COMPARATIVE STUDIES ON GROWTH AND NODULATION OF SUBTERRANEAN CLOVER AND LUCERNE

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Thesis submitted for the degree of
Doctor of Philosophy
in the Faculty of Agricultural Science.

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1965

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

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I INTRODUCTION

One of the most important microbiological processes in agriculture is the production of nodules on the roots of leguminous plants by bacteria of the genus Rhizobium. Through the symbiotic association of plant and bacteria in these nodules the vast reservoir of nitrogen in the atmosphere becomes available either directly or indirectly to plants and animals. In southern Australia and New Zealand symbiotic nitrogen fixation has been widely exploited through the medium of legume-based pastures which have come to play a very important role in agricultural production. In these countries, where low-cost pastoral and crop production form the basis of overseas earnings, and where fertiliser nitrogen is relatively expensive, legumes provide the main source of nitrogen for both crops and pastures.

Under favorable conditions, the amount of nitrogen fixed by legumes may be very high. Sears (1953) has estimated that high producing white clover pastures in New Zealand fix 200-500 lb. of nitrogen per acre each year. On a light-textured soil in the south-east of South Australia Powrie (1964) has measured 150 lb. of nitrogen fixed per acre by a first-year subterranean clover pasture sown on land recently cleared of native scrub.

In spite of losses through leaching, denitrification and export of produce, addition of nitrogen to soil by legume fixation is considerable. In New Zealand, on a

rhyolitic volcanic ash soil, Walker, Thapa and Adams (1959) found that nitrogen accumulated at the rate of 100 lb. per acre each year under white clover pasture. Donald and Williams (1954) showed that on a podzolic soil in New South Wales the nitrogen increment under subterranean clover pasture was linearly related to phosphate application, with 85 lb. of nitrogen accumulating per hundredweight of superphosphate applied.

Largely because of their role in nitrogen fixation and improvement of soil fertility, the use of pasture legumes has extended to many soils previously carrying native vegetation. On this newly-developed country however, conditions are not always favorable for legume nodulation and establishment. For example, on deep, acid sands in South Australia problems in establishment of the two commonly-grown legumes, subterranean clover and lucerne, are well known. At Mt. Compass, in the Adelaide hills, previous trials have shown that lucerne often fails completely, while subterranean clover may be yellow and unthrifty for many weeks after germination. On this and similar acid sands in the south east of South Australia, the failure of legumes to establish has generally been attributed to poor nodulation, and a variety of causes, including low pH, low soil calcium, and poor Rhizobium survival, have been implicated.

Further information on the reasons for legume failure in Mt. Compass sand is needed, particularly on the

effect of calcium and soil pH on legume nodulation. Liming, the addition of calcium carbonate or calcium hydroxide to soil, is a common method of overcoming nodulation problems on acid soils, yet its effects are not completely understood. By raising pH, lime is known to increase the survival and multiplication of rhizobia in the soil; raising pH may also allow better root development of the host. The calcium ion is also required by the legume for growth, and is essential for nodulation; even greater amounts are required for nitrogen fixation. Few experiments have been specifically designed to investigate these effects and their interrelationships in soils where nodulation problems have been encountered. This has been the principal objective in the present study.

Field observations suggest that subterranean clover and lucerne differ in their requirements for growth and nodulation and that lucerne is less tolerant of conditions in these problem sands than is subterranean clover. (Warcup, Hockley, Powrie, unpublished.) This has important agronomic implications on these soils. Lucerne is often a more valuable plant agronomically than is subterranean clover. With its deep-rooted perennial habit, it has the ability to utilise soil water more effectively and survive long periods of drought. The difference in behaviour of the two species is also of intrinsic interest, as it could reflect basic differences in the ability of the species and their associated rhizobia to withstand acid

conditions, and in the need for and ability to absorb calcium.

The present study was undertaken to provide further information on the reasons for legume failure in the acid sands at Mt. Compass, and define more clearly the requirements of lucerne and subterranean clover for optimum growth and nodulation under these conditions.

Initially water culture trials were used to carry out studies of the growth response of subterranean clover and lucerne to varying levels of calcium and hydrogen ions, uncomplicated by soil factors. Further experiments examined the effects of these ions on nodule formation, and the responses obtained were related to the function of calcium in the host species.

The reasons for the failure of the two legumes to nodulate and establish satisfactorily in Mt. Compass sand were examined in a series of field and pot trials and the effects on nodulation of calcium, pH and Rhizobium number on the seed were investigated. The basic information obtained in water culture trials was used in interpreting the results. The effect of temperature on nodulation was also examined in a series of growth room studies, as it was considered that low winter temperatures at Mt. Compass were adversely affecting legume nodulation, and might possibly be interacting with soil chemical factors.

II LITERATURE REVIEW

A. Calcium, pH and Plant Growth.

1. Calcium in soil.

Coleman, Kamprath and Weed (1958) have prepared a comprehensive review on calcium in soil and plant. Calcium is generally the dominant cation in the soil solution, and exchangeable calcium is readily available to plants, although less so from unsaturated montmorillonitic than from illitic or kaolinitic clays or from soil organic matter (Russell, 1961). Release of calcium into the soil solution and uptake by plants also depends on the nature and proportion of complementary exchangeable cations (Black, 1957). Calcium deficiency in soils is usually associated with various acidity effects, and it is often difficult to differentiate one from another (Hewitt, 1952a). Although Albrecht and Smith (1952) consider calcium deficiency prominent in the condition called "soil acidity injury", both Truog (1948) and Aslander (1952) consider that calcium supply is seldom the limiting factor in crop production on acid soils. There appears, however, to be little doubt that calcium is an important factor influencing plant growth, being deficient in at least some acid soils (Colwell and Brady, 1945; Rogers, 1948; Melsted, 1953; Andrew, 1960; Tiver, 1960; Andrew and Norris, 1961).

2. Calcium in the plant.

In the plant, calcium is considered to have a

variety of functions. It is essential for growth of meristems; and Marinos (1962), working with the shoot apex of barley, suggested that calcium is essential for the formation of the cell membrane systems on which the functional integrity of cell metabolism is dependent. Calcium has also been implicated in the activity of enzyme systems in plants (Coleman et al., 1958). The occurrence of calcium as a constituent of cell walls in the form of calcium pectate in the middle lamella has been widely accepted (Hewitt, 1963). Recently Ginzberg (1961) showed that calcium is important in the cell wall as a cross-linking agent in the protein gel of intercellular cement. Florell (1956, 1957) obtained evidence that calcium favoured the formation of, and increased the protein content of, mitochondria. In view of the role of mitochondria in aerobic respiration, and hence salt uptake, calcium appears to have a direct regulating effect on the uptake of other ions.

Calcium is essential for normal growth of plant roots (Sorokin and Sommer, 1940; Wiersum, 1958) and must be continuously available in the nutrient medium to ensure this (Presley and Leonard, 1948; Haynes and Robbins, 1948). Bürstrom (1952, 1954), working with wheat roots, concluded that a minimal amount of calcium was needed for cell division and 10 times that amount for cell elongation; still higher levels were sometimes needed to detoxicate hydrogen ions. He suggested that calcium, in its effects

on roots, may represent an "antiauxin" and as such, enhances root elongation. Bürstrom also found that calcium played an important part in the formation of cell walls of roots. He concluded that growth of cells starts with an increase in tensibility of the walls which is independent of calcium, and that this is followed by a calcium-induced formation, by intussusception, of the elastic components of the final wall. Recent work (Bürstrom, 1964) has suggested that a similar response to calcium occurs during growth of cell walls of pea seedling stems. Calcium is also essential for the formation and growth of root hairs, where it is used for cell wall formation (Cormack, 1949, 1962). In view of the importance of root hairs in nodulation of legumes, this aspect is discussed in greater detail in a following section.

Calcium deficiency appears to have two main effects on plants; it causes stunting of the root system, and affects growing points and younger leaves. Growing points are often killed, young leaves may be severely distorted, and petioles may collapse, especially in clovers (Wallace, 1951; Millikan, 1953; Snaydon, 1962b; Hewitt, 1963). Millikan and Hanger (1964) studied the effects of various levels of calcium on the distribution of Ca^{45} in subterranean clover. Their findings help considerably in accounting for the onset of calcium deficiency symptoms in that plant.

3. Calcium, pH and root hair development.

Root hairs result from retardation in elongation of root epidermal cells and are produced by internal pressure on weaker portions of an unequally hardened cell wall (Cormack, 1962). They develop acropetaly, the first hairs appearing very close to the root apex (Cormack, 1949). Once a certain stage in the development of epidermal cells has been reached, they no longer have the capacity to form hairs.

Farr (1927 a b c, 1928 a b) concluded at the end of a long series of experiments with Brassica Oleraceae that root hairs will not grow at all unless calcium is present in the surrounding solution, and that calcium is used directly from this source for cell wall formation. The hydrogen ion concentration was also shown to be an important factor, but the inhibitory effect of low pH on root hair growth was overcome to some extent by increasing the amount of calcium. Ekdahl (1957a) grew roots of wheat seedlings in nutrient solutions having pH values of 5.5, 6.3 and 7.2, and found that both increasing pH and addition of calcium increased root hair length.

The effects of calcium and pH on root hair development have been extensively investigated by Cormack (1935, 1944). Micro-chemical tests revealed that calcium combined with pectic acid in the hardening of cell walls. Cormack (1959 a, b) used E D T A (Ethylenediamine tetra-acetic acid) in solutions to chelate calcium ions and thus

reduced formation of calcium pectate in the elongating epidermal cell walls. Root hair formation on tomato and Brassica roots was greatly reduced and the root hairs were short and sometimes swollen, while cell walls remained soft and uncalcified. Normal root hair formation resumed when the treated roots were transferred to a solution containing calcium without E D T A. Cormack concluded that the concentration of calcium and the pH of the solution determine the rate at which calcification takes place.

Cormack's findings on the importance of external calcium on calcification of the root hair wall have been questioned by Ekdahl (1953, 1957b) partly on the evidence that roots of most plants develop abundant hairs in moist air in the absence of any external calcium supply. However, Cormack, Lemay and McLachlan (1963) were able to demonstrate that radio-active calcium was translocated from seed to the root hairs of white mustard, corn, and tomato, growing in moist air. Radio-autographs showed conspicuous radio-activity along the walls of the hairs, increasing in concentration near the base. These findings are compatible with the view that gradual calcification is essential for the normal growth and form of the hair.

The length of epidermal cells seems to be inversely related to the length of fully-grown root hairs. The longest epidermal cells are either hairless or produce short hairs, while the shortest epidermal cells produce the longest hairs. Cormack (1935, 1944) found that short cells

were able to calcify their cell walls at an early stage and thus formed root hairs; long cells were not able to do this and remained hairless. Under conditions that retarded calcification (insufficiency of calcium and slightly acid conditions), root hair development was decreased, or was completely absent in extreme cases.

The numerous studies of root hair growth and development have shown clearly that calcium supply and hydrogen ion concentration not only determine the capacity of epidermal cells to produce root hairs, but determine also the shape and length of the hairs themselves.

4. Absorption and movement of calcium.

Uncertainty still exists as to the mechanism of calcium uptake by plant roots. Some workers have considered the process to be one of metabolic uptake (Fried, Noggle and Hagen, 1958; Epstein, 1961; Lopushinsky, 1964). However, Moore, Jacobson and Overstreet (1961a), in experiments with excised barley roots, concluded that calcium uptake was largely non-metabolic.

Varying concepts also exist as to the mode of upward translocation of calcium in the plant stem. It has been commonly accepted that calcium moves in the transpiration stream by mass flow (Hylmo, 1953). This concept of movement by mass flow was recently challenged by Bell and Biddulph (1963) who indicated that in bean plants, tracer calcium moved up the stem by a process of exchange. Recent work by Ahmed (1963) on movement of radioactive

calcium and phosphorus in bean plants supports these findings.

Calcium has long been recognised as an ion which is slowly absorbed and translocated (Overstreet and Jacobson, 1952; Biddulph, Cory and Biddulph, 1959; Ahmed, 1963). The appearance of calcium deficiency symptoms at growing points may be partly attributed to lack of translocation of this element from older plant parts. Gauch (1940); McAlister and Krober (1951); and Biddulph et al. (1959), all report little or no movement of calcium from cotyledons of bean plants to young seedlings, and conclude that the calcium in the cotyledons is largely immobile. Gauch (1940) also noted that although roots of calcium-deficient bean plants contained relatively large amounts of calcium, there was little translocation to other parts of the plant. Calcium moves upward largely in the xylem and there is probably little downward movement in the phloem. In experiments with soybean plants, Biddulph et al. (1959) found that there was little movement of foliar applied Ca^{45} from the leaf, and concluded that the amount translocated in the phloem was very small. Although the findings of Millikan and Hanger (1964) generally support the view that calcium is immobile in the plant, their results also showed that some translocation of Ca^{45} may occur when calcium-deficient subterranean clover plants are provided with sufficient non-radioactive calcium to induce new growth. They found that

Ca^{45}, previously unavailable, was translocated to new leaves produced up to 9 weeks after the non-radioactive calcium was supplied. The mobile Ca^{45} came mainly from the roots.

5. Effects of pH on calcium absorption and requirement.

Cation absorption is greatly affected by hydrogen ion concentration (Hoagland and Broyer, 1940; Fawzy, Overstreet and Jacobson, 1954). This is particularly so with the uptake of calcium and has been the subject of a number of studies. Arnon, Fratzke, and Johnson (1942) examined the uptake of calcium by 5-week-old plants of tomato, lettuce and Bermuda grass placed for 96 hours in a series of nutrient solutions ranging, in graduations of a unit, from pH 3 to pH 9. At pH 3, injury to roots occurred and calcium was not absorbed. At pH 4 and 5 calcium absorption was lower than at higher values, particularly with tomato and lettuce, and the poor growth obtained at these values was thought to be related to the reduced absorption. These studies were continued by Arnon and Johnston (1942), who grew the three plant species for 5 weeks in a similar pH range, but using three levels of calcium (1, 4 and 14 me Ca/l). At pH 3, the hydrogen ion concentration dominated all three calcium levels, and complete failure of growth resulted. Growth of tomatoes and lettuce in solutions maintained at pH 4 and 5 was increased by raising the calcium level in the solution. At pH 6 the lowest calcium level was sufficient for optimum

growth. At pH 5 plant growth in solutions containing 14 me Ca/l was equal to that at pH 6, but at pH 4 plants grown at this level weighed only half as much. Arnon and Johnson (1942) concluded that, within the pH range 4 to 8, fluctuations in hydrogen ion concentration had little effect on plant growth provided an adequate supply of calcium (and other nutrients) was maintained. These conclusions conflict with their experimental results, however, in view of the low plant yield they obtained at pH 4, even at high calcium.

Sutton and Hallsworth (1958) studied the effects of hydrogen ion concentration, and calcium and manganese supply on the yield of lucerne in both agar and water culture. Although they obtained good growth in agar at pH 4 with a high supply of calcium and nitrogen, in water culture the same hydrogen ion concentration was much more toxic, and little response to calcium and nitrogen occurred. This aspect was further investigated in sand culture, and it was concluded that the greater tolerance of low pH shown in agar was due to a pH rise around the plant roots. If this could be maintained, (e.g. in agar) then the plants showed no injury at pH 4. If a high rate of renewal of the nutrient solution prevented a local increase in pH, then the plants were injured. These authors also found that temperature and light intensity affected the incidence of acid-injury symptoms. At pH 4, plants growing at 15°C developed injured root systems, but those

at 25°C grew normally. The effect of light intensity was even more pronounced, and it was only at high light intensities that an interaction was noted between pH and calcium. No clear explanation was given for this effect.

The interaction of calcium and hydrogen ions in determining plant growth was also investigated by Loneragan and Dowling (1958) in their studies of the nodulation of subterranean clover in water culture. Although at pH 3.5 they obtained reductions in root or shoot weight at all calcium levels (0.2 - 20.0 me/l) and at pH 4.0 reduction of root weight at 0.2 me Ca/l, from pH 4.5 to 6.0 there was no significant effect of either calcium or hydrogen ions on the dry weight production of either roots or shoots.

Results of studies by Arnon et al. (1942) and Arnon and Johnson (1942) suggest that higher concentrations of calcium are necessary to support a given rate of absorption at low than at high pH. Jacobson, Moore and Hannapel (1960) using excised barley roots, attempted to explain the effect of calcium on the absorption of monovalent cations, such as H^+. They postulated that the presence of calcium in the solution creates a barrier, probably at the root surface, which is particularly effective in blocking hydrogen ions. Waisel (1963) provided supporting evidence, demonstrating that the effect of calcium was primarily on the selective permeability of the outer cell membrane, probably the plasmalemma. Magnesium was found to be almost as efficient as calcium in preventing

the harmful effects of low pH.

6. Effects of calcium and pH on availability and uptake of other nutrients.

Many studies have shown that calcium enhances the uptake of monovalent ions, such as K^+ and Rb^+, by excised roots (Viets, 1944; Fawzy et al., 1954; Kahn and Hanson, 1957; Jacobson et al., 1960; Jacobson, Hannapel, Moore and Shaedle, 1961; Waisel, 1962; Rains, Schmid and Epstein, 1964) or whole plants (Higdon and Marshall, 1959; Jackson and Evans, 1962), but reduces the uptake of others such as Na^+. Other studies (Moore, Overstreet and Jacobson, 1961b) have shown that calcium considerably depresses magnesium uptake. Calcium has been found to be essential for the uptake of nitrate from an external medium (Nightingale, Addoms, Robbins and Schermerhorn, 1931; Gauch, 1940; Skok, 1941; Burstrom, 1954) while Tanada (1955), working with excised mung bean roots, reported that the uptake of rubidium and phosphorus was greatly enhanced by the presence of calcium.

Hewitt (1952a) has summarised the effects of pH on nutrient availability. In addition to direct injury to plants by hydrogen ions and physiologically impaired absorption of calcium, magnesium, and phosphorus, low pH may increase the solubility of aluminium, manganese, and possibly other heavy metals, to toxic levels in the soil. In addition, low pH may decrease the availability of phosphorus and molybdenum.

7. Varietal differences in calcium requirement and pH tolerance.

Quantitative variations in higher plant requirements for essential elements have been recognised for many years. For instance, temperate legumes generally require a higher level of calcium than grasses growing under the same conditions (Coleman et al., 1958). In recent years, it has been realised that even strains and varieties within a species may vary in their requirement for an element (Vose, 1963b; Gerloff, 1963).

Until recently comparatively few studies of the requirement of calcium by related species had been made. In an unlimed soil low in calcium Andrew and Norris (1961) found that, as compared with four temperate legumes, the higher yields obtained from five tropical legumes were associated with their superior ability to take up calcium. Bradshaw, Lodge, Jowett and Chadwick (1958, 1960), using nutrient culture, studied the response of seven species of grass to levels of calcium varying from 0.25 to 10 me Ca/l. The growth of some species was considerably reduced at the lowest levels of calcium, while others grew equally well at all calcium concentrations.

Snaydon (1962a) and Snaydon and Bradshaw (1962a) observed a highly significant interaction between edaphic ecotypes of Trifolium repens and calcium supply. Ecotypes native to acid upland swards produced greater growth in the acid soil low in calcium, than did ecotypes with a high

calcium requirement from a chalk soil. The reverse was true when the plants from the two populations were grown on the chalk soil. Similar results were obtained for ecotypes of Festuca ovina (Bradshaw and Snaydon, 1959), but Agrostis tenuis did not show any correlation between population origin and soil type or calcium supply. Snaydon (1962b), working in sand culture, determined the response to calcium of Trifolium incarnatum, T. pratense, T. repens and T. hybridum, over the range 0.2 to 6.4 me Ca/l. T. incarnatum showed the greatest response to calcium and was severely affected by low levels of calcium. T. hybridum was the least responsive, but showed a gradual increase in yield with increasing calcium. Snaydon concluded that there was a close relation between response to calcium and edaphic tolerance of the species. The data of Vose and Jones (1963), who worked with varieties of Trifolium repens, support Snaydon's conclusions. Millikan (1953) and J.K. Powrie (pers. comm.) have shown that a similar variation in response to calcium exists amongst varieties of subterranean clover. Kruckeberg (1954) found that the adaptability of certain ecotypes of Streptanthus glandulosus to serpentine soils in California was their tolerance of low calcium levels. Robinson (1942) working with clones of white clover, and Vose (1963a) working with 12 varieties of perennial ryegrass, both found significant differences in calcium content within the clones and varieties respectively examined. The reasons for the differences in calcium uptake

and requirement between species and between ecotypes are not yet clear, nor is the mode of inheritance of these characters understood (Vose, 1963b).

The marked differences in the calcium requirements of strains, varieties and populations of some plants indicate that calcium is a factor to be considered in the edaphic distribution of species. This could be of agricultural significance where legumes are being established on soils of low calcium content, such as the acid sands of southern Australia.

The relationships between ecological distribution of species and soil pH are well known (Small, 1946), but the precise causes of such observed distribution are less well established. The many factors which contribute to the "soil acidity complex" have been summarised by Hewitt (1952a). The need for crop varieties adapted to acid soils has stimulated selection work in this field, and Dessureaux and Ouellette (1958) have selected strains of lucerne tolerant of toxic levels of manganese, which, with aluminium toxicity, is a major factor in the soil acidity complex. Some tolerant genotypes contained less manganese and aluminium, and more calcium in aerial parts than susceptible ones. An increase in the calcium concentration of the nutrient solution reduced manganese toxicity in the lucerne (Ouellette and Dessureaux, 1958). These authors suggest that the rate of uptake of calcium determines the degree of tolerance to manganese and aluminium. Varieties tolerant

of manganese and aluminium toxicities have also been isolated in wheat and barley (Neenan, 1960), oats (Munns, Johnson and Jacobson, 1963), and ryegrass (Vose and Randall, 1962.)

B. Nodulation in Legumes.

Although a number of reviews have appeared in recent years covering various aspects of the interrelationships between leguminous plants and root nodule bacteria (Norris, 1956; Nutman, 1956, 1959a, 1963; Hallsworth, 1958; Allen and Allen, 1958; Loneragan, 1960; Jordan, 1962; Raggio and Raggio, 1962; Vincent, 1962a; Virtanen and Miettinen, 1963; Nicholas, 1963), a brief outline of nodule formation is given as an introduction to discussion of factors which affect nodulation.

1. Occurrence of rhizobia in soil.

Wherever legumes occur naturally, nodule bacteria are found widely in the soil, but their ecology has been little studied. Wilson (1930, 1931) counted Rhizobium trifolii and R. leguminosarum in long term manurial plots which had been free from leguminous crops for at least 10 years. In the test year, the plots were sown to either peas, clover, cowpea, potatoes or oats. Rhizobium number varied from 1 to 10^6 per gram of dry soil, with fewest in plots without legumes or with low pH or low exchangeable calcium. Both kinds of bacteria were stimulated most by clover. According to Nutman (1963) the soil population of nodule bacteria declines in the absence of legumes. At

Woburn (light sandy soil) and Rothamsted (heavy clay) the numbers of Rhizobium trifolii, R. leguminosarum and R. meliloti were counted in permanent clean fallows over a period of several years. In both soils the relative rate of fall in number of each species was fairly constant, with the half-life of population about 35 days. The clay soil supported the larger populations, with R. trifolii being the more abundant and R. meliloti less so.

Greenwood (1964) indicated that in New Zealand R. meliloti are absent from most soils although they may be plentiful on some neutral soils. On the other hand, R. trifolii are much more widespread but are absent or only patchily distributed on acid soils where pasture legumes have not grown previously, e.g. gumland, pakihi and glacial moraine soils. Greenwood found that after liming R. trifolii established and multiplied rapidly in these soils.

Although spore formation in rhizobia has not been definitely proved (Graham, Parker, Oakley, Lange and Sanderson, 1963) some cells can survive under favorable conditions for many years. Jensen (1961) records that cultures of Rhizobium meliloti in sterilised soil remained viable and effective after storage for 30-45 years at room temperature. Similar cultures of R. trifolii and R. leguminosarum were still effective after 10-14 years storage.

2. Multiplication in the rhizosphere.

As the first step in nodulation, nodule bacteria

in the soil nearby are stimulated to multiply in the rhizosphere by secretion of nutrients and growth factors from the root (Nutman, 1963). Rovira (1962) lists the many different substances lost in small amounts from roots. These include phosphorus and calcium, sugars, amino acids, vitamins, organic acids, nucleotides, flavonones, and enzymes. The rhizosphere stimulation is not specific and affects many other soil micro-organisms besides nodule bacteria. It is not confined to leguminous roots, although non-legumes generally stimulate the rhizosphere population to a small degree (Starkey, 1931; Rovira, 1961; Rovira and Stern, 1961). Rhizobia tend to be stimulated more than most other micro-organisms on legume roots. The larger stimulatory effects of legumes compared with those of other plants are not, however, correlated with more copious exudation (Rovira, 1959).

Densities of nodule bacteria in the rhizosphere may be 100 times that of non-rhizosphere populations (Purchase and Nutman, 1957). These densities are generally reached before the young seedling is susceptible to invasion.

Whatever the exact relationship between stimulatory exudates, the environment, and the response of Rhizobium, free living nodule bacteria clearly derive considerable ecological advantage from the presence of the legume host. So far as is known, the advantage to the host of an increased population of rhizobia in the rhizosphere

is only indirect in that it provides a reservoir for infection.

3. Invasion of the root.

Clover roots are usually infected through root hairs at or near a part of the hair which has been deformed or curled by bacterial secretions. Entrance has also been recorded through broken epidermal and cortical cells (McCoy, 1929; Bieberdorf, 1938) and ruptured tissue at the site of rootlet emergence (McCoy, 1929; Allen and Allen, 1940).

The curling is thought to be caused by excretion of IAA (3-indole acetic acid). Kefford, Brockwell and Zwar (1960), in investigations with subterranean clover, showed that exudation of tryptophan may take place from roots and that this could be converted to IAA by nodule bacteria. The evidence on this point is not conclusive, however, as Sahlman and Fahraeus (1962) consider that other soluble substances besides IAA are involved in root hair curling.

The curling of the root hairs, like the rhizosphere stimulation of bacterial numbers, is a non-specific reaction except that it is confined to legumes. All *Rhizobium* secretions act on all hosts, even those from strains which are unable to infect the host (McCoy, 1932; Kefford *et al.*, 1960).

Rhizobia normally invade the curled root hair tip, but the mechanism of infection is not yet clear. The bacteria are known to produce an extra-cellular poly-

saccharide slime (Fahraeus and Ljunggren, 1959; Ljunggren and Fahraeus, 1961) and, if bacteria capable of infection are present, this induces the host to secrete polygalacturonase (PG). The function of the PG is not known, but it may act with IAA to affect the plasticity of the primary wall of the young root hair, and thus assist in penetration. Nutman (1956) suggested that no actual penetration occurs, the root hair wall instead becoming invaginated to form an infection thread. This is possibly preceded by incorporation of the bacteria in the primary wall material. Results of electron microscope studies of clover root hair infection (Sahlman and Fahraeus, 1964) support the invagination hypothesis proposed by Nutman.

An infected hair is usually recognised by the presence within it of a hypha-like infection thread containing the bacteria. The infection thread, consisting largely of cellulose laid down by the host cell, grows at its tip, which is free from cellulose. Growth occurs only when the host-cell is nearby (Fahraeus, 1957; Nutman, 1959b).

Nutman (1959b, 1962) examined the infection of root hairs of young seedlings of 12 species of Trifolium and of Vicia hirsuta, using the Fahraeus glass slide technique (Fahraeus, 1957). He confirmed earlier findings that only a very small number of deformed root hairs are visibly infected, and that a relatively small proportion of infected hairs are associated with nodule formation. Growth of the infection thread may be arrested at an early

stage in the root hair, or later in the cortical cell, and nodule formation consequently fails. The first infections were found to occur at a few well-separated zones or foci on the root, and spread from these points. The number of infected hairs increased exponentially at a rapid rate, but dropped sharply with the appearance of the first nodule.

4. Nodule initiation and development.

The nodule itself is initiated when the penetrating infection thread approaches a preformed tetraploid cell in the cortex (Nutman, 1963). Repeated division of the tetraploid and neighbouring diploid cells then occurs, with branches of the infection threads spreading through the tetraploid cells as they are formed. The mass of cells rapidly differentiates into the young nodule. The uninfected diploid tissue differentiates into nodule cortex, vascular traces and other tissue, while a nodule meristem of both tetraploid and diploid cells provides for further nodule growth.

The bacteria are released from the infection threads into the tetraploid cells, where they multiply rapidly and are then transformed into swollen, and sometimes branched bacteroids. Small groups of bacteroids then become surrounded by membrane envelopes from the host's cytoplasm (Bergersen and Briggs, 1958; Dart and Mercer, 1963; Jordan, Grinyer and Coulter, 1963). It is only in tissue containing bacteroids that nitrogen is fixed.

5. Nodule size and number.

The nodulating habit of a species is broadly related to its rooting habit. Those with strongly developed taproots, e.g. Medicago, nodulate less abundantly than fibrous rooting species such as clovers (Jensen, 1947; Nutman, 1953). This host variation is genetic, and is evident within, as well as between species. High or low nodule numbers on the root may be selected and bred for in the host (Nutman, 1958). It has long been known that nodule size and abundance tend to be inversely related, large nodules occurring sparsely upon the root, and small nodules abundantly (Nutman, 1959a; Jones, 1962). In any one line of host, a compensatory mechanism occurs so that the total nodule volume per plant becomes fairly constant.

Once a nodule has been formed, further root hair infection and nodule formation in the vicinity is greatly reduced (Nutman, 1949, 1956, 1962; Dart and Pate, 1959). This suggests that fully-formed nodules inhibit further nodule formation.

Nutman (1949), working with red clover (Trifolium pratense), and Dart and Pate (1959), working with barrel medic (Medicago tribuloides) seedlings, observed that delaying inoculation resulted in a marked stimulation of nodulation. This occurred mainly on the lateral roots which had developed in the meantime. The stimulation of nodulation was probably due to more root hair sites being available before inhibition by early-

formed nodules restricted further infection. On the other hand, nodules formed earlier on the taproot appeared to exercise a localised and cumulative influence, tending to restrict further nodulation on lateral roots, and also restrict lateral root development itself (Nutman, 1948). Nodules on the lateral roots were found to be less inhibitory than the large earlier-formed nodules on the taproot, and after delayed inoculation, further nodulation continued at a much increased rate (Nutman, 1949). Where inoculation was delayed "clumps" of nodules formed on the later roots (Dart and Pate, 1959) suggesting lowered host resistance to infection.

Nutman (1949, 1956) has suggested that the origin of the inhibitory effect is in the meristematic tissue in both the roots and the nodules. Experimental confirmation of this was obtained by excising nodules, root tips, or nodule meristems, such excision being followed by stimulation of infection in the period immediately following the operation (Nutman, 1952). No inhibitory substance has been isolated so far although Pate (1958) has shown that nodules of several legumes contain large amounts of growth substances.

The alternative hypothesis of a nutritional effect on host plant development and nodulation is equally acceptable at the present time. In this case it is suggested (Dart and Pate, 1959; Nutman, 1962) that certain nutrient substances in their passage down the root are

diverted to the newly-developed nodule meristems, thereby restricting development nearer the root apex.

C. Factors Affecting Nodulation.

Many factors are known to influence nodulation, and those which are directly concerned in this study are discussed in this section.

1. Calcium and pH.

Calcium and pH have been recognised for many years as factors influencing nodulation of legumes. Their effects are of particular interest as they may be closely connected with the effects on plant growth previously discussed. The use of calcium carbonate or calcium hydroxide in nodulation studies, especially in experiments with soil, has prevented determination as to whether beneficial effects are due to addition of calcium, raising of pH, or both (Anderson and Spencer, 1948; Harris, 1961). Both calcium and pH are known to be important for growth and multiplication of rhizobia, and also for nodulation (Tiver, 1960; Loneragan, 1960; Vincent, 1962a). It has recently been shown (Loneragan, 1959) that calcium, in amounts greater than that required for growth of the host plant, is necessary for actual nitrogen fixation.

(a) Calcium and growth of rhizobia.

The question of whether or not calcium is required for growth of Rhizobium has recently become a lively topic among microbiologists. It had been commonly accepted that large amounts of calcium were needed (Norris,

1956), but Loneragan and Dowling (1958), in their investigations on the growth of Rhizobium, failed to obtain any response to added calcium in a medium already containing 0.032 me Ca/l although a marked response in growth of the subterranean clover host plant was obtained. Norris, (1959) was able to maintain a large number of strains in serial subculture in a medium free from detectable calcium, and concluded from this work that Rhizobium did not need calcium in detectable amounts.

In carefully controlled studies, Vincent (1962b) was able to clarify the issue. He showed that the calcium requirement of Rhizobium is low, and deficiency only becomes apparent at less than 0.050 me Ca/l. The magnesium requirement, however, is much higher, and deficiency occurs below 0.200 me/l. Additionally there is a need for total divalent cations of the order of 0.80-1.20 me/l. This can be met by either calcium or magnesium provided both are sufficient for their maximum specific effects.

(b) pH, and survival and growth of rhizobia.

In many nodulation trials involving calcium carbonate application to acid soils the main response appears to have occurred through an increase in Rhizobium survival and multiplication due to increased soil pH (Anderson and Moye, 1952; Vincent and Waters, 1954; Jenkins, Vincent and Waters, 1954; Blair and Bennett, 1960; Mulder and Van Veen, 1960; Parle, 1962; During, Cullen and Mountier, 1963). There are few reports of a

specific response to calcium in nodulation in the field. Spectacular increases in Rhizobium numbers in the rhizosphere of red clover following additions of lime have been recorded by both Mulder and Van Veen (1960) and Rovira (1961). The former workers also noted that the lime had no effect if clover was absent.

Jensen (1942) provided information concerning the influence of pH on the growth of Rhizobium trifolii and R. meliloti, and noted the greater acid tolerance of the former. The critical acid reaction for growth of R. trifolii in water-culture was found to be approximately pH 5.2, while the corresponding figure for R. meliloti was 5.4. R. meliloti however, was more tolerant of alkaline conditions than R. trifolii. Loneragan and Dowling (1958) have verified Jensen's results for R. trifolii, finding growth occurred only above pH 5.0, although after 4 days at pH 4 the cells were still viable.

These data agree with field observations on the natural occurrence of the two species (Greenwood, 1964), and the ability of R. trifolii to become established in acid soils. Vincent and Waters (1954) found that R. trifolii failed to grow in soil at pH 4.8-5.0 but raising the pH to 7.0 with calcium carbonate caused marked improvement. Several workers (Spencer, 1950; Jenkins, Vincent and Waters, 1954; Mulder and Van Veen, 1960) have found that clovers failed to nodulate on acid soils (pH about 5.0) at normal levels of inoculum, but did form

nodules if a very heavy level of inoculum was used. In these cases, the soil was probably too acid to allow multiplication of the bacteria, but not too acid to prevent them from forming nodules if the number of cells around the root was sufficiently high.

(c) Effects of calcium and pH on nodulation.

It is possible that effects of calcium and pH on nodulation are due to interactions on the rhizobial population and the infection process itself. This would make their elucidation difficult. Among the first attempts to explain their action were experiments conducted by Albrecht (1933), who studied the effect of calcium level on nodulation of soybeans over a pH range of 4.0-6.5. The calcium supply was adjusted by mixing different amounts of Ca-H clays of the required pH with quartz sand. At and below pH 5.0 nodules did not form at any level of calcium. Above this, a marked effect of calcium supply on nodulation occurred, as well as an interaction between calcium level and pH. For example, at pH 6.5, less calcium was required to produce a given number of nodules than at lower pH levels, suggesting that at intermediate pH levels (pH 5.5 and 6.0) calcium uptake was depressed by hydrogen ions. Albrecht (1932) also showed that soybeans needed greater amounts of calcium for nodulation than for growth of the host plant, and that the effect of calcium on nodulation was probably through the host plant itself (Albrecht and Davis, 1929).

Jensen (1947), investigating the effect of pH on nodulation of lucerne and subterranean clover grown in sand culture, concluded that nodulation in lucerne was more sensitive to acidity than that in subterranean clover. He observed that nodule numbers on lucerne decreased markedly below pH 5.5 but the numbers on clover were little affected even at pH 4.5. Spencer (1950) found that nodulation of subterranean clover was increased in an initially acid soil (pH 5.0) provided pH was raised and calcium supplied. Either treatment on its own had little effect. Spencer concluded that the main pH effect was probably on multiplication of rhizobia and the calcium effect on nodulation. Powrie (1964) obtained a significant increase in nodulation of lucerne when he applied calcium carbonate to an infertile podzolised sand in the south-east of South Australia. He attributed the response to correction of calcium deficiency raising of pH, or both. Calcium responses in nodulation have recently been demonstrated by Andrew and Norris (1961) who grew five tropical and four temperate pasture legumes (*Medicago tribuloides*, *M. sativa*, *Trifolium fragiferum*, *T. repens*,) in a calcium-deficient sand (pH 5.8). They considered that the addition of calcium carbonate to this soil was primarily one of calcium nutrition of the species, and that there was a greater calcium requirement for nodulation than for plant growth.

The specific role of calcium in nodulation has been greatly clarified by Loneragan and Dowling (1958) who

showed the importance of this element itself, and also its interaction with pH. Their work agrees with and greatly expands Albrecht's (1933) earlier findings. Loneragan and Dowling grew subterranean clover in water culture at five pH levels (4.0, 4.5, 5.0, 5.5 and 6.0) and at four calcium concentrations (0.02, 0.20, 2.0 and 20.0 me/l). At pH 4 or lower no nodules were formed at any calcium concentration, while at 0.02 me Ca/l no nodules were formed at any pH used. Above these critical levels, almost maximum nodulation could be obtained by an increase in either calcium or pH, so that each factor was to a large degree replaceable by the other. Calcium and hydrogen ions in the range of concentrations which produced these marked interactions on nodulation had no measurable effect on plant growth and it was concluded that the calcium requirement for nodulation of subterranean clover was higher than that for growth of the host plant.

Loneragan and Dowling found that hydrogen ions depressed calcium uptake by the plant. They suggested that, in the range of concentrations in which they were replaceable, the effects of calcium and hydrogen ions on nodulation were through their influence on the level of calcium in the plant. The actual stage in nodulation at which calcium exercised its effect was not defined, however. In view of the antagonistic effect that nitrate is known to exert on nodulation (Thornton, 1936; Gibson and Nutman, 1960; Tanner and Anderson, 1963), interpretation of

Loneragan and Dowling's findings are complicated by the presence of 5 me/l KNO_3 in the nutrient solution. Had nitrate been absent, or present at only a low level in the nutrient solution, then nodulation of subterranean clover could well have been different.

2. Rhizobial survival.

Before nodules appear upon the clover root, nodule bacteria in the soil nearby, or from inoculated seed, are stimulated to multiply in the rhizosphere. In Australia and New Zealand the inability of rhizobia to survive either as inoculum on the seed or as free living bacteria in the soil has been an important factor in preventing successful legume nodulation in many soils (Vincent, 1958).

(a) Survival on seed.

In soils suspected of being deficient in the required strain of Rhizobium, legume seed to be sown is inoculated with bacteria from either a peat or agar culture. Rhizobia are known to survive better in peat than in agar (Vincent, 1958). In recent years pelleting of legume seed with a variety of substances, either before or after inoculation, has been used commercially to increase survival and multiplication of the bacteria before nodulation (Brockwell, 1962, 1963).

Root-nodule bacteria are particularly susceptible to death by desiccation. Vincent, Thompson and Donovan (1962) have studied the survival of Rhizobium trifolii

applied to glass beads, and to seeds of subterranean clover. They found the death rate to be rapid, especially in the first few hours after inoculation. Addition of maltose or gum arabic to the distilled water-*Rhizobium* suspension reduced the death rate considerably.

The presence of a toxic factor in the seed coat of legumes was suggested by Lobb (1958) who claimed that the response he obtained to charcoal pelleting of lucerne was possibly due to absorption of an inhibitor. Since then, Thompson (1960, 1961) and Bowen (1961) have demonstrated the presence in the seed coat of subterranean clover, of an antibiotic which is active against a range of rhizobia. Bowen found that inhibition was strong in subterranean clover but weak in lucerne. In field trials, Thompson (1961) found that physical separation of the seed coat and inoculum with various pellets of inert material improved the nodulation of subterranean clover, presumably by protecting the inoculum from the antibiotic. He concluded that this substance could contribute to loss of viability of nodule bacteria on the seed, both in storage and in field sowings. Vincent *et al.* (1962) also found that seed coat inhibitors could reduce survival of *Rhizobium* bacteria on subterranean clover seed, and on glass beads to which a seed-coat extract had been added. Gum arabic coating gave some protective effect and improved survival.

The technique of lime-pelleting of clover and

lucerne seeds has been used for some years and is standard commercial practice on many slightly acid soils in Australia and New Zealand where nodulation failure is a problem (Loneragan, Meyer, Fawcett and Anderson, 1955; Blair and Bennett, 1960; Hastings and Drake, 1960; Walker, 1963; Cass Smith and Goss, 1964). The main function of the small quantity of fine lime round the seed is probably to reduce acidity in the soil adjacent to the seed and thus encourage Rhizobium survival and multiplication. On some soils low in calcium the lime may even supply sufficient calcium for initial nodulation to occur. Lime-pelleted seed may be sown with soluble fertilisers, such as superphosphate, with little damage to the inoculum. Lime-pelleting of seed may not always be beneficial however, as Adams (1964) has shown that a depression in nodulation may sometimes follow its use. But the reason for this depression is not known at present, although it could be due to effects of lime on trace element availability or of seed coat inhibitors on inoculum survival.

Brockwell (1962, 1963), in a reappraisal of seed-pelleting, found that to maintain bacterial viability during storage of inoculated subterranean clover seed, the best coatings were lime, or blood and dolomite, or lime, blood and dolomite attached to the seed with a 45% solution of gum arabic. Peat inoculum was mixed with the adhesive immediately before pelleting. Hastings and Drake (1963) have recently shown the advantage of a ground rock

phosphate (Gafsa) - dolomite pellet over ordinary lime pellets for long storage of seed. Hely (1963) developed a "three-step" method of inoculating and pelleting subterranean clover seed for sowing "problem" hill country in New South Wales. He found that the three-step method was superior to normal one-step methods (e.g. lime pelleting) and provided very high numbers of bacteria (10^6) on each seed. The pellets recommended by Brockwell, Hastings and Drake, and Hely probably perform several functions and may protect Rhizobium against desiccation and seed coat inhibitors, raise soil pH in the vicinity of the seed, and supply certain nutrients to the bacteria.

(b) Survival in soil.

Survival of rhizobia in the soil may be limited by soil physical conditions, soil acidity, microbial or root exudates, or competition with other organisms in the soil or rhizosphere. Various nodulation problems associated with survival of root-nodule bacteria in soil have recently assumed importance in Western Australia and a historical account of the situation there has been given by Parker (1962).

(i) Soil physical conditions.

Although soil physical conditions no doubt play an important part in Rhizobium survival and multiplication, the problem has been little studied. Recently, however, in Western Australia, a problem known as "second-year clover mortality" appeared on certain coarse textured

sands (Marshall and Roberts, 1963; Marshall, Mulcahy and Chowdhury, 1963; Marshall, 1964). It was found that Rhizobium trifolii failed to survive in the period between successful subterranean clover establishment and the subsequent season. A survey indicated that the problem was restricted to coarse-textured grey and yellow sandy soils, and that it increased in severity in areas of lower summer rainfall combined with higher summer temperatures. On red sandy soils (containing haematite) and on other finer textured soils the problem was normally absent. It was suggested that absence of R. trifolii in the top 2" of the problem soils was probably due to the high temperatures (up to 65.5°C in the top 1") which may prevail in mid-summer. Rhizobium lupini survived from season to season, and in the laboratory was comparatively resistant to the effects of high temperature and desiccation in the grey sands. The addition of certain clays (montmorillonite, illite, "fly ash") to sandy soils, both in laboratory studies and in the field protected Rhizobium trifolii from the effects of desiccation, but kaolinite or finely-ground silica failed to do so. The reason for the response to addition of certain clays is not at present known.

Other workers, in laboratory studies, have also shown that strains of rhizobia vary in their tolerance of high temperatures. Bowen and Kennedy (1959) investigated 87 strains and found a considerable death-rate above 40°C; at 40°C the death-rate of R. meliloti was lower than that

of R. trifolii. Graham et al. (1963) found that only 22 of 79 strains tested survived heating at 50°C for 5 minutes and none survived at 60°C for this period. Training, or addition of a variety of organic or mineral compounds did not increase heat resistance.

Little information has been published on the effect of low temperatures on rhizobia in soil. Wilson (1930) at Cornell University, noted a drop in numbers in winter, but a rise again in spring as temperatures rose.

(ii) Microbial "antagonism" and other problems.

Evidence to show that nodulation failure may be due to the inability of native or introduced rhizobia to multiply in soil and to colonise legume rhizospheres has been put forward by several workers in recent years. This inability to multiply and colonise the rhizosphere can be due to a variety of causes.

Nutman (1956) demonstrated that inhibitory compounds could be secreted by clover roots, and Turner (1955) showed that these could be absorbed by added charcoal with consequent increase in nodulation. Poor nodulation of subterranean clover on a yellow podzolic soil in northern New South Wales was described by Hely, Bergersen and Brockwell (1957) as being due to "microbiological antagonism" which prevented normal colonisation of the rhizosphere by the bacteria in the applied inoculum. Greater rhizosphere populations of rhizobia were obtained by the use of increased rates of inoculum or with normal

rates on the sites of log fires.

Beggs (1961, 1962, 1964), investigating the problem of faulty clover nodulation and establishment on the danthonia and browntop-covered Wither Hills near Blenheim, New Zealand, obtained marked responses to soil sterilisation with formaldehyde or "Vapam" (Sodium N-methyl dithiocarbamate dihydrate) and to turf killing with sodium dichloropropionate. Cultivation of the soil resulted in normal nodulation, which also occurred where the plant cover consisted of fern, and danthonia was absent. Parle (1964) showed that the inhibition was directly associated with living danthonia, and suggested that nodulation failure on the Wither Hills was due to a toxic material killing the rhizobia. He was able to extract a material, toxic to rhizobia, from the rhizosphere of danthonia and demonstrate its heat stability. Parle suggested that the toxin was produced either by the danthonia itself or by an organism associated with the danthonia. Two strains of Rhizobium trifolii showed considerable differences in susceptibility to the toxin. Responses to soil sterilisation have also been obtained by Parle (1962) on Galatea sand, a pumice soil in the North Island of New Zealand where lucerne failed to nodulate satisfactorily.

Holland (1962), and Holland and Parker (1962) have investigated the failure of subterranean clover plants to nodulate on certain virgin sandy soils of Western

Australia, where Cass Smith and Holland (1958) had obtained a spectacular nodulation response to sterilisation with formaldehyde or "Vapam". They found that the lack of nodules was largely due to a failure of the nodule bacteria to colonise the soil and/or the rhizosphere. Prolonged high soil temperatures and desiccation were not responsible for this failure which appeared to be due to the influence of other soil micro-organisms. A large number of fungi in the newly-cleared soil, particularly Penicillium spp., inhibited Rhizobium in vitro, while some could also inhibit nodulation under controlled conditions. Soil extracts from the field also showed antibiotic activity against Rhizobium. Development of lateral roots and of root hairs was inhibited by the fungi. Plant symptoms in the field consisted of a reduction of chlorophyll and a marked increase in anthocyanin content of the foliage. Identical symptoms could be produced under controlled conditions with Penicillium. Under established clover pasture the antagonistic group of organisms was very greatly reduced in numbers and activity and normal nodulation took place. Hely et al. (1957) in their investigations of "microbial antagonism" in northern New South Wales also found that the nodulation problem slowly disappeared with pasture improvement.

3. Rhizobium numbers in the rhizosphere.

Rhizobium bacteria are well-known for their ability to proliferate in the rhizosphere of most plants,

leguminous plants showing a larger rhizosphere effect than plants of other families (Rovira, 1961). In their investigations of rhizosphere populations of subterranean clover and lucerne, Purchase and Nutman (1957) found that comparable numbers could be found in water culture, sand culture or agar, and that they exceeded the non-rhizosphere population by about 100 times. When seed was inoculated, the maximum numbers of rhizobia in the rhizosphere were reached at about one week before nodulation commenced.

The total number of nodule bacteria in the rhizosphere do not necessarily bear a close relation to the number of nodules formed. Root hairs are not uniformly susceptible to infection which seems to occur on certain preferred sites or foci (Nutman, 1962). The proportion of root hairs which become deformed is very variable, depending on host species, but rarely reaches 50%. Of the deformed hairs, only a small percentage may become infected, while the proportion of infected hairs which result in nodule formation may also be small (Nutman, 1959b). Thus, nodule formation may be limited by factors other than mere density of the bacterial population.

Thornton (1929), working in soil, and Bhaduri (1951), working in water culture, both found that nodulation of plants was related to the size of the population of the nodule bacteria in the rhizosphere, but not in simple proportion. Purchase and Nutman (1957) showed that nodule numbers increase to a maximum asymptotically with

increase in bacterial density. Sears, Hyde and Greenwood (1955), in field trials on pumice country in New Zealand, found that increasing the inoculum not only increased the proportion of plants which became nodulated, but also increased the mean weight of these plants. Hely (1964), in field experiments on the Southern Tableland of New South Wales, found that survival and dry weight of subterranean clover plants were both significantly increased when inoculum level was raised in three steps, from 1.0×10^2 to 1.0×10^5 bacteria per seed. Further field work in the Snowy Mountains by Hely, Costin and Wimbush (1964) showed that there was a close association between number of early-formed nodules and plant dry weight.

By using a modification of the Fahraeus glass slide technique and by adding avirulent bacteria as a diluent to keep numbers of virulent rhizobia constant in the rhizosphere, Lim (1963) has been able to throw further light on the effects of bacterial numbers on root hair infection and nodulation. Root hair infection in Trifolium parviflorum, T. patens and T. glomeratum was limited by inoculum size in two distinct phases. Before nodulation began, infection was about doubled by doubling the density of the virulent bacteria in the rhizosphere. After nodulation, bacterial density had to be increased much more than twice to double the number of infections. To double infection in the second phase T. parviflorum required a five fold increase in bacterial population,

T. patens 25 fold and T. glomeratum 90 fold. Host susceptibility was considerably lowered by nodule formation. High bacterial numbers in the rhizosphere also promoted early infection and nodulation.

4. Root hairs.

Root hairs are the normal avenue of infection by the nodule bacteria, and the sequence of events in this process has been discussed earlier. A good deal is also known about development and growth of root hairs in plants but, surprisingly enough, little is known of the way in which root hair development affects nodulation. Perhaps the most important work on this problem was carried out by Thornton (1936). Studying lucerne seedlings growing in agar culture, he found that curling occurred mainly in long root hairs, 150-375μ in length. Secretions from Rhizobium bacteria stimulated the production and growth in length of root hairs, as well as their deformation. On the other hand, the addition of sodium nitrate markedly reduced the numbers and length of root hairs, and the percentage which were deformed.

Raggio, Raggio and Torrey (1957) who worked with black wax bean roots, found consistently poor nodulation in agar, but the use of vermiculite or sand resulted in a striking improvement. This was ascribed to the tremendous increase in length and numbers of root hairs which attended the use of the vermiculite or sand.

Since the proportion of infected root hairs which

develop as nodules is small, any reduction in number or length of root hairs will result in fewer opportunities for production of nodules. External factors such as calcium and hydrogen ions, which can have such marked effects on legume nodulation, are known to have considerable influence on the numbers and length of root hairs. Despite the important part played by root hairs in nodulation, the study of their growth and development in connection with nodulation has been somewhat neglected.

5. Temperature.

Little information is available on the effects of temperature on nodule formation. Early work by Jones and Tisdale (1921) showed that temperature was important in the nodulation of lucerne, red clover, field peas and soybeans. Nutman (1949) found that low temperatures delayed nodulation of red clover. Barrios, Raggio and Raggio (1963) found that on excised bean roots, optimum nodulation was obtained at 25°C, while at either 12°C or 33°C no nodules formed. Recently, Hely and Williams (1964) observed that on the Southern Tablelands of New South Wales prompt nodulation of subterranean clover did not occur, even in the presence of abundant inoculum, when establishment was delayed until winter. In pot trials carried out in water baths over a range of temperatures (7, 12, 17, 22, 27 and 32°C) they found that low root temperatures (7 and 12°C) markedly delayed initial nodulation of subterranean clover, and restricted nodule number. At 7°C nodules did

not form on the Mt. Barker variety for 22.5 days, but formed in 7 days at 27°C. No other recent work on this subject has been published and it is a little-explored field.

III EXPERIMENTAL

A. Water Culture Trials.

Calcium supply and the pH of the medium are known to affect the growth and nodulation of legumes in various ways. Furthermore previous experience suggested that lucerne and subterranean clover, the two species under study, might have differing requirements for the two factors. As a first step in the investigation it was decided to obtain basic information on these effects to assist in interpreting results from field and pot trials in Mt. Compass sand.

