Ureide Assay for Measuring Nitrogen Fixation by Nodulated Soybean Calibrated by ^{15}N Methods[1]

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ABSTRACT

We report experiments to quantify the relationships between the relative abundance of ureide-N in root-bleeding sap, vacuum-extracted sap, and hot water extracts of stems and petioles of nodulated soybean (*Glycine max* [L.] Merrill cv Bragg) and the proportion of plant N derived from nitrogen fixation. Additional experiments examined the effects of plant genotype and strain of rhizobia on these relationships. In each of the five experiments reported, plants of cv Bragg (experiment 1), cv Lincoln (experiments 3, 4, 5), or six cultivars/genotypes (experiment 2) were grown in a sand:vermiculite mixture in large pots in a naturally lit, temperature-controlled glasshouse during summer. Pots were inoculated at sowing with effective *Bradyrhizobium japonicum* CB1809 (USDA136) or with one of 21 different strains of rhizobia. The proportions of plant N derived from nitrogen fixation were determined using ^{15}N dilution. In one experiment with CB1809, plants were supplied throughout growth with either N-free nutrients or with nutrients supplemented with 1, 2, 4, or 8 millimolar ^{15}N-nitrate and harvested on eight occasions between V6 and R7 for root-bleeding sap, vacuum-extracted sap, stems (including petioles), and whole plant dry matter. Analyses of the saps and stem extracts for ureides (allantoin plus allantoic acid), α-amino-N, and nitrate, and of dry matter for N and ^{15}N, indicated a positive effect of nitrate supply on concentrations of nitrate in saps and extracts and a negative effect on ureides and on the proportion of plant N derived from nitrogen fixation. The relative abundance of ureide-N in root-bleeding sap, vacuum-extracted sap (100 [ureide-N]/[ureide-N + α-amino-N + nitrate-N]) and stem extracts (100 [ureide-N]/[ureide-N + nitrate-N]) and the proportion of plant N, derived from nitrogen fixation between successive samplings were highly correlated ($r = 0.97$–1.00). For each variable, two standard curves were prepared to account for the shifts in the compositions of N solutes of xylem saps and extracts after flowering which were not related to a change in nitrogen fixation. Relationships between relative ureide-N and the proportion of plant N derived from nitrogen fixation were not affected by plant genotype or by strain of rhizobia. Therefore, assessment of nitrogen fixation by soybean using the ureide technique should now be possible with the standard curves presented, irrespective of genotype or strain of rhizobia occupying the nodules.

Eleven years have elapsed since McClure and Israel (15) proposed a method for estimating the proportion of soybean (*Glycine max* [L.] Merrill) plant N derived from nitrogen

fixation based on the composition of N solutes in xylem (root-bleeding) sap. These authors and others (9, 11, 16, 21) showed that the relationship between P^2 and the relative abundance of ureide-N in xylem sap and extracts of plant parts was highly correlated. Nevertheless, the technique has not been widely adopted as a routine field assay for quantifying nitrogen fixation, but used instead to detect fixation activity or to differentiate treatment effects (22). Reasons for this may include: a general lack of confidence in the calibrations of the technique, because they were done with vegetative plants (16, 21) rather than over a complete growth cycle, or because they relied on the acetylene reduction assay to estimate nitrogen fixation (9, 11); doubts about the applicability of a single set of calibration curves to different genotypes of soybean and to different strains of *Bradyrhizobium japonicum*; uncertainty over sampling protocols and sources of error in the use of the technique; and lack of verification of the technique in field experiments where estimates of nitrogen fixation based on the relative abundance of ureide-N in xylem sap and tissue extracts were compared wtih estimates using other methods, *e.g.* N difference, ^{15}N.

During the past 4 years, we have attempted to resolve these issues. Errors associated with sampling and subsequent treatment of xylem sap have been identified and sampling protocols defined to minimize errors (13). Comparisons of estimates of nitrogen fixation by field-grown plants based on the ureide and other methods are presented in the companion paper (12) and will be the subject of future reports (DF Herridge, MB Peoples, FJ Bergersen, unpublished data). In this paper, we report experiments designed to quantify the relationship between the relative abundance of ureide-N in RBS, VES, and in extracts of the stems (including petioles) of Bragg soybean and Pincr. RBS and VES were sampled at regular intervals throughout the whole growth cycle of soybeans; sampling of stems was terminated during early pod-fill. Pincr, calculated for each interval of time between successive samplings, was considered a more appropriate measure of nitrogen fixation than P for calibrating the point-of-time ureide technique. Additional experiments examined the effect of plant genotype and strain of *B. japonicum* on the relationship between composition of xylem N-solutes and nitrogen

[2] Abbreviations: P, proportion of plant N derived from nitrogen fixation; Pincr, proportion of plant N in a growth period between successive samplings derived from nitrogen fixation; RBS, root-bleeding xylem sap; VES, vacuum-extracted xylem sap; HUP, uptake hydrogenase.

fixation. In each of the experiments, P and Pincr were estimated by the ^{15}N dilution method (5).

MATERIALS AND METHODS

Experiment 1

Calibration of the Relationship between the Relative Abundance of Ureide-N in Xylem Saps and Stem Extracts of Bragg Soybean and Pincr

Plants of soybean (*Glycine max* [L.] Merrill cv Bragg), inoculated at sowing with effective *Bradyrhizobium japonicum* CB1809 (USDA136), were grown in a 3:1 (v/v) mixture of sand and vermiculite in 14-L, free-draining pots in a naturally lit, temperature-controlled glasshouse during summer 1987/1988. Temperatures ranged from 18 to 25°C at night to maxima of 30 to 35°C during the day. Twelve seeds were sown in each pot. Seedlings were thinned to six per pot during vegetative growth and to four per pot at flowering. Positioning of pots resulted in overall densities of 24 and 16 plants/m² of bench during vegetative and reproductive periods, respectively. A randomized complete block design with four replicates was used. Once seedlings had emerged, pots were watered on alternate days during the first 6 weeks or daily thereafter with 2 to 3 L of a N-free nutrient solution (11), supplemented with ^{15}N-nitrate ($K^{15}NO_3$ + $Ca[NO_3]_2$) to give concentrations of 0, 1, 2, 4, or 8 mM. Enrichments of ^{15}N were 0.5 atom % excess (4 and 8 mM nitrate), 1.0 atom % excess (2 mM nitrate), to 2.0 atom % excess (1 mM nitrate).

