A PROCEDURE FOR DETERMINING LEAF STOMATAL DENSITY AND SIZE

The goal of this study was to develop an inexpensive and fast procedure for light microscopy determination of guard cell length and stomatal density of leucaenas, to use in lieu of scanning electron microscopy. The bleach digestion procedure developed appeared to be widely suitable to all *Leucaena* species. Our stomatal survey of the species is not yet complete, but preliminary results showed that guard cells were only present in lower leaflet surfaces of most *Leucaena* species, lower surface guard cells ranged from 180-300 microns in length, and lower surface stomatal densities ranged from 75-500 stomata/mm².

Bleach Digestion Procedure for Leucaena Stomatal Studies

1. Place fresh leaflets in vials of 75% alcohol. Alcohol treatment does not change guard cell size significantly (Eckerson, 1906). Samples may be stored in a refrigerator.

2. Replace the alcohol with full-strength household bleach (5.25% sodium hypochlorite) at room temperature for 1-2 days. Vials containing leaf tissue which still has brown areas should be refilled with fresh bleach and redigested for 1-2 days. Properly digested pinnules are translucent rather than opaque white, and mesophyll cells look mushy under a dissecting microscope (20-40x). Overdigestion causes epidermal layers to become too weak to be dissected properly.

3. Replace the bleach in the vials with water and add a drop of 1% aqueous safranin stain. Samples which are not immediately analyzed should be kept refrigerated.

4. Place cleared leaflets on slides and while looking at them with a dissecting microscope under high power (30-40x), hold them lightly at the tip of the leaflet, laterally cut off the bottom of the leaflet, and sweep out the mesophyll and palisade cells with a dissecting scalpel. Add extra water with a medicine dropper as necessary. Save cleaned epidermal peels if the tissue fragments. If the "epidermal bag" is intact, spread or "butterfly" the peels (Figure 1a). Cover the peels with a cover glass and seal the slip edges temporarily with rubber cement, or permanently with clear nail polish.

5. If the epidermal layers are too fragile to obtain cleaned peels, such as the upper leaflet surface of some *L. leucocephala*, whole leaflets (produced by #4 above) can usually be analyzed directly.

6. Count stomatal densities at 200-400x using a calibrated grid ocular, and measure guard cell length at 600-1000x with a calibrated scale ocular from mid-leaflet areas devoid of large veins. Light microscopy is necessary for whole leaf preparations and is quite adequate for observing peels.

7. Photographs may be taken with phase contrast (Figures 1a-e were taken with mismatched phase contrast rings to enhance contrast).

Comparison of Cast, Cleared Leaflet, and Epidermal Methods

Both positive and negative cast methods can be used to make leaf surface replicates. Cast methods generally suffer from 1) difficulties in cast removal because of trichomes or small leaflet size, 2) cast inaccuracy of fine leaf surface topography or aperture opening (Glinka and Meidner, 1968), and 3) surface topography changes during leaf desiccation (most cast methods employ dried leaves). Cast techniques include those reviewed or developed by Redmann (1985) Weyers and Johansen (1985) and (Kirkwood et al., 1982).

We tested the fingernail polish negative cast technique (Hilu and Randall, 1984) on dried *Leucaena* sp. leaflets because cellulose nitrate (fingernail polish)
becomes cloudy when applied to fresh leaves (Sampson, 1961). Casts of dried \textit{L. greggii} were satisfactory for counting lower stomatal densities, especially when observed with False Nomarski interference contrast microscopy (Figure 1f), rather than with phase contrast (Figure 1e), but were inappropriate for guard cell length measurements since the guard cells had desiccated. The quality of epidermal peels of \textit{L. greggii} (Figure 1b) far exceeded that of the casts.

Fingernail polish casts of upper leaflets of most species correctly showed a total absence of stomata, but rare stomata of species like \textit{L. retusa} (Figure 1d) were easily missed. Lower leaflet casts of most \textit{Leucaena} species were unacceptable because of poor cell definition, particularly in the vicinity of trichomes (as in \textit{L. trichodes} in Figure 1g), perhaps because of light refraction through the hollow trichome casts. Casts were nearly impossible to remove from the surface of thin leaflets of species like \textit{L. esculenta} (leaflets are approx. 1 mm wide).

Cleared whole leaf mounts have been used in several studies (e.g., Tan and Dunn, 1975). Leaves are cleared of pigments and partially digested with agents including glycerol, lactic acid, sodium hydroxide, sodium hypochlorite and chloral hydrate, in order of increasing strength. Air bubbles in cleared leaves interfere with light transmittance and were a common problem, but could sometimes be driven out by depressurization in vacuum under fluid. Heat application speeded clearing.

Cleared whole leaves were fastest to prepare, and often adequate to show the absence of stomata of upper leaflet surfaces of most \textit{Leucaena} species. Bubbles and excessive mesophyll staining were problematic, and not all species lent themselves to whole leaf studies. We compared mean guard cell length measurements of whole leaflets and epidermal peels from the same leaflets in a few accessions and found mean guard cell measurements were as much as 6 % less when taken from epidermal peels than whole leaflets. This difference may have been as much due to difficulty in visualizing the exact borders of guard cells in whole leaflet mounts than an apparent magnifying effect of multiple cell layers.

Epidermal strips have been widely used in stomatal research, especially that on stomatal behavior; however, much of this research utilized plant leaves whose epidermal layers can be mechanically separated without chemical pretreatment. We failed to make any epidermal peels of \textit{Leucaena} leaves solely by mechanical means. We did not locate any literature describing bleach pretreatment to make epidermal peels, although diluted bleach is commonly used as a leaf clearing agent. Hetzer and Vole (1950) utilized boiling aqueous potassium hydroxide in a rather similar manner, however, to prepare peels from dried herbarium specimens of composites.

Discussion

Guard cell length was used by Pan (1985) to separate the diversifolias by ploidy, and is probably useful for identifying chromosome-doubled species or hybrids. Stomatal density was often correlated positively with photosynthetic rates (e.g., Wong et al., 1979) and thus could be used to breed high-yielding leucaenas for areas where water is not limiting. The expected positive relationship between decreased stomatal density and increased water use efficiency is obscured, however, by the number of other factors affecting drought resistance (Parsons, 1979; Sashidhar et al., 1986).

Cast methods which appear worthy of attempting and were not tested during the present study include Sampson's positive cast method (1961) and impression techniques (e.g., McConathy, 1983). The big advantage of Sampson's method is that serial casts can be made from living leaves, and therefore can be used in stomatal behavior studies, while impression methods are fast as they simply involve the pressing of dried leaves onto softened cellulose acetate, etc. which was previously attached to microscope slides.
Figure 1. 1a) Butterfly epidermal peel of *L. cuspidata var. adenosticta* K745 with stomata present only in the lower leaflet surface (left). Trichomes originate from the leaflet margin (11x), b) Closeup (31x) of lower surface stomata from an epidermal peel of *L. greggii* K857, c) Closeup (32x) of lower surface stomata from an epidermal peel of *L. trichodes* K881, d) Upper surface of cleared leaflets of *L. retusa* K502 showing (arrow) rare stomata (14x), e) Lower surface fingernail polish cast of *L. greggii* K857 with phase contrast showing desiccated stomates (19x), f) The same cast pictured in Figure 1e photographed with false Nomarski (19x), and g) Largely unanalyzable lower surface fingernail polish cast of *L. trichodes* K738 (20x). Dark areas are trichome casts.
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References: