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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Determination of *In Vitro* and *In Vivo* Efficacy of Some Bacterial Antagonists Against *Sclerotinia sclerotiorum* (Lib.) De Bary in Sunflowers

Ayçiçeğinde *Sclerotinia sclerotiorum* (Lib.) De Bary'ye Karşı Bazı Bakteriyel Antagonistlerin *In vitro* ve *In vivo* Etkinliklerinin Belirlenmesi

Raziye KOÇAK[1*], Nuh BOYRAZ[2]

Abstract

This study was carried out in 2017-2018 to determine the *in vitro* and *in vivo* activities of some bacterial bioagents against *Sclerotinia sclerotiorum*, which causes root and root-collar rot in sunflower cultivation areas of Konya and Aksaray provinces. Against the two most virulent *S. sclerotiorum* (Hırkatol and Eskil) isolates selected as a result of pathogenicity tests after being isolated and diagnosed from diseased plants which were collected from sunflower cultivation areas in Konya and Aksaray provinces, the antifungal effects of 16 bacterial isolates from the soil in the rhizosphere region of the healthy sunflower plants from the same areas were evaluated. Primarily, the most effective bacterial bioagents were determined by dual culture tests. As a result of the *in vitro* tests, a total of 5 bacterial isolates constituting the largest zone diameter were molecularly identified according to 16S rRNA and were used in pot experiments. The bacteria were identified as *Bacillus cereus, Bacillus simplex, Brevibacterium frigoritolerans, Bacillus toyonensis* (2 isolates) and were coded using the BLAST program of the GenBank database (NCBI). As per *in vitro*, the highest effect in both isolates of *S. sclerotiorum* was observed in *Bacillus cereus* and *Bacillus simplex* with an inhibition rate of 49.19-57.95%. Except for *Bacillus to*yonensis (B1), one of the bacterial species which were tested *in vivo*, all the bacteria reduced or stopped lesion development compared to the control. As a result of the application, the biological control agent completely prevented the growth of both the isolates of *Bacillus cereus* and *Bacillus simplex S. sclerotiorum* in *in vivo* conditions (100%). Efficacy studies have shown that bacterial isolates both cause healthy growth of sunflower plants and significantly prevent disease formation in treated plants when compared to control plants. These results emphasize the importance of such studies as a tool for the development of sustainable agricultural practices that can be easily applied in our region, and also show that *B. cereus* and *B. simplex* in sunflowers can be potential bacterial bioagents that can be used in biological control against *S.sclerotiorum*. In addition, it will be useful to carry out studies on the development of commercial preparations of the bacterial isolates found in the study.

Keywords: Sunflower, White rot, Biological control, *Sclerotinia sclerotiorum*

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*This study was summarized from the Raziye Koçak PhD thesis.

Öz

Bu çalışma Konya ve Aksaray illeri ayçiçek ekim alanlarında kök ve kök boğazı çürüklüğüne neden olan *Sclerotinia sclerotiorum*'a karşı bazı bakteriyel biyoajanların *in vitro* ve *in vivo* etkinliklerini belirlemek amacıyla 2017-2018 yıllarında yürütülmüştür. Konya ve Aksaray illeri ayçiçek ekim alanlarından toplanan hastalıklı bitkilerden izole edilip, tanılaması yapıldıktan sonra patojenisite testleri sonucu seçilen en virulent olan iki *S. sclerotiorum* (Hırkatol ve Eskil) izolatına karşı yine aynı alanlardan sağlıklı ayçiçek bitkilerinin rizosfer bölgesindeki topraktan izole edilen 16 bakteri izolatının antifungal etkileri değerlendirilmiştir. Öncelikli olarak ikili kültür testleri ile en etkili bakteriyel biyoajanlar belirlenmiştir. *In vitro* testler sonucunda en geniş zon çapı oluşturan toplam 5 bakteri izolatının 16S rRNA'ya göre moleküler olarak tanılaması yapılmış ve saksı denemelerinde kullanılmıştır. Bakteriler *Bacillus cereus, Bacillus simplex, Brevibacterium frigoritolerans, Bacillus toyonensis* (2 izolat) olarak teşhis edilmiş ve GenBank veritabanının (NCBI) BLAST programı kullanılarak kodlanmıştır. *In vitro* da *S. sclerotiorum*'un her iki izolatında da en yüksek etki %49.19-57.95 engelleme oranıyla *Bacillus cereus* ve *Bacillus simplex*' de gözlenmiştir. *In vivo* da test edilen bakteri türlerinden *Bacillus toyonensis* (B1) hariç bütün bakteriler kontrole göre lezyon gelişimini azaltmış veya durdurmuştur. Uygulama sonucunda biyolojik mücadele ajanı bakterilerden *Bacillus cereus* ve *Bacillus simplex in vivo* koşullarda *S. sclerotiorum*'un her iki izolatının da gelişmesine tamamen (%100) engel olmuştur. Yapılan etkinlik çalışmalarında bakteriyel izolatların hem ayçiçeği bitkisinin sağlıklı gelişmesine neden olduğu hem de uygulama yapılmış bitkilerde hastalık oluşumunu kontrollerdeki bitkilerle karşılaştırıldığında önemli düzeyde engellediğini göstermiştir. Bu sonuçlar bu tür çalışmaların bölgemizde kolaylıkla uygulanabilecek sürdürülebilir tarım uygulamalarının geliştirilmesi için bir araç olarak önemini vurgulamakta aynı zamanda ayçiçeğinde *S.sclerotiorum*'a karşı *B.cereus* ve *B.simplex*'in biyolojik mücadelede kullanılabilecek potansiyel bakteriyel biyoajanlar olabileceklerini göstermektedir. Ayrıca çalışmada etkin bulunan bakteri izolatlarının ticari preparatlarının geliştirilmesine yönelik çalışmaların yapılması yararlı olacaktır.

