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Heterogenous Stomatal Closure in Response to Leaf Water Deficits Is Not a Universal Phenomenon[1]

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ABSTRACT

The extent and occurrence of water stress-induced "patchy" CO_2 uptake across the surface of leaves was evaluated in a number of plant species. Leaves, while still attached to a plant, were illuminated and exposed to air containing [^{14}C]CO_2 before autoradiographs were developed. Plant water deficits that caused leaf water potential depression to −1.1 megapascals during a 4-day period did result in heterogenous CO_2 assimilation patterns in bean (*Phaseolus vulgaris*). However, when the same level of stress was imposed more gradually (during 17 days), no patchy stomatal closure was evident. The patchy CO_2 assimilation pattern that occurs when bean plants are subjected to a rapidly imposed stress could induce artifacts in gas exchange studies such that an effect of stress on chloroplast metabolism is incorrectly deduced. This problem was characterized by examining the relationship between photosynthesis and internal [CO_2] in stressed bean leaves. When extent of heterogenous CO_2 uptake was estimated and accounted for, there appeared to be little difference in this relationship between control and stressed leaves. Subjecting spinach (*Spinacea oleracea*) plants to stress (leaf water potential depression to −1.5 megapascals) did not appear to cause patchy stomatal closure. Wheat (*Triticum aestivum*) plants also showed homogenous CO_2 assimilation patterns when stressed to a leaf water potential of −2.6 megapascals. It was concluded that water stress-induced patchy stomatal closure can occur to an extent that could influence the analysis of gas exchange studies. However, this phenomenon was not found to be a general response. Not all stress regimens will induce patchiness; nor will all plant species demonstrate this response to water deficits.

For a decade or more, it has been generally accepted that photosynthetic inhibition in the intact leaf caused by plant water deficits is a function of both increases in stomatal resistance and inhibited chloroplast metabolism (7). The distinction between stomatal and nonstomatally mediated photosynthetic inhibition *in situ* has been facilitated by gas exchange analysis in leaves of stressed plants. Concomitant measurement of net CO_2 uptake and transpirational water loss allows for an estimation of C_i[2] at any [CO_2] external to the leaf (5). Comparison of the A:C_i relationship in well-watered and stressed leaves has typically led to the assertion that inhibitions in chloroplast metabolism (*i.e.* the generation of ATP and NADPH and their use by the photosynthetic enzymes) contribute significantly to photosynthetic inhibition and may even be the most significant site of inhibition in the overall photosynthetic process under stress (10, 11, 13).

The validity of this entire line of research has been questioned by some recent studies by Sharkey and Seemann (12) and an initial report by Downton *et al.* (3). Sharkey and Seemann reported that, in bean plants subjected to water deficits, heterogenous ("patchy") stomatal closure was evident. Only patches of the leaves on water-stressed plants were photosynthetically active. Using autoradiograph analysis of $^{14}CO_2$-fed leaves, they deduced that portions of these leaves had stomata that were completely closed; in these portions of the leaf, C_i was thought to be at the compensation point and A was zero. A similar finding was also reported by Downton *et al.* (3), although the autoradiograph analysis presented in that study was on a more microscopic scale. It should be noted that the study by Downton *et al.* (3) confirmed that patchy $^{14}CO_2$ uptake patterns are likely due to heterogenous stomatal closure across the surface of a leaf rather than heterogenous effects of low Ψ_w on chloroplast metabolism in different areas of the leaf. As discussed by Terashima *et al.* (14), the phenomenon of patchy stomatal closure affects A:C_i curves generated from area-based stomatal conductance measurements in several ways. Artifacts are introduced such that the derived A:C_i relationship for the leaf with patchy stomatal closure will not be valid. Patchy stomatal closure has been found to occur in leaves in response to exogenous ABA (2, 14). These ABA studies, along with the work by Downton *et al.* (3) and Sharkey and Seemann (12), have led to the speculation that the A:C_i relationship deduced from gas exchange measurements may be invalid when the plant is subjected to water stress.

Before this significant assertion can be accepted as valid in the general case, further work should be done. In fact, a preliminary study by Bunce (1) did not find that heterogenous stomatal closure occurs in the three different plant species tested to an extent that would influence the calculated A:C_i relationship under water deficits until severe stress levels are reached.

