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Comparative Evaluation of *In-vitro* Bioefficacy of Microbial Bioagents and Novel Chemical Compounds against *Fusarium oxysporum f*.sp. Cubense

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The Assam and North-eastern regions of India recognized as diverse repositories of wild and cultivated banana cultivars, confront a significant threat to banana varieties, Malbhog due to Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (Foc). This study investigates the *in-vitro* bioefficacy of indigenous bioagents and new-generation chemical compounds against

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Foc to develop an integrated module subsequently, in line with the worldwide pursuit of environmentally conscientious and high-performance agricultural practices.In this study, four bioagents viz., *Bacillus vallismortis*, *Bacillus amyloliquefaciens*, *Trichoderma harzianum,* and *Talaromyces pinophilus* were screened *in-vitro* against Foc revealed the highest efficacy of *B. vallismortis* with 68.22 % mycelial growth inhibition of Foc followed by *B. amyloliquifaciens* with (60.05%) and the least inhibition was exhibited by *T.pinophilus* with (50.05%).To identify new generation chemical compounds, namely Propiconazole, Azoxystrobin, Tebuconazole + Trifloxystrobin were tested with a standard check Carbendazim wherein all the chemicals significantly inhibited the mycelial growth of the pathogen over control with Tebuconazole + Trifloxystrobin combination fungicide showing highest percent inhibition of 94% at 0.1% concentration followed by Carbendazim at 0.1%. Our study has identified potential microbial strains and chemical fungicides which can be further explored for the development and exploration of bio-fungicide and chemical fungicide-based biointensive integrated management of Fusarium wilt menace in malbhog banana in the region.

Keywords: Biocontrol; bio-fungicide; chemical fungicide; fusarium wilt; malbhog banana.

1. INTRODUCTION

Bananas and plantains (*Musa* spp. Colla) within the order Zingiberales are significant tropical and subtropical food crops, crucial for the food industry, food security, and income [1]. The term "banana" encompasses various species or hybrids derived from *Musa acuminata* and *Musa balbisiana.* Globally, bananas rank high in export potential and palatability, trailing only rice, wheat, and maize in terms of gross value of output [2]. Cultivation dates back to 4000 BCE in New Guinea and is presently widespread across 150 countries, with major production in Asia, Latin America, and Africa, particularly in India and China. Other top producers include Ecuador, Brazil, the Philippines, Indonesia, Mexico, and Colombia, while major importers include China, the European Union, the United States, Canada, Japan, the Russian Federation, and the Near East countries [3].

Bananas, often termed a 'superfood,' are rich in essential minerals such as potassium, phosphorus, calcium, magnesium, and antioxidants, and provide 1-2.5% protein content, along with fibers, carbohydrates, and vitamins [4]. The Assam and North-eastern region of India stands out as a diverse hub for wild and domesticated banana cultivars. Malbhog banana (AAB) is a popular indigenous cultivar in Assam, prized for its taste, high Total Soluble Solids (TSS), year-round availability, and market demand. However, *Fusarium* wilt disease, caused by *Fusarium oxysporum* f.sp. *cubense* Race 1, poses a threat to Malbhog banana with 30-60% incidence in Assam leading to the gradual replacement of the most valued cultivar by Cavendish varieties [5,6]. *Fusarium* wilt caused by *Fusarium oxysporum f. sp. cubense* tropical race 4 (Foc TR4) is considered to be one of the most devastating diseases [7] Chemical management is often the most relied method, but overuse of chemicals has posed a serious threat to the ecosystem warranting the need to identify safer chemicals and bioagents for agroecosystem [8,9,10].

Carbendazim is the most preferred chemical option for banana growers in Assam. However, the recently proposed ban on the carbendazim group of fungicides warrants safer chemical molecules [11]. Bio-intensive management strategies, incorporating soil antagonists like *Trichoderma* spp. and *Pseudomonas* spp., offer an alternative to chemical fungicides against a wide range of soil-borne pathogens [12,13,14]. Considering the adverse effects of chemical fungicides, Integrated Disease Management (IDM) tools are recommended owing to their direct antagonism and induced resistance for a balanced and natural approach [15,16,17]. Natural biological products with beneficial strains, such as beneficial soil microbes, and endophytes are being explored as new strategies against horticultural disease management [18,19]. Further research is needed to understand the functionality of bioagents, their mode of action, and the identification of antifungal metabolites to enhance the effectiveness of bio-control strategies against Fusarium wilt. With this background, our study aimed at the identification of some native bioagents and new-generation chemical molecules to design integrated modules against the disease.

