Journal of Biological Control, 37(3): 145-149, 2023, DOI: 10.18311/jbc/2023/35546

Research Article

Exploring the impact of cyclic lipopeptides from *Bacillus subtilis* NBAIR-BSWG1 through *in vitro* and *in planta*, studies against *Sclerotium rolfsii*

K. S. ANKITHA[1], T. K. RADHA[2], S. RUQIYA[1], ADITYA KUKRETI[3], N. AARTHI[3], S. NANDITHA[3], R. RANGESHWARAN[3], A. KANDAN[3], G. SIVAKUMAR[3], A. N. SHYLESHA[3], H. C. GIRISHA[3], K. NAGARAJU[3], T. VENKATESAN[3], S. N. SUSHIL[3] and C. MANJUNATHA[3*]

[1]*Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bengaluru – 560065, Karnataka, India*
[2]*ICAR-Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru – 560089, Karnataka, India*
[3] *ICAR- National Bureau of Agricultural Insect Resources, Bengaluru – 560024, Karnataka, India*
**Corresponding author E-mail:* manjuc.nbair@gmail.com

ABSTRACT: *Bacillus subtilis* is a Gram-positive bacterium known for its antagonistic attributes, particularly through the production of various secondary metabolites, including lipopeptides. In this study, we investigated the antagonistic capabilities of *B. subtilis* strain NBAIR-BSWG1 with a focus on assessing the efficacy of NBAIR-BSWG1 in combatting *Sclerotium rolfsii*. Our findings demonstrated substantial inhibitory effects, with 82.73% to 100% reduction in *S. rolfsii* growth when exposed to NBAIR-BSWG1 at concentrations ranging from 50 to 100 µL/mL in poison food technique. In dual culture assay, NBAIR-BSWG1 exhibited a significant 55.50% inhibition of *S. rolfsii*. Moreover, pot experiments revealed a promising 26% reduction in disease incidence. This study underscores the significant role of NBAIR-BSWG1 in controlling *S. rolfsii*, holding substantial potential for developing effective formulations aimed at mitigating the southern blight of tomatoes.

KEYWORDS: Biocontrol, lipopeptides, *Sclerotium rolfsii*

(Article chronicle: Received: 04-08-2023; Revised: 18-09-2023; Accepted: 20-09-2023)

INTRODUCTION

With the rise in human population, there is a rising concern about increasing food requirements and strategies for enhancing food production are highly demanding. A major threat to food production is biotic stress, although we are using chemical pesticides to manage biotic stress in agriculture, these chemical pesticides have detrimental effects on soil fertility, the environment, and even human health (Kumar *et al*., 2022). The application of biological control in plant protection provides us with a safe alternative in reducing the use of toxic chemicals (Yadav *et al*., 2022). *Bacillus subtilis* is documented as a biocontrol agent and represents a model organism for biological study (Guo *et al*., 2015). It is a remarkably distinct bacterial species that is capable of growth under different environmental conditions (Kumbar *et al*., 2017). It can produce several antimicrobial substances that can inhibit the growth of plant pathogens. It antagonizes pathogens by competing for resources such as iron or by secreting secondary metabolites like lipopeptides (Guo *et al*., 2015). Recent studies reveal the role of antifungal lipopeptides like surfactin, fengycin, iturin, etc., in the biocontrol potential of *B. subtilis* (Ramyabharathi *et al*., 2014). Southern blight of tomato caused by *Sclerotium rolfsii* is a highly devastating disease commonly found in subtropics and tropics having a wide host range of legumes, cucurbits, and crucifers. *Sclerotium rolfsii* is a soil-inhabiting omnivorous fungal pathogen infecting a wide range of vegetables (Sultana and Hossain, 2022). Research on lipopeptides produced by rhizosphere colonizing *B. subtilis* has recently caught the attention of many researchers due to its immense potential in biological control and plant growth promotion (Biniarz & Łukaszewicz, 2017). Hence in the present study experiments were conducted to determine the biocontrol potential of lipopeptide-producing NBAIR-BSWG1 against *S. rolfsii*.

ABBREVIATIONS

BSWG *Bacillus subtilis* Western Ghats
HCL Hydrochloric Acid
LB broth Luria Bertani
LPs Lipopeptides
NBAIR National Bureau of Agricultural Important Insects
PDA Potato Dextrose Agar

MATERIALS AND METHODS

Culture and maintenance of NBAIR-BSWG1 and *S. rolfsii*

In our previous studies, we have isolated and characterized the *B. subtilis* strain NBAIR-BSWG1 (Ruqiya *et al*., 2022). The pure culture of isolated NBAIR-BSWG1 was stored in a glycerol stock at -20°C for further studies. The *S. rolfsii* culture used in this study was obtained from the microbial culture collection, at ICAR-National Bureau of Agricultural Insect Resources, Bengaluru, India. This fungal pathogen was maintained on a Potato Dextrose Agar (PDA) medium and stored at -4°C for future studies.

