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Bio prospective management approach for the pathogens associated with micropropagated banana plantlets

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Abstract
During 2014-16, four antagonistic micro-organisms such as *Trichoderma viride, Metarhizium anisopliae, Pseudomonas fluorescens* and *Bacillus thuringiensis* and their consortial formulations were used to suppress *Ralstonia solanacearum,* the causal agent of bacterial wilt disease of micropropagated banana. *In vitro* tests were conducted to evaluate compatibility and the antagonistic ability of all the bioagents and their combinations against wilt pathogen, *R. solanacearum*. Combination of *B. thuringiensis, T. viride, M. anisopliae* and *P. fluorescens* showed significantly highest inhibition (97.06%). The efficacy of the microbe based consortial formulations was also tested for their ability to suppress diseases caused by *R. solanacearum in vivo* in pot grown micropropagated banana plantlets. There was a significant reduction of bacterial wilt disease incidence accompanied by enhancement of yield attributing characters in banana due to application consortial formulation of bioagents applied as root treatment and soil treatment.

Keywords: micropropagated, banana, *Ralstonia solanacearum,* antagonistic microorganisms, consortial formulation

Introduction
Banana is a crop with dual propagation abilities, sexual through seeds and asexual through suckers. Sucker propagation is the only natural means while artificial methods of propagation include macro propagation and micropropagation. Application of micropropagation in banana has the advantages of rapid multiplication, product uniformity in genotypic and phenotypic characters, higher returns due to superior yield. Bacterial wilt disease (Moko) of banana is regarded as one of the most severe diseases affecting banana plants in India, Indonesia and the Philippines (Sequeira, 1998) [24].

Generally, micropropagated plantlets are free from any pests and diseases. But after hardening, when it is transferred from controlled environment to a new environment, then some pathogens are associated with these micropropagated plantlets. To overcome this problem, bio prospective microbial antagonists are incorporated with these plantlets. To have a sustainable agricultural system with minimum contamination and risks to the environment, a consortial formulation of microbial antagonists should be applied to manage disease problems. It can lead us towards a sustainable agricultural system in the future.

The objective of the present study is to screen different antagonistic microorganisms, development of microbial consortia and test for these for their ability to inhibit the growth of bacterial pathogen *R. solanacearum* and reduce diseases caused by them in micropropagated banana and corresponding enhancement of plant growth and yield attributing characters.

Materials and Methods
Microbial isolation
Diseased Micropropagated banana plants showing characteristic symptoms of bacterial wilt were collected for isolation of *R. Solanacearum.* Isolated bacterial culture was preserved in refrigerator at 4°C for subsequent use. The pathogenicity of *R. solanacearum* was conducted by inoculating healthy micropropagated banana plantlets (variety G 9). Characterization of the bacterial pathogen was done following the guidelines described in the Bergey's Manual of Systematic Bacteriology (2012). 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 up to 120 hrs of incubation at 28±1^{0}C, and tabulated for comparison.

Inhibitory effects of bioagents against *R. solanacearum*

The inhibitory effect of the bioagents against *R. solanacearum* was evaluated *in vitro* using Triphenyl Tetrazolium Chloride (TTC) as a basal medium. Assay plates of *R. solanacearum* were prepared by transferring pure culture of the bacterium grown on TTC plates incubated at 28 ±1 ^{0}C for 48 h. A 0.5 cm diameter lawn of fungal bioagent, *T. viride* grown in PDA was transferred to the center of the plates grown earlier with cells of *R. solanacearum*. Following the same procedure, 0.5 cm bit of bioagents, *M. anisopliae, P. fluorescens* and *B. thuringiensis* grown in PDA were scooped out and transferred to the center of TTC plates seeded earlier with *R. solanacearum*. The plates were then incubated at 28±1°C. The inhibitions produced were measured after 72 hr of dual inoculations. The data were converted to percentage of inhibition 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 consortial bio formulation in suppression of bacterial wilt disease of pot grown micropropagated banana plantlets.

