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Soybean Mutants Lacking Constitutive Nitrate Reductase Activity[1]

I. SELECTION AND INITIAL PLANT CHARACTERIZATION

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RICHARD S. NELSON, SARAH A. RYAN[2], AND JAMES E. HARPER
Department of Agronomy (R. S. N., S. A. R.) and United States Department of Agriculture, Agricultural Research Service (J. E. H.), University of Illinois, Urbana, Illinois 61801

ABSTRACT

The objectives of this study were to select and initially characterize mutants of soybean (*Glycine max* L. Merr. cv Williams) with decreased ability to reduce nitrate. Selection involved a chlorate screen of approximately 12,000 seedlings (progeny of mutagenized seed) and subsequent analyses for low nitrate reductase (LNR) activity. Three lines, designated LNR-2, LNR-3, and LNR-4, were selected by this procedure.

In growth chamber studies, the fully expanded first trifoliolate leaf from NO_3^--grown LNR-2, LNR-3, and LNR-4 plants had approximately 50% of the wild-type NR activity. Leaves from urea-grown LNR-2, LNR-3, and LNR-4 plants had no NR activity while leaves from comparable wild-type plants had considerable activity; the latter activity does not require the presence of NO_3^- in the nutrient solution for induction and on this basis is tentatively considered as a constitutive enzyme. Summation of constitutive (urea-grown wild-type plants) and inducible (NO_3^--grown LNR-2, LNR-3, or LNR-4 plants) leaf NR activities approximated activity in leaves of NO_3^--grown wild-type plants. Root NR activities were comparable in wild-type and mutant plants grown on NO_3^-, and roots of both plant types lacked constitutive NR activity when grown on urea. In both growth chamber- and field-grown plants, oxides of nitrogen [$NO_{(x)}$] were evolved from young leaves of wild-type plants, but not from leaves of LNR-2 plants, during *in vivo* NR assays. Analysis of leaves from different canopy locations showed that constitutive NR activity was confined to the youngest three fully expanded leaves of the wild-type plant and, therefore, on a total plant canopy basis, the NR activity of LNR-2 plants was approximately 75% that of wild-type plants. It is concluded that: (a) the NR activity in leaves of NO_3^--grown wild-type plants includes both constitutive and inducible activity; (b) the missing NR activity in LNR-2, LNR-3, and LNR-4 leaves is the constitutive component; and (c) the constitutive NR activity is associated with $NO_{(x)}$ evolution and occurs only in physiologically young leaves.

The mechanism of NO_3^- inhibition of nodulation is unclear, but considerable evidence suggests that the inhibition is due in part to preferential metabolism of NO_3^- at the expense of carbon availability for nodule development and function. One possible way to determine if NO_3^- inhibition of nodulation involves NO_3^- metabolism is to isolate a plant with impaired ability to metabolize NO_3^-, *i.e.* lacking NR[3] activity or NO_3^- uptake capability. A genetically diverse population and a rapid screening technique are required to identify such plants.

Chlorate has long been known to have toxic effects on plant growth, and Åberg (1) proposed that the mode of action in wheat (*Triticum aestivum*) was through reduction of ClO_3^-, likely to ClO_2^-, by the NO_3^--reducing system. Later work indicated that NR was specifically responsible for the reduction of ClO_3^- to ClO_2^- (17, 25). Chlorate also competes with NO_3^- for uptake in *Hordeum vulgare* and *Arabidopsis thaliana* roots (5, 6). Thus, ClO_3^- can act as an analog of NO_3^- during uptake, as well as during reduction by NR. The analog characteristic of ClO_3^- and the resultant toxicity symptoms associated with ClO_3^- reduction suggested that this ion could be used as a screening agent to identify organisms with decreased NO_3^- uptake and metabolism. Although mechanisms of ClO_3^- toxicity other than that involving NR are possible (14, 19, 27), ClO_3^-resistant mutants which are NR deficient have been found in higher plants (7, 22, 26) and plant cell lines (14, 18, 19).

Soybeans (*Glycine max* L. Merr.) have been shown to display characteristic ClO_3^- toxicity symptoms (browning and stunting of leaves) (9, 28), and this was the basis for using a ClO_3^- screen to identify plants defective in NO_3^- metabolism. This paper reports on the selection, from progeny of mutagenized seed, and initial characterization of three soybean lines which have decreased leaf NR activity compared with wild-type plants.

