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Gas Exchange and Phytoluminography of Single Red Kidney Bean Leaves during Periods of Induced Stomatal Oscillations

A DEMONSTRATION OF AN INTEGRATED, SPATIALLY RESOLVING PHYSIOMETRIC TECHNIQUE

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ABSTRACT

This report examines the capabilities of a new approach to the study of gas exchange and electron transport properties of single, intact leaves. The method combines conventional aspects of analysis with an image intensification system that records the spatial distribution of delayed light emission (DLE) over single leaf surfaces. The combined system was used to investigate physiological perturbations induced by exposure of single leaves of *Phaseolus vulgaris* cv 'California Light Red' to a combination of SO_2 (0.5 microliters per liter) and ozone (0.1 microliters per liter). Exposure of one-half of a leaf to this combination induced DLE and stomatal oscillations, but only in the half of the leaf exposed to the combined gases. Examination of phytoluminographs taken during these oscillations revealed distinct leaf patches where the greatest changes in DLE intensity occurred. This phenomenon is interpreted to be evidence that control of stomatal activity of intact plant leaves occurs within discrete leaf areas defined within the vascular network.

Comparison of physiological variables of one leaf with another is routinely confounded by variations in genotype, leaf age, prior environmental history, and even the time of day when measurements are made. In addition, various internal control processes may cause the photosynthetic activity in one area of the leaf to be entirely different from that of another. These variabilities complicate the interpretation of physiological studies. To minimize these variabilities, we have designed an experimental system that conforms to the following criteria. (a) The system must be able to measure properties of single leaves while still attached to the plant; (b) control and treatment must be done simultaneously on the same leaf; (c) the system must provide some means of resolving the photosynthetic activity of small (*e.g.* ~1 mm^2) leaf areas; (d) the system must provide continuous monitoring of electron transport and gas exchange properties of both control and treatment portions of the leaf.

These criteria are met through an experimental design that combines two different technologies. First, gas exchange measurements of each bilateral half of a single leaf are determined with the use of a special two-chambered leaf cuvette. Second, the spatial distribution of electron transport processes over the surface of a single leaf is determined through detection and imaging of DLE[1].

DLE is a plant luminescence that occurs when light-generated photosynthetic intermediates recombine in the dark to produce an electronically excited state of Chl that fluoresces. Although the DLE phenomenon has been studied quite extensively since it was first reported by Strehler and Arnold in 1951 (22), only recently has the sensitivity of DLE of intact plants to environmental effects been studied (17). Bjorn and colleagues have subsequently demonstrated that image-intensified DLE can be used to photograph plant leaves (2, 23). More recently, Blaich *et al.* (4) presented additional data relating DLE images to photosynthetic activity. This process of photographing plants by DLE has been termed 'phytoluminography' (23).

An earlier report from this laboratory (7) presented results that demonstrated some of the capabilities of the combined DLE/gas exchange system. The purpose of the present report is to provide more complete details of this system and to describe how this system meets the criteria enumerated above. Data used to demonstrate this compliance derive from physiological perturbations brought about by controlled exposure of single red kidney bean leaves to a combination of O_3 and SO_2.

MATERIALS AND METHODS

Plant Culture. *Phaseolus vulgaris* L. cv 'California Light Red' was grown from seed in a peat moss:perlite:vermiculite (3:1:1) potting mixture. Plants were cultured in a greenhouse with day and night temperatures maintained at 26°C and 21°C, respectively. The photoperiod was extended to 16 h/d with high intensity discharge sodium vapor lamps. Plants were fertilized twice weekly with a modified Robbins (20) nutrient solution. Analysis of gaseous exchange and investigation of DLE were carried out on intact leaflets chosen from plants 21 to 28 d old.

Gas Exchange Measurements. A clear Plexiglas cuvette was designed to isolate photosynthetic gas exchange and DLE of two halves of a bilaterally symmetrical leaf (Fig. 1). The cuvette consists of two separate 15- × 20- × 6.5-cm compartments, each of which is divided along the long axis by a Plexiglas partition. The two separate compartments can be clamped together with a leaf held between. The closed cuvette has two separate compartments, each of which encloses one-half of a leaf in a volume of 1.0 L. Flow through each chamber was maintained at 2.6 l/min. Closed cell foam insulation provided a leak-free seal between cuvette sections and prevented damage to the petiole and midrib vein. Leaflets were supported between two monofilament grids. All internal surfaces of the cuvette were lined with Teflon film to reduce gas adsorption. One miniature electric fan (Radio Shack) was mounted in each half of the cuvette to facilitate gas mixing and to reduce boundary layer resistance. Temperatures of the leaf and cuvette were measured using type K thermocouple sensors: one sensor was mounted in contact with the adaxial leaf surface and another positioned in the chamber atmosphere.

