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GC-MS Analysis of antimicrobial compounds produced by *Bacillus* spp. against rice sheath rot pathogen *Sarocladium oryzae*

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Abstract
Bacillus species are widely exploited as biocontrol agents because of their efficiency in impeding various plant pathogens with multifaceted approach. In this study, *Bacillus* species were isolated from rhizosphere of rice plants in Southern districts of Tamil Nadu and assessed their potential to control the mycelial growth of *Sarocladium oryzae in vitro* by dual plate and paired dish technique. *Bacillus subtilis* strain BTK1 was confirmed by 16s rRNA and it showed effective in inhibiting mycelial growth of pathogen (75%). Moreover, volatile compounds emitted from the *Bacillus* spp. were found to inhibit the mycelial growth of *S. oryzae*. Further, secondary metabolites from *B. subtilis* (BTK1) were identified as 3-Heptanone, 5-ethyl-4-methyl-, Butanoic acid 2-methyl and 1-propanol 2,2-dimethyl acetate by gas chromatography mass spectrometry (GC-MS). Taken together, these results suggest that *Bacillus subtilis* (BTK1) can be used as a biocontrol agent for rice sheath rot disease caused by *S. oryzae.*

Keywords: *Bacillus spp., biocontrol, Sarocladium oryzae, secondary metabolites, GCMS.*

Introduction
Rice sheath rot disease incited by *Sarocladium oryzae* (Sawada) occurs in all rice growing countries worldwide. In India, the disease caused yield losses of 3–85% depending upon the disease severity. Synthetic fungicides have been extensively used to control diseases caused by these pathogens. However, these chemicals may lead to toxic residues in treated products. Synthetic pesticides can also cause environmental pollution due to their slow biodegradation. Thus, many researchers have focused on the use of biological methods to protect crops from invasion and spread of pathogens. Indeed, management of pathogens by antagonistic microorganisms or their secondary metabolites are now considered as a viable method for disease control (Liu *et al.*, 2007) [20]. One representative candidate is *Bacillus* species belonging to Gram-positive bacteria. *Bacillus* species can produce various kinds of diffusible and volatile compounds with strong inhibitory activity against plant pathogens (Hossain *et al.*, 2016) [13]. These diffusible compounds have low toxicity, high biodegradability, environmentally friendly characteristics (Ongena and Jacques, 2008) [22]. In this view, the objectives of this study were: 1) to characterize bacterial antagonist with strong antifungal activity, 2) to identify volatile and antifungal secondary metabolites.

Materials and Methods
Isolation and identification of *S. oryzae*
The pathogen was isolated from an infected rice plant by tissue segment method. Then the plates were incubated at 28 ± 2°C. The pathogen was identified through morphological and cultural characterization.

Isolation and biochemical characterization of *Bacillus* strains from rhizosphere region
Soil samples were collected from rhizosphere of rice plants from field in different rice growing areas of southern districts of Tamil Nadu, India. After serial dilution, plating was done on Nutrient Agar medium (Himedia) and incubated for 2 days at 30 °C. Pure cultures were maintained on NA medium by picking distinctive colonies obtained after plating at different dilution. A total of 20 isolates were obtained and evaluated for their antagonistic effect against *S. oryzae*.

The bacterial antagonists were identified and characterized based on the diagnostic test detailed in the Bergey's manual for determinative bacteriology (Bergey *et al.*, 1939) [4]. Biochemical test *viz.*, gram reaction, catalase test, voges proskauer, growth in NaCl, growth at 4 ºC and 45 ºC and starch hydrolysis were carried out for confirmation of *Bacillus* spp.

Preliminary screening of *Bacillus* isolates for antagonistic activity against *S.oryzae*

Twenty *Bacillus* isolates were initially screened *in vitro* by the dual culture technique for antagonistic potential against *S.oryzae*. A 9 mm diameter mycelial disc of the pathogen was inoculated towards one edge of Petri plate containing PDA and the bacterial isolate was streaked at the opposite edge. The plates were kept under incubation at 28 ± 2°C and observed for 12-15 days. Per cent inhibition was calculated as: [(C-T)/C] ×100; where, T and C are the diameters in mm of the pathogen in the treatment plates and control plates respectively (Dennis and Webster, 1971) [8]. The tests were performed in triplicates.

