

Original Research Article

Molecular Detection of Antimicrobial Peptide Genes and Identification of Novel Antifungal Compounds of *Bacillus* sp. against *Fusarium oxysporum* f. sp. *cubense* Causing Wilt of 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 a proficient strategy for ensuring banana production around the world. Twenty strains of *Bacillus* sp from various banana rhizosphere regions were analyzed using 16s rRNA and tested against *Foc* for their efficacy under *in vitro*. Among of which three strains B18, B4 and B7 were observed to inhibit the mycelial growth of pathogen. The *Bacillus* sp strains were analyzed 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 identified as *Bacillus cereus* based on biochemical and 16s rRNA region. In addition to antimicrobial peptide gens, the crude antibiotic extract from strain B18 was highly effective against *Foc* possibly owing 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

Bananas are important fruit crops commercially cultivated in many tropical and subtropical areas that still remains to be one among the world's most important fruit crops (Molina and

Valmayor, 1999). It is a vital fruit crop and become the largest cultivation in India. *Fusarium* wilt or Panama disease of banana (*Musa* spp.) caused by *Fusarium oxysporum* f. sp, *cubense* (E.F. Smith) Snyd. and Hans. is the major restraint to banana cultivation and production worldwide (Lin *et al.* 2009). Applying fungicides and resistant cultivars that are resistant to *Fusarium* wilt of banana and these are the management techniques for controlling this disease (Saravanan *et al.*, 2003; Getha *et al.*, 2005). Moreover, loss of non-targeted valuable soil microorganisms due to chemicals, have alerted researchers to the need for an alternating method of management. *Bacillus* species contain as several as 24 varied AMP genes that allow the biosynthesis of antibiotics like iturin, bacilysin, bacillomycin, fengycin, surfactin, mersacidin, ericin, subtilin, subtilosin, and mycosubtilin (Chung *et al.*, 2008; Mora *et al.*, 2011; Rajesh kumar *P et al.*, 2014). Indication of biological control by *Bacillus* spp. against different soil borne plant pathogens has been seen from quite a while. The generation of metabolites with antimicrobial action is one determinant of their capacity to control plant diseases (Silo-suh *et al.*, 2009). The detection of diverse antifungal compounds of *Bacillus* species were investigated by GC-MS and its strong inhibitory effect against pathogenic fungi (Dheepa *et al.*, 2016; Vinodkumar *et al.*, 2017). In this perspective, *Bacillus* species were explored as an effective production of antifungal and antimicrobial peptide genes against *fuarium* wilt of banana. An objective of the present study includes (i) Isolation and characterization of *Bacillus* spp from banana rhizosphere (ii) Detection of diverse antibiotic genes (iii) Identification of antifungal compounds.

Materials and Methods

Isolation of Pathogen (*Foc*)

Pathogen was isolated from different varieties of infected banana. Then the petri plates incubated at room temperature (27±2°C) for 5 days. The Pathogen was identified through morphological and molecular characterization by sequencing 18S-28S rRNA.

52 **Isolation of *Bacillus* spp and screening against *Fusarium oxysporum* f. sp *cubense* (*Foc*).**

53 Twenty strains of *Bacillus* spp were isolated from the rhizosphere soil of banana. Genomic
 54 DNA was extracted (Knapp and Chandlee, 1996) and 16S rRNA gene was characterized with
 55 BCF1 (5'CGGGAGGCAGCAGTAGGGAAT-3') and BCR2 (5'-
 56 CTCCCCAGGCGGAGTGCTTT-3') primer pair as described by Cano *et al.* (1994).
 57 Antifungal activity of the *Bacillus* spp was evaluated through a dual culture technique
 58 (Xiaoning *et al.*, 2014). Plates were incubated at $27 \pm 2^\circ\text{C}$. After 7 days of incubation, percent
 59 inhibition (PI) of mycelial growth was calculated with the formula proposed by Dennis and
 60 Webster (1971).

