

TISSUE DIFFERENTIATION OF THE EARLY AND THE LATE FLOWER BUDS OF *RAFFLESIA PATMA* BLUME

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Abstract: The flower bud of *Rafflesia patma* Blume following the protocorm stage of the flower will undergo rapid cell growth followed by the differentiation stage which lead to the later stage of the flower bud morphogenesis into the floral organs. During this transitional period of the flower bud, we revisited our *R. patma* flower bud microscope slide samples and some images from the previous study in [MURSIDAWATI & SUNARYO, 2012] to examine how the *R. patma* tissue behave prior to the late differentiation stage. We discovered that there are several types of meristematic cells in the *R. patma* early flower bud tissue: The elongated cells in the basal/proximal region of the bud where it in proximity with host xylem, then a column of non-elongated cells where ovary will develop in the later stage (in female flower), and in the apical/distal region of the flower bud, we found a densely packed meristematic cells where in the later flower bud this area will be developed into the protective bracts, perigone lobes, and central disc as later seen in the late flower bud tissue. During the late stage of the flower bud growth, the flower bud also inhibits growth of 1-2 vascular bundles, altering few others host vascular bundles surrounding the flower bud, while on the other side the root vascular bundle growth is just normal. This growth mechanism can minimize the host vasculature damage if multiple buds are growing the same growth direction.

Keywords: histology, holoparasite, parasitic plant, plant development, Rafflesiaceae.

Introduction

Rafflesia growth within its host is shrouded with mysteries. In previous studies, the developmental origin of the organ has been observed by NIKOLOV & al. (2013) using cloning of the RNA isolation from MADS-Box genes (B-class lineages *PISTILLATA* [*PI*] and *APETALA3* [*AP3*], and C/D-class genes *AGAMOUS* [*AG*] and *SEEDSTICK* [*STK*]) using *Rafflesia tuan-mudae* Becc. and *R. cantleyi* Solms-Laubach. Then histologically, the early pre-bud endophyte form within was observed in NIKOLOV & al. (2014) and MURSIDAWATI & al. (2019), showing the uniseriate strands and cell clusters within the host vascular cambium, respectively. In term of the whole process of the flower bud growth, *Rafflesia* requires at least 2-3 years in its meristematic stage inside the host plant of *Tetrastigma* [HIDAYATI & al. 2000]. The process of the flower bud development study by AMINI & al. (2019) in *R. cantleyi* revealed that *Rafflesia* has expressed different signaling transcription factors and genes involved with auxin, cytokinin, gibberellic acid (GA), abscisic acid (ABA), and jasmonic acid (JA). In a tissue culture study by SUKAMTO & MUJIONO (2010), the callus generation of the flower were successfully induced using a synthetic auxins, picloram and auxin-mimicking herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), while at the moment, a cytokinin zeatin was also added but shown no effect in callus growth and

TISSUE DIFFERENTIATION OF THE EARLY AND THE LATE FLOWER BUDS OF RAFFLESIA ...

differentiation. As auxin regulates cell elongation and cytokinin regulates cellular division [TAIZ & al. 2015], how come auxin had more roles in SUKAMTO & MUJIONO (2010) callus generation study while cytokinin did not show any visible effect? Is there any auxin-related cellular differentiation (i.e. elongation) within the *Rafflesia* tissue during early development?

A previous study by MURSIDAWATI & SUNARYO (2012) focused on the flower bud development in more general approach. In the study, there are 3 phases of *Rafflesia patma* growth observed: 1) Phase I – host penetration during early germination stage, 2) Phase II – intrusion/invasive stage, where the flower bud starts to grow and affecting the host vascular tissue morphology, and 3) Phase III – flower establishment stage to matured flower bud prior to anthesis. Also this study, the perigone primordial, the processes primordial of the *R. patma* can be seen. Later updated in MURSIDAWATI & IRAWATI (2017), the fourth phase was added, Phase IV – conductive stage, where the flower bud is already established, taking nutrients from the host (host vascular tissue has been successfully hijacked), and preparing to the anthesis stage. However, this *R. patma* microscopical study in MURSIDAWATI & SUNARYO (2012) has actually more information and data, if the specimen is observed even closer. So using the unused data and available large resolution images from MURSIDAWATI & SUNARYO (2012) figure 3B, and 4A and B (used with permission), extensive new study is performed to analyze the *R. patma* cellular features from the condition of early flower bud differentiation until the later flower bud when the primordial bracts and perigone lobes are developed.

Materials and methods

***Rafflesia patma* samples**

Rafflesia patma bud samples were taken from infected *Tetrastigma leucostaphylum* (Dennst.) Alston ex Mabb root section (to minimize the damage, compared to sampling the stem/aerial flower bud sample) on Pangandaran Natural Reserves, West Java, Indonesia at early and late stages of flower buds growth in 2012 [MURSIDAWATI & SUNARYO, 2012]. The early bud (Figure 2-4 in this study) was approximately 3 months, the later stage (Figure 5, a-c) was 4 months, 5 months, and 5 months (same age). The flower bud age was determined since as it swell on the host periderm. The endophyte – protocorm (primordial flower bud) transitional age inside the host cannot be determine since there was no visual clue to measure. The *R. patma* whole plant age also cannot be defined since, 1) the plant was from its natural habitat and during endophyte stage, the plant has no visual clue since it is microscopical (we couldn't even determine if the buds belong to the same *R. patma* individual) and 2) some *R. patma* within one host plants could be originated from primary infections (parasitic seed invades uninfected host) while the other might come with secondary infections (parasitic seed invades already infected host). Nevertheless, the timeline of *Rafflesia* individual growth stages can be seen in Figure 1.

