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Abscisic Acid and Cytokinin Contents of Leaves in Relation to Salinity and Relative Humidity

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ABSTRACT

The question is raised whether the hormonal modifications in a plant exposed to osmotic root stress result directly from the decrease in water potential of the root environment or from disturbances of the plant's water balance.

Tobacco plants were held for 24 hours under either high or low relative humidities, with or without salt. The amount of abscisic acid in the leaves of salinized plants rose markedly in low, but not in high, relative humidity. No change in the amount of extractable cytokinins was detected in any treatment. It is tentatively suggested that variations in the water content of leaves constitute a primary signal for modification of plant hormonal balance.

Itai and Vaadia (4) were first to propose and support the thesis that water balance in higher plants may be under hormonal regulation. They showed that the amount of cytokinins translocated in the root exudate of plants was drastically reduced after 48 hr of salination and water stress, and that this reduction was proportional to the concentration of NaCl or mannitol in the nutrient solution (5, 14). The idea that cytokinins are involved in the regulation of plant water balance was further supported when Livne and Vaadia (7) found that cytokinins increased stomatal opening and transpiration. It was inferred that root stresses such as caused by drought and salination alter the hormonal balance of the stressed plants and that at least one aspect of this modification would involve a decrease in the amounts of root-synthesized cytokinins which would reach the shoot. An inferred decrease in leaf cytokinins was presumed to reduce stomatal opening and also to arrest vegetative development, thereby improving the water balance of the stressed plant.

While this thesis has not been contradicted in later work, it would seem that the major initial hormonal signal regulating the response of plants to conditions disturbing the water balance is more complex. Recent research points out that ABA closes stomata (8), and that ABA content increases in leaves of plants exposed to water and osmotic stresses (9). It is our hypothesis that in response to such stresses, a decisive change occurs in the ratio between leaf cytokinins and ABA (9) and that the site of the initial hormonal modification may be the leaf. We premised that if the root was the initial site of the plant response to salination, salination-induced hormonal modifications would occur irrespective of the evaporative demands imposed on the plant. In this work we examined the contents of ABA and cytokinins in leaves of plants exposed to salination under high or low relative humidities.

MATERIALS AND METHODS

Fifty-day-old plants of *Nicotiana rustica* growing in 2-liter containers of aerated half-strength Hoagland solution were used. Salination was induced by the addition of 6 g NaCl/liter.

For the high relative humidity treatment, 4 plants, each in its separate container, were placed in a 0.1 mm thick polyethylene bag stretched over a suitable iron frame, the plants and the walls of the polyethylene chamber being sprayed once at the beginning with distilled water. The polyethylene chamber was filled to a height of 5 cm with water, through which 30 liters/min of humidified air, previously passed through water, were bubbled. For the low relative humidity treatment, the chamber was kept dry, and the incoming air was passed through a 5- × 20-cm column of silica gel.

Relative humidity and temperature inside the plastic containers were recorded. The RH[3] in the "high" humidity treatment ranged between 95 and 100% during the course of the experiment, whereas in the "low" humidity treatment, RH fluctuated from 62 to 90%. In the high humidity treatments, maximum day temperature was 32 C and in the low, 31 C. Minimum night temperature for both treatments was 20 C. Light intensity at noon in the greenhouse was 15,800 lux, and at that time the light intensity in the polyethylene containers was 13,500 and 15,000 lux for the high and low humidity treatments, respectively. Each replicate in an experiment included two plants to which 6 g NaCl/liter were added and two plants as controls, in each of the high and low humidity containers. All the experiments were begun at 10 AM by adding the salt to the appropriate plants. The experiments were terminated 24 hr later, and the leaf material from each two experiments was combined, every hormonal extraction thus being obtained from leaves of four plants. Altogether, the experiments were repeated 12 times, yielding material for six separate extracts from each of the four treatments, *i.e.* "high" humidity with salt, "high" humidity without salt, "low" humidity with salt and "low" humidity without salt. Similar results were obtained from all the experiments.

To measure the water content of the leaves, the water saturation deficit was determined. This value is obtained by subtracting the relative water content from 100%, RWC being determined according to Slatyer (12). For WSD determination, one disc, 23 mm in diameter, was taken from each of five

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[3] Abbreviations: WSD: water saturation deficit; RWC: relative water content; RH: relative humidity.

leaves of every plant just before terminating the experiment. All the leaves, except the upper ones less than 10 cm in length, and the oldest leaf were removed for hormone extraction. The midrib was cut from each leaf, and the blades were then quickly frozen in liquid nitrogen and freeze-dried.

