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Comparison of *in Vivo* and *in Vitro* Assays of Nitrate Reductase in Wheat (*Triticum aestivum* L.) Seedlings[1]

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ABSTRACT

The effectiveness of the *in vivo* and *in vitro* assays for nitrate reductase (NR) in estimating the amounts of reduced N made available to plants was tested against the daily increases in reduced N (Nesslerization) actually accumulated by the plant. With growth-chamber-grown wheat seedlings, the average ratio values (input of reduced N as estimated by the *in vitro* assay to actual accumulation of N by the plant) were 3.9 for shoots, 3.7 for the roots, and 4.1 for the entire plant, over a 10-day period. With the *in vivo* assay, the average ratio values were 0.7 for the shoot, 1.8 for the root, and 0.9 for the entire plant. Although the linear regressions between the accumulated N in the plant and the estimated N input (by both *in vitro* and *in vivo* assays) were significant and positive, the *in vivo* assay provided the closest approximation of the actual amount of N accumulated.

The *in vivo* NR assay effectively distinguished between two wheat varieties. The variety known to have the higher percentage of seed protein also had the higher amounts of NR activity.

With seedling wheat leaves, the addition of NADH plus a surfactant increased *in vivo* NR activity approximately 2-fold over comparable controls. Because the tissue contained high levels of nitrate and enzyme, we concluded that reducing potential was the rate-limiting factor in nitrate reduction *in situ* in these growth-chamber-grown plants.

Because NR[3] is the rate-limiting step of NO_3^- reduction to NH_4^+ in cereals, the assimilation of NO_3^- can be used to estimate the amount of reduced nitrogen made available to the plant. This is supported by various studies with wheat (2, 4) and corn (3) that show a highly significant correlation between the amount of N supplied to the plant as estimated by the *in vitro* NR assay and the actual amount of N accumulated by the plant. Although, in most cases, the ratio of estimated N exceeds the actual accumulated N by a factor of 2 to 8, values as low as 0.6 were found during the reproductive phase of wheat (4). The overestimation would be anticipated as the optimum conditions used for the *in vitro* assay would seldom be obtained *in situ*. The underestimation which is most common with mature leaf tissue could result from exposure to NR to inhibitors or proteases during extraction as indicated by the work of Schrader *et al.* (15) and Wallace (17).

The use of the *in vivo* assay for NR, as developed by several laboratories (5, 6, 13, 16), would appear as a desirable alternate to the *in vitro* assay. With young vegetative tissue (seedlings), the *in vivo* assay gives lower values (10–50%) than the *in vitro* assay. With mature tissue, there should be less interference from inhibitors and proteases in the *in vivo* assay than with the *in vitro* assay. However, the critical question remains, which assay gives the better correlation with the actual accumulation of reduced nitrogen?

The major objective of this work was to calibrate the *in vivo* and *in vitro* NR assays against the actual accumulation of reduced N by the plant.

MATERIALS AND METHODS

Plant Material. Three hundred wheat seeds (*Triticum aestivum* L.) cvs. Atlas 66 × Comanche, Comanche, or Lancer were surface-sterilized with 0.26% (v/v) sodium hypochlorite for 20 min and rinsed (four times) with distilled H_2O. The seeds were germinated on wet cheesecloth supported by a nylon mesh frame that fitted into a plastic box (20 × 30 × 10 cm). After adding 750 ml of distilled H_2O, lids were placed on the boxes and the boxes placed in a dark growth chamber at 25 C. After 48 hr for germination, the lids were removed and ungerminated seed and weak seedlings removed leaving approximately 250 seedlings. The water was replaced with 5 liters 0.5 mM $CaSO_4$ and the boxes transferred to an illuminated (fluorescent and incandescent bulbs, 20 klux) growth chamber with a 14-hr photoperiod and 25-C day and 18-C night. The $CaSO_4$ and subsequent nutrient solution were vigorously aerated.

