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In vitro Evaluation of Bacterial Bioagents against *Fusarium* wilt of Banana caused by *Fusarium oxysporum* f. sp. *Cubense*

T. C. Archith[1]*, V. Devappa[2] and R. K. Mesta[1]

[1]Department of Plant Pathology, College of Horticulture, Bagalkot-587104, Karnataka, India
[2]Department of Plant Pathology, College of Horticulture, Bengaluru-560065, Karnataka, India

**Corresponding author*

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A B S T R A C T

The present investigation was undertaken to evaluate potential inhibitory effect of indigenous bacterial biocontrol agents against *Fusarium oxysporum* f. sp. *cubense*, a causal organism of wilt disease in Banana crop. Twelve indigenous bacterial bioagents were isolated from banana rhizosphere soil collected from banana growing fields in and around Mysore, Mandya and Chamarajanagar districts, which is geographically located in Southern part of Karnataka, India. In PCR identification, 16S rDNA test, the size of the amplicon was 360 bp and 450 bp in *Bacillus* spp. and *Pseudomonas* spp. Amongst six isolates of *Pseudomonas* spp, and six isolates of *Bacillus* spp showed significant inhibition against the test pathogen. Among all the *Pseudomonas* isolates tested, Isolate MPB-2 (*Pseudomonas fluorescens*) showed maximum inhibition of 38.89 per cent and *Bacillus* sp CKB-1 showed maximum inhibition of 33.89 per cent. The obtained preliminary results are useful and promising enough for further studies towards isolation and characterization of bacterial bioagents responsible for disease control.

Introduction

Banana (*Musa* spp.) is an important ancient tropical fruit in the world. It belongs to family Musaceae and native to tropical region of Southeast Asia. Banana is cultivated throughout the warm tropical regions of the world and extensively cultivated in Brazil, Ecuador, China, Philippines, Indonesia, Cost Arica, Mexico, Thailand, Colombia and India. It is grown as monoculture or mixed cropping system. The total annual global production of banana is estimated around 113.9 million tonnes (agriexchange.apeda.gov.in). India stands first in the world banana production (26.5 % of the world production). In India, it

is regarded as "fruit of the wise men" and it is grown in an area of 8.74 lakh ha with an annual production of 30 million tones and the average productivity is 35.88MT/ha (http://agricoop.nic.in). Tamil Nadu state has the largest area under cultivation in India, followed by Maharashtra, Gujarat, Andhra Pradesh, and Karnataka. The average yield/hectare is very low, due to abiotic stressors, such as salinity (Willadino *et al.,* 2017) and drought (Said *et al.,* 2015; Nansamba *et al.,* 2020). Another biotic stressor, represented by their primary pests, the banana root borer (*Cosmopolites sordidus*) and the nematodes *Meloidogyne* spp., *Pratylenchus coffeae* and *Radopholus similis* (Monteiro *et al.,* 2020) and disease-causing pathogens, including banana bunchy top virus (BBTV) (Galvez *et al.,* 2020; Sairam *et al.,* 2020), *Xanthomonas vasicola*p.v.*musacearum* causing bacterial wilt (Studholme *et al.,* 2020), *Pseudocercospora fijiensis* causing black Sig Sigatoka (Timm et., 2016) and *Fusarium oxysporum* f. sp. *cubense* (FOC) causing Fusarium wilt (Dita *et al.,* 2018). Among, Panama disease also known as *Fusarium* wilt or vascular wilt incited by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyd. and Hans. is one of the world's most disastrous plant diseases (Siamak and Zheng, 2018) and the disease was believed to have originated in Southeast Asia (Stover, 1962) The latest outbreak of FocTR4 has been confirmed in the Americas affecting the most popular commercial variety which could have jeopardized banana production for decades (Lambert, 2019). *Fusarium oxysporum* f. sp. *cubense* (Foc) causes a typical wilt syndrome on the infected banana plants accompanied by the necrosis and rotting of roots, rhizome, and pseudostem vessels. These symptoms occur between 2 and 5 months after infection of roots (Stover, 1962). The first internal symptom of the disease occurs in the hair roots which are the initial sites of infection. The infection later progresses to the rhizome and pathogen passes through the affected vessels to the new growing shoot (Li, *et al.,* 2017). Use of pesticides and other commercially available fungicides have shown a ray of hope on improving the crop yield but at the same time, the large use of these pesticides and fungicides are bound with various limitations such as loss of soil fertility, contamination of both ground and surface water, biomagnifications, health hazards, etc. which are reported to have deleterious effects on health of all living organisms of the biosphere. Therefore, alternative strategies are being widely employed. One such practice is use of bio-control agents. Research on bio-control agents have expanded in recent past as eco-friendly management of targeted crops. One such area is bio-control activity against *Fusarium* wilt Banana. Hence, the present study was designed and executed towards isolation of indigenous bio-control isolates of *Pseudomonas* spp and *Bacillus* spp from Banana rhizosphere soil as the performance of the introduced bio-control agent may not be always favourable because of competition for space and resources with the already established microorganism in the microcosm. Literature pursued by far have demonstrated positive ray of bio-control agents against *Fusarium* wilt but there is a lack of research dealing with the species used in the study and its commercial application. Consequently, large number of studies demonstrated the potent of isolates bearing significant activity even at harsh conditions (Killani *et al.,* 2011).

