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ANATOMICAL VARIATION ALONG THE LENGTH OF THE *ZEA MAYS* LEAF IN RELATION TO PHOTOSYNTHESIS

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Summary

Anatomical features of the leaf, related to C_4 photosynthetic activity, were examined along the length of the second leaf of 7-day-old plants grown under a diurnal light regime. Changes of significance to photosynthetic activity were observed in cuticle thickness, stomatal size and frequency, mesophyll anatomy and interveinal distance. Anatomical changes within the basal 3 cm of the leaf were attributable to development. Above this region, variation in some anatomical characteristics, notably stomatal frequency and interveinal distance, could only be attributed to ontogenetic differences between parts of the leaf.

Introduction

The leaves of Graminae exhibit a progressive gradient of cell age from the base to the tip and have been used to provide a model for chloroplast development under natural diurnal light regimes (Leech, Rumsby and Thomson, 1973; Hawke, Rumsby and Leech, 1974). Ultrastructural (Leech *et al.*, 1973) and biochemical studies (Leech *et al.*, 1973; Hawke *et al.*, 1974; Leese and Leech, 1976) have shown that a sequential development of chloroplasts occurs from the base to the tip of the second leaf in maize. To achieve a complete understanding of the development of photosynthesis and its control in this system a knowledge of the basic anatomical changes that occur along the maize leaf is needed; changes in the structure of photosynthetic tissue could be reflected in changes in photosynthetic activity. In maize and many other tropical plants 'Kranz' leaf anatomy is highly correlated, if not essential, to C_4 photosynthesis and there is a compartmentation of metabolic pathways between the two chlorenchymatous tissues of the C_4 leaf (Ray and Black, 1979). The synthesis of phosphoenolpyruvate (PEP), the primary acceptor of OC_2 in C_4 photosynthesis, the carboxylation of PEP and the reduction of oxaloacetate occurs in the mesophyll, whilst the decarboxylation of the C_4 dicarboxylate and the reactions of photosynthetic carbon reduction cycle are limited to the bundle sheath cells (Gutierrez, Gracen and Edwards, 1974; Edwards and Huber, 1979). Thus differential rates of development of these two interdependent tissues might explain, or be indicative of, changes in the capacity for CO_2 assimilation along the leaf. Similarly, a knowledge of stomatal development and function is important to the interpretation of the observations made in studies on photosynthesis. For a critical understanding of the development of *in vivo* photosynthetic activity associated anatomical changes must be known.

This paper examines anatomical changes in the cuticle, stomatal apparatus (and

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its resistance to CO_2 diffusion), mesophyll, bundle sheath and vascular tissues along the length of the second leaf of 7-day-old maize plants grown under a diurnal light regime. The significance of these structural features in the regulation of carbon assimilation by the leaf are considered. Also, this anatomical study is used to assess the validity of using regions along the length of a single *Z. mays* leaf as a progressive developmental gradient of the C_4 photosynthetic apparatus.

MATERIALS AND METHODS

Plant material

Caryopses of *Zea mays* (var. L.G. 11, Nickersons Seed Specialists Ltd., Grimsby, U.K.) were washed in running water for 17 h and sown in John Innes No. 2 potting compost. Plants were grown at 25 °C and in an 18 h photoperiod with a photon flux density of 390 μmol m^{-2} s^{-1} produced by a bank of 10, 40 W fluorescent tubes (warm white BIP 1N, Osram-GEC Ltd.). After 7 days plants having a second leaf of 17 cm in length were selected; 17 cm was the mean length of normally developing second leaves. Leaves were measured from the base of the sheath at the mesocotyl insertion to the tip of the lamina.

Leaf anatomy

The second leaf was divided into 1 cm segments for anatomical studies. Sections for light microscopy were cut by hand, stained with either Sudan IV, haematoxylin or phloroglucinol and conc. HCl. From these stained transections, the degrees of cutinization and lignification were determined. Epidermal cell length and breadth and stomatal length were measured from epidermal strips. Interveinal distance and the number of vascular bundles per unit leaf breadth were determined from cleared whole mounts according to the method of Crookston and Moss (1974). Structural details of vascular tissue were examined from microtome sections.