In the trials described in this section a comparative study was made of the effects of calcium and pH on the growth and nodulation of lucerne and subterranean clover in solution culture. Nutrient solutions were used as the growth medium in the experiments, because nutrient availability and pH are most readily controlled by this technique (Hewitt, 1952b).

1. Materials and methods.

All the experiments were conducted in an open-sided glasshouse at the Waite Institute, Adelaide. A general view of an experiment may be seen in Plate 1. Seed of subterranean clover (*Trifolium subterraneum* L. var. Mt. Barker) and lucerne (*Medicago sativa* L. var. Hunter River) was sieved to obtain seed of similar weight. Average weights obtained were:

Subterranean clover - 3.3 mg /seed

Lucerne - 2.5 mg /seed

Seed was surface sterilised by shaking with 95% ethyl alcohol for 3 minutes, followed by 0.2% mercuric chloride for 5 minutes and finally washed with four changes of sterile distilled water. The seed was germinated on stainless steel gauze over dilute nutrient solution (containing 1.0 me Ca/l).

After 9 days, six uniform plants were transferred to each of a number of 2.3 litre polythene pots, painted to exclude light. At this stage lucerne taproots averaged 4.0 cm, and clover taproots 2.5 cm in length. Each plant was supported in a waxed, hardboard ("Masonite") lid by two pieces of split plastic tubing and a small amount of cotton wool, which was removed as plants increased in size. Air-lines entered near the base of each pot and the solutions were aerated for 5 minutes of each hour, the period being controlled with a time switch. All nutrient solutions were prepared in plastic-lined drums and siphoned to the individual pots. The pots were scrubbed and washed twice with distilled water in preparation for each experiment.

Calcium levels were adjusted with standard calcium chloride solution and the required pH was obtained by addition of 0.0146 N H_2SO_4 or 0.04N NaOH solutions. The pH fell with time in the cultures of both species. When plants were small, the pH level of each treatment was adjusted every second day, but as the plants became larger

and the drop in pH greater, daily adjustments were made. The pH level of solutions containing subterranean clover fell quicker than those containing lucerne, and changes of up to 0.4 units were occasionally recorded in pots containing large fast-growing plants. In general, however, the daily variation was much less than this; 0.05-0.10 pH units / day with lucerne, and 0.10-0.15 units / day with clover.

The trials were of factorial design, and all treatments were replicated four times and randomised within each replicate.

At each harvest, plants were removed from the culture solution and rinsed for 30 seconds in distilled water. In Trials I and II tops and roots were separated by cutting immediately below the cotyledons, and in Trial III, leaves, petioles, stems and roots were separated. The material was dried overnight in an oven at 70°C, weighed, and then ground in a stainless-steel micro-hammermill in preparation for chemical analyses.

A 0.05-0.50 g sample of plant material was digested with 7 ml of a HNO_3-$HClO_4$ mixture (500 ml conc. HNO_3 + 83 ml 60% $HClO_4$). Calcium, sodium and potassium levels were determined on an E.E.L. flame photometer, using the method described by Williams and Twine (1960). Phosphorus was determined using an aliquot of the same HNO_3-$HClO_4$ digest. Ten ml of Molybdo-vanadate reagent (500 ml conc. HNO_3 + 500 ml 0.25% NH_4VO_3 + 500 ml 5%

$(NH_4)_6Mo_7O_{24}$) was added to an aliquot of the digest, made up to 100 ml with distilled water, shaken, and allowed to stand for 30 minutes. The colour development was measured on a "Unicam" SP.600 spectrophotometer, in a 1 cm cell at a wave length of 390μ and the value for phosphorus content read from a standard phosphorus curve.

2. Effects of calcium and pH on growth of subterranean clover and lucerne.

(a) Water culture Trial I.

The treatments of this preliminary trial consisted of three calcium levels (0.10, 1.00 and 10.00 me/l) and three pH levels (4.0, 5.0 and 6.0) in factorial combination. The composition of the basal solution is shown in Table 1.

TABLE 1.

Basal Nutrient Solution.

KH_2PO_4	= 0.5 mM	K^+	= 5.5 me/l
		Mg^{++}	= 2.0 "
KNO_3	= 5.0 "	NH_4^+	= 2.0 "
		H^+	= 1.0 "
NH_4NO_3	= 2.0 "	NO_3^-	= 7.0 "
		SO_4^{--}	= 2.0 "
$MgSO_4.7H_2O$	= 1.0 "	PO_4^{---}	= 1.5 "
H_3BO_3	= 0.540 ppm B	$(NH_4)_6Mo_7O_{24}$	= 0.067 ppm Mo
$CuSO_4.5H_2O$	= 0.064 " Cu	$MnSO_4.4H_2O$	= 0.550 " Mn
$ZnSO_4.7H_2O$	= 0.137 " Zn	Fe EDTA	= 5.000 " Fe

Seedlings were planted in the pots on 12th April,

1962. Solutions were changed after 14 days, additions of 3 mM KNO_3 and 1 mM NH_4NO_3 being made to the basal solution at the same time. This raised the K^+ level to 8.50, the NH_4^+ level to 3.0 and the NO_3^- level to 11 me/l of solution.

Three plants from each pot were harvested after 18 days and length of taproot and dry weight of tops and roots were measured. After a further 18 days, a final harvest was taken and similar measurements made.

The complete results of Harvest I are not presented here, but are recorded in Appendix 1.

Results.

Responses to pH in both lucerne and clover appeared 4 days after planting and soon became marked. At pH 4.0, plant growth was severely restricted at all levels of calcium. The root system remained small and individual roots were short, thickened, and brown in colour. Differences in plant growth between pH 5.0 and 6.0 were also obvious, but not as great as between pH 4.0 and 5.0, especially at the higher levels of calcium. After 8 days, growth in both species at 0.10 me Ca/l was visibly less than at higher calcium levels. The differences occurred at all pH levels, but appeared earlier at pH 4.0 and 5.0.

Symptoms of calcium deficiency appeared on lucerne at 0.10 me/l after 12 days and were more prominent and appeared earlier at pH 4.0 and 5.0. Petioles of older leaves collapsed not far below the leaflets, which

gradually died. The new leaves which emerged remained dwarfed and pinched with necrotic tips, while growing points remained stunted or died. Petiole collapse occurred on many plants before any marginal necrosis was present on the younger leaves. Older leaves showed marked anthocyanin accumulation. Root development was reduced, and the roots themselves were short and thickened with necrosis developing near the root tip, particularly at pH 4.0. Calcium deficiency symptoms in lucerne are illustrated in Plate 3.

Symptoms of calcium deficiency appeared much later in subterranean clover than in lucerne and no effect other than reduced growth was seen for 24 days. Again the symptoms occurred only at 0.10 me Ca/l and appeared earlier at pH 4.0 and 5.0 than at pH 6.0. The symptoms were similar to those described for lucerne except that petiole collapse and anthocyanin development were less evident. The effects of calcium and pH treatments after 18 days from planting may be seen in Plates 2, 3 and 4.

When a final harvest was taken 36 days from planting, several clover and lucerne plants at pH 4.0 had died, especially at 0.10 me Ca/l. At this pH, the roots of some plants were growing back up the plastic supports out of the nutrient solution. Acid injury in the form of brown necrotic roots was evident at all calcium levels.

Considerable inter-plant variation in tolerance of low calcium levels was evident, particularly in lucerne. Occasional plants showed only slight symptoms of calcium

PLATE 1.

General view of a water culture experiment in the open-sided glasshouse.

PLATE 2.

Water culture Trial I. Effect of level of calcium on growth of lucerne (upper) and subterranean clover (lower) at pH 4.0, 18 days from planting. Left to right - 0.10, 1.00 and 10.00 me Ca/l.

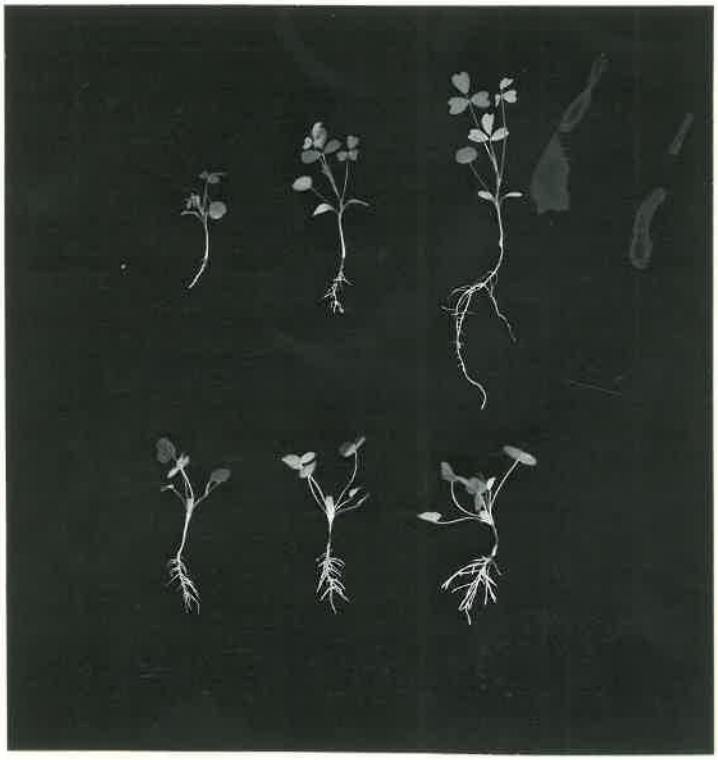
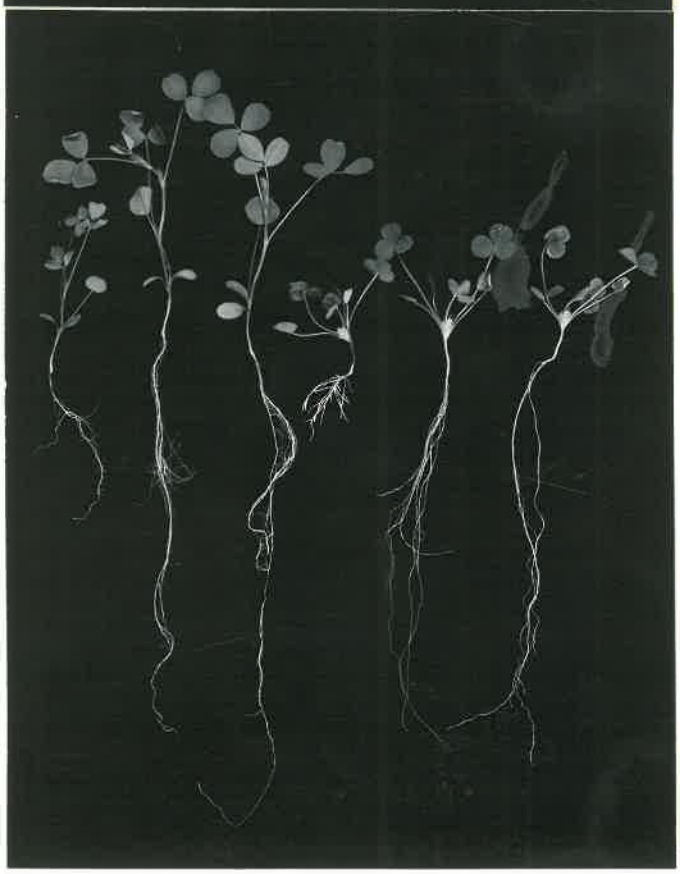


PLATE 3.

Water culture Trial I. Effect of pH on growth of lucerne (left) and subterranean clover (right) at 0.10 me Ca/l, 18 days from planting. Left to right - pH 4.0, 5.0 and 6.0. Note calcium deficiency symptoms in lucerne, but none in clover.

PLATE 4.

Water culture Trial I. Effect of pH on growth of lucerne (left) and subterranean clover (right) at 10.00 me Ca/l, 18 days from planting. Left to right - pH 4.0, 5.0 and 6.0. Note slight phosphorus toxicity symptoms on clover at pH 5.0 and more severe symptoms at pH 6.0.



deficiency while others in the same pot were severely affected.

The trial was reduced in value by the appearance after 12 days of symptoms resembling those of phosphorus "toxicity" on subterranean clover (Plates 4 and 5). The symptoms, similar to those described by Rossiter (1952), first occurred on the unifoliate leaf as necrosis on the distal margins. This gradually spread over the entire leaf surface eventually causing death of the whole leaf. The first and second trifoliate leaves were affected in turn, until only the young leaves were without symptoms. Only high calcium treatments at pH 5.0 and 6.0 were affected at first, but the disorder later extended to the intermediate calcium levels. High temperatures (25-30°C) in late April seemed to intensify the symptoms. In subterranean clover, a significant depression in yield was evident at both harvests where phosphorus toxicity symptoms occurred (Table 2.). This depression completely masked any response to calcium at pH 5.0 and 6.0.

Lucerne seemed more tolerant of high phosphorus levels in solution and showed no foliar symptoms, but at both harvests at pH 6.0 yield was depressed at high calcium. Taproot growth in both species was less affected by phosphorus toxicity than dry matter yield, and growth responses in root length to increased calcium were measured at all pH levels (Table 2 and Fig. 1). No phosphorus toxicity symptoms or effects on yield were

PLATE 5.

Water culture Trial I. Phosphorus toxicity symptoms in subterranean clover, 18 days from planting.

Left - normal. Right - affected.

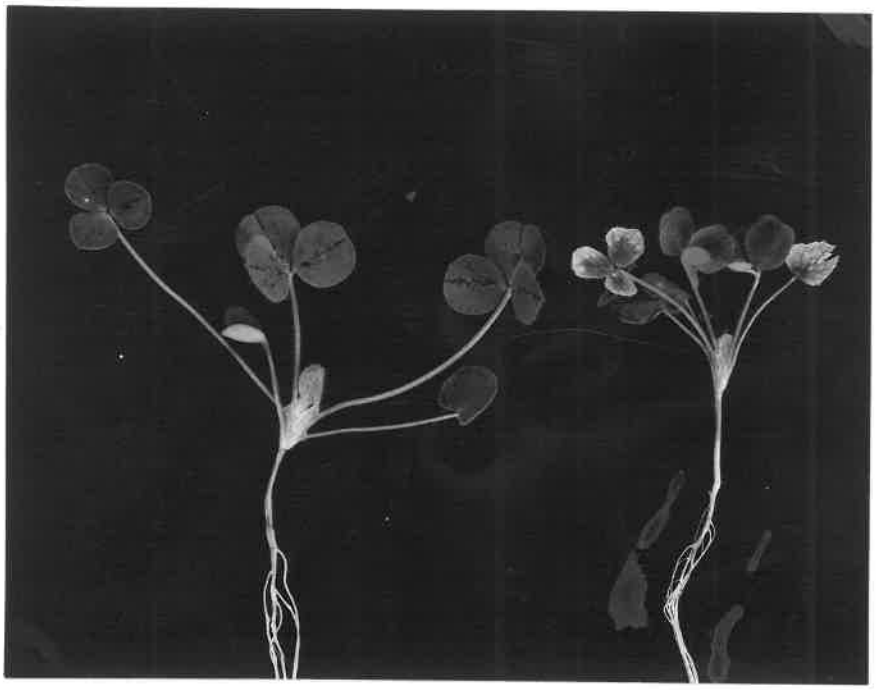


TABLE 2.

Water Culture Trial I - Harvest II.

Treatment pH	Ca (me/l)	Subterranean Clover: Length of Taproot (cm/plt.)	Subterranean Clover: Dry Weight (mg) Tops	Subterranean Clover: Dry Weight (mg) Roots	Subterranean Clover: Relative* Total Yield	Lucerne: Length of Taproot (cm/plt.)	Lucerne: Dry Weight (mg) Tops	Lucerne: Dry Weight (mg) Roots	Lucerne: Relative* Total Yield
4.0	0.10	3.0	51.0	12.3	8.5	2.4	38.8	6.5	2.9
"	1.00	4.2	151.3	51.7	27.3	3.3	134.9	41.7	11.5
"	10.00	4.3	192.4	73.9	35.9	12.2	299.5	116.7	27.0
5.0	0.10	19.0	429.4	121.1	74.2	22.8	442.2	113.3	36.0
"	1.00	21.3	470.3	157.2	84.5	25.2	731.5	265.0	64.6
"	10.00	30.8	310.7	97.7	55.0	34.5	899.9	308.8	78.4
6.0	0.10	27.8	561.6	180.7	100.0	28.5	990.2	297.5	83.5
"	1.00	29.8	473.4	158.9	85.2	37.0	1167.9	374.3	100.0
"	10.00	36.6	312.2	96.3	55.0	40.5	973.8	310.0	83.2

All figures are the mean of 4 replicates

Plant weights are the total from 3 plants.

* Highest yield equals 100. Lower figures are a percentage of this.

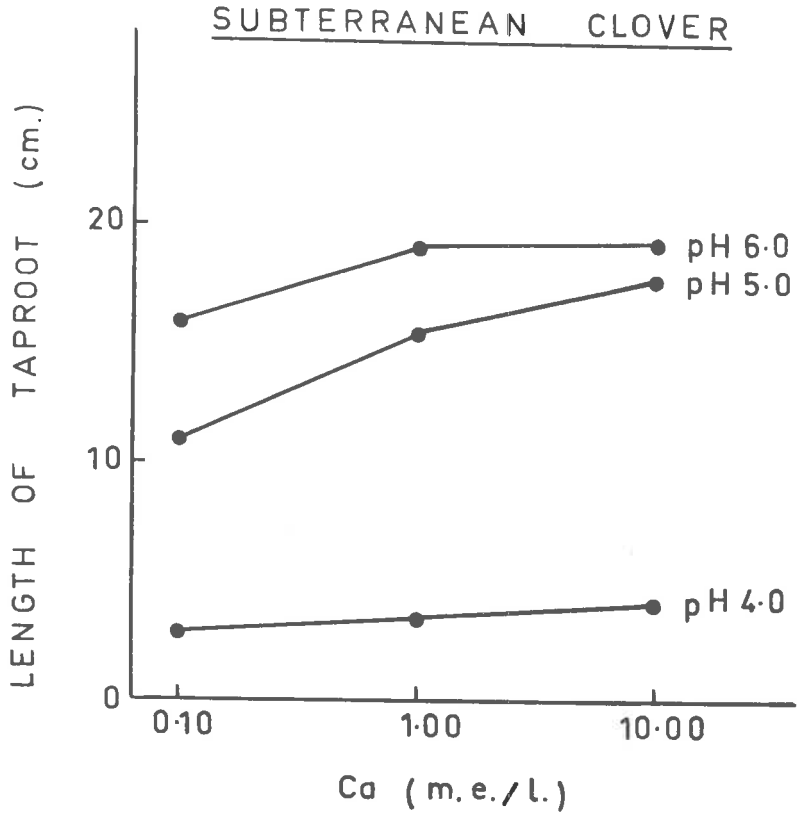
TABLE 2 (cont.)

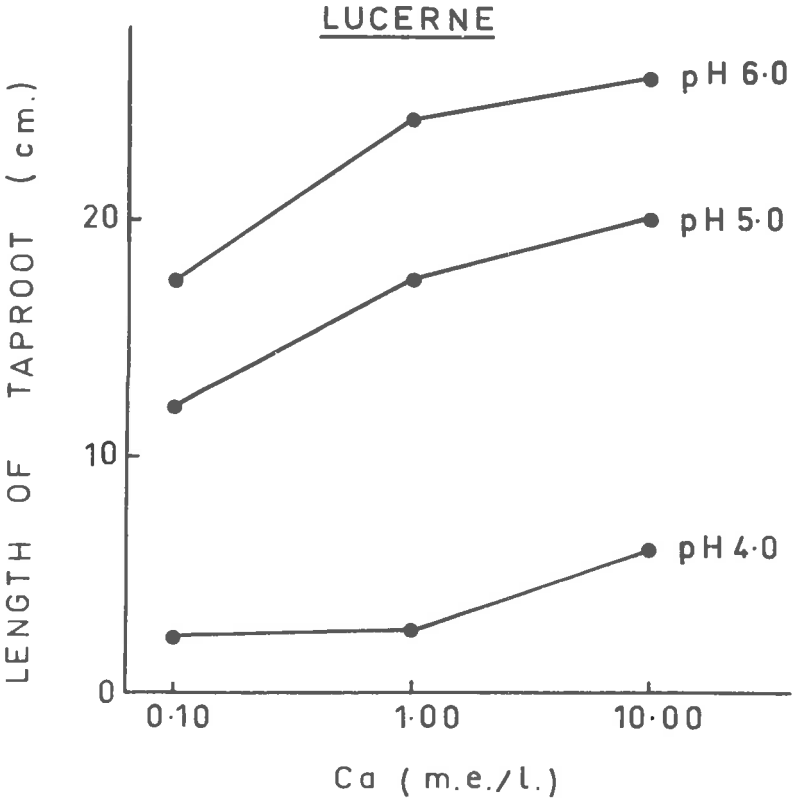
Log Data.

Treatment		Subterranean Clover			Lucerne		
pH	Ca (me/l)	Length of Taproot	Dry Weight Tops	Roots	Length of Taproot	Dry Weight Tops	Roots
4.0	0.10	0.48	1.71	1.09	0.37	1.58	0.78
"	1.00	0.61	2.17	1.70	0.50	2.12	1.61
"	10.00	0.63	2.28	1.87	1.07	2.48	2.07
5.0	0.10	1.28	2.62	2.07	1.35	2.62	1.97
"	1.00	1.32	2.67	2.19	1.40	2.85	2.40
"	10.00	1.49	2.49	1.98	1.54	2.95	2.49
6.0	0.10	1.44	2.74	2.25	1.45	2.99	2.47
"	1.00	1.47	2.67	2.20	1.57	3.06	2.57
"	10.00	1.56	2.49	1.98	1.61	2.98	2.46
Ca, pH L.S.D.	5%	0.05	0.06	0.07	0.08	0.08	0.12
	1%	0.07	0.08	0.10	0.10	0.11	0.16
Ca x pH L.S.D.	5%	0.09	0.10	0.13	0.13	0.15	0.21
	1%	0.12	0.14	0.17	0.18	0.20	0.28

FIGURE 1.

Water culture Trial I. Effect of level of calcium on length of taproot, at three pH levels (Harvest I).


SUBTERRANEAN CLOVER
LENGTH OF TAPROOT (cm.)
20
10
0
pH 6·0
pH 5·0
pH 4·0
0·10
1·00
10·00
Ca (m.e./l.)


LUCERNE
LENGTH OF TAPROOT (cm.)
20
10
0
pH 6·0
pH 5·0
pH 4·0
0·10
1·00
10·00
Ca (m.e./l.)

evident in either species at pH 4.0, and a significant increase in yield to additions of calcium was obtained. In lucerne a yield response to calcium addition was also obtained at pH 5.0.

In an endeavour to alleviate the symptoms attributed to excessive phosphorus in the solution, the nitrogen level was raised from 9 to 14 me/l when the solution was changed, but little reduction of symptoms occurred. At final harvest subterranean clover grown at pH 6.0 contained the abnormally high level of 1.12% P in the tops and 1.43% P in the roots. Rossiter (1952) also recorded figures in excess of 1.4% P in leaves of affected subterranean clover.

In spite of the adverse effect of phosphorus toxicity on yield in the water culture trial, a considerable increase in yield and taproot growth to increased pH was measured in both species at each harvest. At pH 4.0 taproot growth and plant yield at all calcium levels was poor, even though a response to increasing calcium was recorded. A highly significant increase occurred when the pH was raised to 5.0 (Table 2) and a lesser, but significant increase when the pH was raised further to 6.0.

(b) Water culture Trial II.

In order to define the effects of calcium and pH on growth of clover and lucerne more closely, a second water culture trial was prepared, with three calcium levels (0.05, 0.50 and 5.00 me/l) and three pH treatments

(4.5, 5.0 and 6.0). As poor growth at all calcium levels had been obtained at pH 4.0 in Trial I, the lowest pH treatment was increased to 4.5.

The macronutrient content of the basal solution was changed slightly, although a similar micronutrient content was used. Concentrations used were:

K^+	= 8.5 me/l	NO_3^-	= 12.0 me/l
Mg^{++}	= 4.0 "	SO_4^{--}	= 2.0 "
NH_4^+	= 2.0 "	PO_4^{---}	= 1.5 "
H^+	= 1.0 "		

Solutions were changed every 10 days. At the first change the phosphorus level was reduced to 0.5 me PO_4/l and later to 0.25 me PO_4/l in order to alleviate phosphorus toxicity.

Planting was completed on June 9th, 1962. Throughout the trial, seedling growth was slower than in Trial I due to lower temperatures prevailing in the open-sided glasshouse. Average mean air temperatures measured at the Waite Institute Meteorological Station for the period over which Trial I ran was 60.5°F but was only 54.5°F for Trial II.

Two harvests were again taken, at 35 and 60 days from planting. As well as the measurements made in Trial I calcium, potassium, and sodium were determined in the tops and roots and phosphorus in the tops only. No potassium, sodium or phosphorus analyses were conducted on Harvest I. The potassium and sodium analyses were

included as a check on effects of calcium and pH on cation uptake. In order to obtain sufficient plant material, the four replicates were bulked for chemical analyses.

Data from Harvest I are presented in the Appendix 2.

Results.

Growth differences due to both calcium and pH treatment appeared after 5 days. After 9 days, calcium deficiency symptoms at 0.05 me Ca/l began to appear in the unifoliate leaf of lucerne at all pH levels. Symptoms in subterranean clover showed at 0.05 me Ca/l three days later but only at pH 4.5. In lucerne, 16 days from planting, 0.05 me Ca/l treatments showed severe deficiency symptoms at all pH levels, while in subterranean clover, symptoms were severe at pH 4.5, moderate at pH 5, but only slight at pH 6. The effect of calcium and pH treatment on growth at Harvest I is illustrated in Plates 6, 7 and 8. Typical severe calcium deficiency symptoms in lucerne are illustrated in Plate 9.

Deficiency symptoms at 0.50 me Ca/l first showed in lucerne after 47 days, at pH 4.5 and 5.0. Symptoms appeared as collapsed petioles, and reddish older leaves, occasionally purplish-blue underneath. No sign of calcium deficiency other than depressed growth showed in clover at 0.50 me Ca/l until the final harvest; then plants growing at pH 4.5 and 5.0 exhibited shorter, somewhat thickened roots and a few reddish outer leaves.

Water culture Trial II. Effect of level of calcium on growth of subterranean clover (left) and lucerne (right) at three pH levels, 35 days from planting. Left to right - 0.05, 0.50, and 5.00 me Ca/l.

PLATE 6 (upper) pH 4.5

PLATE 7 (centre) pH 5.0

PLATE 8 (lower) pH 6.0

Calcium deficiency symptoms are evident at 0.05 me Ca/l in both species at all pH levels.

PLATE 9.

Water culture Trial II. Calcium deficiency symptoms in lucerne at pH 6.0 and 0.05 me Ca/l, 60 days from planting.

The effects of calcium and pH treatment on taproot length and dry matter production at Harvest II are presented in Table 3 and Fig. 2. Both taproot length and yield were increased by either an increase in pH or calcium level in the solution. At 0.05 me Ca/l growth was poor at all pH levels and many plants of both species had died at final harvest. A significant Ca x pH interaction occurred, and above 0.05 me Ca/l, near maximum yields were obtained in both species by either an increase in pH or calcium level, except at pH 4.5 where a marked species difference occurred. Whereas subterranean clover at 5.00 me Ca/l produced yields 81.9% of the maximum obtained at pH 6.0, lucerne yields were only 40.2% of the maximum.

Roots were more sensitive to low pH (4.5) or low calcium (0.05 me/l) than tops, especially in lucerne. At these low levels taproot growth was reduced, and many roots were thick, brown and brittle, with necrosis and death of meristems at 0.05 me Ca/l.

The results of the plant analyses, presented in Fig. 3 and Table 4, show that calcium uptake was increased by an increase in calcium level in the nutrient solution. Calcium uptake was also increased by an increase in pH. The calcium content of the roots was much less affected by calcium treatment than that of the tops. No significant differences were measured in calcium uptake by the two species.

The level of potassium in the plant tops was not

TABLE 3.

Water Culture Trial II.

Harvest II.

Treatment		Subterranean Clover			Lucerne		
pH	Ca (me/l)	Length of Taproot (cm/plant)	Dry Weight (mg) Tops	Roots	Length of Taproot (cm/plant)	Dry Weight (mg) Tops	Roots
4.5	0.05	3.9	34.8	6.7	8.3	42.4	12.8
"	0.50	10.5	385.0	153.3	15.8	433.4	237.6
"	5.00	20.3	1008.6	444.9	25.1	472.5	350.9
5.0	0.05	7.2	66.5	13.9	12.3	59.4	14.1
"	0.50	17.0	972.5	395.4	26.1	580.1	383.7
"	5.00	22.0	1029.6	418.0	54.9	1133.4	684.0
6.0	0.05	10.0	107.2	20.2	15.9	101.4	27.4
"	0.50	32.6	1296.8	476.8	46.3	1386.3	661.7
"	5.00	36.0	1192.0	454.5	62.3	1286.6	689.6

Plant weights are the total from 3 plants.
All are the mean of 4 replicates.

TABLE 3 (cont.)

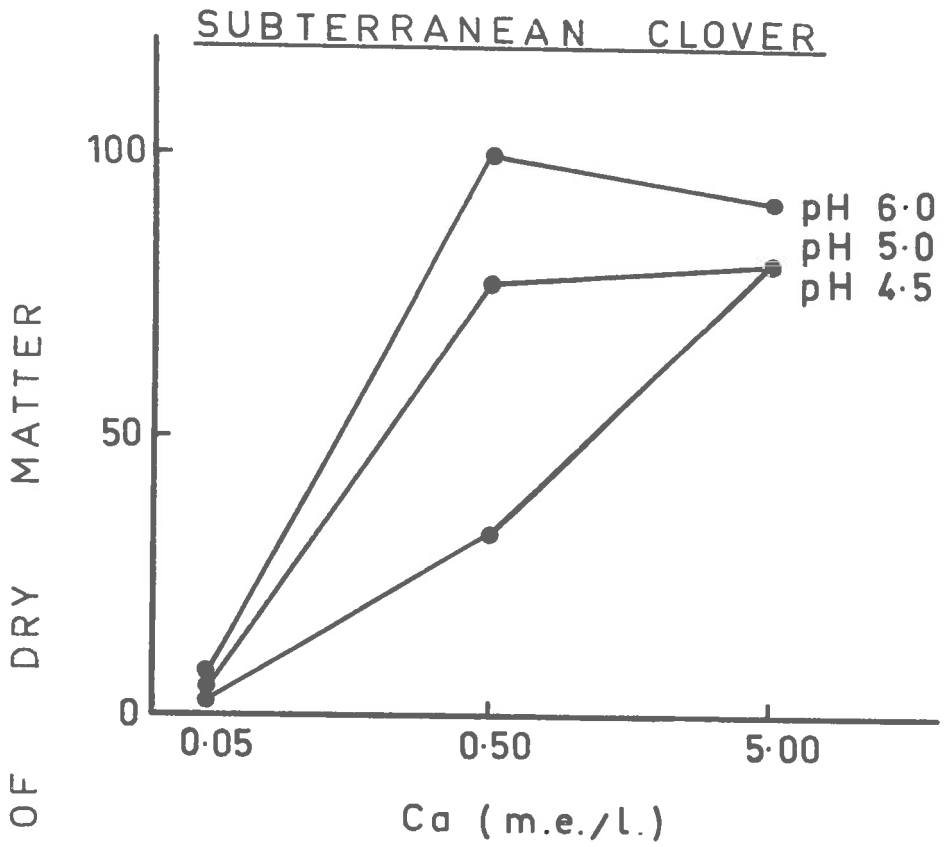
Log Data.

Treatment		Subterranean Clover			Lucerne		
pH	Ca (me/l)	Length of Taproot	Dry Weight Tops	Roots	Length of Taproot	Dry Weight Tops	Roots
4.5	0.05	0.59	1.54	0.77	0.91	1.63	1.10
"	0.50	1.03	2.58	2.18	1.19	2.64	2.38
"	5.00	1.31	3.00	2.65	1.40	2.67	2.54
5.0	0.05	0.85	1.81	1.12	1.08	1.77	1.13
"	0.50	1.23	2.99	2.60	1.41	2.76	2.58
"	5.00	1.34	3.01	2.62	1.74	3.05	2.83
6.0	0.05	1.00	2.03	1.30	1.20	1.96	1.38
"	0.50	1.51	3.11	2.68	1.66	3.14	2.82
"	5.00	1.56	3.07	2.66	1.78	3.11	2.83
Ca, pH L.S.D.	5%	0.05	0.05	0.07	0.08	0.09	0.09
	1%	0.06	0.06	0.09	0.10	0.12	0.13
Ca x pH L.S.D.	5%	0.08	0.08	0.12	0.13	0.15	0.16
	1%	0.11	0.11	0.16	0.18	0.21	0.22

FIGURE 2.

Water culture Trial II. Effect of level of calcium on relative* dry matter yield, at three pH levels (Harvest II).

* Highest yield equals 100. Lower figures are a percentage of this.


SUBTERRANEAN CLOVER
100
50
0
RELATIVE YIELD OF DRY MATTER
pH 6·0
pH 5·0
pH 4·5
0·05
0·50
5·00
Ca (m.e./l.)

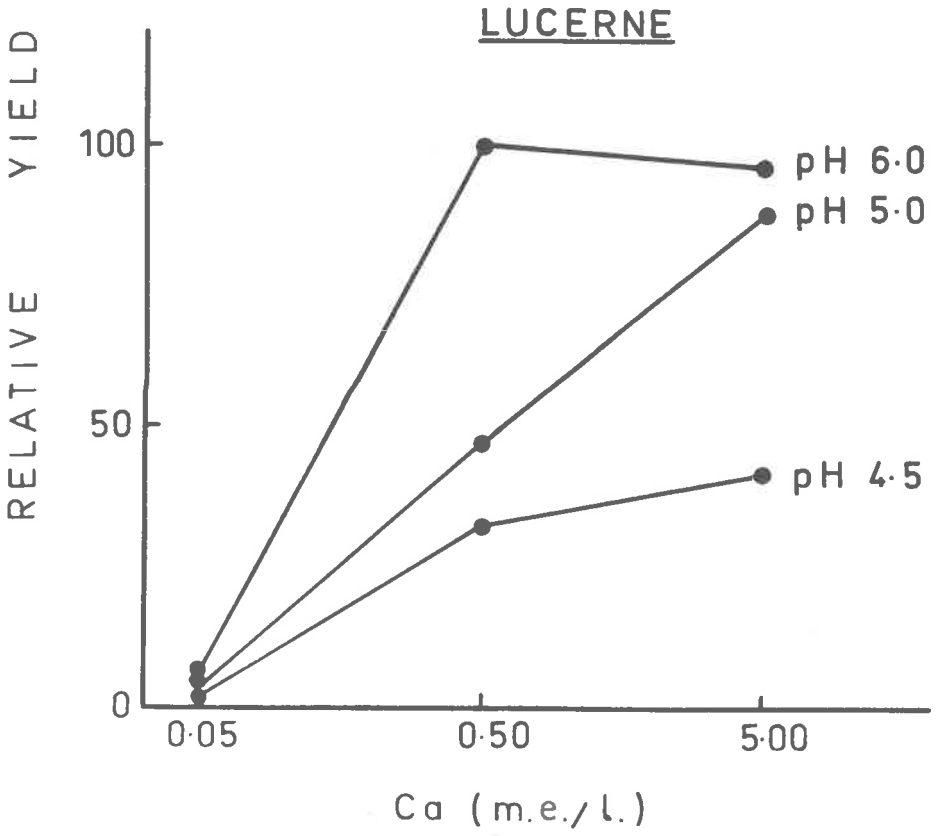
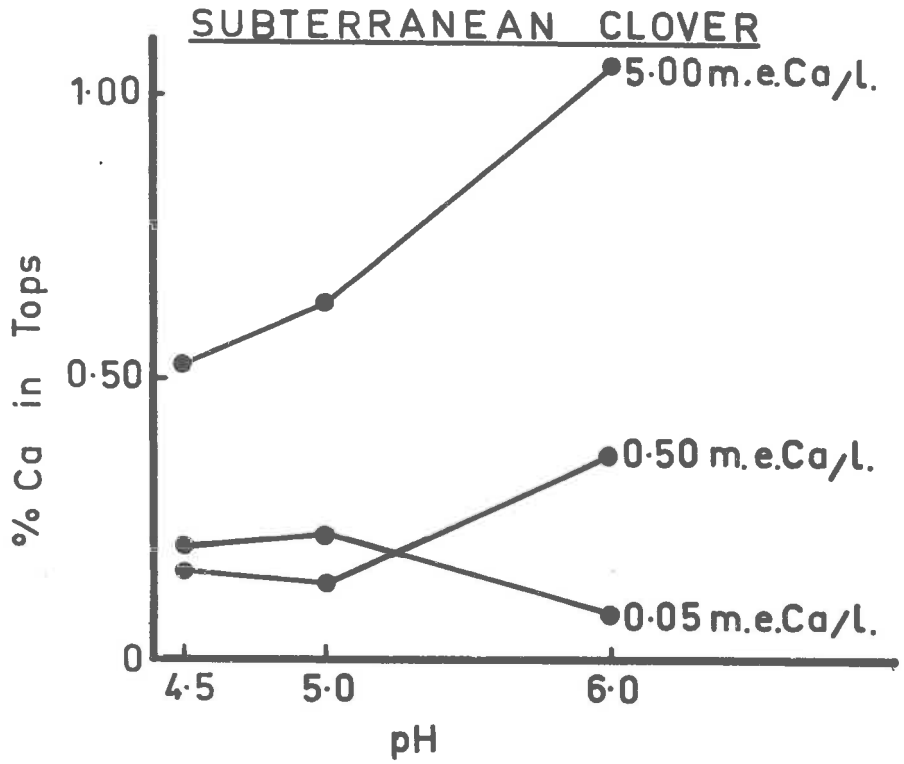

LUCERNE
100
50
0
RELATIVE YIELD
pH 6·0
pH 5·0
pH 4·5
0·05
0·50
5·00
Ca (m.e./l.)

FIGURE 3.

Water culture Trial II. Effect of calcium and pH treatments on level of calcium in tops (Harvest I).


SUBTERRANEAN CLOVER
1·00
0·50
0
% Ca in Tops
5·00 m.e.Ca/l.
0·50 m.e.Ca/l.
0·05 m.e.Ca/l.
4·5
5·0
6·0
pH

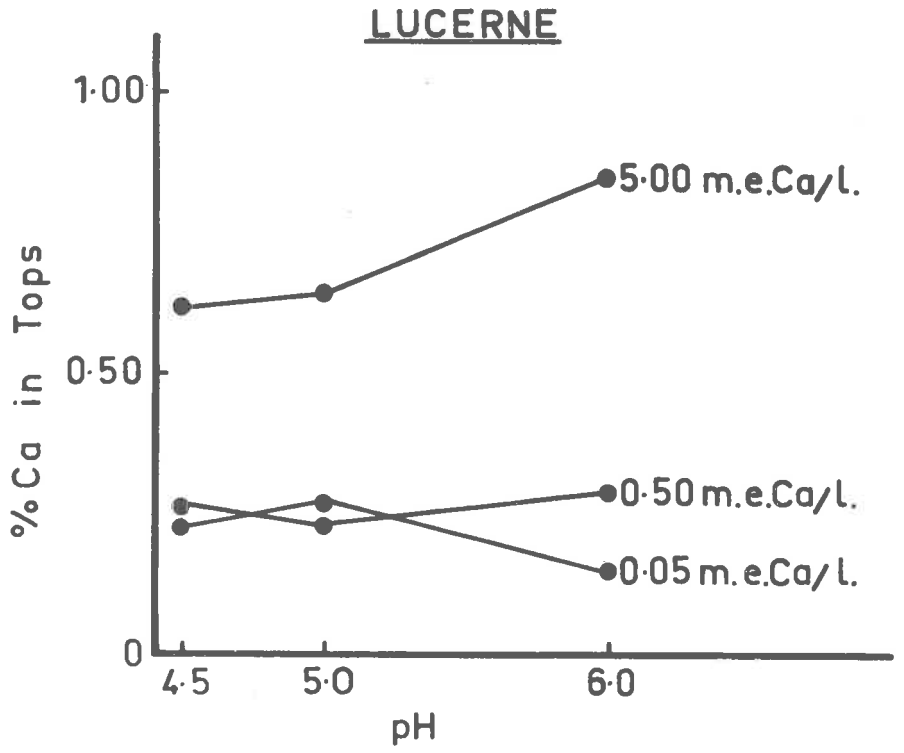

LUCERNE
1·00
0·50
0
% Ca in Tops
5·00 m.e.Ca/l.
0·50 m.e.Ca/l.
0·05 m.e.Ca/l.
4·5
5·0
6·0
pH

TABLE 4.

Water Culture Trial II - Harvest II.

Plant Analyses.

Treatment pH	Ca (me/l)	Subterranean Clover							Lucerne						
		Percentage of							Percentage of						
		Ca		K		Na		P	Ca		K		Na		P
		Tops	Roots	Tops	Roots	Tops	Roots	Tops	Tops	Roots	Tops	Roots	Tops	Roots	Tops
4.5	0.05	0.30	-	4.68	2.07	0.29	0.27	0.78	0.12	-	4.42	2.30	0.18	0.06	0.47
"	0.50	.13	0.21	4.09	3.98	.11	.11	.45	.13	0.21	4.34	2.67	.12	.09	.45
"	5.00	.43	.28	4.26	5.39	.09	.10	.82	.49	.30	4.74	3.78	.11	.07	.62
5.0	0.05	.13	-	5.40	3.09	.31	.11	.81	.19	-	5.93	3.70	.31	.22	.57
"	0.50	.14	.20	4.30	5.15	.14	.11	.81	.16	.19	4.74	4.28	.12	.06	.54
"	5.00	.51	.32	4.34	5.80	.10	.10	1.00	.53	.27	4.96	4.24	.08	.07	.63
6.0	0.05	.15	-	5.20	3.07	.26	.22	.84	.20	-	5.44	5.40	.33	.10	.74
"	0.50	.24	.23	4.02	5.78	.14	.16	.99	.20	.19	4.80	4.56	.10	.07	.66
"	5.00	.79	.35	3.87	6.00	.11	.13	.93	.70	.29	4.68	5.05	.10	.09	.70
Ca, pH L.S.D.	5%	.12	.04	.23	.68	.07	.11	.16							
	1%	.19	.09	.38	1.14	.12	.21	.26							
Species L.S.D.	5%	.09	.04	.19	.56	.06	.09	.13							
	1%	.16	.09	.31	.93	.10	.15	.21							
Ca x pH L.S.D.	5%	.20	.07	.40	1.19	.13	.19	.27							
	1%	.33	.15	.67	1.97	.21	.32	.45							

greatly affected by either calcium or pH level but the level in the roots tended to rise with increase in calcium. On the other hand, sodium level in both tops and roots was considerably reduced by an increase in calcium level.

Phosphorus toxicity symptoms again occurred in subterranean clover, after 35 days. The symptoms were evident only at pH 5.0 and 6.0 and at 5.00 me Ca/l, and were much less severe than in Trial I. By reducing the PO_4^{---} level in the solution to 0.5 me/l and later to 0.25 me/l all plants recovered, although the check in growth slightly depressed the yield of herbage below that obtained at 0.50 me Ca/l at pH 6.0.

Subterranean clover tops still contained high levels of phosphorus, although lower than those measured in Trial I. Lucerne, which failed to exhibit phosphorus toxicity symptoms, was found to contain much less phosphorus than the clover. The phosphorus level in both species was reduced by lowering the pH, but tended to rise at the higher calcium levels.

(c) Water culture Trial III.

In order to make a closer study of effects of various levels of calcium on plant growth, a further trial was conducted with five calcium levels (0.10, 0.25, 0.50, 1.00 and 5.00 me/l) but only two pH treatments (5.0 and 6.0). The basal nutrient solution was similar to that used in Trial II except that 0.3 me PO_4/l was used from the outset.

Seedlings were planted in the glasshouse on September 2nd, 1962. Mild spring conditions resulted in rapid plant growth. The nutrient solution was changed after 13 days and 19 days from planting. Length of taproot was measured in both species at three day intervals during the period of the trial. The first harvest was made 18 days after planting and dry weights of tops and roots were recorded. A second harvest was made 26 days after planting and on this occasion plants were divided into leaves, stems, petioles and roots and their dry weights recorded. The plant material from both harvests was analysed for calcium. Harvest I results are not presented as they do not provide information additional to that obtained at Harvest II.

Results.

Calcium deficiency symptoms appeared in lucerne at 0.10 me Ca/l at both pH levels after 8 days, with odd plants at 0.25 me Ca/l showing symptoms 14 days after planting. In subterranean clover, deficiency symptoms appeared 16 days after planting but only at 0.10 me Ca/l at pH 5.0 and on a few plants at pH 6.0 at the same calcium level.

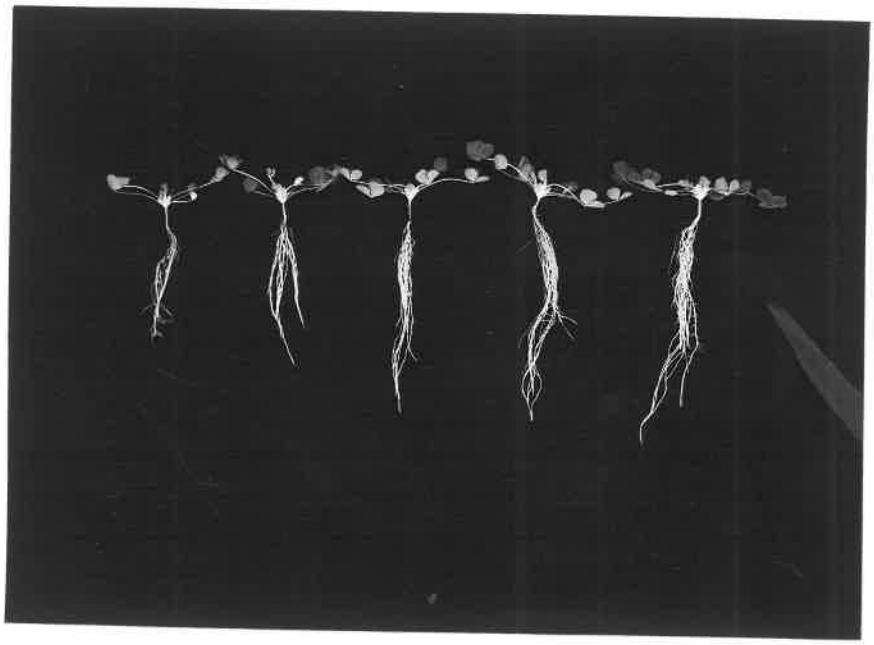
The effects of calcium and pH treatment on growth and dry matter production may be seen in Plates 10, 11, 12 and 13, in Fig. 4 and in Table 5. As in previous trials, the response to pH was very large and interaction with calcium occurred, both species being more susceptible to

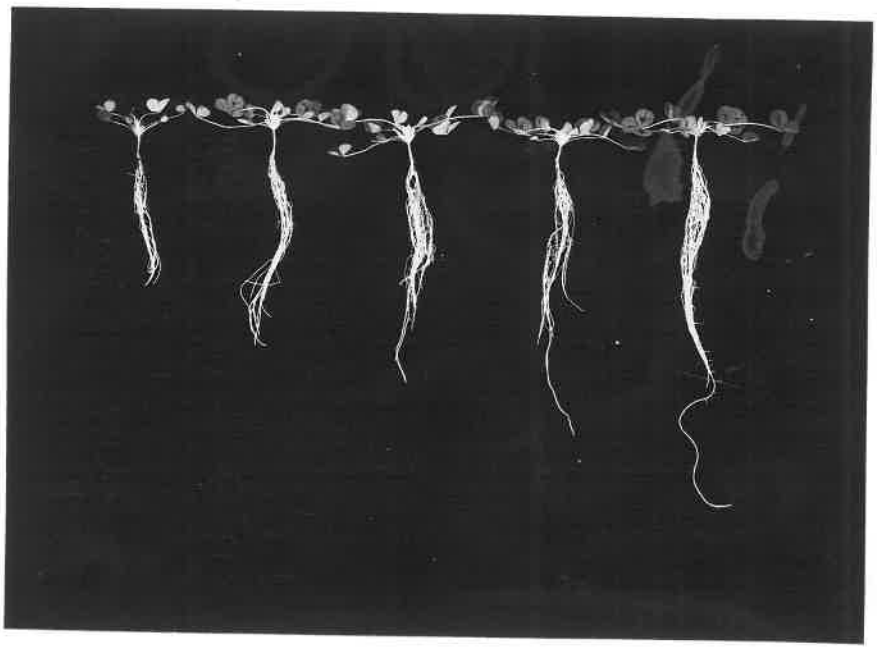
Water culture Trial III. Effect of level of calcium on growth of subterranean clover at two pH levels, 26 days from planting. Left to right - 0.10, 0.25, 0.50, 1.00 and 5.00 me Ca/l.

PLATE 10 (upper) pH 5.0

PLATE 11 (lower) pH 6.0

Note calcium deficiency symptoms at 0.10 me Ca/l at pH 5.0 and 6.0.





Water culture Trial III. Effect of level of calcium on growth of lucerne at two pH levels, 26 days from planting. Left to right - 0.10, 0.25, 0.50, 1.00 and 5.00 me Ca/l.

PLATE 12 (upper) pH 5.0

PLATE 13 (lower) pH 6.0

Note calcium deficiency symptoms at 0.10 and 0.25 me Ca/l at pH 5.0, and at 0.10 me Ca/l at pH 6.0.

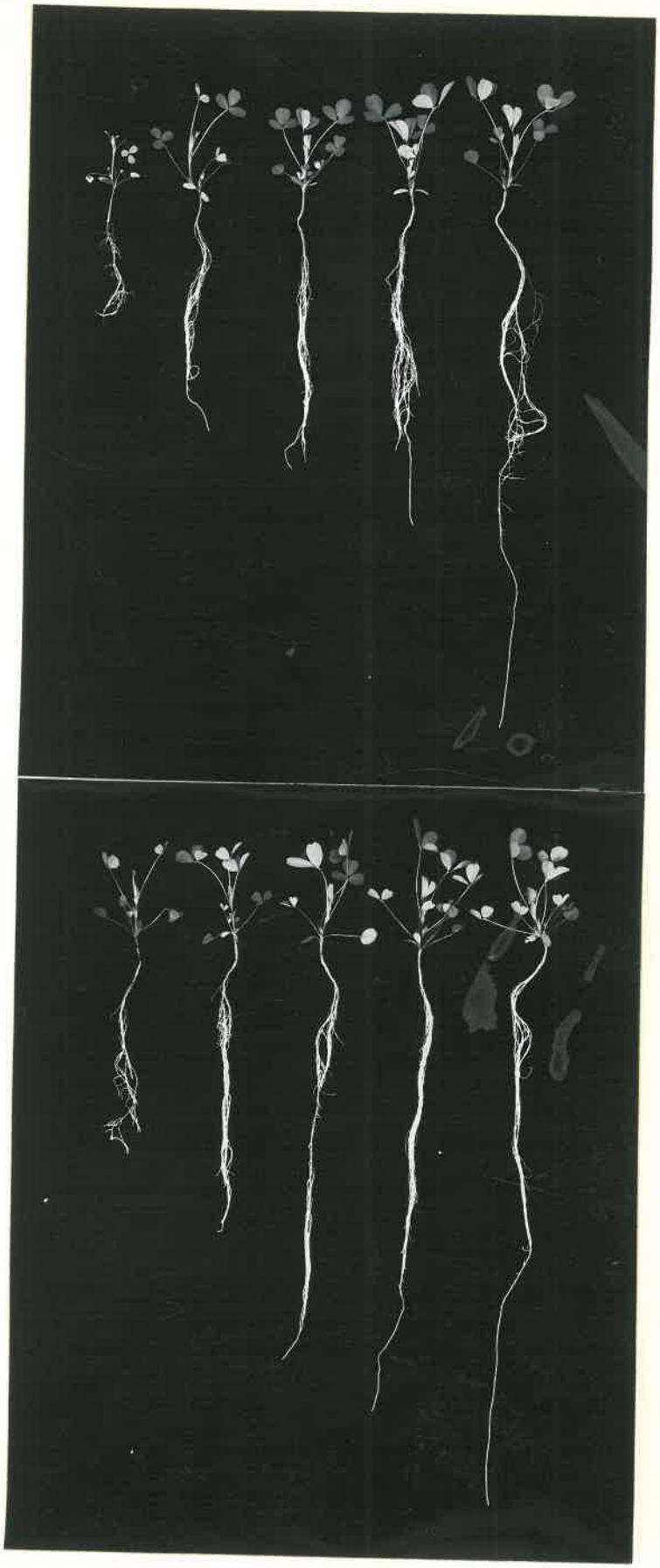


FIGURE 4.

Water culture Trial III. Effect of level of calcium on relative* dry matter yield at two pH levels (Harvest II).

* Highest yield equals 100. Lower figures are a percentage of this.

