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Antagonistic Activity of *Metarhizium anisopliae* (Metschnikoff) Against Phytopathogenic *Fusarium oxysporum* f. sp. *cubense* (Schlecht.) as a Biological Control

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ABSTRACT

The search for the most effective, organic, biological control against fungal-related diseases of economically-important crops such as in banana is still being encouraged in the Philippines. This study compared the antagonistic activity of beneficial endophytic fungi, *T. viride* and entomopathogenic fungi, *M. anisopliae* against *F. oxysporum* (causative agent of Banana Wilt disease) using dual culture method for 10 days incubation period. The study employed Complete Randomized Design (T_1= *T. viride vs. F. oxysporum;* T_2= *M. anisopliae vs. F. oxysporum;* and control plate = *F. oxysporum* alone*)* in five replications. Radial growth and percent inhibition of radial growth (PIRG), as well as Bell rating across treatment groups, were analyzed using ANOVA ($p \leq 0.05$) and T-test ($p \leq 0.05$), respectively. Results showed that both biological control fungi (*T. viride* and *M. anisopliae)* manifested antagonistic activity against *F. oxysporum,* where *T. viride* registered stronger inhibition (PIRG=72.37%; Bell Rating=2) than *M. anisopliae (*PIRG=31.27%; Bell Rating=3). The superior antagonistic action of *T. viride* and *M. anisopliae* against *F. oxysporum* may be attributed to their production of hydrolytic enzymes which facilitated growth inhibition of *F. oxysporum*. To observe stable antagonistic interaction among these fungi, future studies may consider longer incubation period as well as *in vivo* field trial on infected banana crops.

Keywords*: antagonistic assay, T. viride, M. anisopliae, F. oxysporum, biological control, antifungal property*

INTRODUCTION

The attainment of increased crop yield and improved harvest quality are two of the most important goals of the Philippines' Department of Agriculture [DA]. Such mandate ensures that the country's production of staple crops, as well as those important crop commodities for export, are safeguarded by good agricultural practices, program sustainability and farmer's education and training (Department of Agriculture [DA], 2010). The realization of such mandate helps attain the UNESCO's Sustainable Development Goal [SDG] No.1 on Zero Hunger in the Philippines, and for the world's population as well (National Economic Development Authority [NEDA], 2014). Despite these initiatives and programs, one of the most inevitable challenges of sustainable agriculture in the Philippines is on the management of pest, bacterial and fungal diseases like *Fusarium oxysporum*, the main causative agent of Fusarium wilt disease in banana (Food and Agriculture Organization [FAO], 2017.

The Philippines is one of the top exporters of high-quality banana in the world (Rodeo, 2016; Bathan and Lantican, 2010). This high demand is attributed to its high nutritive value and affordability compared to other fruits (Rivera, 2004). At least 80 known banana cultivars (i.e., Cavendish, Lakatan, Señorita, Saba, Cardaba, Latundan, bungulan, among many others) are grown among Philippines' agricultural lands for domestic and international markets (Bathan and Lantican, 2010). In 2015, banana ranked first in terms of production volume among the major fruit crops commercially grown in the country (Rodeo, 2016) and in the first quarter of 2016, the volume of banana production reached 4.26 million metric tons (PSA, 2016). Prior to this

production record, banana production has been dramatically declining since 2010 due to the effect of extreme weather phenomena such as El Niño, strong typhoons as well as the spread of Fusarium wilt disease. These factors caused a shortage in production and reduced yield, particularly the export variety, Cavendish banana. The low output of banana production placed the Philippines at an economic loss (50% drop) especially in its export earnings because the importing countries implement its import restrictions, as well as tightened sanitary and phytosanitary measures through import bans. Up until the present time, the continuous spread of Fusarium Wilt disease is still unresolved because no effective eradication method exists.

A highly destructive disease that targets the vascular system of banana is called Fusarium wilt (a.k.a. Panama Disease) which is caused by a soil-borne fungus *Fusarium oxysporum* f. sp. cubense (Tropical race 4). Pathogenicity of *F. oxysporum* involves invasion of vascular tissue in the host plant resulting in the reduction of water-holding capacity, wilting and eventually death (Ploetz 2006). The use of synthetic chemicals to control its spread is no longer effective against the pathogen because once the cultivar is infected with a pathogen, soil cannot be planted with the infected rhizomes for years. Intensive use of fungicides for the control of diseases has also resulted in an accumulation of toxins in human beings as well as to the environment. Hence, interest has been developed for safer non-chemical methods to control a disease that is effective and causes less risk to human health and the environment. Synthetic fungicides replacement with bio-control agents is an alternative means to control the plant pathogens, produce safety food and reduce environmental pollution.