Stomatal resistance

Both maximum and minimum values of r_s were estimated for each 1 cm segment of the leaf by measuring stomatal apertures in a high CO_2 concentration (2600 mg m^{-3}) to induce partial stomatal closure and a low CO_2 concentration (0 mg m^{-3}) to induce maximal stomatal opening, respectively. The mean number of stomata per unit area in each segment was determined from epidermal strips from both the upper and lower surfaces of five leaves. Mean depth of stomatal pore was determined from transverse sections taken along the length of five leaves. The aperture length and width of 30 stomata in each 1 cm segment were measured on attached leaves enclosed in a chamber mounted on the stage of an inverted microscope (Nikon Model MS, Nippon Kogaku K.K.). A Dyson's reflective parabolic objective (N.A. 0·57 × 40, Vickers-AEI. Ltd.) allowed sufficient working distance between the objective and the specimen to observe the stoma on the leaf enclosed in the chamber. The chamber was maintained at 25 °C, and the leaf was irradiated with a photon flux density of 1500 μmol m^{-2} s^{-1}, and was aspirated with air containing either 2600 mg m^{-3} or 0 mg m^{-3} of CO_2. The equation of Parlange and Waggoner (1970), for eliptical stoma, was used to estimate r_s.

Results

At the leaf base the cuticles of both surfaces were *c.* 0·3 μm thick and stained pale orange with Sudan IV, which is a specific stain for fatty acids (Fahn, 1974). The cuticle thickness increased from *c.* 0·3 to 1·0 μm at 3 cm from the leaf base on both surfaces. The staining of the cuticle changed with the increases in thickness; the cuticle stained deep red with Sudan IV at 3 cm from leaf base suggesting that the fatty acid content of the cuticle increased with maturity. No further changes in the cuticle were observed above 3 cm.

In surface view the epidermal cells were four or five sided on both the upper and lower leaf surfaces and the cells arranged in parallel rows along the long axis of the leaf. The majority of the epidermal cells on the lower surface were similar to those found on the upper surface although one or two rows of smaller cells known as 'short-cells' (Metcalf, 1960) were found above the regions of the vascular bundles. In the ligule region the upper and lower epidermal cells were much smaller and hexagonal in shape. The pattern of change of epidermal cell length and breadth was clearly different for the two surfaces (Fig. 1). The marked decrease

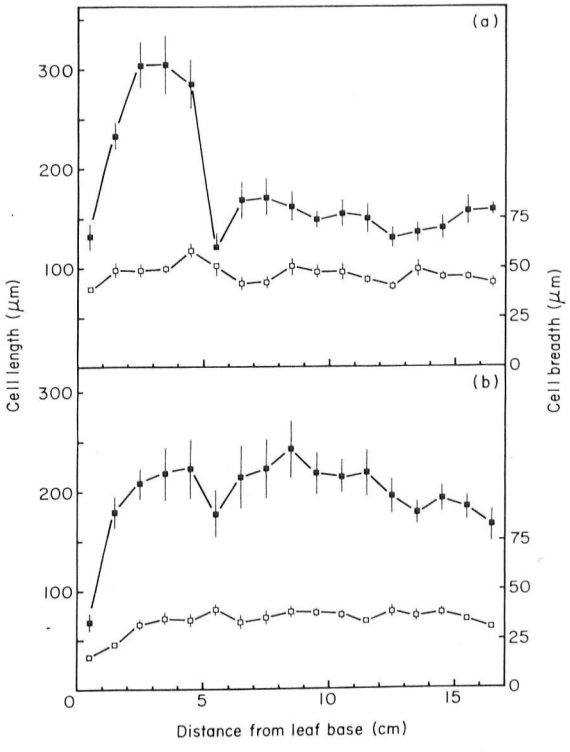

Fig. 1. Changes in epidermal cell length (■) and breadth (□) along the upper surface (a) and the lower surface (b) of the leaf. Vertical bars indicate twice the standard error of the mean, $n = 20$.

in upper epidermal cell length between 3 and 5 cm and the decrease in both the upper and lower epidermal cell length in the ligule region and along the leaf blade must be attributed to ontogenetic differences between the different regions of the leaf, rather than to developmental change.

