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Photosynthetic light gradients and spectral régime within leaves of *Medicago sativa*

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[Plate 1]

By using a fibre-optic probe, light gradients were measured at 450, 550 and 680 nm in sun leaves, 125 μm thick, of *Medicago sativa* L. cv. Armor. The space irradiances immediately beneath the leaf surface were 1.5–2.0 times greater than the incident light for these wavelengths, indicating that *M. sativa* leaves are efficient light traps. Although the palisade was only 60 μm thick, each light gradient declined steeply within this layer. More light appeared to be scattered in forward rather than backward directions and the spectral régime of the light fluxes depended upon their direction of travel within the leaf. Spectra for transmitted light were dependent upon depth within the leaf, whereas back-scattered light consisted of mostly green and far-red light at all depths. PAR (photosynthetically active radiation, 400–700 nm) within both the palisade and spongy mesophyll consisted mostly of green and far-red light, and the spongy mesophyll received only 0.11 of the PAR compared with the midregion of the palisade. Anomalous measurements within the palisade were traced to the epidermis, which was found to act as a mosaic of microlenses that focused light within the palisade layer. In *M. sativa* leaves, the light microenvironment, leaf anatomy and photosynthesis seem to be strongly interrelated.

Introduction

Photosynthesis, at the level of the whole leaf, is presumably a product of the photosynthetic capacity of the individual cell layers, the light régime within those layers and the stomatal control. Although information is accumulating about the photosynthetic properties of the chloroplasts at different depths within leaves (Terishima & Inoue 1984, 1985*a*, *b*), little is known about the intra-leaf light environment and how this is related to leaf anatomy. Presumably there is a close relation between anatomy, photosynthetic light gradients and photosynthesis at the whole-leaf level.

The difficulty in ascertaining the light microenvironment within leaves originates from their complicated optical properties (Vogelmann & Björn 1986). As light enters a leaf, it can be absorbed or scattered so that a light gradient is created. Because the epidermis, palisade and spongy mesophyll layers each have different optical properties (Terashima & Saeki 1983), the amount of light absorption and scattering change as light travels progressively from one layer to the next. Thus both the amount of light and its spectral quality change with leaf depth, and this change is mediated differently as light passes through vertical tubular palisade cells as opposed to more randomly arranged spongy mesophyll cells.

That light gradients are important within leaves is evidenced by a corresponding gradient observed for the photosynthetic properties of chloroplasts. In spinach, near the irradiated leaf surface, the palisade chloroplasts have 'sun' characteristics whereas within the spongy

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mesophyll they have 'shade' characteristics. As one progresses from the top to the bottom of the leaf, there is a gradual change from sun to shade chloroplasts both in electron transport components and chloroplast morphology (Terashima & Inoue 1985*a*, *b*). The functional specialization of chloroplasts probably maximizes energy capture and photosynthetic capacity at different leaf depths. In view of the importance of understanding how the light environment within leaves relates to photosynthesis, we have measured light gradients and spectral régime within the leaf of the common C_3 plant, *Medicago sativa*, by using a relatively new fibre-optic technique.

Fibre-optic microprobe and measurement of internal light

Medicago sativa L. cv. Armor was grown in a greenhouse under a 12h:12h photoperiod and 1500 μmol quanta m^{-2} s^{-1} PAR (400–700 nm). The fifth–eighth fully-expanded leaves from the apex were used in experiments. Leaflets were typically 120 μm thick with approximately equal vertical distances occupied by palisade and spongy mesophyll (figure 1, plate 1). Leaf chlorophyll *a* and *b* contents were 3.15×10^{-3} and 1.20×10^{-4} mg Chl mm^{-2}, respectively, as determined from extinction coefficients published by Arnon (1948).

Fibre-optic probe system

Light distribution and spectral régime were measured in *M. sativa* leaves with a fibre-optic microprobe computerized data-acquisition system described previously (Vogelmann & Björn 1984; Vogelmann *et al.* 1988). Leaves were firmly mounted in a custom-designed holder and irradiated with collimated light from a 150 W xenon arc (Hanovia 901-C1). In all cases the upper (adaxial) epidermis faced the light beam. The fibre-optic probe had a tip diameter of 3 μm and light readings were taken every 2.5 μm as the probe was advanced through the leaf at 5 μm s^{-1} with a high-resolution stepper motor. The amount of light measured by the probe is called the *relative steric energy flux*, which emphasizes the fact that the probes are directional sensors with Gaussian light-acceptance functions (Vogelmann & Björn 1984).

Measurement of light gradients and spectral régime within leaves

Light gradients were determined at 450, 550 and 680 nm by measuring the distribution of transmitted and scattered light with three microprobe sampling orientations (figure 2). These sampling orientations were necessary because of the optical characteristics of the microprobe and the light scattering characteristics of leaves. When the probe was advanced through the leaf at 0°, the distribution of transmitted (collimated and scattered) light was measured. Advancing the probe through the leaf at 30° measured the distribution of forward-scattered light, whereas at 150° back-scattered light was measured. This was replicated six times in three different leaves (two replications per leaf) in regions free of vascular tissue. The data were averaged and light gradients were determined by combining the three sets of measurements to calculate the space irradiance against depth (Vogelmann & Björn 1984). Space irradiance is synonymous with the term *internal fluence rate* and quantifies the amount of light propagated in three dimensions. This is necessary for quantification of light within intensely light-scattering media such as plant tissues (Vogelmann 1986).

