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Reevaluation of Anaerobic Nitrite Production as an Index for the Measurement of Metabolic Pool of Nitrate[1]

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ABSTRACT

The use of anaerobic nitrite production as an index for the measurement of metabolic pool of nitrate was reevaluated using primary leaves of 7-day-old barley and 10-day-old soybean seedlings. The seedlings were grown in nutrient solutions containing 5 to 15 millimolar nitrate. The nitrate-free *in vivo* assay system of nitrate reductase was used for measuring the production of nitrite. Both the duration and extent of nitrite production were dependent on the level of endogenous nitrate in the tissue. At cessation of nitrite production, 30 to 50% of the endogenous nitrate was reduced to nitrite. Nitrate from the tissue leaked continuously into the surrounding medium so that, at cessation of nitrite production, nitrate supply from the tissue was exhausted. The cessation of nitrite production, therefore, may have been caused by the depletion of endogenous nitrate from the tissue. It is concluded that anaerobic nitrite production is not a valid index for the measurement of the size of the metabolic pool of nitrate.

The total amount of nitrite produced in the *in vivo* assay during anaerobic reduction of endogenous nitrate in plant cells and tissues has been used to measure the size of the metabolic pool of nitrate (2, 3, 8). The method was first proposed by Ferrari *et al.* (8), who observed that nitrate reductase activity and energy supply for nitrate reduction were not factors limiting nitrite production in tobacco cells. Hageman *et al.* (9) observed that, in wheat leaves, nitrite production could be stopped by the lack of energy and loss of nitrate reductase activity. Nitrate availability within the tissue is not considered to be limiting to nitrite production (8, 9). However, my preliminary studies with soybean leaves show that, when nitrite production ceased, nitrate supply from the tissue became exhausted. Therefore, in the present investigation, the fate of tissue nitrate during anaerobic nitrite production was followed by analyzing the tissue and the assay medium for both nitrate and nitrite. The results suggest that cessation in nitrite production (nitrate reduction) was caused by the depletion of nitrate from the tissue.

MATERIALS AND METHODS

Plant Material. Seedlings of soybean (*Glycine max* [L.] Merr. cv. Amsoy) and barley (*Hordeum vulgare* L. cv. Numar) were grown in vermiculite and subirrigated with a modified Hoagland solution containing 5 to 15 mM nitrate (14). Seedlings were grown for 7 (barley) or 10 (soybean) days in a controlled environment growth chamber under a 16-h photoperiod at 50% RH and 25 C. Quantum flux at the tops of the plants was 500 μE $m^{-2}s^{-1}$ and was supplied by incandescent and cool white fluorescent lamps. Primary leaves were sampled for assays during the middle of the photoperiod.

Measurement of Anaerobic Nitrite Production. Anaerobic nitrite production (accumulation) was determined by the following methods.

Method A. Leaf-discs from soybean (having a diameter of 9 mm each) or leaf sections from the top 8 cm of barley leaves (measuring approximately 5 × 5 mm or less) were prepared and thoroughly mixed. A weighed leaf sample, approximately 0.3 g (soybean) to 0.4 g (barley), was placed in a 25-ml Erlenmeyer flask containing 10 ml of either 0.1 M K-phosphate (pH 7.5) or 2 mM $CaSO_4$ solution. Preliminary studies showed that increasing the tissue weight up to 0.4 g did not affect the rate or the extent of nitrite production. Chloramphenicol (300 μg/10 ml) was routinely added to the assay medium to stop bacterial contamination. In some studies, 1% (v/v) 1-propanol was added to the assay medium. The flasks containing the tissue (3 replicates per treatment) were placed under vacuum (at 0.2 atm) for 2 min, releasing the vacuum every 30 s. During infiltration, the tissue became waterlogged and sank to the bottom of the flasks. After vacuum infiltration, the flasks were stoppered and incubated in the dark at 28 C in a shaking water bath. Following incubation for different time intervals, both the tissue and the incubation medium were analyzed for nitrate and nitrite.

