# Nitrogen Nutrition and Xylem Transport of Nitrogen in Ureide-producing Grain Legumes[1]

JOHN S. PATE, CRAIG A. ATKINS, SHANE T. WHITE, ROSS M. RAINBIRD, AND K. C. WOO
*Department of Botany, University of Western Australia, Nedlands, Western Australia 6009*

## ABSTRACT

Xylem sap composition was examined in nodulated and nonnodulated cowpea (*Vigna unguiculata* [L.] Walp.) plants receiving a range of levels of $NO_3$ and in eight other ureide-forming legumes utilizing $NO_3$ or $N_2$ as sole source of nitrogen. A $^{15}N$ dilution technique determined the proportions of plant nitrogen derived from $N_2$ in the nodulated cowpeas fed $NO_3$. Xylem sap composition of $NO_3$-fed, nodulated cowpea varied predictably with the relative extents to which $N_2$ and $NO_3$ were being utilized. The ratios of asparagine to glutamine (N/N) and of $NO_3$ to ureide (N/N) in xylem sap increased with increasing dependence on $NO_3$ whereas per cent of xylem nitrogen as ureide and the ratio of ureide plus glutamine to asparagine plus $NO_3$ (N/N) in xylem sap increased with increasing dependence on $N_2$ fixation. The amounts of $NO_3$ and ureides stored in leaflets, stems plus petioles, and roots of cowpea varied in a complex manner with level of $NO_3$ and the presence or absence of $N_2$ fixation. All species showed higher proportions of organic nitrogen as ureide and several-fold lower ratios of asparagine to glutamine in their xylem sap when relying on $N_2$ than when utilizing $NO_3$. In nodulated (minus nitrate) cowpea and mung bean (*Vigna radiata* [L.] Wilczek) the percentage of xylem nitrogen as ureide remained constant during growth but the ratio of asparagine to glutamine varied considerably. The biochemical significance of the above differences in xylem sap composition was discussed.

A number of legumes, especially tropical species, synthesize allantoin and allantoic acid from currently fixed $N_2$ in nodules and use these compounds for transport and storage of N (1, 3, 6, 10). In soybean (*Glycine max* [L.] Merr.) the ureides are more prominent in nodulated plants than in plants grown with $NO_3$ or $NH_4$ salts (8, 11), suggesting a special association between $N_2$ fixation and ureide formation (9), and the possibility of using ureide levels in plant tissues or transport fluids to determine the extent to which a field-grown crop is relying on symbiosis as opposed to combined forms of N from soil or fertilizer. This paper describes xylem sap composition of $N_2$ and $NO_3$-fed cowpea (*Vigna unguiculata* [L.] Walp.) and of other grain legumes which form ureides, and suggests how compositional features of the sap might be used to evaluate the $N_2$-fixing status of nodulated plants grown in the presence of $NO_3$.

## MATERIALS AND METHODS

The species studied were cowpea (*V. unguiculata* [L.] Walp. cv Caloona), mung bean (*Vigna radiata* [L.] Wilczek), soybean (*G. max* [L.] Merr.), cluster bean (*Cyamopsis tetragonoloba* [L.] Taub-

ert), horse gram (*Macrotyloma uniflorum* [Lam.] Verd.), wing bean (*Psophocarpus tetragonolobus* [L.] DC), adzuki bean (*Vigna angularis* [Willd.] Ohivi and Ohashi), rice bean (*Vigna umbellata* [Thunb.] Ohivi and Ohashi) and black gram (*Vigna mungo* [L.] Hepper). A preliminary screening program had shown all species to be rich in ureides.

Plants were grown in pots of heat sterilized sand during summer in a naturally lit glasshouse maintained within the temperature range 20–35 C by supplementary heating (night) and evaporative cooling (day). One set of plants of each species was inoculated with an effective *Rhizobium* (strain CB437, for soybean, strain NGR234 for horse gram, and strain CB756 for all other species) and grown with a N-free culture solution. A matching set of uninoculated plants received culture solution containing 10 mM $NO_3$. By using this level of $NO_3$, sterile sand and effective surface sterilization of seed the plants remained free of root nodules throughout growth. These nonnodulated plants accumulated nitrogen at rates equal to or greater than did nodulated (minus $NO_3$) plants of comparable age. Root xylem (bleeding) sap was collected from 10 to 16 plants of each species in mid-vegetative growth as detailed previously (15). Xylem sap was collected from nodulated plants of cowpea and mungbean throughout growth to relate changes in sap composition to ontogenetic variations in $N_2$ fixation rate.

