UREIDES DETERMINATION IN XYLEM SAP OF *Vicia faba* L. BY THE RHIZOBIAL ISOLATES OF MANANG AND KATHMANDU VALLEY

Bikash Baral and V. N. P. Gupta
Biotechnology Unit, Central Department of Botany, Tribhuvan University, Kirtipur

ABSTRACT

The Nitrogenous compounds translocated to the aerial parts from the nodules are in the form of ureides, allantoin and allantoic acid. The synthesis of ureides occurs mainly in root nodules via the coordination of the plant bacteria association. But still, the occurrence, localization, or properties of the enzymes involved in the assimilation of ureides in shoot tissues has not been resolved so far. We have examined the total amount of the ureides translocated to the aerial parts by the process of xylem bleedings method by taking the allantoin as internal standard as per the principles of Young and Conway. For the determination of ureides, two rhizobial isolates were isolated from Phaseolus vulgaris L. from Manang and Pisum sativum L. from Kathmandu valley. Other different parameters include the shoots length, roots length, nodules number, nodules biomass, plant biomass and the N of stem, leaves and nodules at 20, 30 and 40 days. The ANOVA result showed insignificant result ($P>0.05$) in the different inoculums, shoot length, root length and the nodules nitrogen where it was significant ($P<0.05$) for both rhizobial isolates for nodulation. These ureides have been compared with the total Nitrogen detected from the nodules, stems and the leaves by the help of Micro-Kjeldahl method. The ANOVA result for ureides showed highly significant difference at 99% level of significance for both isolates (Treatment combinations=6.633, Bacterial Blocks= 3.502, $P<0.01$). The overall ureides concentration for KR1 and MR2 was 335.854 mg/l and 338.575mg/l respectively, while total concentration of ureides for KR1 and MR2 was 574.05mg/l and 501.075mg/l respectively at 45 days of plant sowing. The statistical examination of allantoin equivalent also revealed that there was a positive correlation between two rhizobial isolates on different treatment combinations and blocks.

Key-words: Allantoin, assimilation, nitrogenous compound, nodules, rhizobial isolates, ureides, translocation,

INTRODUCTION

The pathway of ureides synthesis involves assimilation of ammonia, produced by nitrogenase, to form glutamine and other amino acids (glutamate, aspartate and glycine) in the infected cells. These are utilized by *de novo* purine nucleotide synthesis in the infected cells to form purine bases (xanthine and hypoxanthine).The ureides, allantoin and allantoic acid, are found in abundance in tropical legumes (Kushizaki *et al.*, 1964; Ishisuka *et al.*, 1970; Fujihara *et al.*, 1977; Matsumoto *et al.*, 1975 and 1977).

The products of N_2-fixation in legume nodules are exported to the leaves and the shoot via the xylem of the plant (Pate and Atkins., 1983). These products are generally the amides asparagine and glutamine or the ureides allantoin and allantoic acid (Atkins, 1991). Ureides can comprise up to 90% of the total nitrogen transported in the xylem of nitrogen fixing tropical legumes (Herridge *et al.*, 1978; Baral., 2009) and can be stored in high amounts in the different plant organs (Matsumoto *et al.*, 1977 a; Streeter, 1979; Layzell and LaRue, 1982). Due to the high concentrations in the vascular system and in certain plant tissues, ureides are interpreted to have an important function in nitrogen transport and nitrogen storage in legumes. In addition to nitrogen from root nodules, N assimilated from soil combined nitrogen and from N cycling within the plant all will be present in the transpiration stream.

The synthesis of ureides mainly occurs in root nodules via the coordination of the plant bacteria association (Atkins and Smith, 2000). After nitrogen fixation in bacteroids of infected root cells, ammonia (NH_3), ammonium ion (NH_4^+), or amino acids are released or transported from the symbiosome into the cytosol, where they are utilized for Gln synthesis (Smith and Emerich., 1993; Day *et al.*, 2001; Lodwig *et al.*,2003). With the formation of Gln, the nitrogen goes via the *de novo* purine synthesis pathway in the plastids (Shelp

et al., 1983) or mitochondria (Atkins *et al* ., 1977), followed by purine degradation via Xanthine in the plastids (Schubert., 1986) or cytosol of infected root cells (Matsumoto *et al.*, 1977c; Shelp *et al.*, 1983). The ureide allantoin was finally synthesized in the peroxisomes of non infected root cells from the purine degradation product uric acid (Hanks *et al.*, 1981). Allantoic acid was probably produced in the smooth ER of the non-infected cells after import of allantoin into this compartment (Hanks *et al.*, 1981). From the place of synthesis, allantoin and allantoic acid are transferred into the cytosol and are then transported to the root xylem and phloem for long distance transport (McClure and Israel, 1979; Streeter, 1979; Atkins *et al.*, 1982).