For measurement of spectral régime, the fibre-optic probe was positioned midway through the palisade layer or midway through the spongy mesophyll at 0°, 30° or 150°. The spectrum

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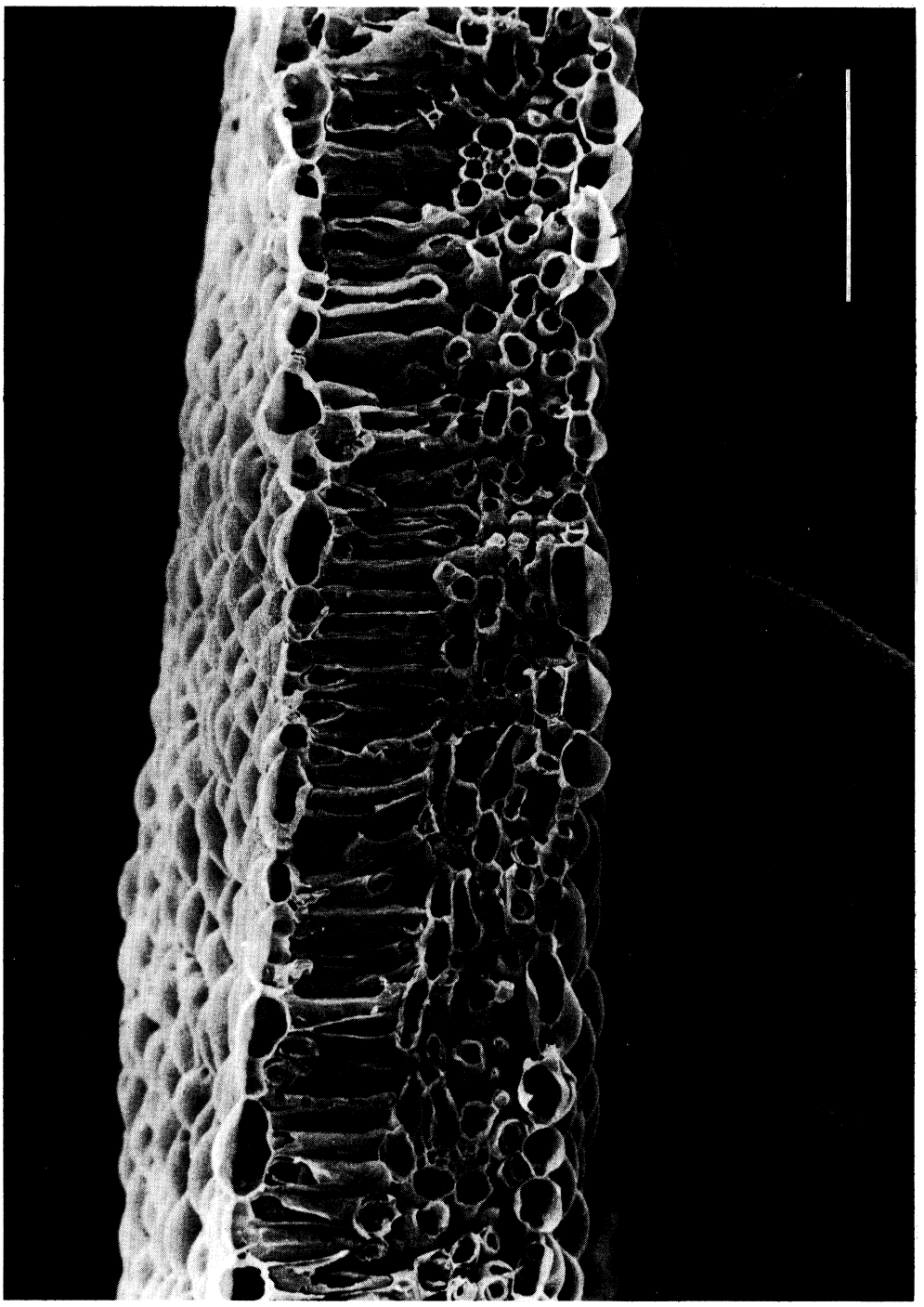

FIGURE 1. Transverse freeze–fracture view of an *M. sativa* leaf. The specimen was prepared by fixation in glutaraldehyde, dehydration in ethanol, freezing and fracturing in liquid nitrogen. The leaf was then critical-point dried and viewed with a JOEL scanning electron microscope. Scale bar 100 μm. Note the plano-convex curvature of the surface of each epidermal cell.

