Doctoral Dissertation (Censored)

博士論文（要約）

Morphological and Molecular Characterisation of Domatium Development in Myrmecophytes

（アリ植物におけるドマティア形成過程の形態学的および
分子生物学的解析）

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Contents

Abstract

Callicarpa saccata (Lamiaceae) and *Tococa guianenesis* (Melastomataceae) are myrmecophytes—or 'ant loving'—plants which possess foliar domatia. Ants live inside the hollow domatia and form relationships of mutual benefit with the plants. The plants provide shelter in exchange for protection from competitors and herbivores. In some myrmecophyte species, domatia are produced by insects; in others, they are produced by a combination of plant and insect activity. The domatia of *C. saccata* and *T. guianensis* form independently of the ants inhabiting them. The aim of this investigation was to characterise the domatia of *C. saccata* and *T. guianensis* from morphological and molecular standpoints, comparing *C. saccata* to a domatia-less relative, *Callicarpa subaequalis.* Though many studies exist into the morphology of various forms of myrmeco-domatia, no studies have been conducted into the molecular basis of domatium development.

Plant tissue was examined using sectioning and micro-computed tomography (CT) to observe the development of *C. saccata* and *T. guianensis* leaves. The mesophyll tissue of domatia does not clearly differentiate into palisade and spongy layers, suggesting that domatia are less specialised for photosynthesis. Various gland types were discovered on the blades and domatia of *C. saccata,* and the blades of *C. subaequalis.* Large, cupulate glands are unique to the inner surfaces of *C. saccata* domatia and are not found on the blades of either *Callicarpa* species. Secretions taken from the inside *C. saccata* domatia were found to contain sucrose, demonstrating that domatia are specialised for attracting ants and maintaining the ant-plant relationship.

Domatia are composed of two individual cavities which are open at their distal ends and closed at their proximal ends. Rather than a curling of the leaf margins, blade tissues are pushed upwards and outwards over petioles to create swollen structures. The boundaries of each domatium are the midvein and lateral veins of each leaf. These structures form due to excess cell proliferation at blade/petiole junctions, which warps the shape of blades. Small cells indicative of cell proliferation were discovered in the distal regions of domatia, overlapping with the sites of blade/petiole junctions. Cells in the proximal regions of domatia were larger.

List of Abbreviations

SAM – shoot apical meristem

DAS – days after sowing

EtOH – ethanol

Genes

AN3 – ANGUSTIFOLIA3

AS1 – ASYMMETRIC LEAVES1

AS2 – ASYMMETRIC LEAVES2

BOP1 – BLADE-ON-PETIOLE1

BOP2 – BLADE-ON-PETIOLE2

BP – BREVIPEDICELLUS

KNAT2 – KNOTTED-LIKE FROM ARABIDOPSIS THAILANA2

KNAT6 – KNOTTED-LIKE FROM ARABIDOPSIS THALIANA6

KNOX1 – Class I KNOX genes; *STM, BP, KNAT2* and *KNAT6* in *A. thaliana*

CHAPTER 1

GENERAL INTRODUCTION

Domatia are plant structures within which arthropods reside. These structures are important components of tropical ecosystems. Two well-studied varieties are acarodomatia, which associate with mites, and myrmeco-domatia, which associate with ants. Plants which associate with ants are known as myrmecophytes, or 'ant plants.' Not all myrmecophytes have myrmeco-domatia. One of the most famous examples of a domatium-bearing myrmecophyte is Vachellia. In the *Vachellia-Psudomyrmex* relationship, first reported in 1966 (Janzen, 1966), ants live inside the thorns of the plants.

The co-evolution of plants and insects has resulted in relationships of mutual benefit, with plants often providing shelter and nutrients in exchange for protection from competitors and herbivores. More than 600 plant species form such relationships (Chomicki and Renner, 2015). The driving force behind the repeated evolution of domatium-facilitated mutualism may be competition, as plants compete for space and ants compete for nutrients and shelter in rich tropical environments. The benefits of ant associations have been reported in *Macaranga* (Fiala *et al.*, 1989): ants live inside the hollow stems of the trees and prune encroaching vines. Some plants, such as *Duroia hirsuta,* form relationships with various ant species. In these cases, the different colonising ant species confer different benefits (Frederickson, 2005).

Domatia may be found on virtually any plant structure but the majority are stem-based. They are divided into two categories: Primary domatia and secondary domatia. Primary domatia are features of ordinary plant anatomy, while secondary domatia are specialised structures which are formed through structural modifications (Benson, 1985). Primary domatia include hollow thorns and stems, while secondary domatia include leaf pouches and swollen roots. On leaves, small domatia may be found at leaf vein axils and are often habitats for mites (Nishida *et al.,* 2006; Walter, 1996; Yamamura, 2007). This research focuses on leaf pouches.

In numerous examples of independent evolution, distantly-related plant families show similar leaf pouch or 'sac-like' domatia in the proximal regions of simple leaf blades. Similar domatia are seen in the Chrysobalanceae, Lamiaceae, Malvaceae, Melastomataceae and

Rubiaceae. Many of these domatium-bearing species have been investigated from anatomical and morphological standpoints (Leroy *et al.*, 2008; Leroy *et al.*, 2010; Bramley, 2009; Nakashima *et al.*, 2016; Vogel, 2012; Kriebel, 2016; Cardenas *et al.*, 2014, Nickol, 1993; Svoma and Moraewetz, 1992; Izzo and Vasconcelos, 2002; Janka *et al*., 2000). Even though the domatia of these species are similar, there are certain interesting differences in domatium structure and also timing of domatium development. In *Maieta guianensis* of the Melastomataceae, domatia develop only in the central regions of proximal leaf blades, while the marginal regions remain flat (Leroy *et al.*, 2008). In *Hirtella myrmecophila* of the Chrysobalanaceae, domatium development is suspended on older branches prior to flowering. This is thought to be a mechanism to protect flowers from damage by insects (Vasconcelos, 2002). Younger branches readily produce domatia. This species is also interesting in that leaf damage results in increased ant recruitment, particularly to young leaves (Romero and Izzo, 2004).

The pouch domatium-bearing species focused on in this investigation are *Callicarpa saccata* of the Lamiaceae family (Fig. 1-1A), and *Tococa guianensis* of the Melastomataceae (Fig. 1-1B). *C. saccata* is native to Borneo and its bipartite, facultative mutualism with ants was first described in 2000 (Janka, *et al.,* 2000). It is the sole foliar domatium-bearing species within its genus. *T. guianensis* is found in South-Central America. Both *C. saccata* and *T. guianensis* are myrmecophytes which live in specialised mutualistic relationships with ants. *C. saccata* associates with ants of the *Technomyrmex* genus (Janka *et al.*, 2000; Nakashima *et al.*, 2016) and *T. guianensis* associates with ants belonging to the genus *Crematogaster* or the genus *Azteca* (Wheeler and Bequaert, 1929). In previous studies, two *Technomyrmex* species were observed inhabiting *C. saccata* (one brown, one black) which showed different behaviour in terms of aggressiveness and scale insect cultivation (Janka *et al.*, 2000). These ants were reported to gain entry to domatia through openings on the abaxial sides of leaf blades, at the distal ends of domatia. Despite being distantly-related, the domatia of both species are similar and are hypothesised to develop through a similar mechanism. In both species, each domatium consists of a cavity on either side of the midvein. The structures are lumpy and uneven, form on the adaxial sides of laminas and are open at the abaxial sides.

In *C. saccata,* it was discovered that while adult ants are found all over leaf surfaces, cocoons and larvae are found mainly inside domatia (Nakashima *et al.*, 2016). This suggests that domatia function as nurseries for the younger and more vulnerable stages of the ant life cycle. In addition, *C. saccata* was found to contain two gland types which are hypothesised to have a role in the secretion of substances which are nutritious to ants (Nakashima *et al.*, 2016). Species within the Lamiaceae family secrete monoterpene-containing oils from capitate and peltate glandular trichomes (Schmiderer *et al.*, 2007; Giuliani *et al.*, 2017) and these secretions are known to deter herbivores. Therefore, the finding of additional gland types within myrmecophytic members of the genus *Callicarpa* is significant and suggests that these glands may have a different function related to sustaining the ant-plant relationship. In exchange for shelter and nutrients, the ants protect the plants from competitors and phytophagous herbivores. This has previously been reported in the genus *Tococa* (Michelangeli, 2006). For this reason, the gland types present in *C. saccata, C. subaequalis* and *T. guianensis* were investigated in the present study.

Other species within the genus *Callicarpa* —including *Callicarpa barbata* and *Callicarpa teneriflora*—have hollow stem domatia (Nakashima, *et al.*, 2016). Some species are myrmecophytes without developing domatia at all, including *Callicarpa subaequalis* (*C. subaequalis*) which was first described in 2008 as a species closely related to *C. saccata* (Bramley, 2008). This species has flat laminas and complete stems. Comparing the interactions of domatium-bearing *C. saccata* and domatia-less *C. subaequalis* provides insight into the evolution of myrmecochory and domatia and the selective advantage domatia may have in tropical climates. In the Amazon Rainforest, there are areas of land consisting entirely of a single tree species, often a species of *Clidemia*, *Duroia* or *Tococa.* These areas are known as 'devil's gardens' and result from other species being pruned by ants, reducing competition in the area. Along with ants and plants, hemipterans and fungi are often involved in these relationships. Inside the domatia of *H. physophora, Allomerus* ants cultivate ascomycete fungi (Ruiz-González *et al.*, 2011).

Unlike galls, domatia are usually formed by the plants alone, although some examples of insect-triggered domatia do exist. In *V. vismiaefolia* (Vochysiaceae), ants excavate the soft tissues of the stem to create habitable living spaces (Wesenberg, 2006). In the genus *Piper,* the stems of myrmecophytic species contain reduced lignified tissues which facilitate ant excavation. Important

for this study is that the domatia of both *C. saccata* and *T. guianensis* form independently of colonising insects. When grown in the absence of ants, domatia develop identically to those of leaves in their natural habitat. This means two things for this investigation. First, that for those of us interested in plant morphology, we can investigate domatia development from a morphological standpoint. Second, that as domatium development has a genetic basis and, in the case of *C. saccata* and *T. guianensis,* is not triggered by insects, we can investigate these domatia from a molecular standpoint without considering insect contribution. Therefore, *C. saccata* and *T. guianensis* provide interesting opportunities for the study of domatium development. The aim of this work was to determine how pouch-like foliar domatia develop from both a morphological and a molecular standpoint, comparing the domatium-bearing *C. saccata* to the domatia-less *C. subaequalis. C. subaequalis* was chosen for this study due to the morphological similarity of its leaves to those of *C. saccata*; both are elliptical in shape, serrated, and covered in soft, red-brown trichomes (Bramley *et al.*, 2008).