In a detailed study of the response of cowpea to nitrate, sets of nodulated and nonnodulated plants were grown at 0, 1, 5, 10, and 20 mM $NO_3$. The cultures were irrigated with the nutrient solutions at least once daily in an attempt to maintain uniform levels of $NO_3$ and other nutrients in the rooting medium. Harvest at 40 days after sowing involved collection of xylem sap from 30 plants of each treatment and separation of plants into leaflets, stem + petioles, and roots for determination of fresh and dry weight and nitrogenous constituents. $^{15}NO_3$ was incorporated at known enrichment (2-10 atm per cent excess) into the culture solutions fed to the nodulated cowpea plants to assess dependence of the plants on $N_2$ and $NO_3$.

**Analyses of Plant Material and Xylem Exudates.** Total N of plant parts was determined by Kjeldahl analysis of dry plant material, using salicylic acid in the digestion mixture if $NO_3$ was present (5). Samples of freshly harvested plant parts were extracted at 0 C in 80% (v/v) ethanol, the extracts evaporated to dryness, partitioned between petroleum ether and water and the water-soluble fraction was used for assay of ureides, amino acids and nitrate. Ureides in tissue extracts and xylem sap were measured colorimetrically as the phenyl-hydrazone of glyoxylate (20). Amino compounds of xylem sap were determined by using a Beckman 118C amino acid analyzer in the "physiological fluids" mode, with low temperatures and lithium-based buffers to resolve the amides glutamine and asparagine and nonprotein amino acids. Nitrate in tissue extracts or xylem sap was analyzed by reduction of a diluted sample on Cd:Cu columns and measuring the resulting $NO_2$ by an automated colorimetric technique (22). $^{15}N$ enriched

plant dry matter was assayed for $^{15}$N content by optical emission spectrometry (16) and the proportions of N coming from NO$_3$, N$_2$ and cotyledon N determined by using an isotope dilution technique (13).

## RESULTS AND DISCUSSION

**Response of Nodulated and Nonnodulated Cowpea to a Range of NO$_3$ Levels.** Contents of dry matter, total N, NO$_3$ and ureide in plant parts from the 0, 1, 5, 10, and 20 mM NO$_3$ treatments were as shown in Figure 1. The isotope dilution analyses indicated that nodulated plants derived a decreasing proportion of their plant nitrogen from N$_2$ fixation with increasing level of nitrate (Table 1). Growth and nitrogen accumulation benefited from nodulation in the 1 and 5 mM NO$_3$ treatments. Dry matter production and N accumulation were similar in nodulated and nonnodulated plants fed 10 mM NO$_3$, but values for these quantities were somewhat higher in nonnodulated than in nodulated plants fed 20 mM NO$_3$. Nitrate represented less than 4% of plant total nitrogen and increased in organs of nodulated and nonnodulated plants with increasing dosage of NO$_3$. Stems + petioles were principal sites of NO$_3$ storage especially at high levels. Ureides represented a small proportion (less than 2%) of plant total nitrogen, and tended to be higher in plants depending principally on N$_2$ fixation (1 and 5 mM NO$_3$ nodulated) than in those depending predominantly or entirely on NO$_3$. Nonnodulated plants fed 10 and 20 mM NO$_3$ accumulated higher amounts of ureide than did comparably fed nodulated plants. Stems and petioles were the major locations for ureide storage in symbiotically dependent plants and in nodulated plants fed high levels of NO$_3$, whereas in all nonnodulated plants greater amounts of ureide were accumulated in leaflets and roots than in the stems + petioles. The concentration of NO$_3$ and ureides per unit of fresh weight of plant parts (Fig. 2) varied considerably with NO$_3$ level and nodulation. Tissue NO$_3$ levels of nonnodulated plants remained relatively constant with increasing NO$_3$ dosage whereas in nodulated plants there were clearly defined increases with increasing nitrate. The ureide levels in organs of both nodulated

and nonnodulated plants changed in a complex manner with NO$_3$ level (Fig. 2).

