PN-AAW-805
ISN 49493

Plant and Soil 79, 77–88 (1984). Ms. 5584
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Composition of bleeding sap nitrogen from lines of field-grown *Phaseolus vulgaris* L.

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Received 13 July 1983. Revised October 1983

Key words Beans Bleeding sap N Nitrate *Phaseolus vulgaris* Ureides

Summary The nitrogenous compounds in the xylem (bleeding) sap of lines of field-grown *Phaseolus vulgaris* L. known to vary in N_2 fixation and yield, were measured during growth with and without N fertilizer. Forty nine-67% of the total sap N was in the form of nitrate in fertilized plants, with low amounts of the ureides, allantoin and allantoic acid (4 - 12%). Ureides contributed between 17 and 38% to the total sap N of non-fertilized plants, with nitrate generally comprising less than 40%. Among the nine lines grown without fertilizer there were significant differences in the % of the sap N as ureides and also in the total μmol N ml^{-1} sap.

The ASN/GLN ratio (mol/mol) was greater in sap collected from the two parental lines given fertilizer compared with non-fertilized plants. However the actual ratio varied between the parental lines. Together the two amides generally comprised between 45–62% of the amino-N in sap samples from the two parental lines with or without N fertilizer.

For each N treatment (fertilized or non-fertilized) there were no obvious differences in sap composition between the high N_2 fixing lines and the low N_2 fixing lines. However there was generally a positive relationship between the rate of N translocation (total N concn. ml^{-1} sap x rate of exudation) and the ranking of the lines on the basis of higher N_2 fixation rates (acetylene reduction), which was to a large extent independent of the source of N available to the plant.

Introduction

A number of economically important legumes belonging to the Phaseoleae, e.g. *Phaseolus* spp., soybean (*Glycine max*) and cowpea (*Vigna unguiculata*), transport large amounts of the ureides, allantoin and allantoic acid, in the xylem stream when fixing atmospheric nitrogen[13, 16, 18]. It has been suggested that the relative ureide content of xylem sap N (% ureide-N of total sap N) might be a useful indicator of nitrogen fixation in soybeans[8, 9] and cowpea[13]. Under controlled environmental conditions positive correlations between the proportion of the plant's N requirements derived from N_2 fixation and the ureide content of the xylem sap N have been reported[9, 13]. When plants are totally dependent on N_2 fixation for their N supplies, the relative contribution of ureide-N to the total sap N is high. When nitrate

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fertilizers are fed to the plants, ureides in the sap decrease and asparagine and nitrate increase[8, 13].

The ureide content of the xylem sap N of common bean (*Phaseolus vulgaris*) has been shown to vary from 13 to 42% of the total sap N in non-nodulated plants grown with different inorganic nitrogen sources[17] and can amount to 80% of the total N in nodulated plants dependent on nitrogen fixation for their N requirements ([4], Thomas and Sprent, unpublished).

In all of the reports cited above plants were grown in growth chambers or greenhouses under controlled environmental conditions. With the exception of one report on soybeans[15] we know little about the transport of nitrogen in field-grown legumes which are ureide producers. The objective of the work reported here was to determine the xylem transport of nitrogen in lines of *P. vulgaris* known to vary in seed yield and nitrogen fixation potentials and grown in the field in the presence and absence of fertilizer nitrogen.

Methods

Plant materials

Seven treatment lines were selected from a population developed to study host factors related to nitrogen fixation in the common bean. The two population parents were 'Sanilac', a Navy bean with low fixation and yield potential and 'Puebla 152 Black', a black bean with high fixation and yield potential. Since the population consisted of 65 near homozygous recombinant families ($F = 0.98$) generated using the inbred backcross line method[1], we were able to select individual lines which closely resembled the 'Sanilac' recurrent parent for certain important traits like plant type, days to flower and maturity, seed color and size, yet differed *inter se* for fixation and yield potential[10, 11]. The two parents were both included twice as treatment lines. The experiment was arranged in a randomized complete block, split-split plot design with 4 replications. Nitrogen fertilizer was the main plot factor, bean lines the sub-plot factor and time of sampling the sub-subplot factor. The two levels of nitrogen fertilizer were 88 kg/ha actual N vs no added N. Fertilizer was applied as urea in sidedressed split applications around 14 and 36 DAP. Treatment lines were arranged in 6.6 m rows 91 cm apart. Within-row spacing was 10 cm between plants. The experimental unit, representing time of sampling, consisted of a 50 cm section of row including five test plants. Experimental units were separated from each other within the row by a 50 cm section of five plants of cv Porrillo Sintetico. Rows were guarded on both ends by similar 50 cm sections.

