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First report of *Colletotrichum fructicola*, causing anthracnose of *Hylocereus* plants, in the Philippines

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Cultivation of dragon fruit plants (*Hylocereus* spp.) in the Philippines has increased over the past years, and its diseases remain one of the major production challenges. In this study, a fungal pathogen, so far unknown in the country, was isolated from *H. undatus* from anthracnose-like lesion. Isolate MBDF0037C-1 was identified as *Colletotrichum fructicola*, based on combined morphological and molecular characterisation. This is the first record of *C. fructicola* causing anthracnose of dragon fruit plants in the Philippines. The isolate caused anthracnose to *H. undatus* and *H. monacanthus*, but not to *H. megalanthus*, in repeated in-vitro and in-vivo pathogenicity tests. Therefore, this study also highlights *H. megalanthus* as a potential source of resistance to anthracnose in dragon fruit breeding programmes.

Key words: *Hylocereus undatus*, *Hylocereus monacanthus*, *Hylocereus megalanthus*, cactus disease, pathogenicity test.

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Evallo E., Taguiam J.D., Bengoa J., Maghirang R., Balendres M.A. (2021): První nález *Colletotrichum fructicola*, původce antraknózy rostlin rodu *Hylocereus*, na Filipínách. – Czech Mycol. 73(1): 79–90.

Pěstování druhů rodu *Hylocereus*, jejichž plody jsou známy jako dračí ovoce, je na Filipínách v posledních letech silně na vzestupu a choroby těchto rostlin zůstávají jednou z velkých výzev pro jejich pěstitele. V této studii je představena patogenní houba, izolovaná z antraknózních lezí *H. undatus* a dosud v této zemi neznámá. Izolát MBDF0037C-1 byl na základě morfologických a molekulárních charakteristik určen jako *Colletotrichum fructicola*; jde o první záznam o výskytu *C. fructicola* coby původce antraknózy těchto rostlin na Filipínách. Testy pathogenity (in vitro a in vivo) prokázaly, že způsobuje antraknózu *H. undatus* a *H. monacanthus*, ale nikoli *H. megalanthus*. Na základě této studie tak lze soustředit pozornost na *H. megalanthus* jako potenciální zdroj rezistence k antraknóze v dalším šlechtění těchto kaktusů, pěstovaných pro dračí ovoce.

INTRODUCTION

Dragon fruit plants (*Hylocereus* spp.) are climbing cacti commonly known for their edible fruits (Britton et Rose 1963). In the Philippines, three *Hylocereus* species – *H. undatus* (red skin, white flesh of the fruit), *H. monacanthus* (red skin and flesh), and *H. megalanthus* (yellow skin, white flesh) – are commercially propagated. The dragon fruit industry is growing in the country, providing consumers with a food and growers with an income. However, the high income offered by dragon fruit cultivation can be hindered by the presence of diseases (Balendres et Bengoa 2019). A major disease of dragon fruit is anthracnose caused by six *Colletotrichum* species, namely *C. aenigma*, *C. gloeosporioides*, *C. siamense*, *C. truncatum*, *C. karstii*, and *C. fructicola* (Awang et al. 2010, Masyahit et al. 2013, Guo et al. 2013, Udayanga et al. 2013, Ma et al. 2014, Vijaya et al. 2014, Meetum et al. 2015, Zhao et al. 2018, Balendres et Bengoa 2019, Abirami et al. 2019, Nascimento et al. 2019). Anthracnose had not been reported from the Philippines.

In 2019, a dragon fruit stem showing dark brown necrotic spots was submitted for identification to the Plant Pathology Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños (PPL-IPB, CAFS, UPLB), Laguna, Philippines. The dragon fruit stem symptoms showed no resemblance with the ones from the reported stem canker. So far, only stem canker caused by *Neoscytalidium dimidiatum* (Taguiam et al. 2020a), red necrotic spot caused by *Epicoccum sorghinum* (Taguiam et al. 2020b), and brown spot caused by *Nigrospora sphaerica* (Taguiam et al. 2020c) had been reported in dragon fruit in the country.

