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THE EFFECTS OF NITRATE NITROGEN AND PHOSPHATE ON THE NODULATION AND NITROGEN FIXATION OF WHITE CLOVER (*TRIFOLIUM REPENS* L.)

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SUMMARY

The effect of nitrate nitrogen and phosphate on nodulation, nitrogen fixation and growth of white clover (*Trifolium repens* L.) plants was evaluated in a series of 'growth cabinet' and glasshouse experiments. Plants of a different age and nodulation status were used.

Nodule dry weight, nodule number and the average nodule weight declined on plants grown in solution cultures containing low concentrations of nitrate nitrogen (0.25 - 0.5mM). The nitrogen fixing activity of plants was also depressed by low concentrations of nitrate nitrogen, in experiments conducted during the winter and spring, but a stimulation in activity was observed in plants grown during the summer. The nature of the response was dependent on the growth response of the host plant to nitrate nitrogen. High concentrations of nitrate nitrogen (4mM) markedly reduced nodulation and nitrogen fixation in all experiments.

Phosphate increased the total nodule weight, nodule number and the nitrogen fixing activity of white clover plants, and several interactions between nitrate nitrogen and phosphate for nodulation and nitrogen fixation were obtained:

(a) Nodule weight, nodule number and the nitrogen fixing activity were more severely reduced by low concentrations of nitrate nitrogen at low phosphate levels than at higher levels.

(b) In one experiment a stimulation in nitrogen fixing activity occurred at the low nitrate nitrogen concentration and the highest phosphate level.

(c) At high levels of nitrate nitrogen no increase in nitrogen fixing activity was observed when higher rates of phosphate were applied.

The effect of nitrate nitrogen and phosphate on the soluble and reserve carbohydrate content, and percent total nitrogen and phosphate in the host plant and nodule material was evaluated and the relationship of the carbohydrate to nitrogen ratio with nitrogen fixing activity determined. The ability of this ratio to explain changes in nitrogen fixing activity is discussed.

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INTRODUCTION

It is a well established fact that the presence of nitrate ions can depress nodule development on legume roots, but recent studies in Queensland (Gates, 1970) have shown that this sensitivity can be ameliorated if phosphate levels are maintained at a high rate. Gates studies involved tropical legumes and their appropriate rhizobial symbionts, and this study was conducted to ascertain whether a similar situation obtains with a temperate species like *Trifolium repens* (White Clover).

Quantitiative information on the effect of nitrate nitrogen and phosphate on nitrogen fixing activity in white clover was also lacking, and an attempt was made to rectify this deficiency by examining the changes in rate of ethylene production per plant or mg nodule (dry weight) with the application of nitrate nitrogen and higher rates of phosphate.

CHAPTER 1

REVIEW OF LITERATURE

The review of literature will for convenience, be handled in four sections. The first will review the process of nodulation, the second the biochemistry of nitrogen fixation while in the third, the effect of combined nitrogen and phosphate on these definite parts of legume-rhizobium symboisis will be discussed. In the final section, the uptake, assimilation and transport of nitrogen compounds will be briefly considered.

1.1 NODULATION

Because of the large volume of literature on this subject, only certain aspects of nodulation in legumes will be dealt with in detail. Other reviews, (Wilson,1940; Nutman, 1956, 1958, 1965a,c; Allen and Allen, 1958; Raggio and Raggio 1962; Stewart, 1966; Dixon, 1969 and Masterton and Sherwood, 1970) contain discussions on the aspects not considered here.

1.2 Root hair infection

The root hairs are the sites of infection by rhizobia bacteria in a large number of legume species, particularly those of the genera *Trifolium* and *Vicia*, (Raggio and Raggio, 1962). However, entrance may also be gained through broken epidermal cells and cortical cells and ruptured tissue at the site of rootlet emergence, (Nutman, 1956; Allen and Allen, 1958).

Root hair elongation and deformation are the first visible indications of the interaction between legume root hairs and rhizobia in most members from the *Trifolium* and *Vicia* genera. Root hair deformations most frequently observed are curling at the tip to produce a shepherd's crook formation and lateral branching, (Nutman, 1965a) It is generally accepted that IAA is responsible in part at least, for these abnormal growth processes. For example,

a curling reaction, though less typical, can be induced by cell free filtrates of nodule bacteria which contain auxins that are active in the Went's pea test, (Chen. 1938). Further, Nutman et al (1945) and Kefford et al (1960) have evidence that rhizobia in culture can oxidise tryptophan which is secreted in small amounts by growing legume roots, (Rovira, 1956) to IAA. Finally, the depressing effects of additions of nitrate nitrogen on root hair curling in Medicago sativa can be overcome in part by adding large concentrations of indole acetic acid to the rooting medium, (Munns, 1968c; Valera and Alexander, 1965). Tanner and Anderson (1964) showed that nitrite produced from nitrate destroyed IAA.

The curling reaction was considered by Nutman (1963; 1965a,b,c) and Raggio and Raggio (1962) to be unspecific. However, more recent evidence, (Fahraeus and Ljunggren, 1968; Dixon, 1969; Yao and Vincent, 1969; and Masterton, 1970) suggests that root hair curling may be more specific. Yao and Vincent (1969) for example, reported that infection threads were almost entirely restricted to markedly deformed hairs and with all hosts, the markedly curled condition was practically restricted to the host plant associated with the virulent homologous rhizobia.

Evidence in support of host involvement in root hair curling has been supplied by Munns (1968b) in experiments designed to measure the effect of NO_3 nitrogen on root hair curling and nodulation in Medicago sativa. Munns demonstrated that pre-inoculation treatment with nitrate markedly reduced the number of curled hairs. Since, in this experiment there was no external effect of NO_3 on the IAA produced by the rhizobia, the factor limiting root hair curling must have been of internal origin, and thus under host control. It is possible that pre-treatment with nitrate resulted either in a cell environment unsuitable for the involvement of the host cell nucleus which has been shown to be closely associated with early stages of infection, (Fahraeus, 1957) or in the production of a factor additional to IAA but necessary for root hair curling. This view is contrary to that of Fahraeus and Ljunggren (1968) who considered the additional factor to be a specific

polysaccharide fraction of the rhizobia cells. However, they have not been able to identify the material.

1.3 Induction of Enzymes in the Host and the initiation of Infection

The passage of bacteria into the root and into the plant cells involves the softening and breakdown of cell wall substances. Nodule bacteria do not produce any of the pectic or cellulolytic enzymes, (Nutman, 1965b) but pectin methyl esterase (PME) and polygalacturonase (PG) have been identified in media where nodulated plants have been grown, (Fahraeus and Ljunggren, 1959). Polygalacturonase synthesis in particular is restricted to those plants with nodule bacteria able to infect them and correlations have been obtained between the amount of enzyme induced and the degree of infectiveness, (Ljunggren and Fahraeus, 1961). Two factors NO_3 nitrogen, (Fahraeus and Ljunggren, 1959) and low pH, (Munns, 1969) which are known to interfere with infection of legumes also reduce the formation, (NO_3 nitrogen) or the activity of polygalacturonase, (low pH). The formation of polygalacturonase can be interpreted as the primary effect of the bacteria on the host, the synthesis of which resulting in a partial depolymerization of the cell wall pectin, which in its turn facilitates the bacterial invasion.

The active principle which induces the formation of the enzyme in the host has been identified as the polysaccharide coat of the bacterium, (Ljunggren and Fahraeus, 1959). It is not certain however, that DNA impurities were not present in the polysaccharide preparations and therefore, the action of bacterial DNA in the induction of the enzyme cannot be ruled out, (Ljunggren, 1961). That the infectiveness of an avirulant variant of clover bacteria can be restored by treatment with DNA from a virulent strain, (Ljunggren, 1961; Balassa, 1960) strongly suggests that cross infectability may be controlled by the constitution of bacterial polysaccharide or its determining nucleotides.

The infection mechanism may be visualised as follows. The bacteria secrete water soluble compounds which are highly specific.

The active principle passes through the cell wall and on reaching the protoplasm, reacts with some specific cell component, probably the root hair cell nucleus. Ljunggren and Fahraeus (1961) considered that the reaction between the bacterial substance and specific cell compound results in the formation of an 'organiser', which governs the production of polygalacturonase.

The exact nature of the 'organiser' is not known. Dixon (1969) suggested that growth substances like IAA and gibberellins may be involved. The nodule inducing principle could alter the metabolism of the root hair cells so that these growth substances would be produced in the required quantities. If the sole inhibitory effect of nitrate on root hair infection is by the oxidation of IAA, and Munns (1968c) and Valera and Alexander (1965) support this, then the inhibition of pectinase production by nitrate is evidence that the production of the enzyme is affected by IAA.

It is clear that the involvement of polygalacturonase in the infection processes has led to the development of the theories on the mechanism of root hair curling and infection thread initiation. However, Lillich and Elkan (1968) and MacMillan and Cooke (1969) were not able to repeat the findings of Ljunggren and Fahraeus. MacGregor, (pers.comm.) also found that the level of polygalacturonase synthesis was not always associated with the degree of infectiveness of the rhizobia bacteria. These workers were highly critical of the methods used in determining the presence of the pectinase enzyme and consider the measurements taken by Fahraeus and Ljunggren (1959) or Ljunggren and Fahraeus (1961) were outside the confidence limits of the technique.

1.4 The Formation of the Infection Thread

After the bacteria enter into the root hair they are enclosed in a tube, called the infection thread. The infection thread grows through the root hair and the cortical cells, and in some way initiates the division of cells within the cortex which then form the nodule tissue.

In the main, three hypothesis have been advanced to explain

the mechanism of invasion and infection thread development. One of these, tactly accepted by most earlier investigators, could be called the penetration theory. This theory implied that the rhizobia actually penetrated the cell wall and grew further through the root hair into the cortex. The cellulose lining of the infection thread was interpretated as a structure laid down as a defence against the bacterial intruder, (Schaede, 1940). However, no cell wall dissolving enzymes have ever been found in the media of rhizobia and no break in the cell wall at the point of entry of the thread has been demonstrated.

In the absence of a chemical explanation, it was then generally supposed that entry was afforded by mechanical means as has been demonstrated for fungi, (Nutman, 1956). But, in a detailed micro-examination of the host's cell wall McCoy (1932) was unable to find any evidence to support such a theory. Nutman (1956) suggested that no actual penetration of the hair occurs, the root hair surface instead becomes invaginated to form an infection thread. Nutman hypothesised that the bacteria could take part in the process of intussusception and build themselves into and through the primary wall of the host cell wall to give an infection thread by invagination. In this way bacteria within the infection thread could still be on the morphological outside of the root, and the infection thread wall would be a continuous with that of the host hair cell wall. Proof of the latter has been provided by a number of workers, including Bergersen and Briggs (1958); Salhman and Fahraeus (1963); Nutman (1959) and Goodchild and Bergersen (1966).

There are according to Dixon (1969) several features in this hypothesis that are not wholly acceptable. First, if the whole cell wall invaginates, it must do so against the hydrostatic pressure of the cell contents. Secondly, no pore can be seen at the point of infection and third, with an open invagination more than one strain of bacteria could be present within a nodule, a feature not yet demonstrated.

Dixon, (1969) considered that the bacteria penetrate to the

inside of the primary wall, where primary and secondary wall material is laid down over the bacterium. It is this material that invaginates and forms the infection thread walls. To invaginate against the tugor pressure of the root hair cell, (Dixon, 1969) the outer portion of the primary wall behind the bacterium is possibly strengthened as well. The hydrostatic pressure of the cell contents could also be reduced by the imbibing of water by the polysaccharide slime associated with the colony of bacteria, (Kefford et al, 1960; Dixon, 1969). These workers considered that the imbibing of water would cause the root hair cytoplasm to retreat from the infection thread and further, provide the stretching force needed to initiate and maintain the growth of the infection thread. The growth of the bacteria within the threads themselves may also provide the stretching force. Goodchild and Bergersen (1966) observed, from electron microscope photographs, bacteria to be tightly packed in the threads. There was also evidence of distortion consistent with the existence of considerable end to end pressure as the bacteria grew in length and divided.

The growth of the infection thread from cell to cell merely involves the imitation of the original infection and growth through the root hair cell. The invagination of the next cell wall is thought to occur at the wall pits. Muhlethaler (1950) has shown that wall pit areas are generally considerably larger than the bacterium and are at first closed only by pectic material [illegible], open net of cellulose fibrils. Maceration experiments on host tissue transversed by infection threads, have shown that the threads are discontinuous from cell to cell (McCoy, 1932), evidence which strongly supports the invagination theory, and the passive movement of bacteria from cell to cell.

The close relationship between the root hair cell nucleus and infection thread initiation and development has already been mentioned in this review. This association continues during the passage of the infection thread through the cortex. The infection thread invariably passes close to or tends to move from the intended path to another closer to the nucleus. These features point to the significance of host control in nodulation,(Fahraeus,1957; Nutman,1959).

1.5 Pattern of Root Hair Infection

Very few root hairs are infected in legumes. Four to five percent of root hairs were infected in lucerne, Medicago sativa (McCoy, 1932) and two percent in white clover, (T. repens), (Fahraeus, 1957). A high percentage, seldom less than 10 to 20% of the infection threads fail to grow in white clover, (Fahraeus, 1957). Purchase (1953; 1958) recorded an equal number of infections and nodules in red clover, thus suggesting closer host control during the root hair infection stage in this species.

Root hairs became infected on the third or fourth day after germination in a large number of Trifolium species, (Nutman, 1965a). The initial infections generally occurred at a few well separated points or zones on the root and not at random. Subsequent infections arose within and near these zones and extended in both directions until a large part of the root became susceptible. The rate of hair infection increased expotentially until the appearance of the first nodule, after which a lower but still expotential rate was observed, (Nutman, 1965a,b).

The spread of infection from centres may be due to the diffusion of activating substances either within or outside the root, (Nutman, 1965a) since the mean number of infections per plant is generally higher when two plants are grown close together than singly.

In an attempt to define the nature and the origin of the principles controlling infection in legumes, Nutman (1965b) evaluated the effect of darkness and decapitation on root hair infection in T. fragiferum and T. pariflorum. Short periods of darkness did not inhibit infection, although the number of infections was reduced in comparison to control plants grown in light. That the number of infections declined steadily with increasing exposure to darkness, suggested that the seed contains 'factors' necessary for infection, (Nutman, 1965b). Decapitation of the top before 4 days after germination or after 4 - 5 days also depressed infection, but did not prevent infection. At four days after germination however, decapitation increased the

number of infections. These results indicate that some factor necessary for infection thread initiation comes from the cotyledon or plumule and that sufficient moves into the root by the fourth day in T. fragiferum to allow normal infection, (Nutman, 1965b).

The distribution of infected root hairs was also changed when seedlings were decapitated at 3 - 4 days. Larger numbers were concentrated near the root tip, a feature not associated with intact plants, where the proximity of an active root meristem could inhibit infection.

The pattern and degree of infection in legume roots is then most probably influenced by stimulatory-inhibitory substances. Nutman (1965b) tentatively proposed that "a precusor of an infection-promoting substance may originate in the plant top and be normally translocated towards the growing root tip, and that an infection-inhibiting substance may be produced in root meristems and move in the opposite direction. The primary zones of infection may reflect the localised transformation of the precusor into active promoter. This may take place in those parts of the root that are regions of incipient meristematic activity, and so give rise to regions of denser infection".

Nutman citied the increase in infections near the root tip on T. fragiferum, when plants were decapitated before four days after germination as evidence for his hypothesis, since the main root apex, through decapitation would have become an 'inhibited' meristem capable of producing promoter substances. The precursor from the cotyledons or hypocotyl would be translocated unaltered into the main root apex in the absence of other incipient meristematic centres. Indeed the failure of intact legumes to produce infections in the first four days may be due to a lack of incipient meristematic centres.

If Nutman's hypothesis is correct, then it underlines the importance of the development of the root as a factor controlling infection patterns and rates.

1.6 Nodule Development

The nodule begins as a proliferation of a small group of cells of the inner cortex of the root. These cells in most legumes are of mixed tetraploid and diploid constitution, the tetraploid cells arising from preformed tetraploid initials that are present before infection, (Wipf, 1939; Wipf and Cooper, 1938; 1940). However there are exceptions, since Kodama (1967), (quoted by Dixon, 1969) has reported that Vicia fabia and some tropical legumes do have diploid nodules.

Proliferation of these cells commences when a tetraploid initial is stimulated into cell division by the proximity of an infection thread. The rhizobia may stimulate the division of cells in the root cortex by contributing a kinetin-like factor, (Torrey, 1961). Once the initial stimulation occurs, the nodule differentiates fully by the rapid formation of an organised meristem that produces mainly tetraploid cells basally and diploid cells toward the outside of the root which forms the cortex in where the vascular traces are differentiated. The central tetraploid zone is enclosed within an endodermis continuous with that of the host root and a separate endodermis may also envelop each vascular trace. Thus the disomatic cells seem to be the actual predetermined sites of infection, (with a few exceptions) but the exact reason for restriction of intracellular infections to tetraploid cells remains obscure.

1.6.1 Bacteria in the Nodules

In some host plants, for example in clovers, bacteria in the infection threads are progagated through the nodule by the invasion of the intracellular spaces by infection threads, while in others by the division of a few infected cells. It is widely accepted that the bacteria, still in the rod form, are released into the host cell at a point where there is no infection thread wall and where the thread is frequently enlargened and spherical in form, (vesicles). The bacteria appear to migrate to the periphery of the thread or vesicle, (Mosse, 1964) before being released by the process of endocytosis into the cytoplasm of the host tetraploid cell,

(Bergersen and Briggs, 1958; Goodchild and Bergersen, 1966; Dixon 1969).

Other less popular theories have been advanced to explain the formation of the envelope about the bacteria and bacteroids. Dart and Mercer (1964) considered that the bacteria emerged from the infection thread without a membrane envelope, the membrane being formed de novo. Mosse (1964) and Jordon, Gringer and Coulter (1963) on the other hand, claimed that the bacteria were enclosed by the endoplasmic membrane.

The role played by the membrane has been fully evaluated by Nutman (1963). It is apparent the membrane prevents the bacteria coming in contact with antibiotic substances in the plant cytoplasm.

1.6.2 Formation of Bacteroids

After being released into the host cell cytoplasm, the bacteria may increase in size and change shape, the nature of the changes being species dependent. In some hosts, the bacteria divide within the membrane so that each membrane contains several bacteria, while in others the membrane behaves as a bacterial membrane and divides with the bacteria, so that each bacterium is enclosed in a separate envelope. The manner in which the rhizobia multiply also appears to be host controlled. In soyabean nodules there are several bacteria within each membrane, (Goodchild and Bergersen, 1966), while in clover and Vicia nodules and possibly lucerne the plant membranes surround each individual bacterium, (Dart and Mercer, 1964; Jordon, 1962; Mosse, 1964).

Other biochemical changes have been recorded with the maturation of rhizobia into bacteroids. Foremost is the synthesis of the enzymes necessary for nitrogen fixation and the production of leghaemoglobin found in the solution of the plant sap or associated with the lipoprotein membranes of the endoplasmic reticulum, (Bergersen and Wilson, 1959). Changes have also been recorded in respiratory pigments, (Bergersen,1969). The total level of cytochromes is higher in soyabean nodule bacteroids, and the bacteroids

do not contain cytochromes a - a_3, cytochrome o or rhizobium haemoglobin, which are associated with the free living form (Rhizobium japonicum). Bacteroids do contain cytochrome c (552 Rhizobium) and the haemoprotein P-450, and the pigments P-420 and P-428. A nonautoxidizable cytochrome c and cytochrome b have been identified in both free living organisms and bacteroids. (Appleby 1962, 1969a,b). Nutman (1965c) recorded bacteroid respiration to be marginally higher, and the bacteroids of actively fixing nodules do not contain appreciable carbohydrate reserves.

These changes are most probably the result of the host cell transferring genetic information to the bacteria or unblocking genetic material already present, but which is repressed in the free living organisms, (Bergersen, 1969). The theory of host cell transfer of genetic information to the bacteria is favoured by Dilworth and Parker (1969). These workers suggested that genetic information, probably encoded in the host as a result of the transfer of symbiosis control to the host from the free living organisms during the evolution of symbiosis, is conveyed to the bacteria by the appropriate host mRNA. Goodchild and Bergersen, (1966) reported that bacteria released from infection threads frequently group adjacent to the host cell nuclei. However, Mosse's (1964) proposal of ultra structure connections between the bacteria and nuclei were not noted.

The environment within the nodule may also account for some of the changes. Changes in the cytochrome complement could be reproduced in culture solutions at low oxygen tension, (Appleby and Bergersen, 1958).

1.7 Nodule Formation and Root Habit

Breeding experiments have shown that selection of host legume plants for high nodule number produces plants with many lateral roots, the reverse being the case for sparcely nodulating plants, (Nutman, 1965a). This close relationship between the rooting habit and nodule formation is also further demonstrated in studies conducted by Nutman (1948). In these seedlings inoculated

with effective Rhizobium cultures had initially a lower complement of lateral roots in comparison to plants not inoculated. It was suggested, (Nutman, 1965a) that some of the lateral root primordia had been occupied by bacteria and had grown out as nodules.

The permanently enhanced rate of infection and nodule formation, which occurs after moderate delays in inoculation, (Nutman, 1949; Dart, 1959) or following release from the initial inhibitory effect of trace levels of NO_3, can also be explained in terms of host - controlled infection and nodule development. The increase in infection potential of the root, results from an increase in the number of lateral roots, each of which bears their own foci for infection and nodule formation or for further root development, (Nutman, 1965a).

These observations point to a common origin or a very closely associated origin of lateral roots and nodules and Nutman (1956; 1965a) suggested that the lateral primordia were metamorphosised into nodules. Recently Wittmann(1968) working with field beans, (Vicia faba) vetch, (Vicia spp) and field peas, (Pisum sativum) found that the nodules were of endogenous origin and possibly transformed lateral root primordia.

Nodules are not however, modified lateral roots, as in the case of non-leguminous nodules, (Fred, Baldwin and McCoy, 1932; Wilson, 1940; Allen and Allen, 1950). Fred, Baldwin and McCoy (1932) in summarising the evidence, concluded that "it is distinctly not a modified lateral root, for it has no central cylinder root cap or epidermis. Furthermore it does not digest its way out from the cortex of the main root, but remains covered with a layer of cortical parenchyma". Allen and Allen (1950) stressed that the nodule is a unique type of root hypertrophy with a cortical origin and an anatomy which differs from that of a secondary root in both structure and function. The original concept, (Thimann, 1936; 1939) that the nodule is a lateral root modified by auxins produced by nodule bacteria is thus no longer tenable.

1.8 Nodule Size and Frequency

The hypothesis of host controlled infection and nodule development at certain foci can also be brought to bear upon the more general problem of variation in nodule size and frequency as affected by bacterial strain, or environmental influences.

The inverse relationship between bacterial virulence and mean nodule size has been established in a larger number of hosts, (Nutman, 1965a). Nutman considered that the nodules formed by the virulent strain tended to inhibit nodule formation to a lesser degree than those of the less virulent strains. Support for the hypothesis was supplied by Nutman (1949; 1952). Excision of effective nodules for example led to an immediate increase in the rate of nodule formation until the deficit was recovered, where upon the rate returned to that characteristic of the host and strain, (Nutman, 1952). A comparison of the effects of excision of different sizes of nodule indicated that larger nodules produced upon poor nodulating hosts, or by less virulent strains are more inhibitory than those formed on abundantly nodulating hosts or by more virulent strains. The strength of the inhibition is related to the activity of the nodular meristem, upon which the size of nodule depends. Excision of the uninfected growing tip of the root also causes an increase in nodulation, thus demonstrating that the root tip is also a controlling influence upon infection, (Nutman, 1965a).

A similar relationship can develop in situations where the number of nodules on hosts is severely limited by environmental factors. The few nodules formed can attain a large size, thus compensating for loss in bacterial tissue through reduction in the number of nodules initiated, (Nutman, 1965a). That bacterial virulence, nodule size, meristematic activity and the morphogenesis of the whole root are closely interrelated was emphasised by Nutman (1965a). He considered that the nodule functions essentially as one meristem among others active in determining the pattern of growth.

1.9 Stimulatory - Inhibitory Factors and Nodulation

The role of inhibitory-stimulatory factors in controlling

infection and the role of growth substances in infection thread development has already been discussed. There is also evidence that stimulatory-inhibitory factors and/or growth substances could be controlling nodule initiation and growth. No nodule formation for example, has been obtained upon roots in fully isolated culture. Raggio et al (1957) reported some success with primary excised cultures, but transfer cultures failed to nodulate. Since nodules formed upon fully etiolated whole seedlings, it is likely that substances synthesized in the leaves are required in nodulation, (Nutman, 1965a). A soluble morphogenic substance produced in bean hypocotyl and cotyledon did exert an influence on nodulation in field beans, (Schaffer and Alexander, 1966). The substance was not however, peculiar to legumes. Schaffer and Alexander (1966) demonstrated that substances in autoclaved coconut water and coconut endosperm were able to replace those in bean cotyledons or hypocotyl.

The absence of nodules on transferred excised root cultures is probably a result of failure of the initial nodule primordium to become activated, (Nutman, 1965) since, while top excision on T. fragiferum seedlings had no effect on infection, no nodules or lateral roots were observed.

Little is known of the requirements for growth factors in the initiation and growth of nodules. Dixon (1969) tentatively suggested roles for IAA and gibberellins. Cytokinins have been shown to promote proliferation of soyabean and pea callus and polyploid mitosis in cultured root segments, (Torrey, 1961; Phillips, 1971). Abscisic acid at concentrations of 1.9×10^{-6} M reduced nodule development in pea plants by 61% without affecting root or shoot growth, root hair curling or infection thread development, (Phillips, 1971). Since abscisic acid also reduced polyploid mitosis in roots, Phillips (1971) concluded that abscisic acid reduced the number of nodules on the plant root by inhibiting the cortical cell divisions required for root nodule formation.

2.1 NITROGEN FIXATION

Biological nitrogen fixation consists of the reduction of nitrogen to ammonia by living organisms. Unlike most other fundamental reactions in nature, nitrogen fixation is not characteristic of major classes of organisms. The reaction is however, distributed through a broad spectrum of micro-organism symbionts, including Rhizobium trifolii. The enzyme system responsible for this reduction is called nitrogenase.

Most of the initial information gained on the nitrogen fixing process has come from studies on free living organisms. However, the information gained from studies using disrupted nodule preparations, (Bergersen, 1967; Bergersen and Turner, 1967; Koch, Evans and Russell, 1967) suggests that the symbiotic system is similar to that present in the free living bacteria, Clostridium pasteurianum and Azotobacter vinelandii. There are distinguishing features, some of which may be associated with the unique environment in the plant cell within which the reaction occurs, (Bergersen, 1969) and these will be highlighted in the review.

A complete review of all literature pertaining to this topic will not be undertaken because of the large volume of literature on the subject. In this section I will attempt to outline the requirements for nitrogen fixation and the probable biochemical pathways. A brief discussion on the possible action of nitrogenase will then follow.

The biochemistry of nitrogen fixation has been the subject of a number of recent reviews including those of Stewart (1966); Burris (1966; 1969); Hardy and Burns (1968); Bergersen (1969; 1971); Fottrell (1968); Postgate (1970); Hardy, Burns, Hebert, Holsten and Jackson (1972) and Streicher and Valentine (1973).

2.2 Site of the Primary Reactions of Nitrogen Fixation

Bergersen (1960); Klucas and Burris (1967) and Kennedy (1966a,b) attempted to ascertain the location of the primary reaction by exposing detached nodules to $^{15}N_2$ for various times,

and then determining the distribution of the label in the bacteroids, cytoplasmic membranes and soluble fractions of the nodule. For soyabean nodules, most of the label accumulated in the soluble fraction, a little in the fraction containing cytoplasmic membranes, (subsequently found to be an artifact) and none in the bacteroids, (Bergersen, 1960; Klucas and Burris, 1967). Initially similar results were obtained for detached serradella (Ornithopus sativa) nodules, (Kennedy, 1966a,b). However Kennedy et al (1966) later reported that the bacteroids from serradella nodules contained substantial quantities of newly fixed nitrogen. It appears that soyabean and serradella nodules differ in the extent to which NH_3 is retained within the bacteroids; 90% compared to 20% of the nitrogen fixed in one minute being detected in the soluble fraction for soyabean and serradella nodules respectively.

Results from studies using bacteroid suspensions obtained from soyabean nodules, (Bergersen and Turner, 1967) supported the evidence obtained by Kennedy et al (1966). Labelled nitrogen was located entirely in the bacteroid fraction. No nitrogen fixation was detected in the soluble fraction. Further, the enzymes necessary for nitrogen fixation have been located in the bacteroids, (Koch, Evans and Russell, 1967b).

The plant components of the legume symbiotic system must then fulfil auxiliary functions.

2.3 Requirements for Nitrogen Fixation in Legume Nodules

Koch, Evans and Russell, (1967a,b) measured the production of NH_3 from N_2 gas in cell-free bacteroid extracts and showed that the omission of an ATP generating system, $Na_2S_2O_4$ or N_2 gas resulted in little NH_3 production. They also demonstrated that the reaction required anaerobic conditions and that the reaction rate was increased by the addition of $MgCl_2$ and creatine phosphokinase, (both components of the ATP generating system).

Thus it would seem that the following are necessary:-

(1) an energy source

(2) a source of reducing power

(3) an active enzyme complex

(4) a suitable reducible substrate, i.e. N_2 gas, acetylene or hydrogen ions.

These requirements are similar to those for cell-free extracts, from free-living N_2 fixing organisms like Clostridium pasteurianum and Azotobacter vinelandii.

2.3.1 Source of Energy

The first experimental evidence in support of the involvement of ATP in nitrogen fixation was furnished by McNary and Burris (1962). These workers showed that arsenate, at concentrations which completely suppressed the formation of acetyl phosphate in free living organisms including Clostridium pasteurianum and thus indirectly ATP synthesis also inhibited nitrogen fixation. Additions of glucose to the clostridial cultures also inhibited nitrogen fixation possibly as a result of the depletion of ATP, brought about by glucose - 6 - phosphate synthesis.

It appears that nitrogenase functions most effectively when ATP is supplied via an ATP generating system, such as creatine phosphate - creatine kinase, or acetyl phosphate or acetokinase which maintain a low steady-state level of ATP and a negligible steady level of ADP. Recent results, however, show that substrate levels of ATP may be used providing correct Mg^{2+}/ATP ratios are maintained, (Kennedy, Morris and Mortenson, 1968; Bergersen, 1971).

In free living organisms the phosphoroclastic reaction provides the immediate energy source for nitrogen fixation. Pyruvate forms acetyl phosphate, which in the presence of ADP yields ATP, acetate, H_2 and CO_2, (Stewart, 1966; Burris, 1966; Hardy and Burns, 1968; Fottrell, 1968; Bergersen, 1969). In symbiotic systems, the oxidative phosphorylation system is the most likely source of ATP, (Bergersen, 1969; 1971). For example, fumarate and succinate, both intermediates of the TCA cycle, restored and enhanced nitrogen

fixation when supplied to bacteroids prepared anaerobically in a medium devoid of 0.3M sucrose, (Bergersen and Turner, 1967). Succinate was found to be a prominent constituent of organic acids leached from bacteroids shaken in a sucrose medium, (Bergersen,1969). No such leakage occurred when the bacteroids were prepared in sucrose since no depression in nitrogen fixation was noted.

Detached nodules, (Bergersen, 1962) and suspensions of bacteroids, (Bergersen and Turner, 1967) also have a requirement for O_2. This is further evidence that substrates are oxidised and that oxidative phosphorylation provides ATP as an energy source. Finally, Bergersen (1966) noted that when 2,4 dinitrophenol was used as an uncoupling agent in oxidative phosphorylation nitrogen fixation was inhibited in nodule breis.

2.3.2 Source of Reducing Power

Pyruvate furnishes not only ATP but also acts as an electron donor for nitrogen fixation in cell free extracts of Clostridium, (Burris, 1966; Stewart, 1966; Fottrell, 1968). The electron might come from the hydrogen generated in this reaction. Free hydrogen, (Burns and Le Comte, 1964; Mortenson, 1964; D'Eustachio and Hardy, 1964) can act as reductant in cell free extracts of free living organisms providing a hydrogenase is present. Free hydrogen cannot however, be utilized in cell free extracts of soyabean nodules. Wong et al (1972) demonstrated that in the absence of any other reductant, the nitrogen fixing activity in soyabean nodule extracts occurs in the presence of H_2, only when the hydrogen donating system from C. pasteurianum was present.

A large number of 'artifical' reductants have been tried. To date, sodium dithionite remains the only known non-biological reductant which functions in substrate amounts and couples directly to nitrogenase. It is an effective reductant for all nitrogenases tested, including Azotobacter, Clostridium and soyabean nodule bacteroid preparations, (Hardy and Burns, 1968; Burris, 1969).

Pyruvate, Ketogluturate, β hydroxy-butyrate, reduced NAD, reduced NADH, succinate and fumarate are unable to replace

dithionite, (Koch *et al*,1967; Bergersen, 1969).

2.4 Electron Transport to Nitrogenase

Bacterial ferredoxin is pivotal in electron transport in anaerobic bacteria. It accepts electrons from pyruvate via the phosphoroclastic reaction and reduces a variety of enzymes including nitrogenase, NAD reductase and hydrogenase. Flavodoxin can replace ferredoxin in vitro. Bacterial ferredoxin has not however, been found in nitrogen fixing extracts of aerobic, facultative or symbiotic nitrogen fixing organisms. The absence of ferredoxin in symbiotic nodules suggests that alternative electron transport systems operate. Evan (1969) and Wong *et al* (1972) have proposed electron transport schemes for nodule bacteroids. The schemes were based on the knowledge that soyabean nodules contain extensive quantities of poly - β hydroxybutyrate, active β hydroxybutyrate dehydrogenase, and that extracts of nodule bacteroids catalyse the reduction of dyes such as benzyl viologen by NADH. Evans (1969) and Wong *et al* (1972) have demonstrated that a mixture containing an active nitrogenase, an ATP generating system, benzyl viologen or a flavoprotein, a catalytic amount of NADH and hydroxybutyrate and the corresponding dehydrogenase catalyses the reduction of acetylene to ethylene. Any dehydrogenase capable of maintaining a supply of NADH could function as a source of reducing power. Other dehydrogenases observed in crude soyabean extracts were, glyceraldehyde -3- phosphate, a NADP specific isocitrate and glucose -6- phosphate dehydrogenases, (Wong *et al*, 1972).

A probable electron transport pathway could be as follows: (Wong *et al*, 1972).

$$\beta\text{-hydroxy butyrate} \xrightarrow{(1)} \underset{\text{other substrate} \searrow}{\text{NAD}} \xrightarrow{(2)} \text{Dye or FMN or FAD} \xrightarrow{(3)} N_2$$

(1) = β hydroxybutyrate dehydrogenase (2) = NADH dehydrogenase (3) = Nitrogenase

A NADH dehydrogenase has been identified in an acetone powder of bacterial cell walls, (Wong *et al*, 1972) and Koch *et al*

(1967) have isolated from bacteroids a brown non-heme iron protein and Koch et al (1970) a flavoprotein similar to azotoflavin. There is some evidence, (Wong et al, 1972) that these factors could well be the natural electron carriers. For example, electrons may be transported from an NADPH-generating system, such as glucose -6-phosphate dehydrogenase through ferredoxin NADP reductase to azotoflavin and bacteroid non-heme iron protein to N_2 via nitrogenase.

2.5 Enzymes

The enzyme nitrogenase has been identified in one or two components obtained in cell-free extracts of free-living organisms. The nitrogenase is present in a component labelled as the nitrogen activating system, while the hydrogenase enzyme, long linked with the evolution of hydrogen in nitrogen fixation, appears to be present in another component, the hydrogen donating system (HDS). In Clostridium pasteurianum, the phosphoroclastic reaction is likewise in the HDS. Both components are necessary for N_2 fixation, (Burris, 1966; Stewart, 1966; Nicholas, 1963; Postgate, 1970). No mention has been made in the literature as to the possibility of dividing cell-free extracts of bacteroids in a similar fashion.

2.5.1 Hydrogenase Enzymes

There appear to be two hydrogenases present, at least in the extracts of free-living organisms. One identified as a 'classical hydrogenase' is carbon monoxide sensitive and ATP independent, while the other is carbon monoxide insensitive but ATP dependent, (Burris, 1969). The 'classical hydrogenase' can accept electrons from ferredoxin and evolve or donate electrons to ferredoxin to be used in nitrogen fixation in Clostridium. Bergersen (1969) has found some evidence for the presence of 'classical hydrogenase' in soyabean nodules, but in the main little reference is made to this enzyme in the symbiotic system. Dixon (1968; 1972) detected a hydrogenase in some Rhizobium bacteroids. The hydrogenase was similar to that found in Azotobacter; it was capable of reducing a range of electron acceptors, but the hydrogenase action was not reversable. Dixon (1972) also demonstrated

that the hydrogenase was concerned with the oxidation of hydrogen with resultant oxidative phosphorylation. An increase in efficiency of nitrogen fixation was observed in those bacteroids possessing the enzyme. In some respects this enzyme conforms to the activity of of the 'classical hydrogenase'.

In contrast to the 'classical hydrogenase', the ATP dependent carbon monoxide insensitive hydrogenase does not require the presence of ferredoxin. The ATP dependent hydrogenase activity remains with nitrogenase during purification of extracts, and both nitrogenase and ATP dependent hydrogenase have similar requirements, (Fottrell, 1968). The nitrogenase (fig 2) and the ATP dependent hydrogenase may be the same enzyme, (Burris, 1966).

2.5.2 Nitrogenase

This enzyme has been fractionated into two components. One component contains iron and molybdenum and has molecular weight of approximately 180,000, the second contains iron and has a molecular weight of about 51,000, (Bergersen, 1971). To date no accurate figure can be quoted as to the actual composition of molybdenum and iron in Fraction 1 or iron in Fraction II.

The enzyme complex is extremely sensitive to O_2 and must be prepared under anaerobic conditions, but unlike its counterpart in extracts from cell-free organisms, the enzyme does not appear to be cold labile, (Bergersen, 1971).

No one has demonstrated an independent function for the Fraction I or Fraction II of nitrogenase. Apparently both must be present to carry out nitrogen fixation. Some tentative proposals have been advanced and these will be discussed.

