

# Relationships between Leaf Water Status, Abscisic Acid Levels, and Stomatal Resistance in Maize and Sorghum

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## ABSTRACT

A new method for abscisic acid extraction and purification was developed to handle large numbers of small (about 125 milligrams fresh weight) samples of leaf discs. This method enabled short term changes in abscisic acid to be followed in single leaves.

Water potentials, stomatal diffusion resistance, and abscisic acid levels were measured in the leaves of maize (*Zea mays* L. var. Wisconsin 575) and sorghum (*Sorghum bicolor*, hybrid NK 145) plants subjected to a drought-recovery cycle under controlled environmental conditions. The levels of abscisic acid began to rise, and the stomata closed, over a narrow range of water potential (-8 to -10 bars) in both species. Abscisic acid levels continued to rise after the stomata closed. The maximum amount of abscisic acid extracted from maize leaves was about twice that from sorghum, but this represented a similar proportional increase over the control level.

In excised leaves little or no change in abscisic acid levels was found within 60 to 120 minutes after stress was applied (phase I). Subsequently abscisic acid levels rose rapidly to about 20 times the prestress level (phase II). Stomatal closure always preceded the phase II increase in abscisic acid.

The aftereffects of stress differed in the two species. In sorghum control levels of both abscisic acid and stomatal resistance were regained within 24 hours of rewatering. In maize abscisic acid levels also fell rapidly, but regained control levels only after 48 hours. The resumption of normal stomatal functioning occurred after a further 24 hours.

Impaired stomatal functioning after stress does not appear to be associated with high residual levels of abscisic acid in the leaf.

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In recent years there have been a number of reports of ABA increasing in water-stressed tissues (3, 17, 28-30). It has also been shown that ABA both inhibits transpiration and induces stomatal closure (6, 19), and some authors have suggested that the aftereffect of water stress on stomatal functioning may be the result of elevated levels of ABA persisting for several days after relief from stress (13, 29). However, Hsiao, in his recent review (11), pointed out that the relative rates of stomatal opening and closing and ABA accumulation and degradation were such as to render the possibility of direct modulation of stomatal response by ABA questionable. Apart from

our preliminary communication (3), information relating  $R$ ,<sup>2</sup> ABA, and  $\Psi$  is lacking, although Zabadal (30) has determined a  $\Psi$  threshold for the rise in ABA in two *Ambrosia* species.

The experiments reported here were undertaken to determine the plant-water status at which ABA increases, the extent to which poststress levels of  $R$ ,  $\Psi$  and RWC are associated with residual levels of ABA, and the relationships between  $\Psi$ , RWC,  $R$ , and ABA levels in attached leaves of maize and sorghum. Further experiments on excised maize leaves were carried out to determine the short term changes in ABA,  $r_i$ , and  $\Psi$  which occurred during the imposition of, and recovery from, stress.

## MATERIALS AND METHODS

**Plant Material.** *Zea mays* L. (var. Wisconsin 575) and *Sorghum bicolor* (hybrid NK 145) plants were grown in peat-sand-vermiculite potting mix (1:1:1, v/v) in 4.5 liter pots in the Climate Laboratory or in a heated greenhouse. Modified Hoagland's nutrient solution (7) was given daily. In the Climate Laboratory day/night temperatures and vapor pressure deficits were 25/20 C and 10/5 millibars, respectively. Daylength was 12 hr and photosynthetically active radiation (400 to 700 nm range) was 150 to 170  $w\ m^{-2}$ . In the greenhouse, daylength was approximately 12 hr, day temperatures were 25 C to 30 C, and the minimum night temperature was 15 C.

At the start of the experiments maize plants had at least 10, and sorghum plants at least 15 fully mature leaves, and new leaves were still emerging. The 8th, 9th, and 10th leaves of maize and the 11th, 12th, and 13th leaves of sorghum plants were used for all measurements, the oldest leaf being designated leaf 1.

Intact plants of maize and sorghum were subjected to water stress by the withholding of nutrient solution, and then rewatered. Measurements were made at various times during the photoperiod. Coincident measurements of ABA,  $\Psi$ , RWC, and  $R$  were always made on the same leaf. Removal of samples for ABA,  $\Psi$ , and RWC determinations had no apparent effect on subsequent measurements.