Method A-1. The method was the same as Method A, except that nitrite accumulation was followed by sequential removal of aliquots (0.05 to 0.1 ml) from the medium of the same assay flasks at intervals. Volume corrections were made whenever sequential sampling was used. Although, with this method, sample to sample variability was reduced, the initial rates of nitrite accumulation in barley leaves were underestimated by as much as 20 to 30% because of retention of nitrite in the tissue. However, at the cessation of nitrite accumulation, the amount of nitrite retained in the tissue was negligible (<5%). Therefore, the values of the size of metabolic pool of nitrate determined by this method were comparable to those determined by Method A.

Method B. This method was also similar to Method A, except that anaerobiosis was achieved by N_2 instead of vacuum infiltration. Barley leaf slices (approximately 0.4 g) were added to flasks containing 10 ml of 2 mM $CaSO_4$ which had been purged with N_2 for 15 min. N_2 was bubbled through the contents of each flask for 3 min after the leaf sections were added. The flask was then sealed with serum caps, purged with N_2 for an additional 1 min, and incubated in the dark at 28 C in a shaking water bath. The rest of the procedure was the same as in Method A.

Nitrate and Nitrite Analysis. The tissue was homogenized with a cold mortar and pestle in eight volumes of cold deionized water. The homogenate was centrifuged at 30,000*g* for 15 min, and the supernatant was used to measure nitrate and nitrite.

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Nitrate was measured as nitrite after enzymic reduction with dissimilatory nitrate reductase obtained from *Klebsiella pneumoniae* (1). Nitrite was determined colorimetrically after color development with 1 ml 1% (w/v) sulfanilamide in 1.5 N HCl and 1 ml 0.02% (v/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride for 15 min. Absorbance was read at 540 nm. Nitrate and nitrite were calculated as μmol per g fresh weight.

RESULTS

Effect of Mode of Anaerobiosis. Figure 1 shows the time course of nitrite accumulation and nitrate loss from barley leaf sections as affected by the mode of anaerobiosis, achieved either by purging with N_2 or by vacuum infiltration. Initially, the rate of nitrite accumulation was somewhat lower with N_2 than with vacuum infiltration, but, after 1 h, similar rates of nitrite accumulation were observed in both treatments. Consequently, under N_2 the total amount of nitrite accumulated remained at a slightly lower level throughout the incubation period than it did with vacuum infiltration. The loss of nitrate from the tissue into the medium was not affected by the method of achieving anaerobiosis. In further studies, therefore, vacuum infiltration was used to achieve anaerobiosis.

Influence of Incubation Medium. Nitrite production (both rate and extent) in soybean leaves incubated in $CaSO_4$ was considerably decreased compared to that in K-phosphate (Fig. 2A). In contrast, in barley leaves nitrite accumulation was not affected, whether the leaves were incubated in $CaSO_4$ or in K-phosphate (Fig. 2B). Propanol also had a different effect on nitrite accumulation in barley and soybean leaves. Whereas no enhancement of nitrite accumulation was observed in barley leaves (Fig. 2B), propanol in soybean leaves stimulated nitrite accumulation considerably in both $CaSO_4$ and K-phospahte (Fig. 2A). The stimulation by propanol was more pronounced in K-phosphate than in $CaSO_4$. Ethanol and 2,4-dinitrophenol also stimulated nitrite accumulation in soybean leaves (data not shown).