2.6 Possible Biochemical Pathways in the Conversion of Nitrogen Gas to the Inorganic Form

The entry of N_2 gas into the biochemical pathways could be by oxidation or reduction. Entry by way of oxidation would result in the formation of N_2O or a compound akin to N_2O. Since it is known that N_2O is a competitive inhibitor to nitrogenase, it is unlikely that oxidation of N_2 gas occurs. Further, the hydration

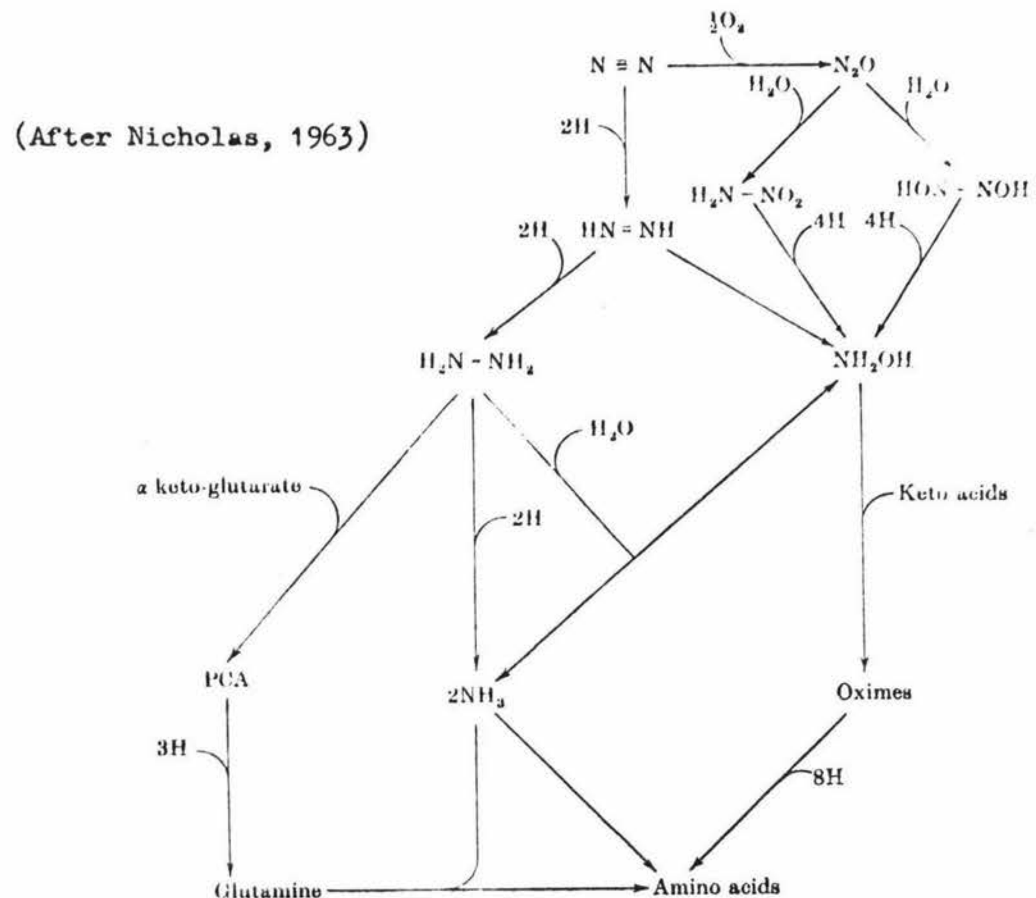


Fig. 7. Possible pathways for biological nitrogen fixation. (PCA = dihydropyridazinone-5-carboxylic acid.)

products of N_2O, nitramide and hyponitrous acid, have not been found to be used by N_2 fixing organisms, thus indicating that their presence as intermediates is remote. Such an oxidation pathway is unlikely, (Nicholas, 1963).

The bulk of evidence supports the view that the initial reactions are reductive, six electrons being involved in the reduction of NH_3. Should two-electron reduction occur, the first product would be a hypothetical diimide, the second a hydrazine and third and last NH_3. In the scheme of Nicholas (Fig1) the diimide could alternatively be hydrolysed to hydroxylamine, the hydrazine to NH_3 and hydroxylamine respectively. Hydrazine could also combine with alpha ketoglutaric acid to form dihydropyridazinone-S-carboxylic acid (P.C.A.). Bach (1957) obtained evidence that P.C.A. might be an intermediate and Virtanen and workers (Nicholas, 1963) produced evidence for the involvement of hydroxylamine as an intermediate.

Nicholas (1963), Burris (1966) and Stewart (1966) could provide only scant evidence for hydroxylamine, diimide, hydrazine or P.C.A. as intermediates. Much evidence, like that of Garcia Rivera and Burris (1962) indicates that they are not intermediates; or if they are intermediates they remain firmly bonded to nitrogenase during the reductive process. The lack of a demonstrable exchange reaction in N_2 fixation would support this concept, (Burris, 1966).

Recent definitive in vitro experiments and in vivo studies demonstrating HD formation with both nitrogenase and model systems, have however provided the first experimental evidence, although indirect, for the occurence of nitrogenase-bound diimide and hydrazine, (Hoch, Schneider and Burris, 1960; Jackson, Parshall and Hardy, 1968). Turner and Bergersen (1969) have demonstrated that the deuterium exchange reaction was dependent on N_2, ATP and a reductant. No enhancement of exchange was observed when substrates for reduction were C_2H_2 or CN, which would not be expected to have enzyme bound intermediates.

2.7 Mechanism of Nitrogenase Activity

The manner in which nitrogen gas is most likely reduced to NH_3 by nitrogenase is shown in figure (2). It can be seen that there are two active sites on the nitrogenase enzyme; an electron activating site (x) and a substrate complexing and reducing site (y). The two sites can be separated on the basis of carbon monoxide and H_2 sensitivity, (Hardy and Burns, 1968). Thus while carbon monoxide inhibits both reduction of N_2 and hydrogen exchange, ('classical hydrogenase' activity) carbon monoxide does not affect the reductant dependent ATPase or ATP dependent H_2 evolution.

Activation of N_2 is undoubtly the most significant physiological aspect of nitrogenase activity. Recent studies indicate that an electron from a suitable donor, including dithionite, ferredoxin or flavodoxin, may react with an oxidised group X_{ox} on the nitrogenase enzyme and utilize ATP to produce ADP and Pi and a reduced group of increased reduction potential (X_{red}).
Das et al (1967) suggested that the X_{red} may exist as a hydride, but the sequence of these initial events is unknown. Prior reduction is favoured, (Hardy and Burns, 1968). Four ATP molecules per pair of electrons transferred appear to be necessary, (Burris, 1969) and molybdenum is involved at this site, (Hardy and Burns, 1968; Bergersen, 1971).

The role of ATP has not been clearly defined. An ATP induced conformational change in nitrogenase during N_2 reduction was proposed by Bulen et al (1965). Recently Kennedy (1970) claimed that ATP did induce conformational changes in the nitrogenase enzyme from R. Lupini. The conformational changes, he considered occurred before substrate reduction, and would generate a reduced chemical species on the enzyme with greater reactive potential, comparable to the conformational states which have been proposed to accompany the electron transfer mediated synthesis of ATP within biological organelles, such as mitochondria and chloroplasts.

Theories on substrate complexing and reduction will not be dealt with in this review. Hardy and Burns (1968) and Hardy et al (1972) have outlined possible schemes.

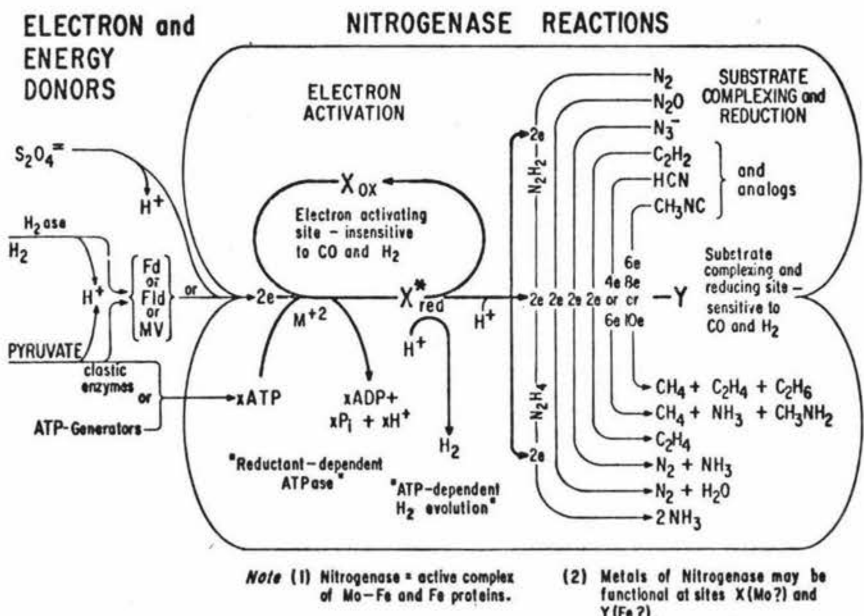


FIG. 2. Scheme for nitrogenase and its reactions based on the electron-activation two-site hypothesis

(After Hardy and Burns (1968)

What has emerged from recent studies then, is the concept that nitrogenase acts as a powerful and versatile redox catalyst, coupling ATP hydrolysis and electron transfer in an integrated reaction. For many workers, electron activation is the limiting reaction in nitrogen fixation.

2.8 Ammonia Assimilation

While in bacteroid suspensions (Bergersen and Turner, 1968) and cell free extracts, (Koch et al, 1967a,b; Bergersen and Turner, 1968) the nitrogen fixation process terminates with ammonia, in nodules the ammonia is rapidly assimilated into amino compounds, (Aprison, Magee and Burris, 1954; Bergersen, 1965 and Kennedy, 1966a,b). The primary assimilation step in serredella and soyabean nodules is the incorporation of ammonia into glutamic acid, (Aprison et al, 1954 and Kennedy, 1966a,b). The presence in bacteroids of isocitric dehydrogenase and glutamic dehydrogenase, (Kennedy et al, 1966 and Mooney and Fottrell, 1968) would enable the reaction to precede by diverting ketoglutarate from the tricarboxylic acid cycle. There is evidence that organic acids of the tricarboxylic acids are only partially oxidised, (Bergersen, 1971).

The existence in nodules of two ammonia pools, one of which was in rapid equilibrium with newly fixed nitrogen and one which was not, has been reported by Bergersen (1965) and Kennedy (1966a). One of these pools may be within the bacteroid or bacteroid plus enclosing membrane and the other maybe in the host cell, (Bergersen, 1971). Thus most transamination reactions as well as the primary assimilation reaction could be mediated by the bacteroids, (Bergersen, 1971).

The amino compounds do not accumulate in the nodule, 80-100% of fixed nitrogen being translocated almost immediately, (Pate, 1958). The main products translocated are aspartic acid, asparagine, glutamine and homoserine, with small quantities of glutamic acid, valine and leucine/isoleucine, (Pate, 1962).

Two alternative theories have been advanced to explain the export of nitrogenous compounds from the nodule. In the

first, it is proposed (Pate, et al 1969) that the pericycle cells leak solutes passively into the apoplast of the stele. Source-sink gradients for amino acids have been demonstrated between the bacterial cells and the nodule cortex, but other analyses have shown that fluids exported from the nodule are many times more concentrated in certain amino acids than are the surrounding donor tissues (Pate et al, 1969). There is also the problem of how nitrogenous solutes and not carbohydrates are discharged into the apoplast. Pate et al, (1969) suggested that the wall protuberances and specialized cytoplasmic structures function in the efficient retrieval from the apoplast of sugars and certain amino acids not destined for export in the xylem.

The second theory proposed by Pate and workers (Pate et al, 1969; Pate and Gunning, 1972) is more fully consistent with the analytical data on amino acid export. Pate and Gunning (1972) contended that the dominant function of the specialized cells of the nodule pericycle (transfer cells) is an active and selective secretion of nitrogenous compounds, in particular amides into the bundle apoplast. Evidence of a glandular activity was obtained from bleeding experiments conducted on detached nodules. Copious bleeding from the cut xylem at the distal end of the nodule occurred immediately and during the next few hours when detached nodules were placed in water. The sap contained amides and certain amino acids up to ten times the concentration of those in the donor nitrogen-fixing tissues of the nodule, but no sugars were detected.

Pate and Gunning (1972) considered that the amino compounds secreted by the transfer cells in the nodules lower the water potential of the bundle apoplast, leading to an influx of water across the endodermis, and the flushing of solutes out to the rest of the plant in the xylem. The rapid removal of amino compounds would also maintain a steep concentration gradient between the bacterial tissues and the transfer cells, thus ensuring a rapid flux of nitrogenous compounds to the nodule pericycle.

2.9 Substrate for Metabolism in Bacteroids

The absolute requirement for a product or products of

photosynthesis for nitrogen fixation has been demonstrated by a number of workers including, Virtanen, Moisio and Burris (1955); Wheeler (1971); Bergersen (1970) and Lawrie and Wheeler (1973) in experiments designed to measure the effect of artificial darkness on nitrogen fixation. Virtanen et al (1955) and Lawrie and Wheeler (1973) detected little nitrogen fixing activity in Pisum plants after 24 hours darkness. Wheeler (1971) found the rate of fixation in Alnus nodules to be 50% of control plants after 12 hours darkness, and no significant activity could be detected after one day. No change in activity was noted during another 72 hours in the dark. Lawrie and Wheeler (1973) were also able to show that the rate of recovery of nitrogenase after dark treatment was dependent on the length of the dark period; the longer the plants were exposed to darkness, the slower the rate of recovery. The rate of recovery of nitrogenase also correlated well with the rate of accumulation of labelled photosynthates in the nodules.

The marked diurnal pattern in nitrogen fixation in both legumes, (Hardy, Holsten, Jackson and Burns, 1968; Bergersen, 1970) and non-legumes, (Wheeler, 1969; 1971) appears to be correlated with the quantity of photosynthates moving from the leaves to the nodules. Where the supply of photosynthates to the nodule is interrupted for example, in the detachment of nodules from soyabean roots, (Bergersen, 1970) and stem ringing of alder plants, (Wheeler, 1971) significant reductions in rates of acetylene reduction, and thus nitrogen fixing activity have been recorded, thus emphasizing the importance of photosynthates in nitrogen fixation.

The increase in nitrogen fixation in Alnus nodules during midday was not correlated with increases in carbohydrate levels in the nodule tissue as a whole, (Wheeler, 1971). It was suggested by Wheeler that a substantial part of the nodule carbohydrates were unavailable for fixation and that maximum rates of fixation were attained only when newly synthesized photosynthates were entering nodules in quantity, producing temporarily high concentrations in infected cells. That more labelled photosynthates were found to accumulate in the younger infected cells, in which the bacteroids

were still multiplying than in cells densely filled with bacteroids in *Pisum sativum* nodules, (Lawrie and Wheeler, 1973) probably explains the failure of Wheeler (1971) to obtain a significant relationship between nitrogen fixation and accumulated sugars. Nitrogen fixing activity was most clearly associated with the densely packed bacteroid cells in *Pisum* and Lawrie and Wheeler (1973) concluded that nitrogen fixation must have been supported by a rapid undetected flux of photosynthate through the densely filled cells. The photosynthates do not accumulate significantly and are metabolized soon after arrival.

Much of the sugar entering nodules, appears from the study of Lawrie and Wheeler (1973) to be used for the support of growth and development of bacteroids. Minchin and Pate (1973) obtained similar results for field peas. Of the photosynthates translocated to the nodule, 17% was consumed in growth, 36% in respiration and 47% was returned to the shoot.

It is generally agreed that glucose, fructose and particularly sucrose are the dominant photosynthates transported to the nodules. That additions of these sugars to soyabean nodule slices enhanced nitrogen fixation, (Bach, Magee and Burris, 1958) is evidence that they are utilized for metabolic purposes in the nodules. The disaccharide, sucrose is probably cleaved to hexoses by a plant invertase, shown by Kidby (1966) to be present in serredalla nodules. The hexoses are then the real substrates which support nitrogen fixation in the bacteroids, (Kidby and Parker, quoted by Bergersen, 1971). Goodchild and Bergersen (1966) have shown that host cell mitochondria are prominent in the periphry of the bacteroids of soyabean nodules, and it is possible that these organelles could be active in the preliminary metabolism of photosynthetic products in nodules, (Bergersen, 1971).

Significant quantities of reserve carbohydrate material are present in the nodules of soyabeans, (Wong and Evans, 1971) *Pisum sativum*, (Lawrie and Wheeler, 1973) and Alnus, (Wheeler, 1971). Bergersen (1971) suggested that poly β hydroxybutyrate might be used by the bacteroids to generate reducing power and ATP during the

basal level of nitrogen fixation, which occurs in soyabean nodules during darkness or after detachment from the root system. However, Wong and Evans (1971) could not demonstrate a direct role for poly β hydroxybutyrate in nitrogen fixation when supplies of carbohydrates to the nodules were reduced through darkness or detachment of nodules. Wheeler (1971) and Lawrie and Wheeler (1973) were also unable to provide evidence of reserve carbohydrates, including starch being used as suggested by Bergersen (1971).

2.10 The Role of Leghaemoglobin

Leghaemoglobin is invariably associated with nitrogen fixing nodules, and because of the strong correlation between leghaemoglobin concentration and nitrogen fixed, many attempts have been made to assign a key role in nitrogen fixation to this pigment. Bergersen (1971) has summarized the proposed roles and concluded that the role of leghaemoglobin which appears most probable is that proposed by Yocum (1964). Yocum suggested that the ventilation of the energy-producing sites in the dense nodule tissue is possibly assisted by the leghaemoglobin solution which surrounds the bacteroids.

Leghaemoglobin is a myoglobin - like haemoprotein and in soyabean nodules there are at least two molecular forms, differing in molecular weight and amino acid composition (Ellfolk, 1961; Ellfolk and Sievers, 1967 and Appleby, 1969c). Both host cells and bacteroids are involved in the synthesis of leghaemoglobin. The host is responsible for the synthesis of the protein (Cutting and Schulman, 1968 and Dilworth, 1969) and the bacteroids the synthesis of haem (Cutting and Schulman, 1968).

Leghaemoglobin has a high affinity for oxygen, being half oxygenated at a pO_2 of 0.05 mm Hg (Appleby, 1962). However, two states of leghaemoglobin exist in soyabean nodule tissue, a ferrous-oxyleghaemoglobin and a ferrous or reduced leghaemoglobin (Keilin and Smith, 1947). Appleby (1969c) has demonstrated that in young soyabean nodules, 20% of the leghaemoglobin was in the ferrous-oxy form, the balance in the reduced form. The proportion of oxyleghaemoglobin rises rapidly with increasing oxygen concentration,

particularly at those oxygen concentrations where nitrogen fixation is inhibited (Bergersen, 1962a,b).

The high affinity of leghaemoglobin for oxygen should then aid the diffusion of oxygen in densely packed nodule tissue. Scholander (1960) has demonstrated that the diffusion of oxygen through a membrane saturated with a solution of haemoglobin or myoglobin was much faster than the diffusion of nitrogen in the same system. Scholander (1960) also showed that oxygen was transported at low concentration gradients, up to eight times more rapidly through haemoglobin solutions than through water.

Since soyabean leghaemoglobin is 80% oxygenated at a pO_2 3 x 10^{-1} mm Hg, and only 15% oxygenated at a pO_2 2 x 10^{-2} mm Hg (Appleby, 1962), the loading and unloading tensions for oxygenation and deoxygenation of leghaemoglobin do not limit the rate of oxygen diffusion to the bacteroids (Bergersen, 1971). Leghaemoglobin is, therefore much suited for the role of faciliating oxygen diffusion under the low concentrations of oxygen which obtain in nodules. The properties of the haemoprotein permit a high flux of oxygen to be maintained through the tissue. However, since the rate of consumption of oxygen by bacteroids is high, the free oxygen concentration in the tissue is maintained at a low level (Bergersen,1971). At normal atmospheric oxygen concentrations, leghaemoglobin maintains a supply of oxygen to the bacteroids at a rate high enough for adequate ATP synthesis, yet not at a rate likely to cause oxygen inactivation of the nitrogenase, or conditions where oxygen might become a competitive inhibitor of nitrogen fixation as a result of the greater utilization of reducing power in respiration.

3.1 EFFECT OF COMBINED NITROGEN ON NODULATION AND NITROGEN FIXATION

The effect of combined nitrogen on nodulation and nitrogen fixation has been the subject of many reports and reviews, including those of Wilson (1940), Nutman (1956; 1965a), Raggio and Raggio (1962) and Stewart (1966).

It is generally accepted that application of high concentrations of combined nitrogen to the growing medium of legumes reduces nodulation and nitrogen fixation. On the other hand, some workers have also obtained evidence to suggest that small applications of combined nitrogen may stimulate nodulation and nitrogen fixation, (Nutman, 1956; 1965a; Raggio and Raggio, 1962 and Stewart, 1966).

The correlation of the degree of nodulation and the rate of nitrogen fixation with the carbohydrate status of the host plant led earlier workers, including Allison (1935), Wilson (1935), Wilson and Wagner (1935) and Wilson (1940) to formulate the hypothesis relating the carbohydrate - nitrogen ratio to the various aspects of the symbiosis. These workers considered that any movement away from a critical carbohydrate - nitrogen ratio would result in a much lowered nodulation and nitrogen fixing activity.

The ability of such a concept to explain the effect of combined nitrogen on legume symbiosis has been questioned by Nutman (1956) and Raggio and Raggio (1962). Nutman (1956) considered that "this relation, however, only summarizes experience and does not provide an explanation". Nutman (1956; 1965a) indicated that an explanation in terms of changes in the growth regulating mechanisms in hosts, in particular, in those factors affecting root morphogenesis and cell phsiology might be more profitable. Stewart (1966) likewise concluded that other internal factors, including growth substances could affect nodulation and nitrogen fixation and that the activity of these factors may be altered through additions of combined nitrogen.

However, applications of combined nitrogen might simply reduce the supply of carbohydrate to nodules by increasing the demand

for carbohydrates in other areas of the plant, as a result of the incorporation of combined nitrogen into amino compounds and protein. Evidence in support of this theory was obtained by Small and Leonard (1969) in studies on the translocation of ^{14}C labelled photosynthate in nodulated pea and subterranean clover. Applications of combined nitrogen reduced the quantity of photosynthate translocated to the nodule, and increased that translocated to the root, presumably as a result of more active root growth.

3.2 Nodulation

The inhibitory effect of large concentrations of combined nitrogen on nodulation have been obtained in pot trials by Thornton and Nicol (1936), Richardson, Jordon and Garrard (1957) and Munns (1968b) for lucerne (Medicago sativa), Darbyshire (1966) for Trifolium repens, Dart and Wildon (1970) for cowpea (Vigna sinensis) and purple vetch, (Vicia atropurpurea) and by Oghoghorie and Pate (1971) for field pea (Pisum arvense). Richardson et al (1957), Dart and Wildon (1970) and Oghoghorie and Pate (1971) have also recorded a stimulation in nodulation at low levels of combined nitrogen in the growing medium.

It is generally agreed that nitrate nitrogen is more inhibititory than other forms of combined nitrogen, including urea, ammonium nitrogen and organic forms, (Richardson et al, 1957; Raggio and Raggio, 1962; Darbyshire, 1966; Dixon, 1969 and Dart and Wildon, 1970). That the inhibitory effect of nitrate nitrogen on nodulation operates externally as well as internally could account for the more severe reductions in nodulation noted when legumes are grown in media containing nitrate, (Raggio et al, 1965, Munns, 1968a,b,c). Tanner and Anderson (1964) showed that IAA produced by the rhizobia is oxidised and 'destroyed' by nitrite formed from the reduction of nitrate. The inhibitory effect of nitrate on nodulation can be reversed in part by exogenous supplies of IAA, (Valera and Alexander, 1965; Munns, 1968b,c).

Thornton (1935) claimed that the reduction in nodule number with application of combined nitrogen is primarily a result of a reduction in the number of curled root hairs. Munns (1968b) has

disputed Thornton's hypothesis. In Medicago sativa, even with application of nitrate, the number of curled root hairs greatly exceeded the requirement for nodulation. On the other hand, Munns found that infection threads outnumbered nodules by a much smaller ratio than curled root hairs. The effect of nitrate on the formation and development of infection threads could then account for much of its effect on nodule number. The action of nitrate is therefore not confined to any one phase of the infection process. Large numbers of infection threads become arrested and disorganised during growth in the presence of nitrate in Medicago sativa, (Munns, 1968b).

Although nodule numbers were found to decline over a range of high nitrate levels in the studies of Thornton and Nicol (1936) and Oghoghorie and Pate (1971) the total nodule weight or volume of bacterial tissue per root and the average size of nodule decreased more markedly.

The response of legume rhizobium symbiosis to combined nitrogen is complicated by other factors, the pH of the medium, (Fletcher, 1959; Mulder and VanVeen, 1960) growth temperatures, (Meyer and Anderson, 1959) and general climatic conditions, including day length and light intensity, (Lyons and Early, 1952; Pate and Dart, 1961). The nodulation response to combined nitrogen is also influenced by the Rhizobium strain used, (Pate and Dart, 1961).

Time of application of combined nitrogen during the growth of legume seedlings is also important. In studies with cowpea seedlings, nitrogen applied in small supplements at sowing benefitted symbiosis by increasing seedling growth rate and the number and size of nodules, (Pate and Dart, 1961). While excessive amounts of nitrogen applied at this stage did depress nodulation, by reducing the number of successful infections on the primary roots, Pate and Dart (1961) considered that the damage may not be permanent since slight reductions in nodule numbers were often offset by increases in nodule size and efficiency. Further, severe restrictions on early nodulation may be offset by a more

extensive and effective nodulation of the lateral root system, providing the levels of applied nitrogen have declined. Such effects were obtained by Dart and Wildon (1970) in later studies with cowpea and purple vetch.

The developing symbiotic association in cowpea was, however, particularly sensitive to combined nitrogen applied during cotyledon unfolding, (Pate and Dart, 1961). These workers suggested that the channelling of photosynthetic products into protein synthesis in the expanding shoot system could have limited the availability of carbohydrate and other growth materials for root and nodule growth. Nodule number was not affected by applications of nitrogen at this stage or later stages in host growth. Munns (1968a) who obtained similar results with lucerne seedlings, suggested that nodule number was being controlled on the one hand by nitrate nitrogen and on the other by the inhibitions from the first established nodules on the initiation of further nodules. Thus in nil combined nitrogen treatments, the initial abundant nodulation impairs further rapid nodulation. Subsequent nodule production could be inhibited approximately equally by prior nodulation the abundant nodulated control plants and by nitrate in the sparcely nodulated plants in the nitrate treatment, (Munns, 1968a). When the effect of nitrate was tested on plants comparable in size and in a number of previously established nodules, nodule production was inhibited by 70 - 80% irregardless of plant age or whether the plants were previously nodulated or not, (Munns, 1968a). These findings further demonstrate that the nodulation response to combined nitrogen is influenced by a multiplicity of factors, including plant growth and previous nodulation status.

3.3 Nitrogen Fixation

Generally combined nitrogen depresses nitrogen fixation, (Allos and Bartholomew, 1959; Moustafa, Ball and Field, 1969; Dart and Wildon, 1970 and Oghoghorie and Pate, 1971). Low levels of combined nitrogen can however, increase nitrogen fixation, (Allos and Bartholomew, 1959; Dart and Wildon, 1970, and Oghoghorie and Pate 1971). The increases in fixation with combined nitrogen fertilization are associated with increases in plant growth, (Allos and Bartholomew, 1959; Dart and Wildon, 1970).

3.4 PHOSPHORUS AND NODULATION AND NITROGEN FIXATION

The much improved growth of the legume component after the addition of phosphate to pasture is indirect evidence of the involvement of phosphate in nodulation and nitrogen fixation. Direct evidence, is however, difficult to find. Van Schreven (1958) and Vincent (1965) have summarized the earlier information available on the effect of phosphate on nodulation and nitrogen fixation, and concluded that phosphate fertilization increases nodulation and nitrogen fixing activity in a ranage of legumes. Gukova and Arbuzova (1969) and Gates (1970) have also reported similar findings and Gates noted that phosphate ameliorated the inhibitory effect of combined nitrogen on nodulation in Stylosanthes humilis. Phosphate can also reduce nitrogen fixation in subterranean clover before it becomes limiting for plant growth (McLachlan and Norman, 1961).

4.1 ASSIMILATION AND TRANSPORT OF NITROGEN IN NODULATED LEGUMES

Pate (1968; 1973) and Oghoghorie and Pate (1972) have reviewed the literature on this subject. Inorganic nitrogen is assimilated at three points in nodulated legumes receiving nitrate nitrogen; in the nodules and in the centres of nitrate reduction in root and shoot material (Fig 3). The activity of the nitrate reductase system in the root and shoot is dependent however, on the availability of nitrate in the rooting medium. At very high levels of nitrate, a pattern of assimilation in field peas dominated by the shoot reductase system is promoted, while at low levels, most assimilation occurs in root material, and the nitrate reductase potential of the shoot is not exploited (Oghoghorie and Pate, 1971; 1972).

Two major transport systems distribute the assimilated nitrogen about the plant. Products from assimilation of nitrate nitrogen in roots, and from nitrogen fixation in nodules, in excess of requirements for growth, are carried to the shoot system in the xylem (pathway1, Fig 3). In field peas (Oghoghorie and Pate, 1972) this

(After Oghoghorie and Pate, 1972)

Nitrogen Transport in a Nodulated Legume

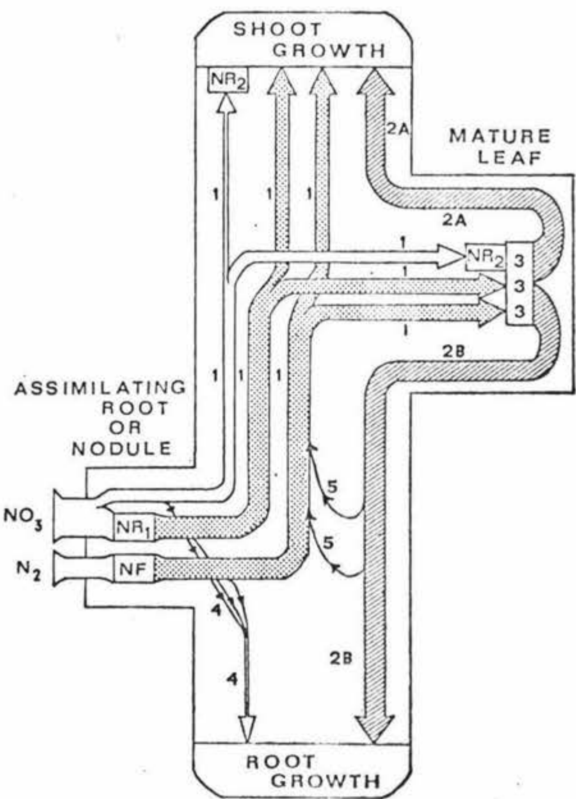


Fig. 3 Scheme illustrating the main features of the assimilatory and transport systems for nitrogen in the field pea. The situation represented is a young vegetative plant, effectively nodulated and growing in the presence of a moderately high level of nitrate in the medium. a) Sites of assimilation: *NF* nitrogen-fixing nodules; NR_1 root-located nitrate reductase; NR_2 shoot-located nitrate reductase. b) Pathways of transport: *1* xylem transport; *2A*, *2B* upward and downward phloem transport respectively; *3* transfer from leaf to phloem of minor veins; *4* direct transfer to distal, non-assimilating parts of root; *5* transfer from downward translocation stream to upward stream of nitrogen in transpiration stream. c) Compounds transported — ▭ free nitrate; ▭ amides, ureide + some amino acids; ▭ variety of amino acids + some amide. Note: The breadth of each pathway gives an approximate indication of its relative significance in transporting nitrogen

pathway carried large amounts of amide nitrogen (asparagine and gluramine) and some ureide nitrogen (allantoic acid) and some amino acid nitrogen (aspartic acid and homoserine) and the level of free nitrate in xylem bleeding sap varying with the availability of nitrate nitrogen in the rooting medium.

Nitrogen compunds are also distributed from mature leaves via the phloem (pathways 2A, 2B Fig 3) to centres of growth in the shoot and root (Oghoghorie and Pate, 1972). Phloem sap collected from petioles and stems of pea plants contained not only amino compounds typical of xylem transport, but also contained relatively high levels of other amino acids, such that the total compliment of amino compounds was in almost correctly balanced proportions for direct incorporation into protein. Pate (1968) and Oghoghorie and Pate (1972) considered this stream of nitrogen to be an extremely important source of nitrogen for young leaves and shoot meristems and for growth in nodulated roots.

In field peas, little organic nitrogen was transferred directly from the nodules to the growing points of the roots, and Oghoghorie and Pate (1972) concluded that the cycling of newly assimilated nitrogen from nodules to the shoot system and back to the root system was an extremely important part of the total transport system operating in plants growing solely on nodule nitrogen (Pathway 1 - 3 2B; Fig 3). The results also indicated that the shoot was just as important in the nitrogen nutrition of the root as the nodules were in providing new supplies of nitrogen to the whole plant.

The relative importance of each transport system would be expected to change with the application of nitrate nitrogen and the level of nitrate in the rooting medium could further affect the operation of various elements of the transport system.

CHAPTER II

EXPERIMENTAL

1.1.1 INTRODUCTION

Several experiments were conducted in this study. The experiments were designed to measure the effect of nitrate nitrogen and phosphate on the various phases of legume-rhizobium symbiosis. The effect of nitrate and phosphate on root hair curling, infection thread development and early nodulation was to be evaluated in Experiments 1 and 2. Experiments 3 and 6 were designed to determine the effect of nitrate nitrogen and phosphate on the nodule number and/or weight and nitrogen fixing activity after clover plants, not previously nodulated, were grown in the nutrient solutions for periods of up to four weeks, (Experiment 3) and six weeks (Experiment 6). The effects of these treatments on nodule development and nitrogen fixing activity in fully nodulated mature clover plants were evaluated in Experiments 4 and 5.

Since Experiments 1 and 2 were conducted under artifical light in a 'growth cabinet' and Experiments 3, 4, 5 and 6 were conducted in the Agronomy Department Glasshouses, the discussion on materials and methods will be divided into two sections, the first dealing with the 'Growth Cabinet' experiments and the second, the glasshouse experiments.

1.2.1 MATERIALS: GROWTH CABINET EXPERIMENTS

1.2.2 Plant

'Grasslands Huia' White Clover (C2478) seed was used in Experiments 1 and 2. The seed was kindly supplied by Mr.J. Hoglund (D.S.I.R. Palmerston North).

1.2.3 Rhizobium

Four *Rhizobium* strains, $^{C}514/1$, $^{C}1/7$, ^{C}TAI and $^{C}5039$ (*Rhizobium trifolii Dang*) known to be effective in nodulating

'Grassland Huia' White Clover were supplied by Mr. Greenwood (D.S.I.R. Palmerston North). The cultures were subcultured regularly (10 day intervals) on to nutrient slopes. A standard Bacto nutrient agar (Difco Bacto Nutrient Agar) was used as the nutrient medium for the rhizobium cultures.

1.2.4 Pots

Clear perspex containers measuring 8 x 8cm on top and 7 x 7cm at the base and 11cm high were used. The containers were painted twice with black Gaydec paint to prevent algal growth in the pots, and covered with a heavy grade aluminium foil to reflect the heat (Plate 1).

Two glass tubes were inserted into the pots, one at the base to permit the inflow of nutrient solution from a reservoir placed at the side of the 'growth cabinet' and the other at the 500ml mark to drain off the overflow (Plate 2).

The tops of the pots were covered with the heavy grade aluminium foil. Twelve holes were punched into the foil at regular intervals to provide the positions for 'planting' the seedlings (Plate 2). Three layers of fine nylon mesh were fixed to the underside of the foil with Araldite to support the seedlings (Plate 2).

1.2.5 Nutrient Solutions

A base nutrient solution of the following composition was used; $CaSO_4$ 4mM, K_2SO_4 2mM, $MgSO_4$ 1mM, FeEDTA 30μM and KCL 10μM. and one ml of the following trace element mixture was added to each litre of nutrient solution:

Boric acid	2.86 gm / litre
Manganese chloride	1.18 gm / litre
Zinc sulphate	0.11 gm / litre
Copper sulphate	0.05 gm / litre
Sodium molybdate	0.025gm / litre
Cobalt chloride	0.05 gm / litre

The $CaSO_4$ was prepared as a 10mM solution when required, the K_2SO_4 and $MgSO_4$ as molar solutions, the FeEDTA as 0.5M solution and KCL as a 0.1M solution. The concentrated solutions were diluted

with distilled water and the nutrient solutions were corrected to pH 6.0 with 0.1N H_2SO_4.

1.2.6 'Growth Cabinet'

The 'growth cabinet' 142cm x 122cm x 46cm high was constructed by the author to standardize the light intensity, photoperiod and temperature during the duration of the experiments.

Lighting was provided by a bank of 'warm daylight' fluorescent tubes and six 25 watt tungsten lamps were also included in the lighting to maintain a balance in red/far red wave lengths. Aluminium foil was placed around the sides of the cabinet to prevent loss of light intensity at the margins of the cabinet. Readings taken with an Eppley pyrrheliometer showed that the photosynthetically active radiation level was about 110 watts m^{-2} or $\frac{1}{4}$ full sunlight. 'Daylight' temperatures were maintained at 24 to 25°C throughout the duration of both experiments by directing cool air into the cabinet. A temperature of 35°C was recorded when the cooling system was not operating. The night temperature was set at 20°C,

A photoperiod of 16 hours light and eight hours dark was used in these experiments.

1.3.1 EXPERIMENTAL

1.3.2 Design

In both Experiments, 1 and 2, a factorial design was used, with two factors nitrate nitrogen and phosphate. There were five levels of nitrate nitrogen and five levels of phosphate in Experiment 1, while in Experiment 2 six levels of nitrate nitrogen and four levels of phosphate.

Two replicates were included in Experiment 1 and four in Experiment 2. Since only two replicates could be run at one time, replication in time was used in Experiment 2.

