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Title: Influence of Soil Acidity upon Nodulation and Growth Characteristics of Trifolium subterraneum L.

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Previous research had identified four serogroups of Rhizobium trifolii indigenous to Abiqua soil (fine, mixed, mesic, Cumulic Ultic Haploxerolls). Nodulation of subterranean clover (Trifolium subterraneum L.) by two of the serogroups, 6 and 36, was influenced by the application of $CaCO_3$ to the soil. The studies described in this thesis showed that liming the soil with either $CaCO_3$, $Ca(OH)_2$, MgO, or K_2CO_3 increased significantly (P = 0.05) the percentage nodule occupancy by serogroup 36 whereas the percentage nodule occupancy by serogroup 6 was decreased by liming, but only significantly (P = 0.05) from applications of $CaCO_3$ or $Ca(OH)_2$. Application of KH_2PO_4 (25 mg P kg^{-1} soil), which did not change soil pH, nor increase P uptake by subclover plants, also increased significantly (P = 0.05) the percentage nodule occupancy by serogroup 36. Application of KH_2PO_4 in combination with $Ca(OH)_2$ produced the same increase in nodule occupancy by serogroup 36 as when the

two materials were applied individually. In contrast, although KH_2PO_4 addition had no influence on percentage nodule occupancy by serogroup 6, a combination of $Ca(OH)_2$ and KH_2PO_4 prevented the negative influence of $Ca(OH)_2$ when the latter was applied alone. Soil population of serogroup 36 consistently and in the majority of cases, significantly ($P = 0.05$) outnumbered serogroup 6 regardless of soil treatment or the outcome of nodulation. Soil chemical and plant analyses provided no evidence that liming was increasing the availability and subsequent uptake of soil P by the subclover plant. Liming did, however, result in significant transformation (30 to 50 mg P kg^{-1} soil) of inorganic P from the apatite and residual P fractions into a NaOH-extractable organic P fraction. Further studies under superior plant growth conditions provided evidence for differential behavior of the indigenous serogroups. Monobasic potassium phosphate, but not $Ca(H_2PO_4)_2$ or $Mg(H_2PO_4)_2$, increased significantly ($P = 0.05$) the percentage of nodules occupied by serogroup 6. All phosphate sources and KCl were found to stimulate the transformation of undifferentiated cells of serogroup 6 into bacteroid form. No such differential effects were observed on serogroup 36. Differences between the two serogroups in the distribution of cell size classes were observed in soil populations. A greater proportion ($\bar{x} = 49\%$) of the population of serogroup 36 passed through a 0.4 μm pore size filter than of the serogroup 6 population ($\bar{x} = 14\%$) when soil was analysed prior to planting.

Influence of Soil Acidity Upon Nodulation and Growth Characteristics of Trifolium subterraneum L.

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CHAPTER I

INTRODUCTION

Subterranean clover (Trifolium subterraneum L.), commonly called subclover, is the most important annual forage legume in Australia and in the Pacific Northwest of the USA. On a worldwide basis, subclover makes the greatest contribution of all the annual clovers to livestock feed production and soil improvement (McGuire, 1985). Subclover's unique seed burying ability (Murphy et al., 1973) makes it well suited for areas with shallow acid soils, with a summer season devoid of precipitation, and where frequent pasture renovation would be difficult and expensive. During the past fifty years, utilization of subclover has increased to provide major support for the successful production of livestock. In this regard, research on both basic and applied aspects of subclover and its microsymbiont Rhizobium trifolii has been quite extensive (Vincent, 1954, 1962; Gibson, 1971; Wilson, 1978; Brockwell, 1980; Knight et al., 1982; McGuire, 1985).

A major attribute of subclover is its ability to utilize atmospheric nitrogen in symbiotic association with the soil bacterium Rhizobium trifolii. As in any legume-Rhizobium association, the effectiveness of the subclover-R. trifolii symbiosis is influenced by numerous abiotic and biotic factors.

Considerable information on the abiotic and biotic factors which can affect the efficiency of legume-Rhizobium symbioses exists. These factors include plant varieties (Caldwell and Vest, 1977), Rhizobium strains (Schwinghamer, 1977), availability of photosynthate to the nodule (Hardy and Havelka, 1975), presence of combined nitrogen (Dazzo and Brill, 1978; Truchet and Dazzo, 1982; Sherwood et al., 1984; Harper and Gibson, 1984), moisture stress

(Sprent, 1972), temperature extremes (Gibson, 1971), and the ability of the soil to satisfy the nutritional requirements of both the legume and the rhizobia (Munns, 1977a; Robson, 1978, 1983).

The acreage of improved subclover pastures currently in production in Australia and the western USA is impressive. However, the species was established originally on marginally productive soils which were inherently acidic (pH 4.5 to 5.5) and/or limiting in phosphorus, sulfur, molybdenum, and occasionally potassium and boron (Jones, 1974; Jackson and Reisenauer, 1984). Many notable Australian scientists devoted significant portions of their research effort toward problems associated with the establishment of this species. From such research, findings were made of worldwide significance to improving the establishment of many legumes (Strong, 1937; Vincent, 1945; Anderson and Spencer, 1948; Loneragan et al., 1955; Gibson, 1964; Dudman and Brockwell, 1968; Chatel and Parker, 1973).

Recently, problems with productivity and/or reestablishment of subclover in both permanent and ley rotations have been reported for a range of soils in Australia (Carter et al., 1982; Reeves et al., 1984; Coventry et al., 1985a). A feature common to both situations is the association between the decline in production of subclover and the lowering of soil pH (Osborne et al., 1978, Bromfield et al., 1983a, b; Haynes, 1983).

It is well recognized that soil acidity can influence the productivity of a legume through its effect on the concentration of mineral nutrients (Munns, 1977a). Low pH, deficiencies of calcium, phosphorus and molybdenum, and

excessive quantities of aluminum, and manganese are the principal soil acidity factors in mineral soils (Schmel et al., 1950; Jackson, 1967; Clark, 1984; Foy, 1984). These factors either individually or in combination may affect any stage of the symbiotic process such as growth and survival of rhizobia in the soil (Bryan, 1923; Peterson and Goodding, 1941; Vincent and Waters, 1954; Mulder and van Veen, 1960; Rovira, 1961; Jones, 1966; Rice et al., 1977; Coventry et al., 1985a,b), multiplication of rhizobia in the rhizosphere (Mulder and van Veen,1960; Rovira, 1961), root hair infection, and nodule development and function (Munns, 1970; Andrew, 1976; Munns et al., 1977; Robson, 1978). Unfortunately, it is difficult to differentiate between the influence of acidity per se and acidity related stresses in a soil system. As a result of this problem, numerous nonsoil studies have been carried out to define more clearly the soil acidity related factors. In solution culture or in defined culture media, effects of pH, calcium, manganese, aluminum and phosphorus can be separated to a degree from each other and from other confounding factors (Munns, 1977b; Keyser and Munns, 1979; Munns and Keyser,1981; Thornton and Davey, 1983a; Beck and Munns, 1984, 1985; Whelan and Alexander, 1986). However, solutions having nutrient concentrations low enough to realistically reflect soil solution present certain problems. For example, solutions are poorly buffered, especially at low phosphorus and base concentrations (Andrew, 1978), and secondly, relative fluctuations in nutrient concentrations are large especially with aluminum (Cooper et al., 1983). Hence, interpretation of results can be difficult, particularly in aluminum toxicity studies because of the reduced toxicity of

aluminum when it is present as a soluble polymer (e.g. after reactions with hydroxyl or phosphate ions) (Blamey et al., 1983). Moreover, it is not easy to extrapolate with any degree of certainty from the nonsoil studies that the factors concerned will limit bacterial growth, nodulation, and/or growth of the host plant in the natural soil environment.

In addition to the complexities resulting from variations in soil chemistry, there is the biological diversity of legume species and rhizobial strains (Munns, 1977b). Differential tolerance of legume species to soil acidity has been clearly established and rhizobial strains also vary significantly in growth rates and in their capacity to nodulate a given host effectively at low pH (Vincent, 1965; Jones, 1966; Russel and Jones, 1975; Munns, 1977a; Bromfield and Jones, 1980; Jones and Morley, 1981; Thornton and Davey, 1983a, b; Thurman et al., 1985; Wood and Cooper, 1984, 1985; Wood et al., 1984a, b; Howeison and Ewing, 1986; Renwick and Jones, 1986).

In the remainder of this review, I will attempt to separate the literature into its major focal points regarding soil acidity effects on legumes and Rhizobium. The intention will be to develop a case that a more integrated approach is required, involving an understanding of the composition of Rhizobium in specific acid soils, the growth characteristics of the plant and the soil chemistry characteristics, if a better understanding is to be achieved.

Effects of Acidity on Rhizobia and Nodulation

The effects of acidity on the growth and survival of rhizobia and nodulation are well documented. Studies in culture media showed that low pH (4.5) increased the lag time, and/or decreased the growth rate of most of the cowpea and Bradyrhizobium japonicum strains tested (Keyser and Munns, 1979). With studies on soil-borne rhizobia, Bryan (1923) found that R. meliloti, R. trifolii and B. japonicum were unable to survive for 75 days in soils below pH 5.1, 4.9, and 4.2 respectively. In more recent studies (Rice et al., 1977; Barber, 1980), evidence has been obtained that the numbers of R. meliloti per gram soil are negatively correlated with soil pH. In other studies, it has been observed that R. trifolii numbers were reduced when soil pH was less than 4.9 (Robson et al., 1970; Rice et al., 1977). It has also been shown that reducing soil acidity by liming enhanced survival and proliferation of R. trifolii both in the absence (Vincent and Waters, 1954; Norris, 1959; Loos and Louw, 1965; Coventry et al., 1985a, b) and in the presence (Mulder and van Veen, 1960; Rovira, 1961) of the host plant. These results support the hypothesis that the effect of soil acidity on nodulation and nitrogen fixation can be a consequence of direct inhibition of Rhizobium survival, colonization and infectiveness (Vincent, 1965; Rice et al., 1977; Coventry et al., 1985a, b). Such possibilities would explain the findings of several authors that an increase in inoculum size can partly counteract inhibitions of nodulation by acidity [cited by Munns (1977b) and Jardim Friere (1977)]. However, other studies have shown that even with large populations established by heavy inoculation, nodulation remains sensitive to acidity

(Munns, 1968; Lie, 1969). In solution culture at pH 4.5, nodulation of peas (Pisum sativum L.) did not occur despite the presence of infective rhizobia (Mulder et al.,1966; Lie, 1969). Evans et al., (1980) reported that no nodules appeared below pH 4.8 in aeroponic culture of peas inoculated with Rhizobium leguminosarum, although 10^5 rhizobia cells/ml were still viable at pH 4.2 in solution culture after four days. Recently, solution studies carried out by Whelan and Alexander (1986) have shown that at pH 4.5, R. trifolii failed to nodulate subclover cv. 'Mt. Barker' and nodules were only formed at pH 4.8 and above. Such failure could not be ascribed to the poor growth of the rhizobia since the numbers were similar at pH 4.5 and pH 5.2 at three days after inoculation. The authors attributed the observed failure to the effect of acidity on the early stage of the infection process (Munns, 1968, 1970; Loneragan and Dowling, 1958; Lowther and Loneragan, 1968, 1970). It was observed that when subclover was transferred from a pH 5.0 solution to a pH 4.0 solution one day after inoculation, few nodules were subsequently formed, but when it was transferred two days after inoculation, there was almost no inhibition. When one reflects on these findings, the majority of these studies were carried out at extremely low pH values where productivity of most temperate legumes would be seriously inhibited.

