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In vitro Screening of Native Banana Rhizospheric Microbes and Endophytes of Assam against *Fusarium oxysporum* f. sp. *cubense*

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A B S T R A C T

A total of 30 native rhizospheric microbes and 7 endophytes had been isolated and purified from rhizospheric soil samples and from roots of healthy banana plants, collected from different banana growing areas of Assam. The screening of 37 rhizospheric microbes and endophytes against *Fusarium oxysporum* f. sp. *cubense* was performed with the help of dual culture technique. The efficacy of the rhizospheric microbes and endophytes were recorded at different intervals from 24 to 120 hours. It was observed that out of 37 isolates, the per cent reduction in growth of *Fusarium oxysporum* f. sp. *cubense* after 120 hours of inoculation was found more than 70% in case of 10 rhizospheric microbes. Of which 3 rhizospheric microbes performed better with a per cent reduction in growth above 80%. However, not a single endophytes could be found promising in inhibition the growth of *Fusarium oxysporum* f. sp. *cubense*. The per cent reduction of growth was recorded minimum 47.3% in endophytes against 86.44% in rhizospheric microbes.

Introduction

The *Fusarium oxysporum* f. sp. *cubense* (Foc) is a devastating fungus causing *Fusarium* wilt in banana. The fungus infects the roots of banana plants, colonizes the vascular system of the rhizome and pseudostem. The infected plant induces characteristic wilting symptoms mostly after 5-6 months of planting and the symptoms are expressed both externally and internally (Wardlaw, 1961, Stover, 1962). The challenging part of the fungus is that it survives in soil for up to 30 years as chlamydospores in infested plant material or in the roots of alternative hosts (Ploetz, 2000). Since the discovery of *Fusarium* wilt of banana, various control strategies like soil fumigation (Herbert and Marx, 1990), fungicides (Lakshmanan *et al.*, 1987), crop rotation (Hwang, 1985, Su *et al.*, 1986), flood –fallowing (Wardlaw, 1961, Stover, 1962) and organic amendments (Stover, 1962) have been evolved and attempted, yet, the disease could not be controlled effectively except by planting of resistant cultivars (Moore *et al.*, 1999). Planting of resistant varieties also cannot be implemented successfully because of consumer preference (Viljoen, 2002). Under these circumstances, use of antagonistic microbes which protect the plants from pathogen and promote plant growth by colonizing and multiplying in both

rhizosphere and plant system has been proved to be a potential alternative approach for the management of *Fusarium* wilt of banana. Biological control of *Fusarium* wilt disease has already become an increasingly popular disease management consideration because of its environmental friendly nature (Weller *et al.,* 2002; Fravel *et al.,* 2003). Biological control of soil borne diseases caused especially by *Fusarium oxysporum* is well documented (Marois *et al.,* 1981; Sivan and Chet, 1986; Larkin and Fravel, 1998; Thangavelu *et al.,* 2004).

Several reports have demonstrated the successful use of different species of *Trichoderma, Pseudomonas, Streptomyces*, non-pathogenic *Fusarium* (np*Fo*) of both rhizospheric and endophytic in nature against Fusarium wilt disease under both glass house and field conditions (Lemanceau and Alabouvette, 1991, Alabouvette *et al.,* 1993, Larkin and Fravel, 1998, Weller *et al.,* 2002, Sivamani and Gnanamanickam, 1988, Thangavelu *et al.,* 2001, Rajappan *et al.,* 2002, Getha *et al.,* 2005, Pushpavathi *et al.,* 2016). Biological control of soil plant pathogens by antagonistic microorganisms is a potential nonchemical means of biocontrol agent against Plant diseases. In the present study efficacy of native potential rhizospheric microbes from banana rhizospheric soil has been tested against Foc *in vitro*.

Materials and Methods

Isolation of Foc

The isolation of the pathogen was carried when the strands have dried. Small sections (3-6 mm long) of dry discoloured vascular strands were submerged into petri- plates with ¼ strength PDA amended with an antibacterial agent streptomycin @ 1.2 ml/240 ml PDA. Generally, *Fusarium* growth appeared from the strands in 2 to 4 days. Single-spore (monoconidial) cultures were prepared from each isolates (Perez Vincent *et al.,* 2014).

