



Antifungal Potential of *Bacillus subtilis* subsp. *inaquosorum* RLS76 for Management of *Fusarium* Wilt Disease of Bt- Cotton

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Abstract

Fusarium wilt is a destructive disease affecting Bt cotton that is caused by *Fusarium oxysporum* f. sp. *vasinfectum*. This seed and soilborne disease pose a significant risk to Bt cotton production worldwide, including India. Therefore, to cope with this problem in an eco-friendly manner, we explored the potentiality of the rhizobacterial isolate possessing antifungal activity as a natural arsenal against the fungal pathogen *Fusarium* wilt of Bt cotton. The rhizobacterial isolate RLS76 inhibited 85.39% of the fungal phytopathogen in the dual culture method. The RLS76 isolate's 16S rRNA partial gene sequence was determined to be *Bacillus subtilis* subsp. *inaquosorum* RLS76, with a closest phylogenetic affiliation of 99.53%. The crude extract of *Bacillus subtilis* subsp. *inaquosorum* RLS76 demonstrated 34.38% inhibition of the growth of the pathogen after a week of incubation by using an agar well diffusion method. *Bacillus subtilis* subsp. *inaquosorum* RLS76 produced volatile organic compounds that inhibited the fungal growth of phytopathogens by 54.84% in a closed environment. Also, GCMS analysis of the crude extract of *Bacillus subtilis* subsp. *inaquosorum* RLS76 identified four compounds, namely Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl); 2,5-cyclohexadiene-1,4-dione, 2,5-Dihydroxy-3-methoxy-6-methyl-; 9,12-Octadecadienoic acid (Z,Z)-,phenylmethyl ester. Out of four compounds, three exhibit antimicrobial activity except 2,5-Dihydroxy-3-methoxy-6-methyl. The RLS76 strain of *Bacillus subtilis* subsp. *inaquosorum* displayed a transition in hue, shifting from blue to orange, surrounding the colony on CAS medium by producing siderophore qualitatively. The quantitative siderophore produced in the crude extract was 86.06 SU



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after 60-72 h of incubation. The RLS76 strain of *Bacillus subtilis* subsp. *inaquosorum* also showed the ability to produce a proteolytic enzyme outside the cell. This enzyme caused a noticeable clear zone around the colony when the bacteria were grown on a skim-milk agar plate. The *Bacillus subtilis* subsp. *inaquosorum* RLS76 also exhibited phosphate solubilization activity with the clear halo around the colonies on NBRIP medium. All these results suggest that *Bacillus subtilis* subsp. *inaquosorum* RLS76 exhibits good biocontrol as well as a plant growth promoting candidate against the phytopathogen of *Fusarium* wilt of Bt cotton.

Introduction

Fusarium wilt disease is a big problem for Bt cotton production globally, including in India. The occurrence of this condition can be attributed to a soilborne fungus known as *Fusarium oxysporum* f. sp. *vasinfectum*. This disease causes significant yield losses and affects the economy of cotton farmers. Common controlling strategies for *Fusarium* wilt disease of cotton include soil conditioning, resistant varieties, crop rotation, and the use of chemical pesticides.¹ Several fungicides are available for controlling *Fusarium* wilt pathogen, but they are expensive and have limited effectiveness. Several investigators reported use of different chemical fungicides for management of *Fusarium* wilt of cotton such as Pyraclostrobin, Mitiram, Captan, hexaconazole,² Carbendazim,^{3,4} Copper oxychloride,⁴ Dithane M-45 Mancozeb, and Thiophanate methyl.⁵

But the application of chemical fungicides imposes drastic problems like affecting the beneficial microbes in soil, the development of resistance among pathogens against fungicides, pollution of the environment, and health problems. As *Fusarium* wilt poses a significant risk to cotton production, it is necessary to look for alternative methods of disease control that do not involve the use of harmful chemicals.

Over the last few years, there has been a growing emphasis on developing alternative and sustainable methods to effectively control plant diseases. These methods often focus on preventing diseases from occurring in the first place, rather than relying on pesticides to control them. Using rhizospheric microorganisms to control plant pathogens is a promising alternative to chemical methods. Rhizobacteria are naturally occurring bacteria that

can be found in the soil surrounding plant roots. Some rhizobacteria exhibit plant growth-promoting (PGPR) and biocontrol properties, making them promising candidates for managing plant diseases. Researchers identified different microorganisms such as *Bacillus* sp.,^{6,7} *Pseudomonas* sp.,⁸⁻¹⁰ *Serratia* sp.,^{8,11} and *Trichoderma* sp.,¹²⁻¹⁵ which would be potential candidates for management of plant diseases instead of chemical fungicides.

There is limited literature on controlling *Fusarium* wilt in Bt-cotton using microorganisms. Despite the work of many researchers in the field of biological control, previous studies have mainly explored the use of endophytic bacteria¹⁶⁻¹⁷ and *Trichoderma* sp.¹⁸⁻¹⁹ for managing *Fusarium* wilt in cotton.

In present research, our prime goals were: i) to test the antifungal activity of a rhizobacterial isolate against the *Fusarium* wilt pathogen by *in vitro* screening. ii) to understand the microbial control mechanism of the efficient rhizobacterial isolate by examining the production of diffusible and volatile organic compounds, siderophore, chitinase enzyme protease enzyme, and phosphate solubilization.

Fusarium wilt Phytopathogen of Bt-Cotton

Fusarium wilt phytopathogen of Bt-cotton which was previously isolated in research work conducted in the Department of Microbiology by freshly activating on PDA agar plate.

Rhizobacterial Bacterial isolate

Rhizobacterial isolate RLS76, which was isolated in an earlier study and had antifungal potential against *Alternaria* sp. was used to study the antifungal activity against a fungal pathogen, *Fusarium oxysporum* f. sp. *vasinfectum*. by activating on freshly prepared nutrient agar.