MATERIALS AND METHODS

Plant Growth and Sampling. *Screening Procedure.* Soybean (*Glycine max* L. Merr. cv Williams) seed, which had been treated for four successive generations with various chemical and physical mutagens (20), were screened for NR-deficient plants. The fourth generation of treated seed (designated M_1) were planted in the field and the bulk harvested M_2 seed were used in the ClO_3^- screen. Seeds were germinated in a field hydroponic system (8) containing nutrient solution A (Table I) plus 0.1 mM $KClO_3$. Screening for ClO_3^- toxicity consisted of visual observation of symptoms on plants 10 to 12 DAP. Symptoms included (a) necrosis of the cotyledon margins and/or (b) chlorosis and necrosis of the unifoliolate leaves with subsequent stunted leaf expansion. Plants were selected if the symptoms displayed for either criterion were

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[2] Present Address: Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, P.O. Box 1600, Canberra, A.C.T. 2601, Australia.

[3] Abbreviations: NR, nitrate reductase; DAP, days after planting; LNR, low nitrate reductase; $NO_{(x)}$, NO and NO_2 collectively.

Table I. *Nutrient Solution Compositions for Field Hydroponic Screening and Growth Chamber Studies*

The basic solutions (A, B, and C) were supplemented with various salts as outlined in "Materials and Methods" for specific studies.

Nutrients	Nutrient Solutions		
	A	B	C
		mM	
$Ca(NO_3)_2$	1.0	5.0	0.0
KNO_3	1.0	5.0	0.0
$MgSO_4$	0.4	2.0	1.0
Potassium phosphate (pH 6.5)	0.2	1.0	0.25
$CaCl_2$	0.0	0.0	2.5
K_2SO_4	0.0	0.0	1.25
		μM	
Fe[a]	9.0	36.0	18.0
KCl	5.1	50.0	25.0
H_3BO_3	2.5	25.0	12.5
$MnSO_4 \cdot 4H_2O$	0.5	6.0	3.0
$ZnSO_4 \cdot 7H_2O$	0.2	2.0	1.0
$CuSO_4 \cdot 5H_2O$	0.05	5.0	2.5
$(NH_4)_6Mo_7O_{24} \cdot H_2O$	0.01	0.015	0.0075

[a] Sodium ferric diethylene triamine pentaacetate (Sequestrene 330 iron chelate, Ciba-Geigy Agricultural Chemicals[4]).

less than those displayed by the bulk of the screened plants. Selected plants were transplanted at 14 DAP into a hydroponic unit containing nutrient solution A (Table I) plus 2.0 mM urea, and individual plants were harvested at maturity to provide M_3 seed for additional screening.

The M_3 plants were subjected to a second ClO_3^- hydroponic screen and, after transplanting resistant plants to the field, leaf NR activity was determined. Parallel to this screen, additional M_3 field-grown plants were analyzed directly for leaf NR activity. Selected M_3 plants were harvested individually at maturity, and resulting M_4 seed, remnant M_3 seed, and wild-type seed were grown in growth chambers in sand with 4 mM KNO_3 for comparison of NR activity. Based on results of the ClO_3^- screens, field NR assays, and growth chamber comparison of selections with wild-type plants, three lines with decreased NR activity (designated LNR-2, LNR-3, and LNR-4) were selected for additional characterization.

Growth Chamber Studies. Growth chamber conditions were 14 h/28°C light periods and 10 h/19°C dark periods. RH was approximately 50% and light flux density (PAR), supplied by fluorescent and incandescent lamps, was 600 $\mu E\ m^{-2}\ s^{-1}$. LNR-2, LNR-3, LNR-4, and wild-type seed were germinated in sand and subirrigated with deionized H_2O. At specified DAP, seedlings were transplanted into 2-L black polystyrene pots (six plants per pot maximum) containing nutrient solution which was continuously aerated. An ion-exchange column (12) was placed in each pot to maintain pH between 5.0 and 7.0.

For the study of NR activity in NO_3^-- and urea-grown plants, seedlings (7 DAP) were transplanted into either 1/2X nutrient solution B (Table I) or 1X nutrient solution C supplemented with 3.75 mM urea (Table I), respectively. Each pot contained four seedlings (LNR-2, LNR-3, LNR-4, and wild type). Nutrient solutions were renewed at 11 DAP. Samples were taken at 15 DAP when first trifoliolate leaves had just reached full expansion and were expected to have maximum measurable NR activity on a g fresh weight basis (11). Two plants within lines were harvested per nutrient treatment, bulked, and the first trifoliolate leaves and roots were assayed for NR activity. There were four replicates per plant line for each nutrient treatment.

Subsequent studies (growth chamber and field) were limited to comparisons between LNR-2 and wild-type plants. This was done since LNR-3 and LNR-4 plants had altered phenotypic characteristics (*i.e.* poor seed set [LNR-4] and slower growth [LNR-3 and LNR-4]) which impaired direct comparison to wild-type plants.

