Growing a plant you cannot see: ex situ propagation of the endoparasite *Rafflesia speciosa* to strengthen in situ conservation

Jeanmaire Molina[1], Mick Erickson[2], Isabel B. Emanuel[2], Stephen Jones[3], Kirsten Kautz[3], Benjamin Gutman[3], Ronniel Pedales[4], Macario Nicer Napulan[5], Marites Muyong[5], Sofi Mursidawati[6], Ngatari Ngatari[6], Pola Geneva A. Bumanglag[7], William McLaughlin[2], Danilo Tandang[8], John Rey Callado[9], Mark Moreno[2], Kyle Wallick[2], James Hill[1], Erika Marie A. Bascos[4], Alysa G. Estopace[4], David Kidwell-Slak[3], Megan Haidet[3], Amy Highland[3], Ray Mims[2], James Adams[3], Lisa Philander[3], Ari Novy[10] & Susan Pell[3]

Abstract

The genus *Rafflesia* R.Br. ex Gray (Rafflesiaceae) produces the world's largest flowers, yet most species are critically endangered, making the flower a symbol of global plant conservation. As a holoparasite confined to the woody vines of its sole host, *Tetrastigma* (Miq.) Planch. (Vitaceae), *Rafflesia* remains notoriously difficult to study and propagate. Its cryptic endophytic life cycle – persisting as microscopic tissue within its host for years before emergence – has long impeded ex situ conservation, such that its cultivation has aptly been described as 'growing a plant you cannot see'. Grafting of infected host material has been the only proven method. After more than a decade of diverse attempts, we report the first viable propagation of *R. speciosa* Barcelona & Fernando-infected pieces sourced from the Philippines at the United States Botanic Garden in Washington, DC – the first documented cultivation of *Rafflesia* in the Western Hemisphere. Our approach combined two complementary methods: (1) rooting infected stem and root cuttings, and (2) grafting infected root tissues onto established *Tetrastigma* vines. Infection was verified using a combined molecular (PCR) and histological diagnostic framework. Notably, one graft produced two *Rafflesia* buds (cupule stage) approximately 2.5 years post-grafting, representing the first evidence of bud initiation outside Southeast Asia. This study (1) establishes replicable glasshouse propagation protocols for *Rafflesia*-infected *Tetrastigma*; (2) demonstrates a practical PCR-histological approach for confirming infection prior to bud

[1] Jeanmaire Molina is an Associate Professor at Pace University.
Address: Pace University, 1 Pace Plaza, New York, USA.
Email: jmolina2@pace.edu
[2] Formerly at United States Botanic Garden, 245 First St SW, Washington, DC 20515, USA.
[3] United States Botanic Garden, 245 First St SW, Washington, DC 20515, USA.
[4] Institute of Biology, University of the Philippines, Diliman, Quezon City, Philippines.
[5] Municipality of Miagao, Iloilo, Philippines.
[6] Bogor Botanic Garden, Jalan Ir. Juanda 13 Bogor-16003, Indonesia.
[7] Wildlife Resources Division of Biodiversity Management Bureau, Department of Environment and Natural Resources, Quezon City, Philippines.
[8] Philippine National Museum, Manila, Philippines.
[9] Department of Science and Technology-Forest Products Research and Development Institute, Laguna, Philippines.
[10] San Diego Botanic Garden, Encinitas, CA, USA.

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emergence; and (3) shows that infected tissues can preserve endophytic germplasm beyond the lifespan of the original scion. These outcomes were achieved despite the formidable logistical barriers of transcontinental permitting and transport, involving a legally protected Philippine endemic and a US-regulated organism. These methods mark a breakthrough in ex situ *Rafflesia* conservation, safeguarding vital germplasm and strengthening the future of in situ recovery efforts, enabling the maintenance of this cryptic parasite within its host until it emerges as one of the world's largest flowers.

Introduction

Rafflesia R.Br. ex Gray is one of the most remarkable plant genera in the world, famed for its enormous carrion-scented flowers, some reaching 1 m in diameter. Unlike most plants, it lacks stems, leaves and roots, living entirely as an endophyte inside its host vine, *Tetrastigma* (Miq.) Planch. (Vitaceae) and emerging only to flower. More than 40 species are currently recognised (POWO, 2025), and all are confined to the diminishing forests of tropical Asia. The Philippines is a centre of diversity, where many species are now critically endangered due to deforestation and other threats (Barcelona *et al.*, 2009; Pelser *et al.*, 2019; Malabrigo *et al.*, 2025).

Rafflesia species are sometimes referred to as the 'panda of the plant world' because of their value as charismatic conservation icon, but despite this, they have proved notoriously difficult to cultivate outside their natural habitat. Because *Rafflesia* persists as microscopic endophytic tissue within its host for years before emergence, its cultivation has aptly been described by one author (M. Erickson) as 'growing a plant you cannot see'. Knowledge gaps, especially around seed biology and germination, remain major obstacles to cultivation and conservation. Although shrews and ants have been observed dispersing seeds (Bänziger, 2004; Nais, 2001; Pelser *et al.*, 2013, 2016), the conditions that allow them to infect a host and establish are still unknown (Wicaksono *et al.*, 2021).

Recent integrative studies highlight the importance of microbiomes and metabolites in understanding *Rafflesia–Tetrastigma* interactions. *R. speciosa* Barcelona & Fernando seeds harbour microbial endophytes similar to those of their hosts, suggesting possible microbial transmission during infection (Molina *et al.*, 2024). Transcriptomic and metabolomic analyses further revealed genes linked to plant growth regulators in seeds (Molina *et al.*, 2023) and shifts in host defence compounds such as benzylisoquinoline alkaloids during infection (Molina *et al.*, 2022). More recently, combined metagenomic and metabolomic profiling uncovered associations between specific bacterial families and polyphenol accumulation, which indicates *Rafflesia* may act like a gall (Molina *et al.*, 2025) to facilitate its own development.

Attempts to propagate *Rafflesia* from seed ex situ, including trials with plant growth regulators and synthetic stimulants, have been largely unsuccessful (Mursidawati *et al.*, 2015; Wicaksono *et al.*, 2016; Molina *et al.*, 2017). How the seeds germinate and infect *Tetrastigma* remains unknown (Wicaksono *et al.*, 2021), though the endophyte can persist vegetatively for years before bud emergence (Bascos *et al.*, 2021). Inoculation experiments using seed, such as those at the United States Botanic Garden (USBG), have yet to yield flowers (Molina *et al.*, 2017). To date, only a single success has been reported: *R. arnoldii* bloomed at Bogor Botanic Garden in Indonesia in 2022, likely resulting from

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a seed inoculation performed in 2010 (S. Mursidawati and D. Latifah, pers. comm., 5 January 2023). The most reliable approach has been grafting *Rafflesia*-infected *Tetrastigma* cuttings onto uninfected rootstocks, which has produced multiple blooms of *R. patma* at Bogor Botanic Garden (Mursidawati *et al.*, 2015). In that case, infected propagules were collected from *Tetrastigma* hosts in the Pangandaran Nature Reserve, approximately 400 km from Bogor, and grafted within the short time window of 24–48 hours after collection – a logistical advantage that likely contributed to the success.

