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The effect of drought on levels of abscisic acid, cytokinins, gibberellins and ethylene in aeroponically-grown sunflower plants

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Abstract. Abscisic acid (ABA), cytokinins and gibberellin-like substances (GAs) were extracted from the roots and shoots of 17-day-old sunflower seedlings which had been droughted or were unstressed. Plants were grown in an aeroponic chamber which allowed for good control over degree of water stress and easy access to roots. Following methanolic extraction of lyophilized material, cytokinins were separated from the acidic growth-regulators on a cellulose PO_4 cationic exchange column. The cytokinins were analysed by paper chromatography and HPLC and the soybean hypocotyl section assay. Semi-purified acidic regulators were chromatographed on SiO_2 columns and HPLC and aliquots assayed with the dwarf rice cv. Tan-ginbozu bioassay for GAs. Fractions known to contain ABA were purified by sequential reverse-phase HPLC of the acid and then of the methyl ester forms followed by quantitation as Me-ABA on GLC-EC. ABA losses were measured by using an internal standard [^{3}H]-ABA). Ethylene production was also monitored in stressed and unstressed seedlings.

The effect of drought on GAs and ethylene was minimal. The ABA levels were markedly higher in droughted plants. Stressed roots had 32 times more ABA than controls. The levels of cytokinins in the shoots of droughted plants were about half those in unstressed shoots, and qualitative differences occurred in the roots. Under stress a large peak of activity was present similar to zeatin glucoside which was not present in the unstressed condition. The results are discussed in relation to drought-effects on metabolism.

1. Introduction

Most mesophytic plants are regularly subjected to periods of water shortage of varying intensity and respond to water shortage of increasing severity first through physiological modifications, then ultimately morphological adaptations. Most sensitive to drought stress are cell expansion and stomatal closure followed by processes such as senescence (Hsiao, 1973). In view of the known effects of plant growth regulators, it has been suggested that modulation of the varied responses to drought may lie with changing levels and the balance of all the growth regulators (Itai and Benzioni, 1976; Vaadia, 1976).

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Confirmation of this idea comes from the observations that cytokinins (CK), abscisic acid (ABA) and even ethylene (Et) can modify stomatal functioning and transpiration (Little and Eidt, 1968; Cooper, and Digby, 1972; Pallas and Kays, 1982). Gibberellins (GA), ABA, CK and Et all influence stem and leaf elongation to varying degrees (Zeroni and Hall, 1984) and senescence is markedly affected by CK, GA and ABA (Leopold and Noodin, 1984; Dhindsa et al., 1982). Also consistent with a role for plant growth regulators in the responses of plants to drought, are the observations that water stress can lower the CK levels in xylem exudate and detached leaves (Itai and Vaadia, 1965), but will cause increases in ABA (Wright and Hiron, 1969) and Et (El-Beltagy and Hall, 1974). Less is known of the response of auxins and GA although there is limited evidence indicating that auxins (Tal and Imber, 1970) and GA (Aharoni et al., 1977) levels may decrease during drought.

There are deficiencies in the literature as it concerns drought and plant growth regulators. Much work has ignored roots and concentrated on shoots and there are very few studies looking at the effects of water stress on more than one growth regulator at any one time, in the same tissue. Further, many investigations dealing with hormones and water stress have looked only at the response in detached tissues. Since changes in the level of one growth regulator can affect the level of another (Evans, 1984) and some growth regulators may be made in one part of the plant and then transported elsewhere (King, 1976), the use of detached tissues may lead to problems when one attempts to extrapolate from data with detached tissues to whole plants.

In the light of this, we thought it was appropriate to study more fully the possibility that the balance of inhibitors and stimulatory growth regulators in the whole plant may be involved in the modification of plant growth and development during and after the onset of drought. We thus looked at the effect of mild drought on the levels of GA, CK, ABA and Et in roots and shoots of intact sunflower plants.

