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Antifungal Activity of *Bacillus* spp. against *Curvularia lunata* causing Grain Discolouration of Rice

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ABSTRACT: Grain discoloration complex disease of rice is an emerging threat to rice crop all over the world and it acquires particular importance as it results in qualitative loss of harvested crop. The study's objective is to evaluate the effective *Bacillus subtilis* strain BS3 is at preventing rice grain discoloration. Twenty *Bacillus* spp. were examined in vitro for their ability to inhibit *Curvularia lunata*. The most effective isolate was chosen based on the antagonism and validated by 16s rRNA. PCR investigation revealed the antibiotic biosynthetic genes that produce bacillomycin, bacilysin, iturin, surfactin, subtilin, mersacidin, subtilosin, ericin, mycosubtilin, and fengycin in the successful isolates. Among the isolates, BS3 inhibited the pathogen's mycelial development the most (48.8%) and had the most antibiotic genes (6). By using gas chromatography mass spectrometry, secondary metabolites from *B. subtilis* (BS3) were discovered as Phenylethyl alcohol, Benzene ethanol, 4-hydroxyquinoline, Pyrrolo (1,2-a) pyrazine, 1-4-dione, and hexahydro-3 (GC-MS). The present investigations used GCMS to find a volatile and antifungal secondary metabolite along with evaluate a bacterial antagonist *B. subtilis* (BS3) with strong antifungal potential that could be employed as a biocontrol agent for rice disease induced by *C. lunata* grain discoloration.

Keywords: *Curvularia lunata*, *Bacillus* spp., secondary metabolites, GCMS.

INTRODUCTION

Grain discolouration is an emerging disease of rice crop, and reduces marketability and superiority of rice (Khamari, 2020). By 2025, the world's population is expected to exceed 8 billion people, and rice production would need to expand by 40% to fulfil rising food demands (Yadav *et al*., 2018; Jena *et al*., 2018). Rice crops are attacked by 50 diseases, including six bacterial, twenty-one fungal, four nematode, twelve viral, and seven other diseases and disorders (Jabeen *et al*., 2012). Arshad *et al*., (2009) reported many fungi are associated with grain discolouration *viz*., *Alternaria padwickii*, *Pyricularia oryzae*, *Bipolaris oryzae*, *Fusarium moniliforme*, *F. graminearum*, *Nigrospora oryzae*, *Phoma sorghina*, *Dichotomophthoropsis nymphacearum*, *Heterosporium echinunulatum*, *Curvularia lunata and Alternaria alternata*. Out of which *Curvularia lunata* (Wakker) Boedign is predominant (Koulagi *et al*., 2011; Persaud *et al*., 2020). The loss due to grain discoloration in rice was estimated approximately about 20 to25 percent (Ghose *et al*., 1960). Baite *et al*., (2019) reported the incidence of grain discoloration ranged from 25 to 92% in different rice varieties. Biological control through the application of antagonistic microorganisms or their bioactive agents is an effective and environmentally safe strategy for plant protection (Sukanya Saechow *et*

al, 2016). When used as a biocontrol agent, *Bacillus* spp. utilizes a multitude of approaches to combat plant infections, including antibiotic synthesis, direct pathogen suppression, plant growth promotion, and induction of defence enzymes (Elanchezhiyan *et al*., 2018). The present study was focused on the *B. subtilis* (BS3) have strong antifungal potential that could be active as a biocontrol agent for *C. lunata* which causing grain discoloration of rice.

MATERIALS AND METHODS

Isolation and identification of *C. lunata.* The pathogenic fungus, *C. lunata* was isolated from diseased rice seeds collected from paddy fields in various districts of Tamilnadu by tissue segment method. Then the cultured plates were incubated at 28°C for seven days. Morphological and molecular analysis were used to identify the pathogen.

