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Biological Control of Lettuce Drop (*Sclerotinia minor* Jagger) Using Antagonistic *Bacillus* Species

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Abstract: *Sclerotinia minor* (*S. minor*) Jagger is a phytopathogenic fungus that causes lettuce drop, a serious problem in lettuce (*Lactuca sativa* L.) production. The control of this pathogen is challenging because of the resistance of sclerotia, which can survive in the soil under favorable conditions. In Bulgaria, the management of lettuce drop relies primarily on the strategic application of synthetic fungicides. To find alternative methods for disease management, four bacterial isolates were screened for antagonism against *S. minor*. This study reports the in vitro evaluation of the antifungal activity of *Bacillus subtilis*, *Priestia megaterium*, *Bacillus safensis*, and *Bacillus mojavensis* against *S. minor*. The molecular identification of the isolates involved in the activity was examined through 16s rRNA sequencing. Isolated bacterial strains produced indole-3-acetic acid (IAA) in a medium supplemented with 0.1% L-tryptophan. The ability of these strains to increase the mobility of phosphorus and zinc was elucidated. The production of siderophores was confirmed on CAS (Chrom azurol S) medium. The inhibitory action of the bacterial growth broth filtrates against *S. minor* was demonstrated, indicating the nature of the molecules involved. The evaluation of antifungal activity was carried out in vitro and in pot experiments. This study determined the effect of growth-promoting rhizobacteria on the development of lettuce. This research focuses mainly on the development of biocontrol strategies for the management of lettuce drop in greenhouses.

Keywords: lettuce; *Bacillus* spp.; biocontrol of white mould; *Sclerotinia minor*; biofertilizers

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1. Introduction

Lettuce (*Lactuca sativa* L.) belongs to the plant family *Asteraceae* and is one of the most widely consumed leaf vegetables worldwide. Among the diseases of lettuce, lettuce drop is one of the most common and destructive diseases in lettuce production, affecting the lettuce producing regions in Bulgaria and other lettuce-growing regions around the world [1]. Diseases are considered a major restrictive factor in lettuce production, with only a few resistant cultivars available [2–4]. The losses due to this disease vary from 10% to 75% depending on cultural (cropping history, irrigation, and cultivation methods) and environmental factors [5]. One of the pathogens that causes lettuce drop is *Sclerotinia minor*, which produces soil-born mycelia only [6]. Current management strategies for *Sclerotinia* sp. in lettuce are similar to the management programs for control, which rely on breeding disease-resistant cultivars and heavily rely on chemical applications.

Sclerotinia sp. produces sclerotia, which can survive in soil for more than 8 years without a host. The sclerotia of *S. minor* are quite small (0.5–2 mm in diameter) and primarily germinate eruptive, infective mycelia that only penetrate lettuce plants nearby. *S. minor* is believed to be a heterothallic, a condition in which male and female reproductive structures are produced in different thalli, and requires sexual mating between two compatible isolates for the production of apothecia. Therefore, the fungus infects plants primarily by hyphae

from the myceliogenic germination of sclerotia [7]. The development of sclerotia also depends on several factors, such as the location of sclerotia along the soil profile, moisture, temperature, and microflora in the soil [1].

In Bulgaria, the management of *Sclerotinia* fungi relies primarily on the strategic application of synthetic fungicides. Fungicide efficacy is affected by disease pressure, weather conditions, the method of application, fungicide overuse, and crop canopy. Resistant lettuce cultivars to *Sclerotinia* are currently not available. In an attempt to find alternative strategies for the management of the disease, four naturally occurring bacterial isolates were screened for antagonism against this pathogen. Biological control results from interactions among microorganisms and includes mycoparasitism, antibiosis, competition for sites and nutrients, and induced resistance. It is one of the most important non-chemical strategies evaluated for the management of several plant pathogens in several cropping systems, including the roughing of wilted plants, crop rotation with Brassicaceae plants, and subsurface drip irrigation [8]. Against diseases, mycoparasites that attack both the sclerotia and mycelia of *Sclerotinia* sp. have been evaluated both under laboratory and field conditions through the utilization of *Trichoderma* sp. [9], *Sporidesmium sclerotivorum* [10], and *Coniothyrium minitans* [11]. *Trichoderma* sp. are perhaps the most widely used mycoparasites and numerous commercial formulations exist [12]. Biocontrol agents currently registered for *Sclerotinia* in lettuce are include the use of *Bacillus subtilis* as a biocontrol agent (BACSTAR™, CLARITY®, SERENADE®). One reason for biocontrol failure is the neutral-to-alkaline pH of lettuce field soils [13]. The other possible reason is that biocontrol for lettuce drop is also incompatible with chemical control because of the effects of fungicides on the biocontrol agent [14]. However, many studies have been conducted to determine the effects of chitinolytic fungi on the growth and development of several fungal pathogens. In 1996, Larena and Melgarejo [15] applied chitinolytic actinomycetes and bacteria for the control of lettuce drop disease, with the possible production of chitinase and β-1,3-glucanase.