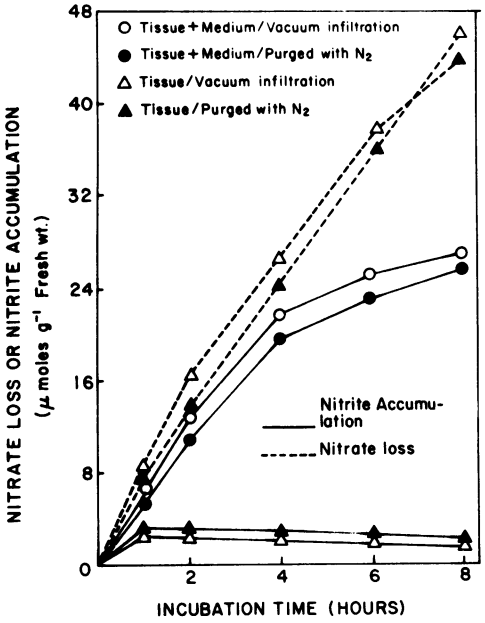

FIG. 1. Time course of nitrite accumulation and nitrate loss from barley leaf sections as affected by method used to achieve anaerobic conditions in the assay medium. Seedlings were grown in 15 mM nitrate for 7 days. Leaf sections were incubated in 2 mM $CaSO_4$ solution. Anaerobiosis was achieved by vacuum infiltration (Method A) and purging with N_2 (Method B). Nitrate loss was determined by subtracting the nitrate remaining in the tissue and total nitrite produced by the tissue at different time intervals from the endogenous nitrate concentration of the tissue at time 0 (93 μmol/g fresh weight). According to *t* test, the differences between the two treatment means at any time interval for each determination were nonsignificant at 5% level of probability.

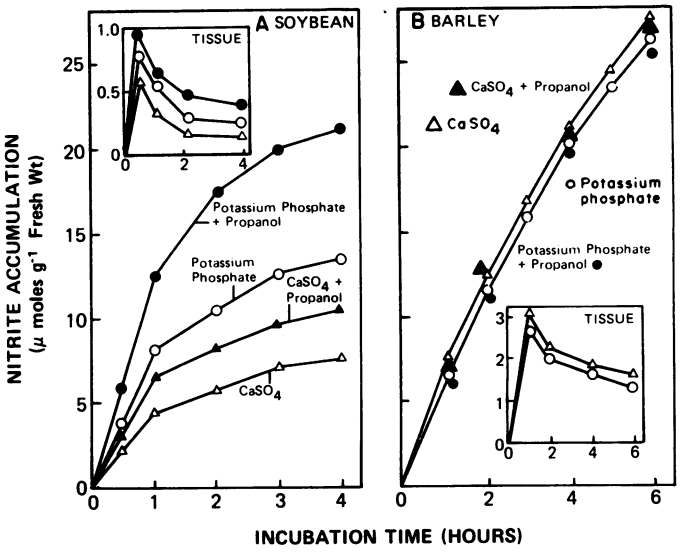

FIG. 2. Effect of composition of the incubation medium on nitrite accumulation (medium + tissue) by leaves of soybean (A) and barley (B) seedlings grown in 15 mM nitrate. Nitrite accumulation was studied by Method A. The inserts show kinetics of nitrite accumulation in the tissue.

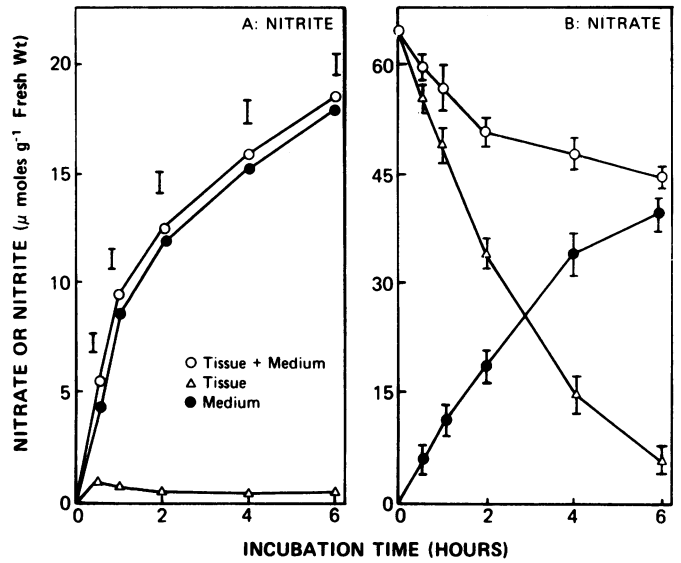

FIG. 3. Time course of nitrite accumulation (A) and nitrate disappearance (B) in soybean leaf discs under dark anaerobic conditions. Seedlings were grown in 15 mM nitrate. Leaf discs were incubated in 0.1 M K-phosphate containing 1% (v/v) 1-propanol, according to Method A. Both the tissue and the medium were analyzed for nitrate and nitrite. Bars on the points represent SD.

