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Characterization of antagonistic and plant growth-promoting traits of endophytic bacteria isolated from bean plants against *Pseudomonas syringae* pv. *phaseolicola*

Fasulye bitkilerinden izole edilen endofit bakterilerin antagonistik ve bitki gelişimini teşvik edici özelliklerinin *Pseudomonas syringae* pv. *phaseolicola* etmenine karşı karakterizasyonu

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ABSTRACT

Pseudomonas syringae pv. *phaseolicola* (*Psp*) is a seed-borne bacterium which causes halo blight disease in common bean. This study aimed the determination of plant growth-promoting traits (such as IAA, phosphate solubilization) and antagonistic potentials (such as siderophore and ammonia productions) of endophytic plant growth-promoting bacteria (PGPB) from healthy bean plants growing in different regions against *Psp in vitro* conditions. A total of 131 bacteria were primarily characterized as putative PGPB and tested for antagonist activity against *Psp* in dual culture tests. Seventy-one bacterial isolates demonstrated antagonistic activities against *Psp* isolate in varying ratios. Among these 71 isolates, 30 representative bacterial isolates from the different regions/fields were selected. On the basis of morphological, physiological, biochemical characteristics and confirmation by MALDI-TOF analyses, 30 endophytic antagonist isolates were identified as belonging to 10 genera, 24 different species. According to results obtained, 10 isolates belong to *Bacillus* spp., 6 isolates belong to *Pseudomonas* spp., 4 isolates belong to *Rhizobium radiobacter*, 2 isolates belong to *Arthrobacter* spp., 2 isolates belong to *Achromobacter spanius*, 2 isolates belong to *Serratia liquefaciens*, 1 isolate belongs to *Acinetobacter calcoaceticus*, *Exiguobacterium* sp., *Microbacterium hydrocarbonoxydans*, and *Ochrobactrum anthropi*. The largest and lowest inhibition zone was produced by endophytic bacterial isolates *Pseudomonas gessardii* (4.85) and *Bacillus licheniformis* (1.35). Among the tested antagonist bacterial isolates, 10 isolates were positive for the production of α-amylase, 7 isolates positive for phosphate solubilization, 29 isolates positive for siderophore production, 11 isolates positive for protease production. All selected bacterial isolates produced IAA and ammonia in relatively varying amounts. *P. gessardii* produced a relatively large amount of extracellular siderophore (5.83), *Exiguobacterium* sp. produced a relatively large amount of extracellular protease (5.25), *P. gessardii* and *O. anthropi* produced a relatively large amount of extracellular IAA (161.39 µg/ml) and *Acinetobacter calcoaceticus* produced a relatively large amount of phosphatase (2.63). This is the first study reporting bean plants harbor endophytes having plant growth promoting activities with antagonistic potential against *Psp*.

INTRODUCTION

Phaseolus vulgaris L., known as the common bean, is a herbaceous, self-pollinated, industrially integrated, rich from vitamins

and proteins and globally important leguminous crop which is grown worldwide for its edible dry seeds or unripe fruit. Beans are the staple food for human and animal nutrition, and it is the most important crop after chickpea and lentil among the grain legumes in Turkey. Annual dry bean production is 31.405.912 tonnes, and green bean production is 24.221.252 tonnes worldwide. Turkey is the fourth country in green bean production worldwide with 1.268.879 tons (FAO 2017).

During the cultivation of bean, many pests and diseases occur and cause economical loses. Bacterial diseases come right after the root diseases of common bean caused by the soil-borne pathogens. Halo blight is a bacterial disease of common bean, caused by *Pseudomonas syringae* pv. *phaseolicola* (*Psp*). Halo blight disease was first discovered in the early 1920s, and rapidly became the primary disease of beans all over the world. Symptoms start with small water-soaked spots on leaves and pods. This infected tissue dies and turns tan-colored and necrotic under arid conditions. Yellow to light green halo surrounds the lesion, distinguishing this disease from the narrow yellow band caused by common bacterial blight disease caused by *Xanthomonas axonopodis* pv. *phaseoli*. This halo symptom is not evident during periods of high temperatures. If infected systemically, young leaflets become curved and chlorotic without the presence of necrotic spots or broad yellow halos. The disease shows up in the places where temperatures are mild and plentiful inoculum is available (Taylor et al. 1979, Webster et al. 1983). Disease agent was reported to occur and caused serious yield loses in different regions of Turkey (Bastas and Sahin 2016, Benlioglu and Ozakman 1993, Bozkurt and Soylu 2001, Donmez 2004).

Even extremely low levels of seed infestation by *Psp* can result in severe epidemics under favorable weather conditions (Webster et al. 1983). Management of bacterial diseases is very hard and sometimes impossible, depending on the severity of the disease on infected plants. Limited success has been recorded following application of copper-based fungicides such as Bordeaux mixture, cupric hydroxide, copper oxychloride, copper sulfate (Saettler et al. 1981, Schwartz and Galvez 1981). Although use of copper-based pesticides are the most common control method preferred by farmer against the disease, frequent uses of fungicides cause the existence of pesticide resistance on pathogens, pesticide residues on the crop, direct and indirect effects of synthetic chemical pesticides on human and nature are the primary limiting reasons for their usage against disease agent. Recently, instead of using chemicals, integrated pest management strategies became an inevitable alternative strategy to manage plant pathogens (Berg and Hallmann 2006, Bozkurt and Soylu 2011, Taylor et al. 1979, Webster et al. 1983).

