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Phloem Glutamine and the Regulation of O_2 Diffusion in Legume Nodules[1]

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The aim of the present study was to test the hypothesis that the N content or the composition of the phloem sap that supplies nodulated roots may play a role in the feedback regulation of nitrogenase activity by increasing nodule resistance to O_2 diffusion. Treating shoots of lupin (*Lupinus albus* cv Manitoba) or soybean (*Glycine max* L. Merr. cv Maple Arrow) with 100 μL L^{-1} NH_3 caused a 1.3-fold (lupin) and 2.6-fold (soybean) increase in the total N content of phloem sap without altering its C content. The increase in phloem N was due primarily to a 4.8-fold (lupin) and 10.5-fold (soybean) increase in the concentration of glutamine N. In addition, there was a decline in both the apparent nitrogenase activity and total nitrogenase activity that began within 4 h and reached about 54% of its initial activity within 6 h of the start of the NH_3 treatment. However, the potential nitrogenase activity values in the treated plants were not significantly different from those of the control plants. These results provide evidence that changes in the N composition of the phloem sap, particularly the glutamine content, may increase nodule resistance to O_2 diffusion and, thereby, down-regulate nodule metabolism and nitrogenase activity by controlling the supply of O_2 to the bacteria-infected cells.

High nitrate levels in soils are known to inhibit both nodule formation and nitrogenase activity in legume nodules (Streeter, 1988). The inhibitory effects of nitrate on specific nitrogenase activity have been classified into those that occur within the first 2 or 3 d (Vessey et al., 1988b; Minchin et al., 1989; Escuredo et al., 1996) and those that result from longer term exposure to nitrate (Minchin et al., 1989; Escuredo et al., 1996). There is widespread evidence that the initial effect of nitrate exposure causes the respiration rate and nitrogenase activity of legume nodules to become severely O_2 limited, as a result of an increase in the nodule resistance to O_2 diffusion and a decrease in the concentration of O_2 in the bacteria-infected cells (Schuller et al., 1988; Vessey et al., 1988a; Minchin et al., 1989; Layzell et al., 1990; Vessey and Waterer, 1992; Hunt and Layzell, 1993; Denison and Harter, 1995; Escuredo et al., 1996). In nitrate-inhibited nodules nitrogenase activity and nodule respiration rates can recover partially or sometimes to rates approximating a pre-decline status by increases in external pO_2 to levels greater than that in air (Minchin et al., 1986, 1989; Schuller et al., 1988; Vessey et al., 1988a; Faurie and Soussana, 1993; Kaiser et al., 1994; Escuredo et al., 1996).

Various hypotheses have been proposed to explain the initial inhibitory effects of nitrate and the role that O_2 plays in the phenomena. For example, within the first few days of exposure, nitrate accumulates in the nodule cortex where it has been proposed to cause water to move out of cortical cells and into the gas-filled intercellular spaces, thus increasing the resistance of the nodule to O_2 diffusion (Sprent et al., 1987; Minchin et al., 1989; Serraj et al., 1995). Nitrate could also enter the central zone of nodules, where it may be converted to nitric oxide, which could bind to leghemoglobin to form nitrosylleghemoglobin, a heme protein that is unable to facilitate the diffusion of O_2 to the bacteroids (Kanayama and Yamamoto, 1990a, 1990b, 1991; Kanayama et al., 1990). However, the latter hypothesis has been recently challenged by a nodule oximetry study (Denison and Harter, 1995) that showed no decrease in the functional leghemoglobin concentration in nodules that are exposed to nitrate.

Other studies have shown that nitrate reduces photosynthate partitioning to nodules (Vessey et al., 1988a, 1988b; Faurie and Soussana, 1993), and because treatments that disrupt the phloem sap supply to nodules (e.g. nodule excision, plant defoliation, stem girdling, or stem chilling) also induce severe O_2 limitation within the infected cells (Minchin et al., 1985; Walsh et al., 1987; Schuller et al., 1988; Vessey et al., 1988a, 1988b; Faurie and Soussana, 1993; Hartwig et al., 1994), nitrate inhibition, through the control of O_2 diffusion, may be mediated by a reduction in the phloem sap supply to nodules. Thus, O_2 diffusion could be affected by either (a) decreasing the pool of soluble sugar in the nodule, thereby altering the osmolarity of cortical cells and causing water to move into intercellular spaces with a resultant increase in the resistance of the nodule to O_2 diffusion (Hunt et al., 1990; Vessey and Waterer, 1992), or (b) decreasing the availability of phloem-delivered water that is required for the export of nitrogenous compounds into the xylem. The latter explanation would be consistent with the observed nitrate-induced accumulation of the N compounds in nodules but would require a feedback control mechanism between the N content of the nodule and

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Abbreviations: ANA, apparent nitrogenase activity; DW_{nod}, dry weight of nodules; EAC, electron allocation coefficient; OLC_N, oxygen limitation coefficient of nitrogenase; PNA, potential nitrogenase activity; pNH_3, partial pressure of NH_3; pO_2, partial pressure of O_2; TNA, total nitrogenase activity.

the resistance of the nodule to O_2 diffusion (Walsh, 1990; Streeter, 1993).