Induction of NR was initiated 8 days after sowing by replacing the $CaSO_4$ solution with 5 liters of a modified (10 mM NO_3^-) Hoagland solution (pH 6) complete with micronutrients. The nutrient solution was changed daily 1 hr prior to sampling. Plants were harvested after 4 hr of illumination and separated into roots and shoots. The seed residue was discarded. The roots were washed thoroughly with distilled H_2O and then immersed in cold (3 C) 0.5 mM $CaSO_4$ for 5 min. The roots were blotted dry with paper towels, weighed, and assayed. The shoots were stored in plastic bags and placed in ice (3 C) prior to weighing and assay.

Enzyme Extraction. Wheat tissue was hand ground with a TenBroeck homogenizer using a ratio of 1:10 (w/v) of extraction medium that contained 25 mM K phosphate (final pH 8.8), 5 mM EDTA, 5 mM cysteine-HCl, and 0.42% (v/v) Neutronyx 600 (nonionic surfactant, Onyx Chemicals, Jersey City, N. J.). For root tissue, the cysteine-HCl was reduced to 0.5 mM. Triplicate samples were taken in all cases.

***In Vitro* Assay.** The procedures described by Scholl *et al.* (14) were used. Duplicate or triplicate assays were made on each sample.

***In Vivo* Assay.** The procedure used for shoot tissue from wheat was a modification from Klepper *et al.* (6). The incubation medium was 0.1 M K phosphate (pH 7.5), 0.1 M KNO_3, and 0.42% (v/v) Neutronyx 600. The shoot material from two to four plants (depending on age and size) was cut into 25-mm²

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[3] Abbreviations: NR: Nitrate reductase; ** and *: significant at 1 and 5% levels, respectively.

sections and suspended in 10 ml of incubation medium and vacuum-infiltrated (two times) as described (6). A stainless steel screen held the tissue below the surface of the assay medium. The samples were then transferred to a shaking water bath and incubated in the dark at 30 C. At time intervals, 0.2 ml of the medium was removed for NO_2^- determination. Because the reactions were linear with time (2 hr), the difference in NO_2^- production (corrected for volume) between zero time and 1 hr of incubation was used to calculate activity. There was no enhancement of activity when N_2 was bubbled through the incubation (assay) medium. With shoot tissue, Neutronyx 600 was superior to 1.5% (v/v) propanol (with or without N_2 bubbling), Pronon 105, Neutronyx 675, Tween 20, Maprofix 0 and 563, and Ammonyx 4080. Neutronyx 622 and Pronon 455 were as effective as Neutronyx 600. When propanol was added, bubbling N_2 through the incubation medium enhanced activity 5 to 10%. The effective surfactants eliminated the lag in NO_2^- production frequently observed with the *in vivo* assay.

With the root tissue from wheat, the same procedure was used except that 1.5% (v/v) propanol was substituted for the Neutronyx 600 and N_2 was bubbled through the incubation medium.

With intact wheat seedlings (seed residue removed), the assay was the same as used for roots, except that one to four plants were added to 10 ml of incubation medium. Because a lag in NO_3^- reduction was noted with the intact seedlings, the difference in NO_2^- produced between 30 and 90 min was used to calculate activities. From three to six replicate samples were assayed each time.

When the effectiveness of NADH as an electron donor for NR was tested, the shoot *in vivo* assay was modified slightly. Expanded leaf tissue (0.2 g) cut into 25-mm^2 sections was added to 5 ml of incubation medium. The NADH (up to 4 mg/ml) was added just prior to vacuum infiltration. Disappearance of NADH was followed by absorbancy loss of aliquots removed at intervals from the incubation medium. Nitrite production was measured concurrently after removing excess NADH by addition of phenazine methosulfate as previously described (14).

Activities. Both *in vivo* and *in vitro* activities were expressed as μmol NO_2^- produced (g fresh wt^{-1} hr^{-1}). Assuming that NR is the rate-limiting step in NO_3^- assimilation, the amount of reduced N (μmol/plant or plant part) supplied per day was estimated by multiplying activity (μmol g fresh wt^{-1} hr^{-1}) × total fresh weight (shoot, root or total plant) × time (24 or 14 hr) × a diurnal correction factor (0.8 or 0.93). Because Canvin and Atkins (1) have provided evidence that leaf tissue does not reduce NO_3^- under dark aerobic conditions, the data for shoots and intact plants were computed using the factor 13 (14 hr × 0.93). A factor of 19.2 (24 hr × 0.8) was used for root material. Data obtained with the excised roots were used to compute a correction factor for the 24-hr root activity period for the intact plants. The diurnal correction factors (0.8 and 0.93) were determined experimentally.

