

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(7): 1563-1569
www.biochemjournal.com
Received: 05-04-2026
Accepted: 08-05-2026

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GC-MS Profiling of endophytic *Bacillus* sp. isolated from Basmati rice and its antagonistic potential against *Rhizoctonia solani* Kuhn

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i7Ss.9270>

Abstract

Rice is one of the most important staple food crops worldwide; however, its productivity is constrained by sheath blight caused by *Rhizoctonia solani* Kühn. The extensive use of chemical fungicides for disease management has led to environmental pollution, fungicide resistance and health concerns, necessitating the development of sustainable biological control strategies. Endophytic *Bacillus* species have emerged as promising biocontrol agents due to their ability to produce diverse antimicrobial metabolites. Therefore, the present study was undertaken to isolate endophytic *Bacillus* sp. from Basmati rice, evaluate the antifungal activity of their crude metabolites against *R. solani* and identify the bioactive compounds through GC-MS. Endophytic bacteria were isolated from stem tissues of Basmati rice (Pusa Basmati-1) and identified as *Bacillus* sp. based on morphological characteristics and Gram staining. Crude metabolites were extracted from bacterial culture using ethyl acetate and evaluated against *R. solani* through the agar well diffusion method at concentrations of 100, 200, 300, 400 and 500 ppm. The results revealed a concentration dependent inhibition of fungal growth, with the highest inhibition observed at 500 ppm, recording 78.79, 78.87 and 68.89 per cent inhibition after 24, 48 and 72 hours, respectively. GC-MS analysis detected forty metabolites in the bacterial extract, including several compounds previously reported to possess antimicrobial properties. The major compounds identified were Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 2,4-Di-tert-butylphenol, 1-Octadecene, Hexadecane, 2,6,11,15-tetramethyl- and Octadecane. The findings demonstrate that endophytic *Bacillus* sp. produces a diverse array of bioactive secondary metabolites capable of effectively suppressing *R. solani*. These metabolites possess considerable potential for development as eco-friendly biocontrol agents for the sustainable management of rice sheath blight and may serve as promising alternatives to chemical fungicides.

Keywords: *Bacillus* sp., Endophytic bacteria, *R. solani*, GC-MS and secondary metabolites

Introduction

Rice is the staple food crop for over half of the global population and is cultivated in more than 100 countries, with Asia contributing nearly 90% of the world's total rice production (Fukagawa and Ziska, 2019) [13]. The various abiotic and biotic factors cause yield reduction in rice. As a major staple crop worldwide, rice is affected by a wide range of fungal, bacterial and viral pathogens (Laha *et al.*, 2017) [25]. The average yield loss of 25-30% per annum in India occurs due to diseases such as blast, brown spot, sheath blight, false smut, bacterial leaf blight and bacterial leaf streak (Jha *et al.*, 2012) [18]. Sheath blight ranks as the second most important fungal disease after blast, causing significant yield loss in rice (Pan *et al.*, 1999) [33]. The disease is caused by the pathogen *Rhizoctonia solani* Kuhn (Anamorphic stage), having a teleomorphic stage *Thanatephorus cucumeris* (Frank) Donk. Sheath blight infection in rice is considered a serious threat in many rice growing regions. Previous studies have indicated that the disease can result in yield losses ranging from 11.1 to 58 per cent, depending on the severity of infection and the rice varieties grown (Chahal *et al.*, 2003) [9]. The sheath blight is controlled by the application of different fungicides. However, the ongoing excessive and indiscriminate use of pesticides has resulted in several adverse effects, such as residual toxicity in food grains, development of fungicide resistance in pathogens, environmental pollution, risks to human and animal health and an overall increase in the cost of cultivation (Groth, 2005) [15].

The continuous reliance on chemical pesticides and their negative impact has created the necessity for sustainable environmental disease management approaches. Biological control has gained considerable attention as an alternative to chemical management strategies. Additionally, such approaches are economically advantageous over time and ensure prolonged crop protection in short duration crops like rice by maintaining their activity throughout the cropping period (He *et al.*, 2021) [16].

Among the various biocontrol strategies, the use of beneficial endophytes as biological control agents has emerged as a highly promising approach for sustainable crop protection (Le Cocq *et al.*, 2017) [26]. Endophytes are microorganisms that inhabit internal plant tissues without causing any visible symptoms of disease and they are known to enhance host defence mechanisms against pathogens (Mousa *et al.*, 2015) [29]. Endophytic bacteria colonise different plant parts including roots, stems, leaves and seeds, making a significant segment of the plant microbiome (Ayilara *et al.*, 2023) [6].

