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Physiological Significance of the *In Vivo* Assay for Nitrate Reductase in Cotton Seedlings[1]

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ABSTRACT

In vivo **assay techniques for nitrate reductase activity were evaluated for their ability to predict nitrate assimilation rates of germinating dark-grown cotton (*Gossypium hirsutum* L.) seedlings. Activity was measured with four *in vivo* assay techniques: either aerobic or anaerobic, and either with or without added nitrate in the assay medium. Assays without added nitrate in the medium allowed closer estimates of reduced N accumulation than did assays with added nitrate. Additionally, in the absence of added nitrate, the anaerobic assay provided a better estimate than the aerobic assay because of O_2-stimulated nitrite reduction. The results suggest that the *in vivo* assay for nitrate reductase best approximated nitrate assimilation rates with only endogenous nitrate as substrate.**

Additional index words: ***Gossypium hirsutum*** **L., Microdiffusion analysis, Nitrate ion electrode.**

THE relationship between the measurable activity of an enzyme and its true activity *in situ* has not been carefully characterized for many enzymes. One prominent exception is nitrate reductase (NR), which presumably catalyzes the rate-limiting step in the nitrate assimilation pathway (1). Many investigators have found a correlation between NR activity (NRA) and accumulation of reduced N (3, 6, 21). However, of the various enzyme assay techniques devised, only two allow estimates of absolute nitrate reduction rates *in situ*: i) following the release of ^{15}N or ^{18}O from labeled nitrate (8, 20) and ii) measuring the difference between nitrate uptake and nitrate accumulation by the tissue (4, 11, 12). The first method is expensive because it requires a mass spectrometer or a neutron activation analyzer. The second is inherently imprecise, in that it often consists of the determination of small differences between large numbers.

Several investigators have developed an *in vivo* assay technique for NR which depends upon the secretion of nitrite by intact tissue (8, 11, 13, 14, 17). In wheat, both *in vivo* and *in vitro* activities were highly correlated with increases in reduced N over an 8-day period (3). The objective of these experiments was to determine *in vivo* assay conditions under which NRA would best approximate the increase in reduced N of cotton seedlings.

MATERIALS AND METHODS

Seeds of cotton (*Gossypium hirsutum* L. 'Deltapine 16') were germinated in plastic trays containing vermiculite moistened with either 0.1 m*M* $CaCl_2$ (control) or 0.1 m*M* $CaCl_2$ plus 100 m*M* KNO_3. The trays were then incubated at 30 C in darkness. NRA was followed for 3 days after imbibition in both control and nitrate-germinated seedlings, or until the early peak of activity had almost disappeared (15). At the end of the germination period, seedlings were removed whole from the vermiculite, washed, lyophilized, weighed, and ground to pass a 40-mesh screen. The dry plant material was analyzed for nitrate and total reduced N.

NRA was determined by methods based on an *in vivo* assay reported earlier (15). In the earlier method, seedlings were infiltrated under vacuum with a solution of 30 m*M* KNO_3, 0.1 *M* phosphate buffer (pH 7.5), and 1% (v/v) 1-propanol, then incubated anaerobically at 30 C. For this report, assays were performed under four conditions: either with or without nitrate added to the incubation medium, and under either air or N_2. Assays were performed in triplicate with groups of five seedlings/replicate. Measured activities of nitrate-germinated and control seedlings were plotted against time for the entire germination period, and the area between the curves was determined graphically for each assay method for differences in cumulative NRA.

Nitrate was extracted with 100 ml water/g dry tissue. Seven replicate samples were placed in test tubes with the water, put into a boiling water bath for 45 min. with occasional stirring, and cooled to room temperature. Analysis was conducted with a nitrate ion electrode (Orion Research Inc.[3]) by the standard addition method, with a standard curve constructed by addition of known amounts of nitrate to the control extract. Previous experiments showed that the nitrate content of control tissue was negligible.

