

STEM DIVERSITY, CAULINE DOMATIA, AND THE EVOLUTION
OF ANT-PLANT ASSOCIATIONS IN *PIPER* SECT.
MACROSTACHYS (PIPERACEAE)¹

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Plants possess a variety of structures that harbor ant nests, and the morphology of these domatia determines the nature of ant-plant mutualisms in a given plant species. In this study, we report on the differences in anatomy between myrmecophytes of *Piper*, which are regularly excavated by an obligate ant mutualist (*Pheidole bicornis*) and nonmyrmecophytes of *Piper*, which consistently have solid stems. Stems of excavated plant species lacked outward evidence of modification; however, striking anatomical differences were apparent between hollow-stemmed species before excavation and the remainder of the solid-stemmed species studied. Prior to excavation by ants, stems of myrmecophytes were characterized by strongly heterogeneous piths in which a large, central area had relatively large cells lacking intracellular crystals with a periphery of smaller cells containing numerous crystals. The domatium excavated by the ants was restricted to the large-celled region. This is the first report of the absence of crystals in ant-excavated portions of stems of myrmecophytes. Cauline domatia became lined with 3–8 cell layers of suberized wound tissue, which may have an impact on nutrient absorption by *Piper* myrmecophytes.

Key words: ant-plant mutualisms; cauline domatia; heterogeneous pith; intracellular crystals; *Pheidole bicornis*; *Piper*; Piperaceae; stem anatomy.

Myrmecophytes are plants that have modified structures in which ant colonies nest, and mutualistic associations between ants and plants have evolved in over 40 plant families throughout the tropics (Davidson and McKey, 1993). Correspondingly, the ecological and evolutionary interactions between ants and plants have produced a number of conspicuous evolutionary novelties (Janzen, 1966; Davidson and McKey, 1993). In plants, these include structures from which ants derive nutrients and those in which they nest. Ants regularly nest in a variety of plant-derived structures (Hölldobler and Wilson, 1990; Huxley and Cutler, 1991; Davidson and McKey, 1993; Orivel and Dejean, 1999), but few species nest in living plants, and fewer still modify living plants for their own use as domatia (Ward, 1991; Moog et al., 1998). Plant domatia are associated with the most long-term, mutually beneficial, and often species-specific associations between ants and plants (Fiala and Maschwitz, 1992). Strong selective forces can result from such close associations, and the

benefits gained by the presence of ant mutualists can profoundly influence domatium morphology (Janzen, 1966; Davidson and McKey, 1993). Although domatia vary greatly among myrmecophytes, few studies have compared the domatia of myrmecophytes with the corresponding organs of congeneric nonmyrmecophytes. In this study, we investigate the comparative morphology of the stems of myrmecophytes with cauline domatia and their relatives that have varying degrees of association with ants.

Bailey (1923) was among the first to stress that the nature and specificity of ant-plant relationships in which ants nest inside plant structures is largely determined by the structure and arrangement of the tissues of the stem. His work revealed a number of modified plant structures that appear to promote ant residency, and he discussed the relative contributions of plant anatomy and ant behavior to domatia formation. Subsequent studies have corroborated Bailey's view that structures of certain plant species facilitate, if not promote, ant residency (Janzen, 1966; McKey, 1984; Fiala and Maschwitz, 1992; Maschwitz et al., 1994, 1996; Moog et al., 2002; Blüthgen and Wesenberg, 2001). For example, some ant plants (i.e., myrmecophytes) have conspicuously swollen organs in which ants regularly nest (e.g., spines of *Acacia*, Janzen, 1967; nodes of *Cordia*, Wheeler, 1942; internodes in *Leonardoxa*, McKey, 1984; leaf rachises in *Tachigali*, Bailey, 1923), and these hypertrophies (Bequaert, 1922) are among the most apparent features of myrmecophytes (exclusive of the ants themselves). Some myrmecophytic species lack hypertrophied domatia; yet most individuals of the species are nearly always inhabited by ants. Although the domatia-bearing organs of these myrmecophytes do not appear outwardly modified, comparative studies have revealed interesting anatomical differences in the homologous structures from species that harbor ants and those that do not (Bailey, 1922a, 1924). For example, Bailey's studies revealed that the exceedingly complex domatia of *Cordia nodosa* and allied species are formed in a fundamentally different manner than the domatia of other congeneric myrmecophytes (Bailey, 1924). Despite these early insights,

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little detailed anatomical work on ant-associated plant parts has been done, with the exceptions of Brouat et al. (2001) and Federle et al. (2001), and even fewer studies have compared the anatomy of myrmecophytes to nonmyrmecophytic congeners. However, comparative studies of myrmecophytes and nonmyrmecophytes permit the discovery of differences that would otherwise go unnoticed in studies of myrmecophytes alone.

In this study, we evaluate the comparative anatomy of stems of *Piper* L. sect. *Macrostachys* (Miq.) C. DC. (Piperaceae), a lineage containing specialized, generalized, and nonmyrmecophytic species. Specialized myrmecophytes in *Piper* are plant species that are nearly always occupied by *Pheidole bicornis* Forel, the specialized ant partner (Longino and Cover, 2004). They have modified organs in which ants nest, they produce pearl bodies that ants eat, and they suffer fitness loss in the absence of ants (Letourneau, 1998). Generalized myrmecophytes house various species of ants, which nest in plant structures that may have minor modifications that result in better nesting sites (Tepe et al., in press). They lack the large-scale pearl body production of the specialized myrmecophytes (Risch and Rickson, 1981), and may or may not suffer fitness loss without the ants. Likewise, the ants may or may not suffer fitness loss without the particular *Piper* species. Similarly, the associations involving these sets of organisms are called specialized or generalized, replacing obligate and facultative, respectively, that we used in previous works (Tepe et al., 2004, in press). These conventional terms are not entirely appropriate for the associations observed in *Piper*; whereas the "obligate" ant-*Piper* association appears to be truly obligate for the ant partner *Ph. bicornis* (Longino and Cover, 2004), the *Piper* species that are occupied by *Ph. bicornis* can survive without the ant partner, albeit with reduced fitness (Letourneau, 1998). This trend has been observed in at least one other ant-plant system (Fonseca and Ganade, 1996).

The neotropical, monophyletic sect. *Macrostachys* includes 50–60 species and is composed of large-leaved shrubs to small trees typical of the deep shade of the rain forest understory (Jaramillo and Manos, 2001; Jaramillo and Callejas, 2004; Tepe et al., 2004). Section *Macrostachys* contains a range of associations with ants including (1) five species of specialized myrmecophytes that are associated with a single ant species, *Ph. bicornis* (Risch et al., 1977; Risch and Rickson, 1981; Risch, 1982; Letourneau 1983, 1998); (2) generalized myrmecophytes in which a number of arboricolous ants nest in some individuals of some populations of at least two *Piper* species (Tepe et al., 2004); and (3) nonmyrmecophytes that do not house ant colonies (14 species are included in this study).