The search for biological control against *Fusarium oxysporum* continues

The wide-ranging occurrence of pathogenic *F. oxysporum* strains is attributed to the pathogen's host species' specificity. Individual isolates cause disease in a narrow range of plant species, thus there are approximately 40 *forma specialis* (f. sp.) of *F. oxysporum*, grouped and defined from a physiological standpoint and the ability of each f. sp. to cause disease in a specific host (Kistler 2001). The number of antagonistic fungi as a biological control against pathogenic fungi is few, with only *Trichoderma viride* and *Ampelomyces quisqualis* as the most common. Thus, the trend now is to explore fungal species that have multiple hosts, such as *Metarhizium anisopliae* that originally attacks insects, but could also be used as biofungicide as well.

M. anisopliae is an entomopathogenic fungus that causes disease in various insects by acting as a parasitoid. It is being used as a biological control to a number of pests such as locust, termites, caterpillars, aphids, wireworms and even mosquitoes (Roberts and St. Leger, 2004; Faria and Wright, 2001; Kabaluk and Ericsson, 2007; Zimmerman, 1992; Zimmerman, 1993). The fungus has been recognized to be an environmentally-safe alternative to synthetic pesticides (Zimmerman 1986). In the last 10 years, few studies evaluated the interaction of *M. anisopliae* against plant phytopathogenic fungi. Soil-isolated *M. anisopliae* promotes growth in tomatoes by acting as an endophytes (Garcia et al., 2011), increased rhizosphere competence in cabbage (Hu and Leger, 2002) and European spruce (Bruck 2005). Meanwhile, very few studies reported the antifungal activity of *M. anisopliae* against plant fungal pathogens. While Chul et al., (1997) investigated the antagonistic activity of *M. anisopliae* against *Botrytis cinerea, Alternaria solani* and *Fusarium oxysporum,* Ravindran et al., (2014) evaluated antifungal activity of *M. anisopliae* against *Cladosporium herbarium, Curvularia clavata* and *Fusarium oxysporum.* Both of these studies listed *F. oxysporum* as a test organism, but there is no mention that the *forma specialis* (f. sp.) was cubense. This information may suggest that both papers did not isolate the strain from a banana wilt diseased plant, and such a research gap is an interesting avenue to investigate, especially that banana wilt disease is now reportedly causing massive damages among tropical varieties today.

This paper evaluates the antagonistic activity of entomopathogenic fungi *Metarhizium anisopliae* against the phytopathogenic fungi *Fusarium oxysporum* f.sp. cubense that causes banana wilt disease. This paper also compared the antagonistic potential of *M. anisopliae* on *F. oxysporum* vs. the most commonly used, efficient fungicide, *Trichoderma viride.*

Specifically, this study aims to answer the following questions: (1) what is the average radial growth (mm) of *F. oxysporum* alone, and in the

presence of 2 fungal antagonists (*T. viride and M. anisopliae*)? (2) What is the average percentage of inhibition of radial growth (PIRG) and Bell rating of the different treatments containing fungal antagonists (*T. viride and M. anisopliae*) against *Fusarium oxysporum?* and (3) Is there a significant difference in the Bell rating and the percent of Inhibition of radial growth (PIRG) between two antagonists against *Fusarium oxysporum*?

MATERIALS AND METHODS

Experimental design

The present study employed Complete Randomized Design (CRD) with equal replications. The control group (represented by **C**) is represented by *Fusarium oxysporum*, without an antagonist. Two treatments were utilized, as follows: **T1:** *Trichoderma viride* vs. *Fusarium oxysporum* and **T2:** *Metarhizium anisopliae* vs. *Fusarium oxysporum*, in 5 replicates (Table 1).

Table 1. Experimental design showing the control group and experimental groups, as well as their corresponding replications and codes.

Treatment	Code	No. of replications (5)	Legends (Treatment+ Replicate)
Control (*F. oxysporum* only, no antagonist fungi)	C	R1	C1R1
		R2	C1R2
		R3	C1R3
		R4	C1R4
		R5	C1R5
Treatment 1 (*T. viride* vs. *F. oxysporum*)	T1	R1	T1R1
		R2	T1R2
		R3	T1R3
		R4	T1R4
		R5	T1R5
Treatment 2 (*M. anisopliae* vs. *F. oxysporum*)	T2	R1	T2R1
		R2	T2R2
		R3	T2R3
		R4	T2R4
		R5	T2R5

Note: *Each replicate is represented by one (1) Petri dish.*

Assignment of the control group and 2 treatments to each Petri plates were done randomly, through a draw by lot method. Experimental endpoints (i.e. dependent variable) refers to the degree of inhibition or antagonistic interaction with the two fungi in the plate, measured in mm. Prior to the actual experiment, two trials were conducted to optimize the methods, minimize contamination and ensure that the experiment will proceed as stated in the literature. In both trials, similar experimental designs and replications were used.

Research environment

All experiments (isolation, fungal culture, and antagonistic evaluation) were performed at the Fungi Microbiology Laboratory, Department of Agriculture 7– Regional Crop Protection Center in Mandaue City, Philippines.

Source of fungal isolates

With the assistance and supervision of a resident plant pathologist in DA RCPC Region 7, the researchers' cultured pure isolates of fungal species used in the experiment. For both fungal antagonists, *T. viride* and *M. anisopliae*, they were sub-cultured from the available pure culture kept and grown in the RCPC Lab. For the fungal pathogen, *F. oxysporum*, it was cultured from banana and was kept in the RCPC lab 1 week prior to use. Isolates were verified by resident mycologists from RCPC lab. Culturing techniques appropriate to each fungus were adopted, with modifications from standard growth protocols of Talapatra et al., (2017), Siametro et al., (2010) and Zimmerman (1993).

Screening by Dual Culture Method

Once the individual culture became available, the dual culture method of Huang and Hoes (1976) (i.e. this culture method is used to tests fungal antagonism, *in vitro*) was prepared (**Fig.1**), and briefly described as follows: 2L of distilled water was poured into the beaker with 78g PDA at 45º-50°C, mixed and melted until it was totally dissolved and, 400 mL of PDA solution was transferred into 500mL Erlenmeyer flask and allowed to cool. Cooled PDA solution was poured into sterilized Petri dishes, half-full and was allowed to solidify before inoculated by the fungi under study. Mycelial disc (6 mm) was cut from the margin of the actively growing colonies of test culture (*Metarhizium anisopliae* and *Trichoderma viride*) and was individually placed near the periphery in one side of the PDA plate. Another disc of 6mm of pathogenic culture (*Fusarium oxysporum*) was placed on the other side of the same plate first opposite to the first disc (see Figure 1).

All pairings were incubated at 28ºC – 30ºC and antagonistic activity was tested after 10d of incubation by measuring the radius (i.e. radial growth) of the *F. oxysporum* colony in the direction of the antagonist colony (**R2**, Fig.1) and the radius of the *F. oxysporum* colony in the control plate (**R1**, Fig.1).

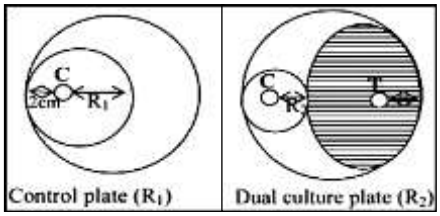


Figure 1. Measurement of radial growth of *F. oxysporum*. **R1**, radius of *F. oxysporum* colony in control plate; **R2,** radius of F. oxysporum colony in dual culture plate; **C,** *F. oxysporum isolate*; **T**, antagonistic fungi isolate (either *T. viride* or *M. anisopliae*)

The two readings (R1 and R2) were transformed into percentage inhibition of radial growth (PIRG) using the formula developed by Skidmore and Dickinson (1976):

$$\%IRG = \frac{(R1-R2)}{R1} x\ 100$$

Where:

R1= is the farthest radial distance grown by the pathogen without the antagonist (control)

R2= represents the radial distance grown by the pathogen in the direction to the antagonist (treatment)

Antagonistic levels based on PIRG was also interpreted following Thanh et al., (2014), as follows:

PIRG ≤ 50%	: Low
50% < PIRG ≤60%	: Medium
60% < PIRG ≤75%	: High
PIRG > 75%	: Very High

Similarly, the interaction between *F. oxysporum* and its antagonists were evaluated following the rating scale of Bell et al., (1982):

Table 2. Rubrics for Bell Rating (after Bell et al., 1982)

