Original research

Cauline domatia of the ant-plant *Humboldtia brunonis* (Fabaceae)

Joyshree Chanam[a,b], Renee M. Borges[b,*]

[a] *National Centre for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bellary Road, Bangalore 560065, India*
[b] *Centre for Ecological Sciences, Indian Institute of Science, Bangalore 560012, India*

ARTICLE INFO

ABSTRACT

We examined the morphology of the cauline domatia of the semi-myrmecophyte *Humboldtia brunonis* at different stages of ontogeny. We observed that the hollow chamber in *H. brunonis* is spontaneously formed by the plant, and suggest this to be a collective effect of both schizogeny and lysogeny, following acropetal lignification of the pith cells as the domatium internode swells up. Unlike some other cauline domatia, there is a self-opening slit that provides access to the domatium chamber. We investigated the micro-structure of the inner wall of the domatia using scanning electron microscopy, and observed that cells that form the inner lining of the domatia cavity have canaliculated, lignified sclerenchyma with numerous plasmodesmata, as reported for the true myrmecophyte *Leonardoxa africana*, which could explain the observed nutrient flux from domatia-dwelling invertebrates into the host plant. We also observed fungal mycelia in ant-occupied domatia, though the role of fungi in this ant–plant system awaits further investigation.

1. Introduction

Myrmecophytes are ant-plants that bear structures called domatia that house ants. These structures are modifications of plant parts that form a safe shelter for ants, and vary greatly in their architecture. They may be simple cavities such as hollow stems and branches in *Macaranga* (Fiala and Maschwitz, 1992b) and *Cecropia peltata* (Wheeler, 1942; Del Val and Dirzo, 2003), or more intricate ones formed by spontaneous swelling and modification of different plant parts, e.g. leaf petioles in *Tachigali paniculata* (Fonseca, 1993), tubers in *Myrmecodia* and *Hydnophytum* (Huxley, 1978), branch internodes in *Leonardoxa africana africana* (McKey, 1989), *Humboldtia laurifolia* (Krombein et al., 1999) and *Humboldtia brunonis* (Gaume et al., 2005), or even leaves in *Dischidia major* (Treseder et al., 1995). While in most plants, domatia are formed spontaneously, in *Vochysia vismiaefolia* (Vochysiaceae), cauline domatia are reported to be induced when ants bite the young twigs (Blüthgen and Wesenberg, 2001).

Many ant species that reside within domatia can also make carton nests (Davidson, 1997; Gaume et al., 2005; Dejean et al., 2007). However, since domatia are ready-made shelters, and relatively safe from both biotic and abiotic risks, they are a very attractive resource to ants, especially in tropical rainforests (Fiala and Maschwitz, 1992a). Brouat and McKey (2000) hypothesised that the availability of these domatia as nesting shelters could have led to greater constancy of ant presence on the host plant, and consequently such plants could start receiving ant-related benefits such as protection against herbivory.

These domatia-related advantages may have led to the evolution of much stronger, and often obligate, mutualistic interactions between the host plants and their ant partners, a classic example of which is the *Pseudomyrmex–Acacia* system (Janzen, 1966).

In addition to domatia, some myrmecophytes also provide food for ants in the form of extrafloral nectar (Shenoy and Borges, 2010; Weber and Keeler, 2013) or food bodies (Fiala and Maschwitz, 1992b). There are also numerous ant-plants that provide only ant-food and no shelter; these are termed myrmecophiles in contrast to myrmecophytes. However, there is no report yet of any case where an obligate or species-specific ant–plant mutualism has been established on the sole basis of food resources provided. It has been suggested that myrmecophytic interactions evolved independently of pre-existing myrmecophilic relations; according to this view, myrmecophytic interactions could have evolved from interactions in which plant cavities were opportunistically inhabited by ants (Ward, 1991; Davidson and McKey, 1993). Domatia, as safe shelters for nesting, and also for tending hemipterans for honeydew (Fiala and Maschwitz, 1992a; Gaume et al., 1998), could have facilitated permanent residence of the ants, and therefore may have been an important factor in the evolution of obligate ant–plant mutualisms (Brouat and McKey, 2000).

Protection against herbivory has been most often cited as an important benefit of having ant partners, and thereby having plant traits such as domatia that could attract protective ants. However, this cannot be generalised for all ant–plant systems (Trager et al., 2010). Many studies now demonstrate that domatia also facilitate flux of nutrients

into the host plant, especially nitrogen, originating from wastes such as excreta and carcasses of domatia-resident ants (Treseder et al., 1995; Sagers et al., 2000; Fischer et al., 2003; Chanam et al., 2014b), or other domatia-resident invertebrates (Romero et al., 2006; Chanam et al., 2014b). This phenomenon of trophic mutualism between the host plant and domatia residents could also contribute greatly to the evolution of myrmecophytism in addition to, or even perhaps in the absence of, protection services from the domatia-inhabitant ants and can explain the maintenance of domatia even before the origin of a protection mutualism with domatia-resident ants (Chanam et al., 2014a,b). Despite the vital role of domatia in ant–plant interactions, detailed studies on the morphology and development of domatia are relatively few (Krombein et al., 1999; Tepe et al., 2007, 2009; Blatrix et al., 2012), as most research efforts have focussed mainly on the dynamics of the interactions between the host myrmecophytes and the domatia-inhabiting ants.

A majority of myrmecophytes have cauline domatia, which are those formed by modifications of branches or stems (Davidson and McKey, 1993; Brouat and McKey, 2000). According to Brouat and McKey (2000, 2001), such domatia are most likely to have evolved in understorey plants with broad leaves. The branches subtending these leaves would have large diameters to accommodate the broad petiole bases of the large leaves, and such thick branches could have larger pith regions. If the pith cells in such branches dry up, the resulting cavity could be opportunistically occupied by arboricolous ants (McKey, 1984). The increase in plant fitness resulting from such opportunistic occupation by ants would eventually lead to selection of bigger cavities, ultimately resulting in the evolution of cauline domatia. Such associations between a plant and its ant occupants may even lead to coevolution of ant and plant partners to form a tight mutualism (Brouat et al., 2001). It has been proposed that greater specialisation of the mutualism led to precocious onset of domatia in plant development with subsequent occupation by symbiotic ants, so that in some obligate myrmecophytes, such as the understorey tree *Leonardoxa africana*, the domatia are expressed when the plant is only 10 cm in height (Brouat and McKey, 2000).

The present study investigates the structure and development of the cauline domatia of *Humboldtia brunonis* Wall. (Fabaceae), a unique semi-myrmecophytic understorey tree, endemic to the tropical wet evergreen forests of the Western Ghats of India (Ramesh and Pascal, 1997). It is one of five species under the genus *Humboldtia*, all occurring in close geographical proximity in the Western Ghats (Ramesh and Pascal, 1997), while a sixth species, *H. laurifolia*, is presently reported only from Sri Lanka, though there are older records of it being present in the Indian Western Ghats (Sanjappa, 1986; Krombein et al., 1999). Of these, *H. brunonis* is a semi-myrmecophyte in which only some individual plants bear domatia, *H. laurifolia* is a true myrmecophyte in which all individuals bear domatia, the myrmecophytic status of *H. decurrens* is not certain, while the rest are non-myrmecophytes (Sanjappa, 1986). The genus *Humboldtia* thus spans a spectrum containing non-myrmecophytic, semi-myrmecophytic and true myrmecophytic species, though the evolutionary trajectory of the domatium trait in this genus is not yet known since the phylogeny of the genus *Humboldtia* is unresolved. While benefits accruing from domatia could lead to selection of domatia-bearing plants, the reverse is also plausible as in the ant-plant genus *Barteria*, in which non-myrmecophytic species were derived from ancestors with specialised domatia (Peccoud et al., 2013). The position occupied by *H. brunonis* as a semi-myrmecophyte in this spectrum of domatium-bearing traits makes the study of this system crucial in our understanding of the evolution of myrmecophytism. In fact, to the best of our knowledge, *H. brunonis* is the only known semi-myrmecophyte among myrmecophytes and has allowed us to investigate the advantages of the domatium-bearing trait in this species (Gaume et al., 2005; Chanam et al., 2014b).

