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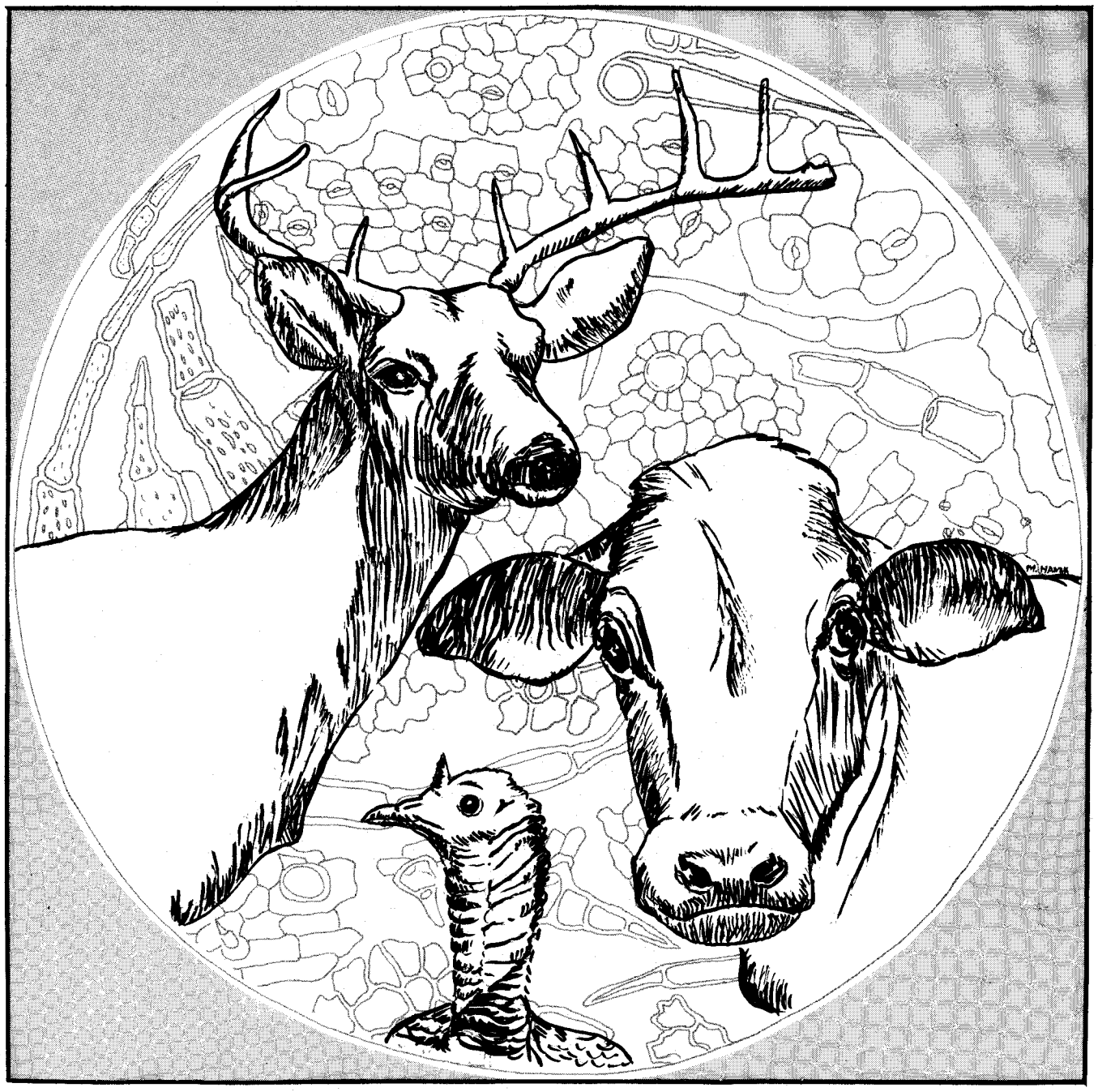
Microhistological Techniques for Food Habits Analyses

Mark K. Johnson, Helen Wofford and Henry A. Pearson



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INTRODUCTION

The use of micro-anatomical features for identification of plants in diets of herbivores has received wide application since the early reports of Baumgartner and Martin (1939), Norris (1943), and Dusi (1949). However, detailed descriptions of specific techniques have not been fully reported. Those attempting to learn how to quantify diets microhistologically have great difficulty without help from experienced technicians.

The purpose here is to describe specific techniques used to prepare and quantify herbivore diet samples for microhistological analyses. It is beyond our scope to provide a detailed taxonomy based on micro-anatomy. However, the variety of plants described here represents probable numbers and anatomical variations that may be normally expected in a diet study. Additional variations of micro-anatomical features exist in other plants. The reader should refer to Metcalfe (1960) for a complete description of plant micro-anatomy. Here we describe the types of anatomical structures useful for identifying plant fragments regardless of the variation in each.

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REFERENCE MATERIALS

The plants described in this report are a select group of those common on longleaf-slash pine-bluestem range of the lower coastal plain in the southeastern United States. Plant names used in this paper follow those provided in the National List of Scientific Plant Names (USDA, SCS 1982).

Some researchers prepare reference and study materials by carefully scraping away tissues to expose the cutinized epidermis on the opposite side (Korschgen 1977). This method is preferable to ripping tissues in a blender when intact leaves are needed for taxonomic description. However, for diet analysis reference materials must simulate the small fragments found in samples following mastication and digestion. Therefore, we prepared reference plants by mixing about 1 g of dry leaves and 20 ml of liquid household bleach at high speeds in a blender.

Hertwig's solution can be used to clear pigments from plant tissues (Cavender and Hansen 1970, Flinders and Hansen 1972, Todd and Hansen 1973). However, soaking samples in household bleach accomplishes the same thing with greater ease (Reynolds et al. 1978).

Some researchers stained plant specimens after decolorizing (Dusi 1949, Voth 1968, Field 1972, Fitzgerald 1976). Although staining aids in distinguishing certain features, some do not stain materials (Stewart 1967, Cavender and Hansen 1970, Hansen 1971, Westoby et al. 1976, Johnson 1979), and we believe it unnecessary.

We used Hoyer's solution for making permanent slides. However, this solution is hygroscopic so that in warm, humid climates slides become sticky and must be stored in low humidity environments for months before they are dry. An alternative plastic mounting medium can be made with lactic acid, phenol crystals, and polyvinyl alcohol. The plastic medium dries within a few days. (Directions for preparing both Hoyer's solution and the plastic medium are in Appendix I).

When permanent slides are not needed, plant fragments can be mounted in a solution of soapy water or water mixed with a small amount of gum arabic. Water infiltrates the tissues quickly and slides can be examined immediately.

Mark K. Johnson is Associate Professor, School of Forestry and Wildlife Management, Louisiana State University, Baton Rouge, LA 70803. Helen Wofford is Research Assistant, Department of Animal and Range Sciences, New Mexico State University, Las Cruces, NM 88003. Henry A. Pearson is Chief Range Scientist, Southern Forest Experiment Station, USDA Forest Service, 2500 Shreveport Highway, Pineville, LA 71360.

PREPARATION OF DIET SAMPLES

Diet samples come from esophageal or rumen fistulated animals, stomachs, rumens, intestines or ceca of dead animals, or fecal droppings. The condition of fragments in esophageal samples is similar to reference material making sample preparation relatively easy. Digested fragments are more difficult to identify since the number of anatomical features per fragment is less usually. Fecal materials exposed to the environment for long periods may be difficult to identify, but decomposition in arid environments may be so slow that fossil dung more than 10,000 years old has been used to obtain a record of an animal's diet (Hansen 1978).

Whenever stomach or rumen samples are available, gross examination with dissecting equipment at low magnifications is recommended before grinding for micro-analysis. Some foods do not have distinctive micro-anatomical characteristics and can only be identified before digestion. On the other hand the presence of some plants can be easily detected by micro-analysis while they may not be detected by gross examination (Yarrow 1979).

DIAGNOSTIC FEATURES

Leaf fragments in diet samples are identified by comparison with fragments and drawings made from reference slides. Drawings can be made by hand or with the aid of a drawing tube attached to a microscope. This piece of equipment allows non-artists to accurately outline the micro-anatomy of plant fragments but all structures do not lie on the same plane so that drawings can not perfectly represent the appearance of fragments in a microscope (Appendix II). Descriptions of plant micro-anatomy from Metcalfe (1960) were used to guide our study of those structures most useful for identification. We generally ignored Metcalfe's descriptions of relative abundance for certain structures since these characteristics are only apparent on large leaf fragments. Therefore, our descriptions are limited to micro-anatomical variations of certain structures.

Micro-anatomy of Monocots

The parallel veins of grasses readily separates leaf tissue into zones, and clearly distinguishes grass leaf fragments from those of dicots. While identifiable tissues from sheath, flower, seed, or culm are often present, leaf blades provide the most useful tissue for microscopic identification of the grasses (Metcalfe 1960). Minor anatomical differences appear on the same leaf, among leaves of the same plant and among different plants. However, micro-anatomical characteristics have been used for taxonomic differentiation of species, genera, and families (Davies 1959, Tateoka 1957, and Metcalfe 1960).

Costal Girder and Bulliform Cells.—The areas directly over and around the veins are termed costal zones while areas between the veins are termed intercostal zones (Metcalfe 1960). Schlerenchymous cells called costal girders surround the veins and are common to grasses from dry habitats (Metcalfe 1960). Bulliform cells are located intercostally and have thinner cell walls than costal girders. They are large, highly-vaculated, epidermal cells. Their function is not known but sometimes they are filled with silica (Esau 1965). Since the arrangement and appearance of costal girder cells are highly variable, we have not found costal girder and bulliform cells to be useful features for identification of grasses.

Long and Short Cells.—There are two other distinct cell types (long and short) in typical epidermis. Long cells are very narrow and run parallel to the vascular bundles. They are a major constituent of the intercostal zone but may also be found over the costal zone. Cell walls may be sinuous or non-sinuous with varying degrees of each (fig. 1). Long cells surround short cells and stomata (Esau 1965). We found that length and width of long cells provide some clues as to the identity of a fragment, but the nature of the cell walls is the primary characteristic we compare with reference materials when trying to identify leaf fragments.

Short cells are dispersed among the long cells and may appear over the costal zones. They are filled with silica or cork. We have found the shapes and sizes of silica cells in the costal areas to be particularly diagnostic.

Metcalfe and Chalk (1950) classified about 20 different shapes of silica cells. Three main shapes we

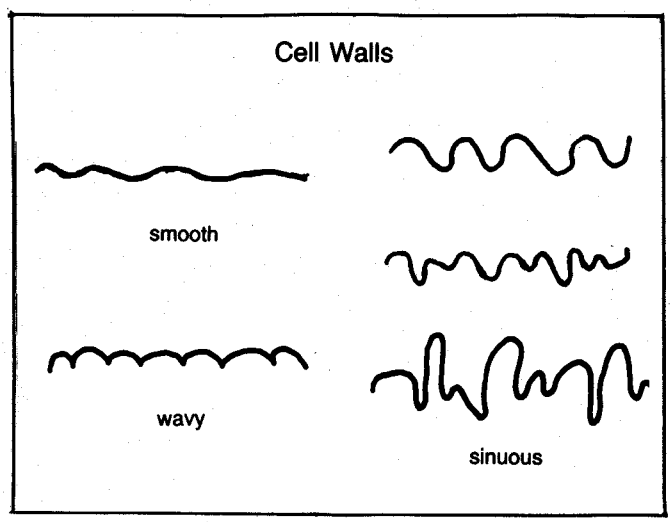

Figure 1.—*Variations in cell walls of grasses.*

have found are dumbbell, non-dumbbell, and dumbbell variations. Non-dumbbell shaped silica cells are square, cuboid, or irregular cuboid (fig. 2). For dumbbell shaped cells in the costal region, length, width, and angular characteristics of the middle and ends aid in identification of species (fig. 3).

For silica cells in the intercostal region, shape and abundance differ among species. They often occur in pairs called companions or couples (fig. 4). Comparison of silica cell size is often useful for distinguishing among species where shapes are similar. However, when size is used for identification a number of different fragments must be examined to account for variation. Size is most useful for distinguishing between genera rather than species. For example, the shape of silica cells found in leaves of *Andropogon, Schizachyrium* and *Panicum* is bone-like but those in *Panicum* leaves are generally smaller.

Stomata.—Stomata of grasses occur intercostally. Openings are surrounded by guard cells that are narrow in the middle and bulbous on the ends. In addition, stomata of grasses are accompanied on their parallel axes by two subsidiary cells (Metcalfe 1960).

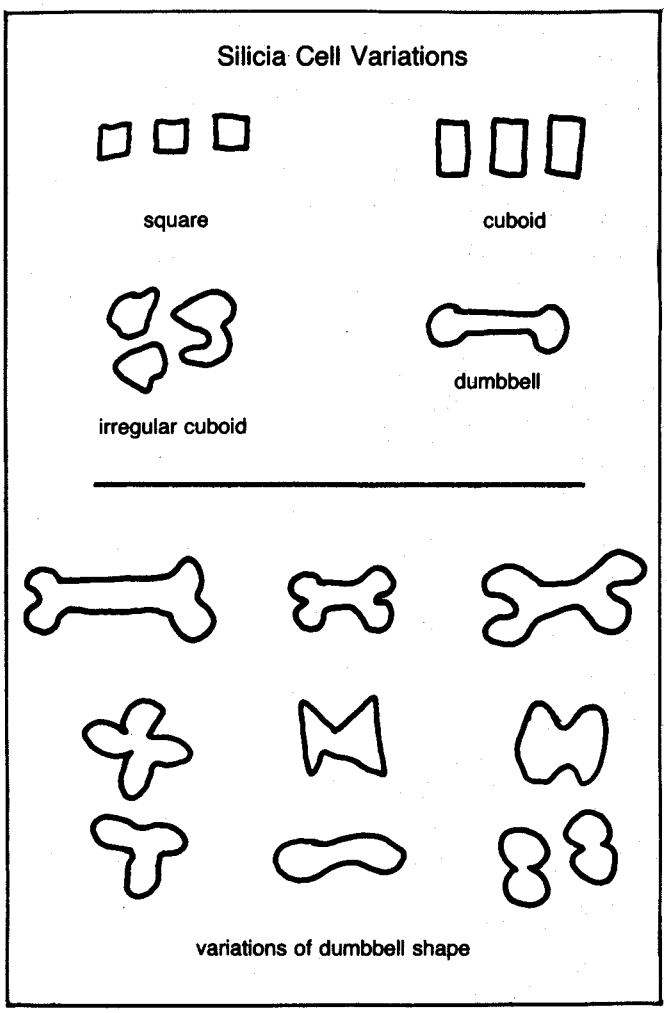

Figure 2.—*Variations in shapes of silica cells observed in grasses.*

It is easier to identify grass species whenever both stomata and silica bodies are present on the same fragment. The size of stomata is not useful for identification except where they are extremely small or large.

Trichomes.—Four types of grass trichomes are useful for identification. They are macrohairs, microhairs, prickle hairs, and papillae (fig. 5). Macrohairs are visible with the naked eye or hand lens. Some are multicellular but the shape of the proximal end is the most outstanding feature. Of all the hair types, microhairs are the least diagnostic because in diet samples the distal cell of bicellular microhairs is often broken, leaving a blunt end (Metcalfe 1960).

Prickle hairs are very common but there are few differences in their appearance among species. Usually located on margins, veins, or between long cells, they simply aid identification by lending a characteristic appearance to fragments. By contrast, prickle hairs vary in shape, location and distribution on leaves so that they may be very useful for taxonomic characterization of species when complete leaves rather than small fragments are examined.

Papillae are protrusions of the epidermal surface which take a variety of shapes. We found that sedges (*Carex* spp.) and pineywoods dropseed (*Sporobolus junceus*) had numerous papillae present.

Micro-anatomy of Dicots

Trichomes.—Trichomes provided a primary means for identifying dicots since they differ more among species than other epidermal cells, crystals or sto-

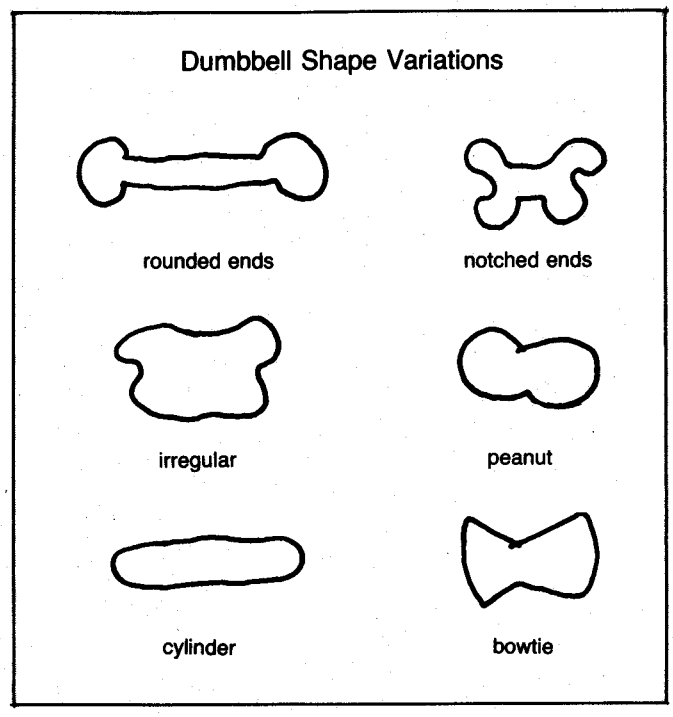

Figure 3.—*Variations in shapes of dumbbell shaped silica cells observed in grasses.*

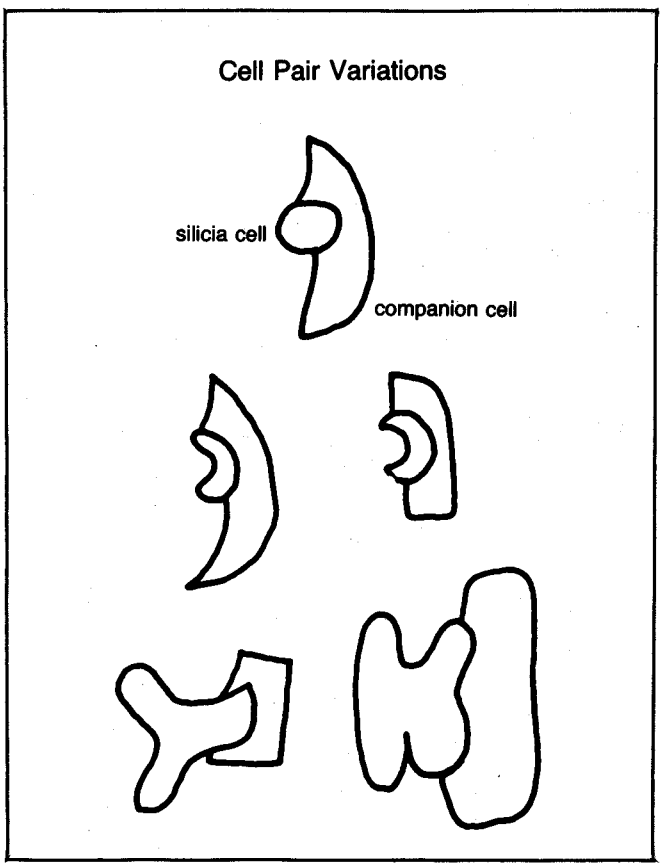

Figure 4.—*Variations in the appearance of silica cell pairs which occur in grasses.*

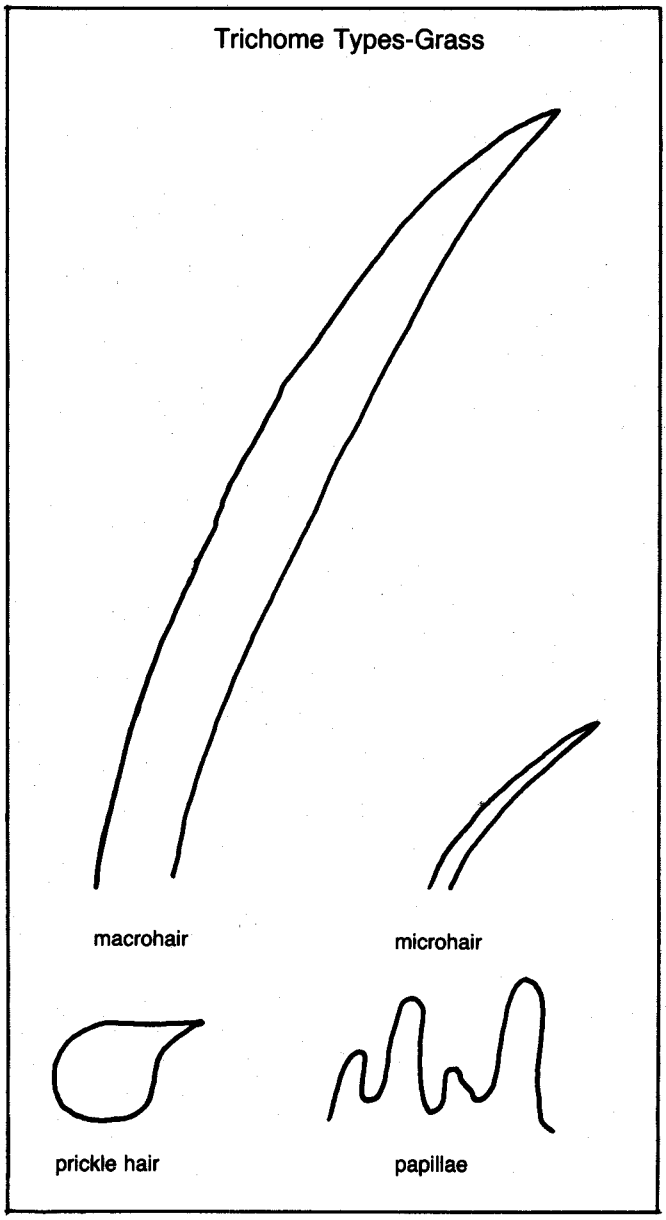

Figure 5.—*Four basic shapes of trichomes commonly found on grass leaves.*

mata. Metcalfe and Chalk (1950) classified families into those having glandular or nonglandular trichomes and also according to the numbers of component cells. Trichome anatomy has been used for generic classification by Rollins (1944), Heintzelman and Howard (1948), Cowan (1950), and Hummel and Staesche (1962). While the shape of plant hairs differs among some taxonomic divisions, there is often considerable uniformity within genera.

We found shape and number of cells making up a trichome to be the best starting point for identifying dicot species. We observed 4 basic kinds of trichomes: ligulate, branched, compound and papillate (fig. 6). Characteristics such as size of the base, shape of the apex, texture of the surface and degree of tapering are useful for identification (fig. 7).

Epidermal Cells.—Unless otherwise indicated, epidermal cell as used here refers only to epidermis of leaves including cells of the upper leaf surface, lower leaf surface, trichome attachment cells, and specialized cells such as glands. Shapes of epidermal cells vary among families, genera, species, and parts of the same plant. Their form is affected by factors such as light intensity and atmospheric humidity. Thus, the size, shape, and walls of epidermal cells may vary from year to year and place to place (Metcalfe and Chalk 1950). In addition, some plants have multiple epidermal layers (Esau 1965).