The seven selected lines and one set of the two parental lines were sampled at the same times. Of the four experimental units per treatment line available for sampling through the season, four were assayed for xylem sap N at 43, 49 and 57 days after planting (DAP). The second set of parental lines were sampled for xylem sap N at additional times of 21 and 63 DAP in order to obtain a more complete set of data.

Seed was pretreated with Thiram fungicide and Diazinon insecticide and planted 2 June 1981 at the Hancock Experiment Station, Hancock, WI in a Plainfield sandy loam tested at 17.9 tons organic matter ha^{-1}. Seeds were dropped 2 per hole and later thinned to one plant per hole to produce a full stand. All experimental units were inoculated at planting with a granular *R. phaseoli* composite of four high-performing strains (CIAT 57, CIAT 75, CIAT 676 and Allen 413-2) supplied by Nitragin Co., Inc., Milwaukee, WI. Regular cultural practices, including sprinkler irrigation by movable pipe, cultivation and application of appropriate pesticides, were followed.

Xylem sap collection and N analysis

The five plants in each experimental unit were decapitated *in situ* midway between the cotyledonary and unifoliolate node with a razor blade and the cut ends rinsed with distilled water and dried with tissue paper. The first few drops of exudate were discarded. These procedures were designed to minimize contamination of the bleeding (xylem) sap from damaged cells. Bleeding sap was collected with hand-held micropipettes for 2 to 20 min, depending on the rate of exudation, until sufficient sap was obtained for a complete analysis (*ca.* 380 μl). Collection of sap samples was started around 10.00 h and was generally completed by 14.00 h. Subsequent analysis of replications within main plots showed that there was no significant effect on the composition of the sap N due to time of sampling. Sap samples were stored on ice before transportation to the lab where they were stored in a freezer (−20 C) until analysis.

Nitrogen in the sap samples was determined by the following methods:

α-NH_2-N. α-amino acid N was measured with ninhydrin reagent after Cramer[5]. Corrections were made for any ammonium present in the samples.

Amide-N was estimated as ammonium after acid hydrolysis in open tubes[17]. Glutamine was used as a standard.

Ammonium-N was determined by a phenol-hypochlorite reaction as described by Bohley[2].

Ureide-N. Allantoin and allantoic acid were measured by a diphenylformazan colourimetric procedure described by Vogels and van der Drift[19], scaled down by a factor of one quarter.

Nitrate-N was measured using the procedure of Cataldo *et al.*[3] scaled down by a factor of one quarter.

Kjeldahl-N. A microKjeldahl procedure was used as described by Bohley[2]. The digestion time was between 30–60 min and there was no increase in absorbance when 200 nmol nitrate was added to 40 or 80 nmol alanine standard. Thus this method does not detect nitrate in the sap samples.

The sum of the α-NH_2, amide, ammonium, and ureide N was generally equal to the total reduced N (Kjeldahl N) ± 10%, indicating that all nitrogenous compounds in the sap samples were accounted for. Results expressed are the means of the four replicate samples collected from each experimental unit.

Analysis of amino acids

Amino acids in the sap samples were separated and identified using a Dionex amino acid analyzer (Model D-300) with lithium acetate elution buffers, pH range 2.75–5.30.

