Dragon fruit calli development and fungal contamination as influenced by activated charcoal

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ABSTRACT

Activated charcoal is often used in plant tissue culture systems to reduce phenolic oxidation and improve cell growth and development. This study investigated activated charcoal's effect on the somatic embryogenesis of dragon fruit (*Hylocereus* species). Nine-month-old seed-derived dragon fruit stems were placed in basal Murashige and Skoog (MS) plates supplemented with 2, 4-D (1.5 mg/L) and activated charcoal (1g/L). Cultures were incubated in 14 h continuous light or under dark condition. MS medium without activated charcoal served as the control. Percent fungal contamination, degree of tissue browning, and callus growth (length, width, and total area) were assessed. A significant decrease in contamination was observed four days after inoculation (dai) in a medium containing activated charcoal regardless of the light condition used ($p<0.05$, T-test analysis). In contrast, no significant difference in fungal contamination between the two treatments was found eight days after incubation (dai). Fungal contaminants' identities were done using a combined morphocultural and molecular analyses. The fungal isolates showed thick and white to grayish mycelia without spore production seven dai in potato dextrose agar medium. DNA sequence analysis showed a high percent similarity (99-100%) of the isolates to two *Diaporthe* species. Activated charcoal did not affect the length, width, and total area of the calli produced. However, a significant decrease in tissue browning was recorded ($p<0.05$). Overall results demonstrated the applicability of activated charcoal in dragon fruit tissue culture to reduce *in-vitro* browning. Additionally, examining further the possible role of fungal contaminants in dragon fruits would be valuable. A hedonic scale that can be used to assess tissue browning in dragon fruit is also described.

Keywords: *Diaporthe* species; *Hylocereus* species; somatic embryogenesis

INTRODUCTION

Dragon fruit (*Hylocereus spp.*) is a fruit crop gaining attention in the Philippine market. The crop's high market value and nutritional and medicinal properties make it popular among farmers and various stakeholders (Rodeo et al., 2018). Dragon fruit is a unique-looking and vine-climbing plant belonging to the Cactaceae family that produces edible fruits (Casas and Barbera, 2002). Dragon fruit contains betacyanin pigments, polyphenols, and flavonoids, which have been reported to have anti-tumor properties (Ortiz-Hernandez et al., 2012), antioxidant (Rebecca et al., 2010), and anti-inflammatory

activities (Bakar et al., 2011; Luo et al., 2014). Some of the commonly cultivated species of dragon fruit are *Hylocereus undatus* (pink-skinned, white-fleshed), *Hylocereus megalanthus* (yellow-skinned, white-fleshed), and *Hylocereus polyrhizus or monacanthus* (red-skinned, red-fleshed) (Ortiz-Hernandez and Carrillo-Salazar, 2012; Mercado-Silva, 2018). Dragon fruits are propagated by seeds, by micropropagation (Mohamed-Yassen, 2002), and, more commonly, through vegetative (stem) cuttings (Ortiz-Hernandez, 1999). However, successful crop production is hampered by many factors. Among these factors are plant diseases. These diseases can negatively affect the yield and fruit quality of dragon fruits (Balendres and Bengoa, 2019). Recently, dragon fruit diseases have been reported in the Philippines (Taguiam et al. 2020a; 2020b; 2020c).

Dragon fruits are mainly propagated by using stem cuttings. These stem cuttings are transported from one region to another and can quickly be established in an area. Nevertheless, vegetative (stem) cuttings could harbor disease causing microorganisms or pathogens. Hence, while large-scale propagation is possible, stem cuttings must be diseased-free to avoid disease epidemics in the field. One of the best methods for large-scale and disease-free crop production is through *in-vitro* tissue culture (Bozkurt et al., 2020; Dahanayake and Ranawake, 2011). Several studies have examined various propagation methods of dragon fruit (Bozkurt et al., 2020). However, currently, little information exists on the protocols for producing high-quality crop planting material via the tissue culture system (Dahayanake and Ranawake, 2011). Moreover, problems such as explant browning remain a constraint in producing the crop via the tissue-culture system.

Activated charcoal has been found to reduce the oxidation of phenolic compounds, which leads to tissue browning and eventually cell death (Thomas, 2008). Several studies found activated charcoal improves plant growth by adsorption of toxic metabolites released by the wounded plant (Liang et al., 2019; Olah, 2017; Pan and Staden, 1998; Shimelis et al., 2015; Thomas, 2008; Wang and Huang, 1976) and increases uptake of growth hormones (Wei et al., 2006; Pan and Staden, 2001). Explant health is important for callus induction. Tissue or explant browning is a challenge in an *in-vitro* culture system. This study provides evidence of the applicability of activated charcoal in decreasing tissue or explant browning in dragon fruit explants. Establishing an excellent *in-vitro* micropropagation technique will be useful for large-scale and disease-free production of dragon fruit. Using stem cuttings became a more popular way in propagating dragon fruit, but pathogen-infested stem cuttings could lead to disease epidemic in the field (Balendres and Bengoa 2019). Although, propagation is quite time-consuming and laborious, especially when using seeds (Trivelleni et al., 2020), a successful *in-vitro* culture system of dragon fruit could lead to the development of potentially disease resistant or high-yielding clones as a result of somaclonal variation.

