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Determination of antagonistic potential of endophytic bacteria isolated from lettuce against lettuce white mould disease caused by *Sclerotinia sclerotiorum*

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Abstract

In this study, biocontrol efficiency of endophytic bacterial isolates obtained from the leaf lettuce (*Lactuca sativa* L.) healthy plants was investigated against white mould disease agent *Sclerotinia sclerotiorum in vitro* and *in vivo* conditions. Antagonistic efficiency of endophytic bacterial isolates was determined to inhibit mycelial growth and sclerotial germination, suppress disease incidence caused by *S. sclerotiorum*. A total of 48 endophytic bacterial isolates were isolated from different tissues of lettuce healthy plants. Mycelial growth and germination of sclerotia of *S. sclerotiorum in vitro* inhibited 18 bacterial isolates. *Bacillus subtilis* and *B. amyloliquefaciens* isolates were found to be the most efficient ones, which significantly inhibited the mycelial growth by 68.1–83.1%, germination of sclerotia by 82.7–89.6% and suppressed disease incidence by 55.7–75% caused by *S. sclerotiorum*. In addition, the fungal mycelium close to the inhibition zone in dual culture was denser and darker in colour. *B. subtilis* and *B. amyloliquefaciens* isolates caused significant morphological alterations in hyphae such as hyphal shrivelling and perforation close to the inhibition zone in dual culture.
Significant suppression in the mycelial growth, sclerotial germination and disease incidence caused by endophytic bacterial isolates indicate that isolates of *B. subtilis* and *B. amyloliquefaciens* could be considered as possible biocontrol agents against soil-borne fungal diseases.

Key words: *Lactuca sativa*, antagonist, biological control, endophytes, *Sclerotinia sclerotiorum*.

Introduction

Leaf lettuce (*Lactuca sativa* L.) is one of the most economically important leafy vegetable crops worldwide. The leading producers of lettuce were Europe and North America; however, since the late 20th century, lettuce production and consumption spread throughout the world. At present, the leading producer of lettuce in the world is China followed by the USA. Turkey, which ranks 10th among the leading lettuce producing countries in the world, produced 500.000 tonnes from 23.335 ha area (FAOSTAT, 2019; http://www.fao.org/faostat/en/#data/QC). This crop is cultivated in several provinces of Turkey and Eastern Mediterranean region accounting for over 45% of Turkish production (Turkish Statistical Institute, 2021; https://biruni.tuik.gov.tr/medas/?kn=92&locale=tr). Lettuce production mainly occurs in three provinces (Adana, Mersin and Hatay) within this region.

The fungal pathogens in the genus *Sclerotinia* are worldwide distributed and attack several vegetable crops including lettuce (Rothmann, McLaren, 2018). *Sclerotinia sclerotiorum* (Lib) de Bary, a destructive fungal pathogen with an extensive host range infecting more than 400 plant species, causes white mould and leaf drop disease on the leafy green lettuce, which has an enormous economic impact on lettuce cultivation causing significant yield losses to growers worldwide (Ni et al., 2014). Disease agent causes remarkable yield losses of up to 60% in individual fields (Hao, Subbarao, 2005). In Turkey, *S. sclerotiorum* is also the predominant soil-borne fungal pathogen determined in the Eastern Mediterranean region surveyed (Soylu et al., 2017).

The most obvious signs of the disease are defined by a watery soft rot accompanied by the appearance of a white fluffy mycelial growth on the crown of infected

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plant. Dark sclerotia are subsequently formed on infected plant parts and soil around roots. Sclerotia are overwintering fungal structures that resist environmental factors and fungicides and remain viable in the soil for many years (Ordóñez -Valencia et al., 2014).

To control lettuce white mould disease agent in Turkey, effective control measures are currently not available. Use of resistant cultivars, solarisation, drip irrigation, crop rotation and soil disinfections were major cultural practices offered for disease management (Collange et al., 2014). However, the fungicides registered against the fungal disease agent do not adequately control the diseases (Zhu et al., 2016). Since the disease agent has a wide host range and produces overwintering sclerotia that survive in soil for a long time, crop rotation is usually not an effective control practice against it (Rothmann, McLaren, 2018). Very few fungicides such as Dicloran, Iprodione and Vinclozolin are registered with a low level of disease control in most production areas (Derbyshire, Denton-Giles, 2016). The efficacies of these fungicides have been reduced due to their frequent uses in the areas, where intensive lettuce cultivation is carried out and due to their rapid degradation in soil (Collange et al., 2014). Public concern over frequent pesticide usage, pesticide residue on lettuce leaves, development of fungicide resistance and the need for a higher level of disease control promote non-chemical approaches for disease agent (Smolińska, Kowalska, 2018).

Because of the harmful side-effects of fungicides on the environment, eco-friendly agricultural practices using bacteria, fungi or actinomycetes have become an alternative to the chemical control of soil-borne pathogens such as *Sclerotinia* (Chen et al., 2016). A number of bacterial biological control agents such as *Paenibacillus alvei* (Fatouros et al., 2018), *Streptomyces* spp. (Chen et al., 2016), *Bacillus subtilis* (Hu et al., 2014) and *Burkholderia pyrrocinia* (Lee et al., 2011) have been successfully used against *S. sclerotiorum*. The mechanisms for the suppression of pathogens by biological control agents include mycoparasitism, antagonism via production of secondary antimicrobial compounds, competition for space and resources, induction of plant resistance and antibiosis (Ahemad, Kibret, 2014).

Plants are hosts to a wide range of beneficial microorganisms such as fungal, bacterial and viral agents. Plant associated beneficial bacteria, also known as plant growth promoting bacteria (PGPB), either colonize epiphytically the rhizosphere (called rhizobacteria) or reside inside the plant tissue in a mutualistic relationship (called as endophytes) (Beneduzi et al., 2012; Sülü et al., 2016; Soylu, Bozkurt, 2019). Since PGPB multiply well enough and produce a wide range of antimicrobial compounds, the use of endophytic bacteria has been of high interest as biocontrol agents for a wide range of soil-borne fungal diseases (Eljounaidi et al., 2016; Santoyo et al., 2016). Bacterial endophytes possess important role in plant growth promotion by increasing nutrient uptake and mineral solubilisation and protecting host plants against their potential pathogens inducing active disease resistance mechanisms (Ahemad, Kibret, 2014).

