Effects of Lime and Calcium on Root Development and Nodulation of Clovers

David Brauer,* Dale Ritchey, and David Belesky

ABSTRACT

Acidic soils can reduce the nodulation of forage legumes. Studies with a Gilpin series silt loam (fine loamy, mixed mesic, Typic Hapludult) from New, WV, were conducted to determine the effects of lime on root development, and to assess effects of soil Ca and pH on nodulation. Liming increased soil pH from 4.8 to 5.3, nodulation, and root growth of white clover (*Trifolium repens* L., cultivar Huia) 28 d after planting. Seedlings from unlimed soil formed fewer indeterminate and determinate roots. Next, soils were amended with either $CaCO_3$ or a mixture of $CaCO_3$ and $CaSO_4$ to achieve a soil pH of 4.7 to 6.1 and soil Ca of 170 to 680 mg kg^{-1} soil. There was a strong quadratic relationship between number of nodules per white clover seedling 28 d after planting and soil pH. Another experiment was conducted to determine if these trends were expressed under field conditions. In 1993, field plots were amended with lime or a coal combustion by-product that supplied Ca as $CaSO_4$ and seeded in 1994 to cool-season grasses. In spring of 1998, plots were drilled with either red (*Trifolium pratense*, L.) or white clover. The nodules per primary root were determined in May (1998, 1999) and August (1998). Number of nodules per primary root was more closely associated with soil pH than soil Ca. These results indicate that changes in nodulation were more closely associated with changes in soil pH than soil Ca.

It is well known that addition of lime to soils will increase the nodulation and growth of legumes (Albrecht, 1932, 1933; Hyland, 1938). Increases in soil Ca may increase nodulation on the basis of the current understanding of the role of Ca in nodulation process. Calcium concentrations in excess of 2 μ*M* were necessary for optimum growth of *Rhizobium* in nutrient media (Balatti et al., 1991). Relatively high concentrations of Ca, i.e., 10 m*M*, were necessary for maximal attachment of *Rhizobium meliloti* to alfalfa roots in nutrient solution (Howieson et al., 1993). Expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* increased with the addition of Ca to the media, especially at lower pH values and in the presence of external Al (Richardson et al., 1988). One nodulation protein, NodO, appears to be a Ca binding protein (Sutton et al., 1994), which suggests that Ca is a regulator of the nodulation process. Nodulation factors increase intracellular Ca levels in root hair cells and alter the fluxes of Ca across the plasma membrane of root hair cells (Felle et al., 1998, 1999). Changes in the cytoskeleton of root hairs in response to *Rhizobium* attachment involve Ca binding proteins (Miller et al., 1997), and thus attachment could be influenced by Ca status of roots. Changes in root physiology and structure early in nodule development also involve Ca. These early events in nodule formulation are similar to those of secondary root formation (Gresshoff, 1993). Improvements in image analysis technology allow researchers to assess the effects of soil conditions on specific root types, rather than just total root length or growth. Recent results indicate that different root types respond to environmental conditions differently (Zobel, 1992).

It is still not clear whether the effects of liming on nodulation are due to changes in soil pH, soil Al, or soil Ca. In mineral soils, plant available Al is most often controlled by soil pH (Thomas and Hargrove, 1984). Therefore the effects of soil pH may be due to changes in plant available Al. At this time there is no adequate way of varying soil pH and plant available Al independently with the Gilpin series soils. Experiments examining the effects of pH and Al on root growth have been conducted using non-soil rooting media (Brauer, 1998, and references therein).

A series of papers (Lynd and Ansman, 1989a, b; Purcino and Lynd, 1986) examined the effects of changes in soil pH and Ca on nodulation of several legume species. There was no significant increase in nodulation with Ca additions to a soil with an initial pH of 6.1. These results may have little bearing on the role of Ca in remediating the effects of acidic soil since the soil pH was relatively high in the unamended soil. Alva et al. (1987, 1990, 1991) attempted to separate effects of Ca, Al, and pH on nodulation. The effects of varying concentrations of Al and Ca on nodulation at several different pH values were assessed for legumes growing in nutrient solution. Their work shows that increases in monomeric species of Al were more closely associated with decreases in growth and N content than other parameters. Nodulation in these experiments was relatively low, even at higher pH and Ca concentrations in the absence of Al, perhaps because the nutrient solutions contained inorganic N. Therefore, it is difficult to conclude that the effects of pH and Al on growth and N content were due to either reduced nodulation or N uptake.

Peanut (*Arachis hypogaea* L.) was grown in a highly weathered Typic Paleudult soil amended with lime ($CaCO_3$), gypsum ($CaSO_4$), or varying ratios of each (Syed-Omar et al. 1990). Nodulation was greatest when Ca was added as gypsum, possibly indicating that changes in soil Ca were mainly responsible for the observed effects. The soils used in this experiment were leached with water after amending, and the addition of Ca as either gypsum or lime resulted in significant decreases in soil Al. The effects of the addition of lime or gypsum may have been due to a reduction in soil Al and/or an increase in soil pH, rather than an increase in soil Ca.

