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Short Communication: Attempted callus induction of holoparasite *Rafflesia patma* Blume using primordial flower bud tissue

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Abstract. *Wicaksono A, Teixeira da Silva JA. 2015. Attempted callus induction of holoparasite* Rafflesia patma *Blume using primordial flower bud tissue. Nusantara Bioscience 7: 96-101.* Callus induction of a holoparasitic plant *Rafflesia patma* Blume was attempted using sections of a young primordial flower bud (syn. knob) of young flower tissue as the explant. The plant is an endangered species and its *ex-situ* conservation is very difficult. Previous attempts in the literature have not been successful, hampered by infection and explant browning. In an attempt to improve these weaknesses, in this study, following the surface disinfection of a primordial flower bud, primordial flower bud sections were cultured on Murashige and Skoog (MS) basal medium supplemented with 2 g/L activated charcoal, 1 mg/L 1-naphthaleneacetic acid and 4 mg/L N^6-benzyladenine in an attempt to induce any growth. All explants browned after 2 h, and even though browning stopped after 4 days, fungal growth continued. The remaining uninfected explants that did not brown were transferred to MS medium with 1.5 mg/L kinetin in an attempt to induce callus. Two out of 6 explants browned within days but did not develop any callus. Future research needs to focus on more robust surface sterilization procedures, a wider selection of antioxidants to reduce browning, a mixed culture with the tissue of a suitable host, *Tetrastigma leucostaphyllum*, to stimulate organogenesis, and testing a wider range of abiotic conditions to establish an effective micropropagation protocol.

Keywords: Activated carbon, flower, parasite, primordial bud, *Rafflesia patma*, tissue culture

INTRODUCTION

Rafflesiaceae is a family of obligate parasites (holoparasites) that rely on nutrients from their host, primarily from the genus *Tetrastigma*, most frequently *Tetrastigma leucostaphyllum*, which is a vine (Susatya 2011), or *T. scariosum* (Veldkamp 2009). Rafflesiaceae, which consists of three genera (*Rafflesia*, *Rhizanthes*, and *Sapria*), is widely distributed in Southeast Asia (Indonesia and Malaysia) (Davis 2008). Unfortunately, several factors are causing natural populations of these plants to drop quickly, including deforestation. According to Kew Royal Botanical Gardens (2015), *Rafflesia arnoldii* (R. Brown) is not included in the IUCN Red List, but is considered to be vulnerable due to disturbance by tourists and collection for traditional medicine, concerns also indicated by Nais (2001). However, one species, *Rafflesia magnifica*, is listed on the IUCN Red List as critically endangered (IUCN 2015). Another reason for the drop in natural populations is because *Rafflesia* is a dioecious plant, i.e., separate male and female flowers (Salleh 2007), with few populations in a designated area (Balete et al. 2010), and most *Rafflesia* plants found in the field are male (Susatya 2011).

Rafflesia patma Blume, the target plant of this study, is found in limited parts of Java and Sumatra Islands, including coastal areas of Pangandaran, West Java, where it is endangered (Mursidawati et al. 2015). It is considered to be vulnerable by the IUCN (Nais 2001). *R. patma* has distinctive features (Figure 1; botanical terms strictly follow Nais (2001)), including a very short ramenta (hair-like structure in the inner area of the hollow floral crown), a flower diameter of 30-60 cm, a slightly raised disk rim, and a small wart of the same color as the upper perigone surface. Compared to *R. arnoldii*, it has tall and coral-branch-like ramenta, large white warts around its upper perigone surface, and a large diaphragm opening exceeding 30 cm (Nais 2001).

There is interest in the ex-situ conservation of *Rafflesia* species. Sabah Parks in Malaysia, known for one *Rafflesia* conservation site in Sabah in the Northern part of Borneo, claimed successful seed germination but the protocol was never released to the public (Nais 2001). In Indonesia, the cultivation of *R. patma* seeds was attempted by incising the *Tetrastigma* stem and by sowing seeds directly into the stem (Mursidawati and Riswati 2009). However, even the addition of strigol, a germination stimulant, did not improve germination (Mursidawati et al. 2015). In addition, no growth was possible, even after 628 days. Sukamto (2001) reported the first micropropagation protocol for *R. arnoldii* using flower explants (specifically from the young primordial flower bud (PFB) and seeds plated on half-strength Murashige and Skoog (½MS; 1962) macro- and micronutrients with varying concentrations of plant growth regulators (PGRs), including 2,4-dichlorophenoxyacetic acid, indole-3-acetic acid, and 1-naphthaleneacetic acid (NAA). We believe that the term PFB, equivalent to

