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Allantoin and Amino Acid Composition in Xylem Exudates of Nodulated and Nitrate-dependent Cowpea Plants

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ABSTRAK

Kesan suntikan Rhizobium dan kepekatan N eksogen [0, 0.5, 1.0, 2.0, 4.0 dan 8.0 mM N sebagai $Ca(NO_3)_2$] ke atas tumbesaran pokok, pembintilan, pengikatan N_2, komposisi asid amino dan kelimpahan ureida dalam eksudat xilem dikaji bagi pokok kacang panjang (Vigna unguiculata L. Walp cv. Kausband) yang ditanam dalam kultur pasir dalam rumah kaca. Tanpa pemberian N, suntikan meningkatkan dengan ketara pengikatan N_2, tumbesaran bahagian atasan pokok dan kepekatan alantoin dalam eksudat berbanding dengan kawalan tanpa suntikan. Nitrogen eksogen, dibekalkan cuma kepada pokok berbintil sahaja, tidak menimbulkan apa-apa faedah ke atas tumbesaran pucuk, akar dan keseluruhan pokok berbanding dengan suntikan tanpa N, tetapi berat dan saiz bintil dan pengikatan N_2 merosot secara ketara dengan pertambahan kepekatan N melebihi 1.0 mM. Walau bagaimanapun, bilangan bintil tidak dipengaruhi oleh kepekatan N. Kepekatan alantoin dan indeks relatif ureida [(ureida-N/ jumlah N dalam eksudat) x 100] merosot secara drastik dengan kepekatan N, tetapi kelimpahan NO_3-N dalam eksudat menunjukkan trend yang bersetentangan. Dalam pokok yang bergantung kepada simbiosis Rhizobium, alantoin adalah hasil eksport yang predominan (94%) dalam eksudat, dengan baki bahan larutan terdiri daripada NO_3-N (4.5%) dan amino-N (1.5%). Di sebaliknya NO_3-N adalah bentuk N utama (87%) dieksport dalam eksudat xilem bagi pokok bergantung penuh kepada NO_3. Walaupun 20 asid amino berlainan dikenalpasti dalam eksudat, glutamina adalah komponen yang terbanyak (65%) dalam tanaman bergantung kepada bintil, dan asparagina adalah asid amino predominan (43%) dalam pokok bergantung kepada NO_3. Berat bintil dan indeks relatif ureida berkorelasi secara tinggi dan positif ($r = 0.959^{**}$). Kepekatan alantoin dan pengikatan N_2 mempunyai korelasi yang rendah ($r = 0.655^{**}$). Kepentingan data ini dalam kajian pengikatan N_2 simbiosis dibincangkan.

ABSTRACT

The effects of Rhizobium inoculation and exogenous N concentrations [0, 0.5, 1.0, 2.0, 4.0 and 8.0 mM N as $Ca(NO_3)_2$] on plant growth, nodulation, N_2 fixation, amino acid composition and ureide abundance in xylem exudates of nodulated cowpea (Vigna unguiculata L. Walp cv. Kausband) were investigated in glasshouse-grown plants in sand culture. Without applied N, inoculation markedly increased N_2 fixation, top growth and allantoin concentration in exudates compared with the uninoculated control. Exogenous N, applied only to nodulated plants, had no beneficial effects on top, root or whole plant growth compared with inoculated control, but nodule mass and size and N_2 fixation decreased substantially with increasing N concentrations exceeding 1.0 mM. However, nodule number was unaffected by any N concentration. Allantoin concentration and relative ureide index [(ureide N/ total N in exudates) x 100] declined drastically with N concentration, but the abundance of NO_3-N in the exudates followed the reverse trend. In plants fully dependent on Rhizobium symbiosis, allantoin was the predominant (94%) export product recovered in xylem exudates, with the remaining N solutes comprising of NO_3-N (4.5%) and amino-N (1.5%). Conversely, NO_3-N was the principal (87%) form of N exported in xylem of NO_3-dependent plants. Although 20 different amino acids were identified in the exudates, glutamine was the most abundant component (65%) in the nodule-dependent crop whereas asparagine was the predominant (43%) amino acid in the NO_3-dependent plants. Nodule mass and relative ureide index were highly and positively correlated ($r = 0.959^{**}$).