Low pH and calcium deficiency tend to occur together in acid soils. Calcium is an important nutrient with an established role in the early stage of infection thread growth or nodule initiation (Loneragan and Dowling, 1958; Munns, 1965; O'Toole and Masterson, 1968; Robson and Loneragan, 1970). It

has been claimed that decreasing soil acidity by liming can facilitate nodulation due to the dual role of lime in increasing soil calcium and soil pH (Spencer, 1950). This argument was supported by the findings that neither increasing soil pH nor increasing calcium alone markedly improved nodulation of subclover; with a combination of both, excellent nodulation was observed. Interaction of calcium and pH on nodulation has been demonstrated with Trifolium species (Loneragan and Dowling, 1958; Small, 1968), Medicago sativa (Munns, 1970), and Glycine max (Vincent, 1965) in controlled media. Soil experiments have given results consistent with a calcium x pH interaction for the two former legume species (Munns, 1965; Vincent, 1965; O'Toole and Masterson, 1968) and Glycine weightii (Lee and Wilson, 1977). Magnesium does not substitute for calcium (Loneragan and Dowling, 1958; Munns, 1970), nor has Mg deficiency yet been found to specifically influence nodulation or nodule function (Munns, 1970).

Munns (1970) has indicated that nodulation of Medicago sativa was inhibited at calcium concentration less than 0.2 mM, regardless of pH. On the other hand, Loneragan and Dowling (1958) have shown that at pH 4 or less, no nodules were formed at any calcium concentration used. At 0.01 mM calcium, no nodules were formed at any pH used. Above these critical values, almost maximum nodulation could be obtained by increasing either calcium concentration or pH. The interaction of calcium and pH is explained in part by the influence of hydrogen ion in reducing the uptake of calcium (Arnon et al., 1942; Sutton and Hallsworth, 1958; Epstein, 1972; Andrew, 1976). Further, it

has been shown that the concentration of calcium in legume roots is low, relative to that in legume tops, and that enhanced calcium nutrition favors increased concentration in the legume tops without affecting calcium concentration in the roots (Andrew, 1976). Recently, Coventry et al. (1985a) have reported that the nodulation of subclover grown in acid soils low in calcium (0.6 - 0.8 meq Ca^{2+}/100 g) increased markedly when soil pH was raised by lime (80% $CaCO_3$). In contrast, the plants growing in unlimed soil had fewer but larger nodules. The increase in nodulation with lime persisted into the third growing season. Although the experiment was not designed to discriminate between the effects of pH and calcium, it is possible that the beneficial effects obtained from liming can be attributed to both an increase in pH and an increase in calcium.

At this point, it is worth digressing to compare Australian and Oregon soils of similar pH, where examples of the former have been reported to contain between 0.6 to 3 meq Ca^{2+}/100g (Bolland, 1985; Coventry et al., 1985a) whereas the latter may contain between 3 and 12 meq Ca^{2+}/100g. Obviously, comparisons of field data have to be treated cautiously within the context of the specific soil situation. Some evidence exists that soil acidity can influence the outcome of nodulation by mixtures of strains of Rhizobium. Some investigators have observed that changing the pH of the growth medium influences the host plant's preference for strains (Jones and Morley, 1981). Ham et al., (1971) observed that B. japonicum, serogroup 123, was the dominant occupant of the nodules of soybeans (Glycine max L.) grown in soils below pH 7.5. In calcareous soils of pH greater than 7.8, serogroup 135 was the dominant nodule

occupant. In white clover (T. repens L.) - R. trifolii associations, Jones et al., (1964) found a higher percentage of effective isolates in nodules of uninoculated plants sown in limed soil than in unlimed soil. However, when studies were conducted using mineral salts agar and an equal mixture of an effective and an ineffective strain of R. trifolii, the effective strain formed the majority of nodules at the lower pH values (Russel and Jones, 1975; Jones and Morley, 1981). Recently, Renwick and Jones (1986) evaluated the nodule occupancy of two cultivars of white clover, 'Milkanova' and of 'S 184'. Three months after inoculating cv. 'Milkanova' with a 1:1 mixture of an effective and an ineffective strain of R. trifolii, the percentages of nodules occupied by the effective strain were 64 and 29 percent in unlimed and limed soil respectively. In contrast, the nodule occupancy by the ineffective strain increased from 4 to 24 percent with mixed nodule infections also increasing from 9 to 22 percent after the soil was limed. No such differences were observed 14 months after inoculation and with cultivar 'S 184'. When plants were inoculated with unequal ratios of the two strains, the strain which dominated the inoculum also dominated the nodules regardless of lime rate. These results support the widely held basis for conventional methods of inoculation with maximum number of cells which aim to 'out-compete' the indigenous population for nodule occupancy (Holland, 1970; Diatloff and Brockwell, 1976). However, in soils which contain large numbers of indigenous rhizobia, the introduced rhizobia have to compete for nodule sites with the already established population in the acid soil. Little attention has been paid to the influence of acidity on the

nodulation by the indigenous rhizobia. Dughri and Bottomley (1983b, 1984) have reported that soil acidity influenced the distribution of indigenous serogroups of R. trifolii in the nodules of uninoculated subclover cv. 'Mt. Barker'. Serogroup 6 occupied 58 percent of the nodules of subclover grown in unlimed soil (pH 5.0), but when the soil was limed with $CaCO_3$ to raise the pH to 6.5, serogroup 6 was absent in the nodules. In contrast, other serogroups appeared with serogroup 36 being a significant nodule occupant in limed soil while it was virtually absent in nodules formed in unlimed soil.

A low level of plant available phosphorus is another important soil acidity related factor about which very little is known regarding effects on the soil rhizobia. An early study showed a positive growth response by soil-borne R. meliloti to phosphate additions (Truesdell, 1917). Keyser and Munns (1979) have demonstrated that in culture media, low phosphate concentration (5-10 μM) can limit the total yield of all cowpea and B. japonicum strains examined, and decreased the growth rate of some. Cassman et al., (1981a) observed differences between strains of cowpea rhizobia and B. japonicum with regard to growth at very low phosphate concentrations (0.05-1 μM). Similar variations have been reported for the ability to store phosphate when presented at high external concentration (Cassman et al., 1981b). Both these properties could be important for successful colonization and nodulation in phosphorus depleted soils.

There are reports in the literature which indicate that phosphate applications can improve nodulation of many legumes (De Mooy and Pesek,

1966; Crush, 1974; Gates, 1974; Munns, 1977a; Singleton et al., 1985; Rickerl and Touchton, 1986). Coventry et al., (1985a) observed an increase in nodule number of subclover in response to phosphate. Experiments of Jones (1986) have shown that an application of either dical-super or superphosphate improved the nodulation of subclover at planting. There are, nevertheless, reports that nodulation is not specially benefited by phosphate (Fellers, 1918; Perkins, 1924; Hallsworth et al., 1964). According to De Mooy and Pesek (1966), contradictions may arise from other soil variables which can interact with phosphate. In their own experiments, maximum nodulation required extremely high levels of applied phosphate and potassium salts.

Aside from low levels of phosphate, acid soils generally have high levels of aluminum (Kamprath, 1973). Studies have shown that aluminum is a potent inhibitor of rhizobial growth in acid culture media by causing either a reduction in multiplication rate through increased division time or cell death at the time of division (Munns and Keyser, 1981). Keyser and Munns (1979) found that 50 μM aluminum in a growth medium adjusted to pH 4.5 increased the lag time or slowed the growth of the strains of cowpea and B. japonicum which were otherwise tolerant of low pH. Such varied responses to acidity and aluminum were also observed among R. trifolii strains (Thornton and Davey, 1983a). Tolerance to acidity did not imply tolerance to aluminum in all cases. When acidity was reduced, the strains were capable of tolerating 40 μM aluminum. In the study of Wood and Cooper (1984), the inhibitory effect of 50 μM aluminum on the multiplication of R. trifolii was overcome by increasing the pH above 6.0,

or by increasing the phosphate concentration from 10 to 100 μM.

Nodulation of both tropical and temperate legumes is reduced or inhibited by aluminum (Carvalho et al., 1981; Carvalho et al., 1982; Murphy et al., 1984; Wood et al., 1984a, b). It is not clear, however, whether this results from an inhibition of rhizobial growth or from an effect on the infection and nodule initiation mechanisms. At this point, it needs to be reiterated that the behavior of aluminum in acid soils can be quite variable. Concentration and form of aluminum in soil solution can be different depending upon the type of acid soil being considered (Holford, 1983,1985; Hue et al., 1986).

Influence of Acidity on the Host Plant

Characterizing the effect of soil acidity on symbiotically grown legumes can be complex. Acid tolerance of legumes varies with the mode of nitrogen nutrition. Substantial evidence has been accumulated showing that when legumes are not dependent on nitrogen fixation, they have the same range of nutritional requirements and acid tolerance as found in other large and diverse plant groups (Munns, 1977b; Andrew, 1978). Plants dependent on nitrogen fixation are generally more acid-susceptible than nitrogen-fertilized plants (Loneragan and Dowling, 1958; Munns, 1965, 1978; Sartain and Kamprath, 1975; Andrew, 1978). A wide range of temperate and tropical pasture legumes, including Medicago, Trifolium and Glycine species, can grow comparatively well at pH 4.0 - 4.5, provided adequate combined nitrogen and calcium are supplied

(Andrew, 1976; Munns, 1965). This has been explained as being due to the sensitivity of either nodule function (Munns et al., 1977; Munns et al., 1979) or the initiation and development of nodulation (Munns, 1968; Lie, 1969) to acid conditions. Hence, it is not surprising that amelioration of soil acidity with lime is often beneficial to the growth of many legumes (Munns and Fox, 1977). However, it is often difficult to identify those specific limiting step(s) that have been alleviated by liming. This could be due in part to the large number of soil chemical properties that are altered when lime is applied. The most commonly cited effects include reduction in the activities of potentially toxic aluminum, manganese, and hydrogen ions and the increased availability of calcium, phosphate and molybdenum (Jackson, 1967).

Sanchez and Uehara (1980) have also advocated that liming increases phosphate availability. Nevertheless, the findings are equivocal since liming has been reported both to increase (Taylor and Gurney, 1965; Griffin, 1971; Ryan and Smillie, 1975), and decrease (Murrman and Peech, 1969; Amarasiri and Olsen, 1973; Haynes and Ludecke, 1981), or not affect (Taylor and Gurney, 1965; Janghorbani et al., 1975) the phosphate that can be extracted from soils. Similarly, the phosphorus content of plants has been observed to increase, decrease or remain unchanged following liming (Abruna et al., 1964; Amarasiri and Olsen, 1973; Janghorbani et al., 1975). Again, this controversial role of liming on phosphate availability can be attributed in part to the wide diversity of acid soil characteristics (Holford, 1983) and to the failure of the extractants commonly used in soil testing to define adequately the chemical environment of

the plant root (Curtin and Smillie, 1983).