In some experiments excised leaves were used to enable stress and recovery to be applied more rapidly than is possible with intact plants. Pre-excision values of  $\Psi$ ,  $r_i$ , and ABA were determined and the leaf then excised and allowed to desiccate in the light. When the stomata closed, the leaf was placed in the shade and subsequent changes in  $\Psi$ ,  $r_i$ , and ABA were

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<sup>2</sup> Abbreviations:  $R$ : stomatal diffusion resistance of the two leaf surfaces in parallel;  $r_i$ ,  $r_u$ : stomatal diffusion resistances of the lower and upper leaf surfaces, respectively; RWC: relative water content; Me-ABA, Me-*t*-ABA: methyl esters of ABA and 2-*trans*-ABA, respectively;  $\Psi$ : leaf water potential.

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followed. Changes during the recovery period were followed after the base of the leaf was recut and kept under water.

**Plant Water Status.** Leaf water potential was measured with a pressure chamber (4, 26) and the relative water content of the leaves was determined by the method of Barrs and Weatherley (1), using a 1-hr floating period.

**Stomatal Diffusion Resistance.** A porometer similar to that described by Kanemasu *et al.* (14), but constructed of stainless steel and with automatic timing (H. G. McPherson and J. S. Talbot, personal communication), was used to determine  $R$ . The diffusive resistances of the upper and lower surfaces of the leaf were determined independently and  $R$  found from the equation

$$\frac{1}{R} = \frac{1}{r_u} + \frac{1}{r_l} \text{ sec cm}^{-1}$$

In the experiments with excised leaves only  $r_l$  was determined. For very high resistances (*i.e.* stomata almost closed) an arbitrary, high transit time was recorded. These appear in the results as  $r_l = 100 \text{ sec cm}^{-1}$  or  $R = 50 \text{ sec cm}^{-1}$ .

**ABA Extraction and Purification Procedures.** A new method for ABA extraction and purification was developed to handle large numbers of small (125 mg fresh weight or less) samples of tissue and small volumes of solvent. The procedure has the advantages of being both rapid (average processing time 1 hr/sample) and sensitive (from 2 ng ABA/sample).

Samples of five leaf discs (15 mm in diameter) were placed in 5 ml of extracting solvent (methanol-chloroform-2 N ammonium hydroxide, 12:5:3, v/v), precooled with dry ice. Samples were stored at  $-15^\circ\text{C}$  for at least 1 week, but could be stored for several months with no apparent change. The original extract was decanted and replaced with 10 ml of fresh solvent. After 24 hr, solvent was again decanted and the combined extract purified (Fig. 1). Over-all recovery from this procedure is 60%. Grinding the leaf discs was unnecessary

since only 5% additional ABA was extracted by subsequently homogenizing and re-extracting the discs.

Known amounts of ( $\pm$ )-ABA were used to monitor recovery at each step of the extraction procedure. No ABA was detected in the chloroform phase partitioned in the presence of  $\text{NH}_4\text{OH}$ . At each partition step at pH 4 the recovery was 45%, giving a total recovery before methylation of 66%. A solution of diazomethane in ether-methanol (9:1, v/v) was prepared (25) and 1.5-ml aliquots of this solution were added to dry extracts. Methylation appeared to be 100% and recovery of Me-ABA from TLC plates was about 92%, giving 60% recovery over-all. This figure was used to calculate endogenous levels of ABA.

Solvent partitioning was carried out in 50-ml conical polypropylene centrifuge tubes by bubbling air into the chloroform phase followed by centrifugation to ensure clean separation of the phases. The chloroform phase was recovered by aspiration through a Teflon and glass capillary directly into the tube used for methylation.

TLC plates coated with Silica Gel GF 254, 250  $\mu\text{m}$  thick, were scribed to give six channels for extracts and three narrow channels for marker Me-ABA. Plates were precleaned by development in chloroform-ethyl acetate (1:1, v/v). (A double development in redistilled ethyl acetate is now recommended.) Extracts were dissolved in dichloromethane or chloroform (redistilled to lower the ethanol content) and applied as a streak to the origin (23). In these solvents Me-ABA does not move from the zone of application. Plates were developed in chloroform-ethyl acetate (9:1, v/v). The zone corresponding to Me-ABA was scraped from the plate, collected in a 1-cm sintered glass filter tube under vacuum and eluted with  $3 \times 150 \mu\text{l}$  redistilled ethyl acetate.