1.3.3 Layout and Randomization

The cabinet area was divided into two replicate blocks, so that any treatment differences, which may have arisen from

Plate 1 'Growth Cabinet'

A 'Warm daylight fluorescent tubes

B Tubing conveying solution from reservoir pots

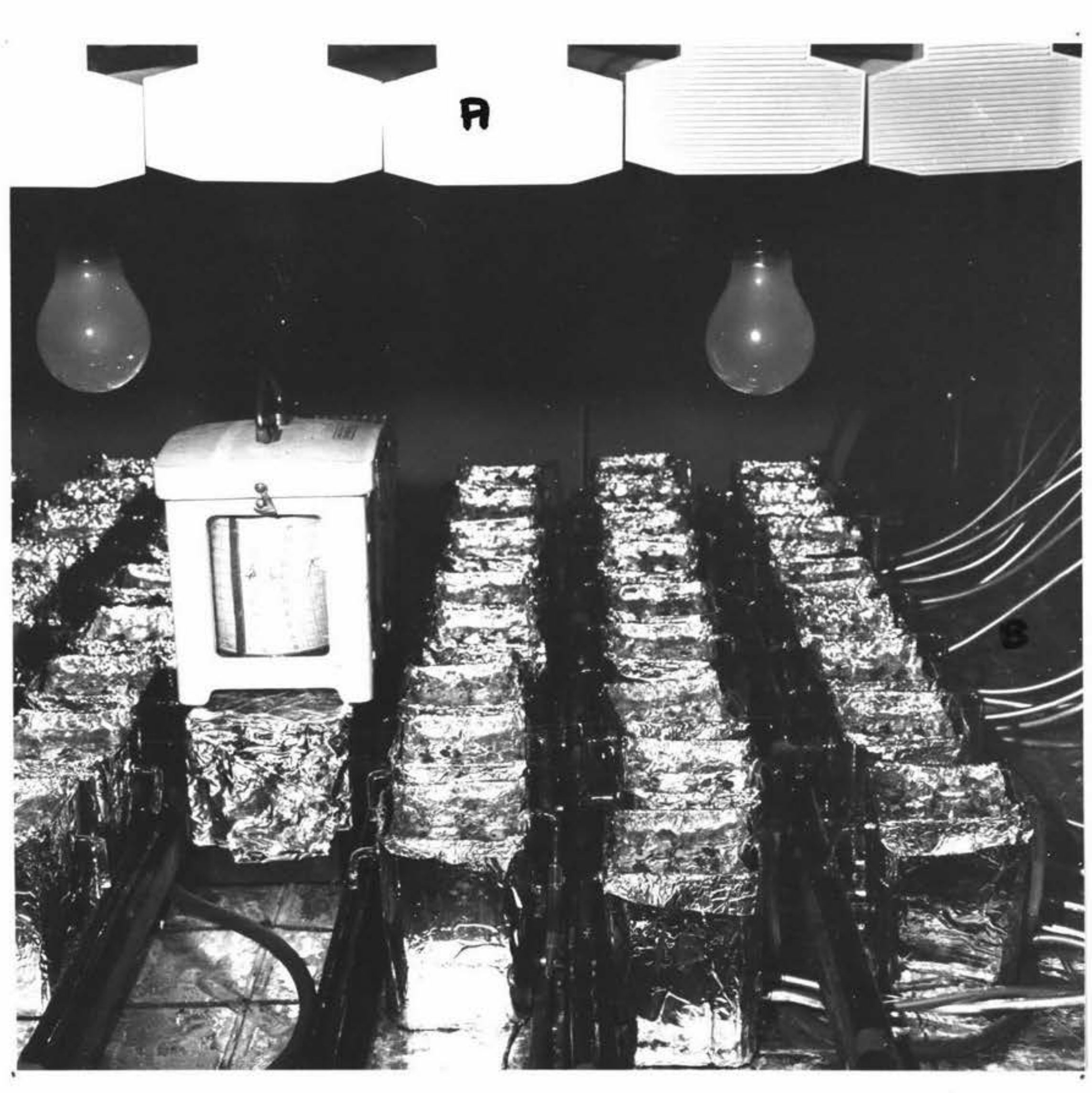

A
B

Plate 2 Close up view of a pot

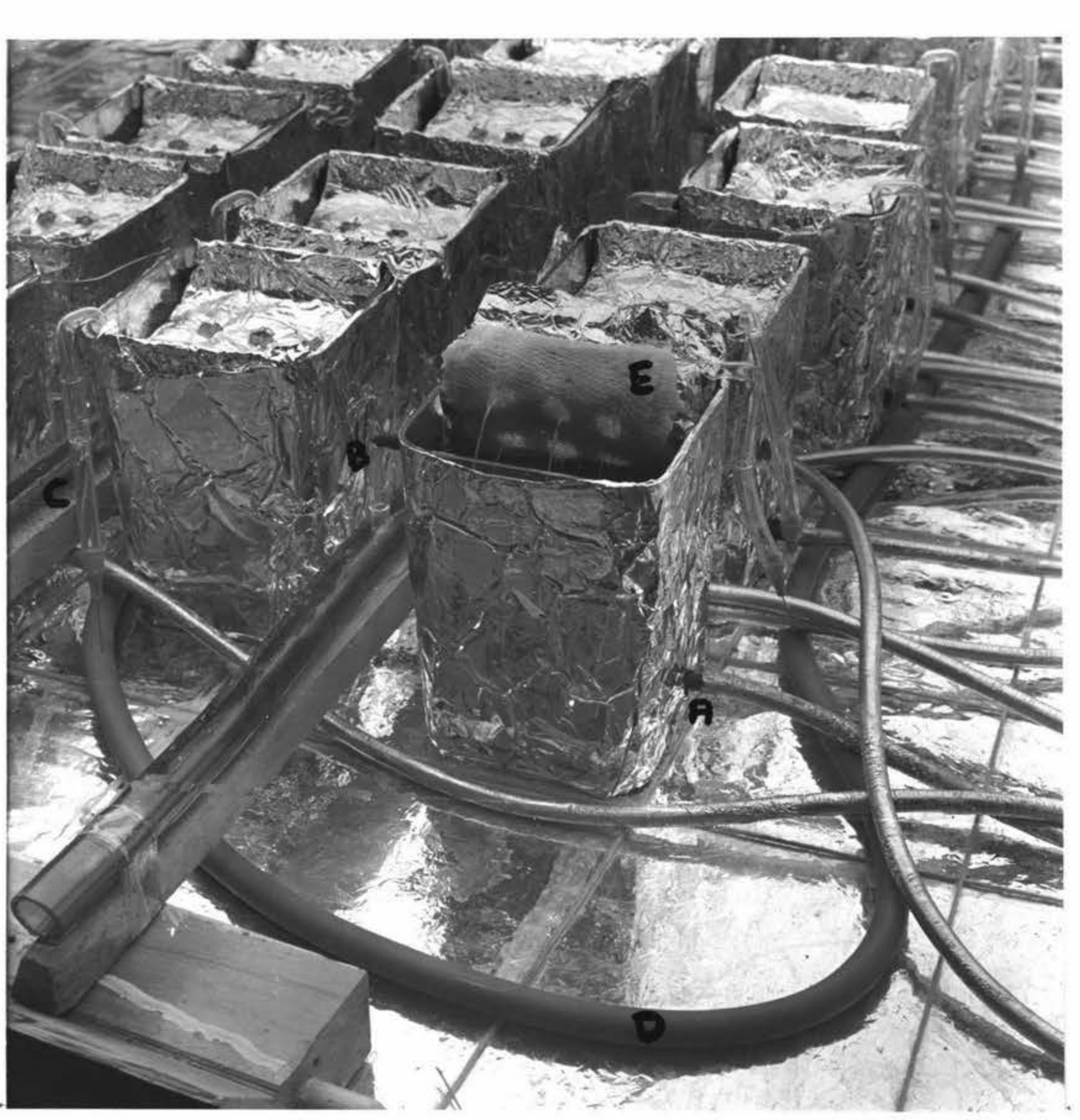
A Inlet

B Overflow

C Aerators

D High pressure rubber tubing air lines

E Fine nylon mesh


E
B
C
A
D

temperature changes in the cabinet, brought about by the introduction of cool air at one end of the cabinet, would be accounted for in the between replicate analysis of variance.

Within each replicate block, the pots were arranged in rows of eight or nine pots (Plate 1). Each treatment was allocated at random within the replicates. The pots were not shifted during the running of each experiment.

1.3.4 Treatments

Nitrogen

The following levels of nitrate nitrogen were used:

	NO_3N (mM)
Experiment 1	0, 1, 2, 4, 8
Experiment 2	0, .1, .5, 1, 2, 4

Phosphate

Phosphate was supplied at the following levels:

	Phosphate (mM)
Experiment 1	0.1, 0.5, 1.0 2.0, 4.0
Experiment 2	0, 0.005, 0.05, 0.5

To prevent the depletion of these concentrations of combined nitrogen and phosphate, the nutrient solutions were added constantly to each pot at a rate of 500mls / pot/ day. The rate of flow was controlled by the height of the reservoir pots, (positioned outside the cabinet) above the pots in the cabinet, and by the length of fine capillary tubing inserted into the tubing conveying the nutrient solution from the reservoirs to the pots. A constant level of nutrient solution in the reservoir was achieved by displacing nutrient solution from a plastic bottle which was inverted over each reservoir pot. The 250ml of nutrient solution in each plastic bottle was renewed every 12 hours.

No attempt was made to collect the overflow from the pots. Plastic tubing positioned under the outlets (Plate 1) conveyed the

solution to a 5cm polythene pipe placed at one side of the 'growth cabinet'. The solutions were then discharged into a sink.

1.4.1 CONDUCT OF EXPERIMENTS 1 AND 2

The 'Grasslands Huia' White Clover seed was sterilized with a 0.1 N $MnC1_2$ solution and then thoroughly rinsed with sterilized distilled water. The seed was then imbibed in aerated sterilized distilled water for 16 hours and transferred to petri dishes lined with moistened filter paper to germinate at room temperature.

For each pot, twelve 24-hour seedlings which showed radicle development were carefully 'planted' into the nylon mesh and the aluminium tops suspended over the surface of each nutrient solution. Before cotyledon unfolding, the nutrient solution was allowed to bathe the seed to prevent dessication and hardening of the testa. After unfolding, however, the foil tops were lifted just clear of the solution to reduce the possibility of salt accumulating on the leaf material.

The nutrient solutions were inoculated immediately with a 5ml suspension of rhizobia* and later at two, four and six days after the commencement of the experiments. The 500 mls of nutrient solution were added constantly to the pots as from 'planting'.

Two plants were harvested from each pot six days after commencement of treatments and the remainder of the plants were harvested six days later.

1.5.1 PREPARATION OF MATERIAL AND METHOD OF ANALYSIS

The plant roots were detached from the seedling tops and placed in a F.A.A. fixing solution.

The number of curled root hairs and infection threads formed on the roots after six days were to be recorded. However, because considerable difficulty was experienced in removing the

(* 5mls of distilled water was added to each of eight culture bottles (two from each strain) and the 40mls of suspension diluted to 250mls)

'rhizosphere', (Dart and Mercer, 1963) which obscured the root hairs, these measurements were not completed. Shaking the roots in water did remove the coatings, but this treatment also damaged the root hairs. Munns (1968d) has reported that brushing the roots with a 2% hemosol solution removed the coatings of micro-organisms and ferric hydroxide on Medicago sativa seedling roots, but this material was not available to the author. It would appear that the 'rhizosphere effect' is associated with plants grown in non aseptic conditions.

1.5.2 Nodule Number

The number of nodules formed on 12 day old seedlings was obtained by viewing the roots under magnification.

1.5.3 Dry weight of Tops

Dry weight of tops was taken after drying the top material in an forced air oven at 80°C for 48 hours.

2.1.0 MATERIALS: GLASSHOUSE EXPERIMENTS

Experiments 3, 4, 5 and 6 were conducted in the Agronomy Department Glasshouses, Massey University.

2.1.1 Plant

To overcome the genetic variability known to be present in White Clover seed, clonal material was used in all of the Glasshouse experiments. Five 'Grassland Huia' White Clover plants were established in May 1972 and in August 1972 each plant was subdivided into twenty plants and each established in a separate pot.

The clonal material used in the experiments was removed from these plants and was established in the following manner. The two inch terminal section of stolons growing on the parent plants were removed and placed into a dilute nutrient solution, (Appendix I 2) contained in large shallow 16 litre trays. The stolon sections were supported in the solutions by placing these through holes which were punched at regular intervals in heavy grade aluminium foil positioned over the surface of the nutrient

solution. Non-absorbent cotton wool was packed between the stolon and foil to prevent the stolon movement.

After a week, the clones were carefully removed from the foil and grouped according to leaf area and size of root system. Where more than one root was present on each stolon section, a scalpel was used to remove those not required. After the grouping and selection of plant material had been completed, the plants were immediately allocated at random to the various treatments.

An attempt was made to prevent nodulation occurring on the roots during the establishment period. Stolon sections were placed in a 1/7 janola-distilled water solution for ten minutes and then thoroughly rinsed with sterilized distilled water. However, this procedure was not entirely successful for some nodules (< 6) did form, probably from infections occurring before washing in the diluted janola solution. It is unlikely that the few formed would have significantly influenced the final nodule number or weight.

2.1.2 Rhizobium Cultures

Four Rhizobium strains, $^{C}514/1$, ^{C}TAI, $^{C}5039$ and $^{C}560$ known to be effective in nodulating 'Grassland Huia' White Clover were supplied by Mr.Greenwood (D.S.I.R. Palmerston North). The cultures were subcultured as described in Chapter II 1.2.3.

2.1.3 Pots

In Experiments 3, 4 and 5, 4.5 litre white plastic liver pails, (purchased from the Longburn Freezing Works) were used. The pots and accompanying lids were coated first with black Duo-plastic Gaydec paint and then with Regency Grey Duo-plastic Gaydec paint to prevent algal growth in pots and heating respectively. A one-inch hole was punched in each of the four corners of the lids and cork stoppers, (each with two holes and split) were pushed into these holes. The corks were designed to provide the support for the growing clover clones.

Cardboard divisions, coated with wax in Experiments 4 and 5 and bitumen paint in Experiment 3, were fastened under each lid.

The divisions effectively divided the pot into four compartments, thereby reducing root entanglement between different plants. However, the structures were removed approximately two weeks after the start of the experiments when fungal growth was observed on them. Perspex or plastic materials would have been more successful, but finance was not available to purchase these materials.

The 600ml containers used in Experiments 1 and 2 were also used in Experiment 6, the inflow and overflow tubes being blocked with wax and sealed with araldite. An inch hole was cut out of the centre of each of the plastic lids which accompanied the containers and the cork stoppers used in Experiments 3, 4 and 5 inserted. The lids were finally covered with aluminium foil.

2.1.4 Aeration

Nutrient solutions in Experiments 3, 4 and 5 were forced aerated. Filtered compressed air was drawn off to each pot by inserting hypodermic needles into high pressure tubing air lines placed between the pots. Rubber tubing attached to the hypodermic needles conveyed the air to the pot lids and glass and plastic tubing was used to conduct air through the solution to the bottom of the pots.

Two types of aerators were used. The more successful aerator was a one inch length of 0.5mm capillary glass tubing attached to the end of the plastic tubing conducting air to the bottom of the pot. The other aerator, used only in Experiment 3, consisted of a ten inch length of plastic tubing, punctured at two inch intervals. The ends of the tubing were connected to a plastic T-piece, which was in turn connected to the air line. The tubing was held at the bottom of the pot by the cardboard divisions. When these divisions were removed, the aerators had to be replaced with the capillary pieces.

In Experiment 6, the solution cultures were not forced aerated, as it was hoped that aeration resulting from mixing of solutions when the pH and nitrogen levels were corrected would be sufficient. The plants did not show any adverse reaction, and

it is considered by the author that forced aeration of solution cultures in small containers at least, is not necessary where White Clover plants are being grown.

2.1.5 Nutrient Solutions

(a) Experiment 3

A base nutrient solution of the following composition was used; $Ca\ SO_4$ 5mM, K_2SO_4 5mM, $Mg\ SO_4$ 1mM, FeEDTA 30μM and KCl 10μM. One ml of the following trace element mixture was added to each litre of nutrient solution.

Boric acid	0.72 gm / litre
Manganese chloride	0.25 gm / litre
Zinc sulphate	0.22 gm / litre
Copper sulphate	0.05 gm / litre
Sodium molybdate	0.025gm / litre
Cobalt chloride	0.05 gm / litre

The $Ca\ SO_4$ was made up as a 10mM solution in 60 litre polyethylene containers and a bulk solution, at twice the required strength of K_2SO_4, $Mg\ SO_4$, FeEDTA, KCl made up in separate 60 litre containers. Equal quantities (2.25 litres) of the $Ca\ SO_4$ solution and the bulk nutrient solution were added to each pot. Distilled water was used throughout the experiments and the pH of the nutrient solutions corrected to pH 6.0 with 0.1N $H_2\ SO_4$.

(b) Experiment 4

A base nutrient solution similar to that used in Experiment 3 was used in Experiment 4 for the first 16 weeks of the pretreatment growth period. However, $Ca\ Cl_2$ was used in place of $Ca\ SO_4$ as the calcium source. For the remainder of the pretreatment growth period and the duration of the experiment, a nutrient solution of double the above strength was fed to the plants.

During the pretreatment growth period, a bulk nutrient of K_2SO_4, $Mg\ SO_4$, FeEDTA and trace elements at eight times the required strength was made and 500mls or 1000mls of the solution diluted to 4000mls with tapwater. The calcium chloride was added as a 4M solution after the bulk solution had been diluted. The

pH of the solution was adjusted to pH 6.5 with 0.1N HCl.

Phosphate was added at an increasing rate during the pretreatment growth period. The object being to maintain a low phosphate status throughout the growth of the clover clones. The rate of supply of phosphate was as follows:

0 ——	8 weeks	0.5 mgP / plant / week
8 ——	12 weeks	1.0 mgP / plant / week
12 ——	16 weeks	1.5 mgP / plant / week
16 ——	20 weeks	2.0 mgP / plant / week
20 ——	22 weeks	2.0 mgP / plant / week

Distilled water was used during the actual experiment and the calcium source changed to Ca SO_4. The Ca SO_4 was made as a 10mM solution in 60 litre polyethylene containers, and the required quantities of K_2SO_4, Mg SO_4, FeEDTA, KCl and trace element mixture added to provide the desired nutrient strength. The nutrient solutions were corrected to pH 6.0 with 0.1N H_2 SO_4.

(c) Experiment 5

During the first 16 weeks of the pretreatment growth period, the nutrient solution supplied was the same as that used in Experiment 4. At 16 weeks the nutrient strength was doubled and the level of phosphate increased to 1mM. This rate of supply was maintained during the remainder of the pretreatment growth period and the experimental period.

As in Experiment 4, distilled water and Ca SO_4 were used during the experimental period and the nutrient solutions were made up as described in Chapter II 2.1.5 (b).

(d) Experiment 6

A base nutrient solution of the same composition as in Experiment 3 was used, the solution being made up as indicated in Chapter II 2.1.5 (b).

2.1.6 Temperature Control

A minimum day temperature setting of 22°C was used, but a glasshouse temperature of 24 - 25°C was normal and on sunny, warm

days, temperatures of 28 - 32°C were not uncommon. The minimum night temperature was set at 18°C.

2.1.7 Control of Insects

The glasshouse was fumigated every ten days with Vapona concentrate (dichlorvos).

2.2.0 EXPERIMENTAL

2.2.1 Design

In Experiments 3, 4 and 6 a factorial design with two factors, nitrate nitrogen and phosphate was used. Five levels of nitrate nitrogen and three levels of phosphate were used in Experiment 3 and 4 and three levels of nitrate nitrogen and three levels of phosphate in Experiment 6. The treatments were replicated three times in all of these experiments.

For Experiment 5, a randomized block design was employed with four levels of nitrate nitrogen and four replicates. Three harvests were included, at two, six and twelve days after the commencement of treatments.

2.2.2 Layout and Randomization

(a) Experiment 3

The thirty pots, (fifteen treatments with two pots per treatment) in each replicate were placed in eight rows on two 6' x 3' glasshouse trolleys. The treatments were allocated at random within each replicate, and every second day were shifted clockwise two positions.

(b) Experiment 4

The fifteen pots in each replicate were placed in four rows on two 6' x 3' glasshouse trolleys. As in Experiment 3, the treatments were allocated at random within each replicate, and were shifted clockwise two positions every second day.

(c) Experiment 5

In this Experiment, the treatments were grouped according to harvest date. The sixteen pots, (four treatments x four

replicates) were placed in four rows on two 6' x 3' trolleys. Each trolley surface was divided into two sections, thereby giving the four replicate blocks. The treatments were allocated at random within each replicate block and shifted two positions every second day.

(d) Experiment 6

The treatments were grouped into three replicate blocks, which were distributed on two 5' x 3' glasshouse trolleys. The treatments were positioned at random within each replicate and were not shifted during the experiment.

2.2.3 Treatments

The following nitrate nitrogen levels were used:

	NO_3N (mM)				
Experiment 3	0,	0.25,	0.5,	1.0,	4.0
Experiment 4	0,	0.25,	0.5,	1.0,	4.0
Experiment 5	0,	0.5,	2.0,	8.0	
Experiment 6	0,	0.2,	2.0		

The concentration of nitrate nitrogen in the solution cultures was checked regularly with the aid of a Corning liquid ion exchange nitrate electrode, coupled to a Radiometer 22 expanded scale ion meter, and the nitrate concentrations were adjusted so that the mV readings corresponded to those of standards at the same concentration. The composition of the standard solutions did not differ from the nutrient solutions and the temperature of the standard solutions and the nutrient solutions were also matched, since solution temperature was found to affect the mV readings.

Interference from the chloride anion was eliminated by using calcium sulphate instead of calcium chloride as the calcium source in all nutrient solutions, where the nitrate concentration was to be checked with the electrode. The affect of organic anions and the bicarbonate ion is not known. Weekly renewal of the nutrient solutions should have maintained the concentration of these ions at a low level. The activity of an ionic species can

also change as the total ionic strength of a solution changes. A 50% decrease in the total ionic strength of the nutrient solution did not however, affect the mV readings for low or high standard nitrate concentrations.

Phosphate

The following rates of phosphate supply were used:

Experiment 3	0.2,	0.5	and	1.5	mgP / plant / week
Experiment 4	2.0,	5.0	and	10.0	mgP / plant / week
Experiment 6	0.2,	0.5	and	1.5	mgP / plant / week

No attempt was made to maintain the concentration of phosphate during the week. Preliminary experiments were conducted to determine the rates of phosphate to be used in the experiments.

2.3.0 CONDUCT OF GLASSHOUSE EXPERIMENTS

(a) Experiment 3

This experiment commenced on July 1 1973. Eight clonal plants, established as described (Chapter 2.1.1) were placed into each of 90 pots and the nutrient solutions inoculated with a suspension of rhizobia. Three different clones were used, one for each replicate in the experiment.

The nutrient solutions were changed weekly and the volume of nutrient solution, the pH and nitrate ion concentration corrected every second day during the week for the first three weeks of the experiment and daily in the fourth and final week. Distilled water was used to replace that water lost from the solution cultures through evapo-transpiration, and the pH of the solutions containing nitrate were corrected to pH 6.0 with 0.1N H_2SO_4. The pH of the nil nitrate control nutrient solutions were corrected to pH 6.8 with 0.1N NaOH to counter the observed rapid decline in pH in these solution cultures.

Four harvests were to be conducted in this experiment at 14, 21, 28 and 35 days after the start of the trial. However, only the first and third harvests were completed and only the data for the third harvest is presented in this thesis. At each

harvest plants were to be removed from one corner of each of two pots allocated to the same treatment. After the first harvest, however, growth differences between plants in each pot were observed. A change in harvesting policy was necessary and since one replicate had to be discarded because of a wilting problem (Plate 3), the decision was made to harvest all remaining plants at the third harvest.

(b) Experiments 4 and 5

'Mature' clonal material, was used in these experiments and although different clones were used in each experiment, the plant material was established at the same time. For each experiment, four clonal plants were placed into each of 50 containers. After four weeks growth, the cork stoppers used to support the stolons were removed to allow unrestricted root growth.

The plant tops were partially defoliated after 14 weeks, and at 18 and 20 weeks the clonal material to be used in Experiment 5 and Experiment 4 respectively was removed from the pots, the excess water removed from the roots and the fresh weight of each plant recorded. The plant material was then grouped according to fresh weight and for each replicate, two similar plants returned to each pot. Before the plants were returned to the pots however, plastic netting (30cm x 38cm) was stapled over the lids. The netting provided a horizontal growing surface for the extending stolons.

Nutrient solutions were renewed fortnightly and the volume of water in pots in both experiments corrected every two days and the pH of the nutrient solutions was adjusted to pH 6.5 once a week. The tap water used in the nutrient solutions during the pre-treatment growth period was alkaline, and the water buffered the downward drift in pH in the nil nitrate solutions.

(i) Experimental Period

The treatments were applied a week after the grouping of plant material in Experiment 5 and two weeks in Experiment 4. The nitrate ion concentration, pH and the volume of nutrient solution

Plate 3 Plant material from Experiment 3 which was discarded after wilting was observed.

were corrected daily and the nutrient solution changed each week. Distilled water was used during the experimental period.

In Experiment 5, plants were harvested 2, 6 and 12 days after the start of the trial (10th. September 1973) and in Experiment 4, the trial was terminated after 12 days (12th.October 1973).

(c) Experiment 6

Plant material from one clone was established as described and one plant placed into each pot and the nutrient solutions were inoculated with a 5ml suspension of rhizobia. The experiment was commenced on the 10th February 1974.

The nutrient solutions were changed weekly and the volume of nutrient solution, the pH and the nitrate ion concentration corrected every second day during the first thirty days of the experimental period. Distilled water was used to replace water lost from the solution cultures through evapo-transpiration, and the pH of the solutions containing nitrate were corrected to pH 6.0 with 0.1N H_2SO_4. The pH of the nil nitrate control nutrient solutions were corrected to pH 6.8 with 0.1N NaOH.

During the last 12 days of the experiment, the nitrate concentration in half of the nutrient solutions containing nitrate was not maintained, but allowed to become depleted. The volume of the nutrient solution and the pH were however corrected every two days, when the nitrate ion concentration, pH and the volume of solution for the remaining pots were being adjusted.

The plant material was harvested six weeks after the start of the experiment.

2.4.0 PREPARATION OF MATERIAL AND ANALYSIS

2.4.1 General

Plants were harvested from early afternoon, when the rate of acetylene reduction was likely to have reached a maximum, (Hardy et al, 1968; Bergersen, 1970). Only one replicate was harvested at a time, the acetylene reduction test being conducted immediately after the harvest of the replicate was completed.

This policy ensured that the acetylene reduction test was completed within two hours of the roots being first detached from the tops. Further, diurnal changes in the rate of acetylene reduction and carbohydrate movement to roots and nodules, (Wheeler, 1969; 1971) would have been accounted for in the between replicate analysis of variance.

After removal from the pots, the plants were thoroughly washed in tap water and rinsed in distilled water. The roots were separated from the tops and both dried with paper towels. In Experiments 4 and 5, where root and nodule sub-samples for the rate of acetylene reduction and for nitrogen, phosphate and carbohydrate analyses were drawn from each plant, the roots were divided approximately in half and the fresh weight of the two sections recorded. Where two sets of plants were harvested per treatment, (Experiment 3) whole plant roots were allocated for the acetylene reduction test or for nitrogen, phosphate and carbohydrate analyses. Both sets of plants were harvested at the same time, so that the relationship between carbohydrate status and rate of acetylene reduction for example, could be determined.

The top, root and nodule material for carbohydrate, nitrogen and phosphate analyses were frozen immediately after being harvested and later transferred to a cold freeze-drier for drying. Nodules were detached from the root systems after freeze drying and the nodule number, (Experiment 3) recorded.

The nodule number and/or weight were determined on only those roots or sections of roots which were set aside for analytical work.

2.4.2 Dry Weights

Dry weights of tops were taken after dreeze drying the plant material for one week. Root and nodule material were also dried for a week before nodule detachment, but after nodule detachment was completed, the root and nodule material were re-dried in a freeze-drier for three days and the dry weights then recorded.

2.4.3 Acetylene Reduction Assay

The procedure used in these experiments was similar to that outlined by Hardy et al (1968); Chu (1971); Dart and Day (1971) and Oghoghorie and Pate (1971), except that in Experiments 4, 5 and 6, the atmospheric gas in the incubation vessels was not withdrawn and replaced with an argon/oxygen gas mixture, as specified by Hardy et al (1968) and other workers. The root and nodule material was incubated in an atmosphere containing both nitrogen gas and acetylene.

A detailed account of the procedure used in the determination of rate acetylene reduction is given in Appendix I 3.

2.4.4 Percentage Soluble Sugars

The soluble sugar content in plant material was determined by a procedure currently in use in the Plant Physiology Division laboratories, D.S.I.R. Palmerston North. The soluble sugars were extracted by suspending freeze-dried plant material in 62.5% methanol for 10-15 minutes. The extraction was done at a temperature of 55°C and the quantity of sugars in the methanol extractant was determined by the phenol-sulphuric acid colorimetric method, (Hodge and Hofreiter, 1962).

A detailed account of the extraction procedure and colorimetric determination of sugars is given in Appendix I 4.

2.4.5 Percentage Starch

The starch content in the plant material was determined by measuring the quantity of glucose released after the incubation of boiled suspensions of plant material with an amyloglucosidase enzyme preparation. The quantity of glucose was determined colorimetrically, by incubating aliquots from the amyloglucosidase digests with a glucose oxidase preparation and o-dianisidine hydrochloride. Sulphuric acid was added after the required incubation time, to produce a magenta colour. Starch determinations were conducted on plant material used previously for soluble sugar determinations.

A detailed account of the procedures used in estimating the starch content in plant tissues is given in Appendix I 5.

2.4.6 Percent Total Nitrogen

The percent total nitrogen in plant material was obtained by Kjeldahl digestion and auto analyser analysis. The Kjeldahl digestion procedure was modified to include nitrate nitrogen in the total nitrogen determination.

2.4.7 Percent Total Phosphate

The percent total phosphate in plant material was obtained by Kjeldahl digestion and auto analyser analysis

2.5.0 STATISTICAL METHODS

2.5.1 Analysis of Variance

The Bar 3 system of linked programmes for regression analysis, written in Fortran 2-D for the IBM 1620 computer was used in the analysis of variance.

2.5.2 Transformation of data

Where the variance was proportional to the mean, i.e. in counts of nodules on the clover roots, a square root transformation was used to stabilize the variance (Snedecor and Cochran, 1967).

2.5.3 Least significant difference (LSD)

The least significant difference for the nitrate nitrogen or phosphate treatment means was calculated as outlined by Snedecor and Cochran (1967)

$$LSD = t_{0.05} \sqrt{\frac{2S}{n}}$$

where s = error mean square

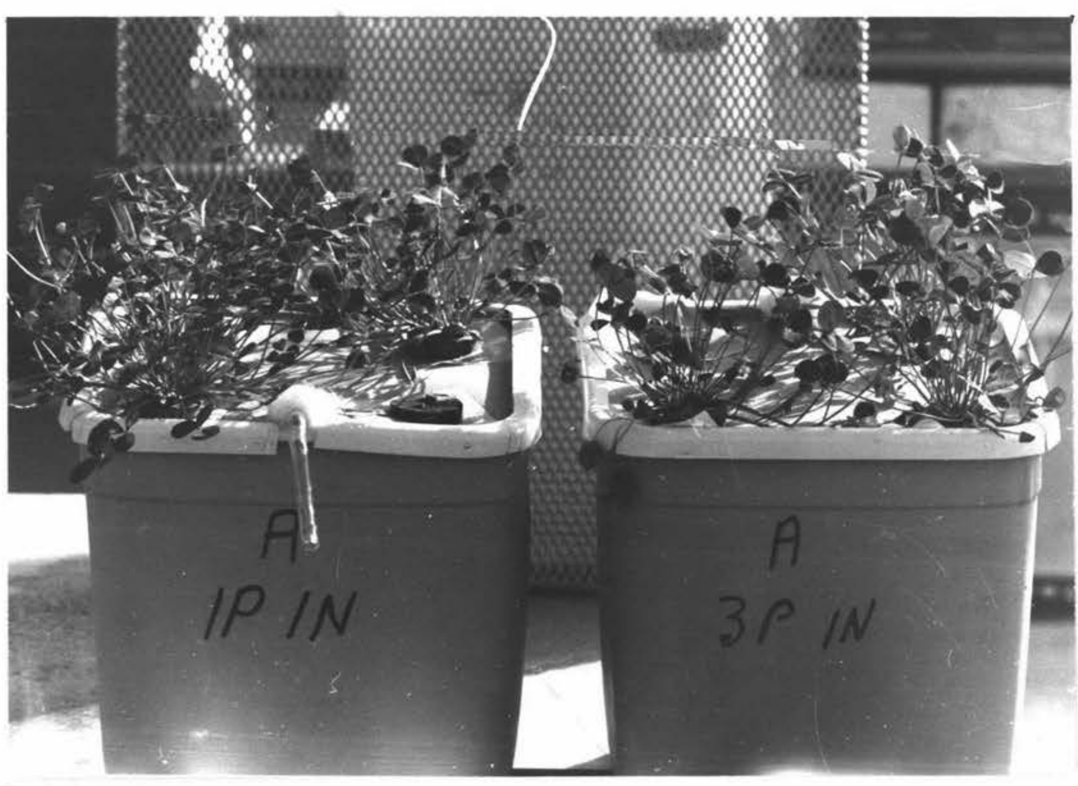
n = number of replications

The LSD was calculated only when the F test for the nitrate nitrogen or phosphate treatments was significant.

Plate 4 Plant material from Experiment 3 showing toxic effect of high levels of phosphate (A).

Plate 5 Comparison of plant material from Experiment 3. Plants in A 1P1N received the low phosphate level, while those in A 3P1N the highest level.

A


A
1P 1N
A
3P 1N

CHAPTER III

RESULTS

For ease of discussion, the results of each experiment have been grouped together.

1 GROWTH ROOM EXPERIMENTS:

1.1 EXPERIMENT 1

1.1.1 Nodule Number

The summary of the analysis of variance for the number of nodules per seedling is presented in Appendix II 1.1. Nitrate nitrogen significantly reduced ($P < 0.01$) the number of nodules, but no significant phosphate treatment effect or nitrate nitrogen x phosphate interaction was recorded.

(a) Nitrate Nitrogen

Table 1 presents the mean number of nodules per plant. The LSD value listed in Appendix II 1.2 indicated that there was a significant difference between the control (nil nitrate nitrogen) and all nitrate nitrogen treatment means. However, there were no significant differences between the nitrate nitrogen treatment means (Appendix II 1.2).

1.1.2 Seedling Top Dry Weight

A highly significant difference ($P < 0.01$) was recorded for the nitrate nitrogen treatment means. However, the phosphate means were not significantly different, and no phosphate x nitrate nitrogen interaction was recorded (Appendix II 2.1).

(a) Nitrate Nitrogen

The growth response of white clover seedlings to applied nitrate nitrogen is significant only between the nil nitrate nitrogen treatment mean and the nitrate nitrogen treatment means (Appendix II 2.2). Increasing the concentration of nitrate in the

Table 1 Nodule number per seedling

Conc. of Phosphate (mM)	Conc. of NO_3 N (mM) 0	1	2	4	8
0.1	17.40	8.60	5.85	7.25	5.60
0.5	15.65	5.85	7.40	7.00	6.75
1.0	16.80	7.40	6.30	6.25	5.15
2.0	15.85	6.95	6.15	5.90	5.45
4.0	20.60	6.25	6.35	5.40	6.10

Table 2 Seedling top weight (mg)

Conc. of Phosphate (mM)	Conc. of NO_3 N (mM) 1	2	3	4	5
0.1	1.13	4.61	4.96	4.25	5.15
0.5	1.26	5.56	3.88	4.00	4.61
1.0	1.09	4.50	4.18	3.65	4.87
2.0	1.15	4.35	3.71	3.65	4.10
4.0	1.20	4.16	3.91	4.26	3.97

nutrient solution did not significantly change the top growth of the seedlings.

1.2 EXPERIMENT 2

1.2.1 Nodule Number

The means for the nitrate nitrogen and phosphate treatments are presented in Fig. 4. Significant nitrate nitrogen and phosphate treatment effects were recorded (Appendix II 3.1), however no interaction between nitrate nitrogen and phosphate was noted.

(a) Nitrate Nitrogen

The addition of nitrate nitrogen to the growing medium significantly reduced the number of nodules on each seedling (Appendix II 3.3). While the means for the 0.5mM and 1.0mM nitrate nitrogen treatments or the 1.0mM and 4.0mM nitrate nitrogen treatments were not significantly different, the differences between all other treatment means were significant at the 5% level of probability.

(b) Phosphate

The means for the 0.005, 0.05 and 0.5mM phosphate treatments were significantly greater than the mean for the nil phosphate treatment. However, no significant differences were obtained between the other phosphate treatment means.

1.2.2 Seedling Top Dry Weight

Application of nitrate nitrogen and phosphate to the solution cultures significantly increased ($P < 0.01$) the growth of the top system of Huia White Clover seedlings (Fig.5). A significant interaction ($P < 0.01$) between nitrate nitrogen and phosphate was also recorded (Appendix II 4.1).

(a) Nitrate Nitrogen

The means for the nitrate nitrogen treatments and the LSD are presented in Appendix II 4.2. The 8.0mM nitrate nitrogen mean which recorded the highest top dry weight, was significantly greater than the means for the 0.5, 0.1 and 0mM nitrate nitrogen

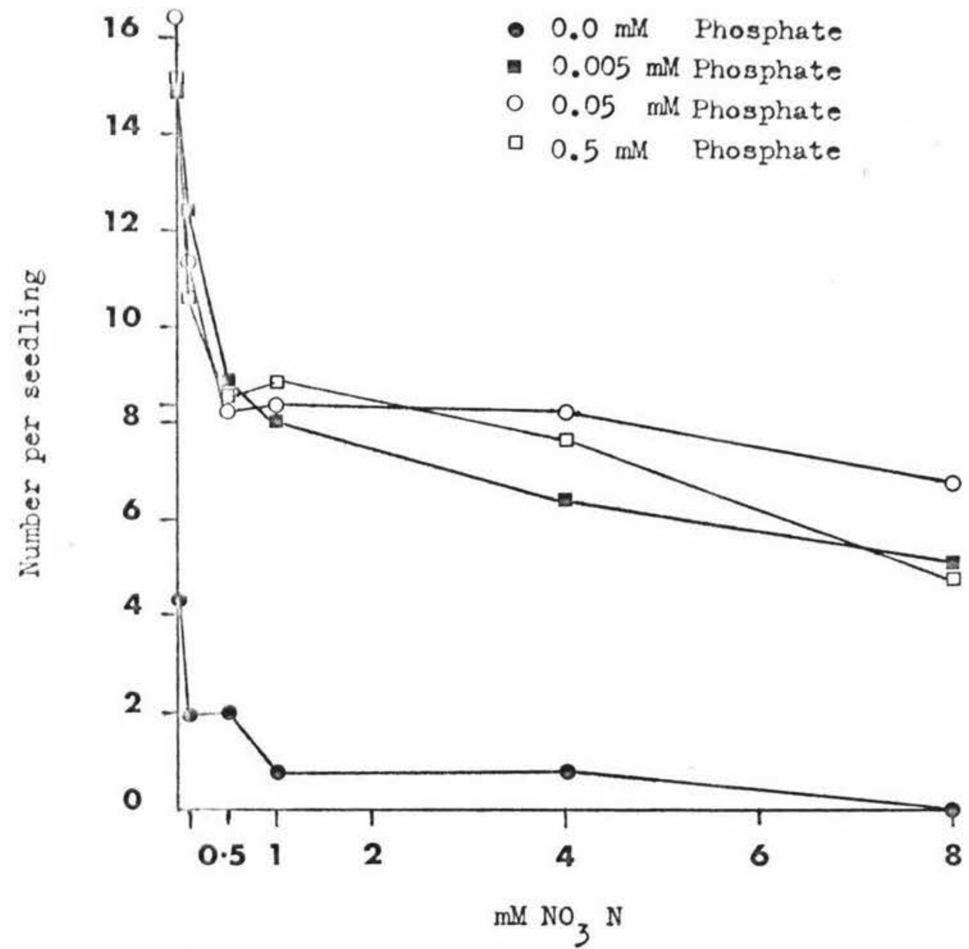


Fig 4 Nodule number per seedling root

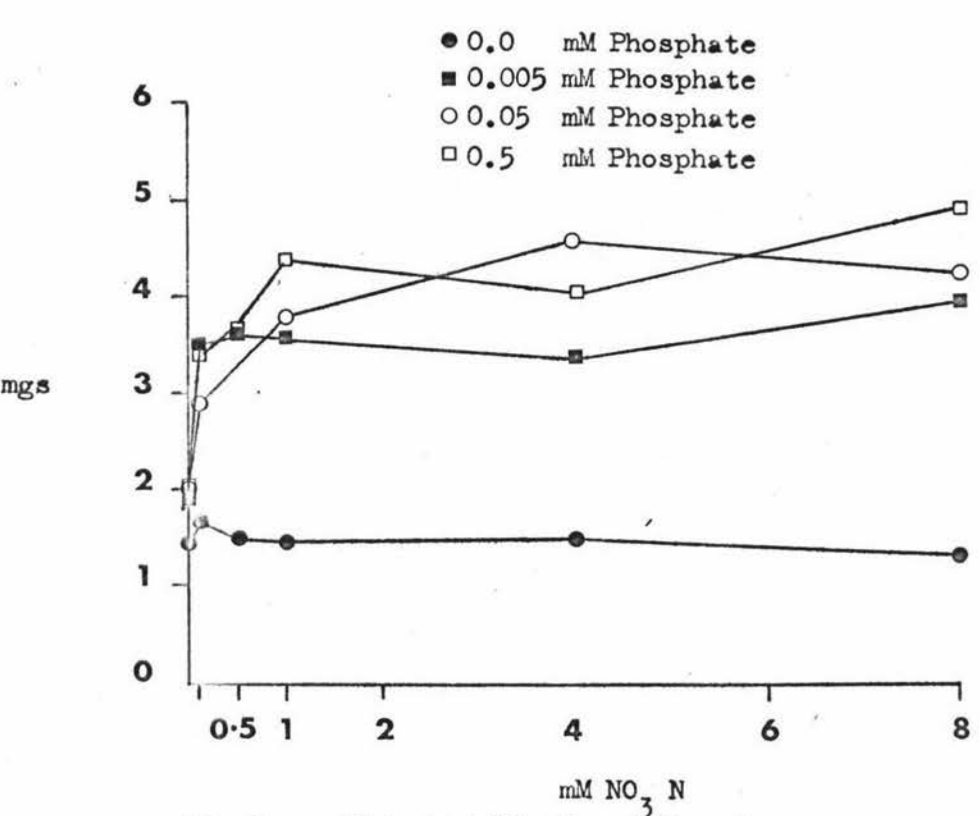


Fig 5 Dry weight of seedling top

levels; and the 4.0, 1.0, 0.5 and 0.1mM nitrate nitrogen means were significantly greater than the 0.0mM control mean.