Members of the genus *Bacillus* are among the most extensively used biological control agents for managing numerous plant diseases. Species such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus pasteurii*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus mycoides* and *Bacillus sphaericus* have considerable potential in suppressing a wide range of phytopathogens (Kloepper *et al.*, 2004; McSpadden Gardener, 2010) [24, 14]. Endophytic *Bacillus* spp. produce volatile organic compounds (VOCs) that inhibit the growth of pathogenic fungi, bacteria and nematodes (Khare *et al.*, 2018) [22].

The biocontrol efficacy of *Bacillus* spp. is largely attributed to their diverse secondary metabolism, which enables the production of a broad spectrum of antagonistic compounds (Stein, 2005) [42]. These bacteria synthesise numerous bioactive metabolites, including antimicrobial peptides, polyketides, lytic enzymes and volatile compounds that directly suppress plant pathogens (Chowdhary *et al.*, 2015) [11].

Although endophytic *Bacillus* spp. have been widely reported as effective biocontrol agents, most studies have mainly focused on their antagonistic potential, while limited studies were conducted to identify the secondary metabolites responsible for pathogen suppression. Therefore, the present study was undertaken to evaluate the antagonistic activity of crude metabolites against *R. solani* and to identify the bioactive compounds present in the metabolites using GC-MS.

Materials and Methods

Isolation of *Bacillus* Endophyte from Basmati Rice

The samples of the basmati rice variety Pusa Basmati-1 collected from the Crop Research Centre, SVPUAT, were thoroughly washed under running tap water to remove adhering soil and debris, followed by surface sterilisation with 70% ethanol for 1 min and 2% sodium hypochlorite for 1 min. The sterilised samples were rinsed three times with sterile distilled water. Subsequently, the sterilised stem tissues were aseptically cut into small pieces and macerated in 10 ml of sterile 0.8% saline solution using a sterile mortar and pestle. Serial dilutions (10^{-1} , 10^{-2} and 10^{-3}) of the homogenate were prepared and 0.1 ml from each dilution was spread onto nutrient agar plates. The inoculated plates were incubated at 30 °C for 24-48 hours and distinct

bacterial colony resembling *Bacillus* spp. were selected and subcultured on fresh nutrient agar plates to obtain a pure culture (Mageshwaran *et al.*, 2022) [27].

Morphological characterisation of isolated Endophyte:

The colony morphology of the bacterial isolates was assessed by observing various characteristics such as shape, margin, elevation, size, texture, appearance, colour and optical properties (Smibert *et al.*, 1994) [41]. Gram staining was performed by preparing a bacterial smear on a clean glass slide, followed by air drying, heat fixation and sequential staining with crystal violet (1 min), iodine (30 s), 95% alcohol (30 s) and safranin (1 min), with distilled water rinsing after each step. The stained smear was examined under a microscope, where Gram-positive bacteria appeared violet and Gram-negative bacteria appeared pink to reddish-pink (Aneja, 2004) [4].

Collection of crude metabolites and the antagonistic activity against *R. solani*:

The isolated endophytic *Bacillus* was grown in nutrient broth at 28 °C for 3 days on a shaker at 180 rpm. The fermented broth was centrifuged at 4000 rpm for 5 min and the supernatant was extracted twice with an equal volume of ethyl acetate by vigorous shaking for 20 min. The organic phase was concentrated using a rotary evaporator at 40 °C to obtain crude extracts (Yadav *et al.*, 2010) [46]. The prepared sample sent to QIMA Quality Inspection India Private Limited, Tamil Nadu, India for analysis.

The extracts were redissolved in ethyl acetate for the preparation of crude metabolites of 100 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm. The crude metabolites are checked for antagonistic potential against *R. solani*. The melted PA is poured into the petri plates and wells are made using a sterile cork borer. About 20 µl of bacterial crude extract was added to each well. Ethyl acetate was used as a negative control. The plates with sterile distilled water were kept as control. The plates were kept at 4 °C for 4 hours to facilitate diffusion of antimicrobial compounds. The test pathogen mycelial disc of 5mm was placed on the PDA plates and then incubated at 28 °C. The inhibition percentage was calculated by the formula:

$$\text{Inhibition percentage} = \frac{C-T}{C} \times 100$$

Where,

C = Mycelial growth in the control plate (cm).