Understanding whether the benefits of lime on the growth and nodulation of legumes are due to effects on soil pH or Ca is of more of concern today than in the

David Brauer, USDA/ARS/SPA, Dale Bumpers Small Farms Research Center, 6883 South State Highway 23, Booneville, AR 72927; Dale Ritchey, and David Belesky, USDA/ARS/NAA, Appalachian Farming Systems Research Center, 1224 Airport Road, Beaver, WV 25813; *Corresponding author (dbrauer@spa.ars.usda.gov).

Published in Crop Sci. 42:1640–1646 (2002).

past. Coal burning power plants generate an array of Ca by-products, varying considerably in their $CaCO_3$ equivalence (Clark et al., 1993). In many cases, especially power plants burning high S coal, the by-products contain substantial quantities of $CaSO_4$. Many power generating companies hope these by-products can be applied to agricultural lands. There are many places in the USA including Appalachia and eastern Oklahoma where soils are acidic and could benefit from application of Ca by-products generated by local coal burning power plants. The effects of soil Ca and soil pH on legume nodulation should be fully understood to use these by-products. The objectives of this research were to determine the effects of lime on the growth of specific types of roots of white clover and to resolve the influence of liming on nodulation of red and white clover relative to changes in soil Ca or Ph.

MATERIALS AND METHODS

General Methods for Growth Chamber and Greenhouse Experiments

Top soil (0–10 cm) of a Gilpin silt loam (fine loamy, mixed, mesic, Typic Hapludult) was collected from a meadow near New, WV (37°48′40″N by 80°58′30″W, altitude 900 m). The soil from this site had not received fertilizer or lime for the last 40 yr. After collection the soil was air dried, ground to pass a 2-mm sieve and stored at room temperature in 120-L covered plastic containers with lids. Before an experiment, a sufficient quantity of soil was removed from the bulk collection and amendments, $CaSO_4$ and $CaCO_3$, added and thoroughly mixed. The surface of the soil was moistened with distilled water as a light spray, and then remixed. Water additions were repeated until the soil moisture reached 80% of field capacity. At this point, the soil was stored in sealed plastic bags at 4°C to prevent loss of moisture. Soil was allowed to react with amendments for 14 to 28 d before use, except where noted. Amendments were added as a find powder ground by mortar and pestle. Changes in soil pH were complete by 14 d after addition of amendments (data not shown).

Planting cones used in these experiments had a slight taper with the top having a maximum diameter of 4 cm. The bottom of the cone was fitted with a #9 size rubber stopper. The cone was then filled within 2.5 cm of the top with soil prepared as described above to produce a soil column of 16 to 17 cm in length. Cones filled with soil were used immediately or the tops and bottoms were covered with plastic film to prevent soil moisture loss.

White clover (*Trifolium repens* L., cultivar Huia) seeds were scarified and germinated for 2 d in sterile distilled water as described previously (Staley et al., 1993). Five 2-d-old seedlings with radicles of about 1 cm in length were planted per cone at a 0.5-cm depth. Immediately after planting the seedlings, 50 g of sterile, acid washed sand was added above the soil column followed by 5 mL of distilled water. The mass of the cone was determined and recorded. Distilled water was added every 2 to 3 d to the top of the soil column to bring the mass of the cone to that recorded on Day 1. Rhizobium (*Rhizobium leguminiosarum* biovar *trifolii*) inoculum was added the day after planting. Inoculum was prepared as described previously (Staley and Morris, 1998) and added as a suspension in distilled water. Typically, the inoculum added 10^8 to 10^9 rhizobial bacteria per cone. The purity and number of rhizobia in the inoculum was verified by serial dilution plating. In Exp. 2 and 3, approximately 7 d after planting, the number of seedlings per cone was reduced to four to ensure a constant number of plants per cone–experiment unit.

All of the plants in a cone were harvested from 2 to 31 d after planting. Plants were harvested over a wide range of days after planting so that the ontogeny of root growth could be followed. Cones were submerged in tap water to saturate the soil to facilitate harvesting of intact root systems. The soil column was then expelled by pushing the rubber stopper out of the cone from bottom to top with the aid of a wooden stave. Plants were washed extensively with tap water to remove most of the adhering soil. Plants were held near the hypocotyl and gently brushed with a fine nylon bristle brush to remove adhering fine soil particles. Roots harvested in this manner had little if any soil contamination (data not shown) and root caps were observed in over 95% of the roots examined microscopically (data not shown), suggesting little if any root damage occurred in the extraction process.