Although antagonistic potential and mode of mechanisms of epiphytic antagonist bacterial isolates have been studied against *S. sclerotiorum* in economically important crop plants such as rapeseed (Kamal et al., 2015), tomato (Abdeljalil et al., 2016) and lettuce (Chen et al., 2016; Fatouros et al., 2018; Oliver et al., 2019), to the best of our knowledge there is no research, which examined the use of endophytic bacterial species identified in this study for biological control of white mould disease of lettuce *in vitro* and *in vivo*. Amongst the endophytic bacterial species, especially *Bacillus* spp., there were the most studied biocontrol agents used for white mould disease.

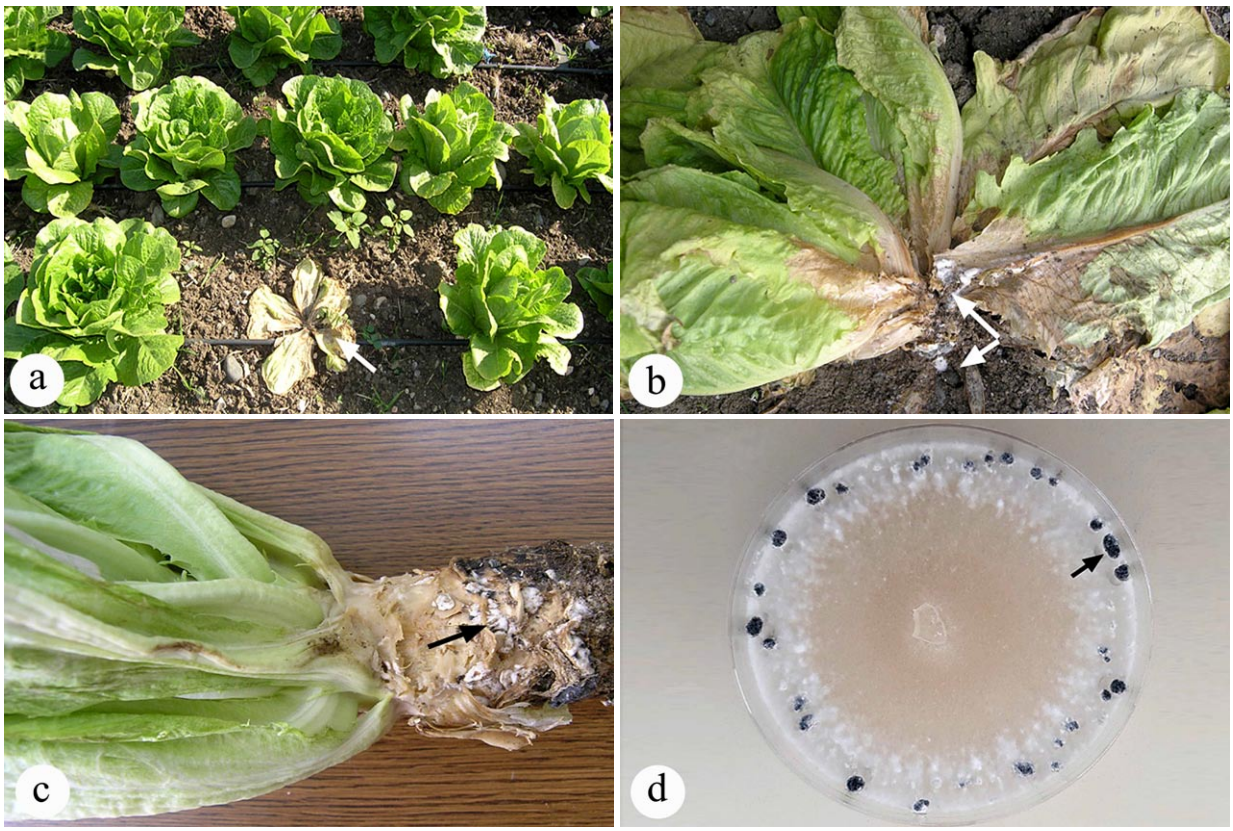
The aim of this study was isolation, characterization and *in vitro* and *in vivo* evaluation of antagonistic properties of endophytic bacteria from inner parts of lettuce healthy plants for utilization as a possible biocontrol agent against white mould disease agent *S. sclerotiorum*. Efficacy of endophytic bacterial isolates on suppression of mycelial growth, the viability of sclerotia and disease incidence were investigated *in vitro* and *in vivo*. The possible mechanism of antagonism was also evaluated under the light microscope.

Materials and methods

Isolation and identification of fungal disease agents. During the disease survey conducted in the 2016–2017 growing season, *Sclerotinia sclerotiorum* was isolated from sclerotia developed on the infected leaf lettuce (*Lactuca sativa*, cultivar 'Göbekli') plants (Figure 1) growing in a small-scale organic farming field in Antakya district (36°19′21″ N, 36°12′14″ E) of Hatay province of Turkey. Sclerotia were removed from infected plants, surface-disinfected and placed on potato sucrose agar (PDA) (Merck, Germany) supplemented with 50 mg ml^{-1} streptomycin sulphate and incubated at 25 ± 1°C temperature for 5–7 days. Isolation from surface-disinfested sclerotia on PDA consistently yielded white mycelia with black sclerotia. For molecular analysis, pure fungal isolates were preserved on PDA plates and kept at 4°C temperature.

