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Pathways of Nitrogen Assimilation in Cowpea Nodules Studied using $^{15}N_2$ and Allopurinol[1]

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ABSTRACT

In the presence of 0.5 millimolar allopurinol (4-hydroxypyrazolo [3,4-*d*]pyrimidine), an inhibitor of NAD:xanthine oxidoreductase (EC 1.2.3.2), intact attached nodules of cowpea (*Vigna unguiculata* L. Walp. cv Vita 3) formed [^{15}N]xanthine from $^{15}N_2$ at rates equivalent to those of ureide synthesis, confirming the direct assimilation of fixed nitrogen into purines. Xanthine accumulated in nodules and was exported in increasing amounts in xylem of allopurinol-treated plants. Other intermediates of purine oxidation, *de novo* purine synthesis, and ammonia assimilation did not increase and, over the time course of experiments (4 hours), allopurinol had no effect on nitrogenase (EC 1.7.99.2) activity. Negligible ^{15}N-labeling of asparagine from $^{15}N_2$ was observed, suggesting that the significant pool (up to 14 micromoles per gram of nodule fresh weight) of this amide in cowpea nodules was not formed directly from fixation but may have accumulated as a consequence of phloem delivery.

The ureides, allantoin and allantoic acid, are the major forms of fixed nitrogen exported from nodules of a wide range of tropical legume symbioses (1). *In vivo* labeling studies with [^{14}C] glycine (3) or $^{14}CO_2$ (9) support the idea that allantoin is formed in nodules from purines. Consistent with these data, high activities of enzymes of *de novo* purine synthesis have been demonstrated in extracts from cowpea (*Vigna unguiculata* L. Walp.) and soybean (*Glycine max* L. Merr.) nodules (4), together with specific purine nucleosidase (8) and enzymes of purine oxidation (IMP:oxidoreductase, xanthine oxidoreductase, and urate oxidase) (3, 6, 21). Although a number of studies using labeled N_2 have confirmed that currently fixed nitrogen is utilized in ureide synthesis (12-16), none has reported the recovery of purine pathway intermediates as products of fixation. As has been pointed out previously (4), the involvement of the purine pathway in the flow of N in nodules is therefore inferred rather than proven. The present study reports the recovery of [^{15}N]xanthine from nodules of cowpea plants exposed concurrently to $^{15}N_2$ and the xanthine oxidoreductase inhibitor, allopurinol.

MATERIALS AND METHODS

Plant Material. Cowpea (*Vigna unguiculata* L. Walp. cv Vita 3) plants, effectively nodulated with *Bradyrhizobium* strain CB756, were grown in closed containers with nitrogen-free nutrient solution as described previously (5). Each 3.5-L liquid culture container comprised five plants with 2.6 L of nutrient solution. The solution was changed every 3 d and was bubbled continuously with ambient air. Plants were used 22 d after sowing, when the initially formed crown nodulation zone had reached maximum size (5) and the specific activity of nitrogenase was greatest (7).

Exposure to $^{15}N_2$. The volume of nutrient solution in the containers was adjusted to 2.8 L leaving a gas space of 0.7 L in which the nodulated zone of each root system was located. Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) treatments involved a final concentration of 0.5 mM in the nutrient solution. Plant stems were sealed to the container lid with Terostat VII (Teroson G.M.b.H., Heidelberg, FRG), and the nutrient solutions and root atmospheres were purged for 5 min with a mixture of 80% Ar:20% O_2 (v/v) prior to the addition of $^{15}N_2$ (99.9 atom % excess) and sealing to give a final atmosphere of approximately 30% N_2 at 35 atom % excess ^{15}N, 20% O_2 and the balance as Ar. The five plants in a container were removed after 0.5, 1, 2 or 4 h exposure to ^{15}N, and the root systems were plunged into liquid N_2 for storage prior to extraction of nodules.