Under iron-limited conditions, prokaryotic cells, certain fungi, and plants produce multiple low-molecular-weight siderophores (often <1000 Da), which are high-affinity chelating agents that solubilize ferric iron in the environment and transport it into the cell [16]. Siderophore-producing bacteria have been used as biocontrol agents to combat plant pathogens [17]. The growth of some species may be inhibited, and this has been attributed to one of the mechanisms by which biocontrol agents act to inhibit the growth of pathogens in the rhizosphere [18]. *Bacillus subtilis* QM3, a siderophore producer investigated in this paper, was illuminated as a potential biocontrol agent [19].

Studies on the biological control of lettuce drop using bacterial antagonists *Pseudomonas chlororaphis* and *Bacillus amyloliquefaciens* were conducted by Fernando et al. in 2007 and Saharan and Mehta in 2008, and a significant reduction in *Sclerotinia* infection was observed [20,21]. Antagonistic bacteria inhibit the germination of ascospores through the production of antimicrobial substances, such as cyclic lipopeptides, including iturin, fengycin, and surfactin [22,23], and siderophores [24] on the growth and development of ascospores. This investigation focuses mainly on the development of biocontrol strategies as the main component of integrated disease management for the management of lettuce drop in greenhouse production. This study reports the in vitro evaluation of the antifungal activity of *B. subtilis*, *P. megaterium*, *B. safensis*, and *B. mojavensis* against *S. minor*.

2. Materials and Methods

2.1. Bacteria

The four bacteria were isolated from soil intercropping with camelina (*Camelina sativa* L.) and vetch (*Vicia sativa* L.) on the experimental field at the Agricultural University Plovdiv. DNA isolation was performed using the HiPurA™ Bacterial Genomic DNA Purification Kit (Canvax, Valladolid, Spain) following the manufacturer's protocol, with the addition of 5 µL lysozyme in the lysis buffer, followed by 20 µL proteinase K. The concentrations of the isolates DNA samples (50–70 ng/µL of DNA) were deter-

mined using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). To achieve accurate species identification, the 16S rRNA gene was sequenced with universal primers (27F and 1492R) [25] at Microsynth (Balgach, Switzerland), and the obtained sequences were compared with world databases (https://blast.ncbi.nlm.nih.gov/ and https://www.ezbiocloud.net/ accessed 25 June 2023). The accession in the NCBI Genbank is under submission (sub14450584/08.06.2024) as follows: *B. subtilis* JAN25-4 (accession OR731944.1), *B. megaterium* JAN33-2 (accession OR731941.1, NCBI submission sub13929898/29.10.2023), *B. safensis* CAM24K3 (accession PP797578.1), and *B. mojavensis* CAM23K1 (accession PP797576.1).

2.2. Amylolytic Activity of Tested Bacillus Strains

The four different isolated strains of the genus *Bacillus* were qualitatively and quantitatively evaluated for the presence of amylolytic activity using the agar diffusion method [26]. The starch agar plate was prepared with the following composition (g/L): wheat starch, 10; peptone, 5; yeast extract, 5; $MgSO_4 \cdot 7H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.01; agar, 15; and pH 6.8 ± 0.2. In the agar diffusion method, 15 cm^3 of the nutrient medium was poured into a petri dish. After solidification in the petri dish, 4 wells with a diameter of 8 mm were made. For each strain, two samples were prepared: CM (culture medium from a 24 h suspension of the strain) and CFS (cell-free supernatant obtained following centrifugation of the culture fluid at 5000× *g* using a Universal 320 R, Marvel Ltd., Plovdiv, Bulgaria). The bacterial suspension or CFS from each isolate was instilled in 100 µL. After incubation at 30 °C for 48 h, the petri dishes were treated with Lugol's solution (3% KI) to form a blue-colored starch–iodine complex. A halo area was observed around the amylolytic bacterial colonies. The result was reported by measuring the diameter of the halo zone in mm.