T = mycelial growth in the treatment plate (cm).

Results

Morphological characterisation of Endophytic Bacteria

The bacteria produced irregular spreading, dull white colonies having umbonate elevation, wrinkled margin and dry texture. Gram staining confirmed that the bacterial isolate was Gram-positive. Based on morphological characteristics, the bacteria were identified as *Bacillus* sp.

Metabolomic Profiling of *Bacillus* sp. by GC-MS

The GC-MS analysis revealed the presence of several compounds in the bacterial extract, including Dodecane, Pentadecane, 1-Tetradecene, Octadecane, 2,4-Di-tert-butylphenol, Hexadecane, 2,6,11,15-tetramethyl-, Cetene, Heneicosane, 1-Octadecene, Cyclo(L-prolyl-L-valine), Hexahydro-3-(1-methylpropyl) pyrrolo[1,2-a]pyrazine-1,4-

dione, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, Hexadecane, 2,6,10,14-tetramethyl-, 1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester, Cycloeicosane, Eicosane, Behenic alcohol, Docosane, Pentacosane, n-Tetracosanol-1, Tetracosane and Octacosanol.

Among the detected compounds, the major peaks were represented by 2,4-Di-tert-butylphenol, 1-Octadecene, Hexadecane, 2,6,11,15-tetramethyl- and Octadecane based on their peak area percentages. Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- recorded the highest

peak area of 5.13 per cent at a retention time of 19.1701 min. Another major compound identified was 2,4-Di-tert-butylphenol, which was detected at a retention time of 13.7646 min with a peak area of 4.58 per cent. The compound 1-Octadecene was detected at a retention time of 17.0529 min with a peak area percentage of 4.03 per cent. Hexadecane, 2,6,11,15-tetramethyl-, was identified at a retention time of 14.0450 min with a peak area of 3.29 per cent, while Octadecane was observed at a retention time of 13.4769 min with a peak area percentage of 3.03 per cent.

Table 1: List of identified metabolites from endophytic *Bacillus* sp. through GC-MS