Xylem sap composition of the treatments (Fig. 3A) showed similarity between nodulated and nonnodulated plants in the percentage of nitrogen exported as NO$_3$ from the root. This was 15–30% in plants fed 1 and 5 mM NO$_3$ and from 55 to 80% for plants supplied 10 and 20 mM NO$_3$. The substantial levels of organic N in xylem sap of all nonnodulated treatments suggested that roots reduced incoming NO$_3$, possibly to a greater relative extent at low than at high NO$_3$ supply. Since nitrate storage was insignificant relative to the total N content of the plants the results also suggested a substantial involvement of the shoots of both nodulated and nonnodulated plants in reduction of the NO$_3$ spilled over to the shoot at the higher levels of NO$_3$. Ureides represented less than 10% of the xylem nitrogen in nonnodulated plants and nodulated plants in which N$_2$ fixation had been suppressed by high NO$_3$, contrasting with the significant levels (25–67% xylem nitrogen) of ureide in xylem sap of nodulated plants fed 0, 1, or 5 mM NO$_3$.

The nitrogenous solutes shown in Figure 3A accounted for 86–91% of the total sap nitrogen determined by Kjeldahl analysis, suggesting that there were no other major solutes of nitrogen present in the xylem.

The amino acid balance of xylem sap from all treatments (Fig. 3B) demonstrated a fairly constant ratio of amide nitrogen (asparagine + glutamine) to amino acid nitrogen, but large differences between treatments in the relative proportions of the sap nitrogen represented by the two amides. Aspartic acid, lysine, valine, leucine, and glutamic acid were the major non-amide constituents in xylem sap of all treatments and these five amino acids together represented 15–20% of the ninhydrinpositive compounds assayed.

**Possible Use of Solute Analysis in Assessing Symbiotic Dependence.** The amount of NO$_3$ recovered from the tissues of nodulated plants reflected the increasing NO$_3$ supply to these plants while the declining amount of ureides reflected declining N$_2$ fixation with NO$_3$ dose (Fig. 1). When expressed as concentrations of solute per unit fresh weight of plant parts (Fig. 2) the data
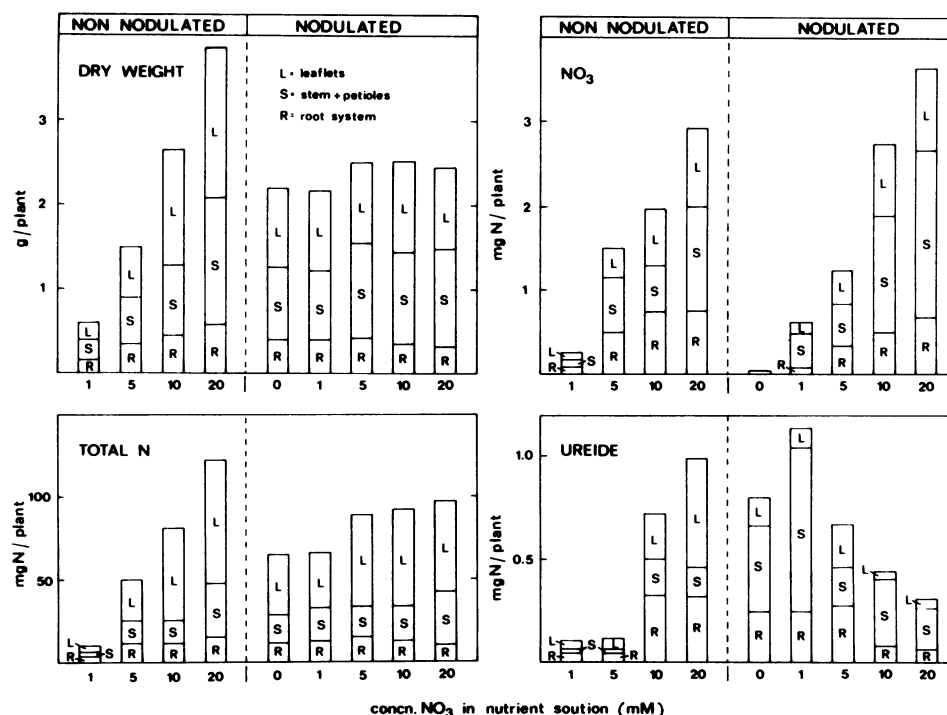