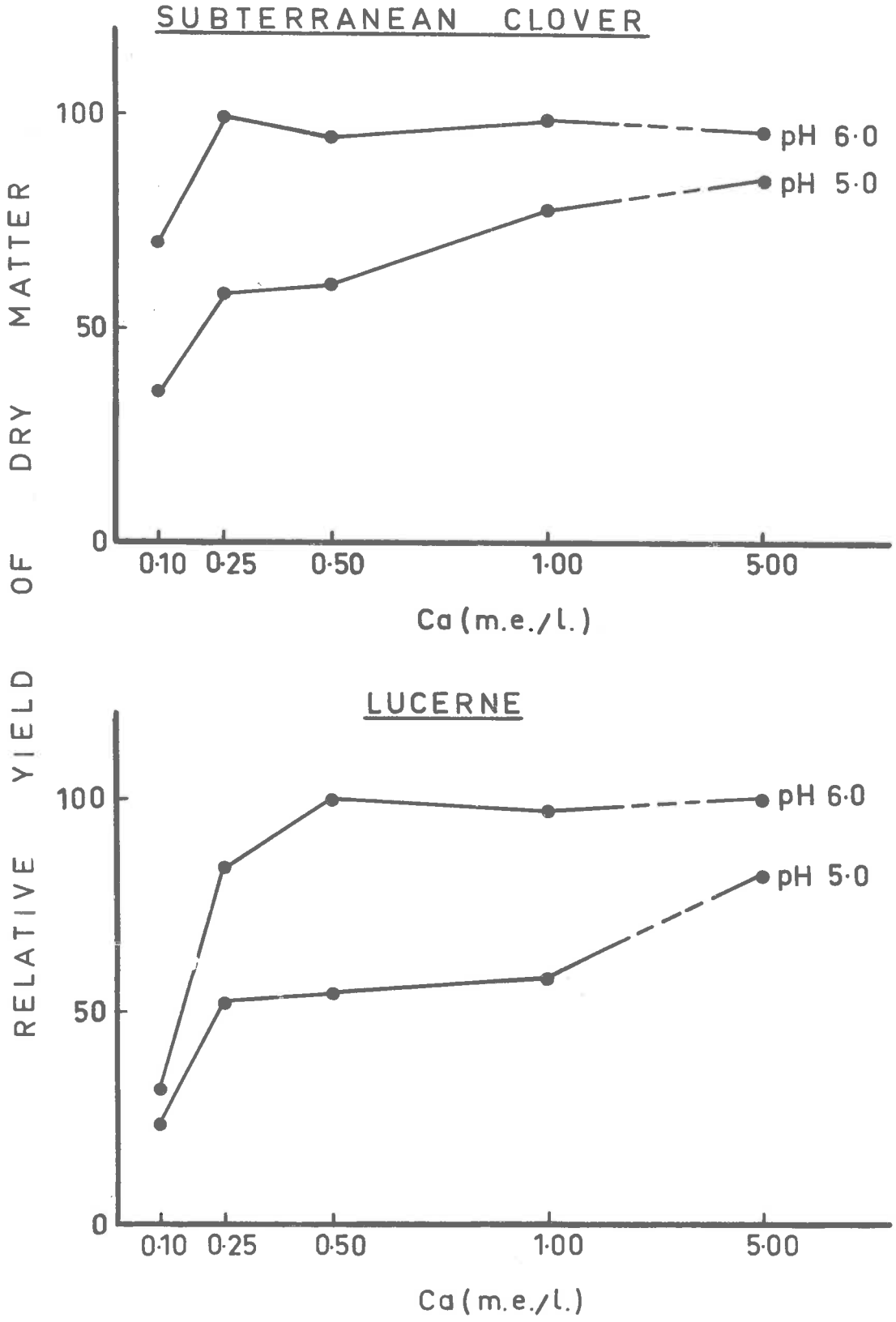

SUBTERRANEAN CLOVER
RELATIVE YIELD OF DRY MATTER
100
50
0
pH 6·0
pH 5·0
0·10
0·25
0·50
1·00
5·00
Ca (m.e./l.)
LUCERNE
RELATIVE YIELD OF DRY MATTER
100
50
0
pH 6·0
pH 5·0
0·10
0·25
0·50
1·00
5·00
Ca (m.e./l.)

TABLE 5.

Water Culture Trial III - Harvest II.

Plant Yield (mg. dry matter, total from 3 plants).

Treatment		Subterranean Clover				Lucerne			
pH	Ca(me/l)	Leaves	Petioles	Stems	Roots	Leaves	Petioles	Stems	Roots
5.0	0.10	46.0	8.5	27.6	27.8	37.0	6.4	33.1	23.9
"	0.25	78.3	16.6	30.5	55.7	98.8	19.3	43.4	63.0
"	0.50	79.8	18.1	30.8	61.1	102.0	17.1	37.7	75.6
"	1.00	101.8	22.2	35.1	82.6	103.2	18.0	37.8	89.0
"	5.00	111.3	25.3	38.7	88.5	145.6	27.9	53.3	123.3
6.0	0.10	86.8	19.9	48.0	62.2	48.8	12.3	46.2	28.7
"	0.25	137.7	30.9	46.8	94.3	160.3	29.0	67.4	101.8
"	0.50	131.5	31.2	44.7	88.2	198.3	33.2	73.3	120.7
"	1.00	135.1	31.0	46.5	94.0	189.3	34.5	64.8	126.0
"	5.00	130.2	29.7	44.8	92.7	196.5	35.2	68.6	125.1

TABLE 5 (cont.)

Log data.

Treatment			Subterranean Clover.				Lucerne			
pH	Ca(me/l)		Leaves	Petioles	Stems	Roots	Leaves	Petioles	Stems	Roots
5.0	0.10		1.66	0.92	1.42	1.42	1.53	0.78	1.51	1.37
"	0.25		1.89	1.21	1.48	1.74	1.97	1.28	1.63	1.78
"	0.50		1.89	1.25	1.48	1.78	2.00	1.22	1.56	1.87
"	1.00		2.00	1.34	1.54	1.92	2.01	1.25	1.58	1.95
"	5.00		2.04	1.40	1.58	1.95	2.16	1.46	1.73	2.09
6.0	0.10		1.93	1.28	1.67	1.79	1.67	1.08	1.66	1.46
"	0.25		2.14	1.49	1.67	1.97	2.19	1.45	1.82	2.00
"	0.50		2.11	1.48	1.64	1.94	2.29	1.52	1.86	2.08
"	1.00		2.13	1.48	1.67	1.97	2.27	1.53	1.80	2.10
"	5.00		2.11	1.47	1.65	1.96	2.29	1.53	1.83	2.09
Ca	L.S.D.	5%	0.08	0.09	0.08	0.09	0.11	0.10	0.08	0.07
		1%	0.10	0.12	0.10	0.12	0.15	0.13	0.10	0.10
pH	L.S.D.	5%	0.05	0.06	0.05	0.06	0.07	0.06	0.05	0.05
		1%	0.07	0.08	0.06	0.07	0.10	0.08	0.07	0.06
Ca x pH	L.S.D.	5%	0.11	0.13	0.11	0.12	0.16	0.14	0.11	0.10
		1%	0.15	0.17	0.14	0.17	0.21	0.19	0.15	0.14

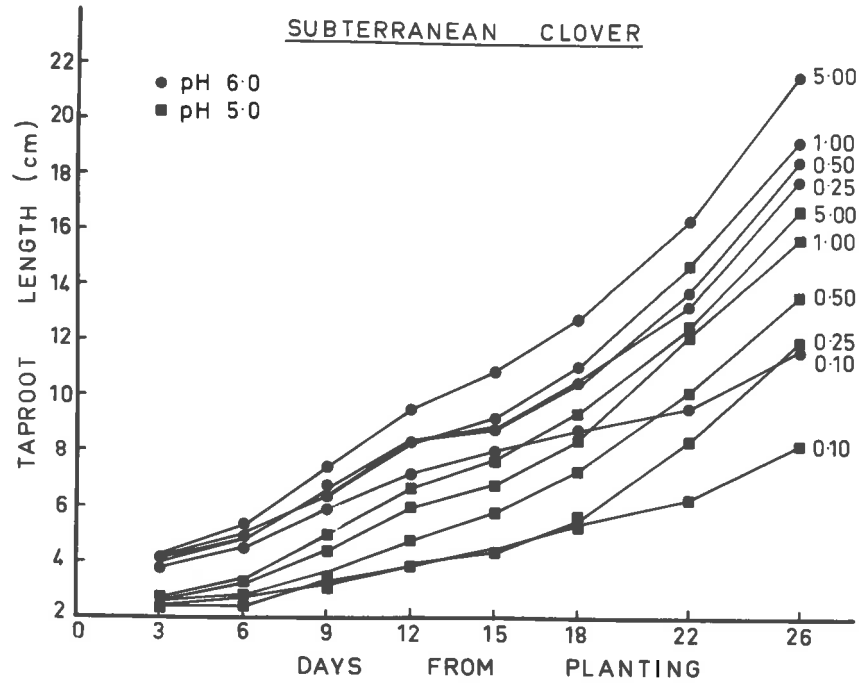
calcium deficiency at the lower pH. With lucerne, near maximum yields were obtained where the calcium level was above 0.25 me/l, but at pH 5.0 yields approached the maximum only at 5.00 me Ca/l. On the other hand, near maximum yields of subterranean clover were obtained above a level of 0.10 me Ca/l at pH 6.0 and above 0.50 me Ca/l at pH 5.0.

Interesting data were obtained from the taproot growth measurements taken every third day (Fig. 5). Taproot growth was generally linear with time, the slight variations probably being due to changes in temperature from day to day. Large variations in rate of growth occurred between treatments. In lucerne, at pH 5.0 high rates of growth were obtained only at 5.0 me Ca/l, but at pH 6.0 these were obtained with calcium levels as low as 0.25 me/l. These differences were not so evident in clover, possibly due to the far greater lateral root development in this species with much less emphasis on taproot growth.

The calcium analyses provided similar results to those obtained in Trial II (Table 6). Calcium level increased with increasing calcium in the nutrient solution, but was depressed by a decrease in pH. Petioles and leaves contained the highest levels of calcium, followed by stems, with a much lower level in roots. In contrast to other parts of the plant, percentage calcium in the roots did not increase greatly with increasing calcium in

FIGURE 5.

Water culture Trial III. Effect of calcium treatment (me Ca/l) on rate of taproot growth, at two pH levels.


SUBTERRANEAN CLOVER
● pH 6·0
■ pH 5·0
TAPROOT LENGTH (cm)
DAYS FROM PLANTING
5·00
1·00
0·50
0·25
5·00
1·00
0·50
0·25
0·10
0·10

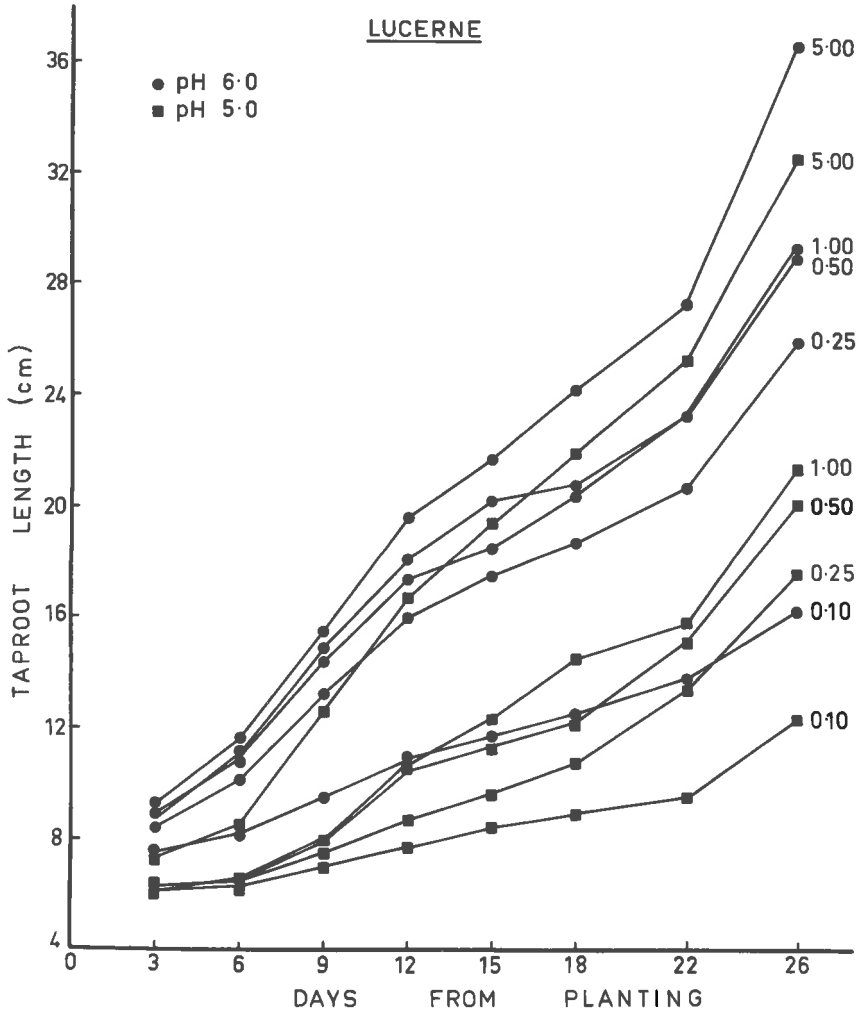

LUCERNE
● pH 6·0
■ pH 5·0
TAPROOT LENGTH (cm)
DAYS FROM PLANTING
5·00
5·00
1·00
0·50
0·25
1·00
0·50
0·25
0·10
0·10

TABLE 6.

Water Culture Trial III - Harvest II.

Percentage Calcium in Dry Matter.

Treatment		Subterranean Clover				Lucerne			
pH	Ca(me/l)	Leaves	Petioles	Stems	Roots	Leaves	Petioles	Stems	Roots
5.0	0.10	0.11	0.19	0.26	0.23	0.10	0.10	0.12	0.21
"	0.25	.16	.32	.33	.28	.12	.22	.15	.21
"	0.50	.18	.32	.30	.29	.18	.32	.26	.23
"	1.00	.17	.32	.33	.25	.22	.31	.33	.28
"	5.00	.50	.65	.58	.37	.70	.61	.70	.29
6.0	0.10	.12	.19	.20	.12	.16	.28	.30	.25
"	0.25	.10	.20	.21	.22	.21	.24	.23	.21
"	0.50	.34	.30	.17	.15	.24	.22	.25	.23
"	1.00	.37	.43	.30	.25	.31	.24	.23	.18
"	5.00	.74	.94	.61	.34	.83	.80	.62	.31
Ca L.S.D.	5%	.12	.16	.16	.10				
	1%	.19	.27	.26	.16				
pH, spp.L.S.D.	5%	.07	.10	.10	.06				
	1%	.12	.17	.16	.10				

the solution.

No phosphorus toxicity symptoms were evident in subterranean clover during the trial. Plant analyses showed that at final harvest phosphorus levels of 0.70 - 1.00% occurred in clover, and levels of 0.50 - 0.90% in lucerne. It was concluded that 0.3 me PO_4/l was probably the maximum level which should be used in these conditions if toxic effects are to be avoided.

(d) Root hair measurements.

When a previous water culture trial was harvested, differences in root hair development were observed between treatments. An unreplicated trial with two calcium levels (0.10 and 5.00 me/l) and two pH treatments (5.0 and 6.0) was therefore conducted to examine root hair development in lucerne and subterranean clover seedlings.

Seedlings were grown in a high nitrogen solution (as used in Trial III) for 7 days, when three plants from each pot were examined for root hairs under a dissecting microscope. The remaining three plants were then transferred to a -N solution and grown for a further 7 days before examination.

The -N basal nutrient solution used was made up as follows:-

KCl	= 2.0 mM	K^+	= 2.1 me/l	Cl^-	= 2.0 me/l
$MgSO_4.7H_2O$	= 1.0 "	Mg^+	= 2.0 "	SO_4^{--}	= 2.0 "
KH_2PO_4	= 0.1 "	H^+	= 0.2 "	PO_4^{---}	= 0.3 "

The micro-nutrient solution was the same as that used in

previous water culture trials.

In the -N solution, root hairs were numerous on lucerne growing at pH 6.0, and were greater in number at high than at low calcium. At pH 5.0, a small number were present on some plants at high calcium, but there were none at low calcium. A similar distribution occurred on subterranean clover, except that at pH 5.0, root hairs were numerous at high calcium, but absent at low calcium. In the +N solution, few root hairs were evident on lucerne with any treatment and those on subterranean clover were reduced as compared with the -N solution.

In view of these responses to calcium and pH, a more comprehensive trial was designed in order to measure the differences in root hair growth already observed.

A factorial design with three levels of calcium (0.10, 0.50 and 5.00 me/l) and two pH treatments (5.0 and 6.0) was used. A larger number of treatments could not be included as the pilot trial had indicated that only about 300 plants could be examined for root hairs in a day. A -N basal nutrient solution was used, similar to that used in the pilot trial. Three plants of subterranean clover and three of lucerne were planted in each pot, after having been germinated and grown for 5 days over a dilute -Ca nutrient solution. At this stage, the taproots of both species were about 1.5 cm in length.

All plants were harvested 4 days after planting.

Although considerable taproot growth had occurred by this time, there was little development of lateral roots. Using a method developed by Bowen and Rovira (1961), the relative number and length of root hairs, and their position of development on the taproot were recorded. Scores of 1, 2 and 3 were given for root hair length, equivalent to 0-100μ, 100-200μ and 200μ+ respectively.

Results.

At pH 6.0, the length of taproot where hairs were present and the number and length of the hairs themselves generally increased in both species with increasing calcium, but were markedly reduced by a decrease in pH. (Figs. 6, 7 and 8, and Plate 14). The root hairs of lucerne were much more sensitive to calcium and pH variation than those of subterranean clover, and did not extend along the taproot for as great a distance. At pH 5.0 root hairs were almost completely absent from lucerne roots whatever the calcium level used while root hairs were present on clover at all calcium levels at this pH but were only long and dense at 5.00 me Ca/l. Statistical data related to this trial are presented in Appendix 3.

3. Effect of calcium and pH on nodulation.

Results of previous water culture trials showed the considerable effect of calcium and pH on subterranean clover and lucerne growth, particularly on root and root hair development. In this experiment the effects of

FIGURE 6.

Effect of level of calcium on length of taproot where root hairs were present, at two pH levels, in water culture.

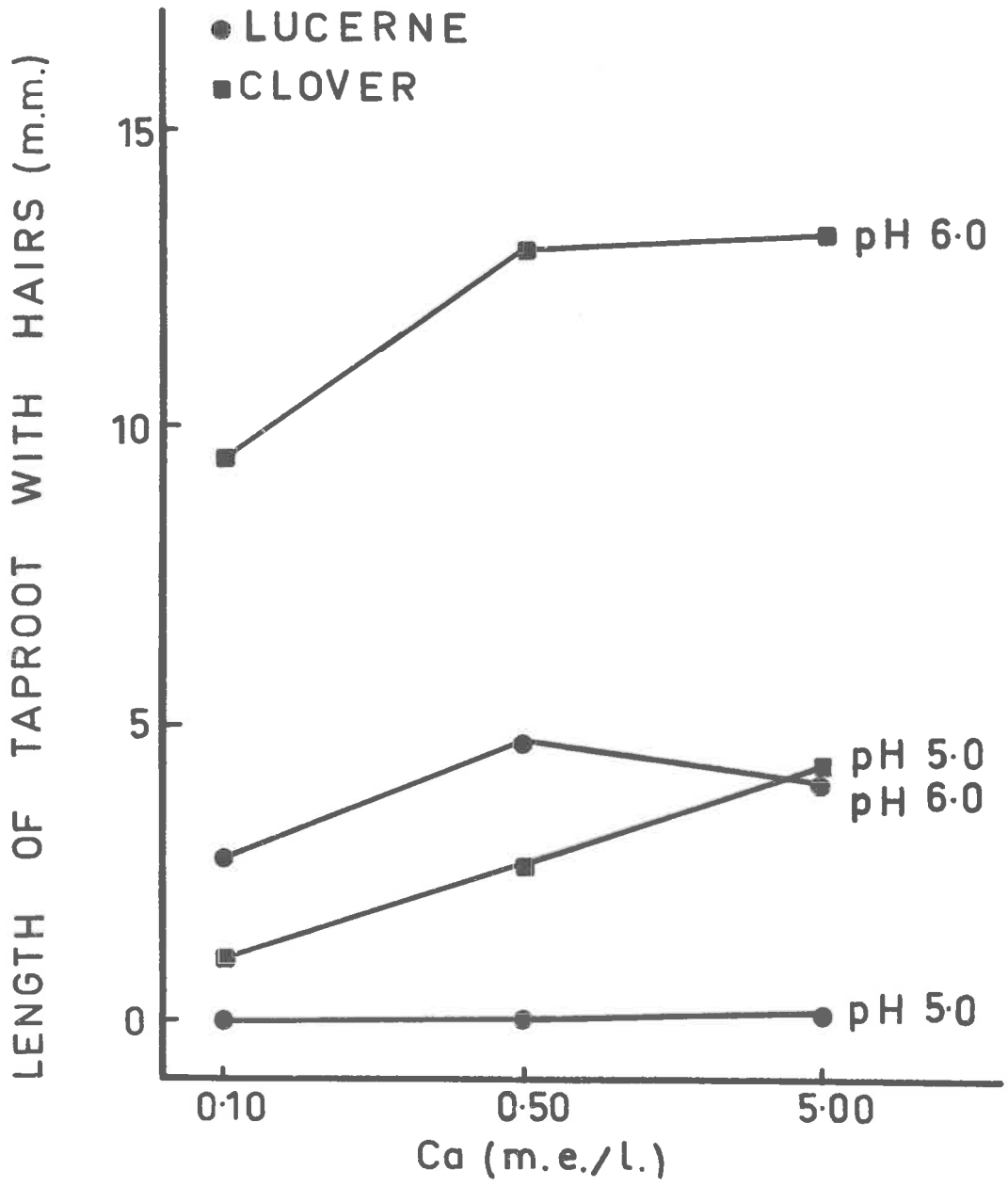
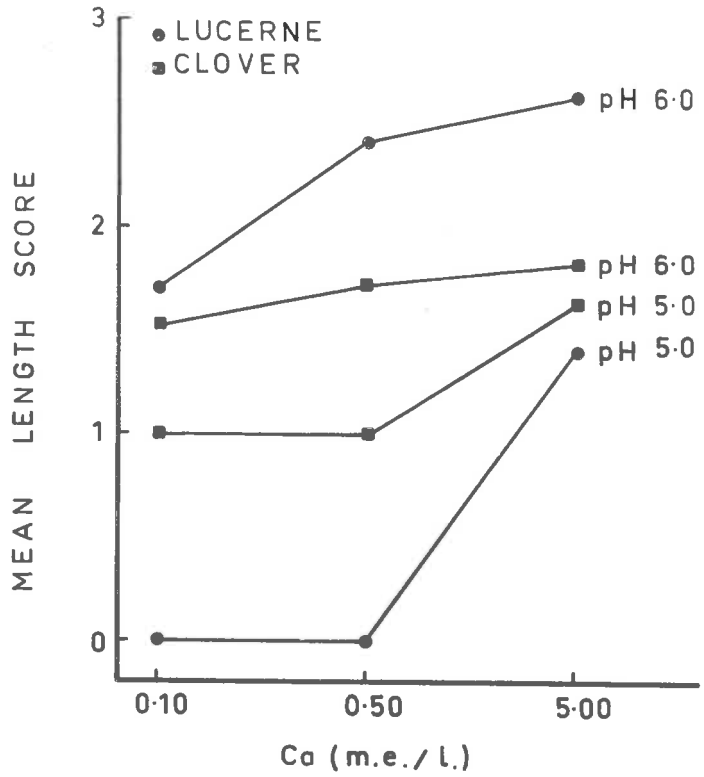

LUCERNE
CLOVER
LENGTH OF TAPROOT WITH HAIRS (m.m.)
15
10
5
0
pH 6·0
pH 5·0
pH 6·0
pH 5·0
0·10
0·50
5·00
Ca (m.e./l.)

FIGURE 7.

Effect of level of calcium on root hair length, at two pH levels, in water culture.

FIGURE 8.

Effect of level of calcium on root hair density, at two pH levels, in water culture.


LUCERNE
CLOVER
pH 6·0
pH 6·0
pH 5·0
pH 5·0
MEAN LENGTH SCORE
3
2
1
0
0·10
0·50
5·00
Ca (m.e./l.)

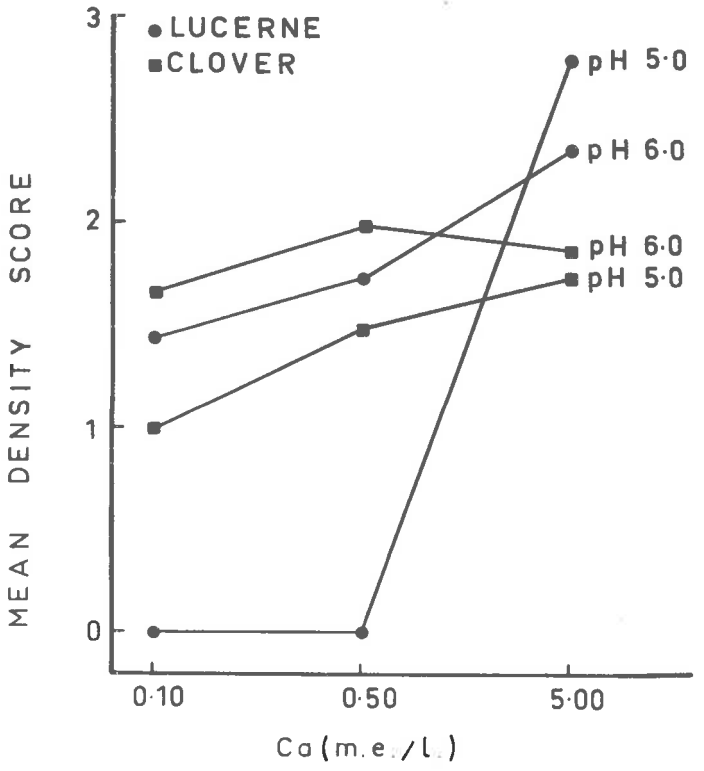

LUCERNE
CLOVER
pH 5·0
pH 6·0
pH 6·0
pH 5·0
MEAN DENSITY SCORE
3
2
1
0
0·10
0·50
5·00
Ca (m.e./l.)

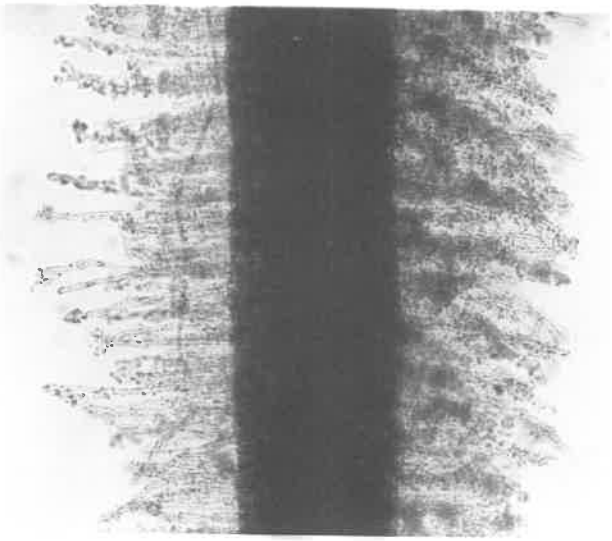
PLATE 14.

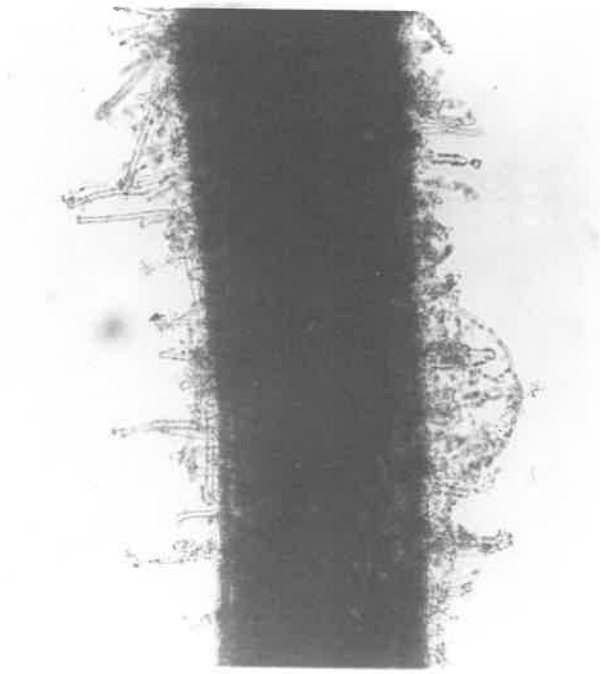
Effect of level of calcium on length and density of root hairs of lucerne in water culture at pH 6.0.

Upper - 5.00 me Ca/l.

Lower - 0.10 me Ca/l.

Magnification - X55.





calcium and pH on nodulation of the two species was studied.

The design of this experiment was similar to that used in Trial III and consisted of five calcium levels (0.10, 0.25, 0.50, 1.00 and 5.00 me/l) and three pH treatments (4.5, 5.0 and 6.0). A similar basal nutrient solution was also used.

Seedlings were planted out on October 9th, 1962 and allowed to grow in the +N solution for 7 days. The basal solution was then changed, and a -N solution, similar to that used in the root hair studies, was introduced.

Bacterial suspensions were prepared on the following day. Agar cultures of Rhizobium trifolii (W 107 ex SU 329 (1956)) and R. meliloti (W 114), both effective strains used for distribution to farmers by the Waite Institute, were each suspended in 1 litre of sterile nutrient solution. 1 ml of this suspension was added to each pot. Plate counts revealed 20 x 10^7 bacteria/ml in R. meliloti and 5 x 10^7 bacteria/ml in R. trifolii suspensions. Although multiplication of rhizobia is known to cease below about pH 5.0, the cells still remain viable in more acid conditions (Loneragan and Dowling, 1958) and the high number of bacteria supplied initially in this trial was expected to be sufficient for nodulation at pH 4.5.

Results.

Nodules appeared on subterranean clover roots

after 4 days. Growth was very slow by this time, and at pH 4.5 nitrogen deficiency symptoms in the form of yellow cotyledons were beginning to develop. Nodules did not appear on the lucerne for a further 4 days. At this stage nitrogen deficiency symptoms were showing on all plants.

Subterranean clover and lucerne plants were harvested 12 days and 15 days respectively from inoculation. At harvest, the number of nodulated plants and nodules per plant were recorded, and observations made on root hair development. The effects of calcium and pH treatment on nodulation are presented in Figs. 9 and 10. Statistical data are presented in Appendix 4.

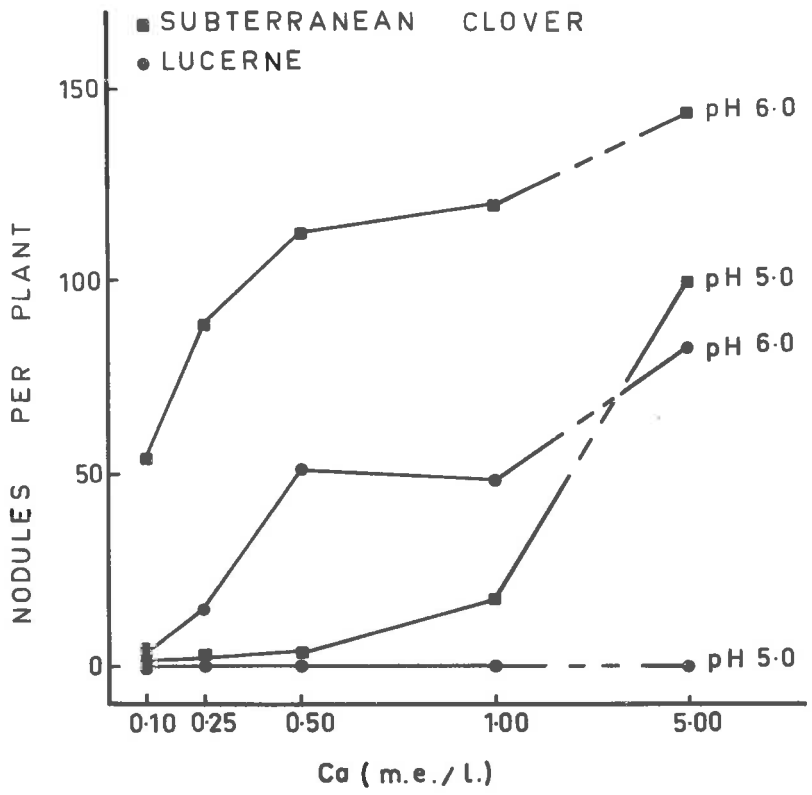
At pH 4.5 no plants nodulated, whatever the treatment. At pH 5.0, a marked species difference occurred. No lucerne plants formed nodules at any calcium level. In subterranean clover nodulation was poor at 0.10, 0.25 and 0.50 me Ca/l, but a progressive increase in numbers of nodulated plants and numbers of nodules per plant occurred as calcium level was increased further. When the pH was raised to 6.0, calcium treatment had little effect on the percentage of nodulated clover plants, but with lucerne, the percentage of nodulated plants was increased from 29.5% at 0.10 me Ca/l to 100% at 0.50 me Ca/l. At pH 6.0 the effects of calcium on nodule number in clover were much larger than on percentage nodulation, and a progressive increase occurred, from 53/plant at 0.10 me Ca/l, to 144/plant at 5.00 me Ca/l.

FIGURE 9.

Effect of level of calcium on nodule number per plant at two pH levels, in water culture.

FIGURE 10.

Effect of level of calcium on percentage of nodulated plants, at two pH levels, in water culture.


SUBTERRANEAN CLOVER
LUCERNE
NODULES PER PLANT
150
100
50
0
pH 6·0
pH 5·0
pH 6·0
pH 5·0
0·10
0·25
0·50
1·00
5·00
Ca (m.e./ l.)

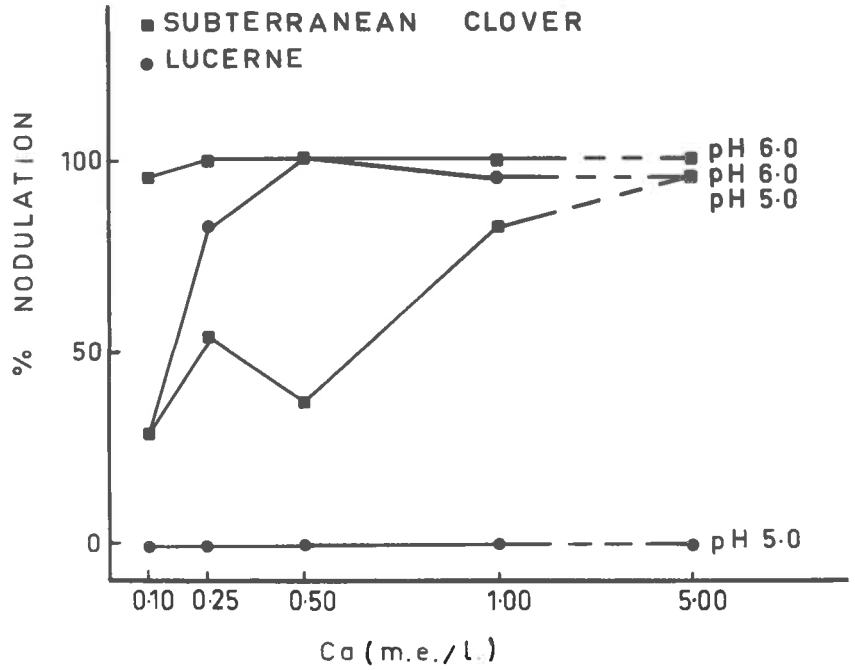
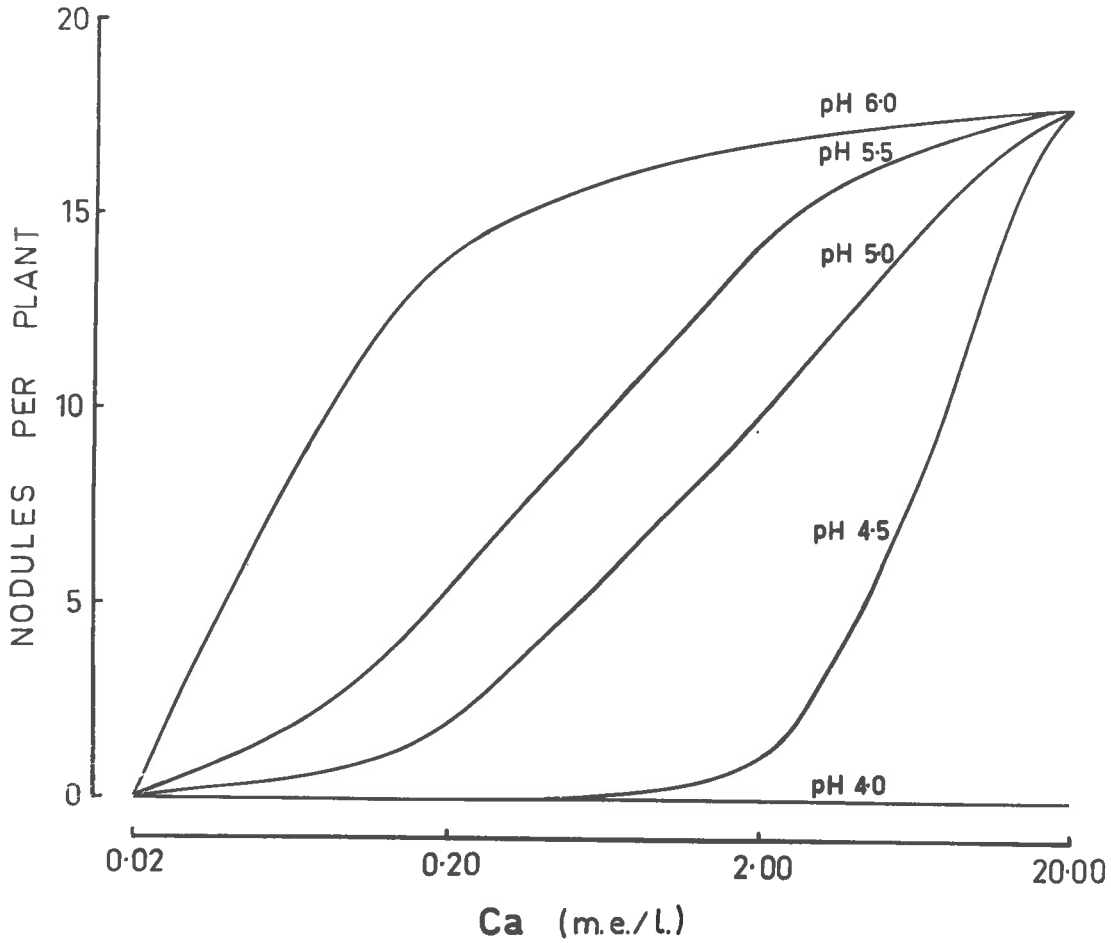

SUBTERRANEAN CLOVER
LUCERNE
% NODULATION
100
50
0
pH 6·0
pH 6·0
pH 5·0
pH 5·0
0·10
0·25
0·50
1·00
5·00
Ca (m.e./ l.)

FIGURE 11.

The interaction of calcium and pH on nodulation of subterranean clover, in water culture. (After Loneragan and Dowling, 1958).


20
15
10
5
0
NODULES PER PLANT
pH 6·0
pH 5·5
pH 5·0
pH 4·5
pH 4·0
0·02
0·20
2·00
20·00
Ca (m.e./l.)

Very few nodules were present on lucerne at 0.10 me Ca/l but a sharp rise to 51/plant occurred at 0.50 me Ca/l with a further rise to 82.5/plant at 5.00 me Ca/l. These increases occurred on plants which often did not differ greatly in root development between treatments.

Visual comparisons of root hair development were made under a binocular dissecting microscope by examining the taproots of four plants (one plant per replicate) from each treatment. The results confirmed the previous observations showing that a lowering of calcium and/or pH levels of the nutrient solution caused a reduction in the length of root covered in hairs and a reduction in length and density of the hairs themselves. The trend was much greater in lucerne, where no root hairs were visible on plants grown at any calcium level at pH 4.5 or 5.0. In subterranean clover, numerous root hairs were visible at pH 5.0 at 5.00 me Ca/l, although none were present at 0.10 me Ca/l at the same pH, or at any calcium level at pH 4.5.

One lucerne plant growing at pH 6.0, in a solution containing 5.00 me Ca/l, produced no nodules, while its companions were profusely nodulated. When the roots were examined, it was found that root hairs were short and fairly sparse on this plant, but numerous on other plants.

4. Discussion of Results.

In the three water culture trials where a study was made of the effects of calcium and pH on growth of

subterranean clover and lucerne, a reduction in the calcium or pH level of the nutrient solution reduced plant growth, and in extreme cases calcium deficiency symptoms developed or acid injury occurred. In water culture trials II and III a large Ca x pH interaction occurred, and the unfavorable effects of low pH on growth could be largely offset by an increase in the calcium level of the solution. In these two trials, at or above a calcium level of 0.25 me/l and a pH of 4.5, near maximum yields of clover could be obtained by an increase in either calcium or pH in the nutrient solution. Lucerne did not behave similarly until the calcium level was doubled and the pH raised to 5.0. At pH 5.0, calcium deficiency symptoms appeared at both 0.10 and 0.25 me Ca/l in lucerne, but occurred only at 0.10 me Ca/l in clover. In trial I, plant growth at all calcium levels at pH 4.0 was very much less than at pH 5.0 and 6.0 and acid injury was evident on roots of both species at all calcium levels.

These results agree with and add to the results obtained by Arnon et al (1942) and Arnon and Johnson (1942) on the effects of calcium and pH on plant growth. Working with tomatoes and lettuce in nutrient solution, they obtained reduced growth at pH levels below 6.0, but at pH 5.0 the effect of acid conditions could be offset by an increase in the calcium level of the solution. At pH 4.0 however, although a response to increased calcium occurred, growth was much less than at higher pH levels.

At pH 3.0 no growth took place and severe acid injury occurred to the roots.

In contrast to the above findings, Loneragan and Dowling (1958) in their water culture studies of the interaction of calcium and pH on growth of subterranean clover found no significant effect of either treatment on dry matter production over the wide range of pH 4.5-6.0 and 0.2-20.0 me Ca/l. As plants were under treatment for only 12 days, it is possible that a favorable root/solution ratio existed for the small plants, allowing sufficient calcium to be taken up for near optimum growth in all treatments. The situation is likely to have changed if the plants had been grown for a longer period. In this study, where clover and lucerne were grown for up to 60 days, large dry weight differences were measured within the range of treatments investigated by Loneragan and Dowling.

Calcium is essential for normal growth of plant roots (Sorokin and Sommer, 1940; Weirsum, 1958), and according to Burstrom (1952, 1954), who worked with wheat roots, ten times more calcium is needed for cell elongation than for cell division. In this study, taproot growth was considerably increased where either calcium level of pH was raised. Measurements of length of taproot at 3 day intervals during the duration of trial III proved a useful method of assessing treatment effects on rate of growth.

The calcium deficiency symptoms observed in

lucerne and subterranean clover were similar to those described for subterranean clover by Millikan (1953). Calcium deficiency symptoms normally occur in the youngest parts of plants; collapse of petioles of the older leaves due to calcium deficiency, observed regularly in these trials, is quite contrary to this. However, Millikan and Hanger (1964) provided an explanation for its occurrence from their studies of the distribution of Ca^{45} in subterranean clover. They found that accumulation of Ca^{45} was quite different in calcium-deficient plants than in those with a normal calcium supply. In calcium-deficient plants much of the newly absorbed Ca^{45} was acquired by young leaves at the expense of the oldest. This resulted in petiole collapse of old leaves, due to lack of calcium buildup in the distal halves of the petioles. Translocation of mobile Ca^{45} from roots to new leaves was also demonstrated.

When plants from trials II and III were analysed it was found that uptake of calcium was increased by either an increase in calcium level in the nutrient solution or an increase in pH. For example, in trial II at 5.00 me Ca/l, clover tops contained 0.43% Ca at pH 4.5, 0.51% Ca at pH 5.0 and 0.79% Ca at pH 6.0. The effect of a high hydrogen ion concentration in reducing calcium uptake has already been noted by Arnon et al (1942) who found that at pH 4.0 and 5.0 calcium absorption by lettuce and tomato was lower than at higher values. Schmehl, Peech and Bradfield (1952)

found that the uptake of Ca^{45} from solution by lucerne was reduced at pH 4.5 and 5.5, as compared with pH 6.5.

Arnon *et al* (1942) suggested that the poorer growth they obtained at pH 4.0 and 5.0 was related to reduced uptake of calcium due to the high hydrogen ion concentration, and it is likely that a similar effect occurred in this study at pH 4.5 and 5.0, where growth was reduced as compared with pH 6.0. The results of the three water culture trials showed that a higher concentration of calcium was necessary to support a given rate of calcium absorption and hence plant growth at low than at high pH. Recently, Jacobson *et al* (1960), and Waisel (1962) have postulated that calcium prevents the harmful effects of low pH by blocking hydrogen ions at the outer cell membrane, probably the plasmalemma.

Many previous studies have shown that calcium enhances the uptake of potassium and rubidium ions from solution, but reduces the uptake of sodium and hydrogen ions (Viets, 1944; Fawzy *et al*, 1954; Kahn and Hanson, 1957; Higdon and Marshall, 1959; Jacobson *et al*, 1960, 1961; Jackson and Evans, 1962; Waisel, 1962; Rains *et al*, 1964). The results obtained from analyses of plant material from trial II supply supporting evidence for these findings. Where calcium level was increased, potassium uptake was also increased, mainly in roots, and sodium uptake was decreased in both roots and tops. This selectivity occurred even where there were only slight

growth responses to calcium. Most previous studies (Viets, 1944; Fawzy et al, 1954; Kahn and Hanson, 1957; Jacobson et al, 1960; Waisel, 1962; Rains et al, 1964) on calcium effects on selectivity for monovalent cations have been short-term experiments with excised roots, and there has been little information available previously on whole plants which have undergone a lengthy period of growth and absorption.

In trials II and III lucerne needed more calcium for growth than did subterranean clover. Symptoms of calcium deficiency appeared earlier on lucerne than on clover, they were generally more severe and occurred at higher calcium and pH levels. The large difference in calcium requirement between the two species is difficult to explain on the basis of calcium uptake, as calcium levels in the tissues were not greatly different. It is possible that more calcium is immobilised in lucerne than in clover, and is unavailable for cell metabolism. Work on this aspect may help to explain the difference in calcium requirement.

At pH 5.0 and 6.0 in trials I and II, phosphorus toxicity symptoms appeared in subterranean clover in higher calcium treatments. Lucerne showed little effect and contained much lower levels of phosphorus in the herbage than did clover. The phosphorus content of the plant material was increased by high calcium chloride in the nutrient solution and by high pH. Over 1% P was

recorded in subterranean clover showing acute symptoms. Little mention is made in the literature of calcium and hydrogen ion effects on phosphorus uptake although Tanada (1955) records that in solution culture, calcium greatly enhanced phosphorus uptake in excised mung bean roots, while McEvoy (1964) found that P^{32} uptake by flue-cured tobacco was significantly increased by raising the pH of the nutrient solution.

High temperatures during trial I seemed to enhance phosphorus toxicity symptoms. Powrie (pers. comm.) has also noted this with subterranean clover in sand and water culture trials. McEvoy (1960) found that in nutrient solution uptake of radiophosphorus by flue-cured tobacco increased significantly with temperature increase from 10°C to 35°C, while Knoll, Brady and Lathwell (1964) in glass house experiments with corn, found that phosphorus uptake increased when soil temperature was increased from 15°C to 25°C.

Phosphorus toxicity has been noted by Rossiter (1952, 1955) in subterranean clover and oats and by Warren and Benzian (1959) in yellow lupins. Both workers recorded phosphorus levels greater than 1% in affected plants. Strains of subterranean clover grown in nutrient solutions are known to differ in their tolerance of high levels of phosphorus. For example, Clare showed more severe symptoms and contained a higher level of phosphorus than the less-affected Bacchus Marsh variety (Powrie,

pers. comm.). Snaydon and Bradshaw (1962b) have shown that natural populations of white clover vary in their response to phosphate, and that the yield of some edaphic ecotypes is depressed by high levels of phosphorus. Noggle and Fried (1960) suggested that the differences in uptake of phosphorus by excised roots of millet, barley and lucerne could be largely accounted for by differences in concentration of phosphorus "carrier" in the plants. Such a difference could possibly be of wide occurrence but further study is required before it could be accepted as an explanation for the differences in phosphorus uptake in clover and lucerne. The reason for the harmful effects of high phosphorus levels is not at present known.

In the experiments where root hair development was examined, it was clear that the length of taproot covered in hairs, and the number and length of the hairs themselves, was highly dependent on the calcium and hydrogen ion concentration of the nutrient solution. Where the calcium level was reduced at pH 6.0, root hair development was also reduced in both species. At pH 5.0, root hairs on clover were long and dense only at 5.0 me Ca/l, and were almost completely absent on lucerne whatever the calcium level.

As far as the author is aware this is the first study of the effects of calcium and pH on root hair development in subterranean clover and lucerne, although observations have been made on a number of other plants

(Cormack, 1949,1962). Farr (1927 a, b, c; 1928 a, b) found that calcium was required in the nutrient solution for the growth of root hairs on Brassica oleraceae, where it was used for cell wall formation. He also showed that pH was an important factor in root hair development, but the adverse effects of low pH could be overcome to some extent by increasing the level of calcium in the solution. Ekdahl (1957a), working with wheat roots, found that root hair length could be increased by increasing the pH or calcium levels in nutrient solution. Cormack (1935, 1944, 1959 a, b) showed that calcium combined with pectic acid in the hardening or calcification of the root hair wall. In the absence of calcium, he found that hair formation on tomato and Brassica roots was greatly reduced, and the hairs were short and sometimes swollen, with soft cell walls.

Lucerne showed a higher calcium and pH requirement for root hair development than did subterranean clover in these trials. This may be due to a greater need for calcium by lucerne in calcification of the root hair wall. The response of the two species to calcium in root hair development parallels the responses in dry matter increase, and indicates some common property of the hair cells and other tissues in respect to calcium need or difficulty of calcium uptake.

In the earlier root hair trial a high level of nitrogen in the nutrient solution caused a large reduction

in root hair development of both species. Thornton (1936) observed a similar effect in lucerne. He found that both the number of root hairs and their length were greatly reduced by addition of sodium nitrate to the solution.

Nodule formation in subterranean clover followed a somewhat similar pattern to that observed by Loneragan and Dowling (1958) for the same species (Figs.9 and 11). They found that almost maximum nodulation could be achieved by increasing either calcium or pH, so long as the calcium was above 0.02 me/l and the pH above 4.0. In this trial, almost maximum nodulation could be obtained in clover by increasing either calcium or pH, so long as calcium was above 0.10 me/l and pH above 4.5. Nodules may have formed at pH 4.5, however, if 20 me Ca/l (as used by Loneragan and Dowling) had been used. In contrast to Loneragan and Dowling's experiments this trial was uncomplicated by the presence of nitrogen in the nutrient solution. A very large species difference in nodule formation occurred. Nodules were much later in appearing on the lucerne roots, and a calcium level of 0.50 me/l was required to obtain near maximum nodulation at pH 6.0. At pH 5.0, no nodules formed even at 5.00 me Ca/l although plants from this treatment in a +N solution produced near maximum yields of herbage.

In contrast to the findings of other workers (Albrecht, 1932; Loneragan and Dowling, 1958; Andrew and Norris, 1961) there was little to indicate that the

calcium requirement for nodulation was higher than for growth of the host plant. For example, the critical calcium level for near maximum plant growth and nodulation of clover at pH 6.0 was 0.25 me/l, and below this both were depressed. However, a higher pH requirement for nodulation was evident in both species. While clover and lucerne at 5.00 me Ca/l grew well at pH 4.5 and 5.0 respectively, they did not nodulate well at this calcium level until the pH was raised to 5.0 and 6.0.

Number of nodules formed on the roots of the two legumes was closely related to root hair number and length. For example, at pH 5.0 or 0.10 me Ca/l where few or no nodules were present on lucerne, root hairs were sparse, short, or completely absent. At 5.0 me Ca/l and pH 6.0 where nodulation was at a maximum, root hairs on lucerne were long and dense. A similar situation occurred with clover. At pH 5.0, root hair development, nodulation and nodule number all increased, and at pH 6.0 root hair development and nodule number increased, with increasing calcium level.

Thornton (1936) observed that where root hairs were less than 150μ in length, little curling occurred. Raggio et al (1957) have ascribed improvement in nodulation in vermiculite and sand to marked increases in length and numbers of root hairs. As the principal means of infection of a clover root is through the root hair, the number of root hairs present, and their length, may largely determine

the percentage of root hairs which curl and nodules which form, so long as bacterial numbers are non-limiting, as was probably the case in the experiments described here. Therefore, in nutrient solution at least, one of the main effects of calcium and hydrogen ions on nodule formation may be through their effects on root hair growth. Not only could this account for the nodulation response to calcium and pH within a species but may largely explain the difference between subterranean clover and lucerne which has been established in these experiments.

