Nitrate Reduction in Leaves of Grapevine and Other Fruit Trees

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Abstract. Optimum conditions for in vivo activity of nitrate reductase (NR) from grape leaves (Vitis vinifera L.) were pH 7.5, 100 mM NO3 substrate, and incubation at 40°C. Pretreatment with low concentrations of NO3 (0.05 M) at room temperature did not increase activity substantially.

Induction of NR by NO3 substrate was investigated. Enzyme activity was maximum at the highest NO3 levels. Intact-tissue assay detected NR in mature leaves of grapevines, walnut (Juglans regia L.), plum (Prunus domestica L.), pears (Prunus communis L.), and sweet cherry (Prunus avium L.) grown under field conditions. In addition, a particulate NR was demonstrated in walnuts and grapevine leaves by in vitro assay.

Despite the importance of nitrogen in the nutrition of grapevines there is little information on their NO3 assimilation and reduction. We know of no investigations of enzymes involved in nitrogen assimilation in grapevines (9, 24).

Early investigations indicated that NO3 is absent in xylem sap and leaves of fruit trees (23). The leaf NR was assumed to be of relatively little importance compared with root NR. However, NR and NO3 are found in all parts of apple seedlings, and are highest in the leaf (16), and NR has been demonstrated in leaves of citrus (2), apples (16), and Prunus (17). To determine the distribution of NR in various parts of grapevines, the enzyme activity in mature leaves and roots of field-grown vines was measured. Since petioles of most grape cultivars accumulate relatively large amounts of NO3 (6), whereas most deciduous fruit trees apparently do not (16, 17, 23), NR activities in leaves of both groups of plants were compared.

Intact-tissue assay of nitrate reductase (NR) has been shown to be a useful technique for measuring enzyme activity in plant species for which cell-free extracts of the enzyme are difficult to obtain (7, 8). Nitrogen uptake and enzyme induction have been studied and characterized in various species, but its assay has not been completely standardized (5). It has been recommended as a tool for diagnosing nitrogen requirements of trees and field crops (3).

In vivo enzyme assays have several limitations (21), including the dependence on absence of endogenous NADH, lower observed activity (in annual plants) than found with in vitro assays, and higher NO3 substrate requirements (10). Bar-Akiva and Sagiv (2), nevertheless, suggested that the enzyme activity measured in situ may be a better estimate of actual enzyme activity in intact plants than that measured using in vitro procedures, which are especially selected to yield maximum activity. Felippe et al. (11) found that in vitro NR activity overestimated the amount of organic nitrogen expected in barley.

Intact-tissue NR has been studied and characterized in various species, but its assay has not been completely standardized (5). This paper reports some of the conditions important for in vivo assay of NR in grapevines. To confirm the in vivo presence of NR in grape and walnut leaves, its presence was also demonstrated using an in vitro procedure.

Materials and Methods

Two-year-old vines of 'Thompson Seedless' (syn. 'Sultanina'), 'Pinot noir', 'Malbec', and 'Zinfandel', (F. vinifera L.) and rootstock cultivars 'St. George' (V. rupestris), 'AxR #1' (Aramon x V. rupestris, Ganzin No. 1), and '99-R' (V. berlandieri x V. rupestris) were utilized in the study. 'Thompson Seedless' and 'Pinot noir' vines were grown in 19 liter metal cans containing a mixture of 2 soil: 2 sand: 1 peat (by vol). Tap water was used to satisfy any additional water requirements. 'Pinot noir' vines were brought to a low N status before the inductive treatment with KNO3 by providing them with tap water only for the first 3 months after bud break. Mature leaves from field-grown 'Thompson Seedless' grapevines and deciduous fruit trees were obtained in July and August from the U.C. Davis Experimental Station farm, where they had been given normal commercial cultural practices. No NO3-inductive treatment was provided to the plants or tissues before the assay, and the only N sources were those in the soil. The fruit trees used for NR assay were: 'Winter Nellis' pear; 'Bing' sweet cherry; 'Santa Rosa' plum; and 'Serr' walnut. For each species 3 or more samples were collected and each assayed in triplicate. For each plant species the most recently fully expanded leaves were used for assays, unless otherwise stated.

