PRELIMINARY STUDY ON ESTABLISHMENT OF *RAFFLESIA CANTLEYI* IN TISSUE CULTURE USING SEEDS AND PRIMORDIAL FLOWER BUD AS EXPLANTS

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ABSTRACT

Rafflesia cantleyi, is a family member of the world's biggest flower, Rafflesiaceae and is a holoparasitic endophyte. This family exhibit extreme modification of their vegetative and reproductive bodies that replace photosynthetic tissues. R. cantleyi consist of flower organ that emerged from its host, Tetrastigma rafflesiae a woody vine from Vitaceae family. Propagation methods of Rafflesia sp. have been studied for conservation through the infected host stem grafting and cutting as well as ex situ seed germination. Micropropagation through tissue culture of other Rafflesia sp. has also been studied, however no successful progress were reported so far. In this study, we determined to develop tissue culture method for R. cantleyi. Primordial flower knob/buds, perianth lobe (petal-like) and seeds of R. cantleyi were collected from Perak, Malaysia and were attempted to introduce into tissue culture. Among the three explants, primordial flower knob/buds and seeds showed high successful rate for clean culture compared to perianth lobe (petal-like). More than 90% clean culture were obtained from surface sterilization of the buds and seeds however low success rate for explants from perianth lobe. The clean culture obtained however, did not showed any growth of callus or seed germination even after more than 6 months observation. The major problem of R. cantleyi in vitro culture was high production of phenolic compound and high browning rates. All samples from buds and perianth lobe were turned to brown as soon as exposed to air after excised to small pieces during surface sterilization. The used of anti-oxidant solutions during surface sterilization and addition of activated charcoal in the culture media have shown no positive effect on the browning of the tissues.

Key words: clean culture, *in-vitro* propagation, phenolic

INTRODUCTION

Rafflesia, is a genus from *Rafflesia*ceae family, with one of its species is known as the world's largest flower with measurement size up to 3 feet (95 cm) across. Being a holoparasite, other than large organ flower, *Rafflesia* does not have a distinct other organ like leaves, roots and stem. The flower organ exhibits highly modify vegetative body to enable them to obtain nutrient from its host instead of making food on their own (Nikolov et al. 2014). The presence of the *Rafflesia* was only noticed when the flower bud emerged from the host, *Tetrastigma* (Vitaceae) (Meijer, 1997; Nais, 2001; Sofiyanti & Yen, 2012). A cabbage like bud takes about 16 months before bloom slowly, near 12 to 48 hour to full bloom.

Rafflesia can be found in South East Asia, mainly in Malaysia, Indonesia, Philippines and Thailand. In Malaysia, *Rafflesia* was reported found in Sabah, Sarawak, Kelantan, Pahang, Terengganu and Perak. In total, five *Rafflesia* species have been reported in Peninsular Malaysia namely *R. cantleyi, R. azlanii, R. kerii, R. su-meiae* and R. *parvimaculata* (Sofiyanti et al. 2016).

Beside its morphological uniqueness, *Rafflesia* was also reported to exhibit medicinal usage in wound healing as practice by locals (Malaysian and Indonesian). In order to balance for ethnomedical, ornamental use and conservation, artificial cultivation procedure need to be studied to avoid over exploitation from the wild. However, the propagation and distribution of *Rafflesia* is still a huge mystery to cover. Previous research notes that the natural distribution of the *Rafflesia* is assisted by small fly known as carrion (blue bottle). These flies attracted to the rotten flesh like smell from the flower and help to spread the seeds from male to the female

flower. For successful pollination to occur, both male and female flower must bloom simultaneously in the same area, the chance of opening is about 8 to 10 days before the flower start to deteriorate (Beaman et al, 1988; http://www.sabahparks.org.my).

