



Beginnings of a plant parasite: early development of *Rafflesia consueloae* inside its *Tetrastigma* host

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Abstract

Main Conclusion Extensive histology of host organs revealed the early events in the vegetative growth of *Rafflesia consueloae* including initial infection site, endophyte distribution, and other developmental events prior to bud emergence.

Abstract The early events in the vegetative development of the holoparasite *Rafflesia* have long remained a mystery. Because its entire vegetative growth occurs within the host body, very little is known about the developmental events prior to emergence of the floral shoot. The goal of this study was to describe the events that occur during the vegetative growth of *R. consueloae*, particularly in the early stages of infection. We performed extensive microtome sectioning of multiple root and stem segments from different *Tetrastigma* host individuals to examine the cytology, distribution, and development of the *R. consueloae* endophyte within the host tissues. We found that *R. consueloae* infection is restricted to the roots of its host. Infection begins within the vascular cambium where the endophyte appears to initially reside prior to their radial spread to the vascular tissues. The tissues obtained from different host individuals had varying degrees of infection alluding to a possible role of host resistance mechanisms and/or varying levels of parasite infectiousness. Endophyte presence in host vines without external manifestations of infection indicates that the parasite may dwell within the host tissues for prolonged periods as small cell clusters without transitioning to the reproductive stage. Furthermore, we found that floral shoots may develop in scarcely infected host tissues indicating that extensive endophyte growth within the host is not a prerequisite to the onset of reproductive development. Overall, our study describes for the first time the developmental events prior to emergence of *R. consueloae* buds from its host.

Keywords Endophyte · Vegetative development · Holoparasitism · Parasite histology · Parasite anatomy

Introduction

The holoparasitic genus *Rafflesia*, known for its putrid, enormous, fleshy flowers, exhibits an extraordinary vegetative development. As all the members lack stems, leaves

and roots, the vegetative body is only composed of endophytic strands embedded in the tissues of the host plant, *Tetrastigma* sp. (Vitaceae) (Nais and Wilcock 1998). In contrast to other angiosperm holoparasites, *Rafflesia* does not develop an external vegetative shoot attached to the host at any stage of its development. Its entire vegetative growth occurs within the host where the endophytic cells spread invasively in the host tissues and eventually lose contact with the original penetration site (Kuijt 1969; Heide-Jørgensen 2008). The parasite only emerges from within the host as a flower bud; thus, only its reproductive growth can be observed by the naked eye.

The anatomy of the *Rafflesia* endophyte has been characterized previously through transverse sectioning of infected *Tetrastigma* roots (Schaar 1898; Brown 1912; Nikolov et al. 2014). The root sections revealed that the parasite initially exists as filaments composed

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of slowly dividing, undifferentiated cells that may likely arise directly from the seed proembryo. Though they are well-integrated into the root structure of the host, the cells of the endophyte can easily be distinguished from the host cells due to the substantial difference in their nuclear size: the nuclei of the parasite cells are two-fold larger than the nuclei of the host cells. Histological techniques that can distinctly stain the nuclei of endophytic cells would therefore be useful not only in characterizing the vegetative development of different species of *Rafflesia*, but also in identifying vine hosts in the early stages of infection.

How the seeds of *Rafflesia* germinate and how the parasite cells enter the host tissues are still unknown. Wicaksono et al. (2021) speculated that the seeds must reach a specific area in the host where a presently unidentified germination stimulant is produced or concentrated. Candidate tissues for the germination site include the vascular cambium (Mursidawati et al. 2019) and the phloem (Molina et al. 2017). It has been speculated that the *Rafflesia* endophyte initially infects the host vascular cambium where it divides together with the cambial cells causing its lateral spread towards the xylem and phloem regions of the infected root (Brown 1912; Mursidawati et al. 2019). Recently, Wicaksono et al. (2021) presented several possibilities regarding the developmental progression of the *Rafflesia* endophyte. One is that, from the vascular cambium, the endophyte may form uniseriate strands (Nikolov et al. 2014), which develop into the incipient floral shoot called protocorm. Another is that the endophyte initially develops as clumps of cells (Mursidawati et al. 2019), which then form the protocorms. Lastly, that the endophyte may initially form uniseriate strands, which develop into cell clumps, and later give rise to the protocorm stages. Presently, there are no empirical studies that can support or invalidate these hypotheses. Our study aims to address this gap of knowledge.

In this paper, we describe for the first time the vegetative development of the smallest *Rafflesia* in the world, *R. consueloae*. Through histology, we were able to characterize the initial stages of parasite development within the tissues of the host up until the early development of the floral shoot. We also describe the anatomy of the root and stem of the *Tetrastigma* host of *R. consueloae* and the extent of infection in the host organs. Moreover, extensive sectioning of multiple root and stem regions from different host individuals allowed us to explore the manner of distribution of the parasite cells within the host and describe how the endophyte grows and spreads within the host. While the exact mechanism of parasite entry into the host still remains unknown, our study provides a glimpse of the events during the early stages of parasite infection.

Materials and methods

Study species and study site

In Pantabangan, Nueva Ecija, Philippines, several populations of *R. consueloae*, grow on *Tetrastigma* vines (~26) in Mt. Balukbok and Mt. Pantaburon (Galindon et al. 2016). Various developmental stages of the flower are found on woody adventitious roots of the host vines, which grow profusely on the forest floor. In the surveyed areas, host vines with and without external signs of parasite growth are present. Infected *Tetrastigma* vines are recognized based on the presence of buds, flowers in bloom, fruits, or scars from past flowering events.

Collection of plant material

Root and stem segments were collected from *Tetrastigma* sp. vines in Mt. Balukbok, Pantabangan, Nueva Ecija (Wildlife Gratuitous Permit No. III-2019-19). Root segments were obtained from eight *Tetrastigma* vines with external manifestation of *Rafflesia* infection (*i.e.*, presence of buds, fruits, or scars from past infection) (Fig. 1a–f) and eight vines without any sign of infection. Stem segments (Fig. 1g), on the other hand, were obtained from five host vines with observable root infection and five vines without externally visible signs of root infection. We limited our sampling on *Tetrastigma* individuals that have already undergone secondary growth because only woody vines have been previously observed to be infected with *R. consueloae* in our study sites. From all the roots and stems sampled, three 10 cm segments were obtained at 1 m intervals, covering a length of 3 m. This length is based on previously documented mean minimum spatial extent of infection (~3 m) based on flower growth observation in *R. cantleyi* populations (Barkman et al. 2017). The diameters of the collected roots ranged from 0.517 to 1.38 cm, while the width (thickest portion) of the sampled stems ranged from 0.8 to 1.24 cm. All collected root and stem segments were cut into smaller pieces and preserved in formalin acetic acid (FAA). It is worth noting that the collection method used caused no significant harm on the host vines (*i.e.*, all vines sampled from are still alive).