Representative isolates (n = 10) on PDA plate were deposited in the Hatay Mustafa Kemal University BİSAK Microbial Culture Collection Centre (isolate Nos. MKUBK-LSs1-10). The identification of representative fungal isolate No. MKUBK-LSs5 was confirmed by analysing matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) MicroFlex LT (Bruker Daltonics, Germany). Fungal isolate was grown in sterile potato sucrose broth (PDB) for 3 days. Then mycelial masses were collected from nutrient broth and subjected for formic acid-ethanol extraction method according to manufacturer's instructions (Kurt et al., 2017). To confirm the morphological and MALDI-TOF MS identification, fungal genomic DNA was extracted from fungal mycelia of representative isolate No. MKUBK-LSs5 by using a DNeasy Plant Mini Kit (Qiagen Inc., Germany) according to manufacturer's instructions. The extracted DNA was then used as template for subsequent amplification using PCR. The internal transcribed spacer (ITS) region of rDNA of representative isolate No. MKUBK-LSs5 was amplified using primer ITS1/ITS4 and directly sequenced. The resulting ITS sequences were deposited in GenBank (accession No. MZ558499).

Isolation and identification of endophytic bacterial isolates. Endophytic bacterial isolates were obtained from leaves, stems and roots of apparently lettuce 48 healthy plants growing in 16 fields in 5 locations in Kırıkhan (36°28′06″N, 36°20′33″E), Reyhanlı (36°14′40″ N, 36°22′22″ E; 36°23′48″, N 36°32′25″ E) and Antakya (36°19′16″ N, 36°12′45″ E; 36°20′26″ N, 36°14′20″ E) districts of Hatay province, Turkey. The entire plants



Note. Typical lettuce white mould and drop disease causing the lower leaves to wilt and drop to the soil surface (a, b); the black sclerotia of *S. sclerotiorum* (arrows) on crown, leaves and stem of infected lettuce plants and soil surface (b, c); mycelial growth and formation of sclerotia (arrow) on PDA (d).

Figure 1. Lettuce plants infected with white mould and drop disease agent *Sclerotinia sclerotiorum*

were flushed with running tap water to remove the soil and rinsed with sterile distilled water (SDW). Roots, stems and leaves of healthy plants were separated. All samples were subjected to surface sterilization procedure and macerated individually in 10 mM $MgCl_2$ (magnesium chloride). Then serially diluted suspensions were spread onto plates with King B (KB) agar (Merck). Surface sterilised healthy tissues were also aseptically cut into small fragments and inside of each tissue were imprinted by touching directly on the KB agar according to Duman and Soylu (2019). All Petri plates were incubated at 25 ± 1°C temperature for 2 days. Based on morphological characteristics, representative bacterial isolates were selected from a single colony, re-streaked on fresh KB agar and maintained at 4°C temperature for routine *in vitro* tests. For long-term storage, all bacterial isolates were kept at −80°C temperature in 40% (v v^{-1}) glycerol solution. For hypersensitive reaction (HR), putative endophytic bacterial isolates were tested on tobacco (*Nicotiana tabacum* L.) plant leaves and potato soft rot test on potato slices. As putative antagonist bacterial isolates, only isolates that did not cause HR and soft rot symptoms were selected.

Following preliminary characterization of putative antagonist bacterial isolates by applying biochemical tests, MALDI-TOF MS based identification was used to confirm the identity of putative antagonist bacterial isolates. In many cases, MALDI-TOF MS based identifications, using peptide mass fingerprint, have shown resolution and reproducibility, which is better than gel-based protein or DNA finger printing techniques (Singhal et al., 2015).

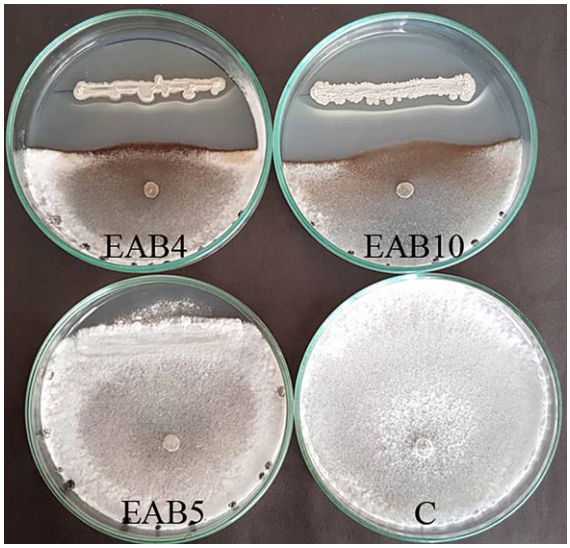
The bacterial mass from the pure colonies developed for 24–36 h on tryptic soy agar (TSA) medium (Merck) was subjected to ethanol-formic acid-pure acetonitrile protein extraction according to manufacturer's instructions. A total of 1 µl of the extract was dropped onto the MALDI target, dried and overlaid with 1 µl matrix solution, as described previously (Duman, Soylu, 2019). Microorganism library was used as software *BIOTYPER*, version 1.1 (Bruker Daltonics GmbH, Germany).

Determination of antagonistic activities of endophytic bacterial isolates on mycelial growth. Antagonistic activity of individual endophytic bacterial isolates was determined on PDA on Petri plates by using a dual culture test (Pane et al., 2012). A loopful of putative bacterial isolates was streaked on Petri plates containing PDA and pre-incubated at 25 ± 1°C temperature for 2 days. Mycelial plugs (5 mm diameter) of the fungal pathogen were placed on the same plates approximately 4 cm away from the bacterial mass 48 h after incubation (Figure 2).

As control plates, mycelial plugs of fungal pathogen without bacterial isolates were used. All Petri plates were incubated at 25 ± 1°C temperature for 4–5 days. The radius of fungal growth towards the bacteria on dual culture and control Petri plates was measured in mm 4–5 days after inoculation and mycelial growth inhibition (MGI) percentage – in relation to the control by using the formula:

$$MGI\ (\%) = [(MGc - MGt) / MGc] \times 100,$$

where MGc and MGt are mycelial growth in control and bacterial isolate drawn Petri plates, respectively.



