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Effects of growth retarding compounds on chlorophyll accumulation and nitrate reductase activity in nitrate induced cucumber cotyledons*

J. S. KNYPL

Department of Plant Physiology, Institute of Physiology and Cytology
University of Łódź

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Abstract

Cotyledons were excised from 5-day old etiolated cucumber seedlings and grown for 24 or 48 h in solutions of plant growth retardants: AMO-1618**,B--Nine, CCC and phosfon D, supplemented with KNO_3 (10^{-2}M) in light. Nitrate reductase (NR) activity was determined *in vivo*.
CCC and Phosfon D at high concentrations had no effect on nitrate reductase activity in 24 h tests. CCC at $5x10^{-2}$ M enhanced NR activity in longer 48 h tests; Phosfon D was inhibitory in that case. AMO-1618 markedly decreased NR activity. B-Nine strikingly enhanced NR activity in KNO_3 induced cytoledons; the effect was positively correlated with the concentration of B-Nine. All the compounds inhibited chlorophyll synthesis.

INTRODUCTION

Nitrate reductase (NADH:nitrate oxidoreductase, EC 1.6.6.1.) is a substrate inducible enzyme which catalyses the first step of assimilation of nitrogen from nitrate, i.e. the reduction of NO_3^- to NO_2^-. The enzyme activity in plants depends upon several genetic and environmental factors (cr. a review by Beevers and Hageman 1969). In isolated embryos of *Agrostemma githago* its activity can be induced by cytokinins (Borriss 1967; Hirschberg et al. 1972; Kende et al. 1971). In fenugreek cotyledons NR activity is synergistically induced by nitrate

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** Abbreviations: NR, nitrate reductase; AMO-1618, 4-hydroxyl-5-isopropyl-2--methyphenyl trimethylammonium chloride, 1-piperidine carboxylate; B-Nine, N,N--dimethylamino-succinamic acid; CCC, (2-chloroethyl) trimethylammonium chloride; Phosfon D, tributyl-2,4-dichlorobenzylphosphonium chloride.

and cytokinins (Rijven and Parkash 1971). Gibberellin and cytokinin, applied simultaneously, can substitute light for maintenance of high NR activity in tobacco leaves (Lips and Roth-Bejer-nano 1969). All the quoted data indicate that NR activity in some plants can be controlled by phytohormones and growth regulators.

Recently it has been found that nitrate reductase in detached cucumber cotyledons can be induced by KNO_3 and/or benzylaminopurine as well in light as in darkness; both compounds act synergistically (Knypl 1973a). Growth retarding compounds including AMO-1618, B-Nine, CCC and Phosfon D inhibit chlorophyll accumulation in detached cucumber cotyledons, the effect being reversed by potassium and decreased by benzylaminopurine (Knypl 1969a). The chlorophyll synthesis inhibitory effect of retardants follows the preceding inhibition of either protein and/or RNA synthesis, presumably in chloroplasts. This study was undertaken in order to examine whether plant growth retardants can effect nitrate reductase activity in the KNO_3-induced cucumber cotyledons.

MATERIAL AND METHODS

Seeds of *Cucumis sativus* L. cv. Delicatess were germinated in the dark at 25° C for five days. Cotyledons were excised from 7-8 cm long seedlings, washed with sterile water, blotted and placed in 9-cm Petri shales lined with two discs of Whatman No. 2 filter paper, previously wetted with 5 ml aliquots of distilled water, KNO_3 (10^{-2}M) or combined solution of KNO_3 and the retardants to be tested. Each of a pair of cotyledons from one seedling was placed in two separate dishes; altogether 22 cotyledons were placed in each dish. Test solutions were supplemented with penicillin G, 100 mg l^{-1}, as an antiseptic.

Petri shales with the cotyledons were placed in a growth room with permanent illumination produced by fluorescent tubes type LW "Flora" 40 W (Unitra-Polam, Warsaw) at constant temperature of 25° C. Light intensity at the tissue level was 1400 lx. The cotyledons were grown either for 24 or 48 h and then used for analyses.