Seven grams of dried leaf material, equivalent to about 70 g fresh weight, were taken for each extraction. The leaf material was homogenized in 70% methanol, centrifuged, and the supernatant passed through Whatman No. 1 filter paper. This was repeated three times; the methanolic supernatants were pooled, and their volumes were reduced under vacuum to 10% of the original. After the pH of the resultant aqueous solution had been brought to 8.6 with NaOH, it was extracted once with petroleum ether to remove inhibitors other than ABA (1). The pH was then adjusted to 2.7 with HCl and the solution extracted three times with ethyl acetate. The ethyl acetate fractions contained the ABA, and the water phase contained the cytokinins.

The combined ethyl acetate fractions were evaporated under vacuum at 40 C. The dry residue was dissolved in 7 ml of absolute methanol and applied to strips of Whatman No. 3 paper which were developed ascendingly in isopropanol-ammonia-water, 10:1:1 (v/v). Sections corresponding to R_F values of 0.60 to 0.80, representing the R_F of *cis*-ABA (Hoffman La Roche, Switzerland), were cut and dried in vacuum to remove all volatiles, then eluted twice with methanol. The eluates were combined and evaporated in vacuum at 40 C, then redissolved in methanol, 1 ml/g dry weight of the original leaf sample.

For bioassay, five eluate replications, each corresponding to 0.2 g fresh leaf weight, were dried in a stream of air. The inhibitory activity in the eluates was estimated by measuring the effect on the elongation of wheat coleoptiles (9). Another part of the eluates was esterified with diazomethane, dissolved in hexane, then injected into a Packard 7400 gas chromatograph using a glass spiral column 1.8 m long × 3 mm internal diameter packed with 1.5% QFl on Gas-chrom Q 60 to 80 mesh. The column temperature was 200 C with injection and detector temperature of 210 C and 200 C, respectively. An electron capture detector was used (11) with radioactive tritium foil. Nitrogen carrier gas was used at a flow rate of 30 ml/min, column inlet pressure 17.4 p.s.i. The electrometer range was 1×10^{-9} ampere. A pulsating voltage of 50 v amplitude, lasting 0.01 millisec (Tektronix pulse generator) was applied at 0.1 millisec intervals to the detector. One microliter of leaf extract, equivalent to 1.750 mg dry weight was injected, except in low humidity saline treatment, where the solution was diluted three times so that 1 μl contained the equivalent of only 0.583 mg dry weight. A calibration curve relating the amount of *cis*-ABA methyl ester injected to the computed area of the recorded peak is shown in Figure 1. The area of a peak was obtained by multiplying the height of the peak by the width of the peak at half height.

To estimate cytokinin activity, the water phase which was separated from the ethyl acetate fraction containing the ABA was passed through a Dowex column as described by Gazit and Blumenfeld (3). The appropriate eluate was dried and redissolved in methanol (70% v/v). Thereafter, 1 ml/g dry weight of the original leaf material was applied on a strip of Whatman No. 3 paper and chromatographed ascendingly in isopropanol-ammonia-water (10:1:1, v/v). After drying, the strips were divided into 10 equal sections and placed in Erlenmeyer flasks for assay by the soybean callus test. Four Erlenmeyers were used for each R_F fraction obtained from chromatograms on which a total of 7 gm dry weight equivalent was loaded. Each Erlenmeyer contained three explants grown on 25 cc nutrient medium. All the explants from one flask were weighed together.

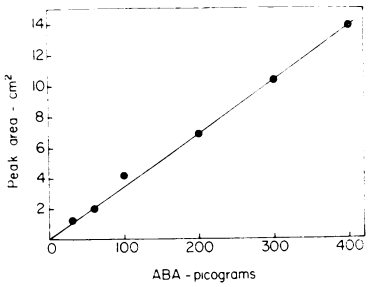

FIG. 1. Relationship between the amount of *cis*-ABA and the area of the recorded peak obtained in gas chromatography.

Table I. *Effect of RH and Salination on WSD of the Leaves and on Transpiration of Tobacco Plants*

The experiment was terminated after 24 hr.