Water, CO_2, and trace hydrocarbons were removed from the

[1] Abbreviation: DLE, delayed light emission.

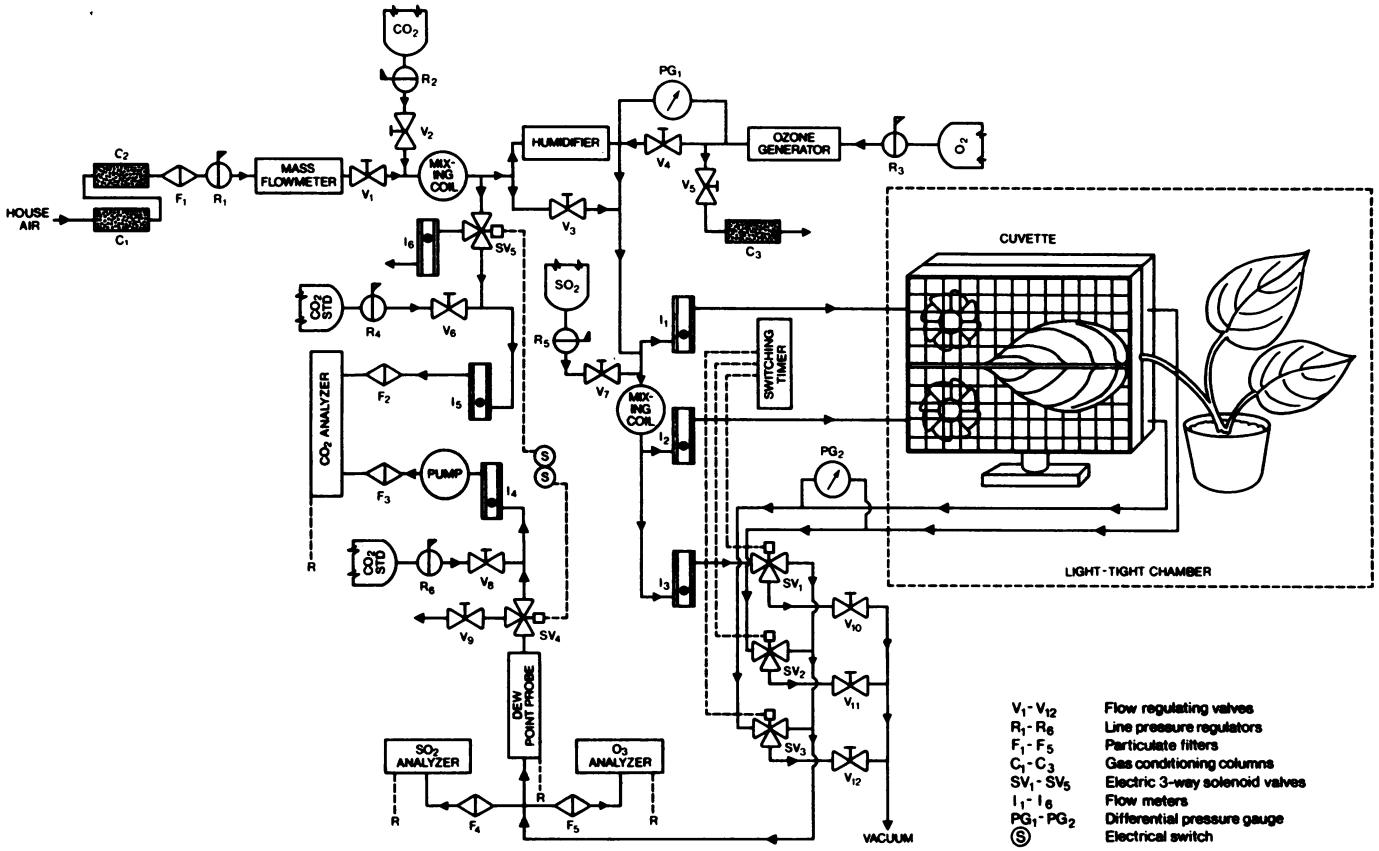

FIG. 1. Schematic of gas exchange circuitry used for the experiments of this report.

supply air by passing the gas stream sequentially through columns of indicating silica gel (Fisher Scientific, 12 mesh), Ascarite II (Fisher Scientific, 20–40 mesh), and molecular sieve (Analabs type 5A). Dew point was controlled by bubbling a proportioned volume of incoming dry air through water. Carbon dioxide and SO_2 from cylinders, and O_3 from an ultraviolet lamp generator were supplied to the air stream via precision metering valves and capillary tubing. All tubing (except capillary) was 0.64 cm o.d. copper or Teflon; connector fittings were brass, stainless steel (Swagelok), or Teflon.

