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Water and Salt Stresses, Kinetin and Protein Synthesis in Tobacco Leaves[1]

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Summary. The capacity of tobacco (*Nicotiana rustica*) leaf discs to incorporate L-leucine ^{14}C into proteins was measured. Leaf discs were obtained from plants which experienced soil water depletion, or which were exposed to a saline or osmotic stress in the root medium. The stresses were brief of relatively short duration and water potential did not decrease below 4 bars in the root media. Leaf discs were sampled 2 hours after stress removal, achieved by reirrigation, or replacement of saline and osmotic solutions with normal nutrient solution. Plants were always turgid when leaves were sampled.

All stressed tissues showed reduced capacity to incorporate L-leucine ^{14}C into protein. The reduction was about 50 % and could not be attributed either to reduced uptake into the discs, or to possible isotopic dilution. Incorporation decreased progressively with leaf age in control discs as well as in stressed leaf discs. At all ages tested, incorporation in stressed discs was lower than that of the control. Full recovery of incorporation capacity in stressed discs was obtained when discs were sampled 72 hours after stress removal but not earlier.

Kinetin pretreatment prior to incubation with labelled leucine partially restored incorporation in stressed discs. The differences in response to kinetin of stressed and control discs suggest a lower endogenous level of cytokinins in the stressed discs. The results were qualitatively similar regardless of the kind of stress given to the plants during pretreatment. This supports the hypothesis that the normal supply of root cytokinins is important in shoot metabolism.

Water deficits apparently enhance leaf senescence (10). In mature leaves of water-deficient plants protein content is lower and protein degradation greater than in well-watered control plants (1, 10). Similar observations are also related to leaf age. As mature leaves age, even in well-watered plants, their protein contents decrease (10).

Leaf senescence may be retarded by certain growth substances. Auxins, gibberellins and particularly kinins are known to be effective in different plant species. In leaves of many annuals, addition of cytokinins retards chlorophyll degradation and promotes amino acid incorporation and protein synthesis (7, 9). Recently, cytokinins have been found in root exudate of several plant species (4, 5). The root exudate of plants recovering from water deficits as well as osmotic and salinity stresses has a much lower cytokinin activity than the exudate of well-irrigated control plants, suggesting that the supply of root cytokinins to leaves is important in regulating shoot metabolism (3).

This paper reports on changes in rates of amino acid incorporation in leaf discs from stressed plants and on recovery of these rates in the presence of kinetin.

Materials and Methods

Single tobacco plants (*Nicotiana rustica*) were grown for about 10 weeks in a cooled greenhouse (20°–30°) in pots containing either 3 kg of a soil-manure mixture (3:2 v/v) or 2 liters half-strength Hoagland solution. Soil-grown plants were used in the water-deficiency experiments and solution-grown plants in osmotic and salinity stress experiments. In water-deficiency experiments the plants were grown normally for 9 weeks and then watering was withheld from half of them; these plants were reirrigated 5 days later when they had reached visible temporary wilting. Leaves were harvested 2 hours after this irrigation, when shoot-turgor recovery seemed to be complete. In other experiments osmotic concentrations of 30 g/l mannitol and salinity of 6 g/l NaCl were reached gradually by addition of 2 equal portions on 2 successive days to the culture solution. After 2 days in the full strength mannitol or salt solutions the plants were transferred to distilled water 2 hours prior to harvest. By the time of harvest they were fully turgid.

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Mature leaves of similar age were used throughout for punching 14 mm discs, avoiding the main veins. The discs were rinsed, dried with filter paper and weighed. Six discs were transferred to 2.5 ml incubation medium and were aspirated with a water suction pump for 2 minutes. When the vacuum was released, the discs became fully infiltrated. The incubation medium contained 0.1 % (w/v) sucrose, 0.01 % (v/v) Tween 80 and 1 to 1.5 uc ^{14}C L-leucine (Schwarz Bio Research, Inc. 200 mc/mmole). Incubation took place at 27° in a shaker under diffused light for 1 hour. The discs were then washed with cold (1°) distilled water and cold 0.05 mM DL-leucine solution and deep frozen instantly in acetone and dry ice. The tissue was ground with quartz sand at 3° with a pestle and mortar, and 1.5 ml 0.5 mM DL-leucine and 1.5 ml of 10 % (v/v) trichloroacetic acid were added to the homogenate. The homogenate was centrifuged at 5000 g for 10 minutes and the pellet was washed twice with 5 % (v/v) trichloroacetic acid. During the second wash the suspension was heated to 90° for 15 minutes, then washed twice with acetone to remove the chlorophyll, dried and dispersed in 1 ml of 20 mM NaOH. Aliquots were counted by liquid scintillation. Protein was determined with the folin phenol reagent after Lowry et al. (6). All analyses were done in duplicate.

