



Antagonistic Effects of Different Soil Isolate Bio-agents against *Fusarium oxysporum* f. sp. *Cubense* TR4 *In vitro* and Molecular Characterizations

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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

Bananas are the earliest fruit crop cultivated by humans from ancient times in India and have great social-economic importance, merged with the country's cultural heritage. Different bio-agents were isolated from the rhizosphere of wilt affected banana plants cultivar Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 areas of Bihar. All isolated bio-agents were tested against FOC TR4 *in vitro* condition by dual culture technique. The maximum inhibition over control at 10 days of inoculation was recorded in *Trichoderma asperellum* 1(Tr1) (64.82%), followed by *Trichoderma asperellum* 2(Tr2) (62.70%), *Aspergillus flavus* (35.00%) and minimum in *Penicillium chrysogenum* (22.62%). The result clearly showed that *in vitro* *Trichoderma asperellum* 1 was highly effective while *Penicillium chrysogenum* was found least effective antagonistic against *Fusarium oxysporum*

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f. sp. *cubense* TR4 *in vitro*. Molecular characterizations of two morphologically different *Trichoderma* spp. were identified as two different strains of *Trichoderma asperellum* 1 and *Trichoderma asperellum* 2.

Keywords: *Fusarium oxysporum* f. sp. *cubense* TR4 (FOC TR4); trichoderma; bio-agents.

1. INTRODUCTION

Banana (*Musa paradisiaca* L.) is a critical herbaceous perennial monocotyledonous plant, which belongs to the family Musaceae. The plant is also known as Kalpatharu, which means herb with all potential uses. It has been believed to be originated from hot tropical regions of South East Asia from cultivar *Musa accuminata* and *Musa bulbisiana*. The banana is the oldest cultivated fruit crop known to humankind. It is also known as the Apple of Paradise. It is also mentioned in the Great Indian epics, Ramayana (2020 BC) and Kautilya's Arthashastra (300-400 BC).

Bananas are unique due to their high calories and nutritive values. As compared to apples, they contain five times more vitamin A and iron, four times more protein, three times more phosphorus, twice the carbohydrates, and the other vitamins and minerals [1]. The origin seems to be either from Malayan, Peninsula, or Asia. Subsequently, this crop was extended to many countries including Ceylon, Costa Rica, Ecuador, Mexico, Honduras, India, Jamaica, Columbia, Panama and several other Eastern countries. The banana crop was mainly cultivated in the humid tropics; it had a history in the subtropic and arid regions in the Middle East. In the 12th century, the banana was the existence in Moorish Spain and Northern Africa [2]. Banana is the world's most valuable fruit crop. In 2011, the total gross production value of US\$44 Billion, and global banana production was nearly 145 Million tons [3].

1.1 About the Panama Wilt

Panama wilt of banana incited by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Synd. and Hans. was first identified in 1874 at South East Queensland, Australia [4]. *Fusarium* wilt of banana incited by *Fusarium oxysporum* f. sp. *cubense* was first time isolated and disease caused by this pathogen was proved [5-6]. The fungus produces a reddish pigment in old culture due to the formation of chlamydospores. These characteristics separate it from other similar species, such as *Fusarium moniliforme* and *Fusarium solani* that also formed large quantities of microspores [6].

Until now, four different races of *Fusarium oxysporum* f. sp. *cubense* has been recorded [7].

Race -1: It affects varieties mainly Pome (AAB) and Silk (AAB) groups of bananas in the world.

Race -2: It attacks to Bluggoe types of bananas and other closely related cooking bananas.

Race -3: Caused diseases in *Heliconia* sp. Not confined to India. It occurs in Honduras, Australia and Costa Rica.

Race -4: All known varieties of banana are susceptible against this race.

Race 4 has divided in two i.e. tropical and subtropical. It produced disease in dwarf Cavendish groups of bananas and those who are susceptible to race I and race II. Subtropical race IV produced disease in dwarf Cavendish groups of bananas in different countries like Canary Islands, Australia, Taiwan and South Africa [8]. Australia and Southeast Asia the TR 4 influences Cavendish groups of bananas in the tropical areas [9].

