Plant Physiol. (1990) 93, 26–32
0032-0889/90/93/0026/07/$01.00/0

Received for publication September 25, 1989
and in revised form December 11, 1989

Comparison between NO_x Evolution Mechanisms of Wild-Type and nr_1 Mutant Soybean Leaves[1]

Lowell Klepper

Department of Agronomy, University of Nebraska, Lincoln, Nebraska 68583-0817

ABSTRACT

The nr_1 soybean (*Glycine max* [L.] Merr.) mutant does not contain the two constitutive nitrate reductases, one of which is responsible for enzymic conversion of nitrite to NO_x ($NO + NO_2$). It was tested for possible nonenzymic NO_x formation and evolution because of known chemical reactions between NO_2^- and plant metabolites and the instability of nitrous acid. It did not evolve NO_x during the *in vivo* NR assay, but intact leaves did evolve small amounts of NO_x under dark, anaerobic conditions. Experiments were conducted to compare NO_3^- reduction, NO_2^- accumulation, and the NO_x evolution processes of the wild type (cv Williams) and the nr_1 mutant. *In vivo* NR assays showed that wild-type leaves had three times more NO_3^- reducing capacity than the nr_1 mutant. NO_x evolution from intact, anerobic nr_1 leaves was approximately 10 to 20% that from wild-type leaves. Nitrite content of the nr_1 mutant leaves was usually higher than wild type due to low NO_x evolution. Lag times and threshold NO_2^- concentrations for NO_x evolution were similar for the two genotypes. While only 1 to 2% of NO_x from wild type is NO_2, the nr_1 mutant evolved 15 to 30% NO_2. The kinetic patterns of NO_x evolution with time were completely different for the mutant and wild type. Comparisons of light and heat treatments also gave very different results. It is generally accepted that the NO_x evolution by wild type is primarily an enzymic conversion of NO_2^- to NO. However, this report concludes that NO_x evolution by the nr_1 mutant was due to nonenzymic, chemical reactions between plant metabolites and accumulated NO_2^- and/or decomposition of nitrous acid. Nonenzymic NO_x evolution probably also occurs in wild type to a degree but could be easily masked by high rates of the enzymic process.

Formation and evolution of NO_x[2] gases (NO and NO_2) from intact soybean (*Glycine max* [L.] Merr. cv Amsoy) leaves has been known to occur for some time (5). Harper (4) later demonstrated that NO_x was evolved during gas purging of the *in vivo* NR assay with soybean. Dean and Harper (2) also showed that winged bean (*Psophocarpus tetragonolobus* [L.] DC. cv Lunita) leaves evolved large quantities of NO_x. Results have shown that NO_2^- must first accumulate within the leaf to a certain level (4, 5) and that NO_x is derived from NO_2^- (2, 3). Klepper (5) earlier concluded that NO_x was formed by chemical decomposition of NO_2^-, nitrous acid and by reactions with plant metabolites. Harper used boiled leaves to stop NO_x formation and suggested that the process was enzymic in nature (4). Since that time a number of papers have been published which provide strong evidence that the NO_x formation and evolution in wild-type soybean leaves is enzymic (2, 3, 9, 10, 14–17). Nelson *et al.* (9) screened for and isolated a mutant from nitrosomethylurea-treated soybeans (cv Williams), which did not contain the two constitutive NR enzymes and did not evolve NO_x during *in vivo* assays (9, 10). They concluded that (a) nitrate-grown wild-type soybean leaves contained both constitutive NR enzymes and the inducible NR and (b) the absence of constitutive NR activities in the mutant were closely associated with NO_x evolution. Since then, this mutant has been designated as nr_1 mutant, and several papers have been published describing the isolation, purification and characterization of the constitutive enzymes from wild-type soybean leaves (13, 15–17). It was shown recently that the constitutive NAD(P):NR enzyme formed NO_x *in vitro* and that the K_m for NO_2^- was lower than the K_m for NO_3^- (3). If the nr_1 mutant could be shown to evolve NO_x without containing the necessary enzyme, it would offer a unique opportunity to compare enzymic NO_x evolution with a nonenzymic process using almost identical germplasm.

Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) leaves can both accumulate NO_2^- and evolve low levels of NO_x (1, 6). This type of NO_x formation is thought to be nonenzymic since neither plant contains a constitutive NR and both evolve low levels of NO_x compared to wild-type soybean leaves. It was reported that rush (*Juncus effusus* L.) carpets used in Japanese houses have the ability to reduce ambient levels of NO_2 to NO (12). This reducing capacity was traced to a low mol wt polysaccharide in the free sugar fraction of the dry rush. Also, dried grass (*Poa pratensis* L.) and ginko (*Ginko biloba* L.) leaves successfully absorbed low levels of NO_2 and liberated up to 70% as NO. This illustrates the chemical reactivity of the gaseous N oxides within plant tissues with the result of NO evolution.

There were three objectives in this study: (a) to verify a portion of previous *in vivo* nr_1 mutant research results (9), (b) to test whether intact leaves of the nr_1 mutant would evolve NO_x by nonenzymic reactions after NO_2^- accumulated, and (c) to compare the known enzymic evolution of NO_x by the wild-type soybean leaves with the proposed nonenzymic evolution of NO_x by the nr_1 mutant.