At noon on days 41, 50, 61, 70, 79, 89, 100, and 110 after sowing, four replicate pots of plants from each of the five nitrate treatments were sampled for RBS and VES and for measurements of whole-plant dry matter, plant N, and ^{15}N concentrations. Phenological development of the plants was noted at each sampling (6). RBS was collected by cutting plants close to ground level and placing sleeves of silicon rubber tubing over the root stumps (23). The sap, exuding under root pressure into each tubing reservoir, was recovered with a Pasteur pipette after 15 to 20 min. RBS from all plants in each replicate pot was bulked, immediately placed on ice, and later frozen until analyzed. Xylem sap from the freshly detached stems (VES) was sampled by applying a mild vacuum (60–70 kPa) to the stem base and progressively cutting off small (3–5 cm) stem segments from the top of the shoot (11, 23). Previous reports indicated that the compositions of N solutes of sap collected by this method were almost identical with those of RBS from the same plants (11, 20). The replicate samples were immediately placed on ice, then frozen until analyzed.

Roots and nodules were washed carefully from the pots and, together with the shoot clippings (stem segments plus leaves) from the same plants which had been collected in large containers during sampling of VES, were dried in a forced-draft oven at 80°C for 48 h and weighed. Whole plant samples were ground to pass through a 1 mm sieve. Concentrations of total nitrogen (% N) in samples were determined by Kjeldahl digestion, followed by distillation and titration (1, 23). The titrated distillates were concentrated and analyzed for ^{15}N as described previously (28). The proportions of plant N

derived from nitrogen fixation (P) were estimated by the ^{15}N dilution technique (5, 24), thus:

$$P(\%) = 100 \left(1 - \frac{[\text{atom } \% \ ^{15}N \text{ excess plant}]}{[\text{atom } \% \ ^{15}N \text{ excess nutrient solution}]}\right) \quad (1)$$

These estimates were then combined with data on the accumulation of plant N to calculate the proportions of plant N derived from nitrogen fixation for each interval of time between successive samplings (Pincr), thus:

$$P\text{incr}(\%) = 100 \ (N_2P_2 - N_1P_1/(N_2 - N_1) \quad (2)$$

where P_1 and P_2 are the proportions of plant N derived from nitrogen fixation at times t_1 and t_2, respectively, and N_1 and N_2 are the N contents of plants at those times.

Stems (including petioles) were sampled from four additional pots from each nitrate treatment on days 41, 50, 61, and 70 after sowing. Samples were oven dried at 80°C for 48 h, ground to pass through a 1 mm sieve, and extracted with boiling H_2O as described previously (9). Extracts were stored at −15°C until analyzed.

Concentrations of ureides (allantoin and allantoic acid) in RBS, VES, and stem extracts were measured as the phenylhydrazone derivative of glyoxylate (31). Nitrate in xylem saps and extracts was measured by the salicylic acid technique (4). The α-amino-N contents of saps were determined colorimetrically with ninhydrin (11, 30), using a 1:1 asparagine:glutamine standard. Procedures for the three analyses are detailed also in Peoples *et al.* (23).

The relative abundance of ureide-N in RBS and VES was calculated as:

$$\text{Relative ureide-}N(\%) = 400a/(4a + b + c) \quad (3)$$

where a, b, and c are, respectively, the molar concentrations of ureides, nitrate, and α-amino-N (ureides contain 4 nitrogen atoms per molecule) (11). The three N solutes contain approximately 90% of total N of xylem sap; amide-N accounts for the remainder. We felt that the time and resources saved by restricting analysis to the three, rather than all four, N solutes outweighed any potential loss of information. Rapid analysis of sap samples and interpretation of treatment effects can be critical in some instances, *e.g.* breeding programs involving large numbers of samples that must be evaluated before selection of lines for further generation can be made. The relative abundance of ureide-N in stem extracts was calculated as:

$$\text{Relative ureide-N} (\%) = 400a/(4a + b) \quad (4)$$

where a and b are, respectively, the molar concentrations of ureides and nitrate (9).

Experiment 2

Effect of Genotype of Soybean on the Relationship between the Relative Abundance of Ureide-N in Xylem Saps and in Stem Extracts and Pincr

Six genotypes of soybean were grown in free-draining pots (6 plants/pot) in a glasshouse during summer 1986/1987, as described above. Genotypes were Bragg, Forrest, Davis, Bos-

sier, and Valder and K466, shown in previous work to have enhanced capacity for nodulation and nitrogen fixation in the presence of nitrate (2). The supply of nutrients and ^{15}N-nitrate treatments were identical with descriptions of experiment 1. Eight pots of each genotype × nitrate treatment were arranged randomly in four complete blocks, i.e. duplicate pots per replicate. For each treatment replicate, 12 plants were available for sampling. Four plants were carefully removed from each of the duplicate pots on d 55 and 63 after sowing for dry matter, N and ^{15}N determinations. RBS and VES were collected from the remaining four plants (two from each pot) on d 59. After extraction of VES, leaflets were removed from the collected shoot clippings. The stems (including petioles) were oven dried, ground, and extracted with boiling H_2O. RBS, VES, and stem extracts were analyzed for ureides, nitrate, and α-amino-N as described above.

Experiments 3, 4, and 5

Effect of Strain of Rhizobia on the Relationship between the Relative Abundance of Ureide-N in Xylem Saps and P

In experiment 3, soybean cv Lincoln were grown in pots (as described above, except that the surface of the potting mixture was covered with a 2-cm layer of ethanol-rinsed polyethylene beads ([Alkathene polyethylene, ICI Australia Ltd] to minimize rhizobial contamination [7]) and inoculated with one of the following strains of rhizobia: *Bradyrhizobium japonicum* CB1809, USDA110, 965*, 29w*, DF395*, DF383*, SM$_1$b*, RGS587; *Rhizobium fredii* USDA191, USDA192, and *Rhizobium leguminosarum* biov. *trifolii* TA1. The latter strain does not nodulate soybean and was included as an indicator of contamination of pots by rhizobia from other pots or from other sources. Strains marked with an * were *ex* EMBRAPA, Brazil. The unmarked strains were from the CSIRO collection, Canberra. After sowing, the four replicate pots of each strain were separated from pots of the other strains to further minimize the risk of contamination. Pots were placed in drip trays, and sterilized N-free nutrients (11) and water were supplied from the base. These measures were effective in avoiding cross-contamination.

At 50 d after sowing, RBS was collected from four of the six plants of each pot as described above. The nodulated root systems from the remaining two plants were removed from the potting mix, separated from the shoot at the first cotyledonary node, and sealed individually in 125 cm^3 conical flasks. Hydrogen evolution in air was measured in one of these systems by gas chromatography using a thermal conductivity detector. Acetylene was introduced into the other flask to measure acetylene reduction by the nodules, i.e. production of ethylene, by gas chromatography and a flame-ionization detector (29). Both were incubated for 30 min at 25°C. The relative efficiency of electron transfer through nitrogenase was determined by the equation of Schubert and Evans (25):

Relative efficiency

$$= 1 - (H_2 \text{ evolved (air)}/C_2H_4 \text{ produced}) \quad (5)$$

All shoot material was retained for measurement of dry matter. RBS was analyzed for ureides, nitrate, and α-amino-N as described above.

