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Antifungal Properties of *Streptomyces bacillaris* S8 for Biological Control Applications

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Soybean (*Glycine max*), a crucial global crop, experiences yearly yield reduction due to diseases such as anthracnose (*Colletotrichum truncatum*) and root rot (*Fusarium* spp.). The use of fungicides, which have traditionally been employed to control these phytopathogens, is now facing challenges due to the emergence of fungicide-resistant strains. *Streptomyces bacillaris* S8 strain S8 is previously known to produce valinomycin t through a nonribosomal peptide synthetase (NRPS) pathway. The objective of this study was to evaluate the antifungal activity of *S. bacillaris* S8 against *C. truncatum* and *Fusarium* sp., assessing its efficacy against soybean pathogens. The results indicate that strain S8 effectively controlled both above-ground and underground soybean diseases, using the NRPS and NRPS-related compound, suggesting its potential as a biological control in plant-microbe interactions. These findings underscore the pivotal role of the stain S8 in fostering healthy soybean microbial communities and emphasize the significance of microbiota structure studies in unveiling potent biocontrol agents.

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Streptomyces strains have been recognized for their ability to produce diverse secondary metabolites with significant roles in plant defense against pathogens (Bentley et al., 2002; Deshpande et al., 1988; Lee et al., 2020). Apart from their well-established role in suppressing plant diseases, *Streptomyces* is increasingly gaining attention as a potential abiotic stress controller through the production of various secondary metabolites (Durán et al., 2018; Harir et al., 2018; Yagüe et al., 2012) The importance on biological control approaches in sustainable agriculture, particularly involving the *Streptomyces* genus have been underscored (Cha et al., 2016; Genilloud, 2017; Kim and Kwak, 2023; Kim et al., 2019; Oskay, 2009). Given the broad spectrum of *Streptomyces* interactions with host plants, these bacteria emerge as an excellent model system for studying plant-microbe interactions (Lindow and Brandl, 2003; Redford et al., 2010). Building upon our previous findings that *Streptomyces bacillaris* S8 exhibits noteworthy antifungal and antibacterial activities while demonstrating suppressive effects on various plant diseases (Jeon et al., 2019, 2021; Kim et al., 2022), the present study aims to elucidate the feasibility of employing these strains as a biological control agent in the intricate dynamics of plant-microbe interactions.

To achieve sterilization of 3 g of soybean seeds (*Glycine max* cv. Daewon), the seeds were aseptically transferred into 50-ml tubes. Subsequently, they underwent a 30-s exposure to 70% ethanol. After, the ethanol supernatant was decanted, and a 1% NaOCl solution was added to the seeds. The seed-NaOCl mixture was vigorously vortexed for 1 min. Following this, the seeds underwent thorough washing with ddH_2O for 1 min, and this rinsing process was

repeated three or more times. Once the rinsing sequence was completed, the seeds were carefully placed within sterilized cotton paper in a Petri dish. The dishes were then incubated for 3 days at 28°C under dark conditions. After germination, the sprouted seeds were transferred to individual plastic square pots (18 cm × 18 cm), ensuring a distance of more than 20 cm between each pot to prevent cross-contamination. The plants were cultivated in a controlled glass greenhouse with a light exposure of 14 h at a temperature of 28 ± 3°C, followed by a dark period of 10 hours at a temperature of 15 ± 3°C. This growth schedule was maintained for a duration of 28 days.

The genome of *S. bacillaris* S8 was previously characterized by Kim and Kwak (2023), underscoring its importance in the synthesis of antifungal metabolites effective against large patch disease (Jeon et al., 2021). To identify potential secondary metabolite gene clusters within this strain, we utilized the antiSMASH 7.0 version tool (https://docs.antismash.secondarymetabolites.org) (Blin et al., 2023). The left flanking and right flanking regions for each deleted gene were amplified using 1 μg of strain S8 genomic DNA and gene-specific primers. The PCR amplification for each gene's left flanking and right flanking regions followed a protocol comprising an initial denaturation at 98°C for 10 min, 30 cycles of denaturation at 98°C for 1 min, annealing at 52-58°C for 30 s (according to the respective primer melting temperature) (Supplementary Table 1), elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. Subsequently, the left flanking and right flanking regions were fused through PCR, involving an initial denaturation at 98°C for 30 s, followed by five cycles of denaturation at 98°C for 8 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. Finally, the target homologous regions, including left flanking and right flanking, were amplified, and the last PCR step adhered to conditions similar to the earlier left flanking and right flanking region PCR, with the annealing temperature set at 60°C. The PCR procedures were executed using the MJ Research RTC-200 Thermal Cycler (Bio-Rad, Hercules, CA, USA).