Note. Strong (*Bacillus amyloliquefaciens* EAB4 and *B. subtilis* EAB10 isolates) and weak (*Sphingomonas yanoikuyae* EAB5 isolate) inhibition of mycelial growth on dual culture plates; C – mycelial growth of pathogen in the absence of antagonist bacterial isolate.

Figure 2. Inhibition of mycelial growth of *Sclerotinia sclerotiorum* by antagonist bacterial isolates in dual culture

Three Petri plates were used for each bacterial isolate, and the dual culture test was repeated at least twice. To characterise the nature of antagonistic effect of diffused metabolite(s) from bacterial isolates, agar plugs taken from the near edge of inhibition zones were placed on fresh PDA plate and incubated at 25 ± 1°C temperature for 3 days. The antagonistic effect was considered fungistatic, if mycelial growth from the plug occurred or fungicidal and if no pathogen growth occurred.

Determination of antagonistic activities of endophytic bacterial isolates on sclerotia viability. Antagonistic activities of the endophytes were also tested on the viability of sclerotia (Soylu et al., 2007). Obtained from the overnight cultures and suspended in SDW at 10^8 CFU ml^{-1} concentration, 5 ml of bacterial suspension was mixed with 10 g of steam-sterilized natural sandy soil and added into Petri plates. Fungal sclerotia were gently removed from 3-week-old PDA culture grown at 25 ± 1°C temperature. Sclerotia (n = 10) were buried at 1 cm depth in inoculated soil. All treated Petri plates were incubated at 25 ± 1°C temperature for 4 weeks. As control, sclerotia in sandy soil treated with SDW was used. The viability of treated sclerotia was assessed, and the number of sclerotia showing mycelial growth on PDA plates was evaluated 4 weeks after incubation at 20°C temperature.

Inhibition percentage of sclerotial germination was calculated by counting germinating sclerotia showing mycelial growth (n = 10) sown into soil by using the formula:

Inhibition (%) = [(GSc − GSt) / GSc] × 100, where GSc and GSt are germinating sclerotia showing mycelial growth in control treatment and bacterial suspension treated sandy soil. For each bacterial isolate, three plates were used, and the experiments were repeated at least twice.

Determination of in vivo antagonistic activities of endophytic bacterial isolates on disease incidence. *In vivo* bioassays studies were conducted in sterile commercial steam-sterilized peat soil (Soylu et al., 2007). Inoculum of *S. sclerotiorum* was prepared by culturing disease agent on oat grains. Oat grains (50 g) were placed in 500 ml flasks, filled with 100 ml PDP solution and autoclaved at 121°C temperature for 30 min. Autoclaved oat seeds in flasks were then inoculated with 20 agar discs (6 mm in diameter) containing fungal mycelia taken from 6-days-old fungal cultures. Flasks were incubated at 25°C temperature in the dark for 2 weeks. As control, inoculum of autoclaved oat seeds without pathogen was considered. To confirm the presence or absence of *S. sclerotiorum* inoculum, inoculated oat grains were cultured on PDA plates and observed for pathogen growth. The resulting fungal oat inoculum was mixed with steam-sterilized peat soil in a sterile plastic bag before sowing the cultivar 'Göbekli' lettuce seeds, as reported previously (Pane et al., 2012). The *S. sclerotiorum* inoculum contained 5.3 CFU g^{-1} soil.

Pots (7 cm diameter) were filled with inoculated and non-inoculated peat soil (100 g). The bacterial suspension (10^8 CFU ml^{-1}) was prepared in nutrient broth (NB) solution, and then each pot (n = 3) was drenched with 20 ml of antagonist bacterial suspension (10^8 CFU ml^{-1}). Experiments were arranged in a growth chamber at 25 ± 1°C temperature following a completely randomized block design. One week after bacterial treatment, the surface sterilized lettuce seeds (n = 20) were sown in the bacterial suspension treated peat soil. The pots were kept covered with plastic stretch wrap film and maintained for 10 weeks. As control treatment, pathogen non-inoculated or inoculated non-amended with the antagonist bacterial suspension soils were considered. The pots were watered with SDW twice weekly. Emerging and surviving healthy plants (without any sign of disease) from each treatment were recorded.

Protection percentage rate was calculated by counting the seedlings that developed and showed signs of disease symptoms from the total seeds (n = 20) planted in soil inoculated with *S. sclerotiorum* by using the formula:

Protection (%) = [(DSc − DSt) / DSc] × 100, where DSc and DSt are diseased seedlings in control treatment and bacterial suspension treated peat soil (Soylu et al., 2007). For each bacterial isolate, three pots were used, and the experiments were repeated at least twice.

Morphological changes caused by endophytic bacteria on hyphal morphology. Determination of antagonistic effect of endophytes on hyphal morphology of fungal pathogen was studied 7 days after inoculation with co-culturing both microorganisms side by side on the same PDA plates. Thin layers (3–4 cm^2) of agar blocks were cut from the adjacent site to the inhibition zone caused by endophytic bacterial isolates, placed on microscope glass slides, and hyphae at the adjacent site to the inhibition zone were examined under Nomarski DIC-equipped light microscope (Olympus BX51, Japan).

Statistical analysis. For all calculations, *SPSS Statistics* for *Windows*, version 17.0 (SPSS Inc., USA) was performed. Data from the *in vitro* and *in vivo* assays were processed with the one-way analysis of variance (ANOVA). When the ANOVA was significant at $P \leq 0.05$, means were separated with Duncan's multiple range test.

Results and discussion

Isolation, identification and determination of antagonistic potential of endophytic bacterial isolates. A total of 48 putative endophytic bacterial isolates were isolated and purified from lettuce 45 healthy plants collected from 15 fields in three districts of Hatay province. To suppress fungal growth using the dual culture test, these isolates were tested individually. Among the tested 48 putative bacterial isolates (data not shown), 18 antagonist bacterial isolates (37.5%) were found to cause inhibition zones by inhibiting the mycelial growth of *S. sclerotiorum* to a varying degree. Among the 18 antagonistic bacterial isolates, 8 isolates (44.4%) obtained from seven fields (wheat-cotton polyculture), 3 isolates (16.6%) from three fields (wheat-maize polyculture), 4 isolates (22.2%) from four fields (carrot-tomato polyculture) and 3 isolates (16.6%) from one field (wheat-pepper polyculture) (Table 1).

