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Nitrate Reductase Activity in Maize (*Zea mays* L.) Leaves

II. REGULATION BY NITRATE FLUX AT LOW LEAF WATER POTENTIAL[1]

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ABSTRACT

Experiments were conducted to determine whether the nitrate flux to the leaves or the nitrate content of the leaves regulated the nitrate reductase activity (NRA) in leaves of intact maize (*Zea mays* L.) seedlings having low water potentials (Ψ_w) when other environmental and endogenous factors were constant. In seedlings that were desiccated slowly, the nitrate flux, leaf nitrate content, and NRA decreased as Ψ_w decreased. The decrease in nitrate flux was caused by a decrease in both the rate of transpiration and the rate of nitrate delivery to the transpiration stream. Upon rewatering, the recovery in NRA was correlated with the nitrate flux but not the leaf nitrate content.

Recovery depended on protein synthesis, since recovery could be prevented in excised leaves if an inhibitor of protein synthesis was present. However, it also depended on a high nitrate flux, since recovery could be prevented if there was no nitrate flux, despite a relatively high, constant leaf nitrate content, a high Ψ_w, and the absence of an inhibitor of protein synthesis.

The synthesis of NRA could be increased at low Ψ_w if seedlings were desiccated in the presence of additional nitrate, which increased the nitrate flux to the leaves. Since the decrease in NRA at low Ψ_w could be relieved by increasing the nitrate flux and recovery also depended on nitrate flux, the inhibition of NRA at low Ψ_w was not controlled by a direct effect of Ψ_w on protein synthesis nor by alterations in the leaf nitrate content, but rather by a decrease in the nitrate flux that in turn regulated the synthesis of the enzyme.

Nitrate reductase activity is sensitive to changes in the water status of plants and is inhibited when the water potential of plants declines (1, 9, 14). Morilla *et al.* (14) concluded that the inhibition was due to a decline in the rate of synthesis of the enzyme rather than an increased rate of degradation or a direct effect of water potential on enzyme activity. NRA[3] varied independently of leaf nitrate content. Since nitrate reductase is inducible by nitrate (2), tissue levels of nitrate appeared to be sufficient for induction of the enzyme. However, Morilla *et al.* (14) could not eliminate the possibility that low Ψ_w could have prevented the movement of nitrate to the induction site, whereas a return to high Ψ_w could have made nitrate available again for induction of NRA. The specific signal(s) regulating NRA at low Ψ_w were not identified.

In maize leaves there are two possible sources of nitrate that might be utilized for the induction of NRA. One is the nitrate within the leaves and the other is the nitrate moving to the leaves from the roots (18). In maize seedlings having adequate water, extractable NRA is regulated more by the nitrate flux from the roots to the shoot than by the nitrate content of the leaves (18). Therefore, the objective of this work was to explore more fully whether either of these forms of nitrate could result in the regulation of NRA at low Ψ_w.

MATERIALS AND METHODS

Plant Tissue, Growth Conditions. Maize plants (*Zea mays* L. Illinois Foundation FR43xFR14A) were grown for 8 to 14 days from seed in Vermiculite and were watered by subirrigation with a modified Hoagland solution as previously described (18). Desiccation was initiated by terminating subirrigation of the plants. Leaf Ψ_w was measured by the isopiestic technique with a thermocouple psychrometer, as previously described (4), in the same tissue used for determination of NRA and nitrate content.

Extraction and Assay of NRA and Leaf Nitrate Content. Samples (1 g) of leaf tissue taken from all the leaves of several plants were ground for 35 sec with a Polytron (Brinkman Instruments, N. Y.) in 6 ml of extraction medium (18). The homogenate was centrifuged at 27,000*g* for 15 min, and the supernatant was used to assay both NRA (17) and leaf nitrate content (13). NRA was expressed on the basis of dry weight. The assays were conducted between the 3rd and 9th hr of the photoperiod when the NRA remained constant within 10%, and endogenous factors regulating the activity of the enzyme were not operating or were constant. All experiments were repeated at least twice.

