

Comparative Induction of Nitrate and Nitrite Uptake and Reduction Systems by Ambient Nitrate and Nitrite in Intact Roots of Barley (*Hordeum vulgare* L.) Seedlings¹

Muhammad Aslam, Robert L. Travis, and Ray C. Huffaker*

Department of Agronomy and Range Science, University of California, Davis, California 95616

The induction by ambient NO_3^- and NO_2^- of the NO_3^- and NO_2^- uptake and reduction systems in roots of 8-d-old intact barley (*Hordeum vulgare* L.) seedlings was studied. Seedlings were induced with concentrations of NaNO_3 or NaNO_2 ranging from 0.25 to 1000 μM . Uptake was determined by measuring the depletion of either NO_3^- or NO_2^- from uptake solutions. Enzyme activities were assayed in vitro using cell-free extracts. Uptake and reduction systems for both NO_3^- and NO_2^- were induced by either ion. The K_m values for NO_3^- and NO_2^- uptake induced by NO_2^- were similar to those for uptake induced by NO_3^- . Induction of both the uptake and reduction systems was detected well before any NO_3^- or NO_2^- was found in the roots. At lower substrate concentrations of both NO_3^- and NO_2^- (5–10 μM), the durations of the lag periods preceding induction were similar. Induction of uptake, as a function of concentration, proceeded linearly and similarly for both ions up to about 10 μM . Then, while induction by NO_3^- continued to increase more slowly, induction by NO_2^- sharply decreased between 10 and 1000 μM , apparently due to NO_2^- toxicity. In contrast, induction of NO_3^- reductase (NR) and NO_2^- reductase (NiR) by NO_2^- did not decrease above 10 μM but rather continued to increase up to a substrate concentration of 1000 μM . NO_3^- was a more effective inducer of NR than was NO_2^- ; however, both ions equally induced NiR. Cycloheximide inhibited the induction of both uptake systems as well as NR and NiR activities whether induced by NO_3^- or NO_2^- . The results indicate that in situ NO_3^- and NO_2^- induce both uptake and reduction systems, and the accumulation of the substrates per se is not obligatory.

The literature pertaining to the physiological bases of the induction of NO_3^- and NO_2^- uptake and their respective reductases contains several conflicting reports. At issue is whether those ions induce only their own uptake and reduction systems or whether they are each capable of inducing either system. The controversy likely stems from at least two inherent complications. First, because NR is constitutively present in the roots (Aslam et al., 1990), they are exposed to both ions when NO_3^- is the sole N source, making it difficult to determine which is the inducer. Second, the situation is further complicated by the fact that NO_3^- appears in tissues of various plant species via oxidation of NO_2^- when the latter is the N source (Lips et al., 1973; Kaplan et al., 1974; Sahulka

and Lisa, 1978; Aslam et al., 1987; Aslam and Huffaker, 1989). Muhammad and Kumazawa (1974) found NO_3^- in rice seedlings supplied with NH_4^+ . These observations must be accounted for in studies of induction of uptake and reduction systems by NO_2^- .

Because NO_3^- and NO_2^- are competitive inhibitors of their respective uptake and apparently share the same transporters and binding sites (Aslam et al., 1992a), the induction of their uptake and reduction systems is especially interesting. The induction of the NO_3^- and NO_2^- uptake systems in the presence of their respective substrates is well established (Jackson et al., 1973, 1974a, 1974b; Goyal and Huffaker, 1986b; Aguera et al., 1990; Siddiqi et al., 1990). In addition, both uptake systems clearly appear to be induced by NO_3^- (Aslam et al., 1992a). In plant species in which the formation of NO_2^- is inhibited, e.g. double-mutant barley (*Hordeum vulgare* L.) seedlings containing neither the NADH nor NAD(P)H NRA, NO_3^- still induced NO_3^- uptake (Warner and Huffaker, 1989). In WO_4^{2-} -treated tissues, in which the formation of an active NR and, hence, of NO_2^- is inhibited, NO_3^- application induced NO_2^- uptake (de la Haba et al., 1990).

The situation regarding the induction of the NO_3^- uptake system by ambient NO_2^- is less clear. Hole et al. (1990) found no induction of NO_3^- uptake by NO_2^- in corn roots. When NO_2^- and NO_3^- were both present in the incubation solution, the induction of the NO_3^- uptake system was delayed in wheat seedlings (Jackson et al., 1974b; Tompkins et al., 1978). When corn seedlings were pretreated with NO_2^- and then transferred to a solution containing only NO_3^- , induction of the NO_3^- uptake system was delayed (Jackson et al., 1973). In contrast, in dwarf bean seedlings, pretreatment with NO_2^- shortened the lag period for the subsequent induction of the NO_3^- uptake system by NO_3^- (Breteler and Luczak, 1982). The initial rates of NO_3^- uptake were higher in NO_2^- -pretreated bean seedlings (Breteler and Luczak, 1982) and tobacco cells (Heimer, 1975) than in those not pretreated with NO_2^- , indicating the development of the NO_3^- uptake system by ambient NO_2^- . Recently, Siddiqi et al. (1992) reported that a 24-h pretreatment with 0.1 mM NO_2^- fully induced the NO_3^- uptake system in barley roots. However, it was not clearly determined in their study whether NO_2^- induced the NO_3^- uptake system per se or whether induction was the

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* Corresponding author; fax 1-916-752-4361.

Abbreviations: CHI, cycloheximide; NiR(A), NO_2^- reductase (activity); NR(A), NO_3^- reductase (activity).

result of NO_3^- produced by the oxidation of NO_2^- . They did not find net accumulation of NO_3^- in NO_2^- -fed barley roots, but the endogenous NO_3^- concentration in their control roots that received no NO_3^- was relatively high. Thus, as they stated, they would not have detected a small increase in NO_3^- concentration from an NO_2^- application.

We previously reported that NRA was induced in barley leaves by either NO_3^- or NO_2^- (Aslam et al., 1987). However, a much higher concentration of NO_2^- than NO_3^- was required for induction. In fact, no induction was found with NO_2^- until NO_3^- appeared, presumably from the internal oxidation of NO_2^- in the leaves.

Studies with roots are less clear. In a preliminary report, we showed that NR was induced in barley roots by both NO_3^- and NO_2^- and also that NO_3^- appeared in roots after feeding with NO_2^- (Aslam et al., 1992b). In contrast, Siddiqi et al. (1992) recently reported that 0.1 mM NO_2^- did not induce NR in barley roots during a 24-h induction period.

