

PRELIMINARY HISTOANATOMICAL STUDY OF *SAPRIA HIMALAYANA* GRIFF. F. *ALBOVINOSA* FLOWER BUDS AND ITS INTERSECTION WITH ITS HOST PLANT *TETRASTIGMA LAOTICUM* GAGNEP.

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Abstract: *Sapria*, a holoparasitic plant of Rafflesiaceae is still understudied in term of anatomical, even more in histoanatomical study. This study aimed to perform preliminary observation on *Sapria himalayana* Griff. f. *albovinosa* morphology on its early and late stage of flower bud development inside its host, *Tetrastigma laoticum* Gagnep. (Vitaceae). The results show the progression from the early flower bud as it starts to grow in the host vascular cambium area towards proximal direction to reach the host xylem and distal direction to reach the host phloem and to reach the next stage of the life cycle, into the late flower bud where differentiation occurs. In late flower bud, some primordial of the organs are visible as the flower bud grows larger distally towards the periderm. This development pattern is similar to the progression of development in the previous studies in *Rafflesia* and *Rhizanthus*, where endophytic growth was observable in both proximal (xylem area) and distal (peridermal). Future comparative study is encouraged, especially to compare between *Sapria* species and between different stages of growth. Although, an efficient method and less invasive way of sampling is encouraged to prevent decline of *Sapria* species in the wild.

Keywords: anatomy, flower, holoparasite, parasitic plant, Rafflesiaceae.

Introduction

Sapria is one of holoparasitic plant in Rafflesiaceae. Its distribution is restricted to the South China, East India, Myanmar, Vietnam, and Thailand region [AHMAD & al. 2020]. Just like two other genera of Rafflesiaceae, *Rafflesia* and *Rhizanthus*, *Sapria* is also a parasite of *Tetrastigma* (Vitaceae) liana. So far, *Sapria* consisted only by four species, *Sapria himalayana* Griff., *S. myanmarensis* Nob. Tanaka, Nagam., Tagane & M. M. Aung, *S. poilanei* Gagnep., and *S. ram* Bänziger & B. Hansen. All grown in specific *Tetrastigma* species as well, such as *S. himalayana* with *T. obovatum* (Laws.) Gagnep., *T. laoticum* Gagnep., and *T. cruciatum* Craib & Gagnep. [ELLIOTT, 1992], *S. poilanei* with *T. laoticum*, *S. ram* with *T. harmandii* Planch. [BÄNZIGER & HANSEN, 1997]. Only *S. myanmarensis* has undocumented *Tetrastigma* species.

Some papers refer *Sapria*, specifically *S. himalayana* to also grow in other Vitaceae, *Vitis* sp. [HUANG & GILBERT, 2004, *cit.* WEAVER, 105]. However, since no study has reported which species of *Vitis*, the speculation remained dubious, especially since some *Tetrastigma* species were once considered as genus *Vitis* until Jules Émile Planchon excluded the genus in 1887 [RAHAYU & al. 2018].

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Previous anatomical-histological study has been done in Rafflesiaceae. In *Rafflesia*, the study was by NIKOLOV & al. (2014a, 2014b), MURSIDAWATI & al. (2019, 2020), MURSIDAWATI & WICAKSONO (2020), and KAMAL & al. (2021). In the same literature by NIKOLOV & al. (2014a, 2014b), there are also anatomical study on *Rhizanthus*, and only in few parts on *Sapria* as endophyte.

Therefore, no study until now has reveal the anatomical development of *Sapria*. All three genera are known as root parasites of *Tetrastigma*, but only *Rafflesia* is known for its aerial form grown in the aerial/erected root of *Tetrastigma* liana, as alternative form to most found terrestrial form [MURSIDAWATI & al. 2021]. Both *Rhizanthus* and *Sapria* until now, only known to grow in terrestrial form.

This preliminary study aim is to reveal the general, semi-microscopic parasitic-host profile of *S. himalayana* Griff. f. *albovinosa* on its host *T. laoticum* on the early bud and later bud. This profile will provide first, preliminary information about *Sapria* and *Tetrastigma* parasite-host relationship. From this study, in the future, the profile between all Rafflesiaceae genera can be made.

