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Role of Nitrate and Nitrite in the Induction of Nitrite Reductase in Leaves of Barley Seedlings[1]

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ABSTRACT

The role of NO_3^- and NO_2^- 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 NO_2^-/gram fresh weight × hour of endogenous NiR activity when grown in N-free solutions. Supply of both NO_2^- and NO_3^- induced the enzyme activity above the endogenous levels (5 and 10 times, respectively at 10 millimolar NO_2^- and NO_3^- over a 24 hour period). In NO_3^--supplied leaves, NiR induction occurred at an ambient NO_3^- concentration of as low as 0.05 millimolar; however, no NiR induction was found in leaves supplied with NO_2^- until the ambient NO_2^- concentration was 0.5 millimolar. Nitrate accumulated in NO_2^--fed leaves. The amount of NO_3^- accumulating in NO_2^--fed leaves induced similar levels of NiR as did equivalent amounts of NO_3^- accumulating in NO_3^--fed leaves. Induction of NiR in NO_2^--fed leaves was not seen until NO_3^- was detectable (30 nanomoles/gram fresh weight) in the leaves. The internal concentrations of NO_3^-, irrespective of N source, were highly correlated with the levels of NiR induced. When the reduction of NO_3^- to NO_2^- was inhibited by WO_4^{2-}, the induction of NiR was inhibited only partially. The results indicate that in barley leaves NiR is induced by NO_3^- directly, *i.e.* without being reduced to NO_2^-, and that absorbed NO_2^- induces the enzyme activity indirectly after being oxidized to NO_3^- within the leaf.

In many plant species, both NR[2] and NiR are induced with either NO_3^- or NO_2^- in the ambient substrate solution (9). It was earlier proposed that NO_2^- was a more specific inducer of NiR since high levels were induced in *Lemna* (23). In radish cotyledons (11) and bean leaves (22), a sequential induction of NR and NiR by NO_3^- indicated that NiR was induced possibly by NO_2^- after its formation from NO_3^- by NR. Evidence has been presented that ambient NO_3^- could induce NiR in NR deficient cells of tobacco (17); however, NO_2^- was not tested as an inducer. Gupta *et al.* (7) showed that NO_3^- induced NiR in wheat embryos during the first 12 h of imbibition of seeds. Induction of NiR was not detected during imbibition of the seeds in the presence of NO_2^-.

Tungstate has been used to inhibit the formation of active NR induced by NO_3^- (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 NO_3^- may induce NiR directly without being reduced to NO_2^-. Recently, Lahners *et al.* (15) reported that both NO_3^- and NO_2^- induced NiR-mRNA in leaves of maize seedlings. Back *et al.* (4) reported that NO_3^- 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 NO_3^- in the environment in which the plants are grown (8). Since the minimum ambient and internal concentrations of NO_3^- and NO_2^- which can induce NiR are not known, low levels of NO_2^- 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 NO_3^- to NO_2^- 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 NO_2^-. 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 NO_2^- can be oxidized to NO_3^- in plant tissues (3, 6, 12, 16). Hence, it is important to determine if the test plants are converting NO_2^- to NO_3^- *in vivo* during the induction period. We recently showed that the induction of NR by the absorbed NO_2^- was likely caused after its oxidation to NO_3^-. Likewise, the induction of NiR by absorbed NO_2^- might also be a result of its oxidation to NO_3^- within the tissue. Furthermore, NO_3^- may be present as a contaminant in NO_2^- solutions (3).

To compare the role of NO_3^- and NO_2^-, the induction of NiR was studied as a function of NO_3^- and NO_2^- 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 NO_3^- is the more likely inducer of NiR even in those supplied NO_2^-, and the induction of NiR may be regulated by the internal concentration of NO_3^- 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

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[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 $m^{-2}s^{-1}$ and was supplied by incandescent and cool white fluorescent lamps. In one experiment (Table I) the seedlings were grown hydroponically in 0.2 mM $CaSo_4$ 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 base down in small glass vials containing 10 mL of 0 to 10 mM $NaNO_3$ or $NaNO_2$ solutions. Sodium salts were used because even the reagent grade KNO_2 contained measurable amounts of NO_3^- as a contaminant; $NaNO_2$ was free of NO_3^-. 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^{-2} s^{-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 NO_3^- and NO_2^-

At the same time when the enzyme activities were assayed, uptake, concentration, and reduction of NO_3^- and NO_2^- 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 NO_3^- and NO_2^- was determined by subtracting the NO_3^- and NO_2^- 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 M Tris-HCl (pH 8.5), 1 mM DTT, 10 μM flavin adenine dinucleotide, 1 μM Na_2MoO_4, 1 mM EDTA, and 10 μM leupeptin (14). The homogenates were centrifuged at 30,000*g* for 10 min, and the supernatants were assayed for NRA, NiRA, NO_3^-, and NO_2^-.

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 KNO_3, 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 NO_2^- formed was determined colorimetrically (20).

