Ecosystems & Development Journal 7(1): 14-20
April 2017 ISSN 2012-3612

Evaluation of the Curative Effect of *Cymbopogon nardus* (L.) Rendle Essential Oil On Fusarium Wilt of Abaca (*Musa textilis* Nee)

[a*]Rebekah Ruth B. Gaña, [b]Jessa P. Ata and [c]Mutya Ma. Q. Manalo

ABSTRACT

Abaca (Musa textilis), a native species in the Philippines, is a reforestation crop integrated in different agroforestry farming systems in the country. In this study, the effect of citronella (Cymbopogon nardus) essential oil was observed to inhibit mycelial growth of Fusarium oxysporum f. sp. cubense (E. F. Smith) W.C. Snyder & H.N. Hansen causing Fusarium wilt disease in abaca in vitro and its disease symptoms in vivo. Four different concentrations (0.001%, 0.025%, 0.05% and 0.1% v/v) were used for the in vitro assay within 21 days of observation. Mycelial growth was significantly inhibited in the 0.05% and 0.1% concentrations. For the in vivo assay, the 0.1% v/v concentration was used to test the efficacy of citronella essential oil in suppressing leaf symptoms of Fusarium wilt disease on Abaca seedlings using the Disease Severity Index (DSI). The Fusarium-inoculated seedlings with citronella essential oil had the highest DSI (2.8), followed by seedlings with inoculation but without essential oil (2.7), seedlings both without inoculation and essential oil (control) (1.6), and seedling without inoculation but with essential oil (1.13). Findings showed that citronella essential oil can lessen the occurrence of morphological symptoms such as leaf spots and anthracnose that are not visible symptoms of the Fusarium wilt disease.

Keywords: abaca, agroforestry, Citronella essential oil, Fusarium wilt,

INTRODUCTION

Abaca (*Musa textilis* Nee), of the family *Musaceae,* is an indigenous plant primarily cultivated for its excellent use in pulp and paper industries, fiber craft and cordage under the supervision of Fiber Industry Development Authority (FIDA) (Philippine Abaca Fiber Market 2015). It is a perennial crop also known as Manila Hemp, a suitable plant for reforestation (Bastasa & Baliad 2005).

The Philippines is the world's largest producer of abaca, contributing to 85% of its production share globally in 2013 (Philippine Abaca Fiber Market 2015). In 2006, total export earnings from abaca manufacturers and fibers reached USD 90.6M. The highest production of abaca that was recorded was in 2004 where it hit 74,465 MT (Lantican 2008). In 2014, the total earnings from abaca products reached USD 79.64M (Orendain 2015). This species is cultivated in 130,000 ha of land by over 90,000 farmers across the country.

In previous years, abaca showed a slight production decline in hectarage despite the increasing demand for pulp and paper and fiber craft industries. Several studies showed that the income derived from abaca production by farmers was only about 50% of its potential use because of low quality and quantity of abaca produce that could be attributed to disease attacks caused by *Fusarium oxysporum f. sp. cubense (E. F. Smith) W.C. Snyder & H.N. Hansen* (Worbs 2002). Disease severity in major abaca-growing areas ranges from 5 — 65% as affected by *Fusarium* wilt, also known as Panama disease, caused by *F. oxysporum* f. sp. *cubense* (SEARCA 2015).

In the Philippines, the demand for abaca, especially in pulp industries continues to increase due to the growing concern for environmental protection and forest conservation which supplied more opportunities for natural fibers, like abaca. Although there has been a high demand for abaca, local production has not met the demand. The abaca industry is solely reliant on traditional varieties. Because of the limited attention given to 'sustained varietal improvement,' the old abaca varieties had "outlived their usefulness" and now had become a vulnerable target for disease devastation (Lalusin & Villavicencio 2014). Despite the fact that Abaca may actually help in slowing down deforestation, it is often overlooked as being a common component in traditional agroforestry systems in central Philippines (Richman 2002).

The use of biological control agents such as essential oils can be used as an alternative technique to combat the Panama disease. Aside from citronella essential oils, *Cymbopogon nardus, Pogostemon calbin, Eugenia aromatic,* and *Vitiveria zizanoides* were tested against *F. oxysporum* f. sp. *cubense* in banana. Results showed that these essential oils were able to affect the said pathogen negatively. Mycelial growth was suppressed when treated with essential oil in different treatments *in vitro*, as *E. aromatica* being the most effective in controlling the growth of the pathogen (Istuanto & Emilda 2011). *Trichoderma* and *Pseudomonas* can also be used to combat *F. oxysporum*. Previous studies show potentials of *Trichoderma* to inhibit growth of pathogens (Dubey *et al.* 2006; Thangavelu *et al.* 2003).

