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RECENT STEREOLOGICAL METHODS FOR ESTIMATING LEAF ANATOMICAL CHARACTERISTICS

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ABSTRACT

The possibilities of recent design-based stereological methods for estimating leaf anatomical characteristics are presented. Reasons for sparse use of these methods in plant anatomy are discussed.

Key words: mesophyll, stereology, stomata, vertical sections.

INTRODUCTION

Recently, a number of new, design-based stereological methods (for review see Gundersen et al., 1988a,b and Cruz-Orive and Weibel, 1990) has been developed. These methods make practically no assumptions about the shape and organization of the structure being studied. Taking into account that biological structures often exhibit some degree of anisotropy or non-homogeneity, design-based stereological methods appear to be very suitable in many biological applications. Their biomedical applications are developing fast, while they are still used very rarely in plant anatomy.

Different opinions can be encountered in this context: The sparse use of stereology in plant anatomy is explained by the lack of clear reasons for getting stereological information or stereological methods are considered to be too complicated and time-consuming; others see the reason in the fact that stereological methods are little known by plant biologists.

The above opinions will be discussed by using the example of leaf anatomical structure evaluation.

LEAF ANATOMICAL CHARACTERISTICS

In the last twenty years much attention has been paid to the relations between leaf anatomy and photosynthesis (Nobel et al., 1975, Chabot and Chabot, 1977, LeCain et al., 1989). Considering the importance of mesophyll and stomata for the transfer of carbon dioxide into the leaf, stomatal and mesophyll characteristics are of primary interest. Stomata are special formations of epidermal cells (Fig. 1) influencing the

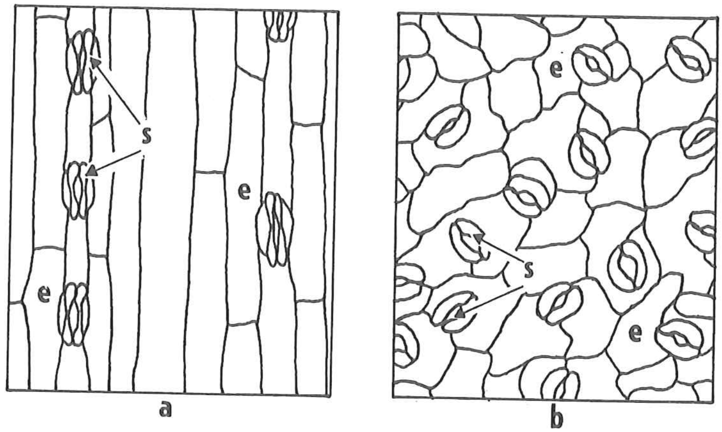


Fig. 1. The surface of (a) a grass leaf and (b) a typical bifacial leaf.
s ... stoma; e ... ordinary epidermal cell

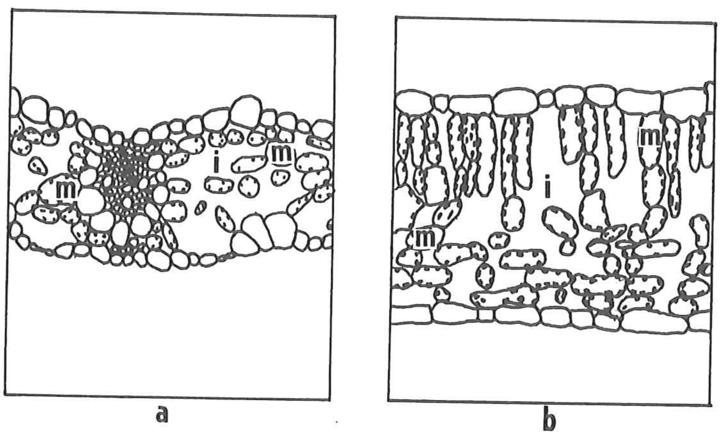


Fig. 2. Transverse section of (a) a grass leaf and (b) typical bifacial leaf.
m ... mesophyll cell; i ... intercellular spaces

flux of carbon dioxide from the external atmosphere into the leaf. The mesophyll (Fig. 2) is a tissue consisting of intercellular spaces and mesophyll cells containing chloroplasts where the process of photosynthesis takes place.