Preparation of experimental material, pretreatment, and in vivo NR assay conditions were as those described by Bilal and Rains (5) except for the following modifications. Leaves were collected, blades removed, washed with distilled water, and blotted dry with paper towels. Sixty to 80 leaf blades were held between 2 blocks of styrofoam by rubber bands and 400 to 600 μm tissue slices obtained using a sliding microtome. These were immediately dipped into ice-cold distilled water, blotted dry with soft tissue paper, and 200-ng samples transferred into precooled reaction mixtures in test tubes. NO3 in the tissue was apparently adequate for enzyme induction, since no time lag in NR activity was observed, so pretreatment with KNO3 was not used.

Ten ml of 0.1 M KNO3 in 0.1 M pH 7.5 phosphate buffer reaction mixture was used per 200 mg tissue. The pH was selected on the basis of preliminary experiments. Propanol was omitted from the reaction mixture since NR activity did not increase appreciably in its presence. N gas was bubbled into the test tubes containing the tissue and reaction mixture, and the reaction was started by placing the test tubes, covered with aluminum foil, in a water bath held at 35° or 40°C. The incu-
bution period was 30 to 60 min. A 200-mg tissue sample in reaction mixture kept in an ice bath for the entire incubation period served as control. A sample was also taken at zero time and saved for NO₂ analysis. No advantage was found in boiling the tissue to stop the reaction; instead, at the end of the incubation period, tubes were shaken for 2-3 sec in a vortex mixer, triplicate 1.5 ml samples were removed, mixed with 0.75 ml each 1% sulfanilamide in 1.5 N HCl and 0.02% N-1-naphthyl-ethylenediamine dichloride, and the absorbance measured at 540 nm. Enzyme activity was calculated as micromoles NO₂ produced per g fresh tissue per hr.

For in vitro NR extraction and assay, 5 g of tissue was ground for 1 min in 100 ml of a mixture of 0.5 M sucrose, 0.35 M K phosphate buffer, pH 7.3, 1 mM MgCl₂, 0.2% bovine serum albumin, and 10 mM ascorbate using a Servall Omni-Mixer. The homogenate was filtered through 8 layers of cotton organdy, 4 layers Miracloth, 1 layer fine-mesh nylon cloth, and centrifuged at 32,800 g for 15 min. The pellet was resuspended in 10 ml of grinding mixture and homogenized with a Teflon homogenizer. This was used for NR assay. After the reaction was completed the tubes were centrifuged at 32,800 g for 15 min, and read in a Zeiss spectrophotometer at 540 nm. Enzyme activity was calculated as micromoles NO₂ produced per g fresh tissue per hr.

Fig. 1. Effect of quantity of fresh tissue on in vivo NR activity. Leaf slices were obtained from 'St. George' grapevines and incubated in 10 ml of reaction mixture. Each point is the mean of 3 replications.

Results and Discussion

To test the validity of the intact-tissue infusion method, from 25 to 200 mg of grape leaf tissue were incubated and NO₂ production measured. NR activity increased linearly over the range 50-200 mg (Fig. 1).

The time course of NR activity was followed by two ways: 1) tissue slices were pretreated in 0.05 M KNO₃ at room temp for 20 min to assure adequate substrate in the tissue before starting the reaction (5), 2) tissue slices were placed directly into the reaction mixture held in an ice bath for 20 min. NO₂ formed in each case was a linear function of time, with no apparent lag period (Fig. 2). These results confirm findings with apricot (17) and apple leaves (16). In contrast, a lag period occurred and a NO₃-induced permease was present (12) in tobacco XD cells, sycamore cells, Penicillium chrysogenum, and Neurospora crassa. The concn of NO₃ in the leaf blades ranged between 0.08 and 0.12% (dry wt). Apparently this provided sufficient substrate to start the reaction when the temperature was raised during incubation. Since the leaf tissue was kept at low temp (ice bath) before incubation, NO₃ probably did not enter the leaves. Evidently, there is no active permease system for uptake of NO₃ in the grapevine.

NR activity in grape leaf tissue increased linearly for 3 hr (Fig. 2) and then increased more slowly. NR induction was similar when apricot (17) and apple leaves (16) were incubated with their petioles in KNO₃ for different periods. Leece et al. (17) attained maximum NR activity of apricot leaf in vitro after 6 hr of petiole immersion in 10 mM KNO₃.