Rating	Description
1	the antagonist completely overgrew the pathogen and covered the entire medium surface
2	the antagonist overgrew the pathogen at least two-thirds of the medium surface
3	the antagonist and the pathogen each colonized one-half of the medium surface and neither organism appeared to dominate the other
4	the pathogen overgrew the antagonist at least two-thirds of the medium surface and appeared to withstand invasion by the antagonist
5	the pathogen completely overgrew the antagonist and occupied the entire medium surface

Data Analysis

Data collected for this study include (a) radial growth (mm) of *F. oxysporum* alone, and in combination with the studied antagonists, (b) percentage inhibition of radial growth (PIRG) and (c) Bell rating. Radial growth measured from 3 independent groups were analyzed using ANOVA, followed by Tukey's HSD test while PIRG and Bell rating were collected from 2 independent groups and were statistically analyzed using two sampled T-test. All data were analyzed using SPSS version 20 at $p \leq 0.05$ level of significance.

Risk and Safety

Prior to the actual conduct of the experiment, the researchers underwent microbiological training on aseptic techniques for fungal research at the RCPC, DA-7 Mandaue City. Caution was emphasized as to the responsible use of microorganisms for research purposes. Considering that the microorganisms involved were pathogenic only to plants and insects but not on humans, the RCPC Plant Pathology division ensured that there is no risk posed for humans during the experiment. However, since it may infect plants, it must be performed in a regulated research laboratory. Laboratory safety was emphasized to avoid inhalation of spores, while disposal of waste and chemicals used in the experiment followed standard laboratory protocol (e.g. autoclaving for 15min at >100ºC). Meanwhile, disposable Petri dishes were soaked in a disinfectant before disposal in a biological hazard bin.

RESULTS AND DISCUSSION

Combative interaction of the antagonists against the pathogenic *F. oxysporum*

Table 3 showed the radial growth of the pathogen *Fusarium oxysporum* without the antagonist – control plate (C); radial growth of the pathogen with *Trichoderma viride* (T1) and radial growth of the pathogen with the *Metarhizium anisopliae* (T2) after 10 days incubation. It can be observed that the results in the five replicates were very close to each other showing consistency of the experimental set-up both for T1 while for T2, only replicate T5R5 registered below 20mm. Results also showed that there was a significant difference (p=0.000) in the means of the radial growth of

F. oxysporum under the three (3) independent set-up, where exposure of *F. oxysporum* to *T. viride* was found to have lowest radial growth of the pathogen. This result suggests that between the two treatments, T1 is more superior in its combative action than T2, manifesting a strong potential as a biological control agent against *F. oxysporum*.

Table 3. Raw data of the radial growth (mm) of *F. oxysporum* exposed to *T. viride* and *M. anisopliae* after 10 days incubation.

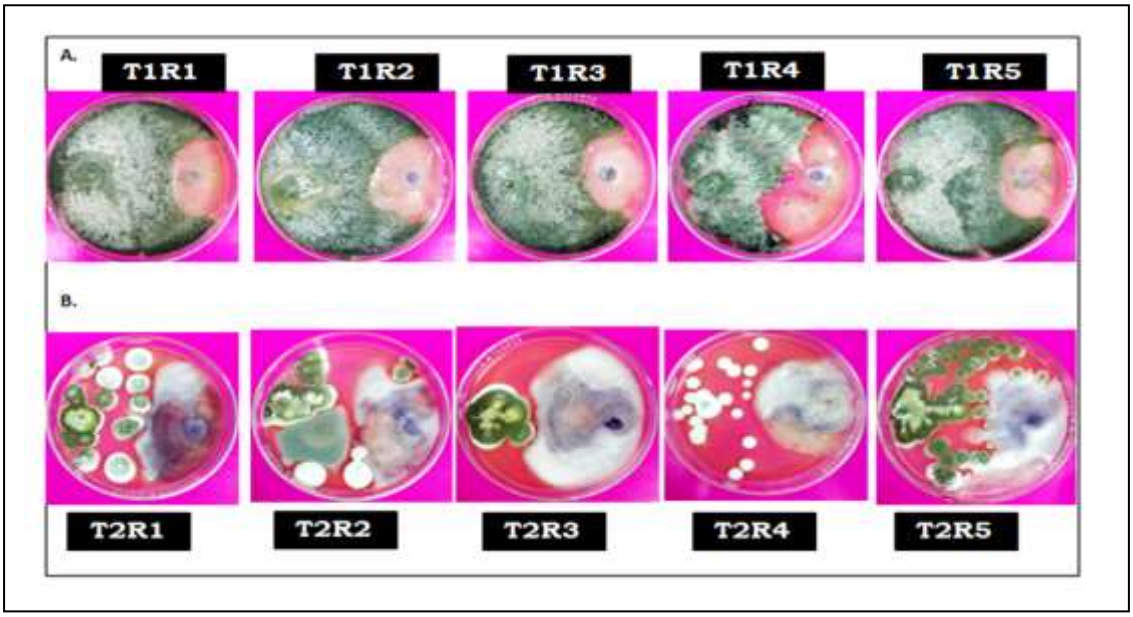
Replicates	Control Plate (mm)	*Trichoderma viride* (mm)	*Metarhizium anisopliae* (mm)	p value
1	32	10	21	0.000*
2	36	16	25	
3	40	9	34	
4	38	10	34	
5	44	6	15	
Mean±S.D. **	38.00±4.47[a]	10.20±3.63[c]	25.80±8. 29[b]	

