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Inhibition by Phenolic Compounds of Cytokinin-Stimulated Betacyanin Synthesis in *Amaranthus caudatus*

J. S. CHALLICE

Long Ashton Research Station, University of Bristol*

Abstract. A number of phenolic compounds have been tested for ability to inhibit the cytokinin-induced synthesis of betacyanin in *Amaranthus caudatus* cotyledons. Under the conditions employed, the compounds responded thus: (1) no inhibition with up to 1 mg ml^{-1} (quercetin 3-rhamnosylglucoside and chlorogenic acid), (2) partial or no inhibition up to 0.1 mg ml^{-1} with greater inhibition at 1 mg ml^{-1} (phloridzin, arbutin, caffeic acid, p-hydroxybenzoic acid and 3,4-dihydroxyphenylalanine) and (3) partial or no inhibition up to 0.1 mg ml^{-1} with complete inhibition at 1 mg ml^{-1} (*o*-coumaric, *m*-coumaric, *p*-coumaric, protocatechuic and ferulic acids and phenylalanine). The results show that if the *Amaranthus* betacyanin bioassay for cytokinins is to give reliable results, then certain contaminating phenolics must be removed beforehand; some difficulties involved in this are briefly discussed.

The most widely accepted criterion for the presence of cytokinin activity in plant extracts is the ability (in the presence of auxin) to induce growth by cell-division in callus cultures of soya bean cotyledon or tobacco pith (*e.g.* WAREING and PHILLIPS 1970). However, due to the experimental difficulties encountered with this method, the quicker and more convenient *Amaranthus* betacyanin bioassay has gained increased acceptance recently (*e.g.* CONRAD 1971, 1974, BIDDINGTON and THOMAS 1973, THOMAS *et al.* 1975a,b BORKOWSKA and RUDNICKI 1975, REDA and RASMUSSEN 1975).

The *Amaranthus* bioassay is based upon the cytokinin-induced formation of betacyanin pigment in the cotyledons and hypocotyls of *A. caudatus* seedlings, incubated in the dark in the presence of tyrosine (KÖHLER and CONRAD 1966, BIGOT 1968, CONRAD 1971, BIDDINGTON and THOMAS 1973). During routine use of the *Amaranthus* bioassay for detecting cytokinin activity in plant extracts at varying stages of purification it was found that some fractions, known to contain cytokinins, gave negative bioassay results. Eventually the reason for this was traced to the presence of contaminating phenolic compounds, although not all instances of such contamination gave rise to inhibition in the bioassay. The present investigations were designed to clarify this situation by finding which phenolics are inhibitory in the bioassay, and at what concentrations.

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**Address:* Bristol BS 18 9AF, U.K.

