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

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SUMMARY

In a glasshouse experiment, single plants of ten-weeks-old white clover (*Trifolium repens* L.) were subjected to two levels of shading and two levels of defoliation. Plant growth, nodulation and nitrogen fixation parameters were collected on six sequential harvests over four weeks.

Losses of roots and nodules resulting from the treatments were probably due to a reduction in photosynthate supply. Decreases in nodule number and hence nodule dry weight per plant were due to nodule decay, sloughing off and non-production, and were related to the losses in root dry weight. Total nodule numbers on both 'control' and 'treated' plants increased with time, due mainly to increases in numbers of medium sized (1-3mm) nodules. Reduction in nodule weight per plant in the 'treated' plants was later reflected in a lower mean nodule weight.

Severe defoliation caused degradation of the pink pigment, leghaemoglobin, an effect which was seen in less than three days from treatment. It also led to a temporary marked decrease in the nitrogen fixing capability of the nodules as measured by the acetylene reduction assay. Recovery of normal activity by the nitrogen fixing system in the defoliated plants took about ten days.

The suitability of acetylene reduction assay for a short term experiment, and the relationship between rates of acetylene reduction and nodule colour are briefly discussed.

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GENERAL INTRODUCTION

The ability of the legume-Rhizobium symbiosis to fix atmospheric nitrogen, hence contributing towards the maintenance of soil fertility is well recognised. Although increasing usage of mineral nitrogen is evident in agricultural practices overseas (Watkin, Williams 1970), in New Zealand legume nitrogen is still by far the most important source of nitrogen in grassland farming.

Nitrogen fixation in a fertile ryegrass-white clover association has been estimated to yield as much as 550 lb N per acre per annum (Sears 1953), and it has been emphasised that for maximum benefit to the grass component in a mixed sward the white clover should be periodically defoliated and have ample light for regrowth (Butler, Greenwood & Soper 1959).

The present study examines the effects of defoliation and shading on nodulation and nitrogen fixation in white clover.

CHAPTER I

REVIEW OF LITERATURE

The legume-Rhizobium symbiosis has two definite parts, i.e. nodule formation and nodule function, each involving a number of distinct and yet interrelated processes. The first section in this chapter reviews the process of nodulation and the functions of the nodules, while the second section discusses the main factors affecting these processes and functions. In a study of this nature it is only possible to cover a fraction of the enormous volume of literature associated with the subject. Attention is therefore centred upon more recent review articles from which most of the references are drawn.

1 NODULATION

The descriptive aspects of nodulation have been reviewed thoroughly by a number of workers (Fred, Baldwin & McCoy 1932; Wilson 1940; Nutman 1956, 1958, 1965; Allen & Allen 1958; Raggio & Raggio 1962; Stewart 1966; Masterton & Sherwood 1970), hence only selected aspects are presented here.

1.1 Physiology of nodule formation

Much of the work in this field has been carried out on various species of the genus Trifolium, and present understanding is based on these observations. It is a composite picture and exceptions to the general pattern can be expected (Nutman 1965).

1.1.1 Root hair infection

The early pre-infection interaction between the host and the nodule bacteria (i.e. stimulus to bacterial multiplication) occurs in the legume rhizosphere. It is a non-specific stimulation of the microbial population by secretions from the host roots. The secretions probably consist of nutrients and growth factors e.g. thiamin and biotin (West 1939; West & Lochhead 1940). Stimulation by non-legumes is generally less pronounced than with legumes (Rovira 1961).

Infection can occur via the host root hairs or elsewhere on the root, but in Trifolium species it is generally through the curled root hairs (Nutman 1958). It is generally accepted that during the infection phase, tryptophane is secreted by the legume root (Rovira 1956) and converted into indolylacetic acid (IAA) by the particular rhizobium species present (Kefford, Brockwell &Zwar 1960), and that the IAA is associated with root hair curling (Nutman 1965). However more recently Masterton et al (1970) cited several workers (viz. Sachlaman & Fahraeus 1962; Haack 1964; Yao & Vincent 1969) whose evidence suggested that root hair curling might be more specific than was suspected and that a non-dialiysable molecule might be involved.

The first specific interaction between the host and the nodule bacteria occurs when the host plant, in response to extracellular bacterial polysaccharide, secretes polygalacturonase, a pectic enzyme (Nutman 1965). The role of this enzyme as a primary cell wall softener which is essential for infection has now been widely accepted (Masterton et al 1970). The specificity of the interaction between the bacterial polysaccharide and the host enzyme induction is closely linked to the host's susceptibility to infection. Several workers (Balassa 1960; Lange & Alexander 1960; Ljunggran 1961 quoted by Nutman 1965), while studying the genetic transformation of rhizobial virulence have obtained evidence to support this point.

The details of the origin of the infection thread and the mechanism of infection have not been resolved. However, the invagination hypothesis first proposed by Nutman (1956) is widely quoted and so far there is no report to the contrary. This hypothesis states that micro-invagination of the host cell wall forms the infection thread without actual penetration and infection of the cytoplast of the root hair (Nutman 1959). The fact that macerated nodular cells could separate freely and are not held by the infection thread was quoted by Nutman in support of his hypothesis. Further supporting evidence obtained by several workers has been summarised by Masterton et al (1970).

Preceding the growth of the infection thread within the clover root cell there is an orderly migration of the host nucleus towards the host root cortex (Fahraeus 1957; Nutman 1959). This points to the significance of host regulation in nodulation.

In lucerne seedling McCoy (1932) reported that only 4-5% of the root hairs were infected with a further 40% deformed but not infected, and that the mean ratio of infected hairs to nodules was 68 : 1. However, in white clover Fahraeus (1957) found that the percentage of

infected hairs was much lower. Furthermore, over 10% of the infection threads within the infected root hairs failed to grow. In red clover (Trifolium pratense), Purchase (1953) noted an almost 1 : 1 ratio in the number of infected hairs to nodules formed which indicated a low level of abortive infection. Thus, at least some aspects of the pattern of root hair infection seem to vary from species to species.

Using the Fahraeus method of slide preparation, Nutman (1958) studied the pattern of root hair infection on twelve small-seeded Trifolium species, and concluded that there was a host resistant period of about 3 - 10 days depending on host species, during which infection was impossible even though there were ample root hairs and viable rhizobia. Also the first infections occurred at a few well spaced points or zones on the root; these were not at random but were sites of potential initiation of secondary roots. Subsequent infections arose within or near to these zones, and later at other points along the root. During this phase, the rate of hair infection increased exponentially until the appearance of the first nodule after which a lower rate continued giving a Mitscherlich curve. This general pattern applied to all the species studied, differing only in the duration of the resistance period and the time to first nodule appearance. These observations were later confirmed by Lim (1963).

The non-random nature of infection and the intense cytoplasmic activity at the site of root hair curling (Fahraeus 1957) have led to the suggestion by Raggio & Raggio (1962) that curling is host-controlled and that the colonies of nodule bacteria occurring around the deformed root hair are the consequence and not the cause of curling.

1.1.2 Rhizobial density and nodulation

Under field conditions low rhizobial density due either to microbial antagonism (Hely, Bergersen & Brockwell 1957), adverse temperatures (Bowen & Kennedy 1959), or low pH (White 1966) can cause nodulation failure.

The early report by Bhaduri (1951) that comparable nodulation could occur over a wide range of inoculum sizes might be a result of not taking rhizobial multiplication into account. Under laboratory conditions, due to multiplication a bacterial density of 10^6 - 10^9 organisms per ml of medium could be reached frequently by the time the seedlings were susceptible to infection (Purchase & Nutman 1957).

Since infection can occur only at discrete foci on the root,

the relationship between virulent bacterial density and nodule number was shown by Nutman and his colleagues to fit Mitscherlich or a compound Mitscherlich curve (Nutman 1958).

1.1.3 Pattern of nodulation

In genetically homozygous species e.g. subterranean clover (Trifolium subterraneum L) the relationship between average nodule size (length) and nodule number is hyperbolic (Nutman 1958), which indicates a constant nodular volume for a particular host-rhizobium combination. This inverse relationship between average nodule size and abundance has also been reported in white clover by Jones (1962), and in red clover by Nutman (1958), but because of their heterozygosity the relationship is more diffuse.

Even when inoculated with an 'effective' strain of rhizobium, individual white clover plants form a heterozygous population exhibiting wide variations in effectiveness. In general, earliness of primary nodule formation is positively correlated with effectiveness. Effectiveness is also correlated with nodule weight but not with nodule number or average nodule size (Masterton & Sherwood 1970).

Differences between primary and secondary nodulation associated with root temperature were reported by Dart & Mercer (1965, see section 3.4.1.a of this chapter).

1.1.4 Relationship between lateral roots and nodules

Fred, Baldwin & McCoy (1932), summarised the early evidence as to whether the nodule is a modified lateral root by stating: "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. Anatomically then, it differs from non-leguminous nodules, many of which are clearly modified roots". This view was supported by Wilson (1940), Bond L. (1948) and Allen & Allen (1958), who stressed that the nodule is a unique type of root hypertrophy with a cortical origin and an anatomy which differs from that of secondary root in both structure and function. The original concept that the nodule is a lateral root modified by auxins produced by the nodule bacteria (Thimann 1936, 1939, quoted from Wilson 1940) has received little subsequent support.

Nutman (1958) quoted a number of workers who had observed that the tetraploid centres which are the primary sites of nodule

initiation are often located near to the lateral root primordia. In experiments with red clover, Nutman (1948) demonstrated the close relationship between the plant's rooting habit and its nodulating habit, in that the number of lateral roots in an uninoculated host plant variety was positively correlated with the number of nodules it would produce when inoculated. In further experiments Nutman (1949) found that the lag period (time to infection) associated with virulent bacterial strains could be reduced by delaying inoculation from about 12 - 30 days after sowing, whereas the rate of formation of later nodules was governed by the size and morphology of the early nodules, a feature related to bacterial virulence. Thus by removing the tip of the first formed nodules he stimulated the rate of subsequent nodule formation. The same result was obtained from the excision of root tips. From these studies he developed the concept that the nodule (and root) meristem is the centre of an inhibitory activity which modifies further nodulation, and that the degree of inhibition varies according to the size of the meristems. These findings were used by Nutman to support his view that the discrete foci where the nodules initiate could not be distinguished physiologically from those centres which gave rise to lateral roots.

More recently Wittmann (1968), working with field beans (Vicia faba), vetch (Vicia spp) and peas (Pisum sativum) found that the nodules were of endogenous origin, being transformed lateral root primordia. However no proliferation of root cortex has been observed.

1.1.5 Nodule development

In the inner cortex of the clover root there are groups of mixed diploid and tetraploid cells which appear to be sites of nodule initiation. The tetraploid cells are probably in excess of the number of nodules formed (Nutman 1958). Nodule differentiation commences only after these centres start cell division, which appears to be stimulated by the approach of the infection thread.

However Kodama (1967), reported that although tetraploid nodules are common, Vicia faba and some tropical legumes e.g. Desmodium fallax, Archis hypogaea, Glycine max, Phaseolus angularis and Vigna sinensis have diploid nodules. It is not known whether the process of infection in these species differs from that in species having tetraploid centres or how significant any differences may be.

Returning to the clover root, cell division and differentiation in the nodule initiation site leads to the formation of a meristem

producing mainly tetraploid cells basally and diploid cells towards the outer zone. An endodermis forms around the tetraploid cells which will form the infected region. The bacteria are released into the host cell via vesicles formed on the infection thread (Nutman 1965), a feature later confirmed by Goodchild & Bergersen (1966). Once inside the host cell the bacteria multiply rapidly and change into bacteroids. Intracellular infection is aided by host cell division during differentiation and initial nodule growth. The bacteroids do not exist freely in the host cell but are enclosed in groups within a membrane which is generally believed to be of host origin (Nutman 1965). However controversy exists as to its exact origin, e.g. from the host plasmalemma in Glycine max nodules (Bergersen & Briggs 1958); from endoplasmic reticulum in Medicago sativa nodules (Jordan, Grinyer & Coulter 1963) or from in situ synthesis in Lupinus luteus nodules (Dart & Mercer 1963). For further references see Masterton & Sherwood (1970).

The commencement of nitrogen fixation in the nodule is associated with the appearance of leghaemoglobin, either in solution or attached to the lipo-protein membrane of the endoplasmic reticulum (Bergersen & Wilson 1959). Leghaemoglobin will be further discussed under section I.2.1.1. Nodules actively fixing nitrogen have little carbohydrate reserves but when fixation is impaired glycogen will accumulate in large quantities (Nutman 1965).

The structure of the nodule has been described in detail by Allen & Allen (1958), and more recently Pate, Gunning & Briarty (1969), using electron microscope have studied its ultrastructure.

2 NODULE FUNCTIONS

The functions of the nodule include nitrogen fixation, translocation of carbohydrate into and nitrogenous compounds out of the nodule, and the general maintenance of the nodular structure. Of these, by far the most documented is the process of fixation.

2.1 Nitrogen fixation

2.1.1 Site of nitrogen fixation : leghaemoglobin

Although Turchin, Berseneva & Zhidkikh (1963, quoted by Masterton & Sherwood 1970) were the first to obtain fixation in extracts of legume root nodules, Bergersen & Turner (1967) using cell free extracts of soya-bean root nodules first demonstrated beyond doubt that the

bacteroids were the agents of fixation.

Leghaemoglobin, the pink pigmented chromoprotein, has been used as an index of nitrogen fixing potential (Virtanen 1955), but Bergersen (1961) suggested that it would be more appropriate as an index of bacteroid concentration. More recently Schwinghamer, Evans & Dawson (1970) have shown that the heme content of pea root nodules is correlated ($r = 0.72$) with ethylene production in the acetylene reduction assay, i.e. with nitrogen fixation.

Since nodule bacteroids washed free of leghaemoglobin can still fix nitrogen (Bergersen & Turner 1967) this suggests that leghaemoglobin does not play a direct role in the fixation process. A solution of leghaemoglobin can transfer oxygen eight times faster than can pure water, suggesting that leghaemoglobin acts as the oxygen remover (Bergersen 1969), thus maintaining low oxygen tension at the site of fixation.

2.1.2 Biochemistry of nitrogen fixation

Advances in the biochemistry of nitrogen fixation have been very rapid; Wilson (1969) gave the general historical background to this field while Stewart (1966) and Burris (1966) reviewed the knowledge as it existed before 1966. In this review only the main features and the more recent developments are presented, and where possible emphasis will be given to results obtained from symbiotic nitrogen fixation. More recent reviews have been presented by Burris (1969) and Postgate (1970).

The extensive literature on the biochemistry of fixation is based mostly upon studies using free living nitrogen fixing micro-organisms (e.g. Clostridium pasteurianum and blue green algae; see Burris 1969). Before 1960 whole cell techniques had mainly been used and although some progress was made, the major breakthrough came with the development of the technique of obtaining high-quality cell free extracts by Carnahan and his co-workers (Carnahan, Mortenson & Castle 1960). Only recently (Bergersen 1966; Bergersen & Turner 1967; Klucas & Burris 1967) has symbiotic nitrogen fixation been successfully reported in disrupted nodule preparations. Most workers agree that many biochemical aspects of fixation in free living micro-organisms is similar to those of symbiotic fixation (Burris 1969; Bergersen 1969).

a. Metabolic pathway in nitrogen fixation

Activated nitrogen may undergo several alternative reactions, viz.

reduction, oxidation, hydrolysis or direct combination with organic compounds (Stewart 1966). From evidence accumulated, most workers now agree that reduction to ammonia is the most probable pathway in symbiotic nitrogen fixation e.g.:

$$N{\equiv}N \longrightarrow HN{=}NH \longrightarrow H_2N\text{-}NH_2 \longrightarrow 2NH_3$$

Nitrogen diimide hydrazine ammonia

b. Key intermediate

The "key intermediate" was defined by Wilson & Burris (1953) as "the compound which represents the end of fixation reaction and the start of assimilation of fixed nitrogen into the carbon skeleton". Under a reduction pathway the key intermediate theoretically could either be diimide, hydrazine or ammonia. As diimide is extremely labile, the key intermediate can only be hydrazine or ammonia, although hydroxylamine can be a possibility for the oxidative pathway (Stewart 1966).

Using radioactive isotopes and cell-free extracts from Clostridium pasteurianum, Carnahan et al (1960) failed to detect any possible key intermediate such as hydrazine (or hydroxylamine) other than ammonia; this led to the postulation of an enzyme-bound intermediate (Burris 1966). Unless there is a six-electron transfer, nitrogenous compounds in the partially reduced form must exist, although they may remain bound to the enzyme and be reduced stepwise until finally reaching the ammonia phase. This hypothesis has been widely accepted (Burris 1966; Jackson & Hardy 1967; Bergersen 1969).

Experimental evidence cited by Stewart (1966), including work done using cell free extracts of Clostridium, soyabean root nodule preparations, and blue-green algae all favoured ammonia as the key intermediate.

c. Requirements for fixation

For fixation to continue under an adequate atmosphere of nitrogen, four major requirements are necessary: a source of reducing power, a source of energy, an enzyme complex and a supply of carbon skeletons for organic combination with ammonia. These will now be discussed.

i. Reducing power

Stewart (1966) pointed out three possible sources of electrons. They were pyruvate, molecular hydrogen and photoreduction. The high concentration of pyruvate that was necessary to support the cell-free extract of Clostridium prepared by Carnahan et al (1960) had been quoted

as evidence in support of this compound as the main source of reducing power. As pyruvate functions in the phosphoroclastic reaction,

$$CH_3CO.COOH + H_3PO_4 \longrightarrow CH_3(CO)H_2PO_4 + CO_2 + H_2$$

producing acetyl phosphate, carbon dioxide and hydrogen, the electron might come from the hydrogen generated during this reaction (Stewart 1966). Alternatively, free hydrogen already in the system could have been the source of electrons. The stimulation of nitrogen fixation activity in the dark by the addition of molecular hydrogen to cell-free extract of Chromatium was demonstrated by Arnon, Losada, Nozaki & Tagawa (1961). Later, this was confirmed by Mortenson (1964) using cell-free extracts of Clostridium.

In the photosynthetic nitrogen fixing bacterium Chromatium, the electron might have came from the photolysis of water molecules as was first suggested by Gest, Judis & Peck (1956 quoted by Stewart 1966). Further evidence in support of this has been reported by later workers (blue-green algae (Anabaena cylindrinca) by Fogg & Than Tun (1960); Rhodospirillum rubrum by Pratt & Frenled (1959); for other references see Stewart 1966).

In a symbiotic nitrogen fixation system, oxidative phosphorylation has been suggested as providing a source of reducing power (Bergersen 1969), hence for this system pyruvate is the most likely source.