Efficacy of volatile organic compounds produced by *Bacillus* spp. against *S. oryzae*

The antifungal volatile compounds produced by effective *Bacillus* isolates were assessed *in vitro* against *S. oryzae* by paired dish technique Laha *et al.* (1996) [19]. The Effective *Bacillus* isolates selected based on dual plate assay was used for further under *in vitro* assay. *Bacillus* spp. was uniformly spread onto Nutrient agar plates (90 mm). Subsequently, a plug (9mm) from the agar of each of the fungi, which were incubated for 12-15 days, was punched and placed onto the center of a fresh PDA plate. A sandwich was made with the PDA medium having fungi on the bottom and the *Bacillus* spp. coated NA on the top. A set of two plates was sealed with parafilm and incubated at 28 °C. The experiment was replicated thrice. After incubation, the per cent inhibition of the pathogen was calculated as proposed by Dennis and Webster (1971) [8].

Extraction and Bioassay of secondary metabolites from effective *Bacillus* spp.

The crude metabolites were extracted as per the protocol described by Prapagdee *et al*. (2000) [24]. The *Bacillus* strains were cultured in Nutrient Broth (NB) and incubated at 28±2ºC for 3 days. The supernatant was collected after 72 hrs (stationary phase) by centrifuging at 8000 rpm for 30 min at 28±2ºC. Then supernatant was adjusted to acidic pH 2.0 with concentrated HCl and the mixture was stirred at 100 rpm in an orbital shaker for 8 hrs at 28±2ºC. Antifungal compounds in culture broth were extracted by adding the equal volume of ethyl acetate and shaken for 2 hrs in an orbital shaker at 200 rpm. Culture broth was extracted twice with ethyl acetate for complete extraction. The solvent fraction that contained antifungal compounds were combined and concentrated by evaporation in the rotary flask evaporator and maintained at 60ºC at 80 rpm. The concentrated crude metabolites of the extracellular antifungal compounds obtained from the culture broth were dissolved in 1 ml methanol: chloroform mixture (1:1) and used for *in vitro* antifungal assay and GC/MS analysis.

The crude compound was processed for secondary screening by agar well diffusion technique to confirm the presence of bioactive metabolites. A nine mm mycelial disc of the sheath rot pathogen *S. oryzae* was placed in the centre of the Petri plate and then 75µl of crude extract of effective *Bacillus* spp. were dropped into the agar well 1cm away from the edge at four sides centering on the fungal disc. The plates were incubated at room temperature and the plates were scored when the mycelium grew over the control disc. Control was maintained with the sterile distilled water instead of crude extract.

Molecular identification of effective *Bacillus* isolate

The *Bacillus* strain BTK1 showing the greatest antagonistic effect was selected among the 20 strains based on *in vitro* antifungal assay and was subjected for molecular characterization. Genomic DNA was extracted and 16S rRNA gene was characterized with BCF1 (5′CGGGAGGCAGCAGTAGGGAAT-3′) and BCR2 (5′-CTCCCCAGGCGGAGTGCTTT-3′) primer pair as proposed by Cano *et al.* (1994) [7]**.** Amplification was done under following conditions: initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 30s, annealing a 52°C for 1min, extension at 72°C for 1 min and a final extension at 72°C for 10 min on a thermal cycler (Eppendrof, Germany). The amplified product was sequenced by Eurofins Genomics India Pvt. Ltd. (Bengaluru, India) and subjected to homology analyses using BLAST program from GenBank database.

Characterization of secondary metabolites by GC-MS

Based on the growth inhibition studies, *Bacillus* spp. extracts were selected and chemical constituents were determined with a Shimadzu Gas chromatography equipped with a mass detector Turbo mass gold containing a Elite-1 (100% Dimethyl Poly Siloxane), 30 m × 0.25 mm ID × 1 mM df. Conditions employed were the following: Carrier gas, helium (1 ml/min); oven temperature programe 110 °C (2 min) to 280 °C (9 min); injector temperature (250 °C); total GC time (45 min). The ethylacetate extract was injected into the chromatograph in 1.0 ml aliquots. The major constituents were identified with the aid of a computer-driven algorithm and then by matching the mass spectrum of the analysis with that of a National Institute of Standards and Technology (NIST) library (Version. 2.0, year-2005). Software used for gas chromatography mass spectroscopy (GC-MS) was Turbo mass-5.1. This work was carried out in center of innovation for excellence, Agricultural College& Research Institute, Tamil Nadu Agricultural University, Madurai.

