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Biogenesis and Cycling of Ureides in Broad Bean (*Vicia faba* L.)

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ABSTRACT

Leguminous plants continuously recycle nitrogenous compounds from the nodules to the aerial parts of the plants through the xylem stream in the form of ureides, allantoin and allantoic acid. These ureides and ureide products, which are intermediates of purine degradation, are the dominant products that define long-distance transport of nitrogenous compounds from the root to the shoot. The oxidation products of *de novo* purine synthesis result in these nitrogenous compounds and nodules are the main sites of their synthesis, which takes place in a coordinated plant-bacteria association (by intracellular bacteria). For the development of nodules, there are different steps, i.e., bacterial infection, nodule organogenesis, and the onset of nitrogen fixation. The occurrence, localization and properties related to the enzymes involved in the assimilation of ureides in shoot tissues remains an unsolved issue. In this study, ureides in plant tissues have been analyzed. The total amount of nitrogen translocated to the aerial parts of faba bean (*Vicia faba* L.) plants was quantified. Other physiological attributes measured at different time intervals (from sowing to harvest) included shoot and root length, symbiotic parameters, plant biomass, and the nitrogen (N) status of the stem, leaves and nodules. KR1 and MR2, two rhizobial isolates, were isolated from *Pisum sativum* var. 'Macrocarpon' and from *Phaseolus vulgaris* (cv. 'Carioca 29') plants, respectively, and were selected from entirely different agro-climatic regions (Kathmandu valley and Manang, respectively). Plants were harvested at regular intervals (every 5 d) for dry matter and xylem-extracted exudates. From the performed experiment, it was concluded that the rhizobial isolate (MR2) of Manang origin growing in adverse climatic and soil conditions was more efficient in fixing atmospheric nitrogen.

Keywords: metabolism, nitrogen uptake, nodule and nodule development, *Rhizobium*, translocation, ureide biogenesis and export
Abbreviations: ALN, allantoinase; **Asn**, Asparagine; **ANOVA**, analysis of variance; **BNF**, biological nitrogen fixation: **DPG**, days post germination; **ER**, endoplasmic reticulum; **EST**, expressed sequence tag; **Gln**, glutamine; **I+N**, *Rhizobium* inocula with nitrogen; **I**, inocula without nitrogen; **KR1**, *Rhizobium leguminosarum* bv. *vicea*; **LSD**, least significant difference; **MR2**, *R. leguminosarum* bv. *phaseoli*; **N**, nitrogen only; **SNF**, symbiotic N_2 fixation; **YEM**, yeast extract mannitol agar

INTRODUCTION

Biological nitrogen fixation (BNF) takes place in the presence of N-fixing rhizobia through the legume-symbiosis process (on a compatible legume; Ferguson *et al*. 2010). One half of the N required for plant growth is available from the legume-bacteria symbiosis (Herridge *et al*. 2008; http://faostat.fao.org/). Mo is required as a cofactor for the nitrogenase complex, while increasing the amount of CO_2 increases Mo adsorption to soil particles, causing a regular decline in N-fixation (Hungate *et al*. 2004).

During the legume-rhizobia interaction, rhizobia (the microsymbiont) live in close co-ordination with the plant (the macrosymbiont) in highly specialized structures (the nodules), fixing nitrogen (N) in return for carbon (C) and amino acids from their host plants (Fred *et al*. 1932; Lodwig *et al*. 2003; Smallridge 2003; Heath and Tiffin 2008). These microsymbionts are initially activated and attracted to plants by aromatic compounds (flavonoids) that are secreted (in the rhizosphere) by those plants during growth. Rhizobia synthesize the symbiotic signaling compounds (inducing the nodulation or expression of Nod factors such as lipochitin oligosaccharides) which induce root hair deformation (Downie and Walker 1999). The signal transduction of the Nod factors require the calcium signaling pathway (activation of calcium and calmodulin-dependent protein kinase; Dunpont *et al*. 2012), which helps the legume develop root hairs. The different spectra of the flavonoids (liberated by the legumes) and strain-specific chemical composition of the Nod factors attract the bacteria to the plants in close vicinity to the plants (Broughton *et al*. 2000), finally inducing nodule formation via root cortex dedifferentiation (Dunpont *et al*. 2012). The association of mutually beneficial species may be evolutionary unstable by natural selection (Trivers 1971), and these stabilizing agents work either before the symbiosis (Mueller *et al*. 2004; Zhang *et al*. 2007) or post-symbiosis (Pellmyr and Huth 1994). For the effective invasion by the bacteria (through an infection thread, a tubular structure) of the legume partner, the secretion of exopolysaccharides, type II and type III secretion systems, is also equally important (Perret *et al*. 2000; Dunpont *et al*. 2012). Moreover, different types of rhizobial strains may also be located on the same nodule (Denison 2003).

Nitrogen fixation in plants is mainly triggered by bacteroids (a rod-shaped bacterium in the root nodules of leguminous plants) by a mechanism of endocytosis as soon as N enters into these microsymbionts (rhizobia). It is assumed – since no studies prove this – that nitrogen allocation in plants is also somehow contributed to by the translocation of small peptides. N is reduced to ammonia by a special enzyme termed the nitrogenase enzyme complex (EC: 1.18.6.1; Ferguson *et al*. 2010; Collier and Mechthild 2012), which enters into the cytosol of the infected nodule cell via the peribacteroid membrane, finally assimilating glutamine (Morey *et al*. 2002; Obermeyer and Tyerman 2005; Masalkar *et al*. 2010). This glutamine then enters into mitochondria and plastids, which utilize it for the *de novo* purine biosynthesis (Smith and Atkins 2002; Boldt and Zrenner 2003; Zrenner *et al*. 2006). Purines rapidly degrade to xanthine which then further oxidizes to uric acid in the cytosol (Datta *et al*. 1991) and this uric acid diffuses further

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from infected to uninfected nodule cells.

The production of ureides (purines the initiators arising by turnover of nucleic acids or by *de novo* synthesis; Todd *et al*. 2006) in broad bean (*Vicia faba*, syn. faba bean; especially the early process) involves a rigorous and tough pathway that takes place within the infected cells by a microsymbiont, the effective rhizobial strains. Initially, purine metabolism passes via a salvage pathway in the developing seedlings, while *de-novo* purine synthesis is the main pathway in the developing nodules (Todd *et al*. 2006). The *de-novo* purine synthesis refers to the synthesis of highly complex molecules from simple molecules such as sugars and amino-acids during which IMP (Inosine monophosphate) is the nucleotide initially formed which is then converted to either AMP (Adenosine monophosphate) or GMP (Guanosine monophosphate) and the precursors of purines for *de-novo* synthesis is glutamine. However, the final process of production takes place in uninfected cells, the interstitial cells, which are readily interspersed between the infected cells (Kaneko and Newcomb 1990; Sprent and James 2007).

