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Pathogens Associated with Micropropagated Banana Plantlets and their Management with Microbial Bioagents

Joli Dutta* and L.C. Bora

Department of Plant Pathology, Assam Agricultural University, Jorhat- 785013, Assam, India
**Corresponding author*

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A B S T R A C T

Four antagonistic microorganisms *Trichoderma viride, Metarhizium anisopliae, Pseudomonas fluorescens* and *Bacillus thuringiensis* and their consortia were used to suppress *Colletotrichum musae* (Berk and Curtis), the causal agent of anthracnose disease of micropropagated banana during 2014-16. The compatibility tests conducted *in vitro* among these bioagents showed that all the bioagents were compatible amongst themselves. The consortia of different antagonists were tested to assay their ability to inhibit the growth of *C. musae in vitro.* The inhibition produced by the consortia of four bioagents *T. viride, P. fluorescens, M. anisopliae* and *B. thuringiensis* was significantly highest against *C. musae* (80.56%). The efficacy of the microbe based consortial formulations was also tested for their ability to suppress diseases caused by *C. musae in vivo* in pot grown micropopagated banana plantlets. There was a significant reduction of anthracnose disease incidence accompanied by enhancement of yield attributing characters in banana due to the application of consortial formulation of bioagents applied as root treatment and soil treatment.

Introduction

Micropropagation is the rapid multiplication of stock plant material to produce a large number of progeny plants under aseptic conditions using modern plant tissue culture methods. An ideal tissue culture raised plant should be free from diseases.

The pathogenic microbes generally get associated with tissue cultured banana plantlets includes, *Fusarium oxysporum f. sp. cubense* (Fusarium wilt or Panama disease); *Pythium sp.* (Damping off); *Rhizoctonia solani* (Root rot); *Ralstonia solanacearum* (Moko disease); *Erwinia carotovora* (soft rot) and *Colletotrichum musae* (Anthracnose). Banana anthracnose caused by *C. musae*, is considered as one of the major constraints to banana production. It deteriorates the quality and nutritive value of the fruits which is unfit for consumption and marketing.

Sunken brown spots develop on ripe fruits with orange acervuli (Lim *et al.*, 2002).

The objective of the present study is to screen different antagonistic microorganisms, development of microbial consortia and test their ability to inhibit the growth of fungal

pathogen, *C. musae* and reduce diseases caused by *C. musae* in micropropagated banana and corresponding enhancement of plant growth and yield attributing characters.

Materials and Methods

Microbial isolation

The infected lesions of micro-propagated banana plantlets were collected for isolation of fungal pathogen, *C. musae.* The isolated culture was preserved in refrigerator at 4°C for subsequent use. Pathogenicity test was conducted in one month old potted micro-propagated banana (var. G 9) plantlets, following injection-infiltration method. Characterization of the fungal pathogen was done following the guidelines described by Alexopolous *et al.,* 1996. The pure culture of microbial bioagents *viz., Trichoderma viride, Metarhizium anisopliae, Pseudomonas fluorescens* and *Bacillus thuringiensis* used in the present study was collected from the culture bank of Programme on Biopesticides, Department of Plant Pathology, Assam Agricultural University, Jorhat.

Evaluation of compatibility among different bioagents and development of microbial consortia

Compatibility among the four microbial bioagents, *viz., T. viride, M. anisopliae, P. fluorescens* and *B. thuringiensis* were tested *in vitro* adopting dual culture essay plate technique (Aspiras and Cruz, 1985) using PDA as basal media. The treatment combinations were : Growth of *T. viride* alone, *M. anisopliae* alone, *B. thuringiensis* alone, *P. fluorescens* alone, *T. viride* + *M. anisopliae*, *T. viride* + *B. thuringiensis, T. viride* + *P. fluorescens, M. anisopliae* + *B. thuringiensis, M. anisopliae* + *P. fluorescens, B. thuringiensis* + *P. fluorescens* and *T. viride* + *M. anisopliae* + *B. thuringiensis* + *P. fluorescens.* The radial growth of each bioagents individually and in combination was recorded upto 120 h of incubation at 28±1 ^{0}C, and tabulated for comparison.

