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# Epidermal focussing and the light microenvironment within leaves of *Medicago sativa*

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The light microenvironment within leaves of *Medicago sativa* L. cv. Armor was related to the anatomy of the epidermis. Leaf epidermal cells had a convex shape and appeared to act as lenses that focussed light within the upper region of the palisade. In leaves irradiated with collimated light, epidermal focussing was demonstrated by ray tracing, photomicrography and fiber optic probe measurements, where lens signatures were observed. No relationship was observed between the location of focal spots within the palisade and chloroplast positioning. Epidermal focussing could be largely eliminated by irradiating leaves with diffuse light or by coating their surface with a thin layer of mineral oil that closely matched the refractive index of the cell walls. Measurement of variable chlorophyll fluorescence after elimination of epidermal focussing on the adaxial leaf surface caused a 19 and 11% decrease in the initial fluorescence level ($F_o$) and maximum fluorescence ($F_{max}$), respectively, whereas similar measurements from the abaxial surface were twice as large. These results suggest that some of the chloroplasts within the leaf may be adapted to local high-light conditions created by the epidermis.

*Key words* – Chloroplast, epidermis, focussing, lens, light, *Medicago sativa*, palisade, photosynthesis, variable fluorescence.

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## Introduction

Whole leaf photosynthesis is presumably a product of the photosynthetic capacity of all the chloroplasts within a leaf, their surrounding light microenvironment and the stomatal control. As light enters a leaf, it is attenuated by absorption and scattering, which create a light gradient across the leaf. The shapes of these gradients are dependent upon a number of characteristics, namely, cell size and shape, organization, pigmentation and the distribution of intercellular air space. Of special importance within green leaves is the preferential absorption of specific spectral regions by light-harvesting pigments, which rapidly deplete light within the blue and red. Consequently, chloroplasts near the upper surface of a leaf not only receive more light than chloroplasts near the lower surface of the same leaf but light of different spectral quality.

The idea that optimal photosynthetic rates at the level of the whole leaf may depend upon the specialization of individual chloroplasts is supported by several studies. Terishima and Inoue (1985a,b) have shown that, in spinach leaves, a gradient exists with leaf depth in the photosynthetic activities of chloroplasts. These chloroplasts also showed a gradient in morphology and changed gradually from sun to shade type chloroplasts from the upper to the lower surface of the leaf (Terishima and Inoue 1985a).

Although leaves of many plants are relatively thin (80–250 μm), attenuation of light by scattering and absorption can create steep internal light gradients. These gradients, measured within alfalfa leaves (*Medicago sativa*) with a fiber optic microprobe (Vogelmann et al. 1989), have shown that 90% of light at 450 and 680 nm was attenuated after passage through the epidermis and the initial 50 μm of palisade. Therefore, most of the

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chloroplasts within the leaf are exposed to a light environment that is enriched within the green and long wave red, and depleted in the blue and red regions of the spectrum. Indeed, the spectral quality was found to be very similar within the palisade and spongy layers, with the spongy mesophyll receiving only about 11% the PAR of the palisade (Vogelmann et al. 1989).

Light does not appear to decrease uniformly with leaf depth; rather, light gradients appear to be very heterogeneous, especially in the palisade of leaves irradiated with collimated light. Some heterogeneity of the light microenvironment may be caused by the positioning of the chloroplasts, and the refraction and reflection of light by intercellular air spaces. However, these are not sufficient to explain the light variability that we have measured with a fiber optic microprobe within leaves of *M. sativa*. In this plant, we have consistently obtained anomalous measurements within the palisade, which indicate an internal concentration of light well above the light levels incident upon the leaf surface. In this paper, we attribute this anomaly to the epidermis, which consists of a layer of convex cells, which appears to focus light into the palisade layer. Moreover, we provide evidence that the chloroplasts are adapted to local high-light conditions created by epidermal focussing.

*Abbreviations* – DCMU, 3-(3,4-dichlorophenyl)-1,1 dimethylurea; $F_{max}$, maximum fluorescence yield; $F_o$, initial fluorescence level; PAR, photosynthetically active radiation; SEM, scanning electron microscopy.

## Materials and methods

### Plant growth

*Medicago sativa* L. cv. Amor was grown in a greenhouse under a combination of 1000 W sodium halide lamps (KSL Sports Flood Light, Vicksburg, MS, USA; Sylvania 1000 W Metal Arc M1000 BU-HDR halide lamps), and General Electric 300 W incandescent flood lamps. Photosynthetic photon flux density (400–700 nm) was 1800 μmol $m^{-2}$ $s^{-1}$, measured with an Optronics 742 spectroradiometer (Optronics Inc. Orlando, FL, USA). The photoperiod was 12 h light/12 h dark. Mature, fully expanded leaflets from upper regions of the plant were used throughout the study.

### Microscopy

Leaflets were cut into 2–3 $mm^2$ pieces and fixed for 2 h at 20°C under vacuum in 2% glutaraldehyde in 0.025 *M* sodium phosphate buffer, pH 6.8. Monitoring the progress of glutaraldehyde fixation of fresh leaf sections observed under a microscope showed that there was no redistribution of chloroplasts during this procedure. Fixed samples were rinsed in the same buffer and dehydrated in a graded ethanol series in 10% steps. Samples were embedded in plastic or processed for scanning electron microscopy (SEM). For SEM, fixed samples were critical point dried with $CO_2$ and sputter-coated with a 15 nm layer of gold. For viewing of leaf cross sectional anatomy, fully dehydrated samples were frozen in liquid nitrogen and fractured with a chilled scalpel (Russin and Evert 1984). The fractured leaf segments were then thawed in absolute ethanol and critical point dried. Samples were examined with a JEOL JSM35C scanning electron microscope at 15 kV.

