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Bacillus amyloliquefaciens subsp. *amyloliquefaciens* RLS19 as a Multifarious Source of Potent Antibiotics and other Secondary Metabolites for Biocontrol of Fusarium Wilt Disease in Bt-Cotton

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Abstract

Fusarium wilt, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (Fov), is a major threat to Bt cotton production worldwide. It can lead to significant yield losses and economic damage. While chemical fungicides have been the traditional method for control, concerns about their environmental and human health impacts have led to increased interest in biocontrol methods. The rhizosphere of plants has often been utilized as a remarkable source for discovering microbial control agents. Keeping this approach in mind, the 45 rhizospheric isolates obtained from a healthy cotton rhizosphere were evaluated against the phytopathogen *Fusarium* wilt of Bt cotton by the dual culture technique. One isolate, RLS19, exhibited promising antifungal activity, suppressing the fungal growth by 89.71% compared to the control. This rhizospheric isolate was identified by using 16S ribosomal RNA gene partial sequencing, and the blast algorithm showed 100% closest phylogenetic affiliation with *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19. The isolate produced volatile metabolites in a closed environment that inhibited the fungal growth by 61.29% which suggests that it releases gaseous compounds that can diffuse through the air and directly harm the pathogen. The cell-free culture filtrate of the isolate also inhibited the fungal growth by 53.13% in an agar well diffusion assay that represented the secretion of non-volatile antifungal compounds that can diffuse through the culture medium and inhibited the pathogen's growth. Siderophore produced by *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 isolate on CAS agar plate showed a change in color of the medium from blue to orange or purple. The siderophore production in the liquid CAS assay of the cell-free culture filtrate was (93.20%) siderophore units, and the optimal harvesting time from the fermentation broth was between 60-72 hours. The isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was also found effective in secreting the chitinase and protease enzymes, which contributed significantly to killing the phytopathogen in *in vitro* conditions. With its multiple potentialities, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 holds great promise as an excellent natural arsenal for biocontrol and plant growth promotion in managing Fusarium wilt in Bt cotton, serving as an alternative to chemical fungicides.

***Key words*:** *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19, *Fusarium oxysporum* f. sp. *vasinfectum,* Volatile metabolites, Diffusible metabolites, Siderophore, Chitinase enzyme, Protease enzyme, Phosphate solubilization

Fusarium wilt is a major disease of cotton, particularly Bt cotton, caused by the soilborne fungus *Fusarium oxysporum* f. sp. *vasinfectum* (Fov). It is a destructive disease that can cause significant yield losses, sometimes reaching up to 100%. *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), was first described by Atkinson in the United States [1]. It can also infect seeds, but seed transmission is low. In India, Fusarium wilt of cotton was reported in 1908 at the Cotton Research Station, Dharwad in the Bombay-Karnataka region [2]. Burgess reported that the Fusarium wilt disease of cotton is worldwide in distribution [3].

The integrated management practices followed for the management of Fusarium wilt of cotton include soil solarization, clean cotton seed, resistant cultivars, crop rotation, and the use of chemical fungicides. Potential control strategies to mitigate Fusarium wilt disease include the use of chemical pesticides. Several researchers reported the application of different chemical fungicides such as Carbendazim [4-5], Thiophanate methyl and Dithane M-45 [4], Copper oxychloride [5], Pyraclostrobin, Mitiram, Captan, hexaconazole [6] successfully used to control Fusarium wilt disease of cotton. But chemical control measures create imbalances in the microbial community, which may be unfavorable to the activity of beneficial organisms and could lead to the development of resistant strains of pathogens and pollution of the environment. As Fusarium wilt poses a growing threat to cotton production, it is imperative to explore alternative methods of disease control that do not involve the use of harmful chemicals.

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Soil is an excellent medium for the growth of various microorganisms because it provides an abundance of nutrients and favorable environmental conditions. The rhizosphere of plants has a natural arsenal that has been utilized as a remarkable source for discovering microbial control agents. It has been proposed that microorganisms obtained from the rhizosphere of a specific crop might possess superior adaptation to the rhizospheric environment of that crop, thereby offering enhanced disease control compared to other plant rhizosphere species [7]. The utilization of rhizospheric microorganisms for the biological control of plant pathogens presents a promising alternative to chemical control methods. There is substantial evidence supporting the effectiveness of microorganisms as potential microbial control candidates in suppressing diseases. In the United States, the U.S. Environmental Protection Agency has registered eight species of microorganisms for commercial use in combating soil-borne plant pathogens [8]. Researchers have recognized rhizobacteria as a natural arsenal for biocontrol and plant growth promotion. Microbial genera from rhizospheric soil, *Trichoderma sp*. [9-10], *Bacillus sp*. [11-12], *Serratia sp*. [13-14], *Pseudomonas sp*. [15-16], have effective biocontrol traits that further need to be explored.

There is a limited amount of literature available on the microbiological control of Fusarium wilt of Bt-cotton, despite the efforts of several researchers working on the biological control of Fusarium wilt in various crops. Previous studies have focused on the use of endophytic bacteria [17-18] and *Trichoderma sp.* [19-20] for the biological control of Fusarium wilt in cotton.

In the present study, our prime focuses were: i) to isolate the phytopathogen responsible for causing Fusarium wilt in Bt-cotton, ii) to isolate rhizospheric bacteria from healthy Bt-cotton plants, iii) to conduct *in vitro* screening of rhizospheric bacteria for antifungal activity against the Fusarium wilt pathogen, and iv) to characterize the *in vitro* microbial control mechanism of efficient rhizospheric bacteria by testing for the production of volatile metabolites, diffusible metabolites, siderophore and hydrolytic enzymes.

MATERIALS AND METHODS

Isolation of phytopathogen

The Fusarium wilt infected Bt-cotton plants (Plate 1) were collected from the fields of Asola village in Parbhani, Maharashtra. The plants were carefully uprooted, placed in sterile polyethylene bags and transported to the research laboratory at the Department of Microbiology, Sant Tukaram College of Arts and Science in Parbhani. To ensure cleanliness, the infected stems and branches of the Bt-cotton were thoroughly washed with sterile water. The infected stem tissues were then cut into small pieces measuring 2-5 mm in size. Using flame-sterilized forceps, these pieces were transferred to sterile Petri plates containing a 0.1% mercuric chloride solution for 30-60 seconds. Afterward, the surface sterilized stem tissues were transferred to another set of sterile Petri plates filled with sterile distilled water. This step was repeated 2-3 times to remove any traces of the disinfectant solution. The outer layer of tissues was swiftly removed using a sterile surgical blade, and small pieces from the central core of tissues in the advancing margin of infection were cut and transferred to Petri plates containing Potato Dextrose Agar (PDA). The Petri plates were then incubated at room temperature for 6 days, following the protocol outlined by Narayanasamy [21]. The isolated pure culture of the phytopathogen was maintained on Potato Dextrose Agar (PDA) and Kings-B agar plates, as well as on a slant, for future use.

