

***Tetrastigma leucostaphylum* (Dennst.) Alston ex Mabb. Partial Wedge Sampling, a New, Less-invasive Solution for Stem-borne *versus* Root-borne *Rafflesia* Identification**

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Tetrastigma (Vitaceae) are a genus of tropical lianas that are hosts for parasitic plants in the family Rafflesiaceae. Since the stem of *Tetrastigma* is creeping and often devoid of leaves, it is often mistakenly visually identified as a root. *Rafflesia* flowers at the ground level (*i.e.* terrestrial flowers) have been documented to grow on *Tetrastigma* roots, but it is unclear whether those that arise aerially emerge from host root or stem organs. In this study, we present a new, less-destructive means of sampling *Tetrastigma leucostaphylum*, which is a host of many *Rafflesia* species. Histological observation of two 250 cm above-ground aerial samples confirmed that both were stems, as shown by distinct anatomical features (compactly arranged vascular bundles with simple phloem bundles) in contrast to roots, which had loosely arranged vascular bundles with ramified phloem bundles. Clearer identification of *Tetrastigma* vine infected with aerial *Rafflesia* is needed to confirm whether the parasitic flower grows on a stem or on an erect aerial root to better understand the biology of this enigmatic *Rafflesia* species that have evaded cultivation and *ex situ* conservation efforts. This new sampling method may be a useful way to identify the *Tetrastigma* vine in the future due to its less invasive nature compared to severing the whole vine.

Keywords: anatomy, parasitic plant, plant morphology, Rafflesiaceae, *Tetrastigma*

INTRODUCTION

Rafflesia (family Rafflesiaceae) is a genus containing the world's largest flowers. All are parasitic on *Tetrastigma*, which are tropical lianas. Although the vast majority of documented studies have shown *Rafflesia* growing terrestrially mostly on the roots of *Tetrastigma*, on occasion, *Rafflesia* has been found to grow on aerial vines relatively

close to the soil surface. Thus far, eight *Rafflesia* species have been reported to grow aerially (Nais 2001; Heide-Jorgensen 2008; Barcelona *et al.* 2008; Susatya 2011; Mursidawati *et al.* 2015; Sofian *Rafflesia*, pers. comm.). The growth of *Tetrastigma* is characteristic of lianas, but the morphology of two organs – the root and stem – are continuous and difficult to differentiate visually. *Rafflesia* is an endophytic holoparasitic plant with a remarkable and highly reduced endophyte (Nikolov *et al.* 2014) during its vegetative stage; in its generative stage, flower anatomy

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is greatly modified (Mursidawati *et al.* 2020). For this reason, the genus is of interest to evolutionary biologists and developmental biologists alike.

The stem and root of the *Tetrastigma* host have a similar visual appearance, even though they appear to produce adventitious roots (Figures 1c and f). A histological analysis would allow for the organ on which *Rafflesia* buds grow to be determined, but the large vine (root or stem) is often left untouched in such analyses simply because destructive sampling would destroy the whole plant; since the vine is infected by *Rafflesia*, sampling it would kill the parasite as well as the host (Mursidawati and Irawati 2017). To date, all documented studies have focused

on *Rafflesia* sampled from the root rather than stem tissue. Those studies and/or all figures in those studies consistently showed anatomical characteristics of roots.

In a previous study of *Rafflesia* (Wicaksono *et al.* 2017), the position of the *Tetrastigma* vine – specifically on individuals infected with *Rafflesia* – was difficult to determine, especially whether the tissue was from the stem or root because roots can sometimes be found erected or located above-ground (Figure 1). In the case of Bogor Botanical Garden's *Tetrastigma leucostaphylum* (Dennst.) Alston, for example, when referring to the *T. leucostaphylum* organ in previous studies – namely, Mursidawati and Sunaryo (2012), Wicaksono *et al.* (2016,