For the profile study of NR activity and $NO_{(x)}$ evolution, LNR-2 and wild-type seedlings were transplanted from the sand medium at 5 DAP into 2X nutrient solution C (Table I) modified to contain 0.25 mM phosphate and supplemented with either 15 mM NO_3^-- or urea-N. Nutrient solutions were renewed at 12 DAP. Sampling began at 5 DAP with the first sample obtained from the sand germination trays. Sampling continued daily to 13 DAP and then every other day to 19 DAP. Single plants from each line were harvested from three randomly chosen pots containing like nutrient solutions and analyzed separately as replicates (except for 5 and 6 DAP samples which were bulked).

Field Study. LNR-2 and wild-type seed were planted in the field on June 2, 1981. The experimental design was a randomized complete block with five replicates. Total canopy NR activity and upper canopy $NO_{(x)}$ evolution data were obtained from leaf samples harvested at 34, 42, 50, and 61 DAP. At each date unifoliolate leaves and trifoliolate leaflets were sampled from all nodes of two plants per plot and bulked within plots by node number (unifoliolate leaf was designated at the first node). The leaf material was then composited by node number into groups of three and each group analyzed for NR activity. Only the youngest three fully expanded leaves were analyzed for $NO_{(x)}$ evolution at these dates. At 71 DAP, total canopy $NO_{(x)}$ evolution as well as canopy NR activity was measured. Senesced leaves were never sampled. *F* tests were performed for each parameter measured.

Assays. *Nitrate Reductase. In vivo* leaf NR assays were performed as described by Nicholas *et al.* (21). Assays were either in the absence ($-NO_3^-$) or presence ($+NO_3^-$) of 50 mM KNO_3. *In vivo* root NR assays were performed according to Crafts-Brandner and Harper (4) using 50 mM KNO_3. *In vitro* leaf NR assays were performed by the method of Scholl *et al.* (24) as modified by Nicholas *et al.* (21).

$NO_{(x)}$ Evolution. Leaf discs for $NO_{(x)}$ assays were obtained using the procedure described by Harper and Hageman (11) for *in vivo* NR analysis. Gas evolution from these leaf discs (200 mg) was measured during incubation in a $+NO_3^-$ *in vivo* NR assay medium as described by Harper (10). Nitrogen carrier gas flow rates were 300 $cm^3\ min^{-1}$. Assays were conducted for 30 min in a shaker bath at 30°C. For wild-type samples, the $NO_{(x)}$ gas evolved was collected by transferring the fritted glass tubes to a fresh trapping solution every 5 min. Data from the six trapping solution tubes were summed to obtain gas evolution values for the 30-min period. For LNR-2 samples, the fritted glass tubes remained in the same trapping solution for 30 min. $NO_{(x)}$ recovered was measured as NO_2^- present in the trapping solution and expressed as NO_2^- equivalents.

RESULTS

Selection of Chlorate-Resistant Mutants. Forty-nine potential NR-deficient plants were selected from 12,000 M_2 seedlings in the first ClO_3^- screen. Thirty-eight of these produced seed. Rescreening of the M_3 plants against ClO_3^-, and direct leaf NR analyses, further decreased the number of possible NR-deficient lines to three (designated LNR-2, LNR-3, LNR-4).

Growth Chamber Studies. *NR Activity.* Nitrate reductase activities of first-trifoliolate leaves of LNR-2, LNR-3, and LNR-4 plants were approximately 50% of wild-type activity when grown on NO_3^-, regardless of assay condition (Table II). In contrast, NR activities of roots were similar for NO_3^--grown LNR and wild-type lines. Nitrate reductase activity was absent in leaves of urea-grown LNR-2, LNR-3, and LNR-4 lines while comparable urea-

Table II. *NR Activity in First Trifoliolate Leaves and Roots from NO_3^-- or Urea-Grown Wild-Type, LNR-2, LNR-3, and LNR-4 Plants*

Samples were from growth chamber plants (15 DAP) grown hydroponically on 7.5 mM N. NADH was utilized as electron donor in *in vitro* assays. Data are means of four replicates ± SD.