We have generated a more extensive base of data from which conclusions about patchy stomatal closure in water-stressed plants can be made. Autoradiographs of illuminated leaves exposed to air containing [^{14}C]CO_2 were made to

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[2] Abbreviations: C_i, internal leaf CO_2 concentration; A, net photosynthesis; Ψ_w, water potential.

determine extent of variation in CO_2 uptake across the laminar surface of a leaf. This "patchiness assay" was used with a number of plant species and with plants subjected to a range of stress regimens.

MATERIAL AND METHODS

Plant Material

The study plants were bean (*Phaseolus vulgaris* L. cv Top Crop), wheat (*Triticum aestivum* L. cv Condor), and spinach (*Spinacea oleracea* L. cv Melody) grown from seed in 1:1 peat:vermiculite mix placed in a growth chamber that had a 10-h light (250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) period and a temperature maintained at 21°C day/18°C night. Spinach, bean, and wheat plants were used after 6, 6, and 7 weeks of growth, respectively.

Plants were subjected to water deficits by withholding irrigation. Extent, and rate of leaf Ψ_w depression varied during these stress episodes. The stress level imposed on the plants ranged from moderate (leaf Ψ_w −1.0 MPa) to severe (leaf Ψ_w −2.6 MPa). Range of stress imposition was varied in some experiments with bean by growing plants in pots of different sizes (1.2-L pots for a fast rate of stress and 3.6-L pots for a slower rate of stress imposition). For all studies, fully expanded nonsenescing leaves were used. Leaf Ψ_w was measured using a pressure chamber (Soil Moisture Equipment Co., Santa Barbara, CA).

[^{14}C]CO_2 Inoculation

A Plexiglas chamber (9.5 × 20 × 13.5 cm) was constructed for leaf inoculation experiments. The chamber had ports with tubing to allow gas to be pumped into and out of the chamber. Water circulating through a 4-cm-high aluminum block with fins that covered the bottom of the chamber, and an internal fan allowed for air temperature in the chamber to be maintained at 22°C during all measurements. Entire bean or spinach leaves could be placed in the chamber while still attached to a plant. The petiole of the leaf extended through a small hole formed at the juncture of the chamber bottom and lid. The leaf was sealed in the chamber by placing clay around the petiole. For wheat leaves, the petiole hole was sealed completely, and rubber gaskets at the lid-chamber interface allowed the leaf blade to be sealed in the chamber when the lid was clamped shut. Leaves were supported above the fan and aluminum fins in the chamber by a series of thin nylon wires.

Leaves were illuminated with saturating light (1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR for spinach and wheat, 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR for bean) provided by a 500-W incandescent floodlight. Before placing leaves in the chamber, the leaf blade was clamped between two pieces of rigid metal screen (0.8-mm diameter wire forming a 12- × 12-mm cross-hatched pattern).

Leaves were allowed to reach steady-state photosynthesis before inoculation with $^{14}CO_2$. Air at ambient conditions (*i.e.* approximately 350 $\mu L/L$ CO_2 and 50% RH) was pumped into the chamber at 400 mL/min while the leaf was illuminated for 30 min. Control experiments indicated that leaves from both well-watered and stressed plants had reached steady-state photosynthesis and maximal stomatal conductance after 15 to 20 min of illumination in the chamber (data not shown). After 30 min, the inflow line of the chamber was switched to receive gas for several minutes (as noted in figure legends) from a compressed gas cylinder that contained air with 342 $\mu L/L$ CO_2 and 10 $\mu Ci/L$ [^{14}C]CO_2 (Gollob Analytical, Berkley Heights, NJ); the specific activity of the radionuclide was 0.65 Ci/mol. After exposure to $^{14}CO_2$, leaves were immediately detached from plants and frozen at −86°C before placement in an autoradiograph development cassette. Clamping the leaves between the wire mesh screens allowed them to stay flat while they were frozen.