Microscopical staining and image analysis

The microtomy results are from the unused microscope slides data (observed on Olympus CX31 optical microscope; Figures 2-4 in this study) and some images (Figure 5 in this study) are reproduced from MURSIDAWATI & SUNARYO (2012) with permission. The microscopical staining followed MA & al. (1993) with safranin staining, which colorized the tissue with tannin, lignin and suberin, and fastgreen, which colorized cytoplasm.

The microtomy procedure was using BERLYN & al. (1976) paraffin method and then sliced (microtomized) with Yamato RV-240 (Yamato Kohki Industrial Co., Ltd., Saitama, Japan) and were sliced for 20 μm thick. This same procedure was later used for later study [MURSIDAWATI & al. 2019; MURSIDAWATI & al. 2020]. The pictures are enlarged and labeled during analysis in this observation using Adobe Photoshop CS6, while no labeling and extensive zooming were performed in MURSIDAWATI & SUNARYO (2012). The zooming in this study were performed to extensively analyze the *R. patma* flower bud cells and primordial tissue during differentiation as well as to observe the effect of the *R. patma* flower bud to the host (*T. leucostaphyllum*) tissue (mostly the vascular tissue: the secondary xylem, vascular cambium, and secondary phloem). The image background and scale bars were edited from MURSIDAWATI & SUNARYO (2012) also using Adobe Photoshop CS6.

Results

The overview of the early flower bud

At 3 month stage, where the flower bud tissue have not yet differentiated into flower organs, the flower bud tissue are composed of three types (based on the cell shape), separated within the proximal (closer to the host secondary xylem), middle, and distal region (closer to the host periderm), as can be seen in Figure 2. The first part is in the proximal region, where the flower bud tissue cells are elongated and proximal-distally oriented. The second part is the tissue with non-elongated cells in the middle. As the shape is more uniform and based on its location, the cell could developed into flower parenchyma tissue which shapes the flower, and in female flower, the middle of the flower is the place where the ovary of the flower developed [NAIS, 2001]. The third part is the tissue in the distal region. The tissue in this area is densely packed, the cell size is small, and highly meristematic. This meristematic cells located in the distal-most region to the small portion of lateral area in the middle region.

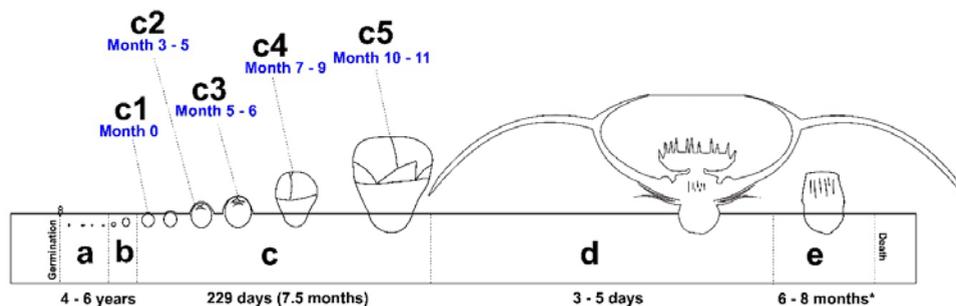


Figure 1. Timeline of a single *Rafflesia* from germination to endophyte stage (a), then to protocorm or primordial flower bud stage (b), flower bud stage (c), anthesis stage (d), fruit stage (e), and death. During flower bud stage, there are sub-stages: Swollen host stage (c1), the flower bud age measurement starts after this stage. Then cupula stage (c2), where the flower bud swells in the host periderm layer, forming the cupula of flower. As the host periderm breaks, the flower bud enters the transitional cupula-bract stage (c3), before the bract fully emerges and becomes bract stage (c4). In the end of flower bud stage, the second transition occurs when the perigone lobes enlarge from the bract in bract-perigone stage (between c4 and c5), and finally the final stage of the flower bud prior anthesis, the perigone stage (c5). Of all age calculation, stage (a) and (b) cannot be determined since the stages are microscopic and has no visual clue. Ages in this figure is based on HIDAYATI & al. (2000), the naming of the flower bud stages is based on SUSATYA (2020). The flower bud ages are based on unpublished personal observation on *R. patma* in Bogor Botanical Garden, Indonesia.