MATERIALS AND METHODS

Plant Culture

Seeds of wild-type soybean (*Glycine max* [L.] Merr. cv Williams) and mutant nr_1 soybean were planted in vermiculite

[1] Published as paper No. 8972, Journal Series, Nebraska Agricultural Experiment Station.

[2] Abbreviations: NO_x, the nitrogen oxide gases, nitric oxide (NO) and nitrogen dioxide (NO_2); NR, nitrate reductase; nr_1, soybean mutant selected for missing constitutive nitrate reductases.

and grown in growth chambers under 16 h light (300 μmol photons m^{-2} s^{-1}, fluorescent/incandescent) with day/night temperatures of 25°C/15°C. Plants were irrigated every other day with a nutrient solution (7) containing 15 mM NO_3^-. Unifoliolate leaves were used for analyses and were assayed 13 to 15 d after planting.

In Vivo Assay for NR Activity

The basic *in vivo* assay medium contained 50 mM KNO_3, 50 mM potassium phosphate buffer (pH 7.5), and 1% (v/v) *n*-propanol. Leaves were cut into 1 cm diameter discs, weighed, and 250 mg placed in 50 mL beakers containing 10 mL assay medium. The beakers and their contents were vacuum-infiltrated (750 mm Hg) twice and then were placed in a shaking water bath (30°C) in darkness. Aliquots (0.2 mL) were taken at appropriate intervals for NO_2^- determination. Several modifications of the assay medium included increasing propanol from 1 to 3% and substituting a nonionic surfactant (Tergitol 15-S-7) for propanol. Tergitol 15-S-7 is a linear ethoxylate (Union Carbide Corp., New York, NY 10017). Oxyrase EC-100 system (Oxyrase, Inc., P.O. Box 899, Asland, OH 44805), which consists of *Escherichia coli* particles (0.2 μm or less) and is used to maintain anaerobic conditions, was also added with 10 mM succinate to the basic assay mixture. All controls without Oxyrase contained 10 mM succinate.

In Vivo NR Assay with NO_x Analysis

The same *in vivo* NR assay medium as described above (with 1% propanol) was also used for this assay. Samples (1–2 g leaf discs) were placed in a 250 mL beaker containing 150 mL assay medium and were vacuum infiltrated twice. After vacuum infiltration, the discs and medium were placed in a 250 mL gas washing bottle. The bottle was then placed in a water bath (30°C) in darkness, and N_2 was purged through the medium at 300 mL/min. The exiting gas from the *in vivo* assay was passed through a H_2SO_4-dichromate oxidizing column to an Aeron NO_2 analyzer as previously described (5, 7). Since NO_x was evolved from fresh leaf tissue and trapped and measured as NO_2^-, NO_x evolution is reported as μmol NO_2^- g^{-1} fresh weight.

NO_x Analyses from Intact Leaves

Soybean stems were cut under water with a razor blade and placed in water-filled 50 mL Erlenmeyer flasks. The trifoliolate bud was removed leaving only the unifoliolate leaves. The intact leaves and Erlenmeyer container were placed inside a 500 mL glass container with two ports, one for entry of the carrier gas (nitrogen) and the other for sample exit. The container was covered with a black cloth for darkness. Nitric oxide was determined by passing the gaseous sample through the H_2SO_4-dichromate oxidizer to the NO_2 analyzer as described earlier (5). Nitrogen dioxide was determined by directing the gaseous sample to the NO_2 analyzer without passing through the oxidizer column (5).

Nitrite Determination

For the *in vivo* assays, aliquots of the aqueous medium were removed at timed intervals and mixed in the Griess-Saltzman reagent. When intact leaves were used, single 1 cm leaf discs were quickly crushed with a glass rod in a test tube containing 10 mM KOH. This was done in subdued light to prevent photosynthetic NO_2^- reduction. The Griess-Saltzman reagent was then added and the color was allowed to develop for 30 min. Absorbance was determined at 540 nm.

Leaf Disc Steaming

Leaf discs (1 cm diameter) contained in a 50 mL beaker were placed in a 500-mL chamber under dark, anaerobic (N_2) conditions until NO_x evolution occurred at a satisfactory level. The leaf discs were quickly removed from the 50 mL beaker, placed in an aluminum 'teaball' suspended over rapidly boiling water for 30 s to 1 min. The discs were quickly removed and replaced into the chamber for NO_x analysis. The entire transfer and steaming operation took 1 to 2 min.

Microwave Treatments

Soybean plants with only the unifoliolate leaves were prepared for NO_x analysis as earlier described. After a sufficient time of dark, anaerobic incubation so that a reasonable level of NO_x was evolving, plants were removed from the NO_x analyzer, placed in the microwave oven for a 10-s treatment, and quickly replaced for NO_x analyses. The entire treatment procedure was completed in 30 to 40 s. All treatments were conducted in a Kenmore microwave oven of 21.4 L volume and energy level of 650 W at 2450 MHz. Only 10 and 15% power levels (65 and 97.5 W) were used for a 10 s period.

All experiments were repeated at least four times. NO_x values normally varied 15 to 20% daily. Nitrite values are the mean of four replicate analyses except where noted otherwise. All data represent typical experiments.

