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No effect of plant growth retarding compounds and growth stimulators on indolo-3-acetic acid oxidase activity in greening cucumber cotyledons*

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Abstract

Cotyledons dissected from 5-day-old etiolated cucumber seedlings were incubated in solutions on AMO-1618 **, B-Nine, CCC and Phosfon D for 48 h in light. In some tests the retardants were applied in mixed solutions with GA_3 or BAP. IAA oxidase was extracted and purified by means of molecular sieving through a bed of Sephadex G-25.

The retardants inhibited chlorophyll synthesis by 50 % or more, and had essentially no effect on IAA oxidase activity per cotyledon basis. GA_3 and BAP also had no effect on enzyme activity in spite of a fact that the compounds stimulated growth of the cotyledons. The crude enzyme extract from B-Nine treated cotyledons showed lower IAA oxidase activity in comparison with the water treated control, the effect being due to a longer lag-phase preceding the initiation of IAA oxidation. KNO_3 strikingly stimulated expansional growth of the cotyledons, the effect being correlated with the accelerated chlorophyll accumulation. KNO_3 had no effect on IAA oxidase activity per cotyledon and decreased it per gram fr wt.

It is concluded that [1] the growth rate of cucumber cotyledons is not correlated with IAA oxidase activity, and [2] the growth retarding compounds do not affect IAA oxidase system is this tissue.

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** Abbreviations: AMO-1618, 4-hydroxyl-5-isopropyl-2-methylphenyl trimethylammonium chloride, 1-piperidine carboxylate; B-9 or B-Nine, N,N-dimenthylaminosuccinamic acid; CCC, (2-chloroethyl) trimethylammonium chloride; Phosfon D, tributyl-2,4-dichlorobenzylphosphonium chloride; BAP, N^6-benzylaminopurine; GA_3, gibberellic acid; IAA-O, IAA oxidase.

INTRODUCTION

Growth retarding compounds AMO-1618, B-Nine, CCC and Phosfon D have been reported to enhance IAA oxidase activity in etiolated 4-day-old cucumber seedlings (Halevy 1963). The effect was inversely proportional to the inhibition of growth in the case of AMO-1618 treated seedlings, and it was mutually antagonized by GA_3. Halevy (1963) has concluded that the retardants exert their effect on plant growth by interacting with the gibberellin on auxin catabolism: GA_3 increases the level of endogenous auxins whereas growth retardants decrease it.

AMO-1618 and CCC strikingly enhanced IAA oxidase activity in coleoptiles and primary leaves of etiolated barley seedling (Gaspar and Lacoppe 1968). AMO-1618 stimulated IAA-O activity in roots of lentil seedlings (Lacoppe and Gaspar 1968). In both plants the enhancement of IAA oxidase activity was inversely proportional to the inhibition of growth, and it has been suggested that AMO-1618 and CCC may retard growth *via* accelerated auxin catabolism.

The data quoted above relate to some reports that a decrease in diffusible auxin follows the CCC treatment in pea (Kuraishi and Muir 1969) and wheat (Norris 1966; Shcherbakov 1969), and that growth inhibitions brought about by CCC and B-Nine can be reversed by exogenous auxin (Knypl 1964; Cleland 1965; Šebánek and Hradilík 1969). However, a reduced content of IAA in plants treated with growth retardants might follow the inhibited auxin synthesis rather that the accelerated auxin catabolism.

According to Knypl and Rennert (1967a,b) CCC and Phosfon D had essentially no effect on IAA oxidase system in vigorously growing cucumber hypocotyl sections when the enzyme activity was calculated per unit of original fr wt instead of the final one. Comparative studies with GA_3 and kinetin revealed that the growth rate of the hypocotyl sections was not directly correlated with the activity of IAA oxidase. Knypl and Rennert (1967) concluded that increased IAA-O activity, expressed per unit of final fresh or dry matter, followed the decreased growth rate, at least as far as cucumber hypocotyl tissue was concerned.

The retardants at concentrations little affecting growth of detached cucumber cotyledons, inhibit chlorophyll and protein synthesis in the tissue (Knypl 1969, 1971). The cotyledons seemed, thus, to be a good object for the re-investigation of the effects of plant growth retarding compounds on IAA oxidase system.

