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Control of *Fusarium oxysporum* f. sp. *cubense* (E.F. Sm.) Snyder and Hansen tropical race 4 causing Fusarium wilt in banana cv. "Lakatan"

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Abstract

This study was conducted to evaluate control strategies using chemical and organic based formulations against *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) causing Fusarium wilt in banana cv. "lakatan". Fungicides such as Dibromo-3-nitropropionamide (10 ml/liter of water) and Tebuconazole +Trifloxystrobin (1 g/liter of water) were effective against Foc TR4 in Lakatan both in vitro and in nursery conditions. These fungicides can be used as effective disinfectants or soil drench that can be included in the implementation of integrated disease management against Foc TR4. Meanwhile, organic-based formulations were found ineffective in the control of the Foc TR4 pathogen.

Keywords - efficacy, fungal pathogen, fungicide, *Musa*, organic

Introduction

Banana is recognized as a major food crop both globally and locally where it is considered as a staple food for some 400 million people. Of all the fruits, it ranks first place in terms of production volume and is among the five most consumed fruits worldwide (INIBAP, 2000). In the Philippines, banana is the leading fruit, a top dollar earner and a major source of income among small-scale farmers.

However, banana production had been confronted with the spread of Fusarium wilt disease caused by the fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc) (E. F. Sm.) Snyder and Hansen, a most devastating disease affecting commercial and subsistence banana production throughout the banana producing areas of the world (Ploetz, 2005). Foc pathogen is one of more than 100 formae speciales of *F. oxysporum* that causes vascular wilts on flowering plants. It contains pathogenic and saprophytic strains that cannot be distinguished morphologically. Colonies grow 4-7 mm/day on Potato Dextrose Agar (PDA) at 24°C, with slight to significant aerial mycelium, and white to purple pigmentation. Sporodochia are tan to orange, and sclerotia are blue and submerged. Micro- and macroconidia are produced on branched and unbranched monophialides. Microconidia are 5-16 x 2.4-3.5 µm, one- or two-celled, oval to kidney-shaped, and are borne in false heads. Macroconidia measure 27-55 x 3.3-5.5 µm, four to eight celled and sickle-shaped with foot-shaped basal cells (Figure 1).Terminal and intercalary chlamydospores are 7-11 µm in diameter, usually globose and are formed singly or in pairs in hyphae or conidia (Ploetz, 2000).

The pathogenic isolates of Foc have been traditionally grouped into four physiological races based on pathogenicity to host cultivars

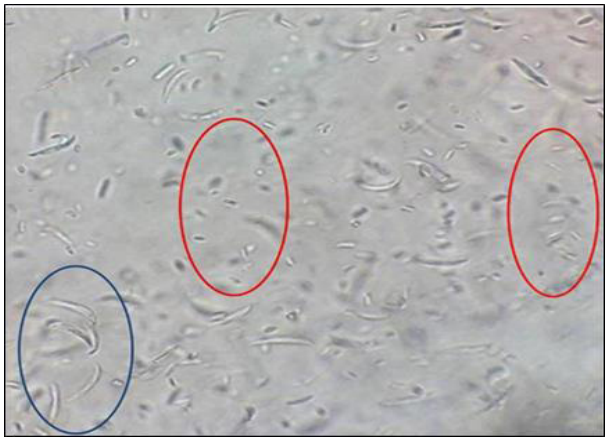

Figure 1. The photomicrograph of the pathogen *Fusarium oxysporum* f. sp. *cubense* isolated from fusarium wilt infected banana showing the typical sickle-shaped Macroconidia (blue circle) and the abundant typical kidney-shaped Microconidia (red circles).

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under field conditions. Race 1 of Foc attacks Gros Michel, Lady Finger (AAB) and Silk (AAB) varieties. Race 2 infects cultivar Bluggoe (ABB), while Race 3 infects *Heliconia* spp. (a close relative of banana). Race 4 is able to attack cultivar Cavendish and all cultivars that are susceptible to Race 1 and Race 2. The disease is destructive because it seriously hampers banana production and is difficult to manage (Gang et al., 2013).