RESULTS AND DISCUSSION

In Vivo NR Assays

N_2-purged *in vivo* assays of wild-type soybean leaf discs had linear rates of NO_2^- accumulation and NO_x evolution (Fig. 1A). The period of assay (210 min) was longer than a normal *in vivo* assay and was intended to show that the wild-type soybean leaf can reduce NO_3^- and form NO_x (primarily NO, nitric oxide) for an extended period with little or no effect upon either the NO_3^- reducing- or NO_x forming-mechanisms. The wild-type leaves produced similar amounts of NO_2^- and NO_x. The sum of the two products from the *in vivo* assay (NO_2^- + NO_x) was assumed to represent the total NO_3^- reduced by leaf discs, so that a total of nearly 70 μmol NO_3^- g^{-1} fresh weight were reduced during the assay period. If a recycling process was present in the leaf tissue which oxidized NO_2^- or NO_x back to NO_3^-, nitrate reduction rates would be higher, and considerably more energy would have been expended without detection.

The *in vivo* assay conducted with leaf discs from the nr_1 mutant (Fig. 1B) indicated that approximately 20 μmol NO_2^- g^{-1} fresh weight accumulated but NO_x was not evolved. In at least 20 separate assays of the N_2-purged *in vivo* NR assay with the nr_1 mutant, NO_x evolution was never detected. This

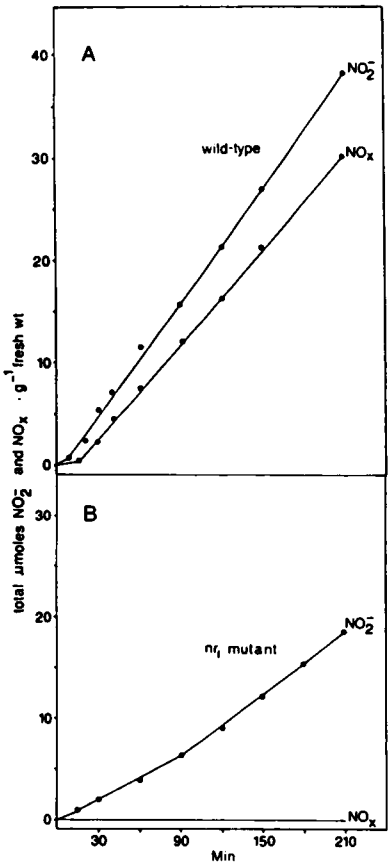

Figure 1. Time course of NO_2^- accumulation and NO_x evolution with N_2 purging during the *in vivo* NR assay. The nonionic surfactant Tergitol 15-S-7 was used in this test. A, Assay with wild type leaves, B, assay with nr_1 mutant leaves.

finding is in agreement with reports by Nelson *et al.* (9). Compared to *in vivo* wild-type NR rates (Fig. 1A), the nr_1 mutant only accumulated 50% of the NO_2^- and had only 30% of total NO_3^- reduction. The lowered NR activity of the nr_1 mutant is undoubtedly due to the absence of the NAD- and NADP-dependent constitutive nitrate reductases. The NAD(P)-NR enzyme is reported to be responsible for the conversion of NO_2^- to nitric oxide in wild-type leaves (3). Normally, the *in vivo* assay with wild-type leaves is not purged with N_2 nor is NO_x measured. Without purging, very little NO_x escapes the aqueous surface of the incubation medium. In past tests, Harper (4) has shown with wild-type leaves that the higher the rate of purging, the higher the NO_x output and the lower the NO_2^- accumulation. This effect is not understood.

The *in vivo* system normally involves vacuum-infiltrated leaf tissue (a partial anaerobic environment) which is incubated in darkness to prevent light from providing energy for NO_2^- reduction. Also, in this laboratory, low levels of Tergitol 15-S-7 have been used for years as an all-purpose surfactant to aid in vacuum infiltration and to increase *in vivo* enzyme activity (7). Other researchers, especially with soybean, have used 1% propanol in the reaction medium (2, 4, 11). Propanol has not been used in this laboratory for two reasons. First, equal NR activity can be obtained with the Tergitol surfactant. Second, during NO_x evolution testing, propanol evaporates from the medium and the vapors blacken the dichromate-sulfuric acid catalytic converter necessary to oxidize NO to NO_2 for detection of NO_x. The surfactant, Tergitol 15-S-7 is water soluble with a sufficiently low vapor pressure so that this reaction does not occur.

The results of the NR *in vivo* assay as shown in Figure 2A were obtained from a normal assay using Tergitol 15-S-7 and no gas purging. These data illustrate a 10-fold difference between leaf NR activities of the nr_1 mutant and wild-type soybeans. NR activity of the nr_1 mutant appeared to cease after 60 min of incubation. In contrast, with N_2-purging of the medium, the nr_1 mutant continued to accumulate NO_2^- (Fig. 1B). There is the possibility that a different nitrogen product was formed by the nr_1 mutant which was not measured or could have inhibited NO_3^- reduction. This apparent inhibition of NO_3^- reduction is not understood.