**Gas-Liquid Chromatography.** Measurement of Me-ABA was performed on a Yanaco G-80 gas chromatograph equipped with a  $^{63}\text{Ni}$  electron capture detector in glass columns (1.5 m  $\times$  6 mm o.d.) packed with 5% OV17 on Chromasorb W (AW-DMCS, 80–100 mesh). The oven was at  $250^\circ\text{C}$  with both injection port and detector at  $300^\circ\text{C}$ , and the flow rate of  $\text{N}_2$  was  $15 \text{ ml min}^{-1}$ . The retention time for Me-ABA was 4.5 min and for Me-*t*-ABA, 5.5 min. The ratio of peak height for Me-ABA in extracts to the peak height for a 200 pg Me-ABA standard injected after every three to five samples was calculated. Levels of ABA were determined from a standard curve relating the ratio of peak heights to pg Me-ABA. Although the absolute response of the detector drifted, the ratio of peak heights remained constant. The amount of Me-ABA injected was as close as possible to 200 pg, except for samples from unstressed tissue which contained approximately 50 pg. Since the area of maize leaves changes very little with desiccation (28) ABA levels were expressed as  $\text{ng cm}^{-2}$  leaf.

The average fresh and dry weights of samples from well-watered leaves were  $19 \text{ mg cm}^{-2}$  and  $3.8 \text{ mg cm}^{-2}$ , respectively, for maize and  $15.5 \text{ mg cm}^{-2}$  and  $3.2 \text{ mg cm}^{-2}$  for sorghum.

## RESULTS

**Experiments with Whole Plants.** In well watered maize plants ABA was present in the leaves at a concentration of  $0.25$  to  $0.5 \text{ ng cm}^{-2}$ ,  $\Psi$  was  $-4$  to  $-6$  bars,  $R$  was less than  $6 \text{ sec cm}^{-1}$ , and RWC was above 96%. In well watered sorghum plants the levels of  $\Psi$  and RWC were the same as in maize, but  $R$  was normally below  $4 \text{ sec cm}^{-1}$  and the endogenous ABA level was rather lower at about  $0.1$  to  $0.2 \text{ ng cm}^{-2}$ . In extracts from some well watered sorghum plants ABA was below the limit of measurement.

The changes that occurred in  $\Psi$ ,  $R$ , and ABA during a stress-recovery experiment with maize are shown in Figure 2. Fol-

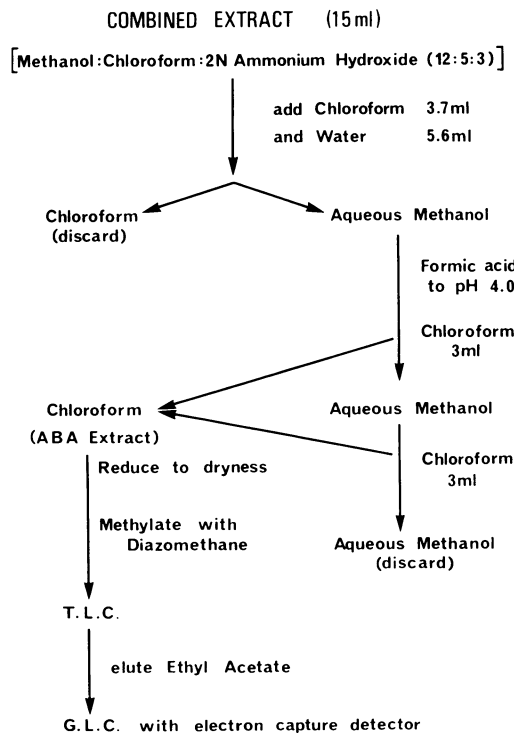


FIG. 1. Flow diagram outlining procedures used for the purification of ABA extracts for gas chromatography.

