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# Nitrate reductase activity and uptake of nitrate and oxygen as affected by nitrate supply to detached maize organs

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## Abstract

Supply of 1, 2, 5, 10 or 20 mM nitrate to detached roots, scutella or shoots from 5- to 6-d-old *Zea mays* L. seedlings increased *in vitro* nitrate reductase (NR) activity in all the organs and NADPH specific NR (NADPH:NR) activity in roots and scutella but not in the shoots. Usually 2 to 5 mM nitrate supported maximum enzyme activity, the higher concentration did not increase it further. The protein content in the roots, scutella and shoots increased up to 5, 2 and 20 mM medium nitrate, respectively. Nitrate uptake also increased with increasing nitrate concentration in roots and shoots, but it increased only slightly in the scutella. In both roots and scutella, methionine sulfoximine had no effect, while cycloheximide and tungstate abolished nitrate induced NADH:NR activity completely and NADPH:NR partially. Methionine sulfoximine increased nitrate uptake by roots and scutella slightly, but other inhibitors had no effect. The depletion of dissolved oxygen from the medium was lower in the presence of nitrate than in its absence or in the presence of ammonium, especially in the scutella.

*Additional key words*: dissimilatory NR, substrate induction, *Zea mays*.

## Introduction

NAD(P)H bispecific nitrate reductase (NR E.C. 1.6.6.2) has been detected in many species including soybean, rice, barley and maize, although the physiological significance of the isoform is not known (Srivastava 1992). The isoform uses either NADH or NADPH as reductant, the activity being several folds higher with NADPH than with NADH (Streit *et al.* 1985, 1987). Although this isoform is usually present along with NADH:NR (E.C. 1.6.6.1) isoform, in tree species *Betula pendula*

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*Abbreviations*: NADH:NR - NADH specific nitrate reductase, NADPH:NR - NADPH specific nitrate reductase.

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(Friemann *et al.* 1991) and in *Erythrina senegalensis* (Stewart and Orebamjo 1979), only this isoform is present. The two isoforms of NR are apparently the products of two different genes (Warner *et al.* 1987) and hence a differences in their regulatory aspects may be expected. With respect to the effect of nitrate supply, in soybean, while NADH:NR is readily induced, NADPH:NR has constitutive expression (Streit *et al.* 1987). On the other hand, NADPH:NR in barley roots is nitrate inducible (Sueyoshi *et al.* 1995). In maize roots, the soluble NR is not detected in the absence of nitrate (De Marco *et al.* 1994).

Thus, there appears to be species related differences in the response of NADPH:NR to nitrate. In the present study, we have examined the responses of NADPH:NR versus that of NADH:NR and of nitrate and oxygen uptake to nitrate supply in detached roots, scutella and shoots of maize seedlings with a view *1*) to demonstrate organ specific differences in the response of two isoforms, if any, and *2*) to link nitrate uptake with nitrate reduction and assimilation.

## Materials and methods

**Plants:** Seeds of *Zea mays* L. cv. Ganga safed-2, purchased from National Seed Corporation, New Delhi, were surface sterilised with 1 % bleaching powder and then washed throughly with sterile distilled water. They were then planted on filter paper in 15 cm Petri plates moistened with half strength Hoagland's solution containing no nitrogen. The seedlings were raised on laboratory benches for 5 to 6 d in diffused light (about 27 W $m^{-2}$) at 25 ± 3 °C.

The roots, scutella and shoots from uniformly grown seedlings were removed and treated further for enzyme induction and nitrate or oxygen uptake.

**Enzyme extraction and assay:** The enzyme preparation was obtained by extracting the freshly harvested organs in cold extraction medium in ratio 1:4 (m/v) containing 1 mM EDTA, 5 mM cysteine and 0.5 % casein in 0.1 M phosphate buffer of pH 7.5. The homogenate was centrifuged (24 000 *g*) for 20 min at 0 to 4 °C. The supernatant was used as enzyme preparation.

The activity of NADH:NR in the extract was assayed according to Srivastava and Ormrod (1984). The NADPH:NR was assayed by the procedure of Dailey *et al.* (1982). The assay mixture contained 1.2 $cm^3$ of 0.1 M phosphate buffer of pH 6.5, 0.2 $cm^3$ of 5 mM $KNO_3$, 0.3 $cm^3$ of 2 mM NADPH and 0.3 $cm^3$ of enzyme preparation in a total volume of 2.0 $cm^3$. The reaction was carried on at 28 ± 1 °C for 30 min and the nitrite produced was determined colorimetrically. NADH:NR activity at this concentration of nitrate and at this pH was only 9 to 21 % of the NADPH:NR activity.