2. MATERIALS AND METHODS

Different laboratory aspects of the experiment were performed in the Department of Plant

Pathology, Assam Agricultural University, Jorhat. The experimental location, Jorhat, is geographically located at 26°44'N latitude and 94°19'E longitude with an elevation of 91m above mean sea level (MSL). It is situated in the Upper Brahmaputra Valley Zone (UBVZ) and experiences a sub-tropical humid climate with summer temperatures around 25-35ºC during the months of May to August and winter temperatures ranging from 7-18ºC from November to February.

Microbial bioagents: Four potentially aggressive bioagents viz., *Bacillus vallismortis, Bacillus amyloliquefaciens, Trichoderma harzianum,* and *Talaromyces pinophilus* were collected from the Biocontrol Laboratory of the Department of Plant Pathology, AAU, Jorhat, Assam, India (Table 1, Fig. 1). The bioagents previously identified and submitted to the National Center for Biotechnology Information (NCBI) gene Bank were collected and preserved at 4±1ºC for further use. Bacterial bioagents were grown utilizing Nutrient Agar (NA) (HIMEDIA-GM001-500G) media and incubated for 48 hours at 28±1ºC. Fungal bioagents were grown in Potato dextrose agar (PDA)(HIMEDIA-MH096) and incubated for 72 hours at 28±1ºC.

Source of pathogen and preservation: The pathogen *Fusarium oxysporum* f.sp. *cubense* Race 1 previously isolated from malbhog banana (AAB genomic group), identified with race-specific primer with NCBI GenBank accession No. OP090365 Foc_AB02 [20] was collected from the Department of Plant Pathology, AAU, Jorhat, Assam, India. The pathogen was transferred to freshly prepared potato dextrose agar (PDA) media and incubated for 72 hours at 28±1ºC. The culture was finally preserved at 4±1ºC in the refrigerator for further use.

Chemical fungicides: Commercial grade four different new generation chemicals viz., Propiconazole, Azoxystrobin, Tebuconazole+ Trifloxystrobin, and Carbendazim were collected from the market for further *in-vitro* test against the pathogen Foc.

In-vitro* bioefficacy assay of bioagents against *Foc: The interaction and *in vitro* antagonistic potential of different bioagents against pathogen Foc were assessed. Fungal bioagents isolated by Saikia, 2022 *viz., Trichoderma harzianum* and *Talaromyces pinophilus* were tested on PDA media by the dual culture plate technique [21]. Mycelial plugs (5 mm in diameter) were collected from actively growing regions with a sterilized cork borer; fungal bioagent and pathogen were placed 4 cm apart on the 9cm Petri plates at the opposite ends and allowed to grow at 28°±1ºC. The pathogen Foc was also grown as a monoculture on their respective media and served as a control. Similarly, bacterial bioagents *viz., Bacillus vallismortis,* and *Bacillus amyloliquefaciens*, were evaluated for their antagonistic effect against the pathogen by dual culture method [22]. Mycelial disc from a seven-day-old culture was placed at the center of the PDA media for Foc pathogen at 28± 1ºC for two days. The bacterial antagonist was inoculated on four sides of the pathogen, 1 cm away from the periphery of the plate. Incubation was done at 28± 2ºC for 72 hours. A monoculture plate of the pathogen was maintained as a control. The *in vitro* dual culture treatments were replicated five times with a completely randomized design (CRD). The growth of the isolates was monitored and observations were recorded at intervals of 24, 48, and 72 hours after inoculation. Radial growth of the treated fungal pathogens was measured and percent inhibition (PI) was calculated against the control plate. The percent inhibition was calculated using the formula as suggested by Vincent [23]:

$$PI = \{(C-T)/C\} \times 100$$

Where:
C = radial growth of the pathogenic fungus in control
T =radial growth of the pathogenic fungus in the presence of an antagonist
PI = Percent Inhibition

In vitro* assay of different chemicals against *Fusarium oxysporum* f.sp. *cubense: The efficacy of chemicals viz., Propiconazole, Azoxystrobin, Tebuconazole+Trifloxystrobin, and Carbendazim were evaluated against the pathogen *Fusarium oxysporum* f. sp *cubense* (Foc) at two different concentrations i.e., 0.1% and 0.2% for the *in-vitro* was assessed by poisoned food technique [24]. A total of 8 treatments including the chemicals with the pathogen as absolute control and Carbendazim, the widely used and recommended fungicide was used as a control check with each treatment replicated five times and was designed in a completely randomized design (CRD). The poison food technique was used where the media was poisoned by the chemicals to get 0.1 and 0.2% concentrations.

