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The Effect of Cytokinins on Nitrate Reductase Activity

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Abstract. Cytokinins in addition to nitrate induce nitrate reductase activity (NRA) in some plants. Effects of cytokinins on NRA was investigated in stem pith parenchyma of kale, intact wheat and barley seedlings and isolated cucumber cotyledons.

The most profound effect on NRA was found in barley and wheat seedlings. NRA in seedlings sprayed with 100 μM 6-benzylaminopurine (BAP) for three subsequent days was increased in leaves and decreased in roots. These changes were further enhanced in seedlings grown in nutrient solution lacking nitrate: NRA in wheat and barley leaves was increased by 57 % and 202 %, respectively, in plants supplied with nitrate the NRA increase was not significant: in wheat and barley leaves by 22 % and 9 %, respectively.

Similar effect of BAP and kinetin was found in kale stem parenchyma and cucumber cotyledons. The cytokinin kinetin or BAP alone increased NRA about twice in kale and three times in cucumber. Addition of nitrate to the medium enhanced the effect of kinetin in kale discs, but the two effects were not additive. Additive effect of nitrate and BAP on NRA was found in cucumber cotyledons in light.

In general NRA was more affected by cytokinins in intact seedlings of wheat and barley as compared to explanted tissue of kale and cucumber, and lack of nitrogen made their effect more expressive.

Most of the nitrogen of higher plants is taken up as nitrate which is reduced to NH_4^+ and simultaneously utilized in sythesis of amino acids and amides. Reduction of nitrate to NH_4^+ is catalyzed by nitrate reductase (NR) and nitrite reductase. NR is the first and rate limiting enzyme involved in reduction of nitrate representing the key regulation site of nitrate utilization. This substrate-inducible enzyme is in a number of plant species subject of hormonal regulation.

Induction of NR activity (NRA) by cytokinin 6-benzylaminopurine (BAP) independent of nitrate was described by BORRISS (1967) in embryos of *Agrostemma githago*. The same plant material was used in a number of other investigations dealing with the hormonal induction of NRA (KENDE *et al.* 1971, HIRSCHBERG *et al.* 1972, KUZNETSOV and LITONOVA 1985, SCHMERDER and BORRISS 1986 *etc.*). The mechanism of enhancement of NRA by cytokinins in *A. githago* is not dependent on nitrate accumulation and is different from substrate induction (KUZNETSOV *et al.* 1979 and others).

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Similar effect of plant growth regulators on NR was found in a number of plants. Cytokinins increase NRA in leaves of *Oryza sativa* (GANDHI and NAIK 1974), *Beta vulgaris* and *Chenopodium album* on light independently of the presence of nitrate (GZIK and GÜNTHER 1984) and in roots of *Phaseolus vulgaris* in absence of nitrate (HÄNISCH TEN CATE and BRETELER 1982). On the other hand cytokinins at low concentrations (10^{-7} M) do not stimulate NRA while at high concentrations (10^{-5} M) in the presence of nitrate they exhibit inhibition of NRA (HIGGINS *et al.* 1974). Kinetin decreased NRA in isolated roots of *Pisum sativum* (SAHULKA 1972) while the cytokinin-active phenylurea derivatives (KARANOV and PAVLOVA 1976) and BAP (PAVLOV *et al.* 1987) induced NRA in leaves of *Hordeum vulgare*. The increase of NRA in leaves of *Hordeum vulgare* and *Triticum aestivum* after application of kinetin was also reported by KOHL *et al.* (1975). Cytokinins also induced NRA in isolated cotyledons of *Cucumis sativus* and *Cucurbita pepo* (KNYPL 1973, 1979) and *Trigonella foenum-graecum* (RIJVEN and PARKASH 1971). Increase of NRA in isolated etiolated leaves of *Zea mays* by cytokinins in the presence of nitrate was described by RAO *et al.* (1984).

Cytokinins and gibberellic acid alone and in combination may substitute for light required for induction of NRA in leaves of *Nicotiana rustica*. Changes of NRA during the day and night and the shift in the daily course of NRA from summer to winter type are probably dependent on the ratio of cytokinins and gibberellins (ROTH-BEJERANO and LIPS 1970). Cytokinins also cause a phase-shift of daily peaks of NRA in leaves of *Capsicum annuum* (STEER 1976). However, it is not known if these changes are a result of translocation or synthesis of cytokinins *in situ*, hydrolysis of cytokinin conjugates or changes of cell membrane permeability. In contrast to *Nicotiana* shoots, the light is required for the induction of NRA in isolated shoots of *Oryza sativa* even in the presence of gibberellic acid and cytokinin (GANDHI and NAIK 1974). The aim of this communication is to compare the effect of cytokinins and nitrate on NRA in different plant materials.