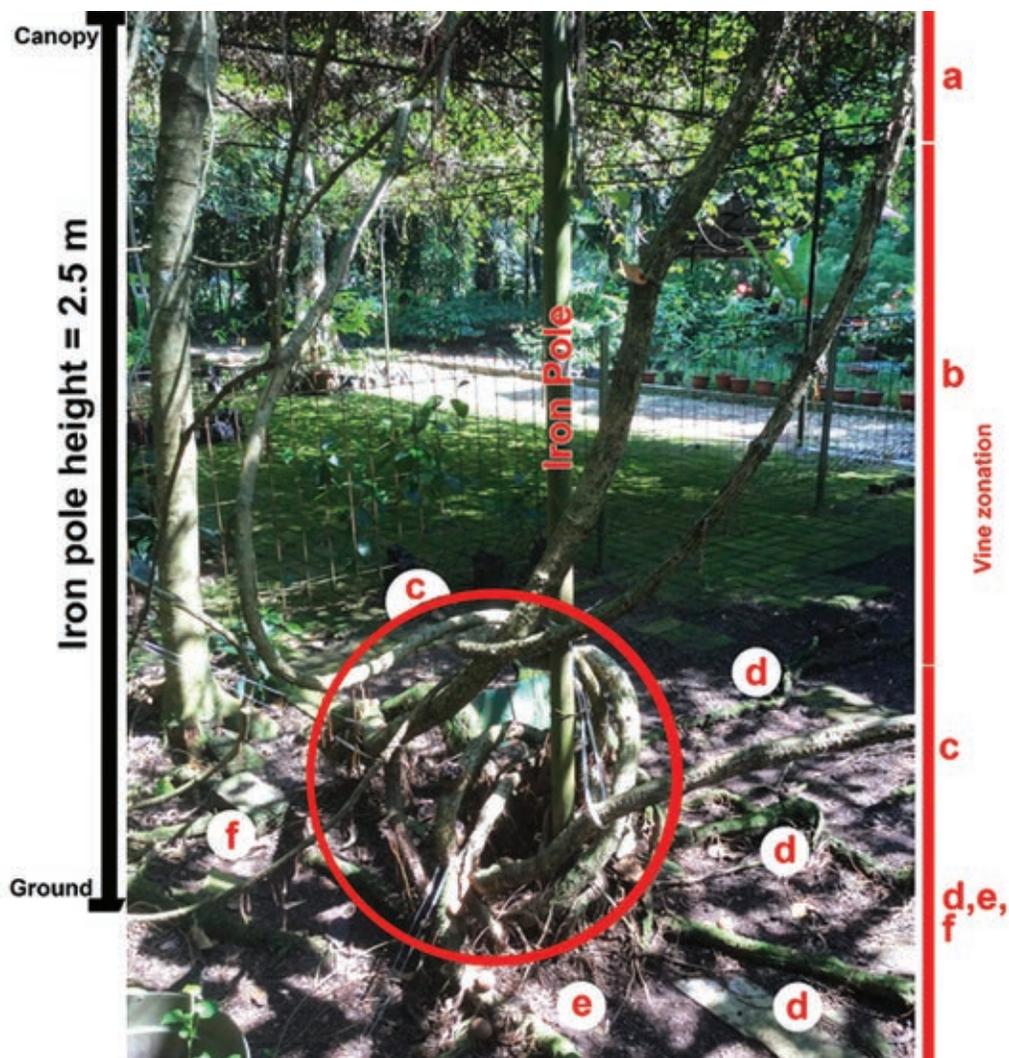


Figure 1. *Tetrastigma leucostaphylum* entangled vines in Bogor Botanical Garden. As it grows surrounding an iron pole, it is visually difficult to determine whether the completely aerial vine in the canopy zone (a), aerial erected vine (b), helical erected vine (c), large log-like vine on the surface of the ground (d), small root-like protrusions extending to the ground (e), or elevated vines (presumably root; f), are actually roots or stems. Although sampling is possible, there is the risk of killing the remaining vine with the *Rafflesia* endophyte inside it, especially if the massive root/stem is cut. *Rafflesia* flower buds are often found in c–f (approximately max. 40 cm from the ground level).

2017), and Mursidawati *et al.* (2019) – a certain level of uncertainty existed regarding the actual organ involved (stem or root). In those cases, the organ that was referred to was based exclusively on visual observations; although Mursidawati *et al.* (2019), in histological studies, revealed the anatomical existence of a periderm layer and open concentric vasculature in secondary growth in which vascular cambium developed. These anatomical features are present in both stems and roots during secondary growth in higher plants (Beck 2010). In *Tetrastigma*, Nikolov *et al.* (2014) reported the existence of vascular cambium on roots with secondary growth, while Pace *et al.* (2018) reported vascular cambium in the stem. In *Rafflesia*, no vascular cambium is found in the grown flower/generative stage (Mursidawati *et al.* 2020).

A study of *Rafflesia tuan-mudae* Becc. inside *Tetrastigma rafflesiae* Miq. was performed by Nikolov *et al.* (2014). Similar to vines in other members of the Vitaceae, such as grapevine (*Vitis* sp.; exact species undocumented), the morphology of the root vasculature is open concentric with xylem in the proximal region of the organ, followed by vascular cambium, then phloem at the distal region of the organ (Gambetta *et al.* 2013). Compared to the stems of *Vitis* sp., the stem vasculature of *Tetrastigma* also has an open concentric arrangement (Pace *et al.* 2018). This stem anatomy is also consistent across genera in the Vitaceae such as *Cayratia* and *Pterisanthes* (Najmaddin *et al.* 2011)

A method needs to be developed that allows for the vine organ of *Tetrastigma* to be determined so that the root and stem can be distinguished to better understand the biological parasite-host nature of the relationship between *Rafflesia* and *Tetrastigma*. Currently, no such efficient method exists. Initially, in a successful *Tetrastigma* grafting study, partial wedges were sampled from aerial (canopy) and erect vines to determine if they were root or stem tissue, then wedges were returned to their original site to allow the vine to regenerate (Mursidawati *et al.* 2015). Based on this newly devised method, the results of tissue/organ identification in this paper will allow for more accurate identification of *Rafflesia* aerial and terrestrial flowers by differentiating *Tetrastigma* organs. Heide-Jorgensen (2008) claimed that *Rafflesia* is both a root and a stem parasite, but no anatomical or histological proof – that we are aware of – exists in the literature that unequivocally shows that the *Rafflesia* growing on a *Tetrastigma* vine is a stem. Here we present a novel methodology for a clearer and more accurate description of the *Tetrastigma* organ that may provide more key characteristics of *Rafflesia* species (or even Rafflesiaceae in general) to be better identified in the future.

MATERIALS AND METHODS

Plant Material

The *T. leucostaphylum* scion was grafted from Pangandaran, West Java, while the rootstock was originally grown in Bogor Botanical Garden (Mursidawati *et al.* 2015). Even though the lower zone of the vine (Figures 1c–f) where the *R. patma* bud is often found and blooms and is believed to be a root, we focused on the erect, aerial vine zone (Figures 1a–b) to obtain samples for organ identification. We sampled four intact vines (in Figure 1b) located 60 cm above the ground, and two aerial vines with cut-ends (Figure 1a) located 250 cm above the ground.

Partial Wedge Sampling, Identification, and Regeneration

T. leucostaphylum has good organ regeneration ability after grafting (Mursidawati *et al.* 2015) and can be propagated through cuttings (Adhityo Wicaksono, pers. observations). Therefore, we believe that if a sample wedge is sliced from the mother (main) vine and returned to its original place, the wedge might reconnect to the mother vine – similar to grafting – allowing the mother vine to continue to thrive and grow. We sampled one wedge per vine (from four vines) located in the Figure 1b region (see Appendix Figure I). This method allows the transverse cross-sections of a histo-anatomical profile of an erect vine to be visually observed, and to confirm if the organ is a root or a stem. We used a blade to partially slice the erected vines in the Figure 1b region, then returned it to its original place from where it was taken (Figure 2). We employed two reference organs – an unused specimen from Mursidawati *et al.* (2019) serving as a root organ (Figure 3a) and a stem organ (Figure 3b) from Mursidawati and Sunaryo (2012) – to identify the *T. leucostaphylum* sample (the wedge). After 3 wk, the sampled erect vine parts were observed to see if the vine had fully regenerated or if it had died. Sampling was performed on 08 Nov 2020, and regeneration was observed on 30 Nov 2020 (rainy season in Indonesia).