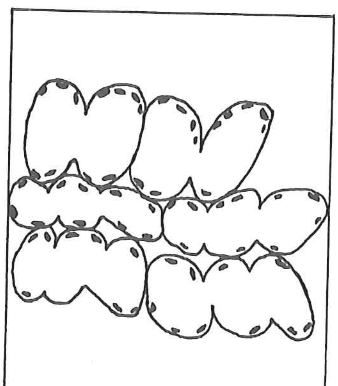
The following parameters are usually measured:

- stomatal frequency (i.e. the number of stomata per mm^2 of leaf area)
- total number of stomata in a leaf
- linear dimensions of stomata, stomatal apertures, and ordinary epidermal cells
- leaf thickness
- proportion of mesophyll or other leaf tissues in the leaf
- volume of intercellular spaces

- number of mesophyll cells
- sizes of cell sections and projections
- surface area of mesophyll cells
- exposed surface area of mesophyll (i.e. the surface area of mesophyll cell walls exposed to intercellular spaces)
- mean cell surface area

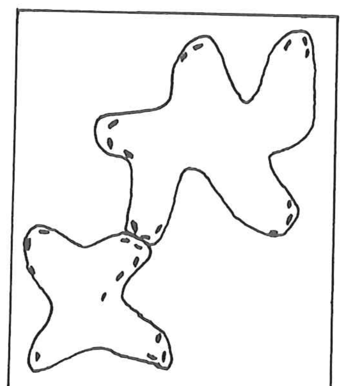
Different methods were used for the measurement of these characteristics. For example, cell number was often measured by counting isolated cells and the volume of intercellular spaces by infiltrating the intercellular spaces with a substance of some kind and determining its volume. Both these methods can hardly be called unbiased. Most frequently, leaf sections and replicas or peals of leaf epidermis were evaluated. Cell sizes were estimated assuming the cells could be modelled by simple geometrical bodies. In my opinion, this approach is not suitable especially for the lobed grass mesophyll cells (Fig.3) and spongy mesophyll cells (Fig.4) which can be very complex in shape. In most cases the representative sampling of leaf segments, sections, cells, etc. was not used. In fact, the sampling procedure was usually not described at all.

It should be stressed here that the leaf structure is non-homogeneous which stems from the well-known fact that there are developmental as well as biochemical and physiological differences in different parts of the leaf. Therefore, it is important to perform proper sampling here.



a

Fig. 3. Lobed grass mesophyll cells.



b

Fig. 4. Spongy mesophyll cells.

SAMPLING AND STEREOLOGICAL ESTIMATION OF LEAF CHARACTERISTICS

In the leaf, it is quite easy to perform the systematic uniform random sampling which is recommended when using the unbiased stereological methods (see Kubínová, 1993,1994). Let us present a simple way of systematic uniform random sampling of leaf segments in a long narrow leaf (e.g. grass leaf) (Fig.5): We can chose a distance between segments to be T (mm) and select a random number z from the set {0,1,...,T-1}. We place the lower edge of the leaf segment at the distance z (mm) from the leaf base. In a flat, broad leaf we can place the grid of central points of leaf segments uniformly at random with respect to the leaf tip (Fig.6).

Such sampling can form a basis for the application of many unbiased stereological methods (for details see Kubínová, 1993, 1994). Stomatal frequency, total number of stomata and their mean sizes can be estimated by examining replicas of leaf segments using an unbiased counting rule (Gundersen, 1977).

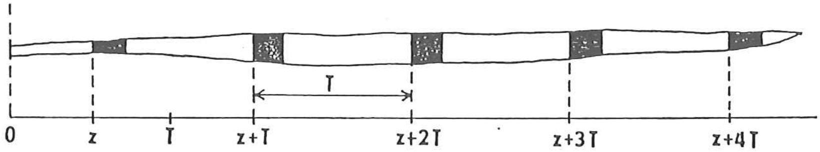


Fig. 5. Systematic uniform random sampling of segments and sections in a grass leaf. Firstly, the distance (T) (mm) between two consecutive sections is chosen. A random number (z) is then selected from the set {0,1,...,T-1}. The transverse sections are made in the positions z, z+T, z+2T, ... For example, if T=40mm, z=20mm, and the leaf length were 200mm, then the transverse sections would be cut at distances of 20mm, 60mm, 100mm, 140mm and 180mm from the leaf base. (Reproduced from Kubínová, 1993, by permission of Oxford University Press.)