**Protein measurement:** Total protein content in fresh material was measured by the method of Lowry *et al.* (1951). One g of fresh sample was extracted with 10 % TCA. The residue was dissolved in 0.1 M NaOH, and the protein content was determined by using Folin-phenol reagent. Bovine serum albumin was used as a standard.

**Nitrate uptake:** The excised organs were removed and washed out after 24 h of incubation in half strength Hoagland's solution containing desired concentration of nitrate. Nitrate content of the medium including the wash out was measured by *Orion* ion concentration meter (*Model 290A, Orion Research Incorporated*, Boston, USA.) equipped with nitrate electrode. The nitrate content of the medium before incubation was also measured. Difference between initial and final measurements was taken as nitrate absorbed by the organs.

**Oxygen uptake:** The depletion of dissolved oxygen by the detached organs was measured also by an *Orion* ion concentration meter equipped with an oxygen electrode (*Model 97-08-99*). The incubation medium without any excised organ served as control.

In each case, 2 to 3 drops of chloramphenicol (1 mg $cm^{-3}$) was added in the incubation medium to prevent bacterial contamination.

## Results

**Nitrate reductase activity:** NADH:NR activity in the roots and shoots increased almost linearly up to 2 mM nitrate in the medium (Fig. 1*A*). Further increase in nitrate concentration, caused only a slight increase in enzyme activity. In scutella also, the enzyme activity increased at higher rate between 0 and 2 mM nitrate, as compared to between 2 and 20 mM concentration of nitrate. NADPH:NR activity, which was detected only in roots and scutella and not in the shoots, increased almost linearly upto 5 mM nitrate, with very little increase thereafter (Fig. 1*B*).

**Protein content:** Increase in protein content varied according to the organ. In roots, it increased upto nitrate concentration 5 mM, with very little increase between 5 and 20 mM (Fig. 1*C*). In scutella, it increased up to 2 mM and remained almost unchanged between 2 and 20 mM. In shoots, however, the protein content increased upto the highest concentration of nitrate used, although the rate of increment was higher between 0 and 2 mM as compared to between 2 and 20 mM.

Table 1. The effect of inhibitors on NADH:NR and NADPH:NR activity [nmol(nitrite) $g^{-1}$(f.m.) $s^{-1}$] in detached roots and scutella from 5- to 6-d-old maize seedlings incubated in half strength Hoagland's solution containing 10 mM nitrate for 24 h in light. Inhibitors were added or nitrate was excluded from the medium as indicated. Values are mean ± S.D. of 6 replicates.

| Treatment | Root | | Scutellum | |
|---|---|---|---|---|
| | NADH:NR | NADPH:NR | NADH:NR | NADPH:NR |
| Control | 0.58 ± 0.02 | 0.30 ± 0.02 | 1.10 ± 0.02 | 0.53 ± 0.02 |
| Methionine sulfoximine (1 mM) | 0.62 ± 0.09 | 0.31 ± 0.03 | 1.07 ± 0.03 | 0.49 ± 0.03 |
| Sodium tungstate (0.1 mM) | 0.22 ± 0.03 | 0.22 ± 0.01 | 0.34 ± 0.06 | 0.28 ± 0.02 |
| Cycloheximide (0.2 mM) | 0.23 ± 0.03 | 0.20 ± 0.01 | 0.41 ± 0.03 | 0.34 ± 0.03 |
| Without nitrate | 0.19 ± 0.01 | 0.17 ± 0.01 | 0.36 ± 0.02 | 0.19 ± 0.01 |

**Nitrate uptake:** In both roots and shoots, nitrate uptake increased almost linearly with the increase in medium nitrate concentration between 1 and 20 mM (Fig. 1*D*). The uptake by the roots was usually 1.4 to 1.9 folds higher than that by shoots. Nitrate uptake by scutella increased only slightly with the increase in medium nitrate concentration between 1 and 5 mM, but it almost remained unchanged between 5 and 20 mM. At 20 mM nitrate, the amount of nitrate absorbed by scutella was only one third of that absorbed by shoots or roots.