There have been suggestions that the N content of the phloem sap delivered to the nodules may play a role in the feedback regulation of nitrogenase activity. Pate (1976) first suggested that amino acids in the phloem may control N_2 fixation but later (Pate and Minchin, 1980) dismissed the role of a feedback mechanism because the soluble end products of N_2 fixation represent such a large proportion of the total N that is accumulated within the plant tissues. However, Parsons et al. (1993) argued that this feedback mechanism may still be valid if the N is supplied in a different form from that produced within nodules or if the N is delivered to a region of the nodule that is normally low in N so that the supply of phloem N would have a major effect on the N content. They proposed that when nitrate is absorbed by roots there is an increase in either the total N content or the concentration of a specific amino acid(s) in the phloem sap that supplies nodules; this provides a feedback signal to nodules to increase their resistance to O_2 diffusion, thereby down-regulating nodule metabolism and nitrogenase activity by decreasing the O_2 concentration in the infected cells.

The present study was designed to test the hypothesis that the N content of the phloem sap that supplies nodulated roots may play a role in the feedback regulation of nitrogenase activity by increasing nodule resistance to O_2 diffusion. A technique involving the supply of NH_3 to the shoot was developed to selectively increase the N content of the phloem without affecting its C content or altering other aspects of nodulated root metabolism. The second aspect of the hypothesis, that nitrate-induced inhibition of N_2 fixation may be mediated by a similar change in the phloem N content, will be examined in subsequent studies.

MATERIALS AND METHODS

Plant Culture

Plants of lupin (*Lupinus albus* cv Manitoba) and soybean (*Glycine max* L. Merr. cv Maple Arrow) were grown in silica sand (grade 16) in pots that could be sealed for gas-exchange studies as previously described (Hunt et al., 1989). Seeds were inoculated at the time of planting with a peat inoculum (0.05 g per seed) containing *Bradyrhizobium lupini* WU 425 (for lupins) or *Bradyrhizobium japonicum* USDA 16 (for soybeans), both strains that are known to lack uptake hydrogenase activity. For the first 10 d after sowing, plants were watered twice daily with a nutrient solution (Walsh et al., 1987) containing 0.5 mM KNO_3. For the remaining growth period, they were watered with the same solution but lacking nitrate. Plants were maintained in growth chambers (model PGV 36, Conviron, Winnipeg, Manitoba, Canada) with a 16-h photoperiod (500–600 μmol quanta PAR m^{-2} s^{-1}) and 80% RH. For lupins, the day/night air temperatures were 20/17°C, whereas soybeans were maintained at a constant 20°C. Lupin and soybean plants were used for experiments in late vegetative growth, which was between 38 and 45 d and 28 and 35 d, respectively, after planting.

Determining an Appropriate Level of NH_3 for Supply to Shoot Systems of Plants

Farquhar et al. (1980) reported that when leaves of *Phaseolus vulgaris* L. were exposed to a pNH_3 in air of greater than about 2.5 nL L^{-1} (i.e. the NH_3 compensation point), they absorb NH_3 from the atmosphere. They showed that the rate of NH_3 uptake increased linearly with pNH_3 between 5 and 50 nL L^{-1} (slope of 0.095 nmol NH_3 m^{-2} leaf s^{-1} [nL L^{-1}]$^{-1}$, Table I, item 1), and this relationship was used to determine the NH_3 concentration required to supply an amount of N that was equivalent to or higher than that normally obtained via primary N assimilation by a lupin plant similar to those used in the present study.

To determine the approximate rate of primary N assimilation in the experimental plants, calculations were carried out as shown in Table I. Given a plant dry weight of 2.5 g (40 d after planting) and a relative growth rate of 0.1 d^{-1}, and assuming an N content in dry matter of 3% of dry weight, we estimated the N assimilation rate to be 7.5 mg N $plant^{-1}$ d^{-1} (Table I, item 2A) or 365 nmol NH_3 m^{-2} s^{-1} (Table I, item 2B). This was close to the value of 8 mg N $plant^{-1}$ d^{-1} that was reported for 37- to 50-d-old lupins (Pate et al., 1981). Given this rate of primary NH_3 assimilation and the defined relationship between the NH_3 concentration and the leaf NH_3 assimilation rate (Table I, item 1), we estimated that a concentration of about 3.8 μL L^{-1} NH_3 would be required in the gas phase to generate a leaf NH_3 assimilation rate equivalent to the current N_2 fixation rate, thereby doubling the overall rate of NH_3 assimilation within the whole plant (Table I, item 3). Consequently, shoots of lupin were exposed to NH_3 that ranged from 5 to 200 μL L^{-1} to determine a suitable concentration to alter whole-plant N status, phloem N content, and nitrogenase activity without inhibiting photosynthesis or causing visible damage to leaves.

Setup for Supplying NH_3 to Plant Shoots

Plants were transferred to a growth chamber (model E8, Conviron) in the proximity of the gas-exchange system. Gas bags (200 L) that were used to supply gases to the

Table I. *The gas-phase NH_3 concentration required to double the NH_3 assimilation rate within a lupin plant*

Item	Value
1. Relationship between the NH_3 uptake rate in a leaf and the NH_3 concentration in the gas phase (nmol NH_3 m^{-2} leaf s^{-1} [μL L^{-1}]$^{-1}$)[a]	95
2. Primary N assimilation rate in a 40-d-old lupin A	
A. mg N $plant$ d^{-1}[b]	7.5
B. nmol NH_3 m^{-2} leaf s^{-1}	365
3. NH_3 concentration required to double the rate of NH_3 assimilation within the plant (μL L^{-1})[c]	3.8

[a] Calculated as the slope of the relationship for *P. vulgaris* L. at 26°C, published in figure 1 of Farquhar et al. (1980). [b] Calculated as whole-plant dry weight (2.5 g) × relative growth rate (0.1 d^{-1}) × N content in dry matter (0.03). Item 2 B incorporated the leaf area of the lupin plants in this study (0.017 m^2 $plant^{-1}$). [c] Item 2 B/item 1.

shoots of the control and treatment plants were constructed from sheets of laminated nylon and polyethylene (Multipak, Montreal, Quebec, Canada) and equipped with an outlet tube, a silicon septum for injection of gases, and glass beads to assist gas mixing. The gas bags were first filled with air, and then a volume of pure CO_2 was injected into each bag to obtain a final concentration of 1500 μL L^{-1} CO_2 in air. In experimental treatments, an additional volume of pure NH_3 was injected into the gas bag to give a final concentration of 5 to 200 μL L^{-1} NH_3 in air.

