INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 26, No. 5, p. 18-26, 2025
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Antifungal activity of endophytic bacteria against *Fusarium oxysporum* f. sp. *cubense* (Foc TR4)

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Article published on May 04, 2025

Key words: Antifungal activity, Endophytic bacteria, Fusarium wilt, Foc TR4, Mycelial growth inhibition

Abstract

People's initiative in managing mangrove refers to the involvement of local communities in planning, implementing, and monitoring mangrove restoration and conservation activities. It has been successful in many nations throughout the world to involve people in managing mangrove forests. This study assessed the community's level of knowledge, awareness, as well as the practices used by the environmental agency and locals in managing mangrove forest that can be used to attain sustainable management. This study used both qualitative and quantitative methods relying on quantitative survey data and qualitative information gathered from the key informant interview respondents. Mangroves are important for preventing erosion, maintaining water quality, and reducing pollution; mangroves also provide a source of income and nourishment for the community, protecting them from natural disasters. The study found that the respondents were aware of the importance of mangroves in terms of wildling/seedling collection, planting, monitoring/protection, and its role in ecological and economic aspects. Further studies are recommended to assess the drivers and barriers of community participation and engagement in mangrove management, assess policy enforcement, and explore the perceptions and preferences of the community regarding guidance and support.

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Introduction

In the early 2000, the 'Cavendish' banana industry was threatened with significantly reduced production due to a devastating disease known as Fusarium wilt which is caused by the fungus, *Fusarium oxysporum* f. sp. *cubense* Tropical race 4 (Foc TR4). This disease might have completely destroyed the banana sector if it was left unchecked, which would have disastrous economic repercussions (Molina *et al.*, 2017). The Philippines national banana export sector approximately employs 320,000 people, with 70% of them working for big corporate producers and 30% being small-scale farmers, according to [Food and Agriculture Organization (FAO), 2017). As of May 2020, the total production declined to 20%, or about 20 million boxes as mentioned by the President of the Philippine Banana Growers Exporters Association (PBGEA) (Alberto, 2020).

Foc TR4 is a soil-borne pathogen that attacks the roots and obstructs the vascular system causing both internal and external symptoms that ultimately cause the plant to lyse and die, thus, affecting the productivity, quality, and profitability of the produce (Maymon *et al.*, 2020).

Integrated management strategies have been implemented to lessen the spread of the disease, however, these methods were not able to provide long-term solutions to the problem. Further, the management of this disease has proven to be difficult as mentioned by Maymon *et al.* (2020).

Utilizing beneficial microorganisms or their products in modern agriculture is based on a thorough understanding of the agricultural context and their capacity to balance our ecosystem (Vaish and Pathak, 2023). The salient feature in utilizing endophytic bacteria as a safe and sustainable approach confers a commendable aspect for their potential in managing fungal plant pathogens (De Silva *et al.*, 2019). Bacterial endophytes generate a wide range of antimicrobial substances that exhibit broad-spectrum antagonism and strong inhibitory actions against several fungal diseases (Lacey and Rutledge, 2022). Additionally, extracellular enzymes and bioactive metabolites may enhance plant resistance and encourage plant development (Olanrewaju and Babalola, 2019). Endophytic microorganism lives within the intercellular spaces of various plant parts such as stems, roots, petioles, and leaves without imposing any apparent symptoms of disease (Strobel, 2003).

With the pressing economic concern of the banana industry due to Foc TR4, this study aims to evaluate the inhibitory effects of bacterial endophytes isolated from 'Cavendish' banana roots against *Fusarium oxysporum* f. sp. *cubense* under *in vitro* conditions.

Materials and methods

Experimental design and treatments

The experiment was laid out following the Complete Randomized Design (CRD) with three replications per treatment with three subsamples per replicate. The following were the treatments under *in vitro* conditions:

T_1 – Negative control (Sterile water)
T_2 – Positive control (Chlorothalonil at 2% per liter)
T_3 – *Serratia marcescens* strain CDA-88
T_4 – *Alcaligenes* sp. RCMD-19
T_5 – *Bacillus cereus* strain MGP-08
T_6 – *Bacillus subtilis* strain RNB-21
T_7 – *Bacillus arachidis* strain MGB-25

Statistical analysis

The Analysis of Variance (ANOVA) was carried out using the Standard Tool for Agricultural Research (STAR) software. Treatment means were compared using Tukey's HSD to statistically determine their significant differences.

