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Investigation on the Role of Nodule Cytokinins in Regulation of Nitrate Reductase Activity of *Phaseolus mungo* (L.)

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Abstract. The effects of some nodular cytokinis, zeatin (Z), zeatin riboside (ZR), N^6 (Δ^2-isopentenyl) adenine (IPA), and N^6 (Δ^2-isopentenyl) adenosine (IPAS) on nitrate reductase (E.C 1.9.6.1) activity of root nodules of *Phaseolus mungo* were investigated. The cytokinis were also tested for their effect on nitrate uptake by nodules. The results show that IPAS is the most effective of all the four cytokinins tested. Z and IPA, which caused higher *in vivo* activity than ZR and IPAS, stimulated uptake of nitrate by nodules. The other two (ZR and IPAS) while inhibiting uptake showed greater *in vitro* activity than Z and IPA. It may be concluded that some cytokinins, in addition to their direct effects on the enzyme, may increase the substrate availability to it, whereas others may have only an direct effect on the enzyme activation or degradation.

There are clear indications of the involvement of cytokinins in nodule initiation and growth (Puppo et al. 1974, Syono and Torrey, 1976), but the specific role of different cytokinins, present in nodules, in nodulation is not well understood. We have recently isolated four cytokinins from root nodules of *Phaseolus mungo* (Jaiswal et al. 1981a,b), and, using authentic samples, have shown their relative significance in nodule initiation and growth (Jaiswal, 1981). Besides being important for cell division and growth, cytokinins are also known to affect enzyme activity (Tavares and Kende 1970, Huang 1977, Kuznetsov

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et al. 1979), but the literature lacks information involving studies with nodule cytokinins. That they might also be associated with the functioning of nodules through their effects on relevant enzyme can thus be a real possibility. Functionally the nodules are mainly concerned with the fixation of molecular nitrogen through the action of the enzyme nitrogenase. This enzyme has recently been shown to be variously affected by nodular cytokinins (Jaiswal et al. 1982, 1983). Nodules are also involved, though not exclusively, in the utilization of nitrate. The concerned enzyme is nitrate reductase, whose presence in the nodules adds to the efficiency of their nitrogen-utilizing systems.

Thus, in the present paper the relative significance of different cytokinins identified in nodules has been investigated with respect to their effects on nitrate reductase activity in nodules of *Phaseolus mungo*.

Materials and Methods

Growth of Plants

Surface-sterilized (with 80% ethanol) seeds of *Phaseolus mungo* (type 9) after thorough washing with distilled water were soaked in solutions of various cytokinins of different concentrations for 8 h. Seeds soaked in distilled water for 8 h were used as a control. The seeds were sowed in acid-sterilized sand contained in earthenware pots, and an inoculum of *Rhizobium leguminosarum* in yeast extract, mannitol, and agar medium (Jaiswal 1981) was added to the sand. The growing plants were supplied with half-strength Hoagland's nurient solution (Jaiswal 1981) on alternate days.

Mode of Treatment

The nodules used for enzyme assay were subjected to cytokinin treatments in two different ways: (1) Nodules were obtained from 23-day-old plants raised from seeds, and treated with different concentrations of cytokinins. (2) Nodules obtained from 23-day-old control plants were treated directly for 3 h with cytokinins of different concentrations containing 5.0 mM KNO_3.

The Assay

The nitrate reductase (E.C. 1.9.6.1) activity was measured, using both *in vivo* and *in vitro* methods, in the nodules obtained from upper (4-cm) portion of primary roots. For the *in vivo* assay, the method described by Harper and Hageman (1972) with slight modifications was adopted. In this, 100 mg of nodules was incubated for 0.5 in 5 ml of the reaction mixture, which contained 0.5 ml of 0.1 M KNO_3, 0.5 ml 5% n-propanol, and 4.0 ml of potassium phosphate buffer (pH 7.4). After 30 min, 1 ml of the reaction mixture was taken out (D_1). The incubated nodules were extracted in phosphate buffer. The extract was centrifuged at 20,000*g*, and the supernatant was used as D_2. In 1 ml each of D_1 and D_2, 1.0 ml of sulphanilamide (1% in 1 NHCl) and 1.0 ml of

0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride were added. The optical density of the pink color so developed was read at 540 nm. The amount of nitrate produced was calculated with the help of a calibration curve drawn by using $NaNO_2$ as standard, and NR activity was expressed as n mol NO^{-2} h^{-1} g^{-1} fresh weight of tissue.

