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The Effect of Exogenous IAA and Kinetin on Nitrate Reductase, Nitrite Reductase and Glutamate Dehydrogenase Activities in Excised Pea Roots

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Abstract. Nitrate reductase (NO_3R) activity, nitrite reductase (NO_2R) activity and $NADH_2$ dependent glutamate dehydrogenase (GDH) activity were followed in extracts from excised pea roots incubated under aseptic conditions for 9 and 24 h in nitrate containing nutrient medium to which IAA was added in concentrations promoting lateral root formation (1×10^{-5}; 3×10^{-5}; 5×10^{-5} M) and kinetin in concentrations which reduce lateral root formation (0.1; 1; 5 mg l^{-1}, that is 4.65×10^{-7}; 4.65×10^{-6} and 2.3×10^{-5} M). NO_3R activity was not influenced by IAA, NO_2R activity was slightly depressed by IAA after 24 h incubation and GDH activity was slightly increased after 24 h incubation in the presence of IAA. Kinetin decreased NO_3R activity significantly both after 9 h and 24 h incubation, slightly increased NO_2R activity after 9 h incubation but slightly decreased it after 24 h incubation, and did not affect GDH activity after 24 h incubation. However, when applied together with IAA, kinetin abolished the promoting effect of IAA on GDH activity. IAA neither reversed nor accentuated the effect of kinetin on NO_2R activity. Nevertheless the depressing effect of kinetin on NO_3R activity was emphasized by the presence of IAA after 9 h incubation. The results obtained indicate that reduced nitrate assimilation due to the depression of nitrate reductase activity caused by kinetin probably contributes to the negative growth effect of kinetin in pea root segments grown in nitrate medium.

In nearly every case reported, the exogenous application of the cytokinins was deleterious to the initiation of roots and elongation of the main axis of roots (*e.g.* SKINNER and SHIVE 1955, DEROPP 1956, DEYSSON 1959, FRIES 1960, HUMPHRIES 1960, HARRIS and HART 1964, FERNQUIST 1966, YANG and DODSON 1970). The exogenous application of auxins also resulted in the inhibition of primary root elongation (see *e.g.* THIMANN 1969) but it enhanced the proliferation of lateral secondary roots in sections of primary roots (*e.g.* TORREY 1950, 1956). Kinetin reduced the effect of auxin on the proliferation of lateral roots in primary pea root sections when applied simultaneously with auxin in nitrate containing nutrient medium (TORREY 1962).

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Little is known, however, about the mechanism of the action of kinetin and about the influence of kinetin and IAA on enzyme activities in roots. In our laboratory when investigating the effects of different compounds on nitrate reductase induction in excised pea roots, we found that kinetin depressed nitrate reductase activity (Sahulka, unpubl. results). WERNER and GOGOLIN (1970) reported similar effects of kinetin on glutamate dehydrogenase. They found that in carrot callus tissues grown on low kinetin concentrations in the presence of IAA, root initiation was preceded by a threefold increase in glutamate dehydrogenase activity. Higher kinetin concentrations which were unfavourable for root growth and initiation decreased glutamate dehydrogenase activity in carrot callus tissues whereas aspartate aminotransferase and isocitrate dehydrogenase activities were not affected. The GDH activity of growing roots was approximately five times as high as that of callus tissues and about ten times as high as that of aged roots.

Glutamate dehydrogenase has been supposed to have a key position in the assimilation of ammonium nitrogen. Nitrogen has however been supplied in experiments with tissue cultures and isolated organs usually in the form of nitrate. Under such conditions, with no other source of nitrogen available, the inhibition of nitrate assimilation would be a sufficient condition to inhibit growth and therefore the inhibition of growth by a growth regulator depressing nitrate reductase activity may be — at least partly — the result of the inhibition of nitrate assimilation, provided the depression of nitrate reductase activity is deeper than the depression of activities of enzymes involved in later steps of nitrogen assimilation.

The aim of this paper is to ascertain the extent to which the growth and morphological effects of IAA and kinetin in roots are correlated with the action of these growth regulators on the activities of enzymes involved in nitrogen assimilation. For this reason we followed the effect of IAA in concentrations which promote lateral root formation and the action of kinetin in concentrations that depress lateral root formation (see TORREY 1962) on the activities of nitrate reductase, nitrite reductase and $NADH_2$ dependent glumatate dehydrogenase. The results obtained indicate that in pea root segments grown in nitrate medium the depression of nitrate assimilation probably contributes to the negative effect of kinetin on lateral root formation.