For light microscopy, dehydrated samples were embedded in Spurr's resin (Spurr 1969) and 2 μm sections were cut in transverse and paradermal planes. These sections were stained with 0.5% toluidine blue O in 0.1% $Na_2CO_3$ for 3 min at 60°C. Chloroplast distribution was mapped in transverse sections by superimposing a micrometer scale along each transversely cut palisade cell, thus dividing the cell into 8.6 μm longitudinal zones, and counting the number of chloroplasts within each zone.

To observe the focussing capabilities of the adaxial epidermis, a shallow paradermal cut was made in fresh leaves with a razor blade so that the palisade was separated from a portion of intact upper epidermal cells. This preparation was then immediately placed so that it protruded over the edge of a microscope slide, adaxial surface facing down. The exposed epidermis was then irradiated with parallel light from the microscope condenser and photographed immediately. Because the isolated epidermal cells deformed rapidly, the time between cutting, viewing and photography was 15 s or less.

### Ray tracing

Cell wall outlines of representative adaxial epidermal cells were traced from projected light micrographs. The paths of individual rays were calculated from Snell's law, using refractive index values of 1.425 for the cell wall (Gausman et al. 1974) and 1.36 for the cell cytoplasm and vacuole (Charney and Brackett 1961). No corrections were attempted for reflection or light scattering.

### Measurement of light within leaves with a fiber optic probe

The distribution of transmitted 550 nm light within *M. sativa* leaflets was measured with a 5 μm diameter fiber optic microprobe (Vogelmann et al. 1988). Leaflets were placed in a custom designed mount and the adaxial surface irradiated perpendicularly with collimated white light from a 150 W xenon arc lamp (Bornman and Vogelmann 1988). Using a high resolution stepping motor, the fiber optic probe was advanced directly through the leaflet from the shaded toward the irradiated surface and light measurements were taken at 2.5 μm increments using a computerized data acquisition system

(Vogelmann and Björn 1984). Thus, local concentrations of light on a microscopic scale were measured almost continuously as the probe passed through the leaf.

To determine if epidermal focussing was the cause of the anomalously high light measurements within the palisade, the internal distribution of transmitted light was measured under 3 experimental conditions designed to optimize or minimize light focussing by the epidermis. Leaves were irradiated with (1) collimated light (internal focussing); (2) collimated light after the adaxial leaf surface was covered with a thin film of mineral oil (reduced focussing); or (3) irradiation with diffuse light (reduced focussing). The rationale for the oil treatment was that the close match between refractive indices of the mineral oil (n = 1.478) and epidermal cell walls (n = 1.425) should eliminate most of the focussing by the epidermal cells. The same is true for the third treatment in which leaves were irradiated with diffuse light, which was created by scattering the xenon beam within an integrating sphere and mounting the leaves on one of the exit ports of the sphere.

The position of the fiber optic probe within the leaf was determined by optical signatures that marked probe entry and exit points. For example, as the probe entered the shaded leaf surface, an abrupt rise in the light readings marked the time point at which the probe entered the leaf. Similarly, as the probe exited the irradiated surface, an abrupt decline in the light readings occurred because the probe is more sensitive when wet than dry. These signatures were correlated with leaf and cell layer thickness measurements taken from fresh leaf cross-sections viewed with a microscope.

### Variable fluorescence

To see if there was any effect of epidermal foccussing upon light-harvesting by chloroplasts within the palisade layer, experiments were done with variable chlorophyll fluorescence in control leaves and in leaves in which epidermal focussing was eliminated with coatings of mineral oil. First, electron flow between photosystems I and II was blocked with $10^{-4}$ *M* 3-(3,4-dichlorophenyl)-1,1 dimethylurea (DCMU, Sigma), which was introduced into the transpiration stream in detached *M. sativa* leaflets incubated for 15–60 min under room light. Treated leaflets were then placed in a custom made holder that held them completely stationary (Fig. 1). They were allowed to dark adapt for 5 min. and then $F_o$ and $F_{max}$ were measured with a Brancker SF-30 fluorometer (Brancker Res. Ltd., Ottawa, Canada). The instrument was zeroed by substituting a piece of black velvet in place of a leaf within the holder. Since the excitation source in this instrument consisted of near parallel 670 nm light, it was possible to examine fluorescence both in the presence and absence of epidermal focussing within the same leaflet. Fluorescence was first measured in a leaflet and then repeated 5 min later in the same leaflet in which the adaxial epidermis was coated, 1 min prior to the second measurement, with a thin layer of mineral oil. This made it possible to examine the effects of removal of epidermal focussing upon $F_o$ and $F_{max}$.