Isolation and purification of Fusarium oxysporum f. sp. cubense (Foc TR4)

Roots from wilted 'Cavendish' banana were washed with running water to remove surface dirt and were cut into 5-10 cm size using sterilized scissors and were surface sterilized using 90% ethanol for 1 minute. The disinfected tissues were washed with

sterile distilled water three times to remove traces of ethanol and were blot dried using sterile tissue paper. The blot-dried tissues were cut longitudinally to expose the pathogen and were planted on the surface of Potato Dextrose Agar (PDA) plates. The culture plates were incubated at room conditions for several days and observed for the growth of fungal mycelia arising from the infected tissues. Hyphal tip transfer technique for purification using PDA medium was employed (Leslie and Summerell, 2006).

Isolation and purification of bacterial endophytes

Freshly collected root samples were cleaned with running water and disinfected with 10% alcohol for 1 minute, immersed in sodium hydrochloride for 4 minutes, and then rinsed 3 times in sterile distilled water. The disinfected root samples were blot-dried using sterile tissue paper.

A sterile mortar and pestle were used to macerate the root samples. To fully release the endophytic bacteria from the root tissues, the macerated samples were incubated for 3 hours at room conditions. Afterwards, 1 ml of extracted sap was collected and diluted with 9 ml sterile distilled water. Further, 1 ml of the suspension was dispensed on the center of the Nutrient Agar (NA) plates was evenly distributed using a sterile glass hockey. The culture plates were incubated for 24-48 hours until colonies of endophytic bacteria were observed. Morphologically distinct bacterial colonies on NA plates were selected and repeatedly streaked on fresh NA plates to obtain single and discrete bacterial colonies for purification and bioassay (Leonardo *et al.*, 2012). A total of 49 distinct colonies of endophytic bacteria were isolated and screened for their potential against Foc TR4 by employing the four-point assay technique.

Molecular identification of Fusarium oxysporum f. sp. cubense and the potential bacterial endophytes

The identity of the fungal pathogen was confirmed *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) by Polymerase Chain Reaction (PCR) technique which was conducted at the Biotechnological Research Service (BRS), Alanib, Lantapan, Bukidnon (Fig. 1).

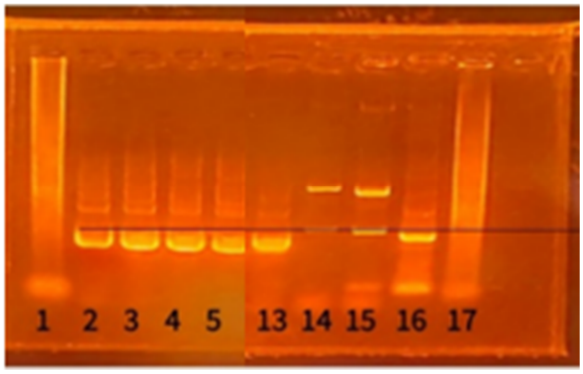

Fig. 1. Polymerase chain reaction (PCR) employing gel electrophoresis with 463 base pairs (bp) of a positive *Fusarium oxysporum* f. sp. *cubense* (Foc TR4) Lin *et al.* (2008). Negative control (Lane 1), Pathogen sample (Lane 2-5), Positive control (Lane 13-16), Water blank (Lane-17)

On the other hand, the bacterial endophytes were sent to the Philippine Genome Center (PGC) Mindanao, Davao City, for DNA extraction, PCR-agarose gel electrophoresis, illumina amplicon sequencing, node selection and identification through 16s ribosomal RNA in NCBI BLASTn system. The five potential isolates were identified as: *Serratia marcescens* strain CDA-88, *Alcaligenes* sp. RCMD-19, *Bacillus cereus* strain MGP-08, *Bacillus subtilis* strain RNB-21, *Bacillus arachidis* strain MGB-25 (Table 1).