Plate 1 Bt cotton crop infected with fusarium wilt disease

Soil samples from the rhizosphere of healthy Bt-cotton fields in village Asola belonging to taluka and district Parbhani, Marathwada region were collected in sterile polyethylene bags and transported to the research laboratory. Each sample in 1 gm quantity was mixed with 100 ml of sterile distilled water and shaken for 2 minutes before allowing the contents to settle. Serial dilutions ranging from 10^{-1} to 10^{-6} were prepared, with the highest dilutions of 10^{-4}, 10^{-5}, and 10^{-6} used for plating. The Pour plate technique was used to plate 0.5 ml of selected dilutions, and all plates were incubated at room temperature for 24-48 hours.

In vitro screening for microbial control agent

A total of 45 rhizobacteria isolates were scrutinized for their ability to inhibit the growth of *Fusarium oxysporum* f. sp. *vasinfectum*, a fungal pathogen of Bt-cotton by the dual culture technique [22]. Petri dishes containing Potato Dextrose Agar (PDA) were prepared and a 5 mm fungal disc from a 5-day-old culture was placed 1 cm away from the center of the plate. In the opposite direction, 1 cm away from the center, a 24-hour-old culture of each rhizobacteria isolate was co-inoculated to maintain an equal distance between the phytopathogen and the isolate. A control plate was kept without inoculation of any rhizobacterial isolate. All plates were then incubated at room temperature for 6 days. The antifungal activity of the rhizobacteria isolates was determined by measuring the percentage of radial growth inhibition [23].

$$\% \text{ PIRG} = \frac{R_1 - R_2}{R} \times 100$$

Where;

R_1: Radius of the fungal phytopathogen colony in the control plate

R_2: Radius of the fungal phytopathogen colony in the direction of the antagonist colony

Identification of microbial control agent

The rhizobacterial isolate RLS76 showing the most excellent antifungal activity was selected for further study and identified using 16S rRNA sequencing. 16S rRNA sequencing was carried out at the Agharkar Research Institute (ARI) Pune, Maharashtra using the following protocol:

The total genomic DNA of rhizobacterial isolate RLS76 was isolated using the GeneElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA, 2.5 mM $MgCl_2$; 1x PCR buffer (Bangalore Genes, Bangalore, India), 200 µM each dCTP. dGTP. dATP. and dTTP 2 pmol of each, forward and reverse primer, and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a final volume of 20 µl FDD2 and RPP2 primers were used to amplify almost the entire 16S rRNA gene, as described previously [24]. The PCR was performed using the

Eppendorf Gradient Mastercycler system with a cycle of 94°C for 5 min: 30 cycles of 94° 60° and 72°C for 1 min each, and a final extension at 72°C for 10 min, and the mixture was held at 4°C The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed three times using 70% ethanol and dissolved in Tris-HCL (10mM, pH 8.0).

The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Brosystems, Foster City, Calif.) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the nearly complete gene [24-25]. The sequencing reaction and template preparation were performed and purified in accordance with the directions of the manufacturer (Applied Biosystems). Samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

In vitro mechanisms of action – A multifaceted approach

To characterize the mechanism of the microbial control agent, the efficient rhizospheric isolate, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was tested for production of volatile metabolites, diffusible metabolites, siderophore production and cell wall degrading enzymes.

Detection of antifungal volatile metabolites

Volatile antifungal metabolites detection of efficient rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was done by using the double plate method [22]. The kings-B agar plate was inoculated with a 5 mm mycelial disc of *Fusarium oxysporum* f. sp. *vasinfectum* and rhizospheric isolate at the center of 90 mm Petri plate separately. Both the inoculated plates were placed facing each other and sealed with cellophane adhesive tape. Control was kept without inoculation of rhizospheric isolate. Procedure repeated for efficient rhizospheric isolate in triplicates. All the plates were incubated at 28 ^{0}C for 6 days. The production of volatile metabolites was then determined by inhibition of *Fusarium oxysporum* f. sp. *vasinfectum* and percentage of radial growth inhibition was calculated using the Whipps formula [26].

$$PGI = \frac{R_1 - R_2}{R_1} \times 100$$

Where;

R_1 is radial growth of the pathogen alone (a control value) and

R_2 is radial growth pathogen in the direction of the antagonist pathogen + antagonist (an inhibition value)

Detection of antifungal diffusible non-volatile metabolites

The antifungal diffusible non-volatile metabolites were detected by a well diffusion assay [27]. Rhizobacterial isolate, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was grown in Kings B broth at room temperature on a rotary shaker at 150 rpm for 36-48 hours to obtain cell-free culture filtrate. Kings B agar plates were then prepared, and three wells were punched on each plate using a sterile Cork borer (10 mm) and labelled according to the rhizobacteria isolates. The fungal mycelial plugs measuring 5 mm from the leading edge of a 3-day old culture of *Fusarium oxysporum* f. sp. *vasinfectum* were punched and placed at the center of the plate. The rhizobacterial isolate broth was centrifuged at 8000 rpm and filtered using a Millipore Syringe filter 0.22 µ (Hi-media) to prepare cell-free culture filtrate for testing of diffusible metabolites. Each well was loaded separately with 100 µl cell-free culture filtrate aseptically. The control was loaded separately with 100 µl sterile distilled water. All the plates were incubated at room temperature for 5 to 6 days. After the incubation, the plates were observed for the zone of inhibition of radial growth and the percentage inhibition was calculated [26].

$$PI = \frac{R_1 - R_2}{R_1} \times 100$$

Where;

R_1 is radial growth by the pathogen in control plate (a control value)

R_2 is radial growth by the pathogen with antagonist (a test value)

Siderophore analysis

Siderophore production of efficient rhizobacterial isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was tested by a universal Chrome Azurol S (CAS) Plate Assay qualitatively and quantitatively using Liquid CAS Assay.

Qualitative detection of siderophore by CAS plate assay

The siderophore production of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was detected qualitatively by using a universal Chrome Azurol S (CAS) Plate Assay [28] with a slight modification [29]. Initially, all the glassware is rinsed with distilled water and dried. 60.5 mg of CAS was weighed accurately and dissolved in 50 ml of distilled water and to this add 10 ml of iron solution (1 mM $FeCl_3.6H_2O$, in 10 mM HCl). 72.9 mg of Hexa decyl tri methyl-ammonium bromide (HDTMA) dissolved in 40 ml of distilled water. The CAS and Iron solution mixture was slowly added to 40 ml of HDTMA with constant stirring to obtain a dark blue color. The basal medium contains 30.24 g Pipes, and 12 g of a 50% (w/w) NaOH to raise the pH to the pKa of Pipes (6.8) and 15 g Agar agar powder in 750 ml of distilled water. All the contents were separately sterilized by autoclaving at 121°C for 15 min. After cooling to 50°C to basal medium 750 ml add 100 ml of the CAS-Fe-HDTMA mixture along with the glass wall and agitate with enough care to avoid foaming.