Studies of induction of NiR are also controversial. WO_4^{2-} , which inhibits the formation of active NR, did not inhibit induction of NiR by NO_3^- in wheat embryos (Gupta et al., 1983), tobacco cells (Kelker and Filner, 1971), and sunflower roots (de la Haba et al., 1990). This suggests that NO_3^- can induce NiR independently of NO_2^- . On the other hand, because NO_3^- may result from the internal oxidation of NO_2^- , the possibility exists that NO_3^- is the real inducer of NiR even in roots supplied with NO_2^- . Our earlier studies also showed that induction of NiR in barley leaves did not occur until NO_3^- was detected in the tissues (Aslam and Huffaker, 1989). Yet, in contrast to the results obtained with leaves, we found that induction of NiR, like induction of NR, in roots occurred with either NO_3^- or NO_2^- in the nutrient solution and before either ion was detected in the root tissue (Aslam et al., 1992b).

In this paper we report that NO_2^- and NO_3^- were equally effective in inducing the uptake systems of either ion, and we demonstrate that NO_2^- will induce NRA and NiRA. We further show that the induction of both uptake and reduction systems occurred several hours before either ion was detected in the roots.

MATERIALS AND METHODS

Plant Culture

Barley (*Hordeum vulgare* L. var CM-72) seedlings were grown hydroponically as described by Aslam et al. (1979). Seedlings were grown in 0.2 mM CaSO_4 in the dark for 6 d. The seedlings were then transferred to aerated 25% full-strength Hoagland solution lacking N (Hoagland and Arnon, 1950) and placed in the growth chamber under continuous light at 25°C and 60 to 65% RH. The PPFD (400–700 nm) at the plant canopy was 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and was supplied with incandescent and cool-white fluorescent lamps.

Induction of Uptake and Reduction Systems

After 24 h in the light, the seedlings were transferred to large volumes (5–10 L) of the induction solution containing 0 to 1000 μM NaNO_3 or NaNO_2 in 25% full-strength Hoagland solution (Hoagland and Arnon, 1950). The seedlings

were then placed in a controlled environment growth chamber set for continuous light, 25°C, and 60 to 65% RH. The induction solutions were analyzed periodically for NO_3^- and NO_2^- , and their concentrations were maintained by adding appropriate volumes of the stock solutions. The depletion of the substrates was never more than 20%. In one experiment, the seedlings were pulsed with 250 μM NO_3^- or NO_2^- for 1 h and then transferred to the N-free solutions. The uptake rates of NO_3^- and NO_2^- and NRA and NiRA were determined at various intervals. In some induction studies, WO_4^{2-} (data not shown) and CHI were also supplied at varying concentrations, as indicated in the respective tables. Sodium salts were used because even the reagent grade KNO_2 contained measurable amounts of NO_3^- , whereas NaNO_2 was free of any NO_3^- .

Measurement of NO_3^- and NO_2^- Uptake Systems

Intact seedlings were used in all experiments. Uptake was determined in a minigrowth chamber set at 25°C, 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity (400–700 nm), and 60 to 65% RH. The growth chamber was part of a fully automatic system described by Goyal and Huffaker (1986a). Uptake was started by placing 15 seedlings in a Pyrex culture tube (25 × 150 mm) containing 50 mL of the appropriate solution. Uptake solutions contained 1.0 mM Mes (pH 6.0), 0.2 mM CaSO_4 , and NO_3^- or NO_2^- as indicated in the figures and tables. All solutions were aerated vigorously to ensure thorough mixing. Aliquots (0.4 mL) for NO_3^- and NO_2^- determination were removed automatically at 1.0- to 3.0-min intervals by the HPLC system. Uptake rates were determined by measuring the disappearance of NO_3^- and NO_2^- from the uptake solutions per unit of time. The rates were computed from determinations of concentration and volume at consecutive sampling times (Goyal and Huffaker, 1986a).

All experiments were repeated at least two times, and the results from representative experiments are shown. In kinetic studies (Fig. 4), the double-reciprocal plots of the uptake rates versus concentration were subjected to linear regression analysis, and the regression equations are shown in the figure legends. All r^2 values were significant at $P = 0.001$. The kinetic constants (K_m , V_{max}) were calculated from these regression equations.

NR and NiR Assays

Roots (about 2 g per treatment) were washed with distilled, deionized water, detached from the shoots at the scutellar node, and homogenized with 4 mL of buffer g^{-1} of root in a chilled mortar and pestle in the presence of acid-washed sand. 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 mM leupeptin (Kuo et al., 1982). The homogenates were centrifuged at 30,000g for 15 min, and the supernatants were used for the assay of NRA, NiRA, NO_3^- , and NO_2^- .

The enzyme activities were assayed by in vitro methods as described by Aslam and Huffaker (1989). NRA was assayed using NADH as the electron donor, and NiRA was assayed using methyl viologen that was reduced by $\text{Na}_2\text{S}_2\text{O}_4$.

NO_3^- and NO_2^- Determination

Both NO_3^- and NO_2^- from the uptake solutions and NO_3^- from the root extracts were determined spectrophotometrically by measuring their A_{210} after separation by HPLC on a partisol-10 SAX anion-exchange column (Thayer and Hufaker, 1980). NO_2^- from the root extracts was determined colorometrically after color development for 15 min with a 1:1 mixture of 1% (w/v) sulfanilamide in 1.5 N HCl and 0.02% (w/v) *n*-naphthylethylenediamine dihydrochloride. The A_{540} was read.

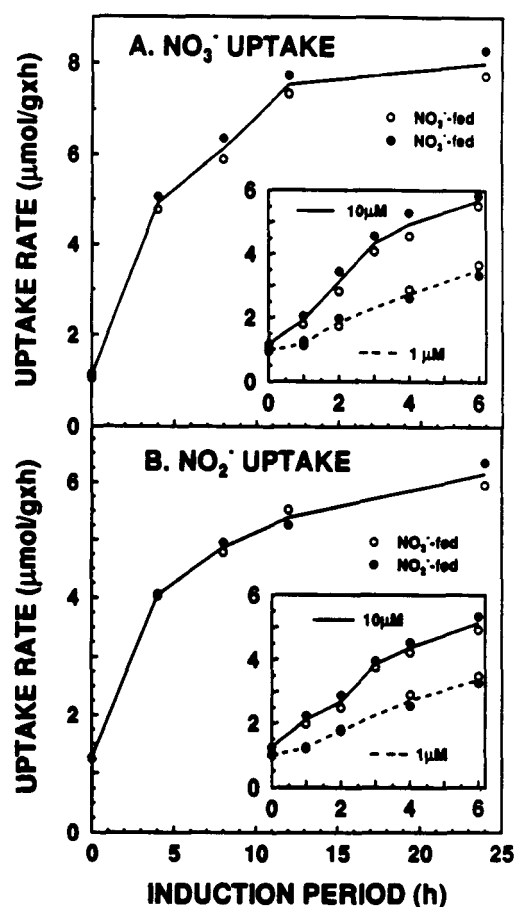


Figure 1. Time course of the induction of NO_3^- and NO_2^- uptake systems in roots of intact barley seedlings induced with 1 and 10 μM ambient NO_3^- or NO_2^- . Seedlings were grown hydroponically in N-free solutions for 6 d in continuous darkness followed by 24 h of continuous light. The seedlings were then transferred to the induction solutions (10 L) containing 1 or 10 μM NO_3^- or NO_2^- in 25% full-strength Hoagland solution and placed in continuous light. The induction solutions were analyzed periodically and were brought to the original concentration by adding appropriate volumes of stock solutions. The depletion of the substrate from the induction solutions was not more than 20%. Uptake rates of NO_3^- and NO_2^- were determined by analyzing the depletion of the ions from 200 μM solutions for 12 min (sampling every 1.5 min) at each time interval. The insets show the lag periods in the induction of NO_3^- (A) and NO_2^- (B) uptake systems at 1 μM (dotted lines) and 10 μM (solid lines) NO_3^- and NO_2^- concentrations.

