Biological Control of Stem Canker in Red-Fleshed Dragon Fruit (*Hylocereus polyrhizus*) by Emulsion Formulation of *Trichoderma asperelloides* PSU-P1

Kim Sreang Dy

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Plant Pathology
Prince of Songkla University
2023

Biological Control of Stem Canker in Red-Fleshed Dragon Fruit (*Hylocereus polyrhizus*) by Emulsion Formulation of *Trichoderma asperelloides* PSU-P1

Kim Sreang Dy

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master Science in Plant Pathology
Prince of Songkla University
2023

Thesis Title	Biological Control of Stem Canker in Red-Fleshed Dragon Fruit (*Hylocereus polyrhizus*) by Emulsion Formulation of *Trichoderma asperelloides* PSU-P1
Author	Miss Kim Sreang Dy
Major Program	Plant Pathology

Major Advisor

.....
(Assoc. Prof. Dr. Anurag Sunpapao)

Examining Committee :

.....Chairperson
(Asst. Prof. Dr. Prisana Wonglom)

.....Committee
(Assoc. Prof. Dr. Anurag Sunpapao)

.....Committee
(Asst. Prof. Dr. Chaninun Pornsuriya)

.....Committee
(Asst. Prof. Dr. Thanunchanok Chairin)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Master of Science Degree in Plant pathology.

.....
(Asst. Prof. Dr. Thakerng Wongsirichot)
Acting Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

.....Signature
(Assoc. Prof. Dr. Anurag Sunpapao)
Major Advisor

.....Signature
(Miss Kim Sreang Dy)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

.....Signature
(Miss Kim Sreang Dy)
Candidate

Thesis Title	Biological Control of Stem Canker in Red-Fleshed Dragon Fruit (*Hylocereus polyrhizus*) by Emulsion Formulation of *Trichoderma asperelloides* PSU-P1
Author	Miss Kim Sreang Dy
Major Program	Plant Pathology
Academic Year	2022

ABSTRACT

Stem canker of red-fleshed dragon fruit (*Hylocereus polyrhizus*) was observed on dragon fruit plantation field in Phatthalung province, southern Thailand. The disease samples were collected and isolated by tissue transplanting method. Pathogenicity was conducted on healthy cladodes of dragon fruits by agar plug method and revealed the fungal isolate PSU-SC02 caused stem canker on cladodes of *H. polyrhizus*. Fungal isolate PSU-SC02 was identified based on morphology and molecular properties of double DNA sequences of internal transcribed spacer (ITS) and β-tubulin (*tub*) as *Neoscytalidium dimidiatum*.

Biological control by *Trichoderma* species are widely used to control several plant diseases. However, environmental conditions affect bio-control agent in the field, development of appropriate formulation is an alternative way to solve this problem. This research aimed to develop a new *Trichoderma asperelloides* PSU-P1 formulation that would be more effective against *N. dimidiatum*. Three vegetable oils, two emulsifier-dispersing agents (Tween 20 and Tween 80) and one source of carbon (dextrose) were tested for carrier additive. Viability and antifungal ability of formulation incubated at ambient temperature and at cool temperature (10^{o}C) were compared. A total of 12 emulsions based was tested for viscosity by evaluating the mixing time. The formulation composed of coconut oil, DW and tween 20 in a ratio of 30:60:10, respectively, showed the great mixing time of 1.14 min significantly lower than that of other formulations ($p<0.05$).

Application of this formulation suppressed canker development with 0.53 cm^2 symptom area compared with control (pathogen alone) 1.65 cm^2. Viability of *T. asperelloides* PSU-P1 conidia was observed by percentage of surface area in PDA plates at day 3, whereas percentage inhibition was observed on colony radii of *N.*

dimidiatum. Viability of this formulation stored at ambient temperature showed percentage of surface area of *T. asperelloides* PSU-P1 ranged 58.08–100% whereas formulation stored at cool temperature showed 70.59–100%. This formulation effectively inhibited *N. dimidiatum* in PDA plates with percentage inhibition rage from 50–77% and 54.2–76.19%, respectively in the same manner with viability. This finding suggested that the emulsion formulation developed in this study was appropriated to store *T. asperelloides* PSU-P1 conidia with high viability and antifungal ability up to 6 months.

Keywords : Morphology, molecular, pathogenicity test, oil based, bio-formulation

Acknowledgement

I would like to express my gratitude to my advisor, Assoc. Prof. Dr. Anurag Sunpapao for this generosity, guidance, innumerable advice, patient, support, encouragement much appreciated, valuable times, and critical comments. I do appreciate your constructive criticisms and discussion through this research work toward the successful completion of this study. I would like to thank the chairperson committee Asst. Prof. Dr. Prisana Wonglom, Assoc. Prof. Dr. Anurag Sunpapao, Asst. Prof. Dr. Chaninun Pornsuriya, and Asst. Prof. Dr. Thanunchanok Chairin for valuable comments, suggestions, and valuable times to the success of this thesis.

I am grateful acknowledgment to the Royal Scholarship under Her Royal Highness Princess Maha Chakri Sirindhorn Education Project to the Kingdom of Cambodia for awarding a scholarship, and the Center of Excellence in Agriculture and Natural Resources Biotechnology (CoE-ANRB) phase 3 research grant, and Agriculture Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University for financial supports throughout this research.

I sincerely appreciate all staffs of the Faculty of Natural Resources, especially Mrs. Sririporn Prompat and Mrs. Parittiya Saejew, International Relations Officers, for the assistance given, kindness, and caregiving for my living in Thailand. Sincerely thanks to Mrs. Paweenuch Chootong for offering great help, providing the information, documents through my study.

Special thanks to my seniors, Miss Suchawadee Kheawleng for helping, guiding, and sharing experiences. Many thanks to my lab mates, and friends for a good friendship, helps, times, and unforgettable memories together during my study. Finally, I wish to thank my beloved family for always supports, well-known with love, and encouragement.

Kim Sreang Dy

Contents

List of Tables

List of Figures

Lists of Published Papers and Proceedings

This thesis contains general summary (introduction, background, information and conclusion) and the following papers which are referred indicated by their roman number.

Research article

Dy, K.S., Wonglom, P., Pornsuriya, C. and Sunpapao, A. 2022. Morphological, molecular identification and pathogenicity of *Neoscytalidium dimidiatum* causing stem canker of *Hylocereus polyrhizus* in southern Thailand. Plants. 11: 504.

Reprint ware made with permission from the publishers

Introduction

Dragon fruit is an economically important crop that could support additive income for many households in Thailand. Saradhuldhat et al. (2009) demonstrated that dragon fruits can grow in practically any type of soils, both upland and lowland in Thailand, and distribute throughout Thailand. It has been known that Thailand locates in tropical and subtropical region where the weather favors for pathogen germination and disease spread (Wonglom et al., 2018). However, study on identification of disease on red-fleshed dragon fruit is rarely limited.

Red-fleshed dragon fruit (*Hylocereus polyrhizus*) belongs to Cactaceae family of climbing cactus. The suitability of tropical climate, rainfall requirements, and soil types may have contributed to grow dragon fruit. According to increasing planting areas and high demand, repetitive dragon fruits in many crops may attract to diseases and pests. There are several diseases have been reported to cause negatively impact on dragon fruit plantation and production (Van et al., 2016). Disease caused by fungi is a major problem for dragon fruit plantation worldwide. For instance, the fungus *Colletotrichum gloeosporioides* was found to cause anthracnose on dragon fruits in Malaysia (Mohd et al., 2013). The fungi *Neoscytalidium dimidiatum* and *Bipolaris* sp. has been reported to cause canker and *Bipolaris* black spot, respectively on dragon fruits in Vietnam (Nguyen and Nguyen, 2015).

Pesticides are used widely in agriculture sector. Those chemical compounds are used by farmers worldwide to protect crops from insects, weeds and diseases. Pesticides are harmful to humans in many ways. Pesticide formulation and application, inadvertent or incidental exposure and residues in food products can have negative health effects (Thapinta and Hudak, 2000). To reduce the intensive use of pesticides, it is a critical need to promote the effective bio-pesticides or biological agents to control agricultural pests in order to replace the chemical pesticides.

Trichoderma species have been found as a successful bio control agent in controlling plant diseases (Kolombet et al., 2008). Using biological control by *Trichoderma* spp. significantly prevents the growth of plant pathogenic microorganisms and regulates the growth rate of plant (Zin and Badaluddin, 2020). Several *Trichoderma* spp., such as *Trichoderma asperellum*, *T. harzianum, T. polysporum, T. viride* and *T. virens* have already been used successfully as biological control agents against a variety of phytopathogenic fungi (Mbarga et al., 2014). *Trichoderma asperelloides* PSU-P1 have been described by previous research as a strong antifungal mechanism against *Stagonosporopsis cucurbitacearum* the pathogen of muskmelon gummy stem blight (Ruangwong et al., 2021).

Formulation development of biological control agents is critical in the production process because it protects the active ingredients (conidia or mycelia of antagonist fungus) from severe pH, low humidity, chemicals, and UV radiation (Locatelli et al., 2018). There are different formulas that can be used to develop formulation such as wettable powder, soluble powders, granules, water in oil emulsions and oil in water emulsions. But solid products or formulation have certain limitation due to the viability of the spores are less and population comes down with storage (Suseela and Anandaraj, 2014). Some research have been suggested should be concentrated the shelf life of the formulation by developing into emulsion to increase shelf life, maximum shelf life with low level of contaminants must be standardized venture. The stored living microorganisms, such as spores, chlamydospores, and fragmented mycelium, can be used in various formulations such as liquid, granules, or powder, and stored for months without losing their efficiency. According to the previous studies (Batta, 1999; 2004), the bio-control potential of the fungus *T. harzianum* (strain Th2), mainly in formulated form in an inverted emulsion, against *Botrytis cinerea* on strawberry leaves and apple fruit. Liquid formulations enable to ensure prolonged shelf life (Sriram et al., 2018), which abilities to keep shelf life

of up to 6 to 9 months (Kolombet et al., 2008) and oil based formulation displayed shelf life longer up to 18 weeks (Rai and Tewari, 2016). However, environmental condition also effect on the abilities and shelf life of active ingredients in formulation (mycelium or conidia), most of farmers in Thailand keep their bio products in both of ambient and cool temperature. Hence, in this study aimed to know the effect of temperature conditions could maintain or enhance the ability of bio products.

Objectives

1. To know the corrected species of pathogen causing stem canker on red-fleshed dragon fruit
2. To receive the most effective formulation of *Trichoderma asperelloides* PSU-P1 against stem canker disease of red-flesh dragon fruit

Result and Discussion

1. Identification of pathogenic fungi causing on red-fleshed dragon fruit

Canker disease caused by *Neoscytalidium* species was observed as the most destructive disease for dragon fruit plantation in Phatthalung province, southern Thailand (Fig. 1). The symptom showed small sunken arrange and later became bigger with arising of plant tissue and mass of black pycnidia (Fig. 1). In this study *Scytalidium*-like fungal isolate PSU-SC02 was isolated from infected tissues. Morphology and molecular tool was used to identify fungal pathogen into species level, and pathogenicity test by agar plug method was conducted to fulfill Koch's postulates. Pathogenicity test showed that after incubation, at day ten black pycnidia developed on surface of *H. polyrhizus* cladodes, and the disease became severe when incubated for 14 days (Fig. 2). Re-isolation by tissue transplanting method revealed the morphology of isolated fungus was similar to PSU-SC02.