Antagonistic activity of NBAIR-BSWG1 under *in vitro* conditions

The antagonistic activity of NBAIR-BSWG1 against *S. rolfsii* was performed using the *in-vitro* dual culture assay. NBAIR-BSWG1 was streaked at one edge of a Petri dish containing solidified potato dextrose agar medium, while a mycelial plug (5 mm) of the fungal pathogen was placed at the exact opposite edge of the same agar plate. Control plates without NBAIR-BSWG1 isolate were maintained simultaneously, experiment was conducted with three replications. The plates were examined for the inhibition of *S. rolfsii* by measuring the radial growth of the fungal colony, and per cent inhibition was calculated (Mardanova *et al*., 2016).

Lipopeptide crude extract from NBAIR-BSWG1 was extracted as described by Ruqiya *et al*., 2022. The extracted lipopeptide was tested against *S. rolfsii* by poison food technique. The crude extract of lipopeptides was mixed with molten PDA medium at 12.5, 25, 37.5, 50, and 100 µL/mL of media. The medium was poured into the Petri plate and allowed to solidify, after solidification, agar plugs of *S. rolfsii* maintained in pure culture were placed at the centre of the plate and incubated at 28ºC. Petri plates without lipopeptide were maintained as control and the assay was conducted in three replicates. Observation of radial mycelial growth of the pathogen was measured and antagonistic efficacy was calculated using the following formula given by Leelasuphakul (Leelasuphakul *et al*., 2008).

Antagonistic potential of NBAIR-BSWG1 against *S. rolfsii* under greenhouse condition

The NBAIR-BSWG1 and extracted lipopeptide were further tested against *S. rolfsii* under glasshouse conditions at ICAR-NBAIR Bengaluru. The *S. rolfsii* was mass cultured in an autoclaved ragi + vermiculite (1:1) mixture containing 10% glucose. The mixture was first thoroughly mixed and moisture adjusted to 15 to 20%. The inoculum was obtained by growing the pathogens in potato dextrose broth and mixed with the mixture at 5%. Autoclavable plastic bags were used for the process, the mixture was used at 5 g per kg of soil. Plastic pots of 5 kg capacity were filled with 3 kg red loamy soil and 15 g of the pathogen mixture was added and mixed, FYM was added at 5 g per kg of soil. The crude extract of lipopeptides was mixed with sterile water at 100 µL/mL and the solutions were used 15 ml per pot. Tomato seedlings (21 days old) of Arka Rakshak were transplanted into each pot (one seedling per pot) in a total of nine treatments with ten replications for each treatment. A foliar spray of NBAIR-BSWG1 and lipopeptide was given at 7 days after transplanting; the experiment was performed by a Completely Randomized Design (CRD). Disease development on each plant was rated after 40 days of growth using the following scale: 0 = no symptoms, 1 = 1 to 20% of plant tissue damage, 2 = 21 to 40% of plant tissue damage, 3 = 41 to 60% of plant tissue damage, 4 = 61 to 80% of plant tissue damage and 5 = 81 to 100% (Kator *et al*., 2015). Additionally, the per cent disease over control and disease index was calculated using the following formula (Kator *et al*., 2015):

$$PDI = \frac{\text{Sum of individual ratings}}{\text{No. of plants examined} \times \text{Maximum disease scale}} \times 100$$

STATISTICAL ANALYSIS

Data obtained from dual culture and poison food assay was arcsine transformed and were subjected to one-way ANOVA and the means were compared by Duncans multiple range test (DMRT), $P<0.05$. All the analysis was done using agricolae package of R software.

RESULTS AND DISCUSSION

Antagonistic activity of NBAIR-BSWG1 under *in vitro* conditions

The biocontrol potential of NBAIR-BSWG1 against *S. rolfsii* was analysed by dual culture and poison food technique. Under dual culture assay, NBAIR-BSWG1 could inhibit the growth of *S. rolfsii* by 55.5% (Figure 1A). The antimicrobial activity of crude extract of the lipopeptides produced NBAIR-BSWG1 by poison food technique showed 43.76, 64.11, 80.92, 82.13, and 100.00 per cent of inhibition of mycelial growth of *S. rolfsii* at lipopeptide concentrations of 12.5, 25, 37.5, 50 and 100 µL/mL, respectively (Figure 1B). Complete inhibition (100%) was observed with 100 µL/mL lipopeptide and at 12.5 µL/mL there was 43.76% inhibition as shown in Table 1. No significant differences were noticed in the inhibition of mycelial growth of *S. rolfsii* when lipopeptide was used at 37.5 or 50 µL/mL.