The qualitative detection of siderophore was conducted by preparing Petri plates using 30 ml of Kings B agar medium to cultivate rhizospheric isolates. Once the medium solidified, it was divided into two equal parts using a sterile surgical blade, with one part being replaced by CAS blue agar (15 ml) and allowed to solidify. The half medium containing Kings B agar was then inoculated with a 24-hour-old rhizospheric isolate, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 near the border of the two mediums. The CAS agar plate that was not inoculated with bacterial culture served as a control. The plates were then incubated at room temperature for a period of 15 days. The change in color of the CAS agar from blue to orange, purple, or dark purplish-red indicated the production of siderophores.

Quantitative detection of siderophore by liquid CAS assay

The quantitative siderophore production was detected using a modified succinate medium based on the method described by Meyer and Abdallah [30]. The medium consisted of the following components per liter: succinic acid (4g), K_2HPO_4 (6g), KH_2PO_4 (3g), $(NH_4)_2SO_4$ (1g), $MgSO_4$ (0.2g), and pH adjusted to 7.0. To initiate the experiment, 0.1 ml of inoculum of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was inoculated into a 250 ml Erlenmeyer flask containing the Succinate medium. The flask was then placed on a rotary shaker incubator and incubated at room temperature for a duration of 72 hours. The supernatant was harvested after 24, 36, 48, 60, and 72 hours and centrifuged at 10,000 rpm in cooling centrifuge at 4°C for 10 min to obtain cell free culture filtrate.

According to Payne [31], the quantification of siderophore production was carried out by mixing 0.5 ml of cell-free culture filtrate with 0.5 ml of CAS solution. To establish a reference, an un-inoculated succinate medium was used. The optical density of both the test and reference samples was measured at 630 nm, and the percentage of siderophore units in the culture filtrate was determined using the provided formula:

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

Where;
Ar = Absorbance of reference at 630 nm
As = Absorbance of test sample at 630 nm

Analysis of cell wall degrading enzymes

The efficient rhizospheric isolates were also screened for cell wall degrading enzymes such as chitinase and protease.

Detection of chitinase enzyme production

In order to assess the production of the chitinase enzyme of an efficient rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was determined by using colloidal chitin agar [32] amended with 0.4% colloidal chitin. The Colloidal Chitin Agar consists of 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g $MgSO_4$, 1g NH_4Cl, 0.5 g NaCl, 0.05 g yeast extract, and 4 g colloidal chitin dissolved in 1000 ml distilled water. The pH of the medium was adjusted to 7.0 and 20 g of agar was added as a solidifying agent. The medium was sterilized at 121°C for 20 min and plates were prepared. A Chitin agar plate was spot inoculated with bacterial suspension and incubated at room temperature for 4 days. The plate was observed for the zone of chitin hydrolysis around the spot inoculated culture.

Protease enzyme assay

The protease production of the efficient rhizobacterial isolates was assessed by spot inoculating the culture of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 on a sterile Skim-Milk Agar plate and allowing it to incubate overnight. The experiment was conducted in triplicate. Following the overnight incubation, the Skim Milk Agar plate was examined for the presence of a halo zone surrounding the rhizobacterial colony, which was then measured in millimeters [33].

Phosphate solubilization assay

In order to assess the ability of growth promotion, the rhizospheric isolate was examined for its capacity to solubilize phosphate using NBRIP agar plates [34]. The NBRIP medium was composed of 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, and 0.1 g $(NH_4)_2SO_4$ in 1000 ml distilled water. The pH of the medium was adjusted to 7.0 using 0.1N HCl. The efficient rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was spot inoculated on the surface of agar plates and incubated at room temperature for 5 days. The colonies exhibiting a clear halo were identified as phosphate solubilizers.

GC-MS analysis of cell free culture filtrate

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of cell free culture filtrate of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was performed in IIT Bombay Powai, Mumbai. The 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, was utilized in conjunction with a VF-5MS (5% phenyl methyl) capillary column measuring 30.0 m × 250 mm × 0.25 µm. Helium was employed as the carrier gas at a rate of 1 µl / min. The column temperature was programmed to begin at 60°C and increase by 5 K/min until reaching 270 °C, where it was held isothermally for 38 minutes. The constituents were identified by comparing their mass spectral data with those found in the National Institute of Standards and Technology (NIST) Library.

RESULTS AND DISCUSSION

Isolation of fusarium wilt phytopathogen

The Fusarium wilt phytopathogen was isolated from an infected Bt cotton stem. After incubation on Potato Dextrose Agar (PDA) plates, phytopathogen produced the whitish mycelial growth characteristics of *Fusarium* sp. (Plate 2).

Plate 2 Isolation of fusarium wilt phytopathogen from infected Bt cotton stem (a) infected Bt cotton plant (b) infected tissue of stem (c) PDA plate inoculated with infected stem tissue (d) isolated fusarium wilt phytopathogen

The macroscopic and microscopic observations of mycelial growth showed boat-shaped macroconidia (slightly tapering apical cells and hooked basal cells 4-celled) microconidia (ellipsoidal and 1-celled) and chlamydospores (globose and usually solitary). Based on these characteristics, the phytopathogen was identified as *Fusarium oxysporum sp*. [35]. The phytopathogen was purified on fresh PDA, Kings B agar plates and also maintained on slants for further use.

