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Enzymes of ammonia assimilation and ureide biogenesis in developing pigeonpea (*Cajanus cajan* L.) nodules

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Abstract. Ammonia assimilatory and ureide biogenic enzymes were measured in the cytosol fraction of pigeonpea nodules during the period 15-120 days after sowing. The activity of enzymes involved in the initial assimilation of ammonia, i.e. glutamine synthetase, glutamate synthase, asparagine synthetase and aspartate aminotransferase, substantially increased activities during the period of plant growth and reached a maximum value around 105 days after sowing. These increases paralleled the increase in nodule mass, nitrogenase activity and ureide content in nodules. Though no regular pattern was obtained for their specific activities, yet these activities when expressed relative to the specific activity of nitrogenase were many fold higher at each stage of development. Similar increases were observed in the activities of enzymes associated with the formation of ureides from purines. In almost all cases, the activities were again maximum around 90-105 days after sowing. The specific activities of nucleotidase, nucleosidase, xanthine dehydrogenase, uricase and allantoinase, when expressed relative to the specific activity of nitrogenase at vegetative, flowering and podsetting stages were again many fold higher indicating the sufficiency of the levels of these enzymes for the biosynthesis of ureides. The data presented are consistent with the proposal that in ureide producing legumes, ammonia is initially assimilated into glutamine, aspartate, etc., which are metabolised for the de *novo* synthesis of purines. The purines are then utilised for the production of ureides by a group of enzymes investigated here.

Keywords. Pigeonpea; *Cajanus cajan*; nodules; enzymes of ammonia assimilation; ureide biogenesis.

Introduction

Many of the tropical legumes including pigeonpea are now known to assimilate most of the ammonium produced from nitrogen fixation into ureides, allantoin and allantoic acid (Herridge *et al.*, 1978; McClure and Israel, 1979; Streeter, 1979; Luthra, *et al.*, 1981; Sheoran *et al.*, 1982). These ureides are synthesised *via* a pathway involving *de novo* purine biosynthesis followed by oxidation and hydrolysis (Atkins *et al.*, 1980; Schubert, 1981; Boland and Schubert, 1982). Production of ureides from a number of nucleotides and nucleosides in cell free extracts from nodules of cowpea (Woo *et al.*, 1980; Atkins *et al.*, 1982), soybean (Triplett *et al.*, 1980) and pigeonpea (Kaur and Singh, 1984) has also been demonstrated. Similarly, studies on subcellular organisation of ureide biogenesis have indicated that a number of enzymes involved in the assimilation of ammonia into amino acids and purines are located in proplastids (Boland *et al.*, 1982; Boland and

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Schubert, 1983; Shelp *et al.,* 1983). Although the biosynthesis of purine intermediates in nodules has not been clearly defined, it is usually assumed that purine biosynthesis in nodules parallels known pathways in animals and microorganisms. This is supported by the presence of phosphoribosyl pyrophosphate synthetase (EC 2.7.6.1) and phosphoribosyl amidotransferase (EC 2.4.2.14), enzymes catalysing two of the initial steps of purine biosynthesis in nodules of soybean (Reynolds *et al.,* 1982a). Similarly, the presence of phosphoglycerate dehydrogenase, serine hydroxymethyltransferase (EC 2.1.2.1) and methylene tetrahydrofolate dehydrogenase was demonstrated in soybean nodules. The levels of these enzymes were found to be much greater in soybean nodules than in nodules of lupin, a legume in which the main products of ammonium assimilation are amino acid amides rather than ureides (Reynolds *et al.,* 1982b). Their activity in nodules increases during onset of nitrogen fixation and ureide production (Schubert, 1981; Reynolds *et al.,* 1982a). Hence, the above evidences indicate that purines synthesised *do novo* in plant cells become precursors for ureides. In our recent reports (Luthra *et al.,* 1983a,b), we have indicated pigeonpea to use ureides as translocatory products of nitrogen fixation to aerial parts for ultimate synthesis of seed proteins. Here, we report the activities of various enzymes assumed to be involved in ammonia assimilation and ureide biogenesis in cytosol fraction of nodules. This has been done mainly to see whether the rates of ammonia assimilation and ureide biogenesis are comparable to the rates of nitrogen fixation during nodule development.