Statistical Analysis

Means differences of the treatment were evaluated with ANOVA by using Duncan's Multiple Range Test at 5% significance (Gomez and Gomez, 1984) [10].

Results and discussion

Isolation and identification of sheath rot pathogen

On PDA, the pathogen produced white, cottony, mycelia growth with large, 7 days after incubation at 28 ± 2 ºC. Based on the morphological characters, the pathogen was confirmed as *S. oryzae*. Upon tissue isolation the pathogen from the locality was brought into pure culture and identified as *S. oryzae* based on morphological, cultural characters in accordance to the description given by Sakthivel *et al*. (2002) [26]

Biochemical characterization of *Bacillus* spp.

Twenty isolates of *Bacillus* spp. were found to show positive results to the following tests conducted *viz*., gram reaction, catalase test, voges proskauer, growth in NaCl, growth at 45ºC and starch hydrolysis. These isolates showed negative result to anaerobic growth and growth at 4 ºC.

Antifungl activity of *Bacillus* spp. against *S.oryzae* under *in vitro*

Twenty isolates of *Bacillus* spp. were tested for their antagonistic activity against *S. oryzae* by dual culture technique. Among the isolates tested, BTK1 was recorded with maximum (75.5%) inhibition of mycelial growth (22.00 mm) of the pathogen at 15 days after inoculation and it was followed by BTN4 which recorded 68.89 per cent inhibition of the mycelial growth over control. BSG1 was found to record the minimum inhibition of mycelial growth of the pathogen at 15 days after incubation which counted for 20 per cent growth reduction over control (Table 1). Correspondingly, Gopalakrishnan and Valluvaparidasan (2006) [11] suggested that, *B. subtilis* isolate 9 was found to be highly effective in inhibiting the mycelial growth of *S. oryzae* by 82.18 per cent.

Table 1: Effect of *Bacillus* spp. on the mycelial growth of *S. oryzae* under *in vitro*

S. No.	Isolates	Mycelia growth (mm)*	Per cent reduction over control (%)
1	BMD1	56.00	37.78 (37.66) **
2	BMD2	49.00	45.56 (43.26)
3	BMD3	60.00	33.33 (35.38)
4	BMD4	39.00	56.67 (48.96)
5	BMD5	65.00	27.78 (31.65)
6.	BMD6	63.00	30.00 (33.53)
7.	BMD7	59.00	34.44 (36.12)
8.	BTN1	40.00	55.56 (48.10)
9.	BTN2	30.00	66.67 (55.01)
10.	BTN3	70.00	22.22 (28.57)
11.	BTN4	28.00	68.89 (56.96)
12.	BTN5	59.00	34.44 (36.12)
13.	BTV1	52.00	42.22 (38.46)
14.	BTK1	22.00	75.56 (60.82)
15.	BKK1	44.00	51.11 (45.68)
16.	BKK2	47.00	47.78 (44.24)
17.	BRP1	69.00	23.33 (28.34)
18.	BRP2	63.00	30.00 (33.66)
19.	BSG1	72.00	20.00 (26.62)
20.	BVN1	68.00	24.44 (30.12)
21.	Control	90.00	0.00 (0.36)
CD (*P*=0.05)		2.86	1.62

Efficacy of volatile compounds from selected *Bacillus* spp. against the growth of *S. oryzae* under *In vitro*

The results on the radial growth of *S. oryzae* due to the production of volatile compounds of *Bacillus* spp. isolates revealed that the maximum 67.78 per cent reduction of 29.00 mm was observed in BTK1 followed by *Bacillus* spp. isolate BTN4 which recorded 60 per cent reduction of mycelial growth (36.00 mm) at 15 days after incubation (Table 2). Antifungal volatile compounds have been demonstrated previously in several pathogen systems. According to Arrebola *et al.* (2010) [3], *B. amyloliquefaciens* PPCB004 could produce acetoin as a major volatile compound which inhibited the growth of several *Penicillium* spp. under *in vitro*. Volatile organic compounds produced by *B.amyloliquefaciens* against *Ralstonia solanacearum* caused 40% reduction in growth of the pathogen (Raza *et al.*, 2016) [25].