## Results

### Leaf anatomy

Under our high-light growth conditions, *M. sativa* leaflets had a one- to two-tiered layer of palisade cells and a highly lacunate layer of spongy mesophyll (Fig. 2A, B). In paradermal section, the adaxial epidermis was revealed as a layer of tightly interdigitated cells (Fig. 2C). Of particular optical interest is the observation that the outer surface of each epidermal cell was convex (Fig. 2A, B, D); in other words, each epidermal cell approximated the shape of a convex lens. Although a transverse view (Fig. 2B) appears to present a variety of epidermal curvatures, these are a result of different planes of section through the cells. In fact, the radius of curvature (16 μm) through the center of each epidermal cell was very similar among cells.

### Focussing of light by the epidermis

To examine directly whether such cells could act as lenses that concentrated light, a portion of upper epidermis was isolated from the palisade by making a shallow paradermal cut with a razor blade. When this region of epidermis was irradiated with collimated light, it was observed to focus light into spots that were positioned in

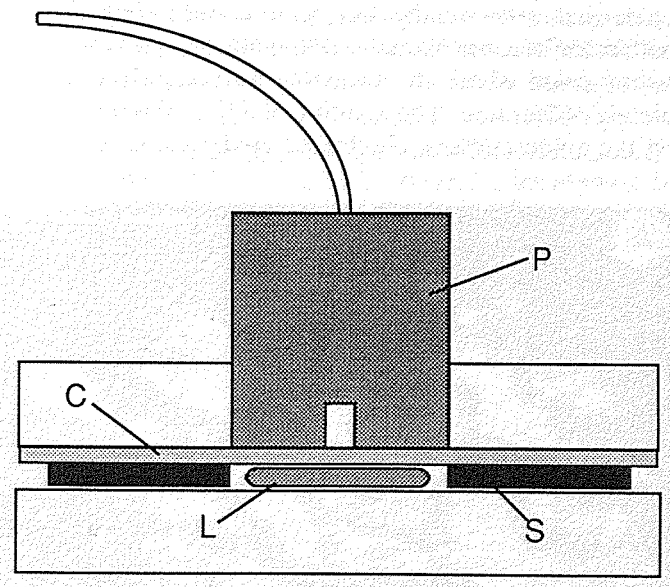


Fig. 1. Assembly for holding leaves stationary under probe of fluorometer. C, cellulose membrane 125 μm thick; L, leaf; P, fluorescence probe; S, spacer 200 μm thick. This accommodated the placement of a thin layer of oil between the cellulose membrane and the leaf sample which eliminated epidermal focussing effects.