Based on the morphological characteristics and MALDI-TOF analysis, antagonist endophytes showing clear antagonism *in vitro* against *S. sclerotiorum* were identified at the species level. Based on MALDI-TOF analysis, endophytic bacteria were identified as *Bacillus* spp. (8), *Stenotrophomona maltophilia* (2) and one each as *Brevibacillus laterosporus*, *Lactobacillus acidophilus*, *Pantoea agglomerans*, *Sphingomonas yanoikuyae*, *Pseudomonas fluorescence*, *Enterobacter cloacae*, *Rhizobium radiobacter* and *Serratia marcescens* (Table 1). The identification of bacterial and fungal species using proteomic approach such as MALDI-TOF MS has proven be a reliable bacterial identification system becoming popular as an alternative to chromatographic and even DNA-dependent molecular methods (Panda et al., 2013). In several studies, MALDI-TOF MS has been found to be more accurate for bacterial species identification than conventional diagnostic methods (Singhal et al., 2015).

Table 1. The antagonistic potential of endophytic bacterial isolates on the inhibition of mycelial growth of *Sclerotinia sclerotiorum in vitro*

Bacterial species	Origin (district)	Mycelial growth mm	Inhibition %
Serratia marcescens EAB1	Hassa	31.0 g	39.6
Bacillus subtilis EAB2	Antakya	16.0 h	68.8
Bacillus subtilis EAB3	Antakya	14.7 hi	71.4
Bacillus amyloliquefaciens EAB4	Kırıkhan	15.0 h	70.8
Sphingomonas yanoikuyae EAB5	Kırıkhan	43.3 b-d	15.6
Stenotrophomona maltophilia EAB6	Kırıkhan	37.7 d-f	26.6
Bacillus amyloliquefaciens EAB7	Antakya	16.3 h	68.2
Bacillus subtilis EAB8	Antakya	13.7 h-i	73.4
Bacillus subtilis EAB9	Antakya	9.3 i	81.8
Bacillus subtilis EAB10	Antakya	8.7 k	83.1
Pantoe agglomerans EAB11	Reyhanlı	40.0 b-e	22.1
Stenotrophomonas maltophilia EAB12	Reyhanlı	38.3 c-f	25.3
Pseudomonas fluorescence EAB13	Reyhanlı	45.0 b	12.3
Bacillus simplex EAB14	Reyhanlı	34.0 fg	33.8
Brevibacillus laterosporus EAB15	Hassa	36.0 e-g	29.9
Enterobacter cloacae EAB16	Hassa	43.0 b-d	16.2
Lactobacillus acidophilus EAB17	Kırıkhan	33.0 fg	35.7
Rhizobium radiobacter EAB18	Antakya	44.0 bc	14.3
S. sclerotiorum (control)	Antakya	51.3 a	0.0

Note. Mean values of mycelial growth given in column followed by the same letters are not significantly different.

Given several proven track records of the accuracy of the MALDI-TOF bacterial identification method, it was assumed that endophytic antagonist bacterial isolates used in this study were identified correctly. Several species belonging to these genera were reported as the most reported as endophytic biocontrol agents (Santoyo et al., 2016; Sülü et al., 2016). In recent years, researchers have started to focus on the biocontrol potential of endophytic bacteria against different plant disease agents (Eljounaidi et al., 2016).

Antifungal activity of endophytic bacterial on mycelial growth and germination of sclerotia. Soil-borne fungal pathogens cause considerable damage to agriculturally important crops, and they have been often targeted in biological control studies. Therefore, antagonistic activities of selected 18 bacterial isolates were further screened against *S. sclerotiorum* (Table 1). Hyphal growth of disease agent was significantly ($P < 0.01$) suppressed by 18 endophytic bacterial isolates obtained from lettuce healthy plant tissues. Among the tested bacterial isolates, the most efficient isolate was found to be *B. subtilis* EAB10, which significantly inhibited the growth of *S. sclerotiorum* (83.1%) from the others (Figure 2). Notably, using dual culture test on PDA, mycelial growth was also suppressed by other isolates of *Bacillus* spp.: *B. subtilis* EAB9 (81.8%), *B. subtilis* EAB8 (73.4%), *B. subtilis* EAB3 (71.4%), *B. amyloliquefaciens* EAB4 (70.8%), *B. subtilis* EAB2 (68.8%) and *B. amyloliquefaciens* EAB7 (68.2%) (Table 1, Figure 2).

In previous studies, epiphytic bacterial isolates of *Pseudomonas* spp. (Soylu et al., 2005; Loewen et al., 2014; Oliver et al., 2019), *Burkholderia* spp. (Lee et al., 2011), *Bacillus* spp. (Soylu et al., 2005; Pane et al., 2012), *Paenibacillus alvei* (Fatouros et al., 2018) and *Streptomyces* (Chen et al., 2016) were isolated from plant surface or soil and found to inhibit *in vitro* fungal growth. However, this is the first study that evaluated the antagonistic potential of endophytic bacterial isolates to manage disease agent on lettuce *in vitro* and *in vivo*.