Two strains of B. *japonicum*, displaying high (CB1809) and low (SM$_1$b) levels of relative efficiency in experiment 3, were used in experiment 4. Plants of soybean cv Lincoln were inoculated with CB1809 or SM$_1$b and supplied throughout growth with either N-free nutrients or with nutrients supplemented with 5 mM nitrate containing K^{15}NO$_3$ (0.913 atom % ^{15}N excess). Conditions of plant culture were in all other respects as described for experiment 3. Plants were sampled during late flowering (R2) and again at pod-fill (R4) for RBS and VES and for total dry matter. Saps were analyzed for ureides, nitrate, and α-amino-N, and concentrations of N and ^{15}N in dry matter were determined.

In a third experiment involving different strains of rhizobia and cv Lincoln, 5 of the 10 replicate pots of each strain were maintained in a glasshouse, and the other five were left outside in brick cold frames. Glass covers were placed over the frames at night or during periods of rain. Under these conditions, night-time temperatures were lower than the temperatures inside the glasshouse (15–22°C *versus* 22–24°C), while daytime temperatures were essentially identical (25–32°C *versus* 27–30°C). Strains of B. *japonicum* used were CB1809 and 10 strains (NA6033, NA6046, NA6099, NA6109, NA6130, NA6160, NA6176, NA6245, NA6314, NA6323) isolated from Northern Thailand and obtained from the University of Chiang Mai. Plant culture and sampling were as described for experiment 3.

RESULTS

Calibration of the Relationship between the Relative Abundance of Ureide-N in RBS, VES, and Stem Extracts of Bragg Soybean and P$_{incr}$

The plants of Bragg accumulated large amounts of N at all levels of nitrate supply (Fig. 1a). The higher concentrations of nitrate, however, produced plants of greater size. Patterns of growth were consistent with those described in previous reports (9, 11), i.e. around 10% of total plant N was accumulated prior to flowering, while 80% accumulated during pod-fill. The five levels of nitrate produced groups of plants which had relied entirely on fixed N (0 mM nitrate), were essentially reliant upon nitrate (8 mM nitrate), or had relied on both sources of N (1, 2, and 4 mM nitrate) (Fig. 1b). The proportions of plant N derived from nitrogen fixation for each time interval between successive samplings (P$_{incr}$) were calculated by combining information summarized in Figure 1, a and b, with Equation 2. The calculated values (Fig. 1c) proved to be similar in magnitude to the cumulative P values, viz. P averaged over all samplings for the 0, 1, 2, 4, and 8 mM nitrate treatments was 100, 78, 64, 31, and 4%, respectively; P$_{incr}$ averaged 100, 79, 67, 37, and 3%, respectively.

Effects of nitrate supply on the composition of N solutes in RBS and VES are summarized in Table I. For each, concentrations of ureides declined and nitrate increased with increasing nitrate supply. Amino-N contents were essentially unaffected by nitrate supply, except at the highest level. Total xylem-N (ureide-N + nitrate-N + α-amino-N) in VES ranged from 74 to 353 mg/mL (overall mean of 113 mg/mL); in
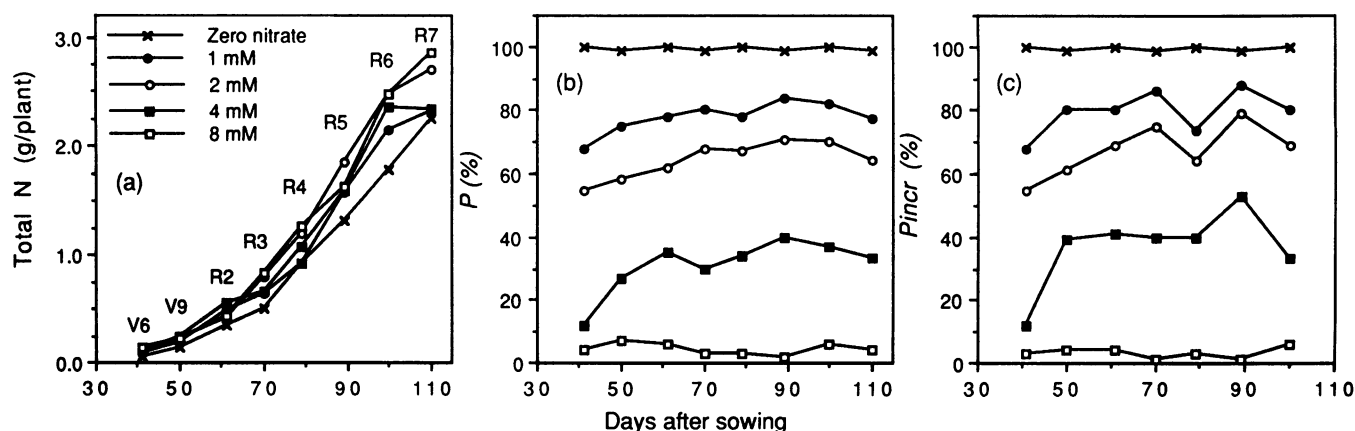
Figure 1. Time courses of the effects of nitrate supply on (a) accumulation of whole plant N, (b) the proportion of plant N derived from nitrogen fixation (*P*), and (c) the proportion of plant N derived from nitrogen fixation for each time interval between successive samplings (*Pincr*), *i.e.* 0–41, 41–50, 50–61 d, etc. Data are for soybean cv Bragg, grown during summer in a sand-vermiculite mixture in large pots in a naturally lit glasshouse. Stages of plant development (6) are shown in (a). Each point is the mean of four replicates, each consisting of four or six plants. Overall CVs were 2.1 and 9.4% for (a) and (b), respectively.

Table I. *Effects of Nitrate Supply on Concentrations of Ureides, Nitrate and α-Amino-N in VES and RBS of Soybean cv Bragg*

Plants were grown during summer in a sand-vermiculite mixture in large pots in a naturally lit glasshouse. Each value is the mean of 32 samples, *i.e.* eight samplings × four replicates. CVs for ureides, α-amino-N, and nitrate were 16, 25, and 28% (VES), and 21, 19, and 25% (RBS).

	Nitrate supply				
	0	1	2	4	8
			*m*M		
Ureides[a]					
VES	1.37	1.21	1.06	0.66	0.31
RBS	2.49	1.93	1.60	1.03	0.40
α-Amino-N					
VES	1.57	1.72	1.60	1.98	3.29
RBS	1.38	1.29	1.33	1.73	2.82
Nitrate					
VES	0.29	1.18	1.68	3.12	5.41
RBS	0.15	1.65	2.49	4.44	6.17

[a] Allantoin + allontoic acid.

