Effects of rhizobium, arbuscular mycorrhiza and lime on nodulation, growth and nutrient uptake of lucerne in acid purplish soil in China

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

Abstract

The effects of inoculation with rhizobium (*Sinorhizobium meliloti*) and arbuscular mycorrhizal fungus (*Glomus intraradices*) and lime application on growth, nodulation and nutrient uptake of lucerne on an acid purplish soil (Udorthent) were investigated in a greenhouse pot experiment. Seedling roots were inoculated with liquid *S. meliloti* inoculant at transplanting, and arbuscular mycorrhizal inoculum was applied to soil in pots before sowing. In the limed treatment, lime was added to adjust soil pH from 5.45 to 6.54. Plants were harvested 2 months after inoculation. Both application of lime and inoculation with mycorrhiza increased nodule numbers and total nodule weight ($P<0.05$) over that with rhizobium alone. This was reflected in increased top growth and top + root growth in plants receiving both lime and mycorrhiza ($P<0.05$). Inoculation with mycorrhiza or rhizobium or lime application tended to improve concentrations of phosphorus and nitrogen in top growth compared with uninoculated plants. Inoculating lucerne with rhizobium and mycorrhiza on these acid soils should improve plant growth but this needs to be verified by field trials.

Introduction

Soil acidity is a significant problem facing agricultural producers in tropical and subtropical regions and limits legume productivity (Bordeleau and Prevost 1994). Soil acidity constrains symbiotic N_2 fixation (Munns 1986), limiting *Sinorhizobium* survival and persistence in soils and reducing nodulation (Graham *et al.* 1982), and causes nutrient imbalance (Foy 1984). In most situations, liming could raise soil pH into the range of 5.7 – 6.5. However, while the beneficial use of lime is well recognised in crop production in southern China (Meng *et al*. 2004), excessive applications of lime or inappropriate timing of application may cause imbalances of soil calcium, potassium and magnesium, resulting in low yields (Walker 2002).

Both mycorrhiza and rhizobium can establish symbiotic associations with roots of lucerne (*Medicago sativa*). The arbuscular mycorrhizal (AM) association is a ubiquitous symbiosis in terrestrial ecosystems. AM fungi are known to have low host specificity, and when associated with higher plants, play important roles in influencing the availability of nutrients with low mobility in soil solution (especially phosphorus) (Smith and Read 1997). Optimum growth of leguminous plants is usually dependent on symbiotic relationships with mycorrhizal fungi and N_2-fixing bacteria (Xavier and Germida 2003). Mycorrhizal infection of plant roots usually stimulates plant growth through effects on nutrient uptake, nodulation and N_2 fixation, and/or water supply (Redecker *et al.* 1997). Lucerne has been found to grow better after inoculation with AM fungi (Nielsen and Jensen 1983).

Compared with *Sinorhizobium meliloti*, AM fungi showed little variation in their colonisation in natural soils covering a wide range of pH (Read *et al*. 1976). However, growth of AM fungi may be stimulated by decreased soil pH (Wang *et al*. 1993), and the formation of extraradical hyphae is influenced by the pH and nutrient status of the soil (Coughlan *et al*. 2000). In a pilot experiment, we showed that inoculation of lucerne with *Glomus intraradices* in acid purplish soil with a pH level of 5.45 stimulated top growth

Correspondence: Yanjun Guo，College of Animal Science and Technology, Southwest University, Chongqing, 400716，P.R. China. E-mail: qhgyj@126.com

more than inoculation with other AM fungi, namely *G. caledonium* and *Acaulospora leavi*. The aims of the present study were to determine whether inoculation with a combination of the fungus *G. intraradices* and *S. meliloti* strain 1.163 would increase growth and nutrient uptake by lucerne seedlings in acid purplish soils, and to assess the potential to use existing biological systems in place of expensive lime applications for site rehabilitation in acid soils.

