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Biological Control of *Sclerotinia sclerotiorum* (Lib.) de Bary, the Causal Agent of White Mould Disease in Red Cabbage, by Some Bacteria

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Abstract

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Sclerotinia sclerotiorum (Lib.) de Bary is the causal agent of white mould, stem, and fruit rot diseases on a wide variety of crop plants including cabbage (*Brassica oleracea* L.) in field and storage. Control of this pathogen by using commercial disease management methods is extremely difficult. Therefore, this study was performed to develop an alternative and effective control method for the diseases by using biocontrol bacteria – *Bacillus subtilis* (strains TV-6F, TV-17C, TV-12H, BA-140 and EK-7), *Bacillus megaterium* (strains TV-103B), and *Bacillus pumilus* (strains RK-103) on Petri plate assays and on red cabbage in pot assays. On Petri plates, all of the tested bacterial strains showed the zone of inhibition against the pathogen fungus ranging 15.00–26.50 mm. Their percentage inhibition rates and lesion length ranged 42.64–79.41% and 0.02–4.50 cm in pot assays, respectively. Consequently, our results indicated that especially *B. subtilis* strains TV-17C, TV-12H, and TV-6F can be used as bio control agent of *S. sclerotiorum* in red cabbage production.

Keywords: plant pathogen; bioagent; *Brassica oleracea* L.

Cabbage, *Brassica oleracea* L., is an herbaceous annual or biennial vegetable from the family Brassicaceae grown for its edible head. Some researchers have accepted that motherland of cabbage is Van region of Anatolia in Turkey and reported on the greatest head cabbages growing in this region (Esiyok 2012).

Several diseases caused by many biotic agents including viruses, bacteria, and fungi are known to attack cabbage plants all over the world causing serious losses in the yield (Srivastava *et al*. 2011). Annual yield losses due to infection exceed several hundred million dollars world over (Purdy 1979). *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most important soil-originated fungal pathogens, and it causes important damage to more than 400 plant species, including cabbage (Fernando *et al*. 2007). The pathogen is the causal agent of white mould stem and fruit rot diseases, and attacks not only crucifers but also a wide variety of other crop plants in the field and storage (Boland & Hall 1994). The affected tissue often turns grey, in wet weather giving rise to fluffy white mould, which is eventually dotted with black sclerotia in the field or storage.

Plant diseases are mostly controlled by the use of chemical pesticides and in some cases by cultural practices. However, the widespread use of chemicals in agriculture has been a subject of public concern and scrutiny due to their potential harmful effects on the environment, their undesirable effects on non-target organisms, development of resistant races

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of pathogens, and possible carcinogenicity of some chemicals (Berg *et al*. 2005; Heydari & Pessarakli 2010). Because the sclerotia persist in soil and the choice of resistant crops is limited, short-term crop rotation is not an effective control measure (Masirevic & Gulya 1992). In addition, the resistance is an important problem of chemical controls. The chemical control of *S. sclerotiorum* using dicarboximide fungicide is economical only if disease concentration reaches 20% (Clarke *et al*. 1993).

Some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. For this reason, recent efforts have focused on developing environmentally safe, long lasting, and effective bio-control methods for the management of plant diseases (Cook & Baker 1983). Biocontrol agents may seemingly represent an environmentally friendly alternative to potent and toxic fungicides, which cannot be broken down in the environment (Abdalla *et al*. 2014). There are many studies on the biological control of *S. sclerotiorum* by using fungal (Krutova 1987; Hannusch & Boland 1995; Mischke *et al.* 1995; McLaren *et al*. 1996; Huang *et al*. 2000; Pieckenstain *et al*. 2001; Zhang *et al.* 2004; Huang & Erickson 2007) and bacterial (Godoy *et al.* 1990; Yuen *et al*. 1991, 1994; Expert & Digat 1995; Boland 1997; Tozlu 2003; Fernando *et al*. 2004; Abdullah *et al*. 2008) biocontrol agents in either laboratory or field-trials. However, many field studies failed to consistently control this pathogen due to the fact that *Sclerotinia* ascospores can disperse for a long distance and then even a reduced number of sclerotia in field can cause significant infection and yield loss (Venette 1998; Hedke *et al*. 1999).