Although subclover has been shown to be tolerant of soil acidity (Munns, 1965; Helyar and Anderson, 1970; Kim et al., 1985), there are reports in the literature indicating positive growth responses to lime. Anderson and Moye (1952) obtained growth response to 8 cwt of lime per acre which was attributed to a release of sufficient molybdenum from the soil for maximum crop production. Jackson et al., (1964) found that 6.7 metric tons of lime per hectare increased the yield and phosphorus uptake of subclover with no additional effect from 13.4 metric tons of lime per hectare. Coventry et al. (1985a, b) have demonstrated that liming (1 to 2.5 tons/ha) improved the yield of subclover grown in acid soils with low exchangeable calcium and high levels of manganese and aluminum. However, liming did not enhance molybdenum availability which was attributed to an absolute deficiency of molybdenum in this soil (Coventry et al., 1985b). In such cases, application of molybdenum together with lime might alleviate the low yield problem (Anderson and Moye, 1952). Petrie and Jackson (1982) observed lime x P and lime x K interactions on the yield of subclover. There are also reports that liming acid soils planted to subclover did not improve the dry matter yield. Bolland (1985) found that an application of two tons/ha of agricultural lime on sandy soils increased the soil pH from 5.5 to 5.8, from 4.9 to 5.6, and from 5.1 to 5.5 at three sites that had been planted to subclover for 10, 20 and 40 years respectively. However, lime had no effect on either dry matter production or seed yield of subclover. When nutrients (phosphorus, potassium, sulfur, copper, zinc, molybdenum, cobalt, manganese and boron) were applied,

the dry herbage yields improved significantly irrespective of the lime treatments. The omission of molybdenum from treatments of 20 and 40 year old pastures reduced herbage yields but had no effect on ten year old pasture. These results support the proposal that in some acid soils application of the growth limiting nutrients is more beneficial to an inherently acid tolerant legume than applying lime to correct the acidity related deficiencies. The response of subclover to lime can also be affected by soil moisture status. Using a soil high in aluminum, Hornsnell (1984) observed a 50% increase in the dry weight of plant tops when lime was added to low moisture treatments (70% of field capacity). In contrast, the response on high moisture treatment (100% field capacity) was negligible (5.6%).

As pointed out earlier, phosphorus deficiency is common in acid soils. It is not surprising therefore, that phosphorus application is very important in improving legume performance in acid soils. The need for phosphorus has been particularly evident in many parts of Australia where the application of phosphatic fertilizer to pasture is followed by spectacularly improved growth of the legume component (Vincent, 1965). The dry matter response of a legume to addition of phosphate is accompanied by a commensurate increase in the nitrogen concentration of the plant tops (Parsons and Davis, 1960; McLachlan and Norman, 1961; Andrew and Robins, 1969; Gates, 1974). It has been calculated that the additional nitrogen fixed consequent on the better growth, resulting from the application of 100 pounds of superphosphate, can amount to 76 pounds of nitrogen annually (Donald and Williams, 1954).

In summary, it is my contention that improved management strategies for legume production in acid soils require findings to be made from a more integrated approach where the composition of soil rhizobia is better characterized, and rhizobia, fertilizer and plant behavior in response to soil acidity are researched simultaneously. Thus, the objectives of this study were to: 1) characterize the effects of lime on the growth and nodulation characteristics of subclover; and 2) enumerate and follow the population dynamics of the indigenous serogroups of R. trifolii in response to soil amendments.

CHAPTER II

FURTHER STUDIES ON THE INFLUENCE OF SOIL ACIDITY UPON NODULATION OF SUBCLOVER BY INDIGENOUS *Rhizobium trifolii*

INTRODUCTION

For many years, factors associated with acid soils have often been diagnosed as impediments to the establishment and subsequent productivity of subterranean clover (Trifolium subterraneum L.). Although liming such soils tends, in general, to alleviate many establishment problems (Anderson and Spencer, 1948; Anderson and Moye, 1952; Loneragan et al., 1955), the critical steps being influenced by such a treatment can be numerous. For example, improvement in establishment and/or growth as a result of liming has been attributed to increased molybdenum availability (Anderson and Moye, 1952; Jackson et al., 1964; Dawson and Bhella, 1972; Petrie and Jackson, 1982) and/or phosphate availability (Jackson et al., 1964; Drlica and Jackson, 1979; Petrie and Jackson, 1982). Spencer (1950), however, suggested that liming had a dual role in increasing both the soil calcium level and soil pH which facilitated nodulation. Subsequently, a higher requirement for calcium in nodulation (Loneragan and Dowling, 1958; Lowther and Loneragan, 1968; O'Toole and Masterson, 1968) than for plant growth (Loneragan et al., 1968) was confirmed as well as an acid sensitive stage identified during root hair infection (Lowther and Loneragan, 1970).

Concerns have also been expressed for the influence of acid soil conditions on survival and proliferation of the microsymbiont, Rhizobium trifolii. Lime improved proliferation in both the absence (Vincent and Waters, 1954; Norris, 1959; Loos and Louw, 1965; Coventry et al., 1985a, b) and in the presence (Mulder and van Veen, 1960; Rovira, 1961) of the host plant. Norris

(1959) raised the issue, however, of whether the increase in divalent cation concentration or the increase in pH was really the critical factor. Recently, reports in the literature have shown variation between strains of R.trifolii regarding their abilities to either grow and/or to nodulate clover singly, or in competitive situations, under acid soil related stresses (Jones, 1966; Russel and Jones, 1975; Jones and Morley, 1981; Thornton and Davey, 1983a, b; Wood and Cooper, 1984, 1985; Wood et al., 1984a, b; Thurman et al., 1985; Renwick and Jones, 1986).

In this connection, the heterogeneous nature of an indigenous R. trifolii population in root nodules of subclover growing in an acidic (pH 5.0) soil was reported (Dughri and Bottomley, 1983a). The composition of the population recovered from root nodules of plants grown in unamended soil was different from that found in nodules of plants grown in the same soil after liming with $CaCO_3$ (Dughri and Bottomley, 1983b, 1984). The objectives of this study were to: 1) determine if liming materials with or without calcium would have the same effect as previously observed; 2) enumerate and to follow the population dynamics of the indigenous serogroups in response to soil amendments; and 3) explore the possibility of other interactive roles of lime independent of calcium of pH per se being the cause of the nodule occupancy changes.

MATERIALS AND METHODS

Four pot experiments were conducted. A randomized complete block design was used with four replications for experiments 1, 2, and 3, and six replications for experiment 4 respectively.

Soil

Surface (0 - 0.3 m) samples of soil from the Abiqua series (Cumulic Ultic Haploxerolls) were collected, mixed thoroughly and while moist (14 ± 0.2 w/w water) passed through a 2 mm mesh screen. Analyses were performed using the standard methods of the soil testing laboratory, Department of Soil Science, Oregon State University (Berg and Gardner, 1978) unless stated otherwise. The soil characteristics are presented in Table 1.

Experiment 1

A comparison was made between the effects of four liming materials ($CaCO_3$, $Ca(OH)_2$, MgO, and K_2CO_3) upon nodule occupancy by indigenous serogroups of R. trifolii. The rates of lime were based on their respective equivalent weights needed to raise the soil pH to 6.5 as determined by lime requirement measurements (Shoemaker et al., 1961). Each lime material (analytical grade) was mixed thoroughly with the soil samples. A supplemental

treatment of KH_2PO_4 added at a rate of 25 mg P kg^{-1} to unlimed soil was included. All the soil samples were brought to, and maintained at a water potential of 0.03 MPa (37 g water per 100 g dry soil) and allowed to equilibrate for four weeks at 21 ± 2°C. For every soil treatment, 0.4 kg soil were placed into each of four plastic-lined pots (0.083 m dia. x 0.11 m height) equipped with a perforated PVC tube (0.018 m dia) extending the full length of the pot to facilitate watering.

Ten to fifteen surface-sterilized seeds of subclover (Trifolium subterraneum L.). cv. 'Mt. Barker' were sown into each pot. The seeds were surface-sterilized following the method described elsewhere (Dughri and Bottomley, 1983a). Each pot was thinned to three plants, when the seedlings were three to 5 cm in height. The plants were grown and maintained under greenhouse conditions previously described (Dughri and Bottomley, 1983a). Ten weeks after sowing (early flowering stage), the plants were harvested and as many nodules as possible were recovered from the tap and lateral root systems of the three plants in each pot.

(i) Evaluation of nodule occupancy. Nodules were surface-sterilized by standard methods (Vincent, 1970) and twenty five nodules were selected at random. For each nodule, triplicate smears were prepared, stained with the non-crossreacting FITC-IgG conjugates of either serogroup 6, 27, or 36 respectively, and occupancy determined by immunofluorescence (Demezas and Bottomley, 1986).

(ii) Enumeration of the soil populations of serogroups 6, 27, and 36.

After harvest, subsamples of soil were taken from each pot, and rhizobia were extracted from 10 g portions using a modification of the method of Demezas and Bottomley (1986). To flocculate the soil colloids in the gelatin ammonium phosphate suspension, 1.83 g of a dry, finely-ground mixture of $CaCl_2 \cdot 2H_20$ and $MgCO_3$ was added (13.3:5 w/w was predetermined to be the most efficient flocculant ratio for Abiqua soil). The population densities of the three serogroups were determined by immunofluorescence (Demezas and Bottomley, 1986). The sequence of extraction and enumeration of rhizobia is summarized in Fig. 1. To evaluate whether the cells counted through immunofluorescence were viable or not, dilution series of the above soil samples were made over the range of 10^{-1} to 10^{-7}. Portions of each dilution were inoculated onto four replicate seedlings of subclover cv. 'Mt. Barker'. Ten weeks after inoculation, the plants were scored for nodulation and nodules were recovered from the final positive dilutions of each treatment. The nodule occupancy by serogroups 6 and 36 was evaluated by immunofluorescence.

Experiment 2

The effects of four phosphate levels (0, 25, 55, and 115 mg P kg^{-1} soil) upon growth and nodulation of subclover 'Mt. Barker' were evaluated. Monobasic potassium phosphate was mixed thoroughly with soil immediately prior to potting and seeding. To the four phosphate treatments, molybdenum (1

mg Mo kg^{-1}) as $Na_2Mo0_4 \cdot 2H_20$ and sulfur (22 mg S kg^{-1}) as K_2SO_4 were applied and a treatment consisting of unamended soil was included. For each treatment, 0.7 kg of soil were placed into each of four pots (0.083 m dia. x 0.22 m height); sowing and seedling thinning were performed as described in experiment one. Eleven weeks after sowing, plants were harvested, nodules were recovered, and nodule occupancy by serogroups 6 and 36 was evaluated as described in experiment one.

Plant analysis. Shoots and roots (devoid of nodules) were separated and oven-dried at 65°C to a constant weight. Plant samples were ground to pass a 1 mm screen, and redried prior to analysis for N, P, K, and S. Samples of root and shoot tissue were digested using the Kjeldahl method (Bremner, 1965) and analyzed for total N and P on a continuous flow analyzer, Model 200, using Technicon Method No. 334-74A/A. Potassium was extracted from composite shoot and root samples by nitric acid digestion followed by perchloric acid digestion (Jackson, 1974) and quantified using a Perkin-Elmer atomic absorption spectrophotometer (Model 4000). Composite shoot samples of each treatment were digested using the method of Tabatabai and Bremner (1970) and analyzed for S (Johnson and Nishita, 1952).

Experiment 3

The results from experiments one and two established a common link between the effects of $Ca(OH)_2$ and phosphate upon nodule occupancy by

serogroup 36. A factorial experiment was designed with two rates of both $Ca(OH)_2$, (0 and 4 g kg^{-1}) and of KH_2PO_4 (0 and 25 mg P kg^{-1}) to evaluate more comprehensively the impact of the treatments on: a) the populations of R. trifolii during the preplant equilibration period; b) selected soil chemical properties; and c) plant growth, P uptake, and composition of nodule occupancy. Soil used in each of the following three experiments was prepared as follows. A 10.5 kg portion of soil was limed with 4 g kg^{-1} of analytical grade $Ca(OH)_2$ and another 10.5 kg was unamended. Both soil samples were maintained at 0.03 MPa water potential and equilibrated for 20 days. Specific treatments in each experiment are described in the relevant subsections below.

(i) Experiment 3a. During the 20 day equilibration period, direct enumeration of serogroups 6 and 36 was determined at zero, six, and 20 days after liming by the immunofluorescence procedures described above.