Materials and methods

Experimental soil

The soil used in the pot experiment was an acid purplish soil (Udorthent) collected from the 0–30 cm horizon of arable land in Beibei borough, Chongqing city, China. The soil was air-dried, ground to pass a 2 mm sieve and sterilised at 120°C for 20 minutes. The soil pH (1:1 soil:water) was 5.45, and the concentrations of available nitrogen and phosphorus (0.03 mol/L NH_4F), extractable potassium, calcium, magnesium and aluminium were 76.8, 32.6, 114.8, 18.4, 3.7 and 0.7 mg/kg, respectively. The soil was fertilised with 6 g lime/kg soil to produce a final soil pH of 6.54. The soil pH level after liming was determined by keeping the soil water concentration at 20%, and incubating for 15 days at 25°C.

Biological materials

Lucerne (*Medicago sativa*) cv. Sanditi seeds were provided by Barenbrug Seed Company in China. *G. intraradices* strain (a collection isolate from calcareous soils in Beijing, China) was cultured on *Trifolium repens*. AM inoculum consisted of rhizospheric soil containing spores, hyphae and mycorrhizal root fragments (approximately 1200 spores per 100 g). *S. meliloti* inoculant used in the study was strain 1.163, supplied by Institute of Microbiology, Chinese Academy of Science. The strain was cultured in yeast mannitol suspension for 4–6 days at 28±1°C. At the time of inoculation, the suspension normally contained between 10^8 and 10^9 cells/ml.

Inoculation process and experimental design

There were 4 plant treatments: A) inoculation with *G. intraradices* and *S. meliloti*; B) inoculation with *S. meliloti* only; C) inoculation with *G. intraradices* only; and D) uninoculated plants (Control). One kilogram of limed or unlimed soil was placed in each pot. AM inoculation dosage was 100 g soil containing mycorrhizal spores per pot in A and C treatments and 100 g sterilised AM inoculum per pot in B and D treatments. Lucerne seeds were surface-sterilised and germinated on wet filter paper in Petri dishes. The healthy seedling roots were dipped in *S. meliloti* inoculant suspension for about 5 seconds, and then transplanted in 15 × 20 cm pots (5 plants/pot). In C and D treatments, the seedlings were directly transplanted into the pot without dipping in *S. meliloti* inoculant. Each treatment had 4 replications. The plants were grown for 2 months in a greenhouse at 25/15°Cand 50/70% RH (day/night). Pot positions were changed every 2 days and water was irrigated when the soil water content was lower than 60% relative water content (5 pots were weighed every 2 days to calculate the relative water content).

Determination of plant yields, nodulation and mycorrhizal colonisation

Two months after sowing, plant height was measured and the above-ground material was removed. This material was sorted into leaves and stems to determine leaf:stem ratio. The below-ground material was thoroughly washed with a fine water spray and separated into nodules and roots. To estimate the root-colonisation by arbuscular mycorrhiza, a subsample of 0.4 g fresh lateral root was randomly taken and thoroughly washed with sterilised water, then cut into approximately 1 cm segments. These were stained with acid glycerol trypan blue (Phillips and Hayman 1970); percent infection was assessed by the gridline intersect method (Giovanetti and Mosse 1980) with 100 counts per slide. The nodules with violet red colour were counted; minute nodules with white colour were not included in the count. All organs were oven-dried at 65°C for 48 h.

Determination of nutrient concentrations in plant material

Plant samples were ground through a 2 mm sieve, then digested by H_2SO_4–H_2O_2. The extract was used to determine N, P and K concentrations. Plant N was determined via the micro-Kjeldahl procedure, K by flamephotometry and plant P colorimetrically by the vanadomolybdophosphoric yellow method. Ca and Mg concentrations of plant material were determined by HCl extraction, followed by atomic absorption. Al concentration was determined by H_2SO_4–$HClO_4$ extraction using the colorimetric method.

Statistical analyses

Data were analysed with 3-way factorial ANOVA tests (SPSS13.0) to observe the effects of lime application, inoculation with *G. intraradices* and *S. meliloti* and their interactions on dry matter yield of plant tops, nodule formation and nutrient absorption of lucerne from acid purplish soils. Differences between treatments were compared with Tukey's multiple range tests. Differences obtained at levels of P = 0.05 were considered significant.