In the five specialized species in sect. *Macrostachys*, the margins of the petioles are appressed to form a closed chamber that comprises the domatium for the resident ants (Tepe et al., in press). Pearl bodies (epidermal cells swollen with lipids, proteins, and carbohydrates) are produced inside the petiole chamber and are consumed by *Ph. bicornis* (Risch and Rickson, 1981; Rickson and Risch, 1984; Fischer et al., 2002; Tepe et al., in press). In most of these species, ants chew a hole into the stem from the petiole cavity, and excavate the stems to create cauline domatia (Risch et al., 1977). Chewing into living plant tissue is a relatively uncommon trait in ants (Ward, 1991), and the pith of many other myrmecophytic species disintegrates spontaneously before inhabitation by ants (Bailey, 1922a). Thus, in addition to the plant morphologies that support specialized ant-plant associations, the behavioral and nutritional requirements of the ants (i.e., dependence on

pearl bodies) are important factors in the establishment and maintenance of the associations. The remaining specialized species, *Piper calcariformis* Tebbs, is also occupied by *Ph. bicornis*, yet the stems remain solid (Tepe et al., 2004). Similarly, stems of the generalized species are solid, and ant nests are restricted to the petioles (Tepe et al., in press).

Myrmecophytes in *Piper* sect. *Macrostachys* lack any outward evidence of obviously modified structures, but certain species are almost always encountered with ant residents and hollow stems. A number of studies have presented thorough descriptions and comparisons of different aspects of *Piper* stems (de Candolle, 1866; Debray, 1886; Van Tieghem, 1908; Chibber, 1912; Hoffstadt, 1916; Rousseau, 1927; Bond, 1931; Duchaigne, 1955), but none have provided a descriptive or comparative account of the stems of *Piper* myrmecophytes. In the present study, we investigated stem morphology and anatomy of myrmecophytic and nonmyrmecophytic species of *Piper* sect. *Macrostachys* to understand the qualities that facilitate occupation by ants. The objectives of this study were (1) to explore the morphology and anatomy of the stems of sect. *Macrostachys*, (2) to provide a comparative account of stem characters among specialized, generalized, and nonmyrmecophytic species, and (3) to examine the relationship between plant vasculature and the passage between the petiole and cauline domatia.

MATERIALS AND METHODS

Samples were collected and observations conducted at 17 locations in Costa Rica during June–August 2000 and May–June 2001 and at 13 locations in Panama during May–June 2003. Vouchers were collected and were deposited in the W. S. Turrell Herbarium at Miami University (MU). Duplicate sets of Costa Rican collections were deposited at the Museo Nacional de Costa Rica (CR) and the Universidad de Costa Rica (USJ). Sets of Panamanian collections were deposited at the University of Panama (PMA) and the herbarium at the Smithsonian Tropical Research Institute (STRI). In total, 121 populations of 21 species were collected, surveyed, and examined. Collections anatomically examined in this study are included in the Appendix.

All plants were surveyed for the presence of resident ants. If present, ant vouchers were either made in the field or recovered from the petioles of pressed plants and stored in 70% ethanol for identification. Ant vouchers are deposited in the Museum of Comparative Zoology at Harvard University. Because *Ph. bicornis* does not abandon the plant during collection, ant vouchers are also present in the plant vouchers of the specialized myrmecophytes.

Stem segments of each field-collected species were fixed in FAA (5% formalin, 5% acetic acid, ~50% ethanol) for 2–4 weeks then transferred to 70% ethanol for long-term storage. Unless otherwise stated, all stem sections are from the internode distal to the third youngest node at approximately one-third the distance to the second-youngest node (Fig. 1). The internode between the second and third youngest nodes was chosen because most branches had at least three leaves, the third leaf was fully expanded on plants observed, and the stems of the myrmecophytes were reliably hollow by the third internode. When available, both unexcavated and excavated stems and nodes were sectioned for hollow-stemmed individuals.

Stems were sectioned using standard hand-sectioning techniques or a Vibratome Series-1000 (Vibratome, St. Louis, Missouri, USA). Separate sections were stained with safranin-fast green and cresyl violet (Gurr, 1965; Dizeo, 1980), examined using standard compound and dissecting light and polarized light microscopy, and photographed with a Nikon 5F (Nikon, Mellville, New York, USA) or a SPOT (Diagnostic Instruments, Inc., Sterling Heights, Michigan, USA) digital camera. Additional sections were stained with Sudan IV to test for suberin and with phloroglucinol to test for lignin (Gurr, 1965).

To study species not encountered in the field and to increase sample sizes for those that were encountered, 1126 herbarium sheets (from MO, MU, NY, U, US) of members of sect. *Macrostachys* were examined. Information that can be unambiguously determined from herbarium material includes solid vs.

