Role of Nitrate and Nitrite in the Induction of Nitrite Reductase in Leaves of Barley Seedlings

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ABSTRACT

The role of NO3− and NO2− in the induction of nitrite reductase (NiR) activity in detached leaves of 8-day-old barley (Hordeum vulgare L.) seedlings was investigated. barley leaves contained 6 to 8 micromoles NO3−/gram fresh weight x hour of endogenous NiR activity when grown in N-free solutions. Supply of both NO3− and NO2− induced the enzyme activity above the endogenous levels (5 and 10 micromolar NO3− and NO2− over a 24 hour period). In NO2−-supplied leaves, NiR induction occurred at an ambient NO3− concentration of as low as 0.05 micromolar; however, no NiR induction was found in leaves supplied with NO3− until the ambient NO3− concentration was 0.5 micromolar. Nitrate accumulated in NO2−-fed leaves. The amount of NO3− accumulating in NO2−-fed leaves induced similar levels of NiR as did equivalent amounts of NO3− accumulating in NO3−-fed leaves. Induction of NiR in NO2−-fed leaves was not seen until NO3− was detectable (30 nanomoles/gram fresh weight) in the leaves. The internal concentrations of NO3−, irrespective of N source, were highly correlated with the levels of NiR induced. When the reduction of NO3− to NO2− was inhibited by WO42−, the induction of NiR was inhibited only partially. The results indicate that in barley leaves NiR is induced by NO2− directly, i.e. without being reduced to NO3−, and that absorbed NO3− induces the enzyme activity indirectly after being oxidized to NO2− within the leaf.

In many plant species, both NR and NiR are induced with either NO3− or NO2− in the ambient solution (9). It was earlier proposed that NO3− was a more specific inducer of NiR since high levels were induced in Lemma (23). In radish cotyledons (11) and bean leaves (22), a sequential induction of NR and NiR by NO3− indicated that NiR was induced possibly by NO2− after its formation from NO3− by NR. Evidence has been presented that ambient NO3− could induce NiR in NR deficient cells of tobacco (17); however, NO2− was not tested as an inducer. Gupta et al. (7) showed that NO3− induced NiR in wheat embryos during the first 12 h of imbibition of seeds. Induction of NiR was not detected during imbibition of seeds in the presence of NO3−.

Tungstate has been used to inhibit the formation of active NR induced by NO3− (25). Although NR induction was largely inhibited by tungstate, NiR formation was not inhibited in wheat embryos (7) or in tobacco cells (13), further indicating that NO3− may induce NiR directly without being reduced to NO2−. Recently, Lahners et al. (15) reported that both NO3− and NO2− induced NiR-mRNA in leaves of maize seedlings. Back et al. (4) reported that NO3− induced spinach NiR-mRNA.

Studies to determine which form of N induces NiR are complicated by several factors. A low endogenous level of NR is often present in plant tissues which may be constitutive or a result of low level contamination of NO3− in the environment in which the plants are grown (8). Since the minimum ambient and internal concentrations of NO3− and NO2− which can induce NiR are not known, low levels of NO2− formed by the endogenous NR might be sufficient to induce NiR over the several h of induction period. This could also be a factor when tungstate is used to inhibit the reduction of NO3− to NO2− by inhibiting the formation of active NR. Our preliminary experiments showed that low levels of NRA were induced in tungstate-fed leaves which might furnish some NO2−. Furthermore, we found in initial studies that NiR induction in leaves was inhibited when the leaves were supplied with 0.5 mM tungstate in the induction solution.

Also important is the fact that NO2− can be oxidized to NO3− in plant tissues (3, 6, 12, 16). Hence, it is important to determine if the test plants are converting NO2− to NO3− in vivo during the induction period. We recently showed that the reduction of NR by the absorbed NO3− was likely caused after its oxidation to NO3−. Likewise, the induction of NiR by absorbed NO3− might also be a result of its oxidation to NO3− within the tissue. Furthermore, NO3− may be present as a contaminant in NO2− solutions (3).

To compare the role of NO3− and NO2−, the induction of NiR was studied as a function of NO3− and NO2− net influx and internal concentration in the leaves. The interaction of these processes on the induction of NiR has not been studied. Evidence is presented that in leaves NO3− is the more likely inducer of NiR even in those supplied NO2−, and the induction of NiR may be regulated by the internal concentration of NO3− in the leaves.