MATERIAL AND METHODS

Changes of NRA were studied using explanted stem pith of *Brassica oleracea* var. *medullosa* TELLG. cv. Krasa, intact seedlings of *Triticum aestivum* cv. Vala and *Hordeum vulgare* cv. Rubin and excised cotyledons of *Cucumis sativus* cv. Palava.

Cylinders 7 mm in diameter were cut off from the stem pith parenchyma of *Brassica* using corkborer and divided into pieces 3 mm high. Sets of six randomly selected explants were placed on 30 ml of medium solidified with 0.8 % (m/v) agar and containing 10 % sucrose (m/v). This control medium was supplemented with the following tested substances: nitrate (0, 5, 10, 20, 40 and 60 mM KNO_3), kinetin (0, 5, 10, 20, 30 and 40 μM) and nitrate plus kinetin in different concentrations (see Fig. 1). Tissues were cultivated under sterile conditions at 25 °C in darkness for two days and then collected for analysis.

Seeds of *Triticum aestivum* and *Hordeum vulgare* were germinated in Petri dishes on wet filter paper at 25 °C in darkness. Three days old seedlings were transferred into vermiculite soaked with Hoagland's nutrient solution No. 3 with (+N) or without (−N) nitrogen supplemented with micronutrients according to ARNON (solution A_4), 5 μg Fe^{2+} l^{-1} as Fe EDTA and 10 μg Mo l^{-1} (ARNON 1938, see LAŠTŮVKA and MINÁŘ 1967). Leaves of plants from the two variants (+N and −N) were sprayed daily from 3rd to 7th day with 0.2 % Tween 80 (v/v). The seedings were grown at 24–29 °C at day (1 500 1x)/night period 16 h/8 h.

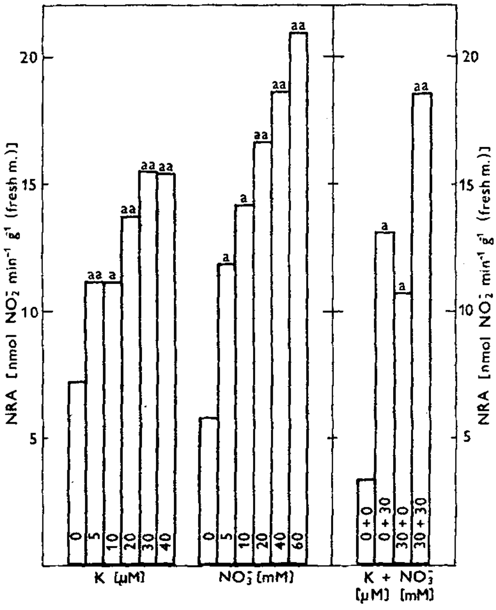

Fig. 1. Nitrate reductase activity (NRA) in stem pith explants of kale cultivated on medium containing different concentrations of kinetin (K), nitrate (NO_3^-) and kinetin plus nitrate (K + NO_3^-) for two days. Significance of differences at $P_{0.05}$ and $P_{0.01}$ are signed a and aa, respectively.

Seeds of *Cucumis sativus* were germinated at 25 °C on wet filter paper. After 6 days the cotyledons were removed, seedlings were rinsed with sterile distilled water and sets of 10 cotyledons were placed into sterile 12 cm Petri dishes on filter paper soaked with distilled water (control) or solution of 100 mM KNO_3, 100 μM BAP or 100 mM KNO_3 plus 100 μM BAP. NRA was estimated after two days of cotyledon cultivation either under illumination (3 500 lx) or in darkness at 25 °C.

Plant material was homogenized at 4 °C in 50 mM Tris-HCl, pH 8.0 (TBS) using tissue : buffer ratio 1 :2.5 (m/v) for kale explants, 1 :4 (m/v) for leaves and roots of wheat and barley (buffer supplemented with 6 mM mercaptoethanol) and 1 :4 (m/v) for cucumber cotyledons. Homogenates were centrifuged at 14 000 *g* for 20 min at 4 °C.

NRA was estimated using *in vitro* method (WRAY and FILNER 1970, GAUDINOVÁ 1983). The activity is expressed in nmol of nitrite formed after 1 min or 1 h incubation by extract corresponding to g of fresh mass (nmol NO_2^- h^{-1} [or min^{-1}] g^{-1} fresh mass). Experiments were 3 to 4times repeated and the results were evaluated by analysis of variance and *t*-test.

RESULTS

The dependence of NRA on concentration of exogenous kinetin (K) and nitrate was estimated after two-day exposure of kale explants to the two agents. In contrast to the nitrate this dependence was not linear in the case of K (Fig. 1). Concentrations 30 μM K and 30 mM nitrate and their combinations were chosen for further experiments. Combined treatment with K and nitrate exhibited synergistic effect (Fig. 1).

