Aydın, 2008). However, the rapid development of the pathogen in the soil, its ability to survive on organic residues for a very long time, and the ability to infect by developing as a vegetative mycelium in the presence of suitable hosts limit the effectiveness of these control methods (Turhan, 2010). For this reason, studies have been carried out in recent years on some control programs to be applied against this disease both in the propagative material and in the soil. It has been reported that the disease can be suppressed by applying some chemicals to tubers / seeds and soil (Errampalli and Johnston, 2001). However, in case of intense inoculum in the soil, it was determined that the applied chemicals could not control the disease sufficiently (Aydın, 2008). Moreover, widespread and improper use of fungicides in the control of the disease causes the pesticide residues in agricultural products to pass to living creatures through nutrition, as well as to pass some of the pesticides to the ground water and the sea through soil, air pollution, to increase disease agents and pests in nature, to destruct predator and antagonist microflora, and to form new resistant pathogen strains by changing the pathogen genes (Delen, 1991; Bora and Özaktan, 1998; Compant et al., 2005; Berg, 2007). Due to such reasons, the insufficiency of methods for controlling soil-borne pathogens and the increased sensitivity on environmental health throughout the world led researchers to work in the field of biological control and to discover new biological control agents within the framework of ecological agriculture. In recent studies, rhizobacteria that promote plant growth are used both as a growth stimulant and as a biological control agent in plants, and very successful results are obtained

(Nandakumar et al., 2001; Walsh et al., 2001; Kageyama and Nelson, 2003; Ongena et al., 2004; Ryu et al., 2004; Jehtonen, 2009). Rhizobacteria come into prominence due to such properties as their ability to change the microflora composition in plant roots, making the plant resistant to various stress factors, being effective on growth hormones (indole acetic acid, ethylene), increasing nutrient intake (siderophore production, binding of free nitrogen in the air or in the soil, ensuring the intake of phosphorus and other nutrients), promoting root development, and accordingly, increasing plant growth and development (Van Loon, 1997; Weller, 1998; Van Loon et al., 1998; Metraux, 2001; Asghar et al., 2002; Vessey, 2003). On the other hand, these microorganisms draw attention with their successful results in disease control by using one or more of the biological control mechanisms such as antibiosis (chitinase, β-1,3-glucanase, antibiotic and cyanide production), competition, hyperparasitism, induced resistance and cross protection (Bora et al., 1994; Smith and Read, 1997; Bora and Özaktan, 1998; Cattelan et al., 1999; Çakmakçı, 2005; Çetinkaya Yıldız, 2007; Genç, 2012).

In this study, possibilities of biological control of *R. solani*, which causes a great economic loss by causing root diseases in many plants including beans, are investigated. For this purpose, the bacteria obtained from the rhizosphere of *Calligonum polygonoides* L. subsp. *comosum* (L'Hér.), which is endemic to Aralik district of Igdir province and which helps to prevent soil erosion of volcanic, sandy and saline soils, were identified with biochemical tests and according to fatty acid profiles. Then, the properties of bacterial strains to fix nitrogen and dissolve phosphorus and potassium were determined and their effectiveness as a candidate for biological control against *R. solani* was evaluated.

MATERIAL AND METHODS

Material

Plant, soil and pathogen material used in the study

Sugar bean variety was used to evaluate the antagonistic effect of bacteria strains isolated against *R. solani* isolates (Rs-pat, B-227 and B-1). Volcanic soil obtained from Taşburun village of Karakoyunlu district, saline soil obtained from Yukarı Çamurlu village of Aralik district, and roots of *Calligonum polygonoides* L. subsp. *comosum* (L'Hér.) endemic to Aralik district and the loamy soil covering these roots were used as isolation material in obtaining the bacteria strains.

Method

Isolation of bacteria strains

For the isolation of bacterial strains from the samples taken, 10 g of soil was weighed and placed in a sterile flask with a volume of 250-300 ml. After that, 90 ml of water was added on the soil and then stirred for 30 minutes. Suspensions were diluted 6 times at 1:10 ratio. 100 µl were taken from the last 3 dilutions and and planted in nutrient agar (NA). The plates were incubated at 27 ^{o}C for 3-4 days. At the end of the incubation period, colonies of different colors and shapes were selected (Saygılı et al., 2006).

Identification of bacterial strains

Characteristics of the bacterial strains were determined by gram reaction test, colony colour onnutrient agar medium, catalase test, oxidase reaction, amylase test, levan colony formation on sucrose nutrient agar and hypersensitive reaction on tobacco leaves. The strains were identified by fatty acid methyl ester (FAME) analysis. Fame profiles were obtained by running samples on a Hewlett Packard Agilent GC 6890 GC fitted with amicroprocessor containing the Sherlock Microbial Identification System (MIDI) Software (V.A. 06. 03). The FAME profiles were compared with the TSBA40 aerobe library (Caesar-TonThat et al., 2007).