Material and Methods

Plant Material

Roots of aseptically germinated *Pisum sativum* seedlings, cv. Raman, were used in the experiments described in this paper. Selected seeds were successively washed with soap and water, rinsed with water, surface sterilized with 96 % ethanol and a saturated solution of chloramin and eventually rinsed several times with sterilized distilled water. The surface sterilized seeds were then germinated on 0.7% agar in Petri dishes at 25 °C in the dark. After six days of germination 3 cm long tip segments of primary roots were excised and transferred to nutrient solutions.

Nutrient Solutions

The incubation of root segments was performed under aseptic conditions in 100 ml Erlenmayer flasks at 25 °C in the dark. The basic standard nutrient solution used in these experiments contained per litre: 242 mg $Ca(NO_3)_2 \cdot 4H_2O$; 42 mg $MgSO_4 \cdot 7H_2O$; 85 mg KNO_3; 61 mg KCl; 20 mg KH_2PO_4; 3 mg FeEDTA; 1 mg $ZnSO_4$; 1.5 mg H_3BO_3; 0.3 mg $Na_2MoO_4 \cdot 2H_2O$; 20 g sucrose. Vitamins were not included because they did not show any influence on tested enzyme activities in our short term experiments. Kinetin was dissolved in 0.01 N KOH by heating and the stock solution obtained was added to the nutrient medium before autoclaving. KOH was equilibrated to the same level in all nutrient solutions in experiments with kinetin. At least 3 independently prepared stock solutions of both kinetin and potassium indoleacetate were used in every series of experiments with these growth regulators. Commercial IAA preparation was repurified before use according to GOOD *et al.* (1956) and the stock solution of potassium indoleacetate (pH approx. 6.0) was prepared from the purified IAA by dissolving in water to which the equivalent amount of 0.1M KOH was added. The stock solution of indoleacetate was sterilized by cold Seitz filtration and the filtrate was added to the nutrient solution just before the beginning of the incubation of the roots. After the incubation, the roots were rinsed with chilled distilled water and their approximate fresh weight was determined.

Homogenization and Extraction

Roots were homogenized in chilled mortars with a fivefold amount of buffer. 0.066 M phosphate buffer pH 7.5 containing 3×10^{-5} M cysteine was used for the extraction when nitrate and nitrite reductase activities were determined and 0.066 M tris-HCl buffer pH 8.0 containing 3×10^{-5} M cysteine was used when glutamate dehydrogenase activity was followed. Homogenates were cleared by centrifugation at 20 000 × g for 25 min and the supernatants obtained were immediately used for the determination of enzyme activities.

Determination of Enzyme Activities

The Determination of Nitrate Reductase (E.C. 1.6.6.1) Activity: Nitrate reductase was assayed using the procedure described by STULEN (1970). The assay mixture (final volume of 4 ml) contained per ml: 90 μmoles of potassium phosphate buffer pH 7.5; 10 μmoles of KNO_3; 75 mμmoles of $NADH_2$; 0.15 ml of root extract. The enzyme reaction was started by adding $NADH_2$ solution which followed immediately after the addition of root extract and was performed at 27 °C for 15 min. A mixture without $NADH_2$ served as a blank. The reaction was stopped by adding activated charcoal (see STULEN 1970) and the NO_2^- produced was determined in the filtrate using 1 % sulphanilamide in N HCl and 0.02 %. N-(1-naphthyl)ethylenediamine dihydrochloride. After 30 min, colour intensity was measured at 540 nm using a Spekol colorimeter.

The Determination of Nitrite Reductase (EC. 1.6.6.4) Activity: The assay mixture (usually a final volume of 3 ml) contained per ml: 70 μmoles of potassium phosphate buffer pH 7.0; 0.33 μmoles of benzyl viologen; 0.2 μmoles of $NaNO_2$; 0.15 ml of root extract. The reaction was started by adding 8 μmoles of sodium dithionite, added just after the root extract; the tubes were then sealed and evacuated. After 20 min incubation at 27 °C, the enzyme reaction was stopped by shaking the tubes. Zero time controls were included. NO_2^- removed from the assay mixture was estimated.