Material and methods

Plant preparation

Host tissue is *Tetrastigma laoticum* Gagnep. infected with *S. himalayana* Griff. f. *albovinosa* (Figure 1). The specimen was taken in March 2019 at Lang Biang Plateau, Vietnam, specifically at Tuyền Lâm Lake, Cam Ly and Nam Ban Protection Forest (similar to the sampling area of the previous study, TRẦN & al. 2018). The plant materials were sent to Ho Chi Minh City Medicine and Pharmacy University, Faculty of Pharmacy for the staining and microscopy sample preparations.

Staining and microscopy analysis

The staining method applies carmine alum and iodine green, aimed to stain the lignified tissue in green while the rest part of tissue in red [LOCQUIN & LANGERON, 2013]. The samples were prepared by slicing manually by hand with razor for approximately 200 µm thick. Then, the samples were soaked in sodium hypochlorite/commercial bleach (NaHClO) for 15 minutes or until the samples were completely discolored, and cleansed with tapwater for 4 times to remove the bleach. To initiate the staining, the samples were soaked in acetic acid 10% for 5 minutes and rinsed, before soaked in carmine alum dye and iodine green for 15 minutes. The samples were then cleansed from the dye in tap water for 2 times and preserved in glycerol 50% until observation under light microscope.

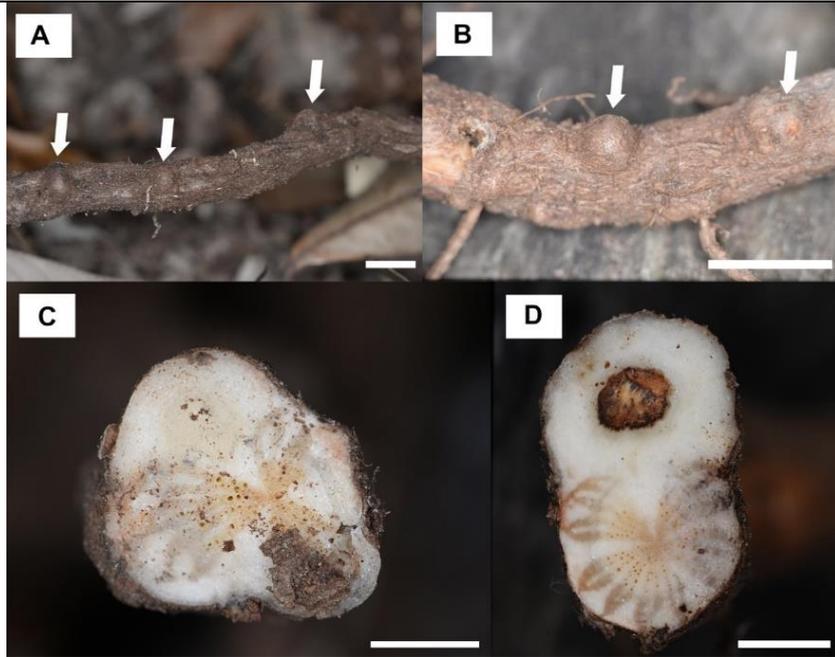


Figure 1. Root of *T. laoticum* with *S. himalayana f. albovinosa* buds at swollen stage (white arrows) (A) and zoomed root area with buds (B). Cross-sectioned root with the early stage of bud (C) and late stage (D). Scale bars = 1 cm (A, B), 0.5 cm (C, D). Photo by Trần Hữu Đăng.

Results

Early bud

At early bud stage (Figure 2), the flower bud (or *Sapria* parasitic tissue, SPT) morphologically resembles an inverted teardrop figure. The proximal part of the flower bud is located between the host secondary xylem vessels, and the distal part is located between the bent host secondary phloem with the farthest distal tip in contact with the host cortex layer. The neighboring phloem bundles of the host have been disfigured by the flower bud distal area growth as it appears to be laterally flattened (Figure 2; PAP). The neighboring xylem bundles of the host, however, only partially disfigured in the proximity area to the flower bud proximal area (Figure 2; PAX).