For the determination of reduced N, 10 replicate 50-mg samples of dry plant tissue from each treatment were digested and refluxed with 1 ml concentrated H_2SO_4 in test tubes in a heated sand bath, until the mixtures became perfectly clear and light straw-colored. The tubes were capped with marbles, to prevent loss of vapors during refluxing. After the solutions cooled, a drop of 30% H_2O_2 was added to each tube, and the digests were allowed to stand overnight. They were then diluted to 100 ml, and 1-ml aliquots were removed for analysis by a modified Conway microdiffusion technique (5, 18). Recovery of ammonia

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[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA, and it does not imply approval to the exclusion of other products that may also be suitable.

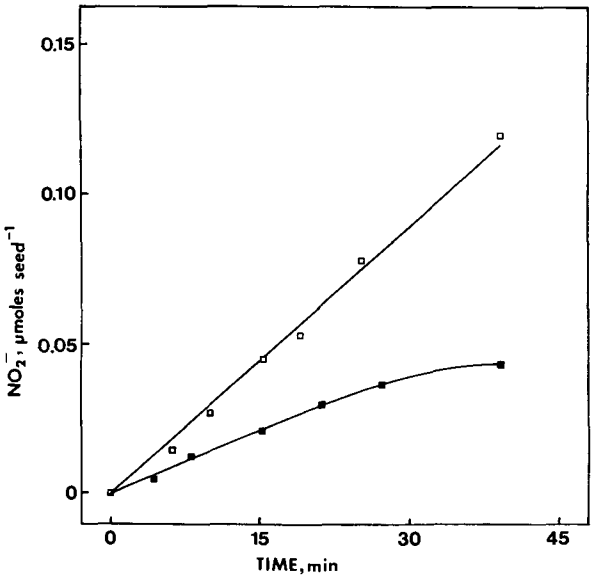

Fig. 1. Nitrite production from 2-day-old seeds germinated on nitrate and assayed either with (□) or without (■) 30 m*M* nitrate in the medium.

from standards in this procedure was essentially stoichiometric. Nitrate in all diluted digests was determined with the nitrate electrode after titration of the samples to pH 3.5 with saturated Ba $(OH)_2$ and centrifugation to remove the precipitated sulfate. Again, standard curves were constructed by use of similarly treated digests of the control seedlings. Tests showed that no nitrate was volatilized as HNO_3 and lost during digestion when refluxing was carefully controlled.

RESULTS AND DISCUSSION

Exogenous nitrate in the medium altered the kinetics of nitrite production during the assay. Activity in 2-day-old nitrate-germinated seedlings was constant with time for the first 30 min without added nitrate, but then fell off as the endogenous nitrate became limiting (Fig. 1). In contrast, activity with added nitrate was initially greater and did not decline with time (Fig. 1). An incubation time of $\leqslant$ 30 min. thus provided reasonable estimates of the initial rate of nitrite production, whether assayed with or without nitrate. These results differ from those of Ferrari, Yoder, and Filner (10), who showed that in cultured tobacco (*Nicotiana tabacum* L.) cells without added nitrate the initial rate of reduction was quite high, but declined within a few minutes. However, in both systems the availability of nitrate, as much as the activity of the enzyme per se or cofactor availability, appeared to be a major limiting factor controlling the rate of assimilation. At the end of the germination period, nitrate content of the cotton seedlings was about 20 mg/g dry wt and thus must have been in a pool unavailable or only slowly available for reduction (10).

To determine which assay method best approximated the rate of accumulation of reduced N, we followed NRA of seedlings grown with and without nitrate. The cumulative differences in NRA over the first 72 hour of germination were obtained by graphical integration and compared to the difference in total reduced N between nitrate-germinated and control seedlings. Nitrate-germinated seedlings apparently contained 259.1 ± 1.3 μmol reduced N/seedling after

Table 1. N contents of cotton seedlings germinated for 72 hours either with or without 100 m*M* KNO_3.

Treatment*	Total reduced N†	Nitrate content	Nitrate remaining after digestion	Actual reduced N‡
	μmol/seedling			
$+NO_3$	259.1 ± 1.3	21.2 ± 0.4	12.3 ± 0.2	250.2 ± 1.4
$-NO_3$	245.0 ± 2.5	0	0	245.0 ± 2.5
Difference	14.1 ± 2.8	21.2 ± 0.4	12.3 ± 0.2	5.2 ± 2.9

* Values are given with standard errors of the means. † Determined by micro-Kjeldahl digestion and microdiffusion analysis; see text for details.
‡ The amount of nitrate reduced during the digestion was separately determined and subtracted from the total reduced N to give actual reduced N.