However, the apparent failure of conventional horticultural attempts may reflect a diagnostic limitation rather than biological impossibility. Because *Rafflesia* remains microscopic within its host for extended periods, infection has traditionally been inferred only from visible bud emergence. In the absence of molecular or anatomical tools capable of confirming endophytic persistence prior to flowering, grafts or seed inoculations may have been prematurely deemed unsuccessful. The long latency between infection and bud initiation likely discouraged repeated trials, and reliance on flowering as the sole indicator of success may have obscured instances of cryptic establishment. Integrating diagnostic methods into propagation efforts is therefore essential to distinguish true absence of infection from undetected persistence within host tissues.

Here, we present a significant milestone in *Rafflesia* cultivation: after more than a decade of attempts (see Molina *et al.*, 2017), we have successfully established the first viable propagation of *R. speciosa*-infected cuttings from the Philippines at the USBG in Washington, DC – the first documented cultivation of *Rafflesia* in the Western Hemisphere. Building on the methods described by Mursidawati *et al.* (2015), our strategy involved two methods: rooting as well as grafting wild-collected, *Rafflesia*-infected root and stem tissues onto established *Tetrastigma* vines at the USBG, with infection verified through both histological and molecular analyses. Notably, one graft produced two *Rafflesia* buds ~2.5 years post-grafting, marking the first evidence of bud initiation ex situ. Though the two buds aborted recently (~5–6 months after discovery), our two methods still represent a major advance in ex situ *Rafflesia* conservation, safeguarding vital germplasm under controlled conditions. Their successful application also holds the potential to transform in situ conservation by enabling the expansion of current ranges and supporting the re-establishment of populations where the species has been extirpated.

Field collection and transport of *Tetrastigma* infected with *Rafflesia speciosa*

The Philippines has more than 15 endemic species of *Rafflesia* (POWO, 2025), all of which are legally protected. Collection is prohibited without permits and the consent of local communities. Gratuitous permits Nos 326 and 338 were granted to J. Molina by the Philippines' Department of Natural Resources Biodiversity Management Bureau. Fieldwork was conducted in Miagao, Iloilo, in January 2023 and August 2024, targeting *R. speciosa* (Fig. 1A) and its hosts, *T. harmandii* Planch. and *T. magnum* Merr. (Fig. 1B). Host plants can only be taxonomically identified when accessible branches bearing foliage are available. In situ, host vines grew under dense tropical montane canopy at 670–853 m elevation, in humid, shaded understory (~4 µmol m^{-2} s^{-1}

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Fig. 1 Flower, habitat and host growth form of *R. speciosa* in situ. **A** Open flower of *R. speciosa* in Miagao, Philippines, measuring approximately 45 cm in diameter. **B** Growth habit of the *Tetrastigma* host in situ, showing mature lianas ascending into the canopy and forming extensive woody stems that support *Rafflesia* infections. **C** Habitat of *Rafflesia* on a steep limestone forest slope, showing the shaded understory and dense secondary growth from which flowers emerge following infection of *Tetrastigma* roots. Photos: Jeanmaire Molina.

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daytime light, Apogee quantum flux, Apogee Instruments) and dense secondary growth conditions (Fig. 1C), with well-drained but organic-rich soils and extensive surface-level adventitious roots. Field conditions were warm and humid, with substantial litter accumulation. These environmental characteristics informed substrate and humidity choices under cultivation. A total of six infected stem pieces and eleven infected root pieces (up to 100 cm in length) were collected each year, along with five uninfected stem cuttings intended for rooting as future rootstocks, as permitted under the gratuitous permits (Fig. 2). For clarity, we use 'piece' to refer to field-collected infected material prior to assignment, 'cutting' for material intended for independent rooting and 'scion' for infected root material used in grafting.

Stem pieces (2.5–7.5 cm in diameter) contained at least two nodes (preferably more) and often displayed existing buds or bud scars from past *Rafflesia* blooms, indicating active infection. Additionally, infected root pieces, which bore early-stage *R. speciosa* buds (i.e. cupule and/or cupule-bract stage) (Susatya, 2020) (Fig. 3), were collected even if nodal regions were not visible. Collected root pieces, to be used as scion material for grafting, were 6–10 mm in diameter. Infected roots extended up to 5 m from the host stem and were typically located at or within 5 cm below the soil surface. When collecting root propagules for grafting, the presence of white, fibrous roots was desirable, but these were difficult to keep alive and hydrated during travel.

All pieces were excised with pruning secateurs, placed in plastic poster tubes

Fig. 2 *Tetrastigma* cuttings prepared for phytosanitary inspection and export to the United States, showing seven infected pieces (left) and five uninfected pieces with foliage (right). All cuttings were wrapped in lightly moistened sphagnum peat moss for transport. Photo: Jeanmaire Molina.

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and dressed with wet sphagnum moss for moisture retention during transport to Iloilo City, Philippines. Shoot–root polarity was recorded for each specimen at the time of collection. Specimens were then cleaned thoroughly with municipal water to remove all soil, in compliance with phytosanitary requirements. Each piece was wrapped in lightly moistened sphagnum peat moss (Besgrow Sphagnum, Hokitika, New Zealand) (Fig. 2), sealed in containers and transported by hand to the USBG production facility (PF) in Washington, DC, following approval of all required Philippine permits and under USDA permit PPQ526, authorising the movement of these regulated organisms into the United States. Transport required five to seven days, including at least two days for permitting in the Philippines (inter-island movement, phytosanitary clearance and export approval), two days of international air travel and USDA inspection on arrival. These logistical delays are a major challenge and have historically caused high mortality of propagules shipped by air cargo (Molina *et al.*, 2017). In this instance, careful collection, packing and hand-carry transport by a dedicated horticulturist ensured that all propagules arrived viable within the shortest feasible timeframe. Continuity of care – from field collection through transport and grafting – was overseen by the same experienced horticulturist, with additional assistance by other authors. The USBG PF was prepared in advance, with sterilised tools, materials and the misting system ready prior to arrival.