Nitrate reductase activity was measured according to an in vivo assay elaborated by Jaworski (1971), and adapted to cucumber (Knypl 1973a). The cotyledons were picked up from Petri shales, washed, blotted, weighed on a torsion balance and placed in penicillin vials containing 5 ml aliquots of a medium consisting of 0.1 M sodium-phosphate buffer, pH 7.5; 0.02 M KNO_3; 5% n-propanol; and two drops of chloramphenicol, 500 mg l^{-1}, as an antiseptic. The vials were stoppered and kept for three hours in darkness at 30° C with occasional shaking. Five cotyledons were placed in each penicillin vial; four replicate NR activity assays were carried out from one Petri dish.

Nitrite released into the medium was determined by treating 0.4 ml aliquots with 0.3 ml each of 1% sulfanilamide in 3 N HCl and 0.02 % of N-l-naphthyl-ethylene diamine hydrochloride. After 20 minutes in darkness the solutions were diluted to 4 or 8 ml with distilled water and the absorbance read at 540 nm. Standard curve for nitrite was made according to Nicholas and Nason (1957).

In foregoing paragraphs the nitrate reductace activity is expressed in nmoles released nitrite for three hours per cotyledon.

Chlorophyl a + b was extracted with 80 % acetone and measured spectrophotometrically (Bruinsma 1963). Fresh weight was determined by weighing the cotyledons on a torsion balance. Each test was repeated three times with two replicates in each series. Other details have been described elsewhere (Knypl 1973a).

RESULTS

Cotyledons grown for 24 h in dist. water released only traces of nitrite. The cotyledons had virtually no NR activity. On the contrary, the cotyledons grown in 10^{-2} M solution of KNO_3 released 55—57 nmoles NO^-_2 $3h^{-1}$ cotyledon^{1-}. AMO-1618 strikingly prevented the rise in NR activity, the effect being positively correlated with concentration of the compound. If KNO_3 solution was supplemented with AMO-1618 at 10^{-3} M and 5×10^{-3}M, the cotyledons produced only 22 and 5 nmoles NO^-_2 $3h^{-1}$ cotyledon^{-1}, respectively. At the lowest concentration tested,

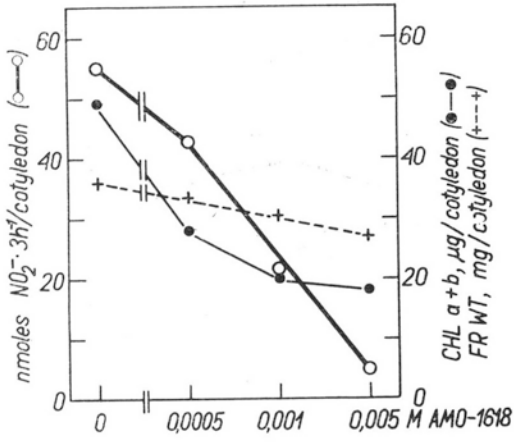

Fig. 1. Inhibitory effect of AMO-1618 on nitrate reductase activity in KNO_3-induced cucumber cotyledons. The cotyledons were grown for 24 h in light of 1400 lx. Each test solution was supplemented with 10^{-2} M KNO_3; there was no NR activity in the cotyledons grown for 24 h in water. Original fr wt of one cotyledon: 20.2 mg.

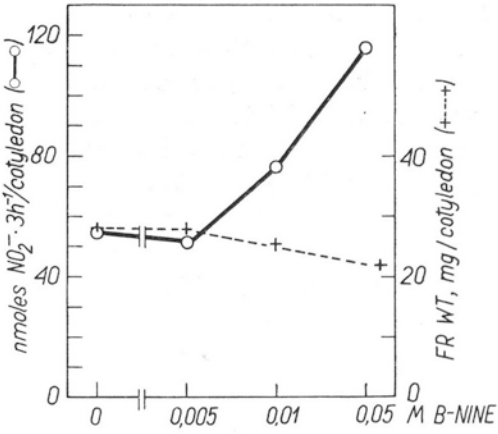

Fig. 2. Enhanced nitrate reductase activity in the cotyledons grown for 24 h in solutions of B-Nine supplemented with KNO_3. Other details as in Fig. 1.