Bacillus subtilis is regarded as an exceptionally potent biocontrol agent, primarily owing to its capacity to generate an array of antimicrobial compounds. Among these compounds are antibiotics, antifungal agents, and bacteriocins, all of

Table 1. Antagonistic activity of lipopeptides produced by *Bacillus subtilis* NBAIR- BSWG1 against *Sclerotium rolfsii*

Concentration µL/mL	Radial mycelial growth (cm)	Percent inhibition
12.5	2.64 (1.91)[b]	43.76
25	1.69 (1.64)[c]	64.11
37.5	0.90 (1.38)[d]	80.92
50	0.84 (1.36)[d]	82.13
100	0.00 (1.00)[e]	100.00
Control	4.70 (2.39)[a]	-

*Same letter denotes that the treatments do not differ significantly and different letters indicate significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$).

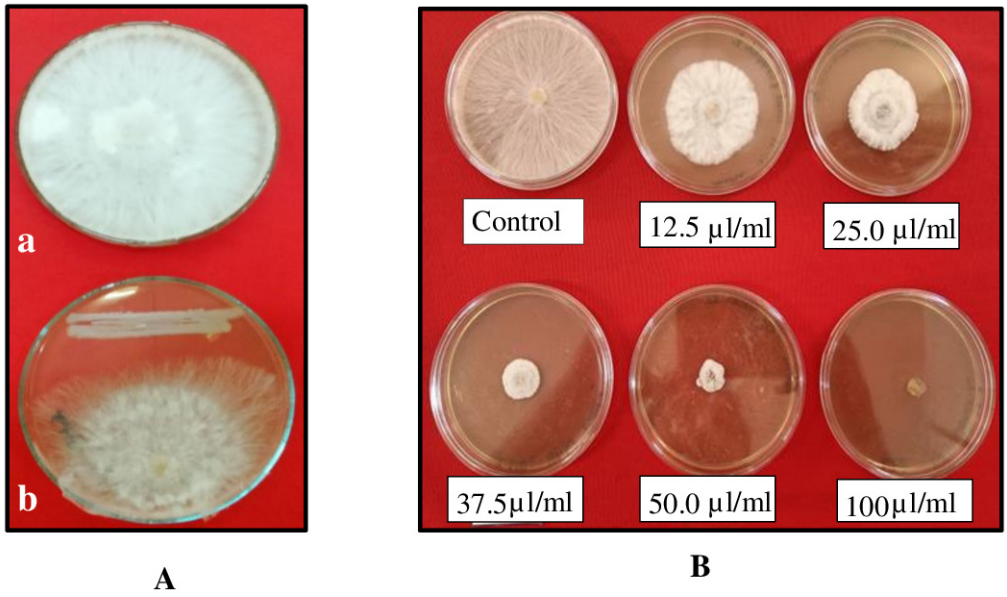

Figure 1. *In vitro* antifungal activity of *Bacillus subtilis* NBAIR-BSWG1 in dual culture and poison food technique against *Sclerotium rolfsii* **A**) Dual culture technique of *B. subtilis* NBAIR-BSWG1 *S. rolfsii*; a) Control - *S. rolfsii* b) Antagonism of *B. subtilis* NBAIR-BSWG1 against *S. rolfsii* **B**) Lipopeptide based poison food technique of *B. subtilis* NBAIR-BSWG1 against *S. rolfsii*.

which have the remarkable ability to inhibit the proliferation and activity of various plant pathogens (Ramyabharathi *et al*., 2020). In recent investigations, it has been revealed that lipopeptides are commonly synthesized by bacteria that are closely associated with plants, particularly within the *Bacillus* group. These lipopeptides exhibit significant potential in mitigating plant diseases, as demonstrated in many studies (Li *et al*., 2012).