Isolation and biochemical characterization of *Bacillus* isolates from rhizosphere region. Soil samples were gathered from the rhizosphere of rice plants in various rice-growing regions of Tamil Nadu. 10 g of rhizosphere soil was transferred to a 250 ml conical flask containing 100 ml of sterile water. After thorough shaking, the antagonist in the suspension was isolated by serial dilution plate method (Pramer and Schmidt 1956). From the final dilutions of 10^{-5} and 10^{-6} one ml of each aliquot was pipetted out, poured into the sterilized Petri plate containing Nutrient Agar Medium. The plates were gently rotated clock wise and anticlockwise for uniform distribution and incubated at room temperature (28±2 °C) for 24 hours. Colonies with characteristics of *Bacillus* spp. were isolated individually and purified by streak plate method on NA medium. The pure cultures were maintained for further studies. A total of 20 isolates were collected and tested for their ability to inhibit *C. lunata* growth. Based on the diagnostic test described in Bergey's manual for determinative bacteriology, the bacterial antagonists were identified and characterised (Bergey *et al*., 1939). Gram reaction, catalase test, voges proskauer, growth in NaCl, growth at 4 ˚C and 45˚C, and starch hydrolysis were performed for *Bacillus* spp. confirmation.

Screening of antagonistic bacteria against *C. lunata* in *in vitro.* The antifungal potency of bacterial isolates was investigated using the dual culture approach (Dennis and Webster, 1971). The fungal mycelial disc was placed at one end of the plate, followed by bacterial antagonists streaking just opposite the pathogen, and the plates were incubated at 25 ± 2 °C. After a 7-day incubation period, the inhibition zone and fungal mycelial growth were measured. The percent inhibition was computed as [(C-T)/C] 100, where T and C are the pathogen diameters in mm in the treatment and control plates, respectively. The tests were carried out in thrice.

Effect of *Bacillus* spp. produced volatile organic compounds against *C. lunata.* Using the paired dish approach, the antifungal volatile compounds produced by the potent *Bacillus* isolates were evaluated *in vitro* against *C. lunata* (Laha *et al.*, 1996). The most effective isolates were selected based on the dual plate assay and then tested in vitro. The experiment was carried out three times. After incubation, the pathogen's percent inhibition was estimated using Dennis and Webster's formula (1971).

Molecular characterization of bacterial strains. DNA was isolated using the modified Cetyl trimethyl ammonium bromide (CTAB) method, Melody (1997) which was confirmed by PCR analysis using the 16S rRNA intervening sequence specific primer pairs 27F (5'- AGAGTTTGATCCTGGTCAGAACGCT-3') and 1492R (5'-TACGGCTACCTTGTTACGACTTCACCCC-3') proposed by Cano et al (1994). In a 20µl reaction tube, 50 ng of total DNA, 5 mM each of the dNTPs, 20 mol each of the forward and reverse primers, and 0.5 U of Taq DNA polymerase were used. Initial denaturation at 95°C for 10 minutes was followed by 30 cycles of denaturation at 95°C for 30s, annealing at 52°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes on a thermal cycler. (Bioserve scientific company, Hyderabad) sequenced the amplified product and used the BLAST tool to search the GenBank database for similarity. Phylogeny investigations were conducted using the Neighbor Joining method with 1000 bootstrap replicates and a cut-off value of 80% based on their 16S rRNA region using the MEGA 6.0 tool and condensed with a cut-off value of 80%.

Antimicrobial peptide detection (AMP). Genes involved in biosynthesis using their unique primers, PCR assays were done to assess the existence of AMP biosynthesis genes such as iturin A, iturin C, surfactin, bacillomycin A, and bacillomycin D.

Characterization of secondary metabolites by GC-MS. The crude metabolites were extracted according to Prapagdee *et al*., (2000) methodologys *Bacillus* spp. extracts were chosen based on the growth inhibition experiments, and chemical components were measured using a Shimadzu Gas chromatography equipped with a mass detector Turbo mass gold containing an Elite-1 (100 percent Dimethyl Poly Siloxane), 30 m 0.25 mm ID 1 mM df. The following conditions were used: Helium (1 ml/min) as carrier gas; oven temperature programed from 110°C (2 min) to 280°C (9 min); injector temperature (250°C); total GC time (45 min). In 1.0 ml aliquots, the ethyl acetate extract was put into the chromatograph. The principal elements were identified using a computer-driven technique and then by comparing the mass spectrum of the study to that of a library from the National Institute of Standards and Technology (NIST) (Version. 2.0, year-2005). Turbo mass-5.1 was utilised for the GC-MS. This work was done at the Center of Innovation, Agricultural College& Research Institute, Madurai.

