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REVIEW

Rafflesia spp.: propagation and conservation

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Abstract

Main Conclusion **The propagation of *Rafflesia* spp. is considered to be important for future development of ornamental and other applications. Thus far, the only successful propagation technique has been grafting. This mini-review succinctly emphasizes what is known about *Rafflesia* species**.

Members of the genus *Rafflesia* (Rafflesiaceae), which are holoparasitic plants known to grow on a host vine, *Tetrastigma* sp., are widely spread from the Malayan Peninsula to various islands throughout Indonesia. The plant's geographical distribution as well as many other aspects pertaining to the basic biology of this genus have still not been studied. The young flower buds and flowers of wild *Rafflesia hasseltii* Suringar, *Rafflesia keithii* Meijer and *Rafflesia cantleyi* Solms-Laubach are used in local (Malaysia and Indonesia) traditional ethnomedicine as wound-healing agents, but currently no formal published research exists to validate this property. To maintain a balance between its ethnomedicinal and ornamental use, and conservation, *Rafflesia* spp. must be artificially cultivated to prevent overexploitation. A successful method of vegetative propagation is by host grafting using *Rafflesia*-impregnated *Tetrastigma* onto the stem of a normal *Tetrastigma* plant. Due to difficulties with culture contamination in vitro, callus induction was only accomplished in 2010 for the first time when picloram and 2,4-D were added to a basal Murashige and Skoog medium, and the tissue culture of holoparasitic plants continues to be extremely difficult. Seeds harvested from fertile fruit may serve as a possible method to propagate *Rafflesia* spp. This paper provides a brief synthesis on what is known about research related to *Rafflesia* spp. The objective is to further stimulate researchers to examine, through rigorous scientific discovery, the mechanisms underlying the ethnomedicinal properties, the flowering mechanisms, and suitable in vitro regeneration protocols that would allow for the fortification of germplasm conservation.

Keywords Holoparasite · Medicine · Metabolite · Propagation · *Rafflesia*

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Introduction

Rafflesia spp. are considered to be some of the most enigmatic flora on the planet. The plants do not have any stem, leaves, roots, or photosynthetic organs. Moreover, they are not visible until the reproductive stage when flowers bud emerge through the surface of the stem of the obligatory host woody vine and open into magnificent and spectacular blooms. It takes up to 21 months to develop from the first visible flower bud to an open bloom (Zuhud et al. 1998) and only 1–18 % of flowers can develop an open bloom (Susatya 2011). *Rafflesia* spp. flowers are among the largest single flowers in the world, up to 150 cm in diameter and up to 11 kg in weight (Rahayu 2003). The flowers, once open, last 4–7 days in *Rafflesia arnoldii*, 5–8 days in *Rafflesia hasseltii*, and 7 days in *Rafflesia rochussenii* (Zuhud et al. 1998). When in bloom, the flower is large, although the size may vary depending on the species, and the largest can reach ca. 100 cm in diameter (Figs. 1a, 2). Within flowers, the thread-like protuberance termed the ramenta is considered to be an important feature for species identification (Fig. 3, explained in Fig. 4). The plant itself is an obligatory parasite (i.e., holoparasite) on its host, which are primarily vines from the genus *Tetrastigma*, mainly *T. leucostaphylum* (Susatya 2011) and *Tetrastigma scuriosum* (Veldkamp 2009). Molecular biological studies have shown that, during the process of evolution, *Rafflesia* has acquired genes from its host by horizontal transfer of mitochondrial genes (Nickrent et al. 2004; Xi et al. 2012) and chloroplast genes since *Rafflesia* has very few intact plastid genes (Molina et al. 2014).

In nature, *Rafflesia* and other plants of the Rafflesiaceae are found in the tropical rainforest region of Southeast Asia, while the genus *Rafflesia* itself is distributed around the Malayan Peninsula, Philippines, to the rainforests of Sumatra, Java, and Borneo with more than 30 species having been identified worldwide, 17 in Indonesia (Indonesian Department of Forestry and Environment 2015). The latest conservation status of known *Rafflesia* species can be observed in Table 1.

Anthropogenic factors that contribute to declining populations of *Rafflesia* spp. are deforestation and harvesting by locals due their perceived medicinal properties (Nais 2001) while limiting biological factors might include the dioecious nature of the plant (i.e., separated male and female flowers on different individual), limited populations, and the fact that the majority of *Rafflesia* flowers found in the field are male (Susatya 2011).

