Environmental and Experimental Biology (2014) 12: 187–197 *Original Paper*

Xylem-mediated channeling of nitrogen in broad bean (*Vicia faba*)

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Abstract

Nitrogenous compounds in leguminous plants translocate in the form of ureides, allantoin and allantoic acid, the oxidation products of *de novo* purine synthesis, from the nodules to the aerial parts. The nodules are the main sites of ureides synthesis through the coordination of the plant-bacteria association. However, aspects related to the occurrence, localization and properties associated with the enzymes involved in the assimilation of ureides in shoot tissues have not yet been fully resolved. In this study, a modified and simplified automated analysis was used to determine allantoin concentration in plant xylem exudates. The total amount of ureides translocated to the aerial parts of faba bean (*Vicia faba* L.) plants was quantified by the stem sap extraction method using allantoin as the internal standard. Other parameters 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. Two rhizobial isolates (KR1 and MR2), isolated from *Pisum sativum* L. var. 'Macrocarpon' and *Phaseolus vulgaris* L. (cv. 'Carioca 29') plants, respectively, were selected from entirely different agro-climatic regions. MR2 accumulated more ureides (587.28 mg L^{-1}) than KR1 (573.33 mg L^{-1}) when assessed at harvest. Plants were harvested at regular intervals for dry matter and stem-extracted exudates. Results were insignificant ($P > 0.05$) for different inocula, shoot and root length and nodule N, but were significant ($P < 0.05$) for both rhizobial isolates during nodulation. The concentration of ureides, which were compared with total N concentrations in nodules, stems and leaves, were significantly different ($P < 0.01$). We conclude that the percentage of N in the form of ureides, however, does not always indicate the ability of the plant to symbiotically fix N_2.

Key words: allantoin, assimilate partitioning, crop productivity, nitrogen transporters, nodules, regulators, *Rhizobium*, ureide biogenesis.
Abbreviations: ANOVA, analysis of variance; BNF, biological nitrogen fixation; DM, dry mass; DPG, days post germination; Gln, glutamine; I+N, *Rhizobium* inocula with nitrogen; I, inocula without nitrogen; KR1, *Rhizobium leguminosarum* bv. *viciae*; LSD, least significant difference; MR2, *Rhizobium leguminosarum* bv. *phaseoli*; N, nitrogen only; QC, quality control; SNF, symbiotic N_2 fixation; YEM, yeast extract mannitol agar.

Introduction

Faba bean (*Vicia faba* L.) is one of the major staple pulse crops grown for dry seeds and green pods throughout the world (Telaye et al. 1994). Beans possess high protein content (26 to 41%) and are thus very important in the combat of nutrient depletion, especially in economically disadvantaged countries. Also, this pulse, together with the microbial symbionts found in the soil in association with the plant, help to recharge the soil by fixing nitrogen from the atmosphere. Hence, finding a better rhizobial isolate of agronomic importance has been a major challenge, especially for crops growing at high altitudes.

Plants demand a high supply of nitrogen for their growth and development, and are used for synthesizing amino acids (the building blocks of proteins) and nucleic acids. The nitrogen status of legumes is a well-documented theme but still has ample aspects that still need to be researched. The synthesis and translocation of nitrogenous compounds that form and translocate from the source to the sink (place of usage) during the nitrogen (N_2)-fixation process takes place by a vascular network (xylem and phloem) that connects the internal parts of plant organs (Tegeder 2014). For the synthesis of amino acids, nitrogenous compounds need to be incorporated into the carbon skeleton of plant metabolites (Udvardi, Poole 2013). The transport (basically of N) is mainly achieved and facilitated by sharing and exchanging signals between plant organs (nodules) moved by the mass flow of solutes (Thompson, Holbrook 2004), via the xylem and phloem (Brenner et al. 2006; Atkins, Smith 2007). The N_2-fixation takes place by the induction of nodule-specific plant genes (genes encoding symbiotic leghemoglobins) with the development of nodules (Ott et al. 2005). Nodules, which are closed vesicles (outer protuberance from the roots), are the main starting point for the synthesis of these amino acids by a close association

with N_2-fixing bacteria (Tegeder 2014). The incorporation of nitrogenous compounds, minerals (both macro and micro) and other nutrients into the plant metabolic pathway are a clear sign that plants have accumulated carbon into its skeleton. This incorporation balances the N : C ratio in a fixed amount within the plant (Layzell et al. 1981; Nicolardot et al. 2001).

The biogenesis of ureides takes place in root nodules, which serve as the source (Rentsch 2007). It involves a rigorous pathway (Atkins, Smith 2000; Kim et al. 2007) that starts with the assimilation of ammonia, which is produced by nitrogenase (EC 1.18.6.1; Eq. 1; Schubert 1995). The reduction of N_2 to ammonia (NH_3) mediated by nitrogenase is labile in oxygen (Udvardi, Poole 2013), and subsequently gives rise to glutamine (Masalkar et al. 2010; Tegeder 2014) and other amino acids (glutamate, aspartate and glycine) within the infected cells. Basically, two enzymes, namely xanthine dehydrogenase (EC 1.1.1.37) and urate oxidase (EC 1.7.3.3), seem to play a key role in the biosynthesis of ureides in legume nodules (Tajima et al. 2004). These ureides, the final dominant nitrogenous products, are then translocated from the nodules to the roots and aerial parts, namely the leaves, flowers and pods or collectively the sink (Rentsch 2007; Collier, Tegeder 2012) where they are finally catabolized (Atkins, Smith 2007).

