EX SITU CONSERVATION OF *RAFFLESIA PATMA* BLUME (RAFFLESIACEAE) – AN ENDANGERED EMBLEMATIC PARASITIC SPECIES FROM INDONESIA

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ABSTRACT

Plants in the genus *Rafflesia* (Rafflesiaceae) bear the largest flowers in the world. Almost all members of this holoparasitic plant family have limited ranges and are rare or threatened. This genus is therefore important from a scientific and conservation perspective.

An *ex situ* conservation collection of a population of *Rafflesia patma* Blume has been monitored at Bogor Botanic Garden (BBG) since 2004, the first time that this has ever been done. Studies have been made, including propagation trials on both seed and vegetative material at BBG. Of all the propagation methods tested, grafting on to the host plant *Tetrastigma scariosum* (Blume) Planch proved to be the most successful for growing *R. patma*, resulting in blooming having occurred seven times since 2010. Grafting is the quickest way to propagate *Rafflesia* in cultivation. This cultivation process provides a new hope for the conservation of this endangered and charismatic genus. This paper describes the methods trialled including both unsuccessful techniques as well as those which resulted in blooming events.

INTRODUCTION

Rafflesiaceae is a family of non-chlorophyllus holoparasites found exclusively on *Tetrastigma* (Vitaceae) (Kuijt, 1969; Barcelona *et al.*, 2009; Sofiyanti & Yen, 2012). As a parasite this family of plants needs a host on which to grow. Indeed, it has no leaves, nor chlorophyll, and is therefore not able to photosynthesise. There are three genera in the family: *Sapnia*, *Rhizanthes* and *Rafflesia* (Mabberley, 2008; Barkman *et al.*, 2003). This last genus is endemic to Southeast Asia and was discovered in 1797 by Louis-Auguste Deschamps in Java. Today, there are 33 species described in *Rafflesia*, some of which have been recorded only once. The most spectacular of all the species is *R. arnoldii*, which forms the largest flower in the world with a diameter of up to one metre (Barcelona *et al.*, 2009). This is not to be confused with the plant in Araceae, *Amorphophallus titanum* (titan arum) which has the largest inflorescence in the world and also originates

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from Southeast Asia. To date, most aspects of the life cycle of *Rafflesia* plants remain unknown, such as the mechanism of germination or seeds dispersal.

Rafflesia patma Blume (Fig. 1) was discovered in 1818 by C.L. Blume on Nusa Kembangan Island along the south coast of Java, Indonesia. Most of the localities of

Fig. 1 Flower of *Rafflesia patma* on *Tetrastigma* root system (top) and of *R. patma* bud on aerial stem of *Tetrastigma* (bottom). Photos: Sofi Mursidawati.

this species are in West Java (Meijer, 1997), but it is also present in Central Java and known historically from Sumatra (Meijer, 1997) (Fig. 2). According to separate studies by Mogea and Nais (both 2001), these regions are threatened by deforestation for palm oil production and natural disasters such as tsunamis. These authors note that buds of *R. patma* are used in local medicinal practices and are therefore subject to intense harvesting. They also state that localities in the south of Sumatra appear to have been heavily impacted by these collection practices. The combined result of these pressures is that *Rafflesia patma* is considered as endangered (EN3cd), according to Mogea (2001), with evidence based on IUCN criteria.

The preservation of this species is a priority for Bogor Botanic Garden (BBG) in Bogor, Indonesia. Indeed, this species is especially rare and Nais (2001) indicates that its distribution is restricted to Sumatra, West Java, Central Java and that it has possibly occurred in Bali. But it seems that the species is now extinct in Sumatra and close to extinction in the other places (Meijer, 1997). Preservation of the species is also urgent due to its importance in local communities as an ingredient used in traditional medicine (Nais, 2001). Previous attempts by various teams to cultivate the plant have not been successful (Nais, 1999; 2001). These disappointing results were explained by a lack of information on its biology. In 2004 the BBG team began several projects dedicated to finding out the biology of this species with the ultimate goal of successfully cultivating it. The purpose of this paper is to present this research.

Fig. 2 Map showing the distribution of *Rafflesia patma* indicated in red (© Sarah Cardinal according to Nais, 2001, map: http://d-maps.com, 2015).

First, we will discuss attempts to cultivate *R. patma*, after which a novel procedure is presented whereby unparasitised *Tetrastigma* were inoculated with a parasitised graft. This last procedure represents a breakthrough in propagating *Rafflesia* specifically and holoparasites in general. These studies provide us with the opportunity to consider the *ex situ* conservation of this species and how it can be managed prior to *in situ* conservation.