CO_2 concentration was determined differentially using an IR gas analyzer (Anarad model AR-600R) calibrated with certified standard CO_2 concentrations (Scott ±1% analyzed). SO_2 and O_3 concentrations were determined using a pulsed fluorescent SO_2 monitor (Thermo Electron Series 43) and a calibrated chemiluminescent O_3 analyzer (Monitor Labs 8410A). Water vapor was quantified using a digital humidity analyzer (EG & G model 911) incorporating a flow-through sensing probe. Water, CO_2, SO_2, and O_3 concentrations could be determined at cuvette inlet and outlet ports utilizing 3-way solenoid valves (Valcor Engineering Corp. No. 54P7605E) to sample at desired time intervals. Sampling lag times between the different instruments were less than 15 s, and instrument time constants were all less than 60 s. In-line flow regulating valves (Dwyer series RM and VF) and a differential pressure gauge (Dwyer Magnehelic series 2000) connected to the cuvette atmosphere enabled flow control measurement and balancing. Total air flow into the system was monitored with an electronic mass flow meter (Matheson model 8143).

Preliminary studies were undertaken to determine leaf boundary layer resistance to water efflux. Calculations were derived from measurements of the steady-state net flux of water vapor transfer from a saturated leaf replica (Whatman No. 4 filter paper). In addition, optimum water vapor transfer from the leaf to the chamber atmosphere was determined by monitoring changes in water vapor flux at different fan speeds. Boundary layer and stomatal resistance values were calculated using the following equation:

$$R = \frac{W_{sat} - W_o}{Tr}$$

where R = total or boundary layer resistance to water efflux from leaf replica or intact leaflet ($s \cdot cm^{-1}$); W_{sat} = saturated water vapor concentration at the temperature of the leaf or leaf replica ($g \cdot cm^{-3}$); W_o = water vapor concentration measured at the cuvette exit ($g \cdot cm^{-3}$); Tr = net flux of water vapor transfer ($g \cdot cm^{-2}s^{-1}$). Tr was calculated according to the relation

$$Tr = (W_o - W_i)^*(F/A),$$

where W_o and W_i are the water vapor concentrations at the cuvette outlet and inlet, respectively; F is the flow rate of the air stream through the cuvette, and A is the leaf area exposed within the cuvette. Water vapor concentrations were derived from Smithsonian tables that related water vapor content to dew point measurements. Typical boundary layer values measured for leaf replicas were less than 0.2 $s \cdot cm^{-1}$. Leaf water vapor conductivities (Fig. 4) were calculated as the reciprocal of leaf resistance measurements.

DLE Detection System. The DLE detection system is similar to that described by Bjorn *et al.* (2, 23) (Fig. 2). Both plant and cuvette were placed in a light-tight box provided with ports for connecting the cuvette to the gas exchange system, for illuminating the sample, and for DLE detection. A 61-cm rotating sectored wheel was driven by a ½-horsepower electric motor rotating at a speed of approximately 40 Hz. DLE produced in the dark between flashes was detected by a Ni-tek NVC/100 image intensifier. The image produced by the image intensifier was either recorded directly on film (Kodak Tri-X) or its intensity observed through the reflex mirror of the camera and monitored with a photodiode.