2.3. Proteolytic Activity of Bacillus Strains

The proteolytic activity of the tested bacteria was tested by inoculating milk agar according to Nabrdalik (2010) [27], containing casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skimmed milk powder 2.5%, and agar 1.5%. The bacterial isolates were pre-activated in TSA broth. The proteolytic activity of each strain was investigated using culture fluid (CM) obtained from a 24 h culture and cell-free supernatant (CFS) obtained following centrifugation of the culture fluid. Wells with a diameter of 9 mm were formed in the agar medium with added skimmed milk, into which 100 µL of the previously prepared cultures and cell-free extracts were added. The samples were cultured at 30 °C and the diameter of the proteolytic zones was measured in mm and reported after 24, 48, and 72 h [28].

3. Quantification of the Synthesized Secondary Metabolites with Phytohormonal Activity

The synthesis of microbial phytohormones by soil microorganisms is associated with signal changes in the root and the stimulation of plant growth. The presence of a particular phytohormone in the microbial culture supernatant is insufficient to prove the molecule's functional role in its interaction with the plant. A number of studies have observed a correlation between plant growth and hormone concentration measured in the culture medium or in colonized plant tissues, both in experimental and in situ conditions [29].

3.1. Quantification of Indole-3-Acetic Acid Using the Salkowski Reagent

For quantification of indole-3-acetic acid (IAA) production, bacterial isolates were grown in a tube containing medium with or without 0.1% (*w*/*v*) L-tryptophan (L-Trp) and were incubated in the dark at 30 °C for 5 days, as described previously by Petkova et al. in 2022 [30]. One milliliter of the cell culture was pelleted using centrifugation at 3000× *g* for 5 min, and 0.5 mL of the supernatant was mixed with 0.5 mL of Salkowski's reagent (2 mL of 0.5 M iron (III) chloride and 98 mL of 35% perchloric acid) [31]. After 30 min, color development (red) was quantified using a spectrophotometer (Unico 1200-Spectrophotometer, Dayton, OH, USA) at 530 nm. A calibration curve using pure indole-

3-acetic acid was established to calculate IAA concentration. IAA production of each bacterial isolate was determined by inoculating L-tryptophan medium containing 0.1% (*w*/*v*) L-tryptophan with and incubating in the dark at 20 °C. After incubation, the IAA produced was quantified spectrophotometrically.

3.2. Screening for Dissolution of Inorganic Phosphates

Screening for the dissolution of inorganic phosphates was performed using Pikovskaya's medium (PVK), as described by Patil in 2014 [32]. The medium contained: glucose, 10 g; $Ca(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4 \cdot H_2O$, 0.002 g; and $FeSO_4 \cdot 7H_2O$, 0.002 g.

3.3. ZnO Dissolution Screening

The isolates were tested for zinc solubilization ability using a modified ZnO medium [33]. The medium included 10.0 g of glucose, 1.0 g of ammonium sulfate, 0.2 g of potassium chloride, 0.2 g of dipotassium hydrogen phosphate, 0.1 g of magnesium sulfate, and 0.1% of insoluble zinc from a ZnO source in 1000 mL of distilled water at a pH of 7.0. From a 24 h culture of isolates, 100 µL were dropped onto 10 mm petri wells containing the medium and incubated at 30 °C for 72 h. Colony diameter and halo areas were recorded. The solubility index was calculated using the clear zone diameter/colony diameter formula [34].

4. Antimicrobial Activity against *Sclerotinia minor*

4.1. Screening for the Production of Siderophores

CAS (Chrome Asurol S) blue agar was prepared by dissolving 60.5 mg of CAS in 50 mL of distilled water and mixing it with 10 mL of iron (III) solution (1 mM of FeCl and 10 mM of HCI). While stirring, this solution was slowly added to 72.9 mg HDTMA (hexadecyl-trimethyl-ammonium bromide) dissolved in 40 mL of water. The resultant dark blue liquid was autoclaved for 20 min. Additionally, a mixture of 750 mL water and 15 g agar was autoclaved, and the pH was adjusted to 6.8 with NaOH. The CAS reaction rate was determined by measuring the intensity of color change in the CAS blue agar from blue to purple or orange.