Suppressive effects of bioagents and their consortia against bacterial wilt (*R. solanacearum*) disease of micropropagated banana plantlets

For preparation of microbe based bio formulation, the antagonists were first grown in their specific media (either PDA or NA). *T. viride* was transferred to PDA slants and incubated at 28±1°C for 48 hour. 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 through stirring it was incubated at 28±1°C for 72 hrs to obtain a concentration of 10^8 cfu/ml. Following the same protocol, bio formulation 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 as follows: *T. viride* alone; *M. anisopliae* alone, *B. thuringiensis* alone; P. *fluorescens* alone, *T. viride + M. anisopliae, T. viride + B. thuringiensis, P. florescence + M. anisopliae, P. florescence + B. thuringiensis, M. anisopliae + B. thuringiensis, T. viride + P. florescence + M. anisopliae, T. viride + P. florescence + B. thuringiensis, T. viride + B. thuringiensis + M. anisopliae, P. florescence + M. anisopliae+ B. thuringiensis, T. viride+ P. florescence + M. anisopliae + 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 distilled 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 @ 1lit /plant. Plants treated with distilled sterile water served as untreated control.

Results and Discussion

Bacterial pathogen associated with micropropagated banana

The bacterial pathogen, isolated from disease infected micropropagated banana plantlets was identified to be *Ralstonia solanacearum*. The morphological, cultural and biochemical studies of the bacterial pathogen revealed that the bacterium is gram negative, positive to oxidase test and could produce cytochrome oxidase enzyme of the bacterial electron transport chain. This enzyme is characteristically produced by a number of microorganisms, including *Neisseria* and *Pseudomonas*, which is a useful differential feature in the classification and presumptive identification of the bacterial genera like *Ralstonia* (Cardinal *et al.*, 1997) [6]. The bacterium could also hydrolyse starch in starch agar medium, positive in Citrate test, ammonia production and negative in Levan production tests. Similar type of results in cultural and biochemical tests were earlier recorded by Hayward (1964) [17] during identification of *R. solanacearum* (Plate I).

Pathogenicity test of the bacterial pathogen, *R. solanacearum* revealed that the bacterium is highly pathogenic, cause complete wilting of the inoculated plants within 5 days (Plate II). In Assam, various isolates of *R. solanacearum* belonging to race-1 and race-3 have been causing serious wilt disease in different crops (Addy *et al.*, 1980 and Nath *et al.*, 1996). [1, 21]

The bacterial wilt pathogen can survive in conducive soil for a very long period and have ability to cause severe epidemic whenever environmental conditions are favorable for growth and multiplication of the pathogen. Its occurrence in an epidemic form has been recorded in other solanaceous crops from many parts of Assam (Bora *et al*., 1996) [3].

Compatibility among different bioagents *in vitro*

The compatibility tests among four different bioagents *Trichoderma viride, Metarhizium anisopliae, Pseudomonas fluorescens* and *Bacillus 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) [5, 11], 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) [4] and Bora *et al.* (2013) [5].

Antagonism of bioagents against *R. solanacearum 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 *R. solanacearum* adopting dual culture method using TTC as basal medium. All the four bioagents produced varying radial growth and showed corresponding suppression against *R. solanacearum in vitro.* The combination of four bioagents *T. viride, P. fluorescens, M. anisopliae* and *B. thuringiensis* produced highest inhibition (97.06%) followed by combination of *T. viride* + *P. fluorescens* + *M. anisopliae* (93.40%) against *R. solanacearum* (Table I, Plate III).

Earlier, similar types of observations have been documented by application of bioagents *Trichoderma* spp., *Metarhizium* spp. under greenhouse and field conditions (Deuri, 2013; Bora *et al.,* 2013) [5, 11]. Das and Bora, (2000) [21] obtained biological control of bacterial wilt in tomato with *T. harzianum*, *T. viride*, *T. koningii* and *Gliocladium virens*.

Metarhizium species are commonly thought of as soil saprophytes and are most frequently found in disturbed habitats like agricultural fields as compared to forest ecosystems (Meyling and Eilenberg, 2007). *M. anisopliae* has antagonistic effects on various plant pathogens, including *Fusarium oxysporum* and *Altarnaria solani* (Kang *et al.*, 1996) as well as against *R. solanacearum* (Bora *et al.,* 2013 and Deuri, 2013) [5, 11].

