



Research Article

***In-vitro* screening of antagonistic potential of native endophyte strains of banana (*Musa* spp.) against *Fusarium oxysporum* f. sp. *cubense* TR4 causing Fusarium wilt of banana**

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Abstract

The experiment was done in the Department of Plant Pathology, RPCAU, Pusa, Bihar during 2021-22 to identify the antagonistic potential of native endophytes of bananas against FocTR4. An *in-vitro* investigation revealed that only four bacterial endophytes, GNBE4, GNBE9, GNBE12, and GNBE14, showed significant antagonistic effects against FocTR4 out of 18 screened endophytes (13 bacterial and 5 fungal) of banana. The GNBE4 (*Alcaligenes* sp.) showed maximum mycelial inhibition (75.18%), followed by GNBE9 (*Stenotrophomonas maltophilia*) (71.90%) and GNBE12 (*Staphylococcus warneri*) (70.69%), while lowest in GNBE14 (*Bacillus subtilis*) (61.07%). Among the fungal endophytes, the maximum mycelial growth inhibition was shown in RzFE and LfFE (42.78%) followed by PsdFE (36.11%), while lowest in PtFE1 (26.67%). Apart from that already identified antagonistic PGPR like *Pseudomonas fluorescens* was obtained from the Department of Microbiology were tested which significantly suppressed the mycelial growth of fungus having a mycelial inhibition of 70.01 %.

Keywords antagonism, banana, endophytes, FocTR4, *P. fluorescens*

Introduction

Banana (*Musa* spp.) is the world's most precious agricultural commodities and chief staple food for >400 million people and annual production is carried out by approximately 148 million tons by 135 countries [1]. Panama wilt is incited by a soil-borne fungal pathogen i.e. *Fusarium oxysporum* f. sp. *cubense* (Foc) is now getting a foremost threat to the production of bananas worldwide. Foc is soil-borne, necrotroph, broad host range, highly virulent, long persistent, survival ability of chlamydospores in soil under adverse climatic conditions for decades [2-3]. Furthermore, non-host crops like weeds act as a reservoir for the survival of the pathogens and therefore it could be considered as a potential source of inoculum [4]. Based on the pathogenicity of Foc over specific banana cultivars, it has been categorized into three races [5] in which race 1 infects to the 'Gros Michel having the genomic group AAA as well as 'Manzano' with a genomic group of AAB, race -2 causes infection to Bluggoe and other banana use for culinary purpose. Race 4 is again categorized into two STR4 and TR4 strains under subtropical regions and also underwater submerged and water scarcity stress [6]. While TR4 with

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VCG-01213/16 group is strongly pathogenic in dwarf cavendish group in the tropical and subtropical zone and therefore considered as the most threatening strain which affects the export potential of banana are around 80% globally [3, 7]. Furthermore, the TR4 is highly virulent on cultivars affected by races 1 and 2 also and other varieties [8]. Globally Fusarium wilt is a serious and notorious disease causing production loss of up to 15% and 47% of the total area under banana cultivation [9]. According to the FAO report, Foc TR4 will create havoc and cause loss of area and production of 160,000 ha and 2.8 million tonnes respectively by the end of 2028. Apart from that direct employment loss of 2.4 lakh banana labour as well as rise in price of banana cost up to 9.2% [10]. Moreover, it has been predicted that FocTR-4 will create havoc and annual losses reach up to US\$10 billion globally by 2040 [11]. However, Foc TR4 has been estimated to disseminate up to 17% of the global banana coverage area by the end of 2040 resulting in a loss of up to 36 million tons which directly correlates with a price loss in the US dollar context of US\$10 billion [12]. In India, the wilt incidence in G-9 has been reported in the majority of the banana cultivating areas of the country including Bihar (especially Purnea and Kathiar). In Bihar, the incidence of Fusarium wilt has been recorded up to 6-65 per cent [13] and disease was also reported in Faizabad district of Uttar Pradesh with a wilt incidence of 30-45 per cent [14]. In 2019-20 presence of strain B2 of FocTR4 has also been reported in Bihar [15]. Apart from that the disease has also been reported in other parts of the country by researchers including Surat district of Gujarat having a wilt incidence of 5-15 per cent and Tamilnadu with a wilt incidence of 15-21 per cent.