For most of our work, we used a period of seven days of drought stress. Using our method of applying water stress, in which we precisely control the amount of water the roots receive in an aeroponic system (Hubick et al., 1982), we are able to reproducibly produce any degree of mild or severe water stress. Many earlier studies investigating the effects of drought tended to subject plants to violent and sudden water stress; a rather unnatural situation. We have attempted to simulate the type of water stress that is more commonly found in nature; that is slow onset of stress. in our system we find that after one week of reduced water supply the sunflower plants reproducibly exhibit typical symptoms of stress (premature senescence of lower leaves, small leaf area, short stems, reduced fresh and dry weight). Another advantage of the aeroponic system is ease of access to completely undamaged roots.

2. Material and methods

2.1. Plant material

Sunflower (*Helianthus annuus* L. cv. Russian) seedlings are grown in trays of 'Terragreen' (baked crushed clay) in a growth chamber: 28°C day/18°C night; 85 Wm^{-2}; 16 h photoperiod; and 85% relative humidity. Seedlings were watered twice daily, once with full-strength Hoagland's solution, and once with deionized water. After seven days the seedlings were transferred to an aeroponic growth system (Hubick et al., 1982) located within a growth chamber. From day seven to day ten the sunflower roots were misted continuously in the aeroponic chamber. Water-stress was induced by interrupting the misting: plants to be stressed were misted for 40 seconds every half hour on day 10 and for 40 seconds every hour on days 11 through 17. Control seedlings (i.e. unstressed) were continuously misted throughout this period. On day 17 seedlings were divided into roots and shoot, frozen in liquid N_2 and lyophilized.

2.2. Leaf water potential

This was estimated, using 6 mm leaf discs taken from the most fully expanded leaves, with a Wescor dew point microvoltmeter in conjunction with C52 Wescor sample chamber. A 20 minute equilibration time was used.

2.3 Analysis of endogenous growth regulators

Lyophilized roots or shoots from ten seedlings were weighed, and extracted with cold 80% MeOH for each treatment or experiment. The subsequent procedure for the purification and assay of CK-like and GA-like substances as well as ABA is outlined in Figure 1. This procedure allows an acidic fraction containing GAs and ABA to be separated from the CK-containing fraction following passage of the extract through an ion exchange column (Taylor and Simpson, 1980). Following assay of the acidic fraction for GA, ABA in the residue, after HPLC purification as the methyl ester form (Taylor et al., 1981), is analyzed on GC-EC. Procedural losses of ABA are corrected for on the basis of recovery of an internal standard (i.e. ^{3}H-ABA). Recoveries of ABA varied from 72 to 81%. Purification of ABA-Me was carried out on a Spectra Physics 8700 HPLC: Whatman Magnum 9 ODS-2 column; isocratic 80% MeOH (pH 3.4); 2 ml min^{-1} GC-EC analysis was on a Varian 3700: 2% OV-17 on a Gas Chrome Q (6′ x 1/8″) packed column; 205°C; gas-flow rate (90% argon: 10% methane) of 60 ml min^{-1}.

Cytokinins were assayed using the Soybean Hypocotyl Section Assay (Manos and Goldthwaite, 1976) and GAs were assayed with a modified Dwarf Rice μ-Drop Assay (Kaufman et al., 1976). The HPLC programs used in the analysis of CK (Taylor et al., 1984) and GA (Koshioka et al., 1983) are given in the figure legends.