Cytological aspects of stomatal development in *Z. mays* have been reported previously (Stebbins and Shah, 1960; Srivastava and Singh, 1972; Ziegler, Shmueli and Lange, 1974). The stomata of both surfaces showed similar structural development and first exhibited pores at *c.* 0·35 cm, although they were neither mature nor functional. On the lower epidermis the stomatal pores were open under all conditions up to 1·75 cm from the leaf base whereas in the upper epidermis the stomatal pores remained open until the ligule region and mature, fully functional stomata were found only in the blade. Although the pattern of change of stomatal length and frequency was similar for both surfaces, their magnitude was significantly different (Figs 2 and 3). In the first 1 cm of the leaf sheath stomatal length was

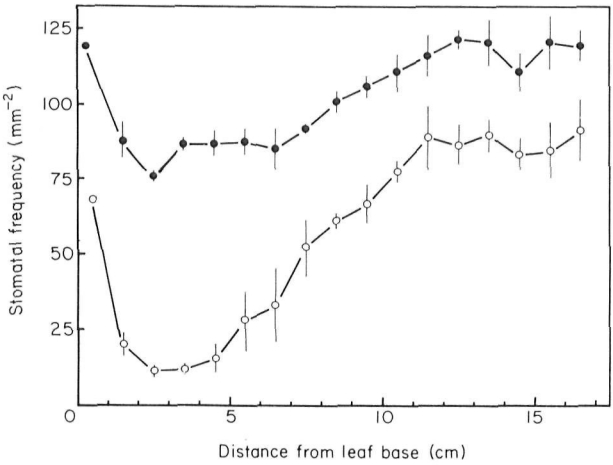

Fig. 2. Changes in stomatal frequency along the upper surface (○) and the lower surface (●) of the leaf. Vertical bars indicate twice the standard error of the mean, $n = 5$.

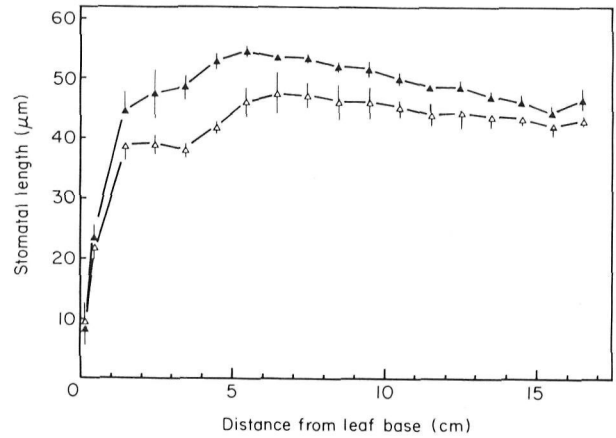

Fig. 3. Changes in stomatal length along the upper surface (△) and the lower surface (▲) of the leaf. Vertical bars indicate twice the standard error of the mean, $n = 30$.

similar for both surfaces, however above 1 cm the stomatal length was significantly greater on the lower epidermis. On both surfaces stomatal length followed an expected developmental pattern until a maximum was reached at the ligule region. However, above the ligule a decrease in the stomatal length was observed. Such

