

Molecular Detection of Antimicrobial Peptide Genes and Identification of Antifungal Compounds of *Bacillus* sp. against *Fusarium oxysporum* f. sp. *cubense* Causing Wilt in Banana

Abstract

Fusarium oxysporum f.sp. *cubense* (Foc) is a serious infectious agent that causes fusarium wilt of banana within tropical and semi tropical regions of India. It is both necessary and critical to find an efficient biocontrol strategy to ensure banana production around the world. Twenty strains of *Bacillus* species from various banana rhizosphere regions were analysed using 16s rRNA and tested against Foc *in vitro*. Among 20 strains, B18, B4 and B7 were observed to inhibit the mycelial growth of pathogen. These three strains of *Bacillus* were analysed through PCR with specific primers for the presence of antimicrobial biosynthetic genes viz., Iturin A, Iturin C, Bacillomycin A, Surfactin and Bacillomycin D. The effective strain B18 was identified as *Bacillus cereus* based on biochemical and 16s rRNA region. In addition to antimicrobial peptide genes, the crude antibiotic extract from strain B18 was highly effective against Foc possibly owing to the presence of diverse antifungal compounds namely, 1- Dodecene, heptadecane, Pyrrolidine, Tetradecane, 1- Hexadecanol and Nonadecane, detected and identified using Gas chromatography and Mass spectrometry (GC/MS).

Keywords: *Bacillus*, *Fusarium oxysporum* f. sp. *cubense*, antifungal compounds, antimicrobial genes, GCMS

Introduction

Banana is commercially cultivated in many tropical and subtropical areas and remains to be one among the world's most important fruit crops (Molina and Valmayor, 1999). *Fusarium*

27 wilt or Panama disease of banana (*Musa* spp.) caused by *Fusarium oxysporum* f. sp. *cubense*
28 (E.F. Smith) “Snyder and Hansen” is the major restraint to banana cultivation and production
29 worldwide (Lin *et al.*, 2009). Application of fungicides and resistant cultivars are the only
30 management techniques available for controlling *Fusarium* wilt in banana. (Saravanan *et al.*,
31 2003; Geetha *et al.*, 2005). The loss of non-targeted valuable soil microorganisms due to
32 chemicals, have alerted researchers to look for alternative methods of management. *Bacillus*
33 species carries 24 varied Anti-Microbial Peptide (AMP) genes that allow the biosynthesis of
34 antibiotics like iturin, bacilysin, bacillomycin, fengycin, surfactin, mersacidin, ericin, subtilin,
35 subtilisin, and mycosubtilin (Chung *et al.*, 2008; Mora *et al.*, 2011; Rajesh kumar *et al.*,
36 2014). The generation of metabolites through bacterial cell culture with antimicrobial action
37 is a proven way to control plant pathogens. (Silo-Suh *et al.*, 2009). The detection of diverse
38 antifungal compounds of *Bacillus* species by GC-MS and their strong inhibitory effects
39 against pathogenic fungi (Dheepa *et al.*, 2016; Vinodkumar *et al.*, 2017). Therefore, *Bacillus*
40 species were considered effective in production of antifungal compounds and antimicrobial
41 peptide genes against *Fusarium* wilt of banana. The objectives of the present study include (i)
42 Isolation and characterization of *Bacillus* spp from banana rhizosphere (ii) Detection of
43 diverse antibiotic genes (iii) Identification of antifungal compounds.

44 **Materials and Methods**

45 **Isolation of Pathogen, *Fusarium oxysporum* f. sp. *cubense* (Foc)**

46 Infected banana plants showing the typical wilt symptom were collected from different
47 varieties of banana in Tamil Nadu. Corm portion was excised from the infected plant. The
48 infected corm were cut into 1cm bits and surface sterilized with 0.1% mercuric chloride
49 (HgCl₂) solution for 30 seconds and washed thrice in the series of sterile distilled water to
50 remove the traces of mercuric chloride, and transferred to sterilized Petri plates containing
51 potato dextrose agar (PDA) medium amended with 1000 ppm of streptomycin sulphate to

52 avoid bacterial contamination (Burgess *et al.*, 1994). Then the Petri plates incubated at room
53 temperature (27±2°C) for 7 days. The pathogen was identified through morphological and
54 molecular characterization by sequencing 18S-28S rRNA.

