

Morphology, Ultrastructure, and Function of Extrafloral Nectaries in Three Species of Caesalpiniaceae

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Light and electron microscopy reveal that the morphologically well-differentiated petiolar nectaries of *Chamaecrista fasciculata*, *Senna hebecarpa*, and *S. marilandica* have an unusually simple anatomy consisting of an epidermis immediately subtended by a mass of small, loosely-packed parenchyma cells. Vascular strands from the petiolar bundles enter the nectary and terminate as phloem within or near this parenchyma. In mature, secreting nectaries, the cuticle of the epidermis extends between the epidermal cells and into the nectary parenchyma, where it occupies, but does not occlude, much of the free space of this tissue. The cutin is not found below the level of the phloem endings and is not found in very young nectaries, but begins to appear when cell expansion occurs. These observations, together with the proximity of phloem to the parenchyma free space and the almost exclusive presence of sucrose in the nectar suggest that, although symplastic transport of sugars may occur, an alternate pathway for secretion is possible whereby sugar diffuses from the phloem, moves through the nectary to the surface without being acted upon by cells in transit, and is released by rupture of the external cuticle and the concomitant activity of foraging ants and other nectar feeders.

INDEX DESCRIPTORS: extrafloral nectaries, morphology, ultrastructure, function, Caesalpiniaceae, nectar.

Extrafloral nectaries (EFNs) come in a variety of shapes and sizes. For example, in the legumes, they can range from the modified trichomes of *Vicia faba* (Figier 1971) to the huge cup-shaped "gigas" types of *Pithecellobium* (Elias 1972). The anatomy of cup-shaped nectaries of *Pithecellobium* and those of some non-legumes such as *Passiflora warmingii* of the Passifloraceae (Durkee 1982, 1983) shows various kinds of internal specialization such as a dedicated vascular supply, a distinct sub-glandular layer, and an epidermis whose ultrastructure and morphology is considerably different from ordinary epidermal cells.

In *P. warmingii*, for example, the epidermis is composed of a multilayer of cuboidal cells derived from the protoderm. These cells are tightly packed, have small scattered vacuoles, an abundance of mitochondria, plastids with some starch deposition, and a ribosome-rich cytoplasm. This hypertrophied epidermis is subtended by a fairly compact array of parenchyma cells, often with deposits of calcium oxalate crystals, and it is within this mass of cells that one finds vein endings of the nectary vascular supply that originate from the petiolar bundle. These endings are found within 3-5 cell diameters from the lowermost epidermal cells and appear as truncated phloem cells unaccompanied by xylem. Plasmodesmata are commonly found between the tightly packed epidermal cells and between these cells and the subtending parenchyma.

Regardless of the morphology of EFNs, the ultrastructural richness and density that characterize their cells have suggested to some workers that the cells are actively involved in processing and transport of nectar sugars (Figier 1971, Wergin et al. 1975, Gunning and Hughes 1976, Fahn 1979). They argue that phloem sugars are transported symplastically and that the epidermal (secretory or glandular) cells and possibly the adjacent parenchyma are engaged in modification of these sugars. The "pre-nectar" is then secreted by either an eccrine (active transport) or granulocrine (exocytotic) system

depending on the species studied. The surface of the nectary typically is covered by a cuticle that usually ruptures, presumably from nectar accumulation between the outer wall of the epidermal cells and the cuticle itself. Nectar thus finds its way to the exterior.

As a rule, extrafloral nectar is a mixture primarily of sucrose, glucose, and fructose with total concentrations that vary from 20-40% (Deuth 1977, Baker et al. 1978, Koptur 1979). Amino acids are frequently present in varying amounts which may relate to the nutritional requirements of visitors to EFNs (Baker and Baker 1975, Baker et al. 1978).

