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Factors Affecting Nitrate Reductase Activity in Some Monocot and Dicot Species

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Activity of nitrate reductase (NR), the first enzyme in the nitrate-assimilation pathway, was estimated in the cotyledons of the sunflower (*Helianthus annuus*) using a standardized in-vivo method. Seedlings were grown in the light on a nitrate medium. Various factors that affect NR activity were optimized, including the molarity and pH of the reaction buffer, nitrate concentration, and use of a surfactant. We also determined whether NADH was required for nitrate reduction. The surfactant propanol (2%) gave the best results, and no NADH supplement was necessary. In a separate study, we compared the effect of various culturing components on in-vivo NR activity among monocot and dicot species, and found that Triton X-100 was the best surfactant for monocots whereas dicots performed better with n-propanol. Monocot species also required additional NADH as an external energy source. Moreover, specific purification procedures were needed to enhance NR activity in dicotyledons. Finally, we also assessed the efficacy of in-vivo versus in-vitro procedures for assaying monocots versus dicots.

Keywords: dicots, *Helianthus annuus*, monocots, nitrate reductase (NR) activity, sunflower

Nitrate reductase (NR), the first enzyme used in nitrate assimilation, is located at the crossroads of two energy-consuming pathways: nitrate assimilation and carbon fixation. The first involves the uptake of nitrate and its subsequent reduction by NR; i.e., nitrate reduction must occur before nitrogen can be used in intermediate metabolism and protein synthesis. All these activities are induced by nitrate, and are modulated directly or indirectly by light.

NR catalyses the reduction of nitrate to nitrite, and is regarded as the rate-limiting and regulatory step in the nitrate-assimilation process. Regulation of NR is complex and involves a hierarchy of transcriptional and post-transcriptional controls (Crawford and Campbell, 1990).

In order to control nitrogen assimilation in green plants, NR must catalyze the reduction of nitrate to nitrite by electron transfer via its prosthetic group FAD and Mo protein (Barber and Notton, 1991; Fido, 1991). In-vivo NR activity depends upon several factors, including the entry of nitrate into the reduction site(s), availability of endogenous NADH for reduction, the amount of NR present in the tissue, and the exit of nitrite to the medium. An in-vivo assay is a rapid, easy, and particularly useful procedure for plant species in which active NR is difficult to assess by standard in-vitro methods (Aslam and Buttery, 1980). Peuke and Tischner (1991) have demonstrated that the former method more accurately reflects a plants status than does an in-vitro assay, in which the enzyme is removed from its natural physiological environment and transferred to optimized conditions. In-vivo assays have been successful in agricultural studies (Blondel and Blanc, 1975) for estimating the optimized level and appropriate time of application for nitrogen on field crops (Chanda et al., 1987), for predicting plant productivity (Johnson et al., 1976), and for screening crop cultivars with high yield potential (Hageman et al., 1976). It has also been employed in research on the source of reducing energy for nitrate reduction (Mann et al., 1979), and for estimating nitrate pools (Ferrari et al., 1973; Saroop et al., 1999).

Although NR was first characterized in higher plants in the early 1950s (Evans and Nason, 1953), its purification was achieved only in the 1970s, primarily because this enzyme is very unstable and requires special care for extraction, isolation, and purification. In-vitro NR assays normally are performed on isolated preparations under optimal conditions, both of which are rarely obtained in situ. Furthermore, the inhibitors or inactivating enzymes present in the tissue (Peuke and Tischner, 1991), which are otherwise spatially separated, are likely to be mixed up during extraction, thereby causing the inhibition of NR activity. Therefore, different additives, e.g., cysteine, bovine serum albumin, or casein, have been recommended (Aguera et al., 1987). However, the particular procedures followed for in-vivo and in-vitro

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assays differ among the various laboratories. Likewise, substrate concentrations, surfactants, tissue thicknesses, pH of the incubation medium, as well as the use of partial or complete anaerobic conditions, also vary when in-vivo assays are devised for each type of plant material. Therefore, the objective of the study presented here was to standardize and optimize the conditions for measuring NR activity in the sunflower (*Helianthus annuus*), and to compare these standards with those that have proven successful in other monocot and dicot species.

