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Original article

Advanced Biocontrol Strategies Utilizing *Bacillus* Species for Sustainable Suppression of *Sclerotinia sclerotiorum*, *Sclerotinia minor,* and *Rhizoctonia solani* in Agroecosystems

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Abstract
This study evaluated the antagonistic activity of *Bacillus* isolates recovered from soil, corn (*Zea mays*) rhizospheres, *Taraxacum officinale,* and sclerotia against three major soilborne fungal pathogens: *Sclerotinia sclerotiorum*, *Sclerotinia minor*, and *Rhizoctonia solani*. Antifungal activity was assessed using dual-culture and culture-filtrate assays evaluating both filter-sterilized and autoclaved filtrates for thermal stability and their effects on fungal growth and sclerotial formation. Among the evaluated isolates, SSB5 demonstrated broad-spectrum antagonistic activity in dual-culture assays, effectively inhibiting mycelial growth across all three target pathogens and suppressing sclerotial development. Isolate BS1 demonstrated selective antagonistic activity, displaying moderate direct inhibition against *Sclerotinia minor* (13.0 mm zone) while lacking activity against *S. sclerotiorum* and *Rhizoctonia solani*. Culture-filtrate assays revealed strain- and thermostability-dependent bioactivity; untreated filtrate of isolate BS3 exerted sustained 100% mycelial growth arrest against *S. sclerotiorum* across 10 days, while retaining moderate heat stability after autoclaving (80.94% at Day 5). Additionally, autoclaved filtrates of BS3, SSB5, and SSB6 induced complete suppression (100%) against *S. minor* at Day 5, though heat-treated SSB5 and SSB6 completely lacked activity (0%) against *S. sclerotiorum*. These findings demonstrate that *Bacillus* isolates, particularly BS3, possess strong antagonistic activity against important soilborne fungal pathogens and highlight their potential as sustainable biocontrol agents for integrated disease management. Offering environmentally friendly alternatives to conventional chemical fungicides.
Keywords. Biocontrol, *Sclerotinia sclerotiorum*, *S minor,* Sclerotia, *Rhizoctonia solani*, Pathogenic bacteria, *Bacillus* spp.

Introduction

Soilborne fungal pathogens pose severe threats to global agricultural productivity, causing substantial yield losses and compromising food security [1]. Among these, *Sclerotinia sclerotiorum*, *Sclerotinia minor*, and *Rhizoctonia solani* represent three of the most destructive phytopathogens worldwide [2, 3]. These pathogens exhibit broad host ranges, attacking vital cash crops including cabbage (Brassica oleracea var. capitata), lettuce (*Lactuca sativa*), and potato (*Solanum tuberosum*) [2, 3]. A primary challenge in managing *Sclerotinia* sp. and *R. solani* is their capacity to form persistent survival structures, such as sclerotia, which remain dormant in soil for extended periods and withstand harsh environmental conditions [2].

Traditionally, crop protection against these soilborne diseases has relied heavily on synthetic chemical fungicides [4]. However, excessive and continuous application of synthetic chemicals causes environmental contamination, toxic residue accumulation in food chains, and the rapid emergence of fungicide-resistant pathogen populations [4, 5]. Consequently, there is an urgent demand for eco-friendly biocontrol strategies to manage disease without degrading agroecosystems [1, 5]. Biological control using antagonistic bacteria offers a promising alternative or complement to synthetic chemicals [1, 6]. Members of the genus *Bacillus* are particularly effective due to their rapid growth, endospore-forming capacity, and ability to produce diverse secondary metabolites [5, 6, 7]. *Bacillus* species suppress fungal pathogens through multiple complementary mechanisms, including direct mycoparasitism, competition for nutrients and space, production of extracellular lytic enzymes and antimicrobial peptides, and emission of volatile organic compounds [7, 8, 9]. Specific *Bacillus* strains have demonstrated marked activity against *S. sclerotiorum* [10, 11] and *R. solani* [3, 12] via mycelial inhibition and volatile production [13].