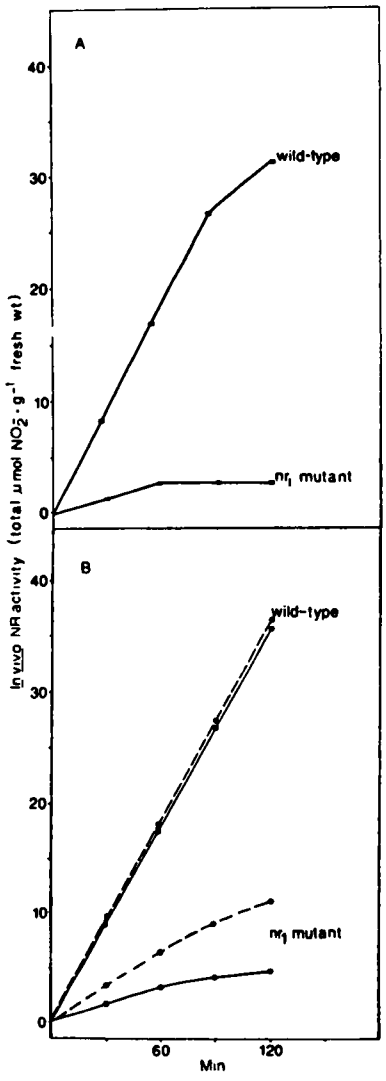

Figure 2. Time course of NO_2^- accumulation by wild type and the nr_1 mutant during the *in vivo* NR assay without N_2 purging. A, Tergitol 15-S-7 in the medium; B, 1% (——) and 3% (– – –) propanol in the assay medium.

An *in vivo* assay was conducted with propanol in the reaction medium to more closely compare with results by Harper (4). Two concentrations of propanol (1% and 3%) were used (Fig. 2B). The wild-type assay responded similarly to both treatments. The nr_1 mutant at 1% propanol had linear activity with time, and again activity began to level off after 60 to 90 min. At 3% propanol, the nr_1 mutant had twice the NR activity as at 1%. This effect was shown repeatedly. It is not known whether the increased propanol aided the exit of NO_2^- from the leaf disc into the solution or prevented further metabolism or conversion of NO_2^-. The nr_1 mutant contains only the NAD-dependent inducible NR (9) which is also present in wild type, but the wild type showed no response to the 3% propanol treatment. With much higher NO_2^- accumulation in wild type, this effect may have been hidden. The overall effects are not clear.

Oxyrase, used to provide anaerobic conditions in the assay medium without purging did increase *in vivo* NR activity 50 to 100% in the nr_1 mutant and by approximately 7% in the wild type (data not shown). The nr_1 mutant NR activity still apparently stopped at 60 to 90 min, while wild-type NR activity continued. Results with Oxyrase were not consistent on a day to day basis. It is not felt that this was due to Oxyrase alone but was primarily a response to small differences in leaf maturity.

From these initial assays comparing rates of NR activity, production of NO_2^- and NO_x, use of Oxyrase, Tergitol 15-S-7 and propanol, it was concluded that differences existed in NO_3^- and NO_2^- metabolism of wild-type and the nr_1 mutant. The nr_1 mutant appears to be more dependent upon anaerobic conditions or N_2-purging for NO_2^- to accumulate in the *in vivo* assay (Figs. 1A and 2, A and B). The differences in the patterns of NR activity in the normal *in vivo* assay and with N_2-purging are not understood.

NO_x Analyses from Leaves

As shown previously, NO_x evolution cannot be detected with the nr_1 mutant during *in vivo* assays (Fig. 1B). However, the nr_1 mutant will evolve NO_x from intact leaves under anaerobic conditions if the leaves are not submerged in an aqueous solution or vacuum infiltrated. Leaves were incubated under anaerobic conditions in darkness with N_2 and tested for NO_x evolution (Table I). The nr_1 rates were considerably lower than wild-type rates. The formation and evolution of NO_x from the nr_1 mutant is thought to be due to chemical reactions of plant metabolites with HNO_2 or simply by HNO_2 decomposition as shown by the following reaction: $2\ HNO_2 \rightarrow NO + NO_2 + H_2O$ (8).

Table I. *Comparison of NO_x Evolution Rates, Threshold NO_2^- Contents, and Lag Times between Wild-Type and nr_1 Mutant Soybean Leaves*

Leaf	NO_x Evolution Rates	Threshold NO_2^-[a]	Lag Time[b]
	μmol NO_x g^{-1} fresh wt h^{-1}	*μmol NO_2^- g^{-1} fresh wt*	*min*
nr_1	1.9 ± 0.3[c]	1.96 ± 0.38	14.4 ± 8.4
Wild type	14.2 ± 1.8	1.53 ± 0.28	6.9 ± 3.6

[a] Threshold NO_2^- equals the endogenous leaf content when NO_x evolution begins. [b] Lag time is the time between initial dark, anaerobic incubation and the onset of NO_x evolution. [c] All values are means ± SE of 10 replications of 14-d-old leaves.

The level of NO_x evolution from the nr_1 mutant leaves is approximately 10 to 20% that from wild-type leaves. The absence of the two constitutive NRs in the mutant resulted in considerable loss of nitrite-forming capacity. The highest rate of NO_x evolution recorded, during any experiment, for the nr_1 mutant leaves was 3.3 μmol NO_2^- g^{-1} fresh weight h^{-1}. This is compared to 12 to 20 μmol g^{-1} fresh weight h^{-1} for wild-type leaves under similar environmental conditions.