MATERIAL AND METHODS

Plant material

Cotyledons dissected from 5-day-old cucumber seedlings, *Cucumis sativus* L. cv. Delicatess, that had been grown in the dark at 25° C were used throughout the study. The cotyledons were placed in 10-cm Petri shales lined with 2 discs of Whatman No. 2 blotting paper wetted with 5 ml portions of test solutions supplemented with penicillin G, 100 mg l^{-1}, as antiseptic. The cotyledons were grown in permanent light of 1,400 lx intensity at the tissue level at 25° C. After 48 h the cotyledons were picked up, washed, blotted and used for assays.

Other details concerning growth of the plants and handling of the cotyledons have already been published (K n y p l 1971, 1973a).

Crude IAA oxidase extracts

Method A: 10 to 20 cotyledons were ground in a pre-chilled porcelain mortar with a pestle and quartz sand in ice-cold 0.05 M phosphate buffer, pH 7.5, supplemented with 0.8 M KCl. Ten ml buffer was used per g fr wt. The brei was stirred in an ice-bath for 10 min and centrifuged at 18 000g for 20 min in a refrigerated centrifuge K-24 (H. Janetzki). The supernatant was filtered through cotton wool to remove contaminating fluffy layer, and either diluted for the IAA-O assay by a colorimetric method or applied to a column of Sephadex G-25.

Method B: In some tests IAA-O activity was determined manometrically. In those tests IAA oxidase was extracted in 0.1 M Na-phosphate buffer, pH 6.1, without KCl; other details were the same as in the Method A.

Gel filtration

Dry Sephadex G-25 (fine) was allowed to swell for one day in several changes of 0.1 M Na-phosphate buffer, pH 6.1. Columns with an inner diameter of 3 cm were filled up with the gel (bed volume approximately of 150 ml; V_o 48—50 ml), and washed with 250 ml of the buffer. 4-ml aliquot of the crude enzyme extract was pipetted on to the top of the bed, drained into the bed and eluted with 500 ml buffer. 5-ml fractions were collected automatically. A_{260}, A_{280}, protein content and IAA-O activity were determined in each fraction.

Manometric assay of IAA oxidase activity

Enzyme activity assay was carried out according to S t u t z (1957) in a Warburg apparatus. 1.0 ml of 0.2 M Na-phospate buffer, pH 6.1, contain-

ing 3 μmoles of $MnCl_2$ and 3 μmoles of 2,4-dichlorophenol was poured into the main vessel of each 15-ml flasks, followed by 1.0 ml of crude enzyme extract. 30 μmoles of IAA in a volume of 1.0 ml was poured into a side-arm. 0.2 ml of 15 % KOH and a strip of blotting paper were placed in the centre cup. After 15 min equilibration at 25° C, IAA was tipped from the side-arm and oxygen uptake recorded each 10 min. for 60 min.

Colorimetric assay of IAA oxidase activity

The assay was carried out according to Goldacre *et al.* (1963). 9 ml of 0.066 M phosphate buffer, pH 6.1, supplemented with $MnCl_2$ (10^{-4} M) and 2,4-dichlorophenol (10^{-4} M) was poured into each of 50-ml bulb-flask wrapped in an alumina foil, followed by 1.0 ml of diluted crude enzyme extract or eluate from Sephadex columns. The mixture was shaken in a water bath (25° C; 90 osc. min^{-1}). 1.5-ml aliquots were sampled at 0 time and in 10 or 15 min intervals, and added to 3 ml of Gordon's and Weber's reagent (1951). The reaction mixture was filtered before measuring of A_{526}, if protein precipitate was formed. A_{526} was converted into μg remaining IAA using a calibration curve prepared with known concentrations if IAA (Fluka AG).

Chlorophyll $a+b$

Chlorophyll was extracted with 80 % acetone and measured spectrophotometrically (Bruinsma 1963).

Protein content

Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as a reference standard. Protein from crude enzyme extracts was precipitated with 5 % (final concentration) of ice-cold TCA and centrifuged. The precipitate was washed twice with 80 % and 96 % ethanol, and solubilized in 0.5 N NaOH. Protein from whole cotyledons was extracted as previously described (Knypl 1971): Total protein from 1 N NaOH extract was re-precipitated with 4 N TCA and solubilized in 0.5 N NaOH.

