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Bacterial Inhibitors of the Fungal Phytopathogen *Sclerotinia sclerotiorum* Isolated from Rhizosphere Soils of Resistant Sunflower Genotypes

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Abstract

Microbial associations can protect cultivated sunflowers (*Helianthus annuus* L.) from being infected by the ascomycete fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary, potentially saving millions of dollars in losses annually. Here, we isolated bacteria from sunflower rhizosphere soil, performed full-length 16S rRNA gene sequencing for identification, and then measured inhibition of *S. sclerotiorum* in co-cultures. Isolated bacterial strains that inhibited *S. sclerotiorum* ($n = 13$) matched six environmental operational taxonomic units (OTUs) sequenced from rhizosphere soils of resistant sunflower genotypes. Four of these six OTUs were detected in sunflower rhizosphere soils from 15 different sites across the Great Plains region of the United States, indicating a broad distribution and potential for use as biocontrol agents. However, the relative

abundance of inhibitor OTUs across 95 sunflower genotypes was not negatively correlated with *S. sclerotiorum* incidence at the Carrington, ND, field evaluation site. All inhibitors we isolated are known to possess antifungal properties and have been tested as potential biocontrol agents in previous studies. Our work shows that soils associated with resistant sunflower plants naturally contain inhibitory bacterial strains. This opens up the potential for using augmentation of locally occurring bacteria as a strategy for biocontrol, potentially by coating seeds or spraying aboveground parts with slurries of one or more of these taxa.

Keywords: antifungal activity, *Bacillus*, biological control, plant pathology, *Pseudomonas*, *Sclerotinia sclerotiorum*, sunflower rhizosphere

Sclerotinia sclerotiorum (Lib.) de Bary is a detrimental fungal pathogen for hundreds of dicotyledonous plant species, causing more than 60 plant diseases (Purdy 1979). This pathogen is notorious for its broad host range and affects many agriculturally

important crops, such as canola (*Brassica napus* L.), soybeans (*Glycine max* (L.) Merr.), and sunflower (*Helianthus annuus* L.), resulting in substantial yield reductions and severe economic losses every year (Bolton et al. 2006). The disease commonly referred to as white mold or stem rot results in symptoms such as wilting, rot, and necrosis, which can devastate entire fields when left unchecked. *S. sclerotiorum* is particularly problematic due to its ability to persist in soil for extended periods as sclerotia—hardened, melanized structures that can germinate under favorable environmental conditions to initiate new infections (Willettts and Wong 1980). As agricultural intensification continues and climate change alters environmental conditions, the prevalence and severity of *S. sclerotiorum* infections are expected to increase (Fones et al. 2020; Velásquez et al. 2018), further stressing the need for effective management strategies. Current techniques to deal with the pathogen have focused either on the use of resistant plant varieties or on the use of chemical control. However, both methods are far from being complete solutions, as, on the one hand, there are unaddressed trade-offs between resistance and yield, limiting full implementation of

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resistant varieties (Duo et al. 2025), and, on the other, the long-term use of fungicides leads to the evolution of resistant pathogenic strains (Amaradasa and Everhart 2016; Lucas et al. 2015). Therefore, the use of biological control has been proposed as an alternative and sustainable way to manage *S. sclerotiorum* stem rot.

Biocontrol agents, which make use of beneficial microorganisms or metabolites to control plant pathogens, are receiving an increasing amount of attention from scientists as a way to effectively provide long-term protection to crops. Biological control using naturally occurring microorganisms offers the potential to be an effective and environmentally friendly way to control disease (Köhl et al. 2019). Numerous studies have demonstrated the feasibility of successful disease control using bacteria and fungi (Li et al. 2003; Rodríguez et al. 2006) to inhibit the growth of plant pathogens through various mechanisms, such as competition, antibiotic production, and induced plant resistance (Köhl et al. 2019). Several microbial taxa, including species from the genera *Bacillus*, *Pseudomonas*, and *Trichoderma*, have demonstrated antagonistic effects against *S. sclerotiorum* in laboratory and field conditions (Kamal et al. 2016; Zhang et al. 2016). Additionally, plant–microbe interactions in the rhizosphere are increasingly recognized for their importance in plant health, enhancing tolerance and resistance to both biotic and abiotic stress factors (Trivedi et al. 2020).

Among the crops affected by *S. sclerotiorum*, sunflower is of particular concern due to its importance in oilseed production globally, particularly in the United States, where the Great Plains region accounts for the majority of commercial cultivation. *S. sclerotiorum* causes three distinct diseases in sunflower: root and basal stem rot/wilt, mid-stem rot, and head rot, each significantly reducing both yield and seed quality (Ekins et al. 2011; Seiler et al. 2017). Recent studies have demonstrated that the relative resistance of sunflower genotypes to *S. sclerotiorum* basal rot covaries with the abundance of certain rhizosphere microbial partners (Pogoda et al. 2024), suggesting that these microbial populations could play a role in disease resistance. In this study, we built on the work by Pogoda et al. (2024) by isolating bacterial isolates from sunflower rhizosphere soils and testing their ability to inhibit *S. sclerotiorum* growth in vitro. We hypothesized that the bacterial strains isolated from the rhizosphere of *H. annuus*-resistant genotypes would match bacterial operational taxonomic units (OTUs) associated with *S. sclerotiorum* resistance and would be present across the core range of sunflower cultivation in the Great Plains region of the United States.