Materials and methods

Plant material

The crop of pigeonpea (*Cajanus cajan* L.) cv. UPAS-120, was raised in sand culture in earthen pots under natural conditions of light and humidity as described earlier (Sheoran *et al.,* 1981). At weekly intervals, each pot was supplied with 250 ml of N-free nutrient solution (Wilson and Reisenaur, 1963). On other days, the pots were irrigated with tap water. The samples were taken at 15 d intervals starting from 15 days after sowing until complete maturity (120 days after sowing).

Estimation of ureides

Ureides, allantoin and allantoic acid in nodules were estimated as described earlier (Luthra *et al.,* 1983b).

Preparation of enzyme extracts

One g nodules were harvested from 10 plants at random and gently ground in a cold mortar with pestle in the presence of 10 ml of 0·1 M potassium phosphate buffer (pH 7·8) containing 0·4 M sucrose, 10 mM dithiothreitol, 10 mM KCl, 1 mM $MgCl_2$, 10 mM ethylenediaminetetra acetate and 1% polyvinyl pyrrolidone (PVP). For nucleotidase and allantoinase, the buffer used was 0·1 M Tris-HCl (pH 7·5). The extract obtained was filtered through cheese cloth and the filtrate centrifuged at 10,000 *g* for 30 min. The resulting supernatant was then passed through a column of Sephadex G-25, and has been designated as cytosol fraction.

Enzyme assays

Nitrogenase (EC 1.18.2.1) activity in freshly harvested nodulated roots was determined as described in an earlier report (Luthra *et al.,* 1983b).

Glutamine synthetase (EC 6.3.1.2), uricase (EC 1.7.3.3) and allantoinase (EC 3.5.2.5) were assayed again as explained earlier (Sheoran *et al.,* 1981). Glutamate synthase (EC 1.4.1.14) was assayed as described by Boland *et al.* (1978). Asparagine synthetase (EC 6.3.1.1) and aspartate amino transferase (EC 2.6.1.1.) were assayed by following the procedures of Rogenes (1975) and Bergmeyer and Bernt (1973), respectively. Xanthine dehydrogenase (EC 1.2.1.37) was measured by the rate of reduction of NAD^+ in the presence of xanthine (Triplett *et al.,* 1982). Nucleotidase (EC 3.1.3.5) was assayed by measuring the release of inorganic phosphate from XMP. The assay mixture in a final volume of 1·5 ml contained 100 mM trismaleate buffer (pH 5·3); 12 mM $MgCl_2$, 3 mM XMP, 1 mg bovine serum albumin (BSA) and enzyme extract. The mixture was incubated at 37°C for 15 min and the reaction terminated by adding 1 ml of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation and the inorganic phosphate estimated in the supernatant by the method of Ozbun *et al.* (1973). Nucleosidase (EC 3.2.2.1) was assayed by coupling the activity of xanthine dehydrogenase present in the enzyme preparation and monitoring NADH production spectrophotometrically at 340 nm (Triplett *et al.,* 1982). In this assay, nucleosidase will hydrolyse its substrate (inosine) to hypoxanthine and sugar. Hypoxanthine will further be oxidised by XDH present in enzyme extract with the production of NADH. The assay mixture in a final volume of 1·5 ml contained 0·6 μmol inosine, 4 μmol NAD^+ and enzyme with all reagents prepared in 0·1 M K-phosphate buffer (pH 7·8).

Each value in various figures is the mean ± S.E. of four independent estimations. The stages at days 45,75 and 105 after sowing have been referred to as vegetative, flowering and pod-filling, respectively.

