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In vitro biocontrol scenarios of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 in response to *Alternaria macrospora*, an *Alternaria* leaf spot phytopathogen of Bt cotton

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ABSTRACT

Alternaria macrospora (Zimm.) is most critical phytopathogen causing *Alternaria* leaf spot disease of transgenic cotton having regional, national, and international significance. In the present research work, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 previously isolated from rhizospheric niches of healthy of Bt cotton plant was screened against *A. macrospora* by dual culture technique. *Bacillus* sp. significantly killed the radial mycelial growth of *A. macrospora* that is, 98.68%. On finding the mechanism, it was revealed that *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 produced diffusible non-volatile secondary metabolites, siderophore, and chitinase enzyme which make contributions to inhibit the mycelial growth of *A. macrospora* during dual culture screening. In addition, the gas chromatography-mass spectrometry evaluation of crude extract discovered the presence of three bioactive compounds specifically acetamide, N-methyl-N-[4-(3-hydroxypyrrrolidinyl)-2-butenyl]-, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, and 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- with the outstanding antimicrobial antibacterial and antifungal activity which contributed in the inhibition of *A. macrospora*.

1. INTRODUCTION

Economically cotton is most crucial cash crop cultivated in all of the cotton growing areas of India and different countries of the world. *Alternaria* leaf spot disease is worldwide in distribution and mentioned [1] a major constraint in cotton production. The blended activity of *Alternaria macrospora* and *Alternaria alternate* results in the development of *Alternaria* leaf spot disease of cotton [2]. The transgenic and non-transgenic cotton hybrid varieties are equally susceptible to foliar diseases such as bacterial blight, *Alternaria* leaf spot, and gray mildew. Now a days in India, majority of the farmers cultivate Bt cotton instead of non-Bt cotton. This has created tremendous competitiveness in hybrid seed production as the production area for hybrid cotton has remained stagnant. In India, *Alternaria* leaf spot disease of cotton is common and reported variable percent disease incidence from South zones (10-35%), North zone (7.33-31.83%), and Central zone (9.66-31.30%). However, some environmental factors such as abiotic and biotic factors interfere the original productivity of crop. Among the biotic factors, several fungal

phytopathogens are responsible to cause different types of foliar diseases and serve as major constraints in crop production. The yield loss up to 33.07% was recorded due to *Alternaria* leaf spot of cotton [3]. Conventionally, the farmers use agrochemicals to protect the crop and increase the crop yield. The tremendous use of agrochemicals leads to develop pest resistance, kills beneficial microflora of soil as well as pollution of soil.

Considering the alarming situation of *Alternaria* leaf spot disease, to avoid yield losses and hazardous effect of agrochemicals, the plant pathologist diverted their research to find out the naturally occurring rhizospheric microflora as biological control agents. The biocontrol is a bio-based, eco-friendly approach which offers a realistic and cost-effective alternative to chemical control for the management of plant pathogens [4]. There are some evidences in which antagonistic bacteria and fungi used as biocontrol agents, such as *Trichoderma* sp. [5,6] *Pseudomonas* sp. [7,8], and *Bacillus* sp. [9-11] isolated from rhizosphere niches of different crop and found effective in controlling plant pathogens.

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To find the solution to control *A. macrospora*, a leaf spot pathogen of Bt cotton following objectives was set: i) To screen *Bacillus* sp. against *A. macrospora* by dual culture technique and ii) to find the mechanism of biocontrol agent involved in controlling *A. macrospora* such as production of non-volatile diffusible secondary metabolites, siderophore production, chitinase activity, and evaluation of crude extract by gas chromatography-mass spectrometry (GC-MS) for volatile metabolites.

2. MATERIALS AND METHODS

2.1 Chemicals, *Alternaria* leaf Spot Pathogen of Bt Cotton, and *Bacillus* sp.

The chemicals used in this study were procured from M/S HiMedia, Mumbai, and CDH analytical/guaranteed grade chemicals and glass-distilled water were used. *A. macrospora*, *Alternaria* leaf spot pathogen of Bt cotton, and *Bacillus* spp., namely, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 (Accession No. MN749553) isolated from infected Bt cotton leaves and rhizospheric soil samples of healthy Bt cotton, respectively, during the previous research work in the Department of Microbiology, Sant Tukaram College of Arts and Science, Parbhani, were used in the present research work.