Materials and Methods

Bacterial collection and isolation

The bacterial strains used in this study were isolated from the rhizosphere soils of resistant *H. annuus* genotypes collected from the North Dakota State University Research and Extension Center, Carrington, North Dakota, in 2017. Microbes were initially acquired via isolation into pure culture by suspending root-associated (rhizosphere) soil from the Carrington field plots in tris-buffered phosphate. Soil suspension dilutions were then plated on Actinomycete Isolation Agar (BD Difco), humic acid vitamin agar (Hayakawa and Nonomura 1987), and VL55 (Sait et al. 2002), a series of tax-specific nutrient media. Each medium type was also adjusted to incorporate a range of carbon concentrations and pH levels to capture a broad range of microbial taxa with diverse nutritional requirements. Cycloheximide was also added to a subset of all plates to discourage growth of fast-growing, saprobic fungi. Inoculated media were incubated at 27°C for 10 to 30 days to allow for the growth of potentially slow-growing taxa. As colonies formed, they were isolated into pure cultures using the media on which they

were initially isolated. Subsets of isolates were cryo-preserved in glycerol in deep freeze (−80°C) for long-term preservation.

Inhibition assays

Cultures of *S. sclerotiorum* and bacterial isolates were cultured separately on plates of a potato dextrose agar (PDA) medium (Difco) or M9 minimal medium, made following manufacturers' recommendations and filled approximately 1 cm deep. Antagonistic activity of bacterial isolates against *S. sclerotiorum* was assessed using a dual-culture assay on PDA plates. A 0.5-cm-diameter plug of one of 83 bacterial isolates and of mycelial growth from 4-day-old *S. sclerotiorum* strain NEB-274 were placed 2.5 cm apart on a fresh PDA plate and left to grow for 14 days at 25°C. After 14 days, negative interactions were scored by growth inhibition at the mycelial interface. The radius of fungal growth was measured and compared with a noninoculated control, consisting of plates incubated with only *S. sclerotiorum* agar plugs and no bacterial isolates. The inhibition percentage of *S. sclerotiorum* radial growth was calculated using the following formula (Rostami et al. 2013):

$$\%I = (D - d) \times 100/D$$

where *I* is the percent inhibition in growth of *S. sclerotiorum*, *D* is the colony diameter of *S. sclerotiorum* in the control plate, and *d* is the colony diameter in the treated plate. This protocol was replicated in triplicate for each bacterial isolate, and inhibition percentages for each bacterial isolate were averaged across the three replicates. Differences in *S. sclerotiorum* growth inhibition (%) among bacterial inhibitors were assessed using the Kruskal–Wallis test. Post-hoc pairwise comparisons were performed using Dunn's test with Bonferroni correction. All statistical analyses were performed using R version 4.3.1 with a significance threshold of *P* < 0.05.

DNA extraction, PCR, and sequencing

DNA from pure cultures was extracted using the Qiagen Power-Soil DNA Isolation Kit according to the manufacturer's protocol. PCR amplification for the full-length 16S rRNA gene was performed using 2 µl of template DNA, 12.5 µl of AmpliTaq Gold 360 MasterMix 5 units/µl (Applied Biosystems), and 1 µl of forward and reverse primers (10 µM) in a C1000Touch Thermal Cycler (Bio-Rad). The 16S rRNA gene was amplified using the domain-specific bacterial primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and universal primer 1391 R (5'-GACGGCGGTGWGTRCA-3'). PCR amplification was carried out using a program of 95°C for 10 min, followed by 35 cycles of 95°C, 1 min; 53°C, 30 s; 72°C, 2 min and 30 s; with a final elongation step of 72°C for 10 min. The PCR product was then checked using gel electrophoresis and sequenced using bidirectional Sanger sequencing by Genewiz (Azenta Life Sciences), generating high-quality reads of approximately 1,300 bp.

Bacterial identification and nomenclature

Bacterial isolates were identified to the genus level based on 16S rRNA gene sequences. The closest phylogenetic relatives at the species level were determined using BLAST (Altschul et al. 1990) searches against the NCBI nucleotide database. Due to the known limitations of 16S rRNA gene sequences for definitive species-level identification (Janda and Abbott 2007), particularly within the genus *Bacillus* (Dunlap et al. 2016), isolates are referred to as *Bacillus* sp. or *Pseudomonas* sp., with their closest phylogenetic match indicated in parentheses based on sequence similarity. Although full-length 16S rRNA sequencing provides higher resolution than short amplicon approaches, it cannot reliably discriminate

between closely related species in which large genomic variability exists among strains sharing >99% 16S rRNA identity.

Phylogenetic analysis of inhibitory bacterial isolates

Geneious (Kearse et al. 2012; Biomatters) was used to edit sequences and trim primers. Phylogenetic trees for inhibiting bacterial isolates were inferred using maximum likelihood and constructed using RAxML v.8.2.12 (Stamatakis 2014). Search parameters were the same for each tree and utilized the GTR + Gamma model for nucleotide substitution, 1,000 iterations of rapid bootstrapping with starting seed 12345, and parsimony-based tree jumping with starting seed 12345. *Escherichia coli* was included as the most distant outgroup, and sequences from the same, and closely related, genera known to be human, animal, and plant pathogens were prioritized to explore their relatedness to our isolates. We also included sequences from closely related genera that provide benefits to plants or that are known to be from soil, aquatic, or epiphytic environments. Our script used for phylogenetic inference, as well as the multiple aligned sequence file, can be found at <https://doi.org/10.5281/zenodo.15476511>.

Distribution in rhizosphere soils

To determine the distribution of cultured isolates within the rhizosphere community, Sanger-derived 16S rRNA gene sequences from inhibitory isolates were matched to 200-bp representative sequences of 97% OTUs from previously published Illumina amplicon sequencing datasets (Pogoda et al. 2024) and to 253-bp sequences of amplicon sequence variants (Bueno de Mesquita et al. 2024) using BLAST with default parameters. Sequence identity and coverage values for all isolate-to-OTU matches and isolate-to-amplicon sequence variant matches are reported in Supplementary Tables S1 and S2, respectively.

