

# Rhythmic Nitrate Reductase Activity in Leaves of *Capsicum annuum* L. and the Influence of Kinetin

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## ABSTRACT

In the expanding leaves of *Capsicum annuum* L. cv. California Wonder, two of the three peaks of nitrate reductase activity associated with the light period exhibit a circadian rhythm that persists in continuous light.

The spray application of kinetin to the whole shoot or to leaves other than the ones used for nitrate reductase assay causes a phase shift in the activity peaks and this has been used in preliminary investigations of the character of the mechanisms controlling the timing of the peaks.

There was some indication that the rate of translocation of nitrate from the roots might be involved. The levels of nitrate moving up the stem after kinetin treatment were more dependent upon the rate of sap flow than on the concentration of nitrate in the sap. For this reason, transpiration rates in whole plants were measured after kinetin treatment but no change in pattern was seen that would correlate with the phase shift in nitrate reductase activity.

The occurrence of nitrate reductase peaks in excised leaves suggested a leaf-based in addition to a root- or stem-based mechanism in the timing of nitrate reductase activity in the leaves.

In previous reports (22-24), the metabolic fate of photosynthetically fixed carbon was shown to be very closely linked in time with the activity of nitrate reductase in leaves of *Capsicum annuum*. Nitrate reductase exhibited three peaks of activity during and immediately after the light period and they were numbered: peak I, the peak observed soon after the end of the photoperiod that was correlated with a rise in carbon flow into amino acids during the last 2 hr of the photoperiod (22); peak II, that observed within 1 hr of the start of the photoperiod; and peak III, the major peak, observed 6 hr after the inductive light-on signal (24).

The resultant periodicity in the supply of reduced nitrogen in the leaf was paralleled directly in the synthesis of amino acids from newly fixed carbon.

A sequence of control mechanisms diverting carbon from the reductive pentose phosphate pathway to amino acid synthesis involved the activation of pyruvic kinase, possibly by  $\text{NH}_4^+$ , resulting from the activity of nitrate reductase (23). Thus, the periodic activity of leaf nitrate reductase ( $\text{NO}_3\text{R}$ ) seemed to play a primary role in this control sequence, and to study it further, some characteristics of two of these nitrate reductase peaks, that at the beginning of the photoperiod (peak II) and that manifest soon after the end of the photoperiod (peak I), have been investigated. Both peaks show entrainment under constant environmental conditions and exhibit phase shifts when the plant is treated with kinetin. Some mechanisms possibly involved with the timing of nitrate reductase activity have been investigated.

There are indications of a leaf-based as well as a root- or stem-based mechanism.

## MATERIALS AND METHODS

**Plant Material.** Seeds of *Capsicum annuum* cv. California Wonder (Arthur Yates & Co. Pty. Ltd., Sydney) were germinated in a seedpan in a glasshouse. After transplanting into pots, they were grown in a controlled environment cabinet with a 10-hr photoperiod (26 C day, 24 C night) with irradiance ( $150 \text{ w} \cdot \text{m}^{-2}$  400-700 nm) from fluorescent lamps as reported previously (24). Nitrogen was supplied daily as nitrate at  $6 \text{ meq} \cdot \text{l}^{-1}$  in a complete nutrient solution.

Throughout this report, zero time is the time of the start of the prevailing photoperiod, or when a photoperiod is replaced by darkness, the time of the expected light-on signal.

**Nitrate Reductase Activity.** The enzyme was assayed in crude leaf homogenates from expanding leaves as reported previously (22) and activity expressed as  $\text{nmol NO}_2^-$  produced/hr. The assay was always complete within 30 min after leaf excision. Samples were single leaves from separate plants selected on the basis of their leaf plastochron index, laminar area, and the same node number (22).

It was not possible to assay replicate samples at the same time when doing time series experiments. However, the variability of nitrate reductase activity from leaves sampled at the same time was small, as shown in Table I. The coefficient of variation was between 14% and 23%. Analysis of variance of the means showed that samples 1, 2, and 3 did not differ significantly from each other but that each differed significantly from sample 4 at the 1% level.