Histology of *Tetrastigma* roots and stems

FAA-fixed root and stem segments were processed in Leica ASP 6025, an automated tissue processor with vacuum and pressure application in an enclosed retort chamber. Additional fixation using 10% Neutral-Buffered formalin was followed by dehydration using ascending-grade ethanol starting with 80%, two changes of 95%, followed by two changes

Fig. 1 Macroscopic images of *Rafflesia consueloae* and its *Tetrastigma* host. **a–b** Woody adventitious roots of *Tetrastigma* bearing *R. consueloae* buds. **c** A fully blooming *R. consueloae* flower attached to the host root. **d–e** Infected *Tetrastigma* roots with newly emerged *R. consueloae* bud (green arrowhead) and scars from past flowering events (yellow arrowheads). **f** Roots collected from host vines with (right) and without (left) externally visible signs of infection. Green arrowhead indicates bud growth. **g** Aerial stem (purple arrowhead) of an infected *Tetrastigma* plant



of 100%. This was followed by three changes of xylene as clearing agent and three changes of paraffin wax at 60 °C for impregnation. These were done in a 17-h overnight cycle program. On the following day, the specimens were embedded in a base mold filled with fresh molten paraffin using Leica Acadia embedding center. All blocks were cooled in cold plate and, after solidification, blocks were trimmed in Leica RM 2235 rotary microtome and cut into 3–4 μm thick sections.

Sections were placed in a 40 °C water bath and then stained using Sakura Prisma automated Hematoxylin and Eosin stainer. Staining process was done following manufacturer’s instructions with some modifications. Process involved warming of sections for 45 min at 70 °C, followed by two changes of xylene for deparaffinization, descending-grade ethanol starting at 100% down to 70% for rehydration. Sections were washed with tap water followed by staining with hematoxylin. Sections were then immersed in 1%

acid + 70% ethanol, blued in ammonia water, and stained with 1% Eosin Y. This was followed by another series of dehydration steps using ascending-grade ethanol starting with 80% ethanol, two changes to 95% ethanol, two changes to 100% ethanol and then two changes to xylene as clearing agent. Stained slides were mounted with a 24 × 24 mm cover glass using a resinous mounting medium. Slides were observed using a light microscope and photographed using Dino-eye camera. The endophyte cells were characterized based on their cell wall, protoplast contents, nuclear size, and distribution in the host tissues. Different stages were photographed and described. We computed percent infection of either the stem or the root by dividing the number of infected root (or stem) microtome sections by the total number of root (or stem) microtome sections, multiplied by 100. We have included the standard error (SE) as the error of the means of the variables measured or computed.

Results

Anatomy of host organs

In this study, we examined a total of 1278 root and 684 stem transverse microtome sections obtained from *Tetrastigma* vines with and without externally visible signs of *R. consueloae* infection. We found that root sections were more infected (Mean ± SE: 61.1 + 11.4%) by the *R. consueloae* endophyte than the stem sections (0%). Of the 684 stem transverse sections (327 from vines with visible root infection and 357 from vines without external signs of root infection) that were examined, none contained *R. consueloae* endophytic cells. We also found that root internal infection does not always manifest externally. Of all root sections examined from plants that had no externally visible signs of infection, about a fifth were still internally infected by the endophyte of *R. consueloae* (Mean ± SE: 22.15 ± 10.9%). On the other hand, all sections from externally infected roots had internal evidence of infection.

Stems from infected and uninfected *Tetrastigma* individuals exhibited similar anatomy (Fig. 2). Secondary vascular tissues flank the central pith on two sides (Fig. 2a, c). The secondary phloem region is composed of sieve elements and parenchyma cells alternating with fiber bands (Fig. 2d). Presence of lignified cells in this region gives the phloem a stratified appearance. Wide vessels were found in the secondary xylem region (Fig. 2a, e). Unlignified parenchyma cells were also seen within the secondary xylem (Fig. 2f). The cambial zone (5 to 7 cell thick) forms distinct layers between the xylem and phloem regions (Fig. 2f). Perivascular fibers are found external to the vascular tissues (Fig. 2a). The cork and cork cambium layers, as well as lenticels,

are easily distinguishable from within the periderm layer (Fig. 2b).

Tetrastigma root anatomy is also composed of the periderm, cortex, secondary phloem, and wood (Fig. 3a, c). As in the stem, the phloem region is composed of sieve elements alternating with fibers (Fig. 3b) and the xylem exhibits vessel dimorphism (Fig. 3c). The cambial zone (with 5 to 8 rows of cells) divides the xylem and phloem regions (Fig. 3d). Clusters of raphide crystals (Fig. 3e) are found in the cortex and rays of the root. Druse crystals (Fig. 3f) are found within the phloem and cortical regions. The xylem rays are 5 to 12 cells wide and the cells contain numerous starch grains (Fig. 3g).

Endophyte morphology and distribution

We found that the endophyte cells of *R. consueloae* are easily distinguishable from the host cells due to their larger nuclear size (Fig. 4a–d). The ovoid nuclei of *R. consueloae* endophyte cells have an average diameter of 14.85 μm (s.d.: ± 1.76 μm, *n* = 50), while the average nuclear size of the host cells is 4.89 μm (s.d.: ± 0.61 μm, *n* = 50). We also observed that the endophyte nuclei, in general, are centrally located while the nuclei of the host cells are found nearer the periphery (Fig. 4a). The walls of endophyte cells were found to be uniformly thin. In the transverse sections, the endophyte cells also have a remarkably different shape (more elongated or fusiform) compared to the cells in the various tissues where they are found.

We found that roots as thin as 0.5 cm (diameter) contained endophytic cells. In the transverse sections, presence of the endophyte was predominantly observed in the secondary phloem, vascular cambium, and secondary xylem regions (Fig. 4). The endophytic strands were absent in the periderm, cortex, perivascular fibers, expanded phloem ray, and were rarely found within the xylem rays. The endophyte was predominantly observed as rows of single cells oriented radially (Fig. 4e–i, m–s) in the transverse sections, while some sections showed the endophyte as single, isolated cells within the parenchymatous phloem and lignified xylem tissues (Fig. 4a, j). In extensively infected roots (Fig. 4s), we observed that more endophytic strands were found in the xylem compared to the cambial and phloem regions. Though the cells of the uniseriate endophyte are more or less elongated, the endophyte cells found within the xylem regions are longer and thinner than those found in the cambial and phloem zones (Fig. 4). Within the secondary xylem, the endophyte was able to grow around vessel elements, positioning itself in between the lignified cells of the xylem (Fig. 4k–o). Longer uniseriate endophytic strands in the phloem mostly traversed the vascular cambium (Fig. 4g–i), while those found within the xylem often occurred as isolated, linear cell clusters (Fig. 4k–o).