Extraction of Nodules and Recovery of ^{15}N-Labeled Solutes. At all stages of extraction and fractionation polypropylene containers, rather than glass, were used to minimize loss of purine bases due to adsorption. Frozen nodules were homogenized in hot 80% (v/v) ethanol and water-soluble materials collected as described earlier (3). Amino acids and ureides were separated with the large capacity cation exchange resin (56 × 0.9 cm) column of a Beckman 118C Amino Acid Analyzer operating in the physiological fluids mode with Li-based buffers. The effluent from the analyzer column was collected directly in 1 or 2 ml fractions, and the amino acids present in individual fractions were identified and measured in assays of 10 µl lots by an ion-exchange HPLC amino acid analyzer technique which also incorporated Li buffers and post-column ninhydrin detection. In this way it was possible to identify fractions containing only a single amino compound. These were then subjected to Kjeldahl digestion and distillation, and the ammonia was recovered for mass spectral measurement of ^{15}N after hypobromite oxidation (10). Where the amount of nitrogen recovered in distillates was below that required by the mass spectrometer (approx. 100 µg N), unenriched ammonium sulfate (of known ^{15}N natural abundance) was added to the distillate prior to oxidation. Ureides (allantoin and allantoic acid) were collected from the amino acid analyzer column, and their ^{15}N contents were determined after identification by an HPLC anion exchange technique (19).

Purine bases, nucleosides, and nucleotides were separated and assayed by HPLC using the reverse phase gradient ion-pairing and ion-suppression procedures described previously (4) except that ammonium phosphate salts were replaced with sodium and potassium phosphate salts in the elution buffer used in the ion-suppression method. Xanthine was collected from the HPLC effluent following ion suppression chromatography (Fig. 1A), combining collections from a number of injections. The pooled material was rechromatographed in both separation systems (Fig. 1, A and B) to check purity and confirm identity by co-chro-

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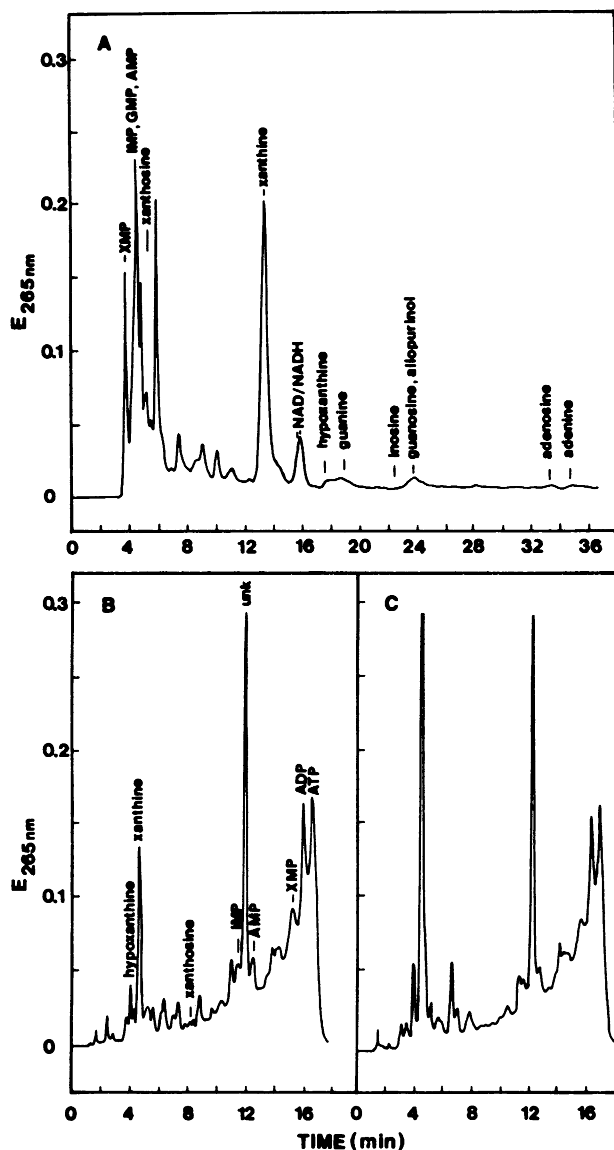