Hence, the objective of this study was to identify the pathogen associated with the anthracnose-like symptom in *H. undatus*, using a combination of morphological and molecular assays. Furthermore, pathogenicity assays were conducted in *H. undatus* and two other *Hylocereus* species, *H. monacanthus* and *H. megalanthus*.

MATERIAL AND METHODS

Fungal isolation and purification. A stem of *H. undatus* showing symptoms similar to anthracnose was obtained from the Municipality of Manolo Fortich, Province of Bukidnon, Philippines. Approximately 3 mm^2 blocks of tissue including the infected and healthy parts, were surface-sterilised using a 10% sodium hypochlorite (NaOCl) solution (v/v, Zonrox, Green Cross Inc., Muntinlupa City, Philippines), washed three times with sterilised distilled water, and air-dried on sterile filter paper inside a laminar flow chamber. The dried stem tissues

were then placed on Petri plates with potato dextrose agar (PDA) medium (Himedia Laboratories, Mumbai, India) and incubated at 28 °C for three days. A pure culture was obtained by means of the single spore method, and the fungus was deposited in the Fungal Repository of the PPL-IPB, CAFS, UPLB with designation MBDF0037C-1.

Morphological and cultural characterisation. A 7-day old culture of MBDF0037C-1 was used for morphological (colony shape and morphology) and cultural (colony colour; conidia size, colour, and shape) characterisations. The conidia characteristics were assessed under a microscope (Olympus CX22, Tokyo, Japan) and photomicrographs were taken using the ToupView software (ToupTek Photonics, Hangzhou, China). Measurements of 40 randomly selected conidia were obtained using ImageJ software (Version 1.51s, Wayne Rasband, National Institute of Mental Health, Bethesda, USA).

Molecular characterisation. Total fungal genomic DNA (gDNA) from a 7-day old culture was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle et Doyle 1987, Cullings 1992). The gDNA was then used as a template in amplifying the partial β-tubulin (*TUB2*), actin (*ACT*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene regions by a polymerase chain reaction (PCR) following the conditions described by Balendres et al. (2020) and Dela Cueva et al. (2018). PCR products were then sent to Apical Scientific Sdn. Bhd. (Seri Kembangan, Malaysia) for DNA sequencing. The obtained consensus sequences were deposited in the NCBI GenBank database and were then compared and aligned with available fungi accessions from GenBank (Tab. 1) using MEGA X software (Kumar et al. 2018). Phylogenetic analysis of the concatenated sequences for the Maximum likelihood (ML) method was performed in MEGA X. The ML tree was generated using the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985), and support values of the tree were evaluated with 1000 bootstrap replicates. For the Bayesian inference (BI) analysis, the jModelTest 2.1.10 programme (Darriba et al. 2012) was used for model selection, and HKY+I was selected as the best nucleotide substitution model based on the Bayesian inference criterion (BIC). The trees were generated using MrBayes v 3.2.7a (Ronquist et Huelsenbeck 2003). Four Markov chain Monte Carlo (MCMC) chains were simultaneously performed with trees starting randomly for 1 million generations. Trees were sampled every 1000 generations and the first 250 generated trees were discarded as the burn in-phase of the analysis. The posterior probability values were determined based on the majority rule consensus tree generated with the remaining 750 trees. Both jModelTest and MrBayes programmes used for the Bayesian inference analysis were utilised at the CIPRES web portal (Miller et al. 2010). Phylogenetic analysis of the concatenated sequences from the ML and BI methods were used to determine the identity of MBDF0037C-1.

Tab. 1. *Colletotrichum* species used in phylogenetic analysis and their corresponding accession numbers.