(ii) Experiment 3b. After the 20 day equilibration period, KH_2PO_4 was added to subsamples of each soil which were potted (0.5 kg pot^{-1}) into four replicates per treatment. Soil samples were taken at time intervals (0, 5, 10, and 15 days) after application of KH_2PO_4. One replicate was utilized at each sampling time and the soil was divided for both soil solution analyses and for determination of extractable Al and P. Soil solutions were recovered from 400 g portions of soil by centrifugal displacement as described by Roseberg et al. (1986) with the following modifications. Soil solutions were composited by treatment and passed through a 0.4 μm pore size millipore filter. Aluminum, and

P were determined colorimetrically by the aluminon (Jayman and Sivasubramaniam, 1974), and molybdate blue (Murphy and Riley, 1962) methods respectively. Calcium, Mg, and K were determined by atomic absorption spectrophotometry.

(iii) Experiment 3c. Different portions of the equilibrated unlimed and limed soil samples were amended uniformly with Mo and S, and selectively with the respective KH_2PO_4 treatment. Soil was potted (0.7 kg pot^{-1}) into four replicates per treatment, and growth, plant analysis, and nodule occupancy were evaluated as described above.

Experiment 4

Results from experiment three revealed that liming the soil with $Ca(OH)_2$ to raise the soil pH to 6.5 did not increase soil solution P, extractable P, nor total P uptake by subclover plants. Hence, an experiment was conducted to: a) determine whether liming had an influence on the distribution of soil P during the preplant period; and b) to evaluate the growth response of subclover to lime under superior growth conditions (larger quantities of soil and two plants per pot).

Three rates of $Ca(OH)_2$ (0, 1, and 4 g kg^{-1}) were used, which adjusted the soil pH to 5.0, 5.8, and 6.4 $\pm$ 0.1 respectively during the 20 day equilibration period. Molybdenum and S were applied to all treatments as described above.

For each replication in each treatment, the amendments were mixed thoroughly and 2.7 kg of soil per pot were brought to and maintained at a water content of 75% of 0.03 MPa (29 g H_2O per 100 g soil). Immediately prior to planting, subsamples were taken from each replication of each treatment and composite samples were prepared for soil solution and P fractionation analyses. Extraction and analyses of soil solution were done as described in experiment 3. The fractionation of inorganic P and organic P was carried out according to the procedure of Hedley et al. (1982) with the following minor modification. Concentrated HCl was used in place of concentrated H_2SO_4 for digesting the residual P remaining after the fractionation scheme (Fig. 2).

Subclover seeds were sown, thinned to two per pot, and grown as described above. Plants were harvested at flowering (10 weeks of age), and yield and analyses of N, P, and K were determined as described above.

RESULTS

Regardless of the composition of the material, liming significantly (P = 0.05) increased nodule occupancy by serogroup 36 (Table 2). Nodule occupancy by serogroup 6 was lower in all lime treatments, yet the decrease was only significant (P = 0.05) in the calcium-containing treatments. Liming did not influence significantly the nodule occupancy by indigenous serogroup 27. There was a concurrent trend for a substantial portion of the nodules still occupied by serogroup 6 in the lime treatments to be now multi-occupied. A phosphate application (25 mg kg^{-1}) , which did not raise the soil pH, also increased significantly (P = 0.05) the percentage nodule occupancy by serogroup 36, but had no effect on nodule occupancy by serogroup 6. Indeed, nodules co-occupied by members of both serogroups 6 and 36 increased substantially as a result of the phosphate application, from approximately one quarter of nodules occupied by serogroup 6 in unamended soil to two thirds after P treatment. Neither the numbers of nodules formed, (ranging between 21 and 42/plant) nor the shoot dry weight yields of the plants were significantly affected by the treatments under these growth conditions (data not shown).

The population sizes of the serogroups were evaluated in post harvest soil samples. Although the populations of serogroups 6 and 36 were of a similar magnitude, (10^4 g^{-1}), in all cases serogroup 36 outnumbered serogroup 6 by 2.5 to 5.5 fold (Table 3). Significant differences (P = 0.05) between the populations of serogroups 6 and 36 were observed in unamended, $Ca(OH)_2$,

K_2CO_3, and KH_2PO_4 treatments. Although there was a nonsignificant trend for the populations of both serogroups 6 and 36 to be greater in the amended soils regardless of treatment, no evidence was obtained for the soil treatments having resulted in selective proliferation of one serogroup over the other. The direct counts of the soil populations were comparable with those obtained by evaluating nodule occupancy of terminal dilutions of plant infection-soil dilution tests (Table 4). These data suggest that the cells of both serogroups 6 and 36 observed by immunofluorescence, were indeed viable, possessed nodulating capability, and made up similar and significant portions of the total soil R. trifolii population.

Experiment 2

Application of 25 mg P kg^{-1} immediately prior to planting resulted in a significant increase ($P = 0.05$) in percentage of nodule occupancy by serogroup 36, but had no influence on the occupancy by serogroup 6 (Table 5). No significant differences were observed between the percentages of nodules occupied by serogroup 36 at any of the three rates of phosphate applied. The percentage of nodules co-occupied by serogroups 6 and 36 was significantly higher ($P = 0.05$) in the treatment receiving Mo and S than in unamended soil, and was further increased ($P = 0.05$) by phosphate application.

Shoot dry weight was increased significantly ($P = 0.05$) by the combined Mo and S application, but no further increase in response to phosphate

application was observed (Table 6). The improved yield is assumed to be due to the Mo application. Although the latter was not measured in plant tissue, S and K concentrations were not affected by the Mo and S treatment and the total uptake of K and S was directly proportional to yield increases observed in response to the treatment. Significant increases in shoot and root P uptake were only observed when 115 mg P kg^{-1} soil was applied. The P concentrations of shoots and roots in all the other treatments ranged between 0.23 to 0.29 percent and 0.14 to 0.2 percent respectively. These values are above the levels considered to be critical for subclover growth.

Experiment 3

Application of a combination of $Ca(OH)_2$ and 25 mg P kg^{-1} soil as KH_2PO_4 resulted in the same increase in nodule occupancy by serogroup 36 which occurred when both materials were applied separately (Table 7). Lime and P in combination, however, prevented the reduced occupancy by 6 which was observed in the presence of lime alone. The incidence of co-occupancy by both serogroups 6 and 36 increased significantly ($P = 0.05$) as a result of P application and was independent of the presence or absence of lime. The direct counts of both serogroups 6 and 36 in the soil during the equilibration period prior to planting revealed that proliferation of serogroups 6 and 36 had occurred during the incubation period (Table 8). However, the population size of serogroup 36 outnumbered serogroup 6 regardless of soil treatment, or the

outcome of nodulation.

No evidence was obtained from either analysis of plant growth or nutrient content to suggest that the common stimulation by lime or phosphate upon occupancy by serogroup 36 occurred concomitantly with lime stimulating P uptake in the plant as a result of soil P becoming more available. A nonsignificant increase in total plant dry weight of 26% in response to P was accompanied by a significant ($P = 0.05$) suppression of yield in response to liming (Table 9). In addition, although total P uptake in the unlimed plus P treatment was 44% greater than the unlimed control, liming suppressed total P uptake by 22% in the minus P treatment. These observations correlate with the values of Bray extractable solution (0.03 N NH_4F and 0.025 N HCl) phosphate obtained on soil samples taken throughout the 15 d period after P addition (Table 10). Liming had reduced the level of extractable soil P by 0.4 mg P kg^{-1} during the 20 d period required for pH adjustment. This magnitude of reduction (0.3 to 0.5 mg P kg^{-1}) was maintained at least throughout the first 15 d after planting. In contrast, P addition raised extractable P by 0.6 to 0.9 mg P kg^{-1} throughout the same 15 d period after P addition. In addition, the level of extractable Al in unamended soil was low (98 ± 6 µmol 100 g^{-1}). Phosphate additions reduced extractable Al by a small amount (4 to 8 µmol 100 g^{-1}) whereas, as expected, lime reduced extractable Al to a level below detection limits.

Experiment 4

Neither yield nor P uptake responses to two different rates of $Ca(OH)_2$ were observed when plants were grown in larger quantities (2.4 kg) of soil (data not shown). Potassium was the only nutrient influenced markedly by liming, with both total content and concentration of K in roots being substantially lower (18 mg g^{-1}) at the higher rate of lime than in unamended soil (27 mg g^{-1}).

Although there was no marked influence of lime on either extractable or solution P (data not shown), the former did affect the portions of P distributed between the inorganic and organic fractions in the preplant soil samples. Regardless of rate, liming increased the percentage of organic P with a concomitant decrease observed in the inorganic P fraction (Table 11). The major portion of the decrease in inorganic P was accounted for in the 1 M HCl extractable P and residual P fractions whereas the concomitant increase in the organic P was observed in the 0.1 M NaOH extractable and residual P fractions (Table 12).

DISCUSSION

The data presented in this study expand upon the previous findings on the effect of $CaCO_3$ on nodule occupancy of subclover by indigenous R. trifolii (Dughri and Bottomley, 1983b, 1984). In those studies, it was hypothesized that the improved nodulation by indigenous serogroup 36 could have been a result of selective proliferation of the latter from a lower population base in the acid soil than serogroup 6. Circumstantial evidence to support this hypothesis has been presented in the literature. Liming has been shown to enhance the proliferation of Rhizobium trifolii both in the absence (Vincent and Waters, 1954; Norris, 1959; Loos and Louw, 1965; Coventry et al., 1985a, b) and in the presence (Mulder and van Veen, 1960; Rovira, 1961; Jones, 1966) of the host plant. The results of this study, however, provided no evidence for this possibility. Serogroup 36 outnumbered serogroup 6 in unlimed or limed soil, independent of sampling time and nodulating success. Although the relative numbers of serogroups 6, 27 and 36 bore no resemblance to the outcome of nodulation, we can infer, since the population densities were significantly different, that the data are revealing preliminary information about 'saprophytic competence' within the indigenous population. Although the latter phrase was introduced to Rhizobium research almost twenty years ago (Chatel et al., 1968), little evidence has been forthcoming until recently (Demezas and Bottomley, 1986) from nonsterile soil studies to suggest that strains do indeed differ in this respect.

The increase in nodules occupied by serogroup 36 as a result of

phosphate addition is noteworthy. Several hypotheses were attractive since, if proven, they would relate concepts of soil fertility, and link other nonsoil Rhizobium research to this phenomenon. 1) Liming the acid soil increased the availability of soil P to either the soil microorganisms and/or the plant and thereby connected the influence of lime to that of P application (Haynes, 1982; Holford, 1985). 2) Both P and lime were reducing a toxic concentration of soil solution aluminum to a level nontoxic to serogroup 36 (Thornton and Davey, 1983a, b: Wood et al., 1984a, b: Wood and Cooper, 1984, 1985). The results of this study revealed that liming did not enhance P uptake by the subclover plant, nor did P reduce an already low soil solution level of Al. Liming however, did stimulate a significant transformation of inorganic P into an organic P form during the preplant equilibration period. The magnitude of this transformation (ca. 30 to 50 mg P kg^{-1} soil) is within the limits of values reported in the literature for soil microbial biomass P which range between 10 and 100 mg P kg^{-1} soil (Hedley and Stewart, 1982; Hedley et al., 1982; Brookes et al., 1984; Sparling et al., 1985; McLaughlin et al., 1986; West et al., 1986). Liming has also been shown to increase soil microbial biomass (Adams and Adams, 1983; Carter, 1986). Our data are supportive of the possibility that liming is resulting in soil inorganic P becoming more available for immobilization by the soil micro-organisms, or liming is simply releasing acidity-related inhibition of P immobilization by the soil microbial population. Studies carried out under nonsoil conditions have shown that a representative of serogroup 36 has inferior capability of growth and survival than a member of serogroup 6 at low

phosphate concentrations typical of Abiqua soil solution. Moreover, 36 was also less efficient in nodulating subclover than 6 under nonsoil phosphate limiting conditions (K.T. Leung, M.S. thesis, 1987, Oregon State University).