FIG. 1. HPLC separation of solutes in extracts of cowpea nodules. A, Reverse phase ion suppression chromatography of an extract of nodules from plants 1 h after exposure to 0.5 mM allopurinol; B, Reverse phase ion pairing chromatography of an extract of nodules from plants 0.5 h after exposure to 0.5 mM allopurinol; C, as for B but 2 h after exposure to allopurinol.

matography with authentic xanthine. The ^{15}N content of the isolated xanthine was determined after Kjeldahl digestion as described above for isolated amino compounds and ureides.

Nitrogenase Assay. Nitrogenase was assayed by acetylene reduction in the rooting atmospheres of containers treated and maintained under the same conditions used in those exposed to $^{15}N_2$, but with omission of the labeled gas. Acetylene was added to 10% (v/v) in the root atmosphere, the pots were sealed, and gas samples were taken after 10 and 20 min for ethylene assay. Although the assay used a static atmosphere, similar assays of acetylene reduction employing a flowing gas stream indicate that in this symbiosis a 10 to 20 min sample period provides maximum, stable values of ethylene production (FD Dakora, CA Atkins, unpublished results).

Xylem Sap Collection. Root bleeding exudate was collected for 30 min following decapitation of all five plants in each duplicate pot of a treatment. The sap was immediately frozen

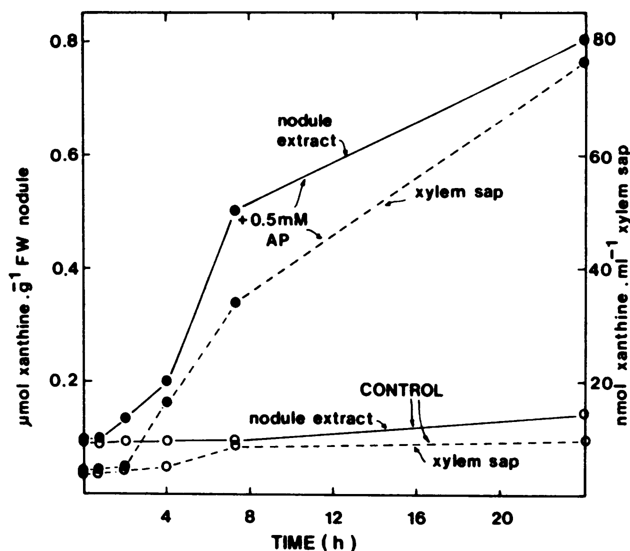

FIG. 2. Effect of allopurinol on levels of xanthine in nodule extracts and in xylem sap collected from cowpea plants.

and subsequently assayed for ureides, purines and amino compounds by the HPLC methods described above.

RESULTS AND DISCUSSION

Exposing the roots of cowpea to allopurinol resulted in a rapid rise in the nodule pool of xanthine and a rapidly increasing level of xanthine in root bleeding xylem sap (Fig. 2). This accumulation of xanthine is consistent with earlier studies (3, 9, 11) showing inhibition by allopurinol of the very active xanthine oxidoreductase present in nodules of cowpea (3) and other ureide-producing symbioses. Some of the applied allopurinol was subsequently recovered in nodule tissue extracts (Fig. 1A) (11), in agreement with a direct inhibition in the organ, but the extent to which allopurinol was transported elsewhere in the plant, ribosylated (11) or otherwise metabolized was not investigated. While the source of the low endogenous level of xanthine in xylem of untreated plants (Fig. 2) has not been defined, the grossly elevated level of this compound in xylem following allopurinol treatment is considered to represent genuine export from the nodule, indicating that purine bases formed from fixed nitrogen may be as readily loaded into xylem as are normally their oxidized products (ureides).

