Plant Physiol. (1979) 63, 269–273
0032-0889/79/63/0269/05/$00.50/0

Analysis of Variability in the *Amaranthus* Bioassay for Cytokinins

EFFECTS OF WATER STRESS ON BENZYLADENINE- AND FUSICOCCIN-DEPENDENT RESPONSES[1]

Received for publication March 7, 1978 and in revised form July 13, 1978

DAPHNE C. ELLIOTT
School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia 5042

ABSTRACT

The use of only the upper part of the hypocotyl and the cotyledons in the *Amaranthus tricolor* bioassay for cytokinins, instead of the whole seedling, was found to reduce the endogenous response and give a higher benzyladenine-dependent response. There is no marked difference in the uptake or metabolism of benzyladenine in whole seedlings compared with that in excised cotyledons.

Analysis of variability in the bioassay showed that water availability to the cut seedlings and to whole seedlings is a major factor in the amounts of betacyanin accumulated during the subsequent induction period. The increase in the amount of betacyanin accumulated in response to benzyladenine, following conditions of water stress, is not correlated with differences in benzyladenine uptake. Endogenous production and fusicoccin stimulation is also increased following water loss by cut seedlings. Possible explanations for this stress induction may be found in responses of active transport to changes in turgor pressure.

Although *pretreatment* of the roots of seedlings with mannitol stimulated *subsequent* induction by excised cotyledons, the presence of mannitol *during* the induction period inhibited the accumulation of betacyanin. This inhibition is not due to any effect on benzyladenine uptake. The susceptibility of amino acid uptake and polysome profiles to water stress suggests that the inhibition of betacyanin synthesis, a process dependent on protein synthesis, may be due to inhibition either of precursor (tyrosine) uptake or of the synthesis or activity of some enzyme in the pathway.

The accumulation of betacyanin in the cotyledons of *Amaranthus* requires either phytochrome (2, 23) or cytokinins (2, 6, 15, 16, 21). Fusicoccin has been found to increase betacyanin synthesis and there is marked synergism between benzyladenine and fusicoccin (10). The use of excised hypocotyl plus cotyledon, instead of the whole seedling, in the *Amaranthus* betacyanin bioassay for cytokinins was suggested by Biddington and Thomas (5). In this paper the use of half-seedlings has been investigated further; the advantage of using this material to give a lower endogenous background and thus a higher percentage response to exogenous cytokinin is shown.

We noticed that there was much greater variability in the results using half-seedlings and that the color development was frequently greater in replicates the longer the time taken in transferring to the induction medium. Experiments to control possible variables led to a number of conclusions about events which influence color development. The effects of temperature and of aging of excised cotyledons in distilled H_2O will be the subject of other papers (11, 13). In this paper experiments showing the importance of the water content of seedlings and of aerobic conditions during pretreatments prior to induction are reported.

MATERIALS AND METHODS

Betacyanin Assay. Seeds of *Amaranthus tricolor* L. var. tricolor (Yates and Co., Sydney, Australia) were germinated in the dark on wet plastic foam in Petri dishes either at 37 C for 46 hr, or at 25 C for 96 hr. Since differences in relative water content of the seedlings at the start of the experiment proved to be important in the magnitude of the response to various treatments, a standardized germination procedure of 1 g seeds/75 ml H_2O per 15-cm Petri dish was adopted. The plastic foam was 0.6 cm deep. Light sources were as previously described (15). After germination, batches of 40 seedlings were transferred to filter paper discs in 9-cm Petri dishes containing 5 ml of incubation medium. When half-seedlings were used (cotyledons plus the top 5-mm hypocotyl), they were cut off with scissors directly onto filter paper (see Fig. 2) or into mannitol solutions (see Fig. 3 and Table IV), or distilled H_2O (see Table II), or pinched off individually using two pairs of fine forceps and transferred to incubation medium directly (see Tables I, III). No attempt was made to remove seed coats, except for some experiments involving radioactivity when seedlings were solubilized in NCS[2]; the seed coats resisted this treatment. The incubation medium was 10 mM Na_2HPO_4-KH_2PO_4 (pH 6.8) containing 5 mM tyrosine, *i.e.* the optimum Na^+/K^+ ratio (10 meq/1, Na^+; 5 meq/1, K^+) and the optimum pH (12). Experiments were conducted in the presence and absence of half-maximal benzyladenine (0.5 μM) (7, 16), usually for an induction period of 24 hr at 25 C in the dark. Homogenization was carried out in 3.33 mM acetic acid (3 ml/40 seedlings) after freezing and thawing. Half-seedlings were homogenized in a Polytron homogenizer (15 sec at half-speed, No. 5) but whole seedlings were best homogenized in a glass-Teflon homogenizer. Cell debris was removed by centrifugation at 38,000*g* for 20 min. Amaranthin was determined by difference spectra ($A_{537\ nm}$–$A_{620\ nm}$) in a Hitachi 176 double beam spectrophotometer, using a molecular extinction coefficient (ϵ) of 5.66×10^4 (23). All treatments were assayed in triplicate. Standard deviation did not exceed ±15%.

Relative Water Content. Water status of seedlings or excised cotyledons was measured as RWC, according to the formula

$$\frac{\text{(Fresh weight-dry weight)}}{\text{(Fully turgid weight-dry weight)}} \times 100$$

(4). Fully turgid weight was measured after floating on distilled H_2O for 4 hr.

Uptake and Metabolism of Labeled 6-Benzyladenine. The up-

[1] This research was supported by grants from the Australian Research Grants Committee (D2 71/15177) and the Flinders University Research Budget.

[2] Abbreviations: NCS: Nuclear-Chicago tissue solubilizer; RWC: relative water content; 9-glucosylbenzyladenine: 6-benzylamino-9-β-D-glucopyranosyl purine; 7-glucosylbenzyladenine: 6-benzylamino-7-β-D-glucopyranosyl purine.

take of benzyladenine by half-seedlings was determined on samples of four half-seedlings (seed coats removed at the beginning of the incubation), after incubation for varying times in the induction medium containing 6-[G-^{3}H]benzyladenine (specific radioactivity 22 mCi/mmol). The benzyladenine was purified as previously described (14). Seedlings were washed briefly in ice-cold water, then for 10 min in ice-cold unlabeled 0.05 mM benzyladenine. Seedlings were blotted gently and transferred to a plastic scintillation vial; 0.1 ml of water and 0.25 ml of NCS were added and the seedlings were incubated overnight at 55 C, tightly capped. After cooling the vials for 30 min at −15 C, 20 μl of glacial acetic acid was added and then 10 ml of xylene-Triton X-114 scintillation fluid (1). Radioactivity of the samples was determined with a Packard Tri-Carb liquid scintillation spectrometer.

Metabolism of benzyladenine was investigated as previously described (15). Chromatography was carried out either on 3MM Whatman paper in butan-1-ol-concentrated NH_3 (specific gravity 0.88)-water (85:5:9, v/v/v) or on Merck TLC plates, Silica Gel 60F 254 precoated (0.25-mm thickness), in butan-1-ol-concentrated NH_3 (specific gravity 0.88)-water (6:1:2, v/v/v, upper phase) or butan-1-ol-acetic acid-water (12:3:5, v/v/v). Benzyladenine, benzyladenosine, 9-glucosylbenzyladenine, and 7-glucosylbenzyladenine were identified by co-chromatography with authentic samples. R_F values on 3MM paper were as follows: 7-glucosylbenzyladenine[3], 0.45; 9-glucosylbenzyladenine, 0.6; benzyladenosine, 0.72; benzyladenine, 0.82. The appropriate areas of the chromatograms were counted directly in toluene scintillator (5 g of 2,5-diphenyl-oxazole and 0.5 g 1,4-bis-[5-phenyloxazol-2-yl]-benzene/toluene) with an efficiency of 8.5%.