Inorganic salts such as ammonium bicarbonate, potassium bicarbonate, sodium bicarbonate and calcium chloride were proven to have inhibitory effect on *F. oxysporum*, *Alternaria*

[a,b,c] *Forest Biological Sciences, College of Forestry and Natural Resources University of the Philippines Los Baños*
**Corresponding author: 1rbgana@up.edu.ph*

alternata and *Botrytis cinerea*, whereas potassium bicarbonate had the highest inhibitory effect at a concentration of 200 mM (Zaker 2014).

This study covers the performance of Citronella as a biological control agent having antifungal properties, *in vitro* and *in vivo*. The main focus of this study is on the growth-inhibiting effect of the citronella essential oil to *Fusarium* wilt disease in abaca seedlings. The application of essential oil in this study is only limited to abaca, thus any experimentation on other species except abaca is not discussed.

The study aimed to help in the conservation of the Philippine abaca as an indigenous crop and an agroforestry species through evaluating the curative effect of citronella essential oil as a biological control agent of the pathogen causing *Fusarium* wilt in abaca. The study determined the most effective essential oil concentration *in vitro* that was used for the *in vivo* assay.

METHODOLOGY

Procurement of the Plant Material

Sixty seedlings of Abaca were procured from the Feeds and Industrial Crops of the Institute of Plant Breeding Crop Protection Cluster, University of the Philippines Los Baños (UPLB). The four-month old abaca seedlings were hybrids (Hybrid No. 7) grown through tissue culture and were virus-resistant.

Preparation of the Fungal Strain

The sub-cultured *F. oxysporum* f. sp. *cubense* obtained from the Philippine National Collection of Microorganisms, BIOTECH-UPLB was further purified and transferred to sterile petri dishes with Potato Dextrose Agar (PDA). The antifungal activity of *C. nardus* essential oil of the Gold in Grass Corporation, extracted through steam distillation was tested through the agar dilution method in a complete random design at the Forest Pathology Laboratory, College of Forestry and Natural Resources (CFNR), UPLB.

Testing the Antifungal Activity of Citronella Essential against *F. oxysporum* f. sp. *Cubense*

In vitro evaluation of the effect of the essential oil on the pathogen

The agar dilution method was done using essential oil incorporated with Tween 20 as the emulsifying agent mixed with PDA. The final concentrations (v/v) were: 0.001%, 0.025%, 0.05%, 0.1%. PDA discs (0.3 cm) from the pure cultures were transferred to PDA plates of different concentrations. As the basis for fungal growth, mycelial growth diameters (MGDs) were recorded after 21 days of incubation for each of the three replicates per concentration. The Antifungal Index (AI) was calculated as (Wang *et al.* 2011):

$$\textit{Antifungal Index \%} = \frac{(D_2 - D_1)}{D_2} \times 100$$

where:

D_1 represents the diameter of the growth zone in the experimental dish, and D_2 represents the diameter of the growth zone in the control dish.

***In vivo* Evaluation of the Effect of Essential Oil in the Control of *Fusarium* Wilt in Abaca Seedlings**

Preparation of F. oxysporum f. sp. cubense spore suspension

Spore suspension of seven-day old pure cultures was prepared in the Microtechnique Laboratory of CFNR. Ten mL of distilled water was poured onto each petri dish and scraped to be filtered with a cheese cloth. The extracted spores from the plates were transferred to 200 mL distilled water. The 10 mL spore suspension (5×10^4 in spores/mL as determined using the hemocytometer) prepared in the laboratory was irrigated and sprinkled to each of the abaca seedlings.

The *in vivo* evaluation was conducted at the Department of Forest Biological Sciences Greenhouse, CFNR-UPLB. The experiment was arranged in complete random block design. The treatments for the *in vivo* assay were the following: Control 1 (without inoculation and essential oil application), Treatment 1 (without inoculation but with 0.1% v/v essential oil application), Treatment 2 (with inoculation but without essential oil), and Treatment 3 (with inoculation and application of 0.1% v/v essential oil). Three replicates with five seedling samples each were used in the study. A total of 30 seedlings were inoculated with the prepared *F. oxysporum* f.sp. *cubense* spore suspension.