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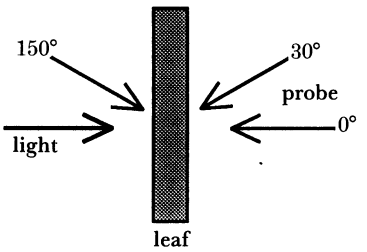

FIGURE 2. Microprobe sampling orientations within an *M. sativa* leaf. Each leaf was positioned so that the adaxial epidermis was irradiated with collimated white light. The distribution of light across the leaf was measured by advancing the microprobe through the leaf at 0°, 30° or 150°. Light readings were taken every 2.5 μm at 450, 550 or 680 nm. Alternatively, the probe was positioned at specific locations within the leaf and the spectrum scanned from 400 to 700 nm.

was scanned from 400 to 700 nm and was normalized by dividing by the output spectrum of the xenon lamp. Spectral space irradiance was determined by combining the measurements at 0°, 30° and 150° in a manner similar to the calculation of light gradients.

LIGHT GRADIENTS WITHIN *M. SATIVA* LEAVES

We chose to measure light gradients at wavelengths where there was strong absorption (450 and 680 nm), and weaker absorption (550 nm). The distribution of transmitted (figure 3) and scattered light (figure 4) through *M. sativa* leaves showed several notable features. The distribution of transmitted light (figure 3) was the product of a complex relation between light scattering and absorption. Here, it is important to remember that fibre-optic probes are directional sensors. When a probe was advanced directly through the leaf, from the shaded toward the irradiated surface, the light measurement obtained was strongly dependent upon the location of the probe within the leaf. When the probe was positioned immediately beneath the adaxial surface, it measured mostly collimated light transmitted through the epidermis. At this location there was little absorption or scattering at the selected wavelengths. In contrast, when the probe was positioned within the spongy mesophyll, it measured only a quantity of light that remained after attenuation by absorption and scattering. Scattering is a particularly important consideration because the probe only measures the light that falls within its acceptance cone. Two important features of the distribution of transmitted light are: (1) 680 nm light was attenuated more rapidly than 550 nm light; and (2) optical discontinuities, evidenced by small rises in the scans, marked probe transition between the spongy mesophyll and palisade layers. One additional point is that data for transmitted light were highly variable. Although this may be expected in view of the fact that a single chloroplast can shade the probe, as will be discussed later, much of this variability was traced to the epidermis.

The distribution of scattered light (figure 4) was measured by advancing the probe obliquely through the leaf from the irradiated (adaxial) surface toward the shaded surface. These measurements are of special importance because scattered light contributes the major proportion of light to the space irradiance within the leaf. Rather strikingly, the amount of scattered 680 nm light fell to less than 10% of its initial level within the vertical distance between the upper epidermis and the initial half of the palisade layer. In contrast, scattered 550 nm light showed a more complex distributional relation including a gradual decline within the palisade layer and a step transition at the palisade–spongy mesophyll boundary.

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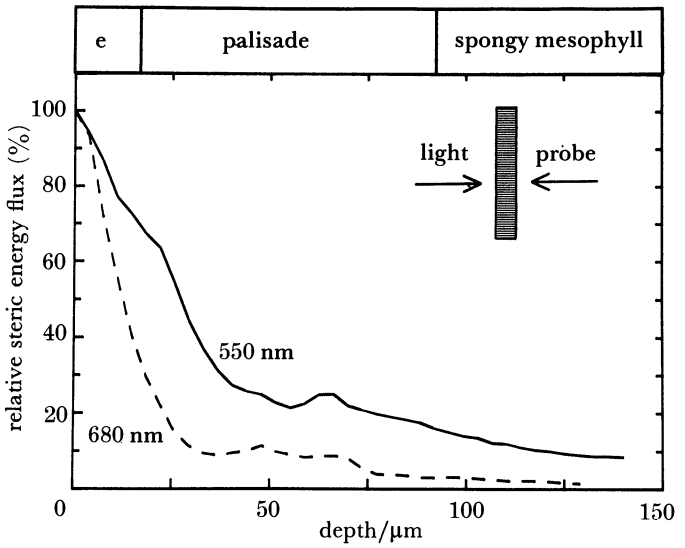

FIGURE 3. Representative scans showing the distribution of transmitted light across an *M. sativa* leaf. The curves are a product of light attenuation by both absorption and scattering. The distribution of 450 nm light was very similar to that of 680 nm and is not shown. The vertical distance occupied by the upper epidermis, palisade and spongy mesophyll is shown at the top of the graph.