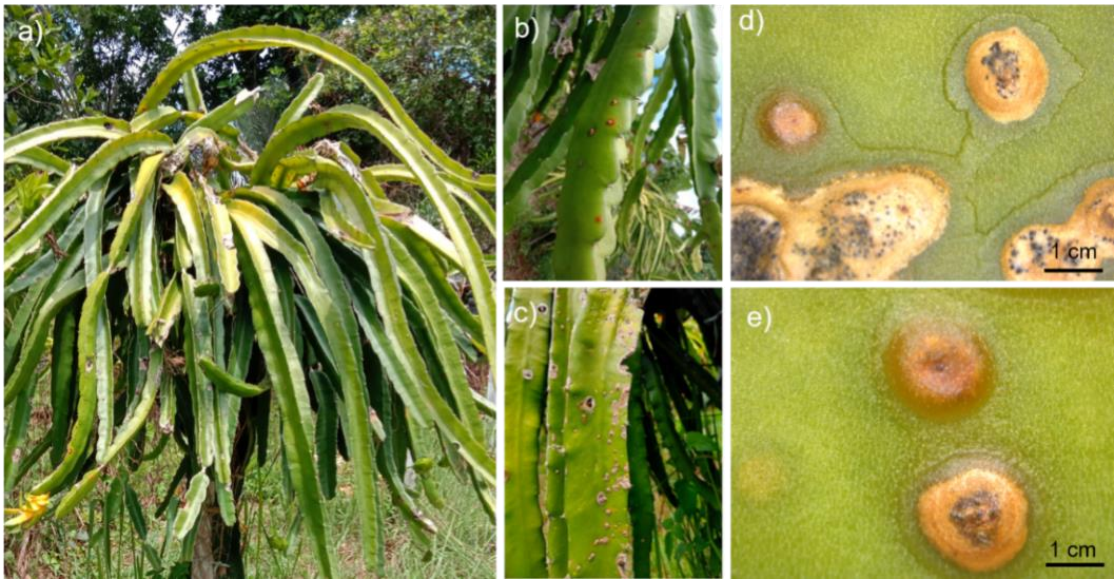

Figure 1 Stem canker disease in dragon fruit plantation (a), whitish yellow spot (b), spots coalesce to form larger spots or lesions (c), pynidia on cladodes of red-fleshed dragon fruit (d and e).

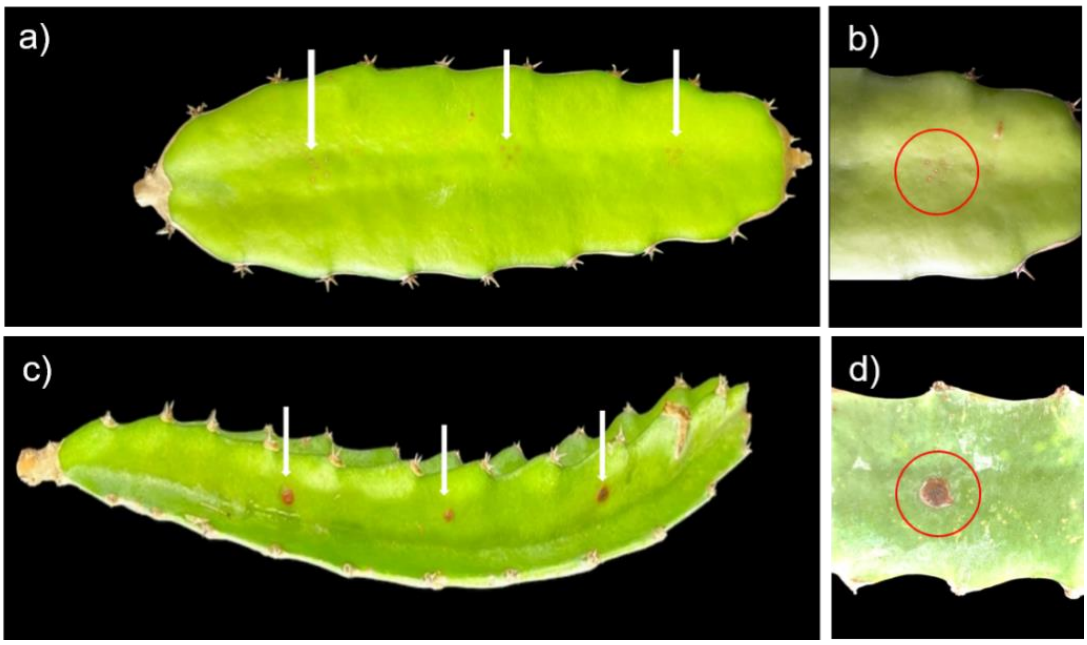

Figure 2 Pathogenicity test PSU-SC02 on red-fleshed dragon fruit cladodes, control (a) and in zoom view (b), inoculated by PSU-SC02 (c) and in zoom view (d). Arrows indicate inoculation points and cycles indicate symptom development.

Morphology characteristic of fungal isolate was observed by Leica S8AP0 stereo microscope and a Leica DM750 compound microscope (Leica Microsystems, Wetzlar, Germany). The isolate PSU-SC02 on PDA showed hairy colony and olive green to greyish colony with dark-grey to black pigmentation. The PSU-SC02 grew quickly and colonized PDA plate within 3 days, the growth rate was 3 cm/day. The PSU-SC02 showed *Scytalidium*-like fungus. The hyphae were brown, branched, septate, and constricted into spore chains before disarticulating into arthroconidia (Fig. 3). These morphologies were typical to *N. dimidiatum* as indicated by previous reports (Turkolmez et al., 2019).

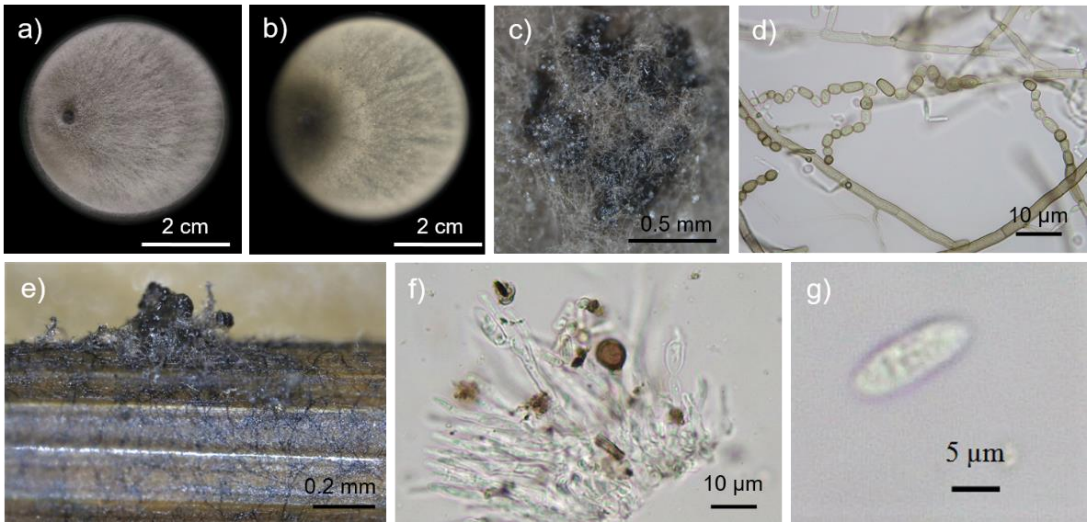

Figure 3 Morphological characteristics of PSU-SC02. Colony growth on PDA in top (a) and bottom view (b) on 3 day-old, Colony developed in to small black conidiomata after 4 week-old on PDA (c), hyphae and arthroconidia (d), pycnidia developed on dried grasses (e), conidiogenouse cells (f) and pycnidial conidia (g).

To confirm *Neoscytalidium* species into species level, double DNA sequences of ITS, and *tub* were used to analyze in this study. Blastn search revealed ITS and *tub* identical to *Neoscytalidium dimidiatum* with 99.43%, and 99.76%, respectively. DNA sequences of ITS and *tub* of PSU-SC02 were deposited in GenBank and acquired accession numbers of LC660640, and LC660642, respectively. Maximum likelihood (ML) tree of combined DNA sequences of ITS and *tub* showed that PSU-SC02 isolate was grouped in the same clade with *N. dimidiatum* 7-H09 (Fig. 4). Therefore, the fungal isolate PSU-SC02 was tentatively identified as *N. dimidiatum*.

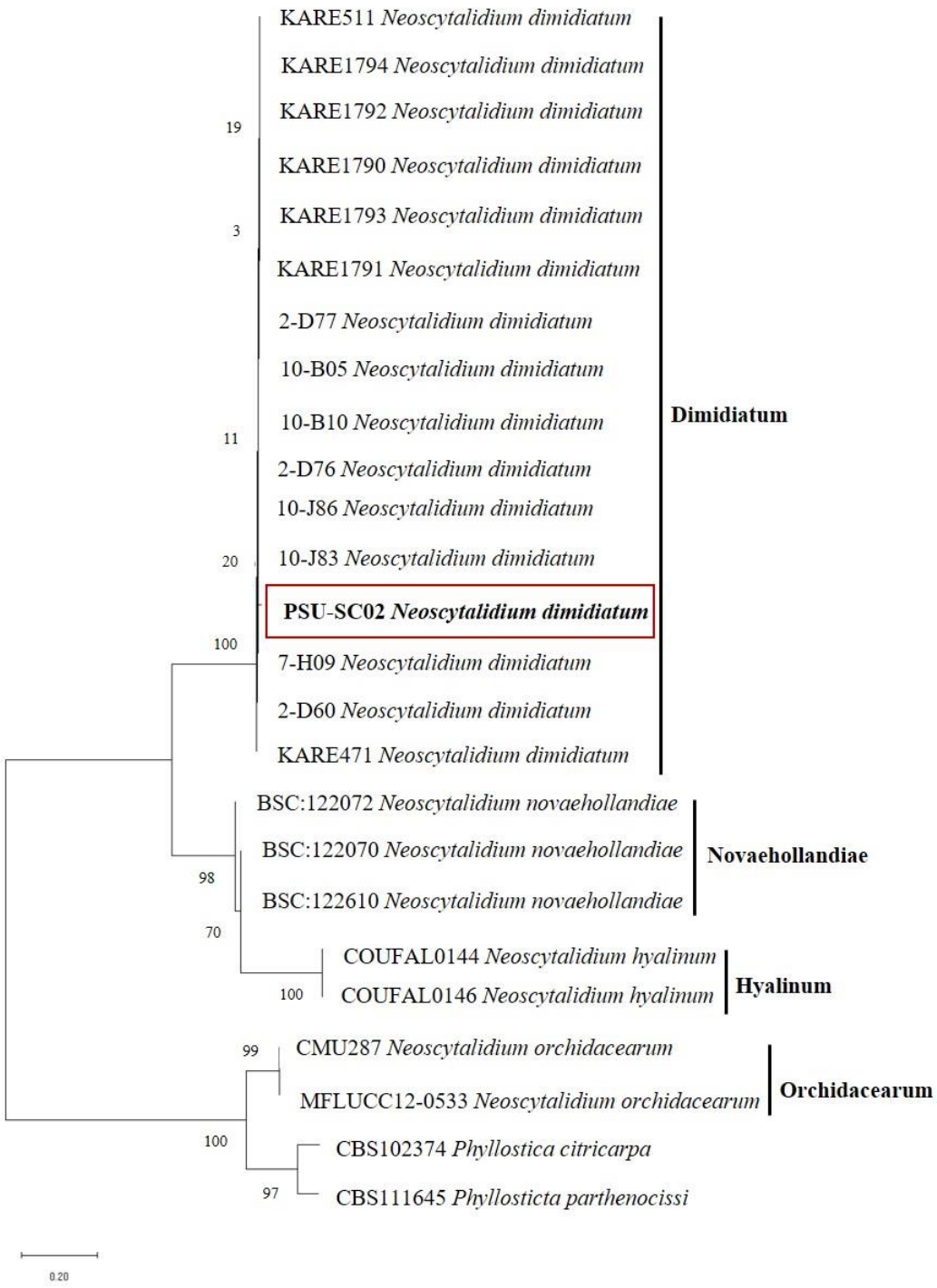

Figure 4 Phylogenetic tree of combined DNA sequences (ITS, and *tub*) of *Neoscytalidium dimidiatum.*

This finding indicated the identification of fungal pathogen by both morphology and molecular study of double DNA sequences. Mohd et al. (2013)

used morphology and single DNA sequence of ITS to identify *N. dimidiatum* causing stem canker on *H. polyrhizus* in Malaysia. Huang et al. (2016) studied morphology and phylogeny of double DNA sequences of ITS and LSU to identify new species of *Neoscytalidium* and named as *N. orchidacearum.* Furthermore, Nouri et al. (2018) also used morphology and multiple DNA sequences of ITS, translation elongation factor 1-α (*tef1-α*) and *tub* to diagnose the *N. dimidiatum* causing canker, shoot blight and fruit rot of almond in California.

