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Water Relations of Cotton Plants under Nitrogen Deficiency

V. ENVIRONMENTAL CONTROL OF ABSCISIC ACID ACCUMULATION AND STOMATAL SENSITIVITY TO ABSCISIC ACID

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ABSTRACT

Suboptimal N nutrition increased the water potential for stomatal closure in water stressed cotton (*Gossypium hirsutum* L.) leaves. This increased sensitivity to water stress had two components, increased accumulation of abscisic acid (ABA) and increased apparent stomatal sensitivity to ABA. Low N increased the threshold water potentials for stomatal closure and ABA accumulation by about 4 bars and 2 bars, respectively. Low N also greatly increased stomatal response to low concentrations of exogenous ABA applied to excised leaves through the transpiration stream. In low N leaves, kinetin decreased stomatal response to ABA to the level observed with high N leaves. Kinetin by itself had little effect on stomata, nor did it alter stomatal response to ABA in high N leaves. The results suggest a cytokinin-ABA balance which is altered by suboptimal N nutrition to favor stomatal closure during stress.

Ambient temperature and N nutrition interacted to alter stomatal response to water stress. Stress-induced ABA accumulation and apparent stomatal sensitivity to ABA were independently affected. The effects of each treatment, and their interaction, could be explained as the net result of changes in both accumulation and apparent sensitivity. Although the results document environmental control of stomatal response to ABA, either altered partitioning of ABA between active and inactive pools, or altered sensitivity of the guard cells, could account for the data.

Suboptimal N nutrition sensitizes stomata to water stress, causing stomatal closure at a higher water potential than normal (10, 12). This early closure is not based upon a difference in leaf turgor (13). Some evidence indicates that low N promotes ABA accumulation during stress in parallel with stomatal closure (12). Closure in both low N and high N plants is accompanied by increased stomatal sensitivity to CO_2 (12), a characteristic considered diagnostic for a role of ABA (16, 17). These results strongly suggest that N effects on stomatal closure during drought are mediated by ABA.

High temperature also affects stomatal closure (15). In cotton, N nutrition and ambient temperature interact to control stomatal responses to water stress (14). However, no data are available to show whether temperature effects on stomata are also mediated by ABA. Answering this question was one goal of the present study.

Trewavas (22) recently suggested that stomatal sensitivity to ABA, rather than the concentration of ABA at the guard cells, may control stomatal closure in water stressed leaves. This proposal is consistent with stomatal sensitivity to CO_2 (12), because it accommodates a role of ABA in stomatal closure (albeit a noncontrolling role). Control of sensitivity to ABA is a concept which has received little attention. Davies (3) and Ackerson (1) reported that water stress increased stomatal sensitivity to applied ABA, but Raschke and Zeevaart (18) found no effect of leaf age on stomatal sensitivity to ABA in immature *Xanthium* leaves. Therefore, our second goal was to determine whether N nutrition and ambient temperature might alter stomatal sensitivity to ABA.

MATERIALS AND METHODS

Plant Growth Conditions. Cotton (*Gossypium hirsutum* L. cv Deltapine 70) plants were grown from seed in a greenhouse in 14-L pots containing a mixture of equal parts of peat moss, sand, and vermiculite. After establishment, plants were thinned to four per pot and watered three times weekly with high N or low N nutrient solutions (containing 5 mM or 1 mM NO_3^-, respectively) as described earlier (14). The high N solution was optimum for growth, and the low N solution supported a growth rate about half the maximum. The greenhouse was cooled by refrigeration, with temperatures ranging from 35°C during early afternoon to 22°C at night. Humidity was not regulated.

Plants were exposed to elevated temperatures in another greenhouse which was uncooled but was ventilated to prevent temperatures from rising above outside temperatures during the day. In this greenhouse daily maximums were 42 ± 2°C and minimums were 28°C. These values are typical for midsummer in Phoenix.