Leguminous plants, especially beans, are also the host of many beneficial microbiomes which can be helpful for nutrient fixation and plant growth promotion. In accordance, plant-associated bacteria can be classified into beneficial, deleterious, and neutral groups based on their effects on plant growth (Dobbelaere et al. 2003). Endophytic bacteria are one of the promising biocontrol agents because they occupy internal living tissues of plants and maybe close to plant pathogens (Berg and Hallman, 2006, Sülü et al. 2016). Bacteria of diverse genera have been identified as plant growth promoting bacteria (PGPB), of which *Bacillus* and *Pseudomonas* species are predominant (Podile and Kishore 2006). PGPB affect plant growth in two different ways, directly or indirectly. The direct promotion of plant growth by PGPB entails through producing several plant growth regulators/ phytohormones (Glick 1995) and facilitating the uptake of certain nutrients from the environment (Glick 1995, Soylu et al. 2018, Vacheron et al. 2013). They can further keep host healthy through the indirect mechanisms by inhibiting fungal or bacterial pathogens, for instance by producing antimicrobial compounds (Glick et al. 2007), siderophores (Compant et al. 2010, Lodewyckx et al. 2002, Reinhold-Hurek and Hurek 2011) against the phytopathogens (Narula et al. 2013, Santoyo et al. 2016). The indirect promotion of plant growth occurs when PGPB lessen or prevent the deleterious effects of one or more phytopathogenic organisms which can be identified and used as in crop protection.

Crop protection mechanisms can be activated by antagonistic substances produced by PGPB inducing resistance to pathogens (Glick 1995). A particular PGPB may affect plant growth by using one or more of these mechanisms. PGPRs, as biocontrol agents can act through various mechanisms, regardless of their role in direct growth promotion enhancement, such as by known production of phytohormones (Patten and Glick 2002), a decrease of plant ethylene levels (Glick et al. 2007) or nitrogen-fixing associated with roots (Döbereiner 1992).

Purposes of this study were to (i) isolate and identify endophytic bacterial population existing in the inner tissues of the healthy common bean plants growing in different regions of Turkey, (ii) to characterize antagonistic effect to inhibit *Psp,* and (iii) to explore their antagonistic and plant growth promoting traits *in vitro* conditions.

MATERIALS AND METHODS

Isolation of endophytic PGPB isolates from healthy plants

Plant materials were collected from Ankara, Burdur, Eskişehir, Konya, and Niğde provinces during 2015 and 2016 production seasons. *Psp* strains were isolated from the plants showing typical disease symptoms. PGPB were isolated from healthy plants sampled from infected fields with the halo blight disease.

Putative endophytic PGPB isolates were isolated from the internal tissues of the roots, stem, leaves, and fruits of healthy bean plants. Each plant material's surface was sterilized with 1% sodium hypochlorite, and after the third surface sterilization, wastewater was plated on Nutrient Agar (NA) plates and incubated for 48 h at 28 °C. After incubation, plates were examined to verify the sterilization process is successful. Surface sterilized plant materials were put into extraction bags, and 2 ml of physiological saline added into each extraction bag. Extractions were prepared by the use of plastic extraction bags to homogenize plant tissues. Dilutions of 10^{-1} and 10^{-2} plant extracts, were plated on NA plates and incubated at 28 °C for 48 h. Each colony was used as one isolate in the NA plates and purified into a new NA plate for further study.

Selection of putative endophytic PGPB isolates

Hypersensitivity reaction test of the endophytic bacterial isolates was carried out by inoculating tobacco leaves, using a highly concentrated bacterial suspension (~10^8 cfu/ml). Effectiveness of the bacterial isolates was evaluated by the absence of disease symptoms and hypersensitivity reaction.

Putative isolates were further checked for potato soft rot test. A loopful bacterial colony was taken from pure fresh culture and spread on surface sterilized potato slices and incubated for the occurrence of any soft rot on potato tissues. Existence of soft rot on potato slices showed the positive effect of pectolytic activities of the tested bacterial isolate.

Incubation of endophytic bacterial isolates on NA media at 37 °C helped us to eliminate human pathogenic isolates.

Finally, all putative endophytic PGPB isolates were tested on bean pods. Bioassays were carried out on healthy bean pods including virulent *Psp* isolate which isolated and identified during the project. A highly susceptible cultivar of *Phaseolus vulgaris* cv. Gina was used for pathogenicity tests. All bean pods were surface sterilized with 1% sodium hypochlorite and dried. Each endophytic bacteria was suspended in sterile distilled water (~10^8 cfu/ml) and inoculated (20 µl) on 5 holes opened by sterile toothpicks on each pod, kept in a plastic box for incubation at room temperature for 3 days. After incubation, all inoculation sites were examined for water-soaked, oily appearance.

Putative endophytic PGPB bacterial isolate(s) which were positive for HR, soft rot, pathogenicity tests and grown at 37 °C were eliminated for further studies.

