Effects of liming and *Sinorhizobium* inoculation on growth, nodulation and nutrient concentrations of lucerne in acid soil

YANJUN GUO[1], NI YU[2], LING YUAN[3] AND JIANGUO HUANG[3]
[1] *College of Animal Science and Technology, Southwest University, Chongqing, P.R.China*
[2] *College of Agronomy and Biotechnology, Southwest University, Chongqing, P.R.China*
[3] *College of Resources and Environment, Southwest University, Chongqing, P.R.China*

Abstract

A greenhouse pot experiment examined the effects of liming and inoculation with *Sinorhizobium* on nodule formation, growth and nutrient concentrations in the shoots of 2 lucerne cultivars, which performed differently when grown in acid soil, in a factorial design with 6 replications. Sanditi (good performer) and Gannong-3 (poor performer) were selected from 6 cultivars in a preliminary experiment and grown in an acid purplish soil (pH 5.45; in water) with and without lime addition and with and without inoculation. *Sinorhizobium meliloti* strain 1.163, an acid-tolerant isolate from a chestnut soil in the north of China, was used as the inoculant. Liming improved growth of Gannong-3 ($P>0.05$) but not of Sanditi, while inoculation increased growth of both cultivars ($P<0.05$). Most of the growth responses occurred in the roots. Very few nodules were formed on the roots of either cultivar without addition of *Sinorhizobium*, while both cultivars nodulated well in the presence of inoculum. Liming increased ($P<0.05$) the number of root nodules/plant but reduced both mean nodule weight and total weight of nodules/plant ($P<0.05$). Liming significantly increased N concentration in shoots of Sanditi and P concentration in Gannong-3. These results indicated that inoculation with acid-tolerant *Sinorhizobium* would boost production of both of the tested cultivars when grown in acid soils. Further testing is required to determine the benefit of liming for Gannong-3.

Correspondence: Jianguo Huang, College of Resources and Environment, Southwest University, Tiansheng Road 1, Beibei, Chongqing 400715, P.R.China. E-mail: jianguoh@hotmail.com

Introduction

Throughout the world, lucerne (*Medicago sativa*) is widely grown in soils with pH 6.5–7.5. Lucerne is more sensitive to acid soils than most other legumes (Baligar *et al.* 1988; María *et al.* 1999), often growing poorly in acid soils because of low pH, Al (aluminium) and Mn (manganese) toxicities and nutrient deficiencies [mainly Ca (calcium), Mg (magnesium), P and Mo (molybdenum)] (Foy 1988). Most *rhizobia* are sensitive to low pH, Al and Mn, and the general infertility of acid soils could inhibit their survival and propagation, plus nodule initiation and development (Munns *et al*. 1981).

Several strategies have been developed to grow lucerne in acid soils, including selection of cultivars tolerant of acidic conditions, inoculation with acid-tolerant *Sinorhizobia* and the application of lime (Bakker *et al*. 1999; Brauer *et al*. 2002; Soto *et al*. 2004). The most common option is liming, which can quickly increase soil pH, alleviate Al toxicity, correct nutrient deficiencies (Lai and Mathur 1989; Littke and Zabowski 2007) and thus promote growth and nutrient uptake by plants (Belkacem and Nys 1997). However, application of lime at inappropriate times or at excessive levels could cause nutrient imbalance in soils and plants, resulting in growth inhibition and yield reduction (Walker 2002).

Legumes differ greatly in growth and nodulation in response to low pH (Tang and Thomson 1996). The selection of acid-tolerant cultivars is a fundamental approach for successful cultivation of lucerne in acid soils (Grewal and Williams 2003). Growth and development of legumes are closely related to nitrogen bio-fixation (Pietsch *et al*. 2007). Nodulation is essential for successful lucerne production and the presence of *Sinorhizobium meliloti*, the organism involved in lucerne nodulation, is a prerequisite to nodule formation in acid soils. However, most strains are quite sensitive to acidic conditions and Al, and are hard to propagate to ensure survival in acid soils. Only a

few are both acid- and Al-tolerant (Rinaudi *et al*. 2006).