Upon arrival at the PF, the infected *Tetrastigma* pieces were subdivided into 32 propagules for cultivation. Some root pieces exhibited lenticel formation in response to excessive moisture and anoxia. All *R. speciosa* buds present at collection died within one

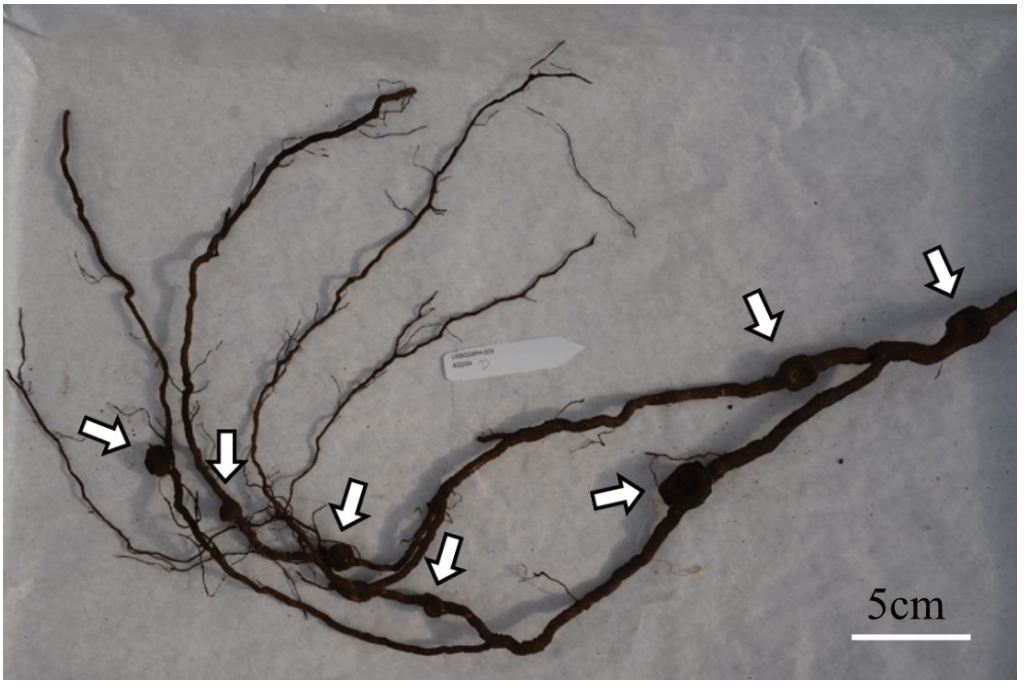

Fig. 3 Sample of *Rafflesia*-infected *Tetrastigma* root cutting collected for propagation. Cuttings bearing early-stage *R. speciosa* buds (arrows) were selected. Scale bar=5 cm. Photo: USBG.

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month of transport. Given the transit period of 5–7 days and associated handling stress, not all pieces arrived in optimal physiological condition. To maximise survival, compromised material was stabilised upon arrival. Basal ends were recut where desiccation or cell death was evident to expose fresh vascular tissue, necrotic portions were removed and pieces were rehydrated under high-humidity conditions to reduce transpiration stress.

Propagation at the USBG

Rooting Rafflesia speciosa-*infected* Tetrastigma *spp. cuttings*

R. speciosa-infected *Tetrastigma* stem cuttings were prepared as semi-hardwood nodal cuttings following general principles outlined in Hartmann *et al.* (2018). Cuttings (10–100 cm) retained 2–4 nodes, leaves were removed to reduce transpiration and the basal end was recut at a 45° angle approximately 2 cm below a node. No exogenous rooting hormone was applied. Cuttings were inserted into a 1:3 (v/v) perlite:milled sphagnum substrate under high-humidity glasshouse conditions to minimise desiccation. Within 2–4 weeks, two cuttings produced callus tissue at the terminal (apical) cut, indicating stem differentiation (Fig. 4). The remaining cuttings that failed to callus were bud-grafted using a single uninfected *Tetrastigma* leaf bud (Fig. 5A) or juvenile leaf via cleft grafting (Fig. 5B), following McMillan Browse (1979), to jump-start photosynthetic growth. Once roots emerged from the base of the propagation pots, cuttings were transferred to Sun Gro® professional growing mix (Metro-Mix™ 560) composed of pine bark, peat and coir for further rooting, before being moved into larger containers for long-term cultivation (Fig. 6).

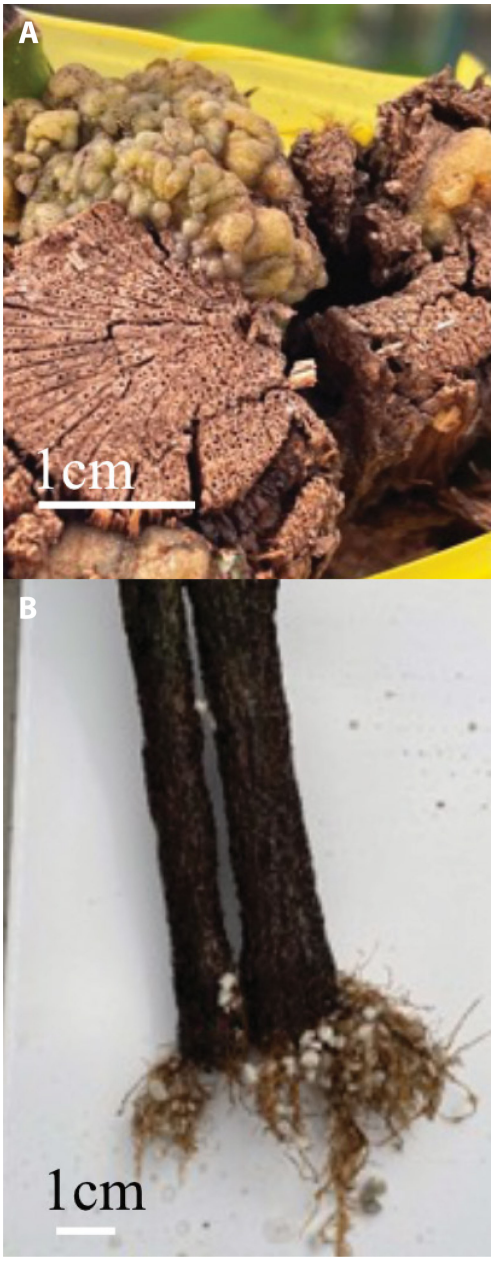

Fig. 4 **A** Callus formation in the apical end. **B** A successfully rooted stem cutting. Photos taken four weeks after rooting. Scale bar=1 cm. Photos: USBG.

A single infected root cutting was also prepared for rooting following Hartmann *et al.* (2018), such that the basal end with older growth was cut at a 45° angle and inserted to a depth of ~10 cm into a 1:3

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Fig. 5 When *R. speciosa*-infected *Tetrastigma* stem cuttings failed to produce a terminal callus within four weeks (indicating unsuccessful rooting), uninfected *Tetrastigma* leaf buds were grafted onto the cuttings to jump-start photosynthetic growth. **A** Axillary bud graft. **B** Juvenile axillary stem graft. Photos: USBG.