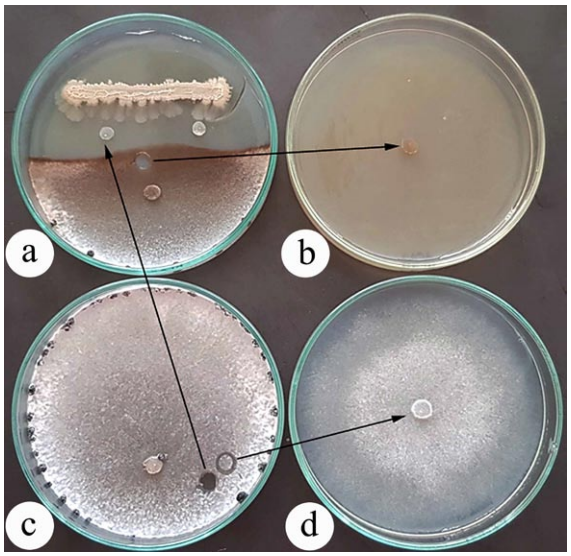
Bacillus species may produce a wide range of secondary metabolites (Zhao et al. 2012). Biocontrol potential of epiphytic bacterial species of *B. subtilis*, *B. amyloliquefaciens* and *B. pumilis*, living in and around

roots of a wide range of crop plants, was tested against root-infecting soil-borne fungal disease agents such as *Macrophomina phaseolina*, *Sclerotinia* spp., *Rhizoctonia solani* and *Fusarium* spp. (Kamal et al., 2015; Abdeljalil et al., 2016; Imriz et al., 2020).

Nowadays, for controlling pathogens, there is considerable interest in developing biological strategies using endophytic biological control agents with antimicrobial activities (Eljounaidi et al., 2016). Chen et al. (2014) isolated endophytic isolate of *B. subtilis* from the rapeseed plant. Bacterial isolate was then evaluated for its suppressive potential on the hyphal growth and sclerotial germination of *S. sclerotiorum*.

On dual culture plate, *B. subtilis* and *B. amyloliquefaciens* isolates caused considerable morphological changes on the fungal hyphae close to the inhibition zone, which were denser and darker in colour (Figures 2 and 3a). When lysed dark mycelial plugs close to the inhibition zone were placed on fresh PDA plate in the absence of antagonist bacteria, fungus typically failed to grow on the PDA plate (Figure 3b). Also, no mycelial growth was observed, when healthy mycelial plugs were placed on the inhibition zone caused by isolates of *B. subtilis* or *B. amyloliquefaciens* (Figure 3a). This test confirms that diffusible metabolite(s) of *B. subtilis* or *B. amyloliquefaciens* isolate were fungicidal to *S. sclerotiorum*. However, typical mycelial growth was observed, when the unaffected white mycelial plugs (Figure 3c) transferred fresh PDA in the absence of antagonist bacteria (Figure 3d).

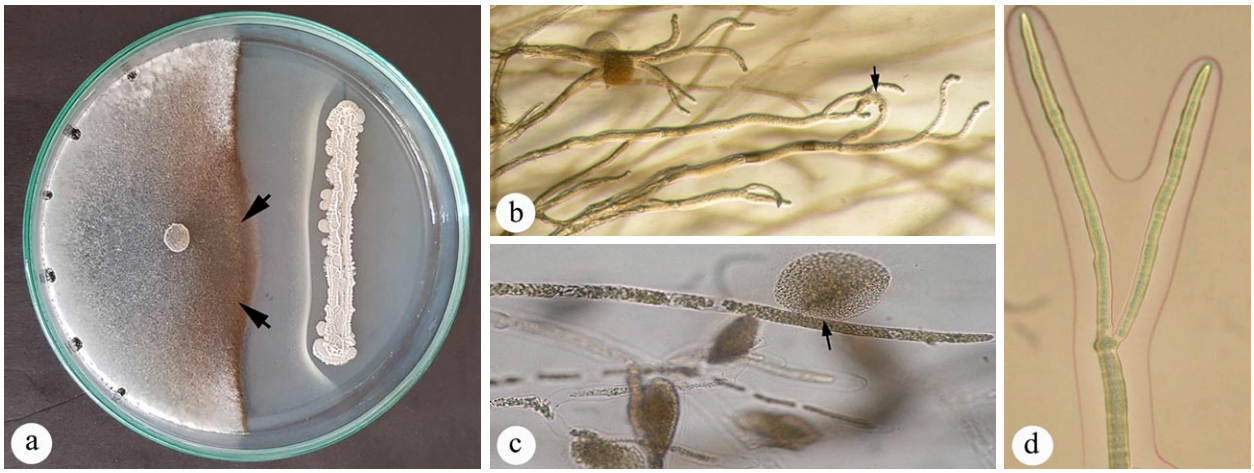
Light microscopic observations on affected hyphae from adjacent site to the inhibition zone caused by *B. subtilis* EAB10 isolate (Figure 4a) showed loss of integrity, hyphal shrivelling and perforation, mycelial degradation and hyphal lysis and fragmentation (Figure 4b, c) compared with thick, elongated and normal mycelial growth in control plates (Figure 4d). In addition, abnormal appearance and leakage of the fungal cell contents from hyphal tips and cells around the inhibition zone were frequently observed (Figure 4b, c). Such abnormalities in fungal hyphae could be caused by bacterial origin secondary metabolites and/or diffusible lytic enzyme(s) such as chitinase, glucanase and proteases synthesised during antagonism suggested earlier (Geraldine et al., 2013). The degenerated hyphae and lysed cells in fungal hyphae strongly supported the role of cell wall degrading enzyme(s) in the antifungal activity of these isolates (Figure 4).



Note. Inhibition of healthy hyphal discs (arrows) on the inhibition zone, where bacterial metabolite(s) gradually diffused (a); the dark coloured affected hyphal disc (*) (b), taken from interaction region adjacent to the clear zone of inhibition caused by *B. subtilis* AEB10 (a), was unable to grow on PDA plate (b) indicating fungicidal activity of the bacterial metabolite(s); mycelial growth of pathogen without bacterial isolate in control plates (c, d).

Figure 3. Fungicidal activity of the inhibition zone caused by *Bacillus* isolate in dual culture

Since chitin and β-glucans are both main constituents of fungal walls of Oomycetes and *Sclerotinia* species (Hernández-León et al., 2015), malformation observed in fungal hyphae caused by *Bacillus* isolates, especially by *B. subtilis* EAB10 and *B. amyloliquefaciens* EAB4, could be one of the possible antagonistic mechanisms against *S. sclerotiorum*. The contribution of cell wall degrading enzymes to antifungal activity



Note. Dark coloured affected hypha at interaction region (arrows) adjacent to the clear zone of inhibition caused by *B. subtilis* AEB 10 (a); morphological deformities and lysis of fungal hyphae (arrow) (b, c); healthy thick fungal hyphae in control plate (d).