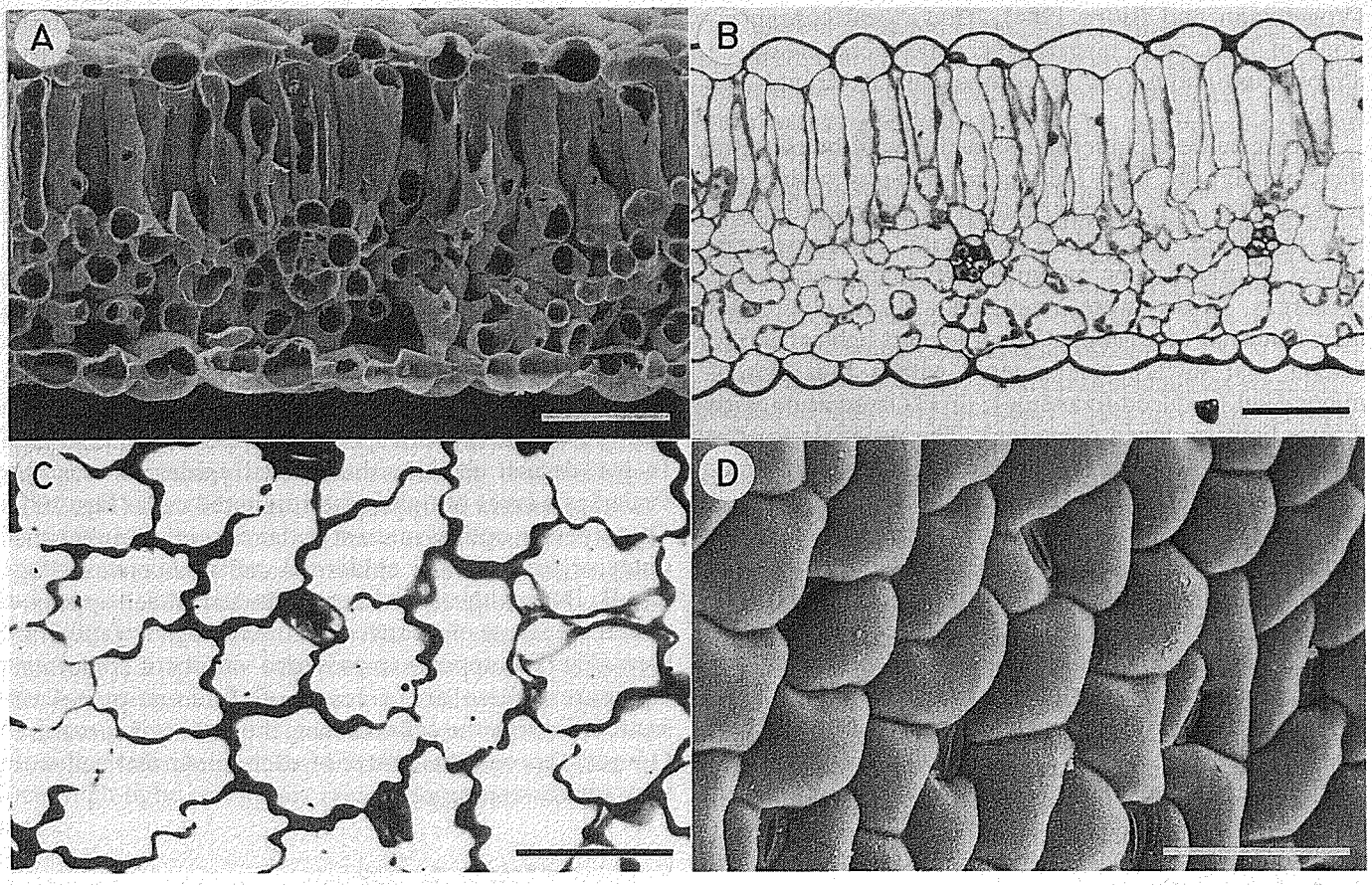


Fig. 2. Leaf and epidermal anatomy of *Medicago sativa*. A, freeze-fracture cross-section; B, transverse section; C, paradermal section midway through the adaxial epidermis; D, surface view of the adaxial epidermis. Scale bars = 50 μm.

a plane below the epidermal cells (Fig. 3). Although direct observation of the lens properties of the epidermis was possible, it was very difficult. Immediately following the creation of the paradermal cut, the isolated epidermal cells rapidly lost their convex shape, and within 15–30 s, the diameter of the focal spots expanded to the point where the focussing advantage was completely obliterated. The addition of a film of water or oil on the epidermis also eliminated epidermal focussing.

Because direct observation of epidermal focussing was so transitory, confirmation of the epidermal lens action was sought using other methods. In the first of these, ray tracing diagrams were constructed in which the path of parallel incident light was followed as it passed through individual epidermal cells (Fig. 4). Setting the angle of incoming light to 90°, a focal point was in fact modeled as the curved surface of each epidermal cell bent the light path (Fig. 4A). Moreover, the posi-

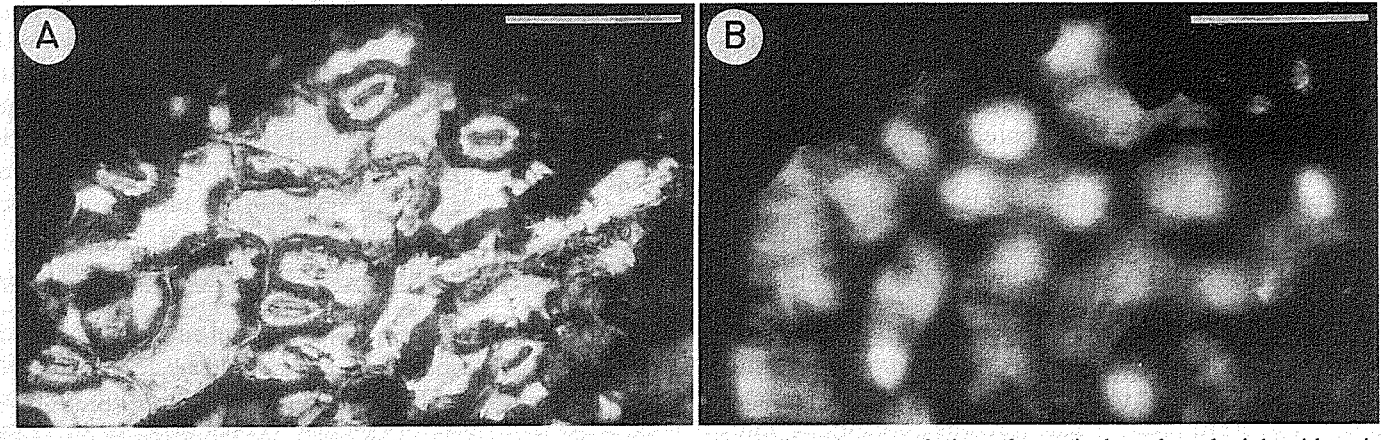


Fig. 3. Focussing of light by the upper epidermis. A shallow paradermal cut was made in order to isolate the adaxial epidermis from the palisade layer. The sample was irradiated with collimated light and photographed with the focal plane of the microscope positioned midway through the epidermis (A) and slightly beneath it (B). Focal spots in 'B' rapidly deformed within seconds as the epidermal cells lost their turgidity. Scale bars = 50 μm.