Identification of putative endophytic PGPB isolates

Putative bacterial endophytes were primarily screened for physiological and biochemical tests such as Gram reaction, oxidase, and production fluorescent pigmentation (Lelliot and Stead 1987). The species identification of PGPB isolates were further confirmed by MALDI-TOF (Bruker Daltonics GmbH, Bremen, Germany) analyses as described by Pavlovic et al. (2012). The bacterial mass taken from the pure colonies of the isolates and obtained from the colonies developed for 24-36 h on Tryptic Soy Agar (TSA) medium was added to Eppendorf tubes with 300 µl of sterile distilled water. Tubes were mixed in the vortex, and the bacteria suspension was obtained. After adding 900 µl of pure ethanol (Merck, Darmstadt, Germany) into the tubes, the mixture was centrifuged at 13.000 rpm for 2 min. After the disposal of supernatant, the remaining pellet was centrifuged for 1 min at 13.000 rpm again. If ethanol residue was observed, the remaining ethanol was carefully removed with a pipette, and the resulting pellet was allowed to dry at room temperature (about 5 minutes). After drying pellet, 30 µl of 70% formic acid (Merck, Darmstadt, Germany) was added and vortexed again at 13.000 rpm for 1 min 30 µl of acetonitrile was added, and the mixture was vortexed at 13.000 rpm for 1 min. Finally, 1 µl of the supernatant was added to the target plate in two replicates, followed by drying at room temperature (approximately 3-5 min), and addition of 1 µl HCCA Matrix on each sample point (α-Cyano-4-hydroxycinnamic acid) and drying.

Brukers Bacterial Test Standard (Bruker Daltonics GmbH, Bremen, Germany) was used as Mass calibration standard. Samples were loaded onto the MALDI-TOF MS (Microflex LT; Bruker Daltonics GmbH, Bremen, Germany) with the software Flex Control Software (Bruker Daltonics GmbH, Bremen, Germany). Each spectrum was obtained with 500 laser beams with the minimum laser power required for ionization of samples in automatic mode. The spectrum was analyzed at m/z (mass/charge) ratio in the range 2-20 kDa. Microorganism library was used as BIOTYPERTM 1.1 software (Bruker Daltonics GmbH, Bremen, Germany).

Determination of antagonistic and plant growth promoting traits of PGPB isolates

Inhibition assays

Dual culture inhibition assay performed on NA plates with fresh overnight cultures of *Psp* and PGPB isolates. PGPB strains spot inoculated on NA plates and incubated at 26 °C

for 24 h. After colony adaptation and growth of PGPB, *Psp* cell suspension (10^8 cfu/ml) was prepared and pulverized onto the Petri surfaces. Then Petri plates incubated at 26 °C for 24-72 h, bacterial colony diameter and inhibition zones caused by each PGBP isolates were measured. Antagonistic Efficiency Index (Ant-EI) for *Psp* growth inhibition was calculated based on the diameter of inhibition zones around the bacterial colonies by using the method of El-Sayed et al. (2014).

Ant-EI (Antagonistic Efficiency Index): [inhibition zone diameter (mm)/bacteria colony growth diameter (mm)].

Assay for α-amylase enzyme activity

Starch Agar plates were inoculated with 24 to 48 h old PGBP isolates on the agar surface and incubated at 26 °C for at least 4 days. Following incubation, Petri plates were flooded with iodine solution. The presence of the clear zone around the bacterial colonies indicates hydrolyzation of starch if blue zone surrounds the bacterial colonies that indicate the starch is still present and has not been hydrolyzed (Schaad et al. 2001).

Assay for ammonia utilization

Endophyte isolates were tested for their ammonia production activity by using peptone water. Freshly grown bacterial cultures were inoculated into sterile 10 ml peptone water solution in test tubes. Incubated at 30 °C with shaking at 120 rpm for 48-72 h. Then, Nessler's reagent (0.5 ml) was added in each tube. When the color changes brown to yellow, that was an indicator of a positive result for ammonia production (Cappuccino and Sherman 1992). The absorbance of suspensions was measured at 530 nm wavelength at spectrophotometer, compared with the standard curve of $(NH_4)_2SO_4$ and added in mg/ml.

Assay for proteolytic enzyme activity

Skim Milk Agar (SMA) is used to demonstrate the proteolytic activity of organisms capable of hydrolyzing casein (Perneel et al. 2007). 2% skimmed milk powder in Luria Broth (LB) Agar was prepared. LB agar inoculated with endophytic bacteria and then incubated at 26 °C for 48 h. Proteolytic bacteria use the enzyme caseinase to hydrolyze casein and form solvable nitrogen variant compounds that cause a clear zone around bacterial colonies. Colorless radiant zones around bacterial colonies indicator of protease production. Enzyme activity zones caused by each PGPB isolates measured and efficiency indices (Pro-EI) for proteolytic activity were calculated based on the diameter of inhibition zones around the bacterial colonies (El-Sayed et al. 2014).

Pro-EI (Proteolytic Enzyme Efficiency Index)= [inhibition zone diameter (mm)/colony growth diameter (mm)].

Assay for siderophore production

Siderophore activity of endophytic bacteria was determined by using the conventional method of Schwyn and Neilands (1987) using blue CAS agar plates containing the chrome azurol S (CAS). Briefly, a loopful of bacterial culture was spot inoculated on blue CAS agar medium and incubated at 28±2 °C for 2 days. The colony with a halo zone of clear to purple-orange color was evaluated as positive for siderophore production. Siderophore production activity showed zones caused by each PGPB isolates measured and efficiency indices (Sid-EI) for siderophore production were calculated based on the diameter of inhibition zones around the bacterial colonies (El-Sayed et al. 2014).