The sole objective of research was to know the total ureides assimilated and translocated to the aerial parts of legume (test plant) in different time intervals (from sowing till the harvest) by the application of different treatment combinations.

MATERIALS AND METHODS

Soil of depth 10-15 cm from two entirely different agro climatic regions (Kathmandu and Manang) was collected and seived on 5mm mesh. Soil pH was adjusted to 6.0-6.5 by lime ($CaCO_3$) application allowing to equilibrate covering with polythene sheet for at least 7d finally shifting it to the pots of 15-16 cm diameter and 18 cm height. *Pisum sativum* and *Phaseolus vulgaris* were grown in the pots and the inner residing *Rhizobium* was isolated from their nodules. These isolated bacteria were used as the source of inocula. For the source of Nitrogen, urea ($Co(NH_2)_2$), 50mgkg^{-1} of soil was employed (Baral, 2009). Plants were watered daily with tap water and twice weekly with a N_2 free nutrient solution.

The two rhizobial isolates (*R. leguminosarum* bv. vicea *and R. leguminosarum* bv. phaseoli designated with KR1 and MR2 resp.) with treatment combinations of four with four replications of each were employed. In each pot different kinds of treatments were performed, i.e. first treatment involved inoculation with Nitrogen, second inoculation without nitrogen, third nitrogen without inoculation and the fourth was without nitrogen and inoculation (i.e. control). For inoculation, 1ml of diluted liquid inoculums was used on 5d of germination. Chemicals employed on this experiment were of analytical grade and obtained from the Sigma Chemical Co.

The plants growing under the aseptic conditions were harvested first at 20 days after planting and repeated at every 10 days ending on 40 days. Firstly, four plants of each treatment were harvested i.e., one plant from one pot with each from four replications. A portion of stem (approximately 3.0 cm) above the ground was taken and grinded finely by the mortar and pestle. The liquid suspension was then filtered using a specific filter paper and the filtrate was finally centrifuged (10,000g, 20 min, 4°c). The supernatant was then pipette out and transferred to determine the ureides.

The principles for the ureides analysis was based on the colorimetric method as described by Young and Conway (1942) , in which allantoin was first hydrolysed under a weak alkaline solution at 100°c to allantoic acid which was then hydrolyzed to urea and glyoxylic acid in a weak solution. Glyoxylic acid thus formed reacted with phenylhydrazine hydrochloride producing a phenylhydrazone derivative of the acid. The final product formed an unstable chromophore with potassium ferricyanide and the colour was read at 522nm using a spectrophotometer.

The reading of standard and samples absorbance was done within a shortest possible time-span, since the colour fades out gradually and the absorbance decreases with time. Therefore, few samples in duplicates were processed in each run. A set of standards and a blank (using dH_2O) in duplicate were processed. For the consistency of the results between the laboratories, a Quality Control (QC) sample was performed at each run and at different dilutions as well.

The concentration of the QC and the other samples was performed using the formula:

$C=(Y-a) \div b \times F.$

Where,

'C' is the concentration of the unknown,

'Y' is the absorbance of the unknown,

'a' and 'b' are the intercept and slope of the standard curve respectively,

'F' is the dilution factor.

RESULTS AND DISCUSSION

Plants with inoculums had a good deal of effective nodules on their root systems at 40DAS. Visible nodules were observed at 20 DAS. For nodules nitrogen, MR2 (O+N) treatment combination showed the highest proportion of nitrogen content (2.88%) than other isolate KR1 (2.78%). Similarly, the nitrogen content of the stem revealed the highest value to be of MR2 on the application of nitrogen without inoculums (2.8%). Likewise, while examining the N of leaf blades, it was revealed that inoculums with N gave maximum value for both isolates (1.78% and 1.96% N for KR1and MR2 respectively) (Fig 1).