As comparison, closer to the *T. laoticum* root cross-sectional core, the xylem appears to be unchanged as the rest of xylem bundles located farther away from the *Sapria* flower bud growth. The parasitic tissue non-affected (normal) root vascular bundle of *T. laoticum* has developed through secondary growth, with ramified secondary xylem area of the vascular bundle (Figure 2; SX) at the proximal point close to the pith (Figure 2; Pi), bordered with vascular cambium (Figure 2; VC) before the phloem, and re-merged in the secondary phloem area of the vascular bundle (Figure 2; SP) close to the cortex layer of the root (Figure 2; Pe/Cx).

The root with secondary growth has its original cortex layer compressed outwards to the epidermis layer, forming a periderm/cork layer, while the new cortex tissue developed from meristematic pericycle tissue along with the vascular cambium [BECK, 2010; GAMBETTA &

al. 2013], hence the labeling of cortex in this figure. Although, the thin layer of the periderm is consistent to NIKOLOV & al. (2014a) study, which found rhytidome in *Rafflesia*-infected *Tetrastigma* only, not *Sapria* and *Rhizanthus*, where the phellogen is originated from outer cortex cells.

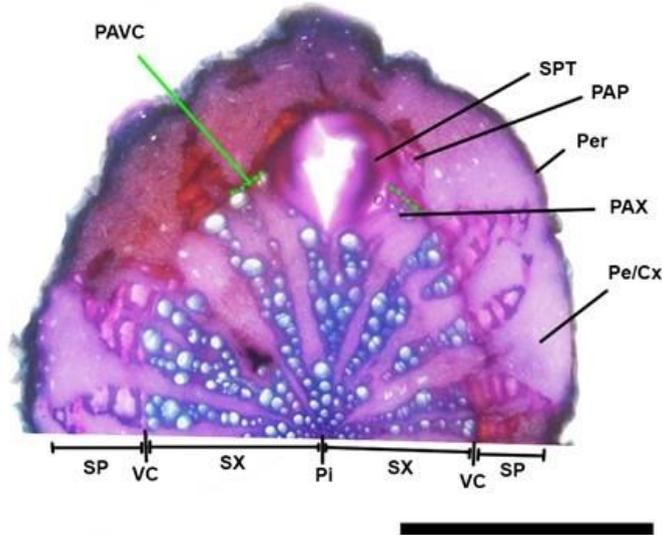


Figure 2. Halved *T. laoticum* root cross-sectional view with early/pre-cupular *S. himalayana* f. *albovinosa* bud (5 month-old), stained with carmine alum-iodine green staining, under light microscope. Pe/Cx – Pericycle/Root Cortex, PAX – Parasite-affected xylem, PAP – Parasite-affected phloem, PACV – Parasite-affected vascular cambium (marked with green dotted lines), Per – Root periderm, SPT – *Sapria* parasitic tissue, SP – Secondary phloem of host, VC – Host vascular cambium, SX – Secondary xylem of host, Pi – Pith (tiny area in the middle of the root cross-section). Scale bar = 0.5 cm. Magnification: 10 × 10. Photo by Nguyễn Thị Ngọc Hương, brightness-contrast-saturation was enhanced by Adhityo Wicaksono.

Late bud

At later stage, the flower bud growth initiates the pre-cupula/swollen stage (analogous to *Rafflesia* life cycle staging by SUSATYA, 2020), where it exhibits gall-like growth that pushes the cortex and periderm area further outwards, swelling the appearance (hence the stage name). At this stage, the bud emergence is already visible by eyes (see Figure 1A and B, white arrows). Despite the flower sex is not yet determined, the possible ovary region (if the flower develops into female or bisexual flower) is already visible in the cross-section (Figure 3; PriOv). The rest of the vegetative structure primordials are also already developed, e.g., primordial central chamber (Figure 3; PriCh) and primordial perigone lobes and bracts (Figure 3; PriPer).