Anahtar Kelimeler: Ayçiçeği, Beyaz çürüklük, Biyolojik mücadele, *Sclerotinia sclerotiorum*

1. Introduction

Sunflower (*Helianthus annuus* L.), which ranks fourth in the world and is one of the most important oil plants, is important in terms of vegetable raw oil production due to the high content of oil (22-50%) in its seeds. Sunflower oil is one of the oils with the highest nutritional value and while 11% of the world's raw vegetable oil production is met from this oil, Turkey is responsible for 50% of this number. In our country, sunflower takes the second place after cotton in cultivation areas of oil crops and sunflower is grown in almost every region of Turkey (Öztürk et al., 2008).

Among the fungal agents which cause root and crown rot in sunflowers, soilborne pathogen *Sclerotinia* species has a great importance (Rasheed et al., 2004). *Sclerotinia sclerotiorum* (Lib.) de Bary is a disease seen in almost every region of the world where sunflower is grown including Türkiye. *Sclerotinia minor* Jagger is another species reported to cause root rot and wilting on sunflowers but is much less common than *S. sclerotiorum* (Baniasadi et al., 2009). *S. sclerotiorum* is a polyphagous and facultative parasite and is the host of more than 400 plant species belonging to 75 families. When the sunflower seedlings reach a certain size, the sclerotia remaining in the soil become infected and crown rot and wilt begins. Sclerotia are the most important asset of penetration. Survival in soil is very variable, but 5 or 6 years is thought to be the upper limit. At first, wilted plants are scattered across the field, then appear in patches in the field (Davar et al., 2012). In an area contaminated with a pathogen, the probability of plants being infected is very high, but the course of the disease is closely related to the soil and water potential in the plant (Vuong et al., 2004).

As sclerotia and fungus that can survive in the soil for a long time produce ascospores responsible for air infection reaching long distances, due to lack of effective chemical control and high sunflower sensitivity, it is difficult to combat, so cultural measures have a very important place (Saharan and Mehta, 2008; Liu et al., 2021). Since the control of *S. sclerotiorum* is insufficient, biological control methods have become unavoidable (Fernando et al., 2005). The use of biological prevention in integrated management systems is important because it is practical to prevent *S. sclerotiorum*'s infection in sunflower, the cost is appropriate and it is not phytotoxic (Gulya et al., 1997; Fernando et al., 2004).

Rhizosphere and rhizoplane microorganisms play an important role in the biological control of soil pathogens. The most common species used as biological control agent are *Pseudomonas* spp., *Bacillus* spp., and *Trichoderma* spp. which are used as biopreparates against many diseases (Weller, 1988; Fira et al., 2018; Yörük and Mirik, 2021). The production of endospores by *Bacillus* spp. against environmental stress conditions provides long survival rates. Biocontrol agent bacteria produce different types of antibiotics and growth promoting compounds that can play a major role in their disease suppressive and growth promoting effects on plants treated with them. (Kloepper et al., 2004; Moeinzadeh et al., 2010).