Despite variation, characteristics of epidermal cells are highly useful for plant fragment identification. Notable features include wall structure, relative size, and relative thickness. Two distinct shapes are angular and contour. Angular cells have pointed or rounded corners, and five, six, or eight straight sides which give the tissue a honeycomb appearance. By comparison, the walls of contour cells fit together like those of a jigsaw puzzle. We found the degree of contour in the walls to be characteristic (fig. 8).

Trichome attachments have different shapes than other epidermal cells, and there is usually a constant

number of cells surrounding each attachment (fig. 9). Glands occur on epidermal tissues of many plants and are usually smaller than other trichomes (fig. 10).

Crystals.—Crystals are formed from secretions of calcium carbonate, calcium oxalate, starch, or silica. While leaves of *Rubus* contain large crystals called druses, those produced by *Lonicera* are usually larger and fewer. Crystals are located in epidermis, vascular tissue, and stemlike tissues around trichome attachment cells. Types of crystals are druses (starshaped), raphides (needlelike), various square or rectangular crystals, and intermediates of druses and squares (fig. 11).

Stomata and Companion Cells.—Stomata are usually most abundant on the lower surface of leaves. However, they may also be located on upper surfaces or on the epidermis of petioles, stems and flowers. The companion cells that surround the guard cells are not generally useful for identification. Exceptions are those that appear to have corner cells at the point where two guard cells meet. These tiny triangular cells are common to *Quercus*, but they are also found in most conifers and ferns.

Frequency and size of stomata are highly variable characteristics that depend on environment, geography, and location on the plant. These characteristics are not useful for identifying small fragments. More useful for identification is the number of epidermal cells that surround stomata. Among the plants in our collections are the patterns outlined by Metcalfe and Chalk (1950): irregular-celled (four or more cells surround the stomata), three unequal-celled (three cells surround stomata, one may be distinctly smaller than the other two), parallel-celled (two cells surround

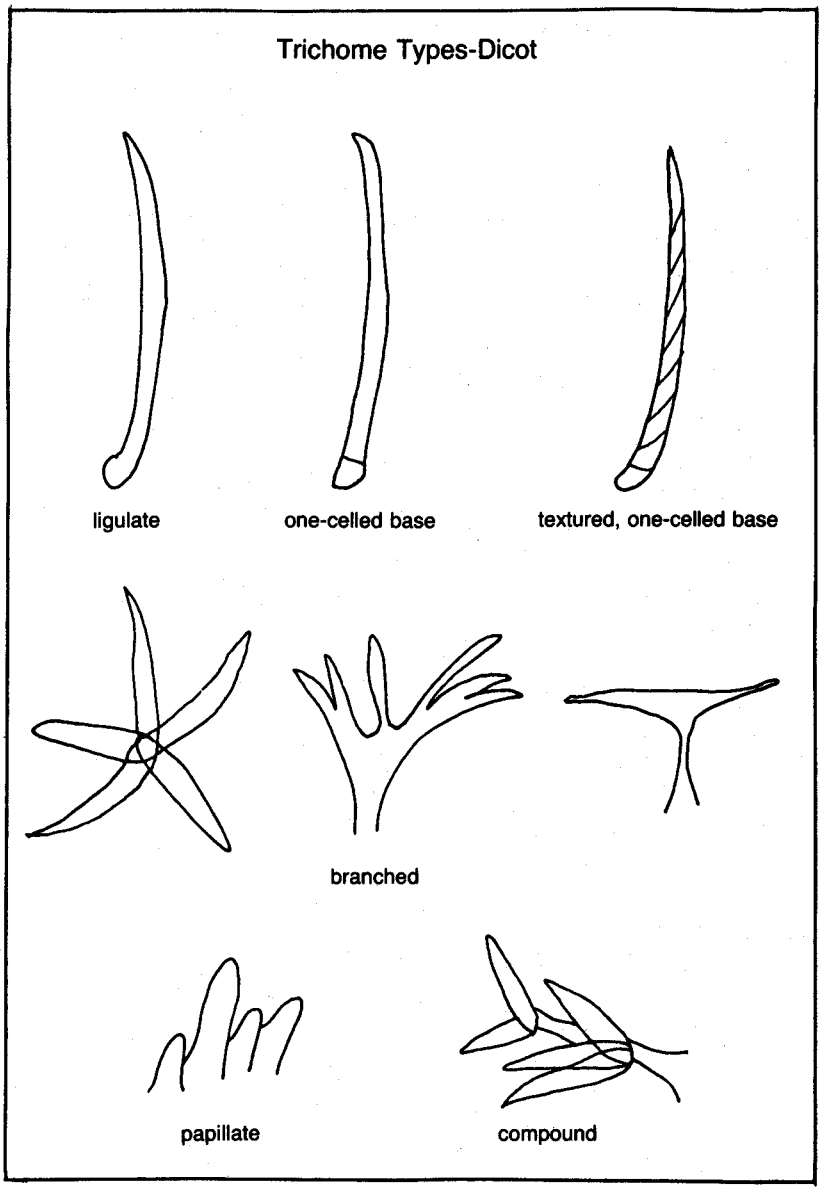

Figure 6.—*Basic kinds of trichomes commonly found on leaves of dicots.*

stomata and their common side is parallel to the long axis of the pore), and cross-celled (two cells surround the stomata and their common wall is at right angles to the long axis of the pore)(fig. 12).

IDENTIFICATION

Whether plant fragments are from reference material or diet samples, they are not uniform in size, shape, or thickness. We mount samples on glass slides ($3'' \times 1''$; cover slips, 20×40 mm) in a layer of medium about 1.0 mm thick. All of the fragments do not lie in the same plane, and some individual fragments curl so different anatomical structures do not lie on the same plane of focus. In addition, tissue thickness causes anatomical structures to be on different planes of focus. Complete view of a fragment requires focusing through tissues until all features are examined. Composite drawings such as those published here do not represent the true appearance of fragments under a microscope, and the necessity of focusing through tissues limits the use of photomicrographs for illustration or as reference material. However, after study, drawings provide excellent reference with reduced effort compared to constant exami-

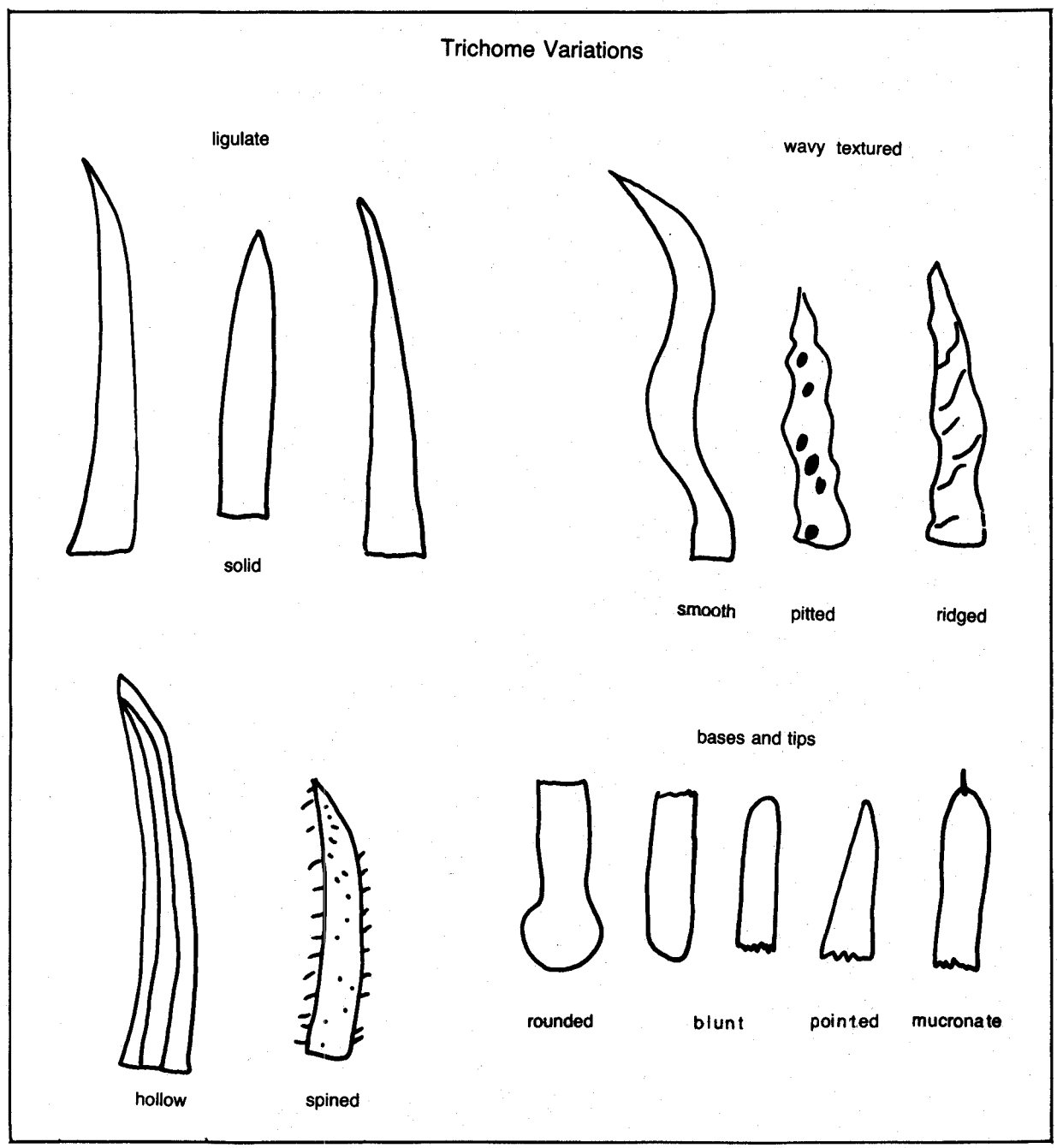

Figure 7.—*Basic characteristics of tips and bases of trichomes usually found on leaves of dicots.*

nation of reference slides which are only needed for confirming uncertain identifications.

Distinguishing monocots from dicots is simple because of the pronounced difference in venation. However, distinguishing among different dicots requires recognition of cell patterns and anatomical structures. Often the presence of a distinctive trichome is sufficient evidence for identifying a fragment. However, some trichomes separate from fragments and in these cases accurate quantification requires association of trichomes with epidermal fragments. For species or parts of leaves without distinctive trichomes, identification depends upon study of cell shape, characteristics of cell walls, the form of stomates, and the presence of crystals. Usually, only one or two micro-anatomical features are needed for identification of dicots.

Identification of grasses, requires study of the cell walls, stomates, and silica cells. In general, fragments of different grasses appear more similar to each other than fragments of dicots. When a sample contains two or more grasses, fragment identification usually requires the presence of at least two distinctive features.

For a large collection of species, it would be difficult to identify plants without large pieces of leaves so that a complete pattern of structures could be examined compared to what can be seen on small fragments. However, practical food habits studies deal with a limited list of plants from which herbivores usually select a few staples during any season. By the process of elimination and intense study of reference plants, small fragments of leaves can be used to identify the foods selected by a herbivore.

QUANTIFICATION

Anthony and Smith (1974) visually estimated the contribution of each species to the numbers of fragments observed on microscope slides. Although this method seems efficient and practical, there is no control over observer bias. Most quantification of plant fragments identified on a microscope slide has been performed by counting fragments or by using some variation of simple counting. Voth (1968) measured the dimensions of each fragment to calculate an area

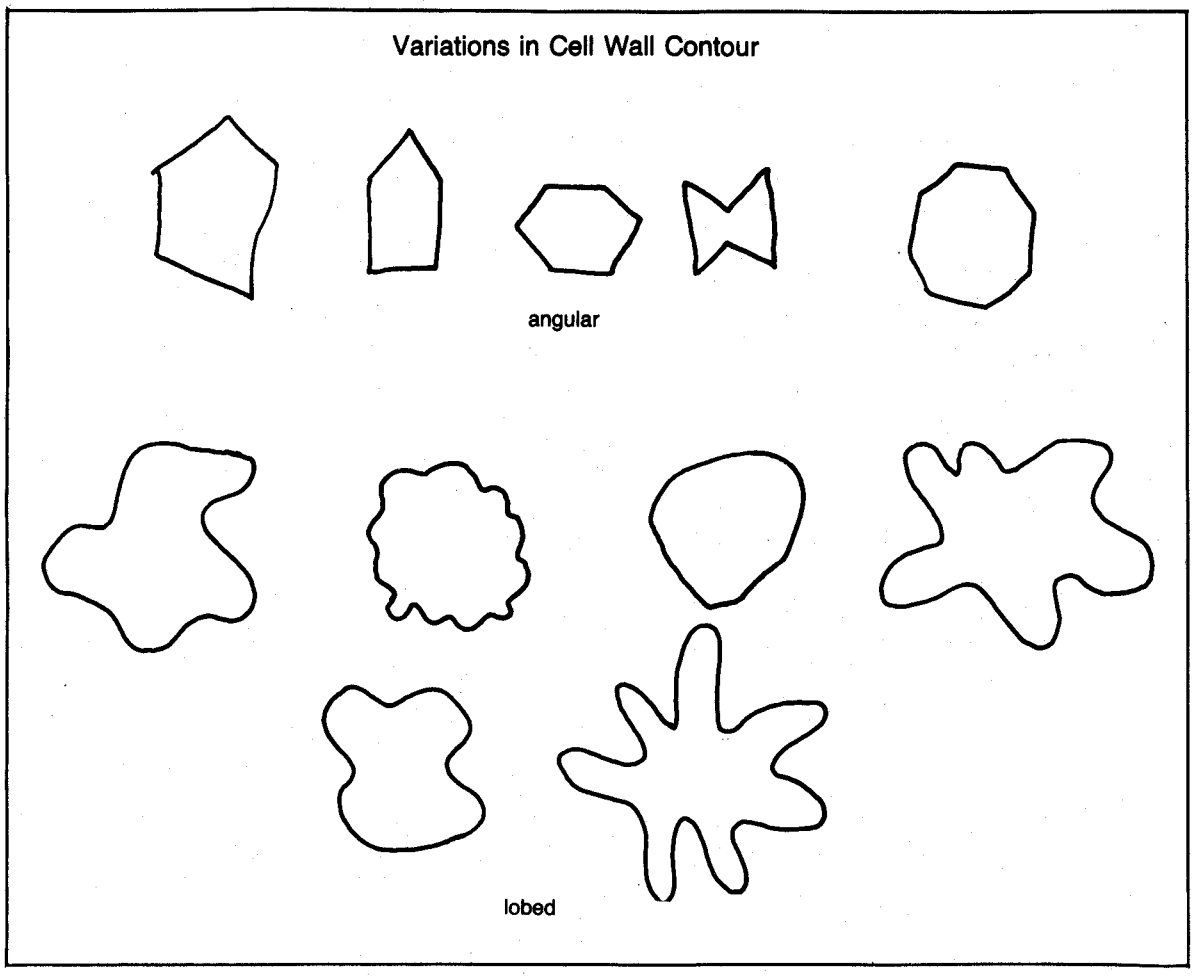

Figure 8.—*Variations in the contour of cell walls in leaves of dicots.*

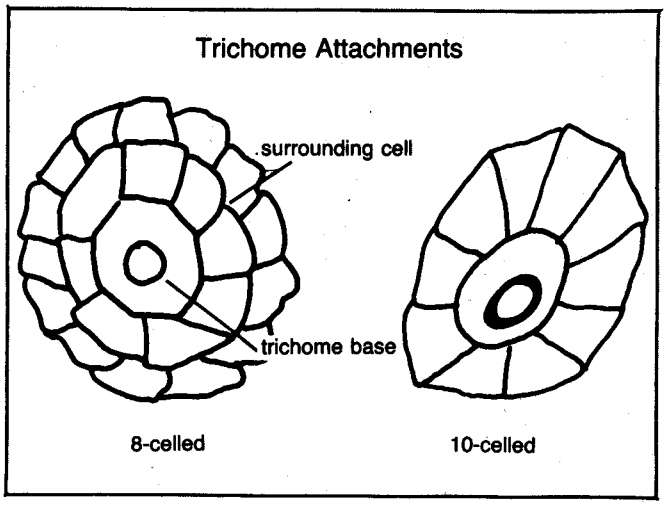

Figure 9.—*Examples of trichome attachments observed on leaves of dicots.*

for each one. Other researchers have counted the numbers of fragments and calculated the relative number for each species (Storr 1961, Ward and Keith 1962, Myers and Vaughan 1964, Bear and Hansen 1966, Sparks 1967, Stewart 1967).

Frequency Sampling

Frequency sampling is nothing more than recording the presence or absence of an item in a sampling unit. The method has been employed for study of wild plant distributions and abundance (Fracker and Brischle 1944), and relative abundance of wild animals (Caughley 1977). Sparks and Malechek (1968) demonstrated that frequency sampling was an accurate alternative to counting each plant fragment when quantifying botanical compositions on microscope slides. As a result the procedure has been used for estimating herbivore diets by a variety of researchers (Flinders and Hansen 1972, Todd and Hansen 1973, Hansen et al. 1973, Dearden et al. 1975, Johnson 1979).

The technique is relatively simple. Microscope slides are made with mixtures of plant fragments ground in a Wiley Mill over a 1.0 mm mesh sieve so that all particles average about the same size. A predetermined number of fields are systematically examined at the same magnification, and the presence of each species is recorded.

Frequency of occurrence is calculated for each species. As long as the amount of ground plant material on each slide is relatively small (1 – 3 fragments per field), average relative frequency of occurrence represents average relative abundance of the different species in the mixture. Since bulk densities of leaves from different plants are about equal, relative abundance provides an estimate of relative dry weight for

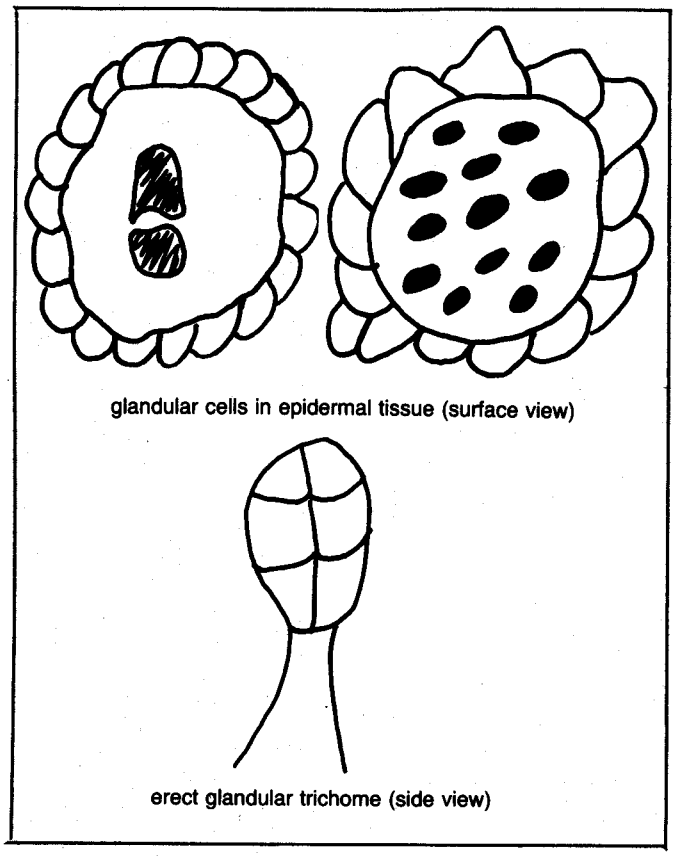

Figure 10.—*Examples of glands observed on leaves of dicots.*

each food in a herbivore's diet (Johnson 1982). A number of published reports demonstrate that, in general, this technique provides accurate estimates of herbivore diets whether study materials are stomach contents or fecal pellets (Hansen et al. 1973, Dearden et al. 1975).

Under the following assumptions each microscope field is treated as a sampling unit: (1) microfragments of plants are randomly distributed on microscope slides, (2) microfragments from different plant taxa are the same average size, (3) dry weight bulk densities of different plant taxa are the same. These assumptions are valid since the distribution, size and average number of fragments per microscope field is controlled in slide making and since there are no significant differences in dry weight bulk densities among the leaf tissues from different plants.

The relationship between particle density and frequency of occurrence is based on finite numbers of plant fragments distributed at random over a microscope slide. A detailed description of the mathematical theory is available in Johnson (1982).

The standard form of the realtionship between frequency and density is

$$F = 1 - e^{-d}, \qquad (1)$$

where F is relative frequency, e is the natural logarithm and d is the mean particle density determined

by the number of fragments (n) and number of microscope fields examined (k) so that

$$d = {}^{n}/_{k}. \quad (2)$$

If fragments from m different plant species are randomly distributed in the microscope fields, the particle density (average number of fragments per field) of each is independent from the others. Relative particle densities (r_i), which are estimates for the relative dry weights of each plant in the diet sample, can be calculated:

$$r_i = \frac{d_i}{\sum_{i=1}^{m} d_i}, \quad (3)$$

where $_i = 1, \ldots, m$ and d_i's are the particle densities for each species. For example, we may simultaneously count the presence or absence of several different taxa in a microscope analysis. Since proper mixing and spreading of microfragments assures randomness and independence of particle distributions, separate estimates for each taxon's particle density may be obtained and relative particle densities calculated.

In practical applications one seeks to estimate the average particle density, which is unknown and not easily estimated. This is done by estimating frequency of occurrence, which is also unknown but easily estimated by recording particle presence or absence, and then estimating density from frequency using equation (1). Estimates for frequency of occurrence are subject to sampling variability.

Relative precision of the estimate for density is also dependent on average frequency (Curtis and McIntosh 1950). As average frequency increases, relative precision increases up to an optimum. Precision decreases for larger particle densities because most or all of the fields contain at least one identifiable plant fragment. This result imposes constraints on the maximum particle density which is allowable on microscope slides. Conversely, low particle densities of rare taxa, which result when microscope slides are made to avoid high particle densities for abundant taxa, require intensive sampling to yield precise estimates of these particle densities. We obtain accurate results in quantifying mixtures when slides are made so that microscope fields contain from 3-5 identifiable fragments.

