**In Vivo Nitrite Reduction in Leaf Tissue of Phaseolus vulgaris L.**

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**ABSTRACT**

Experiments were performed to establish a procedure for *in vivo* measurement of nitrite utilization by leaf tissue of bean (*Phaseolus vulgaris* L. cv. Top Crop).

To measure light-dependent nitrite disappearance, a single disc of leaf tissue was exposed to light for 1 hour at 30 °C while immersed in incubation medium (approximately 0.11 milliliter per square centimeter of leaf area) in the bottom of a tall-form glass beaker. The incubation medium was 100 millimolar phosphate buffer (pH 7.5) with added wetting agent and nitrite. The wetting agent combination of 1% 1-propanol plus 0.05% Neutonyx-600 was used in some experiments for compatibility with established *in vivo* nitrate reductase (NR) assays; however, 0.05% Neutonyx-600 alone was found to be a suitable substitute. Parallel assays run in the dark on related tissue are recommended as a means to determine the amount of nitrite synthesized within the tissue by the NR system. Adding the results of the two assays gives an estimate of total nitrite utilization by the leaf tissue. It was found that 20 millimolar nitrite in the incubation medium was the most suitable level of external nitrite for promoting light-dependent nitrite disappearance. This was also found to reduce, sometimes to zero, the rate of synthesis of nitrite by NR. NR activity declined steadily with advancing age. Except for very young tissue, the rate of nitrite disappearance was independent of age. Nitrite disappearance was completely blocked by diuron.

Enzymic conversion of nitrate to ammonia in higher plants is considered to be a sequential process mediated by two enzymes, NR and NiR (1). Over the past 30 years, NR has been studied extensively by *in vitro* methods, and, more recently, a relatively simple *in vivo* technique (5,7,8) has been developed. The *in vivo* technique is attractive because it permits the processing of a large number of samples at one time, requires no aqueous extraction of the enzymes, and may give a more realistic estimate of actual nitrate reducing activities than *do in vitro* methods (9).

*In vitro* NiR assays using artificial electron donors have been used frequently, but, as yet, only limited attempts to develop an *in vivo* method for NiR have been presented. Hageman et al. (6) indicated that an *in vivo* NiR assay with vegetable marrow had been carried out but provided no details. Ferrari and Varner (3) described their technique for measuring NiR in barley aleurone layers, while Miflin (13) conducted experiments in which nitrite reduction in leaf discs was measured in the presence of various photosynthetic inhibitors. More recently, Klepper (9-12) and Finke et al. (4) have measured the accumulation of nitrite in intact leaf tissue after treatment with various herbicides. Although *in vivo* NiRA was indicated in both cases, it was not characterized to the extent that NR has been.

The limited use of the *in vivo* concept for measuring NiR may be partly due to the fact that NiR is not considered to be the limiting enzyme in the reduction sequence (2). Thus, estimates of NiR may not be expected to bear directly on the question of how rapidly nitrate was being made available for amino acid synthesis. A second concern may be that the contribution of NiRA to the total nitrite pool might not be easily measured. Any nitrite reduction measured could be expected to be in error by the amount of nitrite produced by NiRA in the light. Nevertheless, NiR is an essential enzyme, and any procedure which would simplify its study would be of value. In particular, conditions which cause inhibition of NiRA can result in the accumulation of nitrite in plant tissues with toxic effects to the plant and to animals which may eat the plant.

This paper describes a series of experiments which were performed to characterize some of the apparent properties of nitrite reduction under *in vivo* conditions. The primary goal was to develop a procedure which would be as simple to use as the *in vivo* NR assay and which accounted for NiRA.

**MATERIALS AND METHODS**

Bush bean plants, *Phaseolus vulgaris* L. cv. Top Crop, were sown in vermiculite watered with tap water. Starting 10 days after sowing, the plants were watered daily with 1X Hoagland nutrient solution. The plants were kept in a large growth room under fluorescent plus incandescent lights (approximately 183 µE m⁻² s⁻¹ at the primary leaves) on a 13-h day at 26 C/18 C day/night temperatures with a RH of not less than 50%.