The relative long period of NR activity in the absence of added co-factors and light in grape leaf slices is noteworthy. A comparison of NR activities in intact grape leaf tissue with those in apricot leaves (17) in an in vitro system indicated that the intact grape leaf slices were able to sustain a high level of NR activity without addition of a reductant. This implicates chloroplasts, a rich source of reducing power, as a logical site for location of NR in the cells. Several investigations support that hypothesis (13).

As a further test of the effect of pretreatment with KNO₃ at room temp, 'Thompson Seedless' grape leaves about 2 weeks and 2 months old were both pretreated with 0.05 M KNO₃ for 1 hr at room temp (24°C) or at 3°C. Little difference in NR activity was found (Table 1). Pretreatment with NO₃, as reported by Bilal and Rains (5), was not essential for high NR activity in intact grape leaf.

In agreement with results for other plants (21), optimum in vivo NR activity in grape leaves required high concns of NO₃ substrate (0.1 to 0.2 mM) (Fig. 3). Activity was considerably reduced at 0.4 mM NO₃, indicating that NO₃ concn above the optimum range suppressed NR activity. Accumulated end products of NO₃ reduction may act as feedback inhibitors in roots (20).

The optimum NO₃ concn is usually much lower (10, 21) for in vitro NR activity than for in vivo systems, indicating that one of the factors limiting NR activity may be entry of NO₃ into the cell compartment where the NR enzyme is localized. Rhodes and Stewart (21) found that freezing and thawing
Fig. 3. Effect of substrate (KNO₃) concn in the reaction mixture on the rate of *in vivo* NR enzyme. The reaction mixtures contained 0.1 M K phosphate buffer, pH 7.5, KNO₃ at different concn from 0.02 to 0.4 M and 200 mg of 400 μm width ‘Thompson Seedless’ leaf tissue slices in 10 ml volume. Each point represents the average of three samples incubated at 30°C.

Intact leaf tissue of *Lemna minor* gave levels of NR activity *in vivo* similar to those *in vitro*, suggesting that substrate entry may be facilitated by altering membrane integrity. Increased NR activity in tissue fragments has been obtained also by vacuum infiltration (5) or by chemical treatment (15).

Addition of 1% 1-propanol to the reaction mixture slightly increased NR activity over that of the control (Fig. 4). Conc of 1-propanol greater than 1% sharply decreased NR activity, and, in contrast to findings of Jaworsky (15) and Bilal and Rains (5), 5% 1-propanol in the reaction mixture suppressed NR activity completely. These observations indicate that although propanol may facilitate NO₃ diffusion through cut edges of leaf fragments, it may also have an adverse effect on cells, especially at concn greater than 1%, possibly by allowing phenols and/or inhibitors to come in contact with the enzyme. Also, chlorophyll leakage increased at propanol concn higher than 1%. Streeter and Bosler (22) reported similar findings. Propanol was omitted from the *in vivo* assay even though its presence in the reaction mixture gave some increase in NR activity.

The optimum temp for maximum NR activity in grape leaf tissue slices was 40°C (Fig. 5). *In vivo* and *in vitro* NR assays with most plants have been conducted at 28° or 30°C (2, 4, 5, 21, 22). Bevers et al. (4) found that the induction of NR was temp-dependent and this was partly due to increase in tissue NO₃ level. Temp for maximum NR induction in radish cotyledons and corn seedlings were 31 and 38°C, respectively (4). Temp effects on plant growth and NR activity have recently been reported (18, 19).

NR induction by NO₃ is well known in plants (12). Fig. 6 indicates that it is also an adaptive enzyme in grapevines. The inducible nature of NR was confirmed by growing ‘Pinot noir’ grapevines in a soil-sand-peat mixture without N fertilization for a prolonged period (3 months) before an inductive treatment with NO₃. Immediately after fertilization with 16 mM NO₃, NR activity in mature grape leaves markedly and rapidly increased over leaves from control vines (irrigated with water only), indicating an induction of the enzyme by NO₃. NR induction quickly followed NO₃ fertilization, reaching a level of 15 μmol NO₂/g/hr. This was 2.5 times greater than the original activity of 6.0 μmol NO₂/g/hr.