Tests were also conducted to determine if differences existed between wild type and the nr_1 mutant in lag periods before NO_x evolution and threshold NO_2^- concentrations neccessary for NO_x evolution (Table I). Threshold values were similar for both wild type and the nr_1 mutant. Lag time for the wild type was approximately one-half that of the nr_1 mutant. The largest difference between the two genotypes in these comparisons was in the amount of NO_x evolved, not in time or NO_2^- concentration for initiation of NO_x evolution. These threshold values are larger than reported previously (5) and were most likely due to an improved technique for extracting NO_2^- from soybean leaves. Previously, leaf discs were placed in a test tube in contact with the Griess-Saltzman reagent, and NO_2^- was assumed to diffuse freely from the tissue and react for color development (5). It was found that, if the discs were quickly crushed in distilled water or 10 mM KOH solution prior to addition of the Griess-Saltzman reagent, NO_2^- values increased six- to eightfold. This increased nitrite detection was the same for both wild type and the mutant. Crushing of the discs after incubation of the Griess-Saltzman reagent with the discs did not improve recovery. Loss of nitrite had already occurred. This further demonstrates the high reactivity of NO_2^- inside leaf tissue especially under acidic conditions.

Intact leaves of nr_1 mutant were tested for NO_x evolution for a period of 26 h (Fig. 3). The evolution peaked in 3 to 4 h and gradually decreased thereafter. During this time, a total of 16 μmol of NO_x g^{-1} fresh weight was evolved from the intact leaves. At the end of 5 h, 30% of the total NO_x had been emitted, and NO_2^- content of the leaves was nearly 15 μmol NO_2^- g^{-1} fresh weight (data not shown). It was concluded that internal NO_2^- accumulation was certainly not a limiting factor in this type of NO_x evolution. Although the nr_1 mutant had considerably less capacity to reduce NO_3^-, it accumulated much higher levels of NO_2^- apparently due to its inability to enzymically convert aqueous NO_2^- into gaseous NO. It was previously shown that the nr_1 mutant in the *in vivo* NR assay appears to stop reducing NO_3^- after 60 to 90 min (Fig. 1B); however, NO_3^- reduction apparently continues in the intact leaf at higher rates and longer periods than in the *in vivo* assay.

After 26 h of NO_x evolution and NO_2^- accumulation, even though high humidity was maintained throughout the test period, leaves were badly wilted. Upon removal from the test cylinder, leaves quickly became desiccated and were nearly crisp within an hour. They contained high levels of NO_2^- but no longer evolved NO_x.

Upon comparing NO_x evolution and leaf NO_2^- accumulation of wild type and the nr_1 mutant, it was apparent that

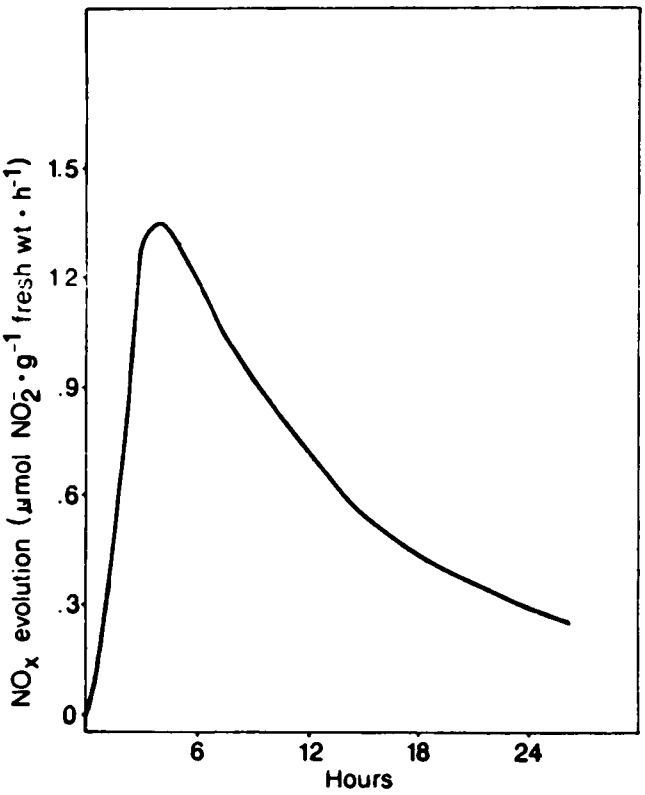

Figure 3. NO_x evolution by intact unifoliolate leaves of the nr_1 mutant over a 26 h period. Leaves were incubated in darkness and flushed with moistened N_2. NO_x level was continuously monitored during the entire incubation period.

these processes in the two genotypes are different. With wild-type leaves, though NR activity is much higher and NO_2^- does accumulate to abnormal levels, enzymic NO_x evolution keeps leaf NO_2^- relatively low compared to the nr_1 mutant. Estimates have been made with wild-type leaves concerning the quantitity of NO_2^- within a leaf at a given time available for NO_x formation and evolution (5, 7). These quantities of NO_2^- would permit wild-type NO_x evolution for only a period of several minutes. However, at the end of 5 h in this experiment (Fig. 3), the nr_1 mutant contained sufficient NO_2^- (15 μmol g^{-1} fresh weight) for over 11 h of nonenzymic NO_x evolution at the highest rate observed, even if no further NO_3^- were reduced.