Despite extensive research on *Bacillus*-mediated biological control, significant gaps remain regarding the comparative efficacy of isolates recovered from distinct ecological niches (such as soil, rhizosphere, and sclerotia) against co-occurring pathogens [14, 9]. Furthermore, the relative contributions of direct bacterial-fungal contact versus cell-free extracellular metabolites require detailed evaluation across variable pathogens [9, 15, 16]. This study investigated: direct antagonistic interactions and sclerotial suppression using dual-culture assays; the thermostable inhibitory potency of culture filtrates against fungal colony growth; and the capacity of selected isolates to degrade sclerotia and reduce disease incidence under soil conditions [9, 5]. The objective is to identify robust, broad-spectrum biocontrol strains for integration into sustainable plant disease management systems [4, 5].

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Materials and Methods

Isolation and Identification Trials

Pure cultures of *Sclerotinia sclerotiorum*, *Sclerotinia minor*, and *Rhizoctonia solani* were isolated from naturally infected *Brassica oleracea* var. *capitata*, *Lactuca sativa*, and *Solanum tuberosum*, respectively. Pathogen isolation and identification were performed as previously described by Trabalsiy *et al.* [17].

Isolation and Screening of Bacillus Isolates

Bacillus isolates were obtained from the rhizosphere soil of actively growing corn (*Zea mays*) plants and from soil-buried sclerotia of *S. sclerotiorum*. Antagonistic activity against the test pathogenic fungi was evaluated using dual-culture plate assays on Potato Dextrose Agar (PDA), according to established methods [18–20]. Antagonistic activity was determined by measuring the width of the inhibition zone (mm) between the bacterial streak and the fungal colony margin after 5 and 10 days of incubation at 25°C. Plates inoculated with the pathogenic fungi alone served as controls.

Effect of Bacillus culture filtrates on fungal colony growth

Culture filtrates of the *Bacillus* isolates were evaluated for antifungal activity against the test pathogens according to the method described by Fayadh *et al.* (1988) [21]. Cell-free culture filtrates were obtained by filtration through a 0.20 µm membrane filter. To assess thermal stability, a portion of the filtrate was heat-sterilized by autoclaving at 121°C and 1 kg/cm^2 pressure for 15 min, while the remaining filtrate was maintained without heat treatment. Both untreated and heat-treated filtrates were incorporated into PDA at 10% (v/v). Antifungal activity was evaluated by measuring the average fungal colony diameter (mm). Percentage growth inhibition was calculated relative to control plates lacking culture filtrate.

Result

A total of 50 *Bacillus* isolates were recovered from rhizosphere soil, plant tissues, and sclerotia. Representative isolates, including BS1, BS2, and BS3 (soil), BP4 (plant), and SSB5 and SSB6 (sclerotia), exhibited differential antifungal activity against *Sclerotinia sclerotiorum*, *Sclerotinia minor*, and *Rhizoctonia solani*. Some isolates showed strong inhibition of both mycelial growth and sclerotia formation, whereas others displayed moderate or weak activity. These findings suggest that the ecological origin of the isolates may influence their antagonistic effect.

Table 1. Bacillus Isolates

Source	Isolate Code
Soil	BS1
Soil	BS2
Soil	BS3
Plant	BP4
Sclerotia	SSB5
Sclerotia	SSB6