2.2. Antifungal Action by Dual Culture Technique

B. amyloliquefaciens subsp. *amyloliquefaciens* strain RLS19 was tested using modified dual culture technique of Skidmore and Dickinson [12] for antifungal activity against *A. macrospora*. In this modified method, the PDA plate was inoculated with 5 mm fungal disc (5-day-old culture) 10 mm away from the center of the agar plate. Similarly, 24 h old *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 culture was streaked in opposite direction, 10 mm away from the center of the agar plate to maintain the equidistance of phytopathogen and antagonist from the center of the agar plate. This dual culture experiment was performed in triplicates. A control plate was maintained without streaking bacterial culture. Both the test and control plates were incubated at room temperature for 6 days.

The antifungal activity was calculated by means of measuring the inhibition of radial mycelial growth of fungal pathogen in test as compared to control in terms of percentage inhibition of radial growth (PIRG).

$$\% \text{PIRG} = \frac{R_1 - R_2}{R_1} \times 100 \quad (1)$$

Where,

R1 – radius of the fungal phytopathogen colony in the control plate

R2 – radius of the fungal phytopathogen colony in the direction of the antagonist colony.

2.3. In Vitro Mechanism of Microbial Control Agent

To search the biocontrol mechanism of microbial control agent, *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 was tested to produce non-volatile diffusible metabolites, siderophore production, cell wall degrading enzymes, as well as volatile organic bioactive compounds with the help of GC-MS analysis.

2.4. Determination of Antifungal Diffusible Non-volatile Metabolites

The diffusible antifungal non-volatile metabolite was tested by agar well diffusion assay [13]. The King's B broth inoculated with

B. amyloliquefaciens subsp. *amyloliquefaciens* strain RLS19 and incubated on a rotary shaker at 150 rpm at room temperature for 48 h to get cell-free culture filtrate. On sterile potato dextrose agar plates, a well was punched 1 cm apart from the center on 90 mm diameter Petri plate using sterile cork borer (10 mm). Five millimeters mycelial disc of *A. macrospora* from leading edge of a 3-day-old culture was punched and kept 1 cm apart from the center and opposite to well in the PDA plate.

Cell-free culture supernatant was prepared from previously inoculated King's B broth culture by centrifugation at 10,000 rpm using cooling centrifuge at 4°C for 10 min. Cell-free culture filtrate was obtained by filtering cell-free culture supernatant using Millipore Syringe Filter 0.22 µ (HiMedia). In aseptic condition, the agar well was loaded with 100 µl cell-free culture filtrate. The control was maintained without loading of cell-free culture filtrate. The inoculated plate was immediately kept in refrigerator to allow diffusion for 2–3 min. Both the plates control as well test were incubated at room temperature for 5–6 days. After the incubation, the plates were observed for the inhibition of radial growth of fungal culture and the % inhibition was calculated [14].

$$PI = \frac{R_1 - R_2}{R_1} \times 100 \quad (2)$$

Where,

R1 is radial growth of the pathogen in control plate (a control value)

R2 is radial growth of the pathogen with antagonist (a test value).

2.5. Siderophore Production

The siderophore production was studied using modified succinate medium [15] consisting (g/l) succinic acid (4), K_2HPO_4 (6), KH_2PO_4 (3), $(NH_4)_2SO_4$ (1), $MgSO_4$ (0.2), and pH (7.0). 0.1 ml of inoculum of *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 was inoculated in 250 ml conical flask containing succinate broth and then incubated at room temperature for 72 h on a rotary shaking incubator.

2.5.1. Qualitative and quantitative estimation of siderophore by liquid CAS assay

During incubation, 2 ml of inoculated succinate broth was aseptically removed in 2.5 ml sterile microcentrifuge tube at regular interval of time. The harvested broth samples were centrifuged at 10,000 rpm for 10 min at 4°C to obtain cell-free culture filtrate. The siderophore production was quantitatively determined using Payne [16] method in which 0.5 ml of CAS solution added with 0.5 ml of cell-free culture filtrate. A reference was prepared using the un-inoculated sterile succinate medium. The optical density of both the test and reference was read at 630 nm. The % siderophore units in the culture filtrate were calculated using the following formula.

$$\% \text{Siderophore Units} = \frac{Ar - As}{Ar} \times 100 \quad (3)$$

Where,

Ar = Absorbance of reference at 630 nm.

