Seasonal Patterns of Nitrate Reductase and Nitrogenase Activities in *Phaseolus vulgaris* L.\(^1\)

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ABSTRACT

The patterns of nitrate reductase activity (NRA) in the leaves (*in vivo* assay) and root nodule nitrogenase activity (*C*\(_2\)H\(_2\) reduction) were investigated throughout the season in field-grown *Phaseolus vulgaris* plants.

Maximal NRA (per g fresh weight) occurred at early stages of leaf development but total activity (per leaf) was maximal when the leaf reached full size. In mature plants, most NRA was associated with the upper leaves. Nitrogenase activity was initiated about 2 weeks after sowing, reached a maximum at flowering (5 weeks after sowing) and declined rapidly thereafter. Nitrogenase activity followed the pattern of nodule development. After flowering, *P. vulgaris* was apparently able to take up and assimilate NO\(_3^-\) as evidenced by the increase in NO\(_3^-\) content of the stem and the high levels of NRA in the leaves. Total plant NRA was maximal after flowering and addition of NH\(_4\)NO\(_3\) to the soil at flowering resulted in even higher levels of NRA through most of the pod-filling period, thus resulting in higher seed yields (59% over control).

It is proposed that *P. vulgaris* can benefit from both N\(_2\) fixation and NO\(_3^-\) assimilation and that nitrate reductase plays an important role in the assimilation of nitrogen after flowering.

Biological N\(_2\) fixation and NO\(_3^-\) assimilation represent the major sources of reduced N for plant growth and seed yields in legume crops. Several environmental and plant factors are known to control the amount of N incorporated through N\(_2\) fixation in *Phaseolus vulgaris* plants (4, 6) and attempts to supplement N\(_2\) fixation with fertilizer N have not succeeded in increasing yields (4, 5, 9). Nevertheless, low levels of combined N during the initial stages of development have been shown to enhance nodulation and N\(_2\) fixation (1, 5).

There are no reports about the relationships between NO\(_3^-\) assimilation and N\(_2\) fixation throughout the season in *P. vulgaris* plants. The seasonal patterns of NO\(_3^-\) uptake and reduction and the patterns of N\(_2\) fixation in soybean plants indicate that the processes of NO\(_3^-\) assimilation and N\(_2\) fixation are successive events, each contributing nitrogen at defined stages of plant development (4, 8, 16). NO\(_3^-\) reduction appears to be more important in certain legumes at the preflowering stages while maximal nitrogenase activities were observed after the decline of NRA\(^3\) (4, 8, 16). Because NO\(_3^-\) is considered the primary source of N available from the soil, the characterization of NO\(_3^-\) uptake and reduction throughout the season in *P. vulgaris* plants, along with the characterization of N\(_2\) fixation, would be highly desirable to obtain the maximum benefit of both atmospheric and soil nitrogen.

The objectives of this work with field-grown *P. vulgaris* plants were: (a) to determine the seasonal profiles of nitrate utilization via NR and of nitrogenase activities using the acetylene reduction method; (b) to study the relationship between the processes of NO\(_3^-\) assimilation and N\(_2\) fixation; and (c) to explore the possibility of using combined nitrogen to complement N fixation to increase bean yields.

MATERIALS AND METHODS

Cultural Procedures. *P. vulgaris* L. (cv. Rico 23) was grown in a red-yellow podzolic (= Ultisol udult) soil at EMBRAPA-Km 47, Rio de Janeiro, Brazil in a randomized complete block design with six replications and four treatments: 1) control-no fertilizer N; 2) 20 kg N ha\(^{-1}\) at sowing; 3) 20 kg N at sowing plus 40 kg N ha\(^{-1}\) at flowering; and 4) 20 kg N applied weekly (7 x 20 = 140 kg N ha\(^{-1}\)). Supplemental liming was not necessary (soil pH 6.0, no aluminum present, and adequate levels of Ca + Mg). Based on soil analysis all treatments received a uniform dressing of 44 kg P ha\(^{-1}\) and 28 kg K ha\(^{-1}\). A mixture of micronutrients (F.T.E., BR-10, Ferroenamel do Brazil, Sao Paulo) mixed with CaCO\(_3\) was applied by pelleting the seeds (3). The seeds were inoculated with a mixture of *Rhizobium* phaseoli strains (F-300 and F-310, EMBRAPA Km 47 culture collection). Each plot consisted of 12 rows (50 cm apart) 10 m long. Each row contained 200 to 240 plants. Irrigation was provided as needed and insects controlled regularly. The mean maximum daily soil temperature at a 15-cm depth was 26.8°C.