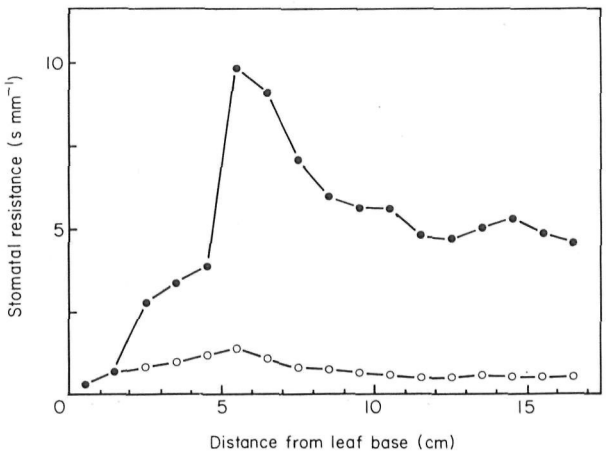

Fig. 4. Estimated changes in stomatal resistance to CO_2 assimilation, r_s, along the length of the leaf in CO_2 free air (○) and air containing 2600 mg m^{-3} CO_2 (●).

ontogenetic variation was also shown by changes in stomatal frequency. Frequency decreased over the first 2 cm of the sheath, presumably due to expansion of the tissue. Above this region there was a progressive increase in frequency. Similar changes in stomatal frequency have also been observed in the mature maize leaf (Heichel, 1971).

The changes in the resistance of the stomata to CO_2 diffusion, r_s, estimated at the two different atmospheric CO_2 concentrations, C_a, are shown in Figure 4. The functional stoma of *Z. mays* showed a decreased aperture in response to increased C_a (Raschke, 1970), thus r_s estimated in $C_a = 0$ will be the minimum whilst r_s estimated in $C_a = 2600\,\text{mg m}^{-3}$ will approach the maximum. The r_s of the basal 2 cm was insensitive to CO_2 concentration, suggesting that the stomata were non-functional. The open pores of these non-functional stomata and the high stomatal frequency would account for the low r_s of these two segments. Above 2 cm the stomata showed an increased capacity to limit CO_2 diffusion by an increased r_s at high C_a. The maximal response of r_s to variations in C_a was obtained at the ligule region. The increase in r_s from leaf base to the ligule at the two C_a levels could be attributed to decreases in stomatal frequency.

At the leaf base the mesophyll is *c.* seven cells thick and composed of six or eight sided cells, which were compactly arranged without intercellular spaces. At *c.* 1 cm from the leaf base the mesophyll cells on the lower side of the leaf were irregular in shape and intercellular spaces were found [Fig. 5(b)]. Within the ligule region the mesophyll cells revert to a compact arrangement with no intercellular spaces. Above the ligule region a well developed intercellular space system was again present. Mature mesophyll cells in the sheath were six or eight sided and 40 to 115 μm across [Fig. 5(a), (b)], whereas in the blade the mesophyll cells were only 20 to 30 μm across [Fig. 6(b), (d)].

The bundle sheath cells were arranged tightly around the vascular bundles in both the sheath and blade [Fig. 6(a)–(d)]. The bundle sheath cells were smaller than the mesophyll cells in the leaf sheath [Fig. 6(a), (c)], but as large or larger in the leaf blade [Fig. 6(b), (d)]. The centrifugal arrangement of the bundle sheath chloroplasts was found 3 cm from the leaf base [Fig. 6(a)]. Within the leaf sheath,