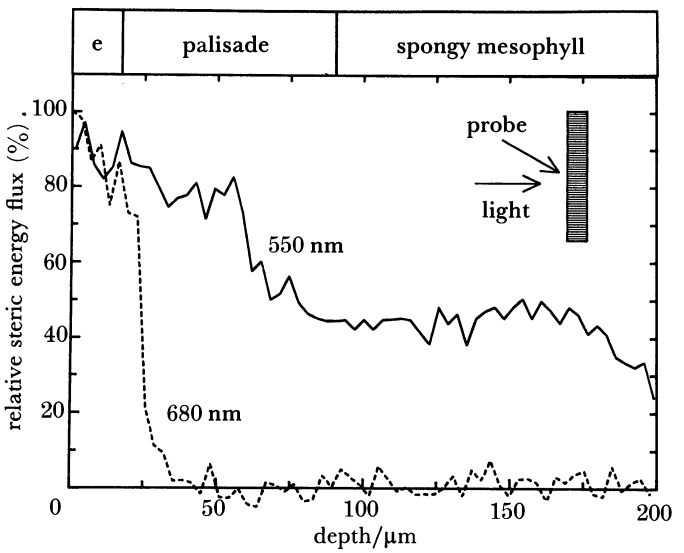

FIGURE 4. Representative scans showing the distribution of scattered light across an *M. sativa* leaf. Scattered 450 nm light was very similar to 680 nm light and is not shown.

The degree of curvature of the distribution of scattered light is indicative of the amount of absorption. When scattered light decreases exponentially with depth, this indicates significant amounts of absorption; whereas a more nearly linear distribution indicates scattering but little absorption. The rapid attenuation of scattered 680 nm light within the palisade indicates that most of the 680 nm attenuation in figure 4 was due to absorption. In contrast, much less light at 550 nm was absorbed and a greater portion of the attenuation of 550 nm light in figure 4 was caused by scattering. It should also be pointed out that quantitatively more 550 and 680 nm light was transmitted than back-scattered. This indicates that scattering within *M. sativa* leaves was neither complete nor isotropic.

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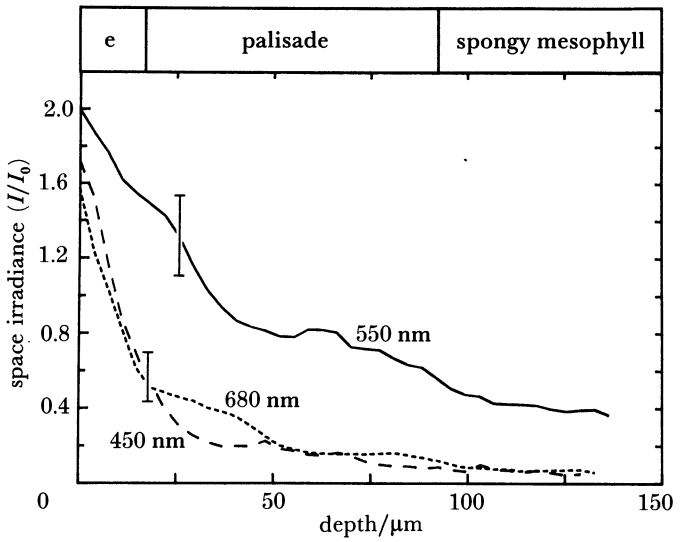

FIGURE 5. Light gradients across an *M. sativa* leaf. Vertical bars indicate standard error between six replicate samples.

Calculation of the light gradients showed a steep decline of the space irradiance at 450 and 680 nm, with a more gradual decrease at 550 nm (figure 5). The space irradiance near the irradiated epidermal boundary was near 1.6 for both 450 and 680 nm light but this decreased to 0.4 or less within the initial 50 μm of the leaf. In fact, most of the light at the three wavelengths was attenuated within the palisade layer. Relatively large variability in the amount of light was measured within the palisade; this would be expected in part on the basis that a single chloroplast can shade the probe. A chloroplast–probe encounter would be a random event and the optical effects would be most striking in a region of the leaf where the incoming light was not yet absorbed or strongly scattered. However, this appears to explain only some of the variability. In some cases, the measurements indicated increasing amounts of light at progressively greater depths beneath the epidermis. This anomaly appears to be caused by the epidermis, a point we will examine below.

SPECTRAL RÉGIME WITHIN *M. SATIVA* LEAVES

Spectral quality of light at different depths within the leaf was measured by positioning the probe at a location midway through the palisade and scanning the spectra of transmitted, forward-scattered and back-scattered light. As shown in figure 6, different levels of light fluxes were passing through the leaf in different directions. For example, the flux for transmitted light (0°) at 550 nm was approximately 3.7 times greater than forward-scattered light (30°) and 20 times that of the back-scattered light (150°). These fluxes had different spectral compositions (figure 7). Whereas transmitted light showed some depletion in the red and blue, back-scattered light consisted almost entirely of green and far-red light. Forward-scattered light (30°) had a spectral composition intermediate between these two extremes.

