

# Nodule and Leaf Nitrate Reductases and Nitrogen Fixation in *Medicago sativa* L. under Water Stress

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## ABSTRACT

The effect of water stress on patterns of nitrate reductase activity in the leaves and nodules and on nitrogen fixation were investigated in *Medicago sativa* L. plants watered 1 week before drought with or without  $\text{NO}_3^-$ . Nitrogen fixation was decreased by water stress and also inhibited strongly by the presence of  $\text{NO}_3^-$ . During drought, leaf nitrate reductase activity (NRA) decreased significantly particularly in plants watered with  $\text{NO}_3^-$ , while with rewatering, leaf NRA recovery was quite important especially in the  $\text{NO}_3^-$ -watered plants. As water stress progressed, the nodular NRA increased both in plants watered with  $\text{NO}_3^-$  and in those without  $\text{NO}_3^-$  contrary to the behavior of the leaves. Beyond  $-15.10^5$  pascal, nodular NRA began to decrease in plants watered with  $\text{NO}_3^-$ . This phenomenon was not observed in nodules of plants given water only.

Upon rewatering, it was observed that in plants watered with  $\text{NO}_3^-$  the nodular NRA increased again, while in plants watered but not given  $\text{NO}_3^-$ , such activity began to decrease. Nitrogen fixation increased only in plants without  $\text{NO}_3^-$ .

Legumes are unique among crop plants in their ability to satisfy their large demand for nitrogen either through absorption and assimilation of inorganic N from the soil solution or by symbiotic fixation of atmospheric N. It has long been recognized (7, 17) that interactions are possible between the processes of (a) absorption, assimilation, and translocation of  $\text{NO}_3^-$ ; (b)  $\text{N}_2$  fixation; and (c) assimilation and translocation of the product  $\text{NH}_3$ .

The reduction and assimilation of  $\text{NO}_3^-$  in higher plants occurs in both above and below ground organs, and the extent to which these parts participate in N assimilation depends on the plant species, the level of  $\text{NO}_3^-$ , and the environmental conditions to which the plant is exposed (18). A number of workers have compared the level of nitrate reductase in the root and shoot or leaves. In some instances, these values are reported on a fresh weight basis, and in other reports, on a protein basis (3, 20); hence, comparisons are difficult. In addition, the amounts of nitrate reductase in the root and shoot depend on the concentration of nitrate used, the nature of the accompanying cation, and on the induction time (23). It appears that, shortly after germination, there is a measurable level of nitrate reductase in both root and shoot. As the plant matures, the activity in the roots disappears, and the upper canopy apparently takes over most of the reduction (28, 29).

Randall *et al.* (21) reported that soybean nodules had high NRA,<sup>1</sup> whereas the roots had no detectable NRA. Leaf NRA is sensitive to changes in the water status of plants, and it is inhibited

when the  $\Psi_w$  of the plant declines (25, 26). There are no reports on the relationships between the nodular NRA and water stress. The objective of this work has been to determine how NRA of alfalfa leaves and nodules as well as nitrogen fixation respond to water stress. In a set of observations, it is shown that nodule NRA increases during water stress.

## MATERIALS AND METHODS

**Plant Material and Growth Conditions.** Seeds of alfalfa (*Medicago sativa* L. cv. "Aragon") were germinated for 3 days on wet filter paper in Petri dishes. Inoculated seedlings were produced by adding samples of a commercial inoculum (Nitragin). Seedlings were then placed into a Perlite-containing seed bed covered with plastic and watered with N-free nutrient solution (8). Afterwards, the plants were transplanted into 20-cm plastic pots also containing Perlite, above which a 6- to 8-mm diameter quartz sand layer was placed and watered 3 days a week with the same N-free solution (1). The plants were grown in a greenhouse with 30/17°C extreme day/night temperatures, but usually about 25°C during the day. RH ranged between 50% and 70%.

One week before the drought experiments, the plants were divided into two lots. One of the lots was watered with the same N-free nutrient solution as before, but the other one received complete nutrient solution (11) containing 20 mM  $\text{NO}_3^-$ . Rewatering was performed with the same solutions. The plants were 3 months old and at the vegetative stage when they were used for drought experiments. Three days before each measurement, the plants were transferred to a growth chamber with a 25/15°C temperature and a 60%/80% RH day/night regime. The light level of photosynthetically active radiation was 200  $\mu\text{E m}^{-2} \text{s}^{-1}$ , and the photoperiod was 16 h.