In this thesis I considered two hypotheses regarding domatium development. The first, that leaf margins curl downwards towards adaxial surfaces, creating habitable spaces on either side of petioles (Fig. 1-2A). This has been demonstrated in *Hirtella physophora* (Leroy *et al.*, 2008). A stage of intermediate curling is observed between flat lamina and enclosed domatium. At this stage, the leaf margins have curled but have not yet attached to the midvein. This 'curling leaf' method was proposed for *C. saccata* (Heckroth *et al.*, 2004), yet no experimental data has been provided. The second hypothesis is that cell proliferation at blade/petiole junctions causes the tissues of the lamina to grow outwards (Fig. 1-2B, C). In the 'outward growth' method, no intermediate stage is observed. Instead, the tissues grow gradually outwards and the domatium cavity becomes larger over time. Eventually, a mature domatium protrudes over the petiole (Fig. 1-2D). In this case, the domatia would develop as structures which are closed at the proximal end, not as open structures which later close. Both 'curling'

and 'warping' likely rely on cell proliferation, with important differences between the locations and directions of division. Domatia that form through curling are expected to have cell proliferation in the marginal regions, while domatia that form through warping are expected to have cell proliferation in the basal regions of the leaf.

To investigate these hypotheses, it was important to analyse areas of cell proliferation in developing leaf primordia. Blade/petiole junctions are important meristematic sites in simple leaves. Cells are supplied from this zone to both proximal and distal regions of the leaf and the proper functioning of the blade/petiole junction is therefore essential for normal leaf organogenesis. In Arabidopsis, this region was shown to be marked by promoter of *ANGUSTIFOLIA3 (AN3),* in addition to the activity of the *CYCLIN D4;2* promoter and *SPATULA* enhancer (Horiguchi *et al*., 2005; Ichihashi *et al*., 2011). Cell proliferation in this region has been visualised using EdU staining (Yin and Tsukaya, 2016), which stains the nuclei of cells in the S phase of the cell cycle, and variation in this site is key for leaf shape diversity (Tsukaya, 2018). Unlike cell division at the SAM, cell division within the blade/petiole boundary is determinate. This site overlaps with the location of domatium development in *C. saccata, T. guianensis* and many other foliar-domatium bearing species. Therefore, the role of this site in domatium development was investigated.

A consideration of the boundaries which separate structurally and functionally distinct plant regions are important in this investigation. The shoot apical meristem (SAM) is the meristematic zone supplying cells to developing leaf primordia. The proliferative function of the SAM is maintained by the expression of the class 1 KNOX (KNOX1) genes: *SHOOTMERISTEMLESS (STM), BREVIPEDICELLUS (BP), KNOTTED-LIKE FROM ARABIDOPSISTHALIANA2 (KNAT2)* and *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA6 (KNAT6)* in Arabidopsis (Vollbrecht *et al.*, 2000). Expression of these genes must be restricted from leaf primordia in order for them to develop normally. This restriction is carried out by a variety of factors, including transcription factors *BLADE-ON-PETIOLE1 (BOP1)* and *BLADE-ON-PETIOLE2 (BOP2)*, acting through *ASYMMETRICLEAVES1 (AS1)* and *ASYMMETRICLEAVES2 (AS2)* which are capable of binding directly to KNOX1 promoters as an AS1-AS2 protein complex (Norberg *et al*., 2005; Ha *et al*., 2007). This distinct zone separates the indeterminate meristem from the determinate leaf primordium.

Simple leaves comprise a flat lamina and rod-shaped petiole. In the *bop1 bop2* double mutant or the *bop1-1* single mutant (Fig. 1-3A, B), ectopic growth of blade-like tissues is observed on petioles. (Ha *et al*., 2003; Norberg *et al*., 2005; Ha *et al*., 2007). This excess tissue is always

produced on the adaxial side of petioles and is due to overproliferation of cells at blade/petiole junctions. As the blade/petiole junction appears to overlap with the site of domatium development, cell proliferation in this region is of interest.

Knowledge of meristematic activity at the SAM and of the boundary region separating it from leaf primordia explains how cells are supplied to produce the leaf. In *C. saccata* and *T. guianensis,* there is an alteration of the proximal-distal axis which produces a leaf anatomy similar to the phenotype observed in mutants causing KNOX1 overexpression or ectopic expression.

The domatia of *Hirtella physophora* and *T. guianensis* do not contain differentiated palisade mesophyll layers and air spaces are largely absent (Leroy *et al.*, 2008; Leroy *et al.,* 2010). *M. guianensis.* In addition to investigating domatium morphology, the structures of *C. saccata* blades and domatia were analysed and compared to the blade tissues of *C. subaequalis.* Such investigation provides additional insight into the function of domatia.

Using techniques such as sectioning and X-ray micro-CT scanning, domatium structure and morphology was investigated. These approaches have the potential to be applied more widely in this field. The comparison between *C. saccata* and *C. subaequalis* considers the evolutionary changes which have occurred following the adoption of myrmecochory within one genus. The comparison between *C. saccata* and *T. guianensis* is able to answer whether the parallel evolution of domatia has occurred through the same molecular mechanisms in distantly related families. These comparisons, and the use of X-ray micro-CT scanning to carry them out, represent strengths of this investigation.

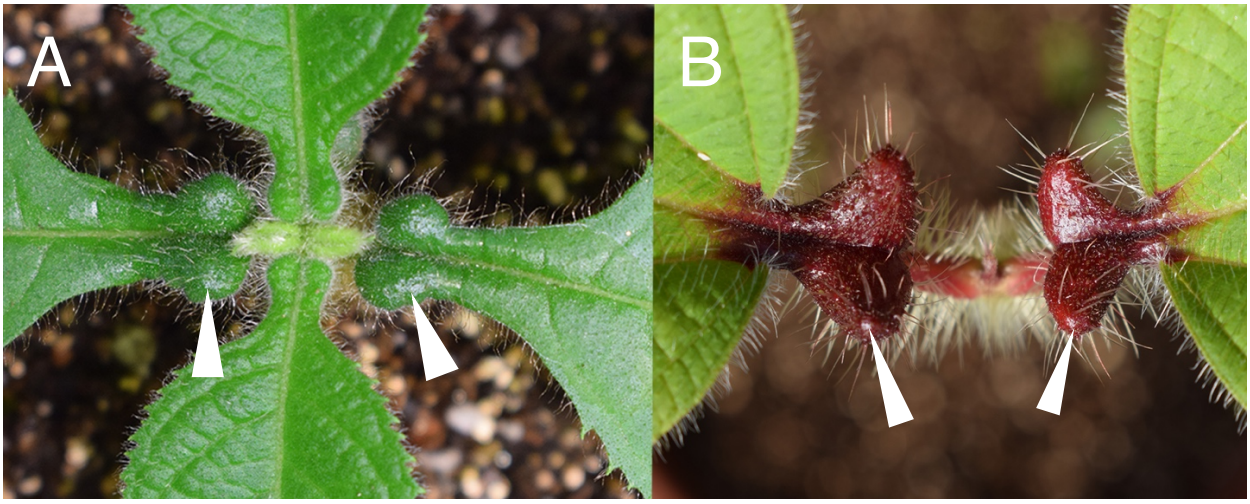

Fig. 1-1: Domatia of materials analysed in the present study. *C. saccata* (A) and *T. guianensis* (B) seedlings are shown. Materials were cultivated in Hongo Campus, The University of Tokyo. Domatia of the oldest leaves shown are indicated by arrowheads.

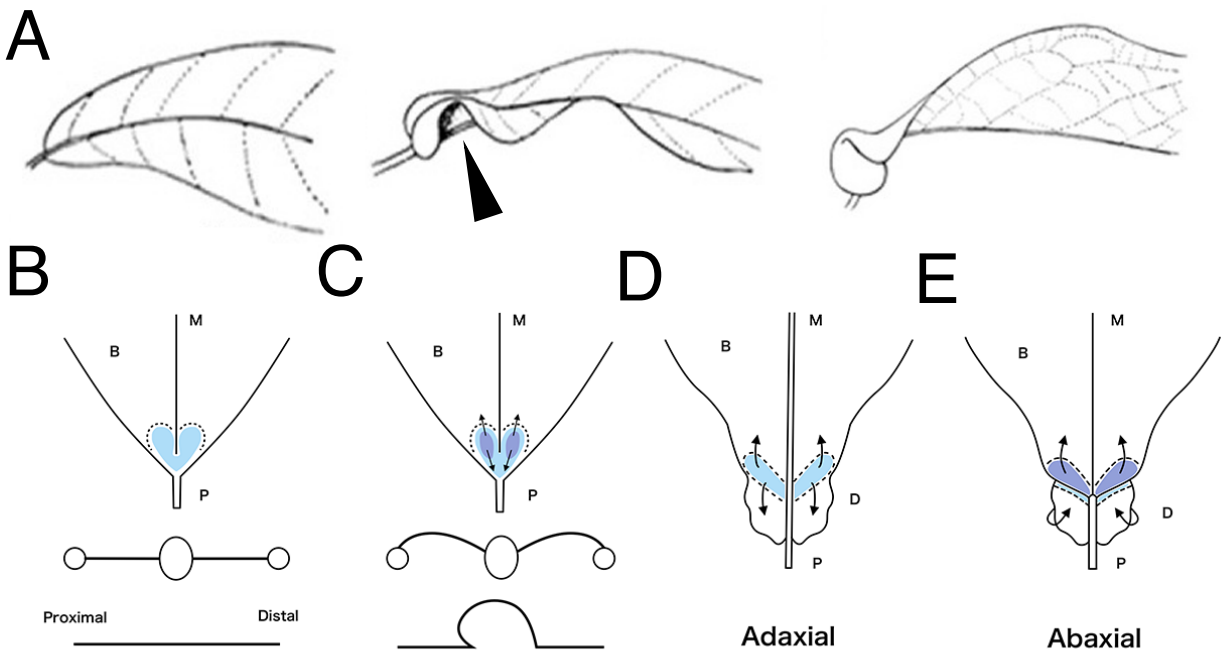

Fig. 1-2: Schematics representing the 'curling margin' hypothesis (A) and the 'outward growth' hypothesis (B). Panel A shows a lateral view of a developing domatium. As margins curl, an intermediate stage is observed between flat lamina and closed domatium. The open, intermediate state is indicated (arrowhead). Images modified from Leroy et al. (2010). B–E show paradermal views of a developing domatium. (B–C) A young leaf, viewed from the adaxial side. Cell division at the blade/petiole junction is shown in pale blue. The blade tissues are pushed outwards to create hollow cavities which increase in size without topological change. Arrows indicate the direction of cell proliferation. The growing tissue is shown in dark blue. A longitudinal view is shown below each image. (D) A mature leaf, viewed from the adaxial side. The site of the blade/petiole junction is shown in pale blue. Arrows indicate the direction of cell division. (E) A mature leaf, viewed from the adaxial side. Cell proliferation on the adaxial side is shown in dark blue. B, D, M and P indicate the blades, domatia, midveins and petioles.

Fig. 1-3: Arabidopsis *bop1 bop2* (B) and *bop1-1* (C) leaves at 28 DAS, compared to a WT leaf of the same age (A). Scale bars = 0.5 cm.

CHAPTER 2

MATERIALS AND METHODS

Specimen collection, growth conditions and measurements

C. saccata and *C. subaequalis* were collected from Betung-Kerihun National Park and Kerihun Nature Reserve, respectively. Seeds of *T. guianensis* were provided by Dr. Gustavo Mori at São Paulo State University. *T. guianensis* specimens were collected and analysed in Brazil under permit numbers SISBIO 53417-1 and SISGEN A86EF37. *C. subaequalis* and *T. guianensis* seedlings were germinated at 23.5℃ on vermiculite or peat moss and watered with water containing HYPONeX plant food. Seedlings of all three species were cultivated at The University of Tokyo. *T. guianensis* seedlings were first cultivated in a growth chamber at 23.5℃, then transferred to a greenhouse at 110 DAS. At the time of measurement of *C. saccata* leaves, leaves without a visible domatium were excluded. *T. guianensis* leaves at each node of 7 individuals were measured over 145 days. The length of leaves at domatium onset were recorded. Domatium onset was defined as the first point at which tissue swelling on either side of the midvein was visible.