MATERIALS AND METHODS

Plant Culture

Barley seedlings (Hordeum vulgare L. var CM72) were grown in vermiculite and sub-irrigated with N-free one-quarter strength Hoaglund solution (10) as described before

1 This work was supported in part by a grant from the U.S. National Aeronautics and Space Agency (NASA NCC2-99).

2 Abbreviations: NR(A), nitrate reductase (activity); NiR(A), nitrite reductase (activity).
(3). The seedlings were grown for 8 d in a controlled environment growth chamber programmed for 16 h light at 25°C and 8 darkness at 15°C. Relative humidity was maintained at 65 to 70%. Light intensity at the top of the seedling canopy was 400 μmol m⁻²s⁻¹ and was supplied by incandescent and cool white fluorescent lamps. In one experiment (Table I) the seedlings were grown hydroponically in 0.2 mM CaSO₄ solution for 5 d in darkness followed by 3 d in continuous light as described before (1).

### Induction of the Enzyme Activities

Induction is defined as the increase in enzyme activity above the initial endogenous activity (2). The tip 9 cm of 10 leaves weighing about 1 g were placed down in small glass vials containing 10 mL of 0 to 10 mM KNO₃ or NaNO₂ solutions. Sodium salts were used because even the reagent grade KNO₃ contained measurable amounts of NO₂⁻ as a contaminant; NaNO₂ was free of NO₂⁻. Tungstate was supplied as specified in the table. The induction of the enzyme activities was carried out at 25°C and 60% RH under light of 400 μmol m⁻²s⁻¹. Both NR and NiR activities were assayed at various intervals. The treatments were run in duplicates and each experiment was repeated at least twice. In time course experiments, the induction solutions were changed after 12 h.

### Uptake, Accumulation, and in Vivo Reduction of NO₂⁻ and NO₃⁻

At the same time when the enzyme activities were assayed, uptake, concentration, and reduction of NO₃⁻ and NO₂⁻ in the leaves were also determined. Uptake of N was determined by following the depletion from the induction solutions (1, 5). In vivo reduction of NO₂⁻ and NO₃⁻ was determined by subtracting the NO₂⁻ and NO₃⁻ content in the tissue from the total taken up at each assay period (1, 5).

### Preparation of Cell-Free Extracts

The leaves were washed with deionized water and homogenized with cold pestle and mortar in four volumes of the extraction buffer. The extraction buffer contained 0.05 mM Tris-HCl (pH 8.5), 1 mM DTT, 10 μM flavin adenine dinucleotide, 1 μM Na₂MoO₄, 1 mM EDTA, and 10 μM leupeptin (14). The homogenates were centrifuged at 30,000g for 10 min, and the supernatants were assayed for NRA, NiRA, NO₂⁻, and NO₃⁻.

### Enzyme Assays

Enzyme activities were assayed by in vitro methods. The assay medium for NR contained 50 μmol potassium phosphate buffer (pH 7.5), 20 μmol KNO₃, 0.8 μmol NADH, and 0.1 mL extract in a final volume of 2.0 mL. The assays were conducted at 28°C for 15 min. The reaction was terminated by addition of 0.1 mL of 1 M zinc acetate, and excess NADH was oxidized by phenazine methosulfate (21). The NO₂⁻ formed was determined colorimetrically (20).

Nitrite reductase activity was assayed by following the disappearance (reduction) of NO₂⁻ from the assay mixture (18). The assay mixture contained 40 μmol potassium phosphate (pH 7.5), 0.5 μmol KNO₂, 0.04 mg methyl viologen, and 0.1 mL extract in a total volume of 1.1 mL. The reaction was started by addition of 0.2 mL of Na₂S₂O₄ solution (8 mg/mL) in 0.1 mL NaHCO₃ and was terminated after 15 min by vigorously mixing the content of the assay tube on a vortex mixer until the methyl viologen was completely oxidized (for 10–15 s). Residual NO₂⁻ in the assay tubes was determined colorimetrically.

### NO₃⁻ and NO₂⁻ Analysis

Nitrate was determined spectrophotometrically at 210 nm following separation by HPLC on a Partisil-10-SAX anion exchange column (24). Nitrite was determined colorimetrically by addition of 2 mL of a 1:1 mixture of 1% (w/v) sulfanilamide in 1.5 N HCl and 0.01% (w/v) naphthyl ethylenediaminedihydrochloride (20). All the results are reported on the basis of fresh weights of the leaves.