Collection of Xylem Sap. Previous work (18) demonstrated that the nitrate concentration of the first 10 μl of xylem sap expressed under pressure from a freshly detopped root system represented the nitrate concentration that was moving through the xylem prior to excision. Therefore, this method was used to collect xylem sap and the nitrate content of the sap was measured by the technique of McNamara *et al.* (13).

Transpiration Measurements. Rates of transpiration were measured by determining the rate of change in weight of pots containing the experimental plants. Corrections for surface evaporation were made by subtracting the rate of change in weight of identically treated containers without plants. Transpiration rates were varied in well watered plants by changing the relative humidity around the leaves as previously described (18).

RESULTS

When 8-day-old maize seedlings were desiccated, there was a steady decrease in NRA as Ψ_w decreased (Fig. 1, A and E). Upon rewatering the tissue with a nitrate-free medium, NRA recovered to 80% of the control level, and Ψ_w also recovered (Fig. 1, A and E). The leaf nitrate content decreased steadily and then remained constant, even when the plants were rewa-

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[3] Abbreviations: NRA: nitrate reductase activity; Ψ_w: water potential; DW: dry weight.

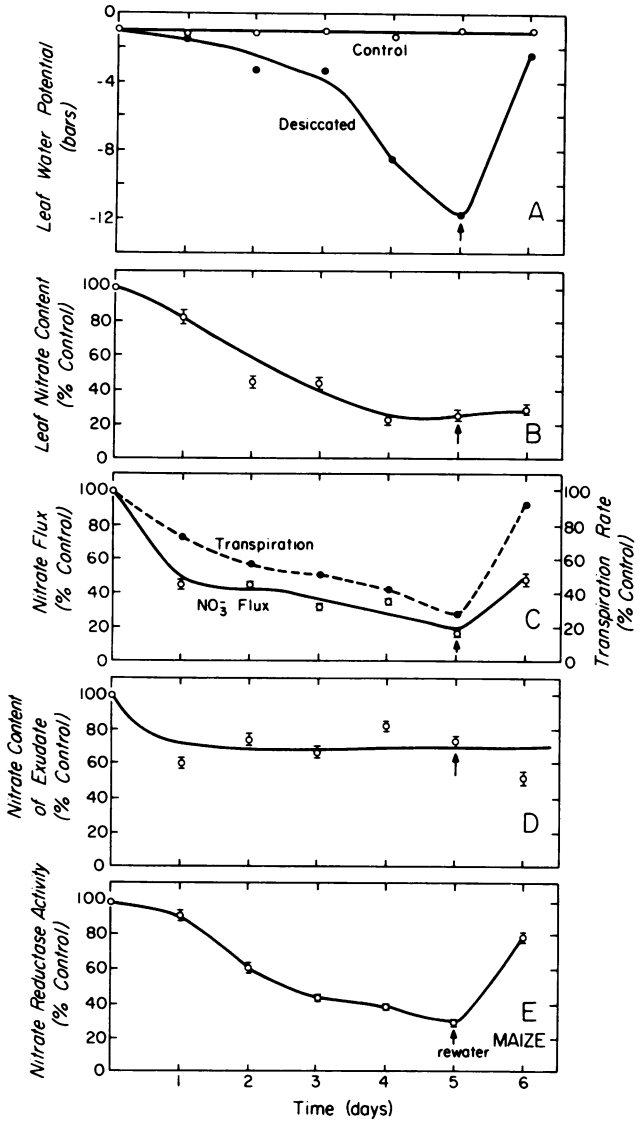

FIG. 1. Effects of desiccation on (A) leaf water potential, (B) leaf nitrate content, (C) transpiration rate and nitrate flux, (D) nitrate content of xylem exudate, and (E) NRA in 8- to 14-day-old maize. Nutrient solution was withheld on day zero, and arrow indicates when the plants were rewatered with a nitrate-free nutrient solution. Data for controls: B: leaf nitrate content, 350 ± 30 μmol·g DW^{-1}; C: transpiration rate, 8 ± 0.3 g·g DW^{-1}·hr^{-1}; D: nitrate content of the xylem exudate, 22.5 ± 2 μmol·ml^{-1}; E: NRA, 250 ± 10 μmol NO_2^-·g DW^{-1}·hr^{-1} for days 1 to 3 and 200 ± 10 μmol NO_2^-·g DW^{-1}·hr^{-1} on days 4 to 6. Bars around points indicate ± one standard deviation. Points represent measurement taken from individual pots containing seven plants each. Nitrate content of the exudate was determined from one plant in each pot.

tered (Fig. 1B). The nitrate content of the xylem exudate also decreased and remained low (Fig. 1D). Transpiration decreased at low Ψ_w but increased upon rewatering (Fig. 1C). The nitrate flux to the leaves, computed from the product of the transpiration rate and the nitrate content of the xylem sap, decreased at low Ψ_w (Fig. 1C), but recovered partially upon rewatering (Fig. 1C). This probably occurred because of residual nitrate in the system in spite of the lack of nitrate in the rewatering solution. Thus, NRA was correlated with nitrate flux but not with leaf nitrate content during recovery.

The decreased rate of water movement through the plant at low Ψ_w may have affected the roots directly so that they were unable to accumulate nitrate as effectively as when transpiration was rapid. This possibility was tested by varying the rate of transpiration in intact, but undesiccated, plants. Unlike the desiccated system, there was no decline in NRA or nitrate flux as

