

Article

# Antifungal Screening of Bioprotective Isolates against *Botrytis cinerea*, *Fusarium pallidoroseum* and *Fusarium moniliforme*

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**Abstract:** The fungi *Botrytis cinerea*, *Fusarium pallidoroseum*, and *Fusarium moniliforme* are the causative agents of several plant diseases and can cause significant crop loss both before and after harvest. Fungicides are employed to control these phytopathogens, but fungicide use has led to an increase in resistance and may negatively affect the environment and human health. Hence, more environmentally sustainable solutions such as biological control methods are needed. The purpose of this study was to screen 22 bacterial isolates for inhibitory activity against fungal phytopathogens. To evaluate antifungal activity, the bacterial isolates were individually spot-inoculated onto Tryptic Soy Agar or de Man, Rogosa, Sharpe agar, and then a plug of fungal-colonized agar was placed onto the center of the isolate-inoculated plate. Plates were incubated at 24 °C for 10 days and fungal growth was evaluated. Nine of the 22 isolates screened inhibited all three fungi; inhibition by these isolates ranged from 51–62%, 60–68%, and 40–61% for *B. cinerea*, *F. pallidoroseum*, and *F. moniliforme*, respectively. Isolates were also screened for biosurfactant activity using the drop-collapse test. *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus thuringiensis* and three *Bacillus amyloliquefaciens* isolates demonstrated strong biosurfactant activity and suppression of all three fungi, and therefore are recommended for further study.

**Keywords:** biocontrol; bioprotective; biosurfactant; phytopathogens; *Botrytis*; *Fusarium*

## 1. Introduction

Crop loss due to phytopathogenic microorganisms has a dramatic impact on the agricultural industry. *Botrytis cinerea* and *Fusarium* species are fungal phytopathogens that cause substantial losses across a wide variety of crops both before and after harvest. In the field, *B. cinerea*, the fungus responsible for the plant diseases gray mold and Botrytis rot, affects over 200 crop species ranging from ornamentals to fruits and vegetables like lettuce, grapes, and strawberries [1,2]. Postharvest, *B. cinerea* causes the rapid decay of fresh produce because of its ability to invade damaged, weak, or rapidly senescing tissue [3], leading to a decreased shelf life. Fusarium Wilt and Fusarium Rot, caused by various *Fusarium* species, also affect a wide array of crops pre-harvest including lettuce, soybeans, strawberries, tomatoes, peppers, potatoes, and oranges [4–6]. *Fusarium* species are also problematic during postharvest storage, particularly in cereal grains and animal feeds [7]. The mycotoxins fumonisin B<sub>1</sub> and B<sub>2</sub>, trichothecenes, and zearalenone are produced by some species of *Fusarium* and can cause disease in humans and animals [8,9]. Control measures to eliminate or mitigate the impact of *B. cinerea* and *Fusarium* species on quality and safety before or after harvest are needed.

Management of fungal phytopathogen pre- and post-harvest often includes the use of chemical fungicides. However, increases in fungicide resistance as well as health and environmental concerns associated with the use of harsh chemicals have created the need for an alternative means of

treatment. The use of bioprotective bacteria pre-harvest or as a postharvest treatment could help to protect crops against fungi. Several bacterial species across a range of genera produce compounds active against phytopathogenic fungi. Synthesis of the antifungal lipopeptides has been identified in species of *Bacillus*, including *Bacillus amyloliquefaciens* [10–12], *Bacillus subtilis* [13,14], and *Bacillus thuringiensis* [15,16]. Some species of lactic acid bacteria (LAB) also produce antifungal compounds; these include organic acids, phenyllactic acid, 3-hydroxy fatty acids, and cyclic dipeptides [17–19]. Additional compounds produced by bacteria that have demonstrated antifungal activity include biosurfactant lipopeptides [20–25]. These bioactive lipopeptides, including surfactin, mycosubtilin, fengycin, and iturin, act on cell membranes, causing membrane disruption and cell leakage [26]. The antifungal activity of bioprotective bacteria in vitro has translated into fungal suppression when applied both in the field and postharvest.

