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Analysis of Bioactive Secondary Metabolites Produced by Endophytic *Bacillus amyloliquefaciens* against Rice Sheath Blight Pathogen *Rhizoctonia solani*

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ABSTRACT

Background: Bacterial endophytes live inside the plant tissues and are known to play a crucial role in the functioning of host plants by influencing their physiology and development. Moreover, this group of bacteria could produce secondary metabolites to suppress plant pathogens and also produce compounds to promote plant growth.

Methods: In this present study, *Bacillus amyloliquefaciens* was isolated from *Oryzae officinalis* and screened for antifungal activity against rice sheath blight pathogen, *Rhizoctonia solani* by dual culture assay. The secondary metabolites produced during the interaction of *B. amyloliquefaciens* with *R. solani* were characterized with pathogen-inoculated control and antagonist inoculated control through Gas Chromatography-Mass Spectrophotometry (GC-MS).

Result: The isolated *Bacillus amyloliqufaciens* displayed antagonistic activity against *Rhizoctonia solani* which showed a 63% inhibiton in the mycelial growth of *R. solani* over control. Analysis of secondary metabolites from the zone of inhibition, confirmed the presence of unique biomolecules including -Propanamine, N,N,2-trimethyl, 1(2H)-Naphthalenone, 3,4-dihydro-6,7-dimethyl, Diethyl Phthalate, Dichloroacetic acid tridecyl ester, Hexadecen-1-ol trans-9, Glycine N-(N-glycyl-L-leucyl) and 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl of antifungal and antimicrobial nature.

Key words: Antifungal activity, *Bacillus amyloliquefaciens*, Endophytic bacteria, Secondary metabolites.

INTRODUCTION

Sheath blight of rice, a destructive plant disease in almost all rice producing areas, is caused by *Rhizoctonia solani* J.G. Kühn (Zheng *et al*., 2021). Management of this disease includes the use of resistant varieties, soil sterilization and chemical control, the last of which is considered the most effective and reliable practice (Liu *et al*., 2018; Senapati *et al*., 2022). However, potential biological control agents (BCAs) are considered safe and environment-friendly for sustainable management of plant diseases. Many scientists have applied non endophytic biocontrol agents like rhizosphere and phyllosphere organisms to suppress plant diseases (Firdous and Subhash, 2017). Recently, endophytic microorganisms have been preferred over non endophytic microorganisms by researchers as biocontrol agents in agricultural fields because endophytes can take shelter and colonize within host plant tissues very easily and they remain protected in their entire life span (Shah *et al*., 2021). After colonizing within host tissues, they show outstanding performance in overcoming both biotic and abiotic stresses faced by the host plant in the natural environment (Abiodun Ajulo *et al*., 2023).

Endophytic biocontrol organisms can suppress phytopathogens by producing different antagonistic metabolites such as hydrolytic enzymes, lipopeptides and other volatile compounds (Jamali *et al*., 2020; Roy *et al*., 2021). Studies have shown that several genera of endophytic bacteria, such as *Bacillus*, *Pseudomonas*, *Burkholderia*, *Strenotrophomonas*, *Enterobacter*, *Micrococcus* and *Serratia* are capable of synthesizing lytic enzymes, including chitinase, protease and β-glucanase, which play a significant role in degradation of fungal cell wall during antagonistic interactions (Kumar *et al*., 2020; Gorai *et al*., 2023).

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In the recent past, *Bacillus* spp. have been well explored for the management of soil-borne and foliar diseases. Endophytic *Bacillus* spp. have been explored for the management of sheath blight pathogen as they are bestowed with beneficial attributes including plant growth promotion and induction of immune response (Firdous *et al*., 2019; Nakkeeran *et al*., 2021;). Exploring the diversity of bacterial endophytes in rice varieties and landraces is crucial for the identification of potential endophytes for plant growth promotion and biocontrol agents. Antimicrobial substances produced by *Bacillus* spp. also play an important role in biocontrol of plant diseases and the functional mechanisms mainly include direct antagonism, induction of plant resistance and aid in the colonization of the plant surface by biocontrol bacteria (Nakkeeran *et al*., 2019; Ali *et al*., 2021). In this context, the current study focussed on analysing the secondary metabolites produced by the *B. amyloliquefaciens* during their ditrophic interaction with *R. solani*.