Sid-EI (Siderophore Production Efficiency Index)= [inhibition zone diameter (mm)/colony growth diameter (mm)].

Assay for phosphate solubilizing activity

Endophytic bacterial isolates were tested for phosphate-solubilizing ability by using PVK media (Kumar et al. 2012). Culture media were prepared by using insoluble phosphate [tri-calcium phosphate $Ca_3(PO4)_2$] with a concentration of 0.5%. Solubilization zones and colony diameters of the bacterium were incubated at 30 °C, measured after 120 h, and efficiency indices (Phos-EI) for phosphate solubilizing were calculated (El-Sayed et al. 2014).

Phos-Index (Phosphate Solubilization Efficiency Index)= [inhibition zone diameter (mm)/colony growth diameter (mm)].

Assay for indol-3 acetic acid (IAA) activity

Production of the phytohormone IAA assayed based on a colorimetric method by using Salkowski reagent (Glickman and Dessaux 1995). Bacterial cultures were grown in 5 ml Nutrient Broth (NB) containing 500 mg/l L-tryptophan (0.5%) at 30 °C for 48 h. Fully grown cultures (1.5 ml) were added into Eppendorf tubes and centrifuged at 5500 rpm for 15 min. The supernatant (1 ml) was mixed with 20 µl of orthophosphoric acid. Subsequently mixed with 4 ml of the Salkowski reagent (1 ml 0.5 M $FeCl_3$ solution, 50 ml 35% of perchloric acid) and incubated for 25 min in the dark at 30 °C. Production of IAA determined as the development of pink-red color, and the absorbance was measured at 535 nm wavelength (Patten and Glick 2002) using a spectrophotometer and compared with the standard curve. The amount of IAA was preprared in a standard curve as µg/ml. Three replicates were performed for each bacterial isolates for IAA synthesis measurement.

Data analysis

Experiments were performed in triplicate Petri dishes for each bacterial isolate and repeated twice. The SPSS statistic program version 17.0 was used for all data involving calculations and the comparison of each isolate for all measurements. The data were statistically analyzed using ANOVA. A Least Significant Difference (LSD) test at a probability level of 0.05 was used to separate the means when the ANOVA F-test indicated a significant effect from the treatments.

RESULTS

Isolation and selection of putative endophytic PGPB isolates

During 2016 and 2017 growing seasons, a total of 120 bean fields in Ankara, Burdur, Eskişehir, Konya and Niğde provinces were surveyed for isolation of *Psp* and PGPB isolates. A total of 343 putative PGPB isolates were subsequently isolated from the different inner parts of bean plants collected during the surveys (Table 1). Most of the isolates were collected from Niğde and Eskişehir provinces. All isolated endophytes were subjected to 37 °C, tobacco HR, potato rotting (pectolytic activity) and pathogenicity tests as explained above. Results were given in Table 1. Following these test, 52 isolates grown at 37 °C, 87 isolates were found positive for tobacco HR, 50 isolates for pectolytic activity on potato slices, and 23 isolates were found to be pathogenic on bean pods. Therefore, a total of 212 bacterial isolates were eliminated and 131 putative endophytic bacterial isolates from different locations retained for *in vitro* antagonistic activities against *Psp*.

Identification of antagonistic bacteria strains

Dual culture assay was used for monitoring the possible inhibitory effect of 131 putative antagonist PGPB isolates against *Psp in vitro* conditions. Seventy-one bacterial isolates, demonstrated antagonistic activities in varying degree against *Psp* isolate. Among these 71 isolates, 30 representative bacterial isolates from the different regions/fields were selected for identification in species level and tested for characterization of antagonistic and plant growth-promoting traits studies (Table 1).

Table 1. The total number of endophytic bacterial isolate collected from different provinces

Provinces	Number of Endophytic Isolates	Growth at 37 °C	Tobacco HR+ Test	Pectolytic Activity	Pathogenicity Test on Bean
Ankara	15	1	6	1	1
Burdur	51	9	15	9	3
Eskişehir	96	18	26	12	8
Konya	74	7	15	14	5
Niğde	107	17	25	14	6
TOTAL	343	52	87	50	23

Morphological, physiological, biochemical characteristics and MALDI-TOF analyses of 30 endophytic antagonist isolates assigned them to 24 bacterial species from 10 genera (Table 2). According to results obtained, 10 isolates belong to *Bacillus* spp., 6 isolates belong to *Pseudomonas* spp., 4 isolates belong to *Rhizobium radiobacter*, 2 isolates belong to *Arthrobacter* spp., 2 isolates belong to *Achromobacter spanius*, 2 isolates belong to *Serratia liquefaciens*, 1 isolate belongs to *Acinetobacter calcoaceticus*, *Exiguobacterium* sp., *Microbacterium hydrocarbonoxydans* and *Ochrobactrum anthropi* (Table 2).

Determination of antagonistic activities

A clear zone of inhibition was observed around each endophytic bacterium in the dual culture assay (Figure 1). The inhibition zone index ranged from 1.35 to 4.85 after three days of incubation. Among the 30 isolates, 11 isolates showed significantly strong inhibition (inhibition indices >3.0), 7 isolates showed moderate inhibition (inhibition indices between 2.0-3.0), 12 isolates showed weak inhibition (inhibition indices >2) (Table 3). The largest and lowest inhibition zone index was produced by endophytic bacterial isolates *Pseudomonas gessardii* (4.85) and *Bacillus licheniformis* (1.35).