Inhibitory effects of bioagents against *C. musae*

The inhibitory effect of bioagents against *C. musae* was evaluated *in vitro* using PDA as basal medium. Assay plates of *C. musae* were prepared by transferring mycelial disc of the pure culture of the fungus on PDA plates and incubated at 28 ±1 ^{0}C for 48 h. Then, 0.5 cm diameter of fungal bioagent, *T. viride* grown in PDA was transferred to the center of PDA plates where *C. musae* was grown earlier. Following the same procedure, 0.5 cm bit of bioagents such as *M. anisopliae, B. thuringiensis* and *P. fluorescens* grown in PDA was scooped out and transferred to the center of PDA plates seeded earlier with *C. musae*. The plates were then incubated at 28±1°C. The inhibitions produced were measured after 72 h of dual inoculations. The data were converted to percentage of inhibitions produced by the bioagents as compared to control.

Based on the percent of inhibitions shown by the antagonists or their combinations *in vitro*, 3 best treatment combinations were selected for their further evaluation as individual or consortia bioformulation in suppression of anthracnose disease of pot grown micropropagated banana plantlets.

Suppressive effects of bioagents and their consortia against anthracnose disease of micropopagated banana plantlets

For preparation of microbe based bioformulations, the antagonists were first grown in their specific media (either PDA, NA or Trichoderma specific medium). *T. viride* was transferred to PDA slants and

incubated at 28±1°C for 48 h. By mixing sterile distilled water to this growth, suspension of *T. viride* @ 10^8 cfu/ml was prepared. A loop of the inoculum was transferred to 1 lit of PDA broth contained in a conical flask and after thorough stirring, after that, it was incubated at 28±1°C for 72 hrs to obtain a concentration of 10^8 cfu/ml. Following same protocol bioformulation of *M. anisopliae*, *B. thuringiensis* and *P. fluorescens* suspensions were prepared to obtain concentrations of 10^8 cfu /ml for each bioagent. For preparation of consortial formulation, individual growth of *T. viride, B. thuringiensis* and *P. fluorescens* were adjusted @ 10^8 cfu/ml and mixed at the ratio of 1: 1. The treatment combinations compared under hydroponic tank conditions are as follows: *T. viride* alone; *M. anisopliae alone, B. thuringiensis* alone; P. *fluorescens* alone*; T. viride* + *B. thuringiensis; T. viride*+ P. *fluorescens; P. fluorescens* + *B. thuringiensis; T. viride*+ P. *fluorescens* + *B. thuringiensis.*

Method of application of treatments

The three best consortial formulations applied as root treatment and soil application methods. For root treatment, properly cleaned roots of micropropagated plantlets were soaked in suspension of antagonists' broth for 1 hour prior to transplanting. Plantlets soaked in sterile water for 1 hour served as untreated control.

Soil treatment was done 30 days after transplanting. Soil near the base of the plants was loosened carefully and diluted suspension of antagonists broth (100 ml broth + 900 ml distilled water) were applied @ 1 lit /plant. Plants treated with sterile water served as untreated control.

Results and Discussion

The fungal pathogen isolated from disease infected micropropagated banana plantlets was identified to be *Colletotrichum musae.* The pure culture of the fungus produced white coloured aerial mycelia, which covered the entire periphery of the PDA plate within 3-4 days of incubation at 28 ± 1^0 C. After 6-8 days of incubation, several black, acervulus-like masses were developed on the culture plates with orange exudates.

Conidia were aseptate, hyaline, mostly ellipsoid, ranging from 10-18 µm and 5-9 µm (average of 14.5-6.9 µm) in size. Similar type of results of cultural and morphological characters was earlier recorded by Lim *et al.,* (2002) during identification of *C. musae.* In the inoculated banana plantlets, some irregular, sunken leaf spots were observed within 7-8 days.

