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Research Article

Identification of secondary metabolites biosynthetic genes, antagonistic activity and potential mechanism of *Bacillus subtilis* NBAIR-BSWG1 in suppression of *Alternaria alternata*

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ABSTRACT: *Alternaria alternata* wreaks havoc on fruit and vegetable production globally, threatening food security by causing black leaf spot disease. *Bacillus subtilis*, a natural inhabitant of soil, is a promising biological control agent for the management of *A. alternata*. In the present study, the antagonistic potential of *B. subtilis* NBAIR-BSWG1 was initially confirmed against *A. alternata* through a dual culture technique with 43.03% inhibition of mycelial growth. Subsequently, we extracted the cell-free extract from the NBAIR-BSWG1 pure culture and assessed its impact on *A. alternata* through the poison food technique and found mycelial growth inhibition of 85.82%. Identification of secondary metabolites biosynthetic genes using specific PCR markers showed the presence of surfactin genes (*sfp, srf AA*) with an amplicon size of 675 bp and 201 bp, respectively. Amplification of fengycin (*fenB*) and iturin (*ituD*) at 670 bp and 423 bp respectively, by using a specific PCR primer confirms the contribution of fengycin and iturin for the antagonistic potential of NBAIR-BSWG1. This study identifies NBAIR-BSWG1 as an effective bacterial biocontrol agent for control of *A. alternata*, unlocks the genetic basis of antifungal activity NBAIR-BSWG1, depicts molecular mechanisms involved in biological suppression of *A. alternata* by NBAIR-BSWG1 paving the way for the development of bioformulations for management of *A. alternata*.

KEYWORDS: Antifungal, biocontrol, gene, lipopeptides, *Bacillus subtilis*

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INTRODUCTION

Alternaria alternata is a phytopathogen known for its ability to infect a diverse array of plant species *viz*., potato, tomato, and tobacco, leading to various symptoms such as rots, leaf spots and blights on different plant parts. The impact of infection can result in substantial yield losses, reaching up to 79% due to early blight damage in tomatoes in Canada (Abbo *et al.*, 2014), and it possesses the potential to cause a 30% reduction in yield along with postharvest losses of up to 10% (Dube, 2014).

Bacillus subtilis is an aerobic, endospore-forming, rod-shaped, Gram-positive bacterium that produces a diverse array of natural weapons, which includes the production of antibiotics, antifungal compounds and enzymes that are bio-active and environmentally friendly agents for disease control, competes with harmful microbes, can directly attack and kill harmful fungi and bacteria (Srivastava *et al.*, 2021). It is a remarkably diverse bacterial species that is capable of growth in different environmental conditions due to the formation of endospores (Kumbar *et al.*, 2017). *B. subtilis* strains can be conveniently prepared and preserved owing to their capacity to generate endospores that exhibit resilience to various physical and chemical interventions. Consequently, they retain their capability to induce defensive reactions in host plants, even when subjected to adverse conditions (Gao *et al.*, 2016). *B. subtilis* synthesizes antibiotics, particularly lipopeptides which exhibit potent antifungal properties with low environmental toxicity and high biodegradability, making them eco-friendly alternatives to chemical pesticides. (Guo *et al.*, 2014; More *et al.*, 2014). In agriculture, Cyclic Lipopeptides (CLPs) *viz*, surfactin, iturin, and fengycin, from *Bacillus* spp. play crucial roles in combating phytopathogens (Ongena & Jacques, 2008). Surfactin promotes the formation of biofilms, aiding in bacterial colonization within its natural environments which serves as a protective barrier as well as induces the secretion of bioactive metabolites which reduces the population of detrimental microorganisms while supporting plant growth. Both iturin and fengycin function as

inhibitors of microbial growth (Wu *et al.*, 2021).

In the previous studies, we found *B. subtilis* strain NBAIR-BSWG1 very effective against *Sclerotium sclerotiorum* (Ruqiya *et al.*, 2022) and *Sclerotium rolfsii* (Ankitha *et al.*, 2023), hence to test its broad-spectrum antifungal activity we attempted to test the bioefficacy of NBAIR-BSWG1 against *A. alternata* by *in vitro* studies using dual culture assay, lipopeptide based poison food technique and also identified secondary metabolite biosynthetic genes using PCR based molecular characterisation. This study investigated the antifungal potential of lipopeptides, a specific class of antimicrobial molecules produced by NBAIR-BSWG1 against *A. alternata*.