RESULTS

Identification of Samples

In this study, four intact vines were visually identified as both root (three samples) and stem (one sample) (see Appendix Figure II and Table I for data). Sampling resulted in samples that were obtained not too deeply so as not to reach the center of the vine (indicated by half of both horizontal and vertical diameters in Appendix Figure I; data can be observed in Appendix Figure I). We identified a stem in the area of Figure 1b, as in Figure 4d (1

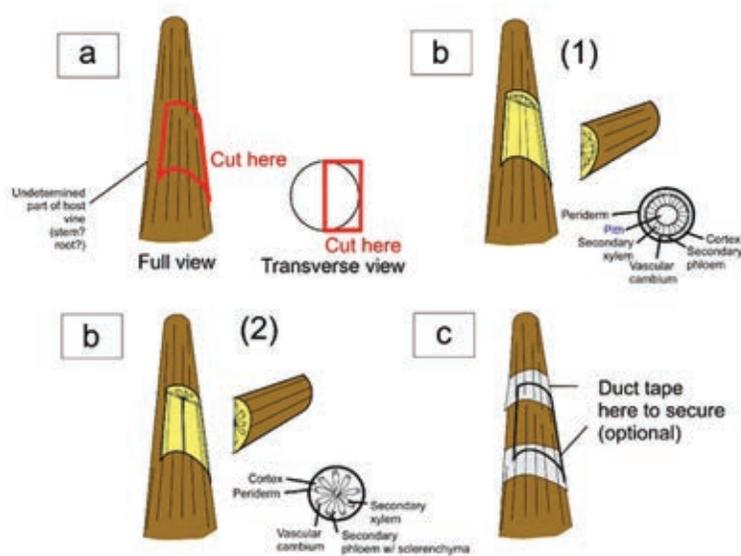


Figure 2. Partial wedge sampling method for *Tetrastigma leucostaphylum* erect vine. In the undetermined vine of the host, the cutting with razor covered a precise rectangular area of the vine side, a. The wedge of the vine, which was taken partially, was peeled out and rapidly documented to determine the histo-anatomical profile of the wedge, whether it is a stem (b1) or root (b2). Finally, the wedge was returned to the original spot, clear duct tape or plastic film can be additionally used to secure the tissue and to prevent drying out (c).

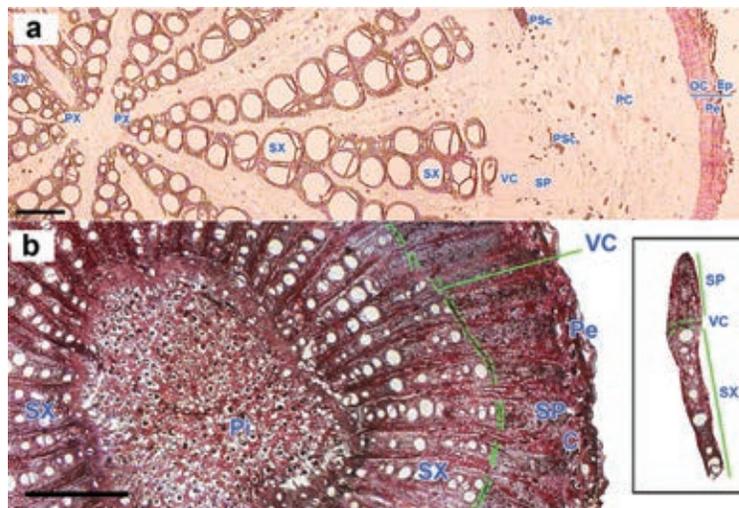


Figure 3. *Tetrastigma leucostaphylum* root (a) and stem (b) transverse anatomy. For the root, it has undergone secondary growth showing the radially arranged open concentric vascular bundles. The stem is shown with radially arranged open concentric vascular bundles surrounding the pith tissue. Inset: the anatomy of a single vascular bundle: protoxylem (PX), phloem sclerenchyma (PSc), pericycle cortex (PC; new cortex), and original cortex (OC) with the epidermis (Ep) forming the periderm (Pe) or cork layer; cortex (C), secondary phloem (SP; dark area), vascular cambium (VC; the area between secondary phloem and xylem), secondary xylem (SX), pith (Pi). For a, microscope zoom 4×10 , scale bar = 1 mm, and picture taken under microscopic conditions indicated in Mursidawati *et al.* (2019) with an unused specimen. For b, scale bar = 724 μ m and picture taken from Mursidawati and Sunaryo (2012) (edited with permission).

for the visual morphology; 2 showing the cut), identified by dense straight vascular bundles – including unified xylem and phloem bundles, and a pith. Surprisingly, we also found roots in the same area (Figure 4e; 1 for the visual morphology; 2 showing the cut), identified by loose vascular bundles with ramified phloem bundles emerging from the xylem bundles, similar to the root cross-section in Figure 4a. Root erect vines are vines that are connected directly to the ground, and stem vines grow from root vines (Figure 4h), often from a lateral shoot. Both fully

aerial (in the canopy) vine (Figure 1a) samples are stems (Figure 4i).

Sample Regeneration

After 3 wk, the returned and sampled vine wedges did not appear to re-attach to the main vine but rather decayed. However, the remaining parts of the erect vines appeared to be alive since the leaves in the stem sample remained healthy (Figure 4f), and the root sample produced new shoots and adventitious roots (Figure 4g) that can grow