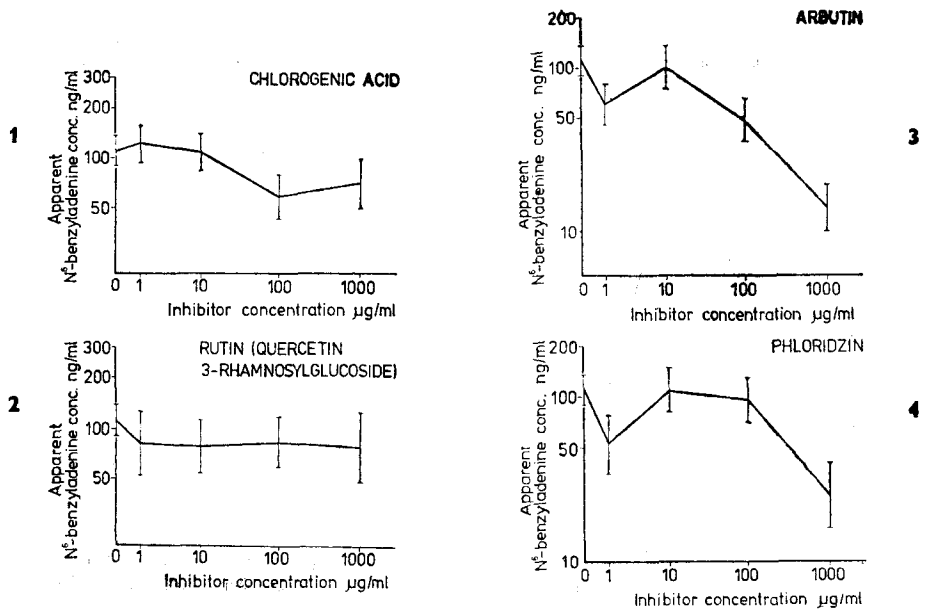
Material and Methods

The procedure used for the *Amaranthus* bioassay was a slightly modified version of that described by BIDDINGTON and THOMAS 1973, based on work of KÖHLER and CONRAD 1966 and BIGOT 1968. The cytokinin sample dissolved in methanol was applied to a 4.25 cm diam. circle of Whatman No. 1 filter paper and air-dried in a 4.8 cm diam. × 1.5 cm deep glass Petri dish with cover (bioassay dish). Ten explants (upper hypocotyl plus cotyledons) of *A. caudatus* seedlings were placed in each bioassay dish on the filter paper circle, previously moistened with 1 ml M/75 phosphate buffer at pH 6.3 containing 1 mg ml^{-1} tyrosine (soln. had to be warmed to dissolve tyrosine).

The following phenolic compounds were tested for their inhibitory properties in the bioassay: chlorogenic acid, rutin (quercetin 3-rhamnosylglucoside), phloridzin (2′, 4′, 6′, 4-tetrahydroxydihydrochalkone 2′-glucoside), arbutin (hydroquinone 0-glucoside), caffeic acid, *p*-hydroxybenzoic acid, 3,4-dihydroxyphenylalanine (DOPA), *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, phenylalanine, ferulic acid, protocatechuic acid. Each phenolic was run at standard levels 1, 10, 100 and 1000 µg plus a blank (20 replicates each) with a single level of 100 ng N^6-benzyladenine used throughout. A set of N^6-benzyladenine standards alone (1, 10, 100 and 1000 ng) was run with each batch for the calibration graph. After extraction of the betacyanin pigment, the amount was determined by measurement of the difference between optical densities at 542 nm and 620 nm, with an appropriate deduction for the blank readings.

Results

Figs. 1—13 show the extent of inhibition of betacyanin synthesis by a range of phenolic compounds, the inhibition being expressed as the apparent concentration (ng ml^{-1}) of N^6-benzyladenine compared with the actual con-



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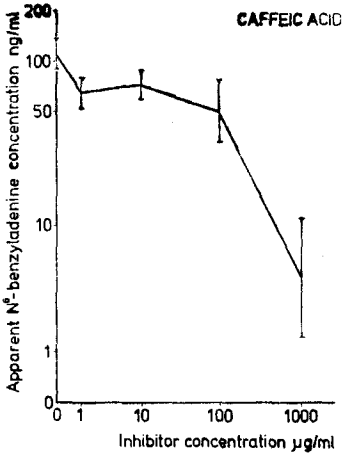
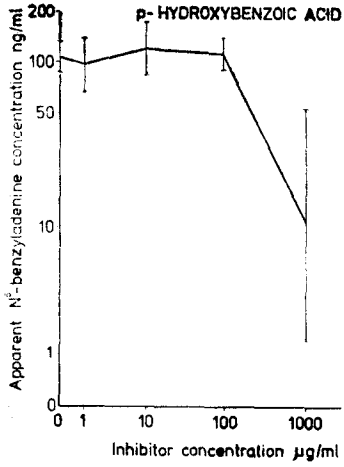
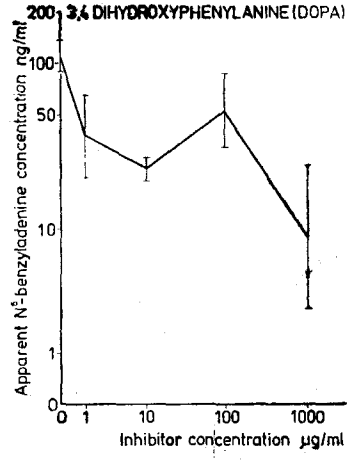

CAFFEIC ACID
Apparent N⁶-benzyladenine concentration ng/ml
200
100
50
10
1
0
0 1 10 100 1000
Inhibitor concentration µg/ml

