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

I. REGULATION BY NITRATE FLUX[1]

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ABSTRACT

The roles that leaf nitrate content and nitrate flux play in regulating the levels of nitrate reductase activity (NRA) were investigated in 8- to 14-day old maize (*Zea mays* L.) plants containing high nitrate levels while other environmental and endogenous factors were constant. The nitrate flux of intact plants was measured from the product of the transpiration rate and the concentration of nitrate in the xylem. NRA decreased when the seedlings were deprived of nitrate. The nitrate flux and the leaf nitrate content also decreased. When nitrate was resupplied to the roots, all three parameters increased.

Attempts to alter the nitrate flux by varying transpiration rates were unsuccessful due to a relatively constant rate of delivery of nitrate to the xylem as transpiration rates fell. However, cooling the roots resulted in a decrease in the nitrate flux. Plants with a lower nitrate flux rapidly lost NRA, although the leaf nitrate content was initially unaffected. If the roots remained cool for a long enough time, the leaf nitrate content eventually decreased. Rewarming the roots increased the nitrate flux, leaf nitrate content, and NRA to control levels. When the nitrate flux in excised shoots was varied in three separate ways, decreasing the nitrate flux to the leaves resulted in a rapid decrease in NRA, although leaf nitrate contents were unchanged.

These experiments show that the nitrate flux to the leaves from the roots plays a much larger regulatory role than the leaf nitrate content in controlling the level of NRA in intact plants.

Nitrate reductase, the first in a series of enzymes that reduces nitrate to ammonia, is sensitive to a number of environmental factors. Activity varies under the influence of light intensity, CO_2 levels, temperature, water availability, and nitrate supply (3). When other environmental factors remain constant, NRA[3] appears to be inducible by nitrate (3, 11).

In tobacco cells, induction is more closely related to the nitrate that is entering the cells than to the nitrate that is stored in the cells (8). Similarly, in intact plants, two sources of nitrate could play regulatory roles for nitrate reductase: one is the nitrate stored in the leaves, and the other is the nitrate coming to the leaves from the roots. No work distinguishes between these possibilities in intact plants, although Meeker *et al.* (13) showed that NRA was more closely correlated with the nitrate in the midrib of maize leaves than with nitrate in the lamina.

Although metabolic factors other than nitrate supply can influence NRA (3), it should be possible to measure the effects of varying nitrate during a portion of the day when NRA would otherwise be constant. Our experiments made use of this concept and were undertaken to determine the regulatory roles for NRA that might be played by the nitrate stored in the leaves and the nitrate flux to the leaves of intact plants.

MATERIALS AND METHODS

Maize plants (*Zea mays* L. Illinois Foundation FR43xFR14A) were grown for 8 to 14 days from seed in Vermiculite in a constant environment chamber (temperature was 30/21 ± 1 C day/night; relative humidity was 39/80 ± 3% day/night; irradiance was 0.2 cal cm^{-2} min^{-1}; photoperiod was 16 hr). Depending on the experiment, 36 seeds were planted 2.5 cm below the surface of 140 g of Vermiculite in a plastic container (11 × 11 × 16 cm), or 12 seeds were planted in 40 g of Vermiculite in a cylindrical aluminum container (6 × 12 cm). The plants were subirrigated daily by immersion in a larger container of modified Hoagland nutrient solution (9). Nitrate depletion was achieved by substituting KCl for KNO_3 and $CaCl_2$ for $Ca(NO_3)_2$ in the nutrient solution (9).

In certain experiments, root systems were rapidly (2 min) cooled while shoots remained at growth temperatures. Hoagland solution (8 C) was poured through the Vermiculite until the desired root temperature was reached. The cold-treated pots were then placed into copper coils through which water (3 C) was circulated. Temperature was regulated at 13.5 ± 0.5 C in the root medium by controlling the flow of chilled water using a thermoregulator placed in one of the pots. Control roots were kept at 29 ± 0.5 C throughout the day, but they cooled to 21 ± 0.5 C at night as the growth chamber temperature changed.