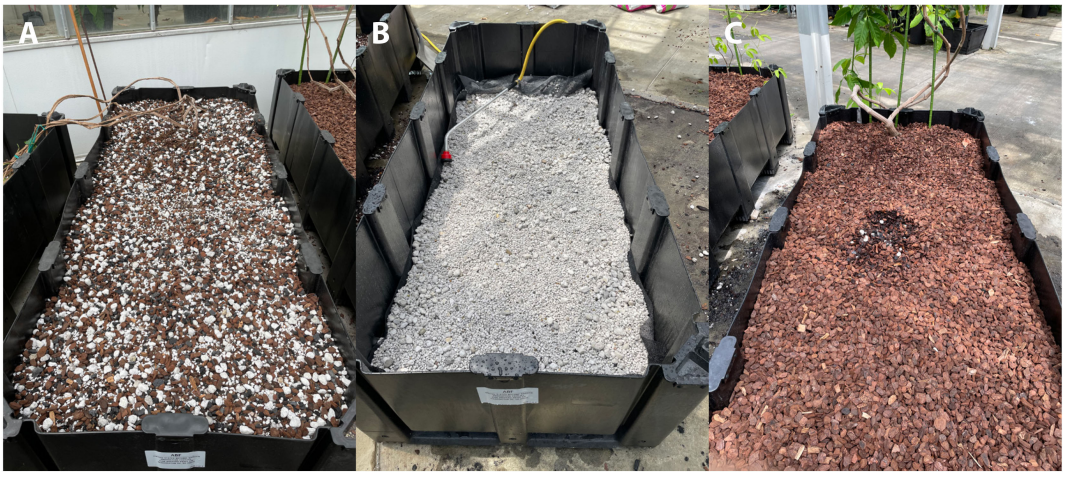

Fig. 6 Containers (2.4 m x 1 m x 60 cm) at the USBG PF filled with a custom soilless medium. **A** A 4:4:3:2:1 (v/v) mixture of pine bark fines, Power Orchiata (New Zealand) pine bark, filter sand, pumice (Hess Pumice, ID, USA) and P1 horticultural charcoal. **B** A 5-cm base layer of coarse pumice. **C** A 5-cm top layer of Power Orchiata bark. Photos: USBG.

(v/v) perlite:milled sphagnum substrate. Bud grafting was not possible because the root tissue was too succulent to hold a bud. Nevertheless, the cutting rooted after approximately six months and produced shoot tissue two weeks later. Rooting was pursued in this case in lieu of grafting because the cutting was ~2 cm in diameter, which is too large for grafting.

All successfully rooted cuttings were established in custom containers measuring 2.4 m x 1 m x 60 cm (DACO Co., Kent, WA, USA) with drainage holes. These containers were filled with a 4:4:3:2:1 (v/v) mixture of pine bark fines, *Pinus radiata* pine bark (Power Orchiata, New Zealand), filter sand, pumice (Hess Pumice, ID, USA) and P1 horticultural charcoal (Fig. 6A), with a 5-cm base layer of coarse pumice (Fig. 5B) and a 5-cm top layer of Power Orchiata bark (Fig. 6C). Trellis arches (2.4 m Monet Garden Arch, Agriframes, USA) were installed at both ends of each container to support canopy growth. Up to two plants were trained per container (one on each end) (Fig. 7A) with stems encouraged to grow along the surface to promote secondary

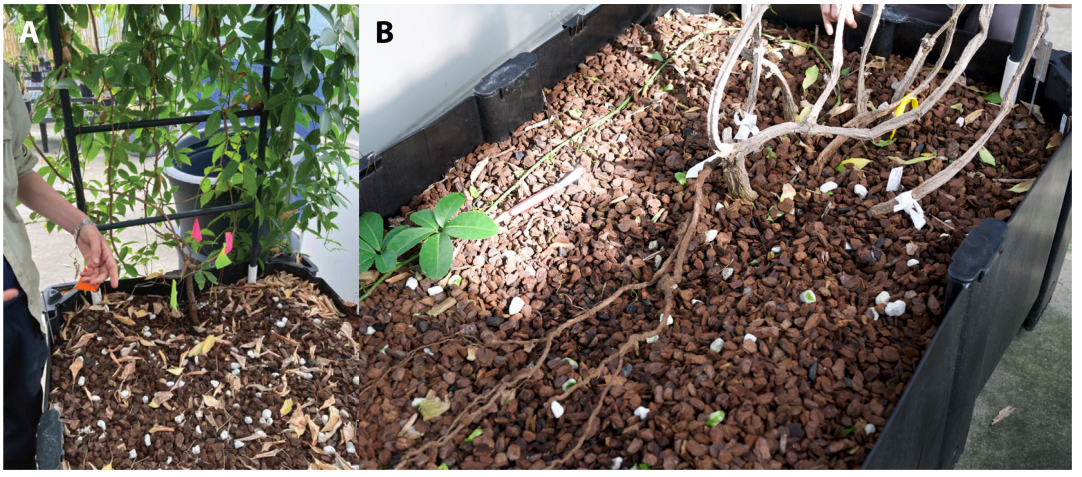

Fig. 7 Infected *Tetrastigma* plants were grown in large pots. Two plants were trained per container, one on each end (**A**) with stems encouraged to grow along the surface (**B**) to promote secondary rooting and maximise aerial root sites for future grafting. Photos: USBG.

rooting and maximise aerial root sites for future grafting (Fig. 7B). Internal media temperatures averaged 24 °C (max. 27.8 °C, min. 20 °C; Disc Mini Temp, Blue Maestro).

Preliminary trials using 100% standard potting soil resulted in failure, with grafted roots rotting prior to establishment, likely due to excessive water retention. In contrast, Orchiata pine bark provided superior aeration while retaining sufficient moisture, enabling graft fusion and secondary root emergence. The shift to bark-based substrates was informed by earlier experiments (M. Erickson, pers. obs., Jan. 2023) and observations by S. Mursidawati and N. Ngatari, both of which highlighted the detrimental effects of waterlogged soils. Beyond preventing rot, bark substrates promoted robust root development, allowed for easier manipulation of roots for grafting and permitted fine control of moisture levels. Functionally, this system mimicked hydroponic conditions with high control and low decomposition risk. However, the substrate will likely require replacement every five to seven years as the Orchiata bark gradually disintegrates.

Overall, stem cuttings proved the most efficient and reliable method for propagating *Tetrastigma* infected with *Rafflesia*, with a 62.5% success rate (Table 1). Two approaches were effective: (1) permitting infected propagules to produce shoots naturally, or (2) bud-grafting semi-hardwood leaf buds at an early developmental stage to jump-start photosynthetic growth. Success was indicated by callus production at cut sites, followed by greening and differentiation into stem tissue.

By contrast, root cuttings required far longer to establish (33.3% success rate) (Table 1) and – based on evidence from viticulture – are not generally recommended (Hartmann *et al.*, 2018).