S.No.	Name of the compound	RT	Peak area %	Molecular formula	Molecular Weight (g/mol)	CID number	Properties	References
1.	Dodecane	7.3155	2.15	C ₁₂ H ₂₆	170.33	8182	Antifungal activity	Bora and Devi (2023) [8]
2.	Dodecane	7.4066	1.01	C ₁₂ H ₂₆	170.33	8182	Antifungal activity	Bora and Devi (2023) [8]
3.	Dodecane	8.0183	1.82	C ₁₂ H ₂₆	170.33	8182	Antifungal activity	Bora and Devi (2023) [8]
4.	Dodecane	8.1021	0.96	C ₁₂ H ₂₆	170.33	8182	Antifungal activity	Bora and Devi (2023) [8]
5.	Pentadecane	10.6183	3.00	C ₁₅ H ₃₂	212.41	12391	Antibacterial, antifungal and Antioxidant activity	Khan and Javaid (2019) [21]
6.	Pentadecane	10.9279	0.78	C ₁₅ H ₃₂	212.41	12391	Antibacterial, antifungal and Antioxidant activity	Khan and Javaid (2019) [21]
7.	Pentadecane	11.2702	2.62	C ₁₅ H ₃₂	212.41	12391	Antibacterial, antifungal and Antioxidant activity	Khan and Javaid (2019) [21]
8.	Pentadecane	11.4013	1.43	C ₁₅ H ₃₂	212.41	12391	Antibacterial, antifungal and Antioxidant activity	Khan and Javaid (2019) [21]
9.	Pentadecane	11.5251	1.44	C ₁₅ H ₃₂	212.41	12391	Antibacterial, antifungal and Antioxidant activity	Khan and Javaid (2019) [21]
10.	1-Tetradecene	12.2497	0.74	C ₁₄ H ₂₈	196.37	14260	Antifungal activity	Bhat <i>et al.</i> (2020) [7]
11.	Octadecane	13.4769	3.03	C ₁₈ H ₃₈	254.5	11635	Antibacterial and antifungal activity	Anacleto-Santos <i>et al.</i> (2023) [3]
12.	2,4-Di-tert-butylphenol	13.7646	4.58	C ₁₄ H ₂₂ O	206.32	7311	Antifungal activity	Varsha <i>et al.</i> (2015) [44]
13.	Hexadecane, 2,6,11,15-tetramethyl-	14.0450	3.29	C ₂₀ H ₄₂	282.5	136331	Antimicrobial and antifungal activity	Patil <i>et al.</i> (2022) [34]
14.	Hexadecane, 2,6,11,15-tetramethyl-	14.1688	0.84	C ₂₀ H ₄₂	282.5	136331	Antimicrobial and antifungal activity	Patil <i>et al.</i> (2022) [34]
15.	Octadecane	14.2890	0.76	C ₁₈ H ₃₈	254.5	11635	Antibacterial and antifungal activity	Anacleto-Santos <i>et al.</i> (2023) [3]
16.	Cetene	14.7769	0.90	C ₁₆ H ₃₂	224.42	12395	Antifungal activity	Edet and Hemalatha. (2023) [12]
17.	Heneicosane	16.0041	2.89	C ₂₁ H ₄₄	296.6	12403	Antimicrobial activity	Vanitha <i>et al.</i> (2020) [43]
18.	Heneicosane	16.4921	2.96	C ₂₁ H ₄₄	296.6	12403		
19.	1-Octadecene	17.0529	4.03	C ₁₈ H ₃₆	252.5	8217	Antifungal and antibacterial activity	Khiraalla <i>et al.</i> (2020) [23]
20.	Cyclo(L-prolyl-L-valine)	17.4461	1.84	C ₁₀ H ₁₆ N ₂ O ₂			Antifungal and antibacterial activity	Sharma <i>et al.</i> (2024) [38]
21.	Heneicosane	18.2618	2.12	C ₂₁ H ₄₄	296.6	12403	Antimicrobial activity	Vanitha <i>et al.</i> (2020) [43]
22.	Hexahydro-3-(1-methylpropyl) pyrrolo[1,2-a]pyrazine-1,4-dione	18.6385	2.40	C ₁₁ H ₁₈ N ₂ O ₂	210.27	6428988	Antifungal activity	Vikram and Hariram (2025) [45]
23.	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	19.1701	5.13	C ₁₁ H ₁₈ N ₂ O ₂	210.27	102892	Antimicrobial activity	Kaari <i>et al.</i> (2021) [19]
24.	Hexadecane, 2,6,10,14-tetramethyl-	18.8772	2.83	C ₂₀ H ₄₂	210.27	102892	Antifungal activity	Sharmin <i>et al.</i> (2025) [39]
25.	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-	19.1176	1.68	C ₁₁ H ₁₈ N ₂ O ₂	210.27	102892	Antimicrobial activity	Kaari <i>et al.</i> (2021) [19]

	methylpropyl)-							
26.	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	19.1686	2.13	C ₂₂ H ₃₄ O ₄	362.5	39180	Antimicrobial, antifungal and antiviral activity	Jayaram <i>et al.</i> (2021) ^[17]
27.	Hexadecane, 2,6,10,14-tetramethyl-	20.3083	1.39	C ₂₀ H ₄₂	282.5	12523	Antifungal activity	Sharmin <i>et al.</i> (2025) ^[39]
28.	Cycloeicosane	20.6980	1.39	C ₂₀ H ₄₀	280.5	520444	Antibacterial and antifungal activity	Aydın Kurç (2023) ^[5]
29.	Eicosane	21.0002	1.39	C ₂₀ H ₄₂	282.5	8222	Antifungal activity	Bhat <i>et al.</i> (2024) ^[7]
30.	Heneicosane	21.0439	1.39	C ₂₁ H ₄₄	296.6	12403	Antimicrobial activity.	Vanitha <i>et al.</i> (2020) ^[43]
31.	Hexadecane, 2,6,10,14-tetramethyl-	21.9215	1.39	C ₂₀ H ₄₂	282.5	12523	Antifungal activity	Sharmin <i>et al.</i> (2025) ^[39]
32.	Behenic alcohol	22.5260	1.39	C ₂₂ H ₄₆ O	326.6	12620	Antibacterial, antifungal and antiviral activity	Reichling (2022) ^[35]
33.	Docosane	22.7299	1.39	C ₂₂ H ₄₆	310.6	12405	Antibacterial, antiviral, and antifungal activity	Rhetso <i>et al.</i> (2020) ^[36]
34.	Heneicosane	22.7700	1.39	C ₂₁ H ₄₄	296.6	12403	Antimicrobial activity	Vanitha <i>et al.</i> (2020) ^[43]
35.	Pentacosane	23.5784	1.39	C ₂₅ H ₅₂	352.7	12406	Antifungal activity	Omoruyi <i>et al.</i> (2014) ^[32]
36.	n-Tetracosanol-1	24.3358	1.39	C ₂₄ H ₅₀ O	354.7	10472	Antibacterial activity	Amudha <i>et al.</i> (2018) ^[2]
37.	Tetracosane	25.8216	1.39	C ₂₄ H ₅₀	338.7	12592	Antibacterial activity and antifungal activity	Naragani <i>et al.</i> (2016) ^[30]
38.	Pentacosane	18.8772	1.39	C ₂₅ H ₅₂	352.7	12406	Antibacterial and antifungal activity	Rhetso <i>et al.</i> (2020) ^[36]
39.	n-Tetracosanol-1	19.1176	1.39	C ₂₄ H ₅₀ O	354.7	10472	Antibacterial activity	Amudha <i>et al.</i> (2018) ^[2]
40.	Octacosanol	19.1686	1.39	C ₂₀ H ₄₂	410.8	68406	Antimicrobial activity	Marumure and Nyila (2025) ^[28]