Effect of Bacillus isolates on mycelial growth and sclerotia formation

In dual-culture assays performed at 25°C, the antagonistic *Bacillus* isolates exhibited variable inhibitory effects on the mycelial growth and sclerotia formation of *Sclerotinia sclerotiorum*, *Sclerotinia minor*, and *Rhizoctonia solani*. Among the tested strains, BS3 demonstrated the strongest direct antagonistic activity against *S. minor* by producing an inhibition zone of 20.6 mm after 5 days, whereas BP4 proved to be the most effective antagonist against *S. sclerotiorum* with a 12.5 mm zone while also displaying strong activity against *S. minor* (20.0 mm). Moderate inhibition against *S. sclerotiorum* was shown by BS2 (8.0 mm), whereas BS1 completely lacked activity against *S. sclerotiorum* (0 mm) despite maintaining moderate inhibition against *S. minor* (13.0 mm). Unlike the *Sclerotinia* species, direct inhibition of *R. solani* was far more restricted across isolates; SSB5 emerged as the primary direct antagonist against *R. solani*, maintaining significant inhibition zones of 12.0 mm at Day 5 and 10.0 mm at Day 10, compared to BP4, which produced only a minor initial zone (5.0 mm).

Beyond direct mycelial inhibition, isolates SSB5 and SSB6 showed distinct dual effects on fungal structure development by suppressing sclerotial formation in *R. solani* (reducing development to "Little" and "Small" by Day 10) and *S. minor,* respectively. (Figures 1,2,3).

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Figure 1. Left: Effect of Bacillus sp. (BP4) on growth and Sclerotia formation in Sclerotinia sclerotiorum Right: Effect of Bacillus sp. (BS3) on growth and Sclerotia formation in Sclerotinia sclerotiorum

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Figure 2. Left: Effect of Bacillus sp. (SSB6) on growth and Sclerotia formation in Sclerotinia minor. Right: Effect of Bacillus sp. (SSB5) on growth and Sclerotia formation in Sclerotinia minor.

Figure 3. Left: Effect of Bacillus sp. (BP4) on growth and Sclerotia formation in Sclerotinia minor. Right Control.

Effect of Bacillus culture filtrates on fungal colony growth

Assessment of *Bacillus* spp. culture filtrates revealed marked, strain- and species-dependent variations in bioactivity against the targeted phytopathogens. Among the untreated filtrates, isolate BS3 showed superior antagonistic potency, exerting complete and sustained mycelial growth arrest (100% inhibition) against *Sclerotinia sclerotiorum* throughout both 5- and 10-day incubation intervals at 20°C (Table 3). High acute susceptibility was likewise observed in *Sclerotinia minor*, where untreated filtrates of BS3, BP4, and SSB6 each induced complete early mycelial suppression (100%) at Day 5; however, this activity attenuated by Day 10 to 85.06 % (12.7mm), 72.94% (23.0mm), and 70.6% (25.0mm), respectively. Conversely, *Rhizoctonia solani* exhibited greater overall tolerance to filtrate exposure, with isolate SSB6 maintaining a steady, moderate level of growth suppression (48.6%, 43.7mm) across both evaluation points (Figures 4,5).

After thermal sterilization by autoclaving, the bioactivity profile showed distinct variations in secondary metabolite thermostability across target organisms (Table 4). Isolate BS3 retained the highest overall heat tolerance across all three fungal species, achieving maximum early inhibition against *S. sclerotiorum* (80.94%, 16.2 mm) and *R. solani* (80.47 %, 16.6_{mm}) at Day 5, which declined moderately to 63.53 % (31.0mm) and 76.1% (20.3mm) by Day 10. In contrast, thermostable bioactive factors from BS3, SSB5, and SSB6 yielded total mycelial arrest (100%) against *S. minor* at Day 5, with BS3 uniquely sustaining complete suppression through Day 10. Notably, while SSB5 and SSB6 retained moderate heat-stable inhibitory activity against *R. solani* at Day 5 (59.29 % and 35.88 %, respectively), their thermostable fractions

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completely failed to inhibit *S. sclerotiorum* (0%), highlighting sharp target-specific constraints in metabolite efficacy. (Figures 6,7,8).