Single-spore isolation for pure culture of Foc isolates

The single spore isolation of Foc was followed after Pérez-Vicente *et al.,* 2014. The single spore isolation of the fungus was obtained by either dilution plating or streaking. In this method, a small scrape of sporulating hyphae collected from cultures grown on ¼ strength PDA plates and dissolved in 10 ml sterile distilled water in test tubes. From the initial spore suspension, a series of dilutions were prepared. One ml of each of the dilution series was then streaked onto water agar and incubated with the lid up overnight at 25^0C. The plates were viewed for germination of conidia. Germinated single conidia were cut from water agar with a surface sterilized scalpel and transferred to new plates of PDA of ¼ strength. Cultures of the fungus growing on PDA medium exhibit abundant fluffy aerial mycelium after 2 days and produce abundant conidia. Additionally, single-spore cultures could also be obtained by dissecting the very tip of single growing hyphae from an older culture grown on PDA. Each single spore isolate was grown on filter paper overlaid on half strength PDA. The colonized filter paper was lifted from the agar plate, dried and stored at 4^0C until further use.

Collection, isolation and pure culture of Rhizospheric Microbes (RM)

The rhizospheric soil samples were collected from the rhizosphere of healthy banana plants from different banana growing locations of Assam (cv. Malbhog). 5g soil samples were collected randomly from a field and mixed together for a composite sample and brought to laboratory for isolation of RMs having antagonistic property. For isolation of rhizospheric microbes, the protocol described

by (Thangavelu and Gopi, 2015) as given below was followed.

One gram of rhizospheric soil collected from different cultivars of banana was transferred to 250 ml conical flasks containing 100 ml of sterile distilled water. The flask was placed in rotary shaker for 10 min at 120 rpm to dissolve the soil thoroughly. From this, 1 ml of the supernatant was taken and serially diluted upto 10^{-5} dilutions. One ml of the dilution such as 10^{-3}, 10^{-4} and 10^{-5} was poured at the centre of sterilized petri plates. Onto such plates, media was poured and rotated clockwise and anticlockwise. For isolating fungal rhizospheric microbial culture potato dextrose agar (PDA) medium is used amended with cyclohexamide to suppress the emergence of bacteria. Finally the plates were incubated at 28^0C for 2 days and observed for emerging colonies. Colonies were purified and glycerol stock of each isolate was maintained at -80^0C for future use. Each fungal colony was purified by single spore isolation technique.

Isolation and pure culture of Endophytes (EP)

For isolation of EPs the protocol described by (Thangavelu and Gopi, 2015) as given below was followed. Banana EPs were isolated from roots of banana germplasm collected from different banana (cv. Malbhog) growing pockets of Assam. The young roots (both from healthy and wilted plants) were selected for collection. The samples of roots were thoroughly washed with tap water to remove dust and soil particles. Then, 5 g of these roots individually immerged in 70% ethanol for 3 minutes, followed by washing with fresh sodium hypochlorite (2.5%) solution for 5 minutes, rinse with 70% ethanol for 30 seconds and subsequently washed five times with sterile distilled water. The aliquots of the sterile distilled water used in the final rinse were spread on tryptic soy agar (TSA) medium plates to confirm the successful sterilization process and the isolates were of endophytic origin. The samples were macerated using sterile mortar and pestle using 1 ml of sterile distilled water and each sample was serially diluted in test tubes containing 9 ml of sterile distilled water. The dilutions such as 10^{-3}, 10^{-4} and 10^{-5} were plated separately on PDA. The plates were then incubated at 28°C for 3 days and observed for the emergence of endophytes and each colony obtained was sub-cultured for further evaluation.