Similarly, results of Hyakumachi (2013) [18] suggest that treatment of tomato roots with the cell-free filtrate of *B. thuringiensis* systemically suppressed bacterial wilt through systemic activation of the plant defence system. Treatment of tomato roots with *B. thuringiensis* culture followed by challenge inoculation with *R. solanacearum* suppressed the development of wilt symptoms to less than one third of the control. This finding suggests that *B. thuringiensis* can be a potential biocontrol agent for the suppression of plant diseases. This disease suppression in tomato plants was due to expression of defence-related genes such as PR-1, acidic chitinase, and β-1, 3-glucanase. Furthermore, the stem tissues of tomato plants with their roots were pre-treated with CF exhibited resistance against direct inoculation with *R. solanacearum*. Misaghi *et al.* (1991) established a negative correlation between the growth of *P. fluorescens* in the rhizosphere and disease incidence in tomato. The inverse relationship between per cent wilt incidence and population sizes of antagonists *viz., P. fluorescens* and *T. viride* spp. was also reported by Kalita (1994).

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

Treatments	***R. solanacearum***	
	Radial growth	**Inhibition**
Control	90.00	0.00 (0.57)
Trichoderma viride	55.6	61.90 (51.89)
Pseudomonas fluorescens	25.5	28.46 (32.22)
Metarhizium anisopliae	47.3	52.70 (46.54)
Bacillus thuringiensis	24.3	27.0 (31.28)
T. viride + *P. fluorescens*	69.0	76.73 (61.11)
T. viride + *M. anisopliae*	75.0	83.37 (65.98)
T. viride + *B. thuringiensis*	62.2	69.10 (56.29)
M. anisopliae + *P. fluorescens*	51.3	57.06 (49.06)
B. thuringiensis + *P. fluorescens*	48.8	54.30 (47.42)
M. anisopliae + *B. thuringiensis*	49.8	55.35 (48.06)
T. viride + *P. fluorescens* + *M. anisopliae*	84.0	93.40 (75.22)
T. viride + *B. thuringiensis* + *P. fluorescens*	75.1	83.50 (66.13)
T. viride + *B. thuringiensis* + *M. anisopliae*	78.0	86.67 (68.75)
P. fluorescens + *M. anisopliae* + *B. thuringiensis*	54.0	60.03 (50.79)
T. viride + *P. fluorescens* + *M. anisopliae* + *B. thuringiensis*	87.6	97.06 (80.17)
S.Ed (±) $CD_{0.05}$	1.43 2.92	

* Data in the parenthesis are angular transformed values

Table 2: Effects of different consortial formulation on disease incidence (%) of potted micropropagated banana plants against *Ralstonia solanacearum*

Treatments	***R. solanacearum***	
	Disease incidence	**Disease reduction**
Root treatment with of *T. viride* + *P. fluorescens* + *M. anisopliae*+ *B. thuringiensis* (EM 1)	36.5 (37.17)	21.96
Root treatment with *T. viride* + *M. anisopliae* + *P. fluorescens* (EM 2)	38.7 (38.47)	19.23
Root treatment with *T. viride* + *M. anisopliae* + *B. thuringiensis* (EM 3)	44.6 (41.91)	12.00
Root treatment of banana plantlets with EM 1 + EM2	33.4 (35.28)	25.92
Root treatment of banana plantlets with EM 1 +EM3	42.5 (40.68)	14.59
Root treatment of banana plantlets with EM 2+EM3	36 36.85)	22.63
Root treatment of banana plantlets with EM1 + EM2+ EM3	29.33 (24.03)	49.54
Soil treatment of banana plantlets with EM 1	33.58 (30.63)	35.69
Soil treatment of banana plantlets with EM 2	34.39 (31.94)	32.94
Soil treatment of banana plantlets with EM 3	37.9 (38.01)	20.19
Soil treatment of banana plantlets with EM 1 + EM 2	22.7 (28.45)	40.26
Soil treatment of banana plantlets with EM 1 + EM 3	20.9 (27.22)	42.85
Soil treatment of banana plantlets with EM 2 + EM 3	22.4 (28.22)	40.75
Soil treatment of banana plantlets with EM 1 + EM2 + EM 3	10.7 (19.08)	59.94
Control	54.6 (47.63)	-
S.Ed (±) $CD_{0.05}$	**0.440.89**	