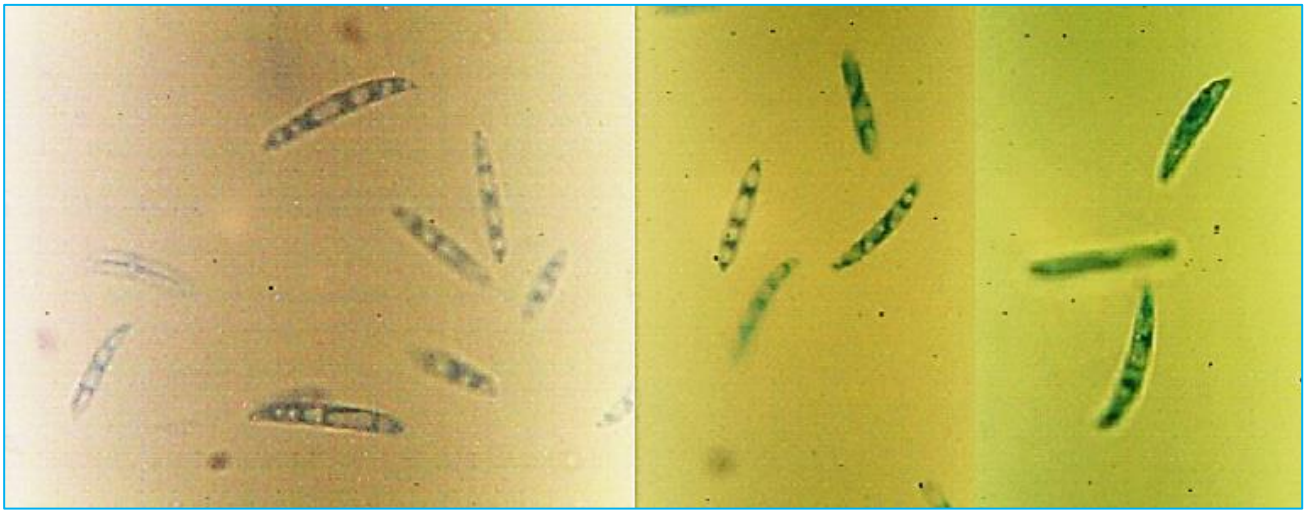

Plate 3 Microscopic study of isolated fusarium wilt phytopathogen of Bt-cotton

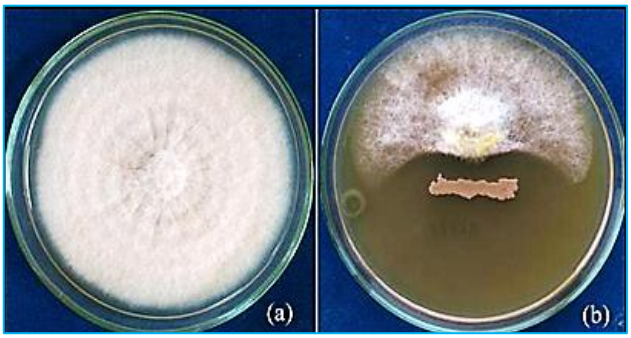

Plate 4 Screening of antifungal activity of rhizospheric isolates against *Fusarium oxysporum* f. sp. *vasinfectum* by dual culture technique (a) Control (b) RLS19

Isolation of rhizospheric bacteria

Forty-five rhizobacteria isolates were isolated and maintained on nutrient agar slants. These isolates were tentatively labeled as RLS1 to RLS45. All the cultures were maintained at 4°C for further use.

In Vitro screening for microbial control agents

Screening against fungal phytopathogen by dual culture method

All the 45 rhizospheric isolates showed variable antifungal activity against the tested phytopathogen. One of the rhizobacterial isolate RLS19, showed significant antifungal potential against the fungal phytopathogen *Fusarium oxysporum* f. sp. *vasinfectum* in a dual culture technique *in vitro*. It was observed that the rhizobacterial isolate RLS19 was found to inhibit the radial mycelial growth of the phytopathogen *Fusarium oxysporum* f. sp. *vasinfectum* whose percent of inhibition was recorded as 89.71% (Plate 4, Table 1).

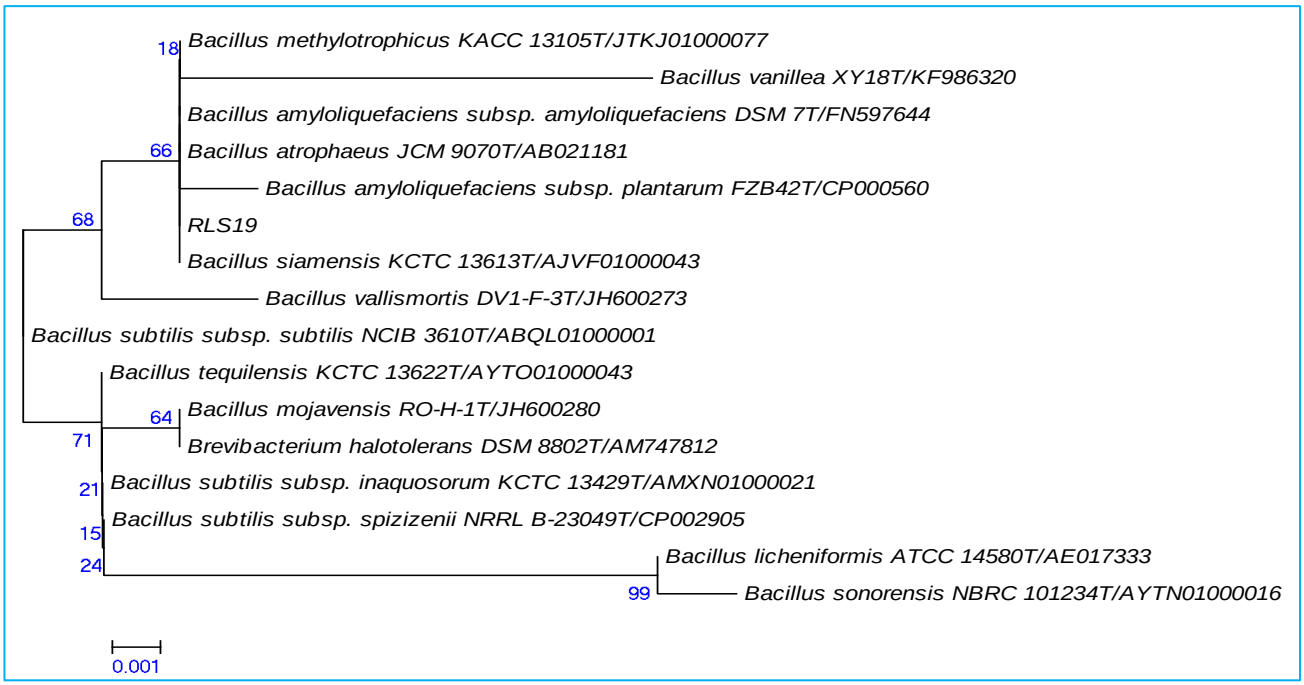

Fig 1 Phylogenetic tree of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

Several researchers working on the bio-management of cotton diseases reported that the endophytic bacterial isolate ME1 belonging to *Pseudomonas sp*. showed the highest inhibitory activity (66.6%) against *Fusarium oxysporum*, a fungal pathogen of cotton using the dual culture technique [36]. According to Murty and Shim [37], the rhizospheric bacterial isolate RZ141 belonging to *Bacillus sp.* was evaluated against the fungal pathogen *Fusarium oxysporum* f. sp. *vasinfectum*

race 4 (Fov4) of Pima cotton (*Gossypium barbadense*) by using the dual culture technique and showed 52% inhibition of mycelial growth. Similarly, three *Bacillus subtilis* strains (LDA-1, LDA-2, and LDA-3) were examined for their potential to act as biocontrol agents against *Fusarium spp*. a fungal phytopathogen of cotton. All *Bacillus* strains inhibited the growth of *Fusarium oxysporum* ICADL2 by more than 70% compared to the control [38]. The highest *in vitro* antifungal activity documented against *Fusarium oxysporum* f. sp. *vasinfectum* of cotton by earlier researchers was 52 to 70%. While in the present research work, the highest antifungal activity recorded against *Fusarium oxysporum* f. sp. *vasinfectum* in the dual culture technique by rhizobacterial isolate RLS19 was 89.71% which is far better. The rhizobacterial isolate RLS19 displayed excellent potential to control *Fusarium sp*., the causative agent of Fusarium wilt in Bt cotton compared to previously recorded research work.