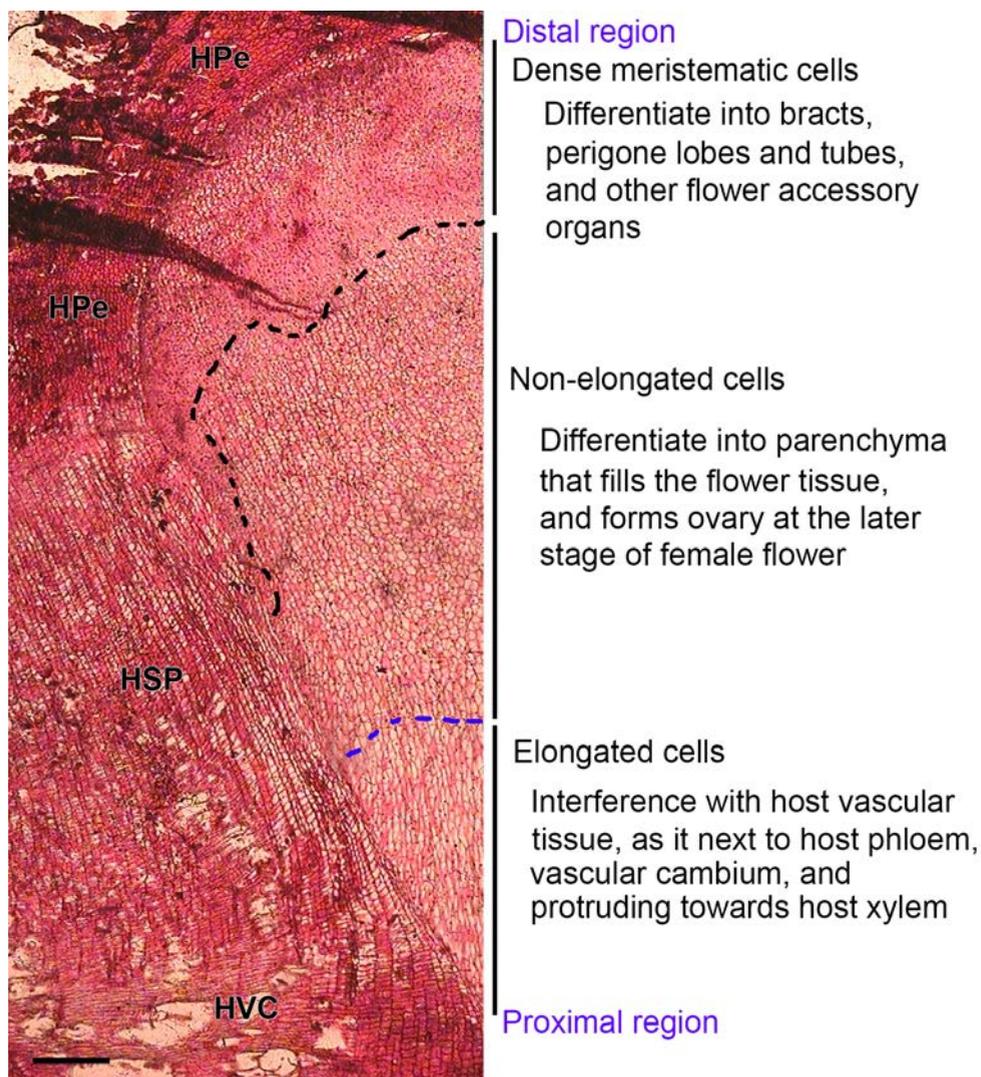


Figure 2. The three cell regions of the early *R. patma* flower bud saffranin-stained sample. At this stage, the cellular regions comprise of the dense meristematic cells at the distal region where it can differentiate into protective bracts, perigone lobes, and central disc (see later at Figure 5), the non-elongated cells at the middle region where the cells grows into parenchyma cells and where ovary supposed to develop at female flower, and the elongated cells at the proximal region where the tissue is pointing towards the host xylem area. HPe – Host periderm, HSP – Host secondary phloem, HVC – Host vascular cambium. Scale bar = 1 mm. The black and blue dots separate the cell regions. The dark area at the distal region is the bent HPe tissue during microtomy slicing. Note: This same image in this figure is enlarged in Figure 3 and 4. The picture are compiled from 4×10 magnification microscopical images. Scale bar = 1000 μm.

The distal region of the *R. patma* early flower bud

The distal region are packed with meristematic tissue. When observed closer in 10×10 magnification, the distal-most region is the where most meristematic cells proliferates, signified by the smaller-sized cells with greater quantity than its surroundings (Figure 3; *R. patma* meristematic distal-most region – RMD). Proximally from the distal region, some cells appears uniform in sizes and appears to have completed their cell divisions, but some are still also appear meristematic (Figure 3; *R. patma* meristematic region – RM).

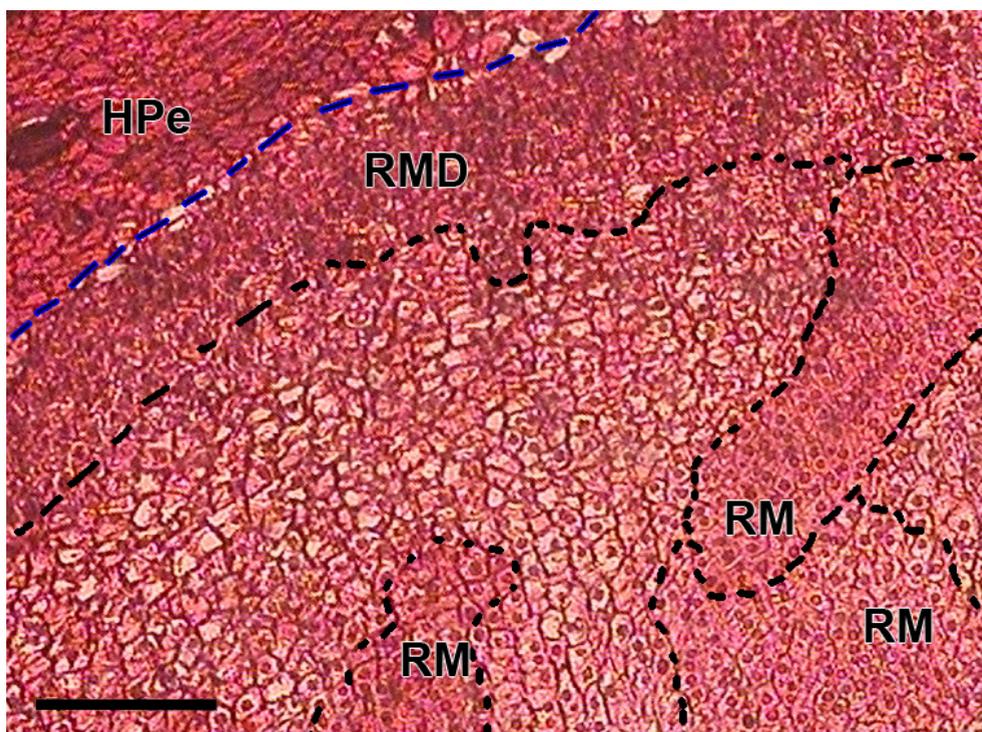


Figure 3. The heavily and dense meristematic part of the *R. patma* flower bud distal (apical) region. This area supposedly grows the flower bud further to the host periderm (HPe) area, as the *R. patma* meristematic distal tissue (RMD) (close to intersection with *T. leucostaphylum* tissue; black-blue dotted intersection) proliferates, The *R. patma* meristematic tissue (RM) shown with densely packed cells bordered with the dotted regions. This are could be where the protective bracts and perigone lobes developed. Note: This figure is the same sample and enlarged from Figure 2. Magnification 10×10. Scale bar = 250 μm.