The formation of nodules is encoded by nodulin (Nod) genes which are first detected at the onset of N fixation (Perlick and Puhler 1993; Quandt *et al*. 1993), hence those that are found in differentiated nodules are referred as late nodulins (leghaemoglobins, sucrose synthetase (EC 2.4.1.13), glutamine synthetase (EC 6.3.1.2) and Uricase II (EC 1.7.3.3) (Gebhardt *et al*. 1986; Bennett *et al*. 1989). Genes that are exclusively expressed during the nodulation process (nodulins) help to fix N (early nodulins help in signal transduction and nodules' overall development, while the late nodulins are only induced at the onset of N-fixation). Broad bean harbors an indeterminate type of nodule, which is a specialized structure comprising several central tissues (histological zones of consecutive developmental stages like an invasion zone, early symbiotic zone, late symbiotic zone and senescent zone) surrounded by a cortex (Vasse *et al*. 1990; Abd-Alla *et al*. 2000; Hartmann *et al*. 2002; Mergaert *et al*. 2003). The broad bean seedlings have somewhat enlarged peroxisomes and tubular endoplasmic reticulum (ER), and ureides are present at a very low level (10% compared with Asn-N) in the xylem transport stream of the stem (Kaneko and Newcomb 1990; Lamberto 2009; Alamillo *et al*. 2010).

Nod genes can be traced by employing several methods such as differential display, differential screening, or subtractive hybridization (Crespi and Galvez 2000), and these *Nod* genes are induced by *Rhizobium Nod* genes (Govers *et al*. 1986). Moreover, technological improvement (hybridization techniques, transformation, etc.) is possible using genetic approaches, including mutants whose nodule development is affected (*Medicago truncatula*; Endre *et al*. 2002; Stracke *et al*. 2002). However, different genes exhibit nodule-specific expression with differences in their spatial and temporal expression profiles (Mergaert *et al*. 2003).

Extensive previous research regarding N-fixation and reciprocal exchange of signals (systemic regulation; Marino *et al*. 2007) between legumes suggests that the successful interaction between bacteria and plants requires the coordinated expression of genes in both partners (microsymbiont and macrosymbiont; Long 1989; Sanchez *et al*. 1991) and that fixed N (in the form of amino acids) is translocated to developing sink tissues such as fruits and seeds (Sanders *et al*. 2009). Two enzymes, namely xanthine dehydrogenase (XDH; EC 1.1.1.37) and urate oxidase (UO; EC 1.7.3.3) are considered to be key enzymes in the biosynthesis of ureides in legume nodules (Triplett *et al*. 1980; Tajima *et al*. 2004). However, some studies show that ureide accumulation occurs in shoots and leaves under severe drought-sensitive legume (soybean) cultivars (Charlson *et al*. 2009). Moreover, during high drought stress more ureides become accumulated in the shoot which is one of the principal causes of nitrogen fixation inhibition (ureide feedback hypothesis; Serraj *et al*. 1999; King and Purcell 2005).

There is no mechanism to select the inflow of reduced N compounds from the phloem which allows a feedback control mechanism regulating nitrogenase activity (Schubert 2007), and this feedback mechanism regulates N-fixation according to the host-plant demand. Amino N transporters in *V. faba* plants have not been studied so far, and are thus the focus of this study. However, different studies regarding functional analysis in broad bean show that ureides accumulate in the shoots and roots (Bai *et al*. 2007).

The objectives of this study were to evaluate how N in the form of ureides is assimilated and cycled to the aerial part of a legume (*V. faba*) at different developmental stages (from sowing to harvest) by comparing those ureides with the total N possessed by the plant in leaves, stems and nodules. In this study, only ureides are considered while other N metabolites (nucleotides, nucleosides, hormones and secondary plant metabolites) are not discussed.

MATERIALS AND METHODS

Source of reagents and chemicals

Chemicals and reagents of analytical grade needed for carrying out the experiments were purchased from Sigma Chemical Co. (St. Louis, USA).

Experimental parameters

Parameters such as nodule number, measurement of plant parts (root and shoot length), nodule biomass, shoot biomass and concentration of N in the stem, leaves and nodules were assessed separately for a total of 40 d. The concentration of ureides in xylem sap was also determined.

Isolation of rhizobia

Soil from two different agro-climatic regions (pH 6.0-6.5) was used to isolate symbiotic bacteria: Kathmandu valley (27°43' 06" N, 85°19' 02" E) and Manang (trans-Himalayan arid region; 28°40' 03" N, 84°15' 01" E). The soil (separated by 5-mm mesh) was added to pots of uniform size (16 ×18 cm) in a greenhouse. These soils (which served as the source of rhizobia) were then used for growing different legumes, namely *Pisum sativum* var. 'Macrocarpon' and *Phaseolus vulgaris* cv. 'Carioca 29' in a greenhouse (28±1°C) at 4 plants pot^{-1}. The respective rhizobial isolates were isolated from the soil collected from two locations (Kathmandu and Manang) using the trap method (Vincent 1970). Seeds were surface sterilized in hydrogen peroxide (4% for 1.5 min) and left to germinate in sterile Petri dishes with a base of a single sheet of Whatman No. 1 filter paper moistened with sterile distilled water. Endogenous nodular *Rhizobium* strains were isolated from *P. sativum* and *P. vulgaris* (described next) and served as the inoculum source.

Rhizobial inoculation in test plant

Faba bean, the test legume, was inoculated with the isolated *Rhizobium* strains using the soil inoculation technique (Somasegaran and Hoben 1994) in a greenhouse with controlled temperature. Urea $[Co(NH_2)_2]$ at 50 mg kg^{-1} of soil was employed as the N source. Plants were watered as needed with distilled water and twice weekly with a N-free nutrient solution. Micronutrients were added to sterile soil as Hoagland's solution (Hoagland and Arnon 1950; Roughley 1984).

Soil testing

Physiochemical parameters of the soil i.e., pH, N, phosphorous (Sharpley 2000), soil organic matter (Bowman 1997) and potassium (Delavalle 1992) were analyzed.

Choice of effective strains

Rhizobial strains of the high Himalaya are particularly neglected and their effectiveness has not been studied in detail. Research on the biogenesis of ureides by rhizobia in legumes is even less stu-

died. The selected locations (Kathmandu and Manang) occupy diverse geographical terrains that are drastically dissimilar in terms of climatic conditions and hence they harbor different rhizobial strains that potentially differ widely in their potency and effectiveness.

Characterization of rhizobial isolates

R. leguminosarum bv. *vicea* and *R. leguminosarum* bv. *phaseoli* inhabiting *Pisum sativum* (KR1; Kathmandu-origin) and *Phaseolus vulgaris* (MR2; Manang-origin) were examined (4 treatments × 4 replications each; Boone *et al*. 2001).

Rhizobial isolates were isolated by raising the legumes in soil from their original sampling sites and the endogenous bacterium (microsymbionts) were isolated by the trap method. Different pots were treated separately i.e., inocula with N (I+N), without N (I), nitrogen only (N) and the control (C), i.e., with tap water only. One mL of diluted liquid inocula was used as standard inocula on 5 days post germination (DPG). Inoculation of the effective rhizobial strains was performed by injecting a pure culture of each *Rhizobium* (KR1 and MR2) on sterile soil prepared in nutrient broth. These rhizobial strains were authenticated by two techniques: the pouch germination method and the soil inoculation method (Somasegaran and Hoben 1994). The effectiveness of the isolated rhizobial isolates was measured by the appearance of nodules and their size in the inoculated soil. A set of controls i.e., sterile nutrient solution as Hoagland's solution (Hoagland and Arnon 1950), was also used in parallel. The production of acid or alkali by the rhizobial isolates was observed every hour through changes in the pH of the broth. Mean generation time was also calculated by employing a spectrophotometer using the following formula (Powar and Daginwala 1982):

$$G = t/N$$

$$= \frac{t \log 2}{\log B_n - \log B_0}$$

where t = incubation time; G = generation time (i.e., doubling time of one cell to two cells); B_o = number of colony forming units CFUs at zero time; B_n= number of organisms after "n" generations; N = number of generations.