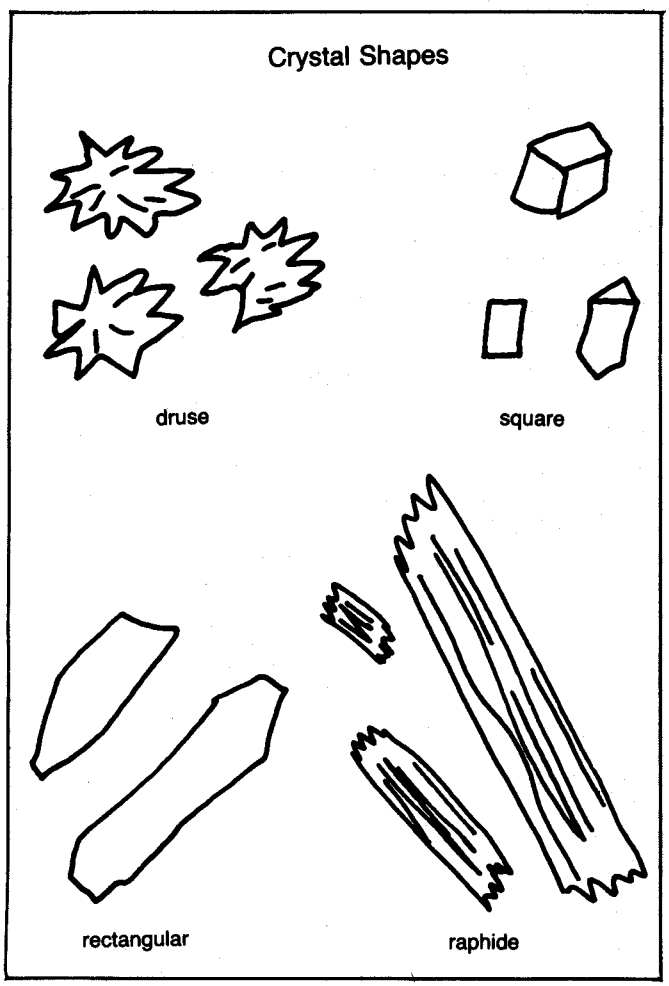

Figure 11.—*Basic examples of crystals observed in leaves of dicots.*

Relative Discernability and Digestion

Since all fragments can not be identified, there have been attempts to account for differences among species as to proportions of fragments that can be

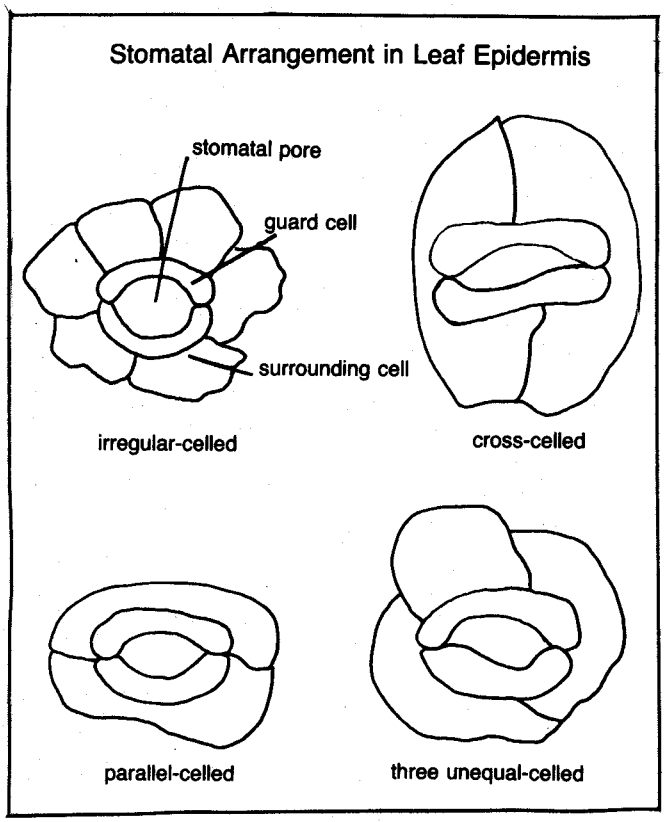

Figure 12.—*Basic arrangements of stomata and surrounding epidermal cells observed in leaves of dicots.*

identified, and to account for the affects of digestion on the discernability of fragments. In addition, for the same weight of leaf material some plants fragment during mastication or sample preparation into more than twice the number of pieces as other plants (Johnson et al. 1983).

Differential digestibility has been widely discussed as introducing bias into diet estimates when fecal samples are used. Some researchers have gone to great lengths to adjust data to account for effects of digestion (Voth and Black 1973) even though reports were not available to suggest that digestion significantly biased analyses. There are no known microorganisms that possess cutin degrading enzymes (Frey-Wyssling and Muhlentahler 1959). The equivalence factors reported by Voth and Black (1973) were related to digestion of tissues underlying the cuticle rather than reduction of the cuticle. Plant fragment identification is based on micro-anatomical characteristics of an indigestible cuticle and cells underlying the cuticle that escape digestion. Leaves are made of epidermis, mesophyll, and vascular bundles. Epidermis is covered by indigestible cuticle which inhibits digestion of mesophyll by blocking passage of microbes into the leaves (Harbers et al. 1981). However, once passage is gained by maceration, mesophyll is readily degraded while epidermis and cells surrounding vascular bundles are degraded next (Aiken 1979). Generally, lignified vascular tissues resist digestion. Theoretically, identification is made only from cutin which retains the impression of epidermal tissues. But undigested groups of cells enhance the visibility of cutinized fragments because the increased thickness causes greater refraction of light. Microhistological identification is easier when both the epidermis and underlying tissues are present.

We recorded numbers of fragments and proportions identifiable for a variety of undigested and digested plants (Johnson et al. 1983). For 47 species tested, digestion increased discernability for 3 and decreased discernability for 9 by more than 10 percent while the other 35 plants were little affected.

Digestion improved discernability for plants having highly suberized, pigmented cell walls, and few trichomes by clearing pigments from tissues making cells easier to see. Digestion decreased discernability for plants having thin cell walls and few stomates or trichomes. Easily identified plant fragments generally had an abundance of features that were easily recognized. Regardless of these affects, we found that they were not dramatic enough to have very much influence on estimates of botanical composition (Johnson et al. 1983). This work helped to explain why estimates of botanical compositions for feeds and feces have usually been significantly similar (Todd and Hansen 1973, Hansen et al. 1973, Johnson and Pearson 1981.)

WOOD FIBERS, MUSHROOMS AND MAST

Wood Fibers

Digesta from animals that browse or gnaw wood may contain fragments that cannot be identified by gross analysis. However, wood fibers can be identified microscopically and often occur in slides made from fecal materials. Identification is performed by comparison with reference materials in the same manner as leaf fragments are identified.

Preparation of reference fibers or wood fragments taken from food habits samples is performed by maceration in a 50:50 mixture of glacial acetic and 30% hydrogen peroxide. Wood fragments should be refluxed in the solution for 1 to 2 hours. The specific time needed should be established through practice; some wood will take longer than others. Variations on this procedure are also available (Panshin and deZeeuw 1980). Materials should be rinsed thoroughly after maceration. Microscope slides can be made and examinations performed as they are for other plant fragments.

Diagnostic features are size, shape, and pitting of fibers. Tracheids are the only cells useful for identification of softwoods. Various features of vessel elements and tracheids as well as the presence or absence of tracheids are diagnostic for hardwoods (Carpenter and Leney 1952, Panshin and deZeeuw 1980).

We know of no authors employing these procedures in food habits studies. We have observed the fibers in fecal pellets of a variety of herbivores but have never attempted quantifying them.

Mushrooms

Herbivores, as well as other animals, eat mushrooms, and proportions in the diet may be significant. Many animals consume mushrooms that are poisonous to humans. Mushrooms tend to concentrate phosphorous and are rich in protein (Miller and Halls 1969, Fogel and Trappe 1978). Both of these nutrients may be lacking in the vegetation of some regions.

The presence of mushrooms in food habits samples complicates quantification. The point analysis method useful for quantifying relative dry weights of macroscopic fragments in stomachs has two requirements; bulk densities of materials should not differ greatly and the form of different items must be similar (Chamrad and Box 1964). For example, the dimensionless quality of point methods is destroyed if large flat pieces of leaves are analyzed in mixtures with small seeds which have a much higher bulk density but a lower probability of occurrence at a point.

These problems can be overcome by hand separation of mushrooms for separate weighing or volume-

tric study, or by microscopic analyses of ground samples. Regression equations can be developed to relate occurrences of mycelia in stomachs to occurrences of plant fragments.

Digestibility of fungal mycelia is nearly complete except where large amounts are ingested in a single meal. We have observed intact mycelia in feces in these cases. However, when mushrooms are eaten in smaller amounts, only spores are present in fecal material. Although indigestible (Fogel and Trappe 1978), spores may not be detected in fecal samples during routine analysis because they are generally tiny. Some can be detected at 100 magnifications but others require 500 to 1000 magnifications before they can be seen.

Proportions of fecal samples made up by spores might be large for small, mycophagous mammals (Tevis 1952), but insignificant for other wildlife; while the proportion of the diet they represent can be more than 30% (unpublished data). Therefore, accurate diet quantification requires developing correlations between amounts eaten and number of spores.

To demonstrate this procedure we separately ground dry mushrooms (*Amantia* sp.) and plant leaves in a Wiley mill through a 20 mesh sieve. Microscope slides were prepared with 14 different amounts of pure plant fragments ranging from 6 – 60 mg. Ten slides were made for each different weight so that 140 slides were used. Slides were also made with 8 different amounts of pure ground mushrooms ranging from 1 – 40 mg and 10 replications were made so that 80 slides were used. We then counted the number of plant fragments (125 ×) (regardless of whether they were discernable fragments) (fig. 13) and the number of spores (500 ×) in each microscope field (fig. 14). Twenty fields were examined for each slide, averages were calculated and grand averages were calculated among the slides for each different weight of material.

Five different test mixtures were made by mixing different proportions of ground mushroom (5 – 30%) with different proportions of leaves from *Schizachyrium tenerum, Eragrostis spectabilis, Desmodium ciliare* and *Lonicera japonica*. Five microscope slides were made for each test mixture. Quantification was performed in two steps. First, the slides were examined separately at 125 × for plant leaf fragments and at 500 × for spores; 20 fields were examined in each case. All plant fragments or spores occurring in each field were counted and an average particle density

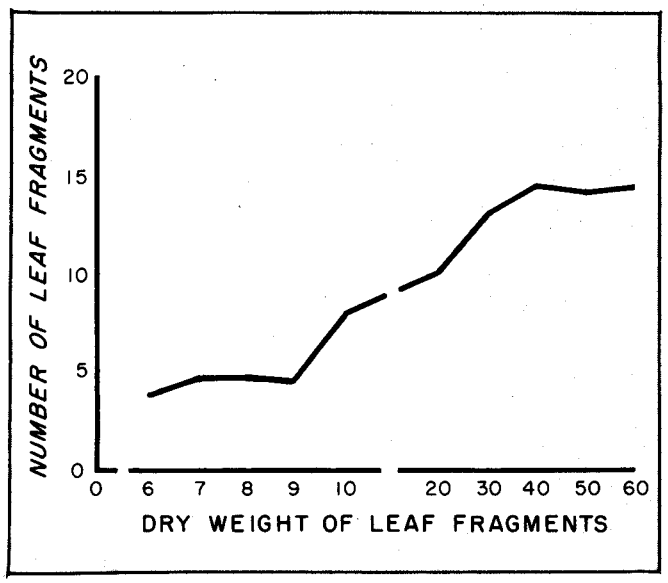

Figure 13.—*Relationship between number of leaf fragments per microscope field (125 ×) to dry weight ($g \times 10^{-3}$) of leaf fragments used to prepare slides having 22 × 40 mm cover slips.*

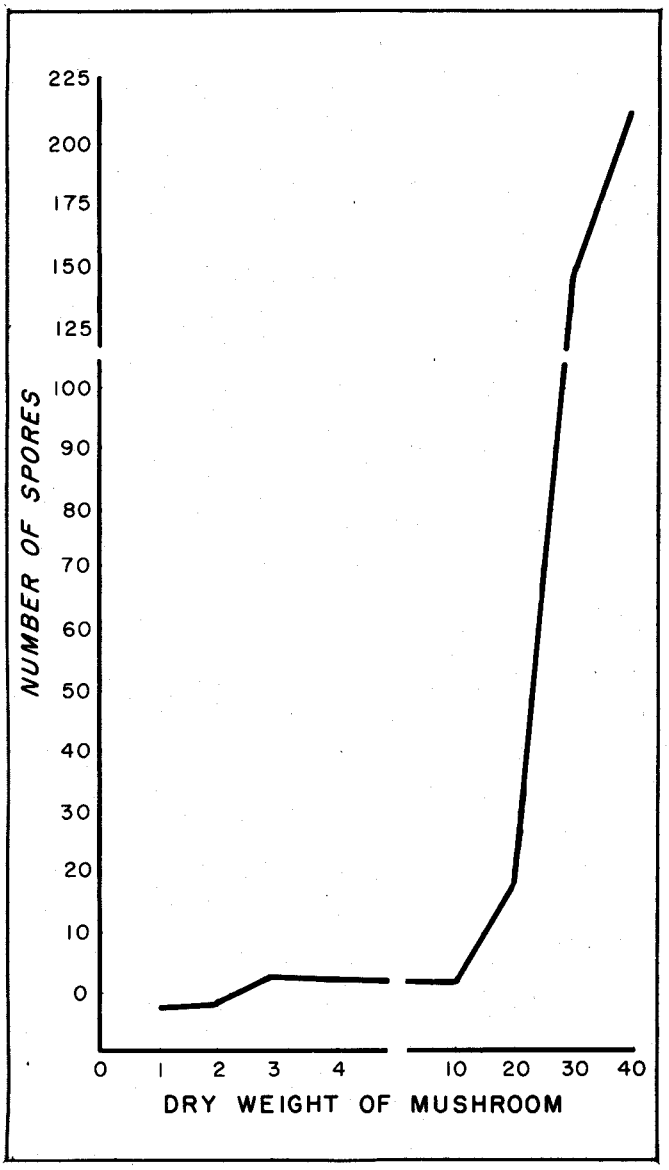

Figure 14.—*Relationship between number of* Amanita *sp. spores per microscope field (500 ×) to dry weight ($g \times 10^{-3}$) of dry mushroom used to prepare slides having 22 × 40 mm cover slips.*

Table 1.—*Comparison of known proportions (%) of plant leaves and mushroom in five test mixtures with estimates obtained from particle densities of leaf fragments and spores*

Species in test mixture	Test	Estimate	Test	Estimate	Test	Estimate	Test	Estimate	Test	Estimate
Schizachyrium tenerum	50	35	40	35	30	18	40	25	20	10
Eragrostis spectabilis	10	10	20	11	15	5	10	6	10	5
Desmodium ciliare	25	34	20	37	15	14	10	24	10	3
Lonicera japonica	10	10	10	4	20	49	10	17	10	4
Amanita sp.	5	11	10	13	20	14	30	28	50	78

was calculated by dividing the total number of plant leaf fragments or spores by the number of fields examined. The dry weight represented by each particle density was estimated by using figure 13 for plant leaves and figure 14 for mushrooms. Relative dry weights of mushroom and plant leaves in the mixtures were estimated by calculating the relative contribution of each from estimates of their dry weights on slides.

The second step in quantification was performed by calculating relative particle densities of leaves according to the procedures described earlier (Sparks and Malechek 1968), and multiplying by the proportion of leaf fragments in each mixture.

Using this procedure known and estimated proportions for mushrooms in the simulated diet were similar except when the proportions of mushrooms were 50% (table 1). At this level the number of spores per field became too numerous for accurate estimates. Based on earlier analyses the number of spores per field from diet sample slides should remain below 20. The accuracy of estimating plant leaf compositions using the same slides is not affected.

Data for leaf particle densities should apply to other situations while data for mushrooms applies only to the species we used. This procedure should be repeated for each mushroom species and each phenological stage.

Mast

Most wildlife husk nuts before eating them, and even though deer often swallow acorns whole, the shells may be regurgitated and spit out. In fragmented form, acorn hulls are difficult to identify and mast (endosperm) is amorphous. Since mast is highly digestible, it is rarely found in fecal material. We have observed mast in fecal pellets of deer and goats that feasted on abundant supplies. In these cases the bulk of material ingested was probably too much for complete digestion.

Regardless of these problems, there are ways to identify the tree that produced the mast found in stomach, rumen or fecal samples. First, all acorns have some trichomes on the hulls. These are brittle, become detached and remain in the digestive tract passing through to the feces. Microhistological examination and comparison with reference material allows identification of species when trichomes are not similar. Quantification can be performed by learning to estimate percentages of mast in mixtures from frequency counts of trichomes on slides (table 2).

Differences in shapes of starch cells in the mast from different acorns have been detected (Korschgen 1981). Reference slides can be made from scrapings of the endosperm and mast in stomachs can be identified from water mounts of the semi-digested starch. This method has not been widely investigated but may be useful for food habits work with a variety of granivores.

QUALITY CONTROL

A technician's behavior and performance can change from day to day because identification is basically a value judgment. This human source of error cannot be overcome through the employ of correction equations or equivalence factors regardless of how they are calculated.

Most plants can be identified through some of the fragments. However, we found it necessary to ignore the presence of any trichomes that were not attached to fragments to avoid over estimation of such species' contribution to diet samples.

Different strategies may be needed for each situation. For example, every fragment of a grass could be identified if it was mixed with dicots. Test mixtures of 2 to 6 different plants are adequate to periodically check a technicians accuracy. Test slides are also valuable for building technician confidence during training periods. There are so many ways to combine plants in mixtures that it is impractical to produce enough tests to simulate every possible combination of foods in actual diet samples. Tests sometimes have to be made after the plants in a mixture are identified, but before they are quantified. Sources of error and uniqueness of mixtures make microhistological techniques both an art and a science (Johnson and Pearson 1981), but a regimented sampling procedure is necessary to provide consistent results.

Table 2.—*Comparison of known and estimated proportions (%) of mast in five mixtures with plant leaf fragments. Mast was quantified by counting acorn trichomes*

Species in test mixture	Test	Estimate	Test	Estimate	Test	Estimate	Test	Estimate	Test	Estimate
Quercus rubra (acorns)	50	54	25	19	25	21	40	40	10	20
Quercus rubra (leaves)	....	....	....	....	25	22	....	....	....	....
Rubus sp.	....	....	....	....	25	29	....	....	40	39
Lonicera japonica	....	....	....	....	....	....	25	27	10	17
Gelsemium sempervirens	....	....	....	....	25	28	15	16	40	24
Smilax rotundifolia	....	....	25	34	....	....	20	17	....	....
Toxicodendron radicans	50	46	50	47	....	....	....	....	....	....

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Appendix I – Directions for Preparation of Mounting Media

Directions for Preparing Hoyer's Solution

Chloral hydrate crystals	200 g
Glycerine	20 ml
Gum arabic (photopurified)	30 g
Tap water	50 ml

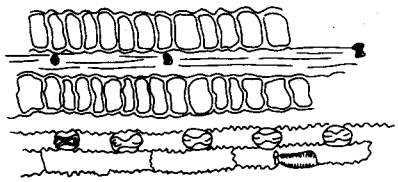
Combine chloral hydrate and glycerine. Add gum arabic and water. Place container in a hot water bath and stir until ingredients are combined.

Directions for Preparing Plastic Mounting Medium

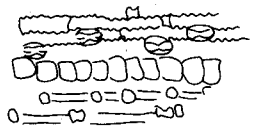
Phenol crystals	400 g
Lactic acid	440 ml
Polyvinyl alcohol beads	150 g
Distilled water	1120 ml

Add phenol crystals to lactic acid and stir until dissolved. Work should be performed in a ventilated hood. Mix polyvinyl alcohol beads with water in a hot water bath. Combine the two solutions and stir while keeping the mixture warm in a hot water bath.

Appendix II – Drawings of Plant Fragments Under Phase-Contrast Microscopy

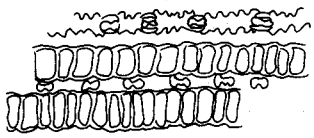


a



b

Purple lovegrass (*Eragrostis spectabilis*) (a) and tridens (*Tridens* spp.) (b)—Stomata are small and have rounded domes. Fragments may have prickles but are without other types of trichomes.



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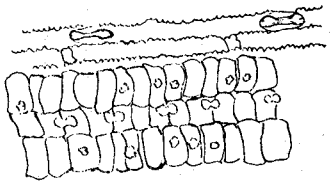
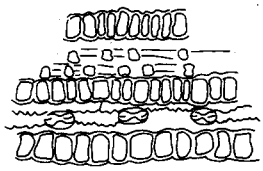
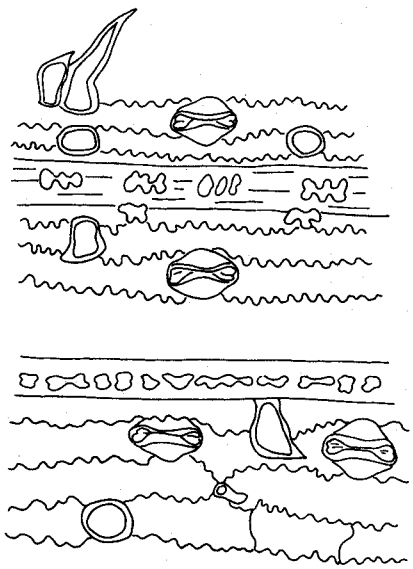


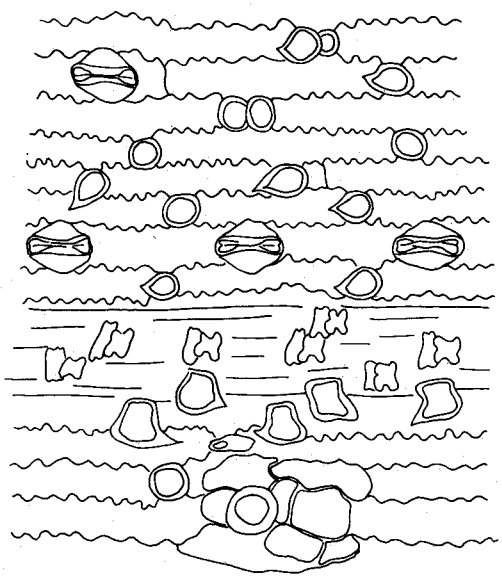
b

Bearded skeletongrass (*Gymnopogon ambiguus*) (a) and switchgrass (*Panicum virgatum*) (b)—Silica bodies are dumbbell-shaped but compared to bearded skeletongrass, switchgrass stomata are larger and adjoining cell walls are tightly sinuous or scalloped.

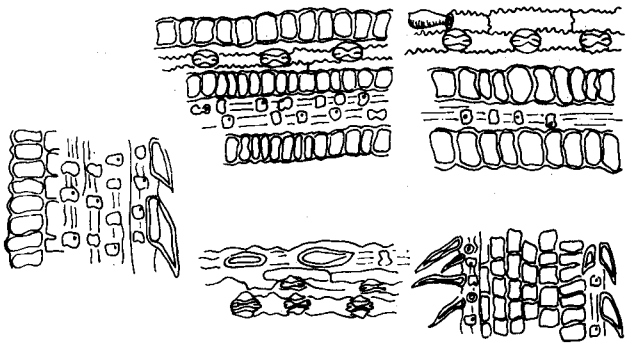


Sedges (*Carex* spp.)—Shiny, papillae are common. Cells are square or rectangular with wavy walls.