Results

Seasonal patterns of the concentration of total N (μmol N ml^{-1} sap) in bleeding sap and rates of N translocation (total μmol N ml^{-1} sap x exudation rate) in the two parental lines ('Puebla 152 BI' and 'Sanilac') grown in the presence and absence of applied N fertilizer are shown in Fig. 1. The concentration of total N was highest in all treatments during early vegetative growth (day 21), particularly in 'Sanilac' given N fertilizer (+N). The concentration of sap N decreased during vegetative growth and early flowering in all treatments before increasing slightly during late flowering and early pod filling (days 49 to 57). During this latter period the total concentration of sap N was higher in both 'Puebla 152 BI' and 'Sanilac' given N fertilizer. The rate of N translocation was generally higher in 'Puebla 152 BI' than in 'Sanilac' with or without N fertilizer, particularly during pod filling. At this time (day 57) the N translocation rate of 'Puebla 152 BI' was between 2.6 (+N) and 3.7 (nil N) times greater than that of 'Sanilac'.

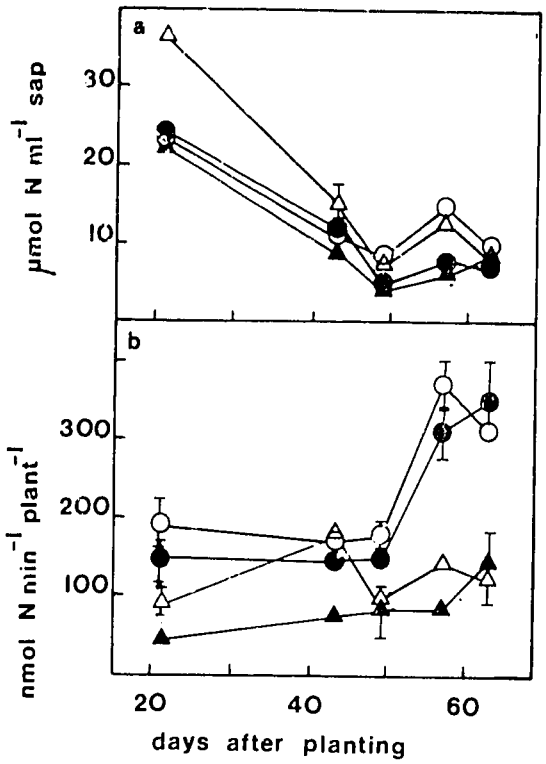

Fig. 1. a) Concentration of total N in bleeding sap and b), rates of N translocation in two parental lines during the season. △, 'Sanilac' + N; ▲, 'Sanilac' nil N; ○, 'Puebla 152 BI' + N; ●, 'Puebla 152 BI' nil N. Error bars included where they exceed symbol size.

The percentage composition of sap N during the development of the two parental lines is shown in Fig. 2. Generally nitrate was the major individual nitrogenous compound throughout development of both lines given N fertilizer. In plants not fertilized, nitrate was the major compound during vegetative growth and early flowering but total reduced N (α-NH_2, amide and ureide N) composed the greater proportion from day 49 onwards.

In non-fertilized 'Puebla 152 BI' the relative contribution of ureides to the sap N increased during vegetative and reproductive growth, peaking around 57 DAP (Fig. 3). In 'Sanilac' however, ureides began to increase only after reproductive growth had commenced. In fertilized plants of both parental lines ureides remained more or less constant during vegetative and early reproductive growth before increasing during pod filling. The maximum contribution from ureides was 27% on day 49 for 'Sanilac' (−N) and 38% on day 57 for 'Puebla 152 BI' (−N). Ammonium-N was a very small component (usually less than 1%) for all treatments throughout the season. In both parental lines the