The potential of antagonistic bacteria to control plant diseases has been demonstrated in several crops. However, the efficiency of biocontrol agents can be improved through the elucidation of the mechanism of action (Fernando *et al*. 2007). As a kind of the biocontrol agents, rhizobacteria (PGPR) indirectly promote plant growth by suppression of diseases caused by pathogens, in addition to some other ways of plant growth enhancement like solubilisation of nutrients such as phosphate, releasing phytohormones, and decreasing heavy metal toxicity (Zhang 2004).

PGPR strains with induced systemic resistance (ISR) activity can be active against a wide range of pathogens (Raupach & Kloepper 1998). Many defense enzymes are involved in the defense reaction against plant pests and pathogens (Liang *et al.* 2011). These include oxidative enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POX), catalase (CAT), and superoxidase dismutase (SOD), which catalyse the formation of lignin, and other oxidative phenols that contribute to the formation of defense barriers for reinforcing the cell structure (Avdiushko *et al.* 1993). They are considered as important biochemical markers in host plant resistance against pest. When plants are invaded by microorganisms or damaged by mechanical injuries, major physiological changes are induced and plant defense enzymes are generally activated (Liang *et al.* 2011). In spite of major advances in our understanding the plant defense response, little information is available on PGPR-mediated induced enzymes (Chen *et al*. 2000).

In this study, we aimed to determine the antifungal activity of seven PGPR isolates against *S. sclerotiorum* on Petri plate assays and the suppression of white mould disease in red cabbage in pot assays, and to investigate the effect of the bacteria on some defense enzymes level in cabbage.

MATERIAL AND METHODS

Plant material, pathogen fungus, and biocontrol bacteria. Red cabbage seeds (*B. oleracea* var. *capitata* L. f. *rubra* (L) (Thell)) obtained from a local market were used in this study. They were stored in refrigerator (5°C) under dry conditions. Pathogenic fungus (*Sclerotinia sclerotiorum*) and biocontrol bacteria (*Bacillus subtilis* TV-6F, TV-17C, TV-12H, BA-140, and EK-7; *Bacillus megaterium* TV-103B; *Bacillus pumilus* RK-103) were obtained from the culture collection unit in the Department of Plant Protection, Faculty of Agriculture at Atatürk University, Erzurum, Turkey. Pathogenic fungus had been isolated from the head rot of red cabbage in Erzurum province, and its morphological characters were identified according to Hasanekoğlu (1991). The biocontrol bacteria had been isolated from the rhizosphere and phyllosphere of wild and traditionally cultivated plants growing in the eastern Anatolia region of Turkey (Kotan *et al.* 2005; Erman *et al.* 2010). Bacteria had been identified according to their fatty acid methyl esters (FAME) profiles using Sherlock Microbial Identification System (Microbial ID, Newark, USA) (Miller 1982). Bacterial cultures were grown on Nutrient agar (NA; Difco, Le Pont de Claix, France) for routine use, and maintained

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in Nutrient Broth (NB; Difco) with 15% glycerol at –80°C for long-term storage. Fungal pathogen was grown on Potato Dextrose agar (PDA; Difco) and maintained on PDA slant cultures at 4°C in the refrigerator until use.

Pathogenicity test of the fungus. Pathogenicity tests of the fungus used in this study were performed both on the head rot of red cabbage and 1-month-old red cabbage stems on container-grown healthy. Healthy cabbage heads were washed under tap water and then surface sterilised with 10% ethanol and inoculated at the centre with a 5 mm PDA (Difco) plug from 5-day-old mycelial cultures growth at 26°C for 7 days. The inoculated heads were kept in plastic containers equipped with wet filter paper and incubated at room temperature for 10 days. Cabbage stems were first surface sterilised and then inoculated as stem assays with wounding. Plants were maintained at 25°C and 95% relative humidity on a 12-h fluorescent under light/dark conditions in a growth chamber for 7 days. Heads and stems inoculated only with PDA plugs were used as controls. There were symptoms on inoculated heads (Figure 1A) and stems (Figure 1B) and no symptoms were observed on control plants. The fungal pathogen was re-isolated from the disease lesions on the inoculated head and stem; this re-isolated pathogen exhibited the same morphological characteristics as those of the original isolates, so Kochs postulates were completed. Based on morphological characters of the re-isolated fungus, *S. sclerotiorum* was identified according to Hasanekoğlu (1991).