Ferredoxin, an electron carrier first named by Mortenson, Mower & Carnahan (1962), who extracted it from a cell-free extract of Clostridium, was later found to be analogous to the plant methaemoglobin reducing factor and also to photosynthetic pyridine nucleotide reductase (Burris 1969). Ferredoxin is a low potential electron carrier (Mortenson 1964), and has been found in all Clostridium extracts so far examined. It has been crystallised and it has a molecular weight of about 5600.

From soyabean root nodule bacteroids, a brown non-haem iron protein was isolated; it may possibly be a very labile ferredoxin and if so it may serve as the natural electron carrier in the symbiotic nitrogen fixation system (Koch, Evans & Russell 1967, quoted by Bergersen 1969).

ii. Energy source

Although in an earlier experiment (Nicholas 1963a) adenosine 5-tri-phosphate (ATP) was shown not to be essential for nitrogen fixation, and that it might even be inhibitory (Carnahan et al 1960), later work (Mortenson 1964) suggested that ATP in fact can be used as a source of

energy for fixation. Stewart (1966), Burris (1966, 1969), and Bergersen (1969) all suggested that during the phosphoroclastic reaction both the reducing power and the energy as acetyl phosphate were furnished by the pyruvate.

That the addition of ATP is an absolute requirement for nitrogen fixation by cell free extract of soyabean bacteroids and that fixation can be prolonged with the incorporation of an ATP generating system (creatine phosphate + creatine kinase) has been widely quoted as evidence of ATP as an energy source, at least in symbiotic nitrogen fixation (Koch, Evans & Russell 1967; Bergersen & Turner 1968). However high levels of ATP (over 2 μ moles/ml) will be inhibitory (Bergersen 1969).

iii. Enzyme complex

The two main enzyme systems, i.e. nitrogenase which catalyses the reduction of molecular nitrogen to ammonia, and hydrogenase which catalyses reversibly the formation of molecular hydrogen from hydrogen ions, have long been postulated (Stewart 1966). Although earlier workers (viz. Gest & Kamen 1949 quoted from Stewart) indicated that the two enzyme systems were synonymous, Mortenson et al (1962) using cell free extracts from Clostridium separated two fractions, one of which was the hydrogen donating system (hydrogenase) and the other a nitrogen activating system (nitrogenase). Nitrogen fixation was only possible when both fractions were present.

Hydrogenase has been detected in all nitrogen fixing systems so far studied, and it is associated with the reduction of ferredoxin. It may be a molybdo-flavoprotein and may also contain iron (Stewart 1966; Burris 1969).

Burris (1969) cited several workers (Mortenson 1966; Bulen & Le Comte 1966; Vandecasteele 1968) who have successfully isolated the nitrogenase complex from cell free extracts of Clostridium and Azotobacter. The two fractions in the nitrogenase complex have yet to be purified. The enzyme complex is cold labile (Dua & Burris 1963) and it has been demonstrated that the cold lability is a characteristic of the iron protein fraction (Moustafa & Mortenson 1968). However Burris and his colleagues (Kelly, Klucas & Burris 1967) have shown that the fractions can be kept for long periods in liquid nitrogen. Burris (1969) presents a summary of the properties of the two fractions.

From cell free extracts of soyabean nodule bacteroids, Klucas, Koch, Russell & Evans (1969) separated two enzyme components, one containing iron and molybdenum, the other iron. These fractions were similar in their reactions to those isolated from the free living

nitrogen fixing systems.

The ability of nitrogenase to reduce a number of triple-bonded substrates, among them acetylene, was first reported by Schöllhorn & Burris (1966) and Dilworth (1966) independently. Later, several workers (viz. Koch & Evans 1966; Koch, Evans & Russell 1967; Hardy, Holsten, Jackson & Burns 1968) demonstrated that the reduction of acetylene to ethylene by the nitrogenase enzyme complex could be used as an index for evaluating the potential nitrogen fixing activities in the symbiotic association. However, more recently Bergersen (1970) cautioned against direct extrapolation of the acetylene reduction assay as a quantitative estimation of nitrogen fixation.

iv. Carbon skeletons

In order for the fixation process to continue, carbon skeletons are needed to remove the ammonia so produced. The most likely organic compound has been suggested to be α-keto-glutarate, as αketo-glutamic acid is the most strongly labelled amino acid in short term fixation experiments using N^{15} with cell free extracts (Wilson 1958 quoted by Stewart 1966). Stewart (1966) has cited evidence to support this view.

2.2 Translocation in nodules

The daily rate of nitrogen fixation in field peas (Pisum arvense) has been estimated by Pate (1958a) to be within the range of 30 - 100 mg nitrogen per gram fresh weight of nodule, and the carbohydrate requirement for fixation has been estimated to be about 3 - 19 mg carbohydrate per mg of nitrogen fixed (Gibson 1966b; Bond 1968). Hence there must exist a very efficient translocation system to remove compounds to and from the nodules. Only recently has the mechanism of nitrogen translocation in the legume root nodule been studied in more detail (Pate 1962; Pate, Gunning & Briarty 1969).

Pate and his colleagues used a 'bleeding' technique whereby exudates were collected from the vascular tissue of intact but detached pea and white clover nodules. In addition they also examined these nodules under electron microscope. From these studies they postulated that the translocation of photosynthate to the nodule and the bacterial tissue is probably by the source-sink flow systems, via the nodule phloem and the symplastic route. For the translocation of the nitrogenous products of fixation from the bacteroids to the nodular bundles, the authors proposed two alternative mechanisms. The first is similar to that described for the root in that the solutes are believed to pass

passively through special compartments in the pericycle cells into the apoplast of the vascular bundles. Certain cytoplasmic structures in the pericycle would retain most of the sugars and certain amino acids, but allow the rest of the solute to move into the xylem, thus maintaining a minimum loss of carbohydrate back to the tops.

The alternative mechanism postulates an active and selective secretion of nitrogenous compounds by the specialised cells of the nodule pericycle into the bundle apoplast. This mechanism is similar to that occurring in some plant and animal gland cells.

Whatever the mechanism of translocation, it is known that the fixed nitrogen does not accumulate in any quantity in the nodule (Bond 1936), and the main products translocated are aspartic acid, asparagine, glutamine and homoserine; smaller quantities of glutamic acid, valine and leucine/isoleucine are also detected (Pate 1962).

2.3 Underground transference of nitrogen

There are at least two mechanisms for the underground transference of nitrogen from legumes. However, controversy exists as to which is the major mechanism.

Virtanen and his colleagues at Helsinki reported that direct excretion of nitrogenous compounds, mainly as aspartic and glutamic acids from the legume root systems, could account for up to 50% of the fixed nitrogen (Virtanen, von Hansen & Laine 1937; Virtanen & Torniainen 1940). The historical background to the controversy in this field was given by Wilson (1940), Walker, Orchiston & Adams (1954) and Stewart (1966); most workers agreed that excretion occurred probably under certain specific climatic conditions (Wilson loc. cit.), and the nature of the rooting medium may be implicated also (Butler per.comm.).

Butler and his colleagues (Butler & Bathurst 1956; Butler, Greenwood and Soper 1959) concluded that the condition required for excretion was too specific to occur under a normal field environment, hence sloughing off and decay of roots and nodules would be the major mechanism of underground transference. However a number of other workers (Strong & Trumble 1939; Dilz & Mulder 1952; Simpson 1965) have reported excretion by legume root systems.

3. FACTORS AFFECTING NODULATION AND NITROGEN FIXATION

3.1. Introduction

The success of a symbiotic association depends on effective nodulation as well as on efficient fixation. The difference in the quantity of nitrogen fixed by effective and ineffective symbiotic associations can be mainly accounted for by the differences in bacterial volume and active duration (Chen & Thornton 1940). Nodulation can be recorded in terms of nodule numbers which reflect the host's susceptibility to infection (infectiveness), and also in terms of nodule weight (or volume) which with its active duration reflects effectiveness.

Absence of nodulation is found in the more primitive genera of the Leguminosae (Mimosoideae and Caesalpinioideae), and has also been reported in mutants of soyabean and red clover (Nutman 1956). Symbiosis between the legume and the rhizobium can fail at many stages. The earlier stages of intracellular incompatability will block nodule formation and the later stages (after nodulation) will cause ineffectiveness resulting in the various types of incompatability known to occur in the legume-Rhizobium symbiosis.

Factors both internal and external to the host plant can influence the symbiosis. Some of the main factors will be discussed in the following sections.

3.2 Presence of an effective rhizobial strain and a susceptible legume host

As the two symbionts can exist independently, for symbiosis to occur an effective rhizobial strain must be brought into direct contact with a susceptible legume host. Frequently, nodulation failure in the field has been attributed to the absence of the bacterial symbiont (Vincent 1958; Cloonan &Vincent 1967), hence the need for inoculation (e.g., Cullen & Ludecke 1966). Antagonism between soil micro-organisms, causing a reduction in effective bacterial density and hence poor nodulation in subterranean clover was noted by Hely, Bergersen & Brockwell (1957). A similar effect was also noted by Khan, Moore & Webster (1968) in lucerne.

The necessity of introducing both the legume and its effective rhizobia into a new environment when establishing the legume for the first time, has been well demonstrated by the case of Lotononis bainesii during its introduction into Australia (Bryan 1961).

3.3 Plant factors

3.3.1 Genetic aspects

As early as 1933, Bjalfv (1933, 1935 quoted by Wilson 1940) suggested that the host was more than just passively supplying carbohydrates to the bacteria; it exerted some definite control over nodulation. Nutman (1946), working with red clover selected a line which was resistant to infection, a resistance he attributed to a recessive gene acting in conjunction with a maternally transmitted cytoplasmic component without which the homozygous recessive condition was lethal. Further work by Nutman (1948, 1949) related the rooting habit of the clover to its nodulating habit (both abundance and earliness) and he considered these characters to be inherent host characters.

Resistance to rhizobial infection was also reported by Lynch & Sears (1952) and William & Lynch (1954) in soyabean. However under prolonged association, complete reversal of the symbiotic situation could result from mutations in both symbionts (Nutman 1956).

Wilson (1939, quoted by Allen & Baldwin 1954) postulated that there was a relationship between the pollinating habits of a legume and its promiscuity with rhizobial strains, thus:

i. self-pollinating legumes tended to be pure lines in which the inherent character permitting symbiosis was absent or was recessive, and

ii. cross-pollinating legumes either had maintained or developed these characters so achieving symbiosis with a large number of rhizobial strains.

However controversy over this relationship has risen since the publication by Norris (1956) on the evolution of the legume-Rhizobium symbiosis. Norris advanced the hypothesis that the tropical legume is the typical one and it represents the ancestral form. Tropical pasture legumes are usually self-pollinated and also exhibit high promiscuity, thus suggesting that this relationship is more of a casual than causal nature. The misconception was a result of earlier studies being centred entirely on temperate and cold-temperate legumes rather than on a wider cross-section of the Leguminosae.

3.3.2 Hormonal aspects

Hormonal effects such as the association of IAA with root hair infection and the inhibitory activities of the nodule meristem have received wide acceptance (see section I. 1.1.1). Other host hormonal effects on nodulation and nitrogen fixation, although suspected (Wilson

1940; Nutman 1956; Raggio & Raggio 1962; Stewart 1966), are less well documented.

Nodule shedding and degeneration is stimulated by flowering and fruiting and this can be delayed by removing the reproductive structures (Pate 1958b). Although competition for nutrients can not be ruled out, the effects noted have been attributed to hormonal actions. Further work by Pate (1958c) confirmed the presence of growth promoters and inhibitors in the nodular tissue. Adverse effects of flowering and frost on nodulation of tropical legumes (Desmodium species) were reported by Whiteman & Lulham (1970) in Australia; however the authors stressed the difference between annual and perennial legumes, and that flowering could not account for all the reduction in nodulation in the perennial tropical legumes they studied.

Raggio & Raggio (1962) postulated a "nodulation factor" which was necessary for successful symbiosis with a specific rhizobial strain and this factor was transmissible by grafting. Experimental evidence was cited to support their hypothesis (Hely, Bonnier & Manil 1953; Bonnier 1958; Raggio, Raggio & Torrey 1957; for other references see Raggio & Raggio 1962). Discoveries by Valera, Concepcion & Alexander (1965) and Schaffer & Alexander (1966) at Cornell provided further evidence for the presence of a nodulation factor which could be transmitted from the cotyledon or replaced by a conconut-water preparation.

On the nitrogen fixation side, Bach, Magee & Burris (1958) found that in detached soyabean nodules, fixation could be maintained by supplying carbohydrate, but such carbohydrate could not completely replace that supplied by photosynthesis. The authors concluded that some other products of photosynthesis could be involved.

3.4 Environmental factors

Environmental factors affecting symbiosis operate mainly through the host's physiology (Wilson 1940; Nutman 1956; Stewart 1966), although they can influence the survival of the symbionts as well. Reviews had been presented by Nutman (1956), Allen & Allen (1958), Raggio & Raggio (1962), Vincent (1965) and Stewart (1966).

3.4.1. Physical aspects

a. Temperature

Extreme temperatures can influence all the stages of the symbiosis by affecting the metabolic activities of the symbionts. It can also affect their survival. Field reports by Bowen & Kennedy (1959) that

strains of rhizobia from Pisum, Trifolium, Medicago, Centrosema and Pultenaea were killed at soil temperature above 40° C had real implications for the sowing of inoculated legume seeds in dry subtropical and tropical conditions.

Early studies by Jones & Tisdale (1921) on the effects of soil temperature (12° - 36°C) on growth and nodulation of lucerne, red clover, field peas and soyabean indicated that different host plants could tolerate different root temperatures, and that root temperatures above the optimum would result in a reduction in nodulation as expressed by nodule weight.

Dart & Mercer (1965) using cowpea (Vigna sinensis Endl. Ex Hassk.) found that variations in temperature could cause differences in the pattern of nodulation of primary and secondary roots. Optimum temperature for nodulation of the primary root was 24°C, whereas that for the secondary roots was 33°C, and the pattern of the secondary root nodulation was almost the inverse of the primary root pattern. Temperature influenced the total fresh weight of nodules per plant, the average nodule size and the distribution of starch within the nodules. The authors suggested poor rhizobial and host plant growth and low root exudation as some of the causes of these temperature effects.

The inverse relationship between high temperature and nodulation was further supported by the experiment of Philpotts (1967), who reported that the percentage of cowpea plants bearing nodules and the number of nodules per plant were both reduced under a high temperature regime (37°C).

Gibson (1967a, 1967b) examining the effects of root temperature on nodulation in subterranean clover seedlings reported that the maximum temperature for nodule formation was 33° C and the minimum was 7°C. The most rapid nodule initiation (2 - 3 days after inoculation) was at 30° C and at the same temperature the rate of nodule appearance was highest. Below 22° C, for each successive fall of 5° C, there was a disproportionate delay in nodule initiation and a marked reduction in the rate of nodule appearance.

That temperature could have differential effects on nodulation and nitrogen fixation was supported by Meyer & Anderson (1959) who reported that, in subterranean clover, fixation but not nodulation was adversely affected by temperature above 25° C. This was later confirmed by Possingham, Moye & Anderson (1964), who found that at 30° C nitrogen fixation in subterranean clover was specifically inhibited, whereas at this temperature, control plants grew well on combined nitrogen (NH_4NO_3).

In addition, the authors reported the presence of a 'dark pigment' and a reduction in pink colour in the nodules 48 hours after treatment.

Low root temperature affects the fixation process less markedly than high temperature (Gukova 1945, quoted by Stewart 1966), in that an increase of 4°C above the optimum inhibited fixation by 50% whereas a decrease of 5°C reduced fixation by less than 5%.

Much other work on the effects of root temperature on symbiotic nitrogen fixation was done by Gibson (1963, 1965, 1966a, 1967a and 1967b), who found that fixation per unit time in subterranean clover at 5°C was only 10 - 17% of that achieved at 18°C, and that when the temperature was over 30°C fixation was also markedly reduced. Both the commencement of fixation and the fixation rate were retarded by a temperature deviating from the optimal range of 22° - 25°C. He concluded that the total amount of nitrogen fixed in any given period was determined in part by the root temperature, the amount of nitrogen previously fixed, the nitrogen percentage at the start of the period and the bacterial strain. Strong interactions between variety and bacterial strain were also noted in his experiments. In a more recent study, Gibson (1969) obtained evidence to suggest that the effects of high root temperature on nitrogen fixation were transient and possibly directed towards some step or steps in the fixation reaction.

The depressing effects of sub-optimum root temperatures on nitrogen fixation have been noted by a number of other workers (viz. Mes 1959; Pate & Dart 1961; Pate 1962; Dart & Mercer 1965; and more recently Roughley 1970). That the leghaemoglobin concentration in cowpea nodules is markedly reduced at root temperatures below 21°C and above 36°C (Dart & Mercer quoted by Masterton et al 1970) might have some bearing on these results.

b. Moisture

In the tropics, studies by Masefield (1952, 1955, 1957, 1958 and 1961) showed that moisture was by far the most important factor influencing nodulation among the tropical legumes. It is thus possible, as suggested by Norris (1956) that low nodulation figures recorded in the literature on tropical legumes were due to a bias in the time of sampling, as most work was done in the dry season. The picture might well be different if the same work could be repeated in the wet season. This view was confirmed by Masefield, who found heavier nodulation in field beans, peas and soyabean under moist conditions irrespective of soil types.

McKee (1961) reported a decrease in nodulation in seedlings of birdsfoot trefoil (Lotus corniculatus) under a low soil moisture regime, but the growth of the tops and the roots was much less affected. Similarly Kawatake, Ishida, Nishimura and Shimura (1962) noted poor nodulation in temperate legumes when the soil moisture content fell below 30% in pot culture. Restriction of root growth under a regime of moisture stress will reduce the number of infectable foci on the root system for nodulation and in extreme cases survival of both symbionts can be affected.

In the field, mechanical shrinkage of the clay under a drying condition could cause losses of nodules from established legumes (Diatloff 1967).

There is little information on the effects of moisture levels on nitrogen fixation per se. Generally speaking, maximum nitrogen fixation would probably be at the moisture potential best suited for plant growth, and this affects nodulation more than fixation. Morley (1961) in his review on subterranean clover stated that the major limiting factor to nitrogen fixation in Australia was inadequate water. The effects of excess water would be in terms of lack of soil aeration (van Schreven 1958).

c. Aeration

Virtanen & Hausen (1936), using water cultures of peas, showed that without aeration the nodule number was high, but that nodules were small and ineffective. Upon aeration there was no change in number, indicating that oxygen did not affect nodule initiation whereas the size of the nodules increased, reflecting an influence of aeration on effectiveness. When pure nitrogen gas was used nodulation was completely inhibited, a result which was confirmed by Bond (1951) and Ferguson & Bond (1954) using soyabean and red clover.