Similar types of results were recorded by (Xiao *et al.,* 2004) up to 21 days after inoculation. Earlier, Meredith (1960) recorded that *C. musae* may form lesions on fruits without skin bruising but produces larger lesions when fruits are damaged. *C. musae* is also responsible for crown rot, blossom end rot, and tip rot of banana (Nazriya *et al.,* 2007).

Compatibility among different bioagents *in vitro*

The compatibility tests among four different bioagents *T. viride*, *M. anisopliae*, *P. fluorescens* and *B. thuringiensis* were made following modified dual culture technique using PDA as basal medium, and was found that the bioagents were compatible amongst themselves. Earlier, Deuri (2013), reported positive compatibility amongst saprophytic antagonists like *P. fluorescens*, *T. viride*, *M. anisopliae.* Similar compatible observations amongst bioagents like *P. fluorescens, T.viride, T. harzianum, M. anisopliae* and *Beauvaria bassiana* was earlier recorded by Bora (2012) and Bora *et al.,* (2013).

Antagonism of bioagents against *C. musae in vitro*

The antagonistic potential of the four compatible bioagents, *viz*., *T. viride, M. anisopliae, P. fluorescens* and *B. thuringiensis* and their consortia were tested against *C. musae* adopting dual culture method using PDA as basal medium. All the four bioagents produced varying radial growth and showed corresponding suppression against *C. musae in vitro.* The combination of four bioagents *T. viride, P. fluorescens, M. anisopliae* and *B. thuringiensis* produced highest inhibition (80.56%) followed by combination of *T. viride, B. thuringiensis* and *P. fluorescens* (68.22%) against *C. musae* (Table 1).

The *Trichoderma* species are extremely versatile biocontrol agents which suppresses the diseases caused by different plant pathogens like Anthracnose and Grey mould in strawberry (Freeman *et al.*, 2004). *Trichoderma* has the ability to produce a series of antibiotics and fungal cell wall-degrading enzymes. These enzymes play important role in mycoparasitism and mycelial lysis of the target pathogenic microbe. The hydrolytic enzyme has been identified includes proteinase (Prb1). The present observation of antagonism of *T. viride* might be as a result of similar type of mechanisms of parasitism (Dagostin *et al.*, 2008).

M. anisopliae has the ability to release three different secondary metabolites *viz*., destruxin A, destruxin E and cytochalasin D. Kang *et al.*,(1996) reported that *M. anisopliae* have antagonistic effects on various plant pathogens, including *Fusarium oxysporium* and *Alternaria solani*.

Table.1 Suppression of radial growth of pathogens by different Microbial bioagents and their consortia *in vitro*

Treatments	*C. musae*	
	Radial growth	**Inhibition**
Control	90.0	0.00 (0.57)
Trichoderma viride	33.3	63.04 (52.61)
Pseudomonas fluorescens	80.3	10.77 (19.08)
Metarhizium anisopliae	37.7	58.04 (49.58)
Bacillus thuringiensis	65.3	27.37 (31.52)
T. viride + P. fluorescens	31.3	65.19 (53.81)
T. viride + M. anisopliae	25.9	71.15 (57.50)
T. viride + B. thuringiensis	30.1	66.48 (54.59)
M. anisopliae + P. fluorescens	36.1	59.82 (50.65)
B. thuringiensis + P. fluorescens	54.1	39.85 (39.12)
M. anisopliae + B. thuringiensis	34.7	61.30 (51.51)
T. viride + P. fluorescens + M. anisopliae	24.5	72.71 (58.48)
T. viride + B. thuringiensis + P. fluorescens	28.6	68.22 (55.65)
T. viride + B. thuringiensis + M. anisopliae	23.2	74.19 (59.43)
P. fluorescens + M. anisopliae + B. thuringiensis	32.9	62.70 (52.33)
T. viride + P. fluorescens + M. anisopliae + B. thuringiensis	17.5	80.56(63.82)
	S.Ed (±) =0.77 $CD_{0.05}$ = 1.57	