When experiments were done with excised shoots, the plants were placed in darkness for 20 min prior to excision. Whole plants were pulled out of the Vermiculite and cut twice between the roots and the blade of the first leaf under freshly degassed water. The shoots were then placed in appropriate solutions under growth conditions.

For uptake experiments with excised shoots, all solutions contained 5 mM K-phosphate at pH 6 and various concentrations of KNO_3. When ABA was used, it was obtained from a fresh stock solution of 1 mM ABA prepared by adding 96 ml of H_2O to 25 mg of ABA dissolved in 1 ml of absolute ethanol. The pH was adjusted to 6, and the solution was stored in darkness at 4 C.

Leaf water potential (Ψ_w) was measured by thermocouple psychrometry using the isopiestic technique as described by Boyer (5).

Extraction and Assay of NRA and Leaf Nitrate Content. Tissue samples (1 g) from all the leaves of several plants were ground for 35 sec with a Polytron (Brinkman Instruments, New York) in 6 ml of extraction medium (15). The homogenate was centrifuged at 27,000*g* for 15 min, and the supernatant was used to assay both NRA (20) and leaf nitrate content (12). All assays

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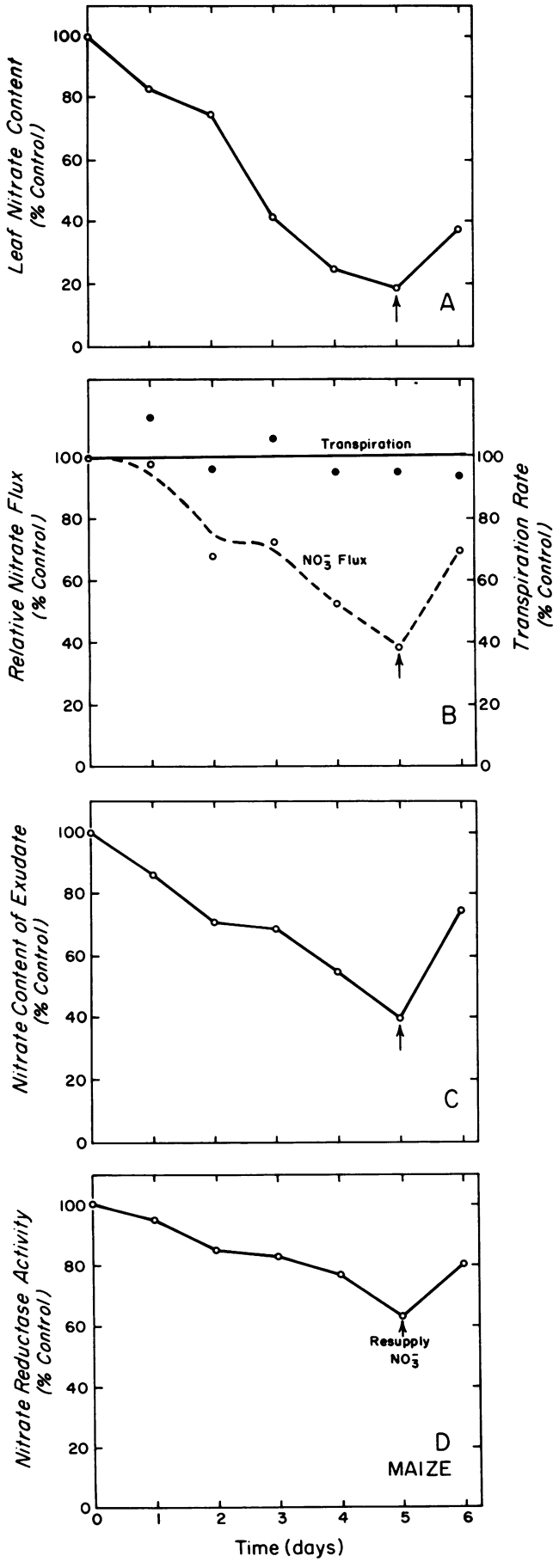
[3] Abbreviations: NRA: nitrate reductase activity; Ψ_w: water potential; DW: dry weight.



were conducted between the 3rd and the 9th hr of the photoperiod, when NRA did not vary by more than 10% in intact plants. The measurements were done in duplicate, and all experiments were repeated at least twice.