Table 2: Effect of volatile organic compounds produced by *Bacillus* spp. on mycelial growth of *S. oryzae*

S. No	Isolates	Mycelial growth @ (mm)*	Per cent reduction over control (%)
1	BMD1	66.00	26.67 (30.68) **
2	BMD2	60.00	33.33 (35.18)
3	BMD4	45.00	50.00 (45.23)
4	BTN1	48.00	46.67 (43.41)
5	BTN2	40.00	55.56 (48.00)
6	BTN4	36.00	60.00 (49.48)
7	BTK1	29.00	67.78 (54.30)
8	BKK1	54.00	40.00 (38.48)
9	BKK2	57.00	36.67 (37.82)
10	BTV1	58.00	35.56 (36.91)
11	Control	90.00	0.00 (0.49)
CD (P=0.05)		2.48	1.76

*Mean of three Replications

** Values in the parentheses are arcsine transformed values

Antifungal activity of crude metabolites from selected *Bacillus* spp. against *S. oryzae* under *in vitro*

The results revealed that the efficacy of crude metabolites of the selected ten isolates of *Bacillus* spp. tested for their antifungal activity against *S.oryzae*. The crude metabolites isolated from *Bacillus* strain BTK1 significantly recorded the maximum (70.00) per cent reduction of mycelial growth followed by BTN4 which recorded 62 per cent reduction of mycelia growth over control. The other isolates were less effective against the pathogen (Fig.1). Similarly, Vinodkumar *et al.* (2017) [33] stated that the crude metabolites of *B. amyloliquefaciens* (VB7 and VB2), reduced the mycelial growth of *S.sclerotiorum* by producing antifungal compounds Pyrrolo, 9-Octadecenoicacid identified by GC/MS.

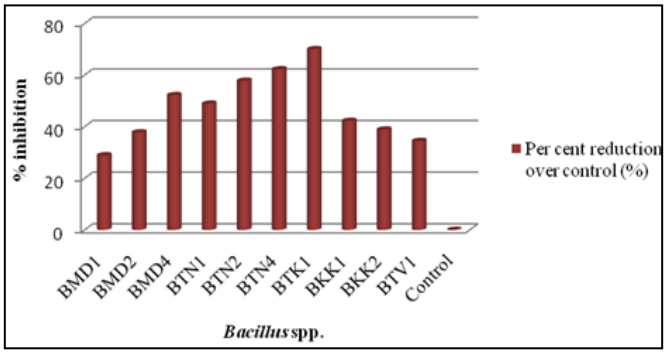


Fig 1: Efficacy of crude metabolites produced by *Bacillus* spp. against *S.oryzae in vitro*

Molecular characterization of the effective antagonistic Bacteria

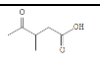
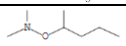
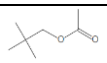
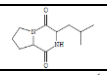
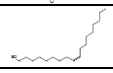
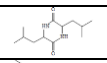
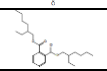
In this study the effective strain BTK1 was identified as *Bacillus subtilis* (GeneBank Accession No. MT007285) with the fragment size of 546bp based on the 16s rRNA intervening sequence specific primers. The sequence showed 99.22 per cent identity with *Bacillus subtilis* sequences available in GenBank. Likewise, Ramyabharathi and Raguchander (2014) [25] reported that the strain EPCO16 was confirmed as *Bacillus subtilis* with the fragment size of 546bp based on the 16s-23s rRNA intervening sequence specific primers.