2. Emulsion formulation development

To control canker disease of red-fleshed dragon fruit, this study aimed to develop formulation of *T. asperelloides* PSU-P1 conidia to control this disease. The emulsion formulation in this present study was developed as oil in water emulsion formulation (Fig. 5). Three different type vegetable oils, coconut oil, palm oil and soybean oil, were tested on the mixing times. Coconut oil spent shorter time for mixing (1.86 min) compared with other treatments (Table 1), and then coconut oil was selected for further test. Six treatments in different ratio of coconut oil and emulsifying agent showed significantly differences in statistical analysis ($P < 0.05$). The best ratio for developing as an emulation formulation was treatment No. 6 which spend minimum mixing time 1.14 min compared with other treatments (Table 2).

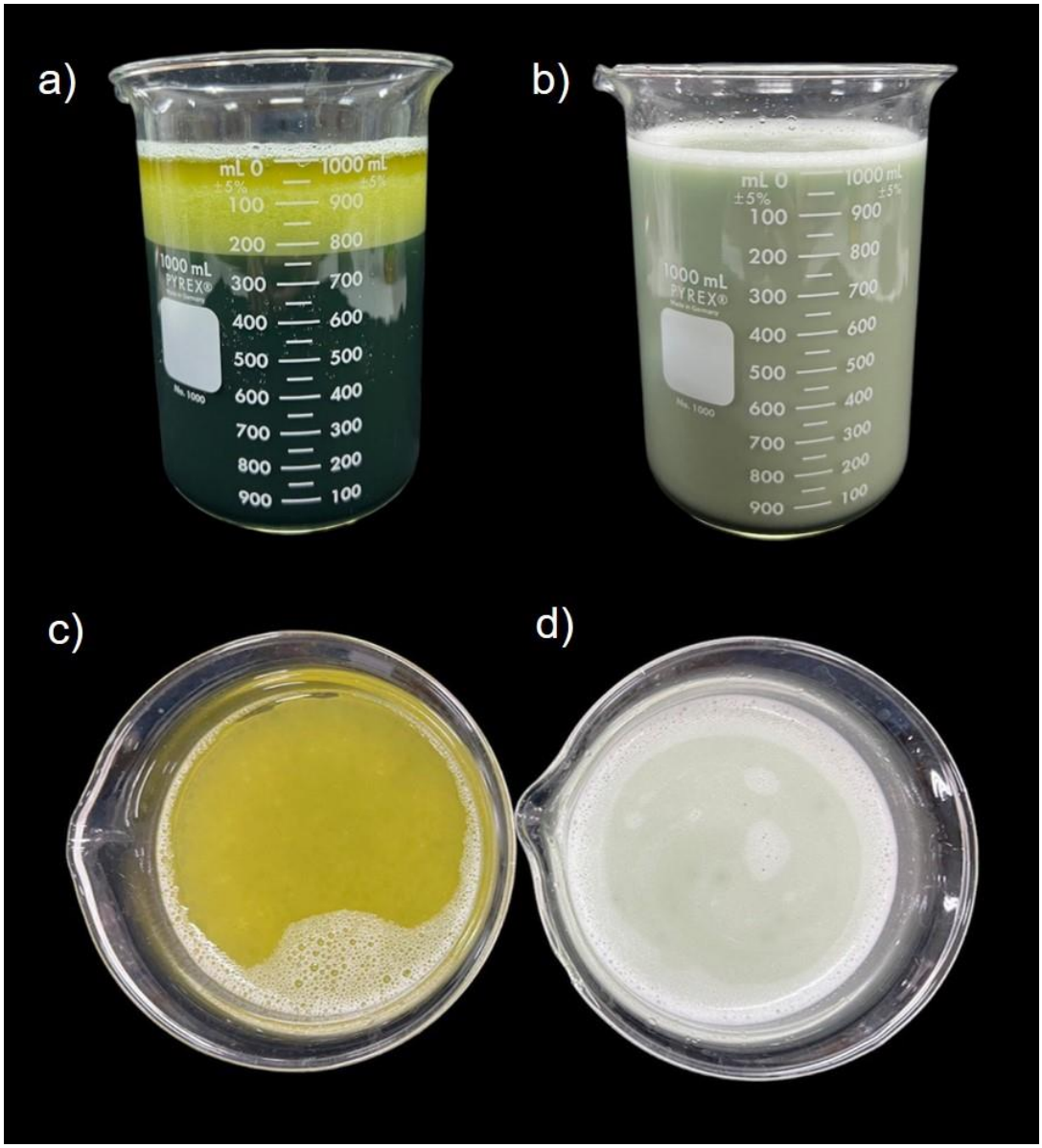

Figure 5 Emulsion formulation of *Trichoderma asperelloides* PSU-P1. At resting stage oil phase separate with distilled water (DW) phase (a and c), after mixing combination of oil and DW phase (b and d).

Table 1 Different oil and emulsifier dispersing agents for emulsion formulation preparation

Form.	Ingredient (mL)						Mixing time (min.)
	Coconut oil	Palm oil	Soybean oil	DW	Tween 20	Tween 80	
1	40	-	-	40	20	-	4.23e ± 012
2	40	-	-	40	-	20	4.46f ± 0.14
3	20	-	-	60	20	-	1.86a ± 050
4	20	-	-	60	-	20	2.84b ± 0.06
5	-	40	-	40	20	-	15.7g ± 0.13
6	-	40	-	40	-	20	15.8g ± 0.06
7	-	20	-	60	20	-	4.20e ± 0.08
8	-	20	-	60	-	20	3.57d ± 0.04
9	-	-	40	40	20	-	16.37h ± 0.01
10	-	-	40	40	-	20	16.27h ± 0.03
11	-	-	20	60	20	-	3.14c ± 0.02
12	-	-	20	60	-	20	3.57d ± 0.06

Form. = formulation. Data were analyzed using one-way analysis of variance (ANOVA) and least significant different among treatments at ($p < 0.05$).

Table 2 Mixing time of different formulation in varying of coconut oil and additive dextrose

Form.	Ingredient					Mixing time (min.)
	Coconut oil (mL)	DW (mL)	Dextrose (g)	Tween 20 (mL)	Spore suspension	
1	40	40	5	20	X	3.21±0.02d
2	40	40	-	20	X	2.29±0.06c
3	45	45	5	10	X	3.58±0.01e
4	45	45	-	10	X	2.25±0.02c
5	30	60	5	10	X	1.25±0.06b
6	30	60	-	10	X	1.14±0.06a

Form. = formulation, X= spore suspension at concentration 1×10^8 conidia/mL is suspended in DW phase. Data were analyzed using one-way analysis of variance (ANOVA) and least significant different among treatments at ($p < 0.05$).

3. Application of formulation suppressed disease development

Ability of emulsion formulation of *T. asperelloides* PSU-P1 in suppressing disease developments in dragon fruit cladodes was tested. Emulsion formulation was diluted in DW with a ratio of 1: 99. The experiment composed of four treatments: i) dropped with 25 µl distilled water alone (T1), ii) dropped with 25 µl spore suspension of *N. dimidiatum* alone (T2), iii) dropped with 25 µl spore suspension of *N. dimidiatum* 24 h and challenged with formulation (T3) and iv) dropped with 25 µl formulation alone (T4). The results showed that application of this formulation showed lesion area of canker 0.53 cm^2 which was significantly smaller than that of inoculated by pathogen alone with area of canker 1.65 cm^2 (Fig.6). Whereas in control showed no canker lesion in both inoculated with DW and emulsion formulation alone. This result suggested that emulsion formulation of *T. asperelloides* PSU-P1 suppressed disease severity of canker on dragon fruit cladodes. This result is in agreement with Syed-Ab-

Rahman et al. (2020) found that the application of emulsion formulations disease severity. Furthermore, the ability of *T. asperelloides* PSU-P1 play an importation action in this test, by defensive mechanisms and antibiosis. And sometime *T. asperelloides* PSU-P1 maybe produce some enzymes that can degrading cell walls of fungi. And some volatile organic compounds (VOCs) produced by *T. asperelloides* PSU-P1 may inhibit growth of pathogen.

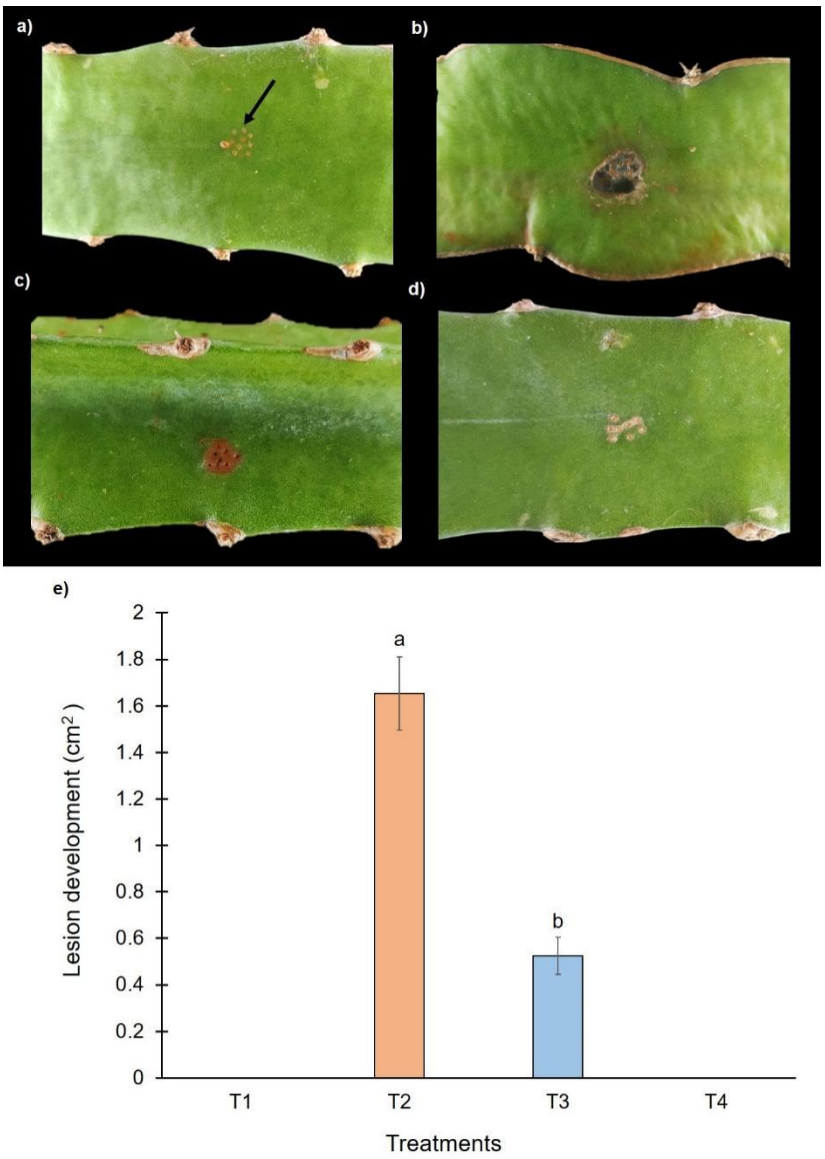

Figure 6 Suppression of canker lesion by emulsion formulation, T1 control (dropped distilled water alone) (a), T2 inoculated by *Neoscytalidium dimidiatum* alone (b), T3 inoculated by *N. dimidiatum* 24 h and challenged with formulation (c), T4 inoculated by formulation alone (d) and lesion development in each treatments. Letters indicate significantly different among treatments according to Tukey's test ($p < 0.05$). Arrow indicates inoculation points.