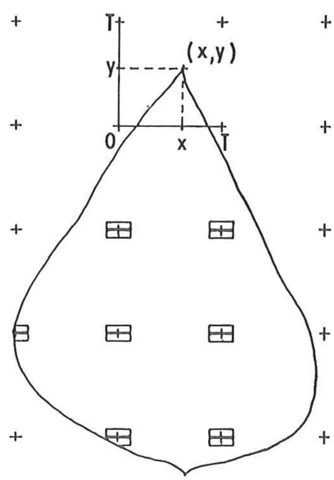


Fig. 6. Systematic uniform random sampling of segments and sections in a flat bifacial leaf. The distance (T) (mm) between the central points of the leaf segments is chosen first. Numbers x and y are then selected at random from the set {0,1,...,T-1}. By placing the leaf tip in the position (x,y), the uniform random position of the grid of central points is ensured. The leaf segments and sections are then cut as indicated in the figure. (Reproduced from Kubínová, 1993, by permission of Oxford University Press.)

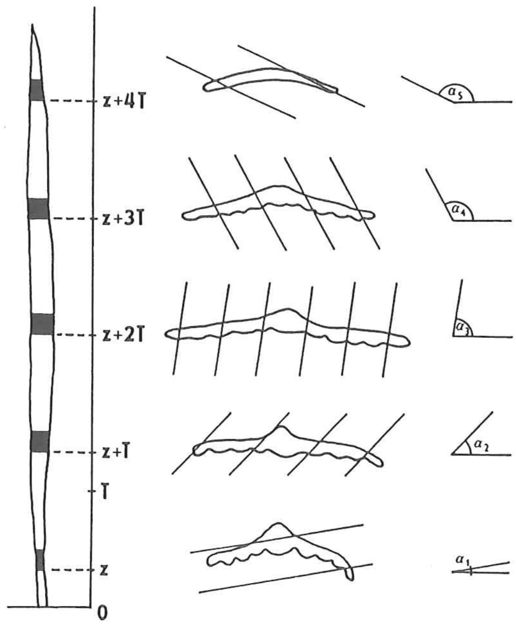


Fig. 7. Construction of vertical sections of the grass leaf. Firstly, the systematic uniform random sampling of leaf segments is done as shown on the left. In the horizontal plane of the first segment, angle α_1 ($0° \leq \alpha_1 < 180°$) is selected uniformly at random (e.g., α_1 is a random number from the set {0°, 10°, 20°,..., 170°}). The vertical sections of the first segment are cut in this orientation. With m segments in the leaf, the orientation of vertical sections in the j-th segment is given by the angle $\alpha_j = \alpha_1 + (j-1)x(180°/m)$ (j=1,...,m). (For example, if m=5 and α_1=10°, then α_2=10°+1x(180°/5)=46°, α_3=82°, α_4=118°, and α_5=154°.) Within the segment, the series of equidistant parallel sections (in the figure illustrated by lines intersecting the sampled segments seen from above) are cut in a way analogous to the one described in Fig.5.
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Transverse sections cut either along the lower edge of leaf segments (narrow leaf) or in the middle of the leaf segments (flat leaf) can be used for estimating the volume density (or proportion) of mesophyll in the leaf by point counting (e.g. Weibel, 1979), the leaf volume or the volume of intercellular spaces by Cavalieri's principle (e.g. Gundersen and Jensen, 1987, Michel and Cruz-Orive, 1988) and the number of mesophyll cells or mean mesophyll cell volume by the disector principle (Sterio, 1984).

In long narrow leaves the above sampling of leaf segments (described in Fig.5) can also be used for generating vertical sections (Fig.7) (for details on vertical sections method see Baddeley et al., 1986). The vertical axis can be represented by the longitudinal axis of the leaf here and so the vertical