Results

Changes in fresh and dry wt of nodules over the entire growth period are illustrated in figure 1A. The fresh wt increased with plant growth, attaining maximum value at 105 days after sowing and decreased thereafter. On the other hand, dry wt increased continuously till the end of the growth period. Total nodule activity (nmol C_2H_4. produced $minn^{-1}$ $plant^{-1}$) similarly increased with growth until 90 days after sowing and then declined till end (figure IB) . However, the specific nodule activity (nmol C_2H_4 produced min^{-1} g^{-1} fresh wt of nodules) exhibited two peaks, one on day 45 and the other on day 90. The ureide concentration in nodules followed the pattern of fresh wt (figure 1C), increasing until day 105 and then decreased. However, at each stage of development, the concentration of allantoin was more than two fold of that of allantoic acid.

Glutamine synthetase activity per plant followed a pattern similar to that of total nodule activity except that the activity in this case peaked at day 105 rather than at day 90 (figure 2A). On the other hand, maximum specific activity was observed at day 15. Thereafter, it declined, remained almost constant till day 45 and again increased till day 75. After dropping at day 90, it increased continuously till the end. Total activity of

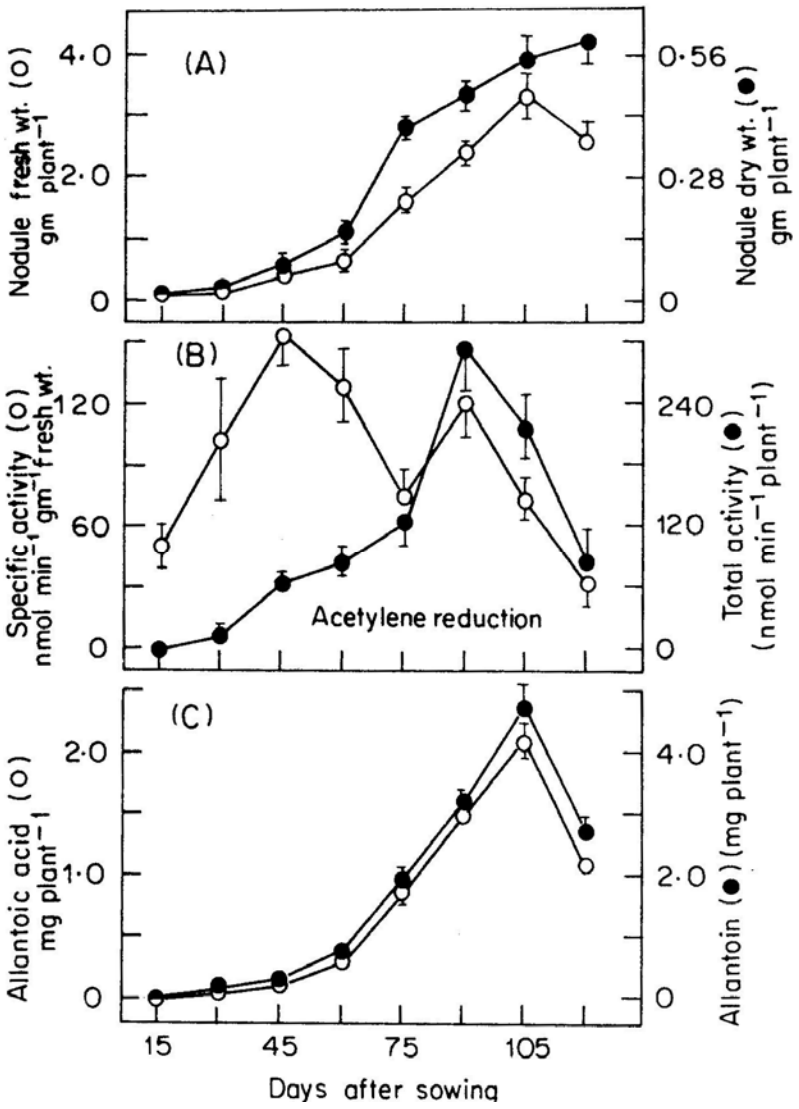

Figure 1. Nodule weight (**A**), acetylene reduction (**B**) and allantoin and allantoic acid content (C) in pigeonpea nodules at different developmental stages.

glutamate synthase (GOGAT) similarly, increased steadily till day 60 and then sharply till day 105 (figure 2B). The activity decreased during the last interval of growth. The specific activity after dropping during the first interval peaked at day 45. After dropping at day 60, it again increased until day 90 and then decreased till the end.

