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Bacterial endophytes mediated suppression of *Fusarium oxysporum* f. sp. *cubense* in banana (*Musa* spp.)

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Abstract
Banana (*Musa* spp) is one among the most important tropical fruits in terms of productivity and trading. Among the various banana cultivars, Ney poovan (AB) is being widely cultivated because of its sweet and sub acid flavour of the fruit. Cultivation and productivity of Ney poovan (AB) is seriously threatened by *Fusarium* wilt caused by destructive soil borne fungi *Fusarium oxysporum* f. sp. *cubense* (*Foc*). In order to assess the virulence of pathogen, in the present study, we characterized the fusarium wilt pathogen collected from four different banana growing regions of Tamil Nadu. PCR amplification of *Fusarium* genomic DNA with Internal Transcribed Spacer region 1 and 4 resulted that all four isolates were confirmed as *Fusarium oxysporum* f. sp. *cubense* (*Foc)*. In order to test verify the antifungal efficacy of select bacterial endophytes *viz.*, *Brachybacterium paraconglomeratum* YEBPT2 (MK263736), *Brucella melitensis* YEBPS3 (MN022548), *Bacillus velezensis* YEBBR6 (MT372157), *Bacillus amyloliquefaciens* YEBFL3 (MT326231) and *Myroides odaratimimus* YEBRT3 (MN082530) of banana against *Foc*, *in vitro* screening was performed by dual plate method. The bacterial endophyte, *Brachybacterium paraconglomeratum* YEBPT2 significantly suppress the mycelial growth of *Foc* up to 61.56 per cent and produce diverse number of antifungal bioactive metabolites like Fluorouracil, Azetidine, Hydroxysulfonyl-3,4,4-trimethyl-2-azetidinone, Quinazoline, Isoxazolemethanol, Pteridine, Formic acid, Ethyl isopropyl phosphonothiolate, Diethyltrisulphide, Acetohydrazide, Pyrazine, Ticlopidine, Hexadecanoic acid, Cyclopentadecane and Propanedioic acid. Hence, *Brachybacterium paraconglomeratum* YEBPT2 based consortia may be developed to contain Fusarium wilt under field conditions.

Keywords: Ney poovan (AB), *Brachybacterium paraconglomeratum*, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) and bioactive metabolites

Introduction
Banana (*Musa* spp.), is one of the most significant staple fruit crops in the world, ranking among the top ten in terms of production and commerce [1]. The banana production and productivity is being constantly threatened by *Fusarium* wilt. *Fusarium* wilt is a soil-borne disease caused by the pathogen, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) [2], *Foc* survives in soil for a long time, infecting xylem vessels and causing wilt and death in infected plants.
Of late, the *Foc* Tropical Race 4 (Foc TR4) has emerged as global threat wherever bananas are being commercially cultivated. Recently, it has been reported in various parts of North India, mostly in Bihar (Katihar and Purnea), Uttar Pradesh (Faizabad), Madhya Pradesh (Burhanpur), and Gujarat (Surat) [3]. However, because of its extreme virulence and wider host range across all genomes of banana including Grand Naine (Cavendish group;AAA), a global trading variety, it is necessary to develop novel and sustainable management strategies to prevent further spread because, chemical protection for the management of *Fusarium* wilt in bananas is not completely reliable and eco friendly viable approach. Fungicides have provided inadequate control levels in the management of *Fusarium* wilt under field conditions. Phosphonate, ambuic acid, carbendazim, carboxin, propiconazole, benomyl, and difenoconazole have all been shown to be harmful to *Foc* under *in vitro* conditions [4, 5, 6, 7]. Only a few publications in planta have shown considerable disease control utilising fungicides (e.g., Carbendazim) [8, 9, 10] or resistance inducers (e.g., indole acetic acid and menadione sodium bisulphite) [11]. Furthermore, scientists have realised the importance of alternative management strategy due to the loss of non-targeted beneficial micro flora and micro fauna as a result of indiscriminate chemical usage. Planting resistant cultivars is also a challenging task due to market preferences [12]. Under these conditions, using antagonistic bacterial endophytes which protect and promote

plant growth by establishing and multiplying in both the rhizosphere and the plant system could be a viable alternative strategy for management of the *Fusarium* wilt of bananas under field condition. Objectives of the present study includes isolation and characterization of *Foc*, identification of effective antagonistic bacterial endophytes and profiling the presence of diverse biomolecules from bacterial endophytes, to exploit against *Foc*.