MATERIALS AND METHODS

Explant source and preparation

The explants were taken from the stems nine-month-old, seed-derived *H. monacanthus* (red-skinned, red-fleshed) and *H. undatus* (red-skinned, white-fleshed) plants. These plants were grown in pots containing sterilized soils and were maintained at the screen

house of the Plant Pathology Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños. Newly-emerged stems (from the main stem) of dragon fruits were cut and placed in tissue paper. In the lab, the stems were transferred in a 125 mL beaker, covered with gauze, and washed in running water for at 30 min. Tissue samples were sterilized in 50% sodium hypochlorite solution (Zonrox, Philipines) for 5 min and were rinsed three times with sterile distilled water. Additional sterilization was done by soaking tissue samples in 70% ethanol for 3 min, and finally, rinsed with sterile distilled water three times. The sterilized tissues were air dried for at 30 min under the laminar flow hood.

Effect of activated charcoal and light on callus induction and tissue browning

The sterilized 2-mm cut explants were inoculated in basal Murashige and Skoog (MS) plates supplemented with 1.5 mg/L 2,4-D and activated charcoal (1g/L). All media had 3% (w/v) sucrose, adjusted to pH 5.7 before adding 0.8% (w/v) agar, and autoclaved at 121℃ for 20 min. Explants that were cultured on basal MS media without activated charcoal served as the control check. The experiment was performed twice and each experiment consists of 4 replicate plates. Callus color changes were observed visually and measured using an arbitrary 9-point and 5-point scales. For the light effect trial, cultures were maintained under 14 hours of cycled fluorescent light and in no light condition (dark) with temperature ranging from 20±5℃. For dark conditions, cultures were covered with foil. Percent contamination was recorded four days and eight days after inoculation in the medium. The type of callus formed, degree of explant browning, and callus growth [measured by length (mm), width (mm), and total area (mm^2)] were collected 28 days after incubation. A paired sample (Independent) T-test was performed using Statistical Tool for Agricultural Research (STAR Nebula) with a 95% confidence level.

Isolation, morpho-cultural, and molecular characterization of fungal contaminants

A 3 mm^2 agar block of hyphae growing from the stem of the dragon fruit explants was transferred onto a potato dextrose agar (PDA) medium. Plates were stored at room temperature (28-30°C) for three days (with 14 hours light in 24 hours cycle). The fungus was then purified and further characterized. Five-mm of the fungal mycelial plug from a seven-day-old culture was transferred to a new PDA medium and incubated (same condition as above). Fungal morphology was assessed using a light microscope (Olympus CX23, Japan and colony characteristics were recorded. For molecular analyses, the fungal genomic DNA was extracted using the procedure of Talbot (2017). The fungal genomic DNA was normalized to 30 ng/µL and used as a template for the succeeding polymerase chain reaction (PCR) assay to amplify the partial sequence of the internal transcribed spacer (ITS) gene region. The PCR assay was performed in MyCycler™ Thermal Cycler (Bio-Rad, USA) in a 15-µL reaction volume. The reaction mix contained 1x PCR Buffer (Invitrogen), 2.0 mM MgCl2 (Invitrogen), 0.2 mM dNTPs (Invitrogen), 0.2 µM each of the forward (ITS4, 5'-TCCTCCGCTTATTGATATGC3') and reverse (ITS5, 5'-GGAAGTAAAAGTCGTAACAAGG-3') primers (White et al., 1990), one U Taq DNA Polymerase (Invitrogen), 1 µL of the fungal genomic DNA, and DEPC-water to volume. The thermal cycling conditions were as follows; initial denaturation at 94 °C for five min, followed by 28 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 s and extension at 72 °C for one min and a final extension at 72 °C for seven min. The PCR product was resolved by gel electrophoresis [1.0% Agarose (Vivantis) 0.5X

Tris-Acetate-EDTA buffer containing one µL GelRed solution (Biotium) (PowerPac™ and Sub-Cell GT, (Bio-Rad Laboratories)]. The PCR product was sent to Apical Scientific Sdn. Bhd. (Malaysia) for DNA sequencing.

Molecular characterization and phylogenetic analysis

A consensus DNA sequence was derived from the forward and reverse sequences using the sequence editing software Geneious. Sequence similarity analysis was performed using the BLASTN program (Zhang and Madden, 1997), based on the highest percent similarity, e-value, and highest query cover. The authentic DNA sequences of the partial sequence of the ITS gene region of 10 *Diaporthe* species (Table 1) were used for comparison of the sequences from the three fungi (MDF1a, MDF1b MDF1c) isolated in this study. *Septoria steviae* (CBS 120132) was used as the outgroup (Jiang et al., 2020). Sequences were aligned using CLUSTALW (Kearse et al., 2012). The phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with 1,000 bootstrap replicates. The analysis was conducted in MEGA X software (Kumar et al., 2018).