As = Absorbance of test sample at 630 nm.

2.6. Detection of Chitinase Enzyme Activity

The chitinase activity of *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 was tested using colloidal chitin agar [17]. The colloidal chitin agar consists of 6 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g $MgSO_4$, 1 g NH_4Cl , 0.5 g $NaCl$, 0.05 g yeast extract, and 4 g colloidal chitin.

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dissolved in 1000 ml distilled water. The pH of the medium was adjusted to 7.0 and 20 g agar was added as a solidifying agent. The medium was sterilized at 121°C for 20 min and plates were prepared. Chitin agar plate was spot inoculated with bacterial culture and incubated at room temperature for 4 days. The plate was observed for the clear zone of chitin hydrolysis around the spot inoculated culture.

2.7. GC-MS Analysis of Crude Extract

The GC-MS evaluation of cell-free culture filtrate was carried out as per Raut and Hamde [18]. The sterile King's B broth was inoculated with *B. amyloliquefaciens* subsp. *amyloliquefaciens* and incubated on rotary shaking incubator at room temperature for 72 h. After incubation, broth culture was centrifuged at 10,000 rpm at 4°C for 10 min to obtain the crude extract. The GC-MS evaluation of crude extract was performed in IIT Bombay, Powai, Mumbai. The system used with specification GC make version Agilent-7890, with FID detector, Head Space injector, CombiPAL autosampler, and MS make version Jeol AccuTOF-GCV with mass range 10–2000 amu and mass resolution 6000 equipped with VF-5MS (5% phenyl methyl) capillary column of dimensions 30.0 m × 250 µm × 0.25 µm and used helium as carrier gas at 1 µl/min. The column temperature was programmed initially at 60°C followed by an increase of 5 K/min to 270°C and was kept isothermally for 38 min. The constituents were identified using comparison of their mass spectral data with the ones from NIST Library.

3. RESULTS AND DISCUSSION

3.1. Antifungal Action by Dual Culture Technique

B. amyloliquefaciens subsp. *amyloliquefaciens* strain RLS19 was efficient in inhibiting *A. macrospora* in dual culture technique whose % inhibition of radial growth was measured and calculated as 98.08 % (Figure 1 and Table 1). The Chi-square test is applied to data

in Table 1. The Chi-square statistic is 27.249, $P < 0.00001$ and the result is significant at $P < 0.05$.

Earlier researchers had also exploited usefulness of *Bacillus subtilis* a bioagent was evaluated against *A. macrospora* of cotton using dual culture technique and substantially inhibited the mycelial growth (73.66%) of the test fungus *in vitro* [19]. *B. subtilis* strain 1 was evaluated *in vitro* against *Alternaria* leaf spot pathogen of cotton using dual culture technique significantly inhibited the mycelial growth (51.68%) of the test fungus over untreated control [6]. Bioefficacy of three bioagents *Trichoderma harzianum*, *B. subtilis*, and *Pseudomonas fluorescens* tested against *A. macrospora* by dual culture technique and results revealed that *B. subtilis* inhibited 71.06% mycelial growth of *Alternaria macrospora* a phytopathogen of cotton [20]. Similarly, *B. subtilis* subsp. *inaquosorum* RLS52 effectively inhibited the radial growth (94.30%) of *Alternaria* leaf spot pathogen of Bt cotton in dual culture technique [21].

Our rhizobacterial isolate *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 inhibited *A. macrospora* in dual culture up to 98.08%, which was far better than the earlier result obtained by Dalpati et al. [19] and Raut et al. [21].

3.2. Determination of Antifungal Diffusible Non-volatile Metabolites

The PDA plates inoculated with 5 mm mycelial disc and agar well inoculated with cell-free culture filtrate of rhizospheric isolate inhibited the mycelial growth of fungal phytopathogen after 6 days of incubation. The results specify that the rhizospheric isolate, *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 was able to secrete some non-volatile diffusible antifungal secondary metabolites which inhibited the mycelial growth of *A. macrospora* up to 61.29% *Alternaria* leaf spot phytopathogen of Bt cotton [Table 2].