Samples 1 and 3 were taken when the increase in peak II  $\text{NO}_3\text{R}$  would be expected. Sample 2 was taken when the increase in peak III would be expected so that the lack of significant difference between them is the anticipated result. However, replacing the normal photoperiod by darkness prevents the ap-

Table I. Variability in Nitrate Reductase Activity

Samples 1 and 2 were from plants in a normal 10-hr photoperiod, but 3 and 4 were from plants where the photoperiod was replaced by darkness on the experimental day.

Sample	Time min.	n	Mean	SEM
			$\text{nmole} \cdot \text{hr}^{-1} \cdot \mu\text{g}^{-1} \text{ protein}$	
1	29	3	10.34	1.42
2	305	4	11.58	1.26
3	54	3	10.01	0.85
4	287	4	5.03	0.45

pearance of peak III (sample 4) (24) and demonstrates a significant decrease in activity from the peak II activity (sample 3). In addition, the lack of significant difference between peak II activity in the light (sample 1) and in the dark (sample 3) suggests that assaying peak II activity in the dark does not introduce any errors. This procedure is convenient in that the decrease of peak II activity is not confused by the occurrence of peak III.

**Nitrate Content of Bleeding Sap.** Sap was collected and assayed as previously reported (22).

**Hormone Application.** All applications were made in aqueous solutions with a wetting agent,  $117 \mu\text{l}\cdot\text{l}^{-1}$  Agral 60 (ICI Australia Ltd.), present. In the phase shift experiments, either the whole shoot or a single leaf was sprayed with  $20 \text{ mg}\cdot\text{l}^{-1}$  kinetin (Calbiochem Pty. Ltd.) but in the leaf-air temperature difference measurements, leaves above the measured leaf were painted with the solution.

**Protein Estimation.** Protein was measured by the Folin phenol method, after precipitation from the crude homogenate by making it 6% w/v with trichloroacetic acid. The pellet was extracted with 0.1 M potassium phosphate buffer (pH 7.5) and protein estimated in this solution. Within experiments, the variability in protein content per unit laminar area was low, with the coefficient of variation being less than 10%.

**Leaf-Air Temperature Differences.** Continuous recording was made with thermocouples consisting of a series of chromal-constantan junctions. Each alternate junction was aspirated in an air stream, with the other junctions held against the leaf surface. The output signal, which was proportional to the difference in temperature between the leaf surface and the air, was amplified and recorded.

## RESULTS

**Persistence of Nitrate Reductase Peaks in Continuous Light.** The persistence of the early morning peak of activity (II) after 24 hr of continuous light has been reported before (24). Figure 1 shows that peak II activity is still apparent, with little indication of damping out, after 50 hr of continuous light. The frequency of the rhythm is close to 25 hr.

It was reported previously (24) that in continuous darkness, peak II is seen only once, at the time of the first expected dawn. The lack of peak II in longer dark conditions was ascribed to a lack of carbon available for protein synthesis in the darkened plants as suggested by Kannangara and Woolhouse (11). For this reason, it was not possible, in support of a circadian rhythm, to

supplement the continuous light data (Fig. 1) with a continuous dark experiment.

Little information on peak I activity has been reported previously, but it may be seen from Figure 1 that it behaves in the same way as peak II in continuous light but the frequency remains at 24 hr. The timing for peak I appears to depend on the time of the light-off signal. For in plants raised under different photoperiod lengths, peak I timing is always correlated with the time of the light-off signal and the timing is not influenced by the number of elapsed hours from the time of the light-on signal.