Table 1. *Diaporthe* species and their corresponding isolates, host, and ITS gene sequences used in this study.
CPC= Culture collection of Pedro Crous, housed at CBS; ATCC=American Type Culture Collection, Historic District, 10801 University

Species	Isolate	Host	Locality	ITS Genbank Accession	Reference
D. passifloricola	CPC 27480	*Passiflora foetida*	Malaysia	NR_147595.1	Crous and Groenewald (2016)
D. ueckerae	CBS 139283	*Cucumis melo*	USA	NR_147543	Udayanga *et al.* (2015)
D. miriciae	BRIP 54736j	*Helianthus annuus*	Australia	NR_147535	Thompson *et al.* (2015)
D. tectonae	MFLUCC 12-0777	*Tectona grandis*	Thailand	NR_147590	Doilom *et al.* (2017)
D. tulliensis	BRIP 62248a	*Theobroma cacao*	Australia	NR_147574	Crous *et al.* (2015)
D. endophytica	CBS 133811	*Schinus terebinthifolius* (leaf)	Brazil	NR_111847.1	Gomes *et al.* (2013)
D. longicolla	ATCC 60325	*Glycine max*	USA	NR_144924	Udayanga *et al.* (2015)
D. tectonendophytica	MFLUCC 13-0471	*Tectona grandis*	Thailand	NR_147591	Doilom *et al.* (2017)
D. yunnanensis	CGMCC 3.18289	*Coffea sp.*	China	NR_152472.1	Gao *et al.* (2017)
D. melonis	CBS 507.78	*Cucumis melo*	USA	NR_103700.1	Gomes *et al*. (2013)
Septoria steviae	CBS 120132	*Stevia rebaudiana*	China	NR_163307	Koehler *et al.* (2019)

Boulevard, Manassas (VA), USA; CBS= Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; BRIP= The Plant Pathology Herbarium, Department of Agriculture, Fisheries, and Forestry, Mareeba Queensland, Australia; MFLUCC= Mae Fah Luang Culture Collection, Mae Fah Luang University, Center of Excellence in Fungal Research, School of Science, Mae Fah Luang University, 333 Moo 1 Muan, Chiang-Rai, Chiang-Rai 57100, Thailand; CGMCC= China General Microbiological Culture Collection, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. ITS= internal transcribed spacers

RESULTS

Culture Establishment

Successful production of dragon fruit calli were observed in MS supplemented with 2,4-D (1.5mg/l) with (Figure 1A) or without the addition of activated charcoal (Figure 1B). Ideal callus with green to pale and friable callus characteristics were produced in cultures without activated charcoal (Figure 1a). White, non-friable to no callus formation were observed in explants placed to activated charcoal (Figure 1b; 1c; 1d).

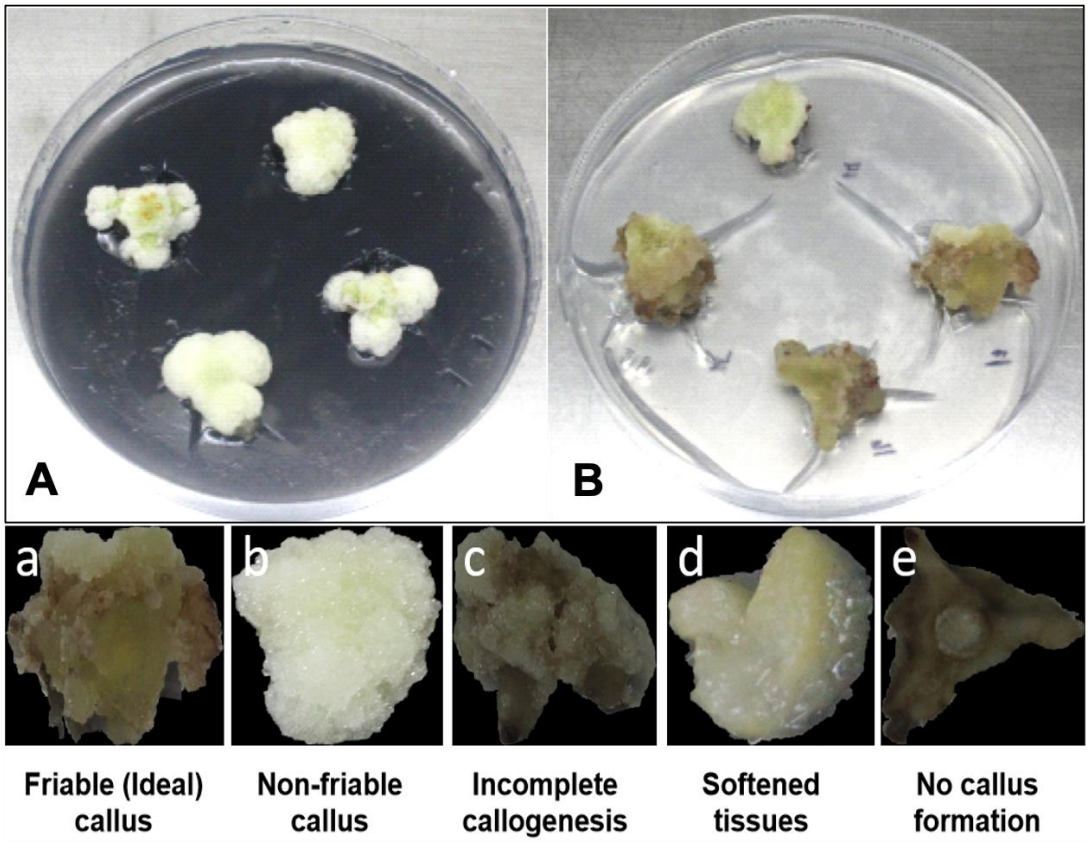

Figure 1. Dragon fruit callus formation in MS with 2,4-D (1.5ppm) supplemented with activated charcoal (A) and without activated charcoal added (B) 28 days after inoculation. Friable calli which are green to pale in color (a) were observed in medium without activated charcoal, while white, non-friable calli were formed (b) in medium exposed to activated charcoal. Dragon fruit explants showing different callus reactions in 2,4-D (1.5mg/l) with (a-b) and without supplementation of activated charcoal (1 g/l) (c-e).