In some experiments after the stress treatment, discs were preincubated in 4 ml of water or kinetin solutions (Sigma, Lot B-0210) and put on a 27° water bath shaker for 13 hours in darkness. Incubation with the label followed this pretreatment as described.

Results

Leaf discs obtained from plants subjected to water deficits, osmotic stress, or salinity, exhibited reduced rates of L-leucine incorporation into protein (table I). The reduction was about 50 %. Protein content and fresh weight were much less altered by the stress in these experiments. The slight increase in protein content in the stressed tissue was probably associated with greater dry weight per disc in these tissues. Nevertheless definite reduction in incorporation was observed no matter how the data were expressed. Most of the experiments were performed on plants subjected to the 3 types of stresses. The results were similar regardless of the kind of stress applied. The data presented in the following discussion include experiments mainly with salt-stressed tissues, but they are representative of the response with water deficient and osmotically stressed tissues.

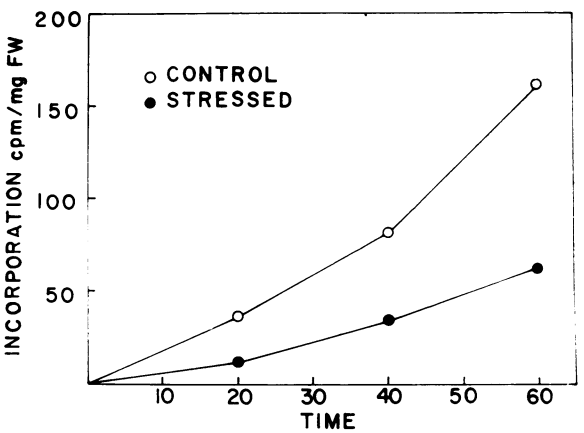

FIG. 1. Incorporation of L-leucine ^{14}C into stressed and control leaf discs as a function of incubation time.

Reduction in incorporation is proportionally similar throughout the incubation period (fig 1). Salt-stressed leaf discs showed relatively similar reduction in incorporation at all the measured intervals.

The observed reduction in incorporation could result from slower uptake of L-leucine ^{14}C into the stressed leaf discs. A check on this possibility is given in table II. In this experiment L-leucine ^{14}C was measured in the trichloracetic acid and acetone washings, and uptake into the tissue was calculated. Uptake into the stressed tissue was lower than into the control. However, incorporation in stressed tissue was much more affected than uptake. Only 29 % of the absorbed leucine was actually incorporated in the stressed tissue, while

Table I. *Incorporation of L-Leucine ^{14}C into Protein in Tobacco Leaf Discs*

Treatments prior to disc sampling included water, osmotic, and salinity stresses to the root system of the intact plant. The L-leucine ^{14}C had a specific activity of 6.6 mc/mmole.

Treatment	Fresh	Protein		
	Expt I Wt,* mg/disc		cpm/disc	cpm/10^2 mg fr wt
Control (soil grown)	42.4	...	1840	4362
Water stress	43.9	...	1005	2286
	Expt II			
Control (solution grown)	33.9	0.018	4370	12866
NaCl	33.7	0.021	1976	5863
Mannitol	40.4	0.026	2870	7110

* Initial weights prior to incubation.

Table II. *Uptake and Incorporation of* L-*Leucine* ^{14}C *into Stressed and Control Leaf Discs*

Uptake was measured by counting the radioactivity in the trichloroacetic acid and acetone fractions, as well as the pellet. Incorporation is the radioactivity of the pellet. The L-leucine ^{14}C had a specific activity of 200 mc/mmole. Free space washing in non-labeled leucine was provided after incubation.

Treatment	Uptake		Incorporation		Incorporation
	cpm/disc	cpm/100 mg fr wt	cpm/disc	cpm/100 mg fr wt	% of uptake
Control	5015	13031	3106	8065	61
Salt stress	3839	9896	1250	3410	29

Table III. *The Effect of Concentration of* L-*Leucine on Incorporation of* L-*Leucine* ^{14}C *into Protein*

Incubation concentrations of L-leucine were 1 μM and 30 μM. Both solutions contained similar radioactivity. The specific activity of the 1 μM solution was 200 mc/mmole and that of the 30 μM was 6.6 mc/mmole.

Treatment	Incorporation			
L-Leucine ^{14}C	1 μM	30 μM	1 μM	30 μM
	CPM/disc		mμmole/disc*	
Control	3225	3534	3.09×10^{-2}	9.17×10^{-1}
Salt stress	1449	1266	1.39×10^{-2}	3.29×10^{-1}

* Calculated on the basis of the specific activities of the solutions.

