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Response of biomass accumulation and nodulation by *Vicia villosa* to soil conditions: Evidence from $\delta^{13}C$ and $\delta^{15}N$ isotopes

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Abstract *Vicia villosa* is an annual legume plant, and is mainly used for green manure by farmers in southwest China. Field growth experiments were performed on six plots. The concentrations of mineral nutrients and soluble sugar, and the changes of carbon and nitrogen isotopic composition within and among organs of *Vicia* were determined. Significant differences in legume growth were found in response to soil type and its moisture conditions. The *Vicia villosa* was relatively well adapted to growth in limestone soils than sandstone soils. The distribution of sugar concentrations and $\delta^{13}C$-differences between roots and leaves indicate that the translocation of sugars from leaves to roots may be restricted by soil drought. Therefore, there was an inhibition of Pi distribution from roots to leaves, resulting in over optimum threshold of N/P ratio. Those may originate from the feedback regulation in the legume, where soluble sugar could not be distributed from leaves to roots. The results of $\delta^{15}N$ values in tissues suggest that there should be different preferential use of nitrogen resource by legume during the formation of nodules: before nodule formation the legume preferentially utilizes inorganic nitrogen from soils, but afterwards the nitrogen should be mainly from N_2-fixation. Our results indicate that the lack of nodulation development, except for S2, should be ascribed to the factor controlling bi-direction nutrient transfer, which should be efficiency of establishment symbiosis with arbuscular mycorrhiza before nodulation formation. It is predicted that the species of *Vicia villosa* should be a legume associated with dual symbiosis with rhizobia and mycorrhiza.

Key words *Vicia villosa*; $\delta^{13}C$; $\delta^{15}N$; isotope; soil

1 Introduction

Vicia villosa is planted during short fallow periods to produce green manure (Piao Hechun et al., 2006) that provides available N for subsequent crops in rotation systems of Guizhou Province of southwest China. Generally, the rotation procedure is as follows: firstly, seeds of *Vicia villosa* are sown into the furrows between the rows of maize in autumn, and then green legumes grown from them are incorporated into the soils, where maize or potato will be planted next spring. Somado et al. (2006) suggested that preceding N-fixing legume of *Crotalaria micans* could meet the N demand of the subsequent food crop, provided that it is fertilized with phosphorus. It is expected that the fertility of *Vicia villosa*, as a green manure, should depend on the capacity of N-fixing. Plants are often exposed to drought stress, which decreases plant productivity. Water limitation reduces nutrient diffusion in soil and leads to a reduced root absorption capacity of crop plants. A precise understanding of factors limiting and regulating nodule formation and nitrogen fixation is important to increase legume productivity and nitrogen fixation (Sprent, 2008).

Symbiotic nitrogen fixation is highly sensitive to soil drought (Serraj et al., 1999). Legumes use feedback systems to regulate the uptake of nutrients that may be available in excess (Parsons et al., 1993). In the feedback systems, photo assimilates in leaves are loaded into the phloem and translocated, mainly as sugars and amino acids, to the roots where they are utilized for the biosynthesis of cellular constituents and the synthesis of storage compounds (Lalonde et al., 1999). Symbiotic nitrogen fixation consumes considerable energy, and thus requires large amounts of assimilates (Schulze, 2004). Photosynthate in the form of sucrose is the ultimate source of carbon, as energy, required for N_2 fixation (Gálvez et al., 2005). In addition, nodules require up to three times more P than the surrounding root tissue, indicating the high demand for P for N fixation. P deficiency can lead to a reduction in both nodulation and symbiotic N-fixation

(Drevon and Hartwig, 1997), and there is increasing evidence that inorganic P is a key regulator for carbon distribution *in vitro* systems (Fredeen et al., 1989). Accumulation or depletion of soluble sugar in legume tissues should result in an increase of N/P ratios over the optimum threshold (14<N/P<16) (Koerselman and Meuleman, 1996). The characteristic ratios of elements determine the basic stoichiometry for the biosphere, allowing us to predict its response to alterations of nutrient availability (Schlesinger, 2004). Thus, links among soluble sugar accumulation or starvation and recycling of N and P_i for regulating photosynthesis in legume under drought remain a contentious issue.