In this study, we observe the growth of the domatia of *H. brunonis*, and investigate their anatomy at different stages of development in

order to understand the formation of the domatium chamber. We then compare these domatia with those of *H. laurifolia* and of *L. africana,* an understorey African myrmecophytic tree found in Cameroon, Gabon, Congo Republic, and mainland Equatorial Guinea (McKey, 1984). Both *Humboldtia* and *Leonardoxa* belong to the basal legume tribe Detarieae (Bruneau et al., 2001), are understorey trees and bear cauline domatia. Therefore, comparing domatia structure in these related genera should provide insights into the structural evolution of such domatia. We also discuss the evolution of domatia in *H. brunonis*, in the context of the generally accepted theory of the evolution of cauline domatia (Brouat and McKey, 2000, 2001).

2. Materials and methods

2.1. Study area and study system

Humboldtia brunonis (Fabaceae) is an understorey tree endemic to lowland tropical wet evergreen forests in the southern Western Ghats ranging from 11°10′N to 13°45′N (Ramesh and Pascal, 1997). It grows in dense multiclonal clusters (Dev et al., 2011), 300 m–1 km in width (Chanam, pers. obs.), that are scattered throughout its distribution range, and is the dominant understorey tree species where it occurs. The individual ramets of a genet have subterranean connections, and therefore appear as separate trees. Multiple genets co-occur, giving rise to clusters with high clonal diversity even within a small spatial scale of less than 5 m (Dev et al., 2011). Several multiclonal clusters often occur in close proximity, separated by 1–2 km. We have referred to such groups of closely occurring multiclonal clusters as populations. Populations are scattered across the now highly fragmented range of this endemic species, separated by 50 km or more. Whether some genets completely lack domatia, or whether domatia occur on at least some ramets of every genet, is unknown. While characterizing variation among genets in the domatium trait would be important in understanding the evolution of myrmecophytism in *Humboldtia*, this is beyond the scope of the present paper. We therefore refer to each ramet as an individual tree or plant in the rest of the paper.

Domatia are not present in all individual trees within a population, but all individual trees produce extrafloral nectar (EFN) on young leaves, stipules and bracts of young floral buds (Shenoy et al., 2012). The domatia have been observed as early as when the plant is approximately 70–80 cm in height (Brouat and McKey, 2000). Unlike most other myrmecophytes, the domatia of *H. brunonis* are occupied not only by ants but also by myriad other invertebrates that include *Braunsapis* bees, an arboreal earthworm, pseudoscorpions and spiders (Rickson et al., 2003; Gaume et al., 2006; Shenoy and Borges, 2008, 2010). Of the 16 species of ants reported from the domatia, only one ant species, *Technomyrmex albipes*, is known to offer significant anti-herbivore protection to its host plant (Shenoy and Borges, 2010). Although a protection mutualism is seemingly absent with respect to the other ant species, trophic benefits accrue to the host plant from its domatia residents, whether these are protective or otherwise (Chanam et al., 2014a). Earlier morphometric studies of *H. brunonis* domatia revealed that the average length of the domatia varies from 8 to 10 cm, the diameter at the widest point ranges between 0.4–0.6 cm, and that occupied domatia are larger in size than those which are not occupied (Shenoy and Borges, 2010).

2.2. Sample collection and observation

Since domatium initiation is not synchronous in a population, newly initiated domatia are rare and scattered across space and time; therefore, we obtained only six samples of newly initiated domatia for observations on domatia ontogeny. However, domatia at varying stages of ontogeny were abundant, and we examined at least 100 samples during the course of our field work. We classified these domatia as (a) very young, (b) young, and (c) mature, and report domatia anatomy in these

classes. Samples of domatia in all these classes were collected across the distribution range of the species and preserved in 70% ethanol. Transverse sections of domatia were cut using a cryotome, and mounted on silane-coated slides, which were then treated with safranin-fast green stains. The anatomy of the domatium was compared with that of the adjacent internode by cutting similar sections of the internode, and observing under a light microscope.

We investigated whether the domatium cavity is formed naturally or is actively excavated by the occupants. For this, we examined unoccupied domatia to determine whether hollow chambers were formed in the absence of ants. We also determined the formation of wound tissue (suberin) in the inner wall of the domatia as a possible response to any excavation of the cavity by ants. We did this by treating the domatia sections with Sudan IV that stains suberin red (Tepe et al., 2007). Domatia samples were also observed under a scanning electron microscope (FEI Quanta 200 ESEM) to determine the surface characterics of the inner wall. For this, portions of alcohol-dehydrated domatia samples were first mounted on double-sided carbon tape (Electron Microscopy Sciences, USA), which were then affixed to aluminium stubs, and placed in a desiccator. Specimens were later gold sputter-coated (Bal-Tec SCD 500, Liechtenstein).

3. Results

3.1. Domatium morphology and modification by inhabitants

The domatium (Fig. 1A) is a modified branch internode. It is swollen towards the distal end, forming a cavity, and tapers towards the proximal end to form a non-swollen solid woody base which is only as thick as the adjacent normal internode. Stem growth in *H. brunonis* appears to be intermittent, and the unit of growth is one internode with a nearly sessile leaf at the terminal node. The apical buds of stems give rise to new internodes only during the seasonal leaf flush that follows the rainy season. The fate of a young internode, whether it develops into a domatium or a normal internode, is decided in the early stages of its development. A young internode that will later develop into a domatium (proto-domatium internode) starts swelling even when the internode is less than 2 cm long, and tender and fleshy. The apical bud arising from the distal node of the proto-domatium internode may later lead to further internodes which may or may not develop into domatium internodes. Therefore, domatia may either be borne singly on a branch or there may be multiple domatia per branch (Fig. 1B). If there are multiple domatia on a branch, they may either be adjacent to one another or separated by normal internodes. During our study, we observed that three was the maximum number of domatia borne in a contiguous series on a single branch. In a few cases, a single branch may have up to five domatia internodes but separated by normal internodes, while in another rare case we observed that there were multiple adjacent branches each of which had multiple domatia, so that in all, there were seven domatia in a small cluster of three branches (Fig. 1B).

We did not find a prostoma, or region of non-lignified cells in the domatium wall that, in *Leonardoxa* (Brouat et al., 2001), facilitates ant excavation of an entrance hole into the domatium. Instead, there is already a preformed self-opening slit (Fig. 1C) at the distal end of each domatium, just before the distal node, even in very young domatia, which often appears to be hidden by two kidney-shaped stipules when the domatium is young (Fig. 1C). The presence of stipules in such an orientation could have benefits such as protection from rain, or making the entrance less obvious to potential predators of the occupants. The self-opening slit is lined by a few layers of dead lignified tissue (Fig. 1D).