Autoradiograph Development

Care was taken to maintain the leaf in a frozen state throughout the steps involved in loading the leaf in an autoradiograph cassette. The following items were loaded (in this exact order, from top to bottom) in a Dupont cardboard x-ray film cassette: Dupont Cronex intensifying screen, x-ray film, plastic (Saran-type) wrap, frozen leaf, filter paper, and, finally, a second intensifying screen. The cassette was kept in complete darkness only when the x-ray film was loaded. Dupont Cronex and Kodak X-Omat RP x-ray film were used interchangeably for all studies. The plastic wrap must be the thinnest commercially available and should not be allowed to bunch up when the cassette is loaded. After the film cassette was loaded, it was returned to a −86°C freezer.

Film was left to expose for varying lengths of time, ranging from 2 to 12 weeks. Exposure times are noted in the appropriate figure legends. After exposure of the x-ray film to the leaves, the resultant negatives were used to develop a positive image. In this report, then, lighter areas of the autoradiograph figures correspond to areas of a leaf where high rates of $^{14}CO_2$ uptake occurred. Contact sheet positives were made using Dupont IHCP or Kodak TP5 photographic film interchangeably. All autoradiograph experiments were repeated at least twice.

Photosynthesis

Gas exchange parameters were measured with an ADC IR gas analysis system (PK Morgan Instruments, Needham Height, MA) at 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. Air at varying [CO_2] was supplied to the leaf chamber from compressed gas cylinders through an ADC GM602 precision gas blender. Net photosynthesis and C_i were calculated from concomitant measures of CO_2 uptake, transpirational water loss, and temperature using equations developed by von Caemmerer and Farquhar (15). Three leaves from different pots were used as replicates for the generation of an A:C_i curve.

RESULTS AND DISCUSSION

An autoradiograph of a well-watered bean leaf is shown in Figure 1A. A homogenous pattern of $^{14}CO_2$ uptake occurred, as evidenced by a homogenous light shade except where the wire mesh screen covered the leaf. Little to no labeled photosynthate was loaded into the vascular system during the $^{14}CO_2$ inoculation period, as evidenced by the dark shade of the major veins. The cross-hatched pattern on this and other autoradiographs caused by the wire screen is similar to the