*Allantoin concentration and N_2 fixation had low correlation coefficient (r = 0.655**). The significance of these data in biological nitrogen fixation studies is discussed.*

Keywords: Asparagine, correlation, cowpea, glutamine, NO_3-N, nodule mass, N-free nutrient solution, relative ureide index, sand culture

INTRODUCTION

Investigations on the composition of nitrogenous solutes in xylem exudates of plants of cowpea (*Vigna unguiculata* L. Walp) and soybean (*Glycine max* L. Merrill) and detailed tracer studies using ^{14}C and ^{15}N reveal that ureides (allantoin and allantoic acid) are products of N_2 fixation (Herridge *et al.* 1978; Matsumoto *et al.* 1976; Matsumoto *et al.* 1977a, 1977b). In many tropical legumes ureides form the major soluble nitrogen pool exported from the nodules to the shoots *via* the xylem stream (Schubert 1986; Peoples *et al.* 1989a; Peoples and Herridge 1990).

The relative abundance of ureides in the xylem sap of legumes is regarded as an indicator of plants' dependency on *Rhizobium* symbiosis in a number of different legumes and can provide a quantitative assay of N_2 fixation. There is a progressive and predictable change in xylem sap composition from non-nodulated plants, which largely export nitrate and amino acids to the shoots when supplied with mineral N, to a xylem stream dominated by ureides in nodulated plants supplied with N-free nutrient solution (McClure *et al.* 1980; Pate *et al.* 1980; Rerkasem *et al.* 1988; Peoples *et al.* 1989b; Herridge and Peoples 1990; Herridge *et al.* 1990).

Although the effects of mineral N application on nodulation, ureide transport and composition of N solutes in xylem exudates of soybean have been widely studied (e.g McClure and Israel 1979; McClure *et al.* 1980; Herridge and Peoples 1990), less is known about the correlation between nodule mass, allantoin concentration, N_2 fixation and the proportional changes in the export products with the plants' decreasing relative dependency on N_2 fixation in other species. This paper examines the spectra of N solutes and amino acid composition exported in xylem exudates of nodulated cowpea plants subjected to a wide range of mineral N concentration supplied in the rooting medium.

MATERIALS AND METHODS

Plant Culture and Maintenance

Four seeds of cowpea (*Vigna unguiculata* (L.) cv. Kausband) were either uninoculated or inoculated with *Rhizobium* strain CB 756 and sown (on 27 December 1989) to a depth of 2 - 3 cm in a sand culture contained in 10 L pots (26 cm diameter x 26 cm height). Each pot contained 16 kg of washed river sand of the following chemical analysis: total N(1.0 g kg^{-1}), total P(58 mg kg^{-1}), K(8.3 g kg^{-1}), Mg(5.4 mg kg^{-1}) and organic matter (3 g kg^{-1}).

After 2 weeks the seedlings were thinned to 2 per pot. The pots were placed in a glasshouse (from 27 December 1989 to 15 February 1990) located at the Department of Field Crops and Grassland Science, Wageningen Agricultural University, The Netherlands. The environmental conditions in the glasshouse were similar to those of previous experiment (Othman *et al.*, 1991). The plants were supplied weekly with 300 ml per pot of basal N-free nutrient solution (pH 6.10) of the following composition (μM): P (200), K (2000), Mg (400), S (1000), Ca (160), Mn (36), B (20), Zn (12), Fe (10), Mo (3.5), Cu (2.5), Cl (2.1), Na (0.8), Co (0.2). The plants were watered daily to field capacity with deionised water. The calibration for the exact volume of water to achieve field capacity (with no excess drainage) was accurately determined prior to the experiment. All nutrients applied (including the applied N) were assumed to be taken up by the plants.

Treatments

The inoculated plants were subjected to 6 concentrations of mineral N solution containing 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mM N [as $Ca(NO_3)_2$]. The uninoculated plants were supplied with 0 mM N. This treatment is referred to as the uninoculated control. The N accumulated by this "reference crop" was used in the calculation of N_2 fixation by total N assays. Each pot received 300 ml of its respective N treatment solution per week.

The 7 treatments were arranged in a randomised complete block design with 4 replications. The data were subjected to an analysis of variance and Duncan multiple range test and least significant difference (LSD).

Plant Harvesting and Xylem Exudate Collection

All plants were harvested 50 days after sowing. At harvest, plant tops were cut below the cotyledonary nodes and a tightly-fitted 3 cm long silicon tube was placed over each stump. Xylem exudates were collected over a period of 1 h. The techniques of exudate collection were the same as the ones described in an earlier experiment (Othman *et al.* 1991). The exudates dispensed into 1.0 ml vials were stored in a freezer (-15° C) until analyses were carried out.