As the light passed through the remaining portion of the palisade layer and half-way through the spongy mesophyll its characteristics were altered further. At a location midway through the spongy mesophyll, the light became more diffuse so that approximately equal fluxes were

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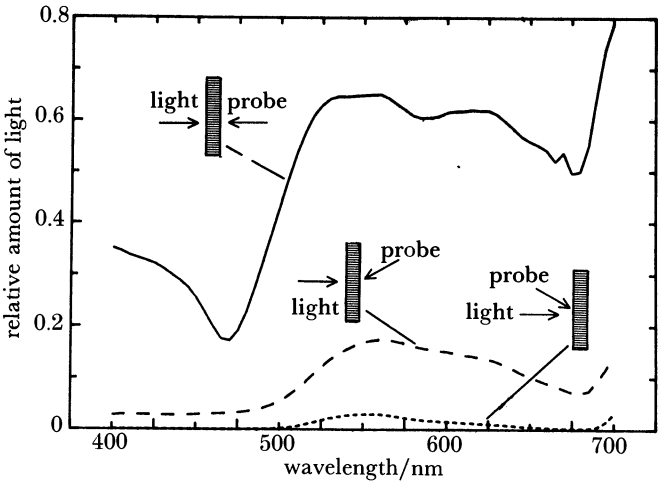

FIGURE 6. Relative amounts of light measured at different sampling orientations within the palisade layer of *M. sativa*. The fibre-optic probe was positioned midway through the palisade at one of the three indicated sampling orientations and the relative amount of light measured between 400 and 700 nm. Spectra have been corrected by dividing by the emission spectrum of the xenon arc beam as it occurs at the leaf surface.

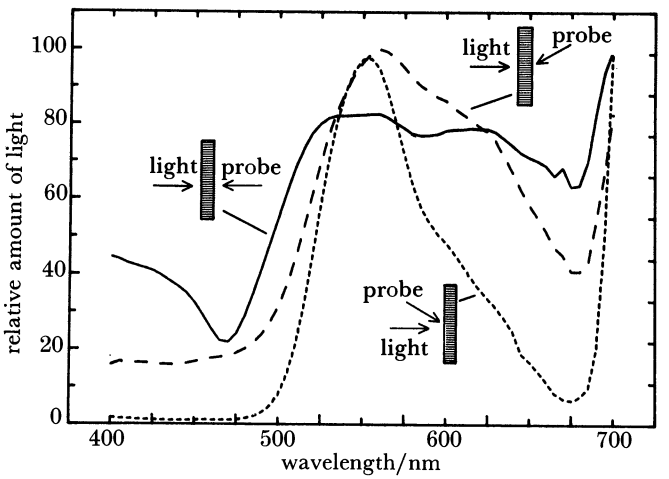

FIGURE 7. Spectral régime at a location midway through the palisade of *M. sativa*. Data from figure 6 have been normalized to 100%.

measured at 0° and 30° (figure 8). However, there was still more light moving in the forward rather than backward direction, as the amount of light measured at 150° was only one seventh that at 0° or 30°. In contrast to the palisade, where there were different spectral compositions between transmitted and back-scattered light, the spectral quality of the light fluxes measured at different directions within the spongy mesophyll was very similar. Regardless of sampling orientation, the spectral régime within the spongy mesophyll consisted almost entirely of green and far-red light (figure 9).

Combining the measurements to calculate the spectral space irradiance shows the difference between the spectral environment within the palisade as opposed to the spongy mesophyll (figure 10). Notable features include: (*a*) enrichment of green and far-red light within the middle of the palisade to 1.4 times higher than levels incident upon the leaf surface; (*b*) the

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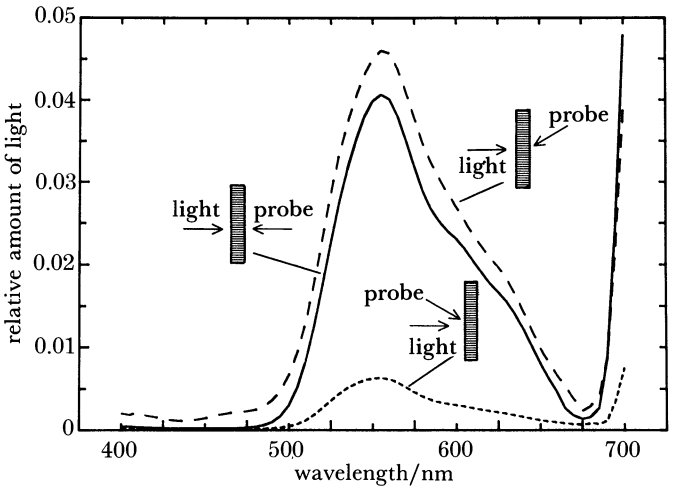

FIGURE 8. Relative amounts of light measured at different sampling orientations within the spongy mesophyll of *M. sativa*. Data were collected in a manner similar to those in figure 6.