Since symbiotic nitrogen fixation requires large amounts of assimilates, it is important to examine controls on the accumulation and depletion of soluble sugar during the nodule development under different moisture conditions. The balance between photochemical reactions in photosynthesis and chemical and biochemical reactions in carbohydrate metabolism should change the isotopic signature of carbon in the carbohydrate pool of plants (Hobbie and Werner, 2004), and plant organs (Peuke et al., 2006). The shoot biomass is always ^{13}C-depleted relative to the root, which is a result of either fractionation processes or an increased proportion of PEPc fixed carbon in root tissues (Brandes et al., 2006). We expected that the differential distribution patterns of soluble sugar should induce differential changes of $\delta^{13}C$ among tissues in feedback system of legume.

Legumes assimilate inorganic nitrogen forms such as NO_3^- and NH_4^+ especially in the early stage of development (Mengel, 1994). Legume species differ in their ability to take up nitrate (NO_3^-) at the degree that soil NO_3^- impairs legume nodule formation and N_2 fixation (Tang et al., 1999). The inhibition of N_2 fixation by nitrate appears to result from the effect of nitrate on nodule development (Fan et al., 2002). Root-induced changes of rhizosphere pH, caused by processes such as differential uptake of anions and cations, root respiration or organic acid exudation, may strongly affect P uptake (Leidi and Rodrĺguez-Navarro, 2000). Hauter and Mengel (1988) suggested that this acidifying process might not be harmful on soils with high $CaCO_3$ concentrations, since the H^+ released into the rhizosphere by roots will be immediately neutralized by $CaCO_3$. It is apparent that legumes are more easily adapted to calcareous soils (Mengel, 1994), where nitrate is the main form of nitrogen (Darrah et al., 1986). Therefore, it is expected that nitrate should be the main inorganic nitrogen source taken up by legumes during early development stages.

The $\delta^{15}N$ values of plants relying solely on N_2 fixation by bacterial symbionts are similar to those of atmospheric N_2 (i.e., close to 0), whereas the $\delta^{15}N$ values in legumes with nitrogen derived from soils should have much wider range (Wanek and Arndt, 2002). Based on differences of ^{15}N abundance between plant-available soil N and the atmosphere N, the proportion of legume N derived from each source can be estimated by using nitrogen isotope composition. As the legume develops, plants switch from deriving their N from soil-borne nitrate to products provided by their bacterial symbionts (Rogers et al., 2006). Therefore, it is necessary to investigate the timing of N fixation during the growth period. In the root nodule, nitrogen fixation generates ammonium. Alternatively, nitrogen taken up from soils is assimilated only in roots (Marschner, 1995), so in either case organic nitrogen in the shoot and root is the product of a single assimilation event (Evans et al., 1996). Legumes might increase more nitrate reductive activity in their shoots than in roots in response to nitrate (Fan et al., 2002), resulting in higher $\delta^{15}N$ values in shoots than in roots. There are not different $\delta^{15}N$ values between root and shoot in legumes supplied only with low NH_4^+ concentration. It is unclear whether N acquisition would differ during the growing season, and there is no a unifying theory to explain related N nutrition of legumes.

To avoid interference by excessive nutrient uptake in fertilization experiments, we used natural soil moisture conditions for our plant growth experiments. The experimental design should result in the varying formation of nodules and biomass. The objective of this study was to investigate the interaction patterns among N, P and C (soluble sugar) in the feedback system of legumes under different moisture conditions, monitored using carbon and nitrogen isotope compositions.

2 Materials and methods

2.1 Sites and sampling

We carried out a field experiment to quantify the changes in concentrations of the relevant nutrients in a legume plant (*Vicia villosa*) grown on six plots with different soil properties (Table 1). All of the six experimental sites are located in the same area (25º15' N and 108º02' E) but are separated from each other over a distance of about 2 km. Among the six experimental plots, three plots (S1, S2, and S3) are underlain by sandstone, but the soil properties in S1 and S2 also are affected by nearby limestone. The other experimental plots are underlain by limestone (L4, L5, and L6). S2 and L4 have a relatively thick soil layer (higher than 1 m), while L5 and L6 are less than 0.5 m in thickness. The sandstone soil types (S1, S2 and S3) are yellowish-red soils (ultisols), and the soils developed on

limestone areas are known as limestone soils (alfisols). S2 and L4 were fallow for 2 years and others were virgin land before the experiments. Despite their thicker profile, S1 and S3 slope by about 15° and 20°, respectively. Every plot has an approximate area of 100 m^2. The growth experiments were performed from 1 April to 14 July: *Vicia villosa* seeds (500 g/100 m^2) were sown on 1 April, and the first sampling was carried out on 16 April, with subsequent samplings every other week. Over 100 sample pairs were collected during the early sampling, but this was reduced to 50 pairs for the last few samples. Legume samples were collected throughout the 100-m^2 plot. Soil samples were collected before experiment; six cores (5 cm in diameter, 0–10 cm in depth) were randomly taken from 100-m^2 plot, and combined to give one sample for each plot. Plant debris, stones and roots were removed, and the soil was sieved with 2-mm sieve. The samples for measuring soil moisture were collected at each sampling time. The fluctuated moisture (Table 1) was the average absolute values of differences between two consecutive soil moisture samples, which were calculated from the first 8 sampling data.

