

# 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  $78,75 \pm 4,75\%$  (biji *R. Arnoldii*) dan  $93,29 \pm 2,67\%$  (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 inoculation has proven to be a challenge. Studies on viability testing of *Rafflesia* seeds aims to ensure viable seeds for supporting *ex situ* seed inoculation 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  $78.75 \pm 4.75\%$  (*R. arnoldii* seeds) and  $93.29 \pm 2.67\%$  (*R. patma* seeds).

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

## 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 inoculation 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 inoculation through host roots has been applied in the *ex situ* conservation area. The success of *R. rochussenii* seed inoculation into *Tetrastigma tuberculatum* host was reported in Bogor Botanic Garden in 1924 (Meijer, 1997). Recently, Nais (2001) reported the success of *R. keithii* seed inoculation into *Tetrastigma diepenhorstii* and *T. tuberculatum*. Available information on germination experiments with seeds of other parasitic plants such as *Striga hermonthica* and *Orobancha ramosa* had shown that these two parasites demonstrated a preference for the exudate of the host species (Bouwmeester et al., 2007). *Orobancha* 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 inoculation 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 inoculations 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. Approximately 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 °C refrigerator. The remaining seeds, which were not used for the initial seed viability test, were packed in air-tight vials and stored in a refrigerator at 4 °C 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 °C (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 30°C 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.

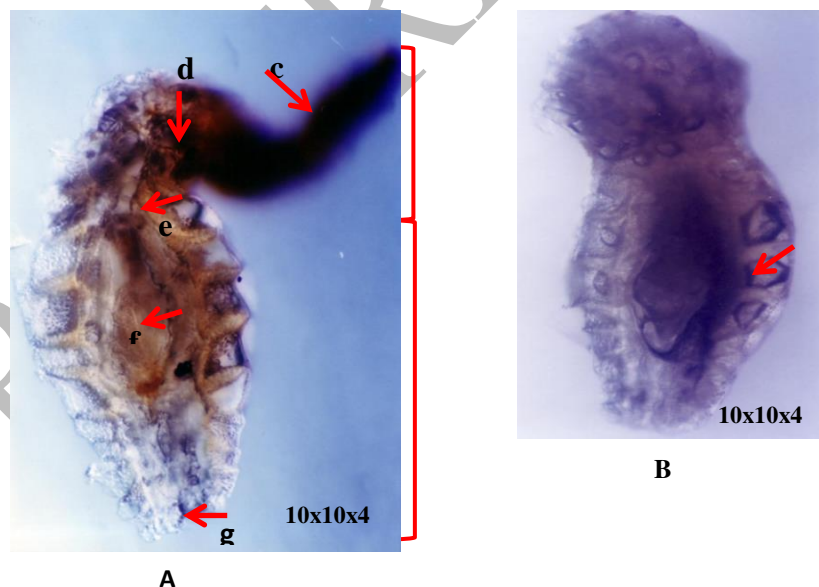
*Rafflesia arnoldii* seeds are brownish and J-shaped (Nais, 2001). The seeds are tiny and had 1-1.5 mm in length and with a mass of 87-97 µg. The seeds also consist of raphal and micropylar sections (Figure 1). The upper parts is smaller near the base of the funiculus, forms about one-third of the seed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

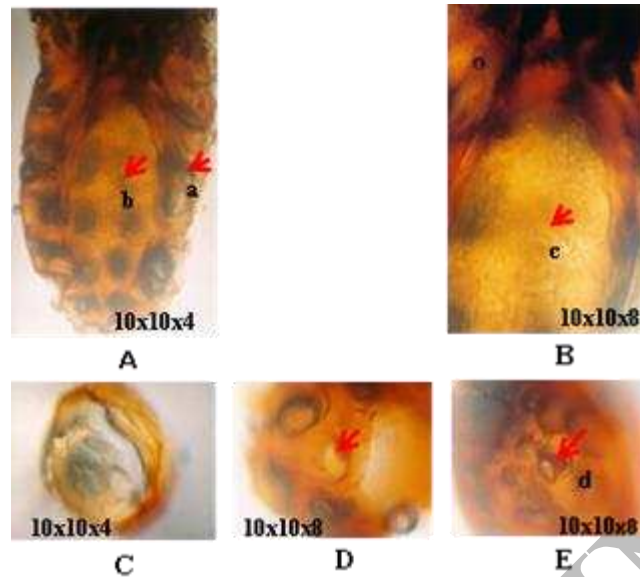
2009). However the funiculus (Figure 1A:c) may act as a funicular aril to attract termites and ants to disperse the seeds (Nais, 2001; Banziger, 2004); additionally, the fruit flesh remains were potentially attached to the aril. The placentation characters of the amphitropous ovules were attached to 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 (Kessler 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).