After harvesting, plants were placed in distilled water in a shallow glass dish illuminated from below by a light box. The individual roots within the plant's root system were separated from each other with the aid of a nylon bristle brush. A ruler was placed next to the root system. Above the dish containing the clover plants was suspended a Dage CCD-72S digital camera (Dage MTI, Michigan City, IN) attached to a developer stand. The digital camera was controlled by a MacIntosh PC equipped with Scion frame grabber (Scion Corporation, Frederick, MD) and NIH-Image software (U.S. National Institutes of Health, 1998). Digital images of root systems were recorded and stored. Root lengths were determined from electronically stored images by means of the distance tool in NIH Image. The presence of a ruler in each image field allowed distances measured in pixels to be converted to centimeters. After images of root systems were recorded, the roots were examined visually and the number of nodules per plant recorded. Plants (shoot and root tissue) were then blotted dry and placed in preweighed glass vials. Plant material was dried for 3 d at 65°C and reweighed so that plant dry matter could be determined. Plant dry matter accumulation varied with harvest dates and treatments similar to that of total root length (date not shown). Since the objectives of these studies were to assess effects on root ontogeny and nodulation, dry matter accumulation per plant is not reported. Dried plant material was finely ground and total N concentration was determined by dry combustion method using Model EA1108, Carlo-Erba CHNS-O Analyzer (CE Elantech, Inc., Lakeview, NJ). Two measures of nodulation were used: nodules per plant and % N content of combined root and shoot tissue. These two measures were found to be good indicators of effective nodulation with white clover seedlings and superior to measurements of acetylene reduction activity (Staley and Morris, 1998).

Experiments 1, 2, and 3

Experiments 1 and 2 were conducted in a growth chamber. Plants were grown with a light/dark regime of 16/8 h at an average irradiance of 300 $\mu mol\ m^{-2}\ s^{-1}$ at 25 and 20°C, respectively. Relative humidity within the chamber was maintained at 60%. The limed soil was amended with 550 mg $CaCO_3\ kg^{-1}$ soil (dry weight). Each experiment was performed twice with each experiment having six replicates. Six replicates were harvested from each treatment at each sample date. The experimental unit was a planting cone. Data from individual plants in a cone for each harvest were averaged to obtain a value for the experimental unit. Analysis of variance of the data indicated no significant effects for experiments and replications and no significant interaction of experiments and repli-

Table 1. Amount of total Ca added and amount added as either $CaSO_4$ or $CaCO_3$ to comprise the seven treatments in Exp. 3.

Treatment number	Ca added		
	Total	As $CaSO_4$	As $CaCO_3$
	mg Ca kg^{-1} Soil (dry weight)		
1	100	0	100
2	400	300	100
3	700	600	100
4	400	0	400
5	700	0	700
6	400	210	190
7	700	420	280

cates with each other or soil treatment (data not shown). Therefore, the data from the two experiments were combined. Reported data are the means from both experiments ($n = 12$) with standard deviations (SD) as an estimate of error.

The objective of Exp. 3 was to assess the effects of independent changes in soil pH and Ca on nodulation by measuring nodules per plant with white clover seedlings grown in soil amended with $CaCO_3$, or a mixture of $CaCO_3$ and $CaSO_4$. This experiment was conducted in the greenhouse. The temperature within the greenhouse was controlled so that the maximum temperatures did not exceed 29°C and the minimum temperature did not fall below 23°C. Supplemental light was not provided. The experiment was conducted in August and October, 1998. Soils were amended with sufficient amounts of $CaCO_3$ or two mixtures of $CaSO_4$ and $CaCO_3$ to increase soil Ca by 100 to 700 mg kg^{-1} soil (dry weight) to yield seven treatments (Table 1). The experimental design was a randomized complete block with six replicates. Data were collected from individual plants and then averaged to yield a value for the experimental unit, planting cone. Analysis of variance indicated no significant effects of experiment, replication and their interaction with soil treatments (data not shown). Therefore, data are reported as means from both experiments ($n = 12$) with (SD) reported as an estimate of error.

Experiment 4: Field Trial

In 1993, an experiment was established in an existing unimproved meadow near New, WV. This field experiment was established within 100 m from where soil was collected for the above experiments. In April, 1994, plots measuring 8 by 3 m were treated with 4600 kg dolomitic limestone ha^{-1}, 8000 kg coal combustion by-product gypsum ha^{-1}, or both amendments. The coal combustion by-product gypsum contained 10% of Ca as $CaCO_3$ and the remainder as $CaSO_4$. A fourth set of plots received no additional Ca. The amount of Ca added to the soil for each treatment appears in Table 2. A sod of tall fescue and orchardgrass was established. The initial design was a randomized complete block with four replications.

Fertilizer was applied from 1993 to 1997 to meet the needs of the forages for these nutrients and to bring soil test levels

Table 2. Addition of Ca comprising various soil amendment treatments applied in 1994 for the field experiment near New, WV (Exp. 4).

Treatment number	Soil amendment	Ca added
		kg ha^{-1}
1	None	0
2	4650 kg dolomite ha^{-1}	977
3	4650 kg dolomite ha^{-1} and 8000 kg ha^{-1} coal combustion by-product	2881
4	8000 kg coal combustion by-product ha^{-1}	1904

Table 3. Soil chemical parameters of the unamended and limed soils in Exp. 1 and 2. Data are means ± SD.