Plant Sampling. Five plants were randomly selected from each plot at weekly intervals. The plants were cut at the soil level and stored on ice until the NRA assay (*in vivo*) was performed. The remainder of the plants were left and the root system plus the nodules used for assays of nitrogenase activities by the acetylene reduction method.

Acetylene Reduction Assay. The root system and nodules of each replicate were shaken to remove adhering soil particles and put into a 300-ml vial, hermetically closed with a rubber stopper through which acetylene was added to give a final concentration of 15% (v/v) in air. After allowing for pressure equilibration, incubation proceeded at room temperature for 1 hr, after which time the amount of ethylene formed was determined by gas chromatography using a Perkin-Elmer gas chromatograph fitted with a Porapak N column (3 mm x 2 m) at 110°C and H\(_2\) flame ionization detection.

NRA Assay. The NRA of leaf sections was determined by the *in vivo* method (8, 10, 11) with modifications. All individual leaves along the plant were separated, superimposed, and small discs taken with a cork borer. Subsamples (0.2 g) were placed into vials containing 5 ml of incubation medium composed of: 100 ml

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\(^3\) Abbreviations: NR: nitrate reductase; NRA: nitrate reductase activity.
RESULTS AND DISCUSSION

NR and Leaf Development. The NRA of a single leaf (2nd trifoliate) determined as a function of leaf development showed that NRA, per leaf, was maximal just after the leaf had reached full size in terms of leaf area (Fig. 1). On the other hand, the NRA per g fresh weight was maximal at early stages of leaf development, when the leaf was about one-third of full size (leaf area basis) and the activity slowly declined with leaf age. Each newly expanded leaf showed a similar pattern of NRA with leaf development. Thus, maximal NRA (per g fresh weight) along the canopy would be associated with the uppermost newly expanding leaves.

Seasonal Potential for NR. The seasonal distribution of NRA, sampling the uppermost fully expanded leaf throughout the season, showed the occurrence of two peaks of activity (per g fresh weight), one at early vegetative growth and a second peak around midpod filling (Fig. 2). This characteristic was associated with the ability of P. vulgaris plants to take up NO\textsubscript{3} efficiently at the postflowering stages (Fig. 3) and contrasts with the continuous decline of both NRA and ability to take up NO\textsubscript{3} by soybean plants after flowering (4, 8, 16). Similar patterns, throughout the season, were observed between the NO\textsubscript{3} content of the stem and the plus or minus NO\textsubscript{3} in vivo NR assays of the uppermost fully expanded leaves (Fig. 3) or the mean NRA activities of all individual leaves (data not shown). Although the minus NO\textsubscript{3} in vivo assay gave a significant correlation with the nitrate content of the stems ($r = 0.76^{*}$) and is probably the best measure of in situ nitrate reduction (15), the addition of NO\textsubscript{3} to the assay medium consistently increased the measured NRA throughout the season (Fig. 3). This suggests that NO\textsubscript{3} reduction by P. vulgaris leaves, under field conditions, may be limited by the availability of NO\textsubscript{3} at the site of reduction. The seasonal distribution of NRA per plant showed that the maximum potential for NO\textsubscript{3} reduction and assimilation in P. vulgaris plants occurred at the postflowering stages (Fig. 4). This appeared to be a reflection of both the higher NR specific activities ($\mu$mol NO\textsubscript{3} hr$^{-1}$ g fresh weight$^{-1}$) and greater leaf mass. The seasonal potential for NO\textsubscript{3} reduction was highest for the plants receiving continuous fertilizer N applications ($\times 20$ kg N ha$^{-1}$). The treatment receiving an additional supplement of N (as NH\textsubscript{4}NO\textsubscript{3}) at the beginning of flowering also resulted in higher levels of total plant NRA through most of the pod filling period and almost equaled the seasonal potential for NO\textsubscript{3} reduction of those plants receiving continuous fertilizer nitrogen throughout the cycle (Fig. 4).

