Fruit brown rot caused by *Neoscytalidium dimidiatum* on *Selenicereus monacanthus* in the Philippines

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Abstract
Multiple fungal pathogens infect economically important fruits, thereby affecting their quality and marketability. Previous research showed that some fungal pathogens that can infect the stems might infect the fruit but show a different symptom. To determine the causal pathogen of a fruit disease of *Selenicereus monacanthus* (Dragon fruit), we used a combination of fungal pathology characterization and molecular biology techniques. This paper presents the pathogenicity of *Neoscytalidium dimidiatum* MBDF36C to *S. monacanthus* resulting in brown rot and canker on fruits and stem, respectively. The paper also demonstrates the *in vitro* inhibition of *N. dimidiatum* MBDF36C by chemicals, including a bio-fungicide containing *Bacillus subtilis*. At seven days post-inoculation, we observed severe browning on *N. dimidiatum* MBDF36C-inoculated fruits but not on stems. Stems exhibited canker-like symptoms. The same fungus was re-isolated from both inoculated diseased fruits and stems, thereby confirming Koch's postulates. The pathogen was identified as *N. dimidiatum* based on its morphology, cultural characteristics, and sequences of the partial ß-tubulin gene. *In vitro* growth of *N. dimidiatum* MBDF36C was also completely inhibited by a bio-fungicide containing *B. subtilis*, isoprothiolane, and mancozeb. This study is the first report of *N. dimidiatum* causing brown fruit rot of dragon fruit in the Philippines. This information could impact the current postharvest fruit handling operations and future studies on dragon fruit disease management.

Keywords: *Bacillus subtilis* QST strain 713, brown rot, dragon fruit, fruit disease, stem canker

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Introduction
Tropical crops are famous for their delicious, water-rich taste and as a cash crop. Therefore, the market is expanding. Many countries such as the Philippines, where these tropical crops are grown, benefit from the global export market. While banana, pineapple, and papaya are the three more commonly exported fruits, minor but emerging tropical fruits are beginning to penetrate the global market. One of which is the edible cactus known as dragon fruit or Pitahaya (*Selenicereus* species) (Casas and Barbera 2002, Nobel 2002). While the dragon fruit market is relatively small compared to banana and pineapple, this budding industry is steadily and increasingly becoming popular. Vietnam is leading the world's dragon fruit export (Mercado-Silva 2018, Tel Zur 2015). Other Southeast Asian countries follow Vietnam's track (Balendres and Bengoa 2019). In the Philippines, the dragon fruit industry over the past decade has grown (Eusebio and Alaban 2018). From a small 12-hectare farm in the early 1990s, the area planted with dragon fruit has reached almost 600 hectares with a volume of production of more than 1800 mt in 2020 (Philippine Statistics Authority 2021). Dragon fruits are consumed as fresh or in various processed and preserved foods (Pascua et al. 2015). However, yield and quality

limiting factors threaten the lucrative dragon fruit industry. In addition, diseases are a significant concern (Balendres and Bengoa 2019).

There are 25 species of plant pathogens causing various diseases in dragon fruits, and more than 90 % of these pathogens are fungi (Balendres and Bengoa 2019). Diseases caused by *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers proved to be problematic in the field once spots developed into canker, leading to rotting and collapse of the stem (Chuang et al. 2012). Although stem diseases can be destructive in the field, some plants can withstand the infection and produce fruit. Therefore, a more pressing concern is understanding when the pathogens infect the fruits. Diseased fruits, e.g., those showing rot and spots, could be devalued because of their appearance or, worst, rejected by buyers or consumers. Diseased fruits may be rejected during export due to the biosecurity rules of the importing countries. The common fungal pathogens that attack dragon fruits are *Bipolaris cactivora* (Taba et al. 2007, Tarnowski et al. 2010) and *N. dimidiatum* (Lan et al. 2012, Ezra et al. 2013, Yi et al. 2015), but no fruit disease has yet been scientifically reported in the Philippines. Fruit diseases are better addressed when the cause of the disease is identified.