However, *Rafflesia* species differ in their sites of emergence: some produce buds on aerial woody portions of the host vine, whereas others, including *R. speciosa*, are primarily associated with root tissues, with buds most often encountered below ground and only rarely produced on aerial stems (J. Molina, pers. obs.) In such cases, propagation from infected root pieces may occasionally be necessary, particularly when tissues

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Table 1 Outcomes of different propagation techniques for *R. speciosa*-infected *Tetrastigma* cuttings and grafts established at the USBG. Shown are survival rates, accession numbers of surviving individuals and confirmation of *Rafflesia* infection status based on molecular (PCR), histological and/or bud evidence. PCR (P) was used as an initial screening tool. Histology (H) and/or bud presence (B) provided confirmatory evidence of infection. Because *Rafflesia* endophytic cells are spatially localised, negative molecular or histological results were interpreted cautiously and considered inconclusive (INC) rather than proof of definitive absence. RSG = root-to-stem graft. RRG = root-to-root graft. LBG = leaf bud graft.

Propagation technique	Propagules	Percentage success (survived/total attempts)	Accession numbers of surviving individuals	*Rafflesia* infection status based on PCR (P), histology (H), bud presence (B); negative results considered inconclusive (INC)
Grafting	RSG	28.6% (4/14)	2023-0071*A, 2024-0454*A, 2024-0454*B, 2017-0203*O	2023-0071*A (INC), 2024-0454*A (P), 2024-0454*B (P, H), 2017-0203*O (H, B)
Grafting	RRG	14.3% (1/7)	2023-0088*A	INC
Cutting	Rooting infected stem cutting (may/may not involve LBG)	62.5% (5/8)	2023-0085*A (LBG), 2023-0085*B, 2023-0090*A (LBG), 2023-0089*A, 2024-0457*A	2023-0085*A (INC), 2023-0085*B (INC), 2023-0090*A (P), 2023-0089*A (P, H), 2024-0457*A (INC)
Cutting	Rooting infected root cutting	33.3% (1/3)	2023-0069*A	INC

are too large or otherwise unsuitable for grafting.

Grafting Rafflesia speciosa-*infected* Tetrastigma

A total of 21 scions of *R. speciosa*-infected *Tetrastigma* spp. were grafted onto established *T. harmandii* and *T. magnum* plants at the USBG PF. These rootstock plants were originally collected in Miagao, Philippines in 2017 (Molina *et al.*, 2017) and maintained in large containers under greenhouse conditions of c. 21–28 °C and 70–80% relative humidity. Grafting was performed irrespective of species because *T. magnum* and *T. harmandii* are graft-compatible. Root-to-stem (RSG) and root-to-root (RRG) grafts were performed, while stem-to-stem grafts were not undertaken in order to prioritise rooting of infected stem cuttings. Although host identity shapes natural *Rafflesia–Tetrastigma* associations, several *Tetrastigma* species show interspecific graft compatibility. Successful unions have been achieved among *T. harmandii*, *T. magnum*, *T. rafflesiae* (Miq.) Planch., *T. voinierianum* (Sallier) Pierre ex Gagnep. and *T. loheri* Gagnep. (W. McLaughlin, M. Erickson, K. Kautz, pers. obs.), indicating broad vascular compatibility within the genus under controlled conditions. However, whether such graft-compatible rootstocks can sustain long-term *Rafflesia* establishment and flowering remains unknown.

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To accommodate space limitations, rootstocks in the PF were regularly pruned, although one specimen in the USBG Conservatory has been allowed to grow into the higher elevations of the room (2017-0203*O), with its canopy reaching natural dimensions of greater than 15 m, similar to that observed under wild conditions. Temperature in the Conservatory is maintained at 18–28 °C with average 75% relative humidity or about 1–1.5 kPa sustained vapour pressure deficit.

Root-to-stem grafting

Infected *Tetrastigma* root propagules were grafted onto established host stems using a modified veneer technique. An incision of approximately 10 mm in length was made into the surface stem tissue at an acute angle, no deeper than one-third of the stem diameter, directly into nodal tissue near the soil line. The basal end of the infected root scion was cut with a Victorinox budding knife (Ibach, Switzerland) to maximise surface contact and cambial alignment. The union was secured with elastic flagging tape (Presco Nursery Flagging Tape, TX, USA) to ensure tight yet flexible pressure. Successful grafts were achieved when root scions of ~6 mm diameter were paired with host stems of ~10 mm diameter, reflecting the recommended 1–3× size ratio between scion and stock. Grafts generally ranged from 15 to 60 cm in length. This root-to-stem veneer graft (Fig. 8A, B) represents a modification of the RRG technique described by Mursidawati *et al.* (2015).

Root-to-root grafting

RRGs were conducted using a cleft graft. A 5-mm incision was made into a host root of comparable diameter, and the basal end of the infected scion was fitted into the cut (Fig. 9). Because *Tetrastigma* roots lack nodes, the graft site was selected where terrestrial roots of appropriate size were available.

Post-grafting care

After establishment, grafted plants were irrigated as needed – typically once per week in cooler months and up to three times weekly during warmer periods. Fertiliser was supplied via continuous liquid feed using municipal water supplemented with a 24:8:16 fertiliser containing micronutrients (Jack's Professional Tropical Foliage).

Independent of graft type, successful unions were indicated by callus formation at the graft interface. Additional considerations include:

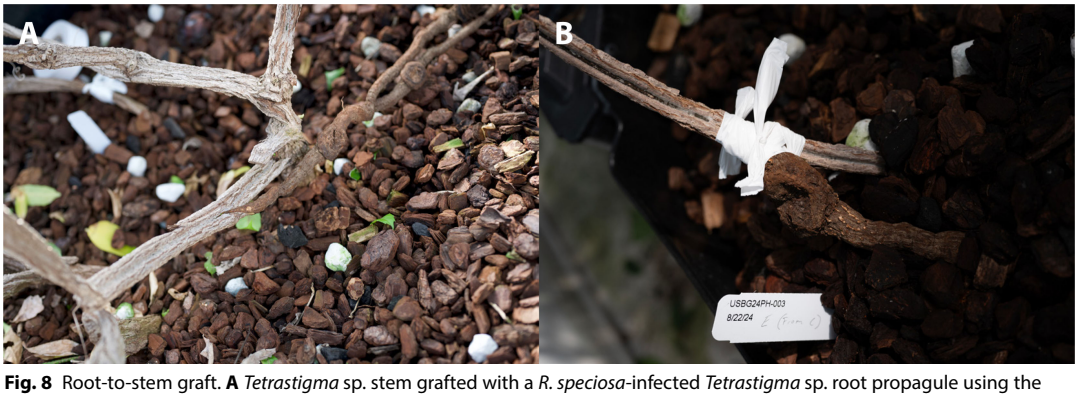

Fig. 8 Root-to-stem graft. **A** *Tetrastigma* sp. stem grafted with a *R. speciosa*-infected *Tetrastigma* sp. root propagule using the veneer technique. **B** Another veneer graft sample following wrapping with elastic flagging tape. Photos: USBG.