The purified molecules were ligated to the pGEM-T easy vector (Promega, Madison, WI, USA) using T4 ligase (Promega) and incubated at 16°C for over 20 h. The ligated constructs were transformed into *Escherichia coli* DH5α via bacterial transformation. Subsequently, the vector and pCRISPomyces-gRNA were digested using *Xba*I (NEB, Ipswich, MA, USA), followed by elution into 30 μl of EB buffer. A 3:1 molar ratio ligation was performed using 400,000 units (400 units/μl) of T4 ligase. The gRNA was cloned into pBHA (2,002 bp), and pCRISPomyces-2 (Cobb et al., 2015) was digested using *Bbs*I (NEB). The resulting fragments were ligated using T4 ligase, connecting the two fragments and subsequently linking them with the gRNA (guide RNA). The resultant plasmids were then transformed into *E. coli* ET12567 containing pUZ802 (Bierman et al., 1992).

The transformed cells cultured and underwent centrifugation (1,914 ×*g*, 15 min), followed by two gentle washes with 10 ml of Luria-Bertani (LB) solution. Each wash was accompanied by centrifugation. The resulting pellet was resuspended in 500 μl of LB broth medium. In a separate E-tube, 500 μl of 2× YT broth medium (10 g yeast extract, 16 g tryptone, 5 g NaCl per liter) was heated at 50°C for 10 min. Subsequently, 10 μl of strain S8 spore stock (10^9 cfu/ml) containing 20% glycerol was mixed with the heated 2× YT broth. The washed *E. coli* (ET12567/pUZ8002) cells were gently mixed with the strain S8 spore stock using a pipette and spread onto mannitol soya agar (SFM; 20 g mannitol, 20 g soya flour per 1 liter) medium (Kieser et al., 2000). After 12 h, the agar plates were supplemented with apramycin (50 μg/ml) and nalidixic acid (25 μg/ml). After 7 days, colonies were transferred twice onto SFM agar containing apramycin (50 μg/ml).

C. truncatum and *Fusarium* sp. were grown onto potato dextrose agar (PDA; 24 g of potato dextrose, 20 g of agar per liter). *C. truncatum* underwent cultivation at 25°C for 10 days, while *Fusarium* sp. was cultured at 27°C for 7 days. The production of the *Fusarium* sp. inoculum as mixture of 99 g of sand and 1 g of oatmeal within a 1-liter plastic bottle, subjected to three cycles of sterilization. Subsequently, this sterilized mixture was inoculated with *Fusarium* sp., previously cultured on PDA medium for 7 days. The inoculated mixture was cultivated at a temperature of 28°C for 3 weeks to facilitate chlamydospore preparation. To assess the biological control efficacy of wild-type strain S8 and mutant strains, each was streaked on mannitol soya agar (SFM; 20 g mannitol, 20 g soya flour per liter), and cultured at 28°C for 5 days. A single colony from each strain was sub-cultured in 5 mL PDK (10 g potato dextrose broth, 10 g peptone per liter) broth at 28°C for 3 days. The cultured cells were then transferred to 200 ml of PDK broth and incubated at 28°C for 7 days, after which cell densities were adjusted to 10^6 cfu/ml.