FIG. 1. Dry matter content and contents of total N, NO$_3$-N and ureide-N in nodulated and nonnodulated cowpea (*V. unguiculata* L. [Walp.]) fed a range of constantly maintained levels of nitrate. All plants harvested at 40 days from sowing.

Table I. *Dependence of Nodulated Cowpea (V. unguiculata) on $N_2$ and $NO_3$ as Determined by an Isotope Dilution Technique*

Plants were harvested at 40 days after feeding on culture solutions containing [15]$NO_3$ at known, constantly maintained enrichment (2–10 atm % excess ^{15}N).

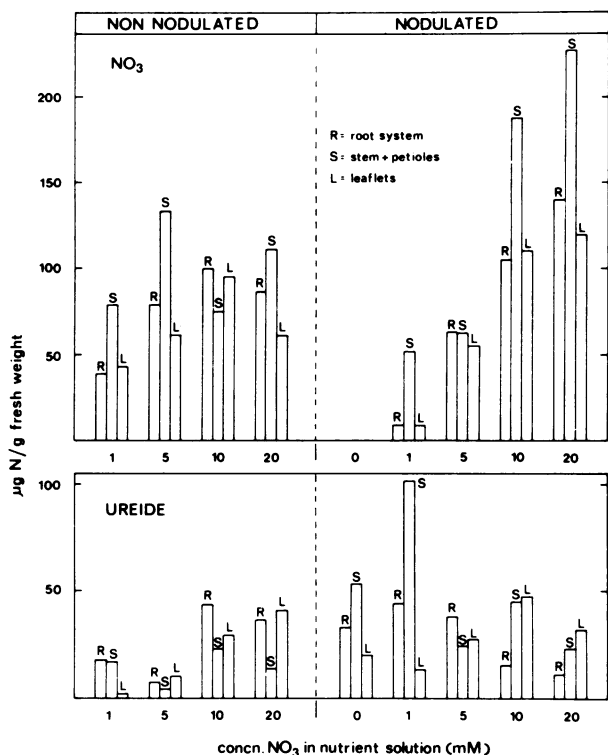| Level of $NO_3$ in Culture Solution | % Total Plant N Derived from | | |
|---|---|---|---|
| | Cotyledons | $NO_3$ | $N_2$ Fixation |
| *m*M | | | |
| 0 | 1.6 | 0 | 98.4 |
| 1 | 1.3 | 9.5 | 89.2 |
| 5 | 1.1 | 33.5 | 65.4 |
| 10 | 1.2 | 81.8 | 17.0 |
| 20 | 1.0 | 85.7 | 13.3 |



FIG. 2. Concentrations of nitrate and ureide in organs of 40-day non-nodulated and nodulated cowpea (*V. unguiculata* L. [Walp.]) fed a range of constantly maintained levels of nitrate.

showed considerable dissimilarity between nodulated and non-nodulated plants and exposed the complex nature of the response of storage systems to the level of supplied $NO_3$. Because of these complexities, the relatively small proportions of total nitrogen as $NO_3$ or ureide in any of the organs and the possibility of considerable diurnal variation in these pools, it seemed unlikely that tissue analysis would be useful for assessing the symbiotic status of nodulated plants utilizing $NO_3$.