NRA was increased in leaves of intact wheat seedlings after spraying with BAP (Fig. 2A). This increase was higher in plants grown without nitrogen (by 57 %) as compared to seedlings irrigated with nitrate-containing solution (by 22 %). Treatment with BAP decreased NRA in roots. This decrease was greater in plants supplied with nitrogen (by 37 %) as compared with seedlings grown without nitrogen (by 22 %).

Similar increase of NRA after spraying with BAP was found in leaves of intact barley seedlings (Fig. 2B). This increase was even more significant in plants grown without nitrogen (by 202 %) in comparison to nitrate-supplied seedlings (by 9 %). NRA in roots was also decreased after application of BAP – more in plants supplied with nitrogen (by 59 %) than in those grown in its absence (by 34 %).

The analysis of variance has shown that some differences (especially in the case of

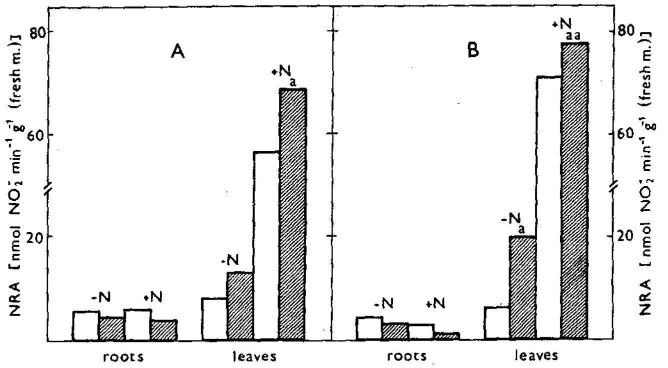

Fig. 2. Nitrate reductase activity (NRA) in 10-day-old wheat (A) and barley (B) seedlings sprayed daily from 6th to 9th day with 100 μM 6-benzylaminopurine in 0.2 % (v/v) Tween 80 (hatched columns) and 0.2% (v/v) Tween 80 alone (open columns). Seedlings were grown in Hoagland's nutrient solution containing (+N) or lacking (−N) nitrate. Significance of differences is signed as in Fig. 1.

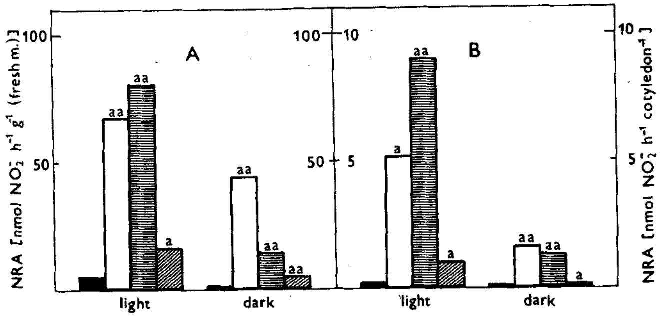

Fig. 3. Nitrate reductase activity (NRA) in excised cucumber cotyledons incubated on filter paper soaked with distilled water (black columns), 100 mM nitrate (open columns), 100 μM 6-benzylaminopurine (obliquely hatched columns) and 100 mM nitrate plus 100 μM 6-benzylaminopurine (horizontally hatched columns) on light and in dark for two days. NRA is expressed per g of fresh mass (A) and per cotyledon (B). Significance of differences is signed as in Fig. 1.

kale explants) were not statistically significant. However, the general trends were always the same.

DISCUSSION

Cytokinins alone or in combination with substrate increase NRA in many plant species, especially when nitrate-induced NRA is not repressed by NH_4^+.

NRA was increased after application of cytokinins in stem pith explants of kale, wheat and barley seedlings and excised cucumber cotyledons. The presence of nitrate is sometimes essential for induction of NRA (RAO *et al.* 1984). However, in some cases NRA is also induced by cytokinins alone (BORRISS 1967, KUZNETSOV *et al.* 1979, HÄNISCH TEN CATE and BRETELER 1982, GZIK and GÜNTHER 1984). According to some authors increase of NRA by cytokinins is accompanied by accumulation of nitrate (SHARMA and SOPORY 1987) while others have found a moderate decrease of nitrate uptake (RAO *et al.* 1984). Induction of NR synthesis by cytokinins probably does not involve substrate induction of NRA as cytokinin may induce NRA under conditions of a complete nitrate depletion (KUZNETSOV *et al.* 1979).

The present results have shown that light is not neccessary for the induction of NRA by kinetin. However, it increased nitrate-induced NR. Some authors found that light is essential for the induction of NRA by cytokinins in *Nicotiana rustica* and *Trigonella f.-g.* (ROTH-BEJERANO and LIPS 1970, RIJVEN and PARKASH 1971) while others reported that it is without effect in *Beta vulgaris* and *Chenopodium album* (GZIK and GÜNTHER 1984).