2. MATERIALS AND METHODS

During survey of banana growing districts in Bihar, collection of soil samples were taken from the rhizosphere of wilt affected banana plant. The depth of soil having 10 cm and diameter at surface was 5 cm taken. Nearly 5-10 g soil was taken, put a tag in a soil sample with the farmer's name, location, and date of collection. The sample was taken in polythene packets and kept airtight. Soil sample brought to the Department of Plant Pathology, Dr. Rajendra Prasad Central Agricultural University (RPCAU), Pusa, Bihar. One gm of a soil sample was taken and serial dilution of 10^{-4} and 10^{-5} was prepared and poured in Rose Bengal agar medium under aseptic condition [10]. All the plates were incubated at $27 \pm 2^\circ\text{C}$. After 72 hrs. of incubation different fungal colonies observed in a compound microscope and prepared a pure culture of them.

For antagonistic effect of *Trichoderma asperellum* 1, *Trichoderma asperellum* 2, *Aspergillus flavus*, and *Penicillium chrysogenum*, the PDA was poured in Petri-plates in equal half [11]. In the first half, inoculated with seven days

old pure culture of each bio agent of 5mm while, in another half, seven days old pure culture of the pathogen poured on PDA directly touching with fungal mycelia. The pathogenic test fungus was placed without any bio-control agent as control plate. Four replications for each treatment were prepared. All Petri plates of each replication were incubated for $28 \pm 2^\circ\text{C}$ until the growth of *Fusarium oxysporum* f. sp. *cubense* TR4 isolates in the control treatment reached up to the margin of Petri-plates. The percent inhibition of linear mycelial growth of pathogenic fungi and bio-control agent was calculated using the formula [12].

Treatment's mean was compared with F-test and L.S.D. at the level of 0.05%.

$$I = \left(\frac{C - T}{C} \right) \times 100$$

Where,

I = Percent growth inhibition
 C = Control Petri plate colony diameter
 T = Treated Petri plate colony diameter
 The percent inhibition data were statistically analyzed by using a completely randomized design (C.R.D)

For molecular characterization of samples will be analyzed by RAPD molecular markers. The genomic DNA extraction of collected isolates of *Trichoderma* will be done separately by using standard 2 % Cetyl trimethyl ammonium bromide (CTAB) extraction method [13]. Spectrophotometer will be used for quantitative and qualitative analysis of the DNA of the test isolates.

3. RESULTS AND DISCUSSION

During survey of Panama wilt affected areas in Bihar, collected soil sample from the rhizosphere of banana. It took 1 gm soil sample to isolate microflora and followed serial dilution at concentrations 10^{-4} and 10^{-5} . After serial dilution, it spread into Rose-Bengal agar medium up to 3-4 days colony of different microflora was observed. Each colony was a different morphological structure and colour. Isolated every colony in PDA and prepared a slide for the identification of microflora. Four diverse microflora found during research work, i.e., *Trichoderma asperellum* 1, *Trichoderma asperellum* 2, *Aspergillus flavus*, and *Penicillium*

chrysogenum. The pure culture was prepared in Petri-plates and test tubes in the PDA medium. Both *Trichoderma* spp. were observed differences in their morphological structure and colony character under a microscope. *Aspergillus flavus* colony colour green, conidia and conidiophores are visible under a microscope. *Penicillium chrysogenum* colony colour initially white and later converted into shades of green. To confirm, the identification of microflora follows available literature.

3.1 Molecular Characterization of Different *Trichoderma* isolates i.e., Tr1 and Tr2

For molecular characterization of *Trichoderma* isolates Tr1 and Tr2 were sent to Indian Institute of Horticulture Research (IIHS), Bengaluru, Karnataka, India. For identification of *Trichoderma* samples used ITS primer. After molecular characteristics these samples having 98% similarity with *Trichoderma asperellum* identify as two different strains of *Trichoderma asperellum* 1 and *Trichoderma asperellum* 2. Results are presented in below-