The response of nodule occupancy by serogroup 6 to soil amendments was more complex than serogroup 36. Although $Ca(OH)_2$ and $CaCO_3$ reduced the nodule occupancy by serogroup 6 thus confirming our previous findings (Dughri and Bottomley, 1983b, 1984), in this study noncalcium-containing liming materials did not reduce the nodule occupancy by serogroup 6 significantly. Moreover, although the presence of phosphate alone had no effect on nodule occupancy by serogroup 6, the presence of P with $Ca(OH)_2$ counteracted the negative effect of the latter material in reducing nodule occupancy by serogroup 6. Exactly how phosphate counteracts the effect of $Ca(OH)_2$ is not known. The results have provided however, evidence for an interaction between pH, calcium, and phosphate on the nodule occupancy by serogroup 6. A further assessment of the behavior of serogroup 6 in response to soil amendment is presented in Chapter III.

In this study the conditions which stimulated occupancy by serogroup 36, but which did not reduce occupancy by serogroup 6 resulted in significant proportions of nodules being co-occupied by both serogroups. These findings support other recent findings that co-occupancy can be substantial in nodules of soil grown legumes (May and Bohlool, 1983; Demezas and Bottomley, 1986; Renwick and Jones, 1986). Obviously, conditions which improve the nodulating

capability of one group of indigenous organisms do not have to result in exclusion of another group from nodules. Increased incidences of co-occupancy have been reported with 1:1 mixtures of two strains of R. trifolii as a result of raising the pH on agar-grown (Jones and Morley, 1981) or the application of lime to field-established (Renwick and Jones, 1986) white clover (T. repens L. cv. 'Milkanova').

Dispersion of Soil (10 g) and Extraction of Bacteria

1. add 50 ml PHG/AP
   (shake 15 min)

2. add 45 ml PHG/AP
   (shake 15 min)

Flocculation of Soil Colloids

1. add 1.8 g $CaCl_2$:$MgCO_3$
   (13.3:5, shake 2 min)

2. leave undisturbed for 1 h

Filtration of Cleared Supernatant

1. filter portions of cleared
   supernatant (0.4 μm pore size,
   nuclepore membranes)

Enumeration of Retained Bacteria

1. treat filter with Rhodamine gelatin
   conjugate (60°C 1 h)

2. stain filter with fluorescent
   conjugate (1 h)

3. destain filter (100 ml of 0.02M
   PB, pH 7.2)

4. enumerate by epifluorescence

Figure 1. Methods for extraction and enumeration of R. trifolii in Abiqua soil.

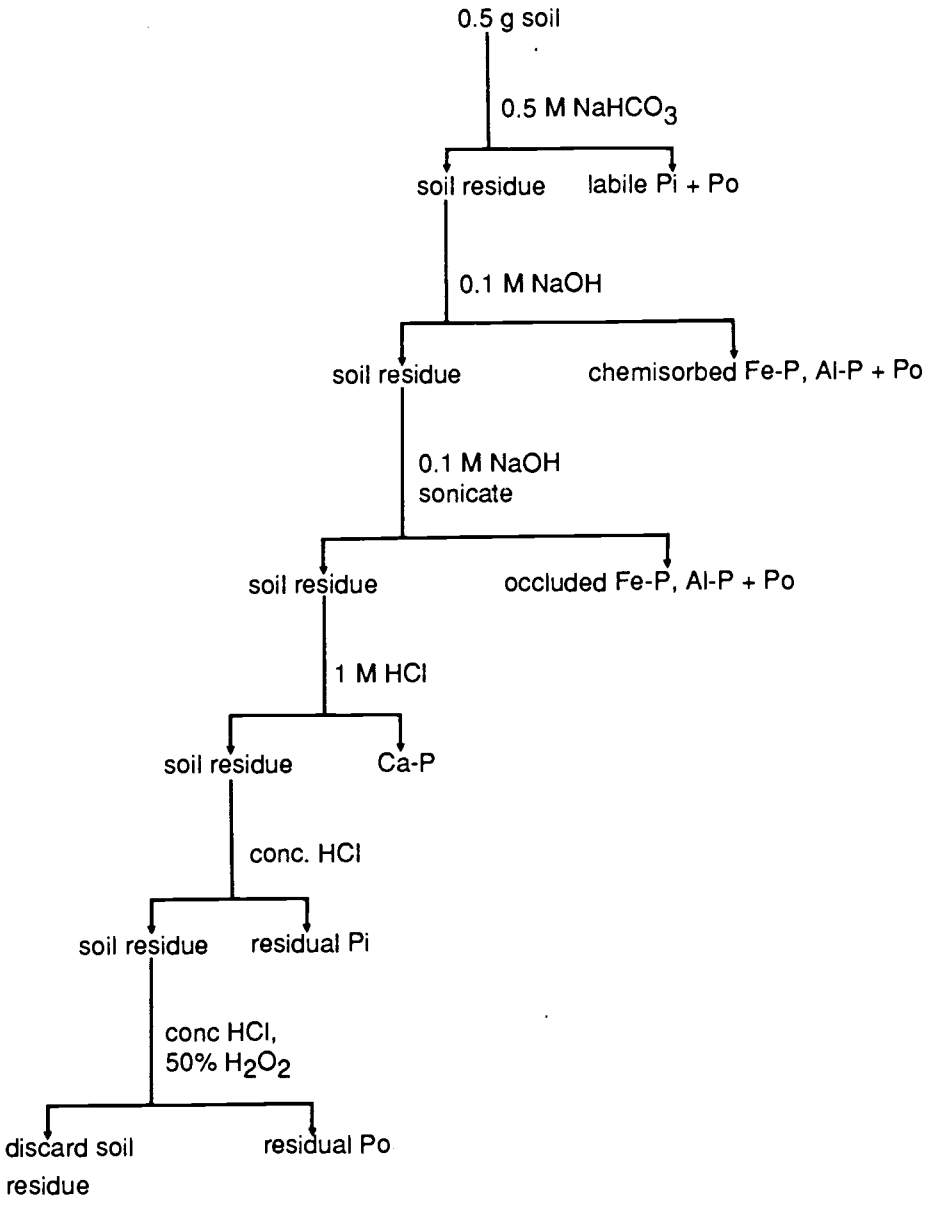

Figure 2. Flow scheme of the fractionation of soil phosphorus.

Table 1. Chemical and physical characteristics of Abiqua soil.†

pH‡		Texture			Organic	Total	Extractable P					Exchangeable Bases			
H_2O	($CaCl_2$)	Sand	Silt	Clay	Carbon	N	Olsen	Bray	KCl-Al§	DTPA-Mn	CEC	K	Ca	Mg	Na
		g 100 g^{-1}					mg kg^{-1}					cmol(+) kg^{-1}			
5.5	4.7	26.2	34.9	38.9	3.0	0.24	5-6	1-5	24.8	79.4	30.6	0.78	12.5	5.6	0.18

†Soil classification: Abiqua SiCl fine, mixed, mesic, Cumulic Ultic Haploxeroll; soil chemical analyses were determined following the standard procedures of the soil testing laboratatory, Dept. of Soil Science, Oregon State University (Berg and Gardner, 1972).

‡Measured in solution/soil ratio of 2:1 (vol/wt); concentration of $CaCl_2$ was 0.01M.

§Extractable Al determined by the Aluminon method after extraction with 1M KCl (Jayman and Sivasubramaniam, 1974).

Table 2. Nodule occupancy by indigenous serogroups of Rhizobium trifolii on subclover 'Mt. Barker' as affected by source of lime and phosphate applications.

Soil Treatment[†]	Nodule occupancy[‡]					
	Serogroups					
	6	36	27	6M	36M	Others[§]
	%					
Unamended	50.0	15.0	17.0	12.0	12.0	35.0
$CaCO_3$	13.0	52.0	18.0	13.0	17.0	34.0
$Ca(OH)_2$	16.5	32.8	20.0	12.0	7.0	52.8
MgO	29.0	46.0	25.0	18.0	19.0	29.0
K_2CO_3	27.0	47.3	26.0	19.0	18.0	31.0
KH_2PO_4	49.3	45.3	28.3	32.3	30.0	23.5
$LSD_{0.05}$[¶]	23.9	13.9	NS	NS	NS	NS

[†]Soil pH values immediately prior to sowing the seed were 5.2, 5.3, and 6.6± 0.2 for unamended, phosphate, and lime amended soils respectively.

[‡]Mean of four replications per treatment; percentages reported for serogroups 6, 27, and 36 include both singly occupied and co-inhabited nodules; 6M represents nodules occupied by combinations of serogroup 6 with 27 and 36; 36M represents nodules occupied by combinations of serogroup 36 with 6 and 27.

[§]Nodules not reacting with fluorescent antibody conjugates specific for serogroups 6, 27, and 36.

[¶]LSD, least significant difference; NS, not statistically significant at P = 0.05.

Table 3. Population densities of indigenous serogroups of Rhizobium trifolii in soil after nodule harvest.†

Soil treatment	Serogroups†		
	36	6	27
	No. x 10^5 g^{-1}soil		
Unamended	0.14a	0.05b	0.01bc
$CaCO_3$	0.29a	0.05ab	0.01b
$Ca(OH)_2$	0.42a	0.18b	0.02bc
MgO	0.33a	0.08a	0.02a
K_2CO_3	0.43a	0.12b	0.01c
KH_2PO_4	0.36a	0.09b	0.01c

†Mean population of four replicates/treatment determined by immunofluorescence; numbers within a soil treatment and not followed by the same letters differ significantly at P = 0.05 according to T- tests for paired comparisons made between serogroups 6 and 36, 6 and 27, as well as 36 and 27.

Table 4. Nodule occupancy by indigenous serogroups of Rhizobium trifolii in the terminal positive dilutions of a plant infection-soil dilution assay.

Soil treatment	Terminal soil dilution with nodulated plants	Nodule occupancy†		
		Serogroups		
		6	36	Others‡
		%		
Unamended	10^{-6}, 10^{-4}	41.3	3.8	66.3
$Ca(OH)_2$	10^{-5}, 10^{-5}	40.0	5.0	64.0

†Mean of two replicate soil samples/treatment.

‡Nodules not reacting with fluorescent antibody conjugates specific for serogroups 6 and 36.

Table 5. Nodule occupancy of subclover 'Mt. Barker' as affected by applications of molybdate (Mo), sulfate (S) and phosphate (P).†

Soil treatment‡	Nodule Occupancy§			
	Serogroups			
	6	36	M¶	Others#
	%			
Unamended	80.0	18.0	15.0	17.0
Mo + S	91.0	29.0	27.0	6.0
Mo + S + 25mg P kg^{-1}	81.5	46.5	39.3	10.1
Mo + S + 55mg P kg^{-1}	92.0	54.0	48.0	4.0
Mo + S + 115mg P kg^{-1}	94.0	45.0	45.0	6.0
$LSD_{0.05}$††	NS	12.8	9.6	8.5

†Mean of four replications/treatment.

‡Molybdenum, S, and P were applied immediately prior to sowing seeds at rates of 1, and 22 mg kg^{-1} soil for Mo, and S respectively.

§Percentages reported for serogroups 6 and 36 include both the singly occupied and co-inhabited nodules.

¶Nodules co-occupied by serogroups 6 and 36.

#Nodules not reacting with fluorescent antibody conjugates specific for serogroups 6 and 36.

††LSD, least significant difference; NS, not statistically significant at P = 0.05.