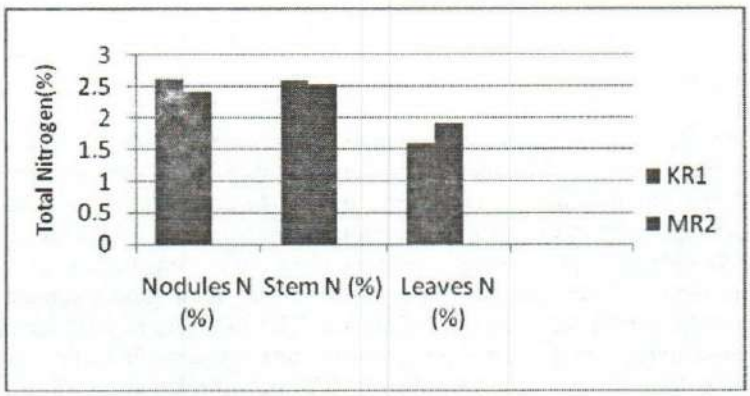

Fig.1. Estimate of N (%) of different parts of the plant

Counting of nodules was done after 20 days of plant age and different treatments gave different results. For both isolates, treatments I-N, I-N and I+N showed maximum number of nodules when observed at 20, 30 and 40 days of plant age respectively. The effect was significant ($P< 0.05$) for both rhizobial isolates KR1 and MR2 for nodulation, but they behaved similar ($P> 0.05$).

The delay in nodulation might be due to low exposure of sunlight and continuous cloud during the initial period of the experiment. The low light exposure leads into the low synthesis of photosynthetas. The carbohydrates supplied by host plants used by bacteriods as a skeleton for incorporation of NH_3 and derive energy via respiration which is utilized in nitrogen fixation and also run their own metabolism (Aslam *et al.*, 1993).

Inoculum of MR2 was found more effective on shoot length than KR1 and ANOVA for shoot length of both rhizobial isolates was found to have significant differences in different treatments and blocks (Treatments= 4.462, Bacterial Blocks= 12.573, $P<0.05$, LSD= 4.566). Increment in length of roots was observed on test plant by supplementation of rhizobial inoculums and analysis of variance revealed insignificant difference within treatment combinations (1.667) while significant difference on blocks (26.102) with LSD value of 8.00 cm.

Firstly, the standard curve of allantoin was obtained at each run of the experiment followed by the significant change in the concentration of the allantoin on the different treatment combinations. The standard curve of the allantoin observed at each run was linear and with the increase of the concentration of the allantoin, the absorbance was also increased (Fig 2).

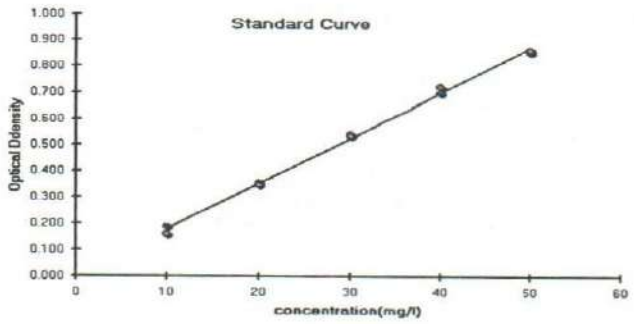

Fig. 2. Standard Curve of the allantoin

The curve of the allantoin was used as the internal standard and according to this curve; the ureides allantoin of the plant was determined at different time intervals. The experiments were calculated separately for both the rhizobial isolates starting from the 20d at an interval of every 5d for 5 times. The concentration of KR1 and MR2 at 20 d was 128.175 and 210.85 mgl^{-1} respectively (Fig. 3) which kept on increasing till the last of the experiment at 45 d (KR1=574.05, MR2=501.075mgl^{-1}). The translocation of the ureides was higher in the inoculums provided of *Pisum sativum* L. than in *Phaseolus vulgaris* L. However, the total ureides content observed for the 6 d of the experiment was higher for MR2 (338.512mg/l) than KR1 (326.316 mg/l) (Figure 5 and 6). Though the trend followed straight upright on the carried experiment, there was a slight decline for the ureides content of MR2 at 40 d.The calculation of f-ratio (P-value: 0.202) and correlation coefficient between the two rhizobial isolates (0.9613 and 0.971 for KR1and MR2 respectively) showed the significant differences. The ANOVA result showed the highly significant difference at 99% level of significance for the both isolates (Treatment combinations=6.633; Bacterial Blocks= 3.502; $P<0.01$).