Characterization of secondary metabolites by GC/MS

The GC-MS analysis revealed that various antifungal compounds were produced by *Bacillus* spp. The crude metabolites of *Bacillus* spp. strains BTK1 and BTN4 were analyzed by GC/MS to detect their production of antimicrobial compounds. The compound identity was confirmed through NIST library 2005. Most important antifungal compounds were detected such as Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro-, 9-Octadecenol, 1-Propanol, 2,2-dimethyl-acetate, Butanoic acid, 2-methyl-, N,N-Dimethyl, 3-Heptanone,5-ethyl-4-methyl-, Phenol, Benzeneacetic acid (Fig.2;Table: 3; Fig.3;Table: 4).

The outcomes of our study are in close agreement with the earlier studies of biocontrol agents against different fungal pathogens. Borick *et al.* (1959) [6] evaluated the antimicrobial activity of fatty acid salts of N-N- dimethyl. Bharose and Gajera (2018) [5] identified that the *B. subtilis* strain JND-KHGn-29-A could inhibited the mycelial growth of *Aspergillus* by producing antifungal compounds such as Bis(2-ethylhexyl) phthalate, Pyrrolo[1,2-a]pyrazine-1,4-dione. These results corresponding with the results of Ramyabharathi and Raguchander (2014) [24] reported that *B. subtilis* EPCO16 strain produced secondary metabolites such as Hexadecanoic acid methyl ester, Dodecanoic acid, Pentadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester belonging to fatty acid having antifungal activity. Pyrrolo also had antifungal activity against the pathogen (Jiang *et al.*, 2014) [15]. Jangir *et al.* (2018) [14] identified that the GC-MS analysis of volatile organic compounds *viz.*, N, N-Dimethyl, 1,2-benzenedicarboxylic acid, 9-octadecenoic acid produced by *Bacillus* spp. might have been responsible for controlling the growth of *F.o.*f.sp.*lycopersici*

Table 3: Identification of Antimicrobial compounds from *Bacillus subtilis* (BTK1) through GC/MS

Peak	Retention Time	Compound Name	Structure	Molecular Formula	Molecular Weight (g/mol)	Peak area %	Activity	References
1	3.032	Pentanoic acid, 3-methyl-4-oxo-		$C_6H_{10}O_3$	130	7.67	Antibacterial activity	Song *et al.* (2015) [29]
2	3.070	L-5-Propylthiomethylhydantoin		$C_7H_{12}N_2O_2S$	188	22.73	Antimicrobial activity	Tahara *et al.* (1979) [32]
5	3.275	N,N-Dimethyl-O-(1-methyl-butyl)-hydroxylam		$C_7H_{17}NO$	131	6.18	Antimicrobial activity	Borick *et al.* (1959) [6]
7	4.388	1-Propanol, 2,2-dimethyl-, acetate		$C_7H_{14}O_2$	130	8.12	Antimicrobial activity	Wijekoon *et al.* (2013) [35]
8	5.474	Butanoic acid, 2-methyl-		$C_5H_{10}O_2$	102	2.21	Antimicrobial	Hayashida-Soiza *et al.* (2008) [12]
11	19.143	1-Hexadecanol		$C_{16}H_{34}O$	242	2.56	Antimicrobial activity	Susanti *et al.* (2013) [30]
13	19.896	Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-		$C_{11}H_{18}N_2O_2$	210	2.14	Antifungal activity	Jiang *et al.* (2014) [15]
15	21.057	9-Octadecen-1-ol, (Z)-		$C_{18}H_{36}O$	268	4.39	Antifungal and antibacterial activity	Hossein *et al.* (2016) [13]
16	21.275	n-Nonadecanol-1		$C_{19}H_{40}O$	284	2.30	Antimicrobial and cytotoxic properties	Kuppuswamy *et al.* (2013) [18]
18	22.339	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-		$C_{12}H_{22}N_2O_2$	226	1.54	Antimicrobial activity	Altaee *et al.* (2017) [2]
21	25.356	Bis(2-ethylhexyl) phthalate		$C_{24}H_{38}O_4$	390	0.74	Antimicrobial activity	Osuntokun and Cristina (2019) [12]
22	25.698	Cyclononasiloxane, octadecamethyl-		$C_{18}H_{54}O_9Si_9$	666	0.96	Antifungal activity	Ahsan *et al.* (2017) [1]