Table 1. NCBI BLASTn sequence identification through 16s ribosomal RNA gene complete chromosome and partial gene sequence of the different endophytic bacteria isolates

Bacterial endophyte isolate code	Percent query cover	Percent identity	Accession length	Sequence identity	Sequence length	Identification
CDA-88	100	99.70	5307415	CP041123.1	705	*Serratia marcescens*
RCMD-19	100	98.04	1443	DQ659618.1	204	*Alcaligenes* sp.
MGP-08	100	100	1160	PV167927.1	507	*Bacillus cereus*
RNB-21	100	100	1135	PP968143.1	464	*Bacillus subtilis*
MGB-25	100	100	1456	PQ813961.1	436	*Bacillus arachidis*

In vitro assay of bacterial endophytes against Fusarium oxysporum f. sp. cubense

The antagonistic activity of the potential bacterial endophytes was evaluated by performing the dual culture plate assay. A mycelial disc of 5 mm in diameter was cut out from the 10-day old culture using a sterile cork borer and placed at the center of the plates. Two bands of each bacterial isolate were streaked, at 3 cm long, parallel to the potato dextrose peptone agar (PDPA) plates and with a distance of 3 cm from the center of the plate. It was aseptically performed in triplicate with three subsamples (Fokkema, 1978). The plates were incubated at room conditions and measured for daily mycelial growth inhibition using the formula by Ji *et al.* (2013).

$$\text{Inhibition (\%)} = \frac{\text{CR - R}}{\text{R}} \times 100$$

Where:

CR - Mean of the radius of fungal colony in control plates CR_1 and CR_2

R - Mean of the radius of fungal colony in test plates R_1 and R_2

Results

Table 2 presents a remarkable mycelial growth inhibition of the different bacterial endophytes against Foc TR4 under *in vitro* conditions using the dual culture plate assay. The statistical analysis provided consistently a significant difference with the Positive Control (T_2). At one day after incubation (DAI), *Bacillus subtilis* strain RNB-21 (T_6) showed the highest mean mycelial growth inhibition of 22.24%, this was followed by *Bacillus arachidis* strain MGB-25 (T_7), *Bacillus cereus* strain MGP-08 (T_5), *Alcaligenes* sp. RCMD-19 (T_4) and *Serratia marcescens* strain CDA-88 (T_3) with statistically similar values of 19.27%, 17.87%, 17.86%, and 16.46%, respectively. On the second DAI, *Bacillus subtilis* strain RNB-21 (T_6) recorded the highest inhibition value of 27.49% which signifies differences with the other treatments while, *Bacillus arachidis* strain MGB-25 (T_7), *Alcaligenes* sp. RCMD-19 (T_4), *Serratia marcescens* strain CDA-88 (T_3) and *Bacillus cereus* strain MGP-08 (T_5) were statistically comparable that obtained the values of 20.70%, 20.70%, 20.00% and 19.65%, respectively. As the observation continued, both bacterial endophytes demonstrated a notable difference in terms of efficacy. At 3 DAI, the highest inhibition of mycelial growth was observed on *Bacillus subtilis* strain RNB-21 (T_6) with a value of 65.17%. This was succeeded by *Alcaligenes* sp. RCMD-19 (T_4), *Bacillus arachidis* strain MGB-25 (T_7), *Serratia marcescens* strain CDA-88 (T_3) with comparable mycelial growth inhibition of 32.93%, 31.70%, and 28.78%, respectively. On the other hand, *Bacillus cereus* strain MGP-08 (T_5), provided the lowest inhibition value of 21.82% as observed (Fig. 2). At 4 DAI, *Bacillus subtilis* strain RNB-21 (T_6) still obtained the highest percent inhibition with a value of 85.46%, followed by statistically comparable inhibition values of *Alcaligenes* sp. RCMD-19 (T_4), *Serratia marcescens* strain CDA-88 (T_3), and *Bacillus arachidis* strain MGB-25 (T_7) that recorded 37.93%, 36.03%, and 33.33%, respectively. Consequently, *Bacillus cereus* strain MGP-08 (T_5) still scored the lowest inhibition activity with 27.42%. Radial mycelial growth inhibition continued to increase at 5 DAI with *Bacillus subtilis* strain RNB-21 (T_6) showing the highest inhibition rate as compared with the other treatments with 94.08%. It was succeeded by a comparable inhibition rate of *Alcaligenes* sp. RCMD-19 (T_4) with 48.82% and *Serratia marcescens* strain CDA-88 (T_3) with 45.13%, but conveyed a statistical difference to *Bacillus arachidis* strain MGB-25 (T_7), and *Bacillus cereus* strain MGP-08 (T_5), with the mean values of 31.25% and 31.69%, respectively. At 6 DAI, *Bacillus subtilis* strain RNB-21 (T_6) consistently exhibited the highest inhibition against the fungal pathogen with the mean value of 105.31%, followed by *Alcaligenes* sp. RCMD-19 (T_4), and *Serratia marcescens* strain CDA-88 (T_3), with comparable inhibition value of 53.94% and 48.93%, respectively. However, it expresses a noteworthy significant difference with *Bacillus arachidis* strain MGB-25 (T_7), and *Bacillus cereus* strain MGP-08 (T_5), having lesser inhibition activity of 34.84% and 33.40%, respectively. Finally, at 7 DAI, the production of antimicrobial substances of the bacterial endophytes is still notable. *Bacillus subtilis* strain RNB-21 (T_6) consistently showed a