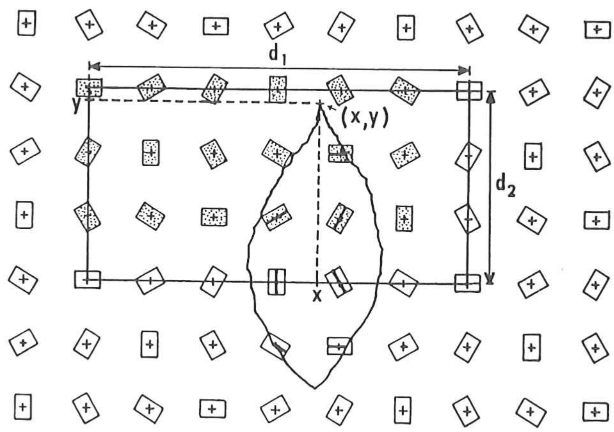

Fig. 8. The construction of vertical sections of the bifacial leaf. Make three transparencies with cut-off windows having different orientations of their longitudinal axes. In each transparency, a unit is formed by 6 x 3 = 18 windows (dotted windows in the figure). The orientations of the windows in the first row of the unit are given subsequently by six angles $\delta + m.30°$ (m=0,1,...,5) and the orientations of windows in the first column by three angles $\delta + n.60°$ (n=0,1,2). Take $\delta = 0°$ for the first transparency, $\delta = 10°$ for the second, and $\delta = 20°$ for the third one (i.e. in the first transparency the first row of the corresponding unit is formed by windows with orientations given by 0°, 30°, 60°, 90°, 120°, 150° and the orientations of the windows of the first column are given by angles 0°, 60°, 120° - see the figure). Now select a random number z from the set {1, 2, 3}. If z = 1, then use the first transparency, if z = 2, use the second one and if z = 3 use the third one. Make a rectangle by connecting the central points in a row of seven neighbouring windows and the central points in a column of four neighbouring windows (see the figure). This rectangle represents the unit area corresponding to the unit of windows. Superimpose the transparency so that the leaf tip coincides with a uniform random point of this rectangle (i.e., with point (x,y), where x is a random number from the set $\{0,1,...,d_1-1\}$ and y is a random number from $\{0,1, ...d_2-1\}$; d_1 is the width (mm) of the rectangle and d_2 is its height (mm)). The leaf segments to be cut are now determined by the windows of the transparency.
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sections are sections parallel to the leaf axis, i.e. perpendicular to the transverse sections of the leaf. In the case of broad flat leaves, it is convenient to chose the vertical axis perpendicular to the leaf surface. (This is not possible in grass leaves because the grass leaf has an uneven surface and tends to curl which makes it impossible to determine unambigously the plane on which the leaf can be flattened.) A special procedure ensuring the isotropic orientation of the leaf segments in a flat leaf can be used

(Fig.8). One central section of each segment, perpendicular to the leaf surface, is then taken and a cycloid test system is superimposed on the section. By counting intersection points between test lines and the surface of cell walls exposed to intercellular spaces, the exposed surface area of mesophyll can be estimated. This parameter is considered to be of great importance in connection with the photosynthetic performance in the leaf. It should be mentioned that the above procedure is extraordinarily suitable for the evaluation of typical bifacial leaves, with palisade and spongy mesophyll (Fig.2b), because it allows one to evaluate these two types of mesophyll separately.

DISCUSSION

The above described stereological procedures can be readily applied to the evaluation of other plant organs (e.g. roots, stems) than leaves - either directly, or after slight modifications. Moreover, other valuable plant parameters than those described above, e.g. the length of veins, branches or roots, parameters describing the distribution of stomata or veins in a leaf, etc. can be estimated. In many cases a number of parameters can be evaluated simultaneously.

Although stereology has a promising future in the quantitative evaluation of plant anatomical structures, it has been used in this field sparsely till now. It may be that many plant biologists are not acquainted with stereological methods, at least with the recently developed unbiased ones. In the community of plant biologists, broader publicity of these techniques should be made (special undergraduate and postgraduate courses, seminars, tutorials, reviews, etc.)

Stereological methods can be considered to be complicated and time-consuming but the procedures described above and elsewhere (Kubínová, 1993, 1994) are not so difficult to apply and often are not more laborious than other, formerly used, biased procedures. However, the necessity of broader theoretical knowledge, required for planning and performing stereological studies, must be stressed. Many biologists would appreciate a kind of a cookbook with recipes telling them exactly what to do. Such a cookbook will be useful, though it does not represent a unique solution because only a limited number of problems can be described. Some kind of cooperation between plant biologists and stereologists would be necessary. Recently, a growing number of papers has confirmed the productivity of such a cooperation in stereological applications to biomedical sciences.

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