Tissue Sampled	NR Assay Condition	Nutrient N Source	Wild Type	LNR-2	LNR-3	LNR-4
			μmol NO_2^-·g^{-1} fresh wt·h^{-1}			
First trifoliolate leaf	+NO_3^- *in vivo*	NO_3^-	26.9 ± 3.9	14.2 ± 3.5	11.1 ± 5.0	12.3 ± 2.8
		Urea	16.0 ± 5.6	0.0	0.0	0.0
	−NO_3^- *in vivo*	NO_3^-	10.4 ± 0.2	6.3 ± 1.6	5.6 ± 2.1	5.4 ± 1.7
		Urea	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
	In vitro	NO_3^-	47.9 ± 2.6	26.9 ± 1.6	26.1 ± 3.6	26.2 ± 4.9
		Urea	18.5 ± 4.7	0.0	0.6 ± 1.0	0.1 ± 0.1
			μmol NO_2^-·g^{-1} dry wt·h^{-1}			
Root	+NO_3^- *in vivo*	NO_3^-	131.0 ± 3.7	129.0 ± 3.1	114.3 ± 10.1	114.4 ± 5.0
		Urea	0.4 ± 0.5	0.0	0.0	0.0

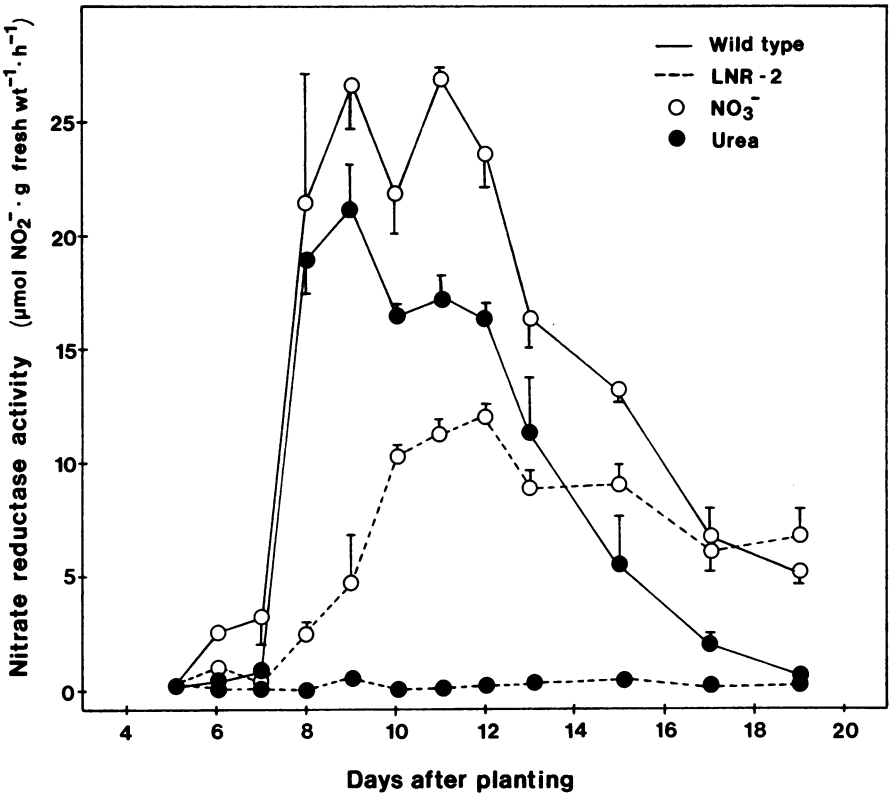

FIG. 1. NR activity profiles for unifoliolate leaves from NO_3^-- or urea-grown wild-type and LNR-2 growth chamber plants. The initial sample was from seedlings grown in a sand germination flat supplied with deionized H_2O. All subsequent samples were from seedlings grown hydroponically on 15 mM urea- or NO_3^--N. Data are means of three replicates ± SE except for values from 5 and 6 DAP samples which were bulked. The SE was within the data point if not shown for means from 7 to 19 DAP.

grown wild-type plants had considerable +NO_3^- *in vivo* and *in vitro* leaf activity (Table II). This activity in urea-grown wild-type plants is considered to be a constitutive NR activity, *i.e.* it occurs in the absence of NO_3^- substrate. The constitutive NR activity was absent in roots of both wild-type and LNR lines.