Small increases in hypoxanthine were also recorded in nodule extracts of allopurinol-treated plants (Fig. 1, B and C) These were 1/10 to 1/20 those of xanthine, indicating that IMP is likely to be metabolized principally through IMP oxidoreductase rather than by an alternative route via inosine. This is consistent with the conclusions of earlier studies on cowpea nodules (21) based on kinetic properties of the purified component enzymes (6) and on the production of XMP in a cell-free system (21).

The nucleotide products of *de novo* purine synthesis (IMP, XMP) or intermediates of their metabolism to bases (xanthosine, inosine) did not accumulate in nodules as a consequence of the inhibition of xanthine (or hypoxanthine) oxidation by allopurinol (Fig. 1). Furthermore FGAR[2] and AICAR, intermediates of the *de novo* purine pathway, identified previously (4) as being formed during purine synthesis with cell-free nodule extracts of cowpea, did not increase in response to allopurinol treatment. Although the HPLC analyses employed would have failed to recover other more labile intermediates of the purine pathway,

[2] Abbreviations: FGAR, formyglycinamide ribonucleotide; AICAR, aminoimidazole carboxamide ribonucleotide.

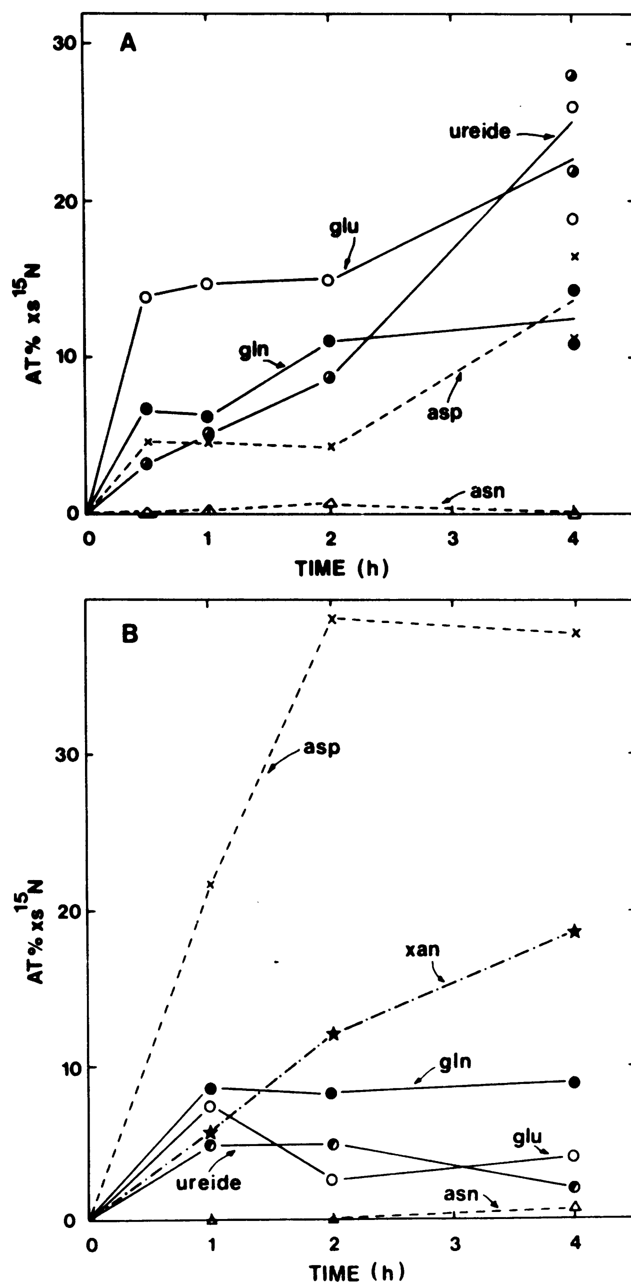

FIG. 3. ^{15}N-labeling of solutes of the soluble nitrogen pool of nodules of cowpea exposed to $^{15}N_2$ (approximately 35 at % excess ^{15}N). A, Control plants; B, plants exposed to 0.5 mM allopurinol in the rooting medium at time zero.