The cavity of a domatium is present only in the swollen portion, and its inner wall is lined by dead pith tissue forming a spongy layer (Fig. 1E), while the base remains solid with pith intact (Fig. 1F). Thus each domatium is a separate chamber, bound by the distal node on one side and the solid base on the other side. Even in cases where multiple domatia are formed adjacent to each other, there is no internal connection between adjacent domatia.

Inhabitants may often modify the domatia. Small ants such as *Tapinoma indicum* cover most of the area of the self-opening slit with carton, and make a small low cylindrical entrance (Fig. 2A) with a diameter just large enough for them to pass through, whereas large ants such as *Polyrhachis* and *Cataulacus* can chew a larger entrance hole. The rims of such openings that are modified by chewing and gnawing develop thick periderms (Fig. 2B) that are visibly different from a natural self-opening slit. In domatia occupied by *Braunsapis* bees, one or more circular openings are excavated through the domatium wall (Fig. 2C) and probably serve as additional entrances. In many cases, a single domatium is co-inhabited by ants and the arboreal earthworm *Perionyx pullus*. In such cases, circular carton discs (Fig. 2D) are built by the ants that divide the domatium into an earthworm chamber and an ant chamber. These earthworms are observed in most cases to occupy the proximal base of the domatium, while the ant nest is towards the distal side, near the self-opening slit.

However, in some domatia, earthworms occupy the distal chamber of the domatia towards the self-opening slit (Fig. 2D), indicating that they could invade an already ant-occupied domatium. In such cases, the ants can no longer access the self-opening slit, and consequently excavate another exit hole through the wall of the domatium.

3.2. Domatium ontogeny

(a) Very young domatia: A very young domatium internode is a small (2–3 cm long), soft, fleshy, and hirsute internode that is slightly swollen compared to the adjacent internode (Fig. 3A). The colour may initially be light pink or a very pale green, but within a few days it becomes brighter green. The self-opening slit is not open yet, and is visible as a small slit blocked by the pith parenchyma from within.

A transverse section of the very young domatium shows a large pith region in the centre, and a considerably constricted vascular region seen as a thin band (Fig. 3B). The pith is ruptured in many places. Cells in the centre of the pith appeared larger than those on the periphery. In the safranin-fast green stained sections of very young domatia (Fig. 3B), the peripheral pith cells are stained green, indicating non-lignification and presence of cytoplasm, whereas the central pith cells do not take up fast green. However, the cell walls are stained by safranin, indicating that these central pith cells have lignified cell walls. This pith tissue then becomes loose and detached from the domatium wall (Fig. 3B).

(b) Young domatia: These are domatia that have reached the average size of full grown domatia, but are still fleshy. The colour is a deeper green, the pubescence on the outer surface disappears, and the domatia have a glossy texture (Fig. 3C). The secondary thickenings of the domatia wall commence during this phase. The self-opening slit is completely opened, and the domatia are often occupied by ants. On opening the domatia, we found that the loose pith cells were mostly absent or remnants of them were attached to the inner domatium wall (Fig. 3D). The pith cells in this phase appear stretched and have thickenings on the cell walls.

(c) Mature domatia: By this phase, the domatia have a hard woody texture (Fig. 3E). The unexpanded base of the domatium internode is often a woody brown, while the distal swollen part of the domatium is green. Most domatia at this stage are occupied by ants or other invertebrates. The loose pith tissue is almost completely absent, and the inner wall of the domatium is lined by a thin spongy layer of the residual pith cells. The transverse sections of mature domatia (Fig. 3F) reveal that the vascular region is compressed, and appears as a thin ring encircling the pith cavity which is formed by the degradation of the pith tissue as the domatium swells.

The inner walls of ant-occupied domatia did not turn red after treatment with Sudan IV, indicating absence of suberised wound tissue that could have indicated active excavation by inhabitants.

In much older domatia, secondary growth appears more prominent;
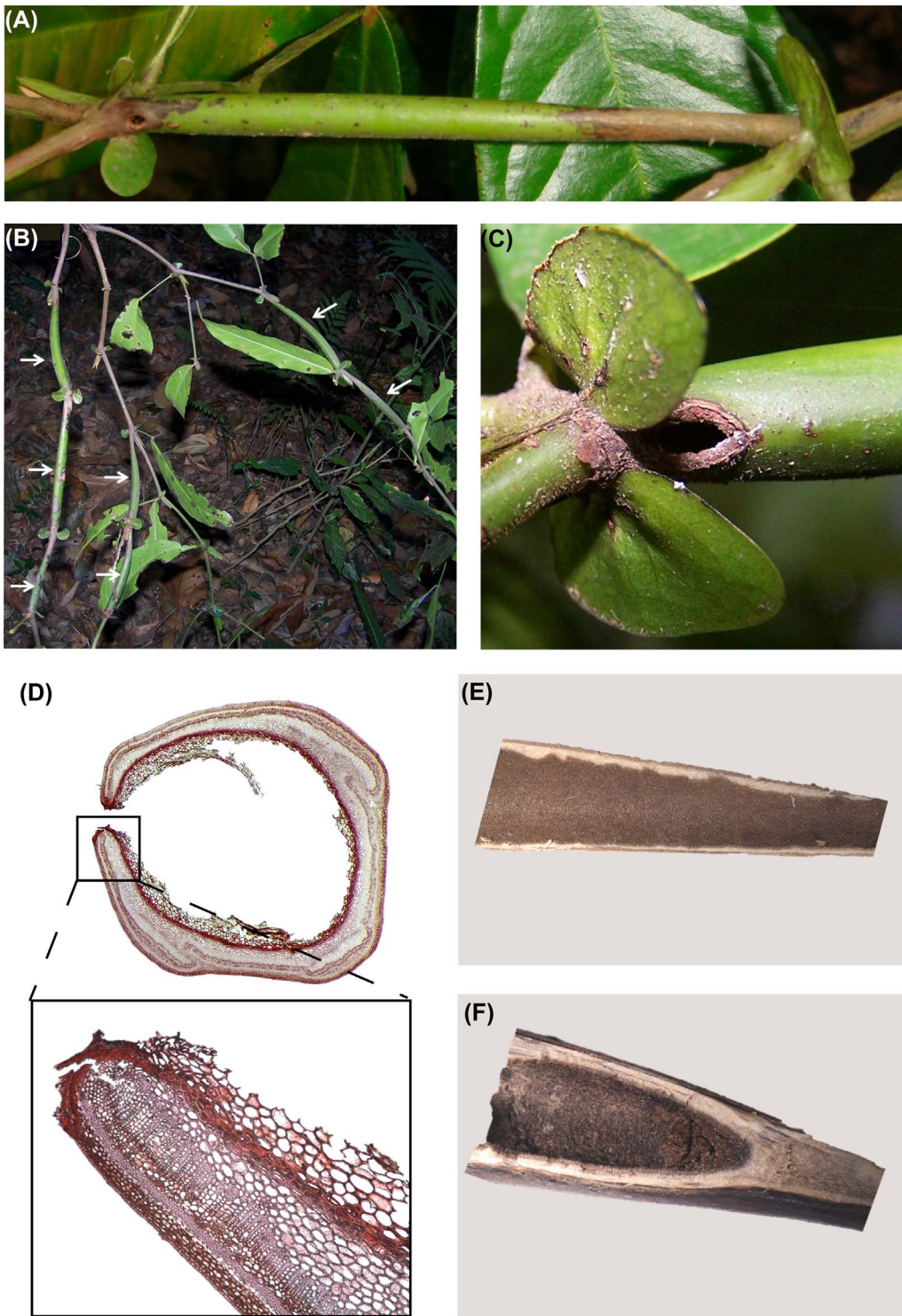
Fig. 1. Domatia of *Humboldtia brunonis* (A) Mature domatium showing the swollen region of the domatium chamber, and a tapering base. The self-opening slit is visible at the distal end of domatium; (B) Multiple adjacent branches each with multiple domatia, indicated by arrows; (C) Close-up of the self-opening slit guarded by a pair of stipules; (D) Transverse section of a domatium through the self-opening slit, showing a thin layer of thick-walled cells lining the inner wall of the domatium as well as the periphery of the self-opening slit (enlarged in box); (E) Inner wall of domatium showing the layer formed by dead pith tissue; (F) Basal portion of a domatium internode, split to show the solid pith region in the basal part of the domatium internode.