RESULTS

Table I gives a comparison of whole seedlings with excised cotyledons of *Amaranthus* in the time course of betacyanin accumulation. The effect of removing the roots has clearly lowered the endogenous production of pigment and inasmuch as the color attained in the presence of benzyladenine is not significantly different, this has increased the benzyladenine-dependent synthesis. The possibility that this increase was due to some difference in uptake or metabolism of benzyladenine was investigated (Table I). With 5 μM benzyladenine, removal of roots has no effect on the correlation between total uptake of labeled benzyladenine with the benzyladenine-dependent betacyanin synthesis, nor any effect on benzyladenine metabolism as judged by percentage conversion to glucosyl derivatives. With 0.5 μM benzyladenine the values for uptake and metabolism in cotyledon explants are not significantly different (*t*-test, $P > 0.1$) from whole seedlings (Fig. 1).

Using the half-seedling assay it was noticed that the pigment accumulation was often greater with the second and third replicate, especially when many assays were being run—and the time taken consequently longer. Seedlings germinated in the dark in closed Petri dishes might be expected to develop an atmosphere high in CO_2. Because of the inverse relationship between high CO_2 and K^+ transport in guard cells (25), it was conceivable that the K^+-dependent betacyanin assay was lower in seedlings taken directly from a CO_2-rich atmosphere and placed in induction medium. However, CO_2 stimulates betacyanin synthesis (Table II).

Table I. Effect of removal of roots on betacyanin accumulation after 6 hr and 24 hr, and on benzyladenine uptake and metabolism after 24 hr

Material[1]	Benzyladenine Concentration	Betacyanin (nmol/seedling)		Total Radioactivity taken up (10^{-4} x dpm/40 seedlings)	Glucosyl-Metabolites (%)
		6 hr	24 hr		
Whole seedling	None	0.021	0.081		
	0.5 μM	0.028	0.307	1.12	57
	5.0 μM	-	0.524	7.91	48
Cotyledon plus hypocotyl	None	0.012	0.027		
	0.5 μM	0.024	0.290	0.86	53
	5.0 μM	-	0.543	10.07	49