The basal/proximal region of the *R. patma* early flower bud

In the middle region of the flower bud, the cell sizes are larger than in the distal region and the sizes are relatively uniform (Figure 4, a and b; see *R. patma* parenchyma tissue – RP) and supposedly matured as parenchyma tissue. In the lateral size, the dense, smaller meristematic cells can also be found where it extended from the distal region (Figure 4b; see *R. patma* meristematic lateral tissue – RML). The flower bud tissue in this study are surrounded by the host (*T. leucostaphylum*) secondary phloem region (Figure 4 and 5; see host secondary phloem – HSP). However, until now the method of *Rafflesia* to obtain nutrient is still unknown.

TISSUE DIFFERENTIATION OF THE EARLY AND THE LATE FLOWER BUDS OF *RAFFLESIA* ...

As the mature *Rafflesia* flower relies on xylem and vascular parenchyma to distribute nutrients [MURSIDAWATI & al. 2020], it is possible that the outermost parenchyma cells of *Rafflesia*, in this case *R. patma* contribute in nutrient gathering from the host phloem close to it. The parenchyma direction at the host-parasitic intersection border of this region is that the parenchyma cells are oriented in parallel to the laterally surrounding phloem.

In the proximal region of the flower bud, the tissue cells are elongated (Figure 4; see *R. patma* elongated cells – RE), with proximal-distal cell orientation. Compared to the parenchyma cells in the middle region, is at the host-parasitic intersection border, the elongated parenchyma cells (we assumed that those are still parenchyma cells despite in different shape) in this region is oriented almost perpendicular to the laterally surrounding host secondary phloem. On the later stage (Figure 5c), these elongated parenchyma cells are fully perpendicular oriented towards the host secondary xylem at the proximal end host-parasitic border.

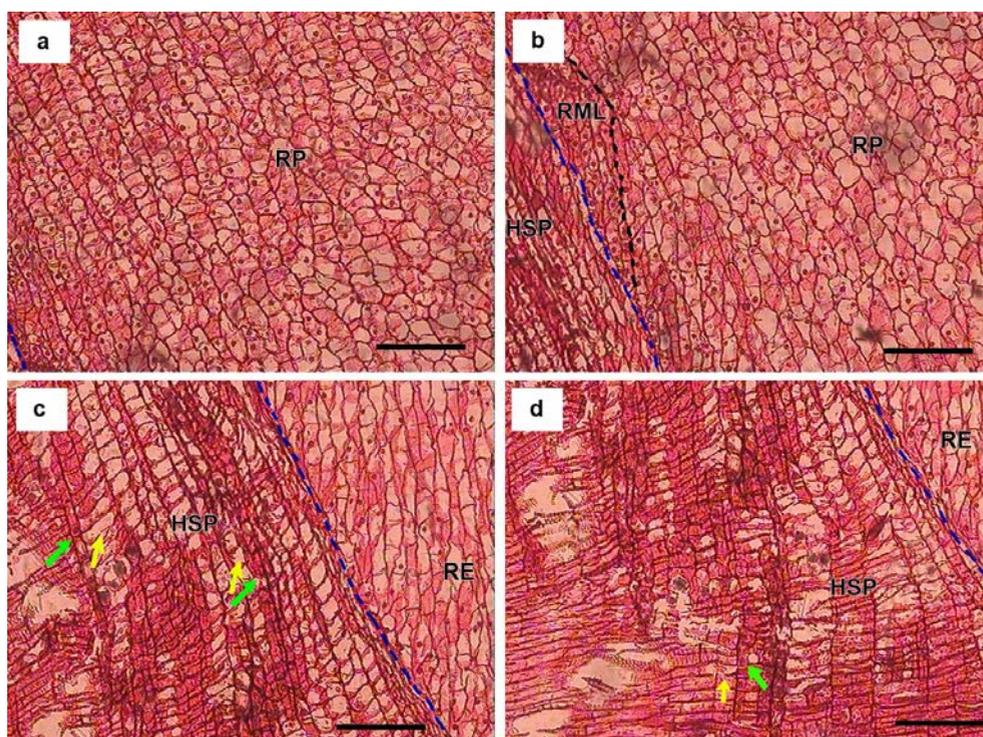


Figure 4. The middle (a and b) and proximal region (c and d) of *R. patma* side-by-side with *T. leucostaphylum* tissue layer. The observed *R. patma* tissue are mostly the non-elongated parenchyma cells (a) at the middle area of the bud tissue. At the intersection area (b) the tissue has the lateral meristematic cells (RML) signified with the smaller size of the cells extended to the distal region, and located close to the host-parasite border (blue dots) next to the *T. leucostaphylum*, and the RP of *R. patma*. Closer to the proximal region (c and d), there are no transitional meristematic cells seen, but the *R. patma* cells are more elongated (RE) and located directly to the host-tissue intersection borders (blue dots) next to the *T. leucostaphylum* (host) secondary phloem (HSP) layer. This HSP layer can be identified by the phloem companion cell (green arrows) and the sieve tube cells (yellow arrows). Note: This figure is the same sample and enlarged from Figure 2. Magnification 10×10. Scale bars = 250 μm.