Material and Methods

Isolation and molecular confirmation of *Fusarium oxysporum* f. sp. *cubense*

The wilt affected banana corms from different banana cultivating regions of Tamil Nadu, India such as Chinnamanur village of Theni province (9° 50' 22.776" N77° 22' 58.0692" E/ Latitude 9.839660/ Longitude 77.382797), Thondamuthur village of Coimbatore province (11°00′35″N 76°49′41″E/ Latitude 10.9905/ Longitude 10.9905), Sathyamangalam village of Erode province (11° 30' 17.1936" N and 77° 14' 18.2184" E. Latitude: 11° 30' 17.1936" N/ Longitude: 77° 14' 18.2184" E) and Athani village of Erode province (11.5232° N, 77.5120° E/ Latitude 11.515219/ Longitude 77.452367), from Tamil Nadu, India were collected (Fig 1).

Fig 1: Vascular discoloration and colony morphology of different isolates of *Foc*

Pathogenic *Fusarium* spp. associated with banana cultivars susceptible to *Fusarium* wilt of banana was isolated as per the protocol [13]. Genomic DNA of *Fusarium* was extracted from the mycelium of pure culture through CTAB method [14].Genomic DNA was used as a template for PCR amplification of *Fusarium* isolates using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer sequence [15]. Programme cycle comprise of initial denaturation (95°C) for 2 minutes, followed by 40 cycles of denaturation (95°C) for 1 minute, annealing at 58°C for 1 minute, extension for 1 minute at 72°C and with a final extension at 72°C. Gel electrophoresis and staining was done by loading 10 µl of PCR product on 1% agarose gel in TAE buffer at 80 V for 50 minutes at 25°C. 1 kb DNA ladder was used to determine the size of amplified genomic products. PCR products were photographed using gel documentation system. Amplified genomic product was sequenced with Eurofins Genomics Biotech Pvt. Ltd., Bangalore, India. The gene homology searches were performed using NCBI BLAST. Sequences were compared with different *Foc* isolates retrieved from Gen Bank database. Newly obtained sequences were submitted in Gen Bank database (New York, USA) and accession numbers were obtained. Phylogenetic tree was performed with MEGA7 software [16]

Antifungal activity of bacterial endophytes against *Foc*

Pure cultures of bacterial endophytes like *Brachybacterium paraconglomeratum* YEBPT2 (MK263736), *Brucella melitensis* YEBPS3 (MN022548), *Bacillus velezensis* YEBBR6 (MT372157), *Bacillus amyloliquefaciens* YEBFL3 (MT326231) and *Myroides odaratimimus* YEBRT3 (MN082530) were collected from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The efficacies of bacterial endophytes were tested under *in vitro* against *F. oxysporum* f. sp. *cubense* (*Foc* 1) through dual culture method. The mycelial disc (9 mm diameter) of seven days old culture of *Foc* 1 was placed at one side of the Petri plate containing PDA medium at 10 mm away from the periphery. Bacterial endophyte (24 h old) was streaked onto the medium 10 mm away from the periphery exactly opposite to the mycelial disc. The plates were incubated under room temperature @ 28±2^{0}C for 7 days. Zone of inhibition against *Foc* KP was measured after 7 days of incubation. Each bacterial isolates were replicated thrice. Each replication consists of 10 Petri plates per replication. The per cent reduction of mycelial growth over untreated control was calculated using the formula $\frac{C-T}{C} \times 100$. The experiment was repeated twice for confirmation.