It is culturally very important to maintain steady population numbers in the wild for conservation purposes and to

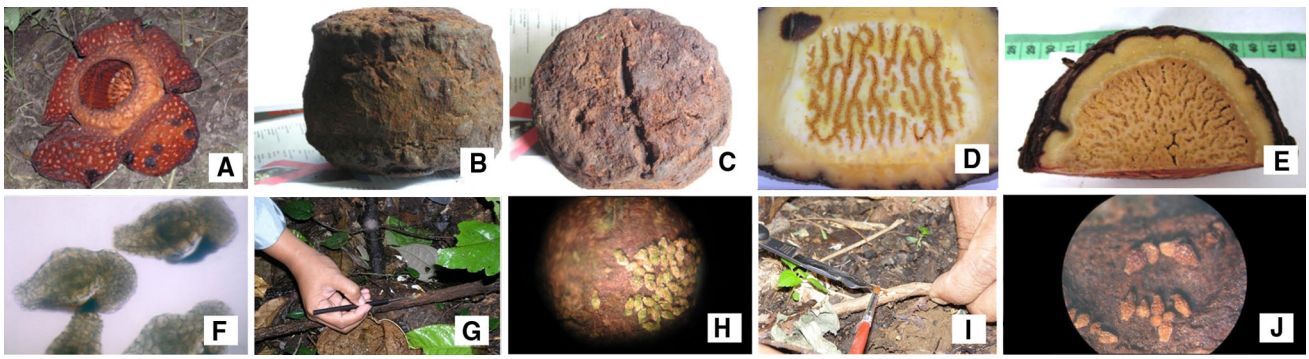

Fig. 1 Flower of *Rafflesia arnoldii* (**a**), with fruit from side view (**b**), and top view (**c**), longitudinal cutting (**d**), and lateral cutting (**d**). Seeds of *R. arnoldii* (**f**) (Mursidawati 2012). Inoculation of *Rafflesia patma* in incised *Tetrastigma* stem on its natural habitat in Pangandaran, West Java, Indonesia (**g**), and no sign of growth in day 628 (**h**), and in Bogor Botanical Garden, West Java, Indonesia (**i**), also no sign of growth in day 218 (**j**) (Mursidawati et al. 2015)

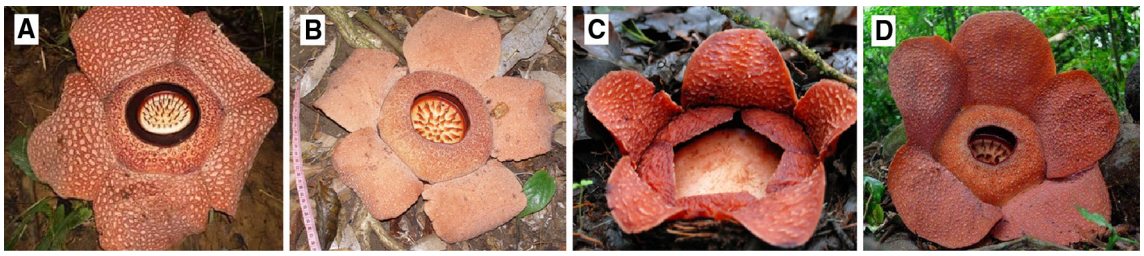

Fig. 2 *Rafflesia arnoldii* (diameter ca. 60–70 cm) (**a**), *R. patma* (diameter ca. 60–70 cm) (**b**), *R. meijerii* (near blooming, diameter ca. 10–14 cm) (**c**), *R. bengkuluensis* (diameter ca. 35–40 cm) (**d**). Photo of *R. arnoldii* taken by Neka Afnidarti, photo of *R. patma* and *R. meijerii* by Sofi Mursidawati, and photo of *R. bengkuluensis* by Noprianto