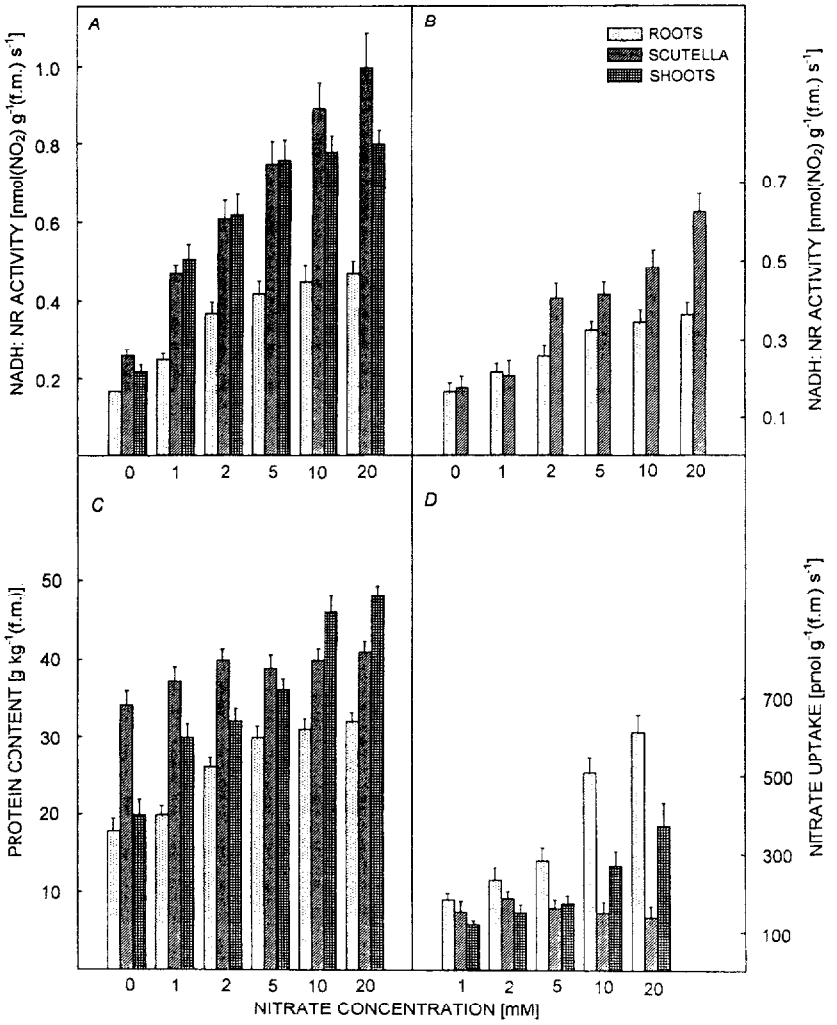


Fig. 1. Effect of different concentrations of nitrate on NADH:NR (*A*) and NADPH:NR (*B*) activities, protein content (*C*) and nitrate uptake (*D*) in detached roots, scutella and shoots incubated for 24 h in half strength Hoagland's solution containing desired concentration of nitrate, in light.

**Effect of enzyme inhibitors:** Methionine sulfoximine had no effect, although, sodium tungstate and cycloheximide abolished NADH:NR induction completely and NADPH:NR partially (Table 1). The protein content was reduced slightly by the cycloheximide (Table 2). Nitrate uptake increased slightly by methionine sulfoximine, while it was unaffected by tungstate or cycloheximide.

Table 2. The effect of inhibitors on protein content [mg $g^{-1}$(f.m.)] and nitrate uptake [pmol(nitrate) $g^{-1}$(f.m.) $s^{-1}$] by detached roots and scutella. Details as in Table 1.

| Treatment | Root | | Scutellum | |
|---|---|---|---|---|
| | protein content | nitrate uptake | protein content | nitrate uptake |
| Control | 29 ± 3.1 | 451 ± 28 | 40 ± 3.0 | 243 ± 14 |
| Methionine sulfoximine (1 mM) | 31 ± 1.7 | 544 ± 42 | 42 ± 2.7 | 289 ± 28 |
| Sodium tungstate (0.1 mM) | 32 ± 2.1 | 463 ± 14 | 40 ± 3.1 | 220 ± 18 |
| Cycloheximide (0.2 mM) | 26 ± 1.5 | 440 ± 24 | 33 ± 1.9 | 231 ± 13 |
| Without nitrate | 17 ± 1.6 | - | 31 ± 2.4 | - |

**Effect of nitrogen on oxygen uptake :** Supply of nitrate inhibited dissolved oxygen uptake as compared to no nitrogen or ammonium supply. This was more apparent in scutella than in roots or shoots (Table 3).