dependable radial mycelial growth inhibition on the culture plate that reached up to 116.37%. This was followed by an intermediate performance of *Alcaligenes* sp. RCMD-19 (T_4), and *Serratia marcescens* strain CDA-88 (T_3), showing inhibition rates of 55.05%, and 50.65%, respectively but conversely significant to *Bacillus cereus* strain MGP-08 (T_5), and *Bacillus arachidis* strain MGB-25 (T_7), that scored 35.86% and 35.85%, respectively.

Considering the over-all efficacy experiment, *Bacillus subtilis* strain RNB-21 (T_6) demonstrated a promising and consistent mycelial growth inhibition capacity against Foc TR4, followed by *Alcaligenes* sp. RCMD-19 (T_4), *Serratia marcescens* CDA-88 (T_3), *Bacillus cereus* strain MGP-08 (T_5), and *Bacillus arachidis* strain MGB-25 (T_7). The results of the *in vitro* assay confirm the efficacy of the different bacterial endophytes against Foc TR4.

Table 2. Mean percent radial growth inhibition of *Fusarium oxysporum* f. sp. *cubense* (Foc TR4) as affected by the different bacterial endophytes at 1, 2, 3, 4, 5, 6, and 7 days after incubation (DAI)

Treatment	Percent growth inhibition						
	1DAI	2DAI	3DAI	4DAI	5DAI	6DAI	7DAI
T_1 - Negative control (Sterile Water)	-	-	-	-	-	-	-
T_2 - Positive control (Chlorothalanil)	17.87^{b}	18.60^{b}	19.51^{c}	20.01^{d}	20.66^{d}	21.40^{d}	21.83^{d}
T_3- *Serratia marcescens* strain CDA-88	16.46^{b}	20.00^{b}	28.78^{b}	36.03^{bc}	45.13^{b}	48.93^{b}	50.65^{b}
T_4- *Alcaligenes* sp. RCMD-19	17.86^{ab}	20.70^{b}	32.93^{b}	37.93^{b}	48.82^{b}	53.94^{b}	55.05^{b}
T_5- *Bacillus cereus* strain MGP-08	17.87^{b}	19.65^{b}	21.82^{c}	27.42^{cd}	31.69^{c}	33.40^{c}	35.86^{c}
T_6- *Bacillus subtilis* strain RNB-21	22.24^{a}	27.49^{a}	65.17^{a}	85.46^{a}	94.08^{a}	105.31^{a}	116.37^{a}
T_7- *Bacillus arachidis* strain MGB-25	19.27^{ab}	20.70^{b}	31.70^{b}	33.33^{bc}	34.25^{c}	34.84^{c}	35.85^{c}
F-test	*	*	*	*	*	*	*
CV(%)	7.62	7.47	6.44	9.33	7.31	6.68	7.40

Means in a column followed by a common letter are not significantly different at 0.05 level using Tukeys HSD test (Tukey, p > 0.05), * - significant; ns - not significant