MATERIALS AND METHODS

Seeds of the sunflower (*H. annuus*) were sorted for uniformity, then thoroughly washed five or six times under running tap water before a final wash with distilled water. The seeds were then soaked in water for 3 h to facilitate germination. Afterward, they were spread evenly on wet filter paper (Whatman) in a clean tray and allowed to germinate in the dark for 36 h. Afterward, the seedlings were transferred to sieve culture dishes and supplied with a modified nutrient solution (Doddema and Telkamp, 1979) containing 0.5 mM K_2HPO_4, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 0.8 mM $CaCO_3$, 70 uM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 0.2 uM $Na_2MoO_4{\cdot}2H_2O$, 0.5% $FeSO_4$, and 0.4% tartaric acid, plus 50 mM KNO_3. The pH was adjusted to 6.8. The dishes were then transferred to a light room (ca. 200 uM m^{-2} s^{-1}) with six fluorescent tubes placed 0.5 m overhead, and the seedlings were allowed to grow for 48 h in continuous light. The time of transfer to the light was considered the 'zero' hour.

In-Vivo Nitrate Reductase Activity

In-vivo NR activity was estimated according to procedures normal to this laboratory (Chanda et al., 1987; Sood et al., 1996; Saroop et al., 1999). Briefly, five pairs of cotyledons were taken from uniformly growing seedlings, cut into small segments with a razor blade, then placed in test tubes containing 25 mM potassium phosphate buffer (pH 7.0), 2% n-propanol, and 200 mM KNO_3. The tubes were then vacuum-infiltrated in the dark for 5 min while the reaction mixture was incubated in the dark for 30 min. The reaction was terminated by putting the tubes in a boiling-water bath for 5 min. Nitrite levels were determined colorimetrically after color development with 1% (w/v) sulphanilamide and 0.02% n-(1-napthyl) ethylenediamine dihydrochloride. Absorbance was read at 540 nm.

In-Vitro Nitrate Reductase Activity

To measure in-vitro NR activity, about 15 cotyledons (500 mg) were excised and homogenized in a mortar at 0 to 4°C in prechilled 0.1 M Tris-HCl extraction buffer (pH 7.8) containing 1 mM cysteine, 0.3 mM ethylene diaminotetraacetic acid (EDTA), and 2% bovine serum albumin. This extract was then cleared by centrifugation at 10,000g for 10 min. The supernatant, which would serve as the enzyme source, was kept in an ice bath until the assay began. The assay mixture, in a 3.5-mL final volume, consisted of 15 mM KNO_3, 1.4 mM NADH, and the enzyme extract. Controls lacked NADH, so enzyme was added to initiate the reaction. After 30 min of incubation at room temperature, the reaction was terminated by adding 1 M Zn acetate, followed by centrifugation for 10 min at 5000g. The amount of nitrite produced was measured as described above. Three replicates were taken for analysis and activity was calculated as nmol of NO_2 produced per hour per gram fresh weight.

RESULTS AND DISCUSSION

In-Vivo Nitrate Reductase Activity

Production of nitrite in vivo can be stimulated by adding surfactants to the assay medium (Hog et al., 1983). Although this practice has been widely used, the mode of action by these agents has not been clarified. Researchers have proposed that surfactants may be able to lower surface tension, uncouple oxidative phosphorylation (Mann et al., 1979), inhibit the further reduction of nitrite (Yoneyama, 1981), or enhance vacuolar membrane permeability (Jaworski, 1971), thereby leading to increased transport of nitrate from a storage pool to a metabolic pool (Lawrence and Herrick, 1982). It is well established that a dark anaerobic environment favors nitrite accumulation during in-vivo assays, and that the nitrite formed under these conditions is not further released.

Because nitrate reductase activity can be promoted via the action of surfactants (Yoneyama, 1981; Chanda et al., 1987), we tested different levels of both Triton X-100 (Fig. 1a) and n-propanol (Fig. 1b) to determine their optimum concentrations in the media. For each surfactant, nitrite production was significantly promoted at low concentrations while at higher concentrations, activity was markedly decreased. In-vivo NR activity was also greater in the presence of n-propanol than