Activity of asparagine synthetase increased steadily until day 45, remained constant during the next interval followed by continuous increase till the end (figure 3A). However, the increase during the last two intervals was much sharper compared to the

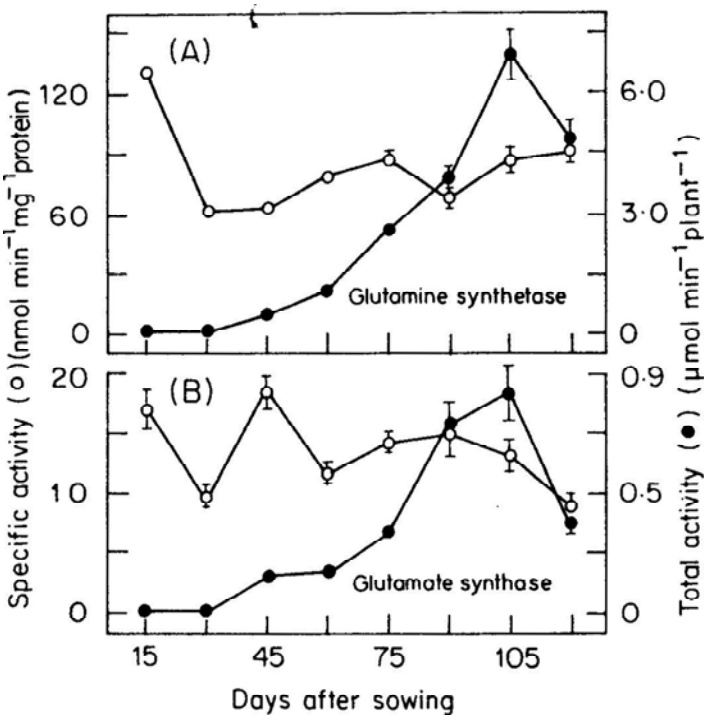

Figure 2. Glutamine synthetase (**A**) and glutamate synthase (**B**) activities in pigeonpea nodules at different developmental stages.

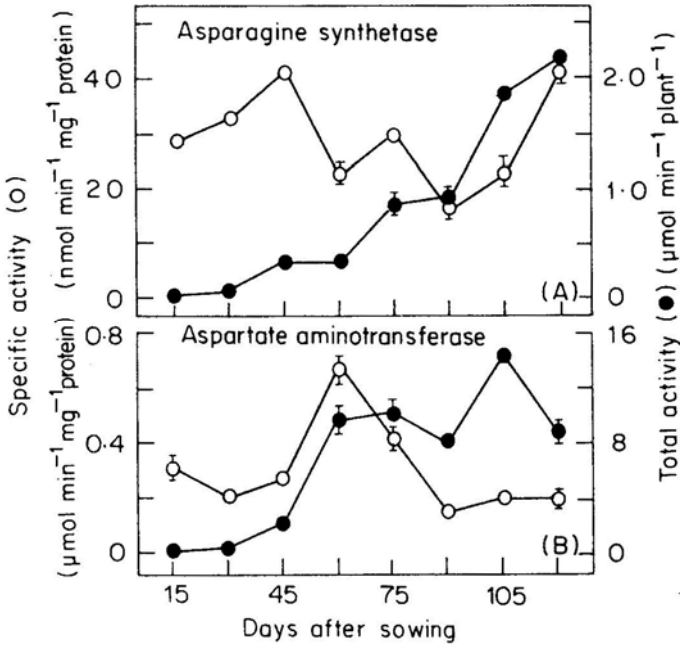

Figure. 3. Asparagine Synthetase (**A**) and aspartate amino transferse (**B**) activities in pigeonpea nodules at different developmental stages.