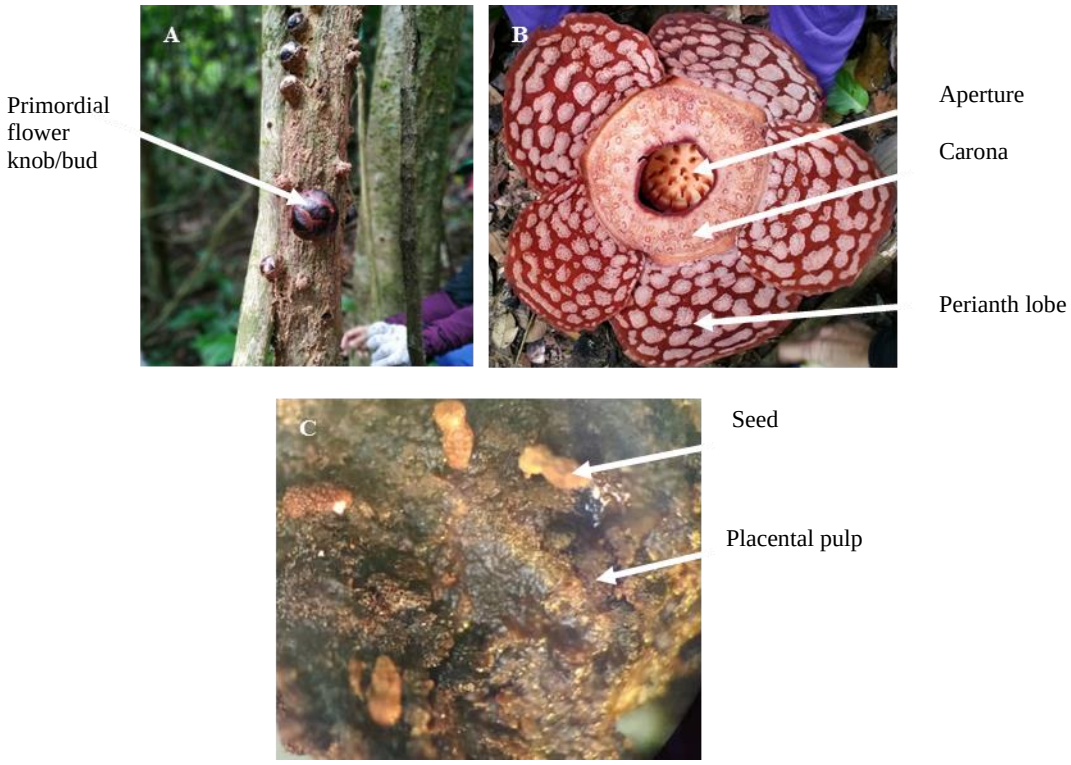
In order to artificially cultivate *Rafflesia*, mature seeds are the important key. Many studies on propagating *Rafflesia* have been reported, however so far, only grafting technique was reported successful. The impregnated *Tetrastigma* with *Rafflesia* seeds were grafted into normal *Tetrastigma* stem (Wicaksono et al. 2016). Another *Rafflesia* spesies, *R. patma*, the seeds were inserted into the incised *Tetrastigma* stem to initiate germination; however the attempt was reported not successful (Mursidawati & Riswati 2009). Other cultivation method such as tissue culture of *Rafflesia* has been reported extremely difficult due to contamination and browning. In this study, another set of attempt to artificially cultivate *Rafflesia cantleyi* from Perak through tissue culture method has been done to study the response of this species in different set of treatments. The purpose of this paper is to share the information on the research done on *R. cantleyi* collected from Perak.

MATERIAL AND METHODS

Samples Collection

Samples collection was made at Gerik, Perak. Three parts from the *Rafflesia*; perianth lobe (a petal-like), seed and primordial flower knob/bud (6-8 diameters) were collected as explants for tissue culture protocol development. The seeds were taken from matured ovary which looked like rotted fruit. Fig. 1 shows the primordial flower knob/bud, full bloom *Rafflesia cantleyi* and seeds under microscope.