Where, C - Mycelial growth of pathogen in control, T - Mycelial growth of pathogen in dual plate technique

Extraction of biomolecules from bacterial endophytes

The crude antibiotics of bacterial endophytes were extracted as per the protocol [17]. Bacterial endophytes were grown in NA broth and incubated at 28 ± 2ºC for 8 days. The supernatant was collected after 8 days by centrifugation at 5,000 rpm for 30 min. Then supernatant was adjusted to acidic pH 2.0 by adding concentrated HCl and the mixture was stirred at 100 rpm in an orbital shaker for 8 hrs. Antifungal compounds in supernatant or culture broth was extracted by adding equal volume of solvent ethyl acetate and shaken vigorously for 1-2 h. Culture broth was extracted twice with ethyl acetate solvent for complete extraction. The solvent fraction that contained antifungal compounds were combined and concentrated by evaporation in the rotary flash evaporator maintained at 600˚C at 80 rpm till the compounds were condensed. The concentrated crude extract of the extracellular antifungal compounds were then dissolved in 1 ml of HPLC grade methanol for *in vitro* antifungal activity assay and GC/MS analysis

Antifungal activity of volatile organic compounds (VOC) / Non volatile organic compounds (NVOC) from effective bacterial antagonists against *Foc* through agar well diffusion method

The melted sterile PDA medium of 15 ml was poured into sterile Petri plates of 9 cm diameter. After solidification, using sterile cork borer of 9 mm diameter, melted agar was removed on all four sides of the Petri plate by leaving 1 cm away from periphery. The 9 mm agar disc from the excised area was removed using a sterile inoculation needle. A 5 mm diameter of *Foc* mycelium from the actively growing edges of 5-day old culture was removed using a sterile inoculation needle and placed at center of the plate. VOC/NVOC compounds extracted from the bacterial endophytes were pipetted into the wells of 9 mm diameter @ 50µl/well. Later, the plates were incubated at 28 ± 2°C for 7 days till the *Foc*1 culture completely colonize the Petri plate in untreated control [18]. Radial mycelial growth and inhibition zone formed by the crude NVOC compounds were measured after 7 days of incubation were replicated three times. Each replication had 10 Petri plates. The experiment was repeated twice for the authenticity of the results. HPLC grade methanol control without VOC/NVOC compounds was maintained as untreated control. Percent inhibition of mycelial growth over control was calculated using the following formula.

$$\text{Per cent inhibition over control} = \frac{C-T}{C} \times 100$$

Where, C - Mycelial growth of pathogen in control, T - Mycelial growth of pathogen in treatment

Results

Molecular confirmation of *Foc* isolates

Amplification of Internal Transcribed Spacer of 4 different isolates of *Foc* for ITS 1 and ITS 4 region with specific primer yielded an expected amplicon size of 560 bp (Fig. 2).

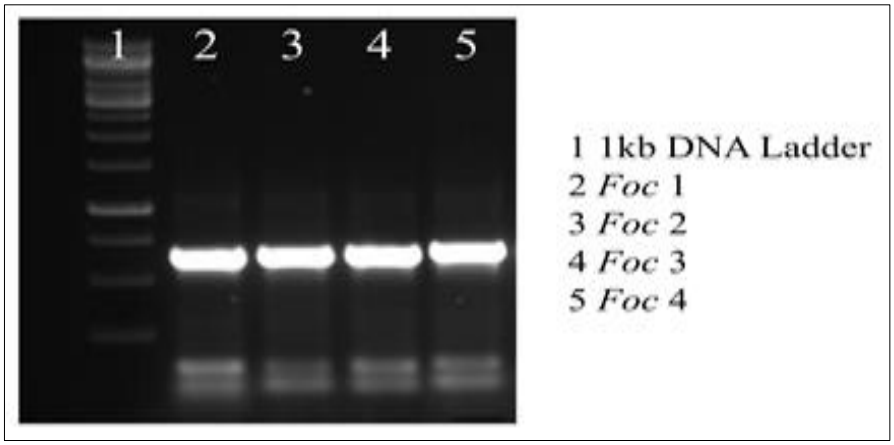


Fig 2: PCR Genomic DNA of different isolates of *Foc* with internal transcribed spacer region