Nitrate. Nitrate concentration of the wheat tissue was determined on an aliquot from the clarified homogenates used for the *in vitro* NR assay by the procedure of McNamara *et al.* (7).

Total Reduced Nitrogen. Triplicate samples of comparable plant tissue were harvested at appropriate intervals to determine the actual accumulation of reduced nitrogen. The samples were dried (70 C) to constant weight prior to digestion with 20% (v/v) H_2SO_4 and 30% (v/v) H_2O_2. The H_2O_2 was added after cooling and as frequently as necessary to obtain clarification. After acidification, the NH_4^+ was determined by Nesslerization in the digestion tubes. This procedure determines only reduced N as addition of NO_3^- during digestion does not increase the amount of N recovered. The amount of reduced N (μmol) accumulated for each plant part or whole seedling per day was calculated by difference from the daily samplings.

RESULTS AND DISCUSSION

With shoot material, the extracted NR activity increased rapidly and linearly for the first 2 days after addition of NO_3^-, slowly for the next 3 days, and remained high and constant for the last 6 days (Fig. 1). A similar pattern was observed with the *in vivo* assay except that the rate of increase for the first 2 days was not constant and a plateau was achieved on the 3rd day. The *in vivo* activity was approximately one-sixth of the *in vitro* activity for the shoots or shoot parts (blades and stems).

With root material, similar patterns of enzyme activity were also obtained with the two assay procedures (Fig. 1). With both assays, highest activity was observed 1 day after the addition of NO_3^- and activity decreased slowly thereafter. Although the decrease in *in vitro* activity can be explained by the decreased stability of the root enzyme with age (11), or the presence of a protease in the extract (17), these explanations would not seem to be applicable to the *in vivo* assay. It is possible that the presence of the propanol in the medium permits one or more proteases to gain access to NR *in vivo*. It is also conceivable that there is an actual decrease in *in situ* NR activity in root tissue with root development. The *in vivo* activity in root tissue was approximately half that of the *in vitro* activity.

The pattern of NO_3^- concentration in the shoot tissue over time was nearly identical with the *in vitro* NR activity pattern (*cf.* Fig. 2 with Fig. 1). Similar coincident patterns of NO_3^- concentration and induced NR activity have been reported for corn roots (10). The data (Fig. 2) show that the capacity of the root and shoot to accumulate NO_3^- and induce NR has been