Figure 1: Samples collected in experimental plot. A) Primordial flower knob/bud of *Rafflesia cantleyi* on *Tetrastigma* stem, B) Full bloom *Rafflesia cantleyi* with 5 perianth lobes, aperture and carona, C) Seeds in placental pulp.



SURFACE STERILIZATION AND CULTURE MEDIA

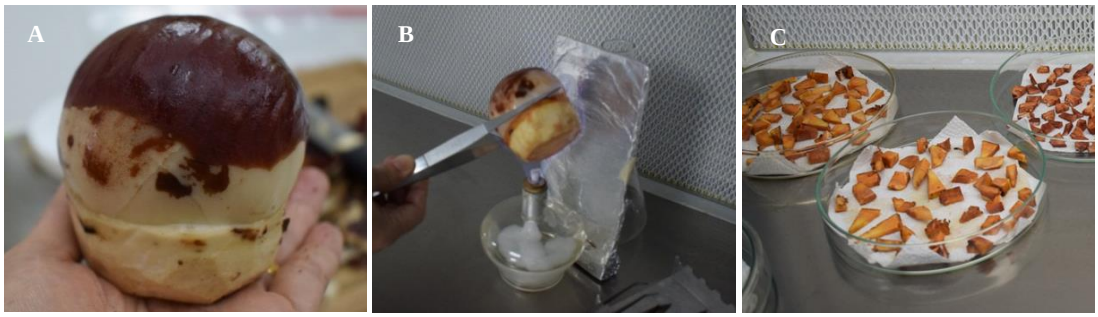
Primordial Flower Knob/Bud

Three buds size ranging between 6-8 cm diameters were collected and surface sterilized with three different methods. The differences between surface sterilization methods used were the Clorox® concentrations and the anti-oxidant solution used. All samples were treated with fungicide for 40 min and 70% ethanol for 2 minutes prior to surface sterilization using Clorox® under laminar flow. The next process followed the details listed in Table 1.

Table 1: Surface sterilization method and the culture media used for samples from primordial flower knob/ buds

Method	Details	Culture media
1	I. Soaked in 70% Clorox® and a few drops of Tween 20 for 20 min II. Cut into smaller pieces (~1.5 cm) and soaked for 30 min into different anti-oxidant solution: (a) Sterilized distilled water, b) 500 mg/L ascorbic acid, c) 1000 mg/L ascorbic acid, d) 2000 mg/L ascorbic acid, e) 500 mg/L Polyvinylpyrrolidone (PVP), f) 1000 mg/L PVP, g) 2000 mg/L PVP III. Cultured into media	I. Murashige and Skoog (MS) basal medium supplemented with 1.0 mg/L 2,4-D and 2.0 g/L activated charcoal II. MS basal medium supplemented with 1.0 mg/L NAA and 2.0 g/L activated charcoal
2	I. Soaked in 50% Clorox® and a few drops of Tween 20 for 20 min II. Cut into smaller pieces (~1.5 cm) and soaked for 30 minutes into different anti-oxidant solution: (a) Sterilized distilled water, b) 500 mg/L ascorbic acid, c) 1000 mg/L ascorbic acid, d) 2000 mg/L ascorbic acid, e) 500 mg/L Polyvinylpyrrolidone (PVP), f) 1000 mg/L PVP, g) 2000 mg/L PVP III. Cultured into media	I. MS basal medium supplemented with 1.0 mg/L 2,4-D and 2.0 g/L activated charcoal II. MS basal medium supplemented with 1.0 mg/L NAA and 2.0 g/L activated charcoal
3	I. Flamed in laminar air flow for three times II. Cut into smaller pieces (~1.5 cm) III. Cultured into media	I. MS basal medium supplemented with 1.0 mg/L 2,4-D and 2.0 g/L activated charcoal II. MS basal medium supplemented with 1.0 mg/L NAA and 2.0 g/L activated charcoal

Figure 2 Surface sterilization process of R. cantleyi bud, A) Bud after removal of outer layer, b) R. cantleyi bud flamed under laminar flow and C) R. cantleyi bud were cut into smaller pieces before cultured into media.