[1]Germination, 37 C for 46 hr

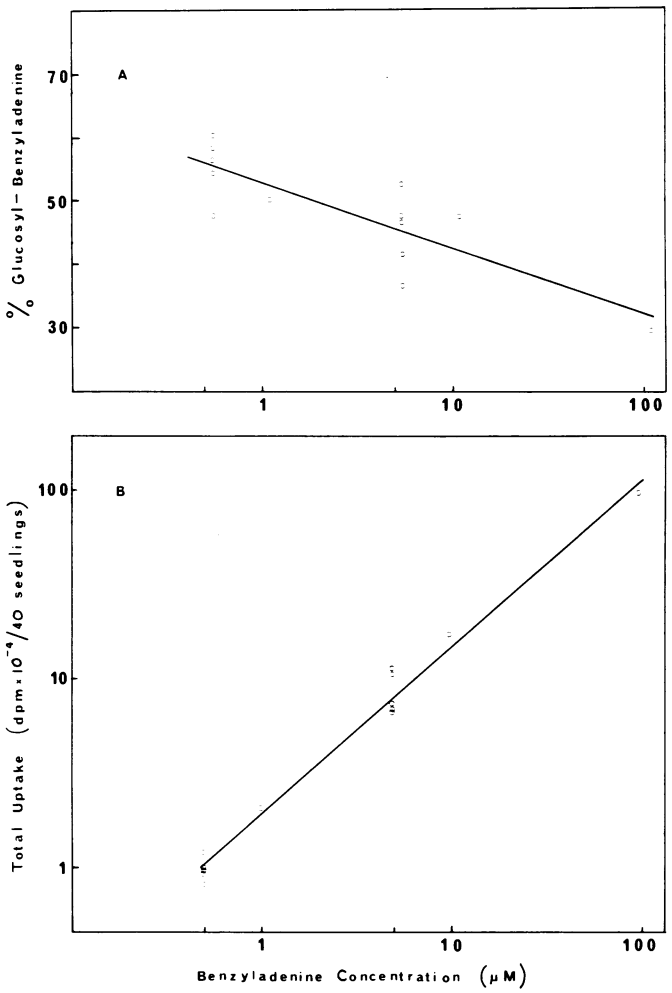

FIG. 1. Relationship between external benzyladenine concentration, total uptake (B), and percentage glucosyl derivatives (A) formed in 24 hr. Whole seedlings were incubated with 6-[G-^{3}H]benzyladenine. Germination: 37 C for 46 hr. Linear regression analysis was carried out on the mean values (assay done in triplicate) of 13 separate experiments. For glucosyl derivative formation (A), a regression coefficient of −10.39 and correlation of −0.83 was calculated. For total uptake of radioactivity (B), a regression coefficient of 0.886 and correlation of 0.99 was calculated.

[3] The confusion noted (15) concerning the R_F value of 7-glucosylbenzyladenine appears to arise over the identification of this compound as the major benzyladenine metabolite in soybean cell cultures. Deleuze *et al.* (9) isolated and identified 7-glucosylbenzyladenine from metabolites of benzyladenine in potato tissue and subsequently assumed that the metabolite from soybean cells was the same on the basis of identical R_F values in some solvents (*e.g.* butan-1-ol-acetic acid-water, 4:1:2, v/v/v) (17). The major metabolite of benzyladenine from soybean cells runs considerably slower than 7-glucosylbenzyladenine in butanol-ammonia (R_F 0.25 on 3MM paper in the system used in this report). The major metabolite of benzyladenine from soybean cells is particularly well separated from 7-glucosylbenzyladenine on silica gel plates, running slower in both the solvents used in this paper. It appears to be a ninhydrin-reacting compound (R. Horgan, personal communication, confirmed by D. C. Elliott, unpublished observations).

A further consequence of removing the cover from the germination Petri dish was a drying effect. Experiments designed to test the effect of water content of seedlings on betacyanin production are shown in Figure 2. There is a clear correlation of time of exposure of cotyledons to ambient humidity with subsequent betacyanin synthesis for endogenous production, fusicoccin-stimulated synthesis, and benzyladenine-dependent synthesis. The germination temperature for this experiment was 37 C and there was the possibility that shock, caused by taking seedlings at 37 C and transferring them to induction media at 25 C, may be contributing to the low early values, with progressive acclimatization to 25 C with time. However, similar experiments using seedlings germinated at 25 C showed the same relationship between exposure to ambient humidity and increased subsequent betacyanin synthesis (*e.g.* Table III, treatment B). This effect could be further increased by the use of PEG 4000 to produce water stress (Table III, treatment D), and a clear correlation between RWC and subsequent betacyanin synthesis was observed (Table III). It was also found that aerobic conditions were necessary for the development of this water stress-induced potential, since wilting in an atmosphere of N_2 had no such effect (Table III, treatment C). Wilted seedlings (Table III, treatments C and D) were subsequently less efficient in the long term total uptake of labeled benzyladenine. This clearly had no correlation with betacyanin accumulation (Table III), and no explanation can at present be offered for it.

Although mannitol pretreatment of the roots of whole seedlings causes a stimulation of subsequent betacyanin induction (data not shown), the presence of mannitol during the induction is inhibitory (Table IV, treatment B). Sucrose is not as inhibitory as mannitol at 150 mM and there is even some stimulation at 25 mM (data not shown). This stimulation is not however as marked as that of sucrose on anthocyanin synthesis (28). There is also some inhibition of betacyanin synthesis when cut seedlings are *pretreated* with mannitol and then transferred to induction medium without mannitol (Table IV, treatment A). In this respect cut seedlings differ from whole seedlings. Their greater sensitivity may be due to some side effects of mannitol following uptake by the cut seedlings (20). The inhibitory effects of mannitol do not appear to be due to inhibition of initial benzyladenine uptake (Fig. 3).