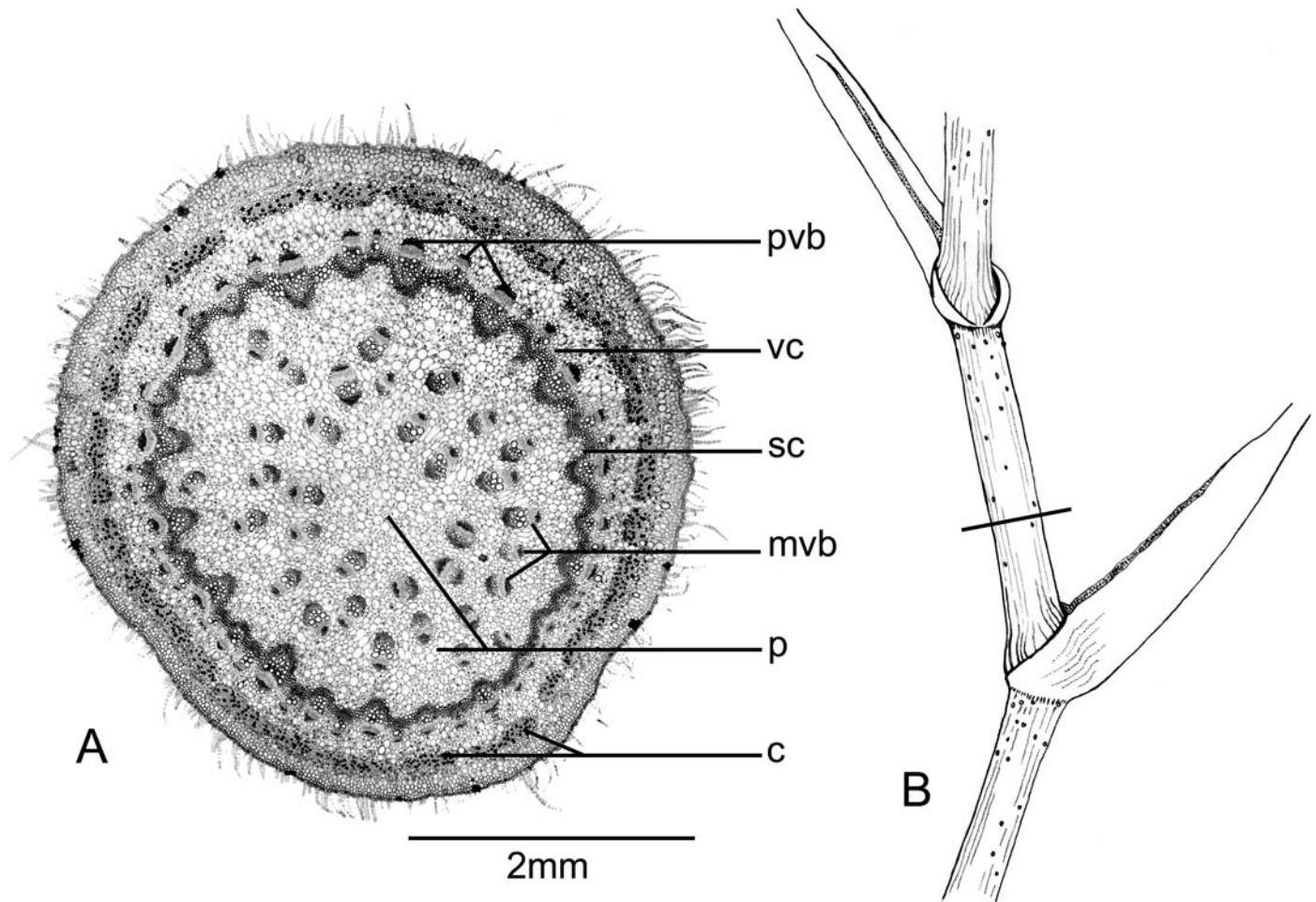


Fig. 1. (A) Cross section of *Piper obtusilimum* stem showing typical arrangement of tissues. c = collenchyma, mvb = medullary vascular bundles, p = parenchymatous pith, pvb = peripheral vascular bundles, sc = sclerenchymatous cylinder, vc = vascular cambium. (B) Typical stem of species of *Piper* sect. *Macrostachys*. Bar indicates location of cross sections taken in this study. Note swollen nodes, sheathing petiole bases, and appressed petiolar margins.

hollow stems, whether the petiole margins were persistent at the time of collection (but not the degree of petiole closure), the presence of pearl bodies inside the petiole cavities, and the remains of specialized ants (e.g., *Ph. bicornis*) in petioles.

RESULTS

General stem structure—The stele of *Piper* stems consists of a peripheral ring of vascular bundles (PVBs) and a number of additional bundles (medullary vascular bundles, MVBs) apparently scattered throughout the pith (Fig. 1). The pith is the part of the stem interior to the PVBs and includes the MVBs. The ring of PVBs undergoes varying degrees of secondary growth in different species, whereas the cambia of the MVBs produce only a few additional cells. Interfascicular cambia of the outer ring produce only nonvascular sclerenchyma; the MVBs lack interfascicular cambia. The quantity and arrangement of MVBs in mature stems are largely determined by stem diameter. For example, narrower stems tend to have fewer bundles arranged in a single ring (Fig. 2). Large stems have numerous bundles arranged into one or more rings, but the

rings are difficult to identify because the rings become increasingly disorganized as stem diameter increases (Fig. 2).

The outer layer of the pith is composed of a continuous ring of sclerenchyma that clearly separates the internal pith from the ring of PVBs (Fig. 1). Interior to the sclerenchymatous ring, the pith is composed of isodiametric parenchyma cells and MVBs. Styloid crystals and starch grains are abundant within the ground tissue of the pith and cortex. Etheral oil cells, sclereids, and secretory cells containing an unidentified, granular substance may also be present in the pith and cortex. Although secretory cells have been reported in the literature (e.g., Bond, 1931), to our knowledge, the nature of their contents remains unknown.

Within the nodes, the vascular cylinder, and correspondingly the pith, increases in diameter. The MVBs move closer to the ring of PVBs, increasing the diameter of the parenchymatous pith center. Vascular bundles in both the peripheral and medullary systems branch profusely in the nodal region, and many members within each system fuse to form two concentric rings; bridges also form between the two rings. Each leaf is supplied with numerous leaf traces (>50 in *P. imperiale*) that depart the stele around the entire node; leaf gaps and traces in