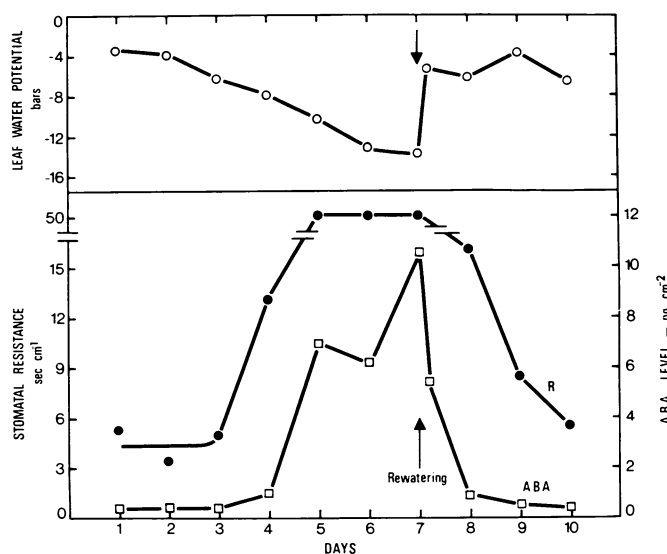


FIG. 2. Changes in  $\Psi$ ,  $R$ , and ABA during a stress-rewetting experiment with maize. All measurements were made 2 hr after the start of the photoperiod, except those immediately following rewatering, which were made 4 hr after the start of the photoperiod. All measurements of  $R$  in excess of 20  $\text{sec cm}^{-1}$  are shown as 50  $\text{sec cm}^{-1}$ . Points are means of up to four measurements.

lowing the cessation of watering (3 hr before the first data points), the normal levels of most processes were maintained for the next 48 hr. On day 4  $\Psi$  had fallen to  $-7.8$  bars, and  $R$  and leaf ABA concentration had both more than doubled from their prestress values. Over the next 3 days  $\Psi$  continued to fall, the ABA concentration to rise, and  $R$  increased to the point where the stomata appeared to be closed ( $R = 50 \text{ sec cm}^{-1}$ ).

After rewatering, on the morning of day 7, there was a rapid rise in  $\Psi$ , which after 2 hr virtually regained its normal level. Levels of ABA also changed rapidly, falling to about half the maximum (pre-watering) value within 2 hr, but it was not until day 9 that prestress levels were regained. The stomata reacted more slowly than either ABA or  $\Psi$ , and on day 8  $R$  had fallen only to 17  $\text{sec cm}^{-1}$ . Subsequently, there was a more rapid reduction in  $R$ , and on day 10 the original level was regained. RWC closely followed the trend of  $\Psi$  (see below).

There was some variation between plants in the rate of recovery of stomatal functioning after rewatering. Additionally, the stomata on the lower surface opened before those on the upper surface. Typical data from two plants are given in Table I; similar results have been found repeatedly.

Sorghum was found to behave in a similar manner to maize as water stress developed. The increases in ABA and  $R$  occurred at a  $\Psi$  of about  $-10$  bars. However, upon rewatering, both ABA levels and the stomata recovered more quickly than in maize, normal values being attained in less than 24 hr.

In the well watered state values of  $\Psi$  and RWC were similar in both species, viz. above about  $-6$  bars and 96%, respectively (Fig. 3). With increasing stress RWC was always higher at any given  $\Psi$  in sorghum than in maize.

In both species there was a threshold value of  $\Psi$  below which ABA levels began to increase (Fig. 4). This value was  $-8$  bars for maize and between  $-8$  and  $-10$  bars for sorghum. As  $\Psi$  fell further, ABA levels continued to increase in both species, but the maximum amounts of ABA found in maize ( $10.7 \text{ ng cm}^{-2}$ ) were twice those found in sorghum ( $5 \text{ ng cm}^{-2}$ ). In both species this represents at least a 20-fold increase over the nonstressed level.

As  $\Psi$  decreased there was no effect on  $R$  until about  $-8$  bars in maize and  $-9$  to  $-10$  bars in sorghum, at which point there was an abrupt increase in  $R$  (Fig. 5). These values of  $\Psi$  are the same as those at which ABA concentrations increased (Fig. 4). However, the transition from open to closed stomata occurred over a narrow range of  $\Psi$ , and this was particularly marked in sorghum, where the intermediate state was seldom found.

**Experiments with Excised Maize Leaves.** The results from a typical excised leaf experiment using maize are shown in Figure 6. Following excision  $\Psi$  fell rapidly to  $-13$  bars and remained at this level. There was little change in ABA level during the first 120 min (phase I) but thereafter a rapid rise was found (phase II). Phase I thus represents a lag phase and although there was some variation it was never found to be shorter than 60 min, considerably longer than the time taken for stomata to close.