After 14 days of pathogen inoculation, the seedlings received a one-time application of 0.1% v/v Citronella essential oil whereby full inhibition of fungal growth was observed for the *in vitro* assay. The one-time application was conducted through spraying a 30mL solution of 0.1% v/v essential oil directly to the leaves and to the soil where the seedlings were planted.

Evaluation for *Fusarium* infection in Abaca seedlings was conducted following the criteria for disease severity in *Fusarium* wilt through the Scales of Leaf Symptom Index as shown in Table 1.

Table 1. Disease diagnosis for Leaf Symptom Index (LSI)

Scale	Diagnosis
1	No streaking or yellowing of leaves. Plant appears healthy
2	Slight streaking and/or yellowing of lower leaves.
3	Streaking and/or yellowing of most of the lower leaves.
4	Extensive streaking and/or yellowing on most or all of the leaves
5	Dead plant

(Source: Mak *et al.* 2004)

The Disease Severity Index (DSI) was computed as:

$$DSI = \frac{\Sigma\ (\textit{Number on scale x Number of seedlings in that scale})}{\Sigma\ (\textit{Number of treated seedlings})}$$

As for the disease diagnosis for all the 60 seedlings under the four different treatments and conditions, the seedlings were rated alongside its numerical rating to determine the disease infection

(Table 2). The index of infection and average rating of the seedlings were taken in order to assess their condition inside the greenhouse.

Table 2. Criteria for Disease Diagnosis measured through Percent Disease Infection.

Rating	Numerical Rating	Percent Disease Infection
1	0	0
2	0.75	1-25%
3	1	26-50%
4	2	51-75%
5	3	76-100%

(Source: Militante and Manalo 1994)

The average rating and index of infection of the seedlings were computed as:

$$Average\ Rating = \frac{\Sigma\ all\ ratings}{Total\ number\ of\ seedlings}$$

$$Index\ of\ Infection = \frac{\Sigma\ all\ numerical\ ratings \times 100}{Total\ number\ of\ seedlings \times 3}$$

Statistical Analysis

Results of the study were analyzed through Analysis of Variance (ANOVA) using the R Studio statistical software version 0.98 (2013).

RESULTS AND DISCUSSION

In Vitro* Evaluation of the Effect of Citronella Essential Oil to *F. oxysporum* f. sp. *Cubense

The results of the computed values of AI and MGD in each of the concentrations were significantly different from each other according to the ANOVA test (Table 3). In general, AI increased as the essential oil concentration increases. The highest Citronella essential oil concentration (0.1%) had the highest AI with 93.76% while the lowest concentration (0.001%) had the lowest AI at 77.83%.

In general, mycelial growth decreased as the essential oil increases. Mycelial growth generally continued to increase up until the 21st day of the experiment as the concentration increases. The treatments Control and 0.1% essential oil application obtained the highest and lowest mycelial growth, respectively (Figure 1).The growth of *F. oxysporum* f. sp. *cubense* was evident in the control alongside the growth of other contaminants.

It can also be said that the greater the concentration of essential oil, the lesser the mycelial growth diameter of the pathogen, *in vitro,* thus, the higher capacity of the oil to infect and suppress the growth of the pathogen.

The pathogen of the *Fusarium* wilt disease, *F. oxysporum* f. sp. *cubense,* was suppressed in all citronella essential oil concentrations *in vitro*. The ability of citronella essential oil to inhibit fungal growth can be attributed to various mechanisms.

Table 3. The effect of Citronella essential oil on the Antifungal Indices (AIs) and Mycelial Growth Diameters (MGDs) on the growth of *Fusarium oxysporum* after 21 days of incubation.

Concentration (% v/v)	MGD (cm)*	Antifungal Index (%)*
Control	3.33 ± 1.04^{a}	-
0.001	0.74 ± 0.10^{b}	77.83 ± 3.03^{a}
0.025	0.39 ± 0.04^{c}	88.26 ± 1.09^{b}
0.05	0.35 ± 0.10^{d}	89.55 ± 3.08^{c}
0.1	0.21 ± 0.13^{e}	93.76 ± 3.89^{d}

**Average values followed by superscript of a different letter are statistically significant (P <0.05) based on the Analysis of Variance (ANOVA) test.*