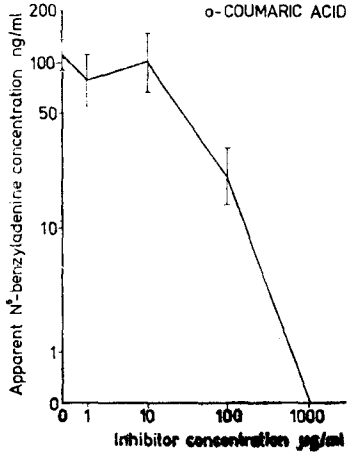
6


p- HYDROXYBENZOIC ACID
Apparent N⁶-benzyladenine concentration ng/ml
200
100
50
10
1
0
0 1 10 100 1000
Inhibitor concentration µg/ml

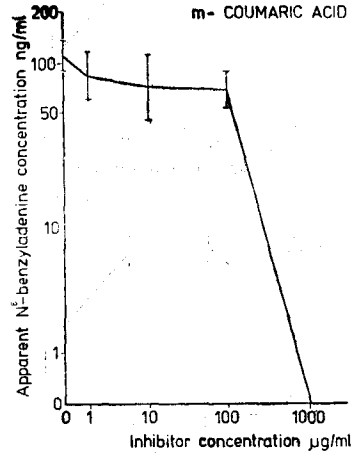
7


3,4 DIHYDROXYPHENYLANINE (DOPA)
Apparent N⁶-benzyladenine concentration ng/ml
200
100
50
10
1
0
0 1 10 100 1000
Inhibitor concentration µg/ml

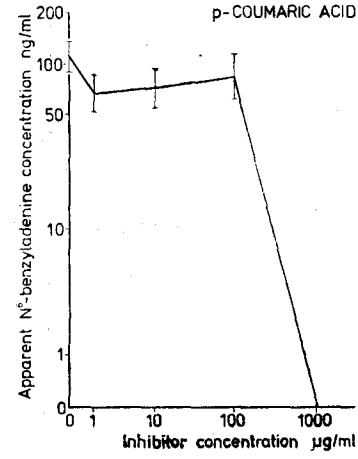
8

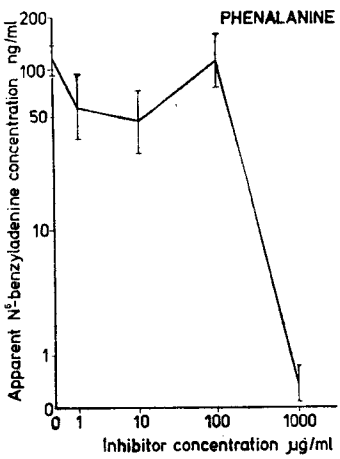

o-COUMARIC ACID
Apparent N⁶-benzyladenine concentration ng/ml
200
100
50
10
1
0
0 1 10 100 1000
Inhibitor concentration µg/ml

9


m- COUMARIC ACID
Apparent N⁶-benzyladenine concentration ng/ml
200
100
50
10
1
0
0 1 10 100 1000
Inhibitor concentration µg/ml

10


p-COUMARIC ACID
Apparent N⁶-benzyladenine concentration ng/ml
200
100
50
10
1
0
0 1 10 100 1000
Inhibitor concentration µg/ml



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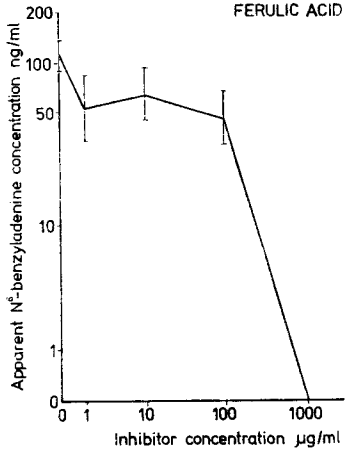

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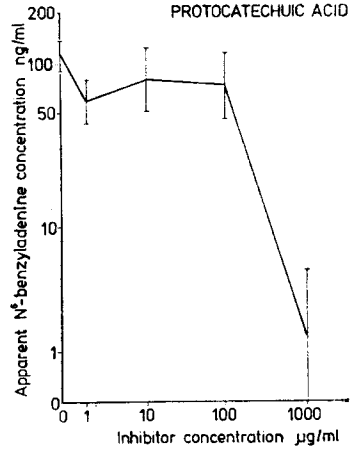

13

Figs. 1—13. Bioassay of cytokinin activity by the *Amaranthus* betacyanin method: inhibition of response to 100 ng N^6-benzyladenine by phenolic compounds. In each bioassay 1 mg of tyrosine was present, with the phenolic inhibitors present at 1, 10, 100 and 1000 µg.
Inhibition of betacyanin pigment synthesis is expressed as the apparent conc. [ng ml^{-1}] of N^6-benzyladenine compared with the actual conc. of 100 µg. Results are plotted on logarithmic scales and 95% confidence limits are indicated by vertical bars.

centration of 100 ng ml^{-1}. Because of the wide scatter of results it was necessary to run 20 replicates at each level of phenolic inhibitor and to transform the data to a $\log_{10}(1 + n)$ scale, prior to computation of the means and 95% confidence limits. The data in Figs. 1—13 is plotted on a $\log_{10}(1 + n)$ scale where n = µg ml^{-1} phenolic inhibitor (horizontal axis) and n = ng ml^{-1} N^6-benzyladenine (vertical axis); for convenience of presentation the original values of n are indicated on the axes. The 95% confidence limits are indicated by vertical bars.

Under the conditions employed there were three general types of response: (1) no inhibition with up to 1 mg ml^{-1} (rutin and chlorogenic acid), (2) partial or no inhibition up to 0.1 mg ml^{-1} with greater inhibition at 1 mg ml^{-1} (phloridzin, arbutin, caffeic acid, *p*-hydroxybenzoic acid and DOPA) and (3) partial or no inhibition up to 0.1 mg ml^{-1} with complete inhibition at 1 mg ml^{-1} (*o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, protocatechuic acid, ferulic acid and phenylalanine).

There is no significant stimulation of cytokinin-induced pigment synthesis by any of the phenolics tested. Results with the highest levels of *p*-hydroxybenzoic acid and DOPA showed a particularly wide scatter; the reason for this is not clear, but the low solubility in aqueous medium could be responsible.

Discussion

It is evident that some, but not all, phenolics act as potent inhibitors of cytokinin-induced betacyanin synthesis from tyrosine in cotyledons and hypocotyls of *Amaranthus* seedlings. The inhibition is of two-fold significance because the results indicate a possible new function for certain phenolic compounds; and because the results mean that the *Amaranthus* betacyanin bioassay for cytokinin activity could give false negative results if the fractions tested are contaminated by certain phenolic compounds. It would need further investigations to establish the existence of *in vivo* inhibition.

A complicating factor is that cytokinins replace light in inducing betacyanin synthesis: within the genus *Amaranthus* some species synthesize betacyanins in the dark, others only in light (KÖHLER 1972b). It is interesting that with chloramphenicol, only the light-induced betacyanin synthesis is inhibited; the cytokinin-dependent synthesis is not blocked (KÖHLER 1972a).

In *Amaranthus*, GIUDICI DE NICOLA *et al.* (1975) found that DOPA functioned as a better precursor of betacyanin than tyrosine whereas GARAY and TOWERS (1966), GURUPRASAD and LALORAYA (1976) found the converse. In the present experiments, each bioassay dish contained 1 mg ml^{-1} of tyrosine and Fig. 7 shows that adding the same amount of DOPA results in much less betacyanin synthesis. There is evidently an optimum amount of tyrosine, necessary for the maximum pigment synthesis; a 10-fold reduction in the amount of tyrosine in each bioassay dish gave a considerable reduction in the pigment synthesized (in the presence of 100 ng N^6-benzyladenine) and a 5-fold increase in the amount of tyrosine (under the same conditions) completely suppressed pigment synthesis.

Phenylalanine (Fig. 11) is, of course, non-phenolic but would be expected to precede tyrosine in the biosynthetic sequence to betacyanins; note the strongly inhibitory properties of this compound. It is interesting to note that while free caffeic acid (Fig. 5) is quite inhibitory at the highest level, the quinic acid ester *i.e.* chlorogenic acid (3-0-caffeoylquinic acid) (Fig. 1) is without any significant inhibitory effect. In the living plant, free phenolic acids (and other phenols) are usually present in either esterified or glycosylated forms, thought to be less toxic to the plant.