Soil chemical parameter	Unamended	Limed
soil pH	4.8 ± 0.1	5.3 ± 0.1
	mg kg^{-1} dry weight	
soil Ca	90 ± 5.1	280 ± 18
soil Al	210 ± 18	33 ± 6

for P and K to optimum levels. In March, 1998, each plot was split and either red or white clover was drilled into existing sod. On 15 May 1998, 7 Aug. 1998, and 14 May 1999, two soil cores measuring 10 cm in diameter and 8 cm long were collected from a row of clover plants in each plot. The cores were sealed in plastic bags and stored at 0 to 4°C overnight. The soil was removed from plants under a stream of water. The number of nodules on primary roots and all nodules in the core were counted. There was some uncertainty if all the nodules in a core could be attributed to the plants in the core. Therefore, only nodules on the primary root are reported here. Similar trends among treatments were found for both parameters, total number of nodules per core and nodules per primary root (data not shown). Significance of treatments (harvests, soil amendments, clover species) was established by analysis of variance. Data from the field experiment regarding forage yields and botanical composition of stands will be the focus of future publications.

Soil Chemical Characterization

Soil samples were air dried and ground to pass a 2.0-mm sieve. Exchangeable K, Ca, and Mg in the soil were extracted with 1 *M* ammonium acetate and determined by atomic absorption spectrometry. Soil exchangeable Al was extracted with 1 *M* KCl and determined by atomic absorption spectrophotometry. Soil pH was determined in 1:1 soil to water suspension using a combination electrodes (Peech, 1965). All analytical measurements were performed in triplicate; triplicate determinations were within 5% of each other.

RESULTS

Experiment 1: Primary Root Growth

Root lengths of white clover seedlings were measured 2 to 15 d after planting in Gilpin soil that was either unamended or limed to raise soil pH from 4.8 to 5.3 (Table 3) to assess effects on primary root growth and to determine when secondary root growth commences. Primary root lengths in unlimed and limed soil increased linearly with time (Fig. 1). Slopes of the regression lines fitted to the data were 1.18 and 0.76 cm d^{-1} for roots from amended and unamended soils, respectively. Thus, primary roots in the limed soil were growing about 55% faster than those in the unamended soil. By the end of 16 d, the primary roots of plants in limed soil were reaching their limit because of the depth of the soil column. Between 10 and 15 d after planting, secondary root growth was observed with plants in both unamended and amended soils.

Experiment 2: Secondary Root Growth

Nodulation and secondary root growth were evaluated 14 to 31 d after planting for white clover seedlings

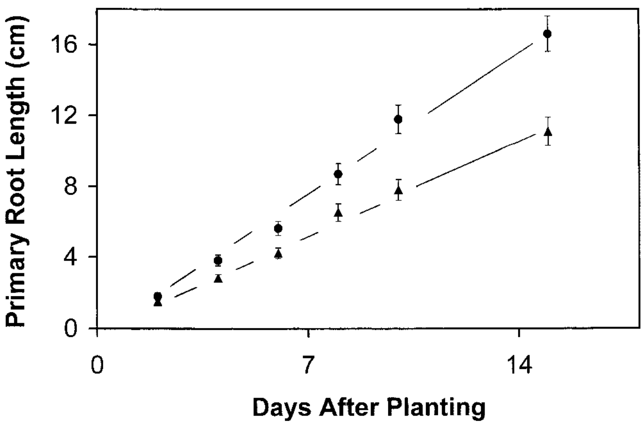

Fig. 1. Effects of liming on primary root length of white clover seedlings (Exp. 1). Primary root lengths were determined from seedlings growing in unamended (triangles) and limed (circles) soil 2 to 15 d after planting. Error bars represent the SD where the error exceeds the size of the data symbol.

growing in unamended and limed soil to assess effects of liming on secondary root growth. There was no significant increase in N content and nodules per plant for plants grown in unamended soils, and N content of plants grown in limed soil between 14 and 31 d after planting (data not shown). There was a strong correlation between days after planting and nodules per plant for plants in the limed soils, nodules per plant = 0.11 (days) + 2.31, $r^2 = 0.946$. Over the experiment, nodules per plant averaged 4.6 ± 0.6 and 0.9 ± 0.2 for plants from limed and unamended soils, respectively. Nitrogen content of plants growing in limed soil was significantly higher than that found with plants in unamended soil, 5.7% ± 0.36 versus 3.7% ± 0.27. These results indicate that liming did increase nodulation of white clover seedlings.

Total root length of plants growing in limed soil was greater than that of plants growing in unamended soil (Fig. 2). There was nearly an exponential increase in total root length with the number of days after planting for seedlings growing in the amended soil. With plants in the unamended soil, root length increased as a linear function of time after planting. Primary root lengths in the limed soil changed little between 14 and 31 d (data not shown), averaging 16.0 ± 0.5 cm plant^{-1}. Primary root length of plants from the unamended soil increased significantly through the experiment from 11.1 ± 0.9 at Day 14 to 14.1 ± 0.9 at Day 31.