To distinguish between the effects of reduced turgor and elevated ABA levels on subsequent stomatal behavior, excised leaves were stressed for various periods up to 120 min and then recut under water. When stress was imposed for less than the duration of phase I,  $r_i$ , and  $\Psi$  regained control values within 45 min (Fig. 7). In leaves stressed for 180 min the phase

Table I. Reduction in ABA Level and Recovery of Stomatal Function in Maize Plants Following Rewatering

For very high stomatal resistances an arbitrary value of 100  $\text{sec cm}^{-1}$  for a single leaf surface was recorded.

	Time after Rewatering	Water Potential	ABA Level	Stomatal Resistance		
				Lower	Upper	Parallel
	hr	bars	$\text{ng cm}^{-2}$	$\text{sec cm}^{-1}$		
Plant 1	0	-13.7	10.70	100	100	50
	24	-6.6	0.68	12.7	43	9.8
	49	-5.9	0.47	9.1	45	7.5
Plant 2	0	-15.7	5.90	73	100	42
	18	-6.2	0.90	20.1	100	17.2
	42	-3.8	0.34	8.2	100	7.6
	67	-6.6	0.25	5.2	10	3.6

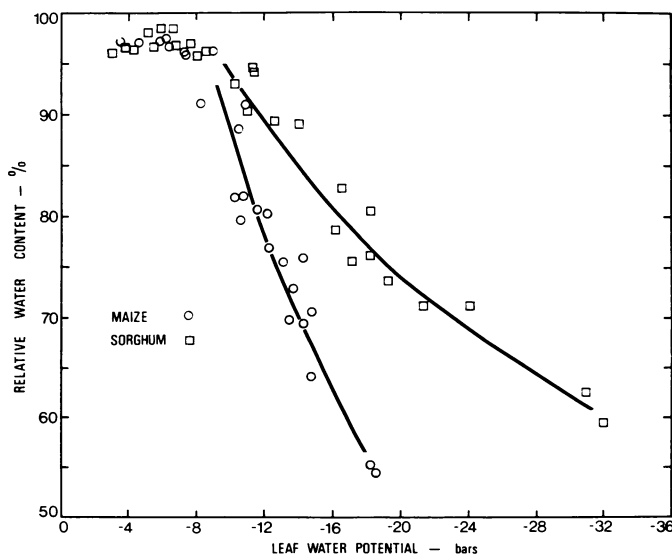


FIG. 3. Relationships between  $\Psi$  and leaf RWC for maize and sorghum.

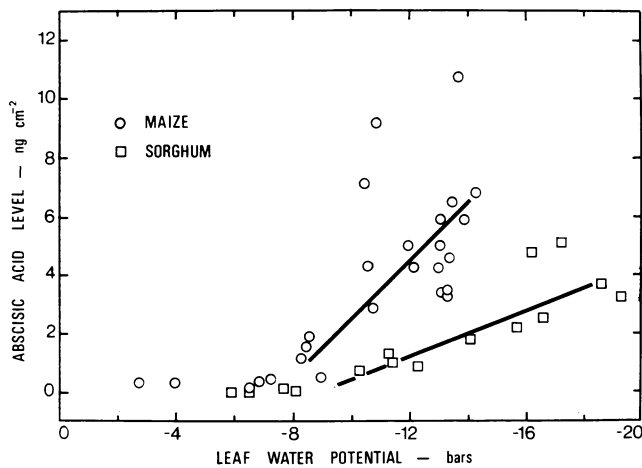


FIG. 4. Relationships between ABA level and  $\Psi$  for maize and sorghum.

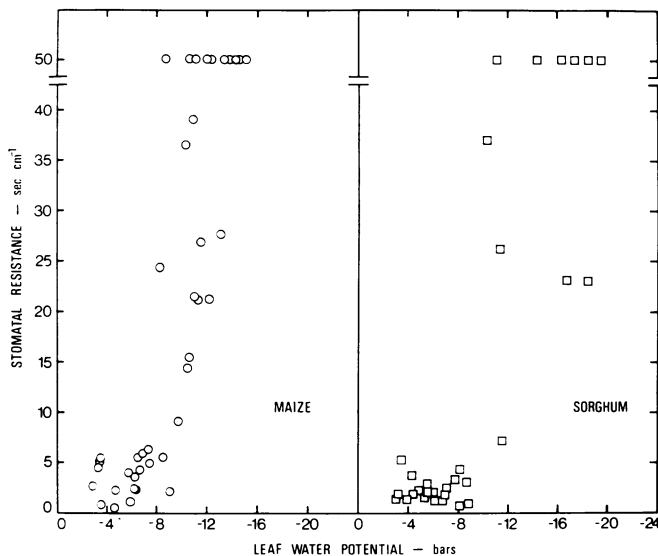


FIG. 5. Relationships between  $R$  and  $\Psi$  for maize and sorghum. Values of  $R$  in excess of 40  $\text{sec cm}^{-1}$  have all been plotted as 50  $\text{sec cm}^{-1}$ .