MATERIALS AND METHODS

Culture of *B. subtilis* and *A. alternata*

The microbial cultures *B. subtilis* strain NBAIR-BSWG1, NBAIR-BSWG2, NBAIR-BSWG3, NBAIR-BSWG4, NBAIR-BSWG5 and *A. alternata* were collected from the microbial culture collection at the Insect Bacteriology Lab, ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru.

Evaluation of antagonistic potential of *B. subtilis* strains against *A. alternata*

Dual culture method

To evaluate the antifungal properties of five *B. subtilis* strains, a dual culture method was employed, where *B. subtilis* was streaked on one side of a Petri dish containing potato dextrose agar and, on another side, an *A. alternata* plug was placed. This enabled direct assessment of the interaction between *B. subtilis* strains and *A. alternata* under controlled conditions. Petri plates without *B. subtilis* strains were maintained as control with three replications per treatment. Plates were incubated at 28ºC for 11 days and radial growth of *A. alternata* was measured after its complete growth on control plates and subsequently inhibition percentage was calculated (Kukreti *et al*., 2023).

Poison food technique

Based on the dual culture assay performance of *B. subtilis* strains we have chosen only NBAIR-BSWG1 for further evaluation of antagonistic activity on *A. alternata* by Poison food technique.

Cell-free extract of NBAIR-BSWG1 was extracted by following the protocol described by Ruqiya *et al.* (2022). To evaluate the antifungal activity of extracted cell-free extract isolated from NBAIR-BSWG1, the poison food technique was employed. The media was poured into the Petri plate and allowed to solidify. After solidification, the crude lipopeptide extract was combined with molten PDA media at concentrations of 12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100 µL/mL. Subsequently, plugs (0.5 mm) of *A. alternata*, maintained in pure culture, were placed on the plates and incubated at room temperature. Petri plates without cell-free extract were kept as controls, and the assay was conducted in three replicates. The radial mycelial growth of *A. alternata* was observed, and antagonistic efficacy was determined using the formula outlined by Lee *et al.* (2008).

Identification of secondary metabolite biosynthetic genes using specific PCR markers

The presence of secondary metabolite biosynthetic genes was validated using specific PCR markers developed by Plaza *et al.* (2015). For lipopeptide gene amplification, the genomic DNA was isolated in five *B. subtilis* strains using a DNeasy Blood and Tissue Kit (Qiagen, Austin, Texas, USA) following the manufacturer's protocol. PCR amplification of selected biosurfactant genes was performed using specific markers in a thermocycler (T100, Bio-Rad, California, U.S.A). Based on the literature surfactin (*srfAA*, *sfp*), fengycin (*fen*B) and iturin (*itu*D), specific PCR were selected and synthesized (Eurofins India Private Limited Bengaluru), details of primers used in this study listed in Table 1. PCR amplification was carried out with a program as follows: PCR conditions for *srfAA*, *spf*, *fenB*, and *ituD* genes involved initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 48°C (srfAA, spf, *fenB*) or 58°C (*itu* D) for 1 minute, and extension at 72°C for 1 minute 30 s. The final extension was performed at 72°C for 10 mins. Gel electrophoresis and documentation (DNR, Mini Lumi, Israel) confirmed the amplification of lipopeptide genes, and purified PCR products underwent Sanger sequencing. Homology matches for obtained sequences were determined using the BLAST algorithm against the nr NCBI database (Manjunatha *et al.*, 2018; Aggarwal *et al.*, 2017, 2018).

Statistical analysis

All *in vitro* experiments were arranged in a completely randomized design and underwent one-way ANOVA. For significant ANOVA results at $P<0.05$, Duncan's Multiple Range Test (DMRT) was applied to distinguish group means. Superscript alphabets in the tables indicate the ranking of treatments according to the DMRT test. The statistical analyses were conducted using the Agricola package in R version 4.3.1. (Kukreti *et al.*, 2023).