Shoots of plants were enclosed in clear, open-bottomed 6-L gas bags that were equipped with inlet and outlet ports. The bags were sealed at the base of the stem with a flexible sealant (Terostat IX, Teroson GmBh, Heidelberg, Germany) (Fig. 1). The appropriate mixture of gases from the 200-L gas bags was supplied to the shoot systems of plants by means of pumps at a rate of 500 mL min^{-1}. Both control and treatment plants were first acclimatized by having the air-CO_2 mixture pass through the shoots for 1 h, after which the in-flow of gases to the shoot of the treatment plant was switched to the air-CO_2-NH_3 mixture. Effluent gases were either vented into the atmosphere (control) or passed through a syringe that was filled with water-soaked glass wool and then bubbled into a volume of water to trap NH_3 (treatment). Treatments lasted 6 h, during which time the nitrogenase activity of the nodulated roots was measured as the H_2 evolution in an N_2:O_2 (80:20, v/v) gas mixture as described below.

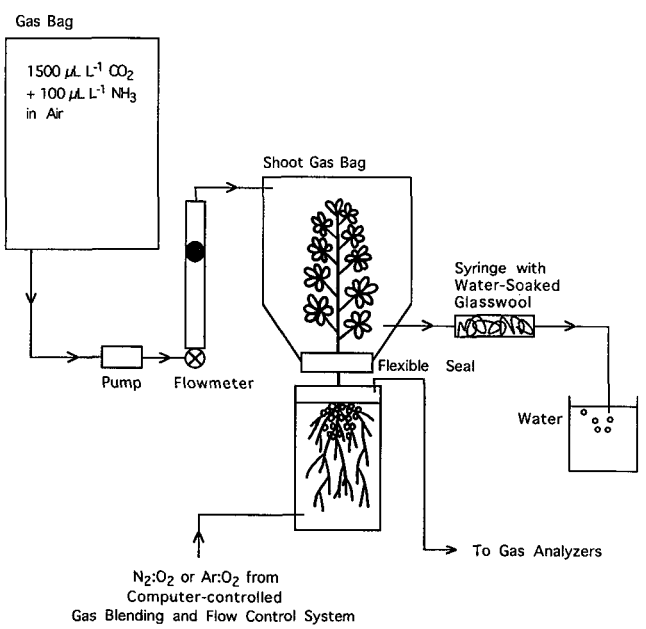

Figure 1. Diagrammatic representation of the system that was used to deliver air-CO_2 or air-CO_2-NH_3 gas mixtures to shoots of nodulated lupins and soybeans. The computer-controlled gas blending and flow-control system and the gas analysis system for the measurement of nitrogenase activity were previously described by Hunt et al. (1989). Only one of the two systems is shown here. See the text for a full description.

Measurements of Nitrogenase Activity and Photosynthetic Rate

The nodulated root systems were sealed for gas exchange and provided with a continuous flow of H_2-free gas, and nitrogenase activity was monitored by measuring the H_2 concentration in the effluent gas stream as described previously (Hunt et al., 1989; Diaz del Castillo et al., 1992). When the gas provided to the pot was an 80:20 mixture of N_2:O_2, H_2 production provided a measure of ANA. ANA stabilized during the 1-h acclimatization period when the air-CO_2 mixture was being passed through the shoots of both the control and treatment plants. After the start of the NH_3 treatment, ANA was measured throughout the 6-h period at 5-min intervals.

Two separate experiments were carried out. In the first experiment, after ANA was monitored for 6 h, the supply of gases to the shoots was maintained, whereas that to the nodulated root system was switched to Ar:O_2 (80:20, v/v). The rate of H_2 evolution under these conditions provided a measure of TNA. As soon as the peak TNA was attained, the O_2 concentration was increased (ramped) linearly from 20 to 100% over a 30-min period (Hunt et al., 1989). The maximum rate of H_2 evolution during the ramp was termed PNA (Diaz del Castillo et al., 1992). Gas bags were then removed from the shoots of plants and the photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) of three leaves from each plant was measured at an irradiance of 500 μmol quanta PAR m^{-2} s^{-1} using a portable IR gas analyzer (model LI-6200, Li-Cor, Lincoln, NE).

In the second experiment, after ANA was monitored for 6 h, the shoot gas bags were removed, nitrogenase measurements ceased, and the plants were used for the collection of phloem sap exudates.

Phloem Sap Collection

At the end of the 6-h treatment period, gas bags were removed from the shoots and phloem sap was collected in both lupin and soybean. In lupin incisions at the base of the stem and on the petioles of leaves 1 to 8 (from the bottom) were used to obtain pure phloem exudates as described previously (Layzell et al., 1981). This was done within 40 min immediately after the treatment. The phloem exudates were immediately stored on ice, and when the collection was completed, samples were stored at −196°C until subsequent analysis.