initial stages. The specific activity increased first till day 45 and then declined at day 60. After showing increase at day 75, it again decreased at day 90. Hereafter, it increased continuously till the end. Total activity of aspartate amino transferase increased steadily with plant growth till day 75 followed by a drop at day 90 and increase at day 105 (figure 3B). The activity again declined during the last interval of plant growth. The specific activity after a slight drop during the first interval., increased continuously to attain peak at day 60. The specific activity then decreased till day 90 followed by slight increase during the later part of plant growth.

Total nucleotidase activity was low, during initial stages of plant growth until day 60 (figure 4A). Thereafter, the activity increased sharply attaining peak at day 90 and then decreased continuously till the end of the plant growth. The specific activity after decrease during initial stages of plant growth increased to attain peak at day 75. Thereafter, the pattern followed was similar to that of total activity. Nucleosidase activity was much less compared to that of nucleotidase at all stages of plant development (figure 4B). Total nucleosidase activity in nodules increased continuously with plant growth attaining maximum value at day 105 and then declined at the last interval. Specific activity, on the other hand, dropped during the first interval and then increased to attain peak at day 75. Another peak in specific activity was observed at day 105.

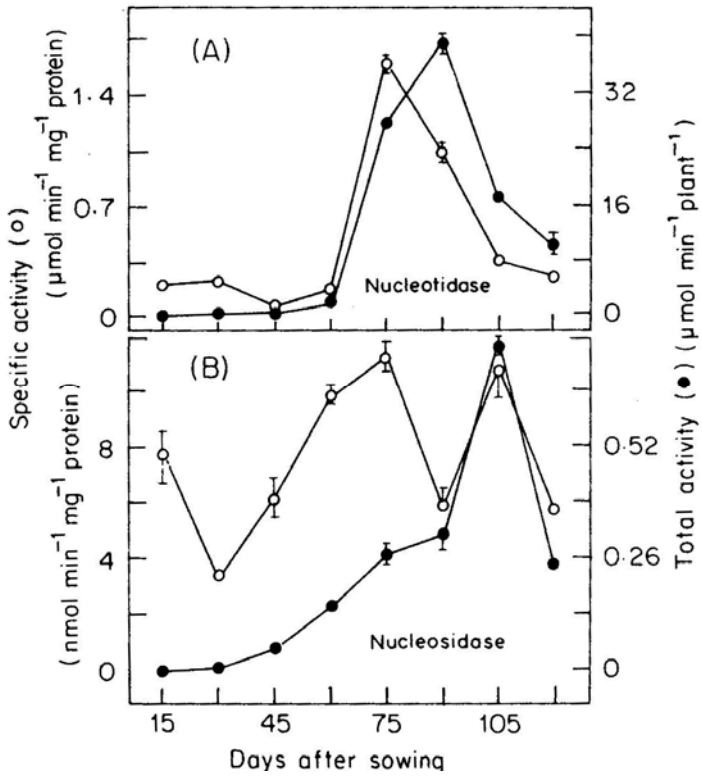

Figure 4. Nucleotidase (**A**) and nucleosidase (**B**) activities in pigeonpea nodules at different developmental stages.

Xanthine dehydrogenase followed a pattern similar to that of nucleosidase. Total xanthine dehydrogenase activity increased continuously till day 105 and then declined (figure 5A). Specific activity again exhibited two peaks one at day 75 and the other at day 105. Total uricase activity increased steadily until day 45 and then sharply attaining maximum value at day 75 (figure 5B). After day 90, the activity decreased till the end of the plant growth. Specific activity exhibited a pattern similar to that of total activity and had its peak at day 75. Allantoinase activity remained low until day 60 (figure 5C). Hereafter, it increased attaining peak at day 105 followed by decline at the last interval. Specific activity after dropping during the first interval increased slightly at day 45. The maximum specific activity was again obtained at day 105.