**Fig. 4.** Effect of 1-propanol in the reaction mixture on *in vivo* NR activity. The reaction mixtures contained 200 mg of 400 μm width leaf slices of ‘St. George’ and different concn of 1-propanol in the range 0 to 10%.

**Fig. 5.** Effect of temp on *in vivo* NR activity. Reaction mixture contained 200 mg of 400 μm width ‘Zinfandel’ leaf slices in 10 ml 0.1 M K phosphate buffer and 0.1 M KNO₃. Each point is the average of 3 samples run in triplicate for 1 hr.

**Fig. 6.** Influence of nitrate fertilization on induction of NR. ‘Pinot noir’ grapevines were fertilized with a single application of 16 μM KNO₃ in 1 liter solution at different dates before assaying for NR. Each point represents the mean of 3 determinations of composite samples obtained from 60 leaves harvested from 6 plants. The leaf slice width used was 600 μm.
of activity more than 3 times that in the control within 24 hr of fertilization and then declining and maintaining a level still above the control for at least 26 days. Fig. 6 indicates that grapevines have an internal regulatory mechanism which modulates enzymatic activity according to physiological need, similar to that found in other plants (12).

NR was found in leaves of all deciduous fruit and nut trees tested. Activity was greatest in walnut and least in sweet cherry (Table 2). The levels of activity in grape leaves using the intact tissue assay were higher than those found with in vitro assay by Klepper and Hageman (16) in apples, Leece et al. (17) in plums, Bar-Akiva and Sagiv (2) in citrus, and by Dirr et al. (8) in blueberries. In the present investigation of NR in leaves of various woody species, no NO3 substrate induction was necessary for enzymatic activity, in agreement with Leece et al. (17). Even so, they could not explain satisfactorily why relatively high NR was present in apricot and apple leaves from young trees in the absence of applied NO3. Most likely, endogenous levels of NR are present in leaves of fruit and nut trees under conditions of normal fertility. The assumption by some horticulturists that NO3 reduction normally takes place only in roots is without foundation. That endogenous NR is present in woody species under normal field conditions is further supported by time-course studies of Leece et al. (17) on field-grown apricot leaves and by data presented earlier on grapevines, both of which showed relatively high initial NR activities. No valid comparisons could be made of NR activities between the different species tested, since optimum assay conditions for each species have not been established and samples were not taken from a sufficiently large representative plant population. The survey was made simply to demonstrate that endogenous NR is present in leaves from a wide range of deciduous horticulture crops.

To confirm the in vivo NR activity in grape and walnut leaves grown under field conditions, NR activity was measured in vitro. The activities obtained from grape and walnut leaves were respectively 0.89 and 1.45 μ moles NO3/g fr wt · hr. Although the assays were made at only one sampling date and with crude enzyme extracts, they clearly demonstrated that NR is present in grape and walnut leaves. The enzyme determination was made in the particulate fraction since we were unable to detect it in the supernatant. This observation is in agreement with various reports indicating the presence of a particulate NR in higher plants (13).

The level of in vitro enzyme activity in grape leaves was lower than that found using the in vivo method. Although no valid comparison between the two assay methods can be made, the in vivo method is simpler to run, and in the authors opinion, reflects more closely what happens in nature. The particulate nature of NR in grapevines and fruit trees makes the in vitro extraction and assay conditions more difficult than for plants in which NR appears in the supernatant fraction (12).

Fig. 7 shows NR activity in roots, petioles and leaves of grapevines and fruit trees grown under field conditions. Samples were taken from 'St. George' rootstock growing in sand culture in a greenhouse. One-half cm root tips and leaf petioles segments and 400-μm-wide slices of leaves under 2 different periods of incubation: 1 hr and 18 hr. NR activities were considerably higher in each of the plant parts after 18 hr than after 1 hr of incubation, suggesting that the availability of NO3 substrate may have limited the reaction. NR activities were considerably higher in leaf tissue than in either roots or petioles. No valid comparison between roots, petioles, and leaves was possible because of differences in the width of tissues slices and structures of plant parts.