Chamaecrista fasciculata Michx. (Caesalpiniaceae), the partridge pea, is a wide-ranging species and is abundant in Iowa. Because it has conspicuous extrafloral nectaries, it became the subject of our study to learn if EFNs in this species affect reproductive fitness. As part of the project, we studied nectar constituents and concentration, but we also decided to examine the morphology and ultrastructure of the nectary. Because these nectaries are cupular, we expected anatomical features similar to those described for *Passiflora warmingii* or *Pithecellobium*. The information obtained led us to investigate two other closely related species, *Senna hebecarpa* Fern. and *Senna marilandica* L., both with prominent EFNs. This paper describes our findings for all three species.

METHODS

Although the species described here were formerly placed in the genus *Cassia*, it is generally agreed that they merit segregation into two different genera, *Chamaecrista* and *Senna* (Gleason and Cronquist 1991). Some treatments suggest that they should be assigned to the family Fabaceae. However, we are following the system of Cronquist (1981) who assigned these genera to the family Caesalpiniaceae.

C. fasciculata and *S. marilandica* were either grown from seed or as seedlings collected along roadsides and maintained under natural and supplemented lighting in the Grinnell College greenhouse. Nectaries from *S. hebecarpa* were obtained from plants growing in a re-

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stored prairie three miles SW of the city of Grinnell. Voucher specimens are on deposit in the Grinnell College Herbarium (GRI).

Non-secreting and mature, secreting nectaries were harvested, cut transversely, lengthwise, or left whole, and fixed in 3% buffered glutaraldehyde, postfixed in buffered osmium tetroxide and dehydrated in a graded acetone series. Specimens selected for scanning electron microscopy (SEM) were critical point dried, sputtered with gold, and examined with a Hitachi S-2300. The remaining samples were embedded in Spurr's resin. For light microscopy (LM), 1-micrometer thick sections were cut with a JB-4 microtome and stained with Toluidine Blue. Sudan Black was used to stain free-hand sections of fresh nectaries to detect lipids. For transmission electron microscopy (TEM), silver-gold sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-300. The Prussian Blue technique described by Evert et al. (1985) was used to trace the extent of free space in the nectary.

Freshly exuded nectar was collected with microliter pipets and frozen until it could be analyzed. It was then assessed chromatographically for the presence and identity of sugars and for ninhydrin-positive substances according to methods described earlier (Durkee et al. 1981). Sugar concentration was measured with an Atago pocket refractometer.

RESULTS

Like many members of this family, *Chamaecrista fasciculata*, *Senna hebecarpa*, and *S. marilandica* have conspicuous nectaries positioned on the adaxial surface of the petiole just above its point of attachment to the stem (Fig. 1). Mature, secreting nectaries of *C. fasciculata* are cup- or boat-shaped (Figs. 1 and 2) while those of *S. marilandica* (Figs. 1 and 3) and *S. hebecarpa* (Figs. 1 and 4) are clavate, the latter with a small stipe. In *C. fasciculata*, the young nectaries are initiated as stubby outgrowths with a convex surface (Fig. 1). Over time, their morphology changes until at maturity, signaled by the onset of secretion, the nectaries appear cup-shaped (Fig. 1). In *S. marilandica* and *S. hebecarpa*, the developing nectaries begin as small peg-like structures and mature into their distinctive club or clavate shapes. In *C. fasciculata*, nectar accumulates in the cup, but in *S. marilandica* and *S. hebecarpa* secretion occurs over the surface of the upper portion of the nectary, appearing as tiny nectar-containing blisters (Fig. 4).

LM shows that the mature, secreting nectary has a simple epidermis immediately below which is a mass of parenchyma. In *C. fasciculata*, this sub-epidermal parenchyma stains deeply and is about 20 cells deep, constituting the bulk of the nectary, but in both *S. hebecarpa* and *S. marilandica*, it is only about 4–5 cells deep and lightly stained. In all three species, these cells are about the size of the epidermal cells, while distinctly larger, lightly stained parenchyma cells comprise the rest of the nectary.

In *C. fasciculata* and *S. hebecarpa* a single vascular bundle containing both xylem and phloem departs from the petiole, while in *S. marilandica*, two or three vascular strands are seen. In all species, after entering the nectary, these strands immediately produce up to 8–10 branches in *C. fasciculata* and *S. hebecarpa*, and up to 20 in *S. marilandica*. As the strands approach the sub-epidermal parenchyma, the xylem is no longer seen and the branches, now composed solely of phloem, proceed to terminate at or within this parenchyma (Fig. 1).