After exudate collection, the root systems were harvested. The sand was washed off the roots, the nodules separated and then counted. The roots, nodules and plant tops were dried in an oven at 75° C for 48 h and weighed. These plant parts were later ground in a Ritz plant grinder, passing through a 1.0 mm mesh screen. The ground plant samples were digested by the micro Kjeldahl techniques and the N concentrations determined on a Technicon autoanalyser.

Determination of Nitrogenous Solutes and Amino Acid Composition in Exudates

The concentrations of allantoin (ureide-N), amino-N and nitrate-N in the exudates were determined colorimetrically on a Vitatron spectrophotometer using the procedures described by Young and Conway (1942), Yemm and Cocking (1955) and Cataldo *et al.* (1975) respectively. These analytical techniques have been modified by Peoples *et al.* (1989a).

The composition of amino acids in the exudates was assayed using an amino acid analyser (Biotronik LC 6000 E) fitted with Durrum DC 6A column (25 cm long and 6 mm diameter). A 250 μL mixture, consisting proportionally of 100 μL of exudate sample, 320 μL diluting buffer (for physiological fluid separations) and 80 μL Norleucine solution (external standards), was injected into the column for analysis.

RESULTS

Top and Root Growth

Without inoculation and devoid of mineral N, cowpea plants showed extremely poor growth of tops and whole plants, yielding about 6.8 and 9.5 g dry matter per pot respectively (Fig. 1a and 1c). However, top and overall plant growth were significantly increased ($P < 0.05$) by inoculation alone without the need for application of mineral N. This increase in dry matter production was 81 and 70% for top growth and whole plant respectively. Inoculated plants showed no positive response to increasing concentration of mineral N. In fact top and whole plant growth showed at first a tendency to increase with N application from 0 to 2.0 mM and then a decline thereafter. However, these increases and decline were non-significant compared with the inoculated control (+ *Rhizobium* + 0 mM N). Unlike top growth, root dry weight was apparently unaffected by either inoculation or applied N (Fig. 1b).

Nodulation

Nodule number, size and mass showed differential responses to inoculation and N application. Unfortunately, the uninoculated plants produced appreciable amounts of small and white-coloured nodules at harvest, indicating a rhizobial contamination in the glasshouse. Nodule mass and size of inoculated plants declined sharply ($P < 0.01$) with increasing concentrations of mineral N applied, but nodule number appeared to be unaffected by N application (Fig. 2), despite a declining trend, due to the large variability in nodule numbers between plants and the large standard errors. Nitrogen concentration of more than 1.0 mM was deleterious to nodulation, with both nodule mass and size greatly suppressed. The reduction in nodule mass and size caused by increasing concentration of applied N was highly significant ($P < 0.01$).

Concentration and Composition of N Solutes in Xylem Exudates

Without applied N, inoculation significantly ($P < 0.05$) increased allantoin concentration compared with the uninoculated control. However, allantoin concentrations declined drastically ($P < 0.01$) from 425 to 50 nmol ml^{-1} when the concentration of mineral N was increased from 0 to 8.0 mM (Fig. 3a). In contrast, the concentration of NO_3-N was the lowest (40 nmol ml^{-1}) at 0 mM N, but increased sharply to 650 nmol ml^{-1} at 4.0 mM N and then levelled off at 8.0 mM N.

The concentrations of amino-N showed erratic trends with applied N between 0 to 2.0 mM, but further increases in N supply had little effect on this soluble nitrogen component. Nonetheless, the concentration of amino-N remained fairly constant at 7 nmol ml^{-1} between 2.0 to 8.0 mM mineral N application.

The composition of soluble N, pool in xylem exudates, expressed as a percentage of total N followed similar patterns as their concentrations described earlier. In plants fully depend-