4. Viability of *Trichoderma asperelloides* PSU-P1 conidia in formulation

Viability of *T. asperelloides* PSU-P1 conidia was observed by colonization of surface area in PDA plates. Viability of *T. asperelloides* PSU-P1 conidia in formulation incubated at ambient temperature and at cool temperature 10°C were compared. Percentage surface area of *T. asperelloides* PSU-P1 was gradually reduced from 0 to 6 months (Fig. 7). Percentage surface area of *T. asperelloides* PSU-P1 was not significantly different between formulation incubated at ambient and cool temperature during 0–2 months. The colonized surface area of *T. asperelloides* PSU-P1 observed from third month of formulation stored at cool temperature (10°C) was 74.3% significantly higher than formulation stored at ambient temperature, with was 69.87% of total area of the petri plates over three days (Fig. 7). Viability of *T. asperelloides* PSU-P1 conidia were well grown for formulation stored at cool temperature (10°C) compared with formulation stored at ambient temperature. Percentage colonized surface area of *T. asperelloides* PSU-P1 incubated at cool temperature at six months was reduced 29.41%, significantly lower than that formulation a ambient temperature 41.92%. A similar study conducted by (Batta, 2004) showed that formulations in invert emulsions (water-in-oil formulations) based on coconut and soybean oils were found to have a viability of 36 months with a 50% reduction in viability after 5.3 months at 20°C. Several studies such as those development by Kumar et al. (2014), Peeran et al. (2014) and Mbarga et al. (2014) were also reported that the invert emulsion formulation of *Trichoderma* spp was better in prolong shelf-life of *Trichoderma* conidia and protected the contaminated from the subsurface in an environment. In this study, *T. asperelloides* PSU-P1 conidia in formulation can be stored for a long time up to half-year.

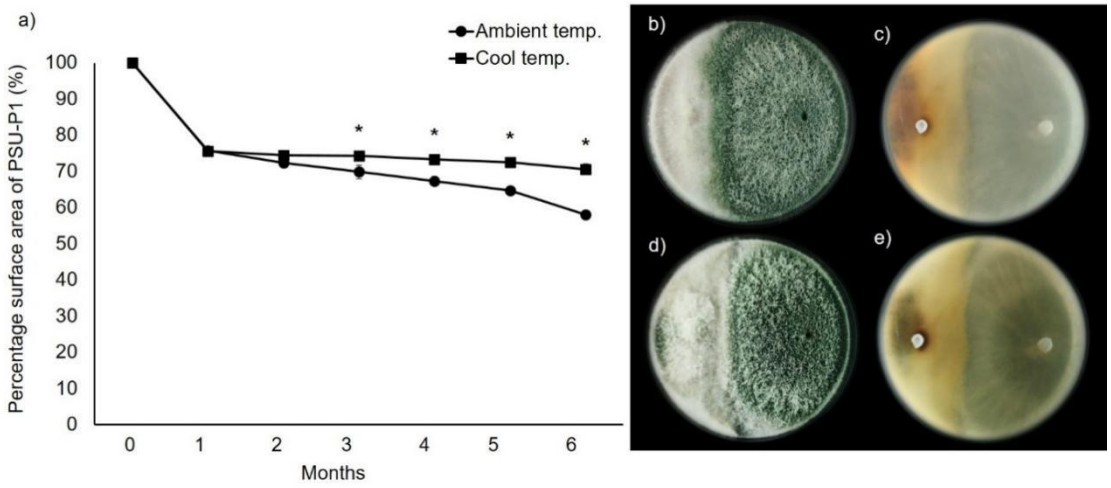

Figure 7 Viability of *Trichoderma asperelloides* PSU-P1 in emulsion formulation is observed by percentage surface area of PSU-P1 against *Neoscytalidium dimidiatum* from 0–6 months (a), colony of *T. asperelloides* PSU-P1 in formulation incubated at cool temperature in top (b) and bottom view (c), and in formulation incubated at ambient temperature in top (d) and bottom view (e). Asterisks indicate significantly different among treatments according to Student t-test ($p < 0.05$).

5. The percentage inhibition of *Trichoderma asperelloides* PSU-P1 in the formulation

The percentage inhibition of *T. asperelloides* PSU-P1 against of *N. dimidiatum* was conducted by dual culture assay on PDA plate. Percentage inhibition of *T. asperelloides* PSU-P1 in formulation incubated at ambient temperature and at cool temperature against fungal growth of *N. dimidiatum* in PDA plate from 0–6 months were compared. The result of antagonistic tests showed that *T. asperelloides* PSU-P1 inhibited the growth of *N. dimidiatum* from 0 to 6 months (Fig. 8). Percentage inhibition were not significantly different between formulation incubated at ambient and cool temperature during 0–2 months. But percentage inhibition were decreased in both temperature. By the formulation stored at cool temperature reduced 21.99% was significantly lower than that of formulation incubated at ambient temperature (27.14%) in the

periods of 6 months (Fig. 8). This may due to low temperature is suitable to preserve active spore of *T. asperelloides* PSU-P1 rather than store at ambient temperature. Błaszczyk et al. (2007) showed the ability of *T. asperelloides* PSU-P1 that inhibited the mycelium growth of pathogen *in vitro* tested and the inhibition rate ranged from 27% to 80% depend on *Trichoderma* species.

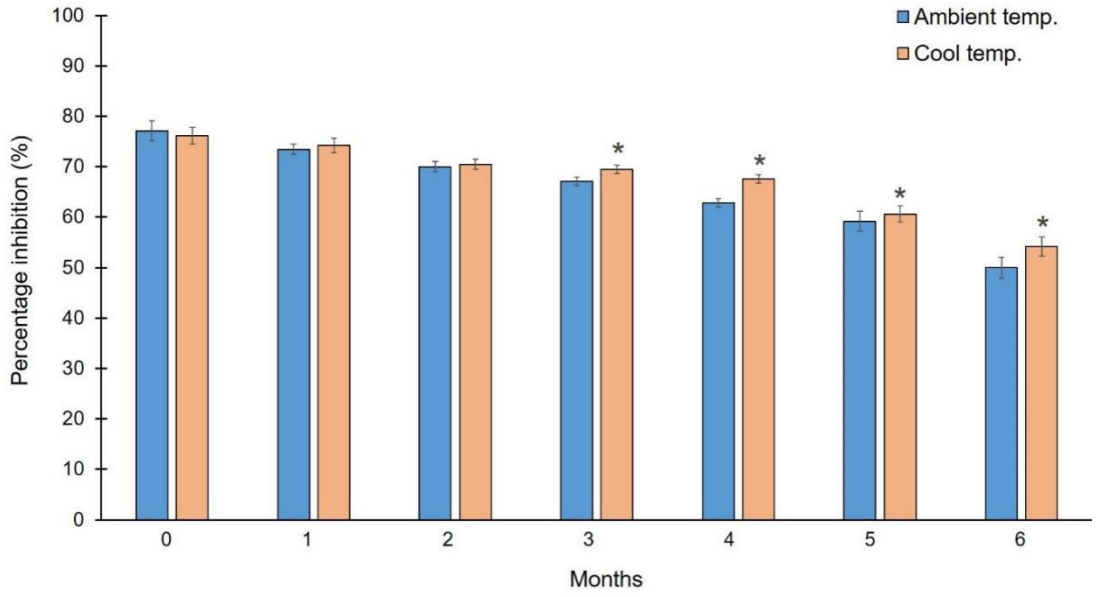

Figure 8 Percentage inhibition of *Trichoderma asperelloides* PSU-P1 in emulsion formulation were observed by colony growth rate of *T. asperelloides* PSU-P1 against *Neoscytalidium dimidiatum* from 0–6 months. Asterisks indicate significantly different among treatments according to Student t-test ($p < 0.05$).

This study is the first contribution to the development of emulsion-based formulation of the *T. asperelloides* PSU-P1 conidia as a biological control agent against *N. dimidiatum*, the pathogen of stem canker on dragon fruit. The goals of this study, to develop a formulation that would increase shelf life and persistence on dragon fruit as well as to improve protection against stem canker have been conducted. The emulsion-based formulation was selected with compose of coconut oil (30%), tween 20 (10%) and *T. asperelloides* PSU-P1 conidia in DW

(60%). Approximately 90% of formulation of antagonistic microorganisms made from active conidia of several *Trichoderma* species (Kaewchai et al., 2009). Formulation of an effective antagonistic fungus *Trichoderma* spp. has been produced and commercialized in Thailand (Kanjanamaneesathian, 2015). However, most formulations were developed as granules (Jin and Custis, 2011) or wettable powder (Fravel, 2005). Only few emulsion formulation was developed from vegetable oils. As well as, all those formulations have various abilities and lifespans. In generally, wettable powder and granule formulations have shelf life about 3 months (Jayaraj et al., 2006) and 4 to 5 months (Sriram et al., 2010). And liquid formulations have longer shelf life up to 6 months (Rai and Tewari, 2016). The most significant formulation types by value in the crop protection market are suspension concentrate, viability and shelf-life long. The same as this investigation, the emulsion formulations were created using spore suspension in high concentrations (1×10^8 conidia/mL) and could be used on a big scale in the field. Our finding of emulsion formulation howled shelf-life long up to a half year which reduced viability only 29.41% at 6 months for the formulation incubated at cool temperature (10^oC). Hence the benefit of emulsion formulation from this study is better adapted to spray without coagulated, which are the method commonly used by farmers. Conidia of *T. asperelloides* PSU-P1 as an emulsion dispersion mixed readily (ready-to-use) with water and conidia remain in suspension.

Concluding remarks

In this study, fungal pathogen causing stem canker on red-fleshed dragon fruit was identified based on morphology and molecular properties of multiple DNA sequences of ITS, and *tub* as *Neoscytalidium dimidiatum*. This finding increased number of pest list of red-fleshed dragon fruit in Thailand. Furthermore, development of a novel emulsion formulation of *T. asperelloides* PSU-P1 in combination of coconut oil and DW showed the most effective formulation against stem canker of red-fleshed dragon fruit and prolong the shelf-life of formulation with high antifungal ability up to 6 months.

References

Batta, Y. 1999. Biological effect of two strains of microorganisms antagonistic to *Botrytis Cinerea* causal organism of gray mold on strawberry. An-Najah University Journal for Research-b Humanities. 13: 67-83

Batta, Y. A. 2004. Postharvest biological control of apple gray mold by *Trichoderma harzianum Rifai* formulated in an invert emulsion. Crop Protection. 23: 19-126.

Błaszczyk, L., Basińska-Barczak, A., Ćwiek-Kupczyńska, H., Gromadzka, K., Popiel, D. and Stępień, Ł. 2017. Suppressive effect of *Trichoderma* spp. on toxigenic *Fusarium* species. Polish Journal of Microbiology. 66: 85.

Fravel, D.R. 2005. Commercialization and implementation of biocontrol. Annual Review of Phytopathology. 43: 337-359.

Huang, S. K., Tangthirasunun, N., Phillips, A. J., Dai, D. Q., Wanasinghe, D. N., Wen, T. C. and Kang, J. C. 2016. Morphology and Phylogeny of *Neoscytalidium orchidacearum* sp. nov. (*Botryosphaeriaceae*). Mycobiology. 44: 79-84.

Jayaraj, J., Radhakrishnan, N.V. and Velazhahan, R. 2006. Development of formulations of *Trichoderma harzianum* strain M1 for control of damping-off of tomato caused by *Pythium aphanidermatum*. Phytopathology and Plant Protection. 39: 1-8.

Jin, X. and Custis, D. 2011. Microencapsulating aerial conidia of *Trichoderma harzianum* through spray drying at elevated temperatures. Biological Control. 56: 202-206.

Kaewchai, S., Soytong, K. and Hyde, K.D. 2009. Mycofungicides and fungal biofertilizers. Fungal Diversity. 38: 25-50.

Kanjanamaneesathian, M. 2015. Bacillus for Rice Cultivation in Thailand. Beneficial Microorganisms in Agriculture, Aquaculture and Other Areas. 29: 81-100.

Kolombet, L. V., Zhigletsova, S. K., Kosareva, N. I., Bystrova, E. V., Derbyshev, V. V., Krasnova, S. P. and Schisler, D. 2008. Development of an extended shelf-life, liquid formulation of the biofungicide *Trichoderma asperellum*. World Journal of Microbiology and Biotechnology. 24: 123-131.

Kumar, S., Thakur, M. and Rani, A. 2014. *Trichoderma*: Mass production, formulation, quality control, delivery and its scope in commercialization in India for the management of plant diseases. African Journal of Agricultural Research. 9: 3838-3852.