RESULTS

Evaluation of antifungal activity of *B. subtilis* strains on *A. alternata*

The antagonistic activity of *B. subtilis* strains against *A. alternata* was evaluated through dual culture and poison

Table 1. List of PCR primers used for validation of antagonistic secondary metabolite biosynthetic genes in *Bacillus subtilis* strains using specific PCR marker developed by Plaza *et al*. (2015)

Sl. No.	Name of genes	Primer sequences	Tm	Amplicon size (bp)
1.	Surfactin (*sfp*)	F-ATGAAGATTTACGGAATTTA	48°C	675
		R-TTATAAAAGCTCTTCGTACG		
2.	Surfactin (*srf*AA)	F-TCGGGACAGGAAGACATCAT	48°C	201
		R-CCACTCAAACGGATAATCCTGA		
3.	Fengycin (*fenD*)	F-AAAGGTGTGTGGAATTGATG	48°C	670
		R-GCTGGTTCAGTT KGATCACAT		
4.	Iturin (*ituD*)	F-TTGAAYGTCAGYGCSCCTTT	58°C	423
		R-TGCGMAAATAATGGSGTCGT		

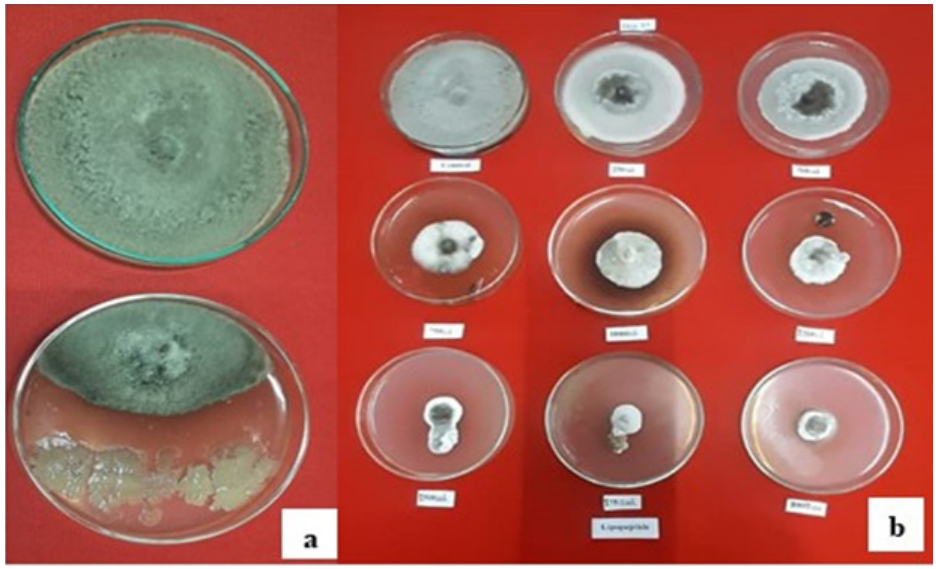

Figure 1. *In vitro* antifungal activity of *B. subtilis* NBAIR-BSWG1 against *A. alternata,* **(a).** Dual culture technique, **(b).** Lipopeptide-based poison food technique.

Table 2. Antagonistic activity of *B. subtilis* strains on *A. alternata* using dual culture inhibition assay

Treatment	*A. alternata* at 11 DAI	
	Average area of mycelial growth*	Per cent inhibition**
NBAIR-BSWG1	50.293	43.03(40.91[a])
NBAIR-BSWG2	59.918	32.13(34.49)[b]
NBAIR-BSWG3	60.850	31.07(33.71)[b]
NBAIR-BSWG4	63.646	27.90(31.83)[b]
NBAIR-BSWG5	64.579	26.85(31.07)[b]
Control	88.280	0(0.00)[c]
CD (0.05)	4.543	
SE(m)	1.458	
SE(d)	2.062	
C.V.	3.910	

DAI - Days after inoculation
* Average area of three replications
** Values in the same column followed by same letter indicates do not differ significantly and different letter indicates significant difference accordingly to Duncan's Multiple Range Test ($P \leq 0.05$)

Table 3. Assessment of the inhibitory effect of lipopeptides produced by *B. subtilis* NBAIR-BSWG1 on *A. alternata* through the poison food technique

Concentration µL/mL	Radial mycelial growth (cm)**	Per cent inhibition
12.5	4.0^{b}	15.60
25	3.2^{c}	31.91
37.5	2.3^{d}	51.06
50	1.7^{e}	63.83
62.5	1.3^{f}	72.34
75.0	1.0^{g}	78.72
87.5	0.8^{gh}	82.98
100	0.7^{h}	85.82
Control	4.7^{a}	-

**Values in the same column followed by same letter indicates do not differ significantly and different letter indicates significant difference accordingly to Duncan's Multiple Range Test ($P \leq 0.05$).