	High RH		Low RH	
	WSD	Transpiration	WSD	Transpiration
	%	ml/day·plant	%	ml/day·plant
With NaCl (6 g/liter)	4.6	93	12.1	116
Without NaCl	1.4	95	7.3	183

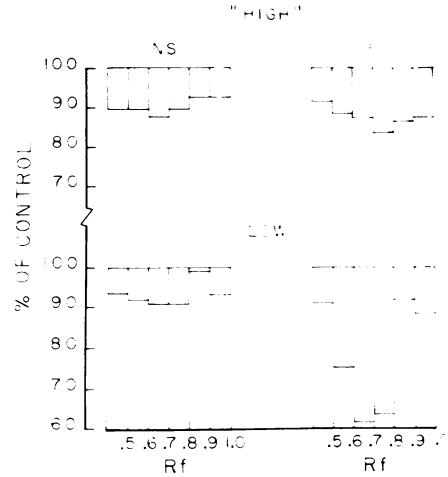

FIG. 2. The extent of inhibition of elongation of wheat coleoptiles, expressed as percentage of coleoptile elongation without inhibitors. Coleoptile elongation in the control was 11.5 mm. In the presence of *cis*-ABA concentrations ranging from 0.1 nM to 1 μM (in 10-fold increments), the percentages of elongation compared to the control were 100.0, 96.0, 68.0, 41.6, and 19.2, respectively. High: high relative humidity; low: low relative humidity; S: 6 g NaCl/liter in half-strength Hoagland; NS: half-strength Hoagland without salt.

Transpiration of the plants was estimated by measuring the amount of liquid lost from the nutrient solution during the 24 hr of the experiment. A very small correction was needed for surface evaporation.

RESULTS

The lowest and the highest water saturation deficits occurred in leaves of plants kept at high and low RH, respectively (Table I). WSD values of leaves from salinized plants at high

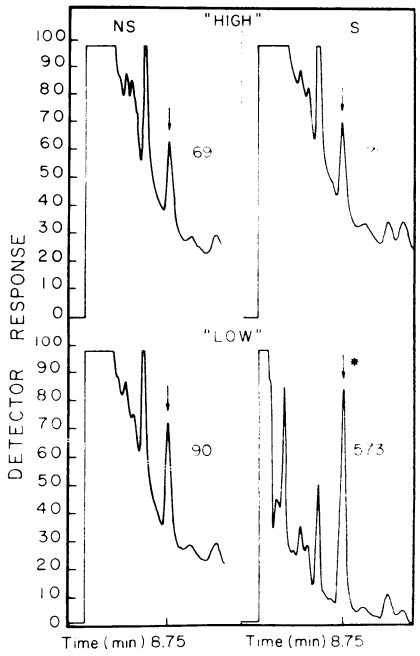

FIG. 3. Separation of ABA in gas-chromatography. Retention time for ABA, indicated by arrow, is 8.75 min. Abbreviations as in figure 2. One microliter of the plant extract, equivalent to 1.750 mg leaf dry weight was injected, except in the low humidity-saline treatment, where the 1 μl injected was equivalent to 0.583 mg dry weight. Numbers at the *cis*-ABA peaks indicate amounts of ABA as 1 pg/mg of leaf dry weight, and represent averages of two injections, material for which was obtained from separate leaf extractions.

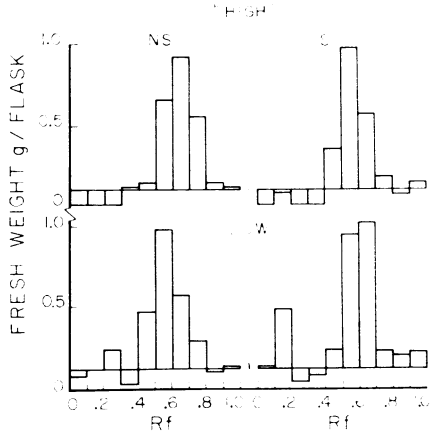

FIG. 4. Cytokinin activity in leaf extracts, assayed by measuring growth of soybean callus. Four Erlenmeyers were used for each R_F fraction obtained from chromatograms on which a total of 7 gm dry leaf weight equivalent was loaded. Each Erlenmeyer contained three explants grown on 25-cc nutrient medium. All the explants from one flask were weighed together. The base line of the histogram represents weight of the control explants grown on nutrient solution alone, without kinetin and leaf extract. Some R_F fractions yielded less callus tissue than the control. Callus yields for different kinetin concentrations were 2.02; 1.13; 0.58; 0.11 g for 5; 0.5; 0.05 and 0 mg/l, respectively.

RH were significantly lower than those of nonsalinized ones at low RH. The salinized plants at low RH appeared wilted initially but regained part of their turgor by the end of the 24-hr experimental period; nevertheless appearing more wilted than the plants at high RH.