MATERIALS AND METHODS

Isolation and molecular identification of rice sheath blight pathogen

Rice plants showing typical sheath blight symptoms were collected from diverse rice-growing areas in Tamil Nadu during 2021-2022. Infected plant tissue were cut into small pieces, sterilized by immersing in 1% sodium hypochlorite solution for 1 minute and then passed through sterilized distilled water. Surface sterilized tissues were dried with sterile filter paper and then transferred to a Petri dishes containing Potato Detrose Agar followed by incubation at 28±2°C for 5-7 days (Sandoval and Cumagun, 2019). The emerging mycelia were purified by single hyphal tip method.

Genomic DNA of *R. solani* was extracted from the mycelium of pure culture through the CTAB method (Griffith and Shaw, 1998) and utilised as a template for PCR amplification using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) primers (White *et al*., 1990). The program cycle comprised of initial denaturation (95°C) for 2min, followed by 40 cycles of denaturation (95°C) for 1min, annealing at 58°C for 1min, extension for 1min at 72°C and a final extension at 72°C. Gel electrophoresis and staining was done by loading 5 µl of PCR product on 1% agarose gel in TAE buffer at 80V for 50min at 25°C. A 1 kb DNA ladder was utilised to ascertain the size of amplified genomic products. The PCR product was sequenced by Sanger dideoxy method in Biokart Pvt. Ltd., Bangalore, India. The obtained DNA sequences were assessed for similarity by comparing with sequences in the NCBI database. Newly obtained sequences were submitted to the GenBank database and accession number was obtained.

Biocontrol agent used for controlling the pathogen

One Gram-positive endophytic bacterial strain, *Bacillus amyloliquefaciens* B6 (GenBank accession no. OQ943610.1), isolated from leaves of *Oryzae officinalis* was used for the present study (Tian *et al*., 2023).

Antagonistic activity of *B. amyloliquefaciens* B6 against the *R. solani*

The efficacy of the bacterial endophyte *B. amyloliquefaciens* B6 against the virulent strain of *R. solani* was assessed through the dual culture method. A 9 mm mycelial disc of a five-day old culture of the pathogen was placed in one side of the Petri plate. Then, exponentially growing bacterial cells were streaked on the opposite side of the agar (PDA) Petri plate. Plates without bacterial inoculated fungal discs were used as control. The percentage of radial growth inhibition (I) was calculated by the formula:

$$I\ (\%) = \frac{C - T}{C} \times 100$$

Where,
C = Diameter of a fungal colony in control plates.
T = Diameter of a colony in treated plates.

GC-MS analysis of bioactive metabolites extracted from the zone of inhibition of *B. amyloliquefaciens* and *R. solani*

Metabolites produced by the endophytic bacteria were characterized by utilizing Gas Chromatography-Mass Spectrophotometry (GC-MS) (GC claurus SQ8C 500 Perklin Elmer, USA), which is available at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The bioactive compounds produced by the endophytic *B. amyloliquefaciens* B6 during the di-trophic interaction with *R. solani* were extracted from the zone of inhibition in the dual culture plate as per the procedure of Cawoy *et al*. (2015). PDA medium from the zone of inhibition of dual culture plate was excised with a sterile scalpel and blended with HPLC-grade acetonitrile in 1:4 ratio (5 g agar in 20 ml of HPLC grade acetonitrile). The mixture was homogenized by sonicating twice for 30 seconds using a power sonicator. The homogenized samples were centrifuged and filtered through sterile What No. 1 filter paper to remove solid particles. The filtrates were dried in a vacuum flash evaporator (Rotrva Equitron Make) and the final product was dissolved in 1 ml of HPLC grade methanol.