Induction of NRA by kinetin in wheat plants was reported by KOHL *et al.* (1975). According to present results the changes of NRA in wheat seedlings after treatment with BAP, *i.e.*, the increase of NRA in leaves and its decrease in roots (Fig. 2A) correspond to those found in barley (Fig. 2B). Changes of NRA in barley induced by BAP (Fig. 2B) are in agreement with the results of KARANOV and PAVLOVA (1976), KOHL *et al.* (1975) and especially of PAVLOV *et al.* (1987), who studied the effect of nitrogen nutrition on NRA. Similar results obtained ZHOLOBAK (1986) for pea plants.

Induction of NRA in excised cucumber cotyledons was described by KNYPL (1973, 1979). The induction by BAP was higher in light. The additive effect of nitrate and BAP indicates that cytokinin (BAP) and nitrate induce NRA by different ways (KENDE *et al.* 1971). Increase in NRA in present experiments was higher when calculated per cotyledon than per g fresh mass probably due to the higher rate of cotyledon fresh mass increase as compared to the increase of NRA. Similar differences were also found by KNYPL (1973).

How long lasts the effect of cytokinin on NRA after its application? Increased NRA in stem pith explants of kale was present for 7 days after cytokinin treatment (GAUDINOVÁ 1988), while in embryos of *Agrostemma githago* NRA declined already after 16 to 48 h. However, in *A. githago* NRA is induced only in certain stages of seed development (KUZNETSOV and LITONOVA 1985).

Most publications deal with substrate induction of NR and its regulation. Much less data are available on its hormonal induction. Information so far available indicates that cytokinins and nitrate induce NRA by different ways (KENDE *et al.* 1971, KUZNETSOV *et al.* 1979) and that induced NRA is most probably the same enzyme (DILWORTH and KENDE 1974) and not two different isozymes. According to KUZNETSOV *et al.* (1979) it is possible that there are two different genes for induction of NR, one of which is activated by nitrate while the other by cytokinin. The same authors also speculate about possible tissue specificity assuming that some tissues contain genes regulated by cytokinins while genes in other tissues are regulated by nitrate or that the same single gene is regulated by both agents in each cell. Results presented here about induction of NRA in explanted kale stem pith show that if the two different genes for NR exist they are not tissue specific, at least in kale. The most recent findings indicate that cytokinin induces synthesis of NR apoenzyme which is activated by nitrate (ZHANG *et al.* 1987).

One way in which plant growth regulators affect plant development is probably their inductive effect on synthesis of enzymes limiting the rate of developmental processes (KUZNETSOV and LITONOVA 1985). There are indications that relative concentrations of different growth regulators are essential in this process while the concentration of one single hormone or one group of plant hormones is less important (ROTH-BEJERANO and LIPS 1970). Activity of NR and probably of some other key enzymes is regulated by multiple hormonal system. Where cytokinins and nitrate exhibit additive effect on NRA the hormone may also accelerate the substrate induced process.

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BOOK REVIEW

PINFIELD, N. J., STOBART, A. K. (ed.): PLANT LIPIDS: TARGETS FOR MANIPULATION. – British Plant Growth Regulator Group, Monogr. No. 17, Parchments (Oxford) Ltd., Bristol, 1988. 110 pp.

The seventeenth Monograph published by the British Plant Growth Regulator Group is devoted to a highly actual theme: plant lipids. It consists of the following contributions: Molecular structure of fatty acid synthesising enzymes from developing seeds of oil seed rape (Slabas, A.R. *et al.*); The biosynthesis of triacylglycerols in oil – seeds with a perspective view on the role of plant growth regulators (Griffiths, G. *et al.*); Lipid body (oleosome) formation and compartmentation of storage fats in oil crops (Theimer, R. R.) – abstract only; The use of tissue culture in the improvement of oil crops (Jones, L. H.); Environmental effects on fatty acid quality (Mazliak, P.); Control of lipid synthesis: Examples of herbicide and auxin effect (Hardwood, J. L.); Regulation of sterol production and the effects on plant cell growth (Goad, L. J. *et al.*), and abstracts of three posters. The first two contributions describe the synthetic pathways for fatty acids and triacylglycerols. Several enzyme components have been purified and their amino acid sequence determined. The fourth contribution gives an important survey of possible use of *in vitro* techniques – clonal propagation, somaclonal variation and mutagenesis, *in vitro* selection, somatic hybridization, embryo rescue, use of haploids and genetic transformation – for the improvement of oil crops. The last three contributions deal with the effect of various environmental (temperature, water) and chemical (IAA, herbicides, paclobutrazol) factors on lipid synthesis.

The publication appeared in the standard small format, printed by offset as is usual with proceedings. It brings very useful information on lipid synthesis and content in oil crops and also some ideas for oil crop improvement.

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