Panama wilt is the most destructive disease, which causes massive economic losses throughout the world and threatens global food security. Due to the non-effectiveness of chemicals pesticides and the non-availability of resistant cultivars has become a quite difficult task to control this disease. Hence biological management using endophytic microorganisms is considered a promising strategy for managing fusarium wilt. Therefore, the present investigation was taken into consideration to carry out in vitro evaluation and identification of the potential native endophytes of bananas for their antagonistic effect against FocTR4 causing Fusarium wilt of banana.

Methodology

Collection of plant sample

Experiments were proceeded in the Department of Plant Pathology, Pusa, RPCAU, Bihar. Samples of banana crops collected from the Banana AICRP farm in Pusa campus. Fresh, healthy and fully matured plants of the age of 6 to 7 month-old banana crops from the cultivar Grand Nane (AAA) were selected and different parts of the samples i.e. roots, leaves, pseudostem, rhizome and matured unripe fruit peel. Similarly, naturally wilt infected exhibiting typical symptoms of Fusarium wilt (internal symptoms in pseudostem with pith region, having reddish-brown discolouration and dryness in pith region) were collected from Purnea district, Bihar.

Isolation, identification, purification and maintenance of a culture of test pathogen

Isolation was done in half strength PDA media according to the protocol described by Dita et. al., [16]. Infected tissues along with some healthy tissues were chopped into tiny fragments (2-3 mm) with the help of a sterilized pointed blade and then transferred to a petri plate containing PDA medium under aseptic conditions followed by incubation at 28±2oC. The pathogen was identified based on cultural and morphological characteristics like that of macroconidia, microconidia and chlamyospores [17].

Isolation, purification and maintenance of endophytes

The protocol for the isolation of endophytes from banana crops was mentioned by Sekhar and Thomas [18]. The samples were thoroughly rinsed in tap water and chopped into tiny fragments and used surfactants i.e. Tween-20 (0.01%) then samples were subjected to disinfecting with 5% sodium hypochlorite (NaOCl) for 20 minutes and rinsed three times with SDW then after 70% ethanol for 1

minute followed by again rinsed 8th times with sterile distilled water (SDW). The sample was crushed in phosphate buffer by using a mortar- pestle. Solutions obtained from tissues sample were serially diluted up to 10³ dilutions. 100 microlitre of aliquots were pipetted from 10¹ and 10³ dilution and plated onto petri plates containing different media for fungal and bacterial endophytes. After plating, it was incubated at 25°C for 48-72 hrs and the appearance of any new colonies was immediately taken out and subcultured to the fresh medium. The bacterial and fungal endophytes have been shown in Figures 1 and 2.