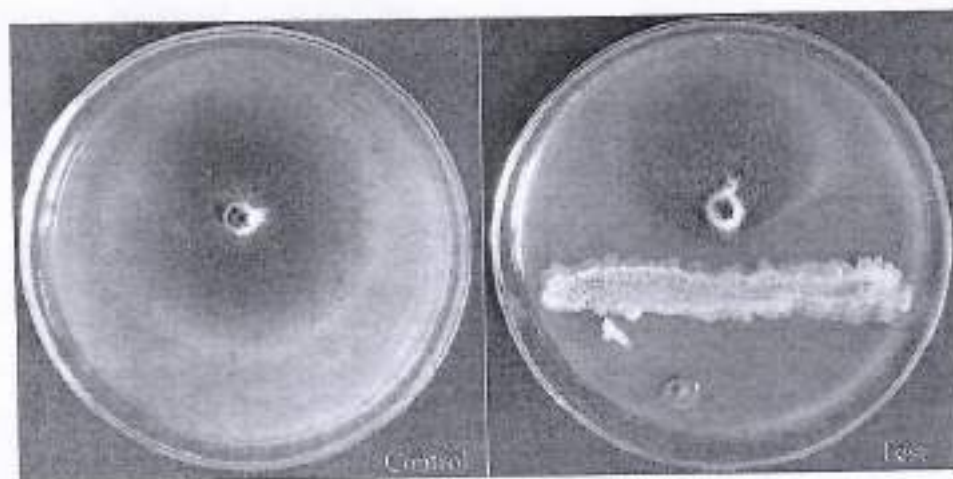


Figure 1: Antifungal action of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 against *Alternaria macrospora* by dual culture technique.

Table 1: Antifungal action of rhizospheric isolate *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 against *Alternaria macrospora* by dual culture technique.

S. No.	Rhizospheric isolate	Radial growth of pathogen in control mm (R1)	Radial growth of pathogen in test mm (R2)	% inhibition of radial growth of pathogen
1	<i>Bacillus amyloliquefaciens</i> subsp. <i>amyloliquefaciens</i> strain RLS19	37±0.81	0.71±0.008	98.08±0.01

±Represent standard deviation of tested sample

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Table 2: Detection of antifungal diffusible non-volatile metabolites by rhizospheric isolates against fungal phytopathogen by agar well diffusion method.

S. No.	Rhizospheric isolate	Radial growth of pathogen in control mm (R1)	Radial growth of pathogen in test mm (R2)	% inhibition of radial growth of pathogen
1	<i>Bacillus amyloliquefaciens</i> subsp. <i>amyloliquefaciens</i> strain RLS19	35±0.81	13.55±0.88	61.29±3.42

±Represents standard deviation of tested sample

The species of *Bacillus* produces lipopeptides as surfactin, iturin, and fengycin had suppressive mechanisms for the phytopathogens which include parasitism, competition, and antibiosis [22]. Similarly, *B. amyloliquefaciens* GA1 produced antibiotics and different secondary metabolites including surfactin, fengycin, iturin A, macrolactin, difficidin, bacillane, and chlorotetaine which contribute in biological management of phytopathogens [23]. *Bacillus velezensis* exhibits genes for synthesizing secondary metabolites such as cyclic lipopeptides and polyketides to induce systemic resistance in plants [24].

The cell-free extract of *B. amyloliquefaciens* CNU14001 strain existed antagonism against phytopathogens which include *S. sclerotiorum*, *Colletotrichum orbiculare*, *Alternaria panax*, *Pyricularia grisea*, *Penicillium digitatum*, and *Botrytis cinerea* [25]. Some researchers applied *Bacillus* sp. to control *Alternaria alternata*, the causative agent of *Alternaria* blight of tomato where biocontrol agent releases diffusible non-volatile metabolites [26]. The cultural filtrate of B11-144 (*B. amyloliquefaciens*) and B11-128 (*B. subtilis*) possessed the most powerful antifungal activity in opposition to three tested *Alternaria* sp. [27].

All these reports were indicative that our rhizospheric isolate, *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 produced diffusible non-volatile secondary metabolites which contributed in inhibition of the mycelial growth of *A. macrospora*. These diffusible non-volatile secondary metabolites needed to be further identified.