In a companion experiment of 3 h length (data not shown), wild-type leaves had evolved a total of 39 μmol NO_x g^{-1} fresh weight and were evolving at a steady state of 12 μmol g^{-1} fresh weight h^{-1} and contained 1.63 μmol NO_2^- g^{-1} fresh weight. This was sufficient NO_2^- for approximately 8 min of NO_x evolution. During the same period, the nr_1 mutant leaves had evolved a total of 2.58 μmol NO_x g^{-1} fresh weight and were evolving at a steady state of 1.1 μmol g^{-1} fresh weight h^{-1}. Leaf NO_2^- content was 6.2 μmol g^{-1} fresh weight. This was sufficient NO_2^- for more than 5.5 h of further NO_x evolution without further NO_3^- reduction. Again, internal NO_2^- concentration of the nr_1 mutant leaves does not appear to be the limiting factor for nonenzymic NO_x evolution. This comparison of the differences in rates of NO_x evolution coupled with leaf NO_2^- content (accumulation and disappearance) clearly provides additional evidence that the two types of soybean leaves are evolving NO_x by completely different systems.

Wild-type and nr_1 mutant leaf NO_x evolution were compared by exposure to dark and light (Fig. 4). High rates of NO_x evolution in darkness by wild-type leaves are completely inhibited by light (5) (Fig. 4). Upon reexposure to darkness, wild-type NO_x evolution resumes to near previous levels. During light exposure, photosynthetic electron flow provides reductant for NO_2^- reduction and removes substrate for NO_x formation. Leaf tissue NO_2^- drops to almost undetectable levels (data not shown). The rapidity of this reaction indicates that the small NO_2^- pool which has accumulated in wild-type leaves in darkness and is being enzymically reduced to NO may also be shunted into chloroplastic NO_2^- reduction.

The nr_1 mutant exhibited a completely different pattern of NO_x evolution (Fig. 4). It only reached 12 to 15% of wild-type NO_x evolution and did not react to light exposure. Two reasons for the insensitivity to light are possible. First, as shown previously, excess NO_2^- (sufficient for hours of NO_x evolution) was already present in the leaf; and second, a portion of this relatively large NO_2^- pool, not capable of being enzymically converted to NO_x, could have been inaccessible to the chloroplast (*e.g.* it could have been in other parts of the cell or other cells of the leaf and was far removed from

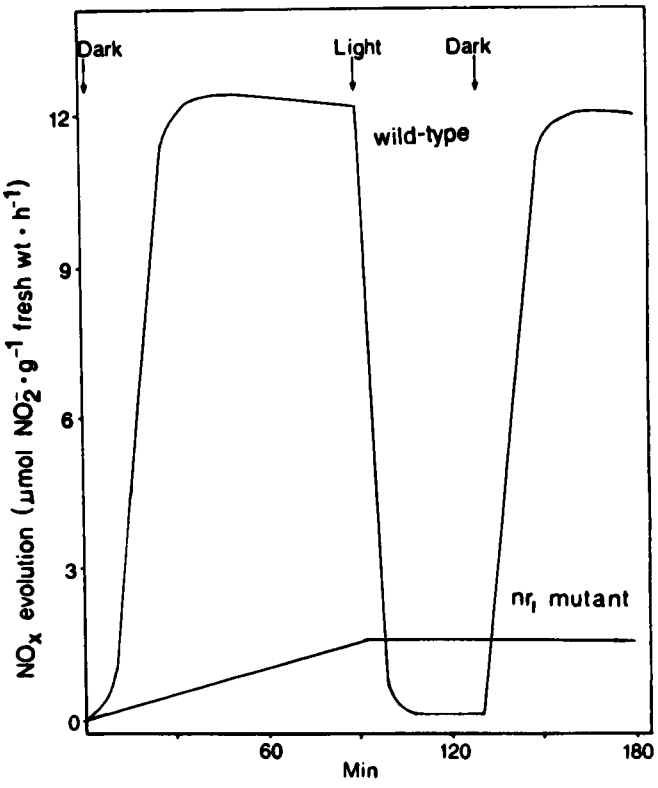

Figure 4. Response of NO_x evolution by wild type and nr_1 mutant leaves to light and darkness. The entire incubation was anaerobic and began with a dark treatment until NO_x evolution rates were easily measureable before the light treatment (100 μmol photons m^{-2} s^{-1}) was begun. Both types of leaves were exposed to the same periods of light and dark.