Stomatal Conductances and Leaf Water Potentials. When plants had five to seven fully expanded mainstem leaves, watering was discontinued and half the plants grown at each N level were transferred to the hot greenhouse. Stomatal conductances (abaxial plus adaxial conductances in parallel) were monitored in early afternooon on the youngest fully expanded leaves, using a Li-Cor LI-1600[1] steady-state porometer (Li-Cor, Inc., Lincoln, NE). Immediately afterward, leaves were detached and their xylem pressure potentials (hereafter equated with leaf water potentials) determined with a pressure chamber. Data shown here are combined from four separate experiments.

It should be noted that the leaf conductances reported in this paper are greater than those reported earlier (12). This difference presumably resulted from the use of the steady-state porometer for the present experiments. In a direct comparison, conductances were much greater when measured with a steady-state device than when measured with a diffusion porometer (8).

Pressure-Volume Procedures. Leaf water potentials for zero turgor were determined from pressure-volume curves (11, 21). A leaf was excised from a well-watered plant, quickly weighed, and

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

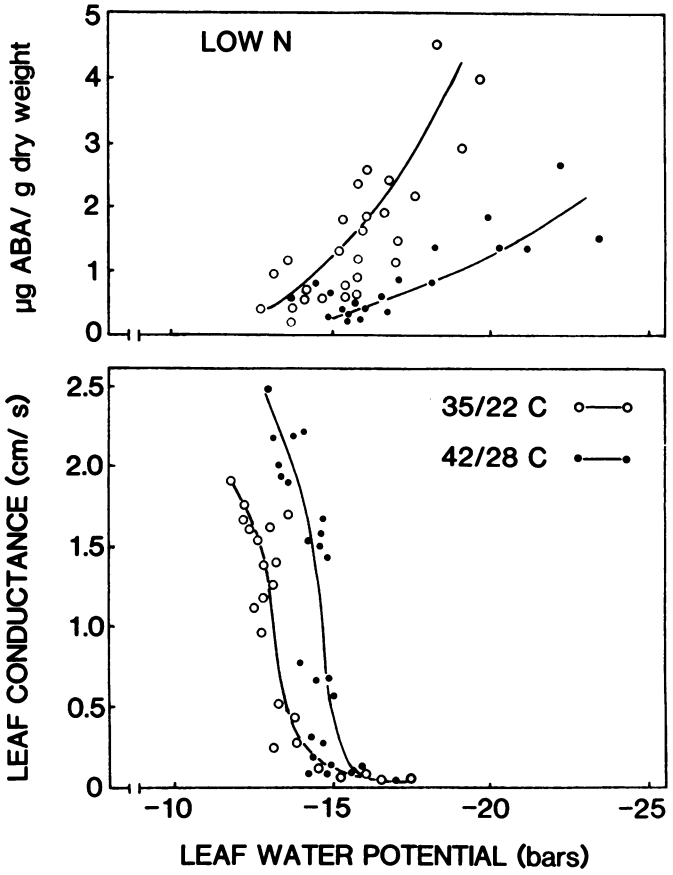

FIG. 1. Stomatal conductances and ABA concentrations of leaves of low-N plants. Plants were grown in the 35/22°C regime, then half were transferred to the hotter regime on cessation of watering. Each point represents an analysis of a single leaf.