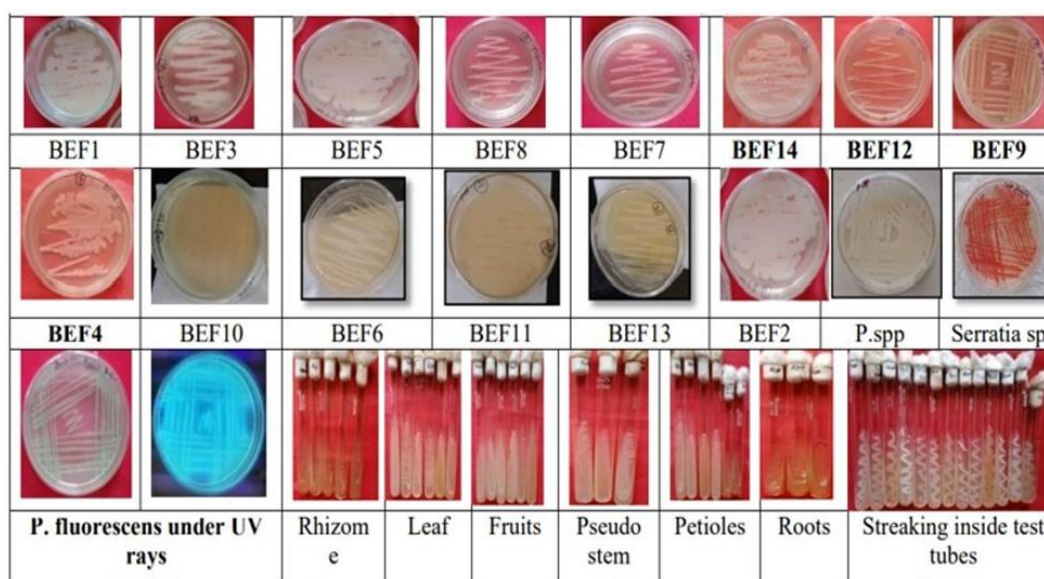


Figure 1. Pictorial representation of different bacterial endophytes isolates from different parts of banana crop cultivar Grand Naine

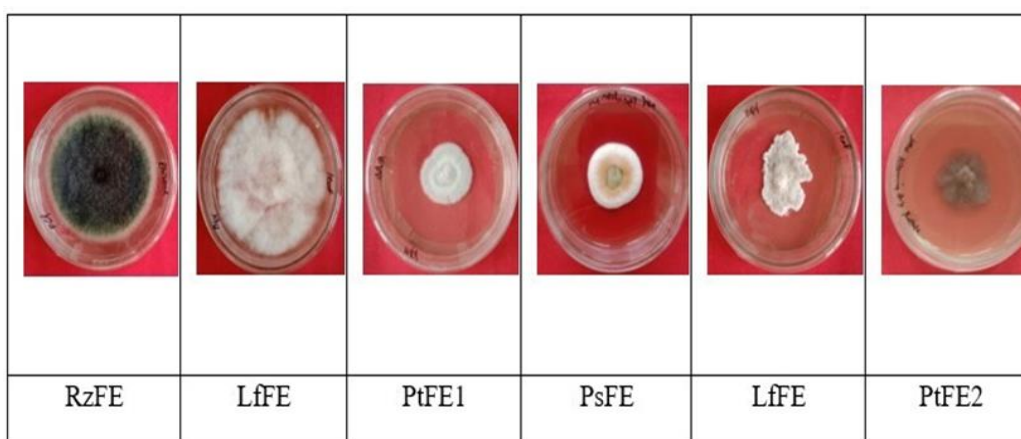


Figure 2. Pictorial representation of fungal endophytes isolated from various parts of Banana crop cultivar Grand Naine

Antagonistic effect of bacterial endophytes against test pathogen

The dual culture technique was performed to find out the antagonistic potential of endophytes against the pathogen Foc TR4 under in vitro conditions [19]. The test pathogen was inoculated in the centre of Petri plates corresponding to the bacteria was streaked on the periphery of the pathogen away from 2.5 cm to the distal end of the plates. A plate containing only FocTR4 was served as a check and incubated at 28± 2°C and per cent inhibition was measured by using the formula described



by Wei et al., [20].

Antagonistic effect of fungal endophytes against test pathogen

The antagonistic potential of endophytes (fungi) was conducted against Foc TR4 through a dual culture technique under lab conditions. Per cent inhibition of radial growth was measured through the following formula as described by Vincent [21]

Statistical Analysis

Data analysis was performed by SPSS software and compared by DMRT ($p < 0.05$) [22].

Results and Discussion

The data presented in (Table1 and Figure 3) revealed that the antagonistic effect was observed in only four bacterial endophytes i.e.GNBE4, GNBE9, GNBE12, and GNBE14 out of 13 bacterial endophytes in which the highest mycelial inhibition per cent of 75.18 % was observed in case of GNBE4 followed by GNBE9with 71.90% and GNBE12 with 70.69%. The lowest mycelial inhibition per cent was recorded in GNBE14 having an inhibition of 61.07%. Apart from that, two PGPR i.e. *P. fluorescens* and *Serratia* spp also tested against the pathogen in which only *P. fluorescens* exhibited the percent mycelial inhibition of 70.01%.