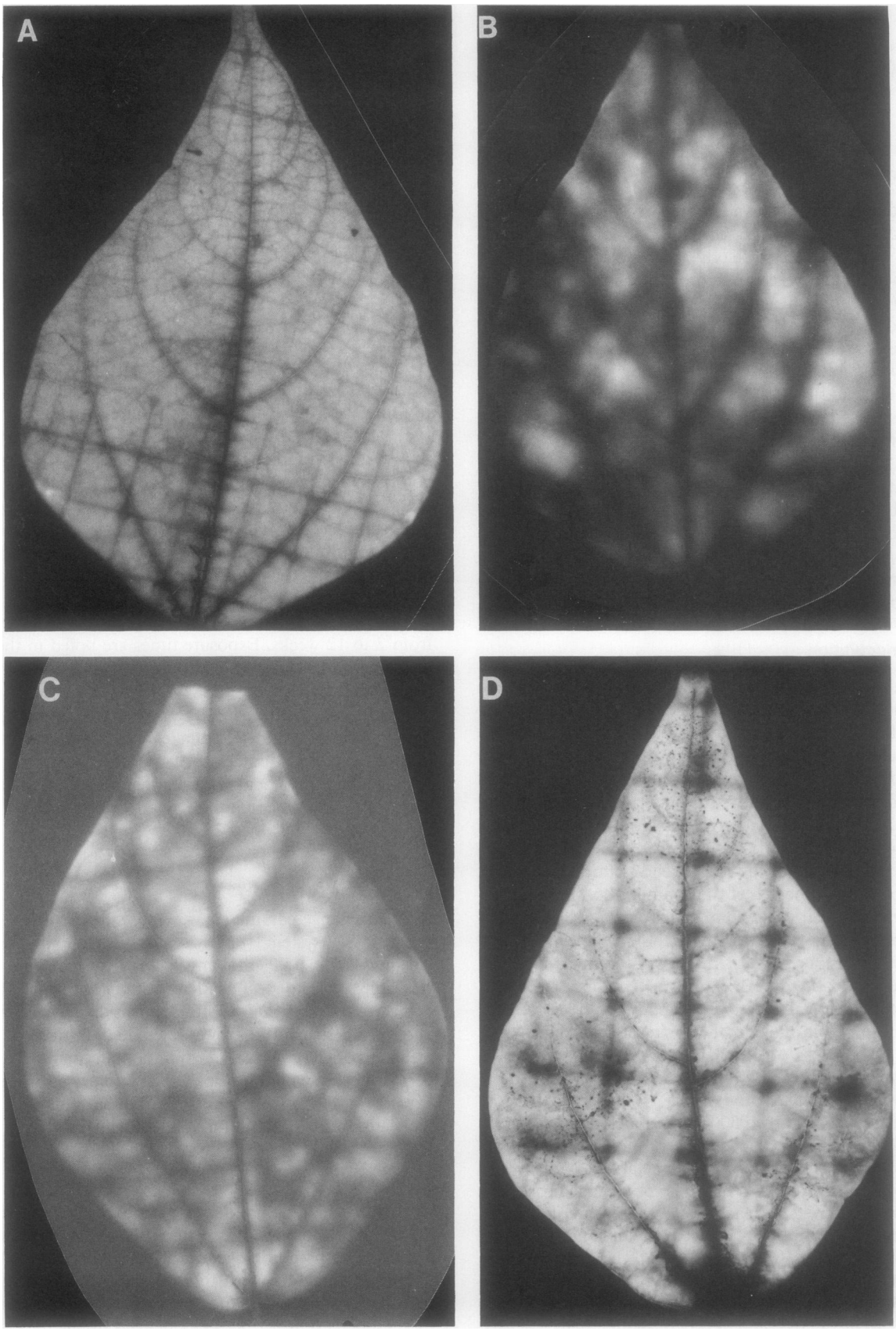

Figure 1. A, Autoradiograph of a well-watered (leaf Ψ_w −0.16 MPa) bean leaf. Darkened areas in this and all other autoradiographs represent areas where no $^{14}CO_2$ uptake occurred. Note the lack of $^{14}CO_2$ uptake in the area of the leaf blocked by the wire mesh screen and in the vascular

shadows caused by the fishing line (used to secure the leaves) in the autoradiographs of Sharkey and Seemann (12). These cross-hatched impressions are an excellent internal control, validating the methodology used to evaluate patchiness in our study. The standard processing of our leaves could possibly allow considerable mixing of cell contents across the face of a leaf. If this mixing occurred, then patchy $^{14}CO_2$ uptake would have been obscured. As was the case with the autoradiographs of Sharkey and Seemann (12), movement of assimilated $^{14}CO_2$ across the face of the frozen leaf likely did not occur during the preparation of our autoradiographs. The impression left by the wire mesh screen as shown in Figure 1A would be absent if there was considerable cell content mixing in the leaf. Patchy $^{14}CO_2$ uptake was noted in leaves of water-stressed bean plants using this assay technique. Autoradiographs were made from leaves of bean plants stressed to −1.22 (Fig. 1B) and −1.04 MPa (Fig. 1C) leaf Ψ_w. In the Figure 1, B and C, patches of the interveinal areas of the leaves appear dark. Little to no photosynthesis occurred in these areas as compared with the areas of the leaf that appear light in the figure. It should be noted that, in addition to the lack of $^{14}CO_2$ uptake noticeable in the vascular areas, and some patches of interveinal area, the cross-hatched pattern where the wire screen blocked uptake is also evident in Figure 1C.

The relative extent of surface where no photosynthetic activity was evident in these leaves was ascertained by weighing areas of paper corresponding to the light and dark regions of these autoradiographs. It was estimated that an average of 31% of the total leaf area was not photosynthetically active in the leaves shown in Figure 1, B and C.

This extent of patchy CO_2 uptake would introduce significant artifacts into an A:C_i curve, as shown in Figure 2. A standard A:C_i curve (Fig. 2A) generated from gas exchange data would indicate that this extent and rate of plant water stress (*i.e.* leaf Ψ_w depression to about −1.1 MPa during 4 d) would result in a substantial nonstomatally mediated inhibition of photosynthetic capacity. When the A:C_i curve is adjusted to account for the 31% of the leaf surface that was not photosynthetically active (Fig. 2B), this relationship is virtually identical in well-watered and stressed leaves. It can be concluded, then, that this extent of water stress does not appreciably inhibit chloroplast metabolism in bean plants.