It is known that bacteria-containing microbial fertilizers and biopesticides are successfully used in agriculture by making many commercial preparates around the world. In Türkiye, although the use of commercial microbial fertilizers in agriculture using local bacterial isolates started, there are no commercial biopesticides yet. Biological control studies are becoming more and more important every day and This causes the studies on prevention to shift in this direction (Kotan, 2014; Kotan and Çelik, 2014; Güldoğan et al., 2022).

In this study, it was aimed to determine the bacterial bioagents against white rot disease in sunflower cultivation areas of Konya and Aksaray provinces and to determine their *in vitro* and *in vivo* activities against the disease.

2. Materials and Methods

2.1. Material

In our study, two isolates of *Sclerotinia sclerotiorum*, which were isolated from diseased sunflower plants grown in Aksaray Province (from Hırkatol/Eskil districts) and with high virulence as a result of pathogenicity test, and İnegöl Alası as sunflower cultivar were used as test pathogens in our study. The bacterial species *Bacillus cereus, Bacillus simplex, Bacillus toyonensis, Brevibacterium frigoritolerans* that were isolated from the rhizosphere and identified were used to determine the antagonistic effectiveness against *S. sclerotiorum*.

2.2. Methods

2.2.1. The isolation and identification of the pathogen

S. sclerotiorum isolates used as a pathogen in the study were collected from different districts of Konya (Karatay, Altınekin, Cihanbeyli, Karapınar, Kadınhanı and Çumra) and Aksaray provinces (Hırkatol, Eskil) as a result of a 2-year survey and a total of 10 isolates were determined. According to Warcup (1958), pure cultures of *S. sclerotiorum* were obtained from samples taken from sunflower fields and plants showing root and root-collar disease symptoms, and the isolates were kept in a refrigerator at 4^0C and used in *in vitro* and *in vivo* experiments (Warcup, 1958).

In order to define *S. sclerotiorum* and to determine its differences from other species of this genus, the width and length of 5 sclerotiums obtained from each isolate grown in PDA for 3 weeks were measured with a caliper and the arithmetic averages of the obtained values were taken (Lucas, 1998; Leslie and Summerell, 2006).

2.2.2. Isolation, selection and identification of bioagent bacteria

For the isolation of bioagent bacteria, soil samples were taken from 17 different sunflower fields in Konya and Aksaray provinces and from the root zone of plants that were better developed than the others, and the soils collected from the root rhizosphere of 3 plants represented each soil sample and bacterial cultures were obtained according to Küsek (2007). Colonies growing on the medium were examined and colonies with different morphological development were selected and inoculated on Nutrient agar (NA) medium until a pure culture was obtained.

The 16 purified isolates were characterized by the MALDI-TOF biotyping method. The selection among the available bacterial isolates were made by the *in vitro* response results shown against *S. sclerotiorum*.

The five isolates, which showed the greatest zone diameters among these bacterial isolates were identified according to 16S rRNA and were used in pot experiments. Molecular identification of the bacteria was conducted; EurX GeneMATRIX Tissue Bacteria Isolation Kit (EURx Ltd., Poland) was used for DNA isolation. Then with Thermo Scientific Nanodrop 2000 (Thermo Scientific, USA), density and quality of the isolates were determined. 16SrRNA gene sequence was amplified in order to ensure that the bacteria. 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') primers were used for PCR amplifications. Band screening of the PCR products was observed in the gel electrophoresis. Amplified products of template DNA were sent to the Macrogen direct sequencing service (Macrogen, Holland) with ABI 3730 XL DNA Analyzer for sequence determination The similarity of the 16S rRNA gene sequence was encoded using the BLAST program of the GenBank database (NCBI).

2.2.3. Determination of antagonistic effects of bacterial isolates against S. sclerotiorum in vitro

In vitro antifungal activity against *S. sclerotiorum* was determined by the dual culture method using PDA growth medium. For this, two discs in 5 mm from 4 to 5-day culture of *S. sclerotiorum* were placed against each other at nearby edge of 9 cm petri plates containing PDA. A 24 h culture of bacterial isolate grown in NA was straked into the middle of the plate. After inoculation of the agar, the petri plates were incubated at 27^0C for 7 days. The barrier formed between the bacteria and the fungus showed that there was an interaction between the two microorganisms and the width of the inhibition zone was measured from the shortest distance between both colonies and evaluated as the inhibition zone (Z_1). The diameter of the zone (inhibition zone) where the bioagents inhibit the growth of phytopathogenic fungal hyphae in the antibiosis interaction was measured in mm, and the value obtained was used to determine the antibiosis efficiency of the candidate bioagent (Xiaoning et al., 2014).