Table I. Time course of the accumulation of NO_3^- in roots of intact barley seedlings supplied with 1 and 10 μM NO_3^- or NO_2^-

For experimental details see legend for Figure 1.

Induction Period	1 μM Substrate ^a		10 μM Substrate ^a	
	NO_3^- fed	NO_2^- fed	NO_3^- fed	NO_2^- fed
<i>h</i>	<i>nmol g⁻¹</i>			
0	9 ^b	9	10	10
1	11	ND ^c	82	12
2	39	10	348	10
3	106	12	865	34
4	199	17	2112	69
6	364	34	3216	111
12	564	65	5832	187
24			8928	369

^a No accumulation of NO_2^- was detected at any time interval. ^b Average coefficient of variation for the data set was 13%. ^c ND, Not determined.

The results are reported on the basis of fresh weights of the roots.

RESULTS**Induction of NO_3^- and NO_2^- Uptake Systems***Time Course of Induction at Low Substrate Concentration*

Both the NO_3^- and NO_2^- uptake systems were induced by either ambient NO_3^- or NO_2^- . At a substrate concentration of 10 μM , the induction patterns for NO_3^- and NO_2^- uptake were similar (Fig. 1). Induction proceeded at a relatively rapid rate during the initial 4 h and then continued at a reduced rate up to 12 h. Maximal activity was approached between 12 and 24 h. At 1 μM concentration, NO_3^- and NO_2^- were also equally effective inducers of both uptake systems (Fig. 1, insets). After a brief lag period, activity increased linearly throughout the 6-h induction period. At 6 h, total activity was about two-thirds of that obtained with 10 μM substrate. Induction levels with 5 μM substrate were similar to those obtained with 10 μM for both systems (results not shown).

NO_3^- accumulation increased with time and in proportion to substrate concentration in roots supplied with 1 or 10 μM NO_3^- (Table I). Conversely, no accumulation of NO_2^- occurred in roots supplied with either 1 or 10 μM NO_2^- during the 24-h incubation period (results not shown). When seedlings were induced with 1 μM NO_2^- , no net accumulation of NO_3^- occurred during the first 3 h, and in the presence of 10 μM NO_2^- , only trace amounts of NO_3^- were detected during the same period. After 3 h, NO_3^- accumulation was low, but appreciable, and was apparently dependent on the concentration of NO_2^- in the induction medium. No accumulation of NO_2^- occurred in NO_3^- -supplied roots during the 24-h induction period (results not shown).

Time Course of Induction at High Substrate Concentration

At 1000 μM substrate, the time course and the level of induction of both systems by NO_3^- were different from those obtained with NO_2^- (Fig. 2). In the presence of NO_3^- , both

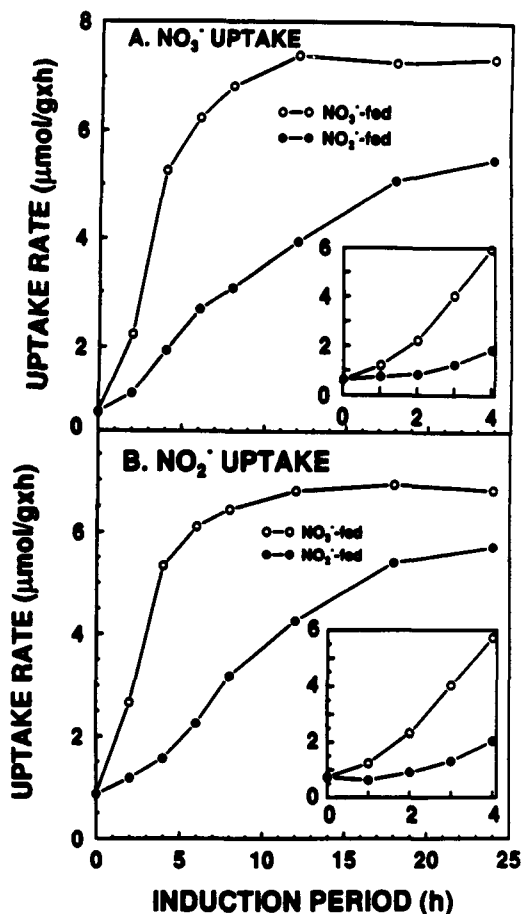


Figure 2. Time course of the induction of NO_3^- and NO_2^- uptake systems in intact roots of barley seedlings induced with $1000 \mu\text{M}$ NO_3^- or NO_2^- . The experimental details are the same as in Figure 1. The insets show the lag periods in the induction of NO_3^- (A) and NO_2^- (B) uptake systems as a function of NO_3^- and NO_2^- concentrations.

uptake systems increased rapidly, with little lag, during the initial 12 h. Thereafter, little increase in activity occurred. In contrast, with NO_2^- , induction was slower but, following a lag period, continued to increase for 18 h before approaching saturation. Induction with NO_3^- reached saturation after 12 h. At that point, uptake induced by NO_2^- was only 50 to 60% of that induced by NO_3^- .

Roots supplied with $1000 \mu\text{M}$ NO_2^- accumulated increasing amounts of NO_3^- during the 24-h induction period (Table II); however, the levels were much less than those in NO_3^- -fed roots. NO_2^- also accumulated in both NO_2^- - and NO_3^- -fed roots, but the accumulation in NO_3^- -fed roots was only 10 to 15% of that in NO_2^- -fed roots. In both cases, the concentration of NO_2^- decreased with time.

Concentration Dependence of Induction

To elucidate further the concentration dependence of induction on NO_3^- and NO_2^- accumulation, seedlings were induced for 4 h in a range of substrate concentrations up to $1000 \mu\text{M}$ (Fig. 3). The induction of both uptake systems was

Table II. Time course of the accumulation of NO_3^- and NO_2^- in roots of intact barley seedlings supplied with $1000 \mu\text{M}$ NO_3^- or NO_2^-

For experimental details and NO_3^- and NO_2^- uptake rates see Figure 2.

