

## Original Research Article

### **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* and molecular characterizations**

#### **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 240 hrs 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* f. sp. *cubense* TR4 *in vitro*. Molecular characterizations of two morphological different *Trichoderma spp.* samples 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

#### **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 that originated 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 and it's botanically known as a *Musa paradisiaca*. It was said to be the Apple of Paradise. It is also mentioned in the Great Indian epics, Ramayana (2020 BC) and Kautilya's Arthashastra (300-400 BC).

Banana pulp per 100 gm has a calorific value ranging from 67 to 137 calories. The ripe banana pulp contains about 70 % water with 23.43 % carbohydrate, 1.03 % protein, 0.48 % fat, 39.6 % K, 0.6 % Ca, 0.1 % Na, 0.03 % Fe and seven types of vitamins, including vitamins-A, B6, C, Thiamin, Riboflavin, Niacin, and Folic acid. Banana provides a more balanced diet compared to other fruits and has a more therapeutic value with low salt, fat content, and cholesterol. 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, Colombia, Panama and several other Eastern countries. The banana crop was mainly cultivated in the humid tropics; it had a history in the subtropical and arid regions in the Middle East. In the 12<sup>th</sup> century, the banana was first identified in Moorish Spain and Northern Africa [1]. 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 [2].

### **About the Panama wilt**

Panama wilt of banana incited by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Syd. and Hans. was first identified in 1874 at South East Queensland, Australia [3]. *Fusarium* wilt of banana incited by *Fusarium oxysporum* f. sp. *cubense* was first time isolated and disease caused by this pathogen was proved [4-5]. The fungus produces a reddish pigment in old culture due to the formation of chlamydospores. The characteristic is used to separate it from other similar species, such as *Fusarium moniliforme* and *Fusarium solani* that also formed large quantities of microspores [5].

Until now, four different races of *Fusarium oxysporum* f. sp. *cubense* has been recorded [6].

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

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

Race -3: Causes 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 banana 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 [7]. Australia and Southeast Asia the TR 4 influences Cavendish groups of bananas in the tropical areas [8].

## Material and Method

During the survey of Panama wilt in Bihar, the collection of a different soil sample from the rhizosphere of Panama wilt affected areas in cultivar Grand naine from different banana growing districts in Bihar. Nearly 5-10 g soil is taken from the rhizosphere and 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 it airtight. Soil sample brings to the Department of Plant Pathology, Dr. Rajendra Prasad Central Agricultural University (RPCAU), Pusa, Bihar. One gm of a soil sample was taken and its serial dilution technique of  $10^{-4}$  and  $10^{-5}$  pore in Rose Bengal agar medium under aseptic condition [9]. All the plates were incubated at  $27\pm 2^{\circ}\text{C}$ . After 72 hrs. observed different fungal colonies and their isolate. Preparations of slides and observe in a compound microscope, prepare a pure culture of separate colonies.

For the testing antagonistic effect of *Trichoderma asperellum* 1, *Trichoderma asperellum* 2, *Aspergillus flavus*, and *Penicillium chrysogenum*, the PDA Petri-plates were distributed in equal half [10]. 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 was put on PDA media directly touching with fungal mycelia. Control plate the pathogenic test fungus placed without any bio-control agent. Four replications for each treatment were done. 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 inhibitions of linear mycelial growth of pathogenic fungi and the bio-control agent were calculated using the formula [11].

Treatments mean were compare 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)

## Result and Discussion

During the 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 media 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.

### Molecular characterization of different *Trichoderma* isolates i.e., Tr1 and Tr2

#### Sample Name

Tr1

#### Sequence

```
TACCTTCTGTAGGGTGACCCTGCGGAGGGATCATTACCGAGTTTACAACCTCCCAA  
ACCCAATGTGAACGTTACCAAACCTGTTGCCTCGGCGGGGTACGCCCCGGGTGC  
GTTCGAGCCCCGGAACCAGGCGCCCCGCCGGAGGAACCAACCAAACCTCTTTCTGT  
AGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAATGAATCA  
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA  
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA  
CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCC  
TCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGGTGCC  
GGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCA  
CAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACCTTTCTG  
AAATGTTGACCTCGGATCAGGTTGGAATTCCAATCGCTGATTTTAAGCATATCAA  
TAAGCGGAGAGAAAAC
```