Table I. *Recovery of ^{15}N from $^{15}N_2$ in Nodule Pools of Glutamine, Ureide, and Xanthine in Plants Treated with or Without 0.5 mM Allopurinol in the Rooting Medium*

Treatment	Compound	Time (h)		
		1	2	4
		µg $^{15}N \cdot g^{-1}$ fresh nodule weight		
− Allopurinol	Glutamine	2.09	1.13	3.45
	Ureides	0.54	1.13	1.72
+ Allopurinol	Glutamine	2.23	2.24	2.69
	Ureides	0.25	0.55	0.18
	Xanthine	0.34	0.87	2.09

it seemed reasonable to conclude that xanthine accumulation had not resulted in feedback inhibition of earlier steps in its synthesis.

Although a number of earlier studies (3, 9, 11) have shown xanthine accumulation in ureide-forming nodules following allopurinol treatment, none has specifically traced the synthesis of purines from fixed N. The ^{15}N-labeling data of Figure 3 clearly demonstrate that xanthine was a direct product of fixation, being formed within and transported from nodules at rates equivalent to those attained for ureides in plants not treated with allopurinol. Despite the fact that some ^{15}N was undoubtedly exported from nodules as ureides and xanthine during the experiment, the amount of labeled N recovered in ureides plus xanthine in nodules of treated plants was about equal to that in the nodule ureide pool of control plants (Table I).

The ^{15}N-labeling pattern among amino compounds and ureides (Fig. 3A) is rather similar to previously published data for soybean nodules exposed to ^{15}N or $^{13}N_2$ (14–16) and suggests, as expected, that glutamine and glutamate serve as precursors for ureides. Despite asparagine being a significant component of the soluble pool of nitrogen in cowpea nodules (average values for the control plants were 14.1 µmol asparagine versus 4.2 µmol glutamine per gram fresh weight) as well as in nodules of other ureide-forming species (15), the ^{15}N-labeling data (Fig. 3) indicated negligible channeling of fixed N into the amide. The pool(s) of this compound in mature cowpea nodules must therefore be derived either from nitrogen transformations in the nodule not closely linked to current fixation or, what seems more likely, from intake of phloem-borne asparagine from the shoot. This is supported by recent analyses of phloem exudate collected from cowpea (17, 18) which indicated that asparagine is indeed a major nitrogenous compound of the translocated assimilate stream of this species.

Attempts to detect asparagine synthetase in extracts of cowpea nodules, by techniques which readily assay the enzyme in nodules from asparagine-forming symbioses (22), have not been successful. Although in soybean nodules the correlation between asparagine synthetase activity and the level of asparagine in the tissue is not always direct (20), in contrast to cowpea, high rates of asparagine synthetase have been demonstrated *in vitro* (13, 20) and the amide shown to accumulate as a significant proportion of the fixed N in $^{15}N_2$ feeding studies (15). It is possible that in cowpea, and perhaps in some other ureide-forming symbioses, the expression of asparagine synthetase in the nodule is specifically repressed, despite its prominence elsewhere in the plant, whether in the assimilation of NO_3^- (2) or in reassimilation of ammonia released from ureides translocated to shoot organs (17). There may therefore be good reasons for classifying legume symbioses in three ways: namely those forming and exporting principally asparagine and to a lesser extent glutamine; those, as here for cowpea, which form principally ureides and glutamine; and those, like soybean, which synthesize and export all three compounds from nodules.