(b) Phosphate

Most growth occurred in the highest phosphate treatment. The mean for the 0.5mM phosphate level was significantly greater than the mean for the 0.005mM and 0.0mM phosphate levels. A significant increase in the growth also occurred between the 0.05 or 0.005mM phosphate means and the 0.0mM phosphate mean (Appendix II 4.3).

(c) Phosphate x Nitrate Nitrogen interaction

The significant increase in top growth with nitrate nitrogen application occurred only in those culture solutions where phosphate was added. Further, the growth response to applied phosphate was more pronounced at higher levels of nitrate nitrogen in the growth medium (Fig.5).

2. GLASSHOUSE EXPERIMENTS

2.2 EXPERIMENT 3 - Harvest 2

2.2.1 Nodulation

The nodulation data are presented in terms of nodule number, nodule weight and the average weight per nodule on a per plant basis.

(A) Nodule Weight

The inclusion of nitrate nitrogen in the growing medium sharply reduced the dry weight of nodules on each clover root (Fig. 7). The reduction in weight was highly significant ($P < 0.01$), (Appendix IV 1.1). Increasing the rate of phosphate on the other hand, significantly ($P < 0.01$) increased nodule weight. No interaction between nitrate nitrogen and phosphate was recorded (Appendix IV 1.1).

(a) Nitrate Nitrogen

The nil nitrate treatment recorded the highest weight of nodule material, the weight of nodule material being reduced to a third of that recorded for the control, by growth in a 4.0mM nitrate nitrogen solution (Fig. 7). The LSD for the nitrate nitrogen means listed in Appendix IV 1.2 indicated that a significant reduction in nodule weight occurred between the following nitrate nitrogen treatment means:

(i) the 0.0mM and the 0.25, 0.5, 1.0 and 4.0mM nitrate nitrogen treatment means

(ii) the 0.25mM and the 0.5, 1.0 and 4.0mM nitrate nitrogen treatment means

(b) Phosphate

The LSD for the phosphate treatment means is presented in Appendix IV 1.3. A significant increase in nodulation occurred between the 0.2mgP / plant / week phosphate level and both the 0.5 and 1.5mgP / plant / week phosphate level. No differences were recorded between the two higher rates.

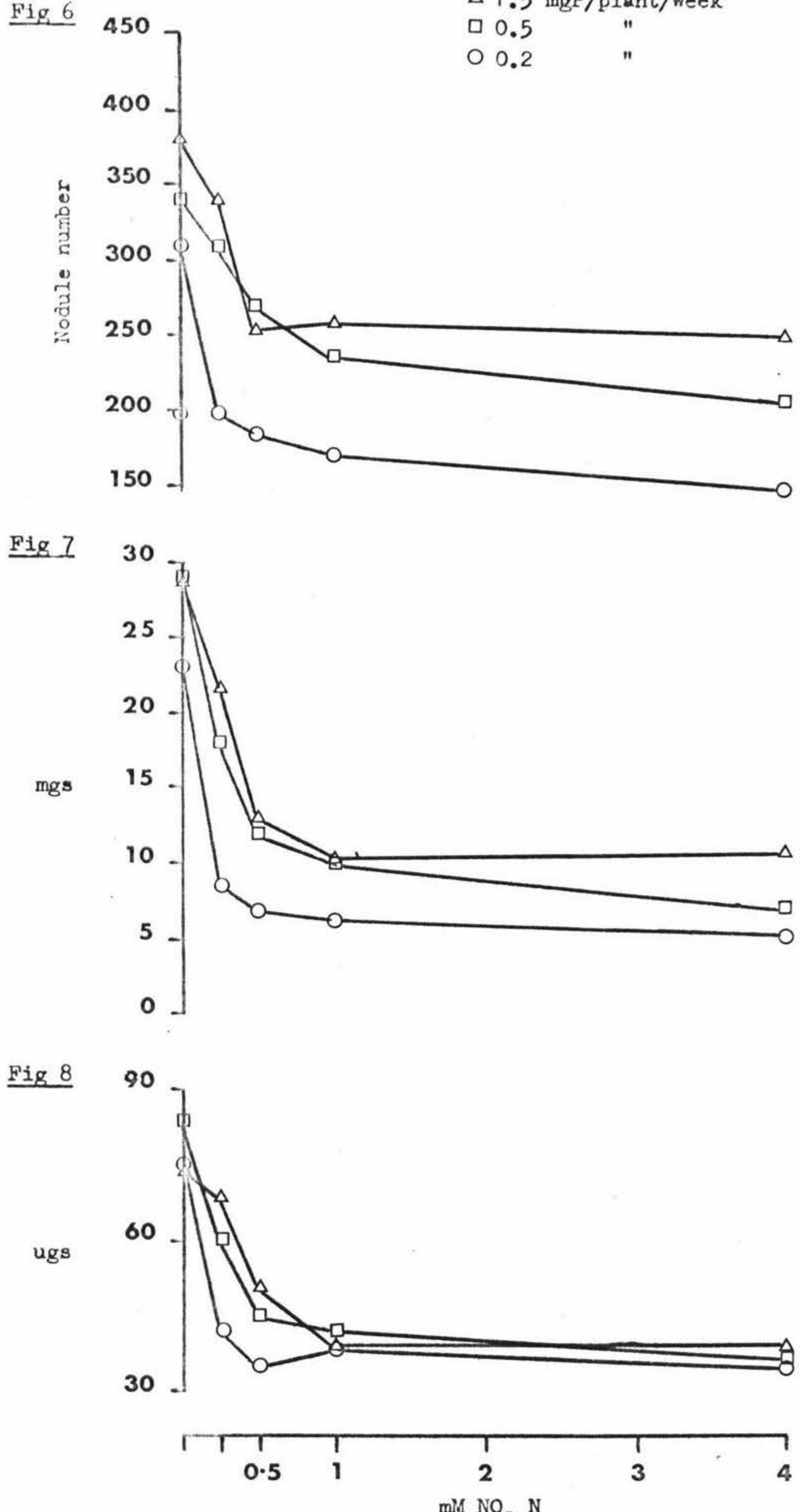


Fig 6
Fig 7
Fig 8
△ 1.5 mgP/plant/week
□ 0.5 "
○ 0.2 "
Nodule number
450
400
350
300
250
200
150
30
25
20
15
10
5
0
mgs
90
60
30
ugs
0·5
1
2
3
4
mM NO3 N

(B) Nodule Number

The summary of analysis of variance for nodule number is presented in Appendix IV 2.1. nodule number per plant root was significantly ($P < 0.01$) reduced by the application of nitrate nitrogen, the number of nodules being reduced to approximately half that of the control, i.e. the nil nitrate treatment, with the application of high levels of nitrate (Fig. 6). Higher rates of phosphate increased the number of nodules (Fig.6), the increase being significant at the 1% probability level (Appendix IV 2.1). No interaction existed between the nitrate nitrogen and phosphate.

(a) Nitrate Nitrogen

As in the case of nodule weight, low levels of nitrate nitrogen in the nutrient solutions (0.25 and 0.5mM NO_3 N) significantly reduced the nodule number. The reduction in nodule number that occurred between the 0.5mM nitrate nitrogen treatment mean and the 1.0 or 4.0mM nitrate nitrogen treatment means was not however, significant (Appendix IV 2.2).

(b) Phosphate

The phosphate treatment means for nodule number and the LSD are presented in Appendix IV 2.3. A significant increase in nodule number occurred between the lowest phosphate level and the two higher levels. There was no significant difference in nodule number between the two higher phosphate levels.

(C) Average Nodule Weight

The average nodule weight data is presented in Fig. 8. Nitrate nitrogen alone affected the average nodule weight (Appendix IV 3.1); the reduction being highly significant ($P < 0.01$). The low rates of nitrate nitrogen were as effective as the higher rates in reducing the average weight of nodules, since no significant differences occurred between the treatment means representing the low and high rates of nitrate nitrogen addition (Appendix IV 3.2).

2.2.2 Nitrogen Fixation

The rates of ethylene production per root system and mg dry weight nodule material were used as an indices of nitrogen fixing activity.

(A) Ethylene produced/Root/Hour

Figure 9 presents the means for the amount of ethylene produced per root per hour and Appendix IV 4.1 a summary of the analysis of variance. Nitrate nitrogen significantly ($P < 0.01$) reduced the rate at which ethylene was produced. One the other hand, additions of higher rates of phosphate significantly ($P < 0.01$) increased the rates of ethylene production. The nitrate nitrogen x phosphate interaction was not just significant at the 5% level of probability.

(a) Nitrate Nitrogen

The nitrate nitrogen treatment means and the LSD are presented in Appendix IV 4.2. The nil nitrate nitrogen treatment mean recorded the highest rate of nitrogen fixing activity. The rate was however, drastically decreased by the low concentrations of nitrate in the nutrient solutions. Further, less severe reductions resulted from the increase in concentration of nitrate. At the highest nitrate nitrogen level, (4.0mM) the rate of ethylene production per root was reduced to 11% of the control nil nitrate nitrogen treatment.

(b) Phosphate

A significant increase in nitrogen fixing activity resulted from the increase in the level of phosphate from 0.2mgP / plant /week to 0.5 and 1.5mgP / plant / week. The difference between the two higher phosphate treatment means was not significant.

(B) Rate Ethylene Production per mg Nodule Dry Weight

Only nitrate nitrogen significantly affected the rate of ethylene produced per mg dry weight of nodule. A summary of analysis of variance is presented in Appendix IV 5.1 and the treatment means are shown in Fig. 10. The computed LSD value listed in

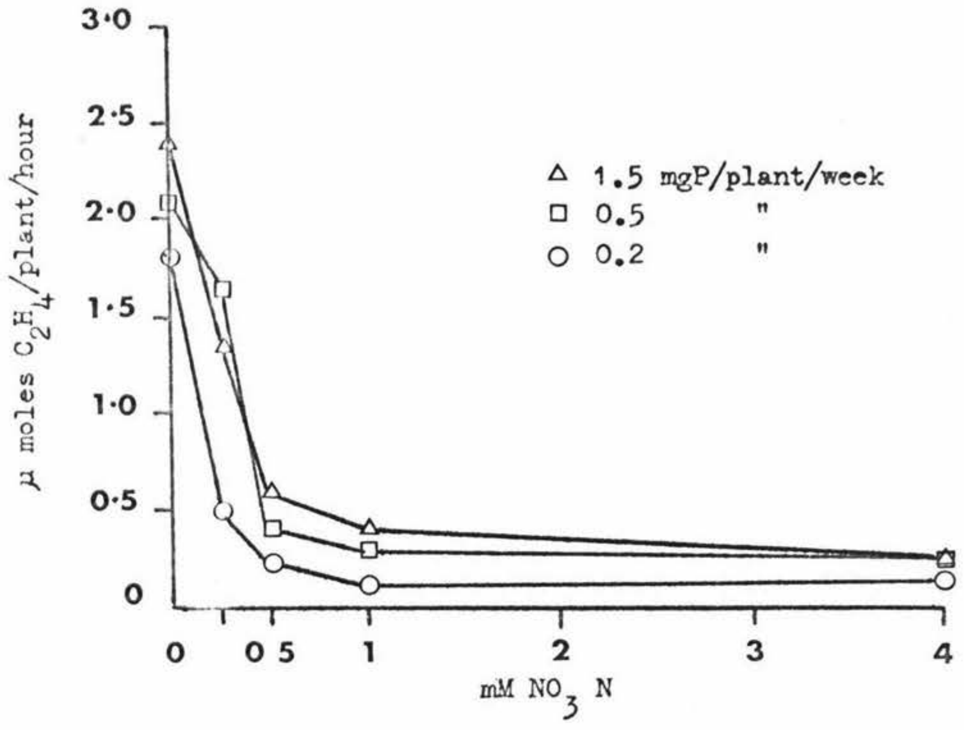


Fig 9 Rate of ethylene produced per plant per hour

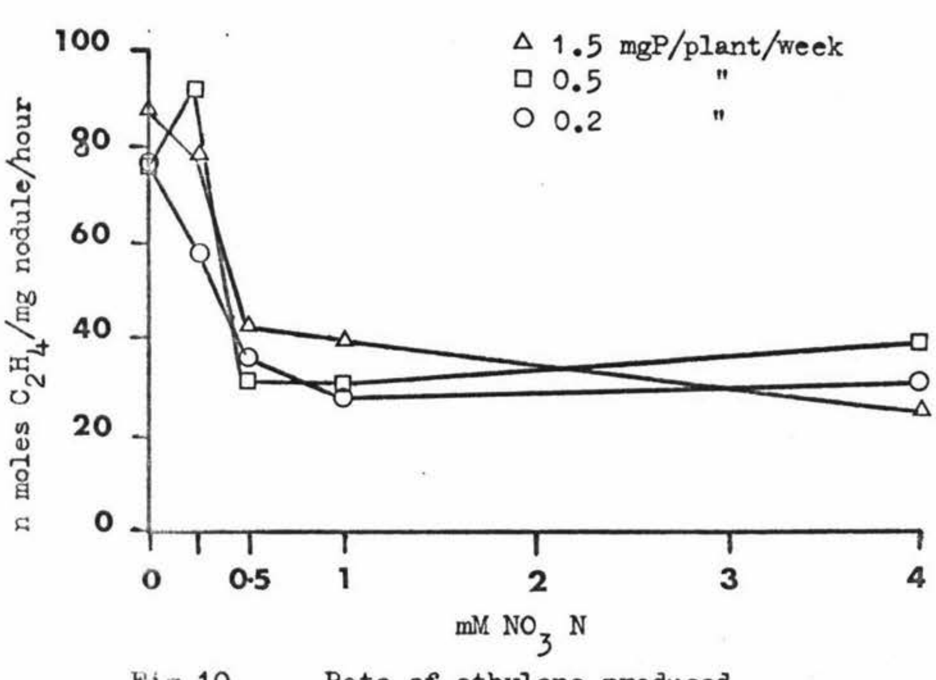


Fig 10 Rate of ethylene produced per mg nodule (dry weight) per hour

Appendix IV 5.2, shows that the 0 and 0.25mM nitrate nitrogen treatment means are significantly different from the 0.5, 1.0 and 4.0mM nitrate nitrogen treatmens means. There were no significant differences between the 0 and 0.25mM nitrate nitrogen means or the 0.5mM and the 1.0 or 4.0mM nitrate nitrogen means. At the highest level of nitrate nitrogen (4.0mM) the rate of ethylene produced per mg dry weight of nodule had been reduced to approximately 40% of the nil nitrate treatment.

2.2.3 Plant Growth

(A) Top Dry Weight

The top dry weight data is presented in Fig. 12 and a summary of the analysis of variance is shown in Appendix IV 6.1. Addition of nitrate nitrogen in the nutrient solutions led to a significant ($P < 0.01$) increase in top growth. Phosphate did not significantly influence top growth, and a nitrate nitrogen x phosphate interaction was not detected.

The highest top weight was recorded for the 1.0mM nitrate nitrogen treatment mean. This mean was not, however, significantly different from the other treatment means where nitrate was added. Significant differences were recorded between the nil nitrate treatment mean and all the nitrate nitrogen treatment means.

(B) Root Weight

Both nitrate nitrogen and phosphate significantly ($P < 0.01$) influenced root growth (Appendix IV 7.1). Fig. 13 shows that the addition of nitrate to the nutrient solutions increased root growth while an increase in the level of phosphate reduced root growth.

As in the case of top growth, no significant differences occurred between the treatment means where nitrate was added. Significant differences were recorded between the nil nitrate treatment mean and all the nitrate treatment means (Appendix IV 7.2).

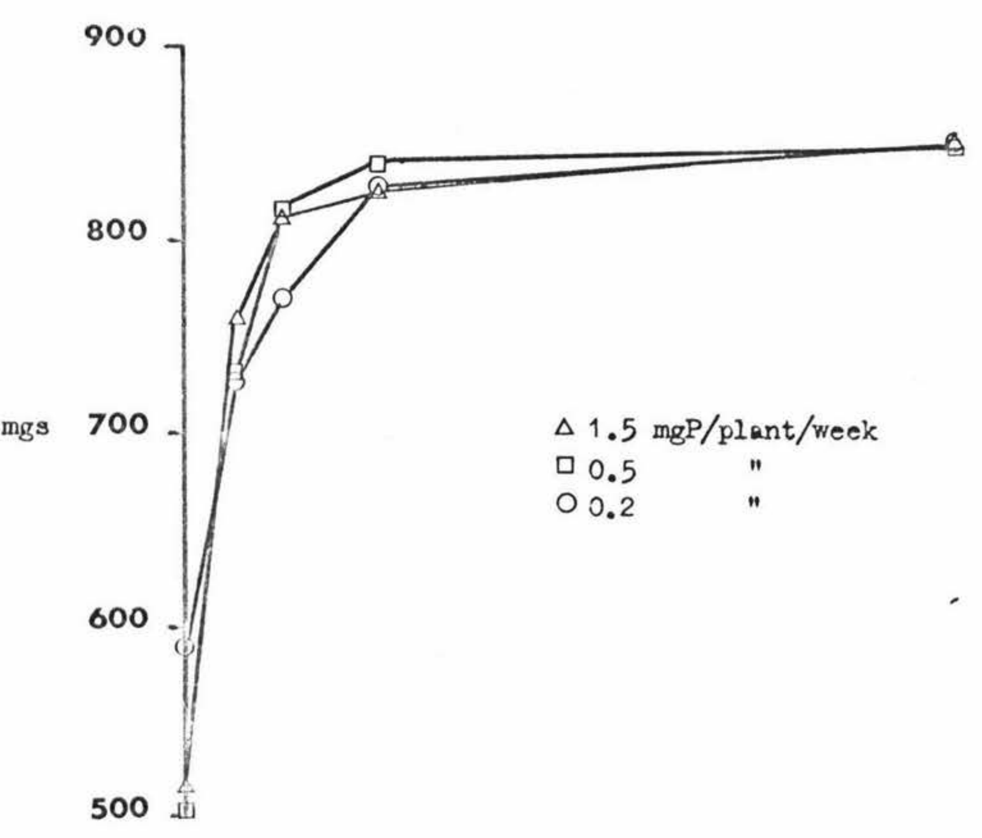
The phosphate treatment means and the LSD are presented in Appendix IV 7.3.

(C) Total plant weight

The analysis of variance is summarized in Appendix IV 14.1 and the mean data for total plant weight is presented in Fig 11.

Nitrate nitrogen significantly increased total plant growth. The means for the nitrate nitrogen treatments and the LSD are presented in Appendix IV 13.2.

Fig 11 Total plant dry weight



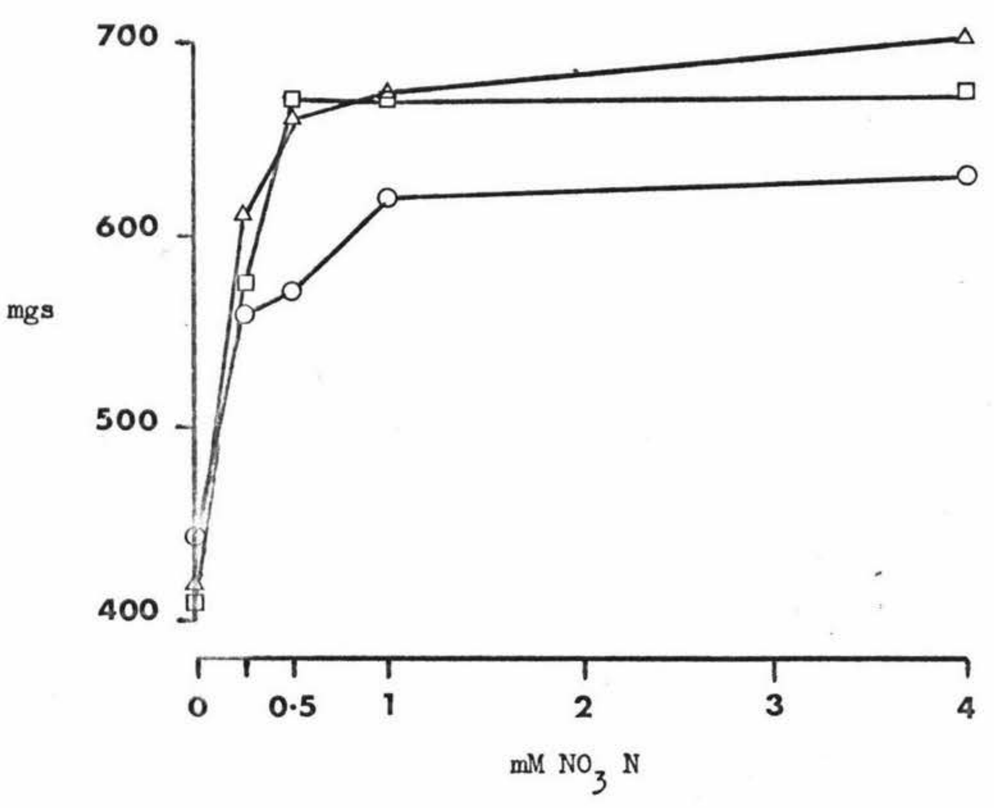


Fig 12 Top dry weight

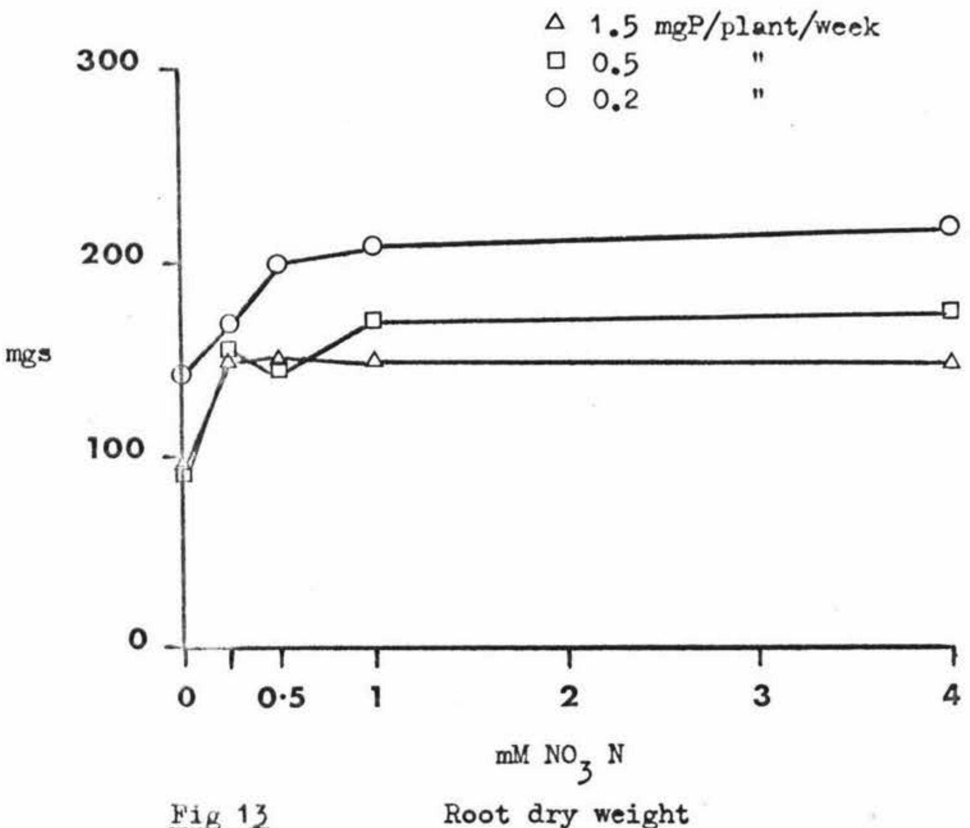


Fig 13 Root dry weight

2.2.4 Percent Total Nitrogen

(A) Top Material

The analysis of variance and mean data for percent total nitrogen are presented in Appendix IV 8.1 (a) and Table 3 respectively. A significant increase in percent total nitrogen resulted from the addition of nitrate nitrogen to the culture solutions. Phosphate did not significantly change the percent total nitrogen level in top material.

Significant increases in percent total nitrogen occurred between the 0 and 0.25 mM nitrate nitrogen treatment means and the 0.5, 1.0 and 4.0mM nitrate nitrogen treatment means (Appendix IV 7.2). There was no significant increase in percent total nitrogen between the 0.0mM and 0.25mM nitrate nitrogen treatment means or between the 0.5mM and 1.0 and 4.0mM nitrate nitrogen treatment means.

(B) Root Material

The inclusion of nitrate nitrogen in the growing medium, and the increase in the level of phosphate increased the percent total nitrogen in the root system, (Table 4). The increases in nitrogen content were significant at the 1% probability level (Appendix IV 8.1 (b). No significant nitrate nitrogen x phosphate interaction was recorded.

(a) Nitrate Nitrogen

Low concentrations of nitrate nitrogen, significantly increased the total nitrogen content of roots. The means for the nitrate nitrogen treatments and LSD are presented in Appendix IV 8.2.

(b) Phosphate

The means for the phosphate treatments and the LSD are presented in Appendix IV 8.3. Increasing the level of phosphate from 0.2mgP / plant / week to either 0.5 or 1.5mgP / plant / week, significantly increased the percent total nitrogen content, however, no increase in content was observed between the two higher phosphate levels.

Table 3 Percent total nitrogen in top material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	3.88	4.02	4.07	4.23	4.15
0.5	3.93	3.98	4.51	4.35	4.82
1.5	4.01	4.09	4.57	4.67	4.31

Table 4 Percent total nitrogen in root material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	2.58	3.21	3.53	3.65	3.51
0.5	2.66	3.60	3.86	4.13	4.02
1.5	2.75	3.28	3.81	4.13	4.29

Table 5 Percent total nitrogen in nodule material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	8.14	7.55	7.02	6.67	6.66
0.5	8.04	7.80	7.79	7.83	7.03
1.5	7.94	6.92	7.51	7.57	8.85

(C) Nodule Material

Neither nitrate nitrogen nor phosphate significantly changed the percent total nitrogen content of nodule material. (Appendix IV 8.1c, Table 5)

2.2.5 Percent Total Phosphate

The data for the percentage total phosphate in top, root and nodule material are presented in Figures 14 A,B, C respectively.

(A) Top Material

A summary of the analysis of variance for percent total phosphate is given in Appendix IV 9.1(a). Phosphate as expected, very significantly affected the phosphate content of the top material; the percent total phosphate increasing with the higher rates of phosphate. Nitrate nitrogen on the other hand, reduced the phosphate content, the reduction being significant at the 5% probability level. (Fig. 14A)

(a) Nitrate Nitrogen

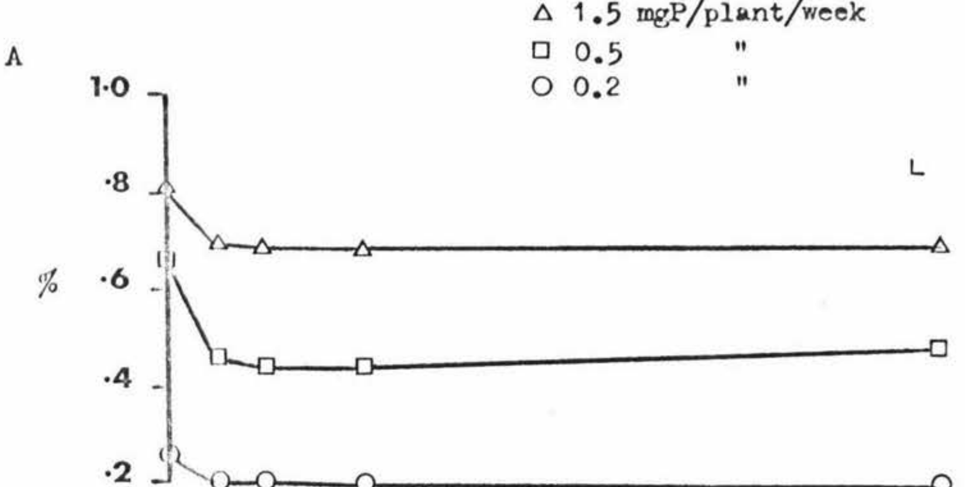
No significant differences were detected between treatment means where nitrate nitrogen was applied (Appendix IV 9.2). The mean for the nil nitrate treatment was however, significantly larger than all treatment means where nitrate was added to the solution cultures.

(b) Phosphate

Significant differences occurred between all phosphate treatment means (Appendix IV 9.3).

(B) Root Material

The mean data for percent total phosphate is shown in Fig. 14B. Inclusion of nitrate nitrogen in the growing medium brought about a significant ($P < 0.01$) decline in phosphate content, while an increase in the level of phosphate very significantly ($P < 0.01$) lifted the percentage total phosphate values. The decline in percent phosphate with the addition of nitrate nitrogen was recorded at the higher levels of phosphate only; no decrease


△ 1.5 mgP/plant/week
□ 0.5 "
○ 0.2 "
A
1·0
·8
%
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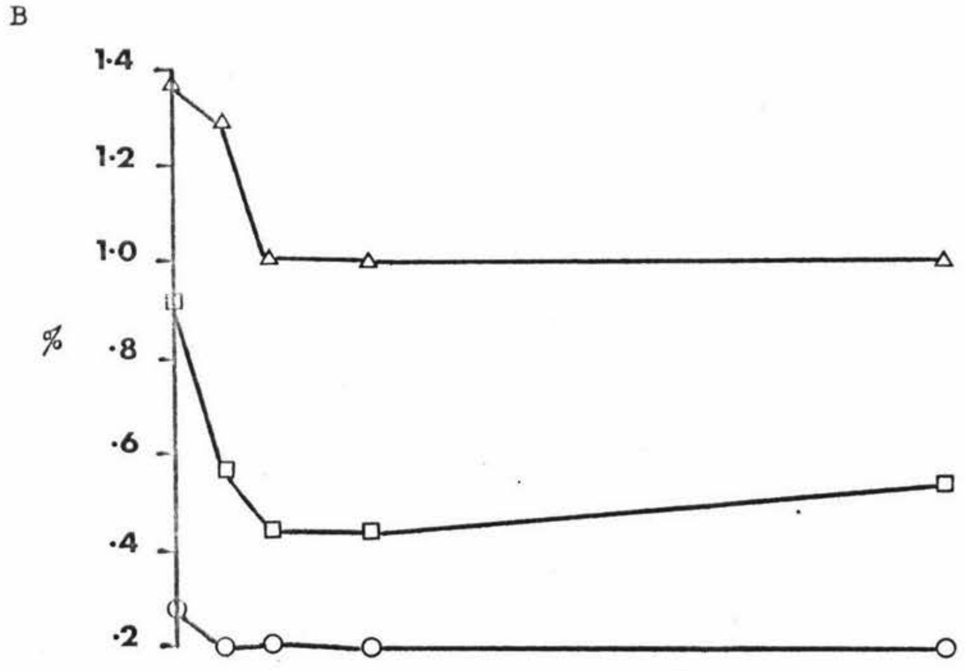
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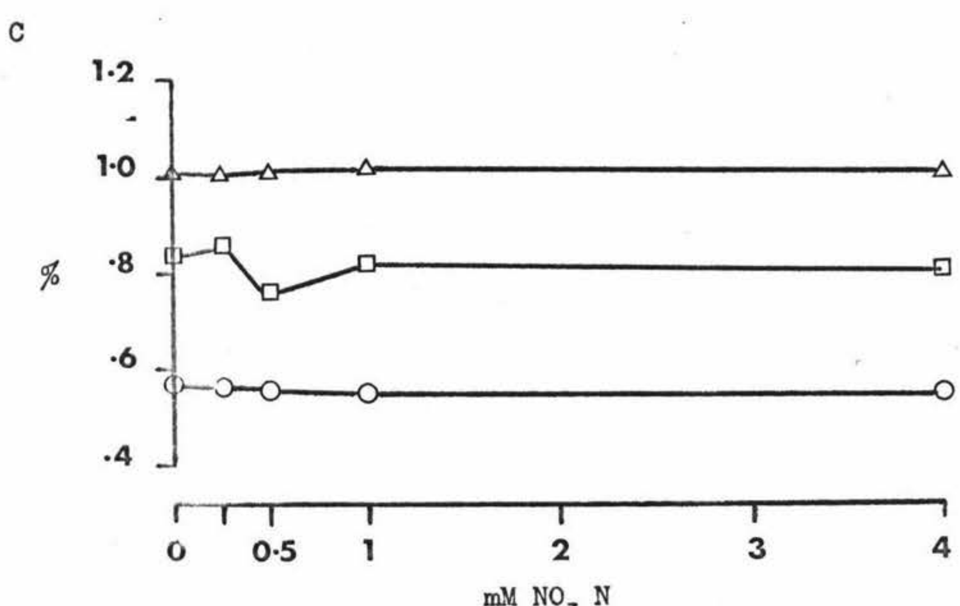
C
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occurred at the lowest phosphate level. This nitrate nitrogen x phosphate interaction was significant at the 5% probability level.

(a) Nitrate Nitrogen

As in the case of the top material, the addition of low concentrations of nitrate nitrogen significantly reduced the percent phosphate in root material. The reduction was however, more severe, significant differences being obtained between the 0.0mM and 0.25mM nitrate nitrogen treatment means and between the 0.25mM and the 0.5mM nitrate nitrogen treatment means. (Appendix IV 9.2)

(b) Phosphate

The means of the phosphate treatments and the LSD are presented in Appendix IV 9.3. Significant differences occurred between all means.

(C) Nodule

Higher percent phosphate values were recorded when the level of phosphate was increased (Fig. 14C). The increase was highly significant ($P < 0.01$), (Appendix IV 9.3 (c) and all phosphate treatment means (Appendix IV 9.3) were significantly different from each other. No change in the percent phosphate level was apparent when nitrate nitrogen was fed to the plants (Fig. 14C), however.

2.2.6 Percent soluble sugars in Top and Root Material

(A) Top Material

The addition of nitrate nitrogen to the nutrient solutions did not cause any consistent changes in the percent soluble sugar levels of top material (Table 6). However, significantly lower levels of sugars were obtained in those plants receiving higher rates of phosphate (Table 6) and (Appendix IV 10.1). The means for the phosphate treatments and the LSD are presented in Appendix IV 10.2.

(B) Root Material

The mean data for the percent soluble sugar level in root material is presented in Table 7. The addition of nitrate

nitrogen, and the increase in the level of phosphate were found to significantly ($P < 0.01$) change the soluble sugar status of the root material. No interaction between nitrate nitrogen and phosphate was recorded, however (Appendix IV 11.1).

(a) Nitrate Nitrogen

The soluble sugar status of the root material increased significantly when low (0.25mM) and high (4.0mM) concentrations of nitrate nitrogen were added to the nutrient solutions. However, the 1.0mM nitrate nitrogen treatment mean was not significantly different from the nil nitrate control treatment mean (Appendix IV 11.2).

(b) Phosphate

The phosphate treatment mean and LSD are presented in Appendix IV 11.3. A significant reduction in soluble sugar level in root material was recorded when the level of phosphate was increased from 0.2mgP / plant / week to 0.5 or 1.5mgP / plant / week. The lowest level of soluble sugars was detected in root material removed from plants receiving the intermediate phosphate level and an increase in the level of phosphate to 1.5mgP / plant / week from 0.5mgP / plant / week significantly increased the percent soluble sugar level.

2.2.7 Percent Starch in Top and Root Material

(A) Top Material

The treatment means are presented in Table 8. A significant ($P < 0.01$) reduction in percent starch resulted from the increase in the level of phosphate (Appendix Iv 12.1). No significant nitrate nitrogen treatment effect or phosphate x nitrate interaction was recorded, however.

A summary of the phosphate treat means and the LSD are presented in Appendix IV 12.2.

(B) Root Material

No significant nitrate nitrogen treatment effects or phosphate x nitrate nitrogen interaction were recorded (Appendix IV 13.1). A significant ($P < 0.01$) reduction in percent starch in root material was obtained in those plants receiving higher rates of phosphate (Table 9). The phosphate treatment means and the LSD are presented in Appendix IV 13.2.

Table 6 Percent soluble sugars in plant top

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	6.06	6.41	6.03	6.25	6.14
0.5	6.00	5.95	5.64	5.19	5.56
1.5	5.65	5.64	5.12	5.53	5.12

Table 7 Percent soluble sugars in plant root

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	4.11	4.49	4.73	4.33	4.61
0.5	3.45	3.79	3.86	3.74	3.97
1.5	3.68	4.42	3.75	3.79	4.26

Table 8 Percent starch in top material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	3.62	2.67	4.28	4.62	4.25
0.5	2.89	3.37	2.68	2.36	2.48
1.5	2.99	2.82	2.02	2.20	2.30

Table 9 Percent starch in root material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	1.69	2.67	2.58	2.39	2.56
0.5	1.33	1.77	1.45	1.34	1.46
1.5	1.00	2.48	1.98	1.31	1.05

2.3 EXPERIMENT 4

2.3.1 Nodule Dry weight

Because of the size of the root system, only those nodules on the section of the root harvested for carbohydrate analyses were detached. The percentage of the total root weight harvested for the carbohydrate analyses was determined, and the total nodule dry weight obtained, by adjusting the recorded nodule dry weight to provide the nodule dry weight for the total root system. The means for the fifteen treatments are presented in Fig. 15 and the summary of the analysis of variance is shown in Appendix V 1.1. Nitrate nitrogen added to the nutrient solutions significantly ($P < 0.05$) changed nodule dry weight, while an increase in the rate of phosphate significantly enhanced the weight of nodule material. No significant nitrate nitrogen x phosphate interaction was observed, however (Appendix V 1.1).