The cutin over the epidermal surface stains lightly with Toluidine Blue and is evident between cells of the epidermis and associated with cell walls throughout the parenchyma. It stains positively with Sudan Black in free-hand longitudinal and cross-sections of fresh nectaries and we have detected traces of Sudan-positive material in the intercellular areas that develop at the onset of nectary expansion.

TEM reveals that, at maturity, all three nectary types share many

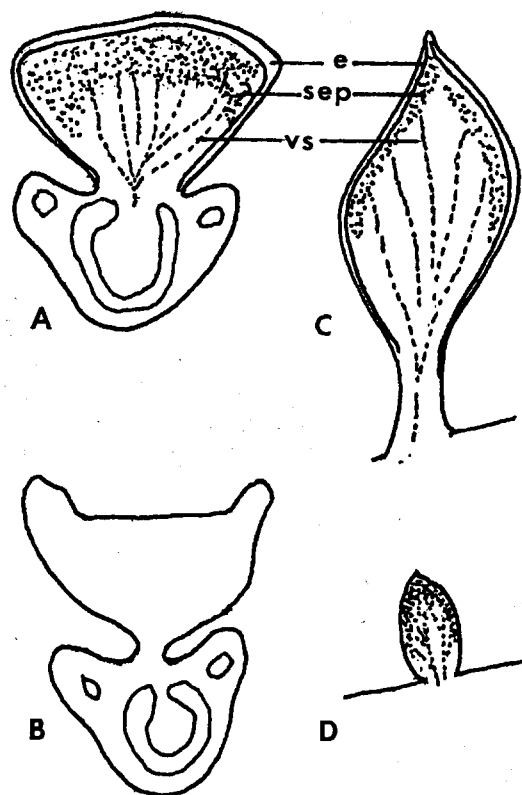


Fig. 1. EFNs of *C. fasciculata*, (A, B) *S. hebecarpa* (C), and *S. marilandica*. (D) showing location of epidermis (e), sub-epidermal parenchyma (sep) and vascular strands (vs). B, C, and D represent configurations of secreting nectaries, while A shows an immature EFN of *C. fasciculata*. Not to scale.

common features. In each, the epidermis consists of a single layer of cells resembling ordinary epidermal cells with a surface cuticle and containing some ER, mitochondria and other organelles, and large vacuoles, often with heavy accumulations of electron-dense material.

Plasmodesmata may occur (data not shown) between these cells, but usually the cells are separated by cuticular flanges along the radial walls, while accumulations of cutin along the inner periclinal walls are common (Fig. 5). Just below the epidermis is the mass of small parenchyma cells. In all three species this tissue, like the epidermis, is composed of cells with a few scattered organelles and large vacuoles. In *C. fasciculata*, the vacuoles contain deposits electron-opaque material. The cutin associated with the epidermal cell walls clearly extends into the sub-epidermal parenchyma and appears to fill much of the intercellular space but does not completely occlude it. The cutin is sometimes seen appressed to the parenchyma cell walls and sometimes dissociated from them (Fig. 6). The cutin can be traced through the parenchyma to about the level of the phloem endings (Fig. 9).

In mature, secreting nectaries of *S. marilandica* and *S. hebecarpa*, the external cuticle is occasionally observed to be separated from the epidermis (Fig. 7). In secreting nectaries of *C. fasciculata*, the epidermis itself in some areas is separated from the underlying cells with the cuticle remaining intact. Here, the epidermal cells become distinctly separated from one another, but remain strung together beneath the cuticular layer (Fig. 8). Although rupture of the cuticle is often detected in many EFNs and has been shown to be the site of nectar release (Findlay et al. 1971), no such disruption was de-

tected with either LM or TEM, although SEM views of all three species showed nectar accumulation beneath the cuticle and droplets on the surface.