By 14 d after planting, seedlings growing in either limed or unamended soil had produced significant amounts of secondary roots. Secondary roots were classified as determinate or indeterminate. Determinate roots arise from either primary or indeterminate roots, have a finite length of less than a few centimeters, and do not produce further branches (Cahn et al., 1989; Fusseder, 1987). Short, determinate roots can be quite numerous, and comprise a significant portion of the total root system. Indeterminate roots are longer than determinate roots and produce branch roots (Zobel, 1992). In these studies, roots with lengths of less than 2.5 cm were classified as determinate. At an early stage of development, a few indeterminate roots will be inaccurately classified.

Between 15 and 31 d after planting, seedlings in the limed soil had significantly more total length of determinate roots than plants in unamended soil (Fig. 3). Between 15 and 21 d after planting, seedlings from both soil conditions produced indeterminate roots. The appearance rate of these roots was initially similar for the two treatments. After 21 d, the length of indeterminate roots increased exponentially with seedlings from the limed soil, while indeterminate roots increased only slightly with plants from the unamended soil. Similar trends were observed when number of determinate and indeterminant roots were compared between limed and unamended soils (data not shown). Results from Exp. 2 indicate that liming significantly increased the growth of various categories of roots at various times during the first 31 d after planting (Fig. 1 and 3). Data also indicated that seedlings in the unamended soil produced the same variety of roots present in seedlings from limed soil.

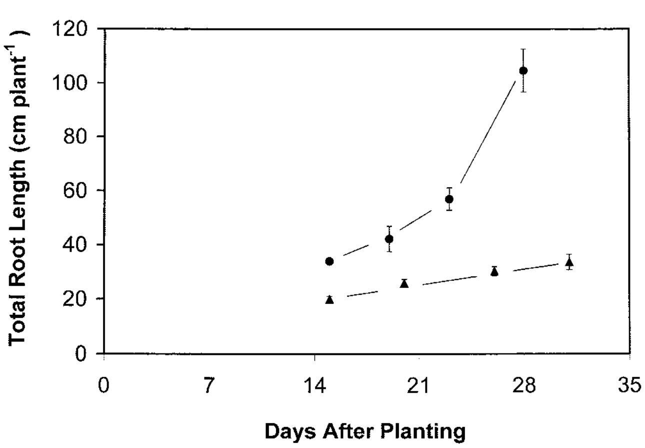

Fig. 2. Effects of liming on total root length of white clover seedlings. Seedlings grown in unamended (triangles) and limed soil (circles) were analyzed for total root length 15 to 31 d after planting. Error bars represent the SD where the error exceeds the size of the data symbol.

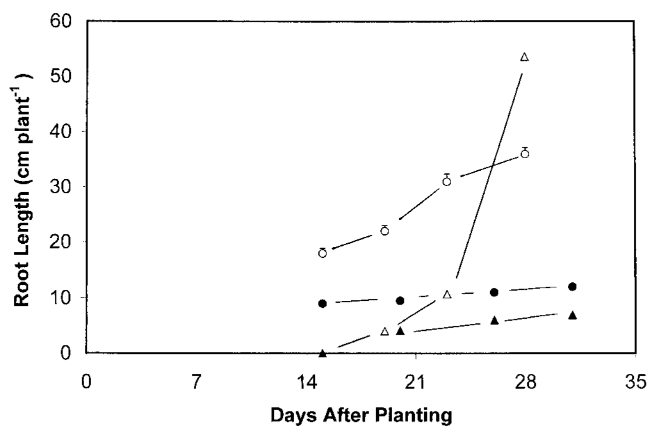

Fig. 3. Effects of liming on the lengths of determinate and indeterminate roots of white clover seedlings. Lengths for determinate (o) and indeterminate (Δ) roots were measured with seedlings grown in either unamended (closed symbols) or limed soil (open symbols) 15 to 31 d after planting. Error bars represent the SD where the error exceeds the size of the data symbol.

Table 4. Effects of soil amendments on soil Ca, soil pH, and nodulation in Exp. 3. A Gilpin series silt loam was amended with either $CaCO_3$ or a mixture of $CaSO_4$ and $CaCO_3$ as described in Table 1. White clover seedlings were harvested 28 days after planting to determine nodules per plant. Data are means ± SD.

Treatment #	Soil pH	Soil Ca	Nodulation
		mg kg^{-1} (dry weight)	nodules per plant
1 (Control)	5.0 ± 0.1	170 ± 12	7.0 ± 0.3
2	4.7 ± 0.1	440 ± 24	6.0 ± 0.3
3	4.5 ± 0.1	680 ± 31	4.2 ± 0.4
4	5.8 ± 0.1	430 ± 18	9.3 ± 0.4
5	6.1 ± 0.1	670 ± 24	9.8 ± 0.5
6	5.0 ± 0.1	420 ± 21	7.2 ± 0.4
7	4.9 ± 0.1	680 ± 28	6.8 ± 0.4

Experiment 3: Effects of Soil Ca and pH on Nodulation

Soils were amended with $CaCO_3$ or mixtures of $CaCO_3$ and $CaSO_4$ to create a range of soil pH values at different soil Ca levels. Soil pH decreased when most of the Ca was added as $CaSO_4$ as in Treatments 2 and 3 (Table 1 and 4). Additions of $CaCO_3$ to increase soil Ca by 400 and 700 mg Ca kg^{-1} soil (Treatments 4 and 5) increased soil pH (Table 4). There was little change in soil pH in Treatments 6 and 7 when Ca was added as nearly balanced mixture of $CaSO_4$ and $CaCO_3$.