2.2 Laboratory analysis

Plant samples for laboratory analysis were dried at 60℃ for 48 h and ground with a mortar and pestle. Total C and N were determined with a CHNS autoanalyser (PE 2400-ll), and P by using nitric-perchloric acid digestion followed by the vanadomolybdate colorimetric method. For soluble sugar determination 0.25 g of air-dried material collected from six plots was extracted four times with distilled water at 75℃. After each extraction samples were filtered (Whatman No. 42 filter paper), and the filtrates were used to determine soluble sugar colorimetrically by using the anthrone reaction (Piao Hechun et al., 2000). The concentrations of C and nutrients were expressed relative to the original dry sample mass. We measured Olsen extractable phosphorus (P) in soil samples extracted with 0.5 M $NaHCO_3$ (adjusted to pH 8.5 with NaOH). The total P was determined after burning 1 g of soil at 550℃ for 2 h and then digesting with 6 M HCl. The Ghosh and Kashyap (2003) method was used to measure the concentrations of NH_4^+-N with 2 M KCl followed by the phenate method, and NO_3^--N with 0.5 M K_2SO_4 followed by the phenol disulphonic acid method. The $\delta^{13}C$ values of tissues and freeze-dried filtrate extracted by water were measured by combustion of 2 mg homogenized samples with CuO (1:50) at 850℃ with a vacuum-combustion system. CO_2 generated in the combustion tubes was separated by cryogenic distillation, collected in breakseals and analyzed with a mass spectrometer (MAT 252). The data are expressed relative to the international standard PDB [as $\delta^{13}C$ ‰ (parts per thousand)]. The $\delta^{15}N$ values of legume tissue and soils were determined with continuous flow-isotope ratio mass spectrometry (EA-IRMS). Each sample was analyzed in duplicate. The standard error of C and N concentrations was always less than 0.01 $g \cdot kg^{-1}$, and the standard deviation of the isotope composition did not exceed 0.1‰ (carbon), or 0.3‰ (nitrogen). Differences between mean values of soil and plant nutrient concentrations were tested for their significance by using linear regression and variance analysis at 5% probability.

3 Results

3.1 Legume growth and nodule formation in relation to soil properties

Differences among 6 experimental plots of the soils were studied (Table 1). The total concentration of soil P is higher in limestone soils than that in sandstone soils, while Olsen P concentrations are similar among the 6 plots (Table 1). The concentration of P is significantly correlated with maximum biomass for each plot (r^2=0.93, P<0.001, n=6). However, P concentrations in leaves are not correlated with the concentrations of soil total P and Olsen P. For instance, S2 has the lowest concentrations of total P and Olsen P, and the highest biomass among 6 plots (Table 1). The values of soil moisture (0–10 cm depth) measured

Table 1 Some soil properties

Plot	$^1C_{org}$ (%)	Total N ($mg \cdot g^{-1}$)	Total P ($mg \cdot g^{-1}$)	Olsen P ($\mu g \cdot g^{-1}$)	pH (H_2O)	Fluctuated moisture (%)	$^2NH_4^+$-N ($\mu g \cdot g^{-1}$)	$^3NO_3^-$-N ($\mu g \cdot g^{-1}$)	$\delta^{15}N$ (‰)
S1	1.92	1.74	0.20	13	5.8	7.3±3.1	37.3±6.0	10.1±2.6	5.1
S2	1.75	1.53	0.15	10	6.7	3.3±2.3	23.4±3.9	13.6±2.9	4.9
S3	3.49	2.54	0.25	19	4.5	6.0±4.2	51.1±7.4	18.5±4.1	4.4
L4	2.32	2.55	0.62	9	7.6	4.6±2.0	18.7±4.4	21.4±10.5	5.9
L5	7.57	8.15	0.65	17	7.4	15.1±9.8	25.9±10.9	31.8±10.7	6.3
L6	4.31	4.22	0.50	14	7.5	4.5±3.2	23.4±4.3	25.1±10.0	5.4