the domatia become more woody, and the self-opening slits, if not maintained by the ants, may become shut with secondary growth tissue.

(d) Normal internodes: The young normal internode adjacent to a very young domatium has a well developed vascular region, which appears as a thick band, unlike that of the domatium. The pith region of this young adjacent internode is intact with no sign of thickening or loosening, and is not as wide as in the domatia (Fig. 3G). In the mature normal internode adjacent to the mature domatium, the pith region is much reduced due to the expanding vascular region (Fig. 3H). The pith cells are intact but reduced in size. Unlike a younger internode, the thickness of the vascular ring in the mature internode is equal to or

often greater than the diameter of the pith it encircles. The secondary growth in the outer surface is much greater in the mature normal internode than in the outer wall of the mature domatium.

3.3. Domatium inner wall

Scanning electron microscopy revealed that the inner wall of a very young unoccupied domatium is lined by collapsed dead pith tissue that appears as flaky sheets covering the underlying layers (Figs. 4A, B). These pith cells have highly pitted walls (Fig. 4C). In some mature ant-occupied domatia, we observed crystals on the surface of the domatium
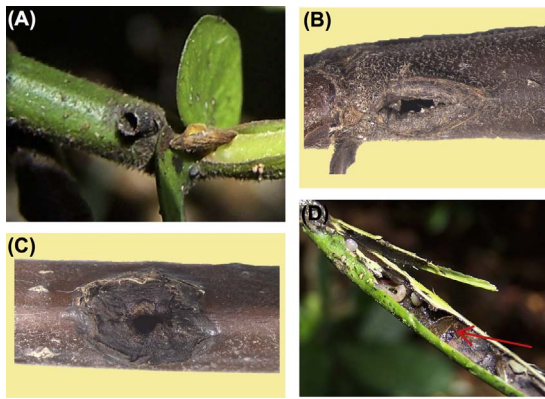
Fig. 2. Modification of *Humboldtia brunonis* domatia by inhabitants (A) A domatium showing modification of entrance by *Tapinoma indicum* ants; (B) Thickened rim of a self-opening slit which has been chewed to enlarge the opening; (C) An opening excavated in the domatium wall, in a domatium occupied by *Braunsapis* bees; (D) The inside of a domatium occupied by earthworm and ants, showing carton disc (indicated by arrow) separating the earthworm chamber (towards self-opening slit) from the ant chamber (towards the base).

inner wall. These crystals were either raphides, styloids or druses (Fig. 5). The flaky sheets of dead pith tissue that were present in very young unoccupied domatia are absent in mature, ant-occupied domatia (Fig. 6A). It appears that, when ants start occupying the domatia, they clear all the loose dead pith tissue within. In some young ant-occupied domatia, the clearing is not complete, and there are patches where the loose pith tissue is adherent to the wall of the cavity, whereas it is scraped off in other patches, revealing the peripheral dead pith layer. Some parts of the surface of the inner wall are lined by a thin sparse mat of thread-like structures that appear to be fungal hyphae (Fig. 6B), penetrating up to one or two layers of cells below the surface of the inner wall. This mat was not present in earthworm-occupied domatia (Fig. 7A). Instead, the inner walls of earthworm-occupied domatia seem to be lined by a thick layer of a substance (Fig. 7B) that could be either earthworm excreta or mucus. Bee-occupied domatia also did not have the putative fungal mats on the inner wall surface.

4. Discussion

This is the first study to report on the detailed morphology of the cauline domatia in the genus *Humboldtia*, to compare their anatomy with that of adjacent normal internodes, and to investigate the onto-geny of cauline domatia formation in the genus *Humboldtia*. In *H. brunonis* the domatia are borne only on some internodes of some stems, unlike the conspecific true myrmecophyte *H. laurifolia* or the related true myrmecophyte *L. a. africana* in which all internodes of all branches are modified to form domatia, and only few "abnormal" internodes do not swell (McKey, 1989; Krombein, 1999; Blatrix et al., 2012). The size of the domatium cavity in *H. brunonis* (ca 9–10 cm in length; Shenoy et al., 2010) is much larger compared to that in *L. a. africana* (ca 3 cm; Blatrix et al., 2012). Unlike the latter, where the length of the internode is almost equally divided into the swollen domatium portion and the solid base (Blatrix et al., 2012), in *H. brunonis,* a much greater portion of the internode is swollen, while the base is relatively shorter, and this is quite similar to that of *H. laurifolia*, as reported in Krombein et al. (1999). The small size of the domatium in *L. a. africana* is thought to be a consequence of the adaptive advantages of expressing domatia very early in ontogeny to house protective ants, which are also selected for their small size (Blatrix et al., 2012). Such early or precocious onset of domatia (i.e., when the plant is only 10 cm tall; Brouat and McKey, 2000) is an advanced trait associated with obligate and specific myr-mecophytic systems as in *L. a. africana* (Brouat and McKey, 2000). In a non-specific myrmecophyte such as *H. brunonis*, the domatia are ex-pressed relatively later in plant ontogeny (when the plant is about

70 cm in height; Brouat and McKey, 2000), and by this time the sapling is big enough to support the larger domatia that are characteristic of this species compared to *L. a. africana*. In *H. brunonis*, only the larger domatia were occupied, and larger-sized domatia (> 8 cm in length and 0.5 cm in breadth) may be preferred by all its occupants (Shenoy and Borges, 2010). The ants associated with *H. brunonis*, with the ex-ception of *Tapinoma indicum* and *Vombisidris humboldticola*, are rela-tively large ants with large colonies. Except for *Vombisidris humboldti-cola* (Zacharias and Rajan, 2004), the ants are not obligate domatium inhabitants, and can nest outside the domatia. However, in a seasonal tropical rainforest, large domatia could be a highly attractive resource offering effective protection from rain for these ants and other in-vertebrates, as well as from desiccation during the lengthy dry seasons for the arboreal earthworm *P. pullus* (Chanam et al., 2014a).