Induction Period	[NO_3^-]		[NO_2^-]	
	NO_3^- fed	NO_2^- fed	NO_3^- fed	NO_2^- fed
<i>h</i>	$\mu\text{mol g}^{-1}$			
0	0.01 ^a	0.01	0.0	0.0
1	1.05	0.05	0.03	1.1
2	3.47	0.10	0.03	2.13
3	7.25	0.16	0.06	3.25
4	10.71	0.21	0.08	2.81
6	15.31	0.36	0.13	1.82
12	28.60	1.05	0.09	1.48
24	42.44	1.52	0.07	0.76

^a Average coefficient of variation for this data set was 9%.

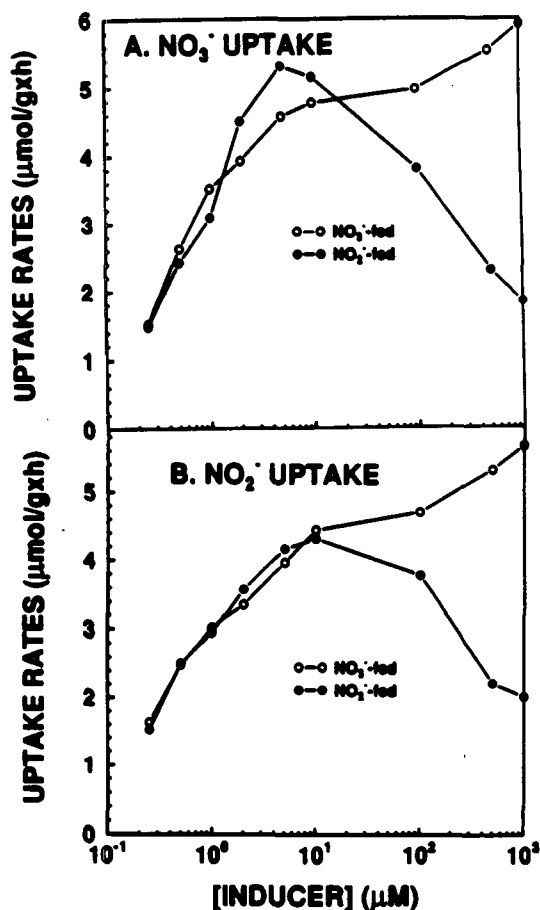


Figure 3. Induction of NO_3^- and NO_2^- uptake systems in roots of intact seedlings supplied with varying concentrations of NO_3^- or NO_2^- for 4 h. For experimental details see Figures 1 and 2. Uptake rates were determined by analyzing the depletion of the ions from $200 \mu\text{M}$ solutions for 12 min (sampling every 1.5 min) at each time interval.

linear up to 5 μM, and then NO₃⁻ transport gradually increased with increasing concentrations up to 1000 μM NO₃⁻. In contrast, the induction of both transport systems significantly decreased at concentrations of NO₂⁻ greater than 10 μM.

The accumulation of NO₃⁻ increased with increasing concentration of NO₃⁻ in the induction solution. On the other hand, only a slight accumulation of NO₂⁻ occurred at NO₃⁻ concentrations greater than 100 μM (Table III). In contrast, no accumulation of NO₃⁻ occurred until roots were supplied with 5 μM and greater NO₂⁻ for 4 h; no accumulation of NO₂⁻ occurred up to 10 μM NO₂⁻. However, at higher concentrations of NO₂⁻ (100 μM and more), both ions accumulated, with the accumulation of NO₂⁻ being much greater than that of NO₃⁻. After 24 h, a significant level of NO₃⁻ had accumulated in roots supplied with 5 μM NO₂⁻, and the accumulation increased with increasing NO₂⁻ concentrations (data not shown).

Kinetics of the Inducible Uptake Systems

The uptake kinetics for both systems induced by ambient NO₂⁻ (Fig. 4) were similar to the kinetics of uptake induced by NO₃⁻. The apparent *K_m* values for the NO₃⁻ and NO₂⁻ uptake systems induced by NO₂⁻ were, respectively, 30 and 64 μM (Fig. 4B). The *V_{max}* of NO₃⁻ and NO₂⁻ uptake systems were, respectively, 6.6 and 8.0 μmol g⁻¹ h⁻¹. Corresponding *K_m* and *V_{max}* values for NO₃⁻-induced systems were about 35 and 45 μM and 8.4 and 6.6 μmol g⁻¹ h⁻¹, respectively, for NO₃⁻ and NO₂⁻ (Aslam et al., 1992a).

Time Course and Concentration Dependence of Induction of NRA and NiRA

NRA and NiRA were also induced by both ions (Fig. 5). However, unlike induction of the uptake systems, induction of the enzyme systems did not show a lag at 1000 μM NO₂⁻ (compare Figs. 2 and 5). The levels of the enzyme activities

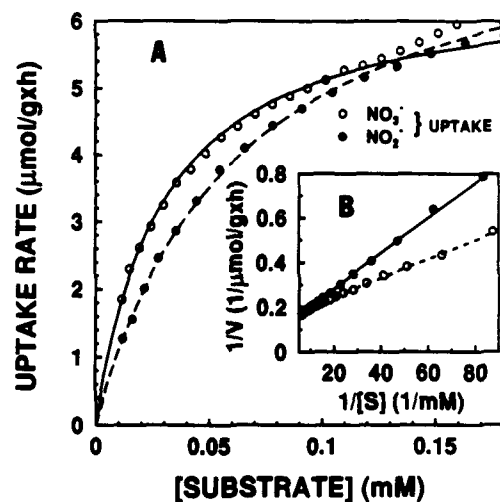


Figure 4. Kinetics of inducible NO₃⁻ and NO₂⁻ uptake systems in roots of intact seedlings induced with NO₂⁻. Seedlings were induced with 1000 μM NO₂⁻ for 24 h as described in Figure 2. Uptake rates were then determined at 3-min intervals by analyzing the depletion of the ions from uptake solutions initially containing 175 μM NO₃⁻ or NO₂⁻. A, Rates of uptake as a function of substrate concentration. The Michaelis-Menten equations are, for NO₃⁻, $y = 6.64 \mu\text{mol g}^{-1} \text{h}^{-1} [S] (0.030 \text{ mM})^{-1} + [S]^{-1}$ and, for NO₂⁻, $y = 7.99 \mu\text{mol g}^{-1} \text{h}^{-1} [S] (0.064 \text{ mM})^{-1} + [S]^{-1}$, where *S* is the substrate. B, Lineweaver-Burk plot of the data in A. The regression equations of the double-reciprocal plots are, for NO₃⁻, $y = 0.15056 + 0.004477x$ ($r^2 = 0.9984$) and, for NO₂⁻, $y = 0.12799 + 0.007834x$ ($r^2 = 0.9978$). The apparent *K_m* values for NO₃⁻ and NO₂⁻, respectively, were 30 and 64 μM, and *V_{max}* values were 6.6 and 8.0 μmol g⁻¹ of root h⁻¹.

induced at 10 μM substrate were about two-thirds of those induced at 1000 μM substrate. Also, whereas the levels of NRA induced by ambient NO₂⁻ were about 60 to 75% of that induced by NO₃⁻ (Fig. 5A), similar levels of NiRA were induced by both NO₃⁻ and NO₂⁻ at each substrate concentration (Fig. 5B). At 1 μM substrate, some induction of NRA occurred (Fig. 5A); however, no NiRA was detected. This was probably due to the lower sensitivity of the NiRA assay.