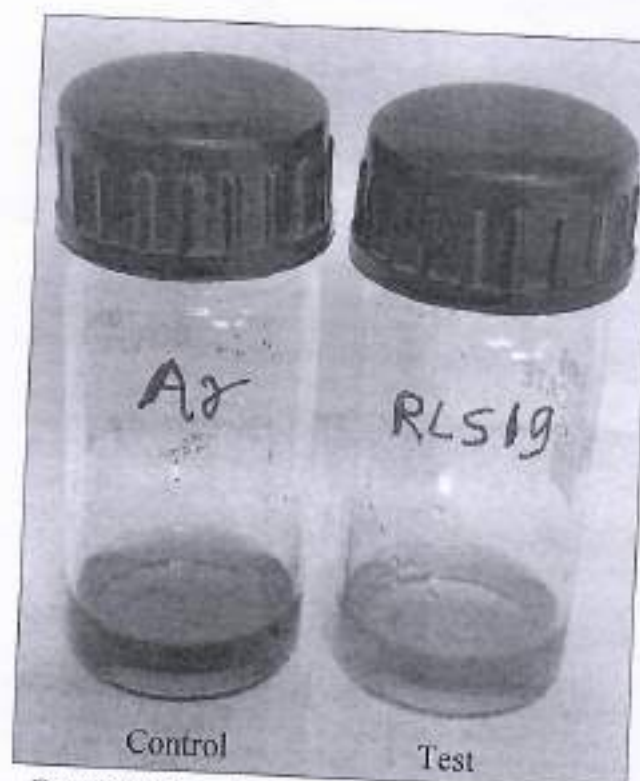
3.3. Qualitative and Quantitative Estimation of Siderophore by Liquid CAS Assay

The fermented succinate broth samples harvested after the specified interval of time (24, 36, 48, 60, and 72 h) and centrifuged at 10,000 rpm in a cooling centrifuge for 10 min to get cell free culture filtrate. In qualitative estimation of siderophore, 0.5 ml cell-free culture filtrate mixed with 0.5 ml of CAS reagent showed the change in color from blue to orange indicates siderophore production (Figure 2).

Similarly for quantitative estimation, the optical density of the test and reference was read at 630 nm using UV spectrophotometer and recorded. The % siderophore unit produced [Table 3] by rhizospheric bacterial isolate in the cell-free culture filtrate was calculated [16]. *B. amyloliquefaciens* subsp. *amyloliquefaciens* RLS19 produced highest siderophore units (93.20%) after 60 h. The subsequent incubation after 60 h there was a slight decrease in % siderophore units and it was due to degradation of produced siderophore.

Some fungi and bacteria are able to produce low-molecular-weight ferric ion chelating compound called siderophore. Members of genus *Bacillus* can secrete a catechol-based siderophore called bacillibactin. The biocontrol agent *B. amyloliquefaciens* GA1 having a gene shown to direct the synthesis the iron siderophore bacillibactin [23]. The genomic analysis of *B. amyloliquefaciens* SQR9 strain identified *dhbE-A* gene which is responsible for the synthesis of bacillibactin and suppresses the different soil-borne fungal phytopathogens [28].

The plant growth-promoting rhizobacterial isolate *B. amyloliquefaciens* strain eks_hnj1 produced 82.35% siderophore units [29]. Furthermore, the siderophore production of *B. subtilis* CTS-Q24 in different media

**Figure 2:** Qualitative estimation of siderophore production by *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 by liquid CAS assay.

such as succinate broth and nutrient broth was studied it was reveals that strain was able to produce 64% and 59% siderophore, respectively, and participates in inhibiting the fungal plant pathogens of chickpea [30]. Similarly, the bioagents and PGP strains of *B. subtilis*-1 and *Bacillus megaterium*-1 evaluated for siderophore production quantitatively, both the strains produced (65.00%) and (50.27%) siderophore units, respectively [31]. *B. subtilis* subsp. *inaquosorum* RLS52 produced 85.04% siderophore unit while the time of harvesting of highest siderophore from fermentation broth was 72 h and with subsequent incubation, there was a decrease in % siderophore units [21].

In the present study, *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 produced 93.20% siderophore units after 60 h, it was found better than the previous researchers Sharma et al. [29] and Raut et al. [21] in terms of siderophore production units. Siderophore production ability of isolate also contributed in mycelial growth inhibition of *Alternaria* leaf spot phytopathogen of Bt cotton.