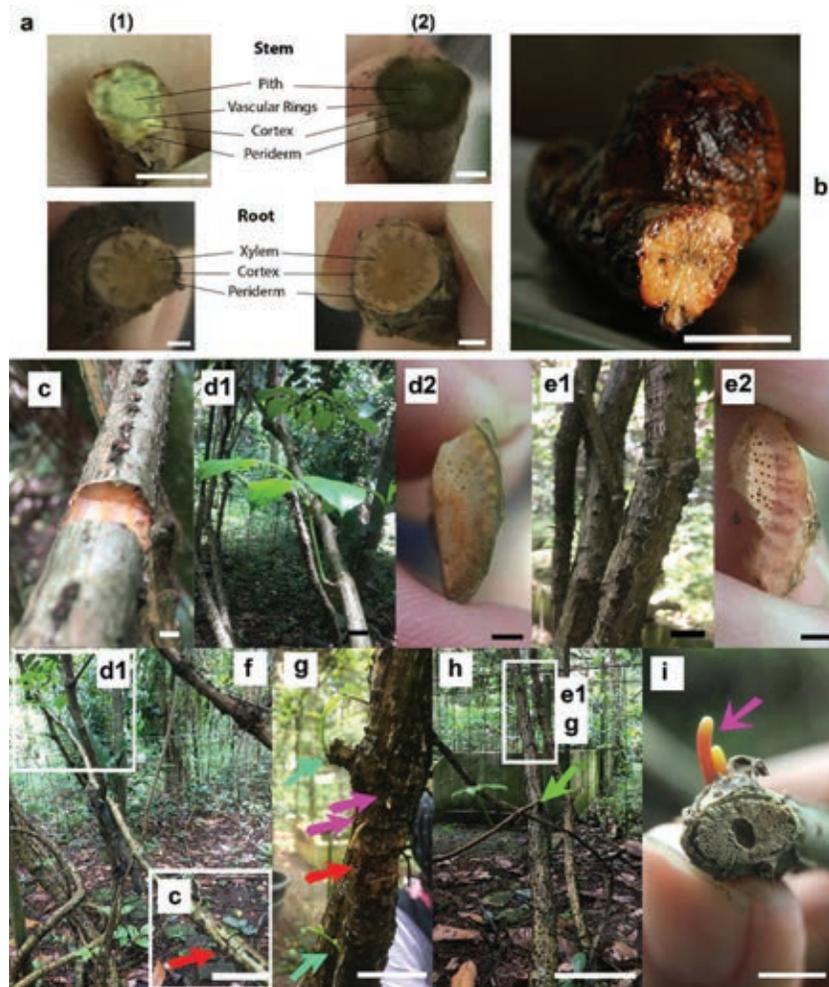


Figure 4. Macroscopic visual anatomical structures from sections of *Tetrastigma leucostaphylum*. The early (1) and late (2) stems (both are aerial; Figure 1a) are distinguishable from the root (early – 1; late – 2; Figure 1e) by its pith (a). The *Rafflesia patma* flower bud from the study of Wicaksono and Teixeira da Silva (2015; unpublished image) was actually from the root of *T. leucostaphylum* since no pith could be visualized at the organ’s core (b; taken from a part in the area of Figure 1f). The inclining (c and d) and erect vines (e) found in the same area as Figure 1b (1.5 m from the ground), despite the similarities as aerial vines, are actually stems (d2, shown with dense vascular bundles with pith; see Figure 3 for comparison) and root (e2, shown with loose vascular bundles; see Figure 2 for comparison) under the partial-wedge sampling of the vines. After 3 wk, the sampled stem area of c (red arrow) can be seen in f and the leaves are still green (the leaves are the same as in d1), implying the stem regeneration. The sampled root area (e1) after 3 wk (red arrow) decayed, but the root is now producing new shoots (cyan arrows) and adventitious roots (purple arrows). If the shoot grows, it can grow into a stem vine (green arrow) ramified from the root vine, which grows erect directly from the ground (h). The aerial stem vine (in Figure 1a) produced adventitious roots (i; purple arrows). Scale bars: a = 2 mm; b = 2 cm; c, d2, and e2 = 0.3 cm; d1 and e1 = 2 cm; f = 5 cm; g = 2 cm; h = 10 cm; i = 0.5 cm. Photos by Adhityo Wicaksono.

into a fully developed vine (Figure 4h). The aerial stem vine also produced adventitious roots (Figure 4i). From this result, it may be assumed that the sampled wedge does not need to be returned to the mother vine since the wedge is small. The returned wedge might dry out, whereas the wounded mother vine heals and continues to grow.

DISCUSSION

Tetrastigma leucostaphylum Stem Structure

Tetrastigma is categorized into the Magnoliopsida group (GBIF Secretariat 2019), which is taxonomically also known as a dicotyledonous plant (USDA 2021). The stem of a dicotyledonous plant during primary growth is initiated from the shoot apical meristem before it grows into a stem (organ) with epidermis in the distal region, a layer of cortex tissue – a set of open concentric vascular bundles (fascicular) composed of primary phloem, vascular cambium, and xylem (distal to proximal, respectively) – and pith tissue in the proximal region (Wang *et al.* 2010). Interfascicular and intrafascicular vascular cambium will develop secondary vasculature during secondary growth, pushing the cortex towards the distal region as secondary phloem develops, and also pushing the pith, making it smaller as secondary xylem endarch develops, similar to roots (Beck 2010).

The aerial vine sample (Figure 4i) reveals anatomical characteristics of stems (Figure 3b), shown by its pith and dense vascular bundle arrangements, with the adjacent distal stacking of xylem and phloem bundles, and the triangular appearance of the phloem bundle instead of being split or ramified, as in the root vine (see Figure 3a). These characteristics were also observed in the wedge sample of an erect vine (Figure 4d2; Appendix Figure IIe1), which clearly shows the pith area of the vine.

Tetrastigma leucostaphylum Root Structure

The root of a dicotyledonous plant during primary growth starts from the root apical meristem (RAM) before it develops into a root (organ) comprised of epidermis on the distal region, followed on the inside by layers of cortex tissue bordered with a layer of endodermis; on the proximal region, there is a central cylinder or stele, *i.e.* a core area with vascular tissues in the center of the root surrounded by a layer of pericycle tissue, and finally the endodermis tissue layer, which lies distally (Beck 2010). The pericycle is semi-meristematic parenchyma tissue that functions during lateral root development and secondary growth differentiation (Chiatante *et al.* 2018; Strock and Lynch 2020). Later on in development, during secondary growth, the root stele of a dicotyledonous plant

will enlarge and push distally, allowing the secondary vasculature to grow (secondary xylem and phloem), the pericycle to differentiate into the vascular cambium, and the phellogen layer – which pushes the endodermis, cortex, and epidermis distally – to develop into periderm (Beck 2010; Campilho *et al.* 2020). The vascular cambium will grow more xylem in endarch development (inwards, the early xylem or protoxylem is directed inwards or distally and the late xylem or metaxylem is outwards or proximally towards the periphery), but less or no more phloem (Beck 2010).

Three wedge samples (Appendix Figures IIe2–4) revealed the anatomical characteristics of roots, despite being sampled from erect vines. In the *Tetrastigma* root (Figure 3a), overall, vascular bundles are loosely arranged and xylem bundles are split from distal ends, with the phloem bundles protruding and merging in the distal tip, forming ramified structures of bundles. These characteristics can be seen in Figure 4e2 (Appendix Figures IIe2–4 for the remaining wedge samples), where the bundle gaps are visible and ramified.