According to evidence presented by van Schrevan (1958), aeration could have a specific effect on fixation besides influencing nodulation. Leghaemoglobin was not formed when oxygen supply was low or lacking (Virtanen & Laine 1945 quoted by van Schreven 1958). However, Stewart (1966) suggested that within the nodule where fixation occurs the oxygen tension (pO_2) must usually be very low and there was little evidence to suggest that oxygen would be essential for the fixation process per se. In fact the contrary was demonstrated by Bergersen & Turner (1967), who showed that nitrogen fixation was only possible

by cell-free extracts of soyabean nodule bacteroids extracted under an atmosphere of argon.

Oxygen will thus inhibit fixation by competing with nitrogen for hydrogen ion acceptors (Stewart 1966). However normal respiration in the plant and rhizobium will have to continue in order to maintain the fixation *in situ*, and since oxygen is required for this process hence indirectly for symbiotic nitrogen fixation. The function of leghaemoglobin as an oxygen remover has been discussed in section I.2.1.1.

d. Light

Light is discussed under two sections, that of light intensity and that of light duration or day length. The effect is mainly an alteration in the supply of carbohydrate to the host (Wilson 1940; Stewart 1966) although a photoperiodic effect cannot be ruled out.

i. Light intensity

There is no report of direct effects of high light intensity *per se* on the survival of rhizobia but high light intensity is frequently associated with high temperature which is detrimental to the rhizobia.

McKee (1959) found that at 25 - 50% of full day light, nodulation and seedling growth were more adversely affected in birdsfoot trefoil than in lucerne or red clover. Nodulation of all species was suppressed when the light intensity fell below 25% of full day light. The reduced nodulation of birdsfoot trefoil at 50% light intensity was equivalent to that of lucerne and red clover at 25% day light. Thus host species could differ in their ability to withstand shading. Similarly Butler and his colleagues (Butler & Bathurst 1956; Butler, Greenwood & Soper 1959), demonstrated that reducing normal day light by 75% inside a glasshouse could cause significant reduction in both root growth and nodulation in white clover, lucerne and *Lotus uliginosus*. That shaded and dark-grown legume seedlings would not nodulate has been well documented in the literature (Schweigen 1932; Wilson 1931; Thornton 1930, see Nutman 1958). Most workers agree that the depressing effects of low light intensity could be overcome by increasing the carbon dioxide content of the air or by foliar application of an energy source e.g., sugar (Stewart 1966).

Nitrogen fixation could be affected by light intensity via the photosynthetic process and the carbohydrate: nitrogen ratio of the plant (see section I.3.4.2 for further discussion). However, van Schrevan (1958) quoted evidence (Orcutt & Fred 1935) that excessive

carbohydrate due to high light intensity could be inhibitory to nitrogen fixation.

ii. Day length

Cartwright (1959) showed that in sand culture, day length alone would not influence the growth habit or the nodulation of red clover or lucerne. However long days could interact with low nitrogen level to cause a decrease in nodulation in lucerne. The reduction in nodulation was thought to be the result of competition for available carbohydrate between the tops and the nodules; the carbohydrate was used by the plant for internode elongation under the longer day length.

There are few experiments on the effects of day length on the pattern of nodulation in legumes. Eaton (1931) and Orcutt & Fred (1935) found no effect of day length on nodulation on soyabean, whereas in Phaseolus, Borodulina (1950 quoted by Nutman 1956) found that varieties responded best when grown under their respective native day length conditions.

Bonnier & Sironval (1956) using controlled environmental conditions reported better nodulation in soyabean grown under a sixteen hours day than under an eight hours day; nodules developing under short days were smaller and ineffective.

As red and far red light is known to regulate photomorphogenic processes they may well be important in affecting nodulation. A specific effect of light on nodulation in peas and beans was reported by Lie (1964), who showed that far red light caused a reduction in nodulation and that this effect was counteracted by red light.

Apart from the early work of Eaton (1931), Orcutt & Fred (1935) and several other workers cited by Nutman (1956), there are few reports on the effects of day length on nitrogen fixation per se. As day length is known to influence plant morphogenesis via photoperiodism, the effects of day length on nitrogen fixation and nodulation can be more complex than a purely carbohydrate-mediated response.

e. Defoliation

Defoliation by cutting or grazing will affect not only the host's physiology but also alter the micro-environment of the plant, thus influencing the symbiosis via both symbionts.

Wilson (1942) found that, with periodic defoliation to half inch in height, approximately 15% of the previously existing nodules in a white clover stand were visibly affected; these nodules were found to

have disintegrated. This was confirmed by Butler & Bathurst (1956) and Butler, Greenwood & Soper (1959); they found that initial effects of defoliation were similar to those of shading but differed in the rate at which these nodules were replaced. The turnover was much faster in the defoliated than the shaded plants. They further suggested that for maximum benefit from the symbiosis white clover requires frequent defoliation and ample light for regrowth; the postrate growth habit of white clover and the production of stolons partly explained the increase in number of new nodules on white clover after defoliation.

Reduction in nodule weight and number due to cutting and grazing was reported by Whiteman & Lulham (1970) and Whiteman (1970a, 1970b). The effect of grazing was more severe due to the return of excreta under such a regime.

Evidence of an adverse effect of defoliation on nitrogen fixation comes mostly from observations showing that, upon defoliation, the nodule colour changes from pink to green or brown (Wilson 1942; Butler et al 1959). As the pink pigment leghaemoglobin was known to be correlated with fixation activities (Virtanen, Erkama, Linkola & Linnasalmi 1947; Schwinghamer, Evans & Dawson 1970), a change in colour signified a loss of or reduction in these activities. More recently Moustafa, Ball & Field (1969) using the acetylene reduction technique showed that defoliation markedly reduced the rate of fixation in white clover, which recovered in about three weeks.

f. Volume of rooting medium and plant density

Nutman (1945) showed that in pot trials, the number of nodules per pot depended on the volume of the rooting medium rather than on the number of plants per pot. Kefford, Brockwell & Zwar (1960) suggested that the 'volume effect' in Nutman's experiment might have been a limitation due to the small amount of tryptophane produced by the host roots. Masefield (1955) sowing field beans, dwarf beans and peas at 1, 2 or 3 seeds per hole with and without a companion crop (maize, oat, barley or foxtail millet) found no evidence to suggest that nodulation was affected by the proximity of the same or different species.

Differences in nodulation between single plants and those grown under sward conditions have not been studied. Allen (1970) has cautioned against extrapolation of results obtained from experiments conducted on agar slopes with legumes seedlings to general field conditions.

3.4.2 Nutritional aspects

Nutrients can affect nodulation and nitrogen fixation in several ways, either directly by influencing the growth and vigor of the symbionts or indirectly by affecting their metabolic activities.

a. Combined nitrogen

Among the nutrients, nitrogen has a special place because it can be taken up by the legume roots in combined forms, whereas in the elemental form it can be fixed in the nodules.

Direct inhibitory effects of combined nitrogen (especially nitrate) were reported by Thornton (1935), Thornton & Nicol (1936) and Virtanen (1953). Thornton and his colleagues suggested that the nitrate acted through the prevention of root hair curling, Virtanen regarded the inhibition as a result of the formation of nitrite from a nitrite-leghaemoglobin complex.

In the field, the major factor governing total nodule number in white clover is, according to Young (1958), the amount of clover root material. This is understandable as the sites for nodule formation are at discrete foci on the roots (Nutman 1958). Young showed that in a mixed sward of white clover and ryegrass, the addition of 36 lb nitrogen per acre in the form of nitro-chalk (15.5% N) increased not only the top growth of clover but also nodulation; with 80 - 100 lb nitrogen per acre there was no change in the number of nodules per gram of root, whereas at 200 lb nitrogen per acre the number of nodules decreases especially those over 2 mm in length.

Cartwright & Snow (1962) using urea sprays on peas, red clover and lucerne found that nodulation was delayed in lucerne and red clover whereas in peas it was inhibited. In all cases plant growth was normal and the adverse effects of urea on nodulation were attributed to the high level of nitrogen within the plants.

Light dressings of fertilizer nitrogen are reported to stimulate fixation (Burgevin & Ronx 1933; Fred & Wilson 1934; Orcutt & Wilson 1935, see van Schreven 1958). Heavy dressings suppress nitrogen fixation but at no stage is the fixation process completely inhibited (Allos & Bartholomew 1955). Similar findings were reported by Stewart & Bond (1961) in the non-legumes Alnus and Myrica.

Although total fixation is reduced, evidence cited by Watkin William (1970) suggests that fixation and transfer of nitrogen per clover unit in a mixed sward are as effective in the presence as in the absence of applied nitrogen. However, this evidence comes from long

term trials, and in the short term the rate of nitrogen fixation could be adversely affected, as was demonstrated by Moustafa et al (1969). They found that the rate of acetylene reduction by white clover plants receiving 80 lb nitrogen per acre in a mixed pasture was about 23 - 30% that of the unfertilized control.

The correlation of nodulation and nitrogen fixation with the carbohydrate : nitrogen ratio in the plant led to the formulation of an hypothesis relating the carbohydrate status of the plant to different aspects of the symbiosis. As early as 1934 Allison & Ludwig had suggested that both nodulation and fixation could be influenced by the host's carbohydrate levels. Wilson (1935) hypothesised that the degree of nodulation and nitrogen fixation was governed by the host's internal carbohydrate to nitrogen ratio. He suggested that each plant would have a critical carbohydrate : nitrogen ratio, any deviation from which would result in relatively poor nodulation and fixation. Stewart (1966) quoted a number of experiments in support of this concept. In spite of the amount of evidence presented, Nutman (1956) cautioned that these findings only summarised results and not the actual causes. Likewise Raggio & Raggio (1962) concluded that the need for carbohydrate was related to the supply of carbon skeletons in the fixation process, so allowing normal host root and nodule growth, and not to its presence as a key factor determing nodulation and fixation.

b. Other elements

Both the macro and micronutrients ordinarily required for normal plant and bacterial growth are essential for proper nodulation. However some elements can interact and are specifically required for fixation. Hewitt (1958) and Hallsworth (1958) have reviewed the micronutrient requirements of legumes.

Several workers cited by Nutman (1956) have shown that phosphorus is essential for normal growth and that an adequate supply enhances the rate of infection as well as increasing nodule density. Potassium stimulates infection under adequate phosphorus supply but is inhibitory when phosphorus is low (Lynch & Sears 1951); nitrogen fixation is similarly affected.

A copper requirement for nodulation has been reported (Hallsworth 1958; Greenwood 1958). At 64 ppm copper, subterranean clover forms large nodules clustered on the main root, whereas at concentrations lower than 0.0064 ppm the clustered form disappears and root growth

is poor. The nodules are small and ineffective. Greenwood also reported an interaction between phosphorus and copper.

Molybdenum and sulphur were shown to have some interactions by Anderson & Spencer (1949). Inadequate molybdenum led to a demand for nitrogen by the plant (subterranean clover), which could not be met by further nodulation, whereas a deficiency in sulphur led to a smaller host demand for nitrogen with an impaired protein synthesis. Nodulation in molybdenum-deficient subterranean clover was just as good as with adequate molybdenum supply and plants with adequate sulphur had more nodules and a higher percentage nitrogen in their tissues. The authors concluded that molybdenum was implicated in the fixation process. Hewitt (1959) also suggested that molybdenum could be involved in the fixation process, since higher concentrations of molybdenum were required by legumes when nitrogen was supplied in the elemental form. It is now certain that molybdenum is associated with at least one of the nitrogenase enzyme fractions (Burris 1969 and Bergersen 1969).

In the tropics Newton & Said (1957, quoted by Masefield 1958) found that addition of molybdenum improved nodulation of groundnuts (Arachis hypogea) on the latosol soils of Java; the possibility of improved host root growth due to the addition of molybdenum, hence providing more nodulation sites, could not be ruled out in this case.

Calcium was found by Loneragan & Dowling (1958) to interact with pH. At pH 4 or below no nodules were formed on subterranean clover at any calcium concentrations, and at a calcium concentration of 0.01 M or less no nodules were formed between Ph 3.5 - 6.0. Rhizobium did not respond to calcium (i.e. forming nodules) until pH reached 5.0. and the specific effect of calcium on fixation was associated with its influence on the metabolites going to the nodules rather than directly in the fixation process. However calcium is required at higher levels than those for either symbiont (Loneragan 1959). Norris (1959) noted that calcium was, at most, a micronutrient for all the subtropical bacterial strains he studied, hence casting doubt on its role as a macronutrient for the rhizobium as was stated by McCalla (1937, quoted by Raggio & Raggio 1962). That there was an absolute requirement for calcium by the rhizobium was demonstrated by Vincent (1962), who showed that a deficiency in calcium led to abnormal cell wall formation in the bacterium.

The concept that tropical legumes did not need lime was first proposed by Norris (1956). This was later supported by Parker &

Oakley (1956) who found that lime pelleting reduced nodulation in lupins (L. luteus), serradella, soyabean and cowpea. The beneficial effects of liming Pueraria phaseoloides on an acid soil reported by Samuel & Landran (1952, quoted by Norris 1958) might be due to the temporary release of trace elements, e.g. molybdenum, and not a direct response to calcium or pH per se. Tropical legumes, e.g. Desmodium, Stylosanthes, Indigofera and Centrosema are reported (Andrews & Norris 1961) to be more efficient in extracting calcium and phosphorus from low fertility soils than most temperate legumes.

Boron is essential for nodule development and when absent, infection is prevented (Mulder 1948, quoted by Nutman 1956). With low levels of boron, nodules are initiated (infection is not impeded), but nodular development is arrested resulting in ineffectiveness. The requirement for boron in the development of the vascular tissue in the nodule could be involved (Brenchley & Thornton 1925, quoted by Nutman 1956). There is no evidence to suggest that boron is required specifically for fixation, although Nicholas (1963b) suggested that it could be involved indirectly in a yet unknown way.

Cobalt was found to be essential to both symbionts for the synthesis of vitamin B_{12} which in turn is essential to enzymic systems (Stewart 1966). Evidence summarised by Reisenaner (1960) showed that lucerne with adequate cobalt fixed more nitrogen than the minus cobalt control. Cobalt is also involved in the synthesis of leghaemoglobin, hence a direct role in the fixation process cannot be excluded (Stewart 1966).

Virtanen (1946, quoted by van Schreven 1958) stated that iron could be required for the synthesis of leghaemoglobin, and he further suggested that iron could be linked to the enzyme systems in the fixation process (Virtanen 1955). The recent work on the properties of the enzymes involved supports these views (see section I.2.1.2.c)

c. pH

The effect of pH may be threefold, i.e. the effect of acidity per se, the effect of a consequent deficiency of molybdenum, phosphorus, carbon dioxide and calcium, or, the consequent toxicity in soils with high levels of aluminium and manganese (Hallsworth 1958; Mulder, Lie, Dilz & Houwers 1966).

Acidity will influence the survival of the rhizobium. White clover rhizobium (R. trifolii) could tolerate a soil pH of 5.5 or less but that of lucerne (R. meliloti) preferred a pH of 6.0 or higher

(White 1966). At pH below 4 the growth of the host root (lucerne) could be restricted hence adversely affecting nodulation (Hallsworth 1958). However this may not apply to tropical legumes. Norris, (1956, 1959) cited evidence to support his hypothesis of the origin of the legume-rhizobium symbiosis, by showing that the tropical legumes (which are the more primitive forms) thrived in an acid soil. Munns (1968) and Lie (1969) found that acidity could specifically inhibit nodulation but the effect was reduced if the test plants were exposed for a short period immediately after inoculation to a nutrient solution of neutral reactions. Once the infection had taken place, nodule development and nitrogen fixation could proceed under the low pH.

Under increasing acidity the levels of molybdenum, phosphorus, calcium and carbon dioxide will decrease whereas those of aluminium and manganese will increase, resulting in adverse effects on the growth of the symbionts. These aspects of legume nutrition have been reviewed by Rorison (1958), Rorison, Sutton & Hallsworth (1958) and Mulder, Lie, Dilz & Houwers (1966).

d. Toxic chemicals

The adverse effects of toxic chemicals have been reviewed by Fletcher & Alcon (1958) and Masterton & Sherwood (1970).

Kerr & Klingman (1960) reported that dalapon at 6 lb per acre or less did not inhibit nodulation in birdsfoot trefoil. Chlorinated hydrocarbon insecticides (DDT, dieldrin and telodrin) at low levels did not affect nodulation in lucerne and soyabean, whereas endrin reduced the nodule size. However, 2, 4 -D and MCPA at 0.1 ppm were reported to reduce the amount of nitrogen fixed in lucerne even when the growth and nodulation were not apparently affected (Nilsson 1957, quoted by Fletcher and Alcon 1958). This could have been due to the fact that, under 2,4 -D, the rhizobia took the form of gram positive rods rather than the usual long rods (Payre & Fult 1947, quoted by Fletcher & Alcon 1958).

Antibiotics produced by soil fungi can cause nodulation failure in subterranean clover grown in the field (Holland & Parker 1966). Toxic effects of legume seed coats to rhizobia were demonstrated by Thompson (1960) and Bowen (1961). The toxins in the white clover seed coats were isolated and identified as myricetin and a tannin (or a mixture of tannins, Masterton & Sherwood 1970). Differences in the degree of toxicity between different legume seeds was noted by Masterton (1965, quoted by Masterton & Sherwood 1970).

3.4.3. Pest and disease aspects

White clover infected with clover phyllody virus was found to produce small, white and ineffective nodules (Joshi 1967). Eeelworm attacks on legumes could be quite serious especially in the tropics, losses up to 50% in nodulation were reported in a plot of *Phaseolus* by Masefield (1958). Competition for nutrients and damage to the root systems were some of the causes suggested. Masefield also noted that, in temperate regions, the nodules of peas, beans and lupins are attacked by the larvae of *Sitona* species.

CHAPTER II

EXPERIMENTAL

The experiment was carried out between 15th December 1969 and 29th March 1970 in a glasshouse at Massey University, Palmerston North, New Zealand (latitude 40° 30').

1. MATERIALS

1.1 Plants

The heterogeneous nature of the commercial New Zealand certified white clover (*Trifolium repens* L.), could give rise to morphologically different plants which would increase the range of variations. To reduce this possible source of variation a line as homogeneous as possible was therefore desirable. White clover strain C 1831, with 10% open pollination was kindly supplied by Dr. P.C. Barclay (D.S.I.R. Palmerston North).