The activities of all these enzymes involving both ammonium assimilation and ureide biogenesis were further compared with nitrogenase at vegetative (figure 6A), flowering (figure 6B) and pod-filling (figure 6C) stages. At each of these stages, all the enzymes required for the initial assimilation of ammonium into glutamine and other

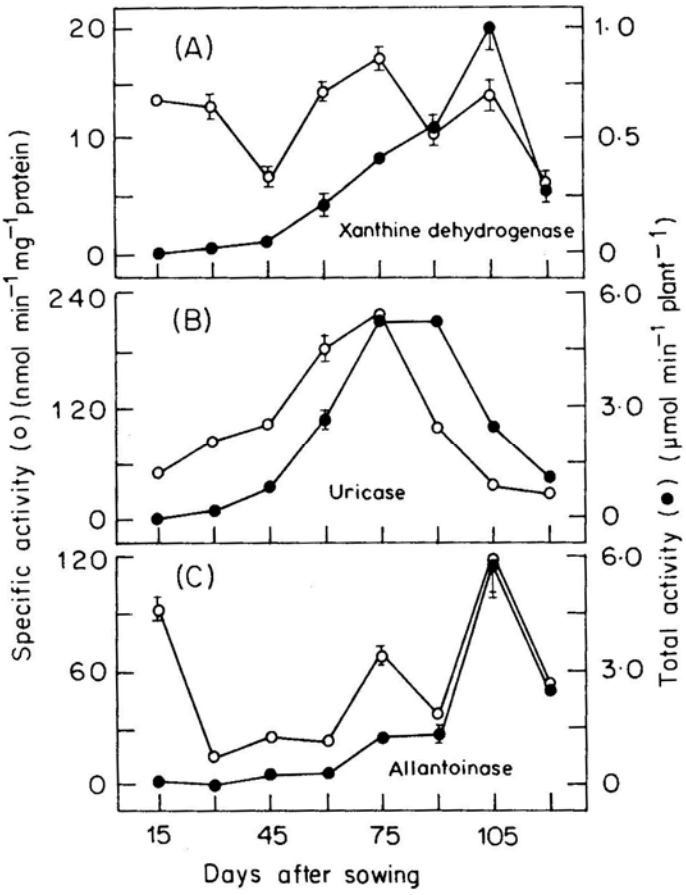

Figure 5. Xanthine dehydrogenase (A), uricase (B) and allantoinase (C) activities in pigeonpea nodules at different developmental stages.