5×10^{-4} M, AMO-1618 reduced the NR activity by about 20%, whereas the chlorophyll content was decreased by about 50% in comparison with the KNO_3 alone treated control (Fig. 1).

B-Nine at 10^{-2} M and 5×10^{-2} M concentrations, in contrast to AMO-1618, strikingly potentiated NR activity. The cotyledons grown for 24 h in KNO_3 solution supplemented with B-Nine at the highest concentration of 5×10^{-2} M released about 120 nmoles NO^-_2 $3h^{-1}$ cotyledon^{-1}, i.e. two times more than the KNO_3 alone treated control (Fig.2).

CCC at 10^{-2} M had no effect on growth of the cotyledons and on NR activity, although it severely decreased the chlorophyll content from about 50 μg cotyledon^{-1} in the KNO_3 alone treated sample to 10 μg cotyledon^{-1}

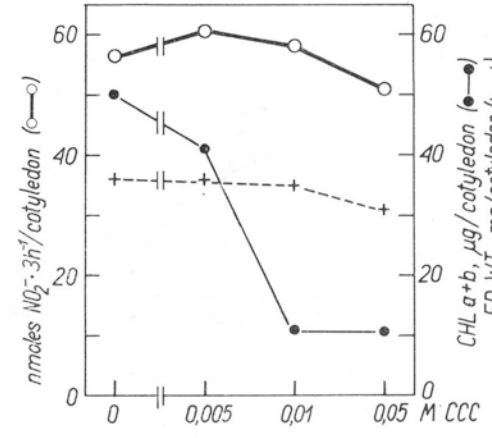

Fig. 3. No effect of CCC on nitrate reductase activity in KNO_3-induced cucumber cotyledons. Details as in Fig. 1.

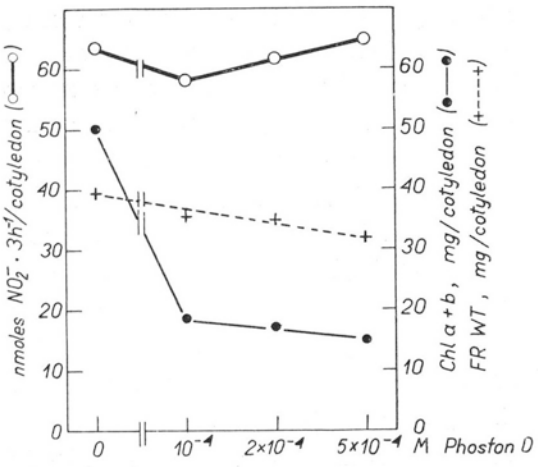

Fig. 4. No effect of Phosfon D on nitrate reductase activity in KNO_3-induced cucumber cotyledons. Details as in Fig. 1.

in the CCC + KNO_3 treated ones (Fig. 3). This growth retarding compound at the highest concentration of 5×10^{-2} M slightly reduced enzyme activity, but the difference was insignificant in comparison with the control.

Phosfon D at 10^{-4} M to 5×10^{-4} M strikingly inhibited chlorophyl synthesis. Despite this fact, the compound had no effect on nitrate reductase activity in the KNO_3-induced cotyledons (Fig.4).