Increasing concentrations of NO₃⁻ or NO₂⁻ in the induction solutions led to increased levels of both enzyme activities in the roots (Fig. 6). The increase in enzyme activity in response to substrate was more rapid at concentrations of less than 100 μM. At greater than 100 μM, little increase in NRA occurred; however, NiRA continued to increase (Fig. 6). Similar levels of NiRA were induced by both NO₃⁻ and NO₂⁻ (Fig. 6B); however, the level of NRA induced by NO₂⁻ was only 60 to 75% of that induced by NO₃⁻ (Fig. 6A).

Induction of Uptake and Reduction Systems after a 1-h Pulse with Substrates

The induction of both the NO₃⁻ and NO₂⁻ uptake systems increased gradually during a 6-h period following a 1-h pulse with 250 μM NO₃⁻ or NO₂⁻ (Fig. 7). In contrast, NRA and NiRA increased only during the initial 2 h (Table IV). Although the levels of NRA were similar in both NO₃⁻ and NO₂⁻-pulsed roots, the levels of NiRA were about 2-fold

Table III. NO₃⁻ and NO₂⁻ accumulation in roots of intact barley seedlings supplied with different concentrations of NO₃⁻ or NO₂⁻ for 4 h

For experimental details and NO₃⁻ and NO₂⁻ uptake rates see Figure 3.

[Substrate]	[NO ₃ ⁻]		[NO ₂ ⁻]	
	NO ₃ ⁻ fed	NO ₂ ⁻ fed	NO ₃ ⁻ fed	NO ₂ ⁻ fed
μM	nmol g ⁻¹			
0	10 ^a	10	0	0
0.25	10	8	0	0
0.50	41	11	0	0
1	267	13	0	0
2	572	12	0	0
5	1224	27	0	0
10	2040	64	0	0
100	5394	122	38	622
500	7465	182	53	1851
1000	9250	224	65	2918

^a Average coefficient of variation for this data set was 13%.

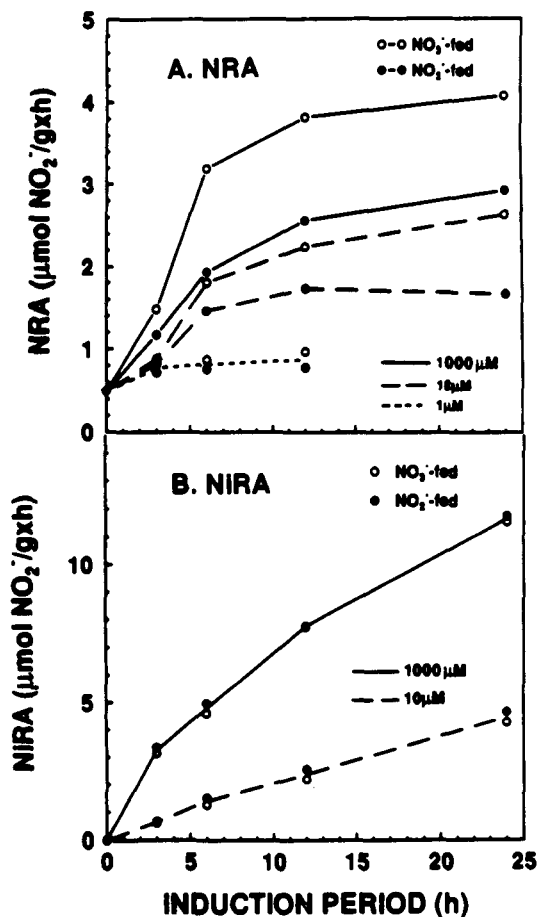


Figure 5. Time course of the induction of NRA and NiRA in roots of intact seedlings supplied with 1 μM (broken lines), 10 μM (dotted lines), and 1000 μM (solid lines) NO_3^- or NO_2^- . Seedlings were grown and induced as described in Figures 1 and 2. The enzyme activities were determined at various intervals during the induction.

higher in roots pulsed with NO_2^- as compared to NO_3^- -pulsed roots. The concentration of NO_3^- in roots pulsed with NO_3^- decreased gradually, whereas NO_2^- taken up by roots pulsed with NO_2^- disappeared during the initial 2 h (Table V).

Effect of CHI on the Induction of Uptake and Reduction Systems

The induction of both NO_3^- and NO_2^- uptake systems as well as NRA and NiRA was inhibited by CHI (Table VI). This indicates that the induction of both uptake and reduction systems by NO_3^- and NO_2^- requires the synthesis of new protein(s).

DISCUSSION

Induction of Uptake Systems

The results presented here support the hypothesis that either ion will induce NO_3^- or NO_2^- uptake. Furthermore, these results, together with the observation that both ions

are competitive inhibitors of the uptake of either ion, suggest that they share the same transporter(s) and binding sites. The alternative to this is that NO_3^- , resulting from the internal oxidation of NO_2^- , is responsible for induction. In fact, we did observe an increase in internal NO_3^- in roots incubated in NO_2^- (Tables I and II). The oxidation of NO_2^- to NO_3^- in several plant species has been reported: barley leaves (Kaplan et al., 1974; Aslam et al., 1987; Aslam and Huffaker, 1989), bean cotyledons (Lips et al., 1973), and pea roots (Sahulka and Lisa, 1978). Muhammad and Kumazawa (1974) reported the appearance of NO_3^- in rice seedlings when NH_4^+ was the substrate. However, equal induction in roots supplied with 0.25 to 2.0 μM substrates (Figs. 1 and 3), when no net accumulation of either ion occurred during the first 2 to 3 h (Tables I and III), indicates that accumulation of either NO_3^- or NO_2^- is not obligatory for the induction of the uptake systems. Siddiqi et al. (1992) also concluded that NO_2^- will