Seasonal Potential for N\textsubscript{2} Fixation. N\textsubscript{2} fixation, as indicated by the acetylene reduction assay, was very low during the first 2 weeks after sowing, reached a peak at flowering and then declined rapidly (Fig. 5A). N\textsubscript{2} fixation in general followed the pattern of nodule development (Fig. 5B). Similar trends have been reported for soybean plants (8, 16). Continuous application of fertilizer
nitrogen (7 × 20 kg N ha⁻¹) reduced both nodule mass and nitrogenase activity per plant. The application of small amounts of fertilizer N at sowing (20 kg N ha⁻¹) had no effect on either nodule mass or nitrogenase activity. Because senescence of nodules right after flowering was characteristic of the cultivar under study, the application of an additional supplement of N at flowering (40 kg N ha⁻¹) did not significantly decrease the amount of N₂ fixed during the season (Fig. 5, A and B). The period of effective N₂ fixation for this cultivar appeared to be relatively short but significant incorporation of N through biological N₂ fixation may have occurred during the flowering stage. From the acetylene reduction data, a N gain of about 15 kg N ha⁻¹ was estimated, for the 3 weeks of maximal nitrogenase activity (Fig. 5A).

Relationship between N₂ Fixation and Nitrate Assimilation. The over-all results indicated that *P. vulgaris* plants benefited from both NO₃ assimilation and N₂ fixation; each process contributing maximally at different stages of plant development. On a plant basis, maximal nitrogenase activities preceded the peak of NRA and this is one of the major differences observed in relation to the patterns reported for soybean plants (4, 8, 16). The ability of the plants to absorb NO₃ after flowering caused the induction of higher levels of leaf NRA (12, 13) and indicated that NR played a major role in the incorporation of N during the postflowering stages of development. This contention was further supported by the stimulatory effects of fertilizer N applied (as NH₄NO₃) at flowering on the levels of NRA. This treatment was also equivalent in terms of total N and seed yields to the treatment receiving continuous N application (Table I) but was significantly higher than the control with no fertilizer N. The results indicated that the addition of fertilizer N at flowering may be beneficial for other *P. vulgaris* cultivars showing N₂ fixation and NO₃ assimilation patterns similar to those described in this paper (Fig. 6).

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**Table I. Total plant nitrogen and seed yields of *P. vulgaris* plants grown under different regimes of nitrogen fertilizer in the field.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total plant nitrogen</th>
<th>Seed yields</th>
<th>kg/ha</th>
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<tbody>
<tr>
<td>1) No fertilizer N</td>
<td>115 b*</td>
<td>546 c</td>
<td></td>
</tr>
<tr>
<td>2) 20 kg N ha⁻¹ at sowing (B)</td>
<td>134 b</td>
<td>682 bc</td>
<td></td>
</tr>
<tr>
<td>3) B + 40 kg N ha⁻¹ at flowering</td>
<td>291 a</td>
<td>868 ab</td>
<td></td>
</tr>
<tr>
<td>4) 7 × 20 kg N ha⁻¹</td>
<td>205 ab</td>
<td>887 ab</td>
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*Within a column, means followed by the same letter are not significantly different at the 5% level (Tukey’s test).*

**Fig. 4.** Seasonal distribution of total plant NRA of field-grown *P. vulgaris* plants. Treatments were: 1) no fertilizer N (O); 2) 20 kg N ha⁻¹ at sowing (□); 3) 20 kg N ha⁻¹ at sowing + 40 kg N ha⁻¹ at flowering (■); and 4) 7 × 20 kg N ha⁻¹ (●). Tukey (*p* = 0.05) = 24.9. Date of flowering indicated by arrow.

**Fig. 5.** Seasonal distribution of nitrogenase activity (A) and nodule weight (B) of field-grown *P. vulgaris* plants. Treatments were: 1) no fertilizer N (O); 2) 20 kg N ha⁻¹ at sowing (□); 3) 20 kg N ha⁻¹ at sowing + 40 kg N ha⁻¹ at flowering (■); and 4) 7 × 20 kg N ha⁻¹ (●). Tukey (*p* = 0.05) for A = 4.47 and for B = 36.65. Date of flowering indicated by arrow.

**Fig. 6.** Relationship between N₂ fixation and nitrate assimilation of field-grown *P. vulgaris* plants. (—–): Nitrogenase activity; (—––):O NRA; (O): no fertilizer N; (■): 20 kg N at sowing + 40 kg N ha⁻¹ at flowering.

Date of flowering indicated by arrow.
LITERATURE CITED