In Vitro Antifungal Activity

The dual culture technique was employed to assess the antifungal efficacy of rhizobacteria isolate RLS76 against the *Fusarium* wilt pathogen of Bt cotton.²⁰ Petri dishes were prepared with potato dextrose agar (PDA). To ensure a consistent separation between the phytopathogen and the antagonist, a 5 mm fungal disc from a 5-day-old culture and a 24-hour-old culture of RLS76 rhizobacteria isolate were both inoculated 1 cm away from the center of the plate. This method was employed to maintain a constant distance between the two organisms. Additionally, a control plate was inoculated with only fungal culture. The plates were left to incubate at room temperature for a duration of 6 days. The antifungal activity of the rhizobacteria isolate was evaluated by determining the percentage of inhibition in radial growth.²¹

$$\% \text{PIRG} = \frac{R1 - R2}{R} \times 100$$

Where, R1 refers to the radius of the fungal colony on the control plate, while R2 refers to the radius of the fungal colony towards the antagonist colony on test pate.

Identification of Biocontrol Agent

The rhizobacterial isolate RLS76, showing antifungal activity by inhibiting the radial mycelial growth of the *Fusarium* wilt pathogen of Bt cotton, was identified by using 16S rRNA partial sequencing at ARI Pune, Maharashtra. The genomic DNA of RLS76 was extracted using Sigma's "GenElute Bacterial Genomic DNA" Kit. polymerase chain reaction was conducted using the RPP2 (CCAAGCTTCTAG CCAA GCTTCTAGACGGITACCTTGTTACGACTT) and FDD2 (CCGGATCCGTCGACAGAGTTTGATCI TGG CTCAG) universal primers to amplify a 1.5 kb fragment for eubacteria.²²⁻²³ The polymerase chain reaction products obtained from the previous reactions were then subjected to the cycle sequencing reaction, using only the SRV3-1 primer. After the reaction, the samples were purified and loaded onto the Avant 3100 Gene Analyzer for sequencing.

Mechanism of Biocontrol Agent

The antifungal activity of biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 was investigated by examining its ability to produce volatile compounds, diffusible compounds, siderophore, chitinase enzyme, protease enzyme, and phosphate solubilizing ability.

Recognition of Extracellular Diffusible Organic Compounds

The agar well diffusion assay was used to detect diffusible organic compounds.²⁴ The biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 crude extract was obtained by culturing in Kings B broth at normal laboratory temperature on a shaking incubator, operating at 150 rpm, for a duration of 36-48 hours. Afterwards, three wells were formed on each of the Kings B plates using a sterilized Cork borer that had a diameter of 10 mm. These wells were subsequently labeled, and the crude extract was carefully loaded into each individual well. Following this, a fungal disc with a diameter of 5 mm, obtained from a fresh culture of *F. oxysporum* f. sp. *vasinfectum* was positioned at the center of the agar plate. The broth culture previously inoculated with *Bacillus subtilis* subsp. *inaquosorum* RLS76 was subjected to centrifugation at 8000 rpm and subsequently filtered using a 0.22 μ (Hi-media) syringe filter. This process was carried out to prepare the crude extract for testing the diffusible organic compounds. The well was individually filled with 100 μl of the crude extract in an aseptic condition, and control was maintained without the inoculation of crude extract. The control and test plate were incubated for 5 to 6 days at room temperature. Following the incubation period, the plates were carefully examined for inhibitory activity and the percentage of inhibition was calculated as described by Whipps.²⁵

$$\text{PI} = \frac{R1 - R2}{R1} \times 100$$

Where, R1 refers to the radius of the fungal colony on the control plate, while R2 refers to the radius of the fungal colony towards the antagonist colony on test plate.

Recognition of Extracellular Volatile Organic Compounds

The extracellular volatile organic compounds of the biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 was tested by using double plate method.²⁰ In this method, a kings-B plate was inoculated with a 5 mm fungal disc of phytopathogen at the center, while the rhizobacterial isolate was inoculated at the center of a 90 mm agar plate individually. The bases of the two plates were then sealed together using adhesive tape. Additionally,

a control plate was maintained without adding the bacterial isolate. This process was repeated three times using the same cultures. All the plates were nurtured at normal laboratory temperature for six days. The volatile organic compounds production was confirmed by measuring the inhibition of Fov, and the inhibition percentage was calculated as described by Whipps.²⁵

$$\text{PGI (\%)} = \frac{R1 - R2}{R1} \times 100$$

Where, R1 refers to the radius of the fungal colony on the control plate, while R2 refers to the radius of the fungal colony towards the antagonist colony on test plate.

Identification of Volatile Bioactive Compounds

The GCMS investigation of the crude extract of biocontrol agent *B. subtilis* subsp. *inaquosorum* RLS76-inoculated Kings B broth and nurtured at normal laboratory temperature for 3 days was conducted at IIT Bombay Powai, Mumbai. The analysis was performed using an Agilent-7890 machine equipped with an FID detector, Head Space injector, Combipal autosampler, and a Jeol Accu TOF GCV MS with a mass range of 10-2000 amu and mass resolution of 6000. A VF-5MS (5% phenyl methyl) capillary column measuring 30.0 m × 250 mm × 0.25 μm was utilized. The carrier gas used was helium at a rate of 1 μl/min. The column temperature was programmed to start at 60 °C and increase by 5 K/min until it reached 270 °C, where it was held isothermally for 38 min. The constituents were identified by comparing their mass spectral data with those present in the NIST library.