For *in vivo* assays conducted in the dark, leaf tissue was usually cut either as discs or as squares into pieces approximately 1 cm⁻²; the pieces were vacuum infiltrated with incubation medium composed of 100 mm phosphate buffer (pH 7.5) and a wetting agent, which was 0.05% Neutonyx-600 (Onyx Chemical Co., Jersey City, NJ) or 1% 1-propanol or both (the combination is the standard in *in vivo* NR assay wetting agent [5]). Vacuum was applied to the extent that vigorous bubbles were released from the tissue or, in the case of media containing Neutonyx-600, until foam just covered the tissue. The vacuum cycle was repeated three times. After infiltration, the tissue was drained damp-dry on a paper towel, transferred to a 125-ml plastic specimen container containing 3 ml fresh incubation medium, placed in the dark, and given gentle, continuous agitation. For *in vivo* assays in the light, a 6-cm diameter disc, cut from the center of a leaf, was weighed and vacuum infiltrated as described above. Unless specified otherwise, the infiltration medium was identical to incubation medium. After infiltration, the disc was blotted damp-dry and placed in the bottom of a tall-form, 400-ml Pyrex beaker. A high precision Cornwall syringe (Becton, Dickinson and Co., Rutherford, NJ) was used to premeasure 3 ml incubation medium into the beaker.

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2 Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; NRA, nitrate reductase activity; NiRA, nitrite reductase activity.
After the tissue was added, the beaker was placed in a water bath 30 cm below the growth room lights (230 µE m⁻² s⁻¹) and incubated for 1 h with agitation at 5-min intervals. Details of tissue source, infiltration, and incubation medium are given in the description of each experiment.

For both dark and light assays, the reaction was stopped by the addition of approximately 50 ml slightly alkaline (pH 10–12), boiling water, and the beaker or vial was held in a hot water bath at approximately 90°C for 20 min. Samples were diluted by weighing to give a final concentration of approximately 50 µM NO₂⁻, and nitrite was measured by combining 1 ml diluted sample with 1 ml 1% (w/v) sulfanilamide in 1.5 M HCl with 1 ml 0.02% (w/v) N-1-naphthylethylenediamine hydrochloride and reading at 540 nm in a spectrophotometer. The amount of nitrite accumulated by the system in the dark was assumed to be a function of NRA.

Disappearance of externally supplied nitrite was determined by subtracting the total amount of nitrite in the tissue incubation medium after the tissue was exposed to light from the nitrite content of a beaker treated identically except for the presence of tissue.

Parallel assays for NRA were run concurrently on separate, but related, tissue to obtain an estimate for nitrite synthesized by NR. This estimate also included any endogenous nitrite which was not included in the tissue at the start of an assay. The sum of this estimate and the measured amount of nitrite that disappeared in the light was initially assumed to approximate total nitrite reduction. Results obtained late in this study indicated that this would result in an underestimate of the NRA contribution. Consequently, we chose to analyze and report the results of some experiments on the basis of nitrite disappearance only. This is noted in the details of each experiment, and the steps required to obtain an accurate estimate of NRA contribution are outlined in the discussion.

RESULTS AND DISCUSSION

The following series of experiments was conducted to establish suitable conditions for measuring in vivo nitrite reduction and to study some properties of the process.

**Effects of Wetting Agents, Nitrite Concentration, and Incubation Temperature on Nitrite Disappearance.** Preliminary experiments indicated that large leaf discs (6-cm diameter) exposed to light for 1 h while immersed in a minimal amount (3 ml) of incubation medium containing a wetting agent and relatively high levels of nitrite (20 mM) at approximately 30°C gave readily measurable levels of nitrite disappearance. At 35°C, activity was greatly reduced. To establish optimum conditions for these factors, tissue from the primary leaves of 21-day-old bush bean plants was exposed to three wetting agent combinations (1% 1-propanol, 0.05% Neutronyx-600, and 1% 1-propanol plus 0.05% Neutronyx-600 [5]), three levels of nitrite (10, 20, and 30 mM NO₂⁻), and two incubation temperatures (25 and 30°C) in a 3 × 3 × 2 factorial arrangement replicated three times.

Since all interactions involving temperature were nonsignificant (P > 0.05), temperature appeared to affect nitrite disappearance independently of wetting agent and nitrite concentration. Increasing the temperature from 25 to 30°C increased nitrite disappearance from 16.28 to 19.93 ± 0.83 µmol NO₂⁻/g fresh weight-h (0.001 < P < 0.005).

In contrast, the nitrite concentration by wetting agent interaction was significant (0.01 < P < 0.05), suggesting that the pattern of the nitrite disappearance response to increasing nitrite concentration depended on the wetting agent used (Fig. 1). In the presence of 1% 1-propanol, nitrite reduction was low at 10 mM NO₂⁻ and increased linearly to its highest value at 30 mM NO₂⁻. With Neutronyx-600 alone as the wetting agent, activity was also low at 10 mM NO₂⁻, but it rose to a peak at 20 mM NO₂⁻ and then leveled off. For the combination of Neutronyx-600 and 1-propanol, no change was observed in nitrite disappearance as nitrite concentration increased.