In general, information on the relationships between lucerne cultivars with variable acid tolerance and *S. meliloti*, particularly acid-tolerant strains, is quite limited. It is also necessary to understand the influence of liming on growth and nutrient absorption by acid-tolerant cultivars of lucerne, and on nodule formation and N_2 fixation by acid-tolerant *S. meliloti*. The objectives of the present experiment were: (i) to assess the growth of lucerne cultivars with different levels of acid tolerance in response to *Sinorhizobium* inoculation and liming; (ii) to assess the influence of liming on nodule formation and development; and (iii) to determine any interactions among lucerne cultivar, *Sinorhizobium* inoculation and liming in relation to nutrient concentrations, particularly N.

Materials and methods

Experimental soil

The cultivated horizon (0–30 cm) of an acid purplish soil (typical Udorthent), which is derived from sandy sedimentary rocks and is widespread in Sichuan Basin, China, was collected, air-dried, ground to pass a sieve with 2 mm openings and then sterilised at 121°C for 30 min. The soil had sandy loam texture, low CEC, low organic matter and pH 5.15 and contained 76.8 mg/kg of 1N NaOH-hydrolysed N, 32.6 mg/kg of Olson P, 114.8 mg/kg of 1N ammonium acetate-extractable K, 14.4 mg/kg of 1N sodium acetate-extractable Ca and 1.24 mg/kg of KCl-extractable Al.

Selection of lucerne cultivars

A field experiment was conducted to select lucerne cultivars with differing production (possibly differing adaptation) on acid soils for use in further pot experiments. The 6 cultivars, Gannong-1, Gannong-2, Gannong-3, Sanditi, Eureka and Defi, which were widely cultivated in the north of China, were grown in the experimental soil for 4 months and shoot weight was recorded at harvest. Prior to sowing, the area was fertilised with 75 kg/ha N (supplied as urea) and 65 kg/ha P (supplied as superphosphate). Standard field management operations such as irrigation and pest and weed control were conducted during the growth period. The cultivar which grew best (Sanditi) as well as the poorest (Gannong-3), considered to be acid-tolerant and acid-sensitive cultivars, respectively, were selected for use in this study.

Experimental design

The experiment was a factorial arrangement of 2 liming treatments (no lime and plus lime) and 2 inoculation treatments (uninoculated and inoculated with *Sinorhizobium*) with 6 replications. In order to obtain 2 levels of soil pH, lime was added to 100 g of soil at about 75% field capacity (w/w) and the soil was incubated in the dark at 25°C for 15 d. The addition of 600 mg lime into 100 g soil raised soil pH from 5.15 to 6.54. Therefore, for liming treatments 12 g lime was mixed with 2 kg of prepared soil, which was placed in each experimental pot (diameter × depth = 12 cm × 20 cm) for growing lucerne seedlings; the blank controls were set up in the same way with no lime added.

Seeds of the 2 lucerne cultivars, Gannong-3 and Sanditi, were surface-sterilised with 1% CaClO solution for 5 min and grown in quartz sands with long day period (14 h light and 10 h dark, light intensity 15 000 lux provided by fluorescent tube) at 25°C for 3 weeks. Healthy seedlings were selected and half had their roots immersed in suspensions of *Sinorhizobium meliloti* 1.163 (*Sm 1.163*) containing 10^8–10^9 cells/ml for about 15 seconds. *Sm 1.163*, supplied by Institute of Microbiology, Chinese Academy Sciences, is acid-tolerant and is widely used in lucerne cultivation in China. Thereafter, 3 seedlings were transplanted into each experimental pot and grown for 3 months in a greenhouse. During the growth period, the seedlings were watered weekly to keep the soil moisture at about 70–75% of field capacity. Each of the 4 treatments (Control, lime added with no inoculation, no lime added with inoculation and lime added with inoculation) was replicated 6 times.