Siderophore Analysis

The qualitative and quantitative method was employed to conduct an analysis of siderophore production of biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76

CAS Agar Plate Method for Siderophore

The universal Chrome Azurol S Plate method²⁶ was utilized to perform qualitative testing of siderophore in *B. subtilis* subsp. *inaquosorum* RLS76. In order to conduct the qualitative detection of siderophores, Petri plates were prepared by using 30 ml of CAS blue agar and allowing it to solidify. The 24-hour-old rhizobacterial isolate *Bacillus subtilis* subsp.

inaquosorum RLS76 was then CAS agar plate was inoculated at its central region. Afterward, the plate was placed in a controlled environment at ambient temperature for a duration of fourteen days. The presence of siderophores was demonstrated by observing a color transformation of the CAS agar medium, which changed from blue to either purple or orange.

CAS Liquid Assay for Siderophore

The investigation of siderophore production was conducted using a modified succinate broth, as per the procedure described by Meyer and Abdallah.²⁷ The medium was composed of the following components per liter: succinic acid (4g), K₂HPO₄ (6g), KH₂PO₄ (3g), (NH₄)₂SO₄ (1g), MgSO₄ (0.2g), and pH adjusted to 7.0. To start the experiment, a 0.1 ml culture of *Bacillus subtilis* subsp. *inaquosorum* RLS76 was introduced into a 250 ml Erlenmeyer flask containing the Succinate medium. The flask was then placed on a shaking incubator and incubated at laboratory temperature for a duration of 24-72 hours. Throughout the incubation period, a 1 ml sample of the broth culture was collected at specific time intervals (e.g., 24, 36, 48, 60, 72 hours). Subsequently, the culture was subjected to centrifugation at 10,000 rpm in a cooling centrifuge set at 4°C for 10 minutes to obtain the supernatant.

The quantification of siderophore production was carried out by combining 0.5 ml solution of CAS with 0.5 ml of centrifuged culture filtrate. To set up a control, a sterile succinate broth was used instead of centrifuged culture filtrate. The OD of the test and control samples determined at 630 nm, and the percentage of SU was calculated.²⁸

$$\% \text{ Siderophore Units} = \frac{Ar - As}{Ar} \times 100$$

Where,

Ar = Absorbance of reference and As = Absorbance of test sample at 630 nm.

Chitinase Enzyme plate assay

The determination of chitinase enzyme production by the biocontrol agent *B. subtilis* subsp. *inaquosorum* RLS76 was conducted using colloidal chitin agar supplemented with 0.4% colloidal chitin.²⁹ The bacterial suspension was used to inoculate the chitin

agar plate, which was then left to incubate at room temperature for a period of 4 days. Subsequently, the plates were inspected to figure out if there was a chitin hydrolysis zone surrounding the culture that was spot inoculated.

Protease Enzyme plate Assay

The assessment of protease enzyme production was conducted by introducing the biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 onto a skim-milk agar plate.³⁰ The plate was inoculated with the rhizobacterial isolate and placed in an incubator at room temperature for a period of 24-48 hours. Following the incubation, the presence of a clear halo zone around the rhizobacterial colony on the skim milk agar plate was observed, and its measurement in millimeters was recorded.

Phosphate Solubilization Assay

The phosphate solubilization ability of the biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 was evaluated using NBRIP agar plate, as described by Nautiyal.³¹ The rhizobacterial isolate was then spot inoculated on the NBRIP agar plate and incubated at normal laboratory temperature for 5 days. A clear halo around the colony was identified as phosphate solubilizers.

Results and Discussion

***In Vitro* Antifungal Activity**

The rhizobacterial isolate RLS76 was found to reduce the fungal growth of the phytopathogen *F. oxysporum* f. sp. *vasinfectum* whose inhibition percentage was recorded as 85.39 % (Photo Plate 1 and Table 1).



Photo Plate 1: Screening of antifungal activity of rhizobacterial isolate against *F. oxysporum* f. sp. *vasinfectum* by dual culture method (a) Control (b) Test (RLS76)

Table 1: Antifungal activity of rhizobacterial isolate against *F. oxysporum* f. sp. *vasinfectum*

Sr. No.	Rhizobacterial isolate	% of inhibition of radial growth of <i>Fov</i>
1	RLS76	85.39

A number of scientists extracted the rhizobacterial bacterial isolates from the disease-free cotton field and examined their antifungal properties against the

fungal pathogen *Fov* using the dual culture method. Among the nine *Bacillus* strains obtained from the rhizosphere of cotton plants, two strains, BP-9 and

BP-13 displayed the highest percentage of killing growth of the fungal pathogen *F. oxysporum* f. sp. *vasinfectum*, with values of 62.50% and 51.25% respectively.³² The rhizospheric isolate belonging to *Pseudomonas* spp. MRh 42 inhibited 66.60% growth of Fov a phytopathogen of *fusarium* wilt disease, in cotton crops.³³ In the same vein, *Saccharothrix algeriensis* NRRL B-24137 exhibited a noteworthy antifungal capacity against the *Fusarium* wilt phytopathogen of cotton in laboratory conditions. It effectively suppressed 68% of the mycelial growth of the pathogen, surpassing the control group.³ Furthermore, *Bacillus subtilis*, a naturally occurring bacterial antagonist, demonstrated a significant inhibitory effect of 47.29% on the growth of the fungal pathogen *Fusarium oxysporum* f. sp. *vasinfectum*, when compared to control.² Similarly, the strain of *Bacillus* spp RZ141 reduced the fungal growth of Fov4 by 52% compared to the control by the dual culture method.³⁴

The highest antimycotic action documented against *F. oxysporum* f. sp. *vasinfectum* by earlier researchers ranged from 47.29 to 68.00 %. However, in the present research work, rhizobacterial isolate RLS76 recorded an 85.39 % reduction in the fungal growth of *Fusarium oxysporum* f. sp. *vasinfectum*. The research work conducted previously indicates that the rhizobacterial isolate RLS76 has shown remarkable efficacy in managing the *Fusarium* wilt disease of Bt cotton.