Antagonistic efficacy of NBAIR-BSWG1 against *S. rolfsii* under greenhouse condition

The greenhouse experiment showed a significant reduction in disease incidence in pots treated with NBAIR-BSWG1 (Figure 2) over positive (diseased) control. Drenching with NBAIR-BSWG1 and inoculating pathogen at the same time (T_1) recorded the lowest Per Cent Disease Incidence (PDI) of 26% (and maximum Percentage Disease Decrease Over Control (PDOC) of 74%) wherein positive control (T_6), it was 100% (Figure 2). Drenching with NBAIR-BSWG1 and inoculating pathogen at different times (T_3, 52% PDI) were found at par with spraying with extracted crude lipopeptide of NBAIR-BSWG1 and inoculating pathogen at the same time (T_2, 56% PDI) and different time (T_4, 48% PDI). Foliar spray with NBAIR-BSWG1 broth and with lipopeptides both at the same (T_7, T_8) or different times (T_9, T_{10}) with pathogen inoculation recorded the least and at par results (Table 2). No phytotoxicity was observed in the foliar application of a crude extract of lipopeptide isolated from NBAIR-BSWG1. Corroborating findings have been documented by Sultana and Hossain (2022), who highlighted that the introduction of *B. subtilis* strain PPB9 into the root system yields a compelling outcome in disease management and plants treated with these strains exhibited a noteworthy reduction in disease impact. The tested rhizobacteria have been proven to suppress the production of oxalic acid by *S. rolfsii* and exhibit the capacity to utilize oxalic acid as a source of growth. Oxalic acid plays a pivotal role in the virulence and pathogenicity of *S. rolfsii*, and the ability of these antagonistic rhizobacteria to neutralize this pathogenic factor holds significant promise for disease control.

Table 2. Efficacy of *Bacillus subtilis* NBAIR-BSWG1 in the management of *S. rolfsii* in tomato plants under greenhouse conditions

Treatments	Treatment details	PDI*	PDOC**
T_1	Pathogen + *B. subtilis* (drenching both at same time)	26 (7.41)^{e}	74
T_2	Pathogen + lipopeptide (drenching both at same time)	56 (9.6)^{c}	44
T_3	Pathogen + *B. subtilis* (drenching both at different time)	52 (9.23)^{cd}	48
T_4	Pathogen + lipopeptide (drenching both at different time)	48 (8.86)^{d}	52
T_5	Negative control (Healthy)	0	-
T_6	Positive control (Diseased)	100 (12.92)^{a}	-
T_7	Pathogen + *B. subtilis* (foliar spray both at same time)	76 (11.22)^{b}	24
T_8	Pathogen + lipopeptide (foliar spray both at same time)	80 (11.53)^{b}	20
T_9	Pathogen + *B. subtilis* (foliar spray both at different time)	72 (10.91)^{b}	28
T_{10}	Pathogen + lipopeptide (foliar spray both at different time)	74 (11.06)^{b}	26

*Values in parentheses in PDI are arcsine-transformed values. PDI, percent disease index; **PDOC, percent disease over control. Means in a column followed by same letters are not significantly different according to DMRT at 5 % level.

Figure 2. *In vivo* bioassary of *Bacillus subtilis* NBAIR-BSWG1 with *Sclerotium rollfsii*. T_1 – *S. rolfsii* + *B. subtilis* NBAIR-BSWG1 (drenching at same time), T_2 – *S. rolfsii* lipopeptide of NBAIR-BSWG1 (drenching at different time), T_3 – S. rolfsii + B. subtilis NBAIR-BSWG1 (drenching at different time), T_4 – *S. rolfsii* + lipopeptide of NBAIR-BSWG1 (drenching at different time), T_5 – Negative control (Healthy), T_6 – Postive control (diseased), T_7 – *S. rolfsii* + *B. subtilis* NBAIR-BSWG1 (foliar spray at same time), T8 – *S. rolfsii* + lipopeptide of NBAIR-BSWG1 (foliar spray at same time), T_9 – *S. rolfsii* + *B. subtilis* NBAIR-BSWG1 (foliar spray at different time), T_{10} – *S. rolfsii* + lipopeptide of NBAIR-BSWG1 (foliar spray at different time).

CONCLUSION

In the present study, our comprehensive evaluation of NBAIR-BSWG1 antagonistic potential against *S. rolfsii* yielded highly promising results, with significant reductions in pathogen growth observed across *in vitro* assays. Pot experiments have provided compelling evidence of the substantial impact of these lipopeptides under glasshouse conditions. This underscores their effectiveness not only in controlled laboratory settings (*in vitro*) but also in *in vivo*. Thus, this study highlights the remarkable bio-efficacy of NBAIR-BSWG1 and its potential as an efficient biocontrol against *S. rolfsii*, which can offer substantial benefits to tomato farmers against the southern blight of tomato. Overall, our findings contribute significantly to integrated disease management strategies for the management of tomato blight.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge The Director of ICAR-NBAIR, Bangalore, and the Head, Department of Microbiology, and the Dean (PGS), UAS, GKVK, Bangalore, for all the support rendered for the smooth conduct of research.