RESULTS AND DISCUSSION

Biological control of plant diseases by beneficial microorganisms is considered to be an environmentally friendly, effective, economical and sustainable approach. Endophytes have a higher antagonistic potential against plant disease than microorganisms isolated from the rhizosphere or soil because they exist in a stable environment inside the plant and can be found in various host plants. Endophytes are implicated in the control of plant disease, plant growth promotion, development of plant tolerance, nitrogen fixation, synthesis of novel bioactive compounds and detoxification of toxic pesticides.

In our current investigation, *R. solani* was isolated from infected sheath blight sample of rice and identified based

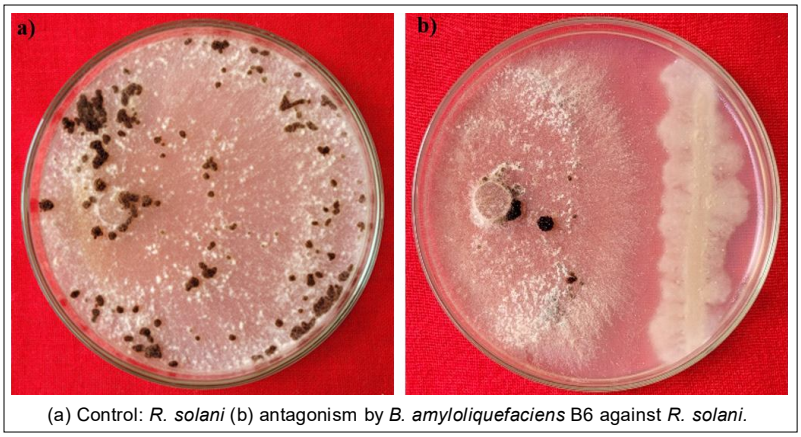
on the sequencing of internal transcribed spacer (ITS) regions. Amplified products of the ITS region of rRNA gene produced a single discrete band of 4 650 bp on a 1% agarose gel. The ITS/ITS4 sequence of the *R. solani* isolate was submitted to NCBI and accession number (OQ940459.1) was obtained which shared over 99.19% similarity with *R. solani* isolate RMPM13 (JF701748.1). Further, endophytic bacterial strain *B. amyloliquefaciens* B6 isolated from *O. officinalis* was tested for its antagonistic activity against the pathogen under *in vitro* conditions. Similarly, Tian *et al*., (2023) isolated a total of 96 endophytic bacterial strains from *O. officinalis*, of which 11 strains performed promising PGP traits on perennial rice seedlings. These 11 strains belonging to the *Enterobacter*, *Bacillus*, *Pseudomonas* and *Kosakonia* species were reported to have utilization potential as biofertilizers for the sustained productivity of perennial rice.

In vitro study revealed significant (63%) inhibition of mycelial growth of the *R. solani* by the test bacterium compared to untreated control (Fig 1). Accordingly, several previous studies have reported the antimicrobial activity of *Bacillus amyloliquefaciens* against various fungal phytopathogens like *Fusarium asiaticum*, *Fusarium graminearum*, *Rhizoctonia solani* and *Botrytis cinerea* (Gupta *et al*., 2016; Boottanun *et al*., 2017; Ntushelo *et al*., 2019). The suppression of *Rhizoctonia* sp. by specific microorganisms is often associated with the formation of secondary metabolites that are toxic to the pathogen (Lee *et al*., 2023). The compounds used for biocontrol of *R. solani* are usually antibiotics or enzymes that lyse the fungal cell wall (Ghasemi *et al*., 2020).