Identification of Biocontrol Agent

The computer software (Applied Biosystems) DNA Sequence Analyzer was used to analyze the partial sequence of the RLS76 isolate's 16S ribosomal RNA gene. The sequence was subjected to comparison with entries in the NCBI GenBank using the blast algorithm, which resulted in the identification of its closest phylogenetic affiliation as 99.53% with *Bacillus subtilis* subsp. *inaquosorum* RLS76. (Fig. 1).

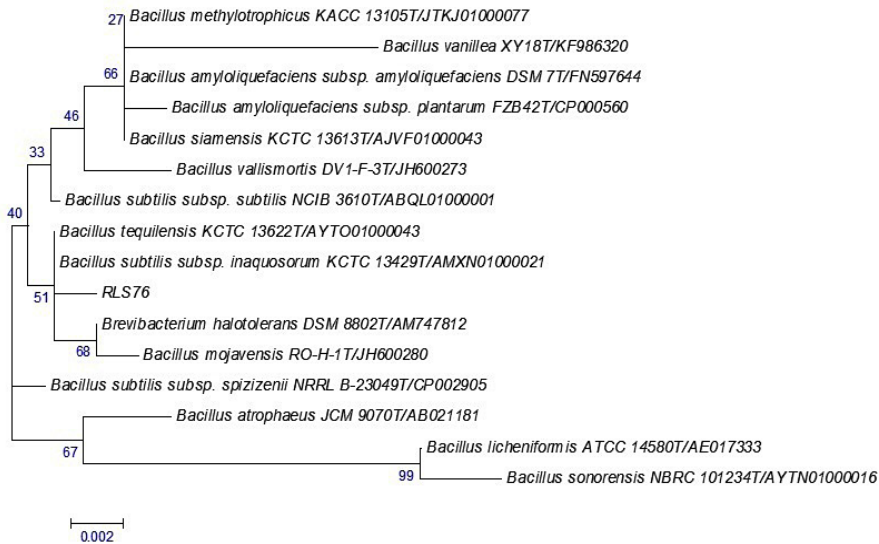


Fig. 1: Phylogenetic tree of *Bacillus subtilis* subsp. *inaquosorum* RLS76.

Mechanism of Biocontrol Agent Recognition of Extracellular Diffusible Organic Compounds

The crude extract of biocontrol agent *Bacillus subtilis* subsp. *immunoserum* RLS76 showed

reduction in fungal growth of 34.38% by producing diffusible non-volatile antifungal organic compounds (Table 2).

Table 2: Production of antifungal extracellular diffusible and volatile organic compound by biocontrol agent against the fungal plant pathogen

Sr No.	Biocontrol agent	Metabolites	Percentage of Inhibition <i>Fusarium oxysporum</i> f. sp. <i>vasinfectum</i>
1	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> RLS76	Extracellular diffusible organic compounds produced by agar well diffusion assay	34.38
		Extracellular volatile organic compound by double plate assay	54.84

The potential of *Bacillus* sp. to inhibit pathogenic fungi is principally based on their production of a variable variety of diffusible non-volatile organic compounds with antimicrobial properties.³⁵ The *B. subtilis* GM2 strain harbors four genes responsible for encoding antimicrobial peptides, namely iturin, bacillomycin, bacitracin, and surfactin. Conversely, the GM5 strain possesses only two genes that encode the nearly same peptides, specifically surfactin and fengycin.³⁶ *B. amyloliquefaciens* DHA55 and *Bacillus amyloliquefaciens* An6 produced multiple bioactive antifungal lipopeptide compounds belonging to iturin, surfactin, fengycin, and bacillomycin and showed significant antifungal activities against *F. oxysporum*.³⁷⁻³⁸ Some strains of *Bacillus* spp. S1301, S1967, S2535, and S2536 had an antifungal effect on the normal development of the *Fusarium oxysporum* f. sp. *vasinfectum* colony, which provided 12.92%, 27.31%, 33.06%, and 30.43% toxic effects respectively.³⁹ The toxic effects were most frequent between the seventh and eighth days of the experiment as compared to the control. The rhizobacterial isolate isolated from tomato rhizosphere *Bacillus cereus* RFP63 showed the highest inhibitory impact (44.5%) on FoI after a week of incubation in the agar well diffusion method by secreting nonvolatile diffusible metabolites.⁴⁰ The rhizobacterium *Bacillus subtilis* F62 reduced the mycelial growth of *Fusarium* sp., which varied from 35.4% to 63.6% measured on the 14th day of the experiment concerning the control.⁴¹

From above reports, it was clear that our biocontrol agent, *Bacillus subtilis* subsp. *inaquosorum* RLS76

showed 34.38% inhibitory activity, which was a lesser inhibitory effect than Russi *et al.* (2022)⁴¹ but produced some kinds of diffusible non-volatiles organic compounds that inhibited the growth of *Fusarium oxysporum*. Further analysis is needed for these diffusible organic compounds.

Recognition of Extracellular Volatile Organic Compounds

The biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 inhibited 54.84 % fungal growth of Fov by producing extracellular volatile organic compounds in a closed environment (Table 2).

The biocontrol of phytopathogens has been found to be significantly influenced by the production of volatile secondary metabolites, including HCN⁴² and ammonia,⁴³ by rhizobacterial isolates such as *Bacillus* and *Pseudomonas* strains.⁴⁴ The antagonistic bacterial strain of *Bacillus* Rz141 showed 24% inhibition of Fov4, a phytopathogen causing *Fusarium* wilt disease of Pima cotton, by producing volatile metabolites.³⁴ Similarly, the two strains of *Bacillus* spp. S1823 and S2536 were evaluated for the production of volatile organic compounds and reduced the growth of Fov by 11.51% and 15.51% respectively, from the fifth day to the last day of the experiment as compared to the control.³⁹ The *Bacillus halotolerans* strains RFP57 and RFP74 isolated from rhizobacterial soil of the tomato crop inhibited 67% and 34% fungal growth of the FoI respectively, in the double plate method by producing volatile metabolites.⁴⁰

The research findings documented in different scientific journals revealed that our results with the rhizobacterial isolate *Bacillus subtilis* subsp. *inaquosorum* RLS76 against *Fusarium oxysporum* f. sp. *vasinfectum* are better than the results documented by earlier research workers by producing volatile secondary metabolites.