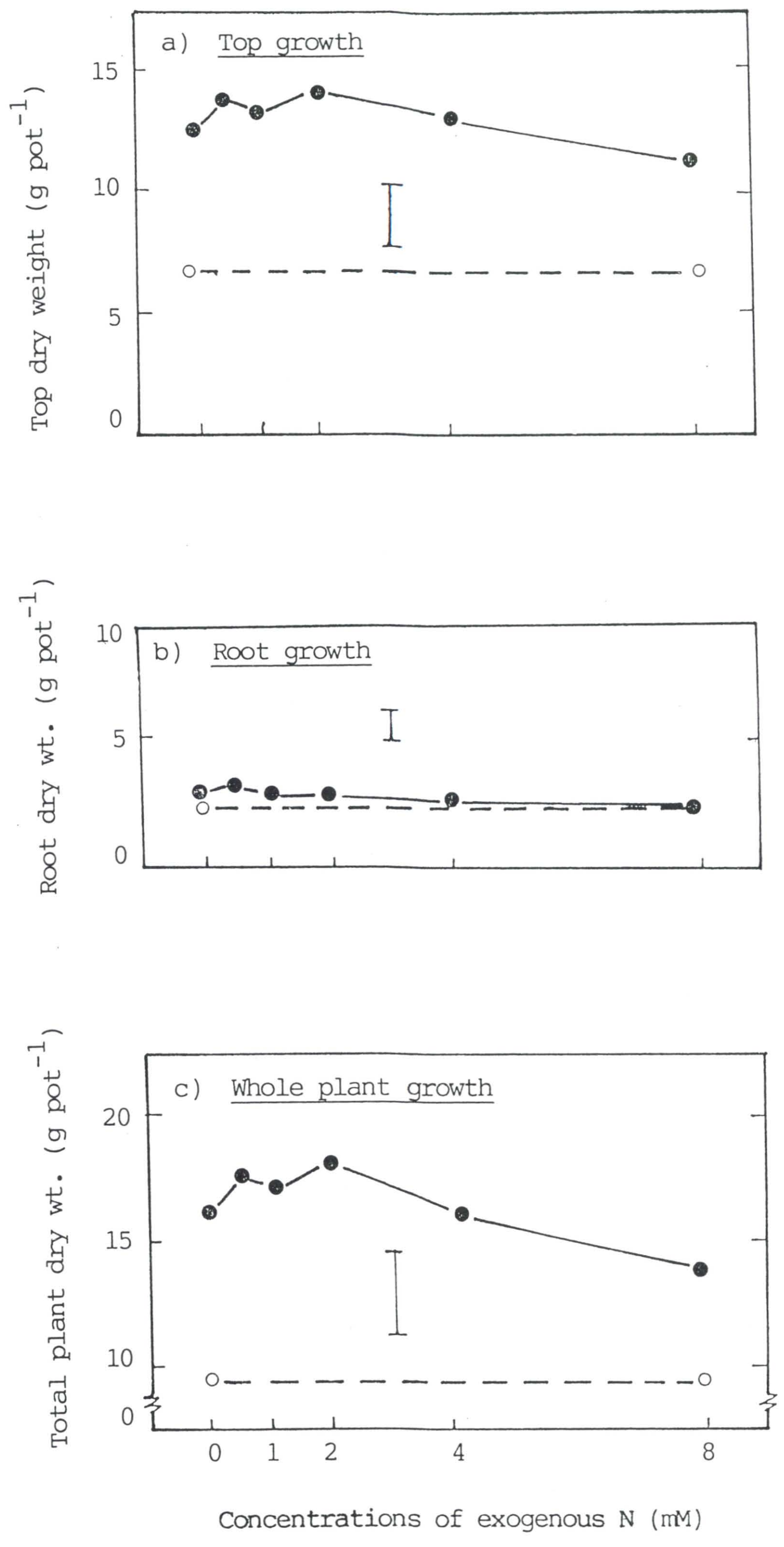

Fig. 1: Effects of Rhizobium *inoculation and exogenous N on top, root and growth of whole cowpea plants. Vertical bars denote LSDs at* $P < 0.05$*.* *(• = inoculated plants, o = uninoculated + 0 mM N)*