4.2. Dual Culture Assay for the Screening of Biocontrol of Syderophore-Produsing B. mojavensis

The bacterial isolate of *B. mojavensis* was tested for in vitro antagonistic activity against *S. minor* using a dual-culture assay, as described by Sakthivel and Gnanamanickam (1986) [35]. *B. mojavensis* was plated in CAS (Chrome Azurol S) blue agar. A fungal disc (≈9 mm^2) was placed in the center of a potato dextrose agar (PDA) plate, which contained 0.7% agar. The plates were incubated at 28 °C for 7 days. The growth of *S. minor* was observed under a microscope and compared with control plates with and without the examined *Bacillus mojavenisis* strain.

4.3. Isolation of Sclerotinia minor Pathogen and Antimicrobial Activity against Sclerotinia minor

The tested bacterial strains were tested to determine their antifungal activities against *S. minor*. This pathogen was originally isolated from infected plants cultivated in a greenhouse in Gradina village, Plovdiv, Bulgaria (42°07′58.8″ N, 25°12′00″ E). White, cottony fungal mycelia were observed on the basal plant surfaces, and black structures called sclerotia were detected within the cottony growth (Figure 1A). The pathogen was isolated and cultivated at 28 °C on yeast extract medium (containing 10 g yeast extract, 20 g glucose and peptone, and 20 g agar) for 7 days. Then, conidia were collected by washing with cold sterile distilled water and were used to prepare an inoculum at a concentration of 1×10^4 spores/mL. *S. minor*-infected plants were used in plant pathogenicity tests according to Koch's postulates for infecting lettuce leaves, as shown in Figure 1B. The 9 mm dicks were cut from the inoculated petri dishes and were used to re-infect the healthy lettuce leaves after surface sterilization (Figure 1B). After infection, the fungal phytopathogen

caused wilting and yellowing of the lower leaves within 5–7 days of incubation, and sclerotia were observed (Figure 1B). Figure 1C shows the healthy lettuce leaves.

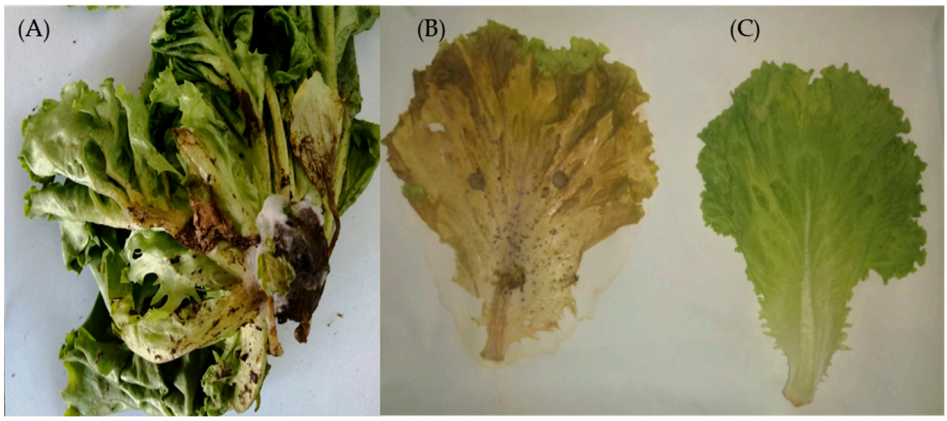

Figure 1. (**A**) *S. minor*-infected plants, (**B**)inoculation of the lettuce leaves after surface sterilization with *S. minor* dicks and symptoms of the disease, and (**C**) the state of a control lettuce leaf.

5. Results and Discussion

5.1. Selection of the Isolated Bacillus Strains According to Their Amylolytic Activity

The first criterion for selecting isolated bacteria is their ability to grow in a medium with starch as the main carbon source. When using solid nutrient media, an indicator of the presence of amylolytic activity is the appearance of lightning around the colonies assimilating starch, which can be observed after treatment with Lugol's solution (Table 1). The amylolytic activity occurring on solid culture media is mainly due to cell-bound amylases, the expression of which depends on multiple factors.