Further studies with bean plants disclosed the significant influence that the rate of stress imposition has on the extent of patchy stomatal closure. Bean plants grown in larger pots than those used for the experiments shown in Figure 1, A to C, were subjected to gradual imposition of water deficits, with leaf Ψ_w declining to the same level (−1.1 MPa) but occurring throughout a more prolonged period of time (17 d instead of 4–6 d). The autoradiograph shown in Figure 1D shows a

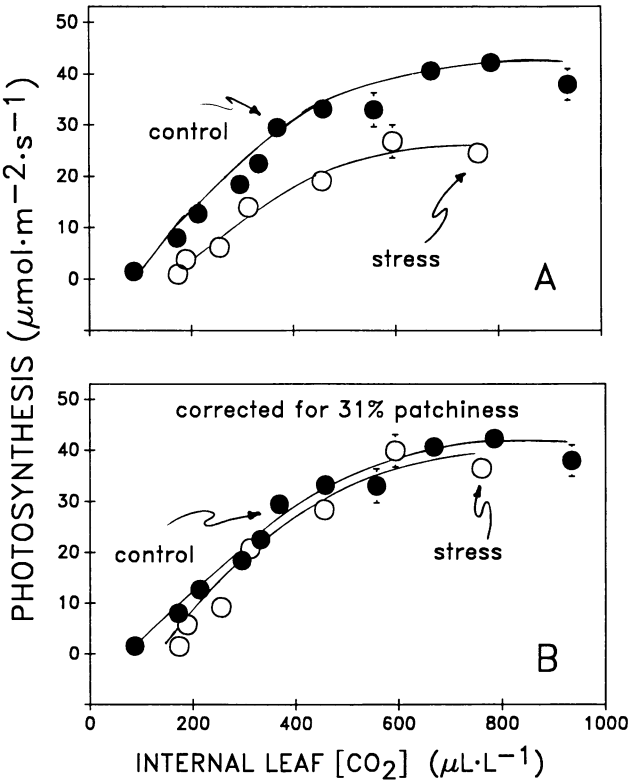

Figure 2. Analysis of the relationship between photosynthesis and internal leaf [CO_2] in leaves of well-watered (●) (leaf Ψ_w −0.2 MPa) and stressed (○) (leaf Ψ_w decline to −1.0 MPa after 4 d of withholding irrigation from the 1.2-L pots) bean plants. A, Standard A:C_i analysis, using equations of von Caemmerer and Farquhar (15) to generate area-based values from gas exchange data. B, Value used in these equations for the effective photosynthetic area was reduced by 31% (based on estimates generated from Fig. 1, B and C). Data points and bars, means ± SE. In many cases, the SE bars are obscured by the points.

homogenous pattern of $^{14}CO_2$ uptake, except in the major veins and underneath the wire mesh screen. It is quite clear from this figure that patchy stomatal closure does not occur in bean plants despite their being subjected to a fairly severe stress level of −1.08 MPa leaf Ψ_w (*cf.* ref. 12), if the stress is induced during a relatively prolonged period of time. This assertion suggests that more work needs to be done to resolve the physiological relevance of water stress-induced patchy stomatal closure. Under field conditions, most crop plants are subjected to slowly developed plant water deficits, *e.g.* 0.5 MPa decline throughout several weeks (6). In the studies that have demonstrated patchy stomatal closure in leaves of water-

system. This leaf was exposed to $^{14}CO_2$ for 3 min, and x-ray film exposure time was 32 d. B, Autoradiograph of a severely stressed bean leaf in which leaf Ψ_w decreased to −1.22 MPa during a 6-d stress period. This leaf was exposed to $^{14}CO_2$ for 12 min, and x-ray film exposure time was 32 d. C, Autoradiograph of a severely stressed bean leaf in which leaf Ψ_w decreased to −1.04 MPa during a 4-d period. This leaf was exposed to $^{14}CO_2$ for 3 min, and x-ray film exposure time was 65 d. D, Autoradiograph of a severely stressed bean leaf in which leaf Ψ_w decreased to −1.08 MPa during a 17-d stress period. This leaf was exposed to $^{14}CO_2$ for 3 min, and x-ray exposure time was 65 d. In a repeat of this experiment, homogenous $^{14}CO_2$ uptake was observed in a leaf from a bean plant brought to −1.1 MPa during a 14-d stress period (data not shown).

stressed plants, the rate of leaf Ψ_w decline was relatively rapid. Sharkey and Seemann imposed a −0.7 MPa leaf Ψ_w on bean in "approximately" 4 d. In the work of Downton *et al.* (3), grapevine, oleander, and red-flowering gum plants were stressed to −1.1 MPa during a 2-d stress period, −1.9 MPa during a 4-d period, and −1.8 during a 6-d period, respectively. The effect of rate of stress imposition on patchiness was not evaluated in these studies.