RESULTS

1. Manometric assays of IAA oxidase activity in crude extracts

AMO-1618, CCC and Phosfon D when applied at concentrations inhibitory for chlorophyll synthesis (Knypl 1969, 1971) had relatively

little effect on growth of the cotyledons and had essentially no effect on IAA oxidase activity in 2-day pre-treatment tests (Table 1).

Gibberellic acid and benzylaminopurine stimulated growth of the cotyledons (Table 1). The both compounds had no significant effect on IAA-O activity per one cotyledon basis.

Table 1

Activity of IAA oxidase in detached cucumber cotyledons greening for 48 h under the influence of growth retardants applied alone or in combination with GA_3 or BAP[1])

Additional compound, M	Treatment [2,3]) M				
	H_2O Control	AMO-1618 2×10^{-3}	B-Nine 10^{-2}	CCC 2×10^{-2}	Phosfon D 2×10^{-4}
	IAA oxidase activity, µl 0_2/20 min/50 mg fr wt				
None	82.7	92.0	64.3	91.2	80.1
GA_3, 10^{-4}	70.9	72.0	60.9	75.5	70.2
BAP, 10^{-5}	80.0	78.4	100.6	99.3	88.3
	IAA oxidase activity, µl O_2/20 min/cotyledon				
None	57.9	57.0	41.0	60.7	52.4
GA_3, 10^{-4}	62.3	54.3	51.8	62.1	54.3
BAP, 10^{-5}	65.3	51.1	76.8	76.3	67.0
	Fresh weight, mg/cotyledon				
None	35.0	31.0	31.9	33.3	32.7
GA_3, 10^{-4}	44.5	37.7	42.5	41.1	38.7
BAP, 10^{-5}	40.8	32.6	38.2	38.4	38.0

[1]) IAA oxidase activity was determined manometrically in crude enzyme extracts.
[2]) Final concentrations of the compounds in mixed solutions are the same as in the sole ones.
[3]) L.S.D at p=0.01: 13.2 µl O_2 per 20 min per 50 mg fr wt, 8.6 µl O_2 per 20 min per cotyledon, and 2.2 mg per cotyledon.

IAA oxidase activity in the cotyledons treated with AMO-1618, CCC or Phosfon D applied in combination with GA_3 was the same as in the GA_3-alone treated reference sample. Only in the GA_3+B-Nine treated cotyledons the enzyme activity decreased in comparison with the both GA_3 and water treated controls.

BAP applied in combination with B-Nine or CCC significantly enhanced IAA-O activity in comparison with the retardants alone or water treated samples as well per unit of fr wt as per cotyledon basis in the case of B-Nine, and per cotyledon in the case of CCC.

Those series of experiments seemd to indicate that of the compounds tested B-Nine decreased IAA oxidase activity. When B-Nine was applied at higher concentration of 0.02 M, then a difference in comparison with the water treated control was more pronounced: Control consumed about 80 μl O_2 per 20 min per 50 mg fr wt (Fig. 1) whereas B-Nine treated sample consumed only 33 μl O_2. Curves representing a kinetics

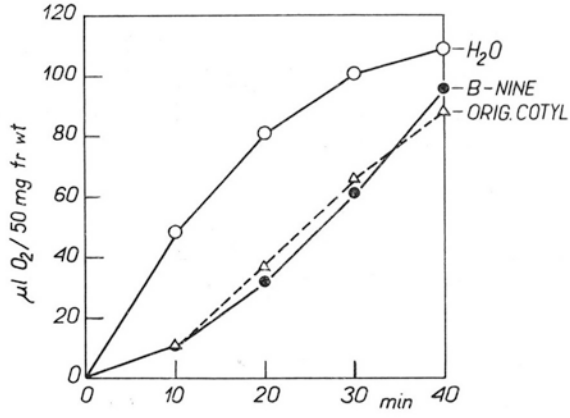

Fig. 1. Kinetics of IAA oxidation by crude IAA oxidase extracts of original cotyledons and detached cotyledons grown for 48 h in light on filter paper wetted with water or B-Nine (0.02 M).

of IAA oxidation clearly show that there is an initial lag-phase in auxin oxidation by crude enzyme extracts from original etiolated cotyledons and the B-Nine treated ones. Such a lag period is due to a presence of low molecular, termostable inhibitors of IAA oxidase (cf. Hare 1964).