**Assay of NRA.** The method used for determination of NRA was the method *in vivo* (5, 10, 12); 0.5 g apical leaves were cut in strips of 2 mm; the strips were infiltrated under vacuum immediately after cutting in 250-ml Erlenmeyer flasks containing 10 ml 50 mM phosphate buffer (pH 7.5), 0.35 M  $\text{KNO}_3$ , and 0.1% (v/v) Triton X-100. The strips were prepared and infiltrated at 0°C. They were then transferred to the incubation medium containing 5 ml identical solution, but excluding Triton X-100, and incubated at 28°C for 4 h. After incubation, 1-ml samples of the medium were removed for nitrite determination. The nodular NRA was determined by detaching the nodules and cutting them with a blade, the samples amount being 0.2 g fresh weight; subsequently, the procedure was as before. Nitrite formed was estimated by adding 1 ml 0.6% sulfanilic acid in 2.4 N HCl, 1 ml 0.5% *N,N*-dimethyl-1-naphthylamine in 4 N acetic acid diluted in 95% methanol, 1 ml 25% sodium acetate (2 M), and 6 ml water. Absorbance at 540 nm was then determined. NRA was expressed as  $\mu\text{mol nitrite formed per g DW and per h}$ .

**Measurement of Nitrate Content of Xylem Sap and Nitrate Flux to Leaves.** Plants were detopped just below the first leaf blade from the bottom and the first 10  $\mu\text{l}$  exudate was collected

<sup>1</sup> Abbreviations: NRA, nitrate reductase activity;  $\psi_w$ , water potential; DW, dry weight.

(25) by forcing exudation under pressure with a pressure chamber (24). Exudate nitrate content was measured by both the enzymic method (14) and the salicylic acid method (6). The nitrate flux to the leaves was computed from the product of the transpiration rate and the nitrate content of the xylem sap (25).

**Measurement of Nitrogen Fixation.** To measure acetylene reduction, plants were placed in assay containers, and acetylene was injected in amounts equivalent to 10% net volume of the container. After 60 min, gas samples were withdrawn and immediately chromatographed. Acetylene and ethylene were separated with a Carlo-Erba model 2300 gas chromatograph equipped with a hydrogen flame ionization detector. The stainless steel column was 1.5 m long and 6.6 mm in diameter and was packed with Porapak R of 80 to 100 mesh. Measurement conditions were similar to those previously described (1).

**Measurement of  $\Psi_w$  and Transpiration.** Transpiration rates were measured by applying the transpiration equation (2). Leaf resistances for upper and lower epidermis were measured on mature leaves with a diffusion porometer (Lambda model LI-60) and leaf temperatures with a thermistor. The difference in water vapor concentration from leaf to air was found by measuring leaf temperature (assuming saturation vapor pressure in the substomatal cavities) and RH with an aspirated psychrometer. Predawn leaf  $\Psi_w$  were measured in the same type of leaves with a pressure chamber (24). The timing for water potential measurements has

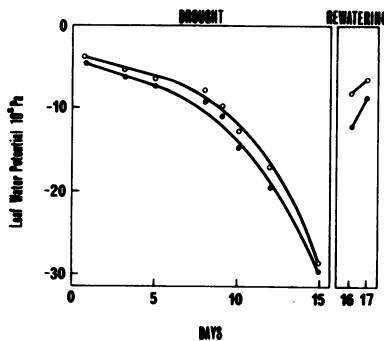


FIG. 1. Course of predawn  $\Psi_w$  for plants subjected to drought and rewatering ( $\bullet$ — $\bullet$ ), water with  $\text{NO}_3^-$ ; ( $\circ$ — $\circ$ ), water without  $\text{NO}_3^-$ . Each point is the average of three measurements made on three different plants for each of three independent experiments.

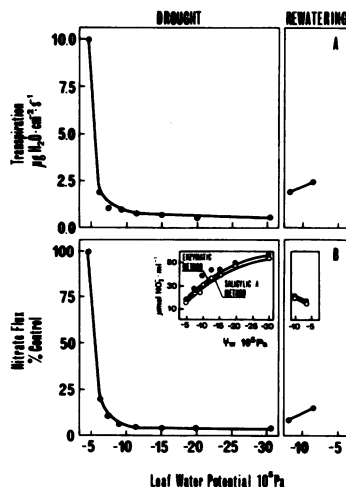


FIG. 2. Effect of predawn leaf  $\Psi_w$  on transpiration (A), nitrate flux (B), and nitrate content of xylem exudate (insert B) for  $\text{NO}_3^-$ -watered plants subjected to drought and rewatering. Each point is the average of three measurements made on three different plants for each of three independent experiments.