Table: 1 Effect of bacterial endophytes over FocTR4 under lab condition

SN.	Bacterial Endophyte	Colony diameter (mm)	Inhibition (%)
1	BEF2	46.82 ±3.25 ^{cd}	47.97±3.61 ^{cd}
2	BEF3	64.69±7.01 ^f	28.12±7.79 ^f
3	BEF4	22.34±0.29 ^a	75.18±0.32 ^a
4	BEF5	66.73±1.25 ^f	25.86±1.39 ^f
5	BEF6	67.98±0.47 ^f	24.47±0.52 ^f
6	BEF7	56.91±8.27 ^e	36.77±9.19 ^e
7	BEF8	49.46±4.78 ^d	45.05±5.31 ^d
8	BEF9	25.29±0.26 ^a	71.90±0.29 ^a
9	BEF10	78.44±1.10 ^g	12.85±1.22 ^g
10	BEF11	74.37±0.23 ^g	17.37±0.25 ^g
11	BEF12	26.38±4.01 ^a	70.69±4.45 ^a
12	BEF13	79.14±3.26 ^g	12.06±3.62 ^g
13	BEF14	35.03±0.55 ^b	61.07±0.61 ^b
14	PGPR-1	74.58±0.88 ^g	17.13±0.97 ^g
15	<i>P. fluorescens</i>	26.99±1.81 ^a	70.01±2.01 ^a
16	<i>Serratia</i> spp	41.81±1.13 ^c	53.55±1.26 ^c
17	Control	90.00±0.0 ^h	0.00±0.0 ^h
	CD at 5%	5.52	6.13
	SE±(m)	1.91	2.13
	CV	6.07	5.34

Similarly, in the case of a total of five fungal endophytes RzEF, PtEF1, PtFE2, PsdFE and LfEF, the maximum mycelial inhibition of 42.78 % and 42.78% was observed in the case of RzFE and LfFE