Prevalence and genotype effects were assessed across 95 sunflower genotypes at one site (Carrington, ND, U.S.A., 2017), with four rhizosphere samples per genotype, two from each of two randomized, complete block replications ($n = 380$; Pogoda et al. 2024). Prevalence, site, and genotype effects were assessed across 10 sunflower genotypes at 15 different sites across the Great Plains region of the United States, again with four rhizosphere samples per genotype, two from each of two randomized, complete block replications ($n = 600$; Bueno de Mesquita et al. 2024). OTU tables in those datasets were rarefied to 5,099 and 6,027 sequences per sample, respectively, and the final sample sizes were 380 and 587, respectively. Genotype effects were tested with negative binomial regression models performed with the 'glm' function in R, with the theta parameter estimated using the 'glm.nb' function from the MASS package (Venables and Ripley 2002), with genotype and field replicate as fixed effects, or site, genotype, their interaction,

and field replicate as fixed effects, respectively. Heritability was calculated as the eta-squared value for the effect size of genotype on OTU relative abundance.

Results

A total of 83 bacterial isolates from sunflower rhizosphere soils were tested in this study.

From our antifungal bioassays, 13 bacterial isolates (15.6%) showed inhibition zones (Fig. 1) against *S. sclerotiorum* (Table 1). The percent inhibition of *S. sclerotiorum* radial growth ranged from 19.89 to 57.15% across the 13 bacterial isolates tested (Table 1; Fig. 2). High variation was observed within replicates of individual isolates, with the coefficient of variation ranging from 0.8 to 116.0%; the two isolates with the highest coefficient of variation values (CRS_405: 87.2%; CRS_202B: 116.0%) each had one replicate showing complete absence of inhibition, whereas the remaining two replicates showed measurable inhibition. Although all 13 isolates showed measurable inhibition of *S. sclerotiorum* growth, a Kruskal–Wallis test comparing the degree of inhibition among isolates showed only marginal differences ($H = 21.04$, $df = 12$, $P = 0.050$). Dunn's post-hoc test with Bonferroni correction revealed no significant differences between any isolate pair, indicating that no single isolate was consistently superior in antagonistic activity. These 13 bacterial isolates corresponded to six OTUs (clustered at 97% similarity) in 16S rRNA marker gene surveys of sunflower rhizosphere soils, with sequence identity ranging from 93.01 to 100% (mean: 98.1%; Supplementary Table S1). *Pseudomonas* and *Bacillus* strains were not closely related to selected human pathogens included in the phylogenetic analysis (Figs. 3 and 4). Most of the *Bacillus* strains that inhibited *S. sclerotiorum* in our study were related to other known probiotic phylotypes, whereas one strain was related to a pathogen of invertebrates (Fig. 4).

The relative abundance of all six OTUs was significantly affected by sunflower genotype at one site with 95 genotypes and five of the six OTUs at 15 sites with 10 genotypes (negative binomial regression, $P < 0.05$). In the multi-site study, genotype and site interacted to affect the relative abundances of all six OTUs. Four (*Pseudomonas*, *Rhodococcus*, *Bacillus*, and *Variovorax*) of the six OTUs were detected at all 15 sites, and *Paenibacillus* and *Sphingobacterium* were detected at six and four sites, respectively (Fig. 5). Of the four OTUs detected at all 15 sites, *Pseudomonas* sp. and *Variovorax paradoxus* had the highest prevalence, as they were found in >40% of samples at each of the 15 sites. The overall prevalence of OTUs ranged from 2 to 83% of samples in the Carrington study (Pogoda et al. 2024) and from 1 to 85% in the multi-site study (Table 1). Heritability scores ranged from 0.37 to 0.67 in the Carrington

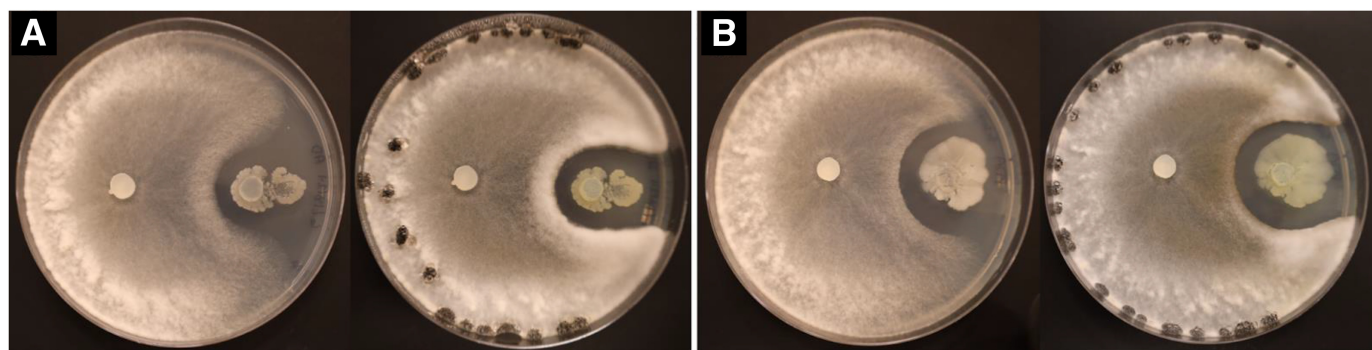


Fig. 1. *Sclerotinia sclerotiorum* culture growth inhibited by bacterial isolates. **A**, CRS_201B, *Bacillus* sp. after 2 (left) and 5 (right) days of growth. **B**, CRS_307, *Bacillus* sp. after 2 (left) and 5 (right) days of growth. See Table 1 for detailed taxonomic assignments based on NCBI BLAST matches.