From five separate weighings the average weight for one hundred seeds was found to be 0.053 g, giving an average value of 5.3 mg per seed. The percentage nitrogen of these seeds was later found to be 4.1%.

Prior to the commencement of the experiment, a germination test was conducted in a Copenhagen germinator. The temperature was set at 22° - 23°C. Average germination percentage was 74% on day 3 and 94% on day 7.

1.2 Rhizobium

Rhizobium strains C 5118 and C 514/1, (*Rhizobium trifolii* Dang.) with known effectiveness were kindly supplied by Mr. Greenwood (D.S.I.R. Palmerston North). A mixture of the two strains was recommended for the inoculation of the plants.

The cultures were received on 5th December 1969 and were subcultured regularly at fortnightly intervals on to nutrient slopes. The nutrient medium used for the rhizobial cultures is presented in Appendix II.1.

In the later stages, standard Bacto nutrient agar was used instead of the above mixture. Details of the inoculation procedures will be discussed under section 4.1 of this chapter.

1.3. Pumice

Fine grade sterilised pumice (97% within the range of 0.5 - 2.0 mm) obtained from a commercial firm was used. A week prior to the commencement of the experiment the pumice was resterilised, using an electric heat steriliser, heating to 185°F for four hours.

Approximately 1.3 - 1.4 kg of the sterilised pumice was used per pot, which after slight shaking left about one inch clearance between the top of the 'soil' surface to the edge of the pot.

1.4 Pots

Black plastic pots measuring 7" x 7" on top, 6" at the base and 7½" high were used. For drainage there was one basal hole and eight side slits; the slits alone were found to be adequate hence the basal hole was sealed with plastic tapes.

2. EXPERIMENTAL

2.1 Design

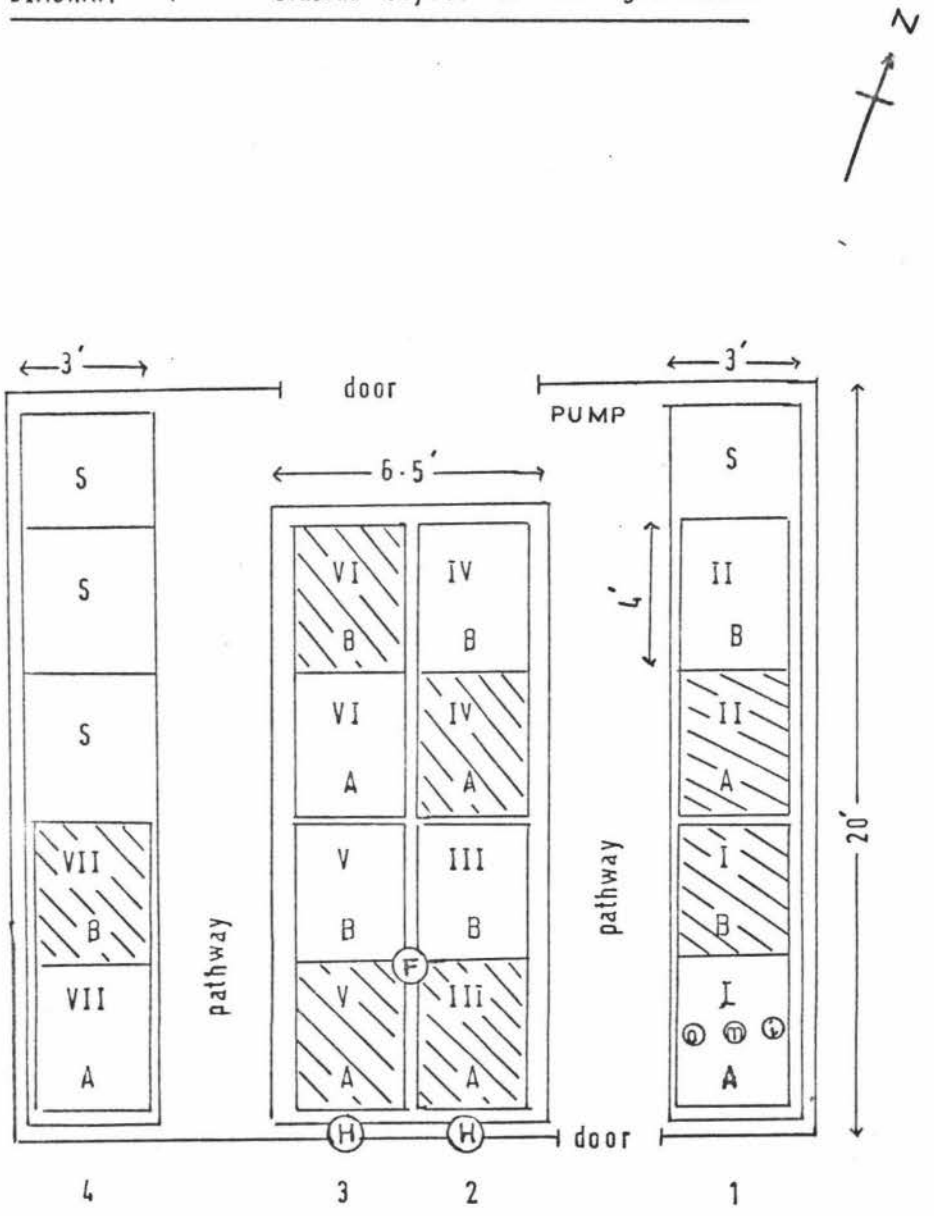
A randomised split-plot design was used, with two levels of light intensity (main plot) and two levels of defoliation. The plants were collected on six sequential harvest dates, which were 3, 6, 10, 15, 22 and 29 days from treatment. The sub-plot consisted of a single plant growing in one pot. There were seven replicates. Details of randomisation procedures will be discussed in section 2.3 of this chapter.

2.2 Layout

Two hundred pots were planted, the extras being used as spares for replacements. The general layout of the glasshouse is shown in Diagram 1. Each of the four benches in the glasshouse was divided into two sections, sections I to VII were the seven replicates, and the balance (marked 's' in Diagram 1) was used to hold the spare pots.

Polythene sheets were placed over the benches which were covered with about one inch of sand, all the pots sitting on inverted petri dishes, hence were not in direct contact with the polythene sheets. The pots were at least five inches away from each other, so reducing mutual shading and ensuring similar edge effects. The general layout of the pots is shown in Plates 1 and 2.

DIAGRAM 1 General layout of the glasshouse



Replicate number I, II, III VII

Plot number A & B

Bench number 1, 2, 3 & 4

Light treatment

Shade treatment

Fan (beneath benches 2 & 3) : (F)

Over head heater : (H)

Light meter reading positions : ⓘ = inner, ⓜ = mid & ⓞ = outer

Place for spare pots : S

2.3 Randomisation of the experiment

Within each replicate the main plot was randomly allocated to either the light or the shade treatments, and the twelve pots within the sub-plot were also randomly allocated to either the undefoliated or the defoliated treatments, such that there were six pots for each sub-plot treatment. The pots were numbered and later they were randomly selected for the six sequential harvest dates.

Prior to the commencement of the treatment all the plants received similar care and handling. From week 2 until the end of the experiment the pots were shifted every second day to a new position (pot 1 was moved to the position of pot 2, which was moved to that of pot 3.... and pot 12 was moved to the vacancy left by pot 1). This minimised localised effects like sun-streaks and differences in drip feed rates. Each time the pots were moved they were also rotated 90^{o} hence not only were their positions but also their directions changed regularly.

2.4 Treatments

2.4.1 Light

Two levels of intensity were imposed;

a) natural illumination under the glasshouse, which was approximately 26% of natural day light in the open, and

b) a lower intensity, using black screens which reduced the illumination to approximately 58% of that in (a).

The intensity was measured by a selenium sense cell light meter (Plate 3), and as the two sensors (head 1 and head 3) differed slightly in their sensitivity all readings were corrected accordingly. Figure II.1 shows the calibration of the two sensors against an Eppley pyrrheliometer (from Plant Physiology Division, D.S.I.R. Palmerston North).

During the course of the experiment five light readings were taken; for each reading head 1 was placed in a horizontal plane at ground level under the noon sun, and head 3 was placed in a horizontal plane level with the foliage. Within each plot, readings were taken from three different positions: 'inner', 'mid' and 'outer' positions in line with the pots along the bench and the mean of these readings was used for the intensity calculation. The positions are shown in Diagram 1.

Assuming there was no deterioration in the selenium cells the two levels of light intensity in terms of m.v. corrected to that of Eppley pyrrheliometer and expressed as percentage of natural illumination at noon on a sunny day are presented in Table II.1.

Figure II.1 Calibration of light meter reading against standard Eppley pyrheliometer

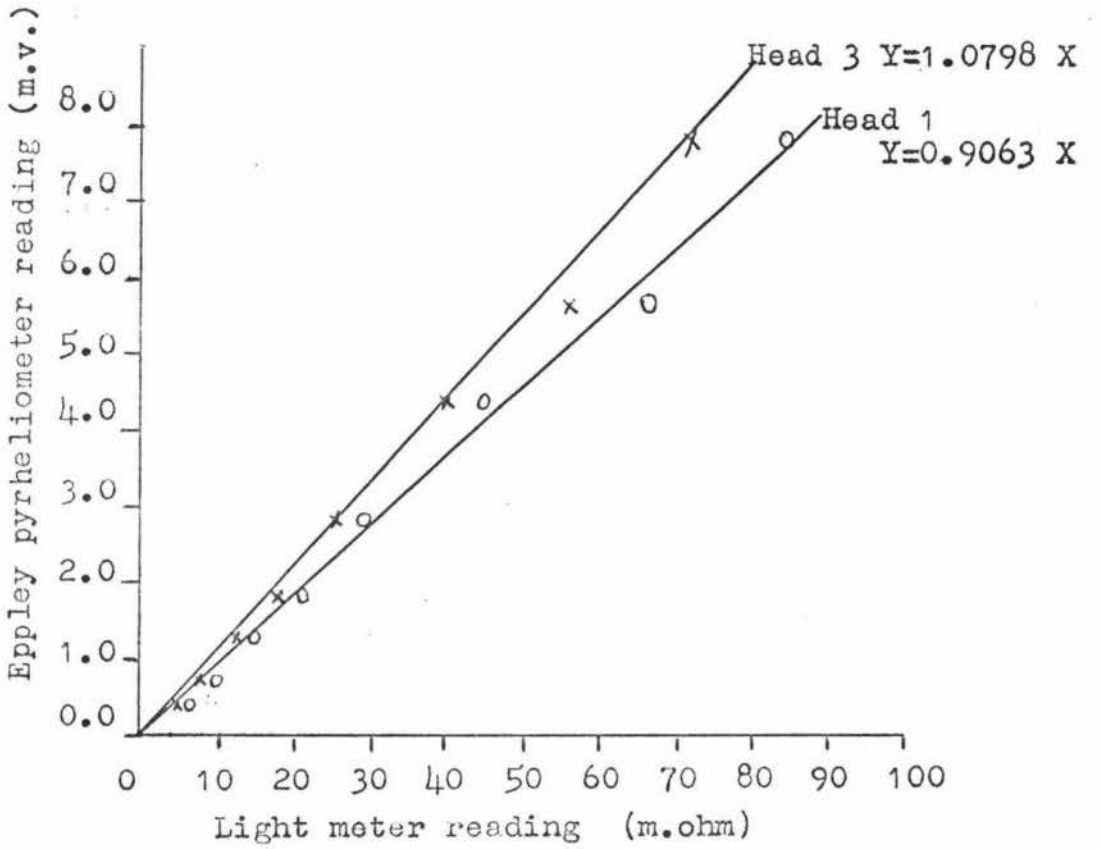


Figure II.2 Average weekly maximum, minimum and noon air temperatures, pot temperature, and relative humidity.

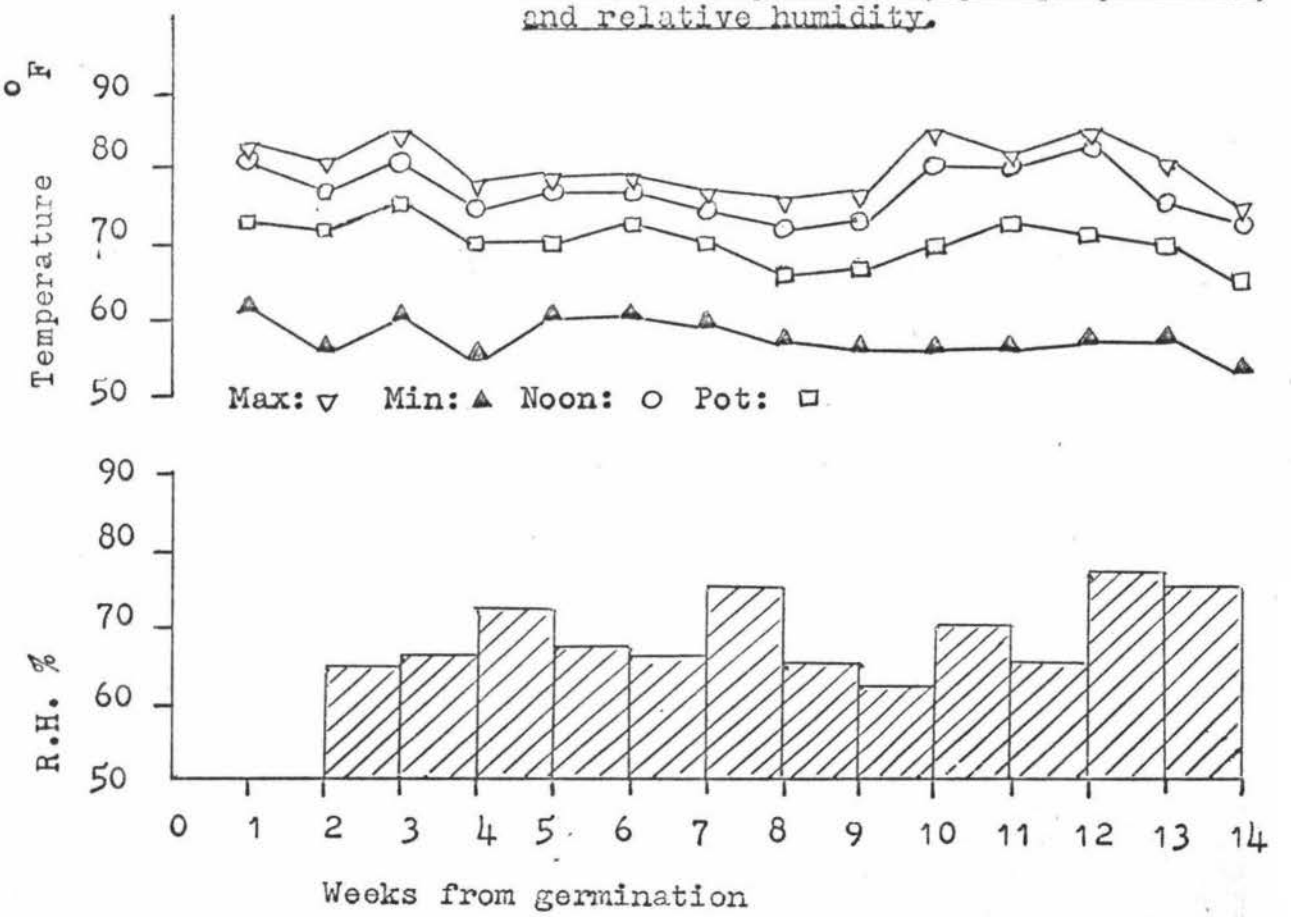


Table II.1 Light intensity readings (m.v.)

Block	Field	Under glass	% to field	Under shade	% to field	% in shade*
I	6.33	1.76	27.8	0.80	12.6	45.3
II	6.38	1.65	25.9	0.81	12.7	48.9
III	6.45	1.89	29.3	0.95	14.8	50.4
IV	6.46	1.66	25.7	1.24	19.2	74.8
V	6.48	1.61	24.9	0.84	13.0	52.4
VI	6.54	1.64	25.1	1.18	18.0	71.8
VII	6.51	1.45	22.3	0.90	13.8	61.9
Average	6.45	1.67	25.8	0.96	14.9	57.9

* Transmission through black screen

Readings were taken between 11.30 a.m. and 12.30 p.m. on a 'cloudless' day.

2.4.2 Defoliation

Two levels of defoliation were imposed, uncut and cut. In the cut treatment, all leaves above 0.3 on the Carlson scale (Carlson 1966a), were removed by cutting off at the base of the petiole. Appendix II.3 illustrates the Carlson scale.

Although the cut treatment was severe, in a preliminary trial all the test plants recovered remarkably well; furthermore this made the treatment more meaningful in terms of quantity of photosynthetic tissue left on the plant for regrowth.

3. PREPARATION FOR EXPERIMENT

3.1 Plant nutrients

The composition of the nitrogen free nutrient solution is presented in Appendix II.2. During the later part of the experiment because of an iron deficiency symptom (detailed in section 4.3.2 of this chapter), a 0.1 M sodium ironetate salt (sequestric acid ferric sodium salt) at 20 ml per litre of nutrient solution was included in the nutrient every second day.

Since the availability of both nutrients (Hallsworth 1958) and

water (Masefield 1958) can influence the process of nodulation, a system of intermittent drip feeding was devised. A system of tubings was set up, part of which can be seen in Plate 2; a diaphragm pump was used to pump the nutrient solution into the reservoir which was a twenty-litre-capacity plastic container on the top of the roof of the glasshouse. A series of tubings with decreasing diameter linked the reservoir to the outlets. Each set of outlets could feed six pots simultaneously. The rate of drip feeding was regulated by a screw clip which could be adjusted to maintain a reasonably uniform rate. The actual flow rate and quantity of nutrient used are discussed under section 4.3.1 of this chapter. The whole system was painted with two layers of paint, one of black which prevented algal growth inside the tubes, and one of silver which reflected some of the light and helped to keep the tubings cool.

3.2 Temperature and humidity control

To keep the glasshouse cool during the summer, corrective measures including spraying the glasshouse roof with white paint, watering the concrete pathway in the glasshouse and removing the insect screens at the side of the glasshouse were carried out. In addition a car fan driven by a 1 h.p. A.C. motor was installed underneath one of the benches and thermostatically controlled at 70°F. A jet of fine water was placed in front of the fan so enhancing cooling and producing an acceptable level of relative humidity (60 - 75%). On exceptionally hot days a sprinkler was placed on the roof top which also helped to reduce the air temperature within the glasshouse. The average weekly maximum and minimum temperatures are presented in Figure II.2; only on two days, both for a short period, did the air temperature in the glasshouse rise to 92°F.