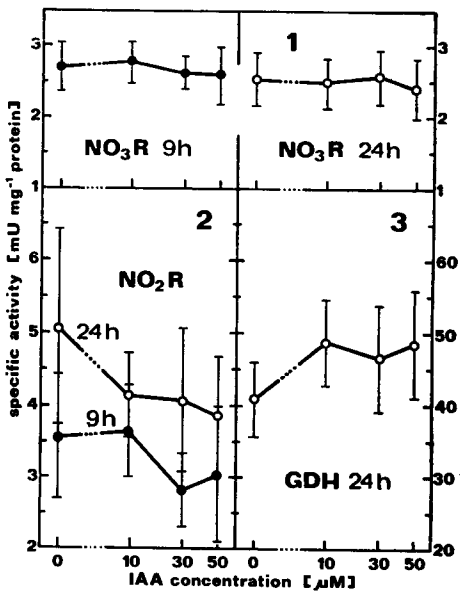
The Determination of Glutamate Dehydrogenase (E.C. 1.4.1.2) activity: GDH activity was determined by measuring the oxidation of $NADH_2$ in the assay mixture. The assay mixture (final volume of 3 ml) contained per ml: 100 μmoles of tris-HCl buffer pH 8.0; 100 μmoles of $(NH_4)_2SO_4$; 167 mμmoles of $NADH_2$; 0.15 ml of root extract; 13 μmoles of potassium α-ketoglutarate. The reaction was started by adding α-ketoglutarate and then extinction readings at 340 nm were taken at room temperature every minute for five minutes after the first minute necessary for the setting of the Optica (Milano) CF 4 spectrophotometer. Mixture without α-ketoglutarate was used as a blank.

Enzyme activities were expressed in mU per mg of protein (one mU = one millimicromole of substrate per min). The presented data are mean values calculated from 8 replicates (independent experiments) with 2 parallel determinations each. 95% confidence intervals are given in the figures for each mean value. Protein determinations were made after POTTY (1969).

Results

At the beginning of the experiments, a suitable sucrose concentration in the nutrient medium was established; nitrate reductase activity served as a criterion. Nitrate reductase activity was not influenced significantly by increasing sucrose concentrations above 2% even after 72 h incubation of roots in nutrient solutions and the 2% sucrose concentration was therefore used in further experiments as standard. The time course of nitrate reductase, nitrite reductase and glutamate dehydrogenase activities were then determined during the first 24 h of incubation in standard nutrient solutions. Nitrate reductase activity increased considerably during the first 8 h and then remained approximately at the same level, nitrite reductase activity increased slowly for a longer period and glutamate dehydrogenase activity did not change significantly.

The effect of IAA and kinetin on enzyme activities was followed after 9 h incubation (just after the upper level of nitrate reductase activity had been reached) and after 24 h incubation (when some of the hormone effects started to be apparent). The results are summarized in Figs. 1 to 6. IAA did not influence nitrate reductase activity (Fig. 1), did not show an explicit effect

