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Scientific Article

VIABILITY TESTS ON THE SEEDS OF *Rafflesia arnoldii* R.Br. AND *R. patma* Blume

Tes Viabilitas Pada Biji *Rafflesia arnoldii* R.Br. dan *R. patma* Blume

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Abstrak

Beberapa spesies *Rafflesia* yang holoparasites terancam oleh hilangnya habitat. Pusat Konservasi Tumbuhan Kebun Raya LIPI telah merintis konservasi *ex situ* menggunakan metode *grafting*. Namun, konservasi *ex situ* menggunakan Inokulasi bibit menjadi bukti sebuah tantangan. Studi pada pengujian viabilitas benih *Rafflesia* bertujuan untuk memastikan bibit yang layak untuk mendukung program konservasi Inokulasi bibit *ex situ*. Tujuan penelitian kami baru-baru adalah: (1) untuk menyelidiki karakter morfologi biji dan (2) untuk menentukan metode untuk pengujian kelayakan. Benih-benih *R. arnoldii* dan *R. patma* yang digunakan dalam penelitian ini. karakter morfologi benih diamati di bawah mikroskop binokuler. Tes kelayakan menggunakan prosedur bioassay adalah sebagai berikut: (1) persiapan reagen; (2) pra-pewarnaan; (3) paparan embrio; (4) pewarnaan, dan (5) penilaian. Penelitian ini menghasilkan: (1) deskripsi morfologi karakteristik biji *Rafflesia*, yaitu benih *R. arnoldii* adalah 2-3 kali lebih besar dibandingkan *R. patma* dan bahwa biji dari dua spesies memiliki Testas yang berbeda, dan (2) teknik untuk pengujian viabilitas bioassay benih *Rafflesia*, yaitu tes kelayakan mengakibatkan diperkirakan persentase kelangsungan hidup 78,75 ± 4,75% (biji *R. Arnoldii*) dan 93,29 2,67% (biji *R. patma*).

Kata kunci :

Abstract

Some of *Rafflesia* species are holoparasites that are threatened by habitat loss. The Center for Plant Conservation Botanic Gardens LIPI has been pioneering *ex situ* conservation using grafting methods. However, *ex situ* conservation using seed innoculation has proven to be a challenge. Studies on viability testing of *Rafflesia* seeds aims to ensure viable seeds for supporting *ex situ* seed innoculation conservation programs. The aims of our recent research were: (1) to investigate the morphological characters of the seeds and (2) to determine a method for viability testing. The seeds of *R. arnoldii* and *R. patma* were used in this research. Morphological characters of the seeds were observed under a binocular microscope. The viability test using bioassay procedures was as follows: (1) reagent preparation; (2) pre-staining; (3) embryo exposure; (4) staining, and (5) assessment. The research resulted in: (1) morphological description of the characteristics of *Rafflesia* seeds, i.e. seeds of *R. arnoldii* are 2-3-times larger than those of *R. patma* and that seeds of the two species have distinct testas, and (2) techniques for bioassay viability testing of *Rafflesia* seeds, i.e. the viability test resulted in an estimated viability percentage of 78.75 ± 4.75 % (*R. arnoldii* seeds) and 93.29 2.67 % (*R. patma* seeds).

Keywords: bud, growth, mortality, population, *R. bengkuluensis*

INTRODUCTION

Rafflesia is a holoparasitic plant, several species of which are deemed to be under threat owing to natural habitat alteration such that an *ex situ* conservation effort is important as a strategy to complement *in situ* conservation. Bogor Botanic Gardens has conducted *ex situ* conservation of *Rafflesia* spp. for over 10 years. It is known that *R. patma* was introduced and bloomed in the Gardens in 1850 and *R. rochussenii* in 1853 (Dakkus, 1957). The current *ex situ* conservation program on *Rafflesia* spp. in Bogor Botanic Gardens has been running since 2004 by seed innoculation and grafting (Mursidawati *et al*., 2006) with ten bloomings to date resulting from grafting (Mursidawati *et al*., 2015).