Materials and Methods

Isolation of native antagonistic rhizosphere bacteria

Antagonistic bacteria were isolated by serial dilution technique. Composite soil sample was collected from rhizosphere of healthy plants. The soils were dried under shade and then used for serial dilution. To get 10^{-1} dilution,

ten grams of soil was dissolved in 90 ml of sterile distilled water. From this, one ml of soil suspension was taken and added to nine ml of sterile distilled water to get 10^{-2} dilution. This was repeated until a final dilution of 10^{-6} for bacteria was obtained. Antagonistic bacteria were isolated on King's B agar medium and LB agar medium by using a dilution of 10^{-6}.

One ml of final dilution of soil suspension was poured into sterilized Petri plates, and then melted and cooled media was poured. Plates were rotated gently on the laminar air flow bench to get uniform distribution of soil suspension in the medium. Subsequently the plates were incubated at 28 ± 2°C and observed at frequent intervals for the development of colonies. One day old individual colonies of gram-negative rod-shaped bacteria on KB agar medium and gram-positive rod shaped on LB agar medium were picked up and purified by streak plate method and stored in NA slants at 4°C for further use.

Identification of Bioagents and *In vitro* evaluation of antagonistic potential of biological control agents

The bacterial bioagents were identified using the molecular tools. Confirmed bioagents by PCR is used the *in vitro* studies. Total of 12 bioagents was tested against *Fusarium oxysporum* f. sp. *cubense* in dual culture technique.

Among them, six isolates were *Bacillus* spp and six were *Pseudomonas* spp. The mycelial disc (5 mm) from 7 days old culture of *Fusarium* was placed on one side of the plate containing PDA medium, one day prior to bacterial streaking and then next day antagonist bacterial strains were streaked on the opposite side of the plate with the help of sterilized inoculation needle. The plates were incubated at room temperature for seven days.

The inhibitory effects of bacterial against linear growth of *Fusarium* are determined. Per cent inhibition of mycelial growth of test pathogen over control was calculated by the formula.

$$\text{Per cent inhibition} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

Statistical analysis

The data obtained in the experiment were statistically analyzed by using completely randomized design (CRD). The data pertaining to percentages were arc sin transformed using Web Agri. Stat. Package 2 developed by ICAR research complex, Goa. The significance of the effect of *Pseudomonas and Bacillus* on growth characteristics were determined by the magnitude of the F value (P = 0.01). Results of the experiment were analyzed following appropriate statistical methods as per the procedure suggested by Panse and Sukhatme (1985).

Results and Discussion

All the bacterial bioagents were isolated using serial dilution method, initial identification of *Pseudomonas* and *Bacillus species* were made using morphological features. Typical colony of *Pseudomonas spp.* showed umbonate, round smooth margins, and appear colourless, translucent on King's B medium and fluorescent when exposed to UV light.