Table 2. Net nitrite production by 1-day-old nitrate-germinated cotton seedlings assayed with or without nitrate and nitrite in the medium.

Treatment*	- Nitrate		+ Nitrate	
	Air	N_2	Air	N_2
	nmol/seedling/h			
- Nitrite	59 ± 3	75 ± 6	220 ± 17	320 ± 26
+ Nitrite	40 ± 5	67 ± 1	89 ± 24	255 ± 20
Difference	19 ± 6	8 ± 6	131 ± 29	65 ± 33

* Nitrate and nitrite concentrations in the medium were 30 m*M* and 50 μ*M*, respectively. The decrease in net nitrite production when nitrite is added to the medium is proportional to NiRA.

72 hours; after correction for the nitrate reduced during digestion, the true reduced N content was 250.2 ± 1.4 μmol (Table 1). Control seedlings contained 245.0 ± 2.5 μmol, for a difference of 5.2 ± 2.9 μmol/seedling (Table 1). The cumulative NRA provided estimates of this difference as follows: i) air — nitrate, 2.7 μmol; ii) air + nitrate, 9.6 μmol; iii) N_2 — nitrate, 5.9 μmol; and iv) N_2 + nitrate, 12.4 μmol (Fig. 2). Thus, the total NRA estimated anaerobically and without nitrate gave closest agreement with measured increases in reduced N. Error estimates for the cumulative NRA, based upon the standard errors of the assay means (Fig. 2), showed that the uncertainty of these predicted values was much less than that of the difference in reduced N content (Table 1).

Two mechanisms are possible for the O_2-mediated decrease in nitrite production: competition with nitrate for reducing power (8, 14), and an O_2 requirement for the further reduction of the nitrite to ammonia (2, 9). In green cotton leaves, the former seems to be more important (14), but the latter phenomenon has been verified numerous times, especially in non-green tissues (2). In dark-grown cotton seedlings germinating on nitrate, nitrite reductase activity (NiRA) could not be measured directly as a loss of nitrite from the medium. However, the decrease in net nitrite production when nitrite was added to the medium served as an indirect assay (14). This indirect method showed that NiRA was doubled by aeration during assay, both with and without exogenous nitrate (Table 2). Thus, even though NiRA was not absolutely dependent upon O_2, it could have accounted for the effect of aeration on the assays.

The role of exogenous nitrate in the assay medium also deserves comment. When nitrate is present, activity is proportional to *in vitro* NRA (7, 19). Thus, the values obtained may represent some measure of the maximum capacity of the seedlings to reduce nitrate, rather than the rate of the process under nonoptimal conditions. In our experience, the latter rate is always less than the former, although in certain tissues at particular developmental stages, the difference may be small (16).