Sectioning

A mature *C. saccata* domatium and the proximal region of a *C. subaequalis* blade were divided into equal halves at the midvein and one half of each domatium was sectioned in a transverse orientation. *C. saccata* primordia of various sizes were sectioned in the same way. To obtain serial images, a 1.6-cm-long leaf primordium was used for tissue sectioning. *T. guianensis* domatia were not divided prior to sectioning. The Technovit® 7100 kit (Heraeus Kulzer GmBH, Hanau, Germany) was used. Samples were fixed overnight in formaldehyde-acetic acid-alcohol (FAA; 225 mL EtOH, 12.5 mL acetic acid, 32.8 mL formaldehyde and 230 mL H_2O) and dehydrated in an ethanol concentration gradient (50% w/v, 50%, 60%, 70%, 80%, 90%, 95% and 100% [30 min each], and 100% [overnight]). Samples were transferred to a 1:1 EtOH and Technovit I solution

for 4 hours, then transferred to a 100% Technovit I solution overnight (at −20°C). The samples were then embedded in moulds using a 14:1 mixture of Technovit 2040 powder and Technovit Universal Liquid was used to attach samples to plastic stands. Sections were made using a microtome (Microm HM360, Dreieich, Germany) at thicknesses of 8–12 µm. Sections were stained with 0.1% (w/v) Toluidine blue dissolved in 10x phosphate buffer solution at pH 7.0. Images of sections were obtained using the 10 x magnification lens of a Leica DM4500B light microscope. Images of glands were obtained using the 100 x magnification lens with Leica Immersion Oil (Leica Microsystems™, Wetzlar, Germany).

Micro-CT scanning

Scanning was carried out at The University Museum, The University of Tokyo, using a ScanXmate B100TSS110 scanner (Comscan Tecno Co., Ltd., Yokohama, Japan). The *C. saccata* sample was a 2-cm-long leaf primordium, while the *T. guianensis* sample was a 1-cm-long domatium. Samples were fixed in FAA (as above) overnight and stained, prior to sectioning, with 1% (w/v) iodine to improve visualisation. The scan parameters were a tube voltage of 100 kV and a tube current of 29 µA. The size of the detector was 1,024 × 1,012 pixels and the resolution was 4.437 µm.

Scanning electron microscopy

Sections of 1 cm^2 were cut from three mature blades of *C. saccata, C. subaequalis* and *T. guianensis.* Three *C. saccata* and *T. guianensis* domatia were split in half and each half was divided into six sections for observation. Samples were fixed in FAA (as above) overnight and dehydrated in a graded EtOH series (50%, 50%, 60%, 70%, 80%, 90%, 95% and 99.5%) for 30 min per step, followed by 100% EtOH overnight. Prior to observation, samples are treated in a 1:1 EtOH and isoamyl acetate solution for 30 min, followed by two washes with 100% isoamyl acetate for 15 mins each. Samples were then dried using a JCPD-5 critical-point dryer (JEOL Datum Ltd., Tokyo, Japan), and mounted for sputter coating (90 s) at 20 mV using a JEC-300FC auto-fine

coater (JEOL Datum Ltd.). Finally, a JSM-6510LV scanning electron microscope was used for observation.

EdU staining

EdU stock was diluted 1000 x in water (for a final concentration of 10 μm). Arabidopsis seedlings were collected at 9 DAS. The first true leaves of the seedlings were removed and incubated in EdU solution at 23°C for 3 hours. After 3 hours, leaves were washed with 90% acetone for 10 minutes, then washed once with PBS. Washed samples were fixed with FAA (overnight). The cycloaddition reaction was carried out using 215 μl EdU reaction buffer, 10 μl CuSO4, 0.6 μl Alexa Fluor azide and 25 μl of 10 x buffer additive. Samples were incubated for 30 minutes. Before observation, samples were washed three times with PBS for 20 minutes per wash.

CHAPTER 3

MORPHOLOGICAL ANALYSES

INTRODUCTION

C. saccata and *T. guianensis* are trees which grow in tropical regions of Borneo and South and Central America, respectively. *C. saccata* was first examined in Betung-Kerihan National Park, Borneo. Both species associate closely with ants and are therefore myrmecophytes. The mutualistic relationships between *C. saccata, T. guianensis* and their respective ant species are facilitated by pouch-like foliar domatia located in the basal regions of leaf blades (Fig. 3-1A, B, E, F). Although *C. saccata* and *T. guianensis* are distantly-related, the domatia of both species are similar; they have lumpy, uneven surfaces, protrude outwards from the adaxial sides of blades and allow ants to gain access to their hollow cavities through openings on the abaxial sides (Fig. 3-1B, F), at the distal ends of domatia closest to the flat laminas. In this investigation, seedlings of *C. saccata* and *T. guianensis* were cultivated in the absence of the ants which would ordinarily colonise them. The aim of this study was to characterise and compare the morphology of *C. saccata* and *T. guianensis* domatia, determining how they develop and if they develop in the same way. In addition to these two domatia-bearing species, a second species of the genus Callicarpa, *C. subaequalis,* was included (Fig. 3-1C, D). *C. subaequalis* is a close relative of *C. saccata,* first examined in Kelian Nature Reserve, Borneo. This species does not possess domatia. In addition to comparing the morphology of domatium-bearing *C. saccata* and *T. guianensis,* I aimed to compare the domatium-bearing species to the domatia-less *C. subaequalis.* This species was chosen for comparison due to the morphological similarity of its leaves to those of *C. saccata* (Bramley, 2008). Despite not developing domatia, *C. subaqualis* is still a myrmecophyte and does still form relationships with ants. Ants build aisle-like structures of organic materials along the midveins of both Callicarpa species (Fig. 3-2). This behaviour was observed on all *C. saccata* leaves studied in Borneo, but only occasionally observed on leaves of *C. subaequalis* (Tsukaya, unpublished observation). *T. guianensis* trees were not observed in their native habitat.

RESULTS

Growth

To observe domatium growth, several *C. saccata* and *T. guianensis* seedlings were grown under laboratory conditions and compared to *C. subaequalis* under cultivation. The first two nodes of all individuals developed flat, domatia-less leaves (*C. saccata,* n = 7 individuals; *T. guianensis,* n = 6), while all leaves at subsequent nodes developed domatia. The domatia of *C. saccata* grew proportionately to leaves, reaching lengths of up to 3.5 cm (Fig. 3-3). Domatium formation was seen under cultivation without symbiosis with ants, clearly indicating that domatium morphology is independent of ants. On average, domatia accounted for 10.8% of the total leaf lengths. In the *T. guianensis* individuals observed, not all leaves developed domatia. On leaves which did produce domatia, the timing of domatium formation varied depending on node (Table 3-1). Four *T. guianensis* plants were measured at 30 weeks after sowing. An additional three plants were measured once they had three nodes. Leaves at higher nodes tended to develop domatia at an earlier stage (based on blade length) than leaves at lower nodes. Leaves at node three produced domatia after reaching an average length of 6.9 cm (n = 8), while leaves at node six were on average 5 cm long (n = 6). During the analysis, a small number of leaves were produced at nodes seven, eight and nine. On average, leaves at these nodes were 3.5 cm in length before producing domatia. On the contrary, no domatium formed on the 87 *C. subaequalis* leaves observed (n = 5).

Domatium development

Figure 3-4 shows developing leaves of *C. saccata* (A–D)*, T. guianensis* (E–H) and *C. subaequalis* (I–L). The domatia are visible in the proximal regions of blades (Fig. 3-4C, D, F–H). Each domatium comprises a cavity on either side of the midvein in both domatium-bearing species. Domatia always protrude outward on the adaxial side, with openings on the abaxial sides through which insects are able to enter, at the distal points of the structure (Fig. 3–1B, F). As small primordia are densely covered in trichomes, small developing primordia cannot be seen with the naked eye. Sectioning was carried out to observe domatia at an early stage of development.

Figure 3-5 shows a structural comparison of a young *C. saccata* domatium (Fig. 3-5A) and a mature *C. saccata* domatium (Fig.3-5B) with the proximal region of a *C. subaequalis* leaf (Fig. 3-5C). Serial sectioning of small *C. saccata* primordia (a single primordium shown in Fig. 3-5D–G) confirmed that domatia are open at the distal ends (Fig. 3-1B, F), and showed that they are closed at the proximal ends. Therefore, domatia develop as structures which are closed at the proximal ends and are not closed at a later stage through curling. Midveins are connected to lateral veins in the proximal regions of blades, where domatia are formed (Fig. 3-5A, B, E–G) midveins marked by 'M' and lateral veins marked by '*'). The tissues of domatia are pushed upwards over these veins and the veins themselves are the margins of the structures. Sections of *C. saccata* (Fig.3-5A, B) and *T. guianensis* domatia (Fig.3-6) of various sizes show that the cavities of domatia gradually increase in size over time without topological change. Small cavities appear (Fig. 3–5D, arrowdead) which are part of the larger open structure.

In order to confirm this mechanism of domatium development, X-ray micro-CT scanning was carried out. Selected images of *C. saccata* (Fig. 3-7A) and *T. guianensis* (Fig. 3-7B) show the structure of leaf primordia from distal to proximal. In both species, the distal region of the blade is flat. Towards the proximal ends, the blades have curved as cell proliferation in the proximal zone warps their structure. Finally, enclosed cavities appear. Two are visible in the proximal region of the *C. saccata* blade, one in the proximal region of the *T. guianensis* blade. This difference is due to the asymmetrical nature of domatia. Through X-ray micro-CT scanning, it was determined that domatia are not seen at the earliest stages of leaf ontogeny (Fig. 3-8) but begin to develop after the formation of separate blade and petiole zones. This indicates that the cell proliferation which forms domatia begins after the establishment of primary organogenesis.

Importantly, the margins of the blade do not curl, but are always contiguous with lateral veins, which are indicated by '*' in Fig. 3-5 and arrowheads in Fig. 3-7. Rather than a curling of the blade margins, the blade tissues are pushed upwards. This indicated that the cell proliferation hypothesis could be correct (Fig. 1-2B). In order to observe the zones of cell proliferation in *C. saccata* leaves, a paradermal section was taken of a mature domatium (Fig. 3-9A). Magnified images of the distal region (B) and proximal region (C) of the domatium showed that cells in the proximal region are larger than those in the distal region, indicating that cell proliferation is

occurring at the blade/petiole junction. Cells in the distal region averaged 592 μm², while cells in the proximal region averaged 1886 μm². This data is supported by the finding of a saucer-like, incomplete form of *C. saccata* domatia which has been observed at the early nodes of young seedlings (Fig. 3-10).

Arabidopsis *bop1 bop2* and *bop1-1* mutants (Fig. 1-3) have phenotypes that are similar to the anatomy of domatium-bearing leaves. In these mutants, excess cell proliferation at blade-petiole junctions produces ectopic blade-like tissues. As the blade-petiole junction is the site of domatium formation in domatium-bearing species, I visualised the cell proliferation in this region using EdU staining to further illustrate the similarity (Fig. 3-10).