Identification of the bacteria by BIOLOG. The identity of the bacteria was confirmed according to BIOLOG systems. Two days before the inoculation of Biolog GP2 plates (Biolog, Hayward, USA), bacterial strains were streaked on Biolog Universal Growth Agar + 25% Maltose agar plates. Each well of Biolog GP2 microtiter plates was inoculated with 125 µl of the bacterial suspension adjusted to appropriate density (10^8 CFU/ml), and incubated at 27°C for 24 and 48 hours. Colour development was automatically recorded using a microplate reader with a 590 nm wavelength filter. Bacteria were identified and their similarity index assessed using BIOLOG420/Databases/GP601 (Holmes *et al.* 1994).

Hypersensitivity test of bacteria. The potential biocontrol bacterial strains were tested for hypersensitivity on tobacco plants (*Nicotina tabacum* L. var. Samsun) as described by Klement *et al*. (1964). The bacterial suspension (10^8 CFU/ml) was prepared in sterile distilled water and infiltrated into the intercostal area of the leaves of tobacco plants by using a 3cc syringe without needle (Becton Dickinson, Franklin Lakes, USA). The inoculated plants were incubated in a completely randomised design on the greenhouse bench for 24–48 h at 20–28°C. The presence of rapid tissue necrosis at the inoculation site was recorded within 24–48 h after infiltration. This test was repeated at least three times for each strain. Sterilised distilled water (sdH_2O) was used as negative control.

Chitinase enzyme activity of the bacteria. Chitinase enzyme activity of the bacteria was determined using colloidal chitin substrate. In this procedure 0.5 ml colloidal chitin and 0.2 ml enzyme solution, which was produced extracelularly by bacteria, were mixed. The nit was subjected to reaction by incubation at 37°C for 30 minutes. Subsequently, after adding staining solutions into the reaction mixture, it was allowed to stand at 80°C for 10 min, and measurement with a T80 UV/VIS Spectrophotometer (PG Instruments Ltd., Leicestershire, UK) was performed against blind sample that was created by putting distilled water, and activity was determined by monitoring absorbance change at 540 nm (Senol *et al.* 2014).

Antifungal activity assays of the bacteria. Petri dishes (9 cm diameter) containing 20 ml of PDA were used for antifungal activity assay. The bacterial strains were grown on NA at 26°C for 24 h to obtain fresh culture for Petri plate assay. The pathogenic fungus was also cultivated on PDA at 30°C for 7 days. PGPR strains were streaked as single line onto PDA medium in Petri dishes and the two adjacent sides of the Petri plates were inoculated with a 6 mm disc of the pathogen fungus simultaneously. The plates were incubated at 27°C until fungal mycelia (control) completely covered the agar surface in control plate. Fungal growth was determined by measuring the diameter of colony radial growth in mm. The percentage inhibition of pathogen fungus by bacterial strains was calculated by using the following formula (Mari *et al.* 1996):

% inhibition = (Control colony diameter treated with only pathogen – Pathogen colony diameter treated with bacteria)/(Control colony diameter treated with only pathogen – Mycelial disc diameter) × 100

The experiment was conducted by using a completely randomised design with 3 replications per treatment. The experiment was performed twice. The data were collected as inhibition zone values in mm in each replication, and evaluated by one-way analysis of variance (ANOVA) by using JUMP 5.0 statistical programme (SAS Institute Inc., Cary, USA).

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Pot assays. The effectiveness of the bacterial strains against *S. sclerotiorum* was tested using red cabbage on pot assays. Seeds of red cabbage were surface sterilised with 2% sodium hypochlorite for 5 min and then three times rinsed in sterile distilled water. Seeds were left to dry on sterile filter paper sheets overnight in the laminar flow hood. Then they germinated in trays (15, 29, 10 cm) containing sterilised 1 : 1 : 2 peat, vermiculite, and perlite. The 28-day-old cabbage seedlings were transplanted into 500 ml (9 cm diameter) plastic pots, one plant per pot in the sterilised field soil. Eventually the plants were transplanted in the greenhouse and placed in a completely randomised design. Each treatment was replicated 6 times. Plants were watered daily, greenhouse temperatures range was 20–28°C.