The bioactive metabolites of *B. amyloliquefaciens* and their di-trophic interaction with *R.solani* were analyzed using GC-MS chromatography. *B. amyloliquefaciens* B6, *R. solani* and their di-trophic interaction were profiled for a total of 30 biomolecules upon elimination of compounds in the PDA medium (control). The antifungal actions of these extracts could be related to various chemical classes, including esters, fatty acids, aldehydes, tertiary amines, alkaloids and ketones. A total of twelve bioactive metabolites were produced by *R. solani* in PDA medium, which were identified as a Naphthalene, squalene, Trans-geranylgeranio, Oleic acid, Hexadecanoic acid 1 4- methyl ester, 1-Hexadecanol 2-methyl, Phenol, Tetradecanoic acid 12 -methyl methyl ester, Dichloroacetic acid, Trichloroacetic acid tridecyl ester and 1-Hexadecane (Table 1).

B.amyloliquefaciens B6 generated a total of 16 compounds in the absence of *R. solani*, of which 11 were unique to the *B. amyloliquefaciens* which include 2,8,9-Trioxa-5-aza-1-silabicyclo [3.3.3] undecane 1-methyl, 1-Undecanol, Trichloroacetic acid dodecyl ester, Pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methylpropyl), 9-Eicosene, (E), 1,3-Benzenedicarboxylic acid bis(2-ethylhexyl) ester, 2-(2-Diethyl amino ethyl amino) ethanol, Dodecyl acrylate, 4(1H)-Pyrimidinone 6-amino-2-methyl-5-nitroso, Hexanedioic acid bis(2-ethylhexyl) ester and Isovaline 3-methyl (Table 2, Fig 2). Solimon *et al*. (2022) reported the compound, Bis (2-ethylhexyl) ester synthesized by both *B. Amyloliquefaciens* and *B. velezensis* possesses antifungal, antimicrobial and antioxidant activities against plant pathogenic fungi. Further, Raut *et al*. (2021) reported the antimicrobial action of Pyrrolo[1,2-a]pyrazine-1,4-dione against *Alternaria macrospora*.

B. amyloliquefaciens B6 synthesized 11 metabolites during its interaction with *R. solani*, of which 7 were formed exclusively during their interaction and they are 2-Propanamine, N,N,2-trimethyl, 1(2H)-Naphthalenone, 3,4-dihydro-6,7-dimethyl, Diethyl Phthalate, Dichloroacetic acid tridecyl ester, Hexadecen-1-ol trans-9, Glycine N-(N-glycyl-L-leucyl) and 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl (Table 3, Fig 3). None of the biomolecule was produced in common by the axenic culture of *B.amyloliquefaciens* B6 and during its interaction with *R. solani*. The compound phenol was shared in common between *R. solani* and its interaction with *B. amyloliquefaciens* B13. The Venn diagram of differentially expressed bioactive metabolites during the interaction of bacterial endophyte *B. amyloliquefaciens* B6 alone, *R. solani* alone and their di-trophic interaction



(a) Control: *R. solani* (b) antagonism by *B. amyloliquefaciens* B6 against *R. solani*.

Fig 1: Dual culture plate method showing inhibition of radial growth of *R. solani* by endophytic *B. amyloliquefaciens*.

Table 1: Diversity of secondary metabolites produced by *R. solani* in PDA medium.

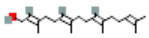
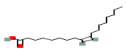
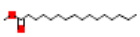
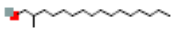
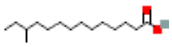
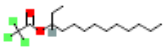
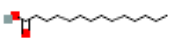
Compound name	Retention time	Peak area percentage	Molecular weight/ Molecular formula	Molecular structure	Function
Naphthalene	7.22	8.04	128.17 $C_{10}H_8$		Antibacterial
Squalene	24.42	19.27	410.7 $C_{30}H_{50}$		Antibacterial
Trans-geranylgeranio	24.24	23.27	290.5 $C_{20}H_{34}O$		Antimicrobial
Oleic acid	25.47	0.54	282.5 $C_{18}H_{34}O_2$		Antifungal
Hexadecanoic acid, 1 4-methyl-methyl ester	10.53	0.67	270.5 $C_{17}H_{34}O_2$		Antimicrobial
1-Hexadecanol, 2-methyl-	21.86	0.72	256.5 $C_{17}H_{36}O$		Antimicrobial
Phenol	4.14	0.78	94.11 C_6H_5OH		Antimicrobial
Tetradecanoic acid, 12-methyl-, methyl ester	10.26	1.95	242.40 $C_{15}H_{30}O_2$		Antimicrobial antioxidant
Dichloroacetic acid	17.96	0.55	128.94 $C_2H_2Cl_2O_2$		Antimicrobial
Trichloroacetic acid, tridecyl ester	4.14	0.78	345.7 $C_{15}H_{27}Cl_3O_2$		Antimicrobial
Tetradecanoic acid	5.01	0.92	228.37 $C_{14}H_{28}O_2$		Antibacterial
1-Hexadecane	29.46	1.15	226.44 $C_{16}H_{34}$		Antimicrobial