55 Isolation of *Bacillus* spp and screening against *Foc*.

56 Twenty strains of *Bacillus* spp were isolated from the rhizosphere of banana. Antifungal
57 activity of the *Bacillus* spp. was evaluated through a dual culture technique (Xiaoning *et al.*,
58 2014). Plates were incubated at 27 ± 2°C. After 7 days of incubation, percent inhibition (PI)
59 of mycelial growth was calculated with the formula proposed by Dennis and Webster (1971).

60 PCR amplification of antibiotic genes

61 Bacterial genomic DNA was extracted (Knapp and Chandlee, 1996) and 16S rRNA
62 intervening sequence specific BCF1 (5'CGGGAGGCAGCAGTAGGGAAT-3') and BCR2
63 (5'- CTCCCCAGGCGGAGTGCTTT-3') primer pair were used to confirm bacterial strain as
64 *Bacillus* species as described by Cano *et al.* (1994). These bacillus species were also
65 analysed for the presence of AMP genes with their respective primer pairs following
66 procedures of Vinod Kumar *et al.*, 2017 and were described below for six genes.

67 Iturin A

68 The forward primer ITUD-1F (5'GATGCGATCTCCTTGATGT3') and reverse primer
69 ITUD-1R (5'ATCGTCATGTGCTGCTTGAG3') were used for amplification of iturin A gene
70 (647 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3min, 40 cycles consisting of 94°C
71 for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 10 min.

72 Iturin C

73 The forward primer ITUC-F (5' TTTCACTTTTGATCTGGCGAT3') and reverse primer
74 ITUC-R (5' CGTCCGGTACATTTTCAC 3') were used for amplification of Iturin C gene
75 (506 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3min, 40 cycles consisting of 94°C
76 for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 10 min.

Comment [SN1]: PLEASE CHECK THE TEMPERATURES for DENATURATION, ANNEALING AND EXTENSION AND THE TIME IN MINUTES AND NUMBER OF CYCLES for PCR for reference Ramanathan, 2007.

I did not understand why the denaturation was done for 40 cycles at 94 degrees after initial cycle of denaturation at 94 for 3 min. (A BIG REVISION in methods, needs explanation for employing 94 degrees temperature for amplification of a gene

I am sorry for not letting you know in the first review, I over looked and was not expected a mistake in standard PCR protocol and primers.

Comment [SN2]: If the PCR conditions are same for the stated five genes and primers used, then it is a good to include the PCR information in the beginning para before the primer information of genes. Or the AMP genes can be placed in a separate table with primer information used for amplification of transcript.

77 Surfactin

78 The forward primer SUR-3F (5'ACAGTATGGAGGCATGGTC3') and reverse primer SUR-
79 3R (5'TTCCGCCACTTTTTCAGTTT3') were used for amplification of surfactin gene (441
80 bp) (Vinod kumar *et al.*, 2017). PCR conditions: 94°C for 3 min, 40 cycles consisting of 94°C
81 for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 10 min.

82 Bacillomycin A

83 The forward primer BACA-F (5' TGAAACAAAGGCATATGCTC3') and reverse primer
84 BACA-R (5' AAAAATGCATCTGCCGTTCC3') were used for amplification of
85 Bacillomycin A gene (344 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3 min, 40
86 cycles consisting of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 10 min.

87 Bacillomycin D

88 The forward primer BACC-1F (5'GAAGGACACGGCAGAGAGTC3') and reverse primer
89 BACC-1R (5'CGCTGATGACTGTTCATGCT3') were used for amplification of
90 Bacillomycin D gene (875 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3 min, 40
91 cycles consisting of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and 72°C for 10 min.