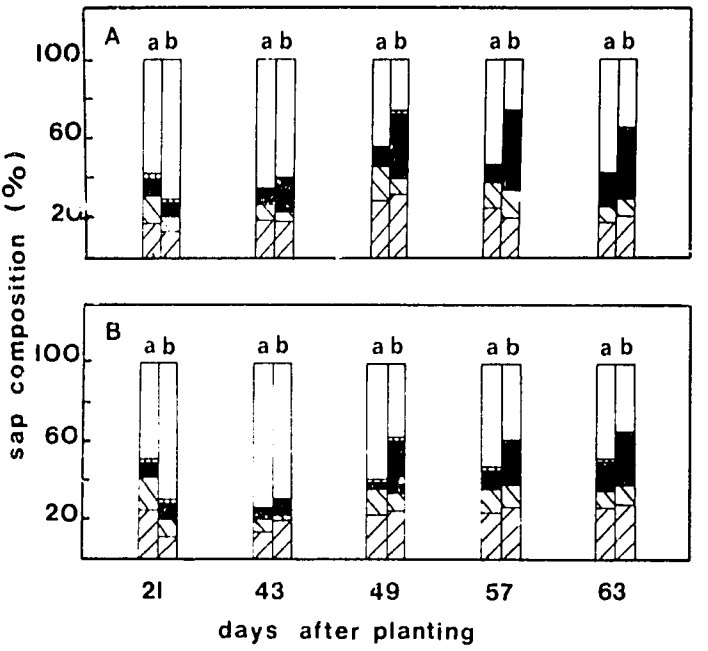

Fig. 2. Relative composition of bleeding sap N of A, Puebla 152 Bl and B, Sanilac during the season. ▨, α-NH_2-N, ▨ amide-N, ■ ureide-N, ▨ NH_4^+-N, □, NO_3^--N. a = fertilized plants, b = non-fertilized plants.

contribution of amino acids (α-NH_2 and amide N) to the total sap N was higher in fertilized than in non-fertilized plants at the initial sample (day 21) but thereafter little difference existed between treatments (Fig. 2). Both 'Puebla 152 Bl' and 'Sanilac' showed seasonal changes in relative amino acid content of xylem sap during development. In 'Sanilac' their initial contribution at day 21 constituted 42% of total sap N in +N plants and 20% in nil N plants. Subsequently, the relative content was similar between fertilizer treatments, decreasing to around 20% at day 43 and later levelling off around 35%. In 'Puebla 152 Bl' amino acids contributed 5–10% more of total sap N in the +N plants than the nil N plants at all sampling dates except at day 63. The relative content increased to a peak around 40–45% at day 49, then decreased steadily to around 30% at day 63. The % of sap N as amides (asparagine and glutamine) was greater in +N plants than in the nil N plants up until day 57 (late flowering and pod filling) when there were no apparent differences between treatments in either line. The relative contribution of amide N to the total sap N averaged around 10–15% during the season. The contribution of amides to the total amino acid N varied during development. In 'Puebla 152 Bl' amides composed on average 62% of the amino acid N in +N and 48% in nil N plants. For 'Sanilac' the corresponding percentages were 53 and 43%.

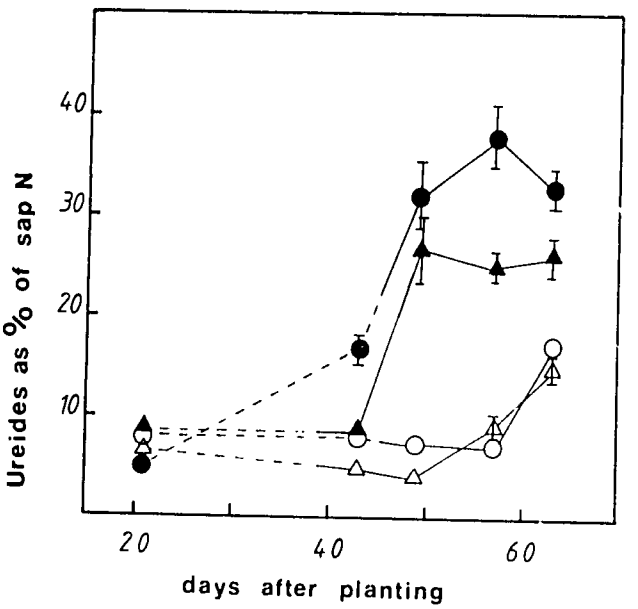

Fig. 3. Percentage of xylem sap N as ureides during growth of the two parental lines. ---- vegetative growth, ——— reproductive growth, △, 'Sanilac' + N; ▲, 'Sanilac' nil N; ○, 'Puebla 152 BI' + N; ●, 'Puebla 152 BI' nil N. Error bars included where they exceed symbol size.