Species	Strain*	GenBank accession numbers			Reference
		ACT	*TUB2*	*GAPDH*	
C. aenigma	ICMP 18686	JX009519	JX010390	JX009913	Weir et al. 2012
C. asianum	ICMP 18696	JX009576	JX010384	JX009915	Weir et al. 2012
C. boninense (outgroup)	CBS 128547	JQ005507	JQ005593	JQ005246	Damm et al. 2012
C. clidemiae	ICMP 18706	JX009476	JX010439	JX009909	Weir et al. 2012
C. fructicola	ICMP 18613	JX009491	JX010388	JX009998	Weir et al. 2012
C. fructicola	ICMP 18120	JX009436	JX010401	JX010041	Weir et al. 2012
C. fructicola	ICMP 18727	JX009565	JX010394	JX010035	Weir et al. 2012
C. horii	ICMP 12942	JX009533	JX010375	GQ329685	Weir et al. 2012
C. kahawae subsp. *kahawae*	CBS 982.69	JX009474	JX010435	JX010040	Weir et al. 2012
C. musae	ICMP 17817	JX009432	JX010395	JX010015	Weir et al. 2012
C. nupharicola	CBS 469.96	JX009486	JX010397	JX009936	Weir et al. 2012
C. siamense	ICMP 12567	JX009541	JX010387	JX009940	Weir et al. 2012
C. tropicale	ICMP 18672	JX009480	JX010396	JX010020	Weir et al. 2012
C. tropicale	CBS 124949	JX009489	JX010407	JX010007	Weir et al. 2012
C. xanthorrhoeae	BRIP 45094	JX009478	JX010448	JX009927	Weir et al. 2012

* BRIP: Culture Collection of the DPI&F Plant Pathology Herbarium, Indooroopilly, Queensland, Australia; CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand.

Pathogenicity test. Three *Hylocereus* species (*H. undatus*, *H. monacanthus* and *H. megalanthus*) were used for the pathogenicity tests. Pathogenicity tests were carried out in vivo (glasshouse assay using rooted-stem cuttings) and in vitro (laboratory assay using detached stems) following the procedures by Taguiam et al. (2020a, 2020b). Briefly, 15 µl of the fungal spore suspension (1×10^8 spores/ml) was inoculated onto wounded and non-wounded inoculation sites. Each stem of the three *Hylocereus* species had a total of 9 inoculation sites, using the triangular shape of the stems. All tests were performed twice, with the same number of biological and technical replicates for each set-up. Disease development was assessed at 7 days post inoculation (dpi). Finally, fungal re-isolation was performed from the diseased inoculated stems using the same procedure mentioned above to establish Koch's postulate. All negative controls were inoculated with sterile distilled water.

RESULTS AND DISCUSSION

Identity of fungus isolate MBDF0037C-1

This study reports *Colletotrichum fructicola* Prihastuti, L. Cai et K.D. Hyde as the causal agent of anthracnose in two dragon fruit species, *Hylocereus undatus* and *H. monacanthus*, in the Philippines. *Colletotrichum fructicola* MBDF0037C-1 was successfully identified using a combined morphological and molecular analysis.

The morphology of *C. fructicola* MBDF0037C-1 closely resembles that of the *C. gloeosporioides* species complex (Barnett et Hunter 1972, Weir et al. 2012, Cannon et al. 2012). The colony of fungal isolate MBDF0037C-1 grown on PDA showed a circular shape, entire margin, and cottony texture. The colour development of the aerial mycelia was initially white, eventually becoming greyish green, starting at the centre, while maintaining white colouration as a halo at 7 dpi (Fig. 1). The same colour was observed on the underside. Further development of the colony at 10 dpi showed the presence of orange spore masses. Conidia observed under the microscope were unicellular, aseptate, hyaline, and oblong in shape. Measurements obtained from 40 randomly selected conidia had a length ranging from 11.40 to 16.75 µm with an average of 14.46 µm, while their width ranged from 4.15 to 6.31 µm with an average of 5.05 µm. Colony and conidia morphology indicate that the fungus belongs to the complex of *Colletotrichum gloeosporioides* sensu lato.