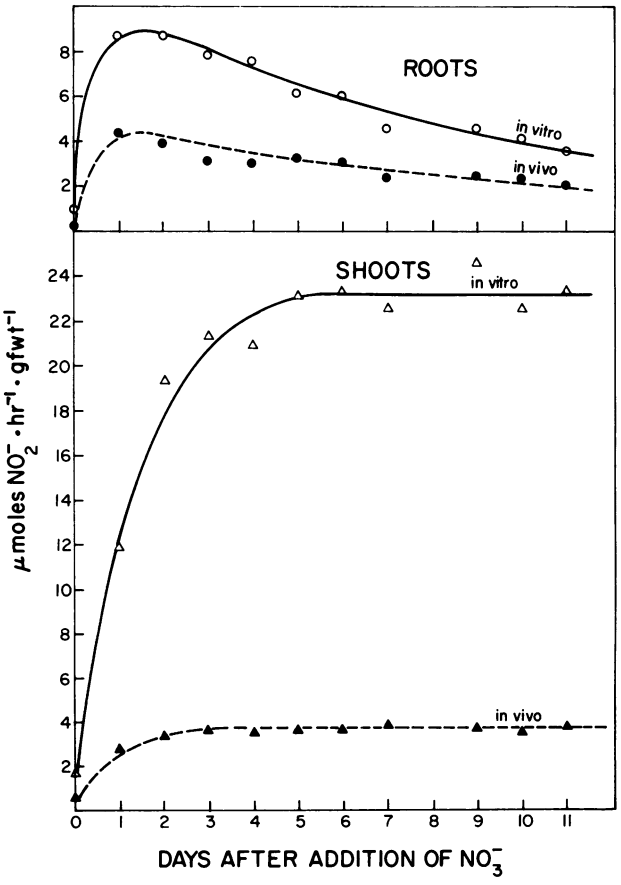

FIG. 1. Patterns of nitrate reductase in shoots and roots of wheat seedlings (cv. Atlas 66 × Comanche) as measured by *in vitro* and *in vivo* assays. The entire plant less the residual seed is represented in the assays.

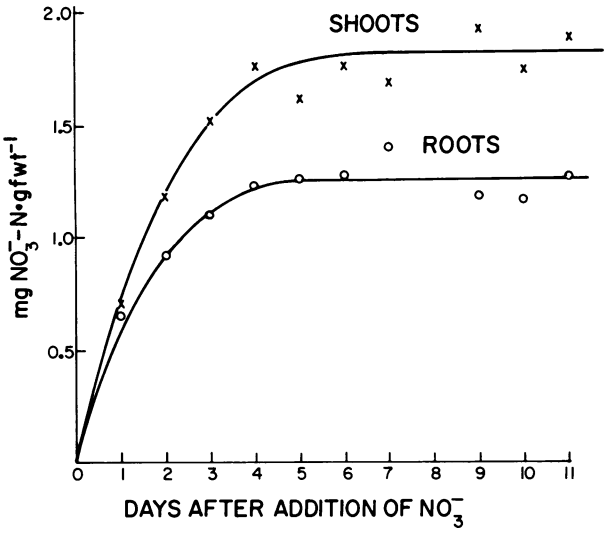

FIG. 2. Patterns of nitrate concentration in roots and shoots of wheat seedlings. The *in vitro* extract (Fig. 1) was used for assay.

saturated under these experimental conditions (10 mM NO_3^- nutrient medium, replaced daily). However; working with corn, Meeker *et al.* (8) found that increases in the NO_3^- concentration of the nutrient medium caused increases in NO_3^- concentrations in the tissue and in the level of NR activity.

The pattern of NO_3^- concentration in the root tissue over time was similar to that of the shoot tissue, except that the saturation level was lower (1.25 mg NO_3^- N g fresh wt^{-1}). In contrast to the shoot tissue, a correlation between NO_3^- concentration and enzyme activity by either method was observed only during the 1st day. Possible reasons for this lack of correlation were discussed previously.

Calibration. The relationships between the amount of reduced N made available to the plant or plant part per day, as estimated by *in vitro* and *in vivo* assays, and the actual amount of reduced N (measured by digestion and Nesslerization of comparable tissue) accumulated by the plant or plant part per day are shown in Figure 3. The correlation coefficients (r) and regression coefficients (b) between *in vivo* and actual, *in vitro* and actual, and *in vivo* and *in vitro* were all positive and significant at the 1% level.

The *in vivo* assay provides a close approximation (one to one basis) of the actual accumulation of reduced N by the intact plant, as the average ratio value (enzyme input/actual) was 0.9, over the 10-day period. However, the *in vivo* assay underestimated the accumulation of N by the shoot (ratio value, 0.7) and overestimated the accumulation of N by the root (ratio value, 1.8). The *in vitro* assay overestimated the actual accumulation of N by ratio values of 4.1 for the intact plant, 3.9 for the shoots, and 3.7 for the roots. This overestimation can be expected because the *in vitro* assay reflects the total amount of extractable enzyme assayed under optimum conditions. These data show that high levels of tissue NO_3^- cause production of high levels of NR and suggest that factors other than enzyme or substrate were limiting NO_3^- assimilation in these growth-chamber-grown seedlings.