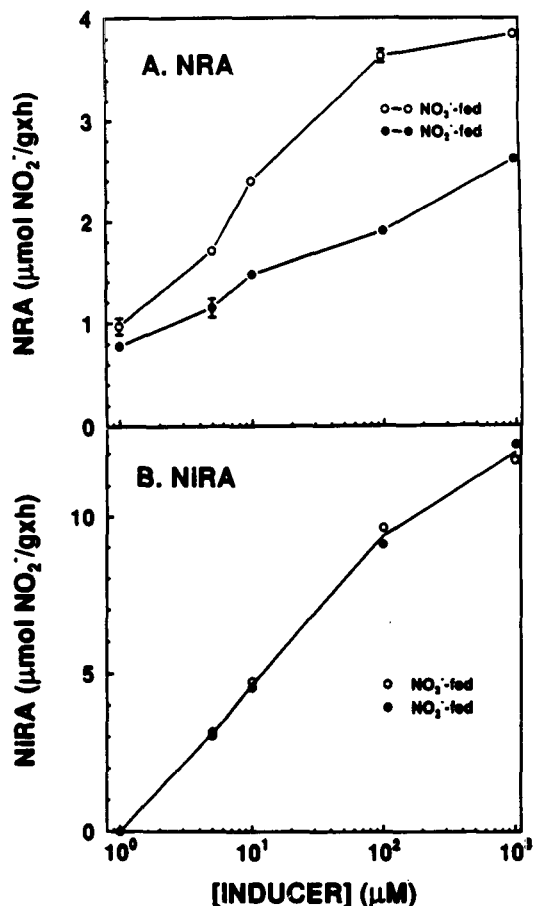


Figure 6. Effect of different concentrations of NO_3^- and NO_2^- on the induction of NRA (A) and NiRA (B) in roots of intact seedlings. Seedlings were induced as described in Figures 1 and 2, except that the induction solutions contained different concentrations of NO_3^- and NO_2^- . The induction solutions were changed after 12 h. Enzyme activities were assayed after 24 h of induction. The depletion of either ion from its respective induction solutions did not exceed 20%. NRA and NiRA in roots of uninduced seedlings were, respectively, 0.32 ± 0.04 and $0 \mu\text{mol of NO}_2^- \text{g}^{-1} \text{h}^{-1}$.

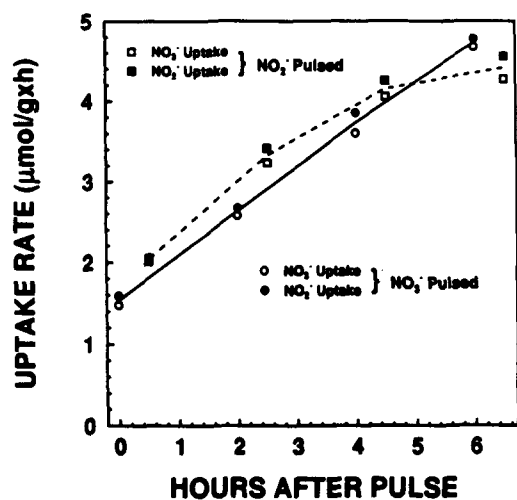


Figure 7. Induction of the NO₃⁻ and NO₂⁻ uptake systems in roots of intact seedlings pulsed with 250 μM NO₃⁻ or NO₂⁻ for 1 h. Uninduced seedlings were supplied with 250 μM NO₃⁻ or NO₂⁻ for 1 h. The seedlings were then rinsed with distilled water and transferred to the N-free solutions. Rates of NO₃⁻ and NO₂⁻ uptake were determined at different intervals as described in Figure 1. See Tables IV and V for corresponding enzyme activities and NO₃⁻ and NO₂⁻ concentrations, respectively.

induce NO₃⁻ uptake, but their results did not rule out the possibility that NO₃⁻, resulting from NO₂⁻ oxidation, could have caused the induction.

The NO₃⁻- and NO₂⁻-induced systems showed similar activity patterns at low substrate concentrations (Figs. 1 and 3); however, they responded differently to increasing substrate concentrations. At higher substrate levels, there was a longer lag in the induction of both systems by NO₂⁻ (Fig. 2), and induction by 1000 μM NO₂⁻ was slower than that by 10 μM NO₂⁻ (Figs. 1–3). This difference is likely due to a toxic effect of NO₂⁻. At 1000 μM NO₂⁻, there was an initial buildup of NO₂⁻ in the roots (Table II). As the NO₂⁻ concentration in NO₂⁻-fed roots decreased with time, a concomitant increase in uptake activity occurred (Fig. 2).

Kinetic data reported here (see Aslam et al., 1992b, for a preliminary report) suggest that the uptake systems are sim-

Table IV. Induction of NRA and NiRA in roots of intact barley seedlings pulsed with 250 μM NO₃⁻ or NO₂⁻ for 1 h

For experimental details and corresponding NO₃⁻ and NO₂⁻ uptake rates see Figure 7.

Period after Pulse	NRA		NiRA	
	Pulsed with NO ₃ ⁻	Pulsed with NO ₂ ⁻	Pulsed with NO ₃ ⁻	Pulsed with NO ₂ ⁻
<i>h</i>	μmol NO ₂ ⁻ g ⁻¹ h ⁻¹			
0	0.80 ^a	0.82	0.85	0.99
2	1.02	1.26	1.28	2.56
4	0.96	1.10	1.49	2.61
6	0.97	1.09	1.30	2.52

^a Average coefficient of variation for this data set was 5%.

Table V. NO₃⁻ and NO₂⁻ concentrations in roots of intact seedlings pulsed with 250 μM NO₃⁻ or NO₂⁻ for 1 h

See Figure 7 for experimental details and corresponding uptake rates. See Table IV for corresponding enzyme activities.

Period after Pulse	[NO ₃ ⁻]		[NO ₂ ⁻]	
	Pulsed with NO ₃ ⁻	Pulsed with NO ₂ ⁻	Pulsed with NO ₃ ⁻	Pulsed with NO ₂ ⁻
<i>h</i>	nmol g ⁻¹			
0	772 ^a	20	0	880
2	689	12	0	0
4	614	10	0	0
6	472	4	0	0

^a Average coefficient of variation for this data set was 8%.

ilar whether induction is by NO₃⁻ or NO₂⁻. For example, the *K_m* values of both systems, when induction was by NO₂⁻ (Fig. 4), were similar to those obtained with induction by NO₃⁻ (Aslam et al., 1992a). Further evidence in support of this hypothesis is the similar competitive, reciprocal inhibition of both NO₂⁻-induced (our unpublished data) and NO₃⁻-induced systems (Aslam et al., 1992a). In *Aspergillus nidulans*, the *crnA* gene product, which is responsible for encoding the NO₃⁻ transporter, is induced either by NO₃⁻ or NO₂⁻ (Unkles et al., 1991). Thus, not only do the two ions apparently share the same transport system, it appears that either will induce that system.

It has been proposed that induction of the uptake systems by NO₂⁻ was a "consequence of NO₂⁻ metabolism rather than NO₂⁻ per se" (Breteler and Luczak, 1982). These investigators reported a decrease in the lag period of NO₃⁻ uptake in roots of dwarf beans pretreated with NO₂⁻. They also suggested that a similar "consequence" causes induction of NO₃⁻ uptake in NO₃⁻-fed roots. Induction of either system by possible changes in N status of the seedlings was ruled out in our study by noting that NH₄⁺ did not promote induction (results not shown).