Seed germination of *Rafflesia* spp. is poorly understood notwithstanding that seed innoculation through host roots has been applied in the *ex situ* conservation area. The success of *R. rochussenii* seed innoculation into *Tetrastigma tuberculatum* host was reported in Bogor Botanic Garden in 1924 (Meijer, 1997). Recently, Nais (2001) reported the success of *R. keithii* seed innoculation into *Tetrastigma diepenhorstii* and *T. tuberculatum.* Available information on germination experiments with seeds of other parasitic plants such as *Striga hermonthica* and *Orobanche ramosa* had shown that these two parasites demonstrated a preference for the exudate of the host species (Bouwmeester *et al*., 2007). *Orobanche* also reacts to strigolactones. The strigolactones produced by the interaction between the parasitic plants and their specified hosts may induce the seed germination of the seeds of *S. hermonthica* and *O. ramosa*; however, the strigolactones alone were not sufficient for successful establishment of the parasitic symbiosis (Bouwmeester *et al.,* 2007).

Seed germination of *Rafflesia* spp. through seed innoculation of the roots of *Tetrastigma* spp. in the living collections of the Bogor Botanic Gardens is one potential method of *ex situ* conservation. Although none of the seed innoculations attempted to date have succeeded, experimentation on seed viability tests are potentially useful for future seed inoculation research. The most appropriate tests for this purpose apply a bioassay technique using tetrazolium chloride. The research described in this paper aimed to (1) investigate the morphological characters of seed, (2) conduct seed viability test using bioassay techniques, and (3) determine the seed viability of *R. arnoldii* and *R. patma.*

MATERIALS AND METHODS

The fruit of *R. patma* was collected from Pangandaran Nature Reserve, West Java, Indonesia and *R. arnoldii* fruit was collected from Sumatra in December 2004. Aproximately 300 seeds were extracted from the fruit of each species.

Seed morphology observation

The seed morphology of *R. arnoldii* and *R. patma* was observed to determine the location of embryos and assess the nature of the endosperm in order to apply the seed viability test properly. The seed morphology was observed under an Olympus binocular microscope. The seed samples were prepared by transverse and longitudinal slicing. Acetocarmin 2.5% was applied for staining (Chang and Neuffer, 1989). The seeds were cleansed a few times using alcohol 70% then the seed coat was peeled off. The outer seed coat characteristics were observed under the microscope.

Seed viability test

The following procedure was adopted from a proven orchid seed viability test (van Waes *et al*., 1986; Seaton and Ramsay, 2005; ISTA, 2015; and Hosomi *et al*., 2011). Owing to constraints in the quantity of seed available, the viability tests used: three replicates (*R. patma*: 100, 89 and 80 seeds in sample P1, P2 and P3 respectively) and four (*R. arnoldii*: 75, 85, 99 and 68 seeds in sample A1, A2, A3 and A4 respectively). Due to the minute size of the seeds and their recalcitrance and to allow less-time consuming, these number of the seeds in each replicate based on sampling without counting the seeds but based on the extrapolating

the 100-seed weight of *R. patma* (1.8-2.1 g) and *R. arnoldii* (8.7-9.7 g); although, this did not allow statistical analysis. The viability test aimed to examine the initial viability of the seeds extracted from fresh fruit and was carried out two weeks after the seed were collected in the field. During this seed extraction process, the fruit/seeds were temporarily stored in 4 ºC refrigerator. The remaining seeds, which were not used for the initial seed viablity test, were packed in air-tight vials and stored in a refrigerator at 4 ºC for ten weeks to determine storage behaviour. The viability test comprised pre-staining, staining and viability determination as follows.

a. Pre-staining

The seed coat was pierced at the raphal portion to expose the embryo. Then the seeds were placed between moist filter papers in petri dishes in a controlled-temperature incubator set at 35 ºC (WTC Binder 78532 Type 4024009900310) for 21 hours until the seeds had fully imbibed. The pre-staining temperature and time were adopted from the premoistening of orchid seeds which aimed for the fully imbibition state of the seeds (Asikin and Handini, pers. comm.).

b. Staining

Taking into consideration the hard seed coat, a 1% concentration of Triphenyltetrazolium chloride was applied in the test rather than a more dilute concentration (ISTA, 2015). A 100 ml quantity of the reagent contained 95 ml 1% 2,3,5-Triphenyltetrazolium chloride and pH buffer 7.0, 2 ml of alcohol 70%, and 3 ml of Tween 80 (Merck) solution (Ercole *et al*., 2015).