**Effect of Kinetin.** Roth-Bejerano and Lips (21) made the observation that kinetin applied to leaves of tobacco altered the diurnal pattern of  $\text{NO}_3\text{R}$  activity. The effect of kinetin on the periodicity of  $^{14}\text{C}$  flow to amino acids in *C. annuum* leaves was investigated, and it was found that early and late in the photoperiod the pattern was altered by kinetin. Accordingly, the response of  $\text{NO}_3\text{R}$  activity to kinetin treatment was examined. Figure 2 shows peak II  $\text{NO}_3\text{R}$  activity in two groups of plants. In one group, leaf number 9 was sprayed with  $20 \text{ mg}\cdot\text{l}^{-1}$  kinetin in aqueous solution at 3 hr after light-on in the previous photoperiod. Leaf number 9 of the control group of plants was sprayed

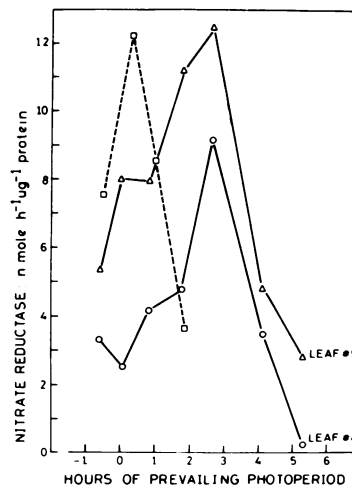


Fig. 2. Timing of nitrate reductase activity peak II in leaves numbers 4 and 9 after kinetin application to leaf number 9 at the 3rd hr of the previous photoperiod. Activity in plants not treated with kinetin ( $\square$ --- $\square$ ). On the experimental day, the prevailing photoperiod was replaced with darkness.

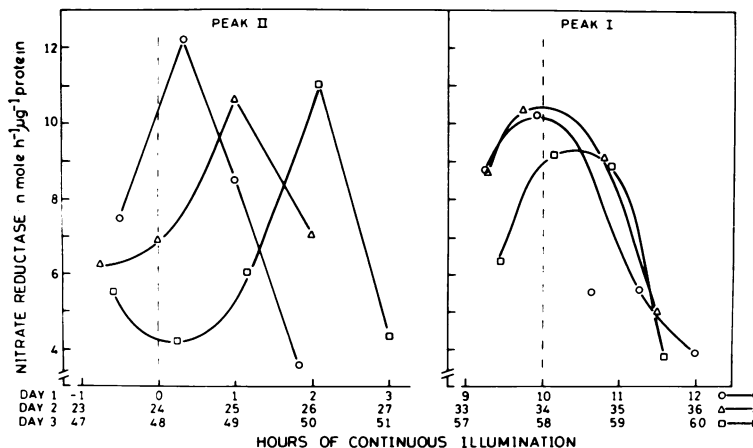


Fig. 1. Persistence of nitrate reductase activity peaks I and II during 3 days of continuous light. A normal 14-hr dark period ended at 0 hr and was followed by 60 hr of light. Vertical dotted lines show the beginning and end of a normal 10-hr photoperiod. Leaf number 5 with leaf plastochron index of 6 was used for peak II and leaf number 8 with leaf plastochron index of 4 for peak I.

with the same solution minus kinetin at the same time. As a result, in the control plants, the peak is exhibited, as usual, during the first hour of the photoperiod, but in the kinetin treatment, activity peaks at 2.5 hr. Peak II activity has been shifted in both leaves, numbers 4 and 9, even though kinetin was applied only to leaf number 9. Thus, the effect of kinetin treatment must be translocated from leaf to leaf. A kinetin spray does not alter the timing of peak III  $\text{NO}_3\text{R}$  activity.

This phase shifting of peak II  $\text{NO}_3\text{R}$  activity by kinetin is illustrated further in Figure 3, together with data for peak I. Both peaks exhibit a phase shift and the degree of shift is dependent upon the timing of the kinetin treatment. It is noticeable that only when kinetin is applied during the photoperiod is a phase shift induced, and that peak I shows an advance while peak II only a delay. Thus, both peaks are shifted toward the middle of the photoperiod and never into the dark period. A single kinetin application has mimicked the signals controlling the timing of both peaks I and II. It is not known how many daily cycles are needed before the normal light-on or light-off signals will reset the  $\text{NO}_3\text{R}$  activity peaks shifted by kinetin.