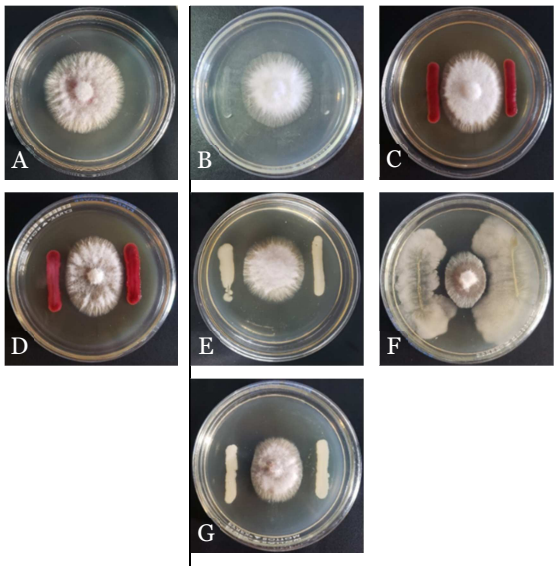

Fig. 2. Radial mycelial growth inhibition of *Fusarium oxysporum* f. sp. *cubense* (Foc TR4) on potato dextrose peptone agar (PDPA) against different bacterial endophytes at three days after incubation (DAI). A. Negative control (Sterile Water) (T_1) B. Positive control (Chlorothalonil at 2%) (T_2), C. *Serratia marcescens* strain CDA-88 (T_3), D. *Alcaligenes* sp. RCMD-19 (T_4), E. *Bacillus cereus* strain MGP-08 (T_5), F- *Bacillus subtilis* strain RNB-21 (T_6), G. *Bacillus arachidis* strain MGB-25 (T_7)

Table 3. Longevity period of inhibition on the growth of *Fusarium oxysporum* f. sp. *cubense* (Foc TR4) as affected by the different bacterial endophytes

Treatment	Number of days
T_1 - Negative control (Sterile Water)	7.11^{d}
T_2 - Positive control (Chlorothalonil)	8.33^{c}
T_3- *Serratia marcescens* strain CDA-88	10.00^{b}
T_4- *Alcaligenes* sp. RCMD-19	11.11^{b}
T_5- *Bacillus cereus* strain MGP-08	7.55^{cd}
T_6- *Bacillus subtilis* strain RNB-21	20.00^{a}
T_7- *Bacillus arachidis* strain MGB-25	7.45^{cd}
F-test	*
CV(%)	3.97

Means in a column followed by a common letter are not significantly different at 0.05 level using Tukeys HSD test (Tukey, p > 0.05), * - significant; ns - not significant

Table 3 shows the longevity period of the different bacterial endophytes to inhibit the mycelial growth of Foc TR4 under *in vitro* conditions. This explains the length of efficacy of a certain BCA against the target pathogen since their biological control

activity has been linked to their ability to produce a wide range of chemically diverse and diffusible metabolites. Statistical analysis manifested a discernible and noteworthy variance in the duration of effectiveness. The mean longevity period ranges from 7.11 to 20.00 DAI. The longest expression of efficacy was observed on *Bacillus subtilis* strain RNB-21 (T_6) because even at 20 DAI, Foc TR4 could not fully develop on the culture plate. This was followed by *Alcaligenes* sp. RCMD-19 (T_4), and *Serratia marcescens* strain CDA-88 (T_3), with significant values compared with the other treatment means that obtained 11.11 and 10.00 days, respectively. On the other hand, *Bacillus cereus* strain MGP-08 (T_5), and *Bacillus arachidis* strain MGB-25 (T_7) are comparable with Negative Control (T_1) that obtained mean values of 7.55, 7.45, and 7.11 days, respectively.

However, Foc TR4 grows faster on the Positive Control (T_2) having a mean value of 8.33 days.

This confirms that *Bacillus subtilis* strain RNB-21 (T_6) could diffuse antimicrobial compounds continually and effectively against the pathogen underscoring their critical significance in achieving successful disease control. In contrast, the release of secondary metabolites of the other endophytic bacteria decreases with time of incubation resulting to a lesser but notable efficacy against Foc TR4.

Discussion

The observed inhibitory effect of the various bacterial endophytes against Foc TR4 is attributed to their capacity to synthesize multiple antimicrobial compounds against diverse pathogens.