Since the nitrogenous solutes of xylem sap were likely to represent current products of N assimilation (14) and since there were substantial differences in sap composition between $N_2$- and $NO_3$-fed plants, an assay system based on xylem sap analysis was regarded as a reliable means of assessing plant dependence on symbiosis in the presence of $NO_3$. Four quantities were selected for diagnostic use in cowpea, namely percentage of xylem sap N as ureide and the ratios (N/N) of ureides + Gln to Asn + $NO_3$, of $NO_3$ to ureide, and of Asn to Gln. The relationship of these quantities to the level of applied $NO_3$ and the plant's dependence on symbiosis were as shown in Figure 4. The proportion of ureide
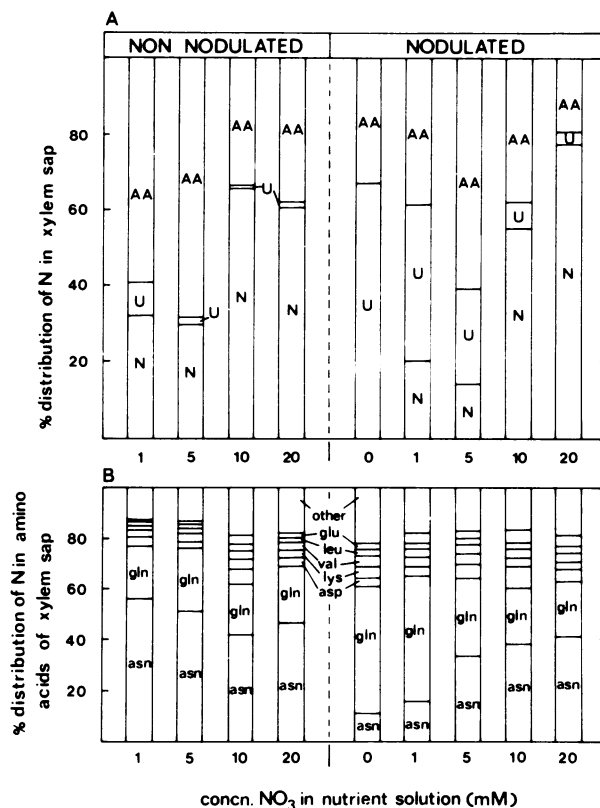


FIG. 3. Xylem sap composition of 40-day, nonnodulated and nodulated cowpea (*V. unguiculata* L. [Walp.]) fed a range of constantly maintained levels of nitrate. A: percentage distribution of xylem N amongst nitrate (N), ureides (U) and ninhydrin-positive amino compounds (AA), these three forms of N representing from 86 to 91% of the total N of the xylem sap. B: relative composition of the amino fraction of the sap.

in the sap and the ratio of ureide + Gln to Asn + $NO_3$ rose predictably with increasing symbiotic activity relative to $NO_3$ assimilation whereas the ratios for $NO_3$ to ureide and Asn to Gln increased with decreasing $N_2$ fixation and increasing reliance on $NO_3$.

The levels of amino acids and ureides in xylem sap of eight additional species were examined (Fig. 5) to determine whether the diagnostic features described above for cowpea were likely to be of value if applied to other ureide-synthesizing grain legumes. In all cases the proportion of xylem nitrogen as ureide was much greater in nodulated (minus $NO_3$) plants than in nonnodulated plants grown with 10 mM $NO_3$. This generalization applied whether ureides were a large (up to 90%) or small (15%, see *Macrotyloma uniflorum*) proportion of the xylem sap N. Similarly in all species Asn to Gln (N/N) ratios were much higher (by 2- to 23-fold) in xylem sap of $NO_3$-fed plants without nodules than in effectively nodulated (minus $NO_3$) plants (Asn to Gln ratio usually considerably less than 1) (Fig. 5). The species therefore behaved similarly to cowpea, although the range of values for the two quantities examined varied widely among the species, and it was not ascertained how the species would respond when relying on both $N_2$ and $NO_3$.