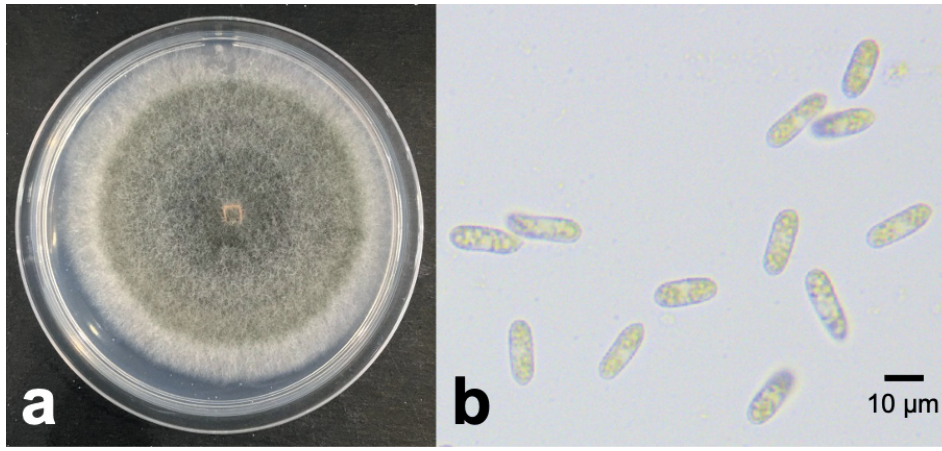

Fig. 1. Colony (**a**) and conidia (**b**) morphology of *C. fructicola* (MBDF0037C-1) in PDA medium, 7 days post inoculation. Photos: E. Evallo (a), J.D. Taguiam (b).