The initiation of synthesis of ureides in root nodules takes place via the intimate coordination and complex interplay between plant (host) and bacteria (symbiont), and involves several processes (Atkins, Smith 2000; Rice et al. 2000). Immediately after N_2 fixation in the bacteroids of infected root cells, NH_3, ammonium ion (NH_4^+), or amino acids are released or transported from the symbiosome to the cytosol, utilizing them for Gln synthesis with the aid of Gln synthetase (Day et al. 2001; Smith, Atkins 2002; Lodwig et al. 2003). This process is then followed by a purine synthesis pathway in plastids or mitochondria (Smith, Atkins 2002; Werner, Witte 2011) and purine degradation via xanthine in plastids or the cytosol of infected root cells (Todd et al. 2006). The ureides are synthesized from purines as allopurinol (a xanthine dehydrogenase inhibitor), which inhibits the further production of ureides (Quiles et al. 2009). The ureide allantoin is finally synthesized in the peroxisomes of non-infected root cells from a purine degradation product, uric acid (Brychkova et al. 2008), through several intermediate steps (Todd et al. 2006; Werner, Witte 2011).

$$N_2 + 16\ ATP + 8\ e^- + 10\ H^+ \rightarrow$$
$$2\ NH_4^+ + H_2 + 16\ ADP + 16\ Pi \quad \text{(Eq. 1)}$$

Following N_2 fixation in legume nodules, the products – especially ureides – are exported to the nodule vasculature, then to leaves and the shoot via the xylem, generally in the form of the amides asparagine (Asn) and glycine (Gln) (Quiles et al. 2009; Sulieman, Tran 2013) or the ureides allantoin and allantoic acid, which are the oxidation products of purine synthesis in nodules (Todd et al. 2006). This fixed N is transported all the way to the active sinks (flowers, pods and leaves) but can leak to adjacent cells of the xylem stream during transport. Accumulating evidence suggests that ureides comprise up to 90% of the total N transported in the xylem of N_2-fixing tropical legumes (Todd et al. 2006) and can be stored in high amounts in different plant organs (Tan et al. 2008). Due to their high concentrations in the vascular system and in certain plant tissues (as much as 59 nM in the paraveinal mesophyll; Matsumoto et al. 1977; Costigan et al. 1987), ureides are believed to have an important function in N transport (2 to 3 mg C mg^{-1} symbiotic N fixed; Valentine et al. 2011) and storage in legumes. The accumulation of ureides in legume nodules under soil-water deficit might trigger a feedback mechanism that results in decreased N_2 fixation (Sinclair, Serraj 1995; Serraj et al. 1999a; Goh, Bruce 2005) and might also be involved in protecting plants against abiotic stress (Werner, Witte 2011). In addition to N from root nodules, N assimilated from soil-combined N and from N cycling within the plant will all be present in the transpiration stream (Stark, Richards 2008); however, the majority of carbon exported to the xylem stream from nodules is in the form of ureides, while this partitioning of nitrogenous compounds requires the action of integral membrane transporters (Rentsch et al. 2007).

The objectives of this study were to understand the extent to which ureides are assimilated and translocated to the aerial part of a legume crop, broad bean (*Vicia faba* L.), at different time intervals or developmental stages (from sowing to harvest) by comparing their concentration to the total N concentration in leaves, stems and nodules. In addition, in a bid to increase nitrogen fixation by legumes and pulse crops growing at high altitudes, which is frequent in Nepal, two new N_2-fixing bacterial strains for broad bean were unveiled and characterized.

Materials and methods

Chemicals and reagents

All chemicals employed in these experiments were of analytical grade purchased from Sigma Chemical Co. (St. Louis, USA).

Experimental design

V. faba (cv. 'Imposa') was used as the model plant to study N_2-fixation by microbial symbionts and the localization of fixed N in the aerial parts. Plants, which were raised in a greenhouse at 28 ± 1 °C and a 16-h photoperiod under natural light intensity, were potted at four plants per pot in 5-L pots for a total of 40 days. In total, four treatments were applied: bacterial inoculum (I); urea [$CO(NH_2)_2$] at 50 mg kg^{-1} of soil as the source of mineral N; inoculum and urea combined (I+N); and the control (C). Plants were watered with N-free water when needed and micronutrients were added as Hoagland's solution (Hoagland, Arnon

1950). More details about the protocol may be found in Baral et al. (2012). This experimental design produced 32 combinations for each rhizobial isolate.

Isolation and selection of effective rhizobial strains

Studies on rhizobial isolates with reference to synthesis of ureides by legumes within Kathmandu and Manang do not exist. Moreover, these locations lie on completely different geographical terrains with vastly different climatic conditions and may harbour rhizobial strains with potent and highly effective N_2-fixing ability, serving as the basal impulse for this research. Manang lies in the trans-Himalayan region from sub-alpine to high Alps with an arid climate, while Kathmandu, a fertile valley, lies in a temperate zone with high humidity. Composite soil samples from a depth of 10 to 15 cm from two different agro-climatic regions (Kathmandu: 27° 43' 06" N, 85° 19' 02" E; Manang: 28° 40' 03" N, 84° 15' 01" E) were sifted through a 5-mm mesh (pH 6.0 to 6.5) in pots 16 × 18 cm in size and transferred to a glasshouse and were used as the source of rhizobial isolates. *Pisum sativum* L. var. 'Macrocarpon' and *Phaseolus vulgaris* L. (cv. 'Carioca 29') plants were raised from viable (89% germination) seeds in a greenhouse (28 ± 1 °C) in 5-L pots, with four plants per pot and nodular rhizobial isolates were isolated using the trap method (Vincent 1970). Two different plant species were selected for trapping compatible rhizobial isolates so as to maximize the success of trapping. Seeds were initially germinated in sterile Petri dishes on filter paper (Whatman No. 1; Sigma-Aldrich) moistened with double distilled water. Only seeds that appeared healthy and seedlings that grew uniformly were used for the experiment.