Bioprotective bacteria have shown antifungal efficacy when applied to crops before harvest. Species of *Bacillus* have reduced the incidence of *Fusarium* disease in maize and tomato plants when applied pre-harvest [27,28] and decreased fruit rot caused by *B. cinerea* on strawberry plants [29]. Biocontrol agents have also suppressed fungal growth on fruits and vegetables when applied postharvest. Various species of *Bacillus* have been shown to reduce *B. cinerea* infection on pears, strawberries, and tomatoes [30–32]. *Lactobacillus plantarum* suppressed *B. cinerea* and *Fusarium graminearum* on cucumbers [33], and *Pediococcus pentosaceus* suppressed the growth of *Penicillium expansum* on pears [34]. Fungal suppression by various bacterial species demonstrates their potential use as biocontrol agents against fungal phytopathogens both before and after harvest.

Several species of *Bacillus* are commercially available as pre-harvest biocontrol treatments for various fruits and vegetables; Serenade<sup>®</sup> Optimum utilizes *B. subtilis* [35], Sonata<sup>®</sup> contains *B. pumilus* [36], and DoubleNickel LC uses *B. amyloliquefaciens* as the active ingredients [37].

The aim of this study was to screen and identify bacterial isolates that could potentially be used for the biological control of *Botrytis cinerea*, *Fusarium pallidoroseum*, and *Fusarium moniliforme*. Of the 22 isolates screened, six showed characteristics that indicated the potential for use as biocontrol agents and are therefore recommended for application-based research.

## 2. Materials and Methods

### 2.1. Fungal Pathogens

All fungi were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Working cultures of *Botrytis cinerea* ATCC 46522, *Fusarium pallidoroseum* ATCC 48152, and *Fusarium moniliforme* ATCC 60846 (teleomorph, *Gibberella fujikuroi*) (Table 1) were maintained on Potato Dextrose Agar (PDA, Becton, Dickson and Company; Sparks, MD, USA) at 4 °C and as fungal-colonized PDA plugs suspended in Potato Dextrose Broth (PDB, Becton, Dickson and Company; Sparks, MD, USA) with 25% glycerol at –70 °C for long-term storage.

### 2.2. Bioprotective Isolates

All bioprotective isolates were provided by BiOWiSH Technologies (Cincinnati, OH, USA). Working cultures of LAB isolates were maintained on de Man, Rogosa, Sharpe agar (MRS; Oxoid; Basingstoke, Hampshire, UK) at 4 °C and all other bioprotective isolates on Tryptic Soy Agar (TSA; Remel, Lenexa, KS, USA) or TSA with 5% NaCl (*w/w*) at room temperature. For long-term storage, isolates were kept in the appropriate growth media (Table 1) with 15% glycerol at –70 °C with the exception of *Bacillus clausii* and *Bacillus firmus*—these two isolates were stored as spore crops at 6 °C. Prior to use in the screening, LAB isolates were inoculated into 10 mL of MRS Broth and incubated at 35 °C for 18–24 h. All other isolates were inoculated into 10 mL of Tryptic Soy Broth (TSB; Remel, Lenexa, KS, USA) or TSB with 5% NaCl (*w/w*) and incubated with agitation on an orbital shaker at either 30 or 35 °C, depending on the isolate, for 18–24 h.

### 2.3. Antifungal Screening

A loopful of prepared bioprotective culture was spot-inoculated in two locations directly across from each other near the perimeter of solidified media; the type of screening media was determined by the growth requirements of the bacterial isolate (Table 1). Using a sterile cork borer, a 9 mm plug was cut from the perimeter of a working culture of the fungal pathogen and placed in the center of the isolate-inoculated plate. Plates were wrapped in Parafilm® and incubated at 24 °C for 10 d. The diameter of the fungal colony between the two isolate inoculation locations was measured daily, beginning on Day 3. A fungus-inoculated plate of each screening media was used as a control. Each test was done in duplicate.