* Data in the parenthesis are angular transformed values

Table.2 Effects of different consortial formulation on disease incidence (%) of potted Micropropagated banana plants against *Colletotrichum musae*

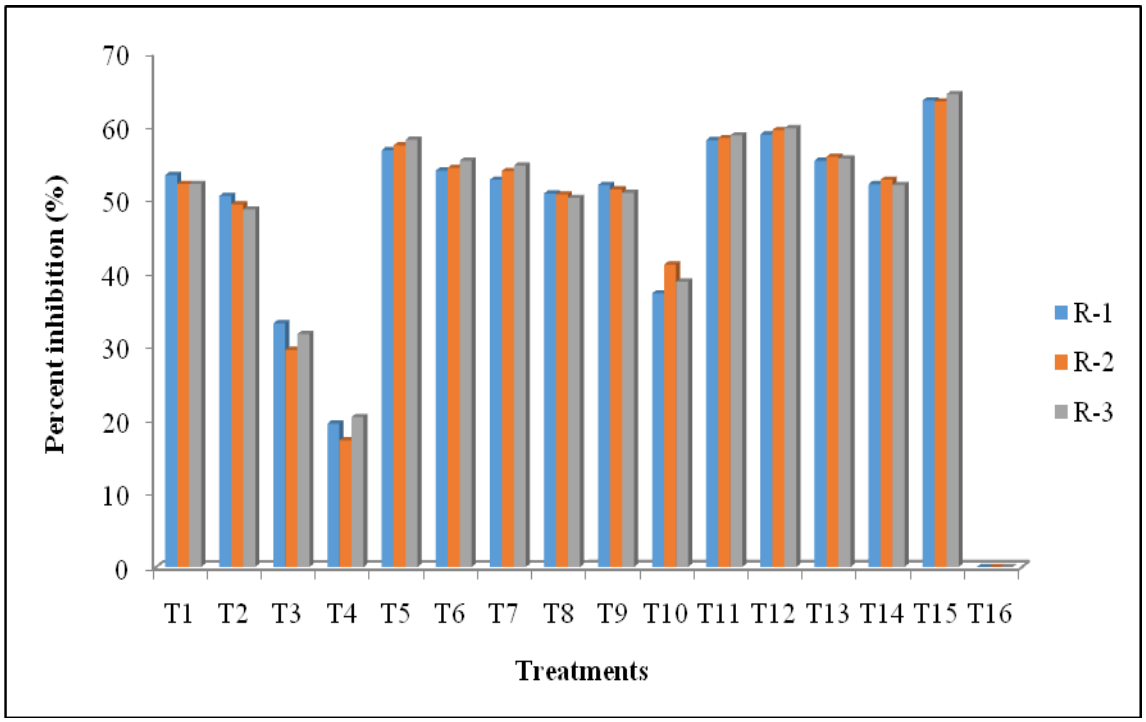
Treatments	*C. musae*	
	Disease incidence	Disease reduction
Root treatment with of *T. viride* + *P. fluorescens* + *M. anisopliae*+ *B. thuringiensis* (EM 1)	28.1 (32.01)	11.74
Root treatment with *T. viride* + *M. anisopliae* + *P. fluorescens* (EM 2)	29.3 (32.75)	9.70
Root treatment with *T. viride* + *M. anisopliae* + *B. thuringiensis* (EM 3)	32.1 (34.50)	4.88
Root treatment of banana plantlets with EM 1 + EM2	23.4 (28.90)	20.31
Root treatment of banana plantlets with EM 1 +EM3	31.2 (31.35)	13.56
Root treatment of banana plantlets with EM 2+EM3	27.6 (31.71)	12.57
Root treatment of banana plantlets with EM1 + EM2+ EM3	18.3 (25.30)	30.24
Soil treatment of banana plantlets with EM 1	22.2 (28.15)	22.38
Soil treatment of banana plantlets with EM 2	23.4 (28.95)	20.18
Soil treatment of banana plantlets with EM 3	28.6 (32.31)	10.91
Soil treatment of banana plantlets with EM 1 + EM 2	15.00 (22.81)	37.10
Soil treatment of banana plantlets with EM 1 + EM 3	17.3 (24.58)	32.23
Soil treatment of banana plantlets with EM 2 + EM 3	20.4 (26.87)	25.91
Soil treatment of banana plantlets with EM 1 + EM2 + EM 3	13.5 (21.55)	40.58
Control	35.00 (36.27)	-
	S.Ed = 0.16 CD0.05 = 0.32	