B. Nodulation Trials in Mt. Compass Sand.

1. Introduction.

Siliceous sands occur widely in South Australia. They are found in the South-East and Eyre Peninsula districts and in scattered areas in the Adelaide Hills. On these sands, difficulty in establishing two commonly-grown legumes, subterranean clover and lucerne, have frequently occurred, more especially in lucerne. Deficiencies of phosphorus, copper and zinc have been recorded in some of these soils (Riceman, 1945, 1948), but nodulation failure has also been responsible for poor establishment. Nodulation failure may be due to a number of causes, including absence of suitable rhizobia from the soil or poor survival of inoculum on seed and/or in soil, due to an adverse physical (heat, cold or desiccation), chemical (acidity, calcium deficiency), or biological (microbial antagonism, antibiotics) environment. The possibility of low pH and calcium levels affecting nodulation as such much also be considered. On deep sands in the Bangham Scrub in the South-East district improvement in both soil calcium and pH levels is necessary for successful nodulation of lucerne (Powrie, unpublished), while at Mt. Compass, in the Adelaide Hills, both lime ($CaCO_3$) and heavy rates of inoculum improve nodulation of subterranean clover (Warcup and Hockley, unpublished).

Field observations suggest that subterranean clover and lucerne differ in their requirements for growth

and nodulation (Warcup, Hockley, Powrie, unpublished). Confirmation of this was obtained in the water culture experiments described in Section A, where lucerne was found to be much less tolerant of low calcium and high hydrogen ion concentrations than subterranean clover. The difference is of agronomic importance because lucerne, with its perennial habit and ability to survive long periods of drought is better suited to deep sands than is subterranean clover.

At Mt. Compass, 45 miles south of Adelaide, where areas of deep, acid sand occur, the legume establishment problem is most acute and both subterranean clover and lucerne are difficult to establish. The Mt. Compass sand was therefore selected for comparative nodulation studies to provide information on the reasons for nodulation failure in the two species and also to allow further investigations of the effects of calcium and pH on nodulation, under field conditions.

(a) Climate.

Mt. Compass has a climate that shows a marked seasonal contrast, with warm to hot, dry summers and cool wet winters. The mean annual rainfall (20 year average) is 31.9 inches, two-thirds of this falling from May to September (Fig. 12). Although no temperature records are available for Mt. Compass, air temperatures measured at Waite Institute (Table 7), indicate the seasonal range.

The mean air temperature during the winter months

is probably less than that prevailing in Adelaide. In 1963, during June, July and August, the average daily air temperature measured at the field plots was 4--5°F below that at the Waite Institute (Fig. 13).

TABLE 7

Average Daily Air Temperatures (°F) 1925-61.

Month	Maximum	Minimum	Mean
January	82.0	60.8	71.4
February	80.0	60.8	70.8
March	78.3	59.5	68.9
April	70.3	54.6	62.5
May	64.0	50.7	57.3
June	59.0	46.9	53.0
July	57.2	45.3	51.3
August	59.1	46.0	52.5
September	63.7	48.4	56.1
October	68.2	51.1	59.6
November	73.7	54.4	64.0
December	78.5	57.9	68.2
Year	69.6	53.0	61.3

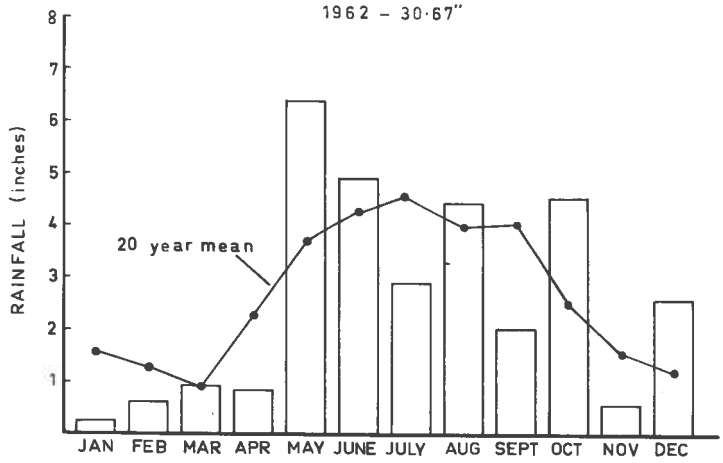
Air temperatures of above 100°F occur occasionally in summer, and a few ground frosts are experienced in winter. At Myponga, a weather station close to Mt. Compass, and receiving 29.3" of rainfall annually, Trumble (1948) has calculated an average growing season of 8.1 months for surface-rooted annual plants. No comparable data are available for Mt. Compass, but the growing season is likely to be similar to Myponga.

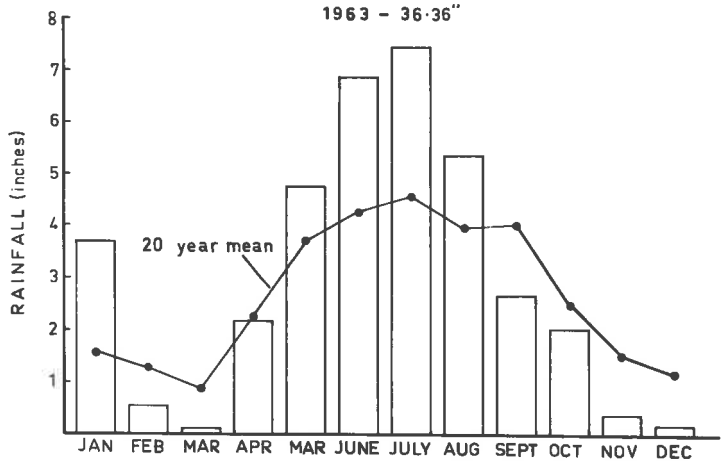
FIGURE 12.

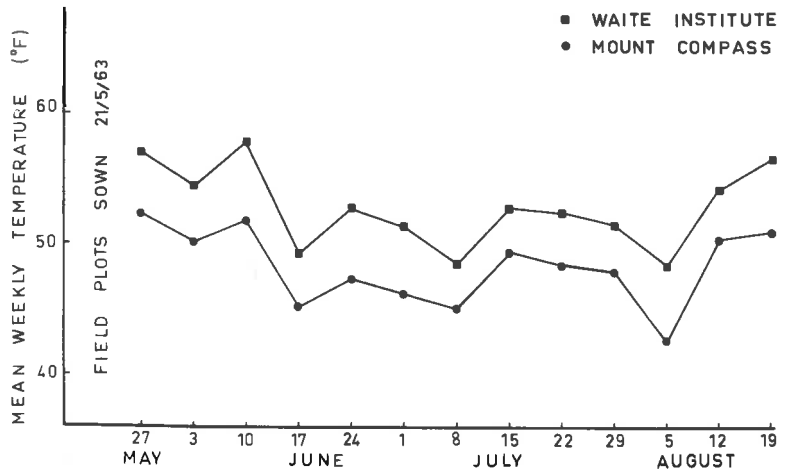
Monthly rainfall at Mt. Compass, 1962 and 1963.

FIGURE 13.

Comparison of air temperatures at Waite Institute, Adelaide, and at field trial site, Mt. Compass, winter 1963.


1962 – 30·67"
RAINFALL (inches)
20 year mean
JAN FEB MAR APR MAY JUNE JULY AUG SEPT OCT NOV DEC


1963 – 36·36"
RAINFALL (inches)
20 year mean
JAN FEB MAR APR MAR JUNE JULY AUG SEPT OCT NOV DEC


WAITE INSTITUTE
MOUNT COMPASS
FIELD PLOTS SOWN 21/5/63
MEAN WEEKLY TEMPERATURE (°F)
27 3 10 17 24 1 8 15 22 29 5 12 19
MAY
JUNE
JULY
AUGUST

(b) Soil.

The soil on which the experiments were conducted at Mt. Compass is a deep sand with marked podzolic characteristics. A typical soil profile is shown in Plate 16. The A horizon consists of about 12" of light grey sand which merges into 30" of leached off-white sand almost free of plant roots and visible organic remains. Below this lies a sharply-defined 6" band of compacted sand stained reddish-brown from an accumulation of organic matter and possibly iron oxides. Below this is several feet of well-drained yellow sand. A mor litter may accumulate if the soil remains undisturbed.

The sand is coarse, with only a trace of clay. Greenland and Ford (1964) by ultrasonic dispersion, separated a light fraction, 4% by weight, which is 40% organic matter and contains 85.3% of the soil carbon. The topsoil has a pH value of 4.90 (4:1 paste), but the pH drops sharply to 4.3 in the zone of organic accumulation at 42" depth. Many soil nutrients are in short supply, including N, P, K, Mg, Zn, Cu, Co, and probably Ca and S. Details of the chemical characteristics of the soil are listed in Table 8.

(c) Vegetation.

The deep sands at Mt. Compass support an open, native scrub vegetation, very rich in species, but now somewhat modified under the influence of fire and domestic animals. The majority of the plants in the vicinity of

PLATE 15.

General view of field trial site, Mt. Compass, showing plots in foreground and native vegetation in the background.

PLATE 16.

Soil profile at field trial site, Mt. Compass.

0	-	12"	Light grey sand.
12	-	42"	Leached, off-white sand.
42	-	48"	Compacted reddish-brown layer of sand.
Below		48"	Yellow sand.

TABLE 8.

Physical and Chemical Characteristics -

Mt. Compass Sand (0-4" layer).

Mechanical analysis:		
Passed through 1000 micron sieve		9.24%
" " 500 " "		73.00
" " 211 " "		17.76
Clay fraction:		trace
Organic carbon (dry combustion method):		0.68%
Nitrogen:		0.036%
C:N ratio:		19.4
Phosphorus:		0.0014%
Cation exchange capacity:		3.16 me%
Total exchangeable bases:		1.46 me%
Exchangeable cations:	Ca^{++}	1.20 me%
	Mg^{++}	0.20 me%
	K^{+}	0.03 me%
	Na^{+}	0.03 me%
	H^{+}	1.70 me%

the trial area are dwarf shrubs with ericoid leaves, giving the whole association a heath-like appearance. Areas of bare ground occur between the shrubs. Eucalyptus baxteri is the dominant tree, growing to the height of 20-25 feet, and Banksia marginata, B. ornata, Xanthorrhoea semiplana, Leucopogon virgatus, Leptospermum myrsinoides, Casuarina paludosa, Isopogon ceratophyllus, Acacia verticillata and A. myrtifolia are the most abundant shrubs. Bracken (Pteridiun aquilinum) is also prominent. A list of plants collected in the vicinity of the plots is recorded in Appendix 5, and specimens of each have been deposited in the Waite Institute Herbarium. A general view of the trial site, with native vegetation in the background, is shown in Plate 15.

(d) Previous trials.

Several studies of legume establishment on Mt. Compass sand have been made in recent years by the South Australian Department of Agriculture, C.S.I.R.O. Division of Soils, and Waite Agricultural Research Institute, and these are briefly summarised here.

(i) Officers of the S.A. Department of Agriculture examined the effect of lime on subterranean clover establishment on the property of Mr. Black, 374 lb. per acre giving better results than 187 lb. per acre.

(ii) Officers of the S.A. Department of Agriculture and the Plant Pathology Department of the Waite Institute laid down fertilizer and Rhizobium inoculation

trials on old cleared ground on the property of Mr. Nitschke. With subterranean clover, apart from an early response to nitrogen, growth of all plots was good and no difference between treatments could be seen.

(iii) Members of the Biology section, C.S.I.R.O. Division of Soils also conducted fertiliser trials on Mr. Nitschke's property, on a site recently cleared from scrub and bracken, and about 200 yards from the Department of Agriculture trial. Neither lucerne nor subterranean clover established well. Work is continuing, and J.R. Harris (personal comm.) has indicated that an organic toxin may be present in the sand, causing reduction of growth of subterranean clover, lucerne and ryegrass seedlings.

(iv) J.K. Powrie of the Agronomy Department of the Waite Institute examined the effect of cobalt on the growth of subterranean clover and lucerne sown in an area adjacent to the C.S.I.R.O. trials. With a basal dressing of 30 cwt. of lime a number of lucerne plants became established, and these were still present three years later. No response to cobalt was recorded.

A further trial on an adjacent site in 1963 showed a marked growth response in subterranean clover to addition of cobalt.

(v) J.H. Warcup and S.R. Hockley of the Plant Pathology Department of the Waite Institute conducted further establishment trials with subterranean clover in

1960 and 1961 alongside the area used by J.K. Powrie. A significant response to increased level of inoculum on the seed and calcium carbonate addition to the sand occurred, as measured by average dry weight per plant five months from sowing.

2. Mt. Compass Field Trial, 1962.

In view of the plant responses obtained by previous workers on addition of $CaCO_3$ to the sand, and to increased level of inoculum on the seed, a field trial was designed to investigate the effect of calcium, pH and level of seed inoculum and their interactions on nodulation of subterranean clover and lucerne.

(a) Site preparation.

The area chosen for the experiment adjoined the plot sites previously established by the C.S.I.R.O. and the Waite Institute and had recently been cleared of scrub and disc-ploughed to a depth of 10 inches. After it had been fenced it was raked smooth and given a basal fertilizer dressing (Table 9) applied by hand on May 17th, 1962.

TABLE 9

Basal Dressing - Mt. Compass.

	Pounds/acre		Pounds/acre
$Na_2HPO_4.2H_2O$ *	260	$CuSO_4.5H_2O$	7
$MgSO_4.7H_2O$	112	$Na_2B_4O_7$	7
K_2SO_4	224	$(NH_4)_6MO_7O_{24}$	2 oz.
$MnSO_4.4H_2O$	14	$CoSO_4.7H_2O$	2 oz.
$ZnSO_4.7H_2O$	7	*Supplying P equivalent to 4cwt. of superphosphate.	

(b) Treatments.

The trial was of factorial design with four replicates of the following treatments.

1.	Legume Species	Mt. Barker subterranean clover	
		Hunter River lucerne	
2.	pH	5.0	
		6.0	
3.	Calcium	Control	Ca_0
		Equiv. 2 cwt. $CaCO_3$	Ca_1 *
		" 4 " "	Ca_2
		" 8 " "	Ca_3 *
		" 12 " "	Ca_4
4.	Inoculum	Normal (1x)	In_1
		Heavy (1000x)	In_2

* Normal inoculum only.

Soil pH was raised by the addition of $MgCO_3$ applied at the rate of 7 cwt. per acre, while calcium was added as $CaSO_4 . \frac{1}{2}H_2O$. To check on the possibility of free-living rhizobia being present in the sand, a Ca_4, pH 6.0 treatment was sown with surface-sterilised, uninoculated seed. In two further treatments, both with heavy inoculum the pH was raised by 4cwt./acre of fine $CaCO_3$ and the proportions of $CaCO_3$ and $CaSO_4$ adjusted to provide a pH of approximately 6.2 and a calcium equivalent of 4 cwt. (Ca_2) and 12 cwt. (Ca_4) of $CaCO_3$ per acre. Thus there were 19 treatments for each species.

The randomised block layout is shown in Fig.14.

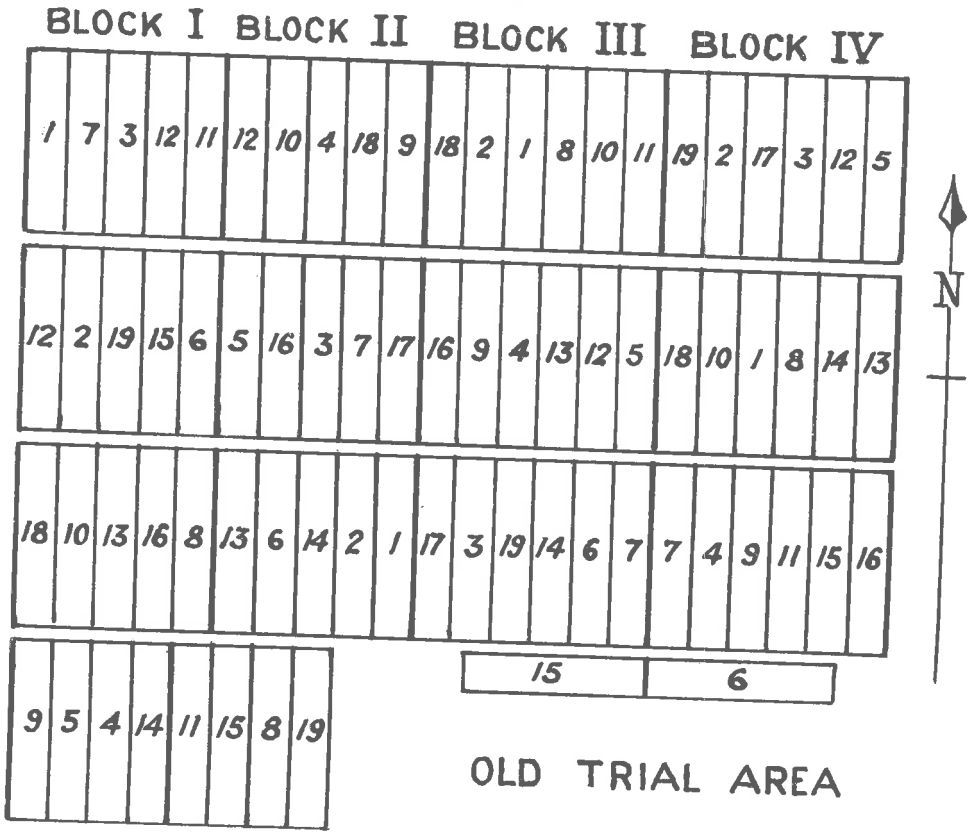
FIGURE 14.

Mt. Compass Field Trial, 1962.

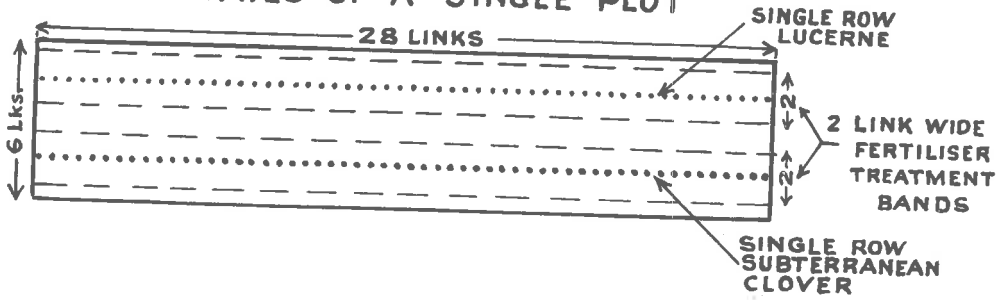
Treatments.

1.	pH 5.0	Ca_0	In_1	11.	pH 6.0	Ca_1	In_1
2.	"	"	In_2	12.	"	Ca_2	In_1
3.	"	Ca_1	In_1	13.	"	"	In_2
4.	"	Ca_2	In_1	14.	"	Ca_3	In_1
5.	"	"	In_2	15.	"	Ca_4	In_1
6.	"	Ca_3	In_1	16.	"	"	In_2
7.	"	Ca_4	In_1	17.	"	"	In_0
8.	"	"	In_2	18.	pH 6.8	Ca_2	In_2
9.	pH 6.0	Ca_0	In_1	19.	"	Ca_4	In_2
10.	"	"	In_2				

Mt COMPASS FIELD TRIAL 1962



DETAILS OF A SINGLE PLOT



A general plan of all field trials conducted in 1962 and 1963 is shown in Fig. 15. Plots measured 28 x 3 links; the two species receiving identical treatment being paired and randomised within each pair. Fertilizers were mixed with a quantity of dry sand and broadcast on a strip 2 links wide down the centre of each plot, and raked in to about 2" depth.

(c) Seed.

Seed of subterranean clover (var. Mt. Barker) and lucerne (var. Hunter River) was sieved to obtain samples with little variation in individual seed size. The following mean weights per seed resulted.

Clover - 5.8 mg./seed

Lucerne - 2.2 mg./seed.

Seed of the same size, and from the same sample was used in all further trials.

(d) Seed inoculation.

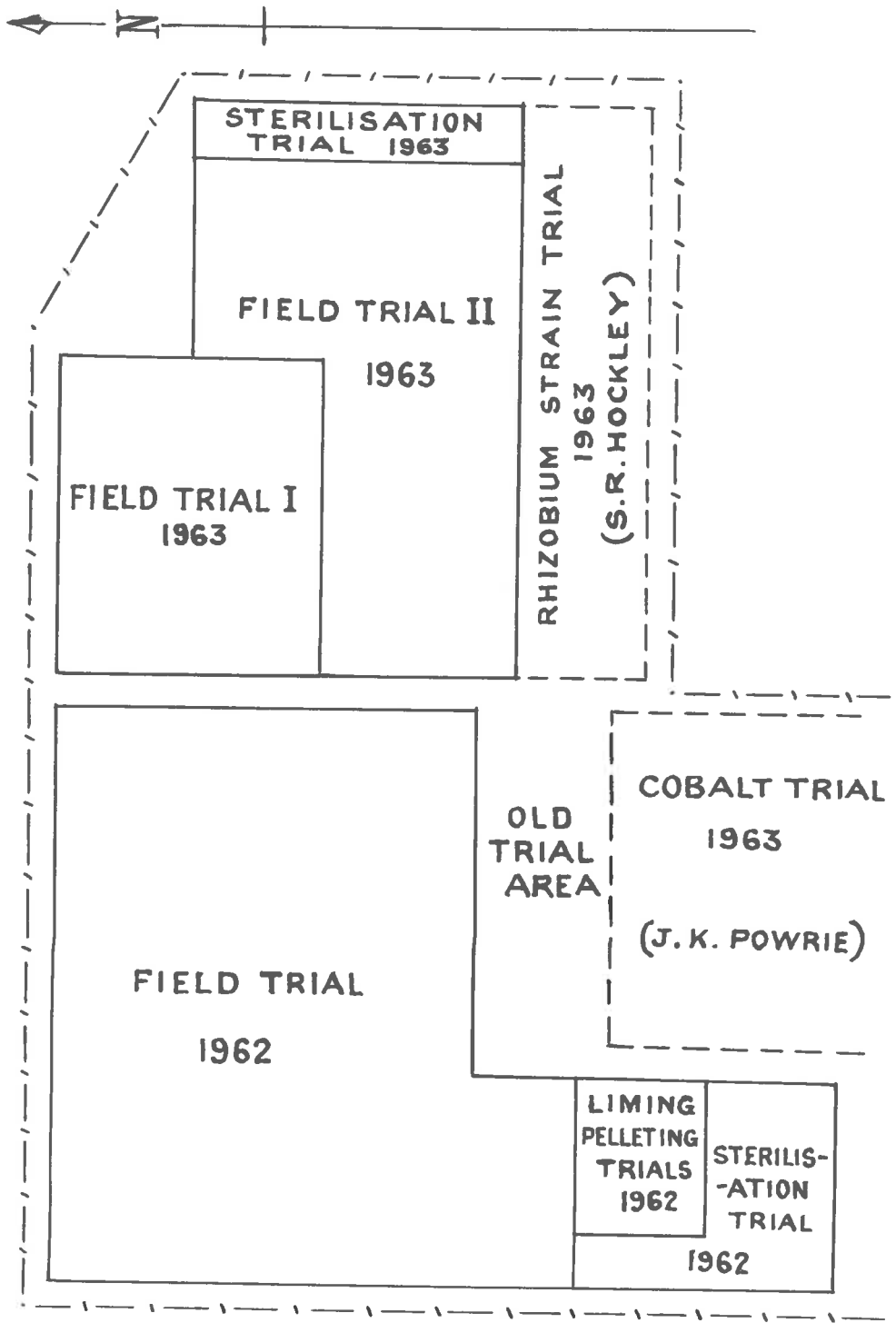
Cultures of Rhizobium trifolii (W 107 ex SU 329 (1956)) and R. meliloti (W 114) were grown on nutrient agar for 5 days. Seed was inoculated 48 hours before sowing and allowed to dry at room temperature in the laboratory. The method used for inoculation was as follows.

Normal inoculum: A culture of inoculum was mixed thoroughly with 850 ml. (1½ pts.) of skim milk. A 4.25 ml. aliquot was added to 113.0 g. (¼ lb.) of seed. Plate counts showed 1,600-2,700 cells/seed on subterranean

FIGURE 15.

Location of field trials at Mt. Compass, 1962 and 1963.

LOCATION
Mt COMPASS FIELD TRIALS



clover, and 3,000/5,000 cells/seed on lucerne.

Heavy inoculum: (x 1,000) The undiluted culture slime was mixed directly with 22.5 g. of seed. Subterranean clover and lucerne had 1 x 10^6 and 3 x 10^6 bacteria/seed respectively, according to plate counts.

The next day, seed was counted into lots of 100, placed in small envelopes and stored in a refrigerator until sowing.

(e) Sowing.

The trial was sown on May 25th, a single row of 100 seeds being sown at $\frac{3}{4}$" depth down the centre of each plot. To facilitate even sowing, the seed was mixed with a small quantity of dry sand. The plots were damp at sowing, having received over 3" of rain in the previous few days.

(f) Emergence and nodulation.

Emergence appeared to be complete within 2 weeks, and when plants were examined on July 2nd (38 days from sowing) there were small nodules on subterranean clover, but none on lucerne.

Movement of sand on part of the plot area damaged young seedlings in the southern end of the trial, especially the lucerne. The northern, more sheltered area was less affected.

(g) Field plot harvesting and nodule counting techniques.

Whole plants were removed at random with a long narrow trowel and placed in labelled plastic bags. The

sand was sufficiently loose to allow removal of the roots with little damage or loss. Plants awaiting examination in the laboratory were stored in a refrigerator at 4°C.

The plants from each plot were washed with tapwater to remove adhering sand. Nodulated and non-nodulated plants were separated and "primary" and "secondary" nodules were counted separately on each nodulated plant. "Primary" nodules were defined as those on the taproot within 1" of the crown of the plant, while all other nodules on both the lower part of the taproot and lateral roots were classed as "secondary". The primary nodules were larger and were observed to form quite early in the seedlings life. Their presence on plant roots was regarded as a measure of effectiveness of the inoculum, pH or calcium treatments.

Where difficulty was experienced in distinguishing between nodules and lobes of an individual nodule, all were classified as single nodules.

(h) Harvests.

Twelve seedlings from each row were removed at random on July 17th, 53 days from sowing. At this stage, all plants had developed a unifoliate leaf, with the first trifoliate leaf beginning to appear on larger subterranean clover plants. Plants were classified into non-nodulated and nodulated, and nodules counted.

A further random sample of 20 plants was harvested from each row on September 4th, 102 days from

sowing and 49 days after the previous harvest, and similar measurements were made of nodulation. Plant tops were rinsed in distilled water and retained for dry weight determination and chemical analyses. Calcium and phosphorus levels were determined by methods already described, while nitrogen levels were determined by the micro-Kjeldahl method. No roots were kept as very considerable effort was needed to remove entangled fragments of organic matter.

Sand blast effects were again evident in the south-west corner and in some plots it was impossible to obtain the required 20 plants. A feature of the lucerne seedlings was their stunted and bluish appearance, even though many were nodulated and the sand had received basal nutrients. Nodulated plants could usually be picked out by their larger size but in no case were they as large as expected of 3 month old plants.

(i) Soil pH measurements.

It is well known that the pH of any soil depends on the conditions of measurement. The Mt. Compass sand does not disperse in water, and a 4:1 paste preparation (sand and distilled water) was used in all pH measurements as it was considered that this approached field conditions more closely than a 1:2.5 or 1:5 suspension used on normal soils. The following are pH readings obtained from four sand-water mixtures.

4:1 = 4.95

1:1 = 5.10

1:2.5 = 5.25

1:5 = 5.45

All readings were made on a Cambridge pH meter with glass and calomel electrodes, after the paste had stood overnight at room temperature.

Soil samples from the field trial were obtained at 0-2" in July and again in December for pH determinations. Three samples were taken from all plots and measurements made on bulked samples. The following results were obtained.

	July	December
Control	4.98	4.93
7 cwt. $MgCO_3$/acre	6.01	5.92
4 cwt. $CaCO_3$/acre	6.78	6.53

The 4 cwt. of fine $CaCO_3$ was expected to raise the pH to 6 but the level obtained was nearer 7. Little change in pH occurred during the experiment in spite of heavy rains in the winter. The plots where $CaSO_4.\frac{1}{2}H_2O$ was added were not measured separately in July, but in December pH levels obtained from +Ca and -Ca plots were similar.

(j) Results.

Significant responses in nodulation to increased level of inoculum and increased pH occurred, but only a slight response to calcium was obtained. A marked

species difference occurred. Complete results from the two harvests, including statistical data, are presented in Appendices 6 and 7.

i. Level of inoculum. The response to level of inoculum was an outstanding feature of the trial (Fig. 16 and Table 10). Where no inoculum was applied, lucerne plants failed to form nodules, although a few secondary nodules gradually appeared on most uninoculated subterranean clover where pH and calcium levels were near optimum. Pronounced responses to inoculation of the seed occurred, especially where 1,000 x normal levels were used. For example, 100% nodulation of clover could be obtained whatever the treatment, provided a high level of inoculum was used. In lucerne, nodulation was poor, even with adequate calcium and a pH of 6.0, except where 1,000 x normal inoculum was applied to the seed.

The dry matter yield at final harvest was greatly affected by wind damage. Consequently the effects of varying nodule number on dry matter production were difficult to assess, although a yield response was apparent in subterranean clover where heavy inoculum was applied to the seed.

ii. Time. In comparison with the first harvest, an improvement in both percentage of nodulated plants and in nodules per plant had occurred at the second harvest in both species, (Fig. 16 and Table 10). This was particularly so in clover where normal inoculum was used,

FIGURE 16.

Mt. Compass Field Trial, 1962 - Effect of level of inoculum on seed on the percentage of nodulated plants, at pH 6.0 and Ca_4 (= 12 cwt. $CaCO_3$/acre).

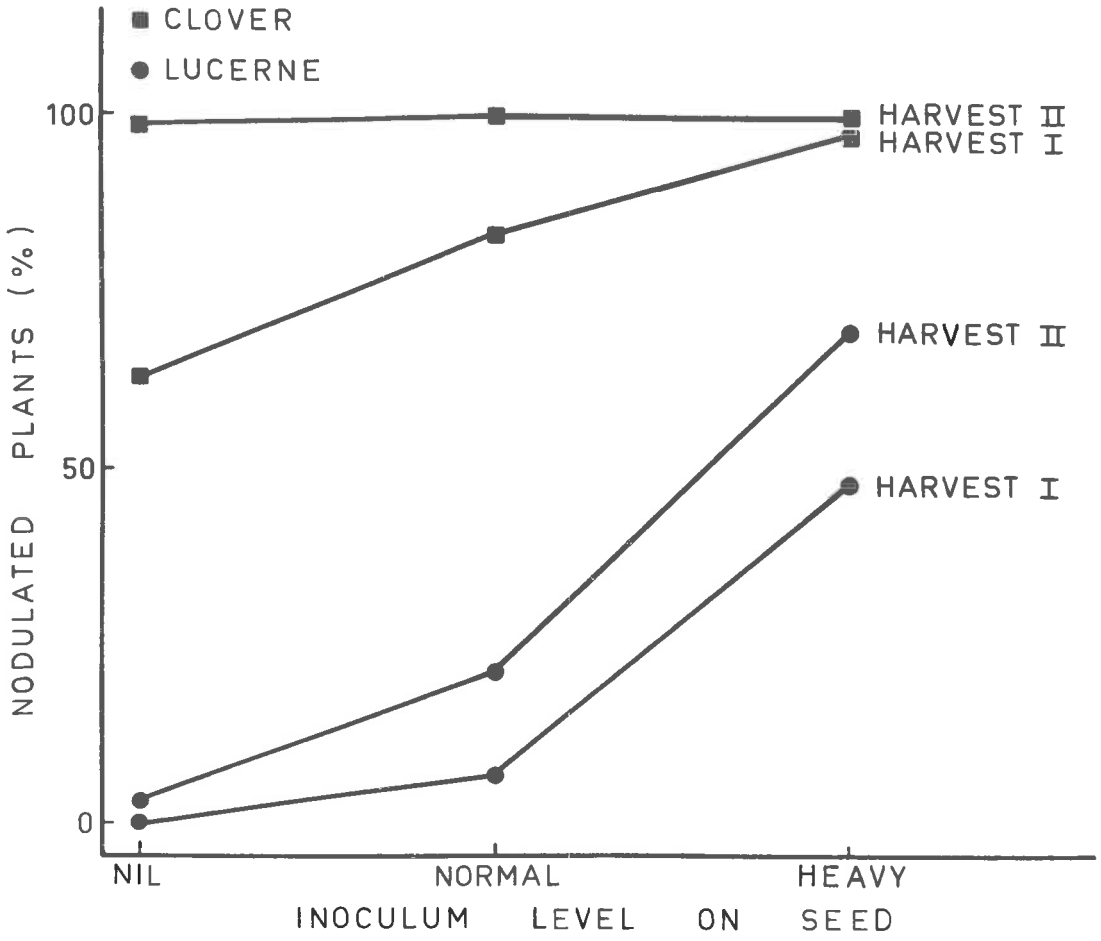

CLOVER
LUCERNE
100
50
0
NODULATED PLANTS (%)
HARVEST II
HARVEST I
HARVEST II
HARVEST I
NIL
NORMAL
HEAVY
INOCULUM LEVEL ON SEED

TABLE 10.

Effect of Level of Inoculum and pH on Nodulation.*

Field Trial 1962.

Treatment	Subterranean Clover			Lucerne		
	% Nod. Plts.	Nods/Plt. Prim.	Nods/Plt. Sec.	% Nod. Plts.	Nods/Plt. Prim.	Nods/Plt. Sec.
		Harvest I.				
No inoculum						
pH 6.0	62.8	0.5	2.8	0	-	-
Normal inoculum						
pH 5.0	67.5	0.9	3.0	0	-	-
pH 6.0	83.7	1.3	4.0	6.8	1.0	0.3
Heavy inoculum						
pH 5.0	100.0	4.0	7.1	18.3	1.1	0.4
pH 6.0	97.7	4.5	6.4	47.9	0.7	1.0
pH 6.8	100.0	3.9	4.5	66.7	1.4	0.5
		Harvest II.				
No inoculum						
pH 6.0	98.7	2.6	6.4	3.1	0.4	1.7
Normal inoculum						
pH 5.0	100.0	2.5	3.0	8.7	0.6	0.9
pH 6.0	100.0	3.3	4.8	21.2	0.9	1.3
Heavy inoculum						
pH 5.0	100.0	4.5	8.2	26.1	2.4	0.6
pH 6.0	100.0	6.9	8.0	69.5	1.7	1.1
pH 6.8	100.0	5.9	7.4	89.5	1.7	1.1

* Mean of 4 replicates, 12 plants/replicate at Harvest I and 20 plants/replicate at Harvest II, all at Ca_4 (= 12 cwt. $CaCO_3$/acre).

and resulted in the differences between the two inoculum levels being considerably reduced when Harvest II was taken.

iii. pH. Nodulation of lucerne was very poor at pH 5.0 whatever the calcium or seed inoculation levels, but progressive improvement occurred at pH 6.0 and 6.8 (Fig. 17 and Table 10). Nodulation of clover at pH 5.0 was much better than that of lucerne, but a response in percentage nodulation (with normal inoculation only) and nodule numbers was still obtained when the pH was raised to 6.0. No further response in clover was obtained at pH 6.8.

iv. Calcium. Calcium responses were small and not significant ($P > 5\%$), but were evident in both species at each harvest. In subterranean clover they occurred at pH 5.0 where, with normal inoculum, an increase in both percentage nodulation and nodule number per plant took place (Table 11). However, where heavy inoculum was used, all treatments were nodulated, and an increase in the number of nodules per plant was the only calcium response. Little increase in percentage nodulation occurred above the minimum calcium level used, although nodule numbers per plant rose with further additions of calcium in some cases.

Nodulation of lucerne was poor where a normal level of inoculum was used, and there was no response to calcium, but with heavy inoculum the percentage nodulation

FIGURE 17.

Mt. Compass Field Trial, 1962 - Effect of soil pH on the percentage of nodulated lucerne, with heavy inoculum, at 53 and 102 days from sowing.

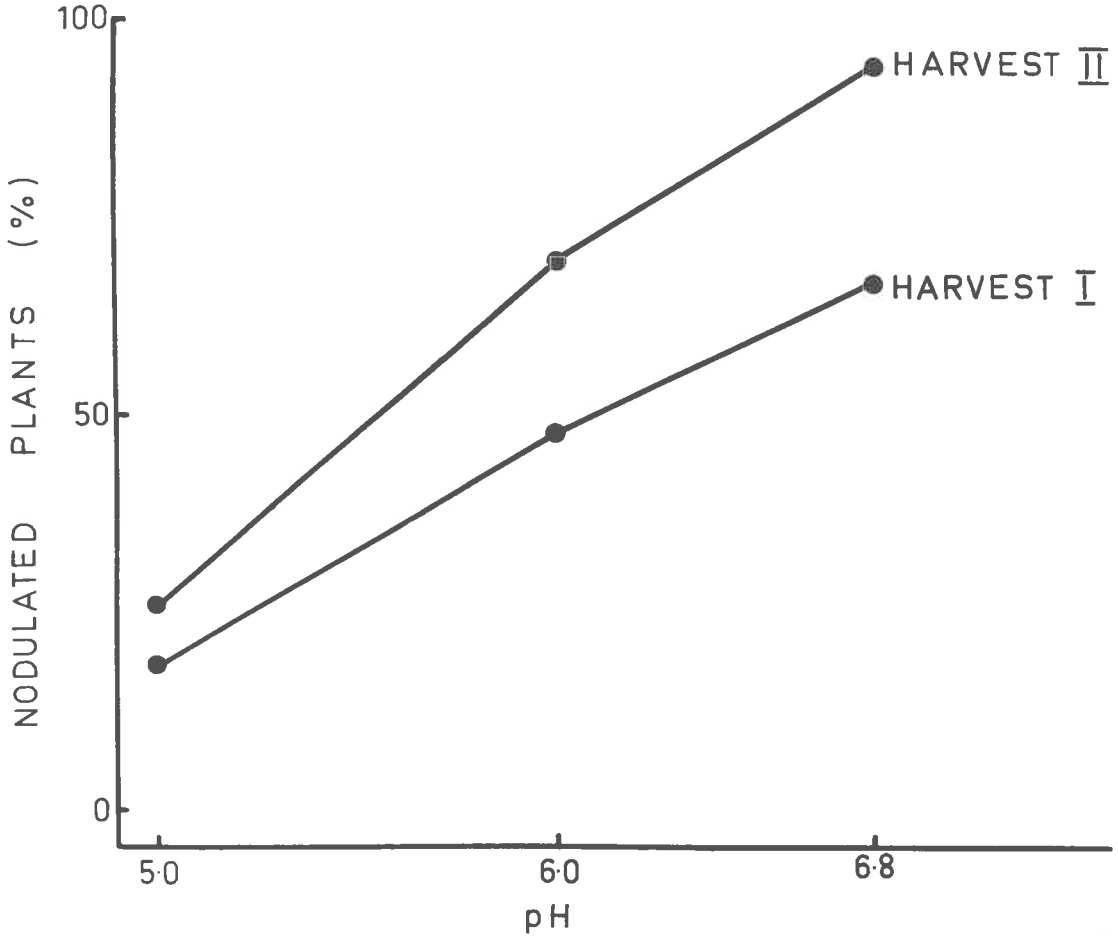

100
50
0
NODULATED PLANTS (%)
HARVEST II
HARVEST I
5·0
6·0
6·8
pH

TABLE 11.

Effect of Calcium on Nodulation - Harvest I.

Field Trial 1962.

Treatment	Subterranean Clover			Lucerne		
	% Nod. Plts.	Nods/Plt. Prim.	Sec.	% Nod. Plts.	Nods/Plt. Prim.	Sec.
pH 5.0.						
Normal inoculum						
Ca_0	54.7	0.9	1.5	2.1	1.0	0
Ca_2	64.6	0.4	2.7	0	-	-
Ca_4	67.5	0.9	3.0	0	-	-
Heavy inoculum						
Ca_0	100.0	3.1	3.5	4.2	1.0	0
Ca_2	100.0	3.5	4.9	10.4	0.9	0.4
Ca_4	100.0	4.0	7.1	18.3	1.1	0.4
pH 6.0.						
Normal inoculum						
Ca_0	89.2	1.1	2.1	8.3	0.5	0.7
Ca_2	75.8	2.2	3.4	6.1	0.8	0.5
Ca_4	83.7	1.3	4.0	6.8	1.0	0.3
Heavy inoculum						
Ca_0	100.0	4.3	5.3	33.4	0.8	0.6
Ca_2	97.9	5.8	3.5	20.8	0.9	0.7
Ca_4	97.7	4.5	6.4	47.9	0.7	1.0

increased with addition of calcium, at both pH levels.

Addition of calcium to the sand increased the level of calcium in the herbage of both species (Table 12).

TABLE 12.

Effect of calcium addition on percentage of calcium in herbage - Harvest II*.

Treatment	Lucerne	Subterranean Clover
Ca_0	1.18	0.97
Ca_2	1.70	1.30
Ca_4	2.22	1.66

* Mean of two pH treatments.

v. Phosphorus. In subterranean clover, plant phosphorus levels ranged from 0.18-0.23%, and were not affected by either calcium or pH treatment. In lucerne, however, the levels were affected by pH, and were 0.21-0.27% at pH 5.0 and 0.36-0.46% at pH 6.0

3. Pot Trials - Mt. Compass Sand.

(a) Pot Trial I.

In order to examine the effects of levels of inoculum, pH and calcium on nodulation of subterranean clover and lucerne in Mt. Compass sand under more controlled conditions, a pot trial was set up in an open-sided glasshouse at the Waite Institute. The treatments used were similar to those of the main field trial at Mt. Compass, and were as follows:-

1. Species: subterranean clover and lucerne.
2. pH levels: 5.0 and 6.0.
3. Calcium: control Ca_0
   equivalent of 8 cwt. $CaCO_3$ Ca_1
   " " 24 " " Ca_2
4. Inoculum: normal x1 In_1
   heavy x1000 In_2

The trial was of factorial design, with 6 replicates, and a total of 144 pots.

i. Method. Sand to a depth of 6" was obtained from an area under virgin scrub, adjacent to the field trial site at Mt. Compass. After air drying, it was sieved through a ¼" sieve. A basal fertilizer dressing, similar to that used in the field trial, and calculated on a soil weight basis, was added to the sand, macronutrients being thoroughly mixed before potting and trace elements added as a solution to each pot. The pH was raised by addition of $MgCO_3$ at the rate of 1.28 g/1000 g sand. Calcium was added as $CaSO_4.\frac{1}{2}H_2O$ (0.93 g and 2.79 g/1000 g sand).

Five inch diameter plastic pots, each holding 1400 g of air-dry sand, were used in the experiment. Three drainage holes at the bottom of each pot were covered with nylon cloth to prevent the sand from escaping. After potting, 170 ml of distilled water were added to each pot. Seed was inoculated as previously, and 13 seeds were sown in each pot at ½" depth, two hours

after inoculation, on August 13th, 1962. Pots were watered regularly with distilled water to constant weight, and the plants thinned to 12 per pot after a fortnight.

The whole trial was harvested on September 25th, 43 days from sowing. Plants were divided into three categories; primary nodulated, secondary nodulated and non-nodulated as defined previously. The three categories are illustrated in Plate 17.

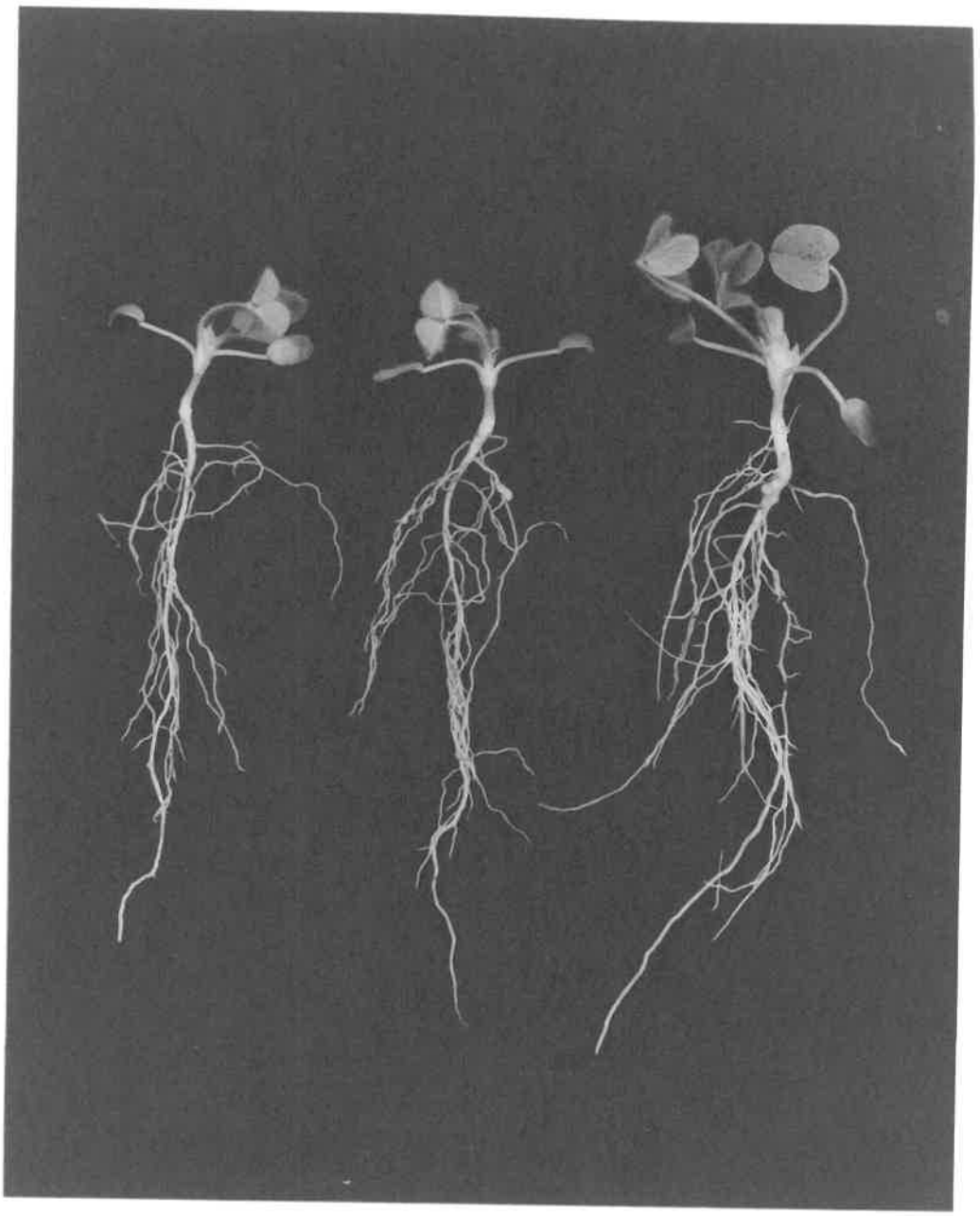
Nodule counts were made, and the tops of each category retained for dry matter, nitrogen, calcium and phosphorus determinations.

ii. Results. Differences in top growth due to level of inoculum and pH treatment were apparent at the time of harvesting (Plates 18 and 19) and were verified by later measurements. No response to calcium was visible at any stage, nor was one found at harvest. Complete results of the harvest are presented in Appendix 8, but the effects of level of inoculum and pH on nodulation are presented in Table 13.

In clover, a highly significant response to heavy inoculum was obtained, both in percentage nodulation and in nodule number per plant. Clover plants from seed receiving heavy inoculum were large, dark-green and vigorous, all treatments having large, pink primary nodules present. Many of the normally inoculated plants were small, yellow and nitrogen deficient with nodules completely absent or present as small, white secondary

PLATE 17.

Nodulation in subterranean clover. From left, non-nodulated secondary nodulated, and primary nodulated plants.



Pot Trial I, Mt. Compass sand.

Effect of pH and level of inoculum on nodulation and growth at harvest (43 days from sowing).

PLATE 18. Subterranean Clover.

Upper - normal inoculum.

Lower - heavy inoculum.

Left - pH 5.0.

Right - pH 6.0.

PLATE 19. Lucerne.

Upper - normal inoculum.

Lower - heavy inoculum.

Left - pH 5.0.

Right - pH 6.0.

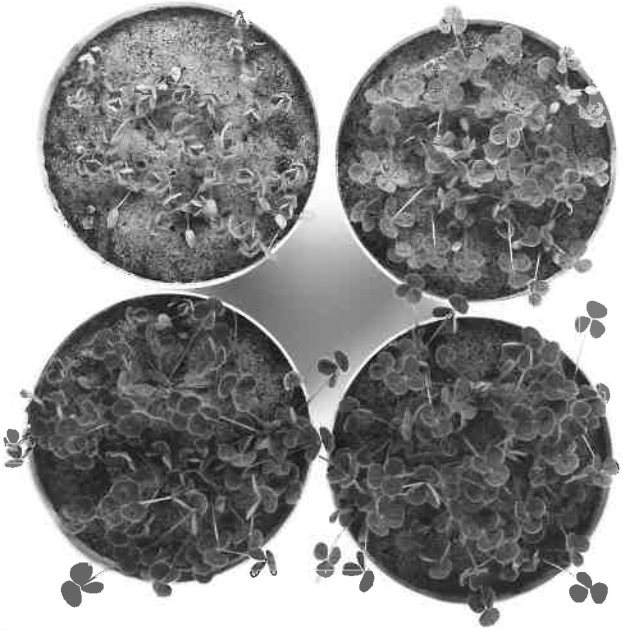


TABLE 13.

Effect of Level of Inoculum and pH on Nodulation.*

Pot Trial I.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
			Subterranean Clover.			
pH 5.0 In_1	23.2	36.1	40.7	5.2	0.7	5.3
In_2	95.2	3.3	1.5	7.6	1.3	-
pH 6.0 In_1	32.3	67.6	0	6.3	13.2	23.9
In_2	99.5	0.5	0	8.6	9.2	-
Significance of difference.						
Inoculum	1%	1%	-	1%	1%	1%
pH	n.s.	5%	-	5%	1%	1%
			Lucerne.			
pH 5.0 In_1	4.0	0	96.0	-	-	-
In_2	28.7	5.6	65.7	4.2	0.1	-
pH 6.0 In_1	30.8	34.6	34.6	5.5	1.5	4.6
In_2	67.2	27.3	5.5	5.5	4.3	8.0
Significance of difference.						
Inoculum	1%	n.s.	-	n.s.	1%	1%
pH	1%	1%	-	1%	1%	1%

* Mean of 3 Ca treatments, 6 replicates, 12 plants/replicate.

nodules, not yet functional or ineffective. The few green plants present were primary nodulated, but nodules were less numerous than those on heavily-inoculated plants. At pH 6.0, in normally inoculated clover, an increase in percentage nodulation and nodule number took place, as compared with pH 5.0. No increase in percentage nodulation occurred where heavy inoculum was used, however, and nearly 100% nodulation was obtained at both pH treatments.

In lucerne, significant improvement in nodulation occurred where inoculum level or pH was increased. At pH 5.0, percentage nodulation of lucerne was extremely poor, unless a high level of inoculum was used on the seed. At pH 6.0 a highly significant improvement occurred, especially where heavy inoculum was used, and a large increase in the percentage of nodulated plants and nodules per plant was recorded. In both species where primary nodules were present a considerable reduction in the number of secondary nodules occurred, as compared with secondary nodulated plants. Secondary nodulated plants sometimes had more than twice as many secondary nodules as primary nodulated plants although their root systems appeared smaller. At pH 6.0, the number of secondary nodules on heavily inoculated clover plants was less than on normally inoculated plants, although the number of primary nodules was greater.

Plant yield and percentage nitrogen in both

species were lower in secondary than in primary nodulated plants, and decreased further in non-nodulated plants (Table 14). The primary nodules, formed at an early stage of growth, were able to commence nitrogen fixation much sooner than the secondary nodules and greater dry matter production resulted. In subterranean clover, the yield of tops was closely correlated with the number of primary nodules present (Fig. 18). In both species, dry matter production was greater at the higher pH level, due to the presence of higher numbers of functional primary nodules.

TABLE 14.

Yield of Dry Matter and Percentage Nitrogen in Tops*.

Pot Trial I.