Table II. Effect of bicarbonate on betacyanin induction

Benzyladenine Concentration	HCO_3^- [1]	Betacyanin (nmol/seedling)
None	-	0.051
0.5 μM	-	0.162
None	+	0.068
0.5 μM	+	0.242

[1] In controls the medium was made 4 mM with respect to KCl; in CO_2 treated experiments, 4 mM $KHCO_3$ was added and CO_2 bubbled through to bring the pH back to 6.8. Germination, 37 C for 46 hr. Half-seedlings used cut into distilled water before transferring to induction medium.

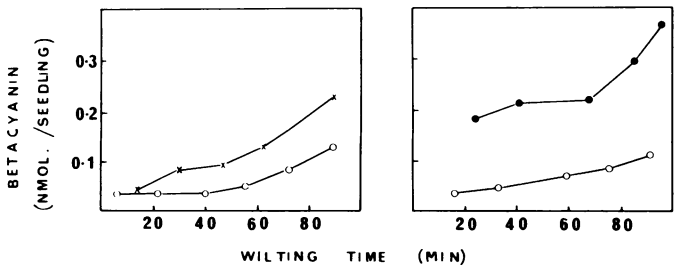

FIG. 2. Effect of wilting of cut seedlings on subsequent betacyanin accumulation. Seedlings were cut directly onto filter paper and exposed to the ambient humidity until transferred at varying times into the induction medium. Curves show time course of endogenous response (○) and response in presence of 0.5 μM benzyladenine (●) or 0.5 μM fusicoccin (×). Germination: 37 C for 46 hr.

Table III. Effect of wilting of whole seedlings in polyethylene glycol and in a stream of nitrogen for 1.5 hr on relative water content, subsequent betacyanin induction, and benzyladenine uptake

Benzyladenine Concentration	Seedling Treatment [1]	RWC (%)	Betacyanin (nmol/seedling)	Benzyladenine uptake (cpm/4 seedlings) 6 hr	24 hr
None	A	82	0.021		
0.5 μM			0.121	244	674
None	B	80	0.033		
0.5 μM			0.183	250	642
None	C	72	0.025		
0.5 μM			0.131	241	477
None	D	68	0.028		
0.5 μM			0.236	213	442

[1] A treatment : seedlings covered in Petri dish for 1.5 hr.
B treatment : seedlings left uncovered in Petri dish for 1.5 hr at ambient humidity with germination water drained off and replaced by fresh distilled water.
C treatment : germination pad drained of germination water and seedlings left uncovered on the drained pad for 1.5 hr in stream of nitrogen.
D treatment : as for B with germination water replaced by 30% (w/v) polyethylene glycol 4000.
Germination, 25 C for 96 hr.

Table IV. Effect of pre-incubation of cut seedlings in mannitol on subsequent betacyanin accumulation, and of mannitol present throughout induction

Mannitol Concentration	Conditions [1]	Induction (%) [2]
None	A	51
25 mM	A	45
150 mM	A	37
400 mM	A	30
None	B	51
25 mM	B	43
150 mM	B	10
400 mM	B	1

[1] A : Seedlings cut into 100 ml each of distilled water or varying [mannitol] for 1 hr before transferring to induction medium minus mannitol additions for 24 hr.
B : Seedlings cut directly prior to adding to induction medium containing varying [mannitol]
Germination, 37 C for 46 hr.

[2] Related to 100% induction given by 5 μM benzyladenine under standard conditions (15).