A layer of sisalcraft was stapled on to the pots which partly reflected the light away. The average pot temperature ranged from 64° - 77° F, while on three occasions only, the temperature reached 82° F. Another sheet of sisalcraft was placed on the top of the pumice; this had an additional function of preventing algal growth on the top of the pumice.

3.3 Light

No attempt was made to increase the light intensity within the glasshouse. Due to reasonably successful temperature control, the white paint on the roof was thinned slightly. This helped to increase the illumination in the glasshouse. However some etiolation of the

petioles were observed, and the leaves were paler than those from field grown plants.

4. GROWING OF EXPERIMENTAL PLANTS

4.1 Germination and inoculation

On 14th December 1969 a suspension of the two rhizobium cultures* was used to soak the scarified seeds overnight. Only large seeds were selected for germination in the Copenhagen germinator. After twenty four hours all the seeds showing radicles were selected and in each pot four such germinating seeds were planted. Seedlings with roots longer than 2 mm were not used so as to avoid possible damage to the root system.

To ensure an adequate supply of Rhizobium in the pumice, five further inoculations were given, one during planting and four more at weekly intervals until the plants were four weeks old. In all cases 5 ml of a diluted inoculant mixture made from one week old cultures was given to each pot. From plate count determinations, the average number of bacteria per McCartney bottle was found to be about 10^{14}, this was diluted to 200 ml therefore each 5 ml lot would have approximately 2.5×10^{12} cells.

(* 20 ml of the suspension were made from four bottles (two from each strain) each of which was shaken with 5 ml of distilled water.)

4.2 Methods of planting, thinning and culling for evenness of size

All germinating seeds were picked up by touching them with a needle, and planted about quarter inch deep into the pumice. The four seeds were planted approximately one and a half inches apart and the seedlings were observed within forty-eight hours after planting.

Seedlings coming through after the fourth day were discarded. At the end of week 1 (22nd December 1969) all the pots were thinned to three plants per pot, and by week 2 (29th December 1969) when the monofoliate leaf was opened, they were thinned to two plants per pot. Thinning was done by digging the roots up and making sure no broken root was left in the pumice by chekcing whether the root cap was still intact. All transplanted seedlings were avoided as transplanting could damage the root system. When the first trifoliate leaf was fully opened the pot was thinned to a single plant. This was completed at week 3.

Until week 3 culling was done within each pot and later, plants with similar sizes were grouped together into different blocks. Block II, IV and VI had larger plants than I, III and V, whereas block VII had the smallest of all. The selection was from a pool of two hundred plants. This grouping would definitely increase the significant difference between replicates in the analysis of variance but within each replicate the variation would be reduced. After week 8 (9th February 1970) no more regrouping was done.

4.3 Care of plants during experiment

4.3.1 Nutrient supply

During week 1 the plants were given 10 ml of quarter strength nutrient solution by hand three times per day (9.00 a.m., 1.00 p.m. and 5.00 p.m.). This was increased to half strength at week 2, but by the end of week 2 some leaves were observed to be rather pale. Since this could have been due to exhaustion of the seed nitrogen, low light intensity or inadequate nutrient strength or a combination of factors, it was decided to give the plants full nutrient strength as from week 3 at four times per day (9.00 a.m., 11.00 a.m., 2.00 p.m., and 5.00 p.m.). The colour of the leaves darkened in about three days.

Drip feeding started at the end of week 3, three times daily (9.00 a.m., 1.00 p.m. and 5.00 p.m.). Each time 10 litres of full strength nutrient were pumped into the reservoir. From a number of collections made at randomly selected outlets, an average of 60 ml per pot was collected, the range was 40 - 70 ml. The drip lasted for about ten minutes each watering. On every third day 20 litres of water were run through the system so flushing the rooting medium of accumulating salts. The nutrient solution fell onto a glass slide in the pot (see Plate 4 for details), which facilitated even spreading. Some slight scorching was observed on leaves that had come into direct contact with the nutrient solution.

4.3.2 Deficiency symptom

On week 4 a deficiency symptom was observed on a few leaves which had typically interveinal yellowing with veins dark green. Iron deficiency was suspected since iron deficiency had been commonly reported in glasshouse experiments even when the nutrient contained an adequate quantity of iron (Hewitt 1952). Consequently an iron

supplement (sodium ironetate salt) was given during one of the routine feedings every second day. The deficiency symptom disappeared in about a week.

However the general paleness of some of the leaves coupled with slightly etiolated petioles were still evident on week 6. This was attributed to the low light intensity inside the glasshouse.

4.3.3 Insect damage

Clover mites (Bryobia spp.) were observed on some plants near to the edge of the glasshouse at week 5. Malathion at the rate of one tablespoon per gallon was used to spray the plants. This provided satisfactory control. Thereafter three more sprays on week 7, 9, and 13 were given.

5. CONDUCT OF THE ACTUAL EXPERIMENT

Treatment started at week 10 (23rd February 1970) when the average leaf area of the plants was 182 cm^2. For the light treatments the shades (as shown in Plate 1), were put into place on the night of 22nd February 1970. These shades were removed at the end of the experiment. On the morning of 23rd February 1970, all the plants allocated to the 'cut' treatment were defoliated. Plate 5 shows the quantity of foliage left as compared with the 'uncut' treatment, and Plate 6 shows the regrowth at harvest 1 (day 3). No further defoliation was imposed.

As shown in the calendar of work (Appendix II. 4), harvest dates were on 3, 6, 10, 15, 22 and 29 days after treatment. An increasing harvesting interval system was adopted instead of an equal interval, because the shorter intervals immediately after the treatment would help to detect more accurately the plant responses to the treatments.

6. PREPARATION OF MATERIAL AND METHOD OF ANALYSIS

6.1 General

Due to the time factor in the acetylene reduction test (the nodules on the washed roots could lose their activities if left for too long) replicates I, II, III and IV were done in the morning and replicates V, VI and VII were done in the afternoon. Leaf area and leaf number were taken the evening before the harvest.

Pumice was very carefully washed from the roots. It was found that the best way was to direct a not-too-strong jet of water into the pot until the nozzle reached the bottom of the pot, so flushing out the

pumice. The plant was lifted out and the remainder of the pumice washed off by dipping the roots into a bucket of water and shaking gently. Pumice particles still attached to the roots were removed during nodule counting.

After cutting the roots into inch segments, the first and the second segments were used in the acetylene reduction test. Later, nodule counts were taken on all the segments, after which the tops, roots and nodules were placed in the oven for dry weight determinations.

6.2 Pumice and leachate

Samples of pumice and leachate were collected for nitrogen determination. A small volume of pumice was collected from the centre of the pot. It was oven dried (85° - 95° C) and stored in a desiccator. The leachate was collected by leaching the pot with 150 ml of the nutrient solution one hour prior to washing of the plants. The leachate was stored in the refrigerator with 1 ml of a 1% toluene.

6.3 Leaf area and leaf number

Leaf area estimation was based on the photosynthetic standards of Williams, Evans & Ludwig (1964). The leaf area standards were cut out and pasted onto a stick and the ratings determined by placing the leaf over the appropriate standard. The ratings and their respective areas are presented in Appendix II.5. Leaf numbers were calculated from the sum of the individual ratings.

6.4 Dry weights

Dry weights of tops, roots and nodules were taken after drying the material in the oven at 85° - 95° C for twenty-four hours. The relative growth rates were calculated as shown in Appendix II.6.

6.5 Percentage nitrogen determination

For all plant material a micro-Kjeldahl digestion unit was used to determine the nitrogen percentage. Each sample consisted of 0.28 gm of oven dried material; however for nodules the entire tissue mass was used. A macro-Kjeldahl digestion unit was also used for the pumice and the leachate. Appendix II.7 summarises the details of the Kjeldahl digestion procedures.

6.6 Nodules

6.6.1 Nodulation

A. General

Nodulation can be recorded either in terms of number of nodules which expresses the degree of host susceptibility to infection or the weight or size of these nodules which if considered with their duration, expresses the degree of nodule effectiveness. The number of nodules was recorded under 'size' and 'colour' categories. In addition nodule density per inch segment of the roots was also recorded.

B. Nodule density

During the preliminary investigation three possible ways of examining the pattern of nodule distribution were considered. Firstly, to identify each root either as the primary or the secondary root and to classify the nodules accordingly so giving the relative densities of the primary and secondary nodules. Secondly, the roots and nodules could be examined in situ; this would represent a spatial distribution. Thirdly, by cutting the roots (both tap and crown roots) into inch segments. The nodules from each inch-segment represent the nodule density within the segment as measured from the base of the plant. Where stolon roots were found they were grouped separately.

After considering the number of plants per harvest (twenty-eight plants) and their sizes, the inch segment method was used. Appendix II.8 shows the numbering of the inch segments and the spaces they represent.

C. Size and colour

The nodules were grouped under three size categories:

a) Large = over 3 mm (measured along the longest axis)

b) Medium = 1 - 3 mm

c) Small = under 1 mm

In each size category there were two colour categories:

i) Pink = less than half of the nodule was green,

ii) Green = more than half of the nodule was green, it also included brown or dead nodules.

The sizes were measured with the aid of a illuminated magna viewer; later, standards were cut out and used. The colour was intensified by passing a stream of coal gas over the nodules for ten

minutes, the carbon monoxide in the coal gas converted the leghaemoglobin into carboxyhaemoglobin and so intensifying the colour (per. comm. A.G. Robertson). Plates 7 and 8 illustrate some of the 'pink' and 'green' nodules. As it was difficult to determine the exact number of nodules in the small size group (under 1 mm) the counts were taken to the nearest 10, and all the countings were done under a five inch magnifying glass.

D. Fresh and dry weights

For each segment the fresh weights of the nodules were taken after drying the nodules several times with blotting paper. The readings were taken to the nearest milligram. In the case of the nodules with sizes under 1 mm the average weight for ten such nodules was determined from a separate exercise, in which five weighings of 50 nodules each were taken giving an average of 2.58 m. gm per ten nodules. This was rounded up to 3 m. gm per ten nodules. The dry weights of 100 small (under 1 mm) nodules were found to be 2 mg., hence nodule dry weight per plant was adjusted accordingly.

6.6.2 Nitrogen fixation

The rate of nitrogen fixation was evaluated using three separate methods.

The first was the commonly used method of dividing the total plant nitrogen yield (after taking away the control nitrogen e.g. from seed and soil) by the weight of the nodules and expressing the result as a daily rate according to the plant age (Wilson 1940). In this method the rate of nitrogen fixation was assumed to be constant and that there were no losses either of plant material or nitrogen from the system. The nitrogen yield was determined by Kjeldahl digestion assay.

The second method was based upon the suggestion by Döbereiner (1966) who compared the efficiency of nitrogen fixation by the nodules from a regression of $\log_{10}$ total plant nitrogen with the nodule weight. Using the equation of linear regression $Y = a + bX$, in which 'Y' represented the logarithm of total plant nitrogen, 'X' the corresponding nodule weight and 'a' the point on the Y - axis where no nodules were formed i.e. $\log_{10}$ weight of the nitrogen from seed or soil, he reasoned that the amount of nitrogen fixed (NF) = Y - a,

i.e.,

$Y = NF + a$

and $Y = a + bX$

$NF = bX$, where b was the slope of the

regression line (regression coefficient), and could be used as an efficiency index for nitrogen fixation. This efficiency index was constant for each species and was independent of the environmental factors. However, Whiteman (1970b) pointed out that this linear relationship would only hold when both the plant and nodule weights were increasing in parallel, and would become curvilinear when nodule sloughing commenced.

The third method was the acetylene reduction assay. Koch & Evans (1967) and Hardy and his colleagues (1968) first reported a more precise method for determining the nitrogen fixing capabilities by measuring the rate of reduction of acetylene (C_2H_2) to ethylene (C_2H_4) by the nitrogenase enzyme system. The reaction being specific, it can be used to measure the instantaneous activity of the nitrogenase system, in contrast to the other two methods which measure the integrated effects of nitrogen fixation over a certain time period.

The information on plant nitrogen content required for the calculation of the first two methods was determined by the micro-Kjeldahl digestion mentioned in section 6.5 of this chapter. Details of the acetylene reduction assay is presented in Appendix II.9.

7. STATISTICAL METHODS

7.1 Analysis of variance

A standard form of analysis of variance for split-plot was used (Snedecor & Cochran 1967 pp 369).

In this thesis for each variable only the mean squares for the six harvests are presented in a summarised form. A computer program was written for the calculation of the split-plot analysis of variance which is detailed in Appendix 2.10.

A further form of analysis of variance based upon a split-split-plot design, using the harvest dates as the additional sub-sub-plot treatment, was used to analyse some variables as required. A modified computer program obtained from the Applied Mathematics Department (coded as ANNA) was used for the actual calculation.

7.2 Transformation of data and decoding

In parameters where the standard deviation in the original scale varies directly as the mean, the values are transformed into logarithms so as to stabilize the variance. Similarly, in counts e.g., nodule numbers, where the variance is proportional to the mean, a square root

transformation is used to stabilize the variance (Snedecor & Cochran 1967). To avoid the problem of transforming very small values (other than zero) the data has been transformed as X x 100, where X is the original value. In the graphs the data has been presented as $\log_{10}$ X or square root X. Where the data has been transformed, minimal amount of decoding is used.

7.2 Coefficient of variation

The calculation of coefficient of variation (C.V.) is as follows:

C.V. = (sq rt (sub-plot error)/sub-plot mean) x 100%

For $\log_{10}$ transformed data, the procedure is according to Snedecor & Cochran (1967 pp 330)

7.3 Regression analysis

Except for those regressions in the acetylene reduction assay all other regression fittings and comparisons between regression lines were carried out on the general statistical program (written jointly by Dr. F. Cockram and Professor R. Munford coded as STATC using the IBM 1600 computer).

CHAPTER III RESULTS

For ease of discussion the results have been grouped under three sections ; 1). plant growth, 2). nodulation and 3). nitrogen fixation.

1. PLANT GROWTH

In this section results from leaf and plant dry weight measurements (top and root only) are presented.

1.1 Leaf measurements

The summaries of the within-harvest analysis of variance for total leaf area per plant, total number of leaves per plant and average area per leaf are presented in Appendix III. 1.1. The highly significant differences between the means of the 'undefoliated' and 'defoliated' plants need no explanation, hence only results of the 'light' treatment are discussed in the text.

1.1.1 Light treatment

Table III. 1.1 presents the mean total leaf area and number per plant. With the exception of leaf number, after three weeks (harvest 5), there was no indication of significant effects on these characters due to shading (see section II. 2.5.1 for details of light intensity levels).

The average leaf size in cm^2 has been plotted against time and is shown in Figure III. 1.1. Average leaf size increased with time and although there was a significant increase in leaf size of the shade-uncut (S/U) plants (a similar trend in the shade-cut (S/C) plants was statistically non-significant), the difference was partly due to a reduction in leaf number under shading. This probably resulted in the significant light x cutting (L x C) interaction at harvests 3, 5 and 6 (Appendix III. 1.1.c.)

1.2 Plant dry weight measurements

The expression 'relative growth rate' (RGR) in mg/mg/day (for details of calculation see Appendix II.2) has been used to describe the dry weight changes. The weight data were transformed using $\log_{10}$ (X x 100) prior to analysis of variance and the summary is presented in Appendix III. 1.2. Table III. 1.2 shows the means of the top and root dry weights. Figure III. 1.2.1 and Figure III. 1.2.2 illustrate the RGR of the dry weights under shading and defoliation

respectively. The analyses of variance for RGR measurements are presented in Appendix III. 1.4.

1.2.1 Light treatment

Although the mean top and root dry weights in the 'shade' plants were generally lower than those of the 'light' plants, statistically significant differences were detected only in harvest 5 ($P < 0.05$) in the top and harvest 5 ($P < 0.01$) and harvest 6 ($P < 0.01$) in the root (Table III. 1.2). The top and root dry weights increased gradually over the experimental period, but the top increased at a decreasing rate (Figure III. 1.2.1.a), whereas the reverse situation occurred for the root (Figure III. 1.2.1.b). Both the top and root RGR of the 'shade' plants were lower than those of the 'light' plants, although the differences were not statistically significant.

1.2.2 Defoliation treatment

The top dry weight increased gradually over the experimental period but at a decreasing rate (Figure III. 1.2.2.a). The 'defoliated' plants had a slightly higher but statistically non-significant RGR than those of the 'undefoliated' plants. For approximately 15 days the roots of the 'defoliated' plants had a lower RGR than those of the control 'undefoliated' plants, a statistically significant difference being detected at day 10. By day 22 the RGR of the 'defoliated' plants (root dry weights) was similar to that of the 'undefoliated' control (Figure III. 1.2.2.b).

1.2.3 Light x Cutting interaction (L x C)

The different responses to shading by the 'undefoliated' and 'defoliated' plants caused a significant L x C interaction for both top and root dry weights at several harvests (Appendix III. 1.2).

1.3 Top to root ratio (T/R)

The T/R ratio was analysed using the untransformed values and the result presented in Appendix III. 1.3.

1.3.1 Light treatment

Overall, shading had no significant effect on the T/R ratio but there was a uniform increase in the ratio up to harvest 4 in the 'shade' plants whereas in the 'light' plants the rate became slower after the 2nd harvest (Table III. 1.3).

1.3.2 Defoliation treatment

As expected, defoliation caused a gross reduction in T/R ratio over the entire experimental period (Table III. 1.3) and like that of the 'shade' plants, the 'defoliated' plants had a more uniform rate of increase in the ratio up to harvest 4 whereas in the 'undefoliated' plants the ratio levelled after harvest 2.

1.3.3 Light x Cutting interaction

There was a tendency (significant at harvest 5 ($P < 0.05$)) for shading to have different effects on the T/R ratio, depending on the defoliation treatment. Thus shading tended to aggravate the drastic effects due to defoliation, whereas with undefoliated plants, shading decreased root growth relatively more than it decreased top growth (Table III. 1.2) causing an apparent increase in T/R ratio (Table III. 1.3).

Table III.1.1 Leaf measurements

a).

Log_{10} leaf area per plant (cm^2)

Harvest	1(3)#	2(6)	3(10)	4(15)	5(22)	6(29)
Treatment						
Light	1.9834	2.1188	2.3861	2.4687	2.6969	2.7665
Shade	1.9478	2.1542	2.3595	2.4713	2.6325	2.7828
	ns	ns	ns	ns	ns	ns +

b).