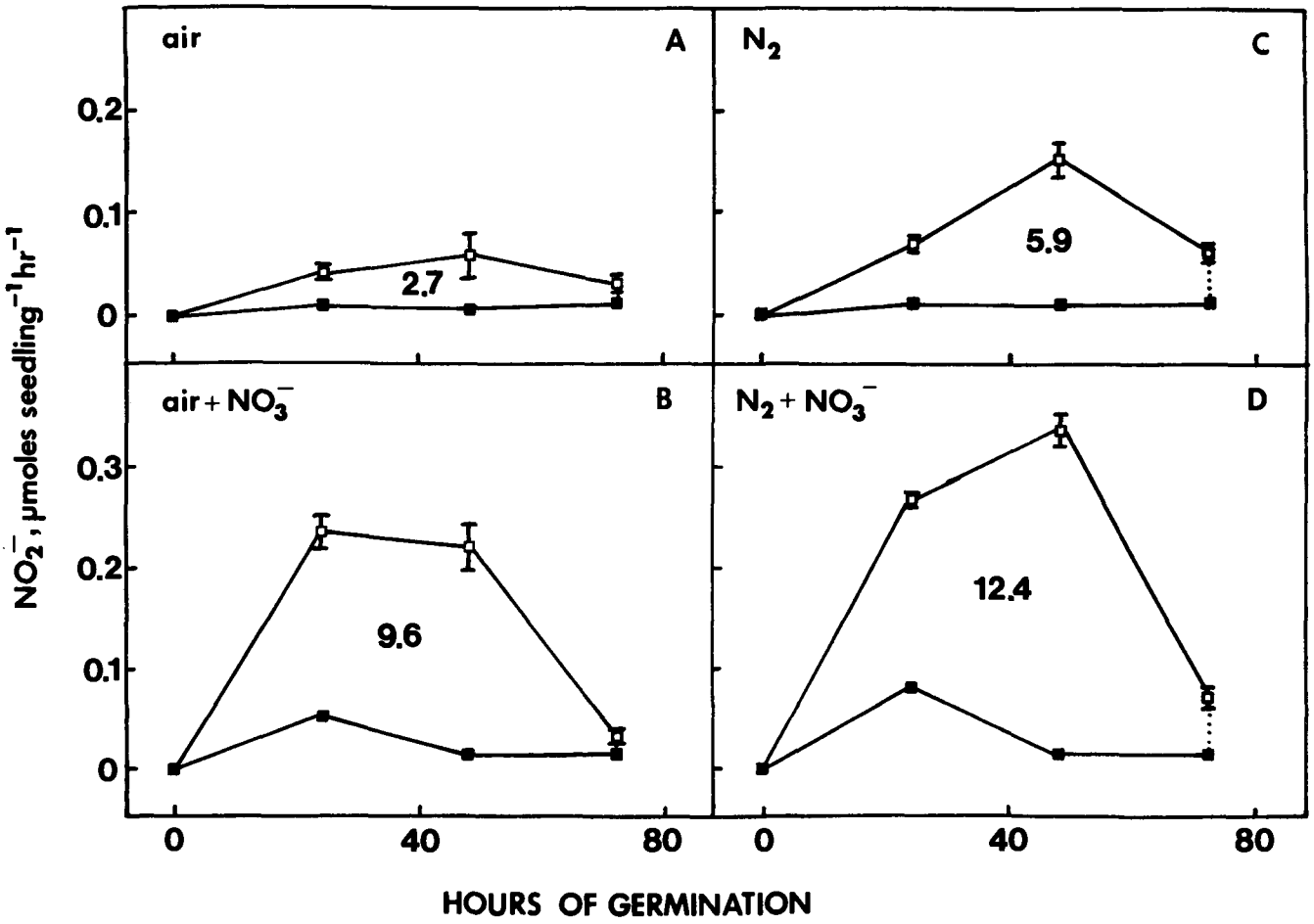

Fig. 2. ***In vivo*** **NRA of nitrate-germinated (□) and control (■) seedlings assayed under four sets of conditions. For each set of assays, the area between the curves was determined and is shown (in units of μmol/seedling). Values for nitrate-germinated seedlings are shown with standard errors of the means. Standard errors for assays of control seedlings were all less than the width of the symbol on the graph.**

The procedure used here to check the validity of the assay results is limited by the ability to resolve small differences between N contents of the nitrate-germinated and control seedlings (Table 1). Experimentally, germinating cotton seedlings nonetheless provide a good means to study the *in vivo* assay in relation to reduced N accumulation, because it is necessary either to assay entire plants or to isolate the assayed organ from translocation of nitrogenous substances. Although leaf tissues display much greater NRA, the viability of isolated tissue (leaf discs) could not be maintained for a suitable period, and the difficulties of selecting and assaying larger plants with sufficiently uniform NRA were substantial. Therefore we did not attempt to compare *in vivo* NRA and reduced N accumulation in green leaves.

However, in such a system, the role of O_2 might be quite different from its role in germinating seedlings (see above), and the anaerobic assay might no longer be the method of choice. It seems unlikely, however, that the role of exogenous nitrate would be greatly different from that described here.

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