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Efficacy Test of Colonized Substrates and Spore Suspension Inoculation of *Fusarium oxysporum f. sp. cubense* TR4 on 'Cavendish' Banana

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***Abstract* – In the advent of the increasing infection of *Foc* TR4 in the Philippines, there is an increasing number of researches being conducted. In several cases, the success of getting positive results from artificial inoculation of *Fusarium oxyxporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4) is variable due to lack of standard artificial inoculation. In this experiment, five methods were compared, namely: root zone inoculation of *Foc* TR4-colonized Corn Grits plus Sand substrate (CGSS), Rice Grain Substrate (RGS), Banana Pseudostem Substrate (BPS), root dipping and drenching of *Foc* TR4 spore suspension (10^6 conidia mL^{-1}). A two-month-old tissue-cultured 'Grand Nain' banana plantlets were used in the study. The results showed that *Foc* TR4 colonized substrates had similarly shorter incubation period of 3.9 to 6.8 days as compared to the use of spore suspension (9.9 to 14.2 days). Fusarium wilt incidences were 100% in the use of CGSS, RGS and spore suspension applied by root dipping. The severity of rhizome discoloration and leaf yellowing was highest in the use CGSS with a rating of 5.4 and 3.7, respectively. Based on the results of the above parameters, the use of *Foc* TR4 colonized CGSS applied at the root zone is the most effective method of artificial inoculation.**

***Keywords* – *Fusarium Oxysporum* f. sp. *cubense* TR4, Cavendish Banana, Substrates, Inoculation Method.**

I. Introduction

Banana and plantain are ranked among the world's most valuable primary agricultural commodities and produced by 135 countries and territories [12]. The global top banana producing countries are India, China, Philippines, and Ecuador. The Philippine banana production was accounted at 2.42 million metric tons in 2018 with an increase of 0.6 percent from the previous year. More than 80% of the bananas (99% Cavendish cultivars) were produced on the island of Mindanao, with Davao, Northern Mindanao, and Soccsksargen as the top regions and Davao del Norte, Compostela Valley and Bukidnon as the top three provinces [11].

However, banana production is seriously threatened by the re-emergence of Fusarium Wilt. The disease is caused by the soil-borne fungi *Fusarium oxysporum* f. sp. *cubense* (*Foc*) and also known as "Panama disease". This fungus wiped out the 'Gros Michel' banana industry in Central America and the Caribbean, in the mid-twentieth century [13]. Once *Foc* is present in the soil, it cannot be eliminated. There are four recognized races of the pathogen based on host susceptibility. Race 1, which was responsible for the epidemics in 'Gros Michel' plantations, also attacks 'Lady Finger' (AAB) and 'SAB) varieties. Race 2 affects cooking bananas such as 'Bluggoe' (ABB) and race 4 is capable of attacking 'Cavendish' (AAA) as well as the other varieties of banana affected by races 1 and 2. These three races have been present on the east coast of Australia for many years, and race 1 is present in WA. Race 4 is further divided into 'sub-tropical' and 'tropical' strains. 'Tropical' race 4 is a more virulent form of the pathogen and is capable of causing disease in 'Cavendish' growing under any condition, whereas 'subtropical' race 4 generally only causes disease on plants growing sub-optimally (cool temperatures, water stress, poor soil). Race 3 affects *Heliconia* spp., a close relative of banana, and is not considered to be a banana pathogen [3]. In the Philippines, the occurrence of the disease is caused by races 1, 2, and recently, by Tropical Race 4 (TR4). Recently, *Foc* TR4 damaged 15,507 hectares in Region XI alone, with Davao del Norte and Compostela Valley as the major provinces affected [5].

Therefore, in the advent of the increasing infection of *Foc* TR4 in the Philippines, there is an increasing number of researches being conducted. In several cases, the success of getting positive results is variable due to lack of standard artificial inoculation method. Hence this study was conducted to select the most effective inoculation method of *Foc* TR4 to the 'Grand Nain' banana plantlets.

II. Materials and Methods

A. *Location and Duration of the Study*

The nursery experiment was conducted at the Fusarium Wilt Research Nursery, University of Southeastern Philippines (USeP), Tagum-Mabini Campus, Mampising, Mabini, Compostela Valley Province from January to March 2016.

B. *Experimental Design and Treatments*

The experiment was laid out in Completely Randomized Design (CRD) with six treatments and four replications. Five plantlets per replication were used in the study.

The treatments were as follows:

T1 - Control (uninoculated).
T2 - *Foc*-colonized corn grits plus sand.
T3 - *Foc*-colonized rice grain.
T4 - *Foc*-colonized banana pseudostem.
T5 - Root dipping on *Foc* TR4 aqueous suspension (10^6 conidia mL^{-1}).
T6 - Drenching of *Foc* TR4 aqueous suspension (10^6 conidia mL^{-1}).