Sqrt leaf number per plant ++

Light	5.41	5.95	7.06	8.34	10.37	11.01
Shade	5.32	5.87	7.03	8.22	8.78	10.03
	ns	ns	ns	ns	*	ns

Figures in parenthesis are days from treatment

+ For all results in the text

** = P<0.01 ; * = P<0.05 ; ns = not significant

++ Sqrt = square root

Figure III.1.1 Average leaf size (L x C interaction)

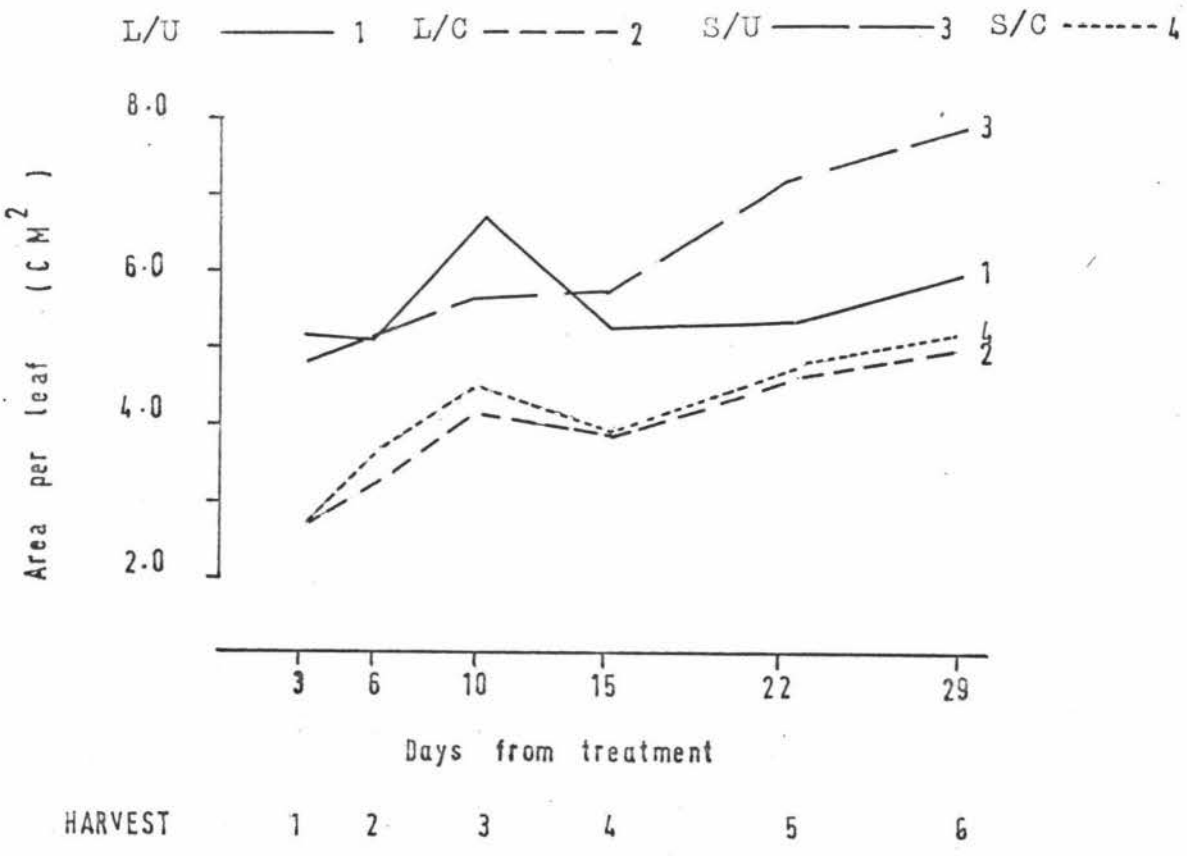


Table III.1.2 Dry weight measurements

a).

Log_{10} top dry weights (mg)

Harvest	1(3)	2(6)	3(10)	4(15)	5(22)	6(29)
Treatment						
Light	2.9582	3.0468	3.2308	3.3469	3.5858	3.6035
Shade	2.9312	3.0045	3.1223	3.2804	3.4043	3.5150
	ns	ns	ns	ns	*	ns
Uncut	3.2403	3.2996	3.4573	3.5690	3.7694	3.7955
Cut	2.6490	2.7517	2.8958	3.0584	3.2208	3.3230
	**	**	**	**	**	**

b).

Log_{10} root dry weights (mg)

Light	2.5280	2.4839	2.5920	2.6031	2.7989	2.8493
Shade	2.4766	2.4577	2.4724	2.5402	2.6587	2.7094
	ns	ns	ns	ns	ns	ns
Uncut	2.5869	2.5357	2.6536	2.7137	2.8949	2.9037
Cut	2.4177	2.4059	2.4107	2.4296	2.5628	2.6550
	**	ns	**	**	**	**

Table III.1.3 Untransformed means of top to root ratio

Harvest	1(3)	2(6)	3(10)	4(15)	5(22)	6(29)
Treatment						
Light	3.13	4.70	4.71	5.66	6.27	6.12
Shade	3.15	3.86	4.82	6.07	5.95	6.70
	ns	ns	ns	ns	ns	ns
Uncut	4.53	6.30	6.44	7.42	7.52	7.87
Cut	1.74	2.26	3.10	4.31	4.70	4.95
	**	**	**	**	**	**

FIGURE III.1.2.1 DRY WEIGHT MEASUREMENTS

(light treatment)

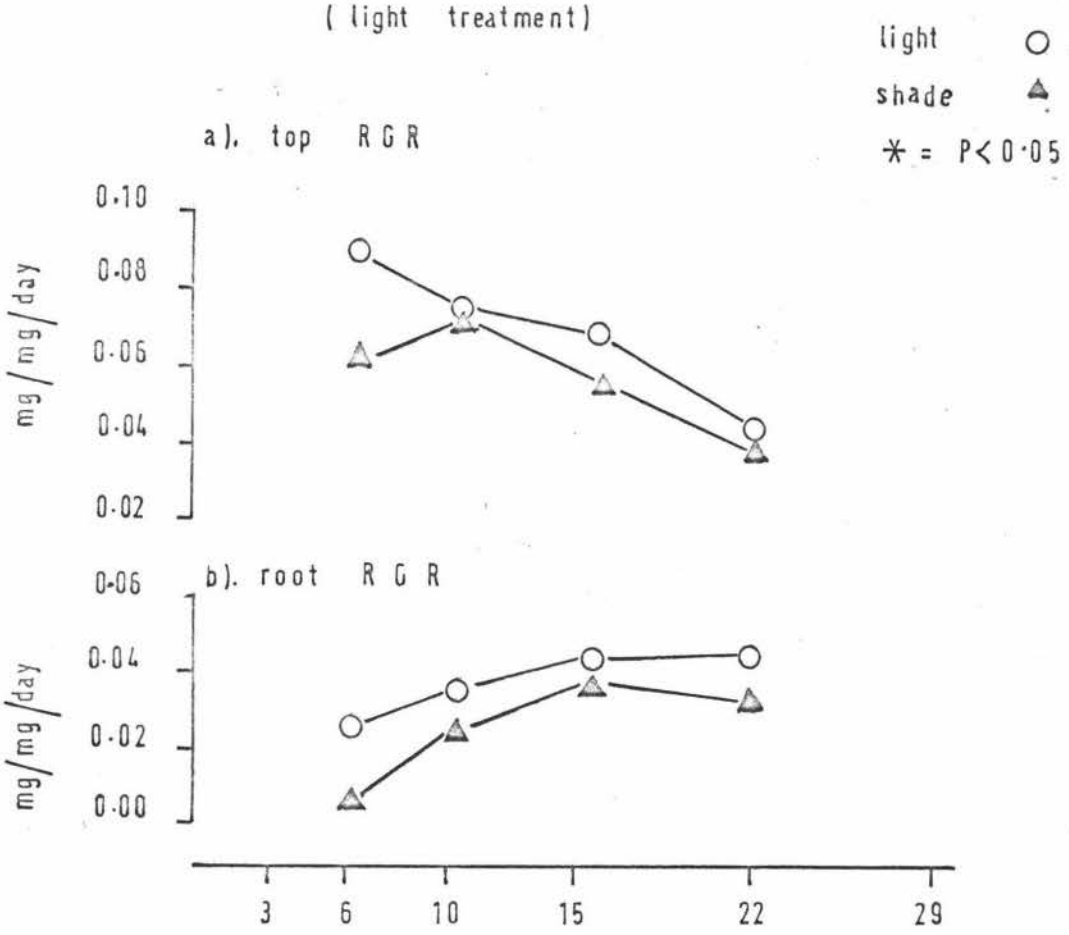
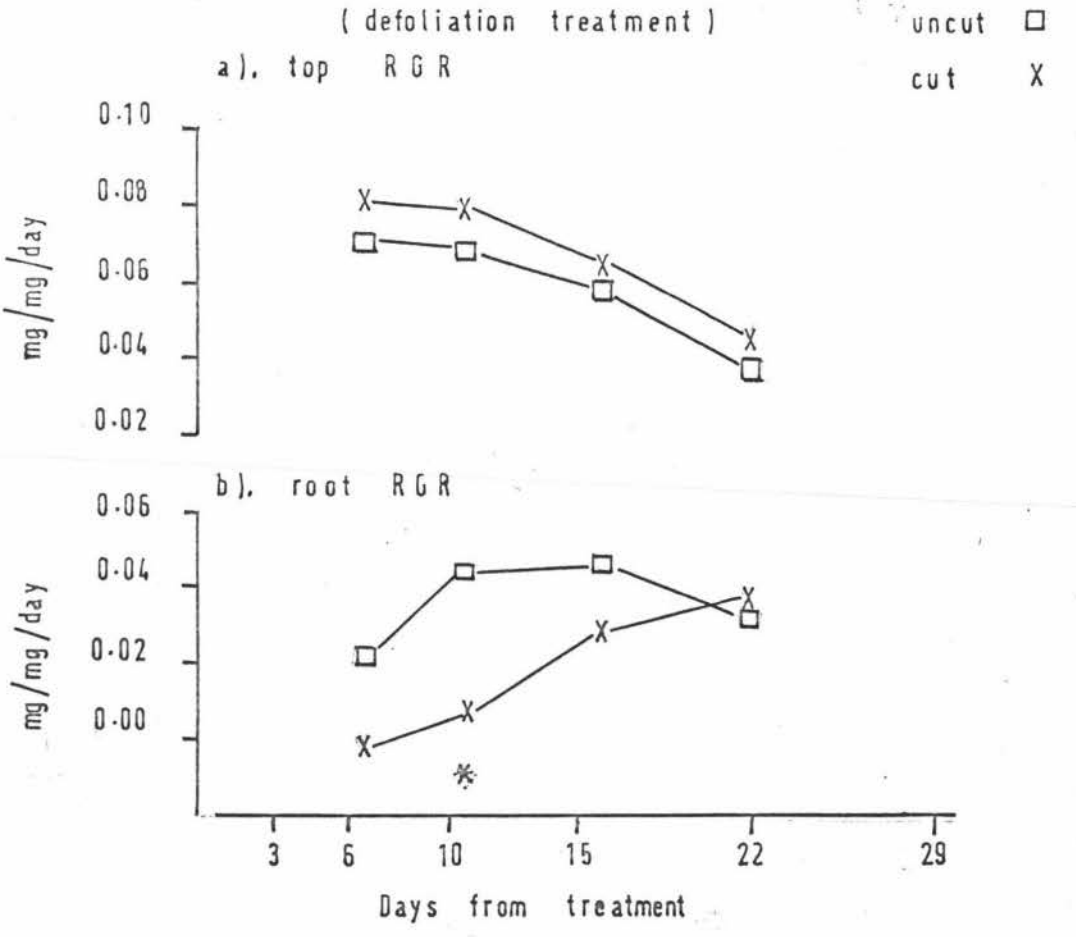


FIGURE III.1.2.2 DRY WEIGHT MEASUREMENT

(defoliation treatment)



HARVEST 1 2 3 4 5 6

2. NODULATION

The nodulation data are presented in terms of nodule number, nodule weight and average weight per nodule on a 'per plant' basis and for the former two components also on a 'per inch' basis. In addition, the number of 'pink' and 'green' nodules have been grouped on a 'per inch' basis.

2.1 Nodule numbers

2.1.1 Nodule numbers per plant

These data were transformed (sqrt (X x 100)) for the analysis of variance. The summary of the analysis is presented in Appendix III. 2.1, and only the transformed (Sqrt X) means are presented in the text.

2.1.1.1 Light treatment

The total nodule number of both 'light' and 'shade' plants increased steadily over the experimental period (Figure III. 2.1.a) the increase being due mainly to nodules in the medium category (Figure. III. 2.1.c). With time, there was a marked increase (from approximately 29% at harvest 1 to 55% at harvest 6 in the light-uncut plants) in the proportion of medium nodules, whereas both numbers of small and large nodules were reduced with time (small nodules decreased from 52% to 39% and that of the large nodules decreased from 17% to 6% for the same time period).

The effect of shading on nodule numbers became apparent from harvest 3 (10 days after commencement of treatment), with a reduction in the numbers of large and medium but not in the number of small nodules.

2.1.1.2 Defoliation treatment

Although the total number of nodules in the 'undefoliated' and the 'defoliated' plants increased with time, defoliation caused a gross reduction in nodule numbers in all categories (Figure III. 2.2). Apart from the absolute loss of nodule number which was apparent even at harvest 1, and a much lower rate of medium nodule formation in the 'defoliated' plants at harvest 4 onwards, the overall pattern in the changes of nodule number for all categories was roughly similar to that described for the light treatment.

2.1.1.3 Light x cutting interaction

No significant L x C interactions were detected for total, medium and small nodule numbers. However there was a significant ($P < 0.05$) interaction at harvest 3 for the large nodule numbers. This could have been caused by a delay for about a week in nodule number reduction in the shade-cut plants. Figure III. 2.2 illustrates the interaction, from which another interesting point emerges. Although there was a gross reduction in the number of large nodules under the light-cut regime (which was evident in less than 3 days from defoliation), the number remained relatively stable with time; whereas under the shade-cut regime there was a drastic and more gradual reduction in the number of large nodules 10 days after the commencement of the treatment.

2.1.2 Nodule number per inch

Nodule distribution patterns for the four treatments are shown in Figure III. 2.4. Three interesting points emerged from these three-dimensional graphs:

1) At the early stages, the number of nodules was highest immediately below the crown, but as the plant grew the highest density (number) shifted towards a region 3 - 6 inches away from the crown. The majority of these nodules were on the crown roots or on laterals arisen from the region near the crown.

2) In the control (light-uncut) plants there were two distinct 'steps' occurring between harvests 2 & 3 and harvests 4 & 5 (these trends can also be seen in Figures III. 2.1 and III. 2.2). However in the shade-uncut plants there was only a single 'step' at harvest 6, and for the defoliated plants the step-wise increments were less distinct.

3) The step-wise trends observed in the light-uncut plants occurred along the entire root zone possessing nodules.

2.1.3 Number of 'pink' and 'green' nodules

The number of 'pink' and 'green' nodules recorded on a per inch basis is shown in Figure III. 2.5.

In the control (light-uncut) plants the nodules were all pink from harvest 1 to harvest 4, and only a few 'green' nodules appeared in harvests 5 & 6. A similar picture was found for the shade-uncut plants. However in the defoliated treatments (i.e. light-cut & shade-cut) the nodules were all green at harvests 1 and 2, but became slightly 'pink' in harvest 3 (these were still classified as 'green' according to

the criterion adopted, see section II. 6.6.1.c). Plates 7 & 8 show the typical nodule colours from harvests 1 and 3 respectively.

At harvest 4, a large number of nodules in all size categories in the defoliated plants showed signs of browning, many with a darker 'ring' at the apex. Some empty nodule 'hulls' were also found. These are clearly shown in Plates 9 & 10. Some nodules presumably had undergone autolysis, and where the nodule had apparently been sloughed off fragments of the nodule 'hull' were occasionally found still attached to the root. This occurred near the crown (i.e. 1st inch segment as shown in Plate 9), as well as on the lateral roots further down the inch segment (that shown in Plate 10 came from the 4th inch segment). In addition, empty 'hulls' were observed from all three size categories, although those from the large grouping were more prominent.

By harvest 5 most of the surviving nodules in the defoliated plants were more than half 'pink' hence were classed under 'pink', the pink region was inevitably at the apex of the nodule (see Plate 10 for example). With time the new growth (pink region) increased in proportion hence when it exceeded half of the nodule length, the nodule was classified as 'pink'. By harvest 6 all the nodules were pink.

In summary, severe defoliation caused immediate (less than 3 days) gross changes in nodule colour, associated with visible loss of some nodules. The 'recovery' of the surviving nodules was completed in 3 weeks.