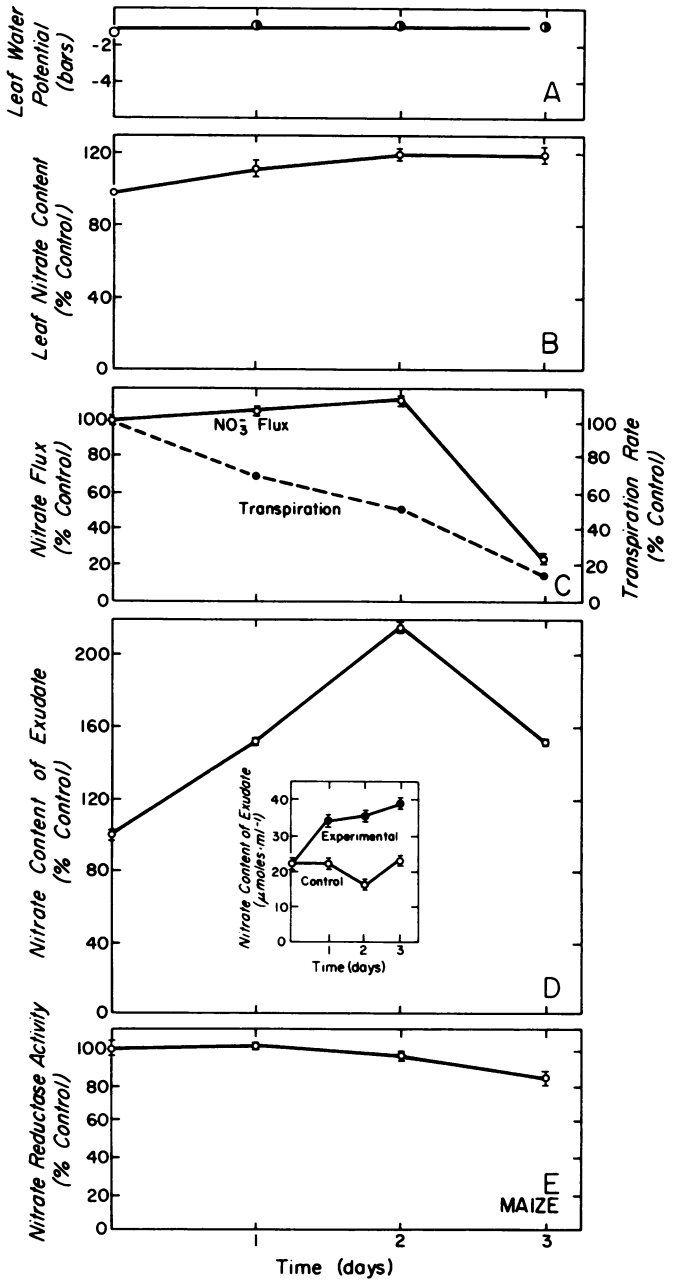

FIG. 2. Effects of varying the relative humidity around the leaves on (A) leaf water potential, (B) leaf nitrate content, (C) transpiration rate and nitrate flux, (D) nitrate content of the xylem exudate, and (E) NRA in 8- to 12-day-old maize. The inset in D gives the changes that occurred in the absolute levels of nitrate in the xylem sap of the plants throughout the experiment. To decrease transpiration, the entire group of plants was subjected to a higher humidity on each day as the experiment progressed. Points represent measurements taken from individual pots containing seven plants each. Nitrate content of the exudate was determined from one plant in each pot. Data for controls: B: leaf nitrate content, 400 ± 40 μmol·g DW^{-1}; C: transpiration rate, 7 ± 0.5 g·g DW^{-1}·hr^{-1}; D: nitrate content of the xylem sap shown in inset; E: NRA, 250 ± 10 μmol NO_2^-·g DW^{-1}·hr^{-1} until day 2 and 225 ± 2 μmol NO_2^-·g DW^{-1}·hr^{-1} on day 3. Bars around points indicate ± 1 SD.