Results

Nodulation and mycorrhizal colonisation

Nodules and AM mycelium were evident at 60 d after transplanting in the roots of lucerne seedlings inoculated with *S. meliloti* and AM fungus (Table 1). Both addition of lime and inoculation with mycorrhiza stimulated number of nodules produced over rhizobium alone (P<0.05) with highest nodule numbers from rhizobium + mycorrhiza + lime (P<0.05). Total nodule weight followed a similar pattern, although mean nodule weight was lower (P<0.05) in limed plots. Infection rate with fungal mycelium was not significantly (P>0.05) affected by inoculation with rhizobium or addition of lime.

Growth and dry matter production

Although the 2 symbiotic associations stimulated top growth of lucerne without lime application, increases were significant only when plants were inoculated with both rhizobium and mycorrhiza (P<0.05) (Table 2). Overall, inoculation had no significant influence on root weight (P>0.05). Plants inoculated with mycorrhiza produced more total dry matter than those on the remaining treatments but differences were not always significant. The only significant effect of lime application was a significant decrease in root weight in uninoculated treatments (P<0.05). Overall, shoot:root ratio was not significantly affected by treatment, but liming significantly (P<0.05) increased this parameter in uninoculated plants.

While there were generally no significant effects of treatment on plant height, plants treated with rhizobium + mycorrhiza + lime were taller (P<0.05) than uninoculated controls without lime (Table 3). Leaf:stem ratio was reduced signifi-

Table 1. Effects of treatment with rhizobium, mycorrhiza and lime on nodulation and mycorrhizal infection rate of lucerne. Mean of 4 pots with 5 plants/pot.

Treatment	Lime	Nodule number (No./pot)	Nodule weight (mg/pot)	Mean nodule weight (mg/nodule)	Infection rate (%)
Rhizobium	-[1]	30.50 d[2]	54.00 c	1.64 a	
	+	81.50 b	81.50 b	1.00 b	
Mycorrhiza + Rhizobium	-	61.00 c	97.75 b	1.62 a	15.29 a
	+	133.25 a	132.75 a	1.04 b	14.18 a
Mycorrhiza	-				14.13 a
	+				12.89 a

[1] '+' and '-' represent with and without lime application.

[2] Means within columns followed by the same letter are not significantly different at P = 0.05 according to Tukey's multiple range tests.

cantly ($P<0.05$) by liming in uninoculated plants but was unaffected by liming on other treatments.

Chemical composition

There were few consistent changes in chemical composition as a result of treatment (Table 4). Liming increased Ca concentration in plant tops in treatments where mycorrhiza was applied ($P<0.05$) and tended to decrease Mg and P concentrations, but these differences were generally not significant.

Discussion

While ineffective nodulation of legumes can be a problem on low pH soils (Munns 1986), this study has demonstrated that the problem can be solved by lime application and inoculating the legumes with rhizobium and mycorrhiza. In this study on acid purplish soils with a pH of 5.45, application of lime to correct the low pH along with rhizobium more than doubled the number of nodules formed with rhizobium alone and increased total nodule weight by 51%. However, this had no significant effect on growth of the plants, indicating that there were other limiting factors for growth. When mycorrhizal fungi were also applied, nodule production was stimulated further, so that total nodule weight was 146% more than with rhizobium alone, with a 53% increase in growth of above-ground parts. It is possible that the mycorrhiza increased the availability and uptake of other nutrients, especially phosphorus, which were previously limiting plant growth. Mycorrhiza has strong mycelia, which expand the area of roots available for absorption of nutrients (especially phosphorus) (Jia *et al*. 2004; Shockley *et al.* 2004), and then stimulate rhizobium infection, improving nitrogen-fixation ability and plant growth (Siviero *et al.* 2008). These responses in both top growth and total weight of lucerne compared with uninoculated plants support the responses reported by Pandey *et al.* (2003) for other legumes. Since the growth response was confined to the above-ground parts rather than roots, there was an increase in the shoot:root ratio. This was achieved without any significant change in the leaf:stem ratio of the plants.