**Table 1.** Bioprotective isolates and fungal phytopathogens.

Isolate	Growth Medium	Incubation Temperature	Screening Medium
Bioprotective isolate			
<i>Bacillus amyloliquefaciens</i> BA1	TSB	35 °C	PDA
<i>Bacillus amyloliquefaciens</i> BA2	TSB	35 °C	PDA
<i>Bacillus amyloliquefaciens</i> BA3	TSB	35 °C	PDA
<i>Bacillus amyloliquefaciens</i> BA4	TSB	35 °C	PDA
<i>Bacillus amyloliquefaciens</i> BA5	TSB	35 °C	PDA
<i>Bacillus clausii</i>	TSB	30 °C	PDA
<i>Bacillus coagulans</i>	TSB	35 °C	PDA
<i>Bacillus firmus</i>	TSB	30 °C	TSA
<i>Bacillus licheniformis</i>	TSB	35 °C	PDA
<i>Bacillus megaterium</i>	TSB	35 °C	PDA
<i>Bacillus pumilus</i>	TSB	35 °C	PDA
<i>Bacillus sphaericus</i>	TSB	30 °C	PDA
<i>Bacillus thiaminolyticus</i>	TSB	30 °C	TSA
<i>Bacillus thuringiensis</i> BT1	TSB	35 °C	PDA
<i>Bacillus thuringiensis</i> BT2	TSB	35 °C	PDA
<i>Lactobacillus amylovorus</i>	MRS	35 °C	MRS
<i>Lactobacillus plantarum</i>	MRS	35 °C	MRS
<i>Oceanobacillus sojiae</i>	TSB w/5% NaCl ( <i>w/w</i> )	35 °C	TSA
<i>Ochrobactrum anthropi</i>	TSB	35 °C	PDA
<i>Pediococcus acidilactici</i>	MRS	35 °C	MRS
<i>Pediococcus pentosaceus</i>	MRS	35 °C	MRS
<i>Serratia plymuthica</i>	TSB	30 °C	PDA
Fungal phytopathogen			
<i>Botrytis cinerea</i>	PDA	24 °C	N/A <sup>1</sup>
<i>Fusarium pallidoroseum</i>	PDA	24 °C	N/A <sup>1</sup>
<i>Fusarium moniliforme</i>	PDA	24 °C	N/A <sup>1</sup>

TSB: Tryptic Source Broth, MRS: Man, Rogosa, Sharpe agar, PDA: Potato Dextrose Agar. TSA: Tryptic Source Agar. N/A<sup>1</sup>: not applicable.

### 2.4. Biosurfactant Screening

Biosurfactant production by bioprotective isolates was evaluated via the drop-collapse test, which was adapted from Turgrul and Cansunar (2005) [38]. Briefly, each bioprotective isolate was cultured as previously described and incubated for 7 d; all isolates except LAB were incubated with agitation on an orbital shaker. After incubation, cultures were centrifuged at 3000 RPM for 15 min to separate the bacterial cells from the culture media; the resultant supernatant was used in the biosurfactant screening.

The surface of a petri dish was coated with mineral oil and allowed to sit at room temperature for at least 1 h. A 20 µL aliquot of bioprotective culture supernatant was placed onto the oiled surface, with the micropipette at a 45° angle relative to the surface of the petri dish. The diameter of the drop was then measured after 2 min. A 20 µL aliquot of fresh media (i.e., TSB or MRS) was treated in the same manner described above to serve as a negative media control.

The presence of a biosurfactant would reduce the surface tension of the bioprotective culture supernatant, thus causing the drop to spread on the oiled surface of the petri dish rather than hold its shape. Therefore, an isolate was considered to be a biosurfactant producer if the diameter of its supernatant drop was significantly larger than the diameter of the appropriate negative media control ( $\alpha = 0.05$ ).

### 2.5. Data Analysis

The antifungal screening was completed once in duplicate. Fungal diameters were averaged and percent fungal inhibition was determined using the following equation:

Calculation for the percent fungal inhibition:

$$\frac{(\text{control fungus diameter}) - (\text{isolate fungus diameter})}{(\text{control fungus diameter})} \times 100\% = \% \text{ inhibition} \quad (1)$$

The biosurfactant screening was completed twice in duplicate. Data were analyzed using Analysis of Variance (ANOVA); analysis was completed with Minitab 16 (Minitab Inc., State College, PA, USA). Dunnett's test was used to identify isolate drop diameters that were significantly larger than the diameter of the negative media control ( $\alpha = 0.05$ ).