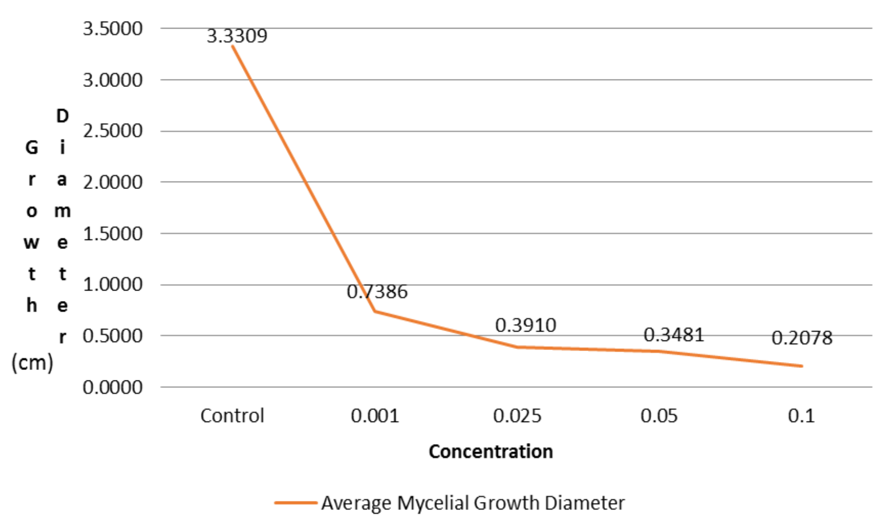

Figure 1. Average Mycelial Growth Diameter (in cm) in each of the Concentration and Control for the *in vitro* assay observed within 21 days.

Anatomical and cytological studies showed that citronella essential oil has the ability to irreversibly "alter" the hyphae and conidia of the pathogen and interfere with the enzymes that are responsible to synthesize cell wall that results to the disturbance of normal growth of the pathogen (Li *et al.* 2013; de Billerbeck *et al.* 2001).

Another possible mechanism of citronella essential oil to inhibit fungal growth is the presence of unique compounds. In general, terpenes and phenolic compounds present among essential oils enable successful inhibition of fungal growth *in vitro.* Its antimicrobial activity is possibly due to its ability to damage 'enzymatic cell systems,' as well as those components related to energy production and synthesis of structural compounds (Sreenivasa *et al.* 2011). In citronella essential oil, the major component citronellal, together with its other components such as α- and β- pinenes, is known to be effective in fungal growth inhibition (Ata and Manalo 2014; Nakahara *et al.* 2003).

The results of the present study showed that the higher the concentration of essential oil is, the more effective it is to inhibit fungal growth. This study showed that the pathogen *Fusarium* can still grow at slower rate in conditions where essential oils are present in small amounts. Previous studies showed similar results where growth of pathogen was entirely suppressed as citronella essential oil increased. (Sreenivasa *et al.* 2011; Monteiro *et al.* 2013; Wang *et al.* 2005; Ata & Manalo 2014; Istianto & Emilda 2011; Katooli *et al.* 2011). Mycelial growth of *Fusarium* was observed at treatment with 250 ppm of citronella essential oil but was completely inhibited at 2000 ppm (Monteiro 2013).

In Vivo Evaluation of the Effect of Citronella Essential Oil to *F. oxysporum* f. sp. *cubense*

Citronella essential oil did not effectively inhibit pathogen development *in vivo*. The inoculated seedlings under Treatments 2 (with inoculation but without essential oil) and 3 (with inoculation and 0.1% v/v essential oil) had seedlings with extensive streaking and/or yellowing on most or all of the leaves (Table 4). Treatment 1 (no inoculation but with 0.1% v/v essential oil), followed by the control, had the most number of seedlings without streaking or yellowing.

The effect of citronella essential oil in seedlings inoculated with the pathogen was not significantly different in terms of DSI (Table 5). Streaking or yellowing of leaves in the seedlings without inoculation but applied with essential oil was not observed as well as in the control. The effect of the pathogen inoculation both in Treatments 2 and 3 was evident in terms of the LSI, considering the yellowing of the leaf apices and margins of the seedlings.

Table 5. Disease Severity Indices (DSI) of the four *in vivo* assay treatments observed within one month.

Treatment	DSI*
0 (Control)	1.6^{a}
1 without inoculation but with essential oil	1.13^{a}
2 with inoculation but without essential oil	2.7^{a}
3 with inoculation and essential oil	2.8^{a}

*Values followed by superscript of a similar letter are not statistically significant *($P < 0.05$)* based on the Analysis of Variance (ANOVA) test.