Determination of total nitrogen

Various parts of tested plants (stem, nodules and leaves; 4 replicates each) were tested for their total N content (%) at different time intervals (20, 30 and 40 DPG) employing the Micro-Kjeldahl method using three sequential steps, digestion, distillation and titration (PCARR 1980), as described in detail next.

1. Digestion

250 mg of ground sample (nodule, stem or leaves) was passed through a 0.425 mm sieve and placed in a dry 300-mL Kjeldahl digestion flask and digested in 6 mL of H_2SO_4 (conc.) with 1 g of mixed catalyst (mixture of $CuSO_4$, K_2SO_4 and metallic selenium; 100: 10: 1, w/w/w). The flask was then heated for 10 min at 70°C for 1 h until a clear apple-green solution appeared in the flask. The digested flask/solution was allowed to cool at room temperature (28°C) for 30 min. To the digest, 60 mL of dH_2O was added, then shaken for a few seconds until the solution was homogenously mixed. As a result, the total N was converted into $(NH_4)_2SO_4$. A blank without plant sample, i.e., H_2SO_4 (conc.) with 1 g of digested mixed catalyst, followed by the addition of dH_2O, was also run for each batch of plant samples digested via this process.

2. Distillation

The diluted digest of the flask was then transferred to a distillation flask (500 mL, Borosil). A 100-mL beaker with 10 mL of boric acid indicator was placed below the nozzle of the condenser in such a way that the end of the nozzle totally lay dipped within the indicator. 30 mL of 40% NaOH solution was added into the distillation flask, and was heated to 70°C. The NH_3 that evolved as a result of reaction was trapped in 10 mL boric acid (4%) indicator changing from pink (of the indicator) to green.

3. Titration

The NH_3 trapped in distillate was titrated with 0.1 N HCl solution and the end point was noted by observing the change in color of the indicator from green to pink. The volume of HCl consumed in titrating distillate was recorded. The volume of acid consumed by both blank and plant samples was recorded, and on the basis of this, the total N content (%) of each plant sample was calculated (PCARR 1980) as:

$$N_2 (\%) = [14\times N\times(S - B)\times 100]/100$$

where N= normality of HCl; S = volume of HCl consumed in each plant sample (mL); B = volume of HCl consumed in the blank (mL); M = weight of plant material (mg).

Statistical analysis

The experiment was arranged in a randomized complete block design. Two rhizobial isolates (KR1 and MR2) with four treatment combinations and four replications of each block were employed for the experiment giving a total of 32 combinations. A test for normality was performed on all data. All statistical analyses were carried out using SPSS version 16.0 (SPSS, Inc., USA) and Excel 2007 (Microsoft Office). All comparisons were performed by applying *t*-tests for independent samples and Duncan's multiple range test (DMRT) at $\alpha = 0.05$ (Somasegaran and Hoben 1994). The *F*-ratio between variables was employed. ANOVA with *post-hoc* LSD (Least significant difference; Baye's LSD) was used to compare the values of two adjacent means.

The independent samples *t*-test was employed for comparing nodule induction and biomass (**Fig. 1**) and total N fixed (**Fig. 5**). The mean ± SD was calculated for the different treatments. The error bar in the figures represents the standard error (±1 SE). All tests in the tables are mean ± SD. Regression analysis between different parameters was also performed.

RESULTS

Physiochemical parameters of soil

A different range of values was obtained while calculating the nutrients of the soil of three locations. The pH of Manang soil was a little more acidic than Kirtipur and Kathmandu soils. The N, P and organic matter contents of the Kathmandu soil were highest. Potassium (K) was highest in the Manang soil (**Table 1**).

Characterization of rhizobial isolates

The growing conditions with the changes in the colony color, colony shape and diameter were observed for the rhizobial isolates. The acidity and basic properties of both were observed. Both rhizobial isolates were milky-translucent with an entire margin, gummy, Gram-negative and acid-producing with 14.1×10^7 and 17.8×10^7 viable cells for KR1 and MR2, respectively (**Table 2**).

Growth and changes in pH

The growth conditions (mean generation time or mean doubling time) of the microsymbiont at different time inter-

Table 1 Physiochemical parameters of the soil from Kathmandu, Manang and Kirtipur.

Parameters	Soil origin		
	Kathmandu	Manang	Kirtipur
pH	6.7	6.3	6.5
N (%)	0.92	0.72	0.81
P (kg/ha)	1012	891	966
K (kg/ha)	2877	3233	2616
Organic matter	6.52	4.34	5.26

Table 2 Characterization of the rhizobial isolates used in this study.

Isolates	Color	Shape	Size/diameter	No. of viable cells (cells ml^{-1})	Gram's test	Acidic/basic
KR1	Milky-translucent	Entire margin	Gummy with convex outer surface, 0.56 mm diameter	14.1 ×10^7	Gram negative	Acid-producing
MR2	Milky-translucent	Entire margin	Gummy with convex outer surface, 0.82 mm diameter	17.8 ×10^7	Gram negative	Acid-producing

Table 3 Mean generation time and change in pH of the *Rhizobia* broth at different time intervals.

Time (h)	Change in pH		Average no. of colonies at 10^{-5} dilution		No. of viable cells (cells ml^{-1})	
	KR1	MR2	KR1	MR2	KR1	MR2
0	6.8	6.8	25	38	8.25×10^7	12.50×10^7
24	6.1	6.3	28	40	9.24×10^7	13.20×10^7
48	5.7	6.1	31	46	10.20×10^7	15.18×10^7
72	5.0	5.4	43	54	14.10×10^7	17.80×10^7
96	4.6	5.3	47	59	17.14×10^7	19.36×10^7

