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Mycofumigation with the endophytic fungi *Fusarium proliferatum* (Matsushima) Nirenberg and *Diaporthe* sp. for the control of banana and mango anthracnose

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ABSTRACT

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Banana and mango are the top commodities in the Philippines that are traded worldwide however, fungal diseases such as anthracnose affect their yield and quality. Environmentally sound control strategies have been explored and one of these is mycofumigation using fungal endophytes. This alternative approach is understudied in the Philippines, hence, the study aimed to evaluate the mycofumigation potential of fungal endophytes collected from Mt. Makiling, Luzon, Philippines and their pathogenicity to banana and mango fruits. In vitro and in vivo mycofumigation assays were conducted with a completely randomized design layout with triplicates per treatment under laboratory conditions.

The fungal endophytes used as mycofumigants, *Diaporthe* sp. and *Fusarium proliferatum,* were comparatively effective in controlling the anthracnose of banana and mango. The mycelial growth for in vitro assay of *Colletotrichum musae* and *Colletotrichum gloeosporioides* were significantly reduced compared to the control when exposed to volatile organic compounds produced by the fungal endophytes. In addition, the results of the in vivo mycofumigation assay against *C. musae* showed *Diaporthe* sp. had a significantly higher inhibition rate (93%) than *F. proliferatum* (67%) when grown on potato dextrose agar plates.

The bioefficacy of the *Diaporthe* sp. and *F. proliferatum* was improved when grown on peanut and corn seed substrates with a reduction of banana and mango anthracnose severity ranging from 82 to 100%. Cultures grown on corn substrate performed better than those grown on peanut. Pathogenicity tests also revealed that these endophytic fungi did not cause disease in the banana

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or mango fruits indicating their potential as good biocontrol agents against *C. musae* and *C. gloeosporioides*.

Keywords: calcium nitrate, soil amendment, anthracnose, *Rhizopus stolonifer, Dioscorea rotundata*

INTRODUCTION

Banana (*Musa* spp.) and mango (*Mangifera indica* L.) are the leading agricultural commodities produced and traded in the Philippines. For exports, the Philippines ranked second (2019) in banana and seventh in mango (2015), with USD1.9 billion (13.2%) and USD91 million (4%) share of the global market, respectively (Workman 2019, UNComtrade 2016). Nevertheless, constant usage of synthetic fungicides is reported to have had carcinogenic impacts on agriculture workers, residual toxicity, ecological pollution, (Nicolopoulou-Stamati et al 2016) and the development of pathogen fungicide-resistance which was observed in Imazalil fungicides (Abdel-Rahim and Abo-Elyousr 2017).

The post-harvest quality of the banana and mango fruits are affected by disease-causing microorganisms such as *Colletotrichum* causing anthracnose which is the major pre- and post-harvest pathogen. It is successful as a post-harvest pathogen because of latent infections in which symptoms become apparent as the fruit ripens (Prusky 1996). Banana anthracnose caused by *Colletotrichum musae* (Berk. and M.A. Curtis) Arx results in 30-40% losses of marketable fruit (Ranasinghe et al 2003). On the other hand, *C. gloeosporioides* (Penzig) Sacc that causes mango anthracnose results in 30-60% yield losses of mango across different countries of the world (Chowdhury and Rahim 2009).

Hot water treatment and the application of synthetic fungicides such as benomyl, thiabendazole (TBZ), prochloraz, and imazalil are commonly practiced for controlling anthracnose diseases (Khan et al 2001, Nelson et al, Perdichizzi et al 2014, Mari et al 2007, Jinasena et al 2011). Nowadays, researchers are eager to discover alternative control measures that are economically viable and ecologically sound such as mycofumigation using endophytes. Mycofumigation is the use of volatile organic compounds (VOCs) produced by fungi to inhibit the growth of phytopathogenic fungi associated with post-harvest diseases. The mycofumigation is convenient since it has no direct contact between the antagonist and the plant product, easily diffuses in closed environments with no residues remaining on the plant product to be consumed. In addition, most of the antimicrobial volatile mixtures exhibit bioactivity against a wide range of microorganisms, including many phytopathogens associated with post-harvest diseases (Gomes et al 2015). Endophytes are any organisms (bacteria or fungi) present inside plant tissues that do not cause visible disease in the plant but show mutualistic, parasitic, and commensalistic relationship with the host through protecting plants against herbivores, insect attacks, or tissue invading pathogens (Singh et al 2011). They have been known as prominent sources of new bioactive constituents such as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenols, and lactones (Yu et al 2010). For instance, various valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic, and anticancer activities were discovered from endophytic fungi which have potential as mycofumigants (Zhao et al 2011).