Nitrite Production and Leakage of Nitrate. The data show that, during nitrite production (Fig. 3A), there was a continuous leakage of nitrate from the soybean leaf discs into the incubation medium (Fig. 3B). After 6 h of incubation, the supply of nitrate from the tissue was almost exhausted, and 38 μmol of nitrate (60% of that originally present in the leaves) were recovered in the incubation medium.

In barley leaves, nitrite production continued for at least 14 to 15 h (Fig. 4). During the same period, nitrate concentration of the tissue decreased continuously and reached a minimal level as

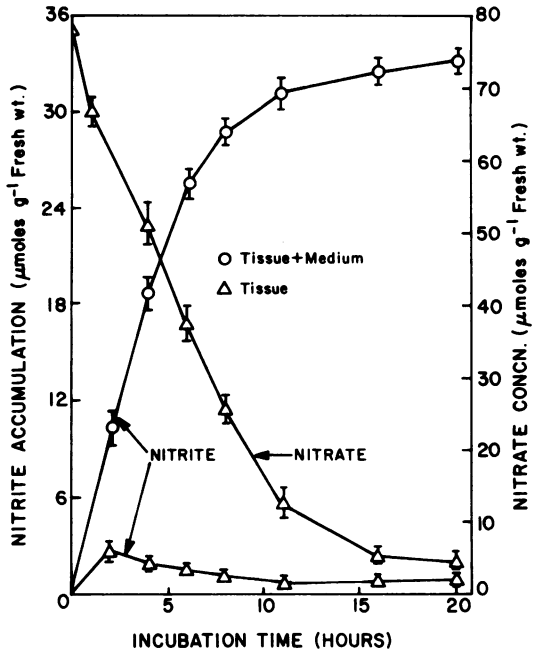

FIG. 4. Time course of nitrite accumulation and loss of nitrate from barley leaf sections in darkness under anaerobic conditions. Seedlings were grown in 15 mM nitrate solution. Leaf-sections were incubated in 2 mM $CaSO_4$ solution, according to Method A. Tissue was analyzed for both nitrate and nitrite, and the assay medium was analyzed for nitrite only. Bars on the points represent SD.

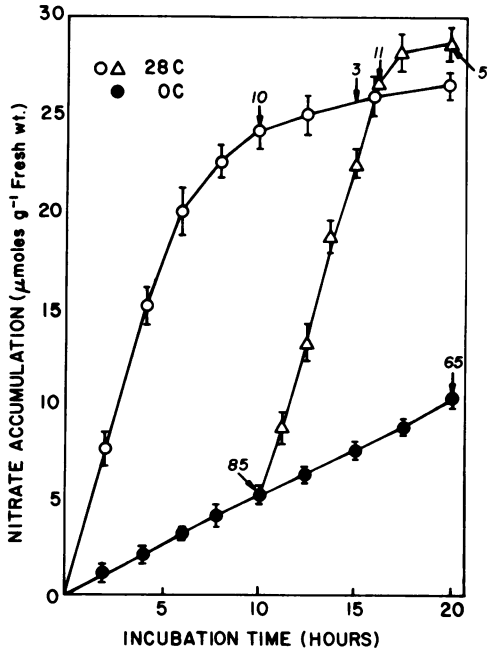

FIG. 5. Effect of cold incubation on anaerobic nitrite accumulation and nitrate loss from barley leaves grown in 15 mM nitrate. Leaf sections were incubated in 2 mM $CaSO_4$ solution, according to Method A-1. One set of flasks was incubated at 28 C (○), the other set at 0 C (●). At the 10th hour, one-half of the flasks from 0 C were transferred to 28 C (△). The values indicated by arrows are percentages of endogenous nitrate (70 μmol/g fresh weight) remaining in the tissue at that particular time of incubation. Bars on the points represent SD.

Table I. *Effect of Endogenous Nitrate on the Duration and Extent of Nitrite Accumulation in Barley Leaves*

Seedlings were grown in nutrient solutions containing 5 to 15 mM nitrate. Anaerobic nitrite production was studied according to method A-1, using 2 mM $CaSO_4$ solution as an incubation medium.