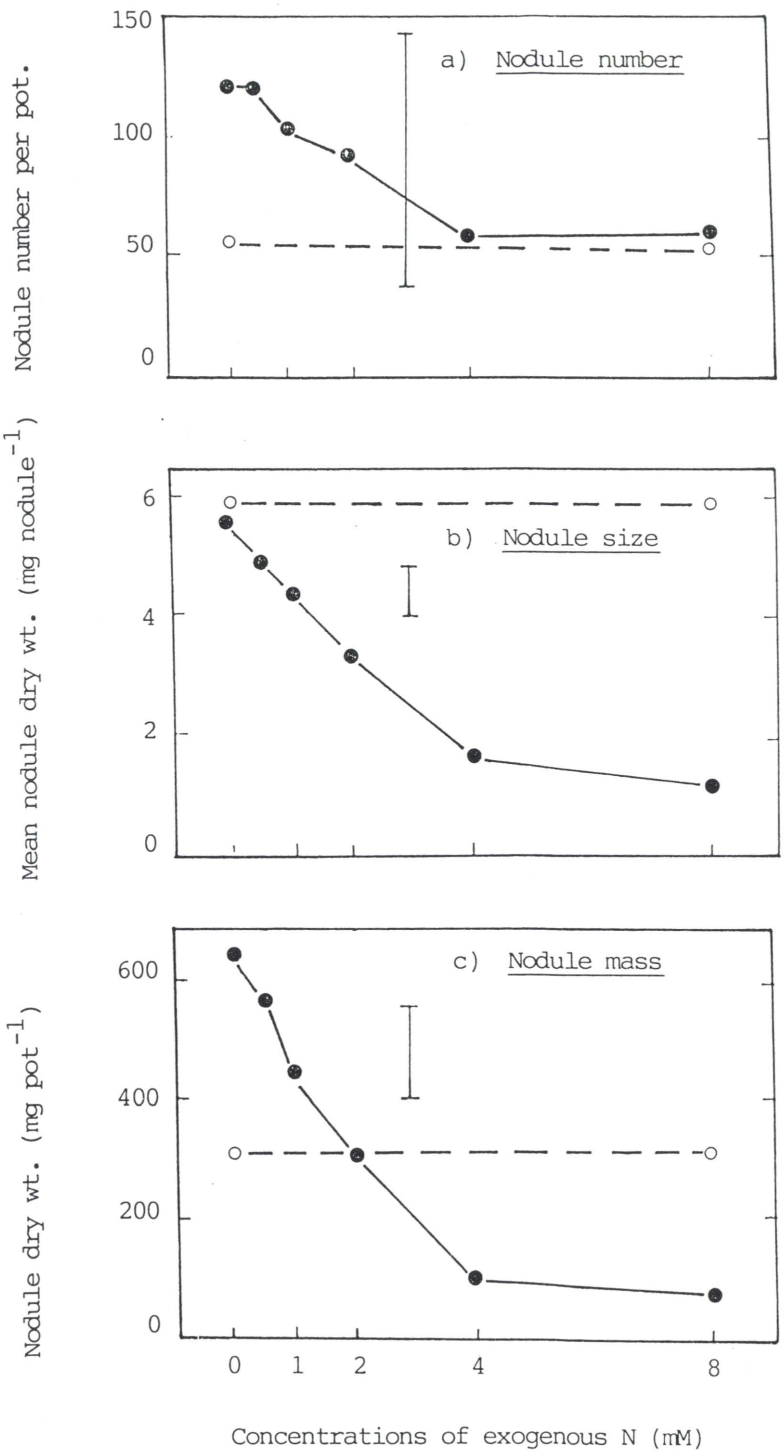

Fig. 2: Effects of Rhizobium *inoculation and exogenous N on nodule number, size and mass. Vertical bars denote LSDs at $P < 0.05$. (• = inoculated plants, o = uninoculated + 0 mM N)*