A greenhouse measuring 5 m in length was utilized to establish distinct zones for both infected and non-infected control groups. Subsequently, a suspension containing the wild-type strain S8 and mutant strains was prepared with a final concentration of 0.1% carboxymethyl cellulose. This

suspension was applied to the proximity of soybean roots and leaves in both groups. Five days post-inoculation, *C. truncatum* was administered to each plant at a rate of 10 ml per plant using a spray method. Conversely, for *Fusarium* sp., a drenching method was employed, administering 10 ml (10^5 spores) of the suspension per plant. Disease severity assessments were conducted at weekly intervals following pathogen inoculation, utilizing a 5-level index. For *C. truncatum*, the severity scale encompassed the following categories: 0 = no significant damage, 1 = spots covering <1-10% of the plant, 2 = spots covering 10.1-15% of the plant, 3 = spots covering 15.1-25% of the plant, 4 = complete plant death (Agam et al., 2019). In the case of *Fusarium* sp., the severity scale was defined as follows: 0 = no symptoms, 1 = mild symptoms (discoloration without visible lesions), 2 = obvious lesions (severe discoloration with lateral root reduction), 3 = severe lesions on the primary and lateral roots and diminished plant vigor, and 4 = stem rotten, plant dead (Chang et al., 2020).

Streptomyces bacillaris S8 has previously been acknowledged for its potent biocontrol capabilities against large patch disease in turfgrass, with the identification of the valinomycin biosynthetic gene cluster (Jeon et al., 2019). Building upon this, our investigation aimed to explore its

Table 1. Putative antibiotic gene clusters in strain S8 predicted by antiSMASH

AntiSMASH	Prediction	Cluster similarity	
		Most similar known cluster	Percentage
nrps1	Hypothetical protein	Lactazole	33
t3pks	Hypothetical protein	Alkylresorcinol	100
nrps2	Synthetase gene (*vlm1*, *vlm2*)	Valinimycin	100
pks1	Transferase	Bafilomycin	94
nrps3	Nrps	Salinomycin	14
nrps4	Peptide synthetase	Bottromycin A2	36
pks2	Pks	Herboxidiene	6
nrps5	Siderophore	Tylactone	10
nrps6	Siderophore	Coelichelin	81
nrps7	Siderophore	Streptobactin	88
nrps/pks1	Pks-malonyl-CoA	Cosmomycin D	31
nrps/pks2	Line *whiE*	Atratumycin	34

Table 2. Bacteria and plasmids used in this study

Strains/Plasmids	Description
Escherichia coli	
DH5α	F^-, Φ80d*lacZ*ΔM15, (*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17* (r_k-, m_k+), *phoA*, *supE44*, *λ*-, *thi-1*, *gyrA96*, *relA1*
ET12567/pUZ8002	*dam-13*::Tn9 *dcm-6 hsdM*, carries RK2 derivative with defective *oriT* for plasmid mobilization, Kan^r
Streptomyces bacillaris S8	
Strain S8. *nrps3*	Strain S8 Δ*nrps3* :: Aprar
Strain S8. *nrps4*	Strain S8 Δ*nrps4* :: Aprar
Strain S8. *nrps/pks2*	Strain S8 Δ*nrps/pks2*:: Aprar
Plasmid/Cosmid	
pCRISPomyces-2	Am^r, *oriT*, reppSG5(ts), *ori*ColE1, sSpcas9, sgRNA cassette
pBHA	Am^r, pUC *ori*, gRNA clone
pCRISP. *nrps3*	pCRISPomyces-2 + Δ*nrps3* editing templated, *nrps3* sgRNA – sSpcas9
pCRISP. *nrps4*	pCRISPomyces-2 + Δ*nrps4* editing templated, *nrps4* sgRNA – sSpcas9
pCRISP. *nrps/pks2*	pCRISPomyces-2 + Δ*nrps/pks2* editing templated, *nrps/pks2* sgRNA – sSpcas9