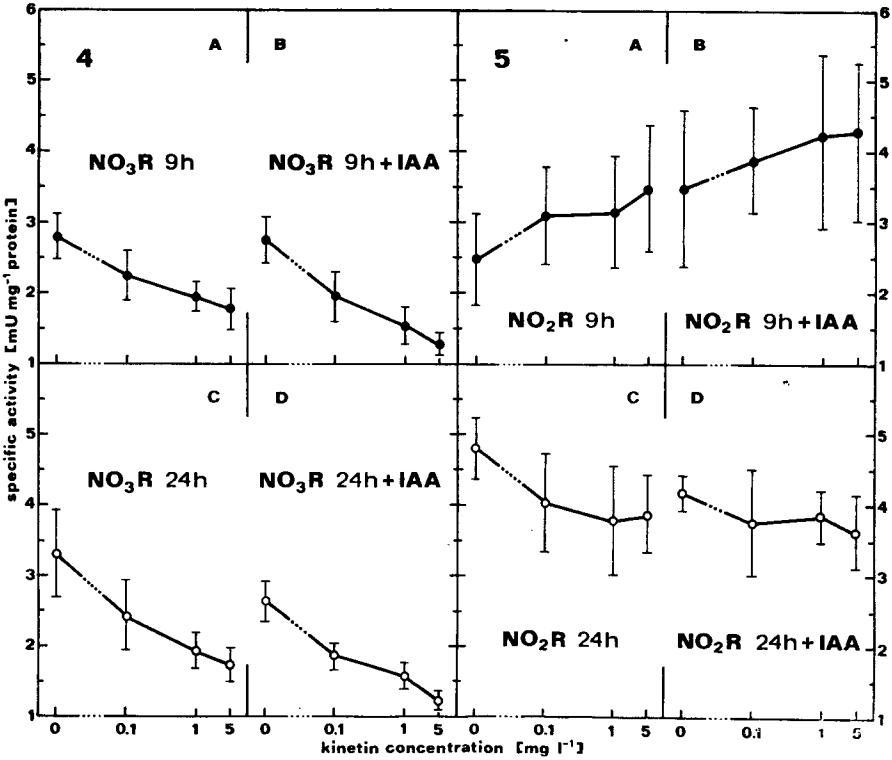


Figs. 1,2,3. The effect of IAA (abscissae) on the specific activity (ordinates) of nitrate reductase (*1*), nitrite reductase (*2*) and $NADH_2$ dependent glutamate dehydrogenase (*3*) after 9 h (●) and 24 h (○) incubation of roots, respectively. Mean values calculated from 8 replicates. Bars on the vertical lines represent 95 % confidence intervals. One milliunit (mU) = one millimicromole of substrate per minute.

on nitrite reductase activity after 9 h incubation, only slightly decreased nitrite reductase activity after 24 h incubation (Fig. 2), and slightly increased glutamate dehydrogenase activity (Fig. 3). Glutamate dehydrogenase activity was always higher in extracts from roots incubated with IAA than without it. Kinetin decreased nitrate reductase activity both after 9 h incubation and after 24 h incubation the depression of activity after 24 h incubation being more pronounced (Fig. 4). Nitrite reductase activity was slightly increased after 9 h incubation with kinetin but slightly depressed after 24 h incubation (Fig. 5). Glutamate dehydrogenase activity was not affected by kinetin alone after 24 h incubation (Fig. 6), however, the promoting effect of IAA on glutamate dehydrogenase activity was abolished by kinetin (Fig. 6). The effect of kinetin on nitrite reductase activity was neither reversed nor accentuated by IAA (Fig. 5). Nevertheless the depressing effect of kinetin on nitrate reductase was slightly emphasized by the presence of IAA after 9 h incubation. The ageing of excised roots in distilled water for 30 h before incubation in nutrient medium increased the effect of kinetin even more. During the inductive phase, the depressive effect of kinetin on nitrate reductase activity was apparent after only 2 h of incubation. 5 p.p.m. of kinetin added to the assay mixture did not influence nitrate reductase activity. The protein level of incubated roots was not influenced by kinetin treatment (Fig. 7).

Discussion

The results have shown that the effects of investigated growth regulators on studied enzymes differ considerably and that different results can be obtained also when the effects of growth regulators on the activity of one enzyme are followed after different periods of incubation — see the effect of kinetin on the activity of nitrite reductase after 9 h and 24 h respectively. Different effects were also reported when IAA and kinetin were applied simultaneously: kinetin acted as an IAA antagonist in the case of glutamate dehydrogenase whereas a moderate synergistic effect of IAA and kinetin was reported with nitrate reductase. Thus, the effects of growth regulators on enzyme activities are diverse and cannot be generalized. The negative effect of kinetin became evident in the experiments described in this paper



Figs. 4,5. The effect of kinetin (abscissae) and IAA on the specific activity (ordinates) of nitrate reductase (*4*) and nitrite reductase (*5*). *A*: 9 h incubation, kinetin alone. *B*: 9 h incubation, kinetin as given in the figure, IAA 3×10^{-5}M. *C*: 24 h incubation, kinetin alone. *D*: 24 h incubation, kinetin as indicated, IAA 3×10^{-5}M. Mean values from 8 replicates. Bars on the vertical lines represent 95 % confidence intervals. One mU = one millimicromole of substrate per minute.

primarily in nitrate reductase. The depression of NO_3R activity appeared soon after the beginning of incubation during the inductive phase. This depression of activity might be caused by the inhibition of a *de novo* synthesis of the nitrate reductase protein, but the total protein level of treated roots was not affected by kinetin. It is of interest that either an enhancement of nitrate reductase activity (Roth-Bejerano and Lips 1970, Rijven and Parkash 1971) or no effect on it (Beevers and Hageman 1969) could be observed in cotyledons and leaves after the application of kinetin or various cytokinins.