Sample Name

Tr1

Sequence

```
TACCTTCTGTAGGGTGACCCCTGCGGAGGGAT
CATTACCGAGTTTACAACCTCCCAAACCCAAT
GTGAACGTTACCAAACCTGTTGCCTCGGCGGG
GTCACGCCCGGGTGCCTCGCAGCCCCGGA
ACCAGGCGCCCGCCGGAGGAACCAACCAAA
CTCTTTCTGTAGTCCCCTCGCGGACGTATTT
CTTACAGCTCTGAGCAAAAATTCAAATGAAT
CAAACCTTTCAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCGCCAG
TATTCTGGCGGGCATGCCTGTCCGAGCGTCA
TTTCAACCCTCGAACCCTCCGGGGGATCG
GCGTTGGGGATCGGGACCCCTCACACGGGT
GCCGGCCCCGAAATACAGTGGCGGTCTCGC
CGCAGCCTCTCCTGCGCAGTAGTTTGCACAA
CTCGCACCGGGAGCGCGGCGCGTCCACGTC
CGTAAACACCCAACCTTTCTGAAATGTTGACC
TCGGATCAGGTTGGAATTCCAATCGCTGATT
TTAAGCATATCAATAAGCGGAGAGAAAAC
```

Identification *Trichoderma asperellum*

Percentage identity 98%

Sample Name

Tr2

Sequence

CTTCCGTAGGGTGAACCTGCGGAGGGATCA
TTACCGAAAAGTTTACAACCTCCCAAACCAATG
TGAACGTTACCAAACCTGTTGCCTCGGCGGGG
TCACGCCCCGGGTGCGTGCAGCCCCGGAA
CCAGGCGCCCCGCCGAGGAACCAACACAC
TCTTTCTGTAGTCCCCTCGCGGACGTATTTCT
TACAGCTCTGAGCAAAAATTCAAATGAATCA
AAACTTTCAACAACGGATCTCTTGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAA

GTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCCGCCAGT
ATTCTGGCGGGCATGCCTGTCCGAGCGTCAT
TTCAACCCTCGAACCCCTCCGGGGGATCGG
CGTTGGGGATCGGGACCCCTCACACGGGTG
CCGGCCCCGAAATACAGTGGCGGTCTCGCC
GCAGCCTCTCCTGCGGAGTAGTTTGCACACTAC
TCGCACCGGGAGCGCGGCGCGTCTACGTCC
GTAGAACACCCAACCTTTCTGAAATGTTGACCT
ACGGATGCAGGTTGGAATCCAA