In early development of the late flower bud (Figure 3A), the central chamber already possesses the streaks that is closely resembles the mature flower [TRẦN & al. 2018], while the perigone-bract primordial remains undifferentiated. In later development of the bud (Figure 3B), central chamber enlarges, other vegetative structures like the central column (Figure 3; PriCC) is now visible, and the primordial perigone-bract layers has formed a protective layer surrounding the central chamber of the flower (and the flower undeveloped diaphragm). These

additional parts will develop into the flower organ of *Sapria*. The number of affected host vascular bundles cannot be seen clearly, however the pattern of the parasitic tissue growth shows the similar trend as in the early bud (Figure 2). The parasite-affected tissue (Figure 3; PAT) has pushed the host vascular bundles further laterally and grows deeply into the host secondary xylem area.

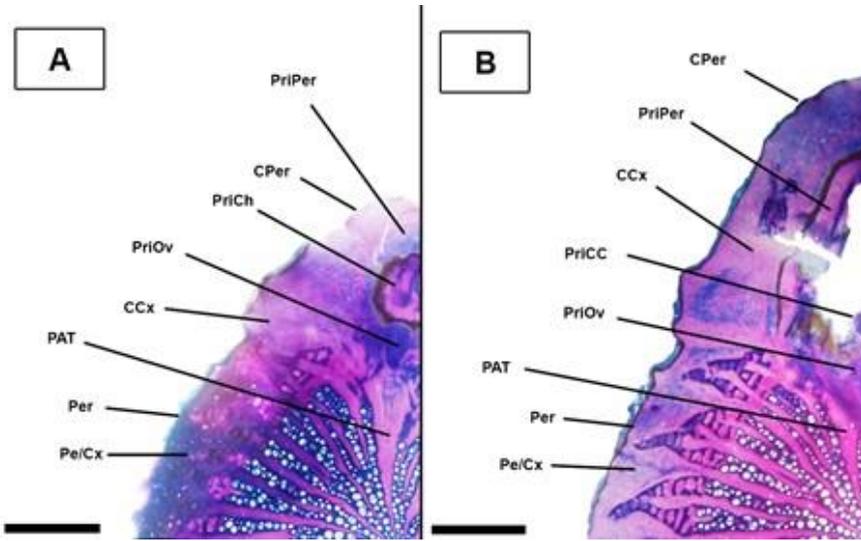


Figure 3. Quarter of *T. laoticum* root cross-section with *S. himalayana* f. *albovinosa* bud in late stage of pre-cupula stage, stained with carmine alum-iodine green staining, under light microscope. At 5 month-old (A) and 7 month-old (B). Pe/Cx - Pericycle/Root Cortex, Per - Root periderm, PAT - Parasite-affected tissue, CCx - Cupular cortex, PriOv - Primordial ovary (if the flower is female), PriCC - Primordial central column, PriCh - Primordial central chamber, PriPer - Primordial perigone lobes-bracts, CPer - Cupular periderm. Scale bars = 0.25 cm. Magnification: 10 × 10. Photo by Nguyễn Thị Ngọc Hương, brightness-contrast-saturation was enhanced by Adhityo Wicaksono.

Discussions

Sapria tissue growth and comparison across Rafflesiaceae

In general, during swollen stage or pre-cupula stage (analogous to *Rafflesia* life stage description by SUSATYA & al. 2020), the parasitic tissue of *Sapria* appears morphologically like an inverted teardrop. This morphology resembles to the floral development stage in the rest of Rafflesiaceae genera, based on observation in *Rafflesia patma* Blume [MURSIDAWATI & WICAKSONO, 2020], *R. azlanii* Latiff & M. Wong [KAMAL & al. 2021], *Rhizanthus lowii* (Becc.) Harms [NIKOLOV & al. 2014b]. The parasitic tissue (Figure 2 and 3; PAT) is centered in the vascular cambium tissue layer of the host root. The proximal region of the parasitic tissue appears to grow towards the core of the root where xylem vessels are abundant, presumably to obtain water and minerals from the host xylem, while the distal region starts to grow and swell towards the surface (direction of the root periderm) and reach the host phloem for nutrition.