Adiova (2019) isolated three promising endophytic fungi - *Tinctoporellus epimiltinus, Fusarium proliferatum* (Matsushima) Nirenberg and *Diaporthe* sp. from plants in Mt. Makiling, Luzon, Philippines, namely *Arcangelisia flava* (L.) Merr, *Macaranga grandifolia* (L.) and *Tetrastigma harmandii* Planch., that were effective in suppressing the growth of *C. musae* in vitro and in vivo. The antifungal property was due to the release of volatile compounds such as phenylethyl alcohol, beta-acorenol and acoradiene from *F. proliferatum;* 2-tridecanon and 1-decanol from *Diaporthe* sp. Few studies have been done to verify the mycofumigation potential of these fungal endophytes. Thus, the study aims to: (1) evaluate the effectivity of the endophytic fungi *F. proliferatum* and *Diaporthe* sp. in controlling banana and mango anthracnose *(*in vitro and in vivo*),* (2) assess the effect of seed substrates on the virulence of endophytic fungi, (3) test the pathogenicity of the endophytic fungi to banana and mango fruits.

MATERIALS AND METHODS

Isolation, Identification, and Maintenance of C. musae and C. gloeosporioides

C. musae and *C. gloeosporioides* were isolated from banana and mango fruits showing typical anthracnose symptoms. Tissue sections were excised from the advancing margin of a lesion including both diseased and healthy parts. The cut sections were surfaced sterilized by dipping in 10% sodium hypoclorite (NaOCl) (Critzer et al 2012) for 3min and rinsed three times in sterile distilled water. Tissue sections were blot dried on sterile filter paper and transferred equidistant onto potato dextrose agar (PDA) plates. After 3 days, when actively growing cells were present and suitable for isolation, the advancing mycelial growth was transferred to PDA slants and plates.

Mass Production of Endophytic Fungi

Endophytic fungi were obtained from the original pure cultures used by Adiova (2019) who isolated *Diaporthe* sp. and *F. proliferatum* from the stems of *Tetrastigram harmandii*, and *Macanga grandifolia,* respectively, which were collected from Mt. Makiling. The revived endophytic fungal isolates were maintained on potato dextrose agar (PDA) plates and incubated at room temperature.

In Vitro Mycofumigation Assay

The ability of the endophytic fungi to inhibit the growth of *C. musae* and *C. gloeosporioides* through the production of VOCs was tested using double-plate assay adopted from Reddy et al (2007) with minor modifications. The bottom plate of the freshly planted mycelial disc of the test pathogen was joined with the bottom plate of the 5-day-old culture of each endophytic fungi and sealed with tape. The control treatment was the test pathogen alone. The set-up was replicated three times per treatment and incubated until the growth of the control treatments covered the petri plate at the optimum of 7 days incubation. Daily measuring of the diameter of the pathogen was done as the average of two diameters for each replicate.

Percentage inhibition was determined following the formula:

$$\text{Percentage inhibition} = \frac{D_c - D_t}{D_c} \text{x} 100$$

where:

D_c = diameter of test pathogen in control treatment
D_t = diameter of test pathogen with endophyte

In Vivo Mycofumigation Assay

Using a micropippete, eight microliters from the adjusted conidial suspensions (10^6 conidia per mL) of *C. musae* were inoculated into wounded banana fruits (Adiova 2019) by dropping the inoculum into the wounded peel surfaces at 3 wounds per fruit. The wounding was done by pricking the fruit surfaces to a depth of approximately one half of the peel's thickness. After which, two fruits were placed separately into sterile polypropylene bags with five plates of 5-day-old cultures of endophytic fungi. The control treatments were uninoculated and inoculated with the test pathogen. The setup had triplicates per treatment and layout in a Completely Randomised Design (CRD). Lesion size per wound was determined and percentage inhibition was computed.

In Vivo Effect of Substrates on the Virulence of Promising Endophytic Fungi

Peanut, rice, and corn seeds were used as substrates for the two endophytic fungi as an alternative media to PDA for mass production. The fungi grown on these substrates were evaluated for the bioefficacy of their performance in controlling the anthacnose in vivo.The seed substrates were boiled for 10-15min. Each medium, weighing 40g was placed into Petri plates and autoclaved twice at 121°C for 30min. After cooling, each Petri plate with a substrate was inoculated with a mycelial disc (~5mm) of 5-day-old pure culture of the endophytic fungi. The fungal cultures were incubated until covering 70-90% of the plates prior to doing the same in vivo mycofumigation assay as previously described.