Chemicals have been used to control major dragon fruit diseases, including those caused by *N. dimidiatum* (Balendres and Bengoa 2019), but given the growing concern over the use of synthetic pesticides and some preference of consumers to organically grown fruits, biopesticides, alongside field hygiene practices, are likely the preferred disease control management. However, some biopesticides can have a phytotoxic effect on fruits (Aifaa et al. 2013). Biopesticides based on microbial antagonists may fill this gap. *Bacillus subtilis* strains have been used against *Bipolaris cactivora* (Bae et al. 2013) and on anthracnose pathogens (Meetum et al. 2017) of dragon fruits. However, reports on large-scale commercial applications are scarce. Testing commercially available biofungicides could expedite the selection of relatively safer chemicals for disease management of dragon fruit.

A diseased fruit of the red-skin, red-fleshed dragon fruit (*S. monacanthus*) from San Juan, Science City of Muñoz, Nueva Ecija, Philippines, was submitted to the laboratory for diagnosis and subsequently to identify the causative agent. Anecdotal claims of fruit diseases in dragon fruits in the country have been made, but are not documented. The disease symptoms have also been linked to fungal pathogens, as reported in the literature. Nevertheless, no empirical evidence to support such claims has been presented. Moreover, insect pests could damage the fruit, and these insect-damaged tissues could be erroneously identified as a fruit disease. This study aimed to isolate and characterize the causative agent of the diseased fruit using combined morpho-cultural and molecular characterization methods.

Materials and Methods

Source of diseased fruit and fungal isolation

The diseased fruit of *S. monacanthus*, showing complex symptoms ranging from white to brown spots, canker-like lesions, browning, and rot, was obtained from San Juan, Science City of Muñoz (15.7295° N, 120.8729° E), Philippines. Fungi were isolated following the method of Balendres et al. (2020). Briefly, infected-fruit tissues (2×2 mm in size) were sterilized in 10 % Sodium hypochlorite solution (v/v, Zonrox, GreenCross Philippines), then washed three times with sterile distilled water at 1 min and 30 sec each. Fruit tissue-cuttings were then blot-dried in sterile tissue paper. Once dried, fruit tissue-cuttings were plated in potato dextrose agar (PDA) medium (HiMedia Laboratories Pvt Ltd, India) and incubated at 28 °C for 7 days. Five fungal isolates (designated as Iso1 to Iso5) were obtained that differed in cultural growth in the PDA medium. However, the pathogenicity assay (see the section below) indicated that only Iso5 was consistently causing disease on the test fruits. Hence, only Iso5 was further assessed and studied. The fungus (designated as isolate MBDF36C) was deposited at the Fungal Repository of the Plant Pathology Laboratory, Institute of Plant Breeding (IPB), College of Agriculture, and Foods Science, University of the Los Baños, Laguna, Philippines.

Morphological and Cultural Characterizations

A pure culture of isolate MBDF36C was obtained and subsequently grown in PDA medium. The fungal morphology and cultural characteristics were assessed following the procedure used by Balendres et al. (2020).

Molecular Identification

Fungal genomic DNA was extracted using the procedure of Doyle and Doyle (1987) and Cullings (1992). The DNA sequence of the partial ß-tubulin gene was amplified following the Polymerase Chain Reaction (PCR) procedure used by Dela Cueva et al. (2018) using primer pair, T1 (5′ AAC ATG CGT GAG ATT GTA AGT) and T22 (5′ TCT GGA TGT TGT TGG GAA TCC) (O'Donnell and Cigelnik 1997). The amplification started with a 3 min initial denaturation at 95 °C, followed by 30 cycles of 94 °C for 1 min, 52 °C for 30 s, 72 °C for 1 min, and a final extension step of 72 °C for 10 min (Dela Cueva et al. 2018). The PCR product was sent to Apical Scientific Sdn. Bhd. (Malaysia) for sequencing and the consensus DNA sequence was then derived from the forward and reverse DNA sequences using the sequence editing software Geneious R9 (Biomatters, New Zealand). First, to identify the pathogen based on % sequence identity, an analysis was performed using the BLASTN program (Zhang et al. 2000) to determine the closest fungal species. A phylogenetic tree was then constructed using the Maximum Likelihood (ML) method based on the T92 (Tamura-3) parameter (Tamura and Nei 1993) with uniform rates of nucleotide substitution supported with 1000 bootstrap replicates (Felsenstein 1985). The analysis was conducted in MEGA version 7 (Kumar et al. 2016). *Neofusicoccum parvarum* CBS 112930 (GenBank No. KX464983) was used as an outgroup.