HISTORY OF THE CULTIVATION OF *RAFFLESIA* IN BOGOR BOTANIC GARDEN

Rafflesia was first cultivated at BBG in 1857 (Nais, 2001) for ornamental purposes. Three species of *Rafflesia* – *R. arnoldii* R.Br., *R. patma* Blume and *R. rochussenii* Teijsm. & Binn. – were transplanted into the Garden between 1850 and 1929. *R. arnoldii* and *R. rochusenii* bloomed in 1853 and 1857 respectively.

The cultivation of *R. patma* was attempted in 1866, 1879 and 1929. Although records have been found which refer to these plantings, there is no record of the cultivation techniques used or subsequent flowering events, so it is impossible to say at present whether or not these plantings were successful.

It is also important to note that in 2010, a new approach was tried at the laboratory in Bogor by a team from the Indonesian Institute of Sciences (LIPI). This was the *in vitro* cultivation of *R. arnoldii* bud tissue. This technique offered a promising solution for a plant with difficult cultivation requirements. The explants survived and the cells multiplied but no differentiation could be observed in the callus (Sukamto & Mujiomo, 2010). The tests were also conducted on *R. patma* with similar results (unpublished data).

RAFFLESIA PATMA CULTIVATION TESTS

In 2004, the Science Research team at BBG began working on developing protocols for the cultivation of *R. patma* for *ex situ* conservation purposes. In addition to presenting this species to the public in the garden, the objective was to extensively multiply the plant for its preservation.

The first cultivation methods tested used seeds of *R. patma*, which were possible to obtain in large quantities from wild populations; indeed, one fruit of *Rafflesia* may contain as many as 270,000 seeds (Nais, 2001). Since the goal was to obtain a large number of plants, this method was the most obvious. Several germination techniques were attempted, all on *in vitro* or on mature *Tetrastigma* plants.

In vitro *germination tests of* Rafflesia patma *seeds alone*

Fresh seeds of *R. patma* were sown on a half-strength Murashige & Skoog (MS) medium (Murashige, 1962) which contains extracts of host plant bark or strigol (GR-24) at low concentrations (0.05–1 ppm). Strigol is a germination stimulant for some parasitic plants.

After 352 days, the seeds were turgid in all media, but had not yet germinated. This experiment suggests that *Rafflesia* seeds have specific requirements that are as yet unknown. It is possible that in nature, germination is induced by specific exudates that are so far unidentified. *Rafflesia* seeds are extremely small, so the presence of a mycorrhizal fungus may also be necessary to induce the breaking of dormancy, similar to seeds from Orchidaceae (Rasmussen, 1995). This experiment must be repeated more rigorously to determine germination requirements.

In vitro *germination tests of* Rafflesia patma *seeds on young* Tetrastigma *plants*

Starting from the hypothesis that seed of *R. patma* needs exudates released by the roots of its host, *Tetrastigma scariosum*, an *in vitro* experiment was conducted in which seeds of *Tetrastigma* and *Rafflesia* were placed separately on a half-strength MS medium. The liana seeds germinated quickly, and within two weeks the roots were well developed. The soaked *Rafflesia* seeds were then added to the *Tetrastigma* cultivation medium. In the three months that followed, the liana seedlings developed significantly, to the point of outgrowing their 11 cm high container. Meanwhile, the *Rafflesia* seeds showed no sign of development and remained thus until the end of the experiment at 24 weeks.

It is understood that most parasite plant seeds need a germination elicitor, a compound released by the host at a particular moment of its life cycle under specific environmental conditions (Nais & Wilcock, 1999). The young *Tetrastigma* may not yet have released the molecule necessary for *Rafflesia* to trigger its germination.

In vivo *germination tests of* Rafflesia patma *seeds on mature* Tetrastigma *plants at Bogor Botanic Garden and in the Pangandaran Nature Reserve*

R. patma seed germination tests were also performed directly on the host. *Tetrastigma scariosum* seedlings, both parasitised and not parasitised, were selected on two sites: at BBG and at a site in the plant's natural environment in Pangandaran Nature Reserve. For this test, the seeds were introduced under the bark of selected hosts in both sites. They were left alone for 218 days at BBG and 652 days at Pangandaran. The seeds were then re-collected and examined under a microscope.