Since variations during growth in proportions of N solutes exported from roots might be a complicating feature in any assay system based on xylem exudates, the extent of such variation was assessed in nodulated cowpea and mung bean, using Asn to Gln ratio and per cent N as ureides in xylem sap as test quantities and relating their variations to ontogenetic changes in specific activity of $N_2$ fixation (Table II). The study indicated that the proportion of nitrogen as ureide in sap was a relatively stable quantity,
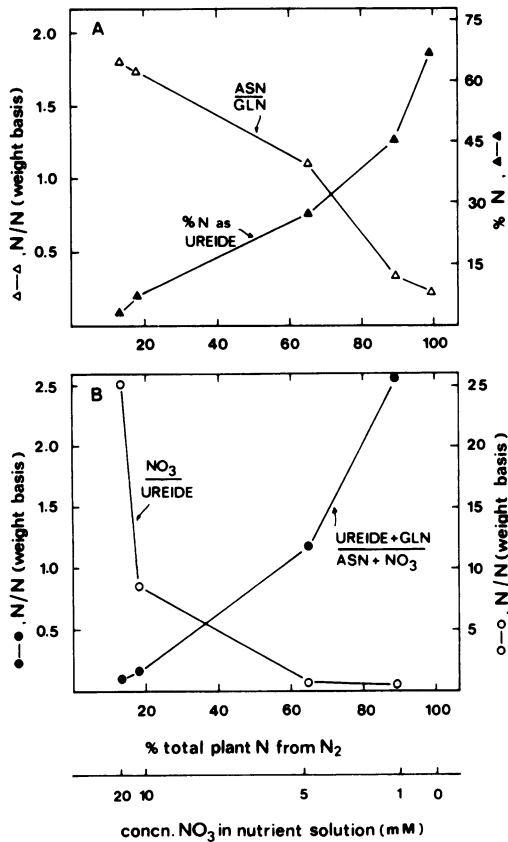
FIG. 4. Relationships between compositional features of xylem sap of 40-day nodulated cowpea and the level of nitrate in the nutrient solution, and the dependence of the plants on $N_2$ fixation as opposed to $NO_3$ reduction. Note the decrease in two of the quantities and the increase in the other two quantities with decreasing fixation of $N_2$ relative to $NO_3$ assimilation.

representing from 80 to 90% during growth of mung bean and from 81 to 95% during growth of cowpea. Asn:Gln ratio, by contrast, varied widely during growth, and there was a tendency for the ratio to be low when rates of nitrogen fixation per unit weight of nodules were at or near maximum.

The results suggested that xylem sap analyses might be used predictably under field conditions to measure symbiotic activity of a ureide-forming legume in the presence of combined N. To be realistic the assay would need to be calibrated carefully under glasshouse conditions as suggested in the present study on cowpea. The xylem solutes to be assayed would then be selected on the basis of these calibration data and the range of levels of combined N likely to be available from the soil during the assay period. With suitable modification for different species and for different stages of growth, the technique might prove reliable and of wide application.

**Metabolism of Nitrogen in Relation to Xylem Sap Composition.** Legume species in which ureides have not been detected (*eg. Lupinus albus* L.) or are present at low level (*e.g. Pisum sativum* L.) typically export organic N from their roots mainly as the amides asparagine and glutamine. Asparagine is usually present at higher concentration than glutamine and xylem sap composition does not change appreciably with the form of N (*e.g. N_2, NO_3^-, NH_4^+*, urea) (2, 21). By contrast all ureide-producing legumes examined so far show very different mixtures of xylem compounds when feeding symbiotically by root nodules or when assimilating a combined source of N. A high proportion of N as ureide and a high level of glutamine relative to asparagine is typical of $N_2$-fed plants, a greatly reduced output of ureide and a
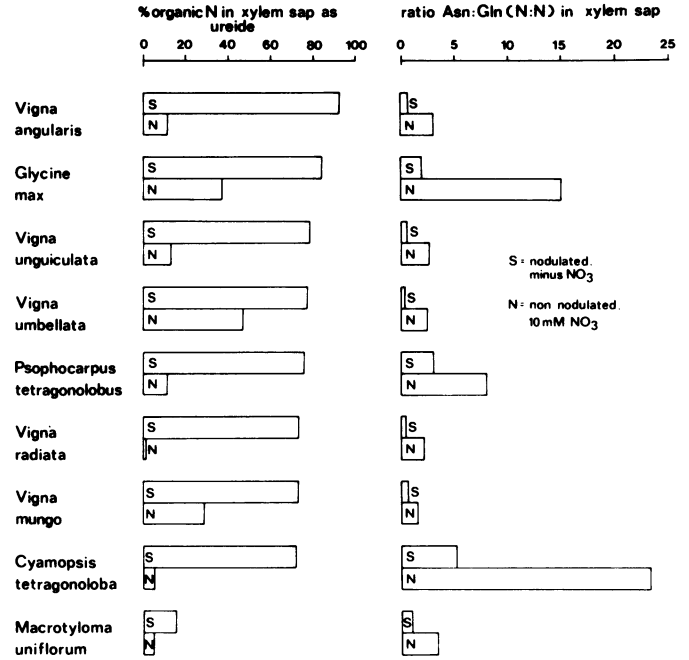