Testing the efficacy of native banana RMs and EPs against Foc

All the native RMs and EPs obtained were screened against Foc pathogen by dual culture plate assay (Dennis and Webster, 1971) Per cent inhibition of mycelial growth of the pathogen was calculated using the formula suggested by Vincent and Budge (1990). A 10 mm diameter mycelia disc was cut from a 5 day old culture of Foc pathogen and placed at 1 cm from the inner edge of 9 cm petri dishes containing PDA agar medium. Each bacterial and fungal antagonist was either streaked or inoculated on the opposite side of the Foc mycelia disc and incubated at 28^0C for 5-7 days. The PDA medium containing Foc alone was maintained as control and three replicates were maintained. The growth of the pathogen and the per cent reduction over control was calculated.

$$\text{Per cent reduction Over control (\%)} = \frac{\text{Growth in control- growth in treatment}}{\text{Growth in control}} \times 100$$

The design followed was Completely Randomized design (CRD) with five replications and three plates per replication was maintained.

Results and Discussion

Isolation of Foc

The fungus exhibited white with purple tinge colony colour, circular shape and smooth margin with abundant aerial cottony mycelia when grown on ¼ strength PDA. Microscopic observation revealed that Microconidia (10-12 × 2.4-3.0 µm), one or two celled, oval to kidney shaped (one or no septation), Macroconidia (27-30 × 3.4- 3.6 µm), four celled (3 septa), and sickle-shaped with attenuated tip and borne on foot shaped basal cell were observed. Chlamydospores (7-9µm dia) both terminal and intercalary with globose shape are formed singly or in pairs in hyphae.

Based on the above cultural and morphological characters the fungus was identified and confirmed as *Fusarium oxysporum* f.sp *cubense* after Booth (1971). The pathogenicity of Foc was confirmed by Koch's postulation.

Collection, isolation and pure culture of native RMs

In an attempt to isolate potential native RMs against Foc, rhizospheric soil samples were collected from different banana growing areas of Assam such as Dibrugarh, Diphu, Sarupeta, Madang, Boko, Dhenubhanga, Dudhnoi, Rangsipara, Shingra, Kukurmara, Bartari and Gossaigoan covering three agro climatic zones. The collected samples were brought to laboratory for isolation, purification and identification of potential rhizospheric microbes. A total of 30 native RM were isolated and purified from 206 soil samples collected. On the other side, a total of 7 EP were also isolated from 110 root samples. The study on the efficacy of the 37 RM and EP isolates against Foc was studied *in vitro* for their potentiality to inhibit/suppress Foc growth (diameter in cm).

The study was recorded at 5 intervals ranging from 24 to 120 hours of inoculation of Foc varied significantly with that of control at all the intervals (Table 1). The observation was recorded upto 120 hours of inoculation as full growth of Foc was observed under control. An increasing trend in the per cent growth reduction of Foc was observed under different treatments at various intervals.

The growth of Foc after 24 hours of inoculation was 0.99 cm under RM 05, which differed significantly from all other treatments. The effect of RM 05 was followed by RM 76 with a growth of 1.03 cm. Next, RM 76 was followed by RM 03 with a growth of 1.04 cm. The per cent reduction in growth under RM 05 was 55% followed by RM 76 with a per cent reduction of 53.18%. Further, RM 76 was followed by RM 03 with 52.75%.

Next, the growth of Foc after 48 hours of inoculation was 1.64 cm under RM 05, which differed significantly from all other treatments. The effect of RM 05 was followed by RM 76 with a growth of 1.66 cm. Next, RM 76 was followed by RM 03 with a growth of 1.69 cm. The per cent reduction in growth under RM 05 was 59% followed by RM 76 with a per cent reduction of 58.50%. Further, RM 76 was followed by RM 03 with 57.75%. Similar observations were recorded after 72 and 96 hours of inoculation.

After 120 hours of inoculation, the growth of Foc was 1.03 cm under RM 05, which differed significantly from all other treatments. The effect of RM 05 was followed by RM 76 with a growth of 1.36 cm.

Thereafter, RM 76 was followed by RM 03 with a growth of 1.41 cm. The per cent reduction in growth under RM 05 was 86.44% followed by RM 76 with a per cent reduction of 82.10% which was followed by RM 03 with a per cent reduction of 81.44%.