(a) Nitrate nitrogen

The means for the nitrate nitrogen treatments and the LSD are presented in Appendix V 1.2. A significant reduction in nodule weight occurred between the 0 and 0.25mM nitrate nitrogen treatments and the 0.5, 1.0 and 4.0mM nitrate nitrogen treatments. However, there were no significant differences between the 0 and 0.25mM nitrate nitrogen treatment means, or between the means for the higher nitrate nitrogen concentrations (Appendix V 1.2).

(b) Phosphate

The highest nodule weight was recorded for the 10mgP / plant / week level of phosphate. The mean for this treatment was significantly greater than the means for the 2 and 5mgP / plant / week phosphate levels, while the means for the two lower rates were not significantly different.

(c) Nitrate nitrogen x Phosphate interaction

Although a significant ($P < 0.05$) interaction between nitrate nitrogen and phosphate was not recorded, an interaction between the two nutrients is apparent (Fig.15). At the 0.25mM nitrate

nitrogen concentration and the highest phosphate level, the nodule weight exceeded that recorded for plants grown in the absence of nitrate nitrogen but at the same level of phosphate. The opposite occurred at the lowest phosphate level, where the application of nitrate (0.25mM) to the nutrient solution markedly reduced the nodule weight. No change was recorded for the medium phosphate level.

When the 1.0mM and 4.0mM nitrate nitrogen treatment means for the three phosphate levels are compared, it can be seen that the addition of the 4.0mM nitrate nitrogen level tended to increase the rate of nodulation at a low phosphate level, whereas for the high phosphate treatment, the rate of nodulation was clearly reduced.

2.3.2 Nitrogen Fixing Activity

(A) Ethylene production per root per hour

Nitrate nitrogen significantly reduced ($P < 0.01$) and the higher phosphate levels, significantly increased the rate of ethylene production per root (Appendix V 2.1). The overall interaction between the nitrate nitrogen and phosphate treatments was not quite significant at the 5% level of probability. The treatment means are presented in Fig. 17.

(a) Nitrate nitrogen

The nil nitrate treatments produced the largest amount of acetylene (Appendix V 2.2), the means being significantly greater than the means for the 0.25, 0.5, 1.0 and 4.0mM nitrate nitrogen treatments. The 0.25mM nitrate nitrogen mean was also significantly greater than the 1.0 and 4.0mM nitrate nitrogen treatment means and the 0.5mM nitrate nitrogen treatment mean was significantly greater than the 4.0mM nitrate nitrogen treatment mean.

(b) Phosphate

Increased rates of ethylene production resulted from the higher levels of phosphate. The most significant increases occurred between the lowest phosphate level and the highest, and between the two higher rates (Appendix V 2.3).

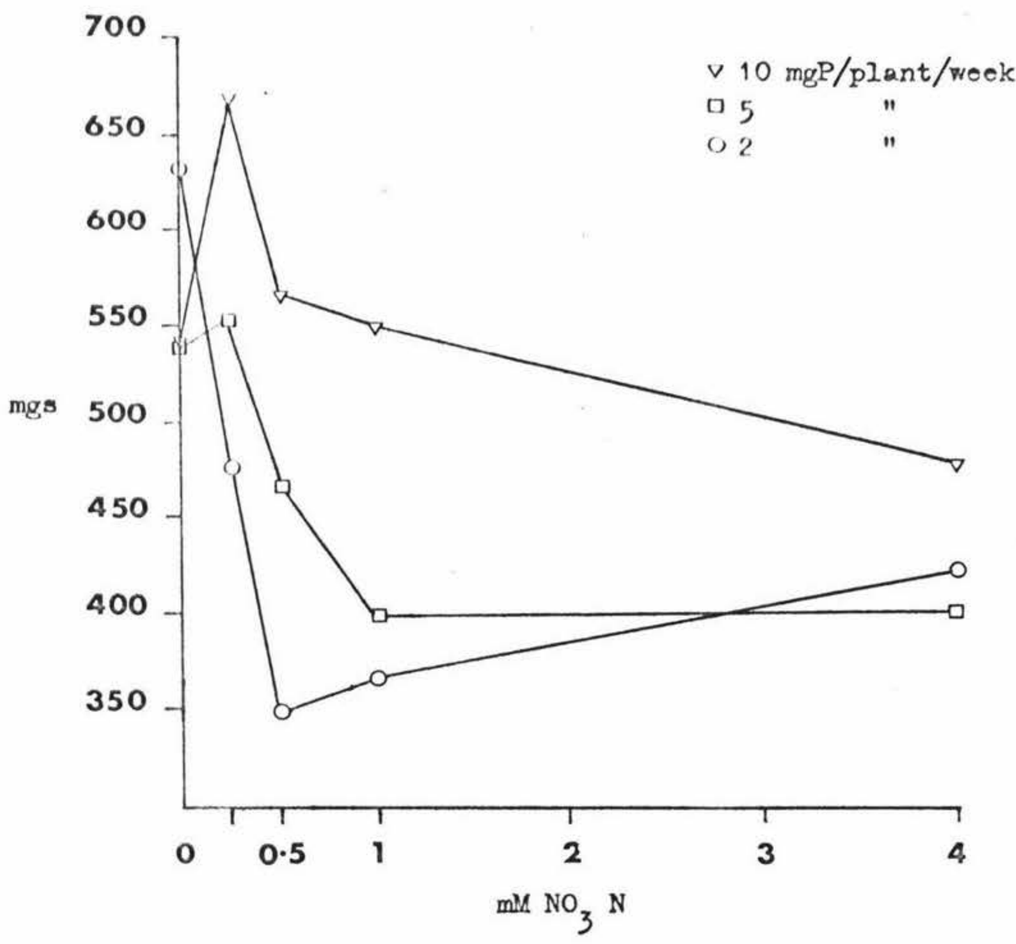

Fig 15 Nodule dry weight per plant

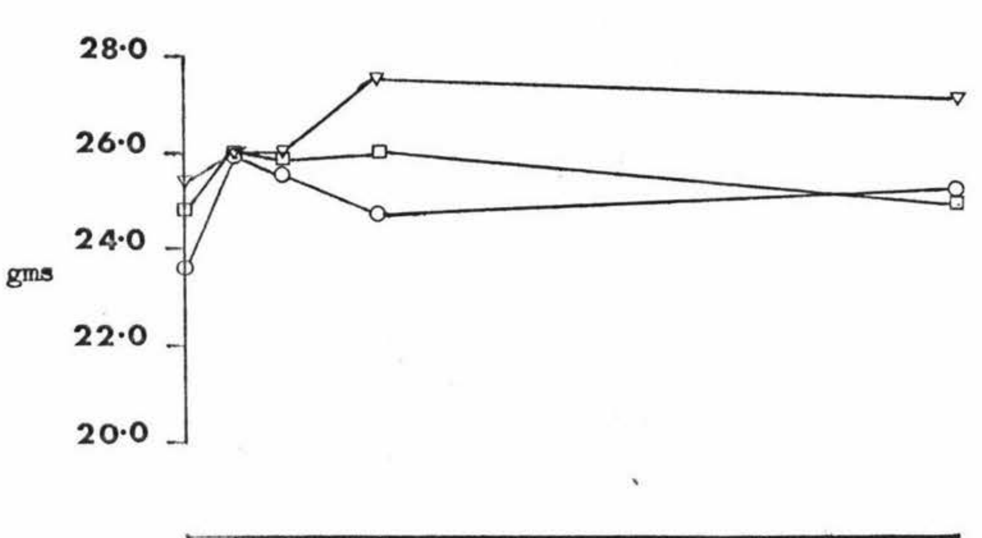

Fig 16 Total plant weight

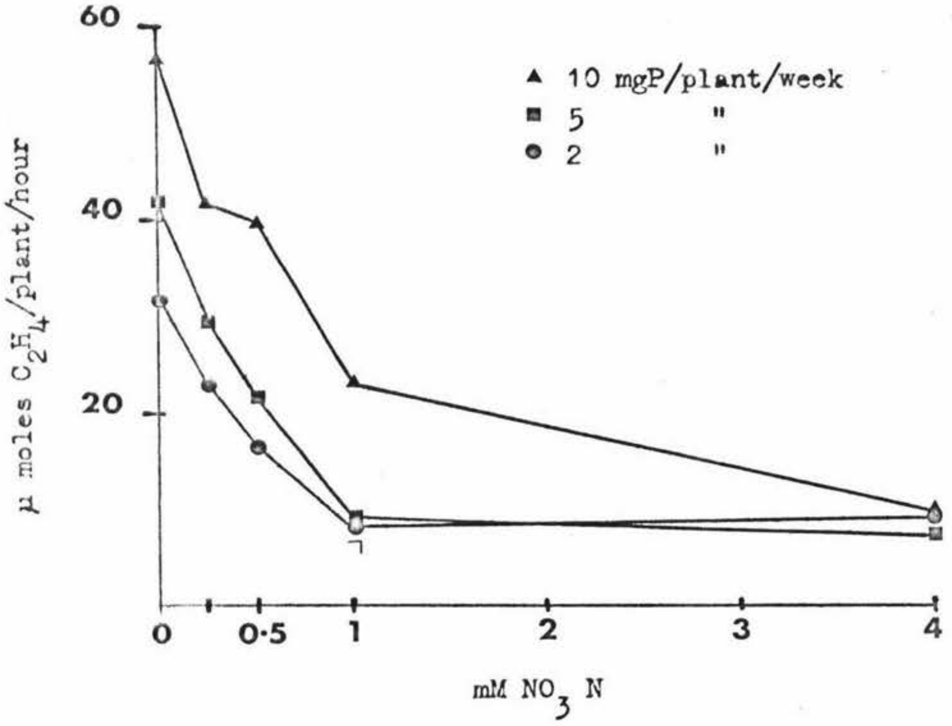

Fig 17 Rate of ethylene produced per plant

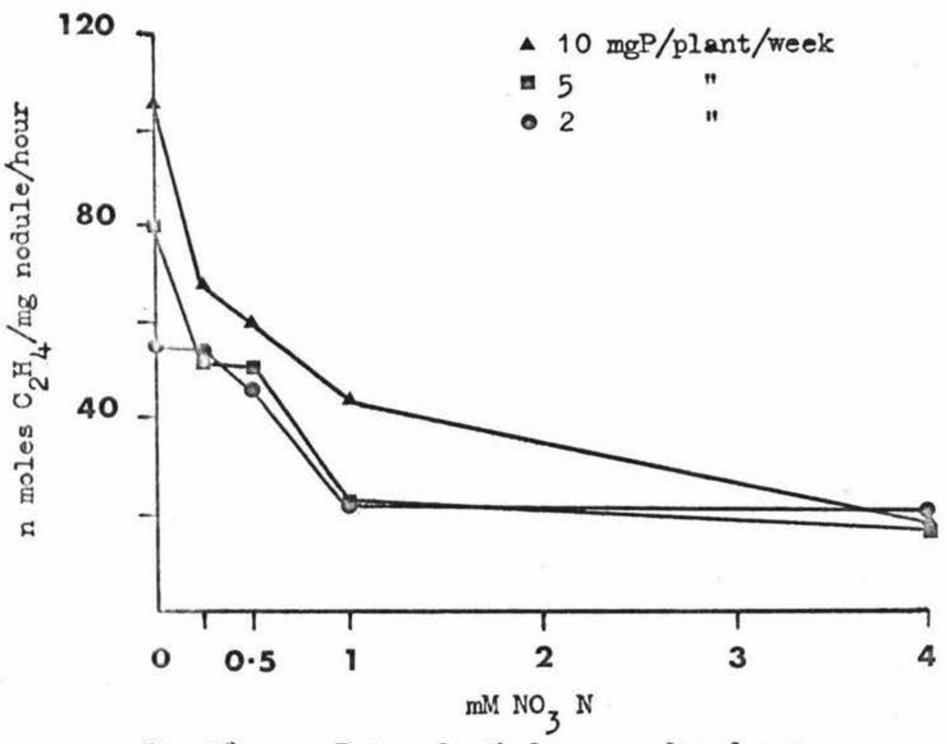

Fig 18 Rate of ethylene produced per mg nodule (dry weight) per hour

(c) Nitrate nitrogen x phosphate interaction

Though not significant, an interaction between the nitrate nitrogen and phosphate treatments was obtained (Fig.17). Noticeably larger phosphate treatment differences were recorded where nitrate was not added to the nutrient solution, these differences declined with the addition of nitrate, until at high levels of nitrate nitrogen, little difference existed between the phosphate means.

(B) Rate of Ethylene production per mg Dry weight nodule

A summary of the analysis of variance is presented in Appendix V 3.1. Significant nitrate nitrogen and phosphate treatment effects and an interaction between the nitrate nitrogen and phosphate treatments was recorded. The means for the treatments are shown in Fig. 18.

(a) Nitrate nitrogen

The addition of nitrate nitrogen to the growing medium of the clover plants significantly lowered the ability of the nodule material to reduce acetylene to ethylene. Significant differences were obtained between the nil nitrate treatment mean and the means for the 0.25, 0.5, 1.0 and 4.0mM nitrate nitrogen treatments and between the 0.25 or 0.5mM nitrate nitrogen treatment means, and the 1.0 or 4.0mM nitrate nitrogen treatment means (Appendix V 3.2).

(b) Phosphate

An increase in the rate of ethylene produced per mg DW nodule occurred when the quantity of phosphate supplied to the plants was lifted. The phosphate treatment means and the LSD are presented in Appendix V 3.3.

2.3.3 Plant Growth

(A) Top Growth

No significant increase in top growth occurred with the addition of nitrate nitrogen to the nutrient solutions. An increase in the availability of phosphate, did on the other hand significantly affect the growth of the top system of the clover

clone. An interaction between nitrate nitrogen and phosphate treatments was also obtained, but was only significant at the 10% probability level (Appendix V 4.1). The treatment means are presented in Fig. 20.

(a) Phosphate

Greater top growth resulted from the increase in the availability of phosphate and significant differences were recorded between the lowest and the highest phosphate treatment means, and the two higher phosphate treatment means (Appendix V 4.2).

(b) Nitrate nitrogen x phosphate interaction

At the lowest phosphate level, the top growth response to nitrate nitrogen was bimodal in form and was characterized by a peak at a low level of nitrate nitrogen, followed by a second peak at the highest level of nitrate nitrogen (Fig. 20). Top growth also peaked at the 0.25mM nitrate nitrogen level and intermediate level of phosphate, but the growth was not markedly reduced when higher rates of nitrate nitrogen were added, and nor did it recover at the highest nitrate nitrogen level. At the highest phosphate level, top growth increased markedly with high concentrations of nitrate nitrogen in the nutrient solutions.

(B) Root Growth

The analysis of variance is summarized in Appendix V 5.1. No significant phosphate treatment effects or nitrate nitrogen x phosphate interaction was obtained, but root growth increased significantly when nitrate nitrogen was applied to the nutrient solutions (Fig.19).

The means for the nitrate nitrogen treatments and the LSD are presented in Appendix V 5.2. There were significant increases in top growth between the 0.0mM nitrate nitrogen treatment means and between the 0.25mM nitrate nitrogen treatment and the 1.0mM nitrate nitrogen treatment mean.

(C) Total plant weight

The analysis of variance is summarized in Appendix V 10.1 and the mean data for total plant weight is presented in Fig 16. Phosphate and nitrate nitrogen significantly increased (P 0.01) the total plant growth.

The means for the nitrate nitrogen and phosphate treatments and the LSDs are presented in Appendices V 10.2, 10.3 respectively.

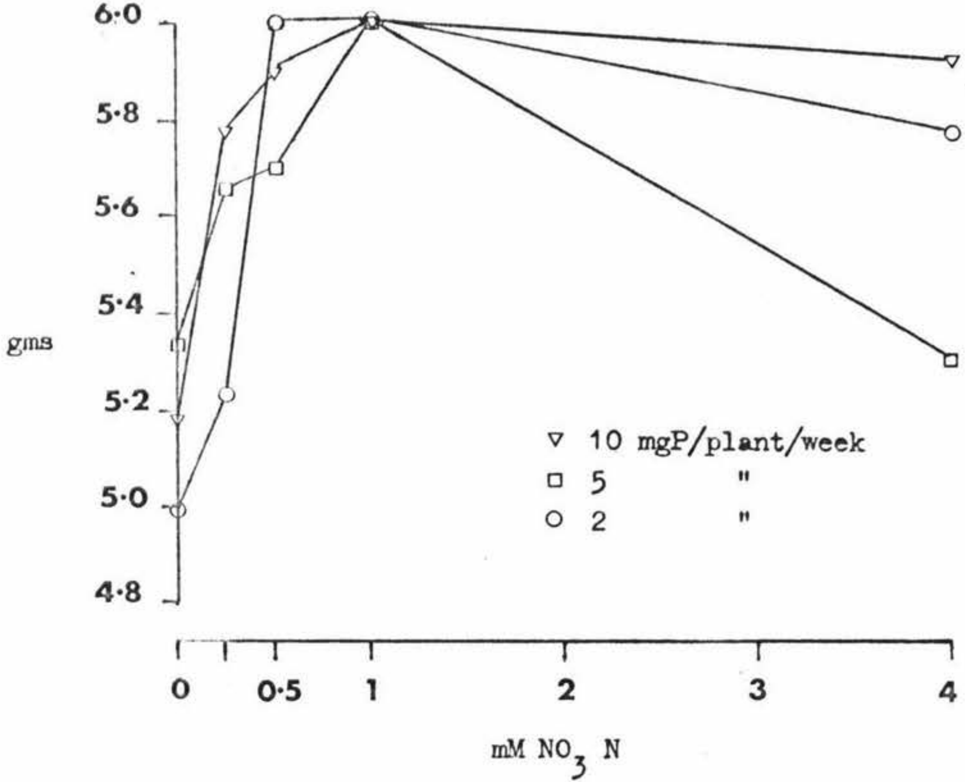

Fig 19 Dry weight of root material

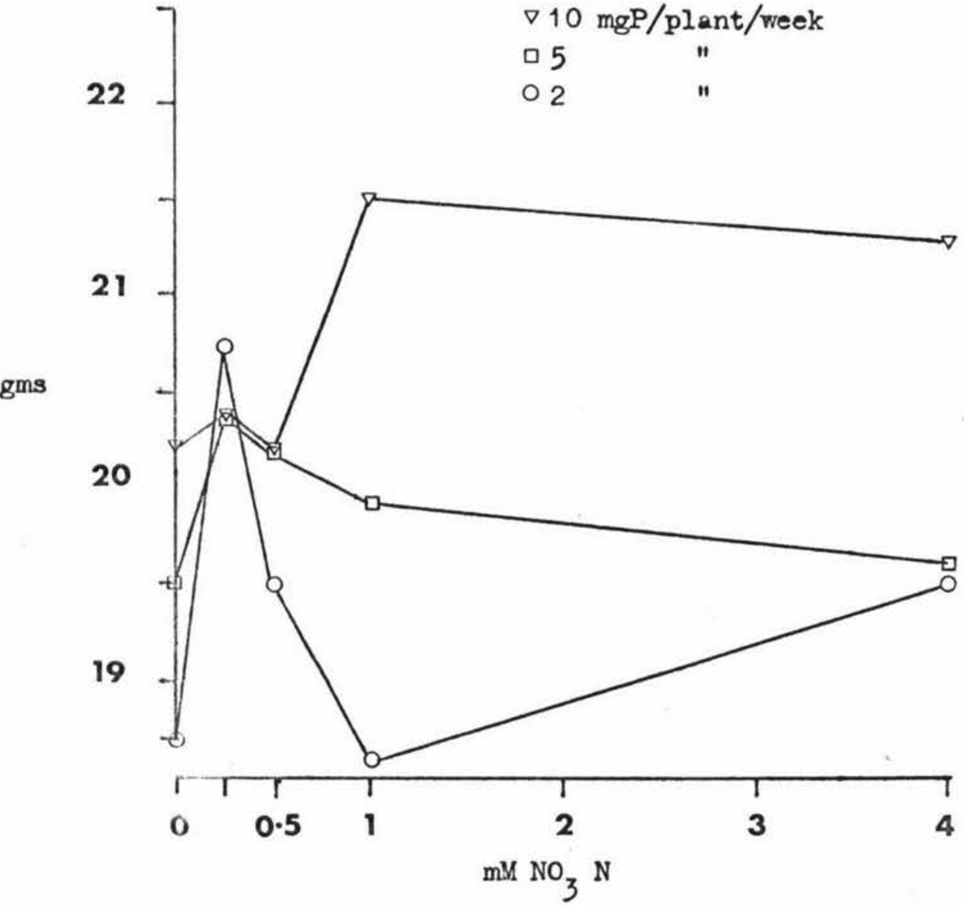

Fig 20 Dry weight of top material

2.3.4 Percent total nitrogen in top (a), root (b) and nodule material (c)

(A) Top Material

A significant ($P < 0.01$) increase in the percent total nitrogen in top material was obtained with the application of nitrate nitrogen. Higher phosphate levels did not however, significantly affect the total nitrogen content of the tissue, but an interaction was obtained between the nitrate nitrogen and phosphate treatments (Fig. 21A; Appendix V 6.1 (a). The means for nitrate nitrogen treatments and LSD are presented in Appendix V 6.2; significant differences occurring between all treatment means.

(a) Nitrate nitrogen x Phosphate interaction

There are three points of interest. First, in the control, nil nitrate treatment, the percentage of total nitrogen declined when the level of phosphate supplied to the plants was increased, while at high levels of nitrate nitrogen (4.0mM) the percent total nitrogen increased at the higher phosphate levels. Finally, phosphate had little effect on the percent total nitrogen level at the two lower nitrate nitrogen concentrations (Fig. 21A).

(B) Root Material

Significant nitrate nitrogen and phosphate treatment affects were recorded, but no interaction was observed (Appendix V 6.1 (b).

(a) Nitrate nitrogen

The percent total nitrogen content increased with the addition of nitrate nitrogen (Fig. 21B). All treatment means were significantly different from each other (Appendix V 6.2).

(b) Phosphate

Overall, the percent total nitrogen content declined when the level of phosphate was increased (Fig. 21B). The lowest phosphate level was only significantly greater than the highest; no significant differences being present between the other means

(Appendix V 6.3).

(C) Nodule Material

No significant treatment effects were recorded (Appendix V 6.1(c). The means for the treatments are presented in Table 12.

2.3.5 Percent Total Phosphate in Top, Root and Nodule Material

(A) Top Material

The analysis of variance is presented in Appendix V 7.1 (a). Nitrate nitrogen did not significantly change the percent total phosphate level, and no interaction between nitrate nitrogen and phosphate was obtained. The level of phosphate in the top material however, was significantly higher when the quantity of phosphate supplied to the plants was increased (Fig. 22A). All phosphate treatment means were significantly different from each other (Appendix V 7.2).

(B) Root Material

The means for the nitrate nitrogen and phosphate treatments are presented in Fig. 22B. Significant nitrate nitrogen and phosphate treatment effects were recorded (Appendix V 7.1 (b), but no interaction between nitrate nitrogen and phosphate was obtained.

(a) Nitrate nitrogen

The means for the nitrate nitrogen treatments are presented in Appendix V 7.2. Addition of nitrate nitrogen to the growing medium significantly reduced the level of total phosphate in root material.

(b) Phosphate

Highly significant increases in the level of phosphate in root material were obtained when the quantity of phosphate supplied to the plants was lifted. All phosphate treatment means were significantly different (Appendix V 7.3).

Table 12 Percent total nitrogen in nodule material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
2	7.48	6.95	7.22	7.10	7.52
5	7.14	7.25	7.32	7.48	7.07
10	7.25	7.16	7.17	7.40	7.55

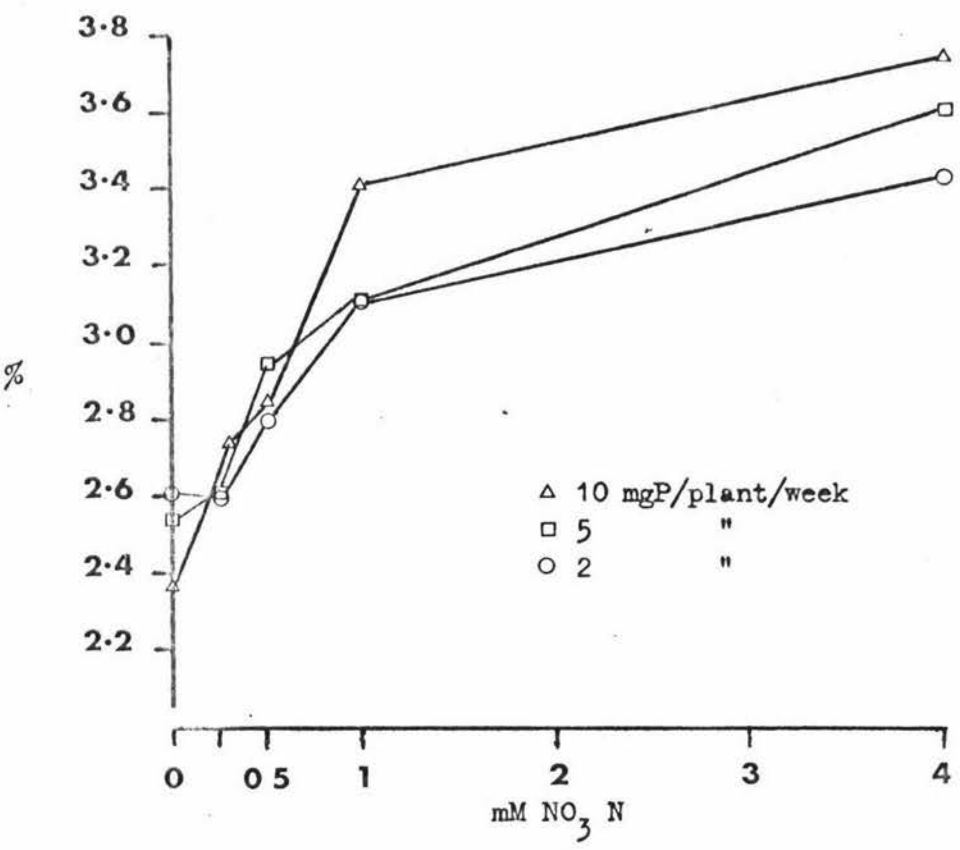

Fig 21 A Percent total nitrogen in top material

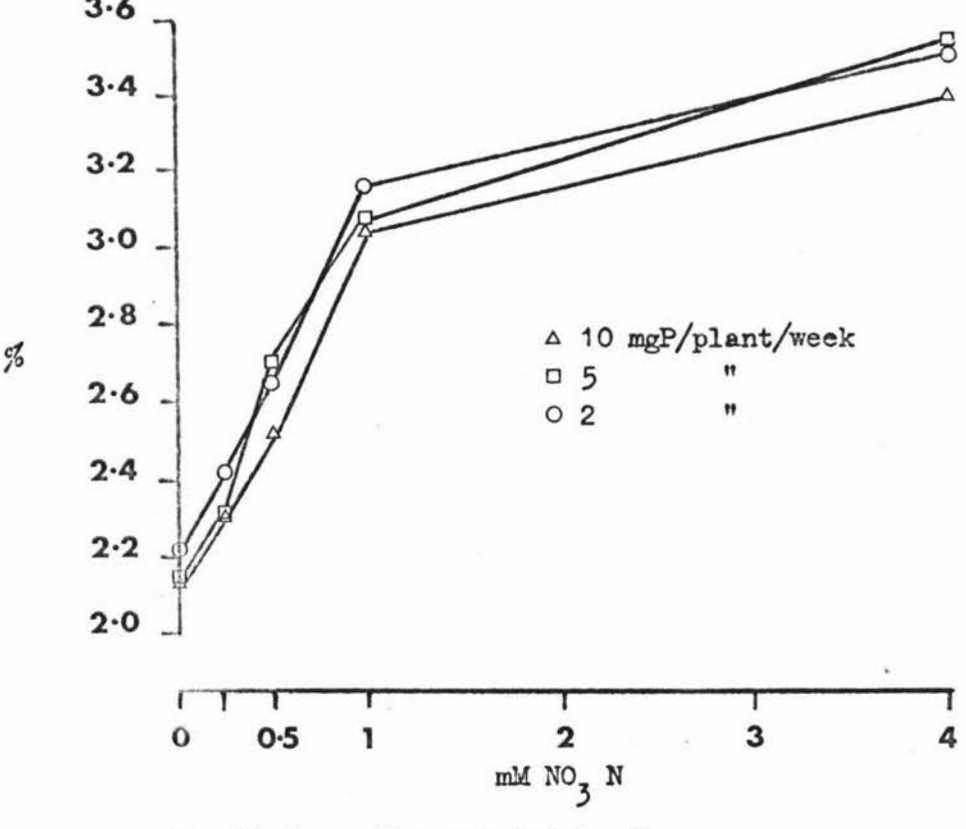

Fig 21 B Percent total nitrogen in root material

Table 13 Percent soluble sugars in top material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
2	8.28	7.72	7.98	7.56	7.23
5	8.10	8.09	8.07	7.93	7.38
10	7.65	8.31	8.00	7.88	7.16

Table 14 Percent soluble sugars in root material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
2	7.03	7.19	6.04	6.25	5.11
5	6.64	6.67	5.74	5.79	4.76
10	6.03	5.29	5.10	4.67	4.01

(C) Nodule Material

As in the case of root material, significant nitrate nitrogen and phosphate treatment affects were recorded (Appendix V 7.1 (c), for nodule material. The level of phosphate in the nodules was reduced by the application of nitrate nitrogen, (Appendix V 7.2) and highly significant increases in phosphate in nodule material followed when the quantity of phosphate in the growing medium was increased (Fig. 23).

2.3.6 Percent Soluble Sugars in Top, Root and Nodule Material

(A) Top Material

The means for the treatments are shown in Table 13. No significant nitrate nitrogen x phosphate interaction was recorded, and phosphate did not affect the level of soluble sugars in top material. A significant decline in the level of soluble sugars did occur with the application of nitrate nitrogen (Appendix V 8.1), the decline in sugar level between the 1.0mM nitrate nitrogen mean and the 4.0mM nitrate nitrogen mean being significant (Appendix V 8.2).

(B) Root Material

A summary of the analysis of variance for percent soluble sugars in root material is presented in Appendix V 8.1 (b). Nitrate nitrogen and phosphate significantly ($P < 0.01$) reduced the percent soluble sugar content, but no interaction was obtained (Table 14).

The nitrate nitrogen treatment means and the LSD are given in Appendix V 8.2 and the phosphate treatment means and LSD in Appendix V 8.3

(C) Nodule Material

A significant ($P < 0.05$) interaction was obtained between the nitrate nitrogen and phosphate treatments, and significant nitrate nitrogen and phosphate treatment effects were also recorded (Appendix V 8.1).

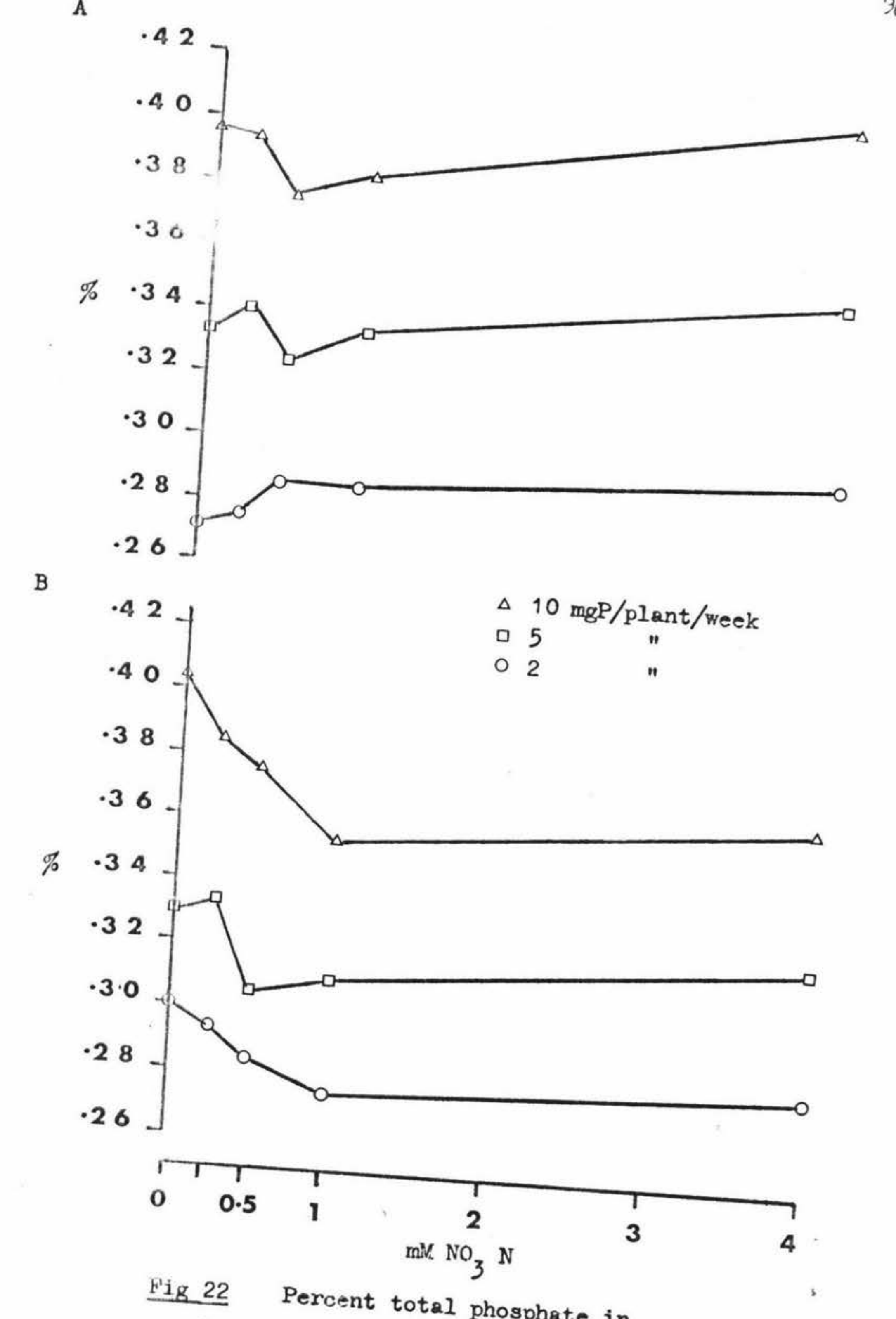

Fig 22 Percent total phosphate in top material (A) and root material (B)

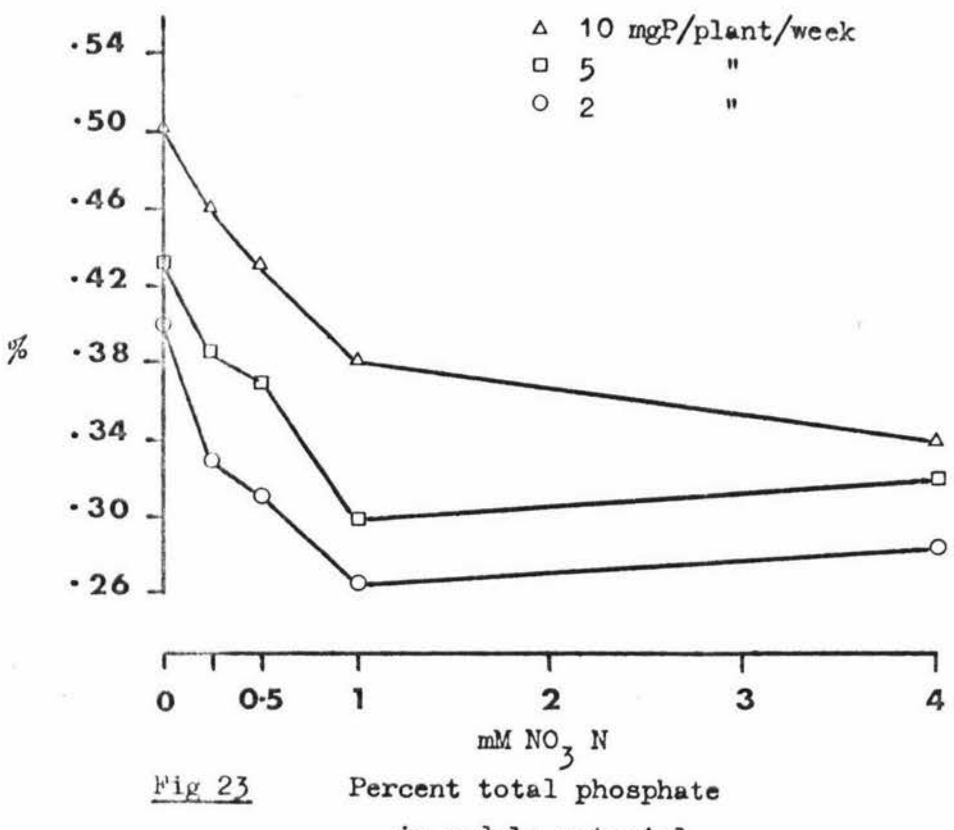

Fig 23 Percent total phosphate in nodule material

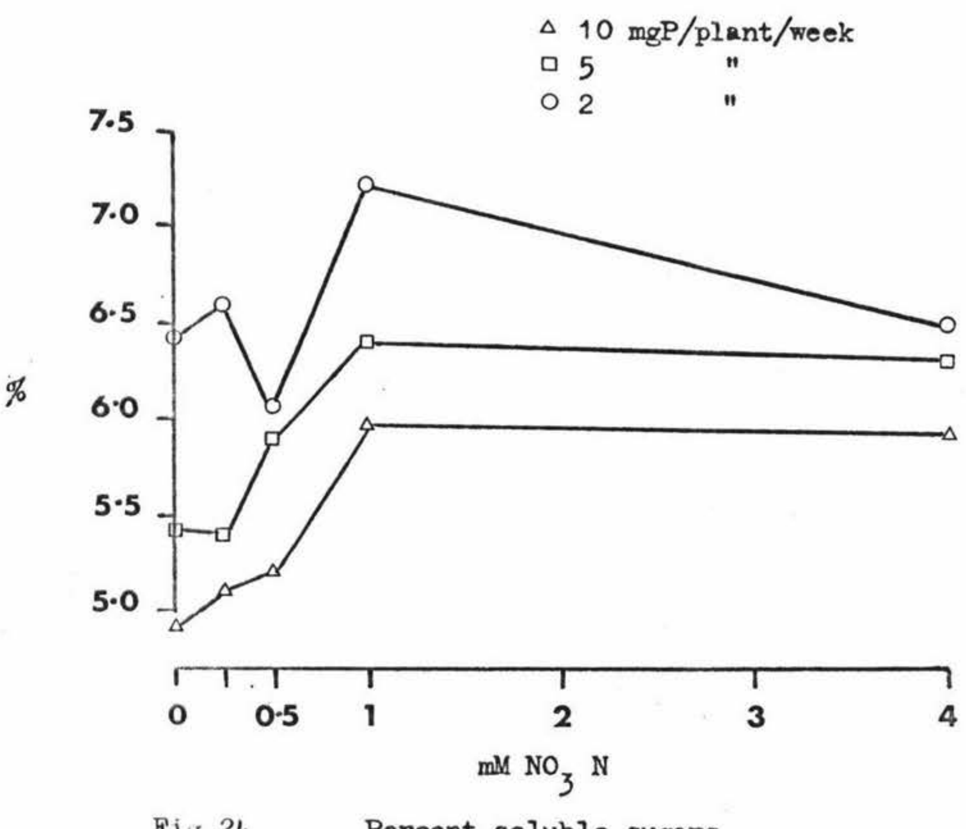

Fig 24 Percent soluble sugars in nodule material

(a) Nitrate nitrogen

The highest level of soluble sugar in nodule material was found in nodules detached from plants which had been grown in the 1.0mM nitrate nitrogen treatment. The sugar level declined steadily with the reduction in concentration of nitrate in the growing medium and was lowest in nodules removed from plants which had not received nitrate nitrogen (Fig. 24).