Authentication, acid production and mean generation time of rhizobial isolates

The two rhizobial isolates (*Rhizobium leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *phaseoli*, isolated from Kathmandu-based *P. sativum* and Manang-based *P. vulgaris*, are respectively designated as KR1 and MR2 throughout the remainder of the manuscript) were used as four treatment combinations and four replications of each, as suggested by Boone et al. (2001). Rhizobial isolates were isolated by gently lifting the legumes in soil from their original sampling sites and the endogenous bacteria (microsymbionts) were isolated by the trap method using a protocol devised by Baral et al. (2012).

Collection of stem sap

The first harvest was performed at 20 days post germination (DPG) and repeated every 5 d until 45 DPG. Four plants of each treatment were uprooted i.e., one plant per pot of each replication every five days starting from 20 DPG were harvested. A portion of the stem (approximately 3.0 cm long) at the base just above the radicle, was ground finely in liquid N_2 by using a mortar and pestle then filtered through Whatman No. 1 filter paper and centrifuged (10 000 × g, 20 min, 4 °C). Vials with stems in phosphate buffer solution were stored at 0 °C until extracted (Herridge 1984). The supernatant was used to determine the ureide content (de Silva et al. 1996; Herridge et al. 1996). Phenols were effectively removed from H-bonded complexes following the addition of 1.5 g of insoluble polyvinylpyrrolidone, which was used to adsorb phenols and thus obtain active soluble enzymes.

Analytical techniques for the estimation of ureides

The experiment used the Herridge and Peoples (1990) protocol with minor modifications, where needed. Briefly, *Vicia faba* L. plants were grown under aseptic conditions, subjected with different treatments (I+N, I, N and C). The plants were uprooted at different time intervals starting from 20 DPG. A portion of the stem (approximately 3.0 cm long) just above ground level was sampled and finely ground with a mortar and pestle with the aid of liquid N_2. One mL of each plant sample (the ground mixture), a quality control (QC) sample (i.e., the test sample for measuring consistency), and standard or distilled water (blank) were pipetted out into 15-mL tubes, treated with 5 mL of distilled water and 1 mL of 0.5 M NaOH followed by vigorous shaking for a few seconds. NaOH was used for color development at room temperature. The tubes were then placed in a boiling water bath for approximately 7 min, then cooled in cold water. To each tube, 1 mL of HCl (0.5 M) was added, adjusting the pH to between 2 and 3 (at higher pH, uric acid forms the duly charged full urate ion) followed by the addition of 1 mL of phenylhydrazine solution. The tubes were again transferred to a boiling water bath for 7 min then left to cool in an ice-cold alcohol bath for 10 min. Fresh solutions of phenylhydrazine HCl and potassium ferricyanide were applied to get better results.

The cooled tubes were treated with 3 mL of precooled HCl (conc.) and 1 mL of potassium ferricyanide. This was performed in a fume cupboard within the shortest possible time span. The solutions were then thoroughly mixed and transferred to 4.5-mL cuvettes at room temperature. The absorbance with a blank (or distilled water) was adjusted at 522 nm. The absorbance for each tube was read exactly 20 min after the addition of potassium ferricyanide. An allantoin standard curve was then plotted against allantoin concentration (mg L^{-1}) on the X-axis versus optical density (at 522 nm) on the Y-axis.

For accurate weighing on a balance, a large quantity of each working standard solution with pH 6.2 (by the drop-wise addition of 0.1N HCl and 0.1N NaOH) was made. For this, 50 mg of allantoin (used as an internal standard) was weighed and then transferred to a 500-mL volumetric flask. This was dissolved in 100 mL of 0.01 M NaOH, and made up to volume with distilled water (dH_2O). The addition of NaOH served only to dissolve allantoin. For preparing 50 mL of the working standards (10, 20, 30, 40, 50 and 60

mgL^{-1}), a stock solution of weight 5, 10, 15, 20, 25 and 30 g was accurately weighed into a 50-mL volumetric flask and made up to volume with dH_2O. Each working standard was stored at 0 °C as a small aliquot in a freezer and only the necessary quantities were thawed while the remainder was discarded.

The analysis of ureides was based on a colorimetric method (Herridge, Peoples 1990), which is a rapid and inexpensive method in which allantoin is converted to allantoic acid followed by hydroxylation to urea and glyoxylic acid in a weak solution. Glyoxylic acid thus formed reacts with phenylhydrazine-HCl producing a phenylhydrazone derivative of the acid which is an unstable chromophore in reaction with potassium ferricyanide; the color obtained was read at 522 nm using a UV/Vis spectrophotometer (6715, Jenway; Bibby Scientific Ltd., Stone, UK).

Spectrophotometric methods used were based on the Rimini-Schryver reaction, which is the condensation of a hydrazine derivative with keto-acids to give a colored product. The absorbance of standard and samples was taken as quickly as possible since the colour fades out gradually and the absorbance decreases with time and thus the process cannot be interrupted in the middle of the experiment. Therefore, a few samples in duplicate were processed in each run using a set of standards and a blank (dH_2O) in parallel. For consistency between the different time periods in the same laboratory, a QC sample was performed at each run with a 20X dilution (Kaito et al. 1977).