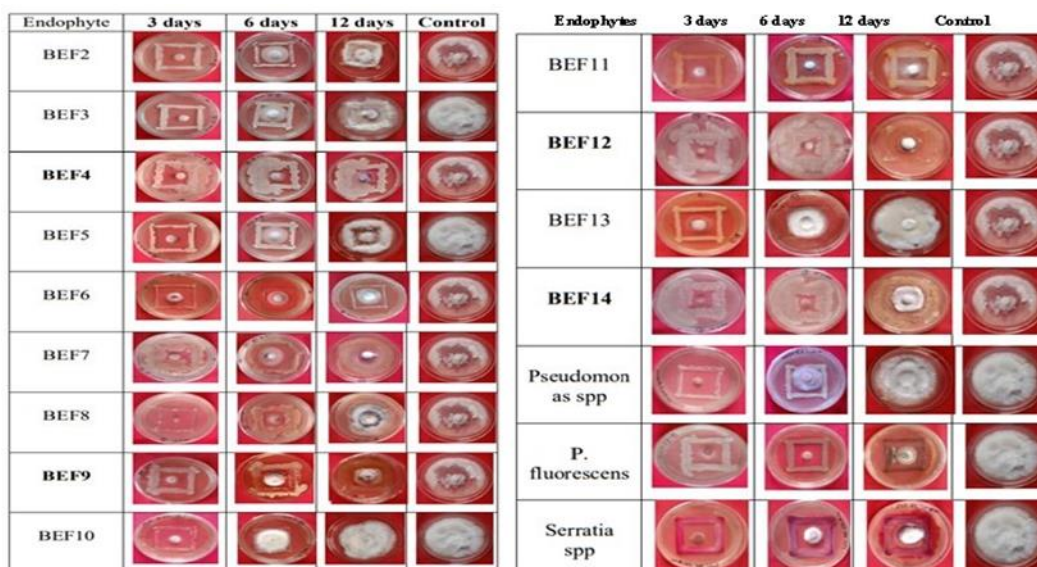


Figure 3. *In vitro* evaluation of bacterial endophytes against FocTR4

respectively which are statistically at par with respect to each other followed by PsdFE with 36.11 %. The lowest mycelial inhibition per cent was recorded in PtFE1 having an inhibition of 26.67 %. The data presented for antagonistic fungal endophytes against FocTR4 was shown in Table 2 and Figure 4. Application of antagonistic microbes i.e. endophytes which defend and trigger plant growth promoting activity through colonization and multiplication inside the plant system might be an alternative strategy to manage the fusarium wilt [23]. Several reports regarding the management of Panama wilt by using different species of both rhizospheric and endophytic microorganisms like *Trichoderma*, *Penicillium*, non-pathogenic races of *Fusarium* sp, *Bacillus* sp, *Streptomyces* sp and *Pseudomonas* sp have been successfully exploited under both in vitro and in vivo condition [24-25]. The research finding of Tan et al., [26] reported that ITBB B5-1 (*Serratia marcescens*) has shown that the radial growth inhibition of Foc race 4 was 95.4% through tip culture assay. The research finding of Fan et al., [27] reported that a total of 813 isolates of bacterial endophytes in which only two YN0904 (*B. amyloliquefaciens*) and YN1419 (*B. subtilis*), the mycelial inhibition under in vitro condition was recorded to 79.6% and 81.3%, respectively. This research finding is contradictory to our research finding in the case of my isolates GNBE-14 (*B. subtilis*) which showed that the percent inhibition of radial growth was observed to be 61.01%.

Zhang et al., [28] experimented and reported that out of 144 endophytic actinomycetes in which *Streptomyces malaysiensis* 8ZJF-21 showed broad-spectrum antifungal activity through culture filtrate assay and revealed the inhibition per cent of radial growth of 73.96 % and inhibited spore germination of Foc TR4 under lab condition. The research finding of Zhang et al., [29] reported that a total of 60 actinomycetes, 17 strains and their culture filtrates showed significant antifungal activity over FocTR4 predominantly and strain BITDG-11 was found to be the strongest mycelium inhibition of 80.48±1.49.

Martin et al., [30] reported that the antifungal bioassay of extracts of culture filterates of *B. amyloliquefaciens* strain CCIBP-A5 significantly inhibited the per cent growth and morphological structure deformities of the mycelium of 25 and 23.46% when the culture filtrate was used in a ratio of 1:10 and 1:100. According to Shen et al., [31] he conducted the experiment and found that out of 60 strains of PGPR, the strain Gxun (*Bacillus siamensis*) showed antifungal activity with maximum inhibition mycelium per cent of 68.8% and also showed that disease intensity was reduced to be 88.26% along with enhancement of fresh weight by 25.36% under pot experiment.

Table 2. Effect of fungal endophytes over Foc TR4 under lab condition

SN.	Fungal endophytes	Colony diameter (mm)	Inhibition (%)
1	RzFE	51.50±4.20 ^a	42.78±4.66a
2	PtFE1	66.00±6.62 ^b	26.67±7.36b
3	PtFE2	60.00±5.98 ^{ab}	33.33±6.64ab
4	PsdFE	57.50±6.23 ^{ab}	36.11±6.92ab
5	LfFE	51.50±0.87 ^a	42.78±0.96a
6	Control	90.00±0.0 ^c	0.00±0.0c
	CD at 5%	8.59	9.54
	SE(m)	2.76	3.06
	CV	7.61	6.52