The amino acids present in sap samples from +N and nil N plants collected on day 63 (pod filling) are shown in Table 1. The amides asparagine and glutamine were the major amino acids, with glutamine predominating in 'Puebla 152 BI' (+N and nil N). However, in 'Sanilac' asparagine was the major amino acid in +N plants, whereas in nil N plants both amides contributed approximately equal amounts to the total amino acids. Apart from the amides there were no great differences in the composition of the amino acids in either line with or without N fertilizer. Other amino acids of importance included arginine, ornithine, γ-aminobutyric acid (GABA), valine, serine, threonine, aspartate and lysine. Together with the two amides these amino acids constituted 90% of the total amino acids in 'Puebla 152 BI' with and without N fertilizer and also in 'Sanilac' with fertilizer. In 'Sanilac' nil N the percentage contribution from these two amino acids amounted to 75% of the total.

The composition of sap N from the 9 lines studied (including the 2 parental lines) was determined at two sampling dates, 43 and 57 DAP. There was no significant difference in the sap composition or the total concentration of N ml^{-1} among lines or between treatments at 43 DAP. Total sap N was 12.24 ± 0.83 μmol N ml^{-1} with α-NH_2-N contributing 18.7 ± 0.5%, amide-N 6.7 ± 0.3%, ureide-N 7.6 ± 0.2%, NH_4-N 0.2 ± 0.08% and NO_3^--N 66.8 ± 0.8%. However, in sap samples

Table 1. Percentage composition of amino acid-N in xylem sap collected from 'Pueblo 152 BI' and 'Sanilac' 63 days after planting

	P498		'Sanilac'	
Amino acid	+N	−N	+N	−N
ASP	3.3	2.0	2.2	9.4
THR	2.4	2.2	2.5	5.2
SER	2.4	2.4	2.9	4.2
ASN	29.4	28.7	37.1	15.3
GLU	1.0	1.0	1.7	3.1
GLN	35.5	44.1	26.7	19.9
ASN/GLN	0.83	0.65	1.4	0.77
GLY	0.2	0.3	0.2	0.7
ALA	0.8	1.1	1.3	2.7
CIT	0.2	0	0	0.7
VAL	2.3	1.9	2.7	4.8
CYS-CYS	0.5	0.4	0.3	0.5
MET	0.2	0.4	0.2	0.2
ILE	1.2	1.0	1.2	2.0
LEU	1.3	1.0	0.8	1.3
TYR	0.4	0.4	0.7	1.3
PHE	0.7	0.6	1.4	2.2
GABA	1.9	1.4	3.1	6.5
TRP	0.6	0.6	1.1	1.1
Ammonium	2.2	0.9	1.5	6.5
OH-LYS	0.2	0.2	0.2	0.7
ORN	3.9	3.5	2.7	0.5
LYS	2.2	1.9	1.6	3.0
HIS	0.4	0	0	0.4
ARG	4.9	2.2	6.7	5.8
PRO	0.5	0.6	0.6	0.8
β-ALA	0.6	0.2	0.8	0.8
Total μmol N ml^{-1}	4.70	3.97	7.61	2.62

For each treatment bleeding sap from 4 replicate samples (5 plants each) was pooled and a 20 μl aliquot applied to an amino acid analyzer. Results are %'s of total amino-N

collected during pod fill (57 DAP), differences were observed between lines and treatments (Tables 2 and 3). Between lines given N fertilizer there were no significant differences ($p = 0.05$) in total sap N or composition; however, rates of exudation were significantly different. Line 21 had the highest exudation rate and 'Sanilac' the lowest (Table 2). Ureides contributed between 6–12% and nitrate 49–57% to the total sap N. Between lines not fertilized significant differences were observed for total sap N and percentage contribution of ureide-N (Table 3). Ureides constituted between 17–38% of the total sap N and there was usually a corresponding decrease in nitrate when ureides increased. Amino acid and amide N remained relatively constant in sap samples from the non-fertilized lines. In all lines and treatments, allantoic acid was the predominant ureide comprising about 64% of the ureide N. Significant differences between all lines and treatments were noted for

Table 2. Relative composition of bleeding sap N collected from fertilized plants 57 days after planting