RBS the range was 100 to 242 mg/mL (mean of 149 mg/mL). The low concentrations of nitrate (4 and 1% of total N in VES and RBS, respectively) measured in the saps of plants grown without nitrate supply most likely reflected traces of nitrate in the water used in the nutrient solutions and inaccurate colorimetric analysis at these concentrations.

Although the averaged concentrations of ureides and nitrate in the saps reflected the level of nitrate supply, a more accurate and responsive measure of the effect of nitrate is found in the expression of ureide-N as a proportion of total N (11, 16, 21) ("Materials and Methods," Equation 3). Similarly, the relative abundance of ureide-N in extracts of plant parts can be calculated (9) ("Materials and Methods," Equation 4). Time courses of the effects of nitrate supply on the relative abundance of ureide-N in xylem saps and in stem extracts are

presented in Figure 2. Patterns of response were similar for both RBS and VES. For plants grown without nitrate supply, relative ureide-N in VES ranged from 68 to 79% (mean of 74%); in RBS, the range was higher at 80 to 91% (mean of 87%). At the highest level of nitrate supply, values ranged from 7 to 19% (mean of 14%), and 6 to 24% (mean of 17%) for VES and RBS, respectively. Figure 3 shows the similarities in the compositions of N-solutes in the two sources of xylem sap. Points shown are drawn from Figures 2, a and b, and represent values for VES plotted against RBS values from the same groups of replicate plants. Clear differentiation of the effects of nitrate supply on the composition of N solutes in stem extracts was observed, *viz.* average values for the relative abundance of ureide-N for the five levels of nitrate supply (in ascending order) were 91, 66, 48, 19, and 6% (Fig. 2c). Data were obtained only for vegetative and early reproductive growth because we anticipate that sampling of stems for relative ureide-N determinations will be restricted to early plant growth when collections of VES or RBS prove difficult.

The proportions of ureide-N in xylem saps and stem extracts (Fig. 2, a, b, and c) were plotted against *P*incr for each sampling interval of the experiment (Fig. 1c) and evaluated by regression analysis. The relationships are summarized in Figures 4, 5, and 6. In each case, there was an apparent shift in relative ureide-N after flowering which did not reflect a shift in *P*incr. Thus, data from the vegetative-flowering (sowing to d 61) and subsequent (d 62–110) samplings were combined. Statistical comparisons of the regression lines for the two subsets of data for both VES and RBS indicated significant effects of intercept (P < 0.001), but not of slope (VES, Fig. 4, a and b), and significant effects of slope (P < 0.05) for RBS (Fig. 5, a and b).

Effect of Genotype of Soybean on the Relationship between the Relative Abundance of Ureide-N in Xylem Saps and Stem Extracts and *P*incr

Statistical comparison of the relationships between relative ureide-N in RBS and VES and *P*incr for the six genotypes of
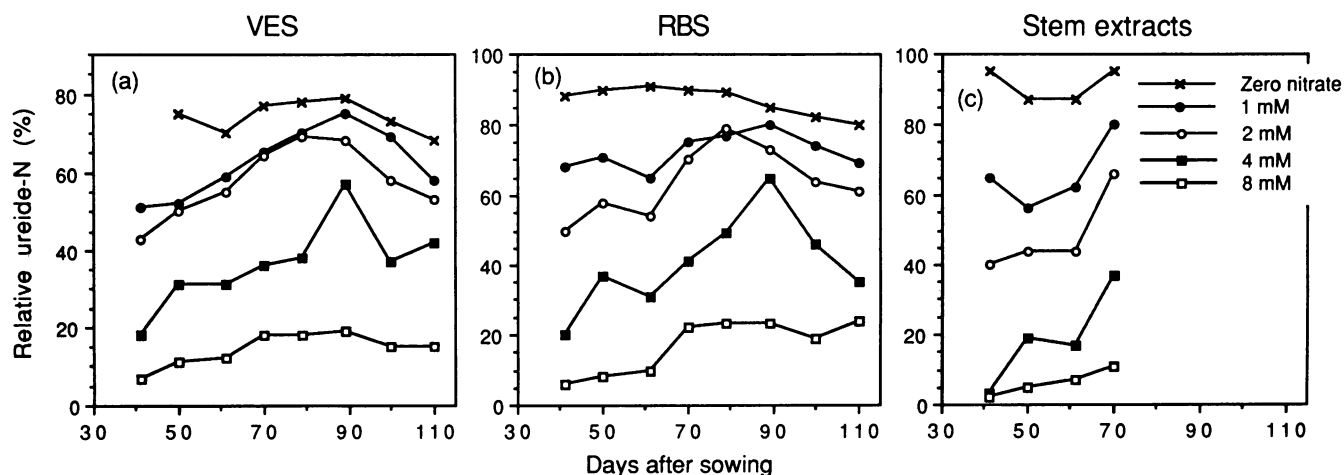
Figure 2. Time courses of the effects of nitrate supply on the relative abundance of ureide-N in (a) VES, (b) RBS, and (c) hot water extracts of stems (including petioles) of glasshouse-grown plants of soybean cv Bragg. Each point is the mean of four replicates, each consisting of four or six plants. Overall CVs were 10.7, 8.7, and 9.3% for (a), (b), and (c), respectively.

soybean showed no significant effects (P > 0.05) (Fig. 7). With stem extracts, however, K466 was different from the other five genotypes. With VES and stem extracts, there was a trend for the relative ureide-N values of the early-maturing genotypes (Valder, Forrest, K466, which were more phenologically advanced at sampling) to be higher than those of the later-maturing genotypes (Bossier, Bragg, Davis) at the same level of Pincr. This is consistent with the effect of plant development on the relationship between relative ureide-N and Pincr (Figs. 4, 5, and 6). For five genotypes, Pincr was affected similarly by the different levels of nitrate supply. The sixth genotype, K466, displayed higher reliance on fixed nitrogen at low to moderate levels of nitrate (1, 2, and 4 mM), relative to the others. Responses overall were consistent with those detailed in experiment 1.

Effect of Strain of Rhizobia on the Relationship between the Relative Abundance of Ureide-N in Xylem Saps and P

In experiment 3, plants of cv Lincoln were inoculated with 11 different strains of rhizobia and assessed for plant growth, relative efficiency of nitrogen fixation, and composition of N-solutes in RBS. The symbioses between Lincoln and the strains ranged from highly effective (CB1809, DF395, 29w, USDA110) through moderately effective (965, USDA191) to ineffective (USDA192) (Table II). However, relative ureide-N values of RBS were constant at around 90% for all strains that formed a nitrogen fixing symbiosis with Lincoln. Plants inoculated with the ineffective USDA192 and nonineffective TA1 did not grow any larger than the uninoculated control plants and lacked sufficient vigor at the time of sampling to yield xylem sap. Relative efficiencies of the effective symbioses ranged from 0.35 to 1.00; composition of N solutes in RBS and shoot mass did not vary in accordance with shifts in relative efficiency.