In the ethylene experiments the plants were subjected to stress by reducing

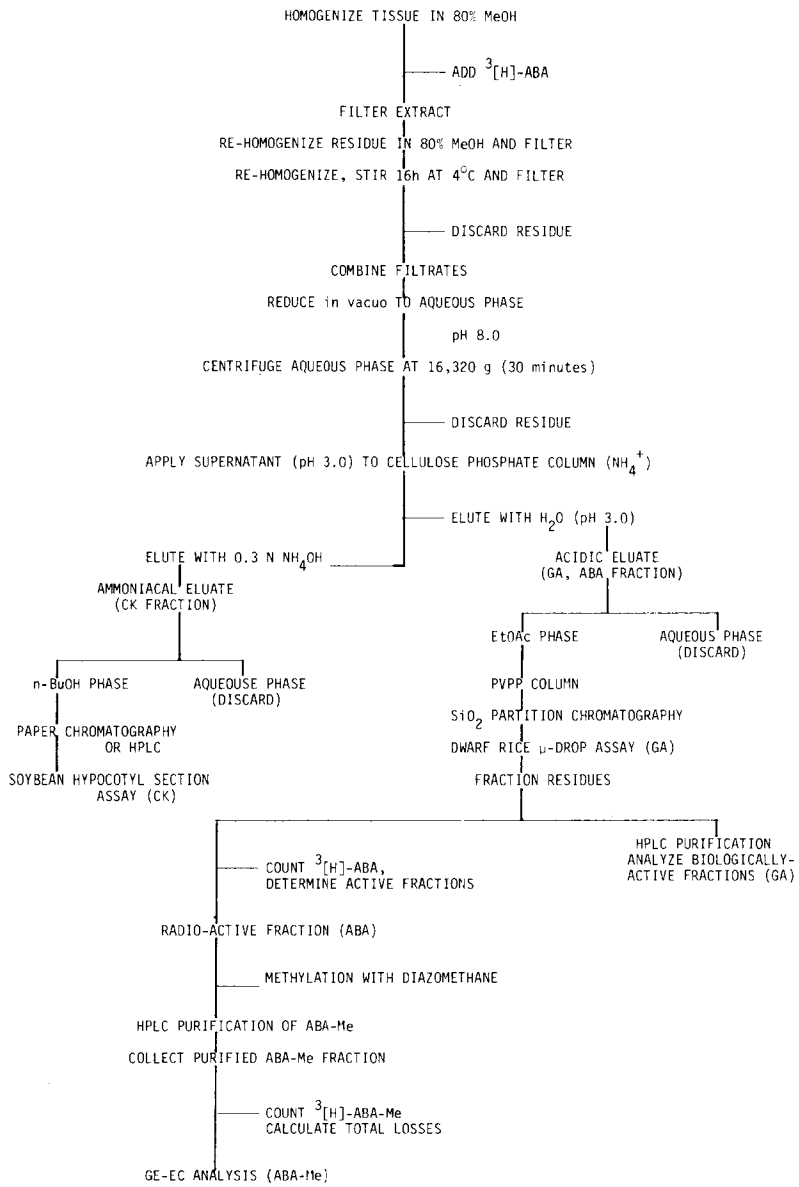

Figure 1. Procedure for extraction, purification and assay of growth regulators from sunflower *Helianthus annuus*.

watering in the aeroponics system. After varying periods of time, two or three uniform plants were selected from each of the aeroponics chambers. The plants were individually placed in beakers of known volume (adjusted to 780 ml with glass beads) and the tops of the beakers were sealed with glass plates and silicone vacuum grease. After $1\frac{1}{2}$ to $2\frac{1}{2}$ hours incubation in an aeroponic chamber in the light, a 50 ml aliquot of the air inside the beakers was removed through a rubber septum capped side arm with a glass, air-tight syringe. The sample was injected into a Poropak S pre-column and the ethylene trapped by cooling the column with a mixture of liquid N_2 and methanol for one minute. The trapped gas was swept onto a $6'' \times \frac{1}{8}''$ o.d. stainless steel column packed with Poropak R by heating the column for 3 minutes in boiling water. The injection took 20 seconds. The Varian 3700 gas chromatograph equipped with a flame ionization detector was maintained under the following conditions: injector temperature 30°; column temperature 40°; detector temperature 120°. Ethylene was quantified with a Hewlett Packard 3390A integrator which had been previously calibrated with ethylene standards of known concentrations. After the ethylene determinations the plants were frozen in liquid N_2 and lyophilized for dry weight determinations except in expt. 1 where plants were dried at 65° for 72 hours. Ethylene was determined in the ambient air in the last two experiments and was 6.76 nl/l and 10.43 nl/l respectively.