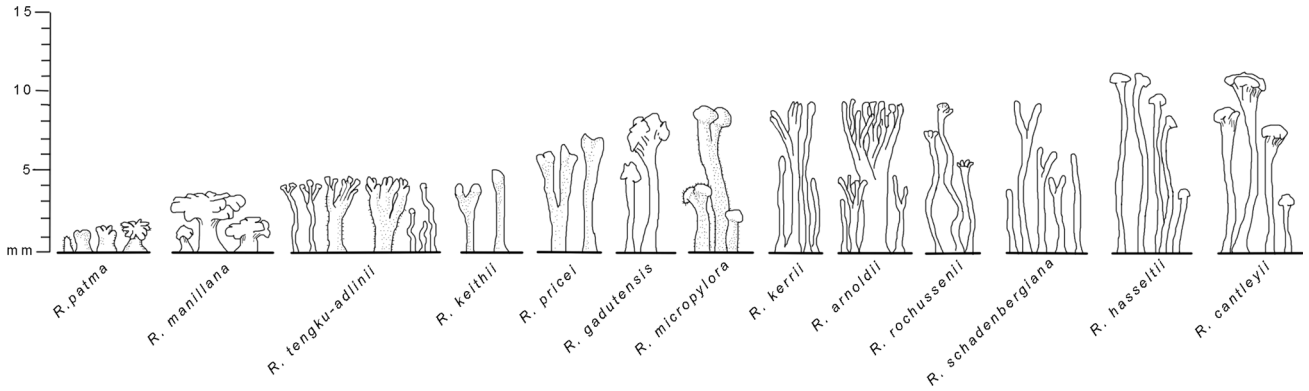

Fig. 3 Different shapes of the ramenta found inside the *Rafflesia* perigone tube. The figure was inspired from Meijer (1997)

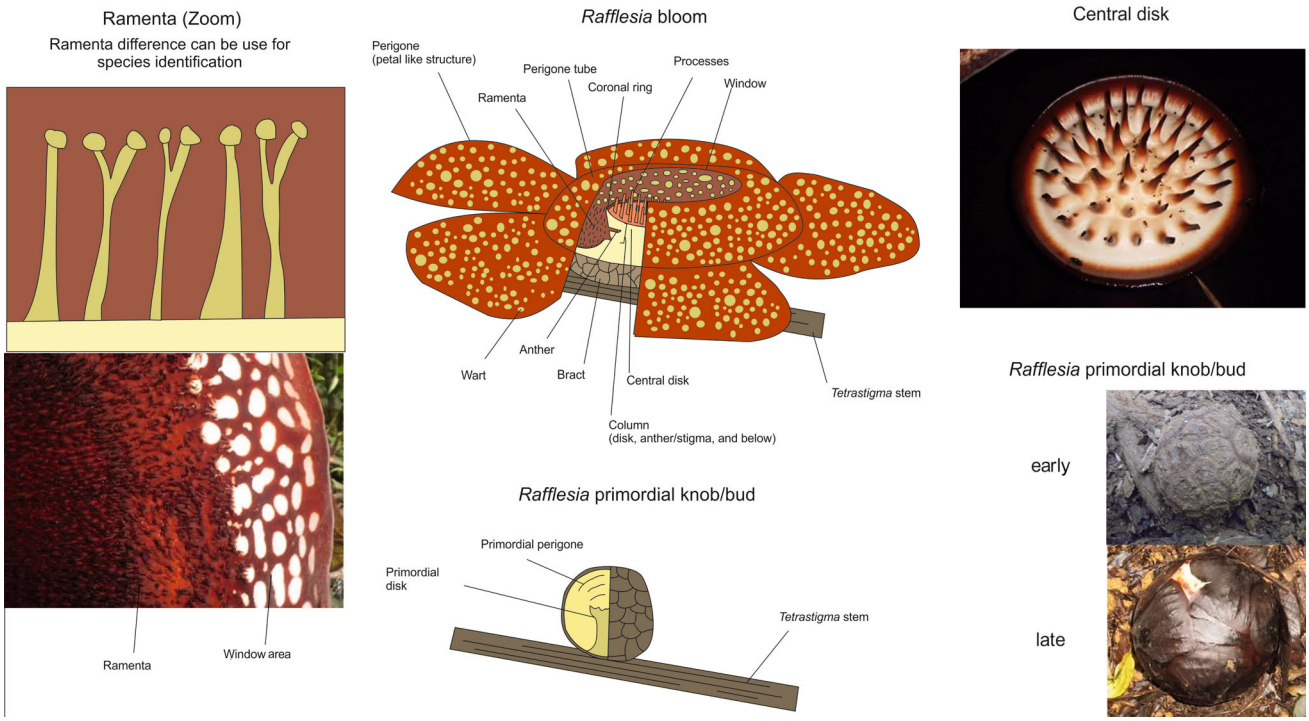

Fig. 4 A closer examination of a *Rafflesia* flower anatomy, including the ramenta, central disk, and late bud. Photo of early bud by Adhityo Wicaksono, and all other photos by Neka Afnidarti. Picture of the ramenta classification from Susatya (2011), with kind permission