Table VI. Effect of CHI on the induction of NO₃⁻ and NO₂⁻ uptake systems and reductases in roots of intact barley seedlings supplied with 100 μM substrates

Seedlings were induced with 100 μM NO₃⁻ or NO₂⁻ solutions containing 0 or 8 μM CHI. NO₃⁻ uptake rates and NRA and NiRA were determined after 6 h of induction. Uptake rates were determined as described in Figure 1.

[CHI]	Uptake Rate		Enzyme Activity	
	NO ₃ ⁻	NO ₂ ⁻	NRA	NiRA
μM	μmol g ⁻¹ h ⁻¹		μmol NO ₂ ⁻ g ⁻¹ h ⁻¹	
NO ₃ ⁻ -fed seedlings				
0	6.02 ^a	5.41	2.52	3.62
8	0.81	0.57	0.51	1.25
NO ₂ ⁻ -fed seedlings				
0	6.41	5.67	1.84	3.39
8	0.75	0.65	0.35	1.06

^a Average coefficient of variation for the data set was 14%.

The mechanism by which NO_3^- and NO_2^- stimulate the induction process in the absence of cellular accumulation of the substrate is an open question. It is plausible that, as NO_3^- or NO_2^- is transported across the plasma membrane by constitutive uptake systems (Behl et al., 1988; Aslam et al., 1992a), either anion may trigger the induction of uptake. A 1-h pulse with either 250 μM NO_3^- or 250 μM NO_2^- was equally effective in inducing both the NO_3^- and NO_2^- uptake systems (Fig. 7), although both ions totally disappeared 2 h after the pulse with NO_2^- (Table V). These results indicate that induction of the uptake systems does not require a continuous supply of NO_3^- or NO_2^- . In contrast, Minotti et al. (1968) and Rao and Rains (1976) suggested that the synthesis of the protein that accelerates NO_3^- uptake depended on the level of NO_3^- entering the roots. In corn roots, a 1-h pulse with 10 to 250 μM NO_3^- accelerated the development of the NO_3^- uptake system similarly during the subsequent 3 h (MacKown and McClure, 1988). MacKown and McClure concluded that the development of the NO_3^- -induced NO_3^- uptake system can be achieved by a small accumulation of endogenous NO_3^- .

Induction of both uptake systems by either NO_3^- or NO_2^- was inhibited by CHI (Table VI). This suggests that induced transport was not the result of activation of preexisting proteins but required the synthesis of new proteins.

Induction of NR

NR was induced by NO_2^- , albeit at levels only 60 to 75% of those induced by NO_3^- , at all substrate concentrations (Fig. 5A). This is in conflict with the results of Siddiqi et al. (1992), who found that NO_2^- did not induce NR. In our study, the accumulation of NO_3^- in the roots supplied with higher concentrations of NO_2^- suggests that enzyme induction may have resulted from NO_3^- produced by the oxidation of absorbed NO_2^- . However, there was no relationship between NR and NO_3^- concentration. In roots supplied with NO_2^- , the accumulation of NO_3^- was much less than in NO_3^- -supplied roots (Table II); yet, NRA was only about 25% lower (Fig. 5A). The induction of NR at 1 μM NO_2^- (Fig. 5A) without significant accumulation of NO_3^- or NO_2^- (Table I) indicates that, similar to induction of the uptake systems, the accumulation of the substrate is not required for the induction of the enzyme activities. Thus, at 1 μM NO_3^- , NRA did not increase after 3 h (Fig. 5A), although NO_3^- accumulation continued to increase (Table I). This is in contrast to the situation in leaves in which a positive correlation was observed between NR induction and NO_3^- concentration and no NRA was detected in the NO_2^- -fed leaves unless NO_3^- accumulated in those leaves (Aslam et al., 1987).

The induction of NRA by both ions was inhibited by CHI (Table VI). This indicates that increased activity was a result of new protein synthesis rather than activation of a preexisting protein. In contrast, Kaplan et al. (1978) reported in vitro activation of NRA by NO_2^- . We were unable to detect any in vitro activation of NRA by NO_2^- (data not shown). Kaplan et al. (1978) reported that the NO_3^- -induced and NO_2^- -activated NRs are different in that the mol wt of NO_2^- -activated NR is lower compared with that of the NO_3^- -induced protein. They postulated that the NO_2^- -activated

component of NR is a constitutive subunit that becomes incorporated into the NR protein during the synthesis of the Cyt *c* component (Kaplan et al., 1979).

Induction of NiR

Unlike NR, both the activity and the time course for NiR induction by NO_2^- were similar to those obtained with NO_3^- (Figs. 5B and 6B). The induction of NiR was not inhibited by WO_4^{2-} in either NO_3^- - or NO_2^- -fed roots (data not shown), indicating that in NO_3^- -fed roots an inhibition of the reduction of NO_3^- to NO_2^- did not affect the induction of NiR. de la Haba et al. (1990) reported that in sunflower roots WO_4^{2-} completely inhibited the induction of NR without affecting the induction of NiR. WO_4^{2-} also did not inhibit the induction of NiR by NO_3^- in barley leaves (Aslam et al., 1987), wheat embryos (Gupta et al., 1983), or tobacco cells (Kelker and Filner, 1971). Like the induction of NRA, the induction of NiR by both NO_3^- and NO_2^- was inhibited by CHI (Table VI), indicating the requirement for new protein synthesis.

The mechanism by which NO_2^- induces NiR is open to speculation. It is possible that NO_3^- produced by the oxidation of NO_2^- in the roots is also the inducer of NiR. However, unlike the induction of NR, the absence of any lag period in the induction of NiR by NO_2^- (Fig. 5B) indicates that NO_2^- induces NiR directly. The greater amounts of NiR in roots pulsed with NO_2^- support this conjecture (Table IV). Although the roots pulsed with NO_3^- had much higher concentrations of NO_3^- , the activity of NiR was lower than in roots pulsed with NO_2^- (Table IV). This is in contrast to leaves in which a positive correlation occurred between NO_3^- concentration and NiR even when the leaves were supplied with NO_2^- (Aslam and Huffaker, 1989).

In summary, our results show that both the NO_3^- and NO_2^- uptake systems and the corresponding reductases are induced by either NO_3^- or NO_2^- . Induction of both uptake and reduction systems occurred well before any NO_3^- or NO_2^- accumulated in the tissue. This eliminates the possibility that NO_3^- , resulting from the oxidation of NO_2^- , is responsible for induction. Inhibition of NO_2^- induction of NR and NiR by CHI indicates that the inductions require synthesis of new protein(s). The results indicate that, in barley roots, the induction of the uptake and reduction systems by NO_3^- and NO_2^- may occur by similar mechanism(s).