Pathogenicity Assay

Ripe fruits and healthy young stem cuttings of *S. monacanthus* used in the pathogenicity assays were sourced from the dragon fruits plants maintained at the IPB. These fruits and stem cuttings were not sprayed with any chemicals. In the detached fruit assay, fruits were washed with tap water, surface-sterilized in commercially available 10 % Sodium hypochlorite solution (v/v, Zonrox, Green Cross, Philippines), and washed three times with distilled water before air drying. Fruits were then placed in a plastic container overlaid with moist tissue paper. Three replicate fruits were used. Each fruit had six wound-inoculated sites (pricked using pipette tip), corresponding to 5 fungal treatments and a distilled water control treatment. 10 µl of spore suspension (>10^6 spores/ml) from seven-day old fungal cultures was injected using a pipette into the inoculation sites. The container was sealed with the plastic cover, and disease development was assessed seven days post-inoculation (dpi). In *in vivo* test, the pathogenicity of *N. dimidiatum* MBDF36C was assessed on three-week-old rooted-stem cuttings of *S. monacanthus* in the screenhouse. The same volume of spore suspension (prepared as above) was inoculated on pre-determined wounded sites. The inoculated sites were then covered with transparent tape to ward off insects or ants from entering the wound and avoid the spore suspension's rapid evaporation. Wounded sites inoculated with distilled water served as the negative control. Disease development was assessed at seven dpi. Re-isolation of the fungus from the diseased fruits and stems was performed to establish Koch's postulates using the same method as described earlier.

In vitro *Chemical Assay*

The effect of a biofungicide containing either *B. subtilis* QST 713, isoprothiolane, mancozeb, citronella oil or propamocarb was assessed *in vitro* using the poisoned food method (Grover and Moore 1962). A five-mm mycelial plug of *N. dimidiatum* MBDF36C was placed at the center of the PDA medium (in Petri plates) containing recommended rates of the test chemicals (Table 1). Pyraclostrobin (Xu et al. 2018) was used as the chemical control. The PDA medium with sterile distilled water only (no chemical) was used as the negative control. The trial was replicated three times. Mycelial growth was measured at three dpi when the fungal growth in the negative control reached the edge of the plate.

The mean percent inhibition was computed, and data were analyzed by the variance (ANOVA) test. Multiple comparisons of means were carried out using Tukey's HSD test.

Table 1. Mean percent growth inhibition of *Neoscytalidium dimidiatum* MBDF36C in potato dextrose agar (PDA) medium amended with sterile distilled water (negative control) and various chemicals at three days post-incubation.

Chemical Treatment	Rate Used[1]	Mean % Growth Inhibition[2]
Pyraclostrobin (chemical control)	1 mL	100 (±0.00)[a]
Bacillus subtilis strain QST 713	2 mL	100 (±0.00)[a]
Isoprothiolane	2.25 mL	100 (±0.00)[a]
Mancozeb	2 g	100 (±0.00)[a]
Citronella oil[3]	1.25 µl	72.22 (0.79[b]
Propamocarb	1.6 mL	0 (±0.00)[c]
Distilled water (negative control)[4]	-	0 (±0.00)[c]

[1]Based on the recommended rate as per product's label and adjusted to rate per 400 mL dH_20 (capacity of Schott bottle). [2]As compared to the fungal growth in PDA medium sterile dH_20, only *in vitro* food poison technique assay (see Materials and Methods). [3]Based on the rate used by Dela Cueva and Balendres (2018).[4]No chemicals added.
Values are mean±standard error of the mean. Means followed by the same letter within the column are not significantly different at 0.05 level by Tukey's HSD test.