92 Extraction and Bioassay of Crude metabolites against *Foc*

93 The isolates of three antagonistic bacteria (B18, B4, B7) were incubated at 28±2° C for 7
94 days. After incubation they were centrifuged at 10000 rpm for 10 -15 min and the supernatant
95 was adjusted to pH 2.0 with concentrated HCl. The mixture was stirred at 100 rpm in an
96 orbital shaker for 8hrs. After shaking, the precipitate was collected by centrifugation,
97 re-suspended in 1ml of 1 M NaOH to adjust the pH 7.0. The resultant suspension was
98 extracted twice with ethyl acetate. The ethyl acetate phase was transferred into the vacuum
99 flash evaporator maintained at 60°C, at 80rpm, till the ethyl acetate fraction gets evaporated.
100 The crude antibiotics was re-suspended in 1ml of methanol – chloroform mixture (1:1) and
101 used for further bioassay and GC/MS analysis. (Dheepa *et al.*, 2016). The bioassay was
102 performed by Agar diffusion method (Hafidh *et al.*,2011). The area of inhibition was also
103 recorded.

104 Characterization of Crude Metabolites by GC-MS

105 Volatile antifungal and anti-bacterial compounds were identified by GC-MS using a column
106 Elite-5MS (100% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25µm df equipped with GC
107 Clarus 500 Perkin Elmer. The turbo mass-gold-Perkin-Elmer detector was used. The carrier
108 gas flow rate was 1 ml per min, split 10:1, and injected volumes were 3µl. The column
109 temperature was maintained initially at 110°C at the rate of 10°C/min-No hold followed by
110 increases up to 280°C at the rate of 5°C /min-9 min (hold). The injector temperature was
111 250°C and this temperature was held constant for 36 min. The electron impact energy was 70
112 eV, Jule line temperature was set at 2000°C and the source temperature was set at 200°C.
113 Electron impact (EI) mass scan (m/z) was recorded in the 45-450 aMU range. Using
114 computer searches on the NIST Ver.2006 MS data library and comparing the spectrum
115 obtained through GC/MS the compounds present in the crude sample were identified. Crude
116 metabolites of the most effective strain of *B. cereus* B18 were analysed by GC-MS for the
117 detection of active antifungal compounds responsible for the inhibition of *Fusarium*
118 *oxysporum* f. sp *cubense* (*Foc*) as proposed by Vinod kumar *et al.*, (2017).

119 Statistical Analysis

120 Data was analysed using IRRITSAT v. /93 software programme developed by International
121 Rice Research Institute (IRRI), Philippines. Means differences of the treatment were
122 evaluated with ANOVA and compared by Duncan's Multiple Range-Test (DMRT) (Gomez
123 and Gomez, 1984).

124 Results and Discussion

125 The *Fusarium* wilt of banana pathogen produced white cottony mycelial growth with
126 microconidia, macroconidia and chlamydospores 7 days after incubation at 20 - 27 ± 2°C.
127 Based on the morphological characters, the pathogen was confirmed as *Fusarium oxysporum*
128 f. sp *cubense*. In Addition, the *F. oxysporum* genus specific primers amplified a fragment of
129 approximately 550 bp corresponding to the region of the 18S-23S rRNA intervening
130 sequence for *F. oxysporum*. The amplified genomic product was partially sequenced at
131 Priority life sciences Pvt. Ltd., Bangalore, India. The rRNA homology searches were
132 performed using the BLAST program through the internet server at the National Centre for
133 Biotechnology Information, USA. The analysis of sequence information for isolates revealed

Comment [SN3]: Author did not provide significance information for any data set presented in the manuscript either in the text or for the tables. Author did not mention the samples, variables replicates, design of the experiment in methods

Comment [SN4]: Please follow the correct temperature or range that was executed during the experimental incubation time