3. Results

Decreasing the rate of misting of aeroponically grown sunflower plants resulted in typical drought stress symptoms. At harvest, leaf water potential of unstressed plants was -0.37 MPa versus -1.23 MPa for stressed plants. A decrease of leaf area compared with continuously misted control plants was observed even after two days. By four days, the cotyledons of stressed plants began to senesce and the primary leaves showed brown lesions. By seven days of treatment, the symptoms were well advanced with only the youngest leaves being dark green. Table 1 shows that, as expected, the shoot/root dry weight ratio decreased from 3.02 in unstressed to 2.27 in droughted plants. Root growth was, therefore, inhibited less than shoot growth by water stress.

ABA levels increased significantly in both roots and shoots (Table 1), stressed roots having 32 times more ABA than unstressed roots. The increase of ABA was 6.7 times in droughted versus control shoots and the level of ABA was 46% greater in stressed roots than stressed shoots.

Changes in total CK activity were significant, but not as dramatic as ABA changes (Table 1). The effect of drought stress on shoots was to lower CK levels by 53%. Because of the stress-induced increase of CK in the roots counteracting the decrease in the shoots, the total level of CK per gram dry weight was unchanged due to stress, but qualitatively, the CKs were different. It can be seen in Figure 2 that the increase of CK activity in stressed roots

Table 1. Levels of CK and GA activity and of ABA in stressed and non-stressed sunflower tissue and mean tissue dry weight. All three hormones were analyzed in three separate experiments-GA and CK were analysed at three extract dilutions. LSDs were calculated following analysis of variance. Means (on the same line) not followed by the same letter are significantly different ($p = 0.05$). Means ± SE are given for tissue weight

Hormone analysis ng/g dry weight	Tissue extracted			
	Unstressed shoots	Stressed shoots	Unstressed roots	Stressed roots
ABA	132.61^{b}	889.08^{c}	40.66^{a}	1301.56^{c}
Cytokinin activity (zeatin equiv.)	19.02^{bc}	8.97^{a}	12.50^{ab}	23.90^{c}
GA activity (GA_3 equiv.)	60.23^{a}	79.11^{ab}	166.72^{c}	119.39^{bc}
Mean tissue weight (ten plants) g dry weight	9.66 ± 1.55	4.00 ± 0.27	3.20 ± 0.47	1.76 ± 0.27

was likely due to the appearance of compounds that chromatographed in the same region as zeatin glucoside. In stressed roots this was a major peak of CK activity not present in unstressed roots. The major peaks of CK activity on paper chromatograms of shoots, both stressed and unstressed, and unstressed roots correspond to the positions of zeatin/zeatin riboside. Stressed and unstressed root tissue was analysed further by HPLC (Figure 3). The major activity corresponded to zeatin riboside, though stressed tissue displayed an earlier peak (fractions 18–20) not present in the unstressed material. This may have corresponded to the glucoside-like activity observed at Rf 0.1–0.4 on the paper chromatograms.

Total free acid GA showed neither a change in level, nor a change in distribution with stress (Table 1, Figures 4 and 5). Overall levels of GA's were higher in roots as compared with shoots. The spectrum of GA's is shown in Figures 4 and 5. On silicic acid partition chromatography (Figure 4), the GA-like activity was located mostly in fraction 2/3, 5/6/7, 9/10, and 11/12. Authentic GA_9, $GA_{4/7}$, $GA_{1/3/19}$, and GA_8, respectively, would be expected to elute in these silicic acid fractions. Fractions 9/10 and 11/12, the major peaks were further analysed by HPLC using a program which separates $GA_{1/3}$ from GA_{19} and GA_8. Peaks corresponding to the positions of $GA_{1/3}$ and GA_{19} were obtained (Figure 5).