Results

Characteristics and Identity of MBDF36C

Isolate MBDF36C was fast-growing, reaching the edge of the PDA medium just after three days of incubation. The arthroconidia were hyaline to dark brown (Figure 1a), thick-walled, one-celled, rod-shaped, and occurred singly or in arthric chains. Initially, the fungus was white with dense white aerial mycelium (Figure 1b) that gradually turned grey to dark with age, usually at seven days from culture (Figure 1c). These morpho-cultural characteristics are those of *Neoscytalidium dimidiatum.* The identity of fungal isolate MBDF36C was further confirmed by the amplification and sequencing of the fungal TUB2 gene. First, the BLASTN search showed a 99.30 % sequence identity with several isolates of *N. dimidiatum* that cause canker or die-back (KF778965, KC357307). The constructed phylogenetic tree based on the ML method supported the identity of MBDF36C as *N. dimidiatum* (Figure 2).

Pathogenicity of N. dimidiatum *MBDF36C*

Only *N. dimidiatum* MBDF36C caused consistent brown rotting symptoms in all three fruit replicates (Figure 1d). Browning started at two dpi from the inoculated sites, which grew larger as days progressed. Despite *N. dimidiatium* being a pathogen known to cause canker or die-back symptoms, no canker-like symptom was observed in the inoculated and wounded sites. However, typical canker symptoms developed at seven dpi on stems in the screen house. No disease or symptoms were manifested in the distilled water-inoculated sites. The same fungus was re-isolated from the diseased fruits and stems from the laboratory and screenhouse assays, thus confirming Koch's postulates.

Inhibition of N. dimidiatum *MBDF36C Growth by Chemicals*

Growth of *N. dimidiatum* MBDF36C in the PDA medium was significantly ($P<0.005$) and completely inhibited by the biofungicide containing either *B. subtilis*, isoprothiolane, and mancozeb (Table 1), which were assayed separately. Substantial and significant growth inhibition (72.22 %) was observed in the PDA medium amended with citronella oil. Propamocarb was not effective against *N. dimidiatum* MBDF36C (growth rate was similar to the control treatment at 90 mm in diameter). The effect of the bio-fungicide containing *B. subtilis*, isoprothiolane, and mancozeb was comparable to that of pyraclostrobin (chemical check).