In addition, the percent inhibition of *S. sclerotiorum* of candidate biological control agents was calculated using the formula of radial growth (Inhibition % = (r1- r2/r1) × 100) specified by Ghildial and Pandey (2008). (r1 represents radial growth of the pathogen without antagonist organism while r2 represents radial growth of the antagonist organism and pathogen).

For each possible bioagent, 3 petri dishes were used and the study was performed in 2 replications. As a control, only *S. sclerotiorum* isolates were inoculated on antibiotic-free PDA media in 2 petri dishes.

2.2.4. Determination of in vivo effectiveness of bacterial isolates against S. sclerotiorum

In order to test the *in vivo* activity of 5 bacteria (*Bacillus cereus, Bacillus simplex, Brevibacterium frigoritolerans, Bacillus toyonensis* (2 isolates)) found to be effective *in vitro* on two isolates of *S. sclerotiorum*, in the open area of Selcuk University Faculty of Agriculture, Department of Plant Protection Research and Application, a pot experiment with 4 replications was established according to the randomized plot design.

The biocontrol agents that were streaked into the nutrient agar were incubated at 28^0C for 48 hours. Some sterile water was poured on the developing bacterial colonies and mixed with a glass baguette. The concentration of the bacterial suspension obtained was adjusted to 10^8 cells/ml. Spectrophotometrically, the absorbance of the suspension was adjusted to 0.1 at 600 nm.

When the plants were at the flowering stage of R2, the shoots localized above the 5 cm of soil surface were made wounds with sterile lancet and the bacterial suspension at a density of $0.5x10^8$ cells/ml was sprayed onto the wounds (Nelson et al., 1988). After the application, the *S. sclerotiorum* 4 mm mycelial disk and moist cotton were placed into the wounds and the wound was wrapped with parafilm. In terms of control plants, sterile water was sprayed onto the wounds and *S. sclerotiorum* 4 mm mycelial disk was placed (Tozlu, 2003). In addition, to determine whether each bacterial isolate has a negative impact in terms of plant growth before the inoculation plant shoots were wounded and the bacterial suspension was sprayed onto the two plants.

The pots were watered with pure water for a week under an open area. The lesions occurring on the shoots were measured with a caliper one week after the inoculation.

2.2.5. Statistical Analysis

The data (lesion length, inhibition zone, percent inhibition ratio) obtained after the study were subjected to ANOVA using SPSS statistical program (SPSS Inc., Version 17.0). The important differences between the treatment means were determined by the Tukey Multiple Comparison Test at 5% significance level.

3. Results and Discussion

3.1. Result

3.1.1 Isolation and Identification of Biocontrol Bacteria

Soil samples from the rhizosphere of plants, which were better grown compared to the others from 17 different sunflower areas in Konya and Aksaray provinces were taken. Each soil sample consisted of 3 different rhizosphere samples.

A total of 16 bacterial colonies, which showed different morphological growth were selected and purified in the isolation studies performed in the laboratory. Five different isolates, which showed strong antagonistic effect against *S. sclerotiorum in vitro* were selected and they were identified by a method based on 16S rRNA gene sequence at species level in BM Labosis Molecular Research Laboratory. The results indicate that the bacterial isolates are closely related to *Bacillus cereus* RP (GenBank access code OQ110614), *Bacillus simplex* RC (GenBank access code OQ116342), *Brevibacterium frigoritolerans* RF (GenBank access code OQ110612), *Bacillus toyonensis* RD (B1) (GenBank access code OQ116344) and *Bacillus toyonensis* RI (B2) (GenBank access code OQ116343). The sequence similarity was 99% when *Bacillus cereus* was concerned and 100% in the others.

3.1.2. Antagonistic Effects of the Selected Bacterial Isolates In vitro

The effectiveness of the 5 bacterial isolates against *S. sclerotiorum in vitro* and the grouping of them according to Tukey Multiple Comparison Test were given in *Table 1*.

It was determined whether or not biological control agent antagonistic bacteria have antibiosis interaction with the two isolates of *S. sclerotiorum*. In the laboratory studies, the most effective strains against *S. sclerotiorum* were shown in *Figure 1* and the least effective strains against *S. sclerotiorum* were shown in *Figure 2*.