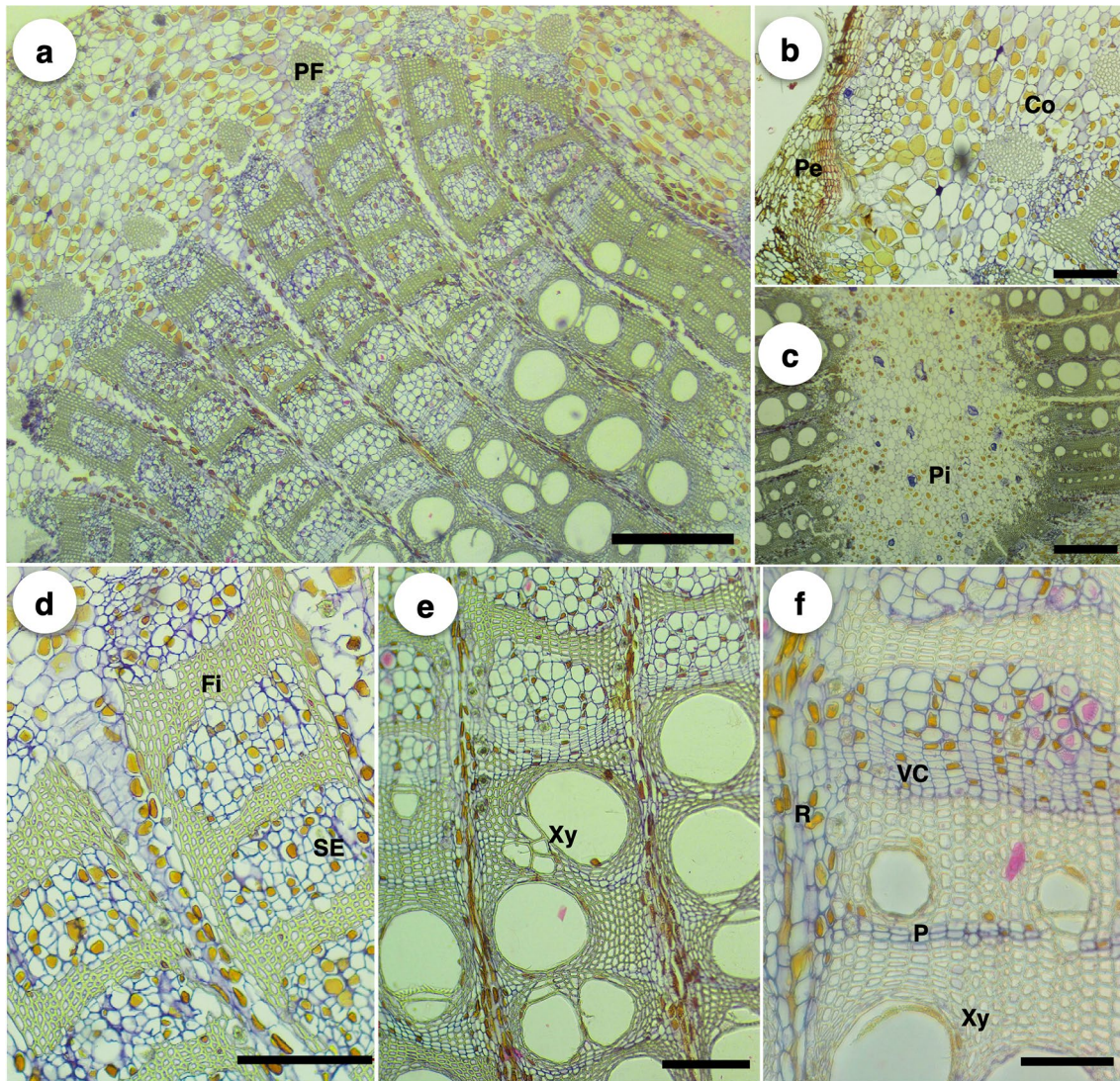


Fig. 2 Micrographs showing stem tissues of the *Tetrastigma* host of *R. consueloae* as seen in transverse sections. **a** Vascular tissues with perivascular fibers (PF) found exterior to the phloem. **b** Periderm (Pe) and cortex (Co). **c** Central pith (Pi). **d** Phloem sieve elements (SE)

and fibers (Fi). **e** Vessel dimorphism in xylem (Xy). **f** Vascular cambium (VC) and parenchyma (P) in xylem (Xy), and ray tissue (R). Scale bars: a, c = 500 μ m; b, e = 200 μ m; d, f = 100 μ m

The root tissue sections that we examined had varying degrees of internal infection. Under the microscope, it was easy to distinguish an uninfected root from an extensively infected one (Fig. 5a–b). Roots of *Tetrastigma* vines without external evidence of *R. consueloae* infection were found to house mostly the single-celled or short, uniseriate endophyte located within the cambial zone (Fig. 5c–e). In these sections, the xylem and phloem areas did not contain endophyte cells which were in stark contrast to the heavily infected roots regions (Fig. 4). Surprisingly, many of the root sections that contained protocorms or buds in the very early stages of development also exhibited the same

endophyte distribution—the parasite cells were confined in or near the vascular cambium (Fig. 5f–g).

Most multiseriate endophytic clusters were observed to occur together with uniseriate endophyte strands in the same root sections though the multiseriate stages were far fewer than the uniseriate strands (Fig. 6). The larger, multiseriate endophyte was mostly found in the xylem region (Fig. 6b–c) with majority of the cell clusters forming within the lignified xylem tissue and pushing their way through the cambium and phloem regions in the more advanced stages. The multiseriate endophyte was also observed in the phloem (Fig. 6a) and near the cambium, but those that were able to develop into the more

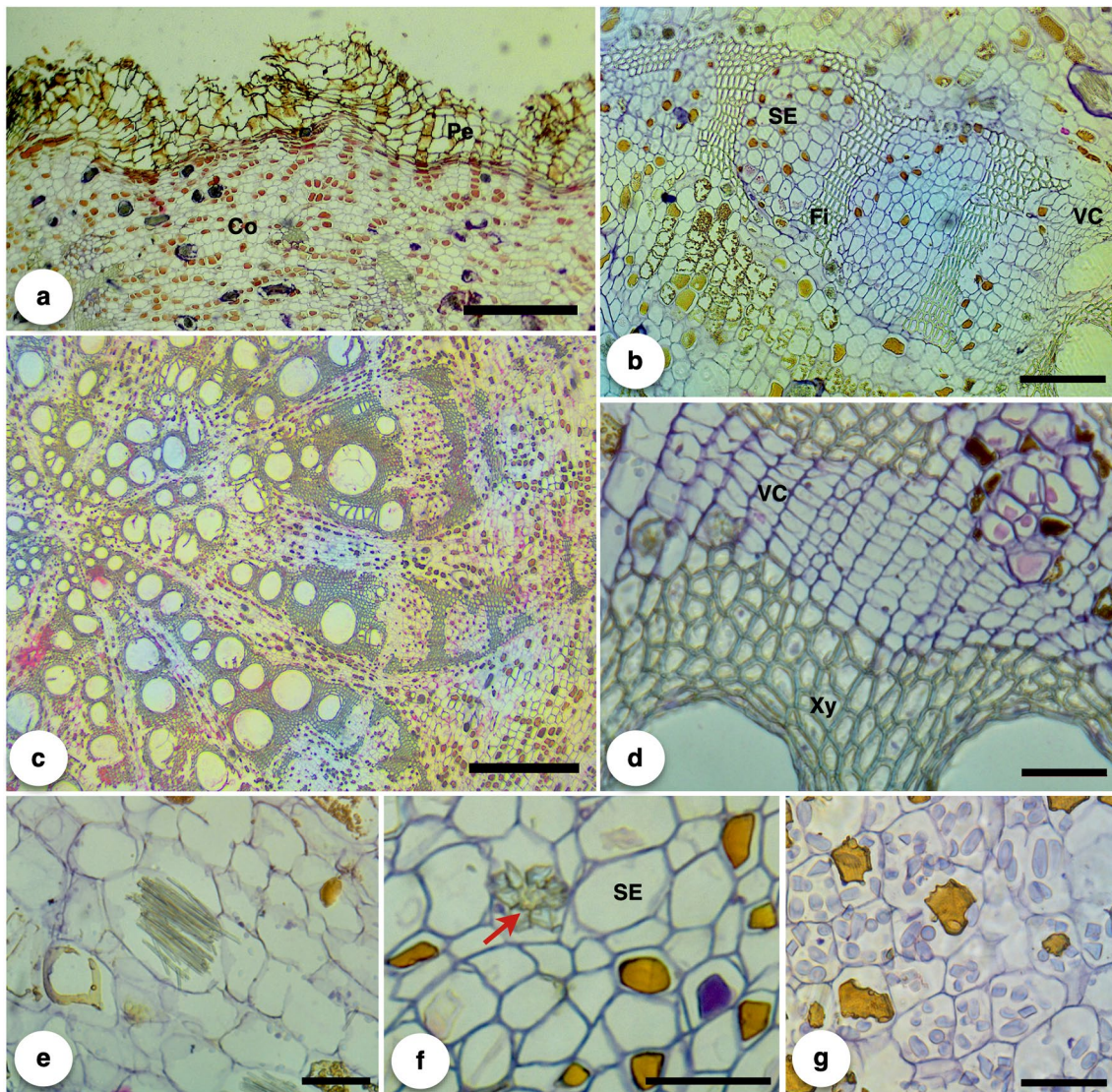


Fig. 3 Micrographs showing root tissues of the *Tetragium* host of *R. consueloae* as seen in transverse sections. **a** Periderm (Pe) and cortical parenchyma (Co). **b** Secondary phloem with lignified phloem fibers (Fi) alternating with sieve elements and companion cells (SE) found exterior to the vascular cambium (VC). **c** Vascular