## 3. Results and Discussion

### 3.1. Antifungal Activity

#### 3.1.1. *Bacillus* Species

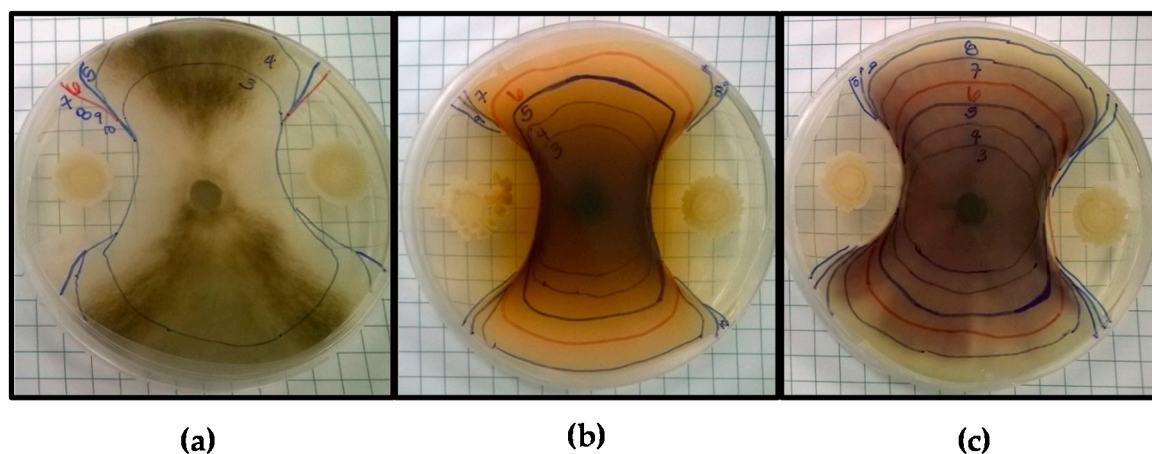
*Bacillus* species have been investigated as possible biocontrol agents because of their ability to synthesize antifungal compounds, including lipopeptides and other antibiotics. Several *Bacillus* species have demonstrated antifungal activity against fungal phytopathogens, including *B. cinerea* and/or *Fusarium* species, in vitro [39–43]. However, antifungal capability is a function of bacterial strain and fungal pathogen. In this study, eight of the *Bacillus* isolates suppressed the growth of *B. cinerea*, *F. pallidoroeseum*, and *F. moniliforme* (Table 2 and Figure 1); *Bacillus megaterium* exhibited the largest zones of inhibition against all three fungi, followed by *Bacillus coagulans* and several strains of *B. amyloliquefaciens*. Fungal inhibition by these isolates ranged from 51–62% for *B. cinerea*, 60–69% for *F. pallidoroeseum*, and 40–61% for *F. moniliforme* (Table 2). Three *Bacillus* isolates inhibited *B. cinerea* but did not suppress the growth of the *Fusarium* species tested. While this screening identified 11 *Bacillus* isolates capable of fungal suppression in vitro; additional testing will be required to determine the efficacy against the phytopathogens when applied to produce.

Inhibition of fungal phytopathogens by *Bacillus* species has not only been observed in vitro but also in planta. The application of *Bacillus* species pre-harvest reduced the incidence and severity of diseases caused by *F. oxysporum* [42], *Alternaria solani*, and *Phytophthora infestans* [44] on tomato plants and *B. cinerea* fruit rot on strawberry plants [29]. Postharvest application of *Bacillus* species has also caused fungal suppression; decay was reduced on pears, tomatoes, bananas, and oranges [12,31,32,45]. Further testing of the isolates used in this study, including challenge studies on various types of produce, is needed to determine their possible use as biocontrol agents.