Structure

As domatia are continuous with leaf blades, it is of interest whether the inner structures of leaf blades and domatia are the same. Indeed in *H. physophora,* mesophyll cells of domatia are not differentiated into palisade and spongy tissues (Leroy et al., 2008). Leroy et al. also reported similar undifferentiated cells in *T.,* the domatia and their trichomes are bright red due to the production of anthocyanin (Fig*guianensis* (Leroy *et al*., 2010). I examined this point in my materials. The outer surfaces of *C. saccata* and *T. guianensis* domatia have adaxial features; darker colouration than the abaxial/inner side, waxy surfaces and dense trichomes. In *C. saccata,* these trichomes are brown. In *T. guianensis*. 3-1E, F). Blades of *C. saccata, C. subaequalis* and *T. guianensis* were sectioned to compare blade tissue structure to that of domatia. The blades of *C. saccata* (Fig. 3-11A), *C. subaequalis, T. guianensis* (Fig. 3-11C) and *C. subaequalis* (Fig. 3-11E, F) all comprise ordinary cell layers; distinct upper and lower epidermis, a palisade mesophyll layer of elongated palisade cells, and a spongy mesophyll layer with air spaces. In comparison, although the epidermal layers and mesophyll layers are identifiable in the domatium tissues of both domatium-bearing species, the mesophyll layers do not contain elongated palisade cells (Fig. 3-11B, D).

Glands

Aqueous droplets were found on the inner surfaces of domatia from the cultivated *C. saccata* individuals. Secretions collected by Kazune Ezaki were analysed using liquid chromatography–mass spectrometry and found to be rich in sucrose, suggesting that they function to attract and nourish ant colonies (Sarath et al, unpublished). To determine which gland types are present in *C. saccata, C. subaequalis* and *T. guianensis,* we observed glands using scanning electron microscopy (SEM).

Species in the Lamiaceae family secrete monoterpene-containing aromatic oils from two types of glandular trichome: capitate and peltate. These secretions function to deter herbivores and therefore these gland types are unlikely to be candidates for the secretion of sucrose. These gland types were observed in both *C. saccata* and *C. subaequalis* (n = 3 leaves of each species). (Fig. 3-13A, B). Previous studies found two additional gland types in *C. saccata;* large, cupulate glands and star-shaped glands (Nakashima *et al*., 2016). Cupulate glands (Fig. 3-13E) averaged 30,877 μm²(n = 20) in area while star-shaped glands (Fig. 3-13D) averaged 3349 μm² (n = 69) the present study, star-shaped glands were also found in *C. subaequalis.* Common to both species are small, round glands (Fig. 3-13C) which were 330 to 695 μm²in area (n = 63).

The small, round glands were composed of 8 cells (Fig. 3-13F). Star shaped glands were composed of a single epidermal basal cell, a single stalk cell, and an eight-cell upper secretory structure surrounded by a storage cavity (Fig. 3-13G). These two gland types were found on adaxial and abaxial surfaces of both *Callicarpa* species, on the inner and outer surfaces of *C. saccata* domatia. The large, cupulate glands comprise a single epidermal basal cell, a single stalk cell and a complicated upper secretory structure (Fig. 3-13H). Unlike the other two gland types, these glands were found exclusively inside the domatia of *C. saccata*—not on the blades of either *Callicarpa* species.

On the abaxial surfaces of blades, small glands were present at densities of 175 per 1 cm² on *C. saccata* and 532 per 1 cm² on *C. subaequalis*. The density of small glands was therefore greater in the domatia-less species. The same was true for star-shaped glands, found in *C. saccata* and *C. subaequalis* at densities of 116 and 949 per 1 cm², respectively. Both glands were found on the abaxial surfaces of domatia: small glands were observed at a density of 52 per 1 cm² and star-shaped glands were observed at a density of 44 per 1 cm².

The small and star-shaped glands were present at similar densities on the adaxial surfaces of blades (637 per 1 cm² in *C. saccata* and 414 per 1 cm² in *C. subaequalis*). The cupulate glands inside domatia were present at a density of 12 per 1 cm². Gland density is shown in Fig. 3-14. Cupulate glands of various sizes were observed (Fig. 3-13I) along with developing star-shaped glands (Fig. 3-13J).

MORPHOLOGICAL DISCUSSION

With the exceptions of leaves at nodes one and two, all leaves of *C. saccata* and *T. guianensis* develop a domatium. The timing of domatium appearance may be important for the ant-plant relationship. Early ant colonisation may be important for protecting younger and more vulnerable leaves from predation. It remains to be determined why, in *T. guianensis,* the timing of domatium appearance varies from node to node. The environmental factors contributing to domatium appearance could be considered in future investigations.

I determined that the cell proliferation which produces domatium tissue occurs at blade-petiole junctions (Fig. 3-9A). Smaller cells were observed in the distal regions of *C. saccata* domatia which overlap with sites of blade-petiole junctions (Fig. 3-9A). Larger cells were observed in the proximal regions, showing that cells have undergone expansion further from the proliferating areas (Fig. 3-9B). Cell proliferation is therefore active between midveins and lateral veins, which form the boundaries of domatia, pushing the tissue upwards (Fig. 3-D–G, Fig. 3-6D–G, Fig. 3-7). This is similar to the excess cell proliferation observed in Arabidopsis *bop1 bop2* and *bop1-1* mutants using EdU staining (Fig. 3-11). Biased cell proliferation, cell proliferation in the distal regions but not proximal regions of domatia (Fig. 3-9A), pushes domatium tissues outwards over petioles. Importantly, this demonstrates that domatia develop as structures which are closed at the proximal ends—the margins do not curl and later attach to close the structures. X-ray micro-CT scanning proved that developing domatia are always closed at the proximal ends and always open at the distal ends. This data is supported by the finding of unusual, incomplete domatia in *C. saccata* seedlings (Fig. 3-10) and the mechanism is shown in Fig. 1-2B–E. This finding contradicts previous descriptions of domatium development in *C. saccata* as a 'curling under' of the leaf margins (Heckroth *et al.*, 2004) and supports the cell proliferation hypothesis in both *C. saccata* and *T. guianensis*. If the warping hypothesis were correct, cell division would be expected in the marginal regions. Polar-biased cell proliferation is able to produce cup-shaped, pouch-shaped or tube-shaped structures in various distantly-related species, including *Cinnamomum camphora* and *Sarracenia purpurea* (Nishida *et al*. 2006; Fukushima *et al*. 2015). The production of sac-like or tubular shapes through cell proliferation may be a common strategy in angiosperm leaves.

In *C. saccata,* excess cell proliferation at blade/petiole junctions produces domatia. This meristematic location has been observed. In studies of Arabidopsis *bop1 bop2* and *bop1-1,* the location, timing and extent of cell proliferation was investigated. *bop1-1* mutant Arabidopsis show prolonged cell division in clusters along petioles (Ha *et al.*, 2003). In future studies, paradermal sections of *C. saccata* and *T. guianensis* domatia of various ages/sizes could be carried out to observe the changing dynamics of cell proliferation in the meristematic blade/petiole tissue over time. In comparison, an investigation into *C. subaequalis* would likely show ordinary cell division to cell expansion transitions, with cell proliferation in the basal regions ceasing earlier than in *C. saccata.*

That the mesophyll of *T. guianensis* domatia does not contain elongated palisade cells was reported in 2010 (Leroy *et al*., 2010). This has also been reported in another member of the Melastomataceae, *M. guianensis,* and in *H. physophora* of the Chrysobalanaceae (Leroy *et al*., 2008). This investigation revealed that the same phenomenon is present in *C. saccata.* The development of similar domatia in these distant families represents an interesting case of parallel evolution, where domatia are less specialised for photosynthesis, which requires a well-differentiated palisade layer, but are specialised to support the ant-plant relationship.

The ant-plant relationship is further supported by sucrose secretion in the domatia. Two gland types were present in both *C. saccata* and *C. subaequalis;* small, round glands and star-shaped glands. The precise gland type which secretes sucrose has not yet been determined and, to date, secretions from *C. subaequalis* have not yet been analysed. If sucrose is secreted from glands present on the blades of *C. subaequalis,* this suggests that sucrose secretion in this family evolved prior to domatia as a mechanism to attract ants and is the basis of myrmecochory in species which do not bear domatia. This idea is supported by the finding of ant-built structures along the midveins of *C. subaequalis* leaves, although this phenomenon was only observed in a single population/tree. (Fig. 3-2B). The lower densities of round (Fig. 3-13C, F) and star-shaped glands (Fig. 3-13D, G) on the abaxial surface of *C. saccata* leaves indicates that the presence of domatia reduces the need for these glands and may therefore make domatia a less expensive way of maintaining the ant plant relationship. In this study, glands were not observed in *T. guianensis.* This suggests that domatia are capable of attracting ants by functioning as habitable spaces,

without the need for sucrose secretions. Cupulate glands (Fig. 3-32E, H, I) were found on the abaxial, but not adaxial, surfaces of *C. saccata.* They were rarely found on leaves and entirely absent from *C. subaequalis.* This interesting distribution suggests a unique role for this gland type which may be related to sucrose secretion. The finding of the sucrose-rich substance in the domatia of a *C. saccata* individual cultivated in the absence of ants shows that there is constitutive sucrose release in this species.

Terpenes are a major component of essential oils secreted by various members of the Lamiaceae family (Shimiderer *et al.*, 2007; Giuliani *et al*., 2017). As they act as insect repellents, the lower density of such glands in *C. saccata,* if they do indeed secrete terpenes, may have evolved to create a more habitable living space for insects. In place of insect repellant, the plant receives protection from the ants.

Between the blades and domatia of *C. saccata,* there are differences in palisade cells and also in gland distribution. This shows that, rather than just excess cell proliferation, additional structural modifications are required to produce these important structures. In the future, other characteristics such as chloroplast density and stomatal density could be analysed. As domatia are less specialised for photosynthesis, it is possible that chloroplast density is lower. Furthermore, stomata density and size may be important factors for maintaining ant colonies. In *H. physophora,* the lower epidermis of domatia have fewer, but larger stomata (Leroy *et al.*, 2008).

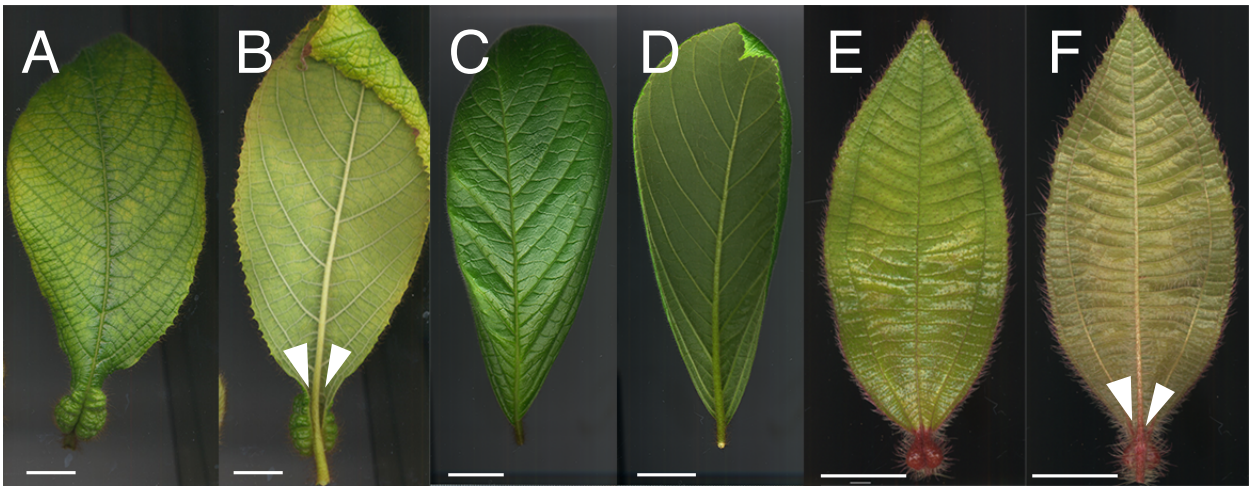

Fig. 3-1: Leaves of *C. saccata* (A, B), *C. subaequalis* (C, D) and *T. guianensis* (E, F) from the adaxial (A, C, E) and abaxial (B, D, F) sides. Domatia are visible in the proximal regions of the *C. saccata* and *T. guianensis* leaves. Domatia are open on the adaxial sides to allow insects to enter (arrowheads). Scale bars = 1 cm.