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LITERATURE CITED

- Aguera E, de la Haba P, Fontes AG, Maldonado JM (1990) Nitrate and nitrite uptake and reduction by intact sunflower plants. *Planta* 182: 149–154
- Aslam M, Harbit KB, Huffaker RC (1990) Comparative effects of selenite and selenate on nitrate assimilation in barley seedlings. *Plant Cell Environ* 13: 773–782
- Aslam M, Huffaker RC (1989) Role of nitrate and nitrite in the induction of nitrite reductase in leaves of barley seedlings. *Plant Physiol* 91: 1152–1156
- Aslam M, Huffaker RC, Rains WD, Rao KP (1979) Influence of light and ambient carbon dioxide concentration on nitrate assimilation by intact barley seedlings. *Plant Physiol* 63: 1205–1209
- Aslam M, Rosichan JL, Huffaker RC (1987) Comparative induction

- of nitrate reductase by nitrate and nitrite in barley leaves. *Plant Physiol* **83**: 579–584
- Aslam M, Travis RL, Huffaker RC** (1992a) Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. *Plant Physiol* **99**: 1124–1133
- Aslam M, Travis RL, Huffaker RC** (1992b) Comparative induction of nitrate and nitrite transporters and reductases by ambient nitrate and nitrite in barley roots (abstract No. 561). *Plant Physiol* **99**: S-94
- Behl R, Tischner R, Raschke K** (1988) Induction of a high capacity nitrate-uptake mechanism in barley roots prompted by nitrate uptake through a constitutive low-capacity mechanism. *Planta* **176**: 235–240
- Breteler H, Luczak W** (1982) Utilization of nitrate and nitrite by dwarf bean. *Planta* **156**: 226–232
- de la Haba P, Aguera E, Maldonado JM** (1990) Differential effects of ammonium and tungsten on nitrate and nitrite uptake and reduction by sunflower plants. *Plant Sci* **70**: 21–26
- Goyal SS, Huffaker RC** (1986a) A novel approach and a fully automated microcomputer-based system to study kinetics of NO₃⁻, NO₂⁻, and NH₄⁺ transport simultaneously by intact wheat seedlings. *Plant Cell Environ* **9**: 209–215
- Goyal SS, Huffaker RC** (1986b) The uptake of NO₃⁻, NO₂⁻, and NH₄⁺ by intact wheat (*Triticum aestivum*) seedlings. I. Induction and kinetics of transport systems. *Plant Physiol* **82**: 1051–1056
- Gupta A, Disa S, Saxena IM, Sarin NB, Guha-Mukherjee S, Sopory SK** (1983) Role of nitrate in the induction of nitrite reductase activity during wheat seed germination. *J Exp Bot* **34**: 396–404
- Heimer YM** (1975) Nitrite-induced development of the nitrate uptake system in plant cells. *Plant Sci Lett* **4**: 137–139
- Hoagland DR, Arnon DI** (1950) The water culture method for growing plants without soil. *Calif Agric Exp Stn Bull* **347**: 32
- Hole DJ, Emran AM, Fares Y, Drew MC** (1990) Induction of nitrate transport in maize roots, and kinetics of influx, measured with nitrogen-13. *Plant Physiol* **93**: 642–647
- Jackson WA, Flesher D, Hageman RH** (1973) Nitrate uptake by dark-grown corn seedlings. Some characteristics of apparent induction. *Plant Physiol* **51**: 120–127
- Jackson WA, Johnson RE, Volk RJ** (1974a) Nitrite uptake by nitrogen-depleted wheat seedlings. *Physiol Plant* **32**: 37–42
- Jackson WA, Johnson RE, Volk RJ** (1974b) Nitrite uptake patterns in wheat seedlings as influenced by nitrate and ammonium. *Physiol Plant* **32**: 108–114
- Kaplan D, Mayer AM, Lips SH** (1978) Nitrite activation of nitrate reductase in higher plants. *Planta* **138**: 205–209
- Kaplan D, Mayer AM, Lips SH** (1979) Effect of cyanide and nitrite on the activity of nitrate reductase. In EJ Hewitt, CV Cutting, eds, *Nitrogen Assimilation of Plants*. Academic Press, New York, pp 315–320
- Kaplan D, Roth-Bejerano N, Lips H** (1974) Nitrate reductase as a product inducible enzyme. *Eur J Biochem* **49**: 393–398
- Kelker HC, Filner P** (1971) Regulation of nitrite reductase and its relationship to the regulation of nitrate reductase in cultured tobacco cells. *Biochim Biophys Acta* **252**: 69–82
- Kuo T-M, Warner RL, Kleinhofs A** (1982) *In vitro* stability of nitrate reductase from barley leaves. *Phytochemistry* **21**: 531–533
- Lips SH, Kaplan D, Roth-Bejerano N** (1973) Studies on the induction of nitrate reductase by nitrite in bean seed cotyledons. *Eur J Biochem* **37**: 589–592
- MacKown CT, McClure PR** (1988) Development of accelerated net nitrate uptake. Effects of nitrate concentration and exposure time. *Plant Physiol* **87**: 162–166
- Minotti PL, Williams DC, Jackson WA** (1968) Nitrate uptake and reduction as affected by calcium and potassium. *Soil Sci Soc Am Proc* **32**: 692–698
- Muhammad S, Kumazawa K** (1974) Assimilation and transport of nitrogen in rice. 1. ¹⁵N-labelled ammonium nitrogen. *Plant Cell Physiol* **15**: 747–758
- Rao KP, Rains DW** (1976) Nitrate absorption by barley. II. Influence of nitrate reductase activity. *Plant Physiol* **57**: 59–62
- Sahulka J, Lisa L** (1978) The influence of sugars on nitrate reductase induction by exogenous nitrate or nitrite in excised *Pisum sativum* roots. *Biol Plant* **20**: 359–367
- Siddiqi MY, Glass ADM, Ruth TJ, Ruffy TW** (1990) Studies of the uptake of nitrate in barley. I. Kinetics of ¹³NO₃⁻ influx. *Plant Physiol* **93**: 1426–1432
- Siddiqi MY, King BJ, Glass ADM** (1992) Effects of nitrite, chlorate, and chlorite on nitrate uptake and nitrate reductase activity. *Plant Physiol* **100**: 644–650
- Thayer JR, Huffaker RC** (1980) Determination of nitrate and nitrite by high-pressure liquid chromatography: comparison with other methods for nitrate determination. *Anal Biochem* **102**: 110–119
- Tompkins GA, Jackson WA, Volk RJ** (1978) Accelerated nitrate uptake in wheat seedlings: effects of ammonium and nitrite pre-treatments and of 6-methylpurine and puromycin. *Physiol Plant* **43**: 166–171
- Unkles SE, Hawker KL, Grieve C, Campbell EI, Montague P, Kinghorn JR** (1991) CRNA encodes a nitrate transporter in *Aspergillus-Nidulans*. *Proc Natl Acad Sci USA* **88**: 204–208
- Warner RL, Huffaker RC** (1989) Nitrate transport is independent of NADH and NAD(P)H nitrate reductases in barley seedlings. *Plant Physiol* **91**: 947–953