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Cytokinin Activity in Water-stressed Shoots[1]

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CHANAN ITAI AND YOASH VAADIA[2]
Division of Life Sciences, Negev Institute for Arid Zone Research, Beer-Sheva, Israel

ABSTRACT

Water stress applied to the plant shoot through enhanced evaporative demands reduced cytokinin activity in extracts of xylem exudate and leaves. This reduction resembled the changes in cytokinin activity caused by water stress applied to the root. Cytokinin activity in detached wilting leaves decreased rapidly. Recovery took place after several hours in a humid chamber. Experiments with ^{14}C-kinetin indicated that the mechanism of the inactivation and its reversal involve a chemical transformation of the cytokinin molecule.

Plants may be water-stressed by limiting water availability to the root through drought, salinity, or osmotic media. Water stress can also be imposed by enhancement of transpiration demands. Plants respond similarly to various water stresses. Similarities lie in RNA and protein metabolism (13) and several developmental and morphogenic features (15). Evidence has been brought (5, 6) on the role of cytokinins in regulating plant response to water stress applied to the root. The work described here attempts to verify and extend the hypothesis that cytokinins play a role in the regulation of plant water stress responses, when transpiration demands are enhanced.

MATERIALS AND METHODS

Six- to 8-week old *Nicotiana rustica* plants were used which, at the beginning of the experimental period, had developed to almost the termination of the vegetative stage. Each plant was grown in 2 liters of half-strength Hoagland solution in a cooled greenhouse.

Cytokinin activity in stem exudate was determined as described earlier (6). The leaf cytokinin extraction and separation method was modified from Heide and Skoog (4). About 100 g of mature leaves, with main veins removed, were frozen in liquid air. The leaves were blended in a Waring Blendor with 1 liter of 70% (v/v) ethanol and then boiled gently for 5 min at 85 C. The cooled extract was filtered, and the pH of the supernatant was adjusted to 8.8. After centrifugation (10,000*g* for 25 min), the precipitate was discarded, and 20 ml of saturated barium acetate were added to the supernatant. Centrifugation was done after an 18-hr period in darkness at 4 C. The precipitate was discarded, the volume of the supernatant was reduced to half, and the pH was adjusted to 2.5 with HCl. A cation exchange column was used for separation (180 ml, Amberlite IR-100 A.R. 20–50 mesh, or Dowex 50W × 4 50–100 mesh). The column was rinsed with 1 liter of 70% (v/v) ethanol and with H_2O. The active substance was eluted with 1 liter of 4 N NH_4OH. The eluate was concentrated to 300 ml under vacuum at 45 C, and the pH was adjusted to 8.0 and extracted twice on a shaker for 20 hr with 200 ml of *n*-butanol. The extracts were then combined and dried under vacuum. The residue was dissolved in 5 ml of water at pH 8. This solution was divided into equal portions and bioassayed by means of soybean tissue culture (9).

The incorporation of ^{14}C-L-leucine into trichloroacetic acid-insoluble leaf disc extracts was measured according to Ben-Zioni *et al.* (2). Kinetin treatment was applied prior to incubation in ^{14}C-L-leucine, by placing leaf discs for 45 min between Whatman No. 3 papers wet with kinetin solution.

Leaf discs were exposed to two extreme relative humidities by placing them for 30 min in desiccators which contained either wet filter paper or $CaCl_2$. Fresh weight change was recorded.

Experiments with labeled kinetin were done as follows. The petiole of a fully expanded leaf was placed in a vial containing 2 ml of 0.5 μc kinetin-8-^{14}C solution (Cal Atomic, Los Angeles, specific radioactivity 24.6 μc/μmole). The 2 ml were taken up by the leaf in 60 to 120 min. The leaf was cut in half, and the middle rib was removed. One half was treated as indicated in each experiment, and the other was put in a humid chamber for the duration of the treatment. On termination of treatment the half-leaf was frozen in liquid air and homogenized with 2 ml of cold ethanol. The homogenate was filtered off, and the residue was washed with 2 ml of cold water. To the combined filtrate 1 ml of chloroform was added. The nonpolar fraction contained no label. The polar fraction was separated and applied to Whatman No. 3 chromatography paper. The chromatogram was developed for 30 cm in an ascending-descending fashion in one of the two solvent mixtures: H_2O-acetic acid-*n*-butanol (1:1:4) or ethanol-chloroform (9:1). The chromatogram was then dried and cut into 10 equal portions which were placed into vials containing dioxane-ethanol-toluene scintillation solution. The vials were then placed into a liquid scintillation counter (Packard TriCarb 3308) and assayed for ^{14}C.