Although both assays are related to the actual accumulation of N by the plant, the *in vivo* assay gives slightly better precision and may reflect more closely the assimilation of NO_3^- *in situ*, as shown by Figure 3. The overestimation for the root and underestimation for the shoot by the *in vivo* assays suggest that reduced N is transported from root to shoot. This would be consistent with the data presented by Pate (12). Based on *in vivo* activities, the contribution of N transported from root to shoot decreased with plant age. The percentages of root activity divided by shoot plus root activity decreased from 54% on day 1 to 40% by day 10. This division of activity between root and shoot is comparable with Pate's findings (12) that approximately 42, 65, and 67% of the total N of exuding xylem sap was present as NO_3^- for *Hordeum*, *Zea*, and *Avena* species, respectively.

The usefulness of the *in vivo* assay in distinguishing between high and low protein wheat genotypes is shown in Figure 4. The Atlas 66 × Comanche plants, a high protein wheat (3.5% N in the seed, dry weight basis) had more NR activity and accumulated more reduced N per plant than did Comanche plants, a low protein wheat (2.7% N). Because Comanche had a larger seed, the difference in total N between seeds of Atlas 66 × Comanche and Comanche was approximately 0.04 mg/seed. This amount of N could account for approximately 12% of the difference in actual N accumulated by the two genotypes by day 9 (Fig. 4). This would indicate that it is the genetic capacity to metabolize NO_3^- rather than the amount of N in the seed that is responsible for the high protein characteristics of Atlas 66 × Comanche.

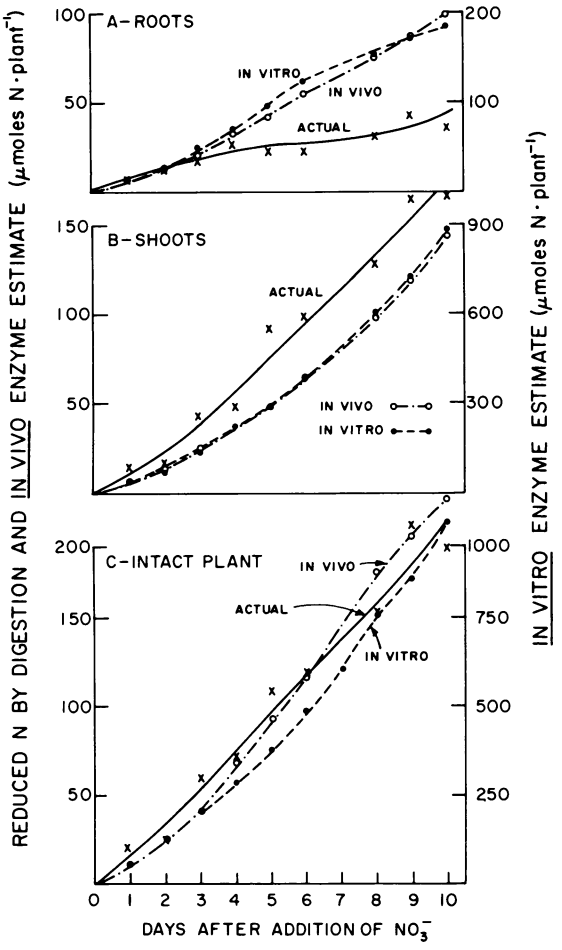

FIG. 3. Correlation between the amount of reduced N made available to the wheat seedling (cv. Atlas 66 × Comanche) or seedling part as measured by *in vivo* and *in vitro* nitrate reductase assays and the actual accumulation of reduced N accumulated by the wheat seedling or seedling part as measured by digestion and Nesslerization. Linear correlation values (r) for the roots, shoots, and total plant were +0.91, +0.98, and +0.96 between the *in vivo* estimates and actual N, and +0.91, +0.98, and +0.98 for the *in vitro* estimates and actual N. Correlation values between the two assays were over +0.99 in all cases. All values were significant at the 1% level. The plant material was the same as used for the data of Figure 1. The intact plant data are not the sums of roots and shoot data.