TABLE 1
Sunflower-associated rhizobacterial isolates inhibiting *Sclerotinia sclerotiorum*, showing the taxonomy, sequence length, percent inhibition, heritability and prevalence in two different rhizosphere soil marker gene datasets, and accession, taxonomy, and percentage identity to closest matches in NCBI GenBank^a

Isolate_ID	OTU_ID	ASV_ID	NCBI accession	Species	Phylum	Seq. length (bp)	Inhibition (%)	Genotype effect (η^2)		Prevalence		NCBI closest match		
								Carrington	Multi-site	Carrington	Multi-site	Accession	Species	% Identity
CRS_205	OTU_275	ASV_806	PV590887	<i>Pseudomonas</i> sp.	Proteobacteria	1,303	57.14	0.37***	0.04***	0.83	0.85	MN602520, CP038438	<i>Pseudomonas koreensis</i> , <i>Pseudomonas fluorescens</i>	99.77
CRS_402	OTU_275	ASV_806	PV590892	<i>Pseudomonas</i> sp.	Proteobacteria	1,305	55.52	0.37***	0.04***	0.83	0.85	MN602520, CP038438	<i>Pseudomonas koreensis</i> , <i>Pseudomonas fluorescens</i>	99.46
CRS_201B	OTU_12372	ASV_10535	PV590884	<i>Bacillus</i> sp.	Firmicutes	1,310	49.54	0.48***	0.01	0.08	0.25	MN826516, AF287011	<i>Bacillus atrophaeus</i> , <i>Bacillus subtilis</i>	100
CRS_405	OTU_275	ASV_806	PV590894	<i>Pseudomonas</i> sp.	Proteobacteria	1,298	49.35	0.37***	0.04***	0.83	0.85	MN602520, CP038438	<i>Pseudomonas koreensis</i> , <i>Pseudomonas fluorescens</i>	99.61
CRS_307	OTU_12372	ASV_10535	PV590889	<i>Bacillus</i> sp.	Firmicutes	1,307	43.65	0.48***	0.01	0.08	0.25	CP146764, PP434512, PP434504	<i>Bacillus velezensis</i> , <i>Bacillus siamensis</i> , <i>Bacillus amyloliquefaciens</i>	99.92
CRS_305	OTU_12372	ASV_10535	PV590888	<i>Bacillus atrophaeus</i>	Firmicutes	1,320	39.44	0.48***	0.01	0.08	0.25	OM534594	<i>Bacillus atrophaeus</i>	99.62
CRS_201	OTU_12372	ASV_10535	PV590883	<i>Bacillus</i> sp.	Firmicutes	1,280	36.69	0.48***	0.01	0.08	0.25	MN826516, AF287011	<i>Bacillus atrophaeus</i> , <i>Bacillus subtilis</i>	99.92
CRS_404	OTU_1672	ASV_1866	PV590893	<i>Variovorax paradoxus</i>	Proteobacteria	1,294	35.99	0.50***	0.02*	0.25	0.72	KF318408	<i>Variovorax paradoxus</i>	99.77
CRS_308	OTU_12372	ASV_10535	PV590890	<i>Bacillus</i> sp.	Firmicutes	1,315	33.63	0.48***	0.01	0.08	0.25	CP146764, PP434512, PP434504	<i>Bacillus velezensis</i> , <i>Bacillus siamensis</i> , <i>Bacillus amyloliquefaciens</i>	100
CRS_204	OTU_4850	ASV_15020	PV590886	<i>Paenibacillus polymyxa</i>	Firmicutes	1,321	32.12	0.45***	0.02	0.16	0.04	JN700208	<i>Paenibacillus polymyxa</i>	99.7
CRS_313	OTU_12372	ASV_10535	PV590891	<i>Bacillus</i> sp.	Firmicutes	1,317	29.5	0.48***	0.01	0.08	0.25	MN826564, MW131662	<i>Bacillus atrophaeus</i> , <i>Bacillus subtilis</i>	99.85
CRS_017	OTU_10441	ASV_13525	PV590882	<i>Sphingobacterium kitahiroshimense</i>	Bacteroidota	1,304	27.44	NA	0.09***	NA	0.01	KC618436	<i>Sphingobacterium kitahiroshimense</i>	99.69
CRS_202B	OTU_978	ASV_3116	PV590885	<i>Rhodococcus</i> sp.	Actinobacteriota	1,281	19.89	0.45***	0.01	0.42	0.22	MN826591, MT549100, MH671642	<i>Rhodococcus qingshengii</i> , <i>Rhodococcus erythropolis</i> , <i>Rhodococcus boritolerans</i>	99.69

^a The table is sorted from top to bottom by inhibition percentage. Multiple closest matches are shown where there are ties in percent identity. Only named species are considered for the closest match. Heritability here is the eta-squared value for the genotype effect (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$). Prevalence is defined as at least 1 sequence present in a sample in the rarefied operational taxonomic unit (OTU) tables from the Carrington ($n = 368$) or multi-site ($n = 587$) datasets. Genotype effect and prevalence values are repeated for the same OTUs. OTU_ID is from Pogoda et al. (2024). NA, Carrington values not reported for CRS_017 because OTU_10441 (Pogoda et al. 2024) shares only 93% identity with the isolate sequence. Heritability = η^2 for genotype effect.

study (Pogoda et al. 2024) and from 0.02 to 0.15 in the multi-site study.