Nodulation of white clover seedlings was assessed by measuring the number of nodules per plant 28 d after planting with white clover seedlings. Nodulation of white clover seedlings in the control (Treatment 1) was relatively high, averaging 7.0 ± 0.3 nodules plant^{-1} (Table 4). The difference between the control in this experiment, and Exp. 1 and 2 was an addition of 250 mg $CaCO_3$ kg^{-1}/soil (dry weight) to raise soil pH to 5.0 to ensure reasonable levels of nodulation in the control. Soils with pH values above the control (Treatments 4 and 5) had higher levels of nodulation. Soils with pH values similar to the control but with higher levels of soil Ca (Treatments 6 and 7) had plants with nodules per plant similar to the control. Plants in amended soils with pH values less than the control (Treatment 2 and 3) had lower levels of nodulation. The relationship between nodules per plant and soil pH conformed to both linear and quadratic equations with the quadratic equation having a greater r^2: nodules per plant = 1.97 (soil pH)^2 + 24.18 (soil pH) − 64.44, r^2 = 0.988. There was no significant linear or quadratic relationship between soil Ca and nodules per plant ($P > 0.10$). These results indicate that nodulation of white clover seedlings was affected more by changes in soil pH than changes in soil Ca.

Table 5. Effects of soil amendments on soil pH, Ca and Al, and nodulation of red and white clover (Exp. 4). Soil amendments in Table 1 were applied in April, 1994. Soil samples were collected in May 1998 and analyzed. Extent of nodulation was evaluated by counting the number of nodules on the primary root to a depth of 10 cm. Nodules per plant are averaged across both white and red clover species, and across the three harvests in May 1998, August 1998 and May 1999. Data are the means ± SD.

Treatment number	Soil pH	Exchangeable Ca	Exchangeable Al	Nodulation
		— mg kg^{-1} dry soil —		nodules per plant
1	4.8 ± 0.1	220 ± 15	210 ± 12	6.8 ± 1.1
2	5.1 ± 0.1	520 ± 38	120 ± 10	19.6 ± 2.0
3	5.0 ± 0.1	520 ± 36	110 ± 8	21.2 ± 2.4
4	4.6 ± 0.1	470 ± 42	190 ± 15	7.8 ± 1.4

Table 6. Summary of the analysis variance of the effects of harvests, soil amendments and clover species on nodulation as measured as nodules per plant (Exp. 4).

Source of error	df[1]	Mean sum of squares	*F*-test	Significance[2]
Harvest (H)	2	1626.1	43.4	$P < 0.001$
Soil Amendment (A)	3	1325.0	35.4	$P < 0.001$
Clover Species (S)	1	25.8	0.0	ns
H × A	6	98.9	2.6	$P < 0.05$
H × S	2	57.1	1.5	ns
A × S	3	74.4	2.0	ns
H × A × S	6	42.3	1.1	ns

[1] df is an abbreviation for degrees of freedom.
[2] Probability that means are different; ns = not significant at $P < 0.10$.

Experiment 4: Field Trial

Nodulation of the primary root of red and white clover plants was followed over a year and half period to determine if trends apparent in the greenhouse studies were expressed under the field conditions. Four treatments from a larger field experiment were used that most closely resembled the conditions of Exp. 3. Soil amendment treatments are summarized in Table 2. Soil chemical properties in May, 1998 are presented in Table 5. Soil in Treatment 1 (control) had low levels of soil pH and Ca and high values for soil Al. Soils in Treatments 2 and 3 had higher levels of both soil Ca and pH than the soil in control plots. Soils in Treatment 4 had higher values for soil Ca than the control, but soil pH and Al were not significantly different from those for Treatment 1.

Number of nodules was significantly affected by harvests and treatments, but not by clover species (Table 6). Therefore, data regarding the effects of harvests and treatments were averaged across species. There was a significant interaction ($P < 0.05$) between harvest and treatment. Over the three harvests, nodules per plant were not significantly different between Treatments 1 and 4 and Treatments 2 and 3 (Table 5). Those soil treatments that increased soil pH and lowered exchangeable Al had greater values for nodules per plant (Table 5).

DISCUSSION

Effects of Liming on Root Growth and Development

The results in Exp. 1 and 2 demonstrated that addition of relatively small amounts of lime increased nodulation and root growth of white clover seedlings. Similar results were found by Staley and Morris (1998). Experiments on these studies were specifically designed to examine the effects of liming on the development and growth of different categories of roots that comprise the root systems of white clover seedlings. There is growing evidence that different roots respond differently to environmental stress (Zobel, 1992). However, the data base for such observations is relatively small. Bushamuka and Zobel (1998) found that different categories of maize (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] roots responded differently to an acidic subsoil. In this study, white clover seedlings growing in the acidic, unamended soil were able to form all the types of roots that comprise the root system of plants growing in limed soil (Fig. 3). The rate of formation of the three different categories of roots was reduced for seedlings growing in the unamended soil. The degree of inhibition among the three types of roots was different for seedlings in the unamended soil as compared to that in the limed soil (Fig. 1–3).