Table IV. *The Effect of Leaf Age and Stress on Incorporation of* L-*Leucine* ^{14}C *into Protein*

The L-leucine ^{14}C had a specific activity of 6.6 mc/mmole.

Leaf age	Control	NaCl	Mannitol
	Incorporation – cpm/100 mg fr wt		
Young	4556	1976	1497
Mature	2190	1029	1230
Old	1460	740	588

Table V. *Incorporation of* L-*Leucine* ^{14}C *into Protein as a Function of Time from Removal of Stress Solution*

The L-leucine ^{14}C had a specific activity of 200 mc/mmole.

Removal of stress solution	Control	Stress	Stress/control
hrs	cpm/100 mg fr wt		% Incorporation
2	14,780	5630	38
24	18,070	8670	48
48	18,790	11,980	64
72	12,700	14,720	116
96	13,250	15,920	121

61 % was incorporated in the control. This is evidence that limitations on the uptake of leucine into the tissue as a whole could not explain the observed reductions in incorporation.

No measurements were made of amino acid pools and specific activities of L-leucine ^{14}C in the leaf tissue during the incubation period. It has been reported that amino acid pools may increase in stressed tissues (1). If the leucine pools in the stressed tissue recorded in table I were significantly larger than those of the control, the observed reduction in incorporation could have been attributed to isotopic dilution rather than to the treatments themselves. To test this point discs of stressed and control plants were incubated in 2 different total concentrations of L-leucine but with similar radioactivity (table III). The relative reduction in incorporation in the stressed tissue was similar in both concentrations. Therefore, any existing pools in the tissue were apparently too small to influence the results. The similarity between the incorporation rates, in terms of cpm from the 2 concentrations suggests that total incorporation was directly proportional to the external concentrations.

Leaf age has significant effect on the capacity of the tissue to incorporate amino acids into pro-

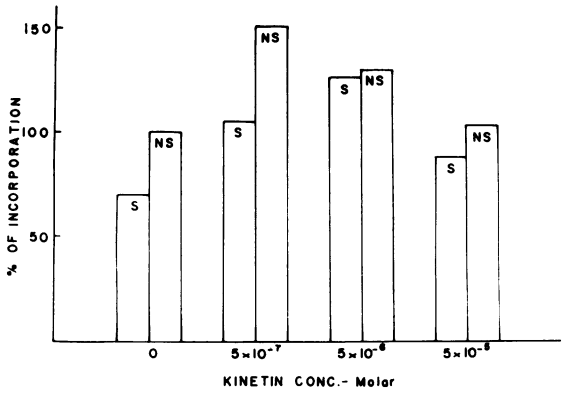

FIG. 2. The effect of kinetin pretreatment on L-leucine ^{14}C incorporation into the protein of tobacco leaf discs. In the stress treatment plant roots were exposed to NaCl solution (100 mM) for 2 days. All data are presented as percent of the non-stressed control where incorporation was 2770 cpm/disc. S = Stressed. NS = Non-stressed.

tein (table IV). In this frame of reference, stressed tissue behaves as if it were older than the control. Progressive reduction in incorporation is observed at all times with increasing leaf age, but at all ages, incorporation in stressed tissue is lower than in the controls.

In the above experiments leaf discs were sampled about 2 hours after the stress solution was replaced with a normal nutrient solution. The data in table V show that if longer time is allowed between removal of the stress solution and leaf sampling, full recovery of incorporation can be achieved. In this case full recovery required about 3 days in nutrient solution following the stress period. In other experiments somewhat shorter periods (36–48 hrs) were sufficient.

Cytokinins are known to retard senescence and enhance amino acid incorporation in leaf tissue. Figure 2 shows that the lost capacity of stressed tissue to incorporate amino acids may be partially restored by pretreatments in kinetin. Kinetin enhances incorporation in both stressed and non-stressed leaves. In non-stressed discs maximum incorporation is observed at a kinetin concentration of 0.5 μM while higher concentrations are less effective. In stressed tissue however, maximum incorporation is observed at a kinetin concentration of 5 μM. The difference in the dose response of stressed and control tissue suggests a lower endogenous level of kinins in the stressed leaf tissue (fig 2).

Discussion

Exogenous kinins retard the senescence of cut leaves and rootless shoots of different plants (4, 5, 9). Aging of excised leaves is likewise retarded when roots are formed on the petioles of such leaves (8). Cytokinins are present in root tips (12) and appear to be translocated to shoots since they are found in xylem exudate (4, 5).