The isolates did not show the same amylolytic activity on all media. This is due to the different genes involved in the breakdown of carbon sources. As a result of the hydrolysis tests, the tested *Bacillus* strains were used, which exhibited the ability to digest wheat starch. Bacteria have extracellular amylase enzyme activity that can hydrolyze starch (amylose and amylopectin). As a result, they can degrade starch, forming a clear zone around the bacterial colonies. The most active bacteria with amylolytic activity were *B. safensis*, with a reported starch digestion area of 29.263 ± 1.1654 mm, followed by *B. subtilis* with an area of 22.365 ± 1.983 mm, and *P. megaterium* with an area of 22.166 ± 1.912 mm. The lowest amylolytic activity was observed in *B. mojavensis* cell-free supernatant, with an area of 15.823 ± 1.028 mm.

Higher amylolytic activity was also observed in the cell-free supernatant of *B. safensis*, with a reported starch digestion area of 12.207 ± 0.725 mm, followed by *P. megaterium* with an area of 11.052 ± 0.828 mm and *B. subtilis* with an area of 10.821 ± 0.938 mm (Table 1).

Table 1. Amylolytic activity of the four *Bacillus* strains. The results are shown as means with standard deviation (n = 3).

No	Bacillus Strain	Amilolytic Activity, mm	
		Cultured Cells	Cell-Free Supernatant
1	*Bacillus subtilis*	22.365 ± 1.983 a	10.821 ± 0.938 a
2	*Priestia megaterium*	22.166 ± 1.912 a	11.052 ± 0.828 a
3	*Bacillus safensis*	29.263 ± 1.1654 b*	12.207 ± 0.725 b*
4	*Bacillus mojavensis*	15.823 ±1.028 c*	9.350 ± 0.813 a*

Note: a, b, and c indicate statistical references, * indicates a highly significant difference with $p < 0.05$.

5.2. Study of Proteolytic Activity of Tested Bacillus sp.

The nature and extent of proteolytic processes are important for preserving good antimicrobial characteristics. The isolates were characterized by the highest proteolytic

activity. *Bacillus* sp. were involved in specific degradation processes of proteins, which are required to fulfil specific functions (e.g., cell cycle regulation, differentiation processes, and stress responses) [36]. Three days after inoculation (at the 72nd hour), the most active bacteria with proteolytic activity were *B. safensis*, with a reported starch digestion area at 72 h of 48.2 ± 0.803 mm for cultured cells and 16.3 ± 0.820 mm for cell-free supernatant. *B. subtilis* and *P. megaterium* also demonstrated very good activity, with zones of 38.6 ± 0.561 mm and 40.6 ± 0.324 mm, respectively. The lowest proteolytic activity was observed in *B. mojavesis*, with an area of 28.3 ± 0.193 mm, as well as its cell-free supernatant showing an area of 10.3 ± 0.563 mm (Table 2).

Table 2. Proteolytic activity of the four *Bacillus* strains. The results are shown as means with standard deviation (n = 3).

No	*B.* Strain	Proteolytic Activity, mm					
		Cultured Cells			Cell-Free Supernatant		
		24 h	48 h	72 h	24 h	48 h	72 h
1	*B. subtilis*	24.0 ± 0.206 a	33.3 ± 0.162 a	38.6 ± 0.561 *a	0	13.0 ± 0.224 b*	11.8 ± 0.321 a
2	*P. megaterium*	25.5 ± 0.218 a	38.9 ± 0.176 a	40.6 ± 0.324 b	0	6.08 ± 0.186 a	12.6 ± 0.052 a
3	*B. safensis*	32.2 ± 0.702 b*	42.4 ± 0.275 b*	48.2 ± 0.803 b*	8.0 ± 0.15	12.5 ± 0.173 b	16.3 ± 0.820 b*
4	*B. mojavesis*	23.4 ± 0.369 a	26.6 ± 0.294 a	28.3 ± 0.193 a	0	7.8 ± 0.204 a	10.3 ± 0.563 a

Note: a and b indicate statistical references, * indicates a highly significant difference with $p < 0.05$.

5.3. Quantification of Indole-3-Acetic Acid (IAA)

The four bacterial strains exhibited weak IAA production. The results obtained are shown in Figure 2. From the results, it can be concluded that the bacteria exhibit a low level of IAA synthesis when 0.1% L-trypophan is present in the medium. The concentrations measured were 0.712 ppm (µd/mL) in *B. subtilis*, 0.848 ppm in *P. megaterium*, the highest concentration of 0.862 ppm in *B. mojavensis*, and the lowest IAA synthesis of 0.304 ppm in *B. safensis*.