A possible criticism of the *Amaranthus* bioassay is that it does not measure that property which is the accepted criterion of cytokinin activity, *i.e.* the ability (as the name implies) to induce cell-division in the presence of auxin. However, it is now known that this is only one of the many other functions of cytokinins. HILL (1973) listed the following additional gross effects dependent on cytokinins: germination of light-requiring seeds, delay of senescence, promotion of lateral bud growth, cell-enlargement in tissue cultures and the control of differentiation (in the presence of auxin). Other apparent functions are described by STEINHART *et al.* (1964), FEIERABEND (1969), FLETCHER (1971), HASHIZUME and IIZUKA (1971), SERVETTAZ *et al.*

(1975), ALVIM *et al.* (1976), BORRISS (1967) and DAS *et al.* (1976). Thus the induction of cell-division is only one of many functions of cytokinins, and the *Amaranthus* bioassay would appear to be just as valid a criterion of cytokinin activity as the callus culture bioassay. Phenolics also inhibit the response to cytokinins in the soya-bean callus bioassay; our results indicate that there is marked inhibition when *p*-coumaric acid, phloridzin, arbutin and chlorogenic acid are present (CHALLICE 1977).

A common step, used during the isolation of cytokinins from plant material, is that of streaking an extract along the end of a sheet of thick chromatography paper, running with a suitable solvent (*e.g.* 2% acetic acid in water), drying, dividing the sheet into 10 segments and the bioassay of each segment. If the cytokinins which are present run to an R_f region not occupied by phenolics, this is fortunate, and the cytokinins will readily be detected by bioassay and high pressure liquid chromatography (HPLC) (CHALLICE 1975, 1976). However, often the contaminating phenolics run to the same general region as the known cytokinins (*e.g.* $R_f \sim 0.6$ to 0.9 in 2% acetic acid-water); in which case quite high cytokinin levels could remain undetected if the phenolics are present in sufficient concentrations. As many common phenolics occur in plants in amounts about a 1000-fold greater than that of endogenous cytokinins, such masking of cytokinin activity could be quite common.

Since cytokinin assay by HPLC depends upon UV-detection, the cytokinin peaks are often obscured by phenolics peaks, of much greater intensity with similar retention times. Some of the phenolic contaminants in leaf extracts of sweet corn (*Zea mays*), field bean (*Vicia faba*), pear (*Pyrus communis* cv.) strawberry (*Fragaria ananassa* cv.) and spring cabbage (*Brassica oleracea*) run to the same general regions as common cytokinins on paper chromatograms run with 2% acetic acid-water and sec-butanol : acetic acid : water (70 : 2 : 28), and on HPLC columns.

Phenolics can be readily detected on paper chromatograms by inspection under a UV-lamp for the presence of fluorescent or absorption spots; additional confirmation can be obtained by use of the diazonium colour reagent (FREEMAN 1952) or Gibb's colour reagent (SMITH 1963). Preliminary absorption of the cationic constituents (which include cytokinins) of a crude leaf extract onto a strong cation-exchange resin, washing with aqueous ethanol and followed by NH_4OH-elution at low temperature is generally successful in removing many of the phenolics originally present, but several cinnamic acid derivatives elute with the cationic fraction (CHALLICE 1976) and their ultimate separation from endogenous cytokinins can be quite difficult. It is therefore regrettable that so many investigations in this field fail to include tests for contaminating phenolics prior to bioassay for cytokinin activity.

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References

ALVIM, R., HEWETT, E. W., SAUNDERS, P. F.: Seasonal variation in the hormone content of willow I. Changes in abscisic acid content and cytokinin activity in the xylem sap. — Plant Physiol. **57** : 474—476, 1976.

BIDDINGTON, N. L., THOMAS, T. H.: A modified *Amaranthus* betacyanin bioassay for the rapid determination of cytokinins in plant extracts. — Planta **111** : 183—186, 1973.

BIGOT, C.: Action d'adenines substituées sur la synthèse des betacyanines dans la plantule d'*Amaranthus caudatus* L. Possibilité d'un test biologique de dosage des cytokinins. — Compt. rend. Acad. Sci. (Paris) Sér. D **266** : 349—352, 1968.

BORKOWSKA, B. RUDNICKI, R. M.: Changes in the levels of cytokinins in apple seeds during stratification. — Fruit Science Reports Skierniewice (Poland) **2** : 1—16, 1975.

BORRISS, H.: Untersuchungen über die Steuerung der Enzymaktivität in pflanzlichen Embryonen durch Cytokinine. — Wiss. Z. Univ. Rostock, Math. nat. Reihe **16** : 629—639, 1967.

CHALLICE, J. S.: Separation of cytokinins by high pressure liquid chromatography. — Planta **122** : 203—207, 1975.

CHALLICE, J. S.: Summary of Research. — Rep. Long Ashton Res. Sta. for 1975 : 45—46, 1976.