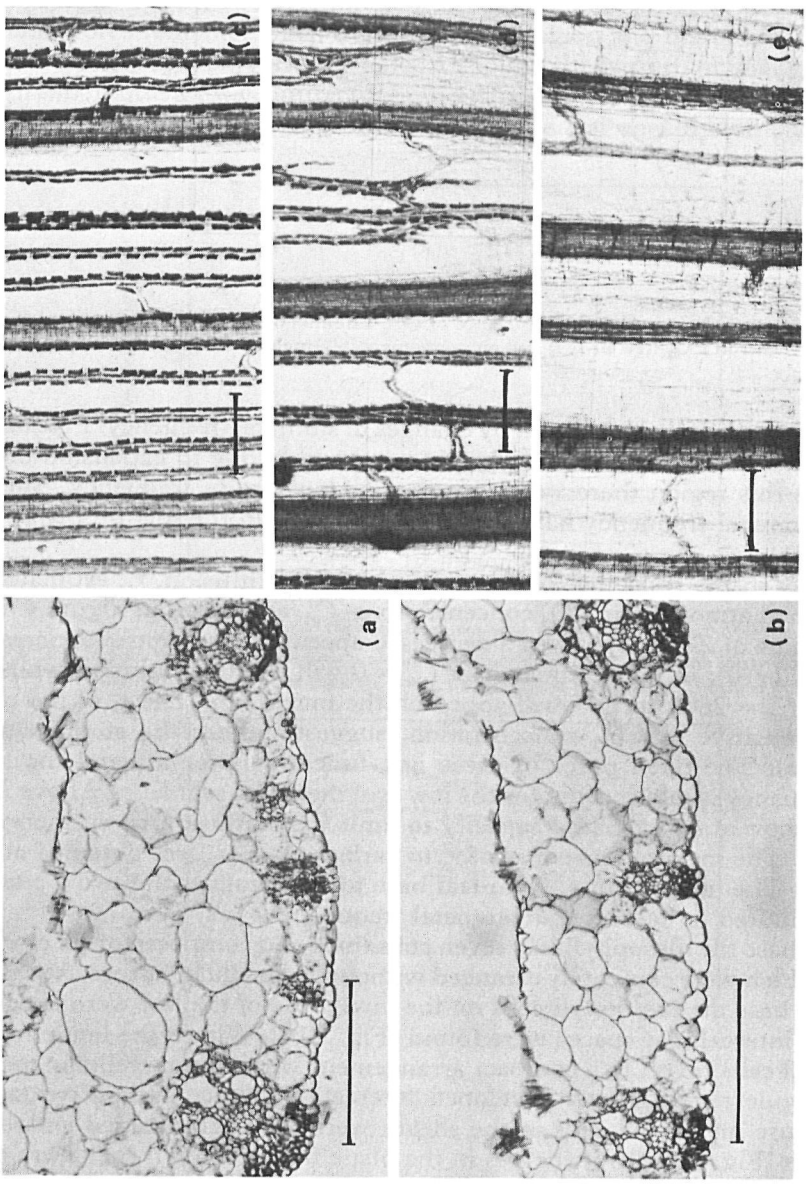

Fig. 5. (a) Transection of leaf sheath 0·5 cm below the ligule region, i.e. 5 cm from leaf base. (b) Transection of leaf sheath showing two large and one small vascular bundle. (c) Surface view of cleared leaf blade segment, 10 cm from leaf base; four or five small vascular bundles are present between pairs of large bundles. (d) Surface view of cleared ligule region. The small bundles can be seen to split at this point. (e) Surface view of cleared leaf sheath segment 3 cm from leaf base showing the alternate arrangement of large and small bundles. A single small bundle is seen between each pair of large bundles. The bars in these micrographs represent a distance of 200 μm.