Early events in nodule formation are very similar to those of secondary root formation (Gresshoff, 1993). The hypothesis that acidic soil conditions interfere with early events in nodule differentiation may seem plausible if seedlings growing in acidic soil are unable to form certain secondary root structures. However, just the opposite was found: seedlings growing in acidic soil were able to form the same categories of roots as seedlings in limed soils (Fig. 3), although rate of formation was slower.

Effects of Changes in Soil Ca and pH on Nodulation

The main thrust of these experiments was to assess whether changes in soil Ca or soil pH were associated with changes in the nodulation of clover plants. In the experiments in this study, soil pH was increased by addition of $CaCO_3$ and soil Ca levels were increased by addition of $CaSO_4$ as a pure chemical or a coal combustion by-product or in combination with $CaCO_3$. There was a very strong negative correlation between soil pH and soil exchangeable Al in all of these experiments (Table 5 and data not shown). The discussion and results ascribed effects to changes in soil pH because soil pH is the parameter that was manipulated. Such references do not exclude the possibility that the effects of changes in soil pH are due in part or totally to changes in soil Al. In fact, many of the effects ascribed to soil pH may be due to soil Al. Brauer (1998) showed that Al is more toxic than protons to white clover roots. However, no attempt was made here to separate the effects of soil pH and soil Al because at present there is no adequate procedure to vary soil pH and exchangeable Al independently.

There was a very strong relationship between soil pH and nodulation of white clover seedlings in which soil Ca and pH levels were manipulated by the addition of $CaCO_3$, $CaSO_4$, or a combination of both (Table 4 and 5). Effects of changes in soil pH on nodulation did not appear to be due to differences in soil P availability. No significant differences in plant available soil P by the Bray I method or in plant P concentration were observed among treatments in these experiments (data not shown). Unlike previous experiments in which $CaSO_4$ addition decreased soil Al (Syed-Omar et al., 1990), the addition of $CaSO_4$ as a pure chemical or a coal combustion by-product to the Gilpin silt loam decreased soil pH and increased soil Al (Table 3 and 4). The differences in effects of $CaSO_4$ on soil pH between this study and Syed-Omar et al. (1990) could be caused by leaching of the amended soils with water in the previous studies. The addition of $CaSO_4$ to soils can lead to a reduction in soil Al when the Ca is allowed to displace Al from exchange sites and Al is leached from that portion of the soil profile (Shainberg et al., 1989). In Exp. 3, there was a strong positive relationship between the degree of nodulation and dry matter accumulation per plant (data not shown). In Exp. 4 (field trial), there was a strong positive relationship between nodulation and clover productivity and fraction of the stand as clover (D.B. Belesky and K.D. Ritchey, unpublished data). These results indicate that nodulation of clovers is affected more by soil pH/soil Al status than soil Ca levels. Such a conclusion is similar to that of Brauer (1998) in which the effects of solution pH, Al, and Ca on growth were examined. These results also suggest that those amendments like lime that change soil pH will be more effective in encouraging the nodulation and growth of clovers than those that change soil Ca like most coal combustion by-products.

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. Tom Staley and his technician Jerry Carter for preparing inoculum and advice regarding amending the Gilpin series soil.

REFERENCES

Albrecht, W.A. 1932. Calcium and hydrogen-ion concentration in the growth and inoculation of soybeans. Agron. J. 24:793–806.

Albrecht, W.A. 1933. Inoculation of legumes as related to soil acidity. Agron. J. 25:512–522.

Alva, A.K., and D.G. Edwards. 1990. Response of lupin cultivars to concentration of calcium and activity of aluminum in dilute nutrient solutions. J. Plant Nutr. 13:57-76.

Alva, A.K., D.G. Edwards, and C.J. Asher. 1991. Effects of acid soil infertility factors in mineral composition of soybeans and cowpea tops. J. Plant Nutr. 14:187–203.

Alva, A.K., D.G. Edwards, C.J. Asher, and S. Suthipradit. 1987. Effects of acid soil fertility on growth and nodulation of soybean. Agron. J. 79:302–306.

Balatti, P.A., H.B. Krishnan, and S.G. Pueppke. 1991. Calcium regulates growth of *Rhizobium fredii* and its ability to nodule soybean CV. Peking. Can. J. Microbiol. 37:542–548.

Brauer, D. 1998. Assessing the relative effects of hydrogen and aluminum ions on primary root growth of white clover seedlings. J. Plant Nutr. 21:2429–2439.

Bushamuka, V.N., and R.W. Zobel. 1998. Maize and soybean tap, basal, and lateral root responses to a stratified acid, aluminum-toxic soil. Crop Sci. 38:416–421.

Cahn, M.D., R.W. Zobel, and D.R. Bouldin. 1989. Relationship between root elongation and diameter and duration of growth of lateral roots of maize. Plant Soil 119:271–279.