Line	μmol N ml^{-1} sap	% of total N ml^{-1} sap					Exudation rate ($\mu l\ min^{-1}\ plant^{-1}$)
		α-NH_2-N	Amide-N	Ureide-N	NH_4^+-N	NO_3^--N	
PB 152 Bl.	14.75	25	13	7	0.5	53	25
21	13.91	25	15	6.5	0.5	50	37
48	11.38	21	15	12	0	51	24
17	11.47	23	10	10	0	57	16
55	11.90	23	16	8	0.1	52	22
9	12.37	23	15	7	0.8	53	24
40	11.04	26	14	9	0.1	49	21
63	12.74	26	14	7	0.2	53	18
Sanilac	13.21	24	13	9	0.8	53	11
L.S.D.	1.94	8	4	5	0.4	7	8**

**Significant at P 0.05

Table 3. Relative composition of bleeding sap N collected from non-fertilized plants 57 days after planting

Line	μmol N ml^{-1} sap	% of total N ml^{-1} sap					Exudation rate ($\mu l\ min^{-1}\ plant^{-1}$)
		α-NH_2-N	Amide-N	Ureide-N	NH_4^+-N	NO_3^--N	
PB 152 Bl.	7.78	20	15	38	0	27	40
21	10.63	22	10	30	0.3	37	20
48	9.97	22	12	29	0	37	26
17	7.02	16	9	18	0.5	55	17
55	5.84	32	13	17	0.5	37	13
9	7.30	27	14	23	0.5	37	14
40	7.19	26	10	28	0	36	11
63	7.20	21	12	34	0	33	20
Sanilac	6.60	27	12	25	0.5	34	13
L.S.D.	0.84**	3.5	1.6	10.0**	1.0	4.9	6.0*

*,**Significant at P 0.1, 0.05 respectively

total sap N, and percentage contribution from ureide and nitrate N (LSD = 1.13, 7.9 and 4.4 respectively).

When lines were compared on the basis of their groupings as high or low fixers (according to AR values[11]) (*i.e.* Group 1 high fixers: 'Puebla 152 BI' 21, 17 and 48; Group 2 low fixers: 9, 40, 55, 63 and 'Sanilac') there were no distinct differences between the composition of the sap N within each treatment (with or without fertilizer, (Tables 2 and 3). For example, high fixers did not necessarily have a high percentage of the sap N in the form of ureides compared with the low fixers (Tables 2 and 3). Similarly low fixers did not necessarily have higher amounts of nitrate in the sap compared with high fixers. However, if one compares rates of N translocation between lines a pattern can be seen (Table 4). High fixers generally had higher rates of N translocation than low fixers.

Discussion

The decrease in the concentration of xylem sap N during vegetative and early reproductive growth observed in parental lines 'Puebla 152 BI' and 'Sanilac,' with or without N fertilizer, was similar to those reported for legumes grown in either growth chambers (*e.g.* beans[17]) or greenhouses (cowpeas[7], soybeans[8]). In those reports and in the work reported here, small increases in the concentration of sap N were observed during pod formation and filling. In field grown soybeans, although the data had more scatter and were only partly expressed on a concentration basis, estimations of xylem sap N also showed an increase during pod filling (data estimated from Streeter[14, 15]). If one assumes that the exudation rate × the concentration of sap N is an indicator of the rate of N translocation from the roots, albeit perhaps an underestimate[12], then 'Puebla 152 BI' shows a marked increase in N translocation during pod filling compared with 'Sanilac' with or without N fertilizer (Fig. 1). This difference may be a factor contributing to the higher yields obtained with 'Puebla 152 BI' compared with 'Sanilac', and is possibly the result of greater and/or prolonged rates of N_2 fixation and assimilation of soil N in 'Puebla 152 BI'.

Even though N was applied in the form of urea and *P. vulgaris* can take up and transport urea in the xylem[6], we did not detect urea in the xylem sap samples. The measurement of greater amounts of nitrate in the sap from fertilized compared with non-fertilized plants suggests that the applied urea was first converted to nitrate in the soil before being taken up by the plants.