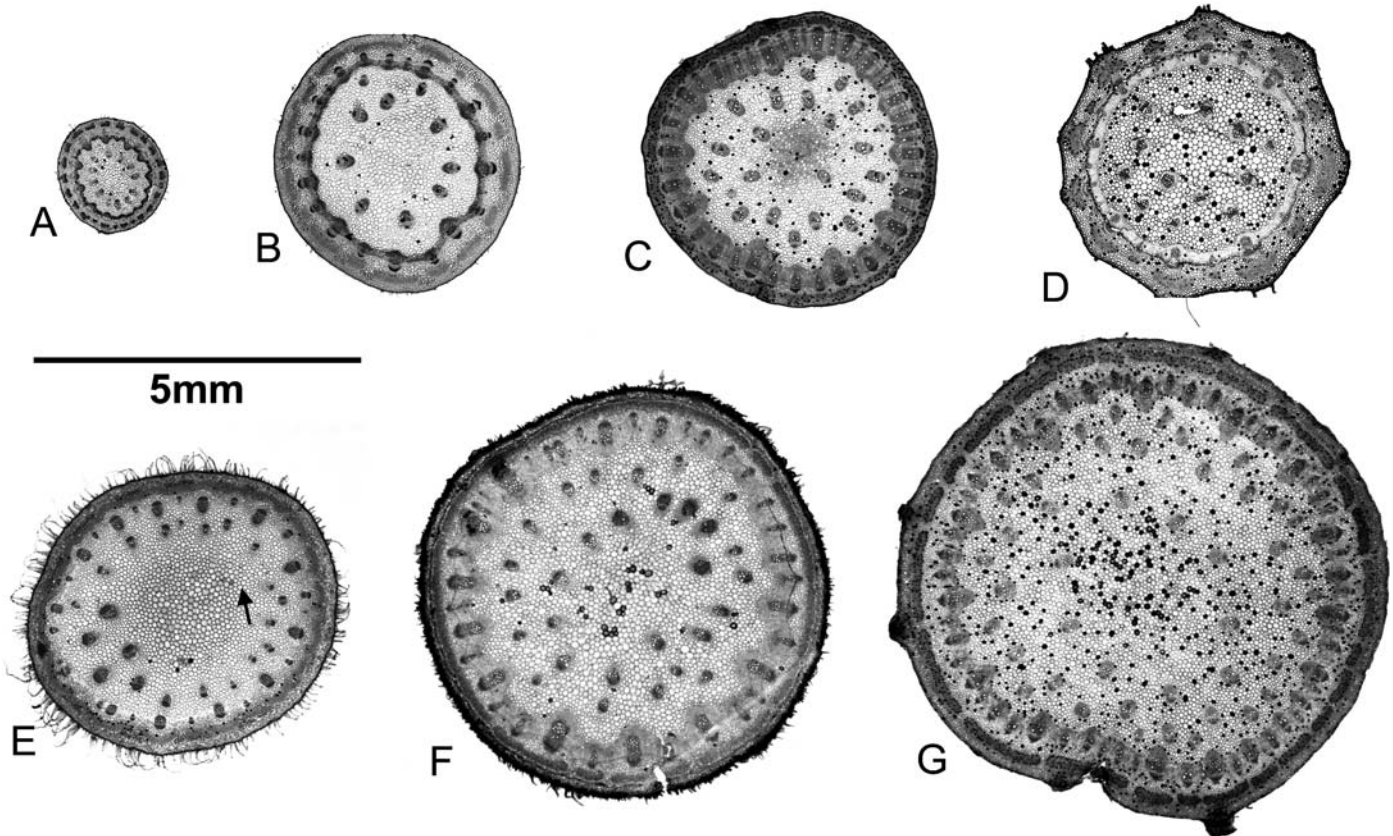


Fig. 2. Sample of stem cross sections of *Piper* sect. *Macrostachys* encompassing the diversity of the section. (A) *P. tuberculatum*, (B) *P. hebetifolium*, (C) *P. maxonii*, (D) *P. daguanum*, (E) *P. cenocladum* (unexcavated stem of a hollow-stemmed specialized myrmecophyte; note change in pith cell size indicated by arrow), (F) *P. campanum*, and (G) *P. imperiale* (generalized myrmecophyte).

Piper have a 1:1 relationship (Fig. 3). Nodes are often enlarged relative to the adjacent internodes to accommodate this large number of leaf traces; expansion of the pith and the stele in nodal regions also contributes to enlargement of the nodes. This enlargement of the nodes gives *Piper* stems their characteristic, knobby appearance. Secondary growth in the nodes is often delayed relative to the sub- and supertending internodes. Additionally, the sclerenchymatous cylinder that marks the boundary of the pith (Fig. 1) is lacking in the nodes (Fig. 3).

Stems of specialized myrmecophytes—Of the five specialized myrmecophytic species surveyed, the stems of all individuals of *P. cenocladum*, *P. fimbriatum*, and *P. sagittifolium* occupied by *Ph. bicornis* are hollow. All occupied individuals of *P. obliquum* are also found with hollow stems, with the exception of those found between 900 and 1250 m.a.s.l. on both the Atlantic and Pacific slopes of the Cordillera de Talamanca in Panama (*P. obliquum*, occupied by *Ph. bicornis*, was collected between sea level and 1350 m.a.s.l. in Costa Rica and Panama), which have consistently solid stems. Stems of *P. obliquum* from the two regions are similar in all parameters except the proportion of pith occupied by MVBs. In contrast, the stems of all ant-occupied individuals of *P. calcariformis* examined are solid, as are the stems of all generalized myrmecophytes and nonmyrmecophytes.

The pith of unexcavated stems of *P. sagittifolium* is strongly heterogeneous (sensu Bailey, 1922a, not Metcalfe, 1979, p. 177), and a sharp distinction is apparent between the small-celled peripheral region and the large-celled central region (Fig. 4). Pith cells in the center of stems are slightly larger in size in most *Piper* species studied; however, the number of large cells, the area of the pith occupied by them, and the abruptness of the transition between small- and large-celled pith is much more pronounced in *P. sagittifolium* than in any solid-stemmed species. Furthermore, needle-shaped crystals or raphides and starch grains that are abundant throughout the piths of solid-stemmed species and in the smaller, peripheral pith cells of *P. sagittifolium* are largely absent in the large-celled region of specialized myrmecophytes in which the stems are excavated (Fig. 4). *Piper cenocladum*, *P. fimbriatum*, and *P. obliquum* also have large areas of large pith cells (Fig. 2), but the transitions between large and small pith cells are more gradual, and the large-celled region is not always as clearly defined as in *P. sagittifolium* (Fig. 2). Nevertheless, a boundary is clearly delimited by a sharp decline in the quantities of starch grains and crystals in the pith cells (Fig. 4). The part of the stem that is excavated is restricted to the large-celled area in all stems observed (Fig. 4). Apart from these four species, strongly heterogeneous pith was not observed in any other species of *Piper* surveyed.