Two of the strains, with high (CB1809) and low (SM₁b) relative efficiencies with Lincoln, were included in a follow-up experiment in which plants were supplied with either N-free nutrients or nutrients supplemented with 5 mM nitrate.
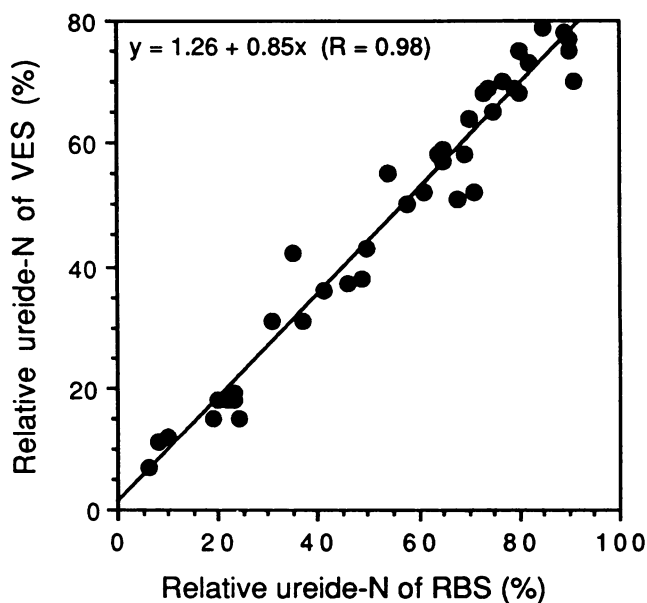
Figure 3. Relative abundance of ureide-N in VES of soybean cv Bragg plotted against relative abundance of ureide-N in RBS collected from the same plants. Values shown represent the means of four replicates, each of four to six plants.

Plants were sampled twice during growth for both RBS and VES. Results are presented in Table III. The 5 mM nitrate reduced P, irrespective of the strain of rhizobia used as inoculant. The relative abundance of ureide-N in RBS was generally higher than in VES, consistent with previous results. For plants sampled during late flowering, effect of strain on relative ureide-N in xylem saps was not significant. At the second (mid-pod-fill) sampling of the plants supplied with nitrate, relative ureide-N of saps was higher in plants inoculated with CB1809 than in plants inoculated wtih SM₁b, despite the identical effect of nitrate in suppression of fixation.

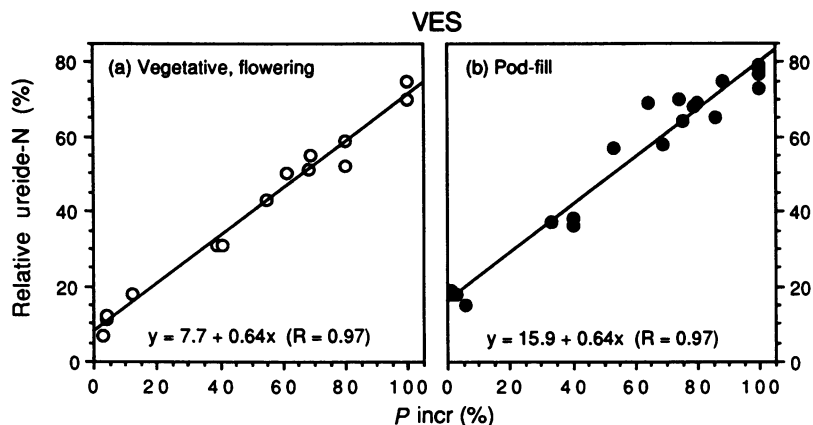A third experiment in this series involved plants of Lincoln,

VES

Figure 4. Relationships between the relative abundance of ureide-N in VES of soybean cv Bragg and *Pincr* during (a) vegetative growth and flowering, and (b) pod-fill. Functions describing the relationships are shown.
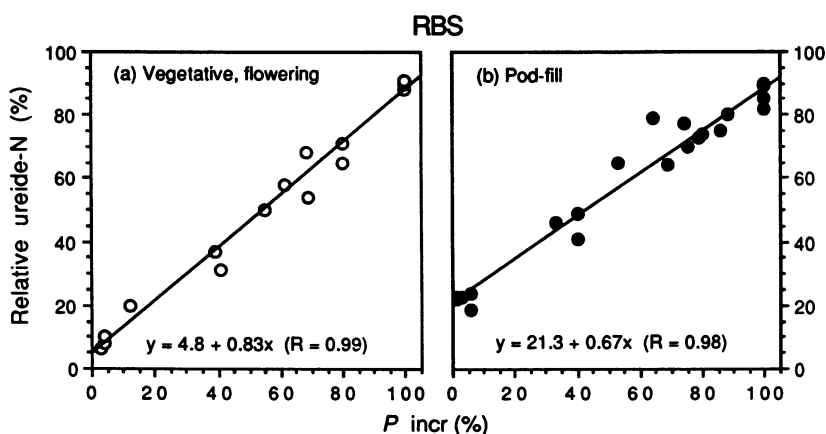
RBS

Figure 5. Relationships between the relative abundance of ureide-N in RBS of soybean cv Bragg and *Pincr* during (a) vegetative growth and flowering and (b) pod fill. Functions describing the relationships are shown.

cultured either in a glasshouse or outside in a cold frame, and inoculated with CB1809 or with one of 10 strains of *B. japonicum* isolated from Northern Thailand. The strains varied in effectiveness (data not shown). Shoot dry weights ranged from 4.4 ± 0.7 (NA634) to 8.3 ± 0.4 g/plant (CB1809) inside the glasshouse and from 2.7 ± 0.7 (NA6130) to 4.2 ± 0.7 (NA6323) in the cold frame. Although relative efficiencies ranged between 0.34 (NA6130) and 1.00 (CB1809, NA6160, and NA6176), relative ureide-N of RBS was constant for plants grown both inside (mean 87, range 82–92) and outside (mean 87, range 85–91) the glasshouse (data not shown). There was no relationship between relative efficiency, shoot growth, and relative ureide-N of RBS (P > 0.05).

DISCUSSION

Three basic requirements must be satisfied before the ureide technique can be used to measure nitrogen fixation with confidence. First, sampling of xylem sap or plant parts should be convenient and, within limits, should not be restricted by crop size or adverse environmental conditions. Second, the composition of N solutes in saps or extracts of plant parts should be calibrated against the proportion of plant N concurrently being derived from nitrogen fixation. Third, the advantages and limitations of the technique should be documented, and protocols for sampling and analysis defined, so that errors and variation are minimized.