RESULTS

Plants were exposed for 30 min to an airstream which caused slight wilting of the leaves. The airstream was turned off and the shoots of both the treated and the control plants were sprinkled with water. After 10 min, leaves recovered their turgor, all the plants were topped, and the mature leaves were frozen in liquid air. A rubber hose was mounted on the stump, and exudate was collected for 6 hr. The amount of exudate did not differ between treatments. The data in Table I demonstrate a reduction in the activity of cytokinin in the leaves and in exudate when the stress was applied to the intact plant. This experiment was repeated three times. The range of the reduction was 39 to 60% in exudate and 22 to 59% in leaves.

Table IIA demonstrates that such a reduction is also observed if the stress is applied to a detached leaf. In this experiment,

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[2] Present address: Volcani Institute for Agricultural Research, Beit Dagan, Israel.

leaves were assayed for cytokinin activity at short intervals, after being detached from the plants. Leaves were placed on the laboratory table with dorsal side up for the time between detachment and freezing. Fresh weight loss was determined prior to freezing. Cytokinin activity decreases as drying proceeds. These experiments were repeated eight times with several modifications. The reduction ranged between 70 and 89% after 10 min and 5 and 52% after drying for 30 min. Table IIB shows that cytokinin activity is partly reversible. In this experiment the leaves were placed in a humid chamber for different intervals after the drying period.

Table III shows the results of ^{14}C-leucine incorporation in discs obtained from leaves which were detached after the stress was applied.

In a further experiment (Table IV) stress was applied by placing leaf discs for 30 min in containers usually used as desiccators. $CaCl_2$ was put into one container and water into the other. Tables III and IV show a reduction in incorporation due to atmospheric stress. This reduction is not reversible by kinetin treatment. The inability of kinetin to bring about recovery of ^{14}C-leucine incorporation (Tables III and IV) does not agree with previous results for leaf discs where stress was applied to the roots through excess salinity or osmotica (5). This disagreement may indicate that, when the leaves are stressed by drying as is done here, cytokinins may be inactivated.

The experiments with labeled kinetin were intended to study the fate of kinetin during stress. Different distribution of the label is observed in stressed and nonstressed leaves (Fig. 1, A, D). Differences in distribution due to stress are eliminated after recovery of the leaves in a humid chamber for 7 hr (Fig. 1, E). The manner of application of the labeled kinetin did not modify the results (Fig. 1, B, C).

Table I. *Cytokinin Activity in Leaves and Exudate of Tobacco Plants*

Stressed plants were exposed to air stream for 30 min.

	Callus Weight	Kinetin Equivalent	Activity
	mg	*μg/g fresh wt*	*%*
Exudate			
Nonstressed	236.2	0.140	100
Stressed	107.5	0.055	39
Leaves			
Nonstressed	115.8	0.044	100
Stressed	87.7	0.026	59

Table II. *Cytokinin Activity in Extracts of 100 g of Detached Leaves as Function of Drying and Recovering Time in Humid Chamber*

Drying Time	Recovery Time	Callus Weight	Kinetin Equivalents	Activity
	hr	*mg*	*μg/kg fresh wt*	*%*
A.				
0	0	0.624	43.4	100
0.15	0	0.558	35.1	81
0.5	0	0.324	22.9	53
B.				
0.5	0	0.254	15.9	36
0.5	18	0.505	31.7	73

Table III. *Incorporation of ^{14}C-L-Leucine into Protein of Leaf Discs Taken from Stressed and Nonstressed Plants and Treated with Kinetin Solution*

Period of incorporation was 1 hr.

	H_2O	Kinetin, 1.0 μg/ml	Percentage of Control
	cpm/100 mg fresh wt		
Nonstressed	13,392	14,868	111
Stressed	12,253	12,667	103

Table IV. *Incorporation of ^{14}C-L-Leucine into Protein of Stressed and Nonstressed Leaf Discs Treated with Kinetin*

The period of incorporation was 1 hr. The concentration of kinetin was 1.0 μg/ml.