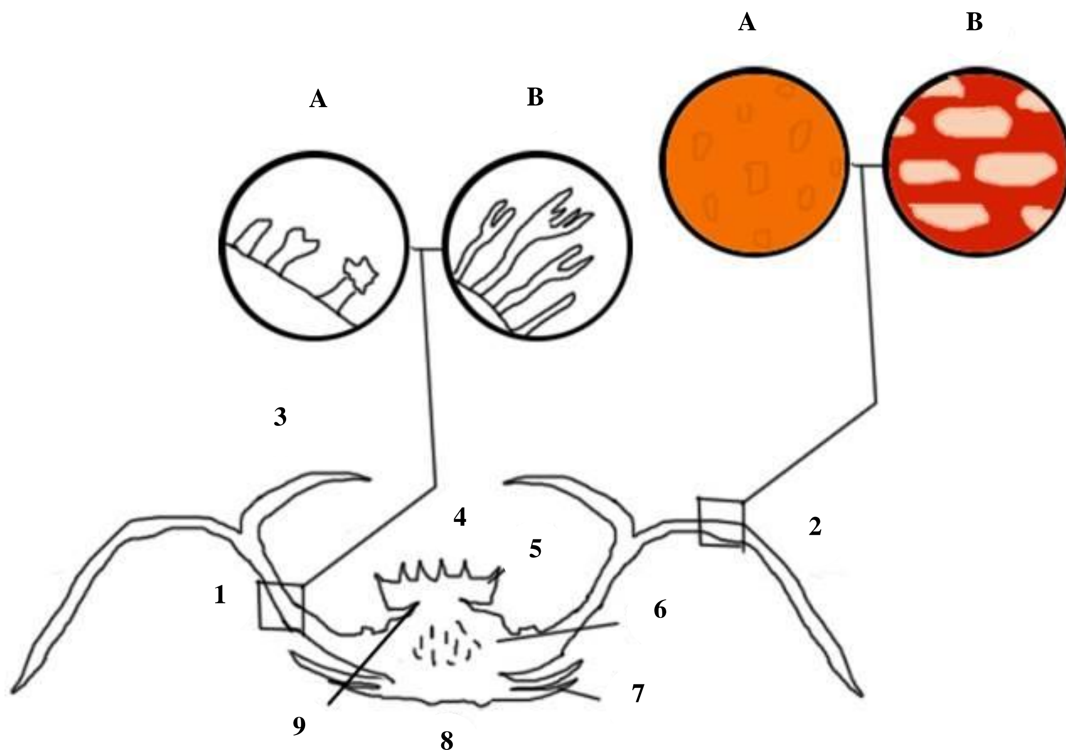

Figure 1. Anatomy – including botanical terms – of a *Rafflesia patma* vs *R. arnoldii* flower according to, and redrawn based on, Nais (2001). (1) Inner surface with ramenta, (A) small ramenta in *R. patma*, and (B) tall and branched ramenta in *R. arnoldii*. (2) Petal-like organ, the perigone lobe; (A) perigone surface warts are both orange in *R. patma*, and (B) red surface of *R. arnoldii* with white warts. (3) Diaphragm. (4) Spiky processus protruding from the disk. (5) Central disk of the flower. (6) In the female flower, this part develops into a fruit that carries small seeds inside (values based on Mursidawati et al. (2015)): fruit of *R. arnoldii* is ~18 cm in diameter with 50 cm length while *R. patma* is 13-15 cm width/length; fresh seed weight for *R. arnoldii* is 87-97 µg and for *R. patma* it is 18-21 µg; seed length for *R. arnoldii* is ~1000-1500 µm while *R. patma* is ~500-900 µm). (7) Outer sheets that encase the flower before blooming. (8) Connection part with the host, *Tetrastigma leucostaphyllum*. (9) Anther in the male flower or pistil in the female flower.