Acid soils often contain excess H and Al and have low availability of Ca, Mg and P (Foy 1984). However, mycorrhiza has the potential to improve the nutrient supply to plant roots in severe conditions and increase the uptake of N, P, K, Ca and Mg in plant tops (Saif 1987). In our study on acid purplish soils, treatment with the combination of rhizobium, mycorrhiza and lime had no significant effect on nutrient concentrations in above-ground parts of the lucerne, despite the increases (53%) in growth discussed earlier. Uptake of nutrients was therefore also stimulated by a similar amount. Interestingly, lime application increased N concentration in top growth of lucerne in uninoculated control plants but reduced P concentration in plots where mycorrhiza was applied. Species of mycorrhiza differ in their response to soil pH (Sano *et al*. 2002). Many studies have been carried out to select appropriate mycorrhiza for inoculating plants in acid soils (Cavallazzi *et al*. 2007). *G. manihotis* was found to be most effective for a range of crops and pastures, at low pH and at a wide range of N, P and K levels (Howeler *et al*. 1987). The positive influence of *G. intraradices* on top growth and uptake of nutrients, especially P and N, in our study, has indicated that it is also effective for inoculation of lucerne on these acid soils.

Andrade *et al*. (2002) found that lime application increased *Sinorhizobium* survival and persistence in acid soils. In our study, it stimulated *Sinorhizobium* infection of lucerne roots, and resulted in significant increases in both nodule numbers and total nodule weight. The reduction in mean nodule weight we recorded was in agreement with the finding of Buerkert *et al.* (1990). The better nodule development would have stimulated effective N_2 fixation, increasing the amount of N available to the plant to support growth. However, there was no significant increase in top growth of uninoculated lucerne plants, with the additional N available causing a significant increase in N concentrations in top growth. This might be a reflection of differing acid tolerance in different lucerne species. Grewal and Williams (2003) suggested that lucerne cultivars differed in their level of acid tolerance and response to lime application. Cultivars with better acid tolerance had no response or an inverse response to lime application. Our early studies also showed that the lucerne cultivar Sanditi had better acid tolerance than other cultivars in acid purplish soils (Guo and Huang 2006). It was interesting that,

Table 2. Effects of treatment with rhizobium, mycorrhiza and lime on dry matter production of lucerne. Mean of 4 pots with 5 plants/pot.

Treatments	Lime	Shoot	Root	Total
			(g/pot)	
Uninoculated	-[1]	0.85 c[2]	0.38 a	1.23 bc
	+	0.90 c	0.19 b	1.09 c
Rhizobium	-	0.95 bc	0.25 ab	1.20 c
	+	0.95 bc	0.29 ab	1.23 bc
Mycorrhiza	-	1.06 abc	0.41 a	1.48 abc
	+	1.24 a	0.39 a	1.63 ab
Mycorrhiza+Rhizobium	-	1.23 ab	0.39 a	1.62 ab
	+	1.30 a	0.39 a	1.68 a

[1] '+' and '-' represent with and without lime application.

[2] Means within columns followed by the same letter are not significantly different at P = 0.05 according to Tukey's multiple range tests.

Table 3. Effects of treatment with rhizobium, mycorrhiza and lime on shoot:root ratio, leaf:stem ratio and height of lucerne. Mean of 4 pots with 5 plants/pot.

Treatments	Lime	Shoot:root ratio	Leaf:stem ratio	Height (cm)
Uninoculated	-[1]	2.17 a[2]	0.96 a	25.66 b
	+	4.76 b	0.79 b	26.98 ab
Rhizobium	-	3.57 ab	0.91 a	26.74 ab
	+	3.33 ab	0.90 a	27.14 ab
Mycorrhiza	-	2.63 b	0.90 a	26.70 ab
	+	3.23 ab	0.90 a	30.51 ab
Mycorrhiza+Rhizobium	-	3.13 ab	0.86 ab	29.01 ab
	+	3.33 ab	0.94 a	32.26 a

[1] '+' and '-' represent with and without lime application.