Selection of nitrogen fixing bacteria strains

Bacterial strains were tested for nitrogen fixing ability using N-Free Solid Malate-Sucrose (Sucrose 10gl^{-1}, L-Malic Acid 5g, $MgSO_4$, H_2O, 0,2g; $FeCl_3$, 0,01 g; NaCl, 0,1 g; $CaCl_2$ $2H_2O$, K_2HPO_4, 0,1g, KH_2PO_4 0,4 g; Na_2MoO_4. H_2O, Agar 18g, pH: 7,2). Strains were streaked on N-free solid malate-sucrose agar. All cultures were incubated for 7 days at 27°C. After incubation, bacterial growth was evaluated as N_2-fixing positive bacteria (Döbereiner, 1989).

Selection of phosphate-solubilizing bacterial strains

The P-solubilizing bacterial strains were screened using National Botanical Research Institutes's Phosphate Growth Medium (NBRIP-BPB; glukose 20g, $Ca_3(PO_4)_2$ 10g, $MgCl_2.6H_2O$ 5g, $MgSO_4.7H_2O$ 0,25g, KCl 0,2g, $(NH_4)_2SO_4$ 0,1g, Bromphenol blue 0,025g, pH:7). 5 ml of NBRIP-BPB medium was transferred to a sterile test tube and autoclaved. The liquid medium was inoculated with 500µl suspension of the bacterial strains. The test tubes were incubated for 14 days at 27 ^oC. At the end of the incubation period, P-solubilizing bacterial strains were detected by the change in colour of liquid medium in test tube (Mehta and Nautiyal, 2001).

Selection of potassium solubilizing bacteria strains

Bacterial strains tested for K-solubilizing activity using Aleksandrow solid medium (5 gl^{-1} glucose, 0,005 g $MgSO_4.7H_2O$, 0,1 g $FeCl_3$, 2 g $CaCO_3$, 3 g waste mica, 2 g $Ca_3(PO_4)_2$, 20 g agar). The strains were cultured on medium. The plates were incubated at 27 °C for 7 days. After incubation, the clear zone formed around colonies were evaluated as phosphate solubilizing positive bacteria (Meena et al., 2015).

Determination of biocontrol effect of bacterial strains against *R. Solaniin vitro*

The interaction between *R. solani* isolates and bacterial strains was studied by the dual culture assay. For this purpose, pathogen was grown for 4-5 days in PDA medium, and candidate antagonist bacteria were grown for 24-48 hours in NA medium. Two discs with a diameter of 4 mm were taken from the cultures of fungal isolates and placed at opposite edges of 9 cm diameter petri dishes containing PDA. Bacteria were incubated at 27 oC for a period of one week by line planting in the middle of the petri dish. Petri dish without bacteria cultivation was used as a control. 2 petri dishes were used for each bacterial strain and the experiment was repeated 3 times (Dönmez, 2015). In the in vitro test, the inhibition zone measurement was carried out when the fungus mycelium filled the petri dish completely. The inhibition zone was

determined by measuring from the edge of the mycelium to the bacterial colony. Percent inhibition rate was determined using the following formula (Çubukçu, 2007).

$$\%\text{Inhibition} = (A-B)/Ax100 \quad (1)$$

A: Diameter of the fungus colony in the control petri

B: Diameter of the fungus colony in the petri with bacteria

Bacterial strains stored at -80 ^{o}C that were grown on Nutrient Agar (NA; Lab-lemco powder 1 g, yeast extract 2 g, peptone 5 g, sodium chloride 5 g, agar 15 g, distilled water 1 L. pH 7.4 ± 0.2) were incubated at 27 ^{o}C for 48 h. Colonies taken from bacterial cultures developed after incubation were transferred into flasks containing nutrient broth (NB; Lab-lemco powder 1 g, yeast extract 2 g, peptone 5 g, sodium chloride 5 g, distilled water 1 L. pH 7.4 ± 0.2). Sucrose was added to the suspensions to enhance the adhesion of the antagonists. Contaminated liquid media were incubated at 140 rpm/min for 24 h in a shaker set to 27 ^{o}C. The bacterial inoculum concentration was prepared to be 1 x 10^7 CFU/ml by diluting with dH_2O.