Three experiments were carried out with ten day old intact plants in which the ethylene production in unstressed was compared with that of stressed plants (Table 2). Apart from a significant stress-induced lowering of ethylene production after 90 hours of stress there was little influence of the drought treatment. We have repeated this work with leaves from older plants, stressed for various times between one and 48 hours, and again did not observe any large or consistent differences in ethylene levels between stressed and unstressed plants (data not presented).

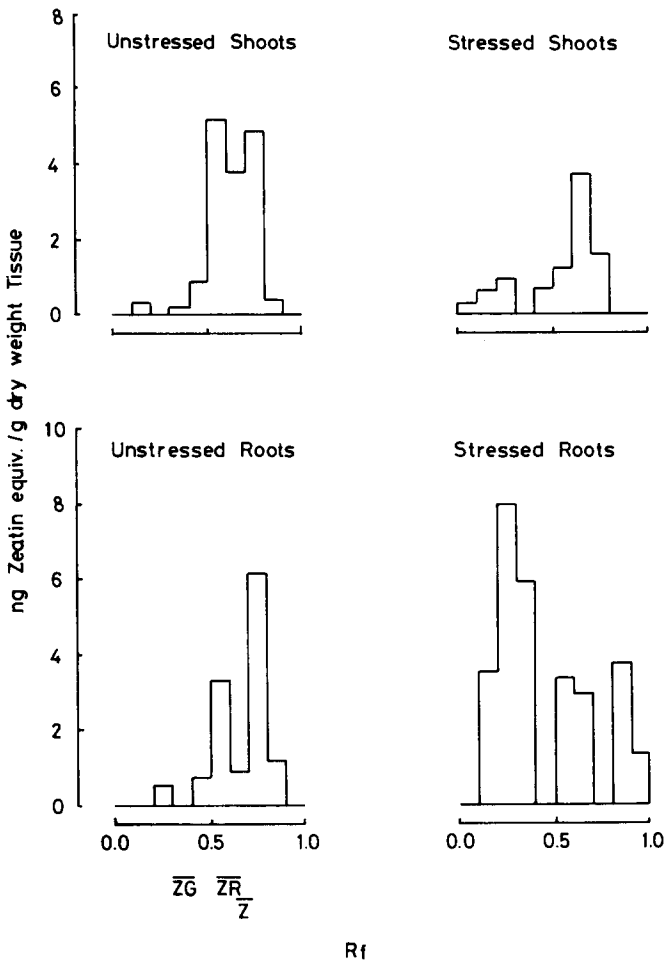

Figure 2. Cytokinin levels in purified extracts of sunflower root and shoot tissue measured by the soybean hypocotyl section assay of three extract concentrations following paper chromatography in i-C_3H_7OH:NH_4OH (30%): H_2O (10:1:1). Tissue from ten seedlings was used for each extract. Z-zeatin; ZR-zeatin riboside; ZG-zeatin-9-glucoside.

4. Discussion

The aeroponic method of plant culture produces material that is relatively unstressed compared with pot culture techniques (Hubick et al., 1982). This system can also be used to subject plants to a very uniform and gradual stress. The technique proved to be effective for providing sunflower plants with leaf water potentials comparable to values previously reported for stressed and unstressed plants (Sionit, 1977). The expected decrease of shoot-root ratio due to insufficient water (Meyer and Boyer, 1981) was exhibited by the aeroponically-grown plants.