DISCUSSION

USE OF EXCISED COTYLEDONS

The use of half-seedlings (cotyledon plus upper part of hypocotyl) of *Amaranthus* is recommended because of the lower endogenous color produced by this material. In addition a clear supernatant is produced after using the Polytron homogenizer in contrast to a cloudy solution under the same conditions using whole seedlings. It is necessary to use the more time-consuming homogenization in a glass-Teflon homogenizer with whole seedlings. In suggesting the use of half-seedlings Biddington and Thomas (5) referred to a number of advantages. Their method involved the removal of seed coats from the cotyledons. We found that after germination at 37 C for 46 hr most of the seed coats fell off either during germination or during subsequent "aging" treatment (11).

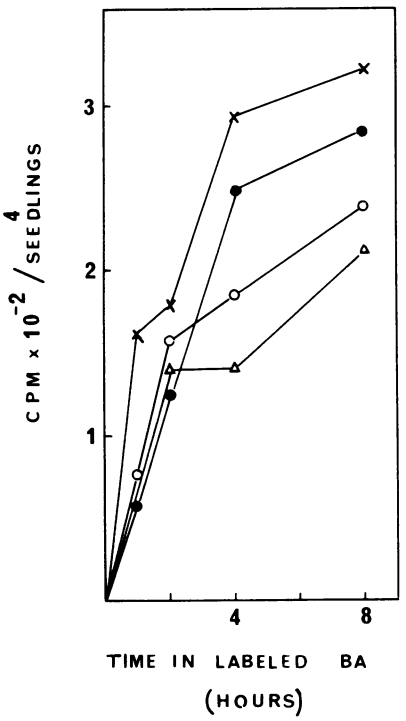

FIG. 3. Effect of 4-hr mannitol pretreatment on subsequent benzyladenine uptake. Seedlings were cut into mannitol solutions of varying concentrations 4 hr prior to incubation as in treatment A, Table IV. Curves show the time course of uptake of labeled benzyladenine after pretreatment in water (○), 25 mM mannitol (●), 150 mM mannitol (×), and 400 mM mannitol (△).

In a number of plant species the root has been implicated as the major source of cytokinins (*cf. e.g.* 19). In the absence of any marked change in the uptake and metabolism of benzyladenine in the presence or absence of roots, it is suggested that the lowering of the endogenous color production by removing roots (Table I) is probably due to the removal of the endogenous supply of cytokinins. The per cent response to red light is also greater in half-seedlings than in whole seedlings (*cf.* Table IV, Experiments 2 and 3 in ref. 12).

EFFECT OF BICARBONATE

The effect of HCO_3^- in stimulating betacyanin accumulation (Table II) can be explained by the malate pH-stat theory (8), whereby alkalinization of the cytoplasm resulting from K^+/H^+ exchange stimulates P-enolpyruvate carboxylase to synthesize malic acid by CO_2 dark fixation. High [HCO_3^-] will increase the level of malate plus H^+ in the cytoplasm and hence, as has been argued by Raven and Smith (25), the H^+/K^+ exchange. The final result of greater K^+ uptake would in effect be the same as the fusicoccin effect in increasing betacyanin synthesis. The same arguments apply to the generation of excess OH^- in NO_3^- assimilation and may explain the effectiveness of nitrate in betacyanin synthesis (12).

EFFECTS OF WATER STRESS

The effects of water stress on enzyme levels in plants have usually been studied by measuring enzyme activity of extracts after plants have been kept for varying lengths of time under defined stress conditions (see ref. 21). In this way Bardzik *et al.* (3) observed a decrease in phenylalanine ammonia lyase and nitrate reductase in maize seedlings with water deficit. Other authors have observed an increase in phenylalanine ammonia-lyase after increasing hydration and/or aeration of barley plumules (26) or radish cotyledons (30). The phenomenon under study by these authors (26, 30) may be more akin to the "aging" effect seen in *Amaranthus* (11) than to any relationship to water stress.