Stem versus Root Anatomy Confusion in *Tetrastigma*, a Liana Plant

The visual observation of a liana plant, where the stem and root grow continuously as a vine, and the fact that sometimes the stem and root grow at the same ground level, makes it difficult to differentiate them based on morphology alone. From this condition, in a *T. leucostaphylum* plant at Bogor Botanical Garden (Figure 1), it is safe to generally assume – based on the levels from the ground – that all above-ground organs are stems (Figures 1a–c), while any organ growing on the ground or below-ground are roots (Figures 1d–f). However, some vine structures at the ground level (approximately 0–40 cm from the soil surface) are twisted and convoluted (Figures 1c–f), making the differentiation of stems and roots difficult. Root morphology has been examined in other members of the Vitaceae, specifically in a study by Gambetta *et al.* (2013) on a grapevine hybrid (*Vitis berlandieri* × *Vitis rupestris*) root. They showed, in a region close to the RAM, that there are only epidermis, cortex, and an undifferentiated stele. Then, in the next region (*i.e.* more distal from the RAM), endodermis, pericycle, xylem, and phloem developed. Distally further away from the RAM, pericycle differentiated into vascular cambium. In the furthest region from RAM with secondary growth, the pericycle formed a periderm layer distally – compressing the endodermis, cortex, and epidermis layer distally – ultimately rupturing it. When observed transversally, the secondary phloem in this region (*i.e.* the region of roots with secondary growth, most distal from the RAM and the most mature

part of the root) was located distally after the vascular cambium, which itself was located distally from the secondary xylem.

In *T. leucostaphylum*, root anatomy (Figure 2) is similar to that of *Vitis* roots described by Gambetta *et al.* (2013). From the core region (transversally), it has a vascular bundle with secondary xylem and secondary phloem, with vascular cambium in between. The pericycle forms a new cortex, while the old cortex together with the epidermis is compressed distally and becomes the cork or periderm layer of the root. The vascular bundle consists of ramifications of the secondary xylem, and at the distal corners of the xylem, there is vascular cambium and secondary phloem with phloem sclerenchyma in this order from the inner to the outer layer. In the core and between the bundles, there are spaces that consist of parenchyma tissue. Compared to the root, *T. leucostaphylum* stem anatomy is dense with vascular bundles and with no spaces (Figure 3). The vascular bundle profile (Figure 3, inset) consists of a straight arrangement of secondary xylem, a thin layer of vascular cambium, then a thick layer of secondary phloem (which is only a thin layer in roots). In the stem core, there is a large space of parenchyma tissue or pith. The specimens used to assess this anatomy were drawn from the top region of the plant (between Figures 1a and b) for the stem and the large root extension (Figure 1f) for the root.

In Bogor Botanical Garden, buds of *R. patma* that infected *T. leucostaphylum* were observed in the lower region of the plant (Figures 1c–f). The *T. leucostaphylum* scion was grafted from Pangandaran, West Java (Mursidawati *et al.* 2015). Many buds grew on the surface of roots (Figures 1e–f), but those that reached anthesis were found only on the large convoluted roots (Figure 1c), based on flowering records from 2010, 2012, 2013, 2014, 2015 (Mursidawati and Wicaksono 2020a), 2018, and 2020 (Mursidawati *et al.* 2019, 2020). The part of the plant that is being compared is the erected vine (Figure 1b) where the aerial *Rafflesia* can be found.

In a similar study, shield budding – in which a small area of the stem with bud was attached to another incised stem to allow the foreign bud to develop in the host stem (*i.e.* a variant of grafting) – requires perfect sealing of the transferred bud in the host stem to prevent drying and to protect the living tissue (*i.e.* the bud) (Samish and Gur 1962). Perhaps, in this case, the returned vine wedge dried because it is too thin (as only a part of the wedge was taken; Appendix Figure I), causing the wedge to dry out.

Using this temporary classification, our objective was to clarify the actual *T. leucostaphylum* organ of the vines mentioned in our previous studies (Mursidawati and Sunaryo 2012; Wicaksono *et al.* 2016, 2017; Mursidawati

et al. 2019). Generally, there is no visual clue as to the *Tetrastigma* organ that is observed, *i.e.* whether it is a stem or a root, and an assumption is usually based exclusively on the position. This is especially pertinent, as the root is considered to be at the ground level (or up to 30 cm above-ground in this case because roots are often entangled with each other, and this is normally where *Rafflesia* buds are found), while the stem is generally found above the root level (*i.e.* > 30 cm). In the study of Mursidawati and Sunaryo (2012), there is more than one vine, but all of them are roots, except for the Figure 1a of the paper (used as Figure 3b in this paper, edited with permission). In Wicaksono *et al.* (2016), most of the organs mentioned refer to the *Tetrastigma* root since that study is about terrestrial *Rafflesia*. In Wicaksono *et al.* (2016), Figure 1g provides an explanation of *T. leucostaphylum* root grafting, and this root is located in the region of Figure 1c in this paper. In addition, Figure 5I of Wicaksono *et al.* (2016) shows tissue culture of still intact *T. leucostaphylum* root with *R. patma* bud, which used root from the area of Figure 1e in this paper. The *T. leucostaphylum* organs described in Wicaksono *et al.* (2017) are roots since they lie on the ground and have anatomical regions described in Figures 1c–f, belonging to the region of ground or terrestrial vines that are mainly composed of roots. Similar to Wicaksono *et al.* (2017), Mursidawati *et al.* (2019) reported that *R. patma* protocorm or primordial young bud and endophyte samples on the roots of *T. leucostaphylum* were found between the regions of Figures 1e–f (also ground or terrestrial vine).

***Tetrastigma* Vine Organ and *Rafflesiaceae* Endophyte Behavior**

All flower buds that grow above host secondary xylem tissue in the vascular bundle of the host root further confirm that the growth of *R. patma* from the endophyte to the flower bud stages is initiated in the root vascular cambium layer, as was stated in Mursidawati *et al.* (2019). Nikolov *et al.* (2014) stated that the *Rafflesiaceae* endophyte could also be found in root xylem and phloem, and in the same study, the endophyte *R. tuan-mudae* Becc. was found at a depth in the root ranging from the cortex (pericycle) to the outer border of the host secondary xylem. Compared to another endophyte, *Rh. lowii* (Becc.) Harms, *Rh. lowii* grew more invasively, surrounding the host xylem vessel elements (sometimes the endophyte encircled the vessel elements), xylem parenchyma rays, vascular cambium, and even phloem. Still in the same study (Nikolov *et al.* 2014), in later flower bud development, instead of growth at the distal point of the host xylem – as was found in this study [Figure 3A of Nikolov *et al.* (2014)] – the *Rh. lowii* flower bud could emerge from the host radial parenchyma tissue between two vascular bundles located adjacent to the host xylem area [Figure 2S of Nikolov *et al.* (2014)]. Compared