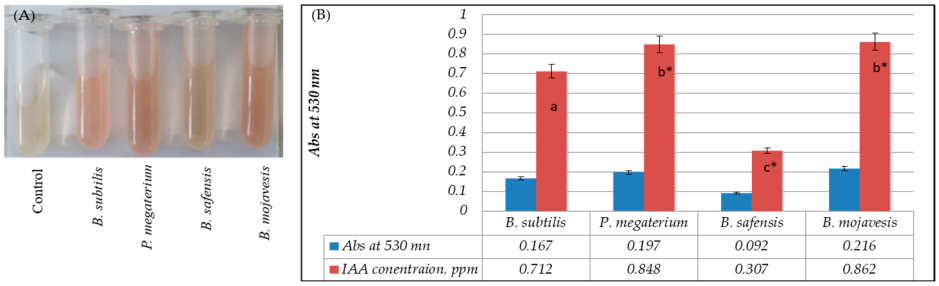

Figure 2. (**A**) Indole-3-acetic acid production by the test bacteria in medium with 0.1% (w/v) L-tryptophan incubated at 30 °C and 150 rpm for 24 h. (**B**) A graph representing the absorbance of IAA at 530 nm and the concentration of indole separated by each strain in ppm. Note: a, b and c indicate statistical references, * indicates a highly significant difference with $p < 0.05$.

The synthesis of microbial phytohormones by bacteria is associated with signaling changes in the root and the stimulation of plant growth. The presence of a particular phytohormone in the microbial culture supernatant is not sufficient to prove the functional role of that molecule in its interaction with the plant. A number of studies have observed a correlation between plant growth and hormone concentration measured in the culture medium or in colonized plant tissues, both in experiments and in situ [29,37].

5.4. Screening for Dissolution of Inorganic Phosphate and ZnO

Acid phosphatases and phytases synthesized by rhizosphere microorganisms are involved in the organic solubilization of soil phosphorus [38]. The strains exhibited high phosphate-solubilizing activity on PVK medium, with solubilization zones greater than

2 cm. The ability of three of the investigated strains to improve the solubility of inorganic phosphates is an important characteristic of PGP microorganisms. The results of the present study show that the cell-free supernatants of *B. subtilis*, *P. megaterium*, and *B. safensis* did not exhibit such activity (Figure 3). The highest dissolution index observed in *B. mojavesis* cell-free culture was 2.670 ± 0.264 cm (Figure 3A). The established ability of the investigated strain to improve the solubility of inorganic phosphates is an important characteristic of PGP microorganisms.

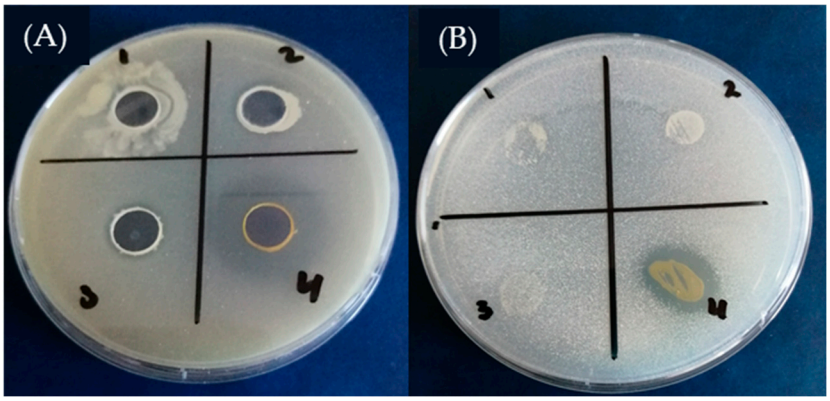

Figure 3. (**A**) Halo zones obtained from the phosphate-solubilization activity of the tested bacteria during cultivation on PVK medium at 28 °C for 3 days. (**B**) Zones obtained from the zinc-solubilizing activity of the tested bacteria when cultured on modified medium containing ZnO. Numbers indicate the Bacillus strains: *B. subtilis* (1), *P. megaterum* (2), *B. safensis* (3), and *B. mojavensis* (4).