The enhancement of lateral root formation due to IAA is accompanied by an enhancement of $NADH_2$ dependent glutamate dehydrogenase activity and the depressive effect of kinetin is accompanied by a reversion of this enhancement. The influence of exogenous IAA on glutamate dehydrogenase activity in roots of intact plants is not known, but GDH activity is higher in older regions of the primary roots of maize seedlings (Reimers and Khavkin 1970). This fact might also be taken as evidence for the involvement of glutamate dehydrogenase, because lateral roots are formed in a higher

number on basal root segments than on tip segments. The higher activity of $NADH_2$ dependent glutamate dehydrogenase could be manifested by a higher assimilation of inorganic nitrogen in seedling primary roots with a high supply of nitrogen in a reduced form but there is little likelihood that a higher GDH activity caused by IAA treatment could function in the same manner

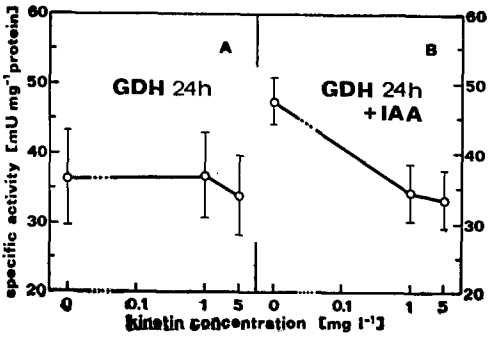


Fig. 6. The effect of kinetin and IAA on the specific activity of $NADH_2$ dependent glutamate dehydrogenase after 24 h incubation *A*: kinetin alone. *B*: kinetin + 3 × 10^{-5}M IAA. Mean values from 8 replicates. I = 95 % confidence intervals. One mU = one millimicromole of substrate per min.

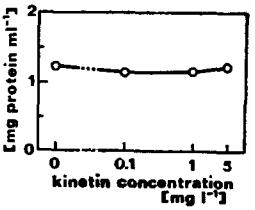


Fig. 7. Protein level in extracts from roots after 24 h incubation. IAA 3 × 10^{-5}M, kinetin as in- dicated in the figure.

in the small excised root segments grown in a nutrient medium containing nitrate as the sole source of nitrogen, if nitrate reductase activity was not enhanced by IAA treatment. In small root segments (TORREY used 0.6 cm segments), no appreciable internal sources of nitrogen could be mobilized. The fact that the *in vitro* nitrate reductase activity was approximately 10 times lower than the *in vitro* glutamate dehydrogenase activity is also in good agreement with this presumption. On the other hand, a decrease in nitrate reductase activity could become evident under such conditions in the depression of nitrogen assimilation and in the subsequent depression of growth processes. Of course, other enzymes can also be involved in the growth effects of kinetin in roots, nevertheless it is probable that the depression of nitrate reductase activity contributes to the negative growth effect of kinetin in pea root segments grown in nitrate containing nutrient medium.

Acknowledgement

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Byla sledována aktivita zmíněných enzymů v extraktech z isolovaných kořenů hrachu inkubovaných v aseptických podmínkách po dobu 9 a 24 h v živném mediu obsahujícím nitrát jako jediný zdroj dusíku, ke kterému byly přidány IOK v koncentracích, které podporují tvorbu postranních kořenů, a kinetin v koncentracích, které brzdí tvorbu postranních kořenů. Kinetinem byla brzděna nejvíce aktivita nitrátreduktázy. Výsledky svědčí o tom, že snížená asimilace nitrátu pravděpodobně přispívá k negativnímu růstovému účinku kinetinu u isolovaných kořenů hrachu pěstovaných v nitrátovém živném prostředí.