the term "knob" used by Mursidawati and Riswati (2009), is equivalent to the flower primordium described by Nais (2001). For all effective purposes, we have employed the term PFB throughout this manuscript. Sterilization of the floral PFB was achieved with a dip in 70% ethanol followed by a dip in 0.1% $HgCl_2$. Seeds were surface sterilized with sodium hypochlorite (NaOCl) before rinsing once with sterile distilled water. In that study, however, the time for each surface disinfection step was not defined, no organogenesis was achieved, and explants browned, despite swelling, while seeds did not germinate, even after 18 months. A more recent attempt by Mursidawati and Handini (2009) used *R. patma* and *Rafflesia meijerii* PFBs of undefined age and infected *Tetrastigma* stem cuttings, which were first cleansed with 70% ethanol, then with NaOCl in three cycles (20% for 20 min, 10% for 10 min, and 5% for 5 min). Explants were placed onto ½MS medium (pH 7.0) to which 20% (v/v) coconut water was added, as well as 4 mg/L N^6-benzyl adenine (BA) and 5 mg/L NAA. Also in the study by Mursidawati and Handini (2009), bacterial and fungal contamination was clearly highly problematic as it contaminated 100% of cultures. Even though explants swelled, they did not develop further, until 24 days. Thus, there are limited, and only unsuccessful, attempts to micropropagate *Rafflesia* species.

Like its host plant *T. leucostaphyllum*, *Rafflesia* species are known to accumulate large amounts of secondary metabolites, primarily alkaloids and phenolic compounds such as nicotine and caffeine (alkaloids) and leucoanthocyanidin derivatives, tannin, and catechin (a flavonoid), as well as phenolic acid (Sofiyanti et al. 2008). *Rafflesia hasseltii* scales accumulate anything from 1-6 times more nicotine, caffeine, tannin, catechin, and phenolic acid than *T. leucostaphyllum* roots and stem (Sofiyanti et al. 2008). This indicates that *Rafflesia* species accumulate secondary metabolites from its host. The extract from *R. hasseltii* has a medical (wound-healing) property (Abdulla et al. 2009) while the flower extract of *Rafflesia kerrii* contains strong antioxidant properties (Puttipan et al. 2014). This further confirms the benefits of *Rafflesia* leading it to be collected from the wild by local populations for medicinal purposes.

Given the ornamental and medicinal properties of *Rafflesia* species, and the endangered status of wild populations, an objective of this study was to induce callus from *R. patma* PFB tissue. To counter the browning of

explants, activated charcoal (AC) was employed since it is known to improve aeration, establish polarity and affect substrate temperature when micronutrients are added, while absorbing toxic compounds such as polyphenols that induce browning (Thomas 2008; Teixeira da Silva et al. 2015a). In addition, in a bid to reduce fungal and bacterial contaminants observed in previous studies, a disinfection procedure that combined a detergent, a fungicide, and flaming, was attempted.

MATERIALS AND METHODS

A single cutting of a *T. leucostaphyllum* stem that was impregnated with *R. patma* (shown with visible PFB; Figure 2) was obtained from the Pangandaran Conservation Park area of West Java in December 2012, after identification and suitable permission. The PFB was transferred within 2 days to the laboratory and maintained in moist tissue paper in a dark plastic bag at room temperature (26-28°C; Bandung, West Java). A single PFB 2 cm in diameter protruding from the *T. leucostaphyllum* stem (Figure 2) served as the explant. Tissue culture experiments were conducted at the Laboratory of Plant Physiology, School of Life Science and Technology of the Institut Teknologi Bandung in Bandung, Indonesia.

Tetrastigma, 1.5 cm in diameter and containing the PFB, was trimmed to just outside of the PFB. It was surface disinfected by a wash in detergent, a rinse under flowing tap water for 60 min, then immersion for 2 h in a water-based 0.5 g/L fungicide solution (Antracol 70WP®, Bayer, India; propineb is the active fungicidal component).