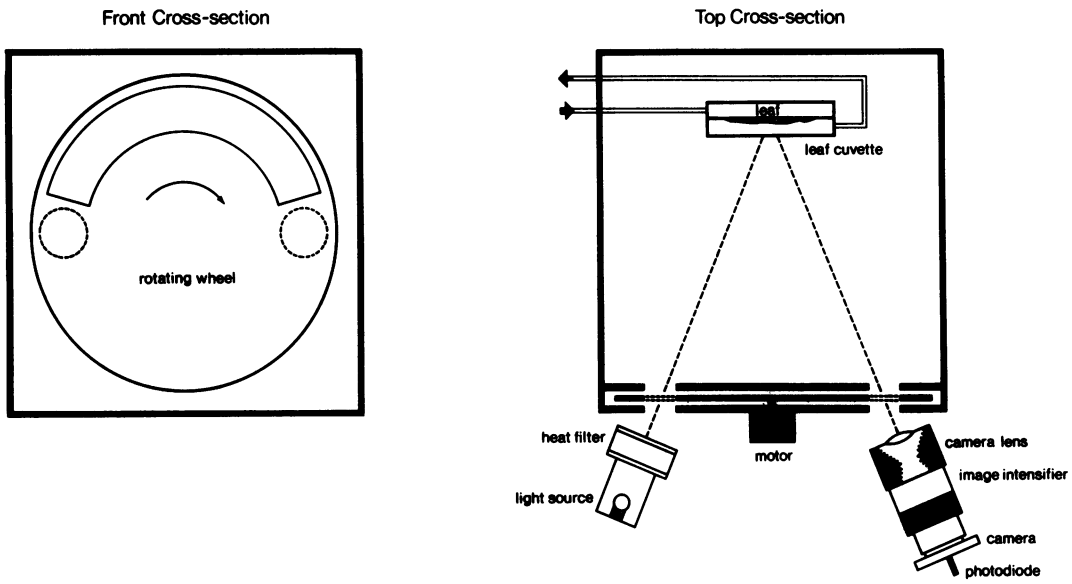

FIG. 2. Schematic showing geometry of plant enclosure used for DLE measurements. See "Materials and Methods" for details.

The latter device provided an analog signal that was recorded on strip chart.

White light illumination was provided by a 1,000-w projector lamp. The light was passed through 10 cm of water to filter out a large portion of the radiant heat. The average light intensity incident on the leaf was approximately 600 $nE \cdot m^{-2} \cdot s^{-1}$ with the wheel rotating, 1,600 $nE \cdot m^{-2} \cdot s^{-1}$ with the wheel stopped in the open position. Illumination with 600 $nE \cdot m^{-2} \cdot s^{-1}$ light was nonsaturating and produced about 50% of the CO_2 uptake produced by 1,600 $nE \cdot m^{-2} \cdot s^{-1}$ light. No difference in CO_2 uptake rates occurred if the incident light was chopped and averaged 600 $nE \cdot m^{-2} \cdot s^{-1}$ or was constant at 600 $nE \cdot m^{-2} \cdot s^{-1}$ with the wheel stopped.

RESULTS

Two halves of a single red kidney bean leaflet were treated to the following protocol: at time = 45 min (see Fig. 3), 0.55 $\mu l/l$ SO_2 was admitted into the upper leaf chamber. A slight increase in DLE occurred at this point. After approximately 10 min, a 10-min pulse of 0.1 $\mu l/l$ O_3 was introduced into both chambers. Shortly after the introduction of O_3, oscillations in CO_2, H_2O, and SO_2 exchange and uptake began, but only in the half of the leaflet that had been previously exposed to SO_2 (*cf.* the solid and broken lines in curves a and b in Fig. 3). The DLE from the upper half of the leaflet also showed oscillations (Fig. 3, curve d).

Calculations based on data obtained from the curves in Figure 3 provided estimates of changes in water vapor, CO_2, and SO_2 exchange that occurred during the period of oscillations. Figure 4 compares the magnitude of changes in CO_2 and SO_2 uptake with changing leaf water vapor conductivity. Whereas the CO_2 *versus* water vapor conductivity curve tended to level off at higher conductivities, the SO_2 uptake appeared to be linear over the same conductivity values.

Extended exposure of other red kidney bean leaves to either gas alone at concentrations similar to those used in this report did not produce the oscillations in gas exchange evidenced in Figure 3. The presence of both gases at once is required.

The oscillations in DLE (Fig. 3, curve d) were synchronous, but almost exactly 180 degrees out of phase with the oscillations in CO_2 uptake; *i.e.* peaks of DLE emission corresponded to troughs of CO_2 uptake. These data suggest that reductions in available CO_2 would cause an increase in DLE, and this is the case: the DLE of bean leaves increased when the cuvette inlet CO_2 concentration was reduced (Fig. 5).

The two DLE images shown in Figure 6 were recorded at the peak (A) and trough (B) in the last oscillation in the DLE curve shown in Figure 3. These photographs demonstrate that changes in DLE intensity did not occur uniformly over the foliar surface. Instead, the observed changes in DLE occurred within discrete areas of the leaf. Close examination of the images revealed that these DLE areas were bounded by the vascular system.