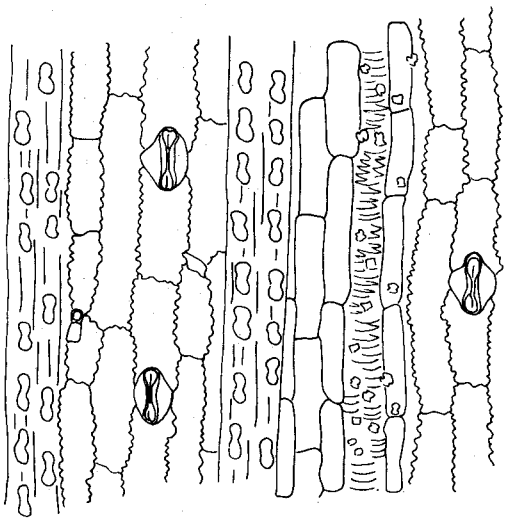




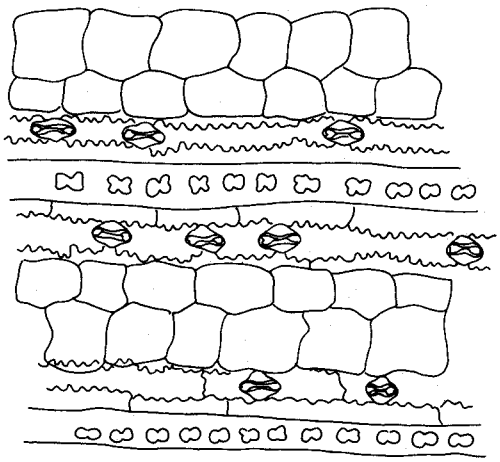
Paspalums (*Paspalum* spp.)—Silica bodies resemble butterflies. Some have cuboid companion cells.

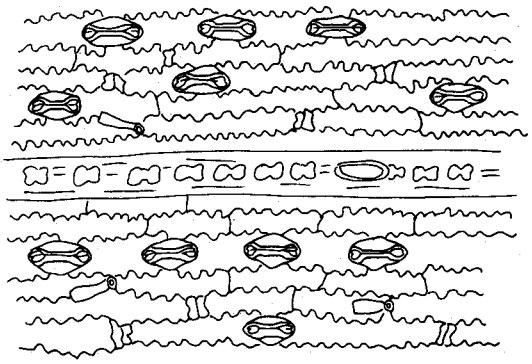


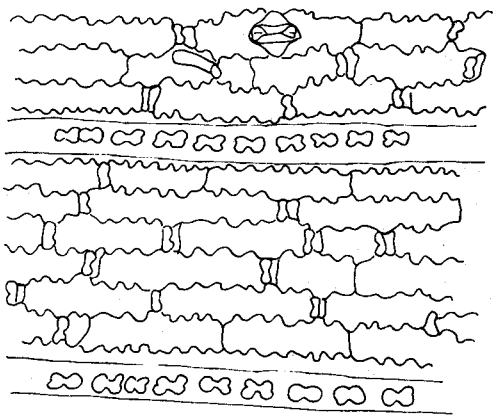
Cutover muhly (*Muhlenbergia expansa*)—Stomata are small with peaked domes. Fragments may have many short trichomes. Costal girders are square.

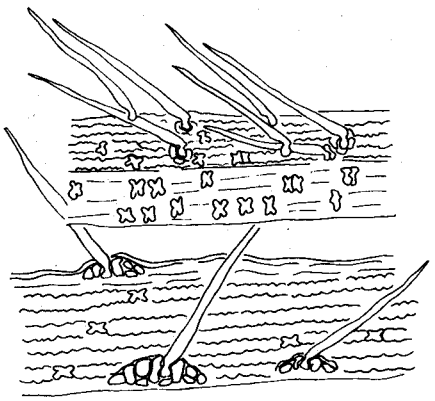


Green silkyscale (*Anthaenantia villosa*)—Stomates are mostly rounded but some are slightly peaked. Most silica bodies look like peanuts.

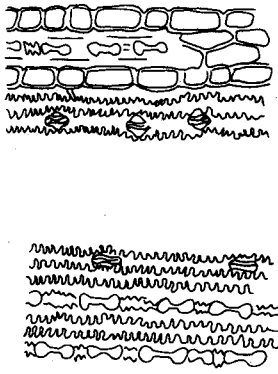




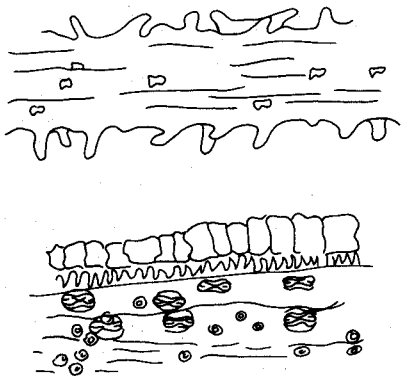




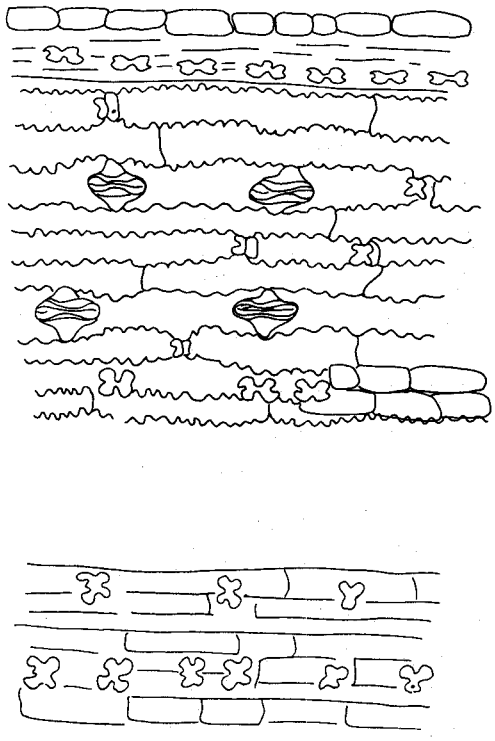
Panic grasses (*Panicum* spp.)—Relatively long bone-shaped silica bodies resemble 8's.



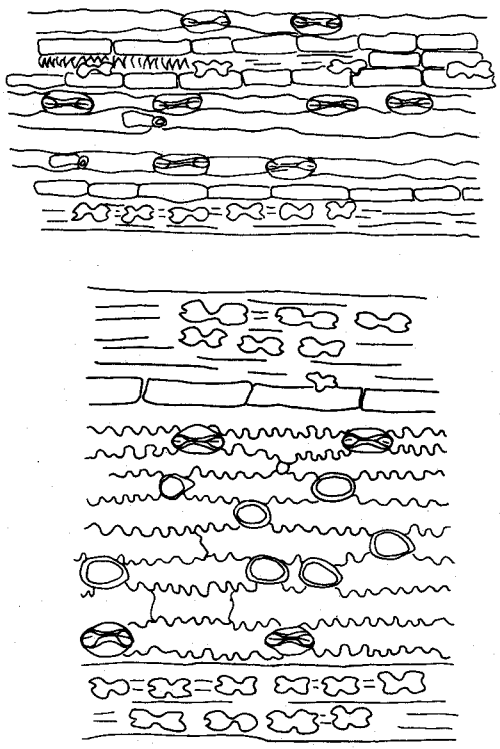
Threeawns (*Aristida* spp.)—Silica bodies are dumbbell-shaped with rounded ends. Stomata have peaked domes and look nearly triangular. Cell walls in the intercostal area are deeply sinuous.



Pineywoods dropseed (*Sporobolus junceus*)—Papillate protrusions of epidermal cells are similar to those of sedges. Vein silica bodies and companion cells form couples. Stomates are small and round.

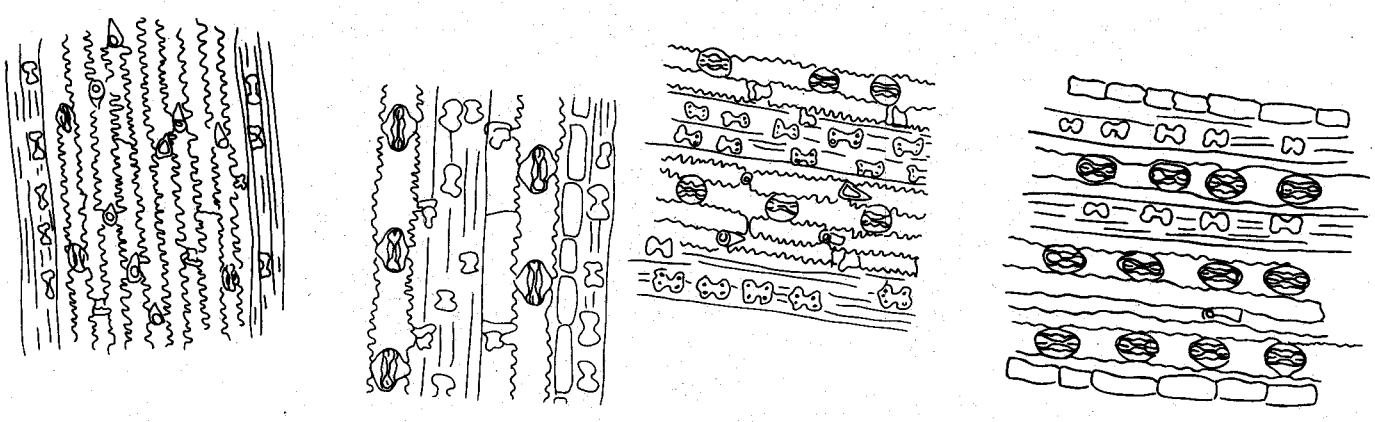


a

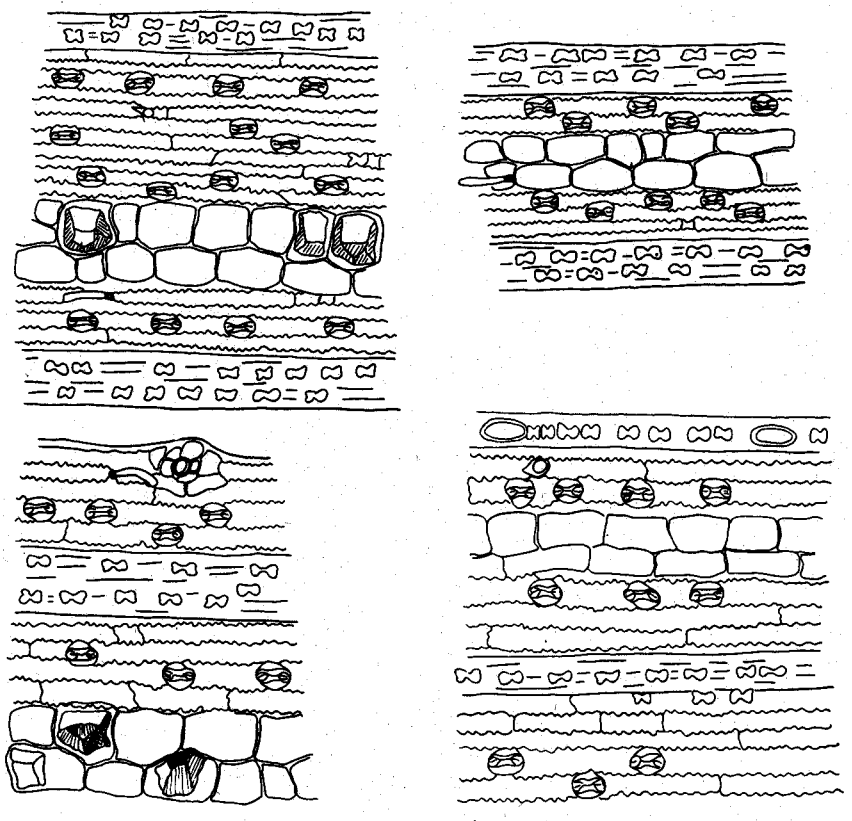


b

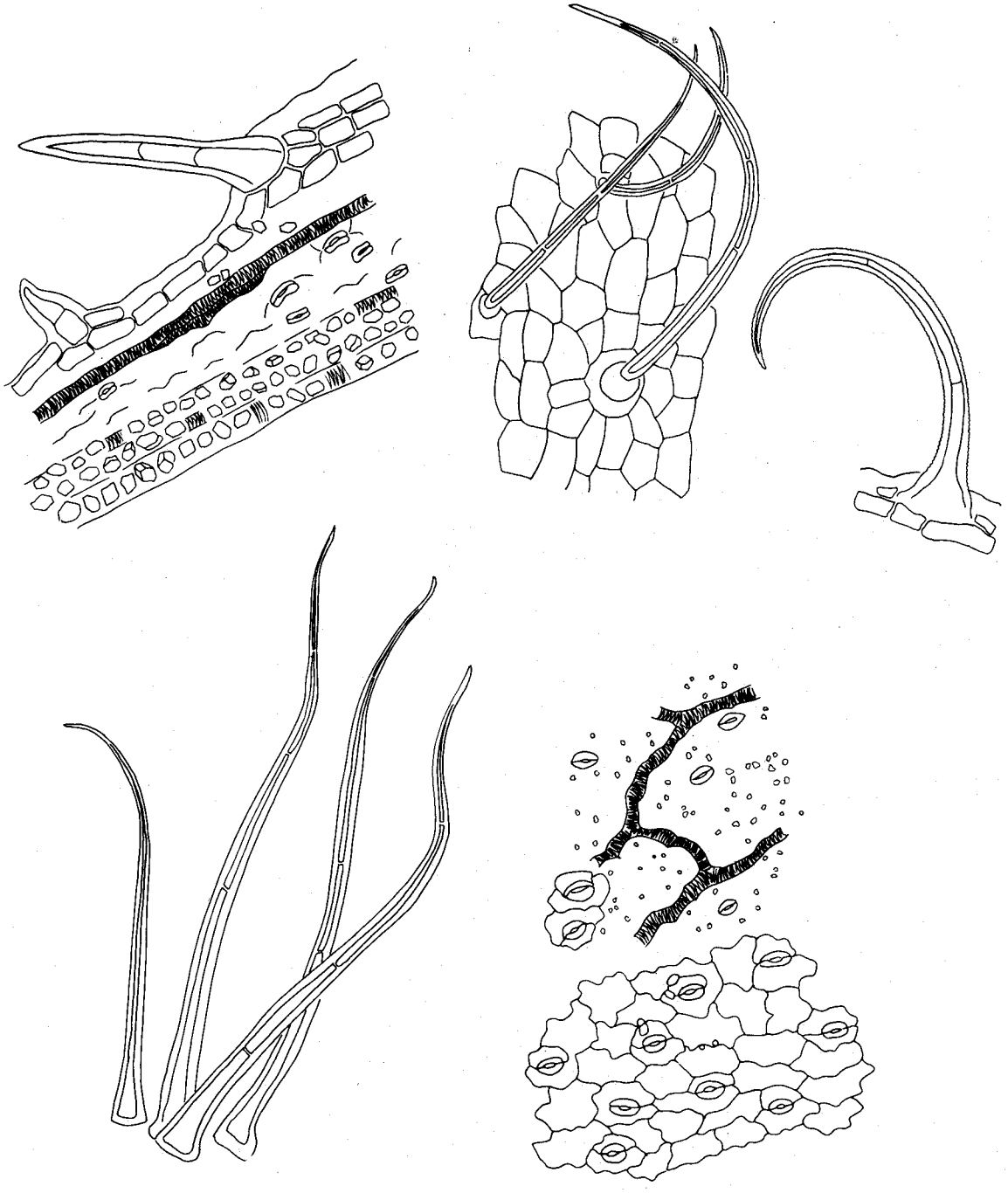
Common carpetgrass (*Axonopus affinis*) (a) and yellow indiangrass (b) (*Sorghastrum nutans*)—Dumbbell-shaped silica bodies are deeply notched. Carpetgrass stomates are triangular while those of indiangrass are oval-shaped.



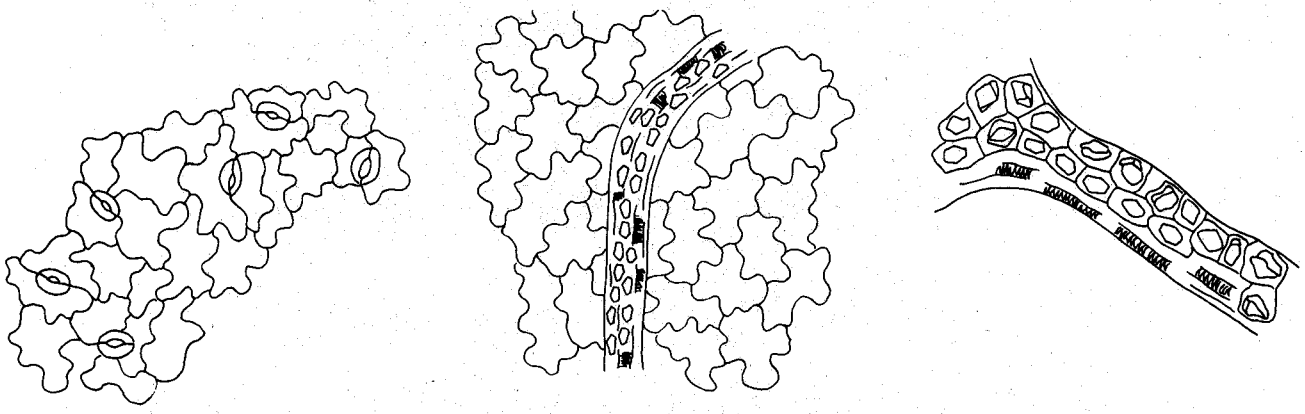
Bluestems (*Andropogon* spp., *Schizachyrium* spp.)—Stomata are triangular. Silica bodies are dumbbell-shaped with notched ends.



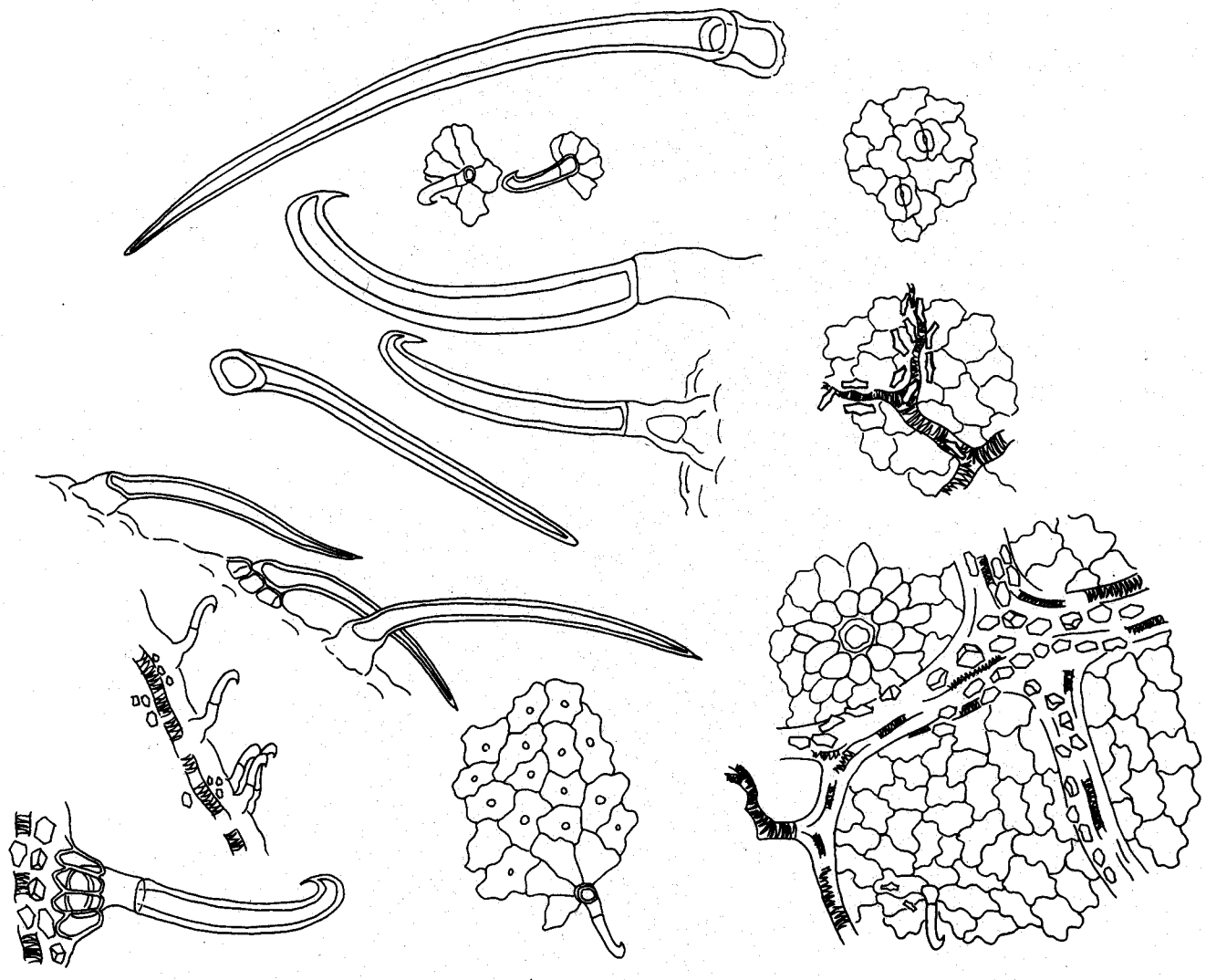
Spike uniola (*Chasmanthium laxum*)—Bulliform cells may contain crystals. Silica bodies are short, small dumbbells that may look like bow ties.



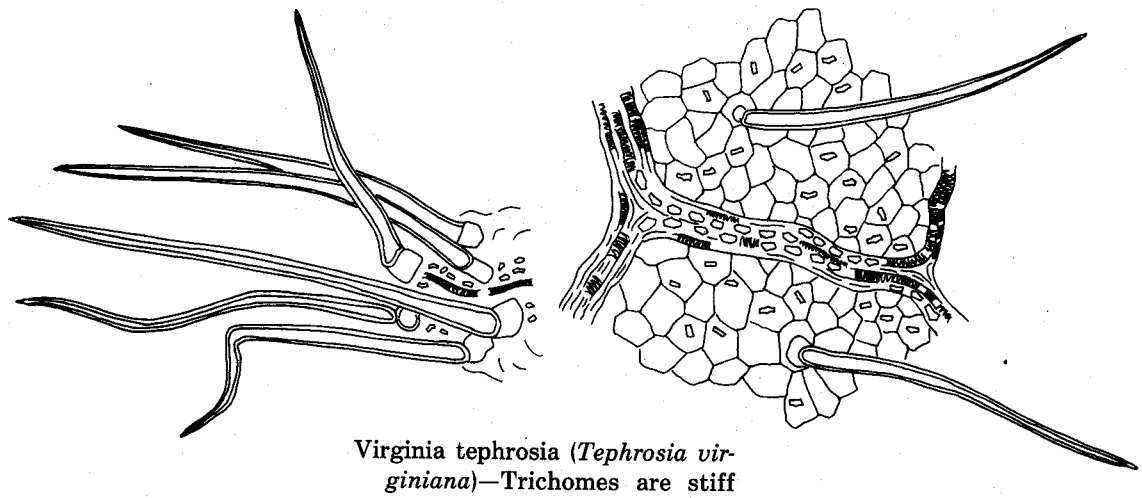
Partridgepea (*Cassia fasciculata*) —Unicellular strap-shaped trichomes are internally segmented. Square crystals are most abundant over veins.