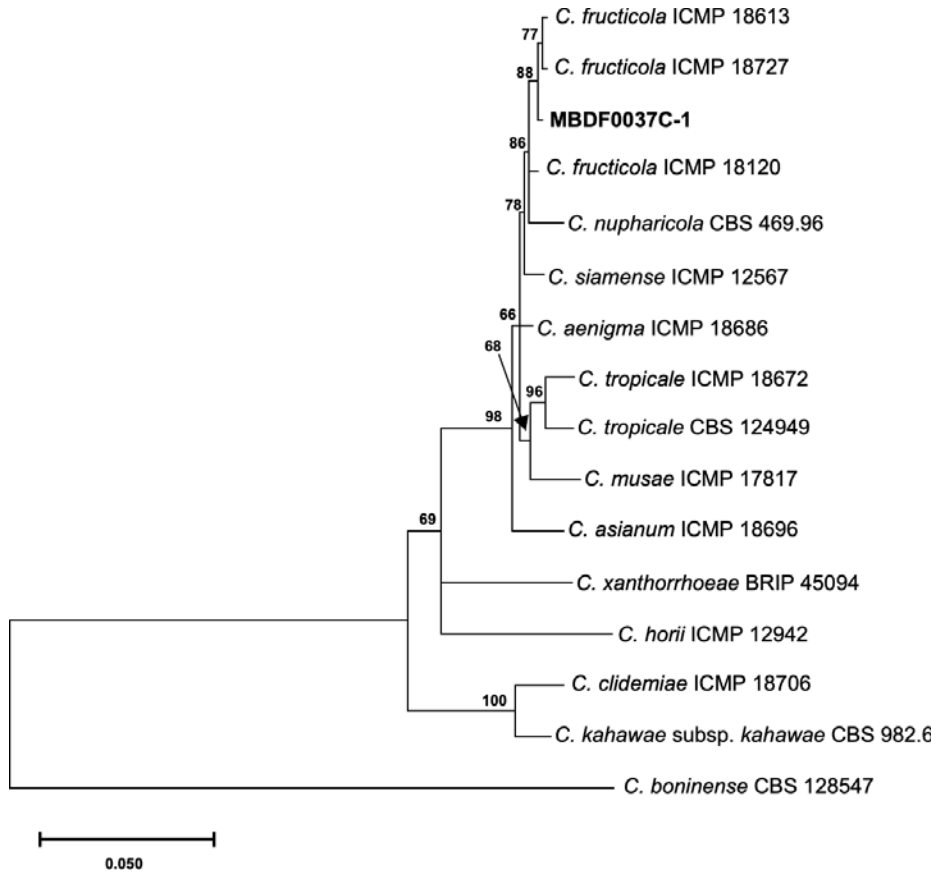

Fig. 2. Maximum likelihood tree generated from concatenated sequences of *ACT*, *TUB2* and *GAPDH* genes of *Colletotrichum* spp. used in this study. Bootstrap support values are indicated on branch nodes. *Colletotrichum boninense* was used as an outgroup.

The identity of MBDF0037C-1 was validated by phylogenetic analysis using the *ACT*, partial *TUB2*, and *GAPDH* genes. The *ACT* (MW767463), *TUB2* (MW767464), and *GAPDH* (MW767465) gene markers of MBDF0037C-1 isolate showed a 100% identity similarity to *Colletotrichum fructicola*. Its identity was further confirmed in both ML (Fig. 2) and BI (Fig. 3) phylogenetic trees with MBDF0037C-1 consistently grouped within the *C. gloeosporioides* clade and sharing the closest identity with *C. fructicola*.