Measurement of Nitrate Content of Xylem Sap. Plants were detopped just below the first leaf blade, and exudate was collected either by permitting free exudation or by forcing exudation under pressure. In the nitrate-depletion experiment, the stump was allowed to exude freely for 10 min after the cut surface had been blotted with damp tissue, dried, and enclosed in an inverted test tube lined with wet filter paper. Xylem exudate (total of 50 μl) was collected from six different shoots.

In all other experiments, the procedure was modified by using a pressure chamber (19) to apply pressure around the undisturbed pot-Vermiculite-root system to provide rapid collection of exudate. The cut end of the stump extended through the top of the chamber and was blotted dry to remove any contaminating material. After sealing the stump into the chamber, pressure was applied and the first 10 μl of the resulting exudate were collected within 4 min after excision. Exudate nitrate content was measured according to McNamara *et al.* (12).

Transpiration Measurements. Transpiration rates were measured by determining the change in weight of containers with plants over a certain period of time. Surface evaporation was corrected by subtracting the change in weight occurring in identically treated containers without plants. When transpiration rates were varied, the plants were placed either in a Plexiglas, humid chamber (33 C air temperature; 100% relative humidity), a partially open, humid chamber (30 C air temperature; 80% RH), in the open growth chamber (30 C air temperature; 39% RH), or in the open chamber with a fan blowing over the leaves. Leaf temperatures were within 1 C of air temperatures.

RESULTS

When plants were watered with a nitrate-free nutrient solution, NRA decreased steadily as the Vermiculite was depleted of nitrate (Fig. 1D). At the same time, leaf nitrate content (Fig. 1A) and the nitrate content of the exudate declined (Fig. 1C). The transpiration rate remained constant (Fig. 1B). The relative nitrate flux to the leaves was computed from the product of the transpiration rate and the nitrate content of the xylem sap after free exudation, and it paralleled the changes occurring in NRA (Fig. 1B). Upon resupplying the plants with nitrate, NRA recovered to 80% of the control. Leaf nitrate content and the nitrate content of the exudate also recovered partially. These data show that NRA was responsive to the nitrate levels in the plants, but do not distinguish whether the leaf nitrate content or the nitrate flux to the leaf regulated the level of NRA, since both were correlated with changes in NRA.

In order to test whether one source of nitrate was more important than the other, it was necessary to vary one relative to the other. This necessitated a more accurate means of measuring the nitrate flux to the leaves of the intact plants, since the free exudation utilized in the first experiment (Fig. 1) might not have provided xylem sap with a nitrate content that was the same as in

FIG. 1. Effects of nitrate depletion on (A) leaf nitrate content, (B) transpiration rate and relative nitrate flux, (C) nitrate content of xylem exudate, and (D) nitrate reductase activity in 8- to 14-day-old maize. Data were taken from plants in the same pots, either control or nitrate-depleted, throughout the experiment. Arrow indicates when pots were watered with a nitrate-containing nutrient solution. Data for controls: A: leaf nitrate content, 350 ± 50 μmol $NO_3^- \cdot g\ DW^{-1}$; B: transpiration rate, 6.6 ± 0.3 $g \cdot g\ DW^{-1} \cdot hr^{-1}$ for the first 6 days and 8.7 $g \cdot g\ DW^{-1} \cdot hr^{-1}$ on day 7; C: nitrate content of xylem exudate, 16 ± 4 $\mu mol \cdot ml^{-1}$; D: nitrate reductase activity, 225 ± 25 μmol $NO_2^- \cdot g\ DW \cdot hr^{-1}$. Standard deviations for this experiment were smaller than size of the data points.

the intact plant. The problem arises because the rate of water flow through the exuding root might have been different from the rate before excision. We tested whether the nitrate content of the xylem exudate was affected by the rate of water flow by forcing exudation at two different pressures around the roots. When successive aliquots of xylem sap were collected from root-Vermiculite-pot systems, the concentration of nitrate was always constant for the first 10 μl but changed when exudation was allowed to proceed further, depending on the rate of flow (Fig. 2). At high flow rates, the final concentration ultimately became lower than in the first 10 μl (Fig. 2). Conversely, at low flow rates, final concentrations became higher. Presumably, the final concentration of nitrate was reached after the original sap had been flushed from the xylem and a new steady state was established. Since the nitrate content of the xylem sap remained constant for the first 10 μl in all plants and was independent of the flow rate (Fig. 2), the initial 10 μl must have represented the sap in the xylem before the shoots had been excised. Therefore, by collecting only the first 10 μl, it was possible to measure the nitrate concentration of the xylem sap of the intact plant without having to reproduce the flow rate of the intact system after excision of the top.