Measurements

At harvest, plants were removed, and below-ground parts were thoroughly washed with a fine water spray and separated into nodules and roots. The nodules with violet red colour were counted; minute nodules with white colour were

not included in the count. Shoot and root samples were oven-dried at 80°C for 48h, weighed individually, ground to pass through a sieve with 2 mm openings and analysed for N by the Kjeldahl procedure, P by molybdenum blue methods followed by colorimetry, K by flame photometry and Ca, Mg and Al by atomic absorption spectrometer after digestion in H_2SO_4–H_2O_2 solution.

Soil was collected from the pots at harvest and routinely prepared for the assessment of exchangeable Ca (extracted by 1 N CH_3COONa) and Al (extracted by 1 N KCl) by atomic absorption spectroscopy. Soil pH was determined by a pH meter (soil:water = 1:1).

Data treatment

All data were subjected to analysis of variance using SPSS model (ANOVA, Duncan's multiple range test and Pearson's correlation coefficient). Differences obtained at levels of P = 0.05 were considered significant.

Results

Soil pH, exchangeable Ca and Al in soil

Soil pH and the size of exchangeable Ca and Al pools at harvest (Table 1) show that liming increased soil pH from 5.45 to 6.54, and exchangeable Ca from 12.42 µg/g to 29.21 µg/g soil (P<0.05). In contrast, exchangeable Al decreased from 1.16 µg/g to 0.31 µg/g soil following lime application (P<0.05).

Table 1. Soil pH and exchangeable Ca and Al in soil.

Treatments	pH	Exchangeable	
		Ca	Al
		(µg/g soil)	
Limed	6.54a[1]	29.21a	0.31b
Unlimed	5.45b	12.42b	1.16a

[1] In each column, means followed by different letters are significantly different at P = 0.05.

Growth of lucerne

Both cultivars produced similar growth responses to liming and *Sinorhizobium* inoculation. Plant biomass increased significantly (P<0.05) as a result of inoculation with most of the response being in root growth. Liming increased growth of Gannong-3 (P>0.05), with the only significant (P<0.05) response being in root growth in the presence of inoculation.

Table 2. Effects of liming and *S. meliloti* inoculation on the growth of 2 lucerne cultivars.

Treatments	Root weight	Shoot weight	Total yield
		(g/pot DM)	
Gannong-3			
No inoculation			
–lime	1.50c[1]	2.79b	4.29c
+lime	1.91c	2.81b	4.72bc
Inoculation			
–lime	2.47b	3.31a	5.78ab
+lime	3.06a	3.43a	6.49a
Sanditi			
No inoculation			
–lime	1.70c	2.98b	4.68bc
+lime	1.57c	3.06b	4.63bc
Inoculation			
–lime	2.66ab	3.53a	6.19a
+lime	2.82ab	3.25a	6.07a
Significant differences:			
Cultivar	NS	NS	NS
Inoculation	**	*	**
Lime	*	NS	NS
Lime × inoculation	NS	NS	NS

[1] In each column, means followed by different letters are significantly different at P = 0.05.

Number and size of nodules

Roots of uninoculated lucerne plants produced very few nodules (data not presented), while inoculated plants produced 24–39 nodules/plant depending on cultivar and liming treatment (Table 3). Both cultivars showed significant (P<0.05) increases in nodule number per plant as a result of liming, with a mean increase of 10.5 nodules per plant. While Sanditi produced more nodules than Gannong-3, mean nodule weight and total nodule weight/plant favoured Gannong-3. Number of nodules per plant was increased by liming, but mean nodule weight and total nodule weight/plant were lower on limed treatments.

Concentrations of mineral elements in shoots

As shown in Table 4, both liming and *Sinorhizobium* inoculation increased N concentrations in

shoots for both cultivars but differences were significant only for Sanditi. Al concentrations were decreased by both liming and inoculation. There was a significant interaction between liming and *Sinorhizobium* inoculation in this response with much greater reduction as a result of liming in the presence of inoculation. Liming increased P concentrations in Gannong-3 ($P < 0.05$).