Growth and reduction of browning using activated charcoal.

Callus growth was similar between explants grown medium with and without activated charcoal, but the browning rate was significantly different. A nine (Figure 2) and five-point (Figure 3) visual hedonic scale was developed based on the varying degree of explant browning observed. A significant decrease in browning of dragon fruit calli was observed in media with activated charcoal (Figure 4). Degree of tissue browning in *H. undatus* calli

exposed to activated charcoal scored 2 or 3 using a 9-point scale (Figure 4a) and 1 or 2 using a 5-point system (Figure 4b). Meanwhile, callus cultured in medium without activated charcoal scored 6 or 7 (9-point scale) (Figure 4a) and 4 (5-point scale) (Figure 4b) on average. Light conditions did not affect the degree of tissue browning (Figure 4). Further, the addition of activated charcoal did not significantly increase the length (Figure 5a), width (Figure 5b), and the total area (Figure 5c) of the calli produced. The average callus length and width values for both treatments ranged from 13.9-16.3 mm and 11.5-12.5 mm, respectively. On the other hand, the callis total area ranges from 380.2 to 550.6 mm^2. Similarly, no significant effect of light condition used on callus growth was observed (Figure 5). Due to the high contamination rate in *H. monacanthus*, data for callus induction and growth were only collected from *H. undatus* explants (see next section).

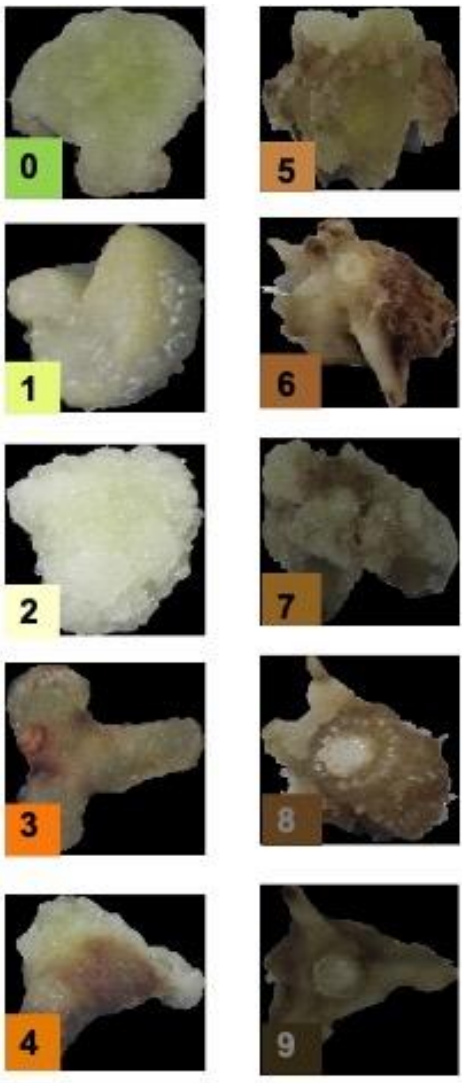

Figure 2. Nine-point (0-9) visual hedonic scale for measuring the degree of tissue browning of dragon fruit explants.

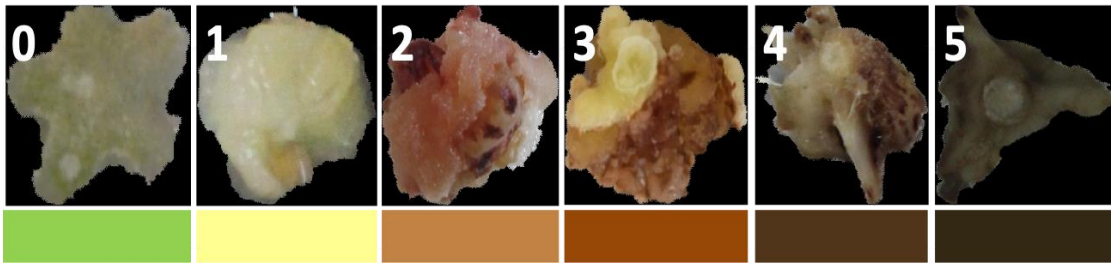

Figure 3. Five-point (0-5) visual hedonic scale for measuring the degree of tissue browning of dragon fruit explants.