3.4. Detection of Chitinase Enzyme Activity

The chitinase production ability of rhizobacterial isolates *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 tested by chitin agar plate assay which shown the zone of chitin hydrolysis surrounding the colony (Figure 3). The zone of chitin hydrolysis surrounding the colony indicates the presence potent chitin-hydrolyzing

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enzyme. The chitinase activity was measured and recorded in mm [Table 4].

An insoluble structural polysaccharide, that is, chitin found within the cell walls of insects and fungi. Chitinases are hydrolytic enzymes which will convert polysaccharide into low molecular mass, soluble, and insoluble oligosaccharides [32]. In general, enzyme chitinase found in organisms useful to reshape their own chitin or degrade and digest the chitin of fungi by breaking the glycosidic bonds in chitin. Chitinolytic enzymes are important due to their biocontrol applications, especially the Chitinases useful in agriculture to manage fungal phytopathogens. The chitinase production ability of *B. amyloliquefaciens* 1st time recorded by researcher Sabry [33].

Some researchers reported that *B. amyloliquefaciens* V656 strain grown in a medium amended with marine waste crab and shrimp shell powder produces the extracellular antifungal enzymes [34]. *B. subtilis* isolated from soil sample having antifungal activity against plant pathogen was evaluated for chitinase activity and showed 0.6 mm zone of clearance surrounding the colony on colloidal chitin agar plate [35]. *Bacillus licheniformis* and *B. subtilis* produced prominent and maximum clear zone of chitin hydrolysis as 14 mm and 11 mm, respectively, on colloidal chitin agar medium [36]. Furthermore, the rhizospheric isolate *B. subtilis* subsp. *inaquosorum* RLS52 having antifungal activity against *A. macrospora*, a leaf spot

phytopathogen of Bt cotton produced 7.3 mm clear halo around the colony [21].

In the present research, *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 produced the 9 mm clear zone of chitin hydrolysis which was better than the results obtained by Raut et al. [21] and Karunya [35] but less chitinase enzyme activity than Abirami et al. [36]. These results indicate that the chitinase enzyme produced by our rhizospheric isolate confirmed the important role in controlling *Alternaria* leaf spot phytopathogen of Bt cotton.

3.5. GC-MS Analysis of Crude Extract

The crude extract of *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 was evaluated to detect the biologically active novel volatile compounds having accountability to suppress the fungal phytopathogens using GC-MS analysis. The novel volatile compound were identified based on a comparison of the peaks of chromatogram with mass spectral data from NIST (2007) library.

B. amyloliquefaciens subsp. *amyloliquefaciens* strain RLS19 shows six peaks in the GC-MS chromatogram. Based on peak abundance three peaks of chromatogram compared to mass spectral data from NIST (2007) Library. The GC-MS spectral results compare with library search successfully identified three compounds. The biologically active compounds with their retention time, molecular weight, molecular formula, structure, and percentage of similarity, and biological activity recorded in Table 5. The GC-MS evaluation of cell free culture filtrate rhizospheric isolate *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 detected three compounds, namely 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-; acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butenyl]-, and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-. Some researchers reported the antifungal activity of 2,5-piperazinedione 3,6-bis(2-methylpropyl) produced by *Lactobacillus plantarum* AF1 against *Aspergillus flavus* ATCC 22546 [37]. The crude extract of *Streptomyces indicensis* after GC-MS analysis revealed major constituents: 3-Pyrrolidin-2-yl-propionic acid; pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-; benzamide, N-propyl pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-; Tetradecane, 2,6,10-trimethyl-5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1,2-b]pyrazine; and chloroxyleneol which showed antifungal activity against *F. oxysporum* [38]. To discover the volatile metabolite a mechanism of antifungal potential, the crude extract of endophyte *B. amyloliquefaciens* BmB 1 having antagonistic activity against *Rhizoctonia* sp. and *Pythium* sp. [39] was analyzed to detect 1 volatile compounds by GC-MS analysis and confirmed the presence of benzene acetic acid; diisooctyl phthalate; octadecanoic acid; pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-; 2,5-Piperazinedione benzyl-6-isopropenyl; pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-