Antagonist strains found to be effective against *R. solani* isolates *in vitro* were tested *in vivo*. The pot medium is made up of soil and perlite at a ratio of 1: 1. The soil mix was sterilized at 121^{o}C in autoclaves for 1 hour and 2 consecutive days and then filled into pots. After the bean seeds were washed in tap water, they were kept in 70% ethyl alcohol for 2 minutes. Then the seeds were kept in pure water for ten minutes and rinsed twice. Washed seeds were kept in bacterial suspensions for 2 hours and then three seeds were planted in pots. Untreated seeds were transferred to pots only after being kept in sterile water. The trial was established according to the randomized blocks trial design with four replications: positive control (clean soil + seed contaminated with pathogen), negative control (clean soil + clean seed) and bacterial application (clean soil + bacteria coded seed + pathogen). In applications involving pathogens, the root part of the plants was opened 3 weeks after planting the plants and 2 fungal discs were placed (Dönmez, 2015).

During the evaluation of the *in vivo* test, the plants in the pot were removed, the disease symptom occurring in the roots of the bean plant was evaluated according to the 0-4 scale (0: No symptom, normal root development, 1: Localized tissue coloration without necrosis, root growth close to normal, 2: Intense color change in tissues, Root growth close to normal, 3: Necrosis in almost all of the roots, partial limitation of the root length, 4: Root rot, serious limitation in root length) (Aydın, 2008; Eken and Demirci, 2004). Disease severity was calculated according to Towsend-Heuberger (1943) formula with values determined according to the scale.

$$\%\ \text{Disease Severity} = [\Sigma(SV \times NP)] \times 100/HSV \times TP \quad (2)$$

SV: Scale value

NP: Number of Plants with the same scale value

HSV: The Highest scale value

TP: Total number of plants

The obtained disease severity (%) results were calculated as % effect values relative to the control with the help of Abbott (1925) formula:

$$\% \text{ Effect} = (X-Y)X*100 \quad (3)$$

X; Disease Severity in the Control

Y; Disease Severity in the Treatments

The obtained data were subjected to variance analysis in the SPSS (SPSS Inc. PASW Statistics version 17) statistical software package and the differences between the applications were determined by DUNCAN test with $P \leq 0.01$ error probability.

RESULTS AND DISCUSSION

Identification of bacterial strains by fatty acid methyl ester analysis

According to the diagnosis results of bacterial strains based on fatty acid methyl ester profiles, 14 different bacteria species (2 *Bacillus mageterium*, 3 *Bacillus atrophaeus*, 1 *Brevibacillus choshinensis*, 2 *Bacillus pumilus*, 1 *Gardonia rubriperticta*, 1 *Microbacterium lacticum*, 1 *Bacillus licheniformis*, 1 *Brevibacillus reuszeri*, 1 *Bacillus thuringiensis israelensis*, 1 *Virgibacillus pantotheticus*, 1 *Bacillus cereus*, 1 *Bacillus filicolonicus*, 1 *Bacillus subtilis* and 1 *Paenibacillus alvei)* were identified as a result of the isolations from salty soils. 18 different bacterial species, which were identified as 3 *Bacillus subtilis*, 6 *Bacillus megaterium*, 2 *Bacillus cereus*, 2 *Pseudomonas stutzeri*, 2 *Sporosarcina psychrophila*, 1 *Bacillus licheniformis*, 1 *Arthrobacter aurescens*, 1 *Raoultella terrigena*, 1 *Panibacillus validus*, 1 *Chryseobacterium indologenes*, 1 *Chryseobacterium indoltheticum*, 1 *Kurthia gibsonii*, 1 *Flavimonas oryzihabitans*, 1 *Cellulomonas fimi*, 1 *Bacillus niacini*, 1 *Kocuria varians*, 1 *Brevibacillus reuszeri* and 3 *Bacillus viscosus*, were determined from the isolations from sandy soils. The results of tests on isolates from volcanic soils revealed 24 different bacterial strains as 1 *Micrococcus luteus*, 3 *Bacillus megaterium*, 1 *Bacillus pumilus*, 2 *Pseudomonas fluorescens*, 3 *Bacillus cereus*, 2 *Bacillus* sp.,1 *Pseudomonas syringae syringae*, 1 *Kluyvera intermedia*, 3 *Bacillus atrophaeus*, 1 *Pseudomonas stutzeri*, 1 *Chryseobacterium indoltheticum*, 2 *Kocuria rosea*, 1 *Brevibacillus chosinensis*, 1 *Pseudomonas putida*, 1 *Paenibacillus polymyxa*, 2 *Brevibacillus centrosporus*, 2 *Sphingobacterium faecium*, 1 *Bacillus subtilis*, 1 *Pseudomonas pseudoalcaligenes*, 1 *Atrobacter globiformis*, 1 *Empedobacter brevis*, 1 *Micrococcus lylac*, 1 *Pseudomonas balearica* and 1 *Bacillus thuringiensis israelensis*.