to *Rafflesia*, in *R. tuan-mudae* (Nikolov *et al.* 2014) and *R. patma* (Mursidawati *et al.* 2019), the endophyte is shallower and only reaches the vascular cambium layer: the *R. patma* endophyte in Mursidawati and Wicaksono (2020a, b) was found 1.0–1.5 mm from the center on an approximately 3.5 mm thick *T. leucostaphylum* root [referring to Figure 19B of Mursidawati and Wicaksono (2020a)] and the *Rh. lowii* flower proximal part was close to the center, approximately 0.5 mm from the center on an approximately 5 mm thick *T. tuberculatum* (Blume) Latiff root [referring to Figure 3A of Nikolov *et al.* (2014)]. Why does *R. patma* tend to grow from the vascular cambium while the growth of *Rh. lowii* can be more invasive (*i.e.* deeper)? Moreover, as vascular cambium tissue undergoes cell divisions that might facilitate endophyte dispersion (Mursidawati *et al.* 2019; Wicaksono *et al.* 2021), can the pericycle (new cortex) of the secondary grown root of *Tetrastigma* facilitate dispersion of the same endophyte? Upon secondary root growth, cortex tissue is pressed to the periderm, and pericycle tissue “becomes” the new cortex (Beck 2010; Gambetta *et al.* 2013). The pericycle is meristematic tissue that contributes to lateral root growth, and in secondary root growth, it contributes to vascular

cambium development and formation of the root periderm (Beck 2010). It is possible, but unproven, that the root pericycle tissue also contributes to endophyte dispersal. This hypothesis of externally-aided endophyte dispersal [vascular cambium in *R. patma*, as in Mursidawati *et al.* (2019); or possibly in pericycle tissue] requires more observations across several *Rafflesia* species and across the Rafflesiaceae. Such additional studies are also needed to confirm the existence of endophyte fragmented strands [endophyte tissue that is scattered in the host tissue and shaped like strands; Nikolov *et al.* (2014)] or cell clusters [endophyte tissue that is scattered in the host tissue and is shaped like cell clumps or clusters; Mursidawati *et al.* (2019)] in other Rafflesiaceae species (Wicaksono *et al.* 2021).

Perspectives on Terrestrial *Rafflesia* vs. Aerial *Rafflesia*: Root vs. Stem Parasite Species?

There have been few recorded sightings of *Rafflesia* species that bloom aerially. In Figures 5a and b, two variants of *R. arnoldii* R. Br. can be observed: aerial (Figure 5a), which appears to be borne from the stem, and terrestrial (Figure 5b), which appears to be borne

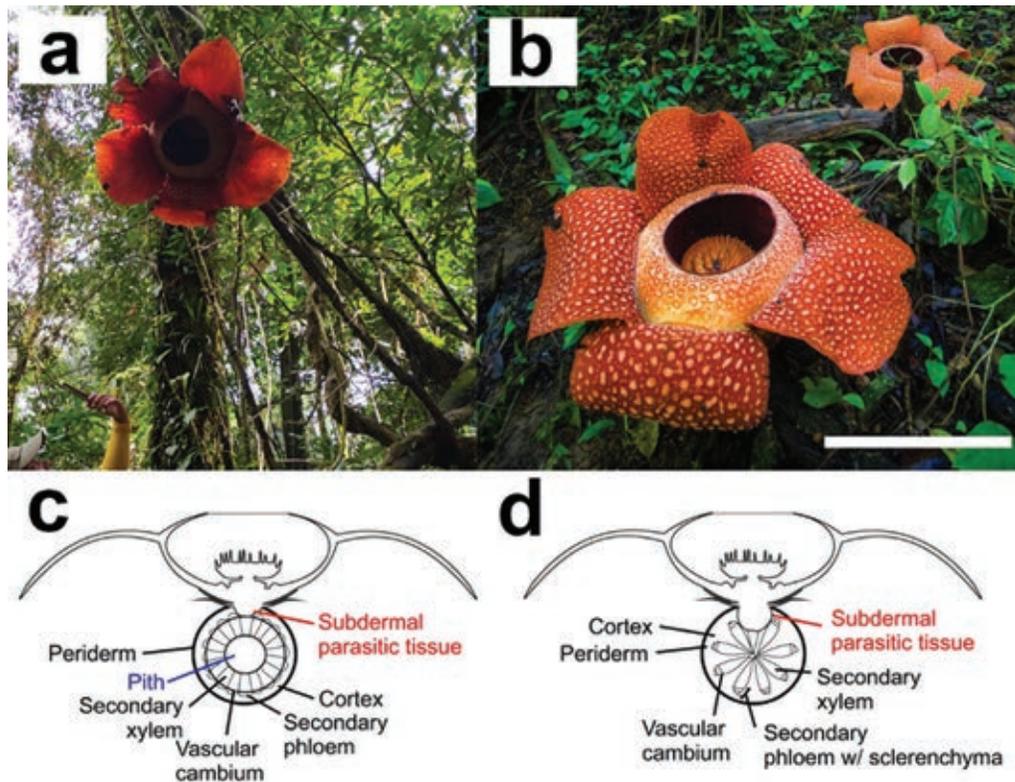


Figure 5. The high aerial *R. arnoldii* R.Br found 3 m above the ground (a), and the commonly found terrestrial *R. arnoldii* grow in the root of *Tetrastigma* (b). The hypothetical schematics of stem-borne *Rafflesia* (c), which requires further investigation, and the known root-borne *Rafflesia* (d). Photos a and b were taken by Sofian Rafflesia and have been used with permission. Illustration d was also taken from Mursidawati and Wicaksono (2020b) with permission. Scale bar: b = 30 cm. Pictures a has no scale bar because the flower was taken from a distance, and illustrations c and d are not to scale.

from the root. According to Susatya (2011), based on *R. hasseltii* Suringar, the aerial bud rarely blooms perfectly due to gravitational pull and, even if it blooms, the period of anthesis will be shorter and the flower will be smaller than a terrestrial bloomer. Nais (2001) found 30 buds of *R. hasseltii* in a single *T. leucostaphylum* stem, expecting the mortality rate of these aerial buds to be high (*i.e.* possibly due to reduced water scarcity). Similarly, *R. keithii* Meijer was found 1 m over the ground, a rare occurrence perhaps due to water transport is likely to be difficult (Nais 2001). Other than *R. arnoldii* and *R. hasseltii*, *R. cantleyi* Solms-Laubach was found growing 2 m above-ground [Susatya (2011); also reported by Nais (2001)], 4 m above-ground for *T. tuberculatum* Latiff. Host (Heide-Jorgensen 2008), and in the Philippines, 10 m above-ground for *R. leonardi* Barcelona & Pelsler, *R. lobata* Galang & Madulid, *R. manillana* Teschem., and *R. speciosa* Barcelona & Fernando (Barcelona *et al.* 2008).