Identification *Trichoderma asperellum*

Percentage identity 98%



1



2



3



4

Plate 1. Isolation of microflora associated with the rhizosphere of banana

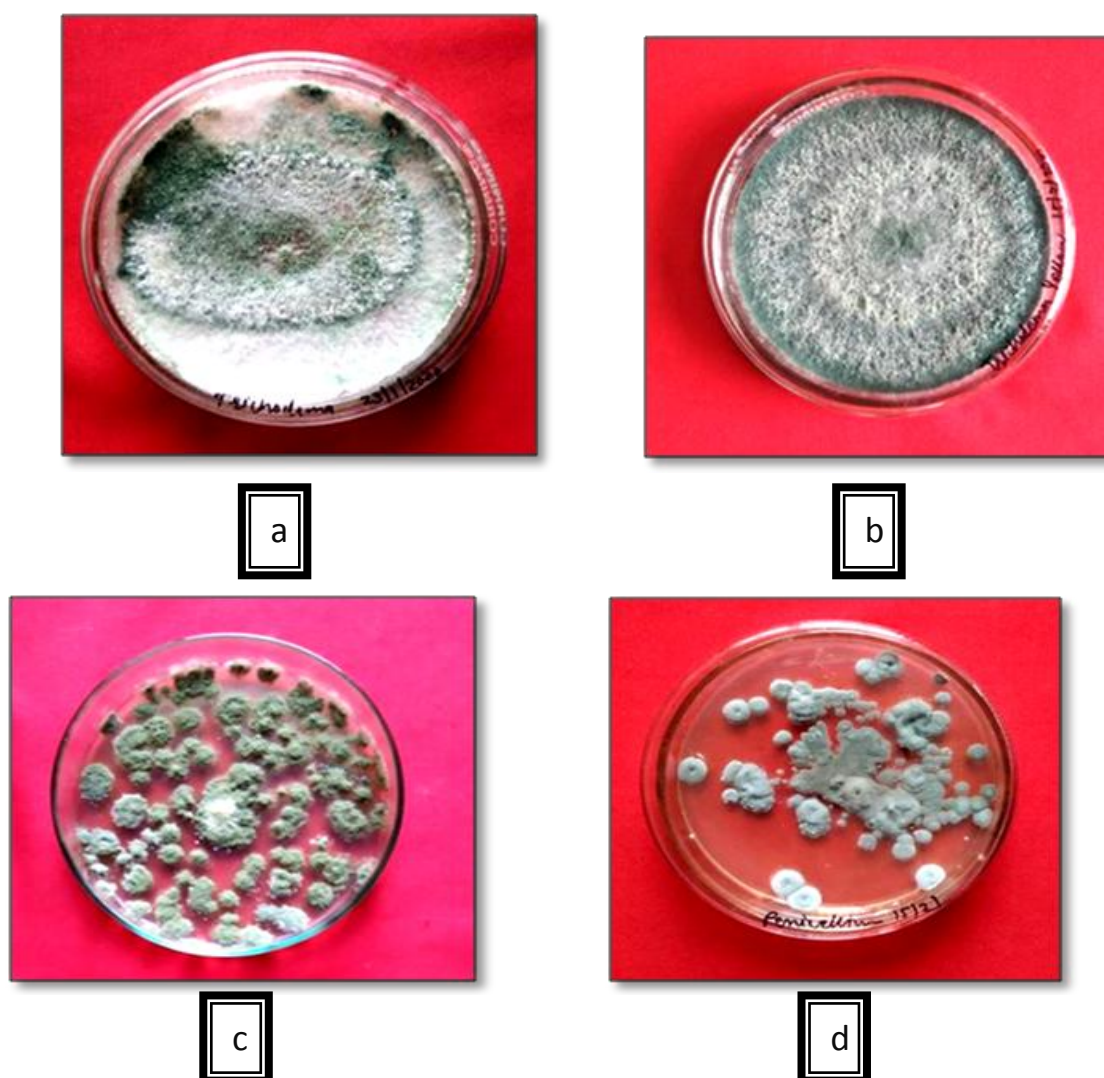


Plate 2. Purification and identification of rhizospheric microflora

a) *Trichoderma asperellum* 1(Tr1)
c) *Aspergillus flavus*

b) *Trichoderma asperellum* 2(Tr2)
d) *Penicillium chrysogenum*

The antagonistic activity of different rhizosphere isolates microflora against the *Fusarium oxysporum* f. sp. *ubense* TR4 was determined by the dual culture technique described [14]. Seven days old pure culture having 5 mm disc of different soil isolates bioagent and isolates of *Fusarium oxysporum* f. sp. *ubense* TR4 were placed equidistance in sterilized Petri plate containing PDA medium. Suitable control was also maintained without any bioagent. The growth of the pathogen was measured at 5 and 10 days of inoculation. Percent inhibition over control at 10 days of the pathogen was

calculated. Data are presented in Table-1. The maximum inhibition over control at 10 days of inoculation was recorded in *Trichoderma asperellum* 1 (64.82%), followed by *Trichoderma asperellum* 2 (62.70%), *Aspergillus flavus* (35.00%) and minimum in *Penicillium chrysogenum* (22.62%). The result clearly showed that *in vitro* *Trichoderma asperellum* 1 was highly effective while *Penicillium chrysogenum* was found least effective antagonistic against *Fusarium oxysporum* f. sp. *ubense* TR4 *In vitro*.

Table 1. Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubeense* TR4 *In vitro*

Bio-agents	Radial growth(mm)*		
	5 days	10 days	Inhibition over control (%) at 10 days
T ₁ <i>Trichoderma asperellum</i> 1(Tr1)	13.3	31.6	64.82
T ₂ <i>Trichoderma asperellum</i> 2(Tr2)	14.1	33.5	62.70
T ₃ <i>Aspergillus flavus</i>	31.3	57.4	35.00
T ₄ <i>Penicillium chrysogenum</i>	33.3	69.4	22.62
T ₅ Control	37.4	89.7	
CD at 5%	1.24	1.21	1.27
S.Em (±)	0.41	0.39	0.41
C.V. (%)	3.15	1.41	1.76