Determination of antagonistic and PGP traits of bacterial isolates

These antagonist bacterial isolates were further tested for the production of α-amylase, siderophore, protease, IAA, and phosphate. A well-developed inhibition zone around the bacterial colonies in CAS Agar, SMA, Pikovskaya media confirmed the bacterial ability for productions of siderophore, protease, phosphate solubilization, respectively. An orange-brown color developed in addition to Nessler's reagent to bacterial culture in peptone water which indicated the bacterial ability to produce ammonia. The appearance of pink color in bacterial culture in NB supplemented with tryptophan following the addition of Salkowski reagent indicated the production of IAA by antagonist bacterial isolates.

Among the tested antagonist bacterial isolates, 10 isolates were positive for the production of α-amylase, 7 isolates positive for phosphatase production, 29 isolates positive for

Table 2. Identification of representative putative endophytic bacterial isolates

Isolate	Bacterial Species	Plant Part	Gram	Oxidase	Florescent
B1.1	*Achromobacter spanius*	P	-	+	-
B4.4	*Pseudomonas koreensis*	L	-	-	+
B6.4	*Rhizobium radiobacter*	R	-	-	-
B9.2	*Rhizobium radiobacter*	S	-	-	-
B11.4	*Bacillus simplex*	S	+	+	-
B12.3	*Bacillus simplex*	S	+	+	-
E1.4	*Acinetobacter calcoaceticus*	L	-	-	-
E5.3	*Rhizobium radiobacter*	R	-	-	-
E6.2	*Achromobacter spanius*	L	-	+	-
E8.4	*Bacillus thuringiensis*	S	+	+	-
E13.3	*Rhizobium radiobacter*	R	-	-	-
E14.6	*Bacillus farraginis*	R	+	+	-
E15.2	*Serratia liquefaciens*	L	-	-	-
E20.2	*Ochrobactrum anthropi*	R	-	+	-
E20.7	*Bacillus megaterium*	R	+	+	-
E21.2	*Exiguobacterium sp*	R	+	+	-
K13.2	*Pseudomonas extremorientalis*	S	-	-	+
K15.1	*Pseudomonas putida*	S	-	-	+
K18.1	*Microbacterium hydrocarbonoxydans*	S	+	-	-
N1.1	*Bacillus licheniformis*	P	+	-	-
N11.3	*Arthrobacter ilicis*	P	+	+	-
N13.11	*Arthrobacter oxydans*	P	+	-	-
N16.1	*Pseudomonas brassicacearum*	S	-	-	+
N17.1	*Bacillus cereus*	P	+	-	-
N17.2	*Bacillus endophyticus*	P	+	-	-
N24.3	*Pseudomonas rhodesiae*	S	-	+	+
N26.2	*Bacillus pumilus*	R	+	-	-
N29.2	*Bacillus siralis*	P	+	-	-
N34.1	*Serratia liquefaciens*	S	-	-	-
N35.1	*Pseudomonas gessardii*	S	-	+	+

P: pods; L: leaf; S: stem; R: root; +: positive reaction for tests; -: negative reaction for tests

siderophore production, 11 isolates positive for protease production. All selected bacterial isolates produced IAA and ammonia in a relatively varying amount (Table 3). Among the tested putative bacterial isolates, *Pseudomonas gessardii* had the highest index values for extracellular siderophore (5.83), *Exiguobacterium* sp. for extracellular protease (5.25), *Pseudomonas gessardii* and *Ochrobactrum anthropi* for extracellular IAA concentration (161.39 µg/ml)) and *Acinetobacter calcoaceticus* for phosphate solubilisation activities (2.63) (Table 3).

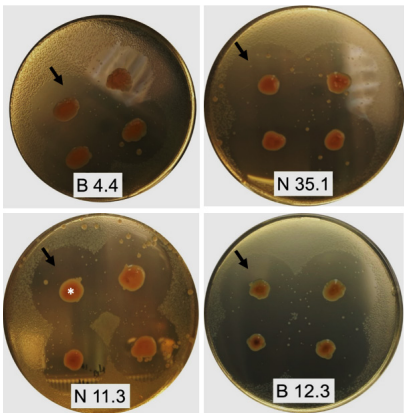

Figure 1. Determination of *in vitro* antagonistic efficacies of endophytic bacterial isolates on *Psp* growth in dual culture test. Note clear zones of inhibition (arrows) were produced by the antagonist bacterial isolates (*) against *Psp* growth in dual culture assay

Table 3. Determinations of antagonistic and plant growth promoting traits of endophytic bacterial isolates obtained from inner tissues of healthy bean plants