tissues (woody cylinder and secondary phloem) and prominent rays. **d** Vascular cambium (VC) found exterior to the xylem (Xy). **e** Cortex showing raphide crystals inside idioblast. **f** Phloem parenchyma tissues (SE) showing a druse-containing cell. **g** Starch grains in cells of rays. Scale bars: a, c = 500 μ m; b = 200 μ m; d–g = 50 μ m

advanced protocorm stage were the ones that had grown initially within or near xylem tissues (Fig. 6d, f). In very rare instances, the endophyte cluster grows towards the ray, but it still remains in direct contact with the xylem tracheary elements (Fig. 6e).

Early development of the floral shoot in the host root

We found that presence of developing protocorms and/or floral shoots is widespread in roots with external signs

of infection (Fig. 7). The basal region of the developing floral shoot is often in direct contact with xylem tissue (Fig. 7b). The cells near the base of the protocorm are more elongated than the cells in the shoot apex and contact zone indicating that cell elongation occurs acropetally (Fig. 7b–c). In contrast to the cells of the early endophyte stages, we observed the cells of the protocorm to divide more rapidly as evidenced by the presence of cells in various stages of mitosis (Fig. 7f). At the surface of the floral shoot apex, cells were seen to divide both anticlinally and periclinally (Fig. 7g). Several layers of flattened cells which have already separated from the apex

of the protocorm during floral shoot development are also visible in the sections (Fig. 7d–e).

Discussion

Overview of *Rafflesia consueloae* endophyte development

The vegetative growth of genus *Rafflesia* has remained one of the most unexplored aspects of its development. Breakthroughs in uncovering the exact mechanism of the parasite cells' entry into the internal tissues of the host have been greatly impeded by the lack of success of in vitro seed germination experiments and difficulty of conducting in vivo time-lapse observation of the initial stages of infection. The early events of endophyte growth and floral shoot development, on the other hand, may be observed through histology of host tissues. In this study, we performed extensive sectioning of the host organs of *R. consueloae* in an attempt to reconstruct the events that occur during its vegetative growth and examine the extent of infection within the host tissues.

Similar to previous observations of the vegetative structure of *Rafflesia* (Schaar 1898; Brown 1912; Nikolov et al. 2014), we found that the nuclei of the endophyte cells are significantly larger than the nuclei of the host cells. The use of the nuclear stain, hematoxylin, and the cytoplasmic stain, eosin allowed easy identification of the endophyte cells as their nuclei appear dark and distinct in the root sections and are roughly three times the size of the nuclei of the host (Fig. 4). It was previously speculated that the larger nuclei sizes in parasitic plants could be a passive consequence of having access to unlimited host resources or a result of host–parasite horizontal gene transfer (Nikolov et al. 2014). Endoreduplication may also occur during the endophyte stage. Occurrence of endoreduplication has been reported in parasitic *Cuscuta* plants (McNeal et al. 2007; Narukawa et al. 2021). For *Rafflesia*, there is a need to perform a thorough karyotype analysis across the genus to confirm if polyploidy is taking place during the early phase of development. Another possible explanation could be related to the loss of housekeeping genes from the plastid genomes and their subsequent transfer to the nuclear genome where they become nonfunctional, as recently discovered in the plant holoparasite *Aeginetia indica* (Chen et al. 2020). Whole genome analyses of *Rafflesia* plants would be useful in confirming this.

In contrast to the report of Brown (1912) wherein the uniseriate endophyte of *R. manillana* was found to be equally distributed in the rays and among the tracheary elements of the xylem, the endophyte of *R. consueloae* was mostly absent from rays (Fig. 5). Serial transverse

sections of the host roots showed that the endophyte is initially growing radially within the vascular tissues (Fig. 4) and we found no evidence of interconnections among the endophytic strands along the length of the root. In the early stages, it appears that the endophyte cells are dividing only in one direction (*i.e.*, periclinally in the root transverse sections) resulting in a radially oriented, filamentous endophyte. Divisions in other planes then occur forming the multiseriate and protocorm stages. Our observations are consistent with the proposed developmental progression of the endophyte by Wicaksono et al. (2021) wherein the endophyte initially forms uniseriate strands which later develop into cell clumps and then transition into the protocorm stage. The protocorm stages were observed to develop within or near the xylem region which is similar to microscopic observations of Mursidawati et al. (2019) wherein growth of the protocorm was seen to originate in the host xylem and then expand towards the exterior tissues.

R. consueloae infection is restricted to roots

Rafflesia plants commonly grow on roots of its host plant but a number of species are known to grow on both stems and roots of their vine hosts. Among the Philippine *Rafflesia* species, *R. leonardi*, *R. lobata*, *R. lagascae*, and *R. speciosa*, have been reported to infect the stems of their hosts (Teschmacher 1842; Barcelona and Fernando 2002; Galang and Madulid 2006; Barcelona et al. 2008; Pelser et al. 2013). These reports are based on visual evidence of flower growth on the stems. Although presence of *R. consueloae* buds was not observed in the stems of its host, we included transverse stem sections in our study to look into the possibility that endophyte growth may reach the host's stem even without external manifestation of infection.

The stem of the host of *R. consueloae* shared similar anatomical characteristics with stems of other species of *Tetrastigma* with the exception of successive cambia production (Pace et al. 2018; Mursidawati et al. 2021), which we did not observe in any of the sections. Anatomical features of the host root are also consistent with findings of previous histological studies on *Tetrastigma* (Brown 1912; Nikolov et al. 2014; Mursidawati et al. 2021). We found no evidence of infection in all the sectioned stem segments, including those that were closest to the root. All tissue regions (from the periderm to the central pith) in the collected stem segments did not contain *R. consueloae* endophyte cells (Fig. 2). Based on these findings, we have reason to believe that host stems are rarely, if at all, infected by *R. consueloae*. However, increased sampling is needed to verify this result.