## RESULTS

### Induction of NiR

Figure 1 shows the time course of the induction of NiR at different substrate concentrations of NO₂⁻ and NO₃⁻. Barley leaves contained 6 to 8 μmol NO₂⁻ g⁻¹ fresh weight h⁻¹ of endogenous NiRA whether grown in vermiculite (washed and unwashed) or hydroponically with a N-free nutrient solution or distilled water (M. A. W. Aslam and R. C. Huffaker, unpublished results). Nitrite reductase activity was increased upon supply of NO₂⁻ or NO₃⁻ (Fig. 1). The enzyme activity induced by exogenously supplied NO₂⁻, at all concentrations, was about one-half of that induced by similar concentrations of NO₃⁻.

![Figure 1](https://plantphysiol.org)

**Figure 1.** Time course of the induction of NiRA in detached leaves supplied with different levels of NO₂⁻ (A) or NO₃⁻ (B) in the induction solutions. Detached leaves from 8-day-old seedlings, grown in vermiculite, were placed in 10 mL of the induction solutions containing 0 (□), 0.2 (●), 1 (○), 2 ([triangle]), 5 (△), and 10 (▲) mM NO₂⁻ or NO₃⁻. The enzyme activities were determined at various times as described in "Materials and Methods."
However, the time course curves of the enzyme activities induced by both NO$_3^-$ and NO$_2^-$ were similar.

**Uptake and Accumulation of NO$_3^-$ and NO$_2^-$**

The time course curves for the uptake of NO$_3^-$ and NO$_2^-$ differed from the NiR induction curves. The uptake of NO$_3^-$ and NO$_2^-$ into leaves was proportional to the concentration of N supplied in the induction solution and continued at a near constant rate through 24 h (Fig. 2, A and B). Similar uptake of NO$_3^-$ and NO$_2^-$ occurred at each concentration applied.

In leaves supplied with NO$_3^-$, measurable amounts of NO$_3^-$ accumulated (Fig. 2D), showing that some of the absorbed NO$_3^-$ was oxidized to NO$_2^-$. In leaves supplied with NO$_3^-$ or NO$_2^-$, the time course curves for the accumulation of NO$_3^-$ resembled the NiR induction curves (compare Fig. 1, A and B, with Fig. 2, C and D).

No induction of NiR occurred in leaves supplied with 0.2 mM or less NO$_3^-$ in the induction solutions (Table I). In these leaves also no NO$_3^-$ was detected. In fact, in leaves supplied with NO$_3^-$, no NiR induction was observed until NO$_3^-$ accumulated in the leaves (Table I). By contrast, NiR induction occurred in leaves supplied with as low as 0.05 mM NO$_3^-$.

NO$_3^-$ was found in NO$_3^-$-fed leaves indicating that all of the reduced NO$_3^-$ was further assimilated to amino N. Also at 1 and 2 mM NO$_3^-$ supply, no NO$_3^-$ was detected in the leaves; however, at 5 and 10 mM NO$_3^-$ supply, the NO$_3^-$ concentration increased up to 1.6 and 5.8 $\mu$mol g$^{-1}$, at 2 and 6 h, respectively, and then decreased gradually with time (data not shown).

**Effect of Tungstate on the induction of NR and NiR**

To test whether NO$_3^-$ induced NiR directly or only after its reduction to NO$_2^-$, the induction of NiR by NO$_3^-$ was studied in the presence and absence of tungstate. In leaves from seedlings previously grown in the presence of WO$_4^{2-}$, NRA was induced after supplying NO$_3^-$ without WO$_4^{2-}$ (Table II). However, the enzyme activity was 45% of that induced in leaves from plants grown in the absence of WO$_4^{2-}$ (3.7 versus 8.3 $\mu$mol NO$_3^-$ g$^{-1}$ fresh weight h$^{-1}$). In contrast, the level of induction of NiR by NO$_3^-$ in leaves from plants grown on WO$_4^{2-}$ was about 86% of that induced in leaves grown without WO$_4^{2-}$ (Fig. 1A and Table II). When the leaves of the seedlings previously grown on WO$_4^{2-}$ were supplied 0.1 mM WO$_4^{2-}$ along with NO$_3^-$, induction of NR was inhibited more than 90%, but little inhibition of NiR induction occurred at this low level of WO$_4^{2-}$ (Table II). While no induction of NR and no in vivo reduction of NO$_3^-$ to NO$_2^-$ occurred with 0.5 and 1.0 mM WO$_4^{2-}$ in the induction solutions, some induction of NiR was still observed (Table II). Similar inhibition of NiR induction by increasing levels of WO$_4^{2-}$ occurred in leaves supplied with NO$_3^-$ (data not shown).