Identification of Volatile Bioactive Compounds

The chromatogram revealed 12 peaks during the GC-MS analysis of the crude extract obtained

from the biocontrol agent, *Bacillus subtilis* subsp. *inaquosorum* RLS76. (Fig. 2). On the basis of spectral data, four compounds were recognized, namely Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl); 2,5-cyclohexadiene-1,4-dione, 2,5-Dihydroxy-3-methoxy-6-methyl- and 9,12-Octadecadienoic acid (Z,Z)-,phenylmethyl ester. Out of four compounds, three show antimicrobial activity except 2,5-Dihydroxy-3-methoxy-6-methyl (Table 3 and Fig. 3, 4, 5, 6 and 7).

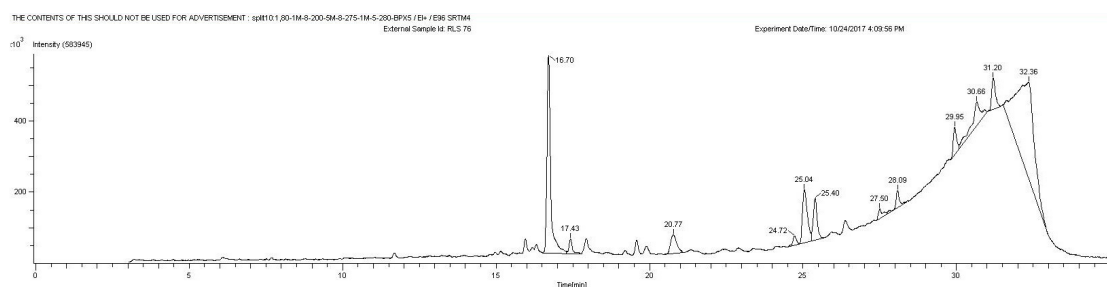


Fig. 2: GC-MS Chromatogram of crude extract of biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76

Table 3: Identification of volatile bioactive compounds in crude extract of biocontrol agent *B. subtilis* subsp. *inaquosorum* RLS76

Retention time	Compound	Formula	Mol. Wt. g/mol	Similarity (%)	Biological activity	Reference
16.75	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	72.5	Anti-microbial Anti-oxidant	45-46
17.43 and 19.59	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	46.9	Anti-bacterial	49,51
20.78	2,5-cyclohexadiene-1,4-dione, 2,5-Dihydroxy-3-methoxy-6-methyl-	$C_8H_8O_5$	184	9.29	Unknown	
24.73	9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester	$C_{25}H_{38}O_2$	370	10.7	Anti-microbial Anticancer	69

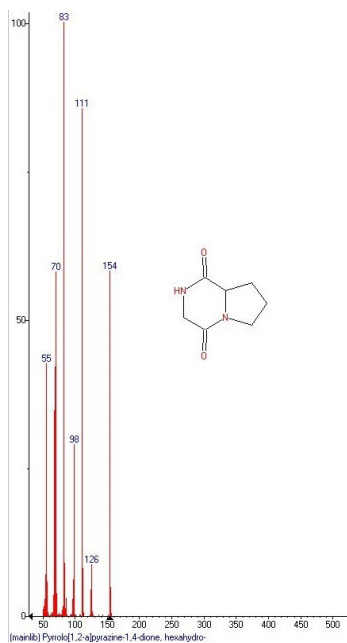


Fig. 3: Mass spectrum of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- with Retention time (RT) = 16.75

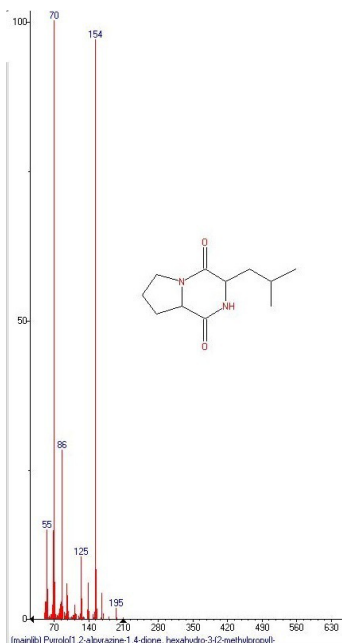


Fig. 4: Mass spectrum of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- with Retention time (RT) = 17.43

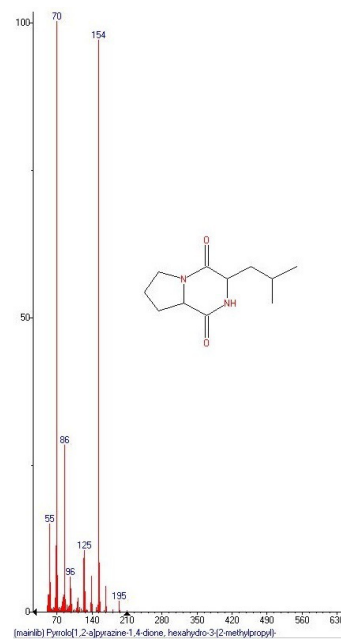


Fig. 5: Mass spectrum of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- with Retention time (RT) = 19.59