Shah and Loomis (10) have shown that during water stress, mature leaves of sugar beets age more rapidly than those of well-watered plants. They have also shown, in agreement with other reports (11), that levels of proteins and RNA in such leaves decrease rapidly. A spray of the cytokinin, 6-benzylaminopurine, on the leaves of the stressed plants prevented such reductions and retarded aging. The data of Shah and Loomis (10) could be interpreted in light of the working hypothesis of Itai and Vaadia (3). The latter observed greatly reduced levels of cytokinins in the exudate of plants recovering from water and salinity stresses as compared to control plants. Their hypothesis stated that metabolic shifts and enhanced aging in shoots of stressed plants may be attributed in part to reduced supply of root kinins.

The data presented in this paper support this hypothesis. The capacity of amino acid incorporation is reduced in stressed tissues (table I). This is so, regardless of the nature of the stresses, which included salinity and osmotic stresses as well as deficient watering. In all of these cases kinin content of root exudate is also reduced markedly (3, and unpublished data). In terms of amino acid incorporation potential, stressed tissue appears to be physiologically older than the control in (table IV). The older the leaf tissue the lower the incorporation in control plants. Water or salt stresses result in an added reduction in amino acid incorporation. The damage to the incorporation capacity in stressed tissues is reversible, provided sufficient time is allowed for recovery from stress. Table V shows that 72 hours are required. This is also the time interval necessary for the recovery of normal levels of kinins in the exudate (3, and unpublished data).

Direct measurement and comparison of kinin levels in stressed and non-stressed leaves cannot be made easily, with the available techniques. However, an indirect indication that endogenous kinin levels are lower in the stressed tissue is provided in figure 2. The dose response curve of the stressed tissue to kinetin is displaced, so that a higher external concentration of kinetin is required to achieve the effect obtained in the control with a lower kinetin concentration. This can be interpreted as an indication that endogenous levels of cytokinins are lower in stressed tissues. Such an interpretation is in agreement with the observations of reduced levels of cytokinins in root exudate of plants recovering from stress (3). Plants may not respond in an identical manner to salinity, osmotic substrates and water shortage. However, the data presented here showed similar reduction in the capacity of the tissues for protein synthesis regardless of the nature of the stress. Similarly, Itai and Vaadia (3, and unpublished results) showed

that the reduction in kinin contents in exudate of plants recovering from stress are independent of the kind of stress applied. This may be interpreted to mean, that due to the stress, plant performance is disturbed and reductions in kinin levels and protein synthesis are only reflections of this disturbance. Alternatively it may be assumed that normal supply of root kinins to the shoot is a prerequisite to normal protein synthesis.

The existence of root hormones has been postulated (2, 13). Recently it became evident that cytokinins and other compounds may well represent these postulated hormones (4, 5). However, the possibility that changes in root environment may modify plant hormonal balance has not been given sufficient attention in stress physiology in general and in water relations and salinity in particular. This paper is an attempt to provide an example of this possibility.

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Literature Cited

1. BARNETT, N. M. AND A. W. NAYLOR. 1966. Amino acid and protein metabolism in bermuda grass during water stress. Plant Physiol. 41: 1222–30.
2. CHIBNALL, A. C. 1939. Protein metabolism in the plant. Yale University Press, New Haven.
3. ITAI, C. AND Y. VAADIA. 1965. Kinetin-like activity in root exudate of water stressed sunflower plants. Physiol. Plantarum 18: 941–44.
4. KENDE, H. 1965. Kinetin-like factors in the root exudate of sunflowers. Proc. Natl. Acad. Sci. 53: 1302–07.
5. KENDE, H. AND D. SITTON. Kinetin and gibberellin-like root hormones and their physiological significance. Proc. N. Y. Acad. Sci. (in Press).
6. LOWRY, O. H., N. J. ROSEBROUGH, L. A. FARR, AND R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–74.
7. OSBORNE, D. J. 1965. Interactions of hormonal substances in the growth and development of plants. J. Sci. Agri. 16: 1.
8. PARTHIER, B. 1964. Proteinsynthese in grünnen Blättern. II. Aminosäure-Inkorporation in die Proteine subzellulärer Strukturen in Abhangigkeit vom physiologischen Zustand der Blätter (Blattalter). Flora 154: 230–44.
9. RICHMOND, A. E. AND A. LANG. 1957. Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science 125: 650–51.
10. SHAH, C. B. AND R. S. LOOMIS. 1965. Ribonucleic acid and protein metabolism in sugar beet during drought. Physiol. Plantarum 18: 240–54.
11. VAADIA, Y., F. C. RANEY, AND R. M. HAGAN. 1961. Plant water deficits and physiological processes. Ann. Rev. Plant Physiol. 12: 265–92.
12. WEISS, C. AND Y. VAADIA. 1965. Kinetin-like activity in root apices of sunflower plants. Life Sciences 4: 1323–26.
13. WENT, F. W. 1938. Specific factors other than auxins affecting growth and root formation. Plant Physiol. 13: 55–80.