Nucleotide sequence of amplicon pertaining to 4 different isolates confirmed the identity of *Foc* KP. The sequences subjected to multiple alignments were submitted in Gen bank and provided with accession numbers *viz*., *Foc*1 OK413391, *Foc*2 OK413392, *Foc*3 OK413396 and *Foc*4 OK413393. Phylogenetic analysis of *Foc* revealed that presence of 2 major clusters. Cluster 1 comprised of 2 sub clusters and sub cluster one comprises *Foc*1 OK413391 and *Foc*2 OK413392. Whereas sub cluster 2 had *Foc*3 OK413396 and *Foc* 4 OK413393 and the cluster 2 comprised of *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) as an out group (Fig. 3).

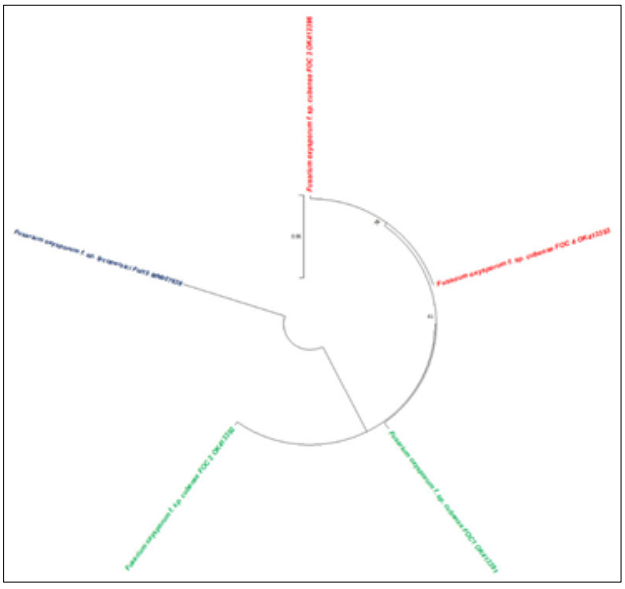


Fig 3: Phylogenetic relationship of different isolates of *Foc*

Antifungal activity of bacterial endophytes from resistant banana cultivar YKM 5 against *Foc* KP

Antifungal activities of all bacterial endophytes were screened against *Foc* under *in vitro* conditions. Screening for antifungal efficacy with 5 bacterial endophytes reflected that *Brachybacterium paraconglomeratum* YEBPT2 (MK263736) reduced the mycelial growth of *Foc* up to 61.56 per cent from all other isolates followed by *Brucella melitensis* YEBPS3 (MN022548) and *Bacillus velezensis* YEBBR6 (MT372157) inhibited the radial mycelial growth of *Foc* up to 58.33 per cent over control. *Bacillus amyloliquefaciens* YEBFL3 (MT326231) and *Myroides odaratimimus* YEBRT3 (MN082530) suppressed the mycelial growth of *Foc* up to 55.1% over untreated control (Fig 4 & Fig 5)