Since soybean plants do not readily bleed phloem, the shoots of the plants were cut off at the base of the stems and immediately re-cut under an EDTA solution consisting of 5 mM EDTA in 5 mM sodium phosphate/disodium phosphate buffer (pH 6.0) (Groussol et al., 1986). The leaves were misted with water and covered with plastic wrap (to prevent transpiration) and the cut stem was immersed in 5 mL of EDTA solution in the dark for 3 h. At the end of the collection period, the volume of the EDTA solution was measured and was stored at −196°C until subsequent analysis.

Determination of C/N Ratios of Phloem Sap

In lupin all of the phloem sap that was collected from a single plant was pooled into one sample. Sugar (Suc) content was measured by refractometry (Layzell et al., 1981) and total amino N was estimated using a modified ninhydrin assay with Asn as a standard (Moore and Stein, 1948).

In soybean Suc was measured in the EDTA phloem exudate using the anthrone method of Graham and Smydzuk (1965). Total amino compounds were determined using ninhydrin (Moore and Stein, 1948) with Asn as the standard, whereas the ureides (allantoin and allantoic acid) were determined by basic hydrolysis of allantoin to allantoic acid, acid hydrolysis of allantoic acid to glyoxylate and urea, and colorimetric determination of glyoxylate after its reaction with phenylhydrazine and ferricyanide (Vogels and Van der Drift, 1970). From these results, the C:N ratios (g/g) of phloem sap were calculated for both lupin and soybean.

Determination of Amino Acid Composition of Phloem

Samples of lupin pure phloem sap (5 μL) and soybean phloem exudate (0.5 mL) from all replicate plants of each treatment were pooled. The pure phloem sap was analyzed directly for amino acid composition using lithium buffers in the physiological fluids mode of an amino acid analyzer (Beckman model 7300, Hospital for Sick Children, Toronto, Ontario, Canada). Precipitation of EDTA from the phloem exudates of soybean was necessary before an analysis was conducted of the amino acids. This was achieved by addition of 60 μL of 1 N HCl/mL EDTA solution (Layzell and LaRue, 1982).

Statistical Analysis

Results were analyzed by one-factor analysis of variance ($P < 0.05$) using a statistical software package (Statview, SE+Graphics, Abacus Concepts, Berkeley, CA).

RESULTS

Identifying the NH_3 Concentration to Provide to Shoots

When lupin or soybean plants had acclimatized in the shoot chamber for 1 h (exposed to 1500 μL L^{-1} CO_2 in air at 500 mL min^{-1}), the concentration of CO_2 in the effluent gases was consistently greater than 350 μL L^{-1}, and the average photosynthetic rates were typically 9 to 12 μmol CO_2 m^{-2} s^{-1} (data not shown).

Various concentrations of NH_3 from 5 to 200 μL L^{-1} were supplied to lupin shoots in the in-flow gas stream to identify the highest concentration that could be provided without causing either leaf damage or a reduction in photosynthesis. At 200 μL L^{-1} NH_3, lesions were observed on the leaves after 6 h of exposure; however, in the range 5 to 100 μL L^{-1} NH_3 there was no noticeable leaf damage (data not shown), and at 100 μL L^{-1} NH_3, the photosynthetic rate was not significantly different from that in the control lupin plants (Table II, treatment 1). At 100 μL L^{-1} NH_3 for 6 h, photosynthesis in soybean was also found to be similar to that in the control population (Table II, treatment 1), and there was no visible effect of the treatment on the soybean leaves.

When lupin shoots were exposed to atmospheres containing 0, 5, 10, 20, or 40 μL L^{-1} NH_3 for 6 h, ANA was unaffected (Fig. 2). However, at 100 and 200 μL L^{-1} of NH_3, ANA started to decrease at 4 h after treatment, and by 6 h it was 48 and 41%, respectively, of the initial ANA (Fig. 2). Since a 6-h exposure to 100 μL L^{-1} NH_3 caused a reduction in ANA without causing leaf damage or reducing photosynthesis, that treatment was selected for subsequent studies with both lupin and soybean.

Specific Nitrogenase Activities in Lupin and Soybean

After shoots of lupin or soybean had been acclimatized for 1 h to an atmosphere of 1500 μL L^{-1} CO_2 in air, the rate of H_2 production by the nodulated root was identified as the initial ANA. Mean initial ANA values of untreated

Table II. *Effects of a treatment of 100 μL L^{-1} NH_3 (500 mL min^{-1} for 6 h) to the shoot systems of lupin and soybean, on the gas-exchange characteristics of nodulated roots and the photosynthetic rates of these plants*

Data for lupin and soybeans are from separate experiments, and values shown are the means (± SE) of six replicate plants. An asterisk (*) beside a value denotes means that are significantly different from controls.

Treatment	Lupin		Soybean	
	Control	+NH_3	Control	+NH_3
1. Photosynthetic rate at photon flux of 500 μE $m^{-2}s^{-2}$ (μmol CO_2 m^{-2} s^{-1})	10.1 ± 0.65	9.7 ± 0.46	11.5 ± 0.69	11.0 ± 0.71
2. Final ANA, at 6 h after treatment (% of initial ANA)	103 ± 3.7	54 ± 4.2*	121 ± 12.3	54 ± 1.7*
3. TNA (% of control TNA)[a]	100	59 ± 5.1*	100	49 ± 5.6*
4. PNA (% of control PNA)[a]	100	107 ± 5.0	100	94 ± 9.7
5. EAC[b]	0.60 ± 0.02	0.63 ± 0.02	0.71 ± 0.02	0.73 ± 0.01
6. OLC_N[c]	0.77 ± 0.01	0.42 ± 0.03*	0.94 ± 0.01	0.50 ± 0.04*

[a] Calculated by random pairing of control and treatment plants. [b] Calculated as 1–(ANA/TNA). [c] Calculated as TNA/PNA.