Pathogenicity Test of Endophytic Fungi in Banana and Mango Fruits

Surface sterilized unripe banana and mango fruits were used for the pathogenicity test of *Diaporthe* sp. and *F. proliferatum*. Mycelial agar discs (~5mm) from the periphery of 5-day-old cultures of *F. proliferatum* and *Diaporthe* sp. were placed on wounded and unwounded tissues. The fruits were incubated in polypropylene bags lined with moist tissue paper for 48h. Observation was done for 7 days after inoculation.

Data Analysis

All data from the experiments except for the pathogenicity test were subjected to Analysis of Variance (ANOVA) and means were compared by Tukey's Honest Significant Difference (HSD) test.

RESULTS AND DISCUSSION

In Vitro Mycofumigation Assay

The study assessed the antagonistic property of the two endophytic fungi against *C. musae* and *C. gloeosporioides* through double plate assay. The VOCs of the endophytic fungi had significant antimicrobial effect on the *C. gloeosporioides* and *C. musae* compared to the control. The endophytic fungi, namely *F. proliferatum* and *Diaporthe* sp. reduced the mycelial growth of *C. gloeosporioides* and *C. musae*. *C. musae* exposed to VOCs of *F. proliferatum* had smaller colony growth (74mm) than *Diaporthe* sp. (80mm) (Table 1). Nevertheless, *F. proliferatum* had comparable inhibition rate (16%) as compared with *Diaporthe* sp. (10%) against *C. musae*.

Table 1. Effect of volatile organic compounds of *Diaporthe* sp. and *F. proliferatum* on the colony growth of *C. musae* and *C. gloeosporioides*

Treatments	Colony growth (mm)		Percent inhibition	
	C. musae	*C. gloeosporioides*	*C. musae*	*C. gloeosporioides*
Pathogen alone	89.0 ^{b}	63.0 ^{a}	0.0 ^{a}	0.0 ^{a}
Co-cultured with *Diaporthe* sp.	80.0 ^{ab}	52.0 ^{a}	10.0 ^{b}	17.0 ^{b}
Co-cultured with *F. proliferatum*	74.0 ^{a}	49.0 ^{a}	16.0 ^{b}	22.0 ^{b}
Pr(>F)	0.0298	0.0298	0.7462	0.8775

Means followed by the same letter are not significantly different at α=0.05

On the other hand, the mean colony growths of *C. gloeosporioides* sub-cultured with the two fungal endophytes were similar with the control (Table 2). *F. proliferatum* showed a higher inhibition rate (22%) on the growth of *C. gloeosporioides* than *Diaporthe* sp. (17%). Although *Diaporthe* sp. and *F. proliferatum* inhibited the growth of *C. gloeosporioides*, there were no significant differences in the mean percent inhibition between the two treatments. According to Cumagun et al (2019), the volatile compounds that possibly inhibit the growth of the *C. musae* were phenylethyl alcohol, beta-acorenol and acoradiene in *F. proliferatum* and 2-tridecanon and 1-decanol in *Diaporthe* sp. The study was also in consonance with Strobel et al (2001) in which the VOCs produced by the endophytic fungus, *Muscodor albus* inhibited the germination of the teliospores of *Tilletia horrida, T. indica*, and *T. tritici* (pathogenic fungi that cause the plant diseases rice kernel smut, wheat kernel bunt and wheat common bunt, respectively). The VOC molecules produced from *M. albus* were 1-butanol and 3-methyl-acetate that reduced the growth of *Cercospora beticola, Fusarium solani, Pythium ultimum, Rhizoctonia solani, Sclerotinia sclerotiorum, Tapesia yallundae*, and *Xylaria* sp.

In Vivo Mycofumigation Assay

Seven days after inoculation, the two endophytic fungi significantly reduced the anthracnose disease severity in banana fruits compared to the control. *Diaporthe* sp. (Table 3) showed a better performance, with a mean lesion size of 14mm^{2} and

inhibition of 93%, compared with *F. proliferatum* with 74mm^2 lesion size and 67% inhibition. The inhibiton may be due to the presence of the VOCs identified by Cumagun et al (2019), which reduced the progression of anthracnose in the banana fruits. The VOCs may affect the sporulation of the pathogen that inhibited the occurrence of the disease. Reports stated by Gao et al (2017) that the VOC, 2-tetradecanone from endophyte strain ZSY-1, affected the spore synthesis system of *Alternaria solani* and has the potential for controlling tomato grey mold and early blight.