DISCUSSION

The combined gas exchange and DLE detection system described meets the four experimental design criteria enumerated in the Introduction. The first design criterion, that the system be able to measure physiological variables of single, attached leaves, is satisfied through the cuvette design that allows an entire leaf to be enclosed and measured without producing a significant stress on the leaf or petiole. The data shown in Figure 3 demonstrate that the cuvette design also fulfills the second criterion: an ability to measure for control and treatment effects in the same leaf. The third and fourth criteria are met through a comparison of gas exchange and DLE phenomena recorded during the experiment.

It is assumed that the oscillations In gas exchange (Fig. 3) are due to synchronized stomatal movements. Although several environmental factors could account for the initiation of stomatal oscillations, including changes in leaf temperature (16), humidity (9), ambient CO_2 concentration (5), and light intensity (5), none of these factors changed until the oscillations began. It is more likely that the perturbation was produced by a biochemical response to the combined SO_2/O_3 exposure. Interactive effects of O_3 and SO_2 on growth of plants are well known (6, 18), and each gas alone has been reported to evoke stomatal movement (1, 3, 8, 12, 15, 19). The stomatal oscillations that occurred after brief exposure of a leaf to O_3 and SO_2 is a further indication that stomatal control mechanisms are particularly sensitive to this combination of pollutants. The biochemical or physiological basis for this response is not known.

The relationship between CO_2 uptake and leaf water vapor conductivity (Fig. 4, curve a) suggests that CO_2 uptake is diffusion-controlled at lower conductivities (uptake increases with diffusion rate) but tends to reach a limiting value at higher conductivity.

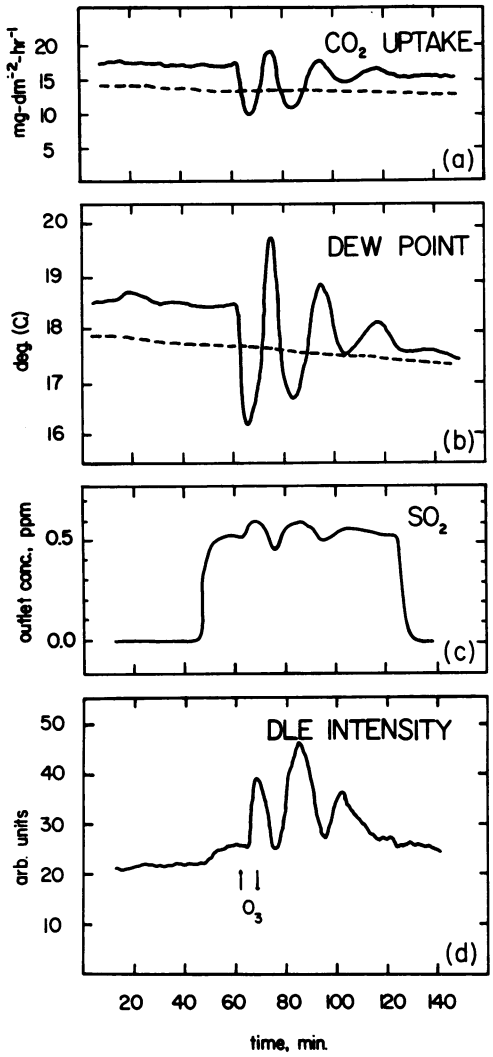

FIG. 3. a and b, CO_2 uptake rate and dew point measured at cuvette outlet for treated upper (——) and control lower (- - -) halves of a kidney bean leaf. c, SO_2 concentration at exit port of upper cuvette chamber. d, Integrated intensity of DLE of upper leaf half. The upper portion of the leaf was exposed to 0.5 μl/l SO_2 at time = 45 min. A brief pulse of O_3 (0.1 μl/l, on and off at times indicated by the arrows on curve d) was introduced into both chambers. Inlet CO_2 concentration was approximately 350 μl/l, cuvette temperature was 23°C, and average light intensity over the leaf was 600 $nE \cdot m^{-2} \cdot s^{-1}$.