Note: $^1C_{org}$=organic carbon; $^2NH_4^+$-N data were calculated from the first 6 sampling times; $^3NO_3^-$-N values were measured only at two sampling times.

here do not exactly reflect the moisture status for plant growth. The sloping and thin soils of plots S1, S3 and L5 may have produced relatively high fluctuation of soil moisture (Table 1), where the plants grown suffered from drought more easily than in other experimental plots. The plant biomass data measured during growth are also different across the 6 plots. The accumulation of biomass in S2 is apparent, and the decline of legume growth in S1 and S3 is 2 and 4 weeks earlier than in S2, respectively. Important growth differences were found in response to soil type, and better growth was observed on limestone soils than on sandstone soils. The formation of legume nodules is normal in S2, fewer nodulations were found in L4 and nodule formation was not observed in the other experimental plots.

3.2 Different distribution patterns of soluble sugar of different tissue types

The decline of growth in S1 and S3 shifted to an earlier time for 2 and 4 weeks, respectively, and typical symptoms of senescence, i.e., leaf yellowing, occurred in S1 and S3, located sandstone areas. In young seedlings the soluble sugar concentrations are similar among the 6 experimental plots in the first sample set, where all of the seedlings are grown similarly. As the leaves are mature, the legume growth almost ceases in S1 and S3. Compared with S2, which produced the highest biomass and normal nodule formation, biomass in S1 and S3 was the lowest, nodules were absent, and the levels of soluble sugar were significantly accumulated in the stems (Fig. 1). The level of soluble sugar gradually increases from leaf to root in S2 and L4 where nodule formations occurred. On average, levels of soluble sugar were relatively low in the leaves of plants grown on limestone soils compared with sandstone soils (Fig. 1). The sugar concentration-differences between roots and stems were significantly correlated with maximum biomass for each plot (Fig. 2), indicating that the concentration of photosynthate affects the growth and nodulation of legumes.

3.3 Changes in carbon isotope composition in response to feedback of sugar

Value of $\delta^{13}C$ in the water-soluble fraction of the leaves where soluble sugar is accumulated will differ from that in tissues where there is soluble sugar starvation. Values of $\delta^{13}C$ in both whole leaves and the water-soluble fractions gradually decreased with growth (data not shown). The $\delta^{13}C$ values in water-soluble fractions extracted from whole leaves are more positive in S2, L4 and L6, and less positive in S1, S3 and L5 relative to the whole plant (Table 2). The latter three plots have higher fluctuations of soil moisture than the former. Paired *t*-tests showed that there were significant differences between L4 and S1 ($P<0.001$), L4 and S3 ($P<0.05$), L6 and S1 ($P<0.01$), and L6 and S3 ($P<0.01$), respectively. Although the relationship between soluble sugar concentrations and $\delta^{13}C$-differences of the whole plant and those of the water-soluble fraction (Table 2) is not statistically significant for all of the 6 plots ($r^2=0.59$, $P<0.10$, $n=6$), water-soluble $\delta^{13}C$ values are more positive in limestone than in sandstone soils (except for L5), where soluble sugar is accumulated in leaves and stems except for S2 (Fig. 1). Although there are no statistically significant differences in carbon isotopic composition in whole leaves (Table 2), there are significant differences among roots in 6 plots, where the values of $\delta^{13}C$ are higher in S1 and S3 than in the others. Therefore, $\delta^{13}C$-differences between roots and leaves are different among 6 plots, and are related significantly and negatively with the maximum biomass for each plot (Fig. 3a) indicating that the level of sugar translocated from leaf to root should affect the root $\delta^{13}C$ value.

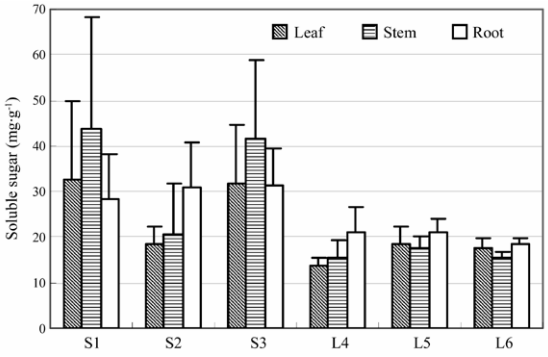

Fig. 1. Distributions of soluble sugar in different legume tissues in the 6 experimental plots.