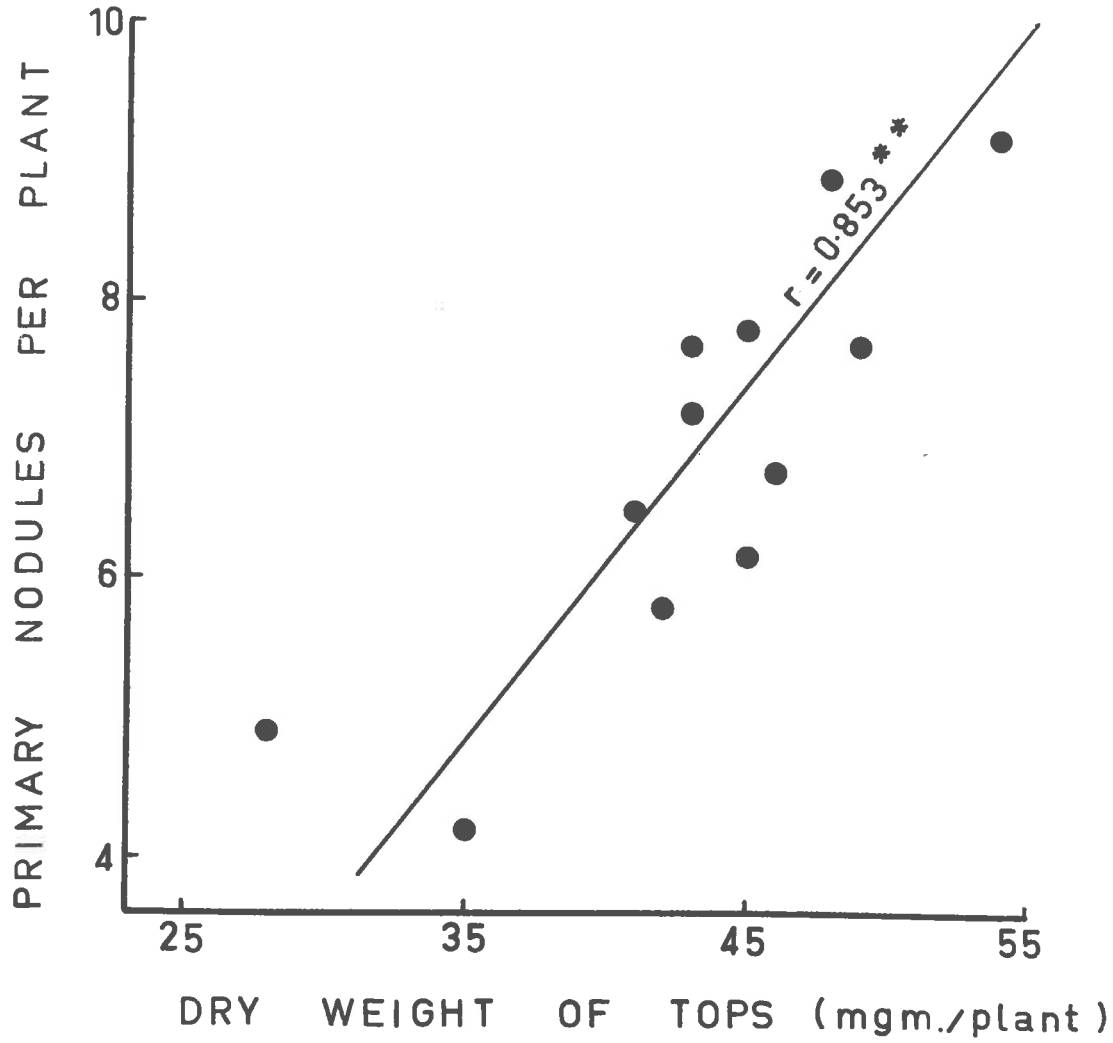
	Subterranean Clover			Lucerne		
	Primary Nod.Plts.	Secondary Nod.Plts.	Non-Nod. Plts.	Primary Nod.Plts.	Secondary Nod.Plts.	Non-Nod. Plts.
D.M.(mg/plant)						
pH 5.0	35	26	22	18	-	11
pH 6.0	44	27	-	21	14	12
% N.						
pH 5.0	5.07	3.01	2.73	4.28X	-	2.73X
pH 6.0	4.55	3.55	-	5.17	3.10	2.57

* Mean of 3 Ca treatments, 6 replicates, 12 plants/replicate.
All normally inoculated, except where marked X.

No response to addition of calcium occurred in either species, except a slight increase in number of

FIGURE 18.

Pot Trial I, Mt. Compass sand - The relation between dry weight of tops and primary nodule number in subterranean clover.


PRIMARY NODULES PER PLANT
10
8
6
4
25
35
45
55
DRY WEIGHT OF TOPS (mgm./plant)
r = 0·853 **

nodules on secondary nodulated lucerne at pH 6.0. In contrast, calcium addition caused a significant depression in primary nodule number (clover $P < 1\%$ lucerne $P < 5\%$), greater at the higher calcium level, in both species at both pH levels (Appendix 8). After harvesting, pH determinations were carried out on sand from all treatments, and the results provided a possible explanation for the depression (Table 15).

TABLE 15.

pH Measurements - Pot Trial I.

Treatment	Actual pH
pH 5 Ca_0	5.05
" " Ca_1	4.62
" " Ca_2	4.44
pH 6 Ca_0	6.35
" " Ca_1	5.75
" " Ca_2	5.54

The pH of the sand-water mixture, as measured with the glass electrode, dropped with increasing additions of $CaSO_4 . \frac{1}{2}H_2O$. The significance and explanation of these unforeseen effects is discussed later.

Calcium levels in the tops of both species were increased by addition of calcium to the sand (Table 16).

Slight phosphorus toxicity symptoms were evident on the unifoliate leaves of some subterranean clover plants at harvesting. Phosphorus levels over 1% were measured in

the tops of some treatments, but the level was reduced where calcium level was raised (Table 17).

TABLE 16.

Percentage Calcium in Tops*.

Pot Trial I.

Treatment	Sub. Clover	Lucerne
Ca_0	0.39	0.38
Ca_1	0.76	0.71
Ca_2	0.89	0.85

* Mean of two pH levels. All primary nodulated plants.

TABLE 17.

Percentage Phosphorus in Tops.
(primary nodulated plants)

Pot Trial I.

Treatment		Sub. Clover	Lucerne
pH 5	Ca_0	1.13	1.30
	Ca_1	1.14	0.88
	Ca_2	0.90	0.82
pH 6	Ca_0	1.20	0.94
	Ca_1	0.90	0.88
	Ca_2	0.76	0.67

An increase in the dry weight of non-nodulated clover and lucerne occurred as calcium level was increased. It would be surprising if this was an actual calcium response, in view of the lack of response to

calcium in nodulation. It may have been due to a reduction in excessive levels of plant phosphorus which occurred as calcium level was increased (Table 18). Phosphorus levels were much higher in non-nodulated than in nodulated plants.

TABLE 18.

Phosphorus Level and Dry Matter Production in Non-nodulated Plants at pH 5.

Pot Trial I.

Treatment	Subterranean Clover		Lucerne	
	D.M. Yield (mg/plant)	% P	D.M. Yield (mg/plant)	% P
Ca_0	21	1.70	9.6	1.72
Ca_1	22	1.53	10.8	1.24
Ca_2	23	1.26	13.1	1.00

(b) Pot Trial II.

It seemed possible that in the previous pot trial any nodulation responses following calcium addition to Mt. Compass sand had been masked by a pH depression caused by the $CaSO_4.\frac{1}{2}H_2O$ application. In order to examine this possibility a second pot trial was designed to include two calcium treatments, with pH adjusted so that no depression occurred when $CaSO_4.2H_2O$ was added. This trial was incorporated with a lime pelleting trial, the results of which are discussed in Section C.

i. Method. Calcium was added as a $CaSO_4.2H_2O$: $CaCO_3$ mixture. Preliminary tests showed that 0.90 g. $CaSO_4.2H_2O$ and 0.12 g. $CaCO_3$ per 1000 g. of sand did not alter pH and supplied calcium equivalent to 8 cwt. $CaCO_3$/acre. The basal fertilizer dressing was revised and the phosphorus level considerably reduced in order to avoid the excessively high levels of phosphorus in the plant which occurred in the previous experiment.

The following basal dressing was used:

Fertilizer	Grams/100 kg.sand	Approx.equivalent per acre to 4".
$Na_2HPO_4.2H_2O$	9.29	2 cwt. (as super).
$MgSO_4.7H_2O$	16.00	2 cwt.
K_2SO_4	16.00	2 cwt.
$Na_2B_4O_7.10H_2O$	0.50	7 lb.
$MnSO_4.4H_2O$	0.50	7 lb.
$ZnSO_4.7H_2O$	0.50	7 lb.
$CuSO_4.5H_2O$	0.50	7 lb.
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.016	3.6 oz.
$CoSO_4.7H_2O$	0.016	3.6 oz.

The trial was of factorial design, the treatments used being as follows:-

Species: Mt. Barker subterranean clover
Hunter River lucerne

Calcium: Control - Ca_0
Equivalent of 8 cwt.$CaCo_3$/acre - Ca_1

pH levels: 5.0

6.5

At each calcium level, three seed treatments (normally inoculated; inoculated and coated with gum arabic; and inoculated, gum arabic coated and lime pelleted) were used, each with two replicates, giving a total of six comparisons of calcium treatment.

The pH of the sand was raised to 6.5 by the addition of $MgCO_3$, while all seed received the normal level of inoculum. Pot preparation, seed inoculation, sowing, and treatment during the trial was similar to that used previously. Initially 220 ml. of distilled water was added to each pot of air-dried sand before sowing. Watering to constant weight was carried out regularly.

Plants were harvested 24 days from sowing. At this stage the third trifoliate leaf was present on subterranean clover plants. Similar measurements to those in the first trial were made.

ii. Results. Responses to addition of calcium appeared in both lucerne and subterranean clover as an increase in percentage of nodulated plants and in the number of secondary nodules per plant. The complete results, including statistical data, are presented in Appendix 9, but effects of calcium and pH treatments are presented in Table 19.

In clover the response to calcium appeared at low pH only, but in lucerne the response occurred at both high

and low pH. The calcium level in the tops was significantly increased in both species by addition of calcium to the sand (Table 20).

TABLE 19.

Effect of Calcium and pH on Nodulation*.

Pot Trial II.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nodulated	Prim. Nodulated Plts. Prim.	Prim. Nodulated Plts. Sec.	Sec. Nodulated Plts.
	Subterranean Clover.					
pH 5.0						
Ca_0	45.8	33.3	20.9	3.5	0.6	2.0
Ca_1	54.2	43.0	2.8	3.5	4.3	7.2
pH 6.5						
Ca_0	76.7	23.3	0	4.4	5.9	13.4
Ca_1	82.0	18.0	0	4.0	7.6	12.0
	Lucerne.					
pH 5.0						
Ca_0	26.4	0	73.6	3.4	0.4	-
Ca_1	33.4	4.2	62.4	3.0	0.1	-
pH 6.5						
Ca_0	53.1	26.6	20.3	3.0	2.1	4.5
Ca_1	65.1	25.2	9.7	2.8	2.3	5.8

* Mean of three pelleting treatments.
Prim. - primary. Sec. - secondary.

It can be concluded from this trial that lack of any response to calcium in Pot Trial I was probably due to pH depression by $CaSO_4 . \frac{1}{2}H_2O$ addition.

TABLE 20.

Percentage Calcium in Tops*.

Pot Trial II.

Treatment	Sub. Clover	Lucerne
pH 5.0 Ca_0	0.63	0.60
Ca_1	0.98	0.89
pH 6.5 Ca_0	0.52	0.54
Ca_1	0.88	0.76

* Mean of three pelleting treatments.

(c) Pot Trial III.

In the 1962 Field Trial at Mt. Compass, the addition of a $CaCO_3$: $CaSO_4.\frac{1}{2}H_2O$ mixture to certain plots, although designed to raise the pH of the sand to 6.2, actually raised it to 6.8. As compared with other plots raised to pH 6.0 with $MgCO_3$, the treatment producing this higher pH resulted in increased nodulation in lucerne, although no effect was apparent on subterranean clover. However, the possibility that this response was not due to pH, but to an effect of the $CaCO_3$: $CaSO_4.\frac{1}{2}H_2O$ mixture in the absence of $MgCO_3$ could not be overlooked. A pot trial was therefore designed to find out whether a similar response in lucerne occurred where the pH was raised to near 6.8 by addition of $MgCO_3$.

i. Method. A factorial trial was designed with the following treatments:

Species: Subterranean clover and lucerne.

pH levels: 4.90, 6.15, 6.60.

Level of inoculum on seed: Normal (x1) and Heavy (x1000).

Each treatment was replicated three times. Pot preparation, basal fertilizers, seed inoculation, sowing and watering was similar to that used in Pot Trial II and all pots received a similar basal $CaCO_3$: $CaSO_4.2H_2O$ dressing designed to maintain pH and add the equivalent of 8 cwt. $CaCO_3$/acre. $MgCO_3$ was used to obtain the higher pH levels in the sand.

For convenience, the trial was conducted in a growth room in conjunction with experiments designed to study the effect of temperature on nodulation. The temperature prevailing during the period of growth was 45°F at night and 60°F during the day, with a 12 hour day-length. A light intensity of 1600-1800 foot-candles was measured at plant level.

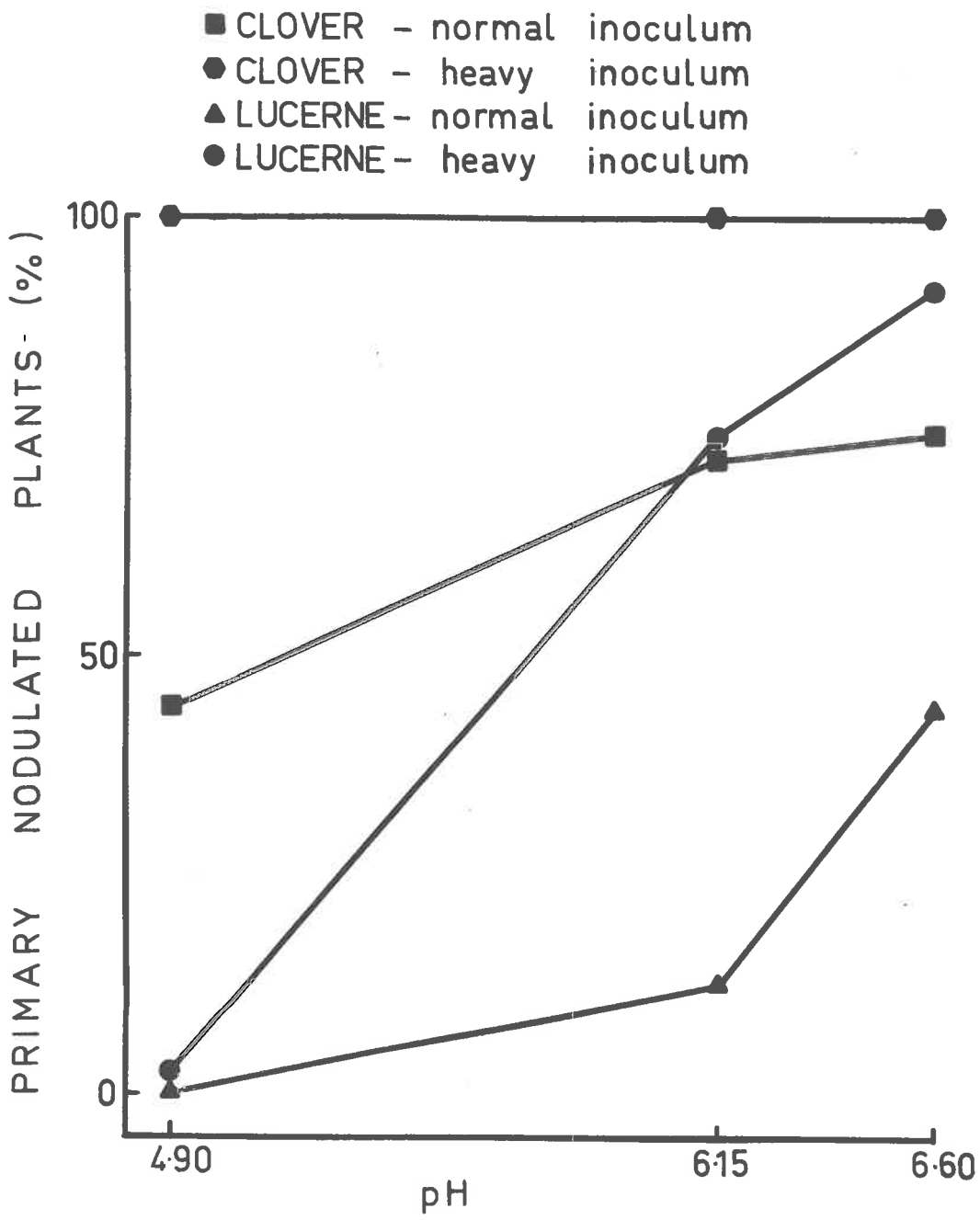
After 35 days, when the clover had developed three trifoliate leaves, all plants were harvested and the number of primary, secondary and non-nodulated plants recorded in each treatment.

ii. Results. The response to pH was similar to that obtained in the field trial. Results are presented in Fig. 19 and in Appendix 10.

While subterranean clover did not give any response above pH 6.15, the percentage of primary nodulated lucerne plants at pH 6.6 was significantly greater than at

FIGURE 19.

Pot Trial III, Mt. Compass sand - Effect of pH on the percentage of primary nodulated plants, at normal and heavy levels of inoculum.


CLOVER - normal inoculum
CLOVER - heavy inoculum
LUCERNE - normal inoculum
LUCERNE - heavy inoculum
PRIMARY NODULATED PLANTS - (%)
100
50
0
4·90
6·15
6·60
pH

6.15. With subterranean clover, no response to pH was obtained where heavy inoculum was used on the seed.

(d) Pot Trial IV.

The standard method used for supplying calcium and raising pH in Mt. Compass sand in previous trials has been by addition of $CaSO_4$ and $MgCO_3$, respectively. In a poorly-buffered soil such as this sand, there is always the possibility that fertilizers may have side effects on plant growth with consequent effects on nodulation. The effect of $CaSO_4$ on soil pH has already been resolved, but effects on uptake of other ions may occur. The aim of this pot trial was to determine the effects on nodulation of various sources of calcium and different methods of raising pH of the Mt. Compass sand.

i. Method. Pot trial preparation was similar to that used for Pot Trial II. The experiment was conducted in a growth room in conjunction with Pot Trial III and was replicated three times. Seed was inoculated at a normal level of inoculum. Treatments are listed in Table 21.

All calcium treatments were at the equivalent of 0.64 g. $CaCO_3$/1000 g. sand, or 8 cwt./acre. As $CaSO_4.2H_2O$ alone was known to depress pH a proportion of the calcium was added as $CaCO_3$, as in Trial II. $CaCl_2$ alone also depressed pH from 4.90 to 3.90, but a mixture of 0.20 g. $CaCO_3$ and 0.33 g. $CaCl_2$/1000 g. sand maintained pH at the required level. A further treatment included a

TABLE 21.

Methods of Adding Calcium and Raising pH in Mt. Compass sand.

Pot Trial IV.

Treatment	Ca addition	pH adjustment.
pH 5.0		
1	Nil	Nil
2	$CaCl_2$	Nil
3	$CaSO_4.2H_2O$	Nil
pH 6.0		
4	Nil	$MgCO_3$
5	$CaCl_2$	$MgCO_3$
6	$CaSO_4.2H_2O$	$MgCO_3$
7	$CaSO_4.2H_2O$:$CaCO_3$	$CaSO_4.2H_2O$:$CaCO_3$
8	Nil	NaOH
9	$CaSO_4.2H_2O$	NaOH

$CaSO_4.2H_2O$: $CaCO_3$ mixture in the proportions 0.33 g. $CaSO_4.2H_2O$: 0.45 g. $CaCO_3$/1000 g. sand. This supplied the required amount of calcium and raised the pH to 6.0.

$CaCl_2$ and NaOH (50 ml. 0.04N NaOH/1000g. sand) were added as solutions and thoroughly mixed with each pot of sand. All other treatments were added in a dry form and thoroughly mixed before potting.

Plants were harvested after 35 days and primary, secondary and non-nodulated plants were recorded and numbers of nodules counted on each plant.

ii. Results. The results are presented in Table 22.

The main point which emerged from the trial was that, apart from the NaOH treatment, little effect on

TABLE 22.

Effect of Forms of Calcium and Various Methods of Raising pH on Nodulation.

Pot Trial IV.

Treatment	pH	Subterranean Clover					Lucerne				
		% Nodulation			Nodules/Plant		% Nodulation			Nodules/Plant	
		Prim.	Sec.	Non-nod.	Prim. Nod. Plts. Prim.	Prim. Nod. Plts. Sec.	Prim.	Sec.	Non-nod.	Prim. Nod. Plts. Prim.	Sec. Nod. Plts.
pH 5.0											
No Ca	4.90	44.4	44.4	11.2	2.0	1.5 4.2	0	0	100.0	-	-
$CaCl_2$	5.09	52.8	44.4	2.8	2.1	4.8 7.0	0	0	100.0	-	-
$CaSO_4$	4.87	54.1	40.8	5.1	2.4	4.0 5.4	0	2.8	97.2	-	-
pH 6.0											
No Ca, $MgCO_3$	6.14	72.2	25.0	2.8	2.6	6.3 8.1	12.3	11.7	76.0	2.0	0.2 1.0
$CaCl_2$, $MgCO_3$	5.84	69.4	27.8	2.8	2.1	5.6 10.7	6.1	0	93.9	2.0	0 -
$CaSO_4$, $MgCO_3$	6.00	66.7	33.3	0	1.7	7.8 11.5	16.7	19.4	63.9	1.5	0.2 1.4
$CaSO_4$, $CaCO_3$	6.11	77.8	22.2	0	2.1	8.7 10.3	22.2	20.7	57.1	1.7	1.0 2.9
No Ca, NaOH	6.26	66.7	33.3	0	2.6	6.5 14.5	0	8.8	91.2	-	- 1.3
$CaSO_4$, NaOH	6.16	75.0	25.0	0	2.7	6.5 12.1	16.7	32.3	51.0	2.4	0.2 1.6
I.S.D. 5%		15.5	14.9	-	1.1	3.2 4.8	20.9	18.5	23.7	0.8	- 1.2
1%		21.3	20.6	-	1.5	4.4 6.6	28.2	22.8	32.6	1.1	- 1.6

nodulation occurred from the use of different forms of calcium or different methods of raising pH. A small calcium response occurred in subterranean clover at pH 5.0 and in lucerne at pH 6.0. The poorer nodulation of lucerne in the $CaCl_2$ treatment at pH 6.0 can be attributed to the lower pH (5.84) of this treatment compared with others.

Where pH was raised by the use of NaOH, nodulation in lucerne was poor and a highly significant calcium response occurred. In contrast, little effect occurred in subterranean clover. Where NaOH was added in the absence of calcium, fine organic matter appeared in a deflocculated state on the surface of the pots. This disappeared where calcium was added as well.

(e) Pot Trial V.

The depression in nodulation of lucerne by NaOH in Trial IV and the marked response following calcium addition suggested that in previous field and pot trials the Na^+ in the basal $Na_2HPO_4.2H_2O$ may have induced or enhanced the responses to calcium nodulation. A pot trial was therefore conducted in an open-sided glasshouse to investigate the possibility.

i. Method. The factorial trial included the following treatments:

Species: Subterranean clover and lucerne.

Sodium: Na_1 - no additional Na.

Na_2 - Na equivalent to 0.093 g. $Na_2HPO_4.2H_2O$/1000 g. sand.

Na_3 - Na equivalent to 0.186 g. $Na_2HPO_4.2H_2O$/1000 g. sand.

Calcium: Ca_0 - no calcium.

Ca_1 - $CaSO_4.2H_2O$:$CaCO_3$ mixture. Ca equivalent to 0.64 g. $CaCO_3$/1000 g. sand.

Each treatment was replicated four times. In two replicates of each sodium treatment the ion was supplied as Na_2SO_4 and in the other two replicates as NaCl. Both salts depressed pH and it was necessary to add a proportion of the sodium as Na_2CO_3 to keep pH constant. ($NaCl:Na_2CO_3$ - 2.56:1.00; $Na_2SO_4:Na_2CO_3$ - 5.34:1.00).

Pot preparation etc. was similar to that used in Pot Trial II. The pH of the sand was maintained at 6.5 by the addition of $MgCO_3$, while a normal level of inoculum was used on the seed.

The pots were sown on September 27th, 1963, and harvested 24 days later, when three trifoliate leaves were present on the clover. Numbers of primary, secondary and non-nodulated plants were recorded and numbers of primary and secondary nodules counted on each plant.

ii. Results. Results are summarised in Table 23. Neither the level nor the form of sodium applied had any effect on nodulation. No significant response to calcium occurred in clover, but addition of calcium reduced the percentage of non-nodulated lucerne and increased the

TABLE 23.

Effect of Sodium on Calcium Responses in Nodulation.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
	Subterranean Clover.					
- Ca Na_1	91.7 (1.31)*	8.3 (0.21)*	0	5.2	4.4	15.5
" Na_2	91.7 (1.31)	8.3 (0.21)	0	4.8	7.0	13.9
" Na_3	93.6 (1.31)	6.4 (0.22)	0	5.0	4.4	12.0
+ Ca Na_1	87.5 (1.20)	12.5 (0.35)	0	5.5	5.7	10.8
" Na_2	91.7 (1.28)	8.3 (0.25)	0	4.5	7.6	15.3
" Na_3	85.4 (1.25)	14.6 (0.27)	0	5.1	5.3	14.8
Na L.S.D. 5%	(0.25)	(0.30)		1.0	2.3	7.0
1%	(0.36)	(0.42)		1.4	3.2	10.2
Ca L.S.D. 5%	(0.21)	(0.24)		0.8	1.9	5.7
1%	(0.29)	(0.34)		1.1	2.7	8.4
Na x Ca L.S.D. 5%	(0.36)	(0.42)		1.4	3.3	9.9
1%	(0.50)	(0.59)		1.9	4.6	14.5

* Arcsin transformation.

TABLE 23 (cont.)

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
		Lucerne.				
- Ca Na_1	56.2 (0.84)*	25.0 (0.51)*	18.8	3.0	1.6	4.6
" Na_2	56.2 (0.84)	27.1 (0.54)	16.7	3.6	2.3	4.3
" Na_3	50.0 (0.78)	39.6 (0.67)	10.4	3.1	2.0	5.4
+ Ca Na_1	58.4 (0.87)	33.3 (0.60)	8.3	2.8	2.9	6.9
" Na_2	62.1 (0.91)	33.7 (0.61)	4.2	3.2	2.6	5.5
" Na_3	56.3 (0.85)	39.6 (0.67)	4.1	2.6	2.9	5.8
Na L.S.D. 5%	(0.17)	(0.16)		0.7	1.4	1.3
1%	(0.23)	(0.22)		1.0	2.0	1.8
Ca L.S.D. 5%	(0.14)	(0.13)		0.6	1.2	1.0
1%	(0.19)	(0.18)		0.8	1.6	1.4
Na x Ca L.S.D. 5%	(0.24)	(0.23)		1.0	2.0	1.8
1%	(0.33)	(0.31)		1.4	2.8	2.5

* Arcsin transformation.

number of secondary nodules on lucerne plants.

4. Mt. Compass Field Trial I, 1963.

Results from Pot Trial II showed that nodulation responses due to calcium addition in Pot Trial I could have been masked by depression of pH in the Mt. Compass sand due to application of $CaSO_4.\frac{1}{2}H_2O$. A temporary depression of pH by $CaSO_4.\frac{1}{2}H_2O$ addition may also have occurred in the 1962 Field Trial, and affected legume nodulation. A further field trial was therefore conducted in order to re-examine the Ca-pH- level of inoculum interaction on nodulation in Mt. Compass sand, taking care to eliminate effects of $CaSO_4.\frac{1}{2}H_2O$ on the pH of the sand.

(a) Site preparation.

An area adjacent to the 1962 Field Trial site was cleared of scrub, fenced and rotary hoed to a depth of 2". A basal fertilizer dressing identical with that used in 1962 was applied to the experimental area and harrowed in.

(b) Treatments.

The trial was of factorial design with four replicates of the following treatments (Fig.20).

Species: Mt. Barker subterranean clover.
Hunter River lucerne.

pH: 5.0
6.0)
7.0) Adjusted with $MgCO_3$.

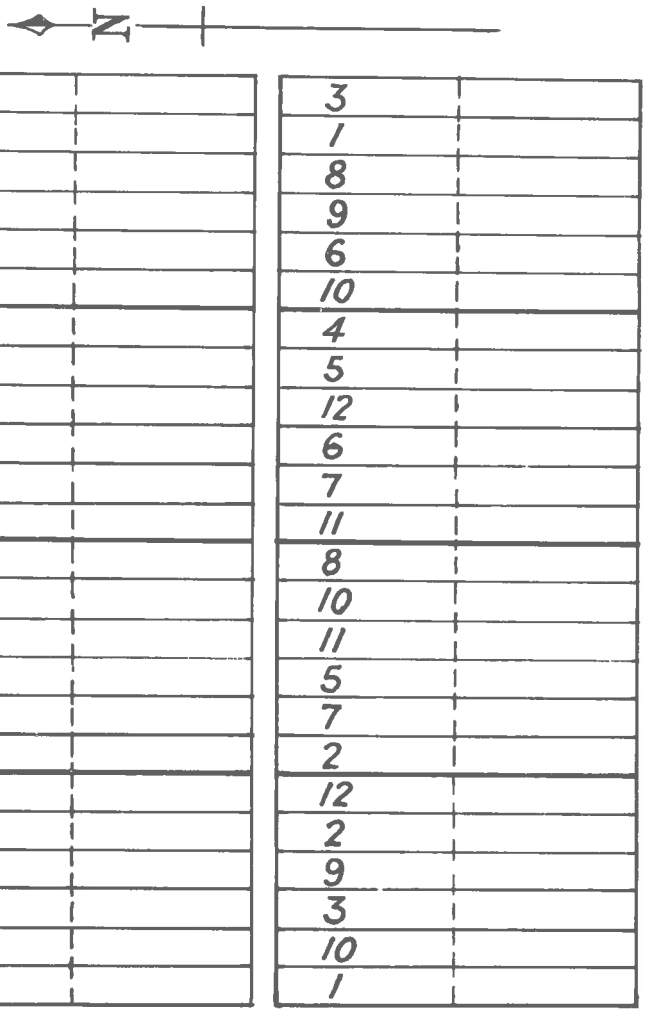
FIGURE 20.

Mt. Compass Field Trial I, 1963.

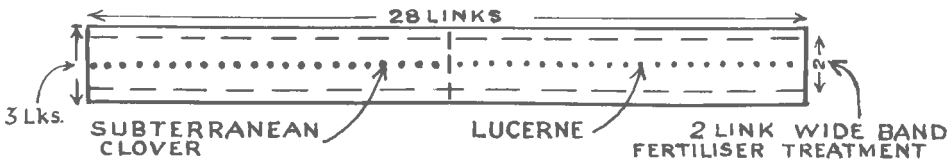
Treatments.

1.	pH 5.0	Ca_0	In_1	7.	pH 6.0	Ca_1	In_1
2.	"	"	In_2	8.	"	"	In_2
3.	"	Ca_1	In_1	9.	pH 7.0	Ca_0	In_1
4.	"	"	In_2	10.	"	"	In_2
5.	pH 6.0	Ca_0	In_1	11.	"	Ca_1	In_1
6.	"	"	In_2	12.	"	"	In_2

MtCOMPASS FIELD TRIAL I
1963



DETAILS OF A SINGLE PLOT



Calcium:	Control	Ca_0
	Equivalent of 8 cwt.CaCO3/acre	Ca_1
Inoculum:	Normal (x1)	In_1
	Heavy (x1000)	In_2

Calcium was added as a $CaSO_4.\frac{1}{2}H_2O$:$CaCO_3$ mixture (1365 lb. $CaSO_4.\frac{1}{2}H_2O$:100 lb. $CaCO_3$/acre). Preliminary tests showed that this mixture had no effect on the pH of the sand. The pH was raised to 6.0 and 7.0 by addition of 7 cwt. and 15 cwt. of $MgCO_3$ per acre respectively. Plots measured 28 x 3 links with fertilizer treatments applied in a band 2 links wide in a similar manner to the previous year's field trial.

(c) Inoculation and sowing of seed.

Seed was inoculated as previously on the afternoon of May 20th, and a single row of seed was sown down the centre of each plot on the morning of May 22nd. Each plot was split at random, and 75 seeds of subterranean clover were sown in one half of the row and 75 seeds of lucerne in the other and covered to the depth of $\frac{3}{4}$".

Most seedlings had emerged within two weeks. Little wind damage occurred to any plots during the period of the trial.

(d) Harvesting.

Twelve seedlings of each species were removed at random from each plot on July 15th, 54 days from sowing. In each treatment, primary, secondary and non-nodulated plants were separated and primary and secondary

nodules counted. Harvesting and nodule counting techniques were similar to those used in 1962.

A further random sample of 12 plants of each species was harvested from each treatment on August 26th, 96 days from sowing and 42 days after the previous harvest, and the same nodulation measurements made. After being rinsed with distilled water, the plant tops were retained for dry weight determination and calcium analysis.

(e) Soil pH measurements.

Soil samples (0-2") were taken from all plots on July 22nd for pH determinations. The following readings were obtained from bulked samples from each treatment.

Treatment.	Actual pH
pH 5.0 Ca_0	5.35
" Ca_1	5.40
pH 6.0 Ca_0	6.30
" Ca_1	6.25
pH 7.0 Ca_0	7.00
" Ca_1	6.92

Addition of calcium as a $CaSO_4$:$CaCO_3$ mixture had little effect on pH in the sand. The pH figure for the control plots was somewhat higher than previous measurements. A similar figure was obtained on resampling. It is possible that local variation in pH may occur at the trial site, or the pH level may vary with season. Over 20" of rain had fallen at Mt. Compass in the three months

previous to sampling and a soluble organic fraction contributing to the low pH in the sand could have been leached out.

(f) Results.

Marked responses in nodulation were obtained to calcium application, and to the raising of soil pH and numbers of rhizobia to the seed. In particular, a much greater calcium response occurred as compared with the 1962 Field Trial, strongly suggesting that some temporary pH depression by $CaSO_4.\frac{1}{2}H_2O$ had occurred previously and had partially masked the calcium response. The complete results of the two harvests, including statistical data, are presented in Appendices 11 and 12.

i. Level of inoculum. As in previous trials, a very large response was obtained to the addition of high numbers of rhizobia to the seed (Table 24).

With clover, a heavy rate of inoculum resulted in nearly 100% primary nodulation regardless of the calcium status or pH of the sand. The number of primary and secondary nodules on each plant was also greatly increased where heavy inoculum was used. At the normal level of inoculum a number of clover plants were unnodulated at first harvest and many more had only developed a few, small, white secondary nodules, not yet functional or possibly ineffective. The importance of primary nodulation on early plant growth was illustrated by the higher mean yield (24 mg/plant) of primary nodulated clover plants than

of secondary nodulated plants (20 mg/plant) at Harvest II. Both had received normal inoculum.

Very few nodules formed on lucerne where a normal level of inoculum was applied to the seed, whatever the soil treatment. The use of heavy inoculum resulted in a high percentage of nodulated plants, especially where the calcium and pH levels in the sand had been raised.

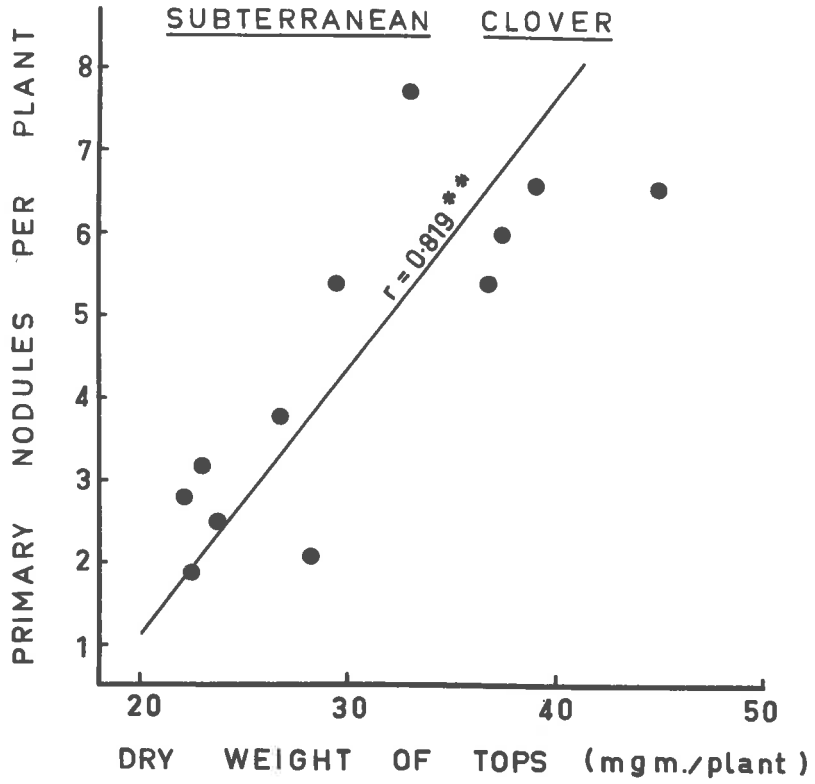
As in Pot Trial I, the number of primary nodules per plant had a marked effect on yield. At the second harvest, the herbage yield from heavily inoculated, primary nodulated clover was 50% higher than from normally inoculated, primary nodulated plants. It would seem highly probably that this was due to the greater number of nodules (and nodular tissue) on the roots of the heavily inoculated plant. Number of primary nodules and herbage yield were closely correlated in both species (Fig. 21).

The number of secondary nodules present on plants of subterranean clover was affected by the presence of primary nodules (Tables 24 and 25). Where primary nodules were present, the number of secondary nodules was much lower than where they were absent. A similar result was obtained in Pot Trial I. This feature was unrelated to size of root system. Generally, the primary nodulated plants were observed to have developed larger roots than secondary nodulated plants.

ii. Time. As in the 1962 Field Trial a gradual improvement in nodulation occurred between the first and

FIGURE 21.

Mt. Compass Field Trial I, 1963 - The relation between dry weight of tops and primary nodule number per plant (Harvest II).


SUBTERRANEAN CLOVER
PRIMARY NODULES PER PLANT
r = 0·819 **
1
2
3
4
5
6
7
8
20
30
40
50
DRY WEIGHT OF TOPS (mg m./plant)

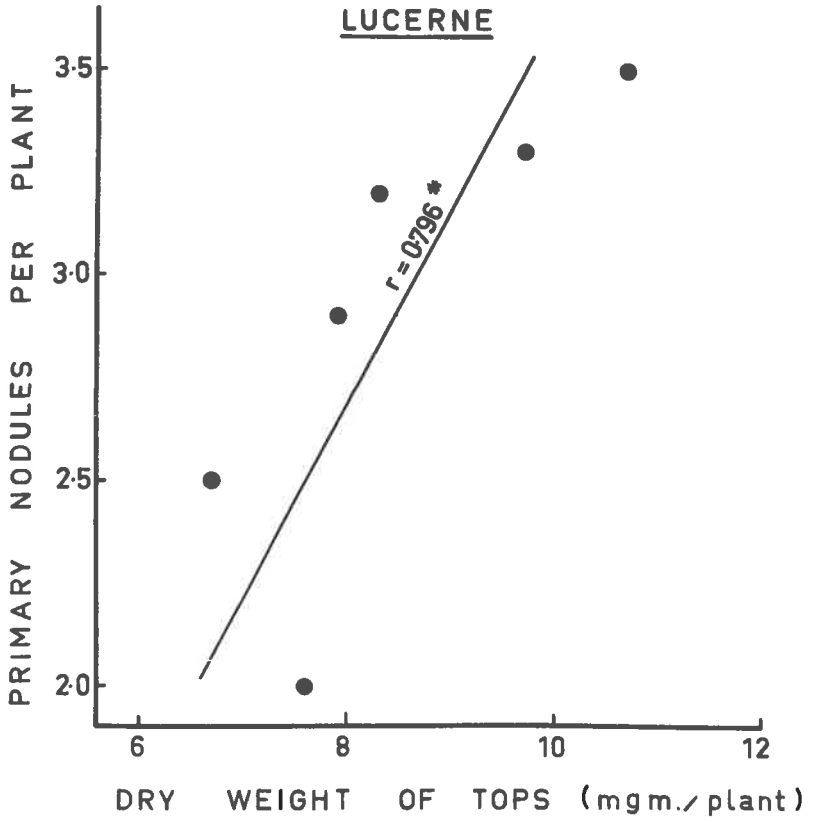

LUCERNE
PRIMARY NODULES PER PLANT
r = 0796 *
2·0
2·5
3·0
3·5
6
8
10
12
DRY WEIGHT OF TOPS (mg m./plant)

TABLE 24.

Effect of Level of Inoculum on Nodulation. *

Field Trial I, 1963.

Inoculum Treatment	% Nodulation			Nodules/Plant			Dry Wgt. Prim.Nod. Plts. (mg/plant)
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	
	Subterranean Clover - Harvest I.						
Normal (x1)	49.7	35.3	15.0	2.1	2.5	3.5	8.8
Heavy (x1000)	97.4	2.6	0	4.9	6.0	-	10.5
	Subterranean Clover - Harvest II.						
Normal (x1)	62.3	36.2	1.5	2.7	3.8	5.7	24.2
Heavy (x1000)	100.0	0	0	6.3	9.9	-	36.7
	Lucerne - Harvest I.						
Normal (x1)	2.4	0.4	97.2	-	-	-	-
Heavy (x1000)	47.2	3.2	49.6	2.3	0.4	-	2.7
	Lucerne - Harvest II.						
Normal (x1)	4.1	5.1	90.8	-	-	-	-
Heavy (x1000)	69.0	8.2	22.8	2.9	0.7	-	8.5

* Mean of Ca and pH treatments, 4 replicates, 12 plants/ replicate.

TABLE 25.

Effect of pH and Calcium on Nodulation of Subterranean Clover (normal inoculum)

Field Trial I, 1963.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
			Harvest I.			
pH 5.35 Ca_0	35.6	20.2	44.2	1.5	0.8	2.3
Ca_1	48.4	39.1	12.5	2.4	1.7	3.0
pH 6.30 Ca_0	44.1	37.4	18.5	1.7	1.4	2.4
Ca_1	53.7	40.5	5.8	2.4	4.2	3.9
pH 7.00 Ca_0	52.6	38.2	9.2	1.6	2.2	3.8
Ca_1	63.5	36.5	0	2.9	4.7	5.8
			Harvest II.			
pH 5.35 Ca_0	52.6	40.8	6.6	2.5	1.0	3.0
Ca_1	61.9	38.1	0	2.8	4.5	5.7
pH 6.30 Ca_0	60.6	37.1	2.3	2.1	4.0	4.5
Ca_1	72.1	27.9	0	3.2	4.8	7.5
pH 7.00 Ca_0	57.0	43.0	0	1.9	3.6	5.2
Ca_1	69.6	30.4	0	3.9	4.8	8.0

second harvests (Tables 24 and 25). In clover, nearly all normally inoculated plants were nodulated at second harvest, although little change occurred in normally inoculated lucerne, which was poorly nodulated at both harvests. It is probable that Rhizobium meliloti added in the normal level of inoculum had failed to survive and multiply. This was supported by R. meliloti survival trials carried out in a growth room and reported later.

The number of nodules on nodulated plants of both species increased considerably between Harvest I and Harvest II, especially secondary nodules formed on lateral roots.

iii. pH. The main pH response in subterranean clover was obtained when the level was raised to 6.30, and only a small further response occurred at pH 7.00 (Table 25). The percentage of nodulated plants was increased, and numbers of primary and secondary nodules rose slightly but produced no consistent increase in plant yield. The use of heavy inoculum resulted in 100% primary nodulation whatever the pH.

The pH response in lucerne was much greater than in clover and the proportion of primary nodulated plants increased sharply from pH 5.35 to 6.30, with a further increase at pH 7.00 (Table 26). The number of primary nodules on each plant was also increased when pH was raised.

TABLE 26.

Effect of pH and Calcium on Nodulation of Lucerne

(heavy inoculum)

Field Trial I, 1963.

Treatment	% Nodulation			Nodules/Plant		Dry Wgt. Tops (mg/plt.)
	Prim.	Sec.	Non-nod.	Prim. Nod. Plts. Prim.	Sec.	Prim. Nod. Plts.
			Harvest I.			
pH 5.35 Ca_0	6.1	0	93.9	-	-	-
Ca_1	56.5	2.1	41.4	2.1	0.1	2.5
pH 6.30 Ca_0	46.2	0	53.8	1.6	0.9	2.5
Ca_1	62.2	7.7	30.1	2.7	0.2	2.7
pH 7.00 Ca_0	44.1	7.5	48.4	2.2	0.3	3.1
Ca_1	67.8	2.1	30.1	2.9	0.4	2.8
			Harvest II.			
pH 5.35 Ca_0	32.1	0	67.9	2.0	0.1	7.6
Ca_1	76.4	10.3	13.3	3.3	0.7	9.7
pH 6.30 Ca_0	63.2	8.9	27.9	2.5	0.7	6.7
Ca_1	79.4	10.5	10.1	2.9	0.7	7.9
pH 7.00 Ca_0	71.8	16.6	11.6	3.2	0.9	8.3
Ca_1	91.0	3.1	5.9	3.5	1.3	10.6

iv. Calcium. In both subterranean clover and lucerne the most striking responses to calcium occurred at pH 5.35 although significant responses also occurred at pH 6.30 and 7.00. In clover, where the response was greater at the lower level of inoculum, the percentage of primary and secondary nodulated plants and numbers of primary and secondary nodules per plant were all increased by addition of calcium (Table 25). At the higher level of inoculum, 100% nodulation occurred whatever the calcium treatment, but calcium application increased the numbers of primary and secondary nodules per plant. The increased numbers of nodules where calcium was applied did not result in a consistent increase in dry matter yield of subterranean clover at either harvest.

In lucerne, the response to calcium occurred at the higher level of inoculum only, as nodulation was uniformly poor with normal inoculum (Table 26). The effect occurred mainly as an increase in the percentage of primary nodulated plants and in the number of primary nodules per plant. At the second harvest a 25% increase in yield of primary nodulated lucerne was measured where calcium had been added. This increase in yield was probably associated with the increase in primary nodule number. Nodulation was poor at pH 5.35 except where calcium had been applied.

A marked pH x Ca interaction occurred in both species. Almost the same percentage of primary nodulated

plants could be obtained by either adding calcium to the sand or raising the pH to 6.30. Where both the pH and calcium levels in the sand were raised percentage primary nodulation rose still further.

The level of calcium in the tops of the two species was more than doubled on addition of calcium to the sand (Table 27). Where no calcium was applied, the level of plant calcium was low, probably bordering on deficiency levels, especially in lucerne, although it tended to increase with increasing pH. The likelihood of calcium being near deficiency levels for plant growth in the virgin sand was further supported by a 33% yield response in non-nodulated lucerne to addition of calcium at pH 5.35 (Table 28). No corresponding comparison for subterranean clover was obtained due to absence of non-nodulated plants. The yield of non-nodulated lucerne was also higher at pH 6.30 than at pH 5.35. This may have been related to the increase in calcium level of the herbage which occurred at pH 6.30.

TABLE 27.

Percentage Calcium in Tops* - (Harvest II)

Field Trial I, 1963.

Treatment		Sub. Clover	Lucerne
pH 5.35	Ca_0	0.78	0.46
	Ca_1	1.72	1.52
pH 6.30	Ca_0	0.88	0.63
	Ca_1	1.66	1.70
pH 7.00	Ca_0	0.96	0.75
	Ca_1	1.79	1.74

* Mean of two inoculum levels.

TABLE 28.

Effect of calcium on yield of non-nodulated lucerne - (Harvest II).

Field Trial I, 1963.

Treatment	% Ca in Tops	Yield (mg/plant)
pH 5.35 Ca_0	0.32	1.8
Ca_1	1.43	2.4
pH 6.30 Ca_0	0.65	2.4
Ca_1	1.57	2.7

5. Root hair - nodulation studies; Mt. Compass sand.

(a) Root hair measurements.

In the water culture trials calcium and pH had a pronounced effect on root hair development in subterranean clover and lucerne, and it was concluded that they may well affect nodulation by this means. In the following study the effect of calcium and pH on root hair development in Mt. Compass sand was examined to ascertain whether similar effects occurred there.

i. Method. Twelve pots of Mt. Compass sand were prepared, using the method described in Pot Trial II. Four soil treatments were used, each replicated three times.

Calcium: Ca_0 and Ca_1 - Ca added as a $CaSO_4.2H_2O$:$CaCO_3$ mixture as in Pot Trial II.

pH: 5.00 and 6.35 - pH raised by the addition of $MgCO_3$.

The trial was conducted in an open-sided glasshouse. Seed of both subterranean clover and lucerne was sown in every pot on October 9th 1963 and thinned soon

after emergence to 6 plants of each species per pot. After 8 days, when clover and lucerne taproots averaged 7 and 8 cm respectively in length, the sand was very carefully washed away from the roots with water. The taproots were examined for root hairs, which were scored for length and density using the method described previously for plants grown in water culture.

ii. Results. Root hairs on both species were more profuse than in water culture, and covered most of the taproot. The careful removal of seedlings from the sand resulted in little visible damage to the hairs. The results are presented in Table 29.

In subterranean clover, root hair length and density were unaffected by calcium treatment, but both were greater where pH had been raised. With lucerne, however, root hairs were generally shorter and less numerous at pH 5.0 and in the absence of calcium than in any other treatment. Addition of calcium or a rise in pH resulted in a marked improvement in both length and density of hairs, but no further response was visible where calcium and pH levels were both raised. The mean length of lucerne taproot with hairs of 300μ or more in length increased nearly 50%, from 2.6 cm to 4.8 cm. when calcium was applied. Although the length of root hairs was comparable on both species, root hair density was much greater on subterranean clover than on lucerne.

TABLE 29.

Effect of Calcium and pH Level on Root Hair Development in Mt. Compass Sand. *

Treatment		Subterranean Clover			Lucerne		
		Length of Taproot (cm)	Mean Scores Root hair length	Root hair density	Length of Taproot (cm)	Mean Scores Root hair length	Root hair density
pH 5.00							
	Ca_0	7.45	2.48	2.10	8.22	2.26	1.35
	Ca_1	6.16	2.49	2.08	7.87	2.55	1.62
pH 5.50							
	Ca_0	7.54	2.61	2.22	8.47	2.65	1.55
	Ca_1	6.67	2.54	2.27	8.12	2.57	1.67
L.S.D.	5%	0.73	0.08	0.28	0.72	0.20	0.48
	1%	1.09	0.12	0.43	1.09	0.31	0.72

* Mean of 6 plants.

(b) Nodulation studies.

The results from the studies of root hair development in Mt. Compass sand suggested that, in lucerne at least, nodulation responses to improvement in calcium and pH status of the sand may be related to effects of calcium and pH on length and number of root hairs. Evidence has already been presented to show that this relationship may exist when plants are grown in water culture. In order to obtain further evidence on the importance of this relationship in Mt. Compass sand, a nodulation trial was designed to re-examine the effects of calcium and pH on nodulation in combination with root hair studies, but eliminating the effects of Rhizobium numbers by supplying high levels in all treatments.

i. Pilot experiment. At pH 5.0 multiplication of rhizobia is likely to be poor, but could be rapid at higher levels. Because of this, high numbers in the sand are essential at low pH, if the bacterial numbers are to be non-limiting in nodulation. In addition, high numbers must be present in the rhizosphere of all plants, and this may be difficult to achieve in ordinary 5" pots containing several plants. Mt. Compass sand could also act as a filter, preventing movement of bacteria to the bottom of the pot if they were surface applied.

Because of the above problems, a pilot trial was conducted to find a way to maintain high bacterial numbers in the rhizosphere of seedlings, in all treatments.

A number of polythene tubes 6" long and 1" in diameter (Fig. 22) were selected in preference to the 5" pots used in previous trials. Each tube held 115 g of sand and was designed to grow a single plant which could be supplied with a known number of bacteria. A $^{3}/_{16}$" plastic (P.V.C.) tube entered each container halfway down so that some rhizobia could be introduced to the lower part of the root system. Nylon cloth secured to the base of each tube allowed free drainage but prevented sand from escaping. Each tube was placed in a glass jar to catch leachate from the sand.