134 that they had a nucleotide sequence identity of 98 - 100 per cent with the *F. oxysporum*.
135 Since, the pathogen was isolated from banana, the identified pathogen was referred as *F.*
136 *oxysporum* f. sp. *cubense*. The partial sequence of *F. oxysporum* f. sp. *cubense* isolates were
137 submitted to the NCBI, GenBank, New York, USA (MF576349). The molecular approaches
138 are rapidly used for species level identification. Various techniques, including rDNA PCR
139 (rDNA-intergenic spacer length polymorphisms) and 16S–23S intergenic transcribed spacer
140 region have been used for identification and differentiation of bacterial species. The 16S rRNA
141 gene sequence analysis has been described to be necessary mainly to detect some misidentification
142 of *Bacillus* and related strains (Wu *et al.*, 2006). In present study, *Bacillus* spp. amplified at 546 bp
143 corresponding region confirmed through 16s rRNA and the effective isolate B18 was
144 sequenced and shows 100% similarity in NCBI database to be identified as *Bacillus cereus*
145 (MH046059) (Fig. 1). Among twenty isolates of *Bacillus* spp screened against *Foc* only three
146 isolates were identified as effective. *B. cereus* strain B18 highly inhibited the growth of
147 mycelium up to 58.89 percent over control (Table 1). Further, 75 µl of crude extracts
148 inhibited the mycelial growth of 407.00 mm² area of *Foc* under *in vitro*. *Bacillus* spp.
149 produce a broad range of AMPs such as cyclic lipopeptides such as iturins, surfactins,
150 fengycins, bacilysin, bacillomycin, mersacidin, and subtilin (Chung *et al.*, 2008; Rajesh
151 Kumar *et al.*, 2014). Mora *et al.* (2011) reported that antimicrobial peptide (AMP)
152 biosynthetic genes surfactin, bacylisin, fengycin, bacylomicin, subtilin and iturin was
153 examined in 184 isolates of *Bacillus* spp., from rhizosphere, and non-rhizosphere regions of
154 Mediterranean farm land area in Spain. Most strains had between two and four AMP genes.
155 Out of ten AMP genes identified from 55 *Bacillus* spp., only *B. amyloliquefaciens* (VB7)
156 highly inhibited the growth of *S. sclerotiorum* (Vinodkumar *et al.*, 2017) that supported the
157 finding of present investigation in Banana. The AMP biosynthetic gene of iturin penetrates
158 cell membranes and it leads to cellular leakage (Ongena and Jacques, 2007). Zhang *et al.*

(2013) reported that bacillomycin produced by *B. cereus* disturbed the plasma membrane of *R. solani* abnormality in conidia and mycelia were also observed. In our study, the 20 isolates of *Bacillus* spp, five AMP genes associated to the antibiotics iturin A, iturin C, bacillomycin A, bacillomycin D, and surfactin were prevalent. The effective *Bacillus* spp., with potential strains of *B. Cereus*, B18 and B4, had five AMP genes responsible for the biosynthesis of antibiotic genes (Table 2). *Bacillus* spp are capable of producing an ample variety of secondary antifungal metabolites that are varied in structure and function. Production of antifungal metabolites determines the ability of *Bacillus* species to control plant pathogens. The crude metabolites of *B. cereus* strains B18 were analysed by GC/MS to identify their production of antifungal compounds (Fig. 2). Mainly vital antifungal compounds detected were, 1-dodecane, heptadecane, pyrrolidine, 1-tetradecene and tetradecane (Table 3). Similarly, the results are also supported by findings of Zhang *et al.*, 2011, which insist the crude lipopeptides extracted from cell-free culture broth of *B. amyloliquefaciens* strain TF28 exhibited a wide-spectrum of antifungal activity, but it strongly inhibited the growth and spore germination of *F. moniliforme*. Crude extracts of *B. subtilis* have been reported to inhibit mycelial growth in *F. oxysporum* f. sp *gerberae* (Suneeta *et al.*, 2016). Dodecanoic acid belonging to fatty acid with antibacterial and antifungal activity (Kumar *et al.*, 2011). Wang *et al.*, 2013 have identified 18 antifungal compounds produced by *B. amyloliquefaciens* W19 strain with high significant antagonistic effect on *F. oxysporum* f. sp *cubense*. Possibly, the biocontrol by bacteria can be owing to the production of AMP genes, antifungal and antibacterial inhibitory compounds.