To test whether this method could detect differences in xylem sap concentrations when the transpiration of intact plants varied, only the first 10 μl of xylem sap were collected under pressure from freshly detopped plants. As the transpiration rate increased, the nitrate content/unit volume of xylem sap decreased (Fig. 3). Thus, the technique for collecting the initial 10 μl of xylem sap permitted nitrate flux to be calculated more accurately for intact plants.

The importance of nitrate flux and leaf nitrate content for regulating NRA was initially investigated by using this new method of collecting exudate and altering transpiration. The

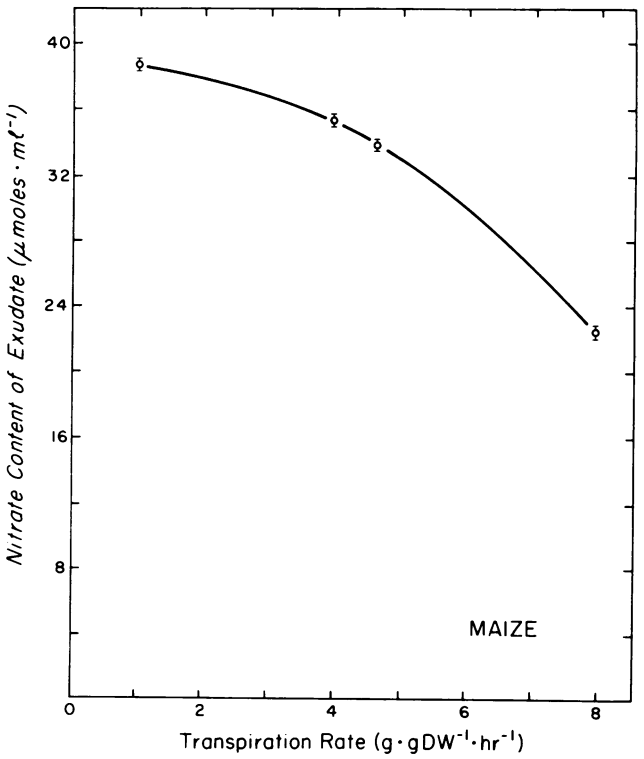


FIG. 3. Relationship between transpiration rates and nitrate content of xylem sap in 11-day-old maize. Nitrate values determined from first 10 μl of xylem sap expressed from freshly detopped roots of plants that had been transpiring at the given rates prior to excision. Measurements were taken within 4 min after excision. Bars around points indicate ± 1 SD.