Bacteria were grown in 50 ml flasks containing 20 ml of TSB medium on a rotary shaker at 27°C for 24 hours. Absorbance of the bacterial suspensions appropriately diluted to 1 × 10^8 CFU/ml in sdH_2O was measured spectrophotometrically at 600 nm. *S. sclerotiorum* was developed in PDA at 25°C for 1 week for pot assays. When plants reached the 3–4th leaf, a 5-mm diameter incision was made in the stem 4 cm from the soil surface. 10^8 CFU/ml bacteria suspension was sprayed and a 4-mm diameter disc of *S. sclerotiorum* was placed in the injury site, and the inoculation point was discovered with parafilm. Control plants were inoculated solely with pathogen fungus, NB, and sterile water. Plants were maintained at room temperature under high humidity for 9 days. The lengths of the lesions forming on the plant stems were measured.

Enzyme assays. Fifteen days after the plants inoculating with the pathogen fungus, bottom leaf samples were taken from each plant per treatment, and were deeply frozen (–20°C). 1 g of the frozen leaf material per treatment was homogenised in a ground glass homogeniser in an ice bath, with 1 ml extraction buffer, 50 mM potassium phosphate, pH 7.0 containing 1.0 mM EDTA and 1% polyvinylpyrrolidone (PVP), according to Nadaroglu and Demir (2009). The homogenates were centrifuged at 13 000 *g* for 5 minutes. The supernatants were assayed for polyphenol oxidase, peroxidase, catalase, and superoxidase dismutase enzyme activities.

PPO activity was measured by monitoring the increase in absorbance at 420 nm in 200 mM phosphate buffer (pH 7.0) containing 25 mM cathecol. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min (Shi *et al.* 2002). POX activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1 mM guaiacol and 0.5 mM H_2O_2 (Janda *et al.* 2003). One unit of POX activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01/minute. CAT activity was measured in the hemolysate. CAT activity was determined at 25°C for 3 min with the substrate H_2O_2 according to the Aebi method (Aebi 1984). The rate of disappearance of H_2O_2 per min in absorbance at 240 nm was determined (Aebi 1984). SOD activity was measured by recording the decrease in optical density of nitro-blue tetrazolium (NBT) dye by samples which were obtained plant homogenates. Three ml of the reaction mixture contained 2 µM riboflavine, 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, and 25 ml hemolysate or milk sample. The reaction was started by adding 60 µl from 100 µM riboflavin solution and placing the tubes

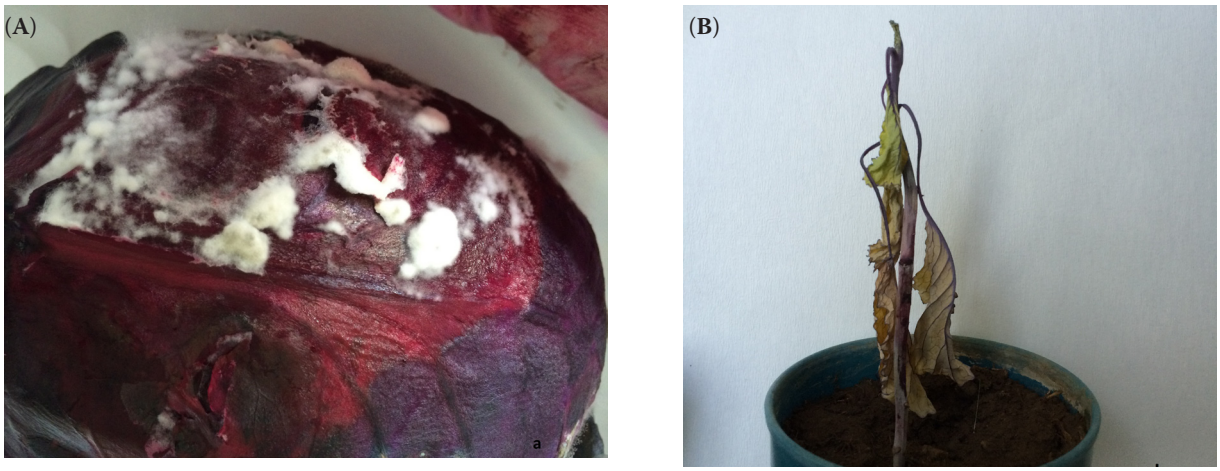

Figure 1. White mould disease on red cabbage head (**A**) and stem (**B**) aused by *Sclerotinia sclerotiorum*