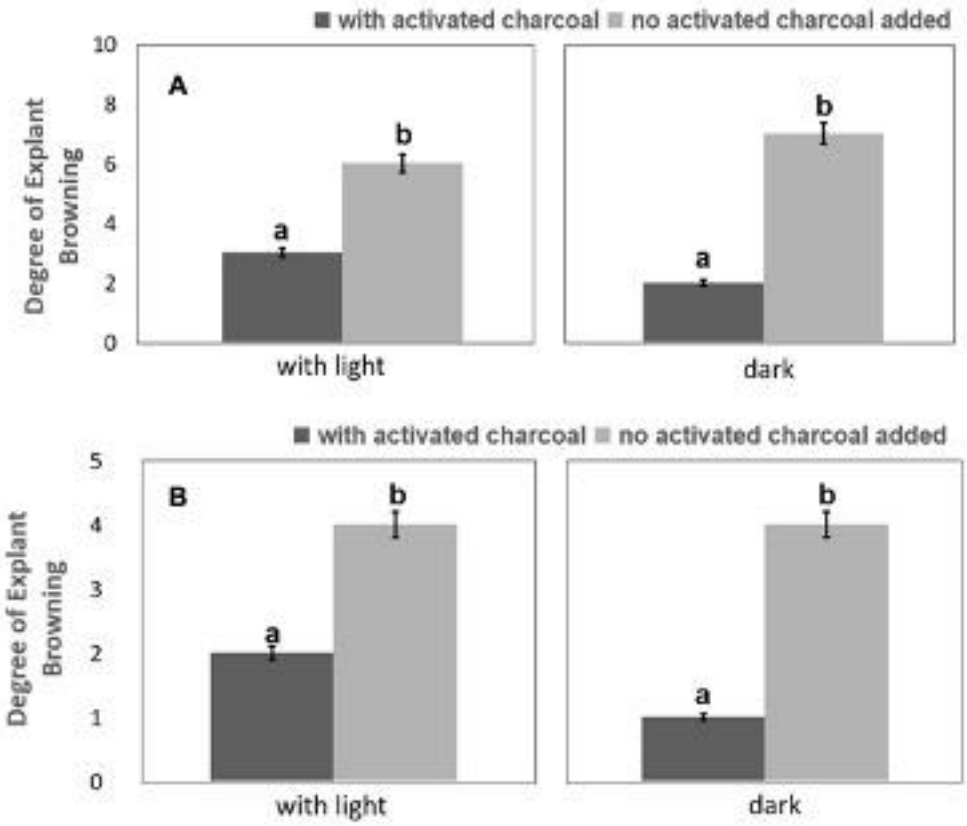

Figure 4. Effect of activated charcoal on the degree of explant browning of dragon fruit calli using 9-point (A) and 5-point (B) arbitrary visual hedonic scale. Bars, within a figure, with different letter indicates means were significant at $p<0.05$ based on T-test analysis. Values represent the mean of the two trials performed.