Foc Tropical Race 4 (TR4) affects banana cultivars that comprise 80% of the world's banana production. The Foc TR4 strain poses a very real threat to the multi-billion dollar global banana trade and the food security of millions of subsistence farmers (Ploetz, 2005). In the Philippines, the pathogen was confirmed to be present in Cavendish cultivars in 2005 particularly in Davao City, Davao del Norte and Davao Del Sur (Molina et al., 2008). Today, the pathogen is continuously spreading particularly in nearby provinces including Compostela Valley, Zamboanga Del Norte, Misamis Oriental, Bukidnon, Saranggani, North and South Cotabato (Generalao et al., 2014, Solpot et al., 2016) which poses a real threat both in export and locally consumed bananas such as Lakatan and Latundan varieties. So far, no Foc TR4 was reported in Luzon and Visayas islands. However, the occurrence of Foc subtropical race 4 (STR4) in Luzon was reported recently by Aguilar-Hawod (2015). This should also be taken into consideration as it can cause severe damage under favorable condition.

Today, effective and widely practical control strategies of Foc TR4 are based on visual monitoring for early symptom appearance, eradication of infected plants, introduction of newly developed resistant cultivars, isolation of infested areas, and strict implementation of quarantine measures to reduce pathogen dissemination.

Furthermore, development of management tactics against the disease is one of the primary concerns in mitigating the outbreak of the disease. The use of the newly developed chemical fungicide as disinfectants and sterilants as well as the use of more safe organic-based agents can be explored for its efficacy as control both *in vitro* and *in vivo* against Foc TR4 pathogen. These tactics can be integrated for the prompt implementation of management practices against the dreadful Fusarium wilt disease.

Methodology

Efficacy Test of Chemical and Organic-Based Formulations Against Foc TR4 *In vitro*

Preparation of Test Treatments

Four (4) commercially available test fungicides were used in the experiment. These were Dibromo-3-nitrilopropionamide, Tebuconazole + Trifloxystrobin, Propineb, and the Benomyl. A botanical broad spectrum fungicide Timorex Gold (*Melaleuca alternifolia* plant extract) was also included in the evaluation *in vitro*.

Meanwhile, organic-based formulations as test treatments were prepared previously in the Department of Plant Pathology, College of Agriculture, University of Southern Mindanao. Preparation was done following the standard procedures. Fermented plant juice (FPJ) was prepared following the procedure described by Miller et al. (2013) with modifications. Two kilograms of camote tops or sweet potato leaves were collected before sunrise as starting material. These were chopped and 1 kg of muscovado sugar was added. The mixture was transferred in a container and covered with a clean paper and tied with a twine. It was kept for seven days in a shaded area to allow fermentation. The juice was extracted and used in the experiment as FPJ formulation. On the other hand, indigenous microorganisms (IMO) was prepared by taking 1 kg of cooked rice and placed in a wooden box covered with a white paper towel. The top was covered with a wire screen and clear plastic sheet to protect the box from animals and rain. This was then buried to partially avoid direct exposure to sunlight, and left undisturbed for a minimum of 4-5 days, allowing the rice to be covered with white mold indicating the presence of indigenous microorganisms. Rice with white molds was transferred into a basin and mixed thoroughly with 1 kg muscovado sugar (1:1). The mixture was transferred into a plastic pail, covered with paper, tied up with twine, incubated at room temperature and allowed to ferment for 7 days. The extracted mixture was then used as IMO formulation.