Table 1

Effect of plant growth retardants on KNO_3-induced activity of nitrate reductase in cucumber cotyledons, grown for 48 h in light

Retardant[1], M	Fr wt, mg cotyledon^{-1}	NR activity, nmoles NO_2^- 3h^{-1} cotyledon^{-1}	Chlorophyll a+b, µg cotyledon^{-1}
None[1] (KNO_3 alone)	47.7[a]	50.7[a]	103.7[a]
AMO-1618, 10^{-3}	49.5[a]	38.6[b]	
B-Nine, 10^{-2}	46.8[a]	79.8[c]	
CCC, 5×10^{-2}	39.9[b]	73.1[c]	25.3[b]
CCC. 10^{-2}	50.2[a]	59.6[d]	76.8[c]
CCC, 5×10^{-3}	46.0[a]	46.2[a]	86.6[c]
Phosfon D, 10^{-3}	31.1[c]	44.4[a]	14.6[d]
Phosfon D, 5×10^{-4}	38.9[b]	28.6[e]	28.2[b]
Phosfon D, 2×10^{-4}	41.4[d]	35.1[b]	35.7[e]
Dist. water	9.3[b]	1.5[f]	38.0[e]

[1]) Each solution of the retardant was supplemented with KNO_3, 10^{-2} M. Differences between two values denoted with different letters are significant at 0.01 probability level. Original fr wt of one cotyledon=20.4 mg.

In a next step of experiments the cotyledons were grown in solution of KNO_3 (10^{-2} M) supplemented with growth retardants for a longer period of time, 48 hours. Results of those tests are summed up in Table 1.

As in the shorter tests, AMO-1618 reduced and B-Nine enhanced the nitrate reductase activity, although the differences in comparison with the KNO_3 alone treated cotyledons were much smaller than after initial 24 hours (cf. Table 1 and Figures 1 and 2). Both growth retardants decreased chlorophyll content (data not shown, cf. K n y p l 1969a).

CCC at 5×10^{-2} M enhanced NR activity by about 150 per cent in spite of a fact that the chlorophyll content was reduced by about 75 per cent in comparison with the respective data for the KNO_3 alone treated control, taken as 100 per cent (Table 1).

Phosfon D at all three concentrations tested (10^{-3} M, 5×10^{-4} M, $2 \times \times 10^{-4}$ M) decreased chlorophyll content, negatively proportionally to the rise of concentration. Despite this fact, Phosfon D at the higest concentration of 10^{-3}M did significantly not affect the enzyme activity. The compound at lower concentration of 5×10^{-4} M decreased NR activity by about per cent (Table 1).

DISCUSSION

Previous study has revealed that the four growth retarding compounds at concentrations that cause about 50 per cent inhibition of chlorophyll synthesis in greening cucumber cotyledons (K n y p l 1969a), markedly inhibit incorporation of ^{14}C-leucine into total proteins of the cotyledons. AMO-1618 (10^{-3} M) had relatively little effect on ^{14}C-uracil incorporation into total RNA of the cotyledons, whereas the other retardants severely inhibited RNA synthesis in 24 h tests (K n y p l 1971). On a basis of those results is has been concluded that the chlorophyll synthesis inhibitory effect of the retardants is a secondary one; the inhibition of greening follows the preceding inhibition of RNA and/or protein synthesis in the cucumber cotyledons.

Recently K n y p l and C h y l i ń s k a (1972a,b) have shown that among four retardants tested, only CCC was found to be active as an inhibitor of greening of intact lettuce cotyledons. And again, only CCC was very active as an inhibitor of total protein synthesis in the cotyledons of 2-day old seedlings. AMO-1618, B-Nine and Phosfon D did inhibit chlorophyll accumulation. Despite this fact, B-Nine and Phosfon D decreased specific activity of the protein fraction by about 20 per cent and by more than 50 per cent in 2- and 5-day old lettuce seedlings, respectively. It thus seems that growth retarding compounds may selectively inhibit the

synthesis of some types of proteins (enzymes) either in cytoplasm, in chloroplasts or in both, the range of selectivity being dependent not only on a chemical structure of the retardant but also on a kind of a plant tissue examined.