The concentration of the QC and the other samples was determined using the following formula (de Silva et al. 1996; Herridge et al. 1996):

$$C = (Y - a) / (b \times F),$$

where C and Y are the concentration and absorbance of the unknown, a and b are the intercept and slope of the standard curve respectively, and F is the dilution factor.

Experimental parameters

Plants were harvested after 20 DPG at 10-day intervals lasting for a total period of 40 days. After each harvest (i.e., every 10 day), multiple experimental parameters [total number of nodules, shoot and root length (longest root), shoot dry biomass (dried in an incubator at 30 °C for three days; Bod Incubator, Prolific instruments, Mumbai, India), stem, leaf and nodule N were determined.

Total nitrogen

The total N concentration (% of dry mass) in plant samples (nodules, stem and leaves) was determined at 20, 30 and 40 DPG with four replications of each treatment using a Micro-Kjeldahl method in three differential steps: wet digestion, distillation and titration for ammonium estimation (ammonia concentration is proportional to nitrogen content in the sample; PCARR 1980; Baral et al. 2012; Muñoz-Huerta et al. 2013).

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. All statistical analyses were carried out using SPSS version 16.0 (IBM SPSS, NY, USA) and Excel 2007 (Microsoft Office; Microsoft Corp., WA, USA). All comparisons were performed by applying t-tests for independent samples and Duncan's multiple range test at $\alpha = 0.05$ (Somasegaran, 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, shoot and root length and total N fixed. Similarly, the *F*-test was employed for comparing ureide concentration with different treatments (bacteria, nitrogen and control). The error bars in the figures represent the standard error (± SE).

Results

Characterization of microbial symbionts

Our analyses reveal that both isolates were Gram-negative, as expected, fast growing, and acid-producing with no significantly different doubling time.

Shoot and root length

MR2 inoculum had a significantly greater effect on shoot length than KR1 (treatments = 8.788 (significant); blocks = 48.326 (significant); $P < 0.01$; $LSD_{0.05} = 11.49$ cm). The root length of test plants inoculated with either rhizobial inoculum increased (treatments = 3.956 (significant); blocks = 153.646 (significant); $P < 0.05$; $LSD_{0.05} = 6.906$ cm) (Fig. 1).

Estimation of ureides

The allantoin curve, which was used as an internal standard by which the ureide (allantoin) content of the plant was determined at various intervals, showed that allantoin concentration decreased over time. Experiments were carried out separately for both isolates from 20 DPG every five days until 45 DPG. The concentration of ureides in plants inoculated with KR1 and MR2 isolates at 20 DPG was 128.175 and 210.85 mg L^{-1}, respectively, which increased by 40 DPG (KR1 = 573.333 mg L^{-1}, MR2 = 587.237 mg L^{-1}). In other words, the translocation of ureides was higher when the *P. sativum* inoculum was used. The MR2 ureide content declined slightly at 40 DPG, which was unexpected. The *F*-ratio (*P*-value: 0.202) and correlation coefficient, assessed separately for the two rhizobial isolates (0.9613 for KR1; 0.971 for MR2), showed highly significant differences ($P < 0.01$). The concentration of ureides increased in the presence of urea as the N source more than in the control (Fig. 2).

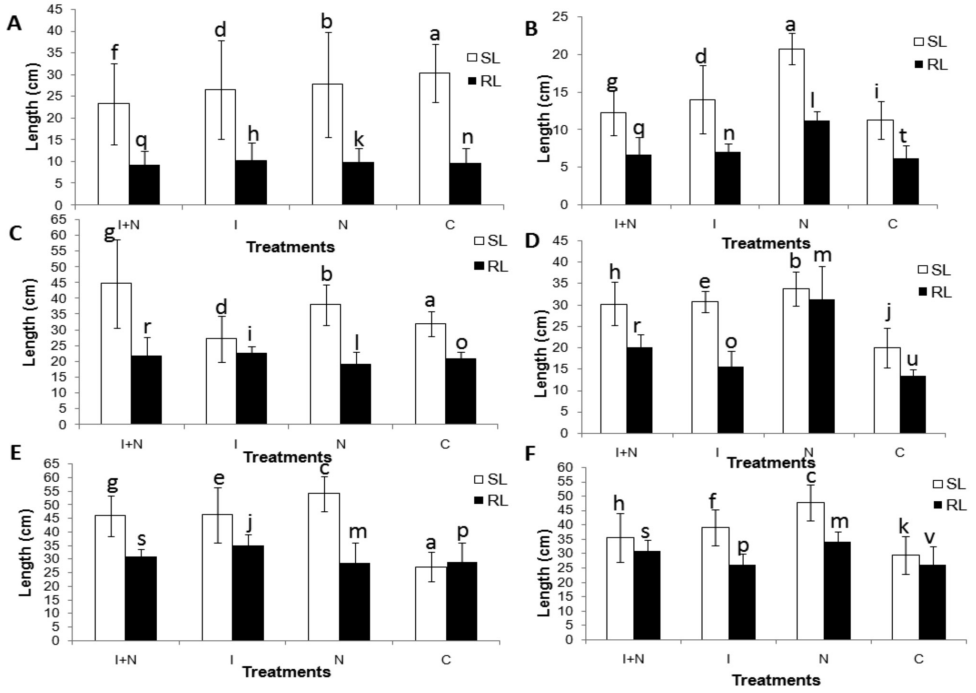

Fig. 1. Shoot length (SL) and root length (RL) of *Vicia faba* on different days after infection by rhizobial isolates KR1 and MR2. DPG = days post germination. n = 16 (biological replicates 4 × analytical replicates 4), mean ± SE. Different letters within treatments for a single rhizobial isolate are significantly different according to DMRT at α = 0.05. White bars = shoot length, black bars = root length. A, C and E were inoculated by KR1 and B, D and F were inoculated by MR2 and sampled at 20, 30 and 40 days after sowing, respectively. Treatments: I+N, inocula with N; I, without N; N, nitrogen only; C, control.