LNR-2 and wild-type unifoliolate leaves were analyzed for NR activity over a 14-d period (Fig. 1). Unifoliolate leaves from urea-grown LNR-2 plants never displayed measurable NR activity, while comparable assays of wild-type leaves displayed significant constitutive activity throughout the experiment, peaking at 9 DAP and declining thereafter. This activity varied from approximately 85% of the activity in NO_3^--grown wild-type plants when unifoliolate leaves were reaching full expansion (8 and 9 DAP), to 70% at 10 through 13 DAP, and 12% at 19 DAP; constitutive NR activity was near zero at 19 DAP. The NO_3^--grown LNR-2 unifoliolate leaves displayed a delayed NR activity profile in comparison with the wild type. At full leaf expansion (9 DAP), LNR-2 activity was only 18% that of NO_3^--grown wild-type activity. This percentage increased rapidly until by 17 DAP the activities were equal and remained so for the rest of the experimental period. Attainment of equivalent activity coincided with the disappearance of constitutive NR activity in urea-grown wild-type plants. Unifoliolate leaves of NO_3^--grown wild-type plants displayed a broad peak in NR activity (8 through 12 DAP) which encompassed both the urea-grown wild-type and NO_3^--grown LNR-2 peaks. Over the 19-d experimental period, unifoliolate leaf

NR activities of urea-grown wild-type and NO_3^--grown LNR-2 plants were 61 and 48%, respectively, that of NO_3^--grown wild-type plants.

$NO_{(x)}$ Evolution. Preliminary studies indicated that fully expanded unifoliolate and first trifoliolate leaves from LNR-2 and LNR-3 plants were unable to evolve measurable $NO_{(x)}$ gases during *in vivo* NR assays (data not shown). In the present study, LNR-2 unifoliolate leaves, regardless of nutrient N source, evolved no measurable $NO_{(x)}$ (Fig. 2). In contrast, unifoliolate leaves of wild-type plants grown on urea or NO_3^- evolved $NO_{(x)}$ gases during the *in vivo* NR assay. In addition, the amount of gas recovered in the wild-type leaf NR assays was the same for plants grown on urea or NO_3^-. Gas evolution from wild-type unifoliolate leaves reached a maximum at 9 DAP, and declined rapidly thereafter (Fig. 2). The constitutive NR activity also reached a maximum at 9 DAP; however, the subsequent decline was slower than the decline in gas evolution (Figs. 1 and 2).

Field Study. *NR Activity.* Over the growing season, NR activity (*in vivo*) in LNR-2 leaves was 50% to 60% of the wild type in the three youngest fully expanded leaves and equal to wild type in the remainder of the lower canopy (Fig. 3). However, considering the whole canopy, LNR-2 plants had approximately 75% of the NR activity in the wild type at each sampling date.

$NO_{(x)}$ Evolution. $NO_{(x)}$ evolution was near zero for the three youngest fully expanded LNR-2 leaves while the respective wild-type leaves always maintained significant activity (Table III). These results (Table III) and those in Figure 3 consistently indicated a relationship between $NO_{(x)}$ evolution and the differential NR activity between wild-type and LNR-2 upper canopy leaves. This relationship was most apparent when canopy profiles were determined for both NR activity and $NO_{(x)}$ evolution (Fig. 4). Only at the top of the canopy did wild-type leaves have greater NR activity than LNR-2 leaves. These leaves were responsible for the emission of ~95% of the total $NO_{(x)}$ recovered from the wild-type canopy.

DISCUSSION

The 49 soybean plants selected in the initial ClO_3^- screen were identified in an outdoor system where light intensity varied daily. Harper (9) showed that ClO_3^- toxicity symptoms in soybean leaves were positively correlated with increasing light intensity. Therefore, some of the original plants selected were likely low light escapes. Furthermore, since seed had been treated for four successive generations with three different mutagens, it was not possible to determine the mutagen responsible for the true ClO_3^--resistant plants.

NR activity of most plant species is usually expressed only when NO_3^- is present in the growth media (2). Lahav *et al.* (16), however, found that soybean plants contained measurable leaf NR activity when grown on urea in the absence of NO_3^-. Leaves of urea-grown LNR-2, LNR-3, and LNR-4 mutant plants did not contain this constitutive NR activity, and when grown on NO_3^- the mutants had approximately 50% of wild-type NR activity (Table II). These results indicated that the decreased NR activity in leaves of NO_3^--grown LNR-2, LNR-3, and LNR-4 plants was due to absence of constitutive NR activity. The results also implied that leaves from NO_3^--grown wild-type plants contained both activities. The presence of constitutive NR activity in urea-grown wild-type plants and absence in LNR-2, LNR-3, and LNR-4 plants was not due to possible differential conversion of urea to NO_3^- in the nutrient solutions since LNR and wild-type plants were grown together in the same pot for this experiment and all plant types had equivalent low levels of tissue NO_3^- (~3% of NO_3^--grown plants). If inducible and constitutive NR fractions are independent, and nutrient N source (urea or NO_3^-) has no effect on the level of constitutive activity, then the sum of the