* Data in the parenthesis are angular transformed values

Table.3 Yield attributing characters of micropropagated banana plantlets due to application of Microbe based bioformulation and their consortia for management of *C. musae*

Treatment	Yield attributing characters				
	No. of leaf per plant	Shoot length (cm)	Shoot girth (cm)	Root length (cm)	No. of roots per plant
Root treatment with of *T. viride* + *P. fluorescens* + *M. anisopliae*+ *B. thuringiensis* (EM1)	15.06	17.56	13.41	18.93	18.00
Root treatment with, T. viride + M. anisopliae + *B. thuringiensis* (EM2)	16.49	17.68	14.41	19.33	17.34
Root treatment with *T. viride* + *M. anisopliae* + *P. fluorescens* (EM3)	15.54	17.55	13.70	18.74	15.89
Root treatment of banana plantlets (RTBP) with EM 1 + EM2	15.00	18.38	12.15	16.34	18.00
RTBP with EM 1 +EM3	16.32	18.50	14.41	19.64	18.34
RTBP with EM 2+EM3	15.50	18.93	13.66	18.67	15.89
RTBP with EM1 + EM2+ EM3	18.42	21.13	15.70	20.00	19.67
Soil treatment of banana plantlets (STBP) with EM 1	15.36	17.87	15.68	19.35	18.34
STBP with EM 2	17.56	19.04	14.21	19.65	18.67
STBP with EM 3	16.33	19.58	15.70	19.95	20.24
STBP with EM 1+ EM 2	14.56	19.04	14.13	18.60	19.00
STBP with EM 1 + EM 3	17.75	21.12	15.43	19.00	18.90
STBP with EM 2 + EM 3	17.78	20.84	15.40	18.00	18.34
STBP with EM 1 + EM2 + EM 3	19.05	21.41	16.77	20.34	20.34
Control	13.99	17.55	12.06	13.34	14.34
	S.Ed (±) =0.83 $CD_{0.05}$ = 1.69	S.Ed (±) =0.68 $CD_{0.05}$ = 1.39	S.Ed (±) =0.56 $CD_{0.05}$ = 1.15	S.Ed (±) =0.92 $CD_{0.05}$ = 1.89	S.Ed (±) =0.81 $CD_{0.05}$ = 1.67

Fig.1 Radial growth of different bioagents and their consortia suppressing growth of *Colletotrichum musae in vitro*