Table 2: Diversity of secondary metabolites produced by *B.amyloliquefaciens* B6.

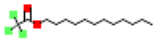
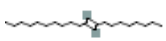
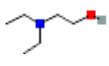
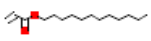
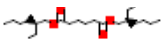
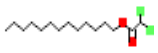
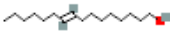
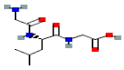
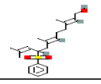
Compound name	Retention time	Peak area percentage	Molecular weight/ Molecular formula	Molecular structure	Function
2,8,9-Trioxa-5-aza-1-silabicyclo [3.3.3] undecane, 1-methyl-	10.20	2.01	232.35 $C_9H_{20}N_2O_2Si$		Antifungal
1-Undecanol	13.74	2.64	172.31 $C_{11}H_{24}O$		Antifungal
Trichloroacetic acid, dodecyl ester	17.90	2.84	331.7 $C_{14}H_{25}Cl_3O_2$		Antibacterial
Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	20.60	2.14	196.25 $C_{10}H_{16}N_2O_2$		Antimicrobial
9-Eicosene, (E)-	21.83	1.80	280.5 $C_{20}H_{40}$		Antifungal
1,3-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester	29.47	2.99	390.6 $C_{24}H_{38}O_4$		Antifungal
2- (2-Diethyl amino ethyl amino) ethanol	24.62	1.23	117.19 $C_6H_{15}NO$		Antifungal
Dodecyl acrylate	24.44	4.49	240.38 $C_{15}H_{28}O_2$		Antifungal
4 (1H)-Pyrimidinone, 6-amino-2-methyl-5-nitroso-	7.17	7.95	154.13 $C_5H_6N_4O_2$		Antimicrobial
Hexanedioic acid, bis (2-ethylhexyl) ester	21.83	1.80	370.6 $C_{22}H_{42}O_4$		Antifungal
Isovaline, 3-methyl-	21.22	1.17	117.15 $C_5H_{11}NO_2$		Antimicrobial

Table 3: Diversity of secondary metabolites produced in the axenic culture of *B. amyloliquefaciens* B6 and its interaction with *R. solani* in PDA medium.

Compound name	Retention time	Peak area percentage	Molecular weight/ Molecular formula	Molecular structure	Function
2- Propanamine, N,N,2- trimethyl-	5.02	1.04	101.19 $C_6H_{15}N$		Antifungal
1 (2H)- Naphthalenone, 3, 4- dihydro-6,7-dimethyl-	10.24	1.02	178.18 $C_{10}H_{10}O_3$		Antimicrobial
Diethyl phthalate	13.95	2.97	222.24 $C_{12}H_4O_4$		Antifungal
Dichloroacetic acid, tridecyl ester	17.94	1.49	311.3 $C_{15}H_{28}Cl_2O_2$		Antimicrobial
Hexadecen-1-ol, trans-9-	21.87	1.04	240.42 $C_{16}H_{32}O$		Antifungal
Glycine, N-(N-glycyl-L-leucyl)-	7.23	4.95	245.28 $C_{10}H_{19}N_3O_4$		Antibacteral
2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl	24.31	8.82	362.5 $C_{21}H_{30}O_3S$		Antimicrobial