61 **PCR amplification of antibiotic genes**

62 **Iturin A**

63 The forward primer ITUD1F (5'GATGCGATCTCCTTGGATGT3') and reverse primer
 64 ITUD1R (5'ATCGTCATGTGCTGCTTGAG3') were used for amplification of iturin A gene
 65 (647 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3min, 40 cycles consisting of 94°C
 66 for 1min, 60°C for 1min, 72°C for 1min and 72°C for 10 min.

67 **Iturin C**

68 The forward primer ITUC-F (5' TTCACCTTTGATCTGGCGAT3') and reverse primer
 69 ITUC-R (5' CGTCCGGTACATTTTCAC 3') were used for amplification of Iturin C gene
 70 (506 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3min, 40 cycles consisting of 94°C
 71 for 1min, 60°C for 1min, 72°C for 1min and 72°C for 10min.

72 **Surfactin**

73 The forward primer SUR3F (5'ACAGTATGGAGGCATGGTC3') and reverse primer SUR3R
 74 (5'TTCCGCCACTTTTTCAGTTT3') were used for amplification of surfactin gene (441 bp)
 75 (Vinodkumar *et al.*, 2017). PCR conditions: 94°C for 3min, 40 cycles consisting of 94°C for
 76 1min, 57°C for 1min, 72°C for 1min and 72°C for 10min.

77 **Bacillomycin A**

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

82 **Bacillomycin D**

83 The forward primer BACC1F (5'GAAGGACACGGCAGAGAGTC3') and reverse primer
84 BACC1R (5'CGCTGATGACTGTTCATGCT3') were used for amplification of
85 Bacillomycin D gene (875 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3min, 40
86 cycles consisting of 94°C for 1min, 62°C for 1min, 72°C for 1min and 72°C for 10min.

87 **Extraction and Bioassay of Crude metabolites against *Foc***

88 The isolates of three antagonistic bacteria (B18, B4, B7) were incubated at 28±2° C for 7
89 days. The crude metabolites were extracted by using ethyl acetate as solvent (Dheepa *et al.*,
90 2016). The bioassay was performed by Agar diffusion method (Hafidh *et al.*,2011). The area
91 of inhibition was also recorded.

92 **Characterization of Crude Metabolites by GC-MS**

93 Crude metabolites of the most effective strain of *B. cereus* B18 were analyzed by GC-MS
94 (Trace GC Ultra DSQ II, Thermo Scientific, Germany) for the detection of active antifungal
95 compounds responsible for the inhibition of *Fusarium oxysporum* f. sp *cubense* (*Foc*) as
96 proposed by Vinodkumar *et al.*, (2017).

97 **Statistical Analysis**

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

102 Results and Discussion

103 The pathogen produced, white cottony mycelia growth with microconidia, macroconidia and
 104 chlamydospores 7 days after incubation at $20 \pm 2^\circ\text{C}$. Based on the morphological
 105 characters, the pathogen was confirmed as *Fusarium oxysporum* f. sp. *cubense*. Additional,
 106 the pathogen was also molecular characterized through PCR sequencing the 18S–28S rRNA
 107 and submitted in NCBI database (MF576349).

108 Among twenty isolates of *Bacillus* spp screened against *Foc* only three isolates were found to
 109 be effective. The isolate B18 inhibited the growth of mycelium up to 58.89% comparative to
 110 the control (Table 1). It was confirmed through 16s rRNA and sequenced shows 100%
 111 similarity in NCBI database, so identified as *Bacillus cereus* (MH046059) (Fig. 1). Further,
 112 75 μl of crude extracts inhibited the mycelial growth of *F. oxysporum* f.sp. *cubense* *in vitro* by
 113 407.00 mm^2 . The 20 isolates of *Bacillus* spp, AMP genes associated to the antibiotics iturin
 114 A, iturin C, bacillomycin A, bacillomycin D, and surfactin were prevalent. The effective
 115 *Bacillus* spp. viz., *B. cereus* strain B18 and B4 both had all the number (5) of AMP genes
 116 responsible for the biosynthesis of antibiotic genes (Table 2). The crude metabolites of *B.*
 117 *cereus* strains B18 were analyzed by GC/MS to identify their production of antifungal
 118 compounds (Fig. 2). The compound identity was confirmed using the National Institute of
 119 Standards and Technology (NIST) library 2006. Mainly vital antifungal compounds detected
 120 were, 1-dodecane, heptadecane, pyrrolidine, 1-tetradecene and tetradecane (Table 3).