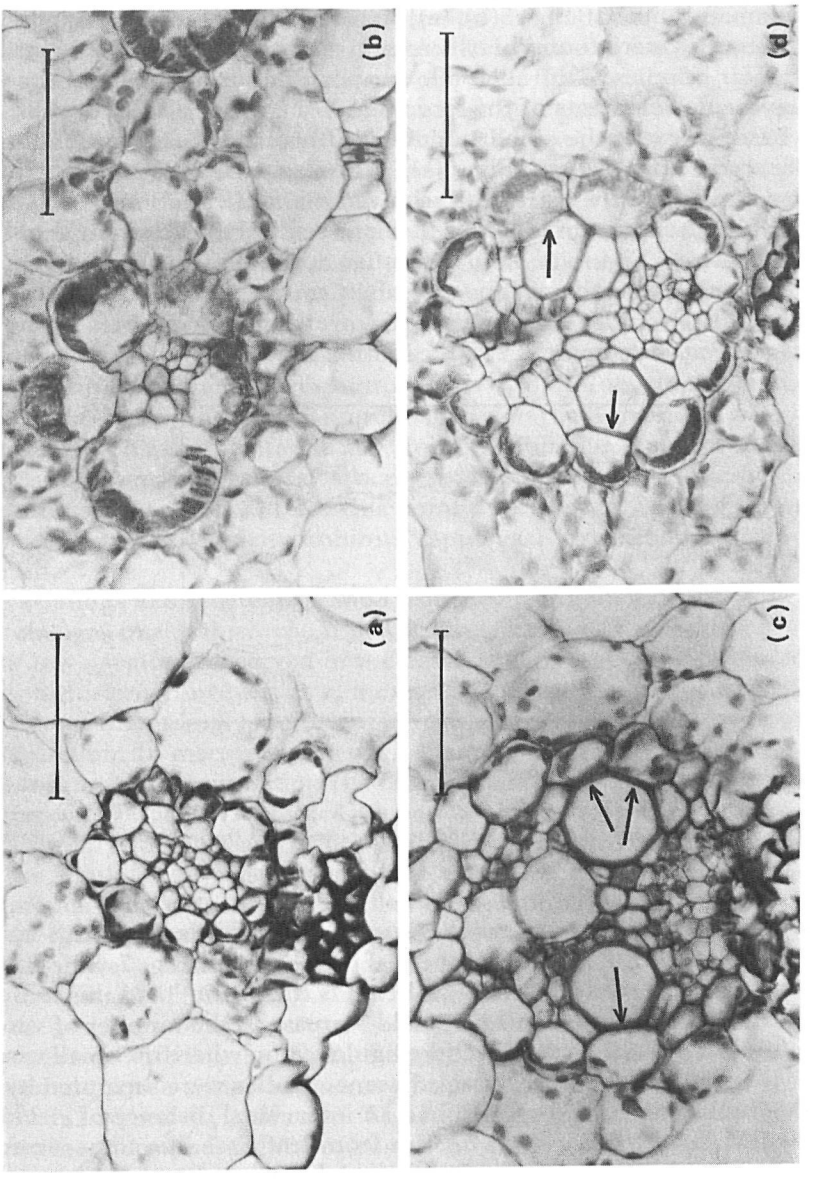

Fig. 6. (a) Transection of small vascular bundle from the leaf sheath; 3·5 cm from leaf base. Centrifugal arrangement of the bundle sheath chloroplasts can be seen. (b) Transection of small vascular bundle from leaf blade; 10 cm from leaf base. (c) Transection of large vascular bundle from leaf sheath; 3·5 cm from leaf base. The 'XyMS-condition' is shown by arrows. (d) Transection of large vascular bundle from leaf blade; 10 cm from leaf base. The 'XyMS-condition' is shown by arrows. The bars in the micrographs represent a distance of 50 μm.

chloroplasts were found only in the mesophyll cells situated next to the bundle sheath.

Two types of vascular bundles, large and small, have been found in the *Z. mays* leaf (Sharman, 1942). Within the basal regions of the leaf sheath large bundles alternate with small bundles [Fig. 5(b), (e)], however within the leaf blade three to eight small bundles were found between each pair of large bundles [Fig. 5(c)]. The large vascular bundles matured at a lower position in the leaf than the small bundles. The vascular elements of the large bundles appeared fully developed at 0·5 cm from base, whereas the small bundles did not show clear differentiation of the vascular tissue until *c.* 3 cm [Fig. 6(a)]. The size, structure and distribution of the vascular bundles in the leaf sheath showed marked variations compared to those of the leaf blade. Structural details of the vascular bundles from the sheath and blade are shown in Figure 6. Large vascular bundles of the sheath generally had incomplete bundle sheaths which were often confluent with the hypodermal schlerenchyma. The bundles were near the lower surface in the sheath [Fig. 5(a), (b)], but midway between the surfaces in the blade.