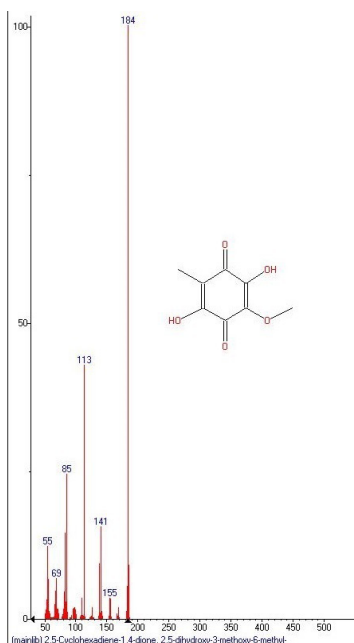


Fig. 6: Mass spectrum of 2,5-cyclohexadiene-1,4-dione, 2,5-Dihydroxy-3-methoxy-6-methyl- with Retention time (RT) = 20.78

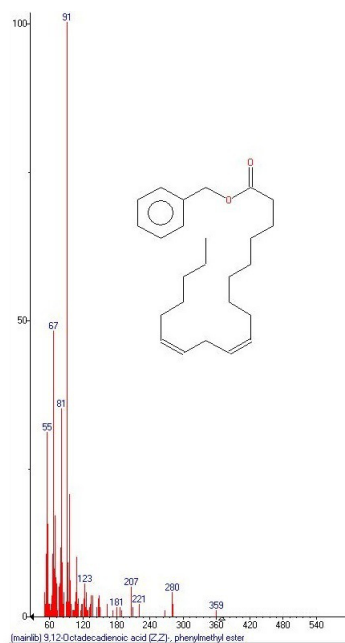


Fig. 7: Mass spectrum of 9,12-Octadecadienoic acid (Z,Z)-,phenylmethyl ester with Retention time (RT) = 24.73

The actinomycetes crude extract from a novel strain of *Streptomyces sp.* strain MUSC 149T displayed remarkable antioxidant activity. Furthermore, chemical analysis confirmed the existence of a volatile metabolite Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- by GCMS with antioxidant properties.⁴⁵ Similarly, *Bacillus tequilensis* MSI45 a sponge associated bacterial strain whose crude extract showed the occurrence of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro- a bioactive antimicrobial secondary metabolite.⁴⁶

The partial characterization of the ethyl-acetate extracts of *Bacillus pumilus* MMM,⁴⁷ *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*⁴⁸ and three strains of *Bacillus sp.* obtained from wild bees honey⁴⁹ was performed using the GCMS and identified Pyrrolo [1,2-a] pyrazine-1,4-dion, hexahydro-3-(2-methylpropyl)- the main bioactive secondary volatile metabolites having antimicrobial properties. Similarly, the GCMS analysis of the crude extracts of *Streptomyces sp.* UTM 1334,⁵⁰ *Streptomyces achromogenes* TCH4,⁵¹ and *Streptomyces sp.* VITGV01⁵² belonging to actinomycetes identified a relatively high amount of aromatic volatile metabolites Pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methylpropyl)- showing great antioxidant activity.

In context to GC-MS analysis, as reported earlier by investigators, the bioactive metabolites Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- have antimicrobial as well as antioxidant activity.⁴⁵⁻⁴⁶ Also, some researchers found the volatile antifungal metabolite Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), formed by *Bacillus subtilis* subsp. *inaquosorum* RLS76, played an significant role in controlling the growth of the Bt cotton phytopathogen Fov.^{47,49,51,52} But the two compounds 2,5-cyclohexadiene-1,4-dione, 2,5-Dihydroxy-3-methoxy-6-methyl- and 9,12-Octadecadienoic acid (Z, Z)-, phenylmethyl ester produced by *Bacillus subtilis* subsp. *inaquosorum* RLS76 were novel metabolites, and no such literature is available.

CAS Plate assay for Siderophore

The biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 showed a transformation in color, shifting from blue to orange surrounding the inoculated colony with secretion and diffusion

of siderophore compounds in CAS blue medium. (Photo Plate 2 and Table 4).

Table 4: Detection of siderophore production qualitatively by biocontrol agent using CAS agar plate method

Sr. No.	Biocontrol agent	Colour of zone
1	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> RLS76	Orange

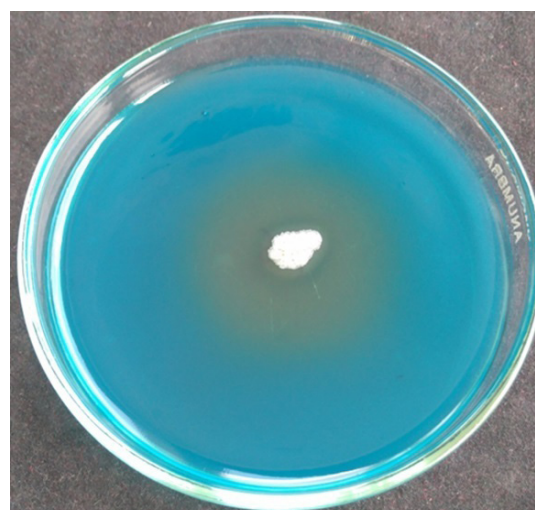


Photo Plate 2: Detection of siderophore production qualitatively by biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 using CAS agar plate method

Siderophores, a small iron chelator molecule with low molecular weight, have a significant impact on inhibiting the growth of the fungal phytopathogen by engaging in iron competition.⁵³ The six strains of *Bacillus* and two strains of *Pseudomonas* showed a color change from blue to an orange halo around the colonies on CAS agar plate after 5 days of incubation.⁵⁴ The XN-04 strain of *Streptomyces alfalfae*, which is a type of actinomycetes, showed its ability to change the blue ferric CAS complex into a vibrant orange color. This change indicates that the strain is capable of producing siderophore.⁵⁵ The *Bacillus licheniformis* YZCUO202005 strain⁵⁶ and *Brevibacillus laterosporus* 301/iK3-2⁵⁷ The blue color of the CAS agar plates was successfully

altered, and the presence of a distinct orange to yellow halo on the medium indicated the production of siderophores.

The outcomes seen clearly demonstrated that our biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 effectively generated siderophore in CAS plates that were modified, resulting in a noticeable alteration of color in the CAS agar plate.