Lastly, preparation of lactic acid bacteria serum (LABS) was done by placing rice wash in a large container covered with clean sheet of paper, and placed in a shaded area for seven

days. After seven days, a sour smell developed forming three layers such as floating compound-rice bran, clear Lactic Acid Serum (LAS), and the starch. The clear (middle) layer was extracted by using a siphon. One (1) part of clear liquid (LAS) was mixed with 10 part of fresh milk. Rice bran was then evenly placed on top of the milk to keep in anaerobic stage without stirring for 5-7 days. Carbohydrate, protein and fat were floated leaving the yellow liquid (serum) which contained lactic acid bacteria. The yellow liquid (serum) was taken and added with muscovado sugar (1:1) for preservation.

Isolation of the Foc pathogen and pathogenicity test to confirm isolates as TR4

The dried vascular lines were taken from Foc infected banana pseudo stem samples and cut into 0.5-1.0 cm long and cultured on half strength PDA through tissue planting technique. The cultures were incubated at room temperature for 2-3 days. Sub-culturing was done to obtain pure culture of the pathogen. To ensure that the isolate was Foc TR4, pathogenicity test was done in Cavendish cultivars. Pathogenicity testing was done by inoculating 1 month-old seedlings with 250 ml of 40,000 ± spores/ ml of Foc TR4 suspension by drenching method. Two root hairs were broken using a teasing needle to create entry point for the pathogen. Three Cavendish seedlings were used in the experiment.

Bioassay Test

For bioassay test, treatments were prepared based on the specified rates. For each test treatment, 100 ml solution was prepared. Melted PDA medium was cooled before adding the desired amount of the test treatments. Evaluation of test treatments against Foc TR4 was conducted using the technique described by Dhingra and Sinclair (1995). Ten (10) ml of melted PDA medium and test treatment mixture were poured into each sterile petri plate. Before dispensing the mixture, it was stirred using a sterile glass rod to dissolve the fungicides and poured into the sterile petri plates to allow to congeal for about one hour. Culture discs from two-week old pure culture of Foc TR4 were obtained using flamed-sterilized cork borer. One disc was placed at the center of the plated PDA medium. Plates were sealed with parafilm, labeled properly and incubated in an upside down position at room temperature for 10 days or until control plates were fully colonized. The efficacy of the different test treatments was evaluated at 3, 5 and 7 days of incubation based on the diameter of the zone of growth of Foc TR4.

The following test treatments and their respective rates were used in the bioassay to test its efficacy against Foc TR4 *in vitro*.

1. Dibromo-3- nitrolopropionamide (10 ml/L of sterile distilled water)
2. Dibromo-3- nitrolopropionamide (20 ml/L of sterile distilled water)
3. Dibromo-3- nitrolopropionamide (30 ml/L of sterile distilled water)
4. Tebuconazole +Trifloxystrobin (1 g/L of sterile distilled water)
5. Tebuconazole +Trifloxystrobin (2 g/L of sterile distilled water)
6. Tebuconazole +Trifloxystrobin (3 g/L of sterile distilled water)
7. Propineb (3 g/L of sterile distilled water)
8. Benomyl (3 g/L of sterile distilled water)
9. Timorex Gold (*Melaleuca alternifolia* plant extract) (0.8 ml/L of sterile distilled water)
10. FPJ (20 ml/L of sterile distilled water)
11. LABS (20 ml/L of sterile distilled water)
12. IMO (20 ml/L of sterile distilled water)
13. FPJ+LABS+IMO (1:1:1) (20 ml/L of sterile distilled water)
14. Control (Sterile Distilled Water)

Efficacy Test of Chemical and Organic-Based Control Formulations Against Foc TR4 *in vivo*

The study on the efficacy of different test treatments for the control of Foc TR4 was carried out using the Randomized Completely Block Design (RCBD) replicated four times with five sample plants per replication per treatment. For the chemical fungicide, only the lower but effective rate per liter of water was used. The two chemical treatments such as Dibromo-3-nitrolopropionamide at 10 ml/liter of water and Tebuconazole Trifloxystrobin at 1 g/liter of water at lower but effective rates were used for further evaluation *in vivo*. Moreover, the treatment IMO and IMO+FPJ+LABS were also included in the *in vivo* evaluation for organic-based control formulations. Though its efficacy was not demonstrated *in vitro*, its effectiveness might be indirect to the pathogen and might be enhanced upon application onto the soil as a drench, and was therefore included in the treatments.