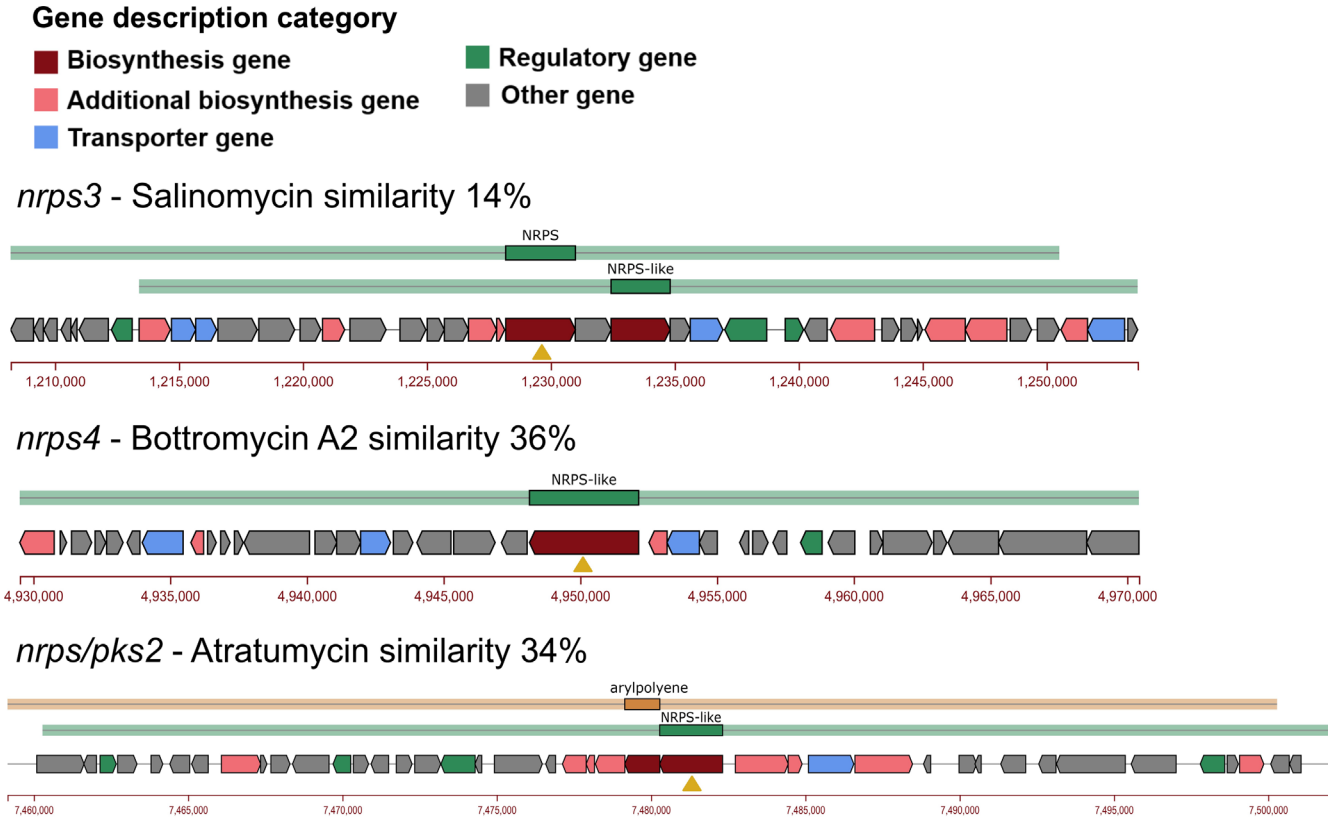

Fig. 1. Schematic depiction of the targeted deletion of a gene utilizing the CRISPR/Cas9 system. The diagram illustrates the distribution of gene functions within the *nrps*-like biosynthesis genes, alongside the specific sites targeted for deletion by the CRISPR/Cas9 system (triangles). The cluster was analyzed using antiSMASH version 7.0 (https://antismash.secondarymetabolites.org/). Mutants were generated through the CRISPR/Cas9 system, employing a plasmid containing a codon-optimized Cas9 from *Streptococcus pyogenes*, as well as *Bbs*I (guide RNA) and *Xba*I (homologous) sites for the incorporation of editing templates.

potential as a biocontrol agent in soybeans, motivated by its relevance to plant health (Kim and Kwak, 2023). The identified gene clusters within the bacteria were found to include genes associated with the production of nonribosomal peptide synthetase (NRPS) or polyketide synthase (PKS) compounds, as detailed in Table 1. Various biosynthesis knockout mutants targeting these clusters were generated using the CRISPR/Cas9 system, to assess their antifungal activities against two soybean phytopathogenic fungi (Table 2).