FIGURE III.2.1 MODULE NUMBER (light treatmen)

light = ○; shade = △ ; * = P< 0·05 ; ** = P< 0·01

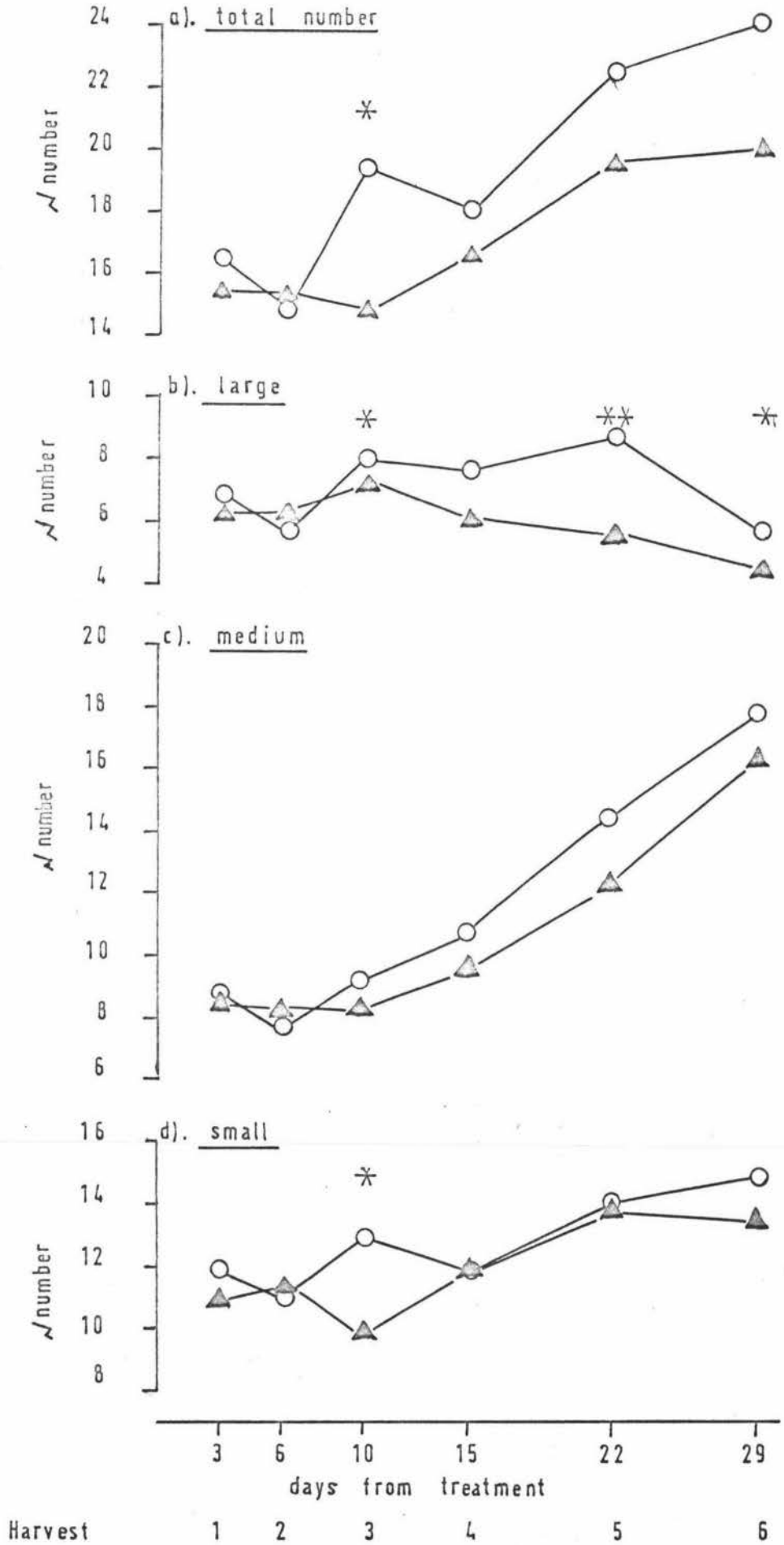


FIGURE III.2.2 MODULE NUMBER (defoliation treatment)

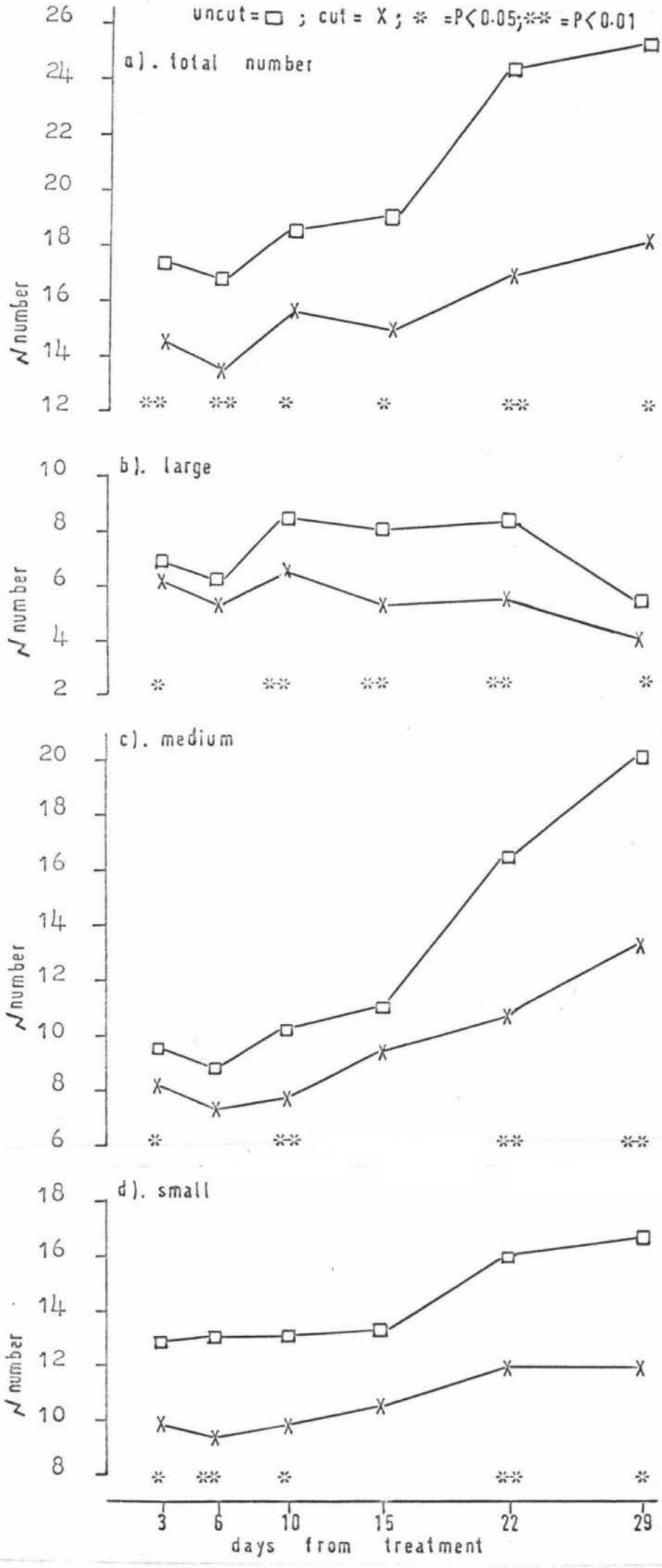


FIGURE III.2.3 Large nodules

Light x cutting interaction

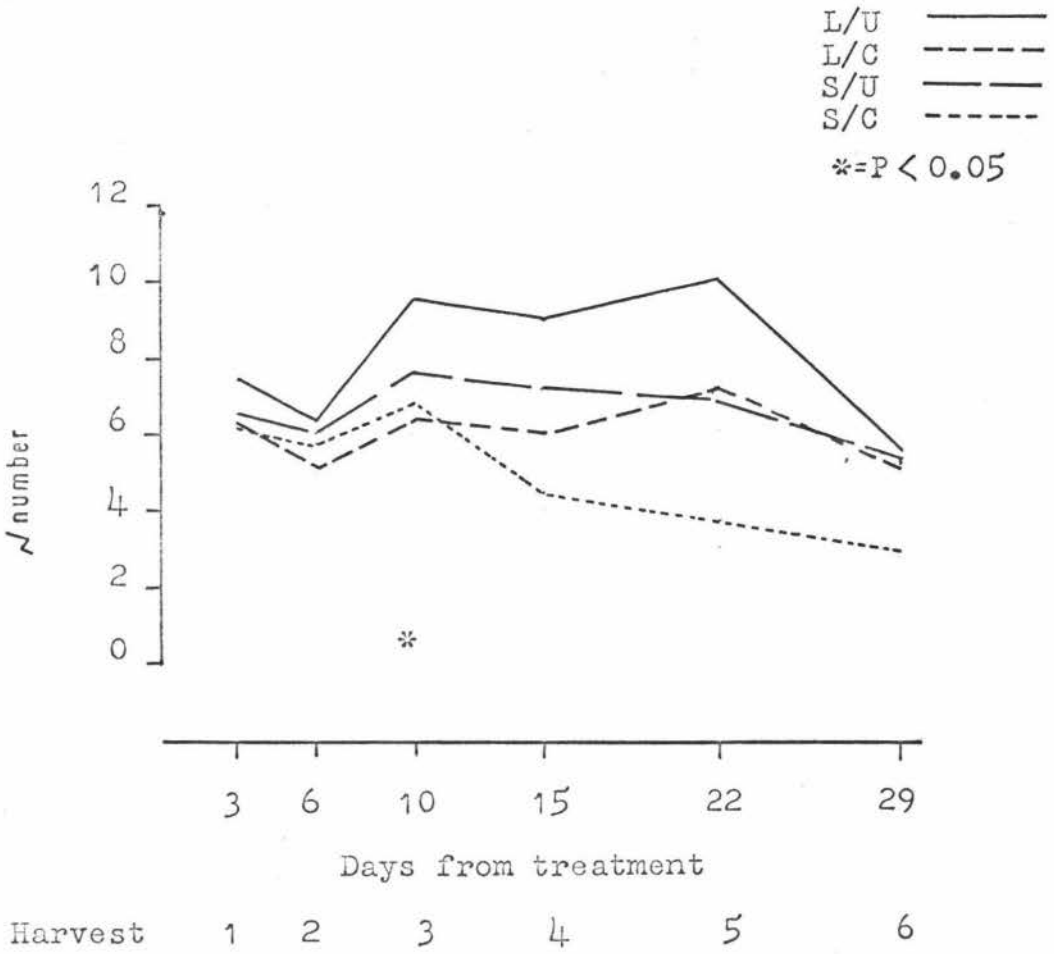


FIGURE III.2.4 NODULE NUMBER DISTRIBUTION

a). light - uncut (control)

numeric (1 .... 7) = root inch - segments measured as inches away from the crown

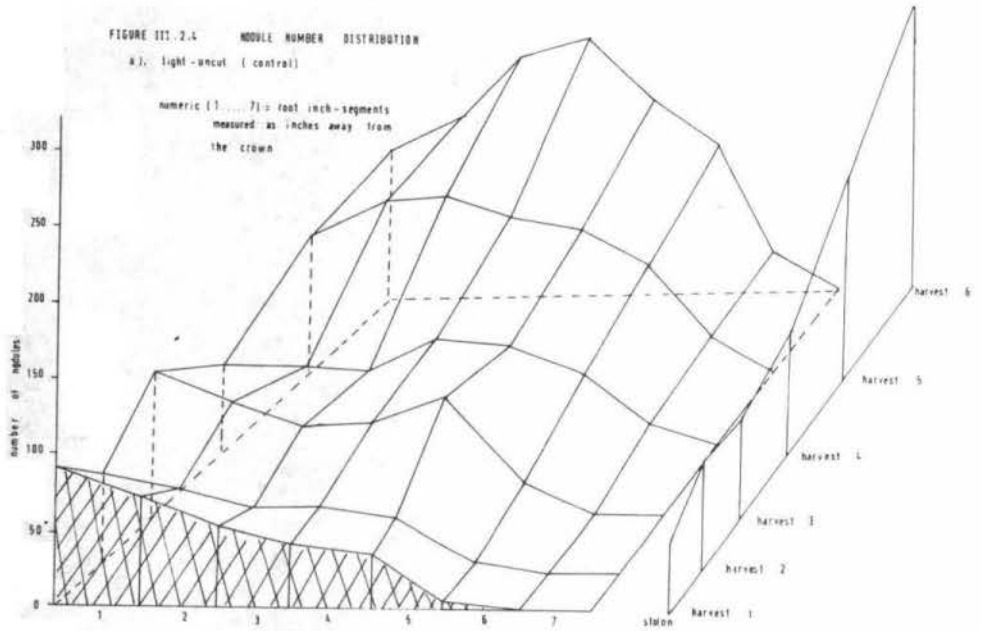


FIGURE III.2.4

b). light - cut

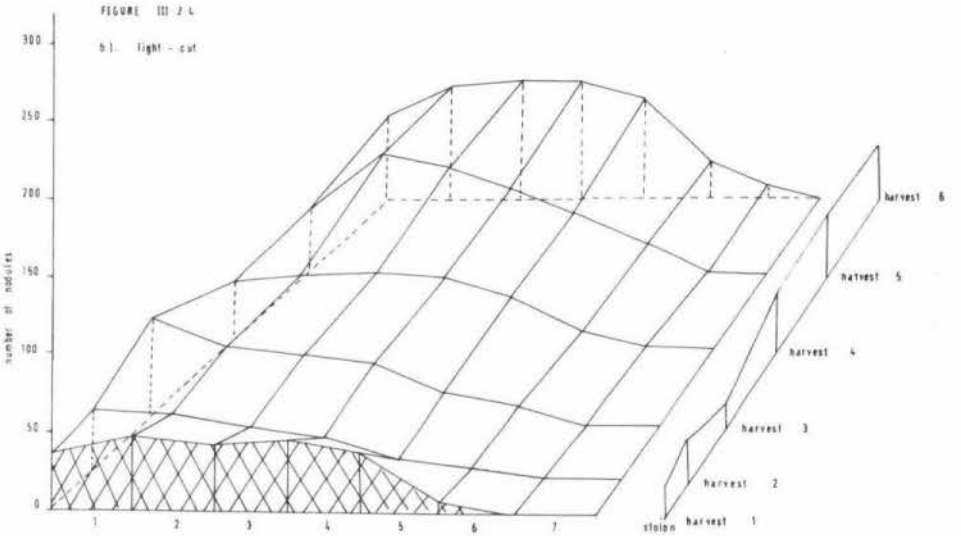


FIGURE III.2.4

c). shade - uncut

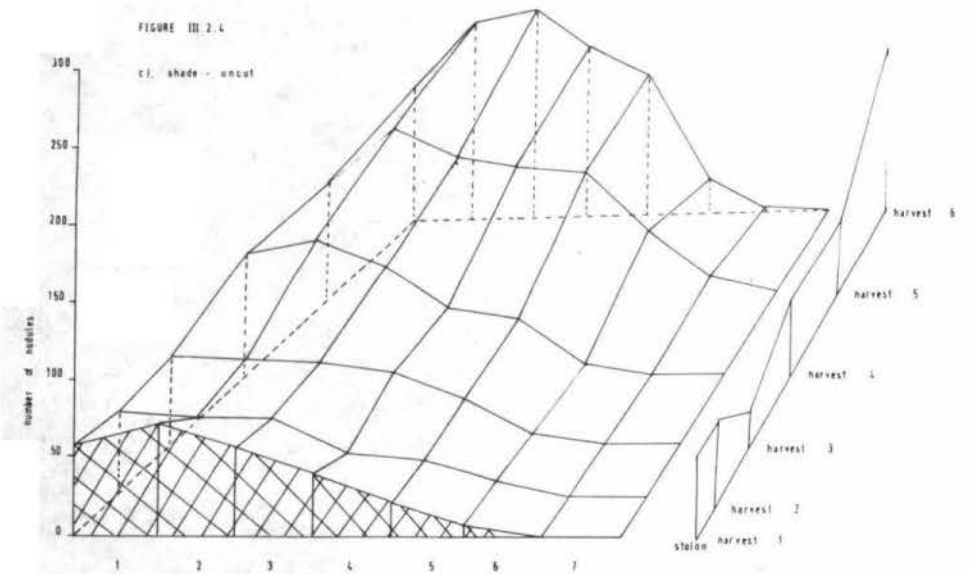


FIGURE III.2.4

d). shade - cut

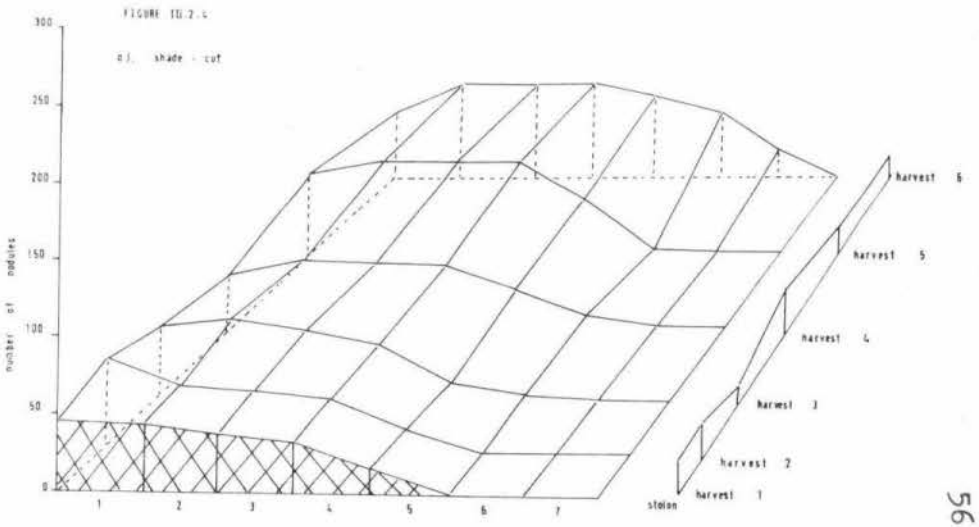
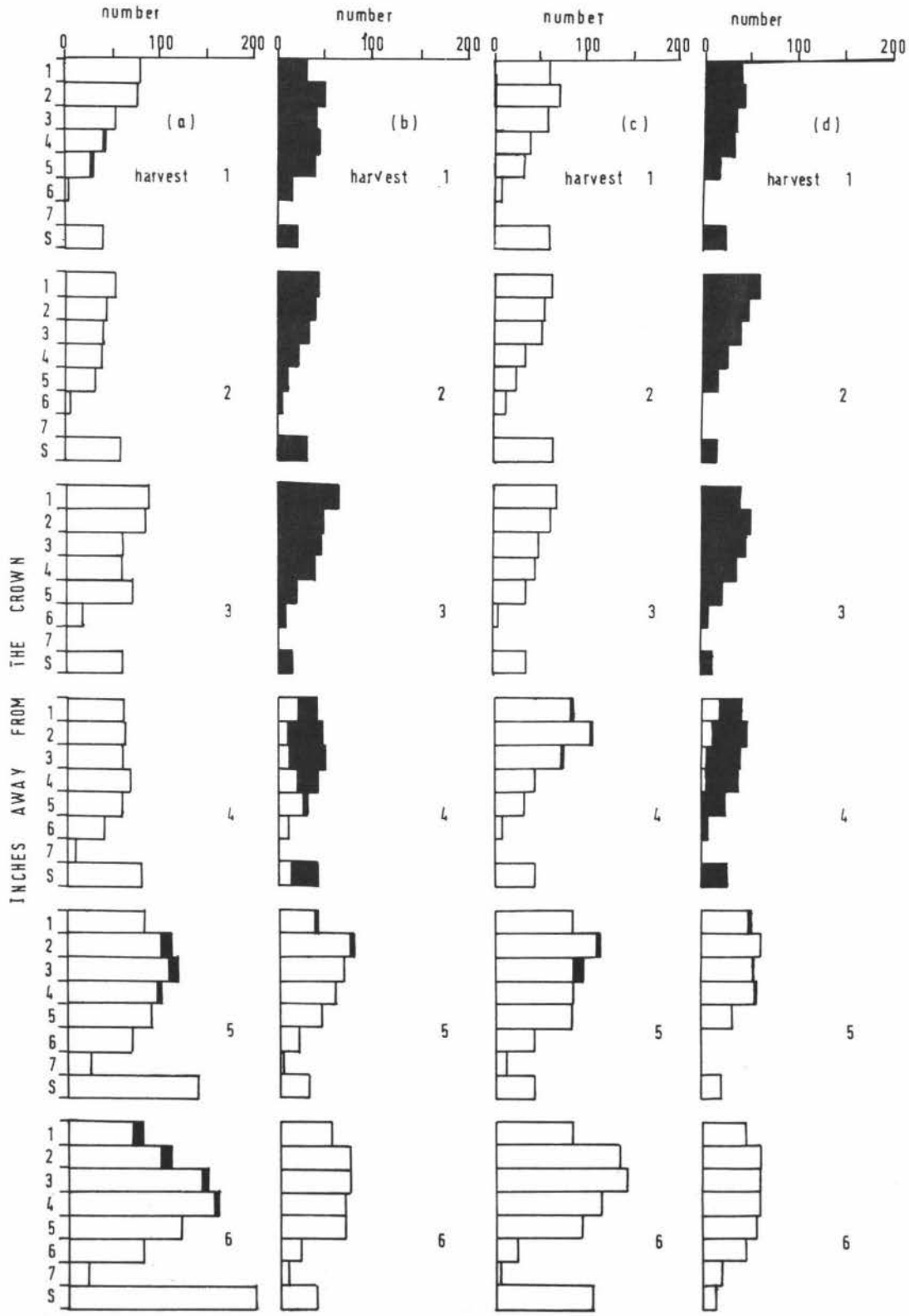


FIGURE III. 2. 5 NUMBER OF 'PINK' AND 'GREEN' NODULES (per inch)

a. light - uncut b. light - cut c. shade - uncut d. shade - cut

(pink = □ ; green = ■ ; S = stolon nodules ; numeric[1 ... 7] = inches away from crown)



2.2 Nodule weight

2.2.1 Nodule dry weight per plant

The summary of the within-harvest analysis of variance is presented in Appendix III. 2.2.1. The expression 'relative growth rate' (RGR) in mg/mg/day (as detailed in Appendix II.2) is used to describe the changes in nodule dry weights. The analysis of variance for RGR is summarised in Appendix III. 2.2.2.