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Table 1. Bacterial strains, their MIS and BIOLOG identification, hypersensitivity (HR), and chitinase (CH-(EU/ml·min)) enzyme activity test results

Strains No.	Isolated from	MIS results	BIOLOG results	SIM	HR	CH
TV-6F	Graminea	*Bacillus subtilis*	*Bacillus subtilis*	0.56	–	65.0
TV-17C	raspberry	*Bacillus subtilis*	*Bacillus subtilis*	0.76	–	67.0
TV-12H	Graminea	*Bacillus subtilis*	*Bacillus subtilis*	0.53	–	74.0
TV-103B	Graminea	*Bacillus megaterium*	*Bacillus megaterium*	0.45	–	58.5
RK-103	apple	*Bacillus pumilus*	*Bacillus pumilus*	0.47	–	65.0
BA-140	soil	*Bacillus subtilis*	*Bacillus subtilis*	0.43	–	65.5
EK-7	rosehip	*Bacillus subtilis*	*Bacillus subtilis*	0.74	–	66.5

SIM – similarity index; – negative reaction

under two 30 W fluorescent lamps for 15 min, then the reaction was stopped by the switching off the light and putting the tubes into dark and the changes in absorbance were determined at 560 nm. The amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions was defined as one unit of SOD activity (Sun *et al.* 1988).

Data analysis. All data in the present study were processed by JUMP 5.0 software and the means were separated by LSMeans Student's tests. The statistical analyses of percentage values in relation to fruit set were performed by using transformed values.

RESULTS

Pathogenicity of the fungus was tested on healthy cabbage heads and stems. The result showed that the pathogen was highly virulent on red cabbage (Figure 1). The bacterial strains used in this study are given in Table 1. According to BIOLOG identification results, TV-6F, TV-17C, TV-12H, EK-7, and BA-140 strains as *B. subtilis*; TV-103B as *B. megaterium*; and RK-103 as *B. pumilus* were also confirmed by the MIS identification results of the bacteria. Their similarity indexes in BIOLOG varied from 0.43 to 0.76. None of them showed hypersensitivity positive reaction on tobacco plants. However, all of them produced chitinase changing from 58.5 to 74.0 EU/ml·minute. The maximum chitinase enzyme activity (74.0) was obtained from *B. subtilis* TV-12H strain (Table 1).

Antifungal activities of the bacterial strains tested against *S. sclerotiorum* on Petri plate assays, and their effect on the incidence of the lesion on the plant stems on pot assays are given in Table 2. According to these results, all the bacteria showed more or less antifungal

Table 2. Antifungal activities of the bacteria against pathogen on Petri plate assays and length of lesion forming on plant stems on pot assays

Applications	Petri plate assays		Pot assays
	inhibition zone (mm)	inhibition rates (%)	lesion length (cm)
B. subtilis TV-6F	26.25 ± 3.061^{A}	76.46 ± 4.805^{A}	0.02 ± 0.004^{C}
B. subtilis TV-12H	24.50 ± 3.674^{A}	77.93 ± 6.005^{A}	0.02 ± 0.009^{C}
B. subtilis EK-7	26.50 ± 0.408^{A}	79.41 ± 0.000^{A}	3.70 ± 1.979^{B}
B. megaterum TV-103B	15.00 ± 0.00^{C}	48.52 ± 3.604^{BC}	4.00 ± 0.00^{B}
B. subtilis TV-17C	17.50 ± 0.408^{BC}	58.81 ± 9.606^{B}	0.02 ± 0.004^{C}
B. pumilus RK-103	16.00 ± 0.816^{BC}	42.64 ± 6.005^{C}	3.16 ± 0.623^{B}
B. subtilis BA-140	19.00 ± 1.632^{B}	60.29 ± 15.611^{B}	4.50 ± 2.677^{B}
sdH_2O	0.00 ± 0.00^{D}	0.00 ± 0.00^{D}	0.00 ± 0.00^{C}
Nutrient Broth	0.00 ± 0.00^{D}	0.00 ± 0.00^{D}	0.00 ± 1.744^{C}
Only pathogen	0.00 ± 0.00^{D}	0.00 ± 0.00^{D}	10.00 ± 4.966^{A}
LSD	3.09	13.55	2.38
CV	0.12	0.17	0.54