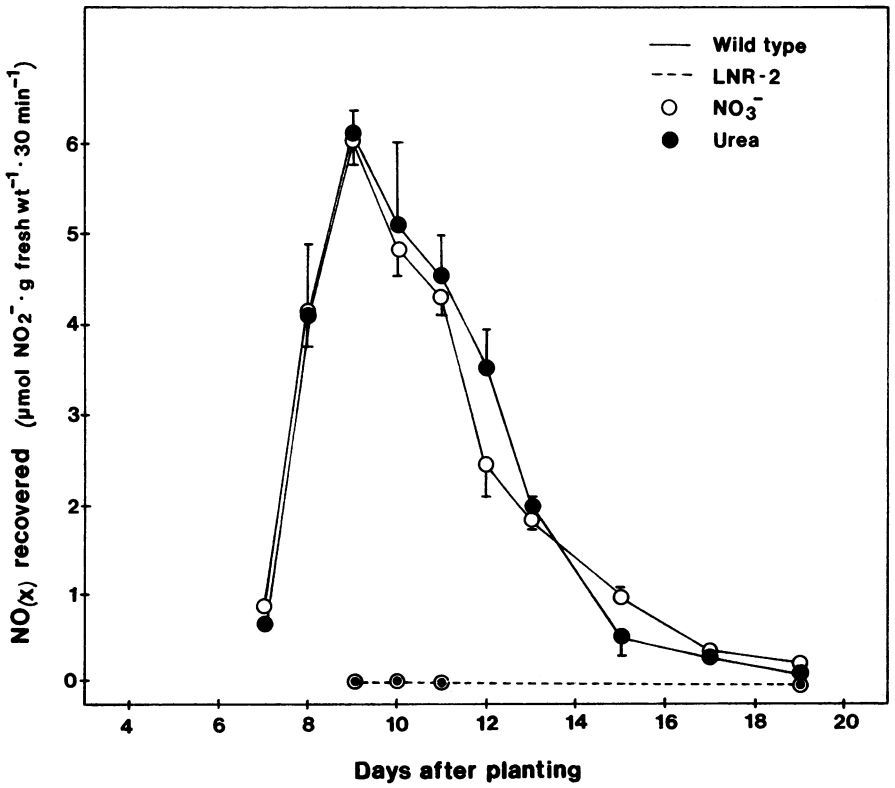

FIG. 2. $NO_{(x)}$ evolution profiles for unifoliolate leaves from NO_3^-- or urea-grown wild-type and LNR-2 growth chamber plants. Plant growth conditions were as indicated in Figure 1 legend. Data are means of three replicates ± SE. The SE was within the data point if not shown. Data for LNR-2 at 9 and 11 DAP were from a separate experiment.