T_1 = *Trichoderma viride*

T_2 = *Pseudomonas florescence*

T_3 = *Metarhizium anisopliae*

T_4 = *Bacillus thuringiensis*

T_5 = *T. viride* + *P. florescence*

T_6 = *T. viride* + *M. anisopliae*

T_7 = *T. viride* + *B. thuringiensis*

T_8 = *P. florescence* + *M. anisopliae*

T_9 = *P. florescence* + *B. thuringiensis*

T_{10} = *M. anisopliae* + *B. thuringiensis*

T_{11} = *T. viride* + *P. florescence* + *M. anisopliae*

T_{12} = *T. viride* + *P. florescence* + *B. thuringiensis*

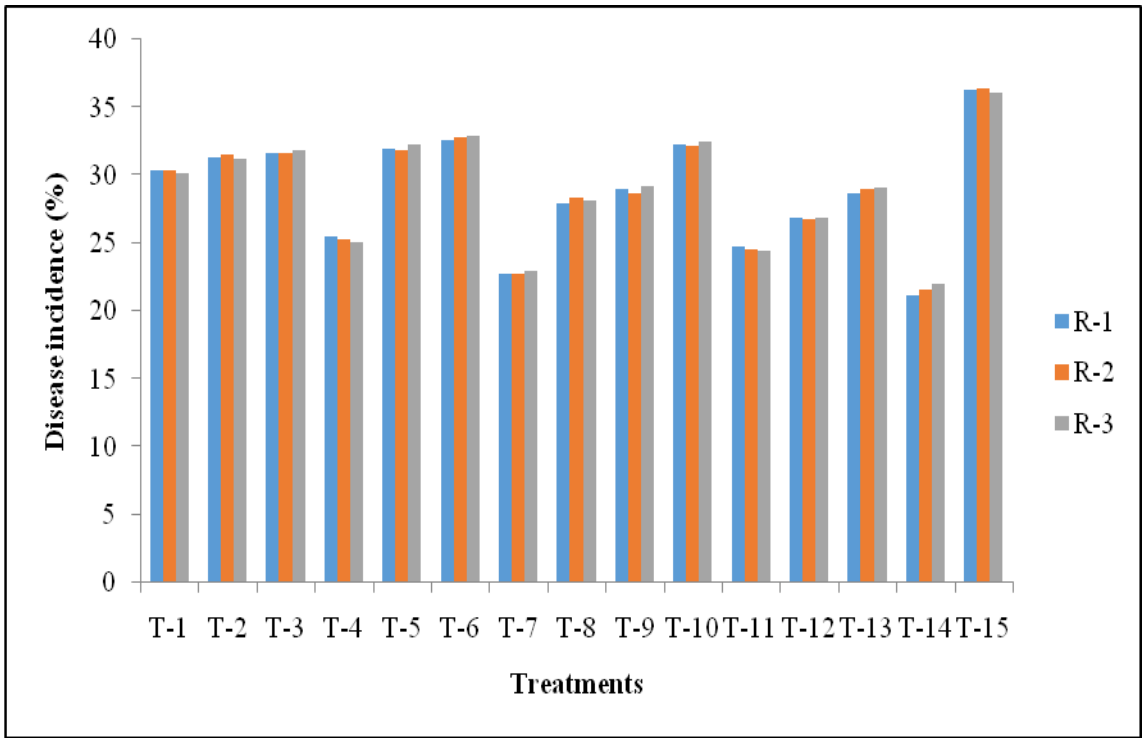
T_{13} = *T. viride* + *B. thuringiensis* + *M. anisopliae*

T_{14} = *P. florescence* + *M. anisopliae*+ *B. thuringiensis*

T_{15} = *T. viride*+ *P. florescence* + *M. anisopliae*+ *B. thuringiensis*

T_{16} = Control

Fig.2 Effects of different microbial consortia on disease incidence (%) caused by *C. musae in* micropropagated banana plantlets



T_1 = Root treatment of banana plantlets with Efficient Microbe 1 (*T. viride, M. anisopliae, P. fluorescens* and *B. thuringiensis*)

T_2 = Root treatment of banana plantlets with EM 2 (*T. viride, B. thuringiensis and M. anisopliae*)

T_3 = Root treatment of banana plantlets with EM 3 (*T. viride, M. anisopliae* and *P. fluorescens*)