Total sample size: n = 5

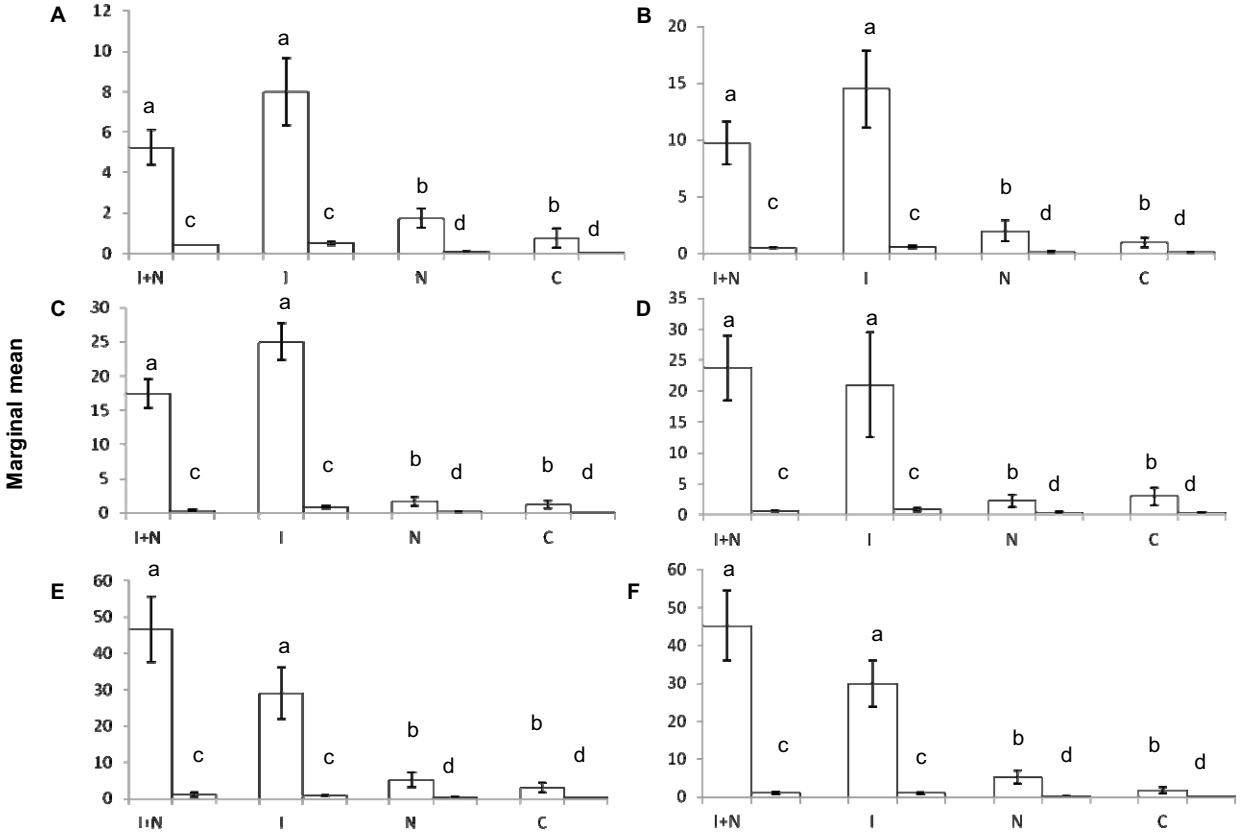

Fig. 1 Nodule induction and biomass on different days after infection by rhizobial isolates KR1 and MR2. DPG = days post germination. n = 4, mean ± SE. Different letters within treatments for a single rhizobial isolate are significantly different according to DMRT at α = 0.05. Plain bars: nodule number; shaded bars: nodule biomass. Values expressed as marginal means on Y-axis and treatments on X-axis: C, control; I+N, *Rhizobium* inocula with nitrogen; I, inocula without nitrogen; N, nitrogen only. A, C and E are the effects on nodule number and nodule biomass shown by KR1 at 20, 30 and 40 DPG, respectively while B, D and E are the effects shown by MR2 at 20, 30 and 40 DPG, respectively.

vals were observed as the fast-growing or the slow-growing species and their pH were accessed. A decrease in pH and an increase in the average number of colonies were observed for both isolates with an increase in time (at 24, 48, 72 and 96 h). The number of viable MR2 (19.36 × 10^7) cells was greater than KR1 cells (17.14 × 10^7) after 96 h (**Table 3**).

Nodule organogenesis

Plants inoculated with the effective bacteria showed the clear presence of nodules (initially circular at 20 DPG) on their root system at 40 DPG (**Fig. 1**). Treatments with inocula showed the greatest number of nodules at harvest (P > 0.05; **Fig 1**).

Total N content

The concentration of N (%) was separately recorded for the nodules, stems and leaves (micro-Kjeldahl method; **Table 4**). The N concentration was highest in nodules, followed by shoot and leaves. The Manang inoculum fixed N more effectively than the Kathmandu bacteria (MR2 > KR1). Plants harvested at 40 DAS accumulated the highest amount of N than plants harvested at 20 and 30 DAS. Differences for nodule and stem N (%) were insignificant but were significant for leaf N (%) (**Fig. 2**).

The concentration of ureides in young seedlings (**Fig. 3**) increased from 2 to 5 DPG, suggesting that leguminous plants are capable of producing ureides without the aid or presence of a microsymbiont. The N content of different plant parts (shoot, leaves and nodules) were analyzed and compared with the response to inoculum source used (KR1 vs MR2). Nodules were found to fix more N, followed by shoot N and leaves N in plants inoculated with KR1 (I) and

Table 4 Total nitrogen content for the two bacterial isolates (KR1 and MR2) at different days of plant growth (DPG).

Tested parts	DPG											
	20				30				40			
	I+N	I	N	C	I+N	I	N	C	I+N	I	N	C
KR1												
LN	1.83 ± 0.590	1.07 ± 0.884	1.16 ± 0.574	1.03 ± 0.262	1.6 ± 0.509	1.67 ± 0.663	1.12 ± 0.490	1.1 ± 0.552	1.92 ± 0.739	1.51 ± 0.465	1.08 ± 0.465	1.014 ± 0.184
SN	2.63 ± 0.755	2.55 ± 0.641	2.41 ± 0.837	2.1 ± 0.668	2.14 ± 0.786	2.61 ± 0.703	2.48 ± 0.885	2.54 ± 0.892	2.77 ± 0.884	2.9 ± 0.633	2.81 ± 0.751	2.63 ± 0.461
NN	2.63 ± 0.470	1.96 ± 0.572	2.62 ± 0.723	2.18 ± 0.899	2.81 ± 0.805	2.61 ± 0.608	2.4 ± 0.697	2.96 ± 0.550	2.9 ± 0.336	2.8 ± 0.559	2.66 ± 0.579	2.9 ± 0.290
MR2												
LN	1.98 ± 1.016	1.66 ± 0.983	1.68 ± 1.087	1.26 ± 0.334	1.90 ± 0.989	1.84 ± 0.101	1.68 ± 0.695	1.26 ± 0.361	2.02 ± 0.715	1.88 ± 0.147	1.61 ± 0.520	1.38 ± 0.188
SN	2.66 ± 0.419	2.48 ± 0.407	2.8 ± 0.425	2.14 ± 0.871	2.74 ± 0.461	2.16 ± 0.409	2.97 ± 0.650	2.54 ± 0.637	2.96 ± 0.625	2.24 ± 0.628	2.65 ± 0.653	2.31 ± 0.228
NN	2.86 ± 0.262	2.71 ± 0.686	2.98 ± 1.041	2.06 ± 0.741	2.16 ± 0.422	2.00 ± 0.637	2.72 ± 0.253	2.66 ± 0.535	2.18 ± 1.187	2.39 ± 0.751	2.94 ± 0.423	2.05 ± 0.953