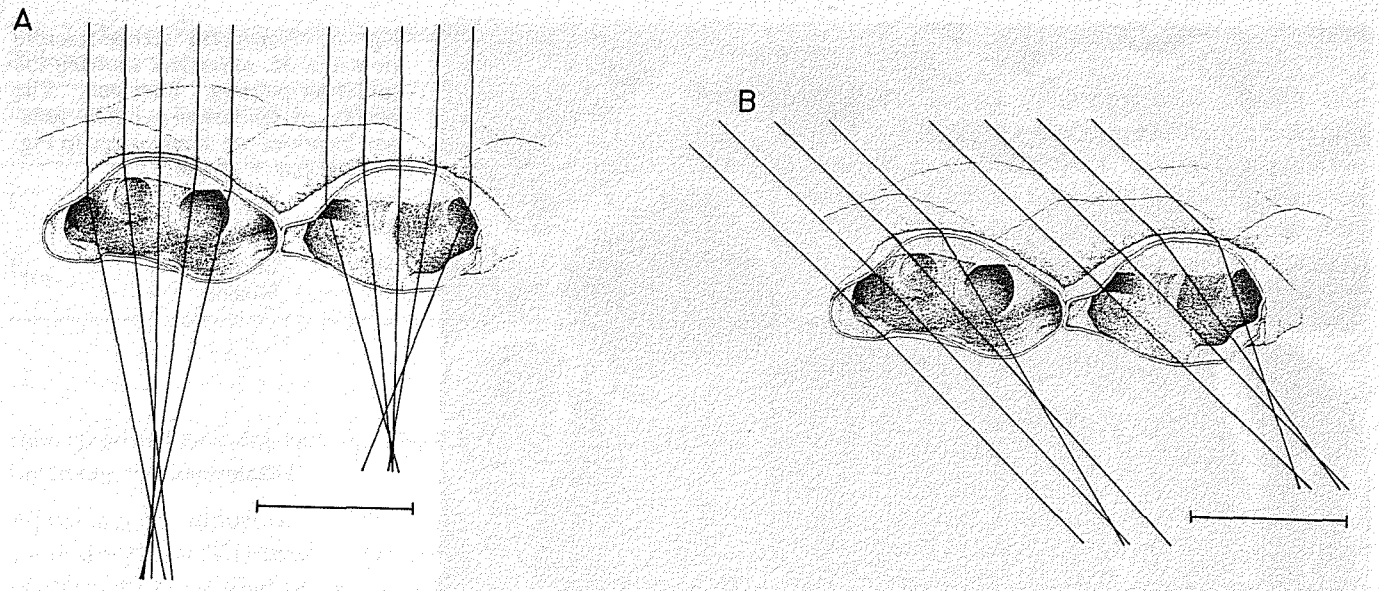


Fig. 4. Ray tracing through adaxial epidermal cells of *M. sativa*. A, with light 90° to the leaf surface; B, with incident light at 45°. The diagram shows the theoretical position of focal spots created by the epidermis. As the rays exit the abaxial wall of the epidermal cells, the path has been drawn with the assumption that the refractive indices of the palisade cytoplasm and vacuole are similar to those within the epidermis. No corrections have been attempted for light scattering which would be caused by the chloroplasts and intercellular air spaces within the palisade. Scale bar = 25 μm.

tion of the focal spot fell 30–50 μm beneath the surface of the epidermis and within the underlying palisade layer. Changing the angle of incident light to 45° appeared to increase the diameter of the focal spot and also displaced it laterally within the palisade (Fig. 4B).

Final confirmation of focussing within the palisade layer was attempted by direct measurement of light within intact leaves using a fiber optic microprobe. This was done by comparing measurements in leaves where focussing was present or reduced by coating leaves with a thin layer of oil or irradiating them with diffuse light. By advancing the probe directly through the leaf from the lower to the upper leaf surface and making continuous light measurements, it was possible to look for the presence of focal spots within the palisade layer. These could be recognized as a peak in the light readings within the palisade as the probe travelled through a focal spot. Because of the small focal spot diameter, passage through such locations by the fiber optic probe was a chance encounter. Under collimated light, peaks of light were observed within the palisade. Out of 25 measurements made in 10 different leaflets, lens signatures were observed in 4 scans. In the best of these (Fig. 5A), the peak light intensity was 60% higher than that of incident light. After correction for the angular sensitivity of the probe, this could indicate a local concentration of light 2 or more times that of incident levels. In addition, the location of the peak at 55 μm beneath the