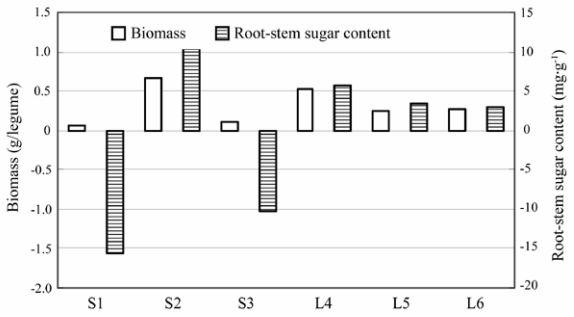

Fig. 2. The relationship of sugar concentration-differences between roots and stems with the maximum biomass for each (g·legume^{-1}) of the 6 experimental plots ($r^2=0.79$, $n=6$).

3.4 Variations in concentrations of N and P and N/P ratios in response to plant growth

On average, the highest concentrations of P and N are in leaves, while those in stems and roots are similar. While P concentrations in roots are not statistically different among the 6 plots, differences in P concentrations occurred between roots and leaves during growth. There is a significant relationship between leaf P concentration and the maximum biomass for each plot (Fig. 3b). Concentrations of N in leaves and roots were similar among the 6 plots except for S1, where leaf N concentration [4.2±1.3 $mg \cdot g^{-1}$(N)] is significantly lower than in other plots [higher than 4.7±0.9 $mg \cdot g^{-1}$(N)].

Table 2 The $\delta^{13}C$ values (‰, mean±SD) of whole leaves and water soluble fraction, and $\delta^{13}C$-differences between whole leaves and water soluble fractions

	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6
Whole leaf (A)	-29.62 (1.57)	-29.92 (0.75)	-29.61 (1.33)	-29.75 (1.31)	-29.77 (1.15)	-29.88 (0.80)
Water soluble (B)	-29.86 (1.69)	-29.69 (0.95)	-29.69 (1.51)	-29.48 (1.37)	-29.87 (1.26)	-29.47 (0.94)
Difference between A and B	0.24 (0.20)	-0.23 (0.27)	0.08 (0.34)	-0.28 (0.20)	0.10 (0.26)	-0.42 (0.35)

The average ratio of N/P is 15.0±1.3 by mass, and is relatively constant during growth in S2, but the ratios quickly increase with growth in S1 and S3. We took S2 with the highest biomass as a reference to assess the statistical differences in N/P ratios in leaves using paired *t*-tests: S3 with the lowest biomass has the most significant difference between S3 and S2 ($P<0.001$), followed by S1, L4 and L6 ($P<0.01$), and finally L5 ($P<0.05$), indicating that the ratio of N/P can be used to indicate the decline of legume biomass. On average, the N/P ratio in leaves is lower than in stems, and the ratio in stems is lower than in roots. The ratio of N/P is negatively and significantly related to maximum biomass for each plot ($r^2=0.81$, $P<0.05$, $n=6$) (Fig. 3c).

3.5 Foliar $\delta^{15}N$ indicator for nodule formation

The $\delta^{15}N$ value of *Vicia villosa* seeds is -0.3‰. Successful seedling establishment after germination requires efficient utilization of both endogenous storage reserves and resources from the environment. At the first sampling time of early seedlings, $\delta^{15}N$ values in roots and leaves in S2 were similar to those in S1, S3 and L4 (Fig. 4), and close to those of seeds. However, the significant increases in $\delta^{15}N$ values resulted from subsequent uptake of soil nitrogen. There was a peak value of $\delta^{15}N$ in roots and leaves in S2, after which the $\delta^{15}N$ value decreased, indicating that legumes switch from deriving their N from soil-borne nitrogen forms to products produced by their bacterial symbionts. There are no significant peak values of $\delta^{15}N$ for roots and leaves in other plots (Fig. 4).

Changes in $\delta^{15}N$ values before nodule formation differ from those after nodule formation in S2 (Fig. 5); the $\delta^{15}N$ value in stems is higher than that of the leaves before nodule formation, while the $\delta^{15}N$ value of stems is lower than that of the leaves after nodule formation (Fig. 5). Interestingly, the dynamics of $\delta^{15}N$ values in S1 (pH=5.8) differ from that in S3 (pH=4.5), despite both being located on sandstone areas, which may be attributed to different preferential nitrogen sources being taken up by the legumes. Average values of $\delta^{15}N$ calculated from the first 6 sampling times are 1.6‰ for roots; 1.5‰ for stems and 2.2‰ for leaves in S3, which are similar to those before nodule formation in S2; while the change in S1 is similar to the pattern after nodule formation in S2, with $\delta^{15}N$ values of 0.8‰ for roots; 0.2‰ for stems and 0.7‰ for leaves.