After 21-hour pre-staining, the seeds were soaked in the 1% Triphenyltetrazolium chloride solution in petri dishes wrapped in aluminium foil to provide darkness. The soaked seeds were placed in a temperature-controlled incubator set at 30ºC in dark conditions until they were fully stained, i.e. 48 hours for *R. arnoldii* seeds and 43 hours for *R. patma* seeds; these staining periods were based on preliminary tests.

c. Seed viability determination

Seed viability determination was observed under a binocular microscope (Nikon SMZ-10A) with viability determined on the basis of the staining pattern in embryos and endosperms (Bhodthipuks *et al.,* 1996; ISTA 2015). Since no information is available for *Rafflesia* seeds, this determination was based on viability tests done on orchid seeds (Van Waes and Debergh, 1986; Hosomi *et al*., 2011). *Rafflesia* seeds were determined as viable if the embryo was red and the endosperm red or more than 50% red. The unstained *Rafflesia* seeds, either pink or pellucid, were determined as not viable or dead. Some of seed samples were used in the unsuccessful in vivo germination trials of *R. patma* seeds on nature *Tetrastigma* plants at Bogor Botanic Gardens (Mursidawati *et al*., 2015).

RESULTS AND DISCUSSIONS

Seed Morphological Characters

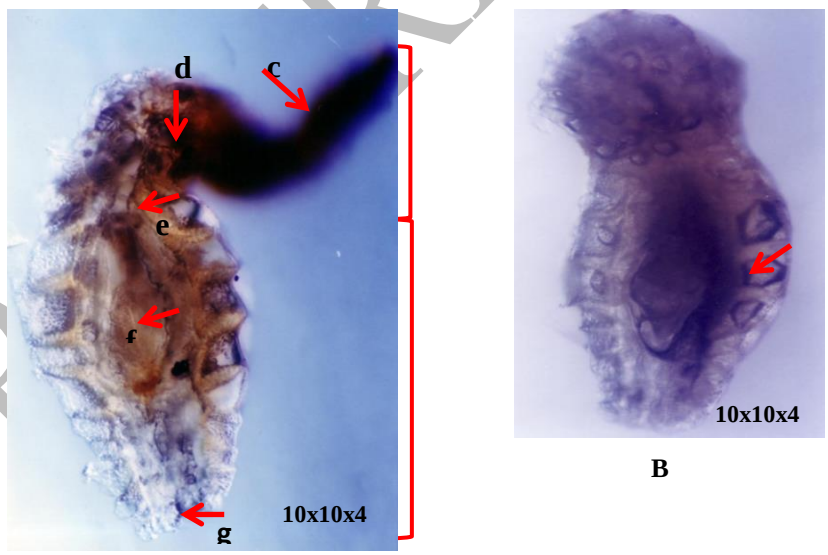
The seeds of *R. patma* are creamy and J-shaped (Nais, 2001). They are minute, 0.5-0.9 mm in length with a mass of 18-21 µg. The seeds are composed of unequal upper and lower sections, i.e. raphal and micropylar portions (Figure 1). The raphal portions are smaller near the base of the funiculus and formed as approximately one-third of the seed coats were curved inward. Seed coats are hard and pitted; the pits are 4-6 angled.

Rafflesia arnoldii seeds are brownish and J-shaped (Nais, 2001). The seeds are tiny and had 1-1.5 mm in length and with a mass of 87-97 µg. The seeds also consist of raphal and micropylar sections (Figure 1). The upper parts is smaller near the base of the funiculus, forms about one-third of the seed coat, and curvs inward. The seed coats are hard with rounded pits.

The seeds of *R. arnoldii* and *R. patma* have some similarities. Four to six sided pits i.e. trapezium, pentagonal to hexagonal on the seed coat surface were present in both species. However, a rounded pit appears only to be present in the seed coat of *R. arnoldii*. The pit characters could be significant characters for taxonomic studies in *Rafflesia* species; e.g. Susatya (2011) differentiated between *R. arnoldii* complex and *R. patma* complex based on their ramenta characters. The characters of the seed coats of these two species show no obvious adaptation to a specific mode of dispersal (Kesseler and Stuppy,

2009). However the funiculus (Figure 1A:c) may act as a funicular aril to attract termites and ants to disperse the seeds (Nais, 2001; Banziger, 2004); additionally, the fruit flesh remains were potentially attached to the aril. The placentation characters of the ampithropous ovules were attached to the wall of many chambers of the ovary (Nais, 2001) as seen then in the fruit (Figure 3).