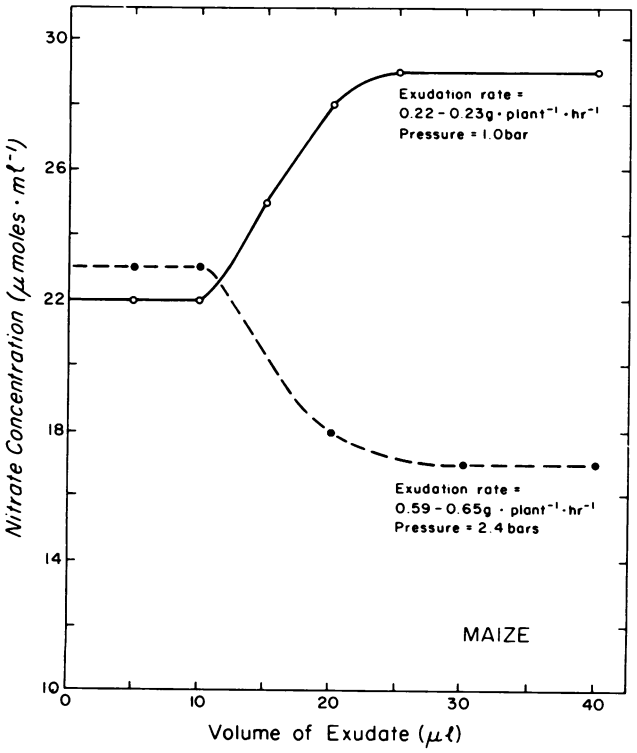


FIG. 2. Relationship between nitrate content of xylem sap and exudation volume from detopped, 11-day-old maize roots exuding at two different rates. Data are from two separate plants. Exudation rates were obtained by applying pressure around a pot-Vermiculite-root system sealed in a pressure chamber. Points indicate the nitrate level in 5-μl aliquots.

nitrate concentration of the xylem sap rose as transpiration rates decreased (Fig. 3). Therefore, nitrate flux and leaf nitrate content remained relatively constant. On the other hand, when roots were cooled without affecting the shoot temperature (as shown by thermocouples inserted in the leaf blades), the flux of nitrate through the intact plants decreased rapidly but leaf nitrate content did not (Fig. 4A). The reduced flux was caused by both a decreased flow of water through the plant and a lower concentration of nitrate/unit volume of xylem sap (Fig. 4B). The NRA in the leaves also decreased within 5 hr after cooling the roots (Fig. 4D). Leaf Ψ_w decreased slightly (Fig. 4 legend), presumably because the resistance to water flow through the cooled roots was higher, but at most this difference could account for only a 5% difference in NRA (14). The leaf nitrate content eventually decreased after 24 hr. Upon rewarming the roots, NRA, nitrate flux, and leaf nitrate content returned to control levels (Fig. 4). These data show that NRA and nitrate flux responded similarly.

The short time of response of leaf NRA to changes in the nitrate flux suggested that it would be possible to vary the nitrate flux in excised shoots and observe changes in NRA. This approach also avoided the complicating behavior of the root system. Therefore, several experiments were conducted to vary nitrate flux in excised shoots by (*a*) varying transpiration by changing the relative humidity around the shoots (Fig. 5), (*b*) varying transpiration by using 10 μM ABA in the uptake solution as an antitranspirant and changing the relative humidity (Fig. 6), and (*c*) keeping transpiration at a high constant rate and varying KNO_3 in the uptake solution (Fig. 7). The effect of the different treatments was determined by measuring NRA after 5 hr under the treatment conditions. Previous work (4, 14) showed that NRA in maize has a half-life of 4 to 4.5 hr. Thus, any perturbation in the rates of synthesis or degradation of NRA