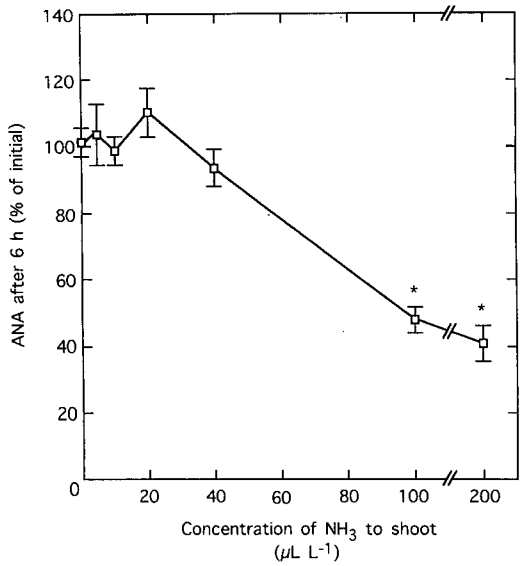

Figure 2. The effect of exposing the shoot of lupin to various NH_3 concentrations on the ANA (H_2 evolution in air) of the nodulated roots. The ANA measurements were made after 6 h of NH_3 treatment and are expressed as a percentage of the initial ANA. Values are means (±SE) of three replicate plants, and asterisks (*) denote means that are significantly different from the initial ANA.

plants were higher in lupin (114 ± 8.2 μmol H_2 g^{-1} DW_{nod} h^{-1}) than in soybean (75 ± 9.8 μmol H_2 g^{-1} DW_{nod} h^{-1}) (Fig. 3) but were typical of the values that were published previously for these species (Hunt et al., 1987, 1989; Diaz del Castillo et al., 1992; de Lima et al., 1994).

In control plants ANA remained relatively constant throughout the 6-h period, the final ANA values being 103 ± 3.7 and 121 ± 12.3% of initial ANA for lupin and soybean, respectively (Fig. 3; Table II, treatment 2). After the final ANA measurement, the gas mixture supplied to the nodulated root system was changed to Ar:O_2 (80:20, v/v) and peak rates of H_2 evolution (TNA) were measured, after which the pO_2 was ramped linearly from 20 to 100% over a 30-min period and the maximum rate of H_2 evolution during the O_2 ramp (PNA) was determined. The mean TNA values for the lupin and soybean control plants were similar (289 ± 8.3 and 297 ± 19.8 μmol H_2 g^{-1} DW_{nod} h^{-1}, respectively; Fig. 3); therefore, the EAC for nitrogenase was lower in lupin (0.60 ± 0.02) than in soybean (0.71 ± 0.01) (Table II, treatment 5).

The PNA for lupin control plants was significantly higher than its TNA, whereas that for soybean was not (Fig. 3). This resulted in a lower OLC_N in lupin (0.77 ± 0.01) than in soybean (0.94 ± 0.01) (Table II, treatment 6). These species differences in OLC_N have been reported previously (Hunt et al., 1989; Diaz del Castillo et al., 1992).

For plants exposed to 100 μL L^{-1} NH_3 to their shoots, ANA remained stable for about 4 h after the start of the NH_3 treatment and declined steadily so that at 6 h the final ANA values were 54 ± 4.2 and 54 ± 1.7% of initial ANA for lupin and soybean, respectively (Fig. 3; Table II, treatment 2). Rates of H_2 evolution in Ar:O_2 (80:20, v/v) showed that TNA was also inhibited to 59 ± 5.1% of control plants in lupin and 49 ± 5.6% of control plants in soybean (Fig. 3; Table II, treatment 3). Therefore, the EAC values in lupin (0.63 ± 0.02) and in soybean (0.73 ± 0.01) were similar to that in the respective control plants (Table II, treatment 5).

Despite the NH_3-induced reduction in ANA and TNA, values for PNA were similar to those in the control plants in both lupin (399 ± 10.7 μmol H_2 g^{-1} DW_{nod} h^{-1}) and soybean (292 ± 23.0 μmol H_2 g^{-1} DW_{nod} h^{-1}) (Fig. 3). Consequently, values for OLC_N were significantly lower than the control plants in both lupin (0.42 ± 0.03) and soybean (0.50 ± 0.04) (Table II, treatment 6). This observation, and the fact that PNA of both lupin and soybean occurred at higher pO_2 values in the treated plants than in the control plants (Fig. 3), showed that the NH_3-treated nodules were much more O_2-limited than the nodules of the control plants.