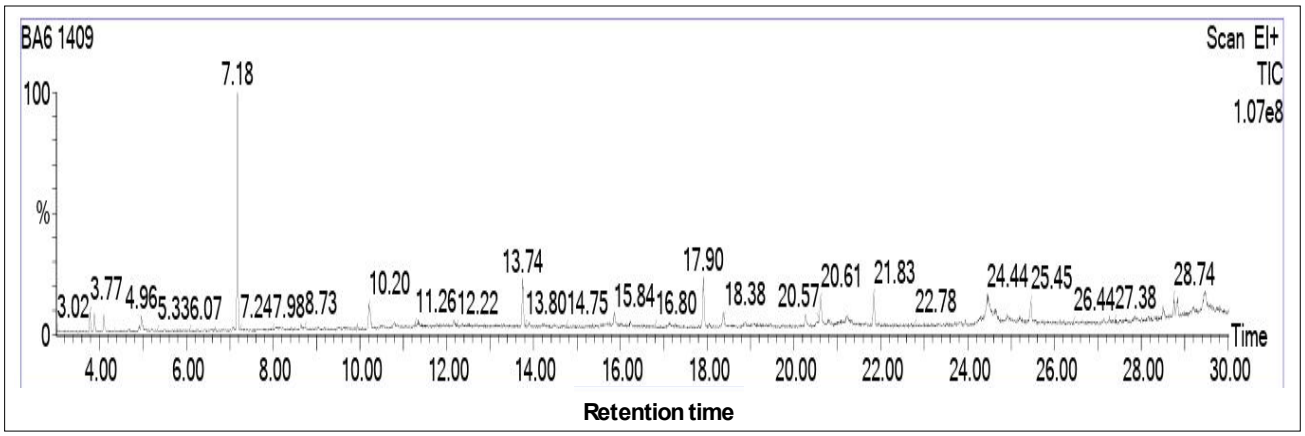


Fig 2: GCMS chromatogram of secondary metabolites produced by *B. amyloliquefaciens* B6.

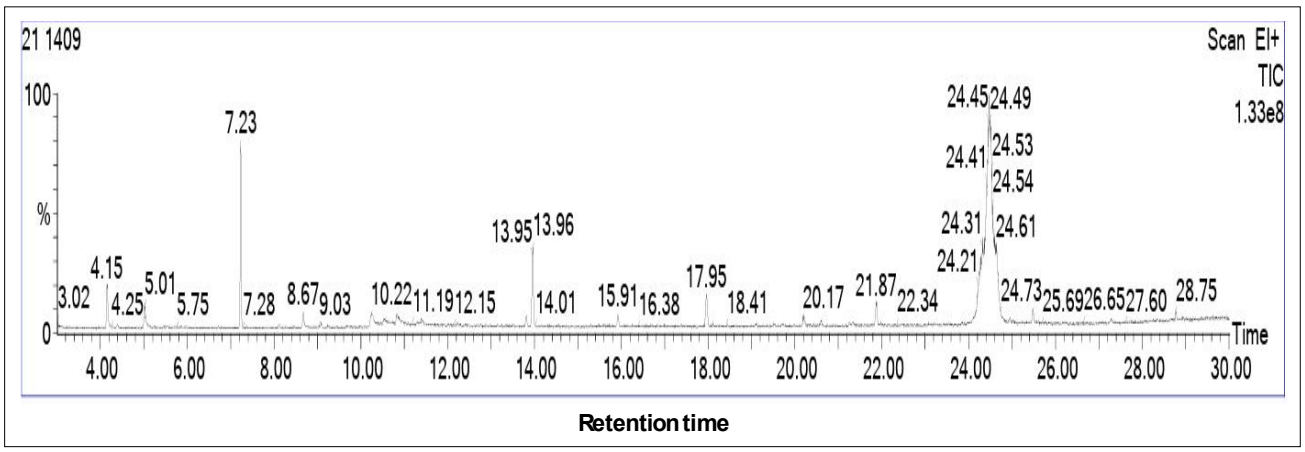


Fig 3: GC MS chromatogram of secondary metabolites produced during the interaction of *B. amyloliquefaciens* B6 and *R. solani*.