According to Susatya (2011; Figures 6a–c), there are three orders of interaction between *Rafflesia*, *Tetrastigma*, and

surrounding trees in a forest: 1) *Rafflesia-Tetrastigma* endophytic parasite and its host interaction, 2) *Tetrastigma* liana interaction with a single tree where it climbs, and 3) *Tetrastigma* interaction if there are multiple trees nearby. It is possible that the *Tetrastigma* root rhizosphere becomes exposed if it climbs on a growing tree that grows faster than *Tetrastigma*, and when the tree becomes tall enough, the lower part (the root) of *Tetrastigma* is pulled upwards and becomes more erect (*i.e.* it becomes an aerial root) (Figure 6d; 1–3). This could lead to the misidentification of *Tetrastigma* stems and roots. This possibility might be further supported if more than one tree is involved since *Tetrastigma* grows across trees (Figure 6d; 4–5). Thus, if the *Rafflesia*-infected *Tetrastigma* root became erect due to the pulling of adjacent trees (caused by their natural growth), the *Rafflesia* flower bud growing in this root would be aerial. In this scenario, where the *Tetrastigma* root is exposed to air due to adjacent tree growth, it is more likely that *Rafflesia* is exclusively a root parasite.

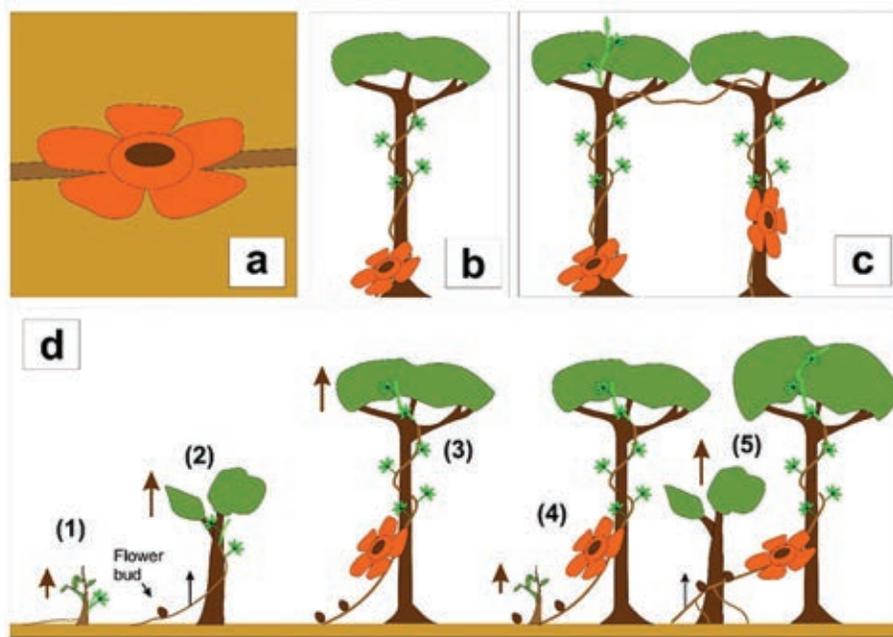


Figure 6. Interactions between *Rafflesia*, *Tetrastigma*, and surrounding trees [inspired from Susatya (2011)] showing the *Tetrastigma* vine and *Rafflesia* bloom (a), the *Tetrastigma* and tree where it climbs to (b), the *Tetrastigma* within a population or community of trees (c), and hypothetical growth as it is affected by nearby trees (d). The *Tetrastigma* initially grows close to a sapling and reaches the sapling crown (1). As the tree grows and since the *Tetrastigma* vine is already attached to the apical region of the tree, the lower part of the *Tetrastigma* is pulled up; thus, when the *Tetrastigma* root is already infected by *Rafflesia*, the root is pulled slightly up, causing the *Rafflesia* bud to grow as semi-aerial flower (2). Later, as the tree matures, its apical growth causes the *Tetrastigma* root to rise further; therefore, when the *Rafflesia* inside the *Tetrastigma* grows, it results in aerial buds (3). Additionally, if another tree sapling nearby is growing in proximity to the *Tetrastigma* root vine (4), when it grows, it may cause the roots of *Tetrastigma* to be pulled even further up, allowing *Rafflesia* to grow at an even higher part on elevated roots (5). Flower size and tree size not to scale.

Limitations, Challenges, and Future Prospects

Despite several sightings of aerial *Rafflesia*, none of those studies characterized the histology or anatomy of the aerial flowers and/or the endophyte's host to confirm if it truly grows in the stem of *Tetrastigma*, or in the root. Previous studies by Nikolov *et al.* (2014) and Mursidawati *et al.* (2019) characterized root-borne *Rafflesia* species histologically. Additional future studies of stem-borne *Rafflesia* flowers using our novel method of sampling will inform how the parasitic tissue interacts with *Tetrastigma* stem tissues, and also if the *Rafflesia* endophytes are actually able to grow or develop on a *Tetrastigma* stem. From Figures 3b (*Tetrastigma* stem) and 5c (possible *Rafflesia* growth on the stem), compared to Figures 3a (*Tetrastigma* root) and 5d (*Rafflesia* growth on the root) – as documented by Mursidawati and Sunaryo (2012) and later by Nikolov *et al.* (2014) – the *Tetrastigma* vascular bundles in the stem are radially more compact than in the root. These differences in vasculature density might affect the endophyte growth of *Rafflesia* from *Tetrastigma* vascular cambium (Mursidawati *et al.* 2019). As root vasculature is loose, could it allow the parasitic endophyte to grow ideally to a mature state, and does the densely packed vasculature of the stem also contribute to the rarity of aerial *Rafflesia*? This question can only be concluded through future observations, despite the challenges involved with sampling above-ground stem tissue. Another challenging requirement is to develop a strong and sharp tool that can be used to collect wedge samples more easily, without causing excessive tissue damage, because aerial *Rafflesia* buds occasionally grow in very thick *Tetrastigma* vines. Moreover, a comparison of apical growth rates between *Tetrastigma* and commonly found trees on the *Tetrastigma* – and which are often infected with *Rafflesia* – in their natural habitat should be studied in order to confirm if the *Tetrastigma* root can be exposed due to the upward growth of an adjacent tree (referring to Figure 6d). Despite the challenges and limitations, this new partial wedge sampling method indicates that the sampled mother vine (both root and stem) is able to continue growing. This signifies that the sampling method is less destructive than severing the whole vine or even removing a full wedge sample (sampling the tissue to the full radius of the vine, and reaching the core of the vine), which may kill the entire *Tetrastigma* plant and the whole *Rafflesia* endophyte if the *Tetrastigma* is infected. This new methodology is particularly useful given the scarcity and conservation concern of most *Rafflesia* species.