This type of movement also can be seen in *Rafflesia* species [MURSIDAWATI & WICAKSONO, 2020; KAMAL & al. 2021] and *Rhizanthus* [NIKOLOV & al. 2014a] where the

parasite tissue grows proximally towards the host root xylem area with the conic-shaped tissue in the host-parasitic attachment/haustorial region.

In the early bud stage (Figure 2), the penetration of the parasitic tissue goes between two xylem area of the vascular bundles, altering the growth of the adjacent vascular bundles, while the remaining vascular bundles continues to grow normally. This is consistent to the *Rafflesia* study in KAMAL & al. (2021), where the early stage of *R. azlanii* bud with size of 2.7 cm, and *R. patma* in MURSIDAWATI & WICAKSONO (2020) only penetrated to single area of host vascular bundles. Assumptively, the number of damaged host vascular bundles in *Rafflesia*-infected *Tetrastigma* is increased by the age of flower bud growth as the parasitic tissue is growing larger and larger, compressing more vascular tissue of the host [MURSIDAWATI & WICAKSONO, 2020]. One vascular bundle is damaged during the cupule stage, two vascular bundles in cupule-bract transition (CBT) stage and three host vascular bundles ruptured by *Rafflesia* in the bract stage for *R. azlanii* and *R. cantleyi* [KAMAL & al. 2021]. In the case of multiple bud formation (as in Figure 1A and B; for *Rafflesia* in MURSIDAWATI & WICAKSONO, 2020), the multiple buds are most likely to grow in the same orientation. This “behavior” is believed to be a strategy to minimize the damage of the host, preventing the death of the host that will ultimately also killing the parasite [MURSIDAWATI & WICAKSONO, 2020].

In initial growth, the primordial tissues develop into the primordial ovary area, followed by the chamber, and perigone lobe-bract primordial area (as shown in Figure 2). In later growth, the floral central chamber expands, the central column matured to then develop the anther/stigmatic region in mature flower, and the laminations by perigone lobes and bracts primordial is becoming visible. This growth pattern resembles the *Rafflesia* at 3-months-old stage, which also considered as early bud growth. The growing meristematic tissue was composed of three regions namely proximal region, middle and distal region, where the proximal region consisted of elongated cells that interference with the host tissues, followed by the middle area, which the location of ovary flower developed at the later stage and distal region where the bracts and perigone lobes developed [MURSIDAWATI & WICAKSONO, 2020].

Host-parasite intersection pattern across Rafflesiaceae

Despite the great resemblances across Rafflesiaceae for the stage in floral accessory growth pattern, the host-parasite intersection area or haustorium in all three genera are slightly different. *Rafflesia* endophyte is distributed in the host vascular cambium area close to the host phloem [NIKOLOV & al. 2014a; MURSIDAWATI & al. 2019], *Rhizanthus* (based on *Rh. lowii*) is distributed from the host phloem, host vascular cambium, and even between the host xylem vessels [NIKOLOV & al. 2014a], and for *Sapria*, the information is still limited as in the study by NIKOLOV & al. (2014a), *Sapria* endophyte was found in the vascular cambium layer of the host. The newest update, however, revealing the growth activity of *Rafflesia* endophyte in the xylem as well [BASCOS & al. 2021], which is similar to *Rhizanthus* [NIKOLOV & al. 2014a]. The reason and mechanisms behind Rafflesiaceae endophytic growth towards the host xylem area require further investigation.

Even in the flower bud stage, the haustorial growth pattern in three genera appears to be distinctive. The proximal meristematic growth in *Rafflesia* appears to be restricted, as the haustorium intersection is found only in the vascular cambium layer and albeit slightly close to the xylem area [MURSIDAWATI & al. 2019; KAMAL & al. 2021].