Table.1 Effect of rhizospheric microbes on growth of Foc

Treatment	Growth of Foc after (dia in cm)	*PRG	Growth of Foc after (dia in cm)	*PRG	Growth of Foc after (dia in cm)	*PRG	Growth of Foc after (dia in cm)	*PRG	Growth of Foc after (dia in cm)	*PRG
	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs	
Rhizospheric microbes										
RM 01+Foc	1.09	50.09	1.72	57.00	2.60	50.00	2.87	52.16	1.55	79.60
RM 03+Foc	1.04	52.72	1.69	57.75	2.27	56.34	2.71	54.83	1.41	81.44
RM 04+Foc	1.06	51.81	1.71	57.25	2.24	57.23	2.85	52.50	1.56	79.47
RM 05+Foc	0.99	55.00	1.64	59.00	2.19	57.88	2.34	61.00	1.03	86.44
RM 9+ Foc	1.25	42.72	2.30	40.00	3.80	26.92	3.40	43.33	2.29	69.86
RM 12+ Foc	1.24	43.63	2.32	42.00	3.50	32.69	3.48	42.00	2.30	69.73
RM 19+Foc	1.21	45.00	1.91	52.50	2.63	49.42	3.13	47.83	1.44	81.18
RM 22+Foc	1.20	45.45	1.98	50.50	2.79	46.34	3.38	43.66	1.86	75.52
RM 25+ Foc	1.30	40.90	2.43	39.25	3.60	30.76	3.55	40.83	2.29	69.86
RM 30+ Foc	1.30	40.90	2.46	38.50	3.80	26.92	3.58	40.33	2.30	69.73
RM 40+ Foc	1.26	42.72	2.38	40.50	3.60	30.76	3.50	41.66	2.39	68.00
RM 45+ Foc	1.29	41.36	2.40	40.00	3.50	32.69	3.53	41.16	2.30	69.73
RM 51+ Foc	1.36	38.18	2.49	37.75	3.60	30.76	3.60	40.00	2.39	68.00
RM 65+Foc	1.22	44.54	2.00	50.00	2.70	48.07	3.09	48.50	1.51	80.13
RM 69+ Foc	1.39	36.81	2.50	37.50	3.90	25.00	3.64	39.33	2.40	68.42
RM 76+Foc	1.03	53.18	1.66	58.50	2.26	57.19	2.46	59.00	1.36	82.10
RM 88+ Foc	1.40	36.36	2.54	36.50	3.80	26.92	3.68	38.66	2.48	67.36
RM 90+ Foc	1.44	34.54	2.58	35.50	3.60	30.76	3.70	38.33	2.50	67.10
RM 97+ Foc	1.49	32.27	2.60	35.00	3.70	28.84	3.76	37.33	2.54	66.57
RM 103+Foc	1.08	50.90	1.67	58.50	2.37	54.42	2.65	55.83	1.55	79.60
RM 116+Foc	1.09	50.45	1.85	53.75	2.60	50.00	3.23	46.16	1.91	74.86
RM 129+Foc	1.50	31.81	2.63	34.25	3.60	30.76	3.80	36.66	2.59	65.92
RM 153+Foc	1.54	30.00	2.67	33.25	3.60	30.76	3.87	35.50	2.60	65.78
RM 160+Foc	1.58	28.18	2.70	32.50	3.70	28.84	3.90	35.00	2.70	64.47
RM 173+Foc	1.60	27.27	2.74	31.50	3.90	25.00	4.00	33.33	2.80	63.15
RM 175+Foc	1.63	25.90	2.78	30.50	3.70	28.84	4.20	30.00	2.90	61.84
RM 189+Foc	1.66	24.54	2.80	30.00	3.60	30.76	4.30	28.33	3.00	60.52
RM 200+Foc	1.69	23.18	2.82	29.50	3.90	25.00	4.50	25.00	3.20	57.89
RM 205+Foc	1.70	22.72	2.85	28.75	3.80	26.92	4.60	23.33	3.40	55.26
RM 206+Foc	1.72	21.81	2.88	28.00	3.70	28.84	4.70	21.66	3.90	48.68
Endophytes										
PTH-E +Foc	1.75	20.45	2.90	27.50	3.70	28.84	4.78	20.33	4.00	47.36
RNG1-E+Foc	1.78	19.09	3.00	25.00	3.90	25.00	4.90	18.33	4.20	44.73
GOS-E+Foc	1.66	18.18	3.20	20.00	3.80	26.92	4.20	30.00	5.00	34.21
RNG2-E+Foc	1.69	24.54	2.85	28.75	3.60	30.76	4.30	28.33	5.60	26.31
GOS5-E+Foc	1.70	23.18	2.80	30.00	3.60	30.76	4.00	33.33	4.00	47.36
ROU-E+Foc	1.72	22.72	2.70	32.50	3.90	25.00	4.60	23.33	5.00	34.21
BRT-E+Foc	1.69	23.18	2.90	27.50	3.80	26.92	4.70	21.66	4.20	44.73
Control	2.20	-	4.00	-	5.20	-	6.00	-	7.60	-
C.D (0.05)	0.007	-	0.086	-	0.020	-	0.017	-	0.011	-