The medium was supplemented with streptomycin sulphate @50ppm to prevent bacterial contamination. Medium without any test chemical was maintained as control. Each Petri plate was inoculated with a culture disc (5 mm diameter) from a week-old pure culture of Foc under laminar airflow and incubated in a BOD incubator at 28±1ºC till full growth was observed in control. The related efficacies of the chemicals were estimated by measuring the radial growth in treatment and control plates.

Observation of colony diameter of the pathogen was recorded when control plates were fully covered with the mycelium of the tested pathogen. Percent inhibition of the mycelial growth was calculated as per the method by Vincent [23].

Statistical analysis: CRD was employed for *in-vitro* studies and pot experiments for statistical analysis of the data. The data were collected for statistical analysis by Fisher's method of analysis of variance. The significance of variance among the data was analyzed by calculating the "F" value and comparing it with the tabulated value of "F" at a 5% level of probability as given by Snedecor and Cochran [25] in the SAS Software. The percentage values were converted after Gomez and Gomez, 1984, and transformed by the angular transformation. The treatment means were compared among themselves by calculating critical difference (CD) as follows:

C.D. at 5%= S. Ed × $t_{0.05}$ or error degrees of freedom

Where the standard error of differences (S. Ed) of the mean was calculated by using the formula:

$$\text{S. Ed. } (\pm) = \sqrt{\frac{2 \times \text{error mean square}}{\text{No.of replication}}}$$

Where, S. Ed. = Standard error of the difference

And $t_{0.05}$ is the table value of students' 't' obtained at the 5% level of probability.

The significance and non-significance of the treatments at a 5% probability level were calculated by multiplying the S. Ed with the appropriate tabulated value for error degrees of freedom. The significance and non-significance of the treatments at a 5% level of probability were calculated by multiplying the S.Ed. with the appropriate tabulated value for error degrees of freedom.

Table 1. List of bioagents with (NCBI) accession numbers

Sl. No	Bioagent	Gene Bank Accession Number
1	*Bacillus vallismortis* BaSv6	OM585584
2	*Bacillus amyloliquefaciens* BaSv11	OM232770
3	*Talaromyces pinophilus* FKz09	ON148010
4	*Trichoderma harzianum* FKz10	MZ959806

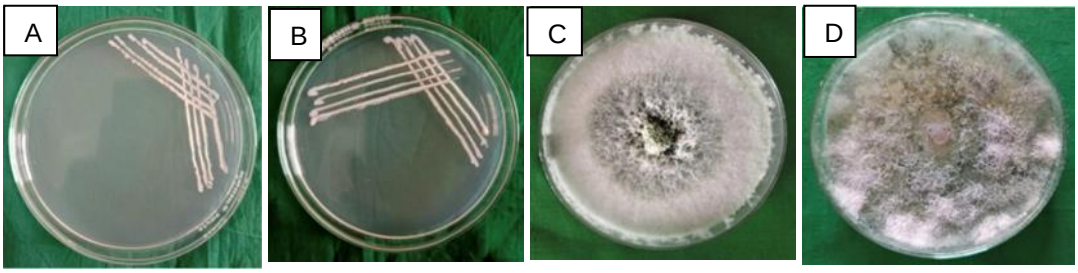

Fig. 1. Pure cultures of bioagents

A. Bacillus vallismortis BaSv6
B. Bacillus amyloliquefaciens BaSv11
C. Trichoderma harzianum FKz10
D. Talaromyces pinophilus FKz09

3. RESULTS AND DISCUSSION

Antagonistic study of selected bio-agents against *Fusarium oxysporum* f.sp. *cubense* by dual culture technique: The study on the efficacy of bio-agents on the growth of Foc (diameter in cm) was recorded at 3 intervals of 24, 48, and 72 hours of inoculation of Foc which varied significantly with that of control at all intervals (Table 2 and Fig. 2). The observation was recorded up to the full growth of Foc observed under control. After 72 hours of inoculation, the growth of Foc was 1.50 cm with inhibition of 68.22% under *Bacillus vallismortis* which differed significantly from all other treatments. The effect of *B. vallismortis* was followed by *B. amyloliquifaciens* with a growth of 1.72 cm with 60.05% inhibition. Thereafter *B. amyloliquifaciens* was followed by *T. harzianum* with growth of 1.83 cm at 58.51% inhibition and lastly *T. pinophilus* with 2.05 cm at 50.05% inhibition. Out of all the tested bioagents, *Bacillus vallismortis* showed 68.22 % growth inhibition of Foc, and the least inhibition was exhibited by *Talaromyces pinophilus* with 50.05%. `