The MVBs of *P. cenocladum*, *P. sagittifolium*, *P. fimbriu-*

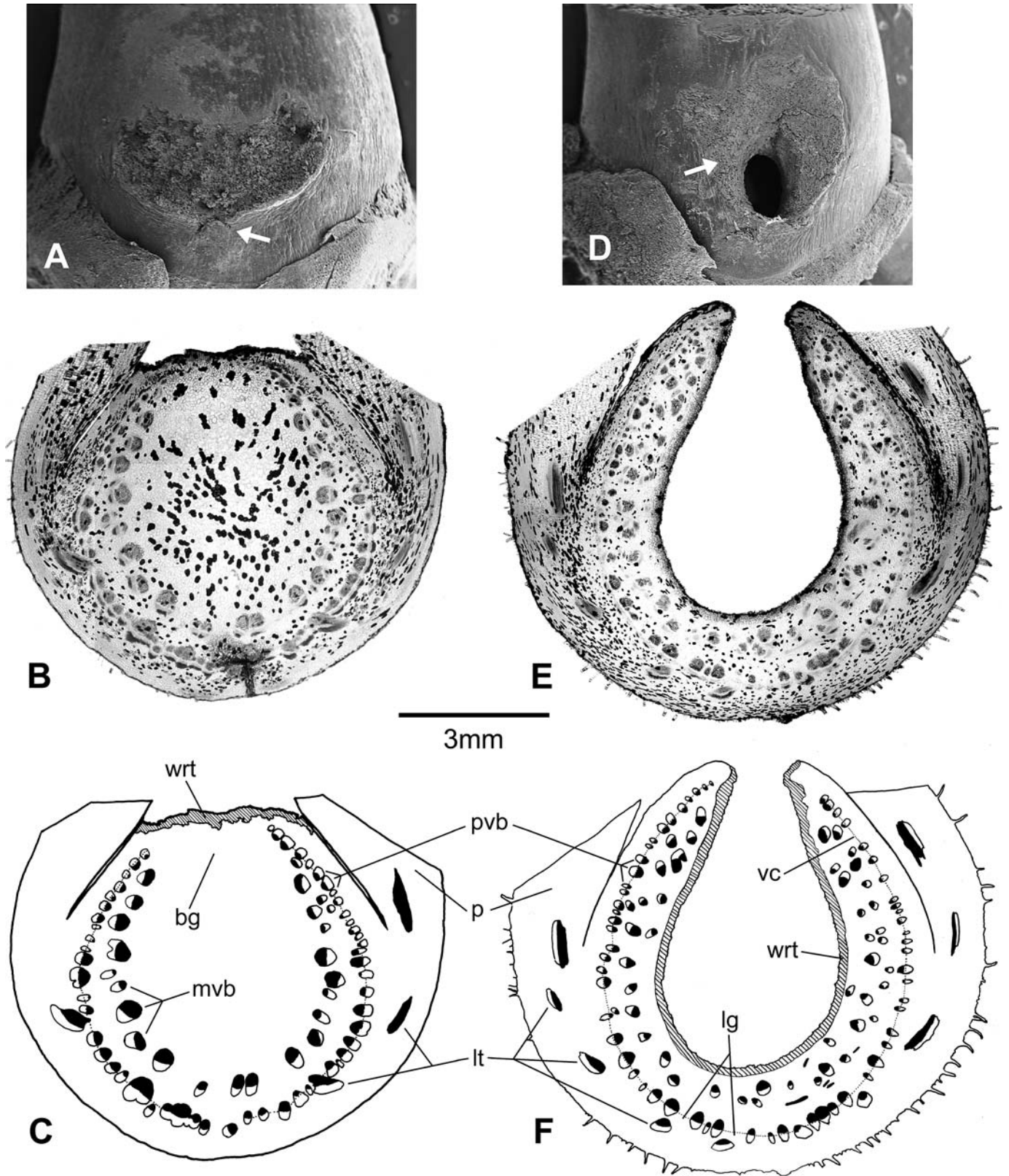


Fig. 3. Nodes of the hollow-stemmed myrmecophyte *Piper obliquum* (collected in Costa Rica). (A) SEM image of an unexcavated stem above the median point of petiole insertion, petiole removed (note minute axillary bud, indicated by arrow), (B) cross section, and (C) illustration of an unexcavated node. Micrographs of this species are obscured by abundant, dark-staining secondary compounds; illustrations show only stem outline and stele. Note the absence of vascular bundles in the semiproximal region (i.e., branch gap). (D) SEM image of an excavated stem and a petiole-stem aperture from same perspective as for (A) (note callus around aperture, indicated by arrow). (E) Cross section and (F) illustration of an excavated node. bg = branch gap, lg = leaf gap, lt = leaf trace, mvb = medullary vascular bundle, p = base of sheathing petiole, pvb = peripheral vascular bundle, vc = vascular cambium, wrt = wound response tissue.

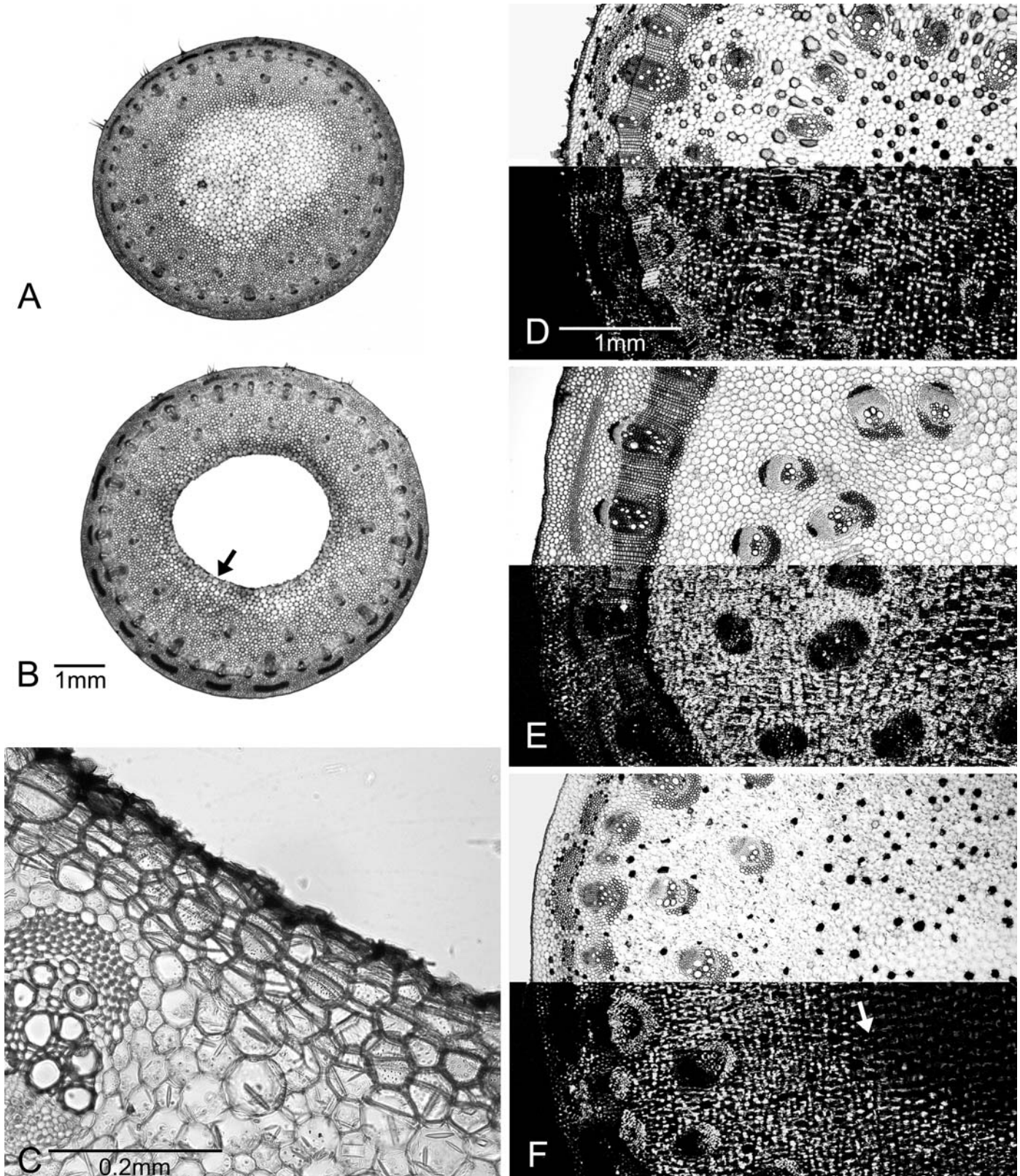


Fig. 4. Stem characters of specialized myrmecophytes and generalized myrmecophytes. (A) Cross section of *Piper sagittifolium* stem before ants have excavated the stem cavity (section from youngest internode); note the strongly heterogeneous pith. (B) Cross section of *P. sagittifolium* stem after excavation by ants (third youngest internode from the stem in [A]). Wound tissue lines the ant-excavated stem cavity, indicated by arrow. (C) Close-up of wound layer from stem of *P. fimbriulatum*. Note additional cell walls and thickened cell walls (visible as a result of observable pits) in wound tissue.

latum, and *P. obliquum* are restricted to the small-celled area and occupy less of the overall pith than they do in stems of comparable diameter with homogeneous pith (i.e., stems that do not become hollow) (Fig. 2). Pith of the solid-stemmed myrmecophyte *P. calcariformis* is largely homogeneous, and only a slight decline in crystals is evident in the center of the stem (Fig. 4). In addition, the ratio of the portion of pith interior to the MVBs to the entire pith is comparable with generalized myrmecophytes and nonmyrmecophytes (i.e., the MVBs extend much farther into the pith than they do in the other four species of generalized myrmecophytes).

The stem cavities, when excavated and fully formed, are lined with several layers of typical wound-response tissue in all individuals observed (Fig. 4). This layer is 3–8 cells thick and is derived from pith cells that undergo several periclinal divisions (without expansion) followed by suberization. The new periclinal cell walls line up, more or less, between adjacent cells resulting in several concentric rings of cell walls around the central cavity. Only cavities in the process of being excavated (i.e., young stems, near the apex of the cavity) do not exhibit any characters typical of wound response. No similar tissues were detected in unexcavated stems.