Fig.1 Growth of Foc under three best treatments after 72 hours of inoculation

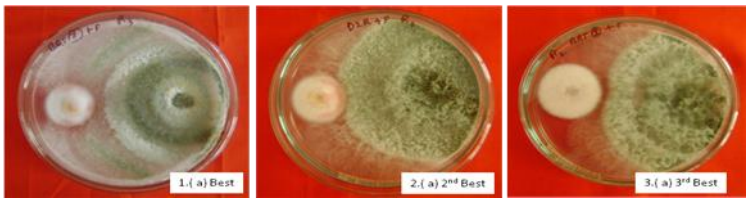
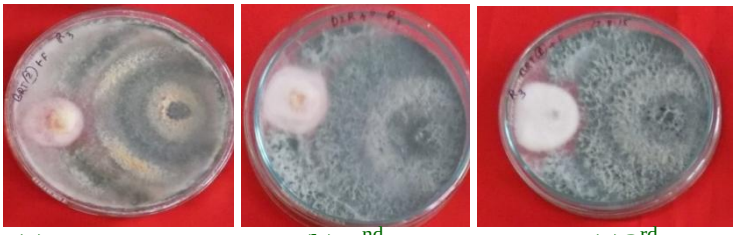
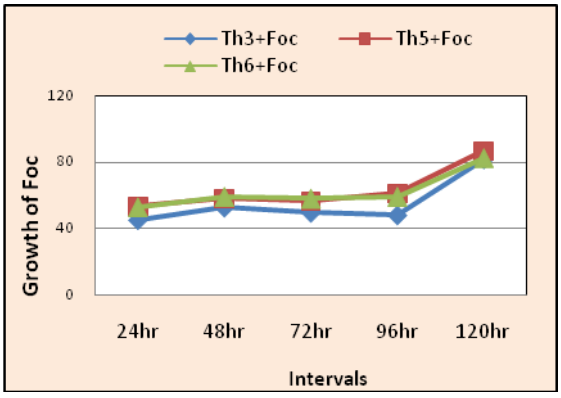


Fig.2 Growth of Foc under three best treatments after 120 hours of inoculation



(a) Best (b) 2nd Best (c)3rd Best

Fig.3 Per cent growth reduction of Foc at different intervals



It has also been observed that the RMs were found to be more effective with that of EPs. In case of the least effective RMs (RM 206), the per cent growth reduction of Foc was observed 48.68% against the best effective EPs (PTH-E) which recorded only 47.36% growth inhibition of Foc. On the other hand, the effect of ten RMs was found to be promising with capabilities of per cent growth reduction of Foc more than 70%. Based on such result, the EPs were not considered for further studies. Moreover, the RMs with a capability of more than 80 per cent Foc growth reduction were considered for further studies.