C:N Ratios and Composition of Phloem Sap That Supplies Nodulated Roots

In plants in which the shoots were supplied with 100 μL L^{-1} NH_3 for 6 h, the C:N weight ratios of the phloem sap

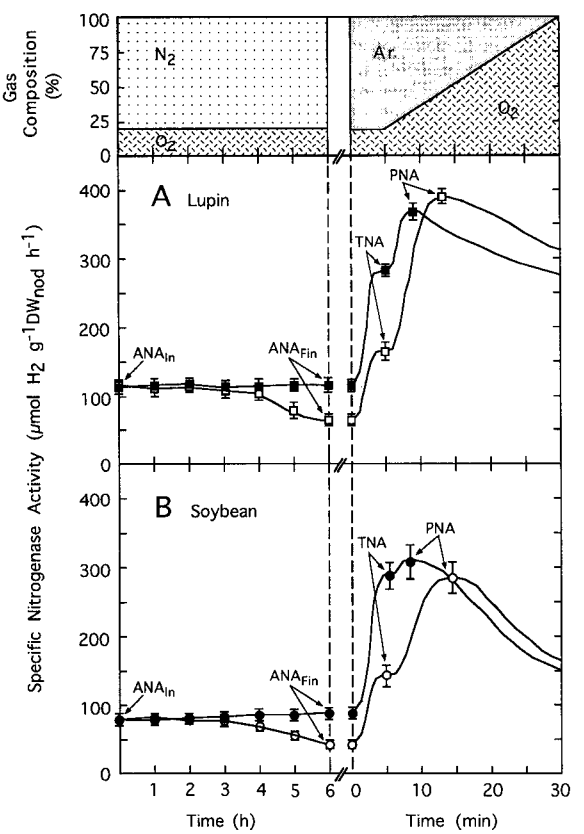

Figure 3. Effect of 100 μL L^{-1} NH_3 (500 mL min^{-1} for 6 h) to the shoot systems of lupin (A) and soybean (B) on the specific nitrogenase activity (μmol H_2 g^{-1} DW_{nod} h^{-1}) of nodules. ANA (H_2 evolution in N_2:O_2, 80:20, v/v) was monitored throughout the 6-h treatment (ANA_{In}, initial ANA; ANA_{Fin}, final ANA), after which TNA (H_2 evolution in Ar:O_2, 80:20, v/v) and PNA (peak H_2 evolution during an O_2 ramp in Ar) were measured. Control (■, ●) and treatment (□, ○) values are means (±SE) of six replicates.

collected from petioles and stem bases were only 19.9 ± 1.3 (lupin) and 5.3 ± 0.5 (soybean), compared with those from control plants, which were 27.4 ± 3.5 and 13.5 ± 1.5 for lupin and soybean, respectively (data not shown). The molar concentration of Suc in the phloem was similar in samples from the control and treated plants, whereas the molar concentration of ninhydrin reactive compounds (lupins) or ureides plus ninhydrin reactive compounds (soybeans) was higher in the samples from the treated than the control plants (data not shown).

A detailed analysis of the amide and amino acid content of the phloem sap samples by HPLC confirmed the pattern observed from ninhydrin measurements (Asn as a standard) but, on average, the total ninhydrin assay resulted in predicted total amino levels of about 8% higher than that obtained by HPLC. Consequently, the HPLC data were used only to provide information concerning the relative amino acid and amide composition of the sap, whereas the ninhydrin assay plus ureide assay was used to provide a quantitative measure of its total N content.

In the phloem Suc was the major organic constituent and accounted for more than 90% of the C in control plants (Fig. 4). In phloem of control plants of lupin, the major N compounds were Asn (55% of total N) and Gln (17% of total N) (Fig. 4A), whereas in control soybean plants, ureides accounted for 26% of total N, and Asn, Gln, Asp, and Glu contributed 15, 6, 9, and 9%, respectively, of the total N (Fig. 4B).

The supply of 100 μL L^{-1} NH_3 (for 6 h) to the shoots of the lupin and soybean plants not only increased the total N level in the phloem but also changed the relative levels of specific N compounds in the phloem. For example, in lupin the N level of pure phloem sap from the NH_3-treated plants was 1.3-fold higher than that in the control sap, a change associated with a 4.8-fold increase in the Gln-N concentration in the phloem (from 0.46 to 2.2 g N L^{-1}). This was accompanied by decreasing the amount of the Asn-N concentration by one-half so that Gln accounted for 62% of total N in phloem sap from the NH_3-treated plants compared with 17% in the control plants (Fig. 4A).

Similarly, in soybean the Gln-N concentration in the phloem exudate of the NH_3-treated plants (12.6 mg N L^{-1}) was 10.5 times higher than that in the control plants (1.2 mg N L^{-1}). The Asn-N and ureide-N content of phloem also increased slightly with the NH_3 treatment, but the proportion of total N that was present in Gln increased from 6 to 23% (Fig. 4B).