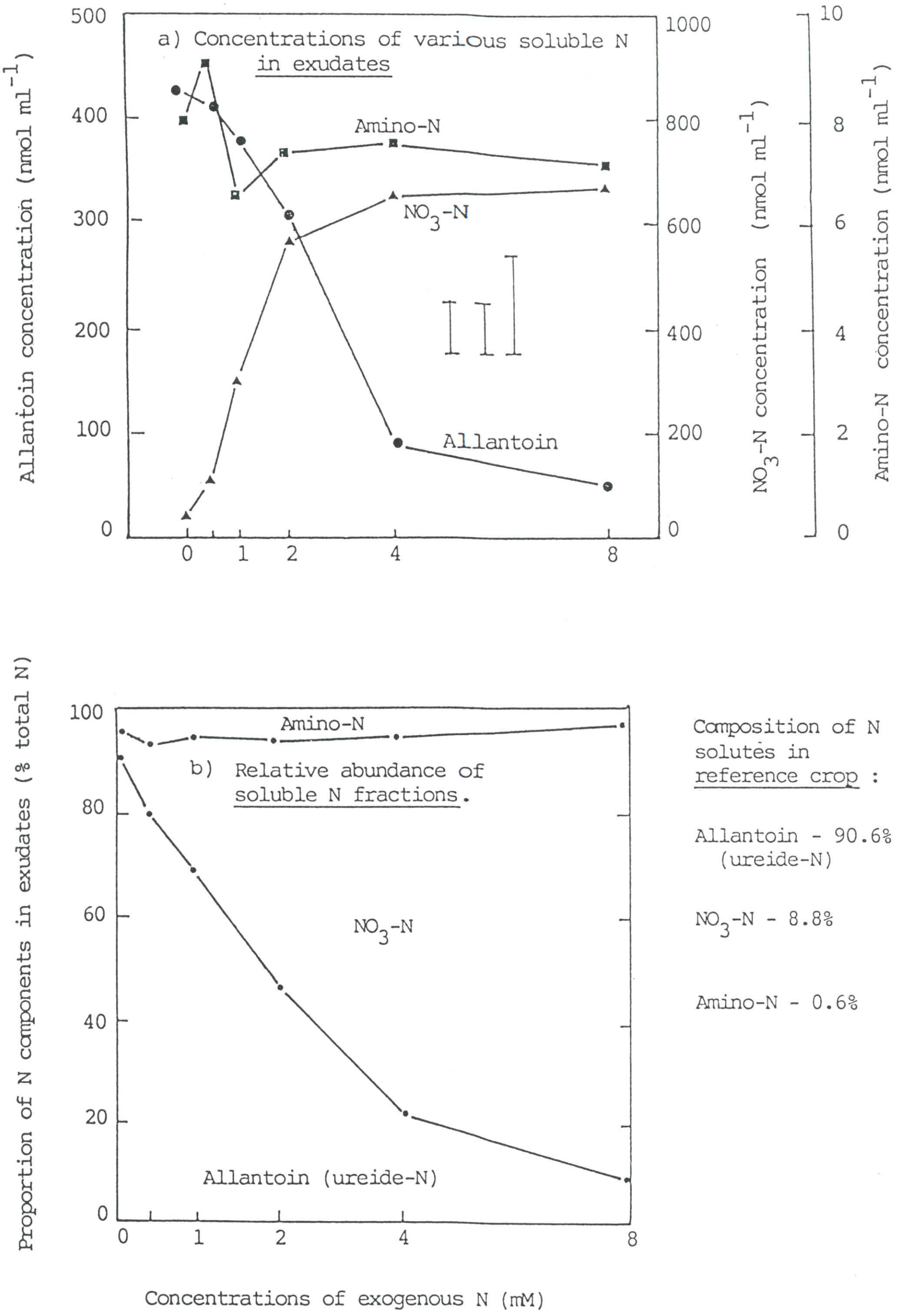

Fig. 3: (a) Concentrations of allantoin, NO_3 -N and amino-N in xylem exudates of cowpea plants and (b) the relative abundance of various soluble N fractions as affected by mineral N application. Vertical bars, from left to right, denote LSDs at $P < 0.05$ for allantoin, amino-N and NO_3 -N respectively. (The soluble N concentration (nmol ml^{-1}) in reference crop: Allantoin (241), amino-N (2.6) and NO_3 -N (31)

ent on symbiosis (*Rhizobium* inoculation + 0 mM N), allantoin (a form of ureide-N) formed the predominant (94%) export product in the exudates with the remainder comprising of NO_3-N (4.5%) and amino-N (1.5%) (Fig. 3b). However, as the supply of exogenous N in the nutrient solution was increased from 0 to 8.0 mM N, the proportion of allantoin declined drastically, tapering to only about 10% at 8.0 mM N when the plants were presumed to be almost fully dependent on mineral N (Fig. 3b). On the other hand, the proportion of NO_3-N increased ($P < 0.01$) rapidly from about 4.5% in the nodule-dependent plants to about 87% in plants dependent on mineral N. The proportion of amino-N in the soluble N pool was relatively small, (ranging between 1.0 and 2.5%) and showed erratic trends with exogenous N concentrations. These inconsistent effects were fairly similar to that of amino-N concentration.

The relative ureide index calculated using the formula :

$$\frac{4 \text{ allantoin conc.}}{(4 \text{ allantoin conc} + NO_3\text{-N} + \text{amino-N})} \times 100$$

(After Peoples *et al.* 1989a)

followed similar patterns as the allantoin composition (%) in Fig. 3b. This index indicated the declining dependency of plants on N_2 fixation as the mineral N was increased from 0 to 8.0 mM.

Composition of Amino Acids

On analysis, the amino-N component was found to contain 20 different amino acids. The most abundant ones, on average, were glutamine (48%), asparagine (13%), aspartic acid (7%), arginine (7%), lysine (4%), histidine (3%) and valine (2%). The other amino acids formed only a minor fraction of the amino-N component.

The application of mineral N caused drastic changes in the composition (percentages) of glutamine, asparagine and aspartic acid, with only marginal variations in the composition of other amino acids (Fig. 4). In fully symbiotic plants and those subjected to 0.5 and 1.0 mM N, glutamine was the predominant (65%) amino acid, with asparagine and aspartic acid comprising about 8 - 10 and 7 - 10% respectively. As the concentrations of mineral N were increased from 1.0 to 8.0 mM, asparagine greatly increased in abundance with glutamine and aspartic acid declining sharply ($P < 0.01$) in proportion. At 8.0 mM N, the major amino acids were asparagine (43%), glutamine (17%), arginine (10%), aspartic acid (5%) and others (25%).