Discussion

This study provides new insights into the potential for naturally occurring rhizosphere-associated bacterial communities to contribute to *S. sclerotiorum* resistance in *H. annuus*. By isolating bacterial OTUs from resistant sunflower genotypes, we identified microbial candidates with potential biocontrol applications. Our findings reinforce the growing body of research suggesting that plant–microbe interactions play a crucial role in disease suppression and plant health and provide potential candidates for biocontrol that should be further studied. The phylogeny of our bacterial isolates revealed that they do not cluster within clades containing well-known human, animal, or plant pathogens. This suggests that these isolates may have low pathogenic potential and could be used in the environment as biocontrol agents with minimal environmental and health risks.

Interestingly, none of the isolated bacterial strains with inhibitory effects toward *S. sclerotiorum* matched the 42 environmental OTUs reported by Pogoda et al. (2024) as negatively associated with *S. sclerotiorum* incidence in sunflower field plots. This discrepancy may be explained, at least in part, by limited statistical power or low abundance of these taxa in the field, which can make detection difficult in microbiome association studies, particularly when microbial heritability is low or varies across environments. This suggests that disease suppression may not be solely attributed to the presence of heritable, naturally occurring microbial communities but could also arise from other mechanisms, such as context-dependent microbial interactions, environmental filtering, or site-specific variation in microbial community structure. These findings highlight the importance of combining functional screening approaches, such as in vitro inhibition assays, with microbiome association studies to more accurately identify microbial taxa with true biocontrol potential.

Despite their association, these environmental OTUs may be difficult to grow in vitro, limiting their direct application. It is also possible that OTUs associated with disease resistance benefit

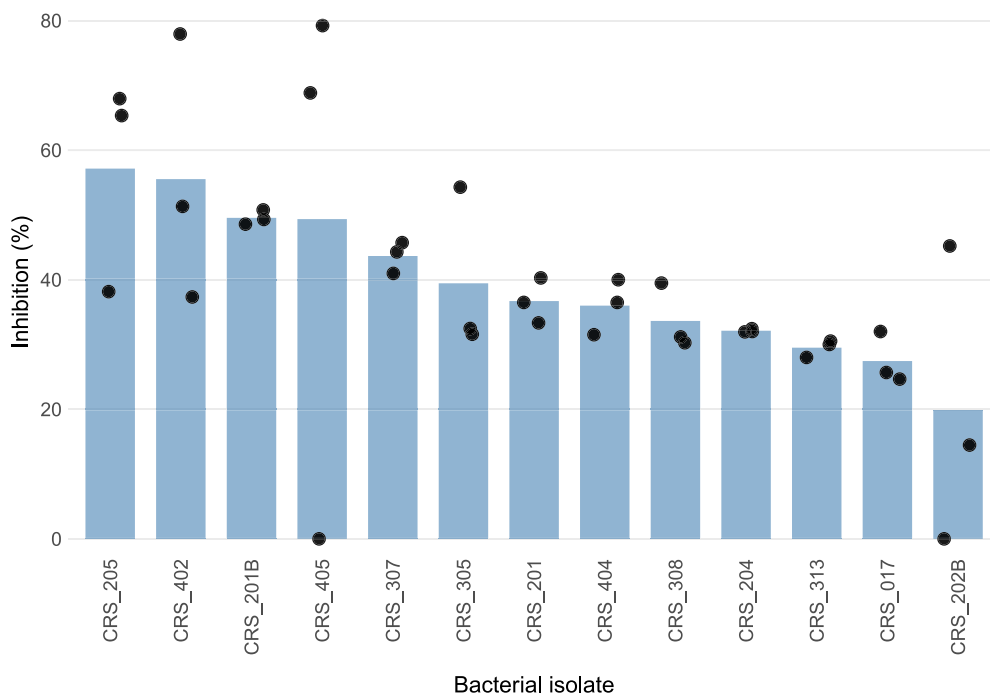
H. annuus growth, thus making the plant more resistant to pathogenic assault, but without having a direct inhibitory effect on *S. sclerotiorum*. Disease-suppressive effects may be synergistic and specific within a specific microbial consortium, rather than the result of a single dominant species, reinforcing the importance of studying microbial interactions (Berendsen et al. 2018). Microbial-mediated resistance may not be solely achieved by directly inhibiting *S. sclerotiorum* but could also involve mechanisms such as competition for resources and the induction of systemic acquired resistance against pathogens. Given the complexity of plant–microbe–pathogen interactions, further work is needed to elucidate the precise roles of microbial OTUs in resistance mechanisms against *S. sclerotiorum*. This highlights the necessity to integrate network and association studies with in vitro inhibition assays to validate biocontrol potential.

Several OTUs were consistently found across multiple sites, especially four of six OTUs found at all 15 sites. These are strong candidates for widespread use, as they have already demonstrated the ability to thrive across diverse climatic conditions ranging from Texas (15.23°C MAT) to North Dakota (4.49°C MAT). However, heritability scores were lower in the multi-site study, likely due to large site effects obscuring genotype effects; however, there were also interactions between genotype and site (Bueno de Mesquita et al. 2024).

Bacillus sp. isolate inhibitors

Bacillus spp. have proven to be some of the most efficient bacteria used for control of fungal phytopathogens (Shafi et al. 2017), including *S. sclerotiorum* (Farzand et al. 2019; Massawe et al. 2018). Exposure to biocontrol agents such as *Bacillus amyloliquefaciens* triggers *S. sclerotiorum* to upregulate genes involved in cell wall and membrane synthesis, including those responsible for (1,3)- β -D-glucan synthesis and ergosterol biosynthesis (Yang et al. 2020). Additionally, autophagy-related genes are activated in response to this interaction. Phylogenetic analysis shows that the inhibitor *Bacillus* strains we isolated belong to a different clade, separate from strains associated with pathogens of human, plant, and invertebrates.