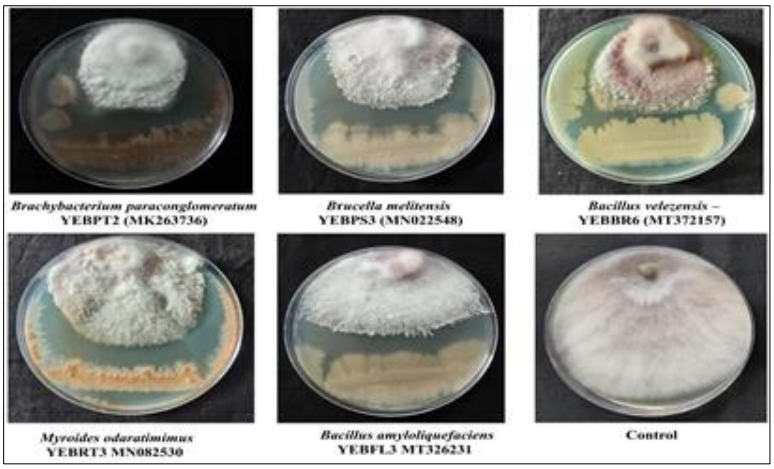


Fig 4: Antifungal efficacy of bacterial endophytes against *Foc*

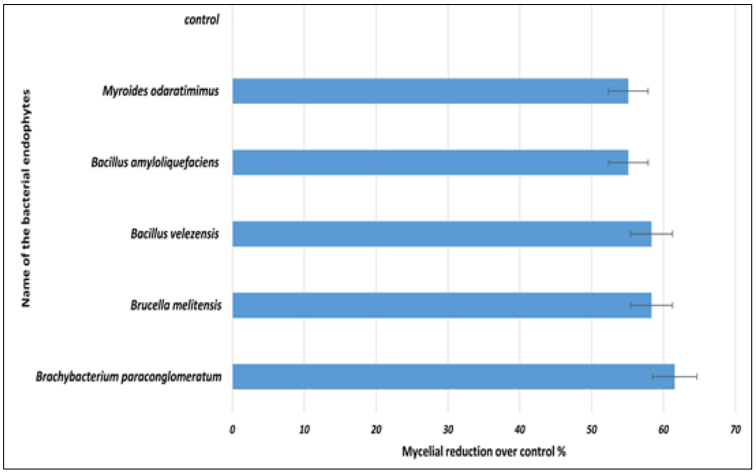


Fig 5: Per cent reduction over control of bacterial endophytes against *Foc*

Antifungal activity of biomolecules extracted from zone of inhibition during the interaction of bacterial antagonists and *Foc* KP

Among all the bacterial endophytes tested, *B. Paraconglomeratum* YEBPT2 produced the maximum inhibition zone compared to other endophytes. Hence, bioactive molecules from the zone of inhibition have been eluted to confirm the antifungal activity against *Foc*. The eluted biomolecules of *B. paraconglomeratum*- YEBPT2 produced 20.2 mm inhibition zone and thus, suppressed *Foc* KP (Fig 6).

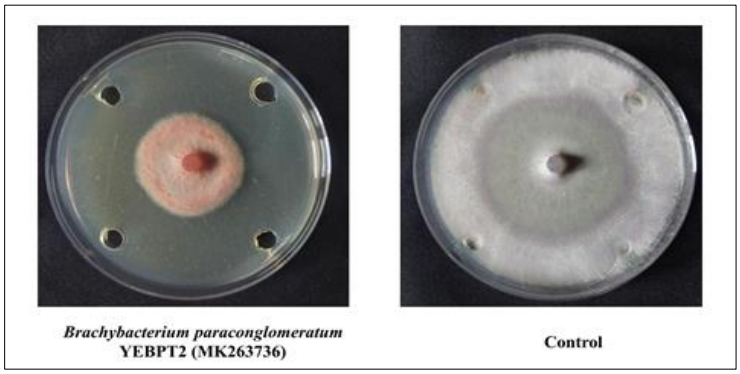


Fig 6: Antifungal efficacy of biomolecule produced by *B. paraconglomeratum*

Characterization of VOC/NVOC biomolecules from the zone of inhibition, produced during ditrophic interaction of bacterial endophytes and *Foc* KP

VOC/NVOC compounds from the zone of inhibition produced during the interaction of effective bacterial antagonists *B. paraconglomeratum*- has been characterized through GC-MS. Fifteen biomolecules have been identified through GCMS, from the crude metabolites of *B. paraconglomeratum* YEBPT2. The compounds are identified as Fluorouracil, Azetidine, Hydroxysulfonyl-3,4,4-trimethyl-2-azetidinone, Quinazoline, Isoxazolemethanol, Pteridine, Formic acid, Ethyl isopropyl phosphonothiolate, Diethyltrisulphide, Acetohydrazide, Pyrazine, Ticlopidine, Hexadecanoic acid, Cyclopentadecane and Propanedioic acid (Fig 7, Table 1).