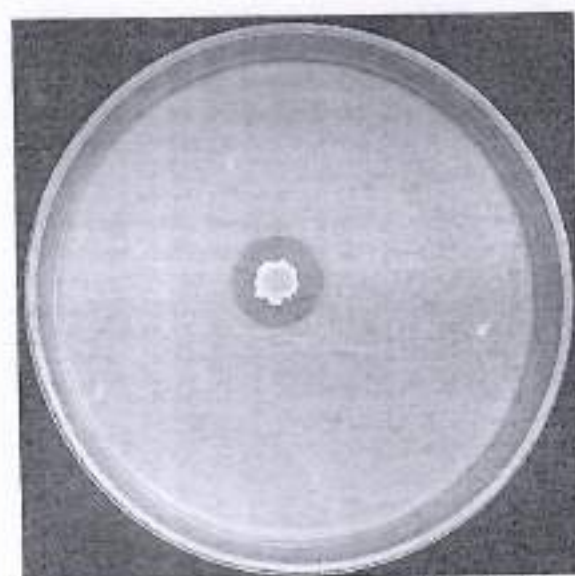


Figure 3: Detection of chitinase enzyme activity of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 by colloidal chitin agar plate assay.

Table 3: Quantitative estimation of siderophore produced by rhizospheric isolate using liquid CAS assay.

S.No.	Rhizospheric isolate	Siderophore units (%)			
		24 h	36 h	48 h	60 h
1	<i>Bacillus amyloliquefaciens</i> subsp. <i>amyloliquefaciens</i> strain RLS19	24.34	76.28	88.14	93.20

Table 4: Detection of chitinase enzyme activity of rhizospheric isolates by colloidal chitin agar plate assay.

S.No.	Rhizospheric isolates	Total zone of hydrolysis including colony (mm)	Diameter of colony (mm)	Diameter zone of chitin hydrolysis (mm)
1	<i>Bacillus amyloliquefaciens</i> subsp. <i>amyloliquefaciens</i> strain RLS19	18	9	9

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Table 5: Biochemical compounds identified in crude extract of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 by GC-MS.

Retention time	Name of the compound	Formula	Structure	Mol. Wt. g/mol	Similarity (%)	Biological activity
16.73	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_8N_2O_2$		154	76.3	Antimicrobial Algicidal
25.07	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	$C_{17}H_{24}N_2O_2$		226	62.3	Antifungal
25.42	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butenyl]-	$C_{11}H_{18}N_2O_2$		210	23.4	Antimicrobial Anti-inflammatory

3-(phenylmethyl); and pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methylpropyl).

Similarly, GC-MS analysis of crude extract of *Bacillus* sp. WG4 reveals the presence of pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro derivative such as pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) as basis of antifungal activity against *Pythium myriotylum* [40]. Two *Bacillus* strains isolated from Krubera-Voronja Cave, the strain 1410WF1-TSA30-2 is 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester and the strain 1350R2-TSA30-6 shown to produce volatile chemical compounds such as pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-, and pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-with antibacterial activity [41]. Furthermore, the characterization of the crude extract of *Bacillus pumilus* MMM by GC-MS and confirms the availability of biologically active compounds pyrrolo[1,2-a]pyrazine-1,4-dione derivatives having inhibitory effect against *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [42]. Crude extract of sponge endosymbiotic *Bacillus* sp. analyzed by GC-MS and reveals the presence of pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, having antibacterial activity [43].

All these reports indicate that *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 produced volatile compounds 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-; Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butenyl]-, and pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- and the inhibitory activity against *A. macrospora*, an *Alternaria* leaf spot phytopathogen of Bt cotton.

4. CONCLUSION

Overall results suggest that *B. amyloliquefaciens* subsp. *amyloliquefaciens* strain RLS19 inhibited the mycelial growth of *A. macrospora*, *Alternaria* leaf spot phytopathogen of Bt cotton. All though searching biocontrol mechanism, the strain was able to produce non-volatile secondary metabolites, siderophore, chitinase enzyme, and volatile organic bioactive compounds which includes pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-; 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-, and acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butenyl]- which contributed in inhibition of mycelial growth of fungal phytopathogen. Due to the presence of multiple potentialities, *B. amyloliquefaciens*

subsp. *amyloliquefaciens* strain RLS19 can serve as an excellent biocontrol candidate for the management of *Alternaria* leaf spot pathogen of Bt cotton and alternative to agrochemicals. Furthermore, this strain needs to test for inhibitory activity in opposition to various phytopathogens of different crops.

5. CONFLICTS OF INTEREST

Authors declared that they do not have any conflicts of interest.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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8. ETHICAL APPROVALS

This study does not involve the use of animals or human subjects.

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