Percentage of Initial Weight	Kinetin Treatment	Incorporation			
		Pretreatment		Post-treatment	
		cpm/disc	*% of control*	*cpm/disc*	*% of control*
96	−	2146	100	4755	100
	+	2872	133	6251	131
75	−	1504	100	3044	100
	+	1744	117	4132	135

DISCUSSION

There are several reports in the literature which indicate that a measurable reduction in cytokinin activity in root exudate is observed when plant roots have undergone a period of water shortage (6) or water flooding (3), or the medium has been changed to impose excess salinity (5), excess osmotica (5), high or low root temperature (14), or a low pH (1). In these cases stress is applied to roots, and changes in shoot behavior are brought about through the effect of the stress on the root. In the experiments reported here an attempt is made to test the effect of direct drying of the leaves on cytokinin activity in exudate as well as in leaves. The results show that the level of cytokinin in both the exudate and the leaves is reduced by an air drying stress (Tables I, II). This is of particular interest since the stress is very much shorter (30 min) than those commonly employed in root stress experiments (1 day to several days). It has been suggested that roots are the site of cytokinin synthesis in plants (11, 18) and that cytokinin biosynthesis in the shoot has so far been reported only in fruitlets (8). Furthermore, clear evidence exists that the shoot depends on root cytokinin (7, 11), and also that various shoot organs compete for cytokinins from the root (12).

It is therefore feasible that biosynthesis in the root ceases at the moment the water tension in the leaf is enhanced. The signal to the site of synthesis may in this case be the changes in water potential which are transmitted through the plant. A different case is the reduction of cytokinin levels in detached leaves, where changes in biosynthesis in the root cannot account for the reduction. The short time of stress necessary to effect such a reduction, the inability of external kinetin to restore amino acid incorporation, and the ability to recover cytokinin level all suggest inactivation as a likely process.

Such inactivation may be a result of either inhibitor accumulation during stress or actual transformation of the cytokinin molecule. Inhibitor accumulation may be responsible for lower cytokinin activity due to stress. Abscisic acid is one of those inhibitors (17) which accumulates during water stress (10, 19, 20). This could explain the data on leucine incorporation. However, several points contradict such an explanation for lower activity of cytokinins in stressed leaves. While cytokinin reduction is rapid, levels of abscisic acid increase only after 4 hr (20). Fur-