Since ants are repelled by the mucus of *P. pullus* (Gaume et al., 2006), the carton partitions built by ants within the domatia could, therefore, serve to restrict the mucus to the portion of the domatium occupied by these earthworms. There could be strong competition be-tween ants and earthworms for domatia occupancy with the mucus repellency effect giving the earthworms a competitive advantage. It is therefore interesting that the earthworms predominate in the domatia in the northern-most portion of the range of *H. brunonis* where the dry season is the longest (Shenoy and Borges, 2010), and that the protection mutualism with ants is restricted to the southern-most portion of the ant-plant's range (Chanam et al., 2014a). The domatia of *H. brunonis* are host to many ants and other invertebrates, and trophic benefits accrue to the host plant from these domatia inhabitants whether they provide protective services or not (Chanam et al., 2014b). Consequently in this system, it is possible that trophic mutualism alone or in addition to protection mutualism is the basis for the maintenance of this myrme-cophytic trait. Therefore, despite the fact that domatia are expressed later in ontogeny, the large size of the domatia could indeed be adap-tive since it allows large numbers of inhabitants (e.g. up to 21 earth-worms, and large colonies of ants) to inhabit domatia. Between 9% (from earthworm inhabitants) and 17% (from all types of residents ants) of nitrogen in tissues nearest the domatium was derived from domatia inhabitants (Chanam et al., 2014b); these benefits may be re-sponsible for the greater fitness of domatia-bearing plants compared to those without domatia in *H. brunonis* populations (Gaume et al., 2005). Consequently, such plants may tolerate non-obligate and non-protective domatia occupants from which they obtain nutritional benefits.

Each of the unoccupied domatia has a hollowed cavity with the dead pith tissue appearing as shriveled membranous flakes on the inner wall, indicating that the cavity is formed spontaneously. This seems similar to what is described for *H. laurifolia* (Krombein et al., 1999) but different from *L. a. africana*, where young internodes are observed to be swollen and "filled with thick pith" which is later excavated and hol-lowed out by ants (McKey, 1984, 1989). The absence of the suberin wound response in the inner wall of the domatia further supports the observation that the domatium cavity is not actively excavated by the ant occupants. The absence of a prostoma, and the presence of a pre-formed self-opening slit instead, may also explain why in this system there is less specificity of occupant identity. The long raphide and styloid-like crystals in the cells that line the inner wall are similar to what is described for the pith cells of other cauline domatia (Tepe et al., 2007), while druse crystals are not yet reported in ant domatia to our knowledge.

Pith parenchyma cells grow and mature faster than other cells, and once mature, they stop dividing (Fahn, 1974, 1990). In the case of a young growing domatium where the stem diameter continues to in-crease, the mature pith cells which are not dividing anymore will be pulled or stretched. The mechanical stress exerted on the pith par-enchyma cells due to the swelling of the domatium could have led to thickening of the walls of the central pith cells (Fahn, 1974, 1990) which is visible in transverse sections of very young domatia (Fig. 3B). As the domatium swells further, these lignified pith cells are pulled
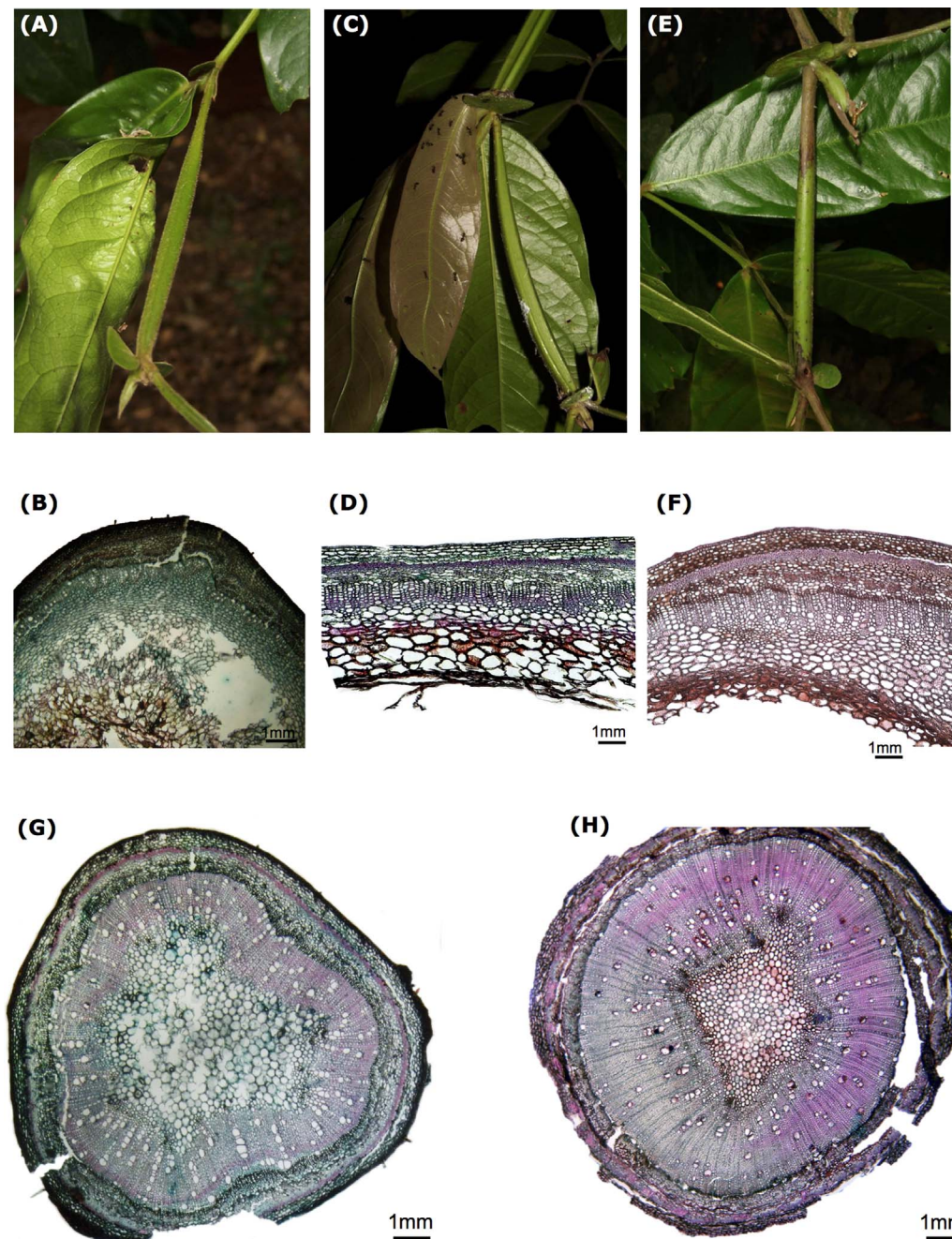
Fig. 3. Morphology of domatia and normal internode of *Humboldtia brunonis* at different growth stages (A) A very young domatium showing fleshy, hirsute wall; (B) Transverse section through a very young domatium, showing partially ruptured pith tissue; pith cells starting to thicken in the central region while the peripheral pith cell are still parenchymatous; the vasculature is constricted and visible only as a thin strip along the wall; (C) A young domatium; (D) Transverse section through a young domatium, showing domatium inner wall lined by dead pith tissue; the pith cells have thickened walls and appear stretched due to the swelling up of the domatium; (E) A mature domatium with woody texture; (F) Transverse section through a mature domatium showing dead pith cells lining the domatium cavity; the vasculature is constricted and visible only as a thin strip along the wall; (G) Transverse section through normal internode adjacent to the very young domatium showing intact pith, and well-formed vasculature. Notice the difference in the proportion of pith area in domatium and normal internode; (H) Transverse section through normal internode adjacent to the mature domatium showing intact pith and well-developed secondary vasculature.

apart, and appear stretched and/or ruptured due to the strain, and hang loose or collapse against the peripheral wall of the domatium cavity. In the more mature domatia, even the peripheral pith cells show lignification. This indicates that lignification of the pith cells starts in the central cells and spreads towards the peripheral cells of the pith, as the domatium continues to grow. These peripheral cells are, however, not apparently pulled apart or ruptured by the swelling of the domatium. Nevertheless, they appear lysed. The cavity formation of the domatium therefore appears to be the collective effect of both schizogeny and lysogeny, following acropetal lignification of the pith cells as the domatium internode swells up. The inner walls of domatia are, therefore, lined by dead peripheral pith cells that have a spongy appearance.