All bacterial agents made inhibition zone in petri plates and showed antagonistic effect. Against the two isolates of *S. sclerotiorum*, *Bacillus simplex* made inhibiton zones of 10.5 mm in Hırkatol isolate and of 10.3 mm in Eskil isolate; *Bacillus cereus* made inhibiton zones of 10.17 mm in Hırkatol isolate and of 10.33 mm in Eskil isolate.

Bacillus toyonensis (B1), made a high inhibition zone (10.17 mm) similar to the other bacteria in Hırkatol isolate, but produced less effect in Eskil isolate. The least inhibition zone measurements were made in *Brevibacterium frigoritolerans* and *Bacillus toyonensis* (B2).

Table 1. The effectiveness of the 5 selected bacterial isolates against S. sclerotiorum in vitro conditions

	***S. sclerotiorum* isolate From Hırkatol**		***S. sclerotiorum* isolate From Eskil**	
Bacterial species	**Inhibition zone (mm)**	**Inhibition ratio (%)**	**Inhibition zone (mm)**	**Inhibition ratio (%)**
Bacillus cereus	10.17 a	57.95 a	10.33 a	50.49 a
Bacillus simplex	10.5 a	54.6 a	10.3 a	49.19 ab
Bacillus toyonensis (B1)	10.17 a	54.44 a	9.5 a	44.51 ab
Bacillus toyonensis (B2)	7.2 b	50.25 a	9.5 a	44.09 ab
Brevibacterium frigoritolerans	7 b	46.5 b	7 b	40.62 b

P≤0.05 (There is no statistical difference between the means expressed with the same letter in the same column)

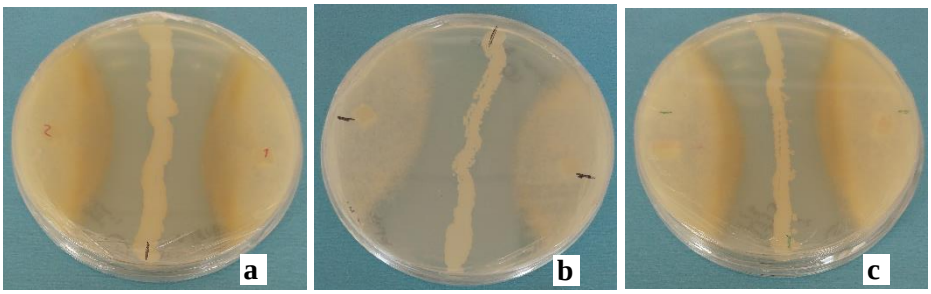

Figure 1. The most effective strains against S. sclerotiorum in vitro a) Bacillus cereus, b) Bacillus simplex and c) Bacillus toyonensis (B1)

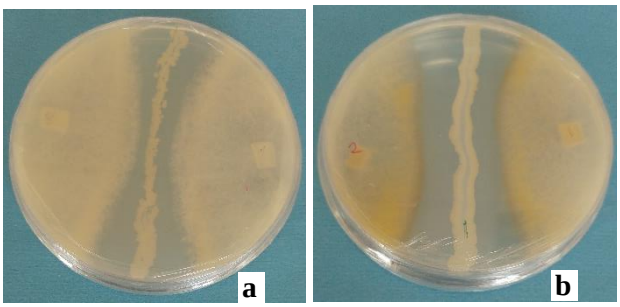

Figure 2. The least effective strains against S. sclerotiorum in vitro. a) Brevibacterium frigoritolerans and b) Bacillus toyonensis (B2)

The percent inhibition ratio of the antagonist bacteria ranged between 38.62% and 57.95%. In terms of Hırkatol isolate, the strongest effect was seen on *Bacillus cereus* with 57.95 %, followed by *Bacillus simplex* with 54.6% and *Bacillus toyonensis* (B1) with 54.44%. However, there was no significant difference between the three bacterial species. *Bacillus toyonensis* (B2) showed 50.25% and *Brevibacterium frigoritolerans* showed 46.5% inhibiton ratios, which are found statistically different. The antagonist bacteria showed similar inhibition in terms of the Eskil isolate, the highest ones were seen in *Bacillus cereus* with 50.49% and *Bacillus simplex* with 49.19%. *Brevibacterium frigoritolerans* was found to be the least effective isolate against *S. sclerotiorum in vitro*. In addition, the bold brown deaths of *S. sclerotiorum*'s mycelia that are close to the inhibition zone (*Figure 1a and 1c*) indicated strong antifungal activity as determined previously by Abdullah et al. (2008) and Rahman et al. (2016).