Mean ± SD (n = 4); LN: leaf nitrogen; SN: stem nitrogen; NN: nodule nitrogen

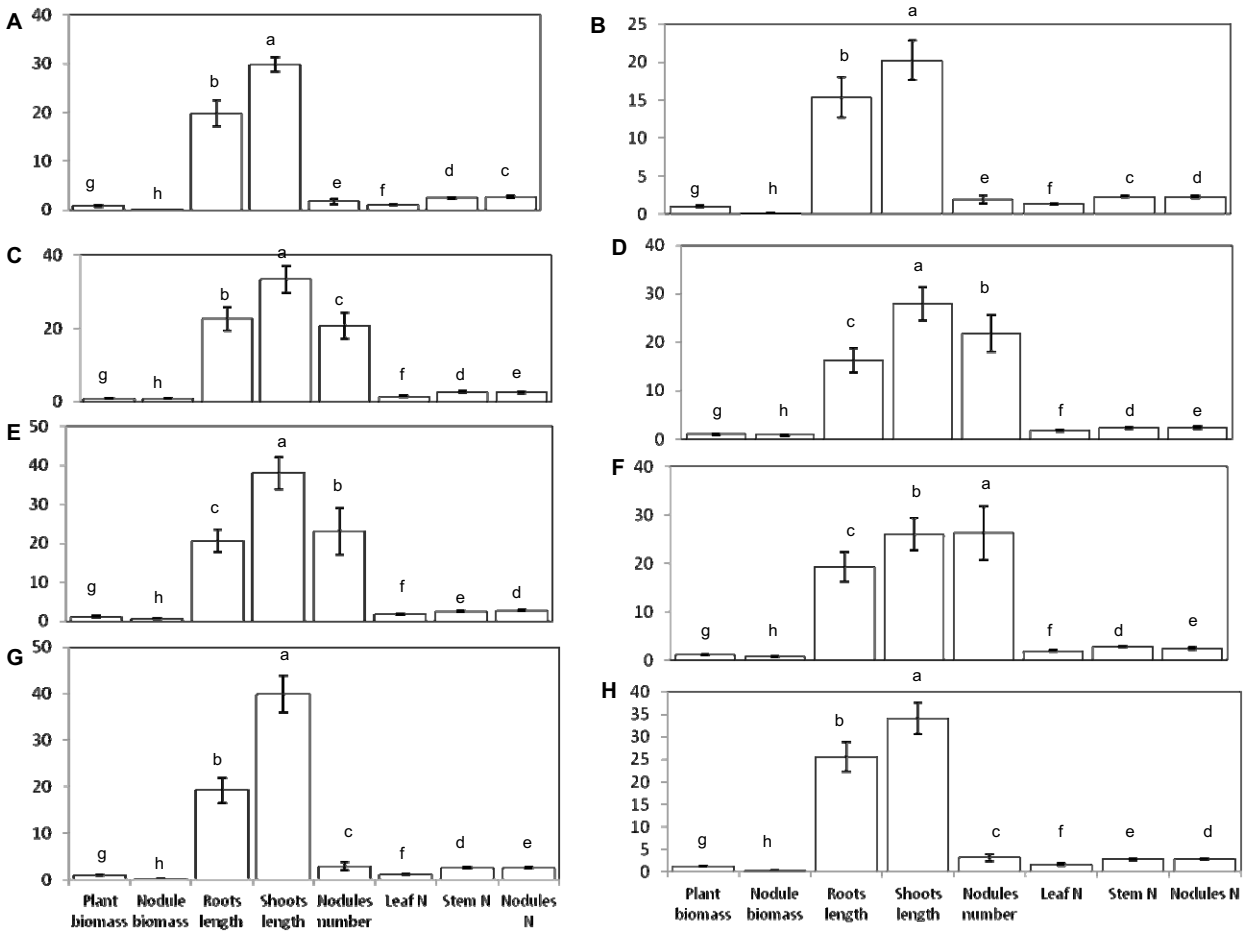

Fig. 2 Comparison of the different parameters of the rhizobial isolates at 40DPG. DPG = days post germination. n = 4, mean ± SE. Different letters within treatments for a single rhizobial isolate are significantly different according to DMRT at α = 0.05. Values are expressed as marginal means on the Y-axis and different experimental parameters on the X-axis. A, C, E and G are the effects shown by KR1 at 20, 30 and 40 DPG respectively while B, D, F and H are the effects shown by MR2 on different experimental parameters at 20, 30 and 40 DPG, respectively.

MR2 (I) alone than in combination with N (i.e., KR1 (I+N) and MR2 (I+N)). MR2 fixed N more effectively than KR1. The N content increased with an increase in the days analyzed (**Figs. 4, 5**). The total ureides content increased from 20 to 45 DAS (except for a slight dip for MR2 (I+N) at 40 DAS and KR1 (I+N) at 35 DAS). KR1 translocated ureides (574.050 mgL^{-1}) more effectively and rapidly than MR2 after 45 DAS (501.025 mgL^{-1}) (**Figs. 4, 5**). The concentration of allantoin, assessed every 22 min at 522 nm, gradually decreased over time during incubation (**Table 5**). However, the allantoin concentration and its effectiveness depend on the conditions of the laboratory environment in which the experiment is carried out. The lowest concentration of allantoin (10 mgL^{-1}) decreased more rapidly than the higher concentration (50 mgL^{-1}). The total ureides content (all treatments combined) increased continuously when assessed at harvest, i.e., at 45 DPG. However, KR1 isolate under the same conditions had a higher ureide content than MR2 (**Table 6**).

A scatter diagram (**Fig. 6A-D**) with the best-fitting trend was plotted between two different parameters to observe their regression value. The nodule and plant biomass dry weight of *V. faba* showed a positive relationship for the treatment I+N (y = 1.048x – 0.524), I (y = 1.025x– 0.174) and N (y = 0.259x– 0.044) but a negative relationship for C (y = -0.020x+0.192). Among the treatments, the relation-

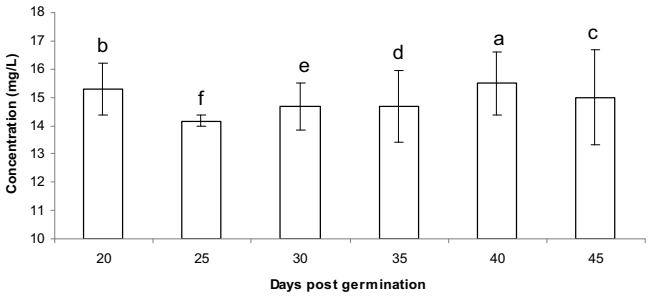

Fig. 3 Total ureide content in the quality control (QC) sample. n = 4, mean ± SE. Different letters indicate significant differences according to DMRT at α = 0.05.