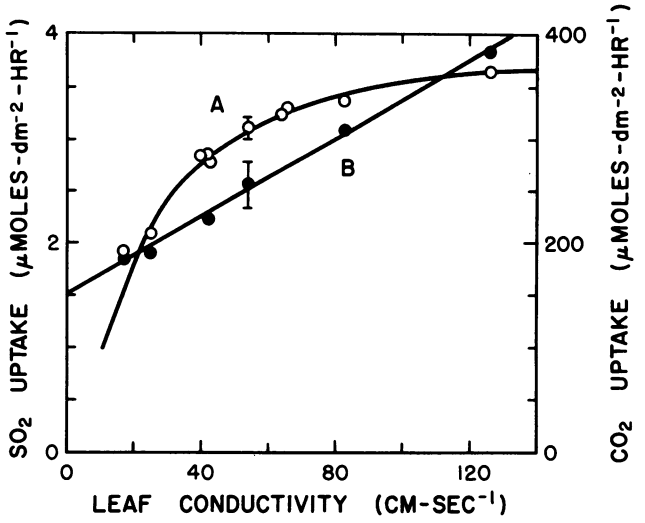

FIG. 4. Relation of CO_2 (curve A) and SO_2 uptake (curve B) to leaf water vapor conductivity. Values were derived from gaseous concentrations determined at the peaks and troughs in curves a (CO_2), b (H_2O), and c (SO_2) of Figure 3. The y-intercept of curve b provides an estimate of passive SO_2 uptake by both the leaf and cuvette surfaces. Measurements made using a blank cuvette indicated that 80 to 90% of this passive uptake was due to the cuvette.

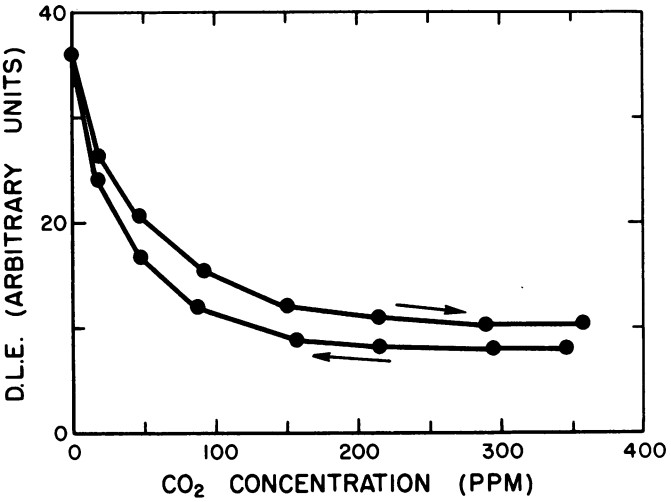

FIG. 5. DLE intensity of a single bean leaflet during the decrease and increase in cuvette inlet CO_2 concentration. The 4.5-fold increase in DLE shown is typical; observed increases range between 3- and 8-fold. Arrows refer to the direction of change in CO_2 concentration. The hysteresis in this curve is most likely due to changes in leaf conductivity and CO_2 uptake over the course of the experiment. Other experimental conditions were similar to those noted in Figure 3.

Inasmuch as the intensity of illumination was not saturating, this curve probably indicates that CO_2 uptake is light-limited at these higher conductivities. In contrast, the relation between SO_2 uptake and water vapor conductivity (Fig. 4, curve b) appears to be linear over the same conductivity range. Curve b is characteristic of an uptake process that is diffusion-limited over the observed conductivity range. Furthermore, the non-zero intercept of curve b represents SO_2 uptake by paths independent of stomatal control (*e.g.* surface adsorption and diffusion through the leaf cuticle). Gas exchange data obtained from an oscillating system can therefore be used to differentiate between stomata-controlled gas exchange phenomena and uptake due to passive mechanisms. Further exploration of the rather sensitive stomatal response to the combined effects of SO_2 and O_3 should prove useful both with respect to understanding the interactive effects of these air pollutants on plants and also to improving our knowledge about stomatal control mechanisms.

The synchrony of oscillations of DLE (Fig. 3, curve d) with the oscillations in transpiration (curve b) and CO_2 uptake (curve a) almost certainly derive from the dependence of electron transport rates through PSII on CO_2 or HCO_3^- (10, 13, 21, 24) and the relation of DLE to PSII electron transport (14). Under conditions of constant irradiance, internal CO_2 concentrations (stomata open) promote electron transport and reduce DLE while decreased internal CO_2 concentrations (stomata closed) inhibit electron transport and increases DLE. This is confirmed by the dependence of DLE on CO_2 concentration (Fig. 5). These results concur with much earlier data presented by Strehler and Arnold (22), but differ from those more recently reported by Stemler and Govindjee (21). Stemler and Govindjee reported decreases in DLE when isolated, broken chloroplasts from corn were suspended in a