Identification of microbial control agent

The 16S ribosomal RNA gene partial sequencing output of RLS19 was analyzed using the DNA Sequence Analyzer computer software (Applied Biosystems). The sequence was compared with National Center for Biotechnology Information GenBank entries by using the blast algorithm, which showed 100% closest phylogenetic (Fig 1) affiliation with *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19.

Table 1 Screening of antifungal activity of rhizospheric isolates against *Fusarium oxysporum* f. sp. *vasinfectum*

S. No.	Rhizospheric isolate	% of inhibition	Sr. No.	Rhizospheric isolate	% of inhibition	Sr. No.	Rhizospheric isolate	% of inhibition
1	RLS1	0.00	16	RLS16	47.70	31	RLS31	12.50
2	RLS2	56.62	17	RLS17	0.00	32	RLS32	6.45
3	RLS3	61.62	18	RLS18	52.86	33	RLS33	35.48
4	RLS4	0.00	**19**	**RLS19**	**89.71**	34	RLS34	21.88
5	RLS5	47.06	20	RLS20	28.57	35	RLS35	19.35
6	RLS6	25.71	21	RLS21	28.48	36	RLS36	20.00
7	RLS7	37.14	22	RLS22	21.21	37	RLS37	0.00
8	RLS8	29.03	23	RLS23	54.55	38	RLS38	6.90
9	RLS9	22.58	24	RLS24	12.50	39	RLS39	3.57
10	RLS10	3.23	25	RLS25	3.13	40	RLS40	0.00
11	RLS11	15.63	26	RLS26	41.38	41	RLS41	6.45
12	RLS12	0.00	27	RLS27	18.75	42	RLS42	9.38
13	RLS13	13.33	28	RLS28	9.68	43	RLS43	13.89
14	RLS14	11.54	29	RLS29	27.27	44	RLS44	16.67
15	RLS15	21.43	30	RLS30	27.27	45	RLS45	6.90

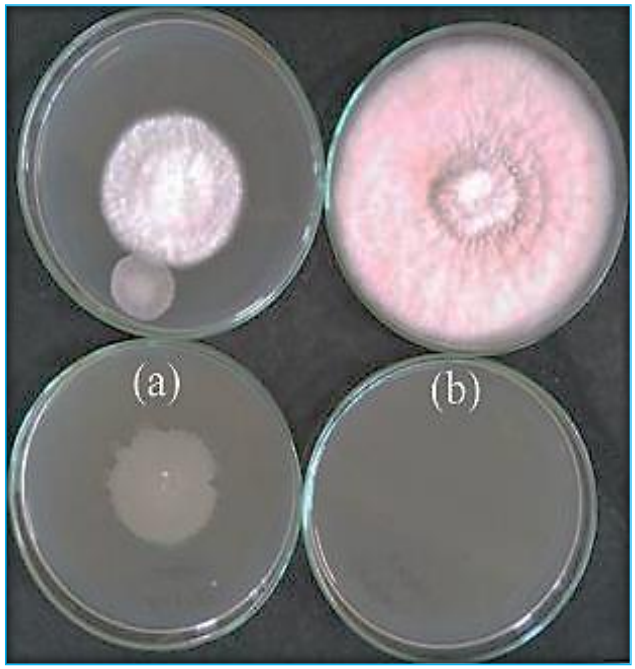

Plate 5 Production of volatile metabolites by rhizospheric isolates against *Fusarium oxysporum* f. sp. *vasinfectum* by Double Plate Assay Method (a) Test (b) Control

In vitro mechanism of microbial control agent

Detection of volatile metabolites

The rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 produced volatile metabolites in a closed environment, which inhibited the phytopathogen *Fusarium oxysporum* f. sp. *vasinfectum* (61.29%) in the double plate method (Plate 5, Table 2).

Table 2 Production of volatile metabolites by rhizospheric isolates against the fungal phytopathogen by double plate assay method

Rhizospheric isolate	% of Inhibition *Fusarium oxysporum* f. sp. *vasinfectum*
Bacillus amyloliquefaciens subsp. *amyloliquefaciens* RLS19	61.29

The volatile secondary metabolites, such as ammonia [39] and HCN [40] produced by rhizospheric bacteria have been reported to play an important role in the biocontrol of phytopathogen. The *Pseudomonas aeruginosa* strain BA5 exhibited the production of volatile antifungal compounds, as demonstrated by the growth suppression of Foc in sealed Petri dishes. The radial expansion of mycelium was notably inhibited (31.11%) in comparison to the control [41]. According to Montalvão *et al.* [42], the antagonistic effect of two *Bacillus* strains, namely S1823 and S2536, against *Fusarium oxysporum* f. sp. *Vasinfectum* fungal pathogen causing Fusarium wilt of cotton by double plate technique remained consistent throughout the assay period, from the fifth day until the final day. The control percentages for S1823 and S2536 were reported as 11.51% and 15.51%, respectively. Also, the study conducted by Murty and Shim [37] showed that *Bacillus* strain Rz141 exhibited the capability to generate secondary volatile organic compounds that significantly influenced the growth and virulence of *F. oxysporum* f. sp. *Vasinfectum* FOV4. Additionally, the growth of FOV4 was observed to decrease by 24% due to the presence of these VOCs. The growth of *F. oxysporum* was significantly hindered by the VOCs emitted from the *Xenorhabdus indica* strain AB After 5 days of

cultivation, the fungal growth was reduced by approximately 60% when compared to the untreated control [43].

Our investigation utilizing the rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 against *Fusarium oxysporum* f. sp. *vasinfectum* has yielded superior results compared to the previously mentioned outcomes. While the inhibition observed due to volatile metabolites for the tested phytopathogen, *Fusarium oxysporum sp*. varied between 15-60%, our findings demonstrated an impressive inhibition rate of 61.29% compared to the previous findings and played an important role in controlling various phytopathogens.