In the present study, we generated three mutant strains in which *nrps/pks2*, *nrps3*, or *nrps4* genes were abolished from the wild-type S8 strain (Fig. 1). Notably, the mutants of strain S8 in the *nrps3* and *nrps/pks2* clusters exhibited reduced efficacy against root rot disease, as evidenced by diminished activity in biocontrol assays (Fig. 2, Supplementary Figs. 1 and 2). Conversely, two distinct mutants showed no significant suppression of the two pathogens, with a statistical significance of P = 1.180e-10 (*** on *C. truncatum*) and P = 7.770e-13 (*** on *Fusarium* sp.) (Fig. 3). These observations lend support to the hypothesis that the NRPS and PKS serves as of antibiotics crucial for protecting the plant.

The biosynthesis genes were identified in *Streptomyces* spp., specifically in *Streptomyces albus* strain BK 3-25, which is known for salinomycin production, and in the deep-sea-derived *S. atratus* SCSIO ZH16NS-80S known to produce atratumycin (Almeida-Paes et al., 2021; Yang et al., 2019). Salinomycin has been reported for its anticancer and antifungal properties against *Paracoccidioides brasiliensis* (Zhou et al., 2013). While information regarding the antifungal effect of atratumycin is limited, its expression level was observed to increase in a co-culture system the fungus *Nigrospora oryzae* roseF7 and *Streptomyces* sp. MW-W600-10 (Siupka et al., 2021). This suggests that two distinct NRPS or PKS related compounds play a role in the antifungal effect against plant pathogens. Furthermore, atratumycin exhibited specific activity against *C. tunca-*

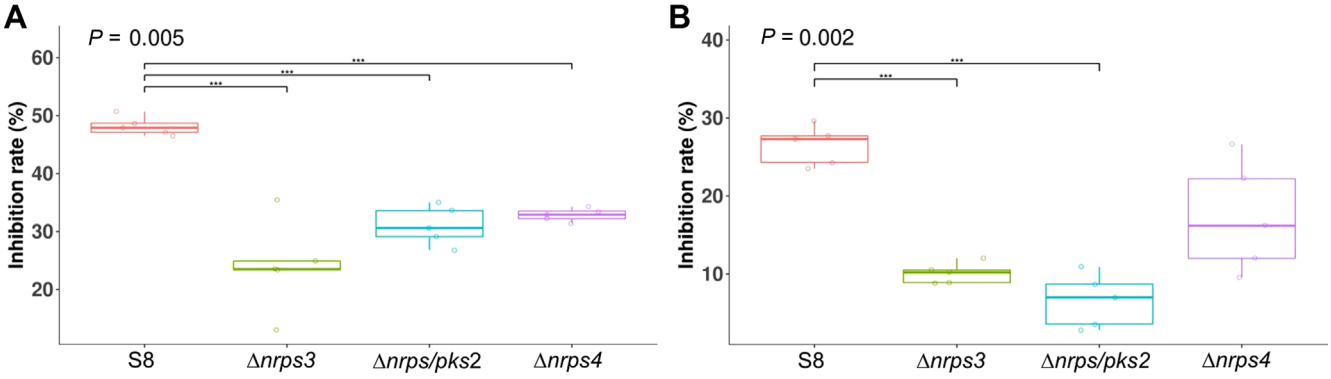

Fig. 2. Antibiotic activity and associated gene clusters in strain S8. The agar plates were incubated at 28°C for 7 days, during which the inhibition zones were carefully measured. Statistical analysis of the test data was conducted using the Kruskal-Wallis rank sum test, followed by the Tukey honestly significant difference (HSD) *post-hoc* test. A significance level of $P < 0.05$ indicates a significant difference in the size of inhibition zones between strain S8 and mutants. The tested pathogens included *Fusarium* sp. (A) and *Collectotruchum truncatum* (B).