Table 2. Severity of anthracnose in wound-inoculated banana fruits exposed to volatile organic compounds of endophytic fungi, 7 DAI

Treatments	Lesion size (mm ^{2})	Percent inhibition
Uninoculated control	0.0^a	0.0^c
Inoculated control	266 .0^c	0.0^c
Diaporthe sp.	14.0^a	93 .0^a
F. proliferatum	74.0^b	67 .0^b
Pr(>F)	<0.0001	<0.0001

Means followed by the same letter are not significantly different at α=0.05

Similar results were also observed by Adiova (2019) that endophytic fungi, namely *Diaporthe* sp. and *F. proliferatum* including *Tinctoporellus epimiltinus*, suppressed the development of *C. musae* on artificially inoculated banana fruits. On the other hand, other endophytic fungi such as *F. oxysporum, F. proliferatum*, *Lasiodiplodia* sp., and *L. theobromae*, showed activities against yeasts while *Xylaria* sp., *F. oxysporum*, *Colletotrichum. tropicale, F. proliferatum*, *Colletotrichum siamense* inhibited gram-negative bacteria. Differences in cell wall composition may also explain this group-directed activity but this requires further testing of their mechanism of action (Moron et al 2018).

In Vivo Effect of Substrates on the Virulence of Promising Endophytic Fungi

Promising endophytic fungi namely *Diaporthe* sp. and *F. proliferatum* as mycofumigants were grown on low cost substrates like seeds to improve biological efficacy. Screening for proper substrate is important since fungal growth and production of VOCs are greatly influenced by the composition of the growth substrates. According to Jackson and Schisler (1992), the C:N ratio not only affects the mycelial and spore production, but also affects the biocontrol efficacy as it also impacts production of various secondary metabolites.

In this study, *Diaporthe* sp. and *F. proliferatum* were grown on rice, corn, and peanut seeds as solid substrates for the mycofumigation assay. Differences in growth responses of *F. proliferatum* and *Diaporthe* sp. were observed for each substrate. Peanut and corn substrates were colonized by *F. proliferatum* and *Diaporthe* sp. at a faster rate which took 8 days to attain the 70-90% colonization of substrates on Petri plates but the growth rate of the endophytic fungi on rice substrates was slower than corn and peanut substrates. *F. proliferatum* exhibited faster growth across different substrates (62mm, 46mm, and 55mm) than *Diaporthe* sp. (59mm, 18mm, and 53mm) for corn, rice, and peanut eight days after inoculation, respectively.

The colony growth of endophytic fungi was influenced by the type of substrate used. *Diaporthe* sp. and *F. proliferatum* were best grown on corn substrate with mean colony growths of 59mm and 62mm, respectively (Table 3). It was followed by peanut with mean colony growths of 53mm and 55mm and lastly, rice seeds with mean colony growths of 18mm and 46mm, respectively. Ezra and Strobel (2003) stated that high levels of a carbon source in the growing medium results in a higher number of VOCs emitted by *Muscudor albus* with dramatic consequences on the ability of the fungus to inhibit or kill the pathogenic fungi. Rice as a substrate was excluded from the succeeding assays because it lacked the requirements for the optimum growth and development of the endophytic fungi due to the low moisture content of the rice grain after sterilization and the thickness of the hull.

Table 3. Growth of *Diaporthe* sp. and *F. proliferatum* on peanut, rice, and corn substrates 8 days after inoculation.

Substrate	Endophytic fungus	Colony growth (~mm)
Peanut	*Diaporthe* sp.	53.0 [a]
	F. proliferatum	55.0 [a]
Rice	*Diaporthe* sp.	18.0 [b]
	F. proliferatum	46.0 [b]
Corn	*Diaporthe* sp.	59.0 [a]
	F. proliferatum	62.0 [a]
Pr(> F)	<.0001	Pr(> F)

Means followed by the same letter are not significantly different at α=0.05

The mycofumigant potential of the endophytic fungi against banana and mango anthracnose was increased when they were grown on corn and peanut substrates. In the in vivo assay, the growth of *C. musae* was severely suppressed 7 days after inoculation when exposed to *Diaporthe* sp. and *F. proliferatum* grown on corn and peanut substrates and significantly different from the control (Table 4). The endophytic fungi showed higher efficacy when grown on corn substrates than peanut substrates even though there was no significant difference in terms of suppression of the pathogen. No anthracnose symptom was observed when exposed to the endophytic fungi grown on corn substrates while slow anthracnose progression was observed in the banana fruits exposed to the endophytic fungi grown on peanut substrates. The reduction of lesion size by the VOCs of *Diaporthe* sp. was comparable to *F. proliferatum.* The inhibition rate of both endophytic fungi ranged from 80-100%.