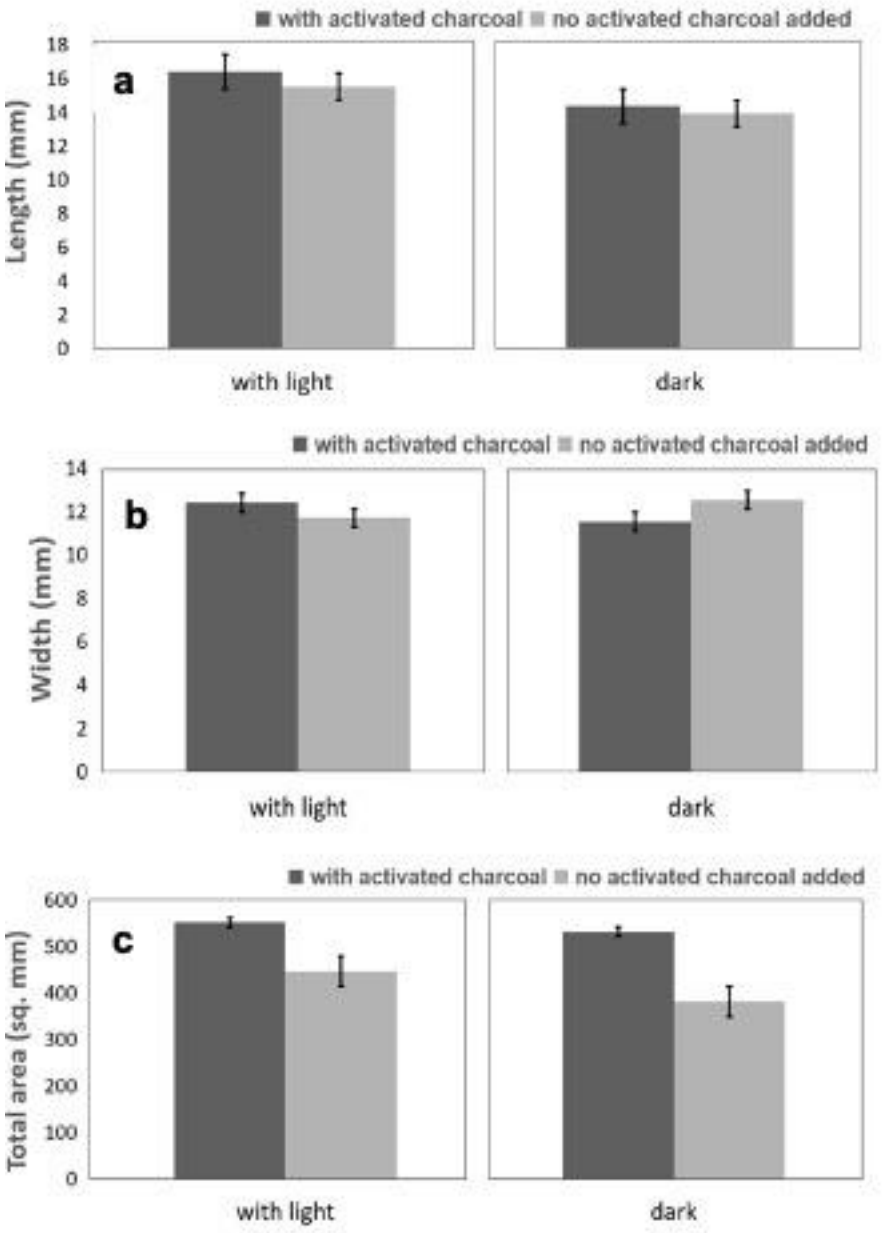

Figure 5. Length (a), width (b), and total area (c) of *H. undatus* calli 28 days after inoculation (dai) in media with and without activated charcoal. No significant difference was found ($p>0.05$) based on T-test analysis. Values represent the mean of the two trials performed.

Fungal Contamination

Significant reduction of contamination of *H. monacanthus* cultures with activated charcoal under 14-hour light (25%) and dark condition (50%) was observed four days after inoculation, compared with untreated cultures (62.5 and 100%) (Figure 6a). The same results were obtained for *H. undatus*. The contamination values for activated charcoal under 14-hour light and dark conditions were 3.1% and 6.3%, respectively, while without activated charcoal were 15.6 18.8%, respectively (Figure 6b). In contrast, an increased contamination rate of both *H.*

monacanthus (87.5-100%) and *H. undatus* (50-75%) explants were observed eight days post-inoculation in both media (Figure 6).

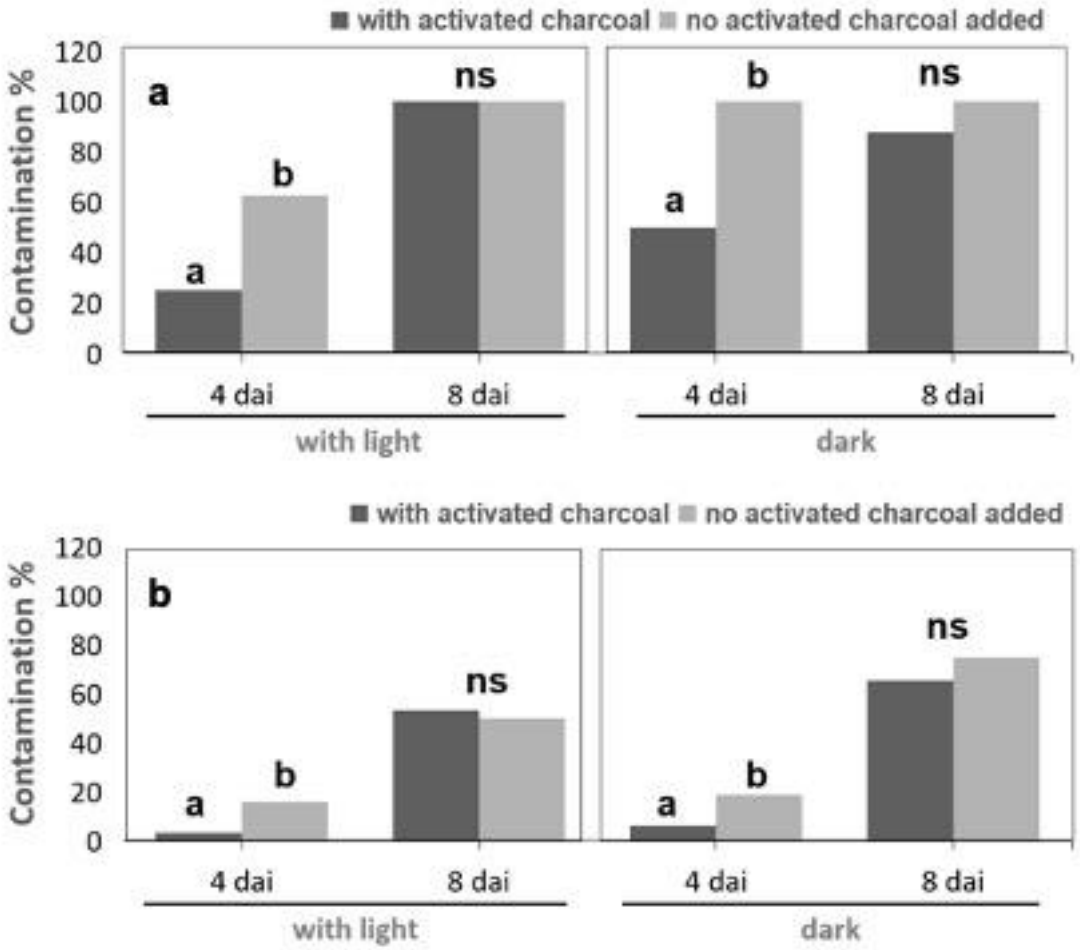

Figure 6. Percent fungal contamination in *H. monacanthus* (A) and *H. undatus* (b) explants four and 8 days after incubation (dai). Means with different letters are significantly different at $p < 0.05$ based on T-test. Values represent the mean of the two trials performed.