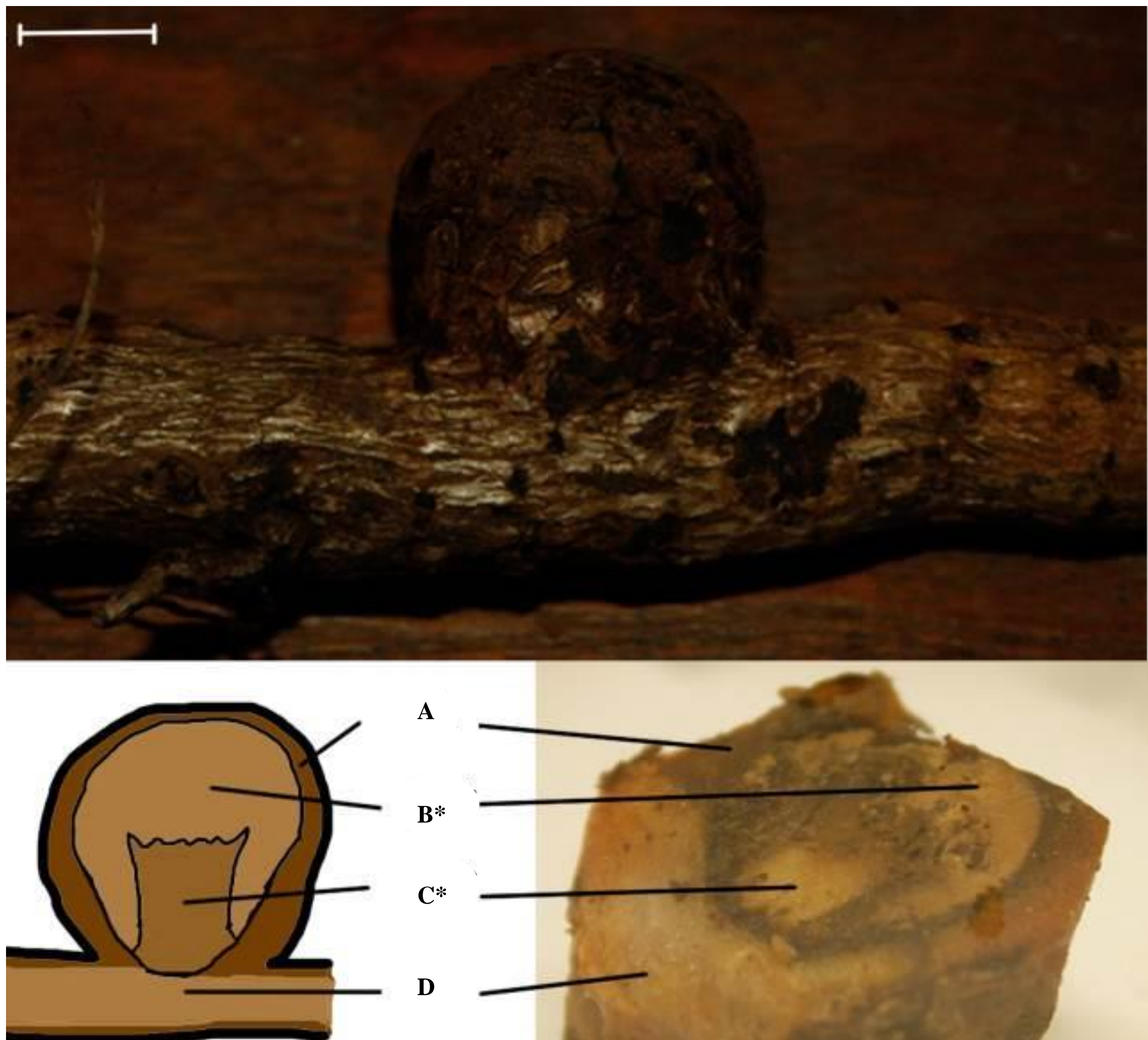

Figure 2. Stem of *Tetrastigma leucostaphyllum* with *Rafflesia patma* primordial flower bud (PFB) intact (top). Incised tissue (bottom) of *Rafflesia* primordial (A) dermal layer of *Tetrastigma* PFB (B*) proto-perigone (which will develop into the perigone; **Figure** 1 (2)) tissue of *Rafflesia*, (C*) primordial processus (which will develop into the processus; Figure 1 (4)) of *Rafflesia*, and (d) *Tetrastigma* heartwood/xylem. * indicates extracted part used for tissue culture. Botanical terminology based on Nais (2001). Bar = 1 cm.