Planting and Maintenance of Test Plants

Tissue-cultured Lakatan plantlets were individually planted in polyethylene bag (10 x 12 inches in size) filled with sterile ordinary garden soil. Application of complete fertilizer (14-14-14) at the rate of 5 g/pot was done in each individual plant 10 days after planting. Maintenance and care of experimental plants like watering and removal of weeds and dead leaves were also done regularly.

Preparation of Inoculum and Inoculation

A two week-old pure culture of Foc TR4 was used for inoculation. Conidia were collected by adding 10 ml of sterile distilled water in the culture, rubbing a brush lightly over the colonies and subjecting the suspension to constant agitation for spore liberation. The spore concentration was determined using a hemacytometer, and diluted with sterile distilled water to a final concentration of 40,000 ± spores/per ml. A month-old test plants were inoculated with 250 ml/pot of spore suspension by drenching method. Prior to inoculation, two root hairs of each seedling were broken using teasing needle and the spore suspension was poured into the test plants. Inoculated roots were covered with soil.

Application of Treatments

Treatments were applied by drenching into the soil medium at 250 ml/pot on five days after the inoculation. Subsequent application of treatments was done late in the afternoon at four cycles of application in seven (7) days interval. A total of 1 L volume of each formulations was applied per plant in all treatments.

Data gathered

Diameter Zone of Growth (DZG)

The mycelial growth of Foc TR4 was measured after two, five and seven days of incubation. The degree of effectiveness of the treatments was based on the following arbitrary rating scales of Gabio et al. (2012).

DZG (mm)	*Degree of Effectiveness*
0	Very Effective (VE)
1- 20	Effective (E)
20.1- 40	Moderately Effective (ME)
40.1- above	Not Effective (NE)

Days to Symptom Appearance (DSA)

The data on days to symptoms appearance were taken by counting the number of days when the characteristic symptoms of Fusarium wilt first appeared on test plants after inoculation.

Percentage Disease Infection (PDI)

Percent infection of Fusarium wilt in all treatments was calculated using the equation below.

$$PDI = \frac{\text{Number of infected plant units}}{\text{Total number of plant units assessed}} \times 100$$

Disease Severity (DS)

Disease severity of Fusarium wilt as affected by the different treatments *in vivo* was determined using a standard evaluation system. The data were estimated by cutting the pseudostem open with a scalpel and assigning a disease severity rating to each plant in each treatment according to INIBAP Technical Guidelines by Carlier et al. (2002).

Rating the internal symptoms caused by Fusarium wilt was according to the following scale:

0 = Corm completely clean, no vascular discoloration
1 = Isolated points of discoloration in vascular tissue
2 = Discoloration of up to one-third of vascular tissue
3 = Discoloration in between one-third and two thirds of vascular tissue
4 = Discoloration of greater than two-thirds of vascular tissue
5 = Total discoloration of vascular tissue

Disease index (DI) was calculated using the formula proposed by Kranz (1988):

$$DI = \frac{\sum (a \times b)}{N.Z} \times 100$$

where:

$\sum (a \times b)$ = Sum of the symptomatic plant and their corresponding score scale
N = Total number of sampled plant
Z = Highest score scale (maximum number of infection category)

Degree of Control (DC)

Percentage degree of control of different treatments *in vivo* for the management of Fusarium wilt in banana was determined using the formula:

$$\% \text{ Degree of Control} = \frac{\% \text{ DI Untreated} - \% \text{ DI treated}}{\% \text{ untreated}} \times 100$$

Degree of Control (%)	*Degree of Efficacy*
81 above	Very Effective
61- 80	Effective
41- 60	Moderately Effective
21- 40	Less Effective
20- below	Not effective

Data Analysis

All treatment means were analyzed by ANOVA and treatment means were compared using Tukey's test (pairwise comparison) of Statistix version 10.