FIG. 5. Relative abundance of ureides and balance between Asn and Gln in xylem sap of a range of ureide-synthesizing grain legumes. The comparisons for each species are between symbiotically effective, nodulated plants grown without nitrate (S) and nonnodulated plants fed 10 mM $NO_3$ (N).

Table II. *Variation with Plant Age and Nitrogen Fixation Activity in the Balance of Asparagine to Glutamine and in the Relative Abundance of Ureides in Xylem Sap of Nodulated Cowpea (V. unguiculata) and Mung Bean (Vigna radiata) Grown in the Absence of Combined Nitrogen*

| Time after Sowing | Asparagine to Glutamine | N as Ureide in Xylem Sap | Rate of $N_2$ Fixation |
|---|---|---|---|
| *days* | *N:N* | *% total N* | *mg N/g fresh weight nodules · day* |
| Mung bean | | | |
| 25–36 | 0.21 | 88 | 7.4 |
| 37–44 | 0.26 | 90 | 9.1 |
| 45–51 | 0.33 | 86 | 4.7 |
| 52–58 | 0.52 | 80 | 3.1 |
| 59–65 | 0.70 | 86 | 4.5 |
| 66–72 | 0.80 | 88 | 5.2 |
| Cowpea | | | |
| 29–39 | 0.18 | 95 | 7.4 |
| 40–46 | 0.16 | 88 | 14.0 |
| 47–53 | 0.34 | 81 | 12.7 |
| 54–60 | 0.69 | 84 | 7.2 |
| 61–69 | 0.75 | 84 | 1.0 |

high level of asparagine to glutamine is typical of plants assimilating $NO_3$ or other forms of combined N. The xylem exports of ureide producing and nonureide-producing legumes are thus closely similar if a combined source of N such as $NO_3$, is being utilized, but widely different if fixed $N_2$ is being exported from root nodules.

Most recent evidence suggests that the amide group of glutamine is the initial product of ammonia assimilation after $N_2$ fixation or $NO_3$ reduction (12) and that this nitrogen serves directly in the synthesis of glutamate (18), and in the synthesis of the amide group of asparagine by a transamidation reaction with aspartate (19). Ureide formation based on purine biosynthesis requires two atoms of amide nitrogen, either as glutamine or asparagine (7, 17),

and a N atom of aspartate to form three of the four N atoms of allantoin and allantoic acid (4), so synthesis of these compounds could be envisaged as competitive with asparagine formation. This suggests that a lower output of asparagine could be expected if ureides were being synthesized than if these compounds were not being formed. The data from xylem sap analysis of cowpea and other ureide-forming legumes support this contention.

Although the above hypothesis may be attractively simple, there are several other possible explanations for the above differences in exported products of $N_2$ and $NO_3$ assimilation in ureide-forming legumes. For example, nodules might simply have low activity of asparagine synthetase relative to roots; there might be basic differences in the availability of four carbon as opposed to five carbon keto acids in nodules and roots, and the nodule and root might behave differently in the relative rates at which the two amides are transported to the xylem. These and other possibilities would need to be explored before the matter could be satisfactorily resolved.