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Fig. 9 Root-to-root graft. *Tetrastigma* sp. root grafted with a *R. speciosa*-infected *Tetrastigma* sp. root propagule using the cleft technique. A 5-mm incision was made into a host root of comparable diameter, and the basal end of the infected scion was fitted into the cut then secured with flagging tape. Photo: USBG.

1. Scion and stock size: small-diameter (< ~6 mm) infected root scions are less viable, especially after extended transport. Pencil-sized scions are recommended, while larger host rootstocks (main woody stem > 40 mm) are preferred as they promote faster callus formation and reduce the risk of scion rot.
2. Grafting location: the scion must be positioned as close as possible to nodal tissue. Fresh grafts must be wrapped tightly to ensure firm cambial contact.
3. Type of graft: veneer grafts are most effective for RSGs, where scion and stock diameters differ, while cleft grafts are preferable for RRGs, when diameters are similar (Mursidawati *et al.*, 2015).
4. Growing medium: a coarse bark substrate (for example Orchiata bark) was critical for graft survival. It balanced aeration and moisture retention, prevented desiccation and reduced rot compared with standard potting soil, which consistently caused graft failure. The bark also supported the development of long, surface-level roots suitable for future grafting.
5. Watering and humidity: newly grafted propagules required daily watering (once or twice) until graft fusion, avoiding direct contact with the graft wound. High ambient humidity was maintained to support establishment by minimising water loss.
6. Root management: new roots tend to grow straight downward. Therefore, to ensure that emerging *Rafflesia* buds develop near the substrate surface, primary roots should be periodically trained and lifted.

In summary, both RSG and RRG are feasible methods for establishing *R. speciosa*-infected *Tetrastigma*. RSG generally yielded quicker callus formation and greater reliability (success rate at 28.6%) (Table 1), while RRG (success rate at 14.3%) (Table 1) was more sensitive to host size and environmental conditions. The use of Orchiata bark as a rooting medium was a critical factor in achieving graft survival. Environmental conditions maintained during transport, propagation, graft establishment and cultivation are summarised in Supplementary Table S1 to facilitate replication of these methods.

Molecular, histological and visual confirmation of *Rafflesia speciosa* infection

The Phire Plant Direct PCR Kit (ThermoFisher, cat. no. F130WH), which enables DNA amplification directly from plant tissue, was used at the PF. Two-millimetre samples were collected with sterile biopsy needles (Miltex, Medex Supply) into 100 µl dilution buffer (included in the Phire Plant Direct PCR kit) with Lysing Matrix A (cat. no. 1169100-CF, MP Biomedicals) and homogenised using a SuperFastPrep™-2 portable bead-beating grinder and lysis system (cat. no. 116012500,

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MP Biomedicals) for 10 s. Sampling was carried out within 5 cm of old growth (as opposed to newly emerged ex situ tissue). One microlitre of the homogenised slurry was used as PCR template, with primers targeting the *matR* region (developed by A. Estopace, unpubl.) and primers designed by J. Molina for the nuclear internal transcribed spacer region. Betaine (1 M final concentration) was added to the master mix to enhance amplification. PCR cycling conditions followed Cheng *et al.* (2016), while visualisation and purification adhered to the protocols of Michel *et al.* (2016). Purified products were sequenced by Genewiz Inc. (South Plainfield, NJ, USA).

For histological studies, 3-mm biopsy cores (Fig. 10) were collected in Formalin Aceto Alcohol (FAA, cat. no. 3210-16, Fisher Scientific) and submitted to VitroVivo Biotech (Rockville, MD), where toluidine blue staining was used in slide preparation. Slides were returned within two weeks and examined using a Panthera C2 microscope (cat. no. BMH4000X, MoticUSA). One sample was also processed for microscopy by E. Bascos, following methods in Bascos *et al.* (2021).

Although no *R. speciosa* buds survived propagation, PCR assays detected *R. speciosa* DNA in five accessions (24-0454B, 23-0090A, 23-0089A, 23-0085A and 2017-0203O), three of which were corroborated by histological evidence, confirming the presence of *Rafflesia* endophytic cells interspersed within host tissues (Fig. 11A–D, Table 1). Endophytic *Rafflesia* cells have pronounced large nuclei (average c. 15 µm) compared with *Tetrastigma* nuclei (average c. 4 µm) (E. Bascos, pers. obs.). Unexpectedly, after lateral root sections were excised from the main roots for PCR/histology sampling, juvenile roots differentiated into stem tissue (Fig. 12A) and regenerated into whole plants after about a month (Fig. 12B), revealing an unanticipated route for asexual propagation of rooted *Tetrastigma* potentially harbouring *Rafflesia* cells. Infected root segments that produce green, photosynthetic shoots could potentially be excised from the parent infected root and replanted as independent propagules. Although there is currently no evidence that *Rafflesia* endophytic cells persist in green tissues, the emergence of photosynthetic shoot tissue from infected woody roots would allow such segments to survive independently after excision, providing a possible strategy for propagating infected host material.

Fig. 10 Biopsy core sample collected for histological analysis. Three-millimetre cores were fixed in Formalin-Acetic Acid-Alcohol (FAA) and submitted to VitroVivo Biotech (Rockville, MD, USA) and to Erika Bascos for microscope slide preparation. Photo: Jeanmaire Molina.

In two cases, PCR assays were positive for *Rafflesia* whereas histological sections did not reveal endophytic cells. This discrepancy likely reflects the highly localised and patchy distribution of *Rafflesia* endophytes within host tissues (Bascos *et al.*, 2021), which reduces the probability of detecting parasitic cells in any single histological section (Molina & Thorogood, 2025). These cases are best interpreted as PCR-positive accessions with histological sampling limitations rather than confirmed absences of infection. Histological identification of *Rafflesia* endophytes within host tissues has been described previously (for example, Bascos *et al.*, 2021; Mursidawati *et al.*, 2019). Here, we