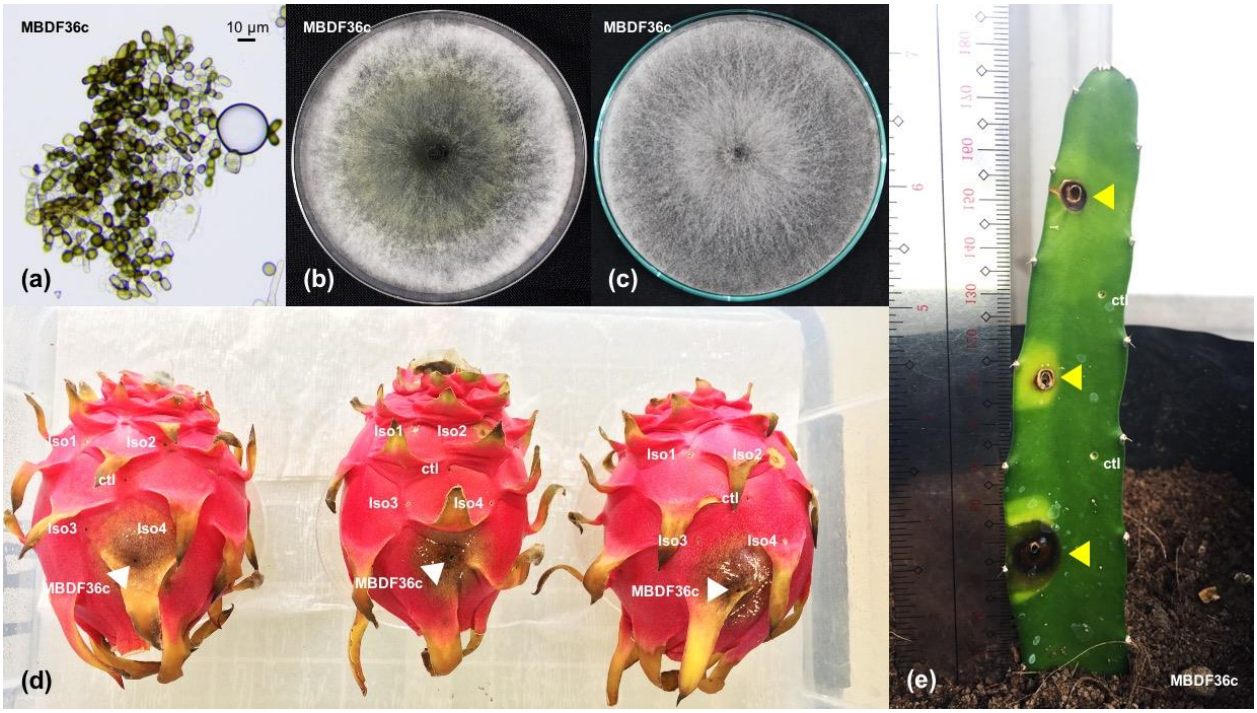

Figure 1. Morphology (a) and cultural characteristics of 3- (b) and 7-day old (c) *Neoscytalidium dimidiatum* MBDF36C in potato dextrose agar (PDA) medium. Inoculation of the pathogen resulted in browning on fruits (d) and canker on the stem (e) within the inoculation point (arrowed). No browning was observed on inoculation points with other fungi (Iso1 to Iso4) and in control (ctl, distilled water) treatments.

Discussion

Bipolaris cactivora (Petr.) Alcorn (Taba et al. 2007, Tarnowski et al. 2010) and *Neoscytalidium dimidiatum* (Lan et al. 2012, Ezra et al. 2013, Yi et al. 2015) have been reported to cause brown spots and rot on fruits of *Selenicereus monacanthus* and *S. undatus*. In this study, fruit brown rot in *S. monacanthus* was caused by *N. dimidiatum* MBDF36C. Black rot and brown rot have been previously reported in Israel (Ezra et al. 2013) and China (Yi et al. 2015). Both diseases were found in *S. undatus* (red skin, white flesh). Here, fruit browning is reported in the Philippines for the first time on the red skin, white-fleshed, *S. monacanthus.* The causative agent, *N. dimidiatum* MBDF36C, also causes canker on dragon fruit stems.

These morphological characteristics of *N. dimidiatum* MBDF36C were consistent with the description of *N. dimidiatum* (Penz.) Crous & Slippers (Crous et al. 2006). This pathogen is also known as *N. hyalinum* (Phillips et al. 2013). However, when Huang et al. (2016) described a related species, *N. novaehollandiae,* the name of the former was changed back to the old name, which is *N. dimidiatum.* Both, nonetheless, are phylogenetically related (Phillips et al. 2013). This pathogen, *N. dimidiatum*, is notoriously known to cause stem canker on dragon fruits (Chuang et al. 2012, Hawa Mohd 2013, Xu et al. 2018), resulting from the collapsing of the plant (Chuang et al. 2012). In this study, the *N. dimidiatum* MBDF36C isolates can also infect the stem and cause canker-like symptoms in *S. monacanthus.* The result suggests that the inoculum during fruit infection likely came from infected stems. Indeed, *N. dimidiatum* can also cause a canker on fruits (Sanahuja et al. 2016, Taguiam et al. 2020).