* Data in the parenthesis are angular transformed value

Table 3: Yield attributing characters of micropropagated banana plants due to application of microbe based bio formulation and their consortia for management of *R. solanacearum*

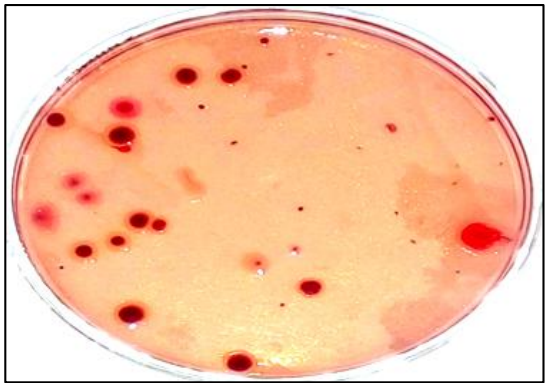
Treatment	Yield attributing characters				
	No. of leaf per plant	Shoot length (cm)	Shoot girth (cm)	Root length (cm)	No. of roots per plant
Root treatment with of *T. viride* + *P. fluorescens* + *M. anisopliae*+ *B. thuringiensis* (EM 1)	16.41	19.97	13.24	13.67	15.67
Root treatment with T. viride + M. anisopliae + P. fluorescens (EM 2)	15.68	19.33	13.47	13.67	17.34
Root treatment with *T. viride* + *M. anisopliae* + *B. thuringiensis* (EM 3)	15.70	19.04	12.50	14.67	18.00
Root treatment of banana plantlets (RTBP) with EM 1 + EM2	15.06	20.25	13.21	13.34	18.00
RTBP with EM 1 +EM3	18.42	19.64	13.38	17.66	18.34
RTBP with EM 2+EM3	16.77	19.97	14.14	18.00	18.67
RTBP with EM1 + EM2+ EM3	16.41	21.13	14.41	18.67	19.67
Soil treatment of banana plantlets (STBP) with EM 1	16.77	19.35	13.64	17.66	18.34
STBP with EM 2	16.75	20.25	14.30	14.67	17.00
STBP with EM 3	15.70	19.95	14.14	15.00	20.00
STBP with EM 1+ EM 2	17.78	20.84	14.13	18.00	19.00
STBP with EM 1 + EM 3	16.41	21.12	13.41	16.34	18.67
STBP with EM 2 + EM 3	17.75	20.25	14.21	17.00	18.34
STBP with EM 1 + EM2 + EM 3	19.05	21.41	14.41	19.00	20.34
Control	14.56	18.74	12.15	12.67	14.34
S.Ed (±)	0.54	0.34	0.38	0.39	0.47
$CD_{0.05}$	1.10	0.71	0.70	0.81	0.96

* Data in the parenthesis are angular transformed value

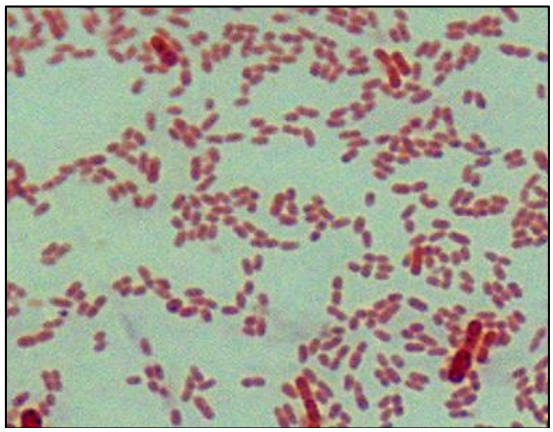
Bioagents and their consortia based formulations for management of bacterial wilt of micropropagated banana