Colony morphologies and diagnostic results of biochemical tests of the isolated bacterial strains whose effectiveness against the pathogen was determined by the *in vitro* experiment are given in Table 1.

Determination of biocontrol effects of bacterial strains against *R. solani* isolates *in vitro*

According to in vitro test results, when the effects of bacteria applications on pathogen isolates are examined; it was determined that among 83 bacterial strains obtained, 11 bacterial strains were effective against B-1, while 13 were effective against B-227 and 17 against Rs-pat. It was determined that the remaining bacterial strains were ineffective on mycelium development of *R. solani* isolates. It was observed that HV20, HV43, HT19, HT21, HT26, HT30, HK3 and HK13 bacterial strains were showing antagonistic effect against three isolates of *R. solani*, while HV25 were having antagonistic effects against B-1 and B-227, HV2 was found to have an antagonistic effect against B-227 and Rs-pat, and HV46 and HK8 against B-1 and Rs-pat isolates. Among the strains, HV21, HV34, HK2, HK7, HK17 and HK32 were

found to be effective only in inhibiting the development of Rs-pat, while HV12, HV13 and HV14 were only effective in inhibiting the development of B-227 isolate (Table 2).

Table 1. Test results of bacterial strains with antagonistic effect on *R. solani* growth *in vitro*

SN	MIS Diagnostic Result	G	K	O	L	A1	T	A	F	P	KR
HV2	*Bacillus megaterium GC subgroup A*	+	-	-	-	-	-	K+	Z+	-	Cream
HV12	*Pseudomonas syringae syringae*	-	K+	+	-	-	-	K+	K+	-	Cream
HV13	*Kluyvera intermedia*	-	K+	+	Z+	-	-	K+	-	-	White, mucoid
HV14	*Bacillus atrophaeus*	+	+	+	Z+	-	-	K+	-	-	Light brown
HV20	*Bacillus megaterium GC subgroup A*	+	+	+	-	-	-	K+	-	-	White
HV25	*Paenibacillus polymyxa*	+	Z+	+	-	-	-	K+	Z+	-	White
HV34	*Bacillus subtilis*	+	+	+	K+	-	-	K+	+	-	White
HV43	*Bacillus thuringiensis israelensis*	+	+	+	-	-	-	K+	+	-	White
HV46	*Bacillus cereus GC subgroup A*	+	Z+	+	-	Z+	-	K+	+	-	White
HK2	*Bacillus subtilis*	+	+	+	-	Z+	-	+	-	-	White
HK3	*Bacillus megaterium GC subgroup A*	+	-	-	-	-	-	+	-	-	Cream
HK7	*Bacillus cereusGC subgroup A*	+	+	+	-	+	-	K+	Z+	-	White
HK8	*Bacillus megaterium GC subgroup A*	+	K+	-	-	-	-	+	+	-	Cream
HK13	*Bacillus licheniformis*	+	+	+	+	-	-	K+	-	-	Cream
HK17	*Panibacillus validus*	+	+	+	-	+	-	Z+	Z+	-	White
HK32	*Bacillus subtilis*	+	+	+	-	+	-	K+	-	-	Cream
HT19	*Bacillus thuringiensis israelensis*	+	+	+	-	-	-	Z+	-	-	white
HT21	*Bacillus cereus GC subgroup A*	+	+	+	+	+	-	Z+	+	-	White
HT26	*Bacillus atrophaeus*	+	+	+	+	+	-	K+	Z+	-	Light brown
HT30	*Bacillus subtilis*	+	+	+	-	+	-	K+	Z+	-	White

SN: Strain no, G: Gram reaction test, K: Catalase test, O: Oxidase test, L: Levan test, A1: Amylase test, T: Hypersensitivity test in tobacco, A: Nitrogen fixing property, F: Phosphorus dissolving property, P: Potassium dissolving property, KR: YDC colony color in the medium, K +: Strong positive result, Z +: Weak positive result, +: Positive result, -: Negative result

When the inhibition zones created by the bacteria whose antifungal properties were tested in the petri experiment, it was determined that the inhibition zone values against Rs-pat isolate varied between 0.2 –1.7 cm. It was observed that the highest inhibition zone against the pathogen was formed by the HV43 bacteria with 1.7 cm. When the results were evaluated in terms of B-1 isolate, it was determined that the inhibition zone values were between 0.2 and 1.4 cm, and for B-227 isolate these values were between 0.1 and 0.93 cm.