In all three species the Prussian Blue technique produced massive deposition of dye crystals in the intercellular spaces of the sub-epidermal parenchyma (Fig. 10). Small crystals were also found in the outer cell wall of the epidermis below the cuticle and in *C. fasciculata* and *S. hepecarpa*, deposition of fine crystals was detectable in the area described as the reticulate region (Holloway 1982) of the cuticular membrane. Control tissue showed no such deposition (Fig. 11).

For both *C. fasciculata* and *S. marilandica*, tests of freshly harvested nectar performed over a week on different plants showed a consistent total sugar concentration of 60–65%. This nectar was almost exclusively sucrose with only traces of glucose and fructose. Ninhydrin-positive substances were undetectable in any of the samples. Because of the heavy rains in Iowa during the study period, we were unsuccessful in obtaining sufficient amounts of nectar from field-grown *S. hepecarpa*.

DISCUSSION

The three nectaries exhibit a lack of internal organization that is unexpected for such conspicuous and well-defined structures. In addition, they have four additional features that are uncharacteristic of EFNs generally:

1) The network of Sudan-positive material found with LM throughout the nectary of all three species corresponds to the pattern of cutin deposition seen with TEM. As far as we know, deposition of the type seen here has not been reported for EFNs although Bhattacharyya and Maheshwari (1971) described a "zone of thick-walled cells" several cell layers below the epidermis in the species of *Cassia* they studied with light microscopy. Some internal cutinization might be expected in those floral nectaries which secrete via modified stomata, as in *Vinca* (Rachmilevitz and Fahn 1973). It is also present in the anticlinal walls of the subsecretory cells in the EFNs of *Aphelandra* spp. (Durkee 1987) and other "flachnektaria" (Zimmermann 1932), but these species do not show the pattern of deposition in the underlying parenchyma that is evident in *Chamaecrista* and *Senna*.

2) The single-layered epidermis does not resemble the secretory epidermis of other EFNs, whether the nectaries are relatively unspecialized, such as secretory trichomes, or highly structured. Cells believed to be secreting nectar are rich in ribosomes, mitochondria and sometimes ER and dictyosomes. They also are closely packed with no intervening space.

In our species, the epidermal cells are unspecialized and tend to become separated as they mature. Except for stomata, it is unusual to find spaces between any epidermal cells, although Esau (1965) has pointed out that they occur in the epidermis of some petals. In many floral nectaries, modified stomata are exit points for the nectar (Durkee et al. 1981, Rachmilevitz and Fahn 1973), but studies so far suggest that EFNs do not operate this way.

In our species, the tendency of the epidermal cells to dissociate and for the epidermis sometimes to separate from the underlying parenchyma is not an artifact of tissue processing, but rather appears to represent the end-point in the maturation of the nectary. A similar

phenomenon was observed by Elias (1983) in the bracteal nectary of *Paeonia*.