Fig. 2. Mean percent inhibition of *Sclerotinia sclerotiorum* radial growth by 13 bacterial isolates. Bars represent mean values; individual points show biological replicates ($n = 3$ per isolate).



***Pseudomonas* sp. isolate inhibitors**

Besides *Bacillus*, *Pseudomonas* is another genus with many described biocontrol agents. *Pseudomonas koreensis* is a promising agent for plant pathogen biological control (Rafikova et al. 2016). *P. koreensis* was first identified from Korean agricultural soils and has demonstrated significant plant growth-promoting properties. Notably, strain IB-4 exhibits antifungal activity, nitrogenase activity, and the ability to synthesize phytohormones such as indole-3-acetic acid and cytokinin-like compounds. Further genomic analysis of *P. koreensis* strain S150 has revealed the presence of genomic islands coding for secondary metabolites, including

lokisin, an unusual cyclic lipopeptide. Mutational studies indicate that lokisin plays a crucial role in the strain's antifungal activity, underscoring its potential as a biocontrol agent (Gu et al. 2020). Phylogenetic analysis shows that the inhibitor *Pseudomonas* strains we isolated belong to a different clade from pathogens of human/plant/invertebrate-related strains. Phylogenetic analyses further aid in distinguishing these beneficial strains from pathogenic ones, facilitating their safe and effective application in agriculture. The complete absence of inhibition in a single replicate of CRS_405 (*Pseudomonas* sp.) and CRS_202B (*Rhodococcus* sp.) may reflect within-culture biological variability, including

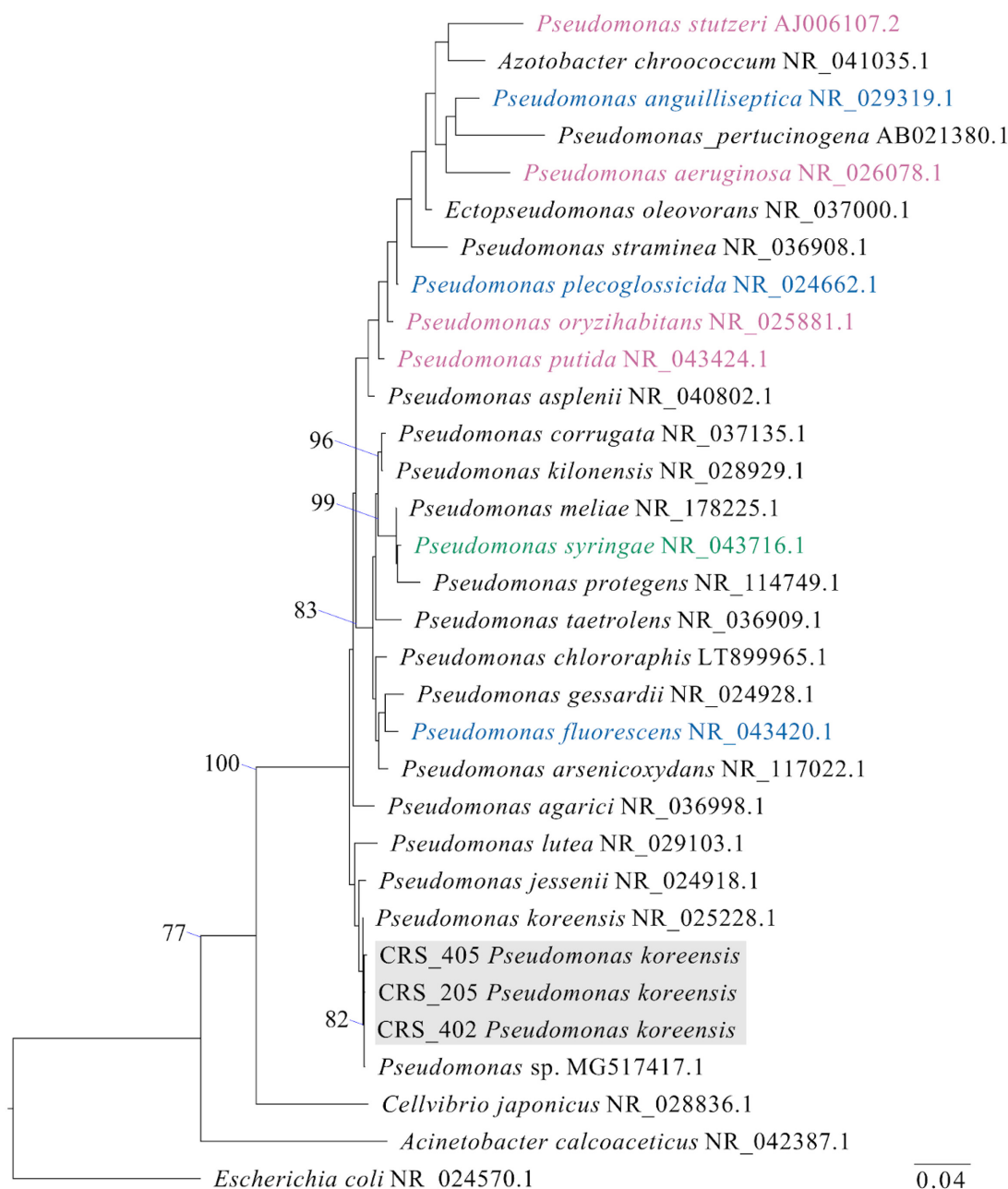


Fig. 3. Maximum likelihood phylogenetic tree of 16S rRNA long-read sequences of *Pseudomonas* sp. isolates. *Pseudomonas* sp. isolates that inhibited *Sclerotinia sclerotiorum* are highlighted in gray. Closest matches are color coded as follows: purple, selected opportunistic human pathogen phylotypes (*P. stutzeri*, *P. aeruginosa*, *P. oryzae*, *P. putida*); green, probiotic phylotypes; blue, aquaculture phylotypes. Isolate labels reflect the closest NCBI BLAST match at the species level; isolates are referred to as *Pseudomonas* sp. in Table 1 to reflect the known limitations of 16S rRNA gene sequencing for definitive species-level identification.

culture-to-culture differences in metabolic activity or, particularly in the case of *Pseudomonas*, stochastic variation in secondary metabolite production known to occur in this genus (Lalaouna et al. 2012). Future work with replicated colony picks could clarify whether this pattern reflects a stable or transient phenotype.