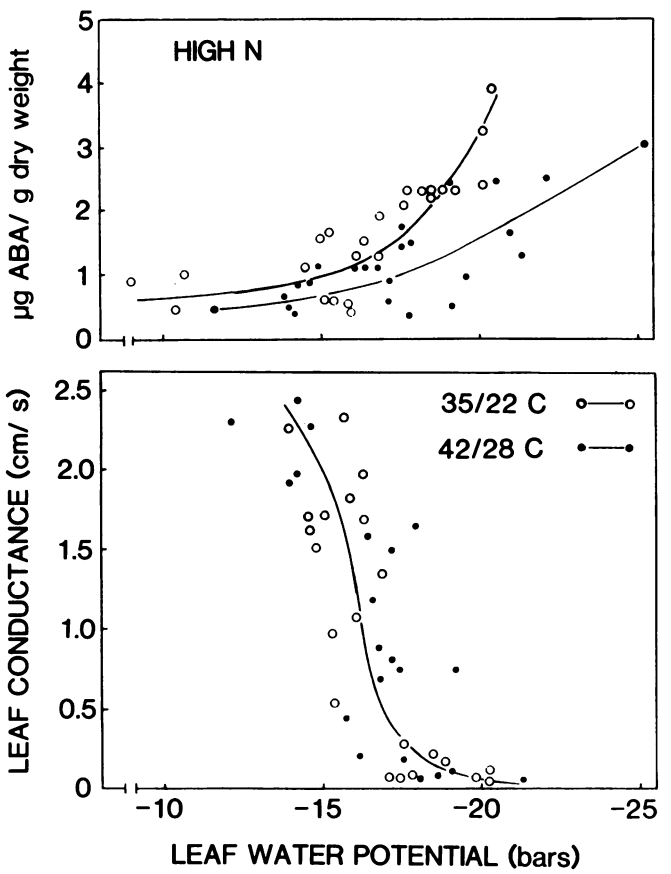

FIG. 2. Stomatal conductances and ABA concentrations of leaves of high-N plants. Details as in Figure 1.

its water potential determined in the pressure chamber. After the initial determination, the leaf was removed from the chamber and allowed to dry slowly in the laboratory. The sequence was repeated until the leaf was badly wilted. The inverse of the balancing pressure was plotted against the weight of water lost from the leaf, and the point of zero turgor was determined by standard graphical methods (11).

Sampling Leaves for ABA Analysis. Water potentials of the youngest fully expanded leaves of stressed plants were determined in early afternoon, and the leaves were immediately frozen on dry ice and lyophilized for ABA analysis. Data shown here are combined from three separate experiments. In two of these experiments, stomatal conductances were recorded before the leaves were detached (see above).

ABA Extraction and Analysis. The dried leaf samples were ground in a mill, then treated as described earlier (4) for extraction and purification of ABA. Purified extracts of ABA were methylated with diazomethane and analyzed by gas chromatography with an electron-capture detector (4). Each sample contained an internal standard of 1 µg mixed (*cis-trans* plus *trans-trans*) isomers of (±)-ABA (Sigma), added at the start of the initial extraction. The amount of extracted ABA was calculated from peak area ratios of *cis-trans* (native) and *trans-trans* isomers. Results were corrected for the amount of the *cis-trans* isomer derived from the internal standard. Recovery of ABA was typically about 80%. This technique worked well when ABA concentrations in tissue were relatively high, but produced considerable scatter as concentrations approached zero.

Stomatal Sensitivity to ABA. Well-watered plants which had five to seven expanded leaves were transferred to a walk-in growth

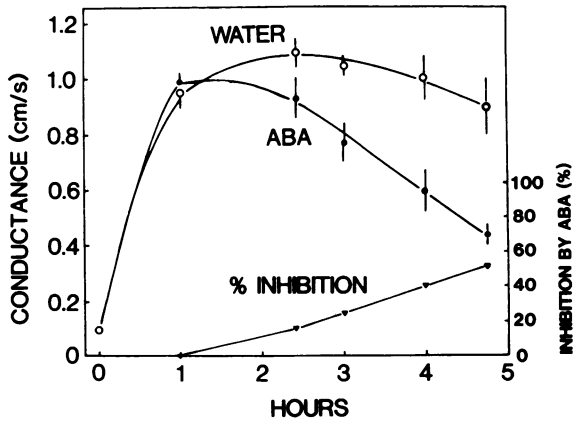

FIG. 3. Time courses for stomatal conductance of excised leaves with their petioles in water or ABA solution. This example is for low-N leaves fed 1 µM ABA. Conductances are means of three leaves, and are shown ± SE.