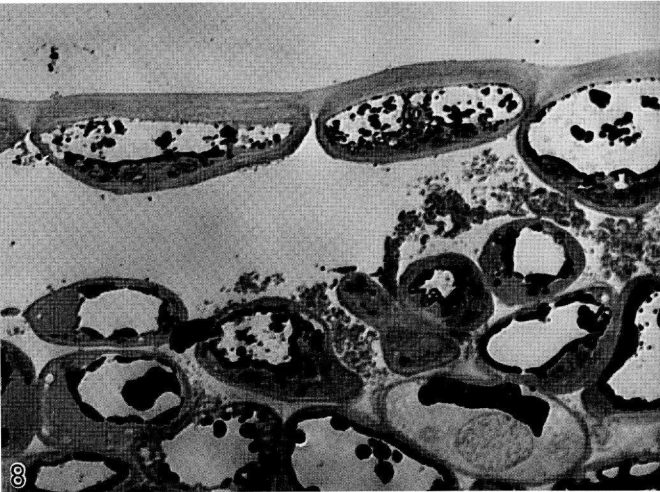
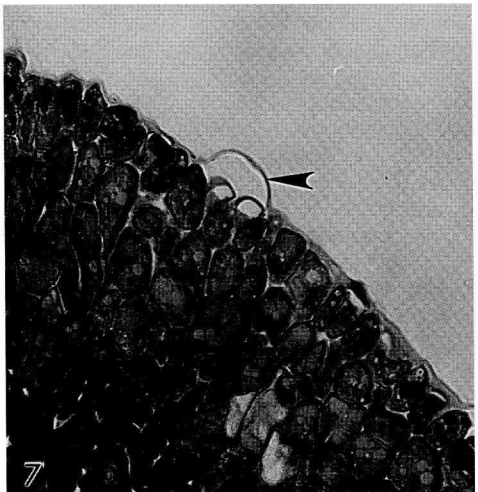
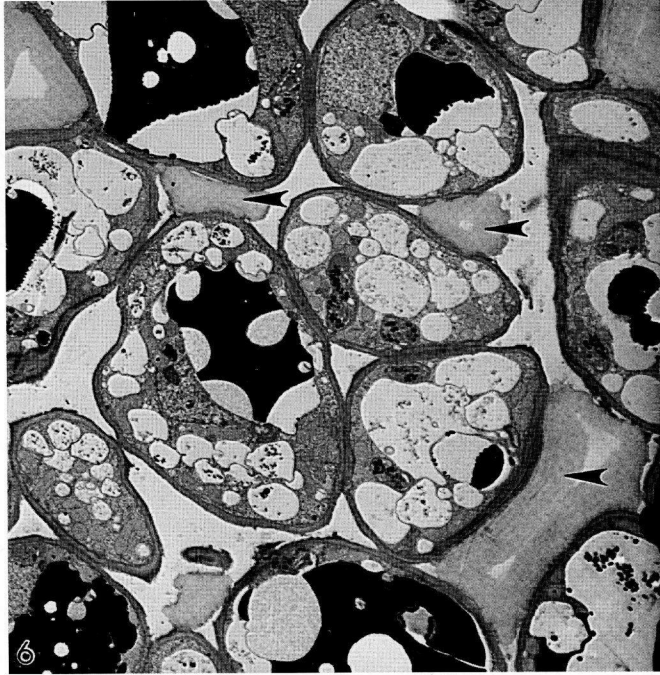
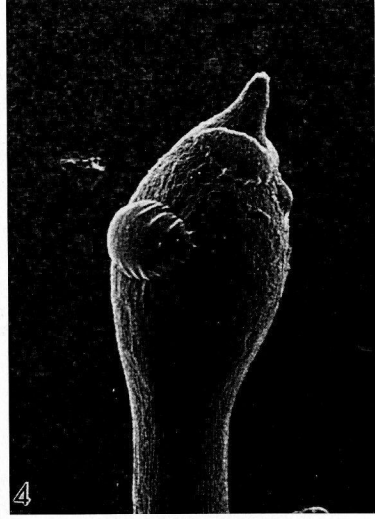
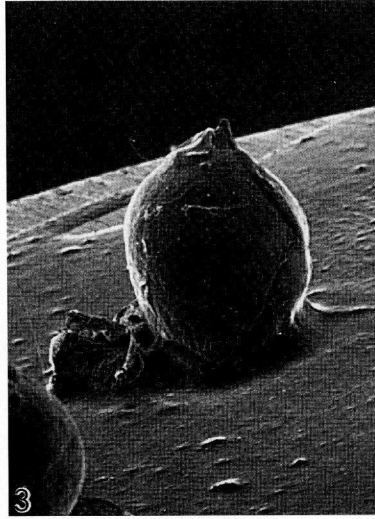
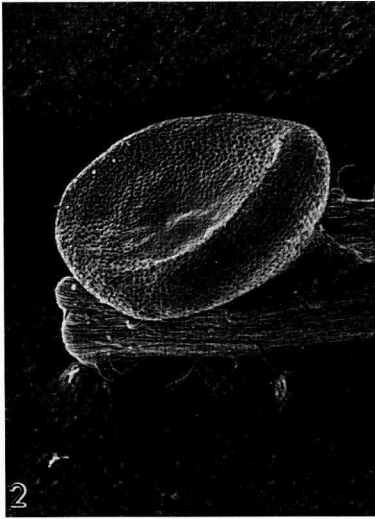
3) As mentioned in the introduction, glucose, fructose, and sucrose are common constituents of extrafloral nectar, but may occur in varying proportion depending on species. In *Aphelandra scabra* (Acanthaceae), for example, fructose and sucrose are consistently dominant while glucose is present only in trace amounts (Durkee 1987). In *Passiflora coerulea*, all three sugars are present, but glucose is more dominant than either fructose or sucrose, while in *P. warmingii*, all three sugars are conspicuous and equally abundant (Durkee 1982). To our knowledge, no strongly sucrose-dominant extrafloral nectar has been reported. Thus, the sucrose nectar of *C. fasciculata* and *S. marilandica* is surprising. The high total sugar concentration of the fresh nectar is also unexpected because those extrafloral nectars that have been analyzed by others show ranges from ca. 20–40%. It has been argued (Frey-Wyssling and Agthe 1950) that there is a correlation between the sugar concentration of nectar and the vascular supply to the nectary so that dilute nectars result when both xylem and phloem are present while more concentrated nectars are supplied by phloem only. This argument may be supported by our study that shows the phloem as the vascular component terminating in or near the sub-epidermal parenchyma.