**DISCUSSION**

**Role of NO$_3^-$ and NO$_2^-$**

Several lines of evidence indicated that NO$_3^-$ rather than NO$_2^-$ induced NiR. (a) At similar fluxes of NO$_3^-$ and NO$_2^-$ (Fig. 2, A and B), the induction of NiR in the presence of NO$_3^-$ was two-times greater than that induced in the presence of NO$_2^-$ (Fig. 1, A and B), although only 60 to 65% of the absorbed NO$_3^-$ was reduced to NO$_2^-$ (Fig. 2, A and C). (b) The time course curves of NiR induction and NO$_3^-$ concentration were similar (compare Figs. 1A and 2C) and were highly correlated (Fig. 3). In contrast, no correlation was found between NO$_3^-$ accumulation and NiR induction. For example, in leaves fed NO$_3^-$ or 2 mM and less NO$_2^-$, no NO$_3^-$ accumulated. In leaves supplied with 5 and 10 mM NO$_3^-$, some NO$_3^-$ accumulated initially then decreased gradually, whereas NiR continued to increase. (c) Induction of NiR was detected at an ambient concentration of NO$_3^-$ that was ten-times lower than that required for induction with NO$_2^-$ (Table I). (d) We have recently shown that NO$_3^-$ is oxidized to NO$_2^-$ in barley leaves, resulting in significant accumulation of NO$_3^-$ in NO$_2^-$ fed leaves (3). Induction of NiR was correlated with the concentration of NO$_3^-$ in leaves fed NO$_3^-$ (Fig. 3) but not with the concentration of NO$_2^-$ (Table I). In fact, no induction of NiR occurred in NO$_3^-$-fed leaves until NO$_3^-$ was detectable (Table I).
Interaction of Pathways of Assimilation

As described above, the internal concentration of NO$_3^-$ seemed to regulate the induction of NiR. The concentration of NO$_3^-$, in turn, was regulated by influx, the induction of NR, and the in vivo rate of NO$_3^-$ reduction (Figs. 1 and 2). At low concentrations of ambient NO$_3^-$ where NO$_3^-$ content plateaued (reduction equaled uptake), the main regulator of NiR induction seemed to be the in vivo activity of NR which determined the concentration of NO$_3^-$. At the higher concentrations of ambient NO$_3^-$, uptake was a greater regulator, since it furnished sufficient NO$_3^-$ to allow full induction of NR and subsequent in vivo NO$_3^-$ reduction, allowing a higher concentration of NO$_3^-$ to accumulate for inducing the enzyme.

Effect of Tungstate

Treatment of plant tissues with WO$_4^{2-}$ has often been used to separate the induction of NR and NiR by NO$_3^-$ and NO$_2^-$ (7, 13). Tungstate is incorporated into NR in place of MoO$_4^{2-}$ making the enzyme inactive (19, 25). The induction of NiR in the presence of WO$_4^{2-}$ would indicate that enzyme activity is induced directly by NO$_3^-$. Such experiments are complicated by the low activity of NR in the tissues after treatment with WO$_4^{2-}$, and WO$_4^{2-}$ can also inhibit the induction of NiR (Table II). In our experiments, seeds were germinated and grown in the presence of WO$_4^{2-}$ to inactivate the endogenous (possibly constitutive) NR (8), and then placed in the induction solutions in the presence of WO$_4^{2-}$. Table II shows the importance of determining the in vivo reduction of NO$_3^-$ along with the induction of NR in relation to the concentration of WO$_4^{2-}$ fed. At a concentration of 0.1 mM WO$_4^{2-}$, NR was inhibited 92%; however, some in vivo reduction of NO$_3^-$ still occurred (Table II). At a WO$_4^{2-}$ concentration of 0.25 mM and above, when NR was inhibited almost 100% and no in vivo reduction of NO$_3^-$ was detected, 52% of full induction of NiR still occurred, indicating that NO$_3^-$ may induce NiR directly. The inhibition of NiR induction by WO$_4^{2-}$ may be due to general toxicity. Induction of NiR was also inhibited gradually when the leaves were supplied with increasing levels of MoO$_4^{2-}$ in the induction solutions (data not shown).