None of the seed from either location showed any sign of development. They were intact and turgescent; green algae covered the surface of some of them. No interaction between the host and the seed of *R. patma* was observed, either in parasitised or unparasitised hosts (Table 1). Cultivation of this species from seed is therefore still a challenge.

Type of test	Result
in vitro germination of *R. patma* seed alone	No germination
in vitro germination of *R. patma* seed on young *Tetrastigma* plants	No germination
in vivo germination of *R. patma* seed on mature *Tetrastigma* plants in BBG and Pangandaran	No germination

Table 1 Results of the three germination tests on *Rafflesia patma.*

Transfer of a parasitized Tetrastigma *plant from Pangandaran to Bogor Botanic Garden*

Concurrently with these germination studies, staff at BBG attempted to cultivate a *Tetrastigma* plant parasitised with *Rafflesia* by transplanting a plant from the wild to the Garden. In 2004, Garden staff received authorisation to collect an individual of *Tetrastigma scariosum* known to be parasitised with *R. patma* from the Pangandaran Nature Reserve and transport it to BBG. A bud of *R. patma* was present on the roots of the vine but did not survive the journey. The host did survive the journey and transplanting process and thrived in the Garden.

In April 2006, a 35 mm bud appeared on the *Tetrastigma* plant. Unfortunately, two months later, a hurricane damaged the vine and the canopy that protected the *Rafflesia*, thereby exposing it to direct sunlight. The bud did not survive. Four years later, in August 2010, eight buds emerged from the roots of the host. Despite a significant mortality rate, two female flowers bloomed the following year, in June and July. Since then, other flowers have bloomed. The transfer may therefore be deemed successful and it can be said that it is possible to cultivate the parasite at BBG. However, the goal is to multiply the plants of *R. patma* for conservation purposes. To this end, grafts were attempted in order to inoculate healthy *Tetrastigma* lianas.

GRAFTING AS A PROPAGATION METHOD FOR *RAFFLESIA PATMA*

Grafting is a method of vegetative propagation used when a plant cannot be reproduced by the traditional methods of cuttings or layering. This operation consists of implanting a bud or other organ from one plant (scion) into the tissues of another plant (rootstock). Both plants need to be the same, closely related or compatible species. Following this operation, the graft will grow from the rootstock; tissues from the two individuals will fuse and will eventually become a single entity. This method enables high-quality plants to be produced quickly and on a large scale. Thus it can be valuable for *ex situ* conservation programmes. This method has been successfully used to propagate numbers of rare *Sorbus* in Scotland (McHaffie *et al.*, 2011); however, to the knowledge of the authors it has not yet been tested on a parasitic plant.

Most *Rafflesia* flowers emerge from the roots or rooted stems of *Tetrastigma*, and the authors have therefore concluded that the roots contain the largest concentration of the vegetative part of the parasite. As a result, new individuals of *R. patma* were collected from root cuttings harvested from *Tetrastigma* growing wild in the Pangandaran Nature Reserve. These root cuttings were then grafted onto *Tetrastigma* plants already present in the Living Collection at BBG. Two grafting methods were used, and each one was selected according to the diameter of the scion and rootstock available: veneer grafting (when the scion and rootstock have different diameters) and cleft grafting (when the diameters are more or less the same size).

The veneer grafting method

Veneer grafting (Fig. 3) is the preferred method for difficult grafts, since the technique is very simple to carry out. It mimics a phenomenon seen quite frequently in nature, such as when two trees grow very close to one another. The diameter of the two roots is of little importance because a contact area of 20–25 mm in length is exposed by removing the bark on the base of the rootstock stem. The scion is prepared in the same way and then placed side by side with the rootstock. The graft is kept in place with tape (Bir *et al.*, 2012).