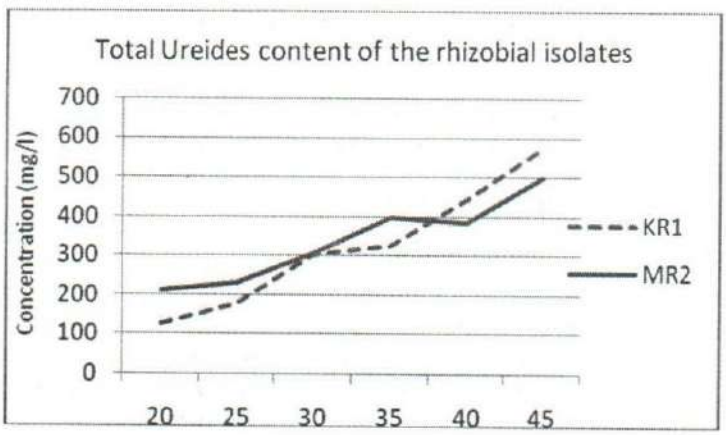

Fig.3. Ureides content of the rhizobial isolates

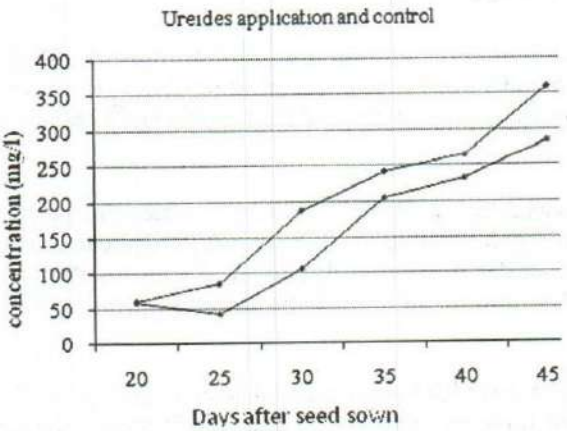

Fig. 4. Concentration of Ureides in the application of Nitrogen and with control

The estimation of Nitrogen (%) was done separately using Micro-Kjeldahl for the different parts of the plants (Nodules, Stem and leaves). The ANOVA result showed the insignificant differences for nodules N (%) (Treatment combinations=1.258; Bacterial Blocks=1.41; P>0.05; LSD=0.251%), stem N (Treatment combinations=1.328; Bacterial Blocks=0.859; P>0.05; LSD=0.202%) and for leaves N (Treatment combinations=4.028; Bacterial Blocks= 0.864; P>0.05; LSD=0.311%).

Ureides were *de novo* synthesized from purines in the cotyledons and as the cotyledons began to senesce, formation of ureides from the pre existing nucleic acids increased and *de novo* purine synthesis declined (Polayes *et al.*, 1984). Over supply of nitrates leads to the production of the shorter chain, incomplete protein compounds that weaken the plant. The concentration of ureides on young seedlings was found to be increased from 2 to 5d of planting which suggests that the leguminous plants are capable of producing ureides without the aid or presence of the microsymbiont (Matsumoto *et al.*, 1977). While the pathway and the sites of the ureides biosynthesis in the young developing seedlings have not been resolved so far.

The accumulation of ammonium ion (NH_4^+) is poisonous to plant cells and is therefore immediately incorporated into the amino acids (Day *et al.*,2001). The main pathway for NH_4^+ assimilation involves two enzymes; glutamine synthase, which catalyses the incorporation of NH_4^+ into glutamate and NADH-glutamate synthase which transfers the amide group of glutamine to α-ketoglutarate (Miflin and Habash, 2002). Assimilated NH_4^+ is exported from the nodules to the shoot.

The variation in nodules number along the compatible host inoculated with different isolates might be due to their variable infectivity and the isolate KR1 with treatment combination of inoculums and nitrogen have the highest infectivity under the prevailing conditions. When nitrate is supplied to an established symbiosis, inhibition of nodulated root N_2 fixation precedes the loss of the potential of bacteroids to fix N_2. The production of ureides occurs in the nodule and the ureides apparently arise from the *de novo* synthesis and subsequent degradation of purines (Woo *et al.*, 1980.)