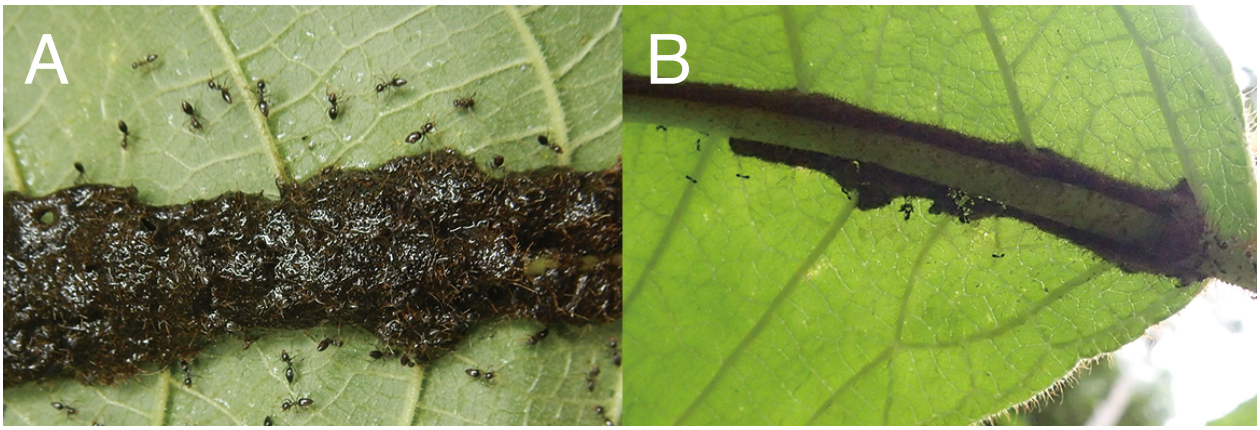

Fig. 3-2: Ant-built structures of organic materials on the abaxial side of *C. saccata* (A) and *C. subaequalis* (B). Photographs taken by Dr. Tsukaya in the native habitats of these species, Borneo.

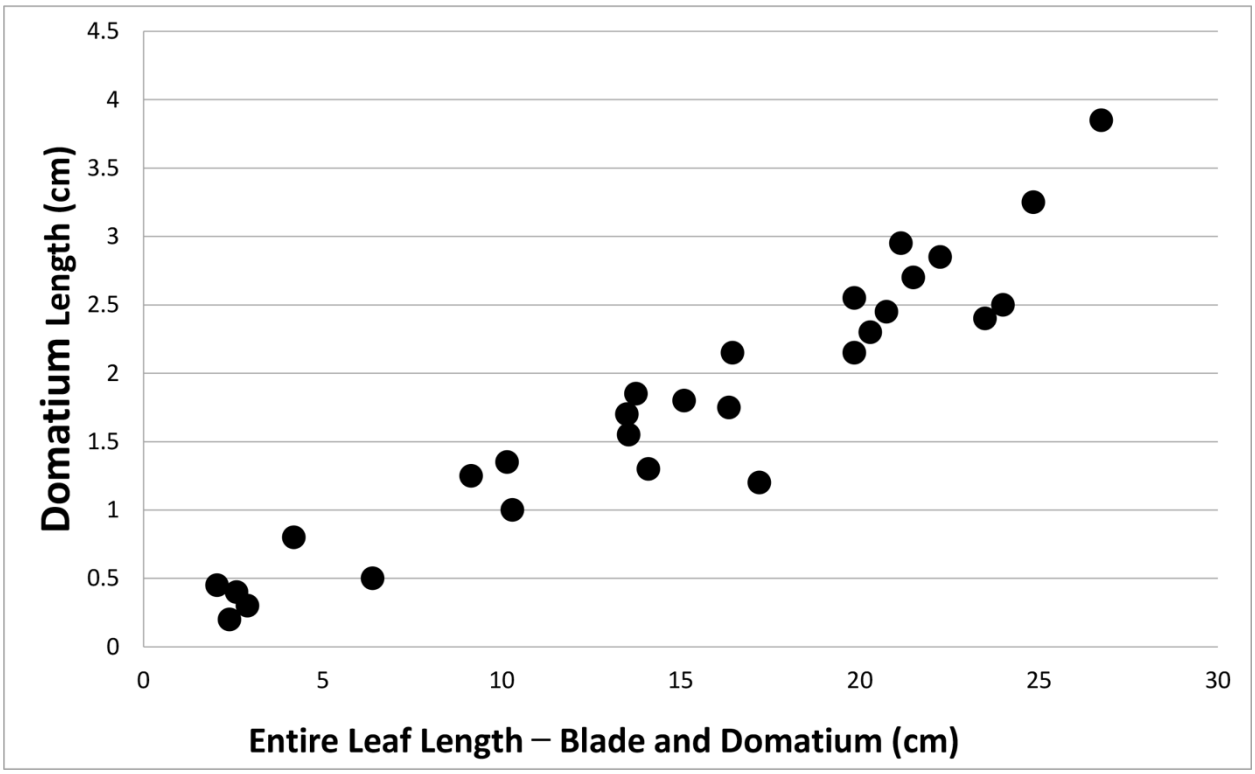

Fig. 3-3: A graph to show the length of *C. saccata* domatia (cm) in relation to the total length of leaves (cm). Domatia are uneven structures and were therefore measured on both sides. The mean of the two was used as 'domatium length' and is shown on the x-axis.

Node number	Length of domatium at onset	Average length
3	8.4	6.86
	7.1	
	5.2	
	8.4	
	5.7	
	7.5	
	7.3	
	5.3	
4	4.5	6.78
	5.6	
	8.3	
	9.9	
	6.5	
	8.1	
	6.7	
	4.7	
5	5.9	6.28
	5.4	
	6.6	
	6.1	
	7.2	
	6.5	
6	2.3	4.95
	9.1	
	6.5	
	5.8	
	3.2	
	2.8	
7	2.9	3.53
	3	
8	2.9	
	5.4	
9	4.6	
	2.4	

Table 3-1: The length of *T. guianensis* leaves (cm) at the onset of domatium ontogeny (n = 6 seedlings). Measurements of leaves at each node are shown separately. Examined *T. guianensis* seedlings were different sizes and had different numbers. of nodes at the time of measuring. Leaves at nodes one and two do not develop domatia and were excluded. Not all leaves at higher nodes had produced a domatium at the time of measuring and these leaves were also excluded from the analysis.

Fig. 3-4: Adaxial views of developing leaves of *C. saccata* (A–D), *T. guianensis* (E–H) and *C. subaequalis* (I–L). The youngest *C. saccata* primordia (A, B) are densely covered in trichomes. Domatia are eventually seen in the proximal regions of *C. saccata* (C, D) and *T. guianensis* (E–H). The leaves of *C. subaequalis* remain flat (scale bars = 0.5 cm).

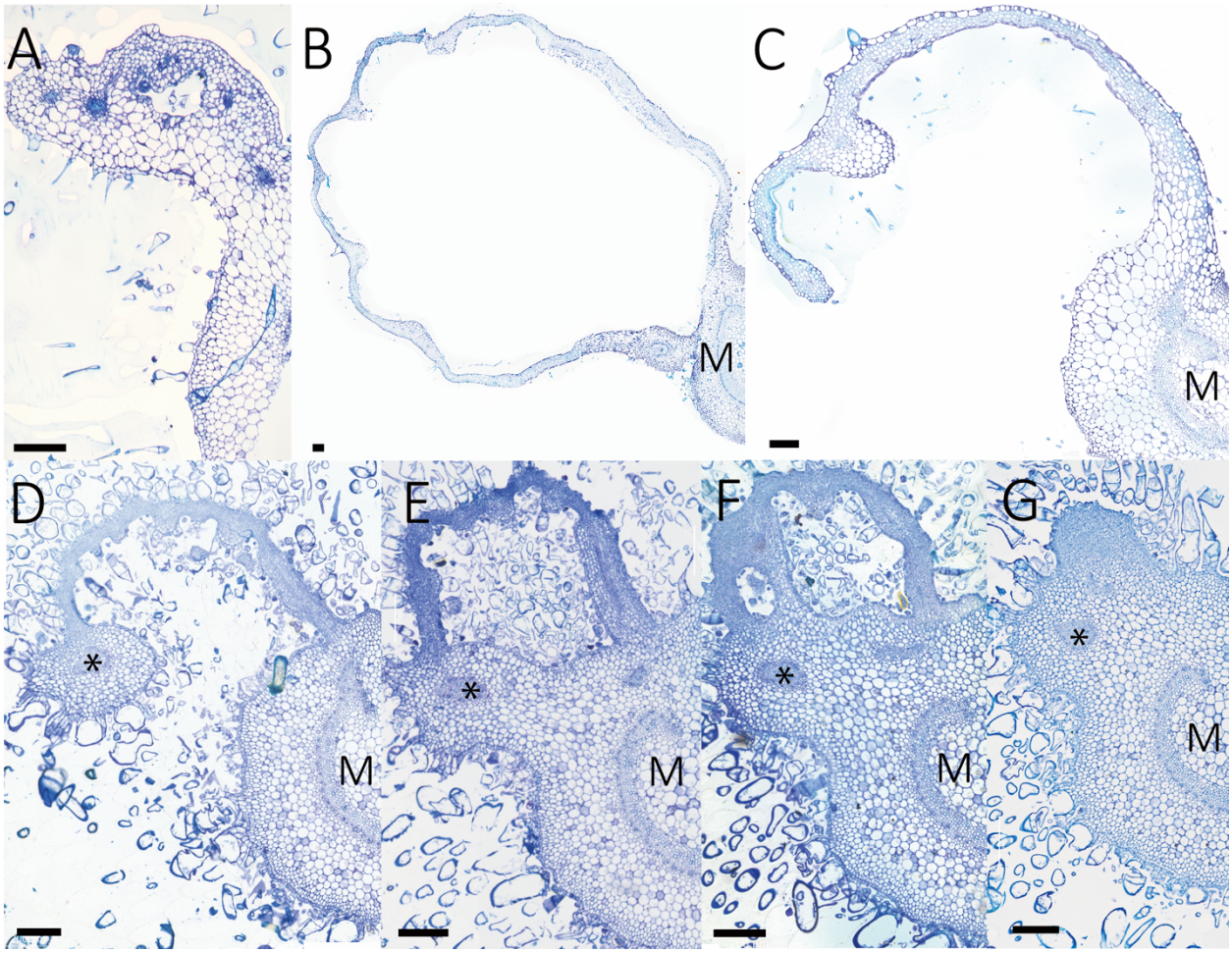

Fig. 3-5: Transverse sections of a mature *C. saccata* domatium (A) and the corresponding proximal region of a *C. subaequalis* leaf blade (B). Each leaf was split in two at the midvein and the proximal region was sectioned. Serial sections of a single *C. saccata* leaf primordia (C–F) show that domatia are open at the distal side, allowing insects to enter (C) and closed at the proximal (F). The hollow cavities (D, E) are produced by cell proliferation which has pushed the tissues upwards. Lateral veins are marked by 'L' in images C-F. Photographs were taken using a Leica DM4500B light microscope at 10× magnification. Images are composite. Scale bars = 200 µm.