Detection of antifungal diffusible non-volatile metabolites

The Kings B agar plates were inoculated with a 5 mm mycelial disc at the center, and the agar well was inoculated with the cell-free culture filtrate of the rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19, inhibited the mycelial growth of *Fusarium oxysporum* f. sp. *vasinfectum*, a phytopathogen responsible for Fusarium wilt of Bt cotton. The inhibition was measured to be 53.13% of the mycelial growth, indicating the secretion of non-volatile diffusible antifungal metabolites in the medium (Plate 6, Table 3).

Table 3 Production of diffusible non-volatile antifungal metabolites by rhizospheric isolate against fungal phytopathogens by agar well diffusion method

Rhizospheric isolate	% of inhibition *Fusarium oxysporum* f. sp. *vasinfectum*
Bacillus amyloliquefaciens subsp. *amyloliquefaciens* RLS19	53.13

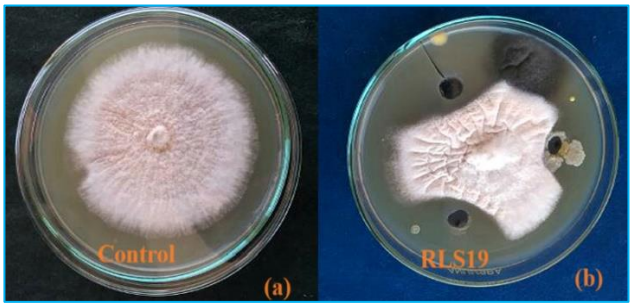

Plate 6 Production of diffusible non-volatile antifungal metabolites of rhizospheric isolates against *Fusarium oxysporum* f. sp. *vasinfectum* by agar well diffusion method (a) Control, (b) *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

Abdallah *et al.* [44], reported that cell free culture filtrate of *Bacillus tequilensis* SV39 in 4 days-old cultures inhibited the growth of *F. oxysporum* f. sp. *lycopersici* by 47.8% whereas 38.7, 38.5, 12.6, and 2% were recorded using filtrates extracted after 3, 7, 2 and 1 day (s) of incubation respectively. Also, the highest reduction (37.9, 45.6, 59.5, and 39.8%) of *F. oxysporum* f. sp. *lycopersici* mycelial growth was recorded using the filtrate of *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 from 2, 3, 4, and 7 days old cultures respectively, as compared to the control by using an agar well diffusion assay. The growth of Foc was significantly inhibited by 25% and 23.46% when exposed to the variable concentrations of diffusible metabolites 1:10 and 1:100 of cell-free culture filtrate of *Bacillus amyloliquefaciens* subsp. *plantarum* strain Ht1-1 respectively, compared to the control [45]. *Bacillus subtilis* strain F62 exhibited varying levels 35.4 to 63.6% of antagonistic activity by agar well diffusion assay against *Fusarium sp.*, a pathogen of Viticulture through the release of diffusible compounds [46]. Similarly, Rafanomezantsoa *et al.* [47], investigated the antifungal activity of *Bacillus spp*. strain RFP63. After 7 days of incubation, it exhibited the most significant inhibition (44.5%) against *Fusarium oxysporum* f. sp. *lycopersici.*

Earlier studies have shown that the highest antifungal activity recorded against *Fusarium oxysporum* f. sp. *vasinfectum* using diffusible non-volatile metabolites, within a range of 25.0 to 63.6%. In the present work, the rhizobacterial isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 exhibited the highest antifungal activity, recording 53.13% inhibition against *Fusarium oxysporum* f. sp. *vasinfectum* using the agar well diffusion method. This particular rhizobacterial isolate RLS19 showed great potential in controlling Fusarium wilt in Bt cotton, surpassing previous research findings except for the work conducted by Russi *et al.* [46]. Based on these reports, it is evident that rhizobacterial isolates, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19, also efficiently secrete diffusible non-volatile metabolites that inhibit the growth of *Fusarium oxysporum* f. sp. *vasinfectum*. Further analysis of these metabolites is necessary.

Table 4 Detection of Siderophore production qualitatively by rhizospheric isolates using modified CAS agar plate method

Rhizobacterial isolate	Colour of zone
Bacillus amyloliquefaciens subsp. *Amyloliquefaciens* RLS19	Purple

Qualitative detection of siderophore by CAS plate method

The rhizobacterial isolate, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19, was inoculated on modified CAS blue agar plates and incubated at room temperature for 15 days, resulting in growth and a noticeable color change from blue to purple. This change in color of the CAS blue agar medium is attributed to the secretion and diffusion of siderophore compounds in the medium (Plate 7, Table 4).

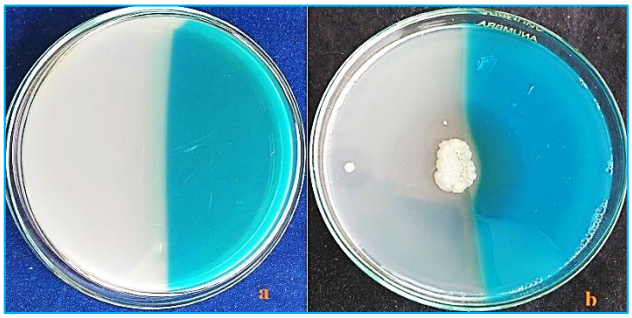

Plate 7 Detection of Siderophore production qualitatively by rhizospheric isolates using modified CAS agar plate method (a) Control (b) *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

Several studies have documented the ability of *Bacillus* species to produce siderophores, which can promote plant growth and act as antagonists against plant pathogens [48-49]. In a study conducted by Shaikh *et al.* [50], six endophytic bacterial isolates exhibited a positive response in terms of siderophore production on CAS agar plates. This response was characterized by a color change in the medium, transforming from blue to a yellowish-orange halo zone surrounding the inoculated colony, indicating siderophore production. These findings were consistent with the observations made by Nithyapriya *et al.* [51] and Jan *et al.* [52] who also noticed a distinct yellowish-orange and purple halo zone on CAS agar

media while studying *Bacillus subtilis* strains LSBS2 and FJ3, respectively.

All of these studies suggest that rhizospheric isolates, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19, efficiently produce siderophore on a modified CAS agar plate.

Quantitative detection of siderophore by liquid CAS assay

The succinate broth inoculated with the rhizobacterial isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 and incubated on a rotary shaker showed yellow colored growth, which indicates the production of siderophore (Plate 8).