Nitrate reductase seems to be synthesized on cytoplasmic 70S ribosomes since the rise of its activity in the induction tests is prevented by cycloheximide and not affected by chloramphenicol (Ritenour et al. 1967; Schrader et al. 1967; Ferrari and Varner 1969; Stewart 1968) in a number of plants, including cucumber (Knypl 1973a). Hence, analyses of the effects of the retardants on this enzyme activity may give us some indirect evidences on their intracellular sites of action on protein synthesis.

In shorter 24 h tests CCC and Phosfon D had no effect on NR activity in KNO_3-induced cotyledons. Since at the concentrations applied the compounds strikingly inhibit protein synthesis (cf. Knypl 1971) it can be inferred that they act, first of all, on protein synthesis in plastids of the cucumber cotyledons. AMO-1618 is possibly an active inhibitor of protein synthesis in both chloroplasts (inhibition of greening) and in cytoplasm, since it strikingly decreased the rise of NR activity in the cotyledons. The enhanced activity of NR in the cotyledons grown for 48 h in the highest CCC concentration is possibly due to the retarding effect of CCC on protein breakdown (cf. Knypl 1969), the same seems to be true for Phosfon D at 10^{-3} M (Table 1).

B-Nine at the range of concentrations very inhibitory for total protein synthesis (Knypl 1971) enhanced the nitrate reductase activity in cucumber cotyledons by about twofold in comparison with the KNO_3 alone treated sample. Interpretation of the result is not easy. Three at least explanations of the fact may be suggested: [1] B-Nine per se induces the enzyme synthesis; [2] it retards enzyme breakdown; or [3] it inhibits NR activity in chloroplasts and causes accumulation of nitrite in the tissue, that is subsequently released into the medium for the intact--tissue NR activity assay. None of these possibilities can be discarded without prior direct experimental prove. Such the tests are being carried out in this laboratory. Preliminary results seem to indicate that B-Nine per se induces nitrate reductase activity in cucumber cotyledons and in senescing leaf discs of kale, the effect being prevented by cycloheximide (Knypl 1973b).

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Author's address:
Doc. dr J.S. Knypl
Department of Plant Physiology,
Institute of Biochemistry and Physiology,
University of Lodz,
ul. Nowopołudniowa 12/16
90-237 Łódź, Poland

Wpływ retardantów na aktywność reduktazy azotanowej w odciętych liścieniach ogórka, indukowanych azotanem

Streszczenie

Liścienie odcięte od 5-dniowych etiolowanych siewek ogórka, hodowano przez 24 lub 48 godz. na świetle w roztworach retardantów wzrostu AMO-1618, B-9, CCC i Phosfonu D, uzupełnionych azotanem potasowym (10^{-2} M). Oznaczano zawartość chlorofilu metodą spektrofotometryczną i aktywność reduktazy in vivo.

W liścieniach, hodowanych w wodzie, nie występuje aktywność reduktazy azotanowej; enzym pojawia się po uprzedniej indukcji azotanem. W ciągu początkowych 24 godz. hodowli, CCC i Phosfon D nie wpływają na aktywność reduktazy azotanowej, chociaż silnie hamują syntezę chlorofilu. Po 48 godz. hodowli, CCC w stężeniu 0.05 M podwyższa aktywność enzymu.

AMO-1618 silnie hamuje, a B-9 podwyższa aktywność enzymu. Po pierwszych 24 godzinach hodowli w liścieniach poddanych działaniu KNO_3 i B-9 w stężeniu 0.05 M aktywność reduktazy azotanowej jest dwukrotnie wyższa niż w kontroli, hodowanej tylko w roztworze KNO_3. B-9 i pozostałe retardanty hamują syntezę chlorofilu.

Ponieważ reduktaza azotanowa jest enzymem syntetyzowanym na rybosomach cytoplazmy, wobec tego wnioskuje się, że CCC i Phosfon D hamują syntezę białek przede wszystkim w chloroplastach, a AMO-1618 w chloroplastach i w cytoplazmie. B-9 prawdopodobnie sam jako taki indukuje aktywność reduktazy azotowej w liścieniach ogórka.