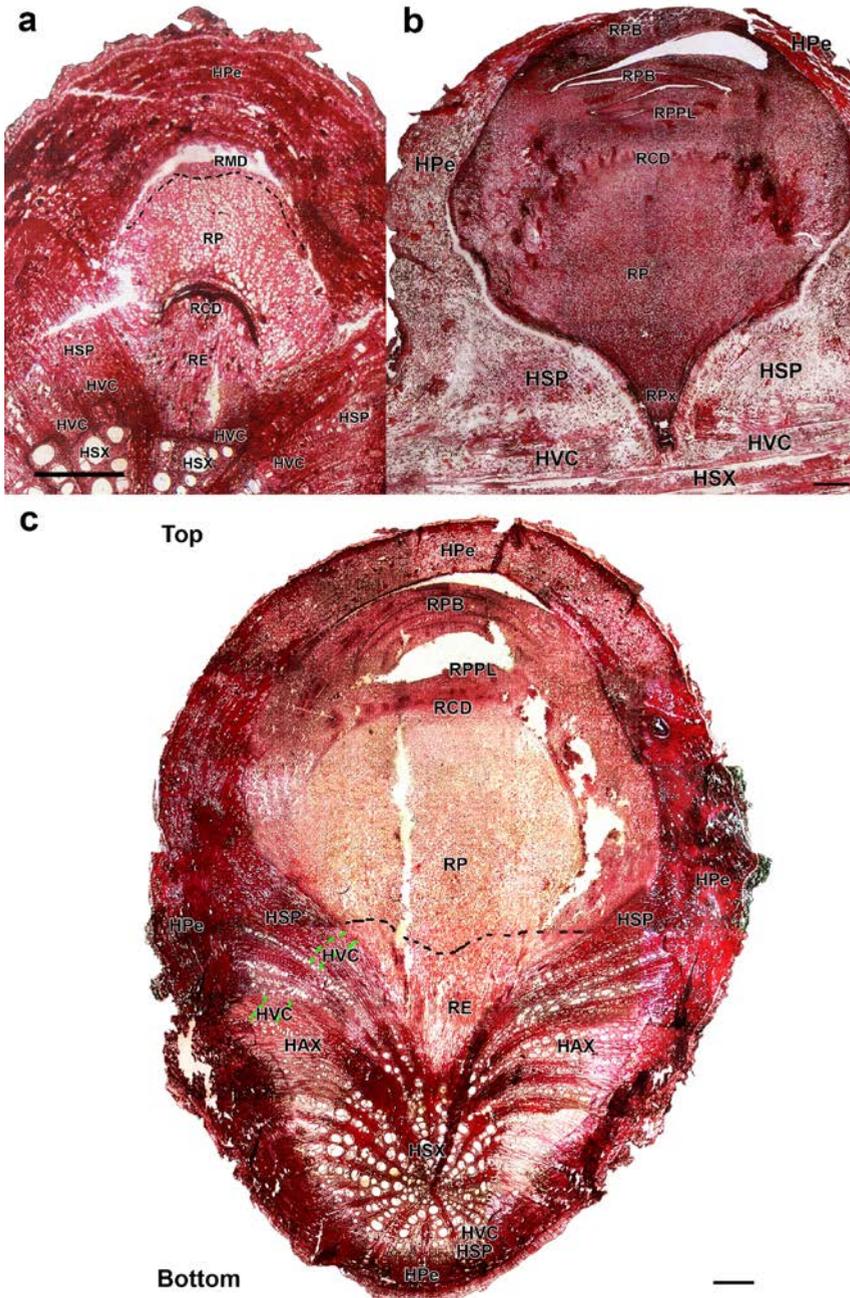


Figure 5. Transition of early to late stage of *R. patma* flower bud development and its interaction with the host, *T. leucostaphyllum* tissue. At early stage (a), the primordial central disc of *R. patma* area (RCD) can be seen distally to the elongated cells (RE) and proximal to the *R. patma* parenchyma cells (RP). At the most distal area of this stage, there are still no bracts or perigone lobes primordial, yet the

TISSUE DIFFERENTIATION OF THE EARLY AND THE LATE FLOWER BUDS OF RAFFLESIA ...

distal meristematic area (RMD) with small-sized dense tissue can be seen. The flower bud is still covered by thick layer of the host periderm layer (HPe). In the proximal region, the *R. patma* flower interrupts the growth of host vascular cambium (HVC), making no phloem growth at the bud area. Later on the later stage (b), for longitudinal cross section and c for transversal cross section, the flower bud elongated cell area (RE) has interfere many part of the host tissue growth. Especially can be seen in c, the vascular cambium (HVC; marked with green dots) area at the top are pushed laterally by the *R. patma* parasitic tissue, and thus are affecting the xylem growth and resulting at the host anomalous xylem area (HAX), as for host secondary phloem (HSP) compared to the bottom part of the *T. leucostaphyllum* where the HVC and HSP are still able to be found as orderly concentric vasculature. In this stage, *R. patma* has more RP, engorging it towards HPe. The RMD has developed RCD, primordial perigone lobes (RPPL), and primordial bracts (RPB). It will continue to grow toward HPe (from c to b), where the bud tissue breaks HPe, revealing the RPB part of *R. patma*. Some cracks can be seen in (a) and (c) due to the fragility of the tissue during microtomy slicing. Pictures are compiled from 4×10 magnification microscopical images. Pictures are reproduced and edited by permission from MURSIDAWATI & SUNARYO (2012). Scale bars = 724.4 μm.