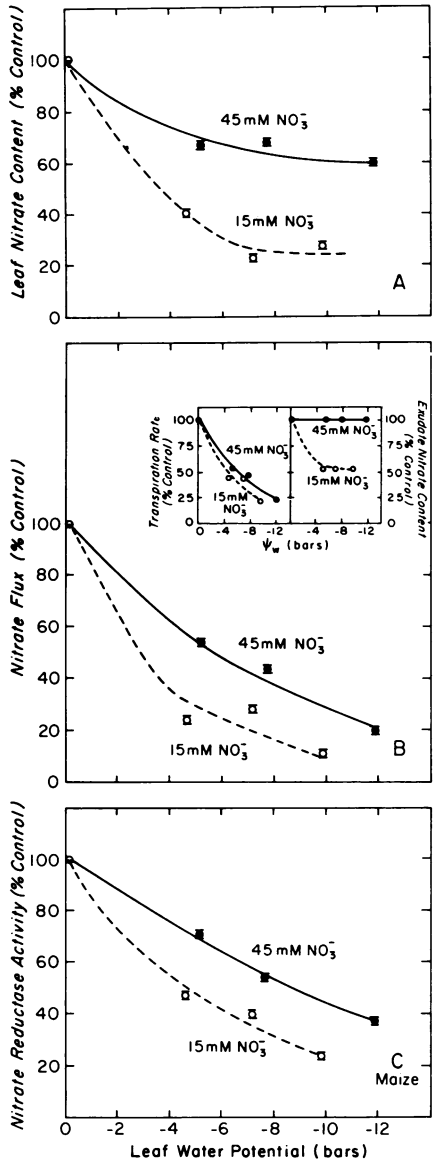

FIG. 3. Effects of decreasing water potentials on (A) leaf nitrate content, (B) nitrate flux, and (C) NRA in maize seedlings with different concentrations of nitrate in the rooting medium. Nutrient solution was withheld on day 8 after planting, and desiccation occurred for 4 days. Nutrient solution containing 15 mM nitrate was supplied to all the plants prior to desiccation, except that nutrient solution containing 45 mM nitrate (25 mM KNO_3 and 10 mM $Ca(NO_3)_2$) was supplied to half of the plants on the last day prior to desiccation. Each point was determined from individual pots containing 10 plants each, except that the nitrate content of the xylem exudate was determined from one plant in each pot. Data for controls: A: leaf nitrate content, 350 ± 30 μmol·g DW^{-1}; B: nitrate flux, 150 ± 20 μmol·g $DW^{-1}\cdot hr^{-1}$; B: inset, transpiration rate, 8 ± 0.3 g·g $DW^{-1}\cdot hr^{-1}$; nitrate content of the xylem sap, 20 ± 2 μmol·ml^{-1}; C: NRA, 210 ± 15 μmol $NO_2^-\cdot g\ DW^{-1}\cdot hr^{-1}$. Bars around points indicate ± 1 SD.

transpiration decreased, except at very low rates of transpiration (Fig. 2). The nitrate flux remained at the control level due to an increase in the nitrate concentration of the xylem sap as transpiration decreased. At very low transpiration rates, the nitrate flux began to decrease (Fig. 2C) and NRA decreased somewhat. The results show that the decreased movement of water through the plant did not directly affect the ability of the roots to deliver nitrate to the shoots, except at very low rates of water movement.