Cardenas, L., J.A. Feijo, J.G. Kunkel, F. Sanchez, T. Holdaway-Clarke, P.K. Hepler and C. Quinto. 1999. *Rhizobium* Nod factors induce increases in intracellular free calcium and extracellular calcium influxes in bean root hairs. Plant Cell 19:347–352.

Clark, R.B., S.K. Zeto, K.D. Ritchey, R.R. Wendell, and V.C. Baligar. 1993. Coal combustion by-product use on acid soil: Effects on maize growth and soil pH and electrical conductivity. p. 131–154. ASA Spec. Publ. 58. ASA, Madison, WI.

Felle, H.H., E. Kondorosi, A. Kondorosi, and M. Schultze. 1998. The role of ion fluxes in Nod factor signalling in *Medicago sativa*. Plant J. 13:455–463.

Felle, H.H., E. Kondorosi, A. Kondorosi, and M. Schultze. 1999. Elevation of the cytosolic free [Ca^{2+}] is indispensable for the transduction of the nod factor signal in alfalfa. Plant Physiol. 121: 273–279.

Fusseder, A. 1987. The longevity and activity of primary root of maize. Plant Soil 101:257–265.

Gresshoff, P.M. 1993. Molecular genetic analysis of nodulation genes in soybeans. Plant Breed. Rev. 11:275–318.

Howieson, J.G., A.D. Robson, and M.A. Ewing. 1993. External phosphate and calcium concentrations and pH but not the products of rhizobial nodulation genes, affect the attachment of *Rhizobium meliloti* to roots of annual medics. Soil Biol. Biochem. 25:567–573.

Hyland, H.L. 1938. Comparison on legume growth in different soil types at varying acidity levels. Agron. J. 30:111–121.

Lynd, J.Q., and T.R. Ansman. 1989a. Effects of P, Ca with four K levels on nodule histology, nitrogenase activity and improved "Spanco" peanut yields. J. Plant Nutr. 12:65–84.

Lynd, J.Q., and T.R. Ansman. 1989b. Soil fertility effects on growth, nodulation, nitrogenase and seed lectin components of jack bean (*Canavalia ensiformis* (L.) D.C.). J. Plant Nutr. 12:563–579.

Miller, D.D., N.C.A. de Ruijter, and A.M.C. Emons. 1997. From signal to form: aspects of the cytoskeleton-plasma membrane-cell wall continuum in root hair tips. J. Exp. Bot. 48:1881–1896.

Peech, M. 1965. Hydrogen-Ion activity. p. 914–926. *In* C. A. Black (ed.) Methods of soil analysis; Part 2, Chemical and microbiological properties. ASA and SSSA, Madison, WI.

Purcino, A.A.C., and J.Q. Lynd. 1986. Soil fertility effects on growth, nitrogen fixation, nodule enzyme activity and xylem exudates of *Lablab purpureus* (L.) Sweet, grown on a Typic Eutrustox. Commun. Soil Sci. Plant Anal. 7:1331–1354.

Richardson, A.E., R.J. Simpson, M.A. Djordjevic, and B.G. Rolfe. 1988. Expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* is affected by low pH and by Ca and Al ions. Appl. Environ. Microbiol. 54:2451–2548.

Shainberg, I., M.E. Sumner, W.P. Miller, M.P.W. Farina, M.A. Pavan, and M.V. Fey. Use of gypsum on soils: A review. Adv. Soil Sci. 9:1–111.

Staley, T.E., and D.R. Morris. 1998. A model system for assessing low-level liming effects on white clover symbiosis development in an acidic soil. Soil Sci. 163:230–240.

Staley, T.E., R.J. Wright, and V.C. Baligar. 1993. Perennial forage legume growth in acidic soils from the major series of the Appalachian hill-lands. J. Plant Nutr. 16:573–587.

Sutton, J.M., E.J.A. Lea, and J.A. Downie. 1994. The nodulation-signalling protein NodO from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. Proc. Nat. Acad. Sci. (USA) 91:9990–9994.

Syed-Omar, S.R., Z.H. Shamsuddin, J.Y. Zuraidah, J.C. Wynne, and G.H. Elkan. 1990. Use of lime, gypsum and their combinations to improve nodulation and yield of groundnut in an acidic soil. p. 275–280. *In* R.J.Wright et al. (ed.) Plant-Soil Interaction at low pH, Proceedings of the second international symposium, Beckley, WV. 24–29 June 1990. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Thomas, G.W., and W.L. Hargrove. 1984. The chemistry of soil acidity. p. 3–56. *In* F. Adam (ed.) Soil acidity and liming, 2nd ed. Agron. Monogr. 12. ASA, CSSA, and SSSA, Madison, WI.

U.S. National Institutes of Health. 1998. NIH-Image, Release 1.62. USNIH, Rockville, MD. Available at http://rsb.info.nih.gov/nih-image/download.html, verified April 25, 2002.

Zobel, R.W. 1992. Root morphology and development. J. Plant Nutr. 15:677–684.