T_4 = Root treatment of banana plantlets with EM 1 + EM2

T_5 = Root treatment of banana plantlets with EM 1 +EM3

T_6 = Root treatment of banana plantlets with EM 2+EM3

T_7 = Root treatment of banana plantlets with EM 1 +EM2+EM3

T_8 = Soil treatment of banana plantlets with Efficient Microbe 1

T_9 = Soil treatment of banana plantlets with EM 2

T_{10} = Soil treatment of banana plantlets with EM 3

T_{11} = Soil treatment of banana plantlets with EM 1 + EM2

T_{12} = Soil treatment of banana plantlets with EM 1 +EM3

T_{13} = Soil treatment of banana plantlets with EM 2+EM3

T_{14} = Soil treatment of banana plantlets with EM 1 +EM2+EM3

T_{15} = Control

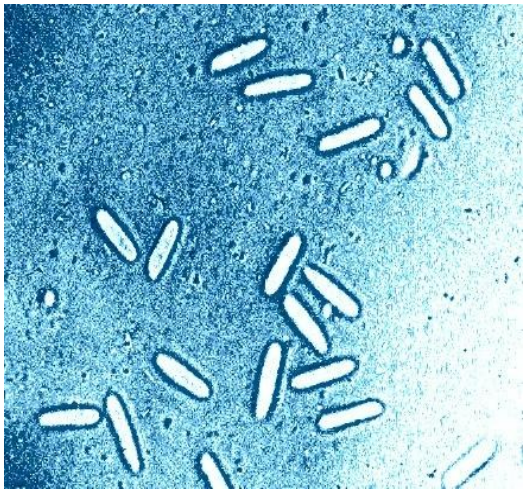
Plate.1 Typical symptom of Anthracnose of micropropagated banana caused by *Colletotrichum musae*

Plate.2 Cultural characterization of the pathogen (*Colletotrichum musae*)

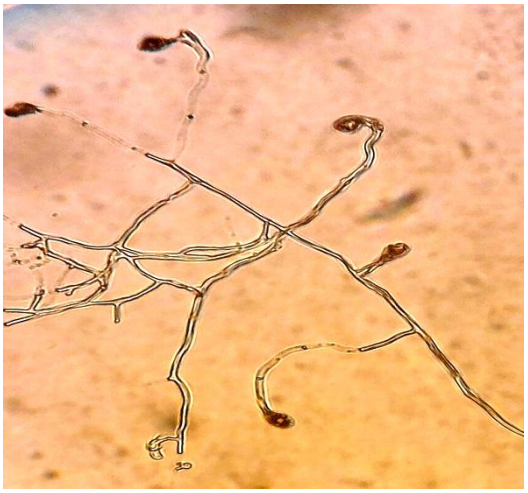
A. Pure culture of *C. musae*

B. Back view of culture plate

Plate.3 Morphological characterization of the pathogen (*Colletotrichum musae*)



A. Conidia of *C. musae*



B. Appressoria formation of *C. musae*

Plate.4 Pathogenicity test conducted with *C. musae* in micropropagated banana

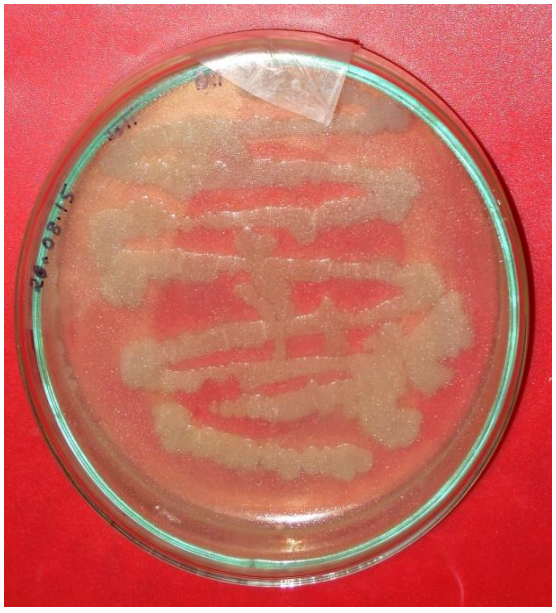
A. Healthy Micropropagated banana plantlets