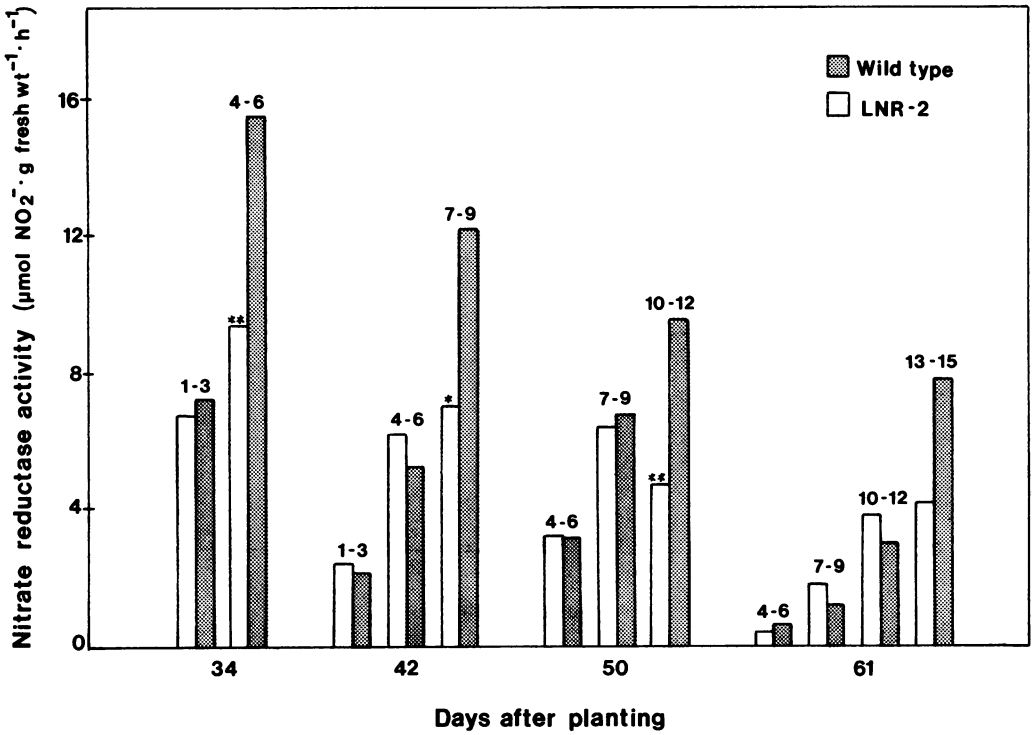

FIG. 3. Canopy nitrate reductase activity for field-grown wild-type and LNR-2 plants at four different sampling dates. Numbers above the bars represent the node numbers of the leaves composited for that particular sample. The unifoliolate leaf was at node one. (*, **), Significantly different from wild type at the 0.05 and 0.01 levels, respectively, according to *F* test.

Table III. *$NO_{(x)}$ Evolution from a Composite Sample of the Three Youngest Fully Expanded Leaves of Wild-Type and LNR-2 Plants Grown in the Field*

Nodes Sampled	Plant Age	Wild Type	LNR-2
	DAP	*μmol NO_2^- recovered · g^{-1} fresh wt · 30 min^{-1}*	
4–6	34	0.77	0.02***
7–9	42	0.90	0.04**
10–12	50	1.16	0.06*
13–15	61	0.23	0.01*

[a] (*, **), Significantly different from wild-type plants at the 0.05 and 0.01 levels, respectively, according to *F* test.

inducible and the constitutive NR activities should equal the total NR activity in leaves of NO_3^--grown wild-type plants. Results in Table II support this proposition. The profiles of unifoliolate leaf NR activity (Fig. 1) also support an additive proposal for the following reasons. First, although in each daily sampling NR activities were not necessarily additive, NR activity under the curves for urea-grown wild-type and NO_3^--grown LNR-2 plants summed to 109% of the total unifoliolate leaf activity from NO_3^--grown wild-type plants. Second, when constitutive activity was disappearing in unifoliolate leaves of urea-grown wild-type plants (~17 DAP), inducible NR activity in LNR-2 unifoliolate leaves equaled NR activity in unifoliolate leaves from NO_3^--grown wild-type plants. Third, the early peak (9 DAP) in NR activity for unifoliolate leaves from urea-grown wild-type plants and the delayed peak (12 DAP) in NR activity for unifoliolate leaves from NO_3^--grown LNR-2 plants were within the broad peak (8 through 12 DAP) in NR activity for unifoliolate leaves from NO_3^--grown wild-type plants.

The lack of NR activity in roots of urea-grown wild-type and mutant plants (Table II) confirmed the previously noted lack of constitutive activity in soybean roots (3). The similar root NR activity of NO_3^--grown wild-type and mutant plants provided further evidence that inducible activity was not affected in the mutant plants (Table II).

The variability in proportion of unifoliolate leaf NR activity in NO_3^--grown LNR-2 plants to NO_3^--grown wild-type plants over time (Fig. 1) indicated that single time point NR assays, normally run when leaves just reach full expansion, might underestimate the total NO_3^--reducing potential of LNR-2 leaves. This indicates the need for multiple measurements when assessing alterations in NR activity.

The $NO_{(x)}$ profile data (Fig. 2) indicated a relationship between the constitutive NR activity and the ability to evolve $NO_{(x)}$ gases. (It should be noted that the identity of the nitrogenous gas(es) evolved from the leaves of soybean during *in vivo* NR assay, suggested to be NO and NO_2 [10, 15], has been questioned [C. Mulvaney and R. H. Hageman, personal communication].) LNR-2 unifoliolate leaves, which did not have constitutive NR activity, never evolved measurable $NO_{(x)}$. Infiltration of leaf discs from urea-grown wild-type and urea- or NO_3^--grown mutant plants with NO_2^-, in the absence of NO_3^- in the assay media, resulted in appreciable $NO_{(x)}$ being evolved from wild-type and only trace levels from LNR-2 leaves (data not shown). Thus, it was concluded that lack of $NO_{(x)}$ evolution from NO_3^--grown LNR-2 plants was not due to compartmentalization of NO_2^- produced by inducible NR activity, but was rather associated with the lack of constitutive NR activity in the LNR-2 plants. The evolution of $NO_{(x)}$ does appear to be an enzymic process (based on boiling studies) (10), although Klepper (15) had previously hypothesized a nonenzymic generation of $NO_{(x)}$ from herbicide-treated soybean leaves. Both possibilities are being investigated in an effort to determine both the relationship between constitutive NR activity and $NO_{(x)}$ evolution and their concurrent loss in the LNR-2 line. In addition, the relationship of constitutive and inducible NR activities to known NADPH- and NADH-NR activities in soybean (13) is under investigation.