*Mean of four replications

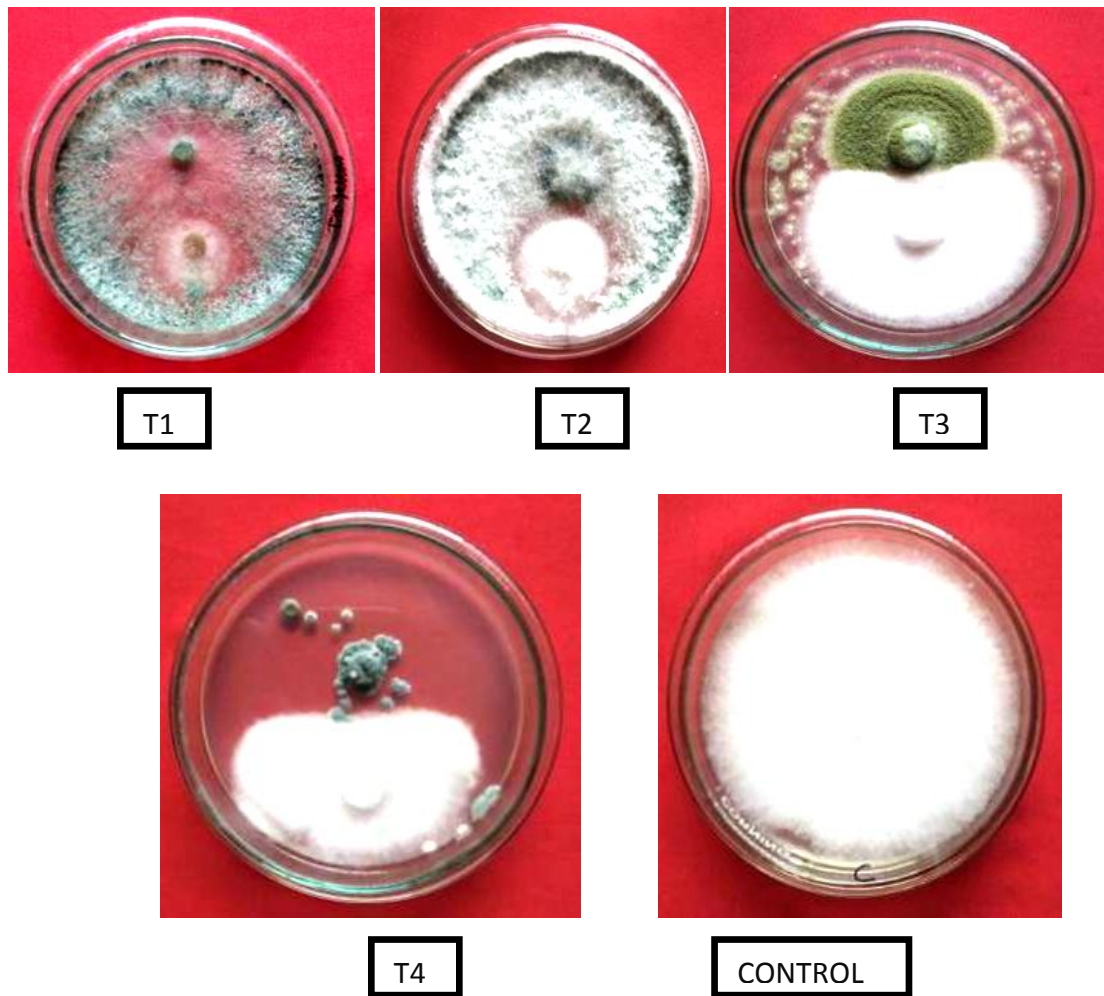


Plate 3. Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubeense* TR4 *In vitro* at 10 days