Table 5 Detection of siderophore production quantitatively by rhizospheric isolate by using liquid CAS assay

Rhizospheric isolate	Siderophore units (%)				
	24 h	36 h	48 h	60 h	72 h
Bacillus amyloliquefaciens subsp. *amyloliquefaciens* RLS19	24.34	76.28	88.14	93.20	87.93

Table 6 Detection of Chitinase enzyme production of rhizospheric isolate by plate assay

Rhizospheric isolates	Total zone of hydrolysis including colony (mm)	Diameter of colony (mm)	Diameter zone of chitin hydrolysis (mm)
Bacillus amyloliquefaciens subsp. *amyloliquefaciens* RLS19	31	17	14

0.5 ml of the cell-free culture filtrate of *Bacillus amyloliquefaciens* subsp. a*myloliquefaciens* RLS19 was mixed with 0.5 ml of CAS solution; a color change from blue to orange (Plate 9) indicates the production of siderophores. The absorbance of the test and reference samples is measured at 630 nm using a spectrophotometer and recorded in (Table 5). The percentage of siderophore units produced by an efficient rhizospheric bacterial isolate in the cell-free culture filtrate is calculated to shows the highest (93.20%) siderophore units. The optimal time for harvesting siderophores from the fermentation broth is between 60-72 hours. With prolonged incubation, there is a decrease in the percentage of siderophore units, which is attributed to the degradation of the produced siderophores.

Plate 8 Growth of siderophore producing *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 in succinate medium

Plate 9 Detection of siderophore production quantitatively by rhizospheric isolates using liquid CAS assay (a) Control (b) *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

The *Bacillus subtilis* strain CTS-G24 has shown siderophore production of 64% and 59% in succinate and nutrient media, respectively [53]. Similarly, the quantitative siderophore production of six isolates was studied using the liquid CAS assay, which showed that EB11 (92.58%), EB4 (88.94%), EB3 (86.02%), EB5 (85.41%), EB8 (84.44%), and EB9 (80.26%) produced siderophore units within 48 hours compared to the control [50]. Additionally, *Bacillus velezensis* strain RC116 exhibited siderophore production that gradually increased with incubation culture time, reaching the highest level of 64% siderophore units at 6 days [54]. In our study, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 produced 93.20% siderophore units after 60 hours, surpassing the results documented by previous researchers. This has proven the role played by the siderophore produced by *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 in reducing the growth of *Fusarium oxysporum* f. sp. *Vasinfectum.*

Spectrophotometric analysis of siderophore

The cell free culture filtrate analysis of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 using UV-VIS spectrophotometry revealed two absorption peaks at 418 nm and 296.50 nm (Fig 2).

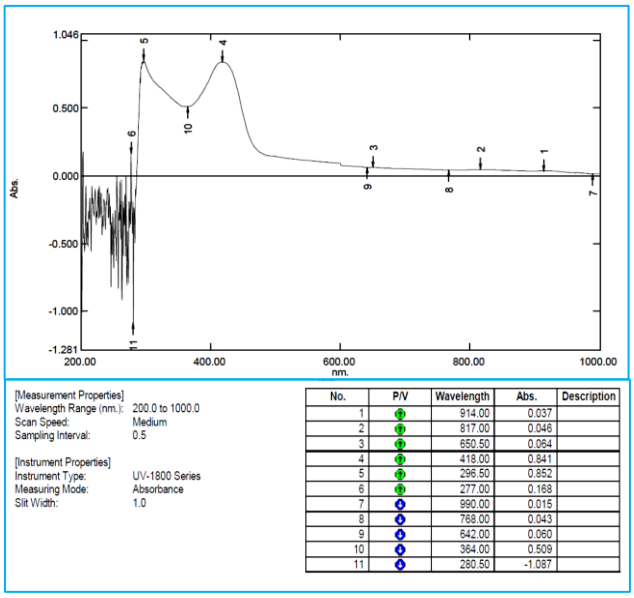

Fig 2 UV-VIS absorption spectra of cell free culture filtrate of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

In a study conducted by Qing and Jian [55], it was observed that the cell free culture of *Bacillus subtilis* QM3 exhibited a strong absorption peak at 380 nm for optimization, while the un-optimized culture showed a peak at 420 nm. Furthermore, the peak area for optimization was significantly larger than that of un-optimization. Patil *et al.* [53], reported that *B. subtilis* produces siderophores in succinate medium, and spectrophotometric analysis of the cell free culture filtrate showed a peak between 420 nm and 450 nm, indicating the presence of ferrate hydroxamate siderophores. Similarly, for *Pseudomoas aeruginosa* FP6, spectrophotometric analysis of the culture in standard succinate medium revealed a peak

between 420 nm and 450 nm, indicating the presence of a ferric hydroxamate siderophore [56]. In our studies too, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 showed approximately the same type of peak between 400 to 420 nm indicating the presence of a ferric hydroxamate siderophore [56].

Analysis of cell wall degrading enzymes

Detection of chitinase enzyme

The chitinase production ability of the rhizobacterial isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 was tested on a chitin agar plate, which showed the zone of clearance around the colony. The zone of clearance around the colony indicated the presence of the chitin hydrolysis enzyme. The chitinase activity was measured in mm (Table 6).

The chitnase enzyme production ability of antagonist *Bacillus subtilis* BCC 6327 on colloidal chitin agar medium was studied and found to secrete chitinase enzyme. The chitinase enzyme has been proven to inhibit the growth and spore germination of the isolated aflatoxigenic fungus [57]. Also, *Bacillus licheniformis* and *Bacillus subtilis* exhibited significant results in terms of chitinase production. *Bacillus licheniformis* produced a clear zone of 14 mm, while *Bacillus subtilis* produced a clear zone of 11 mm [58]. The bacterial strains, EB5 (1.9 mm) and EB3 (1.1 mm), showed solubilization zones on the chitin agar plate after 7 days [50]. Furthermore, Zain *et al.* [36], discovered that four antagonistic bacterial strains, MRh11, MRh21, MRh22, and MRh24, were capable of producing the chitinase enzyme in a solid medium that contained chitin as the sole carbon source.

In the present research, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 demonstrated a clear zone of chitin hydrolysis measuring 14 mm, surpassing the findings made by previous researchers. The chitinase enzyme produced by this isolate from the rhizosphere confirmed its significant role in controlling the phytopathogens of cotton, specifically *Fusarium oxysporum* f. sp. *Vasinfectum.*

Table 7 Production of protease enzyme by rhizobacterial isolates

Rhizospheric isolates	Total zone of hydrolysis including colony (mm)	Diameter of colony (mm)	Diameter Zone of Protease enzyme hydrolysis (mm)
Bacillus amyloliquefaciens subsp. *amyloliquefaciens RLS19*	17	9	8

Plate 10 Production of protease enzyme on skim-milk agar by rhizospheric isolates *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

Detection of protease enzyme

The rhizobacterial isolate, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19, produced the clear zone around the inoculated colony on skim-milk agar (Plate 10, Table 7). The clear halo zone around the colony indicates the production of extracellular protease enzyme.