Bacillus appeared as dry, flat, irregular creamy colony with irregular lobate margins. Based on the morphological appearance and colony characters, total six *Pseudomonas species* and six *Bacillus species* were selected from soil samples. All the isolates were stored in a glycerol stock (25%) at -80 °C. The isolates were subjected to 16S rDNA test of prokaryotes (16s rDNA region).

Table.1 In vitro evaluation of bacterial bioagents against *Fusarium oxysporum* f. sp. *cubense* using dual culture method

Sl. No.	Isolate No.	Radial growth of *Fusarium* in dual culture* (cm)	Per cent inhibition of mycelia growth over control (%)
1	MNB - 2(*Pseudomonas*)	6.75	25.00(30.00)
2	MNB – 3(*Pseudomonas*)	6.65	25.81(30.53)
3	MNB – 4(*Pseudomonas*)	7.12	20.89(27.19)
4	CCB – 2(*Pseudomonas*)	6.53	27.40(31.56)
5	CKB – 2(*Pseudomonas*)	8.50	5.56(13.63)
6	MPB – 2(*Pseudomonas*)	5.56	38.25(38.20)
7	MNB – 1(*Bacillus*)	7.50	16.67(24.09)
8	CCB - 1(*Bacillus*)	6.12	32.00(34.44)
9	MHB - 1(*Bacillus*)	6.95	22.78(28.50)
10	CKB - 1(*Bacillus*)	5.95	33.89(35.60)
11	MMB - 1(*Bacillus*)	6.83	24.11(29.40)
12	MPB - 1(*Bacillus*)	6.25	30.56(33.56)
13	Control	9.00	-
	S. Em ±	0.067	
	CD (1 %)	0.264	

Plate.1a PCR amplification of 16s regions of Bacillus spp. On 1.2 % agarose gel. Amplification exactly at 360bp confirms the organism as *Bacillus* spp.

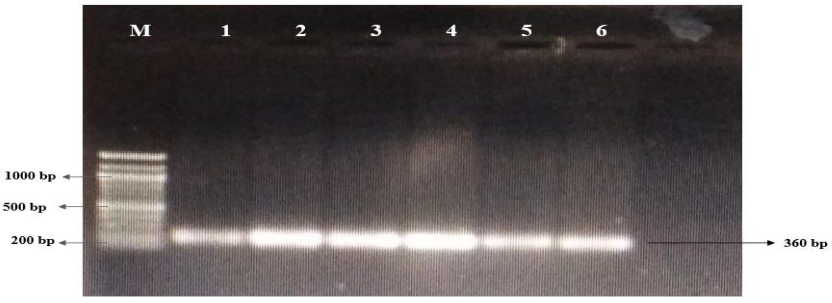


Plate.1b PCR amplification of 16s regions of Pseudomonas spp. On 1.2 % agarose gel. Amplification exactly at 450 bp confirms the organism as Pseudomonas.

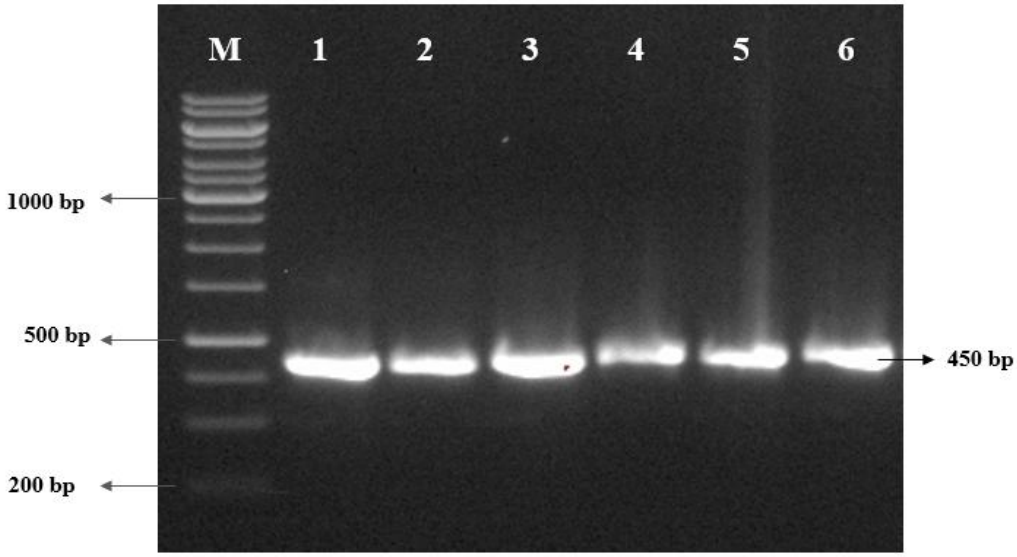


Plate.2a *In vitro* evaluation of antagonistic potential of Pseudomonasisolates against *Fusariumoxysporum* f. sp.*cubense*