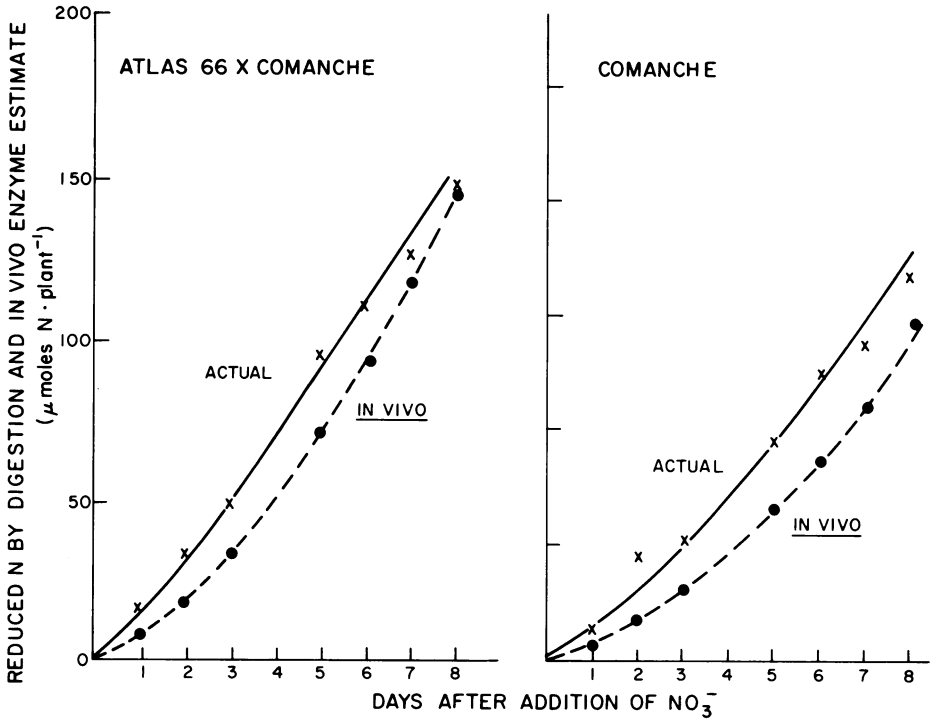

FIG. 4. Correlation between estimated input of reduced N by the *in vivo* assay and actual N for a high protein wheat (Atlas 66 × Comanche) and a low protein wheat (Comanche). Only shoot tissue was assayed.

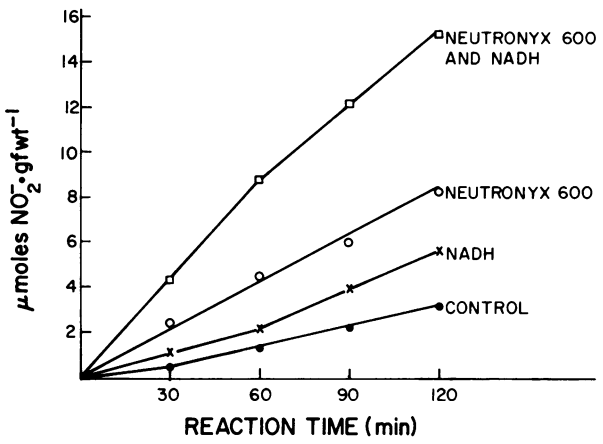

FIG. 5. The effect of addition of NADH to the *in vivo* assay medium with and without a surfactant on the nitrate reductase activity of wheat (cv. Atlas 66 × Comanche) leaves. The concentration of NADH was 2 mg/ml.

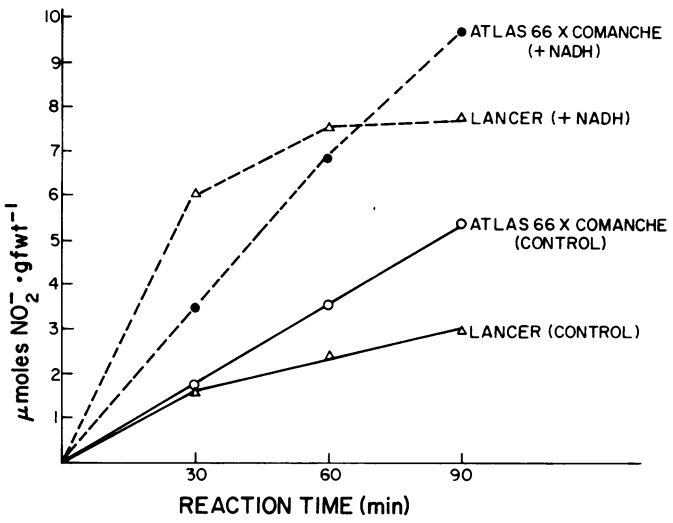

FIG. 6. Effects of NADH on *in vivo* NR activity of two wheat genotypes. Assays were conducted with Neutronyx 600 in the assay medium. The concentration of NADH was 2 mg/ml.