The method of Hageman and Flesher (1960) with slight modification was followed for the *in vitro* assay. The nodules were thoroughly washed, dried with filter paper, weighed, and ground in a chilled glass mortar. The grinding medium contained 0.1 M phosphate buffer (pH 7.4), 5 mM cystein, and 0.3 mM EDTA. Four milliliters of cold grinding medium was added for each gram of the tissue. The supernatant obtained after centrifugation (at 20,000*g* for 15 min) of the homogenate was used to assay the enzyme activity. The reaction mixture contained 0.5 ml (0.1 M) phosphate buffer (pH 7.4), 0.5 ml enzyme extract, 0.5 ml of 0.1 M KNO_3, 0.5 ml of 1 mM NADH (reduced), and, where appropriate, 1.0 ml of cytokinins maintained at different concentrations. The vials containing the reaction mixture were incubated for 30 min at 30°C in an incubator. The activity of the enzyme was expressed in terms of nitrite produced which was estimated as described in the case of the *in vivo* assay.

Determination of Nitrate Uptake

Nitrate uptake by detached nodules obtained from plants raised from untreated seeds was measured using the method of Goldsmith et al. (1973) with slight modification. The nodules were washed with 0.1 M potassium phosphate buffer (pH 6.0); 100 mg of nodules were dipped in a solution containing 4.0 ml of phosphate buffer (containing 0.2 mM KNO_3) and 2.0 ml of different cytokinins maintained at different concentrations. For control, cytokinins were replaced by distilled water. The sets were kept at 30°C for 2 h. Samples of 4.5 ml each were then taken from the solution to which 0.5 ml of concentrated HCl was added. The absorbancy was read at 210 nm by using Beckman DU-2 spectrophotometer. In blanks only phosphate buffer and HCl were used.

Possible leakage of UV-absorbing materials from the nodules was corrected by substracting the OD of the set incubated without nitrate from the OD of the set incubated with NO_3. The nitrate uptake was calculated with the help of standard curve, and percent effect on nitrate uptake of cytokinins was calculated.

All experiments were done in triplicate and repeated twice.

Results

In Vivo *Nitrate Reductase Activity*

In nodules obtained from plants raised from treated seeds, NR activity, while following a similar increasing trend with age as in control (data not included), was stimulated by all the four cytokinins except zeatin riboside (ZR) and N^6 (Δ^2-isopentenyl) adenine (IPA) at 5.0 ppm, which showed almost no effect. On the day (23rd) of optimum activity, the highest (36.6%) stimulation was ob-

Table 1. Nitrate reductase activity (nmole NO_2^- × 10^2 h^{-1} g^{-1} F.W.) in nodules of *P. mungo* raised from seeds treated with various concentrations of zeatin, zeatin riboside, N^6 (Δ^2-isopentenyl) adenine, and N^6 (Δ^2-isopentenyl) adenosine.

	Z		ZR		IPA		IPAS	
Concentration ppm	Enzyme activity	% Change	Enzyme activity	% Change	Enzyme activity	% Change	Enzyme activity	% Change
0.0	28.5 ± 0.82	—	28.5 ± 0.82	—	28.5 ± 0.82	—	28.5 ± 0.82	—
2.5	31.5 ± 0.46	+10.5	35.8 ± 0.70	+25.6	35.0 ± 0.96	+22.8	38.9 ± 1.01	+36.6
5.0	34.4 ± 0.64	+20.7	28.0 ± 0.32	−1.7	29.4 ± 0.39	+2.1	35.2 ± 0.56	+23.5