During the process of preparing stem histological sections, we noticed that the woody stems of the *Tetrastigma* host of *R. consueloae* were significantly harder to cut by

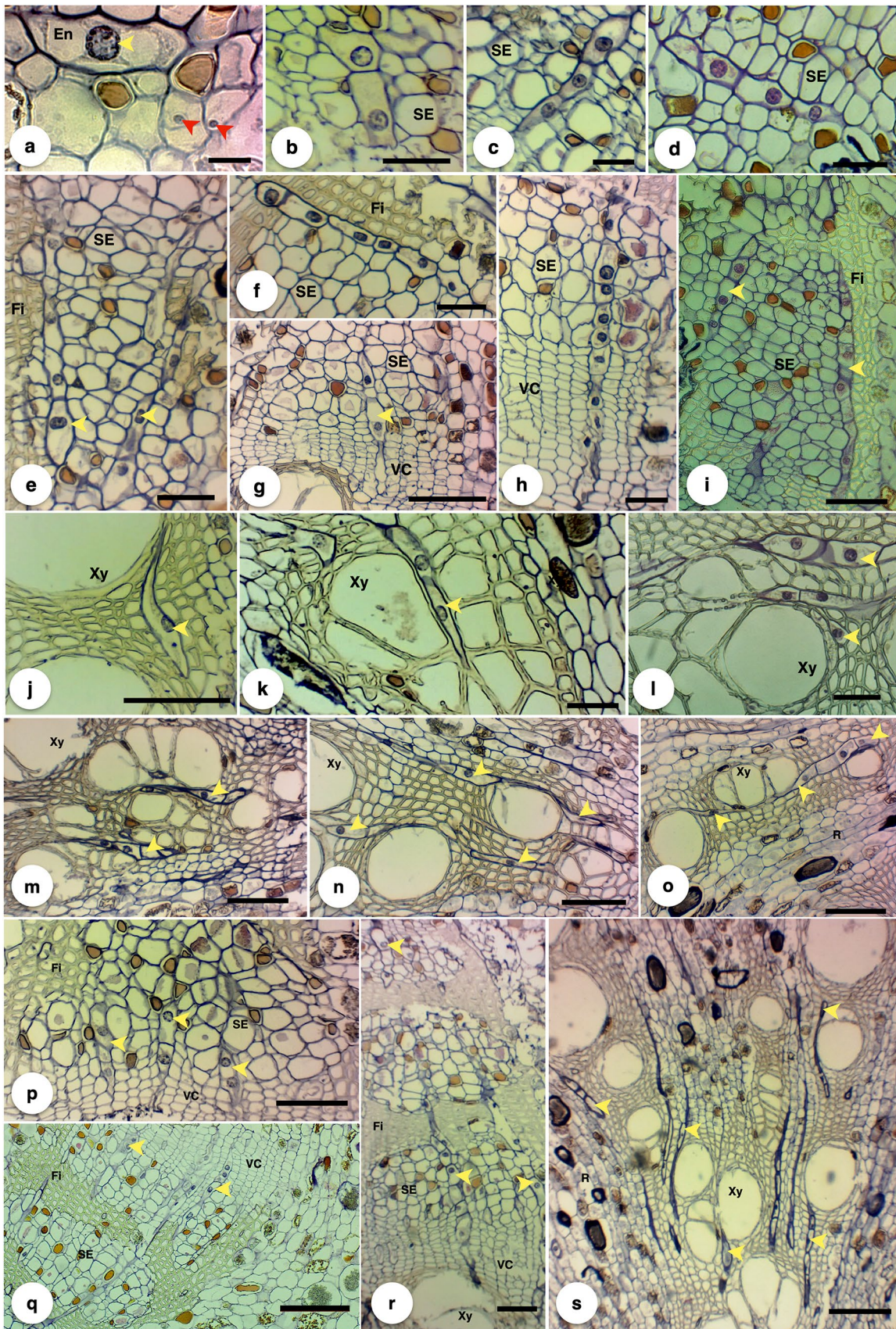


Fig. 4 Micrographs showing single-celled and uniseriate *R. consue-loae* endophyte in *Tetrastigma* host root transverse sections. **a** Size comparison between nucleus (yellow arrowhead) of a single endophyte cell (En) and nuclei (red arrowheads) of host phloem parenchyma cells. **b–d** Endophytic strands with two, three, and four cells growing among host phloem sieve elements (SE). **e–f** Endophytic strands within the sieve elements of the phloem growing in contact with or traversing lignified phloem fibers (Fi) (yellow arrowheads indicate endophyte cells or strands). **g–i** Uniseriate endophyte within the phloem traversing the vascular cambium (VC). **j** A single, elongated endophyte cell growing within xylem tissue (Xy). **k–o** Endophytic strands showing intrusive growth between vessels of the xylem. Endophyte growth was rarely observed in the ray regions (R). **p–q** Endophyte cells growing within and near the vascular cambium. **r** Secondary phloem tissue of the host showing intrusive growth of the endophyte through the fibers. **s** Root xylem tissue showing multiple endophytic strands growing intrusively among vessels and tracheids. Scale bars: a=20 µm; b–f, h, j–m=50 µm; g, i, n–q, s=100 µm; r, t=200 µm

hand and to section compared with the roots. Both the creeping stems and aerial stems had the same relative tissue hardness. On the other hand, the tissues of the woody roots were easily cut and sectioned. The root bark can also be cut using a scalpel without difficulty and can easily be peeled off by hand unlike the bark of the stem, which is firmly attached to the underlying tissues. The hardness of the organ may be a contributing factor as to why *R. consue-loae* infection does not reach the stem—the proembryo from the seed and/or the cells of the endophyte may not be able to penetrate the hard stem tissues (*i.e.*, periderm) of the host vine during the initial stages of infection. Anatomically, we observed that the stem vascular tissues of *Tetrastigma* are densely packed, with the secondary xylem and phloem closely stacked to each other (Fig. 2). In comparison, the *Tetrastigma* root vascular tissues are more loosely stacked (Fig. 3). These features are consistent with findings of previous anatomical studies on *Tetrastigma* (Nikolov et al. 2014; Pace et al. 2018; Mursidawati et al. 2021). With the loose vasculature arrangement in the root of *Tetrastigma*, it is possible that this condition supports more space for the *Rafflesia* endophyte to grow compared to the condition in the stem.

Another likely explanation is that *R. consue-loae* seed germination may require the presence of specific signals that are only produced in the host roots. Parasitic *Striga* plants, for example, require host-generated germination stimulants in the form of strigolactones (Lumba et al. 2017; Runo and Kuria 2018). In the absence of these germination signals, the seeds of *Striga* remain dormant for very long periods (Atera and Itoh 2011). Similar root-derived signals from hosts have been identified as germination stimulants of other root-parasitic plants, such as *Orobanch* spp., *Phelipanche* spp., and *Alectra* spp. (Yokota et al. 1998; Xie et al. 2009, 2010; Flematti et al. 2016). It is highly possible that *R. consue-loae* seeds require a similar germination inducing signal that can only be derived from specific tissues (*i.e.*,

root tissues) of the host. For *Rafflesia* species that can parasitize stems, these signals could be present in both the root and stem of the host. The lack of success of in vitro seed germination and plant tissue culture experiments on *Rafflesia* (Nais 2001; Mursidawati and Handini 2009; Sukanto and Mujiono 2010; Mursidawati et al. 2014; Wicaksono et al. 2016, 2021; Molina et al. 2017) could be due to the absence of such host-derived signals needed for the development of the parasite. Of note, no seed germination was observed after the addition of various plant growth regulators, host bark extracts, peptone, and host exudates to the medium in in vitro experiments (Mursidawati et al. 2014; Molina et al. 2017; Wicaksono et al. 2021) suggesting that a different or novel kind of signal may be required. These observations, along with reports that all successful *Rafflesia* propagation experiments involved inoculation of live hosts (Nais et al. 2015; Wicaksono et al. 2016; Molina et al. 2017), could imply that the still unknown factor required for *Rafflesia* seed germination may only be present in living, woody *Tetrastigma* vines.