C. *Source of Foc TR4*

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A pure culture of *Foc* TR4 was acquired from USeP PCR Laboratory, Tagum City and was sub-cultured on half strength Potato Dextrose Agar (PDA).

D. *Culture Medium Used*

Half strength potato dextrose agar (PDA) medium was prepared following the standard procedure using 10g dextrose, 20g agar, 100g potatoes (unpeeled and diced) and 1000ml distilled water [1].

E. *Pathogenicity Test*

The *Foc* TR4 acquired from USeP PCR laboratory was subjected to a rapid test for pathogenicity using tissue-cultured 'Grand Nain' banana in a test tube to ensure that the pathogen is virulent (Fig 1). The *Foc* TR4 was inoculated by submerging roots of plantlets to aqueous spore suspension at 10^6 conidia mL^{-1}.

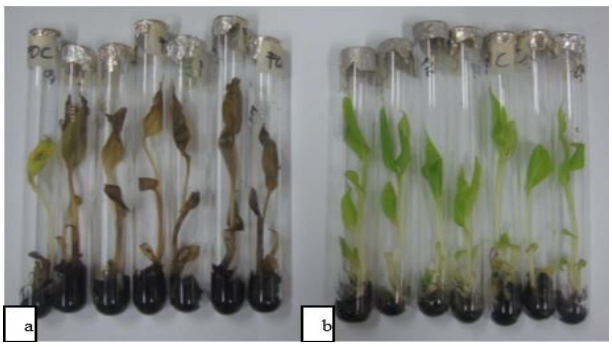

Fig 1. Pathogenicity test of *Foc* TR4 using tissue-cultured 'Grand Nain' 'Cavendish' banana in a test tube. (A) *Foc* TR4 inoculated tissue-cultured 'Grand Nain'; (B) Unino--culated tissue-cultured 'Grand Nain'. Photograph was taken seven days from inoculation.

F. *Preparation of Potting Medium*

The potting medium was prepared using a sandy loam soil and coco coir dust at a ratio of 8:2. This was pasteurized at 82°C for about 30 minutes to eliminate other soil-borne pathogens before inoculation [2]. The soil was allowed to cool before putting it in the prepared plastic pot (18cm diameter). Five kg of the potting medium was placed in every pot.

G. *Preparation of Test Plants*

Two-month-old tissue-cultured 'Grand Nain' were used in the study. It was transferred from the original bag to the plastic pot (18cm diameter) filled with five kilograms of pasteurized potting medium after *Foc* TR4 inoculation [6]. The plantlets carefully handled to avoid excessive root damage during transplanting.

H. *Preparation of Test Substrates*

Corn Grits and Sand substrate

The substrate was prepared by incorporating corn grits and washed river sand at a ratio of 1:10. For every kilogram of this mixture, 400ml of distilled water was added based on the methods of Porter et.al., (2015) with some modifications by Juruena (2014) [10][7]. The 150g substrate was placed in an autoclavable polypropylene bag or flask and sterilized for 25 minutes at 15 psi.

Rice Grain substrate

Rice grain substrate was prepared by floating and removing the unfilled grains and rinsing with tap water. Filled grains were soaked in water for 24h and placed in autoclavable polypropylene bags or flask. Bagged grains were sterilized for 30 minutes at 15 psi. Sterilized grains were again rinsed with distilled water and transferred to a new bag at 150g/bag then sterilized for another 30 minutes at 15 psi.

Banana Pseudostem Tissues Substrate

Fresh and disease-free 'Cavendish' banana pseudostem was collected from the field and was surface sterilized with 10% sodium hypochlorite. It was chopped ($1cm^2$), washed and was placed in autoclavable polypropylene bag or flask at 150g/bag. It was sterilized for one hour at 15psi.

I. *Inoculation of the Substrates with Foc TR4*

Ten pieces of mycelial discs (1cm diameter) from 7 day-old culture of *Foc* TR4 were aseptically inoculated in each bag of the substrate. Each inoculated bag was tightly sealed with a rubber band to avoid contamination then incubated for two weeks at 28-32°C to enhance total colonization of the substrate.

J. *Spore Suspension Preparation*

Aqueous spore suspension was prepared by adding sterile water on seven-day old *Foc* TR4 culture. Spores were dislodged by scraping the growth then strained using four layers of gauze cloth. Spore suspension was standardized into 10^6 conidia mL^{-1} using haemacytometer.

K. *Treatment Application*

The *Foc* TR4 colonized substrates were inoculated by mixing in the top soil at the rate of 150g/pot containing 5kg of soil. Banana plantlets were removed from the original bag and some root tips were wounded by cutting to facilitate infection by *Foc* TR4 before transplanting into the *Foc* TR4 colonized substrate-inoculated pots.