Statistical Analysis. The treatment mean differences were analysed using ANOVA and Duncan's Multiple Range Test with a 5% significance level (Gomez and Gomez, 1984).

RESULTS

Isolation and screening of antagonistic bacteria. In total, 20 bacterial isolates were obtained from various rice field rhizosphere soil samples. Among these, only 10 isolates showed antifungal activity against *C. lunata* using the dual culture technique. The highest percent inhibition zone were observed with isolate *B. subtilis* strain BS3 as shown in Table 1 and Fig. 1 BS3 was therefore selected and used for further biological studies.

Biochemical characterization of *Bacillus* spp. Twenty *Bacillus* spp. isolates were reported to have positive findings in the gram staining, catalase test, voges proskauer, growth in NaCl, growth at 45˚C, and starch hydrolysis tests. Anaerobic growth and growth at 4˚C were both negative for these isolates.

Efficacy of *Bacillus* spp. against *C. lunata* under *in vitro*. By using a dual culture approach, twenty *Bacillus* spp. isolates were investigated for their antagonistic activity against *C. lunata*. Among the isolates studied, BS3 had the greatest (48.8%) inhibition of pathogen mycelial growth (4.6 cm) at 7 days following inoculation, followed by BS11, which had 46.6 percent inhibition of pathogen mycelial development over control. At 7 days following incubation, BS2 was found to have the lowest suppression of pathogen mycelial growth, accounting for a 22% growth reduction above control (Table 1, Fig. 1).

Table 1: Antifungal activity of *Bacillus* spp. against *C. lunata* under *in vitro*.

Sr. No.	Bacterial isolate	Mycelial growth (cm)*	Percentage of inhibition
1.	BS1	6.2^{ef}	31.1
2.	BS2	7.0^{h}	22.2
3.	BS3	4.6^{a}	48.8
4.	BS4	5.2^{ab}	42.2
5.	BS5	6.8^{gh}	24.4
6.	BS6	5.5^{bc}	38.8
7.	B7S	5.2^{ab}	42.2
8.	BS8	6.0^{de}	33.3
9.	BS9	5.0^{ab}	44.4
10.	BS10	5.3^{ab}	41.1
11.	BS11	4.8^{ab}	46.6
12.	BS12	6.5^{gh}	27.7
13.	BS13	6.3^{fg}	30.0
14.	BS14	5.7^{cd}	36.6
15.	BS15	6.6^{gh}	26.6
16.	BS16	6.0^{de}	33.3
17.	BS17	6.9^{h}	23.3
18.	BS18	5.1^{ab}	43.3
19.	BS19	5.4^{ab}	40.0
20.	BS20	6.3^{fg}	30.0
21.	Control	9.0	0.00
CD (0.5) = 0.25 CV% = 2.64			

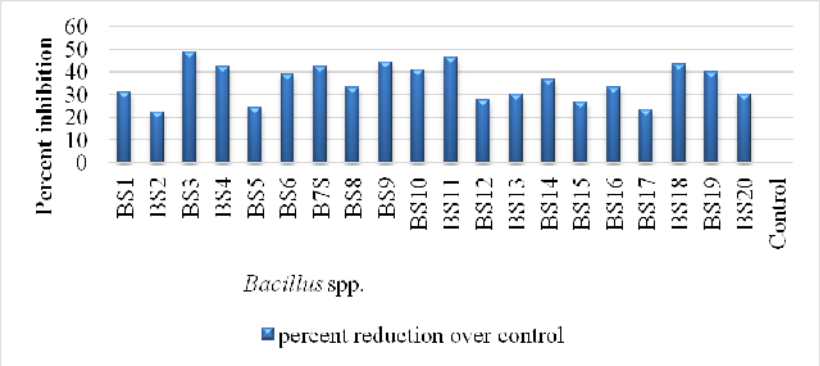


Fig. 1. Antifungal activity of *Bacillus* spp. against *C. lunata* under *in vitro*.