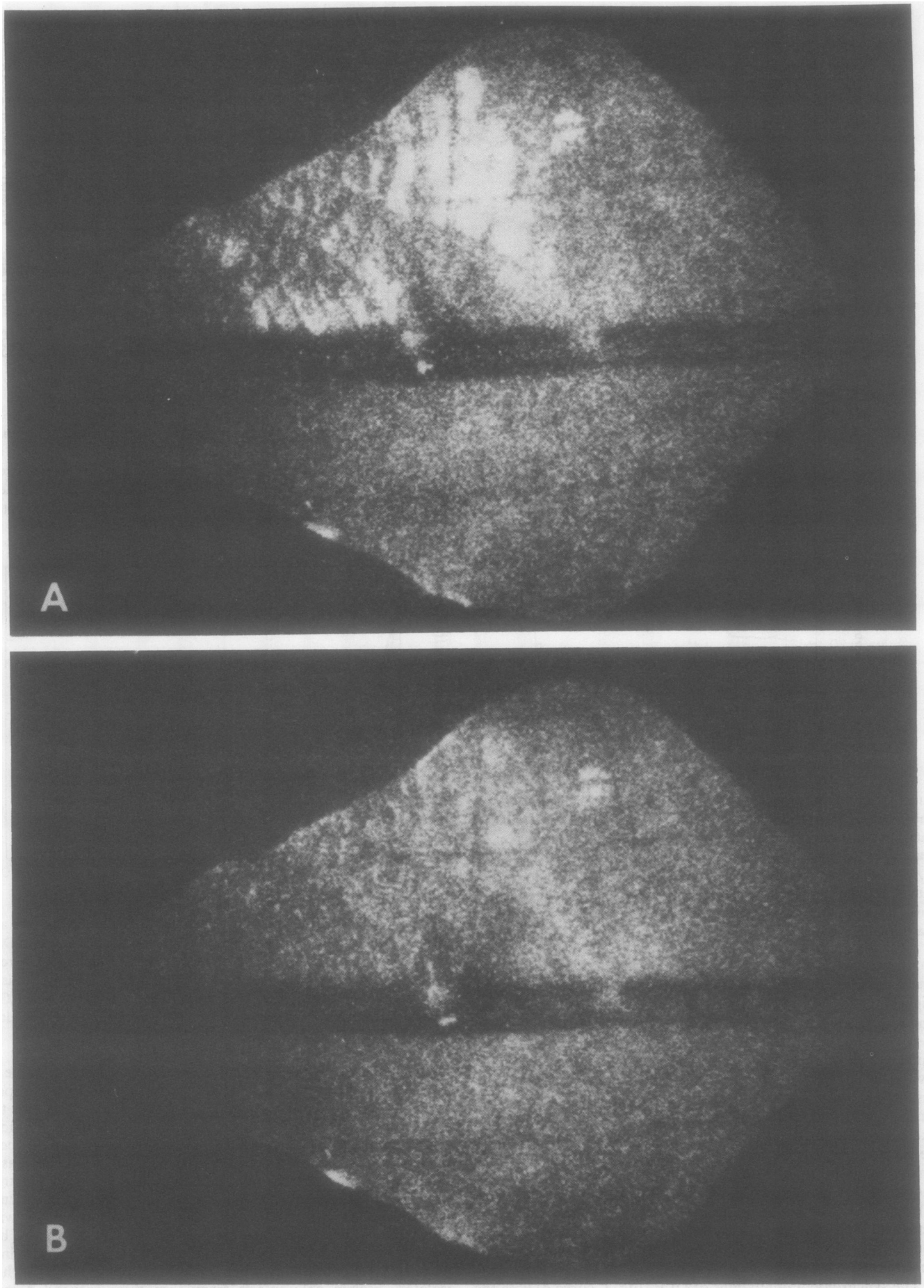

FIG. 6. DLE images recorded during the last oscillation shown in Figure 3. Print A was taken near the peak of emission, while print B was taken during the emission trough. These images demonstrate the discrete localization of leaf areas that undergo the DLE oscillations.

medium depleted of HCO_3^-. The difference between our observations and those of Stemler and Govindjee may be a consequence of investigating intact plants *versus* isolated subcellular fractions.

We conclude from the data presented that localized increases in DLE (Fig. 6) correspond to areas of the leaf where photosynthetic electron transport was decreased, and these decreases were a result of localized depletions in CO_2 that occurred with stomatal closure. Thus, the areas of the leaf where the greatest variations in DLE occurred correspond to areas in which depletions in CO_2 were most pronounced. The phytoluminographs provide evidence that control of photosynthetic activity in this sample may have been determined by the physiological status of relatively independent leaf areas defined within the vascular network. Although evidence for these leaf area domains has not been previously demonstrated by other techniques, we have often observed these features in DLE images of other red kidney bean and soybean leaves. Further investigation of this phenomenon should help clarify what physiological factors are involved in this differential DLE (and presumably stomatal) response.