Index of infection significantly varied among the treatments (Table 6). Seedlings uninoculated with the pathogen but treated with citronella essential oil obtained lowest index of infection. However, the highest index of infection was recorded among inoculated seedlings applied with citronella essential oil.

Cymbopogon essential oils are proven to inhibit the production of mycotoxins (Paranagama *et al.* 2003). Mycotoxins produced by *Fusarium* can cause contamination in *Musa* sp. which can be manifested by leaf symptoms observed *in vivo* (Jimenez *et al.* 1997). However, the overall effect of citronella essential oil against *Fusarium* wilt disease was insignificant in the *Fusarium* inoculated seedlings.

Table 4. *Fusarium* wilt disease diagnosis measured through Leaf Symptom Index (LSI) for all the *In vivo* assay treatments after application of Citronella essential oil.

Scale	Diagnosis	Number of seedlings in Scale			
		Control	1 without inoculation but with essential oil	2 with inoculation but without essential oil	3 with inoculation and essential oil
1	No streaking or yellowing of leaves. Plant appears healthy	6	13	0	0
2	Slight streaking and/or yellowing of lower leaves.	9	2	6	6
3	Streaking and/or yellowing of most of the lower leaves.	0	0	7	5
4	Extensive streaking and/or yellowing on most or all of the leaves.	0	0	2	4
5	Dead plant	0	0	0	0

Table 6. Numerical rating, average rating, and indices of infection of the Abaca seedlings at different treatments after a month of observation.

	Treatments	Numerical Rating*	Average Rating*	Index of Infection*
0	(Control)	1.03 ± 0.21^{a}	2.73^{a}	34.34^{a}
1	without inoculation but with essential oil	0.80 ± 0.13^{b}	2.27^{b}	26.67^{b}
2	with inoculation but without essential oil	1.02 ± 0.15^{c}	2.87^{c}	39.89^{c}
3	with inoculation and essential oil	1.72 ± 0.31^{d}	3.37^{d}	57.22^{d}

**Values followed by superscript of a different letter are statistically significant (P <0.05) based on the Analysis of Variance (ANOVA) test.*

One possible reason for this is the volatility of the essential oil. Essential oils in general are volatile. Components of these essential oils are highly affected by precipitation, temperature, light and humidity. Low air temperature as well as relative humidity provide a good condition for essential oil quality (Pandey 2010). The quality and composition of the oil can also be affected by temperature (Chang *et al.* 2005). As the temperature increased, there was a significant alteration in the composition of most essential oils like rosemary, cardamom, and clove bud (Turek & Stintzing 2013). In particular, components of citronella essential oil, namely: citronellol, citronellal, and geraniol are reduced in drought conditions (Pandey 2010). As these citronella essential oil components are relevant in inhibiting fungal pathogen growth, decreasing their amount brought about by increased temperature would result to reduced ability to inhibit fungal growth *in vivo.*

In the present study, essential oil significantly improved overall health of uninoculated seedlings, showing low index of infection. This low index may be attributed to the essential oil's capacity to reduce other fungal pathogens in the soil (Kishore *et al.* 2007). However, it is possible that the essential oil cannot suppress *Fusarium* in the soil. When seedlings were inoculated with *Fusarium*, treatment with essential oil significantly exhibited more leaf disease symptoms aside from the symptoms of *Fusarium* wilt disease, such as scorching and anthracnose, compared to those untreated with essential oil. These symptoms may be manifestations of phototoxicity of citronella essential oil (Lagey *et al.* 1995). Essential oils, in general can be toxic to the growth of plants (Jha *et al.* 2014). Some may contain furosoumarin derivatives (i.e. psoralen, 8-methoxypsoralen, 5-methoxypsoralen, 4, 5, 8-trimethoxypsoralen) which can induce phototoxic reactions. Ultraviolet radiation and humidity are factors that exacerbate the occurrence of phototoxicity (Lagey *et al.* 1995). High concentrations of essential oils such as Eucalyptus oil may result to burning of leaves (Jha *et al.* 2014). Thus, it is possible that direct application of high concentrations of essential oil onto the leaves may increase the occurrence of burning.

Necrotic symptoms caused by the phototoxicity of citronella essential oil may be also be exacerbated by prevailing conditions in the greenhouse. The greenhouse was not conducive for growth since the building was poorly maintained and some seedlings were subject to direct sunlight. Thus, leaf symptoms brought about by *Fusarium* and the phototoxicity of essential oil increased the index of infection.