**Table 2.** Fungal inhibition of bioprotective isolates against *Botrytis cinerea*, *Fusarium moniliforme*, and *Fusarium pallidoroseum* after 10 d incubation at 24 °C.

Bioprotective Isolate	Inhibition		
	<i>Botrytis cinerea</i>	<i>Fusarium pallidoroseum</i>	<i>Fusarium moniliforme</i>
<i>B. amyloliquefaciens</i> BA1	52%	62%	53%
<i>B. amyloliquefaciens</i> BA2	56%	63%	60%
<i>B. amyloliquefaciens</i> BA3	58%	67%	61%
<i>B. amyloliquefaciens</i> BA4	60%	67%	60%
<i>B. amyloliquefaciens</i> BA5	61%	69%	61%
<i>B. clausii</i>	30%	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>B. coagulans</i>	62%	66%	59%
<i>B. firmus</i>	49%	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>B. licheniformis</i>	N/I <sup>1</sup>	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>B. megaterium</i>	62%	68%	61%
<i>B. pumilus</i>	N/I <sup>1</sup>	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>B. sphaericus</i>	N/I <sup>1</sup>	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>B. thiaminolyticus</i>	50%	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>B. thuringiensis</i> BT1	N/I <sup>1</sup>	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>B. thuringiensis</i> BT2	56%	68%	58%
<i>L. amylovorus</i>	N/I <sup>1</sup>	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>L. plantarum</i>	36%	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>O. sojæ</i>	N/I <sup>1</sup>	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>O. anthropi</i>	N/I <sup>1</sup>	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>P. acidilactici</i>	56%	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>P. pentosaceus</i>	46%	N/I <sup>1</sup>	N/I <sup>1</sup>
<i>S. plymuthica</i>	51%	60%	40%

N/I<sup>1</sup>: no inhibition; fungal colony grew over/around bacterial isolate colony.



**Figure 1.** Antifungal screening of *Bacillus amyloliquefaciens* BA4 against (a) *Botrytis cinerea*; (b) *Fusarium pallidoroseum*, and (c) *Fusarium moniliforme*. Markings indicate the border of the fungal colony each day of incubation, beginning on Day 3 up until Day 10 or the last day of observed growth.

### 3.1.2. Lactic Acid Bacteria

Antifungal activity has also been detected from some LAB species; bioactive compounds produced by LAB include organic acids, phenyllactic acid, 3-hydroxy fatty acids, bacteriocins and bacteriocin-like substances, and cyclic dipeptides [17–19,46–48]. Three of the four LAB isolates used in this study inhibited *B. cinerea*, but not *F. pallidoroseum* or *F. moniliforme*. *P. acidilactici* demonstrated the strongest antifungal activity, followed by *P. pentosaceus* and *L. plantarum*; inhibition was 56%, 46%, and 36% respectively (Table 2). Although these isolates were only effective against *B. cinerea*, other LAB species



have demonstrated broad-spectrum fungal inhibition when used as biocontrol agents on produce, but again efficacy varies between LAB and fungus species.

Pre- and post-harvest application of LAB to agricultural crops have shown mixed results. *P. acidilactici* and *P. pentosaceus* decreased *Fusarium* infection on wheat seeds when germinated on PDA, but failed to significantly reduce disease incidence during greenhouse trials [49]. In contrast, *Lactobacillus* species administered to tomato plants as a seed treatment or soil drench have promoted plant growth and mitigated the impact of *F. oxysporum* in potted trials [50]. When applied as a postharvest treatment, LAB reduced the decay caused by *B. cinerea* and *F. graminearum* on cucumbers [33] and decay by *P. expansum* on apples [51] and pears [34]. Again, additional investigation of the three antifungal LAB isolates identified in this study is needed to evaluate their use as biocontrol agents for the control of phytopathogenic fungi on produce.

