Plant Physiol. (1981) 68, 1488–1493
0032-0889/81/68/1488/06/$00.50/0

Evolution of Nitrogen Oxide(s) during *In Vivo* Nitrate Reductase Assay of Soybean Leaves

Received for publication May 29, 1981 and in revised form July 28, 1981

JAMES E. HARPER
United States Department of Agriculture, Department of Agronomy, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Studies were conducted to quantitate the evolution of nitrogen oxides ($NO_{(x)}$) from soybean [*Glycine max* (L.) Merr.] leaves during *in vivo* nitrate reductase (NR) assays with aerobic and anaerobic gas purging. Anaerobic gas purging (N_2 and argon) consistently resulted in greater $NO_{(x)}$ evolution than did aerobic gas purging (air and O_2). The evolution of $NO_{(x)}$ was dependent on gas flow rate and on NO_2^- formation in the assay medium; although a threshold level of NO_2^- appeared to exist beyond which the rate of $NO_{(x)}$ evolution did not increase further.

The loss of $NO_{(x)}$ from *in vivo* NR assays under gas purging explains partially, but not stoichiometrically, the decrease in NO_2^- accumulation in *in vivo* NR assay medium with young soybean leaves. The lack of stoichiometry between $NO_{(x)}$ evolution and apparent NO_2^- loss suggests that other mechanisms are also involved in loss of NO_2^- or inhibition of formation of NO_2^- during anaerobic and aerobic incubation conditions imposed on the *in vivo* NR assay of soybean. The mechanism of $NO_{(x)}$ evolution under the assay conditions imposed and the relevance of this phenomenon to intact plants remains unclear.

The *in vivo* NR[1] assay is widely used among laboratories and with several plant species, having generally replaced the more time consuming *in vitro* assay. Assay conditions vary considerably among laboratories and optimum conditions are somewhat species-dependent. With soybeans [*Glycine max* (L.) Merr.] the *in vivo* NR assay has been optimized for leaves (10, 13, 17) and roots (7), and the assay has been used to compare estimated reduced N input with actual (Kjeldahl) reduced N accumulation (7, 9, 11). One notable difference between optimized *in vivo* NR assays of soybean roots and leaves was that roots required N_2 purging during assay (7), while N_2 purging of leaves during assay resulted in inconsistent results, ranging from slight stimulation to marked inhibition of nitrite accumulation (unpublished data).

It has been shown that air purging of herbicide-treated soybean leaves results in evolution of NO and NO_2 (collectively $NO_{(x)}$) (16). The reaction mechanism(s) leading to $NO_{(x)}$ evolution are unknown, although Klepper (16) has proposed that accumulated NO_2^- reacts with plant metabolite(s) to form NO and NO_2. Nitrogen oxide (NO) seems to be the primary gas form evolved (16), since NO_2 is known to be readily soluble in aqueous solution, forming NO_2^- and NO_3^-; the equilibrium of the latter two ions being dependent on solution conditions (1).

The above observations suggested that similar evolution of $NO_{(x)}$ gasses may be occurring during gas purging of the *in vivo* NR assay medium. In addition, it was expected that N_2, air, and O_2 purging of the *in vivo* NR assay medium would result in differential accumulation of NO_2^-, due to a more complete blockage of further reduction of NO_2^- to NH_4^+ under more anaerobic conditions. The objectives of this study were to (a) establish why N_2 purging of the *in vivo* NR assay medium resulted in inconsistent NO_2^- accumulation relative to no gas purging, and (b) determine if evolution of $NO_{(x)}$ gasses under anaerobic and aerobic gas purging of the *in vivo* NR assay medium could account for the decrease in nitrite accumulation during NR assays of physiologically young soybean leaves.

MATERIALS AND METHODS

Plant Growth and Sampling

Greenhouse Study. A preliminary study involved NR assays of soybeans [*Glycine max* (L.) Merr. cv. Wells] grown in greenhouse gravel-culture systems, similar to field systems previously described (8). Plants were subirrigated four times daily with a modified one-fourth strength Hoagland solution containing 3.75 mM NO_3^- (8). Natural greenhouse light was supplemented with mercury vapor lamps at a quantum flux of 200 $\mu E\ m^{-2}\ s^{-1}$ (400–700 nm as measured with a LI-170 quantum sensor,[2] Lambda Instruments Co., Lincoln, NE) for a 14-h light period at 27 to 30°C; nighttime temperatures ranged from 17 to 21°C. Plants were sampled at intervals from 11 to 25 DAP for analysis of leaf NR activity. Twenty seedlings were harvested at each sampling time and leaflets from respective nodes were composited and subsampled, 20 leaf discs (1 cm diameter) for each sample (≈0.2 g tissue).

Growth Chamber Studies. Soybean seeds were planted in sand moistened with deionized H_2O and germinated in environmental chambers. Growth conditions were 14 h, 28°C light and 10 h, 18°C dark. Light was supplied by fluorescent and incandescent lamps at a quantum flux of 700 $\mu E\ m^{-2}\ s^{-1}$. Seedlings emerged on day 5 from planting and were either (a) watered on days 7 and 9 with full strength Hoagland nutrient solution (15 mM NO_3^-) and harvested on day 11 directly from the sand trays, or (b) transplanted on day 7 into 2-liter black polystyrene containers (six plants per container) which contained full strength Hoagland nutrient solution. The seedlings which were transplanted were grown under the same environmental conditions described above for an additional 14 days and harvested 21 DAP. Nutrient solutions were aerated continuously and changed 14 and 18 DAP.