The remnant pith cells that form the inner lining of the domatium cavity are similar to those of *L. a. africana* in having 'canaliculated, lignified sclerenchyma with numerous plasmodesmata (intercellular pits)' (Defossez et al., 2011), as observed in the SEM images. This is also very similar to the inner walls of other myrmecophytes (Tepe et al.,

2007; Gegenbauer et al., 2012). Such pitted walls could possibly allow flow of nutrients derived from the domatia occupants into the plant vasculature, and thereby facilitate nutrient flux with domatia inhabitants.

Numerous studies have reported the frequent presence of fungi in ant-occupied domatia, and have suggested the role of these fungi as a third party in ant–plant mutualisms by helping in breakdown of ant wastes in order to facilitate their absorption by the host plant (Moog, 2009; Defossez et al., 2009; 2011; Leroy et al., 2011; Voglmayr, 2011; Mayer et al., 2014). So far, only fungi belonging to the order Chaetothyriales (Ascomycota) have been identified in such interactions (Voglmayr et al., 2011). We did not observe anything like the distinct dark spots of fungal mycelia as reported in *L. a. africana* where the obligately mutualistic ant *Petalomyrmex phylax* repeatedly excretes only on the mycelial patch it is cultivating within the domatium (Defossez et al., 2009, 2011). However, the thread-like structures that form a thin mat covering parts of the inner surface of ant-occupied domatia of *H.*
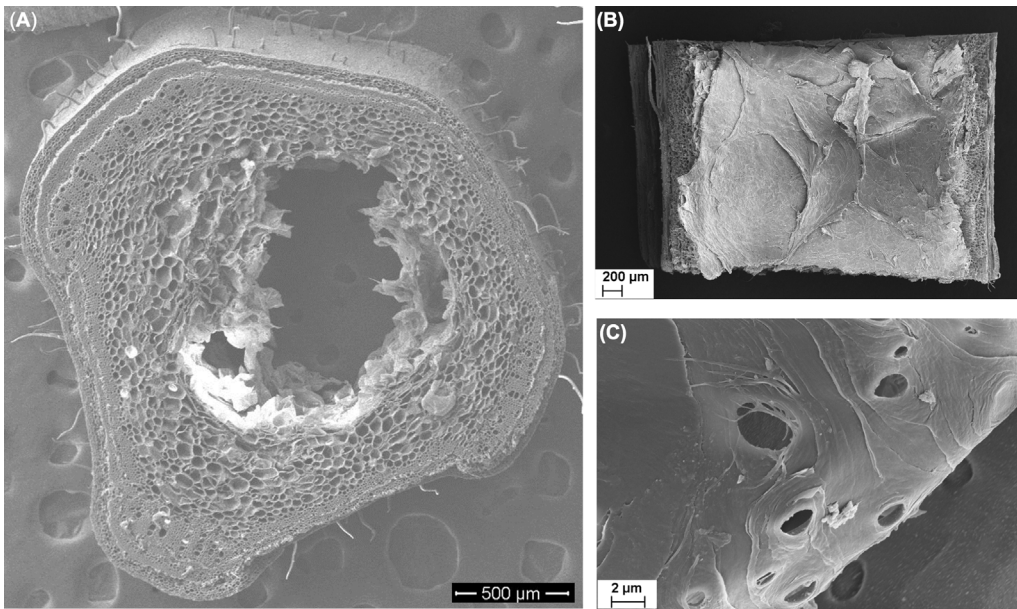
Fig. 4. (A) Scanning electron microscope image of a transverse section of a very young unoccupied domatium showing ruptured pith tissue as the domatium chamber starts to form; (B) Scanning electron microscope image of the inner wall of a young unoccupied domatium. The pith tissue appears as flakes collapsed against the domatium wall; (C) Scanning electron microscope image of the canaliculated walls of the pith cells showing numerous pits.

Fig. 5. Druse crystals on the inner wall of an ant-occupied domatium.

Fig. 6. (A) Scanning electron microscope image of the inner wall of an ant-occupied domatium showing a thin mat of putative fungal hyphae; (B) Close-up image of putative fungal hyphae.

brunonis (Fig. 6B) are fungal mycelia whose identity is in the process of being confirmed (A. Vishnu and R.M. Borges, unpub. data). Such a fungal mat could be involved in the breakdown of ant-derived organic matter, and over a much larger surface area than observed in *L. a. africana*, which is expected in a non-specialised system as *H. brunonis,* where non-obligate ants are not selected for the behaviour seen in *L. a. africana.* Further investigations of ant behaviour inside the domatia of *H. brunonis* would give greater insight into a potential ant–fungus–plant mutualism in this system. The absence of fungal mycelia in earthworm-occupied domatia could be due to earthworm mucus which, with pH values as low as 3 (M. Shenoy and R.M. Borges., pers. obs.), could prevent fungal growth. Earthworm mucus also has a repellent effect on ants (Gaume et al., 2006).