In *Rhizanthus* (based on *Rh. lowii*), the proximal meristematic growth is visible and the haustorium intersection reaches the xylem bundles area [NIKOLOV & al. 2014a]. Based on this

study, *Sapria* appears to exhibit the same pattern with the other Rafflesiaceae member genera. There should be some differences in morphological adaptations of the haustorium, especially since *Sapria* is the only Rafflesiaceae that infects *Vitis*, not only *Tetrastigma* [HUANG & GILBERT, 2003], although no clarification of which *Vitis* species infected by *Sapria* until now. Hypothetically, due to growth in small *Tetrastigma* root area but possess relatively large (host cross-sectional diameter to the flower size), *Rhizanthus* and *Sapria* requires greater anchorage to the host.

Comparatively, *Rafflesia* is often found in larger root area and even in aerial root with large diameter. As *Rafflesia* flower size is also massive [NAIS, 2001], shallower haustorium possibly reduces mechanical damage to the host, thus preventing further stress in the host that will potentially kill it, endangering the parasite.

Future prospects

This study has some limitations, including sample variations and details. More species can be aimed for future sampling, as well as multiple organ sampling during different stage of development or flower organs during blooming/anthesis period. Multiple organ histoanatomical study can be used to understand the different features in the flower or even endophyte inside *Tetrastigma* in a species or across multiple species. This similar concept of study has been done initially by NANTAWAN & SUVIT (2002), and later by MURSIDAWATI & al. (2020) in *Rafflesia*. Moreover, the haustorial attachment differences between all three genera can also be modelled in biomechanics study.

However, this sampling for histoanatomical analysis, requires strategic and optimized sampling, especially due to the rarity of *Sapria* (i.e., *S. himalayana* is considered “Vulnerable”, *S. poilainei* and *S. ram* is considered “Endangered” under IUCN Red List; CHAMCHUMROON & al. 2017). Despite the rest of the species are not considered endangered, Rafflesiaceae, especially based on *Rafflesia*, has complex yet mysterious life cycle that implies if the number in the wild is reduced, the recovery process will take a long period of time [WICAKSONO & al. 2020].

Based on this condition, propagation study of *Sapria*, as in *Rafflesia*, should be greatly encouraged. Also, potential future studies require efficient sampling method with to optimize the amount of data with only small amount of sample and development of less invasive method of analysis to reduce the chance of population disruption during progression of the study.

Conclusions

Sapria has not become a subject of anatomical study, compared to other Rafflesiaceae members, *Rafflesia* and *Rhizanthus*. This study provides brief preliminary overview on *Sapria* histoanatomy, using *S. himalayana* f. *albovinosa* samples, stained using carmine alum-iodine green. The results show the progression of *Sapria* growth from the young, inverted-teardrop-like early flower bud, to the late flower bud that already developed some primordial organs, including flower chamber, central column, and perigone lobe-bract complex.

In term of haustorial intersection, *Sapria* is similar to all Rafflesiaceae genera, *Rhizanthus* and *Rafflesia*, as the proximal growth reaches the xylem area of the host. Although further investigation is required in anatomical samples of all Rafflesiaceae genera in the future to confirm this statement. From the functionality aspect, this deeper anchorage facilitates the large flower growth in small roots, although biomechanical study is required to confirm this hypothesis. Future study to obtain higher details on each organ during different stage of growth

or across different *Sapria* species is encouraged with strategic way of sampling and optimized way to obtain the data.

Notes on contributors

Adhityo WICAKSONO – is a researcher in Generasi Biologi Indonesia (Genbinesia) Foundation as Head of Biotechnology Department in 2018 until now. His main research is on plant science, focused on Rafflesiaceae since 2017 and plant molecular biology. He is also a PhD student in Åbo Akademi University, Finland.

Hữu Đăng TRẦN – is a researcher at Southern Institute of Ecology and Becamex Institute of Research and Development and have experience in most of the forests in Southern Vietnam and few in Northern Vietnam. His conducted research on taxonomy, including characterization of *Sapria himalayana* morphology identification. He and his team acquired the samples in this study.

Syarifah Haniera Sheikh KAMAL – is postgraduate student (MSc) with a special interest in plant anatomy and conservation. She carried out the anatomical study on *Rafflesia* species with the host, *Tetrastigma* and morphology, growth and mortality rate of *Rafflesia* species.

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