The correlation values between the amount of reduced N made available to the plant by the *in vivo* assay and the actual amount of N accumulated by the plant were positive and significant ($r = 0.99$) for both genotypes.

NADH as a Factor Limiting NO_3^- Assimilation. Because these experiments indicated that neither enzyme nor substrate was limiting the *in vivo* assay or the actual NO_3^- assimilation *in situ*, a lack of reductive energy was suggested. Accordingly, experiments were designed to determine whether the addition of NADH to the *in vivo* assay medium would stimulate NO_3^- reduction.

The data (Fig. 5) show that the addition of NADH with or without Neutronyx 600 enhanced NO_3^- reduction over the appropriate controls. After 2 hr, NO_2^- production was 185% and 175% of respective controls when NADH was added with and without Neutronyx 600. When Neutronyx 600 was omitted from the assay medium, half of the NADH (as measured by absorbancy) was still present in the external medium after 120 min. With Neutronyx 600, half of the NADH remained after 60 min, and only 12% was present after 120 min. Nitrite production was 137, 170, 192, and 190% of control (with Neutronyx) when the NADH was added at the rate of 1, 2, 3, and 4 mg/ml of assay medium, respectively. Based on the loss of NADH from the assay medium and the enhancement of NO_2^- production from the addition of NADH, approximately 15% of the NADH was used for NO_3^- reduction.

The pattern of NO_2^- production by another wheat genotype (Lancer), when NADH was added to the assay medium, differed from the pattern of Atlas 66 × Comanche (Fig. 6). The increased production of NO_2^- by both genotypes, in response to

NADH addition, was related to the rate of NADH disappearance from the assay medium. With Lancer, the percentages of NADH that remained in the medium after 20, 60, and 90 min were 13, 11, and 10, whereas the values for Atlas 66 × Comanche were 62, 35, and 10, respectively. Obviously, the rate of NO_2^- production by Lancer (+ NADH) was limited, possibly even during the first 30-min period, by the exhaustion of the added NADH. With Atlas 66 × Comanche, the NADH disappeared more slowly from the media, and the NO_2^- reduction rate was linear. The enhancement of NO_2^- production in both genotypes indicates that at least some of the NADH entered the cells. If one can assume that NAD is not degraded or irreversibly bound, the results with Lancer would suggest that it is the ability to reduce (recycle) NAD that is limiting NO_3^- reduction. Because these plants were supplied with an ample amount of NO_3^- and grown under low light intensities (20 klux), it would appear reasonable that it is the lack of 3-P-glyceraldehyde (6) or malate (9) or the appropriate enzymes rather than NR that is limiting NO_3^- reduction, especially in the case of Lancer. Unfortunately, NAD was not added to verify the assumption that the added NADH was oxidized to supply an ample amount of NAD for recycling by oxidation of organic metabolites.

These observations indicate the metabolic complexity (membrane permeability, cofactor level, and recycling system as well as substrate and NR level) associated with NO_3^- reduction *in situ*. While the *in vitro* assay measures the amount of NR extractable by the procedure used, these *in vivo* assays (Fig. 6) show that factors other than enzyme level could influence NO_3^- reduction *in situ*.

In general, these results are not considered to be applicable to field conditions for the following reasons. First, light intensities would be 2 to 6 times greater under field conditions, which would increase photosynthesis and carbohydrate supply. Second, nitrate concentrations of this magnitude (1.8 mg NO_3^- N g fresh wt^{-1}) are seldon obtained (usually less than 0.1 mg throughout the bulk of the growing season, and no higher than 0.35 mg after heavy N fertilization [4]). Thus, under field conditions, NO_3^- is still considered to be the major limiting factor controlling both level of NR and rate of assimilation (8, 10).

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