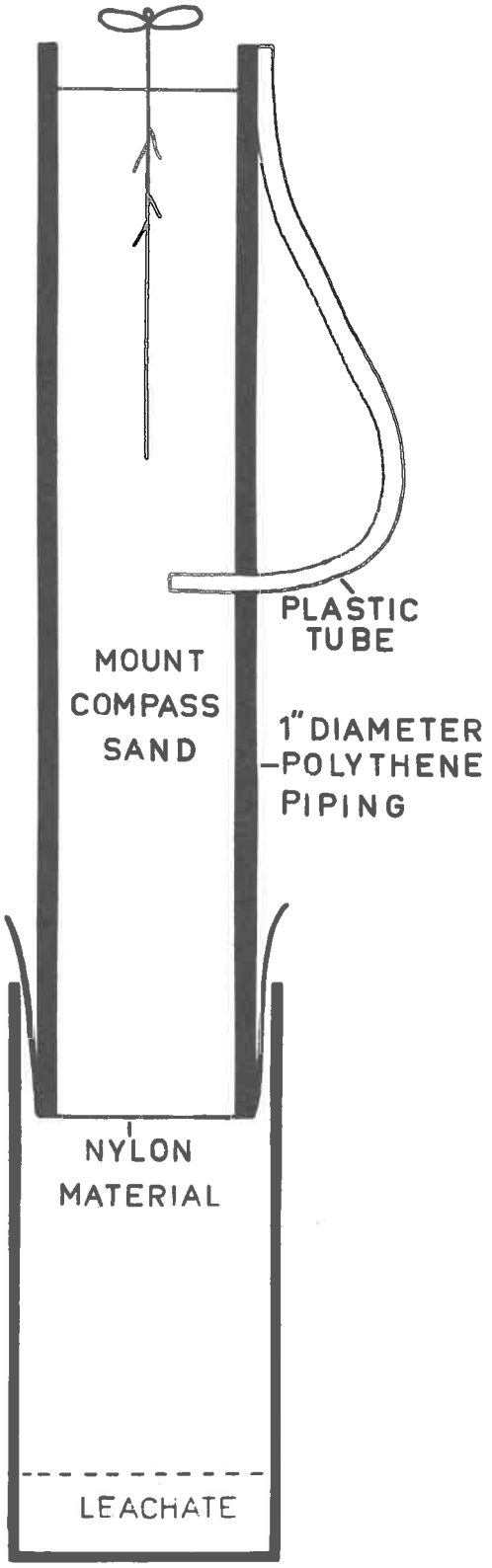
Basal macronutrients were added to the dry sand, and micronutrients were added to each tube in 26 ml of water. All basal nutrients were added at rates similar to those used previously. Two soil treatments were applied. Half the tubes contained sand at pH 5.0 without additional calcium, while the rest contained sand where the pH had been raised to 6.5 with $MgCO_3$ and calcium added as the usual $CaSO_4.2H_2O$:$CaCO_3$ mixture. Three levels of inoculum were used, each replicated 6 times, making a total of 36 tubes of each species.

After sowing, the tubes were transferred to a controlled environment cabinet with a 14 hour day length, and day and night temperatures of 20°C and 19°C respectively. The light intensity at plant level was 4,000 foot-candles.

Five days from sowing all plants had emerged and inoculum treatments were commenced. Cultures of Rhizobium

FIGURE 22.

Design of tube for examining effects of calcium and pH on nodulation in the presence of high numbers of rhizobia bacteria.


PLASTIC
TUBE
MOUNT
COMPASS
SAND
1" DIAMETER
-POLYTHENE
PIPING
NYLON
MATERIAL
LEACHATE

trifolii and R. meliloti were thoroughly shaken each with 50 ml of distilled water. Plate counts revealed that 71×10^9 R. trifolii and 156×10^9 R. meliloti were present in the respective cultures. Aliquots of the suspensions were taken to supply the following numbers of bacteria to each tube.

	R. trifolii	R. meliloti
In_1	71×10^6	156×10^6
In_2	71×10^7	156×10^7
In_3	71×10^8	156×10^8

Each suspension was made up to 20 ml with distilled water before being added to the tube. About 10-15 ml percolated through the sand into the receptable below. As a check on whether the bacteria were moving completely down the tube, samples of the first day's leachate were tested for presence of rhizobia. Three 0.5 ml aliquots were removed from each leachate and added to test tubes containing sterile plants of the appropriate species. Sterile clover and lucerne seedlings were grown in nutrient agar and dilute nutrient solution respectively. After 7 days all test tubes contained nodulated plants, indicating that rhizobia were moving completely through the sand and were likely to be in contact with all parts of the rhizosphere.

Suspensions of bacteria were added to the tubes on each of the following four days, in 20 ml of solution made up of the previous day's leachate and distilled water.

Three-quarters of the bacteria were added at the top of the tube, but a quarter were added halfway down, via the small plastic tube.

Leachate from tubes containing acid sand was brown in colour, probably due to dissolved organic matter, but was quite clear from sand where the calcium and pH levels had been raised. pH readings from the two leachates differed considerably.

pH 5.0 Ca_0 - pH 6.50

pH 6.5 Ca_1 - pH 7.25

No pH differences occurred between the leachates from the two species, or between the three levels of inoculum.

A week after inoculum was first added all plants were harvested and nodules counted. By this time, the unifoliate leaf on each plant was fully developed and the first trifoliate leaf had appeared on some plants.

ii. Results. Nodule numbers were variable from plant to plant in any one treatment and as many as 37 nodules were counted on subterranean clover seedlings.

In subterranean clover, each level of inoculum was satisfactory for nodulation. In lucerne on the other hand at pH 5.0, nodulation was poor at the low level of inoculum, and only 50% of the plants formed nodules.

Nodule numbers in lucerne tended to increase at the high pH and calcium levels (Table 30).

iii. Main experiment. An intermediate level of inoculum (71×10^7 *R. trifolii* and 156×10^7 *R. meliloti*

TABLE 30.

Nodules per plant (mean of 6 plants).

Treatment		Sub. clover	Lucerne.
pH 5.00 Ca_0	In_1	15.8	1.3
	In_2	14.6	6.5
	In_3	12.0	7.5
pH 6.35 Ca_1	In_1	13.0	10.3
	In_2	24.8	14.0
	In_3	17.0	8.5

per tube per day) was used to supply maximum bacteria in a further investigation of nodulation and root hair development in the sand. A technique similar to that described for the pilot trial was used, with similar pH and calcium treatments, but replicated 12 times.

On the day the initial suspensions of inoculum were added to the tubes, six seedlings of each treatment were examined for root hair development. These seedlings had grown in 5" plastic pots beside the tubes in the cabinet.

iv. Results. The mean length and density of root hairs on the taproots of both species was increased where calcium and pH levels had been raised. The improvement was greater in lucerne than in clover. (Table 31).

Nodule numbers were also increased in both species where calcium and pH levels were raised, although variation in nodule number from plant to plant within treatments was considerable (Table 32).

TABLE 31.

Mean Scores of Root Hair Length and Density.

Treatment	Sub. Clover		Lucerne	
	Length	Density	Length	Density
pH 5.0 Ca_0	1.95	2.09	1.88	1.29
pH 6.5 Ca_1	2.38	2.33	2.47	1.95

TABLE 32.

Nodules per Plant.

Treatment	Sub. clover	Lucerne
pH 5.0 Ca_0	10.6	3.7
pH 6.5 Ca_1	15.1	6.5
Significance	$P < 1\%$	$P < 1\%$

Percentage nodulation of clover at pH 5.0 was good even though there were fewer nodules per plant than at the higher pH. In contrast, six of the eleven lucerne plants at pH 5.0 had only one or two nodules on their roots, many fewer than at nearer optimum pH and calcium conditions.

v. Investigations of root hair development and nodulation using a transplanting technique. Seedlings of clover and lucerne were grown for 4 days in Mt. Compass sand. Adjustment of pH and calcium levels induced differences in root hair development. The plants were then carefully transplanted into 6" plastic tubes all filled with acid, untreated sand, and a high level of inoculum was added. However, considerable damage occurred

to root hairs during transplanting and no clear-cut results were obtained.

6. Discussion of Results.

Mt. Compass sand proved to be an excellent medium for the study of the effect of soil calcium, pH and level of inoculum on nodulation of subterranean clover and lucerne. Large responses to varying of all three factors were obtained. Parallel results were obtained from pot and field experiments, although responses to calcium were lower in pots than in the field.

Separation of pH and calcium effects on nodulation by raising pH with $MgCO_3$ and adding calcium as $CaSO_4 . \frac{1}{2}H_2O$ was not straightforward. Depression of pH in the sand by $CaSO_4 . \frac{1}{2}H_2O$ in the 1962 Field Trial and Pot Trial I may have masked calcium responses and Ca x pH interactions in nodulation. In Pot Trial I a significant depression in nodulation was measured where $CaSO_4 . \frac{1}{2}H_2O$ was added. Consequently, only the 1963 Field Trial provided a complete picture of calcium, pH and level of inoculum responses and interactions. However, it is unlikely that $MgCO_3$ and $CaSO_4$ had any side effects other than the effect of $CaSO_4$ on pH. In Pot Trial IV, the use of other forms of calcium ($CaCl_2$, $CaCO_3$) and other means of raising pH (NaOH, $CaCO_3$) showed no significant difference from the standard method in nodulation of clover, but significant differences due to addition of $CaCl_2$ and NaOH were measured in lucerne. The poorer nodulation of

lucerne at pH 6.0 where $CaCl_2$ was used to supply calcium was probably due to the lower actual pH (5.84) in this treatment compared with the others. The addition of NaOH in the absence of calcium also depressed nodulation of lucerne in this trial. In this case, the high level of sodium probably hindered the uptake of calcium ions by the lucerne resulting in poor nodulation, and an improvement when calcium was added. A further pot trial (V) indicated that sodium at the level present in the basal $Na_2HPO_4.2H_2O$ fertilizer was unlikely to be increasing the nodulation responses to calcium addition, as no effect of three levels and two forms of sodium was measured on nodulation.

The apparent pH of a soil, as determined electrometrically on a paste or suspension of the soil made up with water, depends on the salt concentration in the solution and also on the amount of water added to make this suspension. With a material such as Mt. Compass sand, which disperses very poorly, it would seem more meaningful to measure pH on a paste which approximates to moist field conditions, and this was done in all trials. It is well-established (Russell, 1961) that addition of a salt to a soil results in a decrease in the hydrogen ion concentration gradient across the double layer at the soil colloid surface, with a consequent fall in pH of the solution. If rhizobia exist in the solution, and the pH is lowered by addition of a salt such as $CaSO_4.\frac{1}{2}H_2O$, then it may be

expected that the fall in pH would depress multiplication of the bacteria and consequent nodulation. This is likely to have occurred in the 1962 Field Trial and in Pot Trial I, but was countered in later field and pot trials by adding a proportion of the calcium as $CaCO_3$ to act as a neutralising agent.

Nodulation of both species was more affected by level of inoculum on the seed than by either calcium or pH level in the sand. Rhizobium meliloti was completely absent in the field and in the 1962 Field Trial lucerne failed to nodulate when uninoculated seed was used even where calcium and pH levels were both raised. Although R. trifolii was present in the field, numbers were probably quite small, and the strain possibly of lower effectiveness than that used as inoculum. Nodulation of clover was poor and delayed, and few large primary nodulated plants developed where uninoculated seed was used. The difference in occurrence of the two Rhizobium species in the unamended sand is probably due to the greater acid tolerance of R. trifolii, already noted by Jensen (1942) in water culture and by Greenwood (1964) in the field. In New Zealand, according to Greenwood, R. meliloti are absent from most soils, but R. trifolii are much more widespread and are only absent on acid soils where pasture legumes have not grown previously.

In the 1962 Field Trial, application of inoculum to the seed at sowing resulted in a marked improvement in

nodulation, especially where high numbers of bacteria were used. The effect of heavy inoculum on seed was very considerable in all field and pot trials where it was used as a treatment. In subterranean clover, 100% primary nodulation could be achieved with heavy inoculum, whatever the calcium or pH status of the sand, although an increase in nodule numbers per plant still occurred where calcium or pH levels were also raised. Where a normal level of inoculum was used, many clover plants did not form nodules initially but secondary nodules formed later. Nitrogen fixation in the secondary nodulated plants is likely to have commenced later than in those plants with initial primary nodules. Consequently, the primary nodulated plants were larger and darker green, and were significantly higher in dry weight and percentage nitrogen (Pot Trial I). In the 1963 Field Trial the difference in dry weight between primary and secondary nodulated plants was still evident when a harvest was made 14 weeks from sowing.

In lucerne, when a normal level of inoculum was used, nodulation was generally poor, probably due to rapid death of rhizobia in the sand before the seed germinated. Good nodulation was achieved only with heavy inoculum, although nodulation also depended on the calcium and pH status of the sand. For example in the 1963 Field Trial, when neither calcium nor pH levels were raised, nodulation was poor even when high numbers of bacteria were used.

Dry matter production of both species not only depended on whether or not primary nodules were present on the roots, but also on the number of these nodules. Where the number was increased, by increasing soil pH or the level of inoculum, or (in lucerne, in the 1963 Field Trial) by addition of calcium, herbage yield was greatly increased. In Pot Trial I with clover, and in the 1963 Field Trial with both species the number of primary nodules and yield of dry matter per plant were closely correlated. Hely, Costin and Wimbush (1964), from field work in the Snowy Mountains, found that there was a close association between number of early-formed nodules and dry weight in white and alsike clover plants.

Environmental factors at Mt. Compass probably contributed to the close relationship between primary nodules and dry matter production. Soil nitrogen is extremely low, and the plants rely for their early growth largely on nitrogen fixed by primary nodules which form directly from inoculum on the seed. Free living rhizobia are almost completely absent and in the field multiplication of bacteria from the inoculum may be slow in the unfavourable environment of low winter temperatures and coarse sand which easily dries out. In addition it was observed that secondary nodulation was inhibited by the primary nodules on the plant, a phenomenon already closely studied by Nutman (1949) and Dart and Pate (1959). Consequently, the dominance of the primary nodules in

nitrogen fixation probably continued for a much longer period in Mt. Compass sand than in other situations, even though nodulation in both species improved considerably with time.

Several workers (Spencer, 1950; Jenkins, Vincent and Waters, 1954; Mulder and Van Veen, 1960) have obtained marked improvement in nodulation in acid soils where the level of inoculum was increased. Sears, Hyde and Greenwood (1955) who worked on pumice soils in New Zealand found that an increased level of inoculum improved the mean weight of nodulated white clover plants, as well as the proportion which became nodulated. Hely (1964) in field experiments on the Southern Tablelands of New South Wales, obtained increases in both survival and dry weight of subterranean clover plants when inoculum level was raised. None of these workers recorded the effect of level of inoculum on nodule number, however, and this work is among the first critical studies of the inoculum size-nodule number relationship to have been made under field conditions.

Where pH was raised to near 6.0 a considerable increase in percentage nodulation and nodules per plant of both species occurred in all trials. Where the pH was not raised, nodulation of lucerne was poor unless both heavily inoculated seed and (in the 1963 Field Trial) calcium fertilizer were used. Little further response occurred in clover when the pH was raised from 6.0 to 7.0, but

nodulation in lucerne was consistently better at the highest level, both in Pot Trial III and in the 1963 Field Trial. In Pot Trial I, the improvement in number of nodules per plant where pH had been raised was parallelled by an increase in herbage yield of both species, but this response was not evident in the field.

Calcium responses were greater in sand where the pH had not been raised, especially in subterranean clover. Addition of calcium improved both percentage nodulation and the number of nodules on each plant. However, the higher number of nodules was not associated with an increase in dry matter yield, except in lucerne in the 1963 Field Trial, where increases occurred. Where no calcium was added to the sand, the level of calcium in the plant was low, probably bordering on deficiency levels, but was more than doubled in the 1963 Field Trial when the equivalent of 8 cwt. $CaCO_3$ was supplied. Uninoculated lucerne in the same trial increased in yield where calcium was supplied. This suggested that soil calcium was insufficient not only for nodulation but also for plant growth, particularly in lucerne.

Andrew (1960), after carrying out pot trials on a low humic calcium-deficient sand in Queensland, suggested that 1.00% Ca was the critical level for optimum growth of white clover. Later trials on this soil (Andrew and Norris, 1961) showed that the calcium level in lucerne was 0.89% when 2 cwt. of $CaCO_3$ was applied per acre, but

attained 2.10% when a ton of $CaCO_3$ was added. In the 1963 Field Trial, in the absence of calcium, plant calcium levels were less than 1.00% in both species, and were lower in lucerne than in clover. In pot trials, however, calcium levels were much less than in the field, and were below 1.00% even when calcium was added. This difference was possibly because plants in pot trials were harvested at a much earlier stage of growth. Calcium concentration is known to build up with time in subterranean clover (Millikan and Hanger, 1964).

Results from the 1963 Field Trial indicated that comparable nodulation in either species could be achieved by either adding calcium to the sand or raising pH. Where both calcium and pH levels were raised, nodulation increased still further. This interaction between calcium and pH in nodulation confirms for field conditions similar findings in sand (Albrecht, 1933) and water culture (Loneragan and Dowling, 1958).

An important factor to be considered is the mode of action of calcium and pH on nodulation of the two species. Calcium is likely to have had its effect through the plant, as Vincent (1962b) has shown that the level of calcium required for growth of Rhizobium is low, and sufficient of this element for growth of rhizobia was probably supplied from the soil. On the other hand, pH is likely to have had a considerable effect on survival and multiplication of the nodule bacteria in the sand, and

much of the response obtained from raising pH has probably been due to increased bacterial populations in the sand and/or rhizosphere. Supporting evidence for this was obtained from rhizosphere colonisation tests with lucerne, reported in Section C. Here, at a normal level of inoculum, only about 15% of plants grown at pH 5.0 had rhizobia present on their roots, but nearly 50% of the plant roots were colonised at pH 6.5.

Nodulation could also have been affected by pH effects on the plant; for example, the effect of pH on uptake of calcium, or on root hair development. In the 1963 Field Trial, calcium uptake was increased in both species where the pH of the sand was raised from 5.35 to 6.30 and further, to 7.00. This effect of pH on calcium uptake is well-known, and was demonstrated in water culture trials reported in Section A, as well as by a number of other workers (Albrecht, 1933; Arnon et al., 1942; Arnon and Johnston, 1942; Loneragan and Dowling, 1958, Jacobson et al., 1960; Waisel, 1962). Loneragan and Dowling (1958), in water culture experiments, suggested that the main effect of pH on nodulation of subterranean clover was through its influence on the level of calcium in the plant; this effect could also have occurred in the 1963 Field Trial where an interaction was recorded between calcium and pH on nodulation.

Root hair studies showed that in lucerne, an increase in the calcium or pH levels of the sand resulted

in a considerable increase in root hair length and density. If infection occurs mainly in root hairs 150μ or more in length in this species, as observed by Thornton (1936), then improvement of the calcium and pH status of the sand may well provide many more sites for infection and thus increase nodulation.

In clover, little difference in root hair development occurred between treatments. Responses to calcium and pH in the field were less in clover than in lucerne, and if their effects on nodulation were in part through root hair development, then observable differences in that development would be less than in lucerne and were possibly too small to be picked up by the scoring technique.

Controlled environment studies compared root hair development and nodulation in sand at pH 5.0 without added calcium, and in sand where both calcium and pH levels had been raised. Effects of pH on rhizobial survival and multiplication were eliminated by daily application of high numbers of bacteria. Here, improvement in calcium and pH levels caused increases in root hair length and density of 31% and 51% respectively in lucerne, and 22% and 11% respectively in subterranean clover. Similar increases occurred in the number of nodules per plant; 76% in lucerne and 42% in clover. These results again demonstrate the possible association between calcium and pH, root hair development and nodulation.

As in the water culture trials, a marked species difference was evident in the Mt. Compass nodulation trials. Lucerne required a higher pH and calcium level in the sand and a higher level of inoculum on the seed for nodulation than clover. Although well-grown clover plants developed in the field, lucerne plants, even those which were well nodulated, were small and unthrifty and nearly all died in the following summer drought.

The differences in nodulation between the two legume species can be partly accounted for by differences in their root hair development. In clover, where no great differences in root hair development were observed between calcium and pH treatments, the limiting factor was probably the number of rhizobia present in the rhizosphere, and near maximum nodulation could be achieved by use of high numbers of bacteria on the seed. Where normal inoculum was used, however, bacterial numbers were somewhat limiting especially at low pH, and an increase in the number of suitable root hair infection sites by improvement in the calcium and/or pH level in the sand resulted in increased nodulation. On the other hand nodulation in lucerne was probably limited both by the number of rhizobia present in the rhizosphere and number of root hairs suitable for infection. Consequently, only at a high level of inoculum were bacteria present in sufficient numbers for nodulation to occur. However, at low pH and without calcium, root hairs were short and therefore unsuitable for infection;

they were relatively sparse, and nodulation was poor. Good nodulation of lucerne occurred only when root hair development was improved by improvement in calcium or pH level, and a high level of inoculum was used as well.

C. Rhizobium Survival Studies in Mt. Compass Sand.

The field and pot studies of nodulation and growth of subterranean clover and lucerne in Mt. Compass sand showed that improvements in the level of calcium, pH and inoculum were necessary for improved nodulation. However, a number of problems remained, in particular concerned with inoculum level, Rhizobium survival, and practical methods of applying lime. Further investigations of these aspects were carried out.

In previous experiments where a normal level of inoculum had been used nodulation of lucerne was poor, even where calcium had been supplied to the sand and low pH corrected. This suggested that factors other than soil pH were affecting nodulation, possibly through poor rhizobial survival on the seed, or poor colonisation of, and multiplication in, the rhizosphere. Studies were therefore undertaken to investigate the effects of soil sterilisation, low temperatures, and seed pelleting on Rhizobium survival and on nodulation in the sand.

Only two levels of inoculum had been used previously on seed. Although the high level had given very large nodulation responses, clearer definition of the effects of increasing levels of inoculum over a range of values was necessary, both from a theoretical viewpoint and also because of the bearing on practical seed inoculation. A field trial was therefore designed to test the effects of varying levels of inoculum on nodulation

and establishment. The effects of two liming treatments were also examined in order to obtain information on practical methods of raising pH and supplying calcium in Mt. Compass sand.

1. Soil Sterilisation Trials.

Evidence that "microbial antagonism" may prevent rhizobia in the inoculum from multiplying in soil and colonising the rhizosphere of legumes has been put forward by several workers, and relevant papers have already been reviewed. The possibility of "microbial antagonism" affecting nodulation at Mt. Compass could not be overlooked, in view of the poor nodulation obtained with normal inoculum on lucerne seed, and the demonstration of the presence of a "toxin" in the sand by J.R. Harris of C.S.I.R.O. Division of Soils (private comm.). A series of soil sterilisation trials were laid down to investigate the possible existence of an antagonistic microbial population in the sand.

(a) Field trial, 1962.

An area at Mt. Compass sufficient for sixteen 28 x 3 link plots was given a basal fertilizer dressing, followed by a $CaCO_3$:$CaSO_4.\frac{1}{2}H_2O$ mixture equivalent to 12 cwt. $CaCO_3$ per acre, applied as a 2 link wide strip to each plot, and raked in. The mixture was expected to raise the pH to 6.2 but from experience in other trials probably raised it to nearer 7.0. Eight of the 16 plots were sterilised with formalin at the rate of 1 gal.

4% formaldehyde per square yard. Normally inoculated lucerne and subterranean clover seed was sown in separate, single rows in each plot on June 12th 1962, 25 days after application of formalin. Unfortunately, sand blast destroyed most seedlings soon after emergence, so the plots were resown on July 17th. Sand blast again caused widespread damage and no harvest was attempted. At inspection in December 1962, it was noted that some of the surviving plants had made good growth, but no response to formaldehyde application was visible.

(b) Pot trial, 1962.

A pot trial was prepared with the following treatments.

Species: subterranean clover and lucerne.

Sterilisation: control, formaldehyde, and steam.

The trial, of factorial design, was replicated 18 times. The 4" diameter, cylindrical pots were each filled with 1300 g. of Mt. Compass sand, to which had been added a basal fertilizer dressing similar to that used for the field trials, and a $CaCO_3$:$CaSO_4.\frac{1}{2}H_2O$ mixture (0.80 g. and 1.63 g./1000 g. sand respectively) to raise pH to 7.0 and supply calcium.

After all pots were watered, 36 were autoclaved for 1 hour and a further 36 received 44 ml. of 4% formaldehyde per pot (equal to 1 gal./sq.yd.).

On June 12th (21 days after formalin was added) eighteen pots of each sterilisation treatment were returned

to Mt. Compass. 4" diameter cores were removed from prepared plots and replaced by the potted soil (without pots), in which was sown normally inoculated subterranean clover or lucerne seed. On June 13th, seed was sown in the pots still retained in an open-sided glasshouse at the Waite Institute.

Three pots from each treatment in the glasshouse trial were harvested 36 days from sowing, and the final 6 pots 41 days from sowing. The percentage of nodulated plants, number of primary and secondary nodules per plant and dry weight of tops were all measured. No significant response to treatment was found, and all plants were well nodulated (Table 33).

TABLE 33.

Effect of soil sterilisation on nodulation.

Glasshouse pot trial.

Treatment	% Nodulation		Nodules/Plant			
	Clover	Lucerne	Clover Prim.	Clover Sec.	Lucerne Prim.	Lucerne Sec.
Control	100.0	100.0	2.9	15.3	1.1	4.4
Steam	100.0	100.0	2.9	17.7	0.8	5.2
Formaldehyde	100.0	100.0	2.0	10.6	0.8	4.6

Mean of 6 replicates.

Three "pots" of each treatment from Mt. Compass were harvested 35 days from sowing and a further 6 "pots" harvested 84 days from sowing. Similar measurements to those carried out in the glasshouse trial were made, and

no responses to sterilisation occurred. Nodulation of clover was good, but a number of plants in all the lucerne treatments were unnodulated.

(c) Field trial, 1963.

Twelve 9' x 3' plots were topdressed with the usual basal fertilizer dressing and 10 cwt./acre of fine $CaCO_3$, in preparation for a further sterilisation trial at Mt. Compass in 1963. Three treatments were applied, each replicated four times.

Control.

Formaldehyde: 1 gal. 4% formaldehyde/sq.yd.

"Vapam": (NaN-methyl dithiocarbamate dihydrate $CH_3NHC(S)SNa.2H_2O$)
142 ml. of 32.5% a.e. per sq.yd., in 1 gal. water.

The two sterilants were applied on June 21st, and 75 normally inoculated seeds of each of subterranean clover and lucerne were sown in separate rows in each plot on July 14th. Twenty plants from each row were harvested on August 28th and examined for nodulation. No difference was found between control and formaldehyde treated plots. A number of lucerne plants lacked nodules in both treatments. No plants of either species were nodulated in the Vapam-treated plots and root development of the seedlings was poor. It was concluded that residual vapam was still present in the sand at sowing and had caused these adverse effects.

2. Lime Pelleting Trials.

Root nodule bacteria are particularly susceptible to death by desiccation (Vincent, Thompson, and Donovan, 1962). In Mt. Compass sand, which is coarse textured, low in organic matter, and easily dried out, death of seed inoculum by desiccation could be considerable in the field. It could be an important factor in causing poor nodulation where normal levels of inoculum are used. By coating the inoculated seed with a pellet, physical protection of the inoculum from desiccation can be effected, and at the same time the effects, if any, of "microbial antagonism" or "toxins" on Rhizobium survival may possibly be reduced. Two pot trials were therefore sown to examine effects of pelleting on nodulation.

(a) Lime pelleting pot trial.

A factorial trial was designed with four replicates of the following treatments.

Species: subterranean clover and lucerne.

Seed treatments: normal inoculum.
1000 x normal inoculum.
normal inoculum + lime pelleting.

The lime-pelleted seed was prepared as follows - seed was inoculated by the usual method and placed in a conical flask, sufficient dilute gum arabic was added to form a film over all the seed, then finely ground $CaCO_3$ (A.R. grade) was introduced as the seeds were revolved vigorously in the flask.

Pot preparation, basal fertilizing, seed inoculation, sowing and watering was similar to that used in Pot Trial I (Section B) and all pots received a basal $CaSO_4 \cdot \frac{1}{2}H_2O$ dressing designed to add the equivalent of 8 cwt. of $CaCO_3$/acre. The pH was raised to 6.0 in all pots by addition of $MgCO_3$. The improvement of the calcium and pH levels by the above methods was expected to almost eliminate any calcium or pH effects that lime pelleting may have had on nodulation.

After sowing, the pots were placed in an open-sided glasshouse for 24 days before harvesting. Primary nodulated, secondary nodulated and non-nodulated plants were separated after washing, and nodule counts were made.

Results.

A substantial response to lime pelleting was obtained in both lucerne and subterranean clover (Table 34). Increases occurred in the percentage of primary nodulated plants and in the number of primary and secondary nodules per plant. Nodulation of plants from normally inoculated, lime pelleted seed was almost equal to that of the treatment receiving 1000 times normal inoculum.

(b) Seed pelleting trial (Pot Trial II, Section B).

Vincent, Thompson and Donovan (1962) obtained improved survival of *Rhizobium* on glass beads and subterranean clover seed when these were coated with gum arabic. To ascertain whether it was the gum arabic sticker, or the actual lime coating which improved nodulation in the

TABLE 34.

Effect of Lime Pelleting on Nodulation.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
Sub. Clover.						
Normal inoculum	54.1 (0.82)*	24.2 (0.51)*	21.7 (0.47)*	3.6	2.0	5.4
Heavy "	89.3 (1.32)	10.7 (0.19)	0 (0)	6.5	2.7	9.3
Normal + pelleting	81.6 (1.16)	11.4 (0.25)	7.0 (0.19)	5.0	2.8	3.9
Lucerne.						
Normal inoculum	18.8 (0.38)	45.8 (0.74)	35.4 (0.62)	4.6	0.7	3.1
Heavy "	64.6 (0.93)	29.2 (0.57)	6.2 (0.18)	3.3	8.1	9.8
Normal + pelleting	62.9 (0.93)	21.7 (0.47)	15.4 (0.35)	5.0	3.7	8.9
L.S.D. 5%	(0.30)	(0.27)	(0.29)	1.5	1.2	3.6
1%	(0.42)	(0.38)	(0.41)	2.1	1.7	5.1

* Arcsin transformation.

Results are the mean of 4 replicates.

previous trial, a further pot trial was carried out.

i. Method. This trial, conducted in an open-sided glasshouse, formed part of Pot Trial II (Section B). The following treatments, replicated twice, werearranged in a factorial design.

Species:	subterranean clover and lucerne.
Seed treatment:	normal inoculum.
	normal inoculum + gum arabic.
	normal inoculum, gum arabic and lime pelleting.
pH levels:	5.0
	6.5
Calcium:	Control.
	Equivalent of 8 cwt. $CaCO_3$/acre as a $CaCO_3$:$CaSO_4$ mixture.

Gum arabic coated and lime pelleted seed was prepared as previously. All plants were harvested 24 days from sowing, when subterranean clover had reached the three-trifoliate leaf stage. Primary, secondary and non-nodulated plants were separated, and nodules counted on each plant.

ii. Results. In subterranean clover, no significant response occurred to either gum arabic coating or lime pelleting of seed, but at pH 6.5 a highly significant depression ($P<1\%$) in percentage primary nodulation and number of primary nodules occurred where seed was coated or pelleted. However, a marked improvement in the percentage of primary nodulated lucerne plants occurred at

both pH levels when seed was lime pelleted. No response to gum arabic was noted but at pH 6.5 a significant depression in percentage primary nodulation and number of secondary nodules occurred where seed was coated with gum arabic. The results, presented in Table 35, are the mean of the + and - Ca treatments. Full results of Pot Trial II, including statistical data, are presented in Appendix 9. Responses to lime pelleting were marked in both calcium treatments, although a significant response to calcium was also measured in the trial (Table 19 and Appendix 9).

3. Temperature and Nodulation.

Little information is available on the effect of low temperature on Rhizobium multiplication and legume nodulation in the field. According to Wilson (1930) at Cornell, low winter temperatures cause a drop in the number of rhizobia in soil, although a subsequent rise occurs in spring. In laboratory studies, Nutman (1949) observed that low temperatures delayed nodulation of red clover.

During winter at Mt. Compass, temperatures may be sufficiently low to affect multiplication of Rhizobium and consequent nodulation. As legumes in this area are often sown at the beginning of winter (late May-June), seedling emergence is fairly slow and rhizobia on seed are required to survive for a considerable period, until a rhizosphere develops and nodulation can occur. A series of studies were therefore undertaken to examine the effect

TABLE 35.

Effect of Seed Treatment on Nodulation.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
			Subterranean Clover.			
pH 5.0 C.	50.0	39.6	10.4	3.7	1.6	4.0
G.A.	50.0	33.3	16.7	3.5	1.9	3.9
L.P.	50.0	41.7	8.3	3.4	4.0	5.9
pH 6.5 C.	89.6	10.4	0	5.4	5.1	13.2
G.A.	77.1	22.9	0	3.8	6.6	11.9
L.P.	71.3	28.7	0	3.4	8.6	13.1
			Lucerne.			
pH 5.0 C.	8.0	0	92.0	-	-	-
G.A.	6.3	0	93.7	-	-	-
L.P.	75.3	6.3	18.4	3.1	0.3	-
pH 6.5 C.	57.3	29.2	13.5	2.9	2.3	5.8
G.A.	39.6	31.3	29.1	2.9	0.9	3.6
L.P.	80.3	17.4	2.3	2.9	3.6	6.1

C. = Control. G.A. = Gum Arabic. L.P. = Lime Pellet.

of temperature on rhizosphere colonisation and nodulation in relation to seedling growth. At the same time, possible interactions with calcium and pH were investigated.

(a) Method.

The experiments were conducted in a growth room, with a 12-hour day length and a light intensity of 1600-1800 foot-candles at plant level. Pot preparation was similar to that described earlier for Pot Trial II (Section B).

(b) Treatments.

Two experiments were conducted, at temperatures approximately corresponding to Mt. Compass winter and spring conditions, respectively. The temperatures used were:-

Low temperature - 45°F night, 60°F day.

High temperature - 60°F night, 75°F day.

At each temperature, the following treatments were used:

Species:	subterranean clover and lucerne.
pH level in sand:	5.0 and 6.5 - adjusted with $MgCO_3$.
Calcium level in sand:	control.
	equivalent of 8 cwt. $CaCO_3$/acre as a $CaSO_4.2H_2O$:$CaCO_3$ mixture. (0.946 g.:0.100 g./1000 g. sand)
Level of inoculum on seed:	normal.
	heavy - 1000 x normal.

In the low temperature trial, the three replicates were harvested 35 days from sowing, when the clover

had developed three trifoliate leaves. Six replicates were prepared for the high temperature trial. Three of the replicates were harvested at 23 days from sowing, when clover was at the same stage of growth that low temperature plants reached after 35 days. The other three replicates were allowed to grow for the full 35 days before being harvested. At this time, the larger clover plants had developed six trifoliate leaves.

At each harvest, primary, secondary, and non-nodulated plants were separated, nodules counted on each plant, and the tops retained for dry weight measurements.

(c) Results.

At the lower temperature, seedlings of both species emerged in 6 days from sowing, and nodules were visible on clover roots 18 days from sowing. At the higher temperature, seedlings appeared in 3 days and nodules were visible on clover roots 9 days from sowing. At both temperatures, lucerne took 3-4 days longer than clover to produce visible nodules. The nodulation responses to inoculum and pH treatment at the two temperatures are presented in Table 36.

Temperature had a marked effect on nodulation and growth of both species. At low temperature, lucerne plants made poor growth, and were similar in appearance to plants in the field in midwinter (Plate 20). The leaves were small and narrow, and bluish-green in colour. This condition was not so pronounced at high temperature,

TABLE 36.

Effects of Temperature on Nodulation - Lucerne.

Treatment	% Nodulation			Nodules/Plant.		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
Low Temperature - (35 days).						
pH 5.0 In_1	1.4	0	98.6	-	-	-
In_2	1.4	0	98.6	-	-	-
pH 6.5 In_1	13.9	11.1	75.0	1.9	0.4	2.1
In_2	48.6	38.9	12.5	2.0	1.0	2.9
High Temperature - (23 days).						
pH 5.0 In_1	0	0	100.0	-	-	-
In_2	1.4	0	98.6	-	-	-
pH 6.5 In_1	4.1	9.5	86.4	-	-	-
In_2	37.7	38.8	23.5	1.9	0.5	3.3
High Temperature - (35 days).						
pH 5.0 In_1	0	0	100.0	-	-	-
In_2	0	7.0	93.0	-	-	-
pH 6.5 In_1	10.6	42.8	46.6	3.4	0.4	4.6
In_2	38.9	56.9	4.2	3.9	3.5	6.7
Significance of differences.						
Level of Inoculum	1%	1%	-	n.s.	n.s.	5%
pH	1%	1%	-	1%	1%	1%
In x pH	n.s.	n.s.	-	n.s.	n.s.	n.s.
Temperature (35 days)	n.s.	1%	-	5%	5%	1%
Harvests (high temp.)	n.s.	1%	-	5%	5%	1%

Figures are the mean of 2 Ca treatments, 3 replicates/treatment.

TABLE 36. (cont.)

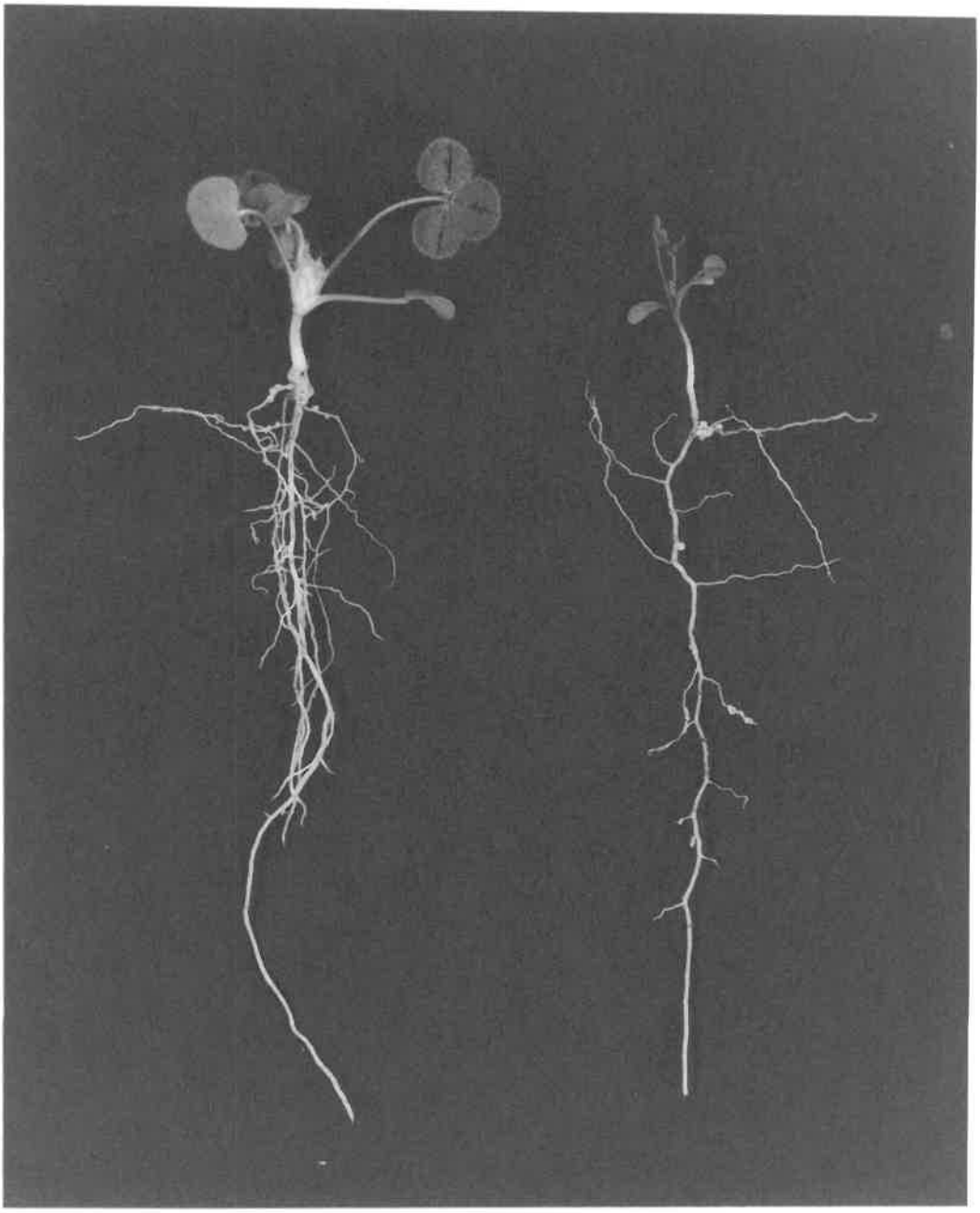
Effects of Temperature on Nodulation - Subterranean Clover.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
	Low Temperature - (35 days).					
pH 5.0 In_1	48.6	34.8	16.6	2.6	2.6	2.9
In_2	98.6	1.4	0	4.5	7.8	-
pH 6.5 In_1	76.4	23.6	0	3.0	8.3	14.2
In_2	100.0	0	0	5.4	7.8	-
	High Temperature - (23 days).					
pH 5.0 In_1	80.3	18.4	1.3	2.9	1.9	8.3
In_2	97.2	2.8	0	4.5	1.9	-
pH 6.5 In_1	87.0	13.0	0	2.4	5.2	12.1
In_2	100.0	0	0	5.5	1.5	-
	High Temperature - (35 days).					
pH 5.0 In_1	72.3	27.7	0	4.3	6.2	10.5
In_2	100.0	0	0	5.4	6.1	-
pH 6.5 In_1	88.8	11.2	0	3.8	17.2	19.9
In_2	100.0	0	0	6.7	18.0	-
Significance of differences.						
Level of Inoculum	1%	1%	-	1%	n.s.	1%
pH	1%	5%	-	5%	1%	1%
In x pH	1%	n.s.	-	1%	n.s.	n.s.
Temperature (35 days)	1%	n.s.	-	1%	1%	1%
Harvests (high temp.)	n.s.	n.s.	-	1%	1%	n.s.

Figures are the mean of 2 Ca treatments, 3 replicates/ treatment.

PLATE 20.

Primary nodulated subterranean clover and lucerne plants at the lower temperature, 35 days from sowing.
Note the small narrow leaves on the lucerne plant.



where plants were larger and leaves almost normal (Plate 21). Growth of clover was less affected by low temperature than that of lucerne and when heavy inoculum was used plants grew normally, but at a slower rate than at high temperature.

At high temperature after 35 days, nodulation in both species was significantly better than that obtained in the same time at low temperature. This was reflected in the much higher plant yields at high temperature (Table 37).

TABLE 37.

Effect of temperature on dry matter yield.

(mg/plant - heavily inoculated, primary nodulated)

Treatment	Sub. Clover	Lucerne
Low temperature - 35 days	264	93
High temperature - 23 days	207	74
High temperature - 35 days	615	270

When temperature was raised, improvement in nodulation of clover occurred only when normal inoculum was used; when heavy inoculum was applied to seed all plants were primary nodulated. At high temperature very few unnodulated plants occurred, and the percentage of primary nodulated plants and nodules per plant were considerably increased. Only a small difference in growth was visible between normally and heavily inoculated clover at high temperature after 35 days (Plate 21). A much

PLATE 21.

Subterranean clover (upper) and lucerne (lower) at the higher temperature, 35 days from sowing. Left to right -

pH 5.0 normal inoculum.

pH 5.0 heavy inoculum.

pH 6.5 normal inoculum.

pH 6.5 heavy inoculum.

Note the small differences between treatments in subterranean clover, but the large differences in lucerne.


SC 9₃
SC 10₃
SC 13₃
SC 14₃


L 9₃
L 10₃
L 13₃
L 14₃

larger difference was visible at the lower temperature.

In lucerne, temperature effects occurred only at pH 6.5, as few plants were nodulated at pH 5.0 whatever the temperature. At the higher temperature a highly significant increase in secondary nodulated plants occurred, and nodule number also increased. At the lower temperature, the weight of tops of nodulated lucerne was little higher than unnodulated plants, indicating that nitrogen fixation was probably low over the two weeks since nodulation.

Nodulation and growth of the two species after 23 days at the higher temperature was similar to that obtained after 35 days at the lower temperature (Tables 36 and 37).

As in previous trials a pronounced response in nodulation and plant growth occurred in clover when heavy inoculum was used on seed, while a smaller response occurred when the pH was raised to 6.5. With normal inoculum, especially at low temperature, many clover plants were small, pale and nitrogen deficient in the early stages of growth. These unthrifty plants were later found to possess no nodules or only small late-developing secondary nodules. When heavy inoculum was used, nearly 100% nodulation was obtained whatever the soil treatment or temperature. Substantial responses to heavy inoculum in nodulation and plant growth also occurred in lucerne, but at high pH only. Hardly any lucerne plants were nodulated

at pH 5.0 whatever the treatment.

No nodulation response to calcium addition was measured at either temperature. This can probably be attributed to a higher ratio of $CaSO_4.2H_2O$ used in the $CaSO_4.2H_2O$:$CaCO_3$ mixture, as compared with Pot Trial II (Section B), where a calcium response was obtained. The pH of the sand was depressed by 0.2 units when calcium was added, and this depression probably masked any nodulation response to calcium.

(d) Rhizobium survival in the rhizosphere of lucerne.

The extremely poor nodulation of normally inoculated lucerne in previous field and pot trials, even where calcium and pH levels in the sand had been raised, could have been due to poor survival of inoculum on seed, or inability of rhizobia to colonise the rhizosphere. Tests were therefore carried out at high and low temperatures for presence of rhizobia in the rhizosphere of unnodulated lucerne to ascertain whether colonisation was occurring and to find out whether temperature affected the process.

Two trials were conducted to test suitable techniques. Because of the very probable presence of other organisms, plate counts were not considered as a practical method. Both Purchase and Nutman (1957) and Rovira (1962) have estimated numbers of rhizobia in the rhizosphere by plant infection techniques, and a modification of their methods was tested in the first trial.

A pot trial with three replicates was prepared, with similar treatments to those in the main temperature trial. Lucerne seedlings were grown in the growth room at the lower temperature (45°F night, 60°F day), and after 35 days were removed from the sand, taking care to avoid damaging roots. Excess sand was lightly washed off the roots with sterile distilled water. A 1 cm. segment of taproot from 2 cm. below the crown was removed from each unnodulated plant and placed in a test tube containing a sterile lucerne seedling. Each sterile seedling was suspended in a nitrogen-free nutrient solution by a fine, stainless steel wire. The mouth of each tube was covered by an aluminium cap.

All tubes were placed in a glasshouse maintained at 70°F and the seedlings examined at weekly intervals for the presence of nodules. Few seedlings formed nodules, even those with root segments from heavy inoculum treatments. It was thought possible that Rhizobium numbers in the rhizosphere were very small and distributed sparsely along the root, and the technique not sensitive enough to provide useful results. The test was repeated in a second trial, and a 3 cm. taproot segment, taken from 1 cm. below the crown, was added to each tube. Again, few nodules formed on the test plants.

In the second trial, a different technique was also tested. A basal fertilizer dressing, similar to that used in Pot Trial II (Section B), was added to Mt. Compass

sand, and calcium added and pH raised to 7.0 by addition of 1,0 g. fine $CaCO_3$/1000 g. sand. The sand was then sterilised for 1 hour in an autoclave. With these treatments the sand was expected to supply optimal nutrient and pH conditions for survival and multiplication of Rhizobium meliloti and at the same time be uncontaminated by free-living rhizobia and other organisms.

Unnodulated lucerne seedlings, carefully removed from the 5" pots and lightly washed over a sieve with sterile distilled water, were transplanted into small waxed cartons, each containing damp sterile sand (450 g. air-dry sand/carton). The seedlings were then watered with sterile distilled water. A waxed lid was placed on each carton, the seedling protruding through a small aperture in the centre (Plate 22). Six unnodulated plants were transplanted from each pot. At the same time, 12 cartons of sterile sand were sown with surface-sterilised seed as a check on contamination during transplanting. The cartons were randomised in a glasshouse maintained at 70°F. Transplanted plants grew rapidly and soon changed from the characteristic stunted, bluish appearance of the low temperature treatment. The cartons were watered when required through the small aperture in the lid, and plants were harvested 28 days from transplanting. Roots were washed and examined for presence of nodules, which were taken as an indication of the presence of rhizobia in the rhizosphere of seedlings at transplanting. No control

PLATE 22.

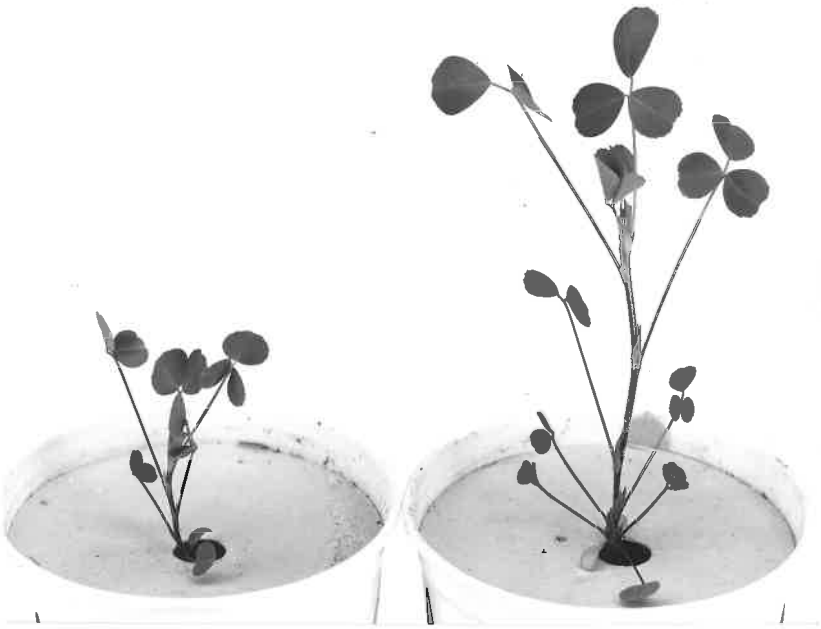
Technique used to check for presence of *Rhizobium meliloti* in the rhizosphere of unnodulated lucerne seedlings.

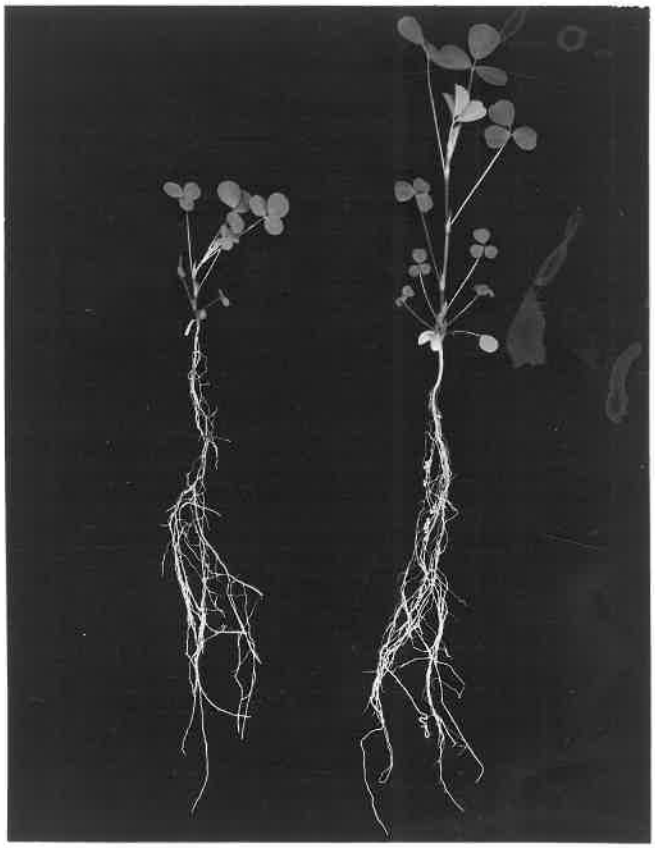
Upper: Waxed cartons containing plants ready for harvest.

Lower: The same plants after harvest.

Left - No *R. meliloti* present (no nodules).

Right - *R. meliloti* present (nodules).





plants were nodulated. At harvesting, nodulated plants were easily observed by their greater size and dark green appearance (Plate 22). The following results were obtained:

	Percentage Nodulation
pH 5.0 In_1	15.3
" " In_2	52.8
pH 6.5 In_1	48.7
" " In_2	95.8
Control	Nil.

The figures are the mean of the two calcium treatments, three replicates of six plants per treatment. No response to calcium occurred.

The results obtained did not provide figures for the actual number of rhizobia on the roots of transplanted plants, only their presence or absence, but the tube tests indicated that numbers were probably low at best. The results showed that the method was sensitive enough to reflect effects of inoculum size and pH on survival of rhizobia in the rhizosphere, and it appeared to be a much more sensitive test than the test tube method. The sterile sand transplanting technique was therefore adopted in the temperature trials already reported. Tests were made on unnodulated lucerne plants at 11, 23 and 35 days from sowing, in both temperature trials. Extra replicates of lucerne were sown to provide plants for testing at 11 and 23 days. Each series of cartons was harvested 21 days

from transplanting. Results are presented in Figure 23.

At low temperature, results were similar to those obtained in the preliminary trial. Few transplants produced nodules where both inoculum level and soil pH had previously been low, but all transplants became nodulated where both had been high. At these two extremes little difference in transplant nodulation occurred at 11, 23 and 35 days from sowing. Where only inoculum level or pH was previously low, intermediate results were obtained. Where a treatment of pH 5.0 and heavy inoculum had been used, transplant nodulation dropped steadily at successive samplings, but with a pH of 6.5 and normal inoculum nodulation tended to rise between the second and third samplings. At 35 days, the two last-mentioned treatments both gave just over 50% nodulation.