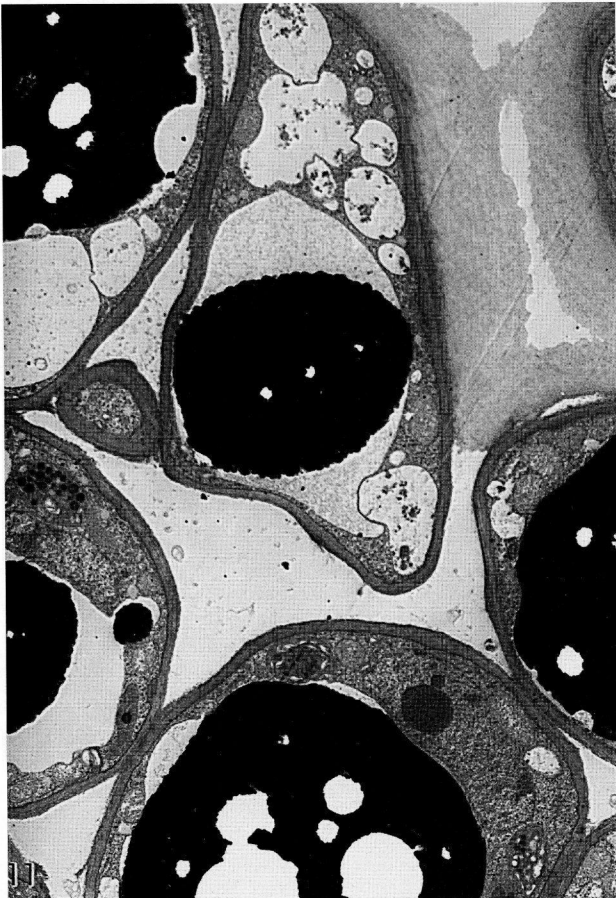
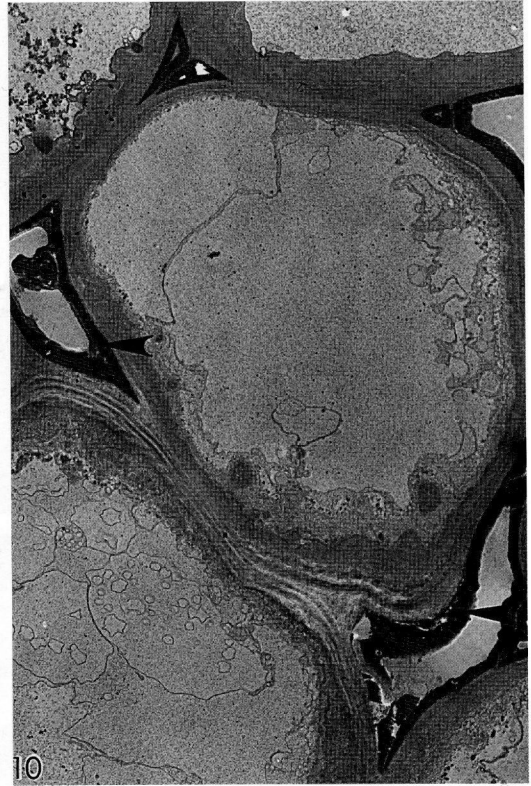
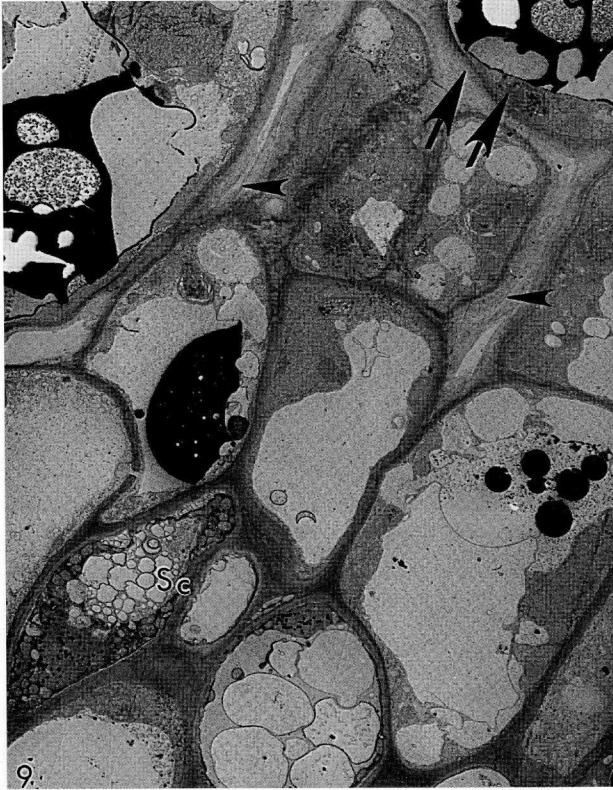
4) The absence of ninhydrin-positive substances is inexplicable since a number of amino acids are commonly found in extrafloral nectar (Baker et al. 1978) and in phloem exudate. Although we were unable to obtain sufficient nectar from *S. hepecarpa* for analysis, the similarities between this EFN and those of the other species examined, lead us to believe that nectar quality will be similar.