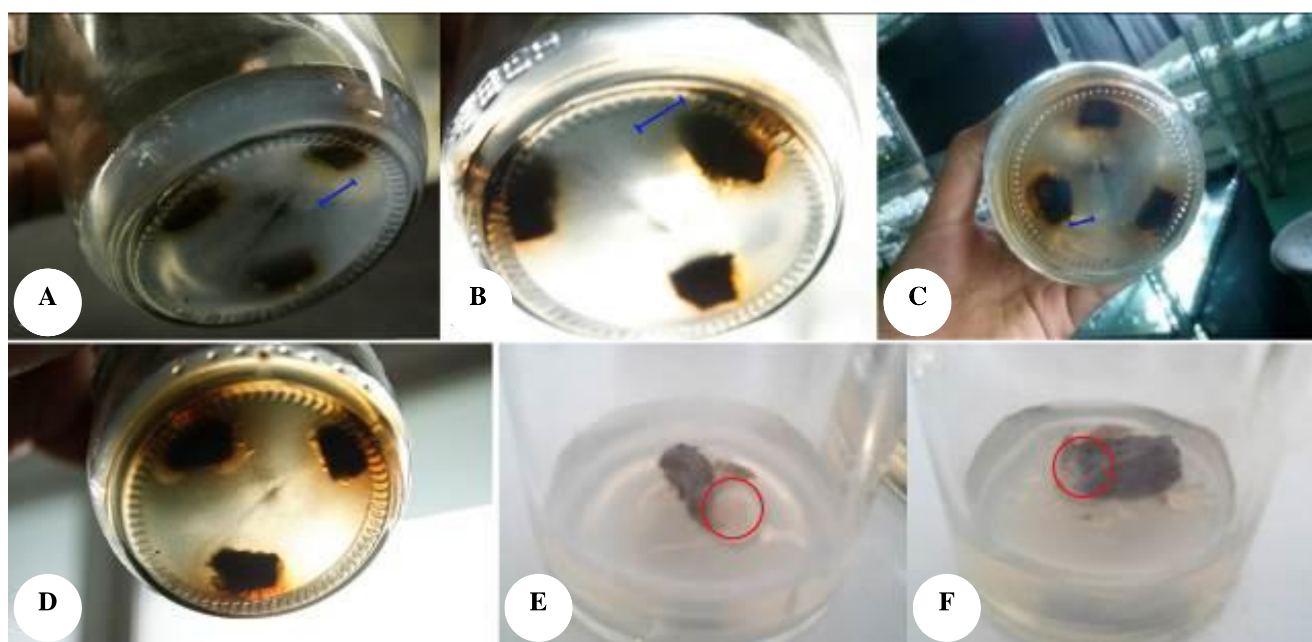

Figure 3. Problems with tissue culture of *Rafflesia patma*. Browning-area spreads around the explants: A. 3 h after initiation, B. 15 h, C. 39 h; blue lines (scale) = 1 cm. Fungal hyphae are detected in top-left and bottom explants (D). Top-right explant is clear of fungal and bacterial infection and can be transferred to callus induction medium. The two remaining explants in callus induction medium were infected by fungi (shown by red circles in E and F).

In a laminar air flow bench, the PFB was dipped in 70% ethanol and flamed for a few seconds until the ethanol completely evaporated. Flaming was repeated twice. In sterilized Petri dishes (Pyrex, Corning, USA) and using sterile scalpel blades, the PFB was removed from the stem and the *T. leucostaphyllum* dermal layer was peeled off using tweezers sterilized in 70% ethanol, leaving only the primordial area (Figure 2). This area, which includes the proto-perigone and primordial processus, was sliced thinly (~2 mm in thickness) and was around 8-10 mm in diameter. These slices served as the explants. Three explants were placed in each jam bottle containing initial growth medium (MS macro- and micronutrients as the base with 1 mg/L NAA, 4 mg/L BA, and 2 g/L AC. Medium was solidified with 7.5 g/L agar after autoclaving at 100 KPa for 17 min and kept in a sterile room before culture initiation. Bottles were transferred to light in a tissue culture chamber (25±2°C; 65-82% relative humidity; 16-h photoperiod; photosynthetic photon flux density of 30 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes). After 4 days, explants were transferred to callus induction medium (MS with 1.5 mg/L kinetin but free of AC). All chemicals and reagents were of tissue culture grade purchased from Sigma-Aldrich (Singapore).

RESULTS AND DISCUSSION

Rafflesia patma is an exquisite terrestrial ornamental plant. However, it is rare and endangered, and methods for its micropropagation are urgently required in order to increase stocks of germplasm that could be made available to botanical gardens and collectors, without threatening natural stands. There have only been two attempts in the literature, both by Indonesian scientists, to tissue culture *Rafflesia* species (Sukamto 2001; Mursidawati and Handini 2009). In both these studies, 100% explant infection and no growth were observed. Our report thus represents the first successful induction of callus from any *Rafflesia* species. Given its extremely rare nature, only a single sample could be collected from a natural park, from which a single PFB was derived (Figure 2). Consequently, these results are preliminary; there are no replicates and no controls, either. That PFB allowed for only 6 explants to be prepared. Using our protocol, despite experiencing fungal and bacterial infection in 4 out of 6 explants, two were infection-free. Browning (tissue oxidation) was observed within 2-3 days. Callus could not be induced from these two explants, which browned and became contaminated (Figure 3E, F).