Results and Discussion

Evaluation of Chemical and Organic-Based Formulations Against Foc TR4 *In vitro*

The results on the evaluation of different chemical and organic-based formulations against Foc TR4 *in vitro* and their corresponding effectiveness rating after 7 days of incubation is shown in Table 1.

The mean diameter zone of growth (mm) of the pathogen as affected by the different test treatments differed significantly (Figure 2a and 2b). Chemical treatments such as Dibromo-3 nitropropionamide and Tebuconazole + Trifloxystrobin at different levels completely inhibited the growth of Foc TR4. Nil growth of the pathogen was observed in 2, 5 to 7 days of incubation, and all were rated very effective (VE).

On the other hand, chemical fungicides, such as propineb and benomyl as well as the organic-based control formulations, such as Timorex, IMO and FPJ+ LABS+ IMO reduced the growth

Table 1. Mean diameter zone of growth (mm) of Foc TR4 as affected by the different chemical and organic-based test treatments at 2, 5 and 7 days after incubation.

Treatment	Diameter zone of growth (mm)[1]			Degree of effectiveness[2]
	2 Days	5 Days	7 Days	
1. Dibromo-3- Nitrolopropionamide (10 mL/L)	0.00^{d}	0.00^{e}	0.00^{c}	VE
2. Dibromo-3- Nitrolopropionamide (20 mL/L)	0.00^{d}	0.00^{e}	0.00^{c}	VE
3. Dibromo-3- Nitrolopropionamide (30 mL /L)	0.00^{d}	0.00^{e}	0.00^{c}	VE
4. Tebuconazole +Trifloxystrobin (1 g/L)	0.00^{d}	0.00^{e}	0.00^{c}	VE
5. Tebuconazole +Trifloxystrobin (2 g/L)	0.00^{d}	0.00^{e}	0.00^{c}	VE
6. Tebuconazole +Trifloxystrobin (3 g/L)	0.00^{d}	0.00^{e}	0.00^{c}	VE
7. Propineb	12.42^{bc}	25.83^{cd}	40.92^{b}	NE
8. Benomyl	12.58^{bc}	19.08^{d}	36.83^{b}	ME
9. Timorex (0.8 mL/L)	12.75^{bc}	28.33^{c}	43.00^{b}	NE
10. FPJ (20 mL/L)	15.00^{ab}	50.25^{a}	71.58^{a}	NE
11. LABS (20 mL/L)	14.67^{abc}	49.75^{a}	67.92^{a}	NE
12. IMO (20 mL/L)	15.58^{a}	40.58^{b}	44.00^{b}	NE
13. IMO+FPJ+LABS (20 mL/L)	12.17^{c}	41.33^{b}	44.17^{b}	NE
14. Control (Sterile distilled water)	16.58^{a}	54.25^{a}	71.25^{a}	NE

[1]Means followed by a common letter are not significantly different at 1 % level using Tukey's Test.
Legend:
[2]VE- Very Effective (0 growth); E- Effective (0.1- 20 mm); ME- Moderately Effective (20.1- 40); NE- Not Effective (40.1 and above).
IMO- Indigenous Microorganisms; FPJ- Fermented Plant Juice; LABS- Lactic Acid Bacteria Serum