### 3.1.3. *Serratia plymuthica*

*Serratia plymuthica* is another species of bacteria that has demonstrated suppression of fungal phytopathogens. Synthesis of antifungal compounds such as pyrrolnitrin, haterumalide, siderophores, chitinolytic enzymes, and proteases has been detected in strains of *S. plymuthica* [52,53]. In this study, *S. plymuthica* suppressed the growth of *B. cinerea*, *F. pallidoroseum*, and *F. moniliforme* (Table 2), making it a possible candidate for additional evaluation. The use of other strains of *S. plymuthica* as a biocontrol agent both pre- and post-harvest has been investigated. Foliar application of *S. plymuthica* reduced disease caused by *B. cinerea* and *Sclerotinia sclerotiorum* in cucumber plants [52] and postharvest treatment of potatoes decreased the severity of dry rot caused by *Fusarium sambucinum* [54].

### 3.1.4. Isolates with No Antifungal Activity

Antifungal activity is not only a function of bioprotective isolates and fungus species. It can also be affected by growth conditions. Culture conditions such as media composition, pH, and incubation period and temperature can affect the production of antifungal compounds [55,56]. In this study, four *Bacillus* isolates, *Lactobacillus amylovorus*, *Oceanobacillus sojiae*, and *Ochrobactrum anthropi* did not suppress *B. cinerea*, *F. pallidoroseum*, or *F. moniliforme*. Although these seven isolates did not inhibit any of the fungi against which they were challenged, this does not mean these isolates are incapable of producing antifungal compounds. The culture media and testing conditions used in this study may not have been optimal for the synthesis of bioactive compounds and further investigation is required to determine if these seven isolates truly do not inhibit *B. cinerea*, *F. pallidoroseum*, or *F. moniliforme*.

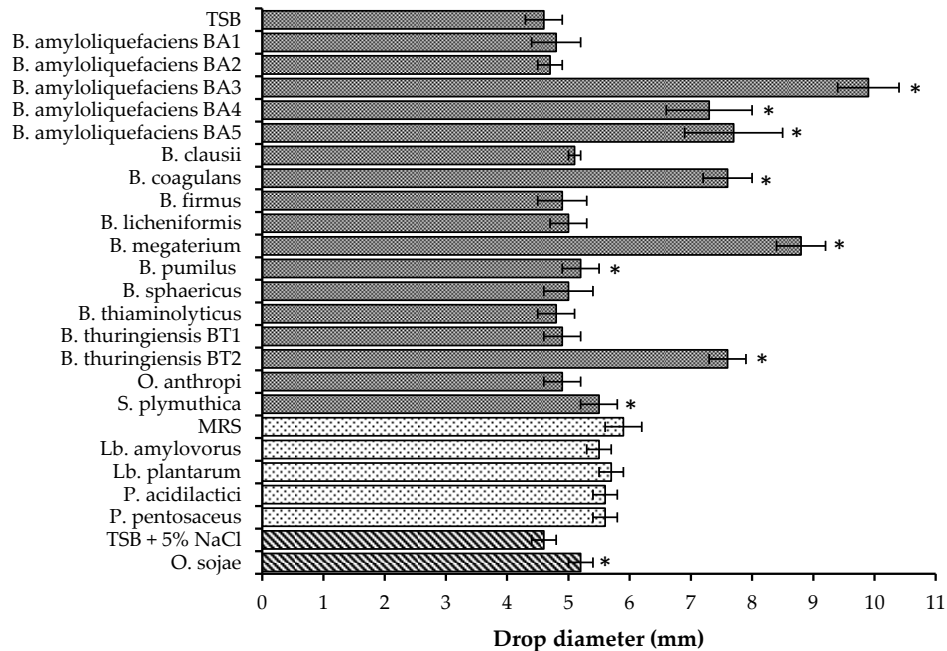
## 3.2. Biosurfactant Activity

### 3.2.1. *Bacillus* Species, *Oceanobacillus sojiae*, and *Ochrobactrum anthropi*

In this study, biosurfactant production was identified in seven *Bacillus* isolates (Figure 2). *B. megaterium*, *B. coagulans*, *B. thuringiensis* BT2 and several *B. amyloliquefaciens* isolates demonstrated both strong biosurfactant activity and suppression of *B. cinerea*, *F. pallidoroseum*, and *F. moniliforme*, implying the ability to synthesize antifungal lipopeptides. The production of antifungal lipopeptides has been identified in other strains of the species of *Bacillus* used in this study, including *B. amyloliquefaciens* [11,12,24], *B. coagulans* [21], *B. megaterium* [23], and *B. thuringiensis* [15,16,57]. *B. pumilus* and *O. sojiae* demonstrated weak biosurfactant activity but no fungal suppression. This suggests that the biosurfactants secreted either did not have antifungal capabilities against the three fungi or that they were not produced in large enough quantities to suppress fungal growth.