The canopy profiles of NR activity for field-grown wild-type

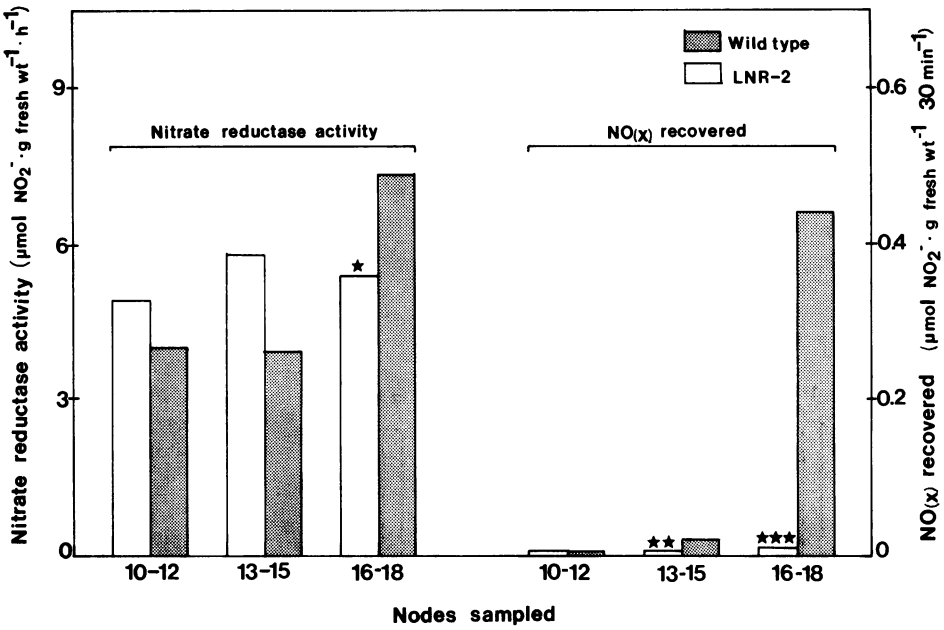

FIG. 4. Canopy nitrate reductase activity and $NO_{(x)}$ evolution from field-grown wild-type and LNR-2 plants at 71 DAP. Numbers on the X-axis represent the node numbers of the leaves composited for that particular sample. The unifoliolate leaf was at node one while the youngest trifoliolate leaf was at node 18. (*, **, ***), Significantly different from wild type at the 0.05, 0.01, and 0.005 levels, respectively, according to *F* test.

leaves (Fig. 3) were similar to those reported previously for cv Beeson (11). Canopy profiles from LNR-2 plants, however, were modified in that the three youngest fully expanded leaves had activities below that of the respective wild-type leaves. This indicated that the normal presence of constitutive NR activity was confined to the youngest leaves in wild-type plants throughout the growing season. This agreed with the NR activity profile results (Fig. 1) which showed that as the unifoliolate leaf aged it lost the constitutive NR activity but maintained inducible activity. The field canopy NR results (Fig. 3) also indicated that total NO_3^--reducing potential by LNR-2 leaves was 75% that of wild type. The LNR-2 plants were likely even more similar to wild-type plants in total NO_3^--reducing potential when one considers that roots of both had similar NR activities (Table II).

The gas evolution data from the field (Table III; Fig. 4) showed that at all sampling dates the youngest leaves of wild-type plants evolved measurable $NO_{(x)}$ and as the leaves aged $NO_{(x)}$ evolution dropped considerably. In addition, as predicted, LNR-2 leaves never evolved measurable $NO_{(x)}$ and, therefore, the missing NR fraction, presumed to be constitutive NR, was again closely associated with the ability to evolve $NO_{(x)}$.

Thus, both growth chamber and field results indicated that the plants selected by ClO_3^- screen and leaf NR analyses had lost a discrete fraction of NR activity in younger leaves. This fraction was designated a constitutive enzyme on the basis of its occurrence in the absence of NO_3^- inducer. This constitutive activity accounted for approximately 25% of total canopy activity and was in some manner linked to the ability to evolve $NO_{(x)}$. The loss of constitutive NR activity in the LNR-2 line did not appear essential to normal plant growth since visual observations in the field indicated similar growth rates to wild type over the growing season. Dry matter and nitrogen analyses of the LNR and wild-type lines are presented in a companion paper (23).

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