The nitrate nitrogen treatment means and LSD are reported in Appendix V 8.2. The 0.0mM nitrate nitrogen mean was significantly greater than the 0.5, 0.25 and 0mM nitrate nitrogen treatment means, but not significantly higher than the 4.0mM nitrate nitrogen treatment mean, while the 4.0mM nitrate nitrogen treatment was significantly higher than the 0.5, 0.25 and 0mM nitrate nitrogen treatment means.

(b) Phosphate

An increase in the supply of phosphate to clover plants reduced the level of soluble sugars in nodule material (Fig.24). The lowest sugar level was obtained in nodule material removed from plants grown at the highest phosphate level, the treatment mean being significantly lower than the means for both lower rates of phosphate (Appendix V 8.3). A significant difference between the means for the two lower phosphate levels was also obtained (Appendix V 8.3).

2.3.7 Percent Starch in Top, Root and Nodule Material

(A) Top Material

The phosphate and nitrate nitrogen treatment means for percent starch are shown in Table 15, and a summary of the analysis of variance is presented in Appendix V 9.1. Nitrate nitrogen and phosphate significantly ($P < 0.01$) changed the starch level in top material, but no interaction between these nutrients was recorded (Appendix V 9.1).

(a) Nitrate nitrogen

A significant reduction in the percent starch in top material occurred when nitrate nitrogen was applied to the rooting medium of clover plants. The LSD for the nitrate nitrogen treatment means is presented in Appendix V 9.2.

(b) Phosphate

The starch level in the top material was lower in those plants which had received higher levels of phosphate (Table 15). All phosphate treatment means were significantly different from each other (Appendix V 9.3).

(B) Root Material

A summary of the analysis of variance is presented in Appendix V 9.1. Nitrate nitrogen and higher phosphate levels, significantly ($P < 0.01$) reduced the percent starch level in root material. No significant interaction between nitrate nitrogen and phosphate was obtained (Table 16). The means for the nitrate nitrogen treatments and phosphate treatments are shown in Appendix V 9.2 and 9.3 respectively.

(C) Nodule Material

No significant phosphate treatment effect was obtained and no interaction between nitrate nitrogen and phosphate was recorded. However, nodules removed from plants which had been grown in solutions supplied with nitrate nitrogen had significantly ($P < 0.01$) lower starch levels (Table 17).

Significant differences were obtained between all the nitrate treatment means, with but one exception. The difference between the 1.0mM nitrate nitrogen treatment mean and the 4.0mM nitrate nitrogen treatment mean was not significant at the 5% probability level (Appendix V 9.2).

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Table 15 Percent total starch in top material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
2	16.08	15.77	14.35	13.55	13.43
5	14.96	14.45	13.19	12.40	11.59
10	14.94	12.96	12.21	10.66	10.47

Table 17 Percent total starch in nodule material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
2	7.10	6.13	4.66	3.61	4.02
5	7.50	6.35	6.41	3.26	3.53
10	7.53	7.14	5.89	4.33	3.24

Table 16 Percent total starch in root material

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
2	5.08	4.34	4.42	4.38	4.06
5	5.37	3.66	4.19	4.08	3.85
10	4.59	3.71	3.53	3.28	3.57

2.4.1 EXPERIMENT 5

The rate at which nitrate nitrogen inhibited nodulation and nitrogen fixation was studied in this experiment. However, only within harvest comparisons of treatment means were made.

2.4.2 Nodule Dry Weight

No significant change in nodule weight was recorded on clover roots, when plants were harvested two and six days after the application of nitrate nitrogen to the nutrient solutions (Fig. 28) and (Appendix VI 1.1). However, after 12 days growth in nutrient solutions containing nitrate, nodule production was significantly reduced ($P < 0.01$). For harvest three, the highest nodule weight was recorded in the nil nitrate control treatment and the lowest for the 2.0mM nitrate nitrogen treatment. The increase in nodule weight obtained when the concentration of nitrate nitrogen was increased to 8.0mM from 2.0mM was not significant (Appendix VI 1.2).

2.4.3 Ethylene production per plant per hour

The analysis of variance for each harvest is presented in Appendix VI 2.1. The rate of ethylene production per plant at harvest was not significantly changed by the addition of nitrate nitrogen to the growing medium, but with time and with the uptake and utilization of nitrate nitrogen by the plants, very significant ($P < 0.01$) reductions in rates of ethylene production were recorded (Appendix VI 2.1).

The nitrate nitrogen treatment mean and LSDs for harvests 2 and 3 are presented in Fig. 26 and in Appendix VI 2.2.

2.4.4 Ethylene production per mg DW nodule per hour

The rate of ethylene production per mg DW nodule was not significantly reduced by the application of nitrate nitrogen at harvest 1, but at harvest 2 and 3, significant reductions in activity were recorded (Appendix VI 3.1). As was the case for rate ethylene production per plant, the reductions became more significant with time (Appendix VI 3.1) and (Fig. 27).

At harvest 2, no significant differences were recorded between the treatments where nitrate was present in the growing medium. The 2.0mM nitrate nitrogen treatment mean was however, significantly lower than the 0.5mM nitrate nitrogen treatment mean at harvest 3 (Appendix VI 3.2).

2.4.5 Top dry weight

No significant changes in top dry weight were recorded at any harvest, when nitrate nitrogen was added to the growing media of 'mature' nodulated clover plants (Appendix VI 4.1). A summary of the treatment means is presented in Fig. 30.

2.4.6 Root dry weight

A reduction in root dry weight, just significant at the 5% level of probability, was recorded for the third harvest (Appendix VI 5.1). No significant changes in root weight were recorded in harvest 1 and 2. The treatment means and LSD for harvest 3 are presented in Fig. 31 and Appendix VI 5.2.

2.4.7 Total plant weight

No significant changes in total plant growth were recorded at any harvest, when nitrate nitrogen was fed to 'mature' nodulated clover plants. (Appendix VI 9.1) and (Fig. 29).

2.4.8 Percent total nitrogen in Top, Root and Nodule Material

The percent total nitrogen levels presented in this experiment are for harvest 2 and 3; no analyses were conducted on plant material from harvest 1.

(A) Top Material

As expected, the addition of nitrate nitrogen significantly increased the percent total nitrogen level in top material (Appendix VI 6.1). The treatment means and the LSD for percent total nitrogen are presented in Table 18A and Appendix VI 6.2.

Fig 26 Rate of ethylene produced per plant per hour

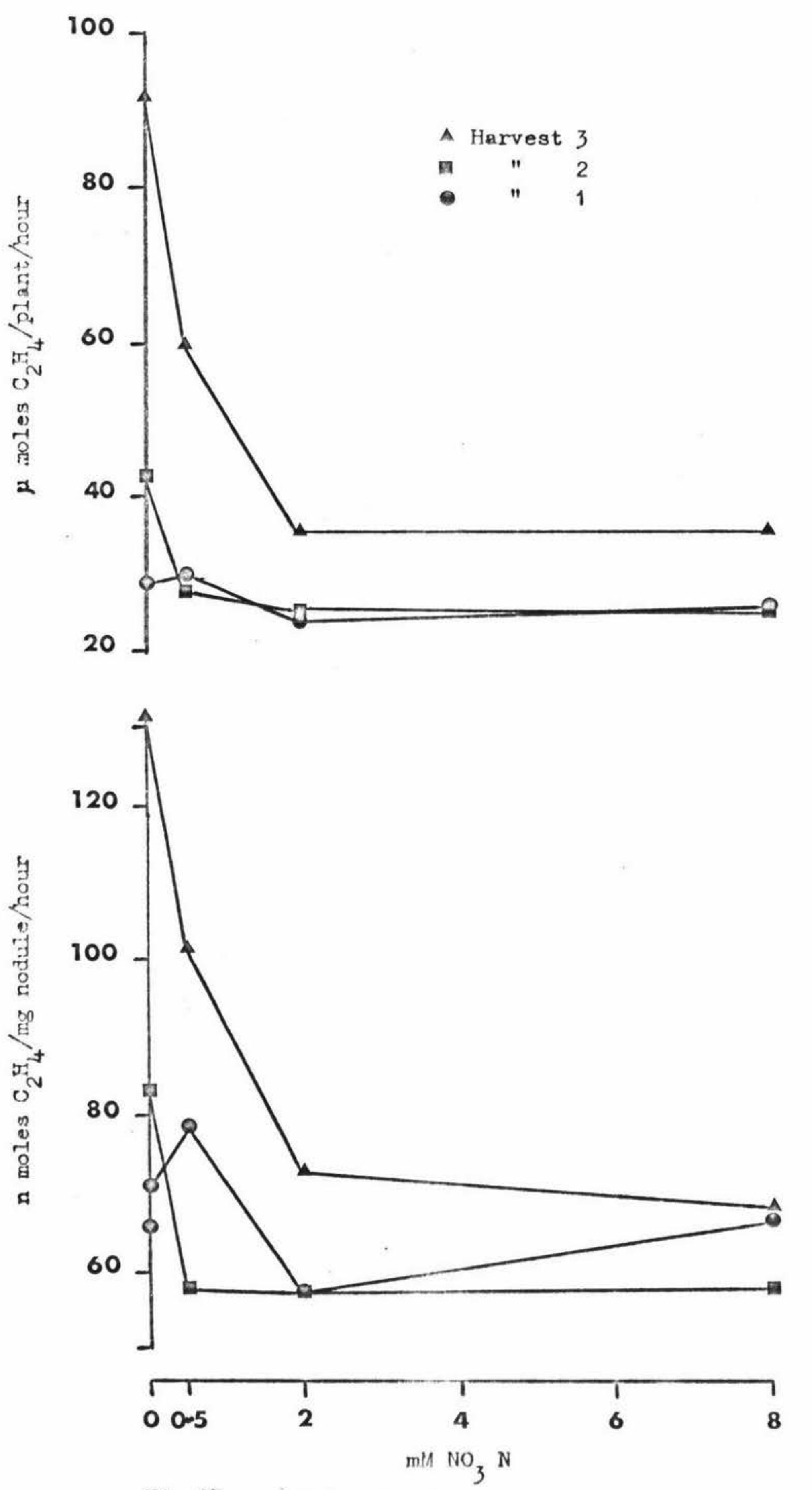

Fig 27 Rate of ethylene produced per mg nodule (dry weight) per hour

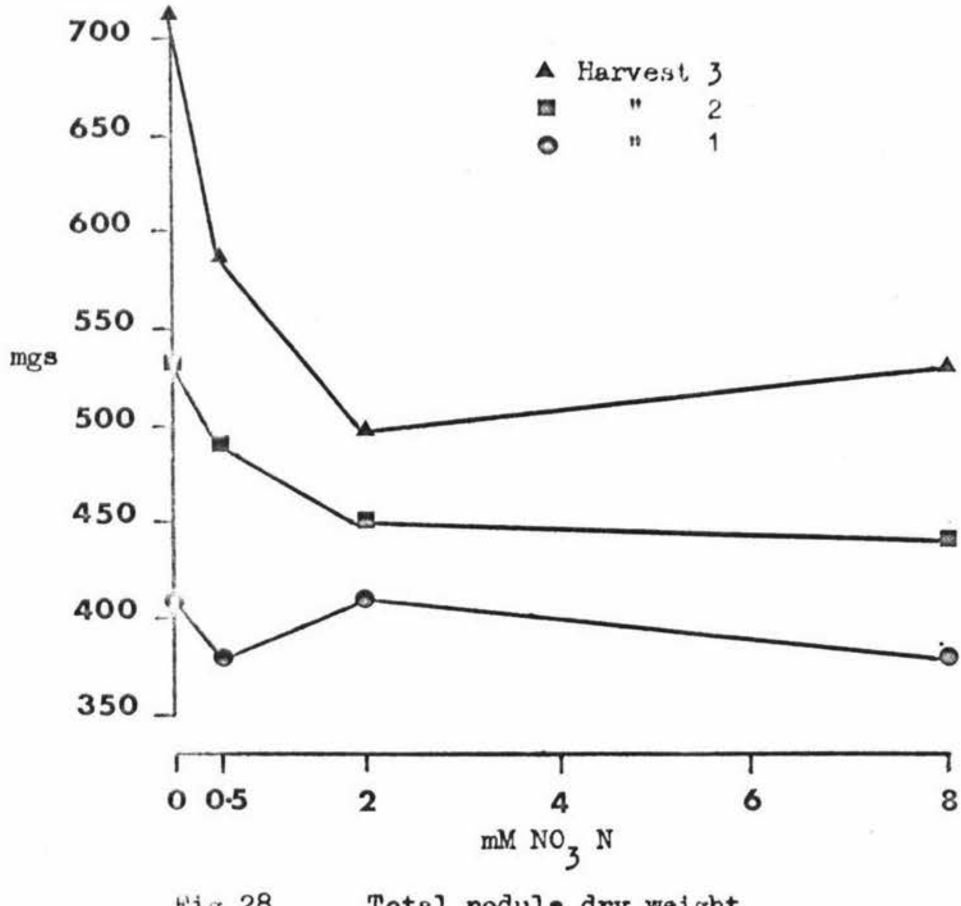

Fig 28 Total nodule dry weight

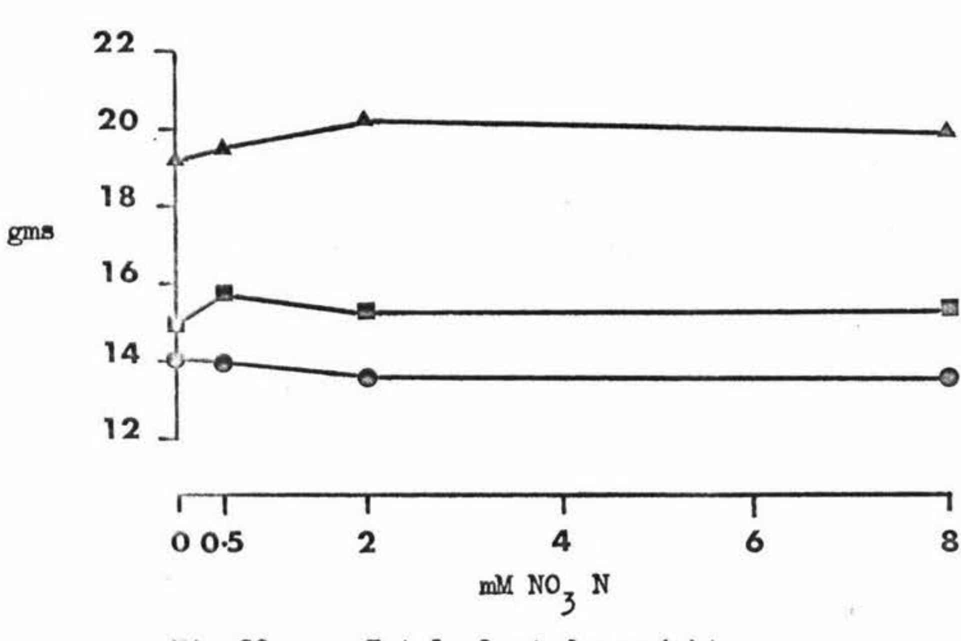

Fig 29 Total plant dry weight

Fig 30 Top dry weight

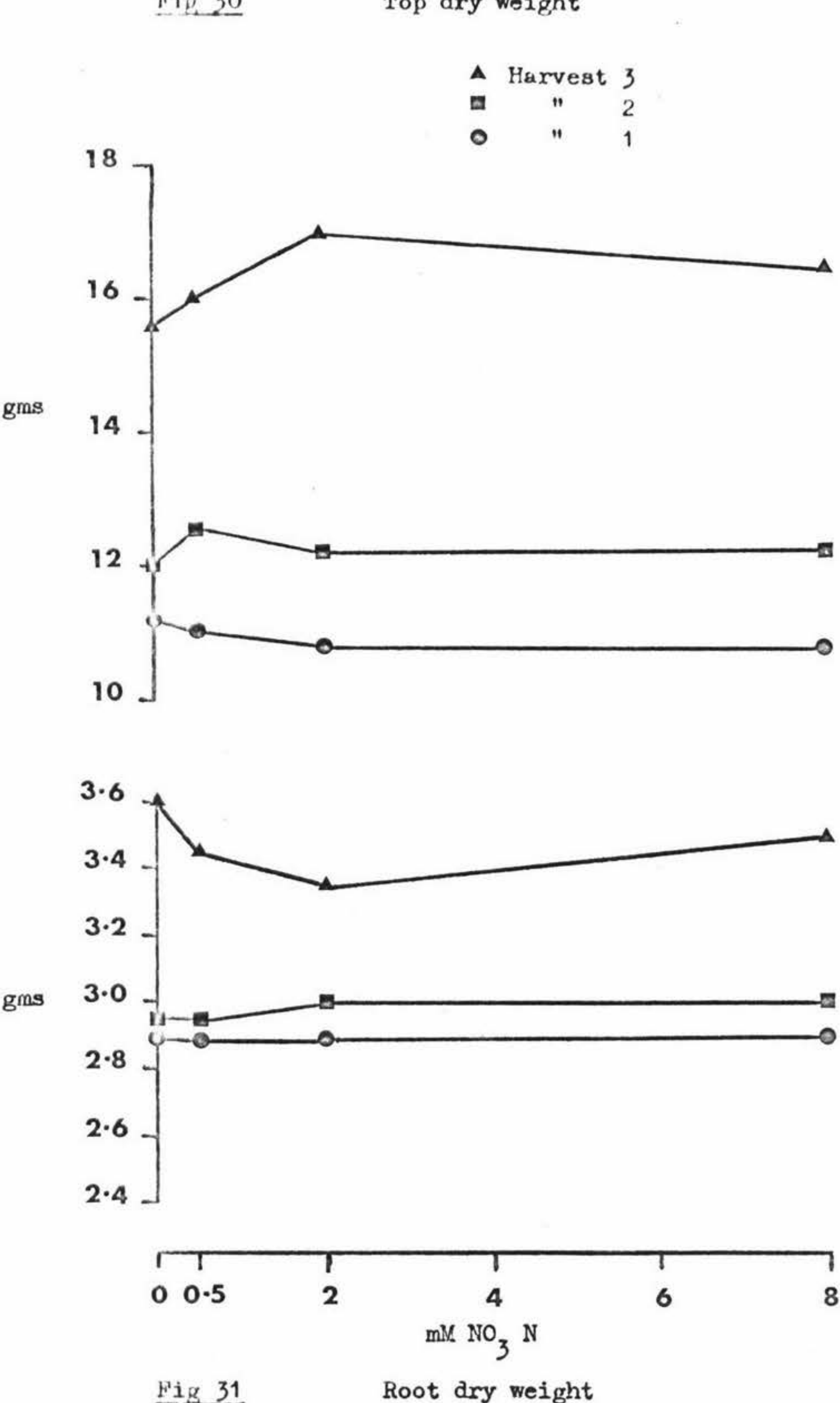

Fig 31 Root dry weight

(B) Root Material

A summary of the treatment means is given in Table 18B. Significantly ($P < 0.01$) higher percent total-nitrogen values were obtained where nitrate nitrogen was added to the growing media of clover plants. The treatment means and the LSD for percent total nitrogen in root material are presented in Table 18B and Appendix VI 7.2.

(C) Nodule Material

While an increase in percent total nitrogen was recorded in nodule material which was removed from plants after six days growth in nutrient solutions containing nitrate nitrogen (Table 18C), no significant change in percent total nitrogen was obtained in nodule material removed from plants which were harvested twelve days after the commencement of the nitrate treatments (Appendix VI 8.1).

The nitrate nitrogen treatment means and LSD for harvest 2 are presented in Appendix VI 8.2.

Table 18 Percent total nitrogen in top, root and nodule

	Conc. of NO_3 N (mM)			
	0	0.5	2.0	8.0
A Top material				
Harvest 2	2.98	3.31	3.50	3.54
Harvest 3	2.96	3.33	3.84	3.84
B Root material				
Harvest 2	2.18	2.59	2.55	2.67
Harvest 3	2.18	2.41	2.83	2.75
C Nodule material				
Harvest 2	7.02	7.37	7.36	7.42
Harvest 3	7.42	7.40	7.67	7.51

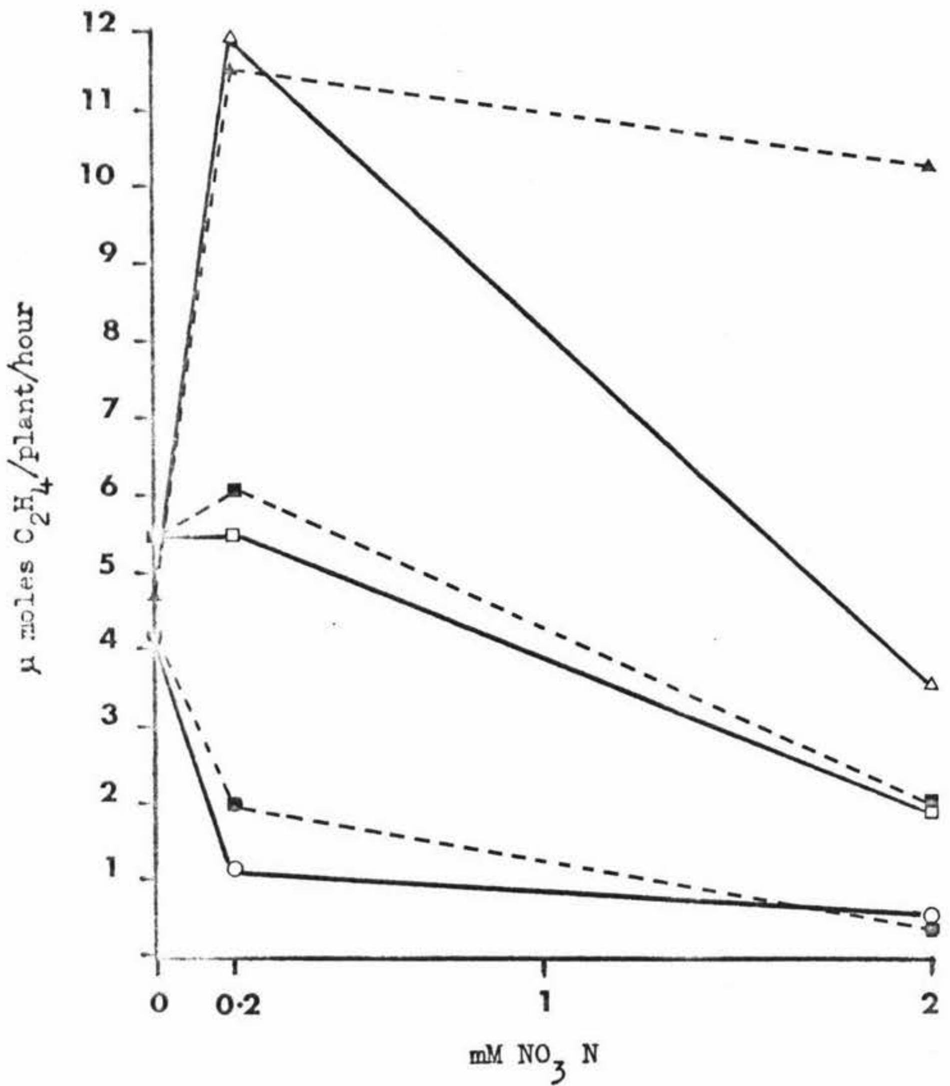

Fig 32 Rate of ethylene produced per plant per hour

▲ 1.5 mgP/plant/week
△ 1.5 "
■ 0.5 "
□ 0.5 "
● 0.2 "
○ 0.2 "
----- NO_3 N not corrected during the last 10 days of the experiment

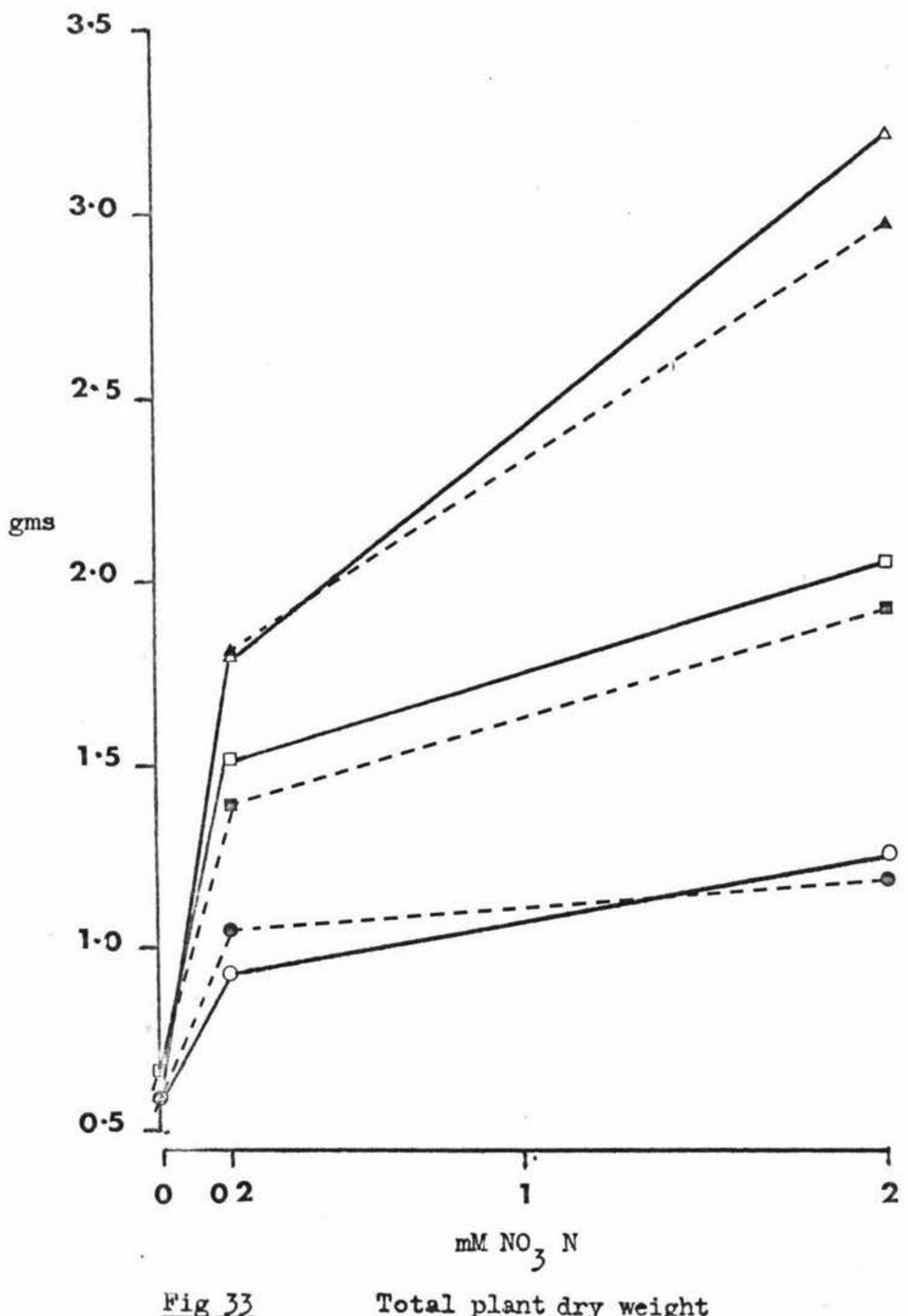

Fig 33 Total plant dry weight

▲ 1.5 mgP/plant/week
△ 1.5 "
■ 0.5 "
□ 0.5 "
● 0.2 "
○ 0.2 "
------ NO_3 N not corrected during the last 10 days of the experiment

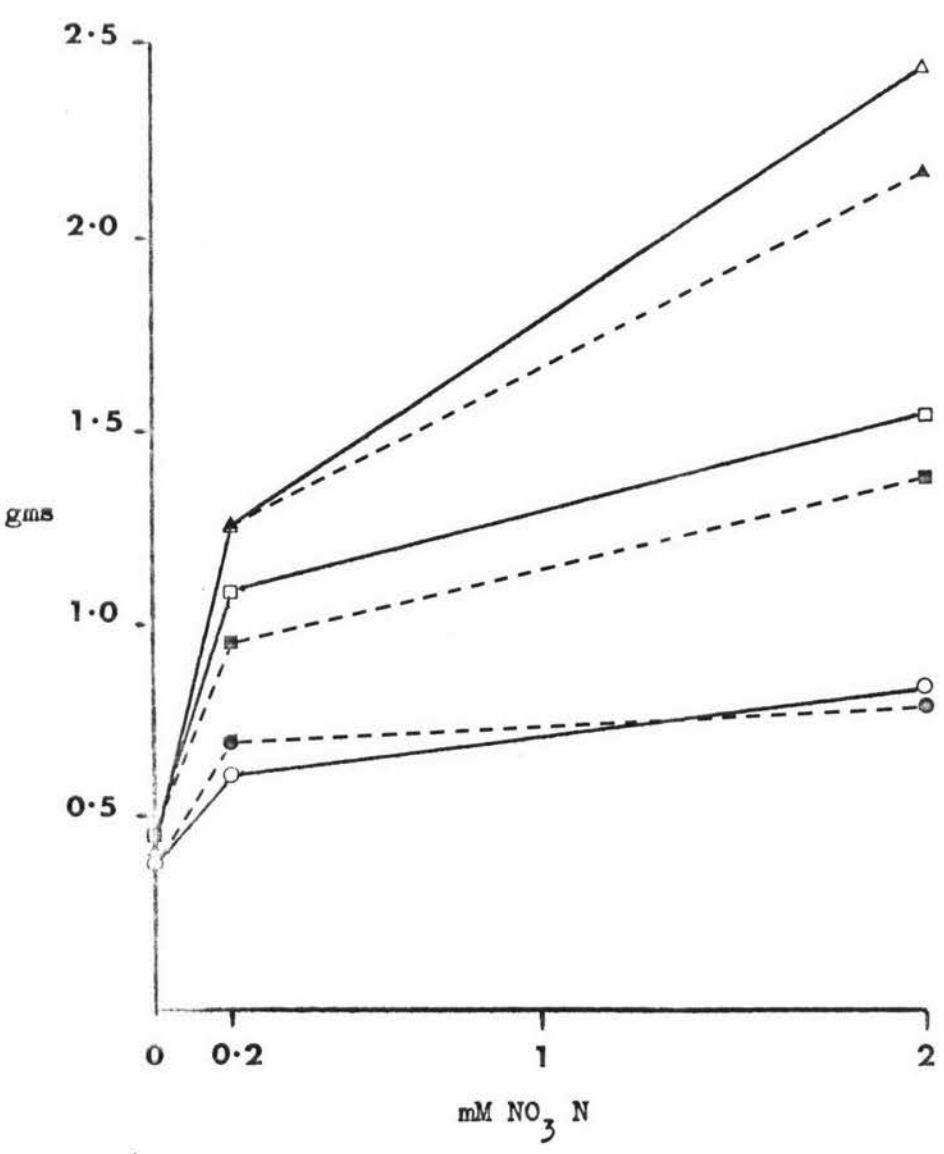

Fig 34 Dry weight top material

▲ 1.5 mgP/plant/week
△ 1.5 "
■ 0.5 "
□ 0.5 "
● 0.2 "
○ 0.2 "
------ NO_3 N not corrected during the last 10 days of the experiment

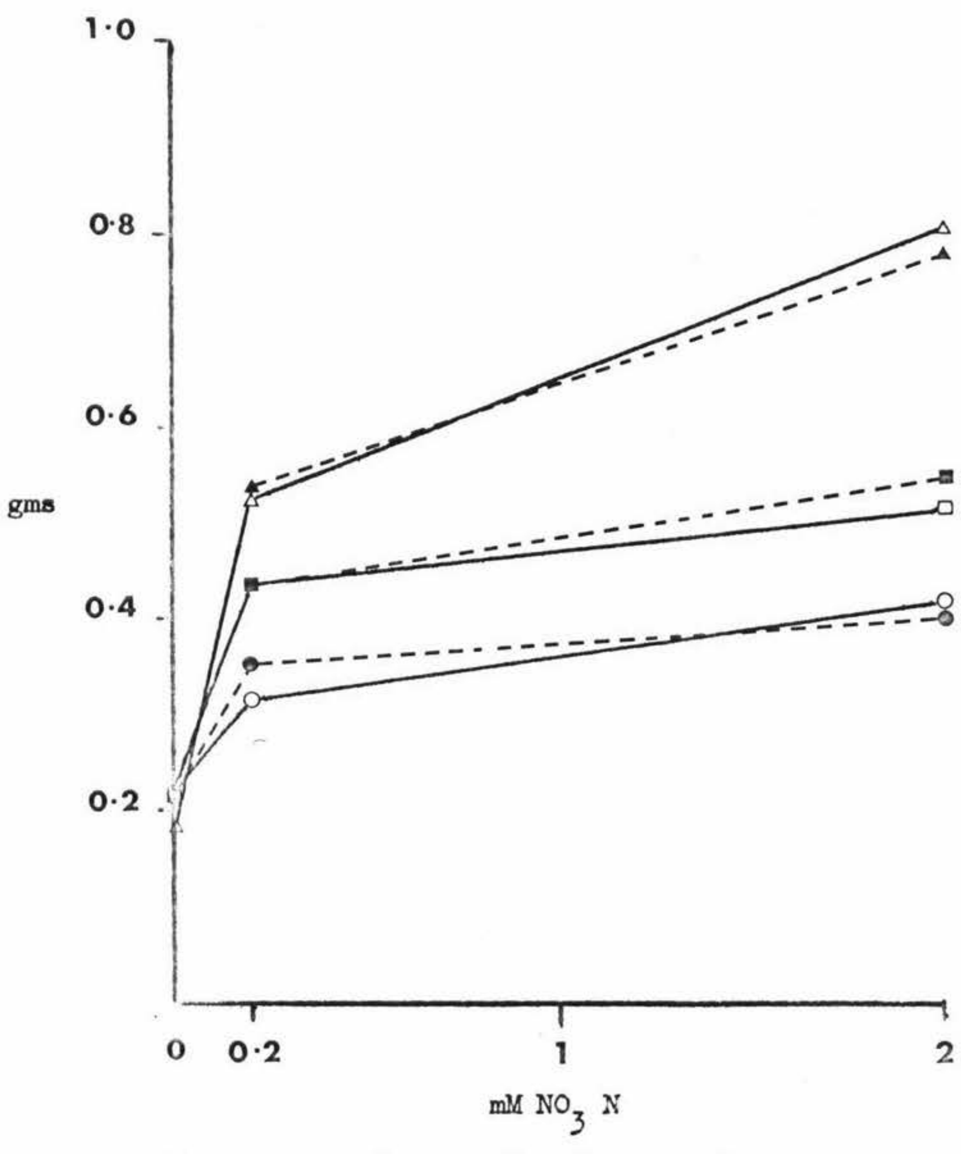

Fig 35 Dry weight of root and nodule material

▲ 1.5 mgP/plant/week
△ 1.5 "
■ 0.5 "
□ 0.5 "
● 0.2 "
○ 0.2 "
- - - - - NO_3N not corrected during the last 10 days of the experiment

2.5.1. EXPERIMENT 6

Because Experiment 6 was conducted late in this study, there was not sufficient time for a detailed statistical analysis of the data.

The treatment means for rate of ethylene produced per plant per hour, total plant weight, top dry weight and root dry weight are presented in Figs 32, 33, 34 and 35 respectively.

Table 19 Top to root ratio Experiment 3

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.25	0.5	1.0	4.0
0.2	3.08	3.32	2.88	2.86	2.78
0.5	4.33	3.61	4.63	3.94	4.06
1.5	4.30	4.14	4.44	4.54	4.80

Table 20 Top to root ratio Experiment 6

Level of Phosphate mgP/plant/week	Conc. of NO_3 N (mM) 0	0.2	2.0
0.2	1.68	1.92	2.00
0.5	2.04	2.50	2.94
1.5	2.28	2.40	3.00

CHAPTER IV

DISCUSSION

For convenience, the discussion will be handled in four sections. In the first section, the effect of nitrate nitrogen and phosphate on nodulation will be discussed, while in the second and third, the effect of these treatments on nitrogen fixation and plant growth will be considered. The role of the carbohydrate to nitrogen ratio and the effect of nitrogen and phosphate treatments on the carbohydrate, nitrogen and phosphate content of plant and nodule material will be discussed in the final section.

1. NODULATION

In their review, Raggio and Raggio (1962) concluded, that low rates of nitrate nitrogen (around 5 to 14ppm but even up to 50 ppm NO_3 N) can be beneficial for nodulation in legumes. The results obtained in several experiments in this study, where plant material of a different age and nodulation status were used, do not support their conclusion since low concentrations of nitrate nitrogen (0.25, 0.5mM) in solution culture significantly reduced the weight of nodule material (Fig 7). For example, in Experiment 3, the nodule weight of clover clones was reduced to 60, 40, 30 and 27% of the nodule weight of plants grown in solution cultures without nitrate nitrogen, when concentrations of 0.25, 0.5, 1.0 and 4.0mM nitrate nitrogen were applied respectively (Fig 7). Much of the reduction in nodule weight occurred then at nitrate nitrogen concentrations below the levels considered by Raggio and Raggio (1962) to be stimulatory and similar results to those recorded for Experiment 3, were also obtained in Experiments 4 and 5, where nitrate nitrogen was added to the growing media of nodulated 'mature' clover clones (Figs 15 and 28).

Further reductions in nodule weight occurred when the concentration of nitrate nitrogen was increased to 4mM in Experiment 3 (Fig 7). However, the 4.0mM nitrate nitrogen mean for nodule weight was only significantly different from the mean for the 0.25mM

nitrate nitrogen level (Fig 7). Similar responses were also recorded, where high levels of nitrate nitrogen were added to 'mature' clover clones (Figs 15 and 28). However, in the latter experiments, the nodule weight obtained from plants receiving 1.0 or 4.0mM nitrate nitrogen levels were slightly but not significantly higher than those recorded for the 0.5mM nitrate nitrogen treatment.

Nodule weight is significantly reduced when large concentrations of nitrate nitrogen are applied to the medium of growing white clover plants. Thornton and Nicol (1936) and Oghoghorie and Pate (1971) have also recorded marked reductions in nodule weight when large concentrations of nitrate nitrogen were added to lucerne (Medicago sativa) and field peas (Pisum arvense L.) respectively.

An evaluation of the number of nodules present on the root system of white clover plants after treatment with a series of nitrate nitrogen concentrations, was undertaken in Experiments 1, 2 and 3. In the growth room experiments, very significant reductions in nodule number were obtained, when Huia White clover seedlings were grown for ten days in the presence of nitrate nitrogen. Concentrations of nitrate nitrogen as low as 0.1mM significantly reduced the nodule number, and the nodule number steadily declined as the level of nitrate nitrogen was increased, so that in Experiment 2 only a third of nodules counted on nil nitrate control plants were present on plants grown in the 8.0mM nitrate nitrogen solution culture (Fig 4). In Experiment 2, significant differences were recorded between most nitrate nitrogen treatment means, but in Experiment 1, because higher rates of nitrate nitrogen were used, significant treatment mean differences were recorded only between the nil nitrate treatment mean and the means for the nitrate treatment. Munns (1968b) also found that nodule number was significantly reduced when lucerne seedlings were grown for short periods in dilute (0.02 and 0.2mM) nitrate nitrogen solutions.