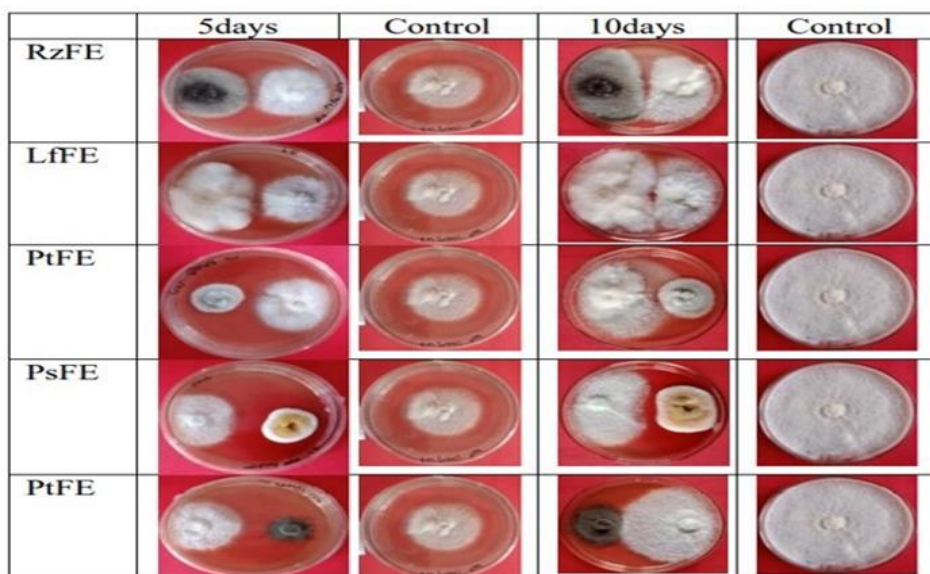


Figure 4. In vitro evaluation of fungal endophytes against FocTR4

The research findings of Shahzad et al., [32] in vitro study revealed that RWL-1 strains (*B. amylolequifaciens*) showed maximum growth inhibition activity of 79.19 ± 3.8 against *F. oxysporum* f. sp. lycopersici as compared to control. According to Yadav et al., [33] the experiment was conducted to evaluate the antagonistic activity of strains CSR4 (*Bacillus licheniformis*) and they found that the strain inhibited the significant inhibition (%) of radial growth of FocTR4 of 77.59% through dual plate assay and also found to be a reduction of disease incidence of 10% along with increased defence-related enzyme in inoculated plant with CSR4 strains. According to research findings of Taribuka et al., [34] in vitro study reported that out of four fungal endophytes, only *Trichoderma gamsii* showed the per cent mycelial inhibition of 60.61% and in greenhouse conditions and the *T. asperellum* and *T. harzianum*swn-2 would repress the disease severity of 8.33%. According to research findings of Sudantha et al., [35] in vitro study reported that six isolates of the endophytic fungi *Trichoderma* spp including m, *T. viride*, *T. koningii*, *T. harzianum*, *T. hamatum*, *T. areoviride* and *T. piluliferum*. Among them, the maximum mycelial growth inhibition was observed in *T. koningii* at 45.70 % followed by *T. harzianum* at 43.60% and the least inhibition was recorded in *T. piluliferum*



with 40.10% against Foc. The research finding of Puig and Cumagun [36] reported that a total of 155 fungal endophytes were tested against FocTR4 using the dual culture method. The mycelial inhibition percentage was found to be more than 75% in the case of *Penicillium* CGP116, *Trichoderma* CGP.106, *Fusarium* CGP.150, *Pestalotiopsis* CGP.117 and *Schizophyllum* CGP.119 against FocTR4. Apart from that it also produced amylase, cellulase, chitinase and protease enzyme and concluded that *Schizophyllum* CGP119 exhibited antagonistic ability over FocTR4.

Conclusion

The present investigation demonstrated that bacterial endophytes i.e. GNBE4 is found to be best and exhibit mycelial inhibition per cent of 75.18 % followed by GNBE9 (71.90%) and GNBE12 (70.69%), while lowest in GNBE14 (61.07%) under in vitro condition. It can be re-evaluated in a pot experiment to figure out its antagonistic activity against Foc to manage Fusarium wilt in bananas. Among the fungal endophytes, the maximum mycelial growth inhibition was observed in RzFE and LfFE at 42.78%. Endophytes have a crucial role in plant disease management due to the vast majority of secondary metabolites like peptides, quinones, phenols, alkaloids, steroids, terpenoids and flavonoids as well as hydrolytic enzymes like chitinases and β -1,3 glucanase are responsible for growth inhibition and degradation of cell wall of pathogen. Indirectly, it shows biological control through competition, antibiosis, siderophore production and SAR induction in plants. Furthermore, there is a need to be focused on the commercialization of consortium development, determination of dose and method of application. Much attention towards metagenomics to understand the genetic diversity of endophytes and the presence of a diverse array of genes, which are responsible for the synthesis of secondary metabolites, has to be sequenced and characterized which ultimately could broaden the feasibility and applicability of endophytes for management of wilt disease in field condition and minimize the losses caused by them.

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