For studies involving 11-day-old plants, the unifoliolate leaves from 20 plants were divided into two groups, one leaflet from each

[1] Abbreviations: NR, nitrate reductase; $NO_{(x)}$, refers collectively to nitric oxide (NO) and nitrogen dioxide (NO_2); DAP, days after planting.

[2] Mention of a trademark, vendor, or proprietory product does not constitute a guarantee or warranty of the vendor or product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other vendors or products that may also be suitable.

plant in each group. Six subsamples, consisting of a 1-cm diameter leaf disc from each of 20 leaflets, were taken and weighed (≃0.2 g samples) from each of the two groups of leaflets. The resulting 12 subsamples were considered equivalent, and four assay conditions, three replications per assay, were compared with a given set of plants. Due to the inherent variability of the plant material from one sampling time to the next (slight variation in growth chamber conditions, germination rates, etc.) each experiment had internal controls against which like experiments were normalized.

For the study involving 21-day-old plants, 10 plants were composited for a sample and unifoliolate leaves and 1st, 2nd, and 3rd trifoliolate leaves were analyzed separately. Both of the unifoliolate leaflets were sampled while the two lateral leaflets from each of the trifoliolate leaves were sampled. Six subsamples, as described above, were taken for each age of leaves. This provided material for three replications and two assay treatments within each leaf age, unifoliolate through 3rd trifoliolate.

ASSAYS

Nitrate Reductase. Nitrate reductase activity was determined using an *in vivo* assay technique described previously (17). Assays were either in the absence or presence of added NO_3^- (50 mM) as indicated in tables and figures, and all samples were vacuum-infiltrated prior to assay. The assay treatments were no gas purging (standard NR assay) and purging with nitrogen, air, oxygen, or argon (Union Carbide Corp., Linde Div., New York) at flow rates of 50, 100, 200, or 300 cm^3 min^{-1}. Nitrite formation in the incubation medium during the 30-min dark incubation period was measured with the Griess-Saltzman reagents as described previously (17).

$NO_{(x)}$ Evolution. Evolution of $NO_{(x)}$ gasses during the *in vivo* NR assay of growth chamber-growth plants was monitored under the various gas phases and flow rates designated above. The gas phase was metered (Matheson 603 flow tubes) to a manifold connected to either three or six *in vivo* NR assay tubes (25 × 150 cm). The *in vivo* NR assay tubes were foil-covered to exclude light and fitted with gas-tight rubber stoppers through which inlet and outlet 16-gauge needles were inserted. The inlet needle was inserted to a point near the bottom of the tube such that the gas phase was bubbling through the solution containing the leaf discs. The outlet needle extended just through the rubber stopper (above the liquid medium) and was attached to a glass column containing glass beads coated with a sulfuric acid-dichromate solid oxidizer as described by Klepper (16). After passing the $NO_{(x)}$ gas phase through the oxidizer column, the NO_2 gas formed was trapped by bubbling through a single fritted glass dispersion tube into 20 ml of trapping solution (7.5 g tartaric acid, 0.75 g sulfanilamide, 0.025 g *n*-1-naphthylethylene-diamine diHCl, and 0.025 g disodium 2-naphthol-3,6,-disulfonate per liter). The efficiency of the single oxidizer column and single trapping solution were both greater than 95% as determined by sequentially passing the gas phase through two oxidizer columns and then into a series of trapping solutions. Although $NO_{(x)}$ collectively refers to NO and NO_2, preliminary experiments indicated that only trace amounts of NO_2 were actually evolved from the *in vivo* NR assays as determined by passing the gas phase directly into trapping solution without going through an oxidizer column, the latter serving to oxidize NO to NO_2. Since NO_2 is readily soluble in water ($2\ NO_2 \rightarrow NO_2^- + NO_3^- + 2H^+$), it is unlikely that NO_2 would escape from the plant cells and aqueous *in vivo* NR assay medium even if it were formed in the leaf tissue.

A time course of $NO_{(x)}$ gas evolution was conducted by transferring the fritted glass dispersion tubes to fresh trapping solution every 5 min over a 30-min interval. Color was allowed to develop for at least 10 min before measuring *A* at 540 nm. The $NO_{(x)}$ trapped was determined by comparison with secondary standards prepared with sodium nitrite and expressed as μmol NO_2^- g^{-1} fresh weight 30 min^{-1}. No correction was made to account for the portion of NO_2 which was possibly converted to NO_3^- in the trapping solution. Therefore, the values presented for $NO_{(x)}$ evolution will be in all likelihood an underestimation (see Allen [1] for discussion of NO_2 reactions in Griess-Saltzman reagents).

RESULTS

Nitrite accumulation in the *in vivo* NR assay medium was either increased (in old leaves) or decreased (in young leaves) by N_2 gas purging of the assay medium, compared with controls in the absence of gas purging (Table I). Air purging resulted in less NO_2^- accumulation in the NR assay medium regardless of physiological leaf age, compared with controls in the absence of gas purging. Accumulation of NO_2^- under air purging was greater than (in young leaves) or less than (in old leaves) that observed with N_2 purging.