Efficacy of *Bacillus* spp. volatile compounds against the growth of *C. lunata in vitro*. At 15 days after incubation, the results on the radial growth of C. lunata due to the production of volatile compounds by *Bacillus* spp. isolates revealed that BS3 had the highest reduction of 61.62 percent (3.3 cm), followed by *Bacillus* spp. isolate BS11, which had the lowest reduction of 53.48 percent (4.0 cm) (Table 2).

Table 2: Effect of VOC produced by *Bacillus* spp. on *C. lunata* mycelial growth.

Sr. No.	Bacterial isolate	Mycelial growth (cm)*	Percentage of inhibition
1.	BS3	3.3[a]	61.62
2.	BS8	5.4[c]	37.20
3.	BS11	4.0[b]	53.48
4.	BS18	5.0[c]	41.86
5.	BS19	6.3[d]	26.74
6.	Control	8.6	00.00
CD (0.5) = 0.12 CV % = 1.34			

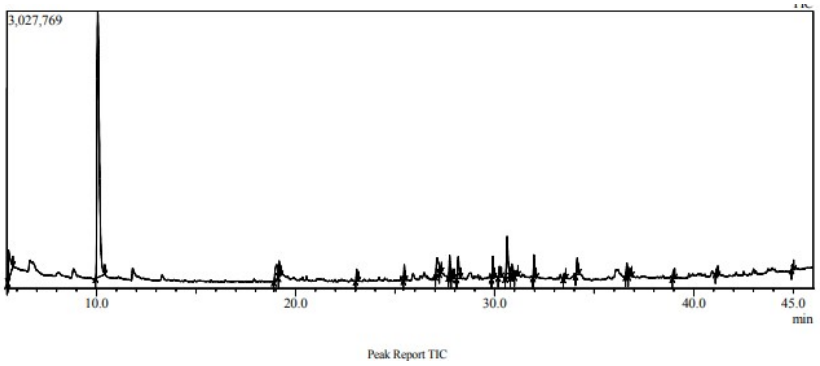
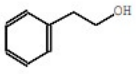
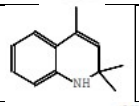
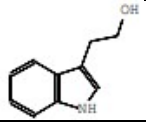


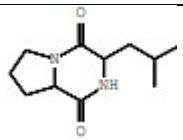
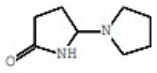
Fig. 2. Characterization of secondary metabolites by GC/MS.

Characterization of the effective antagonistic bacteria at the molecular level. Based on the 16s rRNA intervening sequence specific primers, the effective strain BS3 was identified as *Bacillus subtilis* (GeneBank Accession No. MZ080626) with a fragment size of 1492 bp (Fig. 2). The sequence was found to be 100 percent identical to *B. subtilis* sequences in Gene Bank.

Characterization of secondary metabolites by GCMS. *Bacillus* spp. produced a variety of antifungal chemicals, according to GC-MS analyses. GCMS was used to examine the crude metabolites of virulent *Bacillus* spp. isolate BS3 in order to detect the synthesis of antibiotic chemicals. Antifungal chemicals such as phenylethyl alcohol, benzene ethanol, 4-hydroxyquinoline, Pyrrolo (1, 2-a) pyrazine, 1-4-dione, and hexahydro-3 (Table 3, Fig. 3,).

Table 3: Identification of secondary metabolites from *B. subtilis* (BS3) through GC/MS.

Peak	Retention Time	compound Name	Structure	Molecular Formula	Molecular Weight (g/mol)	Peak area %
3	10.065	Phenylethyl alcohol		C8H10O	122	55.62
4	19.025	Benzenethanol, 4-hydroxy		C8H10O2	138	3.67
5	19.175	Quinoline, 1,2-dihydro-2,2,4-trimethyl		C12H15N	173	2.08
8	27.113	Tryptophol		C10H11NO	161	3.39

12	28.172	Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3		C11H18N202	210	2.70
13	29.908	5-Pyrrolidino-2-pyrrolidone		C8H14N2O	154	1.70

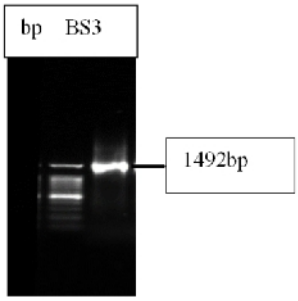


Fig. 3. PCR amplification of *Bacillus subtilis* strain BS3.