In the present case there are two effects of water deficit to consider:

Effect of Water Stress during Induction. When water stress is produced by an external osmoticum (mannitol) *during* the induction process a marked inhibition is observed even at concentrations well below those causing plasmolysis (0.35 M). Protein synthesis is readily reduced by mild water stress, as is uptake of amino acids (20), so that the uptake of the precursor tyrosine and the synthesis of enzymes of the betacyanin pathway may well be affected. Mannitol is known to have side effects and may be taken up slowly by tissues (20), and, inasmuch it has been shown (22) that mannitol inhibits phenylalanine ammonia lyase completely at 150 mM, it is possible that there is a direct effect on the activity of enzymes in the betacyanin pathway.

Effect of Wilting Pretreatment on Subsequent Induction. The increase in *subsequent* betacyanin accumulation after wilting pretreatment either of whole or cut seedlings under aerobic conditions, at ambient humidity, or by the application of PEG or mannitol to the roots of whole seedlings, could be explained in a number of ways. Water stress clearly allows the development of a potential for subsequent betacyanin accumulation (when induction takes place under nonstressed conditions), rather like the effect of water stress in creating a situation of "stored growth." This potential may follow from responses of active transport (K^+ uptake) to changes of turgor pressure (18) or from changes in levels of certain enzymes (20) (*e.g.* the development during the pretreatment of some component limiting for subsequent over-all betacyanin accumulation such as a transport protein, or an enzyme in the synthesis pathway). Finally, the increased tyrosine level during water stress (see *e.g.* 29) may be in a pool more accessible for betacyanin synthesis than is the exogenous tyrosine applied in the induction medium when water stress is relieved. This reasoning has been applied to the oxidation of proline during water stress (27).

Whatever the mechanism, the potential that is developed during water stress clearly results subsequently in increased betacyanin accumulation.

Acknowledgments—The technical assistance of J. Stapledon is gratefully recorded. Generous gifts of fusicoccin (A. Ballio, Rome; E. Marrè, Milan), 6-benzylamino-9-β-D-glucopyranosyl purine (J. Ueyanagi and Y. Kuwada, Takeda Chemical Industries Ltd., Osaka) and 6-benzylamino-7-β-D-glucopyranosyl purine (R. Horgan, Aberystwyth) are also acknowledged.

LITERATURE CITED

1. ANDERSON LE, NO MCCLURE 1973 An improved scintillation cocktail of high-solubilizing power. Anal Biochem 51: 173–179
2. BAMBERGER E, AM MAYER 1960 Effect of kinetin on formation of red pigment in seedlings of *Amaranthus retroflexus*. Science 131: 1094–1095
3. BARDZIK JM, HV MARSH JR, JR HAVIS 1971 Effects of water stress on the activities of three enzymes in maize seedlings. Plant Physiol 47: 828–831
4. BARRS HD 1968 Determination of water deficits in plant tissues. *In* TT Kozlowski, ed, Water Deficits and Plant Growth, Vol I. Development, Control and Measurement. Academic Press, New York, pp 235–368
5. BIDDINGTON NL, TH THOMAS 1973 A modified *Amaranthus* betacyanin bioassay for the rapid determination of cytokinins in plant extracts. Planta 111: 183–186
6. BIGOT C 1968 Action d'adénines substituées sur la synthèse des bétacyanines dans la plantule d'*Amaranthus caudatus* L. Possibilité d'un test biologique de dosage des cytokinines. CR Acad Sci Ser D 266: 349–352
7. CONRAD K 1971 Zur Spezifität des *Amaranthus* - Cytokinintests. V. Adenin- und Adenosinderivate. Biochem Physiol Pflanzen 162: 327–333
8. DAVIES DD 1973 Control of and by pH. Symp Soc Exp Biol 27: 513–529
9. DELEUZE GG, JD MCCHESNEY, JE FOX 1972 Identification of a stable cytokinin metabolite. Biochem Biophys Res Commun 48: 1426–1432
10. ELLIOTT DC 1976 Cytokinin action in enzyme induction is a membrane-related phenomenon. Abstracts, 9th International Conference on Plant Growth Substances, Lausanne, pp 86–87
11. ELLIOTT DC 1979 Analysis of variability in the *Amaranthus* betacyanin assay for cytokinins: effects of "aging" excised cotyledons. Plant Physiol 63: 274–276
12. ELLIOTT DC 1979 Ionic regulation for cytokinin-dependent betacyanin synthesis in *Amaranthus* seedlings. Plant Physiol 63: 264–268