Other isolate inhibitors

Variovorax paradoxus is a Gram-negative, aerobic bacterium known for its metabolic versatility, enabling it to degrade a wide array of organic compounds (Satola et al. 2013). Although its

antagonistic activity against phytopathogen *P. syringae* has been documented (Hong et al. 2015), to our knowledge, there were no prior reports of it exhibiting antagonistic activity against *S. sclerotiorum*. This finding expands the potential application of *V. paradoxus* in biocontrol strategies against diverse fungal pathogens.

Paenibacillus polymyxa is a well-established plant growth-promoting rhizobacterium (Ali et al. 2021). Studies have shown that various strains of *P. polymyxa* produce antimicrobial compounds effective against pathogens such as *Fusarium oxysporum*, *Botrytis cinerea*, and *Fusarium fujikuroi* (Khan et al. 2020). The antifungal

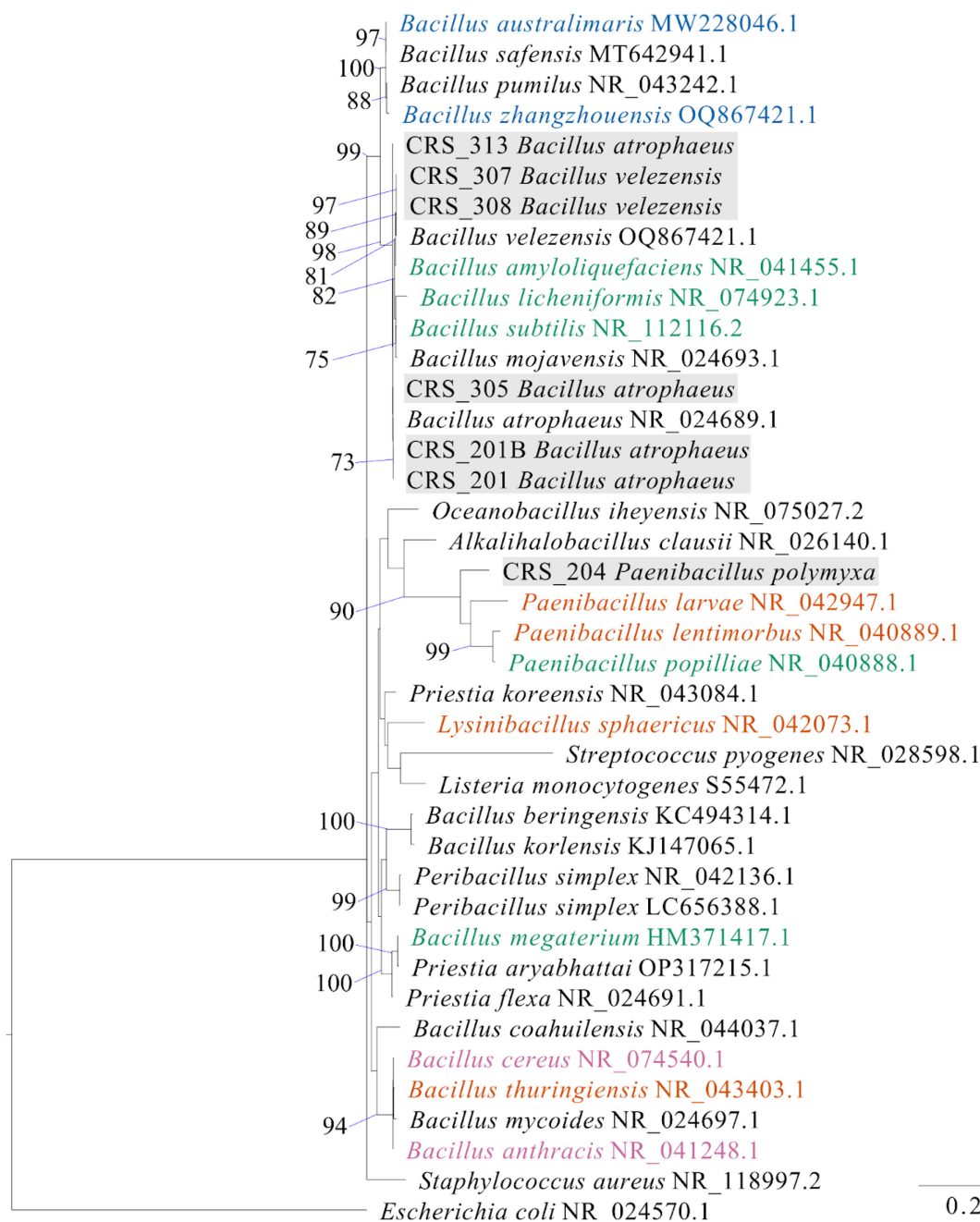


Fig. 4. Maximum likelihood phylogenetic tree of 16S rRNA long-read sequences of *Bacillus* sp. isolates. *Bacillus* sp. isolates that inhibited *Sclerotinia sclerotiorum* are highlighted in gray. Closest matches are color coded as follows: purple, selected human pathogen phylotypes (*B. cereus*, *B. anthracis*); orange, pathogen of insect or invertebrate phylotypes; green, probiotic phylotypes; blue, aquaculture phylotypes. Isolate labels reflect the closest NCBI BLAST match at the species level; isolates are referred to as *Bacillus* sp. in Table 1 to reflect the known limitations of 16S rRNA gene sequencing for definitive species-level identification.

activity is attributed to the production of lipopeptides, including fusaricidin, paenimycin, and pelgipeptin, which disrupt fungal cell membranes (Dobrzyński and Nازیبło 2024).