Table 3. Effect of liming on nodulation of 2 lucerne cultivars inoculated with *S. meliloti.*

Treatments	Nodules/ plant	Mean nodule weight	Total nodule weight
		(mg/nodule FW[2])	(mg/plant FW)
Gannong-3			
–lime	23.9c[1]	23.0a	547.8a
+lime	34.7a	11.1b	381.8b
Sanditi			
–lime	28.4b	14.0b	411.9b
+lime	38.6a	6.1c	230.6c
Significant differences:			
Cultivar	**	**	**
Lime	**	**	**
Lime × cultivar	**	**	**

[1] In each column, means followed by different letters are significantly different at P = 0.05.
[2] FW = fresh weight.

Discussion

The results of this study have done little to confirm our hypothesis that Sanditi was an acid-tolerant cultivar while Gannong-3 was acid-sensitive. Neither of the lucerne cultivars responded significantly in growth in response to liming, even though liming markedly increased pH and lowered exchangeable Al levels. While Gannong-3 appeared to show a growth response, the differences were not significant ($P>0.05$). However, this tendency to respond to liming does add some weight to the suggestion that Gannong-3 is more sensitive to acidic soil conditions than Sanditi. Xu *et al.* (2006) reported that pasture legumes acidified soils to some extent and differed in their abilities to efflux organic acids. The acid-tolerant species were usually tolerant of high Al levels in acid soils, since they were able to efflux organic acids into culture mediums to form chelates with Al, thereby alleviating toxicity (Foy and Lee 1987). Taking into account the close relationship between pH and Al in soils, it seems reasonable to suggest that Gannong-3 might be more susceptible than Sanditi to high Al levels in acid soils. One of the reasons for the apparent growth improvement of Gannong-3 following lime addition could be the reduction in exchangeable Al levels in the treated soil. The reduction in

Table 4. Concentrations of N, P, K, Ca, Mg and Al in the shoots of 2 lucerne cultivars in response to liming and inoculation with *S. meliloti*.

Treatments	N	P	K	Ca	Mg	Al
			(g/kg DM)			
Gannong-3						
No inoculation						
–lime	30.52c[1]	5.21b	31.52b	13.12a	1.78a	1.55a
+lime	32.93bc	8.44a	31.73b	13.13a	1.67a	1.13b
Inoculation						
–lime	31.94bc	5.56b	33.64ab	13.64a	1.56a	0.76c
+lime	34.32b	7.37a	32.71b	13.86a	1.63a	0.24d
Sanditi						
No inoculation						
–lime	25.73d	7.12ab	38.45a	14.17a	1.66a	1.65a
+lime	33.05bc	7.36ab	38.72a	13.95a	1.84a	1.22b
Inoculation						
–lime	34.07bc	7.15ab	35.27a	14.62a	1.73a	0.87c
+lime	38.28a	7.41ab	33.44ab	14.64a	1.76a	0.34d
Significant differences:						
Cultivar	**	**	**	NS	NS	NS
Inoculation	**	NS	NS	NS	NS	**
Lime	**	*	NS	NS	NS	**
Lime × inoculation	**	NS	NS	NS	NS	**

[1] In each column, means followed by different letters are significantly different at P = 0.05.

Al levels in shoots of plants grown on the limed treatments would support this hypothesis.