Legend: *Control plate= F. oxysporum only*
** Statistically significant ANOVA result (p<0.05)*
*** Different letter superscript indicate statistical significance in Tukey's HSD (p<0.05)*

The degree of antagonism exhibited by *T. viride* and *M. anisopliae* versus the pathogen *F. oxysporum* is reflected in the Percent Inhibition of Radial Growth [RIPG] and Bell rating of the pathogen as confronted by the antagonists through the dual culture technique **(Table 4, Fig. 2).** In terms of percent inhibition of radial growth [PIRG], T1 (72.37%) registered a higher PIRG than T2 (31.27%), suggesting that T1 (*T.viride* vs. *F.oxysporum*) has a more pronounced inhibition against pathogen than T2. The result may also indicate that *T. viride* is a stronger biological control, statistically speaking, than *M. anisopliae* towards *F. oxysporum* (p=0.010) and registered a high level of antagonism to *F. oxysporum* (60% < PIRG ≤75%, Thanh et al., 2014). The combative action of *T. viride* agrees with the study of Siameto et al., (2010) on the antagonistic effect of *T. harzianum* on the mycelia growth of pathogenic fungi like *F. oxysporum f. sp. phaseoli*.

Table 4. Percent inhibition of radial growth (PIRG) and Bell rating of different antagonists (T1 = *T. viride* and T2 = *M. anisopliae*) against the pathogen, *F. oxysporum*

T1	PIRG (%)	Bell Rating	T2	PIRG (%)	Bell Rating	P value (p<0.05)
T1R1	68.75	2	T2R2	34.37	3	
T1R2	55.56	2	T2R2	30.56	3	
T1R3	77.50	2	T2R3	15.00	4	
T1R4	73.68	2	T4R4	10.53	4	
T1R5	86.36	1	T5R5	65.91	2	
Mean±S.D.	72.37±11.4			31.27±21.8		0.010*
Interpretation (PIRG)	High level of antagonism			Low level of antagonism		
Mean±S.D.		1.8±0.45			3.2±0.84	0.016*



**T-test significant at $p \leq 0.05$*

Figure 2. Comparative antagonistic interaction is shown in Treatment 1 (first row Petri dishes, A= *Trichoderma viride versus Fusarium oxysporum*) in five replicates and Treatment 2 (second row Petri dishes, B= *Metarhizium anisopliae versus Fusarium oxysporum*) in five replicates. Visual analysis shows the defined area occupied by *T. viridae* vs *F. oxysporum* (first row) vs dispersed colonies forming in *M. anisopliae,* but still able to manifest antagonism against *F. oxysporum* (see Table 4).

Bell rating for T1 registered a mean of 1.8 vs T2 (3.2). By interpretation, a Bell rating of ~2.0 indicates that the antagonist (*T. viridae*) outgrew the pathogen (*F. oxysporum*) by at least 2/3 of the medium surface, while in the case of T2, *M. anisopliae* outgrew *F. oxysporum* by at least ½ of the entire medium surface. These Bell rating results suggest that *T. viride* manifested a stronger antagonistic property than *M. anisopliae* (p=0.016). The results of the present study corresponds to the findings of previous works where *Trichoderma* species exhibited maximum inhibition and such behavior is attributed to its ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients (Siameto et al., 2010; Sharon et al., 2001).