As expected, transpiration was higher at low than at high RH, both in salinized and non-salinized plants, being highest in low RH, nonsalinized plants. At high RH transpiration was about the same in both salinized and nonsalinized plants.

Figure 2 illustrates the effects of RH and salinity on the ABA-like inhibitor content of leaves as assayed by the inhibition of elongation of wheat coleoptiles. In leaves of salinized plants grown under low RH, the assay indicates a substantial increase in inhibitor content; this did not occur in similar plants grown under high RH nor in nonsalinized plants.

The results obtained by gas chromatography (Fig. 3) agree with the patterns obtained by bioassay. In the high humidity treatment the *cis*-ABA content of leaves from salinized and nonsalinized plants was identical, about 70 pg/mg dry weight of the original sample. In the low humidity treatment, the *cis*-ABA content of leaves from salinized plants was about six times greater than that in nonsalinized plants; 573 *versus* 90 pg/mg dry wt, respectively. We have followed in detail the pattern of the salt-induced rise in leaf-ABA. Our findings, to be published elsewhere, indicate that ABA content of tobacco leaves rises continuously up to the 4th day after salination. ABA-content declines thereafter to a steady level which is significantly higher than that detected in the control plants.

The soybean bioassay for cytokinins (Fig. 4), did not reveal any differences in any of the leaf extracts of plants held for 24 hr at either high or low RH, with or without 6 g NaCl/liter in the nutrient solution.

DISCUSSION

The low relative water saturation deficits found in leaves of salinized plants at high RH suggest that this treatment relieved the water stress in the plants. This agrees with the observation of Nieman and Poulsen (10) that high RH relieves almost completely the suppressive effect of salinity on the growth of cotton plants.

In repeated experiments, no differences could be observed in the extractable cytokinin content of leaves of salinized plants and nonsaline controls at either high or low humidities. This may seem to contradict reports of Itai and Vaadia (4, 14) and Itai *et al.* (5) who found a marked decrease in the cytokinin content of the root exudate of tobacco plants after 48 hr of salination. There are some possible explanations for this apparent discrepancy, one being that changes in leaf cytokinins may not yet be observable 24 hr after salination. In addition, the amount of cytokinins in the root exudate collected from the cut stem after the stress was relieved (5) may not be correlated to the amount of extractable cytokinins in the leaves. Another possible explanation relates to the findings of Blumenfeld (2) and Gazit and Blumenfeld (3). They observed that while no cytokinin activity was found in the methanolic extract of avocado mesocarp, this activity, as assayed by soybean-cotyledon callus, did appear in the aqueous fraction after either acid hydrolysis or passage through Dowex 50 (H^+) ion exchange columns. They suggested that the aqueous phase contained a "bound" cytokinin, inactive in the soybean callus bioassay, but liberated by acid hydrolysis. It may be that salination changed cytokinins from "free" to "bound" forms, and that this was not observed by us because "bound" cytokinins were freed during extractions in this study. This possibility is worth further exploration in the light of the recent proposal that cytokinins are inactivated in leaves exposed to enhanced evaporative demands (6).

We have already shown (9) that the ABA content of leaves of osmotically stressed plants changed within a short time after the exposure of plants to osmotic stresses. This phenomenon was also seen in the present work, where a marked increase in

the *cis*-ABA content was observed in leaves of salinized plants exhibiting high WSD at low RH. Why, then, was there no increase in the ABA content of leaves of salinized plants at high RH? The answer probably concerns the mechanism by which a modification in the plant's water-balance brings about a response. Wright and Hiron (13) found that ABA rises rapidly in detached wilting leaves, and Itai and Vaadia (6) reported a rapid decline in cytokinin activity in such leaves. It seems, indeed, that a drastic change in the hormonal balance may take place in the leaf, irrespective of root environment. We suggest that variations in the leaf-water content constitute the primary signal which brings about modifications in the hormonal balance of the plant. Alternatively, our results do not negate the possibility that salination may first be recognized by the roots which would produce the primary, salination-induced hormonal signal to the leaf. This signal would then effect an increase in leaf ABA only when the leaf-water content declines below a certain optimum. Even this alternative, however, places the major regulatory role of plant response to salination with the water content of the leaf. Thus when the leaf-water content does not decline far from the optimal, as in the saline-high humidity treatment, ABA content in the leaves does not rise, despite the saline environment of the root.

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