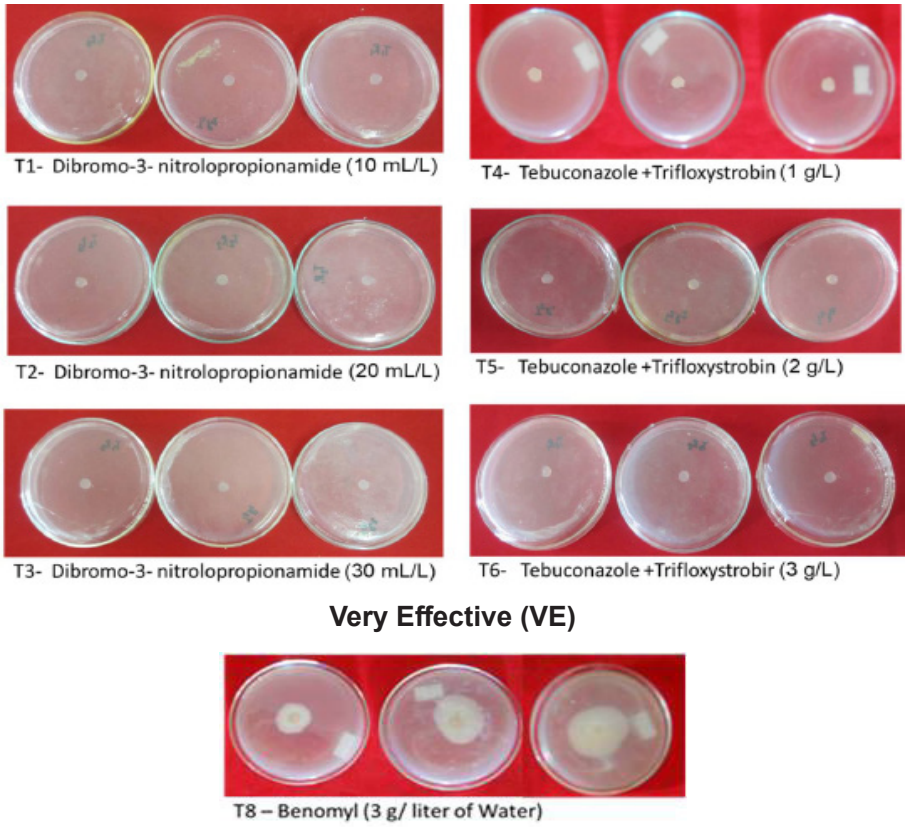

Figure 2a. *In vitro* assay of test treatments which were rated very effective (VE) and moderately effective (ME) againts the pathogens Foc TR4.

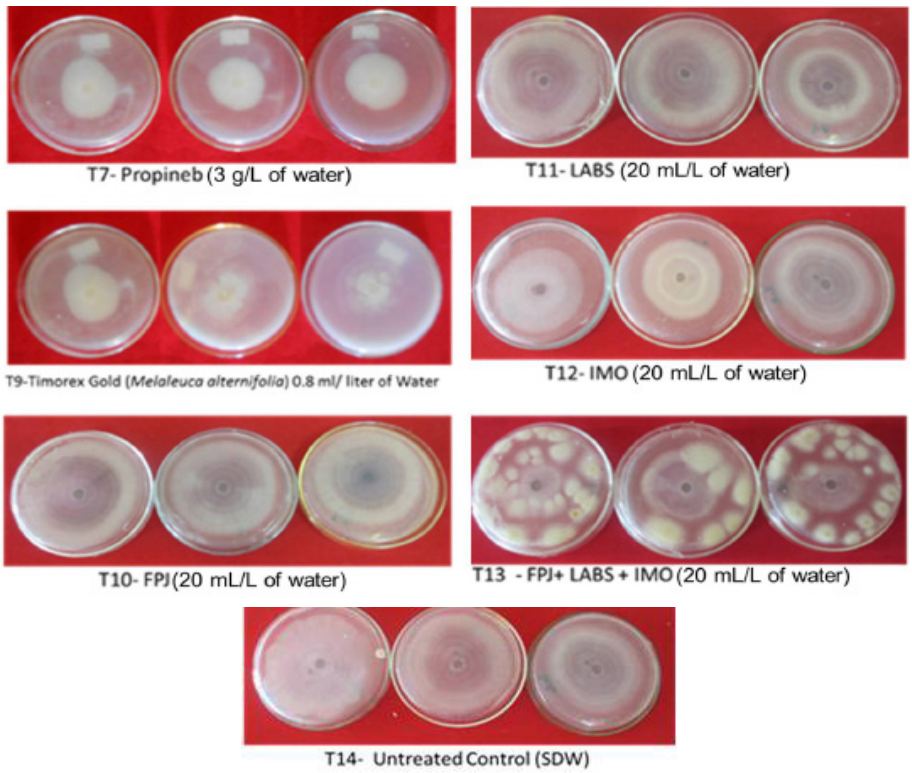

Figure 2b. *In vitro* assay of test treatments which were rated not effective (NE) againts the pathogens Foc TR4.