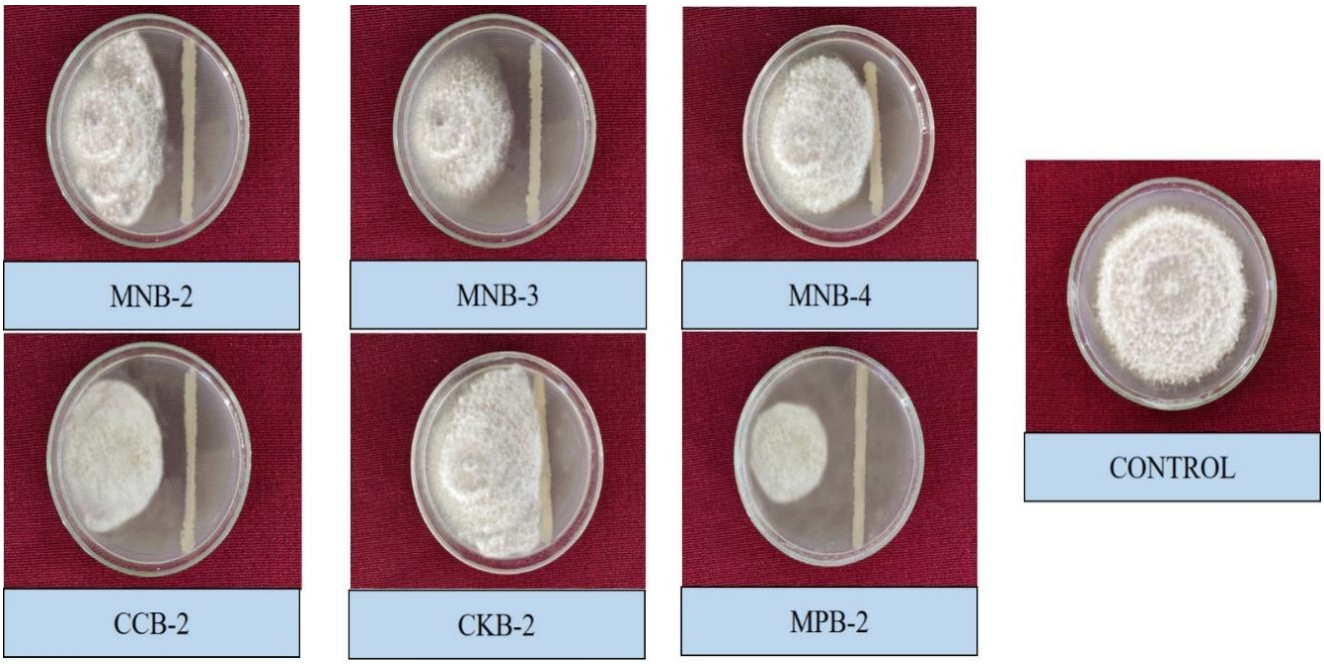
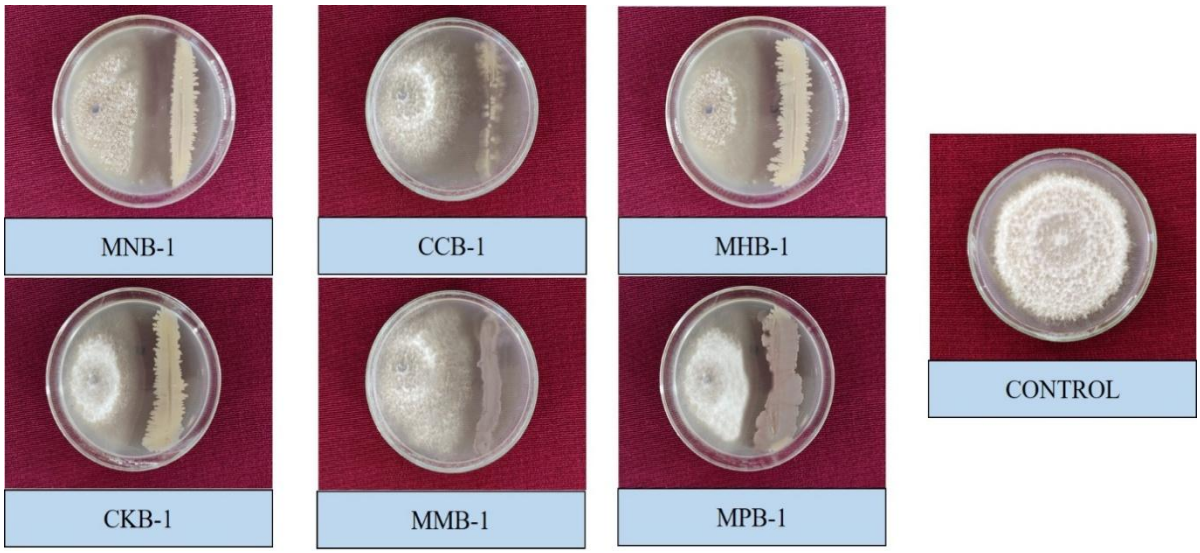