13. ELLIOTT DC 1979 Temperature-dependent expression of betacyanin synthesis in *Amaranthus* seedlings. Plant Physiol 63: 277–279
14. ELLIOTT DC, AW MURRAY 1972 A quantitative limit for cytokinin incorporation into transfer ribonucleic acid by soya-bean callus tissue. Biochem J 130: 1157–1160
15. ELLIOTT DC, AW MURRAY 1975 Evidence against an involvement of cyclic nucleotides in the induction of betacyanin synthesis by cytokinins. Biochem J 146: 333–337
16. ELLIOTT DC, AW MURRAY, GT SACCONE, MR ATKINSON 1972 Use of structural analogues in the study of cytokinin action. *In* DJ Carr, ed, Plant Growth Substances, 1970. Springer-Verlag, Heidelberg, pp 459–466
17. FOX JE, J CORNETTE, G DELEUZE, W DYSON, C GIERSAK, P NIU, J ZAPATA, J MCCHESNEY 1973 Formation, isolation, and biological activity of a cytokinin 7-glucoside. Plant Physiol 52: 627–632
18. GUTKNECHT J 1968 Salt transport in *Valonia*: inhibition of potassium uptake by small hydrostatic pressures. Science 160: 68–70
19. HENSON IE, PF WAREING 1977 An effect of defoliation on the cytokinin content of buds of *Xanthium strumarium*. Plant Sci Lett 9: 27–31
20. HSAIO TC 1973 Plant responses to water stress. Annu Rev Plant Physiol 24: 519–570
21. KÖHLER K-H, K CONRAD 1966 Ein quanitativer Phytokinin-Test. Biol Rdsch 4: 36–37
22. KOPP M, B FRITIG, L HIRTH 1977 Phenylalanine ammonia-lyase activity of protoplasts: *in vitro* inhibition by mannitol and sorbitol. Phytochemistry 16: 895–898
23. PIATELLI M, M GIUDICI DE NICOLA, V CASTROGIOVANNI 1969 Photo-control of amaranthin synthesis in *Amaranthus tricolor*. Phytochemistry 8: 731–736
24. RASCHKE K 1975 Stomatal action. Annu Rev Plant Physiol 26: 309–340
25. RAVEN JA, FA SMITH 1977 Characteristics, functions and regulation of active proton extrusion. *In* E Marrè and O Ciferri, eds, Regulation of Cell Membrane Activities in Plants. North Holland Publ Co, Amsterdam, pp 25–42
26. SAUNDERS JA, JW MCCLURE 1973 Acetylcholine inhibition of phytochrome-mediated increases in a flavonoid and in phenylalanine ammonia-lyase activity of etiolated barley plumules. Plant Physiol 51: 407–408
27. STEWART CR, SF BOGNESS, D ASPINALL, LG PALEG 1977 Inhibition of proline oxidation by water stress. Plant Physiol 59: 930–932
28. THIMANN KV, YH EDMONDSON, BS RADNER 1951 The biogenesis of the anthocyanins. III. The role of sugars in anthocyanin formation. Arch Biochem Biophys 34: 305–323
29. THOMPSON JF, CR STEWART, CJ MORRIS 1966 Changes in amino acid content of excised leaves during incubation. 1. The effect of water content of leaves and atmospheric oxygen level. Plant Physiol 41: 1578–1584
30. TOMÉ F, E BELLINI 1974 Phenylalanine ammonia-lyase activity in excised radish cotyledons: effects of water availability, fusicoccin and kinetin. Plant Sci Lett 3: 413–418