**Translocation of Nitrate.** The level of nitrate in the bleeding sap of decapitated *C. annuum* stems reaches a maximum during the early part of the photoperiod (22). It was thought that this maximum might be correlated with the occurrence of the early morning peak (II) of  $\text{NO}_3\text{R}$  activity in the leaves. If such a correlation exists, it could be expected that the phase shift of  $\text{NO}_3\text{R}$  activity caused by kinetin treatment might also be seen in the nitrate content of bleeding sap. When this was measured, it was found that nitrate per unit volume of sap remained fairly constant throughout the day but the volume of sap varied. Figure 4 shows that the amount of sap  $\text{NO}_3\text{N}$  collected from previously kinetin-treated plants exhibited a different pattern from the control group with the former having a shoulder in the 2nd and 3rd hr of the photoperiod that is absent in the control, but these differences were not statistically significant. In addition, using decapitated plants removes the short term influences of the shoot on root activity, such as leaf-based control of the rate of the transpiration stream. For these reasons, it was considered that this shoulder in sap flow caused by kinetin treatment needed to be re-examined using whole plants.

**Diurnal Transpiration Patterns.** Barrs and Klepper (3) made the observation that *C. annuum* plants sometimes showed a weak, rapidly damped cycling in transpiration rate on transfer from dark to light. This cycling could influence the influx of nitrate to the leaf during the early part of the photoperiod. Consequently, to assess the influence of transpiration rate on  $\text{NO}_3\text{R}$  activity patterns, leaf-air temperature differences were

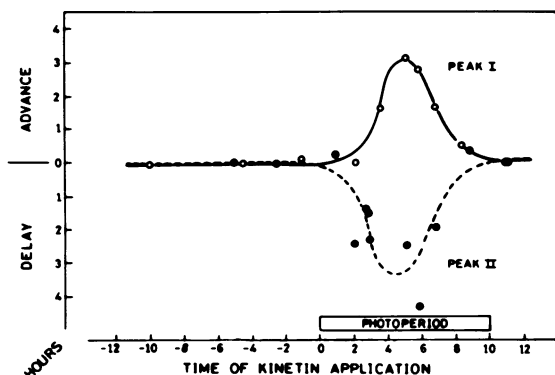


FIG. 3. Phase shift in the timing of nitrate reductase activity peaks I and II induced by spraying the shoots with  $20 \text{ mg} \cdot \text{l}^{-1}$  kinetin. Time zero for kinetin application is the start of the 10-hr photoperiod. Where a kinetin application has not altered the timing of the peaks by comparison with control plants, the point is placed at zero on the ordinate. Changed timing is shown as advances or delays in hours.

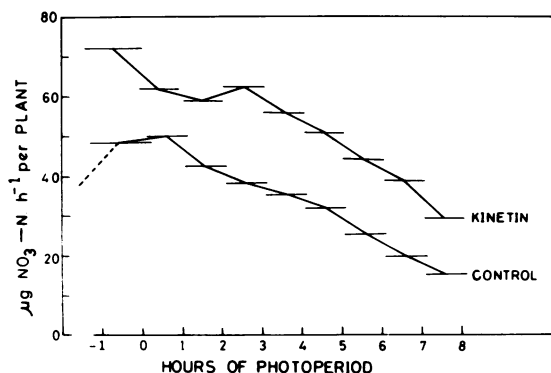


FIG. 4. Nitrate-N content of bleeding sap collected at hourly intervals from decapitated plants. Values are the means of four plants. One group of four was sprayed with kinetin on the 5th hr of the previous photoperiod and the control group was sprayed with a solution minus kinetin.

measured. Ehrler *et al.* (6) demonstrated that in cotton leaves, leaf temperature and transpiration rate had similar patterns when transpiration was oscillating with frequencies of less than an hr. Leaf-air temperature differences, hereafter called transpiration for brevity, for *C. annuum* are shown in Figure 5.

The rapidly damped cycling (Fig. 5a) in the first 2 hr of the photoperiod is of the same form reported by Barrs and Klepper (3) and it is seen to be preceded by cycling in transpiration in the last 3 hr of the dark period. This pattern has been found consistently. In continuous illumination (Fig. 5c), the same pattern is seen in transpiration, indicating an entrained control of stomatal aperture.