Perianth Lobe (Petal-Like)

Perianth lobes from a full bloom *R. cantleyi* were collected from the experimental plot and surface sterilized using the method in Table 2. The samples were cut to 5 cm x 5 cm and soaked in 1000 mg/L ascorbic acid at 4ºC overnight. All samples were treated with fungicide for 40 min and 70% ethanol for 2 min prior to surface sterilization using Clorox® under laminar flow. The rest of the procedures were as listed in Table 2.

Table 2: Surface sterilization method and the culture media used for samples from perianth lobe

Method	Details	Culture media
1	I. Soaked in 40% Clorox® and a few drops of Tween 20 for 20 min II. Cut into smaller pieces (~1.5 cm) III. Cultured into media	MS basal medium supplemented with 1.0 mg/L 2,4-D and 2.0 g/L activated charcoal
2	I. Soaked in 50% Clorox® and a few drops of Tween 20 for 15 min II. Cut into smaller pieces (~1.5 cm) and soaked in 2.0 g/L PVP solution III. Cultured into media	
3	I. Soaked in 35% Clorox® and a few drops of Tween 20 for 20 min	

	II. Cut into smaller pieces (~1.5 cm) and soaked in 2.0 g/L PVP solution
	III. Cultured into media

Seeds

Mature fruits were collected from the experimental plot and seeds were separated from the placental pulp under microscope before surface sterilized using the method in Table 3.

Table 3: Surface sterilization methods used for seeds and the culture media

Method	Details	Culture media
1	I. Soaked in 70% Clorox® and a few drops of Tween 20 for 20 min II. Blotted dried III. Cultured into media	I. MS basal medium supplemented with *Tetrastigma* bud extract (1.0 g/L) II. MS basal medium supplemented with 1.0 mg/L NAA III. MS basal medium supplemented with 1.0 mg/L BAP

All samples were kept in dark condition in the growth room. The temperature was set at 24 ± 2ºC and observed every week for 3 months.

RESULTS AND DISCUSSION

Few weeks after surface sterilization, the percentage of clean culture from three different samples was summarized in Table 4. Among the three, samples from seeds and primordial flower knob/buds showed high percentage of clean culture, followed by perianth lobe. High percentage of clean culture from buds was probably due to explants used were encapsulated by multiple outer layer such as layers of bracts, perianth lobe and carona. The part used from the bud as samples were central axis, disk, inner layer of perianth lobe and carona. The samples from bud were protected from outside contaminant hence the potential for contamination reduced. The percentage of clean culture obtained were more than 90% from bud and seeds. Method 3 from bud shows the highest percentage of clean culture obtained and shorter time during surface sterilization process. The bud's outer surface was flamed for a few seconds to remove the contaminant without destructing the tissue within. After flamed and removal of outside layer, the inside tissue was still fresh and no changes of colour were observed indicated that the tissue was still viable. In contrast with perianth lobe, the percentage of clean culture were only 0 - 21% due to the fact that the perianth lobe is exposed to outside contaminant and was also seen with scar possibly made from ant and other insect infection during sample collection in the field. As for sample from seeds, originally they were also encapsulated by fruit structure, however in this study we found the mature fruit in a condition where decaying already started where some part of the seeds has been exposed. However, due to the high concentration of Clorox® used and the seeds have an epidermis of large cells with tough durable cell walls (Ng 2019), clean culture was successfully obtained.