Mean values in the same column by the same letter are not significantly different to the LSMeans Student's test ($P < 0.01$)

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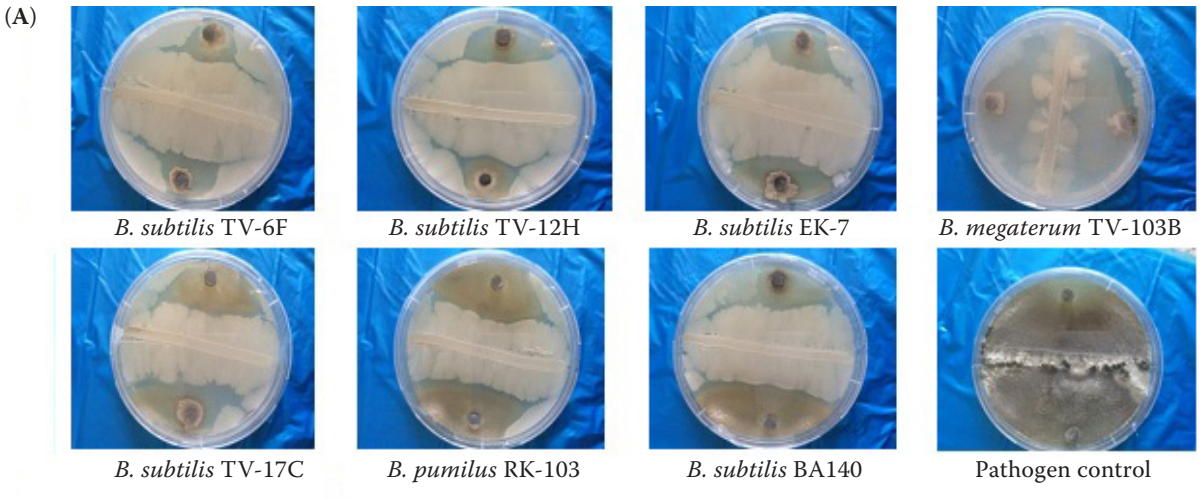

Figure 2. Effectiveness of tested bacterial strains against *Sclerotinia sclerotiorum in vitro* (**A**) and *in vivo* (**B**)

1– biocontrol agent + pathogen; 2 – only Nutrient Broth; 3 – only sterile water; 4 – only pathogen

activity against pathogen on Petri plate assays. Their inhibition zone (mm) and percentage inhibition rates varied between 15.00–26.50 mm and 42.64–79.41%, respectively. The highest inhibition zone values were obtained from *B. subtilis* EK-7 (26.50 mm), *B. subtilis* TV-6F (26.25 mm), and *B. subtilis* TV-12H (24.50 mm) (Table 2 and Figure 2A). The percentage inhibition rates (%) for *B. subtilis* EK-7, *B. subtilis* TV-12H, and *B. subtilis* TV-6F were 79.41, 77.93, and 76.46%, respectively (Table 2). All bacterial strains significantly reduced the disease severity determined as the lesion length (cm) compared to the control (only pathogen application). Furthermore, totally three bacterial strains (*B. subtilis* TV-6F, TV-12H,

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Table 3. Effect of the treatment on enzyme activity of plants on pot assays

Applications	PPO	POX	SOD	CAT
B. subtilis TV-6F	0.656 ± 0.004^{E}	101.794 ± 2.489^{F}	3.566 ± 0.047^{H}	4.655 ± 0.730^{D}
B. subtilis TV-12H	0.890 ± 0.148^{D}	159.516 ± 13.477^{D}	3.633 ± 0.047^{H}	5.125 ± 0.519^{D}
B. subtilis EK-7	0.533 ± 0.047^{F}	171.683 ± 2.307^{D}	8.300 ± 0.081^{C}	5.621 ± 0.017^{CD}
B. megaterum TV-103B	3.333 ± 0.06^{A}	327.703 ± 5.458^{A}	30.500 ± 0.374^{A}	30.169 ± 6.996^{A}
B. subtilis TV-17C	0.680 ± 0.000^{E}	216.104 ± 7.827^{BC}	4.566 ± 0.047^{G}	3.383 ± 0.427^{D}
B. pumilus RK-103	1.376 ± 0.049^{C}	137.896 ± 4.899^{E}	8.333 ± 0.899^{C}	6.694 ± 4.612^{BC}
B. subtilis BA-140	0.630 ± 0.008^{EF}	143.500 ± 9.341^{E}	6.233 ± 0.492^{E}	5.637 ± 0.005^{CD}
sdH_2O	0.666 ± 0.016^{E}	209.310 ± 0.441^{BC}	5.233 ± 0.169^{F}	4.619 ± 0.807^{D}
Nutrient Broth	0.846 ± 0.004^{D}	203.399 ± 3.233^{C}	7.133 ± 0.249^{D}	6.860 ± 1.311^{CD}
Pathogen	2.083 ± 0.004^{B}	220.234 ± 1.668^{B}	22.433 ± 0.169^{B}	14.268 ± 2.314^{B}
LSD	11.65	13.51	0.66	5.88
CV	0.06	0.04	0.04	0.37