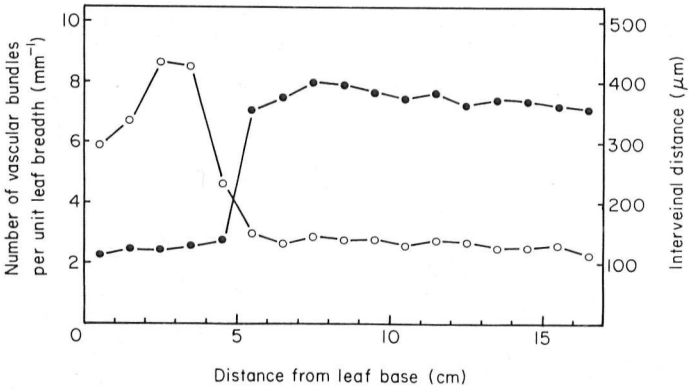

Fig. 7. Changes in the number of vascular bundles per unit leaf breadth (●) and in the interveinal distance (○) along the length of the leaf. Standard errors were less than 1 % of mean values.

The changes in the number of vascular bundles per unit leaf breadth and the interveinal distance along the length of the leaf are shown in Fig. 7. The number of bundles per unit leaf breadth in the leaf sheath is *c.* 2·5 mm^{-1} and this increased to *c.* 7·5 mm^{-1} in the leaf blade. This threefold increase in the number of vascular bundles per unit leaf breadth occurred at the ligule region where the small vascular bundles split [Fig. 5(d)]. At the leaf base adjacent bundles were separated by four to seven mesophyll cells [Fig. 5(b)] and had an interveinal distance of *c.* 295 μm. This distance increased to 433 μm at *c.* 3 cm from leaf base due to expansion of the mesophyll cells. Above 4 cm the interveinal distance showed a progressive decrease, reaching 125 to 140 μm in the leaf blade. The decreases in the interveinal distances were consistent with the number of mesophyll cells separating the adjacent vascular bundles, which was two or three at the ligule region [Fig. 5(a)] and was always found to be two within the leaf blade [Fig. 6(b)].

Discussion

The first objective of this study was to describe anatomical changes, which could affect photosynthetic activity, along the length of the second leaf in the 7-day-old *Z. mays* plant. Cuticle thickness, stomatal size and frequency and the presence of intercellular spaces within the mesophyll tissue are all features which will influence the gas exchange characteristics of the leaf. The cuticular thickening observed within the basal 3 cm of the leaf would be expected to increase the cuticular resistance to CO_2 diffusion. The cuticle at 3 cm appeared to be fully developed and would thus provide a large resistance to CO_2 diffusion. Above 3 cm the major pathway for CO_2 diffusion would be through the functional stomata.

The absence of intercellular spaces in the basal cm would seriously limit rates of CO_2 diffusion to the sites of CO_2 assimilation because rates of diffusion are lower in liquid rather than gaseous phase. In addition, the path length of liquid phase diffusion for CO_2 would be greater in the sheath than in the blade because of the presence of large non-photosynthetic parenchyma cells around the chlorenchyma. It is possible that the high respiratory activity of the developing tissue in this region (Miranda, Baker and Long, unpublished data) will maintain a high internal CO_2 concentration and obviate the need for CO_2 diffusion from the atmosphere. Above the basal 1 cm, intercellular spaces were found in the mesophyll and there was continuum of air spaces from this point to the ligule. Lack of intercellular spaces within the ligule region prohibits rapid gaseous exchange between blade and sheath.