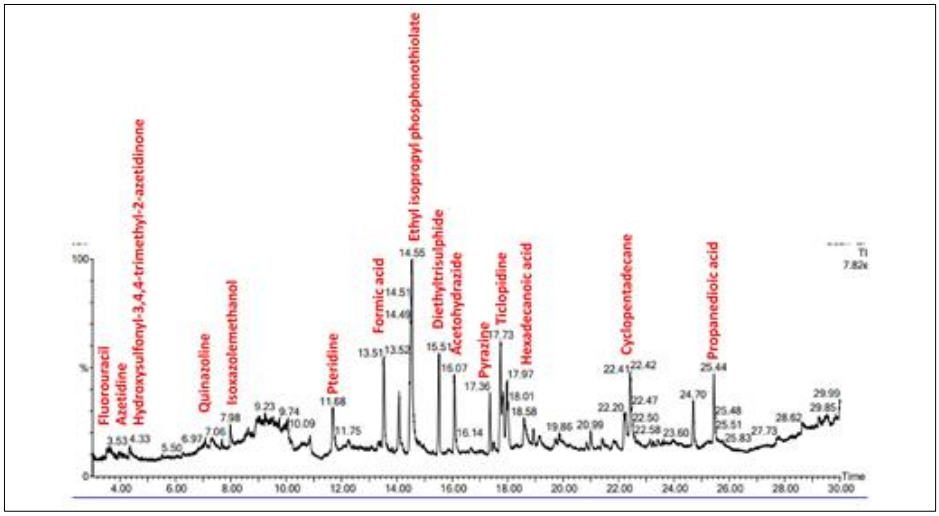
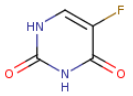
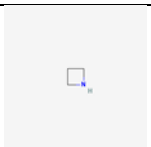
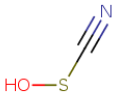
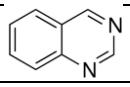
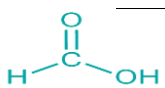
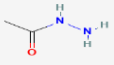
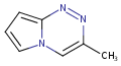
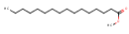
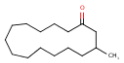


Fig 7: Diversity of antifungal biomolecule produced by *B. paraconglomeratum*

Table 1: Bioactive metabolites produced by *B. paraconglomeratum*

S. No	Retention time	Name of the compound	Peak area percent	Structure	Biological activity	Reference
1	3.088	Fluorouracil	0.611		Antifungal (inhibit fungal RNA and DNA synthesis	[35]
2	1.253	Azetidine	1.253		Antifungal	[36]
3	4.069	Hydroxysulfonyl-3,4,4-trimethyl-2-azetidinone	4.069		Antimicrobial	[37]
4	7.680	Quinazoline	1.424		Antimicrobial	[38]
5	7.975	Isoxazolemethanol	7.975		-	-
6	11.677	Pteridine	1.311		Antifungal	[39]
7	13.523	Formic acid	2.066		Antimicrobial	[40]
8	14.813	Ethyl isopropyl phosphonothiolate	14.813			
9	15.513	Diethyltrisulphide	1.808		Antifungal	[41]
10	16.069	Acetohydrazide	16.069		Antifungal	[42]
11	17.734	Pyrazine	1.735		Antimicrobial	[43]

12	17.969	Ticlopidine	1.407			
13	18.580	Hexadecanoic acid	1.426		Antifungal	[44]
14	22.416	Cyclopentadecane	2.655		Antifungal	[45]
15	24.697	Propanedioic acid	1.009			