The concentration of ureides in young seedlings increased from 2 to 5 DPG, suggesting that leguminous plants are capable of producing ureides without the aid or presence of a microbial symbiont. The N_2-fixing ability of KR1 and MR2 at 40 DPG showed that the latter was more effective in fixing atmospheric N and translocating it in the form of ureides to the aerial parts. However, plant age significantly affected symbiotic effectiveness, peaking at the fourth week of plant growth. The translocation of ureides was higher in those plants inoculated with MR2 at 40 DPG (587.237 mg L^{-1}), while plants inoculated with KR1 could only translocate 573.333 mg L^{-1} of ureides at 40 DPG (Fig. 2).

Estimation of nitrogen content

The N (%) of nodules, stems and leaves was estimated using the Micro-Kjeldahl method (Fig. 3). Differences were insignificant for nodule N (%) (treatments = 0.870 (insignificant); blocks = 0.164 (insignificant); $P > 0.05$; $LSD_{0.05}$ = 1.022%), stem N (treatments = 1.230 (insignificant); blocks = 0.747 (insignificant); $P > 0.05$; $LSD_{0.05}$ = 0.898%) and leaf N (treatments = 6.390

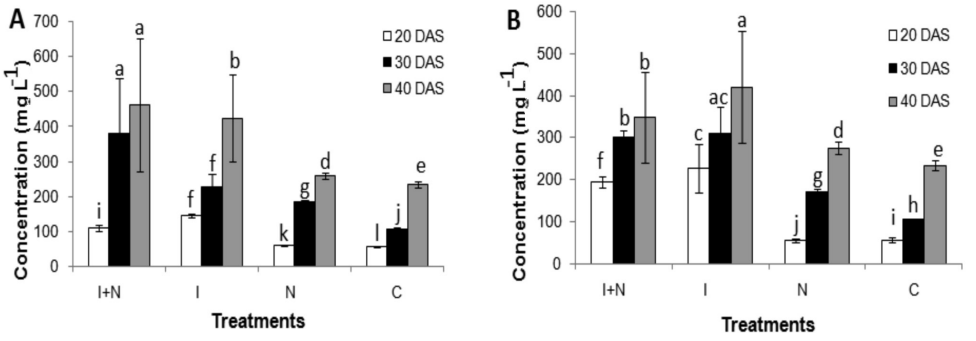

Fig. 2. Concentration of ureides in *Vicia faba* with the application of bacterial inoculum, nitrogen and with control. DPG = days post germination. n = 16 (biological replicates 4 × analytical replicates 4), mean ± SE. (A) Inoculated by KR1 and (B) inoculated by MR2 at 20, 30 and 40 days after sowing (DAS). Different letters indicate significant differences for each isolate separately according to DMRT at α = 0.05. Treatments: I+N, inocula with N; I, without N; N, nitrogen only; C, control.

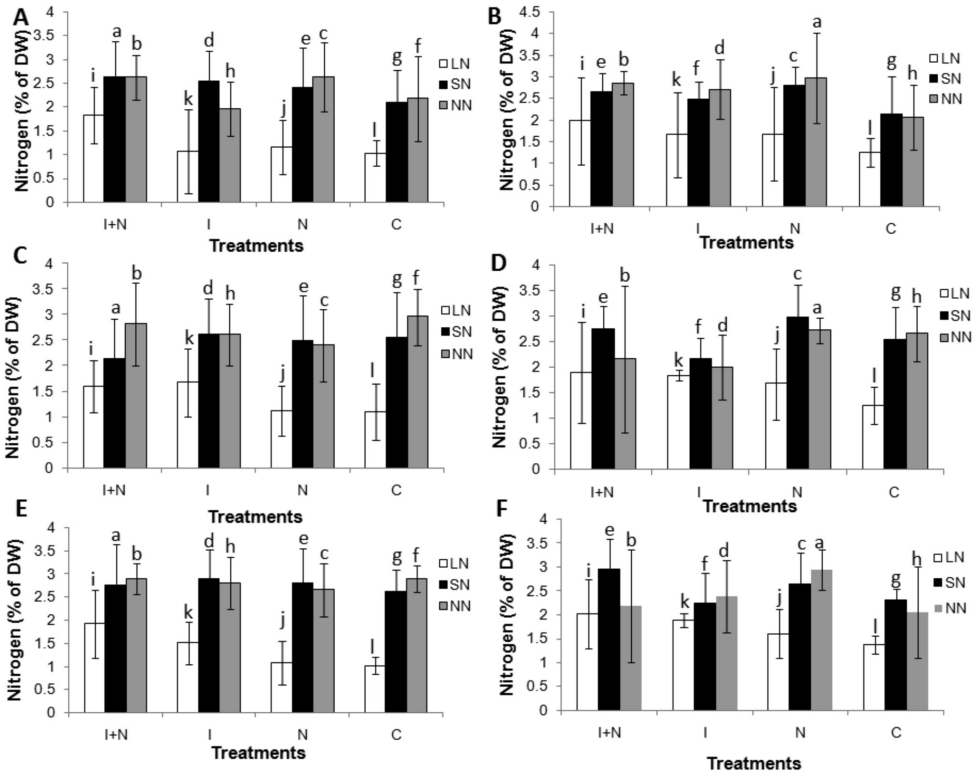

Fig. 3. Total fixed N by *Vicia faba* using an inoculum of two rhizobial isolates KR1 and MR2. DPG = days post germination, n = 16 (biological replicates 4 × analytical replicates 4), mean ± SE. Different letters within treatments for a single rhizobial isolate within each treatment are significantly different according to DMRT at α = 0.05. White bars = leaf N (LN), black bars = shoot N (SN), grey bars = nodule N (NN). A, C and E were inoculated by KR1 and B, D and F were inoculated by MR2 at 20, 30 and 40 days after sowing, respectively. Treatments: I+N, inocula with N; I, without N; N, nitrogen only; C, control.