Subsequent studies showed that purging of the *in vivo* NR assay of leaf discs with either N_2 or air resulted in $NO_{(x)}$ evolution during a 30-min assay (Fig. 1). There was an initial lag in rate of $NO_{(x)}$ evolution with both N_2 and air purging, and maximum evolution rates were obtained within 15 min incubation time. The maximum rate of $NO_{(x)}$ evolution was sustained through 30 min with the exception of the $-NO_3^-$-NR assay purged with N_2 where $NO_{(x)}$ evolution rate declined. This decline appeared real in that the experiment was repeated twice more with identical trends.

Evolution of $NO_{(x)}$ gas from the *in vivo* NR assay medium was dependent on the flow rates of both the N_2 and air purge gasses (Fig. 2). Highest $NO_{(x)}$ evolution occurred at the 300 cm^3 min^{-1} N_2 flow rate (Fig. 2A) and N_2 gas purging resulted in more $NO_{(x)}$ evolution than did air purging, at each respective flow rate (Fig. 2, A and B). A lag in $NO_{(x)}$ evolution rate was again noted with both gas sources and with all flow rates, which in part was attributed to temperature equilibration going from an ice bath into the 30°C water bath for incubation. Rates of $NO_{(x)}$ evolution were nearly identical to those shown in Figure 2 when exogenous NO_3^- was omitted from the *in vivo* NR assay and the samples were subjected to the same gas phases and flow rates (data not shown).

No specific effect of gas flow rate on NO_2^- accumulation in the *in vivo* assay medium was evident when N_2 was the gas phase (Table II). With air as the gas phase, NO_2^- accumulation in the *in vivo* assay medium decreased when air flow rate increased from 50 to 100 cm^3 min^{-1} with both ± NO_3^--*in vivo* assays; further

Table I. *Effect of Gas Purging on +NO_3^--In Vivo Nitrate Reductase Activity of Soybeans*

Plants were grown in the greenhouse. Gas flow rates were approximately 100 cm^3 min^{-1} and NO_2^- formed in the assay medium was measured. Values presented are means ± SD of three replications.

Leaf Sampled	Days After Planting	Gas Phase		
		None	N_2	Air
		μmol NO_2^- g^{-1} fresh wt 30 min^{-1}		
Unifoliolate	11	8.6 ± 0.3	4.4 ± 1.2	6.8 ± 1.2
Unifoliolate	15	3.5 ± 0.4	4.5 ± 0.1	0.9 ± 0.1
1st trifoliolate	15	6.8 ± 0.9	4.1 ± 0.3	3.8 ± 0.6
Unifoliolate	18	6.9 ± 0.3	7.4 ± 0.1	3.8 ± 0.7
1st trifoliolate	18	9.9 ± 0.4	7.1 ± 0.3	8.1 ± 0.3
2nd trifoliolate	18	7.4 ± 0.2	3.2 ± 0.4	4.4 ± 0.3
Unifoliolate	25	3.4 ± 0.2	4.8 ± 0.2	1.0 ± 0.2
1st trifoliolate	25	5.0 ± 0.4	6.5 ± 0.6	2.6 ± 0.8
2nd trifoliolate	25	7.3 ± 0.3	5.4 ± 0.6	5.0 ± 0.5
3rd trifoliolate	25	7.5 ± 0.4	4.3 ± 1.3	4.5 ± 1.2

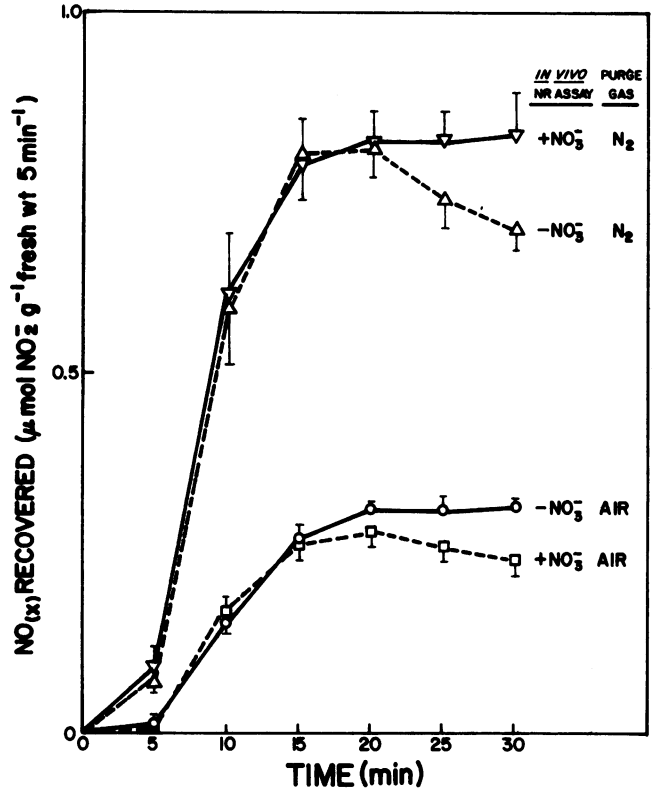

FIG. 1. Evolution of $NO_{(x)}$ gasses during N_2 or air purging of $\pm NO_3^-$-*in vivo* NR assays of soybean leaflets. Unifoliolate leaflets of growth chamber-grown plants were sampled 11 days after planting. Gas flow rates through the *in vivo* NR assay medium were 200 cm^3 min^{-1}. Each point represents the $NO_{(x)}$ trapped during the preceding 5-min interval. Values are means ± SD of three replications. The SD was within the data point where not shown.