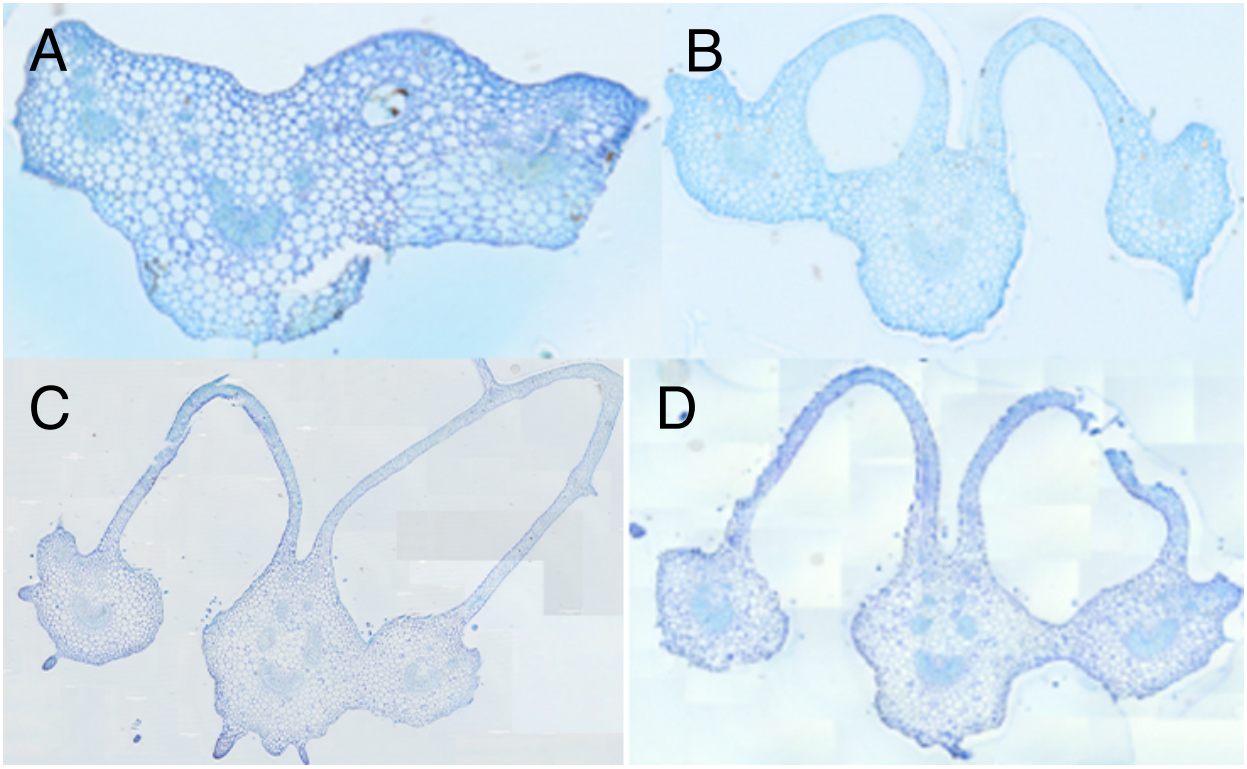

Figure 3-6: Transverse sections of *T. guianensis* domatia (A–D) of various sizes. The domatia were sectioned whole. As in *C. saccata*, the cavities of domatia gradually increase in size as the leaves grow, resulting in the production of swollen structures, one on each side of the midvein. The lateral veins mark the boundaries of domatia (*). Spaces between lateral veins and midveins in seen in some sections (arrowheads) due to the uneven nature of domatia and the distal positions from which sections were taken. Photographs were taken using a Leica DM4500B light microscope at 10× magnification. Images are composite.

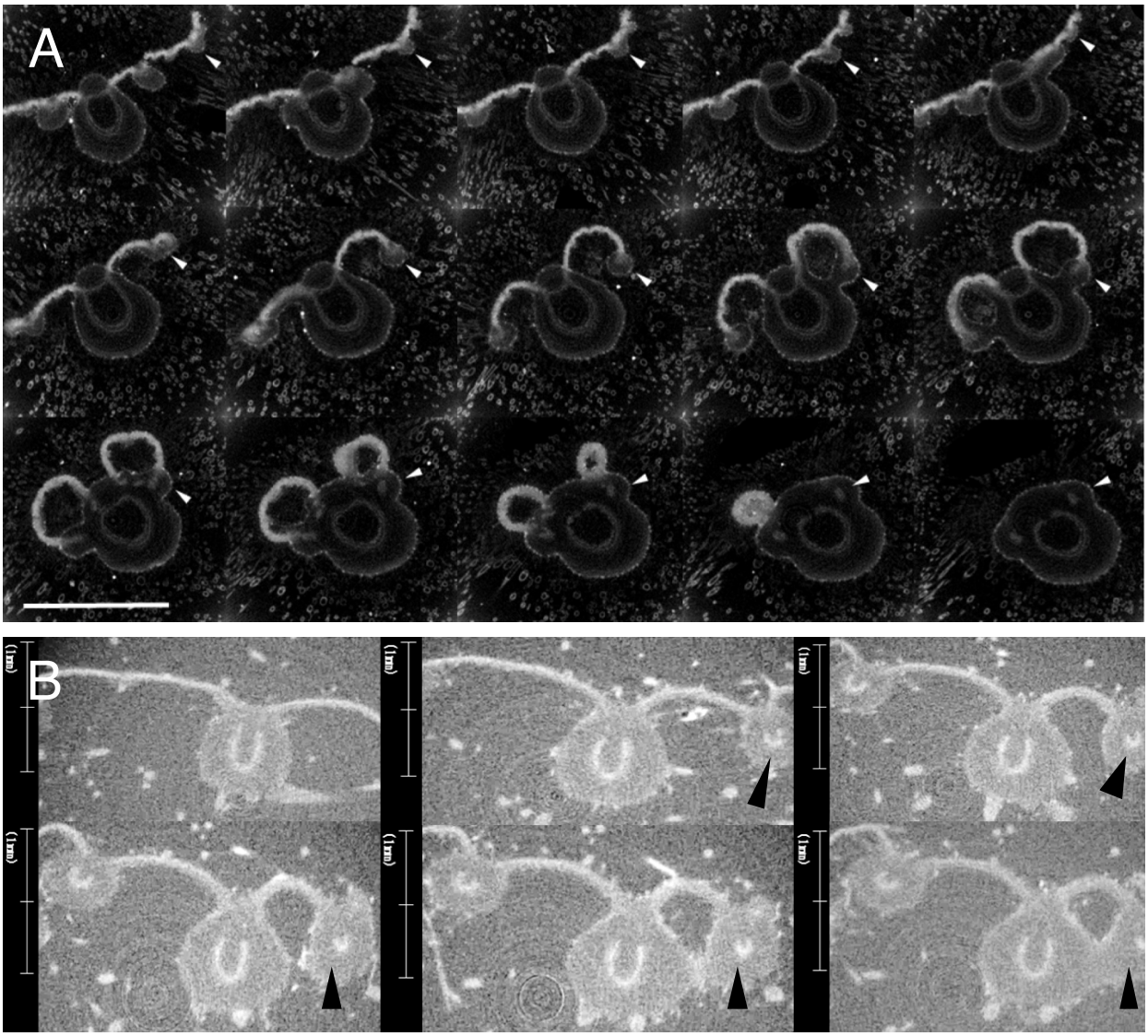

Fig. 3-7: X-ray micro-CT scanning of a 2 cm long *C. saccata* leaf primordium (A) and a 1 cm *T. guianensis* domatium (B) from the distal end (top left) to the proximal end (bottom right). At the distal ends, the blades are relatively flat. Towards the proximal ends, the blade tissues are curved, as cell proliferation has warped their shapes. Arrowheads indicate lateral veins, which can be seen approaching the midveins in the proximal region of both species. In the *C. saccata* leaf primordium images, two cavities appear which gradually close towards the petiole, proving that *C. saccata* domatia develop as structures which are closed at the proximal end. Scale bars = 1 mm.

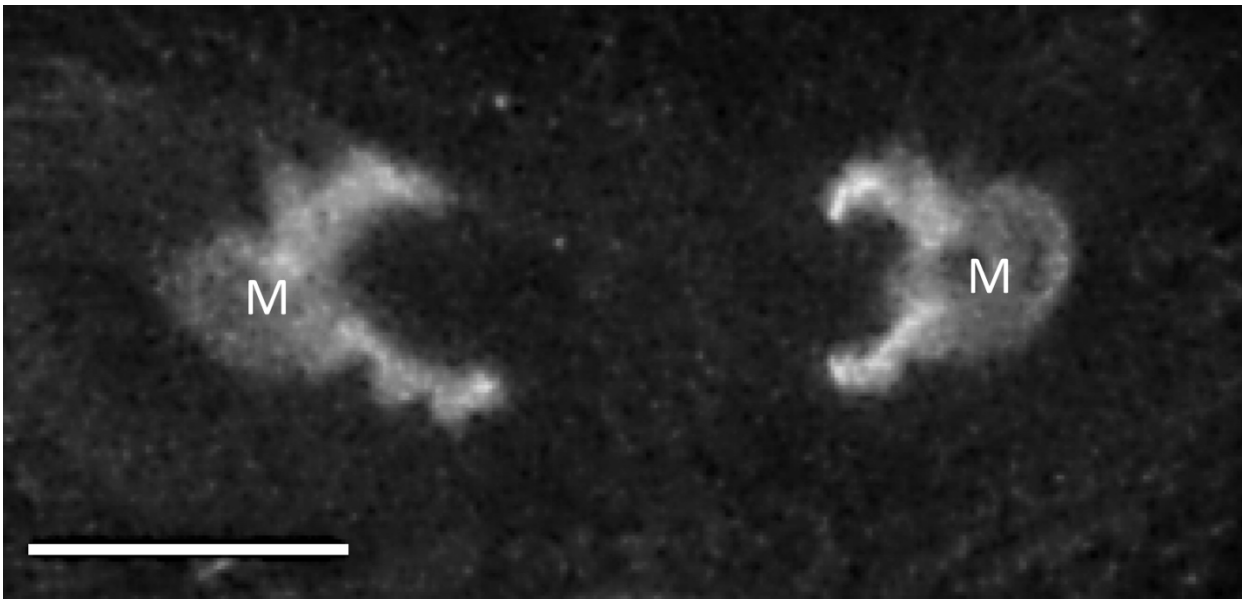

Fig. 3-8: A young *C. saccata* leaf primordia, observed through CT scanning (transverse view). No domatia are observed at this early stage. Midveins are indicated by 'M'. Scale bar = 0.5 mm.