ship was more effective in treatment I (55.9%) followed by I+N (45.8%) and the regression coefficient between nodule biomass and plant biomass was 81% ($R^2 = 0.812$). Similarly, a relationship was plotted between shoot length and root length in the four treatments. The trend was linear in I+N ($y = 1.063x+10.76$), polynomial (order 3) in I ($y = 0.968x+11.79$), logarithmic in N ($y = 18.17\ln(x) - 17.50$) and polynomial (order 2) in C ($y = -0.053x^2+2.285x+5.923$). Among the treatments, I ($R^2 = 0.833$) showed a good relationship of 83.3% followed by I+N (72.3%; $R^2 = 0.723$) and the regression coefficient between shoot length and root length was 91% ($R^2 = 0.913$; **Fig. 6B**). Similarly, the regression between nodule biomass and nodule number was linear in I+N ($y = 0.017x+0.270$), N ($y = 0.058x+0.091$) and C ($y = 0.109x - 0.022$), and exponential in I ($y = 0.400e^{0.032x}$). Among the treatments, I ($R^2 = 0.980$) showed a highly positive relationship followed by C ($R^2 = 0.883$) and the regression coefficient between nodule biomass and nodule number was 95% ($R^2 = 0.955$; **Fig. 6C**). Similarly, a polynomial (order 2) trend for I+N ($y = 0.000x^2+0.273x+152.2$) and a linear one for I ($y = 0.968x+68.77$) was observed

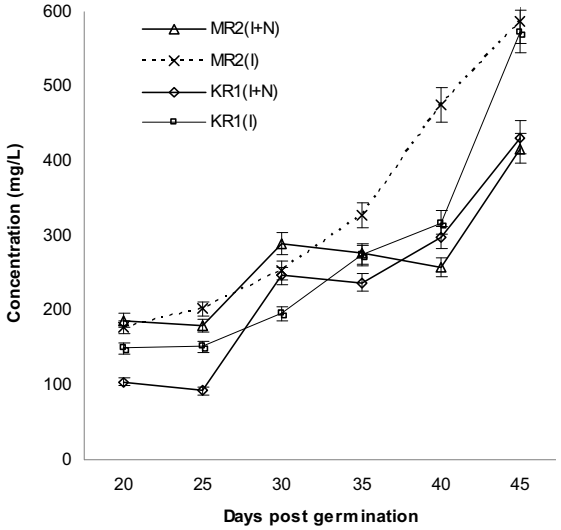

Fig. 5 Comparison of N fixed and translocated in the form of ureides by the rhizobial isolates, MR2 and KR1 (inocula alone and with N).

when total ureides – fixed and translocated between the two rhizobial isolates KR1 and MR2 – were plotted. Among the treatments, I+N (92.8%; $R^2 = 0.928$) showed a good relationship followed by I (9.04%; $R^2 = 0904$) and the regression coefficient between DPG and total ureides fixed by KR1 and MR2 was 81% ($R^2 = 0.81$) and 93% ($R^2 = 0.934$; **Fig. 6D**).

The colony characteristics of *Rhizobium*, their authentication and nodule induction are represented in **Fig. 7**). Initially, the nodules were more or less round (observed as small, creamy white bulges; **Fig. 7F**), but they turned into finger-like projections (branched or bilobed) over time. Infected and uninfected cells in the meristem at the apex

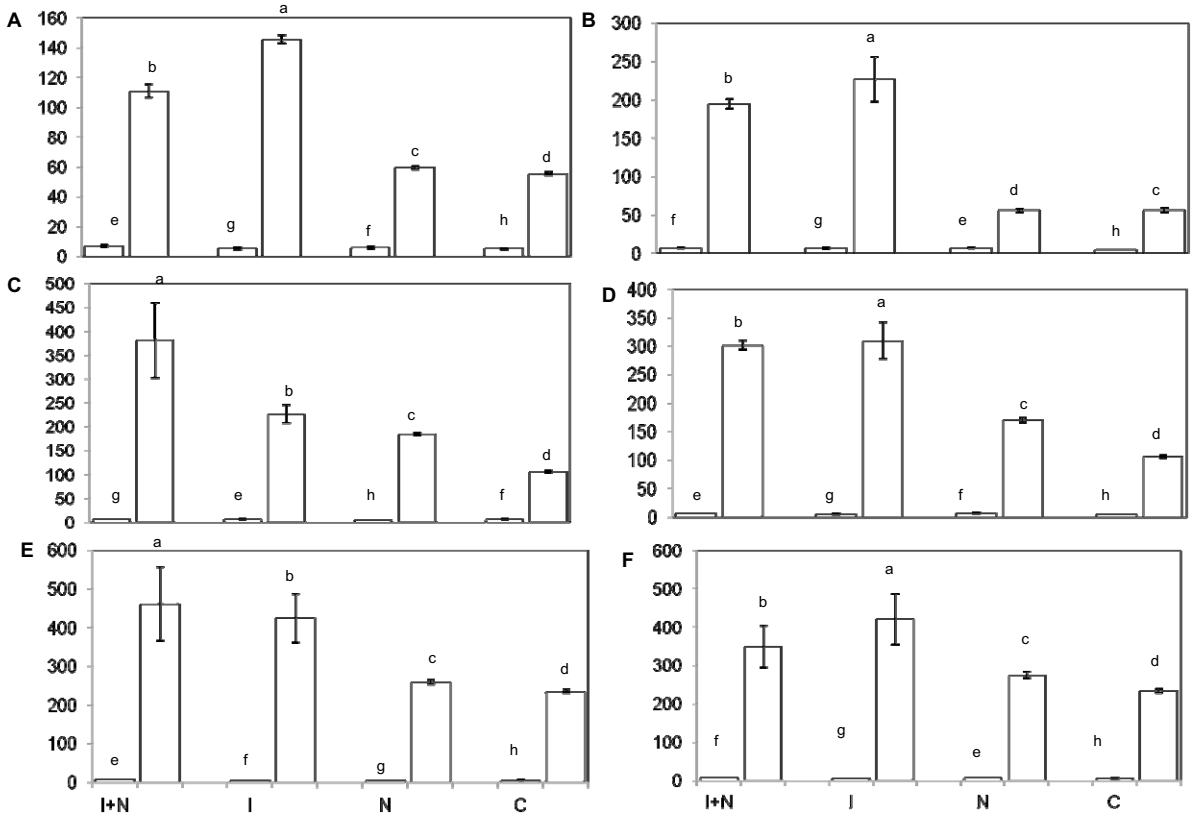

Fig. 4 Comparison of total N (%) of the plant (nodule, stem and leaf) and total ureides (mg/L) at different DPG. DPG = days post germination. n = 4, mean ± SE. Different letters within treatments for a single rhizobial isolate are significantly different according to DMRT at α = 0.05. Plain bars: total N; shaded bars: total ureides. Values expressed as marginal means on the Y-axis and treatments on the X-axis: C, control; I+N, *Rhizobium* inocula with nitrogen; I, inocula without nitrogen; N, nitrogen only. A, C and E are the effects shown by KR1 at 20, 30 and 40 DPG, respectively while B, D and E are the effects shown by MR2 on total N and total ureides at 20, 30 and 40 DPG, respectively.

Table 5 Effect of time interval (min) on incubation of allantoin at 522 nm.