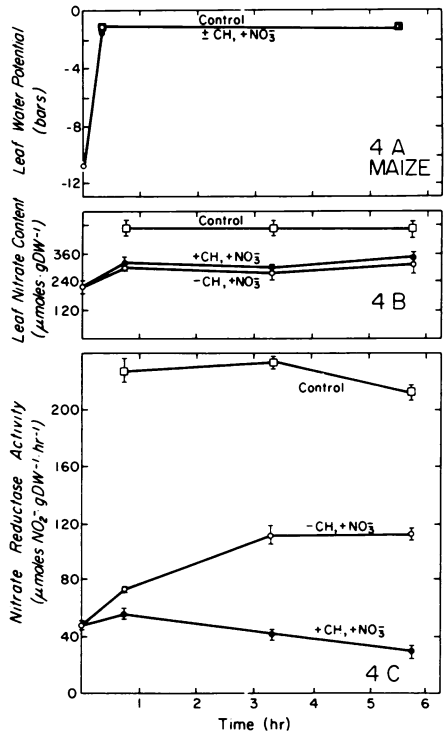

FIG. 4. Effects of cycloheximide upon the recovery of (A) leaf water potential, (B) leaf nitrate content, and (C) NRA in rapidly rehydrated leaves from 14-day-old plants that had been unwatered for 6 days. Leaves were rehydrated by excision under degassed water containing buffer, nitrate, and, for half the leaves, cycloheximide (50 $\mu g\cdot ml^{-1}$). Controls consisted of excised 14-day-old leaves that had been well watered throughout. The time axis indicates the time after excision. Bars around points indicate ± 1 SD.

To determine whether NRA was limited by the availability of nitrate for induction of the enzyme at low Ψ_w, the nitrate flux was increased at low Ψ_w by supplying the seedlings with 3-fold (45 mM) the usual nitrate (15 mM) on the day prior to the onset of desiccation. The nitrate flux at low Ψ_w was approximately doubled by this treatment (Fig. 3B). This difference was not caused by altered rates of transpiration, but rather by changes in concentration of nitrate in the xylem sap (Fig. 3B, inset). Leaf nitrate content at low Ψ_w was higher in the seedlings with supplemental nitrate than in those without the supplement (Fig. 3A). NRA also was higher in the seedlings with supplemental nitrate (Fig. 3C). Therefore NRA responded to nitrate even though Ψ_w was low.