Growth Nitrate Level	Initial Rates of Nitrite Accumulation	Tissue Nitrate Concentration[a]		Total Nitrite Accumulation[a]	Cessation Time[b]
		Initial	Final		
mM	*μmol/g·h*	*μmol/g fresh wt*			*h*
5.0	2.4	10.4 ± 0.8	1.2 ± 0.3	5.0 ± 0.9	3
7.5	3.6	22.5 ± 1.9	2.0 ± 0.4	11.5 ± 1.0	6
10.0	4.3	41.0 ± 1.7	3.2 ± 0.4	21.2 ± 1.1	9
15.0	6.5	70.5 ± 1.8	4.8 ± 0.6	32.5 ± 1.7	14

[a] Means ± SD.

[b] Cessation time is the time when nitrite production ceased.

nitrite production ceased. These data show that, at cessation of nitrite production, about 30 to 40% of the endogenous nitrate was reduced to nitrite (Figs. 3 and 4).

Effect of Endogenous Nitrate. The data in Table I show that both the duration and the total amount of nitrite produced were functions of endogenous nitrate level of the barley tissue. As the endogenous nitrate concentration in the tissue was increased, the time required to attain a plateau in nitrite production also increased proportionately. The total amount of nitrite produced was also directly proportional to the level of endogenous nitrate. In all cases, the nitrate concentration in the tissue at cessation of nitrite production was less than 10% of the initial levels (Table I).

Effect of Cold Incubation. The incubation of barley leaf sections at a cold temperature (0 C) under anaerobic conditions greatly reduced the rate and quantity of nitrite produced compared to that produced by incubation at 28 C (Fig. 5). However, when the flasks, after 10 h of cold incubation, were transferred to 28 C, nitrite production was accelerated, and both the rate and total amount of nitrite produced were similar to those of leaves incubated at 28 C from time zero. After 10 h of incubation at 0 C, 85% of the endogenous nitrate was still present in the tissue, whereas, at 28 C, 90% of the endogenous nitrate disappeared from the tissue during the same period. Even after 20 h of incubation at cold temperature, the tissue still contained 65% of the endogenous nitrate, indicating that, at a cold temperature, loss of nitrate from the tissue is much reduced.

DISCUSSION

The measurement of the metabolic pool of nitrate is based on the assumptions that (a), the cessation of nitrite production is caused by the complete reduction of nitrate from the metabolic pool and (b), leakage of nitrate from the tissue into the surrounding medium does not affect the measurement of the size of metabolic pool of nitrate (8). Inasmuch as metabolic nitrate is considered to be a fraction of the total nitrate in the tissue (8), large amounts of nitrate should still be present in the tissue when nitrite production ceases. The evidence presented here indicates that, as nitrite production ceased, nitrate supply from the tissue was essentially exhausted (Figs. 3 and 4). This implies that cessation in nitrite production may have been caused by the depletion of nitrate from the tissue rather than by the complete reduction of the metabolic nitrate. In contrast, Ferrari *et al.* (8) observed that most of the nitrate was still present in tobacco cells as cessation in nitrite production was approached. However, Hageman *et al.* (9) have shown recently that the system used by Ferrari *et al.* (8) probably was not completely anaerobic, and cessation of nitrite production could have been caused by the lack of complete anaerobiosis in the assay system. The presence of O_2 in the system of Ferrari *et al.* (8) may have not only prevented the leakage of nitrate from tobacco cells (6) but also inhibited nitrate reduction

(4, 15), thus causing apparent cessation in nitrite production. Hageman *et al.* (9) also concluded that, in wheat leaves, availability of nitrate is not a limiting factor in causing cessation of nitrite production. They observed that similar amounts of nitrite were produced whether the tissue was incubated in medium containing or in medium lacking nitrate. However, they did not determine the nitrate concentraton of the tissue. In the study reported herein, although at the time of cessation in nitrite production endogenous nitrate supply from the tissue was exhausted (Fig. 4), exogenous nitrate had no marked effect on nitrite production (data not shown). These data are in contrast to those previously reported by Aslam *et al.* (3) and Ferrari *et al.* (8), who observed higher rates of nitrite production when nitrate was added in the infiltration medium. This stimulation in nitrite production on the addition of massive doses of nitrate to a partially anaerobic system could have been due to the diversion of the reducing equivalents from NADH towards nitrate rather than towards O_2 (16).