When leaves younger than the measured leaf are treated with  $20 \text{ mg} \cdot \text{l}^{-1}$  kinetin at the fifth hr of the photoperiod, then transpiration during the rest of that photoperiod is lower than previously (Fig. 5b) but there is little difference from a control trace during the following dark period and photoperiod.

Despite the persistence of the normal transpiration pattern in continuous light, and hence, the possible correlation of peak II  $\text{NO}_3\text{R}$  activity with transpiration cycling early in the photoperiod, this was not supported by a correlation between transpiration and  $\text{NO}_3\text{R}$  activity patterns in response to kinetin. There was no indication of a shift in the transpiration pattern after kinetin treatment that would accord with the shift in peak I and II  $\text{NO}_3\text{R}$  activity. Over-all, this lack of consistent correlation between the transpiration patterns and  $\text{NO}_3\text{R}$  activity patterns after different treatments suggests that control of  $\text{NO}_3\text{R}$  activity by compounds arriving in the leaf in the transpiration stream (e.g. nitrate and hormones) is of minor importance or is supplemented by other control mechanisms.

**Experiments on excised Leaves.** Leaves excised at the axil, 3 hr before the prevailing time of light-on and placed in either water or one-tenth nutrient solution containing  $0.6 \text{ meq} \cdot \text{l}^{-1}$  nitrate  $\text{l}^{-1}$  in the dark will exhibit a peak II  $\text{NO}_3\text{R}$  activity within 1 hr of the expected time of light-on (Fig. 6). This is not a response to excision, for leaves excised 2 hr after the expected light-on time will not show a peak in activity. These results suggest a leaf-based timing control, independent of immediate nitrate supply to the leaf, in addition to the root- or stem-based control indicated by the translocation of the kinetin effect from leaf to leaf (Fig. 2).

## DISCUSSION

The experimental design of studying the control of C and N metabolism in a leaf throughout the photoperiod and using time-based differences rather than induced perturbations (e.g. short term light-dark transitions) has yielded results of interest from

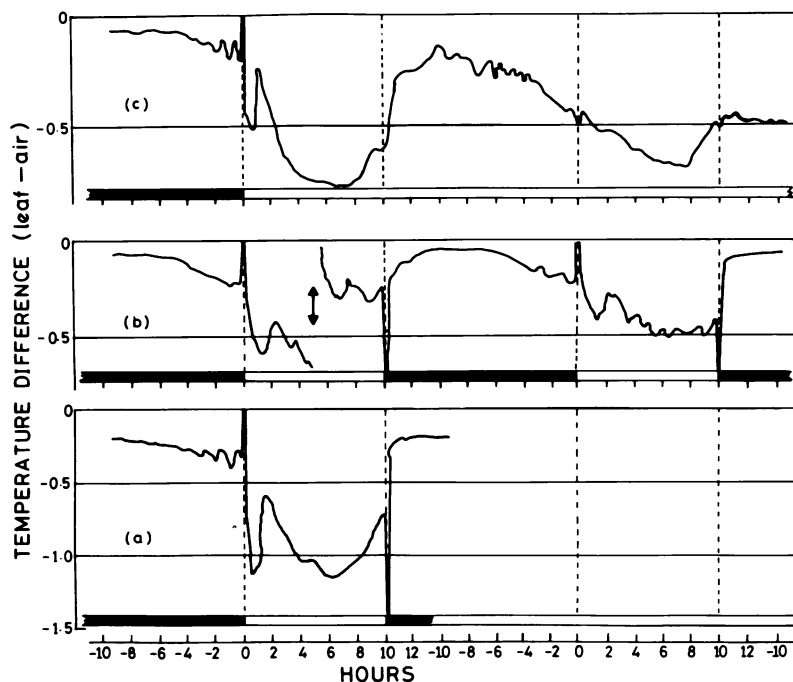


FIG. 5. Leaf-air temperature difference (transpiration) traces. Plants were grown and measurement made in a controlled environment cabinet. (a): normal untreated plant over 24 hr; (b): trace when leaves younger than the measured leaf (number 3) are treated with kinetin at the 5th hr of the first photoperiod (arrow); (c): trace over 48 hr, when a dark period is replaced by continuous light.