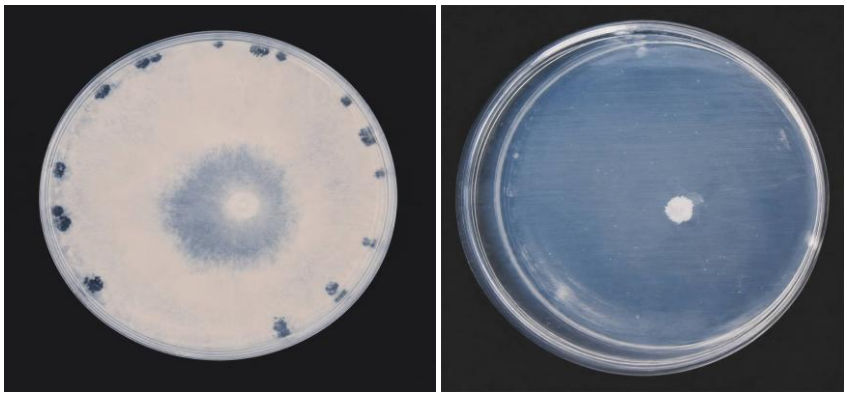

Figure 4. Effect of Bacillus isolate (BS3) culture filtrate on the growth and Sclerotial formation of S. Sclerotiorum. Left (control), Right (Effect of Bacillus sp)

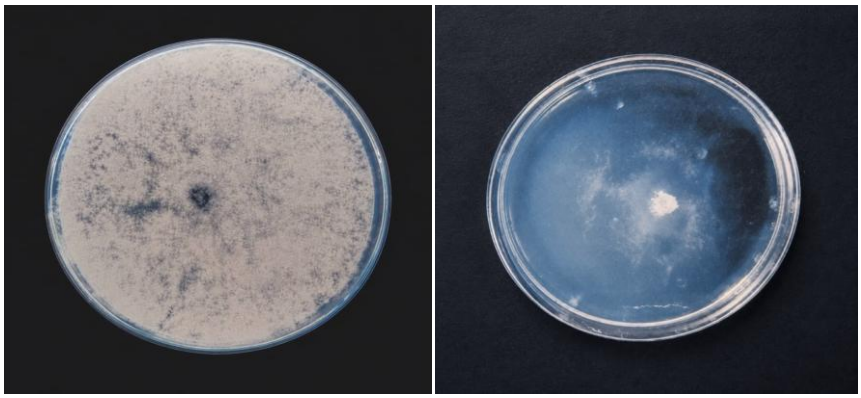

Figure 5. Effect of Bacillus isolate (BS3) culture filtrate on the growth and Sclerotial formation of Sclerotinia minor. Left (control), Right (effect on Sclerotinia minor)

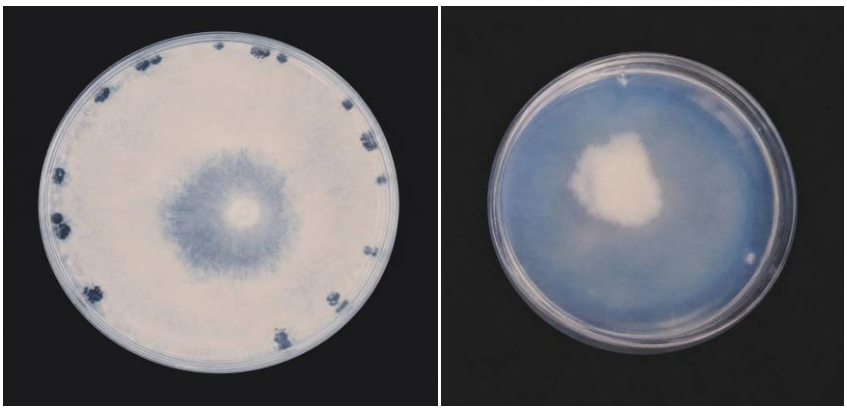

Figure 6. Effect of the heat-sterilized culture filtrate from Bacillus isolate (BS3) on the growth and Sclerotial formation of S. Sclerotiorum. Left (control), Right (Effect of Bacillus spp.).