Although the highest level of nitrite disappearance occurred with propanol only (20, 30, and 30 mM NO₂⁻), it was not significantly different (P > 0.05) from the Neutronyx-600 results obtained at 20 and 30 mM NO₂⁻. However, since the rate of nitrite disappearance was apparently still rising at 30 mM NO₂⁻ in the presence of l-propanol, we decided to study this effect further.

Six levels of nitrite (10, 20, 30, 40, 50, and 60 mM) were used with 1% 1-propanol in 100 mM phosphate buffer (pH 7.5). Tissue was from fully expanded trifoliolate leaves, and each treatment was replicated four times. The resulting mean activities in terms of nitrite disappearance were 21.0, 29.3, 29.0, 30.4, 21.6, and 21.4 ± 1.1 µmol NO₂⁻/g fresh weight-h, respectively. The rates of nitrite disappearance at 20, 30, and 40 mM were not significantly different (P > 0.50) from each other, but they were substantially higher (P < 0.001) than the effects of the other three nitrite concentrations, which were also not significantly different (P > 0.50). These results indicate that activity peaks in the 20 to 40 mM range. However, it is likely that the results at the higher levels are unreliable, since dilution factors become extreme.

The nitrite disappearance values in this experiment were higher than those in the previous experiments, but this was probably due to the use of trifoliolate tissue, which was shown several times in preliminary experiments to be more active than primary leaves from the same plant.

**Effect of Nitrite Concentration on NRA in the Presence of Neutronyx-600 or Propanol Plus Neutronyx-600.** The tissues nor-
mally contained considerable amounts of nitrate (approximately 0.5 mg nitrate N per g fresh weight). This condition maintained induction and synthesis of NR and NiR. Under these circumstances, it was assumed that NR would be functioning and that it would add a certain quantity of nitrite to the assay system. This assumption was tested by using a modification of the standard in vitro assay for NRA conducted in the dark, as described in “Materials and Methods.” The experiment was a 2 × 3 factorial with two wetting agent combinations (0.05% Neutronyx-600 and 0.05% Neutronyx-600 plus 1% 1-propanol) and three levels of nitrite (0, 10, and 20 mM NO₃⁻). Six replicates were run at each of the six treatment combinations. Discs (4.5 cm in diameter) were cut from the primary leaves of 14-day-old plants. The discs were separated into two groups and infiltrated with buffer containing the wetting agent typical of their ultimate treatment but without nitrite. After infiltration, the discs were drained damp-dry on a paper towel and then added to 3 ml premeasured incubation medium containing the appropriate combination of wetting agent and nitrite. Nitrite production was measured as the increase in nitrite over a 1-h period.

Analysis of the results showed that there was no significant \( P \approx 0.25 \) interaction between the wetting agent and the nitrite, indicating that these factors acted on NRA independently. Specifically, Neutronyx-600 plus 1% 1-propanol was consistently more active \( (P < 0.001) \) than was Neutronyx-600 alone in facilitating NRA in this assay (Fig. 2), a situation which we have found is true of the normal in vivo NRA assay, as well. Increasing levels of nitrite, from 0 to 20 mM, caused a very significant linear decrease \( (P < 0.001) \) in NRA. In fact, we have found that NRA could be completely suppressed in some instances by 20 mM nitrite in the incubation medium, but the exact conditions under which this occurs are, as yet, unknown. Ordinarily, the NRA contribution to the nitrite pool was, at most, 8 to 9% of the amount of nitrite that disappeared when Neutronyx-600 plus 1-propanol was the wetting agent and, at most, 3% when Neutronyx-600 alone was used.

**Effect of Tissue Age on Nitrite Disappearance and NRA.** This experiment was conducted to examine the effects of tissue age on nitrite disappearance and NRA concurrently. Primary leaf tissue of plants from seeds sown at 3-day intervals and ranging in age from 12 to 33 days was used. For the nitrite disappearance assay, tissue from one primary leaf from each of four plants in each age group was infiltrated with 100 mM phosphate buffer (pH 7.5) containing 0.05% Neutronyx-600 plus 1% 1-propanol and incubated in a solution which carried, in addition, 20 mM NO₃⁻. Standard NRA assays were carried out on tissue from the alternate primary leaf of the same set of plants. Due to accidental loss, the replicates for each of the 18-, 21-, and 24-day-old samples were reduced to three for the nitrite disappearance portion of the experiment.