When the effect of bacterial applications on Rs-pat isolate was examined, it was determined that HV43 was 65% effective in preventing the disease, while HT30 was 64% effective and HT26 was 54%. When the effects of the applications on B-227 were evaluated, the highest effect was obtained from HV43 strain with a rate of 53%. When the success of bacterial strains was evaluated in terms of B-1 isolate, it was found that HV43 was the most

effective with 55% inhibition rate. When the results are evaluated in general, the highest effect on all three fungus isolates was obtained from HV43 strain with 53-65% inhibition rate (Table 2).

Table **2.** *In vitro* activities of candidate antagonist bacteria strains against *R. solani* isolates

		Rs- pat		B-227		B-1	
Strain No	**MIS Diagnostic Result**	**Inhibition Zone (cm)**	**Inhibition Rate (%)**	**Inhibition Zone (cm)**	**Inhibition Rate (%)**	**Inhibition Zone (cm)**	**Inhibition Rate (%)**
HV-46	*Bacillus cereus GC subgroup A*	0.16 h	18.00 h	--	--	0.20 fg	23.10 e
HK-17	*Panibacillus validus*	0.26 g	20.50 gh	--	--	--	--
HK-32	*Bacillus subtilis*	0.16 gh	24.00 fgh	--	--	--	--
HK-7	*Bacillus cereus GC subgroup A*	0.20 g	24.76fg	--	--	--	--
HK-2	*Bacillus subtilis*	0.30 g	26.40 efg	--	--	--	--
HV-21	*Bacillus cereus GC subgroup A*	0.33 fg	28.63 ef	--	--	--	--
HV-2	*Bacillus megaterium GC subgroup A*	0.93 b	29.46 ef	0.46 cd	45.46 b	--	--
HV-20	*Bacillus megaterium GC subgroup A*	0.50 ef	31.86 de	0.36 de	26.43 fg	0.36 def	27.23 e
HK-13	*Bacillus licheniformis*	0.63 de	31.96 de	0.36 de	34.70 d	0.66 bcd	35.50 cd
HK-8	*Bacillus megaterium GC subgroup A*	0.86 bc	36.83 cd	--	--	0.76 bc	34.73cd
HV-34	*Bacillus subtilis*	0.33 fg	37.66 cd	--	--	--	--
HK-3	*Bacillus megaterium GC subgroup A*	0.70 cd	37.70 cd	0.43 cd	35.56 d	0.83 b	41.33 b
HT-19	*Bacillus thuringiensis israelensis*	0.70 cd	40.16 c	0.43 cd	31.66de	0.40 def	35.50 cd
HT-21	*Bacillus cereus GC subgroup A*	0.76 bcd	41.00 c	0.90 a	40.46 c	0.56 bcde	39.66 bc
HT-26	*Bacillus atrophaeus*	1.50a	54.10 b	0.76 ab	48.83 ab	0.50 cdef	38.83 bc
HT-30	*Bacillus subtilis*	1.63a	63.96 a	0.93 a	50.40 a	1.26 a	51.20 a
HV-43	*Bacillus thuringiensis israelensis*	1.63a	64.70 a	0.63 bc	52.90 a	1.40 a	55.36 a
HV-25	*Paenibacillus polymyxa*	--	--	0.23 def	23.93 g	0.26 efg	33.03 d
HV-14	*Bacillus atrophaeus*	--	--	0.13 ef	24.76 fg	--	--
HV-13	*Kluyvera intermedia*	--	--	0.23 def	29.16 ef	--	--
HV-12	*Pseudomonas syringae syringae*	--	--	0.30 de	28.90 ef	--	--
Control (+)		.0000 h	.0000 ı	.0000 f	.0000 h	.0000 g	.0000 f

*Means with the same letter are not significantly different according to Duncan's multiple range test at p≤0.001.

Determination of biocontrol effects of bacteria strains against *R. solani* ısolates *in vivo*

In vivo test results were obtained by evaluating the disease symptom occurring in the roots of the bean plant according to the 0-4 scale. Disease symptom was observed in plants included in the positive control application inoculated with the pathogen and the average disease severity index was determined as 93.7%. There was no disease symptom in the plants in the negative control group. When the effect of bacterial applications on B-227 isolate was examined, it was seen that HT21, HT30 and HV43 strains prevent the disease by 100% and have the highest

antagonistic effect. HV2, HT26, HV25 and HV20 strains were also found to inhibit the development of disease successfully, with 91.1%, 84.4%, 66.7% and 40% inhibition rates, respectively. Among the strains, HV13 was found to be in the same group with the positive control with 93.7% disease severity and it was observed to be unsuccessful in preventing the development of the disease (Table 3).