Further studies focused on evaluating the extent of patchy stomatal closure that occurs when plants of crop species other than bean are subjected to water stress. Autoradiographs were also made from wheat and spinach leaves exposed to $^{14}CO_2$. It should be noted that the mesophyll anatomy of the bean leaf is considered "heterobaric," whereas spinach and wheat leaves are "homobaric" (2, 4, 14). In heterobaric leaves, extensions of bundle sheath cells to the upper and lower epidermis delimits the mesophyll laterally. Diffusion of CO_2 across these vascular extensions, *i.e.* from one areola to another, is restricted. Patchy CO_2 assimilation patterns have been reported to be associated with variable stomatal closure occurring in different areolas of a heterobaric leaf (2, 14). This effect and extent of "apparent" inhibition of chloroplast metabolism due to ABA application as deduced from A:C_i curves was substantially less evident in homobaric leaves (14). Based on these findings, it has been asserted that patchy stomatal closure may occur only in heterobaric leaves. However, large nonphotosynthetic areas much larger than individual areolas have been noted in homobaric leaves after ABA application (14). These larger patches appear similar to the water stress-induced patches of the nonphotosynthetic area in bean leaves noted here and by Sharkey and Seemann (12).

Homogenous $^{14}CO_2$ uptake patterns were found in leaves of well-watered and water-stressed spinach and wheat plants (Figs. 3 and 4). Apparently, patchy stomatal closure does not occur in response to water stress in all plant species, even at a substantial level of stress imposed rather quickly (leaf Ψ_w −1.5 MPa after 4 d of stress; Fig. 4B). Patchy $^{14}CO_2$ uptake was noted in wheat, however, when 10 μM ABA was added to the transpiration stream (Fig. 4C).

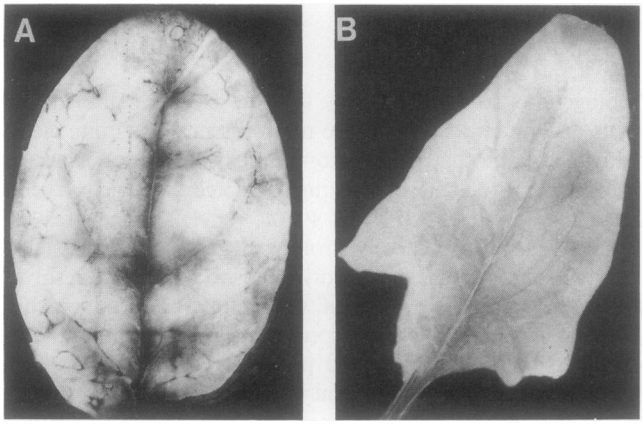

Figure 3. A, Autoradiograph of a well-watered (leaf Ψ_w −0.2 MPa) spinach leaf. This leaf was exposed to $^{14}CO_2$ for 3 min, and x-ray exposure time was 65 d. B, Autoradiograph of a stressed spinach leaf in which leaf Ψ_w decreased to −1.1 MPa during a 3-d period. This leaf was exposed to $^{14}CO_2$ for 4 min, and x-ray exposure time was 3 months. In other experiments, homogenous $^{14}CO_2$ uptake patterns were found in leaves of spinach stressed to −1.5 MPa leaf Ψ_w (data not shown).