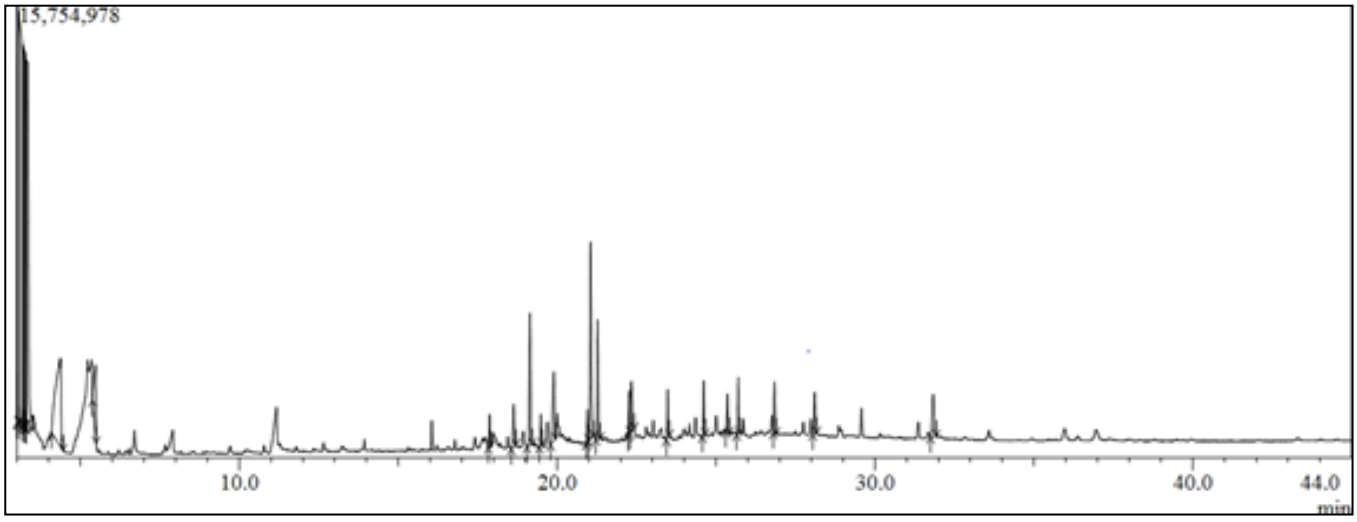


Fig 2: Gas chromatogram of antimicrobial compounds identified from *Bacillus subtilis* (BTK1) through GC/MS

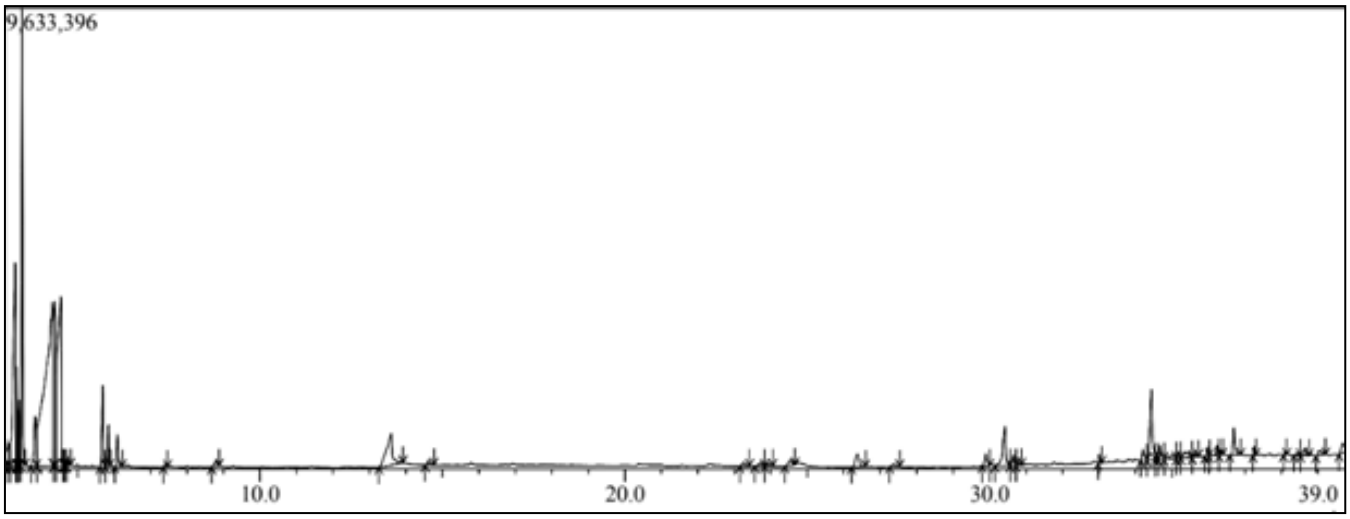
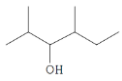
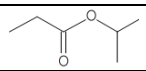
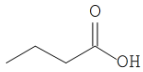
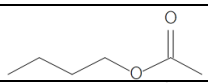
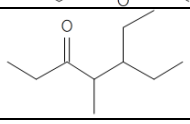
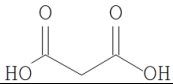
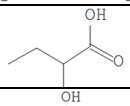
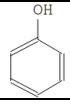
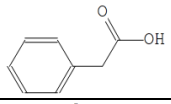
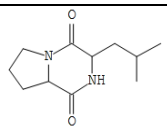