Microtomy and histology can detect presence of the endophyte in hosts without external signs of infection

Our study allowed detection of the *R. consue-loae* endophyte in *Tetrastigma* vines without any external manifestation of infection. On the outside, the roots of these seemingly uninfected host plants were completely devoid of buds or scars. However, several root segments from these plants were found to contain endophytic cells in their tissues. Notably, we did not find any developing protocorms in root sections obtained from vines without external *Rafflesia* growth. The said roots were found to exhibit one of the following patterns of endophyte distribution: (1) single-celled or short, uniseriate endophyte solely found in the cambium area, or (2) numerous uniseriate endophyte scattered within the vascular tissues, or (3) a combination of numerous uniseriate and few small, multiseriate endophyte clusters within the vascular tissues. This suggests that the *R. consue-loae* endophyte could exist within the host root tissue as small, slowly dividing groups of cells without immediately transitioning into the reproductive protocorm stage. For the internally infected vines we encountered in this study, it was determined through further observations that bud growth had ensued in some of them 4–5 months after the histology studies were performed. Nonetheless, some of these vines remained free from external *Rafflesia* growth after half a year. Our study provides the first evidence that *R. consue-loae* may remain in its vegetative form for more than half a year before reproductive development ensues. It is possible that this pre-reproductive period may last longer as

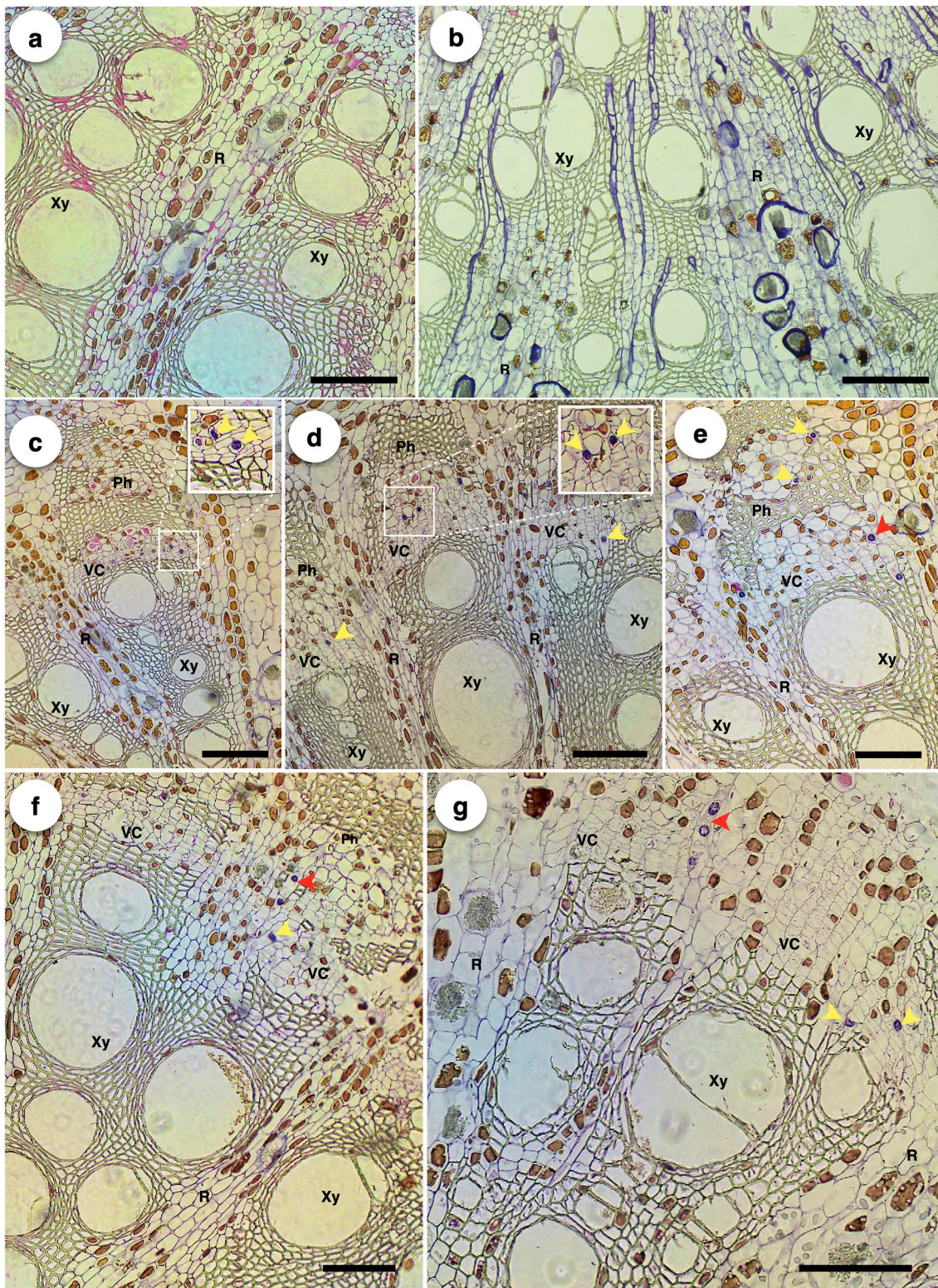


Fig. 5 Varying degrees of *R. consue-loae* infection observed in transverse sections of the host root. **a** Root tissue completely devoid of endophyte cells. **b** Intrusive, widespread growth of the endophyte in the root xylem (Xy). **c–e** Single-celled endophyte (yellow arrowheads) within the vascular cambium (VC) and phloem (Ph), and uniseriate endophyte (red arrowheads) growing towards the phloem of *Tetragymna* roots without externally visible signs of *R. consue-*

loae infection. The endophyte was noticeably absent from the xylem. **f–g** Single-celled endophyte (yellow arrowheads) within the vascular cambium and short uniseriate endophyte (red arrowheads) growing towards the phloem found several micrometers away from host root regions containing protocorms. The xylem and ray (R) regions are completely devoid of endophyte cells. Scale bars: a–b, d–f=200 μm ; c=300 μm ; g=100 μm