REFERENCES

Biniarz, P., and Łukaszewicz, M. 2017. Direct quantification of lipopeptide biosurfactants in biological samples via HPLC and UPLC-MS requires sample modification with an organic solvent. *Appl Microbiol Biotechnol*, **101**(11): 4747-4759. https://doi.org/10.1007/s00253-017-8272-y PMid:28432441 PMCid:PMC5442266

Guo, S., Li, X., He, P., Ho, H., Wu, Y., and He, Y., 2015. Whole-genome sequencing of *Bacillus subtilis* XF-1 reveals mechanisms for biological control and multiple beneficial properties in plants. *J Ind Microbiol Biotechnol*, **42**(6): 925-937. https://doi.org/10.1007/s10295-015-1612-y PMid:25860123

Kator, L., Hosea, Z. Y., and Oche, O. D. 2015. *Sclerotium rolfsii*: Causative organism of southern blight, stem rot, white mold and sclerotia rot disease. *Ann Biol Res*, **6**(11): 78-89. https://doi.org/10.1016/j.crmicr.2021.100094 PMid:35024641 PMCid:PMC8724949

Kumar, S., Sindhu, S. S. and Kumar, R. 2022. Biofertilizers: An ecofriendly technology for nutrient recycling and environmental sustainability. *Curr Res Microb Sci*, **3**: Article 100094.

Kumbar, B., Mahmood, R., Narasimhappa, N. S. 2017. Identification and molecular diversity analysis of *Bacillus subtilis* from soils of western ghats of Karnataka using 16S rRNA bacterial universal primers. *Int J Pure Appl Biosci*, **5**(2): 541-548. https://doi.org/10.18782/2320-7051.2721

Leelasuphakul, W., Hemmanee, P., and Chuenchitt, S. 2008. Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. *Postharvest Biol Technol*, **48**(1): 113-121. https://doi.org/10.1016/j.postharvbio.2007.09.024

Li, L., Ma, J., Li, Y., Wang, Z., Gao, T., and Wang, Q. 2012. Screening and partial characterization of *Bacillus* with potential applications in biocontrol of cucumber Fusarium wilt. *Crop Prot,* **35**: 29-35. https://doi.org/10.1016/j.cropro.2011.12.004

Mardanova, A. M., Hadieva, G. F., Lutfullin, M. T., Khilyas, I. V. E., Minnullina, L. F., Gilyazeva, A. G., Bogomolnaya, L. M., and Sharipova, M. R. 2016. *Bacillus subtilis* strains with antifungal activity against the phytopathogenic fungi. *J Agric Sci*, **8**(1): 1-20. https://doi.org/10.4236/as.2017.81001

Ramyabharathi, S. A. M., and Raguchander, T. 2014. Jonathan EI. Isolation and characterization of novel *Bacillus* strain from cut flowers in Tamil Nadu. *Pestol*, **38**: 4. https://doi.org/10.1007/s10658-020-02087-6

Ramyabharathi, S. A., Meena, K. S., Rajendran, L., Raguchander, T., and Jonathan, E. I. 2020. Potential of a rhizobacterium *Bacillus subtilis* (Bbv 57) on *Fusarium oxysporum* f. sp. *gerberae* and *Meloidogyne incognita* infecting gerbera grown in protected cultivation. *Eur J Plant Pathol*, **58**: 615-632.

Ruqiya, S., Girisha, H., Manjunatha, C., Rangeshwaran, R., Kandan, A., Sivakumar, G., Kumar, M. K. P., Pramesh, D., Shivakumara, K., Venu, H., Nanditha, S., Ankitha, K. S., Aditya, K., Aarthi, N., and Sushil, S. N. 2022. Biocontrol potential and molecular characterization of lipopeptides producing *Bacillus subtilis* against *Sclerotinia sclerotiorum*. *J Biol Control*, **32**(4): 251-221.

Sultana, F., and Hossain, M. M. 2022. Assessing the potentials of bacterial antagonists for plant growth promotion, nutrient acquisition, and biological control of Southern blight disease in tomato. *Plos One*, **17**(6): Article 0267253. https://doi.org/10.1371/journal.pone.0267253 PMid:35675341 PMCid:PMC9176874

Yadav, R., Singh, S., and Singh, A. N. 2022. Biopesticides: Current status and future prospects. *Proc Int Acad Ecol Enviromental Sci*, **12**: 211-233.