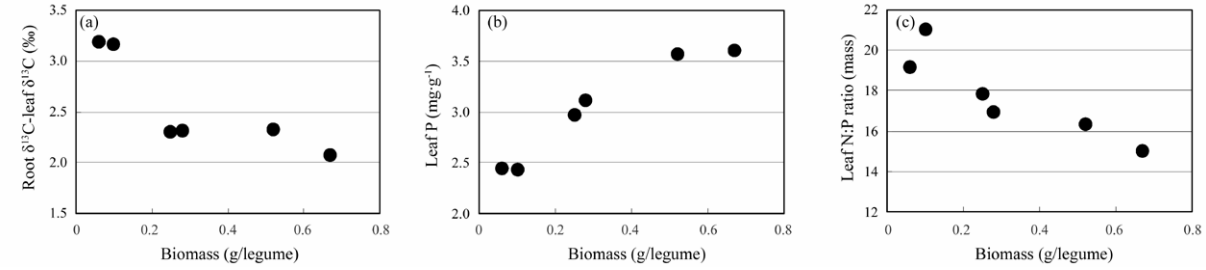

Fig. 3. The relationships of the maximum biomass for each plot ($g \cdot legume^{-1}$) with $\delta^{13}C$-differences between roots and leaves ($r^2=0.70$) (a); and leaf P concentrations ($r^2=0.93$) (b); and leaf N/P ratios ($r^2=0.81$) (c).

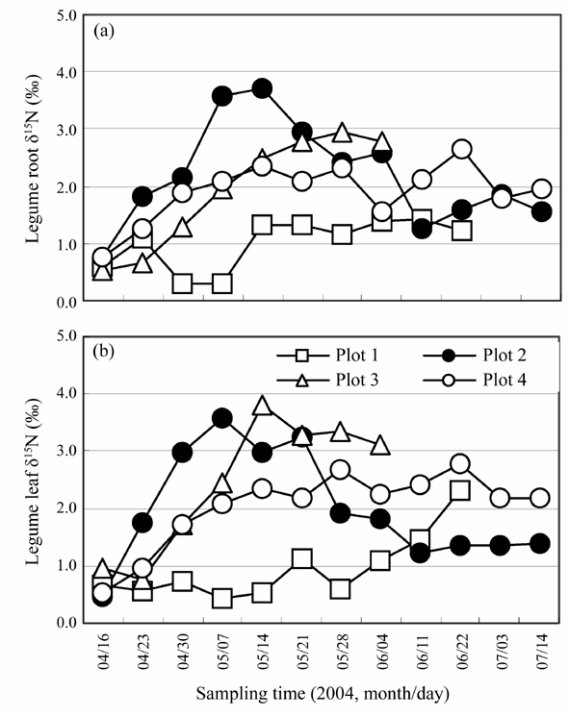

Fig. 4. Seasonal changes in $\delta^{15}N$ for roots and leaves in legume growth in the 4 experimental plots (including S1, S2 and S3, and L4).

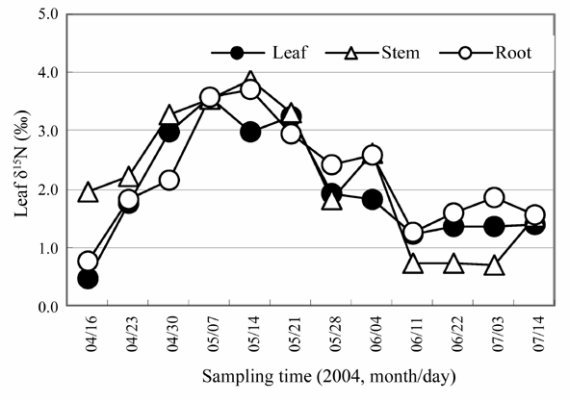

Fig. 5. Seasonal changes in $\delta^{15}N$ values in legume organs during growth in S2.

4 Discussion

4.1 Legume growth and nodule formation in relation to soil conditions

Our experimental results indicate that the legume *Vicia villosa* grows more easily in limestone soils than in acid soils. Acidication of the rhizosphere is a typical response of plants to P deficiency (Marschner, 1995), even when plants are supplied with NO_3^- (Leidi and Rodrĺguez-Navarro, 2000). The increase in rhizosphere pH would decrease P uptake, which has an optimum pH between 5 and 6 (Schachtman et al., 1998). Legumes release H^+ into rhizosphere (Kouas et al., 2008), which should be useful for P uptake in calcareous soils (Hauter and Mengel, 1988).