CAS Liquid assay for Siderophore

The succinate broth inoculated with the biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76

and incubated on a shaking incubator showed yellow colored growth (Photo Plate 3a). The color of the mixture consisting of 0.5 ml crude extract of *Bacillus subtilis* subsp. *inaquosorum* RLS76 and 0.5 ml of CAS solution changed from blue to orange (Photo Plate 3b). The percentage of siderophore units generated in the culture filtrate without cells was determined to be 86.06% after a duration of 60-72 hours (Table 5). During the subsequent incubation period, the quantity of siderophore units declined as a result of the degradation of the generated siderophore.

Table 5: Production of siderophore quantitatively by biocontrol agent by using liquid CAS assay

Sr. No	Biocontrol agent	Siderophore units (%)				
		24 h	36 h	48 h	60 h	72 h
1	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> RLS76	17.34	40.15	70.04	78.02	86.06



Photo Plate 3: (a) Growth of siderophore producing biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 in succinate broth (b) Detection of siderophore quantitatively by *Bacillus subtilis* subsp. *inaquosorum* RLS76 using liquid CAS assay.

The rhizobacterial isolate *Bacillus pumilus* MK5 produced 51.36% siderophore units after 72 hours of incubation by liquid CAS assay.⁵⁸ Novel strains

of *Bacillus cereus* Wah1,⁵⁹ *Brevibacillus brevis* GZDF3,⁶⁰ *Bacillus subtilis* subsp. *inaquosorum* RLS52,⁶¹ and *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19⁶² also showed maximal siderophores production of 97.76, 29.67, 85.04, and 93.20% SU respectively. Also, the siderophore production of the *Bacillus siamensis* Gxun-6 strain⁶³ and *Brevibacillus laterosporus* 301/IK3-2⁵⁷ was detected by a liquid CAS assay. The tested strain was able to produce 56.43% and 50.00% siderophore units after 48 h of incubation respectively, showing that the bacteria had strong siderophore-producing ability.

In the present study, the *Bacillus subtilis* subsp. *inaquosorum* RLS76 strain produced 86.06% siderophore units after 72 h. These results were far better than the results documented by earlier research workers, except for *Bacillus cereus* Wah1 strain.⁵⁹

Chitinase Enzyme Plate assay

The biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 showed a clear halo of

distinct chitin hydrolysis surrounding the inoculated colony on colloidal chitin agar. The existence of the chitin hydrolysis enzyme is shown by the clear

area surrounding the colony. The measurement of chitinase enzyme activity on the plate was recorded in millimeters (Table 6).

Table 6: Production of different enzymes by biocontrol agent

Sr. No.	Biocontrol agent	Hydrolytic enzyme activity	Total zone including colony (mm)	Diameter of colony (mm)	Diameter clear halo (mm)
1	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> RLS76	Chitinase enzyme activity on colloidal chitin agar	29	13	16
		Protease enzyme activity by skim milk agar	18	11	7
		Phosphate solubilization using NBRIP medium	20	15	5

Chitin, an integral part of the fungal cell wall, is a plentiful natural polysaccharide consisting of a linear polymer of repeating $\beta(1,4)$ -N-acetylglucosamine units. In nature, some microorganisms have the ability to use the substrate chitin by using enzymes called chitinases. The chitinase enzyme production ability of *Bacillus spp.* BT42 medium supplemented with colloidal chitin formed a noticeable clear zone surrounding the colony, indicating extracellular chitinase enzyme.⁶⁴ Similarly, the *Streptomyces alfalfae* XN-04 strain on the colloidal chitin agar plate exhibited a clear halo of chitin hydrolysis of the inoculated bacterial culture.⁶⁵ Also, *Bacillus tequilensis* strains B2 and B3 showed chitinase-producing ability on medium amended with colloidal chitin showing, a clear zone of clearance surrounding the inoculated colonies.⁶⁵ This demonstrates the capability of these bacterial isolates to break down chitin, a crucial component of the fungal cell wall in *Fusarium oxysporum*.

In this study, the production of the chitinase enzyme by biocontrol agent *Bacillus subtilis* subsp. *inaquosorum* RLS76 was seen the visible zone of chitin hydrolysis. This confirms the significant role of the chitinase enzyme in controlling the phytopathogens *Fusarium oxysporum* f. sp. *vasinfectum* in cotton.

Protease Enzyme Plate assay

The biocontrol strain RLS76 of *Bacillus subtilis* subsp. *inaquosorum* exhibited the ability to produce extracellular protease enzyme, as evidenced by the

clear halo around the inoculated colony on a skim-milk agar plate (Photo Plate 4 and Table 6).

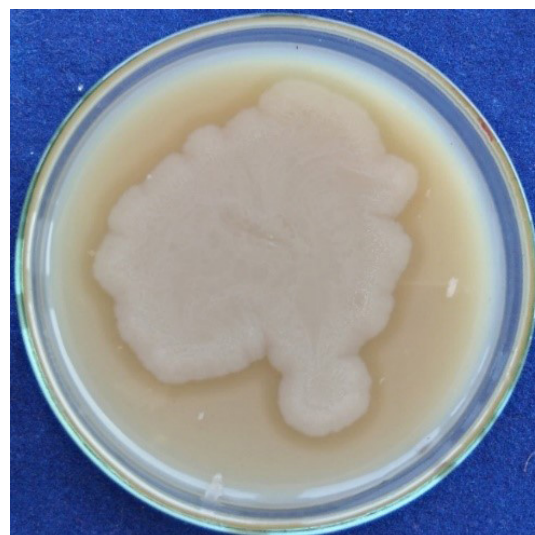


Photo Plate 4: Detection of protease enzyme activity on skim-milk agar by *Bacillus subtilis* subsp. *inaquosorum* RLS76

Proteases are the most important enzymes produced by rhizobacterial microorganisms and used to control fungal phytopathogens. The *Streptomyces alfalfae* XN-04 grow well on medium amended with casein as a source of substrate, and a zone of clearance surrounding the colony indicated the protease enzyme production.⁶⁵ Similarly, the strains of *Bacillus tequilensis* B2, B3, and B4 produced

protease enzyme activity on a milk agar plate, which was indicated by the clear zone surrounding the inoculated cultures.⁶⁵ Also, the PDA agar plate added with skimmed milk and spot inoculated with *Bacillus licheniformis* YZCUO202005 showed a visible clear halo zone of protein degradation surrounding the colony, indicating a strong protease enzyme activity.⁶⁶

All these reports support the idea that the protease enzyme produced by *Bacillus subtilis* subsp.

inaquosorum RLS76 played a significant role in inhibiting the mycelial growth of *Fusarium* wilt phytopathogen of Bt cotton.