Regression analysis of the nitrite disappearance results indicated a moderate linear decline \( (P \approx 0.05) \) in nitrite disappearance with age (Fig. 3A). However, when the 12-day results were removed from the analysis, it was found that nitrite disappearance was more or less constant over the 15- to 33-day period (Fig. 3A).

NRA in bean leaves was previously shown to be maximum

![Fig. 2. Effect of nitrite concentration and wetting agent on NRA in primary leaves of P. vulgaris. Symbols represent Neutronyx-600 only (0.05%) (□) and 1-propanol (1%) plus Neutronyx-600 (0.05%) (△), and they indicate the mean NRA ± 1 SE.](image)

![Fig. 3. Effect of age of primary leaves on nitrite disappearance and NRA in P. vulgaris. A, Nitrite disappearance (μmol NO₃⁻/g fresh weight. h) = 21.60 - 0.1649 age (days) for all age groups (——); nitrite disappearance = 17.45% when 12-day results are removed (——). B, NRA (μmol NO₃⁻/g fresh weight. h) = 3.782 - 0.1078 age (days). Symbols indicate the mean NRA at each time ± 1 SE.](image)
when the leaves were quite young and, subsequently, to decline with age (5). In this experiment, as expected, the NRA assay showed an extremely significant ($P < 0.001$) linear decline in activity with advancing age (Fig. 3B). From this, it was evident that the plant tends to maintain its ability to reduce nitrite even as it loses the ability to convert nitrate to nitrite. Since nitrite has toxic properties, this is the most logical relationship of the two enzymes.

**Effect of Diuron and Nitrite on Nitrite Disappearance and NRA in Light and Dark.** Since diuron is known to be an effective inhibitor of photosynthesis, it could be expected to interfere with the light-driven process of nitrite reduction. Mittin (13) reported such an inhibition but was not convinced that the normal PS II pathway was the only way for energy transfer to convert nitrite to ammonia. More recently, Klepper (12) and Finke et al. (4) showed that diuron treatment resulted in the accumulation of nitrite in leaf tissue exposed to light. It was apparent that NRA was greater than NiRA under these conditions. Preliminary trials indicated that similar conclusions could be obtained using the procedure described here. We decided to conduct an experiment which would test several of the concepts relating to nitrite utilization and NRA.

Fully expanded trifoliolate leaves selected at random from 25-day-old plants provided the tissue for this experiment. For 15 days prior to the assay, the plants were given 13-h days under a 400-W high pressure sodium lamp (General Electric; Duraglow, 230 µE m$^{-2}$s$^{-1}$ at the level of the primary leaves) located in a laboratory. Temperature was approximately 22°C, and humidity was not controlled. The sodium lamp was also used as a light source for the assays in this experiment. The various treatments provided at the time of the assay are summarized in Table I. Diuron was included in the infiltration medium of those tissues that were to receive diuron also during the assays.

When the assay was conducted in the light, use of incubation medium alone resulted in essentially no measurable nitrite (Table I). Under the conditions of this treatment, both NR and NiR would be expected to function, and, since NiRA is substantially greater than NRA, it should use up all the nitrite produced by NR. With the addition of diuron to the incubation medium in the light, the amount of nitrite produced was typical of NRA levels. In this instance, it was expected that nitrite reduction would be inhibited by diuron (4), with a resulting accumulation of nitrite. The third treatment with incubation medium containing nitrite was the proposed in vivo assay for nitrite disappearance, and it typically used approximately 19 µmol NO$_2^-$/g fresh weight-h. Finally, tissue was exposed to light in the presence of both diuron and nitrite. This resulted in an apparent net loss of a small amount of nitrite, but the value obtained was not measurably different from zero.

Conducting the assay in the dark with incubation medium alone, the standard in vivo NRA assay (5) typically produced approximately 4 µmol NO$_2^-$/g fresh weight-h. When diuron was added to the incubation medium, the resulting NRA was not significantly different ($P > 0.1$) from this value. Addition of nitrite instead of diuron to the incubation medium caused a decrease in nitrite over the course of 1 h. The amount was small in comparison to the corresponding treatment in light and possibly was due to a carryover of a limited amount of light-generated reducing agents. When diuron and nitrite were both present in the dark assay, there was no net gain or loss of nitrite. The latter two treatments in the dark were examples of the situation mentioned in the second experiment (described above), where no net synthesis of nitrite was measured. Otherwise, the effects of these treatments were consistent with results reported in earlier parts of this paper and elsewhere (4, 9–12).