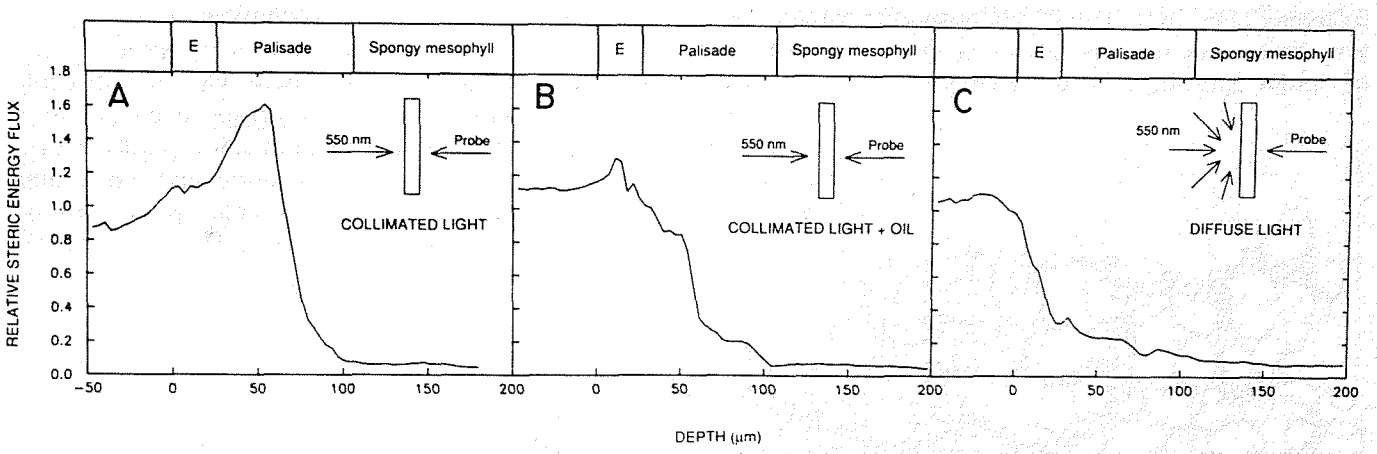


Fig. 5. The amount of 550 nm light measured with a fiber optic probe within *M. sativa* leaves. A, leaf irradiated with collimated light; B, adaxial epidermis coated with a thin layer of oil to eliminate epidermal focussing and irradiated with collimated light; C, leaf irradiated with diffuse light. The boundaries of the upper epidermis, palisade, and spongy mesophyll are shown at the top of the graph. As the fiber optic probe exits from the upper epidermis, the amount of measured light decreases because the probes are less sensitive when they are dry than wet. Note prospective lens signature in 'A' as indicated by the peak of light measured within the palisade layer.

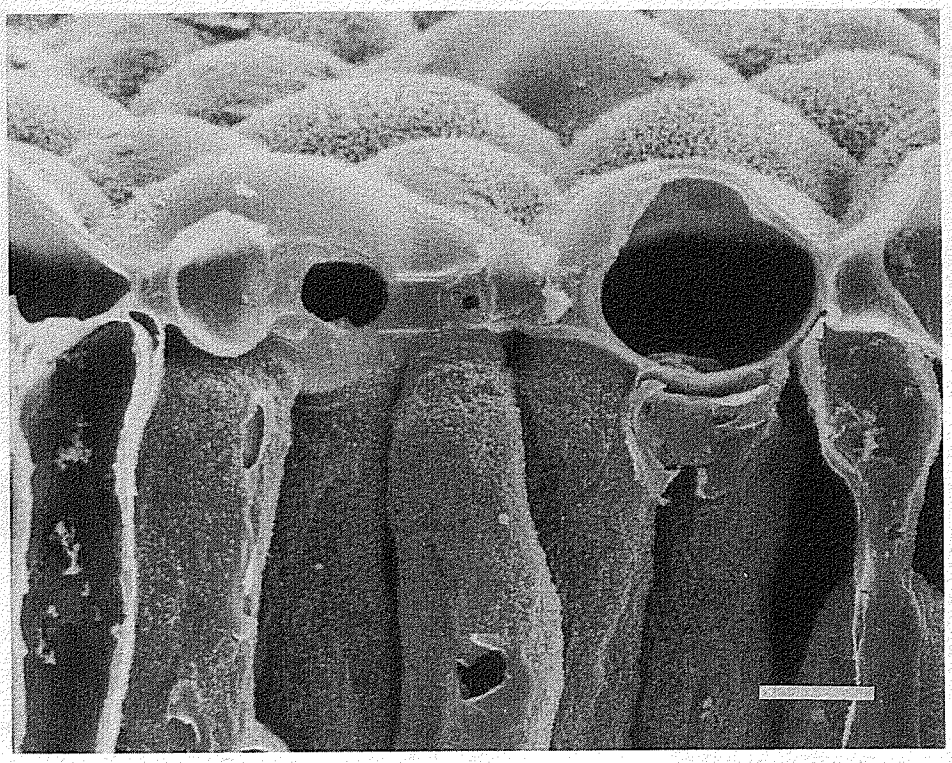

Fig. 6. Transverse freeze-fracture view of a *M. sativa* leaf showing the epidermis-palisade interface. The variety of epidermis-palisade junction locations are summarized in Fig. 7. Scale bar = 10 μm.

epidermis was close to the focal depth predicted by ray tracing diagrams.

When isolated epidermal strips were observed under a microscope, a close match between the refractive index of the epidermis (n = 1.425) and that of a layer of oil (n = 1.478) spread over the epidermis removed most of the focussing advantage. This treatment also removed the presence of lens signatures in fiber optic probe measurements within the palisade layer of intact leaves. Out of 25 measurements, aside from small light fluctuations within the epidermis (Fig. 5B) no lens signatures were recorded. Similar results were obtained when the leaflets were irradiated with diffuse light, which should also greatly reduce the amount of internal focussing (Fig. 5C). These results support general observations made over the last 2 years in which hundreds of similar scans have been collected.

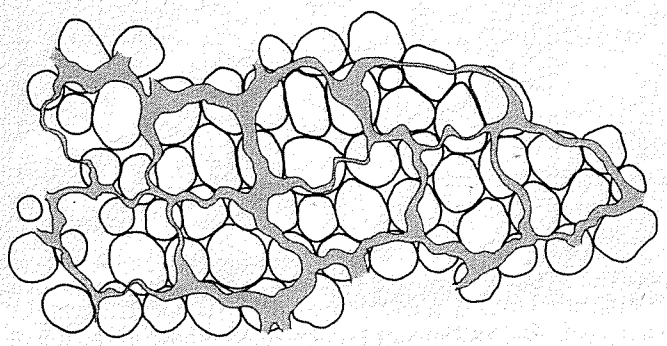

Fig. 7. Location of palisade cells beneath individual epidermal cells. Outline of epidermal cell walls is shown by the shaded area.