DISCUSSION

Nowadays, Several researchers have demonstrated the antifungal potential of *Bacillus* sp. by producing antibiotics and secondary metabolites, and there is growing need for a safe and environmentally friendly alternative to conventional fungicides for plant disease control. The antagonistic effect of *Bacillus* isolates against *C. lunata* was investigated in vitro in this work, and the strain BS3 was found to have the greatest antagonistic capability against *C. lunata* in limiting mycelial growth. Sukanya Saechow *et al*., (2016) discovered that isolate *B. subtilis* (BAS114) isolated from soil has considerable antagonistic activity against *C. lutana* (*in vitro*), inhibiting radial growth by 50.67 ± 1.15 percent. Rahma and Kristina (2021) shown that rhizobacteria can compete with *C. lunata*. In this context, the antagonistic effects of *Bacillus* strains against *B. oryzae* were investigated *in vitro*, and the strain BS5 was found to have the greatest antagonistic potential against *B. oryzae* in terms of limiting mycelial growth. Similarly, Using *Bacillus* strains, Shrestha *et al.,* (2016) exhibited mycelial growth suppression of many plant diseases, including rice pathogens. Antimicrobial peptides such as fengycin, iturin, surfactin, zwittermycin, bacillomycin, and bacteriocin are secreted by *Bacillus* sp. (Ramarathnam *et al*., (2007); Xie *et al*., (2009)). Cell leakage is caused by AMPs that create gaps in the cellular membrane, reduce callose deposition in the fungal cell wall, disturb the lipid bilayer structure, and cause deformity of fungal hyphae due to significant morphological changes, among other things (Krishnan *et al.,* 2015). These causes inhibition and death of the mycelia.

According to our findings, the isolate BS3 showed amplification of five genes: bacD, ituC, ituA, srfA, and bamC, suggesting it could produce bacilysins, iturins, subtilosins, surfactin, and bacillomycin. Vinodkumar *et al*., (2017) established the existence of eleven antimicrobial peptides in diverse Bacillus strains, including subtilin, subtilosin, bacilysin, bacillomycin, surfactin, and iturins, and documented their significance in *Sclerotinia sclerotiorum* hyphal abnormalities. We theorised that the presence of these cyclic lipopeptides was involved in *B. oryzae* suppression. The antifungal properties of VOCs produced by 10 endophytic *Bacillus* strains isolated from *E. ulmoides* were studied by Xie *et al*., (2020). By the third day of treatment, *B. subtilis* strain DZSY21 had significantly suppressed *C. lunata* mycelia growth, and the inhibition rate had reached 36.2 percent by the seventh day. By using gas chromatography mass spectrometry, secondary metabolites from *B. subtilis* (BS3) were discovered as Phenylethyl alcohol, Benzene ethanol, 4-hydroxyquinoline, Pyrrolo (1,2-a) pyrazine, 1-4-dione, and hexahydro-3 (GC-MS).Similiarly, the GC-MS study of volatile organic chemicals produced by *Bacillus* spp., such as N, N-Dimethyl, 1,2-benzenedicarboxylic acid, and 9-octadecenoic acid, identified by Jangir *et al*. (2018), may have been responsible for limiting the growth of *F.o.f.sp.lycopersici*. Pyrrolo demonstrated antifungal properties against the infection as well (Jiang *et al*., 2014). According to Gajera (2018), the *B. subtilis* strain JNDKHGn-29-A can suppress Aspergillus mycelial growth by generating antifungal chemicals such Bis(2-ethylhexyl) phthalate and Pyrrolo[1,2-a] pyrazine-1,4-dione.

CONCLUSION

One of the most promising techniques for more reasonable and safe crop management strategies is the use of helpful microbes. We collected antagonistic bacteria from soil, which exhibited significant antagonistic activity *in vitro* against *C. lutana*. As a result, we revealed that *B. subtilis* (BS3) efficiently inhibited *C. lunata* mycelial growth at a rate of 48 percent by producing volatile compounds and a wide array of secondary metabolites with varied structure and function. The ability of these species to manage plant disease is determined by their production of antibacterial compounds.

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Conflict of Interest. None.

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