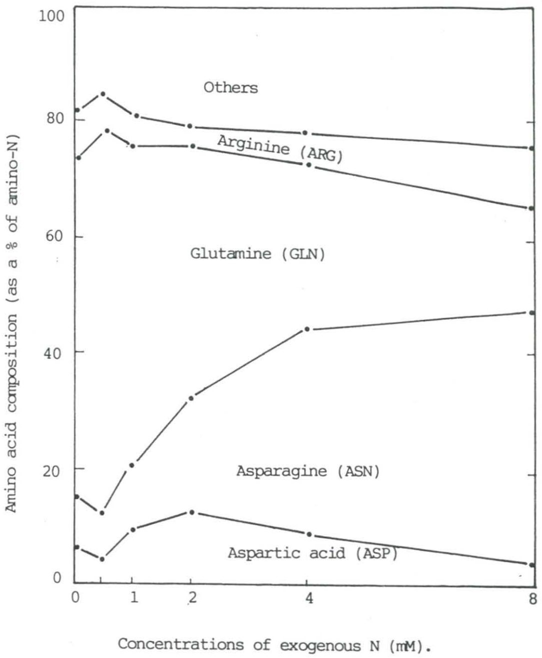

Fig. 4: Changes in the composition of predominant amino acids (asparagine and glutamine) in xylem exudates of nodulated cowpea plants subjected to various concentrations of exogenous N. (The amino acid composition in the reference crop: GLN (60%), ARG (6.6%), ASN (4%), ASP (3%) and others (26.4%).

Estimation of N_2 Fixation by Total N Assays

The total N recovered in the plant tops, roots and nodules was summed up, giving the N content in the whole plant. In the uninoculated control (reference crop), the whole plant total N was largely derived from the sand culture medium and from seeds, with the basic assumption that no symbiotic fixation occurred. The second basic assumption was that all plants, irrespective of N treatments or inoculation, took up the same amount of N from the planting medium.

TABLE 1
Effects of applied N on N content in whole plant and N_2 fixation

Applied N conc. (mM)		N in whole plant (mg pot^{-1})	N from fertiliser (mg pot^{-1})	N from sand/ ref. crop* (mg pot^{-1})	N from symbiotic fixation (mg pot^{-1})
0	Uninoculated	156 b	0	156*	-
0	Inoculated	532 a	0	156	376 a
0.5	"	563 a	29.4	156	378 a
1.0	"	521 a	58.8	156	306 ab
2.0	"	550 a	117.6	156	276 b
4.0	"	632 a	235.2	156	240 b
8.0	"	569 a	470.4	156	0 c (-57)

* Amount of N from reference crop (uninoculated + O N) = 156 mg pot^{-1}.
Figures in the same column followed by the same letters are not significantly different at $P < 0.05$.

TABLE 2
Correlation coefficients between nodule dry weight, allantoin concentration, relative ureide index (RUI) and N_2 fixation (total N assay)

Parameters	Correlation coefficients (r)
Nodule d. wt and RUI$^+$ (%) (mg pot^{-1})	0.959 **
Nodule d. wt and allantoin conc. (mg pot^{-1}) (nmol ml^{-1})	0.900 **
Nodule d. wt and N_2 fixation (mg pot^{-1}) (mg N pot^{-1})	0.731 **
Allantoin conc. and RUI$^+$ (%) (nmol ml^{-1})	0.914 **
Allantoin conc. and N_2 fixation (nmol ml^{-1}) (mg N pot^{-1})	0.655 **
RUI$^+$ (%) and N_2 fixation (mg N pot^{-1})	0.736 **

** Significant at $P < 0.01$
\+ RUI = relative ureide index

With inoculation and N application, the whole plant N was derived from 3 sources, namely from symbiotic fixation, from the applied mineral N and from the sand culture medium. Since the amount of N derived from fertiliser and reference crop were known, N from symbiotic fixation was calculated from the difference between N in whole plant and N from the other 2 sources (Table 1).

Nitrogen from fixation was significantly reduced by N application only when the concentration exceeded 1.0 mM, compared with the inoculated control. A further increase in mineral N concentration caused a further reduction in N_2 fixation, although this detrimental effect was not as prominent as that of relative ureide index outlined in the previous section. Without applied N, inoculation significantly increased N_2 fixation compared with no inoculation. However, applied N between 0.5 and 1.0 mM had little effect on N_2 fixation compared with the inoculated control. As the applied N concentration was increased further from 2.0 to 8.0 mM, the plants' symbiotic activity decreased progressively and was completely inhibited at 8.0 mM N, when the plants were fully dependent on mineral N for nutrition.