Phosphate Solubilization Assay

The *Bacillus subtilis* subsp. *inaquosorum* RLS76 exhibited phosphate solubilization activity with the clear halo around the colonies (Photo Plate 5). The diameter of a clear halo surrounding the inoculated colony was measured in mm and found to be 5 mm (Table 6).

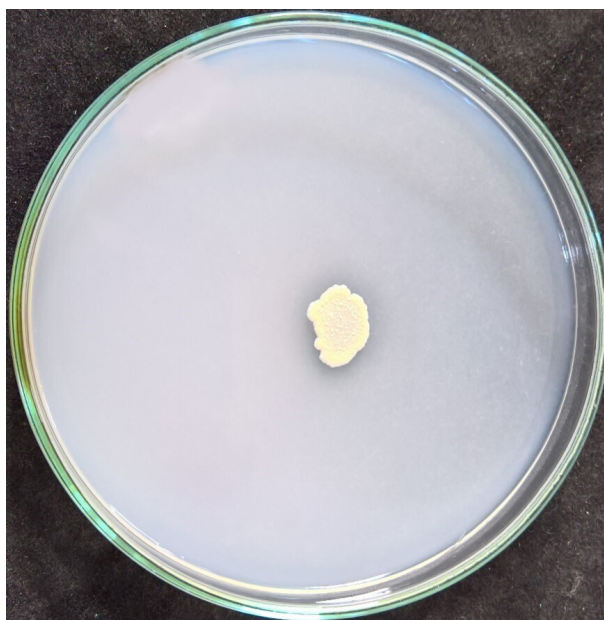


Photo Plate 5 : Phosphate solubilization assay of *Bacillus subtilis* subsp. *inaquosorum* RLS76 by using NBRIP medium.

Plants need phosphorous for their growth and development. The soil contains both organic and inorganic compounds of phosphate, but the majority of them are inactive and therefore not accessible to plants. Some rhizospheric microorganisms are able to solubilize organic and inorganic phosphate and make it available to plants.⁶⁶ Similarly, the four strains of *Sporolactobacillus laevolacticus* exhibited a halo zone of tri-calcium phosphate solubilization on Pikovskaya's agar plates, measuring over 5 mm in diameter after 9 days of incubation.⁶⁷

Also, the *Streptomyces alfalfae* XN-04 exhibited robust growth on NBRIP medium supplemented with insoluble organic phosphate. This leads to

the formation of a clear halo zone surrounding the colony, indicating the hydrolysis of the substrate and showcasing its capability to solubilize phosphate.⁵⁵ Khatoon and Khan⁶⁸ showed that thirteen rhizospheric isolates solubilized phosphate on NBRIP agar medium supplemented with tri-calcium phosphate. Similarly, *Bacillus licheniformis* YZCUO202005 showed a visible halo zone of phosphate solubilization on NBRIP medium.⁵⁶

All these reports gave positive evidence that *Bacillus subtilis* subsp. *inaquosorum* RLS76 possesses the capability to transform insoluble phosphate into soluble phosphate. This transformation is crucial in facilitating the growth promotion of Bt cotton crop.

Conclusion

The effectiveness of the rhizobacterial isolate *Bacillus subtilis* subsp. *inaquosorum* RLS76 as a biocontrol agent against *Fusarium oxysporum* f. sp. *vasinfectum*, a phytopathogen causing *Fusarium* wilt in Bt cotton, was proved in the present study. The findings clearly showed that the rhizobacterial isolate RLS76 effectively suppressed the radial growth of the phytopathogen *Fusarium oxysporum* f. sp. *vasinfectum*, with an impressive inhibition rate of 85.39%. The rhizobacterial isolate displayed various direct and indirect mechanisms for biocontrol and plant growth promotion, including diffusible nonvolatile antifungal organic compounds and volatile organic compounds. The GC-MS analysis of the crude extract of the isolate identified four compounds, namely Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl); 2,5-cyclohexadiene-1,4-dione, 2,5-Dihydroxy-3-methoxy-6-methyl- and 9,12-Octadecadienoic acid (Z,Z)-,phenylmethyl ester. Out of four compounds, three show antimicrobial activity except 2,5-Dihydroxy-3-methoxy-6-methyl. Also, the rhizobacterial isolate was able to produce siderophore and showed strong chitinase, protease and phosphate solubilization activity. Based on the findings mentioned earlier, it has been concluded that our rhizobacterial isolate, *Bacillus subtilis* subsp. *inaquosorum* RLS76 demonstrates remarkable potential as a biocontrol agent and plant growth-

promoting candidate in the effective management of *Fusarium* wilt disease in Bt-Cotton.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Not applicable

Ethics Statement

Not applicable

Authors' Contribution

1. Laxman S. Raut: Formulated and executed the analysis, conducted all practical tasks, and drafted the manuscript. 2. Sanjay M. Dalvi: Assessed and revised the manuscript. 3. Ravindra R. Rakh: Devised and planned the analysis, and formatted the manuscript according to journal guidelines.

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