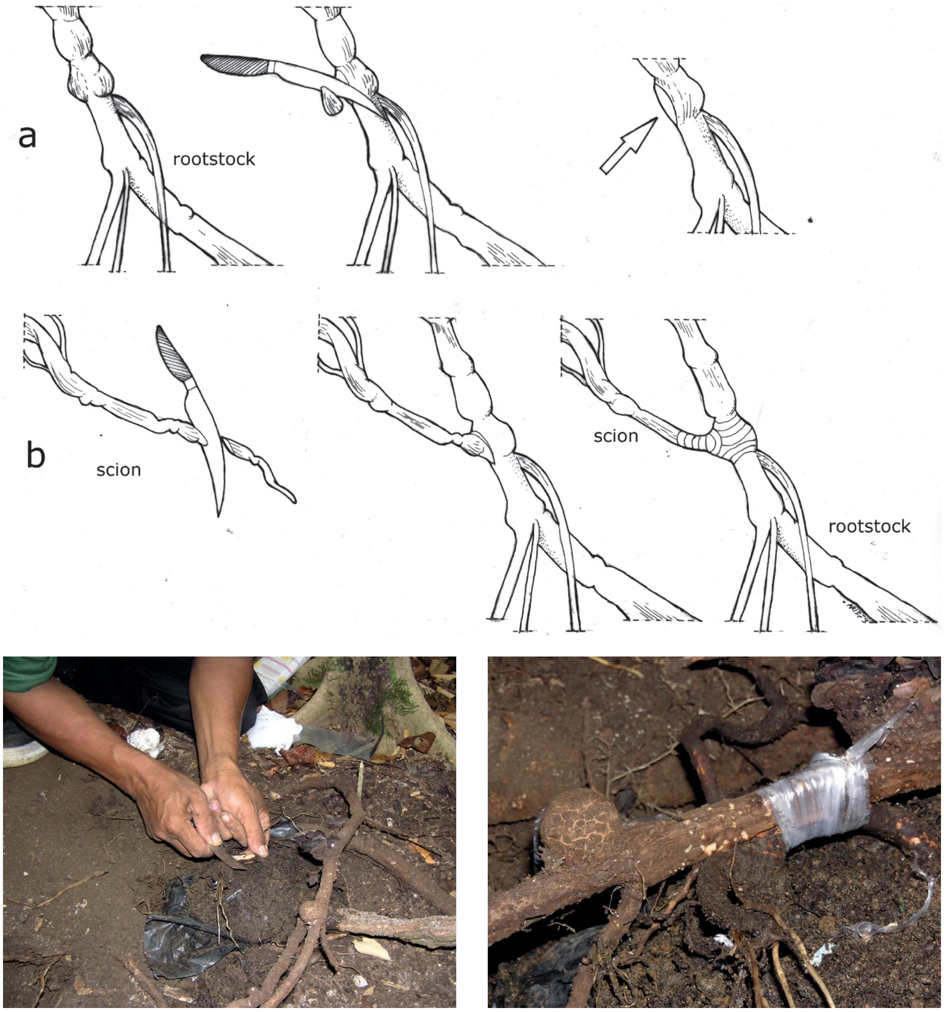

Fig. 3 Veneer grafting method. a: Original rootstock, preparation by removing bark, and exposed graft site (arrow); b: Scion preparation, connection to rootstock and finally the secured and sealed graft (a+b diagrams drawn by Arif Setiawan); pictures of the veneer graft tested in Bogor Botanic Garden. Photos: Sofi Mursidawati.

The cleft grafting method

Cleft grafting (Fig. 4) is probably the most popular form of grafting; it is a method commonly used for flowering and fruiting trees. In this process, the diameter of the rootstock must be the same as that of the scion. The rootstock is cut at an angle twice so that the incision presents an inverted V with compression surfaces from 10 to 25 mm long. The scion is cut and split to fit the rootstock accurately (same angle, same length) and the two parts are then joined. A good contact between the two cambia is required for them to fuse properly. The final phase of the graft involves securing the scion to the rootstock with tape (Bir *et al*., 2012).