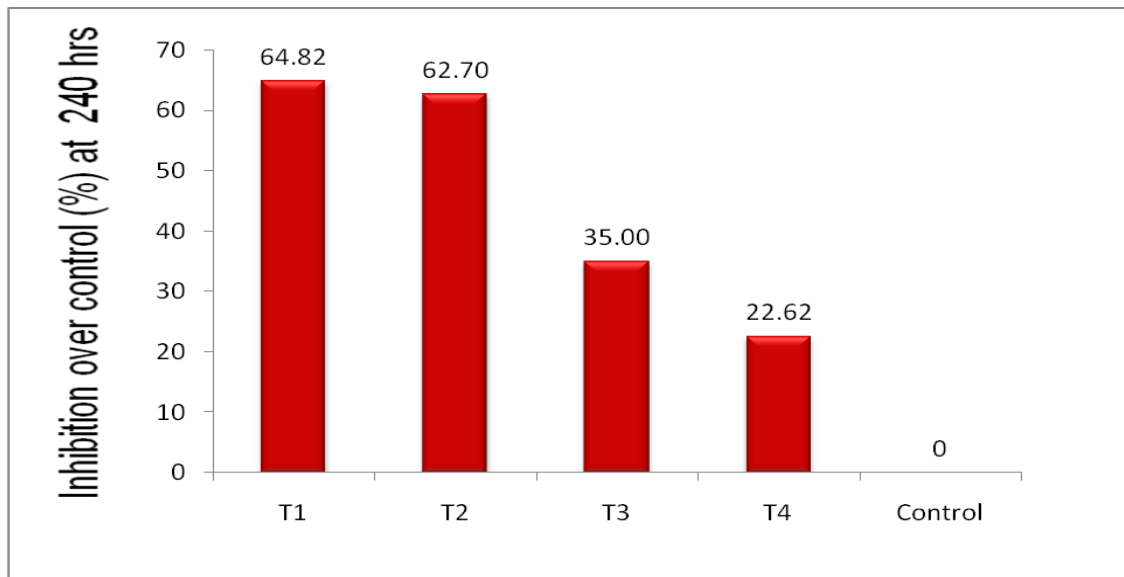


Fig. 1. Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro*

An experiment *in vitro* and in the glasshouse in which the result found that the different *T. harzianum* isolates TH, TH 13 and UH inhibited the growth of fungus FOC TR4 of the isolate under lab condition. However, *Fusarium oxysporum* f. sp. *cubense* inoculated banana seedlings above same treatment apply in the glasshouse. Then, it was not found effective against FOC TR4 [15]. New rhizospheric strain of *Trichoderma* sp. NRCB3. This was combined with the endophyte *Trichoderma asperellum* Prr2 and successfully tested against Panama wilt of banana in the field [16]. *Trichoderma harzianum* prevents up to 75.5% hindrance growth of the fungal pathogen when incubation for 72 hrs at $28\pm 2^{\circ}\text{C}$ *in vitro*. While, in pot conditions, *Trichoderma harzianum* will prevent disease severity in banana [17]. Experiment *in vitro* and in the poly house to determine the effects of *Trichoderma asperellum* (B01) against the Panama wilt. Maximum 84.85% inhibition of radial growth was found in *Trichoderma asperellum* (B01) *in vitro* while in poly house condition spore suspension of *Trichoderma asperellum* (B01) was apply then significantly reduced Panama wilt incidence percentage up to 94.4% in comparison to control [18]. Evaluated ten strains of *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *cubense* (FOC) *in vitro* and all strains of *Trichoderma harzianum* produced volatile metabolites that inhibit the growth and development of the *Fusarium oxysporum* f. sp. *cubense* (FOC). Among the ten strains of *Trichoderma harzianum* F116 strain, the most compelling antagonist *in vitro* has a potential

bioagent for biological control [19]. Studies to be found the effect of 3 isolates were *Aspergillus* against *Fusarium oxysporum* f. sp. *cubense* (FOC) *Aspergillus* spp. strain PD2, PD4, and PD5 prevent fungal mycelia growth of FOC up to 37.31, 26.52 and 12.04% respectively [20].

4. CONCLUSION

The antagonistic activity of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro* was determined by dual culture technique. The maximum inhibition over control at 10 days of inoculation was recorded in *Trichoderma asperellum* 1 (64.82%), followed by *Trichoderma asperellum* 2 (62.70%), *Aspergillus flavus* (35.00%) and minimum in *Penicillium chrysogenum* (22.62%). The result clearly showed that *in vitro* *Trichoderma asperellum* 1 was highly effective while *Penicillium chrysogenum* was found least effective antagonistic against *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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