Indigenous strains of *Trichoderma viride* were also investigated for its antagonistic activity by Naglot et al., (2015) against two important tea fungal pathogens namely *Pestalotia theae* and *Fusarium solani* and was found to also exhibit the reduction in the radial growth. Such interaction was due to extracellular antifungal metabolites that caused morphological changes such as hyphal swelling and distortion. In another study, Talapatra et al., (2017) purport that antagonistic activity of T. viride can be due to its suppression ability by producing hydrolytic enzymes or by the production of antibiotics that diffuses into cell walls that dissolves cell fragments of the host cells triggering more physiological changes and rapid growth inhibition.

T. viride inhibited the growth of the target organisms through its ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients. Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens. A second mechanism of pathogen control that *Trichoderma* displayed is mycoparasitism. Microscopic observation of the interaction region between *F. oxysporum* with *T. viride* or *M. anisopliae* showed that the mycelia of the antagonist fungi grew on the surface of the pathogens always coiling round their mycelia and later penetrating the cell walls directly without formation of appresorium structures. The pathogen's mycelia then disintegrate suggesting enzyme action Metcalf and Wilson (2001) and Sharon et al., (2001).

Until the present, there is no existing scientific study that reported pathogen overgrowth percentage of *M. anisopliae* against pathogenic fungi. However, insect pathogenic fungi *Metarhizium anisopliae* was recently explored for its antifungal activity against phytopathogenic fungi *Fusarium oxysporum, Cladosporium herbarum* and *Curvularia clavata.* Result of the study found that *Metarhizium anisopliae* reported the highest inhibitory activity against *C. herbarum* and lowest to *F. oxysporum* (Ravindran et al., 2014). The cited study attributed such antifungal action to some enzymatic mechanisms or toxic metabolites produced by the fungus (Jiang et al., 2002) specifically that among the secondary metabolites, destruxin (DTX) is physiologically active in killing other fungal species (Vey et al., 1982). In the study of Jia et al., (2016), compounded antagonistic effects were exhibited between entomopathogenic fungus *Metarhizium anisopliae* and insecticide chlorantraniliprole on host mortality and enzyme activities on the grasshopper. Given this scenario, *M. anisopliae* can be further explored by combining it with other endophytic fungi for its synergistic activity in controlling pathogens.

Meanwhile, the differences in the mycelia growth of the two antagonistic fungi in this present study clearly show the distinct actions exhibited on *F. oxysporum* such that *T.viride* is more active than *M. anisopliae*. Such slow growth of the pathogen *F. oxysporum* made it also easily overpowered by the antagonist fungi as seen in **Fig. 2**. Entomopathogenic fungi such as *M. anisopliae* require long periods to induce sufficient insect mortality (Jia et al., 2016) thus may also exhibit the same growth pace (i.e. slower growth) on the potato dextrose agar to interact with the pathogen, *F. oxysporum.*

CONCLUSION

In this *in vitro* test of the endophytic fungi *Trichoderma viride* and entomopathogenic *Metarhizium anisopliae* against disease-causing Banana wilt pathogen *Fusarium oxysporum*, antagonistic activity was observed. However, between the two antagonists, *T. viride* was found to manifest a higher level of antagonism than *M. anisopliae* as evidenced by lower radial growth of *F.oxysporum* in the presence of *T. viride*, as well as a higher level of antagonism (PIRG) and lower Bell rating of *T. viride* than *M. anisopliae.*

Although *M. anisopliae* exhibited a lower level of antagonism than the commonly used biological control, *T. viride*, it still exhibited a considerable extent of inhibition against the *F. oxysporum*. This may suggest that in the absence of *T. viride*, an alternative fungal biological control (*M. anisopliae*) which may also exhibit antagonistic activity against *F. oxysporum* can be utilized by farmers.

RECOMMENDATIONS

The following recommendations are hereby proposed to fully understand the mechanism of antagonism in these fungal species:

1. Further isolation of endophytes from sheaths, leaves, and roots of economically important crops like banana should be conducted to search for more isolates with high antagonistic potential.
2. For *in vivo* experiments, this study suggests that long time exposure of the endophytes or antagonists to the host plant must be assessed to ensure that the endophytes or antagonists will have already established its growth into the host plant.
3. Other inoculation methods of the endophytes or antagonists should be done to fully assess their antagonistic potential towards other phytopathogenic species like *F. oxysporum* in the greenhouse to prove results obtained in the laboratory.
4. Studies regarding the required or optimal amount of inoculant of the antagonists in greenhouse assays must be conducted.

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