Conclusion

Management of *Fusarium* wilt of banana usually relies on several control methods. Hence, a study was conducted to understand the role and efficacy of antimicrobial genes and antifungal compounds from *Bacillus* spp by extracting crude metabolites. GC/MS analysis of

184 crude metabolite extract of the effective *Bacillus cereus* strain B18 revealed active antifungal
185 compounds responsible for inhibition of *FOC*. Results showed five amplified *AMP* genes
186 from all *Bacillus* spp. Therefore, the antifungal and antibacterial compounds identified
187 through present investigation would help to control the *FOC* pathogen causing wilt in banana.

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S.No	Isolates	Radial growth of pathogen (mm)*	Percentage of inhibition
1.	B1	51.00 ^{gh}	43.33 (41.16) ^{cf}
2.	B2	64.00 ^b	28.89 (32.51) ^k
3.	B3	60.00 ^c	33.33 (35.26) ^j
4.	B4	40.00 ^k	55.56 (48.19) ^b
5.	B5	50.00 ^h	44.44 (41.81) ^c
6.	B6	58.00 ^{de}	35.56 (36.60) ^{hi}
7.	B7	42.00 ^j	53.33 (46.90) ^c
8.	B8	57.00 ^e	36.67 (37.26) ^h
9.	B9	47.00 ⁱ	47.78 (43.72) ^d
10.	B10	53.00 ^f	41.11 (39.87) ^g
11.	B11	48.00 ⁱ	46.67 (43.08) ^d
12.	B12	59.00 ^{cd}	34.44 (35.93) ^{ij}
13.	B13	51.00 ^{gh}	43.33 (41.16) ^{cf}
14.	B14	52.00 ^{fg}	42.22 (40.52) ^{fg}
15.	B15	47.00 ⁱ	47.78 (43.72) ^d
16.	B16	59.00 ^{cd}	34.44 (35.93) ^{ij}
17.	B17	69.00 ^a	23.33 (28.88) ^l
18.	B18	37.00 ^l	58.89 (50.12) ^a
19.	B19	63.00 ^b	30.00 (33.20) ^k
20.	B20	64.00 ^b	28.89 (32.51) ^k
21.	Control	90.00	0.00

264

265 **Table 1. Efficacy of *Bacillus* spp against *Fusarium oxysporum* f. sp *cubense* under in**
 266 **vitro**

267

S. No	Isolates	Bacillomycin D	Bacillomycin A	Iturin A	Iturin C	Surfactin
1	B1	+	+	+	+	-
2	B2	+	+	+	+	+
3	B3	-	-	+	-	+
4	B4	+	+	+	+	+
5	B5	+	+	+	+	+
6	B6	+	+	+	-	+
7	B7	+	+	+	+	+

8	B8	+	+	+	+	+
9	B9	+	-	+	-	+
10	B10	-	-	+	-	+
11	B11	+	-	-	-	+
12	B12	-	-	+	-	+
13	B13	+	+	+	-	+
14	B14	+	-	-	-	-
15	B15	-	-	-	-	+
16	B16	+	-	+	-	+
17	B17	+	+	+	+	+
18	B18	+	+	+	+	+
19	B19	+	+	+	+	+
20	B20	+	-	+	+	+

268 (+) = Positive (-) = Negative

269 **Table 2. Detection of antimicrobial biosynthetic genes among of *Bacillus* spp. through PCR**

S. No	Retention time (min)	Chemical name	Molecular formula
1.	2.55	1-Dodecene	C ₁₂ H ₂₄
2.	13.97	Heptadecane	C ₁₇ H ₃₆
3.	18.75	Pyrrolidine	C ₂₂ H ₄₁ NO ₂
4.	22.49	1-Tetradecene	C ₁₄ H ₂₈
5.	32.48	Tetradecane	C ₁₇ H ₃₆

Table 3. Antifungal compounds produced by *Bacillus cereus* strain B18 and identified by GC-MS.