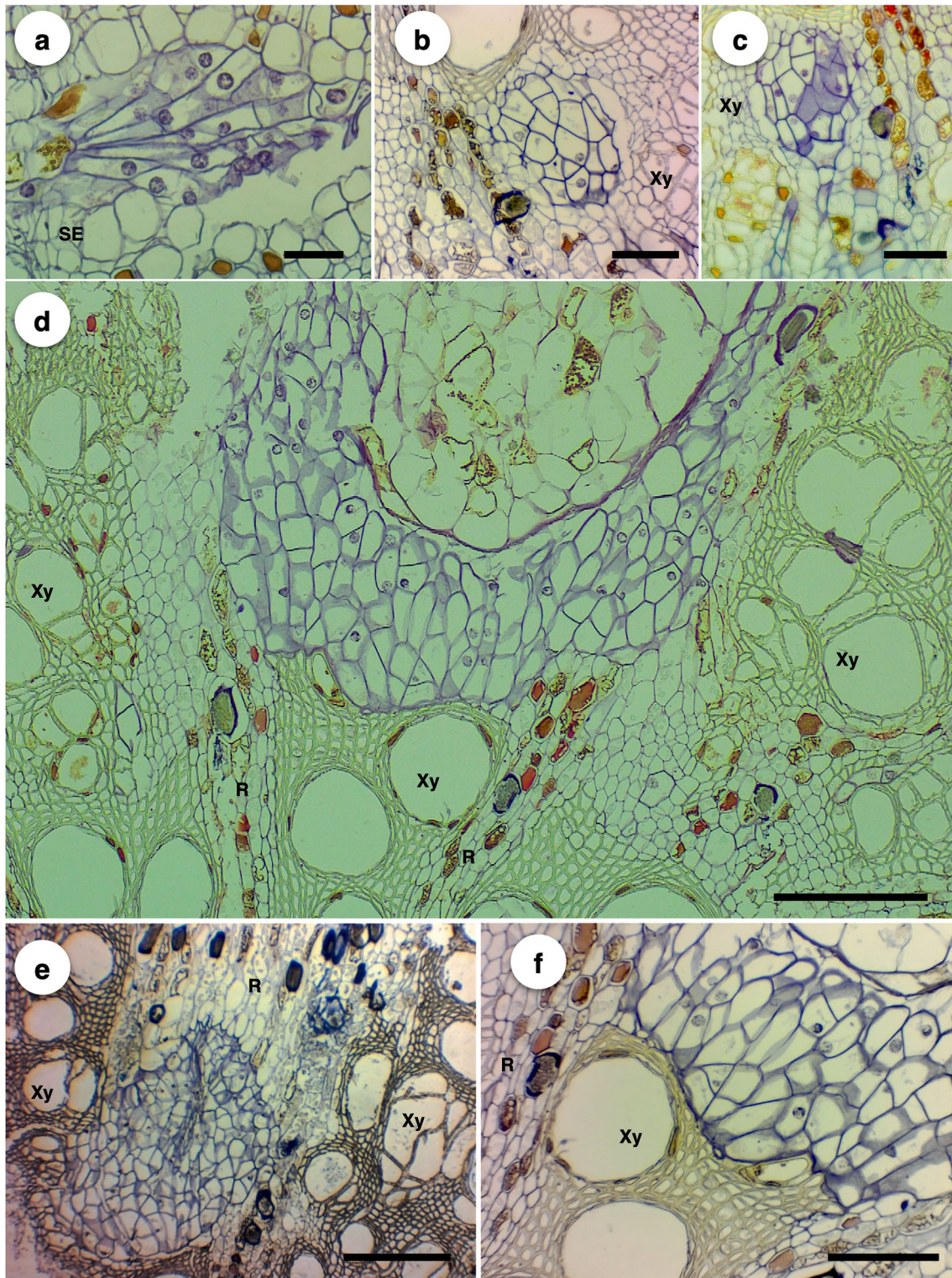


Fig. 6 Multiseriate *R. consueloae* endophyte in transverse sections of *Tetragium* roots. **a** A multiseriate endophyte found among the sieve elements (SE) of the phloem. **b–c** Oval- and spatulate-shaped endophyte growing within the xylem (Xy). **d** A larger endophyte occu-

pying a huge portion of the host xylem. **e** A globular endophyte in the ray (R) region. **f** A close up of the cells from a large endophyte growing within xylem tissues. Scale bars: a=50 µm; b–c, f=100 µm; d=200 µm; e=300 µm

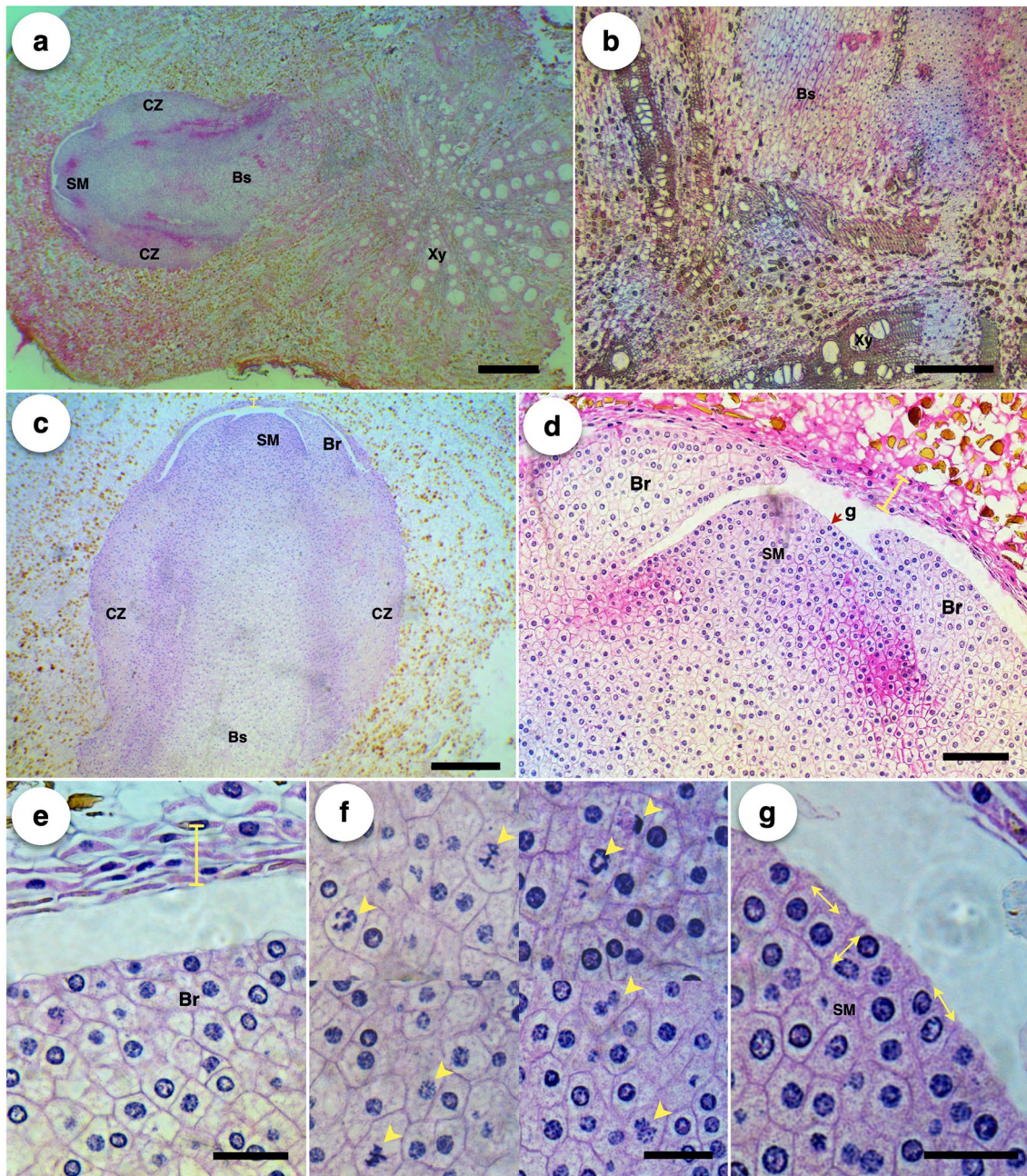


Fig. 7 *R. consueloae* floral shoot development within the host root. **a** Floral shoot emerging from the host xylem (Xy) with the shoot meristem (SM), contact zones (CZ) and basal region (Bs) labeled. **b** Elongated cells of the basal region (Bs) in direct contact with the host xylem. **c** Longitudinal section of a young floral shoot with developing bracts (Br) and increasingly elongated cells towards the basal region (Bs). **d–e** Layers of flattened cells surrounding the top part of

the floral shoot after separation from the apex of the protocorm during development. **f** Floral shoot cells (arrowheads) in various stages of mitosis. **g** Cells on the surface (labeled 'g' in **d**) of the shoot meristem (SM) that have undergone anticlinal and periclinal divisions (double arrowheads). Scale bars: a–b = 1000 μm ; c = 500 μm ; d = 200 μm ; e–g = 50 μm

observations made on growth of *R. patma* estimated that this period may last up to 3 years (Hidayati et al. 2000).