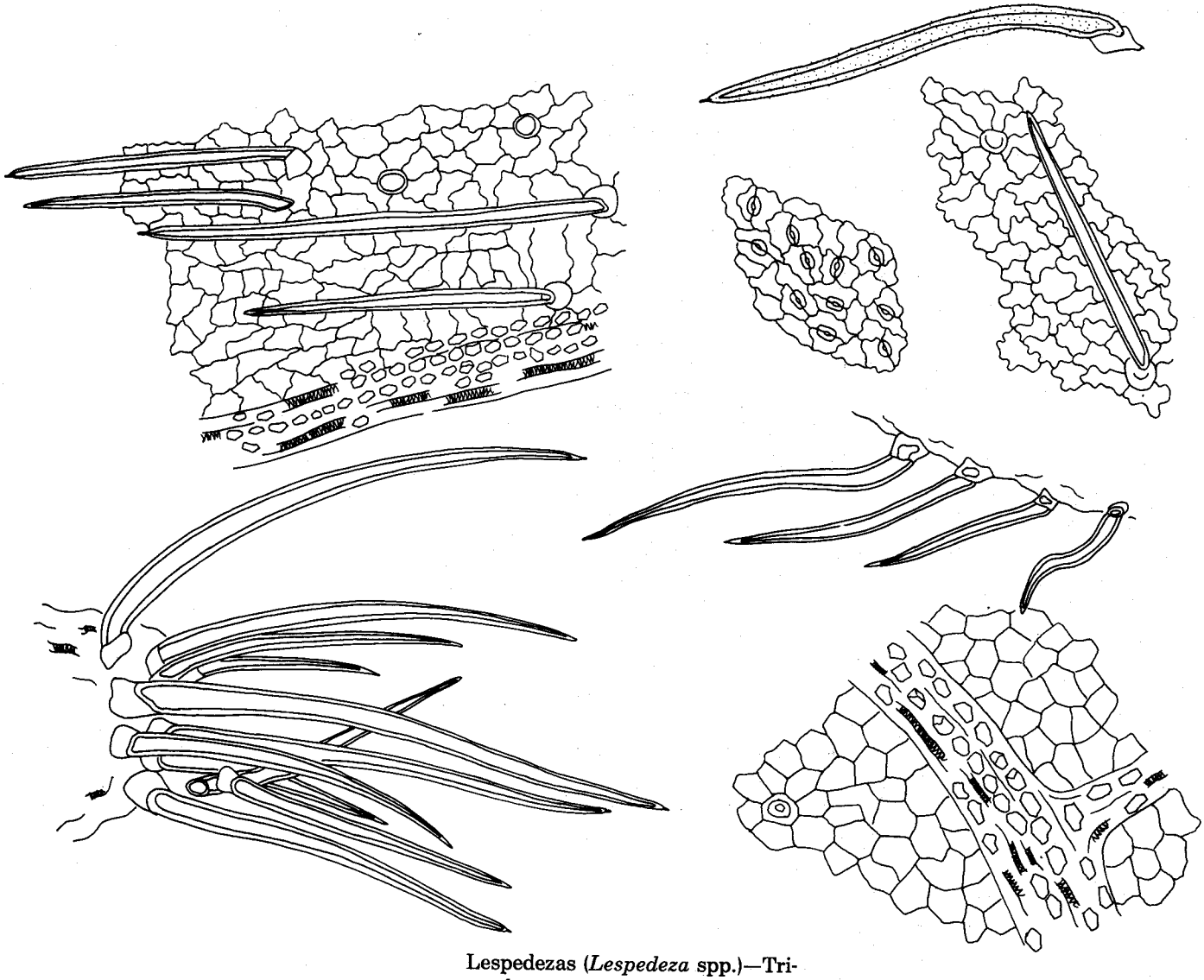
Pigeonwings (*Clitoria mariana*)—Fragments are without trichomes and numerous square crystals cover the veins.



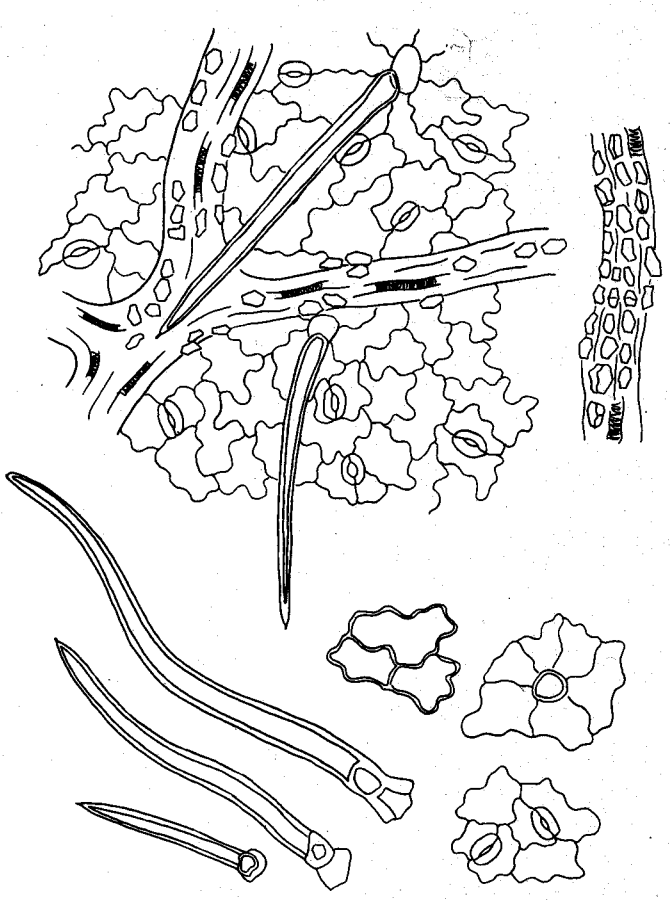
Littleleaf tickclover (*Desmodium ciliare*)—Three types of trichomes can be observed; one has a mucronate tip, two have hooked tips. All trichome bases are separate, single cells. Square crystals cover the veins.



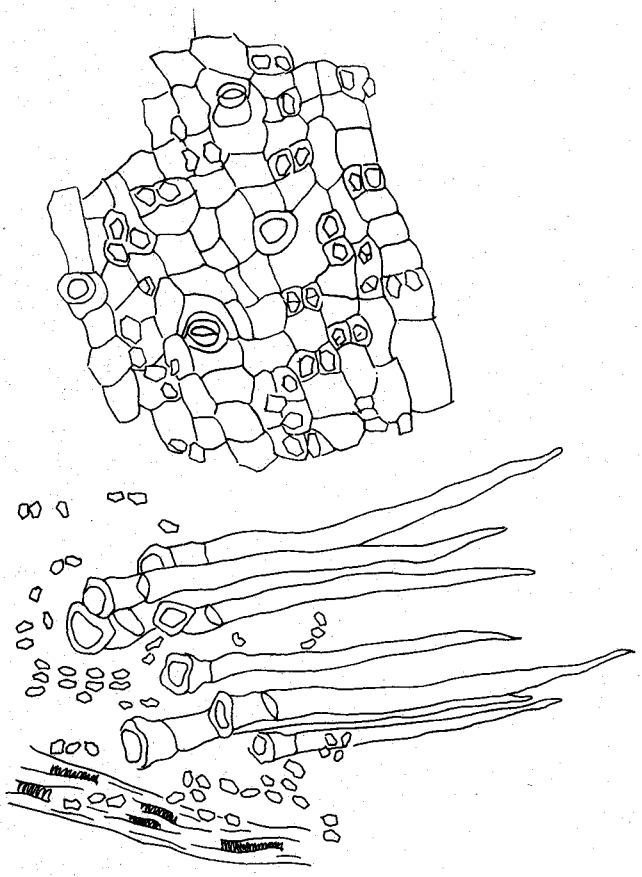
Virginia tephrosia (*Tephrosia virginiana*)—Trichomes are stiff and tapered with mucronate tips, and have a 1-celled, round base. Square and rectangular crystals are abundant.



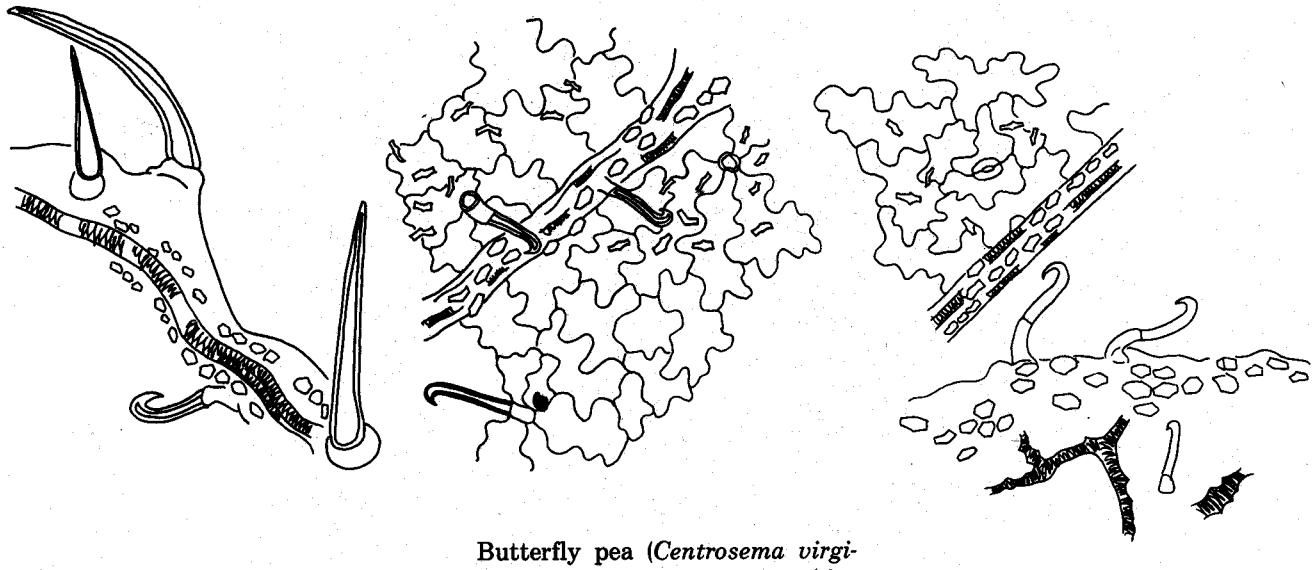
Lespedezas (*Lespedeza* spp.)—Trichomes are flexible with mucronate tips. Surfaces of trichomes are often ciliate in *L. virginica*; sometimes ciliate in *L. hirta*.



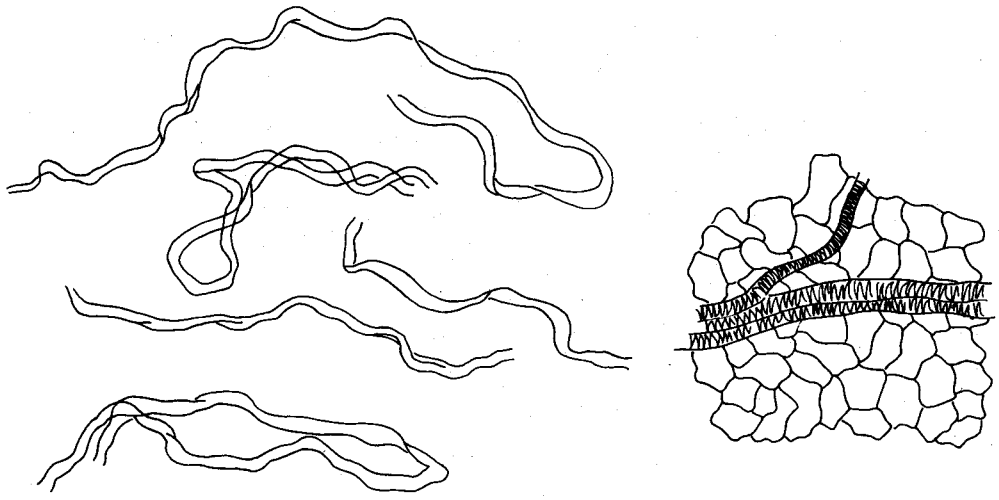
Downy milkpea (*Galactia volubilis*)—Trichomes are stiff with a mucronate tip and 1-celled base. Intercostal cells are puzzle-like. Square crystals are mostly over the veins.



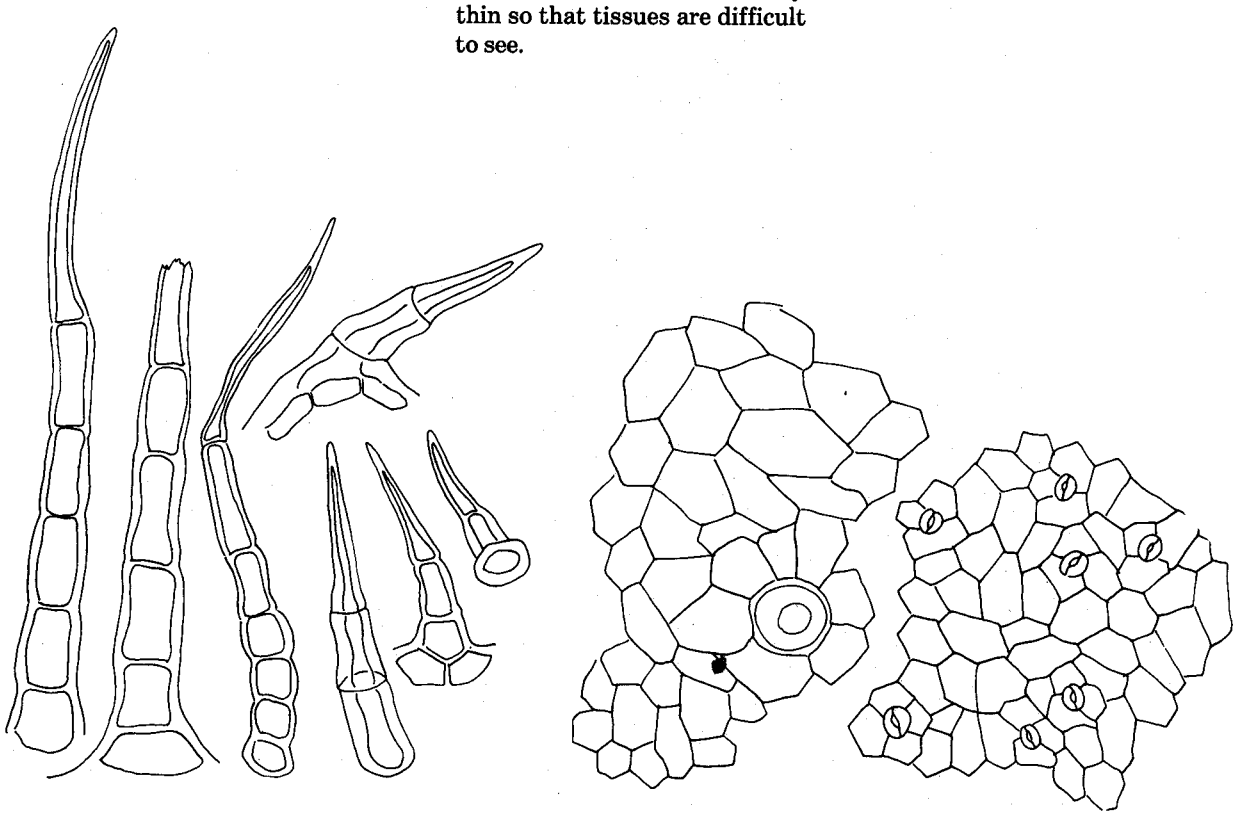
Pencilflower (*Stylosanthes biflora*)—Trichomes appear wilted and have a 1-celled base. Square crystals are very abundant.



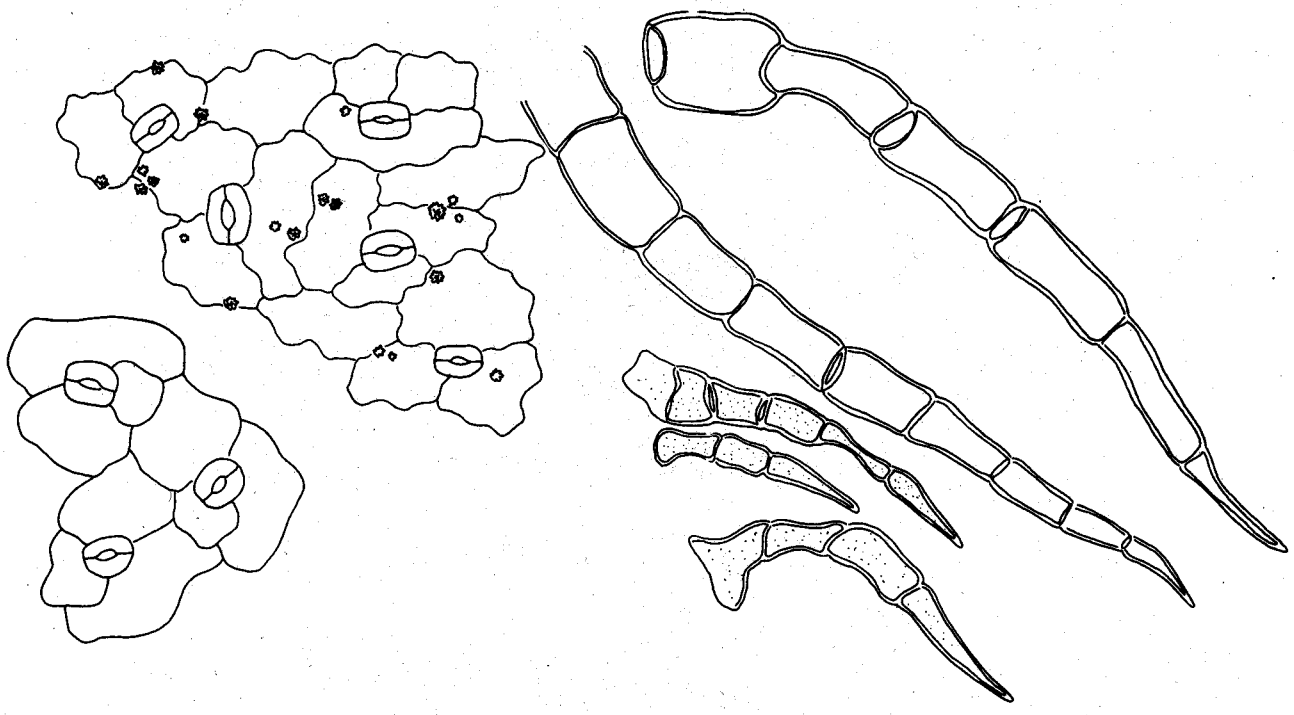
Butterfly pea (*Centrosema virginianum*)—This plant resembles *Desmodium* and *Galactia*. Two types of trichomes are present; one is large with mucronate tips, the other is smaller with hooked tips. Square and rectangular crystals are present.



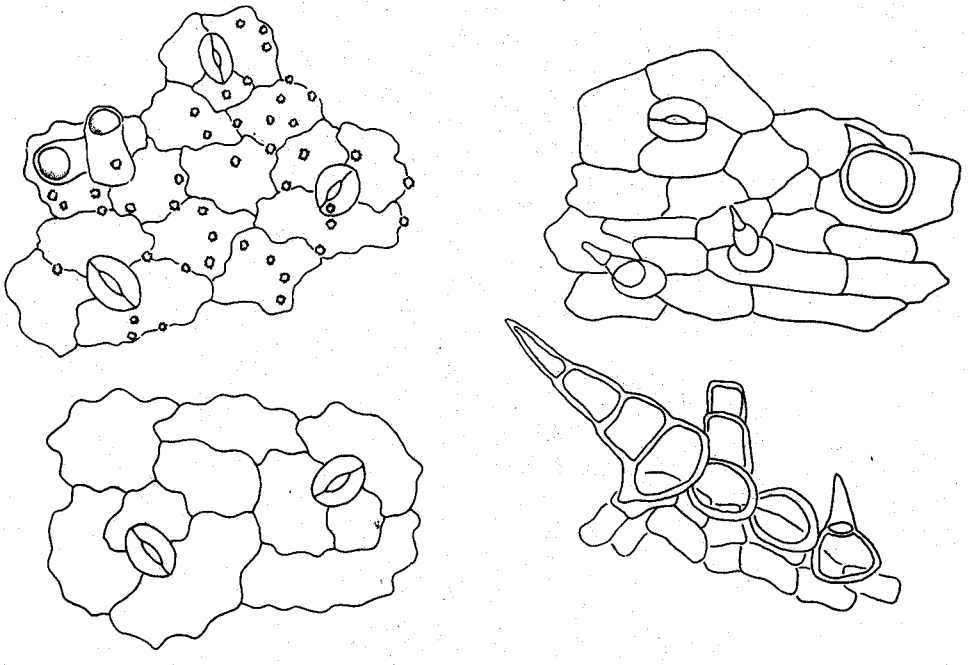
Hymenopapus (*Hymenopappus artemisiifolius*)—Flattened, ribbon-like trichomes look like spaghetti. No crystals appear in tissue. Cells walls are very thin so that tissues are difficult to see.



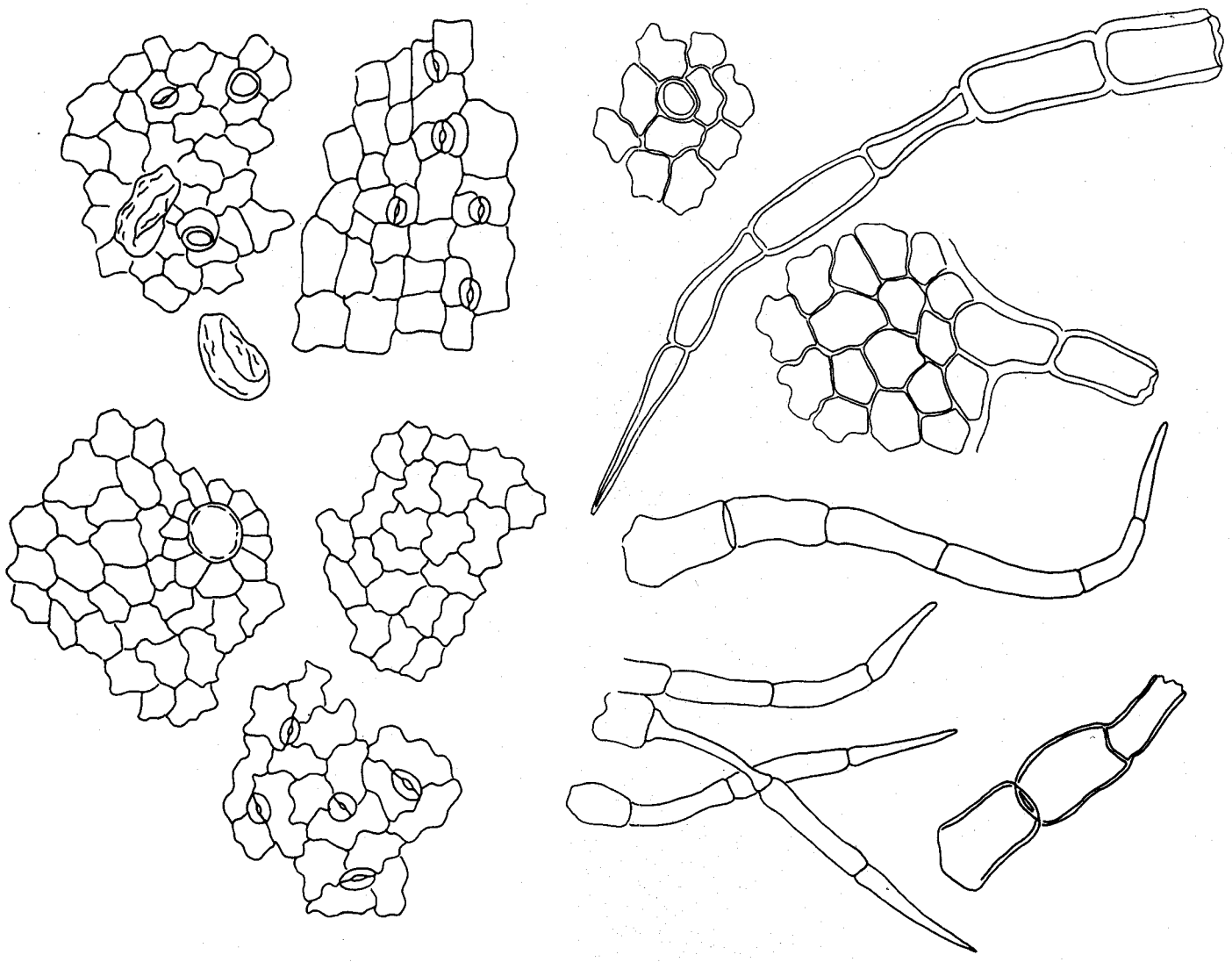
Goldenrod (*Solidago rugosa*)—Ligulate, segmented trichomes have thick walls. Flared bases are usually broken off. Intercostal cells are angular.