Table 2: *In vitro* efficacy of crude metabolites of *Bacillus* sp. against *R. solani* through the agar well diffusion method

S.No.	Concentration (ppm)	24 hrs Mycelial growth (cm)	Percent reduction over control	48 hrs Mycelial growth (cm)	Percent reduction over control	72 hrs Mycelial growth (cm)	Percent reduction over control
1.	100	2	39.39 ^e (38.85)	4.1	42.23 ^e (40.52)	7.0	22.22 ^e (28.11)
2.	200	1.6	51.52 ^d (45.84)	3.8	46.16 ^d (42.78)	6.4	28.89 ^d (32.49)
3.	300	1.3	60.61 ^c (51.10)	2.8	60.55 ^c (51.07)	4.9	45.56 ^c (42.43)
4.	400	1	69.70 ^b (56.57)	1.9	73.23 ^b (51.07)	3.4	62.22 ^b (52.05)
5.	500	0.7	78.79 ^a (62.55)	1.5	78.87 ^a (58.82)	2.8	68.89 ^a (56.08)
6.	Solvent	2.90	11.75 ^f (20.02)	6.7	5.87 ^f (62.61)	8.3	7.41 ^f (15.75)
7.	Control	3.30	0.00 ^g (0.00)	7.1	0.00 ^g (14.00)	9.0	0.00 ^g (0.00)
	CD (P = 0.05)	0.06	1.722	0.147	1.671	0.19	1.946

*Mean of three replications

*Values in the parentheses are arcsine transformed values

In Vitro* efficacy of crude metabolites of *Bacillus* sp. against *R. solani

The Agar well diffusion against *R. solani* was conducted with crude metabolites at concentrations of 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm. The mycelial growth was recorded every 24 hours until the pathogen covered the entire plate in the control. The response of *R. solani* to crude metabolites varied markedly with concentration. The highest concentration 500 ppm, was the most effective, restricting mycelial growth to 0.7 cm and resulting in 78.79 per cent inhibition after 24 hours. This was followed by 400 ppm, which recorded 69.70 per cent inhibition. Lower

concentrations of 300, 200 and 100 ppm showed comparatively lower inhibition of 60.61 per cent, 51.52 per cent and 39.39 per cent, respectively.

After 42 hours, the treatments containing 400 and 500 ppm metabolites continued to suppress fungal growth effectively, resulting in more than 70 per cent inhibition. The 300ppm treatment also showed considerable antifungal activity, whereas the lower concentrations exhibited only moderate suppression. At 72 hours, the highest inhibition was recorded at 500 ppm with 68.89 per cent inhibition, followed by 400 ppm with 62.22 per cent inhibition and 300 ppm with 45.56 per cent. Lowest inhibition was observed at

200 ppm with 28.89 per cent and 100 ppm with 22.22 per cent. The solvent also showed maximum fungal growth of

8.3 cm, highlighting the effectiveness of higher metabolite concentrations in suppressing *R. solani*.