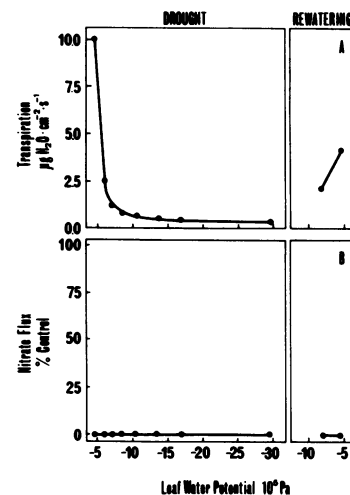


FIG. 3. Effect of predawn leaf  $\Psi_w$  on transpiration (A) and nitrate flux (B) for plants watered without  $\text{NO}_3^-$  subjected to drought and rewatering. Each point is the average of three measurements made on three different plants for each of three independent experiments.

been discussed by several authors (13, 15). In the present work, we were interested in referring the measured parameters to one daily plant water status measurement, and we decided to measure predawn  $\Psi_w$ . This represents the maximum potential the plant reaches after nightly recovery. Besides, by measuring predawn potentials, the differences between nodule and leaf  $\Psi_w$  would be minimal.

## RESULTS

The course of predawn leaf  $\Psi_w$  during drought and rewatering is shown in Figure 1. In Figures 2A and 3A, the values of transpiration are given as function of leaf  $\Psi_w$ . As can be observed when the plants were subjected to water stress, transpiration rates decreased until a minimum steady value was reached (about  $-10 \cdot 10^5$  Pa). In the plants watered with  $\text{NO}_3^-$ , the flux of  $\text{NO}_3^-$  to the leaves decreased together with the diminishing of transpiration rates (Fig. 2B), whereas, as expected in plants watered without  $\text{NO}_3^-$ , the presence of  $\text{NO}_3^-$  in xylem sap was not detected (Fig. 3B). After rewatering, a partial recovery of such parameters was observed (Figs. 2A, 2B, and 3A). The insert of Figure 2B shows the changes of  $\text{NO}_3^-$  concentration of xylem sap during drought and recovery as determined by the enzymic and salicylic acid methods. As can be observed, the  $\text{NO}_3^-$  concentration in xylem sap increases with drought, while it decreases with rewatering. Correlation between both methods was quite good. Leaf NRA decreased with water deficits, particularly in plants watered with  $\text{NO}_3^-$  (Figs. 4A and 5A). Leaf NRA recovery was quite obvious on rewatering, especially in the same  $\text{NO}_3^-$ -watered plants. Unlike the behavior of leaves, the nodular NRA increased as water stress progressed (Figs. 4B and 5B); although in plants watered with  $\text{NO}_3^-$ , NRA began to decrease when  $-15 \cdot 10^5$  Pa was reached. This phenomenon was not observed in the nodules of stressed plants previously watered without  $\text{NO}_3^-$  (Fig. 5B). During rewatering, it was noticed that, in plants watered with  $\text{NO}_3^-$ , the NRA increased again, whereas in plants watered without  $\text{NO}_3^-$ , such activity began to decrease.

In well watered plants, acetylene reduction was significantly higher in plants watered without  $\text{NO}_3^-$  (Fig. 5C) than in plants watered with  $\text{NO}_3^-$  (Fig. 4C). With increasing water stress, nitrogen fixation was affected significantly (Figs. 4C and 5C). Upon rewatering, a partial recovery of nitrogen fixation was observed only in plants watered without  $\text{NO}_3^-$ .

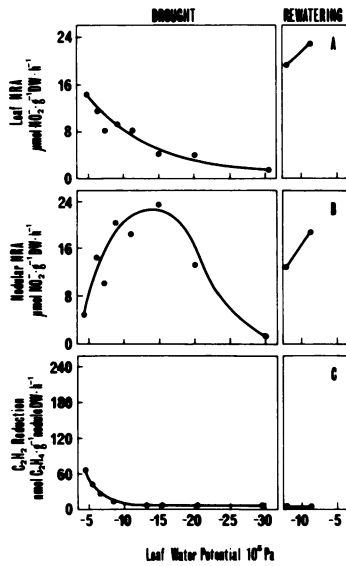


FIG. 4. Effect of predawn leaf water potential on leaf NRA (A), nodule NRA (B), and acetylene reduction activity (C) for  $\text{NO}_3^-$ -watered plants subjected to drought and rewiring. Each point is the average of three measurements made on three different plants for each of three independent experiments.