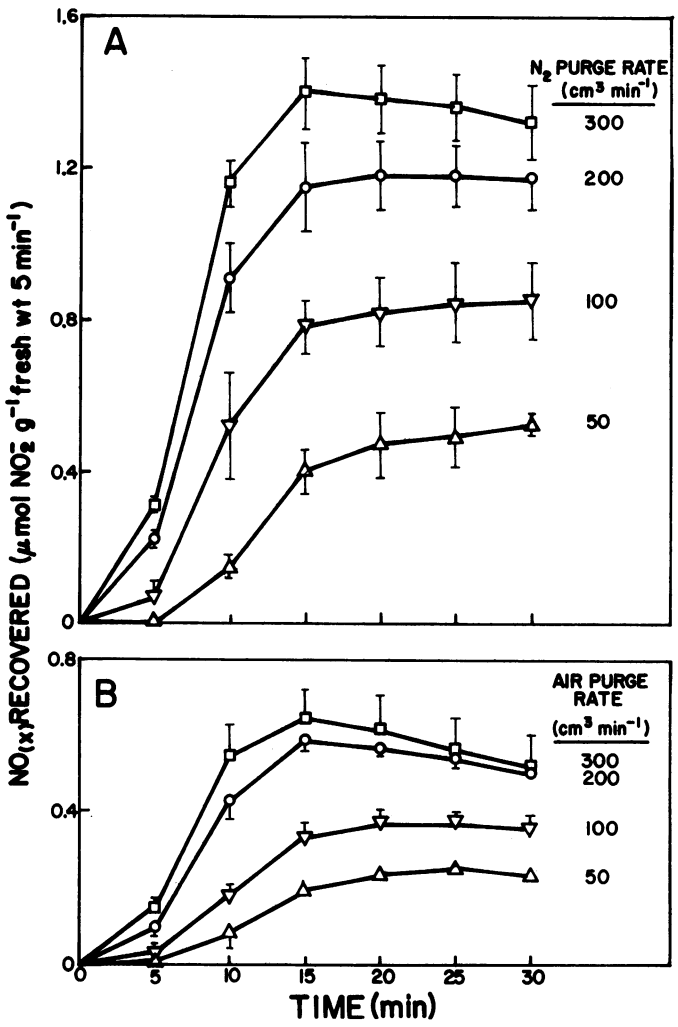

FIG. 2. Effect of gas flow rate on $NO_{(x)}$ gas evolution during N_2 or air purging of *in vivo* NR assays of soybean leaflets. The purge gas and *in vivo* NR assay condition were, respectively, N_2 and $+NO_3^-$ (A), air and $+NO_3^-$ (B). Other details, with exception of gas flow rates, were as given in Figure 1 legend.

changes were not obvious at 200 and 300 cm^3 min^{-1} flow rates. The cumulative $NO_{(x)}$ recovered as NO_2^- from the gas phase consistently increased with each incremental increase in flow rate, regardless of gas phase (N_2 or air) or *in vivo* assay condition ($\pm NO_3^-$ in the incubation medium) (Table II). The lower recoveries of $NO_{(x)}$ with the 50 cm^3 min^{-1} flow rate, compared with the 300 cm^3 min^{-1} flow rate, was not accounted for by residual $NO_{(x)}$ remaining in the *in vivo* NR assay media. (Recovery of $NO_{(x)}$, following removal of the leaf discs at the end of the 30 min assay period and purging the solution with N_2 for an additional 15 min, was 0.19 and 0.06 μmol NO_2^- g^{-1} fresh weight for the 50 and 300 cm^3 min^{-1} N_2 flow rates, respectively.)

The decrease in NO_2^- accumulation in the *in vivo* NR assay medium due to gas purging did not appear to be solely due to loss of NO_2^- as $NO_{(x)}$. On a percentage basis, and comparing within the $-NO_3^-$-*in vivo* NR assay with increasing flow rates, the recovery of NO_2^- in the *in vivo* NR assay medium following gas purging plus the $NO_{(x)}$ recovered during gas purging ranged from 65 to 116% of the NO_2^- recovered in the *in vivo* NR assay medium without gas purging (Table II). A similar trend occurred with the $+NO_3^-$-*in vivo* NR assay with N_2 purging, while with air purging the recoveries were more constant (mean of 86%) over the various flow rates and *in vivo* NR assays in the absence and presence of added nitrate (Table II). The results of purging with N_2 or air indicated that anaerobic conditions were more conducive to $NO_{(x)}$ evolution during the *in vivo* NR assay. Results with O_2 (100%) and argon purging further substantiated that $NO_{(x)}$ evolution was greater under more anaerobic conditions (Fig. 3).

Argon or N_2 purging of the *in vivo* NR assay medium resulted in a similar decrease in NO_2^- accumulation, while O_2 or air were intermediate in effect to that of the control without gas purging and the completely anaerobic treatments (argon and N_2) (Table III). While O_2 or air had similar effects on NO_2^- accumulation in the *in vivo* medium, O_2 resulted in less $NO_{(x)}$ evolution than did air. The complete anaerobic treatments (N_2 and argon purging) resulted in similar $NO_{(x)}$ evolution, while both anaerobic treatments resulted in considerably greater $NO_{(x)}$ evolution than did aerobic treatments (air and O_2 purging).