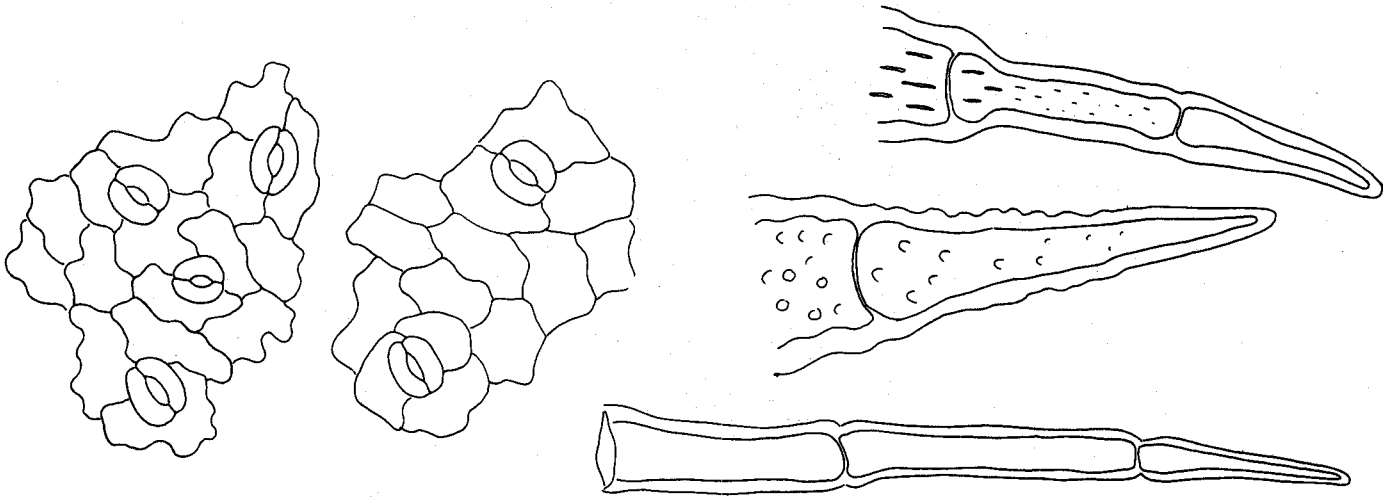
Thickleaf coreopsis (*Coreopsis lanceolata*)—Segmented trichomes are ligulate. Small druses have slightly lobed cells.



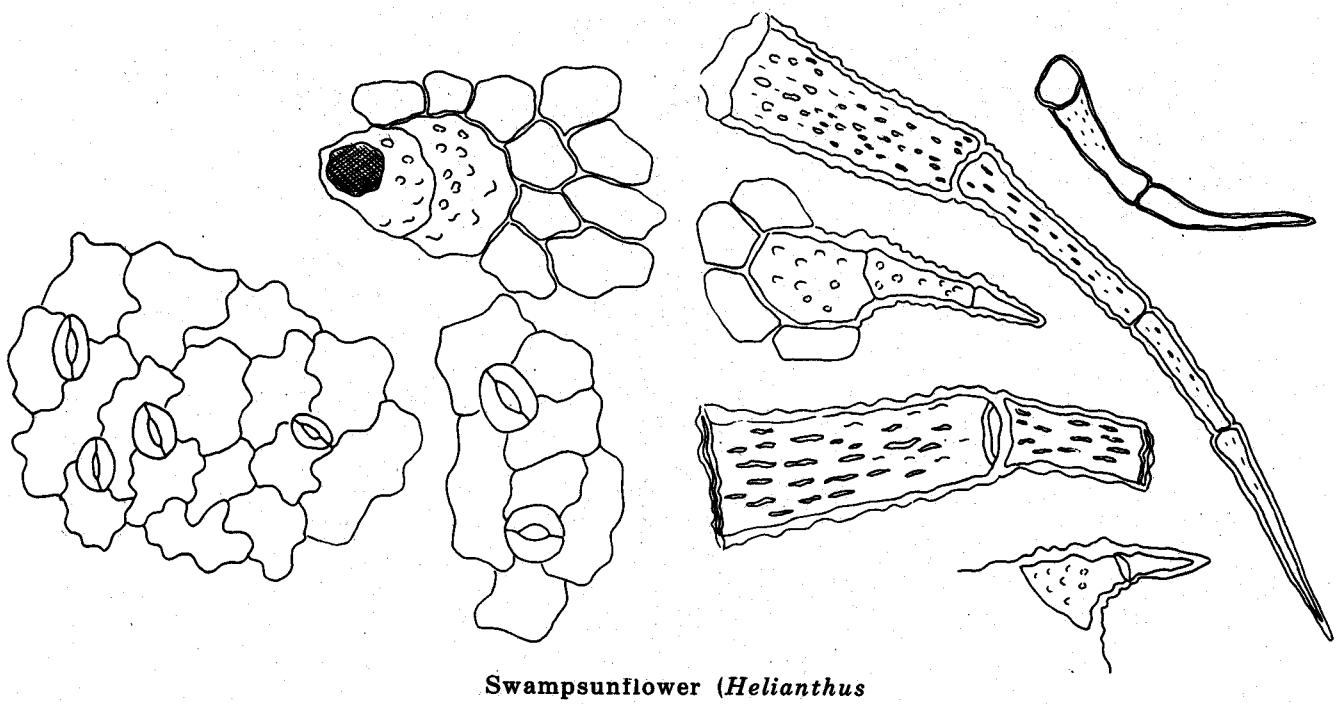
Grassleaf goldaster (*Heterotheca graminifolia*)—Segmented trichomes are ligulate. Very small druses occur intercostally with slightly lobed cells.



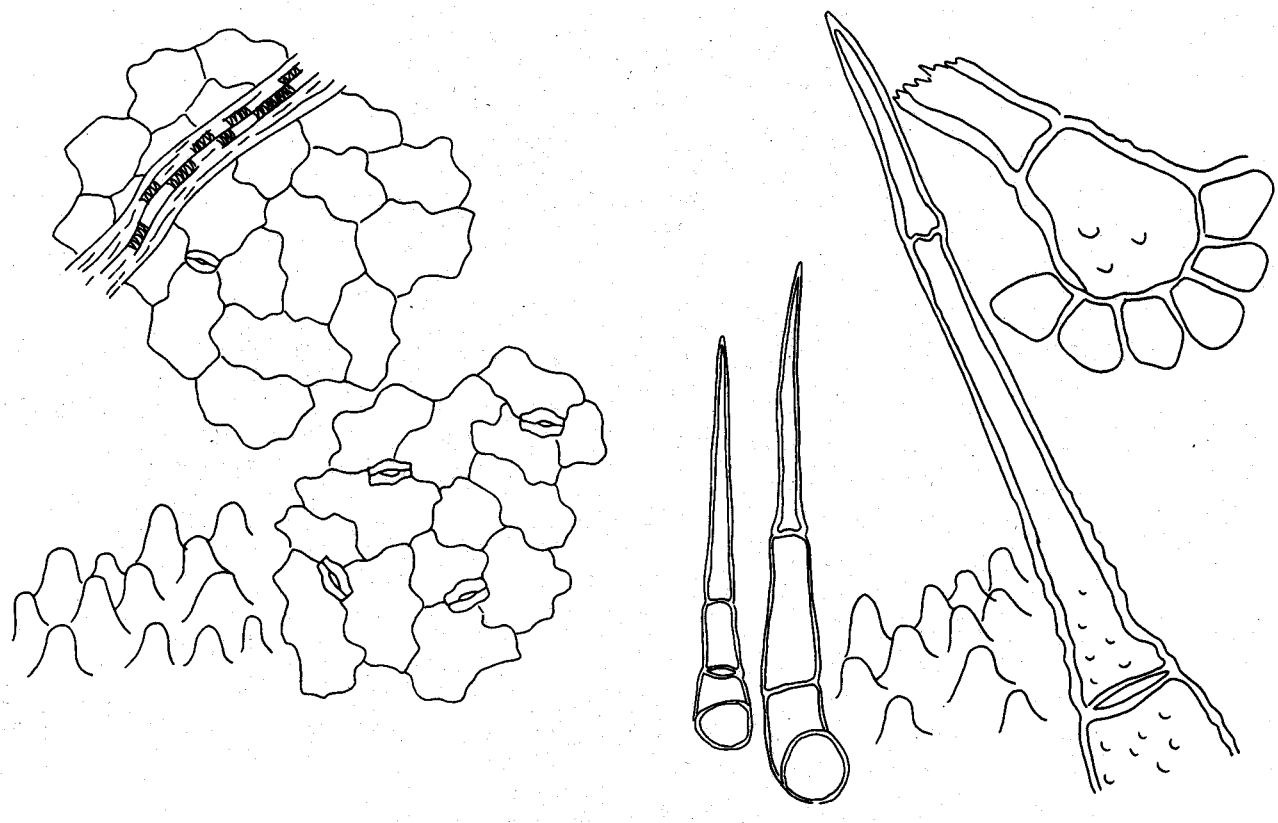
Eupatoriums (*Eupatorium* spp.)—Ligulate, segmented trichomes and silica flowers are present. Intercostal cells are angular with wavy walls.



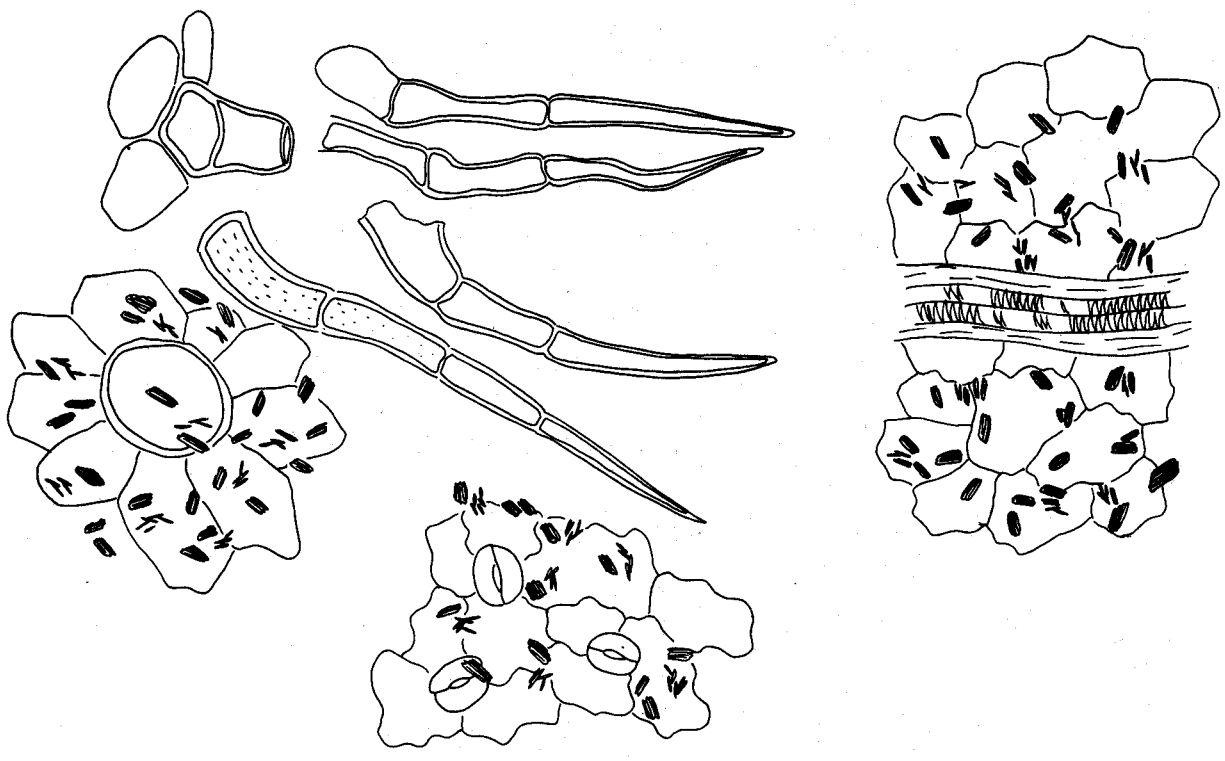
Slender rosinweed (*Silphium gracile*)—Trichomes and tissues area similar to those of swamp sunflower.



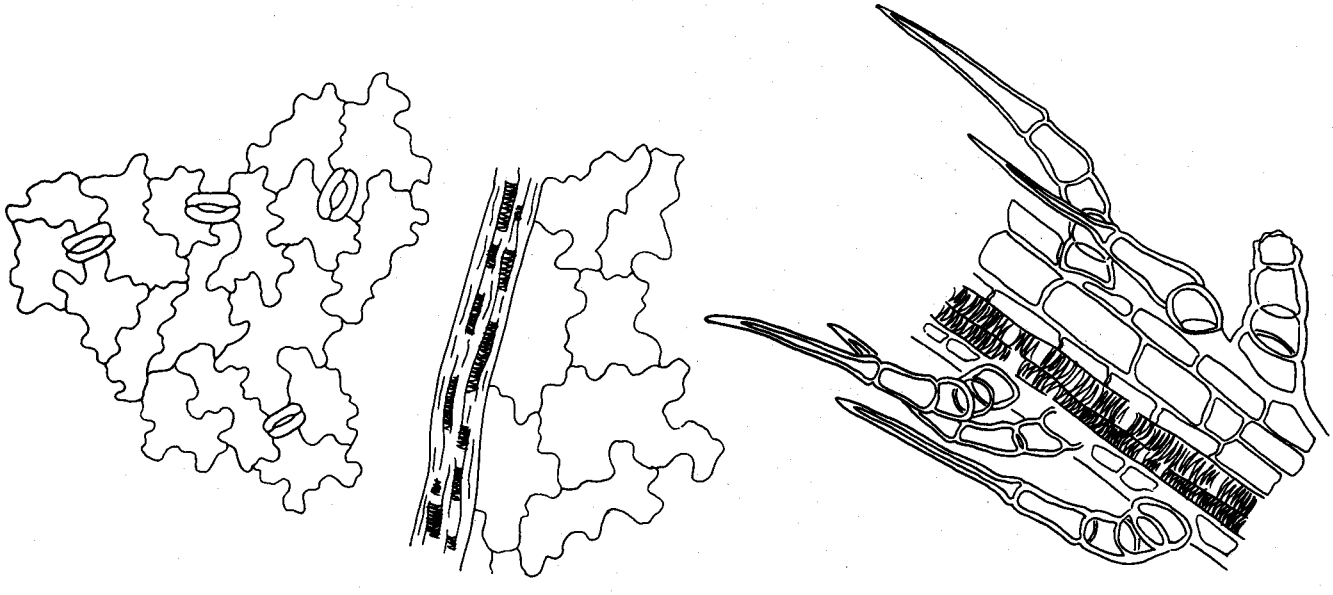
Swampsunflower (*Helianthus angustifolius*)—Large, segmented trichomes have thick bumpy surfaces. Distinct attachment cells in tissue are made up of large thin-walled cells and druses are present.



Blackeyed susan (*Rudbeckia hirta*) —Large, ligulate, segmented trichomes are present.

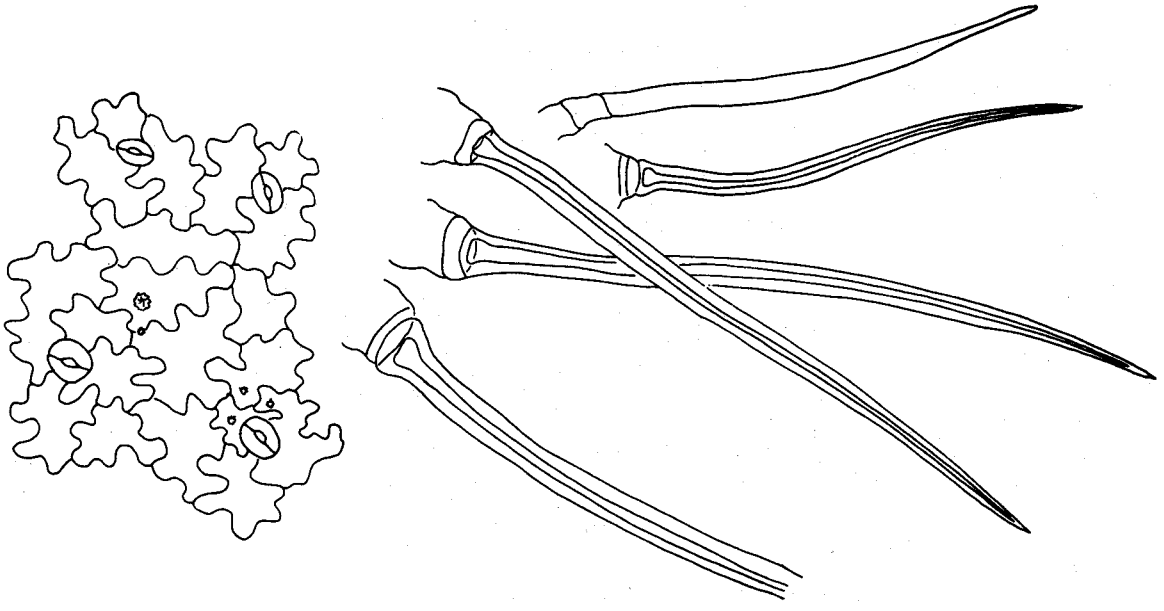


Plantainleaf coneflower (*Rudbeckia grandiflora*)—Medium, ligulate, segmented trichomes are present. Small raphides are very abundant.

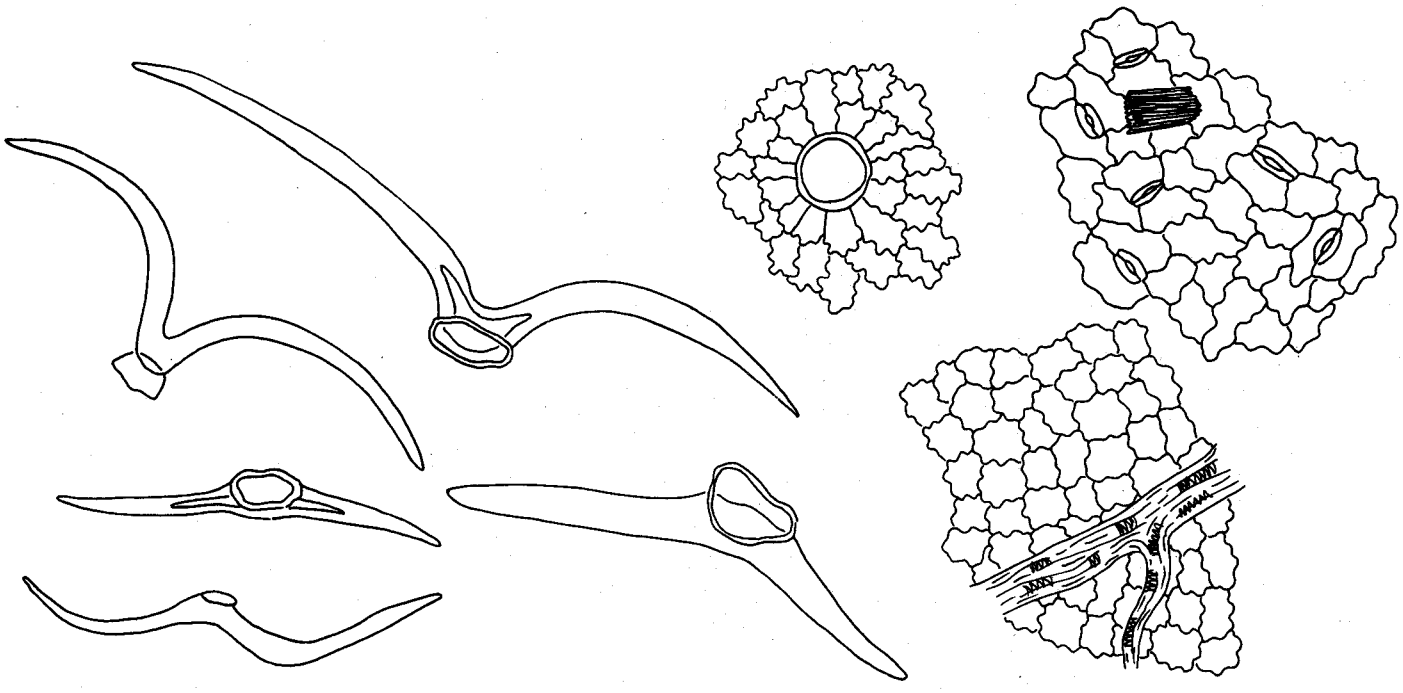


Daisy fleabane (*Erigeron strigosus*)—Segmented trichomes appear jointed. Leaf cells are usually puzzle-like while stem cells are rectangular and parallel.