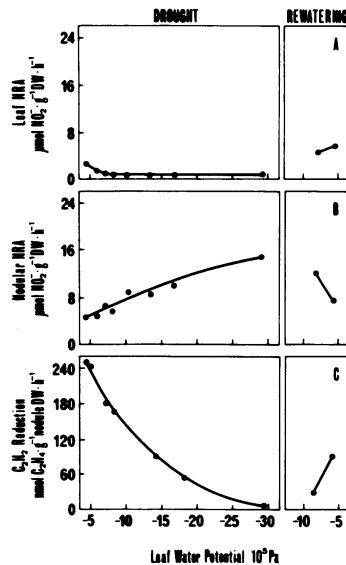


FIG. 5. Effect of predawn leaf water potential on leaf NRA (A), nodule NRA (B), and acetylene reduction activity (C) for plants watered without  $\text{NO}_3^-$  subjected to drought and rewiring. Each point is the average of three measurements made on three different plants for each of three independent experiments.

## DISCUSSION

Results show that in plants watered with  $\text{NO}_3^-$ , leaf NRA at high  $\Psi_w$  (Fig. 4A) was correlated with the nitrate flux (Fig. 2B) that in turn regulated the rate of synthesis of the enzyme (26). There is considerable evidence that rates of protein synthesis decrease as  $\Psi_w$  decreases and increase as  $\Psi_w$  increases (9). Nitrate reductase activity in maize leaves has been shown to decrease at low  $\Psi_w$  due to a decline in the rate of synthesis of the enzyme rather than an increased rate of degradation or a direct effect of  $\Psi_w$  on enzyme activity, whereas in recovery, increased activity was due to increased synthesis (16). Moreover, according to these authors, the possibility that low  $\Psi_w$  would prevent the movement

of nitrate to the induction site should not be eliminated, whereas a return to high  $\Psi_w$  can make nitrate available again for induction of nitrate reductase. Therefore, nitrate plays an important role in regulating the levels of leaf nitrate reductase activity, thus explaining the higher levels of activity which are found in plants watered with nitrate (Fig. 4A). During recovery, an increase of activity can be observed as nitrate flux increases again (Figs. 2B and 4A). The lack of nitrate flux to the leaves in plants watered without  $\text{NO}_3^-$  can explain the extremely low NRA in these plants (Figs. 3B and 5A).

Contrary to the behavior of leaves, the nodular NRA increased with water deficits both in plants watered with  $\text{NO}_3^-$  as in those which had not received  $\text{NO}_3^-$  (Figs. 4B and 5B). In the first case, an important decrease of activity began once  $\Psi_w$  reached  $-20 \cdot 10^6$  Pa, while in plants watered without  $\text{NO}_3^-$ , NRA kept increasing constantly even under extreme values of water deficits. Quite likely, the increased nodular NRA which is observed down to  $-15 \cdot 10^6$  Pa in  $\text{NO}_3^-$ -watered plants could be caused by increasing  $\text{NO}_3^-$  concentration in xylem sap (Fig. 2B, insert). Nevertheless, this and other possibilities would need to be explored before the matter could be satisfactorily resolved. Nitrate reductase has been described either as assimilatory where the physiological activity is primarily the ultimate production of ammonia for metabolism or as dissimilatory, or by the preferred term respiratory, where the nitrate is primarily an electron acceptor in place of oxygen as found mainly in bacteria. The root nodule bacteria have been shown to have both types of nitrate reductase functions (19, 22, 27). Quite likely, the alfalfa nodule NRA may function under water stress in a dissimilatory mode. Indeed, respiration of many tissues increases during drought (4), and the nodule NRA could reflect a coupling to respiration.

In conclusion, present data provide insight into the nitrogen reduction system of alfalfa and show that nodule NRA increases during water stress.