On the other hand, the two endophytic fungi showed a comparable effect in reducing pathogen inoculated mango anthracnose disease severity compared to the inoculated control mango fruits (Table 4). The uninoculated control did not exhibit observable anthracnose symptoms (Figure 1). Nine days after inoculation, mango fruits inoculated with *C. gloeosporioides* being exposed to VOCs of *Diaporthe* sp. showed the highest growth inhibition (100%) followed by the inoculated fruits of *C. gloeosporioides* that were exposed to *F. proliferatum* (98%). Although the means of percent inhibition of samples varied, the effects were statistically not significantly different between the two endophytic fungi (Figure 1).

Table 4. Severity of anthracnose in wound-inoculated banana fruits exposed to volatile organic compounds of endophytic fungi grown in corn and peanut substrates

Treatments	Lesion size (mm ^{2})		Percent inhibition	
	banana	mango	banana	mango
Uninoculated control	0.0 ^{a}	0.0 ^{a}	0.0 ^{b}	0.0 ^{b}
Inoculated control	287 .0 ^{c}	336.0 ^{b}	0.0 ^{b}	0.0 ^{b}
Corn				
Diaporthe sp.	0.0 ^{a}	0.0 ^{a}	100 .0 ^{a}	100.0 ^{a}
F. proliferatum	0.0 ^{a}	0.0 ^{a}	100 .0 ^{a}	100.0 ^{a}
Peanut				
Diaporthe sp.	0.0 ^{a}	0.0 ^{a}	100 .0 ^{a}	100.0 ^{a}
F. proliferatum	48 .0 ^{b}	3.0 ^{a}	83 .0 ^{a}	98.1 ^{a}
Pr(>F)	0.0001	<.0001	<0.0001	0.0003

Means followed by the same letter are not significantly different at α=0.05

C.musae and *C. gloeosporioides* that were isolated from the treated banana and mango that showed no progression of symtoms during the experiment were recovered when plated on PDA which indicated that the endophytic fungi exhibited a fungistatic mode of action. The VOCs of endophytic fungi did not kill the pathogen instead they only reduced the virulence of the pathogen resulting in no visible anthracnose symptoms.

The results also conformed with Cumagun et al (2019) demonstrating the significant growth reduction of *C. musae* by *Diaporthe* sp. and *F. proliferatum* during in vivo evaluation and no significant differences on the performance of both endophytic fungi regardless of media used. Mercier and Jimenez (2004) believed that the production cost of *M. albus* as a biological control agent in agricultural applications was low and could be cultivated on readily available substrates such as rye grain. *M. albus* growing on autoclaved rye produces 2-methyl-1-butanol (48.5%) as the major component in the headspace along with isobutyric acid (14.9%) and ethyl propionate (9.63%) as the second and third major components, respectively. The mechanism of the growth inhibition by the endophytic fungi VOCs may be the alteration of the respiration and the cell permeability of the pathogen. For example, *Fusarium oxysporum*'s growth and respiration was inhibited by endophytes' VOCs through cell membrane damage (Macías-Rubalcava et al 2018).

Pathogenicity Test of Endophytic Fungi

After seven days of incubation, necrotic lesions were observed on the inoculation site of banana and mango for *Diaporthe* sp. and *F. proliferatum,* but the lesions did not progress indicating that the promising endophytic fungi were not pathogenic. The pathogenicity test suggested that *F. proliferatum* and *Diaporthe* sp. were promising biocontrol agents against *C. musae* and *C. gloeosporioides* and safe to apply as mycofumigants without any harmful effect to postharvest banana and mango fruits.

Figure 1. Severity of anthrac*nose on banana and mango fruits wound-inoculated with Colletotrichum musae* (top) and *Colletotrichum gloeosporioides* (bottom) when exposed to volatile organic compounds (VOCs) of *Diaporthe* sp. and *F. proliferatum* which were grown in corn (left) and peanut (right) substrates. a. uninoculated control, b. inoculated control, c. *Diaporthe* sp., d. *F. proliferatum*

CONCLUSION

Mycofumigation using endophytic fungi is an efficient technique to reduce the anthracnose severity of banana and mango. In vivo mycofumigation assay using corn and peanut substrates increased the bioeffecacy of the endophytic fungi against the anthracnose that ranged from 80 to 100% inhibition. In addition, *C. musae* and *C. gloeosporioides* were recovered when plated on PDA which indicated that endophytic fungi exhibited a fungistatic mode of action. On the other hand, the pathogenicity test revealed that the endophytic fungi did not cause visible diseasein banana and mango fruits indicating *Diaporthe* sp. and *F. proliferatum can be used as* effective mycofumigants against *C. musae* and *C. gloeosporioides.*

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