II increase occurred and on rewatering  $r_i$  remained high after  $\Psi$  had risen to control levels.

### DISCUSSION

**Responses to Stress.** In both maize and sorghum the stomata closed, and ABA levels rose, at rather high levels of leaf water status, viz., in the  $\Psi$  range  $-8$  to  $-10$  bars (Fig. 2), or about 96% RWC (Fig. 3). There are few reports in the literature relating ABA and  $\Psi$  levels. Zabadal (30) found that ABA rose abruptly between  $-10$  and  $-12$  bars  $\Psi$  in two species of *Ambrosia*, and Loveys and Kriedemann (17) found a 26-fold increase in ABA when the  $\Psi$  of *Vitis vinifera* leaves fell from  $-2$  to  $-10$  bars. Our results confirm the finding of Zabadal that there is a threshold value of  $\Psi$  below which ABA levels increase abruptly.

Although in both species the thresholds for increase in  $R$  and ABA appear to coincide (Figs. 4 and 5), stomatal closure occurred over a narrower range of  $\Psi$  than the increase in ABA. After stomata appeared to be closed, ABA levels increased

further, suggesting that stomatal closure does not require a large increase in ABA and that the amount of ABA produced in leaves is well in excess of that required to bring about stomatal closure.

The lag period before the rise in ABA in excised maize leaves suggests that ABA synthesis is not necessarily involved in the induction of stomatal closure. This is contrary to the suggestion of Wright (29). However, ABA has been shown to act directly on the guard cells (6, 21) and in epidermal strips

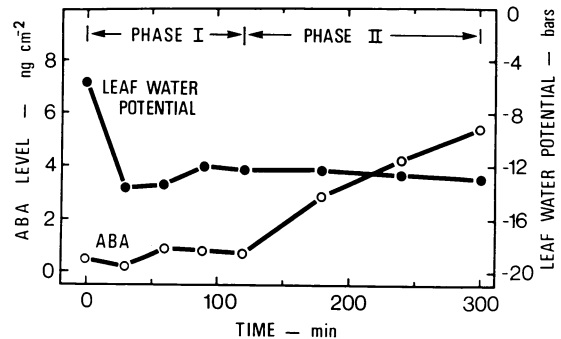


FIG. 6. Time course for changes in  $\Psi$  and ABA level following leaf excision and desiccation. The data represent individual samples from a single leaf of a well watered, greenhouse-grown maize plant. ABA level ( $\circ$ );  $\Psi$  ( $\bullet$ ).

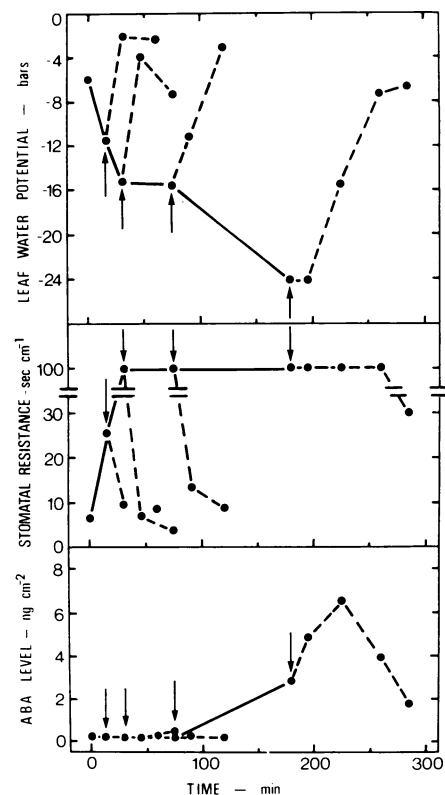


FIG. 7. Effect of short periods of stress on the subsequent recovery of the stomatal resistance of the lower surface. Leaves excised from plants grown in the Climate Laboratory were recut under water after 15, 30, 75, or 120 min of stress and the recovery of  $\Psi$  and  $r_i$  were compared with changes in ABA level. Stress period (—); recovery period (---); arrows indicate times of rewatering.

the relationship between stomatal pore size and log ABA concentration is linear over four orders of magnitude (10, 21). It is therefore the concentration in and around these cells, rather than the over-all level in the leaf, which is related to stomatal closure.