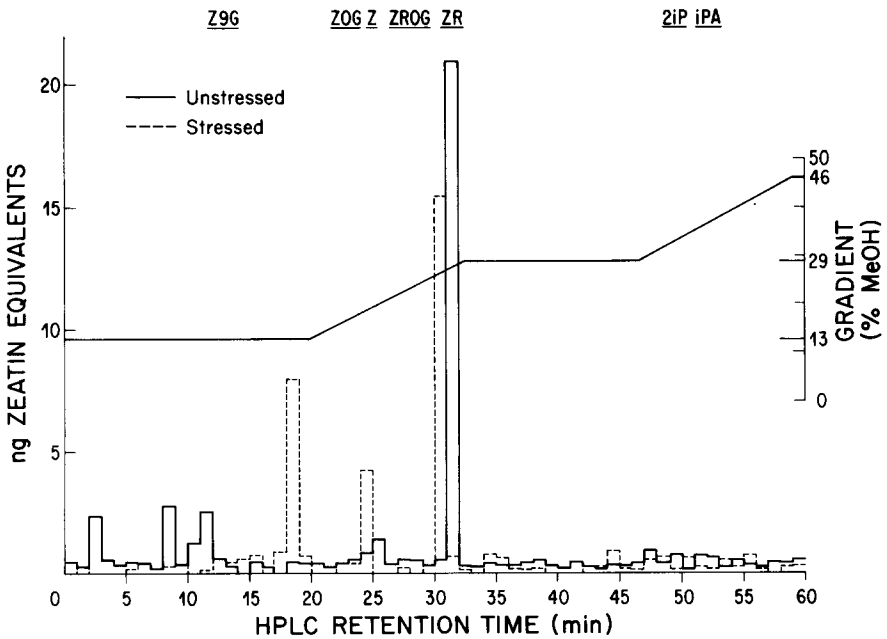

Figure 3. Cytokinin activity in sunflower root tissue after purified extracts were run on a Waters Bondapak C18 column, three ml min^{-1}, three ml fractions. Solvent program (methanol: H_2O) and the position of known CKs are indicated. Z-zeatin; ZR-zeatin riboside; Z9G-zeatin-9-glucoside; ZOG, riboside-O-glucoside; ZORG-zeatin riboside-O-glucoside; 2iP-N^6-isopentenyladenine; iPA-N^6-isopentenyl adenosine.

The increased level of ABA in shoots of droughted sunflower plants was higher than increases previously reported for sunflowers (Walton et al., 1976). The increase of 32 times in droughted roots was ten times greater than Walton et al., (1976) found. Changes in hormones in roots have usually been overlooked. In roots, ABA seems to be involved in regulation of permeability to ions and water (Cram and Pitman, 1972; Glinka, 1980) and possibly other events (Evans, 1984). In addition to the role of ABA in stomatal control, exogenous ABA modified the shoot growth of unstressed wheat plants so they resembled droughted plants in several ways (Quarrie and Jones, 1977). Along with these changes, the increase of ABA in the roots might be involved in morphological changes by selective inhibition of different root meristems during drought stress. Changes in root morphology changes during drought have indeed been observed (Hurd, 1974).

The level of CK in sunflower shoots decreased by 53% during seven days of stress. This was similar to the decrease observed in detached wilted tobacco after one hour of stress (Itai and Vaadia, 1971). CKs may be produced in the roots (Itai and Voadia, 1965) and stress reduces the concentration of cytokinins in xylem exudate (Itai and Vaadia, 1965). It has been speculated that