Nitrite reductase activity was assayed by following the disappearance (reduction) of NO_2^- from the assay mixture (18). The assay mixture contained 40 μmol potassium phosphate (pH 7.5), 0.5 μmol KNO_2, 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 $Na_2S_2O_4$ solution (8 mg/mL) in 0.1 M $NaHCO_3$ 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 NO_2^- in the assay tubes was determined colorimetrically.

NO_3^- and NO_2^- 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) sufanilamide 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 NO_3^- and NO_2^-. Barley leaves contained 6 to 8 μmol NO_2^- 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 Aslam and RC Huffaker, unpublished results). Nitrite reductase activity was increased upon supply of NO_3^- or NO_2^- (Fig. 1). The enzyme activity induced by exogenously supplied NO_2^-, at all concentrations, was about one-half of that induced by similar concentrations of NO_3^-.

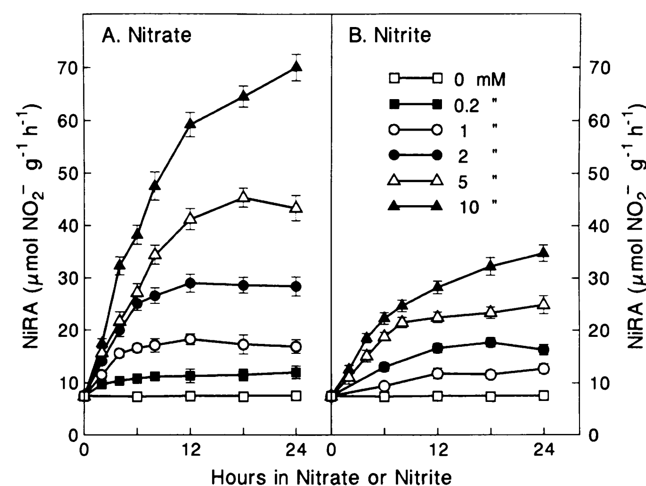

Figure 1. Time course of the induction of NiRA in detached leaves supplied with different levels of NO_3^- (A) or NO_2^- (B) in the induction solutions. Detached leaves from 8-day-old seedlings, grown in vermiculite, were placed in 10 mL of the induction solutions containing 0 (□), 0.2 (■), 1 (○), 2 (●), 5 (△), and 10 (▲) mM NO_3^- or NO_2^-. 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_2^-, measurable amounts of NO_3^- accumulated (Fig. 2D), showing that some of the absorbed NO_2^- was oxidized to NO_3^-. 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_2^- in the induction solutions (Table I). In these leaves also no NO_3^- was detected. In fact, in leaves supplied with NO_2^-, 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 NO_2^- 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_2^- supply, no NO_2^- was detected in the leaves; however, at 5 and 10 mM NO_2^- supply, the NO_2^- concentration increased up to 1.6 and 5.8 μmol g^{-1}, at 2 and 6 h, respectively, and then decreased gradually with time (data not shown).

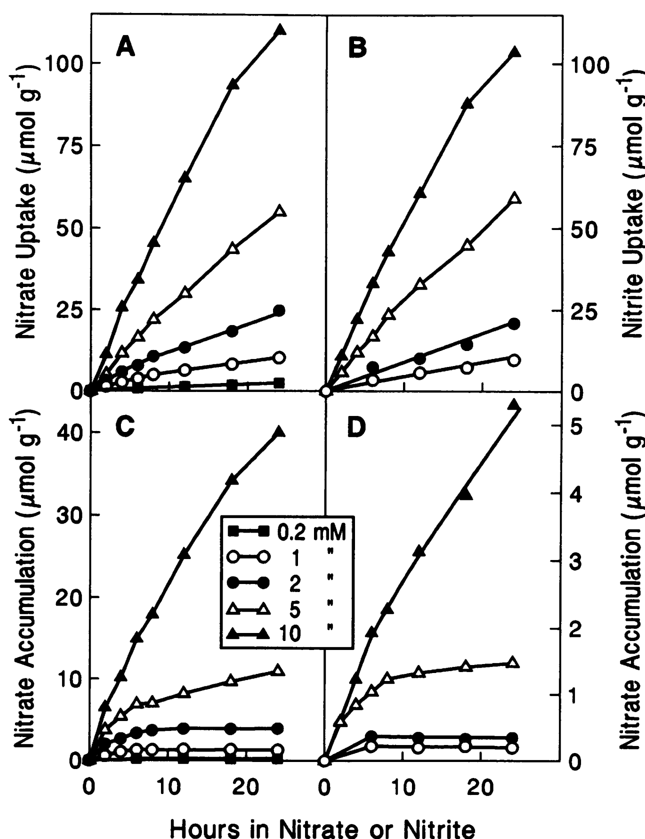

Figure 2. Time course for the uptake of NO_3^- (A), NO_2^- (B), and content of NO_3^- (C, D) in detached leaves supplied with 0.2 (■), 1 (○), 2 (●), 5 (△), and 10 (▲) mM NO_3^- (A, C) or NO_2^- (B, D) in the induction solutions. For experimental details, and corresponding NiRA data see Figure 1. The vertical scale in D is expanded eight times as compared to that in C.