Results presented here and earlier (7) demonstrate that the combination of phytoluminography and dual stream gas exchange analysis supplements data obtained through more conventional physiometric means. Two new capabilities are provided by this combination. First, the dual stream cuvette provides a means of applying environmental perturbations to selected areas of single leaves and of measuring the resulting responses in gas exchange properties of perturbed and nonperturbed areas of the same leaf. Second, phytoluminography determines the precise location and homogeneity of changes in photosynthetic activity that occur either normally or as the result of environmental perturbations. Phytoluminography can be used to discern the spatial and temporal uniformity of photosynthetic activity of intact plant leaves. This capability may have applications in the study of plant-pathogen interactions, senescence, and other environmental or physiological factors that affect photosynthetic activity in a nonhomogeneous manner. Most important, observations made using this system are immediate and nondestructive. However, before phytoluminography can be used as an effective physiometric tool, a more efficient means of evaluating DLE images will have to be developed. This problem is currently being approached through the application of digital image processing techniques.

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LITERATURE CITED

1. Beckerson DW, G Hofstra 1979 Stomatal responses of white bean to O_3 and SO_2 singly or in combination. Atmos Environ 13: 533–535
2. Bjorn LO, AS Forsberg 1979 Imaging by delayed light emission (phytoluminography) as a method for detecting damage to the photosynthetic system. Physiol Plant 47: 215–222
3. Black VJ, MH Unsworth 1980 Stomatal responses to sulfur dioxide and vapor pressure deficit. J Exp Bot 31: 667–677
4. Blaich R, O Bachman, I Baumberger 1982 Studies of photosynthesis inhibition by phytoluminography. Z Naturforsch 37c: 452–457
5. Cowan IR 1977 Stomatal behavior and environment. Adv Bot Res 4: 117–228
6. Dochinger LS, FW Bender, FL Fox, WW Heck 1970 Chlorotic dwarf of eastern white pine caused by an ozone and sulfur dioxide interaction. Nature 22: 476
7. Ellenson JL, RG Amundson 1982 Delayed light imaging for the early detection of plant stress. Science 215: 1104–1106
8. Evans LS, IP Ting 1974 Ozone sensitivity of leaves: relationship to leaf water content, gas transfer, gas transfer resistance and anatomical characteristics. Am J Bot 61: 592–597
9. Farquhar GD, IR Cowan 1974 Oscillations in stomatal conductance—the influence of environmental gain. Plant Physiol 54: 769–772
10. Good NE 1963 Carbon dioxide and the Hill reaction. Plant Physiol 38: 298–304
11. Deleted in proof
12. Hill AC, N Littlefield 1969 Ozone. Effect on apparent photosynthesis, rate of transpiration and stomatal closure in plants. Environ Sci Tech 3: 52–56
13. Izawa S 1962 Stimulatory effect of carbon dioxide upon the Hill reaction as observed with the addition of carbonic anhydrase to reaction mixture. Plant Cell Physiol 3: 221–227
14. Lavorel J 1975 Luminescence. *In* Govindjee, ed, Bioenergetics of Photosynthesis. Academic Press, New York, pp 223–317
15. Majernik O, TA Mansfield 1970 Direct effect of SO_2 pollution on the degree of opening of stomata. Nature 227: 377–378
16. Mederski HJ, LH Chen, RB Curry 1975 Effect of leaf water deficit and nonstomatal regulation of net CO_2 assimilation. Plant Physiol 55: 589–593
17. Melcerek PK, GN Brown 1977 Effects of chill stress on prompt and delayed chlorophyll fluorescence from leaves. Plant Physiol 60: 822–825
18. Menser HA, HE Heggestad 1966 Ozone and sulfur dioxide synergism: injury to tobacco plants. Science 153: 424–425
19. Rich S, NC Turner 1972 Importance of moisture on stomatal behavior in plants subjected to ozone. J Air Pollut Assoc 22: 718–721
20. Robbins WR 1946 Growing plants in sand cultures for experimental work. Soil Sci 62: 3–22
21. Stemler A, Govindjee 1973 Bicarbonate ion as a critical factor in photosynthetic oxygen evolution. Plant Physiol 52: 119–123
22. Strehler BL, W Arnold 1951 Light production in green plants. J Gen Physiol 34: 809–820
23. Sundbom E, LO Bjorn 1977 Phytoluminography: imaging plants by delayed light emission. Physiol Plant 40: 39–41
24. West J, R Hill 1967 Carbon dioxide and the reduction of indophenol and ferricyanide by chloroplasts. Plant Physiol 42: 819–826