ZnO is insoluble in water and soluble in most acids [39]. The carboxylic groups of organic acids can complex metal cations and displace anions to convert insoluble forms of Zn, such as ZnO, into more soluble forms [40]. Several laboratory studies have reported that microbes, which produce siderophores or CO_2, may contribute to environmental Zn solubilization, depending on the species of organism and their growth conditions [41,42]. *B. mojavesis* exhibited Zn solubilizing activity, whereas the remaining strains showed no zinc-solubilizing ability (Figure 3B).

5.5. The Production of Siderophores

A color change to yellow was ovserved in *P. megaterum* (2), *B. safensis* (3), and *B. mojavensis* (4), with a zone of 0.323 ± 0.078 cm, 0.167 ± 0.032, and 0.598 ± 0.233 cm, respectively (Figure 4A). Exogenous application of the bacterial siderophore on two layers of CAS/PDA agar to fungal cultures resulted in decreased colony size, increased filament length, and changes in the hyphal branching pattern (Figure 4B,C).

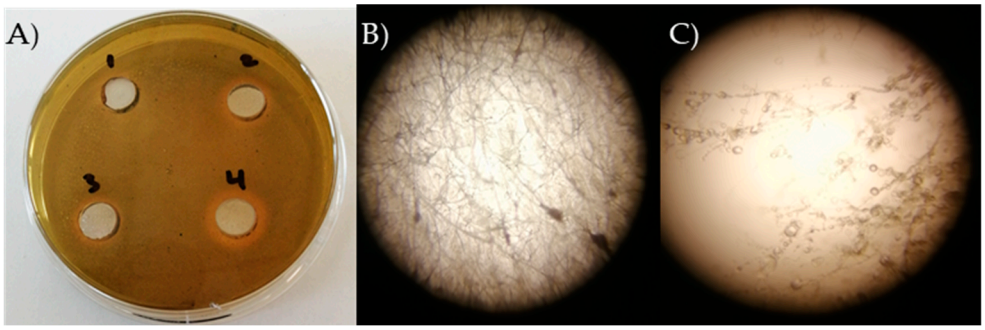

Figure 4. (**A**) Siderophore production by *B. subtilis* (1), *P. megaterum* (2), *B. safensis* (3), and *B. mojavensis* (4). Strains were evaluated based on the halo zone observed on the CAS agar plate. (**B**) *S. minor* filament growth and (**C**) changes in the hyphal branching pattern after treatment with *B. mojavensis* siderophores on two layers of CAS/PDA agar.

In the current investigation, siderophore production was detected using the CAS reagent, which revealed a color change from blue to orange for *P. megaterum* (2), *B. safensis* (3), and *B. mojavensis* (4). This result is in accordance with the findings of De Los Santos-Villalobos (2012) and Mehri et al. (2012) [43,44], which reported that different doses of zinc and magnesium also play a major role in the induction of siderophore production, as evidenced by the formation of yellow–green fluorescent pigment around them on nutrient agar medium. Several studies revealed that siderophores are involved in stimulating plant growth and protecting the plants against various biotic stresses [45] and phytopathogens, including *Sclerotinia sclerotiorum* (Lib.) de Bary [46].

5.6. In Vitro Antifungal Activity of the Tested Bacillus Strains

Many active bacterial strains have been described as biocontrol agents due to their common ability to produce various antifungal metabolites. The antifungal activity of the four tested strains inhibited *S. minor* and the calculated inhibition effects are presented in Figure 5. From the obtained results, all four strains of the tested bacteria completely inhibit the growth of *S. minor* (Figure 5).