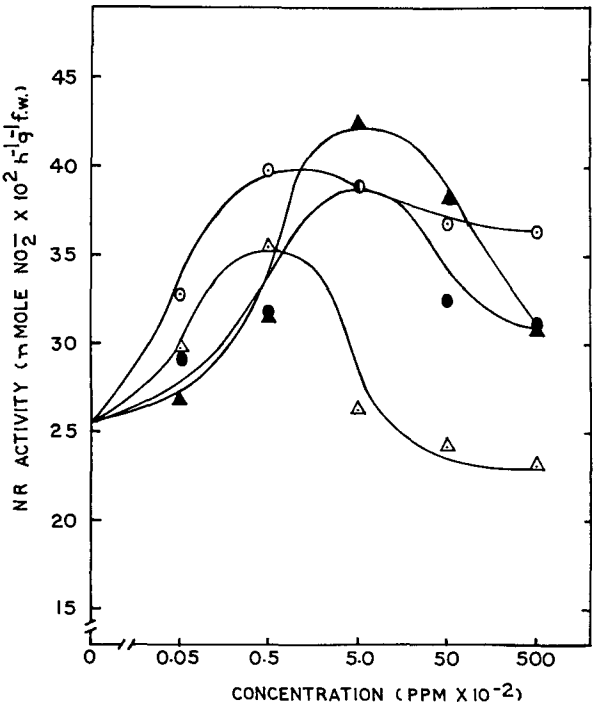

Fig. 1. Nitrate reductase activity in detached root nodules (obtained from plants raised from untreated seeds) directly treated with different concentrations of Z (○——○), ZR (●——●), IPA (△——△), and IPAS (▲——▲) in *P. mungo*.

tained with N^6 (Δ^2-isopentenyl) adenosine (IPAS) at 2.5 ppm. Its higher concentration was relatively less stimulatory. At their respective optima, the ribosides of Z and IPA were more stimulatory than zeatin (Z) and IPA (Table 1).

In the nodules treated directly with cytokinins, NR activity was affected in the same way as in case of nodules obtained from plants raised from pretreated seeds. All the cytokinins showed concentration-dependent stimulation except IPA, which proved inhibitory at higher concentrations. Relative stimulation at the respective optima were 65.2, 54.1, 51.5, and 37.9% by IPAS, Z, ZR, and IPA, respectively (Fig. 1).

In Vitro *NR Activity*

Cytokinin treatment of the cell-free extracts from nodules of the plants raised from untreated seeds showed similar results as obtained with nodules directly treated *in vivo* (Figs 1, 2). With Z and IPA the *in vitro* assay (in extracts treated with cytokinins), however, showed lesser stimulation (20.6% and 25.3%) at their respective optimal concentration than obtained in case of assay with whole nodules. On the other hand, enhancement with IPAS and ZR, at their respective optima, showed considerable improvement (76.6% and 62.5%) over that obtained with the whole nodule (Figs. 1, 2).

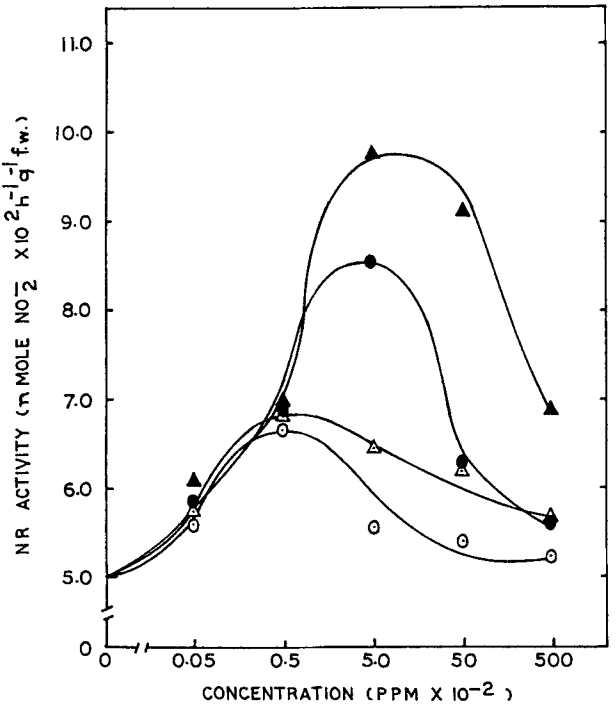

Fig. 2. Nitrate reductase activity in cell-free extracts of nodules (obtained from plants raised from untreated seeds) in the presence of different concentrations of Z (○——○), ZR (●——●), IPA (△——△), and IPAS (▲——▲) in *P. mungo*.