Table 6. Dry matter yield and nutrient concentration and uptake of subclover 'Mt. Barker' as affected by molybdate, sulfate, and phosphate.†

Plant Part	Soil treatment‡	Dry weight	Nutrient Concentration				Nutrient uptake			
			N	P	K	S	N	P	K	S
		g pot^{-1}	mg g^{-1}				mg pot^{-1}			
Shoot	Unamended	2.0	31.8	2.9	19.9	2.0	62.1	5.7	38.9	3.8
	Mo + S	2.6	36.0	2.3	20.4	2.0	94.2	5.9	53.5	5.1
	Mo + S + 25mg P kg^{-1}	2.6	32.9	2.4	21.5	2.1	86.4	6.3	57.6	5.3
	Mo + S + 55mg P kg^{-1}	2.6	34.6	2.9	21.2	2.1	89.6	7.6	56.0	5.7
	Mo + S + 115mg P kg^{-1}	3.1	36.2	3.4	23.2	2.6	108.7	10.2	70.2	7.9
	$LSD_{0.05}$§	0.6	NS	0.7	NS	ND	16.4	2.3	15.1	ND
Root	Unamended	0.24	23.7	2.5	11.5	NM¶	5.9	0.6	2.8	NM
	Mo + S	0.43	27.0	1.5	13.2	NM	11.6	0.6	5.7	NM
	Mo + S + 25mg P kg^{-1}	0.31	23.0	1.4	11.2	NM	7.4	0.5	3.5	NM
	Mo + S + 55mg P kg^{-1}	0.37	24.7	1.7	14.4	NM	9.4	0.7	5.3	NM
	Mo + S + 115mg P kg^{-1}	0.42	28.1	3.9	19.2	NM	11.6	1.6	8.1	NM
	$LSD_{0.05}$	NS	NS	1.0	ND		NS	0.5	ND	

†Mean of four replications/treatment, with the exceptions that K and S were analyzed on composite samples.

‡Molybdenum, S, and P were applied as described in Table 5.

§LSD, least significant difference; NS, not significantly different at P = 0.05; ND, statistical analyses not determined.

¶NM, values not measured.

Table 7. Nodule occupancy of subclover 'Mt. Barker' as affected by lime and phosphate applications.†

Soil treatment‡	Nodule dry weight	Nodule Occupancy§			
		Serogroups			
		6	36	M¶	Others#
	mg plant^{-1}	%			
Unlimed, -P	16.1	44	19	8	45
Unlimed, +P	17.6	34	38	15	41
Limed, -P	17.4	25	36	8	47
Limed, +P	17.2	35	38	15	43
$LSD_{0.05}$††	NS	10.9	11.6	1.8	NS

†Mean of four replications/treatment.

‡Soil pH values immediately prior to sowing seeds were 5.2 and 6.4± 0.1 for unlimed, and limed soils respectively; P rate equal to 25 mg P kg^{-1} soil was applied immediately prior to sowing; Mo, and S were applied to all treatments just prior to sowing at rates of 1 and 22 mg kg^{-1} soil respectively.

§Percentages for 6, and 36 include both singly occupied and co-inhabited nodules.

¶Nodules co-occupied by serogroups 6 and 36.

#Nodules not reacting with fluorescent antibody conjugates specific for serogroups 6 and 36.

††LSD, least significant difference; NS, not statistically significant at P = 0.05.

Table 8. Population sizes of Rhizobium trifolii serogroups 6 and 36 during the soil incubation after liming and prior to planting.

Time (d)	Soil treatment	Serogroups† 6	36
		Number x 10^5 g^{-1} soil	
0	Control‡	0.24	0.46
7	Unamended§	0.34	0.55
	Limed¶	0.36	0.58
20	Unamended	0.85	1.25
	Limed	0.82	1.21

†Mean of three replications for control at 0 day; mean of 2 replications per treatment at 7 and 20 days.

‡Unamended soil prior to incubation.

§Unamended soil maintained at 0.03 MPa water potential and at 21 ± 2°C.

¶Soil limed with $Ca(OH)_2$ and maintained identical to the unamended treatment. Soil pH values were 6.6 and 6.4 ± 0.1 at 7 and 20 days after liming respectively.

Table 9. Total dry matter yield and nutrient uptake of subclover 'Mt. Barker' as affected by lime and phosphate applications.†

Soil Treatment‡	Total plant dry weight§	Total nutrient uptake¶		
		N	P	K#
	g pot^{-1}	mg pot^{-1}		
Unlimed, -P	1.9	67.2	4.5	39.0
Unlimed, +P	2.4	82.4	6.5	50.0
Limed, -P	1.3	53.7	3.7	35.0
Limed, +P	1.7	58.5	4.4	33.6
$LSD_{0.05}$††	0.6	NS	NS	ND

†Mean of four replications/treatment.

‡Soil pH values immediately prior to sowing seeds were 5.2 and 6.4 ± 0.1 for unlimed and limed soils respectively; P rate equal to 25 mg P kg^{-1} soil was applied immediately prior to sowing; Mo and S were applied to all treatments just prior to sowing at rates of 1 and 22 mg kg^{-1} soil respectively.

§Sum of shoot and root dry weights.

¶Sum of shoot and root nutrient uptake.

#Composite plant samples were analyzed for shoot and root K.

††LSD, least significant difference; NS, not statistically significant at P = 0.05; ND, not determined.

Table 10. Influence of the addition of phosphate upon extractable P in unlimed and limed soils.†

Soil treatment	Days after P application			
	0	5	10	15
	mg P kg^{-1}			
Unlimed, -P	0.7	0.9	1.1	0.9
Limed, -P	0.3	0.6	0.6	0.5
Unlimed, +P	1.4	1.5	1.8	1.8
Limed, +P	0.9	0.4	0.8	0.8

†Phosphate at 25 mg P kg^{-1} dry soil was applied after 21 days of equilibration; soil pH values were 5.3 and 6.4 ± 0.2 for unlimed and limed soils respectively.

Table 11. Influence of lime upon the distribution of phosphorus in Abiqua soil.†

Lime Rate	Phosphorus Fractions‡		
	Inorganic	Organic	Total
	mg P kg^{-1}		
g kg^{-1}			
0	866 (64.2)	483 (35.8)	1349
1	852 (61.3)**	537 (38.7)**	1389
4	834 (61.9)*	514 (38.1)*	1348
$LSD_{0.1}$	2.0	2.0	
$LSD_{0.05}$	2.8	2.8	

†Soil was maintained at 75% of 0.03 MPa capacity and 21 ± 2°C; soil pH values prior to P fraction analysis were 5.4, 5.8 and 6.4 ± 0.1 for 0, 1 and 4 g $Ca(OH)_2$ kg^{-1} soil respectively.

‡Mean of duplicate composite samples per treatment; fractionation of inorganic and organic P was carried out according to the procedure of Hedley et al. (1982); values enclosed in parentheses represent the percentage in relation to total P.

**, *Significantly different from the unlimed treatment at P = 0.05, and P = 0.1 respectively.

Table 12. Distribution of phosphorus in samples of unlimed and limed Abiqua soil.†

P fractions	Lime rate (g kg^{-1}) 0	1	4	Percent change§ due to lime application
		mg P kg^{-1}		
Inorganic P (Pi)				
0.5 M $NaHCO_3$	17.4 (1.3)‡	16.2 (1.2)	18.9 (1.4)	0
0.1 M NaOH	304.1 (22.5)	318.3 (22.9)	310.6 (23.0)	+0.5
1 M HCl	124.6 (9.2)	120.4 (8.7)	117.3 (8.7)	-0.5
Residual	419.6 (31.1)	397.2 (28.6)	387.3 (28.7)	-2.5
∑Pi fractions	865.7 (64.2)	852.1 (61.3)	834.1 (61.9)	-2.6
Organic P (Po)				
0.5 M $NaHCO_3$	44.8 (3.3)	37.3 (2.7)	39.3 (2.9)	-0.5
0.1 M NaOH	299.3 (22.2)	362.9 (26.1)	322.7 (23.9)	+2.8
Residual	138.9 (10.3)	136.8 (9.9)	151.5 (11.3)	+0.3
∑Po fractions	483.0 (35.8)	537.0 (38.7)	513.5 (38.1)	+2.6
Total P (Pi + Po)	1348.7	1389.1	1347.6	

†See Materials and Methods for details of soil treatments.

‡Values in parentheses represent the percentage of total soil P.

§Difference in percentage between the unlimed and the mean of the percentages of the two lime levels.

CHAPTER III

CATION AND PHOSPHATE INFLUENCES ON INDIGENOUS SEROGROUPS OF *R. trifolii*: EFFECTS ON SOIL POPULATION, NODULATION AND MORPHOLOGY WITHIN THE NODULES

INTRODUCTION

Subterranean clover (Trifolium subterraneum L.) is the most important annual forage legume on both the Australian continent and in the Pacific Northwest of the U.S.A. (McGuire, 1985). The majority of the acreage was established originally on marginally productive soils which were either inherently acidic (pH 4.5 to 5.5) and/or limiting in phosphorus, sulfur, molybdenum and occasionally potassium and boron (Jones, 1974; Jackson and Reisenauer, 1984). In a few well documented cases, establishment was further impeded by indigenous R. trifolii which were predominantly ineffective on subclover (Ireland and Vincent, 1968; Holland, 1970; Jones et al., 1978; Hagedorn, 1978; McGuire et al., 1978). Hence, the benefits of applying phosphate (Cass Smith, 1939), lime (Anderson and Spencer, 1948), molybdenum (Anderson, 1942) and the appropriate inoculant technology (Cass Smith, 1939; Jenkins et al., 1954; Loneragan et al., 1955) to the establishment of subclover were recognized early.

In recent years, problems with productivity and/or reestablishment of both permanent and ley rotation subclover pastures have become widely documented in Australia. Thirty to forty years of annual superphosphate applications and the build-up of soil nitrogen has resulted in pH declines accompanied by increases in aluminum and manganese to potentially toxic levels (Donald and Williams, 1954; Williams and Donald, 1957; Williams and David, 1976; Williams, 1980; Bouma et al., 1981; Carter et al., 1982; Bromfield et al., 1983a, b; Coventry et al., 1985a). These observations have led researchers

to reexamine the tolerance of subclover to soil acidity-related factors (Osborne et al., 1981; Jarvis and Robson, 1983a; Kim et al., 1985; Alva et al., 1986a, b). Concerns about the sensitivity of R. trifolii to acid soil conditions have also resurfaced (Coventry et al., 1985b; Jones and Curnow, 1986).

In regard to this problem, it is now well recognized that the nature of acid soils can be quite diverse which is reflected in the variable, and often unpredictable responses of both the soil chemistry and crop response to liming (Haynes, 1982; Holford, 1983, 1985; Curtin and Smillie, 1983, 1984, 1986a, b; Sorn-srivichai et al., 1984). In addition, cultivars of subclover can vary in response to acidity related stresses such as manganese (Osborne et al., 1981); and aluminum (Kim et al., 1985), and show variation in the magnitude of cation/anion imbalances in plant tissue (Jarvis and Robson, 1983b). To further complicate matters, variations in growth, nodulation, and competitive nodulation by different strains of R. trifolii to acid soil related stresses have been documented (Russell and Jones, 1975; Hagedorn and Caldwell, 1981; Jones and Morley, 1981; Thornton and Davey, 1983a, b; Wood and Cooper, 1984, 1985; Renwick and Jones, 1986).