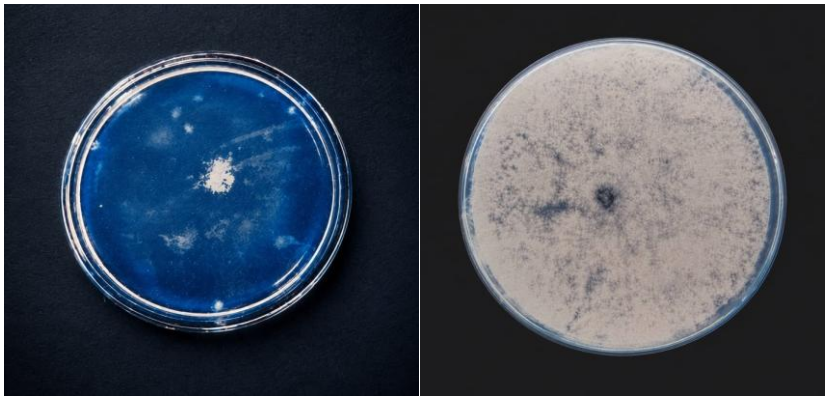

Figure 7. Effect of the heat-sterilized culture filtrate from Bacillus isolate (BS3) on the growth and Sclerotial formation of Sclerotinia minor. left(control), Right (Effect of Sclerotinia minor)

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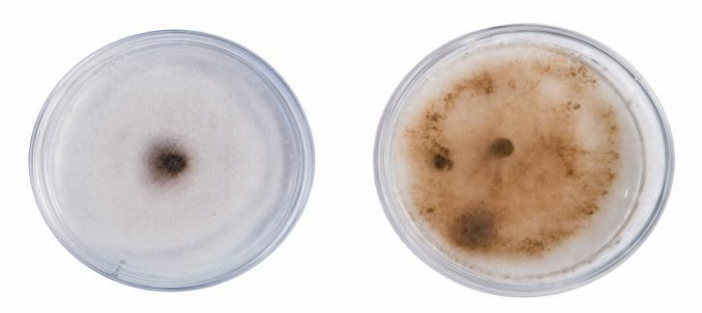

Figure 8. Effect of the heat-sterilized culture filtrate from Bacillus isolate (BS3) on the growth and Sclerotial formation of Rhizoctonia solani. Left(control), Right (effect on R. solani)

Table 2. Direct antagonistic activity and sclerotial suppression by Bacillus isolates against Sclerotinia sclerotiorum, Sclerotinia minor, and Rhizoctonia solani in dual-culture assays at 25 °C

Bacillus isolates	No. Of incubation days	Inhibitory effect on growth and Sclerotia formation of pathogenic fungi								
		S. sclerotiorum			*S. minor*			*R. solani*		
		Inhibition zone (mm)	Sclerotia formation		Inhibition zone (mm)	Sclerotia formation		Inhibition zone	Sclerotia formation	
			Number	Size		Number	Size		Number	Size
BS1	5	0	0	0	13	0	0	0	0	0
	10	0	Many	Large	13.7	Very few	Small	0	few	Small
BS2	5	8	0	0	16	0	0	0	0	0
	10	8	Moderate	Medium	14	Very few	Very Small	0	0	0
BS3	5	10.6	0	0	20.6	0	0	0	0	0
	10	10.6	Moderate	Medium	17.6	few	Small	0	0	0
BP4	5	12.5	0	Small	20	0	0	5	0	0
	10	10	few	0	19	Very few	Small	0	few	Small
SSB5	5	6.6	0	0	15	0	0	12	0	0
	10	6.6	Moderate	Medium	15	Very few	Small	10	few	Small
SSB6	5	8.3	0	0	7.6	0	0	0	0	0
	10	8	Moderate	Medium	5.6	few	Medium	0	Many	Large
Control	5	0	0	0	0	0	0	0	0	0
	10	0	Many	Large	0	Many	Medium	0	Many	Large