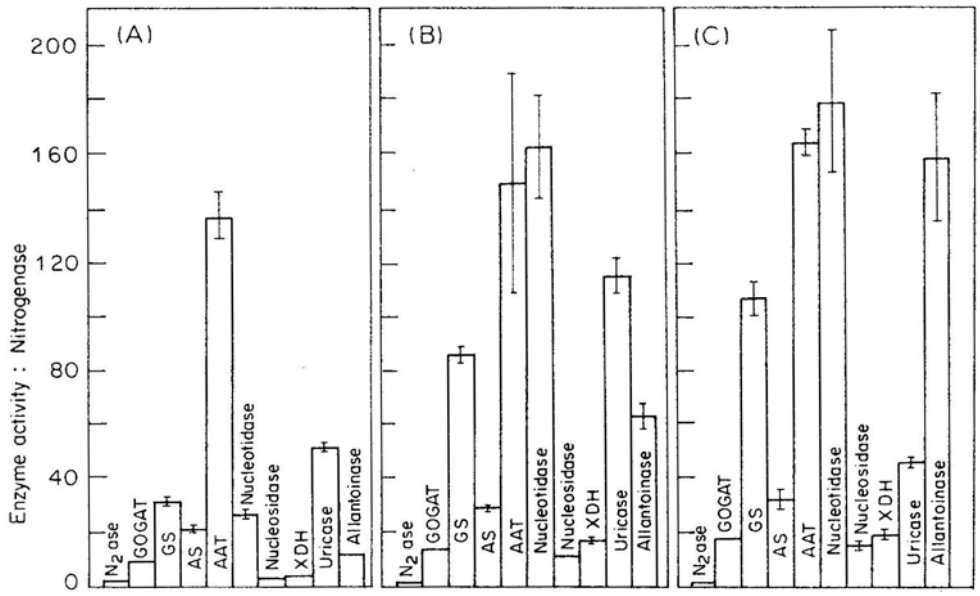

Figure 6. Specific enzyme activities relative to nitrogenase activity in pigeonpea nodules at vegetative (**A**), flowering (**B**) and pod-filling (**C**) stages.

amino acids as well as for the biosynthesis of ureides from the products of purine synthesis were present in quite high amounts compared to nitrogenase.

Discussion

According to current concepts, the primary assimilation of recently fixed nitrogen occurs through the action of glutamine synthetase (Rawsthorne *et al.,* 1980). The glutamine formed in the process can serve as the source of amino group for the synthesis of glutamate, aspartate, asparagine, other amino acids and purines as well (Schubert, 1981). In soybean, this enzyme activity parallels acetylene reduction during the growth and development of nodules (Schubert, 1981). Here also, total glutamine synthetase activity followed the pattern of acetylene reduction, total nodule mass and ureide concentration in the nodules. Similarly, the other enzymes involved in the incorporation of NH_3 into amino acids, *i.e.* glutamate-oxoglutarate aminotransferase, asparagine synthetase and aspartate aminotransferase showed a continuous increase in general., and followed closely the changes in glutamine synthetase activity and acetylene reduction. After initial decrease at day 30, the specific activity of all these enzymes increased in parallel during the vegetative stage of crop growth. When the specific activity of each of these enzymes was expressed relative to specific activity of nitrogenase at vegetative (figure 6A), flowering (figure 6B) and pod-filling (figure 6C) stages, the activity of each of the enzyme was much more compared to that of nitrogenase indicating that these activities are sufficient to take care of the recently fixed nitrogen in nodules.

Ureides in nodules are now known to be synthesised from purine degradation (Atkins *et al.,* 1980; Woo *et al.,* 1980; Triplett *et al.,* 1980; Boland and Schubert, 1982), involving the enzymes xanthine dehydrogenase, uricase and allantoinase. These three enzymes increased in activity during the growth and development of nodules and paralleled the increase in nodule mass, acetylene reduction and glutamine synthetase activity; activity showing peak at day 105 in the case of xanthine dehydrogenase and allantoinase and day 90 in case of uricase. Except the initial stages of growth, the specific activity also increased about 3 to 5 fold during the active period of nitrogen fixation. Similarly when their levels were expressed relative to specific nitrogenase activity at three stages of growth, the activity in each case was many fold higher compared to nitrogenase indicating again that their levels in nodules are sufficient to produce ureides from current nitrogen fixation.

Not much is known about the enzymes, nucleotidase and nucleosidase in legume plants except for a recent report (Tove *et al.,* 1983), where 5'-nucleotidase and nucleosidase were found to be about 50 and 2-fold higher, respectively in nodules of soybean compared to those present in pea. When their activity was monitored during the entire growth and development of pigeonpea nodules, they were again found to closely follow the same pattern as was observed for other enzymes (figure 4A, B). The specific activity in the case of nucleotidase peaked at day 75, whereas, in nucleosidase, the activity peaked at day 75 and 105. The specific activity was again maximum during the period of active nitrogen fixation. When their levels were expressed relative to that of nitrogenase of vegetative, flowering and pod-filling stages, the activities were many fold higher than that of nitrogenase, again indicating their sufficiency and involvement in the process of nitrogen fixation and ureide biosynthesis.