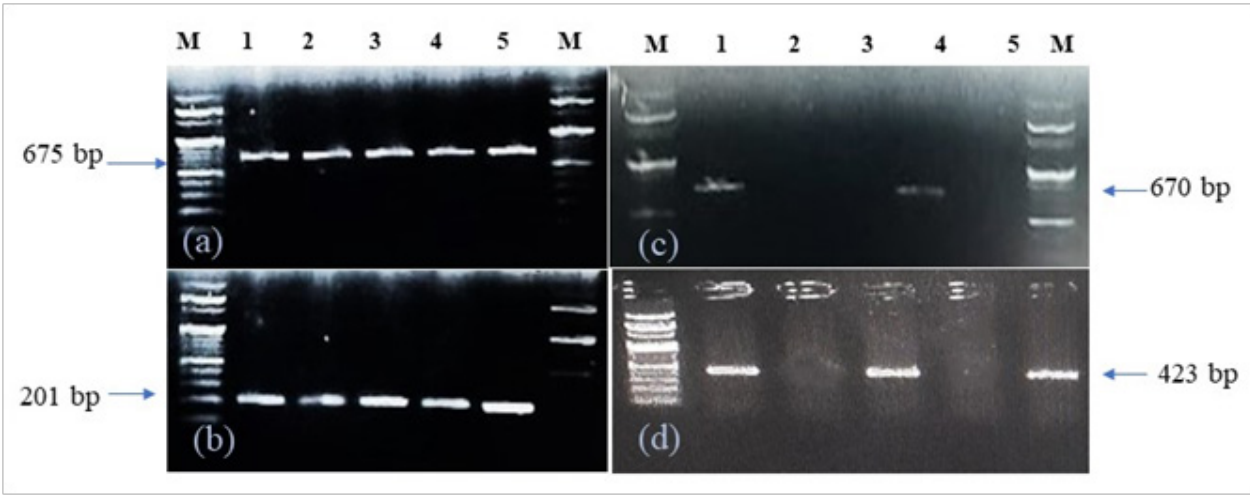

Figure 2. Gel electrophoresis pictures showing amplification of **(a)** surfactin (*spf*), **(b)** *srf*AA, **(c)** *fen*B, **(d)** *ituD*, in *B. subtilis* isolates, M = 100 bp ladder, (1) NBAIR-BSWG1, (2) NBAIR-BSWG2, (3) NBAIR-BSWG3, (4) NBAIR-BSWG4, (5) NBAIR-BSWG5.

food technique. Results of the dual culture method indicated that NBAIR-BSWG1 inhibited the mycelial growth of *A. alternata* with an inhibition percentage of 43.03% compared to control followed by NBAIR-BSWG2 (32.13%), NBAIR-BSWG3 (31.07%), NBAIR-BSWG4 (27.90%) and NBAIR-BSWG5 (26.85%) (Figure 1a, Table 2).

In the poison food technique, cell-free extract isolated from NBAIR-BSWG1 at concentrations of 12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100 µL/mL exhibited inhibition percentage of 15.60 %, 31.91 %, 51.06 %, 63.83 %, 72.34, 78.72, 82.98, 85.82 % respectively (Figure 1b, Table 3). The highest inhibition percentage was observed at 100 µL/mL concentration of cell-free extract.

Validation of secondary metabolite biosynthetic genes using specific PCR markers

The genes responsible for the biosynthesis of secondary metabolite in five *B. subtilis* strains were molecularly validated using specific PCR primers developed by Płaza *et al*. (2015). Surfactin gene (*sfp*, *srf* AA) was amplified in all five selected strains of *B. subtilis* (Figure 2a, Figure 2b) at an annealing temperature of 48°C, with amplicon size of 675 bp and 201 bp respectively, Sanger sequencing and BLAST analysis shown exact match with surfactin gene submitted by Płaza *et al*. (2015) in NCBI database. The presence of fengycin gene was tested in the genomic DNA of all five isolates of *B. subtilis* using specific primers (*fen-B*) at an annealing temperature of 48°C. The result showed that the gene got amplified only in NBAIR-BSWG1 and NBAIR-BSWG4 strains with an amplicon size of 670 bp (Figure 2c), Sanger sequencing and BLAST analysis confirms the matching of gene sequences with the fengycin gene in NCBI database. Iturin gene (*ituD*) was amplified in NBAIR-BSWG1, NBAIR-BSWG3 and NBAIR-BSWG5 at an annealing temperature of 58°C, with amplicon size of 423 bp (Figure 2d), Sanger sequencing and BLAST analysis also shown exact match with iturin gene