Table 3. The effect of candidate on the antagonist bacteria severity of root rot disease in the pot trial

		Rs-pat		B-227		B-1	
Strain No	**MIS Diagnostic Result**	**Disease Severity (%)**	**Effect (%)**	**Disease Severity (%)**	**Effect (%)**	**Disease Severity (%)**	**Effect (%)**
HV-46	*Bacillus cereus GC subgroup A*	66.65 c	28.90 b	--	--	62.48 d	33.35 d
HK-17	*Panibacillus validus*	100.00 a	-6.70 d	--	--	--	--
HK-32	*Bacillus subtilis*	100.00 a	-6.70 d	--	--	--	--
HK-7	*Bacillus cereus GC subgroup A*	100.00 a	-6.70 d	--	--	--	--
HK-2	*Bacillus subtilis*	100.00 a	-6.70 d	--	--	--	--
HV-21	*Bacillus cereus GC subgroup A*	95.83 a	-2.28 d	--	--	--	--
HV-2	*Bacillus megaterium GC subgroup A*	97.90 a	-4.47 d	8.30 hg	91.10 ab	--	--
HV-20	*Bacillus megaterium GC subgroup A*	60.40 c	35.50 b	56.23 e	40.00 d	60.38d	35.60 d
HK-13	*Bacillus licheniformis*	100.00 a	-6.70 d	83.30 ab	11.06 gh	91.63 a	2.20 g
HK-8	*Bacillus megaterium GC subgroup A*	91.65 ab	2.21 cd	--	--	75.00 bc	20.00 ef
HV-34	*Bacillus subtilis*	.0000 d	100.00 a	--	--	--	--
HK-3	*Bacillus megaterium GC subgroup A*	56.23 c	39.98 b	62.48 de	33.35 de	64.55 cd	31.15 de
HT-19	*Bacillus thuringiensis israelensis*	83.30 b	11.12 c	79.15 bc	15.50 fg	79.15 b	15.50 f
HT-21	*Bacillus cereus GC subgroup A*	.0000 d	100.00 a	.0000 h	100.00 a	0.00 g	100.00 a
HT-26	*Bacillus atrophaeus*	8.30 d	91.10 a	14.55 g	84.43 b	25.00 e	73.30 b
HT-30	*Bacillus subtilis*	.0000 d	100.00 a	.0000 h	100.00 a	0.00 g	100.00 a
HV-43	*Bacillus thuringiensis israelensis*	.0000 d	100.00 a	.0000 h	100.00 a	0.00 g	100.00 a
HV-25	*Paenibacillus polymyxa*	--	--	31.23 e	66.65 c	44.55 e	52.43 c
HV-14	*Bacillus atrophaeus*	--	--	68.73 cde	26.70 ef	--	--
HV-13	*Kluyvera intermedia*	--	--	93.73 a	-.0400 h	--	--
HV-12	*Pseudomonas syringae syringae*	--	--	70.80 bcd	24.50 ef	--	--
Control (+)		93.73 ab	.0000 cd	93.73 a	.0000 h	93.73 a	.0000 g
Control (-)		.0000 cd	.0000 cd	.0000 h	.0000 h	.0000 g	.0000 g

*Means with the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.001$.

When the mean disease severity indexes determined for the Rs-pat isolate were evaluated, it was found that seven of the bacterial strains (HK2, HK7, HK13, HK17, HK32, HV2, HV21) were not effective against the pathogen. HV34, HV43, HT21 and HT30 strains had 100% effect on the disease and it was determined that they were the most effective antagonist microorganisms against the disease. The mean disease severity index of plants where HT26 bacteria was applied was determined as 8.3 and the effect on disease development was determined as 91.1%, and in this respect, it was recorded as a very effective application in preventing the disease. It was determined that HK3 strain had a 40% potential to prevent disease, while HK8, HT19, HV46 and HV20 bacteria prevent development of disease at lower rates (2.1% - 35.5%) compared to other applications (Table 3).

When the biocontrol trial results of the B-1 isolate were evaluated, no disease symptom was observed in plants to which HV43, HT21 and HT30 bacteria were applied, and all strains were found to be quite successful with 100% effective rate against the pathogen. HV25 and HT26 bacteria applications are accepted to be successful by preventing disease over 50% rate (57.7% - 73.3%) (Table 3).