For inoculation by root dipping, wounded roots of banana plantlets were submerged in spore suspension for 2h before transplanting. In the case of inoculation by drenching, banana plantlets were similarly wounded and transplanted then spore suspension was drenched at the root zone at the rate of 150mL per pot immediately after transplanting (Fig. 2).

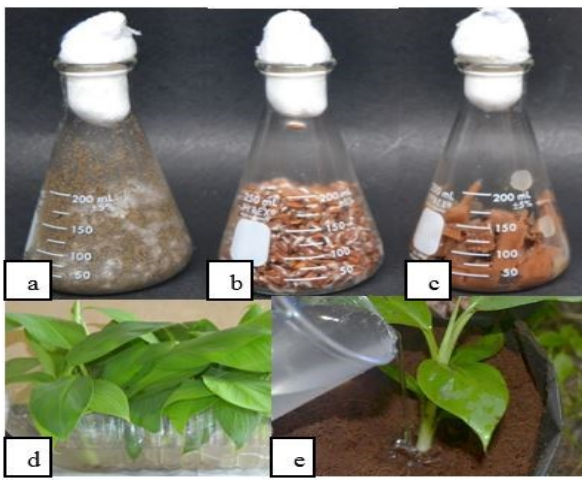

Fig. 2. Different methods of *Foc* TR4 inoculation a. *Foc*-colonized CGSS b. *Foc*-colonized RGS c. *Foc*-colonized BPS d. Root dipping on aqueous *Foc* TR4 spore suspension (10^6 conidia mL^{-1}) and e. Drenching of aqueous *Foc* TR4 spore suspension (10^6 conidia mL^{-1}).

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L. *Care and Maintenance*

Cultural practices such as fertilization, watering, weed control, leaf pruning and pseudostem sanitation were done in the experiment.

M. *Biosafety Protocol*

The potting media and infected banana plantlets used in the experiment was decontaminated by heat sterilization for 60 minutes in the Fusarium Wilt Facility in University of Southeastern Philippines at Mabini Unit before disposal.

N. *Data Gathered*

Incubation Period was recorded as soon as the earliest symptoms of Fusarium wilt appeared. Splitting of the pseudostem was monitored daily as well as the appearance of yellow streaks and wilting symptoms. The rating for rhizome discoloration and leaf yellowing was based on Mohammed and co-workers (1999) with some modifications by Juruena (2014) [8] [7].

Table 1. Rating scale for severity of leaf yellowing.

Scale	Description
1	No yellow discoloration of leaves.
2	Yellowing of older leaves.
3	Yellowing of all older leaves and slight discoloration of younger leaves.
4	All leaves turn yellow.

Table 2. Rating scale for rhizome discoloration.

Scale	Description
1	No vascular discoloration.
2	Isolated points of discoloration in vascular tissue.
3	Discoloration up to between 1/3 of vascular tissue.
4	Discoloration of between 1/3 and 2/3 of vascular tissue.
5	Discoloration greater than 2/3 of vascular tissue.
6	Total discoloration of vascular tissue.

Based on severity of rhizome discoloration and leaf yellowing manifested by *Foc* TR4, the disease index for each of the symptoms was computed using the formula below:

$$\text{Disease Severity Index (DSI)} = \frac{\Sigma(\text{Number on scale} \times \text{Number of plantlets in that scale})}{\Sigma\ (\text{Number of treated plantlets})} \times 100$$

III. Results and Discussion

The root zone application of *Foc* TR4 colonized CGSS and RGS at 150g/5kg soil resulted in 100% Fusarium wilt incidence. Root dipping to for 2h in aqueous spore suspension of *Foc* TR4 (10^6 conidia mL^{-1}) likewise attained 100% Fusarium wilt within 30 days from inoculation (Table 3).

Incubation period was short (3.9-6.8 days) in plantlets inoculated with colonized substrates as compared to the use of aqueous spore suspension applied by root dipping and drenching which had 9.9 and 14.2 days, respectively. The difference in incubation period can be attributed to the type of the inoculum used. Colonized substrate contain already actively growing vegetative and reproductive stages of *Foc* TR4 while spores in the aqueous suspension has yet to undergo germination, ingress and hyphal extension prior to colonization resulting in symptom expression. Dercks' *et al.* (2008) reported that substrate inoculation is more suitable for trials than inoculation by poured spore suspensions [4].

Inoculation with *Foc* TR4 colonized CGSS resulted in the highest severity rating of rhizome discoloration (5.4) and leaf yellowing (3.7). Leaf yellowing is a secondary symptom resulting from *Foc* infection in the rhizome and vascular tissues indicated by discoloration, thus when rhizome and vascular tissues are severely infected, leaf yellowing as a consequence, will likewise be severe. The establishment of fungus in the xylem vessel induces the formation of tyloses. These transformations may affect the translocation of water and nutrients from roots to the leaves which affects many biotic functions [11].