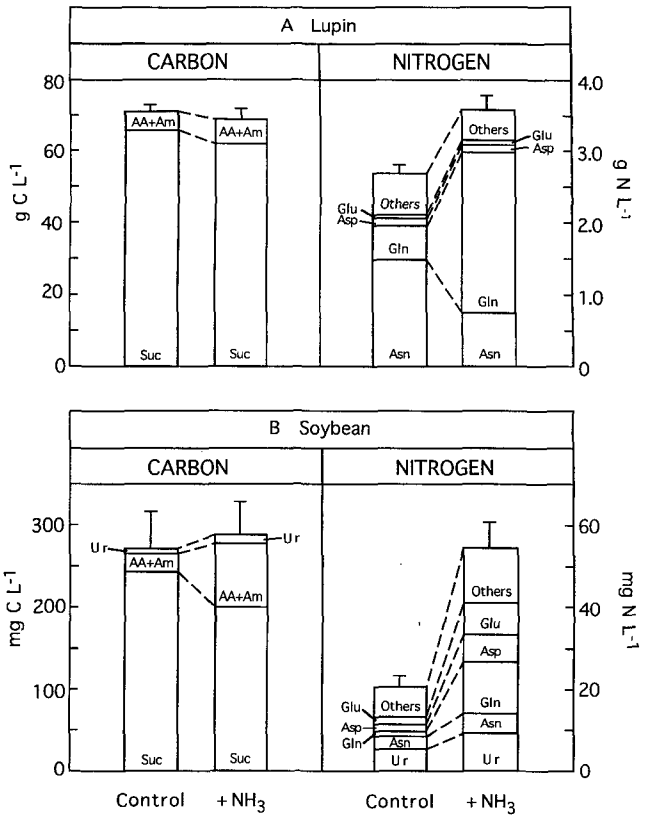

Figure 4. The relative contribution of Suc, amino acids (AA), amides (Am), and ureides (Ur) to the composition of C and N in phloem sap collected from lupin (A) or soybean (B). The phloem exudate was collected from plants in which the shoots had previously been exposed to 6 h of CO_2-enriched air without (control) or with 100 μL L^{-1} NH_3 (treatment). The total height of each bar represents the total concentration (±SE, n = 6) of C or N in the pure phloem sap for lupin or in the EDTA phloem exudate for soybean.

DISCUSSION

Does Phloem N Content Affect the Resistance of a Nodule to O_2 Diffusion?

The treatment of 100 μL L^{-1} NH_3 to the shoots of lupin and soybean plants resulted in a decrease in the C:N (weight) ratios of phloem sap, due largely to a 4.8-fold (lupin) to 10.5-fold (soybean) increase in the concentration of Gln-N in the phloem sap that supplies the nodulated root (Fig. 4). Associated with these changes in the phloem sap composition was a decline in nitrogenase activity (ANA and TNA) that began within 4 h of the NH_3 treatment and reached 54% of initial ANA within 6 h of the start of the treatment (Fig. 3; Table II). However, PNA values in treated plants were not significantly different from those of the control plants (Fig. 3; Table II), showing that the NH_3 treatment inhibited nodule metabolism and N_2 fixation by causing the nodules to be more O_2-limited. For example, with the NH_3 treatment, the OLC_N declined from 0.77 ± 0.01 to 0.42 ± 0.03 in lupin and from 0.94 ± 0.01 to 0.50 ± 0.04 in soybean (Table II). These results support the hypothesis that changes in the N composition of phloem sap-supplying, nodulated roots may play a role in the feedback regulation of nitrogenase activity by altering nodule resistance to O_2 diffusion.

Verification of the NH_3-Feeding Technique

The NH_3 treatment was chosen as a means of modifying the N content of the phloem sap supplied to nodules without altering the leaf photosynthetic rate, the C content of the phloem, or the N metabolism in the nodulated root. In this regard, it has advantages over the other methods that have been used to alter phloem sap composition, including

defoliation (Heim et al., 1993; Hartwig et al., 1994), depodding (Fujita et al., 1991), debudding (Oti-Boateng et al., 1994), Ar:O_2 treatment (Pate et al., 1984), 100% O_2 treatment (Parsons and Baker, 1996), or nitrate or ammonia fertilization to roots (Parsons and Baker, 1996).

In 1980, Farquhar and co-workers showed that plants are able to take up NH_3 from the atmosphere if it is present at a concentration that is greater than their NH_3 compensation point. Presumably, the pathway of NH_3 entry into the leaf cells would be via Gln synthetase/glutamate synthase within the photorespiratory N cycle, a pathway that has been estimated to operate at about 10 to 20 times the rate of primary N assimilation within a plant (Canvin, 1981). In the present study the shoots were exposed to a humid atmosphere with a steady-state partial pressure of CO_2 between 350 and 1500 μL L^{-1}; therefore, photorespiration would likely be greatly reduced, possibly freeing up the N capacity for assimilation of the NH_3 provided within the atmosphere. Using the relationship between the NH_3 uptake rate and the NH_3 concentration reported by Farquhar et al. (1980), we estimated that the rate of NH_3 uptake by leaves would be equivalent to the primary N assimilation rate at a pNH_3 of about 3.8 μL L^{-1} (Table I). Therefore, at 100 μL L^{-1} NH_3, the NH_3 uptake rate may be as much as 25 times the primary N assimilation. This enhanced rate of N assimilation in the leaves resulted in a 1.3-fold (lupin) to 2.6-fold (soybean) increase in the total N content of phloem and a 4.8-fold (lupin) to 10.5-fold (soybean) increase in the concentration of Gln-N in the phloem.

It was possible that the NH_3 treatment used here would alter whole-plant N assimilation to a greater extent than that which may occur under natural conditions. Nevertheless, the regulatory processes being examined were likely to operate under conditions that plants may experience during normal growth and development, since the large increase in NH_3 assimilation was localized to the shoot, and the timing of the effects on nodulated root metabolism was consistent with the rate of phloem delivery to nodulated roots. For example, studies by Kouchi et al. (1985) showed that it took 3 to 4 h for labeled C that was fed to a leaf of soybean to be delivered to the nodules. This time course fits well with the time required for the first observed decline in nitrogenase activity, and it suggests that nodules are able to rapidly alter their resistance to O_2 diffusion in response to changes in the phloem N content.

Previous studies (Fensom et al., 1990) have provided evidence that the NH_3 supply to the shoot may block phloem transport by inhibiting phloem loading; however, those experiments involved the use of NH_3 concentrations 500 to 10,000 times greater than that in the present study. Moreover, in our experiments there was no effect of the NH_3 treatment on the photosynthetic rate or the rate of phloem exudation, both phenomena that would probably be sensitive to reductions in phloem loading (Herold, 1980).