Mbarga, J. B., Begoude, B. A. D., Ambang, Z., Meboma, M., Kuaté, J., Schiffers, B. and Ten Hoopen, G. M. 2014. A new oil-based formulation of *Trichoderma asperellum* for the biological control of cacao black pod disease caused by *Phytophthora megakarya*. Biological Control. 77: 15-22.

Mohd, M. H., Salleh, B. and Zakaria, L. 2013. Identification and molecular characterizations of *Neoscytalidium dimidiatum* causing stem canker of red-fleshed dragon fruit (*Hylocereus polyrhizus*) in Malaysia. Journal of Phytopathology. 161: 841-849.

Nguyen, H. T. and Nguyen, H. V. 2015. Management strategies of major pitaya diseases in Vietnam. Management. 473: 129-142.

Nouri, M. T., Lawrence, D. P., Yaghmour, M. A., Michailides, T. J. and Trouillas, F. P. 2018. *Neoscytalidium dimidiatum* causing canker, shoot blight and fruit rot of almond in California. Plant Disease. 102: 1638-1647.

Peeran, M.F., Nagendran, K., Gandhi, K., Raguchander, T. and Prabakar, K. 2014. Water in oil based PGPR formulation of *Pseudomonas fluorescens* (FP7) showed enhanced resistance against *Colletotrichum musae*. Crop Protection. 65:186-193.

Rai, D. and Tewari, A.K., 2016. Shelf life studies of different formulations based on *Trichoderma harzianum* (Th14). Biological Research. 7: 1-5.

Ruangwong, O. U., Wonglom, P., Phoka, N., Suwannarach, N., Lumyong, S., Ito, S. I. and Sunpapao, A. 2021. Biological control activity of *Trichoderma*

asperelloides PSU-P1 against gummy stem blight in muskmelon (Cucumis melo). Physiological and Molecular Plant Pathology. 115: 101663.

Saradhuldhat, P., Kaewsongsang, K. and Suvittawat, K. 2009. Induced off-season flowering by supplemented fluorescent light in dragon fruit (*Hylocereus undatus*). International Society for Southeast Asian Agricultural Sciences. 15: 0859-3132.

Saravanakumar, K., Yu, C., Dou, K., Wang, M., Li, Y. and Chen, J. 2016. Synergistic effect of *Trichoderma*-derived antifungal metabolites and cell wall degrading enzymes on enhanced biocontrol of *Fusarium oxysporum f.* sp. *cucumerinum*. Biological Control. 94:37-46.

Sriram, S., Palanna, K.B. and Ramanujam, B. 2010. Effect of chitin on the shelf-life of *Trichoderma harzianum* in talc formulation. Indian Journal of Agricultural Sciences. 80: 930.

Syed-Ab-Rahman, S.F., Carvalhais, L.C. and Omar, D. 2020. Development of plant-based emulsion formulations to control bacterial leaf blight and sheath brown rot of rice. Heliyon. 6:03151.

Thapinta, A. and Hudak, P. F. 2000. Pesticide use and residual occurrence in Thailand. Environmental Monitoring and Assessment. 60: 103-114.

Turkolmez, S., Dervis, S., Ciftci, O. and Dikilitas, M. 2019. First report of *Neoscytalidium dimidiatum* causing shoot and needle blight of pines (*Pinus* spp.) in Turkey. Plant Disease. 103: 2960-2961.

Van H., N., Hieu, N. T., Hanh, T. T. M., Uyen, D. T. K. and Dien, L. Q. 2016. Emerging infectious diseases and insect pests of dragon fruit, passionfruit, citrus, longan. Management. 1: 630.

Wonglom, P., Thithuan, N., Bunjongsiri, P. and Sunpapao, A. 2018. Plant-parasitic algae (*Cephaleuros* spp.) in Thailand, including four new records. Pacific Science. 72: 363-371.

Zin, N. A. and Badaluddin, N. A. 2020. Biological functions of *Trichoderma* spp. for agriculture applications. Annals of Agricultural Sciences. 65: 168-178.

Appendices

Materials and Methods

1. Emulsion formulation development

Trichoderma asperelloides PSU-P1 was obtained from Culture Collection of Pest Management, Faculty of Natural Resources, Prince of Songkla University. *Neoscytalidium dimidiatum* (SC02) and *T. asperelloides* PSU-P1 were cultured on potato dextrose agar (PDA), incubated at ambient temperature (28 ± 2^{o}C) for 2 days before used in further methods. Spore suspension was prepared in 10 mL with sterile distilled water from ten days old culture of *T. asperelloides* PSU-P1 (Kheng and Omar, 2005). The stock suspension was then serially diluted in order to prepare a spore suspension of 1×10^{8} spores/ml concentration. The number of spores in the suspension was quantified by direct microscopic counting using hemocytometer (Absher, 1973). The emulsion formulation in this present study was developed as oil in water type by *T. asperelloides* PSU-P1. Conidia of *T. asperelloides* PSU-P1 harvested from 10 days old cultured on PDA medium plates by scraping colonies on agar surface using sterile scalpels and then suspended in sterile distilled water. The concentration of conidia in the aqueous phase of the emulsion was 1×10^{8} conidia/mL added into the emulsion during preparation according to the technique developed by previous research (Batta, 1999, 2004). Three different type vegetable oils, coconut oil, palm oil and soybean oil with two emulsifying agents (Tween 20 and Tween 80) were tested on the mixing times (Table 1). And then vegetable oil that spend the shortest mixing time was selected for further test. Six treatments in different ratio of coconut oil, emulsifying agent and dextrose were prepared (Table 2) and tested for the best ratio which pended minimum mixing time for developed as an emulation formulation. The emulsion were stored in glass bottles at ambient temperature and cool temperature (10^{o}C)

for the duration of the viability study. The viability and percentage inhibition, were measured every month in the period of six months.

Table 1 Different oil and emulsifier dispersing agent for emulsion formulation preparation

Form.	Ingredients (mL)					
	Coconut oil	Palm oil	Soybean oil	DW	Tween 20	Tween 80
1	40	-	-	40	20	-
2	40	-	-	40	-	20
3	20	-	-	60	20	-
4	20	-	-	60	-	20
5	-	40	-	40	20	-
6	-	40	-	40	-	20
7	-	20	-	60	20	-
8	-	20	-	60	-	20
9	-	-	40	40	20	-
10	-	-	40	40	-	20
11	-	-	20	60	20	-
12	-	-	20	60	-	20

Form. = formulation

Table 2 Mixing time of different formulation in varying of coconut oil and additive dextrose

Form	Ingredients				
	Coconut oil (mL)	DW (mL)	Dextrose (g)	Tween 20 (mL)	Spore suspension
1	40	40	5	20	X
2	40	40	-	20	X
3	45	45	5	10	X
4	45	45	-	10	X
5	30	60	5	10	X
6	30	60	-	10	X

Form. = formulation, X= spore suspension at concentration 1×10^8 conidia/mL is suspended in DW phase.

2. Application of formulation suppressed disease development

Fresh, healthy clades of red-fleshed dragon fruit were used in this tested. Emulsion formulation was diluted in DW with a ratio of 1: 99. Experiment composed of four treatments: i) dropped with distilled water alone (T1), ii) dropped (25 µl) with spore suspension of *N. dimidiatum* alone (T2), iii) dropped (25 µl) with spore suspension of *N. dimidiatum* 24 h and challenged with formulation (T3) and iv) dropped (25 µl) with formulation alone (T4). The treatments were incubated for 10 days at 28±2°C in moisture boxes. The effectiveness was evaluated by measuring the pathogen-lesion diameter (cm) that formed around wounds made on the cladodes surface 5–7 days after inoculation by ImageJ software.

3. Viability of *Trichoderma asperelloides* PSU-P1 conidia in formulation

The viability activity of *T. asperelloides* PSU-P1 in the formulation were assessed a period of 6 months. Determined the colonization, competition with the *N. dimidiatum* by cultured on potato dextrose agar (PDA) culture medium. Autoclaved sterilized filter paper discs with a diameter of 0.4 cm were uniformly moistened with 1% emulsion (1ml+99 ml) of sterile water (Senkov et al., 2022). A filter paper was placed on PDA medium at a distance of 4 cm from a 0.4 cm diameter *N. dimidiatum* agar plug cut from 3 days old culture. Petri plates with both cultures were stored at ambient temperature (28 ± 2°C). Viability was measured on the third day from the area of the colonized surface of *T. asperelloides* PSU-P1 and expressed as a percentage of the total surface area of conidia in plate. All experiments were repeated at least once.

4. Percentage inhibition of *Trichoderma asperelloides* PSU-P1 in the formulation

To test antifungal activity of *T. asperelloides* (PSU-P1) against *N. dimidiatum* filter paper discs method was conducted. The processed were similar as method described in viability test. Each treatment composed of 3 plates (tri replicates) and the experiment were repeated twice. The tested plates were incubated at ambient temperature for 3 days. Colony radii of pathogen were measured and converted to percentage of inhibition as formula (Rahman et al., 2009).

$$\textbf{Inhibition}(\%) = (\frac{\textbf{R1} - \textbf{R2}}{\textbf{R1}}) \times \textbf{100}$$

Where, R1 is the colony radii of pathogen in the control plates, and R2 is the colony radii of pathogen in dual culture plate.

5. Statistical analysis

Data of result on mixing time was subjected to by one-way analysis of variance (ANOVA). Significant different among treatments of lesion

development was analyzed by Tukey's test, whereas significant among treatments of viability and percentage inhibition was analyzed by Student's T-test at 95% significant level ($p< 0.05$).

6. References

Absher, M. 1973. Hemocytometer counting. Academic Press. In Tissue culture. 395-397.

Batta, Y. 1999. Biological effect of two strains of microorganisms antagonistic to *Botrytis Cinerea* causal organism of gray mold on strawberry. An-Najah University Journal for Research. 13: 67-83

Batta, Y. A. 2004. Postharvest biological control of apple gray mold by *Trichoderma harzianum* Rifai formulated in an invert emulsion. Crop Protection. 23: 19-26.

Kheng, P.P. and Omar, I.C. 2005. Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate. Songklanakarin Journal of Science and Technology. 27: 325-336.

Rahman, M.A., Begum, M.F. and Alam, M.F. 2009. Screening of *Trichoderma* isolates as a biological control agent against *Ceratocystis paradoxa* causing pineapple disease of sugarcane. Mycobiology. 37: 277–285.

Senkovs, M., Dzierkale, M.T., Rimkus, A., Grigs, O. and Nikolajeva, V. 2022. Application of a posttreatment to improve the viability and antifungal activity of *Trichoderma asperellum* biomass obtained in a bioreactor during submerged cultivation. Biology. 11: 1610.

Paper

Morphological, Molecular Identification and Pathogenicity of *Neoscytalidium dimidiatum* Causing Stem Canker of *Hylocereus polyrhizus* in Southern Thailand

Dy, K.S., Wonglom, P., Pornsuriya, C. and Sunpapao, A

Reprinted from Plants (2022) 11: 504

plants

MDPI

Article

Morphological, Molecular Identification and Pathogenicity of *Neoscytalidium dimidiatum* Causing Stem Canker of *Hylocereus polyrhizus* in Southern Thailand

Kim Sreang Dy [1], Prisana Wonglom [2], Chaninun Pornsuriya [1] and Anurag Sunpapao [1,*]

[1] Agricultural Innovation and Management Division (Pest Management), Faculty of Natural Resources, Prince of Songkla University, Hatyai 90110, Songkhla, Thailand; dykimsreang168@gmail.com (K.S.D.); chaninun.p@psu.ac.th (C.P.)
[2] Faculty of Technology and Community Development, Thaksin University, Pa Payom 93210, Phatthalung, Thailand; prisana.w@tsu.ac.th
* Correspondence: anurag.su@psu.ac.th

Abstract: Red-fleshed dragon fruit (*Hylocereus polyrhizus*) is commonly cultivated in Thailand, especially in southern Thailand, where the weather favors plant growth and development. In 2021, stem canker of *H. polyrhizus* was observed in a dragon fruit plantation field in Phatthalung Province, southern Thailand. Small, orange circular spots developed on the stem of *H. polyrhizus*, which later became gray, and the lesion expanded with a mass of conidia. *Scytalidium*-like fungus was isolated from infected tissues. Based on morphology and phylogenetic analyses of internal transcribed spacer (ITS), nuclear large subunit (LSU) and β-tubulin (*tub*) sequences of fungal isolates, the fungus was identified as *Neoscytalidium dimidiatum*. Pathogenicity tests revealed that this isolate caused stem canker on the stem of *H. polyrhizus*, similar to that observed in the field. Knowledge of the diagnosis of plant diseases is an important step for managing plant diseases and therefore, this finding provides basic information for the development of appropriate strategies to manage stem canker disease on *H. polyrhizus* plants.