The comparative growth of 3 best RMs after 72 and 120 hours after inoculation (Fig. 1 and 2). The variations in the per cent growth reduction of Foc under best three treatments were presented in Fig. 3

The rhizosphere encompasses the millimeters of soil surrounding a plant root where complex biological and ecological processes occur. The growth of banana plants is closely related to the bio- diversity in the rhizosphere. Under constant supply of normal nutrition, the roots of the banana plants are well developed. The exudates from the root hairs or root tip promote the growth of rhizosphere organisms

(Bais *et al.*, 2006), which in turn enhance the biodiversity of soil. In our study, 206 rhizospheric soil samples collected from rhizosphere of healthy banana plants adjacent to infected fields from different locations of Assam (cv. Malbhog).

A total of 110 banana root samples were collected from different banana growing locations of Assam adjacent to wilted fields for isolation of EPs. Reports of collection of EPs from healthy as well as diseased plant are available and the report of Cao *et al.*, (2014) indicated that a higher percentage (50%) of antagonistic actinomycetes was found in healthy plants, especially in the roots. However, they reported that the EPs isolated from diseased plants had higher degree of diversity. These results are in conformity with the findings of Reiter *et al.*, (2002). In an interesting note Cao *et al.*, (2014) further added that no difference in terms of efficacy was evident amongst the EPs isolated from wilted and healthy plants.

In the present investigation altogether 30 RMs and 7 EPs were collected and their efficacy was tested against Foc. Though the effect of the RMs and EPs varied significantly with that of control at different intervals from 24 hours to 120 hours. After 24 hours the per cent reduction was observed highest (55%) in RM 05 followed by RM 76 (53.18%). Similar trend in reduction percentage was observed under 48, 72, 96 and 120 hours after inoculation with RM 05 was the best followed by RM 76.

Moreover, the per cent reduction in growth of Foc after 120 hr of inoculation was found more than 70% in case of 10 RMs. And the best 3 were selected for further studies. Not a single endophyte could show their promise for growth inhibition of Foc. The per cent reduction of growth was recorded minimum 47.3% in EP against 86.44% in RM. Probability of the result may be explained by considering which might be due to many reasons, *viz*., occurrence of lower population densities of endophytes than rhizospheric microbe (Hallmann *et al.*, 1997, Rosenblueth and Martínez-Romero, 2004), shifting of the status of endophytes from endophytic nature to pathogenic depending upon developmental stages of the plant (Stone *et al.*, 2000 and Schulz and Boyle, 2005).

The promising 3 RMs were found to possess similar cultural and morphological characters based on the cultural and morphological characteristics the isolates were identified as *Trichoderma* spp. after Rifai, 1969 which were further confirmed as *Trichoderma harzianum* Rifai. at Agharkar Research Institute, Pune.

The result of the present study revealed fair amount of efficacy against Foc *in vitro* and a large number of reports support our findings (Sundaramoorthy and Balabaskar, 2013, Otadoh *et al.*, 2011, Barari, 2016).

The potentiality of *Trichoderma harzianum* as one of the most efficient biocontrol agent is considered due to its high reproductive capacity, ability to survive under unfavourable conditions, efficiency in nutrient utilization, capacity to modify the rhizosphere, strong aggressiveness against the pathogenic fungi and efficiency in promoting plant growth and defence mechanisms (Misra and Prasad, 2003).

The result of dual culture test revealed two exceptional RMs- *Ceratocystis frimbrata* and *Rhizopus arrhizus*. These two RMs are pathogens causing diseases. But from the experiment it was recorded that they could inhibit the growth of Foc sufficiently as compared to other RMs and EPs. This nature of antagonism may be due to availability of non-pathogenic form of the fungus such as

non-pathogenic form of *Fusarium* against Foc. Moreover sometimes pathogens may act as biocontrol agent. There are references of *Penicillium* sp. acting as biocontrol agent against Phytophthora root rot of Azalea and citrus (Fang and Tsao, 1995). Therefore, further studies on *Ceratocystis frimbrata* and *Rhizopus arrhizus* as biocontrol agents may open up new areas of research.

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