The antagonistic bacterium *B. vallismortis* and *B. amyloliquififaciens* exhibited 68.22 and 60.05 percent higher inhibition than other bioagents *Trichoderma harzianum* and *Talaromyces pinophilus* when compared against Foc control plate suggesting their superiority. Earlier Kaur et al. [26] also established *Bacillus* spp. working against the growth of Foc and the species was also effective against fruit rot of King Chilli up to 83.22% [27]. *Bacillus velezensis* YEBBR6 and *Bacillus licheniformis* were found effective against Foc with percent inhibition of 63.3% and 77.59% [28,29] .*Bacillus* spp. a multi-functioning bacteria acting as a bioagent, and plant growth enhancer and is known for its wide range of secondary metabolites [30]. The pathogen inhibition by both species was mainly attributed to antimicrobial metabolites, antibiotics that might have led to morphological deformities, and disintegration of fungal cells [31]. The fungal antagonist *T. harzianum* exhibited an inhibition percentage of 58.51% and that of *T. pinophilus* was 50.0 % similar to the earlier results (Kumari et al*.,* 2014), [32]. The inhibition zone developed suggested the presence of diffusible non-volatile compounds with an antagonistic effect. *Trichoderma* spp. are well-established antagonists with excellent efficacy against a wide range of fungal and bacterial phytopathogens [33] with varied modes of action *viz.*, mycoparasitism, competition for space and nutrition, antibiosis or action of secondary metabolites or elicitation of plant defence mechanisms in inhibition (Bora et al*.,* 2023).

***In vitro* screening of new-generation fungicides:** Four different new-generation chemical fungicides were evaluated singly against Foc to find out the in vitro efficacy in inhibiting the growth of the fungus. The results presented in Table 3 and Fig. 3 show all the chemicals significantly inhibited the mycelial growth of the pathogen over control.

Among the fungicides tested, Tebuconazole+ Trifloxystrobin exhibited the highest inhibition of 100% and 94 % at concentrations of 0.2% and 0.1% respectively, followed by Carbendazim (0.1%) with percent inhibition of 93% on mycelial growth of the pathogen over control. Azoxystrobin and Propiconazole showed percent inhibition of 88% and 91% respectively at 0.2% concentration.

Table 2. *In vitro* efficacy of different bioagents against *Fusarium oxysporum* f.sp. *cubense* in dual culture assay

Sl. no.	Treatments	Mycelial growth (cm)*	Percent (%) inhibition over control
		72(hrs)	72(hrs)
1	*Bacillus amyloliquifaciens*	1.72	60.05
2	*Bacillus vallismortis*	1.50	68.22
3	*Trichoderma harzianum*	1.83	58.51
4	*Talaromyces pinophilus*	2.05	50.05
5	Control	4.00	0
	SE(±d)	0.0132	
	CD (p =0.05)	0.297	

**Mean of five replication*

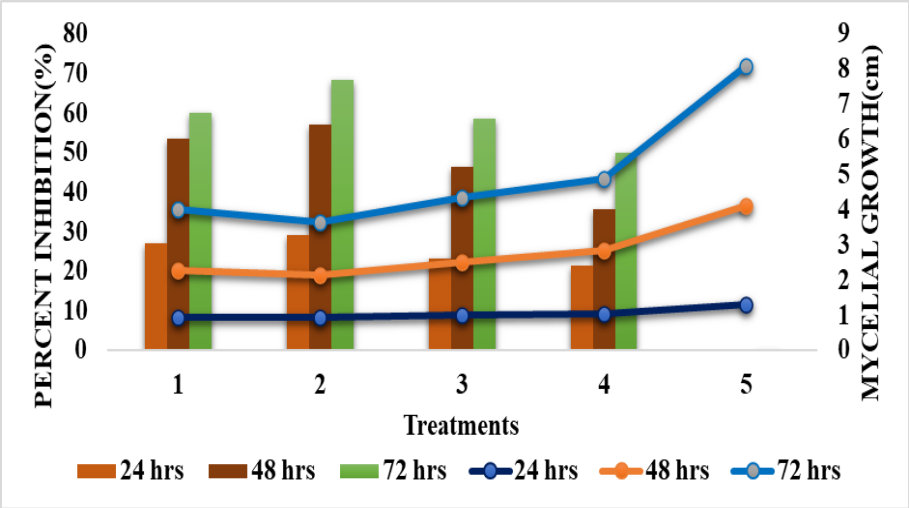

Fig. 2. Graphical representation of effect of bioagents on Foc mycelial growth(cm) and percent inhibition over control (%) at 24 hours interval