[2] Means within columns followed by the same letter are not significantly different at P = 0.05 according to Tukey's multiple range tests.

Table 4. Effects of treatment with rhizobium, mycorrhiza and lime on nutrient concentrations in top growth of lucerne. Mean of 4 pots with 5 plants/pot.

Treatments	Lime	N	P	K	Ca	Mg	Al
				(g/kg)			
Uninoculated	-[1]	30.42 b[2]	3.64 c	30.14 ab	15.62 ab	2.60 ab	0.148 a
	+	36.28 a	4.32 ab	32.20 a	15.09 ab	2.48 abc	0.128 a
Rhizobium	-	35.84 a	4.00 abc	31.12 ab	15.19 ab	2.74 a	0.141 a
	+	34.57 ab	4.09 abc	31.29 ab	16.73 a	2.50 abc	0.150 a
Mycorrhiza	-	34.55 ab	4.13 abc	29.69 ab	13.90 b	2.43 bc	0.213 a
	+	34.09 ab	3.70 bc	28.53 b	16.75 a	2.25 c	0.154 a
Mycorrhiza+	-	32.88 ab	4.54 a	30.47 ab	14.13 b	2.24 c	0.153 a
Rhizobium	+	31.48 ab	3.50 c	29.91 ab	16.75 a	2.24 c	0.158 a

[1] '+' and '-' represent with and without lime application.

[2] Means within columns followed by the same letter are not significantly different at P = 0.05 according to Tukey's multiple range tests.

when mycorrhiza was applied, P concentrations in top growth were reduced, though not always significantly, following lime application.

Acknowledgements

The authors are grateful to Professor Yuan Ling for her help in statistical analysis of the results. This research was supported by National Basic Research Plan of China (2007CB108901) and National Science Foundation of China (30800802).

References

Andrade, D.S., Murphy, P.J. and Giller, K.E. (2002) Effects of liming and legume/cereal cropping on populations of indigenous *rhizobia* in an acid Brazilian Oxisol. *Soil Biology and Biochemistry,* **34**, 477–485.

Bordeleau, L.M. and Prevost, D. (1994) Nodulation and nitrogen fixation in extreme environments. *Plant and Soil*, **161**, 115–124.

Buerkert, A., Cassman, K.G., Piedra, R. De La and Munns, D.N. (1990) Soil acidity and liming effects on stand, nodulation, and yield of common bean. *Journal of Agronomy*, **82**, 749–754.

Cavallazzi, J.R.P., Filho, O.K., Stürmer, S.L., Rygiewicz, P.T. and Mendonça, M.M. (2007) Screening and selecting arbuscular mycorrhizal fungus for inoculating micropropagated apple rootstocks in acid soils. *Plant Cell Tissue and Organ Culture*, **90**, 117–129.

Coughlan, A.P., Dalpe, Y., Lapointe, L. and Piche, Y. (2000) Soil pH-induced changes in root colonization, diversity, and reproduction of symbiotic arbuscular mycorrhizal fungus from healthy and declining maple forests. *Canadian Journal of Forest Research*, **30**, 1543–1554.

Foy, C.D. (1984) Physiological effects of hydrogen, aluminum and manganese toxicities in acid soils. In: Adams, F. (ed.) *Soil Acidity and Liming*. 2nd Edn. (American Society of Agronomy: Madison, WI).

Giovanetti, M. and Mosse, B. (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist*, **l84**, 489–500.

Graham, P.H., Viteri, S.E., Mackie, F., Vargas, A.T. and Palacios, A. (1982) Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. *Field Crops Research*, **5**, 121–128.

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.

Guo, Y.J. and Huang, J.G. (2006) Nutrients absorption and nodulation ability of alfalfas in acid purple soil. *Plant Nutrition and Fertilizer Science (in Chinese with English abstract)*, **12**, 97–103.

Howeler, R.H., Sieverding, E. and Saif, S. (1987) Practical aspects of mycorrhizal technology in some tropical crops and pastures. *Plant and Soil*, **100**, 249–283.