Table 1 Latest available status of *Rafflesia* species in the wild

Status	Species
Indeterminate	*R. borneensis* Koorders; *R. ciliata* Koorders; *R. witkampii* Koorders (Nais 2001); **R. atjehensis* Koorders (Susatya 2011)
Low risk	**R. pricei* Meijer (Susatya 2011)
Vulnerable	**R. arnoldii* R. Brown; *R. cantleyi* Solms-Laubach; *R. keithii* Meijer (Nais 2001)
Rare	*R. kerrii* Meijer (Nais 2001)
Endangered	*R. manilana* Teschemacher; *R. schadenbergiana* Goeppert; *R. tengku-adlinii* Mat Saleh & Latif (Nais 2001); *R. manillana* Tesschem (IUCN Red List 1998)
Critically endangered	*R. magnifica* Madulid Tandang & Agoo (IUCN Red List 1998; Madulid et al. 2008); **R. patma* Blume; **R. rochusseni* Teijsm. & Binn.; **R. tuan-mudae* Beccari; **R. hasseltii* Suringar; **R. zollingeriana* Koorders; **R. gadutensis* Meijer; **R. micropylora* Meijer; **R. bengkuluensis* Susatya et al.; **R. lawangensis* Mat-Salleh, Mahyuni, *et* Susatya (Susatya 2011)

* Species that occur exclusively in Indonesia

propagate *Rafflesia* artificially to produce a reliable, constant, and unthreatened source of material for ethnomedicinal and ornamental use, for private collectors, or for educational purposes in eco-tourism. By gaining a deeper understanding of how to multiply giant holoparasitic *Rafflesia* flowers, which is the key objective of this paper, future studies would then be able to explore other potentials of this exquisite and rare ornamental plant genus.

Antioxidant and ethnomedicinal properties of *Rafflesia* spp

Several *Rafflesia* spp. are used ethnomedicinally and there are no proven (i.e., by science or clinical trials) medicinal properties, only limited biological activities. Even so, in the latter case, the compounds and secondary metabolites responsible for these activities remain unknown. *Rafflesia patma* is used to purify the uterus after childbirth, stop bleeding excessively in menstruation, is used as an aphrodisiac for women, and, when added to cinnamon and nutmeg, stops bleeding after childbed and strengthens weak organs; it is also sold as a trade-marked herbal medicine "Padma Sari" in Java; *R. hasseltii* is used to expedite delivery in childbirth by the Sakai tribe in Riau, Sumatra, Indonesia while *R. arnoldii* is used especially during and after childbirth, to promote delivery and recovery, and also as an aphrodisiac (Burkill 1935; Heyne 1987; Rahayu 2003). Malaysians consider *Rafflesia* spp. to have medicinal properties, but their use is purely as ethnomedicine (Nais 2001). The methanolic extract of young flower buds and flowers of *R. hasseltii* has wound-healing properties in rats (Abdulla et al. 2009) and inhibits the growth of *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Wiart et al. 2004), while the water and ethanol (5:95, v/v) or water and polypropylene glycol (1:1, v/v) extract of dried flowers for 1 day of *R. kerrii* Meijer has antioxidant property (Puttipan and Okonogi 2014). The extract (petroleum ether, followed by ethyl acetate, and finally ethanol) of *Rafflesia cantleyi* Solms-Laubach dried flowers inhibited the growth of Gram-positive bacteria (Azizan et al. 2011). Two alkaloids (nicotine and caffeine) and three phenolic compounds (catechin, proanthocyanidin, and phenolic acid) were detected in the methanolic extracts of *R. hasseltii* bracts as well its host, *T. leucostaphylum* (Sofiyanti et al. 2008). Alkaloids and phenolic compounds have anti-inflammatory, antioxidant, and anti-cancer properties (Fürst and Zündorf 2014; Li et al. 2014; Cozzolino 2015; Lewandowska et al. 2016). To the authors' knowledge, no other research on the medicinal properties of *Rafflesia* spp. exists to support these ethnomedicinal uses.