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## LITERATURE CITED

1. APARICIO-TEJO P, MF SANCHEZ-DIAZ, JI PEÑA 1980a Nitrogen fixation, stomatal response and transpiration in *Medicago sativa*, *Trifolium repens* and *T. subterraneanum* under water stress and recovery. *Physiol Plant* 48: 1-4
2. APARICIO-TEJO P, MF SANCHEZ-DIAZ, JI PEÑA 1980b Measured and calculated transpiration in *Trifolium repens* under different water potentials. *J Exp Bot* 31: 839-843
3. ASLAM M, A OAKS 1975 Effects of glucose on the induction of nitrate reductase in corn roots. *Plant Physiol* 56: 634-639
4. BRIX H 1962 The effect of water stress on the rates of photosynthesis and respiration in tomato plant and loblolly pine seedlings. *Physiol Plant* 15: 10-20
5. BRUNETTI N, RH HAGEMAN 1976 Comparison of *in vivo* and *in vitro* assays of nitrate reductase in wheat (*Triticum aestivum* L.) seedlings. *Plant Physiol* 58: 583-587
6. CATALDO DA, M HAROON, LE SCHRADER, VL YOUNGS 1975 Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun Soil Sci Plant Anal* 6: 71-80
7. DE MOOY CJ, J PESED, E SPALDON 1973 Mineral nutrition. In BE Caldwell, ed, *Soybeans: Improvement, Production and Uses*, Chap 9, Monograph 16. American Society Agronomy, Madison WI
8. EVANS HJ 1974 Symbiotic nitrogen fixation in legume nodules. In TC Moore, ed, *Research Experiences in Plant Physiology*. Springer-Verlag, Berlin, pp 417-433
9. HSIAO TC 1973 Plant responses to water stress. *Annu Rev Plant Physiol* 24: 519-570
10. HEUER B, Z PLAUT 1980 Reassessment of the *in vivo* assay for nitrate reductase in leaves. *Physiol Plant* 43: 306-312
11. HEWITT EJ 1962 Sand and water culture methods used in the study of plant nutrition. *Tech Commun Vol 22* Farham Roy Bucks Common Agric Bur
12. JAWORSKI EG 1971 Nitrate reductase assay in intact plant tissues. *Biochem Biophys Res Commun* 43: 1274-1279
13. KLEPPER B 1968 Diurnal pattern of water potential in woody plants. *Plant Physiol* 43: 1931-1934
14. MCNAMARA AL, GB MEEKER, PD SHAW, RH HAGEMAN 1971 Use of dissimilatory nitrate reductase from *Escherichia coli* and formate as a reductive system for nitrate assays. *Agric Food Chem* 19: 229-231
15. MERINO J, F GARCIA-NOVO, M SANCHEZ-DIAZ 1976 Annual fluctuation of water

- potential in the xerophytic shrub of the Doñana biological reserve (Spain). *Oecol Plant* 11: 1-11
16. MORILLA CA, JS BOYER, RH HAGEMAN 1973 Nitrate reductase and polyribosomal content of corn (*Zea mays L.*) having low leaf water potentials. *Plant Physiol* 51: 817-824
  17. PATE JS 1973a Physiology of the reaction of nodulated legumes to environment. In PS Nutman, ed. *Symbiotic Nitrogen Fixation in Plants*, Chap 27. Cambridge University Press, Cambridge
  18. PATE JS 1973b Uptake, assimilation and transport of nitrogen compounds by plants. *Soil Biol Biochem* 5: 109-119
  19. PAYNE WJ 1973 Reduction of nitrogenous oxides by microorganisms. *Bacteriol Rev* 37: 409-452
  20. RADIN JW 1975 Differential regulation of nitrate reductase induction in roots and shoots of cotton plants. *Plant Physiol* 55: 178-182
  21. RANDALL DD, WJ RUSSELL, DR JOHNSON 1978 Nodule nitrate reductase as a source of reduced nitrogen in soybean. *Glycine max.* *Physiol Plant* 44: 325-328
  22. RIGAUD J, EG BERGERSEN, GL TURNER, RM DANIEL 1973 Nitrate dependent anaerobic acetylene-reduction and nitrogen fixation by soybean bacteroids. *J Gen Microbiol* 77: 137-144
  23. SAHULKA J 1977 Influence of pretreatment with different cations on anaerobic nitrite production by excised *Pisum sativum* roots. *Biol Plant (Prague)* 19: 113-128
  24. SCHOLANDER PF, HT HAMMEL, ED BRADSTREET, E HEMMINGSEN 1965 Sap pressure in vascular plants. *Science* 148: 339-346
  25. SHANER DL, JS BOYER 1976a Nitrate reductase activity in maize (*Zea mays L.*) leaves. I. Regulation by nitrate flux. *Plant Physiol* 58: 499-504
  26. SHANER DL, JS BOYER 1976b Nitrate reductase activity in maize (*Zea mays L.*) leaves. II. Regulation by nitrate flux at low leaf water potential. *Plant Physiol* 58: 505-509
  27. SIK T, A KENDOROSI, I BARABUS, Z SRAB 1975 Nitrate reductase and effectiveness of *Rhizobium*. In WE Newton, CJ Nyman, eds. *Proceedings of the First International Symposium on Nitrogen Fixation*, Vol. 2. Washington State University Press, Pullman, Washington, pp 374-382
  28. WALLACE W 1974 Purification and properties of a nitrate reductase inactivating enzyme. *Biochim Biophys Acta* 341: 265-276
  29. WALLACE W 1975 A re-evaluation of nitrate reductase content of the maize root. *Plant Physiol* 55: 774-777