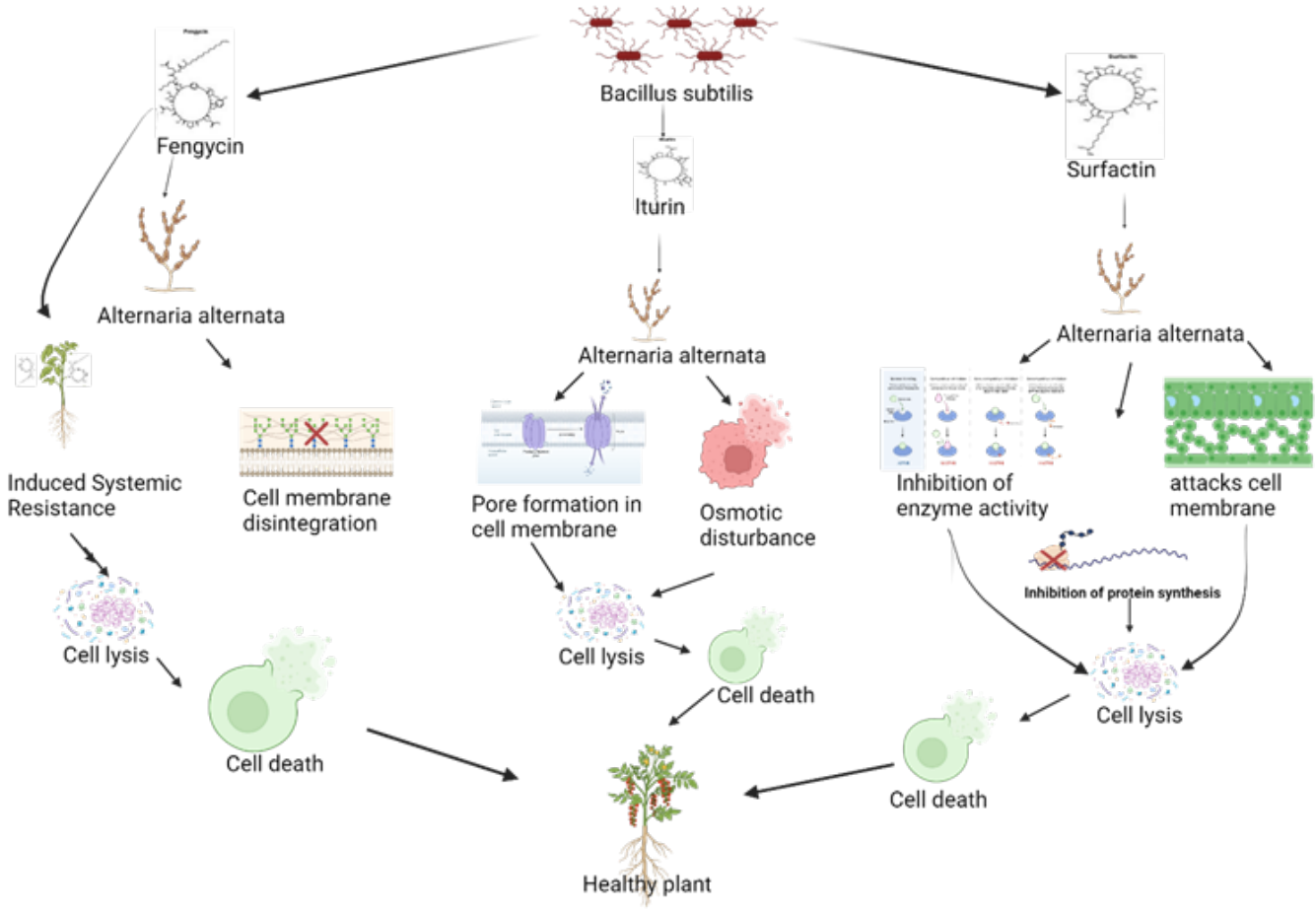

Figure 3. Graphical abstract (hypothetical model) depicting biocontrol mechanisms of surfactin, fengycin and iturin in *Bacillus subtilis* NBAIR-BSWG1 against *Alternaria alternata.*