What Aspect of Phloem Composition Regulates Nitrogenase Activity?

Several previous studies have provided indirect evidence that the composition of the phloem sap-supplying nodules may be involved in the feedback regulation of nitrogenase activity. For example, nitrogenase activity decreased following pod removal in soybean (Fujita et al., 1991) and debudding in faba bean (Oti-Boateng et al., 1994), and this was attributed to a decrease in N requirements by the plant and an increase in the pool size of soluble N. Silsbury et al. (1986) used a split-root system with subterranean clover and found that the application of combined N to one-half of the root system resulted in an inhibition of nitrogenase activity on both sides, indicating that phloem-translocatable compounds were involved in the inhibition. In another study using faba bean, Oti-Boateng and Silsbury (1993) showed nitrogenase activity to be inhibited to 30 to 50% of the initial values within 48 h of increasing the soluble N pool of nodules by supplying Asn to plants, either through cut roots or through the stem by direct injection.

A recent study by Parsons and Baker (1996) has provided the first evidence regarding the relationship between phloem sap composition and nitrogenase activity. In that study, nitrogenase activity was inhibited by exposing lupin nodules to nitrate or ammonium for 2 weeks prior to the nitrogenase activity measurements and phloem sap assays. The ammonium treatment, but not the nitrate treatment, was associated with an increase in the Gln concentration in phloem; however, no information was provided concerning whether the observed reductions in nitrogenase activity were associated with an O_2 limitation of nodule metabolism. The severity and long duration of the inhibitory treatments used by Parsons and Baker (1996) made it difficult to ascertain whether the observed changes in phloem sap composition played a causal role in the inhibition of nitrogenase activity.

The shoot-NH_3 treatment used in the current study overcomes some of these limitations and offers some of the clearest evidence to date that (a) the N content of phloem sap plays a role in the feedback regulation of nitrogenase activity, and (b) the feedback regulation of phloem N on nitrogenase activity is mediated by an increase in nodule resistance to O_2 diffusion and the imposition of a severe O_2 limitation of nodule metabolism. In addition, it presents the possibility that, if a specific nitrogenous compound is acting as a "signal molecule," Gln is a likely to be that compound.

An increase in Gln levels was observed in both lupin (an amide exporter) and soybean (a ureide exporter), therefore, it may not be necessary for the "signal molecules" that are involved in regulating nitrogenase activity to be different in ureide and amide symbioses, as hypothesized by Parsons et al. (1993). In both groups of legumes Gln synthetase and glutamate synthase are used in the assimilation of NH_4^+; however, neither Glu nor Gln is the major product of fixed N that is exported from legume nodules. When ureide synthesis is inhibited by allopurinol, soybean or cowpea nodules will accumulate intermediates and downregulate nitrogenase activity rather than export Gln or Glu (Atkins et al., 1988, 1992). In lupins the Gln that is present within the xylem and phloem sap is probably associated with the N that is continuously cycling through the plant and not with the recent products of N_2 fixation (Parsons and Baker, 1996). Since Gln is not exported in large quan-

tities from nodules, its presence in the phloem in a high concentration may indicate to the nodule that the plant has an alternate source of NH_3 in a tissue with adequate supplies of reductant and ATP that are needed for NH_4^+ assimilation. This may provide a basis for down-regulation of nodule metabolism and N_2 fixation.

Comparison of the Shoot NH_3 Treatment and the Ar:O_2 Treatment

A number of environmental and physiological treatments such as plant disturbance, nodule excision, stem girdling, nitrate fertilization, drought, or C_2H_2 exposure have been reported to inhibit nitrogenase activity by causing an increase in the resistance of the nodule to O_2 diffusion (Hunt and Layzell, 1993). However, for most of these inhibitory treatments, O_2 limitation of nodule metabolism is not the only causal factor associated with reduced nitrogenase activity. In most cases a portion (from 30–80% or more) of the inhibition can be attributed to a reduction in PNA, i.e. the maximum nitrogenase activity that can be supported at an optimal O_2 concentration (Vessey et al., 1988a; Sung et al., 1991; Diaz del Castillo et al., 1992).

Excluding the NH_3 treatment used in the present study, extended exposure to Ar:O_2 is the only inhibitory treatment that has been described that inhibits ANA and TNA but does not affect PNA. Therefore, the NH_3 treatment is similar to the Ar:O_2 treatment because it seems to reduce nitrogenase activity solely by regulating the supply of O_2 to the infected cells. It is interesting that the Ar:O_2 treatment is, in many ways, "opposite" to the NH_3 treatment. The Ar:O_2 treatment rapidly blocks N_2 fixation (without affecting nitrogenase activity, at least initially), and thereby reduces the rate of N assimilation within the nodule. On the other hand, the shoot-NH_3 treatment increases the N assimilation rate in the above-ground portions of the plant. In lupin Ar:O_2 exposure results in a 50% reduction in the phloem N concentration within 24 h, largely due to a decrease in the Asn content; the Gln content of phloem was not affected (Pate et al., 1984). In contrast, the shoot-NH_3 treatment dramatically increased Gln and either decreased (lupin) or slightly increased (soybean) Asn.

Further studies are needed to determine how seemingly opposite treatments may both be involved in the feedback regulation of nitrogenase activity by control of O_2 diffusion. A better understanding of these treatment effects may provide the breakthrough that is needed to understand the complex interrelationships among C metabolism, N assimilation, phloem sap composition, and infected cell O_2 concentration in N_2-fixing legume nodules.

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