(significant); $P < 0.01$); blocks = 0.213 (insignificant); $P > 0.05$; $LSD_{0.05} = 0.822\%$). MR2 had a higher total N content (2.88%) than KR1 (2.78%). Similarly, the total N content of the stem was highest for MR2 following the application of urea (2.8%). The percentage of N in leaf blades was maximum for I+N for both isolates (1.78 and 1.96% N for KR1 and MR2, respectively; Fig. 3).

Nodulation

Inoculated plants had many effective nodules (determinate nodules) on their root systems at 40 DPG. Visible nodules were observed at 20 DPG but were only counted after 20 DPG (Fig. 3). Treatments I, I and I+N showed maximum number of nodules for both isolates (KR1 and MR2) at 20, 30 and 40 DPG ($P > 0.05$).

Symbiotic effectiveness

The effectiveness of the two inocula tested (KR1 and MR2) at 40 DPG could be ranked (I+N > I > N > C), although differences were insignificant ($P > 0.05$; $LSD_{0.05} = 0.479$). The formation of nodules (i.e., the number of nodules) by both rhizobial isolates was significantly different on different days of the experiment (treatments = 34.992 (significant; $P < 0.01$); blocks = 19.438 (significant; $P < 0.01$); $LSD_{0.05} = 13.99$). Shoot biomass was recorded every 10 d starting from 20 DPG. For KR1, the highest value recorded was for treatments I+N, N and N at 20, 30, and 40 DPG, respectively and for MR2, highest values were for I+N, C and C at 20, 30 and 40 DPG, respectively, although differences were insignificant (treatments = 0.492; blocks = 1.368; $P > 0.05$; $LSD_{0.05} = 0.6418$ g; Fig. 4). The nodules formed by MR2 were two-fold larger than the KR1-induced nodules, suggesting that MR2 was a highly effective strain.

Discussion

The *Rhizobium*-legume symbiosis is the only and pivotal source for injecting a bulk amount of nutrient N into agricultural systems (*ca*. 40 million t of N $year^{-1}$; Udvardi, Poole 2013), representing an economical and environmentally friendly alternative to chemical fertilizers. This biological nitrogen fixation (BNF) provides about 65% of the total biosphere's available N (Lodwig 2003). Legumes contribute approximately 30,000 t of N annually in Nepal (Maskey et al., 2001), recharging the soil N pool every year. Plant productivity in regions with extreme climates, such as in Nepal, are limited by very low soil fertility (Maskey et al. 2001). The efficient N supply through microsymbionts (by inducing *nod* genes; Rolfe, Gresshoff 1988; Gage 2004) may help in stabilizing food production, recharging

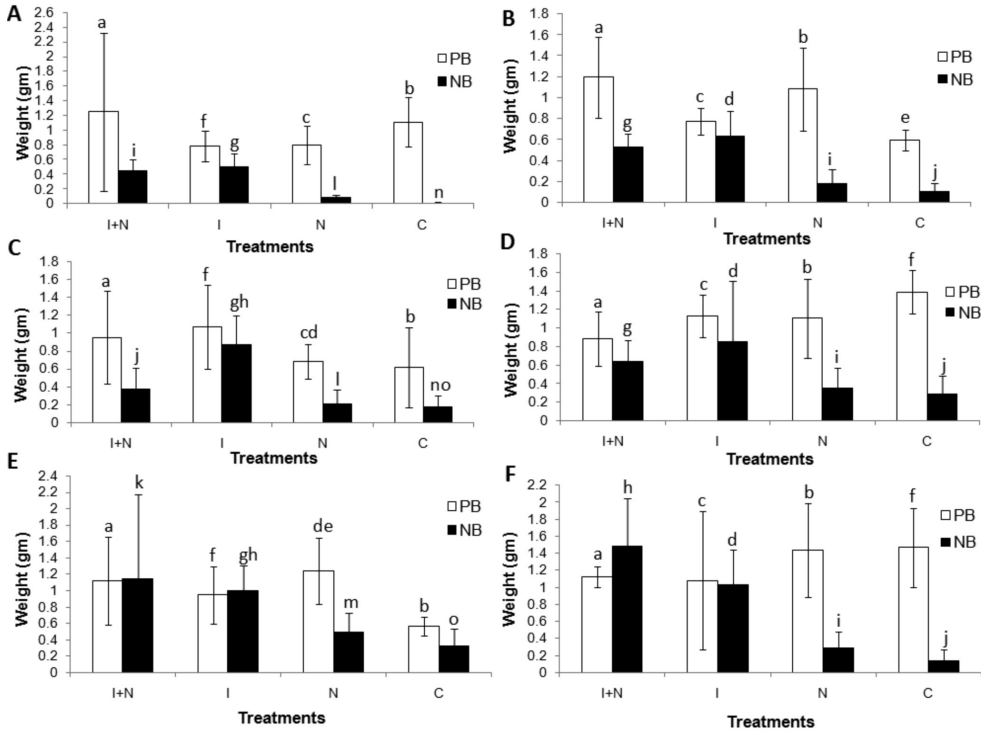

Fig. 4. Plant dry biomass and nodule dry biomass on different days after infection by rhizobial isolates KR1 and MR2. DPG = days post germination. n = 16 (biological replicates 4 × analytical replicates 4), mean ± SE. Different letters within treatments for a single rhizobial isolate are significantly different according to DMRT at α = 0.05. White bars = plant biomass (PB), black bars = nodule biomass (NB). A, C and E were inoculated by KR1 and B, D and F were inoculated by MR2 at 20, 30 and 40 days after sowing, respectively. Treatments: I+N, inocula with N; I, without N; N, nitrogen only; C, control.