The major and consistent outcome from this study was the marked growth responses following inoculation in both cultivars. Interestingly, the growth responses following inoculation were largely confined to the roots, with 66% (Gannong-3) and 75% (Sanditi) of the overall growth response occurring in the roots. In contrast, liming produced non-significant growth responses in Gannong-3 (about 25%) and no response in Sanditi. Following inoculation with acid-tolerant *Sinorhizobium,* plants changed from producing virtually no nodules to producing significant numbers (24–39 nodules per plant) on their roots. The nitrogen fixed by these nodules would have contributed to the significant plant growth responses in inoculated plants. However, the interaction between liming and nodule formation is intriguing. In both cultivars, liming increased the number of nodules produced but these were much smaller than those in unlimed plots, resulting in less total weight of nodules in limed plots. The larger nodules in unlimed plots might reflect an inhibition of nodulation, with fewer opportunities to nodulate resulting in larger nodules as the system compensated. Edmeades *et al.* (1981) found that liming improves soil nitrogen mineralisation, and the resulting higher available nitrogen for plants obviates the need for early nodulation, limiting nodule development, which might partly explain why liming decreased total nodule weight. The differences in nodulation of the two cultivars also are of interest. While the preliminary study suggested that Gannong-3 was acid-sensitive, it produced a much greater weight of nodules than Sanditi in both the presence and the absence of lime. However, maximum total DM yield in both cultivars was similar as was the total amount of N contained in the shoots.

The increase in N concentrations in shoots of lucerne following liming contrasts with the generally smaller increases from inoculation and reductions in Al concentrations. The reduction in Al concentrations in shoots would be a function of the lower exchangeable Al, available for plants, in the limed soil. However, the increase in N levels in shoots following liming conflicts with the greater weight of nodules formed on roots of unlimed plants. This suggests that there is no significant relationship between total nodule weight per plant and nitrogen-fixing ability and N levels in plant shoots. Tajima *et al.* (2007) reported that nitrogen-fixing ability of root nodules was closely related with their size, with medium-sized nodules fixing most N. It appears that the nitrogen increase in shoots of limed plants is a function of both nitrogen-fixing ability of the plants and soil nitrogen availability, especially during the early growing stages (Edmeades *et al.* 1981).

Liming can either increase (Holford and Crocker 1994) or decrease (Mendoza *et al*. 1995) available P in soils. The lime effect on P absorption by plants is highly dependent on the types of soils and plants (Lemare and Leon 1989), with liming producing variable effects on available P in soils and P uptake by plants (Prasad 1992). Our results suggest that Gannong-3 benefited from liming by increasing P uptake from the soil while Sanditi was unaffected. This again suggests that Sanditi is more tolerant of acidic soil conditions than Gannong-3.

From the present study, we conclude that acid-tolerant *Sinorhizobium* should be applied to both lucerne cultivars, Sanditi and Gannong-3, when grown on acid soils to obtain maximum growth. However, responses of these lucerne cultivars to liming during the early growing stages are not clear. Further long-term experiments are needed to evaluate the responses to liming, particularly of Gannong-3, in terms of growth and nodule production.

Acknowledgements

Financial support was provided by the National Basic Research Plan of China (2007CB108901) and the Doctor's Initial Fund of Southwest University in China.

References

Bakker, M.R., Kerisit, R., Verbist, K. and Nys, C. (1999) Effects of liming on rhizosphere chemistry and growth of fine roots and of shoots of sessile oak (*Quercus petraea*). *Plant and Soil*, **217**, 243–255.

Baligar, V.C., Wright, R.J., Fageria, N.K. and Foy, C.D. (1988) Differential responses of forage legumes to aluminum. *Journal of Plant Nutrition*, **11**, 549–562.

Belkacem, S. and Nys, C. (1997) Effects of liming and gypsum regimes on chemical characteristics of an acid forest soil and its leachates. *Annual Science Forest*. **54**, 169–180.

Brauer, D.K., Ritchey, K.D. and Belesky, D.P. (2002) Effects of lime and Ca on root growth and nodulation of clovers. *Journal of Plant Nutrition*, **5**, 1640–1646.

Edmeades, D.C., Judd, M. and Sarathchandra, S.U. (1981) The effect of lime on nitrogen mineralization as measured by grass growth. *Plant and Soil*, **60**,177–186.

Foy, C.D. (1988) Plant adaptation to acid, aluminum toxic soils. *Communications in Soil Science and Plant Analysis,* **19**, 959–987.

Foy, C.D. and Lee, R.H. (1987) Different aluminum tolerance of two barley cultures related to organic acids in their roots. *Journal of Plant Nutrition,* **10**, 1089–1101.