Isolate	Bacterial Species	Ant-EI	Sid-EI	Pro-EI	Phos-EI	IAA (µg/ml)	Ammonia (µg/ml)
B1.1	*Achromobacter spanius*	3,57 ^{BC}	1,77 $^{D-I}$	0,00 ^{G}	0,00 ^{F}	67,69 ^{IJ}	91,82 $^{J-L}$
B4.4	*Pseudomonas koreensis*	4,25 ^{AB}	2,25 $^{C-G}$	0,00 ^{G}	0,00 ^{F}	110,88 $^{D-G}$	105,76 $^{F-I}$
B6.4	*Rhizobium radiobacter*	1,97 $^{F-J}$	2,39 $^{C-F}$	1,50 ^{EF}	0,00 ^{F}	103,29 $^{E-H}$	63,94 ^{PQ}
B9.2	*Rhizobium radiobacter*	1,67 $^{H-J}$	1,22 ^{HI}	0,00 ^{G}	1,35 ^{E}	126,20 $^{B-F}$	172,59 ^{B}
B11.4	*Bacillus simplex*	1,90 $^{F-J}$	2,28 $^{C-G}$	1,52 ^{EF}	0,00 ^{F}	108,55 $^{D-G}$	108,18 $^{E-H}$
B12.3	*Bacillus simplex*	4,35 ^{AB}	3,50 ^{B}	0,00 ^{G}	0,00 ^{F}	112,35 $^{D-G}$	115,15 $^{C-F}$
E1.4	*Acinetobacter calcoaceticus*	3,08 $^{C-E}$	1,32 $^{G-I}$	0,00 ^{G}	2,63 ^{A}	145,04 $^{A-C}$	118,79 $^{C-E}$
E5.3	*Rhizobium radiobacter*	1,44 ^{IJ}	1,47 $^{F-I}$	0,00 ^{G}	0,00 ^{F}	124,67 $^{C-F}$	174,44 ^{B}
E6.2	*Achromobacter spanius*	1,93 $^{F-J}$	2,66 $^{B-D}$	3,94 ^{C}	0,00 ^{F}	109,43 $^{D-G}$	93,33 $^{J-L}$
E8.4	*Bacillus thuringiensis*	3,07 $^{C-E}$	1,78 $^{D-I}$	0,00 ^{G}	0,00 ^{F}	138,03 $^{A-D}$	84,24 $^{K-M}$
E13.3	*Rhizobium radiobacter*	2,17 $^{F-J}$	2,71 $^{B-D}$	0,00 ^{G}	0,00 ^{F}	161,39 ^{A}	89,7 $^{J-L}$
E14.6	*Bacillus farraginis*	2,24 $^{E-I}$	1,78 $^{D-I}$	4,56 ^{B}	0,00 ^{F}	84,65 $^{G-I}$	121,52 ^{CD}
E15.2	*Serratia liquefaciens*	2,02 $^{F-J}$	2,08 $^{C-H}$	5,25 ^{A}	0,00 ^{F}	41,91 ^{J}	101,52 $^{G-J}$
E20.2	*Ochrobactrum anthropi*	3,22 ^{CD}	1,34 $^{G-I}$	0,00 ^{G}	0,00 ^{F}	135,77 $^{A-D}$	187,78 ^{A}
E20.7	*Bacillus megaterium*	2,63 $^{D-F}$	2,65 $^{B-E}$	0,00 ^{G}	0,00 ^{F}	132,92 $^{A-E}$	119,09 $^{C-E}$
E21.2	*Exiguobacterium sp*	1,96 $^{F-J}$	1,63 $^{F-I}$	0,00 ^{G}	0,00 ^{F}	75,08 ^{HI}	109,7 $^{D-G}$
K13.2	*Pseudomonas extremorientalis*	2,45 $^{D-H}$	2,96 ^{BC}	2,12 ^{D}	0,00 ^{F}	61,68 ^{IJ}	107,88 $^{E-H}$
K15.1	*Pseudomonas putida*	3,56 ^{BC}	1,67 $^{F-I}$	0,00 ^{G}	0,00 ^{F}	145,4 $^{A-C}$	113,94 $^{D-F}$
K18.1	*Microbacterium hydrocarbonoxydans*	2,52 $^{D-G}$	1,65 $^{F-I}$	0,00 ^{G}	0,00 ^{F}	137,01 $^{A-D}$	126,06 ^{C}
N1.1	*Bacillus licheniformis*	1,35 ^{J}	1,37 $^{G-I}$	1,69 $^{D-F}$	0,00 ^{F}	155,79 ^{AB}	95,45 $^{I-K}$
N11.3	*Arthrobacter ilicis*	4,23 ^{AB}	1,69 $^{E-I}$	0,00 ^{G}	0,00 ^{F}	101,75 $^{F-H}$	126,06 ^{C}
N13.11	*Arthrobacter oxydans*	3,07 $^{C-E}$	1,53 $^{F-I}$	1,88 ^{DE}	2,50 ^{AB}	92,11 $^{G-I}$	167,41 ^{B}
N16.1	*Pseudomonas brassicacearum*	3,05 $^{C-E}$	1,09 ^{I}	0,00 ^{G}	2,06 $^{B-D}$	73,04 ^{HI}	96,36 $^{H-J}$
N17.1	*Bacillus cereus*	2,28 $^{E-I}$	0,00 ^{J}	0,00 ^{G}	0,00 ^{F}	69,12 ^{IJ}	81,52 $^{L-N}$
N17.2	*Bacillus endophyticus*	1,77 $^{G-J}$	1,53 $^{F-I}$	0,00 ^{G}	0,00 ^{F}	62,25 ^{IJ}	68,18 $^{O-P}$
N24.3	*Pseudomonas rhodesiae*	1,98 $^{F-J}$	1,23 ^{HI}	1,83 ^{DE}	0,00 ^{F}	75.0 ^{HI}	55,56 ^{Q}
N26.2	*Bacillus pumilus*	1,92 $^{F-J}$	1,62 $^{F-I}$	0,00 ^{G}	1,74 ^{DE}	74,26 ^{HI}	70,91 $^{N-P}$
N29.2	*Bacillus siralis*	1,89 $^{F-J}$	2,25 $^{C-G}$	0,00 ^{G}	1,88 ^{CD}	69,61 ^{IJ}	67,58 ^{OP}
N34.1	*Serratia liquefaciens*	1,91 $^{F-J}$	1,35 $^{G-I}$	2,08 ^{D}	2,25 $^{A-C}$	131,39 $^{A-F}$	77,27 $^{M-O}$
N35.1	*Pseudomonas gessardii*	4,85 ^{A}	5,83 ^{A}	1,32 ^{F}	0,00 ^{F}	161,39 ^{A}	64,55 ^{PQ}