Fig 3: Gas chromatogram of antimicrobial compounds identified from *Bacillus* spp. (BTN4) through GC/MS

Table 4: Identification of Antimicrobial compounds from *Bacillus* spp. (BTN4) through GC/MS

Peak	Retention Time	Compound Name	Structure	Molecular Formula	Molecular Weight (g/mol)	Peak area %	Activity	References
3	3.289	1-propanol 2,2-dimethyl acetate		$C_7H_{14}O_2$	130.18	10.86	Antimicrobial	Matysiak *et al.* (2019) [21]
4	3.340	Propanoic acid 2-methyl		$C_6H_{12}O_2$	116	1.54	Antimicrobial	Ertürk *et al.* (2016) [9]
6	3.402	Butanoic acid		$C_4H_8O_2$	88.11	1.69	Antimicrobial	Kai *et al.* (2009) [16]
7	3.482	Acetic acid, butyl ester		$C_6H_{12}O_2$	116	9.08	Antimicrobial	Seddek *et al.* (2019) [28]
9	4.308	3-Heptanone, 5-ethyl-4-methyl-		$C_{10}H_{20}O$	156	26.05	Antifungal	Wheatley *et al.* (1997) [34]
10	4.383	Propanedioic acid		$C_3H_4O_4$	104	6.59	Antimicrobial	Seddek *et al.* (2019) [28]
11	4.545	Butanoic acid 2-methyl		$C_5H_{10}O_2$	102.13	13.75	Antimicrobial	Hayashida-Soiza *et al.* (2008) [12]
17	6.090	Phenol		C_6H_6O	94	0.93	Antifungal	Teresa *et al.* (2014) [32]
20	13.601	Benzeneacetic acid		$C_8H_8O_2$	136	3.94	Antifungal, Antimicrobial	Kim *et al.* (2004) [17]
29	30.416	Pyrrolo(1,2-a) Pyrazine 1,4 dione, hexahydro-3		$C_{11}H_{18}N_2O_2$	210	2.46	Antifungal activity	Jiang *et al.* (2014) [15]

Conclusion

One of the biggest ecological challenges to be faced by microbiologists and plant pathologists in the near feature is the development of environmentally friendly alternatives to chemical pesticides for combating crop diseases. The use of beneficial microorganisms is considered as one of the most promising methods for more rational and safe crop management practices. In this respect, we attempted to isolate antagonistic bacteria with strong antifungal activity against *S. oryzae*. As a result, we found that *Bacillus subtilis* (BTK1) effectively inhibited the mycelial growth of *S. oryzae* by producing volatile compounds and a wide variety of secondary metabolites that are diverse in structure and function. Production of antimicrobial metabolites determines the ability of these species to control plant disease.

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