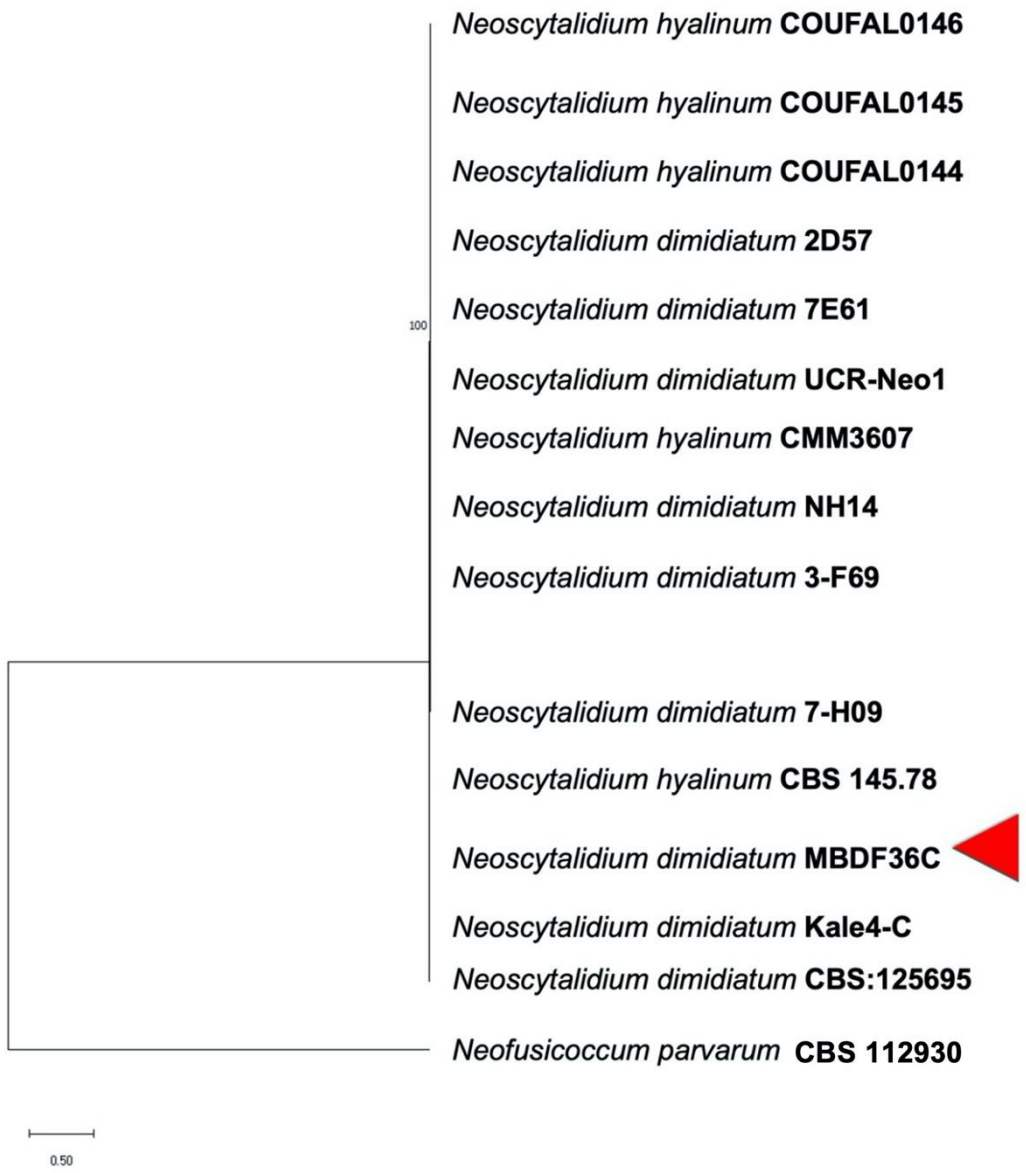

Figure 2. Phylogenetic analysis by Maximum Likelihood (ML) method using the TUB2 sequence of *Neoscytalidium dimidiatum* MBDF36C (arrowed) Philippine isolate (red arrowhead) and other *N. dimidiatum* (syn. *N. hyaline*) isolates. The evolutionary history was inferred using the ML method based on the T92 (Tamura-3) parameter (Tamura and Nei 1993) with uniform rates of nucleotide substitution supported with 1000 bootstrap replicates (Felsenstein 1985). The analysis was conducted in MEGA version 7 (Kumar et al. 2016). The tree was rooted to *Neofusicoccum parvarum* CBS 112930 (GenBank No. KX464983).

Chemicals are used to mitigate severe infection of dragon fruit diseases in the field. Nevertheless, information on what chemicals and their effectivity are not widely reported. Azoxystrobin and difenoconazole have been shown to control anthracnose and stem canker (Noegrohati et al. 2019). Hexaconazole, tebuconazole, flusilazole, and pyraclostrobin inhibit *N. dimidiatum* growth (Xu et al. 2018). These chemicals are known to suppress the spore germination and fungal growth either by affecting the fungal cell walls or inhibiting mitochondrial respiration. In this study, isoprothiolane and mancozeb completely inhibited the growth of *N. dimidiatum* MBDF36C. These results were comparable to pyraclostrobin and corroborated with the previous findings of Xu et al. (2018). While the result promises chemicals that can be used for control, it is not without a disadvantage from an environmental and health perspective. Therefore, attempts have been made to select a relatively eco-friendly approach, e.g., biopesticides in the form of essential oils.

Cymbopogon essential oil has been previously used to control dragon fruit anthracnose (Aifaa et al. 2013). However, essential oil higher than 2 % was phytotoxic and was not recommended for treatment. Why a relatively higher concentration is phytotoxic remains unknown but it might have something to do with the complex interactions of multiple chemical components in the essential oil that need to be characterized (Brokl et al. 2013). In this study, *N. dimidiatum* MBDF36C growth was strongly inhibited by citronella essential oil at a 1.25 uL/mL concentration. In