Activity Degree Scale: (Very Few): Number from 1 to 3, (Few): Number from 4 to 5, (Moderate): Number from 6 or more, (Many): Covers more than half of the plate, (Very Many): Covers most of the plate. Sclerotia Formation Scale: (Very Few): <25% plate Coverage, (Few): 25% coverage, (Moderate): 50% coverage, (Many): >50% Coverage, (Very Many): coverage of most of the plate.

Table 3. Inhibitory effect of unsterilized culture filtrates of antagonistic Bacillus isolates on mycelial growth and inhibition percentage of Sclerotinia sclerotiorum, Sclerotinia minor, and Rhizoctonia solani after 5 and 10 days of incubation at 20 °C

Bacillus isolate	Incubation period (days)	*S. sclerotiorum*		*S. minor*		*R. solani*	
		Mean mycelial growth (mm)	Inhibition (%)	Mean mycelial growth (mm)	Inhibition (%)	Mean mycelial growth (mm)	Inhibition (%)
BS2	5	85	0	85	0	64.7	24
	10	85	0	85	0	85	0
BS3	5	0**	100	0**	100	17*	80
	10	0**	100	12.7**	85	20.5**	75.88
BP4	5	35.7**	58	0**	100	38**	55.3
	10	66**	22.35	23**	72.94	61.6*	27.6
SSB5	5	11**	87.06	0**	100	41.0**	51.8
	10	30.3**	64.35	22.5**	73.5	50.5*	40.6
SSB6	5	68.2**	19.8	0**	100	43.7**	48.6
	10	73.7**	13.3	25**	70.6	43.7**	48.6
Control	5	85	0	85	0	85	0
	10	85	0	85	0	85	0

*T-test at the 0.05 significance level *. T-test at the 0.01 significance level ***

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Table 4. Inhibitory effect of autoclaved (thermostable) culture filtrates of antagonistic Bacillus isolates on mycelial growth and inhibition percentage of Sclerotinia sclerotiorum, Sclerotinia minor, and Rhizoctonia solani after 5 and 10 days of incubation at 20 °C

Bacillus isolate	Incubation period (days)	*S. sclerotiorum* Mean growth (mm)	Inhibition (%)	*S. minor* Mean growth (mm)	Inhibition (%)	*R. solani* Mean growth (mm)	Inhibition (%)
BS2	5	85	0	24.2*	71.53	85	0
	10	85	0	85	0	85	0
BS3	5	16.2**	80.94	0**	100	16.6**	80.4
	10	31**	63.6	0**	100	20.3**	76.1
BP4	5	48.7**	42.71	24.2**	71.53	85	0
	10	61	28.3	72**	15.29	85	0
SSB5	5	85	0	0**	100	34.6**	59.3
	10	85	0	13**	84.71	70**	17.7
SSB6	5	85	0	0**	100	54.5**	35.9
	10	85	0	29.5**	65.3	65**	23.6
Control	5	85	0	85	0	85	0
	10	85	0	85	0	85	0

*T-test at the 0.05 significance level *. T-test at the 0.01 significance level ***