The results of this series of experiments indicate that it is possible to measure nitrite reduction in green bean leaf tissue directly. Moreover, by observing certain general conditions, it should be possible to extend the procedure for use with leaf material from other plants such as soybean and grasses.

Since the nitrite disappearance portion of the assay is based on subtraction of the postincubation nitrite concentration from a known initial value, any modification which maximizes the differences will tend to increase the sensitivity of the assay. In particular, the assay should be run as long as is practical, and the initial concentration of nitrite should be as low as is needed to provide maximum activity (for example, an assay based on 20 mM NO$_2^-$ is 1.5 times as sensitive as one based on 30 mM NO$_2^-$). The tissue mass should be uniformly exposed to the light source, and the ratio of tissue mass to incubation medium volume should be maximized while still ensuring that the tissue is completely wetted by the fluid. This has been accomplished in trials by curling a leaf to fit, without overlap, inside a capped vial which was then rotated slowly at a right angle to the light source to expose the tissue entirely to the light. As an added advantage, this system required a very small volume of fluid to keep the leaf surface wet. By using tall vials or capped test tubes, the elongated leaves of grasses could be similarly processed.

It has also been found that the sensitivity of the assay does not depend, within limits, on the size of the tissue mass, provided other conditions are scaled accordingly. For example, a mean nitrite disappearance of 31.2 ± 1.3 µmol NO$_2^-$/g fresh weight-h was obtained when 1.6-cm-diameter discs were cut from a trifoliolate leaf and incubated in 0.25 ml incubation medium in a shell vial of appropriate size. Both the tissue and the incubation medium were weighed to four decimal places. (This weighing procedure improved accuracy and has been adopted as standard practice to overcome occasional variations in the delivery volume of the dispensing syringe.) Although the use of a smaller volume of fluid has the advantage of reducing dilution factors, this is counterbalanced by the smaller tissue samples being less representative of larger leaves or plants. Note, in general, that, since nitrite concentration decreases as the assay proceeds, it is important that none of the above factors be carried to such an extreme that this decrease would materially influence the course of the reaction. Initially, we assumed that the production of nitrite by NR, as measured in a standard in vivo NRA assay, would be added to the nitrite disappearance values to arrive at a total nitrite reduction figure. To obtain the NRA correction factors, parallel assays were conducted on the leaf tissue remaining after removal of the large disc used in the nitrite disappearance assay. The subsequent discovery that high levels of nitrite in the incubation medium significantly decreased, and sometimes eliminated, NRA and that this was dependent in part upon the choice of wetting agent resulted in a reassessment of this procedure. Specifically, the

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Table I. Effects of Diuron and Nitrite on NR and Nitrite Disappearance in Light and Dark

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Light</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM</td>
<td>-0.02 ± 0.01</td>
<td>+3.96 ± 0.73</td>
</tr>
<tr>
<td>IM + diuron</td>
<td>+3.49 ± 0.50</td>
<td>+4.98 ± 0.55</td>
</tr>
<tr>
<td>IM + nitrite</td>
<td>-19.36 ± 1.12</td>
<td>-1.61 ± 0.35</td>
</tr>
<tr>
<td>IM + diuron + nitrite</td>
<td>-0.92 ± 0.31</td>
<td>-0.06 ± 0.40</td>
</tr>
</tbody>
</table>

* Mean activity given as nitrite used (−) or produced (+) ± 1 SE.

* IM, Incubation medium.
incubation medium used in the NRA part of the assay must be identical to that for the nitrite disappearance assay, so that the results of the two procedures can be combined to give nitrite reduction measurements.

We also decided initially that 1% 1-propanol plus 0.05% Neutronyx-600 would be used as the wetting agent for the nitrite disappearance assay, inasmuch as this combination had been established as the optimum wetting agent for NRA assays in Phaseolus tissue (5). However, since Neutronyx-600 alone is apparently sufficient wetting agent to facilitate nitrate reduction and results in relatively low NRA in the presence of high nitrite, it is recommended as the preferred wetting agent. Moreover, in applications where a minor error in the estimation of total nitrite reduction would be acceptable, measurement of nitrite disappearance in the presence of 0.05% Neutronyx-600 would be a close approximation. This would also save the work involved in running the parallel NRA assays and would be only slightly more complex than the standard in vivo NRA assay.

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LITERATURE CITED