To distinguish whether NRA at low Ψ_w was controlled by the nitrate flux or by direct effects of low Ψ_w on protein synthesis, leaves having low Ψ_w were excised to remove sources of nitrate in the lower portions of the plant, and the excised leaves were rehydrated in the presence or absence of exogenous nitrate. NRA increased considerably when exogenous nitrate was present (Fig. 4C), although there was little increase in leaf nitrate content (Fig. 4B). If the protein synthesis inhibitor, cycloheximide, was present, NRA did not increase (Fig. 4C). If nitrate was withheld from the leaves during recovery, NRA also did not increase, although no inhibitor of protein synthesis had been supplied and Ψ_w recovered quickly to the control level (Fig. 5). The leaf nitrate content was approximately the same as in plants rewatered in the presence of nitrate (Fig. 5).

DISCUSSION

The results show that NRA at low Ψ_w was controlled by the nitrate flux which in turn regulated the rate of synthesis of the

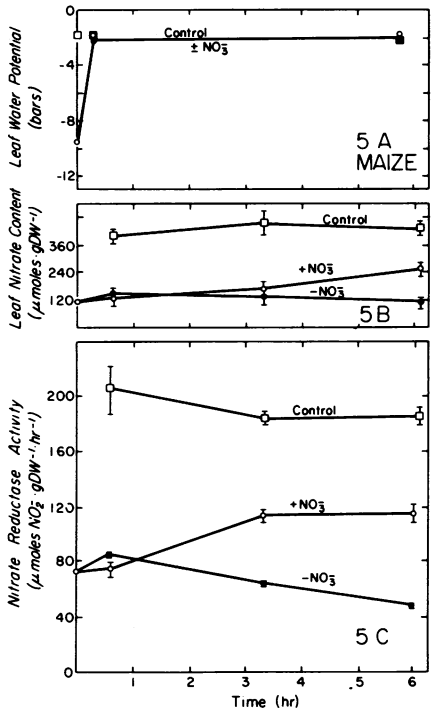

FIG. 5. Effects of the presence or absence of nitrate upon the recovery of (A) leaf water potential, (B) leaf nitrate content, and (C) NRA in rapidly rehydrated leaves from 14-day-old plants that had been unwatered for 6 days. Leaves were rehydrated by excision under degassed water containing buffer and, for half the leaves, nitrate (12.5 mM). Controls consisted of excised 14-day-old leaves that were well watered throughout. The time axis indicates hours after excision. Bars around points indicate ± 1 SD.

enzyme. There is considerable evidence that rates of protein synthesis decrease as Ψ_w decreases and increase as Ψ_w increases (8). Also, NRA has been shown to decrease at low Ψ_w because of decreased rates of synthesis of the enzyme and recover because of increased synthesis (14). Decreased NRA at low Ψ_w was not caused by altered rates of enzyme degradation or direct effects of low Ψ_w on the enzyme (14).

Nevertheless, it has been unclear whether these changes in protein synthesis and NRA in particular result from a direct effect of low Ψ_w on protein synthesis or whether the changes arise indirectly from alterations in the signals that in turn control the synthesis of specific proteins at low Ψ_w. Thus, in our experiments, low Ψ_w may have acted directly on protein synthesis or an associated process, with decreased NRA as a consequence. Alternatively, low Ψ_w may have altered nitrate availability that in turn could have controlled the synthesis of NRA, since NRA is known to be induced by nitrate (2).

Two experiments showed that the second alternative was operating in desiccated maize. First, NRA was higher at low Ψ_w if nitrate availability increased (Fig. 3). If low Ψ_w had regulated NRA by acting directly on protein synthesis, an improvement in the availability of nitrate should not have affected the rate of synthesis of NRA, since Ψ_w was not changed. Second, the recovery of NRA was prevented in rehydrated maize leaves by depriving the tissue of an external source of nitrate (Fig. 5). If low Ψ_w had regulated NRA by directly inhibiting protein synthesis, rehydration of the tissue should have released the inhibition and increased NRA should have been observed. The lack of recovery of NRA in the absence of a nitrate flux indicates that synthesis of the enzyme remained low. Indeed, NRA decreased during recovery of Ψ_w in the absence of a nitrate flux. This lack of enzyme synthesis was confirmed with the inhibitor of protein synthesis, cycloheximide, which caused an effect on NRA virtually identical with that caused by the absence of a nitrate flux (*cf.* Figs. 4 and 5).