**Figure 1.** *Rafflesia patma* seed in a longitudinal section; length: 500-900  $\mu\text{m}$ ; weight:18-21  $\mu\text{g}$ . (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  $\mu\text{m}$ ; weight: 87-97  $\mu\text{g}$ . (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



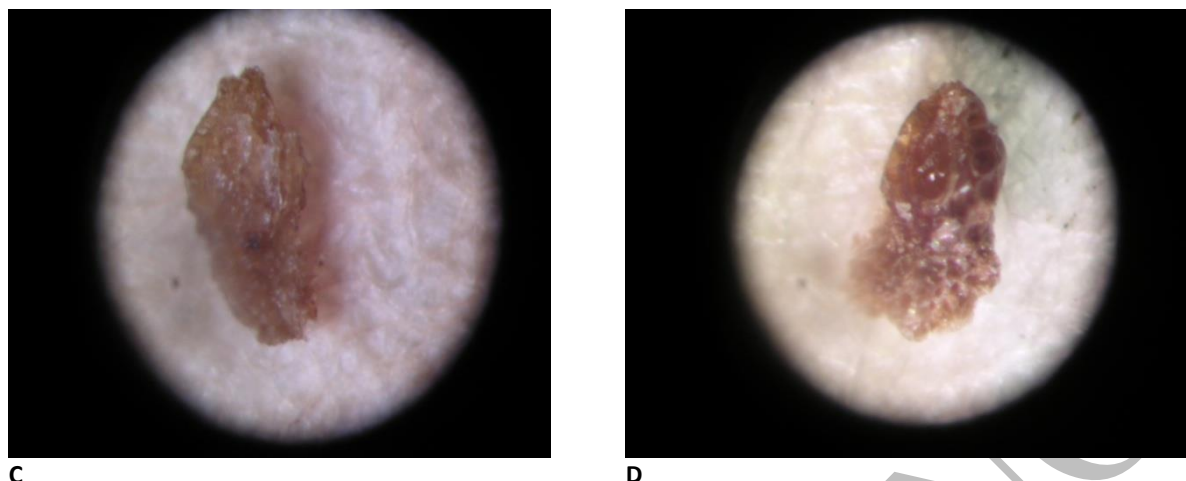
**Figure 3.** Fruit of *Rafflesia patma* in a transverse section

### Seed Viability Tests

Seeds in which the red staining distributed into the embryo and the red embryo was determined as viable as shown in Figure 4 (ISTA, 2007). The viability test resulted in an estimated viability percentage of  $78.75 \pm 4.75\%$  (*R. arnoldii*) and  $93.29 \pm 2.67\%$  (*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 desiccation-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 inoculation (seed germination) experiments have not yet succeeded. Referring to other parasitic plants such as *Striga*, *Orobancha* 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 inoculation in *ex situ* conservation effort.



**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 respective 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 inoculation. 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 Government of Bengkulu Province and the Research Chamber of Bengkulu Province.