Jia, Y.S., Gray, V.M. and Straker, C.J. (2004) The influence of *rhizobium* and arbuscular mycorrhizal fungi on nitrogen and phosphorus accumulation by *Vicia faba*. *Annals of Botany*, **94**, 251–258.

Meng, C.F., Lu, X.N., Cao, Z.H., Hu, Z.Y. and Ma, W.Z. (2004) Long-term effects of lime application on soil acidity and crop yields on a red soil in Central Zhejiang. *Plant and Soil*, **265**, 101–109.

Munns, D.N. (1986) Acid soils tolerance in legumes and *rhizobia*. *Advances in Plant Nutrition*, **2**, 63–91.

Nielsen, J.P. and Jensen, A. (1983) Influence of vesicular-arbuscular mycorrhiza fungi on growth and uptake of various nutrients as well as uptake ratio of fertilizer P for lucerne (*Medicago sativa*). *Plant and Soil*, **70**, 165–172.

Pandey, D., Singh, J.P., Kashyap, N. and Dwivedi, G.K. (2003) Response of vesicular arbuscular mycorrhizae (AM), *rhizobium* and phosphorus sources on nodulation, growth and yield of pea variety VL-7. *Crop Research*, **25**, 333–336.

Phillips, J.M. and Hayman, D.S. (1970) Improved procedures for cleaning roots and stain parasitic and vesicular-arbuscular fungi for rapid assessment of infection. *Transactions of the British Mycological Society,* **55**, 158–161.

Read, D.J., Koucheki, H.K. and Hodgson, J. (1976) Vesicular-arbuscular mycorrhiza in natural vegetation systems. I. The occurrence of infection. *New Phytologist*, **76**, 415–431.

Redecker, D., Vonberneswordtwallrabe, P., Beck, D.P. and Werner, D. (1997) Influence of inoculation with arbuscular mycorrhizal fungi on stable isotopes of nitrogen in *Phaseolus vulgaris*. *Biology and Fertility of Soils,* **24**, 344–346.

Saif, S.R. (1987) Growth response of tropical forage plant species to vesicular-arbuscular mycorrhizae. *Plant and Soil*, **97**, 25–35.

Sano, S.M., Abbott, L.K., Solaiman, M.Z. and Robson, A.D. (2002) Influence of liming, inoculum level and inoculum placement on root colonization of subterranean clover. *Mycorrhiza*, **12**, 285–290.

Shockley, F.W., McGraw, R.L. and Garrett, H.E. (2004) Growth and nutrient concentration of two native forage legumes inoculated with *Rhizobium* and *Mycorrhiza* in Missouri, USA. *Agroforest Systems*, **60**, 137–142.

Siviero, M.A., Motta, A.M., Lima, D.S., Birolli, R.R., Huh, S.Y., Santinoni, I.A., Murate, L.S., Castro, C.M.A., Miyauchi, M.Y.H., Zangaro, W., Nogueira, M.A. and Andrade, G. (2008) Interaction among N-fixing bacteria and AM fungi in Amazonian legume tree (*Schizolobium amazonicum*) in field conditions. *Applied Soil Ecology*, **39,**144–152.

Smith, S.E. and Read, D.J. (1997) *Mycorrhizal symbiosis*. (Academic Press: London).

Walker, R.F. (2002) Growth and nutritional responses of bare-root Jeffrey pine on a Sierra Nevada surface mine to mine-site applications of fertilizer and lime. *New Forest*, **24**, 225–238.

Wang, G.M., Stribley, D.P., Tinker, P.B. and Walker, C. (1993) Effects of pH on arbuscular mycorrhiza I. Field observations on the long-term liming experiments at Rothamsted and Woburn. *New Phytologist,* **124**, 465–472.

Xavier, L.J.C. and Germida, J.J. (2003) Selective interactions between arbuscular mycorrhizal fungi and *Rhizobium leguminosarum bv. viceae* enhance pea yield and nutrition. *Biology and Fertility of Soils,* **37**, 261–267.

(Received for publication March 20, 2009; accepted September 7, 2009)