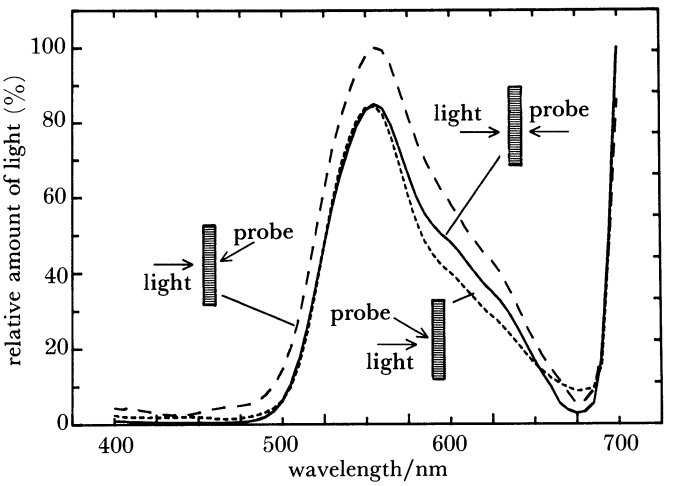

FIGURE 9. Spectral régime at a location midway through the spongy mesophyll of *M. sativa*.

presence of about nine times more photosynthetically active radiation (400–700 nm) in the middle of the palisade as opposed to the middle of the spongy mesophyll; and (*c*) the almost complete depletion of blue and red light by the palisade layer.

OPTICAL ANOMALIES WITHIN THE LEAF

Although there were relatively steep light gradients across *M. sativa* leaves at most of the photosynthetically active wavelengths, these gradients were not completely uniform between neighbouring palisade cells. There was often large variability in the amount of light measured within adjacent palisade cells in scans of transmitted light. Anomalous measurements included the peculiar case where there appeared to be more light in the upper palisade than within the epidermis (figure 11). Because we could not explain these data in a straightforward manner, we directed our attention to leaf anatomy as a potential source of this anomaly. As shown in

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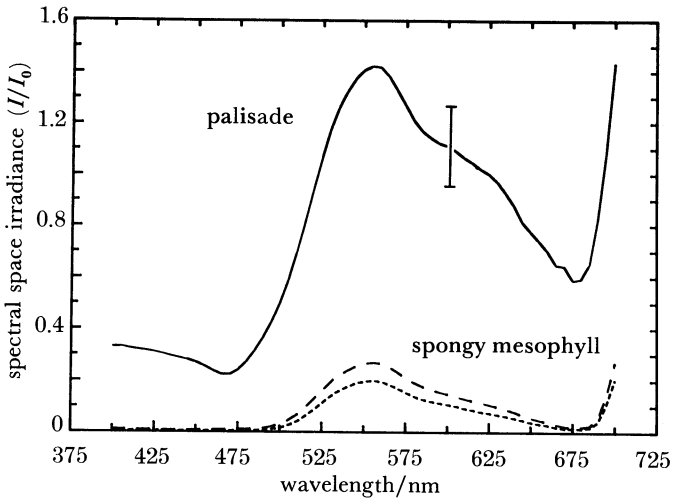

FIGURE 10. Spectral space irradiance at locations midway through the palisade and spongy mesophyll of *M. sativa*. The spectral space irradiance was calculated as the ratio between the space irradiance inside to that outside the leaf for each wavelength.

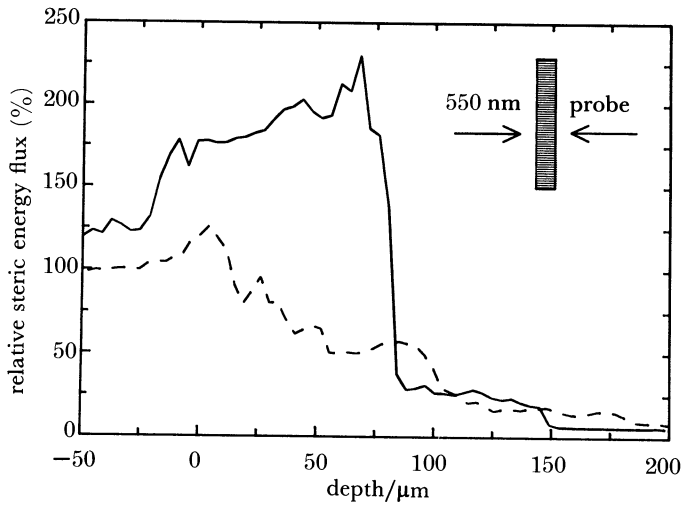

FIGURE 11. Extreme examples of variability in measurements of transmitted light within the palisade.

figure 1, the adaxial epidermis of *M. sativa* leaves is composed of a mosaic of epidermal cells with plano-convex shaped surface walls. Passage of collimated light through this layer should severely distort the uniformity of the radiation field, since the difference between the refractive indices of air ($n = 1.000$), the cell walls ($n = 1.425$), and sap ($n = 1.36$) converts each epidermal cell into a lens. Indeed, ray-tracing diagrams indicated that, not only could each epidermal cell focus light to a small spot, but also the calculated focal plane happened to fall within the upper region of the leaf in the palisade layer (figure 12). These results were verified by examining fresh paradermal leaf sections under a microscope, and each epidermal cell was observed to concentrate light from the condenser lens into a small spot. Moreover, the location of the predicted focal plane coincided with the region within the leaf where we encountered the largest variability in our measurements. At greater depths, the measured light gradients