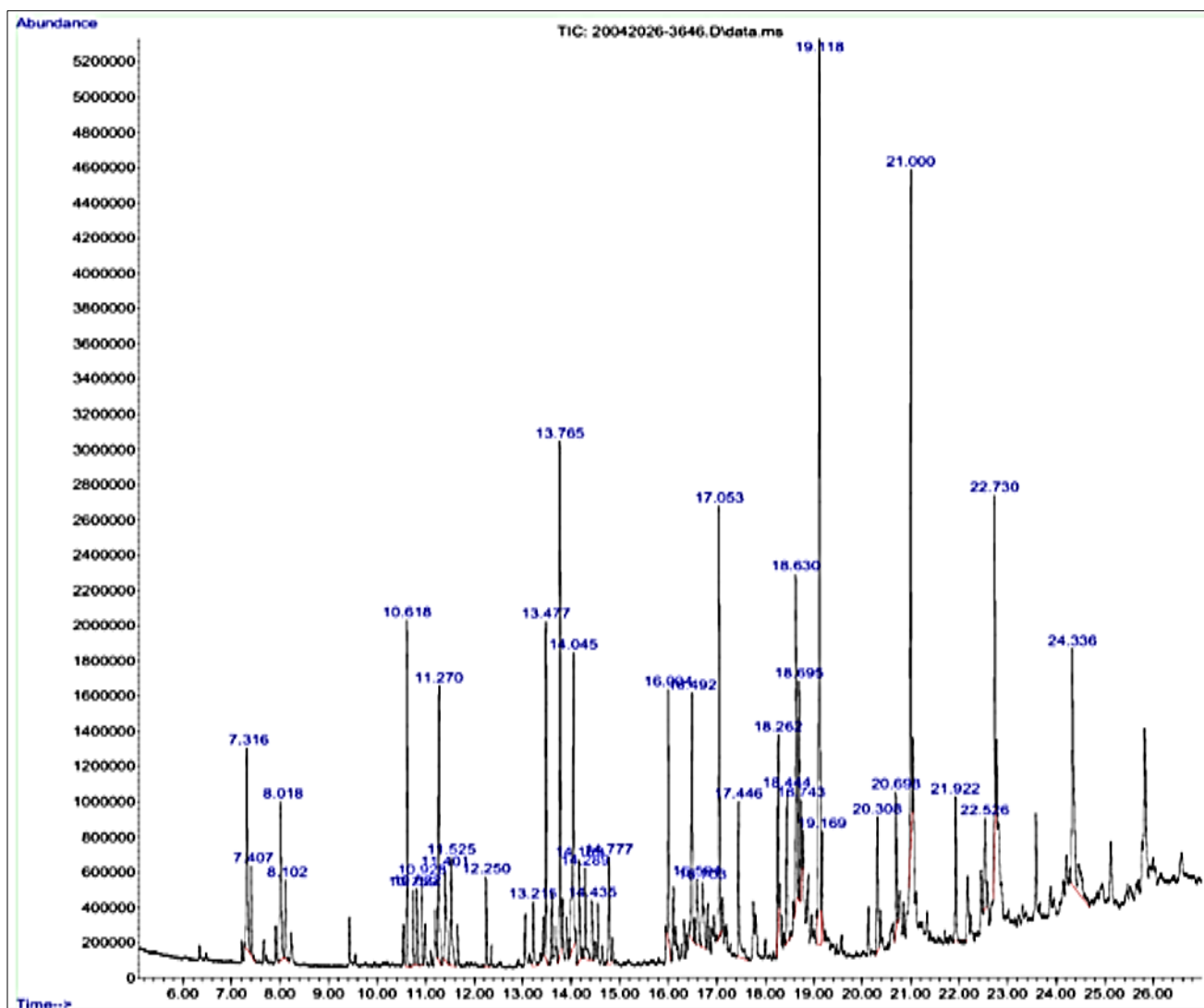


Fig 1: GC-MS Chromatogram of Secondary Metabolites Produced by Endophytic *Bacillus*