The reduction in nodule number with the application of high concentrations of nitrate nitrogen was not as marked in Experiment 3 (Fig 6); the decline did not parallel the decrease in nodule weight which occurred under high nitrate conditions. For example, on applying nitrate nitrogen concentrations of 0.25, 0.5, 1.0 and

4.0mM, the nodule number was reduced to 82, 69, 64 and 59% of the number found on no nitrate control plants (Fig 6). Thornton and Nicol (1936), Pate and Dart (1961) and Oghoghorie and Pate (1971) also reported that nodule number was less markedly affected than either nodule weight or volume of nodule tissue. Pate and Dart (1961) suggested that nodulation, as characterized by nodule number became less sensitive to reduction by nitrate as the host plant aged. Munns (1968a) and Dart and Wildon (1970) have obtained similar results. For example, Munns noted that the nodule number in the first 'crop' of nodules produced on lucerne seedling roots was severely reduced by nitrate whereas the nodule number in a later 'crop' was not affected. Dart and Wildon (1970) reported that the number of nodules on the primary roots of cowpea plants were reduced when different forms of combined nitrogen were applied at high concentrations, but no reduction in nodule number was detected on the secondary roots. Munns (1968a) claimed that with time the experiments cease to be a simple test of the effect of nitrate on nodule production; rather they became comparisons between abundantly nodulated plants in nitrate free medium, and sparcely nodulated plants in medium containing nitrate. When Munns (1968a) tested the effect of nitrate on plants of comparable size and degree of nodulation, nodule production was inhibited by 70 - 80%, regardless of plant age or whether the plants were previously nodulated or not.

Nutman (1956; 1965) has published evidence that the inhibitory effect of an established nodule originates in the nodule meristem, and that larger nodules, because of the more active nodule meristem are more inhibitory than smaller nodules. The mean average weight of nodules in the nil nitrate treatment was significantly greater than the mean average weight of nodules in the 0.25, 0.5, 1.0 and 4.0mM nitrate nitrogen treatments (Fig 8) and the mean average weight of nodules in the 0.25mM nitrate nitrogen treatment was significantly greater than the 0.5, 1.0 and 4.0mM nitrate nitrogen treatments. Thus, in Experiment 3, the inhibition of further nodule formation would be much greater in the nil nitrate treatments, and since most nodules appeared in the second flush or on secondary

roots, features which both Munns (1968a) and Dart and Wildon (1970) reported, the true effect of combined nitrogen and nitrate nitrogen in particular was hidden, due to the vigorous restraint offered by the prior heavy nodulation. Such results suggest that nodule number alone is not a satisfactory guide to the effect of nitrate nitrogen on nodulation in legumes; nodule weight or volume of nodule tissue are better indices. It is possible that the stimulation in nodule number by low levels of nitrate nitrogen recorded by Richardson et al (1958) and McConnell and Bond (1957) may have been due to the inhibitory effect of prior nodulation on further nodule development in the control treatments.

There are other reasons why nodule number in the experiments in this study; in particular the growth room experiments, was markedly reduced by low concentrations of nitrate. The concentration of the nitrate nitrogen in the medium was maintained during the duration of the experiments and local depletion of nitrate was also prevented by using vigorously aerated solution cultures. The supply of nitrate with time, did decrease in Experiment 3 as a result of increased plant growth and a faster rate of removal of nitrate nitrogen which may have contributed slightly to the less inhibitory effect of nitrate on nodule number recorded in this experiment.

Phosphate generally increased the extent of nodulation in these experiments. A very significant increase in nodule number resulted from the application of phosphate to nutrient solutions in Experiment 2. However, an increase in phosphate concentration from .005mM to .05 or 0.5mM did not significantly increase the nodule number at low nitrate nitrogen levels, although there appeared to be some advantage in applying the higher rate at high levels of nitrate nitrogen.

Gates (1970) suggested that the demand upon phosphate seed reserves during nodule development could be considerable, with intense competition occurring between the nodule and host seedling for this supply of phosphate under conditions of limited external supply of phosphate. Nodule number was severely reduced when phosphate was not supplied to the medium (Fig 4). Presumably very low rates of phosphate could induce similar but less severe effects. The severity of the phosphate deficiency increased as nitrate nitrogen

was applied and no nodulation was recorded at very high nitrate nitrogen concentrations (Fig 4).

In Experiment 3 significant increases in nodule weight and nodule number were recorded where the phosphate level was increased from 0.2mgP / plant / week to 0.5mgP / plant / week, but no significant increase occurred when the rate of phosphate was increased to 1.5mgP / plant / week, from the 0.5mgP / plant / week. The increase in nodule weight which occurred, was a result of the increase in number of nodules formed, not in an increase in the average weight, since the means for the three phosphate treatments were not significantly different from each other (Fig 8). Further work is obviously required to verify this finding. Application of higher rates of phosphate to 'mature' clover clones also stimulated nodule growth, more particularly where nitrate nitrogen was applied as well (Fig 15).

No interactions between nitrate nitrogen and phosphate were recorded for nodule weight, nodule number or average nodule weight. However, there are several trends that justify comment. Firstly, nodule weight, nodule number and the average weight of nodules are more severely reduced by low concentrations of nitrate nitrogen at the low phosphate level than at higher phosphate levels (Figs 6, 7 and 8), and secondly for mature clover plants in particular, the level of nitrate nitrogen required to produce the lowest nodule weight, increases with increasing phosphate supply (Fig 15). Thus at higher phosphate levels 'mature' white clover plants may be better equipped to overcome the inhibitory effect of low or moderate levels of nitrate in the growing medium. However, at the high nitrate nitrogen concentrations the advantage disappeared, largely as a result of the increase in nodule weight at the lower phosphate level (Fig 15) and the continued reduction in nodule weight at higher phosphate levels. Further evaluation of the effect of nitrate nitrogen and phosphate on 'mature' nodulated white clover plants is warrented. The above study was not designed to measure changes in nodule weight and observations of change after longer periods of treatment would provide more substantial evidence.

2. NITROGEN FIXATION

Allos and Bartholomew (1959); Raggio and Raggio (1962); Dart and Wildon (1970) and Oghoghorie and Pate (1971) have reported that low concentrations of nitrate nitrogen (around 5 to 14ppm and even up to 50ppm) can be beneficial for nitrogen fixation. In this study, the rate of ethylene produced per root per hour was used as an index of the rate of nitrogen fixation and the results obtained in Experiments 3, 4 and 5 show that low concentrations of nitrate nitrogen (0.25 or 0.5mM) significantly reduced the rate at which ethylene was produced. Higher concentrations of nitrate nitrogen further significantly depressed the rate of ethylene production, but the 1.0mM nitrate nitrogen mean was not significantly lower than the 4.0mM nitrate nitrogen mean in Experiments 3 and 4 (Figs 9 and 17). At the 4.0mM nitrate nitrogen concentration, the rate of ethylene production was reduced to 11% of the rate for the control nil nitrate treatment, in Experiment 3 and to 20% in Experiment 4; an indication that at high levels of nitrate nitrogen, nitrogen fixation is markedly depressed, even in plants fully nodulated before the application of combined nitrogen. Allos and Bartholomew (1959); Moustafa, Ball and Field (1969); Dart and Wildon (1970) and Oghoghorie and Pate (1971) have also reported a marked depression in nitrogen fixation when high levels of combined nitrogen were applied to the growing medium of legumes.

The results obtained in Experiment 6 are in direct contrast to those obtained in Experiment 3 since the rate of ethylene production was 32% higher than that recorded for the control treatment at the 0.2mM nitrate nitrogen treatment (Fig 32). This result is similar to those reported by Allos and Bartholomew (1959); Raggio and Raggio (1962); Dart and Wildon (1970) and Oghoghorie and Pate (1971) and, it is probable that the change in response to nitrate nitrogen is associated with a change in climatic conditions. Since Experiment 3 was conducted during the winter months, and Experiment 6 during the summer months, light intensity and in particular, day length would have differed between the two experiments.

Nitrogen fixation is not immediately affected when nitrate nitrogen is fed to nodulated clover plants, since in Experiment 5, no significant changes in the rate of ethylene production were noted between the nitrate treatments 48 hours after the addition of nitrate nitrogen (Fig 26). After six days however, the rate of ethylene production was significantly lower at the 0.5mM nitrate nitrogen level, but no further decline in activity resulted from the addition of higher concentrations of nitrate nitrogen. The decline in the rate of ethylene production became more severe after 12 days; the means for the 0.5, 2.0 and 8.0mM nitrate nitrogen treatments being significantly lower than the mean for the control treatment. The decline in nitrogen fixing activity with time is possibly associated with an increase in the utilization of combined nitrogen for the growth of new leaf, stem and root material.

The rate of ethylene produced per plant per hour increased significantly at the higher levels of phosphate in all Experiments (Figs 9 and 17). Similar results have been obtained by other workers (Van Schreven, 1958; Vincent, 1965 and Gukova and Arbuzova, 1969) for a range of legumes including soya-bean, peas, horse beans and subterranean clover. The increased requirement for phosphate may be linked with the high requirement for ATP in nitrogen fixation, and Bergersen (1971) suggested that restricted inorganic phosphate supply may affect the formation of ATP in nodules.

No significant interactions between nitrate nitrogen and phosphate were recorded for the rate of ethylene production per root in Experiment 3 or 4, but in Experiment 6, the nitrogen fixing activity at the 0.2mM nitrate nitrogen level was markedly influenced by the level of phosphate supplied (Fig 32). The increased sensitivity of nitrogen fixation to low levels of nitrate nitrogen in association with low phosphate levels was also apparent in Experiment 3 (Fig 9). It would appear that nitrogen fixation is more severely depressed when small concentrations of nitrate nitrogen are added to the growing medium

of restricted phosphate supply, and when plant growth and therefore demand for nitrogen is low. The relationship between plant growth and nitrogen fixation will be discussed in a later section.

The reduction in the rate of ethylene produced per mg nodule (dry weight), (Fig 10) in the presence of increasing concentrations of nitrate nitrogen in Experiment 3 was not as marked as that recorded for the rate of ethylene produced per plant. It can be concluded therefore that where legume - rhizobium symbiosis develops in the presence of nitrate nitrogen, the reduction in nitrogen fixing activity is due mainly to a decline in the weight of actively fixing nodule material (Fig 7). However, a different response is obtained where nitrate is added to nodulated legumes. A very significant decline in the ability of nodule material to reduce acetylene to ethylene was recorded in Experiments 4 and 5, and it is likely that this decline in the activity of established nodules caused much of the decrease in the rate of ethylene produced per plant (Fig 18 and 27).

There appears to be no advantage in supplying higher rates of phosphate to legumes when high concentrations of nitrate nitrogen are also supplied, since the rate of ethylene produced per mg nodule (dry weight) per hour was not increased with higher rates of phosphate at the 4.0mM nitrate nitrogen concentration in Experiment 4 (Fig 18). Several factors may have contributed to this response. Firstly, the level of phosphate in nodule material rose only slightly with higher phosphate rates at this nitrate nitrogen concentration (Fig 23). Secondly, top growth was greatly enhanced at these levels of phosphate and nitrate nitrogen and it is possible that the inhibition of nitrogen fixation was correspondingly more severe.

3. PLANT GROWTH

Very significant changes in total plant growth were recorded when nitrate nitrogen was added to the nutrient solutions and where the level of phosphate was increased. For example, phosphate significantly increased the growth of plants in Experiment 4 (Fig 16) and Experiment 6 (Fig 33), while nitrate nitrogen stimulated the growth of plants in Experiment 3 (Fig 11), Experiment 4 (Fig 16) and Experiment 6 (Fig 33). Nitrate nitrogen did not significantly increase plant growth when applied to the media of 'mature' white clover clones in which phosphate and other essential nutrients were not limiting (Experiment 5, Fig 29). In these plants, symbiotic nitrogen fixation was furnishing all the nitrogen needed in growth; combined nitrogen, when applied, merely replaced that source, the level of nitrogen fixation being decreased (Fig 26). The carbohydrate requirements for symbiotic nitrogen fixation and the assimilation of nitrate nitrogen are also similar, since if a lower carbohydrate requirement for the assimilation of combined nitrogen existed, more carbohydrate would have been available for growth in the nitrate treated plants. This in turn would have lead to higher relative growth rates in the nitrate treated plants, and in terms of dry weight, an increasing difference between the control and nitrate treated plants. Gibson (1966) and Minchin and Pate (1973) have also demonstrated that the carbohydrate requirements for nitrogen fixation and nitrate assimilation are similar in subterranean clover and pea plants.

A greater utilization of carbohydrate in nodulated plants can occur during the earlier periods of growth when the symbiotic system is being developed (Gibson, 1966). The growth response recorded for plants grown in the presence of nitrate nitrogen in Experiments 3 and 6 probably reflects the greater utilization of carbohydrate in heavily nodulated plants. The delay in nitrogen fixing activity in the nil nitrate treated plants could have also contributed to the failure of these plants to gain dry weight as rapidly as the nitrate treated plants. Gibson (1966) also observed that shoot growth in subterranean clover was more affected than root growth. This reduction in total photosynthetic capacity of the plants would have

an obvious effect on the future development in terms of dry matter increase. While no growth response to nitrate nitrogen was recorded in Experiment 5, nitrate nitrogen significantly increased the growth of fully nodulated 'mature' plants which had previously been grown at low phosphate levels and in the absence of nitrate (Fig 16). Since at the low phosphate level, the rate of ethylene production per plant (Fig 17) was significantly lower than that for the high phosphate treatments, the growth of the clover clones in the nil nitrate treatments would have been limited by the availability of symbiotically fixed nitrogen. Gukova and Arbuzova (1969) observed that in soyabeans, peas and horse beans, low phosphate levels inhibited fixation of atmospheric nitrogen more than the utilization of combined nitrogen. The delay in obtaining the higher nitrogen fixing capacity in plants grown in the nil nitrate treatment when the phosphate level was increased could explain the growth response to nitrate nitrogen recorded at the higher rates of phosphate (Fig 16).

The effects of phosphate and nitrate nitrogen on top and root growth were also observed. Very significant changes occurred when nitrate nitrogen and phosphate were added to the growing medium of clover plants. An increase in the phosphate supply significantly increased the growth of the top system of plants grown in Experiment 2 (Fig 5), Experiment 4 (Fig 20) and Experiment 6 (Fig 34); while nitrate nitrogen stimulated the growth of the top system of plants grown in Experiment 1 (Table 2), Experiment 2 (Fig 5), Experiment 3 (Fig 12) and Experiment 6 (Fig 34). As expected, no increase in top growth occurred in Experiment 5, when nitrate nitrogen was supplied to fully nodulated 'mature' clover clones (Fig 29) but the nitrate nitrogen main treatment effect, (averaged over all phosphate levels) was also non-significant in Experiment 4, although different top growth responses to nitrate nitrogen were recorded between the three phosphate treatments (Fig 20). While this interaction was only significant at the 10% level of probability the resulting trends are of interest. At the lowest phosphate level, the top growth response to nitrate nitrogen was bimodal in form and was characterized by a peak at a low level of nitrate nitrogen, followed by a second peak at the highest level of nitrate nitrogen. Top growth also peaked

at the 0.25mM nitrate nitrogen concentration and intermediate level of phosphate, but the growth was not markedly reduced when higher rates of nitrate nitrogen were added to the nutrient solutions, nor did growth recover at the highest nitrate nitrogen level. At the highest phosphate level, top growth increased appreciably with high concentrations of nitrate nitrogen in the nutrient solutions (Fig 20).

The bimodal growth response recorded at the low level of phosphate was probably the net effect of nitrogen uptake and nitrogen fixation over the twelve day growing period. The occurrence of the peak at low levels of nitrate nitrogen was dependent on this nitrogen being additive to that obtained in nitrogen fixation. Indeed, the efficiency of nodule material in reducing acetylene to ethylène was not significantly lower than that recorded for the nodules from control nil nitrate plants, at the 0.25mM nitrate nitrogen concentration and low phosphate level (Fig 20). At the higher phosphate levels on the other hand, top growth was similar in the control and 0.25mM nitrate nitrogen treatments. The supply of inorganic nitrogen was no longer complementary to that obtained in nitrogen fixation, since the nitrogen fixing activity of the nodules was significantly reduced by the presence of nitrate ions (Fig 20).

The trough between the two peaks which is apparent at the low phosphate level, was probably the result of the suppression of nitrogen fixation by the 1.0mM nitrate nitrogen concentration. The second peak corresponds to the growth response to high concentrations of nitrate nitrogen. At higher phosphate levels no noticeable decrease in plant growth occurred at the 1.0mM nitrate nitrogen level, although the nitrogen fixing activity of plants declined at this level of nitrate nitrogen. Combined nitrogen appears to replace the loss of symbiotically fixed nitrogen more effectively at high phosphate rates. This response was probably not observed at low phosphate levels because insufficient phosphate was present for optimal nitrate reductase activity, nitrogen metabolism and plant growth (Gukova and Arbuzova, 1969).

The very marked increase in top growth which occurred at high levels of nitrate nitrogen and phosphate (Fig 20) was also

observed in Experiment 2 (Fig 5) and Experiment 6 (Fig 34). Cartwright (1972) also reported positive top growth responses with high levels of combined nitrogen and phosphate. The increase in growth of leaf material in lucerne was associated with an increase in the activity of the pentose phosphate pathway in young expanding lucerne leaves (Cartwright, 1972). The pentose phosphate pathway is generally considered to be a major source of NADPH, (required for reductive biosynthetic processes) pentoses and other intermediates required for nucleic acid synthesis and growth (Cartwright, 1972).

Root growth was higher in plants fed nitrate nitrogen in Experiments 3, 4 and 6, while no change in growth was recorded in harvest 1 and 2 in Experiment 5. A significant reduction in growth at the 2.0mM nitrate nitrogen level was however, recorded in harvest 3. It is generally agreed that the growth of root systems of legumes on nodule nitrogen alone is somewhat inferior to that of nodulated plants receiving some additional inorganic source of nitrogen and it is conceivable that one of the main beneficial effects of the nitrate nitrogen is to provide a direct and readily utilizable source of nitrogen for root growth. Most of the nitrogen required in root growth in pea plants using only symbiotically fixed nitrogen is obtained from the shoot system, negligible amounts being transferred directly from the nodule to the root, (Oghoghorie and Pate, 1972).

Root growth responses to phosphate varied between different experiments. In Experiment 4, for example, where the level of phosphate supplied to 'mature' clover clonal plants was increased, phosphate did not significantly change root growth. A decline in root growth was actually recorded in Experiment 3, but in a similar experiment conducted during summer months (Experiment 6) an increase in growth was recorded at higher levels of phosphate. No satisfactory explanation can be advanced to cover these results. Gibson (1966) has noted that nodule development in subterranean clover was associated with a reduction in the rate of extension in the main root, and a reduction in the number of lateral roots formed. While nodule development was promoted at high levels of phosphate in Experiment 3, the increase in nodulation at high nitrogen levels would not be expected to have greatly influenced root growth at those levels (Fig 13). On the other hand, toxic levels of

phosphate may have arisen in roots at the higher phosphate levels. Top growth in the control nil nitrate treatment was depressed at the two higher phosphate levels (Fig 12) and a toxicity symptom described by Rossiter (1952) was very much in evidence in the older leaves. No such toxicity was recorded for the lowest phosphate level (Plates 4,5) Williams (1948) considered that the growth depression with high phosphate supply resulted from a decrease in the synthesis of nucleoproteins and Rossiter (1952) concluded that for subterranean clover, internal concentrations in excess of 1.2 - 1.4% total phosphorus in leaves were associated with toxicity symptoms. Applications of combined nitrogen were found to overcome the phosphate toxicity in subterranean clover, by promoting plant growth and a 'dilution' effect, whereby the internal concentration of phosphorus did not become critically high (Rossiter, 1952). The toxicity was not noted in the leaves of plants in Experiment 3 receiving nitrate nitrogen and the percent phosphate level did decline (Fig 14A). However, while percent total phosphate values were high in leaf material the values for the root material were much higher and remained at 1.0% even when high concentrations of nitrate nitrogen were applied (Fig 14B). Root growth may then have declined at the higher phosphate levels because of the presence of toxic levels of phosphate. Root growth in Experiment 6 (Fig 35) was not increased by phosphate at the control nil nitrate treatment, and similar toxicity symptoms were observed in leaf material at the higher rates of phosphate. However, when nitrate nitrogen was present in the growing media, root growth increased with higher rates of phosphate. The increased growth resulting from the applied nitrate may have affected a dilution of the phosphate concentration in the root material. Although the same rates of phosphate supply were used in Experiments 3 and 6, it is also possible that lower rates of phosphate absorption into roots occurred in Experiment 6, since the solution cultures were not continually aerated.

Allos and Bartholomew (1959) and Hoglund (1973) concluded that the host plant growth response to combined nitrogen may determine the nitrogen fixation response to combined nitrogen. The results obtained in this study substantiate their findings. Generally,

applications of low concentrations of nitrate nitrogen to growing media of white clover plants, reduced the nitrogen fixing activity of the plants. However a stimulation in nitrogen fixation was recorded at the 0.2mM nitrate nitrogen concentration and the highest rate of phosphate in Experiment 6 (Fig 32). The stimulation in nitrogen fixation was associated with a marked increase in plant growth (200% higher than control plants grown at the same level of phosphate) and is probable that an increased demand for nitrogen resulted from the higher growth rate, and that with time, the demand for nitrogen greatly exceeded the supply of inorganic nitrogen. Symbiotically - fixed nitrogen could meet the deficit between plant demand and the supply of inorganic nitrogen.

Although an increase in plant growth also occurred with the application of low concentrations of nitrate nitrogen in Experiment 3, plant growth at the 0.25mM nitrate nitrogen concentration was only 50% greater than that of the control plants grown at the same rate of phosphate (Fig 11).

At higher nitrate nitrogen concentrations, inorganic nitrogen provides most of the nitrogen required in plant growth and nitrogen fixation is severely restricted. This is apparent in Experiment 5, (Harvest 2 and 3) where plant growth at the highest nitrate nitrogen concentration was not significantly higher than that recorded for the 0.5mM nitrate nitrogen concentration (Fig 29) and where the rate of ethylene production was significantly lower at the higher nitrate nitrogen concentration (Fig 26). The inhibitory effect of moderately high nitrate nitrogen concentrations (2.0mM) on nitrogen fixation can be offset however by the growth response of the plant to the combined nitrogen. In Experiment 6, the rate of ethylene production in plants grown in nutrient solutions containing a 2.0mM concentration of nitrate nitrogen and the highest level of phosphate, was not much lower than the value recorded for the plants grown in the nil nitrate nutrient solutions and at the same level of phosphate (Fig 32). Plant growth for the nitrate treated plants was approximately 450% higher than that recorded for the control plants (Fig 33). At the same nitrate nitrogen concentration, but at the

lowest phosphate level, nitrogen fixation was severely depressed, since the supply of phosphate limited plant growth and therefore the demand for nitrogen.

A stimulation in nitrogen fixation and nodulation can also be obtained where high concentrations of combined nitrogen in growing media are not maintained (Fig 32). The increase in nitrogen fixing activity with the depletion of nitrate nitrogen was most significant in plants grown at the highest nitrate nitrogen concentration and phosphate level, and where the rate of plant growth and therefore the demand for nitrogen were high. Nitrogen fixation did not increase in plants grown at the lower phosphate levels since the supply of phosphate limited plant growth, nodule development and prevented an increase in the nitrogen fixing activity of established nodule material (Fig 32). At the lower nitrate nitrogen concentration and the highest phosphate level, symbiotic nitrogen fixation was providing most of the nitrogen required in plant growth (Fig 32).

Control of the level of nitrate nitrogen in the growing medium is necessary if the effects of nitrate nitrogen on nodulation and nitrogen fixation are to be interpreted correctly. It is probable that the stimulation in nodule development on Stylosanthes humils reported by Gates (1970), when concentrations of 15 and 30ppm of combined nitrogen and high rates of phosphate were added to a light textured nutrient deficient soil, occurred as a result of a fall in soil nitrate levels as plant size increased.

THE CARBOHYDRATE - NITROGEN RATIO

Much has been written about the relationship of the carbohydrate - nitrogen ratio with nodulation and nitrogen in legumes (Wilson, 1940; Stewart, 1966). In this study, the effect of nitrate nitrogen and phosphate on the relationship was evaluated.

The percent total nitrogen level in top and root material was found to increase significantly in plants fed nitrate nitrogen (Tables 3, 4; Figs 21A,B), although for top material in Experiment 3, the mean for the 0.25mM nitrate nitrogen treatment was significantly greater than that for control treatment (Table 3). The percent total nitrogen levels did not however, increase in proportion to the nitrate nitrogen concentrations in the growing media, thus indicating that luxury consumption of combined nitrogen did not occur in these experiments. In nodulated plants receiving no nitrate nitrogen, the percentage of total nitrogen in dry matter of shoots was consistently higher than in roots, thus giving the shoot system a disproportionately large share of plant total nitrogen. Root material from plants receiving only symbiotically fixed nitrogen possess a sub-optimal nitrogen status, a feature also noted by Bouma (1970) for nodulated subterranean clover plants; and it is probable that this factor contributes to the slower growth of plants receiving no combined nitrogen (Bouma, 1970; Oghoghorie and Pate, 1972) and that one of the main beneficial effects of combined nitrogen is to provide a direct and readily utilizable source of nitrogen for root growth, in addition to that which is made available to the root via the translocatory system of the shoot system, (See Fig 3 and Oghoghorie and Pate, 1972).

The increase in the percent total nitrogen level in top and root material with the application of nitrate nitrogen is a reflection of the nitrate reductase activity, and greater synthesis of amides and amino acids. Considerable quantities of energy would be used in these processes, and it is conceivable that sugars, normally used to support the growth of nodules and nitrogen fixation, are redirected to the leaves and roots. The percent total nitrogen values for top and root material in Experiments 3 and 4 were both significantly but

negatively correlated with the nitrogen fixing activity (Appendix I 7).

The percent total nitrogen level of top material was also affected by the phosphate level (Table 3, Fig 21A). At high levels of nitrate nitrogen, phosphate increased the nitrogen content significantly, but in the control treatments the phosphate effect was influenced by the age and pretreatment status of plant material. Phosphate increased the percent total nitrogen content in top material from the nil nitrate treatments in Experiment 3, but reduced the nitrogen content in top material in Experiment 4. An increase in growth rate and demand for nitrogen occurred when the phosphate level was raised in Experiment 4, and the temporary delay in attaining a higher nitrogen fixing capacity could have caused the lower nitrogen content. In Experiment 3 on the other hand, the phosphate treatments were imposed over a longer period, and the percent nitrogen level in top material reflected the activity of the nitrogen fixing system. Bergersen (1970) and Schwinghamer et al (1970) have reported significant correlation coefficients between the nitrogen fixing activity of legumes and the total nitrogen in plant tops. The percent total nitrogen level in root material from Experiment 3 also increased when phosphate levels were raised, (Table 4) but in Experiment 4, the total nitrogen content declined in all the nitrogen treatments (Fig 21B).

In Experiments 3 and 4, neither nitrate nitrogen nor phosphate significantly changed the percent total nitrogen content of nodule material (Tables 5, 12). However, nodule material obtained from plants fed nitrate nitrogen and harvested six days after the treatments commenced, contained significantly higher total nitrogen levels. A reduction in demand for symbiotically fixed nitrogen, or a shortage of "carbon skeletons" for amino acid synthesis in nodules may have contributed to the accumulation of nitrogen, and it is possible that the nitrogen fixing activity and nodule growth declined as a result of the higher nitrogen levels. However, no significant differences were noted at the third harvest (12 days) and further experimental work is required to ascertain whether the percent total nitrogen level does accumulate for short periods in nodule material after the addition of nitrate nitrogen.

The carbohydrate status of plant material in Experiments 3 and 4 was determined by measuring the soluble sugar (sucrose and hexoses) and starch levels. The soluble sugar content in top material in Experiment 3 was not significantly reduced with the application of nitrate nitrogen (Table 6) but in Experiment 4, the level was significantly lower at the highest nitrate nitrogen concentration (Table 13). The soluble sugar content in root material (Experiment 4) on the other hand, was reduced by small concentrations of nitrate nitrogen (0.5mM); with further significant reductions as the concentration of nitrate nitrogen was increased (Table 14). In Experiment 3, higher soluble sugar levels were recorded in root material from plants receiving nitrate nitrogen; but the 1.0mM nitrate nitrogen mean was not significantly higher than the mean for the control treatment, although it was significantly lower than the means for the 0.25 and 4.0mM nitrate nitrogen treatments (Table 7).

The reduction in soluble sugar levels in top and root material was probably the result of increased utilization of sugars for nitrate assimilation, amide, amino acid and protein synthesis (Orcutt and Wilson, 1935). An increase in soluble sugar content can occur, however when the supply of soluble sugar increases as a result of a larger leaf area and higher photosynthetic activity, or where the rate of utilization of soluble sugars declines. The increase in sugar concentration in root material receiving the 0.25mM nitrate nitrogen concentration (Experiment 3) probably occurred because the nitrate level was insufficient for utilization of the additional sugar formed. This explanation does not apply however, to the increase at the 4.0mM nitrate nitrogen treatment. A reduction in nitrate reductase activity in root material may have occurred at the highest nitrate level. Oghoghorie and Pate (1972) concluded that "at very high levels of nitrate, the nodulated legume may mimic those non-leguminous plants shown to be incapable of reducing nitrate in their roots". An examination of the activity of the nitrate reductase enzyme in shoot and root tissue at low and high nitrate nitrogen concentrations is warrented.

At higher phosphate levels, the level of soluble sugars in top and root material was significantly lower. Tingey Fites and Baharsjah (1974) showed that compounds such as phosphate which stimulated glycolysis and the pentose phosphate pathway, also stimulated nitrate reductase activity. The reduction in sugar concentration at higher phosphate levels is a reflection of the greater utilization of sugars in nitrate reduction, amide and amino acid synthesis and protein synthesis.

In Experiment 4, the percent starch level in top and root material was significantly reduced when nitrate nitrogen was supplied to the plants and when the level of phosphate was increased (Table 15,16). Significant quantities of starch could have been hydrolysed to provide substrate for nitrate assimilation and protein synthesis and it is also probable that the level of starch synthesis declined with the reduction in the surplus of soluble sugars in both top and root material.

It has been suggested that the degree of nodulation and nitrogen fixation in a legume is governed by the internal carbohydrate to nitrogen ratio, (Wilson, 1940; Stewart, 1966) and that any deviation from a 'critical ratio' causes sub-optimal nodulation and nitrogen fixation. The soluble sugar and metabolizable carbohydrate (soluble sugar + starch) status and the total nitrogen concentration for the whole plant were determined and the soluble sugar or metabolizable carbohydrate to nitrogen ratio calculated. Both ratios were significantly and positively correlated to the nitrogen fixing activity of nodules (Appendix I 6).

While it is clear that the ratio is highly correlated with nitrogen fixing activity, it is probable that the information only summarises experience (Nutman, 1956) and that the carbohydrate - nitrogen relationship of the plant is not causally connected with any of the functions of symbiotic nitrogen fixation. The mechanism by which the carbohydrate - nitrogen relationship in the leguminous host plant exerts its influence is still a matter of dispute. One theory that has gained widespread acceptance, indicates that combined nitrogen reduces the supply of carbohydrates to nodules because of

increased protein synthesis in top and root material. Wilson (1940) considered that the simplified "carbohydrate" mechanism was too limited a concept since it neglected that equally important function of nitrogen in the plant. However, there has been little support for the other concept that the regulatory influence of the carbohydrate - nitrogen relationship is comparable to that of a hormone. Some evidence has been provided in support of the former theory. Small and Leonard (1969) for example, showed that the proportion of photosynthate translocated to nodules in subterranean clover and pea plants declined when nitrate nitrogen was supplied. However, Small and Leonard (1969) did not estimate the level of soluble sugars in leaf, root or nodule material or the nitrogen fixing activity of the nodules. The keystone of all the carbohydrate hypotheses is the assumption that combined nitrogen reduces the level of soluble carbohydrate in the host. Few experiments have been conducted to check this assumption and the results reported above, suggest that nitrate nitrogen does not depress the soluble sugar content of the host plant, although the metabolizable carbohydrate levels were lower, with the reduction in percent starch in top and root material. Nitrate nitrogen then causes a reduction in 'reserve' carbohydrates and not readily utilizable carbohydrates in the host plant.

Orcutt and Wilson (1935) have reported a decline in the level of reducing sugars and sucrose in the sap of soyabean plants which were fed nitrate nitrogen; the level of sugars in the leaf, stem and root material were affected differently. In leaf material the level of reducing sugars in the cell sap decreased with the addition of large concentrations of nitrate nitrogen. A decline in sugar content also occurred in stem and root material at the low concentrations of nitrate nitrogen, but at higher concentrations the sugar levels increased. Orcutt and Wilson (1935) also observed that plants which did not receive nitrate nitrogen, suffered from nitrogen 'hunger' and that nitrogen fixation was just commencing in these plants when the plant material was harvested for sugar analyses. A surplus of sugar would be expected where nitrogen is

limiting protein synthesis. If an allowance is made for the surplus at the nil nitrate and lowest nitrate nitrogen treatment, then the results reported by Orcutt and Wilson (1935) are similar to those recorded in Experiment 3.

The suggestion by Allison and Ludwig (1934) that the soluble sugars are used primarily for top growth and not for root growth and indirectly nodule growth when nitrate nitrogen is supplied, must be rejected. The top to root ratio in plants harvested in Experiment 3 (Table 19) and Experiment 6 (Table 20) did not increase with the addition of nitrate. Wilson (1935) and Thornton and Nicol (1936) also failed to detect any relationship between the change in top to root ratio and the growth of nodules.

If the supply of photosynthates to nodule material declines with the application of nitrate nitrogen, then lower levels of soluble sugars in nodule material might be expected. However, an increase in the soluble sugar content was noted in nodule material receiving nitrate nitrogen (Fig 24). The increases were significant at the 1.0 and 4.0mM nitrate nitrogen treatments for the two highest phosphate levels and at the 1.0mM nitrate nitrogen treatment and the lowest phosphate level. A reduction in the soluble sugar level was recorded however, at the 4.0mM nitrate nitrogen treatment and lowest phosphate level; this decline being associated with an increase in nodule growth (Fig 24).

The higher levels of sugars could have resulted from an increase in the translocation of soluble sugars to nodule material, or from a decline in the utilization of sugars in nodule growth (Fig 15) and nitrogen fixation (Figs 17 and 18). The latter theory is more acceptable, since Small and Leonard (1969) have demonstrated a reduction in translocation of sugars to nodules with the application of nitrogen. The results obtained (Fig 24) also suggest that the growth of nodule material and the nitrogen fixing activity are not limited by the supply of soluble sugars, and that the change in translocation pattern recorded by Small and Leonard (1969) may have arisen from a reduction in 'sink' activity in nodule material. Lawrie and Wheeler (1973) concluded that the main 'sink' for accumulation of

photosynthates was in the infected tissue. However, there was considerable variation in the accumulation of labelled photosynthates in individual infected cells. Younger infected cells showed a marked accumulation of photosynthates, while the cells densely filled with bacteroids showed relatively little accumulation. The reduction in nodule growth (Fig 15) may have contributed most to the accumulation of sugars, but soluble sugars not utilized in nodule growth would be available for nitrogen fixation (Lawrie and Wheeler, 1973).

The quantity of sugars translocated to nodules is sufficient to meet the requirements for growth and nitrogen fixation, even when nitrate nitrogen is supplied to the plants. There is evidence that one or more factors inherently associated with plant growth are capable of inhibiting nodule development and nitrogen fixation. The relative growth of different plant organs is determined by the distribution and activity of stimulatory and inhibitory growth factors as well as by assimilation rate and nutrient uptake, and the explanation of the correlation of nodulation and nitrogen fixation with the carbohydrate to nitrogen ratio may follow from a better understanding of the hormone control of plant growth (Nutman, 1965). It is also clear that the amount of nodule growth and the nitrogen fixing activity in nodule material is related to the nitrogen requirements of the plant. The host is thus not called upon to support a larger amount of symbiotic tissue than necessary to maintain growth.

A reduction in 'sink' activity in nodules may have also caused the decline in the percent total phosphate levels in nodules from plants supplied with nitrate nitrogen (Fig 23). That the phosphate levels in nodule material were significantly correlated with the nitrogen fixing activity ($r = .838$ and 0.801 for rate of ethylene produced per plant or mg nodule (dry weight) per hour respectively), is evidence in support of the hypothesis that the increased requirements for phosphate in legumes may be linked with the high requirement for ATP in nitrogen fixation (Bergersen, 1971). Other workers (van Schreven, 1958) have reported similar correlations between phosphate levels in nodule material and nitrogen fixing activity.

CONCLUSION

1. Low concentrations of nitrate nitrogen (0.2 - 0.25mM NO_3 N) caused either an increase or a reduction in nodulation and nitrogen fixation in white clover plants. The nature of the response was dependent on the growth response of the host plant to nitrate nitrogen and the degree to which the resulting demand for nitrogen exceeded the supply of inorganic nitrogen.

2. Large concentrations of nitrate nitrogen caused marked reductions in nodule weight, nodule number and the average weight of nodules and also severely depressed the nitrogen fixing activity. At these concentrations, inorganic nitrogen supplied most of the nitrogen required for host plant growth.

3. Phosphate increased the total nodule weight, nodule number and nitrogen fixing activity.

4. Interactions between nitrate nitrogen and phosphate were observed for nodulation and nitrogen fixation. The total nodule weight, nodule number, the average weight of nodules and the nitrogen fixing activity were more severely reduced by low concentrations of nitrate nitrogen at the low phosphate level than at the higher phosphate levels. On the other hand, an increase in nitrogen fixing activity can occur at low concentrations of nitrate nitrogen, providing that phosphate and other factors are not limiting plant growth and therefore the demand for nitrogen.

Although higher rates of phosphate either partially or fully overcame the inhibitory effect of low concentrations of nitrate nitrogen on the nitrogen fixing activity of nodules, no such increases were observed when higher rates of phosphate were added in association with high concentrations of nitrate nitrogen.

5. Nitrogen fixation was most immediately reduced when nitrate nitrogen was supplied to nodulated clover plants. The decline in nitrogen fixing activity with time appeared to be associated with an increase in the utilization of combined nitrogen for the growth of new leaf, stem and root material.

6. Nodule number alone, is not a satisfactory guide to the effect of nitrate nitrogen on nodulation in legumes.

7. In seedlings and newly established clonal material, plant growth was greater in those plants which received nitrate nitrogen, and higher levels of phosphate when the treatments commenced at inoculation. A growth response to nitrate nitrogen and phosphate was also obtained when these treatments were supplied to nodulated plants where the rate of nitrogen fixation was limiting the availability of nitrogen for growth. Nitrate nitrogen does not however, increase plant growth when supplied to nodulated clover plants in which phosphate and other essential nutrients were not limiting symbiotic nitrogen fixation. In such cases it can be concluded that the carbohydrate requirements for the assimilation of nitrate and symbiotic nitrogen fixation are the same.