Consortial formulations of three best bioagents were prepared namely EM 1(*T. viride*, *M. anisopliae*, *P. fluorescens* and *B. thuringiensis*), EM 2 (*T. viride*, *M. anisopliae* and *P. fluorescens*) and EM 3 (*T. viride*, *B. thuringiensis* and *M. anisopliae*) and applied as root treatment and soil treatment in pot grown micropropagated banana plantlets for management of bacterial wilt disease. Soil treatment with EM 1 + EM 2 + EM 3 showed the best result in controlling bacterial wilt with least disease incidence (10.7%). Highest disease reduction over control (%) was recorded in Soil treatment with EM1 + EM2 + EM 3 (59.94) (Table II, Plate V). The enhancement of yield attributing characters followed the trend of disease suppression as a result of the he 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 (Table III).

The bacterial wilt disease incidence of micropropagated banana decreased significantly accompanied by significant increase of yield attributing characters in plants treated with consortia of different bioagents. Lowest disease incidence was exhibited by the consotial formulation of *T. viride*, *M. anisopliae*, *P. fluorescens* and *B. thuringiensis*.



A. Pure culture



B. Gram negative cells of *R. solanacearum*

Acknowledgements

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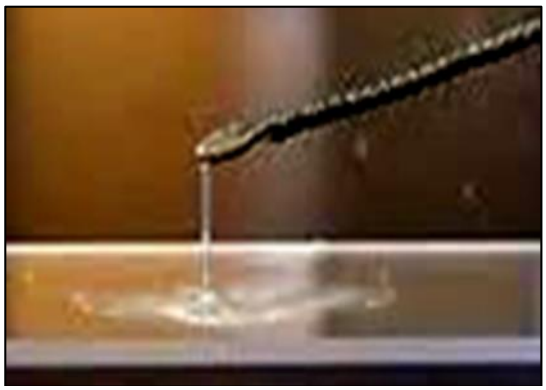
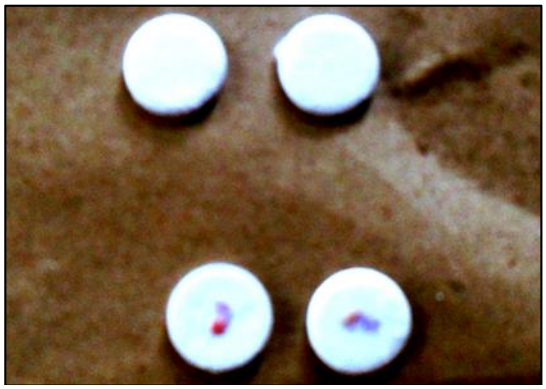
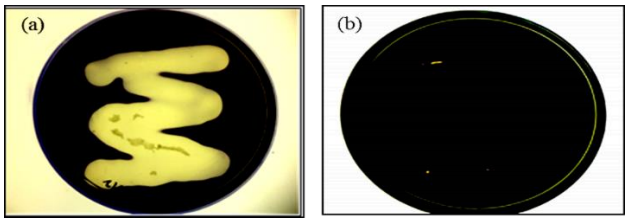


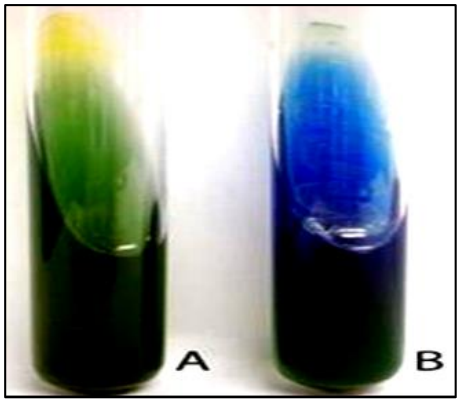
C. KOH test (+ve)