The nodules of plants incorporated a major proportion of their fixed N into ureides, which recently fixed N was exported from nodules to the shoot in the xylem largely as ureides, and that recent photosynthate donated carbon to the synthesis of ureides in the nodulated roots (Herridge *et al.*, 1978). The relative ureide content or xylem sap from plants totally dependent upon N_2 fixation was shown to be insensitive to changes in the exudation rate and the total N concentration of xylem sap brought about by diurnal changes in environmental factors (McClure *et al.*, 1980).

Thus, the different rhizobial isolates have the different Nitrogen fixing ability and the isolates of the same group also need not have the same fixing ability. Elements like P, Ca, Mo, Co and B deficiency, soil moisture, drought, soil acidity, excessive mineral, extreme temperature, , excessive defoliation and insects and nematodes all contributes to the nitrogen fixing aspects of the bacteria (Baral, 2009).

CONCLUSION

The rhizobia-legume symbiosis is the major source of nutrient nitrogen into agricultural systems and represents an economical and environmental friendly alternative to chemical fertilizers. It was revealed that both the isolates were gram negative, as expected, fast growing, and acid producing with no significantly different mean generation time.

Analysis of their symbiotic effectiveness revealed that the MR2 – rhizobial isolate belonging to the field soils of Manang , was comparatively more efficient for fluxing fixed or nutrient nitrogen, in terms of ureides or total, into the French bean when seed-inoculated. Between the two rhizobial isolates there was no significant difference for in marginal mean ($P>0.05$) as analyzed for the cases like plant N, plant biomass, nodules N, and nodules biomass.

Translocation of the total ureides was found higher for KR1- rhizobial isolate belonging to the field soils of Kathmandu, in comparison to MR2 – obtained from the field soils of Manang. However, the comparative study of N content of nodules, shoot and stem plants inoculated with MR2 showed higher percentage of total N that indicated relatively higher degree of effectiveness. While the ureides translocation data (at 45 days of plant growth) showed the maximum value for KR1 with origin into the field soils of Kathmandu.

Thus it is apparent that the present investigation helped open an array of avenues to work on high altitude legumes and their micro-symbiont. Nutrient nitrogen available in the natural habitat / soils, with source in Legume—*Rhizobium symbiotic system*, stands special relevance at high altitudes. The agro-ecosystem of *high* Himalayas is unique for Legume—*Rhizobium symbiotic system* since the state of nutrient nitrogen determines its productivity. An insight into natural systems, including legume—*Rhizobium* symbiosis, with likely impacts of climate change, is desirable and poses a challenge to Rhizobilogists.

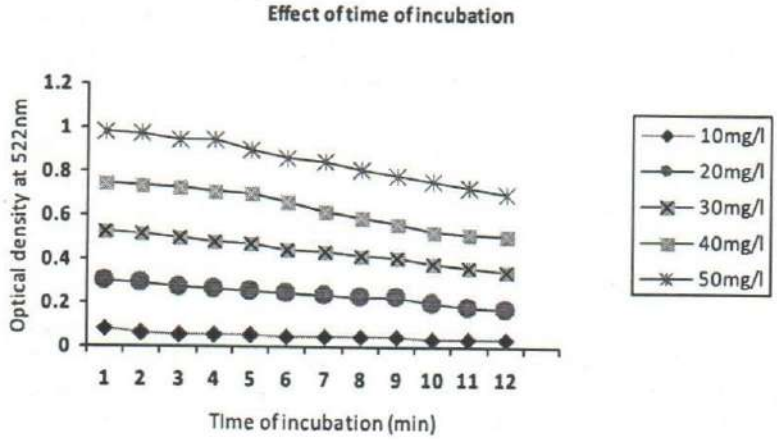

Fig. 4. Effect of time of incubation of allantoin

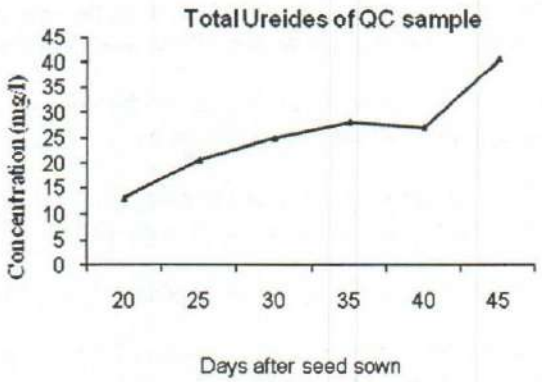

Fig.6 Graph for Quality Control (QC) sample

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