121 *Bacillus* spp. produce a broad range of AMPs such as cyclic lipopeptides such as iturins,
 122 surfactins, fengycins, bacilysin, bacillomycin, mersacidin, and subtilin (Chung *et al.*, 2008;
 123 Rajesh Kumar *et al.*, 2014). Mora *et al.* (2011) reported that antimicrobial peptide (AMP)
 124 biosynthetic genes surfactin, bacilysin, fengycin, bacillomycin, subtilin and iturin was
 125 examined in 184 isolates of *Bacillus* spp., from rhizosphere, and non rhizosphere soil from
 126 Mediterranean land area of Spain. Most strains had between two and four AMP genes.

Furthermore, ten AMP genes were found from 55 *Bacillus* spp., among *B. amyloliquefaciens* (VB7) was to be highly inhibited the growth of *S. sclerotiorum* (Vinodkumar *et al.*, 2017). The AMP biosynthetic gene of iturin penetrates cell membranes and it which leads to cellular leakage (Ongena and Jacques, 2007). Zhang *et al.* (2013) reported that bacillomycin produced by *B. cereus* disturbed the plasmamembrane of *R. solani* abnormality in conidia and mycelia were also observed. *Bacillus* spp are capable of producing a 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. Crude extracts of *B. subtilis* have been reported to inhibit mycelial growth in *Fusarium oxysporum* f. sp *gerberae* (Suneeta *et al.*, 2016). Dodecanoic acid belonging to fatty acid with antibacterial and antifungal activity (Ashwanikumar *et al.*, 2011). Wang *et al.*, 2013 have identified 18 antifungal compounds produced by *B. amyloliquefaciens* W19 strain with high significant antagonistic effect on *Fusarium oxysporum* f. sp *cubense*. Possibly, the biocontrol by bacteria can be owing to the production of both antifungal and antibacterial inhibitory compounds.

Conclusion

Fusarium oxysporum f. sp. *cubense* (*Foc*) is causing *Fusarium* wilt of banana and its management is difficult to realize which usually relies of several control methods. Hence, a study was to understand the antimicrobial genes and antifungal compounds from *Bacillus* spp by extracting crude metabolites. GC/MS analysis of crude metabolite extract of the effective *Bacillus cereus* strain B18 and all *Bacillus* spp was showed AMP genes. So, antifungal compounds and AMP genes were which would control the pathogen.

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S.No	Isolates	Radial growth of pathogen (mm)*	Percentage of inhibition
1.	B1	51.00 ^{gh}	43.33 (41.16) ^{ef}
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) ^e
6.	B6	58.00 ^{de}	35.56 (36.60) ^{hi}
7.	B7	42.00 ^j	53.33 (46.90) ^c
8.	B8	57.00 ^c	36.67 (37.26) ^h
9.	B9	47.00 ⁱ	47.78 (43.72) ^d
10.	B10	53.00 ^t	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) ^{ef}

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

218

219 **Table 1. Efficacy of *Bacillus* spp against *Fusarium oxysporum* f. sp *cubense***