CONCLUSION

The sampling of erect and aerial (canopy) vines using partial wedge sampling reveals that roots might also be

found among erect vines, apart from the stem. From our result, the sampled part (wedge or partial wedge) does not have to be returned in order for the mother vine to survive and continue growing, although – if returned – the wedge tends to dry out. As the stem occupies the full aerial and canopy region of *T. leucostaphylum*, the erect root might result from external factors such as an upwards pull by the growth of adjacent plants/trees on which the *Tetrastigma* climbs. However, actual observations of *Tetrastigma* with an aerial vine where a *Rafflesia* flower grows using this new sampling method are required to confirm if the aerial *Rafflesia* is growing in a *Tetrastigma* stem, or if it is growing in an erect and exposed *Tetrastigma* root.

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NOTES ON APPENDICES

The complete appendix section of the study is accessible at <http://philjournsci.dost.gov.ph>

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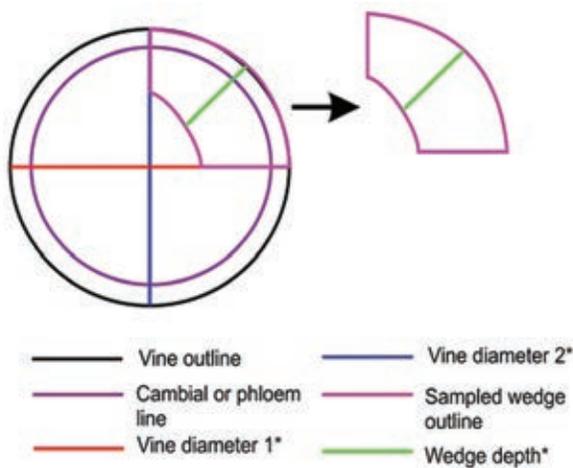


Figure I. A schematic of the vine and sampled part. Note that the sampled wedge (magenta) depth (green) does not pass through the central area of the vine (the core; intersection of two perpendicular diameters, red and blue); hence, it is only a partial wedge and not a full wedge (*i.e.* not cut to the center). Wedge sampling has to reach the vascular cambium or phloem ring (purple line) of the vine for root/stem identification of the vine. Details of the partially sampled wedge can be found in Appendix Table I, which covers the measured values (indicated as asterisks, *) of the characters described above.

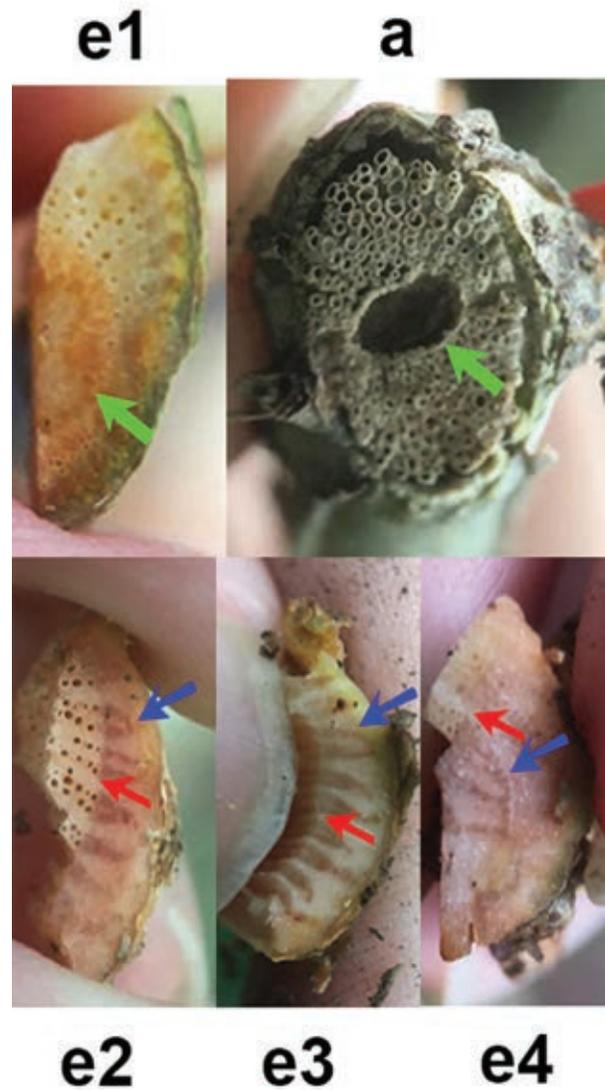


Figure II. The collected vine wedges taken from erect (e1–4) vines compared to the dead aerial (a) vine. The stem vine is characterized visually by the existence of pith in the core area (green arrows), while root vine is characterized visually by the ramified phloem bundles (blue arrows) and gap (red arrows) between xylem bundles. Note: e1 is the same part in Figure 4d2, e1 is to Figure 4i, and e2 is to Figure 4e2.

Table I. The sampled wedge depth and comparison to the sampled vine (mother vine) cross-sectional diameters.

| No. | Sampled wedge depth (cm) | Vine diameter 1 (cm) | Vine diameter 2 (cm) | Vine organ |
|-----|--------------------------|----------------------|----------------------|------------|
| 1 | 0.6 | 1.5 | 1.5 | Stem |
| 2 | 0.65 | 3 | 3 | Root |
| 3 | 0.75 | 2.5 | 2.5 | Root |
| 4 | 0.9 | 12 | 2 | Root |

Note: 1) only erect aerial vines data (Figures 1b and 4c–f) is included for this sampling, as the canopy aerial vine (Figure 1a) is documented in full cross-section (Figure 4i); 2) see Appendix Figure 1 for illustrations.