Sphingobacterium kitahiroshimense has been identified as a component in a microbial consortium able to promote plant growth and confer protection against fungal pathogens (Swiontek Brzezinska et al. 2022). Although the specific antifungal mechanisms of *S. kitahiroshimense* are not extensively documented, its role within microbial communities suggests a synergistic effect in enhancing plant resistance to diseases.

Rhodococcus qingshengii is recognized for its plant-stimulatory capability and inhibits the plant-pathogenic fungus *Fusarium oxysporum* in vitro (Kuhl et al. 2021). The antagonistic activity is likely due to the production of bioactive compounds that impede fungal growth, although the exact metabolites and mechanisms require further elucidation.

Conclusions

Our study showed that bacterial inhibitors of the fungal pathogen *S. sclerotiorum* can be isolated from the rhizosphere soils of disease-resistant sunflower genotypes. These bacterial isolates are not closely related to known pathogenic strains. Further research into their modes of action and efficacy in field conditions will be instrumental in developing integrated disease management strategies. The next step will be the introduction of single or combined strains into a sterile growth medium for in vivo sunflower infection assays in greenhouse experiments. The bacterial strains could be added directly to the soil or applied as a seed coat. Future work employing whole-genome sequencing would allow for more precise taxonomic placement of these isolates and provide access to the full biosynthetic gene cluster repertoire relevant to biocontrol activity. Such

research is critically important to protect sunflowers and other major crops from devastating pathogens.

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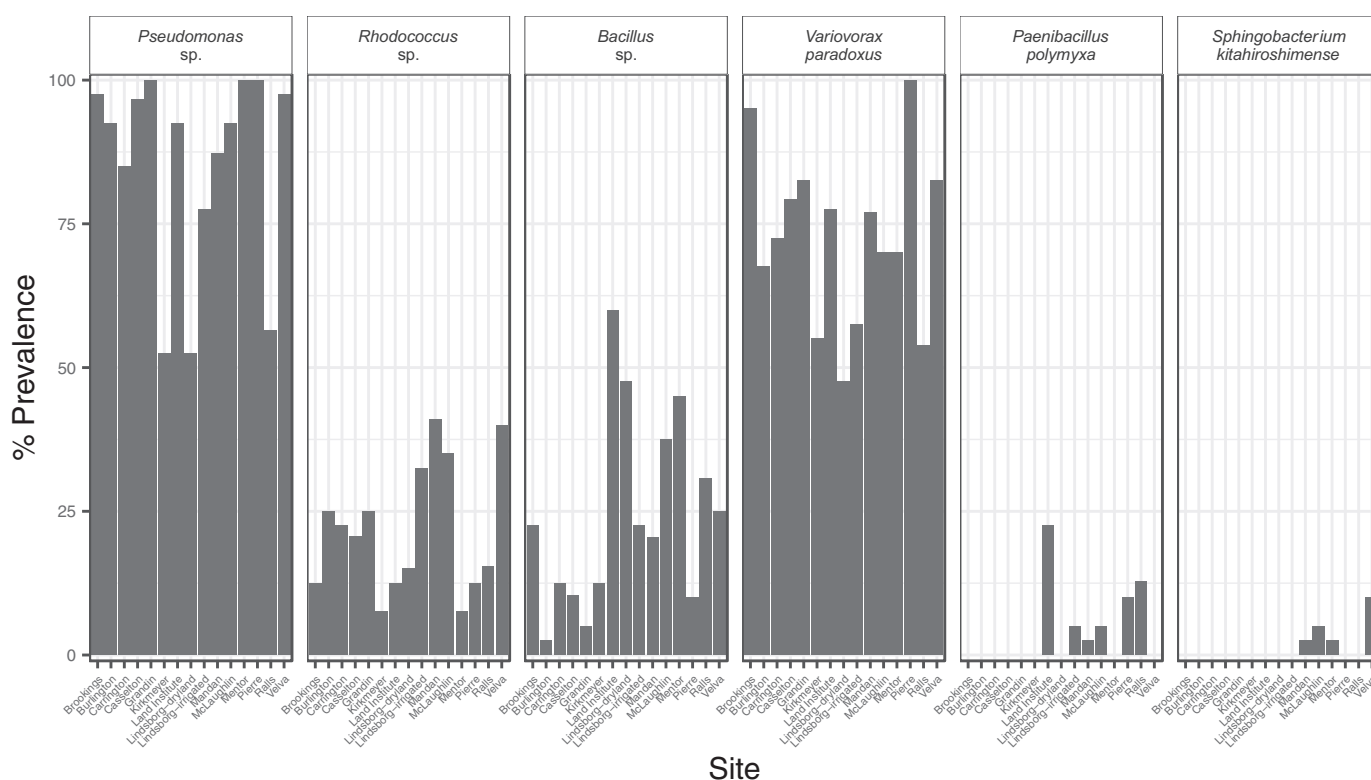


Fig. 5. Prevalence (percentage of samples present) of the six inhibitor operational taxonomic units (OTUs) at 15 sites across the Great Plains region of the United States. Sites had 29 to 40 samples from a total of 10 sunflower genotypes.

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