of Foc TR4 with a DZG mean ranging from 36.83 to 44 mm, but were rated not effective (NE) except for Benomyl which was rated moderately effective (ME). No significant reduction in the growth of Foc TR4 was observed as affected by FPJ and LABS with comparable DZG mean to the untreated control (SDW) ranging from 67.92 to 71.58 mm and were rated not effective (NE).

Evaluation of Chemical and Organic-based formulations against Foc TR4 *in vivo*

The effects of chemical and organic-based control formulations against Foc TR4 on the days to symptom appearance, percentage and severity infection and the degree of effectiveness and control of test treatments are shown in Table 2.

Significant differences were observed among treatment means. Results revealed that chemical fungicides Dibromo-3-nitrolopropionamide and Tebuconazole +Trifloxystrobin (1 g/L) completely suppressed the Foc TR4 pathogen and caused nil infection in Lakatan after four cycles of treatment application. Both treatments showed 100% degree of control and were both rated as very effective (VE). Meanwhile, the two organic-based control formulations, such as IMO and combination of IMO+ FPJ+ LABS were found not effective (NE). However, IMO+ FPJ+ LABS delayed the days to symptom appearance of Fusarium wilt at three days, reduced the severity of infection and percentage infection with 42% and 87.50% as compared to untreated control with 50% severity infection and 100% percentage infection, respectively. The effect of IMO was comparable to the untreated control with 100% severity and percentage infection and 2% degree of control. The untreated control inoculated with Foc TR4 exhibited typical Fusarium wilt symptoms such as yellowing and pseudostem splitting after 28 days of inoculation (Figure 3). The internal symptoms of the infected Lakatan banana test plants included vascular discoloration in the corm (Figure 4).

The above results suggested the

Table 2. Mean number of days to symptom appearance, percentage and severity of infection of Fusarium wilt as affected by the two chemical fungicides and organic-based control formulations and the degree of effectiveness and control of test treatments[1]

Treatments	Days to Symptom Appearance**	Percentage of Infection**	Severity of Infection**	Degree of Control (Dc)	Degree of Effectiveness[2]
Dibromo-3-nitropropionamide (10 ml/L)	00.00^{c}	00.00^{c}	00.00^{c}	100.00	VE
Tebuconazole +Trifloxystrobin (1 g/L)	00.00^{c}	00.00^{c}	00.00^{c}	100.00	VE
IMO	28.75^{b}	100.00^{a}	49.00^{ab}	2.00	NE
IMO+FPJ+LABS	31.00^{a}	87.50^{b}	42.00^{b}	16.00	NE
Untreated Control	28.00^{b}	100.00^{a}	50.00^{a}	00.00	NE

** highly significant.

[1]Data based on the average of five plants per treatment per replicate. Means followed by a common letter are not significantly different at 1 % level using Tukey's Test.

Legend:

[2]VE- Very Effective (81% above DC); E- Effective (61- 80 % DC); ME- Moderately Effective (41- 60% DC); LE- Less Effective (21-40% DC); NE- Not Effective (20% below DC).