To determine if the effects of N_2 or air on $NO_{(x)}$ evolution were reversible, a time course study was conducted in which the *in vivo* NR assay medium was alternately subjected to 20 min N_2 and 20 min air (Fig. 4). The effects of N_2 and air were reproducible through two complete cycles of alternating gas phase with N_2 resulting in consistently greater $NO_{(x)}$ evolution than did air. The overall trend of $NO_{(x)}$ evolution within each gas phase was to reach maximum evolution at 20 to 25 min after the start of the incubation with a gradual decline through the remaining assay period (through 80 min). The evolution of $NO_{(x)}$ under N_2 purging was greater for the $+NO_3^-$-NR assay than for the $-NO_3^-$-NR assay during the interval of 50 to 60 min and again at 70 to 80 min, a result consistent with the trend noted during the 20 to 30 min interval in Figure 1. With air, this differential in $NO_{(x)}$

Table II. *Effect of Gas Flow Rate on NO_2^- Formation and $NO_{(x)}$ Evolution During In Vivo NR Assay of Soybean Leaflets*

Unifoliolate leaflets of growth chamber-grown plants were sampled 11 DAP. Values presented are mean ± SD (cumulative over a 30-min assay period) of three replications. The recovery of $NO_{(x)}$ in the absence of gas purging was determined at the end of the 30-min assay period by removing the leaf discs and then purging the *in vivo* NR assay media for 15 min at 300 cm^3 N_2/min. Ninety-five % of the $NO_{(x)}$ came off in the first 5 min and less than 0.2 μmol NO_2^- g^{-1} fresh weight was recovered with either the $+NO_3^-$ or $-NO_3^-$ media.

Gas Phase	Flow Rate	NO_2^- formed		$NO_{(x)}$ Recovered as NO_2^-		$\frac{NO_2^- (Gas) + NO_{(x)} (Gas)}{NO_2^- (No Gas)} \times 100$	
		$-NO_3^-$ assay	$+NO_3^-$ assay	$-NO_3^-$ assay	$+NO_3^-$ assay	$-NO_3^-$ assay	$+NO_3^-$ assay
	cm^3 min^{-1}	μmol NO_2^- g^{-1} fresh wt 30 min^{-1}				%	
No gas	0	8.6 ± 0.7	12.7 ± 0.7				
N_2	50	2.8 ± 0.6	7.7 ± 0.3	2.7 ± 0.8	2.2 ± 0.3	65	78
	100	2.8 ± 0.1	7.4 ± 0.5	4.0 ± 0.7	4.1 ± 0.6	78	91
	200	3.2 ± 0.1	6.8 ± 0.9	5.8 ± 0.2	5.3 ± 0.4	105	95
	300	3.5 ± 0.2	6.8 ± 0.2	6.5 ± 0.3	6.4 ± 0.3	116	104
Air	50	6.4 ± 1.4	10.3 ± 0.7	1.1 ± 0.2	1.1 ± 0.1	87	89
	100	4.7 ± 0.2	8.7 ± 0.6	2.1 ± 0.3	1.8 ± 0.2	78	82
	200	3.9 ± 0.3	8.8 ± 0.4	3.2 ± 0.1	2.5 ± 0.4	82	88
	300	3.9 ± 0.3	8.6 ± 0.4	3.7 ± 0.5	2.9 ± 0.4	88	91

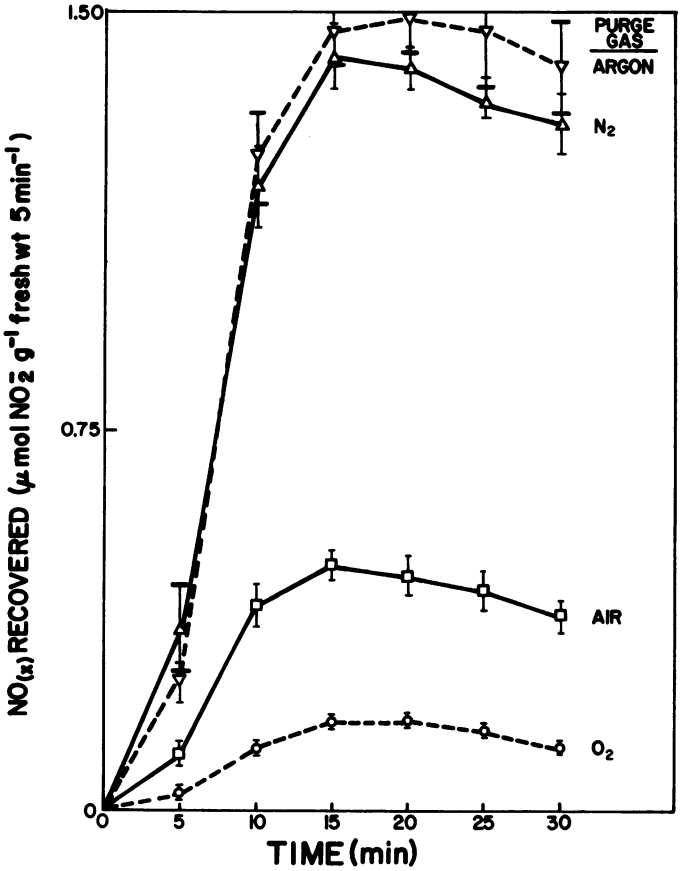

FIG. 3. Effect of aerobic or anaerobic gas purging on $NO_{(x)}$ evolution during $+NO_3^-$-*in vivo* NR assay of soybean leaflets. Other details were as given in Figure 1 legend.

evolution responses to + and $-NO_3^-$-NR assay media was not evident (Fig. 4).