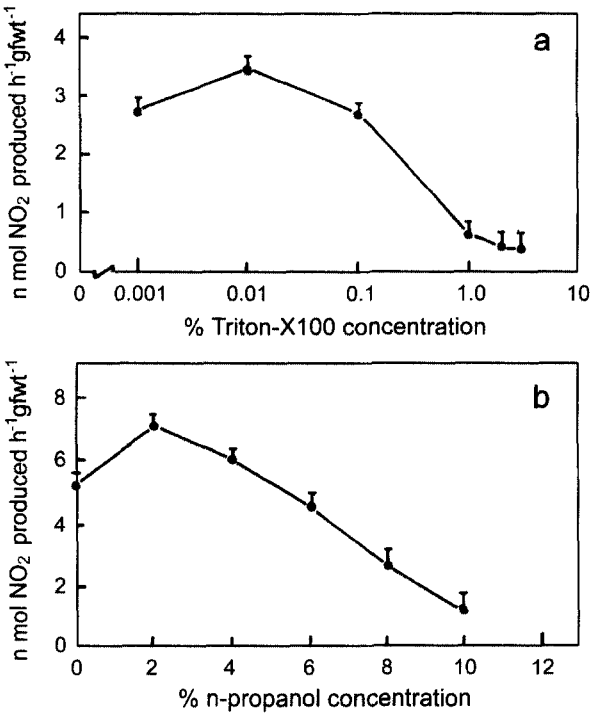

Figure 1. Effect of different concentrations of a) Triton X-100 and b) propanol on in-vivo nitrate reductase activity of sunflower seedlings. Vertical bars represent ± SD.

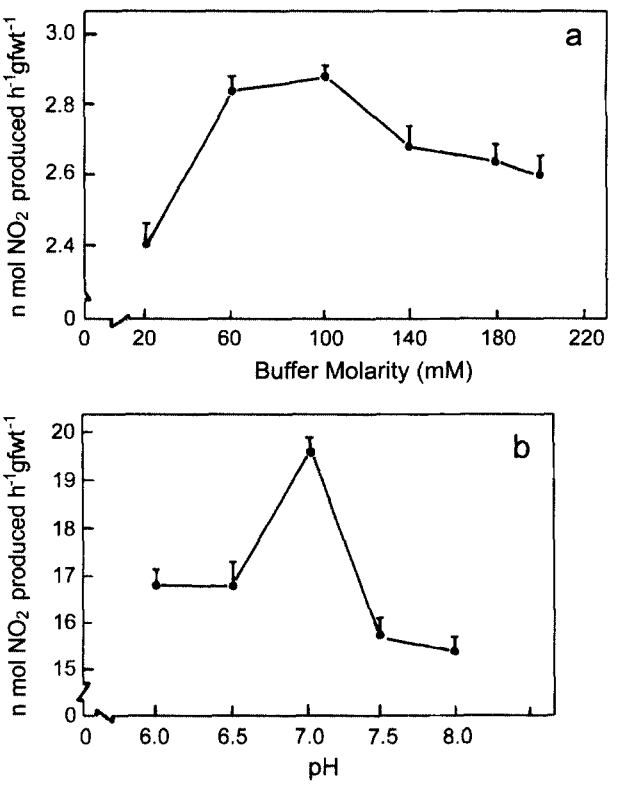

Figure 2. Effect of different concentrations of a) buffer molarities and b) pH on in-vivo nitrate reductase activity of sunflower seedlings. Vertical bars represent ± SD.

with Triton X-100, the maximum being reported with 2% n-propanol. Hence, we chose to use that particular level and surfactant for all subsequent analyses. The inhibition of activity observed here with Triton X-100 suggests that modes of action vary for different surfactants. Therefore, it is essential to determine the compound best-suited for each plant species.

We also studied the relationship between in-vivo NR activity and the pH and molarity of the assay medium. The effect of buffer molarity is shown in Figure 2a. At low concentrations, very little activity was present; optimum activity occurred at 100 mM. Higher buffer concentrations clearly inhibited activity, probably due to the increasing osmotic potential of the incubation solution. We also found that the optimum assay pH for promoting in-vivo nitrate reduction was 7.0 (Fig. 2b), with activity being considerably decreased at both lower and higher pH levels.