IMO-Indigenous Microorganisms; FPJ-Fermented Plant Juice; LABS- Lactic Acid Bacteria Serum

Untreated Control: No treatment applied but inoculated with Foc TR4

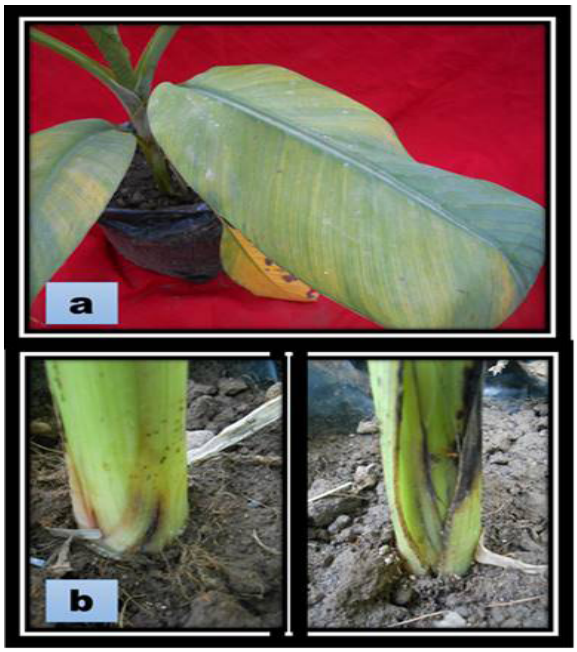

Figure 3. Yellowing of leaves (a) and splitting of pseudostem (b) 35 days after inoculation with Foc TR4 in Lakatan banana.

Figure 4. The experimental plants and the longitudinal section of the pseudostem of Lakatan bananas after four cycles of application of the two chemical fungicide and organic-based control agents against Foc TR4 showing healthy and corm discoloration.

effectiveness of chemical fungicides Dibromo-3-nitropropionamide (10 ml/L) and Tebuconazole +Trifloxystrobin (1 g/L) both *in vitro* and in nursery conditions. Dibromo-3-nitropropionamide or DBNPA is an industrial broad spectrum biocide for the control of algae, bacteria, and fungi (Dow Chemical Company, 2013). The efficacy of the above fungicide to control plant disease, particularly, the citrus greening caused by *Candidatus liberibacter* asiaticus was demonstrated by Zhang et al. (2010). Furthermore, the chemical was also found out to destroy cellulosic microorganisms responsible for the deterioration of papers (Roman et al., 2013). It could therefore be used to enhance paper preservation. The above findings therefore demonstrated the potential of the DBNPA as an effective disinfectants and soil drench to prevent further spread of Foc TR4 pathogen.

On the other hand, Tebuconazole+ Trifloxystrobin, is a broad spectrum fungicide used to treat pathogenic and foliar plant fungi. This fungicide is a potent inhibitor of fungal spore germination with high levels of activity against many fungal pathogens within the Ascomycete, Deuteromycete, Basidiomycete, and Oomycete classes (US- EPA, 1999).

The above results conformed with the findings of Gabio et al. (2012), that Triadimenol+Tebuconazole, the same chemical group in the treatment used in this study, was effective against Foc TR4 in Cavendish banana both *in vitro* and *in vivo*/nursery conditions applied in leaf axil and as a soil drench at the rate of 1 ml/L.

Conclusion

The chemical fungicides tested such as the Dibromo-3-nitropropionamide (10 ml/liter of water) and Tebuconazole+Trifloxystrobin (1 g/L of water) were found effective for the control of Foc TR4 *in vitro* and in nursery conditions. Organic-based control formulations such as Indigenous Microorganisms (IMO) and the combination of Indigenous Microorganisms (IMO)+Fermented Plant Juice (FPJ)+Lactic Acid Bacteria Serum (LABS) were found not effective against the pathogen. The effectivefungicides therefore could be used as disinfectant and soil drench to control and prevent the further spread of the pathogen in other banana production areas where Foc TR4 was usually absent particularly in nearby municipalities or provinces. The use of chemical fungicides such as Dibromo-3-nitropropionamide and Tebuconazole+Trifloxystrobin as disinfectants or soil drench is highly recommended to be part of the tactics in the implementation of integrated disease management. The efficacy of these fungicides under field condition could also be carried out.

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