Uptake of Nitrate

Correct assessment of the NR activity measured by the *in vivo* method is complicated from the facts that (1) the method does not take into account the question of permeability barrior of the tissue to NO_3^-, (2) the enzyme is substrate-inducible, and (3) cytokinins are known to alter permeability of plant tissue. It therefore seemed important to study their (cytokinins) affect on nitrate uptake.

Cytokinin treatments variously affected uptake of NO_3^- in detached nodules (Table 2). Zeatin at all the concentrations stimulated the uptake, and the maximum increase obtained was 20.8% at 5.0×10^{-2} ppm. N^6 (Δ^2-isopentenyl) adenine was also stimulatory at lower concentrations (maximum increase by 12.5%), but it slightly inhibited uptake at higher concentrations. Ribosides of Z and IPA proved inhibitory to nitrate uptake, causing maximum inhibition by ca 8.0% at 500×10^{-2} ppm (Table 2).

Discussion

While the present investigation has been mainly concerned with the study of role of nodule cytokinins in regulation of nitrate reductase activity, preliminary

Table 2. Effect of different concentrations of zeatin, zeatin riboside, N^6 (Δ^2-isopentenyl) adenine, and N^6 (Δ^2-isopentenyl) adenosine on nitrate uptake by nodules of *P. mungo* (percent of change).

Cytokinin concentration ppm × 10^{-2}	Z	ZR	IPA	IPAS
0.05	+14.1	−2.1	+12.5	+2.2
0.5	+12.5	−4.2	+6.2	—
5.0	+20.8	−6.4	−1.2	−4.3
50	+18.7	−6.4	−2.3	−6.5
500	+16.6	−8.5	−3.5	−8.7

studies on seedling growth have been important in demonstrating that the different cytokinins used had no differences in their relative effects on seedling growth. Thus, the response observed with respect to enzyme activity can be safely considered as having been caused independently of the effects of cytokinins on growth and general metabolism of the plant (Jaiswal 1981).

Functionally, the main activity of nodules is nitrogenase-dependent nitrogen fixation. However, another enzyme, not exclusive to nodules, that is important for utilization of nitrogen nutrients is nitrate reductase. A study of the responses of this enzyme to treatments of different cytokinins should thus be significant from a functional standpoint. In studying the responses of nitrogenase it was observed that of the four cytokinins used some showed concentration-dependent stimulation whereas others proved inhibitory, and the effects were independent of their effects on the growth of rhizobial population (Jaiswal et al. 1982). As regards nitrate reductase, all the four cytokinins proved stimulatory both for directly treated nodules and for those obtained from plants raised from treated seeds. In both the cases, IPAS seemed more effective than other cytokinins. Relative to the *in vivo* assay, the *in vitro* assay showed much higher activity with ZR and IPAS than with Z and IPA. In this context it is interesting to note that the cytokinins (Z and IPA) that caused relatively higher *in vivo* activity also stimulated (at relevant concentration) uptake of nitrate by nodules, whereas the other two (ZR and IPAS) inhibited it. This variation between *in vivo* and *in vitro* assays would thus seem to suggest that in the assay method followed, assay of *in vivo* activity can be affected to a large extent by the rate of nitrate uptake. This is understandable because, besides being the substrate, nitrate is also known to induce the synthesis of enzyme (Beevers and Hageman 1969, Ashley et al. 1975, Chantorotwong et al. 1976). The direct effect of cytokinins on the enzyme in addition to their effects on nitrate uptake is clearly demonstrated from the observed stimulation of the activity in the *in vitro* system. Many enzymes, including nitrate reductase, have earlier been shown to be stimulated by different cytoknins (Penner and Ashton 1967, Samedova et al. 1977, Khan 1979, Kuznetsov et al. 1979, Jaiswal et al. 1983). Stimulation of enzymes in general by cytokinins is explainable from the fact that they stimulate RNA synthesis (see Audus 1972, Kuznetsov et al. 1979), affect protein synthesis (Maab and Klambt 1979, de Boer and Feirabend 1978), or inhibit protein degradation (Tavares and Kende 1970, Shibaoka and Thimann 1970).

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