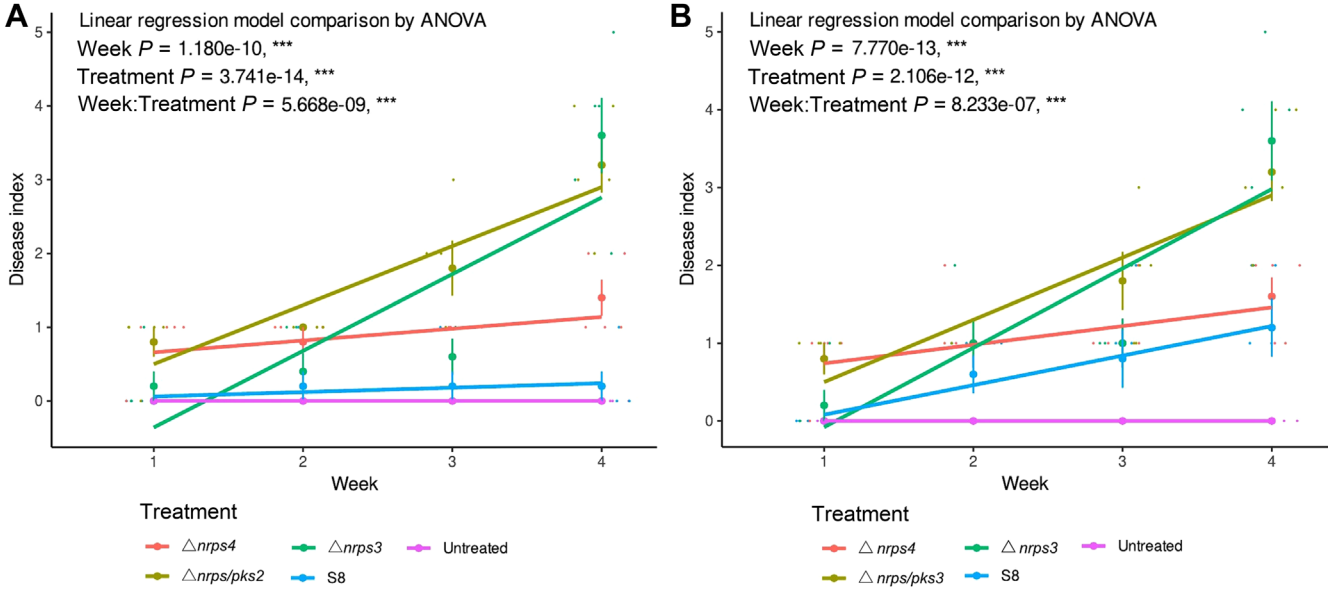

Fig. 3. Effectiveness of biocontrol measures against fungal diseases. *Collectotrichum truncatum* and *Fusarium* sp. were inoculated in soybean plants during the vegetative stage at 28 days. Plants were treated with a 10 ml drench of bacterial stocks (10^6 cfu/ml: strain S8, Δ*nrps3* and Δ*nrps4*, Δ*nrps/pks2*) in 0.1% carboxymethyl cellulose solution (v/v). (A) *Fusarium* sp. was inoculated with a chlamydospore stock at 10^5 spores/g. (B) *C. truncatum* with a conidia stock at 10^5 spores/g. Each spore stock was applied in a 10-ml volume. The disease index was assessed on a scale of 0 to 5 after 4 weeks ($n = 12$), and statistical analysis was performed using linear regression ANOVA.

tum and *Fusarium* sp., suggesting its promise as a strain-specific biocontrol agent for the S8 strain.

The diminished biocontrol activity of specific mutants underscores the importance of these antibiotic clusters in combatting plant diseases. This study contributes valuable insights into the potential use of *Streptomyces* strains for sustainable crop disease management, with far-reaching implications for agricultural practices. Additionally, the findings provide a foundation for basic research in environmental biological control, offering potential strategies for controlling soybean diseases and enhancing overall plant health.

Conflicts of Interest

No potential conflict of interest relevant to this article was

reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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