Figure 4. Changes in the structural integrity of fungal hyphae adjacent to the inhibition zone caused by *Bacillus subtilis* AEB10 isolate

against different plant disease agents and sclerotia forming fungal disease agents has been suggested for certain isolates of *B. subtilis, B. amyloliquefaciens* and *B. mojavensis* (Ahemad, Kibret, 2014; Youcef-Ali et al., 2014). In previous studies (Gupta et al., 2001), *Pseudomonas* sp. and *Burkholderia cepacia* have been reported to cause similar mycelial deformities and inhibition of mycelial growth of charcoal rot disease agent (*M. phaseolina*) and white mould disease caused by *S. sclerotiorum* following the production of antifungal metabolites *in vitro*. Cell wall degrading enzymes such as chitinase and glucanase produced by *B. subtilis* isolates may degrade the major fungal cell wall composed of chitin, glucosidic bonds and β-1,3-glucan (Kumar et al., 2012; Hernández-León et al., 2015). Myriocin, produced by *B. amyloliquefaciens* LZN01 isolate, was reported to suppress the mycelial growth of *Fusarium oxysporum* f. sp. *niveum*. Microscopical observation revealed that myriocin decreased the membrane fluidity and destroyed the membrane integrity. Severe morphological changes, including conidial shrinkage, the appearance of larger vacuoles and inhomogeneity of electron density, were reported to occur in myriocin-treated cells (Wang et al., 2021).

The antagonistic effects of all bacterial isolates on the viability of over-wintering fungal structure called sclerotia was also assessed (Table 2). Among 18 isolates, 13 bacterial isolates significantly inhibited myceliogenic germination of fungal sclerotia compared to the control treatment. The highest inhibitory activities (82.8–89.7%) against sclerotial germination was shown by isolates of *B. subtilis* EAB10, EAB8 and *B. amyloliquefaciens* EAB4. In previously conducted study (Ji et al., 2013), *B. amyloliquefaciens* cell-free cultures suppressed mycelial growth and germination of sclerotia of *S. sclerotiorum*.

Table 2. The antifungal effect of endophytic bacterial isolates on the germination of sclerotia of *Sclerotinia sclerotiorum in vitro*

Bacterial species	Number of germinating sclerotia	Inhibition %
Serratia marcescens EAB1	6.7 ef	31.1
Bacillus subtilis EAB2	4.7 cd	51.7
Bacillus subtilis EAB3	3.7 bc	62.1
Bacillus amyloliquefaciens EAB4	1.7 a	82.8
Sphingomonas yanoikuyae EAB5	8.3 f-h	13.8
Stenotrophomona maltophilia EAB6	6.7 ef	31.1
Bacillus amyloliquefaciens EAB7	2.7 ab	72.4
Bacillus subtilis EAB8	1.3 a	86.2
Bacillus subtilis EAB9	2.3 ab	75.9
Bacillus subtilis EAB10	1.0 a	89.7
Pantoe agglomerans EAB11	7.7 e-g	20.7
Stenotrophomonas maltophilia EAB12	8.0 f-h	17.3
Pseudomonas fluorescence EAB13	9.0 gh	6.9
Bacillus simplex EAB14	6.7 ef	31.1
Brevibacillus laterosporus EAB15	6.0 de	38.0
Enterobacter cloacae EAB16	8.7 gh	10.4
Lactobacillus acidophilus EAB17	6.7 ef	31.1
Rhizobium radiobacter EAB18	9.3 gh	3.5
Sterile distilled water (SDW) only	9.7 h	–

Note. Values are the mean number of germinating sclerotia showing mycelial growth over an initial number of sclerotia (n = 10) placed into soil; mean values followed by the same letters are not significantly different.

Suppression of disease development in vivo. Most of the conducted biological control studies for white mould disease focused on the antagonistic efficacy of bacterial isolates *in vitro* tests. In this study, bacterial isolates were also investigated for disease suppression *in vivo*. In control treatment, fungal agent caused significant disease incidence on lettuce seedlings. The number of germinated seeds (%) and seedling development were significantly higher in inoculated soil drenched with the suspension of endophytic bacterial isolates (Table 3).

Table 3. The antagonistic potential of endophytic bacterial isolates on the disease incidence caused by *Sclerotinia sclerotiorum in vivo*

Bacterial species	Number of surviving plants	Protection %
Serratia marcescens EAB1	7.0 e	24.9
Bacillus subtilis EAB2	11.0 f	48.0
Bacillus subtilis EAB3	13.3 gh	61.3
Bacillus amyloliquefaciens EAB4	13.0 f-h	59.5
Sphingomonas yanoikuyae EAB5	3.7 a-c	5.8
Stenotrophomona maltophilia EAB6	5.7 c-e	17.3
Bacillus amyloliquefaciens EAB7	12.3 fg	55.5
Bacillus subtilis EAB8	13.7 g-i	63.6
Bacillus subtilis EAB9	14.7 hi	69.4
Bacillus subtilis EAB10	15.7 i	75.1
Pantoe agglomerans EAB11	4.7 a-d	11.6
Stenotrophomonas maltophilia EAB12	6.0 de	19.1
Pseudomonas fluorescence EAB13	4.0 a-d	7.5
Bacillus simplex EAB14	7.0 e	24.9
Brevibacillus laterosporus EAB15	5.3 b-e	15.0
Enterobacter cloacae EAB16	4.3 a-d	9.2
Lactobacillus acidophilus EAB17	7.3 e	26.6
Rhizobium radiobacter EAB18	3.3 ab	3.5
S. sclerotiorum	2.7 a	0.00

Note. Values are the number of emerging and surviving healthy seedlings over an initial number of seeds (n = 20) sown into soil inoculated with *S. sclerotiorum*; mean values followed by the same letters are not significantly different.