3.1.3. The effectiveness of the bacterial agents against Sclerotinia sclerotiorum in vivo

The effectiveness of candidate bioagent bacteria, which were found to be effective *in vitro* in the pot experiment, against *S. sclerotiorum* isolates was tested and the lesion lengths they formed as a result of the *in vivo* test are given in *Table 2*.

Table 2 The effectiveness of the selected candidate bioagent bacteria against Sclerotinia sclerotiorum isolates in vivo.

Bacterial species	*S. sclerotiorum* Hırkatol Isolate		*S. sclerotiorum* Eskil Isolate	
	Lesion length (cm)	**% Inhibition ratio***	**Lesion length (cm)**	**% Inhibition ratio***
Bacillus cereus	0 a	100	0 a	100
Bacillus simplex	0 a	100	0 a	100
Bacillus toyonensis (B2)	5.83 b	31	5.67 bc	12
Brevibacterium frigoritolerans	6 b	30	3.17 b	51
Bacillus toyonensis (B1)	8.67 c	-4	7.17 c	-12
Control	**8.33 c**	-	**6.4 c**	-

P≤0.05 (The means that are followed with the same letter are not statistically different in the same column)
* % Inhibition ratio = (Control-Treatment) /Control x 100

The inhibition effectiveness of the bacterial isolates against *S. sclerotiorum* used in this study was found to be different and the differences were statistically significant. All the bacterial isolates with the exception of *Bacillus toyonensis* (B1) significantly reduced or stopped the lesion development. After the treatment, *Bacillus cereus* and *Bacillus simplex* among the selected bioagent bacteria completely stopped (100%) the *S. sclerotiorum*'s two isolates development *in vivo* (*Figure 3a and 3b*).

The other bacterial agents, which reduced the lesion development in Hırkatol isolate compared to the control (8.33 cm) were *Bacillus toyonensis* (B2) (5.83 cm with an inhibition ratio of 31%) and *Brevibacterium frigoritolerans* (6 cm with an inhibition ratio of 30%). In terms of Eskil isolate, *Brevibacterium frigoritolerans* (3.17 cm with and inhibition ratio of 51%) was first (*Figure 3c*) and *Bacillus toyonensis* (B2) (5.67 cm with and inhibition ratio of 12%) was second compared to the control (6.14 cm) (*Table 2*). However, the isolate *Bacillus toyonensis* (B1) was ineffective against *S. sclerotiorum*'s both isolates compared to the control (*Figure 4a and 4b*). It was recognized that this could be related to some situations, which were responsible for stopping the antibiosis mechanism. Among these could be the lack of antibiotic production, the disappearance of the antibiotic shortly after the production and the short area of the effectiveness in plants. Because the production of bioactive secondary metabolites varies depending on the species or strains of microorganisms and their cultural conditions as determined by Jose et al. (2011) and Wang et al. (2011). It was recognized that the environmental conditions such as pH and temperature have effects on the growth and antibiotic production of the different bacterial species (Vijayakumari et al., 2013). It was previously determined that *Bacillus* species have a pH optimum range of 6.5 to 7.5 and a temperature optimum range of 5 to 30°C (Guimaraes et al., 2004).

In addition, the application of only bacterial agents to the wounds created in sunflower plants as a control *in vivo* resulted in no lesion development. This shows that the bacterial strains used in sunflower plants do not produce any kind of disease.

It was reported that *Bacillus* species in general and more specifically the species *B. subtilis, B. cereus, B. amyloliquefaciens, B. pumilus*, and *B. megaterium* are effective against *S. sclerotiorum* and other soil origin fungal pathogens (Georgakopoulos et al., 2001; Soylu et al., 2005; Zhang and Xue, 2010; Onaran and Yanar 2011; Ji, 2013; Mansour et al., 2008; Ajilogba et al., 2013; Tozlu et al., 2016). When the isolates were considered at species level, *B. cereus, B. simplex, B. toyonenensis* and *Brevibacterium frigoritolerans* were the most effective respectively and they showed the inhibition ratio differing from 12% to 100% (*Table 2*).