Keywords: morphology; molecular identification; pathogenicity test; pitaya

Citation: Dy, K.S.; Wonglom, P.; Pornsuriya, C.; Sunpapao, A. Morphological, Molecular Identification and Pathogenicity of *Neoscytalidium dimidiatum* Causing Stem Canker of *Hylocereus polyrhizus* in Southern Thailand. *Plants* **2022**, *11*, 504. https://doi.org/10.3390/plants11040504

Academic Editors: Rafał Ogórek and Agata Piecuch

Received: 26 January 2022
Accepted: 9 February 2022
Published: 12 February 2022

1. Introduction

Dragon fruit (*Hylocereus* spp.) is native to Latin America's tropical and subtropical forest regions, including North, Central, and South America. Dragon fruit, of the genus *Hylocereus*, belongs to the Cactaceae family of climbing cactus [1] and is known by several names, including pitaya, pitahaya, and strawberry pear. Dragon fruit enriched with micronutrients is in high demand and is being promoted as a healthy fruit [2]. Currently, dragon fruit is classified into one of three varieties: *H. polyrhizus*; *H. undatus*; or *Selenicereus megalanthus* [3]. The suitability of a tropical climate, rainfall requirements, and soil types may have contributed to the growth of dragon fruit, especially red-fleshed dragon fruit [3].

Due to increased planting areas and high demand, dragon fruit in many crops attract disease and pests. Several diseases have been reported to negatively affect dragon fruit plantations and production [4]. Disease caused by fungi is a major problem for dragon fruit plantations worldwide [3,5]. For instance, the fungus *Colletotrichum gloeosporioides* was found to cause anthracnose on dragon fruit in Malaysia [3]. The fungi *Neoscytalidium dimidiatum* and *Bipolris* sp. have been reported to cause canker and bipolaris black spot, respectively, on dragon fruit in Vietnam [5]. Furthermore, the fungus *Gilbertella persicaria* was recently reported to cause flower rot on red-fleshed dragon fruit in Thailand [6].

Dragon fruit is an economically important crop that can support additive income for many households in Thailand. Saradhuldhat et al. [7] demonstrated that dragon fruit could grow in practically any type of soil, both upland and lowland in Thailand, and

they are distributed throughout Thailand. Thailand is located in tropical and subtropical regions where the weather favors disease spread [8,9]. Several emerging diseases have been isolated and reported to cause diseases in several plant species in this area in the past 5 years [10–17]. However, the identification of fungal pathogens causing diseases on *H. polyrhizus* in Thailand is rarely documented. During 2021, stem canker of red-fleshed dragon fruit (*H. polyrhizus*) was observed in a cultivation field in Phatthalung Province, southern Thailand. Therefore, this research aimed to identify pathogens causing stem canker in Thailand based on morphology, molecular properties, and pathogenicity tests.

2. Results

2.1. Symptom Observation

Sunken brown necrosis was observed on the stems of *H. polyrhizus* at the cultivation field in Phatthalung Province, southern Thailand (Figure 1a). The whitish-yellow spots turned orange to brown with age. The spots coalesced to form larger spots or lesions and were surrounded by yellow halos (Figure 1b,c). The spots turned into necrotic water-soaked lesions and produced black pycnidia on the stem of *H. polyrhizus* (Figure 1d,e). The fungus was directly isolated and cultured on PDA for further study.

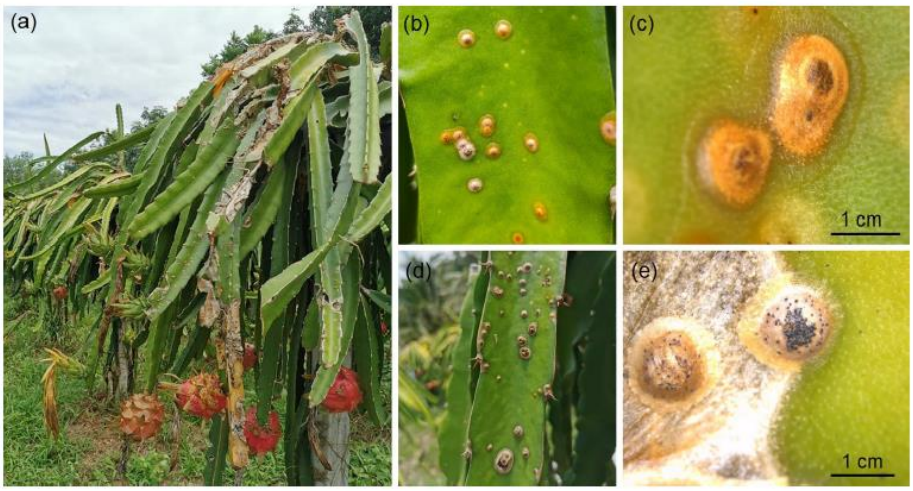

Figure 1. Stem canker of *Hylocereus polyrhizus* in the field (**a**), whitish-yellow spots, coalesced to form larger spots or lesions (**b**), spots or lesions surrounded by yellow halos (**c**), black pycnidia on stem of *H. polyrhizus* (**d**,**e**).

2.2. Morphology of Fungal Isolate

The fungal isolate PSU-SC02 obtained from PDA stock in Section 2.1 showed hairy colonies and olive-green to grayish colonies with dark gray to black pigmentation on PDA (Figure 2a–c). PSU-SC02 reached a diameter of 9 cm on PDA plates within 3 days, and the growth rate was 3 cm/day. The morphology of the PSU-SC02 isolate showed *Scytalidium*-like fungus. The hyphae were brown, branched, septate, and constricted into spore chains before disarticulation into arthroconidia. The arthroconidia were ellipsoid to ovoid in shape and hyaline to dark brown with thick walls and septate arthrospores, 3.1–18.0 µm long × 3.8–10.3 µm wide (n = 20, av = 10.0 ± 3.7 × 5.4 ± 1.8) µm (Figure 2e). Pycnidia had rarely developed on PDA after 4 weeks of incubation and developed on dried Napier grasses within 1 week (Figure 2d,f). Conidiogenous cells were observed in pycnidia that developed on dried Napier grass (Figure 2g). Pycnidial conidia were aseptate, ellipsoidal to nearly fusiform, and 8.5–15.5 µm long × 3.5–5.7 µm wide (n = 20,

av = 12.5 ± 1.7 × 4.7 ± 0.6) µm (Figure 2h). The fungal isolate was deposited in the Culture Collection of Pest Management, Faculty of Natural Resources, Prince of Songkla University Thailand, with accession number PSU-SC02.

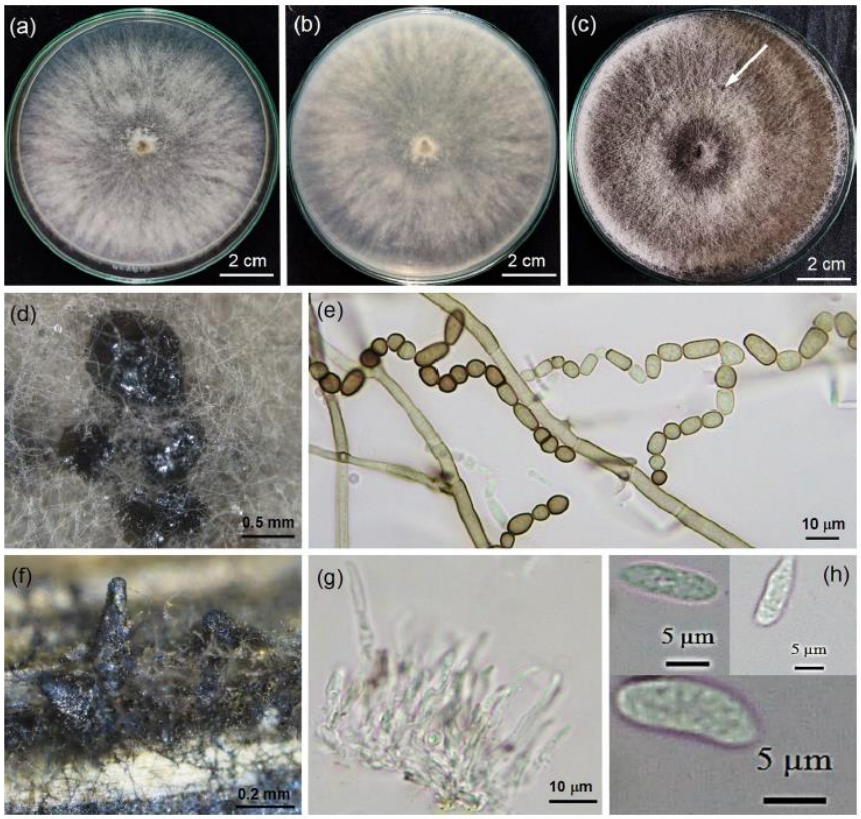

Figure 2. Morphological characteristics of PSU-SC02 on *Hylocereus polyrhizus*, 3-day-old colony on PDA in top (**a**) and bottom view (**b**), 4-week-old colony on PDA developed small black conidiomata ((**c**), arrow), zoom view of conidiomata (**d**), hyphae and arthroconidia (**e**), pycnidia developed on dried Napier grasses (**f**), conidiogenouse cells (**g**), and pycnidial conidia (**h**).

2.3. Molecular Identification

The PCR products of ITS, LSU, and *tub* were approximately 917, 1326, and 411 base pairs (bp) long, respectively. A BLAST search (https://blast.ncbi.nlm.nih.gov, accessed on 10 December 2021) revealed ITS, LSU, and *tub* sequences identical to those of *Neoscytalidium dimidiatum*, with 99.43%, 100%, and 99.76% identity, respectively. The DNA sequences of ITS, LSU, and *tub* of PSU-SC02 were deposited in GenBank and acquired accession numbers LC660640, LC660641, and LC660642, respectively. The maximum likelihood (ML) tree of the combined DNA sequences of ITS, LSU, and *tub* showed that the PSU-SC02 isolate grouped in the same clade as *N. dimidiatum* CBS 251.49 (Figure 3). Therefore, fungal isolate PSU-SC02 was identified as *N. dimidiatum*.

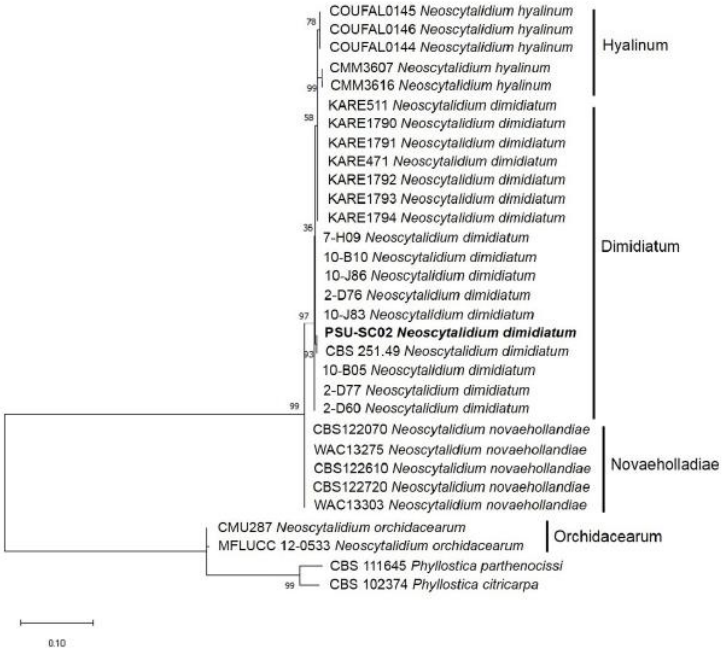

Figure 3. Phylogenetic tree of combined DNA sequences (ITS, LSU, and *tub*) of *Neoscytalidium dimidiatum* and related species acquired from Genbank constructed by maximum likelihood with 1000 bootstrap replications. Bold letters indicate the sample from this study. *Phyllostica parthenocissi* and *P. citricarpa* were used as out groups.