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## REFERENCES

- Banziger, H. 2004. Studies on hitherto unknown fruit and seeds of some Rafflesiaceae and unknown method to manually pollinate their flowers for research and conservation. *Linzer Biologische Beitrage* 36: 1175-1198.
- Bhodthipuks, J., P. Pukittayacamee, S. Saelim, B.S.P. Wang and S.L. Yu. 1996. ASEAN Forest Tree Seed Centre Project: Rapid Viability Testing of Tropical Tree Seeds: Rapid viability testing of tropical tree seeds. ASEAN Forest Tree Seed Centre. Saraburi, Thailand.
- Bouwmeester, H.J., C. Roux, J.A. Lopez-Raez and G. Becard. 2007. Rhizosphere communication of plants, parasitic plants and AM fungi. *Trends in Plant Science* 12: 224-230.
- Cardoso, C., C. Ruyter-Spira and H.J. Bouwmeester. 2010. Strigolactones and root infestation by plant-parasitic *Striga*, *Orobanche* and *Phelipanche* spp. *Plant Science* 180: 414-420.
- Chang, M.T. and M.G. Neuffer. 1989. A simple method for staining nuclei of mature and germinated maize pollen. *Stain Technology* 64: 181-184.
- Dakkus, P.M.W. 1957. An Alphabetical List of Plant Species Cultivated in The Hortus Botanicus Bogoriensis. *Pertjetakan Archipel*. Bogor.
- Ercole, E., M. Rodda, M. Girlanda and S. Perotto. 2015. Establishment of a symbiotic in vitro system between a Green Meadow orchid and rhizoctonia-like fungus. *Bio-protocol* 5: 1482.
- Gold, K. and F. Hay. 2008. Identifying desiccation-sensitive seeds. *Technical Information Sheet-Millennium Seed Bank Partnership Kew* 10: 1-2.
- Hidayati, S.N., W. Meijer and J.L. Walck. 2000. A contribution to the life history of the rare Indonesian holoparasite *Rafflesia patma* (Rafflesiaceae). *Biotropica* 32: 408-414.
- Hosomi, S.T., R.B. Santos, C.C. Custadio, P.T. Seaton, T.R. Mark, . and N.B. Machado-Neto. 2011. Preconditioning *Cattleya* seeds to improve the efficacy of the tetrazolium test for viability. *Seed Science and Technology* 39: 178-189.
- International Seed Testing Association (ISTA). 2015. *International Rules for Seed Testing*. Basserdorf, Switzerland.
- Kessler, R. and W. Stuppy. 2009. *Seeds: Time Capsules of Life*. Second Edition. Firefly Books Inc. New York.
- Meijer, W. 1997. Rafflesiaceae. *Flora Malesiana* 13: 1-42.
- Mursidawati, S., D. Latifah, E. handini, Ngatari and M.K. Riswati. 2006. Program konservasi *Rafflesia* di Kebun Raya Bogor. *Warta Kebun Raya* 6: 3-9.
- Mursidawati, S. 2012. Fruit and seed morphology of *Rafflesia patma* and *R. arnoldii*. *The Botanic Garden Bulletin* 15: 20-29.
- Mursidawati, S. and Sunaryo. 2012. Anatomical study of endophytic *Rafflesia patma* inside the host *Tetrastigma* sp. *The Botanic Garden Bulletin* 15: 71-80.
- Mursidawati, S., Ngatari, Irawati, S. Cardinal and R. Kusumawati. 2015. Ex situ conservation of *Rafflesia patma* Blume (Rafflesiaceae) – an endangered emblematic parasitic species from Indonesia. *Sibbaldia: Journal of Botanical Garden Horticulture* 17: 9-14.
- Nais, J. 2001. *Rafflesia of the worlds*. Sabah-Park Malaysia.
- Seaton, P. and M. Ramsay. 2005. Growing orchids from seeds. *The Board of Trustees of The Royal Botanic Gardens, Kew*.
- Shamrov, I.I. 2006. Cellular type of endosperm development. In: T.B. Batygina (ed.), *Embryology of Flowering Plants: Terminology and Concepts*. Second Volume: Seed. Science Publishers, Plymouth UK. p 121-126
- Susatya, A. 2011. *Rafflesia* pesona bunga terbesar di dunia. Direktorat Kawasan Konservasi dan Bina Hutan Lindung Kementerian Kehutanan, Jakarta.
- Teryokhin, E.S. 2006. Embryo. In: T.B. Batygina (ed.), *Embryology of Flowering Plants: Terminology and Concepts*. Second Volume: Seed. Science Publishers, Plymouth UK. p 173-174.



- Van Waes, J.M. and P.C. Debergh. 1986. Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western Europe orchids. *Physiologia Plantarum* 66: 435-442.
- Vijayaraghavan, M.R. and K. Prabhakar. 1984. The endosperm. In: B.M. Johri (ed.), *Embryology of angiosperms*. Springer-Verlag, Berlin.
- Way, M. and K. Gold. 2008. Assessing a potential seed collection. Technical Information Sheet-Millennium Seed Bank Project Kew 2: 1-2.
- Westwood, J.H., J.I. Yoder, M.P. Timko and C.W. dePamphilis. 2010. The evolution of parasitism in plants. *Trends in Plant Science* 15: 227-235.
- Yoder, J.I. and J.D. Scholes. 2010. Host plant resistance to parasitic weeds; recent progress and bottlenecks. *Current Opinion in Plant Biology* 13: 478-484.

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