Time (min)	Concentration of standard allantoin (mgL^{-1})				
	10	20	30	40	50
1	0.080 ± 0.140	0.298 ± 0.300	0.529 ± 0.160	0.747 ± 0.450	0.984 ± 0.220
2	0.063 ± 0.310	0.290 ± 0.240	0.522 ± 0.160	0.735 ± 0.040	0.971 ± 0.310
3	0.059 ± 0.210	0.273 ± 0.470	0.501 ± 0.310	0.726 ± 0.220	0.950 ± 0.140
4	0.053 ± 0.420	0.266 ± 0.210	0.483 ± 0.380	0.713 ± 0.640	0.940 ± 0.520
5	0.051 ± 0.140	0.251 ± 0.280	0.471 ± 0.240	0.696 ± 0.650	0.902 ± 0.840
6	0.047 ± 0.480	0.246 ± 0.630	0.450 ± 0.014	0.660 ± 0.320	0.861 ± 0.660
7	0.047 ± 0.140	0.238 ± 0.290	0.439 ± 0.310	0.618 ± 0.620	0.841 ± 0.240
8	0.046 ± 0.670	0.231 ± 0.540	0.422 ± 0.310	0.593 ± 0.390	0.813 ± 0.640
9	0.046 ± 0.250	0.231 ± 0.310	0.410 ± 0.380	0.568 ± 0.830	0.783 ± 0.960
10	0.038 ± 0.190	0.200 ± 0.350	0.382 ± 0.390	0.531 ± 0.460	0.751 ± 0.880
11	0.038 ± 0.630	0.181 ± 0.370	0.360 ± 0.210	0.522 ± 0.117	0.728 ± 0.690
12	0.038 ± 0.210	0.170 ± 0.570	0.350 ± 0.380	0.510 ± 0.160	0.698 ± 0.370

Mean ± SD (n = 4).

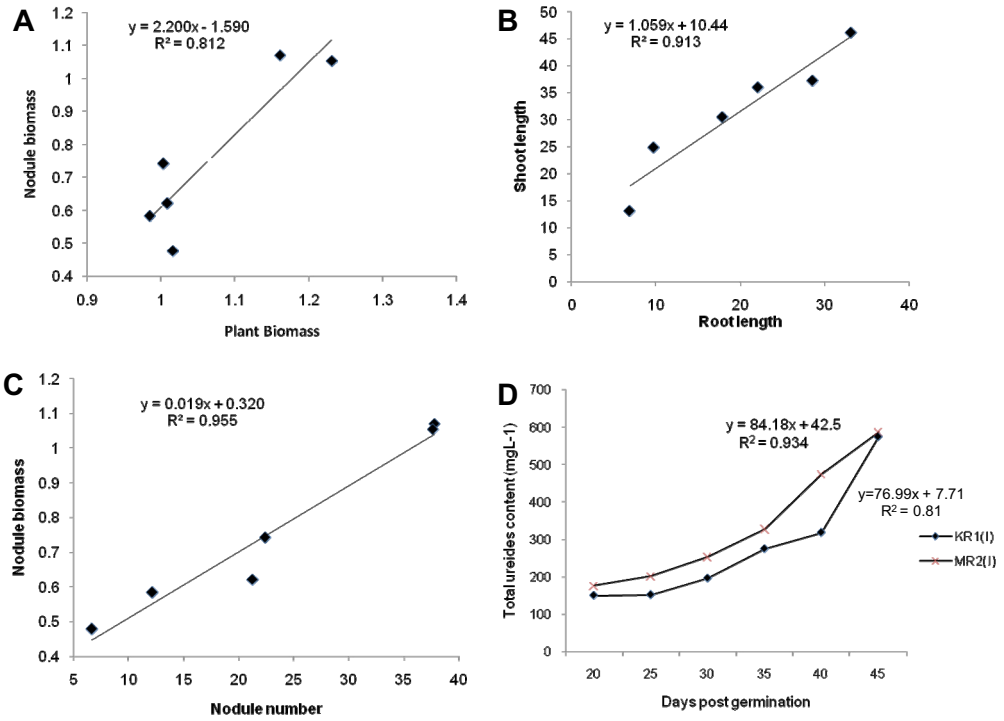

Fig. 6 Correlation between several parameters as affected by inoculation (mean of KR1 and MR2 at 20, 30 and 40 DPG). Plant biomass = g; root length = cm.

Table 6 Ureides content of the rhizobial isolates (total treatments combined).

Total number of days	Ureides content (mgL^{-1})	
	KR1	MR2
20	128.175 ± 0.363	210.850 ± 0.241
25	181.825 ± 0.245	229.950 ± 0.323
30	354.475 ± 0.364	305.625 ± 0.257
35	368.375 ± 0.339	398.655 ± 0.687
40	443.112 ± 0.287	364.925 ± 0.354

Mean ± SD (n = 4).

and the proximal grown cells were of indeterminate type (proof not shown).

DISCUSSION

Nodule organogenesis is initiated by the host plant roots when phenolic flavonoid compounds are exuded into the rhizosphere, a chemical signaling pathway (Redmond *et al*. 1986; Dunpont *et al*. 2012), which is ultimately regulated by changes in plant gene expression (up-and-down regulation). As the plants are invaded by the compatible bacteria, the bacteria undergo rapid multiplication, the infected cells finally go through endoduplication cycles (replication of genomic DNA without mitosis or cytokinesis) to form big structures housing thousands of bacteroids (Kondorosi *et al*. 2000; Jones *et al*. 2007). Leguminous plants such as *Vicia faba* form indeterminate type of nodules: first cell division events occur anticlinally within the inner cortex, followed by periclinal divisions in the endodermis and pericycle (with a persistent meristem and obvious developmental gradient; Ferguson *et al*. 2010), giving rise to the finger-like projections at maturity. The products of N-fixation (the ureides allantoin and allantoate) are key molecules in the transport and storage of nitrogen in legumes. In shoots and leaves from *Phaseolus vulgaris*, plants using symbiotically fixed nitrogen, the level of ureides coincides with that of nitrate-supported plants during the non-flowering stage which increased unexpectedly at the onset of flowering (Diaz-Leal *et al*. 2012). As soon as the legume-*Rhizobium* symbiosis is established, the bacterium shuts down NH_4^+ assimilation (Smallridge 2003). Immediately as the symbiosis is achieved, the bacteriods are not further accessed to the outer environment to meet their need like carbohydrates and minerals (sulphur and phosphorous) to maintain their effective metabolism and nitrogen fixing ability (Krusell *et al*. 2005; Delmotte *et al*. 2010), hence they must rely on the plant products for addressing their nutritional needs.