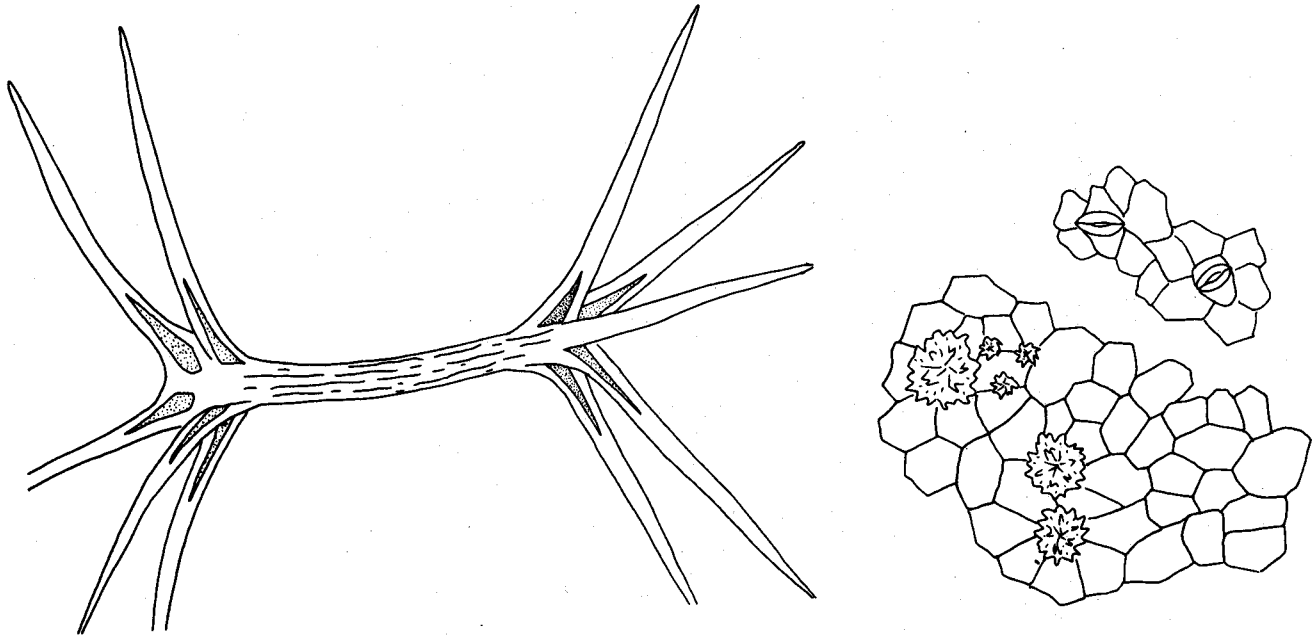
Savoryleaf aster (*Aster linariifolius*)—Short, fat segmented trichomes, and some nipple-like pappillae are present. Intercostal cells are angular and occur in layers so that cell walls overlap each other.



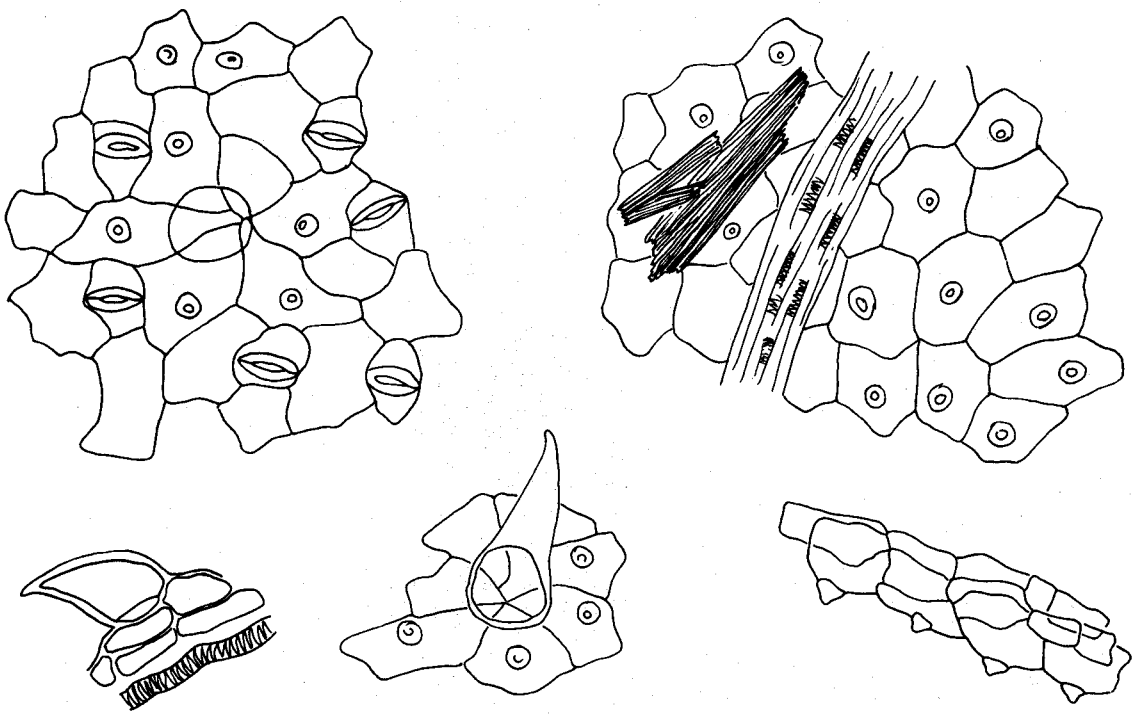
Elephantsfoot (*Elephantopus tomentosus*)—Long, tapered, thin trichomes have 2-celled bases. Puzzle-like intercostal cells contain small druses.



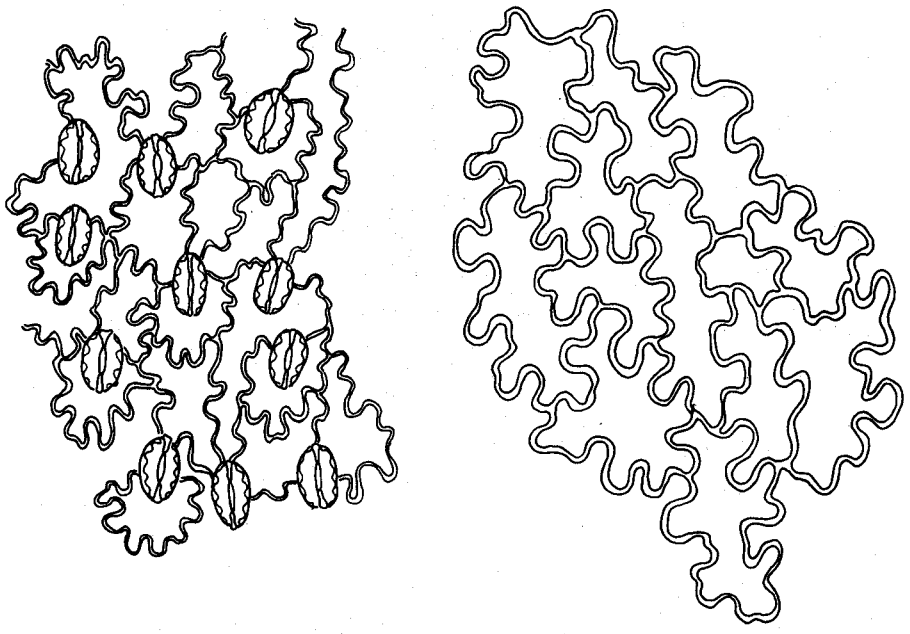
Flowering spurge (*Euphoria corollata*)—There are two types of trichomes; one is branched and similar to dogwood trichomes. Trichome attachments are surrounded by many cells.



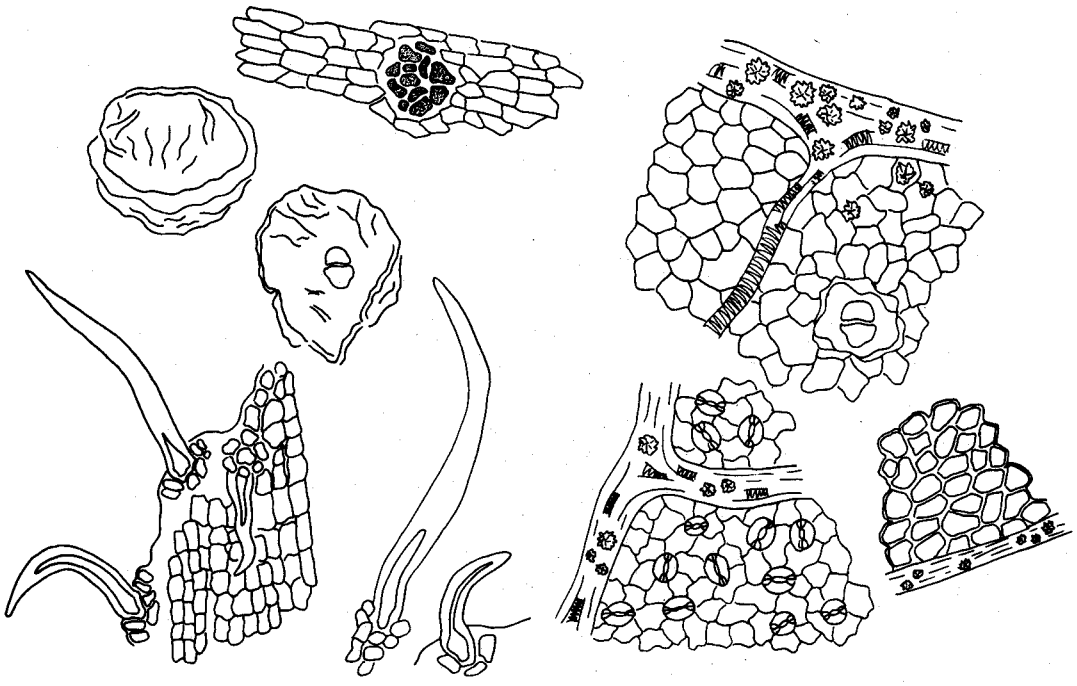
Croton (*Croton* spp.)—Compound, stellate trichomes are often attached by a common stalk. Angular cells have very thin walls so that tissues are hard to see.



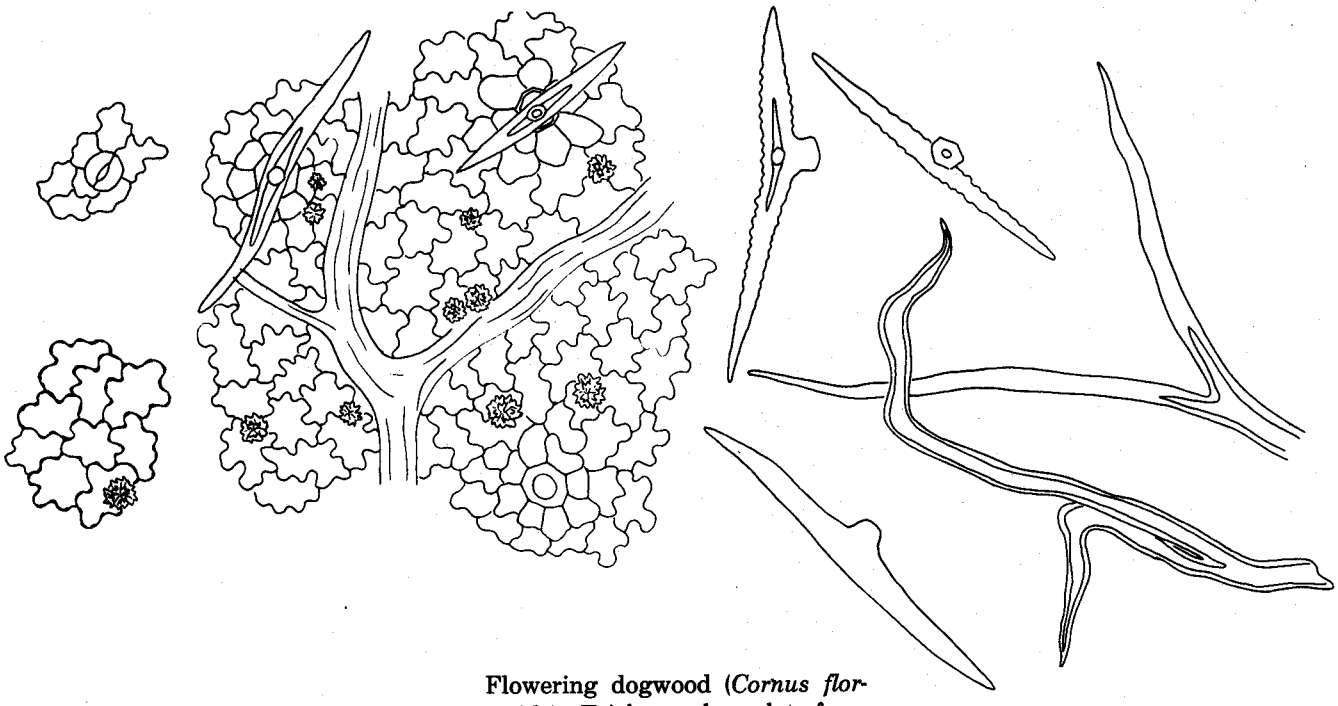
Poor-joe (*Diodia teres*)—Few trichomes occur on leaf margins. Each intercostal cell contains a nipple-like papilla. Raphides are present.



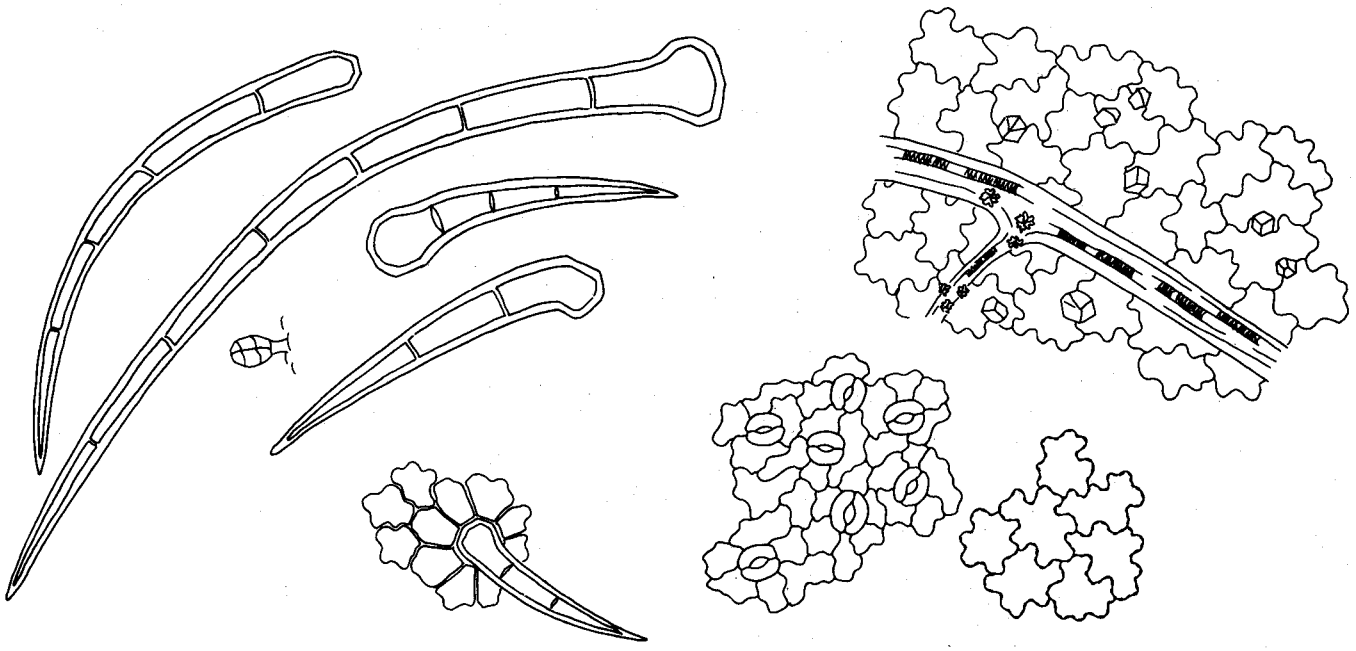
Bracken fern (*Pteridium aquilinum*)—This distinctive tissue is made of deeply lobed cells. Stomata corners have triangular cells.



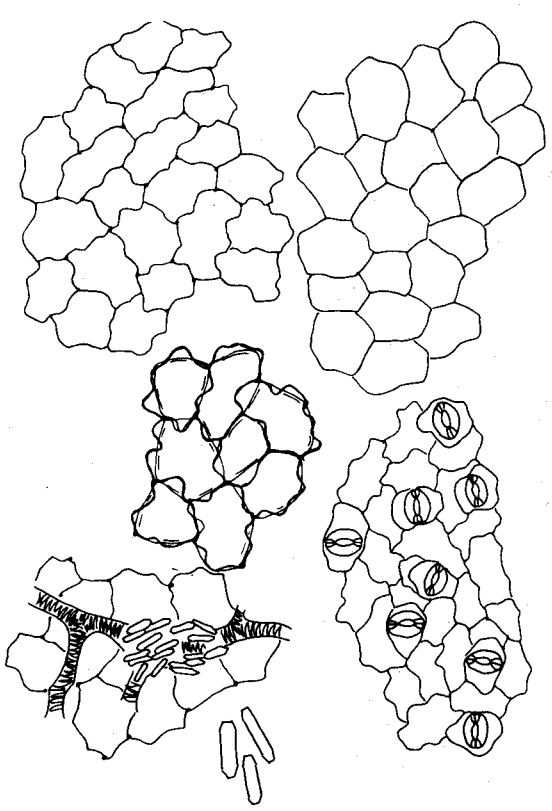
Southern waxmyrtle (*Myrica cerifera*)—Trichomes are not abundant and usually occur only on leaf margins. Druses are common.



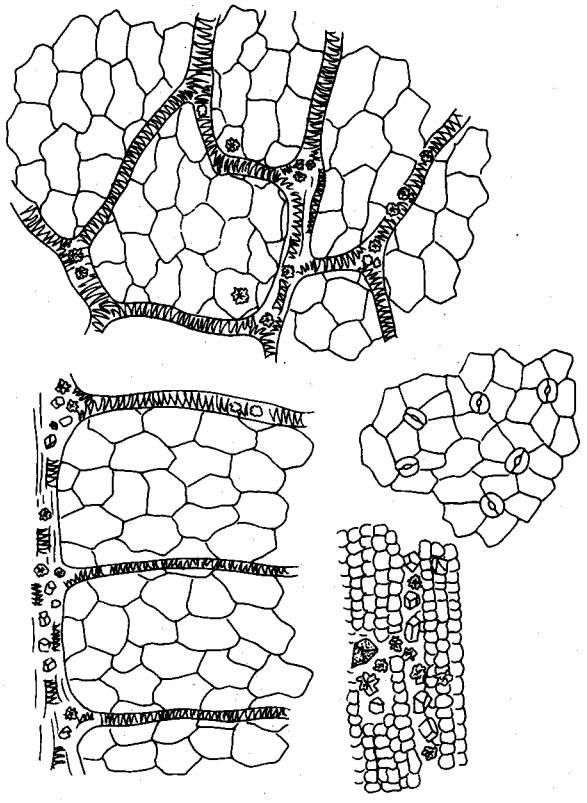
Flowering dogwood (*Cornus florida*)—Trichomes branch to form a 't' and attachment cells are mostly 8- sided. Druses are common.



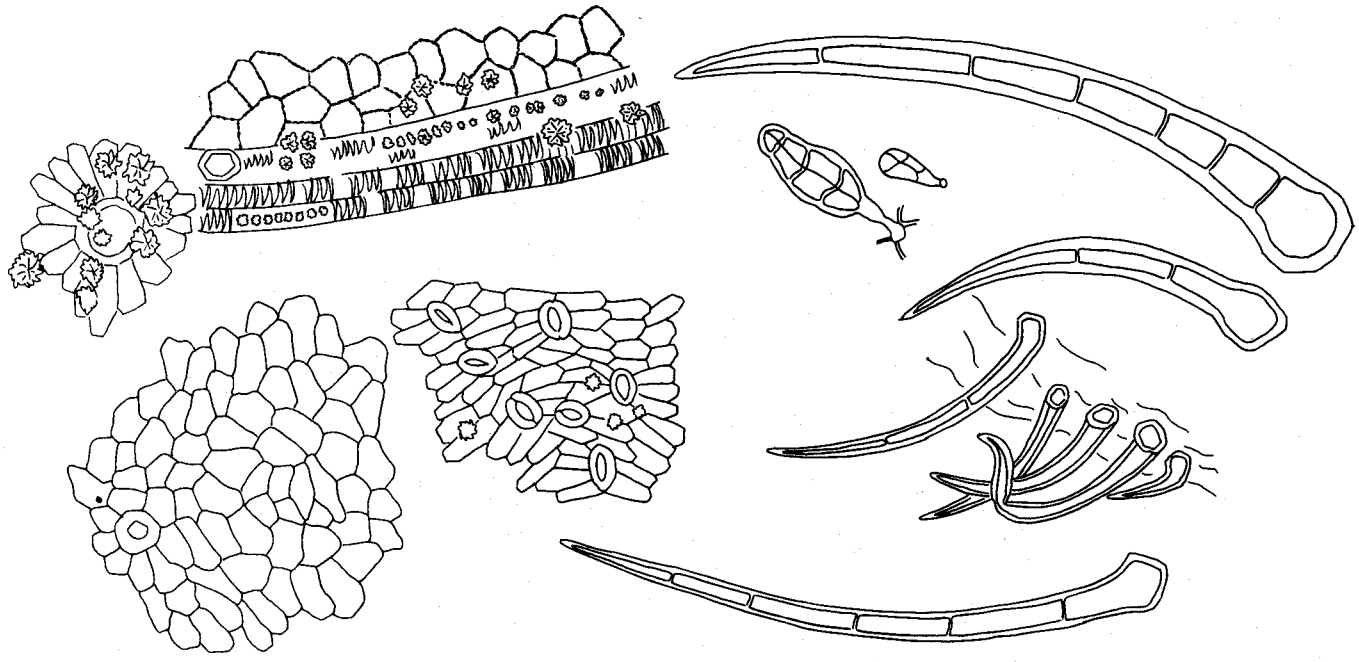
Poison ivy (*Toxicodendron radicans*)—Ligulate and glandular trichome bases are usually 6-sided. Druses occur over veins and square crystals occur between the veins.