Ant-EI: Antagonistic Efficiency Index; Sid-EI: Siderophore Production Efficiency Index; Pro-EI: Proteolytic Enzyme Efficiency Index; Phos-EI: Phosphate Solubilisation Efficiency Index. Mean values followed by different letters within each column were significantly different according to Least Significant Difference (LSD) test (P≤0.05)

DISCUSSION

Recently, several research has focused on the exploration of varied agroecological and biological niches for the existence of beneficial bacterial and fungal microbioms (El-Sayed et al. 2014). Healthy or wild plants, as the greatest potential resource for acquiring novel microorganisms and their products, are likely to harbor unique endophytic bacterial communities (Gamal-Eldin et al. 2008). These findings agree with the well-known observations that many microbiomes belonging to different genera (Podile and Kishore 2006) are capable of a resident endophytic phase of growth within the healthy host. In the current study, endophytic bacterial isolates with *in vitro* antagonistic and PGP traits were

isolated from apparently healthy bean plants from different agroecological regions in Turkey. These potential PGPR isolates were enumerated and screened *in vitro* for a broad spectrum of plant growth-promoting abilities as well as for antagonistic potential against bacterial halo blight disease agent *Psp*.

On the basis of morphological, physiological, biochemical characteristics and MALDI-TOF analyses, among 30 endophytic antagonist isolates tested *in vitro*, most antagonists belonged to the species of *Bacillus* and *Pseudomonas*. It was reported that *Bacillus* and *Pseudomonas* spp. occurs commonly on rhizosphere and aerial plant parts (Gibbins 1978) as endophtyically or epiphytically (Völksch et al. 1992). The different species belonging *Bacillus* and *Pseudomonas* have been reported to inhibit several fungal and bacterial plant pathogens (Beer et al. 1984, El-Goorani and Beer 1991, Völksch et al. 1993) and it is an effective competitor in biological prevention of frost injury (Lindow et al. 1983). Different species of *Pseudomonas* were reported to produce a variety of metabolites (Leisinger and Margaff 1979), some of which were implicated in the biological control of plant pathogenic fungal and bacterial disease agents (Dowling and O'Gara 1994). With this respect, antagonistic bacterial isolates belonging to *Pseudomonas* and Bacillus genera will be of interest as PGPB against *Psp* in future biological control experiments in the field.

Ojiambo and Scherm (2006) showed in their research that there was no difference in effectiveness of antagonist bacteria between studies conducted in the greenhouse versus the field between soil-borne versus aerial diseases or among those of low, medium, or high disease infection. Their study also showed that organisms which have short-lived offspring expressed more effective antagonism than those does not show this feature.

Besides that, Scots pine root-associated mycorrhizae-bacteria and wheat associated bacteria against *Fusarium culmorum*, *Rhizoctonia solani*, and *Botrytis cinerea* was tested by Dilfuza (2011). Production of some extracellular lytic enzymes by bacteria screened for their antagonistic activities. Results of the study showed that wheat-associated bacteria possessed more antagonistic activity compared to mycorrhizal hyphae. *Arthrobacter ilicis* KNCL24 isolate was one of the isolates that used during the study.

Another study conducted by Barrows-Broaddus et al. (1985) showed that *Arthrobacter* sp. isolates suppress disease emergence and this helped plants to close their wounds faster and more comfortable during the infection stage, that eliminates the propagation of *Fusarium moniliforme* var. *subglutinans* on slash pines.

Morrissey et al. (1976) found an *Arthrobacter* sp. which actively lyses *Fusarium roseum* cells by secreting chitinase (E.C. 3.2-1.14, chitin glycanohydrolase), enzyme which is essential for the hydrolysis of chitin. Enzyme production paralelled growth; liberation of enzyme took place during the log phase with the maximum yield being obtained at the stationary phase.

Another study conducted by Kang et al. (2009), tested for certain *Acinetobacter calcoaceticus* isolate that promotes plant growth by producing organic acids such as malic, succinic, and citric acids and lowering the endogenous abscisic acid contents. *A. calcoaceticus* strain has significantly promoted cucumber plants for grow better. The PGPR application had better shoot length, biomass, and chlorophyll contents compared to controls.

In another study, Deinema et al. (1985), showed that *Acinetobacter* strain 210A had accumulated large amounts of phosphate in soils, which can tolerate nutrient uptake deficiencies in poor conditioned soils.