The composition of the amino acid N in the parental lines was similar to that reported for other legumes grown in the presence or absence of

Table 4. Estimated rates of N translocation of bean lines differing in N_2 fixation potential* (57 DAP)

N_2 fixation*	Line	N translocation rate[z] nmol min^{-1} plant^{-1} +N	−N
High	'Puebla 152 BI'	369	311
	21	515	213
	48	273	259
	17	184	119
Low	55	262	76
	9	297	102
	40	232	79
	63	229	145
	'Sanilac'	119	86
LSD ($p < 0.05$)		108	68

*As measured by acetylene reduction assay[11].
[z]N translocation rate = total nmol N μl^{-1} sap x rate of exudation (μl min^{-1} plant^{-1}).

nitrate (*e.g.* beans[17], soybeans[8,14], cowpeas[13]). Generally the amides, asparagine and glutamine, constitute over 60% of the total amino acid-N with relatively large amounts of all or some of the basic amino acids (arginine, lysine and histidine). One exceptional report is that of Cookson *et al.*[4] who found serine/threonine as the major amino acids in sap of non-nodulated *P. vulgaris* fed ammonium or nitrate. However amides, although detected in sap samples were not quantified.

Data obtained from growth chamber and greenhouse studies have indicated that when ureide-producing legumes are dependent on N_2 fixation for their N supplies the percentage of ureide N in the xylem sap is high whereas asparagine and nitrate or ratios of ASN:GLN are low[8,9,13]. When plants are dependent on nitrate (fertilizer N) these relationships are reversed *i.e.* low ureides, high levels of asparagine and nitrate[8,13]. When fertilized and non-fertilized beans were grown in the field in the current studies, these relationships were shown to be true only during and beyond reproductive growth. Prior to reproductive growth (up to 43 DAP) the ratio of nitrate to ureide for example, was high (mean value ~9) in xylem sap from both fertilized and non-fertilized lines. During pod formation and pod filling this ratio varied from 4 to 8 in fertilized and from 0.7 to 3 in non-fertilized plants (calculated from Tables 2 and 3). A similar pattern of changes in sap composition during development was noted for non-fertilized soybeans grown in the field[14,15]. These results indicate that in field grown legumes where the sources of nitrogen are likely to change during the season (*e.g.* depletion of soil N, slow establishment of N_2 fixation in

the presence of soil nitrate), the time of sampling will be a critical factor in any attempt to use the composition of xylem sap N as an indicator of N_2 fixation potential. In addition, the ratio of ASN:GLN, although greater in fertilized than in non-fertilized plants as suggested by Pate *et al.*[13], was sufficiently variable between the two parental lines (Table 1) that it is doubtful that this ratio could be used as an indicator of N_2 fixation in Phaseolus.

It is of interest to note that the relative contribution of ureide-N to the sap N of non-fertilized 'Puebla 152 Bl' increased steadily during vegetative and reproductive growth. On the other hand ureides increased in sap of 'Sanilac' only during reproductive growth (Fig. 3). This could be interpreted as being the result of earlier nodulation and/or establishment of N_2 fixation in 'Puebla 152 Bl' compared with 'Sanilac' grown under the same field conditions.

Work with soybeans indicated there was little evidence for cultivar effects on the relative ureide content of sap N[9]. With lines of *P. vulgaris* differing in fixat.on and yield, however, significant differences were observed for percentage ureide in sap samples from non-fertilized treatments (Table 3).

A positive relationship was obtained between the rate of N translocation and the ranking of the bean lines on the basis of N_2 fixation (AR rates, Table 4). The trends observed were similar with or without the addition of N fertilizer suggesting that this positive relationship is independent of the N source available to the root system for export to the shoot. If this is so then it is tempting to suggest that rates of N translocation may be of use in screening trials. Further work is needed to characterize the factors affecting sap exudation rates such as soil water potential and salt concentrations before any widespread applicability of this is claimed.

Acknowledgements We thank M. Baer and K. Jokinen for expert asssitance. This works was supported by USDA/S & E Competitive Research Grant No. 59-2551-0-1-445-0.

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