Since higher concentrations of essential oil cannot only inhibit growth of pathogen but also may cause photoxicity in plants, it is noteworthy to consider avoiding application to essential oil directly onto the leaves under direct sunlight.

Pathogen development can be further observed in host plants such as abaca and banana when healthy roots are submerged and/or dipped in conidia suspension for two or more hours (Mak *et al.* 2004; Banihashemi & deZeeuw 1975).

CONCLUSION

Citronella essential oil is an effective means to inhibit the growth of the pathogen *F. oxysporum* f. sp. *cubense* causing *Fusarium* wilt disease in abaca seedlings. At 0.05% v/v and 0.1% v/v, citronella essential oil can significantly inhibit fungal growth of pathogen, *in vitro.* Furthermore, an increase in the concentration of the essential oil, generally increases its antifungal property.

Efficiency of citronella essential oil to inhibit symptoms of *Fusarium* wilt disease may be affected by its volatility. Exposure to high temperature and relative humidity may decrease important components (citronellol, citronellal and geraniol) of the oil responsible for its antifungal performance may result to reduction of the essential oil's capacity to inhibit fungal growth.

While precautions need to be observed to avoid phototoxicity, higher concentration of essential oil may be used *in vivo* to effectively inhibit leaf necrotic symptoms brought about by the pathogen *Fusarium* and other physiopaths.

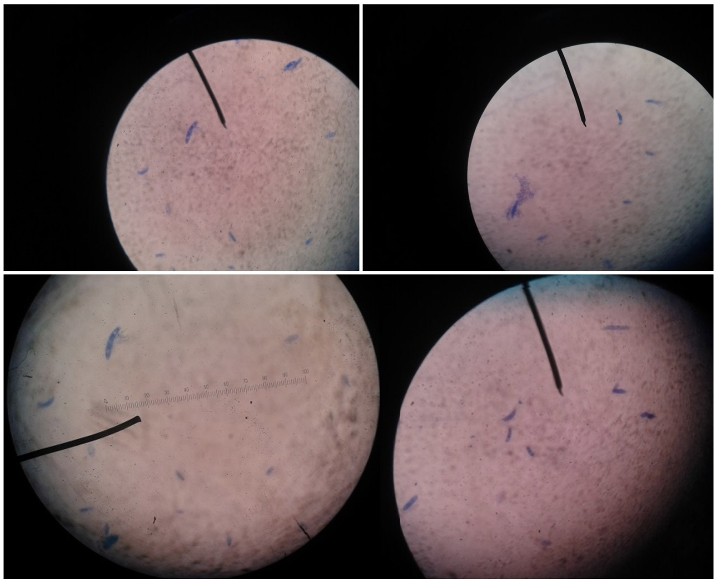

Figure 1. Microscopic view of the macroconidia of Fusarium *oxysporum* cubense 400 times magnification

RECOMMENDATION

As an effective strategy for *Fusarium* wilt disease management, it is recommended that fungal pathogen-resistant varieties should be developed in the country. The need for the development of fungal pathogen-resistant Abaca varieties should be addressed, specifically against *F. oxysporum* f. sp. *cubense.* Although the abaca seedlings procured from the Institute of Plant Breeding UPLB were virus-resistant, these seedlings were still susceptible to the *Fusarium* wilt disease. In order to lessen the disease occurrence of the said pathogen, further research on resistant varieties of abaca against certain pathogens must be done.

Increasing the concentration of the essential oil necessary to yield significant results for the *in vivo* assay is recommended. A limitation of this present research was the level of concentration used for the *in vivo* assay. Other studies suggest that increasing the concentration up to 0.5% v/v is effective for the field tests whereas, the highest concentration used in this study was only 0.1%. This is mainly due to the scarcity of the citronella essential oil.

The recommendations that were mentioned can at the very least help in the improvement of research in the country in relation to *M. textilis*. While there have been other initiatives on improving the quality and quantity of the Philippine abaca, biological control agents such as citronella essential oil play a promising role in furthering the research and management strategies in the country.

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ACKNOWLEDGMENT

The authors would like to acknowledge the support of Mr. Cyrus Pelayo of the Gold in Grass Company and Dr. Antonio Lalusin of the Crop Protection Cluster, Institute of Plant Breeding, BIOTECH, UPLB.