The seeds of *R. patma* and *R. arnoldii* are endospermic. The embryo is ovate and plump in the micropylar region (Figure 1A and 2A). It appeared to be minute or embedded in the cellular endosperm that provides the main nutrient reserves. According to Bewley and Black (1992), the embryos of parasitic plants are mostly minute as Nais (2001) mentioned a fruit of *Rafflesia* may contain up to 270,000 tiny seeds; these tiny seed size may relate to the seed dispersal strategies (Kesseler and Stuppy, 2009) for the survivorship of parasitic plants. In the cellular type of endosperm, cell walls are formed around each protoplast as it completes a mitotic division of the primary endosperm nucleus which results from the fusion of one sperm with the endosperm nuclei of the embryo sac following fertilization (Vijayaraghavan and Prabhakar, 1984; Shamrov, 2006). Mursidawati (2012) has confirmed the presence of reserve nutrient accumulation in mature *R. arnoldii* and *R. patma* seeds. Moreover, this may explain why the basal part of the embryo may modify into haustorial-nutritive tissue to nourish the embryo during seed germination when the seeds come into contact with the root surface of *Tetrastigma* spp. This is the situation in some other taxa of parasitic flowering plants but the embryology of Rafflesiaceae has not been observed yet (Teryokhin, 2006). These results confirmed the endophytic development of *Rafflesia* spp. in the host plants, *Tetrastigma* spp. (Mursidawati and Sunaryo, 2012). Based on these observations of seed morphology, the embryo exposure for our seed viability test used the piercing method, focused on the raphal region of the seed (ISTA, 2015).



A B

Figure 1. *Rafflesia patma* seed in a longitudinal section; length: 500-900 µm; weight:18-21 µg. (A) a= raphal portion, b= micropylar portion, c= funiculus, d= chalazal swelling, e = schlerenchymatous seed coat with 4-6-squared pits, f= embryo with a cellular endosperm, g= micropyle; (B) a hexagonal pit (red arrow).

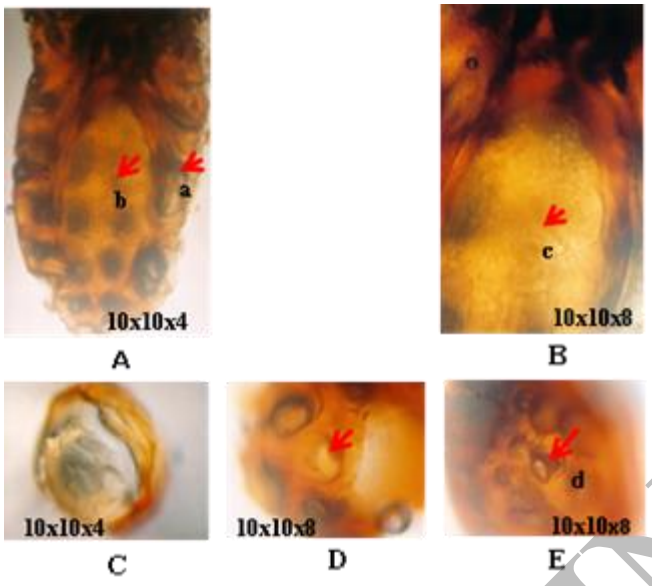


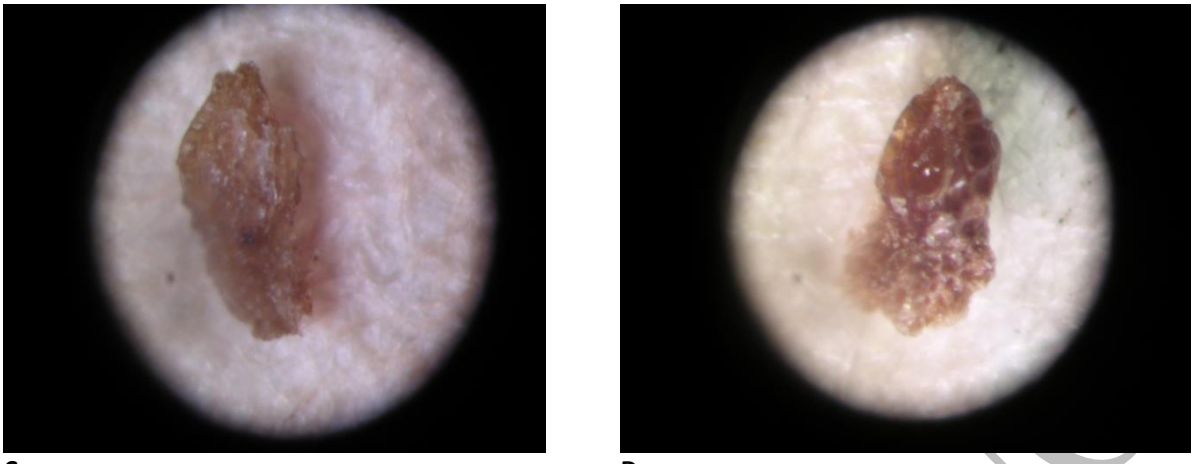
Figure 2. *Rafflesia arnoldii* seed; length: 1000-1500 µm; weight: 87-97 µg. (A-B) a=seed coat with rounded to 4-6-squared pits, b, c= embryo with a cellular endosperm; (C) a transverse section of the seed; (D) a rounded pit; (E) micropyle, d= the central micropylar canal