Among the numerous factors, soil drought would be responsible for the reduction of P distribution from roots to leaves, and the accumulation of water-soluble sugars by plant cells (Rhizopoulou et al., 1997). Sucrose-phosphate synthase (SPS) is a key enzyme in the regulation of sucrose synthesis (Geigenberger et al., 1999). SPS is important for acclimation to water stress, since sucrose synthesis is important for the ability of plants to cope with water deficits (Geigenberger et al., 1999). Therefore, soil drought should be responsible for the increased ratios of N/P, and the limitation of biomass during the growth legumes in experimental plots except for S2.

4.2 Legume growth and nodulation in relation to feedback system in legume

The variations of N/P ratios in tissues were mainly determined by using the significant differences between N and P in translocation patterns from roots to leaves. Although the concentrations of soluble sugar in leaves are significantly and negatively correlated with those of leaf total P, leaf P could account for part of the variance in concentrations of leaf sugar across all the experimental plots. This indicates that there must be another factor affecting the transport of P from roots to leaves. P_i must be delivered to each cell, and then to the various subcellular compartments through P_i transporters (Javot et al., 2007). Here the main question arises whether there is a factor controlling the transfer of P from root to leaf. From the results obtained here, it should be expected that there is an inhibition of P_i transport from roots to leaves, and it may originate from the feedback system in the legume, where soluble sugar could not be translocated from leaf to root.

The distribution of photosynthate among competing sinks is a major factor influencing whole plant productivity and crop yield, and is largely regulated by sink-located transport and transfer processes. Therefore, it is suggested that since source activity drives sink metabolism, photosynthetic control must be responsive to the needs of the whole plant and optimal use of C and mineral nutrient resources (Paul and Foyer, 2001). As the accumulation of soluble sugar is a function of the balance between photosynthesis and use by the growth processes of the plant, the inability to establish new sink capacity results in accumulation of assimilates in leaves and stems, as observed in S1 and S3. The low rates of sucrose synthesis during P_i deficiency due to low demand from sinks restrict the delivery of P_i to the chloroplast and

limit the rate of photosynthesis (Pieters et al., 2001). This may explain the decrease of P_i levels from root to leaf as sugar levels decrease from leaf to root. This result can also be estimated from the significant and positive relationship of sugar concentration-difference between root and stem with leaf P concentration (r^2=0.88).

Legume roots growing in soils become naturally infected with indigenous arbuscular mycorrhizaal (AM) fungi (Goss and de Varennes, 2002; Sprent and James, 2007). Arbuscular mycorrhizl fungi are primary belowground sink of host C resources in the dual symbiosis with root nodules. This allows AM establishment and the subsequent enhancement of P nutrition (Mortimer et al., 2008). That bi-dirctional nutrient transfer should benefite nodular formation and host development (Mortimer et al., 2008). When the dual symbiosis is established, it results in improved nodulation in soybean and greater biological nitrogen fixation. However, the effects on nodulation are detected more than 14 days after plant emergence (Autunes et al., 2006; Mortimer et al., 2008). In our case, the visible nodule formation in S2 soile was after 30 days. As the leaves matured, the legume growth almost ceased in S1 and S3 soils, which originated from herb and C_3 grassland. It is well known that many non-mycorrhizal plants are herbs occurring in disturbed habitats, resulting in the lack of mycorrhizal fungus inoculum in some habitats, (Brundrett, 2009). A key feature of mycorrhizal colonization is bi-directional nutrient transferring between plants and fungi (Piao Hechun and Liu Congqiang, 2011). One mechanism of overcoming P limitation is the formation of mycorrhizae, a symbiotic relationship between the legume and arbuscular mycorrhizal fungus. Therefore, it is possible that this should be ascribed to lack of establishment of host plant with AM fungi, especially S1 and S3 soils, where bi-direction nutrient transfer was signicantly impeded. Unforturnatly we did not measure the colonization level in legume growing stage. Further work is needed to identify colonization level with AM, where more intensive sampling is required to resolve the mycorrhizal status of *Vicia villosa.*