2. Colorimetric assay of IAA oxidase activity in purified enzyme extracts

IAA oxidase can be purified from low molecular inhibitors by several methods (Hare 1964). The simplest mean of purification of crude enzyme extracts seems to be a technique of molecular sieving through Sephadex G-25 bed (Gamburg 1966).

The cotyledons that had been grown for 48 h in light in solutions of growth retarding compounds or KNO_3 were extracted according to a method A, and passed through a column of Sephadex G-25. Table 2 presents the effects of the compounds on growth, protein content and chlorophyll accumulation. KNO_3 (10^{-2} M) strikingly stimulated growth and greening. AMO-1618 was the only one of the retardants that slightly but significantly inhibited growth. CCC enhanced growth; Phosfon D and B-Nine were not effective in this respect.

In spite of a little or none effect on growth, the retardants inhibited chlorophyll synthesis (Table 2), the effects being comparable to those ones reported earlier (Knypl 1969).

A typical elution profile from a column of Sephadex G-25 is presented in Fig. 2. Protein was eluted in fractions No. 12 to 17 with a peak in a fraction No. 14. All IAA oxidase activity was eluted in frations No. 12-15 with a peak in a fraction No. 13. Low molecular UV absorbing

Table 2

Effect of retardants and KNO_3 on growth, protein content and chlorophyll synthesis in detached cucumber cotyledons grown for 48 h in light

Treatment, M	Fr wt, mg/cotyledon	Protein, µg/cotyledon	Chlorophyll $a+b$, µg/cotyledon
Water	35.9	926	40
AMO-1618, 10^{-3}	31.8	910	14
B-Nine, 10^{-2}	35.7	1,060	21
CCC, 10^{-2}	39.3	935	17
Phosfon D, 2×10^{-4}	35.3	1,060	27
KNO_3, 10^{-2}	58.0	1,170	130
Original cotyledons	20.0	1,060	0
Detached cotyledons grown for 48 h in darkness	28.6	825	0
L. S. D., p=0.01	2.1	100	4.1

compounds were eluted in fractions No. 20 to 35 with minor peaks in fractions No. 24, and 29-30.

Elution profile of IAA oxidase and protein from cotyledons treated with other compounds was similar to that one presented in Fig. 2. Nevertheless, because six columns were used at once, the peaks from three columns were shifted for one fraction, i.e. the IAA-O activity peak occurred in a fraction No. 14 instead of a fraction No. 13 as shown in Fig. 2.

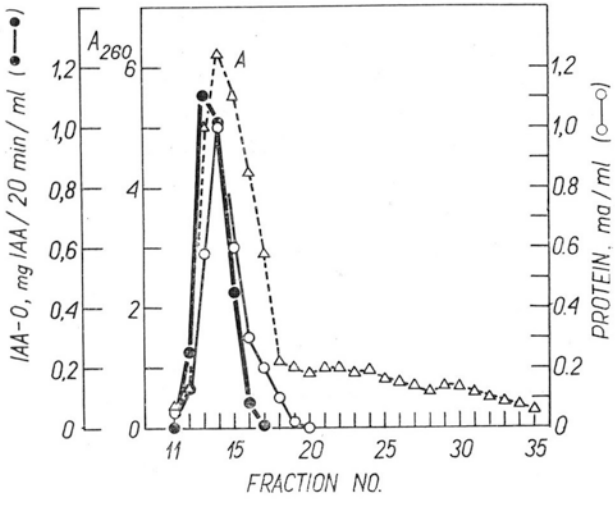

Fig. 2. Typical elution profile of IAA oxidase of greening cucumber cotyledons from a column of Sephadex G-25. Enzyme was extracted from water treated cotyledons, greening for 48 h. 5 ml fractions were collected; enzyme activity is expressed in mg IAA oxidazed for 20 min per ml eluate.