During the relatively short time course of these experiments, allopurinol treatment did not alter the rate of nitrogenase activity measured by acetylene reduction (Fig. 4). Furthermore, the overall level of ^{15}N labeling of the major nitrogenous solutes of nodules exposed to $^{15}N_2$ over a 4-h period was essentially similar in treated and control plants (Fig. 3, Table I), suggesting that N_2 reduction was likewise relatively unaffected by either allopurinol (*per se*) or the accumulation of xanthine. Triplett (23) has shown that longer term (2-week) repeated exposure of nodulated root systems of a number of ureide-forming legumes to allopurinol results in severe chlorosis and senescence of nodules. The cause of this is not known, but since it can be relieved by added NO_3^- (23) it may be concluded that extended exposure to the inhibitor specifically reduces N_2 fixation. Interestingly, a number of non-

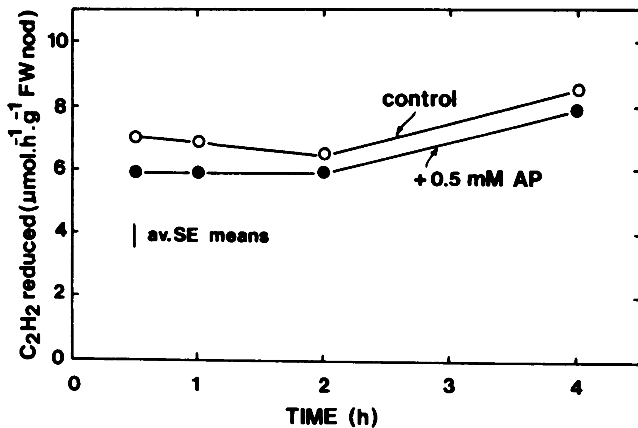

FIG. 4. Effect of allopurinol on rates of acetylene reduction by intact nodulated cowpea root systems.

ureide-forming legume symbioses are not affected in this way by allopurinol treatment (23) and, although the entry or metabolism of the drug in these species has not been proven, the observations together suggest that the apparently specific effect of allopurinol on nitrogenase activity in ureide-forming legumes might result from inhibition of ureide biosynthesis. While measurements of nitrogenase at times greater than 4 h after exposure to allopurinol might show a direct effect of the inhibitor, one obvious possibility is that, if in the longer term allopurinol continues to be transported through xylem, xanthine oxidizing enzyme(s) in shoot organs would become progressively inhibited, thus precluding utilization of the xanthine-N exported from the nodule. Eventually, this would induce progressive N deficiency and possibly result in nodule senescence. Resolution of these questions requires further investigation.