Criticism has been extended to many of the nonsoil acidity related studies on clovers for not using concentrations and ratios of ions more typical of soil solution (Blamey et al., 1983; Jarvis and Hatch, 1985; Kim et al., 1985; Alva et al., 1986a, b). In the previous chapter, no evidence was found that liming the Abiqua soil was enhancing soil P uptake by the subclover plants. Of some surprise, therefore, was the lack of a significant plant growth response to added

P despite the extremely low extractable P in the soil. In addition, although the influence of lime and phosphate on nodule occupancy by serogroup 36 seemed relatively simple, the factors influencing nodule occupancy by serogroup 6 seemed more complex with evidence indicating pH x cation x phosphate interactions.

The objectives of this study were: 1) to evaluate the P requirements of subclover in Abiqua soil under superior plant growth conditions; and 2) to further characterize the influence of P and associated cations upon the soil population and nodulating characteristics of serogroups 6 and 36.

MATERIALS AND METHODS

A greenhouse experiment was performed using surface samples of soil from the Abiqua series, which were collected and screened as described in Chapter II.

Experimental Design and Phosphate Treatments

Two rates (0 and 55 mg P kg^{-1}) and three sources [$Ca(H_2PO_4)_2 \cdot H_2O$, $Mg(H_2PO_4)_2$, and KH_2PO_4] of phosphate were used. For each phosphate source, a corresponding minus phosphate control treatment was included containing an equivalent amount of the respective cation as the chloride salt ($CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, and KCl respectively). Molybdenum and S were applied to all treatments at rates of 1 and 22 mg kg^{-1} respectively. Molybdenum was applied as $Na_2MoO_4 \cdot 2H_2O$, while the cation associated with the sulfate salts corresponded to the respective phosphate source ($CaSO_4 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ and K_2SO_4 respectively). For each replication in each treatment, the amendments were mixed thoroughly with 2.7 kg soil, placed in plastic pots (0.19 m dia. x 0.17 m height), brought to, and maintained at a water potential corresponding to 75% of 0.03 MPa (equivalent to 29 g H_2O per 100 g soil), and allowed to equilibrate for 7 days at 21 ± 2°C. To minimize moisture loss, each pot was covered with a plastic sheet. The pots were arranged in a randomized

complete block design with six replicates under the greenhouse conditions described earlier.

Preplant Measurements

After the equilibration period, and immediately prior to planting, soil was taken from each pot, mixed thoroughly and returned to the pots. Subsamples were taken for soil solution analyses, and for enumeration of serogroups 6 and 36 by immunofluorescence using the procedures described in Chapter II with the following modifications. Lactic acid and formaldehyde were added to the supernatants obtained after flocculation to a final concentration of 0.1% (v/v) and 2% (v/v) respectively. The supernatants were filtered through 0.4 µm pore size Nuclepore filters. The numbers of cells of serogroup 6 or 36 on each filter were determined by immunofluorescence.

Planting and Post Harvest Measurements

Unless specified, sowing of seeds, growing and harvesting of plants, and post harvest determinations were carried out as described in Chapter II. Each pot was sown with 10 to 15 surface-sterilized seeds of subclover cv. 'Mt. Barker' which were later thinned to two per pot. After nine weeks, the plants were harvested and soil samples of each treatment were taken from each pot, and composited for direct enumeration of serogroups 6 and 36 by immuno-

fluorescence. Dry weights, and N, P, and K contents of the shoots and roots were determined as described in Chapter II.

Nodule occupancy by serogroups 6 and 36 was determined by immunofluorescence as described in Chapter II. Nodule smears positive to either serogroup 6 or 36 were evaluated for the percentage of cells observed to be in either the undifferentiated or in the bacteroid form. Short rod-shaped cells (2.8 to 3.5 µm in length; 1.0 to 1.4 µm width) were scored as undifferentiated cells; pleiomorphic or elongated, irregular rod-shaped cells (>3.5 µm in length) were scored as bacteroids.

RESULTS

Both the total dry matter yield and shoot dry-weight of subclover plants were increased significantly ($P = 0.01$) by the addition of 55 mg P kg^{-1} soil regardless of source (Table 13). Increases in root dry-weight to the addition of phosphate were consistent but not statistically significant. No significant difference in nodule number was noted. Despite the marked growth response, addition of 55 mg P kg^{-1} soil resulted in only a small increase in either Bray extractable P, (2 to 5 mg P kg^{-1}) or in the Olsen bicarbonate extractable P (6 to 10 mg P kg^{-1}).

All P sources significantly ($P = 0.01$) increased the N and P content of shoots although they had no significant effect on either shoot N or P concentrations (Table 14). Applications of KCl and KH_2PO_4 resulted in 15 to 20 percent, and 25 to 30 percent increases respectively, in shoot K concentrations over the other non-K treatments. In the case of roots, the differences in K concentrations were even more dramatic ranging between 48 and 69 percent greater than the respective non-K treatments.

Although no interaction between the phosphate source and growth of the plants was observed, the percent nodule occupancy by serogroup 6 was influenced by the phosphate source. Fifty-one percent of the nodules formed in the KH_2PO_4 treatment were occupied by serogroup 6 (Table 15). This value was significantly greater ($P = 0.05$) than all the non-phosphate and also the $Ca(H_2PO_4)_2$ treatments. In contrast, percentage nodule occupancy by

serogroup 36 was decreased by the addition of phosphate, with significant differences only observed for potassium and calcium phosphate treatments. There was no significant difference in nodule occupancy by serogroup 36 observed within phosphate sources. The morphologies of 6 and 36 within nodules of a specific treament were often quite different (Table 16), with the former being influenced by the treatment. Occupants of serogroup 36 were predominantly found in bacteroid form, regardless of treatment. The morphology of serogroup 6 inside the nodules was influenced by the treatments. With the exception of the KCl treatment, 95% of the cells of serogroup 6 in 56 to 60 percent of the nodules occupied by 6 were undifferentiated (Table 16). Both calcium and magnesium phosphates resulted in a significant increase ($P = 0.1$) in the proportion of serogroup 6 occupants in the bacteroid form suggesting a role for phosphate, replaceable with K, in facilitating the transformation of cells of serogroup 6 into the bacteroid form inside the nodule.

In either preplant or post harvest soil samples, serogroup 36 out-numbered serogroup 6 and the numbers were not differentially affected by any soil treatment (Table 17). Differences in cell size between the two serogroups, however, were also apparent. In general, a greater percentage of the total number of cells of serogroup 36 than of serogroup 6 passed through a 0.4 μm filter in both preplant and post harvest samples. In the preplant soil samples, no influence of treatment was observed on the proportions of 36 retained on 0.4 μm and 0.2 μm filters, whereas the absolute numbers and percentage of serogroup 6 cells passing through a 0.4 μm filter were greater in the phosphate treatments.

However, in the post harvest samples, the differential influence of treatment on the size classes of serogroup 6 was no longer apparent. Distribution of the size classes of serogroups 6 and 36 was also determined in unamended soil which had been equilibrated over the same time period as the experimental treatments. In this case, both the absolute number and the percentage of 0.2 μm filter-retained cells of serogroup 36 were similar to those values obtained from treated soil (ca.40%). The percentage of serogroup 6 retained on the 0.2 μm filter was equal to 21% of the total and therefore more similar to the values obtained in the phosphate ($\bar{x}$ = 19.4%) than in the nonphosphate ($\bar{x}$ = 8.9%) treatments.

The data suggest that cations and/or chloride, but not phosphate can be implicated in the transition of the cells of serogroup 6 to a larger size class. In this regard, analyses of soil solutions showed that the ionic concentrations of both Ca^{2+} and Mg^{2+} were raised in all of the nonphosphate treatments by at least two-fold, whereas phosphate treatments had no effect on the soil solution cation concentrations despite equivalent amounts of cations having been added (Table 18).

DISCUSSION

The findings presented here further illustrate the complexities of the factors influencing nodulation by the indigenous serogroups and especially serogroup 6. The data illustrate an interaction between cations and phosphate upon the soil population during the preplant incubation, an influence of specific phosphate source on the percent nodule occupancy by indigenous serogroup 6, and an influence of phosphate on bacteroid development by members of serogroup 6, which could be substituted by K ion.

The reason for nonphosphate salts influencing the percentage of the preplant population of serogroup 6 capable of passing through a 0.4 μm filter and a lack of influence on serogroup 36 is unknown, but worthy of some comment. Although numerous studies have shown that a significant percentage of soil microbes can be abnormally small in size (Casida, 1971; Bae et al., 1972; Balkwill et al., 1975; Jenkinson et al., 1976; Lundgren, 1984: Bakken, 1985; Bakken and Olsen, 1987; Olsen and Bakken, 1987), this study is the first of its kind to show different size classes within an identifiable species and, more specifically, a different distribution within specific serogroups of the species. Bae and Casida (1973) observed that additions of water to air-dried soil resulted in an increase in the size of 'small' cells without any significant increase in cell numbers, and which was accelerated by the addition of NH_4Cl. Recently, Bakken and Olsen (1987) have found that only 0.2% of small cells in soil (diameter <0.4 μm) were viable as determined by recoverability on nutrient agar.

Obviously, the question of the significance of this cell size distribution on the nodulating capabilities and autecology of R. trifolii requires more intensive study.

The differential influence of the source of P on the nodulation success by indigenous serogroup 6 has not been recorded previously in Rhizobium literature. Munns (1977a) and Robson (1978) have both commented on the lack of information on mineral requirements of rhizobia and their influence on saprophytic and nodulating behavior in soil. Beck and Munns (1984, 1985) recorded that certain strains of R. meliloti required higher levels of Ca^{2+} to grow at soil solution P levels than in luxury P - containing growth medium. There is a precedence for cation by P interactions in P uptake in other prokaryotes (Rigby et al., 1980; Poindexter, 1984) and in higher plants (Robson et al., 1970). Representatives of serogroups 6 and 36 have different critical P levels, 0.45 μM and 1 μM respectively, for growth under bacterial growth medium conditions where the concentrations of Ca^{2+} and Mg^{2+} cations are somewhat similar to those found in unamended soil solution reported here (K-T., Leung, M.S. Thesis 1987, Oregon State University, Corvallis). Hence, further work is required to evaluate the possible significance of cation x P interactions on P uptake and nodulation by representatives of these two serogroups.

Although there are numerous reports on the influence of host and Rhizobium genotype on bacteroid morphology, the only other study where influences of growth conditions impacted differentially on bacteroid development by two R. trifolii strains involved a temperature variable (Gibson, 1963; Pankhurst and Gibson, 1973). Two strains of R. trifolii, names NA3O and TA1,

differed in their temperature requirements for bacteroid transformation. Above a critical temperature, strain NA30 would not transform into bacteroids in contrast to TA1. Analogies can be drawn to this study where serogroup 36 was able to transform effectively into bacteroids under the same conditions where serogroup 6 was having difficulty. The results of this study suggest that efficient transformation of serogroup 6 cells from the undifferentiated to a bacteroid form required a higher K concentration than that of serogroup 36. The exact role of K in transformation is obscure and beyond the scope of this study. It is possible, however, that the effect of K was through the plant as a result of increasing translocation of photosynthate to the nodules (Mengel et al., 1974; Barta, 1982). Alternatively, K might have a direct effect on the cells of serogroup 6. Gober and Kashket (1986), observed that Bradyrhizobium sp. strain 32H1 required a high external K level under low $p0_2$ to maintain a low membrane potential, which is characteristic of this strain when fixing N_2. Perhaps there are analogies here for a role of K in the transformation of cells of serogroup 6 into the N_2 fixing bacteroid form.