The late development of flower bud

At the late flower bud, it appears that the primordial central disc are formed first (Figure 5a; the bud age: 4 months), and in the next stage, the primordial structures of early processus are formed (Figure 5b and c; the bud age: 5 months; see RCB – *R. patma* primordial central disc). Then, the primordial of protective layers, the perigone lobes and bracts are formed (Figure 5a to c and to b; where the *R. patma* distal meristematic region – RMD in Figure 5a, developed into *R. patma* primordial perigone lobes – RPPL, and *R. patma* primordial bracts – RPB in Figure 5c; and finally in Figure 5b, the bract becomes exposed as the host periderm – HPe breaks; also known in cupule-bract transition stage in SUSATYA (2020) where the emerging bract color is still pale). Despite the bud ages are the same, bud in Figure 5b could emerge first probably determined by how thick is the peridermal layer (the root cork) of the host at the bud growth area. Also at the later stage, the proximal region of the flower bud appears to forms a pointed tissue towards the host secondary xylem region (Figure 5b and c). It shows that instead of occupying the entire region of xylem as sinker cells (observed in *Cytinus*; DE VEGA & al. 2007; and *Pilostyles*; KUIJT & al. 1984), *R. patma* proximal region “sinks” only towards 1 or 2 vascular bundles as shown in Figure 5a (approximately it attached to 2 vascular bundles area) and 4c (approximately attached to 1 vascular bundle area). However, despite the low coverage of the host vascular bundle, the flower bud proximal growth greatly alters the growth and positioning of the host vascular cambium and host secondary phloem (Figure 5a and c). On the other hand, the flower bud lateral growth also presses the host vascular cambium laterally, to the point that the host vascular cambium anomalously form abnormal xylem growth (Figure 5c; see host anomalous xylem vasculature – HAX) and the host secondary phloem in the affected area bent and grows laterally. In Figure 5c, it can also be seen the great difference between the *R. patma* affected *T. leucostaphyllum* tissue (top area) and the unaffected *T. leucostaphyllum* tissue where the vascular bundles are uninterrupted and growth concentrically as normal (from proximal to distal: secondary xylem, vascular cambium, secondary phloem, and periderm).

Discussion

The composing cell differences in early flower bud different regions

Rafflesia patma cellular division rate seems to be differ on each stage. During endophyte stage, the *Rafflesia* cells can be distinguished from its surrounding host cells by its large nuclei appearance (also seen in this study, where *R. patma* cells are more visible than its surrounding host cells), which might lead to possession of large genome size, leading to slow growth due to longer time required for genome replication [NIKOLOV & al. 2013]. At this later stage (this study), it appears that the cell division of *R. patma* cells happen more rapidly. It is not yet clear if there a change in molecular level which regulates this shift of cellular proliferation rates.

The distal region of the early stage of the flower bud (age: 3 month) are composed mainly by the meristematic cells. This part serves as the “apical part” in analogue to the other plant where the apical meristem. The middle region is composed of the non-elongated parenchyma cells, while in the proximal region is composed of the elongated parenchyma cells. Based in the study in MURSIDAWATI & al. (2019), *R. patma* grows from the host vascular cambium, forming the “basal part” in the proximal region of the *T. leucostaphylum* tissue where the host secondary xylem can be found, then the rest of growth are occurred distally towards the host secondary phloem layer, and finally to the periderm. At early stage (endophytic-protocorm transitional stage, as in MURSIDAWATI & al. 2019), the proximal cells of *R. patma* is still not as elongated as in the early flower bud growth in this study. However, the distal region is already composed by the meristematic, proliferating cells.

Apparently, parenchyma tissue in the early stage between the middle and the proximal region has difference in orientation towards the laterally surrounding host-parasitic border. It is unclear if there are differences of functions between the parenchyma tissue in the middle and in the proximal region. The host-parasitic border parallel parenchyma tissue in the middle are laterally surrounded by host secondary phloem. It is possible, that the parenchyma cells could help to acquire the nutrients from the host tissue. The case in *Cytinus*, despite the parasitic tissue possess its own parasitic xylem and phloem, no xylem-xylem and phloem-phloem [DE VEGA & al. 2007], thus it is proposed that the parenchyma tissue is helping the nutrient distribution by cellular wall between the host and parasite via apoplastic flow continuum [COETZEE & FINERAN, 1987; KUO & al. 1989]. DE VEGA & al. (2007) also mentioned that the parenchyma cells with thickened walls may act as transfer cells, providing interaction between host and parasite, helping with nutrient absorption, transport, and distribution of the photoassimilates between the host and parasite. In the *Cytinus* study by DE VEGA & al. (2007), no parenchymal cell shape difference and orientation detected, as it just serves as the endophytic tissue surrounding the parasitic xylem and phloem, adjacent to either host phloem or xylem. It is unclear if the cell shape and orientation have specific application to the *Rafflesia* bud. Reflecting to this condition, it is possible that the proximal region parenchyma of early flower bud helps with photoassimilates absorption, transport, and distribution from the adjacent host phloem via the apoplastic flow mechanism as in *Cytinus*, or it points proximally as it specifically absorbs the photoassimilates from the host phloem. Additionally, as the elongated parenchyma cells are oriented proximal-distally and seemingly pointed toward the xylem, Figure 5b and c show that the contact are with xylem is not actually visible (no visible intrusion or side-by-side intervention) compared to the direct phloem contact which is just laterally in the bud proximal region. The proximal point of late flower bud is rather conical in shape, suggesting that it could be analogous to the root function and if the absorption occur only on the elongated cell of the bud, its function is possibly be to regulate as well to optimize the nutrient absorption despite the abundance of the host secondary

phloem in the middle-proximal region of the bud and avoid too much absorption when the flower bud grows bigger which could affect the host condition. Up to date, it is known that *Rafflesia* does not store starch on its tissue, compared to the surrounding host tissues (NIKOLOV & al. 2014; observed at the same late flower bud stage in WICAKSONO & MURSIDAWATI, 2020), indicating that *Rafflesia* directly takes the sugar photoassimilates from its host and this requires intake regulations.