## LITERATURE CITED

1. ATKINS CA, DF HERRIDGE, JS PATE 1978 The economy of carbon and nitrogen in nitrogen-fixing annual legumes. Experimental observations and theoretical considerations. In Proc FAO/International Atomic Energy Agency Advisory Group meeting on "Potential use of isotopes in the study of biological dinitrogen fixation", Vienna, Austria pp 211-242

2. ATKINS CA, JS PATE, DB LAYZELL 1979 Assimilation and transport of N in nonnodulated ($NO_3$-grown) Lupinus albus L. Plant Physiol 64: 1078-1082

3. ATKINS CA, RM RAINBIRD, JS PATE 1980 Evidence for a purine pathway of ureide synthesis in $N_2$-fixing nodules of cowpea (Vigna unguiculata (L.) Walp.). Z Pflanzenphysiol 97S:249-260

4. BEEVERS L 1976 Nitrogen Metabolism in Plants. Edward Arnold, London, pp 118-121

5. EASTIN EF 1978 Total nitrogen determination for plant material containing nitrate. Anal Biochem 85: 591-594

6. HERRIDGE DF, CA ATKINS, JS PATE, RM RAINBIRD 1978 Allantoin and allantoic acid in the nitrogen economy of the cowpea (Vigna unguiculata [L.] Walp.) Plant Physiol 62: 495-498

7. KAPOOR M, ER WAYGOOD 1962 Initial steps of purine biosynthesis in wheat germ. Biochem Biophys Res Commun 9: 7-19

8. McCLURE PR, DW ISRAEL 1979 Transport of nitrogen in the xylem of soybean plants. Plant Physiol 64: 411-416

9. MATSUMOTO T, M YATAZAWA, Y. YAMAMOTO 1977a Distribution and change in the contents of allantoin and allantoic acid in developing nodulating and nonnodulating soybean plants. Plant Cell Physiol 18: 353-359

10. MATSUMOTO T, M YATAZAWA, Y YAMAMOTO 1977b Incorporation of $^{15}N$ into allantoin in nodulated soybean plants supplied with $^{15}N_2$. Plant Cell Physiol 18: 459-462

11. MATSUMOTO T, M YATAZAWA, Y YAMAMOTO 1977 Effects of exogenous nitrogen-compounds on the concentrations of allantoin and various constituents in several organs of soybean plants. Plant Cell Physiol 18: 613-624

12. MIFLIN BJ, PJ LEA 1977 Amino acid metabolism. Annu Rev Plant Physiol 28: 299-439

13. OGHOGHORIE CGO, JS PATE 1971 The nitrate stress syndrome of the nodulated field pea (Pisum arvense L.). Techniques for measurement and evaluation in physiological terms. In TA Lie, EG Mulder, eds, Proc Tech Mtg on Biol Nitrogen Fixation of the International Biological Programme (1970) Plant Soil (Special Vol) pp 185-202

14. PATE JS 1980 Transport and Partitioning of nitrogenous solutes Annu Rev Plant Physiol 31: In press

15. PATE JS, PJ SHARKEY, OAM LEWIS 1974 Phloem bleeding from legume fruits. A technique for study of fruit nutrition. Planta 120: 229-243

16. PERSCHKE H, EA KEROE, G PROKSCH, A MUEHL 1971 Improvements in the determination of nitrogen-15 in the low concentration range by emersion spectroscopy. Anal Chim Acta 53: 459-463

17. ROBERN H, D WANG, ER WAYGOOD 1965 Biosynthesis of nucleotides in wheat. I. Purines from $^{14}C$-labelled compounds. Can J Biochem 43: 225-235

18. ROBERTSON JG, MP WARBURTON, KJF FARNDEN 1975 Induction of glutamate synthase during nodule development in lupin. FEBS Lett 55: 33-37

19. SCOTT DB, JG ROBERTSON, KFJ FARNDEN 1976 Ammonia assimilation in lupin nodules. Nature 263: 703-708

20. TRIJBELS F, GD VOGELS 1966 Degradation of allantoin by Pseudomonas acidovorans. Biochim Biophys Acta 113: 292-301

21. WALLACE W, JS PATE 1965 Nitrate reductase in the field pea (Pisum arvense L.) Ann Bot 29: 654-671.

22. WOOD ED, FAJ ARMSTRONG, FA RICHARDS 1967 Determination of nitrate in seawater by cadmium copper reduction to nitrite. J Mar Biol Assoc 47: 23-31