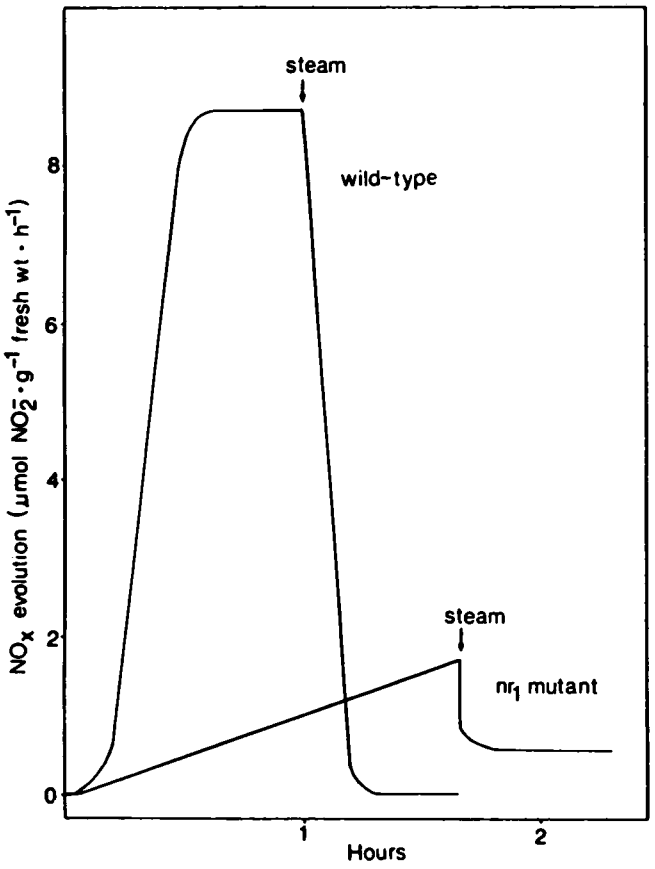

Figure 5. Response of NO_x evolution by wild type and nr_1 mutant leaves to steam treatments. Leaf discs (1 cm) were given dark, anaerobic treatments until NO_x evolution reached near maximal rates, then transferred to a 'teaball' for 30 s steam treatments and immediately retested for NO_x evolution.

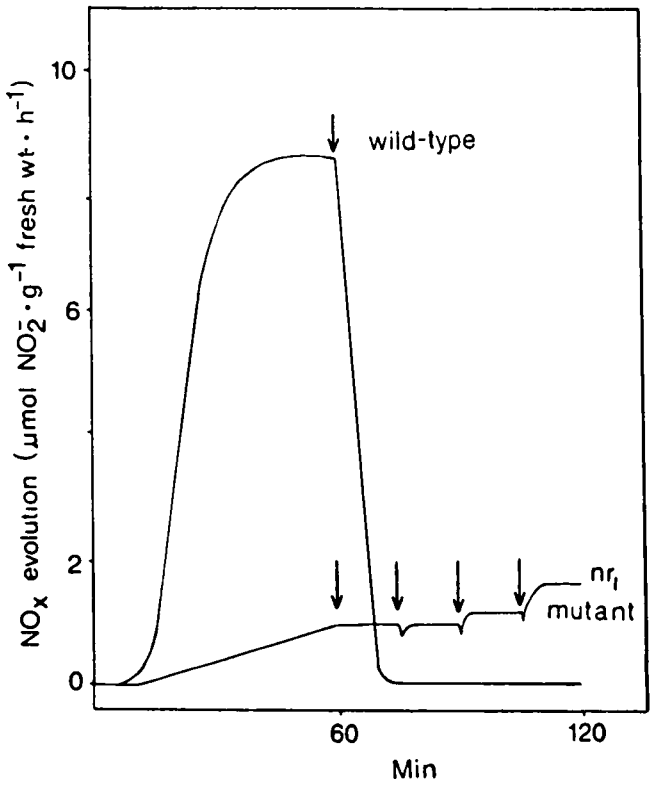

Figure 6. Response of NO_x evolution by wild type and nr_1 mutant intact leaves to microwave denaturation treatments. Arrows denote times of microwave treatments. Initial NO_x evolution was begun using dark, anaerobic conditions. The leaves were transferred to the oven for 10 s treatments and then transferred back for NO_x analyses.

the site of chloroplastic NO_2^- reduction). The nr_1 mutant does contain NO_2^- reductase and under normal circumstances NO_2^- is photosynthetically reduced and does not accumulate. Wild-type leaves demonstrated the typical sigmoid pattern of NO_x evolution quickly reaching a level state. NO_x evolution by the nr_1 mutant always slowly increased linearly until reaching a lower plateau.

In all tests comparing NO_x evolution, wild-type leaves always evolved more than the nr_1 mutant. However, when the products of NO_x evolution were compared, differences were found. With wild type, usually 98 to 99% of the NO_x is composed of NO (2, 4, 5). Very little NO_2 escapes the aqueous interior of the leaf since it is completely water soluble ($2\ NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2\ H^+$) (5). The nr_1 mutant repeatedly evolved 15 to 30% of NO_x as NO_2 from intact leaves. This source of NO_2 could have been due to HNO_2 decomposition according to the reaction previously mentioned (8), while wild type would have more NO_2^- enzymically converted specifically to NO.

Heat Treatment

In an earlier report (4) it was first suggested that NO_x evolution was an enzymic process, because it was completely inhibited by boiling the leaf discs for 5 min. Heat treatment of the leaf discs with 30 s steam stopped wild-type NO_x evolution (Fig. 5). The same treatment with the nr_1 mutant decreased NO_x evolution by only 50%, indicating that it is less heat-labile than the wild type. One min of steam treatment completely inhibited the mutant. In both tissues, NO_3^- reduction and photosynthetically driven NO_2^- reduction ceased with the 30-s steam treatment (data not shown). The typical kinetic pattern of NO_x evolution by the two types is again demonstrated in this experiment (Fig. 5).