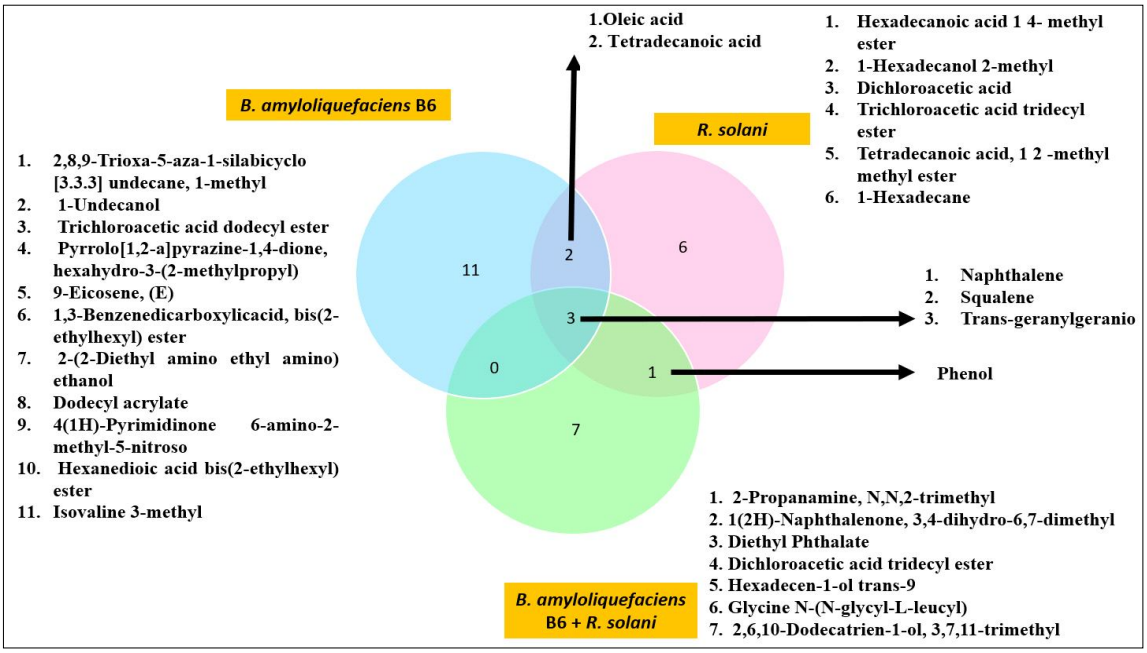


Fig 4: Comparative distribution of metabolites produced by *B. amyloliquefaciens* B6, *B. amyloliquefaciens* B6 + *R. solani* and *R. solani* alone.

revealed three common biomolecules *viz*., Naphthalene, Squalene and Trans-geranylgeranio (Fig 4). Raza *et al*., (2016) reported the antibacterial nature of naphthalene compound produced by *B. amyloliquefaciens* against *Ralstonia solanacearum*. Shahzad *et al*. (2017) discovered the endophytes capability to produce secondary metabolites provided additional support to plants and increased plant development, increasing their resilience to biotic and abiotic challenges.

CONCLUSION

In summary, a bacterial strain *B.amyloliquefaciens* B6 which was isolated from *O. officinalis* showed inhibitiory activity against the rice sheath blight pathogen, *R. solani*. Antifungal secondary metabolites from endophytic *B. amyloliquefaciens* B6 was responsible for the suppression of *R. solani* under *in vitro* conditions. Controlling plant pathogen diseases in a safe, effective and alternative manner has become increasingly crucial for improving the quality of agricultural products. Compared to chemical control, biological control using antagonistic microorganisms, such as bacteria, is a long-term approach to inhibiting plant pathogens.

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Conflict of interest

The authors declare that they have no conflict of interest.

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