Sampling

The ease with which xylem sap could be collected as RBS from glasshouse-cultured soybean (9, 15) facilitated the initial development of the ureide technique (16). Collection and analysis of RBS has been used subsequently in field studies of nitrogen fixation in the humid tropics (19). In many field environments, however, sampling sap by this method is difficult (27), and in some instances, impossible (10). Prewatering field plants may stimulate root bleeding (27), although, in the majority of cases, prewatering is inconvenient and could generate unreliable results through effects on the soil environment. The similarities between compositions of N solutes in the soluble fraction of the stem and petioles and RBS, together with the high concentrations of soluble N in these plant parts, provided the rationale for sampling the stem and petioles, rather than RBS, as a means of assessing nitrogen fixation activity of plants (9). The procedure, which involved the collection, drying, grinding, and laboratory extraction of plant material prior to analysis, was time-consuming, particularly during pod fill when plants were large (10). It is possible also that cellular contents and stored N compounds are extracted as well as transport (xylem) compounds. The tissue extract method, therefore, may not provide as sensitive a measure of nitrogen fixation activity as a procedure developed solely on the basis of xylem sap analysis. Nonetheless, it may represent the only method of sampling plants in the field in some
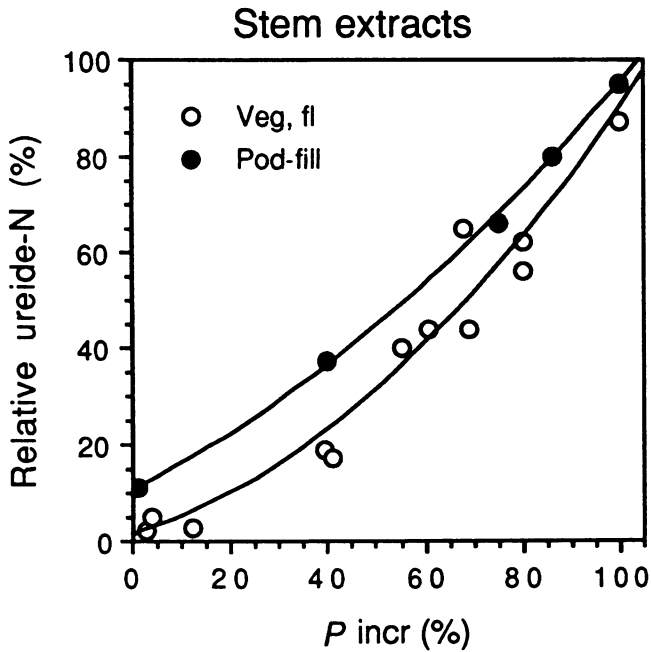
instances, *e.g.* in young, vegetative plants. Extraction of xylem sap from detached shoots with a mild vacuum overcame the sampling problems of RBS and avoided the laborious and time-consuming steps involved in extraction of N solutes from stems (11). VES was similar to RBS in composition of individual amino compounds and in the proportions of major N
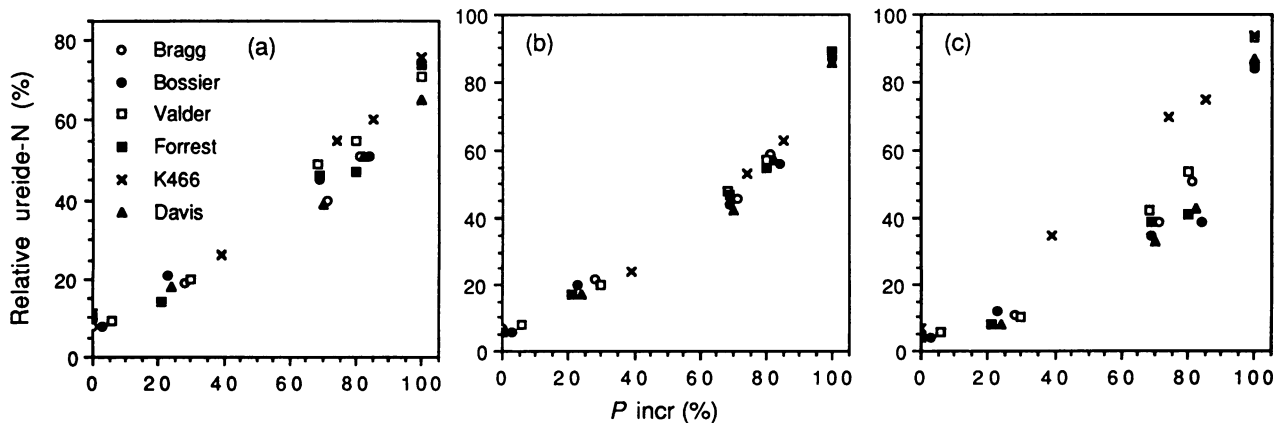
Stem extracts

Figure 6. Relationships between the relative abundance of ureide-N in stem extracts of soybean cv Bragg and *P*incr during (a) vegetative growth and flowering, and (b) pod-fill. Functions describing the relationships are:

$$y = 1.38 + 0.311x + 0.0057x^2$$

($R = 0.98$) for vegetative, flowering plants

$$y = 10.7 + 0.504x + 0.0034x^2$$

($R = 1.00$) for plants in pod-fill.

solutes. The technique proved reliable also in studies of soybean growing in diverse environments, *e.g.* during drought, without the need for prewatering.

Calibration

Experiments to calibrate the ureide assay have been reported (9, 11, 16). Each provided useful information although each had specific limitations. McClure *et al.* (16) assessed *P* by the ^{15}N dilution technique, analyzed RBS only, and restricted observations to vegetative plants. Herridge (9, 11) calibrated relative ureide-N in extracts of plant parts and in both RBS and VES against *P* over complete growth cycles of Bragg soybean. In those experiments, the acetylene reduction assay (29) was used to estimate nitrogen fixation activity and, from that, *P*. It was assumed that the quantitative nature of the relationship between acetylene reduction and nitrogen fixation was unaffected by level of nitrate supply, and that the five levels of *P* induced by the five levels of nitrate supply did not change during the course of plant growth. While there is some justification for the first assumption (26), it is possible that *P* was influenced by increasing plant size (*e.g.* 16; Table III), although every attempt was made to ensure that the plant roots were exposed to a constant supply of nitrate. Variations of this type would have been most likely in the case of the 1, 2, and 4 mM nitrate treatments (11) and would have resulted in errors in the final calibrated regressions.