Reproductive biology and propagation of *Rafflesia*

Pollination and seed dispersal

Rafflesia spp. flowers are dioecious (Fig. 4) and their corpse-like scent attracts insects such as flies, although the precise organ and compounds responsible for this mechanism remain unknown. As the pollen of *Rafflesia* spp. is sticky, it can attach to the body of pollinators, primarily insects, allowing the transfer of pollen from male flowers to the stigma of female flowers. After pollination, fruit develop (Fig. 1b, c), in some cases by agamospermy (i.e., without pollination), but in such cases the fruit contain sterile seeds (Nais 2001). Large animals can serve to disperse seed (Meijer 1997). *Rafflesia* spp. have a hard mature fruit with rows of microseeds (Fig. 1d, e), which are gray-brown and peanut or pod-shaped (Fig. 1f). *Rafflesia arnoldii* and *R. patma* Blume seed are 500–1500 μm long and weigh 18–97 μg (Mursidawati 2012).

Tetrastigma stem grafting and cutting

Rafflesia was successfully propagated by grafting a *Rafflesia*-infected *Tetrastigma* stem from Pangandaran, West Java, onto an uninfected *Tetrastigma* rootstock in Bogor Botanical Garden. Veneer grafting and cleft grafting were successful for *R. patma* (Supplementary Fig. 1, 2): it took 2 years from 2007 until bud emergence and 3 years until flower blooming using veneer grafting but 6 years until flower bloom after cleft grafting in 2006 (Mursidawati et al. 2015). According to Nais et al. (2015), grafting is more successful than cuttings, but both are more effective than seed inoculation of *Tetrastigma* rootstock although details have not yet been released.

Ex situ seed germination and attempts at inoculating

As mentioned by Meijer (1997) in Flora Malesiana, early attempts to cultivate *Rafflesia* spp. were already performed artificially or by human intervention. Earlier records indicate that attempts to cultivate *Rafflesia* were made at Bogor Botanical Garden in Indonesia in 1850 and succeed in 1857 (Nais 2001). Three species were transferred from their natural habitat: *R. arnoldii*, *R. patma* and *R. roschussenii* Teijsm. & Binn. *Rafflesia patma* was successfully repotted in 1866, 1879, and 1929. *Rafflesia patma* and its host *T. scuriosum* were transferred from Pangandaran coast, West Java to Bogor Botanical Garden in 2004. The first *R. patma* bud emerged in 2006 but failed to develop into a full blooming flower until 2010 (Mursidawati et al. 2015). Attempts to propagate *Rafflesia* by seeds have taken place in Bogor, Indonesia under controlled greenhouse

conditions since 1930, but with only negative results to date. Meijer in 1997 [citing Teijsmann (1856a, b, 1858), Dokters van Leewen (1929) and Meijer (1958)] and unpublished observations by the authors of this review note that it takes 3–4.5 years for *Rafflesia* seeds to complete their life cycle while *Rhizanthes lowii* Beccari (Harms), another member of the Rafflesiaceae, takes around 200–255 days from emergence from the host tissue to open flowers (3.7–4.1 cm in diameter) (Meijer 1997). The life cycle of other genera in the Rafflesiaceae remains unknown (Meijer 1997).

An early in vitro seed germination trial was performed by Sukamto (2001) using *R. arnoldii* seeds obtained from Bengkulu and Lampung, Indonesia and stored at 5 °C. Under laminar air flow, the surface of seeds was wiped with sodium hypochlorite (NaOCl) and washed several times with sterile distilled water before placing seeds on solid ½ MS medium with seven variations of plant growth regulator (PGR) combinations: control, 1 mg/L BA, 1 mg/L BA + 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L BA + 0.5 mg/L NAA, 1 mg/L BA + 0.5 mg/L IAA, 1 mg/L Kinetin + 0.5 mg/L 2,4-D, and 1 mg/L Kinetin + 1 mg/L 2,4-D. Seed cultures were stored under photoperiodic room (dark and light cycles). No growth was observed even after 18 months. Several recent attempts have been made to germinate *R. patma* seeds in *T. leucostaphylum* host stem, although no growth was detected after 628 days after placing seeds directly on *Tetrastigma* stem skin or by adding them to an incision in the stem (Fig. 1g, h) (Mursidawati and Handini 2009). The same test was repeated in Bogor Botanical Gardens, but seeds did not germinate, even after 218 days (Fig. 1i, j). Biotechnological approaches to seed germination have included the in vitro culture of seeds in bottles containing half-strength Murashige and Skoog (1962; ½ MS) medium with *T. scuriosum* callus (Fig. 5a–d) and in *T. leucostaphylum* bark extract to which 0.05–1.0 mg/L Strigol (GR-24, strigolactone, a stimulant) was added to ½ MS medium, but seeds did not germinate, but cultures were also not contaminated (Fig. 5e–h is control) (Mursidawati et al. 2014, 2015).