The source of nitrate that controlled NRA was the nitrate moving to the leaves from the roots rather than the nitrate content of the leaves. In intact plants, NRA recovered upon rewatering even though leaf nitrate content remained constant (Fig. 1). The nitrate flux increased during recovery, however. On the other hand, when the nitrate flux was kept low during rehydration, NRA did not recover despite a constant nitrate content of the leaves (Fig. 5).

The primary cause of the decreased nitrate flux at low Ψ_w was the decrease in the ability of the roots to supply nitrate to the transpiration stream. When the decreased nitrate transport by the roots was coupled with the decreased transpiration at low Ψ_w, the combined effects on the nitrate flux were large (Fig. 1). When Ψ_w is low, root transport of ions to the xylem may be affected both by soil-ion availability (20) and by the ability of roots to absorb and transport ions to the shoot (6, 15, 16). However, since a higher nitrate flux occurred when supplemental nitrate was present around the roots at low Ψ_w (Fig. 3), the roots must have been capable of transporting additional nitrate. Thus, soil-ion availability may have been more limiting than root activity for nitrate transport at low Ψ_w in our experiments.

It might be argued that changes in NRA at low Ψ_w reflected over-all changes in the nitrogen nutrition of the plants that resulted in altered rates of protein synthesis. However, this interpretation is not consistent with the results of the recovery experiments (Figs. 4 and 5). The recovery experiments showed that NRA increased within 3 hr even though the nitrate content of the leaves was virtually unchanged. Therefore, NRA was immediately responsive to the increased nitrate flux, but the over-all nitrogen status of the leaves remained essentially the same.

The inhibition of transpiration at low Ψ_w, when taken alone, could not account for the decreased nitrate flux to the leaves, since nitrate delivery to the xylem was almost constant for a wide range of transpiration rates if water was adequately supplied to the roots (Fig. 2). Although the nitrate flux was generally independent of the rate of transpiration (3, 5, 12), nitrate delivery decreased when transpiration was very low (Fig. 2). This phenomenon has been noted by others (7, 10, 12), but it is not clear why it occurs. Equally unclear is the reason NRA decreased only slightly under these conditions (Fig. 2). We assume that nitrate also may have moved to the shoot by means other than the transpiration stream and, therefore, would have been undetected by our measurement of nitrate flux. This hypothesis is supported by the large loss in NRA that we previously observed (18) when all forms of nitrate movement to the leaves were prevented by withholding nitrate from the roots of plants having adequate water.

Since NRA is controlled by a number of factors other than nitrate flux, including both environmental factors and endogenous regulators (2), it was important to conduct the experiments under conditions that were not confounded by these phenomena. In the present work, care was taken to assay NRA in plants of the same age in light, under constant environmental conditions (except for water and nitrate availability), and during a time when NRA was nearly constant. Thus, environmental and endogenous factors should not have affected the results.

The conclusion that NRA is regulated by nitrate flux at low Ψ_w does not imply that direct effects of Ψ_w on protein synthesis are entirely absent. We cannot exclude the possibility that the synthesis of enzymes other than nitrate reductase might be affected by some type of direct alteration of protein synthesis by low Ψ_w. However, such a direct effect was not large enough to control NRA in our experiments.

It has been proposed that low water availability to plants may

cause enzyme activities to change because of changes in the free energy of water within the cells (11, 19), changes in spatial relationships of membrane systems, volume changes, and concentration effects resulting from losses of water, or decreases in the water of hydration surrounding macromolecules (8). None of these phenomena appear to apply to nitrate reductase. NRA responded to the flux of the inducer of the enzyme at low Ψ_w, which in turn was controlled by root uptake and transpiration effects. Although NRA is the first enzyme for which the desiccation-altered signal has been identified, the uniqueness of the signal for NRA makes it unlikely that the enzyme complement of a cell responds solely to a general signal produced at low Ψ_w. Rather, the regulatory mechanisms operating at low Ψ_w may be specific for each enzyme and varied in character.