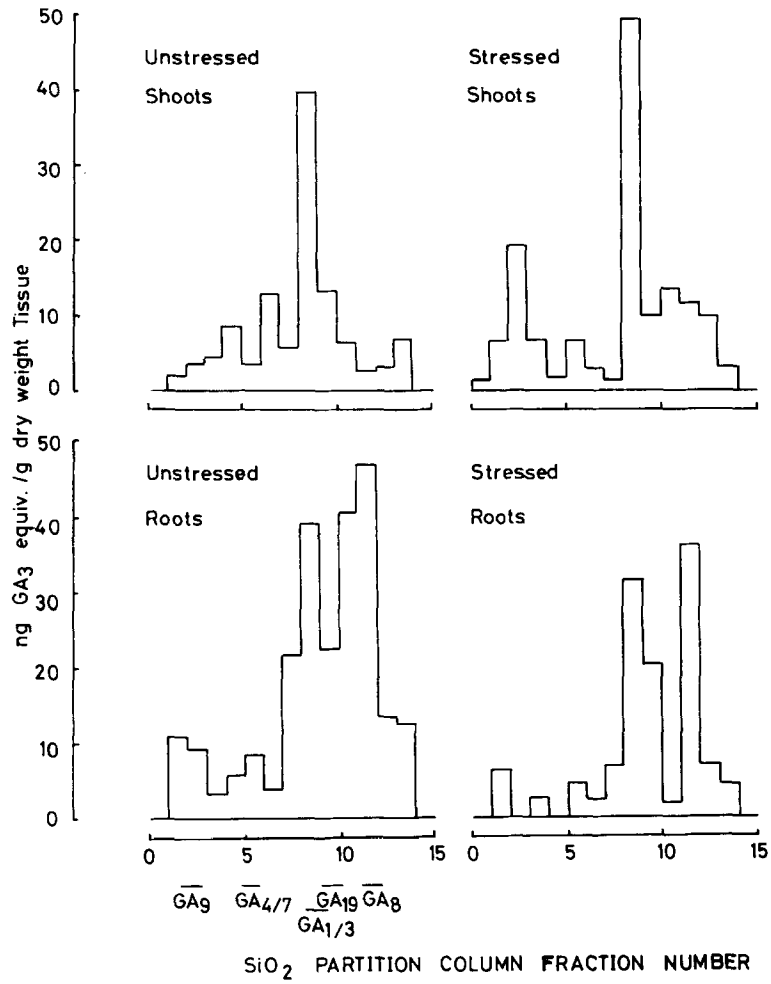

Figure 4. Levels of GA (as determined by bioassay in serial dilution on dwarf rice cv. Tan-ginbozu) in purified extracts of root and shoot tissue from sunflower chromatographed on a gradient eluted SiO_2 partition column (tissue from ten seedlings/extract).

stress either inhibits CK production in the roots or inhibits their transport out of the roots (Van Staden and Davey, 1979). Our results support an inhibition of transport out of roots without a decreased synthesis. However, there is little information about the effect of drought stress on CK activity within the roots themselves. Most reports have been concerned with the effect of salt stress on CK transport from roots. However, salt stress is not identical to drought stress.

Detached roots appear to be capable of producing glucoside-like compounds of CK which may act as storage forms to reduce the excess of

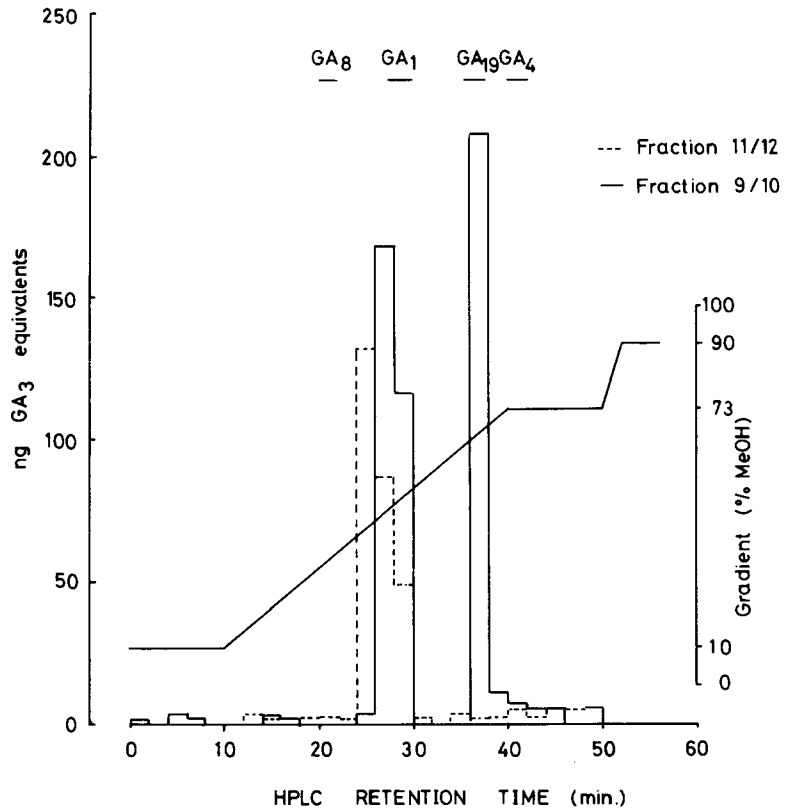

Figure 5. GA activity from fractions 9/10 and 11/12 (Figure 4) run on a Whatman Partisil-10 ODS-2 analytical column, two ml min^{-1}, two min fractions. Solvent program (methanol: H_2O (pH 3.4)) and the position of known GAs are indicated.