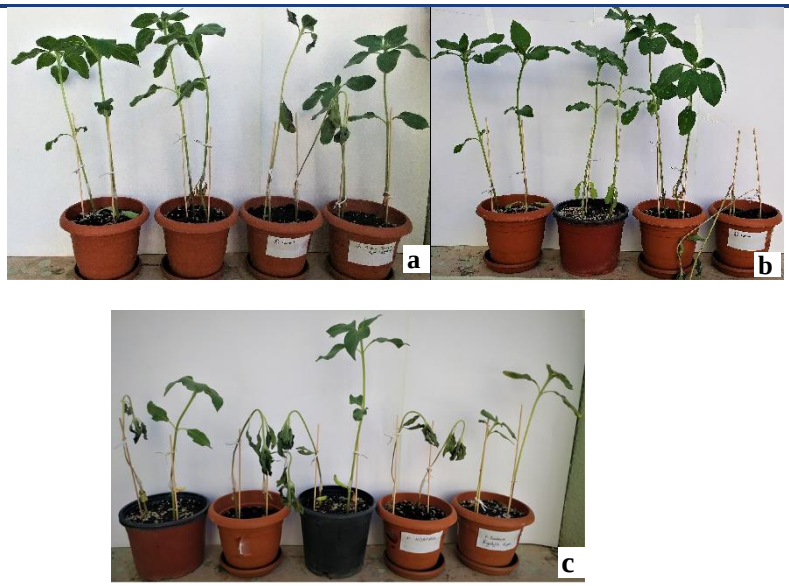

Figure 3. The bacterial isolates, which showed strong inhibition of Sclerotinia sclerotiorum in vivo. Bacillus cereus (a), Bacillus simplex (b) and Brevibacterium frigoritolerans (c).

Figure 4. The bacterial isolates, which showed low inhibition of Sclerotinia sclerotiorum in vivo. Bacillus toyonensis (B2) (a) and Bacillus toyonensis (B1) (b).

3.2. Discussion

Bacillus cereus is very closely related to *Bacillus thuringiensis* (Han et al., 2006). *Bacillus thuringiensis* is a biopesticide used worldwide and a well-known entomopathogen bacterium. At the same time, *B. thuringiensis* and *B. cereus* have been shown to be effective biological control agents against *S. sclerotiorum* in the studies performed earlier (Duncan et al., 2006; Zeng et al., 2012; Gao et al., 2014; Ouhaibi-Ben Abdeljalil et al., 2016). The data obtained in earlier studies are similar to our results and *B. cereus* has been shown to be the most effective bioagent with 57.95% inhibition zone in our study.

Kamal et al. (2015) determined in the study conducted on canola that against *S. sclerotiorum* crown rot disease, *B. cereus* and *B. subtilis* antagonistic strains significantly reduced the mycelium development and completely inhibited the sclerotinia germination *in vitro*.

There is a relationship between *Bacillus simplex* and *Bacillus subtilis*, they are both present in the soil for the same ecological position, and they compete with each other (Sikorski and Nevo, 2007; Earl et al., 2008). However, *B. subtilis* shows strong antibacterial, antiviral and antifungal activity with surfactin produced, and inhibits the activity of many *Bacillus* species including *B. simplex* in soil by antagonistic interaction. This condition is believed to be related to the metabolites released into the environment. The studies performed showed that *B. toyonensis* colonies are usually eradicated by *B. subtilis* (Rosenberg et al., 2016). Many earlier studies performed showed that *B. simplex* promotes plant growth (Gutiérrez-Luna et al., 2010; Hassen and Labuschagne, 2010; Erturk et al., 2010) and some studies classified *B. simplex* as a PGPR (Ash et al., 1991; Xu and Côte, 2003). *B. subtilis* species are used as biological control agent widely by the bacillibactine produced as a siderophore and they have the other

PGPRs' effects. In a study performed, *B. subtilis* strain produced faster inhibition zone compared to *B. simplex*, both of which were isolated from the rhizosphere of different plants (Schwartz et al., 2013). However, both strains showed antifungal activities, which can be used as a potential biocontrol agent. There have been quite a few studies, which used *B. simplex* as a phytophathogen *in vitro*, however, our results indicated that *B. simplex* can be used against *S. sclerotiorum* as a strong biocontrol agent.

B. toyonensis, belongs to the group *B. cereus* (Jiménez et al., 2013) and produces toyocerin active ingredient. It has an economical importance as a probiotic bacterium and it has been used as a supplementary material in animal feed for long time (Williams et al., 2009). In a study similar to ours, strains isolated from healthy tomato plants have been identified as *B. toyonensis* and *B. cereus* by 16S rRNA gene sequence analysis (Rocha et al., 2017). These strains showed strong antagonistic effects against *Fusarium* wilt (*Fusarium oxysporum* f. sp. *lycopersici)* in dual culture technique *in vitro* and stopped the mycelial development completely.