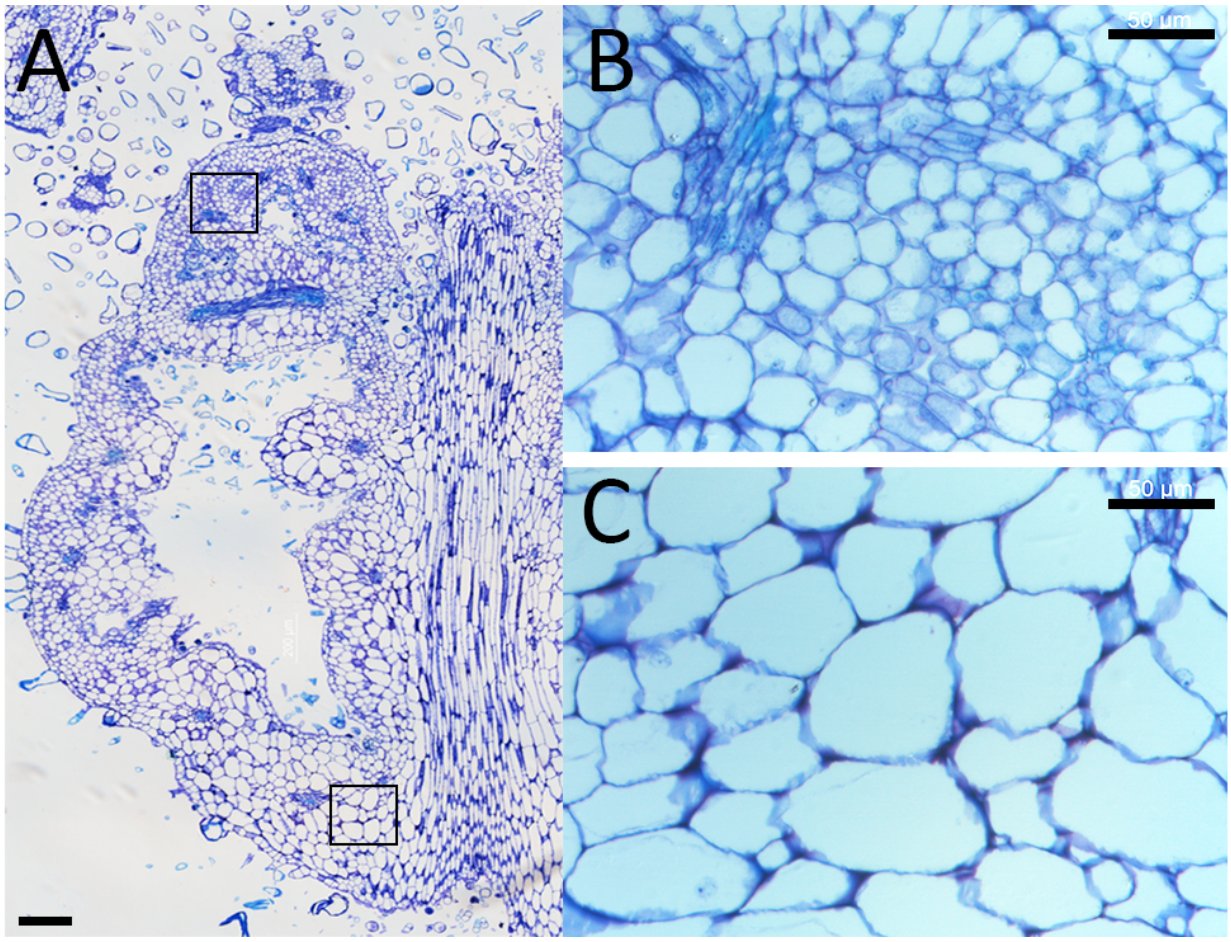

Fig. 3-9: One half of a *C. saccata* domatium. A paradermal section is shown in panel A (scale bar = 200 µm). The blade, domatium and midvein are indicated by the letters B, D and M. Magnified images of the distal (B) and proximal (C) domatium regions show that cells closer to the blade-petiole junction are smaller (scale bars = 50 µm), indicative of cell proliferation in this area. This biased cell proliferation results in domatium growth towards the petiole.

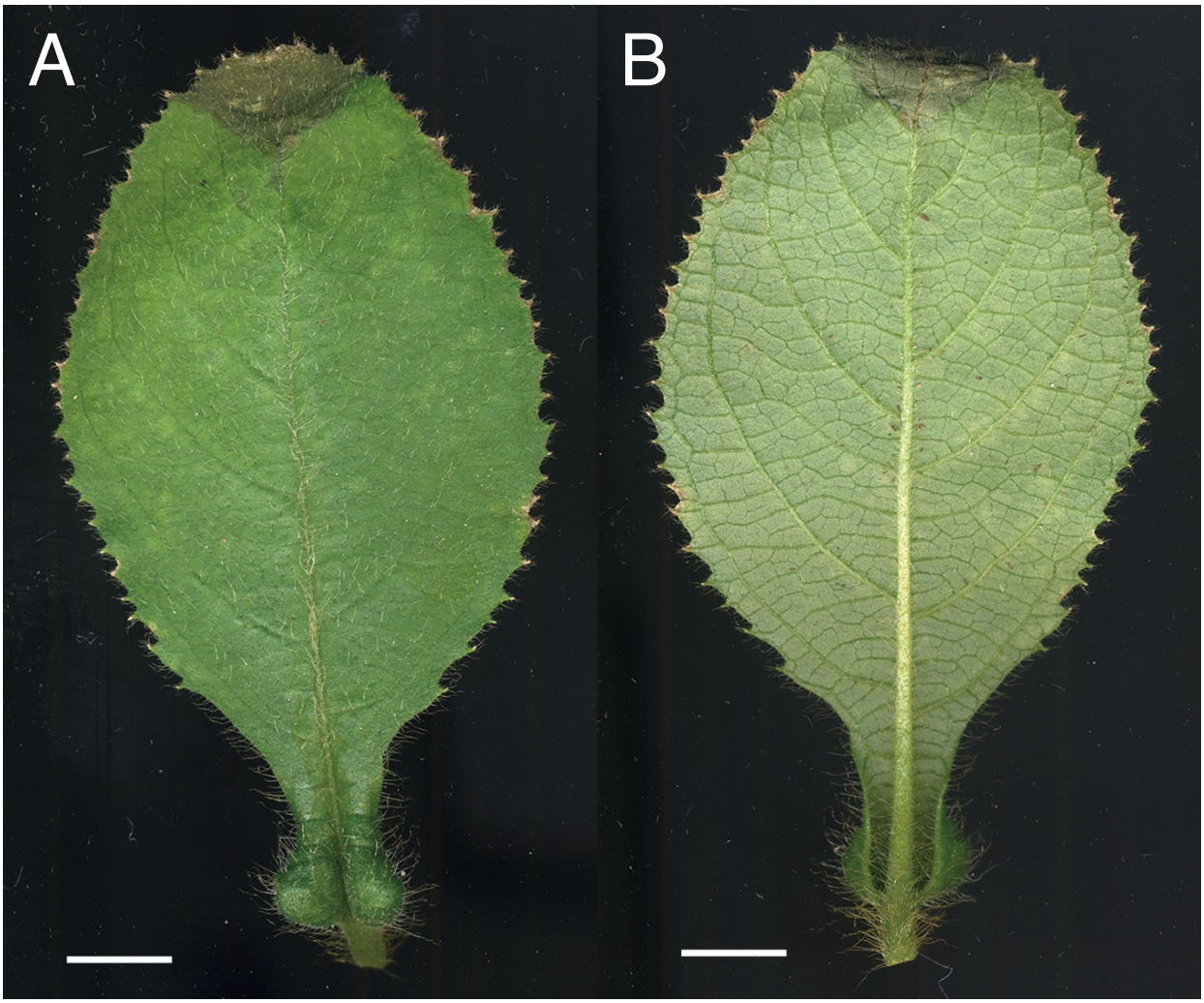

Fig. 3-10: A leaf taken from an early node of young *C. saccata* seedling, shown from the adaxial (A) and abaxial (B) sides. Leaves of this seedling had unusual, incomplete forms of domatia (circled). Scale bars = 1 cm.

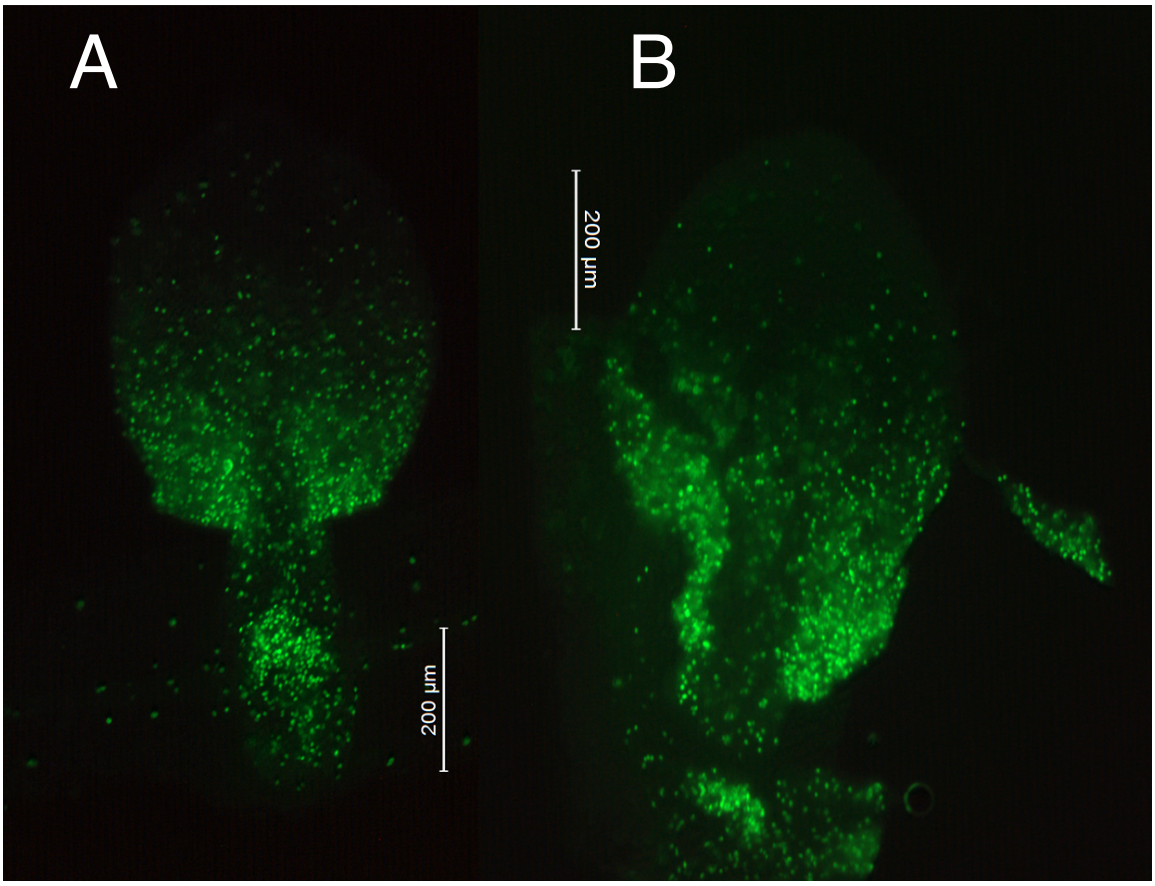

Fig. 3-11: EdU staining of Arabidopsis WT (A) and *bop1 bop2* (B) leaves at 10 DAS. Scale bars = 200 µm.