In summary, several lines of evidence indicate that NO$_3^-$ is a more likely inducer of NiR in barley leaves than is NO$_2^-$. In contrast, the induction of NiR in the presence of ambient NO$_2^-$ seems to be a result of its oxidation to NO$_3^-$ within the leaf. The induction of NiR seems to be regulated by the internal concentration of NO$_3^-$ in the leaf.

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**Table I. Induction of NiRA and Uptake and Content of NO$_3^-$ and NO$_2^-$ in Detached Barley Leaves as a Function of Concentration of NO$_3^-$ or NO$_2^-$**

Detached leaves from 8-d-old seedlings, grown hydroponically in 0.2 mM CaSO$_4$ solution, were placed in 10 mL of aqueous solution containing 0 to 2 mM NO$_3^-$ or NO$_2^-$ and incubated in light. Enzyme activities, NO$_3^-$ and NO$_2^-$ uptake and content were determined after 6 h. Means ± SD are given.

<table>
<thead>
<tr>
<th>NO$_3^-$ or NO$_2^-$ Supplied</th>
<th>Uptake</th>
<th>NO$_3^-$ Content*</th>
<th>NiRA</th>
<th>NO$_3^-$-fed</th>
<th>NO$_2^-$-fed</th>
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<tr>
<td>mm</td>
<td>µmol g$^{-1}$</td>
<td>µmol g$^{-1}$ h$^{-1}$</td>
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<td>0.05</td>
<td>0.13</td>
<td>0.13</td>
<td>0.05</td>
<td>0</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>0.10</td>
<td>0.26</td>
<td>0.25</td>
<td>0.10</td>
<td>0</td>
<td>9.9 ± 0.3</td>
</tr>
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<td>0.20</td>
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<td>0.15</td>
<td>0</td>
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<td>0.50</td>
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<td>0.03</td>
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</tr>
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<td>1.00</td>
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<td>2.84</td>
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<td>0.08</td>
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<td>5.51</td>
<td>1.18</td>
<td>0.16</td>
<td>16.6 ± 0.3</td>
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</table>

*NO$_2^-$ was not detected in the leaves from any of the above treatments.

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**Table II. Effect of Different Levels of Tungstate on the Induction of NR and NiR Activities by NO$_3^-$ in Detached Leaves**

Detached leaves from 9-d-old seedlings, grown in vermiculite and irrigated with N-free Hoagland solution containing 0.5 mM WO$_4^{2-}$, were placed in 10 mL of the induction solution containing increasing levels of NO$_3^-$ (10–20 mM) along with increasing concentrations of WO$_4^{2-}$. Enzyme activities were assayed after 6 h of induction in light as described in "Materials and Methods." The initial NR and NiR activities were 0.06 and 7.2 µmol NO$_3^-$ g$^{-1}$ h$^{-1}$, respectively.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>WO$_4^{2-}$</th>
<th>NO$_3^-$*</th>
<th>Uptake</th>
<th>Content</th>
<th>Reduction</th>
<th>NIRA</th>
<th>NiR</th>
<th>µmol NO$_3^-$ g$^{-1}$ h$^{-1}$</th>
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</thead>
<tbody>
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<td>µmol g$^{-1}$</td>
<td>µmol g$^{-1}$</td>
<td>µmol g$^{-1}$</td>
<td>µmol g$^{-1}$</td>
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<tr>
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</tr>
<tr>
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<td>0.3</td>
<td>0.06 ± 0.01</td>
<td>10.1 ± 0.7</td>
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</table>

*NO$_3^-$ uptake in detached leaves is a function of transpiration, and WO$_4^{2-}$ inhibited transpiration. Therefore, to eliminate NO$_3^-$ uptake as a variable, the leaves supplied with higher levels of WO$_4^{2-}$ were also supplied increasing concentrations of NO$_3^-$ in the induction (uptake) solutions.

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I I-
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and B, 2, W, M, 40 50 60

LITERATURE CITED