In Palupuh, Bukittinggi, West Sumatra, Indonesia, Joni Hartono claimed the successful propagation of *R. arnoldii* by seeds (Koos 2011; personal communication M. Apriza Suska, owner of Suska Nursery in Ciawi, West Java), but instead of by making an incision on the *T. leucostaphylum*

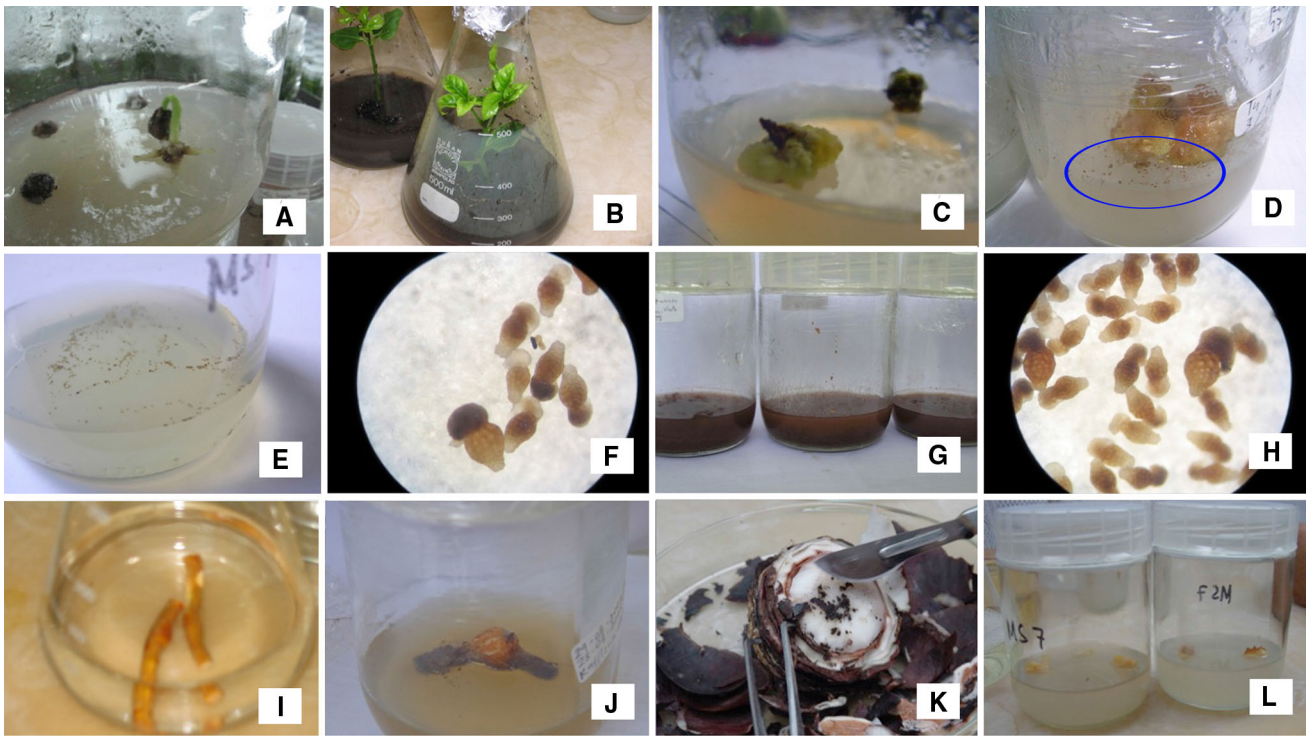

Fig. 5 *In vitro* germination of *Tetrastigma leucostaphylum* seeds (**a**) until seedling is grown and ready for callus generation (**b**). Callus of *Tetrastigma* (**c**) and with seeds of *Rafflesia patma* Blume placed in the same medium (shown in *blue circle*) (**d**). Finally, no growth occurred. Control MS medium with *R. patma* seeds spread on the surface (**e**) and no growth is detected in day 352 (**f**). MS medium with extract of *Tetrastigma* (**g**) and also no growth in day 352 (**h**). Infected *Tetrastigma* stem with *R. patma* young knob is prepared (**i**) and in 18 days the knob is swollen (**j**). Preparation of discuss (area of ovary/androecium) of *Rafflesia meijerii* (**k**) and no change occurred in the 62nd day (**l**) (Mursidawati and Handini 2009; Mursidawati et al. 2015). Photos (personal documents of Sofi Mursidawati)

stem of two 3-year-old plants, the seeds were only spread over the surface three times a year, and the flower bud emerged only after 5 years.