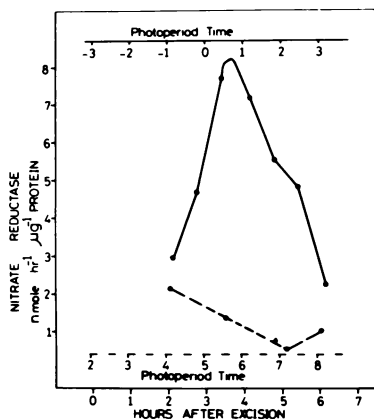


FIG. 6. Occurrence of nitrate reductase peak II activity in excised leaves. Zero hr on the photoperiod time axes is the time of lights-on in the normal 10-hr photoperiod in which the plants were raised. Plants were excised 3 hr before lights-on (●) and excised 2 hr after the lights-on time (○). Activity (Δ) in an attached leaf from the same population as (○). During the experimental day, plants were kept in darkness.

*C. annuum*. From this and previous reports, it is evident that within the prevailing photoperiod, leaf metabolism can differ considerably from hr to hr. The elucidation of control mechanisms in this way has the advantage over induced perturbation studies in that the findings are likely to be a good description of how the plant behaves in natural photoperiodic regimes in the field, when other major environmental constraints are not the predominant influence.

The step function of the light-on and light-off signal in the controlled environment cabinet may be thought to impose a signal on the plant that it does not experience in naturally illuminated environments where the change in light intensity is a gradual process. However,  $^{14}\text{C}$  flow to amino acids, peak II  $\text{NO}_3\text{R}$  activity and transpiration cycling patterns occur in plants brought from the glasshouse to the controlled environment cabi-

net just before light-off. Either a single stepped light-off signal is sufficient to cause the patterns in all three phenomena, in many cases more likely linked to light-on than light-off, or they are present in naturally illuminated plants.

The activity of pyruvic kinase seems to play an integral part in controlling the flow of carbon into amino acids. This enzyme is activated when  $\text{NO}_3\text{R}$  activity is high in the leaves, perhaps with  $\text{NH}_4^+$  as the modulator (23, 24). Thus, factors controlling the periodicity in the activity of  $\text{NO}_3\text{R}$  can control, fairly directly, carbon flow to amino acids.

Circadian rhythms in  $\text{NO}_3\text{R}$  activity have been reported previously in wheat (27) and *Chenopodium rubrum* (4). In wheat,  $\text{NO}_3\text{R}$  activity shows peaks 24, 48, and 72 hr after transfer of dark-grown plants to continuous light. A similar entrainment of the rhythm by dark to light transition was seen in seedlings of *C. rubrum* with a frequency of 18 hr, but in this species, germinated in continuous light, another rhythm was also present which was not reset by a transition to darkness. An interesting parallel is also available in the different rhythms of  $\text{CO}_2$  output from *Lemna perpusilla* when grown on different N sources (10). It is suggested that minor shifts in the N metabolism of the cells might result in these modifications of the  $\text{CO}_2$  output rhythms.

This study of *C. annuum* has suggested that two minor peaks of  $\text{NO}_3\text{R}$  activity exhibit a circadian rhythm linked to the light-on and light-off signals. Phase shifting of both peaks of enzyme activity by the application of kinetin to leaves other than the sampled one suggests that the timing stimulus is translocated from leaf to leaf. Investigations on the effects of plant hormones on leaf  $\text{NO}_3\text{R}$  activity have shown that cytokinins stimulate activity synergistically with nitrate (12, 18) and with  $\text{GA}_3$  (20) and this will replace a light requirement for induction in the latter case (13). This involvement of cytokinins in the control of  $\text{NO}_3\text{R}$  activity makes it possible that the timing mechanism is, at least partially, root- or stem-based. Current evidence on the translocation of cytokinins has been reviewed by Gordon *et al.* (8) and some data support the acropetal movement from roots to shoots. Another pertinent finding, in leaves of *Populus x robusta*, is that cytokinin levels exhibit a peak just after light-on

and, sometimes, in the middle of the photoperiod (9, 26). The peak associated with light-on occurred in attached or detached leaves, as well as in plants in the field, so that it is not clear whether cytokinin translocation, *in situ* synthesis, or release from a bound form is involved.