8. The top growth response in nodulated plants to nitrate nitrogen was bimodal in form when a range of nitrate nitrogen concentrations were added in association with a low level of phosphate. The bimodal growth response was a reflection of the net effect of nitrogen uptake and nitrogen fixation over the growing period.

Marked increases in top growth occurred at the high levels of nitrate nitrogen and phosphate, providing the environmental conditions did not limit plant growth.

9. Evidence was produced in support of the theory that root growth in nodulated legumes is inferior to those receiving some additional source of combined nitrogen.

10. The percent total nitrogen level in top and root material was negatively correlated with the nitrogen fixing activity.

11. The soluble carbohydrate or metabolizable carbohydrate to nitrogen ratio of host plants were significantly correlated with the nitrogen fixing activity. There was however, little evidence that nitrate nitrogen reduces the soluble carbohydrate levels in the host plant.

12. Soluble sugars were observed to increase in nodule material from plants receiving high concentrations of nitrate nitrogen.

13. The phosphate level in nodule material was positively correlated with nitrogen fixing activity.

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Appendix I 2 Nutrient solution for establishing clonal material

$CaCl_2$	2mM
K_2SO_4	2mM
$MgSO_4$	1mM
Fe EDTA	30 uM
$K_2H_2PO_4$	0.1mM
KNO_3	0.1mM

Micro - nutrients, 1ml of the trace element mixture (Chapter II 2.1.5).

Appendix I 3 Acetylene Reduction Technique

The measurements of ethylene production during incubation of samples with acetylene were made on a Shimadzu series GC-4B gas chromatograph, fitted with a flame ionization detector and:

(a) a column packing 'Poropak T', 80-120 mesh; column length 2 metres (Experiment 3) and 25cm in Experiments 4, 5 and 6; column diameter 3mm.
(b) nitrogen gas flow rate of 25ml per minute
(c) column temperature of 100°C
(d) gas sample of 1.0ml (Experiment 3) and 0.5ml in Experiments 4, 5 and 6

Procedures

Freshly cut root or root segments were blotted dry and placed in labelled 200ml 'Vegemite' screw sealed jars. In Experiment 3, the air in the bottles was completely replaced with an argon (80%)/oxygen (20%) gas mixture before incubation, but this procedure was not continued in Experiments 4, 5 and 6; and root samples were incubated in an nitrogen gas-acetylene atmosphere. Even at low concentrations acetylene is a powerful competitive inhibitor of nitrogen fixation (Schollhorn and Burris, 1967) and Hardy et al (1968) observed that the decrease in acetylene reduction rate resulting from a failure to replace air with an oxygen/argon mixture was only 10 to 20%.

Twenty mls of the atmospheric or argon/oxygen mixture gas in the incubation vessels were withdrawn and replaced with 20mls of acetylene gas. The roots were incubated for a given period, normally 30 minutes, although a 20 minute incubation period was used in Experiment 4. The rate of acetylene reduction was found to be linear over these periods.

At the end of the incubation period, gas samples were withdrawn in 1ml disposable plastic syringes, and the samples stored by pushing the needles into rubber stoppers. Regular shaking of the labelled vessels insured that the gas was uniformly distributed throughout the vessels during the incubation period, and particularly when the gas samples were withdrawn.

Calculation of ethylene production in the acetylene reduction assay of nitrogen fixation

Ethylene produced was calculated from the C_2H_4/C_2H_2 gas liquid chromatograph peak height ratio in the sample compared to the ratio in a standard mixture (20 units acetylene/1 unit ethylene) in 200ml incubation vessels.

Assume:

	mls C_2H_4	mls C_2H_2	Pk ht ratio
Standard	x	y	A
Sample	u	z	B

Volume of C_2H_2 injected for incubation = X

Gas mixture ratio x/y gives a pk ht ratio A

" " " u/z gives a pk ht ratio B

$$\frac{x/y}{u/z} = A/B$$

$$u = \frac{Bxz}{Ay}$$

$$= Bkz \qquad \text{where } k = \frac{x}{Ay}$$

$$= X - z$$

$$= X - \frac{u}{Bk}$$

$$= \frac{X}{1 + \frac{1}{Bk}}$$

$$u = \frac{X.B.K.}{1 + BK}$$

This gives u in mls C_2H_4 at the temperature and pressure at which C_2H_2 was measured into the incubation vessel.

1 mole gas $\frac{22.4}{1000}$ ml at $0^{\circ}C$ and 1 atmsopheric pressure

$$= \frac{22.4}{1000} \times \frac{1}{P} \times \frac{273 + T}{273}$$ ml at $T^{\circ}C$ and P atmospheric pressure

u in µ moles $$= \frac{X.B.K.P. \quad 273 \quad 1000}{1 + B.K. \; 22.4 \; (273 + T)}$$

u in µ moles $$= \frac{X. \; ^{a}/_{b}. \; K. \; F}{1 + {^{a}/_{b}}. \; K}$$

where X = vol. C_2H_2 used in the incubation

a = C_2H_4 pk ht in sample

b = C_2H_2 pk ht in sample

K $= \frac{x}{Ay} = \frac{\text{ml } C_2H_4 \text{ in standard}}{\text{Pk ht ratio in std x mls } C_2H_2 \text{ in standard}}$

F $$= \frac{P \text{ (atmos)} \times 273 \times 1000}{22.4 \times (273 + T)}$$

$= 41.6$ at 1atmos. and $20^{\circ}C$

A programme was written by Mr R. Scott, (for the Hewitt- Packard calculator), to calculate this.

Appendix I 4 A summary of the procedure for determination of soluble sugars

Reagents

(1) Methanol extractant (62.5%): 150ml distilled water and 250ml methanol.

(2) Phenol 5%: 5gms of phenol in 100mls of distilled water

(3) Sucrose standard: Dissolve 2.5gm of sucrose in 20mls of water and dilute to 25mls with distilled water.

(4) Concentrated sulphuric acid:

Procedure

(1) 50mg of freeze dried plant material was weighed into screw capped culture tubes (125 x 16mm tubes and caps with teflon liners) and 5mls of 62.5% methanol added.

(2) The tubes were placed in a 55°C water bath for 15 minutes, cooled in tap water and centrifuged at 2000rpm for 15 minutes.

(3) Four mls of the supernatant were removed and added to 5ml of chloroform in screw capped culture tubes. The methanol and chloroform were thoroughly mixed, by vigorously shaking the tubes. The tubes were centrifuged for 5 minutes at 2000rpm to clarify the two phases.

(4) Fifty µl of the upper phase was added to 1ml of 5% phenol and 4mls of concentrated sulphuric acid directed onto the surface of the liquid to ensure adequate mixing and heating. The test tubes were allowed to cool slowly to room temperature and the absorbances read at 490nm.

(5) Standards were prepared by adding 25 and 50 µl of 10% sucrose to 4ml of 62.5% methanol and extracting with chloroform as before. These amounts of sucrose corresponded to 5% and 10% (dry weight) of soluble sugars for the 50mg samples. Readings for the samples were multiplied by x 5/4 before comparison with standards.

Appendix I 5 A summary of the procedure for determination of starch

Reagents

(1) 0.5m Sodium acetate buffer pH 4.5: 41.3mls of glacial acetic acid is made up to 450ml with water and adjusted to pH 4.5 with 20% sodium hydroxide. Dilute to 500mls.

(2) Amyloglucosidase reagent: Dissolve Sigma amyloglucosidase (A 7255) 1mg/ml in the sodium acetate buffer. Filter through Whatman No. 1 paper and store in a refrigerator.

(3) Tris/Glycerol buffer: 30.2g Tris is dissolved in 400ml water, and the pH adjusted to pH 7.0 with conc, hydrochloric acid. Dilute to 500mls and add 330mls of A.R. glycerol and store in a refrigerator.

(4) Glucose Oxidase/Peroxidase reagent (G.O.P.):

60mgs of Sigma glucose oxidase (*Aspergillus niger*), 6mg of Sigma peroxidase (horse radish) and 20mg of O-Diansidine hydrochloride were dissolved in 200mls of Tris/Glycerol buffer.
Note: The O-Diansidine hydrochloride is best dissolved in about 50mls of the buffer with warming. Dilute to 200mls and stir in the glucose oxidase and peroxidase until dissolved. Store in a brown bottle in a refrigerator.

(5) Starch standard: Suspend 100mg BDH Analar soluble starch in 10ml of distilled water. Place in a boiling water bath until 'dissolved'. Dilute to 50ml, i.e. 2mg starch per ml. Prepare at least weekly and reheat before used if stored at 0-4°C.

(6) Glucose standard: 0.25g D-glucose in 250ml distilled water.

Procedure

(1) The residue from the 62.5% methanol extraction was suspended in 100% methanol, the tubes capped and placed in a boiling bath for about 5 minutes. The tubes were then cooled in tap water, and centrifuged at 2000rpm. The supernatant was decanted carefully and the extraction repeated.

(2) Two mls of distilled water were added to the residue of these extractions, and the tubes were capped and placed in a boiling water bath for 15 minutes to gelatinize the starch. After cooling, 2mls of 0.5M sodium acetate buffer and 1.0ml of the amyloglucosidase preparation were added, and the tubes placed in a 55°C water bath and incubated for 60 minutes. The tubes were cooled, the contents diluted to 10mls and the tubes centrifuged at 2000rpm for 10 minutes.

(3) The amount of glucose released was determined by incubating 0.05 to 1ml (depending on starch concentration) of the diluted amyloglucosidase digest in a final volume of 1ml of water plus 2mls of the GOP reagent at 37°C for 60 minutes. Tubes containing D-glucose go a brown colour during this incubation. Five mls of 18N sulphuric acid was added to the tubes to produce a magenta colour, and the absorbances were read at 540nm.

Glucose standards (25 and 50 µl equivalent to 25 and 50 µg D-glucose in 1ml of water) were also prepared.

The level of D-glucose (µg) in the digests was calculated and the starch content obtained by multiplying the D-glucose value by 0.9.

Reaction Mechanism

(1) Gelatinized starch $\xrightarrow[55^{\circ}C,\ 60\ minutes]{amyloglucosidase}$ D glucose

(2) D-glucose + FAD $\xrightarrow[37^{\circ}C,\ 60\ minutes]{glucose\ oxidase}$ gluconolactone + $FADH_2$

(3) $FADH_2$ + O_2 $\xrightarrow{spontaneous}$ FAD + H_2O_2

(4) O-Dianisidine H_2O_2 $\xrightarrow{peroxidase}$ 3,3-dimethoxy-4, 4-diiminodipheno quinone (Magenta in H+)

Appendix I 6 Carbohydrate to nitrogen ratio (Experiment 4)

	df	Soluble carbohydrate to nitrogen	Metabolizable carbohydrate to nitrogen
Rate of ethylene per root	43	0.615	0.639
Rate of ethylene per root	43	0.681	0.705

Appendix I 7 Correlation of percent total nitrogen with nitrogen fixing activity

Rate of ethylene/root	df	% nitrogen top	% nitrogen root
Experiment 4	39	.646	.767
Experiment 3	28	.535	.782
Rate of ethylene/mg nodule			
Experiment 4	39	.742	.799
Experiment 3	28	.538	.637

Appendix II 1.1 Analysis of variance for number of nodules per seedling

(sq rt transformed data)

Source	df	MS	F	
Replicate	1	3.285×10^{-3}	0.036	ns
NO_3 N	4	5.356×10^{1}	58.68	**
Phosphate	4	2.797×10^{-2}	0.306	ns
N x P	16	6.446×10^{-2}	0.706	ns
Error	24	9.127×10^{-2}		

Appendix II 1.2 LSD for nitrate nitrogen treatment means

(sq rt transformed data)

Conc. of NO_3 N (mM)	0	1.0	2.0	4.0	8.0	LSD (t 0.05)
Mean	4.16	2.65	2.53	2.52	2.41	0.278

Appendix II 2.1 Analysis of variance for seedling top weight

(natural data)

Source	df	MS	F	
Replicate	1	1.152	1.618	
NO_3 N	4	2.064×10^{1}	28.982	**
Phosphate	4	6.601×10^{-1}	0.927	ns
N x P	16	2.788×10^{-1}	0.392	ns
Error	24	7.120×10^{-1}		

Appendix II 2.2 LSD for nitrate nitrogen treatment means: Top dry weight

(natural data)

Conc. of NO_3 N (mM)	0	1.0	2.0	4.0	8.0	LSD (t 0.05)
Mean	1.17	4.64	4.13	3.96	4.54	0.779

Appendix II 3.1 Analysis of variance for number of nodules per seedling

(sq rt transformed data)

Source	df	MS	F	
Replicate	3	7.731×10^{-1}		
NO_3 N	5	5.547	43.179	**
Phosphate	3	2.415×10^{1}	188.005	**
N x P	15	1.198×10^{-1}	0.932	ns
Error	69	4.012		

Appendix II 3.2 LSD for nitrate nitrogen main treatment effects: nodule number

(sq rt transformed data)

Conc. of NO_3 N (mM)	0	0.1	0.5	1.0	4.0	8.0	LSD (t 0.05)
Mean nodule number	3.461	2.847	2.519	2.353	2.195	1.741	0.253

Appendix II 3.3 LSD for phosphate main treatment effects: nodule number

(sq rt transformed data)

Conc. of phosphate (mM)	0	0.005	0.05	0.5	LSD (t 0.05)
Mean nodule number	0.909	2.981	3.091	2.988	0.206

Appendix II 4.1 Analysis of variance for seedling top dry weight

Source	df	MS	F	
Replicate	3	6.468×10^{-1}		
NO_3 N	5	6.437	22.798	**
Phosphate	3	2.576×10^{1}	91.251	**
N x P	15	1.089	3.856	**
Error	69	2.823×10^{-1}		

Appendix II 4.2 LSD for nitrate nitrogen treatment means top dry weight

Conc. of NO_3 N (mM)	0	0.1	0.5	1.0	4.0	8.0	LSD (t 0.05)
Mean top weight	1.829	2.853	3.072	3.303	3.378	3.616	0.375

Appendix II 4.3 LSD for phosphate treatment means: top dry weight (natural data)

Conc. of Phosphate (mM)	0	0.005	0.05	0.5	LSD (t 0.05)
Mean top weight	1.475	3.321	3.506	3.732	0.306

Appendix IV 1.1 Analysis of variance for nodule weight

Source	df	MS	F	
Replicate	1	3.960×10^2	22.76	**
NO_3 N	4	3.718×10^2	21.37	**
Phosphate	2	1.317×10^2	7.57	**
N x P	8	7.993	0.50	ns
Error	14	1.740×10^1		

Appendix IV 1.2 LSD for nitrate nitrogen means: nodule weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean nodule weight (mg)	27.00	16.14	10.50	8.98	7.92	5.17

Appendix IV 1.3 LSD for phosphate means: nodule weight

Phosphate level mgP/plant/day	0.2	0.5	1.5	LSD (t 0.05)
Mean nodule weight (mg)	10.02	15.33	16.97	4.00

Appendix IV 2.1 Analysis of variance for nodule number (sq rt transformed)

Source	df	MS	F	
Replicate	1	1.890×10^{-3}	0.00	
NO_3 N	4	1.803×10^{1}	16.12	**
Phosphate	2	2.578×10^{1}	23.05	**
N x P	8	7.674×10^{-1}	0.69	
Error	14	1.119		

Appendix IV 2.2 LSD for nitrate nitrogen treatment means: nodule number

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean nodule number	18.53	16.71	15.33	14.85	14.17	1.31

Appendix IV 2.3 LSD for phosphate treatment means: nodule number

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean nodule number	14.11	16.47	17.17	1.02

Appendix IV 3.1 Analysis of variance for average nodule weight

Source	df	MS	F	
Replicate	1	5.741×10^3		
NO_3 N	4	1.645×10^3	12.91	**
Phosphate	2	2.746×10^2	2.16	ns
N x P	8	7.378×10^1	0.58	ns
Error	14	1.274×10^2		

Appendix IV 3.2 LSD for nitrate nitrogen treatment means: average nodule weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean nodule weight (mg)	77.8	57.2	43.7	40.2	37.9	13.98

Appendix IV 4.1 Analysis of variance for rate ethylene produced per root per hour

Source	df	MS	F	
Replicate	1	1.121×10^{-2}	[illegible]	[illegible]
NO_3 N	4	3.873	66.61	**
Phosphate	2	5.607×10^{-1}	9.65	**
N x P	8	1.156×10^{-1}	1.99	ns
Error	14	5.814×10^{-2}		

Appendix IV 4.2 LSD for nitrate nitrogen means: moles C_2H_4/root/hour

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean C_2H_4/rt/hr	2.098	1.18	0.41	0.28	0.23	0.094

Appendix IV 4.3 LSD for phosphate main effect: moles C_2H_4/root/hour

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean C_2H_4/rt/hr	0.567	0.942	1.005	0.073

Appendix IV 5.1 <u>Analysis of variance for rate of ethylene production per mg DW nodule per hour</u>

Source	df	MS	F	
Replicate	1	3.142×10^3	15.91	**
NO_3 N	4	3.567×10^3	18.07	**
Phosphate	2	2.916×10^2	1.48	ns
N x P	8	1.729×10^2	0.88	ns
Error	14	1.975×10^2		

Appendix IV 5.2 <u>LSD nitrate nitrogen means: C_2H_4/mg dry weight nodule/hour</u>

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean C_2H_4/mgDW/hr	80.4	76.1	37.7	32.1	32.2	17.40

Appendix IV 6.1 Analysis of variance for top dry weight

Source	df	MS	F	
Replicate	1	5.410×10^4	9.87	
NO_3 N	4	5.613×10^4	10.25	**
Phosphate	2	6.120×10^3	1.12	ns
N x P	8	2.260×10^3	0.41	ns
Error	14	5.478×10^3		

Appendix IV 6.2 LSD for nitrate nitrogen means: top dry weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean (mg)	424.7	582.0	635.0	656.2	650.2	91.66

Appendix IV 7.1 Analysis of variance for root dry weight

Source	df	MS	F	
Replicate	1	8.112×10^2	3.44	
NO_3 N	4	4.046×10^3	17.14	**
Phosphate	2	7.494×10^3	31.75	**
N x P	8	3.141×10^2	1.33	ns
Error	14	2.361×10^2		

Appendix IV 7.2 LSD for nitrate nitrogen means: root dry weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean (mg)	112.2	157.5	164.5	176.5	172.8	19.0

Appendix IV 7.3 LSD for phosphate means: root dry weight

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean (mg)	188.2	143.6	138.4	14.7

Appendix IV 8.1 Analysis of variance for percent total nitrogen in top, root and nodule material

Source	df	top MS	top F		root MS	root F		nodule MS	nodule F	
Replicate	1	1.496×10^{-2}			2.083×10^{-3}			5.201×10^{-1}		
NO_3 N	4	3.238×10^{-1}	5.68	**	1.766	53.91	**	4.574×10^{-1}	0.93	ns
Phosphate	2	1.997×10^{-1}	3.50	ns	4.236×10^{-1}	12.93	**	9.152×10^{-1}	1.86	ns
N x P	8	7.625×10^{-2}	1.34	ns	5.082×10^{-2}	1.55	ns	8.282×10^{-1}	1.68	ns
Error	14	5.704×10^{-2}			3.275×10^{-2}			4.918×10^{-1}		

Appendix IV 8.2 LSD for nitrate nitrogen means: % total nitrogen

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean %						
Top	3.94	4.04	4.38	4.41	4.43	0.296
Root	2.66	3.36	3.73	3,97	3.94	0.224

Appendix IV 8.3 LSD for phosphate treatment means: % total nitrogen

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean %				
Root	3.29	3.65	3.65	0.174

Appendix IV 9.1 Analysis of variance for percent total phosphate in top, root and nodule material

Source	df	top MS	top F		root MS	root F		nodule MS	nodule F	
Replicate	1	4.720×10^{-2}	10.19		1.330×10^{-5}			2.430×10^{-3}		
NO_3 N	4	2.111×10^{-2}	4.56	**	1.109×10^{-1}	15.02	**	2.372×10^{-3}	0.32	ns
Phosphate	2	6.310×10^{-1}	136.23	**	2.031	275.03	**	7.023×10^{-1}	94.24	**
N x P	8	2.769×10^{-3}	0.60	ns	2.487×10^{-2}	3.37	*	1.292×10^{-3}	0.17	ns
Error	14	4.632×10^{-3}			7.385×10^{-3}			7.452×10^{-3}		

Appendix IV 9.2 LSD for nitrate nitrogen means: % total phosphate

Level of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean %						
Top	0.582	0.460	0.448	0.440	0.451	0.084
Root	0.857	0.690	0.558	0.528	0.567	0.106

Appendix IV 9.3 LSD for phosphate means: % total phosphate

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean %				
Top	0.215	0.498	0.716	0.065
Root	0.217	0.589	1.114	0.082
Nodule	0.554	0.819	1.084	0.083

Appendix IV 10.1 Analysis of variance for percent soluble sugars in plant top

Source	df	MS	F	
Replicate	1	4.889×10^{-1}	6.37	
NO_3 N	4	1.739×10^{-1}	2.27	ns
Phosphate	2	1.579	20.56	**
N x P	8	8.480×10^{-2}	1.10	ns
Error	14	7.680×10^{-2}		

Appendix IV 10.2 LSD for phosphate means: percent soluble sugars in top material

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean percent	6.18	5.61	5.41	0.266

Appendix IV 11.1 Analysis of variance for percent soluble sugar in roots

Source	df	MS	F	
Replicate	1	1.145		
NO_3 N	4	2.880×10^{-1}	5.89	**
Phosphate	2	1.251	23.61	**
N x P	8	6.664×10^{-2}	1.36	ns
Error	14	4.885×10^{-2}		

Appendix IV 11.2 LSD for nitrate nitrogen means: percent soluble sugar in root material

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean percent	3.75	4.23	4.11	3.95	4.28	0.274

Appendix IV 11.3 LSD for phosphate means: percent soluble sugars in root material

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean percent	4.45	3.76	3.98	0.212

Appendix IV 12.1 Analysis of variance for percent starch in top material

Source	df	MS	F	
Replicate	1	2.840×10^{1}	128.42	
NO_3 N	4	2.908×15^{1}	1.32	ns
Phosphate	2	9.013	40.76	**
N x P	8	3.272×10^{-1}	1.48	ns
Error	14	2.212×10^{-1}		

Appendix IV 12.2 LSD for phosphate means: percent starch in top material

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean percent	3.89	2.76	1.56	0.451

Appendix IV 13.1 Analysis of variance for percent starch in root material

Source	df	MS	F	
Replicate	1	3.239×10^{1}	106.61	
NO_3 N	4	8.056×10^{-1}	2.65	ns
Phosphate	2	2.513	8.27	**
N x P	8	2.018×10^{-1}	0.66	ns
Error	14	3.038×10^{-1}		

Appendix IV 13.2 LSD for phosphate means: percent starch in root material

Phosphate level mgP/plant/week	0.2	0.5	1.5	LSD (t 0.05)
Mean percent	2.38	1.47	1.56	0.529

Appendix IV 14.1 Analysis of variance for total plant weight

Source	df	MS	F	
Replicate	1	6.816×10^4	10.572	
NO_3 N	4	9.014×10^4	13.981	**
Phosphate	2	1.628×10^3	0.253	
N x P	8	2.579×10^3	0.400	
Error	14	6.447×10^3		

Appendix IV 14.2 LSD for nitrate nitrogen treatment means: total plant weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean plant weight (mgms)	536.8	739.6	799.3	832.6	823.0	99.44

Appendix V 1.1 Analysis of variance for nodule dry weight

Source	df	MS	F
Replicate	2	1.767×10^4	1.39
NO_3 N	4	4.255×10^4	3.34 *
Phosphate	2	1.341×10^4	4.034 *
N x P	8	1.341×10^4	1.05 ns
Error	28	1.275×10^4	

Appendix V 1.2 LSD for nitrate nitrogen means: nodule dry weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean (mg)	571.1	566.5	406.8	438.5	434.5	109.0

Appendix V 1.3 LSD for phosphate means: nodule dry weight

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean (mg)	449.5	472.8	560.6	84.5

Appendix V 2.1 Analysis of variance for rate of ethylene produced root/hour

Source	df	MS	F	
Replicate	2	4.331×10^{2}	12.16	
NO_3 N	4	1.739×10^{3}	48.85	**
Phosphate	2	1.089×10^{3}	30.59	**
N x P	8	7.586×10^{1}	2.13	ns
Error	28	3.562×10^{1}		

Appendix V 2.2 LSD for nitrate nitrogen means: rate of ethylene produced root/hour

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean	43.3	31.1	25.8	13.3	8.7	5.76

Appendix V 2.3 LSD for phosphate means: rate of ethylene produced root/hour

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean	17.7	21.67	34.0	4.46

Appendix V 3.1 Analysis of variance for rate ethylene produced mg nodule DW per hour

Source	df	MS	F	
Replicate	2	6.956×10^2	5.16	
NO_3 N	4	5.326×10^3	39.50	**
Phosphate	2	1.505×10^3	11.16	**
N x P	8	3.093×10^3	2.29	*
Error	28	1.348×10^2		

Appendix V 3.2 LSD for nitrate nitrogen means: rate ethylene produced per mg nodule DW per hour

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean	80.1	58.5	52.3	29.5	18.6	11.21

Appendix V 3.3 LSD for phosphate means: rate ethylene produced per mg nodule DW per hour

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean	40.0	44.3	59.0	8.68

Appendix V 4.1 Analysis of variance for top dry weight

Source	df	MS	F	
Replicate	2	2.548×10^{1}	39.54	
NO_3 N	4	1.232	1.91	ns
Phosphate	2	6.675	10.36	**
N x P	8	1.284	2.00	ns
Error	28	6.444×10^{-1}		

Appendix V 4.2 LSD for phosphate means: top dry weight

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean (gms)	19.4	19.9	20.7	0.60

Appendix V 5.1 Analysis of variance for root dry weight

Source	df	MS	F	
Replicate	2	3.237	14.45	
NO_3 N	4	1.094	4.88	**
Phosphate	2	8.845×10^{-1}	0.39	ns
N x P	8	1.553×10^{-1}	0.69	ns
Error	28	2.241×10^{-1}		

Appendix V 5.2 LSD for nitrate nitrogen means: root dry weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean (gms)	5.16	5.55	5.87	6.09	5.65	0.46

Appendix V 6.1 Analysis of variance for percent total nitrogen in top, root and nodule material

Source	df	top MS	top F		root MS	root F		nodule MS	nodule F	
Replicate	2	1.389×10^{-2}	0.72		5.600×10^{-3}	0.46		1.541×10^{-1}	1.19	
NO_3 N	4	1.783	92.87	**	2.654	217.45	**	9.777×10^{-2}	0.75	ns
Phosphate	2	4.742×10^{-2}	2.47	ns	4.838×10^{-2}	3.96	*	1.134×10^{-2}	0.09	ns
N x P	8	4.633×10^{-2}	2.41	*	6.529×10^{-3}	0.53	ns	1.123×10^{-1}	0.86	ns
Error	28	1.920×10^{-2}			1.221×10^{-2}			1.300×10^{-1}		

Appendix V 6.2 LSD for nitrate nitrogen treatment means: % total nitrogen

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean top	2.51	2.65	2.87	3.24	3.60	0.134
Mean root	2.17	2.35	2.63	3.10	3.49	0.107

Appendix V 6.3 LSD for phosphate treatment means: % total nitrogen

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean root	2.79	2.76	2.68	0.083

Appendix V 7.1 Analysis of variance for percent total phosphate in top, root and nodule material

Source	df	top MS	top F		root MS	root F		nodule MS	nodule F	
Replicate	2	4.667×10^{-5}	0.23		5.089×10^{-4}	1.785		3.356×10^{-4}	0.41	
NO_3 N	4	5.722×10^{-4}	2.81	ns	1.469×10^{-3}	5.16	**	2.820×10^{-2}	34.14	**
Phosphate	2	4.376×10^{-2}	214.70	**	3.049×10^{-2}	106.97	**	4.546×10^{-2}	55.04	**
N x P	8	1.739×10^{-4}	0.85	ns	1.817×10^{-4}	0.64	ns	7.939×10^{-4}	0.96	ns
Error	28	2.038×10^{-4}			2.850×10^{-4}			8.260×10^{-4}		

Appendix V 7.2 LSD for nitrate nitrogen means: % total phosphate

Conc. of NO_3 N	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean root	0.344	0.336	0.322	0.312	0.321	0.016
Mean nodule	0.451	0.392	0.372	0.316	0.319	0.028

Appendix V 7.3 LSD for phosphate treatment means: % total phosphate

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean top	0.283	0.334	0.391	0.011
Mean root	0.286	0.320	0.375	0.013
Mean nodule	0.319	0.363	0.429	0.022

Appendix V 8.1 Analysis of variance for percent soluble sugars in top, root and nodule material

Source	df	top MS	top F		root MS	root F		nodule MS	nodule F	
Replicate	2	2.188			1.676×10^{-1}	0.89		9.200×10^{-2}	1.04	
NO_3 N	4	9.947×10^{-1}	4.42	**	5.362	28.69	**	1.496	16.92	**
Phosphate	2	9.704×10^{-2}	.43	ns	6.697	35.83	**	4.92	55.62	**
N x P	8	1.617×10^{-1}	.72	ns	1.464×10^{-1}	0.78	ns	2.113×10^{-1}	2.39	*
Error	28	2.249×10^{-1}			1.869×10^{-1}			8.843×10^{-2}		

Appendix V 8.2 LSD for nitrate nitrogen treatment means: % soluble sugars

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean top	8.01	8.04	8.02	7.79	7.26	0.46
Mean root	6.57	6.38	5.63	5.57	4.62	0.42
Mean nodule	5.60	5.70	5.73	6.53	6.25	0.29

Appendix V 8.3 LSD for phosphate treatment means: % soluble sugars

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean root	6.33	5.92	5.02	0.32
Mean nodule	6.57	5.89	5.43	0.22

Appendix V 9.1 Analysis of variance for percent total starch in top, root and nodule material

Source	df	top MS	top F		root MS	root F		nodule MS	nodule F	
Replicate	2	1.316	2.10		1.439×10^{-1}	0.43		3.612	6.00	
NO_3 N	4	1.938×10^{1}	30.89	**	2.191	6.49	**	2.547×10^{1}	42.33	**
Phosphate	2	2.140×10^{1}	34.12	**	2.049	6.07	**	1.033	1.72	ns
N x P	8	5.638×10^{-1}	.90		1.639×10^{-1}	.49		9.435×10^{-1}	1.57	ns
Error	28	6.273×10^{-1}			3.374×10^{-1}			6.015×10^{-1}		

Appendix V 9.2 LSD for nitrate nitrogen treatment means: % total starch

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean top	15.33	14.39	13.25	12.20	11.83	0.765
Mean root	5.01	3.90	4.05	3.91	3.83	0.56
Mean nodule	7.38	6.54	5.65	3.73	3.60	0.75

Appendix V 9.3 LSD for phosphate treatment means: % total starch

Phosphate level mgP/plant/week	2	5	10	LSD (t 0.05)
Mean top	14.64	13.32	12.25	0.59
Mean root	4.46	4.23	3.74	0.434

Appendix V 10.1 Analysis of variance for total plant weight

Source	df	MS	F	
Replicate	2	4.688×10^{1}	77.679	
NO_3 N	4	3.273	5.214	**
Phosphate	2	8.184	13.036	**
N x P	8	1.254	1.998	ns
Error	28	6.277×10^{-1}		

Appendix V 10.2 LSD for nitrate nitrogen treatment means: total plant weight

Conc. of NO_3 N (mM)	0	0.25	0.5	1.0	4.0	LSD (t 0.05)
Mean plant weight (gms)	24.63	26.04	25.82	26.20	25.78	0.76

Appendix V 10.3 LSD for phosphate treatment means: total plant weight

Level of phosphate mgP/plant/week	2	5	10	LSD (t 0.05)
Mean plant weight (gms)	25.02	25.54	26.47	0.57

Appendix VI 1.1 Analysis of variance for nodule weight

Harvest 1

Source	df	MS	F	
Replicate	3	5.065×10^3	3.51	ns
NO_3 N	3	1.070×10^3	0.74	ns
Error	9	1.445×10^3		

Harvest 2

Source	df	MS	F	
Replicate	3	9.621×10^3	5.31	
NO_3 N	3	6.804×10^3	3.75	ns
Error	9	1.812×10^3		

Harvest 3

Source	df	MS	F	
Replicate	3	9.986×10^3	5.32	
NO_3 N	3	3.585×10^4	19.11	**
Error	9	1.876×10^3		

Appendix VI 1.2 LSD for nitrate nitrogen treatment means: nodule weight

Conc. of NO_3 N (mM)	0	0.5	2.0	8.0	LSD (t 0.05)
Harvest 3 mean (mg)	713.08	589.28	497.48	531.83	69.28

Appendix VI 2.1 Analysis of variance for rate of ethylene production per root per hour

Harvest 1

Source	df	MS	F	
Replicate	3	2.046×10^{2}	4.484	
NO_3 N	3	3.280×10^{1}	0.78	ns
Error	9	4.225×10^{1}		

Harvest 2

Source	df	MS	F	
Replicate	3	2.830×10^{1}	0.70	
NO_3 N	3	2.856×10^{2}	7.07	**
Error	9	4.340×10^{1}		

Harvest 3

Source	df	MS	F	
Replicate	3	1.733×10^{1}	0.34	
NO_3 N	3	2.841×10^{3}	55.72	**
Error	9	5.099×10^{1}		

Appendix VI 2.2 LSD for nitrate nitrogen treatment means: moles C_2H_4/root/hour

Conc. of NO_3 N (mM)	0	0.5	2.0	8.0	LSD (t 0.05)
Harvest 2 mean	42.94	27.73	25.62	25.24	10.16
Harvest 3 mean	91.93	60.00	35.57	35.83	11.42

Appendix VI 3.1 Analysis of variance for rate of ethylene production per mg DW nodule material

Harvest 1

Source	df	MS	F
Replicate	3	4.985×10^2	3.76
NO_3 N	3	3.143×10^2	2.37 ns
Error	9	1.328×10^2	

Harvest 2

Source	df	MS	F
Replicate	3	6.006×10^1	0.63
NO_3 N	3	5.115×10^2	5.36 *
Error	9	9.540×10^1	

Harvest 3

Source	df	MS	F
Replicate	3	4.857×10^2	3.57
NO_3 N	3	3.389×10^3	24.92 **
Error	9	1.360×10^2	

Appendix VI 3.2 LSD for nitrate nitrogen treatment means: C_2H_4/mg DW nodule/hour

Conc. of NO_3 N (mM)	0	0.5	2.0	8.0	LSD (t 0.05)
Harvest 2 mean	80.33	57.58	57.30	58.30	15.62
Harvest 3 mean	131.55	101.85	73.13	68.80	18.65

Appendix VI 4.1 Analysis of variance for top dry weight

Harvest 1

Source	df	MS	F
Replicate	3	1.033×10^1	74.20
NO_3 N	3	1.986×10^{-1}	1.43 ns
Error	9	1.392×10^{-1}	

Harvest 2

Source	df	MS	F
Replicate	3	1.004×10^1	49.168
NO_3 N	3	4.680×10^{-1}	2.29 ns
Error	9	2.042×10^{-1}	

Harvest 3

Source	df	MS	F
Replicate	3	1.587×10^1	38.24
NO_3 N	3	1.161	2.79 ns
Error	9	4.149×10^{-1}	

Appendix VI 5.1 Analysis of variance for root dry weight

Harvest 1

Source	df	MS	F	
Replicate	3	4.302×10^{-1}	35.29	
NO_3 N	3	1.072×10^{-2}	0.88	ns
Error	9	1.219×10^{-2}		

Harvest 2

Source	df	MS	F	
Replicate	3	5.032×10^{-1}	23.58	
NO_3 N	3	1.277×10^{-2}	0.59	ns
Error	9	2.134×10^{-2}		

Harvest 3

Source	df	MS	F	
Replicate	3	6.526×10^{-1}	55.80	
NO_3 N	3	4.690×10^{-2}	4.01	*
Error	9	1.169×10^{-2}		

Appendix VI 5.2 LSD for nitrate nitrogen treatment means: root dry weight

Conc. of NO_3 N	0	0.5	2.0	8.0	LSD (t 0.05)
Mean gm	3.613	3.448	3.355	3.433	0.173

Appendix VI 6.1 Analysis of variance for percent total nitrogen in top material

Harvest 2

Source	df	MS	F
Replicate	3	1.149×10^{-2}	0.73
NO_3 N	3	2.634×10^{-1}	16.77 **
Error	9	1.571×10^{-2}	

Harvest 3

Source	df	MS	F
Replicate	3	2.523×10^{-3}	0.07
NO_3 N	3	7.923×10^{-1}	21.67 **
Error	9	3.366×10^{-2}	

Appendix VI 6.2 LSD for nitrate nitrogen treatment means: percent total nitrogen in top material

Conc. of NO_3 N (mM)	0	0.5	2.0	8.0	LSD (t 0.05)
Harvest 2 mean percent	2.98	3.31	3.50	3.55	0.200
Harvest 3 mean percent	2.96	3.33	3.84	3.84	0.293

Appendix VI 7.1 Analysis of variance for percent total nitrogen in root material

Harvest 2

Source	df	MS	F
Replicate	3	3.139×10^{-2}	2.37
NO_3 N	3	1.940×10^{-1}	14.63 **
Error	9	1.327×10^{-2}	

Harvest 3

Source	df	MS	F
Replicate	3	1.389×10^{-2}	0.64
NO_3 N	3	3.654×10^{-1}	16.86 **
Error	9	2.167×10^{-2}	

Appendix VI 7.2 LSD for nitrate nitrogen treatment means: percent total nitrogen in root material

Conc. of NO_3 N (mM)	0	0.5	2.0	8.0	LSD (t 0.05)
Harvest 2 mean percent	2.18	2.59	2.55	2.67	0.184
Harvest 3 mean percent	2.18	2.41	2.83	2.75	0.235

Appendix VI 8.1 Analysis of variance for percent total nitrogen in nodule material

Harvest 2

Source	df	MS	F
Replicate	3	9.475×10^{-2}	2.66
NO_3 N	3	1.371×10^{-1}	3.86 *
Error	9	3.557×10^{-2}	

Harvest 3

Source	df	MS	F
Replicate	3	8.354×10^{-2}	1.39
NO_3 N	3	6.306×10^{-2}	1.39 ns
Error	9	4.550×10^{-2}	

Appendix VI 8.2 LSD for nitrate nitrogen treatment means: percent total nitrogen in nodule material

Conc. of NO_3 N (mM)	0	0.5	2.0	8.0	LSD (t 0.05)
Harvest 2 mean percent	7.02	7.37	7.36	7.42	0.302

Appendix VI 9.1 Analysis of variance for total plant dry weight

Harvest 1

Source	df	MS	F
Replicate	3	1.494×10^{1}	112.36
NO_3 N	3	2.294×10^{-1}	1.73 ns
Error	9	1.330×10^{-1}	

Harvest 2

Source	df	MS	F
Replicate	3	1.475×10^{1}	71.51
NO_3 N	3	4.254×10^{-1}	2.06 ns
Error	9	2.062×10^{-1}	

Harvest 3

Source	df	MS	F
Replicate	3	2.291×10^{1}	46.86
NO_3 N	3	7.690×10^{-1}	1.57 ns
Error	9	4.888×10^{-1}	