**Position of palisade cells and chloroplasts**

To see if there was any anatomical arrangement within the palisade relative to the position of epidermal focal spots, serial paradermal sections were used to construct a superimposition of epidermal cell wall outlines and the underlying palisade cells (Figs 6 and 7). From this it was apparent that the palisade cells did not follow any pattern in their arrangement below the epidermal cells. Rather, they were located directly in line with the transverse axis of an epidermal cell, anywhere along the inner periclinal wall, directly below one of the interdigitated lobes of the epidermal cell, or even below a junction of the 2 or more epidermal cells.

Analysis of the vertical distribution of chloroplasts (Tab. 1) indicated that chloroplasts were spread evenly throughout the palisade and there was no noticeable organization with respect to the prospective location of focal spots. The apparent random chloroplast arrangement was not an artifact of fixation and embedding procedures because monitoring chloroplast position with a microscope during this process showed no visible chloroplast movements.

Tab. 1. Vertical distribution of chloroplasts within the palisade.

| Depth from leaf surface (μm) | Number of chloroplasts per palisade cell ± SE |
|---|---|
| 0–17 | 1.7±0.9 |
| 17–34 | 1.8±0.9 |
| 34–52 | 1.8±0.9 |
| 52–69 | 1.8±0.8 |

Tab. 2. Fluorescence induction values for *M. sativa* leaves. N = 25 leaves.

| Parameters | Leaf surface | Mean ±SE | % Change (oil-control) ±SE |
|---|---|---|---|
| $F_o$ | adaxial | 2994±382 | −18.6± 8.2 |
| | abaxial | 3261±337 | −36.7±16.2 |
| $F_{max}$ | adaxial | 5363±323 | −11.3±11.3 |
| | abaxial | 5391±306 | −27.5±12.6 |

**Does epidermal focussing have any effects upon light-harvesting by chloroplasts?**

Fluorescence induction curves of detached leaves treated with DCMU made it possible to examine some of the light-harvesting properties of leaves in the presence and absence of epidermal focussing. The approach for this experiment was similar to that of the fiber optic probe measurements in that the focussing advantage could be removed by a thin layer of mineral oil. Fluorescence parameters ($F_o$, $F_{max}$) were first measured in a leaflet, and then again in the same leaflet coated with mineral oil. The results show that removal of epidermal focussing decreased both $F_o$ and $F_{max}$ by 19% (Tab. 2, Fig. 8). This indicates that removal of focussing resulted in less apparent light-harvesting. Similar data were obtained when a thin film of water was used in place of oil so that the observed effects were consistent with a change in the leaf optical properties and were not related to an artifact caused by the oil. A decrease in the fluorescence signals was found with oil treatment of either the adaxial or abaxial epidermis. In fact, the greatest decrease in $F_o$ and $F_{max}$ was observed (37%) when the lower epidermis was coated with oil. This large difference may be related to greater convexity of the abaxial epidermal cells.

## Discussion

In *M. sativa,* the convex epidermal cells strongly distorted the uniformity of the incoming radiation field and created concentrated light spots within the palisade layer. Although epidermal focussing has been described in the past for a few plants, it has been attributed to special environmental conditions or physiological adaptations. For example, Haberlandt (1914) described epidermal focussing but attributed the physiological role to providing the leaf with a mechanism for detection of light direction. More recently, Bone et al. (1985) and Lee (1986) examined epidermal focussing in tropical forest understory herbaceous plants. They suggested that epidermal focussing may be a special adaptation to the low light environment on the tropical forest floor. Even though the ambient light within the tropical understory is largely diffuse, mathematical modeling of light propagation through the epidermal cells of these plants suggested that the epidermis could concentrate diffuse light. For example, within leaves of *Anthurium warocqueanum,* which possesses highly convex epidermal cell walls, collimated light may be concentrated 20-fold and diffuse light 2-fold by the epidermis (Bone et al. 1985).

The existence of light focussing by epidermal cells in *M. sativa* and many other plants (Vogelmann et al., unpublished data), indicates that this phenomenon is not restricted to special conditions within the tropics and is widespread. Although many questions can be posed about the adaptive significance of epidermal focussing, this is beyond the scope of this article. But it should be mentioned that morphological features often serve more than one function. Convex epidermal cells not only focus light, which could be of some advantage under shade conditions, but also increase capture of light from low angles by minimizing specular reflection from the leaf surface. Both could be of advantage under