soil N reserves, and increased yield of agricultural crops (Dahal, Dahal 1998; Hartwig 1998). Thus, the soil nutrient content (micro- and-macro nutrients) together with other characteristics should be considered in conjunction with the selection of appropriate plant and symbiotic microorganisms (Maskey et al. 2001). Consequently, some symbiotic characteristics of rhizobial isolates obtained from the field soils of Kathmandu and Manang were compared.

The carbon sink is related to nodule growth and legume BNF, especially in faba bean (Lawrie, Wheeler 1975). The N_2 fixing kinetics based on nodule biomass (0.05 g N g^{-1} nodule DM day^{-1}; Boote et al. 2002, 2008), provides evidence that C is allocated to and used by nodules, incorporating these processes for potential legume BNF. Legume BNF is less sensitive to ammonium than nitrate (Bollman, Vessey 2006), and the N_2 fixing rate of nodules depends on the amount of C supplied to the nodules (Haase et al. 2007; Voisin et al. 2007). In the present study, plant height was maximum when mineral N was supplied, followed by bacterial inocula, suggesting that the plants readily absorbed available N in the soil (until exhaustion) rather than fixing it through an energy-consuming process (Fig. 1). However, the longer roots of the experimental plants when N was supplied can be explained by the healthy growth of these plants and active mineral absorption activity that could be used to maintain physiological activities of the plant. In addition, legumes harbour small bio-factories on their roots, the nodules, which discharge N_2 through a complex process. In the legume-*Rhizobium* symbiosis, plants supply amino-acids to bacteroids, while bacteria reciprocally supply ammonium to the plants enabling the synthesis of amino acids, making both reliant on each other in the amino-acid cycle (Lodwig et al. 2003). Thus, legumes enjoy a competitive advantage in the trade of reduced nitrogen provided by microbial symbionts over other plants.

The level of ureides differs between drought-sensitive and drought-tolerant lines of legumes, especially soybean, with greater levels in the former (Vadez, Sinclair 2001). During scarce water deficit, these ureidic legumes hoard ureides in plant tissues and N_2 fixation is inhibited (Alamillo et al. 2010). Exogenously applied ureides may increase ureide concentration 5- to 8-fold in legumes (King, Purcell 2005). However, experiments that employ drought-sensitive or drought-tolerant faba bean lines are scarce. The concentration of ureides in a plant's aerial parts (basically the leaves) is dependent on the water conditions of the soil during N_2 fixation (inhibition of nitrogenase activity restricts N_2 translocation; Serraj et al. 1999b). The exact determination of ureides in N-fed crops sometimes becomes difficult, rendering false data. Thus, in such a prevailing scenario, sap nitrate status is a proven method to detect over-fertilized plants (Muñoz-Huerta et al. 2013).

Moreover, the factors that inhibit the flow of nutrients and other products from phloem to nodules also inhibit the flow rate of fixed N_2 from nodules to the aerial parts (Serraj et al. 1999b), causing the accumulation of nitrogenous products in the nodules themselves. Our study confirms this observation. Also, when the aerial translocation of ureides was analyzed, the treatment combination of inoculum and nitrogen (i.e., I+N) and inoculum alone (i.e., I) were efficient for both isolates, suggesting that the broad bean plants were able to channel N in the form of ureides (Fig. 2). Moreover, MR2 was a more effective rhizobial symbiont, as it fixed more total ureides in broad bean when seed were inoculated (Baral et al. 2012).

Translocation of total ureides was higher in plants inoculated with MR2 than in KR1. Moreover, ureide content continued to increase with plant development until plant harvest (40 days), which indicates that plants actively fixed N_2 and that N was translocated as ureides to active sinks, i.e., aerial parts, including leaves. Such elevated expression of ureides in plants shows the active fixation of N_2, which may be regarded as a specific metabolic feature of symbiosis in such ureide-forming legumes (Smith, Atkins 2002). A similar experiment on soybean shoots found elevated levels of ureides in sinks (pods and leaves) during the reproductive stage and also in response to the application of boric acid (Vadez et al. 2000). It has now been widely accepted that nitrogenous compounds are stored mostly as ureides in seeds, while they break down into other products in germinating seeds (Duran, Todd 2012). Further research is needed to ascertain how long these plants continue to channel N to the sinks (aerial parts), while the ureide content of pods also needs to be analyzed. This experiment suggests that these two Himalayan isolates are somewhat effective in influxing or injecting atmospheric N_2 into the biological cycle. However, substantial evidence using various microbial symbionts from higher elevations and analyzing several physiological parameters is desired to further validify this claim.