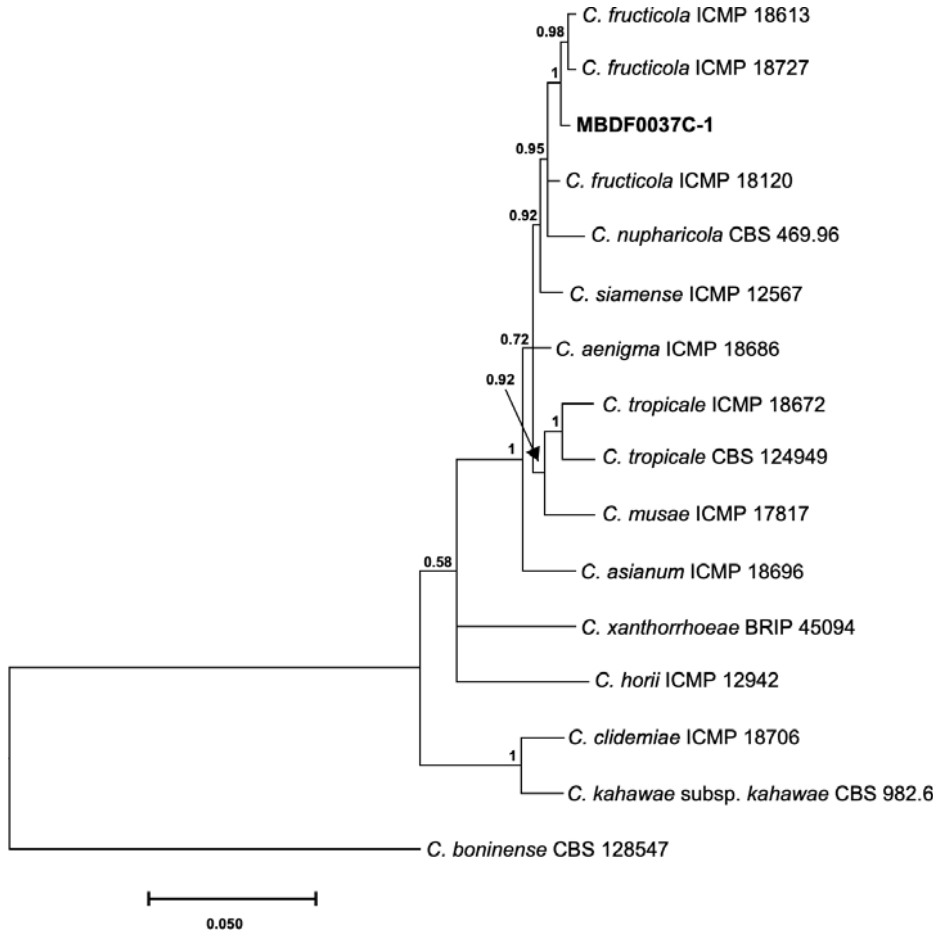

Fig. 3. Bayesian inference tree generated from concatenated sequences of *ACT*, *TUB2* and *GAPDH* genes of *Colletotrichum* spp. used in this study. Posterior probability values >0.5 are indicated on branch nodes. *Colletotrichum boninense* was used as an outgroup.

This species is primarily found in Asia, although some reports come from elsewhere in the world. *Colletotrichum fructicola* has a diverse host range, including fig in Germany, coffee in Thailand, sea lavenders from Israel, cacao and *Tetragastris* in Panama, apple and strawberry in the US and Brazil, Asian pear in Japan, avocado in Australia, papaya in India, bean and cowpea in Iran, and yam in Nigeria (Prihastuti et al. 2009, Weir at al. 2012, Atghia et al. 2015, Saini et al. 2016, Fuentes-Aragon et al. 2018).

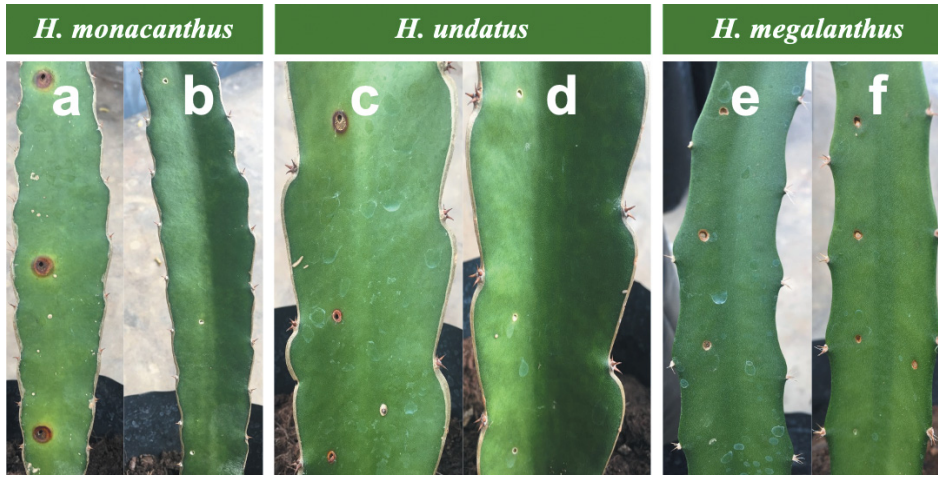

Fig. 4. In-vivo pathogenicity test of *Colletotrichum fructicola* MBDF0037C-1 in *Hylocereus* spp. at 7 days post inoculation. Infecting lesions were observed in *H. monacanthus* (**a**) and *H. undatus* (**c**), while no symptoms were observed on *H. megalanthus* (**e**) or the control (**b, d,** and **f**).
Photo: J.D. Taguiam and E. Evallo.