chamber set at 30°C with the lights off. In some experiments, the plants had been pretreated in the hot greenhouse for 2 d. After about 30 min in darkness to allow stomatal closure, the uppermost fully expanded leaf blades were trimmed to about 50 cm^2. The leaves were excised with the petioles under water and transferred to test tubes containing a known volume of water. Stock solutions of kinetin and (±)-ABA (both from Sigma) were added to produce specified concentrations, and the lights were turned on. Photosynthetically active radiation at leaf level was 570 µE m^{-2} s^{-1}, measured with a Li-Cor 190SB quantum sensor. Leaf conductances were monitored with the steady-state porometer as described earlier. Transpiration rates were calculated from the loss of water

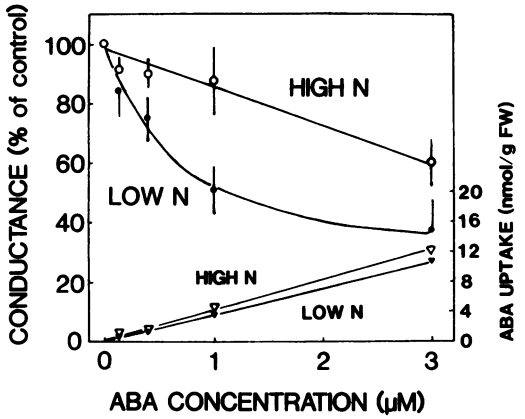

FIG. 4. Dependence of ABA uptake and stomatal conductance upon ABA concentration. Conductances (○, ●) are means of three leaves, and are shown ± SE. Uptake values (▽, ▼) are means of two independent trials, and are typical of several experiments.

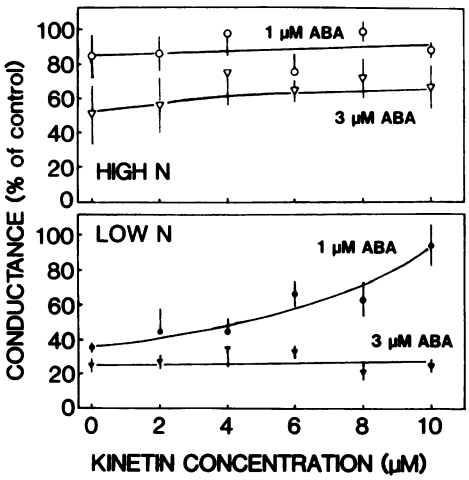

FIG. 5. Interaction of ABA and kinetin on conductance. Values are means of three leaves, and are shown ± SE.

Table I. *Effects of Ambient Temperature Regime and N Nutrition on Apparent Stomatal Sensitivity to ABA*

Excised leaves were given ABA at 1 μM through the transpiration stream. Conductances are shown as percentages of the corresponding controls (water only). Values are means of three leaves, and are shown ± SE.

Temperature	Conductance	
	High N	Low N
°C	*% of control*	
35/22[a]	84 ± 14	53 ± 2
42/28	46 ± 13	49 ± 16

[a] Daily maximum/minimum.

from the tube during incubation. Fresh weights of blades were measured at the end of the incubation.

RESULTS

ABA Accumulation and Stomatal Conductance. Stomatal closure and ABA accumulation during water stress were strongly affected by the ambient temperature regime. In low N plants, elevated temperatures decreased by 2 bars the water potentials for stomatal closure and for accumulation of ABA (Fig. 1). In addition, elevated temperatures greatly decreased the total amount of ABA accumulated during stress. In high N plants, temperature had little effect upon stomatal response to stress, but it again shifted the threshold for ABA accumulation and decreased the amount of ABA accumulated (Fig. 2).

Comparison of Figure 1 and Figure 2 shows that low N increased the water potential for stomatal closure by about 4 bars in the lower temperature regime and 2 bars at the higher temperatures. The water potentials for zero turgor were −18.0 ± 0.5 bars in high N plants and −18.9 ± 0.8 bars in low N plants. The stomatal effects thus could not be assigned to differences in leaf turgor. This interaction of N and temperature is similar to that reported earlier (14).