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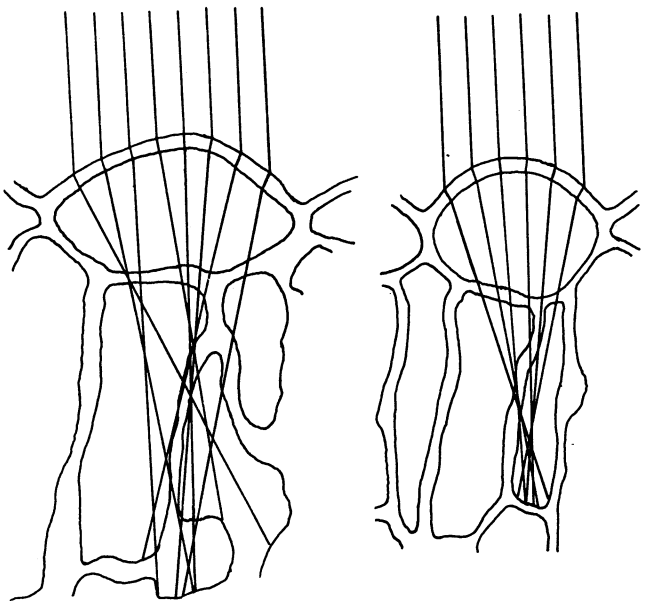

FIGURE 12. Ray tracing through epidermal cells of *M. sativa*. The refractive indices used for the cell wall and cytoplasm were 1.425 and 1.36, respectively.

became more uniform, indicating that these heterogeneities were rapidly attenuated by absorption and scattering by the underlying cells.

DISCUSSION

Examination of the internal light environment within the leaves of *M. sativa* has revealed some notable features. Light scattering and internal reflection can trap light within the leaf so that the internal space irradiance can exceed that of incident light. In *M. Sativa* leaves, the I/I_0 values near the irradiated surface of 1.7, 1.55 and 2.0 for 450, 550 and 680 nm light, respectively (figure 5), agree reasonably well with theoretical studies based upon light-scattering equations (Fukshansky-Kazarinova *et al.* 1986) formulated by Kubelka-Munk. For example, in greened cotyledons of *Cucurbita pepo*, the calculated space irradiance near the irradiated surface was 1.2 and 1.5 for 450 and 660 nm, but this rose to 2.7 for more weakly absorbed light at 730 nm (Seyfried & Fushansky 1983). More recently, Kaufmann & Hartmann (1988) have devised a new experimental approach and, in bifacial leaves from three different plants, they have measured surface photon fluxes of 1.26–2.01 for 548 nm and 1.12–1.69 for 672 nm light. Allowing for differences in experimental approaches, and the varying optical properties of the plant materials, these values may indicate typical space irradiances found near leaf surfaces.

The light gradients found within the palisade were steeper than expected, especially in view of the fact that this layer was only 60 μm thick, and that the tubular shape of the cells should minimize light scattering. In *M. sativa*, the gradients at 450 and 680 nm dropped to 15% of their initial values within the first half of the palisade cell. This suggests that in studies of leaf fluorescence, where strongly absorbed excitation light is utilized, the returning fluorescence signal may originate mostly within the initial 50 μm or so of the leaf tissue.

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It is interesting to point out that the gradient in photosynthetic activities measured across a 600 μm thick spinach leaf (Terashima & Inoue 1985*b*), was much more gradual than the light gradients measured in *M. sativa* (125 μm thick). Although light-gradient information for spinach is lacking, the relatively gradual changes in the measured photosynthetic activities suggest that chloroplast properties may develop in response to the total PAR present within the leaf as opposed to the amount of light present at a specific wavelength. Along this line, it has been suggested that photosystem I and II stoichiometry and composition may be regulated by the amount of electron flow between the two photosystems (Glick *et al.* 1985; Melis 1984). Such a regulatory mechanism would integrate both the spectral quality and amount of light at different depths within a leaf. It is interesting to note that even in the very thin leaves of *M. sativa*, the PAR decreased by about 80% from the middle of the palisade to the middle of the spongy mesophyll, a distance of only 60 μm.

It should be emphasized that chloroplasts may have to adapt to light conditions much more extreme than those predicted on the basis of measured light gradients. Light trapping within leaves by scattering alone will create conditions where chloroplasts could be exposed to space irradiance values several times those of full sunlight. Epidermal features can greatly complicate the situation. In *M. sativa*, the epidermal cells act as lenses that concentrate light within the palisade and this may be intercepted by specific chloroplasts. At present it is difficult to estimate exactly how much light is concentrated by focusing in *M. sativa*, but in tropical understory species with similar plano-convex shaped cells, modelling studies suggest that they may concentrate an incident beam of collimated light up to 20-fold (Bone *et al.* 1985). Although epidermal focusing was described as early as 1914 by Haberlandt, the physiological significance and possible effects upon photosynthesis are currently unknown. It is intriguing to speculate over the possible photosynthetic significance of such cells in plants and appropriate experiments are currently in progress. Whatever their function, their presence increases variability in the light gradient tenfold higher than that in leaves with a topographically flat epidermis.