another study, Dela Cueva and Balendres (2018) found that citronella essential oil higher than 1.25 uL/mL concentration, although helpful in mitigating anthracnose symptoms, can negatively affect the quality of pepper fruits. Thus, future studies involving citronella essential oil in dragon fruit disease management would need to include the effect of the oil on the quality of the fruits and stems. Biological control agents, e.g., *Bacillus subtilis*, have also been shown to inhibit fungal growth. They can also reduce the virulence of anthracnose pathogens (Meetum et al. 2017). In this study, a bio-fungicide containing *B. subtilis* QST 713 was found to inhibit the *in vitro* growth of *N. dimidiatum* MBDF36C completely. The inhibitory-growth effect was comparable to the chemical check, pyraclostrobin. However, the *in vitro* assay may not reflect the actual efficacy of the biofungicide in the field. Hence, future screenhouse and field studies would need to incorporate the comparison of rate, timing, and frequency of biofungicide application. Information could lead to a better understanding of how to maximize the use of the biofungicide to manage diseases (stem canker and fruit brown rot) caused by *N. dimidiatum*. Propamocarb did not inhibit the growth of the pathogen and the mechanism is yet to be determined.

In conclusion, *Neoscytalidium dimidiatum* MBDF36C causes fruit rotting and stem canker in dragon fruit. The fungal pathogen was identified using morphological, cultural, pathogenicity, and molecular characterization. This study also demonstrates the potential of the biofungicide containing *B. subtilis* in managing diseases caused by *N. dimidiatium.* However, further screenhouse and field trials are warranted to further underpin the chemical's efficacy.

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Statement on conflict of interest
The authors declare that they have no conflict of interest.

Authors contribution
MAB wrote the manuscript, analyzed the data, conceived and designed the research study. JDT and EE conducted the experiments, analyzed the data, and co-wrote the manuscript. JE and CC collected the samples and co-wrote the manuscript.

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