Thus, the first experiment reported here was designed to verify and improve on those previous calibrations. Sampling of Bragg spanned the period between early vegetative growth (V6) and physiological maturity (R7), involved the collection of RBS, VES, and stem extracts, and relied on the ^{15}N dilution technique to determine *P* and *P*incr. The calibrated relationships between relative ureide-N in the saps (Figs. 4 and 5) and stem extract (Fig. 6) and *P*incr are essentially consistent with those previously reported (9, 11, 16). Although there was an apparent shift in relative ureide-N in both RBS and VES once flowering had ceased which was not related to a shift in *P*incr, only two regression functions were needed, rather than the complex set of curves previously proposed (11). The relative

Figure 7. Effect of soybean genotype on the relationships between the relative abundance of ureide-N and *P*incr in (a) VES, (b) RBS, and (c) stem extracts. All plants were grown during summer in a sand-vermiculite mixture in large pots in a naturally lit glasshouse. Each point is the mean of four replicates. CVs were 13.4, 10.4, 11.4, and 10.4% for *P*incr, relative ureide-N in VES, RBS, and stem extracts, respectively.

Table II. *Effect of Strain of Rhizobia on the Relative Efficiency of Nitrogen Fixation and on the Relative Abundance of Ureide-N in RBS of N_2-Dependent Plants of Soybean cv Lincoln*

Inoculated plants were grown in a 1:1 mixture of sand and vermiculite and were supplied throughout growth with N-free nutrients. Plants were sampled at 50 d after sowing. Values shown are the means ($\pm$ SE) of four replicates, each comprising two (relative efficiency), four (RBS) or six plant-samples (dry weights).

Strain of Rhizobia	Relative Efficiency[a]	Shoot Dry Wt	RBS Composition		Relative Ureide-N
			Ureide	Amino-N	
		g/plant	mM		%
CB1809	1.00	4.1 ± 0.9	4.3 ± 0.3	1.8 ± 0.1	90 ± 0.8
USDA110	0.98	3.5 ± 0.6	2.8 ± 0.7	1.7 ± 0.4	86 ± 0.8
965	0.59	2.9 ± 0.7	2.2 ± 0.2	1.1 ± 0.1	89 ± 0.7
USDA191	0.56	2.0 ± 0.6	3.0 ± 0.3	1.3 ± 0.1	90 ± 0.8
29w	0.51	3.8 ± 0.6	4.4 ± 0.2	2.7 ± 0.1	87 ± 0.4
DF395	0.50	3.9 ± 0.6	3.9 ± 1.0	2.0 ± 0.1	89 ± 0.3
DF383	0.50	3.2 ± 0.5	3.4 ± 0.1	2.1 ± 0.1	87 ± 0.8
SM₁b	0.49	3.3 ± 0.7	4.8 ± 0.2	2.8 ± 0.1	87 ± 0.5
RGS587	0.35	3.4 ± 0.6	4.3 ± 0.4	2.2 ± 0.1	88 ± 1.5
USDA192[b]	ND[c]	1.2 ± 0.3	ND	ND	ND
TA1[d]	ND	1.4 ± 0.3	ND	ND	ND
Uninoculated	ND	1.3 ± 0.2	ND	ND	ND

[a] Relative efficiency calculated as: 1-(H_2 evolved [air]/C_2H_2 produced).   [b] Strain ineffective on cv Lincoln.   [c] Not done.   [d] Strain did not nodulate cv Lincoln.

Table III. *Effects of Strain of Rhizobia, Plant Age, and Nitrate Supply on P and on the Relative Abundance of Ureide-N in RBS and VES of Soybean cv Lincoln*

Inoculated plants were grown in a 1:1 mixture of sand and vermiculite and were supplied throughout growth with either an N-free nutrient solution or with nutrients supplemented with 5 mM nitrate containing $K^{15}NO_3$ (0.913 atom % ^{15}N excess). Values shown are the means ($\pm$ SE) of four replicates, each comprising two plant samples.

Nitrate Supply/ Strain of Rhizobia	Late Flowering (R2)			Pod-Fill (R4)		
	P	Relative ureide-N in		P	Relative ureide-N in	
		RBS	VES		RBS	VES
		%				
0 mM nitrate						
CB1809	100	84 ± 2.2	64 ± 4.0	100	87 ± 1.9	78 ± 0.5
SM₁b	100	82 ± 2.0	67 ± 1.8	100	84 ± 1.6	72 ± 2.1
5 mM nitrate						
CB1809	49	49 ± 2.8	43 ± 1.4	72	67 ± 3.8	70 ± 0.5
SM₁b	50	48 ± 2.7	43 ± 1.3	71	50 ± 3.6	51 ± 2.2

ureide-N value of RBS was generally higher (maximum of 91%) than that of VES (maximum of 79%) from the same plants (Fig. 2; cf. 11).

Limitations, Errors

Sources of error associated with sampling of stems for extraction of N solutes and of whole shoots for extraction of VES have been examined (9, 11, 13), and protocols for sampling, treatment of samples, and chemical analysis are now well documented (see also 3, 23). However, the ureide technique has restricted application to the measurement of nitrogen fixation in field studies if the relationships between relative ureide-N and P are specific for each combination of plant genotype and strain of *B. japonicum*. McClure *et al.*

(16), in a study involving 11 cultivars of soybean from a range of maturity groups, found no effect of cultivar on the relative abundance of ureide-N in RBS of plants either fully dependent on fixed nitrogen or essentially relying on mineral N sources. Hansen *et al.* (8) examined the composition of N solutes in both RBS and VES of Bragg and three mutants of Bragg over a range of P; their data indicated insignificant differences. Results of our studies (Fig. 7) are consistent with those already reported, and we conclude that the technique can be used in comparisons of different genotypes of soybean.

The effect of strain rhizobia on the correlated relationship between relative ureide-N in xylem sap and P is more confusing. McClure *et al.* (16) concluded that the relationship was likely to be unaffected by strain of rhizobia involved in nodulation of the host, although they cautioned that a wider range of strains should be included in additional studies before the possibility of a strain effect could be dismissed. Minamisawa *et al.* (17) examined the role of the HUP system of root nodules on the transport of fixed N in the xylem of soybean. Although the ratio of the amides, Asn and Gln, to amino acids was slightly altered, the ratio of ureide-N to total N was unaffected by strain of rhizobia and the presence or absence of HUP. Neves *et al.* (18), on the other hand, provided evidence of large effects of rhizobial strain on the levels of ureides in the xylem stream mediated through the HUP system. If that were so, changes in the ratio of ureide-N to total N may reflect changes in both the relative efficiency of the strain and in P rather than just the latter. Our results did not support those of Neves *et al.* (18); rather, for the 21 different strains involved in experiments 3, 4, and 5, they indicated a lack of relationship between strain effectiveness, strain relative efficiency, and composition of N solutes in xylem sap (Tables II and III). Culture of the plants in open-air cold frames which were covered only at night or during

rain (cf. 18) did not affect xylem sap composition. In our studies, the amide-N component of xylem sap was not measured and the relative abundance of ureide-N is the proportion of approximately 90% of total N as ureides. Neves et al. (18) included amide-N in their calculations and have presented evidence subsequently of a negative relationship between strain relative efficiency and the relative abundance of amide-N in sap (14). Possibly, the effect of strain on N solute composition may not have been as great if amide-N had not been included in their calculations.