Stems of generalized myrmecophytes and nonmyrmecophytes—Stems of the remaining species of sect. *Macrostachys* are consistently solid, barring occasional damage by coleopteran or lepidopteran stem borers, which was easily distinguished by meandering cavities (cavities excavated by *Ph. bicornis* are straight) with abundant frass. Stems of generalized myrmecophytes and nonmyrmecophytes range from less than 2 mm in diameter in *P. tuberculatum* to greater than 12 mm in *P. imperiale* (Fig. 2). Large pith cells are present in relatively small numbers in the centers of several solid-stemmed species (<25 in *P. melanocladum* vs. >300 in *P. sagittifolium*; also see Fig. 2A vs. 4A). Furthermore, the proportion of the pith interior to the MVBs is much smaller in all generalized myrmecophytes and nonmyrmecophytes than in any of the specialized myrmecophytes except *P. calcariformis* (Fig. 2). Starch grains and crystals are present in abundance throughout the piths of these species (Fig. 4D) or they slightly decline in abundance in the pith center (Fig. 4E).

Nodal anatomy and the petiole–stem aperture—At each node, *Ph. bicornis* excavates a passage that connects the petiolar chamber to the subtending internodal chamber (Fig. 5) and is consistently located just above the median point of petiole insertion. The aperture is excavated through the branch gap associated with the axillary bud (Fig. 3). Although the buds are minute and not strongly vascularized, each one has an associated branch gap in the peripheral and medullary rings of vascular bundles (Fig. 3). The passages are lined with the same type of wound-response tissue lining the stem cavity; callus develops at the outer edge of the aperture, and, in older stems where leaves have senesced, the callus grows to close the apertures. Ants presumably remove callus continuously from a particular aperture as long as it is being actively used.

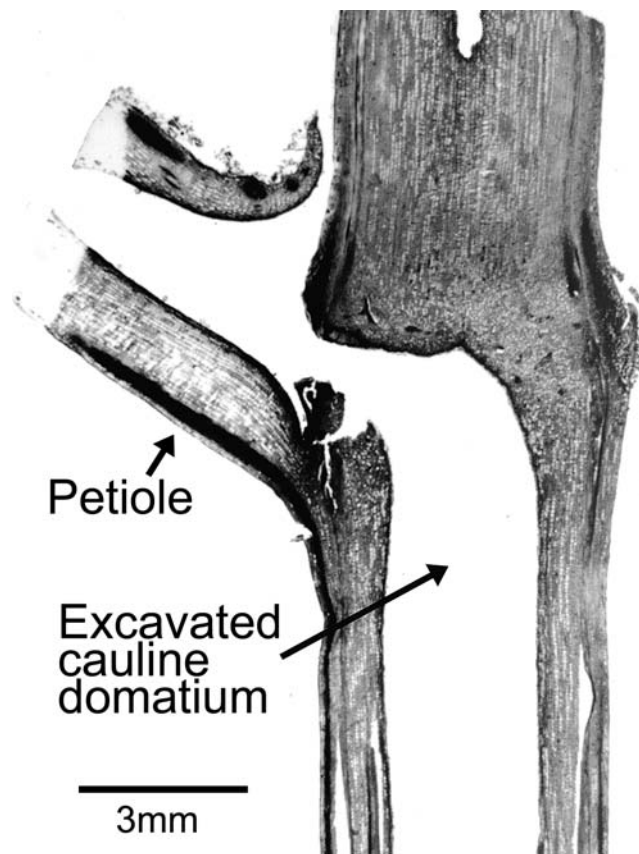


Fig. 5. Median longitudinal section through a recently excavated node of *Piper sagittifolium*. Excavation begins when ants chew an entrance hole from the petiole chamber into the stem. It then progresses basipetally, but it slows as the cavity nears the next older node. Nodes are eventually excavated completely to produce continuous cauline domatia.

DISCUSSION

It has long been known that the stem structure of myrmecophytes can directly influence the excavation of cauline domatia by ant mutualists (Bailey, 1923). The present comparative study revealed that this observation holds true in *Piper* sect. *Macrostachys*. Several traits typical of stems that ants excavate are largely absent in stems of other species.

Hollow stems—Pith, which functions in mechanical support in young shoots and in storage, is typically homogeneous in nonmonocotyledonous angiosperms, with a slight increase in cell size toward the center of the stem. Distinctly heterogeneous pith, however, appears to be a common feature of many myrmecophytes with cauline domatia, including the myrmecophytes of *Piper* sect. *Macrostachys* that we observed. Bailey (1922a) compared solid and hollow portions of stems of eight species of African myrmecophytes (one species each of

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(D–F) Bright field (upper) and polarized light (lower) micrographs of stems showing pith-cell size (bright field) and the distribution of intracellular crystals (polarized) across pith. (D) *P. biseriatum* (generalized myrmecophyte), (E) *P. calcariformis* (solid-stemmed specialized myrmecophyte), and (F) an unexcavated stem of *P. obliquum* (hollow-stemmed specialized myrmecophyte). Note the abrupt decline in crystal density in the center of the stem of *P. obliquum*, indicated by arrow.

Sarcocephalus and *Vitex* and two species each of *Barteria*, *Cuviera*, and *Plectronia*) and in portions of the stems with the pith still intact, found heterogeneous pith in all species. The same pattern of heterogeneous pith has been observed in several SE Asian myrmecophytes, including *Neonauclea* (Rubiaceae; Razafimandimbison et al., 2005), a few *Macaranga* species in which the pith does not dry up (e.g., *M. hosei* and *M. pruinosa*, Euphorbiaceae; Moog et al., 2003), and *Spatholobus bracteolatus* (Fabaceae; Moog et al., 2003). Although *Ph. bicornis* excavates the pith in *Piper* myrmecophytes (Unoccupied plants in the field and grown in the greenhouse in the absence of ants have solid stems; W. Kelly, Mesa State University, personal communication), the pith of many other myrmecophytic species disintegrates spontaneously prior to inhabitation by ants (Bailey, 1922a). In the African myrmecophytes studied by Bailey (1922a), as in *Piper*, the stem cavities are restricted to the large-celled portion of the pith. The patterns of pith excavation by ants and the disintegration of pith observed in myrmecophytes suggest that heterogeneous pith makes possible, or perhaps facilitates excavation by ants or that selection has favored minimal investment in a part of the stem that is likely to be lost in these species. This observation is corroborated by Bailey's (1922a) studies of several species (i.e., *Barteria fistulosa*, *Cuviera* spp., and *Plectronia* spp.) in which only hypertrophied parts of stems with heterogeneous piths were occupied by ants, whereas unmodified parts of stems with homogeneous piths remained solid. Moreover, two species in Bailey's (1922a) study did not have outwardly modified stems (i.e., no hypertrophies), but had cauline domatia associated with heterogeneous pith: *Barteria dewevrei* (Passifloraceae) and *Vitex staudtii* (Verbenaceae). Cross sections of hollow-stemmed *Piper* species strikingly resemble stems of these latter two species. The absence of crystals, however, has not been reported previously in association with heterogeneous pith. Within *Piper*, strongly heterogeneous pith and the corresponding lack of crystals in the large-celled pith center appears to be restricted to the four species regularly inhabited and excavated by *Ph. bicornis*.