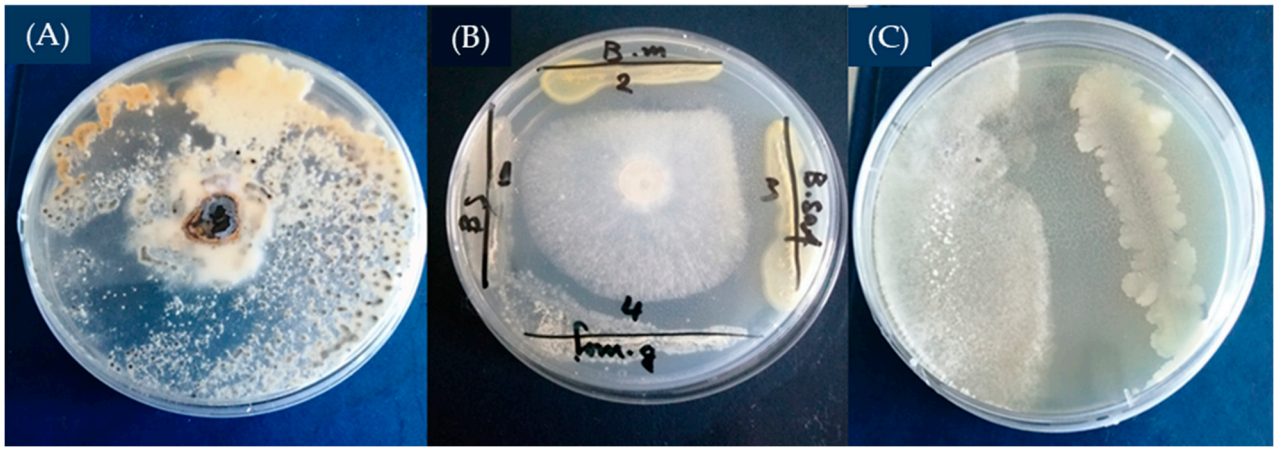

Figure 5. Antifungal activity of the tested strains against *S. minor* on PDA for 7 days of incubation at 28 °C. (**A**) *Sclerotinia minor* grown on PDA for 5 days of incubation at 28 °C. (**B**) Antifungal activity of cultured cells of the tested strains: 1—*B. subtilis*, 2—*P. megaterium*, 3—*B. safensis*, and 4—*B. mojavensis* against *S. minor*. (**C**) Antifungal activity of *B. mojavensis* against *S. minor*.

S. minor produces mycotoxins, which are unsuitable for human consumption. *S. minor* has become one of the most economically important and studied fungal plant pathogens. Environmental pollution caused by the excessive use of chemical pesticides has led to a considerable shift toward biological control using antagonistic microorganisms (Figure 5).

In vivo, the antibacterial efficacy of *B. mojavensis* against *S. minor* in lettuce exhibited inhibition on the seventh day after infection (DAI) (Figure 6). On the seventh DAI, symptoms were observed in plant tissues not treated with beneficial bacteria. In the control plants, the fungal pathogen caused 100% infection, with lesions on the lettuce plants (Figure 6). In the presence of *B. mojavensis*, infection was suppressed, with a 10% infection rate (Figure 6). These results demonstrate the promising potential of *B. mojavensis* CAM23K1 for the control of *S. minor* infection in lettuce and expand our knowledge of the application of bacteria for the biological control of pathogens.

In the literature, effective management of *Sclerotinia* lettuce drop involves considering records of disease incidence during several growing seasons. Cultural management practices, including crop rotations with resistant non-host plants, altering tillage practices, and using cover crops, can help to reduce the risk of disease development [46]. Garza et al. 2002 reported that crop rotation and no-tillage in soybeans are practically the most successful strategies for preventing *Sclerotinia* stem rot in soybeans [47]. The pathogen has more than 400 host plants and requires around 5 years of rotation of two non-host crops of *Sclerotinia*

to decrease the infection level in sunflowers [48]. Research into bacterial antagonists is gaining acceptance and application. Bacterial antagonists, such as *Trichoderma* sp., *Bacillus amyloliquefaciens* BS6, and *Pseudomonas* sp. DF41 strains, have well-studied mechanisms that play a role in the suppression of *Sclerotinia* infection under both field and greenhouse conditions [46,49,50]. These strains produce extracellular metabolites, including protease, hydrogen cyanide (HCN), alginate, and lipopeptides, which suppress the development of *Sclerotinia* [51].

Figure 6. Antifungal activity of the tested strains against *S. minor* on the 7th DAI in pot experiments.

In conclusion, *Bacillus* sp. can promote plant growth and suppress phytopathogens by producing amylolytic enzymes, proteolytic enzymes, and siderophores. In the pot trials, when lettuce plants were grown in soils contaminated with *S. minor*, a 100% pathogen attack was achieved when plants were without *B. mojavensis* CAM23K1 treatment. Lettuce treated with the bacteria experienced an 80% reduction in pathogen effects. The antagonistic *B. mojavensis* CAM23K1 suppressed *S. minor* disease in the pot experiment and promoted lettuce growth and development. Further experiments will involve the application of these strains under greenhouse conditions with *Sclerotinia* infestation. The suppression of the phytopathogen by the tested bacterial strains will be further investigated through microbiological and metagenomic analyses.

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