Apart from isolates *Sphingomonas yanoikuyae* EAB5, *Pantoea agglomerans* EAB11, *Pseudomonas fluorescence* EAB13, *Enterobacter cloacae* EAB16 and *Rhizobium radiobacter* EAB18, amendment of infested soil with 13 different bacterial isolates significantly promoted plant survival in comparison to pathogen treated soil without bacterial inoculation (control treatment) (Table 3). Six isolates increased the number of surviving seedlings more than 50%. In addition, the numbers of surviving seedlings in the soil treated with isolates of *B. subtilis* EAB8, EAB9 and EAB10 were significantly higher ($P < 0.05$) than those observed in the soil amended with other isolates.

Conclusions

1. Among the tested 48 putative bacterial isolates, 18 antagonist bacterial isolates were found to cause inhibition zones by inhibiting the mycelial growth of *Sclerotinia sclerotiorum* to a varying degree in dual culture.

3. Based on the morphological characteristics and MALDI-TOF mass spectrometry (MS) analysis, endophytic bacteria were identified as *Bacillus* spp. (10), *Stenotrophomona maltophilia* (2) and one each as *Pantoea agglomerans*, *Sphingomonas yanoikuyae*, *Pseudomonas fluorescence*, *Enterobacter cloacae*, *Rhizobium radiobacter* and *Serratia marcescens*.

4. The highest mycelial growth inhibition was caused by *B. subtilis* EAB10 (83.11%), which was followed by isolates of *B. subtilis* EAB9 (81.82%), *B. subtilis* EAB8 (73.37%), *B. subtilis* EAB3 (71.42%), *B. amyloliquefaciens* EAB4 (70.78%), *B. subtilis* EAB2 (68.83%) and *B. amyloliquefaciens* EAB7 (68.19%).

5. On dual culture plates, all *B. subtilis* and *B. amyloliquefaciens* isolates caused considerable morphological changes such as loss of integrity, hyphal shrivelling and perforation, mycelial degradation and hyphal lysis and fragmentation on the fungal hyphae close to the inhibition zone. Vitality test confirmed that diffusible metabolite(s) produced by all *B. subtilis* and *B. amyloliquefaciens* isolates were clearly fungicidal to *S. sclerotiorum*.

6. Among 18 isolates, 13 bacterial isolates also significantly inhibited myceliogenic germination of fungal sclerotia compared to the control treatment without bacterial inoculation. The isolates of *B. subtilis* EAB10, EAB8 and *B. amyloliquefaciens* EAB4 were recorded to possess the highest inhibitory activities (82.8–89.7%) against sclerotial germination.

7. Bacterial isolates were also investigated for disease suppression *in vivo*. Apart from isolates of *Sphingomonas yanoikuyae* EAB5, *Pantoea agglomerans* EAB11, *Pseudomonas fluorescence* EAB13, *Enterobacter cloacae* EAB16 and *Rhizobium radiobacter* EAB18, amendment of infested soil with 13 different bacterial isolates suppressed disease incidence by 55.7–75%.

8. Although the nature and identity of the antifungal compound(s) were not studied in detail, overall results suggest that suppression of disease incidence caused by *S. sclerotiorum* might be resulted by the production of antifungal compound(s), which caused inhibition in mycelial growth, sclerotial germination, disease incidence and morphological deformities of fungal hyphae. These observations are significant in the light of the attractive role of *Bacillus* spp. in the biocontrol of *S. sclerotiorum*. Significant suppression in the mycelial growth, sclerotial germination and disease incidence caused by endophytic bacterial isolates indicates that isolates of *B. subtilis* and *B. amyloliquefaciens* could be considered as possible biocontrol agents against soil-borne fungal disease.

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Sėjamosios salotos endofitinių bakterijų antagonistinis potencialas nuo salotų sklerotinio puvinio sukėlėjo *Sclerotinia sclerotiorum*

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Santrauka

Įvertintas iš sėjamosios salotos (*Lactuca sativa* L.) išskirtų endofitinių bakterijų biologinės kontrolės efektyvumas nuo sklerotinio puvinio sukėlėjo *Sclerotinia sclerotiorum in vitro* ir *in vivo* sąlygomis. Nustatytas endofitinių bakterijų antagonistinis veiksmingumas slopinant micelio augimą bei skleročių daigumui ir sklerotinio puvinio sukėlėjo *S. sclerotiorum* sukeliamų ligų paplitimui *in vitro* bei *in vivo*. Iš įvairių vizualiai sveikų salotų audinių iš viso buvo išskirti 48 endofitinių bakterijų izoliatai. *S. sclerotiorum* micelio augimą ir skleročių daigumas *in vitro* slopino 18 bakterijų izoliatų. Nustatyta, kad veiksmingiausi buvo *Bacillus subtilis* ir *B. amyloliquefaciens* izoliatai, kurie reikšmingai 68,1–83,1 % slopino micelio augimą, 82,7–89,6 % skleročių daigumą ir 55,7–75 % *S. sclerotiorum* paplitimą *in vitro* bei *in vivo*. Be to, dviguboje kultūroje netoli slopinimo zonos grybo micelis buvo tankesnis ir tamsesnės spalvos. Dviguboje kultūroje netoli slopinimo zonos *B. subtilis* ir *B. amyloliquefaciens* izoliatai sukėlė reikšmingų morfologinių hifų pakitimų, pavyzdžiui, susitraukimą ir perforaciją.
Endofitinių bakterijų reikšmingas micelio augimo, skleročių daigumo ir sklerotinio puvinio paplitimo slopinimas rodo, kad *B. subtilis* ir *B. amyloliquefaciens* izoliatai gali būti naudojami per dirvožemį plintančių grybinių ligų biologinei kontrolei.

Reikšminiai žodžiai: antagonistai, biologinė kontrolė, endofitai, *Lactuca sativa*, *Sclerotinia sclerotiorum*.