The solid stems of *P. calcariformis* (a specialized myrmecophyte) lack all of the anatomical characters that distinguish the hollow-stemmed species. They are virtually identical to stems of any generalized or nonmyrmecophytic species of sect. *Macrostachys* with stems of similar size. The difference in stem structure between *P. calcariformis* and the remaining specialized myrmecophytes is not completely unexpected. Preliminary phylogenetic evidence suggests two to four independent origins of specialized myrmecophytes within sect. *Macrostachys*, and *P. calcariformis* represents one of the independent origins (Tepe et al., 2004). Similarly, crystals are found throughout the pith in the stems of *P. obliquum* collected in Panama. Both solid-stemmed Panamanian and hollow-stemmed Costa Rican collections were inhabited by *Ph. bicornis*. It should be noted that Costa Rican and Panamanian *P. obliquum* may represent two distinct lineages. The currently recognized circumscription of *P. obliquum* (Tebbs, 1989) is problematic and may include a number of recognizable segregate taxa that warrant specific recognition (Callejas, 2001; Jaramillo and Callejas, 2004; E. Tepe, personal observation). Most species of sect. *Macrostachys* are soft-wooded shrubs with large, parenchymatous piths. It is unlikely that dense pith, scattered MVBs, and occasional sclereids could prevent ants from excavating the stems; nevertheless, we have

not encountered ant-excavated stems in any specimen with homogeneous pith and with stems of comparable size and softness to those that are excavated. A sharply reduced number of styloid crystals, however, is observed in the center of all hollow-stemmed species; thus it is possible that crystals function as a deterrent to excavation. Studies are needed to determine whether crystals are also absent from ant-excavated tissues of other myrmecophytes.

Entrance to cauline domatia—The concept of a prostoma sensu Brouat et al. (2001) is that of a membranous area on a hollow plant organ that is thinner relative to adjacent areas and that lacks vascular or other lignified tissues, and is exemplified in the myrmecophytes of *Cecropia* (Bailey, 1922b) and *Leonardoxa* (Brouat et al., 2001). *Piper* myrmecophytes do not have true prostomas because unexcavated stems are solid, yet the position of the aperture between the stem and petiole cavities is uniform in all nodes of all hollow-stemmed species. The area where the petiole–stem aperture is excavated has some characters of a prostoma in that it lacks lignified tissue (vascular elements and the sclerenchymatous cylinder) and an arm of large-celled pith extends into the cortex (Fig. 3). Furthermore, like a true prostoma, it remains free of tough tissues after the remainder of the stem has gone through considerable secondary growth, thereby extending the window of colonization (Bailey, 1922b; Brouat et al., 2001; Federle et al., 2001). However, a prostoma implies a specialized structure that facilitates access of mutualist ants into the interior domatia, and it is not clear that this is the case in *Piper*; rather, we believe that ants with a proclivity toward excavation are taking advantage of a universal plant structure—the branch gap—to gain access to the interior of the stem. Whether a prostoma is present or not, the location of the entrance holes in most myrmecophytes is strongly influenced by plant vascular anatomy as evidenced in previous studies (Bower, 1887; Bailey, 1922a, b, 1924; Brouat et al., 2001; Federle et al., 2001; Moog et al., 2002). Likewise, the location of the entrance holes in *Piper* is closely correlated with the vascular system of the stem.

Cauline domatia, wound tissue, and nutrient absorption—Wound-response tissue in the cauline domatia of *Piper* myrmecophytes was first noticed by Risch et al. (1977) in *P. cenocladum*. In the present study, we detected a multilayered wound tissue lining the excavated cauline domatia of all four species of hollow-stemmed specialized myrmecophytes. The cell walls of the wound response layer are thickened (Fig. 4C) and suberized, making the layer—like the bark of woody plants—impervious to gasses, liquids, and the invasion of the plant body by fungi and other pathogens (Bloch, 1941, 1952). The wound layer was fully developed by the third-youngest internode of all individuals studied. Similar wound tissue lines the ant-excavated lateral cavities of the West African *Vitex staudtii* (Verbenaceae; Bailey, 1922a). The wound tissue that lines cauline domatia is presumably produced in response to the trauma of excavation by ants. In myrmecophytes in which ants come in contact with the living plant body through, for example, the excavation of living plant tissue, the uninhibited exchange of nutrients between ant and plant is possible as documented in several cases (Rico-Gray et al., 1989; Sagers et al., 2000; Fischer et al., 2003). Special structures are required for nutrient absorption in domatia that are formed by the plant

and are not excavated (e.g., roots in leaf pouches of *Dischidia major*, Treseder et al., 1995).

The absorption of nutrients from ant detritus or CO₂ from ant respiration by *Piper* myrmecophytes has been suggested (Risch et al., 1977) and more recently tested (Letourneau, 1998; Fischer et al., 2003). Letourneau (1998) introduced fertilizer into the base of occupied stems (the area of the cauline domatia where the majority of ant-associated detritus accumulates) and found no significant difference in net leaf production over 2 years between experimental and control plants. Our observations further explain the negative results of Letourneau (1998) in that the experimental nutrients were introduced into the oldest part of the stem where the stem cavity is lined with a fully developed layer of wound tissue.

Fischer et al. (2003) elegantly demonstrated that *Piper* specialized myrmecophytes do absorb nutrients from ants when they fed ants with ¹⁵N-enriched food and found that the majority of the ¹⁵N was absorbed through the stem rather than the petiole or leaf blade. The majority of the ¹⁵N was absorbed in the upper half of the plants and after absorption, was transported acropetally and basipetally as evidenced by signal in the youngest unoccupied tissue and in the roots, respectively. Our observations of wound tissue lining cauline domatia supports the explanation of the patterns of nutrient absorption reported by Fischer et al. (2003) in that wound tissue is present in older stems, but not present or not yet fully developed in younger, recently excavated stems where the nutrients were absorbed. Fischer et al. (2003) reported an absence of wound tissue in the stem cavities of *P. fimbriulatum*, but the age of the stem sectioned was young (V. Mayer, personal communication). If the wound tissue in *Piper* stems does in fact constitute an effective barrier between the stem cavity and living plant tissues as it does in other plants (Bloch, 1941, 1952), then it is indeed likely that the discrepancy between ant distribution and ¹⁵N incorporation into plant tissues reported and explained by Fischer et al. (2003) could be the result of localized absorption of ¹⁵N in recently excavated cavities and subsequent intraplant translocation.

Piper calcariformis and Panamanian *P. obliquum* do not have cauline domatia; thus, all areas of plant in contact with ants are covered with epidermis. Given that most of the ¹⁵N absorbed by *Piper* plants studied by Fischer et al. (2003) was absorbed by the stem, it would be interesting to know whether these two specialized myrmecophytes with only petiolar domatia absorb fewer nutrients than their hollow-stemmed relatives.