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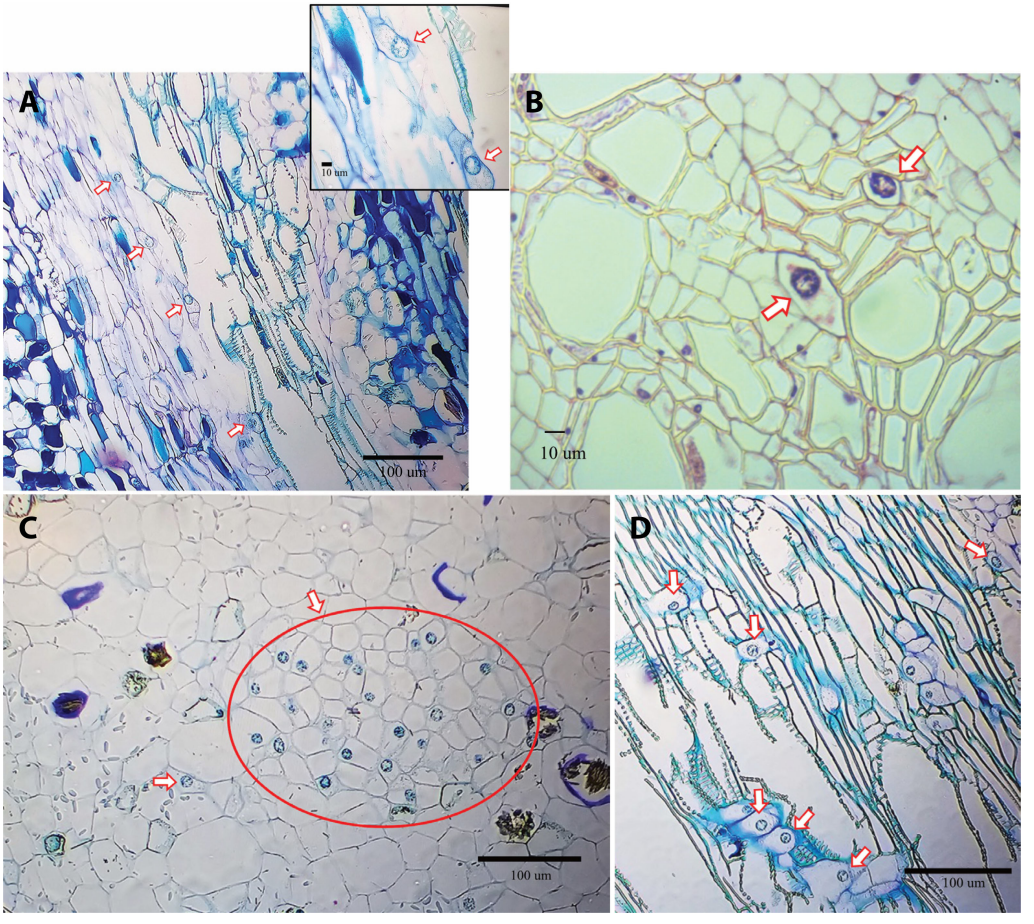

Fig. 11 Micrographs (longitudinal sections) showing *Rafflesia speciosa* endophytic cells (marked with arrows/outline) interspersed in *Tetrastigma* sp. host tissues. **A** Accession 2024-0454B propagated via RSG with toluidine blue staining processed by VitroVivo Biotech (Rockville, MD, USA), at 100x magnification; inset at 400x to show two *Rafflesia* cells up close. **B** Sample from same accession processed by E. Bascos with hematoxylin and eosin staining. Endophytic *Rafflesia* cells have pronounced larger nuclei (average c. 15 um) compared with *Tetrastigma* nuclei (average c. 4 µm). **C** Accession 2017-0203O stem (grafted with infected 2023-Tet5 root scion in Jan. 2023) stained with toluidine blue. A clump of *Rafflesia* cells (red outline) within host parenchyma are evident at 100x magnification. This was sampled ~1 cm from one of the visible *Rafflesia* buds (cf. Fig. 13). **D** Another sample from 2017-0203O stem showing *Rafflesia* cells among host xylem cells, 100x. Photos A, C, D: Jeanmaire Molina; B: Erika Bascos.

extend these approaches by incorporating PCR as a practical and sensitive screening tool for detecting *Rafflesia* DNA in ex situ grafted material prior to bud emergence. For future replication, we recommend a tiered diagnostic framework consisting of initial PCR screening followed by targeted histological sampling of PCR-positive tissues.

In January 2023, a root-to-stem veneer graft was performed on a mature USBG Conservatory accession of *Tetrastigma cf. magnum* (2017-0203*O), derived from a green softwood stem cutting collected in 2017 from a vine that showed no visible *Rafflesia* buds (Molina *et al.*, 2017). Although pre-graft molecular screening was not performed and latent infection cannot be definitively excluded, anatomical studies have not documented *Rafflesia* endophytes in actively growing green shoots, even in species that produce flowers

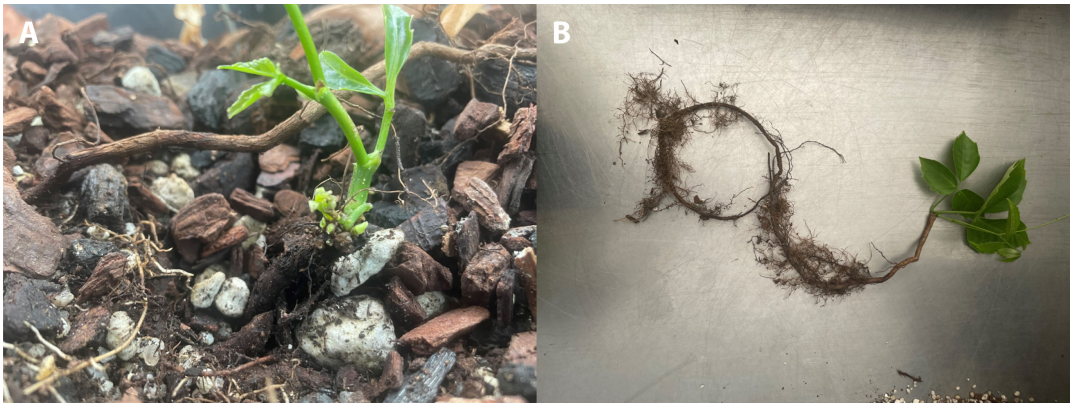

Fig. 12 Adventitious shoot formation from infected *Tetrastigma* roots. **A** Unexpectedly, after lateral root sections were excised from the main roots for PCR/histology sampling, juvenile roots differentiated into stem tissue. **B** This eventually regenerated a whole plant after about a month, revealing an unanticipated route for asexual propagation of rooted *Tetrastigma* potentially harbouring *Rafflesia* cells. Photos: USBG.

from aerial woody stems (E. Bascos, pers. obs; A. Wicaksono, pers. comm). Thus, systemic persistence of endophytes in the young shoot tissues from which this accession was derived is considered unlikely based on current evidence (Bascos *et al.*, 2021).