Dr. Jamili Nais claimed the successful germination of *Rafflesia* in *Tetrastigma* by placing seeds in a shallow incision of the *Tetrastigma* stem from artificially (manually) pollinated fruit to improve fertile seed production and reduce the chances of agamospermous fruit with sterile seeds (Nais et al. 2015). However, the detailed methods are currently being patented by Sabah Parks. Thus, the chances to germinate *Rafflesia* species from seeds are low if a small amount of seeds are used. Seed fertility and compatibility with *Tetrastigma* might also be factors affecting the outcome and further studies are required.

Micropropagation by tissue culture

Attempts to culture *Rafflesia* spp. in vitro through tissue culture since 2001 have all failed. The first attempt by Sukamto (2001) used explants from *R. arnoldii* young flower buds 10 cm in diameter and seeds cultured in ½ MS medium with seven PGR combinations used as explained above for in vitro seed germination. The outer layer of flower buds was disinfected with 70 % ethanol, 0.1 % mercuric chloride (Hg_2Cl), and rinsed with sterile water under a laminar air flow (no other details, including exposure period, were indicated). Slices of surface disinfected buds served as explants and placed in media as for seed germination. The only change observed was explant browning and swelling after 18 months and no change in morphogenesis. The second recorded, but unsuccessful, attempt used explants from 1 cm diameter cuttings of *Tetrastigma* with *R. patma* flower buds (Fig. 5i, j) and the perigon (a petal-like structure in *Rafflesia*) of *R. meijerii* (Fig. 5k, l) in ½ MS medium containing sterilized coconut water (pH 7, 200 mL), 4 mg/L BA and 0.5 mg/L NAA after surface disinfection with NaOCl in three steps: 20 % for 20 min, 10 % for 10 min and 5 % for 5 min and a rinse with sterile water after each NaOCl step (Mursidawati and Handini 2009). A subsequent trial in 2010 used 0.1, 0.5, 1.0, and 5.0 mg/L 2,4-D and picloram (4-amino-3,5,6-trichloropicolinic acid), separately or in combination, but 1 mg/L 2,4-D and 0.5 mg/L picloram in MS solid medium successfully induced callus in *R. arnoldii* explants in MS medium and all callus resulting from the treatments had compact structures (Sukamto and Mujiono 2010; Supplementary Fig. 3). *Rafflesia arnoldii* flower buds (2 cm in diameter) originating from Bengkulu (Sumatra, Indonesia), were disinfected a soak in 1 % Teepol (detergent containing 4-nitrophenol and chlorine hydrate) in a glass beaker with a magnetic stirrer for 10 min, 10 % NaOCl for 10 min, 5 % NaOCl for 10 min, and three rinses with sterile water in a laminar hood. Buds were cut horizontally and vertically into 5 × 1.5 mm explants. After placing on media, cultures were incubated in the dark at 27 °C and subcultured every 30 days. All callus treated with 0.1 mg/L zeatin and 1, 3, 5 and 10 mg/L 2,4-D or picloram did not regenerate. Callus was maintained in culture for over 3 years, some of which was covered with thread-like strands on the top surface (22.22 %) after treatment with 10.0 mg/L 2,4-D (Supplementary Fig. 3f). These thread-like strands from callus resemble fungal hyphae that penetrate plant tissue using lytic enzymes (Mayer 2006), similar to thread-like strands in the tissue of *R. arnoldii* after embedded within the host (*Tetrastigma*) root or stem (Sukamto and Mujiono 2010).

Rafflesia patma tissue culture is hindered by tissue browning and infection by endophytic mircoorganisms, even if 2 g/L activated charcoal (AC), used to adsorb polyphenolic compounds, was added to MS basal medium containing 1 mg/L NAA and 4 mg/L BA (Wicaksono and Teixeira da Silva 2015; Supplementary Fig. 4). However, until 39 h of culture, browning and fungal growth were not observed, but fungal growth occurred when explants were subcultured to callus induction medium (AC-free MS + 1.5 mg/L kinetin) although no further browning was observed (Wicaksono and Teixeira da Silva 2015). Secondary metabolites released by *R. patma* explants in vitro causing browning may have antifungal property since Wiart et al. (2004) demonstrated the antifungal and antimicrobial properties of *R. hasseltii* whole extract (extracted using methanol). Refaei et al. (2011) tested whole flower parts (perigone/petal, upper well wall, raised disk, disk vertical spines, and buds) of *R. cantleyi*, all sliced into 1 cm^2 fragments and surface disinfected with 97 % ethanol for 1 min, 2.6 % NaOCl for 3 min, and 97 % ethanol for 30 s before placed in water-based agar plates and potato dextrose agar plates and incubating in 25–28 °C to observe the growth of endophytic fungi. Refaei et al. (2011) confirmed eight strains from three endophytic fungal genera (*Colletotrichum*, *Cytospora*, and *Gliocladiopsis*), which may explain the ease with which *Rafflesia* in vitro cultures become infected easily.