Different concentrations of nitrate in the assay mixture also influenced in-vivo activity (Fig. 3). The inclusion of NO_3 enhanced the rate of nitrite production compared with the activity recorded in the absence of exogenous nitrate. A level of 400 mM nitrate produced a four-fold increase while at higher concentrations, activity was slightly inhibited. Distinctly higher enzyme activities

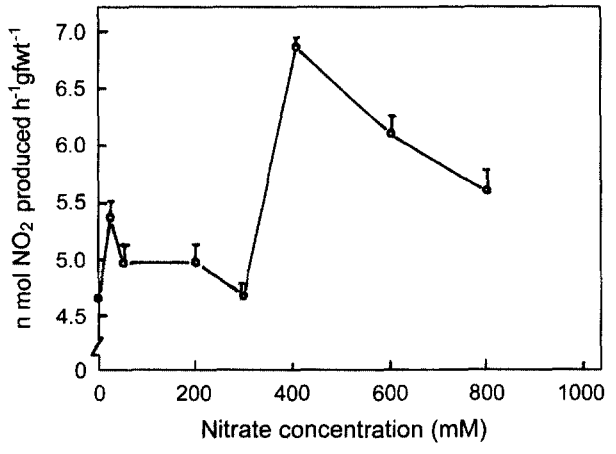

Figure 3. Effect of different nitrate concentrations on in-vivo nitrate reductase activity of sunflower seedlings. Vertical bars represent ± SD.

have been reported under anaerobic assay conditions (Mann et al., 1979; Chanda et al., 1987). This was also demonstrated in the current investigation. Finally, in our study of the effect of time on in-vivo NR activity, we noted that activity was linear over intervals of up

Table 1. Factors affecting in-vivo nitrate reductase activity in different monocot and dicot species. (×) indicates not required; (∨) indicates required.

Plant	pH	n-propanol	Triton X-100	KNO_3	NADH
wheat	7.4	×	0.25% (v/v)	200 mM	∨
bajra	7.4	×	1.00% (v/v)	200 mM	∨
radish	7.0	2.0%	×	200 mM	×
mustard	7.5	2.5%	×	200 mM	×
sunflower	7.0	2.0%	×	400 mM	×

to 60 minutes.

Based on these results, therefore, we suggest that maximum NR activity in vivo is achieved when the growth medium is supplemented with 2% n-propanol, 400 mN nitrate, and a 100-mM potassium phosphate buffer adjusted to a pH of 7.0.

In-Vitro Nitrate Reductase Activity

In-vitro studies of various species have shown that NR activity is inhibited by substances present in the plant. In maize, this inactivator is a protease (Yamaya et al., 1980). The isolation of active enzymes can be interfered with by both the presence of phenolic compounds and the high activity of phenol oxidase (Echevarria et al., 1984). In fact, some researchers have added insoluble polyvinyl pyrolidine to remove those phenolic compounds (Loomis, 1974). The addition of reducing substances, e.g., cysteine, inhibits the formation of such products. Aguera et al. (1987) have reported that the use of BSA or casein also can enhance extraction and improve the stability of NR in a number of species. The added protein protects the enzyme from inactivation and from proteolytic enzymes, and also removes phenols from the extracts. Nevertheless, despite using various extraction buffers in our tests, none of the additives described above could protect the enzyme from being inactivated or denatured.

Comparison of In-Vivo and In-Vitro Assays for Monocot Versus Dicot Species

In the final portion of this study, in-vitro and in-vivo assays were compared for their effectiveness in a number of monocot and dicot species. The methods had been standardized and were evaluated with bajra (*Pennisetum americana*), wheat (*Triticum aestivum*), radish (*Raphanus sativus*), sesame (*Sesamum indicum*), mustard (*Brassica juncea*), and sunflower (*H. annuus*). Although some culturing factors produced similar effects in both species types, others had very specific influences. For example, during the in-vivo assays, the surfactant Triton X-100 promoted maximum NR activity in monocots, whereas n-propanol was much more successful with dicots. This may have been a result of the difference in cell-wall contents between monocots and dicots. Likewise, wheat and bajra (both monocots) required external reductant NADH to adequately estimate activity while the dicot species needed no additional NADH (Table 1). For the in-vitro assays, NR activity could be detected in monocot species by extracting the enzyme in a phosphate buffer supplemented with EDTA, cysteine, and BSA. Such additions were able to protect the enzyme from denaturation and also stabilized it. In contrast, although a number of buffers were tested with the dicots (e.g., sunflower and sesame), none could provide sufficient protection (data not shown).

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