In conclusion, assessment of nitrogen fixation by soybean using the ureide technique should now be possible with the standard curves presented, irrespective of cultivar involved or strain of rhizobia occupying the nodules.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Bergersen FJ** (1980) Measurement of nitrogen fixation by direct means. In FJ Bergersen, ed, Methods for Evaluating Biological Nitrogen Fixation. John Wiley & Sons, Chichester, UK, pp 65–110

2. **Betts JH, Herridge DF**(1987) Isolation of soybean lines capable of nodulation and nitrogen fixation under high levels of nitrate supply. Crop Sci 27: 1156–1161

3. **Boddey RM, Pereira JAR, Hungria M, Thomas RJ, Neves MCP** (1987) Methods for the study of nitrogen assimilation and transport in grain legumes. Mircen J 3: 3–22

4. **Cataldo DA, Haroon M, Schrader LE, Youngs VL** (1975) Rapid colorimetric determination of nitrate in plant tissues by nitration of salicylic acid. Comm Soil Sci Plant Anal 6: 71–80

5. **Chalk PM** (1985) Estimation of N₂ fixation by isotope dilution: an appraisal of techniques involving ¹⁵N enrichment and their applications. Soil Biol Biochem 17: 389–410

6. **Fehr WR, Caviness CE, Burmood DT, Pennington JS** (1971) Stage of development descriptions for soybeans, Glycine max (L.) Merrill. Crop Sci 11: 929–931

7. **Gibson AH** (1980) Methods for legumes in glasshouses and controlled environment cabinets. In FJ Bergersen, ed, Methods for Evaluating Biological Nitrogen Fixation. John Wiley & Sons, Chichester, UK, pp 139–184

8. **Hansen AP, Peoples MB, Gresshoff PM, Atkins CA, Pate JS, Carroll BJ** (1989) Symbiotic performance of supernodulating soybean (Glycine max [L.] Merrill) mutants during development on different nitrogen regimes. J Exp Bot 40: 715–724

9. **Herridge DF** (1982) Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. Plant Physiol 70: 1–6

10. **Herridge DF** (1982) Use of the ureide technique to describe the nitrogen economy of field grown soybeans. Plant Physiol 70: 7–11

11. **Herridge DF** (1984) Effects of nitrate and plant development on the abundance of nitrogenous solutes in root-bleeding and vacuum-extracted exudates of soybean. Crop Sci 24: 173–179

12. **Herridge DF, Bergersen FJ, Peoples MB** (1990) Measurement of nitrogen fixation by soybean in the field using the ureide and natural ¹⁵N abundance methods. Plant Physiol 93: (708–716)

13. **Herridge DF, O'Connell P, Donnelly K**(1988) The xylem ureide assay of nitrogen fixation: sampling procedures and sources of error. J Exp Bot 39: 12–22

14. **Hungria M, Neves MCP** (1987) Cultivar and Rhizobium strain effects on nitrogen fixation and transport in Phaseolus vulgaris L. Plant Soil 103: 111–121

15. **McClure PR, Israel DW** (1979) Transport of nitrogen in the xylem of soybean plants. Plant Physiol 64: 411–416

16. **McClure PR, Israel DW, Volk RJ** (1980) Evaluation of the relative ureide content of xylem sap as an indicator of N₂ fixation in soybeans. Phant Physiol 66: 720–725

17. **Minamisawa K, Arima Y, Kumazawa K** (1983) Transport of fixed nitrogen from soybean nodules inoculated with H₂-uptake positive and negative Rhizobium japonicum strains. Soil Sci Plant Nutr 29: 85–92

18. **Neves MCP, Didonet AD, Duque FF, Dobereiner J** (1985) Rhizobium strain effects on nitrogen transport and distribution in soybeans. J Exp Bot 36: 1179–1192

19. **Norhayati M, Sudin MN, Chong K, Faizah AW, Herridge DF, Peoples MB, Bergersen FJ** (1988) Adaption of methods for evaluating N₂ fixation in food legumes and legume cover crops. Plant Soil 108: 143–150

20. **Pate JS** (1980) Transport and partitioning of nitrogenous solutes. Annu Rev Plant Physiol 31: 313–340

21. **Pate JS, Atkins CA, White ST, Rainbird RM, Woo KC** (1980) Nitrogen nutrition and xylem transport of nitrogen in ureide-producing grain legumes. Plant Physiol 65: 961–965

22. **Patterson TG, LaRue TA** (1983) Nitrogen fixation (C₂H₂) and ureide content of soybeans: ureides as an index of fixation. Crop Sci 23: 825–831

23. **Peoples MB, Faizah AW, Rerkasem B, Herridge DF** (1989) Methods for Evaluating Nitrogen Fixation by Nodulated Legumes in the Field. ACIAR Monograph No. 11, Australian Centre for International Agricultural Research, Canberra, Australia

24. **Peoples MB, Sudin MN, Herridge DF** (1987) Translocation of nitrogenous compounds in symbiotic and nitrate-fed amide-exporting legumes. J Exp Bot 38: 567–579

25. **Schubert KR, Evans HJ** (1976) Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc Natl Acad Sci USA 73: 1207–1211

26. **Schuller KA, Day DA, Gibson AH, Gresshoff PM** (1986) Enzymes of ammonia assimilation and ureide biosynthesis in soybean nodules: effect of nitrate. Plant Physiol 80: 646–650

27. **Streeter JG** (1972) Nitrogen nutrition of field grown soybean plants. I. Seasonal variations in soil nitrogen and nitrogen compositions of stem exudate. Agron J 64: 311–314

28. **Turner GL, Bergersen FJ** (1983) Natural abundance of ¹⁵N in root nodules of soybean, lupin, subterranean clover and lucerne. Soil Biol Biochem 15: 525–530

29. **Turner GL, Gibson AH** (1980) Measurement of nitrogen fixation by indirect means. In FJ Bergersen, ed, Methods for Evaluating Biological Nitrogen Fixation. J Wiley & Sons, Chichester, UK, pp 111–138

30. **Yemm EW, Cocking EC** (1955) The determination of amino acids with ninhydrin. Analyst 80:209–213

31. **Young EG, Conway CF** (1942) On the estimation of allantoin by the Rimini-Schryver reaction. J Biol Chem 142: 839–853