Light resulted in markedly less NO_2^- accumulation and no $NO_{(x)}$ evolution during the *in vivo* NR assay compared with the normal dark assay (Table IV). This led to the conclusion that $NO_{(x)}$ evolution was at least in part dependent on NO_2^- accumulation in the tissue. The low level of NO_2^- accumulation in the light was likely due to further reduction of NO_2^- to NH_4^+ as suggested previously (3).

Table III. *Effect of Aerobic and Anerobic Conditions on NO_2^- Formation and $NO_{(x)}$ Evolution During a $+NO_3^-$-In Vivo NR Assay of Soybean Leaflets*

Other experimental details as indicated in Table II legend.

Gas Phase[a]	NO_2^- Formed	$NO_{(x)}$ Recovered	$\frac{NO_2^- (Gas) + NO_{(x)} (Gas)}{NO_2^- (No Gas)} \times 100$
	μmol NO_2^- g^{-1} fresh wt 30 min^{-1}		%
None	12.1 ± 0.5		
O_2	9.6 ± 0.6	0.9 ± 0.0	86
Air	10.1 ± 0.9	2.4 ± 0.3	103
N_2	6.9 ± 0.6	6.6 ± 0.4	111
Argon	6.8 ± 0.4	6.3 ± 0.2	109

[a] Gas flow rates were 200 cm^3 min^{-1} with each gas phase.

The evolution of $NO_{(x)}$ for soybeans was greater from younger, recently expanding, leaves of 21-day-old plants than from older leaves, regardless of gas phase (Table V). Consistent with results from 11-day-old plants (Table III), $NO_{(x)}$ evolution by the younger leaves of 21-day-old plants was greater when the *in vivo* NR assay conditions were more anaerobic (*i.e.* O_2 < air < N_2) (Table V). Only trace levels of $NO_{(x)}$ evolution were detected from the oldest leaves (unifoliolate) under N_2 purging and no $NO_{(x)}$ evolution was detected when air or O_2 were gas phases.

The younger leaves (3rd trifoliolate) also exhibited the highest rates of NR activity, again regardless of gas phase (no gas, N_2, air, or O_2) (Table V). There was no effect of N_2 or air, compared with no gas purging, on NO_2^- accumulation in the *in vivo* NR assay medium with the youngest trifoliolate leaves (3rd trifoliolate, Table V). This was in contrast to the inhibitory effect of N_2 and air on NR activity at an earlier growth stage (11-day-old plants,

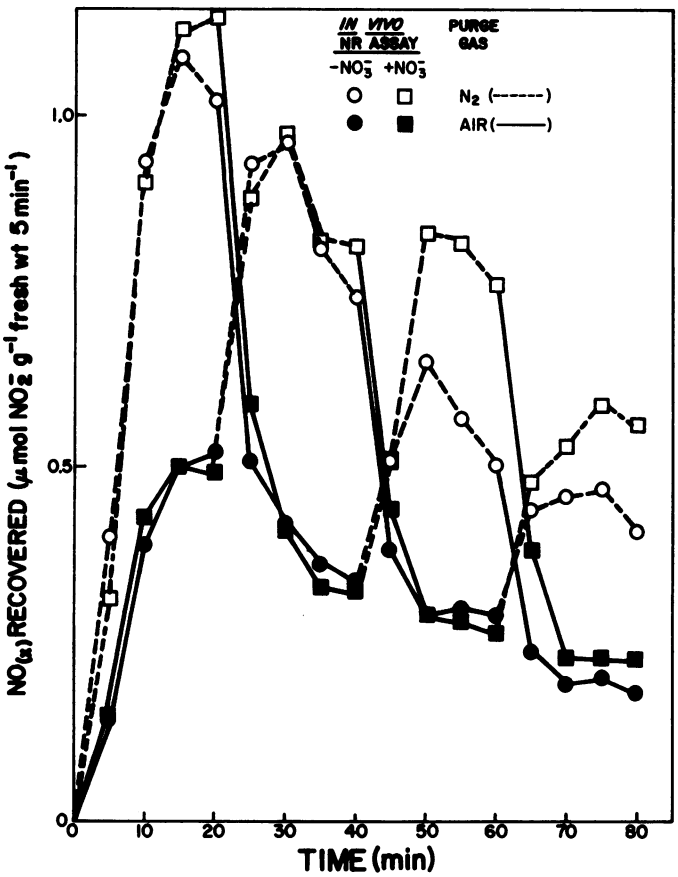

FIG. 4. Effect of alternating N_2 and air purging on $NO_{(x)}$ evolution during $\pm NO_3^-$-*in vivo* NR assay of soybean leaflets. Other details were as given in Figure 1 legend, with exception that plants were sampled 12 days after planting.

Table IV. *Effect of Light and Dark on NO_2^- Formation and $NO_{(x)}$ Evolution During a $+NO_3^-$-In Vivo Assay of Soybean Leaflets*

Other experimental details as indicated in Table II legend.

Parameter Measured	Gas Phase[a]	Assay Condition	
		Light	Dark
		μmol NO_2^- g^{-1} fresh wt 30 min^{-1}	
NO_2^- formed	None	0.5 ± 0.1	9.2 ± 0.8
	N_2	0.6 ± 0.1	6.4 ± 0.1
	Air	0.2 ± 0.1	7.0 ± 0.5
$NO_{(x)}$ recovered	N_2	0.0	3.5 ± 0.2
	Air	0.0	1.6 ± 0.1

[a] Gas flow rates were 200 cm^3 min^{-1} with each gas phase.