There are two steps by which ureides are degraded in plant tissues. First, allantoin degrades to allantoic acid by allantoinase (EC 3.5.2.5; Vadez and Sinclair 2000). Allantoic acid undergoes further degradation by two pathways that involve allantoate amidinohydrolase (EC 3.5.3.4; Shelp

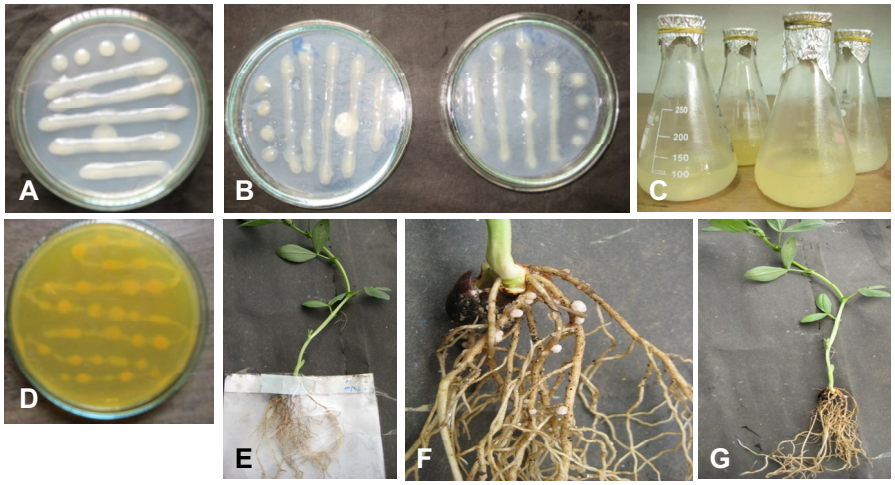

Fig. 7 Isolation, culture and authentication of rhizobial isolates. Soil sample from Kathmandu and Manang district of Nepal was collected as a source of *Rhizobia* and bacteria were isolated using trap method. (**A**) Pure culture of KR1; (**B**) pure culture of MR2; (**C**) broth culture of KR1; (**D**) acid-producing nature of KR1 at 5 d on YMA-BTB medium; (**E**) authentication of *Rhizobium* (KR1) by pouch culture method; (**F**) nodule induction by KR1 at 20 days; (**G**) nodule induction by MR2 at 30 d (I+N).

et al. 1984) and manganese-dependent allantoate amidohydrolase (AAH; EC 3.5.3.9; Lukaszewski *et al*. 1992; Stebbins and Polacco 1995), more sensitive to drought. An increment in leaf Mn results in lower levels of ureides in leaves (Vadez and Sinclair 2000, 2001).

Studies involving the enzymatic and physiological properties of ureide degradation are complicated and these compounds, especially ureidoglycolate by ureidoglycolate amidohydrolase (EC 3.5.3.19) and ureidoglycine by ureidoglycolate amidohydrolase (EC 3.5.3) in French bean (*Phaseolus vulgaris* L.) (Todd and Polacco 2004; Muñoz *et al*. 2006; Todd *et al*. 2006; Brychkova *et al*. 2008; Werner *et al*. 2010) are quite unstable and rapidly generate to urea (in acidic or basic pH; Werner *et al*. 2010). However, the major challenge lies in understanding the complex biogeochemical pathways of the N cycle in different agricultural systems and how management practices can harbor them. Moreover, the underlying complex mechanisms through various metabolic changes taking place within plant cells remains unclear.

Different developmental stages of legumes influence different ureide levels in legumes such as *Phaseolus vulgaris*, broad bean, etc., both in N-fixing and in nitrate-fed plants. This development is further mediated by the tissue-specific regulation of ALN (allantoinase; EC 3.5.2.5; Diaz-Leal *et al.* 2012), causing a rise in ureide levels in the non-nodulated legumes which may also be due to the recycling of N from proteins or nucleic acids in tissues undergoing drought-induced senescence as the source of ureides (Alamillo *et al*. 2010).

Places (especially alpine) which are relatively lower in N-input, symbiotic N-fixation budget is relatively high (Thomas *et al*. 1998). The total N availability in the atmosphere (*ca.* 4×10^{21} g; Mackenzie 1998) is available in very low amounts (<1%) to very few organisms (<1%; Galloway *et al*. 2003), and because of the energy required to break its heavy bond dissociation energy (NN; bond length: 110 pm; bond energy: 945 $kJmol^{-1}$), it makes it difficult for plants to trap N from the atmosphere and utilize it in their metabolic pathway. This difficulty can either be addressed through a high temperature process or it requires soil microbes to break and utilize it (Galloway *et al*. 2003). In greenhouse-grown plants (*Robinia pseudoacacia* L.) inoculated with specific rhizobial strains (*Rhizobium* sp.), ureide-N comprised *ca.* 8% of the total soluble-N in the xylem sap (Kaneko and Newcomb 1990).

One of the biggest problems lies in the resolution of N cycles across this ecosystem (Hedin *et al*. 2009), and microbes (especially N-fixing bacteria like *Rhizobium* within specific organs called nodules in a considerably complex process called symbiosis) play a pivotal role in fixing substantial atmospheric N (unusable form) to a readily usable form. Plants have a special ability to adapt symbiotically in highly adverse climatic and soil conditions (Svenning *et al*. 1991; Jacot *et al*. 2000) esp. the cold, frost conditions, and acidic soils. Hence, the interference cannot be made on a whole on the low and decreasing productivity based on the availability of N in the soils of high altitudes. The legumes growing in such soils and climatic conditions take up a higher amount of N and incorporate it in their metabolic structure. The reduction in symbiotically fixed N yields with increasing elevation is mainly due to the decline in the proportion yield of legumes and not in the percentage of N derived from symbiosis in the individual legume because symbiotic effectiveness is high at highest altitudes (Jacot *et al.* 2000).

During and by the pod-filling period, most of the ureides have already been transferred from mature nodules (particularly in soybean and cowpea), from the nodules to the aerial parts. Moreover, most of the ureides remain in the xylem sap as a soluble N in the infected cells while the appearance of abundant intact endoplasmic reticulum (tubular) with marked enlargement of peroxisomes they are still present in uninfected cells of the root nodule (Kaneko and Newcomb 1990). The process of translocation of ureides might also be confined to a certain number of cells (late symbiotic zone) within particular regions of the N-fixing nodule (Kaneko and Newcomb 1990). The symbiotic N-fixation process is highly sensitive to soil water deficit (Serraj *et al*. 1999; Vadez and Sinclair 2001). The symbiotic properties between the microsymbiont and macrosymbiont finally results in high crop productivity (Sinclair *et al*. 2000) and also determines the differential sensitivity of N-fixation. Evidence regarding the ultrastructure specialization of mature nodules for the production of ureides and the undergoing mechanism for their synthesis within cells has not been revealed so far.

CONCLUSIONS

Legumes are considered as future sustainable biofuel sources because of their high seed oil content (Scott *et al*. 2008). N is considered to be the main limiting factor for plant growth and development (Ferguson *et al.* 2010). Further research is required to understand the relative advantage of different pathways for the ureide production and degradation in *Vicia faba* and also to determine the best pathway under specific cropping conditions. The impact of ureide nutrition to plants needs to be better understood and its diverse role in plants should be assessed. Furthermore, the

detailed mechanism of N uptake and metabolism within a plant (especially ureide metabolism including ureide transporters, internal transport, storage and possible route of enzymatic purine ring hydrolysis) also need to be addressed. There is also a high demand to introduce differential expression profiling tools that functions depending on genome and high throughput EST-sequencing, so that the nodulin genes and their functions can easily be traced out. Finally, we conclude that more efficient rhizobial strains (especially those of the high Himalayas) need to be investigated and introduced to the Nepalese farming system to promote and develop better strategies for knowledge-based crop improvement in farming in an agricultural country such as Nepal. Legume-based technology (for choosing highly infective *Rhizobium* with great N-fixing ability) should also be developed to enhance the Nepalese cropping system while developing a common sustainable framework of natural resources and further prevent environment deterioration.

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