In our previous study, the lack of plant growth response to applied phosphorus seemed unusual, considering the low extractable P status of the Abiqua soil and the high affinity and capacity of this soil for inorganic P (Leung and Bottomley, unpublished observations). The possibility that a P effect on nodulation had occurred through the plant, yet a potential yield response was subsequently overshadowed by other growth limitations, convinced us that

larger volumes of soil, superior plant growth conditions, and improved fertilizer application procedures should be adopted. In this regard, we were successful in obtaining a yield response to the phosphate applications (Table 13). We can only speculate upon the impact, if any, of either the changes occurring in the proportions of nodules occupied by different serogroups, or the bacteroid transformation which occurred in response to P and/or K fertilization, on the N_2 fixing capability of the plants. Singleton et al. (1985) have reported that an effect of P on yield of soybeans was not consistent and was dependent on the nature of the inoculant strain.

Some aspects of the study contradict with our earlier findings. The positive influence of P on nodulation by serogroup 36 reported in Chapter II was not observed. Furthermore, KH_2PO_4 resulted in an increase in percent nodule occupancy by serogroup 6, whereas no influence was observed in experiments reported in Chapter II. Again, an analogy can be drawn to results published from recent soybean studies. Kosslak et al. (1983) and Kosslak and Bohlool (1984) reported that the primary nodulation of soybean by USDA 138 suppressed further nodulation by a secondary inoculum, USDA 110, applied 2 to 7 d later. The suppression of the secondary inoculum was more complete under suboptimum winter growth conditions, and less complete when the plants were growing more vigorously under summer conditions where more nodules were formed on the plants. In contrast to our earlier studies, better growth conditions produced an improved yield per plant, a yield response to phosphate, two to three fold more nodules per plant and a greater overall incidence of

bacteroids in nodules. Perhaps it can be hypothesized that the improved growth vigor of the host plant and the increasing requirement for N_2 fixing nodules overrode the influence of P on the nodulating capabilities of serogroup 36.

In this study, large volumes of soil were brought in from the field in rather cold climatic conditions and soil manipulation was rather extensive in order to mix fertilizers thoroughly. Recent literature on soil biomass evaluations has drawn attention to the perturbations in biomass which can occur as a result of conventional sampling, screening, mixing and preincubation prior to biomass assays (Sparling et al., 1985; McLaughlin and Alston, 1985; Carter, 1986; McLaughlin et al., 1986; West et al., 1986). One can only compare these concerns with the extensive manipulations which are routinely carried out in order to thoroughly equilibrate fertilizer materials with soils (Borkert and Barber, 1985a, b; Fox et al., 1986; Yao and Barber, 1986). In retrospect, since our results provide evidence for an influence of mineral nutrients and soil fertility concepts upon the soil microflora, more attention will need to be given to develop techniques which satisfactorily address both microbiological and fertility concepts when studying legume-Rhizobium interactions in soil.

Table 13. Dry matter yield and nodule number of subclover 'Mt. Barker' as affected by various sources of phosphate.

Soil Treatment‡	Nodule Number	Dry Matter Yield†		
		Root	Shoot	Total§
	No. pot^{-1}	g pot^{-1}		
P_o - $CaCl_2$	139	0.44	1.9	2.3
P_o - $MgCl_2$	152	0.42	1.8	2.2
P_o - KCl	157	0.46	1.7	2.2
P_1 - $Ca(H_2PO_4)_2$	149	0.52	2.6	3.1
P_1 - $Mg(H_2PO_4)_2$	136	0.49	2.4	2.9
P_1 - KH_2PO_4	144	0.50	2.4	2.9
$LSD_{0.05}$¶	NS	NS	0.3	0.3
0.01	NS	NS	0.5	0.5

†Values are means of six replications/treatment.

‡Mo, S, and P sources were applied to all treatments one week prior to sowing seeds at rates of 1, 22 and 55 mg kg^{-1} dry soil respectively.

§Sum of the shoot and root dry weights.

¶LSD, least significant difference; NS, not statistically significant.

Table 14. Nutrient concentration and uptake by subclover 'Mt. Barker' as affected by various sources of phosphate.†

Plant Part	Soil Treatment‡	Nutrient					
		N	P	K	N	P	K
		mg g^{-1}			mg pot^{-1}		
Shoot							
	P_o - $CaCl_2$	29.9	1.6	25.1	48.2	3.0	46.8
	P_o - $MgCl_2$	28.3	1.9	25.6	51.0	3.4	47.0
	P_o - KCl	27.1	1.9	29.9	46.4	3.2	51.6
	P_1 - $Ca(H_2PO_4)_2$	25.8	2.1	23.6	65.9	5.3	60.9
	P_1 - $Mg(H_2PO_4)_2$	26.6	2.0	23.2	63.0	4.7	55.2
	P_1 - KH_2PO_4	26.2	2.0	29.5	64.0	4.9	72.5
	$LSD_{0.05}$	NS	NS	ND	6.7	0.7	ND
	$LSD_{0.01}$§	NS	NS	ND	8.9	1.0	ND
Root							
	P_o - $CaCl_2$	23.1	2.0	17.0	10.5	0.9	7.6
	P_o - $MgCl_2$	23.0	2.1	13.6	10.0	0.9	5.8
	P_o - KCl	23.0	2.2	23.1	10.6	1.0	10.6
	P_1 - $Ca(H_2PO_4)_2$	21.6	2.2	13.7	11.3	1.1	7.1
	P_1 - $Mg(H_2PO_4)_2$	22.3	2.2	17.6	11.2	1.1	8.6
	P_1 - KH_2PO_4	22.0	2.1	26.7	11.1	1.1	13.4
		NS	NS	ND	NS	NS	ND

†Mean of six replications/treatment for N and P values; mean of duplicate composite samples for K values.

‡Mo, S, and P were applied to all treatments as described in Table 13.

§LSD, least significant difference; NS, not statistically significant; ND, statistical analyses not determined.

Table 15. Nodule occupancy by indigenous serogroups of R. trifolii on subclover 'Mt. Barker' as affected by various sources of phosphate.

Soil Treatment†	Nodule Occupancy‡			
	Serogroups			
	6	36	M§	Others¶
	%			
P_o - $CaCl_2$	26.0	31.3	4.7	47.3
P_o - $MgCl_2$	31.3	25.3	4.0	47.3
P_o - KCl	34.7	34.7	9.3	40.0
P_1 - $Ca(H_2PO_4)_2$	33.3	18.0	3.3	52.0
P_1 - $Mg(H_2PO_4)_2$	42.0	20.0	2.0	40.0
P_1 - KH_2PO_4	51.3	21.3	8.7	36.0
$LSD_{0.05}$#	14.5	8.7	NS	NS

†Soil treatments are as described in detail in legend to Table 13.

‡Values are means of six replications/treatment; percentages reported for serogroups 6 and 36 include both the singly occupied and co-inhabited nodules.

§Nodules co-occupied by serogroups 6 and 36.

¶Nodules not reacting with fluorescent antibody conjugates specific for serogroups 6 and 36.

#LSD, least significant difference; NS, not statistically significant.

Table 16. Influence of soil treatment on the proportion of the nodules which were occupied by undifferentiated or bacteroid forms of the indigenous serogroups of R. trifolii.

Soil treatment†	Serogroups‡			
	6		36	
	U§	B¶	U	B
	%			
P_o - $CaCl_2$	56.2	43.8	5.7	94.3
P_o - $MgCl_2$	59.5	40.5	12.7	87.3
P_o - KCl	23.2	76.8	6.0	94.0
P_1 - $Ca(H_2PO_4)_2$	31.7	68.3	9.7	90.3
P_1 - $Mg(H_2PO_4)_2$	36.7	63.3	2.8	97.2
P_1 - KH_2PO_4	43.2	56.8	11.8	88.2
$LSD_{0.05}$	24.8	24.8	NS	NS
$LSD_{0.1}$	20.5	20.5	NS	NS

†Soil treatments were as described in Table 13.

‡Values are means of six replications/treatment.

§Ninety five ± 5 percent of the cells per field which reacted to the fluorescent antibody conjugates specific to either serogroup 6 or 36 were in undifferentiated form.

¶Five to 100 percent of the cells per field which reacted to the fluorescent antibody conjugates specific to either serogroup 6 or 36 were in bacteroid form.

NS, not statistically significant.

Table 17. Populations of serogroups 6 and 36 of R. trifolii prior to planting and after nodule harvest.

Soil treatment†	Filter pore size (μm)					
	0.4		0.2		Total	
	Serogroups					
	6	36	6	36	6	36
	No x 10^{-5}/g dry soil					
Preplant§						
P_o - $CaCl_2$	3.2 (86.5)‡	15.5 (64.0)	0.5 (13.5)	8.7 (36.0)	3.7	24.2
P_o - $MgCl_2$	6.1 (93.8)	12.2 (43.6)	0.4 (6.2)	15.8 (56.4)	6.5	28.0
P_o - KCl	3.9 (92.9)	13.1 (47.5)	0.3 (7.1)	14.5 (52.5)	4.2	27.6
P_1 - $Ca(H_2PO_4)_2$	4.5 (80.4)	19.6 (59.6)	1.1 (19.6)	13.3 (40.4)	5.6	32.9
P_1 - $Mg(H_2PO_4)_2$	4.9 (86.0)	9.1 (40.6)	0.8 (14.0)	13.3 (59.4)	5.7	22.4
P_1 - KH_2PO_4	4.0 (75.5)	17.5 (51.5)	1.3 (24.5)	16.5 (48.5)	5.3	34.0
Post harvest¶						
P_o - $CaCl_2$	2.5 (71.4)	20.3 (56.5)	1.0 (28.6)	15.6 (43.5)	3.5	35.9
P_o - $MgCl_2$	5.2 (83.6)	18.6 (63.7)	0.9 (16.4)	10.6 (36.3)	6.1	29.2
P_o - KCl	2.9 (76.3)	20.6 (54.6)	0.9 (23.7)	17.1 (45.4)	3.8	37.7
P_1 - $Ca(H_2PO_4)_2$	2.9 (70.7)	27.5 (57.5)	1.2 (29.3)	20.3 (42.5)	4.1	47.8
P_1 - $Mg(H_2PO_4)_2$	5.3 (86.9)	22.1 (49.7)	0.8 (13.1)	22.4 (50.3)	6.1	44.5
P_1 - KH_2PO_4	3.5 (79.5)	12.8 (80.5)	0.9 (20.5)	3.1 (19.5)	4.4	15.9

†Soil treatments were as described in Table 13.

‡Values enclosed in parentheses report percentages in relation to the particular serogroup total.

§Composite samples taken after equilibration for 7 d at 75% of field capacity (0.03 MPa) and at 21 ± 2°C.

¶Composite samples taken immediately after harvesting of the plant.

Table 18. Influence of phosphate upon the chemical composition of soil solution prior to planting.†

Soil treatment‡	Elements				
	P	Mn	Ca	Mg	K
	μM		mM		
Unamended	ND	0.6	0.95	0.54	0.32
P_o - $CaCl_2$	ND	0.5	2.25	1.25	0.26
P_o - $MgCl_2$	ND	0.7	2.25	1.25	0.33
P_o - KCl	ND	0.6	2.21	1.23	0.35
P_1 - $Ca(H_2PO_4)_2$	0.65	0.2	1.08	0.58	0.29
P_1 - $Mg(H_2PO_4)_2$	0.97	0.1	1.08	0.58	0.29
P_1 - KH_2PO_4	0.81	0.0	0.99	0.54	0.23

†Values are means of duplicate composite samples. Extraction of soil solution was carried out after equilibration for 7 d at 75% of field capacity (0.03 MPa) and at 21 ± 2°C.

‡With the exception of the unamended treatment, Mo and S were applied to all treatments a week prior to extraction of soil solution and at rates of 1 and 22 mg kg^{-1} dry soil respectively; phosphate was applied at the same time with Mo and S at a rate of 55 mg P kg^{-1} dry soil.

ND, not detected.

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