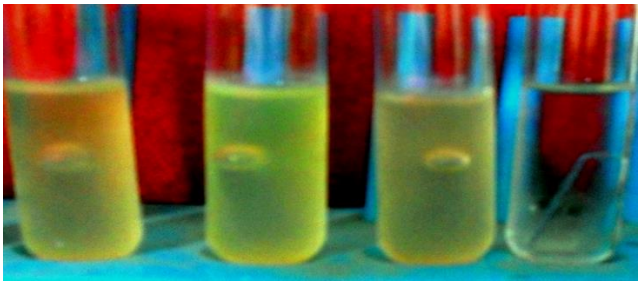
D. Results of oxidase test (+ve)



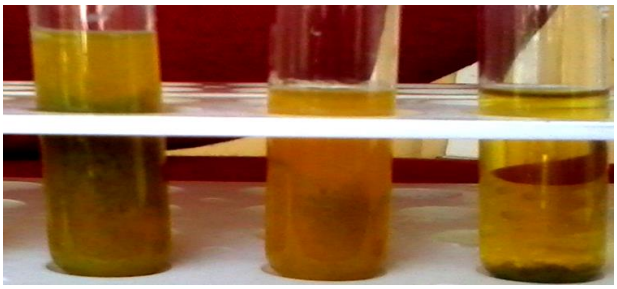
E. Starch hydrolysis tests (a) positive result (b) Negative result



F. Positive Citrate utilization test for *R. solanacearum* (A); Control (B)



G. Positive denitrification test for *R. solanacearum*; C-control



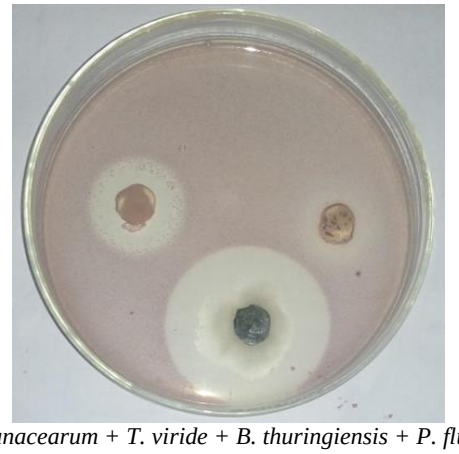
H. Positive Ammonia production test for *R. solanacearum*; C-Control

Plate I: (A-H). Biochemical tests for the identification of the bacteria

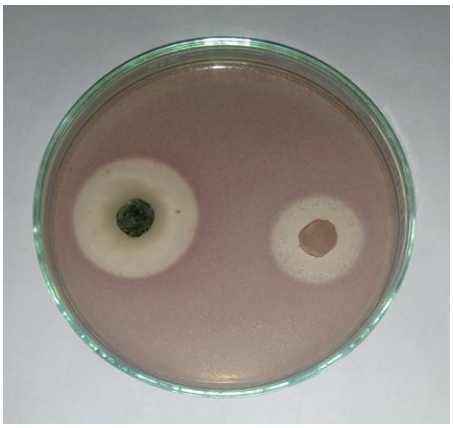
Healthy Micropropagated banana plantlet

Wilted micropagated banana plantlet

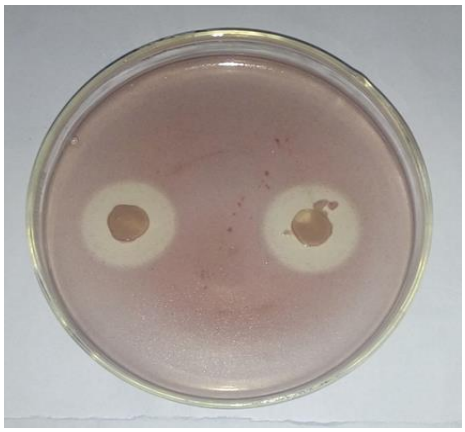
Plate 2: Pathogenicity test conducted with *R. solanacearum* in micropropagated banana