Domatium production in *L. a. africana* imposed heavy costs on the plant early in ontogeny owing to greater investments of primary wood in domatia compared to the non-swollen domatia bases, especially since every internode bears a domatium from an early phase of plant ontogeny, thereby requiring greater investment for support (Blatrix et al., 2012). In *H. brunonis*, a similar study has not yet been done; however, the cost is likely to be lower than that for *L. a. africana* since in *H. brunonis*, domatia are borne only on some internodes of the plants, and they are expressed at a relatively later stage in plant ontogeny. However, as mentioned earlier, both trophic (Chanam et al., 2014b) and non-trophic benefits (Gaume et al., 2005) accrue to *H. brunonis* plants that bear domatia, and such plants have higher fruit set compared to
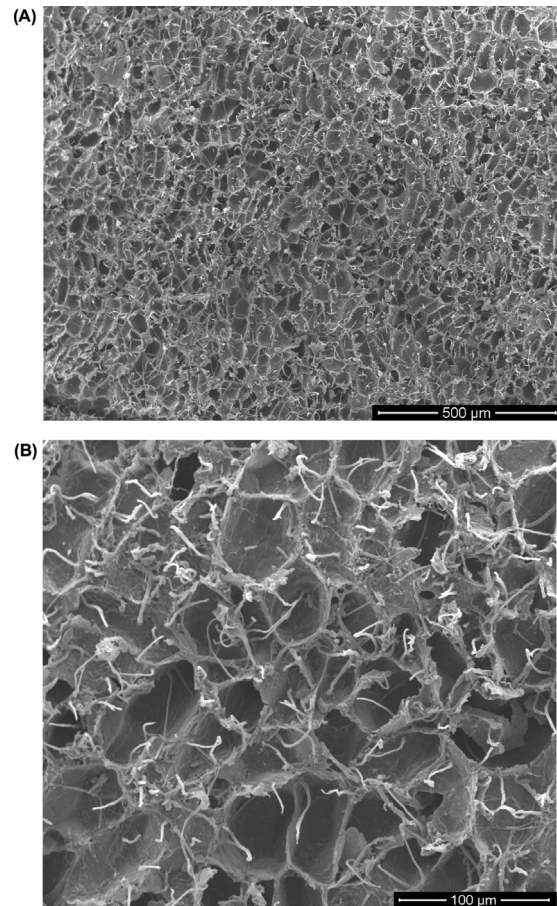
non-domatia bearing plants (Gaume et al., 2005). Therefore the cost of domatia is likely offset by the increased fruit set of domatia-bearing plants.
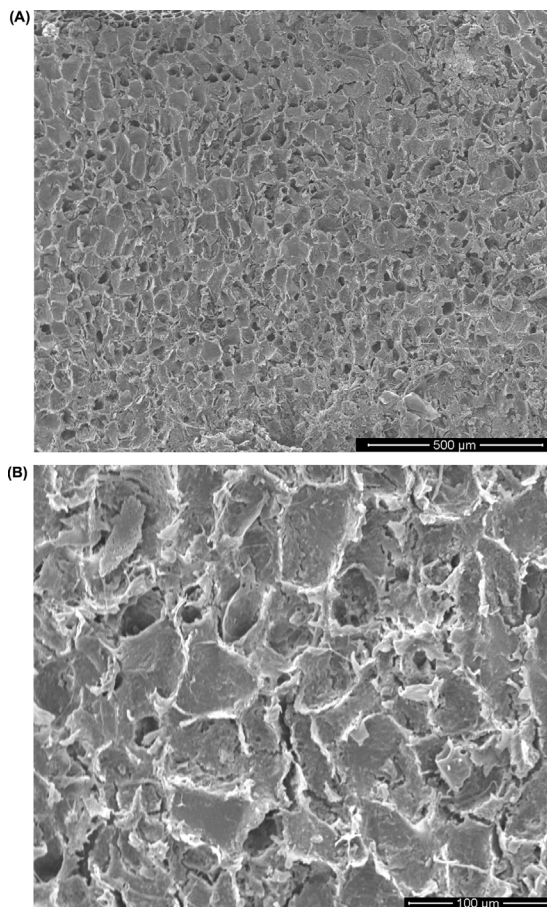
Fig. 7. (A) Scanning electron microscope images of the inner wall of an earthworm-occupied domatium; (B) Close-up of the inner wall of an earthworm-occupied domatium showing a thick layer of a substance that could be either earthworm excreta or mucus.

5. Conclusion

The present study is the first to describe the mechanism of cauline domatia formation in the genus *Humboldtia* as a collective effect of both schizogeny and lysogeny, following acropetal lignification of the pith cells as the domatium internode swells up, with descriptions of domatia at different developmental stages, and comparisons with normal internodes, i.e., those not bearing domatia. Our findings contribute to our present understanding of spontaneous formation of domatia in myrmecophytes, and the role of domatia as a shelter not only for protective ants, but also for other inhabitants that provide nutrition to the host plant.

Acknowledgements

References

Blüthgen, N., Wesenberg, J., 2001. Ants induce domatia in a rain forest tree (*Vochysia vismiaefolia*). Biotropica 33, 637–642.

Blatrix, R., Renard, D., Djieto-Lordon, C., McKey, D., 2012. The cost of myrmecophytism: insights from allometry of stem secondary growth. Ann. Bot. 110, 943–951.

Brouat, C., McKey, D., 2000. Origin of caulinary ant domatia and timing of their onset in plant ontogeny: evolution of a key trait in horizontally transmitted ant-plant symbioses. Biol. J. Linn. Soc. 71, 801–819.

Brouat, C., McKey, D., 2001. Leaf-stem allometry, hollow stems, and the evolution of caulinary domatia in myrmecophytes. New Phytol. 151, 391–406.

Brouat, C., Garcia, N., Andary, C., McKey, D., 2001. Plant lock and ant key: pairwise coevolution of an exclusion filter in an ant–plant mutualism. Proc. R. Soc. B 268, 2131–2141.

Bruneau, A., Forest, F., Herendeen, P.S., Klitgaard, B.B., Lewis, G.P., 2001. Phylogenetic relationships in the Caesalpinioideae (Leguminosae) as inferred from chloroplast *trn*L intron sequences. Syst. Bot. 26, 487–514.

Chanam, J., Kasinathan, S., Pramanik, G.K., Jagdeesh, A., Joshi, K.A., Borges, R.M., 2014a. Context dependency of rewards and services in an ant–plant interaction: southern sites favour the mutualism between plants and ants. J. Trop. Ecol. 30, 219–229.

Chanam, J., Sheshshayee, M.S., Kasinathan, S., Jagdeesh, A., Joshi, K.A., Borges, R.M., 2014b. Nutritional benefits from domatia inhabitants in an ant–plant interaction: interlopers do pay the rent. Funct. Ecol. 28, 1107–1116.

Davidson, D.W., McKey, D., 1993. The evolutionary ecology of symbiotic ant-plant relationships. J. Hymenopt. Res. 2, 13–83.

Davidson, D.W., 1997. The role of resource imbalances in the evolutionary ecology of tropical arboreal ants. Biol. J. Linn. Soc. 61, 153–181.

Defossez, E., Selosse, M.A., Dubois, M.P., Mondolot, L., Faccio, A., Djieto-Lordon, C., McKey, D., Blatrix, R., 2009. Ant-plants and fungi: a new threeway symbiosis. New Phytol. 182, 942–949.

Defossez, E., Djiéto-Lordon, C., McKey, D., Selosse, M.A., Blatrix, R., 2011. Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. Proc. R. Soc. B 278, 1419–1426.

Dejean, A., Corbara, B., Orivel, J., Leponce, M., 2007. Rainforest canopy ants: the implications of territoriality and predatory behavior. Funct. Ecosyst. Comm. 1, 105–120.

Del Val, E., Dirzo, R., 2003. Does ontogeny cause changes in the defensive strategies of the myrmecophyte *Cecropia peltata*? Plant Ecol. 169, 35–41.

Fahn, A., 1974. Plant Anatomy, second ed. Pergamon Press, Oxford.

Fahn, A., 1990. Plant Anatomy, fourth ed. Pergamon Press, Oxford.

Fiala, B., Maschwitz, U., 1992a. Domatia as most important adaptations in the evolution of myrmecophytes in the paleotropical tree genus *Macaranga* (Euphorbiaceae). Plant Syst. Evol. 180, 53–64.

Fiala, B., Maschwitz, U., 1992b. Food bodies and their significance for obligate ant-association in the tree genus *Macaranga* (Euphorbiaceae). Bot. J. Linn. Soc. 110, 61–75.

Fischer, R.C., Wanek, W., Richter, A., Mayer, V., 2003. Do ants feed plants? A ^{15}N labelling study of nitrogen fluxes from ants to plants in the mutualism of *Pheidole* and *Piper*. J. Ecol. 91, 126–134.