The relationship between stomatal conductance and ABA accumulation was altered by N nutrition and temperature. In high N plants at 35°C, conductance was decreased about 25% when the ABA level had reached 1 μg/g dry weight (Fig. 2). Low N and high temperature each seemed to sensitize stomata to ABA, so that closure was 90% complete or more at an ABA level of 1 μg/g dry weight (Figs. 1 and 2).

N. Nutrition and Stomatal Sensitivity to ABA. The altered relationship between conductance and accumulated ABA could result from changes in partitioning of ABA between active and inactive pools or from changes in stomatal sensitivity to ABA in the active pool. The active pool is generally equated with that ABA released from mesophyll cells and carried to the guard cells by the transpiration stream (2, 15, 18, 25). Thus, we applied ABA to excised leaves via the transpiration stream to test for sensitivity differences.

In the standard procedure adopted, leaves were excised in darkness with their petioles under water, and ABA was added to the water in which the petioles were immersed. Then the lights were turned on to initiate stomatal opening and ABA uptake. This procedure virtually eliminated the formation of air embolisms upon cutting. In the water controls, conductance increased rapidly for 1 h, then remained approximately constant for 2 to 3 h before beginning to decline slowly (Fig. 3). The effects of ABA became apparent after about 1 h of light. Inhibition of stomatal opening steadily increased with time as greater amounts of ABA were transported to the leaf blade (Fig. 3). All further tests were run with a standard incubation time of 3 to 4 h. With this incubation time, the conductances achieved in this system varied from 0.9 to 1.2 cm/s for low N leaves and 1.0 to 1.4 cm/s for high N leaves.

Leaves from low N and high N plants were affected differently by increasing concentrations of ABA. Stomatal conductance of low N leaves was sharply decreased by concentrations as low as 0.1 μM (Fig. 4). High N leaves required an ABA concentration 10 to 20 times greater to obtain the same effect. If conductance were considered in terms of actual uptake of ABA (calculated from water uptake and ABA concentration), then this difference in sensitivity would be slightly greater, because the greater stomatal closure in low N leaves slightly restricted their transpiration rate (Fig. 4). Stomatal closure to the degree shown in Figure 4 had only a small effect on uptake because, in the growth chamber where the leaves were incubated, the boundary layer tended to control transpiration rate.

Reversal of Stomatal Sensitivity to ABA. In low N leaves, stomatal response to low concentrations of ABA could be almost completely blocked by concurrent application of kinetin in the transpiration stream (Fig. 5, lower half). At 1 μM ABA, kinetin concentrations up to 10 μM progressively reduced the degree of stomatal closure until the ABA had only a slight effect. Kinetin could not reverse closure induced by 3 μM ABA, suggesting that the nature of their interaction was competitive in some way. In high N leaves, the effects of ABA were again much less than in low N leaves, and added kinetin had only slight influence (Fig. 5, upper half). Kinetin by itself did not alter stomatal conductance by more than 10% at either N level in these tests.

Temperature and Stomatal Sensitivity to ABA. Temperature interacted with N nutrition to alter apparent stomatal sensitivity to ABA. When high N plants were subjected to high temperatures, stomata of excised leaves showed increased sensitivity to ABA (Table I). In low N leaves, though, high temperature did not increase sensitivity beyond the level induced by the low N itself (Table I).

DISCUSSION

The results in this paper show that N nutrition alters both the accumulation of ABA during stress and the relationship between stomata and ABA content (Figs. 1 and 2). Changes in apparent stomatal sensitivity to applied ABA in excised leaves (Fig. 4) are consistent with changes in the relationship between ABA accumulation and conductance in intact stressed plants. It seems likely that both components of stomatal response to stress (*i.e.* ABA accumulation and apparent stomatal sensitivity to ABA) contributed to the N effect on stomatal closure.