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LITERATURE CITED

1. ATKINS CA 1982 Ureide metabolism and the significance of ureides in legumes. *In* NSS Rao, ed, Advances in Agricultural Microbiology. Oxford and IBH, New Delhi, pp 53–88
2. ATKINS CA, JS PATE, GJ GRIFFITHS, ST WHITE 1980 Economy of carbon and nitrogen in nodulated and non-nodulated (NO_3-grown) cowpea (*Vigna unguiculata* [L.] Walp.). Plant Physiol 66: 978–983
3. ATKINS CA, RM, RAINBIRD, JS PATE 1980 Evidence for a purine pathway of synthesis in N_2-fixing nodules of cowpea (*Vigna unguiculata* L. Walp.). Z Pflanzenphysiol 97: 249–260
4. ATKINS CA, A RITCHIE, PB ROWE, E MCCAIRNS, D SAUER 1982 *De novo* purine synthesis in nitrogen-fixing nodules of cowpea (*Vigna unguiculata* L. Walp) and soybean (*Glycine max* L. Merr). Plant Physiol 70: 55–60
5. ATKINS CA, BJ SHELP, J KUO, MB PEOPLES, JS PATE 1984 Nitrogen nutrition and the development and senescence of nodules on cowpea seedlings. Planta 162: 316–326
6. ATKINS CA, BJ SHELP, PJ STORER 1985 Purification and properties of inosine monophosphate oxido-reductase from nodules of cowpea (*Vigna unguiculata* L. Walp). Arch Biochem Biophys 236: 807–814
7. ATKINS CA, BJ SHELP, PJ STORER, JS PATE 1984 Nitrogen nutrition and the development of biochemical functions associated with nitrogen fixation and ammonia assimilation of nodules on cowpea seedlings. Planta 162: 327–333
8. ATKINS CA, PJ STORER, BJ SHELP 1986 Specific purine nucleosidase from cowpea nodules. *In* W Wallace, SE Smith, eds, Proceedings of the 8th Australian Nitrogen Fixation Conference. Australian Institute of Agricultural Science Occasional Publication No. 25, Melbourne, p 19
9. BOLAND MJ, KR SCHUBERT 1982 Purine biosynthesis and catabolism in soybean root nodules. Incorporation of ^{14}C from $^{14}CO_2$ into xanthine. Arch Biochem Biophys 213: 486–491
10. FIEDLER R, G PROKSCH 1975 The determination of nitrogen-15 by emission and mass spectrometry in biochemical analysis: a review. Anal Chim Acta 78: 1–62
11. FUJIHARA S, M YAMAGUCHI 1978 Effects of allopurinol [4-hydroxypyrazolo-(3,4-*d*)pyrimidine] on the metabolism of allantoin in soybean plants. Plant Physiol 62: 134–138
12. HERRIDGE DG, CA ATKINS, JS PATE, RM RAINBIRD 1978 Allantoin and allantoic acid in the nitrogen economy of the cowpea (*Vigna unguiculata* [L.] Walp). Plant Physiol 62: 495–498
13. HUBER TA, JG STREETER 1985 Purification and properties of asparagine synthetase from soybean root nodules. Plant Sci 42: 9–17
14. MEEKS JC, CP WOLK, N SCHILLING, PW SHAFFER, Y AVISSAR, W CHIEN 1978 Initial organic products of fixation of [^{13}N]dinitrogen by root nodules of soybean (*Glycine max*). Plant Physiol 66: 139–141
15. MINAMISAWA K, Y ARIMA, K KUMAZAWA 1986 Characteristics of asparagine pool in soybean nodules in comparison with ureide pool. Soil Sci Plant Nutr 32: 1–14
16. OHYAMA T, K KUMAZAWA 1978 Incorporation of ^{15}N into various nitrogenous compounds in intact soybean nodules after exposure to $^{15}N_2$ gas. Soil Sci Plant Nutr 24: 525–533
17. PATE JS, MB PEOPLES, CA ATKINS 1984 Spontaneous phloem bleeding from cryopunctured fruits of a ureide-producing legume. Plant Physiol 74: 499–505
18. PEOPLES MB, JS PATE, CA ATKINS 1985 The effect of nitrogen source on transport and metabolism of nitrogen in fruiting plants of cowpea (*Vigna unguiculata* (L.) Walp). J Exp Bot 36: 567–582
19. RAINBIRD RM, JH THORNE, RWF HARDY 1984 Role of amides, amino acids, and ureides in the nutrition of developing soybean seeds. Plant Physiol 74: 329–334
20. REYNOLDS PHS, MJ BOLAND, DG BLEVINS, KR SCHUBERT, DD RANDALL 1982 Enzymes of amide and ureide biogenesis in developing soybean nodules. Plant Physiol 69: 1334–1338
21. SHELP BJ, CA ATKINS 1983 Role of inosine monophosphate oxidoreductase in the formation of ureides in nitrogen-fixing nodules of cowpea (*Vigna unguiculata* L. Walp). Plant Physiol 72: 1029–1034
22. SHELP BJ, CA ATKINS 1984 Subcellular location of enzymes of ammonia assimilation and asparagine synthesis in root nodules of *Lupinus albus* L. Plant Sci Lett 36: 225–230
23. TRIPLETT EW 1986 Two indirect methods for detecting ureide synthesis by nodulated legumes. Plant Physiol 81: 566–571