A relationship between nodule dry weight, allantoin concentration relative ureide index and N_2 fixation was computed to establish the correlation of these nodule activity parameters (Table 2).

Nodule dry weight and relative ureide index were highly and positively correlated (r = +0.959**). Although nodule dry weight, allantoin concentration and N_2 fixation were also highly correlated, the coefficients of correlation were much lower than those of nodule mass and relative ureide index. Similarly, allantoin concentration and N_2 fixation, and relative ureide index and N_2 fixation were positively and significantly ($P < 0.01$) correlated, but the former two nodule activities had the lowest coefficient of correlation (r = +0.655**).

DISCUSSION

Legume plants often respond to *Rhizobium* inoculation where effective native rhizobia are lacking in numbers and weak in colonisation of root zones. Farmers and investigators often apply low rates of nitrogenous fertilisers to inoculated legume crops mainly to promote initial growth prior to nodule initiation. Our present data indicated that mineral N supply had no beneficial effects on early growth when cowpea plants were well nodulated (Figs. 1 and 2). In fact mineral N exceeding 1.0 mM was detrimental to nodulation, with nodule mass and size adversely affected (Fig. 2) followed by depressed N_2 fixation (Table 1). The negative value of N_2 fixation in the 8.0 mM N treatment in Table 1 was associated with the accidental rhizobial contamination of the uninoculated reference crop which consequently overestimated the N derived from the growth medium.

Over the years much research emphasis has been focussed on direct and indirect methods of estimating symbiotic N_2 fixation of field-grown crop legumes, using the ureide analysis or ^{15}N depleted dilution techniques as quantitative assays (e.g. Norhayati *et al.* 1988; Rerkasem *et al.* 1988; Pereira *et al.* 1989; Herridge *et al.* 1990; Peoples and Herridge 1990). Although several methods of assessment of N_2 fixation are available (Peoples *et al.* 1989a), ^{15}N methodology is currently the most accurate but expensive. The ureide analysis technique employed in the present study is an indirect method but simple, sensitive and inexpensive, requiring no excavation of nodulated root systems as opposed to acetylene reduction assays. The ureide analysis offers an alternative assessment procedure with practical applications and which allows researchers to compare accurately differences between treatments in respect of plant dependency on N_2 fixation where other N sources (soil-N and fertilizer-N) exist and affect nodule functioning. Since tropical legumes relying on N_2 fixation export ureide-N from the nodulated root systems to the shoots and mineral N absorbed by the roots is exported in the form of NO_3-N, this biochemical discriminating factor makes quantitative assays much simpler. The extremely abundant ureide-N (allantoin) and the low level NO_3-N recovered in xylem exudates of plants fully dependent on *Rhizobium* symbiosis obtained in the present study (Fig. 3) distinguished that from NO_3-dependent crops in which NO_3-N was the principal export product. In the latter case the proportion of allantoin in exudates was small and nodule activity was markedly impaired or completely inhibited (Fig. 3b, Table 1). This is consistent with other observations on cowpea (Herridge *et al.* 1978; Pate *et al.* 1980; Peoples *et al.* 1985; Elowad *et al.* 1987; Peoples and Herridge 1990).

The declining trends in allantoin abundance, nodule mass and N_2 fixation in response to exogenous N application correlated strongly with one another and were interdependent (Figs. 2 and 3, Tables 1 and 2). These results suggest that allantoin concentration (and relative ureide index) are reliable indicators of nodule activity and the plants' dependency on N_2 fixation. However, difficulties in collecting root-bleeding xylem sap from root stumps of field-grown plants, as experienced by Streeter (1972), may limit the widespread adaptation of this technique. However, alternative techniques of recovering xylem sap have more recently been developed (Herridge *et al.* 1988; Peoples *et al.* 1989a). The exceptionally large proportion of glutamine in exudates of nodule-dependent plants and the abundant asparagine in NO_3-fed crop is another biochemical determinant of nodule activity (Fig. 4) in response to applied N (or other factors, see Pate *et al.* 1980). The amino acid analysis may be particularly useful in studies with peanut or other legumes which are known to be non-ureide exporters, but transport amides from their nodules (Peoples *et al.* 1986, 1987; Norhayati *et al.* 1988).

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