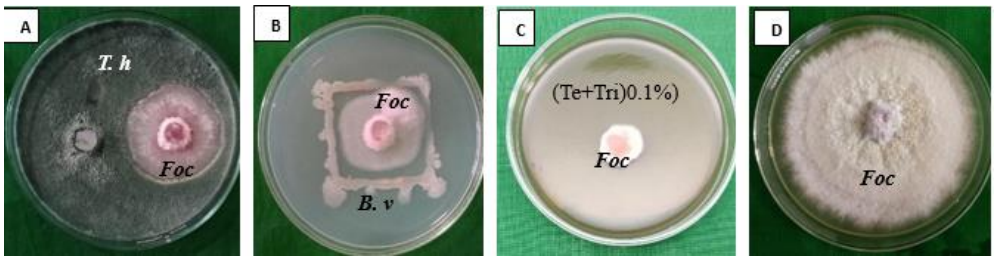

Fig. 3. Inhibition of *Fusarium oxysporum* f.sp. *cubense* (Foc) by most effective bioagents and *chemicals*

A. T. harzianum (Th)+Foc
B. B. vallismortis(B.v)+Foc
C. (Tebuconazole+Trifloxystrobin) 0.1% (Te+Tri 0.1%)+Foc
D. Control(Foc)

Table 3. *In-vitro* efficacy of different chemicals against mycelial growth of *Fusarium oxyporum* f.sp. *cubense*

	Treatments	Mycelial growth (cm)*	Percent (%) inhibition over control
T1:	Propiconazole (0.1%) + *Fusarium oxyporum*	1.00	88
T2:	Propiconazole (0.2%) + *Fusarium oxyporum*	0.80	91
T3:	Azoxystrobin (0.1%)+ *Fusarium oxyporum*	1.67	82
T4:	Azoxystrobin (0.2%)+ *Fusarium oxyporum*	1.05	88
T5:	Tebuconazole+Trifloxystrobin (0.1%) + *Fusarium oxysporum*	0.64	94
T6:	Tebuconazole+Trifloxystrobin (0.2%) + *Fusarium oxyporum*	0.00	100
T7:	Carbendazim(0.1%) + *Fusarium oxyporum*	0.62	93
T8:	Control	9.00	-
	SE(±d)	0.042	
	CD (p =0.05)	0.089	

**Mean of five replication*

Mishra et al. [34] carried out *in-vitro* screening of commonly used commercially available potential systemic fungicides (Topsin M and Carbendazim) against *Fusarium oxysporum* f. sp. *lycopersici* at three different concentrations (50 ppm, 100 ppm, and 200 ppm) which revealed that Carbendazim at 200 ppm has the strongest antifungal potential. Earlier Niwas et al. [35] evaluated the fungicides viz. carbendazim, azoxystrobin, and propiconazole *in-vitro* against *Fusarium oxysporum* f. sp. *cubense* where the results revealed that among the fungicides used, carbendazim @ 500 and 750 ppm significantly inhibited the mycelial growth of Foc followed by azoxystrobin. While in another study by Somu et al*.* [36] reported the control of *Fusarium oxysporum* f.sp. *cubense* (Foc) up to 100% by the usage of Carbendazim, Carboxin, Propiconazole and Benomyl. Azoxystrobin showed a moderate growth inhibition in conformity. New generation chemicals *viz.,* Propiconazole is known to have inhibitory function in fungal cell membrane ergosterols formation, through the blockage in the action of 14-α-sterol demethylase [37-39]. Azoxystrobin and other Strobilurins cause blockage of electron transport and inhibit mitochondrial respiration [40-41]. Tebuconazole is a demethylase inhibitor (DMI) interfering in the cell-building process and Trifloxystrobin obstructs respiration in plant pathogenic fungi. Carbendazim restricts the biosynthesis of DNA during fungal cell division [2].

4. CONCLUSION

In conclusion, our study highlights the efficacy of native bioagents (*Bacillus* spp., *Trichoderma,* and *Talaromyces*) and new-generation chemical fungicides (Propiconazole, Azoxystrobin, Tebuconazole+ Trifloxystrobin, and Carbendazim) against *Fusarium oxysporum* f.sp*. cubense*, *Bacillus vallismortis* showed outstanding biocontrol potential with 68.22% growth inhibition. Propiconazole, especially at 0.2%, demonstrated 100% inhibition among the chemicals tested. This study has identified bioagents *Bacillus* spp. and chemical Propiconazole as management options. However, field study coupled with a basic understanding of the compatibility of the bioagent and chemical, in-depth biocontrol mechanism identifying bioactive metabolites, and finally development of a bio-intensive module combining both the control options can provide a sustainable solution on a real-time basis against Foc in banana. Further, developing a consortium of all the effective microbes could address the fusarium wilt in organic production systems as well as new sustainable green technology.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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