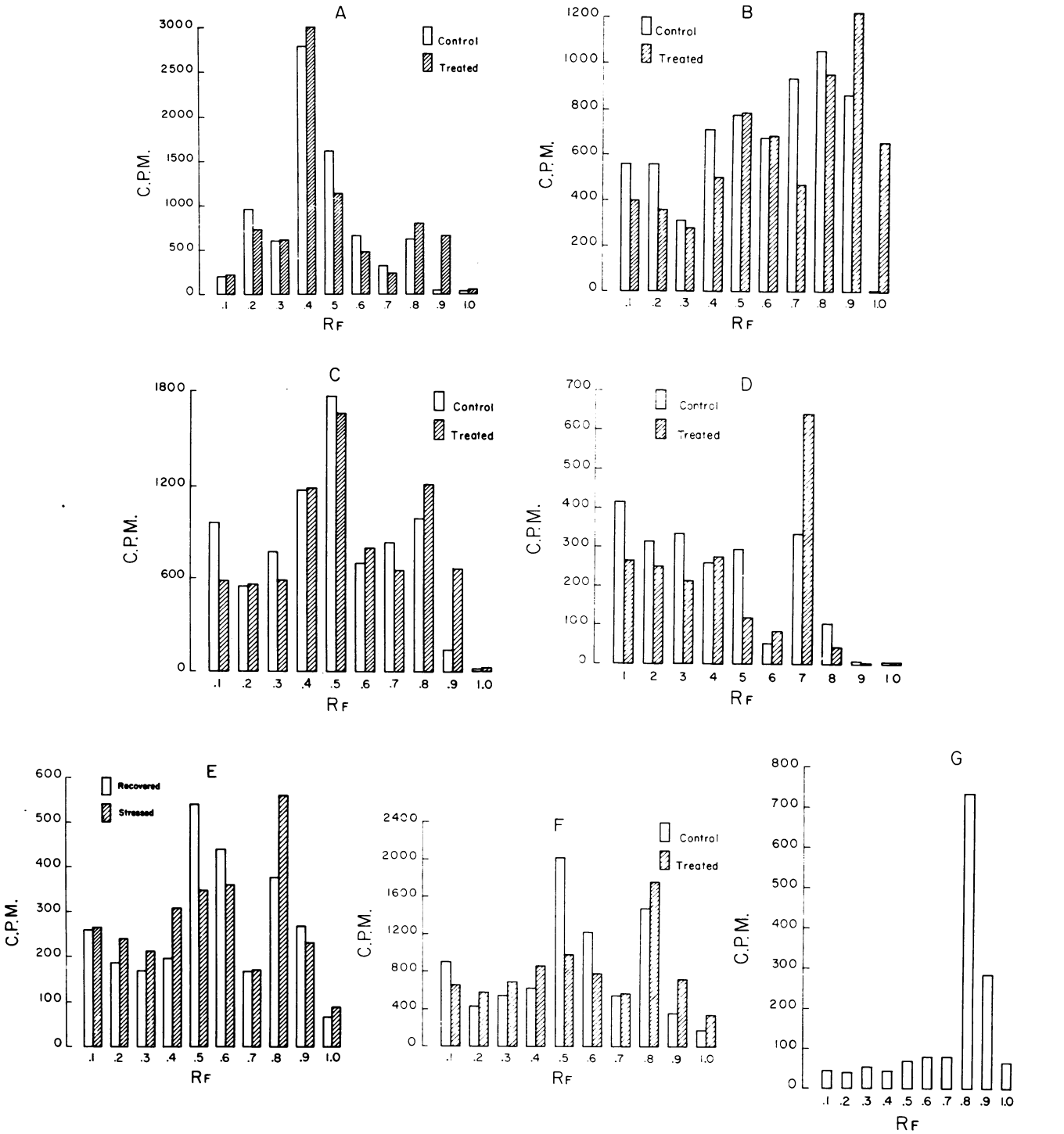

FIG. 1. Distribution of ^{14}C in chromatograms of ethanolic leaf extracts. The leaf was fed 0.5 μc of ^{14}C-kinetin prior to treatment. Solvent used was 1-butanol-H_2O-acetic acid (4:1:1). Treatments given: wilting for 30 min (A); stress after labeling of 12 hr through petiole (B); as in B but label applied by spray (C); as in A, but chromatogram developed in different solvent system (chloroform-ethanol, 9:1) (D); recovery of stressed leaves in humid chamber for 6 hr (E); heat treatment of 49 C for 2 min (F); chromatogram of kinetin solution (G).

thermore, it is unlikely that both abscisic acid and cytokinins will be extracted into a nonpolar fraction (butanol) from a basic aqueous solution.

Kinetin is a synthetic substance which has never been isolated from plant material. The possibility that kinetin is inactivated in the same manner as endogenous cytokinin still has to be proved. Nevertheless, the fate of ^{14}C-kinetin has been followed to see whether stress modifies the distribution of the label in pretreated leaves. In these experiments differences were found in distribution of the label in stressed and nonstressed leaves (Fig. 1). The R_F 0.8 to 1.0, in which accumulation of label in stressed leaves is evident, corresponds with the R_F of kinetin in three different solvents. The substance corresponding to R_F 0.5 to 0.6 which is sometimes lower in stressed plants is as yet unknown and may be a degradation product. The data indicate that inactivation of cytokinins in stressed leaves is possible.

Some evidence of the inactivation of cytokinin as a result of high temperature treatment was reported by Mothes (11). He showed that a short heat treatment applied to leaves causes rapid yellowing, suggesting inactivation of leaf cytokinins. Figure 1G shows that heat treatment to the leaves affects the distribution pattern of ^{14}C-kinetin extracted from the leaves. The changes in distribution in treated leaves resemble those observed for the water-stressed leaves (Fig. 1A).

In plants the interactions between leaves and roots must be closely regulated, and several hormones may be involved. It has been proposed that the root cytokinins play a regulatory role in shoot metabolism (3, 7, 11, 12, 16). If leaves depend on roots for their supply of cytokinins, factors which would affect cytokinin synthesis in the roots would in due course influence cytokinin-dependent processes in leaves. Factors which have been shown to affect cytokinin levels in the xylem exudate stream are root stresses of various kinds (1, 3, 5, 6, 13). This paper shows that, when the stress is applied directly to leaves, cytokinin levels are affected. Although in cases of both root and shoot stress the levels of cytokinins are similarly reduced, the manner in which the reduction occurs may be different. It is possible that under shoot stress, inactivation of cytokinins present may be more common than under root stress.

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