Table 3. Effect of nitrogen suply on dissolved oxygen uptake [pg $g^{-1}$(f.m.) $s^{-1}$] by detached roots, scutella and shoots incubated in half strength Hoagland's solution containing desired nitrogen sources (10 mM) for 20 h in light.

| Nitrogen source | Oxygen uptake | | |
|---|---|---|---|
| | shoots | roots | scutella |
| None | 186 ± 15 | 201 ± 25 | 217 ± 29 |
| Nitrate | 142 ± 14 | 126 ± 10 | 108 ± 22 |
| Ammonium | 175 ± 11 | 178 ± 11 | 189 ± 17 |

## Discussion

The experiments demonstrate that the levels of NADH:NR in roots and scutella of maize seedlings are higher than those of NADPH:NR. Further, the supply of nitrate increases both the isoforms, although the increase in NADH:NR is higher as compared to that of NADPH:NR. It may, therefore, be suggested that NADPH:NR in roots and scutella is partially constitutive and partially inducible. This suggestion is supported by the observation that while tungstate and cycloheximide, the inhibitors of active NR formation (Deng *et al.* 1989) and protein synthesis respectively, abolished the nitrate induced NADH:NR activity, they did so only partially to NADPH:NR, especially in the scutella. As mentioned in the introduction, while NADPH:NR is constitutive in soybean (Streit *et al.* 1987), it is inducible in barley

(Sueyoshi *et al.* 1995). In maize, there is a subcellular differentiation, while plasmalemmal NADPH:NR is constitutive, the cytosolic one is inducible (De Marco *et al.* 1994). Apparently our enzyme preparation from scutella was a mixture of both plasma-lemmal as well as cytosolic and hence both constitutive as well as inducible properties of the isoform were observed. The plasmalemmal NR may be associated with some functions other than the reduction of nitrate, such as acquisition of nitrate or other nutrient ions or in the dissimilation of nitrate (Srivastava 1992). Since the isoform was not involved in nitrate assimilation, it was only partially inducible by the nitrate. Ferrario *et al.* (1995) by using transgenic tobacco plants have demonstrated that constitutive NR had no role in nitrate assimilation.

The inhibitory effect of cycloheximide and of tungstate on NADH:NR or NADPH:NR was as expected. Methioinine sulfoximine an inhibitor of glutamine synthetase, however, did not inhibit activity of either of the isoforms. Apparently the glutamate dehydrogenase pathway, which is believed to be operational in maize roots (Sengar and Srivastava 1990), took care of the ammonium, the potential end product of nitrate reduction in the presence of methionine sulfoximine.

There was not any correlation between nitrate absorption and NR induction (either of the isoforms) specially in scutella. While enzyme activity increased with increasing concentration of nitrate, the nitrate uptake was only slightly increased and only up to 2 mM medium nitrate. As recorded in the present study, ineffectiveness of cycloheximide (Li and Oaks 1993) or of tungstate (Miyagi *et al.* 1992) on nitrate uptake and increase of uptake by methionine sulfoximine (Lee *et al.* 1992) have been reported earlier. The cycloheximide increased nitrate uptake by potato tuber slices (Palmer 1982).

The data provide some scope for discussing the possible physiological role of NADPH:NR in particular and NR in general in maize roots and scutella. If nitrate assimilation was the only function of NADPH:NR, then a positive correlation between the inducibility of the enzyme and the increase in protein content, the eventual product of nitrate asimilation, was expected. However, such a correlation was generally lacking, specially in scutella. Also the nitrate uptake by scutella did not increase much with nitrate supply. Thus, in scutellum which is essentially a storage organ, either of the isoforms of NR might be involved in some functions other than the acquisition and assimilation of nitrate. The scutella are situated deep inside the seed where anaerobic conditions might necessitate the NADPH:NR at least to catalyse the transfer of electrons to an alternate (to $O_2$) acceptor, which is nitrate in this case. In our study, the supply of nitrate suppressed the acquisition of dissolved oxygen from the medium, specially in the scutella. Obviously, the nitrate acted as an alternate electron aceptor and hence the requirement of dissolved $O_2$ was lowered. A dissimilatory role of NR has been suggested in flood tolerant rice plants during annoxia (Garcia-Novo and Crawford 1973). In maize also, the anaerobiosis increases nitrate reduction (Gray and Cresswell 1983, Srivastava unpublished). Also the nitrate receptor protein, reported to be present in plasma membrane, binds with the respiratory inhibitors azide and cyanide (Mertens *et al.* 1995). These aspects are under further investigations, to elucidate the role of NADPH:NR in dissimilatory release of $O_2$.

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