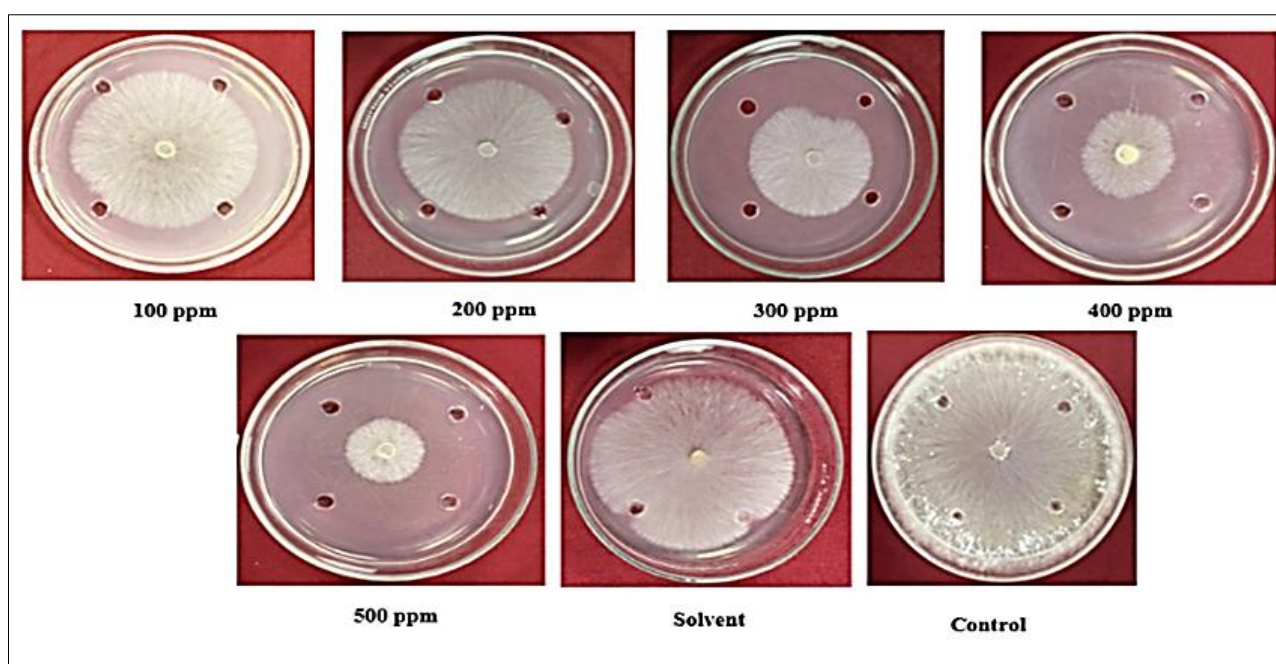


Fig 2: Efficacy of crude metabolites at different concentrations on *R. solani*

Discussion

The GC-MS profile of endophytic *Bacillus* sp. confirmed the production of numerous secondary metabolites with antifungal and antibacterial characteristics. The endophytic bacteria contained several compounds previously reported from biologically active endophytic bacteria. Previous studies by Kaviyarasan *et al.* (2025) ^[20], Sirivella *et al.* (2025) ^[40], Njoroge *et al.* (2024) ^[31], Shabanamol *et al.* (2021) ^[37] and Alvionita *et al.* (2020) ^[1] have similarly reported the detection of diverse antimicrobial metabolites from endophytic bacteria through GC-MS analysis. The identification of several common metabolites between the present and earlier studies suggests that endophytic *Bacillus* sp. possess metabolite profiles associated with antifungal activity and support its potential as promising biocontrol agents against *R. solani*.

The endophytic *Bacillus* sp. effectively inhibited the mycelial growth of *Rhizoctonia solani*. The antifungal activity increased with increasing metabolite concentration, with 500 ppm exhibiting the greatest suppression of fungal growth. The results obtained in the present investigation are similar to the findings of Zheng *et al.* (2021) ^[47], who reported that crude metabolites from an endophytic *Bacillus velezensis* isolate inhibited the mycelial growth of *R. solani*. Similarly, Chiranjeevi *et al.* (2021) ^[10] observed that crude metabolites of *Bacillus subtilis* significantly inhibited the growth of *Rhizoctonia bataticola* and caused structural damage to the fungal hyphae.

Conclusion

GC-MS analysis of *Bacillus* sp. identified several bioactive metabolites, with Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- as the predominant compound, followed by 2,4-Di-tert-butylphenol, 1-Octadecene, Hexadecane, 2,6,11,15-tetramethyl- and Octadecane. The crude metabolites of *Bacillus* sp. exhibited maximum antifungal activity against *R. solani* at 500 ppm, recording 68.89 per cent inhibition, followed by 400 ppm with 62.22 per cent, 300 ppm with 45.56 per cent, 200 ppm with 28.89 per cent and 100 ppm with 22.22 per cent inhibition.

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