2.4. Neoscytalidium dimidiatum Causing Stem Canker

To fulfill Koch's postulates, a pathogenicity test was conducted on the stem of *H. polyrhizus*. Use of the agar plug method showed that *N. dimidiatum* PSU-SC02 caused cankers on healthy stems of *H. polyrhizus* after incubation in a moist box for 7 days (Figure 4). After 10 days of incubation, black pycnidia developed on the surface of *H. polyrhizus*, and the disease became severe when incubated for 14 days. Reisolation using the tissue transplanting method revealed that the morphology of the isolated fungus was similar to that of PSU-SC02.

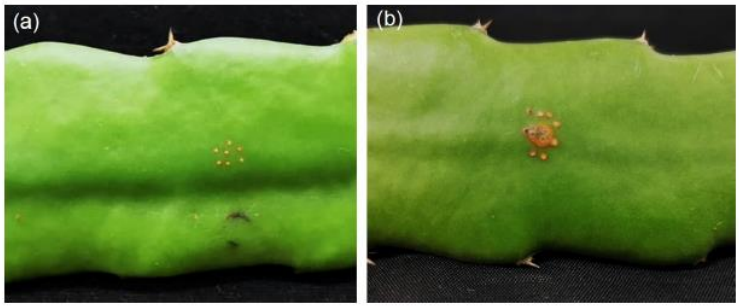

Figure 4. Pathogenicity test of *Neoscytalidium dimidiatum* PSU-SC02 on stem of *Hylocereus polyrhizus*, control (**a**), and PSU-SC02-inoculated stem (**b**).

3. Discussion

Canker disease caused by *Neoscytalidium* species is considered the most destructive disease for dragon fruit plantations worldwide and affects dragon fruit production, resulting in losses of up to 60–80% of market value [18]. In this study, we used both morphological and molecular tools to identify fungal pathogens at the species level and pathogenicity tests to fulfill Koch's postulates. Based on morphology and phylogenetic analyses of ITS, LSU, and *tub* sequences in this study, the fungal pathogen causing stem canker on *H. polyrhizus* was identified as *N. dimidiatum*.

Neoscytalidium dimidiatum has been reported to be a fast-growing fungus that commonly reaches a diameter of 9 cm on Petri dishes within 3 days of incubation, as previously described by Mohd et al. [3] and Turkolmez et al. [19]. Our results are in agreement with previous research; the *N. dimidiatum* PSU-SC02 colony on PDA reached a diameter of 9 cm in 3 days. Arthroconidia of our strain (*N. dimidiatum* PSU-SC02) were diverse in size and shape, as previously reported for *N. dimidiatum* by Nouri et al. [20]. Pycnidia of *N. dimidiatum* have been observed on PDA with irregular shapes, singly or in aggregate after 4 weeks of incubation [20]; these characteristics were also observed in our study. Pycnidia was successfully developed on sterile dried grasses covered on WA (Figure 2f), as observed in previous studies [21]. Furthermore, *N. dimidiatum* PSU-SC02 from our study showed hyaline conidiogenous cells, and pycnidial conidia were aseptate, hyaline, and ellipsoidal to fusiform. These morphologies were typical of *N. dimidiatum*, as indicated by previous reports [3,19,22].

To confirm the *Neoscytalidium* species at the species level, multiple DNA sequences of ITS, LSU, and *tub* were analyzed in this study. It is known that the identification of fungal pathogens relies on both morphology and molecular properties. Mohd et al. [3] used the morphology and single DNA sequence of ITS to identify *N. dimidiatum* as causing stem canker on *H. polyrhizus* in Malaysia. Huang et al. [23] studied the morphology and phylogeny of double DNA sequences of ITS and LSU to identify a new species of *Neoscytalidium* and named it *N. orchidacearum*. Furthermore, Nouri et al. [20] also used the morphology and multiple DNA sequences of ITS, translation elongation factor 1-α (*tef1-α*), and *tub* to diagnose *N. dimidiatum* as causing canker, shoot blight, and fruit rot of almond in California. Based on our study, the morphology and molecular characteristics of ITS, LSU, and *tub* successfully identified *Scytalidium*-like fungi causing canker on *H. polyrhizus* as *N. scytalidium*.

Currently, fungi in the genus *Neoscytalidium* comprise four species, namely *N. dimidiatum*, *N. hyalinum*, *N. novaehollandiae*, and *N. orchidacearum*. *N. dimidatum* causes diseases in several plant species: canker of *Ficus* trees in Egypt [24]; root rot of sweet potato in Brazil [25]; shoot and needle blight of pines (*Pinus* spp.) in Turkey [26]; and shoot blight of citrus in Jordan [27]. Furthermore, *N. dimidiatum* has also been reported to cause canker on *H. polyrhizus* in Taiwan [28], Malaysia [3], and China [29]. However, there are no previous reports of *N. dimidiatum* causing canker on *H. polyrhizus* in Thailand. To our knowledge, this is the first report of *N. dimidiatum* as a fungal pathogen of canker on *H. polyrhizus* in Thailand.

4. Materials and Methods

4.1. Sample Collection and Pathogen Isolation

A total of ten symptomatic stem canker samples of *H. polyrhizus* were collected from a dragon fruit plantation field in Phatthalung Province, southern Thailand (7°45′24.2″ N, 99°58′47.2″ E), kept in a plastic bag in an ice box and taken to a plant pathology laboratory, where isolation was subsequently conducted. The isolation of fungal pathogens was conducted by tissue transplantation according to the method of Pornsuriya et al. [14]. Small pieces (2–3 mm) of infected tissue were surface disinfected with 0.5% sodium hypochlorite (NaOCl) [13], rinsed three times with sterilized distilled water (DW), air-dried, placed on water agar (WA), and incubated for 24 h at 28 ± 2 °C. Hyphal tips were cut and transferred to potato dextrose agar (PDA), incubated at ambient temperature (28 ± 2 °C) with natural light–light cycle and subsequently used for further methods.

4.2. Morphology Study

The morphology of fungal colonies is determined by their ability to grow on PDA, with varied observations of colony traits, such as color, size, and shape. In this study, three plates were incubated at 28 ± 2 °C, and the diameters of colonies were measured daily until the colonies reached the edge of the plate. The growth rate per day on PDA of fungal isolates was calculated. The growth rate experiment was repeated twice. The general morphological characteristics of the fungal isolates were observed using a Leica S8AP0 stereomicroscope (Leica Microsystems, Wetzlar, Germany) with 10× magnification and a Leica DM750 compound microscope (Leica Microsystems, Wetzlar, Germany) with 40× magnification. Mycelial plugs (0.5 cm) were cut from the edges of 3-day-old colonies of fungal isolates and placed on WA covered with sterile dried Napier grasses as substrates to develop pycnidia structures according to previous studies [13,21].

4.3. DNA Extraction and PCR Amplification

Fungal isolates were cultured on PDA for 2 days and subjected to DNA extraction by the mini-preparation method [30]. DNA quantification was observed by 1% agarose gel electrophoresis. PCR amplification of internal transcribed spacer (ITS), nuclear large subunit (LSU), and β-tubulin (*tub*) was amplified by using ITS1/ITS4 [31], LR0R/LR5 [32], and Bt2a/Bt2b [33] primer pairs, respectively. The PCR mixture was composed of 20 pmol of primers, DNA template, nuclease-free DW, and 2 × OneTaq® 2X PCR master mix with standard buffer (Biloabs, New England, MA, USA). The Thermal Cycler (Bio-Rad Laboratories, CA, USA) was run with the following settings: initial denaturation at 94 °C for 30 s; 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 60 s; extension at 68 °C for 1 min; and final extension at 68 °C for 5 min. PCR products were stained with novel juice (GeneDireX, Taoyuan, Taiwan), separated by 1% agarose gel electrophoresis and observed on an LED Transilluminator (GeneDireX, Taoyuan, Taiwan).

PCR products were sequenced at the WARD MEDIC sequencing service in Thailand. The DNA sequences of ITS, LSU, and *tub* were searched for in the Blast program (National Center for Biotechnology Information, NCBI). The DNA sequences were aligned by Clustal W, and a phylogenetic tree was constructed by MEGA X [34] with a maximum likelihood of 1000 bootstrap replications. DNA sequences of fungal isolates and related species were obtained from GenBank (Table 1) to construct the phylogenetic analyses. DNA sequences were deposited in GenBank to obtain accession numbers.

4.4. Pathogenicity Test

The fungal isolate was cultured on PDA for 7 days and subjected to inoculation of the stem of *H. polyrhizus* using the agar plug method [16]. Four stems of *H. polyrhizus* (4 replications) were prepared for inoculation, and the experiment was repeated twice. The stem of *H. polyrhizus* was disinfected with 70% ethanol and wounded with fine needles (0.5 cm in diameter). An agar plug of fungal isolate was cut from a 7-day-old colony and directly placed on wounded *H. polyrhizus* stems. Inoculation of PDA alone via agar plugs was used as a control. The inoculated samples were then incubated in a moist chamber to maintain humidity (85% RH), 28 ± 2 °C, with a natural light–dark cycle for 7 days. The development of canker was observed and photographed. Infected tissue samples were reisolated via the tissue transplanting method as described in Section 4.1, and morphology was observed via microscopy as explained in Section 4.2.

Table 1. DNA sequences used to generate a phylogenetic tree acquired from GenBank with accession numbers.

Taxa	Isolate	Host, Region	Accession Numbers		
			ITS	LSU	*tub*
Neoscytalidium dimidiatum	2-D60	*Ficus carica*, USA	MG021571	–	MG021514
	2-D76	*Prunus dulcis*, USA	MG021583	–	MG021480
	2-D77	*P. dulcis*, USA	MG021584	–	MG021481
	7-H09	*P. dulcis*, USA	MG021587	–	MG021484
	10-B05	*P. dulcis*, USA	MG021589	–	MG021486
	10-B10	*P. dulcis*, USA	MG021591	–	MG021488
	10-J83	*P. dulcis*, USA	MG021595	–	MG021492
	10-J86	*P. dulcis*, USA	MG021596	–	MG021493
	CBS 251.49	*Juglans regia*, USA	KF531819	DQ377923	FM211166
	KARE471	*P. dulcis*, USA	MG021601	–	MG021498
	KARE511	*P. dulcis*, USA	MG021608	–	MG021505
	KARE1790	*P. dulcis*, USA	MG021578	–	MF991145
	KARE1791	*P. dulcis*, USA	MG021579	–	MG021476
	KARE1792	*Prunus dulcis*, USA	MG021580	–	MG021477
	KARE1793	*P. dulcis*, USA	MG021581	–	MG021478
	KARE1794	*P. dulcis*, USA	MG021582	–	MG021479
	PSU-SC02 *	***Hylocereus polyrhizus*, Thailand**	**LC660640**	LC660641	**LC660642**
N. hyalinum	CMM3607	*Jatropha curcas*, Brazil	KF234542	–	KF254925
	CMM3616	*J. curcas*, Brazil	JQ927342	–	KF254931
	COUFAL0144	*Nopalea cochenillifera*, Brazil	MH251953	–	MH251969
	COUFAL0145	*N. cochenillifera*, Brazil	MH251954	–	MH251970
	COUFAL0146	*N. cochenillifera*, Brazil	MH251955	–	MH251971
N. novaehollandiae	CBS 122070	*Grevillea agrifolia*, Australia	–	–	MT592759
	CBS 122072	*Adansonia gregorii*, Australia	–	–	MT592761
	CBS 122610	*Acacia synchronicia*, Australia	–	–	MT592762
	WAC13275	*Mangifera indica*, Australia	GU172400	–	–
	WAC13303	*M. indica*, Australia	GU172398	–	–
N. orchidacearum	CMU287	*Cattleya* sp., Thailand	KY933091	KY933092	–
	MFLUCC 12-0533	Orchidaceae, Thailand	KU179865	KU179864	–
Phyllostica citricarpa	CBS 102374	*Citrus aurantium*, Brazil	FJ538313	DQ377877	–
Phyllostica parthenocissi	CBS 111645	*Parthenocissus quinquefolia*, USA	EU683672	–	–

* Bold letters indicate samples from this study.