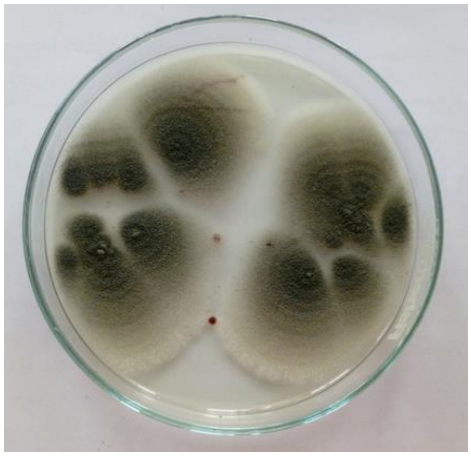
A) *R. solanacearum* + *T. viride* + *B. thuringiensis* + *P. fluorescens*



B) R. solanacearum + T. viride + B. thuringiensis

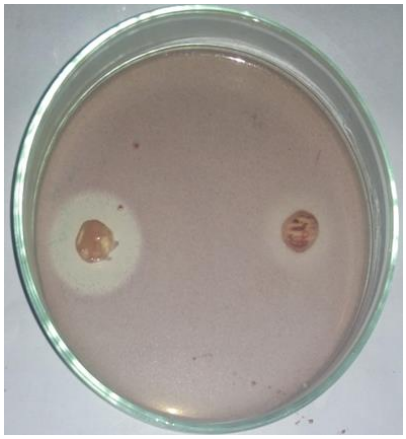


C) R. solanacearum + B. thuringiensis

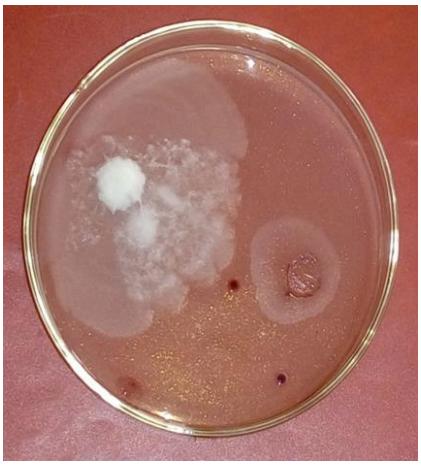


D) *R. solanacearum + M. anisopliae*

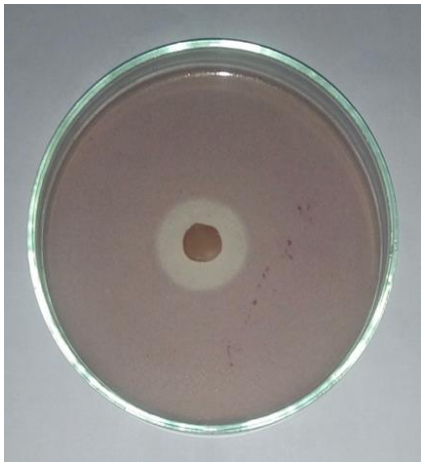
Plate 3: (A-D). Antagonistic effect of different bioagents against *R. solanacearum*



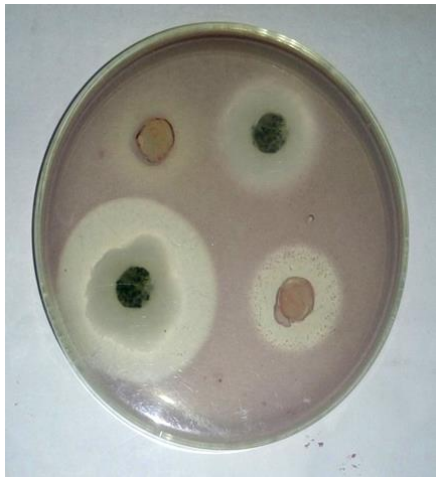
E) *R. solanacearum + B. thuringiensis + P. fluorescens*



F) *R. solanacearum + T. viride + P. fluorescens*



G) *R. solanacearum + P. fluorescens*



H) *R. solanacearum + T. viride + B. thuringiensis + P. fluorescens + M. anisopliae*

Plate 3: (E-H). Antagonistic effect of different bioagents against R. solanacearum

Plate 4: General view of experimental area

Plate 5: Application of consortial formulation for controlling bacterial wilt disease

References

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