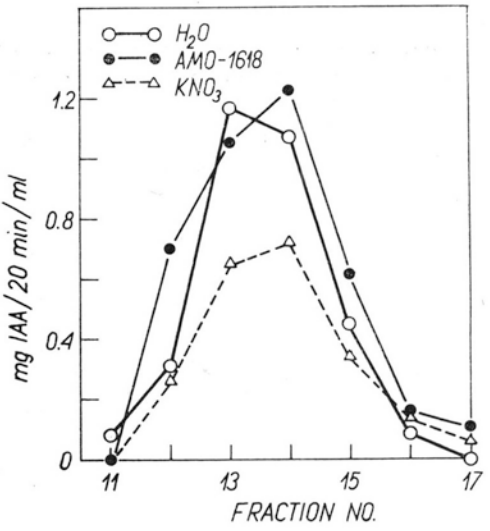

Fig. 3. IAA oxidase activity in eluates from columns of Sephadex G-25. Detached cucumber cotyledons were grown for 48 h in light on filter paper wetted with water, AMO-1618 (10^{-3} M) or KNO_3 (10^{-2} M). 4 ml aliquots of crude enzyme extracts (Method A; gram fr wt per 10 ml) were applied to the columns and eluted with phosphate buffer (0.1 M; pH 6.1). 5-ml fractions were collected; IAA oxidase activity is expressed in mg oxidized IAA per 20 min per ml eluate.

The fractions No. 12-17 were pooled, IAA oxidase activity determined and computed per 10 mg of fr wt or per cotyledon basis. As it can be seen from the data of Table 3, IAA-O activity in the cotyledons grown for 48 h in light on water wetted filter paper increased from original 510 and 1,020 to 700 and 2,400 μg oxidized IAA per h per 10 mg fr wt and per cotyledon basis, respectively. KNO_3 and all retardants had no significant effect on the enzyme activity per cotyledon basis. AMO-1618 and Phosfon D increased, and CCC and B-Nine decreased enzyme activity per unit of fr wt; the differences were, however, insignificant in comparison with the control (at $p = 0.01$). KNO_3 strikingly decreased IAA-O activity per 10 mg fr wt, the effect being a consequence of the stimulated growth of the cotyledons (cf. Table 2).

In Figure 3 IAA activity in each of the fractions 11-17 from Sephadex G-25 columns is presented for H_2O, AMO-1618 and KNO_3 treated cotyledons. It is clearly seen that there is no significant difference between H_2O and AMO-1618 grown cotyledons, and that KNO_3 strikingly decreased enzyme activity. That is becaue 1 ml of the eluate corresponds to a unit of final fr wt: Homogenates were made by grinding 1 gram of the tissue per 10 ml of the buffer and 4 ml aliquots were placed on the column. Since KNO_3 treated cotyledons were larger

Table 3

Activity of IAA oxidase in detached cucumber cotyledons grown for 48 h in light under the influence of growth retarddants or KNO_3[1)]

Treatment, M	µg oxidized IAA h^{-1} per 10 mg fr wt	µg oxidized IAA h^{-1} per cotyledon
Water (Control)	700	2,430
AMO-1618, 10^{-3}	760	2.440
B-Nine, 10^{-2}	590	2,110
CCC, 10^{-2}	580	2,270
Phosfon D, 2×10^{-4}	800	2,800
KNO_3, 10^{-2}	460[2)]	2,550
Original cotyledons[3)]	510[2)]	1,020[2)]

[1)] Enzyme extracts were purified by means of gel filtration through Sephadex G-25 beds; fractions No. 12-17 were pooled and IAA oxidase activity determined colorimetrically.

[2)] The values are significantly different from the water treated control ($p=0.01$).

[3)] Original cotyledons were analysed immediately after dissecting from 5-day-old etiolated seedlings.

than the control ones (Table 3), 4-ml aliquot of the enzyme extract corresponds to about 7 and 11 cotyledons for KNO_3 and water treated samples, respectively.

DISCUSSION

Halevy (1963), Gaspar and Lacoppe (1968), Lacoppe and Gaspar (1968) have shown that retardants stimulate IAA oxidase activity, the effect being inversely proportional to the inhibition of the growth rate of cucumber, barley and lentil seedlings. The data were interpreted as indicating that the phenomenon of decreased growth might be caused by accelerated auxin catabolism by the growth retarding compounds. Such a suggestion implies that there exists a strict inverse correlation between the growth rate and the activity of IAA oxidase in plants. However, it is not the case (cf. a review by Hare 1964).