submitted by Płaza *et al*. (2015) in NCBI database. The specific biocontrol mechanisms of surfactin, fengycin and iturin in *B. subtilis* NBAIR-BSWG1 strain in suppression of fungal cells was represented through a hypothetical model in graphical abstract (Figure 3), the diagram depicts that the lipopeptides are involved in cell membrane damage, osmotic disturbance and inhibition of enzyme and protein synthesis.

DISCUSSION

In the global scenario, *A. alternata* is a devastating plant pathogen in fruits and vegetables, it is a latent fungus that develops during cold storage of fruits and vegetables and becomes visible during the marketing duration, thereby also causing post-harvest loss (Abbo *et al*., 2014). Biological management of this pathogen stands as the best choice considering human health and environmentally friendly nature. Bacterial biological control agents are most commonly used due to their endospore formation which helps to work in a wide range of environments (Priyanka *et al*., 2018). *B. subtilis* is extensively employed for plant disease management owing to its effective antimicrobial capabilities (Chandrasekaran *et al.*, 2016). In this study, we used *B. subtilis* NBAIR-BSWG1 on *A. alternata* by dual culture method and found 43.03% inhibition.

Cell-free extract from the NBAIR-BSWG1 exhibited 85.82% growth inhibition with a concentration of 100 µL/mL of lipopeptide by poison food technique. This is consistent with the findings of Ruqiya *et al*. (2022) and Ankitha *et al*. (2023) similarly reported the efficiency of strain NBAIR-BSWG1 against *S. sclerotiorum* and *S. rolfsii*, respectively. Ahmad *et al*. (2023) also indicated that *B. subtilis* Y17B and its lipopeptide show an antagonistic effect against *A. alternata* in both dual culture and poison food technique. The lipopeptides present in cell-free extract were found to disrupt fungal cell membranes, leading to leakage of cellular contents and cell death.

Molecular analysis revealed the presence of a diverse range of antagonistic secondary metabolite biosynthetic genes with distinct antifungal properties in *B. subtilis* strains using PCR. In the present study surfactin, *sfp* and *srfAA* were amplified at amplicon sizes of 675 bp and 201 bp respectively.

Surfactins display the ability to create pores and ion channels, disrupting the typical integrity and permeability of the cell membrane lipid bilayer by inserting themselves into it. They chelate mono and divalent positively charged ions, leading to trans-membrane ion influxes that result in cell death due to cell disruption (Etchegaray *et al.*, 2008), fengycin (*fenD*) was also amplified at an amplicon size of 670 bp, which interacts with the lipid bilayer, causing changes in the structural integrity and permeability of the membrane. (Etchegaray *et al.*, 2008). Among the antifungal lipopeptides, iturins cause osmotic perturbations by forming ion-conducting pores that result in the leakage of K^+ and other vital ions, precipitating cell death (Kim *et al.*, 2020). These results are illustrated using a hypothetical model where, the role of surfactin, fengicin, and iturins produced by *B. subtilis* NBAIR-BSWG1on *A. alternata* is described as graphical abstract.

CONCLUSION

Bacillus subtilis-derived lipopeptides offer a promising biocontrol alternative for managing *Alternaria* infections in plants. Their diverse structures and potent antifungal activity of lipopeptides provide a broad spectrum of protection against fungal pathogens. In present study, the presence of antimicrobial compounds unequivocally underscores the antagonistic potential of *B. subtilis* NBAIR-BSWG1 on *A. alternata*. Its multifaceted mechanisms of action and broad-spectrum activity render NBAIR-BSWG1 a highly promising candidate for the creation of eco-friendly bioformulations, tailored to efficiently combat a wide array of plant pathogens, including *A. alternata*. The current research elucidates the intricate molecular mechanisms underlying the biocontrol efficacy of NBAIR-BSWG1 and holds significant promise for the utilization of the same as a potent tool in the biological control of *Alternaria* diseases.

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