Variations in stomatal size and frequency along the length of the leaf accounted for changes in r_s, which could modify the rate of CO_2 diffusion into the leaf. The very low r_s values, observed in the basal 2 cm of the sheath, were unlikely to limit CO_2 diffusion. However, as r_s increases with leaf development stomata are likely to become increasingly important in regulating the capacity for photosynthetic CO_2 assimilation. In mature leaves of C_4 plants r_s has been shown to be a major factor in limiting CO_2 assimilation (Ludlow and Wilson, 1971; Akita and Moss, 1972; Gifford, 1974).

The morphological and anatomical transition from the sheath to the blade occurs at the ligule. The sheath and blade exhibited large differences in the sizes of chlorenchymatous cells, the number of mesophyll layers, interveinal distance and in the size, structure and distribution of the vascular bundles. The leaves of C_4 plants have a low interveinal distance, usually below 160 μm (Takeda and Fukuyama, 1971; Crookston and Moss, 1974; Hattersley and Watson, 1975) due to the presence of only two mesophyll cells between adjacent vascular bundles (Crookston and Moss, 1974; Laetsch, 1974). The large interveinal distance recorded for the sheath segments of the second leaf of *Z. mays* shows deviations from this typical C_4 character. The leaf blade exhibits typical 'Kranz' anatomy. In transections the sheath consisted of about seven layers of mesophyll cells, however chloroplasts were observed only in the mesophyll cells adjacent to bundle sheath cells, thus it would appear that only a fraction of the mesophyll cells may be capable of photosynthesis. The vascular bundles within the leaf sheath are situated close to the lower epidermis. Such structural organization would facilitate light and CO_2 availability to the chlorenchymatous tissue since only the lower surface of the leaf sheath is exposed to the external atmosphere. The upper surface of the leaf sheath was always rolled over the inner leaves.

Although the sheath lacks typical 'Kranz' anatomy it did possess two types of

chlorenchymatous cells, mesophyll and bundle sheath, which were in intimate association, a feature considered essential for C_4 metabolism (Ray and Black, 1979). Leaf tissue of other species has been shown to exhibit non-classical 'Kranz' anatomy whilst employing C_4 metabolism for CO_2 assimilation (Hattersley, Watson and Osmond, 1977). On the basis of the C_4 acid decarboxylating mechanism found in the bundle sheath cells C_4 plants have been divided into three groups: NADP-malic enzyme, NAD-malic enzyme and PEP-carboxykinase species (Gutierrez *et al.*, 1974; Hatch, Kagawa and Craig, 1975). These three biochemically distinct C_4 metabolic groups have been associated with certain structural characteristics, such as bundle sheath chloroplast position and the degree of granal stacking within these chloroplasts (Hatch *et al.*, 1975). *Z. mays* is known to be a NADP-malic enzyme species, in which the bundle sheath chloroplasts tend to be agranal and are situated in a centrifugal position (Hatch *et al.*, 1975). Hattersley and Watson (1976) have shown that the condition where there are no cells between the metaxylem vessel elements and the laterally adjacent sheath cells of large bundles in grass leaf blades, i.e. the 'XyMS-condition', is a characteristic of NADP-malic enzyme species. This 'XyMS-condition' was present in the large bundles of leaf sheath and blade of the second leaf of *Z. mays* [Fig. 6(c), (d)]. This anatomical evidence suggests that C_4 metabolism should be present within the leaf sheath and blade of *Z. mays* leaf.

The second objective of this study was to utilize anatomical information to assess the validity of using regions along the length of a single *Z. mays* leaf as a progressive developmental gradient of the C_4 photosynthetic apparatus. Changes in anatomical characteristics expected during the normal course of leaf development are limited to the basal 3 cm of the leaf. Above this region many anatomical changes were clearly ontogenetic rather than developmental. The most striking example of this was found at the ligule, where features such as interveinal distance, cell size and mesophyll anatomy show marked discontinuities. In conclusion, the use of the single *Z. mays* leaf in studies of development of aspects of the photosynthetic processes, which are dependent on anatomical features, may be complicated by ontogeny.

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