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 μmol NO_2^- 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 NiRA 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_2^- (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_2^- accumulation and NiR induction. For example, in leaves fed NO_3^- or 2 mM and less NO_2^-, no NO_2^- accumulated. In leaves supplied with 5 and 10 mM NO_2^-, some NO_2^- accumulated initially then decreased gradually, whereas NiRA 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_2^- is oxidized to NO_3^- 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_2^- (Fig. 3) but not with the concentration of NO_2^- (Table I). In fact, no induction of NiR occurred in NO_2^--fed leaves until NO_3^- was detectable (Table I).

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.

NO_3^- or NO_2^- Supplied	Uptake		NO_3^- Content[a]		NiRA	
	NO_3^-	NO_2^-	NO_3^--fed	NO_2^--fed	NO_3^--fed	NO_2^--fed
mM	*μmol g⁻¹*				*μmol NO_2^- g^{-1} h^{-1}*	
0.0	0	0	0	0	7.9 ± 0.4	7.9 ± 0.4
0.05	0.13	0.13	0.05	0	8.8 ± 0.5	7.7 ± 0.4
0.10	0.26	0.25	0.10	0	9.9 ± 0.3	8.0 ± 0.5
0.20	0.47	0.58	0.15	0	10.5 ± 0.2	8.3 ± 0.6
0.50	1.33	1.26	0.32	0.03	12.6 ± 0.0	9.4 ± 0.2
1.00	2.97	2.84	0.65	0.08	14.4 ± 0.3	10.5 ± 0.2
2.00	5.48	5.51	1.18	0.16	16.6 ± 0.3	11.2 ± 0.3

[a] NO_2^- was not detected in the leaves from any of the above treatments.

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_2^- g^{-1} h^{-1}, respectively.

Treatments		NO_3^-			Enzyme Activities	
WO_4^{2-}	NO_3^-[a]	Uptake	Content	Reduction	NRA	NiRA
mM		*μmol g⁻¹*			*μmol NO_2^- g^{-1} h^{-1}*	
0.00	10	36.0 ± 0.2	27.4 ± 1.7	8.6	3.74 ± 0.07	32.6 ± 1.1
0.10	12	35.6 ± 0.3	32.8 ± 1.2	2.8	0.31 ± 0.01	27.4 ± 0.3
0.25	15	39.8 ± 0.7	38.9 ± 2.7	0.9	0.13 ± 0.01	16.8 ± 0.2
0.50	17	39.3 ± 0.3	38.6 ± 1.2	0.7	0.06 ± 0.03	12.6 ± 0.3
1.00	20	39.4 ± 0.4	39.1 ± 1.2	0.3	0.06 ± 0.01	10.1 ± 0.7

[a] 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.

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 regulant, 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.

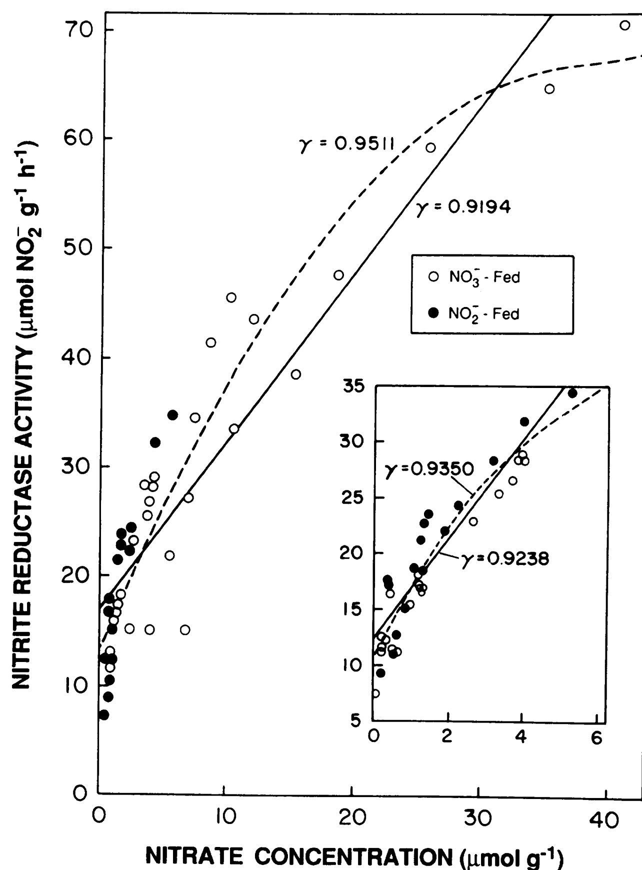

Figure 3. Linear (——) and quadratic (- - - - -) regressions of NiRA *versus* NO_3^- concentration of the leaves supplied with 0.2, 1, 2, 5, 10 mMNO_3^- (O) and 1, 2, 5, 10 mM NO_2^- (●). The data from Figures 1, A and B, 2, C and D, were plotted. The inset shows the relationship in the leaves accumulating low levels of NO_3^-. The data for leaves supplied with 5 and 10 mM NO_3^- were excluded. The correlation coefficients (γ) were significant at P = 0.001.

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