Plate.2b *In vitro* evaluation of antagonistic potential of *Bacillus* isolates against *Fusarium oxysporum* f. sp.*cubense*



PCR amplification was observed in all the twelve isolates during 16S rDNA test with the amplicon size of approximately 360 bp and 450 bp in *Bacillus* spp. (Plate 1a) and *Pseudomonas* spp.(Plate 1b), thus the isolates were confirmed as *Pseudomonas* spp. and *Bacillus* spp. The antagonistic effect of six biocontrol agents were evaluated against *Fusarium oxysporum* f. sp. *cubense* and the results are presented in Table 1**.** All the *in vitro* evaluation of *Pseudomonas spp.* against *Fusarium oxysporum* f. sp. *cubense* growth is represented in the Table 1 and Pate 2a. Analysis of antagonistic activity of isolated bioagents showed significant difference among the isolates collected. Maximum inhibition was observed in MPB-2 (38.89 %), followed by CCB-2 (27.78 %), MNB-3 (26.11 %), MNB -2 (25.00 %) and MNB – 4 (20.89 %). The lowest inhibition zone was observed in CKB – 2 (5.56 %). *Bacillus spp.* also recorded various zone of inhibition against *Fusarium oxysporum* f. sp. *cubense* under *in vitro* condition. Among the isolates CKB-1 showed maximum inhibition of 33.89 per cent followed by CCB-1 (32.00 %), MPB-1 (30.56 %), MMB-1(24.11 %), MHB-1 (22.78 %) and the least zone of inhibition was observed in MNB-1 (16.67 %). Zone of inhibition produced by the bioagents were documented (Plate 2b). The results were supported by Saravanan *et al.*, (2004) who conducted the study to know the efficacy of *Pseudomonas fluorescens* on *Fusarium* wilt pathogen in banana rhizosphere. All the strains of *Pseudomonas fluorescens* isolated from banana rhizosphere had significant inhibitory action on the growth of *Fusarium oxysporum* f. sp. *cubense*.

Among the strains Pfm of *Pseudomonas fluorescens* had higher inhibitory action than other strain. Similarly, Killani *et al.*, (2011), who reported that *Bacillus subtilis* successfully inhibited the growth of all the soil borne fungal pathogens isolated from cowpea *in vitro*. Shobha and Kumudini, 2012 showed Bacillus isolates-controlled *F. oxysporum* growth irrespective of the antagonistic method used. Bacterial bio agents are the promising alternative strategies for management of *Fusarium* wilt. As far the results demonstrated the positive ray of bio-control agents against *Fusarium* wilt. The potent of the current isolates showed some extent of inhibition indicting a need of screening more beneficial

bacteria for obtaining significant effect against the pathogen.

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