Discussion

Biological control mediated by antagonistic *Bacillus* species represents a promising approach for the sustainable management of soilborne phytopathogens and may reduce reliance on synthetic chemical pesticides [1, 2]. In the present study, *Bacillus* isolates recovered from rhizosphere soil, plant tissues, and pathogen survival structures exhibited distinct, pathogen-dependent bioactivity. Soil isolate BS3 exhibited the highest overall antagonistic efficacy among the tested isolates. In dual culture, BS3 produced prominent inhibition zones against *S. sclerotiorum* (10.6 mm) and S. *minor* (20.6 mm). Moreover, its cell-free culture filtrate completely inhibited the growth of S. *sclerotiorum* (100%) and strongly suppressed *S. minor* growth (100% at Day 5 and 85% at Day 10). Members of the genus *Bacillus* are well known to produce diverse antimicrobial compounds, including the lipopeptide families iturins, fengycins, and surfactins, which are associated with membrane-active and other antagonistic mechanisms in plant disease biocontrol [2, 3]. The observed activity of BS3 is therefore consistent with the broad antimicrobial potential previously reported for antagonistic *Bacillus* strains; however, the specific compounds responsible for the activity of BS3 remain to be determined. A notable difference was observed in the activity of BS3 against *R. solani*.

In the dual-culture assay, BS3 produced no measurable inhibition zone (0 mm), whereas its cell-free culture filtrate reduced pathogen growth by 80.0% and 75.88% at Days 5 and 10, respectively. This contrasting response suggests that extracellular inhibitory compounds present in the culture filtrate contributed to the observed activity against *R. solani*. Differences between direct dual-culture and cell-free filtrate assays may arise from variation in compound production, concentration, diffusion, or accessibility under the respective assay conditions [3,4]. Previous studies have demonstrated that *Bacillus* strains can suppress *R. solani* through extracellular antimicrobial compounds, including lipopeptides such as iturin A [4]. Plant-associated isolate BP4 also demonstrated substantial antagonistic activity, particularly against *S. minor*, for which its cell-free filtrate produced 100% inhibition at Day 5, and against *S. sclerotiorum*, against which it produced a 12.5-mm inhibition zone in dual culture. The decline in BP4 filtrate-mediated inhibition over the 10-day incubation period suggests reduced persistence or stability of one or more extracellular inhibitory compounds under the assay conditions. However, the specific mechanism underlying this decline cannot be established from the present experiments.

Isolates SSB5 and SSB6, recovered directly from sclerotia, exhibited targeted antagonistic activity. In dual culture, SSB5 was the only isolate to produce a measurable inhibition zone against *R. solani* (12.0 mm at Day 5), while also suppressing sclerotial development in *S. sclerotiorum*. Antagonistic *Bacillus* strains can employ multiple mechanisms, including antibiosis and the production of extracellular hydrolytic enzymes such as glucanases and proteases, in addition to lipopeptide production [3, 5]. The strong activity of the SSB5 cell-free filtrate against *S. sclerotiorum* (87.06% inhibition at Day 5) further supports the potential of bacteria recovered from sclerotia as a source of antagonistic microorganisms for biological control. Overall, the pronounced antagonistic activity exhibited by BS3 and SSB5, particularly in the cell-free filtrate assays, identifies these isolates as promising candidates for further evaluation as biological control agents against soilborne phytopathogens. Further characterization of their extracellular inhibitory compounds and evaluation under controlled and field conditions would be necessary to determine their biocontrol potential and applicability.

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Conclusion

The findings of this study demonstrate that specialized *Bacillus* strains isolated from diverse ecological niches, particularly rhizosphere soil and pathogen survival structures, possess potent, pathogen-dependent biocontrol efficacy against major soilborne phytopathogens. Among the evaluated isolates, rhizosphere-derived isolate BS3 and sclerotia-derived isolate SSB5 emerged as standout candidates, exhibiting exceptional mycelial antagonism and complete or near-total growth suppression via their extracellular secondary metabolites. By confirming that these bacterial cell-free filtrates can drastically inhibit pathogen proliferation without direct cellular contact, this work underscores the critical role of diffusible antimicrobial compounds in *Bacillus*-mediated plant protection. These results position BS3 and SSB5 as highly promising, environmentally sustainable bio-inoculants capable of reducing reliance on synthetic chemical pesticides. Future research will focus on characterizing the specific chemical profiles of their extracellular metabolites and validating their performance under greenhouse and field conditions to bridge the gap between in vitro discovery and commercial agricultural application.

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