cytokins that would normally be transported to shoots (Van Staden and Smith, 1978). Substances with the properties of cytokinin glucosides have been found in the roots of several species (Yoshida and Oritani, 1972). One of the effects of stress on sunflower, then, may be the reduction of cytokinin transport to the shoot due to the production of storage forms which remain in the roots.

There may be antagonistic interactions between ABA and CK. ABA promotes stomatal closure and senescence; CK tends to increase transpiration and delay senescence (Thimann and Satler, 1979; Cooper and Digby, 1972). However, the sites of action may not be the same. In transpiration Pallas and Box (1970) explained the action of kinetin as affecting the turgor potential of epidermal cells surrounding the guard cells, whereas ABA directly affects guard cell turgor (Raschke, 1979).

ABA can affect the metabolism of ^{14}C-kinetin. Application of ABA to

Table 2. Effect of water stress on ethylene production of intact sunflower plants. Plants were stressed by reducing the watering regime from continuous root misting in the aeroponic chamber, to 40 seconds of misting every half hour for the first day, to 40 seconds per hour for the remainder of the experiment

Duration of stress (h)	Expt. no.	Unstressed ($nl\,g\,DW^{-1}\,h^{-1}$)	Stressed C_2H_4 ($nl\,g\,DW^{-1}\,h^{-1}$)
2	3	101.9	80.2
4	3	97.7	153.4
24	3	47.6	38.3
24	2	32.9	10.8
48	2	29.6	20.4
96	1	113.1	58.5[a]
120	1	37.2	39.0

[a]Denotes significantly different from unstressed ($p = 0.05$).

detached *Rumex* leaves caused a 30% decrease of conversion of kinetin into adenine (Back et al., 1972). More radioactivity remained at the R_f of ^{14}C-kinetin after wilting of tobacco leaves (Itai and Vaadia, 1971). Perhaps the appearance of 'bound' cytokinin in sunflower roots may be a function of the ABA/CK interaction controlling the level of CK at the production site at a time when the action of the CK might result in harmful consequences to the plant.

Little is known about the interactions of drought and GA's. An early report (Livne and Vaadia, 1965) found that transpiration of barley was promoted by GA and CK. However, later it was found that GA had no effect on transpiration in oat leaves (Luke and Freeman, 1968); nor did GA have any effect on metabolism of ^{14}C-kinetin (Back et al., 1972) or ABA-induced stomatal closure (Horton, 1971).

It might be expected that during a period of slowed growth, the levels of growth promoters, such as GA, would decrease. Although this occurred in wilted detached lettuce leaves (Aharoni et al., 1977), we did not find this to be the case in droughted intact sunflower plants. However, observations on the effects of CK on transpiration (Bengtson et al., 1977) and senescence (Thimann and Satler, 1979) suggest that intact plants may react rather diferently from their isolated parts. Thus, it may also be true that changes of endogenous GA's in intact stressed plants and detached stressed plants parts could be different. In any case the effects of drough stress on GA metabolism may be very complex (Greenwood 1981).

A number of studies following the earlier report of El-Beltagy and Hall (1974) (see Wright, 1980) found more ethylene in water and osmotically stressed than unstressed tissue. Wright's 1980 work showed that in droughted excised wheat leaves, there are complex interactions of ethylene production with other hormones. Again, many of these studies were with excised organs. Our work with intact sunflower plants showed no evidence for a water stress-induced rise in ethylene production.

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