5. Conclusions

Herein, we identified the fungal pathogen causing stem canker in *H. polyrhizus* in Thailand. Based on the morphological characteristics and molecular properties of multiple DNA sequences of ITS, LSU, and *tub*, the pathogenicity tests revealed that the fungal pathogen *N. dimidiatum* caused stem canker in *H. polyrhizus*. Knowledge of the diagnosis of plant diseases is important for disease control and management. In order to determine and verify appropriate methods to manage stem canker disease on *H. polyrhizus* plants, further studies are needed in the near future.

Author Contributions: Conceptualization, K.S.D. and A.S.; methodology, K.S.D.; software, K.S.D.; validation, P.W., C.P. and A.S.; formal analysis, K.S.D.; investigation, K.S.D.; resources, A.S.; data curation, A.S.; writing—original draft preparation, K.S.D.; writing—review and editing, P.W., C.P. and A.S.; visualization, A.S.; supervision, A.S.; project administration, A.S.; funding acquisition, A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science, Research and Innovation Fund (NSRF) and Prince of Songkla University (Grant No. NAT6505003M and NAT6505003b).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The DNA sequences data obtained in this study have been deposited in GenBank with accession numbers for ITS (LC660640), LSU (LC660641), and *tub* (LC660642).

Acknowledgments: The authors would like to give special thanks to Prince of Songkla University and the Center of Excellence in Agricultural and Natural Resources Biotechnology (CoE-ANRB) phase 3, Faculty of Natural Resources, Prince of Songkla University.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Blancke, R. *Tropical Fruits and Other Edible Plants of the World: An Illustrated Guide*; Cornell University Press: Ithaca, NY, USA, 2016.
2. Masyahit, M.; Sijam, K.; Awang, Y.; Satar, M.G.M. First report on bacterial soft rot disease on dragon fruit (*Hylocereus* spp.) caused by *Enterobacter cloacae* in Peninsular Malaysia. *Int. J. Agric. Biol.* **2009**, *11*, 659–666.
3. Mohd, M.H.; Salleh, B.; Zakaria, L. Identification and molecular characterizations of *Neoscytalidium dimidiatum* causing stem canker of red-fleshed dragon fruit (*Hylocereus polyrhizus*) in Malaysia. *J. Phytopathol.* **2013**, *161*, 841–849. [CrossRef]
4. Van, H.N.; Hieu, N.T.; Hanh, T.T.M.; Uyen, D.T.K.; Dien, L.Q. Emerging infectious diseases and insect pests of dragon fruit, passionfruit, citrus, longan. *Management* **2016**, *1*, 630.
5. Nguyen, H.T.; Nguyen, H.V. Management strategies of major pitaya diseases in Vietnam. *Management* **2015**, *473*, 129–142.
6. Ruangwong, O.-U.; Kunasakdakul, K.; Wonglom, P.; Dy, K.S.; Sunpapao, A. Morphological and molecular studies of a rare mucoralean species causing flower rot in *Hylocereus polyrhizus*. *J. Phytopathol.* **2022**, 1–7. [CrossRef]
7. Saradhuldhat, P.; Kaewsongsang, K.; Suvittawat, K. Induced off-season flowering by supplemented fluorescent light in dragon fruit (*Hylocereus undatus*). *J. Int. Soc. Southeast Asian Agric. Sci.* **2009**, *15*, 0859–3132.
8. Wonglom, P.; Thithuan, N.; Bunjongsiri, P.; Sunpapao, A. Plant-parasitic algae (*Cephaleuros* spp.) in Thailand, including four new records. *Pac. Sci.* **2018**, *72*, 363–371. [CrossRef]
9. Thithuan, N.; Bunjonsiri, P.; Sunpapao, A. Morphology and behavior of gametes and zoospores from the plant-parasitic green algae, *Cephaleuros* (Chlorophyta, Ulvophyceae). *Pac. Sci.* **2019**, *73*, 403–410. [CrossRef]
10. Chairin, T.; Pornsuriya, C.; Thaochan, N.; Sunpapao, A. *Corynespora cassiicola* causes leaf spot disease on lettuce (*Lactuca sativa*) cultivated in hydroponic systems in Thailand. *Australas. Plant Dis. Notes* **2017**, *12*, 16. [CrossRef]
11. Pornsuriya, C.; Ito, S.; Sunpapao, A. First report of leaf spot on lettuce caused by *Curvularia aeria*. *J. Gen. Plant Pathol.* **2018**, *84*, 296–299. [CrossRef]
12. Nuangmek, W.; Aiduang, W.; Suwannarach, N.; Kumla, J.; Lumyong, S. First report of gummy stem blight caused by *Stagonosporopsis cucurbitacearum* on cantaloupe in Thailand. *Can. J. Plant Pathol.* **2018**, *40*, 306–311. [CrossRef]
13. Daengsuwan, W.; Wonglom, P.; Sunpapao, A. First report of *Lasiodiplodia theobromae* causing spadix rot in *Anthurium andraeanum*. *J. Phytopathol.* **2020**, *168*, 129–133. [CrossRef]
14. Pornsuriya, C.; Chairin, T.; Thaochan, N.; Sunpapao, A. Identification and characterization of *Neopestalotiopsis* fungi associated with a novel leaf fall disease of rubber trees (*Hevea brasiliensis*) in Thailand. *J. Phytopathol.* **2020**, *168*, 416–427. [CrossRef]
15. Wonglom, P.; Sunpapao, A. *Fusarium incarnatum* is associated with postharvest fruit rot of muskmelon (*Cucumis melo*). *J. Phytopathol.* **2020**, *168*, 204–210. [CrossRef]
16. Daengsuwan, W.; Wonglom, P.; Arikit, S.; Sunpapao, A. Morphological and molecular identification of *Neopestalotiopsis clavispora* causing flower blight on *Anthurium andraeanum* in Thailand. *Hortic. Plant J.* **2021**, *7*, 573–578. [CrossRef]
17. Suwannarach, N.; Khuna, S.; Kumla, J.; Cheewangkoon, R.; Suttiprapan, P.; Lumyong, S. Morphology Characterization, Molecular Identification, and Pathogenicity of Fungal Pathogen Causing Kaffir Lime Leaf Blight in Northern Thailand. *Plants* **2022**, *11*, 273. [CrossRef]
18. Hong, C.F.; Gazis, R.; Crane, J.H.; Zhang, S. Prevalence and epidemics of *Neoscytalidium* stem and fruit canker on pitahaya (*Hylocereus* spp.) in South Florida. *Plant Dis.* **2020**, *104*, 1433–1438. [CrossRef] [PubMed]
19. Turkolmez, S.; Dervis, S.; Ciftci, O.; Serce, C.U.; Dikilitas, M. New disease caused by *Neoscytalidium dimidiatum* devastates tomatoes (*Solanum lycopersicum*) in Turkey. *Crop Prot.* **2019**, *118*, 21–30. [CrossRef]
20. Nouri, M.T.; Lawrence, D.P.; Yaghmour, M.A.; Michailides, T.J.; Trouillas, F.P. *Neoscytalidium dimidiatum* causing canker, shoot blight and fruit rot of almond in California. *Plant Dis.* **2018**, *102*, 1638–1647. [CrossRef]
21. Pipattanapuckdee, A.; Boonyakait, D.; Tiyayon, C.; Seehanam, P.; Ruangwong, O. *Lasiodiplodia pseudotheobromae* causes postharvest fruit rot on longan in Thailand. *Australas. Plant Dis. Notes* **2019**, *14*, 21. [CrossRef]
22. Yi, R.H.; Mo, J.J.; Wu, F.F.; Chen, J. Fruit internal brown rot caused by *Neoscytalidium dimidiatum* on pitahaya in Guangdong province, China. *Australas. Plant Dis. Notes* **2015**, *10*, 1–4. [CrossRef]
23. Huang, S.K.; Tangthirasunun, N.; Phillips, A.J.; Dai, D.Q.; Wanasinghe, D.N.; Wen, T.C.; Kang, J.C. Morphology and phylogeny of *Neoscytalidium orchidacearum* sp. nov. (*Botryosphaeriaceae*). *Mycobiology* **2016**, *44*, 79–84. [CrossRef] [PubMed]
24. Al-Bedak, O.A.; Mohamed, R.A.; Seddek, N.H. First detection of *Neoscytalidium dimidiatum* associated with canker disease in Egyptian *Ficus* trees. *For. Pathol.* **2018**, *48*, e12411. [CrossRef]
25. Mello, J.F.; Brito, A.C.Q.; Motta, C.M.S.; Vieira, J.C.B.; Michereff, S.J.; Machado, A.R. First report of *Neoscytalidium dimidiatum* causing root rot in sweet potato in Brazil. *Plant Dis.* **2019**, *103*, 373–374. [CrossRef]

26. Turkolmez, S.; Dervis, S.; Ciftci, O.; Dikilitas, M. First report of *Neoscytalidium dimidiatum* causing shoot and needle blight of pines (*Pinus* spp.) in Turkey. *Plant Dis.* **2019**, *103*, 2960–2961. [CrossRef]
27. Alananbeh, K.M.; Al-Qasim, M.; Gharaibeh, A.; Al-Hiary, H.A. First report of shoot blight caused by *Neoscytalidium dimidiatum* on citrus in Jordan. *Plant Dis.* **2020**, *104*, 571. [CrossRef]
28. Chuang, M.F.; Ni, H.F.; Yang, H.R.; Shu, S.L.; Lai, S.Y.; Jiang, Y.-L. First report of stem canker disease of pitaya (*Hylocereus undatus* and *Hylocereus polyrhizus*) caused by *Neoscytalidium dimidiatum* in Taiwan. *Plant Dis.* **2012**, *96*, 906. [CrossRef]
29. Xu, M.; Peng, Y.; Qi, Z.; Yan, Z.; Yang, L.; He, M.-D.; Li, Q.-X.; Liu, C.-L.; Ruan, Y.-Z.; Wei, S.-S.; et al. Identification of *Neoscytalidium dimidiatum* causing canker disease of pitaya in Hainan, China. *Australas. Plant Pathol.* **2018**, *47*, 547–553. [CrossRef]
30. Saitoh, K.; Togashi, K.; Arie, T.; Teraoka, T. A simple method for a mini-preparation of fungal DNA. *J. Gen. Plant Pathol.* **2006**, *72*, 348–350. [CrossRef]
31. White, T.J.; Bruns, T.; Lee, S.; Tayler, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. In *PCR Protocols: A Guide to Methods and Applications*; Innis, A.M., Gelfelfard, D.H., Snindky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
32. Vilgalys, R.; Hester, M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *J. Bacteriol.* **1990**, *172*, 4239–4246. [CrossRef]
33. Glass, N.L.; Donaldson, G.C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* **1995**, *61*, 1323–1330. [CrossRef] [PubMed]
34. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamaru, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]

VITAE

Name Kim Sreang Dy

Student ID 6410620001

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Agronomy)	Royal University of Agriculture	2019

Scholarship Awards during Enrolment

1. The Royal Scholarship under Her Royal Highness Princess Maha Charkri Sirindhorn Education Project to the Kingdom of Cambodia for Master Degree Program.
2. The Center of Excellence in Agricultural and Natural Resources Biotechnology (CoE-ANRB) phase 3 research grant

List of Publication and Proceeding

Dy, K.S., Wonglom, P., Pornsuriya, C. and Sunpapao, A. 2022. Morphological, molecular identification and pathogenicity of *Neoscytalidium dimidiatum* causing stem canker of *Hylocereus polyrhizus* in southern Thailand. Plants. 11: 504.