Although fibre-optic probes are intrusive, many of the current measurements can be verified by other experimental approaches and it appears to be a reliable technique; we hope that it can be used in other ways (e.g. fluorescence) to provide additional information relating to photosynthesis. We would like to point out that, although the light environment within *M. sativa* leaves may be similar to that within other leaves, it is difficult to say exactly what internal light régime would be present within a leaf with a different anatomy, such as a different C_3 plant, or in C_4 and CAM plants. In view of the wide variety in leaf structure and pigmentation among plants, it is tempting to speculate that there may be a close relation between leaf structure, light gradients and the photosynthetic properties of the chloroplasts. All of these probably contribute to photosynthesis at the whole leaf level.

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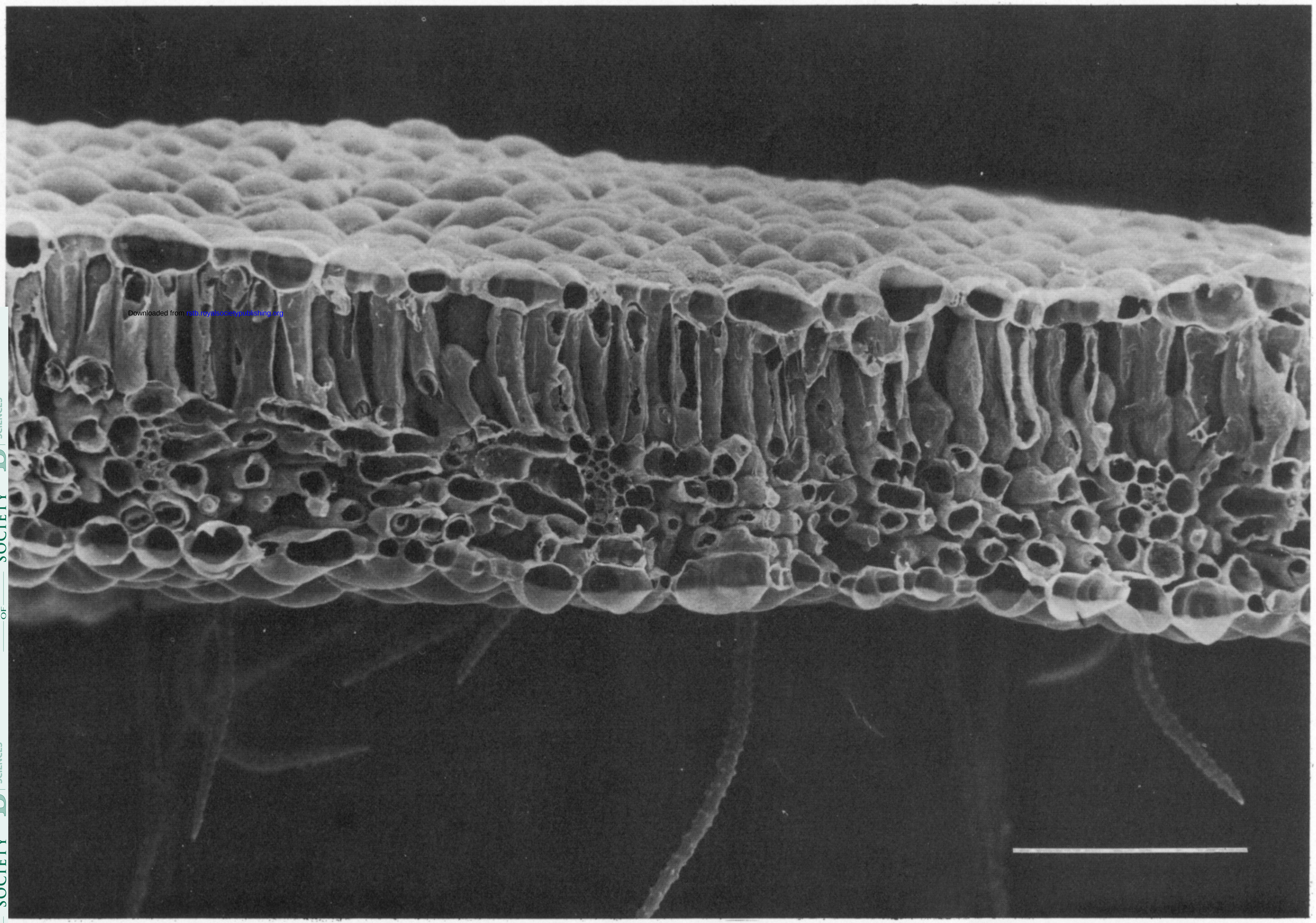

FIGURE 1. Transverse freeze–fracture view of an *M. sativa* leaf. The specimen was prepared by fixation in glutaraldehyde, dehydration in ethanol, freezing and fracturing in liquid nitrogen. The leaf was then critical-point dried and viewed with a JOEL scanning electron microscope. Scale bar 100 μm. Note the plano-convex curvature of the surface of each epidermal cell.

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