Conclusions—Comparative studies make possible the discovery of novel structures or novel modifications of existing structures in plants and can provide insights into key characters and evolutionary trends implicated in the origin of myrmecophytism. This study contributes to our understanding of the plant traits that permit or facilitate the occupation of myrmecophytes by ant mutualists and to our understanding of nutrient absorption by myrmecophytes—a field about which we know very little—by providing details of the anatomical characters that may affect nutrient absorption. Finally, our results add to our understanding of the evolution of plant traits in general and in the context of interaction with other organisms by illustrating the plasticity of pith after secondary growth provides structural stability to the stem. Furthermore, the parallel evolution in several lineages of *Piper* myrmecophytes of the same suite of traits in stems that are excavated,

apparently associated with the benefits of ant residents, exemplifies how one organism involved in an interaction can influence the evolution of another. This study joins others (Bailey, 1922a; Brouat et al., 2001; Federle et al., 2001) in which anatomical character states unique to the myrmecophytes have been detected in a given lineage. In the otherwise comparable stems of species of *Piper* sect. *Macrostachys*, unexcavated stems of hollow-stemmed specialized myrmecophytes have markedly heterogeneous piths. Heterogeneous pith appears to be a widespread character among myrmecophytes that is associated with domatia in which the ants nest. Within the pith cells of *Piper* myrmecophytes, the distribution of crystals and starch grains is largely restricted to the small-celled pith that is peripheral to the larger-celled center, a trait that may facilitate excavation of the pith center by the ant residents. Similarly, holes that ants chew to access the center of the stem are excavated through the branch gap associated with the axillary bud at each node, conforming to the pattern of a close association of cauline domatia with plant vasculature observed in most myrmecophytes. Heterogeneous pith and, perhaps more importantly, the absence of crystals in the stem center appear to be important factors in the excavation of cauline domatia by ant residents. Whether the domatia of myrmecophytes become hollow spontaneously or are excavated by the ant residents, a growing body of evidence suggests that the location and extent of the cavities, as well as the entrances to those cavities, are largely determined by stem characters.

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APPENDIX. Accessions of *Piper* sect. *Macrostachys* that were studied anatomically. Collectors are EJT = E. J. Tepe and AE = Armando Estrada (CR). MOBOT numbers are Missouri Botanical Garden accession numbers for plants cultivated in the garden. All vouchers are deposited at MU. Duplicate sets of Costa Rican collections are held at CR and USJ, and sets of Panamanian collections are held at PMA and STRI.

Species—Collection/accession no.; Source (country: province)

- Piper arboreum* Aubl.—EJT 377; Costa Rica: Puntarenas. *P. arboreum* Aubl.—EJT 1040; Panamá: Panamá. *P. arboreum* Aubl.—EJT 620; French Guiana: Cayenne. *P. arboreum* var. *falcifolium* Yunck.—EJT 1039; Panamá: Panamá. *P. auritifolium* Trel.—EJT 94, 102, 103, 104, 427; Costa Rica: Heredia. *P. auritifolium* Trel.—EJT 178, 179; Costa Rica: Cartago. *P. biseriatum* C.DC.—EJT 77, 95, 438; Costa Rica: Heredia. *P. biseriatum* C.DC.—EJT 140, 141; Costa Rica: Guanacaste. *P. biseriatum* C.DC.—EJT 183; Costa Rica: Cartago. *P. biseriatum* C.DC.—AE 2398, EJT 468; Costa Rica: Alajuela. *P. calcariformis* Tebbs—AE 2397; Costa Rica: Alajuela. *P. calcariformis* Tebbs—EJT 1009; Panamá: Chiriquí. *P. campanum* Yunck.—EJT 1033; Panamá: Panamá. *P. campanum* Yunck.—EJT 1048; Panamá: Coclé. *P. cenocladum* C.DC.—EJT 90, 92, 98, 99, 105, 428, 430, 431, 435, 436, 440; Costa Rica: Heredia. *P. cenocladum* C.DC.—EJT 144; Costa Rica: Guanacaste. *P. cenocladum* C.DC.—EJT 180, 185; Costa Rica: Cartago. *P. cenocladum* C.DC.—EJT 393; Costa Rica: Alajuela. *P. cordulatum* C.DC.—EJT 975; Panamá: Chiriquí. *P. cordulatum* C.DC.—EJT 1011; Panamá: Colón. *P. cordulatum* C.DC.—EJT 1016; Panamá: Panamá. *P. daguanum* C.DC.—EJT 1044; Panamá: Panamá. *P. euryphyllum* C.DC.—EJT 410; Costa Rica: Cartago. *P. fimbriulatum* C.DC.—EJT 115, 119, 321, 343, 352; Costa Rica: Puntarenas. *P. fimbriulatum* C.DC.—EJT 971; Panamá: Panamá. *P. gibbosum* C.DC.—EJT 168, 170; Costa Rica: San José. *P. gibbosum* C.DC.—EJT 4115; Costa Rica: Cartago. *P. gigas* Trel.—EJT 1000; Panamá: Chiriquí. *P. hebetifolium* Burger—EJT 448, 454; Costa Rica: Alajuela. *P. imperiale* (Miq.) C.DC.—EJT 97, 100, 106, 107, 419, 432; Costa Rica: Heredia. *P. imperiale* (Miq.) C.DC.—EJT 169, 182; Costa Rica: San José. *P. imperiale* (Miq.) C.DC.—EJT 401; Costa Rica: Alajuela. *P. imperiale* (Miq.) C.DC.—EJT 473; Costa Rica: Puntarenas. *P. imperiale* (Miq.) C.DC.—EJT 989; Panamá: Veraguas. *P. imperiale* (Miq.) C.DC.—EJT 1055; Panamá: Chiriquí. *P. marsupiatum* Trel. & Yunck.—MOBOT 931716; Ecuador. *P. maxonii* C.DC.—EJT 370; Costa Rica: Puntarenas. *P. melanocladum* C.DC.—EJT 134, 426; Costa Rica: Heredia. *P. obliquum* Ruiz & Pav.—EJT 114, 345, 351, 385, 386; Costa Rica: Puntarenas. *P. obliquum* Ruiz & Pav.—EJT 173; Costa Rica: San José. *P. obliquum* Ruiz & Pav.—EJT 974, 1022; Panamá: Panamá. *P. obliquum* Ruiz & Pav.—EJT 1008, 1067; Panamá: Chiriquí. *P. obtusilimbus* C.DC.—MOBOT 930887; Ecuador. *P. sagittifolium* C.DC.—EJT 116, 120, 126, 320, 326, 327; Costa Rica: Puntarenas. *P. sagittifolium* C.DC.—MOBOT 931714; Unknown. *P. sasaimanum* (Miq.) C.DC.—EJT 1055; Panamá: Chiriquí. *P. tuberculatum* Jacq.—EJT 1061; Panamá: Panamá.