Another method used to inactivate the conversion of NO_2^- to NO_x and to compare differences between the processes of NO_x evolution between wild type and nr_1 mutant was denaturation by microwave treatments (Fig. 6). In initial studies, it was quickly determined that a relatively small amount of microwave energy (15% energy level for 10 s) completely inactivated NO_3^- reduction and photosynthetically driven nitrite reduction. NO_x evolution by the wild-type soybean leaf with a 10-s treatment at 10% energy level (65 W) was inactivated by 60 to 70% and completely inactivated by a single treatment at the 15% level. The nr_1 mutant leaves were treated four times successively with the 15% level energy for 10 s periods. NO_x evolution was not affected by the first two treatments and actually increased with the last two treatments even though the leaves were partially cooked. These data offer further proof that the nr_1 mutant evolves NO_x by chemical reactions rather than an enzymic system as is contained in wild-type leaves.

CONCLUSIONS

The nr_1 mutant with N_2-purging during the *in vivo* NR assay accumulated NO_2^- but did not evolve NO_x. This is consistent with previous reports that the nr_1 mutant did not contain the two constitutive NRs, one of which is capable of converting NO_2^- to NO_x (3, 9, 10, 15, 17). The nr_1 mutant intact leaf or leaf disc, however, is capable of nonenzymically forming and evolving NO_x under dark, anaerobic conditions. The mechanism for this NO_x formation by the nr_1 mutant is proposed to be due to chemical reactions between N oxides and plant metabolites and/or decomposition of nitrous acid.

A different mechanism for the formation of NO_x within the nr_1 mutant is supported by data from the comparison experiments showing at least six differences from the wild type. First, lower levels of NO_x are evolved after approximately twice as long a lag time. Second, the kinetics of the NO_x evolution are vastly different. Third, higher levels of NO_2^- are found in the leaves (even though the mutant contains less NO_3^- reducing capacity). Fourth, different proportions of NO and NO_2 are evolved. Fifth, NO_x evolution by the mutant is not affected by light incubation. Sixth, NO_x evolution by the mutant is much less suceptible to heat denaturation. On the basis of these data, it is likely that a certain amount of nonenzymic NO_x evolution can occur in wild-type leaves. However, this mechanism would be easily masked by the high rates of enzymic NO_x evolution.

ACKNOWLEDGMENT

I would like to thank Dr. Jim Harper, USDA-ARS, University of Illinois, for furnishing seeds of the nr_1 mutant soybean.

LITERATURE CITED

1. **Churchill KA, Klepper LA** (1979) Effects of ametryn on nitrate reductase activity and nitrite content of wheat. Pest Biochem Physiol **12:** 156–162
2. **Dean JV, Harper JE** (1986) Nitric oxide and nitrous oxide production by soybean and winged bean during the *in vivo* nitrate reductase assay. Plant Physiol **82:** 718–723
3. **Dean JV, Harper JE** (1988) The conversion of nitrogen oxide(s) by the constitutive NAD(P)-nitrate reductase enzyme from soybean. Plant Physiol **88:** 389–395
4. **Harper JE** (1981) Evolution of nitrogen oxide(s) during *in vivo* nitrate reductase assay of soybean leaves. Plant Physiol **68:** 1488–1493
5. **Klepper LA** (1979) Nitric oxide (NO) and nitrogen oxide (NO_2) emissions from herbicide treated soybean plants. Atmos Environ 13: 537–542
6. **Klepper LA** (1980) Nitrite disappearance from excised green leaves in darkness (abstract No. 626). Plant Physiol **65:** S–113
7. **Klepper LA** (1987) Nitric oxide emissions from soybean leaves during *in vivo* nitrate reductase assays. Plant Physiol **85:** 96–99
8. **Nelson DW, Brenner JM** (1970) Gaseous products of nitrite decomposition in soils. Soil Biol Biochem **2:** 203–215
9. **Nelson RS, Ryan SA, Harper JE** (1983) Soybean mutants lacking a constitutive nitrate reductase activity I. Selection and initial plant characterization. Plant Physiol **72:** 503–509
10. **Nelson RS, Streit SL, Harper JE** (1986) Nitrate reductases from wild-type and nr_1 mutant soybean leaves II. Partial activity, inhibitor, and complementation analyses. Plant Physiol **80:** 72–76
11. **Nicholes JC, Harper JE, Hageman RH** (1976) Nitrate reductase activity in soybeans I. Effects of light and temperature. Plant Physiol **58:** 731–735
12. **Nishimura H, Hayamizu T, Yanagisawa Y** (1986) Reduction of NO to NO_2 by rush and other plants. Environ Sci Technol **20:** 413–416
13. **Robin P, Streit L, Campbell G, Harper JE** (1985) Immunochemical characterization of nitrate reductase forms from wild-type (cv Williams) an nr_1 mutant soybean. Plant Physiol **77:** 232–236
14. **Ryan SA, Nelson RS, Harper JE** (1983) Soybean mutants lacking constitutive nitrate reductase activity II. Nitrogen assimilation, chlorate resistance, and inheritance. Plant Physiol **72:** 510–514
15. **Streit L, Harper JE** (1986) Biochemical characterization of soybean mutants lacking constitutive NADH:nitrate reductase. Plant Physiol **81:** 593–596
16. **Streit L, Martin BA, Harper JE** (1987) A method for the separation and partial purification of the three forms of nitrate reductase in wild-type leaves. Plant Physiol **84:** 654–657
17. **Streit L, Nelson RS, Harper JE** (1985) Nitrate reductases from wild-type and nr_1-mutant soybean leaves I. Purification, kinetics and physical properties. Plant Physiol **78:** 80–84