In a plant-bacterium symbiosis, the plant supplies amino acids to the microbial symbiont, which helps them to shut down their ammonium assimilation while the microbial symbiont helps to recycle the amino acids back to the plant for asparagine synthesis (Lodwig et al. 2003). This process helps the bacteria remain associated with the plant, and also prevents the plants from being dominant over the rhizobium in symbiosis (Lodwig et al. 2003). The membrane proteins (transporters) involved in the transport of N in faba bean have not yet been characterized, although three transporters (GmUPS1-1, GmUPS1-2 and PvUPS1) in common bean involved in the transport of ureides are localized in the plasma-membranes of nodules (Collier, Tegeder 2012). Moreover, the xylem loading of these N compounds from the vasculature of nodules remains an unsolved issue (Collier, Tegeder 2012). In this study, nodule N was significantly higher in most plants at 30 and 40 DAS (Fig. 3A, B, C, E) with both rhizobial inocula, which might be a good indicator of hoarding sufficient N as reserve by the nodules before channeling to the aerial parts (Fig. 3). Regarding N content of nodules, shoot and stem, plants inoculated with MR2 showed a higher percentage of total N which indicates a relatively higher degree of effectiveness. In most cases in this study, nodules possessed more N than other parts (shoot or leaves) (Fig 3B, C, E), suggesting that nodules play a vital role in N_2 fixation. The amount of N in a shoot can be used to indicate that the fixed N_2 is channeled through the xylem to pods, which are an active N sink (Atkins, Smith 2007). Thus, the nodules (in this case) may act as a transient storage pool of the fixed N_2 and translocated N (Diaz-Leal et al. 2012). This N test was done using the Micro-Kjeldahl digestion method (PCARR 1980). However, the N measurement employing Kjeldahl digestion method only measures nitrogen bound to organic components (proteins, amino acids, nucleic acids) and ammonium in the sample, while other forms of nitrogen (nitrate and nitrite) cannot be measured through this method (Muñoz-Huerta et al. 2013). Thus, for a clear picture of actual N content, a modified standard Kjeldahl procedure employing the addition of salicylic acid prior to digestion followed by sodium thiosulfate for nitrate reduction is desired (Lee et al. 1996; Labconco 1998). Moreover, the lower N_2-fixation values represent the lower biomass production of plants and the nodules (compare Fig. 3 and Fig. 4) as suggested by Maskey et al. (2001). The use of these two rhizobial isolates resulted in significant differences in plant N, plant dry biomass, nodule N and nodule dry biomass (Baral et al. 2012) (Fig. 4).

Isolate MR2 was more efficient than KR1 in terms of influxing N under environmentally stressed conditions (MR2 was isolated from the high Himalayan region), as assessed by longer shoots and roots. This might suggest that the oxygen status rather than the nitrogen status is responsible for the induction of genes needed for nitrogen fixation (*nif* genes) and associated processes, the *fix* genes (Dixon, Kahn 2004). At a high oxygen concentration and the absence of leghaemoglobin, the nitrogenase in bacteroids might be damaged by oxygen diffusion, limiting the N_2-fixing ability and the carbohydrate supply (Sheehy et al. 1984). The distribution of N may be altered in the long-distance transport pathway and its symplasmic discontinuity (Tegeder 2014). Thus, such alterations during the transport of N to the shoot as observed in this study, rather than to the root, might also be possible (comparing Fig. 1 and Fig. 3). Ureide catabolism, although equally responsible for every organism, responds to manganese (Mn) fertility levels, especially in soybean leaves (Vadez, Sinclair 2000; Todd et al. 2006). Genotypic variations were found in soybean cultivars to which Mn was applied to soil and this Mn apparently had stimulatory effects on N_2-fixation in soybean plants grown under water deficit (Vadez et al. 2000; Sinclair et al. 2003). However, a detailed

molecular pathway regarding the catabolism of ureides and ureides transporters (other than GmUPS1-1, GmUPS1-2 and PvUPS1 in common bean; Collier, Tegeder 2012) has yet to be identified. Moreover, a partitioning in C and N distribution in the vegetative and reproductive parts of a legume occurs, rendering the developing seeds a potential sink for assimilates (Thorne 1985). Our results indicate that ureide-N concentration varies with the *Rhizobium* strain used and also depends on the plant part analyzed. Also, the soil mineral N is a potent inhibitor of N_2-fixation, hence, the assessment of soil N before and after plant harvest is highly recommended (Hartwig 1998; Herridge et al. 1998).

Conclusions and future research directives

This study presents proof of the symbiotic effectiveness of rhizobial strains isolated from two entirely different regions, with more efficient N_2 fixing ability of the rhizobial strain isolated from the higher elevation. The reliable and versatile technique used in our study may have great potential for field studies in legumes of different geographical terrains, and may be an important asset for a first-assessment of the N_2-fixing capability of numerous legume species grown under field conditions. The N_2-fixing ability of the micro-symbiont from this high altitude could also be further justified by employing other legumes restricted to the high Himalayas. Thus, the present investigation opens up an array of recent advances in studies on high altitude legumes and their microbial symbionts. Additional research is needed for the genetic discourse of rhizobia inhabiting higher altitudinal soils, to elicit the potential of the Himalayan rhizobial strains discovered in this study, and to document the organization of rhizobia in the rooting zones of legumes. Hence, to better understand the symbiosis between a plant and bacteria, molecular, cellular, biochemical and physiological methods need to be integrated to develop a holistic approach with predictive models of BNF systems in legumes.

Acknowledgements

The authors thank Mr. Sishir Panthi, Mr. Trilok Thakur and Mr. Deepak Mahat of the Central Department of Botany, Tribhuvan University for their supportive role during the research period. The authors declare no conflicts of interest.

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Received 1 August 2014; received in revised form 29 September 2014; accepted 24 November 2014