This suggestion, however, have not been documented or studied and it is still possible if the non-elongated parenchyma also takes role in nutrient absorption as in some certain condition (Figure 5c) the non-elongated parenchyma position is closer to the host phloem rather than the elongated ones which are closer to the host xylem vascular bundles. This penetration towards between the host xylem vascular bundle regions might provide better grip as the bud grows into fully developed flower, hence making it more analogous to the taproot. Additionally, despite there are no sign of xylem intrusion, the bud elongated parenchyma cells closer to the host xylem could serve as water collector. Also this elongated parenchyma cell morphology is also similar to the vascular parenchyma cells as in MURSIDAWATI & al. (2020), further suggesting its function in nutrient distributions from host.

In term of differentiation of the elongated parenchyma cells, it is unclear if auxin plays some role in the cellular elongation from early condition of parenchyma cells shown in MURSIDAWATI & al. (2019). Cell elongation by auxin is mediated by loosening the cell wall, allowing more water into the cell [MAJDA & ROBERT, 2018]. As the most proximal region elongated cells are also located in proximity to the host xylem as the water source, this positioning might also explain how the parenchyma cells get elongated possibly by auxin regulation. Auxin has been studied to initiate the flower primordium [ALABADI & al. 2009; FAN & al. 2015], but the cytokinin affects flower bud formation, size, ovule formation, and seed size [VAN DER KRIEKEN & al. 1989; BARTRINA & al. 2011]. However, it is unknown if the auxin is synthesized on the distal region of *Rafflesia* (analogue to the apical shoot; TAI & al. 2015), or host-originated. The elongation of the cells also require further physiological studies. As the *in vitro* study of *Rafflesia* is extremely hard to perform (so far, only SUKAMTO & MUJIONO (2010), has succeeded in callus generation but not followed by somatic embryo or organ generation), new procedure is required to observe the effect of added auxin and cytokinin to the flower bud of *Rafflesia*.

Differentiation of processes (central disc), perigone lobes, and bracts

The flower accessory organs of *R. patma* differentiation in this study appears to be in the later stage of flower bud (after 4 month old). In Figure 5a, the first to differentiate is the central disc, which later (Figure 5b and c), forms the spiky processes. At the same time (Figure 5c, then b), the primordial perigone lobes developed close to the central disc. Within this region of development, the perigone tube and its diaphragm will grow in much later stage. Compared to the perigone lobes, the bract developed first. This is because the bract serves as the protective layer to the flower once it emerged. According to SUSATYA (2020), the flower bud of *Rafflesia* starts as in cupula/cupule stage, then bract-cupule transition where the bract is emerged as a pale early bract, which later hardened and darkened in the next stage, bract stage where the bract is fully emerged and covering the flower bud distal region. Then the bract-perigone (lobe) transition stage where the bract revealed the perigone lobe within partially, perigone stage where the perigone lobe fully enlarged and revealed, and finally the *Rafflesia* blooms/anthesis stage. The fully emerged and hardened bract provides protective shell to the flower. Later, the protective early perigone lobe with smooth, waxy abaxial will open first before the late perigone lobe with rough, leathery abaxial [MURSIDAWATI & al. 2020].

The affected host vascular growth at later stage of flower bud

As previous studies [NIKOLOV & al. 2014; MURSIDAWATI & al. 2019], *Rafflesia* tend to grows in the *Tetrastigma* with secondary growth. Using the reference of the grapevine (*Vitis* sp.; also Vitaceae as *Tetrastigma*) root growth, which has the same anatomy, as the root grows from its primary growth stage to the secondary growth stage, the root cortex along with the endodermis will be pressed distally, forming the cork or periderm, the pericycle fills the portion where the cortex was as it moves distally as well, and contributes to the vascular cambium development (reference from hybrid grapevine, *Vitis berlandieri* × *rupestris* in GAMBETTA & al. 2013). It was known in the dicot secondary growth of root, the pericycle plays important role in periderm and vascular cambium development, aside to its role to develop lateral roots [BECK, 2010]. This anatomical details are important to determine the anatomical location of the *Rafflesia* flower bud.

During the later stage of flower bud (Figure 5, from a to c, to b), it appears that the distal growth will dominate, resulting to the smaller bud tissue in the proximal region and larger in the distal region. The impact of flower bud extensive growth in the late stage can be seen in both Figure 5a and c. The *R. patma* flower bud, as it grows from the vascular cambium towards the host periderm [MURSIDAWATI & al. 2019] will press the surrounding *T. leucostaphylum* vascular cambium layer and secondary phloem. As *T. leucostaphylum* vascular cambium layer pressed laterally, the organogenesis of the new vascular tissue will be altered. The new xylem located proximally from the flower bud will be no longer generated from the vascular tissue and similarly, no phloem will be generated from the pressed vascular tissue. Leaving only xylem in the *Rafflesia*-inhibited vascular bundle. The surrounding vascular bundle will be pressed laterally, causing irregularity in the vascular cambium phloem and xylem generation (Figure 5c; top area). Interestingly, host secondary phloem will be generated even longer surrounding the late flower bud proximal area, hence assumingly provide the flower with more nutrients. This alteration of host can be also observed in the other parasitic plant infestation of *Phoradendron crassifolium* (Pohl. ex DC.) Eichler on *Tapirira guianensis* Aubl., where its xylem lumen size are decreased, higher density of embolized vessel, higher vessel density, taller and wider rays, fibers with thinner cell wall, and the xylem growth bent laterally due to the parasitic growth [TEIXEIRA-COSTA & CECCANTINI, 2015]. The difference here is the xylem is mostly affected, especially *P. crassifolium* is a hemiparasitic plant.