The interaction of N nutrition and temperature on stomatal closure during stress can also be explained as a net result of changes in both components. In high N plants, high temperature decreased ABA accumulation but increased apparent stomatal sensitivity to ABA (Fig. 2; Table I). These two changes balanced each other, and stomatal response to stress was unchanged overall (Fig. 2). In low N plants, high temperature decreased ABA accumulation and did not further increase apparent stomatal sensitivity to ABA (Fig. 1; Table I). As a result, stomatal closure was inhibited overall (Fig. 1).

Although apparent stomatal sensitivity to ABA, as determined in excised leaves, is consistent with the behavior of stomata in intact stressed plants, there are other possible explanations for the relationship between conductance and ABA. Stomatal closure results from the secretion of ABA into the active pool from an inactive pool (2, 15, 25). Thus, the relationship between conductance and bulk ABA content must be controlled in part by partitioning between the two pools. Partitioning follows pH gradients, with ABA tending to be transferred to the compartment with the higher pH (5, 9). Thus, if N nutrition alters pH gradients within a cell or a tissue, it might also alter partitioning of ABA. Indeed, increased nitrate reduction raises intracellular pH (19). Based upon this reasoning, high N leaf cells should accumulate a greater fraction of total ABA in the inactive pool, and release less into the active pool. This is also consistent with the observed behavior. However, intracellular pH changes are presumably small, on the order of 0.1 pH unit (see Ref. 6 for an example involving excess cation uptake by roots). We do not know to what extent pH changes might contribute to apparent stomatal sensitivity to ABA.

The simplest interpretation of stomatal behavior in excised leaves is that it reflects behavior *in vivo*. However, comparison of ABA uptake for a given degree of closure with ABA accumulation in stressed intact plants shows that stomata of stressed intact plants were apparently more sensitive. In low N leaves, the amount of ABA([+]-isomer only) causing 50% closure when applied exogenously was associated with virtually complete closure when generated endogenously and accumulated. In high N plants, the disparity was even greater. Whether ABA uptake was altered by time (Fig. 3) or concentration in the transpiration stream (Fig. 4), apparent stomatal sensitivity to absorbed ABA remained about the same. Data from other laboratories (1, 18) also suggest lower stomatal sensitivity to exogenous ABA (in unstressed excised leaves) than to endogenous ABA (in stressed leaves). One possible explanation is that water stress sensitizes stomata to ABA (1, 3). Comparisons of ABA sensitivity between stressed and unstressed leaves may therefore be inappropriate. However, excised leaves may partition the incoming ABA between inactive and active pools or metabolize it to inactive compounds. If so, then these processes could affect apparent stomatal sensitivity to the applied dose. Because of these problems, the mechanism by which environment controls apparent stomatal sensitivity to ABA cannot be deduced from these experiments.

Interactions between ABA and cytokinins on stomata are a recurring but controversial idea in the literature (*e.g.* 7, 23). In our work, kinetin prevented stomatal responses to ABA in excised low N leaves but not in leaves of plants grown under optimum conditions (Fig. 5). This difference indicates that N nutrition may affect stomatal behavior by altering the balance between ABA and cytokinins. Significantly, low N is known to decrease cytokinin levels in plants (20, 24, 26).

Trewavas (22) suggested a model for action of growth substances in which both the concentration of effector and the number of active receptor sites can determine a response to that effector. The simplest form of this model equates the number of receptor sites to the sensitivity of the tissue to the effector. In excised cotton leaves, clearly low N increased stomatal response to applied ABA, but the system is not easily interpreted in terms of the model. Assuming that the effect of kinetin arises at the guard cells, the interaction between kinetin and ABA suggests that receptor sites which bind ABA might be affected by a cytokinin. Again, however, effects of kinetin on ABA partitioning or metabolism are possible. An explanation of this interaction will probably await identification of the ABA receptor site.

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