Despite high percentage of clean culture from both bud and seeds samples, the response of these samples in tissue culture media were frustrating. The buds were turned brownish as soon as it exposed to the air, even during the surface sterilization process in laminar flow. Few approaches to reduce the browning were conducted such as using anti-oxidant solution during surface sterilization as well as supplementation of activated charcoal in the culture media however no positive result were achieved (Fig. 3A). The used of activated charcoal was to absorb quinines, which are highly reactive oxidant species (ROS) and other compounds that induces oxidation and browning (Thomas 2008). According to previous research on R*afflesia* tissue culture, the browning of the tissues was the major problem (Wicaksono et al. 2015).

In this study, the browning tissue were later died even we sub cultured a few times into fresh media. Other alternative taken were sub culturing some of the samples in MS liquid media, after surface sterilization and kept in 4ºC to see whether the browning can be reduced, however, no positive response were observed(Fig. 3B).

As for the seeds, no sign of growth observed in all media even in the MS basal media supplemented with the host (*Tetrastigma*) powder. The cultures were kept in the growth chamber for monitoring. However after a few months, some of the culture started to contaminate by fungus. Other study on *Rafflesia arnoldii*, the seeds did not grow even after 18 months in culture media (Sukamto 2001) and *ex-situ* germination trial on *Rafflesia patma* also did not show any germination after 628 days even with germination stimulant (Mursidawati et al. 2015). Germination of *Rafflesia* has never been observed so far on soils, moist paper or even on disk of *Tetrastigma* stems (Ng 2019) and these show limited knowledge on the *Rafflesia* seed germination process. For this study, longer culture duration is probably required until method to stimulate germination of *Rafflesia* seeds *in vitro* discovered. Therefore, an extension of experiment on manipulation of different culture media, hormone and culture condition for both *Rafflesia* seeds and primordial flower knob/bud would probably be required.

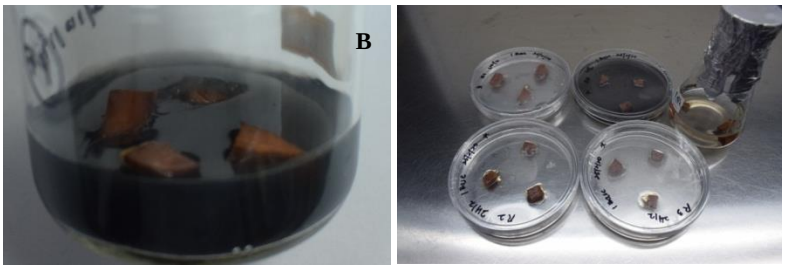
Overall, even though callus induction or seed germination from *R. cantleyi* was not able to be reported in this paper, however method for surface sterilization of *R. cantleyi* from bud and seed can be used as future reference.

Table 4: Percentage of clean culture, browning and condition of the samples after two weeks in culture

Samples	Method	Clean Culture (%)	Browning (%)	Condition
Primordial flower knob/bud	1	77	100	No callus, no response
	2	96	100	No callus, no response
	3	100	100	No callus, no response
Perianth lobe (petal)	1	0	100	No callus, no response
	2	0	100	No callus, no response
	3	21	100	No callus, no response
Seed	1	100	N/A	No germination, no response

Figure 3: A) R. cantleyi primordial flower knob/bud turned brown in culture media with activated charcoal B) R. cantleyi in different media treatment

A **B**



CONCLUSION

As of current result, no callus or seed germination was induced yet though the clean culture of *R.cantleyi* was successfully obtained. *R. cantleyi* primordial flower knob/bud surface sterilization method using flame was considered successful with high percentage of clean culture obtained and this method has low destructive effect to the inner tissue compared to Clorox®. However, other approach to reduce tissue browning, induce callus and seed germination are needed for future study. In this study, the use of anti-browning agent such as PVP, ascorbic acid and activated charcoal were unable to stop the browning of the *R.cantleyi* tissue. Even though no callus induced or seed germination of *R. cantleyi* observed, this study will contribute to the collection of research finding regarding the *Rafflesia* species.

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