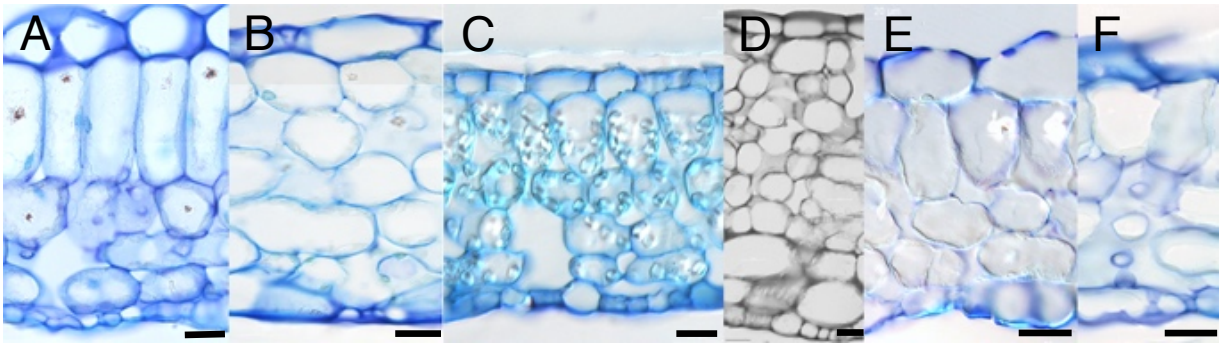

Fig. 3-12: Sections compare the blade tissues of *C. saccata* (A), *T. guianensis* (C) and *C. subaequalis* (E, F) to the domatium tissue of *C. saccata* (B) and *T. guianensis* (D). Blade tissues comprise ordinary cell layers. In comparison, domatia lack dorsoventrally elongated palisade cells. The blade tissues of *C. saccata* and *T. guianensis* were taken from the center of the laminas. The *C. subaequalis* blade tissues were taken from proximal (E) and distal (F) regions to demonstrate that the entirety of the *C. subaequalis* blade is ordinary. Scale bars = 20 µm.

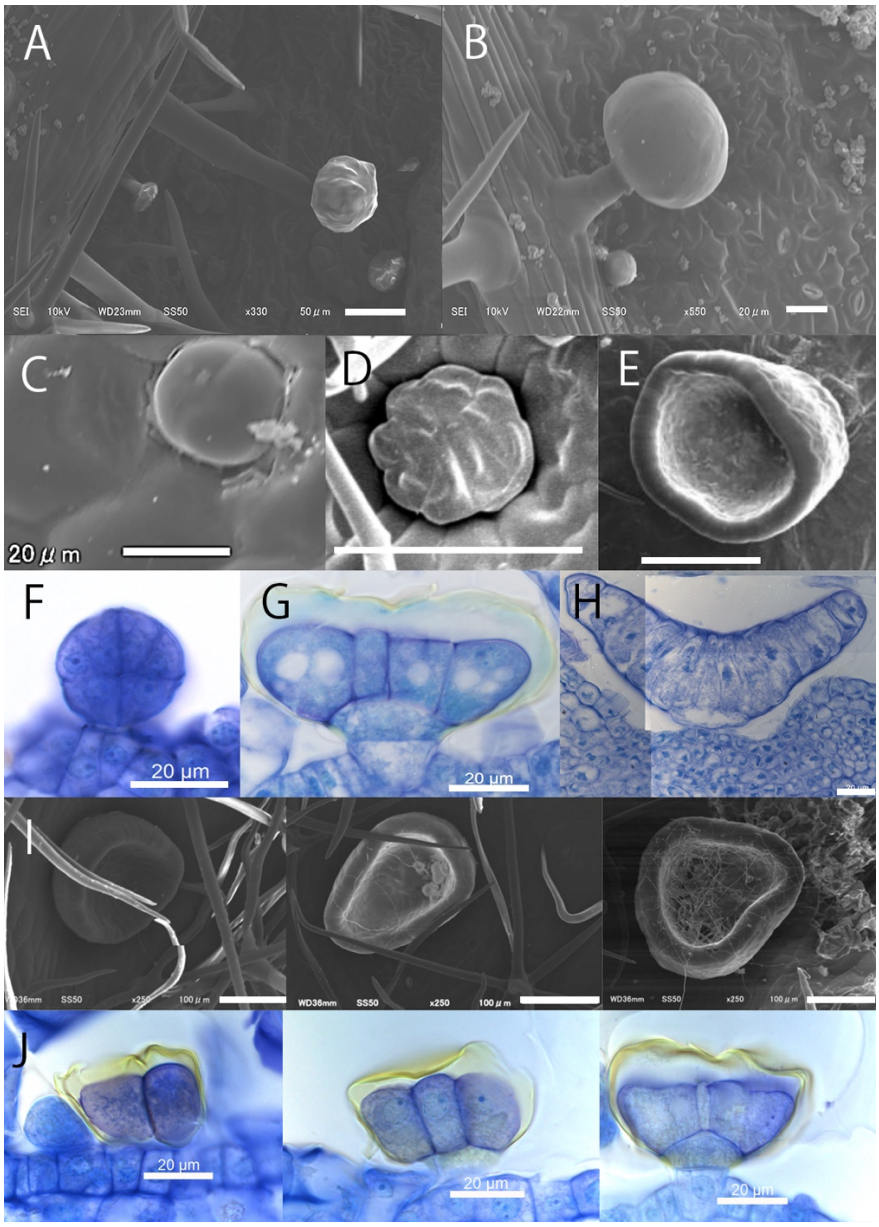

Fig. 3-13: SEM images of a peltate (A) and a capitate (B) glandular trichome positioned on the adaxial surface of a *C. subaequalis* blade (scale bars = 20 µm). Three gland types were found in *C. saccata:* small, round glands composed of eight cells (C, F), star-shaped glands composed of a single epidermal basal cell, a single stalk cell and an right-cell upper secretory structure (D, G) and complex cupulate glands (E, H). Of these three, round and star-shaped glands were also found in *C. subaequalis.* Cupulate glands of various sizes were found on the inner surface of *C. saccata* domatia (I) and images of developing star-shaped glands were observed in the same location through sectioning (J). Scale bars on SEM images = 20 µm, on sectioning images scale bars = 100 µm.

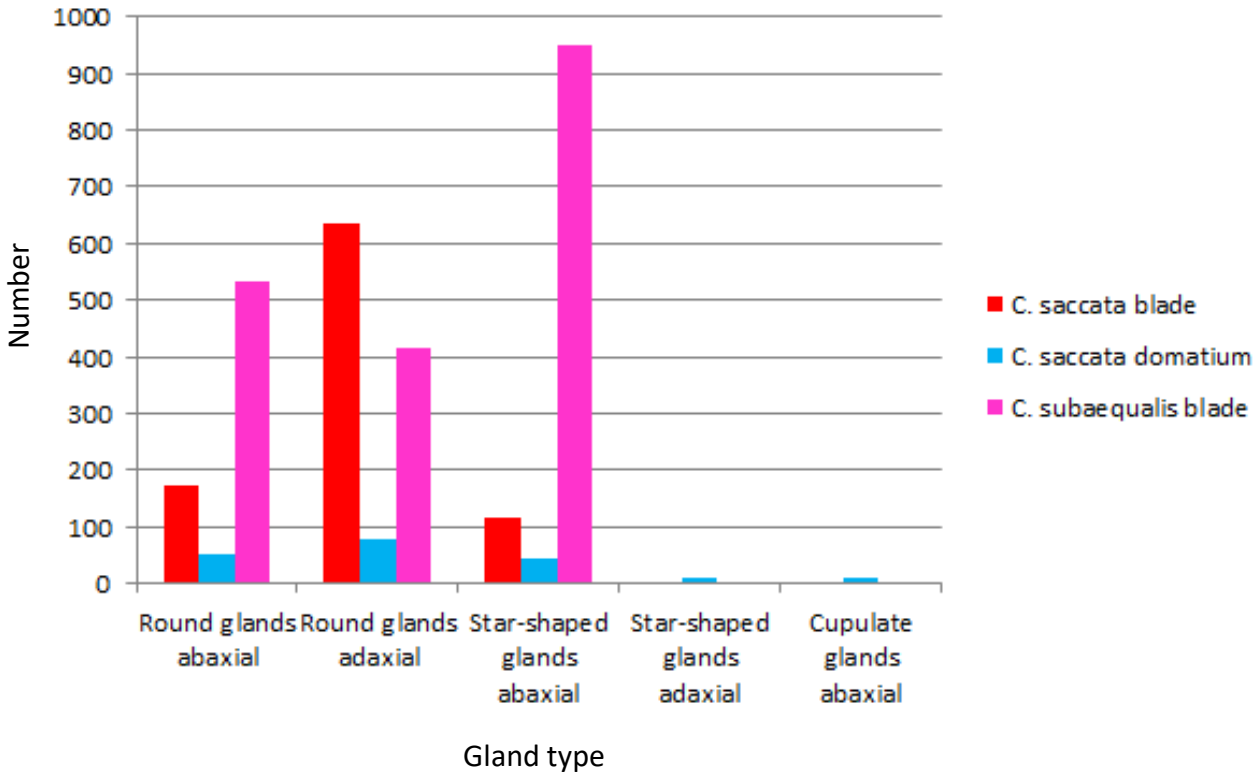

Fig. 3-14: The number of small glands, star-shaped glands and cupulate glands in different locations; *C. saccata* blade (red), domatium (blue) and *C. subaequalis* blade (pink). The numbers of small glands and round glands are shown for both abaxial and adaxial surfaces (n = 3). Cupulate glands were not observed on the adaxial surfaces.

CHAPTER 5

GENERAL DISCUSSION

As domatium development in *C. saccata* and *T. guianensis* occurs independently of colonising insects and is therefore developmentally determined, I investigated the morphological and molecular mechanisms of these plants which produce these unusual structures. *C. subaequalis,* a domatia-less relative of *C. saccata,* was included in the study for comparison.

In this study, it was determined that domatium formation in *C. saccata* and *T. guianensis* occurs due to excess cell proliferation at the blade/petiole junctions of myrmecophyte leaves. The 'warping' hypothesis of this study was therefore confirmed. In the future, it would be interesting to compare the morphologies of domatia formed through warping to domatia formed through curling, identifying the different zones of cell proliferation and comparing the cell division directions. This study discovered the involvement of the meristematic blade/petiole junction in domatium growth. The involvement of other meristematic sites in domatia formed through warping and domatia formed through curling is one possible future avenue of investigation. In *C. saccata* and *T. guianensis,* cell proliferation in the proximal direction is fundamental to domatium growth. Comparing *C. saccata* to both a domatia-less relative and a distantly-related species with morphologically similar domatia provided interesting insights into domatium formation.

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