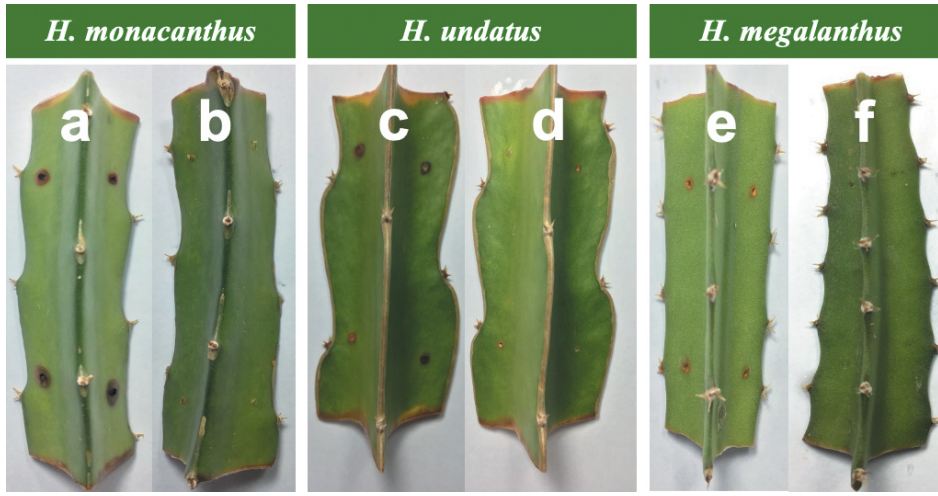

Fig. 5. In-vitro pathogenicity test of *Colletotrichum fructicola* MBDF0037C-1 infecting *H. monacanthus* (**a**) and *H. undatus* (**c**) at 7 days post inoculation. No symptoms were observed on *H. megalanthus* (**e**) or the control (**b, d,** and **f**). Photo: J.D. Taguiam and E. Evallo.

Pathogenicity test

The pathogenicity test performed in non-wounded tissues produced no infections. However, in wounded tissues, the development of symptoms at wounded sites inoculated with fungal isolate MBDF0037C-1 was observed only in two *Hylocereus* species in the in-vivo tests (Fig. 4). The isolate induced symptoms of sunken, necrotic, dark brown lesions with faint chlorotic halos on both *H. undatus* and *H. monacanthus* (Figs. 4a and 4c). Moreover, the presence of light to dark brown pycnidia was also observed on the marginal linings of the lesions on the infected *H. undatus* and *H. monacanthus* stems. Similar results were obtained in the in-vitro detached stem assays (Fig. 5), where stems showed sunken necrotic dark lesions with slight water soaking at the inoculated sites of *H. undatus* and *H. monacanthus*. No disease symptoms were observed in *H. megalanthus* in either in-vivo or in-vitro trials. No disease developed in any negative controls. A fungus having the same cultural and morphological characteristics of MBDF0037C-1 was re-isolated from the diseased tissues, applying Koch's postulate. While this study is the second report of *C. fructicola* as a dragon fruit pathogen worldwide, after the report in *H. undatus* by Udayanga et al. (2013), this is the first time that *C. fructicola* was found pathogenic to *H. monacanthus*.

Colletotrichum species causing anthracnose have also been reported from Thailand, Brazil, China, the US, Malaysia, and India (Guo et al. 2013, Masyahit et al. 2013, Meetum et al. 2015, Nascimento et al. 2019, Abirami et al. 2019). Our study indicated that *H. undatus* and *H. monacanthus* are indeed susceptible to *Colletotrichum* spp., particularly to *C. fructicola*. A study from Brazil showed that *C. gloeosporioides* caused anthracnose in *H. megalanthus* (Takahashi et al. 2008), but it was unclear whether the disease was caused by *C. gloeosporioides* sensu lato or by *C. gloeosporioides* sensu stricto due to the lack of molecular data. Hence, the inability of *C. fructicola*, as shown in this study, to cause infection in *H. megalanthus* could indicate potential resistance of *H. megalanthus* to the disease and could thus potentially be used in dragon fruit breeding programmes as a source of resistance to stem anthracnose.

This is a new record of *Colletotrichum fructicola* causing stem anthracnose in *Hylocereus undatus* and *H. monacanthus* in the Philippines. The resistance of *H. megalanthus* to anthracnose caused by *C. fructicola* is also reported. With accurate identification of the causal agent of stem anthracnose, effective management strategies may now be formulated to maximise the potential high revenues offered by dragon fruit.

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