With the evidence of phase shifts by kinetin treatment reported above, experimental design in investigations of hormones on NO<sub>3</sub>R activity should differentiate between activation and inhibition of enzyme activity and phase shifting of pre-existing activity peaks.

Study of the timing mechanism must involve the whole plant. Intermediates have been sought between a basic oscillator and NO<sub>3</sub>R activity. Published evidence suggests that NO<sub>3</sub>R activity is influenced by a small metabolic pool of nitrate and not by large storage pools (7, 14). With the replenishment of metabolic pools by translocated nitrate in mind, the timing of nitrate moving up the stem was studied in bleeding sap. Although kinetin-treated plants showed an extra shoulder in sap volume that could be correlated with the shifted peak II, no such indication was seen that would correlate with peak I. Although the level of nitrate moving up the stem is mainly dependent upon the volume of sap flow, there was no indication from transpiration measurements that NO<sub>3</sub>R activity responds directly to changes in the magnitude of the transpiration stream. Perhaps this is not surprising, for a direct response would have NO<sub>3</sub>R activity changing with each small change in environmental conditions. However, there have been instances where environmental perturbations (*e.g.* 1 hr of one-tenth the prevailing light intensity) have caused short frequency cycling (90 min) of NO<sub>3</sub>R activity (unpublished).

In summary, the data reported here on the patterns of transpiration and NO<sub>3</sub>R activity do not support the idea that the timing mechanism external to the leaf resides in the process of nitrate translocation from the roots to the leaves.

The occurrence of peak II NO<sub>3</sub>R activity in excised leaves suggests a timing mechanism within the leaves in addition to a mechanism external to the leaf. Insight into the leaf-based timing mechanism requires information about the behavior of NO<sub>3</sub>R that is not available for *C. annuum*, such as the role of inactivating systems. Wallace (31) has demonstrated the occurrence of a NO<sub>3</sub>R-inactivating enzyme from maize seedlings, and the occurrence of a similar system in *C. annuum* controlling the loss of activity from all three peaks of NO<sub>3</sub>R needs to be investigated. It must be pointed out that there is no indication that the peaks of activity are the result of variable proteolytic activity in the leaf homogenates after extraction (the inclusion of casein or phenylmethylsulphonyl fluoride with the homogenizing medium has not altered the results).

The model of Pavlidis (19) provided for the generation of circadian rhythms from populations of interacting short frequency oscillators. In *Chenopodium rubrum*, Wagner *et al.* (30) have shown the presence of short term oscillations in adenine nucleotide contents which together give a circadian rhythm in energy charge (ATP + 0.5 ADP/ATP + ADP + AMP) (1, 2) and a circadian rhythm in NADPH/NADP ratio (28). ADP is known to inhibit NO<sub>3</sub>R from tomato (16) and spinach leaves (5), and rhythmic changes in adenine nucleotides could be involved in the rhythms of NO<sub>3</sub>R activity. Furthermore, the energy charge concept may be linked with a membrane oscillator (29) that could be a timing mechanism for circadian rhythms (17, 25). Changing membrane permeability toward nitrate would serve as an important control for a substrate-inducible NO<sub>3</sub>R, and in this way, hormone effects on NO<sub>3</sub>R activity could be mediated by membrane permeability (15). Thus, further investigations on the leaf-based and root-based timing of peaks I and II of NO<sub>3</sub>R activity will be on the levels of hormones within, and arriving at, the

leaves, together with some estimations of the adenine nucleotide and pyridine nucleotide status of the leaves throughout the photoperiod.

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