Fig 1. The effect of strain HV43 on B-227 isolate

Fig 2. The effect of strain HT21on B-227 isolate

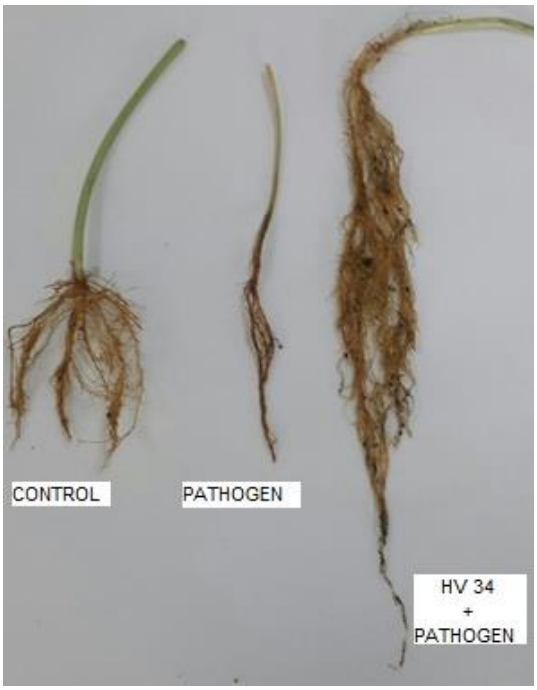

Fig 3. The effect of strain HV34 on Rs-pat isolate

Fig 4. The effect of strain HT30 on B-1 isolate

In vivo trial results were grouped according to the potential of bacteria strains in inhibiting the pathogen, and those with an effect between 70% and 100% were considered "very successful" while those with an effect between 50% and 70% were considered "successful".

In this study, it was observed that all 7 strains of bacteria, which were determined to have antagonistic effects on three isolates of *R. solani*, belong to the genus *Bacillus*. It was determined that 4 different bacterial strains, namely *Paenibacillus polymyxa* (B-1 and B-227), *Panibacillus validus* (Rs-pat), *Pseudomonas syringae syringae* and *Kluyvera intermedia* (B-227), are all among the bacterial strains with antagonistic effects. In studies similar to this study, it has been reported that different *Bacillus* species suppress the development of soil borne fungal pathogens *in vitro*. *Bacillus sp.* isolated from soil by Ulukuş (1988) was reported to be inhibiting the development of *V. dahliae*. It was reported by Amer et al. (1997) that *Bacillus thuringiensis* causes morphological distortion and lysis in the hyphae of *Pythium ultimum* and *F. oxysporum*, which causes disease in tomatoes. It was found by Tekin (2004) that antagonists belonging to *Bacillus* species significantly inhibit pathogens that cause disease in the roots of pepper plant. Soylu et al. (2005) reported that fluorescent *Pseudomonas* spp. and *Bacillus* spp. show antagonistic effect against disease agents and prevent the disease to a high extent when used in the control against soil-borne *S. sclerotiorum* and *R. solani*, which cause infection in peppers and tomatoes. It was reported by Abd-Allah et al. (2006) that *B. subtilis* applications prevent disease incidence by causing the change of lipid fractions in the plant. Fluorescent strains of *Pseudomonas* and *Bacillus* species were reported by Çubukçu (2007) to significantly reduce the growth of *V. dahliae*, *Verticillium* wilt factor in cotton plants.

It was found that seven of the bacteria strains (HK2, HK7, HK13, HK17, HK32, HV2, HV21) that were effective against the pathogen *in vitro*, were not effective *in vivo*, and contrary to expectations, they caused a higher rate of disease than positive control. Likewise, it was reported by Berg et al. (2001) that *Serratia plymuthica R12* application increased the symptoms of *Verticillium* wilt statistically in the strawberry plant under greenhouse conditions, compared

to positive control. Similarly, in another study (Vestberg et al., 2004) investigating the effectiveness of applications of *Glomus mosseae BEG29, Bacillus subtilis M, Trichoderma harzianum DB11, Pseudomonas fluorescens C7r12* and *Gliocladium catenulatum* (Gliomix) against diseases caused by *P. cactorum* and *P. fragaria* pathogens in strawberry, it was reported that the applications could not control the disease, and even the disease severity increases as a result of some applications. Another striking result of this study in line with these findings is the inconsistency between *in vitro* and *in vivo* test results of some bacterial strains, which is frequently encountered in biological control studies. It has been reported by Papavizas and Lewis (1983) and Fravel (1998) that there are many findings of incompatibility between *in vitro* antibiosis studies and *in vivo* biocontrol. In a study conducted by Duczek (1994) on the control of *Bipolaris sorokiniana* pathogenic fungus, no relationship was reported between greenhouse and field trials in terms of the efficiency of bacteria strains. Shtienberg and Elad (2002) reported that *in vitro* and *in vivo* results may differ in biological control studies. It was reported by Mercado-Blanco et al. (2004) that there is no relationship between *in vivo* and *in vitro* effectiveness of bacteria strains tested in the inhibition *Verticillium* wilt. In addition, it is known that the content of the medium used in dual culture assays performed to determine the antagonistic effects of bacteria *in vitro* is important. It has been determined that the antibiotic production of microorganisms varies according to the carbon sources contained in the medium (Shanahan et al., 1992; Nielsen et al., 1998; Yoshida et al., 2001). In addition, it was stated that antibiotic production is greatly affected by environmental factors and in case antibiotic production is related to the enzyme, results cannot be obtained by *in vitro* tests in the absence of the enzyme substrate (Fravel, 1998).