Discussion

The vascular wilt of banana caused by *Foc* is a most destructive soil borne disease across the globe. None of the fungicides are effective in suppressing *Foc.* Transfer of identified R genes through classical and molecular breeding from wild genotypes to consumer preferred commercial varieties are not successful, due to polyploidy, sterility, poor seed set and germination besides heterozycosity [19]. Hence, identifying environmental friendly sustainable alternative for the management of *Fusarium* wilt of banana is need of hour. Bacterial endophyres can modulate the host immune response by production of several volatile and non volatile biomolecules. In the present study, we investigated the antifungal efficacy of bacterial endophytes and diversity of biomolecules produced by bacterial endophytes. Molecular assay on the confirmation of *Fusarium* wilt pathogen through ITS 1 and 4 confirmed the presence of *Foc*. Likewise, ITS region has been used as molecular marker to confirm the identity of *Foc* infecting *Musa* spp. (ABB) in Southern Mexico. Further, in the present study, phylogenetic analysis of *Foc* isolates with ITS region showed variation among the investigated isolates. Similarly Wong *et al.*,2019 [20] analysed the phylogenetic relationship of Foc TR4 in Peninsular Malaysia based on transcription elongation factor (TEF-1) sequences and reported the genetic variability among the isolates. In our study, the bacterial endophytes were tested against *Foc* under *in vitro* condition to understand the antifungal activity and revealed that actinobacteria, *B. paraconglomeratum* - YEBPT2, *B. melitensis* YEBPS3, an *α-Proteobacteria* and *B. velezensis* - YEBBR6 (MT372157) belonging to *Bacilli*, inhibited the mycelial growth by more than 60%. *B. velezensis* CT32 inhibited the mycelial growth of *Verticillium dahliae* and *F. oxysporum*, causal agents of strawberry vascular wilt up to 66.67 per cent and Souza *et al*.,2014 [21] identified 4 bacterial endophytes with antifungal activity among 122 bacterial endophytes isolated from two banana cultivars grown in *Foc* contaminated field. Wang *et al*., 2013 [22] harnessed 6 bacterial endophytes with antifungal action against *Foc* from 57 bacterial endophytes isolated from banana grown in soils with heavy pressure of *Foc*. Bacterial endophytes with potential antifungal and growth promotional properties were isolated from banana by Weber *et al*., 2007 [23]. Antifungal nature of *Bacillus* spp., against *Foc* has been confirmed by several workers [24]. *B. amyloliquefaciens* isolated from the banana plants exposed to maximum disease pressure caused by *Foc* promoted banana growth and antifungal action against *Foc* by producing antimicrobial peptides and volatile organic compounds. Bacterial endophytes and other growth promoting microorganisms produce several kinds of volatile and non-volatile organic compounds during their growth and development, the biomolecules can suppress the pathogen growth and promote the plant growth [25]. In the present study, the biomolecules produced by bacterial endophyte, *B. Paraconglomeratum* were characterized through GCMS analysis and revealed that 15 different biomolecules with different biological activity. The biomolecule, Fluorouracil have antimicrobial activity which can suppress the plant pathogen growth by inhibit fungal RNA and DNA synthesis [26].

Asif *et al*., 2018 [27] reported that broad spectrum antifungal activity of Azetidine against several filamentous fungus. Saundane *et al*., 2012 [28] reported that antimicrobial activity of Hydroxysulfonyl-3, 4, 4-trimethyl-2-azetidinone. Quinazoline have significant antifungal and antimicrobial activity against several plant and human pathogens [29]. Antifungal activity of formic acid against *F. oxysporum* was reported by El-Hasan *et al*., 2008 [30]. Fernando *et al*., 2005 [31] reported that inhibitory effect of antifungal volatile organic compounds produced by P. *chlororaphis* – PA23 against *S. sclerotiorum*. In addition, Gao *et al*., 2017 [32] also confirmed the antifungal effect of VOCs produced by endophytic bacteria, *B. velezensis* ZSY-1 against *Alternaria solani* and *Botrytis cinerea*. Nakkeeran *et al*., 2020 [33] reported the antibacterial activity of biomolecules produced by *B. subtilis* BIO3 and *B. amyloliquefaciens* BS2. VOC and NVOCs of *B. amyloliquefaciens* (VB7) effectively inhibited the *Botrytis cinerea* causing *Lilium* leaf blight [34]. Hence, effective bacterial endophytes based consortia may be developed to contain *Fusarium* wilt under field conditions for environmentally viable and sustainable approach.

Conclusion

Effective bacterial endophytes based consortia may be developed to contain *Fusarium* wilt under field condition for environmentally viable and sustainable approach. Increasing the population density of beneficial endophytes by application at hardening stages may offer protection to the young plants in the early growth stages and extend the life of planting materials.

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