The alteration of *T. leucostaphylum* root tissue by the *R. patma* bud tissue during late development occurred in 1-2 vascular bundles area only, leaving their vascular growth completely inhibited, few adjacent vascular bundle to grow with anomalous xylem area and elongated phloem area (Figure 5c, top). The rest of the root however, are continued to grow normally (Figure 5c, bottom). This strategy of minimizing host vascular damage probably is to allow the host to be alive as *Rafflesia* flower is massive (hence required large amount of nutrients), with chances of other flowers bud is growing in the same host plant (Figure 6). This 1-2 vascular inhibition (compared to *Phoradendron* where it affects multiple vascular bundles) reduced the chance of the host *T. leucostaphylum* death, which may lead to the death of the entire *Rafflesia* endophytes inside the host. In *Rafflesia* (referring to Figure 6), the scale of host vasculature damages are increased if multiple buds are growing in the different directions. However, if multiple buds are growing in the same direction (in series), the vasculature damages are assumed to be minimal. This condition would apply as a comparison of multiple buds grown in the same host root size. If the host root is larger, with more vascular bundles, the damage could be even more minimalized.

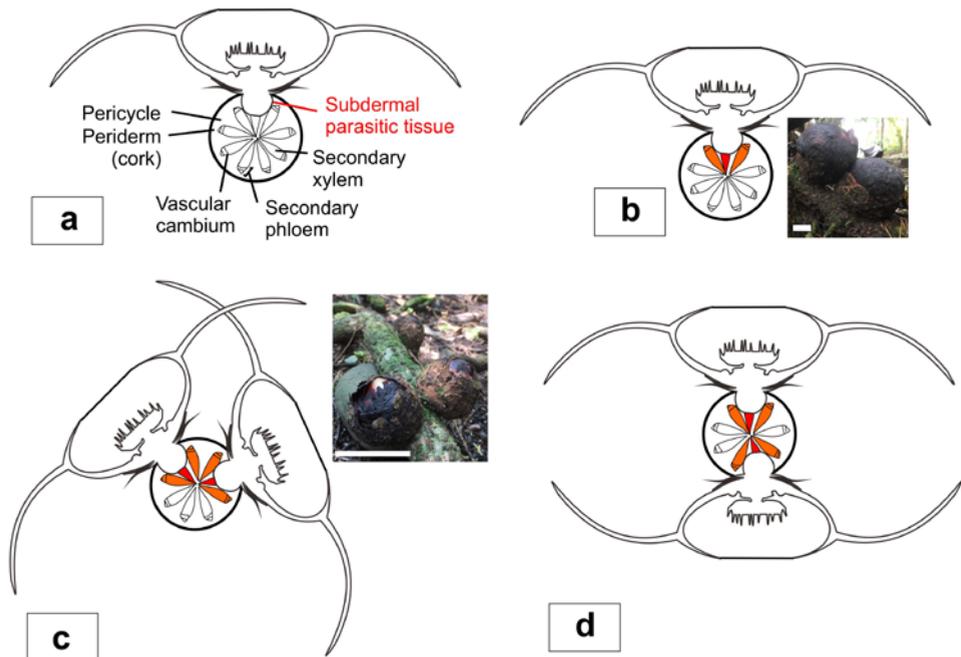


Figure 6. Interaction of *Rafflesia* and its host root in general. The host, *Tetrastigma* organ where *Rafflesia* grows is the root which has developed under secondary growth (it has cork layer, vascular cambium, the pericycle fills the area where cork previously is, and the epidermis and endodermis are no longer exist) (a). In this study, a single *Rafflesia* flower grows in a single vascular bundle cambium region (b), suppressing the secondary phloem and xylem growth in this one respective bundle (red), and affecting the neighboring bundles growth pushing laterally to make the bundles to become adjacent to the parasitic tissue (orange). It is suggested that their secondary phloem tissues become nutrient providers to the parasitic tissue. In case of multiple buds growth within the same vasculature line (in series and the same direction), the flower can also grow (inset; photo taken from September 2017). Other case in multiple buds growth, if both buds growth in some angle of angle difference (c) (and the inset; photo taken from July 2018), that means more damage to the host and more vascular bundles are affected. However, as *Rafflesia* affects the host vasculatures in at least 3 bundles, same host vascular damages as in (c) if both flowers are in opposite directions despite different scale of possible host tissue truncations (d). The host damage will be increased if more buds are growing in multiple direction angles, less damage is assumed if there are more buds bud in the same growth direction. The host stem size in a, b, and c are assumed at the same size. If the host size is larger, the host vasculature damage could be different. Photos are taken by Adhityo Wicaksono and are unused data. Scale bars = 5 cm.

Conclusion

The flower bud of *R. patma* has differences in the early stage and the late stage of development. On the early flower bud, the bud has three types of cells: densely packed and meristematic distal region, non-elongated parenchyma in the middle region, and elongated parenchyma in the proximal region. The distal region is contributing to flower first protective layers (bract and perigone lobes) development as well as the flower accessory organs (central disc with the processes). The non-elongated parenchyma in the middle region fills in the

flower tissue, helps in possible nutrient transport and distribution from the surrounding host secondary phloem. However, the elongated parenchyma might be more specifically to absorb nutrients from the host and provides structural grip, making it analogous to the root. The late flower bud develops primordial central disc first, followed by the primordial bract and perigone lobes. Also on the later stage of flower bud development, the flower bud enlarged and inhibits the growth of 1-2 host vascular bundle, leaving only the early xylem. Surrounding it, the host vascular growth are altered heavily with enlarged secondary phloem region and anomalous xylem region developed. On the other side of the host however, no disturbance is occurred and the root vascular tissues grow normally. This strategy of minimalizing vascular damage is assumed to prevent host death if multiple *Rafflesia* bud is emerging at the same time in the same direction.

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Conflict of interests

All authors declare no conflict of interests.

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TISSUE DIFFERENTIATION OF THE EARLY AND THE LATE FLOWER BUDS OF *RAFFLESIA* ...

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