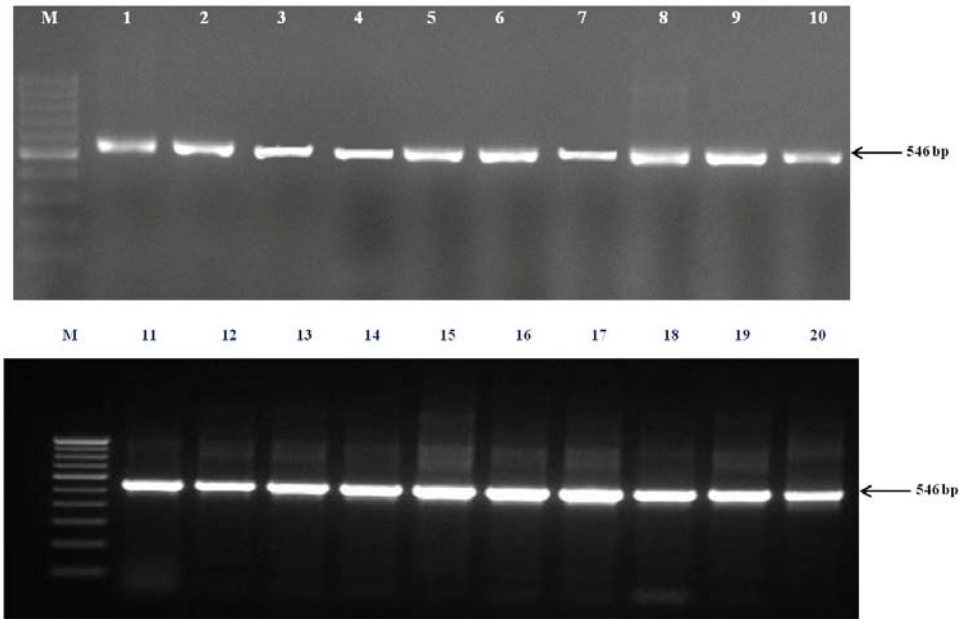


Fig. 1. PCR amplification of 16S-rRNA region of *Bacillus* isolates

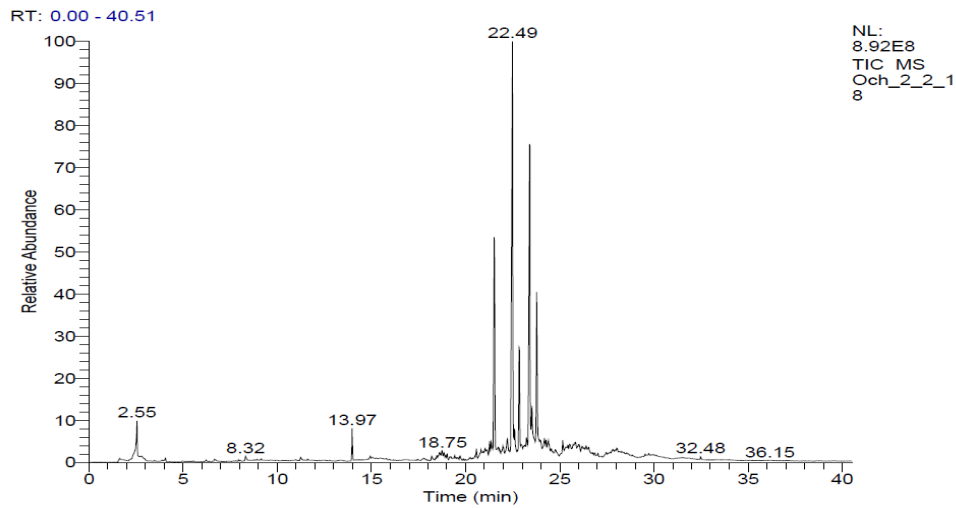


Fig. 2. Total Ion Chromatogram (TIC) of antifungal compounds identified from *B. cereus* strain B18 using GC/MS.