At high temperature, rhizobial survival in the rhizosphere, as assessed by the transplanting technique, was higher than at low temperature, but similar differences to those occurring at low temperature were measured between treatments.

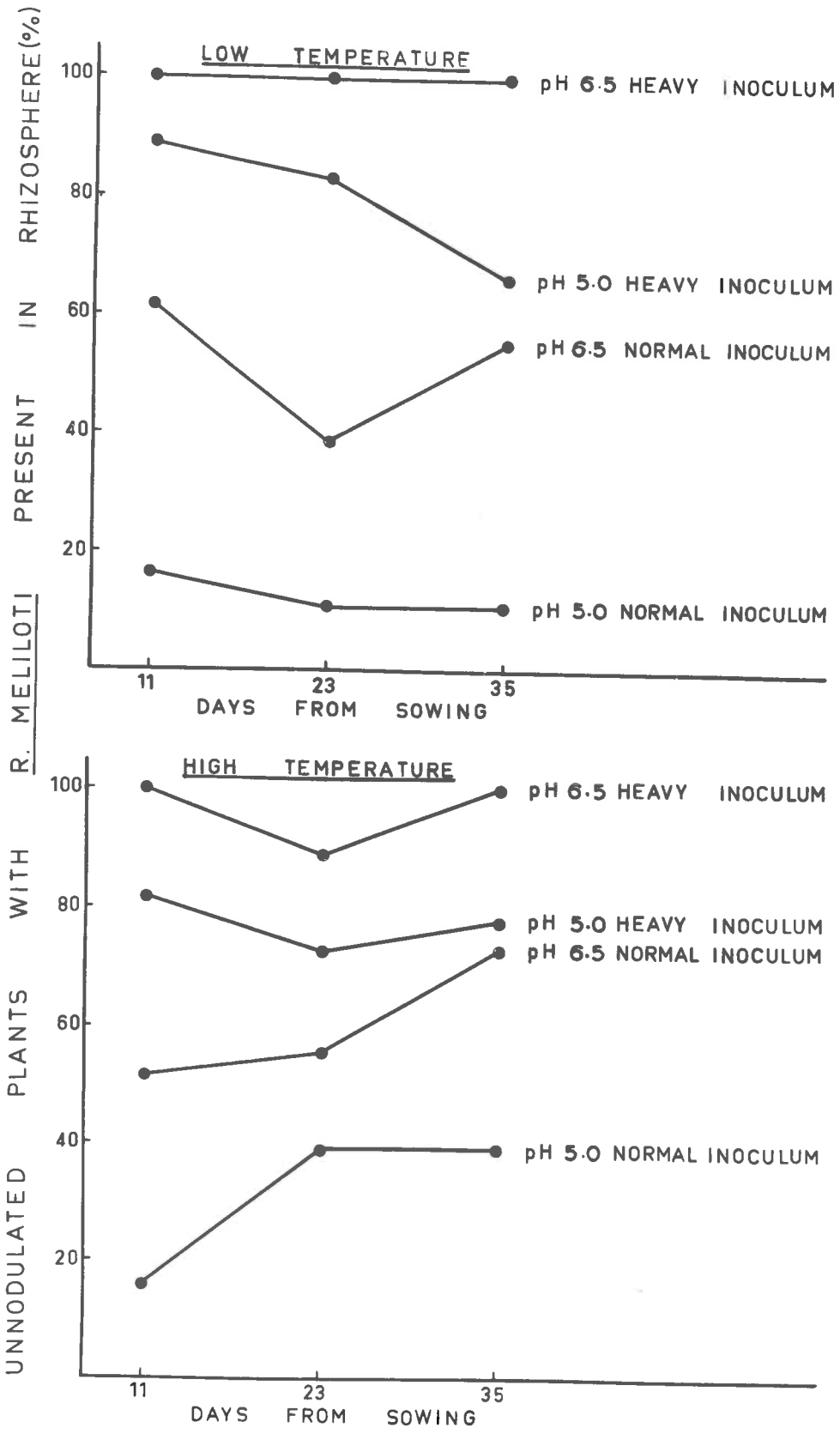
No control seedlings were nodulated, indicating that contamination was most unlikely to have occurred, and that the nodules were formed by rhizobia already present on the roots of the transplanted seedling.

4. Mt. Compass Field Trial II, 1963.

Previous field and pot trials showed that in Mt. Compass sand a substantial improvement in nodulation

FIGURE 23.

Effect of temperature, level of inoculum on seed, and soil pH on survival of Rhizobium meliloti in the rhizosphere of unnodulated lucerne, at three intervals from sowing.


LOW TEMPERATURE
R. MELILOTI PRESENT IN RHIZOSPHERE (%)
100
80
60
40
20
pH 6.5 HEAVY INOCULUM
pH 5.0 HEAVY INOCULUM
pH 6.5 NORMAL INOCULUM
pH 5.0 NORMAL INOCULUM
11
23
35
DAYS FROM SOWING
HIGH TEMPERATURE
UNNODULATED PLANTS WITH
100
80
60
40
20
pH 6.5 HEAVY INOCULUM
pH 5.0 HEAVY INOCULUM
pH 6.5 NORMAL INOCULUM
pH 5.0 NORMAL INOCULUM
11
23
35
DAYS FROM SOWING

could be obtained by supplying high numbers of rhizobia on the seed. In order to define the effects of increasing level of inoculum more closely, further study was needed to provide both basic information and a basis for practical recommendations on inoculation of legume seed in Mt. Compass and similar sands. In this trial, two levels of peat inoculant and four levels of agar inoculant were used. As a positive response to seed pelleting had been obtained in previous pot trials, a lime-pelleting treatment was also included.

At present, there are three practical methods which may be used by farmers to supply calcium and raise pH in soil to assist legume nodulation. These are:

1. Lime pelleting of seed.
2. Lime in drill row, 1-2 cwt./acre with seed.
3. Heavy broadcast liming, ½-2 tons/acre before sowing.

Generally, the first two methods are used where the soil is above pH 5.5 for lucerne and above pH 5.0 for subterranean clover, and where calcium deficiency is not marked, if present at all. In more acid soils, however, they are not always successful and heavier rates of liming, though costly, are sometimes necessary.

At Mt. Compass, the pH and calcium levels are sufficiently low to cast doubts on the effectiveness of lime pelleting as a means of obtaining successful nodulation of clover and lucerne. In observational trials,

sowing of lime pelleted, normally inoculated seed in soil with only basal fertilizers added at Mt. Compass in 1962 resulted in nodulation failure in lucerne, and poor results in clover. On the other hand, plants nodulated well in adjacent plots which had received 10 cwt. of $CaCO_3$/acre as a band mixed to 4". Lucerne in these plots survived the summer drought and grew well in the second year. J.K. Powrie was also successful in establishing lucerne in a neighbouring trial when he applied 1½ tons $CaCO_3$/acre.

In view of the above observations, two contrasting methods of liming, light drill-row application and heavy broadcasting were decided upon, in order to obtain information on the best practical method of supplying calcium and raising pH. The liming and level of inoculum treatments were combined in a multiple-factor trial of factorial design.

(a) Treatments.

The following treatments were used:

Species: subterranean clover and lucerne.

Liming: 4 cwt./acre in drill row

1 ton/acre broadcast and rotary-hoed to 4" depth.

Inoculum: normal (x1)

x10

x100

x1000

normal + lime pelleting.

In addition, two peat inoculum treatments (x1 and x10) were used, but only on broadcast-lime plots. Each treatment was replicated four times, making a total of 96 plots. Each plot measured 9' x 3'. The trial layout may be seen in an accompanying site plan (Fig. 24).

(b) Fertilizer and lime application.

The site was cleared and rotary-hoed in a similar manner to Field Trial I, 1963. Broadcast-lime treatments were applied to appropriate plots on May 14th, 1963, and the whole plot area rotary-hoed to 4" depth. A basal fertilizer dressing, identical with that used in previous field trials was then applied and harrowed in. Drill-row lime was applied with the seed at sowing. Agricultural limestone was used throughout. The fineness of grinding is indicated by the following particle size measurements:

Retained on	1000	micron	(.0394")	sieve	8.8%
"	"	500	"	(.0197")	"	24.6%
"	"	211	"	(.0083")	"	32.1%
Passed through	211	"	"	"	34.5%

(c) Seed inoculation, and Sowing.

Rhizobium cultures were grown on nutrient agar for 5 days and seed inoculated in the afternoon of May 20th, allowed to dry at room temperature and stored overnight in a refrigerator. The method used for inoculation was similar to that used in previous trials and is summarised in Table 38.

FIGURE 24.

Mt. Compass Field Trial II, 1963.

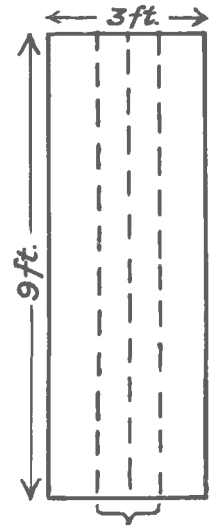
Treatments.

1, 13.	Lime in drill row	x 1	agar inoculum
2, 14.	" " " "	x 10	" "
3, 15.	" " " "	x 100	" "
4, 16.	" " " "	x 1000	" "
5, 17.	" " " "	x 1	agar inoculum + lime pellet.
6, 18.	Lime broadcast	x 1	agar inoculum
7, 19.	" "	x 10	" "
8, 20.	" "	x 100	" "
9, 21.	" "	x 1000	" "
10, 22.	" "	x 1	agar inoculum + lime pellet.
11, 23.	" "	x 1	peat inoculum
12, 24.	" "	x 10	" "

Mt COMPASS FIELD TRIAL II
1963

BLOCK III		BLOCK IV	
22	8	17	24
2	18	21	14
4	13	19	16
23	20	9	6
12	3	11	7
21	18	24	3
4	14	15	13
20	7	10	6
19	23	9	12
22	1	17	5

BLOCK I		BLOCK II	
11	15	15	23
5	8	5	3
1	20	10	17
16	18	1	2
2	14	8	6
10	19	2	9
24	4	16	12
7	13	11	22
12	1		18
10	15		7
2	5		13
17	11		4
6	2		14
23	3		19
8	9		24
16	22		20



THREE SEED ROWS
7 INCHES APART

DETAILS OF A
SINGLE PLOT

TABLE 38.

Method of Seed Inoculation.

Inoculum Level	Skim Milk added to Culture (ml.)	Aliquot for 113.4g. Seed (ml.)	Cells per Seed (plate counts) Sub.Clover	Lucerne
Normal (x1)	852.0	4.25	1,630	4,730
x10	85.2	4.25	15,000	53,300
x100	8.5	4.25	93,000	400,000
x1000	Culture slime added directly to 22.5g. seed		770,000	3,330,000

Inoculation with peat powder was carried out by mixing .0875 g. or .8750 g. of powder with 22.5 g. of seed, adding 1 ml. of skim milk and again mixing thoroughly before allowing to dry. The above levels provided the recommended and 10 times the recommended rate of inoculum respectively on the seed. Actual numbers of bacteria on each seed were not determined for peat inoculum.

The trial was sown on May 21st, 24 hours after the seed was inoculated. Two hundred seeds were sown in three 9' rows, 7" apart, in the centre of each plot, and covered to ¾" depth. The rows were made with a ring roller, which consolidated the sand at the same time. To facilitate sowing, a small amount of dry sand was mixed with the seed, while drill-row lime was also added in the appropriate plots.

(d) Soil pH measurements.

On July 22nd, samples were obtained from all

plots for soil testing. A 0-4" sample was obtained from the broadcast-lime plots, while 0-1", 1-2" and 2-3" samples were obtained directly from the rows of the drilled-lime plots. The following figures were obtained:

Treatment		pH
1 ton broadcast	=	6.80
4 cwt. in drill row 0-1"	=	7.02
1-2"	=	5.99
2-3"	=	5.49

(e) Harvests I and II.

On July 15th and August 26th, 55 and 97 days respectively from sowing, 20 plants were removed at random from each plot as previously. Primary, secondary and non-nodulated plants were separated, and primary and secondary nodules counted on each plant.

(f) Harvests III and IV.

On October 15th, 147 days from sowing, 12 plants were removed from each plot for determination of herbage production. At this harvest it was noticed that lucerne which had received 1 ton of lime, broadcast, was making better growth than that which had received only 4 cwt. of lime in the drill row. This difference in growth seemed to be associated with differences in root development; taproot formation was much better where lime was broadcast. A fourth harvest of six plants per plot was therefore obtained on November 11th to assess the effects of liming on root development of both species.

Plants were carefully removed with a spade, and those with forked or straight roots separated. After drying, tops and roots were retained for dry weight, calcium, phosphorus and manganese determinations. Manganese was determined by the periodate method (Piper, 1942), while calcium and phosphorus were determined as previously.

Forked-rooted plants were defined as those in which a taproot could not be identified 2-3" below the crown. The roots of these plants were generally much-branched, small, and brown in colour.

Straight-rooted plants had produced a long, straight white taproot for at least 6" below the crown. Brown roots were much less evident.

In lucerne, the different categories were easily separated, but clover roots were more difficult to classify, because of a general abundance of fibrous, branched roots in all plants. Immediately before Harvest IV soil samples were obtained at 0-2", 2-4", 4-6" and 6-8" depths from the two liming treatments. In the drilled-lime treatment, samples were taken along the actual plant rows.

(g) Results.

Significant nodulation responses to inoculum level and liming treatment were evident, and a considerable species difference again occurred. The results of Harvest I are presented in Table 39, while Harvest II results are presented in Appendix 13.

TABLE 39.

Mt. Compass Field Trial II, 1963, Harvest I.

Subterranean Clover.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
Drilled lime.						
x 1 inoculum	76.9 (1.07)*	20.7	2.4	2.3	5.2	5.7
x 10 "	97.6 (1.40)	2.4	0	3.6	8.0	-
x 100 "	100.0 (1.47)	0	0	4.9	9.0	-
x 1000 "	100.0 (1.47)	0	0	5.4	9.1	-
x 1 + lime pellet	71.1 (1.00)	25.2	3.8	2.5	3.3	4.6
Broadcast lime.						
x 1 inoculum	74.2 (1.04)	24.4	1.4	2.1	3.0	5.8
x 10 "	88.7 (1.24)	6.6	4.8	3.1	7.2	9.0
x 100 "	100.0 (1.47)	0	0	4.7	7.1	-
x 1000 "	100.0 (1.47)	0	0	5.6	7.9	-
x 1 + lime pellet	74.5 (1.03)	25.5	0	2.5	4.6	4.9
x 1 inoc. (peat)	92.7	7.3	0	3.5	6.8	5.9
x 10inoc. (peat)	100.0	0	0	3.7	7.7	-
Inoculum L.S.D.5%	(0.16)			0.4	1.9	
1%	(0.22)			0.6	2.6	
Lime L.S.D.5%	(0.10)			0.3	1.2	
1%	(0.14)			0.4	1.7	
In x L L.S.D.5%	(0.22)			0.6	2.7	
1%	(0.31)			0.8	3.7	

* arcsin transformation.

Note. Peat inoculum results were not statistically analysed.

TABLE 39. (cont.)

Mt. Compass Field Trial II, 1963, Harvest I.

Lucerne.

% Nodulation			Nodules/Plant		
Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
68.5 (0.97)*	5.9	25.6 (0.52)*	2.8	0.6	4.6
86.5 (1.19)	1.0	12.5 (0.35)	3.2	0.7	-
98.8 (1.44)	1.2	0 (0)	4.0	1.1	-
97.7 (1.40)	1.1	1.2 (0.06)	4.1	1.3	-
87.8 (1.20)	3.4	8.8 (0.26)	2.9	0.5	-
15.9 (0.36)	3.0	81.1 (1.15)	1.6	0.2	-
60.6 (0.89)	7.1	32.3 (0.60)	1.9	0.6	-
84.9 (1.18)	2.5	12.6 (0.34)	3.1	1.3	-
97.4 (1.40)	1.3	1.3 (0.06)	3.5	1.4	-
63.4 (0.92)	3.9	32.8 (0.60)	2.2	0.2	-
58.1	6.2	35.7	2.1	0.2	1.3
65.8	9.8	24.4	2.1	0.2	2.2
(0.15)		(0.17)	0.5	0.4	
(0.21)		(0.23)	0.7	0.5	
(0.10)		(0.11)	0.3	0.2	
(0.13)		(0.15)	0.5	0.3	
(0.22)		(0.24)	0.8	0.5	
(0.29)		(0.33)	1.0	0.7	

* arcsin transformation.

Note. Peat inoculum results were not statistically analysed.

At Harvest I, nearly all clover plants were nodulated whatever the treatment, but both inoculum level and lime treatment had a marked effect on the proportions of primary and secondary nodulated plants, and the number of primary and secondary nodules formed on each plant. In contrast to clover, many lucerne plants lacked nodules where only normal or 10 times normal agar inoculum was used. In both species, as inoculum level was increased, the percentage of primary nodulated plants and the number of primary and secondary nodules per plant also increased (Figs. 25 and 26). However, about 30 times more inoculum was required on lucerne than on clover seed to achieve a similar percentage of primary nodulated plants.

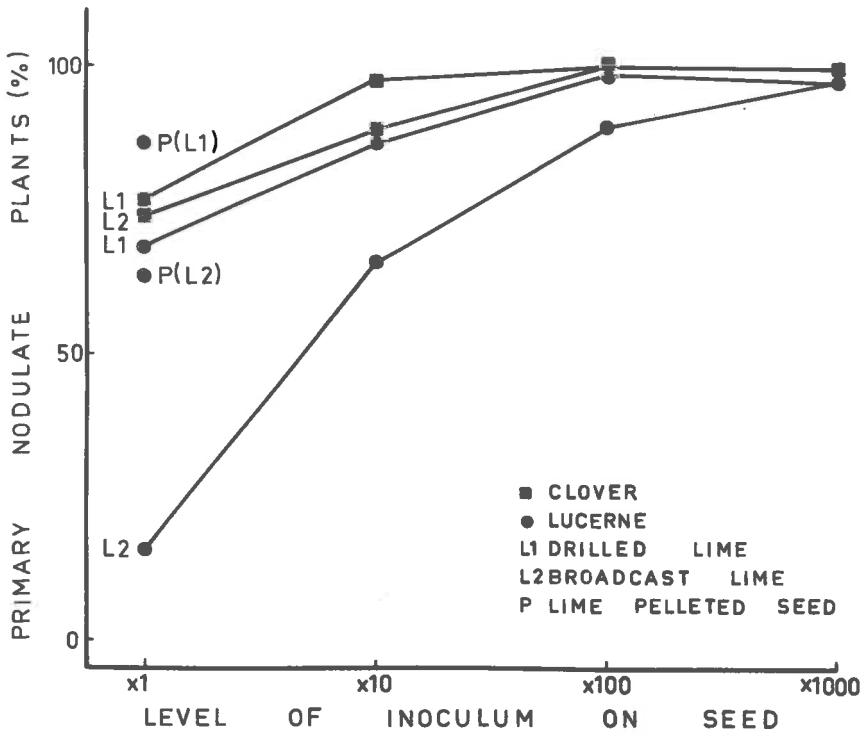
The method of applying lime to the sand affected nodulation considerably in both species (Figs. 25 and 26). Where lime was applied in the drill row, the percentage of primary nodulated plants, and the number of nodules on each plant were significantly higher than where lime was broadcast. This difference was marked in lucerne at normal inoculum. Only 18.9% of lucerne plants in broadcast-lime plots were nodulated at first harvest, while 74.4% were nodulated in drill-row lime plots. To obtain equivalent nodulation, about 10 times more inoculum was necessary with lucerne in broadcast than in drill-row lime plots. The difference in inoculum requirement was less for subterranean clover, however, and only about 5 times more inoculum was required in broadcast-lime plots

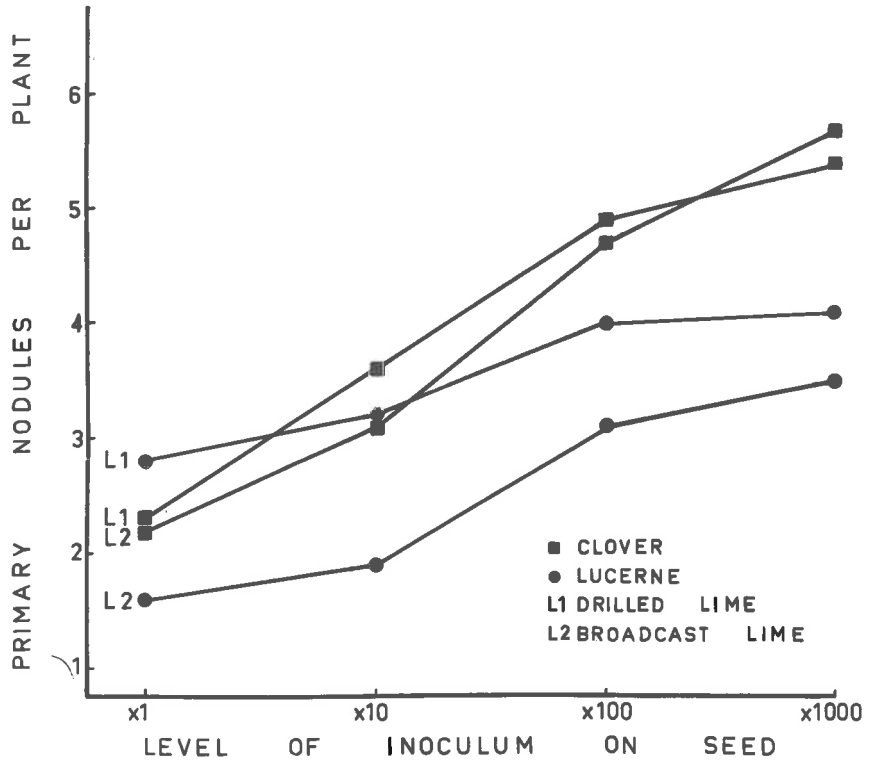
FIGURE 25.

Mt. Compass Field Trial II, 1963. Effect of level of inoculum on seed on the percentage of primary nodulated plants, with two methods of liming (Harvest I).

FIGURE 26.

Mt. Compass Field Trial II, 1963. Effect of level of inoculum on seed on the number of primary nodules per plant, with two methods of liming (Harvest I).


PRIMARY NODULATE PLANTS (%)
100
50
0
P(L1)
L1
L2
L1
P(L2)
L2
CLOVER
LUCERNE
L1 DRILLED LIME
L2 BROADCAST LIME
P LIME PELLETED SEED
x1
x10
x100
x1000
LEVEL OF INOCULUM ON SEED


PRIMARY NODULES PER PLANT
6
5
4
3
2
1
L1
L1
L2
L2
CLOVER
LUCERNE
L1 DRILLED LIME
L2 BROADCAST LIME
x1
x10
x100
x1000
LEVEL OF INOCULUM ON SEED

to obtain equivalent nodulation.

Lime pelleting of subterranean clover seed had no effect on nodulation; in contrast, a highly significant increase in percentage nodulation occurred with lucerne. The percentage of primary nodulated lucerne plants from normally inoculated, lime pelleted seed was equal to that where 10 times normal inoculum was used without pelleting. The response occurred in both liming treatments but was more pronounced where lime was broadcast. Lime pelleting of lucerne seed did not increase nodule number, however.

As with agar inoculum, the application of a high level of peat inoculum to seed resulted in increased nodulation in both species, as compared with the recommended rates. Peat and agar inoculums used in this trial cannot be directly compared as the number of bacteria on seed inoculated with peat was not counted. Peat inoculum appeared superior to agar inoculum at "equivalent"levels, but this was possibly due to a greater number of bacteria applied per seed in peat than in agar inoculum.

As in previous field trials at Mt. Compass, nodulation of both species improved considerably between the first and second harvests (Table 39 and Appendix 13). The large differences in nodulation due to inoculum size and lime treatment were still apparent at the second harvest, however.

Considerable differences in plant growth due to inoculum treatment occurred in both species during the trial, even in young plants (Plates 23 and 24). In particular, plots of normally inoculated lucerne contained few vigorous plants, and many of the non-nodulated plants had died by the time a second harvest was taken. With heavy inoculum, most lucerne plants were large, dark green and vigorous. Herbage yields obtained at Harvest III, although somewhat variable, generally increased in parallel with increased inoculum size on the seed (Appendix 13). As in previous trials, yield was probably influenced greatly by the number of primary nodules present on each plant. A relationship was evident between the number of primary nodules/plant at Harvest II and plot yield at Harvest III (Fig. 27).

A feature of the herbage yields was the significantly lower production ($P<1\%$) of both species from the drilled-lime than from broadcast-lime plots, even though better nodulation had occurred with drilled lime. The difference in yield between liming treatments was associated with considerable differences in root development, observed first at Harvest III and later verified at Harvest IV. The results of the latter harvest are presented in Table 40.

In drilled-lime plots, root development of lucerne was poor, with no obvious taproot and many short, brown, branched roots (Plate 25). Where lime was broadcast

Mt. Compass Field Trial II, 1963. Effect of level of inoculum on growth of subterranean clover and lucerne, 147 days from sowing. Plots in centre foreground:

Left - heavy inoculum.

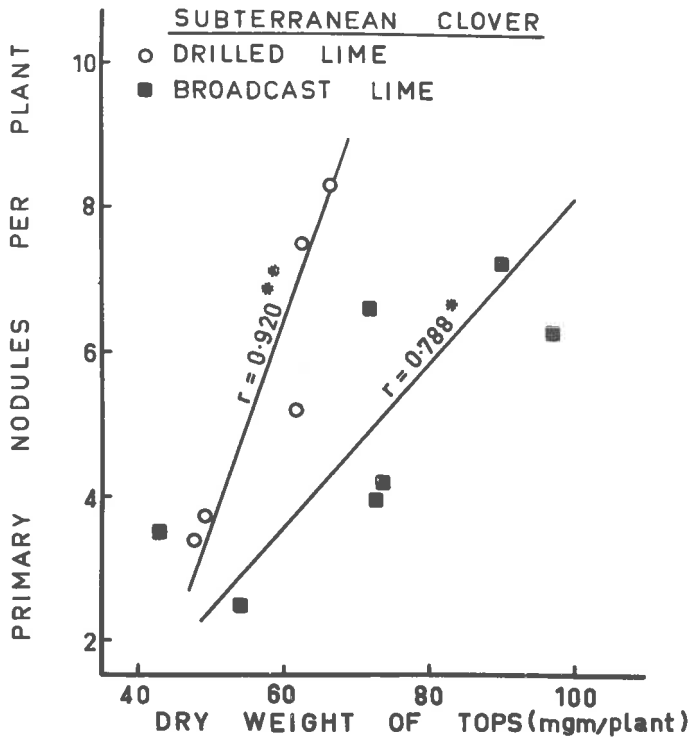
Right - normal inoculum.

PLATE 23 (Upper): Subterranean clover.

PLATE 24 (Lower): Lucerne.

FIGURE 27.

Mt. Compass Field Trial II, 1963. Relation between dry weight of tops (Harvest III) and primary nodule number (Harvest II) at two levels of liming.


SUBTERRANEAN CLOVER
DRILLED LIME
BROADCAST LIME
PRIMARY NODULES PER PLANT
10
8
6
4
2
r = 0·920 **
r = 0·788 *
40
60
80
100
DRY WEIGHT OF TOPS (mgm/plant)

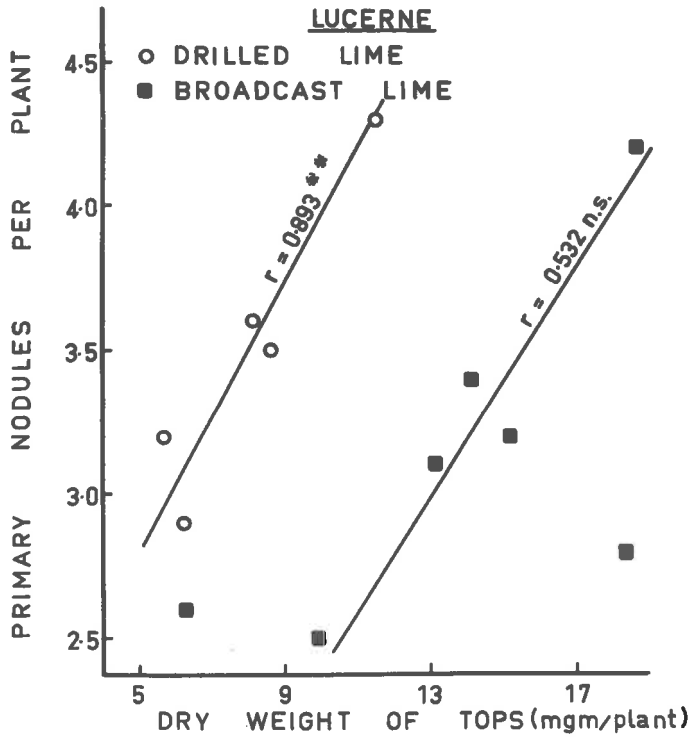

LUCERNE
DRILLED LIME
BROADCAST LIME
PRIMARY NODULES PER PLANT
4·5
4·0
3·5
3·0
2·5
r = 0·893 **
r = 0·532 n.s.
5
9
13
17
DRY WEIGHT OF TOPS (mgm/plant)

PLATE 25.

Mt. Compass Field Trial II, 1963. Effect of liming on root development of lucerne.

Left - 4 cwt of lime in drill row.

Right - 1 ton of lime broadcast and rotary-hoed to 4" depth.

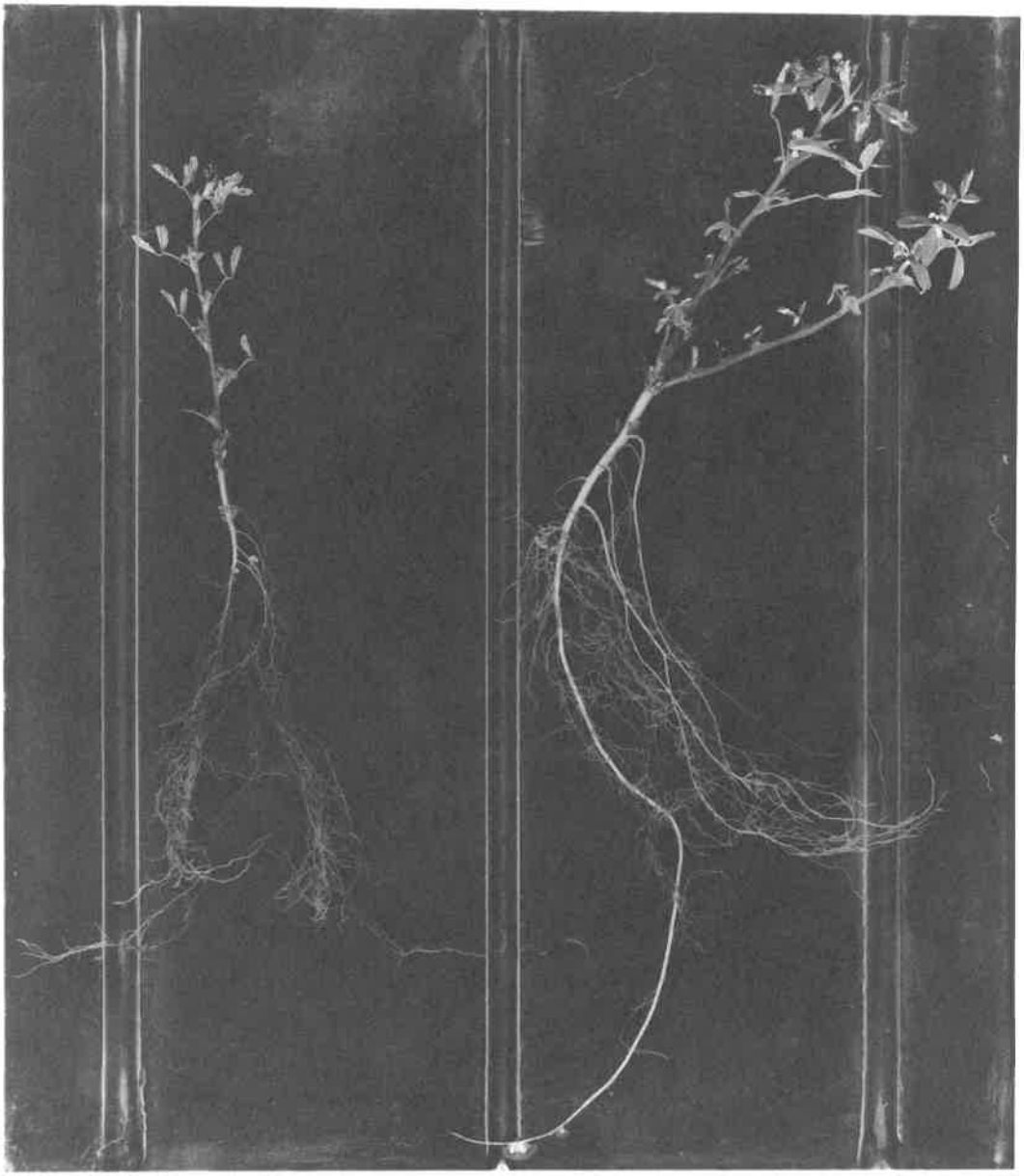


TABLE 40.

Effect of Liming on Root Development and Plant Yield.

Treatment	% of Plants with straight roots	% of Plants with forked roots	Yield of Tops (gm./plant) straight rooted	Yield of Tops (gm./plant) forked rooted	Yield of Roots (gm./plant) straight	Yield of Roots (gm./plant) forked
			Lucerne			
Drilled Lime	15.6	84.4	.130	.070	.137	.070
Broadcast Lime	73.9	26.1	.167	.078	.168	.074
			Subterranean Clover.			
Drilled Lime	76.2	23.8	.880	.490	.209	.134
Broadcast Lime	97.0	3.0	.876	.717	.177	.142

taproot development was strong and few brown roots were evident. Although less pronounced, a similar trend occurred in clover. Where roots of both species were forked, the yield of tops and roots was much less than where roots were straight.

Results of chemical analyses of herbage from Harvest IV are presented in Table 41. The level of calcium in the tops of both species was considerably higher where 1 ton of lime was broadcast than where 4 cwt. was drilled. No clover roots were analysed, but in lucerne, the level of calcium in roots was much lower than in tops, and was not affected by lime treatment. The phosphorus level was little affected by lime treatment, although in clover tops the level was somewhat

lower where lime was drilled. Manganese levels in both tops and roots of lucerne were much higher from plots where lime was drilled than from plots where it was broadcast.

TABLE 41.

Effect of lime treatment on calcium, phosphorus, and manganese levels in herbage.

Treatment	% Ca		% P		Mn (p.p.m.)	
	Tops	Roots	Tops	Roots	Tops	Roots
Sub.Clover						
Drilled lime	0.56	-	0.13	-	-	-
Broadcast lime	1.06	-	0.20	-	-	-
Lucerne.						
Drilled lime	1.05	0.34	0.24	0.17	86	54
Broadcast lime	1.79	0.33	0.25	0.19	32	30

The results of the pH determinations are presented in Table 42.

TABLE 42.

Effect of lime treatment on pH at various depths.

Sample depth (in.)	Drilled lime	Broadcast lime
0-2	pH 7.03	pH 7.30
2-4	5.90	6.56
4-6	5.15	5.70
6-8	5.13	5.40

A large drop in pH occurred below 2" depth in the rows of drilled lime. Below 4" and between the rows, the band of lime had no effect on pH. On the other hand,

pH levels were higher at all depths in the broadcast-lime plots, due to lime application.

5. Discussion of Results.

Sterilisation of Mt. Compass sand with steam and formaldehyde failed to improve nodulation of subterranean clover and lucerne, although large responses to similar sterilisation treatments have been obtained on certain other soils where legume nodulation problems have occurred (Cass Smith and Holland, 1958; Holland, 1962; Beggs, 1961, 1963, 1964; Parle, 1962). This lack of improvement in nodulation suggests that an antagonistic microbial population in the soil is unlikely to be one of the causes of poor nodulation of legumes in Mt. Compass sand. However, Harris (pers. comm.) has demonstrated that an organic toxin in Mt. Compass sand may cause a reduction in growth of subterranean clover, lucerne and ryegrass. It is conceivable that toxic material could be produced from plant remains in the sand, and, unaffected by soil sterilisation, still affect plant growth, and possibly nodulation. Further work on this aspect would seem necessary.

The marked response to raising temperature above winter level in growth room studies strongly suggests that at Mt. Compass low winter temperature may be an important factor affecting Rhizobium survival and nodulation. Only in the first three weeks following sowing in 1963 were air temperatures at the field trial site comparable with those prevailing in the growth room during

the low temperature trial (Fig. 13). For the next eight weeks the mean temperature was nearly 6°F below that in the low temperature trial, and did not rise again until early August. Therefore, the effect of low temperature on nodulation in the field may be greater than that obtained in growth room studies.

In the growth room, the time taken for seedlings to germinate and form nodules at low temperature (45°F night, 60°F day) was twice that taken at high temperature (60°F night, 75°F day). The delayed germination and seedling growth at low temperature may have had a marked effect on Rhizobium survival and multiplication, especially of R. trifolii, as lucerne took 3-4 days longer to form nodules than clover. The transplanting technique developed for lucerne in this study showed that at high temperature, improvement occurred in colonisation of the rhizosphere by R. meliloti, suggesting that at the lower temperature, Rhizobium survival and multiplication was reduced. The improvement in rhizosphere colonisation of lucerne at high temperature was reflected in improved secondary nodulation and nodule number per plant, but at pH 6.5 only.

The transplanting technique showed that at pH 5.0 and with a normal level of inoculum, very few of the 4,000 cells on each lucerne seed could be recovered from the rhizosphere 11 days after sowing, although when inoculum level or pH was raised, relatively greater

rhizosphere colonisation occurred. However, at pH 5.0 nearly all lucerne plants were unnodulated when harvested from the pots, whatever the temperature or inoculum level. Presumably some other factor, such as lack of suitable root hair sites, was limiting nodulation at this pH. It is also possible that at pH 5.0 rhizobia may be present in the rhizosphere, but in insufficient numbers to infect root hairs and form nodules.

In clover, the greatest response to high temperature occurred in normally inoculated plants, where a large improvement in percentage primary nodulation occurred, although nodule number increased significantly at both inoculum levels when temperature was raised. Hely and Williams (1964), in pot trials carried out in water baths over a range of temperatures, found that low root temperatures (45° and 54°F) markedly delayed initial nodulation of subterranean clover, and restricted nodule number. At 45°F, nodules did not form on the Mt. Barker variety for 22.5 days, but formed in 7 days at 81°F. These findings are similar to those obtained for subterranean clover in this experiment, where nodules did not form for 18 days at 45°F night, 60°F day, but formed in 9 days from sowing at 60°F night, 75°F day. Low temperatures in the field may well have contributed to the high proportion of unnodulated and secondary nodulated plants of both species which occurred where a normal level of inoculum was used on the seed. Hely and Williams (1964) found that

on the Southern Tablelands of New South Wales, prompt nodulation of subterranean clover did not occur, even in the presence of abundant inoculum, when establishment was delayed until winter. At Mt. Compass, it would seem desirable to sow legumes as soon as possible after autumn rains occur, i.e. late April or May, to take full advantage of the warmer temperatures so that rapid germination and nodulation is promoted. Late sowing, in June or July, is likely to be affected by low temperatures with delayed germination, poorer rhizobial survival and multiplication, and consequently greater chance of nodulation being delayed or absent. All the field trials at Mt. Compass were bordering on late sowings and were probably adversely affected by low temperature in June and July, especially lucerne.

In Field Trial II, 1963, as in previous trials, there were large nodulation responses to increasing the level of inoculum on the seed, and these were correlated with dry matter yields of the plants. Both the percentage of primary nodulated plants and the number of primary and secondary nodules on each plant were increased with increased inoculum. The relation between inoculum size and nodulation was not simple, but the number of primary nodules formed was approximately proportional to the log of the number of bacteria supplied. The greatest increase in percentage primary nodulation in both species occurred when the inoculum size was increased to 10 times normal.

Thornton (1929) working in soil, and Bhaduri (1951) working in water culture, both found that nodulation of legumes was related to the size of the rhizobial population in the rhizosphere. More recently Purchase and Nutman (1957) and Lim (1963) in laboratory studies, have defined the relationship more closely. Lim showed that root hair infection was limited by inoculum size in two distinct phases. Before nodulation began, infection was about doubled by doubling the number of virulent bacteria in the rhizosphere, but after nodules were formed, bacterial density had to be increased much more than twice to double the number of infections. However, as Nutman (1959b) points out, nodulation may be limited by factors other than bacterial density and does not necessarily bear a close relationship to bacterial numbers in the rhizosphere.

In Field Trial II, 1963, a definite relationship between inoculum size and nodule number has been established. This appears to be one of the first studies of the inoculum size-nodule number relationship made under field conditions. Hely (1964), in field experiments on the Southern Tablelands of New South Wales, reports of a consistent and significant increase in both survival and dry weight of subterranean clover plants when inoculum level was raised in three steps, from 1.0×10^2 to 1.0×10^5 bacteria per seed. However, he does not publish any data on nodule number per plant at the various inoculum

levels.

To obtain comparable nodulation, about 30 times more R. meliloti than R. trifolii were required on the seed. This bears out observations from previous trials of the poorer nodulation of lucerne in Mt. Compass sand where pH and calcium levels had been raised, and when approximately equivalent numbers of R. meliloti and R. trifolii had been used on respective seeds.

Drilled liming proved superior to broadcast liming in nodulation of both species. pH and calcium levels in the sand are unlikely to have been limiting in either treatment; the pH was 7.0 in the row at 0-1" depth where lime was drilled, and 6.8 at 0-4" depth where lime was broadcast. Results from previous trials (Section B) indicate that these levels are both optimum for nodulation. The difference in nodulation is most probably a reflection of the effect of liming on Rhizobium survival in the sand. Over 34% of the limestone was fine material ($<$ 211 micron) and the increased nodulation from the drill-row lime may have been an effect on rhizobial survival by this fraction, concentrated near the inoculated seed. R. meliloti was affected more than R. trifolii. To obtain comparable nodulation between the two liming treatments, 10 times as many R. meliloti were required in the broadcast-lime plots as in the drill rows, but only 5 times as many R. trifolii were needed.

The way in which the fine material could affect

Rhizobium survival is not clear. It is possible that one of the effects may be the provision of carbon dioxide in the sand, which is low in organic matter and in which microbiological activity is probably reduced by low winter temperatures. Low carbon dioxide levels have been shown to affect growth of nodule bacteria (Lowe and Evans, 1962) and nitrogen fixation (Mulder and Van Veen, 1961). Hely (1964), in large field trials on the Southern Tablelands of New South Wales, obtained significant responses in nodulation, seedling survival and dry weight of tops when fine lime was added in the drill row with inoculated, pelleted subterranean clover seed. He also suggested that the response was due to additional carbon dioxide in the soil.

Fine lime may alternatively protect the root nodule bacteria in some way, possibly against desiccation, to which they are particularly susceptible (Vincent, 1962a), although this should not be so important at Mt. Compass in winter. In Western Australia, the inability of *R. trifolii* to survive the summer in certain coarse-textured sands has been overcome by the addition of fine particle materials, mainly clays, with the inoculated seed (Marshall and Roberts, 1963; Marshall, Mulcahy and Chowdhury, 1963; Marshall, 1964). The fine fraction may even protect rhizobia against organic toxins shown to be present in Mt. Compass sand by Harris (pers. comm.)

The probability that the fine fraction of the lime in the drill row increased Rhizobium survival was supported by the results of the lime pelleting trials, both in pots and within the main field trial. In both cases large increases in percentage nodulation occurred with pelleting of lucerne seed even where both calcium and pH levels in the sand were almost certainly not limiting. In the field the response was greater in the broadcast-lime plots, as rhizobial survival was already improved by fine lime in the drill row plots.

Except in the initial pot trial no response to lime pelleting was obtained in subterranean clover. This is possibly due to the better survival of R. trifolii in the sand. Alternatively, seed coat inhibitors may have been present, and counteracted any Rhizobium response to lime pelleting. Thompson (1960, 1961), Bowen (1961), and Vincent et al. (1962) have all demonstrated that seed coats of various legumes contain substances which inhibit Rhizobium multiplication. This inhibition was found to be strong in subterranean clover but weak in lucerne.

No improvement in nodulation was obtained from gum arabic coating alone; in contrast, a significant depression in nodulation of both species was recorded at pH 6.5 following its use. Weak solutions of gum arabic are sometimes known to reduce inoculum survival and cause poor nodulation (Brockwell, 1963), and this effect may have occurred here.

That the function of lime in legume establishment at Mt. Compass is not solely the raising of pH and supplying of calcium for legume nodulation and nitrogen fixation was shown by the marked response in root development to a heavy application of lime. Short, brown, branched roots developed on plants where only 4 cwt. of lime was applied in the drill row, but where lime was broadcast at 1 ton per acre and rotary hoed to 4" depth plants developed long, straight, white taproots. Lucerne, a deep-rooted plant with a well-developed taproot, was affected by lime placement to a greater extent than subterranean clover, which is predominantly a surface feeder with branched roots.

In drilled lime plots, where nodulation was superior to that in broadcast-lime treatments, plant yield responses to increasing primary nodule numbers were low, as growth was limited primarily by poor root development. On the other hand root development in broadcast lime plots was satisfactory, yield was determined mainly by the amount of nodular tissue present on the roots, and a greater growth response to increasing primary nodule number occurred.

Root development had a considerable effect on summer survival of lucerne. When the field trial was inspected on April 24th, 1964, 11 months from sowing, only occasional plants in drilled lime plots had survived, whereas survival of plants in broadcast lime plots was

good, especially where heavy inoculum had been used. Failure of nodulated lucerne in previous trials to survive a summer drought was undoubtedly due to poor root development of the plants. Even with subterranean clover, second-year seedling establishment was superior in broadcast-lime plots. Although examination of all clover plots for nodules was not carried out, the difference was probably due to poorer seedling nodulation in the acid soil between the rows of lime. In broadcast-lime plots, all second-year clover seedlings which were examined were primary nodulated.

The adverse effects of acid soil conditions on legume root development have been observed by a number of previous workers (Watenpaugh, 1936; Pohlman, 1946; Schmehl, Peech and Bradfield, 1950, 1952; Fox and Lipps, 1955; Hourigan, Franklin, McLean and Bhumbla, 1961; Shoop, Brooks, Blaser and Thomas, 1961; Kehoe and Curnow, 1963), and the marked effects of lime in improving root development have been reported. In pot trials conducted by Watenpaugh (1936), Pohlman (1946), and Schmehl _et al_. (1952), a common finding was that liming the lower layers of the soil markedly affected root distribution of lucerne. When lucerne roots met an acid layer, normal taproot development ceased and further root growth was poor and in a lateral direction. When the lower soil layers were limed, normal taproot development occurred.

The findings of these workers fit in well with

the observed effects of lime placement on root development at Mt. Compass. Soil tests indicated that while broadcast lime had raised pH even to the depth of 8", drilled lime had no effect below 4" and acid conditions still prevailed between the rows.

The effects of liming on root development have been variously attributed to lowering toxic levels of hydrogen ions (Watenpaugh, 1936; Hourigan *et al*, 1961), manganese (Schmehl *et al*, 1950), or aluminium (Shoop *et al*. 1961; Kehoe and Curnow, 1963), in the soil. None of the responses reported are attributed solely to correction of a calcium deficiency, although Schmehl *et al*. (1952), using Ca^{45}, found that the rate of absorption of calcium by lucerne growing in nutrient solution was markedly reduced in the presence of aluminium ions, and, to a lesser degree by manganese and hydrogen ions. Vlamis (1953) verified the toxic effects of all three ions on root growth in his experiments with lettuce and barley in water culture. In water culture trials of the present study, growth of roots of both lucerne and subterranean clover was reduced at lower pH levels, especially at pH 4.0 and 4.5.

At Mt. Compass, where the sand is almost completely lacking in clay minerals and is highly leached, aluminium and manganese levels are unlikely to be high. The manganese content of the lucerne ($<$100 p.p.m.) although lowered when a heavier rate of lime was applied

to the sand, was not approaching toxic levels and is unlikely to have affected plant growth. Kipps (1947) found that in a savannah soil at Canberra, lucerne grew normally where plant manganese levels were below 200 p.p.m., while Vose and Jones (1963) found that high yields of white clover were associated with manganese levels of less than 100 p.p.m. On the other hand, the sand in drilled-lime plots was quite acid except in the immediate vicinity of the actual band of lime, and it seems possible that the high hydrogen ion concentration was reducing root development. Also, calcium levels in the tops of plants from drilled-lime plots were considerably lower than those from broadcast-lime plots. In view of the large responses obtained in root elongation to increasing the calcium level in previous water culture trials (Section A), it seems possible that low calcium as well as a high hydrogen ion concentration, was limiting root development in Mt. Compass sand.

IV GENERAL DISCUSSION.

Field experiments and pot trials have shown that a number of factors limit the nodulation and establishment of subterranean clover and lucerne in Mt. Compass sand. Both pH and calcium levels of the sand were low for optimal nodulation, and large responses in percentage nodulation and nodule number per plant were obtained when the levels were raised. In Field Trial I, 1963, comparable nodulation in either species could be achieved by adding calcium to the sand or raising pH; where both calcium and pH levels were raised nodulation increased further. In order to interpret these results, basic information on the effect of calcium and pH on growth and nodulation of the two species was obtained by means of water culture trials. Variation in calcium and pH levels of the nutrient solution caused marked growth responses in both subterranean clover and lucerne in these trials. Critical levels of 0.25 me Ca/l and pH 4.5 for clover and of 0.50 me Ca/l and pH 5.0 for lucerne were established for the conditions prevailing in the experiments. At or above these critical levels, an increase in either calcium or pH level produced near maximum yields of herbage. One of the main reasons for the effect of pH on growth appeared to be its influence on calcium uptake, which was depressed at the lower pH levels used (4.5 and 5.0). The interaction of calcium and pH on plant growth and calcium absorption is already known (Arnon *et al.*,

1942; Arnon and Johnson, 1942; Moser, 1942; Sutton and Hallsworth, 1958; Loneragan and Dowling, 1958; Jacobson et al., 1960; Waisel, 1962), but their effects on subterranean clover and lucerne have not been closely defined previously.

In water culture, root hair development was highly dependent on the calcium and pH levels of the nutrient solution. A large species difference occurred, lucerne being more sensitive to the lowering of both calcium and pH levels than subterranean clover. Although the effects of calcium and pH on root hair development has been noted in other plants (Cormack 1949, 1962), it has been little studied in legumes. This is surprising, in view of the importance of root hairs as paths of invasion of roots by rhizobia during nodulation, and of the known effects of calcium and pH on nodulation. In nutrient solution, highly significant responses in nodulation to calcium and pH treatments were obtained, with lucerne again being more severely affected by low calcium and pH levels. These responses were closely related to effects of calcium and pH on root hair length and number.

In Mt. Compass sand, root hair development was much less affected by variation in calcium and pH level than in nutrient solution, but again a large species difference occurred. In lucerne, both root hair length and density were considerably increased when calcium or pH levels were increased, but responses in clover were

small. Similar differences also occurred in nodulation of the two species following improvement of calcium and pH levels in the sand. Lucerne showed a greater response to both treatments than did subterranean clover.

The evidence from experiments in both water culture and Mt. Compass sand suggest that effects of calcium and pH on nodulation of lucerne and subterranean clover occur partly through effects on root hair development, and that these are probably associated with effects on calcium uptake by the plant. However, direct evidence of this is difficult to obtain, and further studies are required. A modification of the Fahraeus glass slide technique (Fahraeus, 1957) may allow direct observation of effects of calcium and pH on root hair development and on nodulation simultaneously.

In water culture, a higher pH level was required for nodulation than for growth of the host plant. This higher pH requirement for nodulation, which occurred in both species, has not been previously reported. There was little evidence of a higher requirement of calcium for nodulation than for growth, however. This is surprising in view of results of other workers (Albrecht, 1932; Loneragan and Dowling, 1958; Andrew and Norris, 1961), who all found that a higher level of calcium was required.

Although large responses to increased pH and calcium levels occurred in Mt. Compass sand, the most

important factor limiting nodulation of both species was the number of rhizobia on the seed and/or in the rhizosphere. Consequently, inoculation of seed, addition of fine lime in the row in contact with inoculated seed, and lime pelleting of normally inoculated seed (lucerne only) all resulted in significant increases in nodulation. Evidence has been presented to show that the effects of these three treatments (where calcium was non-limiting) was almost solely one of increasing Rhizobium number on the seed, in the case of inoculation; or increasing survival on the seed, and survival and multiplication in the sand or in the rhizosphere in the case of lime treatments. In addition, it is likely that nodulation responses which occurred when soil pH was raised or temperature was increased were also partly due to increased survival and multiplication of Rhizobium bacteria in the sand and/or rhizosphere.

A considerable difference in the natural occurrence of R. trifolii and R. meliloti in Mt. Compass sand and in the number of each species required for nodulation was apparent. In results obtained from the 1962 Field Trial, it was evident that native R. meliloti were almost completely absent while R. trifolii were present in low numbers. This difference is likely to be due to the greater acid tolerance of the latter species, a fact observed by Jensen (1942) in water culture. In Pot Trial III and Field Trial I (1963) little improvement in

nodulation occurred in clover when soil pH was raised from nearly 6.0 to near 7.0, but nodulation of lucerne was considerably better at the higher level. This difference could again be due to differences in acid tolerance of the two Rhizobium species, although effects of pH on root hair development may also have been involved.