As a result of the study, it was observed that the activities of bacterial strains were generally higher *in vivo*. To obtain an effective result in biological control with plant diseases *in vivo* depends on the interactions of host (morphological and genotypic changes depending on the plant variety, plant growth period), pathogen (virulence and pathogenicity difference), antagonist bacteria (population density, production of enzymes, hormones and antimicrobial substances) and environmental factors (pH, temperature, humidity, soil texture, abiotic factors such as inorganic and organic matter content) (Landa et al., 2004). Among the microbial communities, the most competitive species that are best adapted to the living environment, create population differences by forming more colonies, thus rapidly colonizing plant roots and exhibiting an effective biocontrol feature against disease agents (Boudyach et al., 2001; Rezzonico et al., 2007). It was reported that changes in environmental factors such as temperature, humidity and light do not affect the underground part of the plant faster and at a higher level than the part above ground, therefore the bacteria strains applied to the rhizosphere region are more effective than those applied to the part of the plant above ground (Hsieh et al., 2005). It was also reported that a successful rhizosphere colonization for microorganisms is associated with root exudation of the plant. It was determined that the structure of the exudates changes depending on the plant variety, the growth period of the plant and its exposure to stress, and this situation causes differences in the colonization of various bacterial communities. Exudates secreted into rhizosphere by plants are formed up of various carbohydrates, amino acids and organic acids, and these secretions are used as food source by some bacteria in the

region and affect their colonization (Lugtenberg and Dekkers, 1999; Bais et al., 2006; Haichar et al., 2008).

According to the results obtained from the pot experiment, it was observed that some bacteria applications resulted in no disease and the root part of the plant developed very well compared to the control. Therefore, bacteria strains which were determined as successful have attracted attention with their biocontrol effects as well as their plant growth enhancing properties. These results were thought to be due to the fact that the bacteria strains applied to the seed stimulate the systemic resistance of the plant, and its abilities to fix nitrogen and solubilizing phosphorus. Studies on this subject show that PGPRs prevent the formation of diseases in plants by pathogenic microorganisms with some mechanisms they possess, and at the same time, they are widely and successfully used to promote plant growth (Hoitink, 1986; Slezack et al., 1999; Heungens and Parke, 2000; Estevez de Jensen et al., 2002; Anandhakumar and Zeller, 2004). As a result of the stimulation of systemic resistance by antagonist bacteria, it was determined that enzymatic changes that provide resistance against diseases occur in plants, and that the activities of enzymes such as *phenylalanine ammonialyase, lipoxygenase, hydroperoxidase, peroxidase, polyphenol oxidase* increase (Chen et al., 2000; Ongena et al., 2004; Silva et al., 2004). In addition, it was reported that microbial antagonists produce antimicrobial compounds such as pyrrolnitrin, 2,4-diacetylphloroglucinol, hydrogen cyanide, phenazine-1-carboxylic acid, pyoluteorin, mycobacillin, oomycin A, subtilin, bacilysn and iturin A, and inhibit pathogen development by producing enzymes such as chitinase, sellulase and glucon that decompose cell wall (Chen et al., 2000; Tambong and Höfte, 2001; Krechel et al., 2002; Ramamoorthy et al., 2002). It was demonstrated in various studies that bacterial strains increase plant growth and play a role in preventing pathogen development by producing plant hormones, such as indole acetic acid, giberallic acid, cytokinin and ethylene, by fixing nitrogen asymbiotically, and by mineralizing phosphorus and other nutrients that cannot be taken from the soil (Gutierrez-Manero et al., 2001; Dobbelaere et al., 2002; Şahin et al., 2004). It was determined that indole acetic acid production by antagonist bacteria is effective in root elongation and development of the plant, and there is a positive relationship between indole acetic acid and root development (Asghar et al., 2002; Khalid et al., 2004). Bacterial strains, which were found to be effective in this study, are thought to suppress the pathogen by using one or more of these mechanisms.

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