Katsumi et al. (1967) have found that helminthosporol like GA_3 decreased IAA oxidase activity in cucumber seedlings per unit of fr wt of the hypocotyl tissue, but had no effect on the enzyme activity per the whole cucumber seedling. Moreover, IAA-O activity in cucumber was decreased not only by GA_3 but also by IAA (Katsumi and Sano 1968). The results was interpreted as being a consequence of the accelerated growth, induced by GA_3 or IAA.

Detached, greening cucumber cotyledons were chosen for this study because growth retarding compounds little affect growth of the tissue. In the same time the object is sensitive to the action of retardants in that sense that RNA, protein and chlorophyll synthesis is effectively arrested (Knypl 1969, 1971). Moreover, recent studies have revealed

that AMO-1618 and Phosfon D strikingly inhibit activity of nitrate reductase in the KNO_3 induced cotyledons, whereas B-Nine *per se* induces *de novo* synthesis of nitrate reductase (Knypl 1963a,b).

This study revealed that none of the growth retarding compounds increased IAA-O activity per whole cotyledon basis when purified enzyme extracts were used for analyses. Although KNO_3 strikingly accelerated growth of the cotyledons, it had no effect on the enzyme activity per cotyledon basis. In the same time it strikingly decreased IAA oxidase activity per unit of final fresh weight. It is no doubt that the decrease of enzyme activity follows the accelerated growth: Absolute enzyme activity in the water and KNO_3 treated samples, grown for 48 h in light, is the same (Table 3). This activity is more "diluted" in the KNO_3 treated cotyledons weighing 58 mg than in the control ones weighing 36 mg.

IAA oxidase activity in the cucumber cotyledons is not correlated with the growth rate. Basing on the results of the experiments reported here it can be concluded that growth retarding compounds do not primarily affect the activity of IAA oxidase *in vivo* (cf. Knypl and Rennert 1967a,b).

Acknowledgments.

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Brak wpływu retardantów i stymulatorów wzrostu na aktywnoćć oksydazy kwasu indolilo-3-octowego w zieleniejących liścieniach ogórka

Streszczenie

Liścienie, odcięte od 5-dniowych etiolowanych siewek ogórka, hodowano przez 2 dni na świetle w roztworach retardantów wzrostu AMO-1618, B-9, CCC i Phosfonu D; stosowano również GA_3, benzyloaminopurynę i KNO_3. Aktywność IAA oksydazy oznaczano manometrycznie w nieoczyszczonych wyciągach enzymatycznych, lub kolorymetrycznie po oczyszczeniu ekstraktów na kolumnach z żelem Sephadex G-25.

Retardanty — słabo wpływając na wzrost, silnie zahamowały syntezę chlorofilu. Aktywność IAA oksydazy, w przeliczeniu na jeden liścień, nie uległa istotnym zmianom w porównaniu do kontroli, hodowanej w wodzie destylowanej. GA_3 i BAP również nie zmieniły aktywności enzymu, chociaż pobudziły wzrost liścieni. Nieoczyszczone ekstrakty z liścieni hodowanych w roztworze B-9 wykazują słabszą aktywność IAA oksydazy; jest to spowodowane występowaniem inhibitorów, powodujących lag-fazę w procesie utleniania IAA.

KNO_3 silnie stymuluje wzrost liścieni i syntezę chlorofilu; mimo to KNO_3 nie wpływa na aktywność IAA oksydazy w przeliczeniu na liścień, chociaż obniża aktywność enzymu w przeliczeniu na jednostkę świeżej masy tkanki.

Wnioskuje się, iż [1] aktywność oksydazy kwasu indolilo-3-octowego nie jest skorelowana z szybkością wzrostu liścieni, oraz [2] retardanty wzrostu nie wpływają bezpośrednio na aktywność IAA oksydazy, a tym samym ich hamującego wpływu na wzrost nie można tłumaczyć przyśpieszeniem katabolizmu auksyn.

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