In Field Trial II, 1963, where pH was unlikely to have been limiting, 30 times as many R. meliloti as R. trifolii were needed on each seed to obtain equivalent nodulation in clover and lucerne. The reason for this difference is not clear. Tests for presence of rhizobia in the rhizosphere indicated that at normal levels of inoculum, the ability of R. meliloti to survive and invade the rhizosphere of lucerne was poor, although it was improved by an increase in temperature. The greater improvement in nodulation of lucerne due to addition of fine lime in the drill row, or to lime pelleting of seed, compared with subterranean clover suggests that R. meliloti was more sensitive to some conditions prevailing in the sand than R. trifolii; perhaps to low carbon dioxide levels in the sand, or to organic toxins, factors already discussed in Section C.

Highly significant responses to increased inoculum level were obtained in both species, in the form of higher percentage nodulation, increased primary nodulation and higher numbers of primary and secondary nodules on each plant. In Field Trial II, 1963, and in

earlier trials, inoculum level and primary nodule number were closely related, although a greater increase in nodule number occurred in clover than in lucerne with equivalent increase in inoculum level.

A fairly precise measurement of effects of inoculum level and other treatments on nodulation was obtained by dividing nodulated plants into primary and secondary nodulated, and counting the individual nodules. By use of this technique, the importance of obtaining primary nodulation of seedling legumes at Mt. Compass became apparent, and in Field Trials I and II, 1963, close correlations were obtained between primary nodule number and dry matter production, even 14 weeks after sowing.

Results from growth room studies strongly suggest that nodulation of legumes at Mt. Compass may be limited by low winter temperatures. At the lower temperature used in this study, comparable with winter temperatures at Mt. Compass, both seedling emergence and nodulation took twice as long as at the higher temperature which was approximately equivalent to autumn or spring temperatures. Nodulation of both species was considerably reduced at the lower temperature. The effect of temperature on nodulation has received little attention from previous workers on legume nodulation. These findings indicate that it may be of considerable importance, especially where winter sowing is practised, and deserves

further study.

The marked effect of lime placement on root development in Field Trial II, 1963, left no doubt that the beneficial action of lime at Mt. Compass was not limited to improvement in nodulation. Good nodulation in both species could be obtained with an application of 4 cwt. of agricultural lime in the drill row, but this treatment did not allow normal root development of the seedlings, especially of lucerne, which was much more sensitive to lime placement than clover. Examination of the trial in 1964 showed that survival of nodulated lucerne in the sand depended largely on the development of a good root system in the first few months from sowing. Most plants in drill-row lime plots had died during the summer drought, probably due to their poor root development.

It is clear that limited application of lime in the drill row is inadequate for good root development of legumes in Mt. Compass sand. Greater amounts should be broadcast and cultivated in so that acidity is reduced throughout the top few inches of sand. It is likely that a lesser amount would be required for subterranean clover than for lucerne, in view of its greater tolerance of acid conditions for growth and nodulation, and of its surface-rooting habit.

An outstanding feature of the whole study has been the considerable difference in behaviour of the two

species in most experiments. Lucerne required higher pH and calcium levels for growth, root hair development and nodulation than subterranean clover, both in water culture and in Mt. Compass sand, while formation of nodules on lucerne occurred several days later than on clover. Nodulation and growth of lucerne was more affected by low temperature than that of clover. In addition, root development in lucerne was more affected by lime placement than in clover. The marked differences in behaviour of *R. trifolii* and *R. meliloti* have already been discussed. It is clear therefore that *Rhizobium meliloti* and *Medicago sativa* are more sensitive in a number of respects to factors affecting growth and nodulation than *Rhizobium trifolii* and *Trifolium subterraneum*.

The studies presented here have revealed in some detail the basis for the complex interplay of edaphic and climatic factors, plant and bacterial genotype and methods of management which determine the success of an attempt to introduce pasture legumes into the unfavourable soil environment at Mt. Compass. Further work on a number of aspects would be profitable but of special value would be a continued study of the factors affecting the survival of rhizobia in Mt. Compass sand or a similar soil.

In addition to their intrinsic interest the results obtained in this work should be of value in forming the basis of practical recommendations on the establishment of lucerne and subterranean clover on acid

sands both in this and other localities. At the same time it should be stressed that while modification of the soil environment would seem to be a useful method of extending the range of these species there could well be opportunities for the selection or breeding of plant and rhizobial strains which are better adapted to these soil conditions. Such an approach might avoid or at least minimise the expense inevitably involved in modifying the soil environment.

V

SUMMARY.

The failure of subterranean clover (*Trifolium subterraneum* L. var. Mt. Barker) and lucerne (*Medicago sativa* L. var. Hunter River) to nodulate and establish on a deep acid sand at Mt. Compass, South Australia, was investigated by means of field experiments, and pot trials carried out in glasshouse and growth room. In addition, studies were made of the effects of calcium and pH on growth and nodulation of these plants in water culture, and the information obtained used in interpretation of the results from the trials in Mt. Compass sand. In all experiments, comparison was drawn between the requirements of the two legumes for nodulation and growth.

In water culture critical levels of 0.25 me Ca/l and pH 4.5 for subterranean clover, and of 0.50 me Ca/l and pH 5.0 for lucerne were established, below which growth was inhibited and calcium deficiency symptoms appeared. At, or above, these critical levels almost maximum plant growth could be obtained by an increase in either calcium concentration or pH. Within the range where calcium and pH were interchangeable growth was affected by pH largely through effects on calcium uptake.

Length and density of root hairs were highly dependent on the calcium and pH level of the nutrient solution, and a marked species difference in response was evident. Root hair production in lucerne was much more sensitive to lower calcium and pH levels than subterranean

clover. Large nodulation responses to calcium and pH treatment were obtained and these were related to effects on root hair development. A higher pH level was required for nodulation than for growth of the host plant, but there was little evidence of a higher calcium requirement for nodulation.

Nodulation in Mt. Compass sand was limited by low soil calcium and pH, low Rhizobium numbers, and low winter temperatures. No effect of soil sterilisation was observed. Substantial responses in percentage nodulation and nodule number per plant were obtained following increase in pH and calcium levels, and these were related to root hair development of the hosts. It is suggested that effects of calcium and pH on nodulation occur partly through their influence on root hair development of the host plant.

The most important factor limiting nodulation of both species in Mt. Compass sand was Rhizobium number; and inoculation of seed, addition of fine lime with seed, or lime pelleting of seed (lucerne only) all resulted in a marked increase in nodulation by increasing Rhizobium number on seed, in soil, or in the rhizosphere. Part of the nodulation response to increased soil pH and temperature is also attributed to effects on Rhizobium number.

In the field, increases in inoculum level caused very large increases in percentage nodulation and nodule number per plant in both species. Inoculum level and

number of primary nodules per plant were closely related. Close correlations were also obtained between primary nodule number and dry matter production, and the importance of obtaining primary nodulation of seedling legumes at Mt. Compass was demonstrated.

In a growth room at a lower temperature (45°F night - 60°F day), seedling emergence and nodulation took twice as long as at a higher temperature (60°F night - 75°F day) and nodulation was considerably reduced. It is suggested that at Mt. Compass, nodulation is reduced by low winter temperatures.

Good nodulation of both species was obtained with 4 cwt. of agricultural lime concentrated in the drill row, but only when 1 ton was broadcast and rotary-hoed to 4" depth was root development normal. Survival of lucerne over summer depended largely on good root development. It is suggested that the extra lime reduced acidity in the mass of the sand and improved calcium uptake.

Rhizobium meliloti and Medicago sativa were both more sensitive to a number of the factors examined, which affected nodulation and growth, than R. trifolii and Trifolium subterraneum. R. meliloti was less tolerant of low pH and was absent in virgin sand (pH 5.0), whereas R. trifolii appeared to be present in small numbers. Even where pH was favorable, 30 times more R. meliloti than R. trifolii were needed on seed for equivalent nodulation in the two hosts. Rhizosphere colonisation by R. meliloti

was poor at a normal level of inoculum, when grown at 45°F night, 60°F day, but improved by raising temperature to 60°F night, 75°F day.

Lucerne required higher pH and calcium levels for plant growth, root hair development, and nodulation than subterranean clover, both in water culture and in Mt. Compass sand. Other differences noted were that formation of nodules on lucerne occurred several days later than on clover, and that in the field, lucerne needed more lime for optimum root development than subterranean clover.

VI ACKNOWLEDGEMENTS.

I wish to thank my supervisors, Mr. J.K. Powrie, Agronomy Department, and Dr. J.H. Warcup, Plant Pathology Department, Waite Agricultural Research Institute for the advice, guidance and constructive criticism they have freely given throughout this study.

Appreciation is also extended to Professor C.M. Donald, Mr. K.P. Barley, and other members of the Agronomy Department staff for their help and advice; to Messrs. S.R. Hockley, D.J. Williams and M.J. Gray for advice and assistance in experimental work; to Mr. D. Messant for advice and assistance concerning statistical analyses; to Miss Robyn Danks for assistance with preparation of figures; to Messrs. K.P. Phillips and B. Palk for the photography, and reproduction of plates and figures; and to Mrs. R. Dodman and Mrs. S. Edmonds for typing the draft and final manuscripts respectively.

This study was made possible by the award of a Commonwealth Scholarship by the Australian Government; this assistance is gratefully acknowledged.

Finally I wish to thank Lincoln College, University of Canterbury, New Zealand for generous financial assistance while on leave from the staff.

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APPENDIX 1.

Water Culture Trial I - Harvest I.

Treatment pH	Ca (me/l)	Subterranean Clover: Length of Taproot (cm/Plt.)	Subterranean Clover: Dry Weight (mg) Tops	Subterranean Clover: Dry Weight (mg) Roots	Subterranean Clover: Relative Total Yield	Lucerne: Length of Taproot (cm/Plt.)	Lucerne: Dry Weight (mg) Tops	Lucerne: Dry Weight (mg) Roots	Lucerne: Relative Total Yield
4.0	0.10	2.9	34.7	9.5	35.7	2.4	29.4	3.6	17.9
"	1.00	3.5	39.5	13.2	42.6	2.6	34.8	7.5	22.9
"	10.00	4.1	53.9	16.6	57.0	6.0	52.1	12.2	34.8
5.0	0.10	10.9	76.4	27.8	84.2	12.0	92.8	24.4	63.4
"	1.00	15.5	71.4	27.4	79.9	17.5	101.3	30.1	71.0
"	10.00	17.8	65.6	21.6	70.5	20.0	110.6	23.9	74.0
6.0	0.10	15.9	89.3	34.4	100.0	17.4	152.7	32.1	100.0
"	1.00	19.1	78.9	29.5	87.5	24.1	143.0	32.9	95.2
"	10.00	19.2	63.9	24.2	75.8	25.9	116.3	26.6	77.3

All figures are the mean of 4 replicates.
Dry weights are the total from 3 plants.

APPENDIX 1 (cont.)

Log Data.

Treatment		Subterranean Clover			Lucerne		
pH	Ca (me/l)	Length of Taproot	Dry Weight Tops	Dry Weight Roots	Length of Taproot	Dry Weight Tops	Dry Weight Roots
4.0	0.10	0.46	1.54	0.98	0.37	1.47	0.55
"	1.00	0.54	1.59	1.11	0.41	1.52	0.86
"	10.00	0.61	1.68	1.16	0.77	1.71	1.07
5.0	0.10	1.03	1.88	1.43	1.07	1.92	1.34
"	1.00	1.18	1.84	1.42	1.20	1.99	1.41
"	10.00	1.25	1.81	1.33	1.28	2.02	1.33
6.0	0.10	1.20	1.95	1.54	1.24	2.18	1.49
"	1.00	1.28	1.89	1.45	1.38	2.15	1.52
"	10.00	1.28	1.80	1.34	1.41	2.06	1.42
Ca, pH L.S.D.	5%	0.05	0.07	0.09	0.09	0.10	0.12
	1%	0.07	0.10	0.12	0.12	0.13	0.16
Ca x pH L.S.D.	5%	0.09	0.12	0.15	0.15	0.16	0.21
	1%	0.13	0.17	0.20	0.21	0.22	0.28

APPENDIX 2.

Water Culture Trial II - Harvest I.

Treatment pH	Ca (me/l)	Subterranean Clover: Length of Taproot (cm/plt.)	Subterranean Clover: Dry Weight (mg) Tops	Subterranean Clover: Dry Weight (mg) Roots	Subterranean Clover: Relative Total Yield	Lucerne: Length of Taproot (cm/plt.)	Lucerne: Dry Weight (mg) Tops	Lucerne: Dry Weight (mg) Roots	Lucerne: Relative Total Yield
4.5	0.05	3.1	28.3	5.4	14.6	7.0	32.3	7.3	21.4
"	0.50	6.1	92.7	34.4	54.9	9.3	84.2	28.0	60.7
"	5.00	12.2	135.5	56.0	82.7	18.2	86.9	41.4	69.4
5.0	0.05	6.5	55.0	11.1	28.5	9.0	35.0	7.2	22.8
"	0.50	8.9	143.2	54.2	85.2	16.5	104.5	34.2	75.0
"	5.00	12.4	154.9	62.0	93.7	24.2	106.2	44.3	81.4
6.0	0.05	9.2	86.3	18.9	45.4	11.4	45.3	11.6	30.8
"	0.50	14.4	152.0	58.7	91.0	26.3	113.3	43.1	84.6
"	5.00	18.8	167.3	64.3	100.0	32.8	132.4	52.5	100.0

All figures are the mean of 4 replicates.
Plant weights are the total from 3 plants.

APPENDIX 2 (cont.)

Log Data.

Treatment pH	Ca (me/l)	Subterranean Clover Length of Taproot	Subterranean Clover Dry Weight Tops	Subterranean Clover Dry Weight Roots	Lucerne Length of Taproot	Lucerne Dry Weight Tops	Lucerne Dry Weight Roots
4.5	0.05	0.49	1.44	0.72	0.84	1.51	0.86
"	0.50	0.78	1.97	1.53	0.96	1.92	1.45
"	5.00	1.08	2.13	1.75	1.26	1.94	1.62
5.0	0.05	0.81	1.73	1.03	0.94	1.53	0.85
"	0.50	0.95	2.15	1.73	1.22	2.01	1.53
"	5.00	1.09	2.19	1.79	1.38	2.01	1.64
6.0	0.05	0.96	1.93	1.27	1.05	1.64	1.06
"	0.50	1.15	2.18	1.76	1.42	2.05	1.62
"	5.00	1.27	2.22	1.80	1.51	2.12	1.71
Ca, pH L.S.D.	5%	0.05	0.06	0.07	0.06	0.07	0.07
	1%	0.07	0.08	0.10	0.08	0.10	0.10
Ca x pH L.S.D.	5%	0.09	0.10	0.12	0.10	0.13	0.12
	1%	0.12	0.13	0.17	0.13	0.18	0.17

APPENDIX 3.

Root Hair Development - Water Culture.

Treatment			Subterranean Clover			Lucerne		
pH	Ca (me/l)		Length of taproot with hairs (mm)	Mean length score	Mean density score	Length of taproot with hairs (mm)	Mean length score	Mean density score
5.0	0.10		1.0	0.50	0.50	0	0	0
"	0.50		2.7	1.00	1.44	0	0	0
"	5.00		4.3	1.64	1.92	0.9	1.63	2.87
6.0	0.10		9.5	1.55	1.68	2.7	1.70	1.43
"	0.50		13.0	1.74	1.97	4.7	2.39	1.74
"	5.00		13.3	1.83	1.88	4.0	2.69	2.47
Ca	L.S.D.	5%	1.8	0.31	0.35	2.5	0.67	0.47
		1%	2.4	0.43	0.48	3.7	1.02	0.71
pH	L.S.D.	5%	1.4	0.25	0.28	-	-	-
		1%	2.0	0.35	0.39	-	-	-
Ca x pH	L.S.D.	5%	2.5	0.43	0.49	-	-	-
		1%	3.5	0.60	0.68	-	-	-

All figures are the mean of 4 replicates, 6 plants/replicate.

APPENDIX 4.

Nodulation - Water Culture (log data).

Treatment			Nodules/Nodulated Plant	
pH	Ca (me/l)		Subterranean Clover	Lucerne
4.5	0.10		0	0
	0.25		0	0
	0.50		0	0
	1.00		0	0
	5.00		0	0
5.0	0.10		0.33	0
	0.25		0.48	0
	0.50		0.66	0
	1.00		1.04	0
	5.00		2.00	0
6.0	0.10		1.74	0.47
	0.25		1.95	1.11
	0.50		2.05	1.55
	1.00		2.07	1.64
	5.00		2.16	1.90
Ca	L.S.D.	5%	0.25	0.50
		1%	0.34	0.70
pH	L.S.D.	5%	0.16	-
		1%	0.21	-
Ca x pH	L.S.D.	5%	0.35	-
		1%	0.48	-

All figures are the mean of 4 replicates,
6 plants/replicate.

APPENDIX 5.

Vegetation - Mt. Compass.

Trees, shrubs.

Acacia verticillata
A. myrtifolia
Banksia marginata
B. ornata
Boronia filifolia
Conospermum patens
Casuarina paludosa
Dillwynia floribunda
Eucalyptus baxteri
Hibbertia virgata
H. sericea
Isopogon ceratophyllus
Leucopogon virgatus
Leptospermum myrsinoides
Pimelia humilis
Platylobium obtusangulum
Xanthorrhoea semiplana

Herbs.

Centrolepis aristata
C. strigosa
Crassula pedicellosa
C. sieberiana
Cicenda filiformis
Goodenia geniculata
Helichrysum scorpioides
H. obtusifolium
H. blandowskianum
Hydrocotyle callicarpa
Hypolaena fastigiata
Juncus bufonius
Kennedya prostrata
Lagenophora stipitata
Laxmannia sessiliflora
Luzula campestris
Microseris scapigera
Millotia tenuifolia
Opercularia turpis
Patersonia glauca
Podosperma angustifolia
Pteridium aquilinum
Rutidosis multiflora
Scirpus antarticus
Senecio quadridentatus
Stuartina muelleri
Viola sieberiana
Wahlenbergia gracilenta
Zaluzianskia divaricata

Exotics.

Aira caryophylla
Briza maxima
Cerastium viscosum
Erodium botrys
Leontodon leysseri
Poa annua
Rumex acetocella
Trifolium arvense
T. glomeratua
Vulpia myuros

APPENDIX 6.

Mt. Compass Field Trial 1962.

Harvest I - Subterranean Clover.

Treatment		% Nodulation	Nodules/Plant Primary	Nodules/Plant Secondary
pH 5.0	Ca_0 In_1	54.7	0.9	1.5
	" In_2	100.0	3.1	3.5
	Ca_1 In_1	72.7	1.0	2.6
	Ca_2 In_1	64.6	0.4	2.7
	" In_2	100.0	3.5	4.9
	Ca_3 In_1	66.7	0.8	3.5
	Ca_4 In_1	67.5	0.9	3.0
	" In_2	100.0	4.0	7.1
pH 6.0	Ca_0 In_1	89.2	1.1	2.1
	" In_2	100.0	4.3	5.3
	Ca_1 In_1	74.6	1.2	2.6
	Ca_2 In_1	75.8	2.2	3.4
	" In_2	97.9	5.8	3.5
	Ca_3 In_1	75.8	1.3	3.5
	Ca_4 In_1	83.7	1.3	4.0
	" In_2	97.7	4.5	6.4
	" In_0	62.8	0.5	2.8
pH 6.8	Ca_2 In_2	100.0	4.3	6.7
	Ca_4 In_2	100.0	3.9	4.5
Significance of differences.				
Inoculum		1%	1%	1%
Calcium		n.s.	n.s.	n.s.
pH		5%	5%	n.s.
In x pH		5%	n.s.	n.s.
In x Ca		n.s.	n.s.	n.s.
Ca x pH		n.s.	n.s.	n.s.

All figures are the mean of 4 replicates
12 plants/replicate.

APPENDIX 6.

Mt. Compass Field Trial 1962.

Harvest I - Lucerne.

Treatment		% Nodulation	Nodules/Plant Primary	Nodules/Plant Secondary
pH 5.0	Ca_0 In_1	2.1	1.0	0
	" In_2	4.2	1.0	0
	Ca_1 In_1	2.1	1.0	0
	Ca_2 In_1	0	-	-
	" In_2	10.4	0.9	0.4
	Ca_3 In_1	0	-	-
	Ca_4 In_1	0	-	-
	" In_2	18.3	1.1	0.4
pH 6.0	Ca_0 In_1	8.3	0.5	0.7
	" In_2	33.4	0.8	0.6
	Ca_1 In_1	6.4	0.7	0.7
	Ca_2 In_1	6.1	0.8	0.5
	" In_2	20.8	0.9	0.7
	Ca_3 In_1	8.8	0.5	0.5
	Ca_4 In_1	6.8	1.0	0.3
	" In_2	47.9	0.7	1.0
	" In_0	0	-	-
pH 6.8	Ca_2 In_2	65.9	1.3	0.6
	Ca_4 In_2	66.7	1.4	0.5
Significance of differences.				
Inoculum		1%	1%	1%
Calcium		n.s.	n.s.	n.s.
pH		1%	5%	1%
In x pH		n.s.	n.s.	n.s.
In x Ca		n.s.	n.s.	n.s.
Ca x pH		n.s.	n.s.	n.s.

APPENDIX 7.

Mt. Compass Field Trial 1962.

Harvest II - Subterranean Clover.

Treatment			%Nodulation	Nods./Plant		Dry wgt.tops (mg/plant)	% Ca
				Prim.	Sec.		
pH 5.0	Ca_0	In_1	92.6	1.9	2.4	51	-
	"	In_2	100.0	6.2	5.6	68	1.02
	Ca_1	In_1	98.8	2.8	2.9	57	-
	Ca_2	In_1	94.5	2.5	3.3	51	-
	"	In_2	100.0	7.3	6.1	78	1.36
	Ca_3	In_1	97.5	2.2	3.0	65	-
	Ca_4	In_1	100.0	2.5	3.0	43	-
	"	In_2	100.0	4.5	8.2	56	1.73
pH 6.0	Ca_0	In_1	100.0	3.3	3.2	60	-
	"	In_2	100.0	5.8	7.4	71	0.91
	Ca_1	In_1	99.0	3.3	3.5	56	-
	Ca_2	In_1	100.0	4.4	4.6	51	-
	"	In_2	98.8	6.9	7.6	66	1.24
	Ca_3	In_1	96.3	3.4	5.1	51	-
	Ca_4	In_1	100.0	3.3	4.8	51	-
	"	In_2	100.0	6.9	8.0	68	1.58
	"	In_0	98.7	2.6	6.4	50	-
pH 6.8	Ca_2	In_2	100.0	7.0	6.9	69	1.51
	Ca_4	In_2	100.0	5.9	7.4	56	1.77
Significance of differences.							
Inoculum			-	1%	1%	-	-
Calcium			-	n.s.	n.s.	-	-
pH			-	1%	n.s.	-	-
In x pH			-	n.s.	n.s.	-	-
In x Ca			-	n.s.	n.s.	-	-
Ca x pH			-	n.s.	n.s.	-	-

All figures are the mean of 4 replicates,
20 plants/replicate. (Reps. bulked for Ca analyses)

APPENDIX 7.

Mt. Compass Field Trial 1962.

Harvest II - Lucerne.

Treatment			%Nodulation	Nods./Plant Prim.	Nods./Plant Sec.	Dry wgt.tops (mg/plant) Nod.	Dry wgt.tops (mg/plant) Non-nod.	% Ca
pH 5.0	Ca_0	In_1	1.4	0	1.0	-	8	-
	"	In_2	21.1	1.4	1.2	20	10	1.45
	Ca_1	In_1	6.7	0.9	0.7	-	10	-
	Ca_2	In_1	11.1	1.4	0.6	-	10	-
	"	In_2	31.0	1.5	0.6	10	8	1.89
	Ca_3	In_1	7.9	2.7	0.3	-	9	-
	Ca_4	In_1	8.7	0.6	0.9	-	12	-
	"	In_2	26.1	2.4	0.6	20	9	2.34
pH 6.0	Ca_0	In_1	25.4	1.2	0.5	18	9	-
	"	In_2	53.3	2.0	0.7	13	14	0.91
	Ca_1	In_1	25.8	1.7	0.8	16	8	-
	Ca_2	In_1	21.7	1.6	2.1	14	10	-
	"	In_2	51.7	1.5	0.8	10	7	1.50
	Ca_3	In_1	21.5	0.7	1.2	9	7	-
	Ca_4	In_1	21.2	0.9	1.3	17	11	-
	"	In_2	69.5	1.7	1.1	17	7	2.10
	"	In_0	3.1	0.4	1.7	14	12	-
pH 6.8	Ca_2	In_2	73.6	1.5	1.1	14	8	2.08
	Ca_4	In_2	89.5	1.7	1.1	13	8	2.20
Significance of differences.								
Inoculum			1%	1%	-	-	-	-
Calcium			n.s.	n.s.	-	-	-	-
pH			1%	n.s.	-	-	-	-
In x pH			n.s.	n.s.	-	-	-	-
In x Ca			n.s.	n.s.	-	-	-	-
Ca x pH			n.s.	5%	-	-	-	-

All figures are the mean of 4 replicates,
20 plants/replicate. (Reps. bulked for Ca analyses.)

APPENDIX 8.

Pot Trial I - Mt. Compass Sand.

Treatment	% Nodulation			Nodules/Plant			Dry Wgt.Tops(mg/plt.)		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.	Sec.	Non-nod.
				Subterranean Clover.					
pH 5.0 Ca_0 In_1	26.6	40.6	32.8	6.5	1.5	5.8	41	24	21
" In_2	98.5	1.5	0	7.8	1.4	-	45	-	-
Ca_1 In_1	9.8	31.1	59.1	4.9	0.2	4.1	28	26	22
" In_2	92.6	3.0	4.4	7.7	1.1	-	43	-	-
Ca_2 In_1	33.2	36.5	30.3	4.2	0.4	5.9	35	27	23
" In_2	94.5	5.5	0	7.2	1.4	-	44	-	-
pH 6.0 Ca_0 In_1	29.0	71.0	0	6.8	13.0	25.7	46	26	-
" In_2	98.6	1.4	0	9.2	5.6	-	54	-	-
Ca_1 In_1	37.2	62.8	0	5.8	12.3	19.8	42	29	-
" In_2	100.0	0	0	8.9	11.0	-	48	-	-
Ca_2 In_1	30.6	69.4	0	6.2	14.3	26.3	45	27	-
" In_2	100.0	0	0	7.7	11.1	-	49	-	-
Significance of differences.									
Inoculum	1%	1%	-	1%	1%	1%	1%	1%	-
Calcium	n.s.	n.s.	-	1%	n.s.	n.s.	n.s.	n.s.	-
pH	n.s.	5%	-	5%	1%	1%	5%	5%	-

Mean of 6 replicates, 12 plants/replicate.

APPENDIX 8 (cont.)

Treatment	% Nodulation			Nodules/Plant			Dry Wgt.Tops(mg/plt.)		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.	Sec.	Non-nod.
				Lucerne.					
pH 5.0 Ca_0 In_1	7.9	0	92.1	-	-	-	-	-	9.3
" In_2	36.1	2.8	61.1	4.1	0	-	18	-	9.8
Ca_1 In_1	1.4	0	98.6	-	-	-	-	-	10.2
" In_2	23.6	9.7	66.7	4.7	0	-	20	-	11.3
Ca_2 In_1	2.8	0	97.2	-	-	-	-	-	13.5
" In_2	26.4	4.2	69.4	3.7	0.3	-	17	-	12.7
pH 6.0 Ca_0 In_1	36.1	38.9	25.0	6.1	1.6	3.8	21	14	12
" In_2	75.0	22.2	2.8	6.1	3.5	7.6	22	12	-
Ca_1 In_1	23.0	27.5	49.5	5.5	0.8	4.8	19	15	11
" In_2	57.0	33.3	9.7	5.2	4.6	7.0	23	14	-
Ca_2 In_1	33.3	37.5	29.2	4.8	2.1	5.2	24	16	14
" In_2	69.6	26.4	4.0	5.2	4.7	9.5	22	15	-
Significance of differences.									
Inoculum	1%	n.s.	-	n.s.	1%	1%	n.s.	n.s.	-
Calcium	n.s.	n.s.	-	5%	n.s.	5%	n.s.	n.s.	-
pH	1%	1%	-	1%	1%	1%	1%	1%	-

Mean of 6 replicates, 12 plants/replicate.

APPENDIX 8.

Pot Trial I, Mt. Compass Sand.

Chemical Analyses of Tops.*

Treatment		% Ca		% N			% P	
		P.N.P.	N.N.P.	P.N.P.	S.N.P.	N.N.P.	P.N.P.	N.N.P.
				Subterranean Clover.				
pH 5.0	Ca_0 In_1	0.39	0.56	5.18	3.00	2.47	1.13	1.70
	Ca_1 In_1	0.79	0.97	5.50	3.19	2.87	1.14	1.53
	Ca_2 In_1	0.85	1.18	4.54	2.83	2.84	0.90	1.26
pH 6.0	Ca_0 In_1	0.38	-	5.09	3.50	-	1.20	-
	Ca_1 In_1	0.73	-	3.87	3.42	-	0.90	-
	Ca_2 In_1	0.93	-	4.69	3.74	-	0.76	-
				Lucerne.				
pH 5.0	Ca_0 In_2	0.38	0.48	5.14	-	2.94	1.30	1.72
	Ca_1 In_2	0.70	0.84	4.12	-	2.55	0.88	1.24
	Ca_2 In_2	0.83	0.96	3.57	-	2.70	0.82	1.00
pH 6.0	Ca_0 In_1	0.37	0.43	5.16	2.87	2.58	0.94	1.13
	Ca_1 In_1	0.72	0.80	5.19	3.28	2.81	0.88	0.80
	Ca_2 In_1	0.86	0.93	5.17	3.15	2.31	0.67	0.62

* Replicates bulked before analysis.

P.N.P. = Primary nodulated plants. S.N.P. = Secondary nodulated plants.
N.N.P. = Non-nodulated plants.

APPENDIX 9.

Pot Trial II - Mt. Compass Sand.

Treatment	% Nodulation			Nodules/Plant			Dry Wgt.Tops(mg/plt.)		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.Nod. Plts.	Sec.Nod. Plts.	Non-nod. Plts.
				Subterranean Clover.					
pH 5.0									
Ca_0 C.	41.6 (0.70)*	37.5 (0.65)*	20.9	3.4	0.4	2.3	18.1	16.9	16.8
" G.A.	45.8 (0.74)	25.0 (0.52)	29.2	3.5	0.2	1.7	19.2	16.2	15.7
" L.P.	50.0 (0.78)	37.5 (0.65)	12.5	3.6	1.3	1.9	20.5	17.7	14.6
Ca_1 C.	58.4 (0.86)	41.6 (0.70)	0	4.0	2.8	5.6	20.1	17.4	-
" G.A.	54.1 (0.82)	41.7 (0.70)	4.2	3.4	3.5	6.0	20.4	18.6	-
" L.P.	50.0 (0.78)	45.8 (0.74)	4.2	3.2	6.7	9.9	20.1	17.5	-
pH 6.5									
Ca_0 C.	91.7 (1.31)	8.3 (0.21)	0	5.2	4.4	15.5	23.5	20.8	-
" G.A.	62.5 (0.91)	37.5 (0.65)	0	3.9	5.3	12.7	23.8	20.6	-
" L.P.	75.9 (1.05)	24.1 (0.51)	0	4.0	7.9	11.9	23.7	19.6	-
Ca_1 C.	87.5 (1.20)	12.5 (0.36)	0	5.5	5.7	10.8	22.7	18.9	-
" G.A.	91.7 (1.31)	8.3 (0.21)	0	3.6	7.9	11.0	22.4	14.5	-
L.P.	66.7 (0.95)	33.3 (0.61)	0	2.8	9.3	14.3	23.1	20.8	-
Pelleting L.S.D. 5%	(0.17)	(0.18)	-	0.6	1.7	3.2	-	-	-
1%	(0.23)	(0.25)	-	0.9	2.4	4.7	-	-	-
Calcium L.S.D. 5%	(0.19)	(0.21)	-	0.7	1.9	3.7	-	-	-
1%	(0.27)	(0.29)	-	1.0	2.7	5.4	-	-	-
Pelleting x Ca L.S.D. 5%	(0.33)	(0.36)	-	1.3	3.4	6.5	-	-	-
1%	(0.47)	(0.51)	-	1.8	4.7	9.3	-	-	-

APPENDIX 9 (cont.)

Treatment	% Nodulation						Nodules/Plant			Dry Wgt. Tops (mg/plt.)		
	Prim.		Sec.		Non-nod.		Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.Nod. Plts.	Sec.Nod. Plts.	Non-nod. Plts.
					Lucerne.							
pH 5.0												
Ca_0 C.	0	(0)*	0	(0)*	100.0	(1.47)*	-	-	-	-	-	10.1
" G.A.	4.2	(0.15)	0	(0)	95.8	(1.37)	-	-	-	-	-	8.6
" L.P.	75.0	(1.04)	0	(0)	25.0	(0.52)	3.4	0.4	-	11.9	-	10.6
Ca_1 C.	16.1	(0.41)	0	(0)	83.9	(1.15)	3.8	0	-	-	-	9.6
" G.A.	8.3	(0.29)	0	(0)	91.7	(1.26)	2.5	0	-	-	-	10.1
" L.P.	75.7	(1.05)	12.5	(0.27)	11.8	(0.35)	2.8	0.2	-	11.8	-	9.5
pH 6.5												
Ca_0 C.	56.2	(0.84)	25.0	(0.52)	18.8	(0.43)	3.0	1.6	4.6	13.7	12.6	-
" G.A.	29.2	(0.57)	33.3	(0.58)	37.5	(0.62)	3.3	1.0	2.2	12.4	10.3	-
" L.P.	73.8	(1.03)	21.6	(0.48)	4.6	(0.16)	2.8	3.7	6.7	13.9	12.5	-
Ca_1 C.	58.4	(0.86)	33.3	(0.61)	8.3	(0.29)	2.8	2.9	6.9	13.4	10.0	-
" G.A.	50.0	(0.78)	29.2	(0.56)	20.8	(0.47)	2.5	0.7	4.9	12.7	10.9	-
" L.P.	86.8	(1.19)	13.2	(0.36)	0	(0)	3.0	3.4	5.5	14.5	11.1	-
Pelleting												
L.S.D. 5%		(0.13)		(0.28)		(0.22)	0.9	1.2	2.0	-	-	-
1%		(0.19)		(0.41)		(0.32)	1.3	1.7	3.1	-	-	-
Calcium												
L.S.D. 5%		(0.15)		(0.32)		(0.26)	1.0	1.3	2.3	-	-	-
1%		(0.21)		(0.48)		(0.37)	1.5	2.0	3.6	-	-	-
Pelleting x Ca												
L.S.D. 5%		(0.26)		(0.55)		(0.44)	1.8	2.3	4.0	-	-	-
1%		(0.37)		(0.84)		(0.64)	2.7	3.4	6.3	-	-	-

* Arcsin transformation. Mean of 2 replicates, 12 plants/replicate.
C. = Control. G.A. = Gum Arabic. L.P. = Lime Pellet.

APPENDIX 9.

Pot Trial II - Mt. Compass Sand.

Percentage Calcium in Tops. *

Treatment			Subterranean Clover	Lucerne
pH 5.0	Ca_0	C.	0.63	0.60NN
		G.A.	0.63	0.65NN
		L.P.	0.63	0.55
	Ca_1	C.	1.00	0.93NN
		G.A.	0.92	0.88NN
		L.P.	1.01	0.86
pH 6.0	Ca_0	C.	0.53	0.53
		G.A.	0.49	0.53
		L.P.	0.53	0.55
	Ca_1	C.	0.92	0.79
		G.A.	0.94	0.71
		L.P.	0.79	0.79
Pelleting L.S.D.		5%	0.21	0.18
		1%	0.49	0.40
Calcium L.S.D.		5%	0.17	0.14
		1%	0.40	0.33
Pelleting x Ca L.S.D.		5%	0.30	0.25
		1%	0.69	0.57

C. = Control. G.A. = Gum Arabic. L.P. = Lime Pellet

* Calcium figures are all from primary nodulated plants except where marked NN (non-nodulated plants).

APPENDIX 10.

Pot Trial III - Mt. Compass Sand.

Effect of pH on Nodulation.

Treatment	Subterranean Clover			Lucerne		
	% Nodulation			% Nodulation		
	Prim.	Sec.	Non-nod.	Prim.	Sec.	Non-nod.
pH 4.90						
x 1 In	44.4 (0.72)*	44.4	11.2	0 (0)*	0	100.0
x 1000 In	100.0 (1.47)	0	0	2.8 (0.13)	2.8	95.5
pH 6.15						
x 1 In	72.2 (1.01)	25.0	2.8	12.3 (0.27)	11.7	76.0
x 1000 In	100.0 (1.47)	0	0	75.0 (1.04)	25.0	0
pH 6.60						
x 1 In	75.0 (1.04)	22.2	2.8	43.7 (0.71)	11.1	45.2
x 1000 In	100.0 (1.47)	0	0	92.3 (1.33)	5.1	2.6
L.S.D. 5%	(0.15)	-	-	(0.16)	-	-
1%	(0.20)	-	-	(0.21)	-	-

* Arcsin transformation.

All figures are the mean of 3 replicates, 12 plants/replicate.

APPENDIX 11.

Mt. Compass Field Trial I, 1963.

Harvest I.

Treatment	% Nodulation			Nodules/Plant			Dry Wgt. Tops (mg/plt.)
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.Nod. Plts.
			Subterranean Clover.				
pH 5.35							
Ca_0 In_1	35.6 (0.63)*	20.2 (0.46)*	44.2 (0.72)*	1.5	0.8	2.3	8.6 (0.98)+
" In_2	100.0 (1.47)	0 (0)	0 (0)	4.2	2.6	-	10.5 (1.06)
Ca_1 In_1	48.4 (0.76)	39.1 (0.66)	12.5 (0.31)	2.4	1.7	3.0	7.8 (0.94)
" In_2	90.4 (1.33)	9.6 (0.17)	0 (0)	4.4	6.9	-	10.1 (1.04)
pH 6.30							
Ca_0 In_1	44.1 (0.72)	37.4 (0.65)	18.5 (0.38)	1.7	1.4	2.4	8.2 (0.96)
" In_2	97.9 (1.42)	2.1 (0.08)	0 (0)	4.6	4.6	-	10.7 (1.07)
Ca_1 In_1	53.7 (0.82)	40.5 (0.68)	5.8 (0.21)	2.4	4.2	3.9	9.2 (1.01)
" In_2	98.2 (1.42)	1.8 (0.07)	0 (0)	5.5	8.6	-	9.8 (1.03)
pH 7.00							
Ca_0 In_1	52.6 (0.81)	38.2 (0.66)	9.2 (0.26)	1.6	2.2	3.8	9.8 (1.03)
" In_2	100.0 (1.47)	0 (0)	0 (0)	4.7	5.1	-	11.5 (1.09)
Ca_1 In_1	63.5 (0.93)	36.5 (0.62)	0 (0)	2.9	4.7	5.8	8.9 (0.99)
" In_2	98.1 (1.42)	1.9 (0.08)	0 (0)	5.7	8.2	-	10.3 (1.05)
Ca, Inoc.							
L.S.D. 5%	(0.16)	(0.17)	(0.18)	0.5	1.2	1.2	(0.04)
1%	(0.22)	(0.23)	(0.25)	0.7	1.6	1.7	(0.06)
pH							
L.S.D. 5%	(0.14	(0.15)	(0.15)	0.4	1.0	1.0	(0.04)
1%	(0.19)	(0.20)	(0.22)	0.6	1.4	1.4	(0.05)
Ca, In x pH							
L.S.D. 5%	(0.28)	(0.29)	(0.31)	0.8	2.1	2.1	(0.07)
1%	(0.39)	(0.40)	(0.43)	1.1	2.8	2.9	(0.10)

APPENDIX 11 (cont.)

Treatment	% Nodulation			Nodules/Plant			Dry Wgt.Tops (mg/plt.)	
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.Nod. Plts.	Non-nod. Plts.
				Lucerne.				
pH 5.35								
Ca_0 In_1	0	0	100.0 (1.47)*	-	-	-	-	1.8 (0.44)+
" In_2	6.1	0	93.9 (1.34)	-	-	-	-	1.7 (0.44)
Ca_1 In_1	4.6	0	95.4 (1.36)	-	-	-	-	2.0 (0.47)
" In_2	56.5	2.1	41.4 (0.69)	2.1	0.1	-	2.5	- -
pH 6.30								
Ca_0 In_1	0	0	100.0 (1.47)	-	-	-	-	1.9 (0.46)
" In_2	46.2	0	53.8 (0.82)	1.6	0.9	-	2.5	1.9 (0.46)
Ca_1 In_1	4.2	0	95.8 (1.39)	-	-	-	-	2.0 (0.47)
" In_2	62.2	7.7	30.1 (0.51)	2.7	0.2	-	2.7	- -
pH 7.00								
Ca_0 In_1	1.6	2.1	96.3 (1.38)	-	-	-	-	2.1 (0.52)
" In_2	44.1	7.5	48.4 (0.77)	2.2	0.3	-	3.1	- -
Ca_1 In_1	4.2	0	95.8 (1.37)	-	-	-	-	2.0 (0.47)
" In_2	67.8	2.1	30.1 (0.57)	2.9	0.4	-	2.8	- -
Ca_0 Inoc. L.S.D. 5%			(0.18)	0.7	-	-		(0.04)
1%			(0.25)	1.0	-	-		(0.06)
pH L.S.D. 5%			(0.16)	0.6	-	-		(0.04)
1%			(0.21)	0.8	-	-		(0.05)
Ca_0 In x pH 5%			(0.32)	1.2	-	-		(0.07)
1%			(0.43)	1.7	-	-		(0.10)

* Arcsin transformation.
\+ Log data.

Mean of 4 replicates, 12 plants/replicate.

APPENDIX 12.

Mt. Compass Field Trial I, 1963. Harvest II

Treatment	% Nodulation			Nodules/Plant			Dry Wgt.Tops (mg/plt.)	
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.Nod. Plts.	Sec.Nod. Plts.
				Subterranean Clover.				
pH 5.35								
Ca_0 In_1	52.6 (0.81)*	40.8 (0.69)*	6.6	2.5	1.0	3.0	23.4 (1.39)+	16.1 (1.23)+
" In_2	100.0 (1.47)	0 (0)	0	5.4	7.2	-	36.6 (1.56)	- -
Ca_1 In_1	61.9 (0.90)	38.1 (0.66)	0	2.8	4.5	5.7	22.1 (1.34)	18.4 (1.25)
" In_2	100.0 (1.47)	0 (0)	0	5.4	11.5	-	29.4 (1.47)	- -
pH 6.30								
Ca_0 In_1	60.6 (0.89)	37.1 (0.65)	2.3	2.1	4.0	4.5	28.1 (1.45)	23.5 (1.36)
" In_2	100.0 (1.47)	0 (0)	0	6.0	8.7	-	37.3 (1.58)	- -
Ca_1 In_1	72.1 (1.01)	27.9 (0.55)	0	3.2	4.8	7.5	22.7 (1.36)	19.7 (1.28)
" In_2	100.0 (1.47)	0 (0)	0	6.6	11.7	-	44.7 (1.64)	- -
pH 7.00								
Ca_0 In_1	57.0 (0.85)	43.0 (0.71)	0	1.9	3.6	5.2	22.4 (1.36)	17.3 (1.26)
" In_2	100.0 (1.47)	0 (0)	0	6.6	9.7	-	39.0 (1.59)	- -
Ca_1 In_1	69.6 (0.98)	30.4 (0.56)	0	3.9	4.8	8.0	26.6 (1.43)	24.7 (1.40)
" In_2	100.0 (1.47)	0 (0)	0	7.9	10.7	-	32.9 (1.51)	- -
Ca, Inoc.								
L.S.D. 5%	(0.08)	(0.08)		0.8	1.5	2.0	(0.08)	(0.13)
1%	(0.11)	(0.11)		1.1	2.0	2.7	(0.12)	(0.18)
pH								
L.S.D. 5%	(0.07)	(0.07)		0.7	1.3	1.7	(0.07)	(0.11)
1%	(0.09)	(0.10)		1.0	1.7	2.3	(0.10)	(0.15)
Ca, In x pH								
L.S.D. 5%	(0.13)	(0.14)		1.4	2.6	3.4	(0.15)	(0.22)
1%	(0.18)	(0.19)		1.9	3.5	4.7	(0.20)	(0.31)

APPENDIX 12 (cont.) Harvest II

Treatment	% Nodulation			Nodules/Plant			Dry Wgt.Tops(mg/plt.)	
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.	Prim.Nod. Plts.	Non-nod. Plts.
				Lucerne.				
pH 5.35								
Ca_0 In_1	0	0	100.0 (1.47)*	-	-	-	- - +	1.8 (0.45)+
" In_2	32.1	0	67.9 (1.01)	2.0	0.1	-	7.6 (0.93)	2.0 (0.47)
Ca_1 In_1	5.3	5.5	89.2 (1.26)	-	-	-	- -	2.4 (0.53)
" In_2	76.4	10.3	13.3 (0.27)	3.3	0.7	-	9.7 (1.02)	- -
pH 6.30								
Ca_0 In_1	2.3	10.0	87.7 (1.27)	-	-	-	- -	2.4 (0.53)
" In_2	63.2	8.9	27.9 (0.49)	2.5	0.7	-	6.7 (0.88)	- -
Ca_1 In_1	5.0	12.4	82.6 (1.17)	-	-	-	- -	2.7 (0.56)
" In_2	79.4	10.5	10.1 (0.27)	2.9	0.7	-	7.9 (0.94)	- -
pH 7.00								
Ca_0 In_1	7.8	0	92.2 (1.29)	-	-	-	- -	3.4 (0.62)
" In_2	71.8	16.6	11.6 (0.25)	3.2	0.9	-	8.3 (0.93)	- -
Ca_1 In_1	4.2	2.5	93.3 (1.33)	-	-	-	- -	2.6 (0.53)
" In_2	91.0	3.1	5.9 (0.18)	3.5	1.3	-	10.6 (1.06)	- -
Ca_2 Inoc.								
L.S.D. 5%			(0.23)	0.6	-	-	(0.10)	(0.07)
1%			(0.31)	0.8	-	-	(0.14)	(0.10)
pH								
L.S.D. 5%			(0.20)	0.5	-	-	(0.09)	(0.06)
1%			(0.27)	0.7	-	-	(0.12)	(0.08)
Ca_2In x pH								
L.S.D. 5%			(0.39)	1.1	-	-	(0.18)	(0.12)
1%			(0.53)	1.5	-	-	(0.25)	(0.17)

* Arcsin transformation.
\+ Log data.

Mean of 4 replicates, 12 plants/replicate.

APPENDIX 12.

Mt. Compass Field Trial I, 1963.

Harvest II.

Percentage Calcium in Tops. *

Treatment		Subterranean Clover	Lucerne
pH 5.35	Ca_0 In_1	0.66	0.32NN
	" In_2	0.90	0.60
	Ca_1 In_1	1.66	1.43NN
	" In_2	1.78	1.60
pH 6.30	Ca_0 In_1	0.86	0.65NN
	" In_2	0.90	0.60
	Ca_1 In_1	1.63	1.57NN
	" In_2	1.69	1.82
pH 7.00	Ca_0 In_1	0.91	0.67NN
	" In_2	1.01	0.82
	Ca_1 In_1	1.72	1.50NN
	" In_2	1.86	1.98
Calcium, Inoculum	L.S.D. 5%	0.11	0.31
	1%	0.25	0.70
pH	L.S.D. 5%	0.13	0.37
	1%	0.31	0.86
Ca, In x pH	L.S.D. 5%	0.19	0.53
	1%	0.43	1.22

* Calcium analyses are on primary nodulated plants except those marked NN (non-nodulated plants).

APPENDIX 13.

Mt. Compass Field Trial II, 1963.

Harvest II.

Treatment	% Nodulation			Nodules/Plant		
	Prim.	Sec.	Non-nod.	Prim.Nod.Plts. Prim.	Prim.Nod.Plts. Sec.	Sec.Nod. Plts.
			Subterranean Clover.			
Drilled lime						
x1 inoculum	88.0 (1.21)*	12.0	0	3.4	6.2	7.7
x10 "	98.7 (1.44)	1.3	0	5.2	11.3	-
x100 "	100.0 (1.47)	0	0	7.5	12.2	-
x1000 "	100.0 (1.47)	0	0	8.3	14.0	-
x1 + lime pellet	92.1 (1.32)	6.7	1.2	3.7	4.4	6.4
Broadcast lime						
x1 inoculum	75.9 (1.05)	24.1	0	2.5	6.2	6.3
x10 "	97.5 (1.40)	2.5	0	4.2	9.2	-
x100 "	100.0 (1.47)	0	0	6.6	12.5	-
x1000 "	100.0 (1.47)	0	0	7.2	13.5	-
x1 + lime pellet	83.9 (1.16)	16.1	0	3.5	4.7	6.3
x1 inoc. (peat)	85.7	14.3	0	4.0	8.3	8.8
x10inoc. (peat)	100.0	0	0	6.3	13.6	-
Inoculum						
L.S.D. 5%	(0.13)			0.8	2.1	
1%	(0.17)			1.1	2.9	
Lime						
L.S.D. 5%	(0.07)			0.5	1.3	
1%	(0.10)			0.7	1.8	
In x L						
L.S.D. 5%	(0.17)			1.2	3.0	
1%	(0.23)			1.6	4.0	

* Arcsin transformation.

Note: Peat inoculum results were not statistically analysed.

APPENDIX 13 (cont.)

Mt. Compass Field Trial II, 1963.

Harvest II.

% Nodulation			Nodules/Plant		
Prim.	Sec.	Non-nod.	Prim Nod.Plts. Prim.	Prim Nod.Plts. Sec.	Sec.Nod. Plts.
		Lucerne.			
74.9 (1.04)*	17.7 (0.43)*	7.4 (0.23)*	3.2	1.1	4.8
90.0 (1.26)	7.5 (0.24)	2.5 (0.12)	3.6	2.1	3.3
100.0 (1.47)	0 (0)	0 (0)	4.3	3.3	-
100.0 (1.47)	0 (0)	0 (0)	3.5	2.3	-
89.0 (1.25)	7.0 (0.23)	4.0 (0.17)	2.9	0.8	5.1
45.8 (0.74)	9.9 (0.31)	44.3 (0.72)	2.6	0.4	2.9
74.5 (1.04)	14.8 (0.39)	10.7 (0.31)	3.1	1.7	3.7
86.0 (1.19)	14.0 (0.37)	0 (0)	3.3	2.6	5.6
97.6 (1.40)	2.4 (0.11)	0 (0)	4.2	3.5	6.0
78.2 (1.08)	14.2 (0.37)	7.6 (0.28)	3.2	0.9	5.1
51.8	20.9	27.3	2.5	0.8	3.7
74.7	14.9	10.4	2.8	1.4	4.7
(0.13)	(0.12)	(0.14)	0.4	0.6	1.3
(0.17)	(0.17)	(0.19)	0.6	0.8	1.9
(0.08)	(0.08)	(0.09)	0.3	0.4	0.9
(0.11)	(0.11)	(0.12)	0.4	0.5	1.2
(0.18)	(0.18)	(0.19)	0.6	0.8	1.9
(0.24)	(0.24)	(0.27)	0.8	1.1	2.6

* Arcsin transformation.

APPENDIX 13 (cont.)

Mt. Compass Field Trial II, 1963.

Harvest II.

Dry Weight of Tops (gm/plant).

	Subterranean Clover		Lucerne	
Drilled lime.				
x 1 inoculum	0.478	(2.67)*	0.057	(1.74)*
x 10 "	0.618	(2.77)	0.081	(1.87)
x 100 "	0.623	(2.78)	0.115	(2.05)
x 1000 "	0.663	(2.82)	0.086	(1.92)
x 1 + lime pellet	0.490	(2.66)	0.062	(1.77)
Broadcast lime.				
x 1 inoculum	0.540	(2.70)	0.063	(1.67)
x 10 "	0.735	(2.85)	0.131	(2.05)
x 100 "	0.720	(2.85)	0.141	(2.12)
x 1000 "	0.900	(2.94)	0.187	(2.27)
x 1 + lime pellet	0.433	(2.63)	0.151	(2.17)
x 1 inoc. (peat)	0.728	-	0.099	-
x 10 " "	0.970	-	0.184	-
Inoculum L.S.D. 5%		(0.12)		(0.21)
1%		(0.17)		(0.28)
Lime L.S.D. 5%		(0.08)		(0.13)
1%		(0.11)		(0.18)
In x L L.S.D. 5%		(0.18)		(0.30)
1%		(0.24)		(0.40)

* Log data.