Identities of fungal contaminants

Fungal mycelia with white to grayish growth were isolated (Figure 7) from all infected dragon fruit cultures. The fungi were fast-growing, with mycelial growth reaching the edge of the plate on the 7th-day post-incubation (Figure 7A). Thick mycelia with no spores formed were observed (Figure 7B) from the seven-day-old cultures. Culture and morphology characteristics resemble those of *Diaporthe* species. Three isolates (MDF1a, MDF1b, and MDF1c) were chosen for sequence analysis based on their unique morphocultural characters. Analysis of the DNA sequence of the partial sequence of the ITS-rDNA gene region using the BLASTN software showed high similarities (99-100%) of the isolates to *D. passifloricola* and *D. tectonae* (Table 2). The phylogenetic tree (Figure 8) further supported close similarity of isolate MDF1a and MDF1b to *D. passifloricola* and isolate MDF1c to *D. tectonae*.

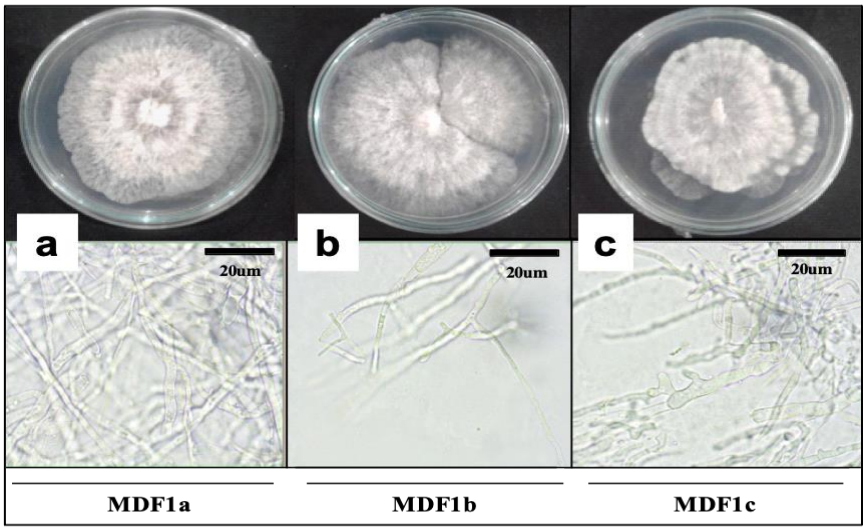

Figure 7. Cultural (A) and morphological (B) characteristics of seven-day-old *D. passifloricola* (a and b) and *D. tectonae* (c) in potato dextrose agar medium (PDA).

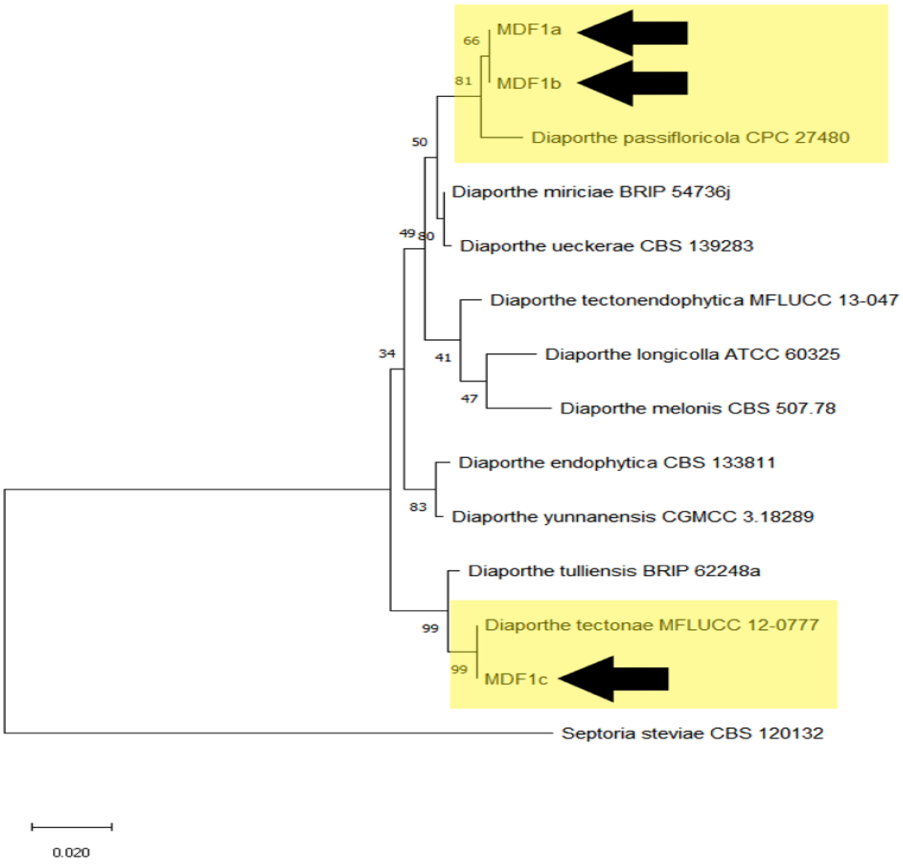

Figure 8. Phylogenetic tree based on the maximum likelihood analysis of the partial sequence of the ITS gene of the three fungal isolates (MDF1a, MDF1b, and MDF1c) from this study and other *Diaporthe* species. *Septoria steviae* CBS 120132 served as an outgroup.