*Bacillus* species also produce other compounds with antifungal activity, including antibiotics [22,41,58,59], proteinaceous substances [43,60], siderophores [22,61], and chitinolytic enzymes [27,40,62]. In this study, five *Bacillus* isolates demonstrated antifungal activity, but biosurfactant secretion was not detected; this indicates either the production of antifungal lipopeptides at low levels or that antifungal activity was not due to synthesized lipopeptides, but to some other

bioactive compound. Biosurfactant production and antifungal activity were not detected in four *Bacillus* isolates and *O. anthropi*. The lack of both fungal inhibition and biosurfactant activity suggests that these strains did not secrete antifungal lipopeptides.



**Figure 2.** Biosurfactant activity of bioprotective isolates. Error bars indicate standard deviation. Media used are designated by bars with corresponding patterns. Cultures grown in TSB (cross-hatched), MRS (white), and TSB + 5% NaCl (diagonal lines). Asterisks indicate drop diameters significantly larger than that of the media control ( $\alpha = 0.05$ ).

### 3.2.2. Lactic Acid Bacteria Isolates

Biosurfactant production was not detected in the four LAB isolates used in this study (Figure 2), but this does not mean that these isolates are incapable of producing biosurfactants. Biosurfactants produced by bacteria can either be excreted or remain cell-bound. Gudiña et al. [63] found that *Lactobacillus coryniformis*, *Lactobacillus paracasei*, *L. plantarum* and *Leuconostoc mesenteroides* produced cell-bound biosurfactants at higher levels than excreted ones. Cell-bound biosurfactant production has been observed in several species of *Lactobacillus* [64], including *L. plantarum* [65]. The drop-collapse test used in this study to determine biosurfactant production was only able to detect excreted biosurfactants and not those which are cell-bound; rescreening of LAB isolates with the addition of an extraction step may yield different results.

### 3.2.3. Serratia Plymuthica

The *S. plymuthica* isolate used in this study demonstrated weak biosurfactant activity (Figure 2) and suppressed the growth of *B. cinerea*, *F. pallidoroseum*, and *F. moniliforme* (Table 2). The production of antifungal compounds including pyrrolnitrin, haterumalide, siderophores, chitinolytic enzymes, and proteases has been identified in strains of *S. plymuthica*. This suggests that antifungal activity may have been due to the production of antifungal lipopeptides at low levels, the synthesis of some other type of bioactive compound, or a combination of lipopeptides and antibiotics.

## 4. Conclusions

The present study identified bacterial isolates with antifungal and/or biosurfactant activity. Of the 22 isolates tested, nine isolates—eight *Bacillus* isolates and *S. plymuthica*—suppressed the growth of

*B. cinerea*, *F. pallidoroseum*, and *F. moniliforme* and six isolates—three *Bacillus* and three LAB—inhibited only *B. cinerea*. Biosurfactant production was detected in many of the *Bacillus* species which showed antifungal activity, suggesting the production of antifungal lipopeptides. Based on the results of this screening, six isolates—*B. amyloliquefaciens* strains BA3, BA4, and BA5, *B. coagulans*, *B. megaterium*, and *B. thuringiensis* BT2—are recommended for further evaluation. Additional evaluation should include screening with a larger panel of environmentally isolated fungal strains and characterization of the antifungal compounds produced by the bioprotective isolates. Finally, an investigation into the isolates' antifungal efficacy on produce, both before and after harvest, is needed to evaluate their potential use as biocontrol agents in the agricultural industry.

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