In this study, *Brevibacterium frigoritolerans* (*Bacillus frigoritolerans*) which belongs to the genus *Bacillus*, produced less inhibition zone compared to the other bacteria and showed 46.5% inhibition ratio. Similarly, *B. frigoritolerans* strain isolated from Aloe vera rhizosphere is able to solubilize phosphate and produce high amounts of IAA *in vitro* (Tara and Saharan, 2017). This strain has a potential to be used as biological control agent by inhibiting the pathogen growth by 38.6% siderophore production (Tsavkelova et al., 2007; Tara and Saharan, 2017). Siderophores inhibits the spore formation and the disease symptoms of fungal pathogens by absorbing the Fe-III. (Montesinos et al., 2002). Some *Pseudomonas* species with the ability of plant growth promotion have stronger siderophore production than *Bacillus* species (Kannahi and Kowsalya, 2013). Our results indicated that *B. frigoritolerans* has a strong siderophore production potential and it can be used as a potential bioagent.

In recent years, there have been studies associated with *B. frigoritolerans* in Turkiye. For example, soil samples from different orchards in Aydın province were collected and the bacterial diversity among these soil samples were investigated. The isolates were identified as *Brevibacterium frigoritolerans* and *B. cereus* according to 16S rRNA gene sequence analysis (Yörükçe et al., 2017). It has been stressed that the importance of these isolate from soils in ecosystem management and agricultural applications is very high. Consequently, the *Bacillus* strains have been frequently met because the bacteria in this group have the potential to survive in many different ecological niches (Connor et al., 2010).

Effective antagonist bacteria, when colonized on the rhizosphere, seeds or wounds in the plants, they produce antimicrobial substances (siderophore, protease, ammonia etc.) and provide suppressive effects. For antibiotic substances to be effective, they have to be close to the pathogen (Paulits and Belanger, 2001). It is known that some antagonists have the ability to produce more than one antibiotic (Bacon et al., 2015; Cawoy et al., 2011). For example, *Bacillus cereus* strain UW85 have the ability to produce antibiotics zwittermycin and kanamycin. To be able to produce more than one antibiotic provides a great advantage in the competition among microorganisms in soil. In addition, antagonists that possess mechanisms other than antibiotic production have the ability to suppress diseases in greater areas (Pal and Gardener, 2006).

It is clearly understood that *in vitro* and *in vivo* pot and field experiments are necessary to be able to find fungal and bacterial isolates that have the biological control potential. It is necessary that more detailed studies are required for the determination of the mechanisms used by the isolates that we have employed in this study to suppress the pathogens.

Detailed knowledge of bioagents will allow their specific application in biocontrol and will lead to the production of strains that produce sufficient amounts of pharmaceutically biotechnologically active substances.

Recently, it has been demonstrated that bacteria and fungi that belong to different taxonomical groups produce different bioactive substances and it has been reported that many successful studies have been performed in this area. The study that we have performed show important results. However, it should not be forgotten that there are many points that need to be emphasized in order to put them into practice.

More detailed studies for the identification of the successful isolates at species level and suitable formulations for the mass production of the bioagents are required. The biocontrol agents only make up 1% of the total pesticide sales (Fravel, 2005). It is understood that chemicals govern the agricultural industry. Formulation and agricultural usage methods determine the effectiveness and the sales of the commercial products (Arora et al., 2010; Mishra et

al., 2015). For this reason, after these studies, it should be focused on why biological control agents are not effective in the field scale. As the new data are obtained better biopesticide formulations can be developed. For this reason, it is predicted that these microorganisms will play a great role in the 21st century agriculture.

4. Conclusions

In this study, while a high (100%) activity inhibiting mycelial growth of *S. sclerotiorum* was exhibited by *B. cereu*s and *B. simplex*, the antifungal effect in other isolates was observed to vary between 12-51% when evaluated in general. These results provided important data for the application of effective bioagents in the control of *Sclerotinia* crown rot.

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Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

There is no conflict of interest between the article authors.

Authorship Contribution Statement

Concept: Koçak, R., Boyraz, N.; Design: Koçak, R., Boyraz, N.; Data Collection or Processing: Koçak, R., Boyraz, N.; Statistical Analyses: Koçak, R.; Literature Search: Koçak, R.; Writing, Review and Editing: Koçak, R., Boyraz, N.

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