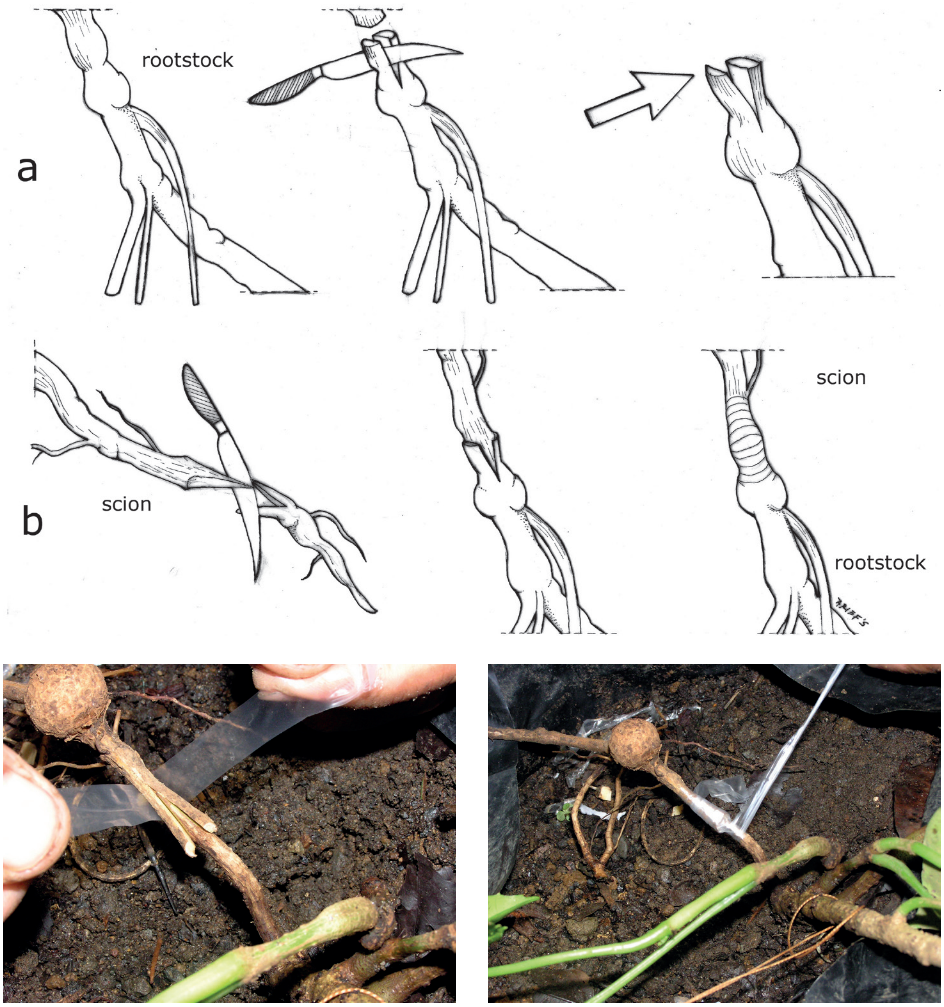

Fig. 4 Cleft grafting method: a: Root stock cutting in grafting preparation; b: Cutting of scion in preparation for joining, insertion and sealing the graft with tape (a+b diagrams drawn by Arif Setiawan); pictures of the cleft grafting method tested in Bogor Botanic Garden. Photos: Sofi Mursidawati.

RESULTS

Several tests were carried out using these two grafting techniques. Some grafts were not successful or showed no sign of *Rafflesia patma* development afterwards. However, two trials were successful and these are described below.

Veneer grafting test

The first successful test was carried out in February 2007 at BBG. A root of a host plant, parasitised with *R. patma* and taken from the original habitat in Pangandaran, was grafted to the stem of a healthy *Tetrastigma* plant growing in the living collection using the veneer grafting method. The parasite bud on the scion did not survive, but the graft itself was a success. The cut healed quickly as new roots developed and as the diameter increased on the grafted side.

In June 2009, a new *Rafflesia* bud appeared on the rootstock. It developed, and a male flower bloomed in June 2010.

Cleft grafting test

A cleft graft was attempted in 2006 at BBG. The team used a scion from *Tetrastigma* roots parasitised with *R. patma*, taken from the natural environment, and a healthy rootstock from the Garden. Again, the *Rafflesia* buds present on the scion did not survive the journey. However, a female flower eventually bloomed in November 2012 on the rootstock.

As a result of these two successful tests, we can say that grafting is a viable method for propagating *R. patma* individuals. Further tests are needed to determine its utility with other parasitic species.

CONCLUSION

This paper has described three techniques tested in order to find efficient ways to propagate the parasitic and endangered plant *Rafflesia patma* at BBG for its conservation.

The first method, *in vitro* seed germination, proved to be unsuccessful and encouraged the team to turn towards other methods. The second method was the straightforward cultivation of a parasitised host in the Garden. This experiment showed that with a minimal amount of care during the transfer of the plant, it is possible to maintain the parasitised host and to get the *Rafflesia* to flower several times afterwards. Building on this success, the team worked to try to multiply the plant vegetatively for *ex situ* conservation purposes.

Finally, the third method used grafts to transfer a plant of *R. patma* from one *Tetrastigma* plant to another. This technique was successful, resulting in several blooming events since 2010. The grafting process is a first for the propagation of holoparasites, and gives new hope for the conservation of *R. patma* specifically and *Rafflesia* in general.

This success comes at a critical time. The natural habitat of *R. patma* is rapidly disappearing. This is not a unique situation for either this species or the Pangandaran Nature Reserve. These propagation studies and the novel technique of grafting parasitised hosts onto non-parasitised ones have the potential to conserve other endangered Rafflesiaceae and holoparasites.

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