Since nitrate first accumulates in the metabolic pool and only excess nitrate is accumulated in the storage pool (3, 12), at low nitrate supply to the tissue most of the nitrate absorbed is expected to accumulate in the metabolic pool, whereas, at higher nitrate supply (saturation conditions), the proportion of the absorbed nitrate accumulating in the metabolic pool will be low. If under anaerobic conditions, nitrate in the metabolic pool is the only source of nitrate available for reduction, then in tissue with low levels of endogenous nitrate, a higher proportion of the endogenous nitrate should be recovered as nitrite and *vice versa*. However, the evidence indicates that the total amount of nitrite produced was directly proportional to the level of endogenous nitrate in the tissue (Table I), suggesting that, under anaerobic conditions, nitrate from the storage pool also becomes readily available for reduction. A similar conclusion was also reached by Subbalakshmi *et al.* (16), who observed that, in intact leaves of wheat and rice, about two-thirds of the endogenous nitrate was reduced under anaerobic conditions.

During cold incubation, solute loss from the tissue is accelerated (13), whereas *in vivo* reduction of nitrate to nitrite is prevented (10). The incubation of the tissue at cold temperature, therefore, should allow nitrate to leak into the surrounding medium without concomitant nitrite production (8). Similar amounts of nitrite production by the tissue, whether preincubated at cold temperature or incubated continuously at 28 C (Fig. 5), would then suggest that, during cold preincubation, leakage of nitrate occurred from the storage pool only. However, during cold incubation, the loss of the endogenous nitrate from the tissue was negligible (Fig. 5). During cold incubation, the leakage of nitrate from the tissue was prevented, and, on warming the tissue, nitrite production resumed and ceased only when nitrate supply from the tissue became exhausted (Fig. 5).

It is well documented that Ca^{2+} or Mg^{2+} is necessary for the maintenance of membrane selectivity of ion uptake and ion retention by plant cells (7). These divalent cations also prevent the loss of solutes induced by anaerobiosis (6). Incubation of leaf sections in medium containing Ca^{2+} should, therefore, prevent the leakage of nitrate from the tissue, and, consequently, more nitrate should be available for anaerobic reduction yielding more nitrite. However, Ca^{2+} decreased nitrite production in soybean leaves, whereas, it had no effect in barley leaves (Fig. 2). In contrast, a monovalent cation (K^+) stimulated nitrite production in soybean leaves (data not shown). Heuer and Plaut (11) also observed that, in alfalfa leaves, K^+ stimulated and Ca^{2+} inhibited nitrite accumulation. They argued that Ca^{2+} altered the membrane permeability so that more nitrite was retained by the tissue. However, they did not determine the nitrite content of the tissue. Evidence indicates that Ca^{2+} does not help prevent the leakage of nitrate (data not shown) and nitrite (Fig. 2) from the soybean leaf discs, suggesting that the effect of Ca^{2+} on anaerobic reduction of nitrate may be more complex than simply maintaining membrane integrity.

How propanol, ethanol, and 2,4-dinitrophenol stimulate anaerobic nitrite production is speculative. It is presumed that these chemicals either increase the entry and accessibility of nitrate within the cell (5, 8), or increase the leakage of nitrite from the cell (5). However, the differential effect of propanol in soybean and barley leaves and the fact that the leaves incubated in propanol accumulated more nitrite (Fig. 2) indicate the complexity of the influence of alcohols on anaerobic nitrite production.

In summary, the results presented here indicate that (a), nitrite production ceased when nitrate supply from the tissue was exhausted; (b), the duration and extent of nitrite production were directly proportional to the level of endogenous nitrate in the tissue; and (c), nitrite production (both the rate and quantity) was differently affected in soybean and barley leaves by Ca^{2+} and propanol. I concluded that anaerobic production of nitrite is not a valid index for the measurement of the size of the metabolic pool of nitrate in higher plants.

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