B. Infected Micropropagated Banana plantlets

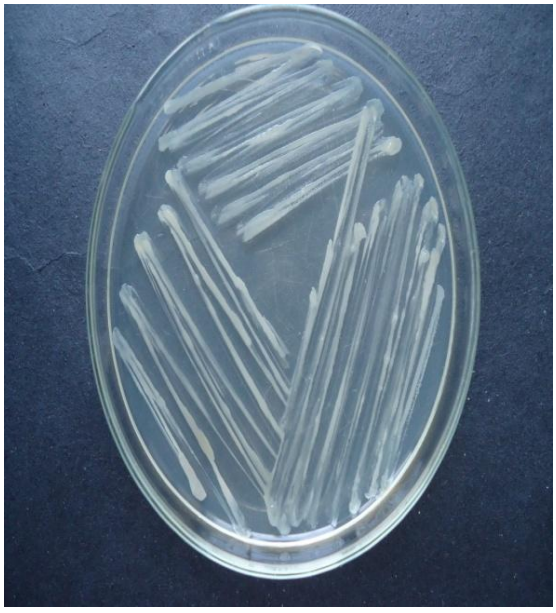
Plate.5 Growth of different microbial antagonists

Metarhizium anisopliae

Trichoderma viride



Bacillus thuringiensis



Pseudomonas fluorescens

Plate.6 Antagonistic effect of different bioagents against *C. musae*

A) *C. musae* + *M. anisopliae*

B) *C. musae* + *T. viride*

C) *C. musae* + *M. anisopliae* + *B. thuringiensis*

D) *C. musae* + *P. fluorescens* + *M. anisopliae*

Plate.7 General view of experimental area

Plate.8 Application of consortial formulation for controlling Anthracnose disease

The fluorescent pseudomonads antagonize plant pathogens by producing a range of metabolites like antibiotics (Fravel, 1988), siderophores (Loper and Buyer, 1991) and other substances such as cyanide (Voisard *et al.*, 1989). The main mechanism of the antagonism of *P. fluorescens* seems to be competition with pathogenic microorganisms for iron by release of siderophores which are secondary metabolites with an affinity to Fe^{3+} (Kloepper *et al.*, 1993). *Pseudomonas aeruginosa* isolates showed potential antagonistic activity against *C. musae* causing anthracnose fruit rot of banana (Ranathunge *et al.*, 2014). *P. aeruginosa* is capable of producing volatile substances and diffusible substances with antifungal properties that significantly inhibited the mycelia growth of *C. musae*.

Arokia Raj (2000) found that the bacterial antagonist *B. subtilis* could significantly reduce mycelial growth of *C. musae*. Similar observations were also made with *Bacillus spp.* antagonistic to *Colletotrichum spp*. (Sariah, 1994; Rahman *et al.*, 2007). The Bacillus genus includes some species which are known to be endophytically active, and could play a key role in the biocontrol of pathogens like *C. musae*. *B. subtilis* and *B. amyloliquefaciens* were reported effective for the control of plant pathogens, due to production of iturin a cyclic lipo-polypeptide. The effective use of *Bacillus* as a biocontrol agent against *C. musae* on curcuma was reported by Mahadtanapuk *et al.*, (2007).

Bioagents and their consortia based formulations for management of anthracnose of micropropagated banana

Three best microbes based consortial formulations were applied as combinations of root and soil treatment for management of anthracnose of micropropagated banana. Consortial formulations of three best bioagents were prepared namely EM 1 (*T. viride*, *M. anisopliae*, *P. fluorescens* and *B. thuringiensis*), EM 2 (*T. viride*, *B. thuringiensis* and *M. anisopliae*) and EM 3 (*T. viride*, *M. anisopliae* and *P. fluorescens*) and applied as root treatment and soil treatment in pot grown micropropagated banana plantlets for management of anthracnose disease. Soil treatment with EM 1 + EM 2 + EM 3 showed the best result in controlling anthracnose with least disease incidence (21.55%). Highest disease reduction over control (%) was recorded in Soil treatment with EM 1 + EM 2 + EM 3 (40.58%) (Tables 2 and 3; Figs. 1 and 2)).

The enhancement of yield attributing characters followed the trend of disease suppression as a result of the number of leaves per plant, shoot length, shoot girth, root length and number of roots per plant of micropropagated banana plantlets increased in Soil treatment with EM 1 + EM 2 + EM 3.

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