PPO – polyphenol oxidase; POX – peroxidase; SOD – superoxide dismutase; CAT – catalase; mean values in the same column by the same letter are not significantly different to the LSMeans Student's test ($P < 0.01$)

and TV-17C) were highly effective in preventing the disease and inhibiting the formation of lesions (Table 2 and Figure 2B).

The effect of several plant defense-related enzymes including SOD, POX, CAT, and PPO were monitored in the red cabbage leaves on pot assays (Table 3). The study revealed that there was a significant increase or decrease in the levels of PPO, POX, SOD, and/or CAT in red cabbage treated with the biocontrol bacteria compared to the controls including only sterile water, NB, and pathogen applications. Especially, the levels of PPO, POX, SOD, and CAT activities in the leaves of red cabbage treated with *B. megaterium* TV-103B were significantly higher compared to only pathogen application. However, the enzymes activities in the leaves of red cabbage treated with *B. subtilis* TV-6F, TV-12H, and TV-17C strains were lower than if only pathogen was applied (Table 3).

DISCUSSION

Totally 7 bacterial strains belonging to *Bacillus* genera were tested for antifungal activities against *S. sclerotiorum.* All of the tested bacterial strains showed more or less antifungal activity against the pathogen both on Petri plate and pot assays. The most effective bacteria were *B. subtilis* TV-6F, TV-12H, and TV-17C strains. These bacteria showed the inhibition zone varying between 15.00–26.50 mm on Petri plate assays (Table 2 and Figure 2A) and were highly effective in preventing the disease and inhibiting the formation of lesions on pot assays (Table 2 and Figure 2B).

The above-given bacterial strains have been selected for the present study because of their bio-agent properties against various plant pathogenic bacteria and fungi, and their micro-fertiliser properties inhibiting growth of some plants as shown in our previous studies (Bastas *et al.* 2012; Turan *et al.* 2014). The applications of PGPRs in our experiments demonstrated their ability to promote plant growth and yield and to suppress disease on plant naturally and/or artificially infected by bacteria and fungi. These bacterial strains can be used both as bio-pesticides and bio-fertilisers in agriculture (Kotan *et al.* 2014).

Ekinci *et al.* (2014) stated that *B. subtilis* TV-17C application increased plant growth parameters such as fresh shoot weight, dry shoot weight, root diameter, root length, fresh root weight, dry root weight, plant height, stem diameter, leaf area, and chlorophyll contents of cauliflower transplant. In addition, with *B. subtilis* TV-17C PGPR applications, the amount of organic acids increased from 9.63% to 186.02%. In addition, the highest values for tartaric, butyric, and citric acids were obtained in *B. subtilis* TV-17C bacterial application. Similar results were also reported in previous studies.

In a study conducted to determine the amino acids, organic acids, and hormone contents, antioxidant enzyme and other enzyme activities of some bacterial applications, the highest values for cysteine, valine, methionine, tryptophan, isoleucine, leucine, butyric acid, and maleic acid were obtained for *B. subtilis*

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TV-17C application. In addition, high alkaline phosphatase activity (ALPEA) was found in *B. subtilis* TV-17C (Günes *et al.* 2015).

Out of 17 PGPR strains tested in our previous study, *B. subtilis* BA-140 used herein had significant effects on controlling fungi species including *Fusarium sambucinum*, *F. oxysporum,* and *F. culmorum* causing potato dry rot in storage (Kotan *et al.* 2009). However, *B. subtilis* BA-140 strain was much less effective than *B. subtilis* TV-6F, TV-12H, and TV-17C strains in this study.