Other inoculation methods had Fusarium wilt infection ranging from 85% to 100%. In addition, the incubation period ranged from 6.8 to 14.2 days while, the rating for rhizome discoloration ranged from 2.3 to 2.9 and leaf yellowing ranged from 2.4 to 2.6 (Table 3, Figure 3 and 4).

Table 3. Summary of means for Fusarium wilt incidence, incubation period, and severity on 'Grand Nain' banana plantlets subjected to the different inoculation methods[1].

Treatments	Disease Incidence[2] (%)**	Incubation Period (days)**	Disease Severity	
			RDI**	LSI**
Negative Control	0^{d}	0.0^{a}	1.0^{d}	1.0^{d}
CGSS	100^{a}	3.9^{b}	5.4^{a}	3.7^{a}
RGS	100^{a}	4.6^{b}	3.3^{b}	3.2^{b}
BPS	85^{c}	6.8^{bc}	2.7^{bc}	2.4^{c}
Root dipping	100^{a}	9.9^{cd}	2.9^{c}	2.6^{c}
Drenching	95^{b}	14.2^{d}	2.3^{cd}	2.6^{c}

[1] Average of 20 banana plantlets per treatment. Data were Arc sine transformed prior to analysis of variance.
[2] Cummulative data up to 30 days from inoculation.
**Significant at 1% level

Fig. 3. Severity of rhizome discoloration of 'Grand Nain' banana plantlets a. Negative Control (uninoculated) b. *Foc*-colonized CGSS c. *Foc*-colonized RGS d. *Foc*-colonized BPS e. Root dipping on aqueous *Foc* TR4 spore suspension (10^6 conidia mL^{-1}) and f. Drenching of aqueous *Foc* TR4 spore suspension (10^6 conidia mL^{-1}).

Furthermore, Daly and Walduck (2006) explained that internal symptoms first become obvious in the xylem (water conducting) vessels of the roots and the rhizome. These turn a reddish-brown to maroon color as the fungus grows through the tissues. Occasionally, the discoloration first appeared yellow in plants showing early stages of infection. When cut in cross-section the discoloration appeared in a circular pattern around the center of the rhizome where the infection concentrates due to the arrangement of the vessels. As symptoms progressed into the pseudostem, continuous lines of discoloration were evident when the plant is cut longitudinally [3].

Thus, the result of the study produced typical infection process of Fusarium wilt on its host wherein internal symptoms of the hosts is first established such as discoloration of roots and rhizome and eventually leading to pseudostem colonization causing leaf yellowing starting on the older to younger leaves and wilting of the whole plant.

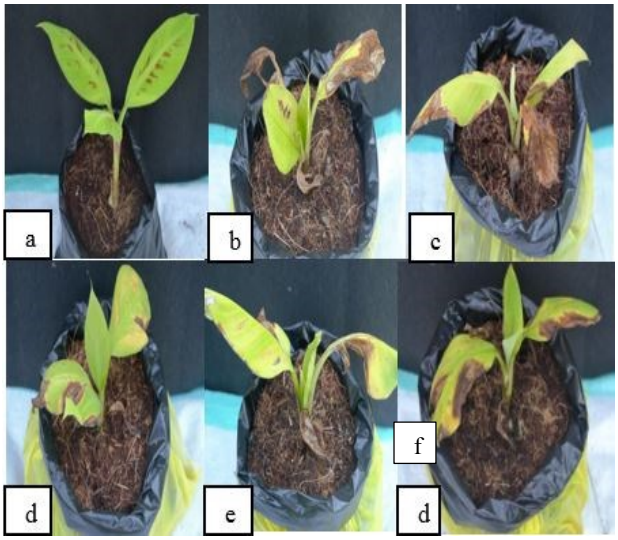

Fig. 4. Severity of leaf yellowing on 'Grand Nain' banana plantlets a. Negative Control (uninoculated) b. *Foc*-colonized CGSS c. *Foc*-colonized RGS d. *Foc*-colonized BPS e. Root dipping on aqueous *Foc* TR4 spore suspension (10^6 conidia mL^{-1}) and f. Drenching of aqueous *Foc* TR4 spore suspension (10^6 conidia mL^{-1}).

IV. Conclusion

The efficacy test of *Foc* TR4 colonized substrates and spore suspension inoculation of *Foc* TR4 (10^6 conidia mL^{-1}) on two-month-old 'Grand Nain' banana plantlets revealed that *Foc* Corn Grits and Sand Substrate was the most effective method over the other methods based on highest disease incidence (100%), disease severity and earliest symptom appearance (3.9 days).

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