Tables I–IV). Nitrogen gas purging of older leaves (unifoliolate, 1st and 2nd trifoliolate) compared with respective leaf age analyzed without gas purging, resulted in a stimulation of NO_2^- accumulation in the *in vivo* NR assay medium, while the $NO_{(x)}$ evolution with N_2 purging was progressively less with older leaves (Table V). Oxygen purging resulted in less NO_2^- formation in the *in vivo* NR assay medium at all leaf ages relative to respective controls without gas purging. Thus, the negative effect of O_2 (100%) purging on *in vivo* NR activity was evident with young and old (11- and 21-day-old) plants as well as for a range of leaf ages within the same plant (21-days-old). In contrast, effects of N_2 and air on NR activity were variable, being dependent on plant and leaf age.

Table V. *Effect of Leaf Age on NO_2^- Formation and $NO_{(x)}$ Evolution During In Vivo NR Assay of Soybean Leaflets*

Growth chamber-grown plants were sampled 21 DAP. Other experimental details as indicated in Table II legend.

Parameter Measured	Gas Phase[a]	Leaflet			
		Unifoliolate	1st trif.	2nd trif.	3rd trif.
		μmol NO_2^- g^{-1} fresh wt 30 min^{-1}			
NO_2^- formed	None	0.6 ± 0.1	2.1 ± 0.2	6.4 ± 0.3	11.2 ± 0.8
	N_2	1.6 ± 0.0	3.9 ± 0.1	7.5 ± 0.5	11.2 ± 0.2
	Air	0.4 ± 0.0	1.9 ± 0.1	6.6 ± 0.7	11.4 ± 0.4
	O_2	0.4 ± 0.0	1.9 ± 0.1	4.8 ± 0.5	6.5 ± 0.4
$NO_{(x)}$ recovered	N_2	0.1 ± 0.0	0.2 ± 0.1	0.8 ± 0.1	3.1 ± 0.1
	Air	0.0	0.0	0.2 ± 0.1	1.3 ± 0.2
	O_2	0.0	0.0	0.2 ± 0.0	0.2 ± 0.0

[a] Gas flow rates were 200 cm^3 min^{-1} with each gas phase.

DISCUSSION

The similar evolution of $NO_{(x)}$ under conditions which resulted in different levels of NO_2^- accumulation in the medium ($\pm NO_3^-$ *in vivo* assay comparisons, Tables I and II) indicated that conditions other than NO_2^- concentration were regulating or limiting $NO_{(x)}$ evolution. However, the light study (Table IV) did indicate that it was essential to have NO_2^- accumulation in the medium before $NO_{(x)}$ evolution occurred. The association of greater amounts of $NO_{(x)}$ evolved and lesser amounts of NO_2^- accumulated during *in vivo* NR assays under N_2 purging, compared with air purging (Tables I and II), seemed to support that evolution of $NO_{(x)}$ was at the expense of NO_2^- accumulation. However, the lack of stoichiometric changes in the NO_2^- accumulation and $NO_{(x)}$ evolution precluded any strong conclusions that $NO_{(x)}$ was formed at the expense of NO_2^- accumulation in the *in vivo* NR assay medium.The conversion of a portion of the NO_2 (gas) to NO_3^- when trapped by the Griess-Saltzman reagent (1) would account for the lower than stoichiometric recoveries of NO_2^- from the *in vivo* assay medium. However, since certain treatments already account for more $NO_{(x)}$ trapped as NO_2^- than would be expected if NO_2^- from the NR assay medium was being quantitatively lost as $NO_{(x)}$, applying a correction to account for possible NO_3^- formed from NO_2 would cause further disparity in a stoichiometric recovery.

The mechanism of $NO_{(x)}$ evolution during the *in vivo* NR assay under gas purging is unknown. It has been postulated that NO_2^- may nonenzymically react with plant metabolites with resulting disappearance as gaseous forms of nitrogen following herbicide treatment (15). However, when soybean leaf discs were infiltrated with NO_2^-, boiled for 5 min, and incubated with N_2 purging, no $NO_{(x)}$ evolution occurred (data not shown). This suggested that an enzymic reaction was responsible, since the unboiled control infiltrated with NO_2^- and purged with N_2 gas results in $NO_{(x)}$ evolution. Evidence is available that NO_2^- can be enzymically reduced to NO by using reduced Cyt *c* oxidase derived from beef heart (5). Nitric oxide and nitrous oxide have been identified as products of NO_2^- reduction by *Pseudomonas aeruginosa* Cyt oxidase (ferrocytochrome *c*-55:oxidoreductase, EC 1.9.3.2) during dissimilatory denitrification (19) involving the following possible reaction sequence: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. No reports were found concerning this type of pathway occurring in higher plants exhibiting assimilatory pathway of NO_3^- utilization. The lower amounts of $NO_{(x)}$ evolved from air and O_2 purged *in vivo* NR assays could possibly be due to further assimilation of NO_2^- during dark aerobic conditions as shown by several workers (2, 12, 14, 18). However, other work (6) reported that excised leaves do not assimilate $^{15}NO_2^-$ into amino acids under dark

aerobic gaseous environment. These results were obtained with different plant species which could explain the differences observed. The fact that NO_2^- accumulated under O_2 purging in the dark with soybean leaf discs (Table III) is in contrast to a recent report using soybean (18) and to the extreme sensitivity of NO_2^- accumulation in wheat leaves when incubated in air (4). These conflicting results may be due to differences in assay conditions and to species differences in control of the NO_3^-/NO_2^- reduction system.