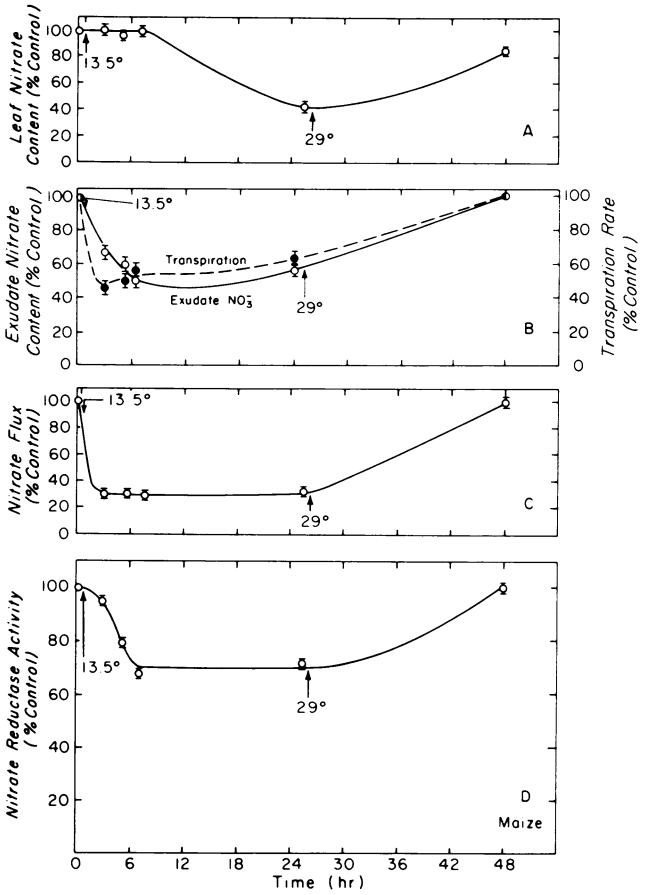


FIG. 4. Effects of cooling and rewarming the roots of intact 11-day-old maize on (A) leaf nitrate content, (B) transpiration rate and nitrate content of xylem exudate, (C) nitrate flux, and (D) nitrate reductase activity. Points represent determinations from separate pots each containing 10 plants. One plant was used from each pot to determine the nitrate content of the xylem sap. Arrows indicate when the temperature of the roots was changed. Leaf temperatures were the same in both treatments as measured by thermocouples inserted into the leaves. Leaf Ψ_w for the controls was -1.2 ± 0.2 bars and for the plants with the cooled roots was -1.6 ± 0.2 bars. Data for controls: A: leaf nitrate content, 380 ± 40 μmol·g DW^{-1}; B: transpiration rate, 5 ± 0.2 g·g DW^{-1}, nitrate content of xylem sap, 24 ± 4 μmol·ml^{-1}; C: nitrate flux, 115 ± 15 μmol·g $DW^{-1}\cdot hr^{-1}$; D: nitrate reductase activity, 160 ± 20 μmol NO_2^-·g $DW^{-1}\cdot hr^{-1}$. Bars around the points indicate ± 1 SD.

should have been evident within 5 hr. Regardless of the way the flux of nitrate was varied, the maintenance of high NRA for 5 hr required a high nitrate flux (Figs. 5-7). There was no relationship between the leaf nitrate content and NRA. Leaf nitrate remained high and virtually constant for the three experiments, despite large differences in nitrate flux.

DISCUSSION

Many investigators have shown that nitrate reductase is induced by nitrate (3), although nitrite may also be involved (11). Since the only source of nitrite is nitrate in intact plants, nitrate is likely to play an important role in regulating the levels of NRA. The nitrate content of plant tissue is determined by the rate at which nitrate enters the tissue and the rate at which nitrate is lost by the tissue. In leaves, there is no substantial loss of nitrate other than that which is reduced by nitrate reductase. Therefore, only two sources of nitrate can act in a regulatory role for the enzyme: (*a*) the nitrate that is stored in the leaves, and (*b*) the

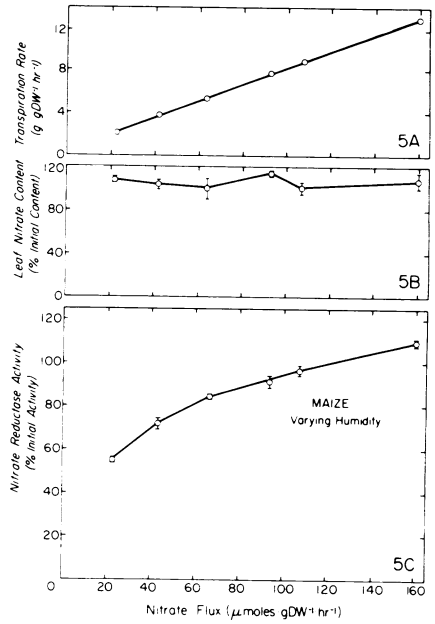


FIG. 5. Effects of nitrate flux on maintenance of nitrate reductase activity and leaf nitrate content of excised 11-day-old maize roots having different transpiration rates. A: transpiration rate: varied by changing the relative humidity around the leaves as described under "Materials and Methods." B: leaf nitrate content: initial content, 366 ± 18 μmol·g DW^{-1}. C: nitrate reductase activity: initial activity, 212 ± 5 μmol NO_2^-·g $DW^{-1}\cdot hr^{-1}$. Nitrate reductase activity was determined initially and 5 hr after excision. All uptake solutions contained 12.5 mM KNO_3. Bars around points indicate ± 1 SD.