Phylloplane associated bacteria *Microbacterium hydrocarbonoxydans* shows antimicrobial activity against *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Gaeumannomyces graminis* var. *tritici* (Wang et al. 2015) besides this bacterium shows plant growth promoting activities on commercial wheat varieties (Batool et al. 2016). Also, soil remediation and weed control properties were investigated by Barghouthi and Salman (2010). Surprisingly *M. hydrocarbonoxydans* bacterium is also carried diamino acid in the cell-wall peptidoglycan of BAS69T is lysine and of BNP48T is ornithine. The significant menaquinones are MK-11 and MK-12 based on their crude oil degrading ability (Schippers et al. 2005).

Matthijs et al. (2014) showed in their study that mupirocin is a broad spectrum antibiotic has polypeptide structure which was isolated from *Pseudomonas fluorescens* NCIMB 10586. Study was based on the phylogenetic distribution of mupirocin producing strains of *Pseudomonas* genus. The *mmpD* gene of showed the mupirocin gene cluster. They all five *mmpD*+ isolates produced mupirocin and were strongly antagonistic against *Staphylococcus aureus*. In their study *Pseudomonas gessardii* was one of the isolates showed large amounts of mupirocin production.

Dilfuza et al. (2015) showed that microbial indole-3-acetic acid (IAA) played an important role in the regulation of root growth and plant biomass and antagonistic activity on controlling root-rot of cotton which is caused by *F. solani*. In their study even very low quantities of IAA such as 0.01 and 0.001 µg/ml can lower the disease incidence and increase

the plant growth. It shows that bacterial secreted IAA plays a significant role in salt stress tolerance and may be involved in induced resistance against root rot disease of cotton.

In conclusion, bean plant hosts several active endophytic bacterial species without any side effect to plant. The data reported here clearly describes the characterization of endophytic bacterial isolates which exhibit excellent abilities to antagonize the halo blight disease agent *Psp* by exhibiting several antagonistic and plant growth-promoting mechanisms including production of siderophore, protease, ammonia, IAA production, and phosphate solubilization activities. Further studies are needed to determine the exact mode of action of each endophytic bacterial isolate, which will help to improve consistency of a commercial biocontrol product in the field conditions.

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ÖZET

Pseudomonas syringae pv. *phaseolicola* (*Psp*) fasulye bitkisinde Hale yanıklığı hastalığına sebep olan tohum kökenli bir bakteriyel hastalık etmenidir. Bu çalışmanın amacı, farklı bölgelerde yetişen sağlıklı fasulye bitkilerinden izole edilen endofitik bitki gelişimini teşvik eden bakterilerin (PGPB) *Psp* üzerine antagonistik (siderofor ve amonyak üretimi gibi) ve bitki gelişimini teşvik eden mekanizmalarının (IAA üretimi, fosfatı indirgeme özelliği gibi) *in vitro* koşullarda karakterizasyonudur. Araştırma sonunda elde edilen 131 PGPB arasında 71 izolat *Psp* etmenine karşı değişen oranlarda engelleme zonu oluşturmuş olup, bu izolatları temsilen 30 izolatın teşhisleri, antagonistik ve bitki gelişimini teşvik eden mekanizmaları araştırılmıştır. Morfolojik, fizyolojik, biyokimyasal ve MALDI-TOF analizler, elde edilen izolatların 10 cins 24 farklı türe ait olduğunu göstermiştir. Bu izolatlar arasında 10 izolat *Bacillus* spp., 6 izolat *Pseudomonas* spp., 4 izolat *Rhizobium radiobacter*, 2 izolat *Arthrobacter* spp., 2 izolat *Achromobacter spanius*, 2 izolat *Serratia liquefaciens*, 1'er izolat *Acinetobacter calcoaceticus*, *Exiguobacterium* sp., *Microbacterium hydrocarbonoxydans* ve *Ochrobactrum anthropi* olarak teşhis edilmiştir. *Psp*'ye karşı en yüksek engelleme zonu *Pseudomonas gessardii* (4.85) izolatı tarafından, en düşük engelleme zonu ise *Bacillus licheniformis* (1.35) tarafından oluşturulmuştur. Test edilen izolatlardan 10 izolat α-amylase pozitif, 7 izolat fosfataz pozitif, 29 izolat siderofor üretmiş, 11 izolat proteaz pozitif bulunmuştur. Tüm izolatlar IAA ve amonyak üretimi açısından değişken değerlerde pozitif bulunmuştur. En yüksek siderofor üretimi *P. gessardii* (5.83 indeks değeri), en yüksek protease üretimi *Exiguobacterium* sp. (5.25 indeks değeri), en yüksek IAA üretimi *P. gessardii* ve *O. anthropi* izolatları (161.39 µg/ml), en yüksek fosfor çözme kapasitesi *Acinetobacter calcoaceticus* izolatı (2.63 indeks değeri) tarafından gösterilmiştir. Bu çalışma ile ilk kez fasulye bitkisinde bitki gelişimini teşvik eden ve *Psp*'ye antagonistik etkiye sahip endofit bakteriler elde edilmiştir.

Anahtar kelimeler: fasulye, hale yanıklığı, antagonist, bitki gelişimi teşvik eden bakteriler, endofit, biyolojik mücadele

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