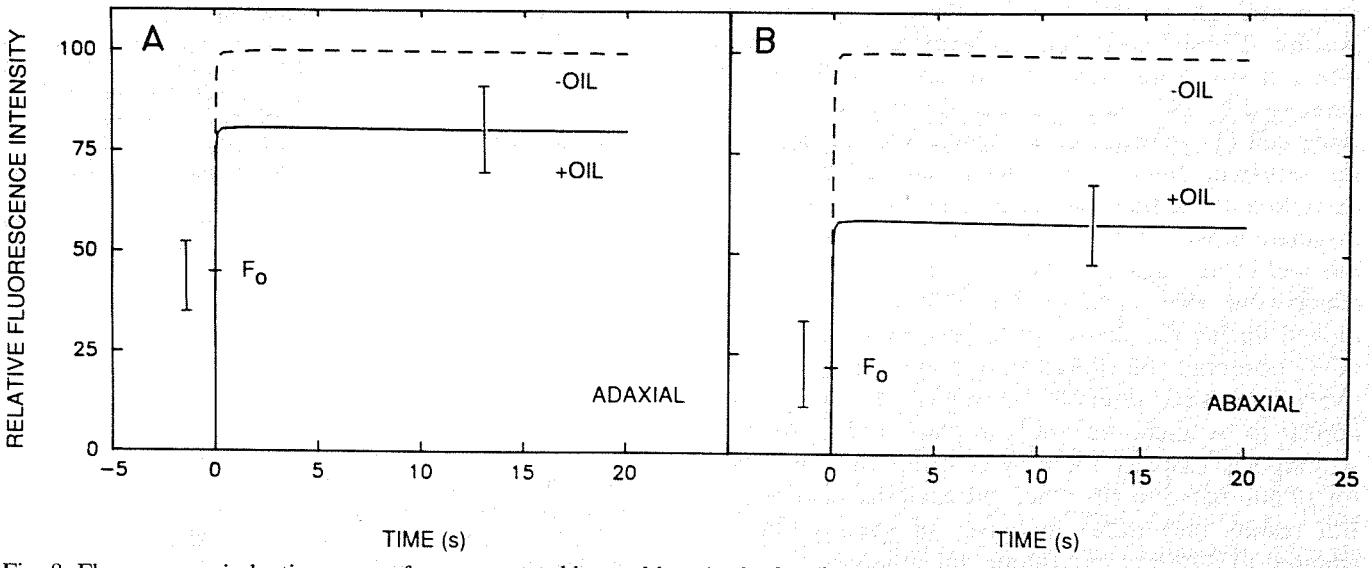


Fig. 8. Fluorescence induction curves from upper and lower *M. sativa* leaf surfaces plus or minus a thin coating of oil to eliminate epidermal focussing effects. SE bars show the variation observed for $F_o$ and $F_{max}$. N = 25 leaves.

different environmental conditions. Regardless, convex epidermal cells do concentrate light within the leaf and this appears to affect the light-harvesting properties of the chloroplasts.

How much do epidermal cells concentrate light in *M. sativa*? Although we have attempted to substantiate experimentally the presence of epidermal focussing in different ways, none are ideally suited to answer this question. Ray tracing only approximates the position and width of focal spots, paradermal preparations for photography are extremely labile and direct measurement with a fiber optic probe underestimates the amount of light. This is so because the probes have an angular light sensitivity function and only capture light that falls within their limited acceptance cone. However, taking into account probe acceptance function, curvature of the epidermal cells, cell refractive indices, probe diameter and other variables, computer modelling indicates that a focal spot in which light was concentrated to 2–3 times incident light would be required to give the peak intensities recorded in Fig. 5A. Other plants may have larger epidermal focussing effects (Bone et al. 1985).

Most importantly, fluorescence induction curves implicate a possible linkage between epidermal focussing in *M. sativa* and photosynthetic light-harvesting. Oil coatings not only eliminate focussing but also decrease reflection from the leaf surface so that more light is able to enter the leaf. This means that more light can enter the leaf for excitation of fluorescence and more fluorescence can escape from the leaf. Thus, the real differences between $F_o$ and $F_{max}$ after removal of lensing may be greater than those measured (Tab. 2, Fig. 8). Nonetheless, the finding that a layer of oil decreased both $F_o$ and $F_{max}$ is compelling evidence that some of the chloroplasts may be adapted to a high-light environment within focal spots in the palisade, i.e. removal of focussing results in less apparent light-harvesting. Indeed, fluorescence may maximize the detection of microenvironmental light conditions within the palisade. This supposition is based upon light gradient data in *M. sativa,* where it was found that the amount of 680 nm light decreased by 90% after passage through a single palisade cell (Vogelmann et al. 1989). This suggests that the returning fluorescence signal, elicited by 670 nm excitation of the fluorometer used in these studies, may originate mostly from the chloroplasts near the epidermis and in the region of the focal plane. Although it is conceivable that some of the chloroplasts may have moved during the fluorescence pretreatment, we have never observed chloroplast movement during this relatively short time interval. Moreover, the chloroplasts appear to be anchored firmly in place within the cells. Attempts to displace them by centrifugation at 1 000 *g* for 10 min failed to alter their intracellular distribution. But results may differ in leaves of species that are adapted to different environmental situations.

Another consequence of epidermal focussing is that the focal spots would not be stationary; rather, they would move laterally should the angle of light incidence deviate from perpendicular (Fig. 4B). Thus, different populations of chloroplasts would be exposed to high-light conditions with changing angles of light incidence. Although solar tracking by *M. sativa* leaves eliminates this to a certain degree, flutter by wind could cause rapid and large fluctuations in the amount of light on the microscopic scale within the leaf. How the chloroplasts would respond to this is unclear. But it is clear that the epidermis can create an extremely heterogeneous light microenvironment within leaves. Chloroplasts may adapt, not only to gradients in light quantity and spectral quality across leaves, but to local concentrations of light to levels well above full sunlight with rapid temporal fluctuations related to angles of light incidence. Although the epidermis is the first cell layer that light must penetrate in leaves, its potential importance to photosynthesis has largely been neglected and it may play a significant role in photosynthetic adaptations to the ambient light environment.

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