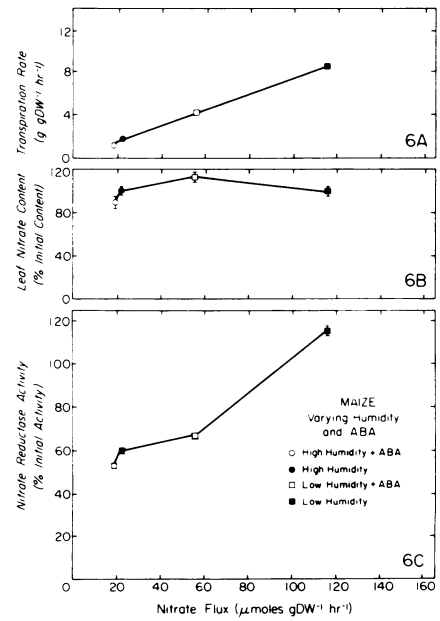


FIG. 6. Effects of nitrate flux on maintenance of nitrate reductase activity and leaf nitrate content in excised 11-day-old maize shoots with different transpiration rates. A: transpiration rate. B: leaf nitrate content: initial content, 362 ± 35 μmol·g DW^{-1}. C: nitrate reductase activity; initial activity, 196 ± 10 μmol NO_2^-·g $DW^{-1}\cdot hr^{-1}$. Nitrate reductase activity was determined initially and 5 hr after the shoots were excised. All uptake solutions contained 12.5 mM KNO_3. Plants treated with ABA were fed 10 μM ABA in the uptake solution. Low humidity was 39% relative humidity at 30 C. High humidity was close to 100% at 33 C. Bars around points indicate ± 1 SD.

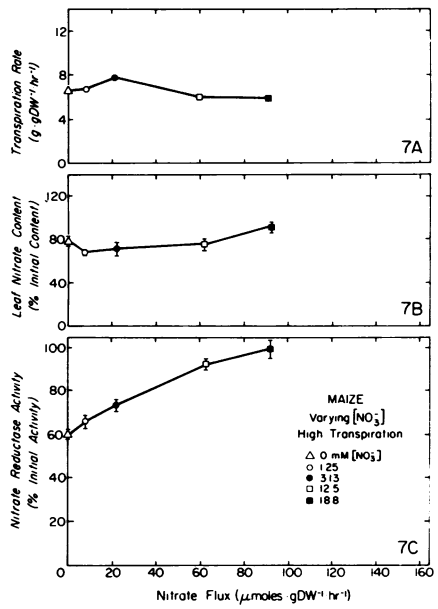


FIG. 7. Effects of nitrate flux on maintenance of nitrate reductase activity and leaf nitrate content in excised 11-day-old maize shoots with high transpiration rates. A: transpiration rate: kept high by exposing shoots to relative humidity of 39% at 30 C. B: leaf nitrate content: initial content, 316 ± 21 μmol·g DW^{-1}. C: nitrate reductase activity: initial activity, 255 ± 13 μmol NO_2^-·g $DW^{-1}\cdot hr^{-1}$. Nitrate reductase activity was determined initially and 5 hr after excision. Nitrate flux was altered by varying the nitrate content of the uptake solutions. Bars around points indicate ± 1 SD.

nitrate that is moving to the shoots from the roots. The data show that the nitrate flux to the leaves plays the largest role in regulating NRA. No matter how the nitrate flux was varied in either intact or excised shoots, NRA responded in the same way: as the flux decreased, NRA decreased.

Others have shown that NRA does not necessarily reflect the nitrate content of plant tissue. In cultured tobacco cells, Heimer and Filner (8) found that NRA increased while nitrate was present in the exogenous medium, but fell as soon as the nitrate was removed, even though total cellular nitrate levels were relatively constant. Similar relationships between exogenous nitrate supply and NRA have also been demonstrated in excised maize leaves (21), terminal buds of peas (10), roots (2, 6, 15, 18), and cotton ovules (17).

The present work is the first to measure nitrate flux directly in intact plants and relate it to NRA. While it may be difficult to visualize how enzyme induction could be responsive to the flux of an inducer, it should be noted that the NRA extracted from maize was in excess of that required to reduce the nitrate moving to the leaves, often by a considerable amount. Therefore, the incoming nitrate should have been rapidly reduced and the size of the pool of nitrate at the inducing sites should have been highly responsive to the flux of nitrate to the leaf cells. The nitrate flux then controlled the induction of the enzyme.