Future potential and biotechnological interventions

Rafflesia spp. have potential as medico-pharmaceutical ornamental plants (Fig. 6). The callus of *Rafflesia* spp. Producing secondary metabolites could be multiplied via bioreactors and harvested for medicine. Despite the long history and botanical fascination of these plants, much has still to be leant about the basic biology and conventional propagation of these species. The micropropagation of *Rafflesia* spp. has, as its first two immediate

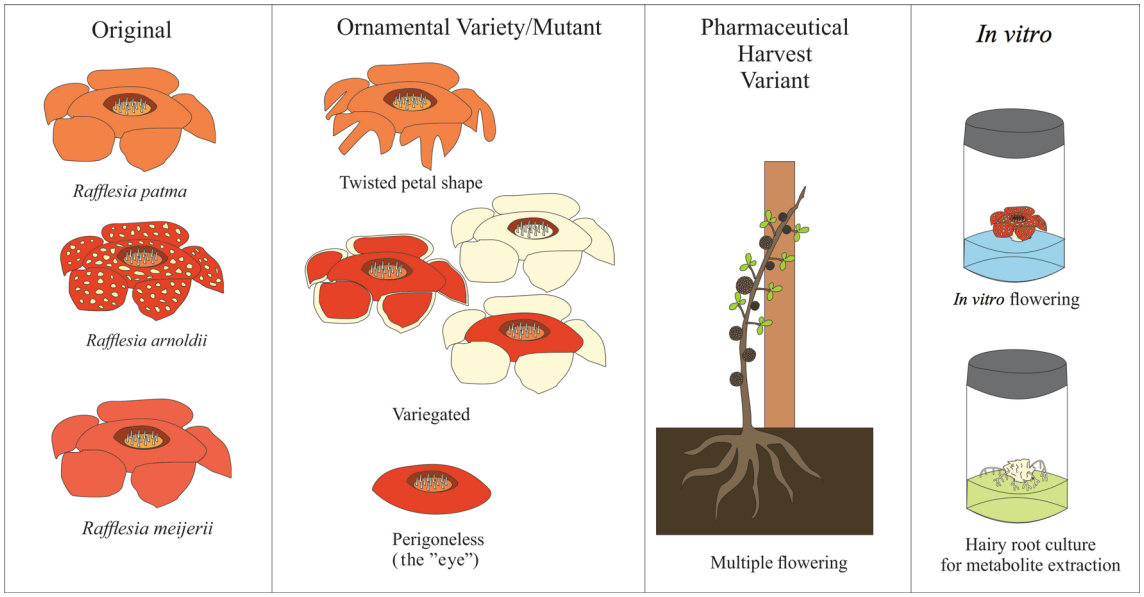

Fig. 6 Future potential for *Rafflesia* breeding and propagation. On the *left* are the original *Rafflesia* species. Through mutagenesis or artificial pollination, a novel color variety might be produced. Through breeding, a variety of *Rafflesia* that produces more knobs (but smaller and more compact) can be produced for the extraction or pharmaceutically and medicinally important metabolites. Furthermore, in vitro flowering for in vitro hybridization or as gifts, as well as hairy root cultures for mass production of secondary metabolite production, is envisaged

objectives, to devise effective and repeatable genotype-independent protocols that effectively eliminate fungal and bacterial contamination (Supplementary Fig. 5), which itself might be challenging given the fact that the plants flower on the forest floor. Thus, the use of surface disinfected seeds or zygotic embryos may be a promising source of material given that artificial pollination is now possible (Nais et al. 2015). Once these two first hurdles can be overcome, indirect organogenesis through callus induction and direct organogenesis should be the next immediate focus of biotechnological endeavors to artificially mass propagate these species. Only then can objectives such as plant breeding or genetic modification be attempted.

Author contribution statement All four authors contributed equally to the development of ideas, writing, figures and editing of the manuscript.

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