CHALLICE, J. S.: Summary of Research. — Rep. Long Ashton Res. Sta. for 1966, in press, 1977.

CONRAD, K.: Zur Spezifität des *Amaranthus*-Cytokinintests V. Adenin-und Adenosinderivate. — Biochem. Physiol. Pflanzen **162** : 327—333, 1971.

CONRAD, K.: Ein sensibilisierter *Amaranthus*-Cytokinintest (AT 74). — Biochem. Physiol. Pflanzen **165** : 531—535, 1974.

DAS, V. S. R., RAO, I. M., RAGHAVENDRA, A. S.: Reversal of abscisic acid induced stomatal closure by benzyl adenine. — New Phytol. **76** : 449—452, 1976.

FEIERABEND, J.: Formation of the photosynthetic apparatus during germination and its control. — In: METZNER, H. (ed.): Progress in Photosynthesis Research. Vol. 1. Pp. 280—283. Tübingen 1969.

FLETCHER, R. A., MCCULLAGH, D.: Benzyladenine as a regulator of chlorophyll synthesis in cucumber cotyledons. — Canad. J. Bot. **49** : 2197—2201, 1971.

FREEMAN, J. H.: Separation and identification of polymethylol phenols by paper chromatography. — Anal. Chem. **24** : 955—959, 1952.

GARAY, A. S., TOWERS, G. H. N.: Studies on the biosynthesis of amaranthin. — Canad. J. Bot. **44** : 231—236, 1966.

GIUDICI, DE N., AMICO, M. V., SCIUTO, S., PIATTELLI, M.: Light control of amaranthin synthesis in isolated *Amaranthus cotyledons*. — Phytochemistry **14** : 479—482, 1975.

GURUPRASAD, K. N., LALORAYA, M. M.: Betacyanin biosynthesis in the isolated hypocotyls of *Amaranthus caudatus*. — Planta **130** : 185—188, 1976.

HASHIZUME, T., IIZUKA, M.: Induction of female organs in male flowers of *Vitis* species by zeatin and dihydrozeatin. — Phytochemistry **10** : 2653—2656, 1971.

HILL, T. A.: Endogenous Plant Growth Regulators. — Edward Arnold, London 1973.

KÖHLER, K.-H., CONRAD, K.: Ein quantitativer Phytokinin-Test. — Biol. Rundschau **4** : 36—37, 1966.

KÖHLER, K.-H.: Action of inhibitors of protein and nucleic acid synthesis on light-dependent and kinetin-stimulated betacyanin synthesis. — Phytochemistry **11** : 127—132, 1972a.

KÖHLER, K.-H.: Photocontrol of betacyanin synthesis in *Amaranthus caudatus* seedlings in the presence of kinetin. — Phytochemistry **11** : 133—138, 1972b.

REDA, F., RASMUSSEN, O.: A modified *Amaranthus* betacyanin test for cytokinin bioassay. Biol. Plant. **17** : 368—370, 1975.

SERVETTAZ, O., CASTELLI, D., LONGO, C. P.: The effect of benzyladenine on anthocyanin accumulation in excised sunflower cotyledons. — Plant Sci. Letters **4** : 361—368, 1975.

SMITH, B.: Investigation of reagents for the qualitative analysis of phenols. — Chalmers Tek. Högskol. Handl. Nr. 263, 1963. (Avd. Kemi Teknol. 41).

STEINHART, C. E., MANN, J. D., MUDD, S. H.: Alkaloids and plant metabolism VII. The kinetin-produced elevation in tyramine methylpherase levels. — Plant Physiol. **39** : 1030—1038, 1964.

THOMAS, T. H., CARROLL, J. E., ISENBERG, F. M. R., PENDERGRASS, A., HOWELL, L.: Separation of cytokinins from Danish cabbage by column chromatography on insoluble polyvinylpyrrolidone. — Physiol. Plant. **33** : 83—86, 1975.

THOMAS, T. H., CARROLL, J. E., ISENBERG, F. M. R., PENDERGRASS, A., HOWELL, L.: A simple inexpensive, high pressure liquid chromatographic method for separating cytokinins in plant extracts. — Plant Physiol. **56** : 410—414, 1975.

WAREING, P. F., PHILLIPS, I. D. J.: The Control of Growth and Differentiation in Plants. — Pergamon, Oxford 1970.