Serratia species is known to produce several hydrolytic enzymes like chitinase, protease, lipase, and cellulase and toxins such as serotonin, prodigiosin, and other secondary metabolites leading to fungal cell wall degradation, mycelial collapse, atrophy, deformation, perforation, bulging and bursting in the hyphae. Additionally, the bacterium use fungal hyphae as a means for translocation by attaching its cells, migrate, and eventually killing the fungal hyphae (Hover *et al.*, 2016).

According to Bharucha *et al.* (2013) *Alcaligenes faecalis* strain recovered from rhizospheric soil of banana plant has shown the ability to produce two forms of siderophores, hydroxamate and catecholate that have biocontrol to fungal diseases, promoting plant growth and increase mineral nutrient content in seedlings, resulting in better plant growth. These antifungal metabolites were effective against, *Fusarium oxsyporum, Aspergillus niger, Alternaria alternata*, and *A. flavus*.

Moreover, *Alcaligenes faecalis* was reported as fungistatic bacterium due to their ability to produce hydroxylamine to exert antifungal activity against *Colletotrichum gloeosporoides* (Yokohama *et al.*, 2013) In contrast, they have also fungicidal effect by excretion of fungicidal siderophore, bavistin, that has a role in biocontrol of pathogenic fungi (Sayyed *et al.*, 2009).

Moreover, glucanase and chitinase enzymes were produced by *Alcaligenes* sp. that are effective against *Phytophthora cinnamomi, P. nicotiane* and *Rhizoctona solani* (Kavroulakis *et al.*, 2010).

Bacillus species has various mechanisms against fungal growth such as, competition for nutrients and space to meet their nutritional requirements, antibiotic production, and enzyme production as well (Castillo *et al.*, 2013). Bacillomycin is a known toxin produced by *Bacillus* species that disrupt the integrity of the fungal cell membrane, resulting in cytoplasm leakage, hyphal death and inhibit spore germination (Andric *et al.*, 2020). Lytic enzymes such as chitinases, cellulases, proteases and glucanases degrade the structure of the cell wall. Chitinases breaks down the glycosidic bond of chitin, the main component of the fungal cell wall (Jadhav *et al.*, 2017).

Glucanases hydrolyze the polysaccharide *B*-glucans which is a second major polysaccharide in the cell

wall of plant pathogenic fungi (Dewi *et al.*, 2016). The secreted enzymes of bacteria can therefore play an important role in the lysis of the cell wall that occurs during the endophytic bacteria and pathogen interaction (Kenneth *et al.*, 2019). Many *Bacillus* species include *B. arachidis, B. cereus, B. thuringiensis, B. subtilis, B. licheniformis, B. amyloliquefaciens, B. sapensis, B. pumilus, B. velezensis,* and other species produce chitinases that have shown inhibitory effects to *Fusarium oxysporum* (Brzezinska *et al.*, 2020).

Conclusion

Endophytic bacteria have become a leading candidate in controlling plant pathogens. Its beneficial application as a biological control agent (BCA) in agricultural system results in the reduction of pesticide use, which has detrimental effects on human, animal, and the environment.

Based on the study, bacterial endophytes isolated from the roots of 'Cavendish' banana demonstrate a promising inhibition capacity against the radial mycelial growth of Foc TR4 with varying degree of efficacy and longevity. The five potential isolates were identified based on molecular analysis as *Serratia marcescens* strain CDA-88, *Alcaligenes sp.* RCMD-19, *Bacillus cereus* strain MGP-08, *Bacillus subtilis* strain RNB-21, and *Bacillus arachidis* strain MGB-25.

Among the bacterial endophytes, *Bacillus subtilis* strain RNB-21 (T_6) provided excellent performance in terms of inhibition rate and longevity of effectiveness against the fungal pathogen. Although, other isolates of bacterial endophytes showed lesser capacity in suppressing Foc TR4 but still found to be effective against this pathogen under *in vitro* conditions.

Acknowledgments

By the grace of God, the author gratefully thanks his co-authors for their expertise that helped make this study a success. Sincerest gratitude is also extended to: Mr. Mhar L. Loquez, Ms. Sheryl M. Bayang, Louie T. Palarao, Merian G. Gamorot, Mr. Regiez Novem P. Idulsa, Mr. Fheb Bryan Perez and Mr. Eric Maniscan for their valuable contribution during the conduct of the experiment. This research was funded by the first author in order to complete his degree, Doctor of Philosophy in Plant Pathology.

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