Shaikh *et al.* [50] documented that those six isolates, namely EB15 (16.60 mm), EB12 (14.00 mm), EB11 (13.60 mm), EB1 (12.60 mm), and EB4 (11.80 mm), showed the production of the protease enzyme. Similarly, the activity of proteases was observed in six strains, namely MRh1, MRh6, MRh20, MRh22, MRh42, and MRp1, suggesting their potential role in antagonizing the Fusarium pathogen [36]. Additionally, the production of protease enzyme was confirmed in *Bacillus velezensis* strain RC116 and *Bacillus subtilis* strain FJ3 on skim-milk agar [52-54].

All these reports support the idea that the protease enzyme produced by *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 measured 8 mm diameter zone of hydrolysis and displayed an important role in inhibiting the growth of the phytopathogen *Fusarium oxysporum* f. sp. *vasinfectum*.

Phosphate solubilization assay

The rhizobacterial strain *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 showed phosphate solubilization activity, as evidenced by the presence of a clear halo surrounding its colonies (Plate 11). The diameter of the clear halo around the bacterial growth measured 6 mm (Table 8).

Table 8 Phosphate solubilization assay for rhizobacterial isolates by using NBRIP medium

Rhizospheric isolates	Total zone of phosphate solubilization including colony (mm)	Diameter of colony (mm)	Diameter clear halo of phosphate solubilization (mm)
Bacillus amyloliquefaciens subsp. *amyloliquefaciens* RLS19	18	12	6

The phosphate solubilization of endophytic isolate EB9 obtained from cotton leaf exhibited significant qualitative phosphate solubilization. This was evident through the formation of a halo zone on Pikovskaya's agar medium, with a diameter of 4.0 mm [50]. Similarly, Zain *et al.* [36], discovered that eight bacterial strains, namely MRh1, MRh4, MRh6, MRh17, MRh31, MRh37, ME1, and ME4, were also capable of solubilizing phosphate, as indicated by the formation of halo zones on Pikovskaya's agar medium. Nithyapriya *et al.* [51], observed a clear zone of phosphate solubilization around the colonies of *Bacillus subtilis* strain LSBS2 on Pikovskaya agar, with a maximum zone of 0.5 mm diameter. Furthermore, Jan *et al.* (52], found that the *Bacillus subtilis* strain FJ3 exhibited the ability to solubilize tricalcium phosphate in large quantities, as

evidenced by the halo zone formation around the colony. All these reports gave evidence that the *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19, converted insoluble phosphate to soluble phosphate which played an important role in the growth promotion of the Bt cotton plant.

GC-MS analysis of cell free culture filtrate

The rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens RLS19* showed 6 peaks in the GC-MS chromatogram (Fig 3). Based on peak abundance, three peaks of the chromatogram were compared to mass spectral data from NIST (2007 Library). The GC-MS spectral results compared with the library search successfully identified three compounds. The active principles with their retention time (RT), molecular formula, molecular weight (MW), percentage of similarity and biological activity were recorded (Table 9). The GC-MS analysis of cell free culture filtrate rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 detected three compounds namely Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro- ($C_7H_{10}N_2O_2$); 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- ($C_{12}H_{22}N_2O_2$); and Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]- ($C_{11}H_{18}N_2O_2$) (Fig 4-6).

Table 9 Biochemical compounds identified in cell free culture filtrate of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

Retention time	Compound	Formula	Mol. Wt. g/mol	Similarity (%)	Biological activity	Reference
16.73	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	76.3	Anti-microbial Algicidal	[59-60]
25.07	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	$C_{12}H_{22}N_2O_2$	226	62.3	Antifungal	[61-62]
25.42	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-	$C_{11}H_{18}N_2O_2$	210	23.4	Anti-microbial Anti-inflammatory	[63-64]

Plate 11 Phosphate solubilization assay for rhizobacterial isolates *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 by using NBRIP medium

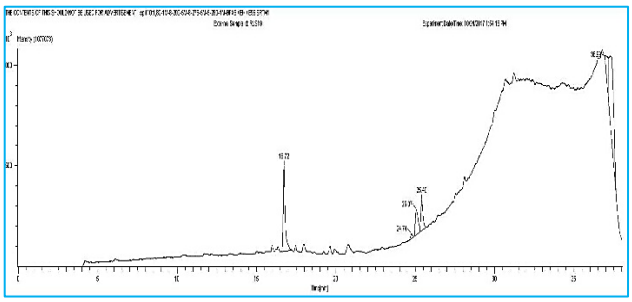

Fig 3 GC-MS chromatograms of cell free culture filtrate of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19

The GC–MS spectra obtained for the extracted fraction of *Streptomyces sp*. VITMK1 was identified as pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methyl propyl)- which exhibited potent antibacterial and antifungal properties [65]. Kiran *et al.* [60], reported that pyrrolo[1,2-a] pyrazine1,4-dione, hexahydro- is a potent antioxidant agent isolated from *Bacillus tequilensis* MSI45 exhibiting antimicrobial and antifungal activity against various pathogenic bacteria and fungi. The ethyl acetate fraction of *P. aeuriginosa* (MPP EB 4) revealed the presence of two compounds, namely Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-, and 2,5-Piperazinedione, 3,6-bis(2-methyl propyl)-, with retention times of 12.68 and 19.36, respectively [66]. Awan *et al.* [62], reported that the n-hexane fraction of the *Bacillus subtilis* strain (BS-01) produced extracellular metabolites Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2- methylpropyl)- and dichloromethane fractions of extracellular metabolites produced Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- detected by GC-MS analysis. All these reports suggest that the rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 produced volatile secondary metabolites having antibacterial and antifungal potential, which aid in controlling the fugal pathogen *Fusarium oxysporum* f. sp. *vasinfectum.*

CONCLUSION

The overall findings indicate that *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 effectively suppressed 89.71% of the mycelial growth of *Fusarium oxysporum* f. sp. *vasinfectum*, a phytopathogen responsible for Fusarium wilt disease in Bt cotton, as compared to the control in a dual culture assay. During the investigation of its biocontrol mechanism, the strain proved its ability to produce both volatile and non-volatile secondary metabolites, siderophore, chitinase enzyme, protease enzyme, and phosphate solubilization. Gas Chromatography-Mass Spectrometry (GCMS) analysis of the cell-free culture filtrate revealed the presence of volatile organic bioactive compounds such as pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- ($C_7H_{10}N_2O_2$); 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- ($C_{12}H_{22}N_2O_2$); and acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]- ($C_{11}H_{18}N_2O_2$) which contributed to the inhibition of fungal phytopathogen's mycelial growth. Notably, this strain not only inhibits the growth of the fungal phytopathogen but also promotes the growth of Bt cotton crops. With its multiple potentialities, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 holds great promise as an excellent candidate for biocontrol and plant growth promotion in managing Fusarium wilt in Bt cotton, serving as an alternative to chemical fungicides. In the forthcoming investigation, it is imperative to analyze the identification of diffusible secondary metabolites generated by this particular

strain using Liquid Chromatography-Mass Spectrometry (LC-MS).

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