Others (1, 7, 8, 10) have proposed the existence of two pools of nitrate in plant cells—a large storage pool, possibly located in the vacuole, and a smaller, rapidly exchanged, rapidly metabolized pool in the cytoplasm that may be involved in the induction of NRA. The reason that little response of NRA to leaf nitrate content was observed in the present work is probably that the leaf nitrate content reflected primarily the vacuolar pool, which was sequestered. Clear evidence of this sequestration was shown by the experiment involving chilled roots (Fig. 4). The leaf nitrate content required 24 hr to decrease 60% or approximately 240 μmol·g DW^{-1}. This was equivalent to a net average flux from the vacuole to the cytosol of 10 μmol·g $DW^{-1}\cdot hr^{-1}$. On the other hand, nitrate fluxes of 150 μmol·g $DW^{-1}\cdot hr^{-1}$ were common in the transpiration stream. At nitrate fluxes of 20 to 40 μmol·g $DW^{-1}\cdot hr^{-1}$, NRA was considerably decreased (Figs. 5–7). Thus, the nitrate flux via the transpiration stream was often at least ten times the nitrate flux that could be supplied by the vacuoles, which indeed would have been depleted of nitrate after only a few hours if they lost nitrate at higher rates. Rapid depletion of the vacuolar nitrate was never observed in the present work.

In several cases, the nitrate content of the leaves showed no change during the experiment (Figs. 4–7). During these times, the incoming nitrate was quantitatively reduced by nitrate reductase and the nitrate flux represented a measure of the *in vivo* activity of the enzyme. Clearly, the activity of nitrate reductase was limited by substrate availability *in vivo*. Substrate availability was in turn controlled by the ability of the roots to supply nitrate.

In view of the large number of environmental and metabolic factors known to affect NRA (3), it is not possible to compare the importance of regulation by nitrate flux with that of other regulatory phenomena. The approach of the present work was to hold the environment and plant age constant and to conduct experiments at times of the day when there would usually be very little change in NRA, so that endogenous factors were presumably not affecting the system or were constant. Furthermore, for experiments extending for periods of days, assays were made at the same time each day. All the experiments were conducted in the light to assure the rapid reduction of nitrite, the product of nitrate reductase.

An assumption underlying this work was that the nitrate flux to the leaves was represented by the nitrate moving in the transpiration stream. Thus, the product of the transpiration rate and the nitrate content of the xylem sap permitted measurement of the nitrate flux in intact plants. Nevertheless, nitrate might have been transported toward the shoot in tissues other than xylem, although phloem is unlikely to be involved (16). Since transpiration accounts for the largest water flux by far, the nitrate carried by the transpiration stream probably represented the largest component of the nitrate flux whenever transpiration was appreciable. This interpretation is supported by the eventual decline in leaf nitrate content that occurred when nitrate flux was low (Fig. 4), as would be expected if nitrate flux had been adequately estimated from the nitrate in the transpiration stream. When transpiration was low, however, the other transport modes for nitrate would be increasingly significant.

The nitrate flux to the shoot was controlled primarily by both the rate of transpiration and the rate of delivery of nitrate to the transpiration stream by the roots. The latter was more important, since nitrate delivery to the xylem was fairly constant regardless of rates of transpiration and hence nitrate flux tended to remain constant. Thus, it was simple to affect NRA by affecting root activity by cooling, but it was difficult to affect NRA by altering transpiration.

These results suggest that, in field situations, environmental perturbations that influence the uptake of nitrate by roots, especially if they also influence transpiration, should have a rapid effect on NRA. An example is the chilling of soil which frequently occurs early in the growing season. The content of nitrate in the tissue should protect the plant against decreases in external nitrate to a small extent by slowly releasing nitrate to nitrate reductase. Since this pool of nitrate is not large, however, its contribution should eventually become negligible, and the over-all nitrate availability to NRA should reflect only the nitrate flux to the shoots from the roots.

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