220

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

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	+	-	+	+	+

222 (+) = Positive (-) = Negative

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

S.no	Rt (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
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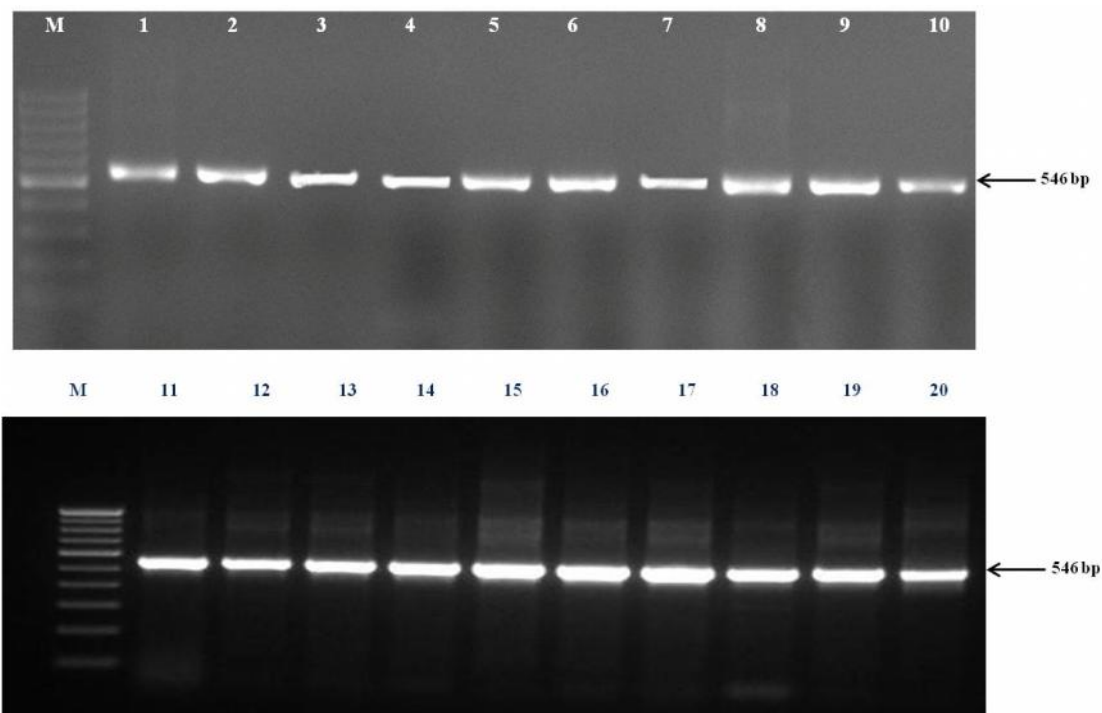


Fig. 1. PCR amplification of 16S-rRNA region of *Bacillus* isolates

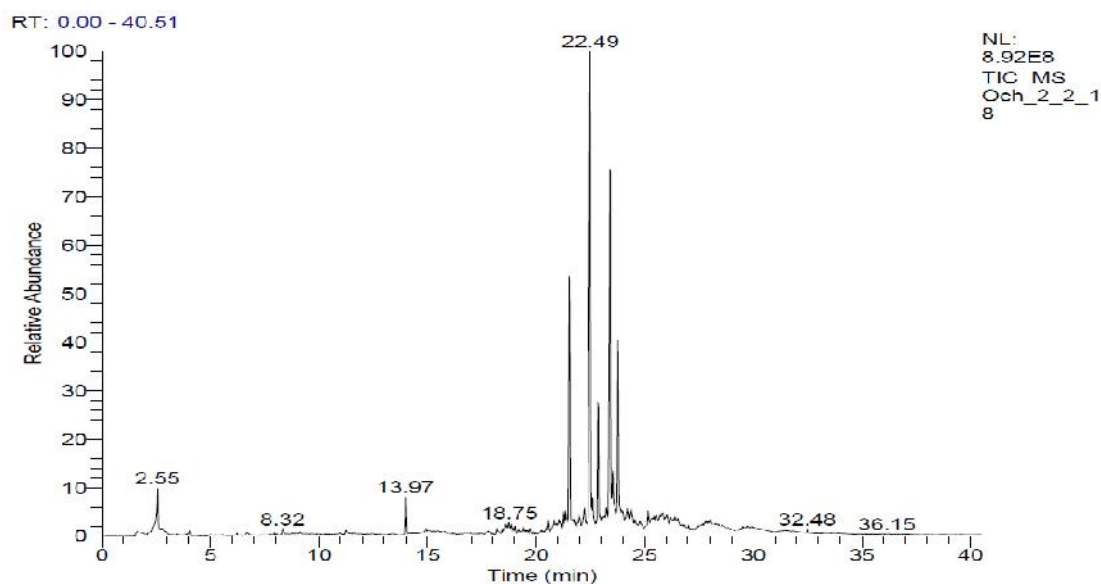


Fig. 2. Total Ion Chromatogram (TIC) of antifungal compounds identified from *B. cereus* strain B18 using GC/MS.