Bacillus species as a group offers several advantages over other bacteria for the protection against pathogens because of their ability to form endospores, and because of the broad-spectrum activity of their antibiotics (Abdalla *et al.* 2014). There are many studies with similar results in which *Bacillus* species showed a strong antimicrobial activity against *S. sclerotiorum* (Zazzerini *et al.* 1987; Tu 1997). *Bacillus* spp. could reduce the apothecium formation by its application into the soil (Lyth *et al.* 1993).

Chitin is a major structural component of most fungal cell walls (Peberdy 1990; Selitrennikoff 2001). The enzyme could be used directly in the biological control of microorganisms (Gomes *et al.* 2000) or indirectly using purified protein (Gomes *et al.* 2001) or through gene manipulation (Tsujibo *et al.* 2000). All the bacterial strains used in this study produced chitinase enzyme. The enzyme has antifungal activity against pathogen and is expected to be used in agriculture as biocontrol agent. Some of the most potent chitinase producers are *Serratia marcescens*, *Streptomyces* sp., and *Trichoderma harzianum*. Chitinolytic enzymes have been considered important in the biological control of soil-borne pathogens because of their ability to degrade fungal cell walls, a major component of which is chitin (Bartnicki-Garcia 1968). Senol *et al*. (2014) have reported that chitinase enzyme produced from *B. subtilis* TV-125, which has antifungal activity against *Fusarium culmorum*, can be used in agriculture as biocontrol agents against fungal pathogens.

There are many studies showing that Rhizosphere bacteria treatments are one of the most potential biological control agents in the plant disease protection, and may improve plant growth (Turan *et al*. 2014). Hoitink (1986) has reported that chemical application is generally not successful against soil-borne pathogens whereas bio-control agents are well colonised in the rhizosphere and have no toxic effects on the leaves like chemicals, and besides controlling the disease they also have positive effects on the plant development.

In the present study, PPO, POX, SOD, and CAT activity was significantly decreased in the inoculated plants treated with the bacteria except for *B. megaterium* TV-103B as compared to the control treated solely with pathogen. Leite *et al.* (2014) have stated that the activities of SOD and POX enzymes and the lignin and soluble phenol contents are more intense when collected nearer to the region of entry of the pathogen. Liang *et al*. (2011) reported that two of the PGPR strains, *Pseudomonas* strains 13 and 63-28, significantly induced the plant defense enzymes both locally and systemically in cucumber against seedling damping-off. Fernando *et al*. (2007) stated that spray application of *Pseudomonas chlororaphis* (PA-23), *B. amyloliquefaciens* (BS6) or *Pseudomonas* sp. (DF41) helped control stem rot of canola by reducing petal infestation of *Sclerotinia* through both direct antimicrobial action and/or induction of plant defense enzymes.

In this study, the maximum PPO, POX, SOD, and CAT enzymes activity was obtained from plants treated with *B. megaterium* TV-103B. This bacterial strain was not very effective in disease control in pot assays. However, levels of PPO, POX, SOD, and CAT enzyme activities in leaves of red cabbage treated with *B. subtilis* TV-12H, TV-17C, and TV-6F were significantly lower than in the case of solely pathogen application on pot assays. Furthermore, *B. subtilis* TV-17C, TV-12H, and TV-6F strains were also very effective in disease control, and significantly reduced the lesion length as compared to the control in pot assays. For this reason, the effectiveness of these bacteria may be related to their direct antifungal action against the pathogen, but not against the induction of plant defense enzymes.

Our results indicate that *B. subtilis* TV-12H, TV-17C, and TV-6F were effective bacterial strains against *S. sclerotiorum*. Microbial formulations consisting of these bacteria have direct potential for the control of *S. sclerotiorum* that is the causal agent of white mould stem and fruit rot diseases, but also positive effects on plant development of a wide variety of crop plants. In the future, we plan to prepare a commercial preparation after making a good carrier consisting of organic material with long shelf life for the most effective bacterial strain. In the midst of these obstacles, the antagonistic bacteria *B. subtilis* TV-12H, TV-17C, and TV-6F can be commercialized for management of *S. sclerotiorum* in both agricultural and horticultural crops.

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