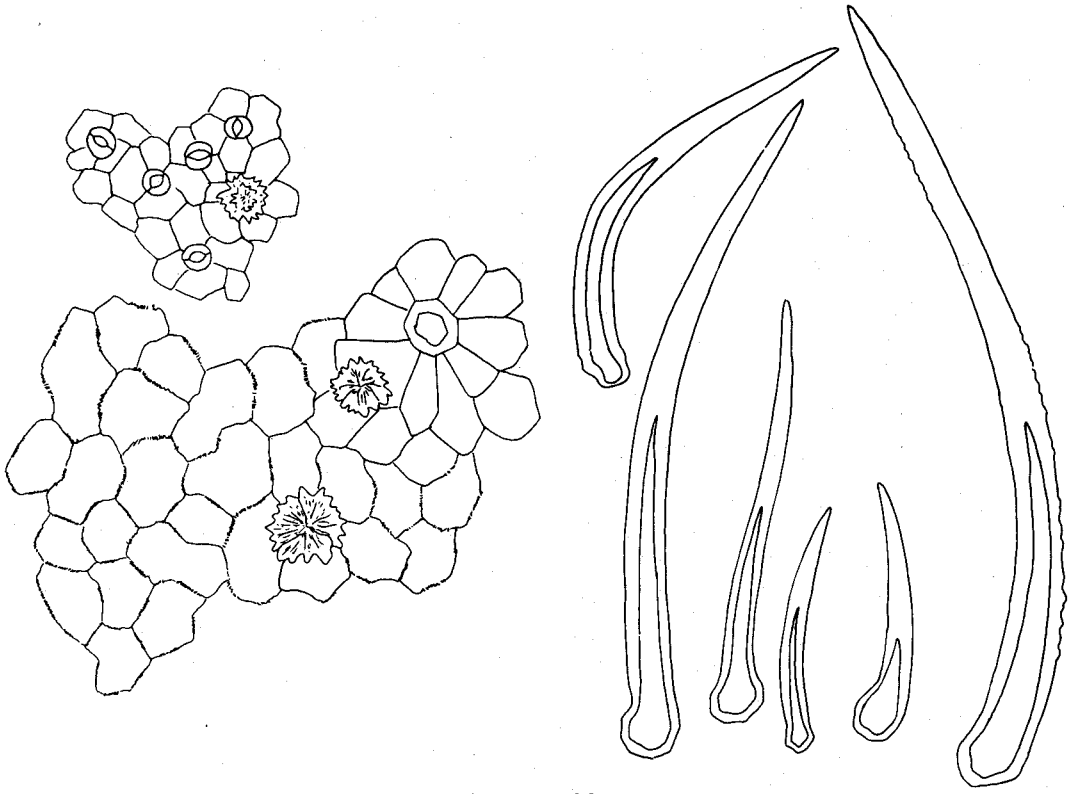
Carolina jessamine (*Gelsemium sempervirens*)—There are few trichomes so that tissues are essentially glabrous. Crystals are small and shaped like prisms.



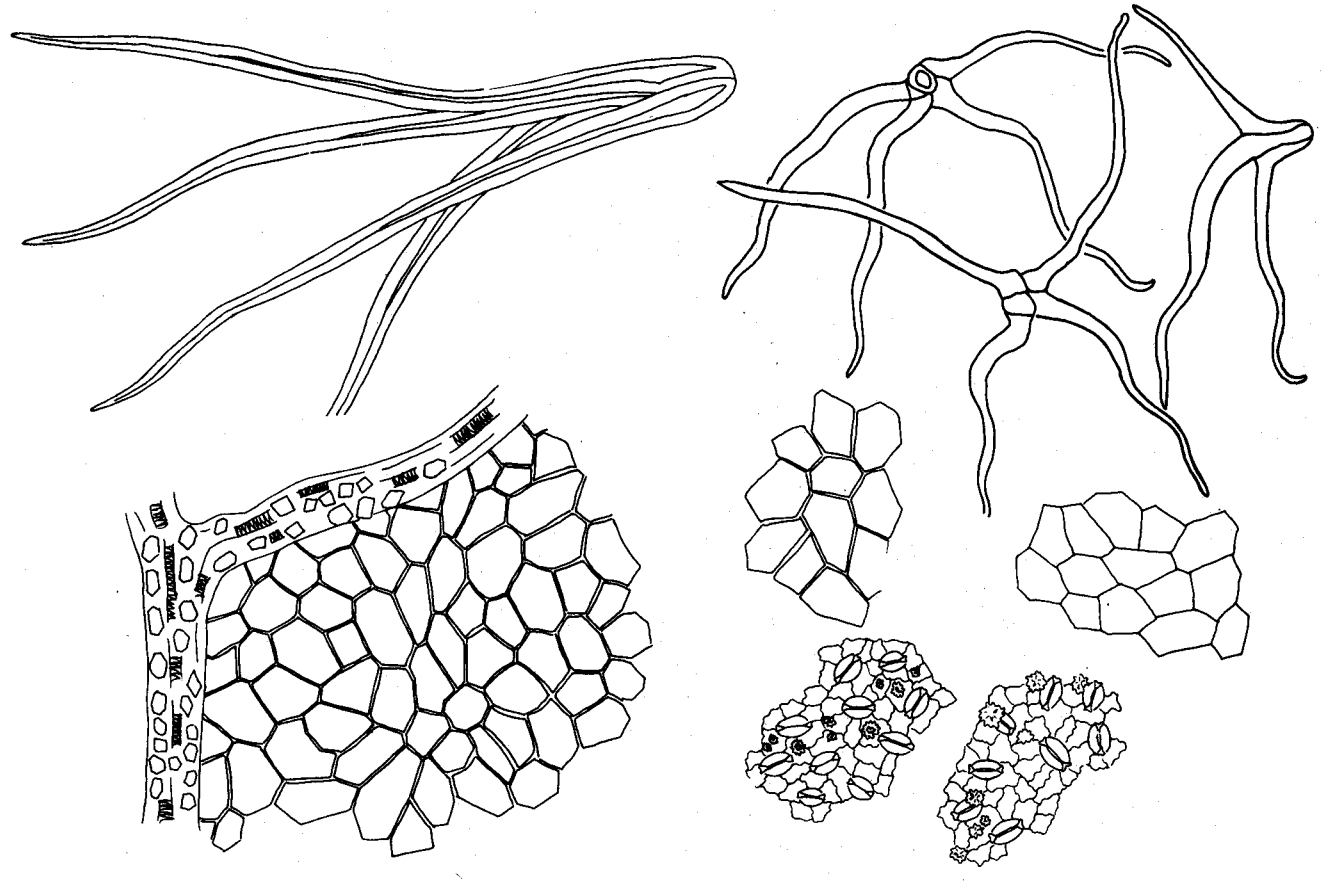
Rattanvine (*Berchemia scandens*) —There are no trichomes. Druses occur intercostally and square crystals are found over the veins. Woody tissue has small round cells and contains both types of crystals.



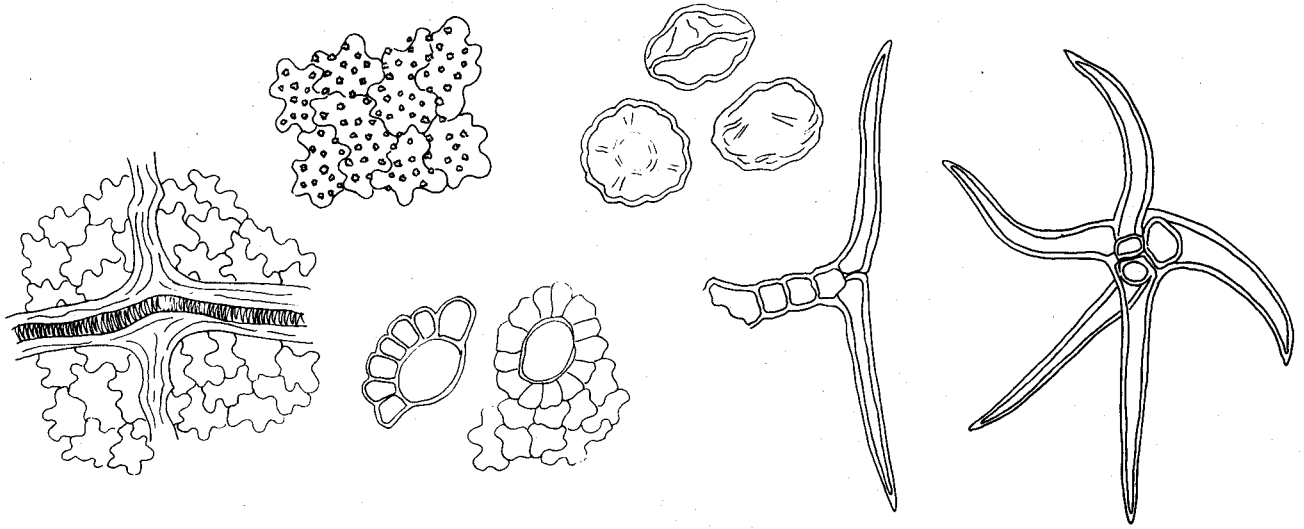
Sumac (*Rhus copallina*)—Ligulate, internally segmented trichomes have swollen bases that are usually 6-sided. Cells fit together to resemble alligator skin.



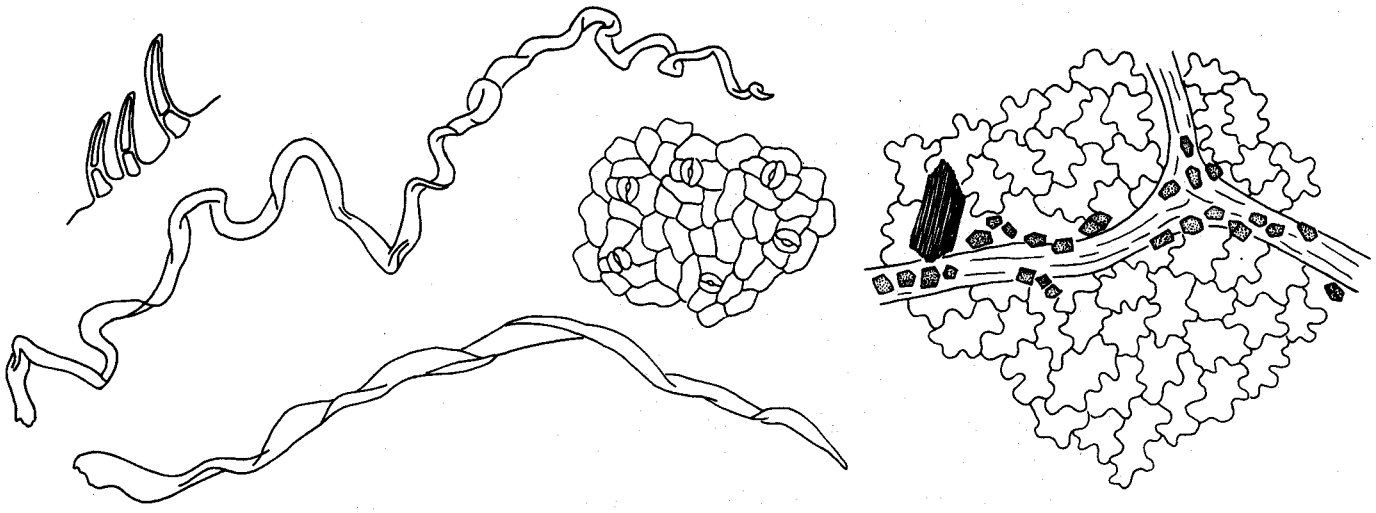
Japanese honeysuckle (*Lonicera japonica*)—Ligulate, hollow trichomes have 6-sided attachment cells. Large crystals are not abundant.



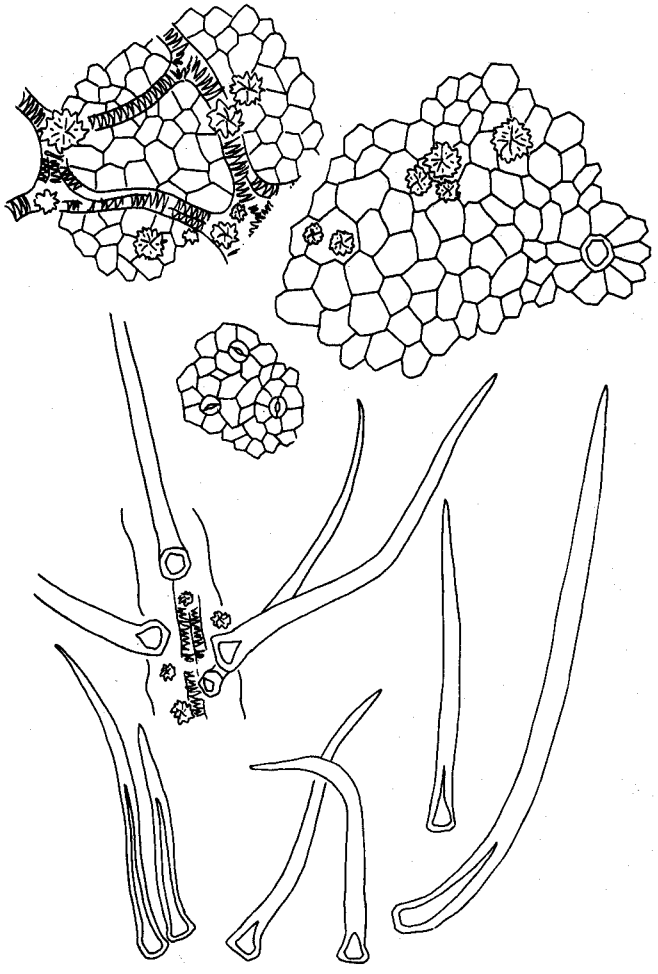
Oaks (*Quercus* spp.)—All species have compound stellate hairs that vary in size. Small druses are usually abundant. Some species have a few square crystals.



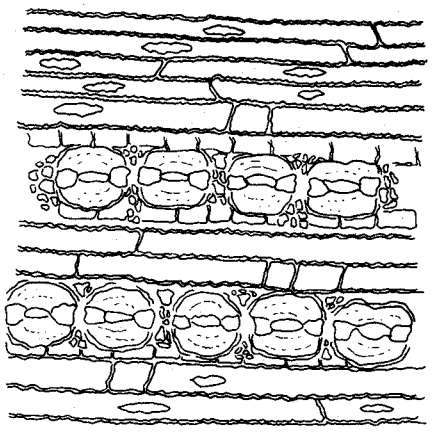
American beautyberry (*Callicarpa americana*)—Some trichomes are branched or compound but most are stellate.



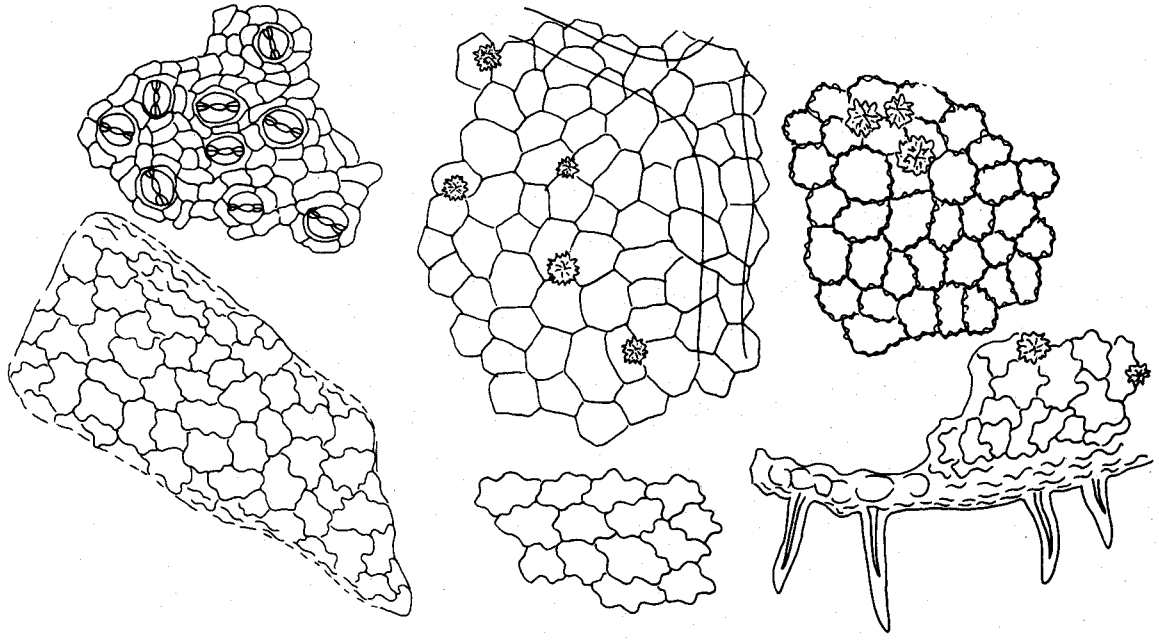
Wild grape leaves (*Vitis* spp.)—Trichomes are ribbon-like. Square crystals and druses are usually along the veins, and large raphides are present intercostally.



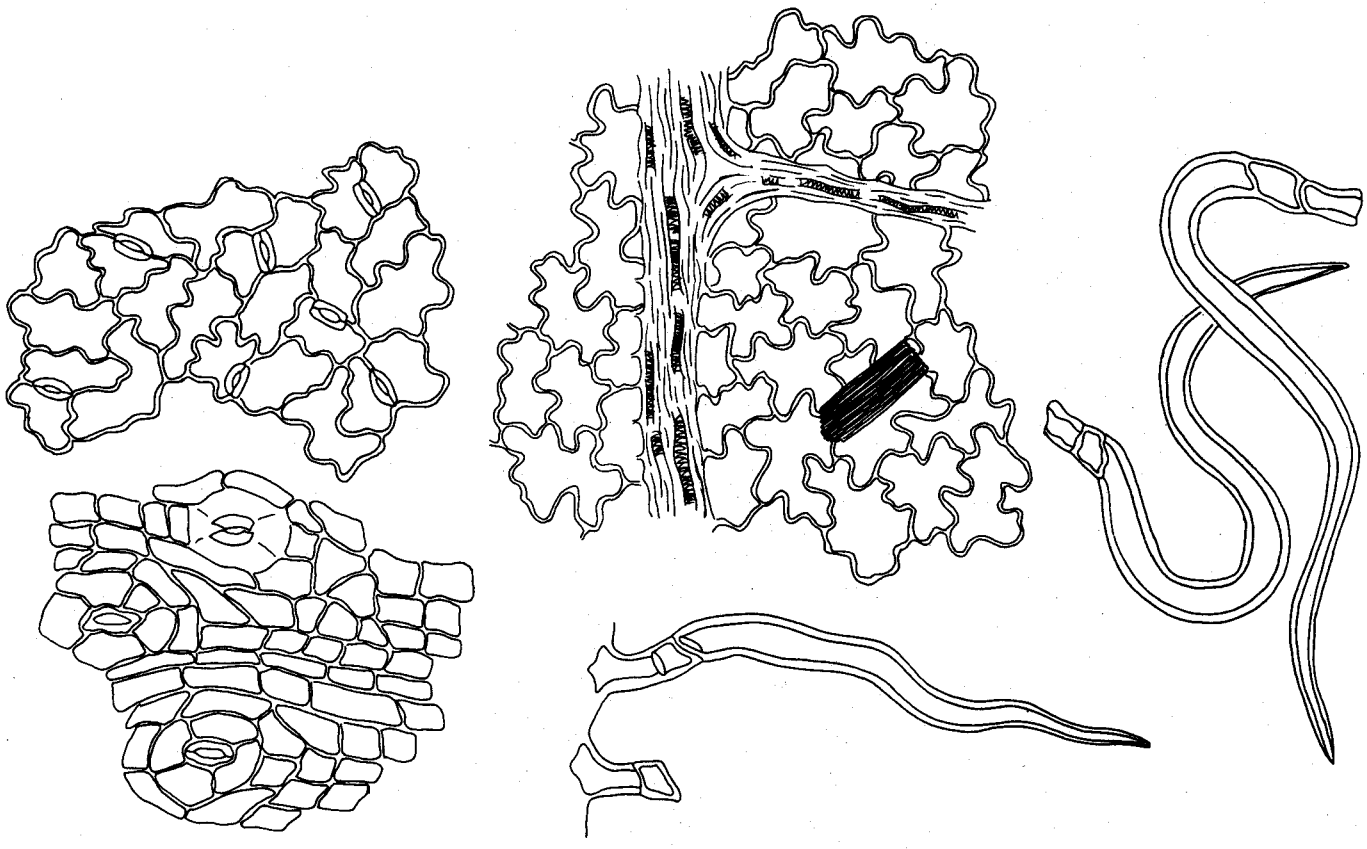
Dewberries (*Rubus* spp.)—Medium to small druses occur in tissues with angular cells. Trichomes are ligulate.



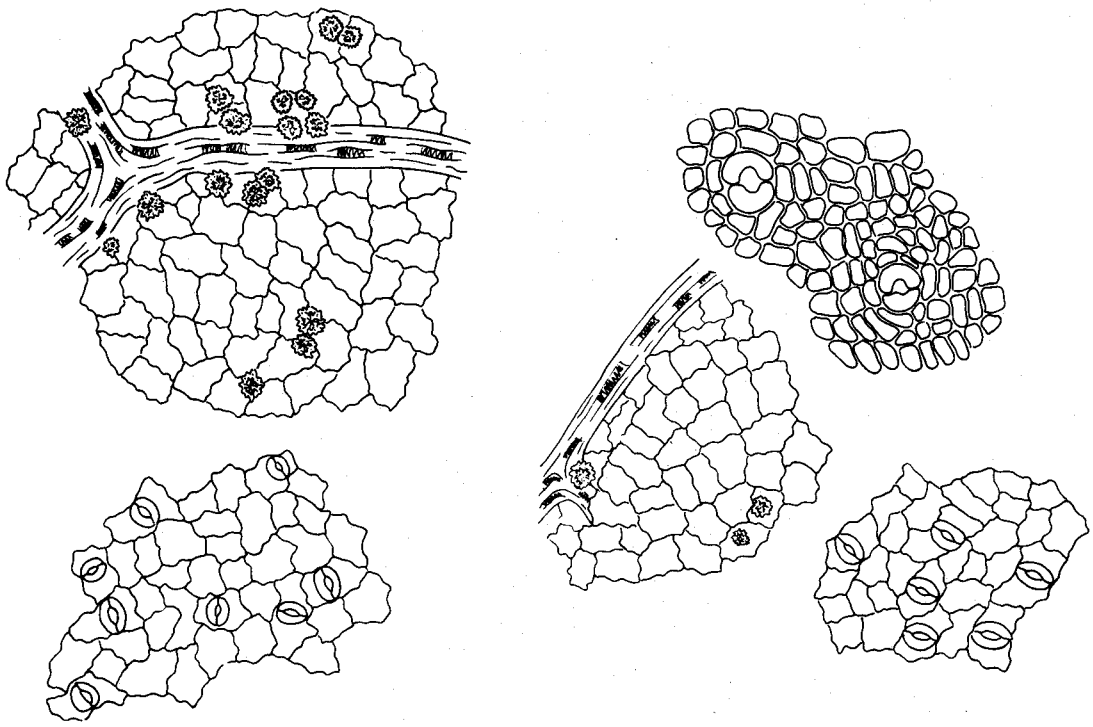
Pine (*Pinus* spp.)—Large stoma are deployed in rows with parallel intercostal cells. There are triangular cells in stoma corners.



Yaupon (*Ilex vomitoria*)—Short, ligulate trichomes are present on leaf margins, but not abundant. Medium and small druses are present.



Greenbriars (*Smilax* spp.)—*S. glauca* has small pappilae while *S. pumila* has long, ligulate trichomes with 2-celled bases. Raphides are present in both species.



Blueberries (*Vaccinium* spp.)—Fragments are without trichomes. Druses are present intercostally with square crystals over the veins.

Johnson, Mark K.; Wofford, Helen; Pearson, Henry A. Microhistological techniques for food habits analyses. Res. Pap. SO-199. New Orleans, LA: U.S. Department of Agriculture, Forest Service, Southern Forest Experiment Station; 1983. 40 p.

Techniques used to prepare and quantify herbivore diet samples for microhistological analyses are described. Plant fragments are illustrated for more than 50 selected plants common on longleaf-slash pine-bluestem range in the southeastern United States.

Keywords: Herbivore diets, plant fragments, micro-anatomy, longleaf-slash pine-bluestem range.

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