Even allowing for possible differences between species, there is considerable variation in the absolute amount of ABA reputedly required to initiate stomatal closure. The data of Kriedemann *et al.* (16) suggest ( $\pm$ )-ABA concentrations of 4.7 ng cm<sup>-2</sup> for bean leaves and 9.2 ng cm<sup>-2</sup> for maize, whereas from Cummins *et al.* (6) a value of 0.06 ng cm<sup>-2</sup> for barley can be calculated. Differences in the concentrations of the feeding solutions (50  $\mu$ M and 0.1  $\mu$ M) may partially account for this. The absolute amount of ABA required for the control of stomatal aperture may therefore be very small.

There is some evidence that ABA may occur in discrete compartments in the cell (5, 18, 22). One can postulate that when  $\Psi$  reaches the threshold value, a redistribution of ABA occurs. The reduction in ABA at sites of accumulation might in turn act as the trigger for enhanced ABA synthesis. Even the small amounts of ABA in unstressed maize leaves (0.5 ng cm<sup>-2</sup>) would, if uniformly distributed, give an over-all concentration of 0.1  $\mu$ M.

The closure of stomata over a 2- to 3-bar range of  $\Psi$  has been shown for maize (2, 24, 27) and sorghum (27). Sorghum has been reported to show stomatal closure over a wider range of  $\Psi$  than maize (2, 24). Differences in the absolute value of  $\Psi$  at which closure occurred may be attributable to the conditions under which the plants were grown, but in all cases closure was initiated at a slightly higher  $\Psi$  in maize than in sorghum.

Neumann *et al.* (20) interpret the inflection point in the  $\Psi$ -RWC curve as being approximately the point of zero turgor. This point in our data (Fig. 3) corresponds to the  $\Psi$  at which  $R$  and ABA levels increase. The shape of the  $\Psi$ -RWC curve can be affected by environmental conditions (9, 15), which may account for the differences in published values of the  $\Psi$  and RWC at which stomata close. Sanchez-Diaz and Kramer (24) published  $\Psi$ -RWC curves for maize and sorghum grown under a higher temperature and vapor pressure deficit than ours ("water saturation deficit" equals (100 - RWC)). The general form of their curves is similar to ours in that maize lost more water than sorghum when desiccated to any given level of  $\Psi$ . However, at any given  $\Psi$  their plants had a lower RWC.

The differences in the  $\Psi$ -RWC curves (Fig. 3) may reflect differences in the sensitivity of the two species to stress, sorghum being the less sensitive. In sorghum the critical turgidity for cell damage will be more slowly approached (12), and we have previously shown (7) that sorghum is more resistant than maize to ultrastructural damage at low  $\Psi$ .

**Recovery from Stress.** The more rapid recovery of sorghum stomata than maize after rewatering found in the present experiments confirms the earlier findings of Glover (8), but is at variance with those of Sanchez-Diaz and Kramer (24). The latter used younger plants and this, along with the differences in environment referred to previously, may account for the difference in response.

Leaves which had been stressed for only a short period behaved differently from those in which a phase II increase in ABA level had occurred. In the former the stomata reopen shortly after relief from stress, whereas high resistances persisted in the latter even after ABA had returned to control levels. Since  $\Psi$  and RWC recovered rapidly in all leaves the continued high resistance cannot be attributed to reduced turgor. Neither can the aftereffect of stress be attributed directly to high residual levels of ABA (13, 29) although high levels of

ABA during the stress period might cause changes in guard cell function which are only slowly reversed.

**Conclusion.** It appears that the patterns of response to stress in maize and sorghum are rather similar. Stomatal diffusion resistance and ABA rise at slightly lower levels of  $\Psi$  in sorghum, and this species also appears to recover more rapidly upon rewatering. In both species levels of ABA continued to rise after the stomata closed. There is a lag of at least 60 min before an increase in ABA level can be detected in maize. Hence, a redistribution of endogenous ABA rather than synthesis may be involved in the initial closure of stomata. Impaired stomatal functioning after stress does not appear to be associated with high residual levels of ABA in the leaf.

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