4.3 $\delta^{13}C$ indicator for carbon distribution pattern

Our experimental results suggest that when substrate utilization exceeds assimilate, the $\delta^{13}C$ in the water-soluble fraction will increase relative to the whole plant. Therefore, the limitation of legume growth may have been caused by limited assimilate in L4 and L6, and by limitation of substrate utilization in S1, S3 and L5, indicating that soil drought should affect the balance between substrate utilization and assimilate during legume growth. The concentration of leaf soluble sugar in L5 is lower than in S2, but $\delta^{13}C$-differences between whole leaves and water-soluble fractions in L5 (Table 2) are close to those in S1 and S3. Limited growth may have caused the accumulation of soluble sugar in L5. By contrast, soluble sugar is not accumulated in L4 and L6 (Fig 1), where the substrate utilization exceeds assimilate. The carbon isotope composition in the water-soluble fraction in legumes grown on L4 and L6 located in limestone areas is less negative than that on S1 and S3 located in sandstone areas. Production, rather than the utilization of photosynthates may have limited growth on L4 and L6 located in limestone areas (except L5). As indicated by Pieters et al. (2001), the relative proportion of inorganic P (P_i) associated with carbon distribution may be an important factor for determining the balance between assimilate and utilization of substrate.

Previous observations have generally shown that roots are enriched in ^{13}C by 1‰–2‰ relative to leaves. It has been proposed that these patterns may be attributed to isotopic differences between the dominant biochemicals within roots and leaves. Storage carbohydrates become relatively ^{13}C enriched during synthesis and/or transport from leaves to roots (Savidge and Blair, 2004). Terwilliger et al. (2001) suggested that the decrease of $\delta^{13}C$ values during growth could be explained by a change in the relative proportion, the activity of phosphoenolpyruvate carboxylase (PEPc) and Rubisco in leaves. The ^{13}C enrichment of organic matter in roots is a result of either fractionation processes or an increased proportion of PEPc fixed carbon in sink tissues (Brandes et al., 2006). Therefore, the significant different $\delta^{13}C$ values between roots and leaves should have resulted from the proportions of phosphoenolpyruvate between leaves and roots in the 6 plots. Furthermore, the proportions of photoassimilative distributions in roots could not have exceeded those of PEPc fixed carbon in S1 and S3, resulting in higher $\delta^{13}C$-differences between roots and leaves than in other plots.

4.4 N strategy in legumes

It is quite difficult to assess the contributions of different soil nitrogen forms to legume tissue $\delta^{15}N$ variability. Additional independent non-isotopic data are needed for interpretation of $\delta^{15}N$ results (Högberg, 1997). The pattern of $\delta^{15}N$ in S1 is similar to that in S2 after nodulation when ammonium is generated by nodules, suggesting that legume grown on S1 may take up more ammonium than nitrate. However, it is not clear why fractionations occur in S2 after nodulation and in S1, where the $\delta^{15}N$ values in stems are lower than in roots.

In calcareous soils, NH_4^+ is deprotonated which

may result in NH_3 volatilization to the atmosphere, and NO_3^- is almost the sole nitrogen form (Darrah et al., 1986) and taken up by plant roots. The soil pH below 5 may result in a lack of nitrification (Krull and Skjemstad, 2003). Marschner et al. (1991) pointed out that in a soil with high cation exchange capacity, the use of NO_3^- should be further favoured because of the relative immobility of NH_4^+. It is already known that some species adapted to calcareous soils prefer nitrate-nitrogen, while some species adapted to acidic soils prefer ammonium-nitrogen (Gigon and Rorison, 1972). Therefore, it is expected that *Vicia villosa* in calcareous soils should preferentially uptake nitrate before nodulation.

If inadequate N_2 fixation was the factor that limited legume growth on acid soils, plants would show symptoms of nitrogen deficiency (Munns et al., 1981), such as the yellow discoloration of leaves that occurred in S1. The soil pH of S1 is 5.8, which should be favorable for legumes. However, in S1 located on the upper hillside, nitrate is easily lost in run off. Therefore, NO_3^- concentration is the lowest among the 6 plots (Table 1), and leaf N concentrations are significantly lower than those in other plots. These results may suggest that the main nitrogen form taken up by the legume grown in S1 is NH_4^+, which might be the non- preferential nitrogen form for legume grown. Yellow discoloration of leaves also occurs in S3. Variations in $\delta^{15}N$ values in S3 tissues are similar to those in S2 and L4 during the early growth stages, indicating that the nitrogen form taken up by legume growth in S3 should be similar to that in S2 and L4 before nodulation. The soil pH is 4.5 in S3, which is located low down on the hillside, and native nitrate is absent. However, there is mobile nitrate from the upper hillside that provided a relatively high nitrate concentration (Table 1) for legume growth.

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