Fonseca, C.R., 1993. Nesting space limits colony size of the plant-ant *Pseudomyrmex concolor*. Oikos 67, 473–482.

Gaume, L., McKey, D., Terrin, S., 1998. Ant–plant–homopteran mutualism: how the third partner affects the interaction between a plant-specialist ant and its myrmecophyte host. Proc. R. Soc. B 265, 569–575.

Gaume, L., Zacharias, M., Grosbois, V., Borges, R.M., 2005. The fitness consequences of bearing domatia and having the right ant partner: experiments with protective and non-protective ants in a semi-myrmecophyte. Oecologia 145, 76–86.

Gaume, L., Shenoy, M., Zacharias, M., Borges, R.M., 2006. Co-existence of ants and an arboreal earthworm in a myrmecophyte of the Indian Western Ghats: anti-predation effect of the earthworm mucus. J. Trop. Ecol. 22, 341–344.

Gegenbauer, C., Mayer, V.E., Zotz, G., Richter, A., 2012. Uptake of ant-derived nitrogen in the myrmecophytic orchid *Caularthron bilamellatum*. Ann. Bot. 110, 757–765.

Huxley, C.R., 1978. The ant-plants *Myrmecodia* and *Hydnophytum* (Rubiaceae) and the relationships between their morphology, ant occupants, physiology and ecology. New Phytol. 80, 231–268.

Janzen, D.H., 1966. Coevolution of mutualism between ants and acacias in Central America. Evolution 20, 249–275.

Krombein, K.V., Norden, B.B., Rickson, M.M., Rickson, F.R., 1999. Biodiversity of domatia occupants (ants, wasps, bees, and others) of the Sri Lankan myrmecophyte *Humboldtia laurifolia* Vahl (Fabaceae). Smith. Contrib. Zool. 603, 1–34.

Leroy, C., Séjalon-Delmas, N., Jauneau, A., Ruiz-González, M.X., Gryta, H., Jargeat, P., Corbara, B., Dejean, A., Orivel, J., 2011. Trophic mediation by a fungus in an ant–plant mutualism. J. Ecol. 99, 583–590.

Mayer, V.E., Frederickson, M.E., McKey, D., Blatrix, R., 2014. Current issues in the evolutionary ecology of ant–plant symbioses. New Phytol. 202, 749–764.

McKey, D., 1984. Interaction of the ant-plant *Leonardoxa africana* (Caesalpiniaceae) with its obligate inhabitants in a rainforest in Cameroon. Biotropica 16, 81–99.

McKey, D., 1989. Interactions between ants and leguminous plants. In: In: Stirton, C.H., Zarucchi, J.L. (Eds.), Advances in Legume Biology, Monogr. Syst. Bot. Miss. Bot. Gard. 29. pp. 673–718.

Moog, J., 2009. The Associations of the Plant-Ant *Cladomyrma* with Plants in Southeast Asia, Doctoral Dissertation. Frankfurt (Main) University.

Peccoud, J., Piatscheck, F., Yockteng, R., Garcia, M., Sauve, M., Djiéto-Lordon, C., Harris, D.J., Wieringa, J.J., Breteler, F.J., Born, C., McKey, D., 2013. Multi-locus phylogenies of the genus *Barteria* (Passifloraceae) portray complex patterns in the evolution of

myrmecophytism. Mol. Phylogenet. Evol. 66, 824–832.

Ramesh, B.R., Pascal, J.P., 1997. Atlas of Endemics of the Western Ghats (India): Distribution of Tree Species in the Evergreen and Semi-evergreen Forests. French Institute of Pondicherry, Pondicherry, India.

Rickson, F.R., Rickson, M.M., Ghorpade, K., Norden, B.B., Krombein, K.V., 2003. Invertebrate biodiversity (ants, bees, and others) associated with stem domatia of the Indian myrmecophyte *Humboldtia brunonis* Wallich (Magnoliophyta: fabaceae). Proc. Entomol. Soc. Washington 105, 73–79.

Romero, G.Q., Mazzafera, P., Vasconcellos-Neto, J., Trivelin, P.C.O., 2006. Bromeliad-living spiders improve host plant nutrition and growth. Ecology 87, 803–808.

Sagers, C.L., Ginger, S.M., Evans, R.D., 2000. Carbon and nitrogen isotopes trace nutrient exchange in an ant–plant mutualism. Oecologia 123, 582–586.

Sanjappa, M., 1986. A revision of the genus *Humboldtia* Vahl. (Leguminosae: Caesalpinioideae). Blumea 31, 329–339.

Shenoy, M., Borges, R.M., 2008. A novel mutualism between an ant-plant and its resident pollinator. Naturwissenschaften 95, 61–65.

Shenoy, M., Borges, R.M., 2010. Geographical variation in an ant–plant interaction correlates with domatia occupancy, local ant diversity, and interlopers. Biol. J. Linn. Soc. 100, 538–551.

Shenoy, M., Radhika, V., Satish, S., Borges, R.M., 2012. Composition of extrafloral nectar influences interactions between the myrmecophyte *Humboldtia brunonis* and its ant associates. J. Chem. Ecol. 38, 88–99.

Tepe, E.J., Vincent, M.A., Watson, L.E., 2007. Stem diversity, cauline domatia, and the evolution of ant–plant associations in *Piper* sect. *Macrostachys* (Piperaceae). Am. J. Bot. 94, 1–11.

Tepe, E.J., Kelley, W.A., Rodriguez-Castañeda, G., Dyer, L.A., 2009. Characterizing the cauline domatia of two newly discovered Ecuadorian ant plants in *Piper*: an example of convergent evolution. J. Insect Sci. 9, 27.

Trager, M.D., Bhotika, S., Hostetler, J.A., Andrade, G.V., Rodriguez-Cabal, M.A., McKeon, C.S., Osenberg, C.W., Bolker, B.M., 2010. Benefits for plants in ant-plant protective mutualisms: a meta-analysis. PLoS One 5, e14308.

Treseder, K.K., Davidson, D.W., Ehleringer, J.R., 1995. Absorption of ant-provided carbon dioxide and nitrogen by a tropical epiphyte. Nature 375, 137–139.

Voglmayr, H., Mayer, V., Maschwitz, U., Moog, J., Djieto-Lordon, C., Blatrix, R., 2011. The diversity of ant-associated black yeasts: insights into a newly discovered world of symbiotic interactions. Fungal Biol. 115, 1077–1091.

Ward, P.S., 1991. Phylogenetic analysis of pseudomyrmecine ants associated with domatia-bearing plants. In: Huxley, C.R., Cutler, D.F. (Eds.), Ant-Plant Interactions. Oxford University Press, Oxford, pp. 335–352.

Weber, M.G., Keeler, K.H., 2013. The phylogenetic distribution of extrafloral nectaries in plants. Ann. Bot. 111, 1251–1261.

Wheeler, W.M., 1942. Studies of neotropical ant-plants and their ants. Bull. Mus. Comp. Zool. Harvard Coll. 90, 1–262.

Zacharias, M., Rajan, P.D., 2004. Vombisidris humboldticola *Vombisidris humboldticola* (Hymenoptera: Formicidae): a new arboreal ant species from an Indian ant plant. Curr. Sci. 87, 1337–1338.