The variable percentage recovery of NO_2^- as $NO_{(x)}$ may involve two distinct and separate effects of N_2. First, in older leaves which did not evolve $NO_{(x)}$ (Table IV), N_2 resulted in a stimulation of NO_2^- accumulation in the *in vivo* NR assay medium. This may also have occurred in younger leaves but was masked due to evolution as $NO_{(x)}$. Second, the evolution of $NO_{(x)}$ from young leaves was dependent on N_2 flow rate but independent of NO_2^- concentration (Table II). Thus, N_2 may be having a positive effect on NO_2^- accumulation in the *in vivo* assay due to more complete blockage of NO_2^- conversion to NH_4^+, which is independent of flow rate, and a negative effect on NO_2^- accumulation in the *in vivo* assay due to $NO_{(x)}$ evolution, which is dependent on flow rate. This explanation appears consistent with all the data obtained.

The results obtained strongly point out that use of the *in vivo* NR assay to estimate *in situ* NR activity must be viewed with caution. It is obvious that assay conditions selected by various laboratories can markedly affect the amount of NO_2^- that accumulates in the *in vivo* NR assay medium. Plant species and leaf age within a species can also affect NO_2^- accumulation during *in vivo* NR assays. The *in vivo* NR assay of soybean leaves currently in use by our laboratory (17) does not involve gas purging and is still the assay of choice, since $NO_{(x)}$ does not appear to be evolved in the absence of gas purging.

Acknowledgment—The author acknowledges Dr. L. A. Klepper, University of Nebraska, Lincoln, for helpful discussions and for supplying the disodium 2-naphthol-3,6-disulfonate.

LITERATURE CITED

1. ALLEN JD 1973 A review of methods of analysis for oxides of nitrogen. J Inst Fuel 46: 123–133
2. ASLAM M, RC HUFFAKER, DW RAINS, KP RAO 1979 Influence of light and ambient carbon dioxide concentration on nitrate assimilation by intact barley seedlings. Plant Physiol 63: 1205–1209
3. BEEVERS, L, RH HAGEMAN 1972 The role of light in nitrate metabolism in higher plants. Photophysiology 7: 85–113
4. BEEVERS L, RH HAGEMAN 1980 Nitrate and nitrite reduction. Biochem Plants 5: 115–168.
5. BRUDVIG GW, TH STEVENS, SI CHAN 1980 Reactions of nitric oxide with cytochrome *c* oxidase. Biochemistry 19: 5275–5285
6. CANVIN DT, CA ATKINS 1974 Nitrate, nitrite, and ammonia assimilation by leaves: effects of light, carbon dioxide and oxygen. Planta 116: 207–224
7. CRAFTS-BRANDNER SJ 1980 Nitrate reduction by roots of soybeans [*Glycine max* (L.) Merr.] seedlings grown on high levels of nitrate. MS Thesis. University of Illinois, Urbana-Champaign
8. HARPER JE 1971 Seasonal nutrient uptake and accumulation patterns in soybeans. Crop Sci 11: 347–350
9. HARPER JE 1974 Soil and symbiotic nitrogen requirements for optimum soybean production. Crop Sci 14: 255–260
10. HARPER JE, RH HAGEMAN 1972 Canopy and seasonal profiles of nitrate reductase in soybeans [*Glycine max* (L.) Merr.]. Plant Physiol 49: 146–154
11. HATAM M, DJ HUME 1976 Relations between nitrate reductase activity and nitrogen accumulation in soybeans. Can J Plant Sci 56: 377–384
12. ITO O, K KUMAZAWA 1978 Amino acid metabolism in plant leaf. III. The effect of light on the exchange of ^{15}N-labeled nitrogen among several amino acids in sunflower discs. Soil Sci Plant Nut 24: 327–336
13. JAWORSKI EG 1971 Nitrate reductase assay in intact plant tissues. Biochem Biophys Res Commun 43: 1274–1279.
14. JONES RW, RW SHEARD 1978 Accumulation and stability of nitrite in intact aerial leaves. Plant Sci Lett 11: 285–291
15. KLEPPER L 1974 A mode of action of herbicides: Inhibition of the normal process of nitrite reduction. Nebraska Exp Sta Res Bull 259: 1–42
16. KLEPPER L 1979 Nitric oxide (NO) and nitrogen dioxide (NO_2) emissions from herbicide-treated soybean plants. Atmospheric Environ 13: 537–542
17. NICHOLAS JC, JE HARPER, RH HAGEMAN 1976 Nitrate reductase activity in soybeans [*Glycine max* (L.) Merr.]. I. Effects of light and temperature. Plant Physiol 58: 731–735
18. REED, AJ, DT CANVIN 1981 Nitrate and nitrite assimilation by leaves of four plant species. Plant Physiol 67: S-76
19. WHARTON DC, ST WEINTRAUB 1980 Identification of nitric oxide and nitrous oxide as products of nitrite reduction by pseudomonas cytochrome oxidase (nitrite reductase). Biochem Biophys Res Commun 97: 236–242