The two biggest hurdles of our protocol were browning and explant contamination. Browning takes place as a result of the oxidation of tissues caused by the presence of phenolics and tannins that inhibit growth, and with dark coloration the result of quinine formation; quinines are highly reactive oxidant species (ROS) that are toxic to plant tissues (Sansberro et al. 2003; Ahmad et al. 2013). Tissue oxidation is usually accompanied by a burst in the activity of antioxidative enzymes (e.g., Kaewubon et al. 2015). Some pathogenic fungi can protect themselves against ROS when a large number of phosphorelay sensor

kinases produce antioxidant enzymes and release metabolites with an antioxidant function (Aguirre et al. 2006). Due to the prolonged duration of contamination, it is also suspected that metabolites that contribute to browning may inhibit the growth of contaminants for several days until browning stopped and at which point fungi started to grow. This hypothesis is supported by a study by Wiart et al. (2004) in another *Rafflesia* species, *R. hasseltii*, which had been screened for its antimicrobial and antifungal properties. The broadest spectrum of activity of the extract was able to counter the growth of several bacteria, including *Bacillus cereus*, *Bacillus substilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

The greatest problem and limiting factor in *Rafflesia* tissue culture studies is the limited amount of available tissue. Consequently, large-scale trials, as for other crops, are not possible. Thus, using a limited pool of explants, the main issues of explant contamination and browning need to be tackled. AC absorbs quinines and other compounds that induce oxidation and browning (Thomas 2008). Despite the addition of AC to our medium, tissue browning could not be prevented. However, it might be prevented or delayed by frequent rinsing of explants in sterile distilled water, frequent subculture to fresh medium, use of liquid media with or without paper rafts, the use of polyvinylpyrrolidone, the addition of ascorbic acid, L-cysteine, potassium citrate, HCl, dithiothreitol, silver nitrate or glutathione as antioxidative agents, or culture in the dark (Sathyanarayana and Varghese 2007; Ahmad et al. 2013; Teixeira da Silva 2013). Should there be sufficient explants, such alternatives should be tested.

The issue of the elimination of explant infection is more complex because wood-borne fungi may exist in the host *T. leucostaphyllum*. However, no endophytic fungi have yet been identified in *T. leucostaphyllum* or *R. patma*. In *Rafflesia cantleyi*, eight strains of an endophytic fungal species, belonging to three genera (*Colleotrichum*, *Cytospora*, and *Gliocladiopsis*), were detected (Refaei et al. 2011). It is possible that similar endophytic fungi may also be growing in *R. patma*. A phytopathological assay is thus required in the future to confirm this. Alternative ways to eliminate, or reduce, infection by microorganisms include: use of higher concentrations of NaOCl, use of mercuric chloride (risk of greater tissue necrosis), use of younger tissues, smaller explants (risk of greater tissue damage), the use of other tissues (e.g., flower tissue about to bloom, since it is actively growing, is easy to collect and is large) or increasing the surface area of explants to allow for greater exposure to sterilants (e.g., Teixeira da Silva et al. 2015b).

Finally, since *Rafflesia* species are obligate holoparasites, there is interest in cultivating the host species under aseptic conditions, which would be the first step towards assisting the reduction in infection of in vitro cultures of *Rafflesia*. To this end, Surya and Idris (2011) were able to induce the germination of *Tetrastigma rafflesiae* in vitro, and also induce callus in MS medium, without contamination, in the presence of NAA or 2,4-D, and best in the presence of AC. However, browning caused by tissue damage, was problematic.

The ability to successfully propagate *R. patma* through callus induction, and then eventually through direct organogenesis and somatic embryogenesis, would provide alternative pathways for the conservation and mass production of this holoparasitic plant. This would then allow cellular-symbiotic behavior to be studied in more detail. Although this study was unable to induce callus, and thus constitutes a negative result, it sheds more in-depth details about how explants respond in vitro, and how contamination occurs (a wave of bacteria followed by a wave of fungus/fungi). This paper does, however, show at least three novelties: a) the BA+NAA combination was not successful in inducing callus; b) we were able to stop tissue browning in 4 days; c) the use of AC is insufficient to stop tissue browning; d) no explant contamination was observed for 4 days, i.e., contamination was temporarily halted, even though contamination is usually visible within 1-2 days.

Negative results from an important backbone of the scientific process because they allow scientists to formulate new hypotheses based on those negative results (Teixeira da Silva and Giang 2014; Teixeira da Silva 2015). It is our desire that these negative results may serve as an additional catalyst for positive callus induction or organogenesis in *Rafflesia* species.

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