The time course data presented here support the proposal earlier envisaged from *in vitro* studies (Kaur and Singh, 1984) that nitrogen assimilation in pigeonpea occurs *via de novo* purine biosynthesis and subsequent purine oxidation to ureides, allantoin and allantoic acid. Reynolds *et al.* (1982a) placed the various enzymes involved in the overall process into four distinct groups: (i) the ammonia assimilatory enzymes, glutamine synthetase, glutamate: oxoglutarate aminotransferase, asparagine synthetase and aspartate aminotransferase; (ii) the regulatory enzymes involved in the process *of de novo* purine biosynthesis; (iii) enzymes involved in the supply of glycine and 1-carbon fragments to purine biosynthesis and (iv) the enzymes involved in the subsequent conversion of purines into ureides. The first group of enzymes is responsible for the initial assimilation of ammonia leading to the formation of glutamine, glutamate, aspartate and asparagine which are utilised by the enzymes of groups (ii) and (iii) for *de novo* purine biosynthesis. Though there is no certainty about the end product of purine biosynthesis (Atkins *et al.,* 1982; Boland and Schubert, 1982), it seems to be IMP as a cytosolic NAD dependent IMP oxidoreductase has now been detected in nodules of cowpea (Shelp and Atkins, 1983). Furthermore, in nodules of cowpea, metabolism of IMP to form XMP, xanthosine and xanthine in the presence of allopurinol accompanied by NAD reduction provides convincing evidence for the presence of IMP oxidoreductase and IMP to be the end product of *do novo* purine biosynthesis in nodules. Shelp *et al.* (1983) further observed the synthesis of IMP to be associated with plastids in cowpea nodules. This suggests that *in vivo,* IMP is effectively transferred across the plastid outer membrane. Once in cytosol, it is converted to XMP by IMP

oxidoreductase which in turn can be converted ultimately to xanthine by nucleotidase and nucleosidase. Xanthine could then be oxidised to uric acid by xanthine dehydrogenase, and uricase and allantoinase can ultimately convert uric acid to ureides which are the transport products in xylem sap of these legumes. The presence of all these enzymes in sufficient quantities in pigeonpea nodules is consistent with the hypothesis that all these enzymes are involved in the production of ureides from the products of nitrogen fixation as shown in figure 7.

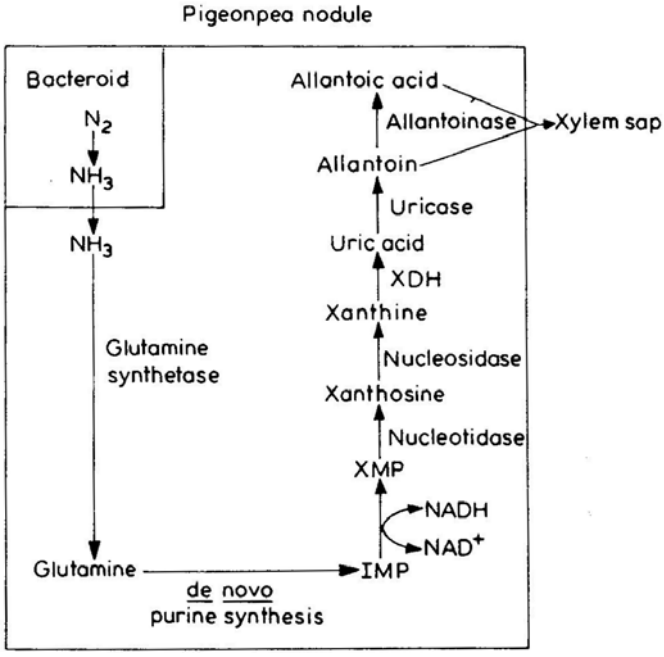

Figure 7. Reaction sequence leading to the formation of allantoin and allantoic acid from purines in pigeonpea nodules.

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