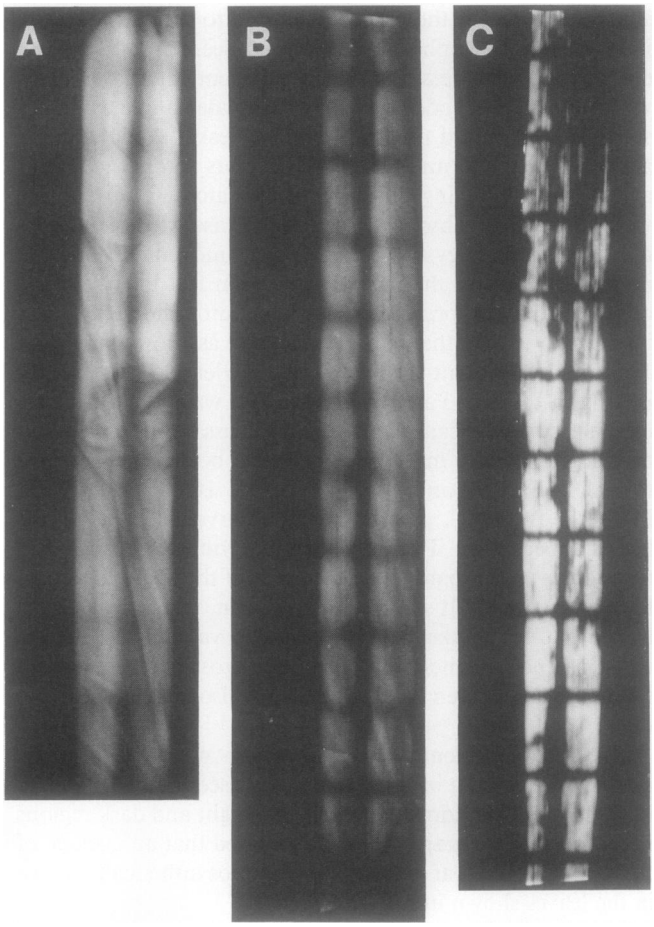

Figure 4. A, Autoradiograph of a well-watered (leaf Ψ_w −0.2 MPa) wheat leaf. This leaf was exposed to $^{14}CO_2$ for 3 min, and x-ray film exposure time was 16 d. The slightly "swirling" pattern was caused by the plastic film wrap not being completely flat when this autoradiograph was developed. B, Autoradiograph of a stressed wheat leaf in which leaf Ψ_w decreased to −1.5 MPa during a 4-d stress period. This leaf was exposed to $^{14}CO_2$ for 4 min, and x-ray film exposure time was 14 d. In other experiments, homogenous $^{14}CO_2$ uptake patterns were found in leaves of wheat stressed to −2.6 MPa (data not shown). C, Autoradiograph of a detached wheat leaf incubated in 10 μM ABA for 35 min before and during exposure to $^{14}CO_2$. This leaf was exposed to $^{14}CO_2$ for 3 min, and x-ray film exposure time was 68 d. Detached wheat leaves incubated in water before and during exposure to $^{14}CO_2$ had homogenous uptake patterns (data not shown).

CONCLUSIONS

The conclusions that can be made from the observations noted in this report are rather straightforward. Previous studies (3, 12) have demonstrated patchy CO_2 uptake patterns in

leaves of plants subjected to rates of leaf Ψ_w depression that are rarely encountered by crop plants growing under agronomic conditions in the field. We extended the work done in these previous studies by demonstrating that patchiness does not occur in plants subjected to a relatively gradual rate of stress imposition. Also, the results shown here indicate that patchy stomatal closure is not a universal phenomenon occurring in all plants subjected to rapid leaf Ψ_w depression. Patchy CO_2 uptake was not found to occur in spinach or wheat plants, even when the stress level was substantial and the stress was imposed quickly. Previous gas exchange work in this and other laboratories has indicated that this level of water stress has substantial effects on chloroplast metabolism *in situ* in both wheat and spinach (8, 9, 11). The work presented here suggests that artifacts induced by patchy stomatal closure likely did not influence these results.

Application of ABA to plants has been found in other studies (2, 14), and here, to result in patchy stomatal closure. These results should be interpreted with caution. No evidence has been presented to document a correlation between patchy stomatal closure and changes in endogenous levels of ABA. In light of the findings reported here that heterogenous CO_2 uptake does not occur in all plant species under stress, or under all stress regimens, the contention that patchy stomatal closure may be a generalized response to either low water potentials or increases in endogenous ABA that arise during the induction of plant water deficits may not be valid. Further work should examine whether this phenomenon occurs in crop plants exposed to the slowly developed water deficits that occur in the field.

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