**Identification** *Trichoderma asperellum*

**Percentage identity** 98%

**Sample Name**

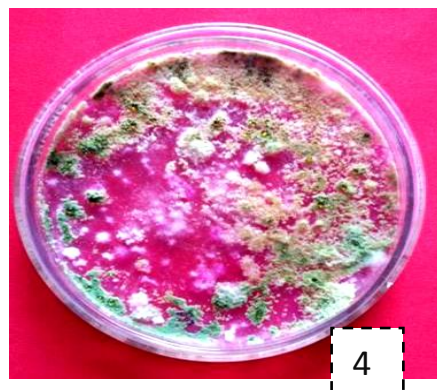
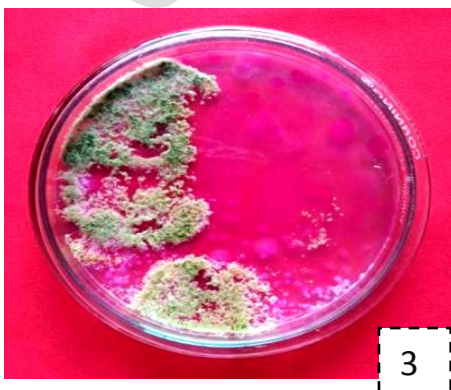
Tr2

**Sequence**

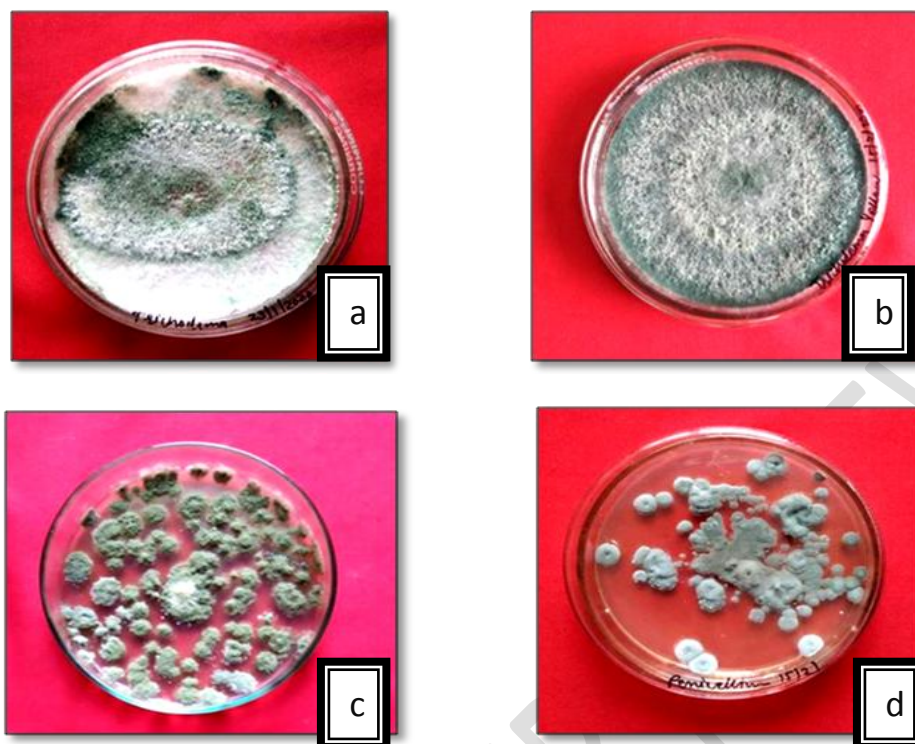
```
CTTCCGTAGGGTGAACCTGCGGAGGGATCATTACCGAAAGTTTACAACCTCCCAA  
ACCCAATGTGAACGTTACCAAACCTGTTGCCTCGGCGGGGTCACGCCCCGGGTGC  
GTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCACACTCTTTCTGT  
AGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCA  
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA  
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA  
CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCC  
TCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGGTGCC  
GGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGGAGTAGTTTGCA  
CTACTCGCACCGGGAGCGCGGCGCGTCTACGTCCGTAGAACACCCAACCTTTCTGA  
AATGTTGACCTACGGATGCAGGTTGGAATTCCAA
```