In grapevines, NO3 normally accumulates in the petioles (6). This suggests that the bulk of supply of NO3 in the vine is transported to the aerial part of grapevines and reduced in the leaf blade.

Table 2. In vivo NR in leaves from young fruit trees and grapevines grown under field conditions.2

<table>
<thead>
<tr>
<th>Species</th>
<th>In vivo NR activity (μ moles NO3/g fr wt · hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pear (Winter Nellis)</td>
<td>2.11</td>
</tr>
<tr>
<td>Sweet cherry (Bing)</td>
<td>0.11</td>
</tr>
<tr>
<td>Plum (Santa Rosa)</td>
<td>1.04</td>
</tr>
<tr>
<td>Walnut (Serr)</td>
<td>4.02</td>
</tr>
<tr>
<td>Grapevine (Thompson Seedless)</td>
<td>3.06</td>
</tr>
</tbody>
</table>

2 Each reading represents triplicate samples of 60 leaf blades composited from 10 plants. All samples were taken in August.

![Fig. 7. In vivo NR activity in different parts of 'St. George' grapevines. Samples were taken from 'St. George' rootstock growing in sand culture in a greenhouse. One-half cm root tips and leaf petioles segments and 400-μm-wide slices of leaves under 2 different periods of incubation: 1 hr and 18 hr. NR activities were considerably higher in each of the plant parts after 18 hr than after 1 hr of incubation, suggesting that the availability of NO3 substrate may have limited the reaction. NR activities were considerably higher in leaf tissue than in either roots or petioles. No valid comparison between roots, petioles, and leaves was possible because of differences in the width of tissues slices and structures of plant parts. In grapevines, NO3 normally accumulates in the petioles (6). This suggests that the bulk of supply of NO3 in the vine is transported to the aerial part of grapevines and reduced in the leaf blade.

Literature Cited

Endogenous Gibberellin-like Activity in Cranberry at Different Stages of Development as Influenced by Nitrogen and Daminozide

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Abstract. Gibberellin (GA)-like activity in ‘Early Black’ cranberry (Vaccinium macrocarpon Ait.), as affected by N and treatment with succinic acid-2,2-dimethylhydrazide (daminozide) was measured at different growth stages. Cranberry buds and young leaves contained relatively high levels of GA activity. Activity in the leaves of uprights declined during July, coinciding with active elongation of the stems and increased in August, after terminal bud formation. Leaves of runners contained relatively high levels of GA activity, and may represent important sources of GA production during active runner growth. Plants receiving the lowest level of N had the highest GA-like activity in both runners and uprights. High N treatment increased vegetative growth, which may have diluted endogenous GAs. Leaves of uprights of daminozide-treated plants had higher GA-like activity than control plants, at the low and medium N rate. Daminozide-treated plants produced significantly more runners than control plants, except at the high N rate.

Cranberry flowers are borne on short vertical stems, or uprights, that arise from long prostrate vines or runners that form an intertwining mat over the surface of the cranberry bog. Terminal buds begin to form at the tip of the upright in late July. The terminal bud is a mixed bud which continues to develop throughout the winter and resumes growth in the Spring, 7.5 to 9 cm of new growth is added to the old upright and flowering occurs. The new growth is completed in July and a new terminal bud is formed thus completing the Spring cycle. Terminal buds on the runners normally produce new fruit the following season.

In order to obtain good annual commercial yields of cranberry, fruit production and vegetative growth must be in balance. Although N may increase the yield of cranberries, it can also overstimulate vine growth, leading to decreased yield. Less than 30% of cranberry flowers normally set fruit (9); therefore, the potential for higher yield exists. GA increases fruit set in cranberry, but even low concns can also seriously inhibit subsequent flowering and greatly increase the amount of vegetative growth (2, 12). In this respect, GA and N produce similar effects on the cranberry.

Daminozide retards growth and promotes flowering in many fruit crops, and may interfere with the action of natural GA (13, 14). The balance of reproductive and vegetative growth in the cranberry may be influenced by these 3 growth-controlling agents, and may reflect GA-like activity at different stages of development.

Materials and Methods

Samples of current season’s growth of uprights and runners from ‘Early Black’ cranberry plants were collected at biweekly intervals from an experimental bog at the Cranberry-Blueberry