We tentatively conclude that the EFNs of *C. fasciculata*, *S. hepecarpa*, and *S. marilandica* represent a very simple type of nectary which begins as localized cell proliferation just beneath the petiolar epidermis and develops into the form characteristic of each species. It has a simple epidermis, a distinctive sub-epidermal parenchyma, and its own vascular supply. In immature nectaries, the cells are typically tightly packed, but as cell expansion takes place with the concomitant development of intercellular spaces, there is a gradual accumulation of cutin in these spaces. Internal cutinization has received relatively little attention. However, deposition of cutin-like material on internal cell surfaces was observed in the apical meristems of *Ricinus communis* and other species (Scott and Lewis 1953) and in leaves of *Citrus sinensis* (Scott et al. 1948). In their plants, the deposition was barely discernible in the intercellular spaces of young tissue, but became clearly defined as the tissues matured. In a later paper, Scott (1964) argued that such internal cutinization/suberization was evidence of a wound reaction resulting from the severing of plasmodesmatal connections between cells. If these workers are correct, it may be that, during cell expansion in these nectaries, breaks occur in some of the plasmodesmatal connections to trigger the wound reaction described by Scott (1964). This would be manifested as an early deposition of cutin on the cell walls abutting extracellular space, as has been observed in these nectaries with LM. The deposition becomes more abundant as the nectary expands further and matures.