**Identification** *Trichoderma asperellum*

**Percentage identity** 98%



**Plate- 1.1. Isolation of microflora associated with the rhizosphere of banana**



**Plate-1.2. Purification and identification of rhizospheric microflora**

a) *Trichoderma asperellum* 1(Tr1)

b) *Trichoderma asperellum* 2(Tr2)

c) *Aspergillus flavus*

d) *Penicillium chrysogenum*

The antagonistic activity of different rhizosphere isolates microflora against the *Fusarium oxysporum* f. sp. *cubense* TR4 was determined by the dual culture technique described [12]. Seven days old pure culture having 5mm disc of different soil isolates bioagent and isolates of *Fusarium oxysporum* f. sp. *cubense* 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 120 hrs and 240 hrs (10 days) of inoculation. Percent inhibition over control at 240 hrs of the pathogen was calculated. Data are presented in Table-1.1. The maximum inhibition over control at 240 hrs 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*.



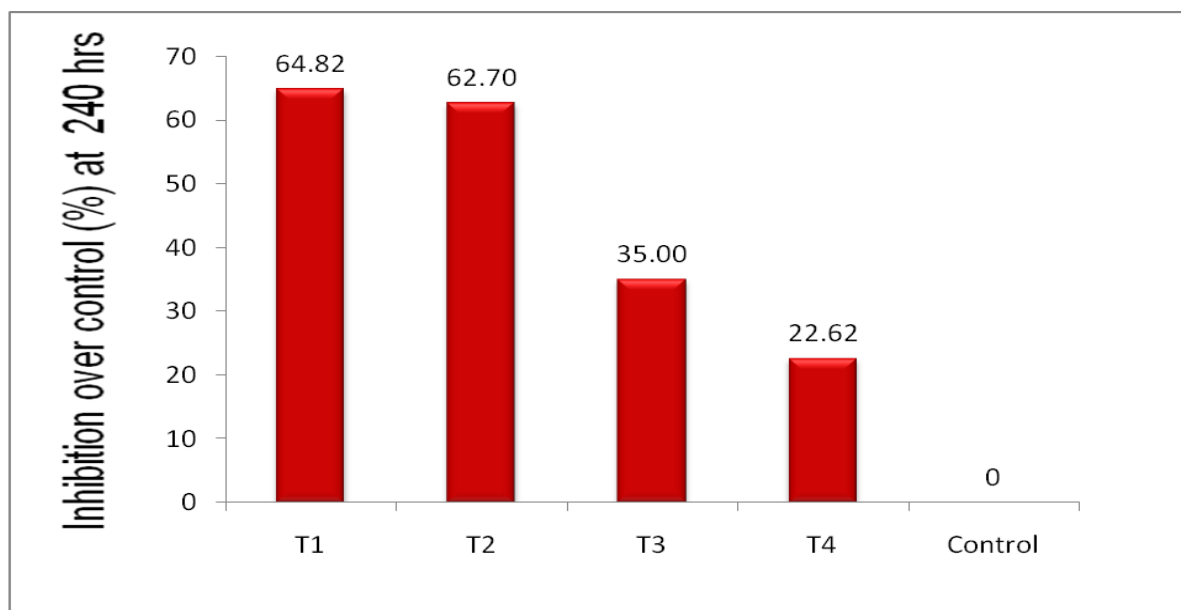
**Plate-1.3. Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro* at 240 hrs**

**Table-1.1. 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***

Bio-agents		Radial growth(mm)*		
		120hrs	240 hrs	Inhibition over control (%) at 240 hrs
T <sub>1</sub>	<i>Trichoderma asperellum</i> 1(Tr1)	13.3	31.6	64.82
T <sub>2</sub>	<i>Trichoderma asperellum</i> 2(Tr2)	14.1	33.5	62.70
T <sub>3</sub>	<i>Aspergillus flavus</i>	31.3	57.4	35.00
T <sub>4</sub>	<i>Penicillium chrysogenum</i>	33.3	69.4	22.62
T <sub>5</sub>	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
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\*Mean of four replications



**Fig-1.1. 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 [13]. 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 [14]. *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 [15]. 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 [16]. 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 [17]. 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 [18].

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