Grewal, H.S. and Williams, R. (2003) Liming and cultivars affect root growth, nodulation, leaf to stem ratio, herbage yield and elemental composition of alfalfa on an acid soil. *Journal of Plant Nutrition*, **26**, 1683–1696.

Holford, I.C.R. and Crocker, G.J. (1994) Long-term effects of lime on pasture yields and response to phosphate fertilizers on eight acidic soils. *Australian Journal of Agricultural Research*, **45**, 1045–1062.

Lai, S. and Mathur B.S. (1989) Effect of long-term fertilization, manuring and liming of an Alfisoil on maize, wheat and soil properties: I. maize and wheat. *Journal of Indian Society of Soil Science*, **37**, 717–724.

Lemare, P.H. and Leon, L.A. (1989) The effects of lime on adsorption and desorption of phosphate in five Colombia soils. *Journal of Soil Science*, **40**, 59–69.

Littke, K.M. and Zabowski, D. (2007) Influence of calcium fertilization on Douglas-fir foliar nutrition, soil nutrient availability, and sinuosity in coastal Washington. *Forest Ecology and Management*, **247**,140–148.

María, F., Balagué, L.J., Sowinski, S.C., Wegener, C., Segundo, E., Abarca, F.M., Toro, N., Niehaus, K., Pühler, A., Aguilar, O.M., Martínez-Drets, G. and Lagares, A. (1999) Isolation and characterization of alfalfa — nodulating rhizobia present in acidic soils of Central Argentina and Uruguay. *Applied and Environmental Microbiology*, **4**, 1420–1427.

Mendoza, R.E., Collantes, M.B., Anchorena, J. and Cainzos, S. (1995) Effects of liming and fertilization on forage yield and vegetation in dry heath soils from Tierra del Fuego. *Journal of Plant Nutrition*, **18**, 401–420.

Munns, D.N., Hohenberg, J.S., Righetti, T.L. and Lauter, D.T. (1981) Soil acidity tolerance of symbiotic and nitrogen fertilized soybeans. *Journal of Agronomy*, **73**, 407–410.

Pietsch, G., Friedel, J.K. and Freyer, B. (2007) Lucerne management in an organic farming system under dry site conditions. *Field Crops Research*, **102**, 104–118.

Prasad, R. (1992) Effect of liming on yield of soybean and nutrient availability in acid soil. *Journal of Indian Society of Soil Science*, **40**, 377–379.

Rinaudi, L., Fujishige, N.A., Hirsch, A.M., Banchio, E., Zorreguieta, A. and Giordano, W. (2006) Effects of nutritional and environmental conditions on *Sinorhizobium meliloti biofilm* formation. *Research in Microbiology*, **15**, 867–875.

Soto, M.J., Dillewijn, P., Martínez-Abarca, F., Jiménez-Zurdo, J.I. and Toro, N. (2004) Attachment to plant roots and *nod* gene expression are not affected by pH or calcium in the acid-tolerant alfalfa-nodulating bacteria *Rhizobium* sp. LPU83. *FEMS Microbiology Ecology*, **48**, 71–77.

Tajima, R., Lee, O.N., Abe, J., Lux, A. and Morita, Shigenori (2007) Nitrogen-fixing activity of root nodules in relation to their size in peanut (*Arachis hypogaea* L.). *Plant Production Science*, **10**, 423–429.

Tang, C. and Thomson, B.D. (1996) Effects of solution pH and bicarbonate on the growth and nodulation of a range of grain legumes. *Plant and Soil,* **186**, 321–330.

Walker, R.F. (2002) Growth and nutritional responses of bareroot Jeffrey pine on a Sierra Nevada surface mine to minisite applications of fertilizer and lime. *New Forests*, **24**, 225–238.

Xu, J.M., Tang, C. and Chen, Z.L. (2006) The role of plant residues in pH change of acid soils differing in initial pH. *Soil Biology and Biochemistry*, **38**, 709–719.

(Received for publication November 22, 2007; accepted March 7, 2008)