In the last few years, a number of *Rafflesia* seed inoculation and host grafting experiments were reported. In Sabah, Malaysia, a local resident was reported to have successfully

inoculated *T. leucostaphylum* vines with *R. keithii* seeds. As described in Molina et al. (2017), bud growth on the host root was observed two to seven years after placing the seeds within shallow incisions on the root bark. Mursidawati et al. (2015) and Wicaksono et al. (2017), on the

other hand, reported to have propagated *R. patma* through veneer grafting or cleft grafting of its host, implying that the endophyte in infected parts has a way of spreading to uninfected rootstock. In all these reports, none of the “uninfected” host vines used were checked for presence of *Rafflesia* endophyte cells before carrying out the experiments. If the endophyte can indeed persist in the host root for a long time without developing into floral shoots, then internally infected host individuals without external manifestation of *Rafflesia* growth can easily be misidentified as uninfected. Thus, it would be best if propagation efforts through seed inoculation or grafting be supplemented with checking for presence of endophytic cells prior to the experiment proper. This can be done through histology of host tissues or alternatively, a protocol for molecular detection of the endophyte within the host tissue may be developed. This will help to accurately test whether the subsequent bud growth is a result of the inoculation/grafting procedure or due to pre-existing endophytic cells within the host tissues.

Parasite cells must reach the vascular cambium prior to proliferation, but extensive vegetative growth need not precede reproductive development

It has been previously speculated that the *Rafflesia* endophyte initially infects the host vascular cambium where its cells divide together with the cambial cells causing their lateral spread towards the xylem and phloem regions of the infected root (Brown 1912; Mursidawati et al. 2019). Our results suggest that endophyte cells may indeed reside within the host cambium prior to invasive growth towards the xylem cylinder and secondary phloem. A number of root transverse sections showed the endophyte confined exclusively within the vascular cambium, with the wood and phloem regions completely devoid of parasite cells (Fig. 5c). This supports the hypothesis of Mursidawati et al. (2019) that the endophyte cells of *Rafflesia* initially form small meristematic cell clusters among the fusiform initials of the vascular cambium. The subsequent periclinal divisions of the fusiform initials as they form axial secondary xylem and phloem likely resulted to the radial spread of the endophyte cells as seen in the root transverse sections. The shorter filamentous endophyte in both the xylem and phloem regions, which were radially oriented in the transverse sections, was often observed to contain cells in contact with or entirely found within the vascular cambium (Fig. 5e–g), indicating that they likely originated from it. All these imply that cells from the seeds of *R. consueloae* must be able to reach beyond the tissues of the root bark to initiate infection in the host.

We also found that the occurrence of a limited degree of infection (*i.e.*, endophyte growth limited to the vascular cambium only) does not prevent the development of more

advanced reproductive stages within the same tissue. Several sections that included developing protocorms were found to contain isolated endophyte cells solely in the cambium and nearby phloem regions. These sections markedly differ to those with a much higher degree of infection (Fig. 5). This suggests that extensive growth of the endophyte within the host vascular tissue is not a prerequisite to the onset of reproductive development. Floral shoot development may ensue as long as a group of endophyte cells in contact with the vascular tissues, particularly the xylem, could continue dividing.

Host root tissues had varying degrees of infection

We found that the abundance and distribution of the endophyte in the root tissues obtained from host individuals with and without visible external signs of infection varied widely. As discussed in the previous section, the extent of infection ranges from just a few isolated endophyte cells confined in the cambium area to widespread growth of numerous filamentous and multiseriate cell clusters in the entire vascular area. There could be several possible explanations for the differences in the degree of infection observed in the roots examined. One is that the sections with fewer endophyte cells growing exclusively within the cambium region were obtained from more recently infected roots. As aforementioned, the vascular cambium is the hypothesized initial site of endophyte infection. This would explain why many of the roots obtained from host plants without externally visible signs of infection only had endophytic cells within the vascular cambium region.

Another intriguing possibility is that the roots with limited endophyte growth may have been infected for a longer time but these host individuals may have higher resistance to the infection. This remains plausible because there were host roots that already had *R. consueloae* floral shoots but the growth of the endophyte in the sections was only confined to the vascular cambium. Host resistance to plant parasite infections has been recently compared to the kind of the resistance that plants employ to defeat microbial pathogens, suggesting that plant parasites are regarded by their hosts as they would plant pathogens (Delavault 2020; Su et al. 2020). As such, different host individuals may have varying capacities to prevent infection or limit its extent. This type of resistance has been demonstrated by the hosts of *Striga* plants. The parasite is unable to penetrate the endodermis of resistant cowpea cultivars, which were found to produce resistance (R) proteins that can cause growth arrest or necrosis of the parasite cells within host roots (Li and Timko 2009). Moreover, Brown (1912) and Nikolov et al. (2014) found that some *Tetrastigma* hosts can hinder the growth of Rafflesiaceae protocorms by clogging vessel elements near the base of the protocorm with mucilage or by forming

a phellogen-like layer around the protocorm which would then block the parasite's access to host resources. We did not find any degenerating protocorms in any of the root sections examined but the varying levels of endophyte growth may allude to the possibility that some host individuals may possess traits that can limit the degree of infection prior to reproductive development of the parasite.

The differences in the abundance and distribution of the endophyte within the host tissues could also be related to the number of seeds that were involved in the infection process. It has been previously shown through nuclear DNA genotyping of *R. cantleyi* and *R. tuan-mudae* buds that a single *Tetrastigma* vine can house up to 13 different genotypes, indicative of multiple infections (Barkman et al. 2017). Using a similar microsatellite genotyping approach, Pelser et al. (2017) also showed that a single host individual can be infected with multiple *R. lagascae* genotypes. Roots with higher endophyte abundance may have been initially penetrated by a greater number of parasite cells from multiple seeds. Alternatively, different parasite genotypes could have varying degrees of infectiousness, leading to the differences in the extent of endophyte growth detected in the roots.

Conclusion

Cells contained in the seeds of *R. consueloae* must reach the root vascular cambium of its *Tetrastigma* host to initiate infection. These endophytic cells may remain confined within the cambium area or spread radially in surrounding tissues if they are able to divide together with the meristematic cambial initials. The parasite may remain in the vegetative stage for long periods. Thus, host individuals without external *Rafflesia* growth may not necessarily be free of infection. Furthermore, development of *R. consueloae* floral shoot may ensue even in the absence of widespread growth of its endophyte within the host root indicating that extensive vegetative growth is not a prerequisite to the onset of reproductive development. It is clear that the study of the early development of *Rafflesia*—an often neglected area of research—must be explored further as it will help us understand how *Rafflesia* and its host modulate each other's development as well as how they may interact at the cellular and molecular level.

Author contribution statement All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Erika Marie A. Bascos, Edwino S. Fernando, Melizar V. Duya, and Lillian Jennifer V. Rodriguez. The first draft of the manuscript was written by Erika Marie A. Bascos and all authors commented

on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability statement The data generated and/or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest All authors declare no conflicts of interest.

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