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LITERATURE CITED

1. Bardzik, J. M., H. V. Marsh, Jr., and J. R. Havis. 1971. Effects of water stress on the activities of three enzymes in maize seedlings. Plant Physiol. 47: 828-831.
2. Beevers, L. and R. H. Hageman. 1969. Nitrate reduction in higher plants. Annu. Rev. Plant Physiol. 20: 495-522.
3. Bowling, D. J. F. 1968. Active and passive ion transport in relation to transpiration in *Helianthus annuus*. Planta 83: 53-59.
4. Boyer, J. S. 1971. Nonstomatal inhibition of photosynthesis in sunflower at low leaf water potentials and high light intensities. Plant Physiol. 48: 532-537.
5. Emmert, F. H. 1972. Effect of time, water flow, and pH on centripetal passage of radiophosphorus across roots of intact plants. Plant Physiol. 50: 332-335.
6. Greenway, H., P. G. Hughes, and B. Klepper. 1969. Effects of water deficit on phosphorus nutrition of tomato plants. Physiol. Plant. 22: 199-207.
7. Hooymans, J. J. M. 1969. The influence of the transpiration rate on uptake and transport of potassium in barley plants. Planta 88: 369-371.
8. Hsiao, T. C. 1973. Plant responses to water stress. Annu. Rev. Plant Physiol. 24: 519-570.
9. Huffaker, R. C., T. Radin, G. E. Kleinkopf, and E. L. Cox. 1970. Effects of mild water stress on enzymes of nitrate assimilation and of the carboxylative phase of photosynthesis in barley. Crop Sci. 10: 471-473.
10. Jensen, G. 1962. Relationship between water and nitrate uptake in excised tomato root systems. Physiol. Plant. 15: 791-803.
11. Kramer, P. J. 1969. Plant and Soil Water Relationships: A Modern Synthesis. McGraw-Hill Book Company, New York.
12. Lopushinsky, W. 1964. Effect of water movement on ion movement into the xylem of tomato roots. Plant Physiol. 39: 494-501.
13. McNamara, A. L., G. B. Meeker, P. D. Shaw, and R. H. Hageman. 1971. Use of dissimilatory nitrate reductase from *Escherichia coli* and formate as a reductive system for nitrate assays. Agric. Food Chem. 19: 229-321.
14. Morilla, C. A., J. S. Boyer, and R. H. Hageman. 1973. Nitrate reductase activity and polyribosomal content of corn (*Zea mays* L.) having low leaf water potentials. Plant Physiol. 51: 817-824.
15. Parrondo, R. T., R. C. Smith, and K. Lazurik. 1975. Rubidium absorption by corn root tissue after a brief period of water stress and during recovery. Physiol. Plant. 35: 34-38.
16. Pitman, M. G., U. Lüttge, A. Laüchli, and E. Ball. 1974. Effect of previous water stress on ion uptake and transport in barley seedlings. Aust. J. Plant Physiol. 1: 377-385.
17. Scholl, R. L., J. E. Harper, and R. H. Hageman. 1974. Improvements of the nitrite color development in assays of nitrate reductase by phenazine methosulfate and zinc acetate. Plant Physiol. 53: 825-828.
18. Shaner, D. L. and J. S. Boyer. 1976. Nitrate reductase activity in maize (*Zea mays* L.) leaves. I. Regulation by nitrate flux. Plant Physiol. 58: 499-504.
19. Slatyer, R. O. 1967. Plant Water Relationships. Academic Press, New York.
20. Viets, F. G., Jr. 1972. Water deficits and nutrient availability. *In*: T. T. Kozlowski, ed., Water Deficits and Plant Growth. Academic Press, New York. pp. 217-239.