Callus formation was observed at the graft interface prior to scion senescence, indicating partial cambial fusion and temporary vascular continuity. The infected root scion remained viable for approximately two months before senescing. In late June 2025 (~2.5 years post-grafting), two *Rafflesia* buds (~2 cm diameter, cupule stage) (Susatya, 2000) were observed: one ~5 cm above the graft site and another ~23 cm above (Fig. 13). Similar observations at Bogor Botanic Garden (S. Mursidawati and N. Ngatari, pers. comm.) indicate that infected scions may fuse and later die, yet *Rafflesia* buds can emerge on the rootstock one to two years afterwards, suggesting sustained scion survival is not required once endophytic tissue has crossed the graft interface. The spatial and temporal relationship between grafting and bud emergence strongly supports graft-mediated transmission of endophytic *Rafflesia* cells,

Fig. 13 Root-to-stem veneer graft of *T. magnum* stem accession 2017-0203O (grafted with infected root scion 2023-Tet5) resulting in *Rafflesia* bud emergence. Two *Rafflesia* buds (~2 cm diameter, cupule stage) were observed in June 2025, ~2.5 years after grafting, providing evidence of successful graft-mediated transmission of *Rafflesia* infection. The graft site is indicated by a red arrow in the lower left. Photos: USBG.

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which likely traversed the graft union during the period of scion viability and subsequently persisted within the rootstock prior to bud initiation. We hypothesise that transmission occurred via intercellular spread along the host vascular cambium (Nikolov *et al.*, 2014; Mursidawati *et al.*, 2019), potentially across callus tissue reconnecting the graft interface (Melnyk & Meyerowitz, 2015). While graft-mediated transmission is the most parsimonious explanation, definitive confirmation will require future studies incorporating pre-graft PCR and histological screening of recipient plants.

Although upward movement of endophytic tissue from the graft interface into mature stem tissues is supported by the spatial distribution of buds observed here, available evidence does not support systemic colonisation of newly differentiated juvenile shoots. Histological studies conducted by E. Bascos on green shoots branching from graft-mediated infected vines did not detect *Rafflesia* endophytic cells (unpublished data). In addition, PCR screening of woody stem tissues from the Conservatory *T. cf. magnum* rootstock post-bud emergence (J. Molina, pers. obs.) did not detect *Rafflesia* DNA. These observations suggest that while localised spread within established woody tissues may occur, systemic invasion of actively growing juvenile shoots is unlikely based on current evidence. Further targeted sampling across developmental stages will be necessary to clarify the limits of endophytic movement within host architecture.

Because PF rootstocks are smaller owing to frequent pruning, we hypothesise that the larger, more vigorous Conservatory rootstock, reflected in its larger size and more extensive canopy, may have facilitated bud induction. The *T. cf. magnum* in the Conservatory grows from a ~10.2 m^2 mixed planting bed, at least four times the surface area of the containers at the PF, and extends upward to the canopy walk. It fully covers a trellised 1.1-m rail over a length of 5.4 m with unobstructed southern exposure, and additionally extends into the canopy of a *Ficus lutea* tree as well as along a densely planted north-facing rail approximately 15 m in length. As a result, this plant possesses substantially more than twice the total foliage area of any *Tetrastigma* individual maintained in the PF. Enhanced host vigour may therefore have increased resource availability (for example, carbohydrate or nutrient supply), potentially facilitating endophytic proliferation and bud initiation. Similar relationships between host vigour and parasite growth have been reported in other systems (for example, *Cuscuta chinensis*: Li *et al.*, 2012; *Phelipanche ramosa*: Moreau *et al.*, 2016).

Following bud discovery in late June 2025, subsequent regular measurement and documentation revealed slow growth (approximately 20–30%, corresponding to <1 cm increase over 3–5 months) (Fig. 14A), prior to cracking of the cupule and exposure of the *Rafflesia* bracts (Fig. 14B). Development continued briefly, with gradual expansion of the fissure and increasing bract pigmentation; however, in both cases, visible signs of rot appeared within one month. Following the initial onset of rot, tissue necrosis progressed rapidly throughout the bud (Fig. 14C) over the course of several days. Once failure was evident, each bud was dissected for further analysis (Fig. 14D). Although the buds ultimately failed, PCR and histological evidence suggest persistence of endophytic *Rafflesia* cells within host tissues, but the factors regulating subsequent proliferation and bud initiation remain unknown (Hidayati *et al.*, 2000; Bascos *et al.*, 2021).

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Fig. 14 Bud development over months. **A** Cupule stage, 28 July 2025. **B** Cupule breaking open, revealing *Rafflesia* bract tissue, 7 October 2025. **C** Cupule-bract stage with rot progression into *Rafflesia* tissue (arrow) and bracts darkening, 14 October 2025. **D** Dissection and sampling of bud revealing a mix of rotten and live tissue including bracts. Photos: USBG.

Conclusion

For more than a decade, ex situ cultivation of *Rafflesia* has eluded success – until now. We report the first propagation of the Philippine endemic *R. speciosa* outside Asia, achieved at the USBG. Two complementary methods are now confirmed viable: (1) rooting infected stem or root cuttings and (2) grafting infected roots onto uninfected hosts, which ensures rapid vascular fusion and establishment. This study demonstrates that the grafting technique pioneered by Mursidawati *et al.* (2015) can be replicated for Philippine *Rafflesia*, specifically *R. speciosa,* outside its native range. The same approach has since been applied at the USBG to propagate *R. lagascae* Blanco-infected *T. loheri* collected in July 2025, with histological and PCR analyses planned in a year to confirm the presence of viable parasitic cells. This marks a paradigm shift in conservation horticulture: unlike conventional grafting, which rarely involves root-to-root unions, this approach uniquely relies on infected *Tetrastigma* roots as scions capable of transmitting endophytic tissue across graft interfaces. Thus, in practice, inserting a wedge of infected host tissue – ideally containing an early *Rafflesia* bud – into a matching cut on the rootstock, securing it to prevent desiccation and allowing the 'wedge-transplant' to heal may be enough for the parasite to establish.

The emergence of *Rafflesia* buds under controlled glasshouse conditions represents a significant advance in ex situ cultivation. The most parsimonious explanation is that endophytic cells traversed the graft union during scion viability and persisted within the rootstock to initiate bud development, indicating that grafting can maintain host–parasite continuity beyond the lifespan of the original scion. Although both buds aborted 5–6 months after discovery, the causes of failure remain unclear and warrant investigation into host physiology, disease pressures and environmental cues regulating bud induction. Continued progress will require coordinated in situ–ex situ collaboration, pre-graft diagnostic screening of recipient plants, long-term monitoring of graft unions (including interspecific combinations) and replicated trials across institutions to optimise transmission and sustain long-term *Rafflesia* establishment.

Through careful field collection, strong partnerships between local experts and international botanical institutions, innovative horticultural techniques, and rigorous molecular and histological verification, we have established a reliable pathway to sustain *Rafflesia* in cultivation across continents. This demonstrates that even the world's most cryptic floral parasite can be cultivated, marking a turning point in conservation

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horticulture: after years of trials, practical methods now exist to safeguard these botanical icons ex situ and extend their survival beyond vanishing forests.

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