Studies such as those by Wergin et al. (1975) in which a secretory

Figs. 2–8. 2–4 SEM views of the three species studied. 2. *C. fasciculata* × 40. 3. *S. marilandica*. × 30. 4. *S. hepecarpa*. with blistered surface × 40. 5. Nectary of *S. marilandica* showing epidermis with cutin accumulation (arrowheads) in intercellular areas of epidermis and underlying parenchyma. × 4000. 6. Nectary of *C. fasciculata* with cutin deposit (arrowheads) in intercellular area of the sub-epidermal parenchyma. × 2100. 7. LM view of the nectary epidermis of *S. hepecarpa* showing uplifted cuticle (arrowhead), epidermis, and underlying parenchyma. × 400. 8. TEM view of mature nectary of *C. fasciculata* with detached epidermis × 2000.





epidermis and an underlying parenchyma have been implicated in the symplastic transport of pre-nectar, show epidermal cells with dense cytoplasm and very high frequencies of clustered plasmodesmata between these cells and between them and the underlying small, dense, tightly-packed parenchyma cells. In our species, neither nectary epidermis nor parenchyma are notably different from ordinary epidermal cells and parenchyma. Plasmodesmata are not unusually abundant anywhere in the nectary. Because the epidermal cells of our three species are ultrastructurally unspecialized and, as the nectary matures, often become separated from one another, first by accumulations of cutin-like material between the cells and later by distinct gaps, we believe that these cells do not function in the secretion process and although symplastic movement of sugars through the parenchyma cannot be completely discounted, this tissue, like the epidermis, also does not have the ultrastructural features of tissue associated with secretion.

We suggest that the nectar may be derived directly and primarily from the phloem and move through the free space of the nectary without being modified by parenchyma or epidermal cells. The presence of Prussian Blue throughout this area suggests that a clear path is available and the cutin may partially insulate these cells from contact with the nectar. As sucrose is unloaded from the phloem, possibly by simple diffusion (Patrick 1997), its initial accumulation would further expand the intercellular space, exert pressure on the epidermal layer and even rupture the cuticle, with release of nectar. It is at this stage, for example, that the nectary surface of *C. fasciculata* probably collapses to produce the cup shape characteristic of the secreting mode.

It is difficult to explain the unusually high concentration of total sugar in the nectar of these species if the phloem is the direct source of these sugars because, in the few examples of phloem exudate which has been tested (Ziegler 1975, Hall and Baker 1972, Hayashi and Chino 1985), the sugar concentration is considerably lower. There is a need for more comparative analyses of phloem contents and nectar from our species and others similar to that done by Baker et al. (1978) for *Ricinus*.

Other factors may be involved here. The dominant presence of sucrose in the nectar suggests that hydrolysis of this sugar by the nectary cells is minimal. Thus, the driving force for continued sucrose unloading could be the physical removal of sucrose as it is being released. In the greenhouse, regular misting of plants would be sufficient to remove exuded nectar. Under natural conditions, rain could accomplish this, but frequent harvesting of nectar by ants and other nectar feeders, perhaps resulting in destruction of the epidermis and some underlying tissue by chewing, may also facilitate movement of nectar through the parenchyma to the surface. Although consideration of this kind of interaction between ant and nectary is rarely found in the literature, it may be an important part of the nectar-release process.

We believe that the EFNs described in this paper are quite different from nectaries that have been studied thus far. Their distinctive morphology masks an anatomical simplicity that is uncommon in such structures, while their ultrastructural features suggest that neither the epidermis nor the underlying parenchyma are involved in the secretory process. The extensive presence of cutin is also unusual. These EFNs may offer an ideal system for the study of phloem unloading uncomplicated by the activity of intervening cells. In ad-

dition, they present an opportunity to investigate the processes leading to internal cutin deposition.

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Figs. 9-11. *C. fasciculata*. 9. Sieve element-companion cell complex (Sc) near termination of cutin deposition (arrowheads). Nectary surface in direction of arrows. $\times 2500$. 10. Accumulation of Prussian Blue (arrowheads) in free space of sub-epidermal parenchyma. $\times 4000$. 11. Free space of sub-epidermal parenchyma, control sample. $\times 4000$.

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