Figure 3. Fruit of *Rafflesia patma* in a transverse section

Seed Viability Tests

Seeds in which the red staining distributed into the embryo and the red embryo was detemined as viable as shown in Figure 4 (ISTA, 2007). The viability test resulted in an estimated viability percentage of 78.75 ± 4.75% (*R. arnoldii*) and 93.29 ± 2.67% (*R. patma*). These results demonstrated that the viability of the seeds of *R. arnoldii* and *R. patma* were high, based on bioassay-germination test. The unviable seeds may be either dry or empty seeds (Fig. 4C) indicating the dessiccation-sensitivity of seeds of *R. arnoldii*.

Further research is required to confirm these results, i.e. by applying a 'cut test' as adopted from cut tests applied to orchid seeds, i.e. fresh, plump embryos indicate viable seeds (Seaton and Ramsay, 2005; Way and Gold, 2008). The cut-test aims to examine the physical quality of the seeds by cutting 10-20 seeds of the sample examined. The results of the cut-test may indicate the most frequently occurring full, empty/dried, infested and immature seeds (Way and Gold, 2008). However, the results could also be doubtful because seed innoculation (seed germination) experiments have not yet succeeded. Referring to other parasitic plants such as *Striga*, *Orobanche* and *Phelipanche* spp. (Bouwmeester et al., 2007; Westwood *et al.*, 2010; Yoder and Scholes, 2010; Cardoso *et al*., 2011), the stages of host recognition by *Rafflesia* seeds and the possible strigolactone – dependent - *Rafflesia* seed germination may give a hint as to best approach to seed innoculation in *ex situ* conservation effort.



C **D**

Figure 4. Seed viability of *Rafflesia patma* (A-B) and *R. arnoldii* (C-D). (A) *R. patma* seeds before staining, (B) after staining, (C) a dessication-sensitive seed of *R. arnoldii* indicating not-viable seed, (D) a viable seed of *R. arnoldii*.

The embryo portion that was much smaller than the seed coat portion in *R. arnoldii* and *R. patma* seeds may indicate that both seeds of *R. arnoldii* and of *R. patma* are likely to be desiccation sensitive, i.e. recalcitrant (Gold and Hay, 2008). This was reinforced by the finding of a high moisture content (i.e. 19.43% on average, based on 24 mg initial fresh weight) after 10-weeks of refrigerated storage. Cryopreservation could be an appropriate technique to maintain the viability of *Rafflesia* seeds.

CONCLUSIONS

The seeds of *Rafflesia arnoldii* and *R. patma* are distinguishable by the dimensions of the repective seeds and the presence of rounded pits in the seed coat surface of *R. arnoldii* but not of *R. patma*. The *ex situ* conservation of *Rafflesia* spp. encounters many challenges such as ignorance about seed germination characteristics when the seeds come in contact with the roots of their host plants. The potential recalcitrance of *Rafflesia* seeds may restrict the success possibilities for the seed innoculation. Therefore, we should respect their natural habitats as their optimum seed banks. Future research and conservation collaboration opportunities could benefit from applying Banziger's manual pollination method in the natural habitats with the potential to increase fruit-set 6-10 fold. We hope that the completion and implementation of a Strategy and Action Plan for Rafflesiaceae Conservation (SRAK) 2015-2020 may facilitate future research and conservation collaboration between the Center for Plant Conservation Botanic Gardens LIPI, the Ministry of Environment and Forestry, the Goverment of Bengkulu Province and the Research Chamber of Bengkulu Province.

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