Table 2. Percent similarities of the fungal isolates associated with the tissue-cultured dragon fruit.

Isolate/Sample	Species	ITS (Accession)
MDF1a	*D. passifloricola* CPC 27480	99.20% (NR_1475.95.1)
MDF1b	*D. passifloricola* CPC 27480	99.20% (NR_147595.1)
MDF1c	*D. tectonae* MFLUCC 12-0777	100% (NR_147574.1)

DISCUSSION

This study showed activated charcoal (AC) reduces tissue browning of dragon fruit explant. The findings were consistent with the previous results obtained in other plants, such as potato (Buckseth et al., 2018), sugarcane (Shimelis et al., 2015), carrots (Pan and Staden, 2001), and grapevine (Olah, 2017). The arbitrary visual hedonic scales used in this study could be used in future status to assess callus browning intensity. Both the 5- and 9-point scale were able to discern the browning variation. Some calli were formed, but the presence of AC in the media affected the friability (ideal) of the calli. Activated charcoal did not effect on the length, width, and total area of the calli produced compared to the control check. Activated charcoal has been shown to adsorb auxin and cytokinin from culture media, thus rendering them inactive (Weatherhead et al., 1979). Activated charcoal can also absorb α-naphthyl acetic acid (NAA), up to 300 mg/l, (Weatherhead, 1979), dichlorophenoxyacetic acid (2, 4-D) (Ebert and Taylor, 1990), and 6- benzyl amino purine (BA) (Ebert et al., 1993). Three cytokinins, 6-furfuryl-aminopurine, 6-benzyl amino purine, and 6-(γ,γ-dimethylallylamino) purine, were likewise adsorbed at a concentration of 10 mg/l (Weatherhead et al., 1979) by AC. Thus, the addition of activated charcoal in the media possibly interfered with the growth and callus development of dragon fruit explants through adsorption of the hormone and nutrients available in the medium.

A significant decrease in the dragon fruit cultures' contamination was also documented four days after inoculation (dai) using activated charcoal. Aside from reducing phenolic oxidation, activated charcoal (AC) is known to adsorb undesirable/inhibitory substances and nutrients found in the medium (Pan and Staden, 2001; Thomas, 2008). Activated charcoal in the media might have influenced the environment necessary for the growth of specific microorganisms (Weatherhead et al., 1978). However, no significant effect of AC in contamination was observed eight dai. A higher contamination rate was observed in *H. monacanthus* (87.5-100%) than in *H. undatus* (50-75%) callus cultures. The relatively higher rate of contamination in *H. monacanthus* might be associated with the rich microflora present in the initial explant, which was not effectively removed during the sterilization process. AC's activity is still unclear, but some workers believe that AC gradually releases certain adsorbed products, such as nutrients and growth regulators, which become available to plants (Thomas, 2008). This gradual release of adsorbed nutrients by activated charcoal might have supported microorganisms' growth in the cultures. Hence, activated charcoal for reducing contamination rate in tissue culture is ineffective in the long run.

Molecular analyses of the ITS region of the fungal contaminants found in the dragon fruit cultures revealed that the isolates were highly similar to two *Diaporthe* species: *D. passifloricola* and *D. tectonae*. Although these fungi were not associated with any disease of dragon fruit stem explants used in this study, *Diaporthe* species are known to cause diseases on a wide range of host plants. *Diaporthe* species cause root and fruit rots, dieback, cankers,

leaf spots, blights, decay, and wilt (Mostert et al. 2001; van Rensburg et al. 2006; Thompson et al. 2011; Udayanga et al. 2014; Dissanayake et al. 2015). For example, *D. ampelina* is one of the pathogenic species best known as the causal agent of Phomopsis cane, leaf spot, and grape yield losses in temperate regions (Erincik and Madden 2001). At the same time, *D. phaseolorum* and *D. longicolla* are reported to be pathogenic to soybean (Santos et al., 2011). The occurrence of fruit rot disease in dragon fruit caused by *Diaporthe* sp. was also reported in China (Tsai et al., 2001). Recently, stem rot disease in *H. undatus* caused by *D. phaseolorum* has been reported (Karim et al., 2019). However, the two *Diaporthe* species found in this study was not associated to any symptom in the explant. These two species are considered as an endophyte in dragon fruit, unless proven pathogenic to dragon fruits in future studies.

CONCLUSION

The addition of AC in the tissue culture media reduced callus browning regardless of presence or absence of light. Both the 5- and 9-point visual hedonic scale can be useful for future callus development studies. Activated charcoal does not inhibit fungal contamination. However, although callus were formed, AC (at 1 g/L) negatively affects callus friability. Therefore, determining the optimum concentration of activated charcoal that can significantly reduce tissue browning without hindering callus friability would be worthwhile. *Diaporthe passifloricola* and *D. tectonae* were also identified as fungal contaminants in dragon fruit tissue culture and are considered endophytes in dragon fruit stem. This is the first report of the association of these two *Diaporthe* species in stems of dragon fruits. Nevertheless, the two species' pathogenicity to dragon fruit plants would be tested to further elucidate their interaction with dragon fruits.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors. The research did not contain any experiment that uses animals or humans.

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