2.2.1.1 Light treatment

Shading decreased total nodule weight, the effect becoming significant after two weeks (Figure III. 2.6a). In the 'light' plants the total nodule RGR reached a maximum of 0.0620 mg/mg/day by day 15, whereas in the 'shade' plants the RGR remained low (around 0.0105 to 0.0130 mg/mg/day) until about day 22 when it reached a similar level to that of the control. These trends are illustrated in Figure III. 2.6b.

2.2.1.2 Defoliation treatment

Defoliation caused a marked reduction in total nodule dry weights (Figure III. 2.6.c). The overall trend in the rate of nodular growth in the defoliated plants, as indicated by RGR was similar to that described for the 'light' treatment.

2.2.1.3 Light x cutting interaction

Only at harvest 1 (day 3) was a significant L x C interaction detected (Appendix III. 2.2.1): this could have been due to the different effects caused by defoliation upon 'light' and 'shade' plants.

2.2.2 Nodule weight per inch (fresh weight)

Nodule fresh weight per inch is shown in Figure III. 2.7, indicating that both the pattern of distribution and the step-wise increment in nodule weight with time are very similar to that described for nodule number (see section III. 2.1.2).

2.2.3 Average weight per nodule

The summary of the within-harvest analysis of variance is presented in Appendix III. 2.3.2.a. There were no significant light x cutting interactions.

2.2.3.1 Light treatment

Figure III.2.8.a shows the effects of shading on the average nodule dry weights which are plotted against time in days. The nodules from the 'shade' plants were lighter than those of the control 'light' plants from about 15 days onwards although only the results from harvest 5 were statistically significant ($P < 0.01$).

2.2.3.2 Defoliation treatment

Defoliation also caused a reduction in average weight per nodule (Figure III.2.8.b), and the effect seemed to start earlier (day 10) than that caused by shading. This could have been due to the reduction in large nodules as shown in Figure III. 2.1.b and Figure III. 2.2.b, for defoliation and shading respectively.

FIGURE III.2.6 NODULE DRY WEIGHT PER PLANT

light = ○ shade = △

uncut = □ cut = X

* = P<0.05 ** = P<0.01

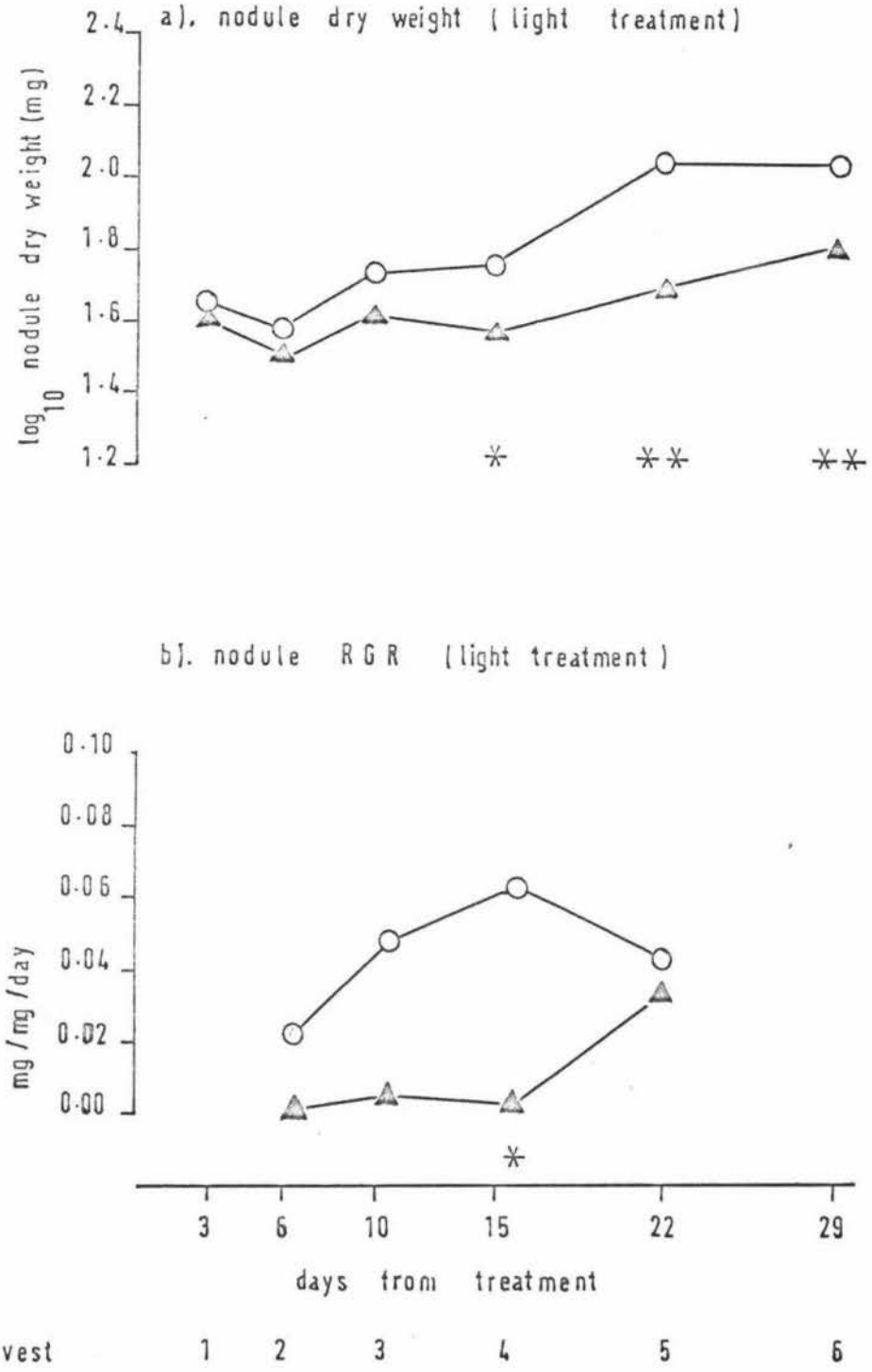
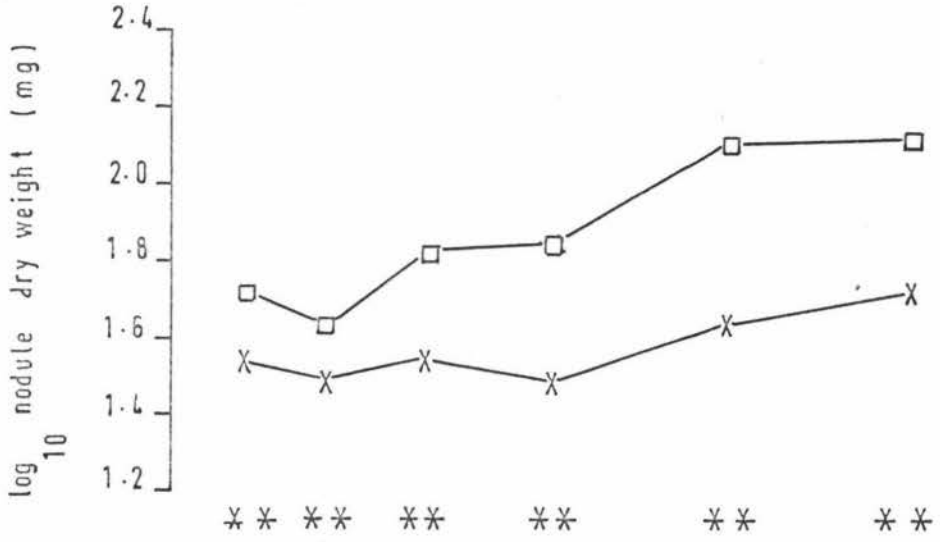


FIGURE III.2.6 (continue)

c). nodule dry weight (defoliation treatment)



d). nodule RGR (defoliation treatment)

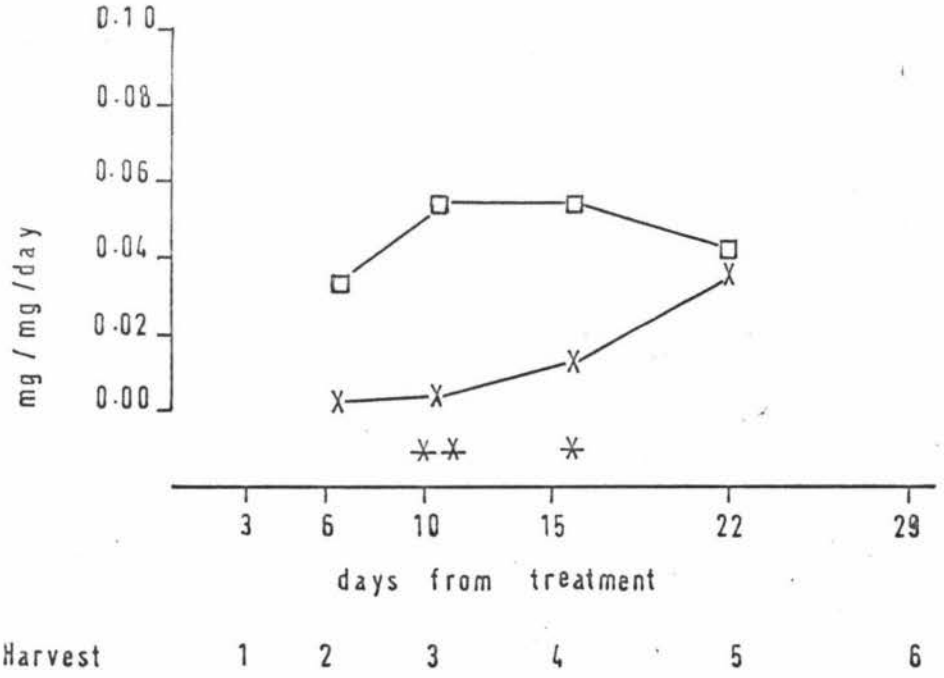


FIGURE III.2.7

NODULE WEIGHT DISTRIBUTION

a). light - uncut (control)

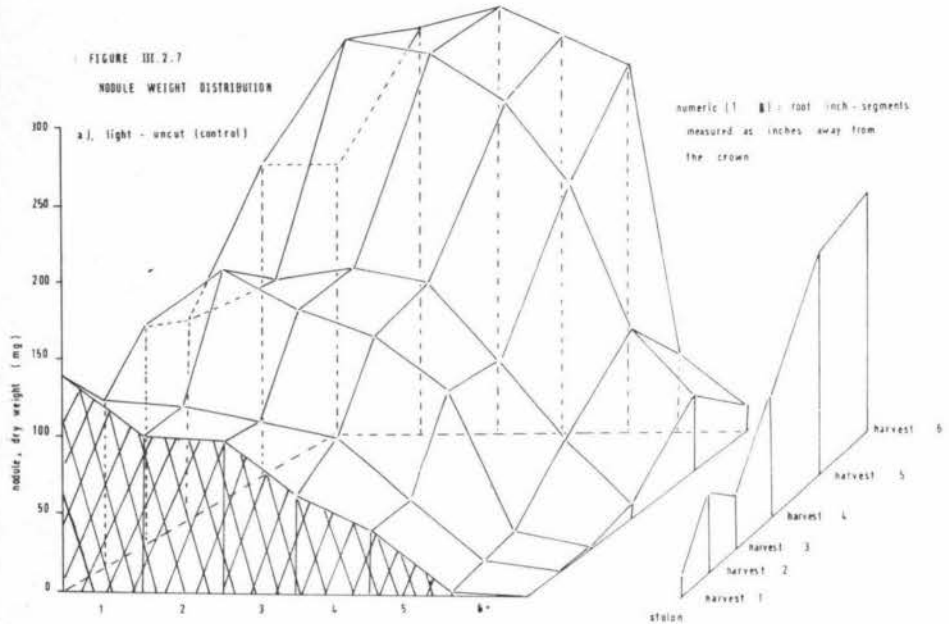


FIGURE III.2.7

b). light - cut

segment 6+ = total nodule dry weight from segments 6 & 7

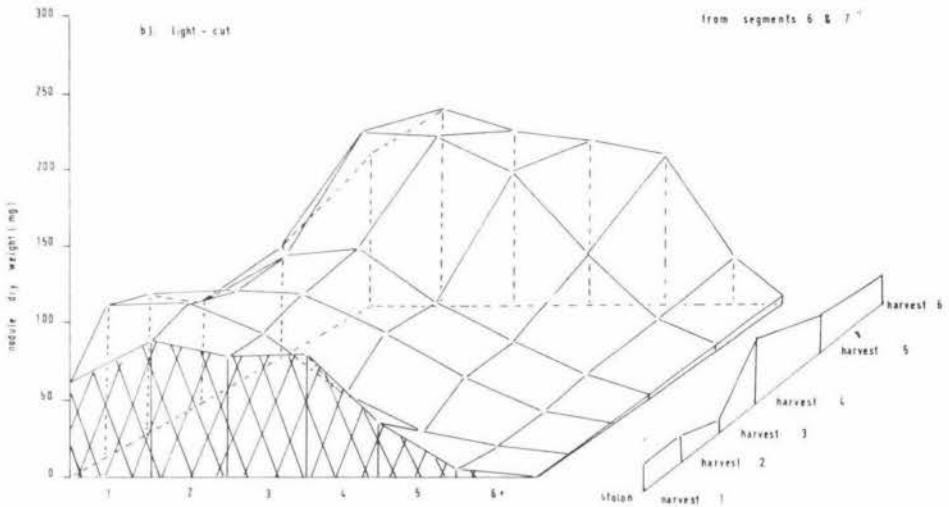


FIGURE III.2.7

c). shade - uncut

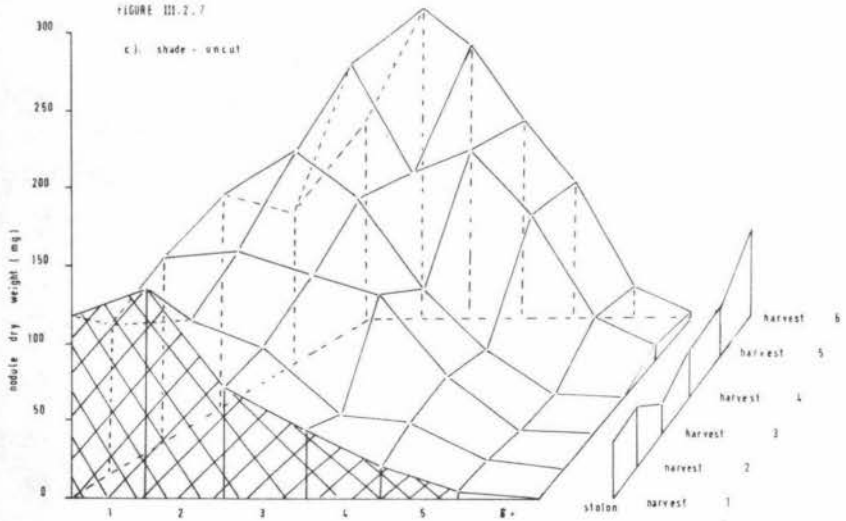


FIGURE III.2.7

d). shade - cut

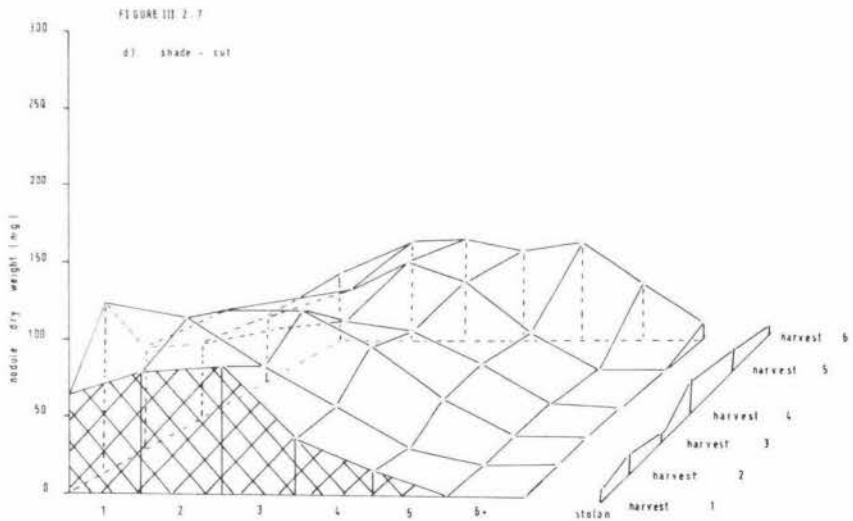
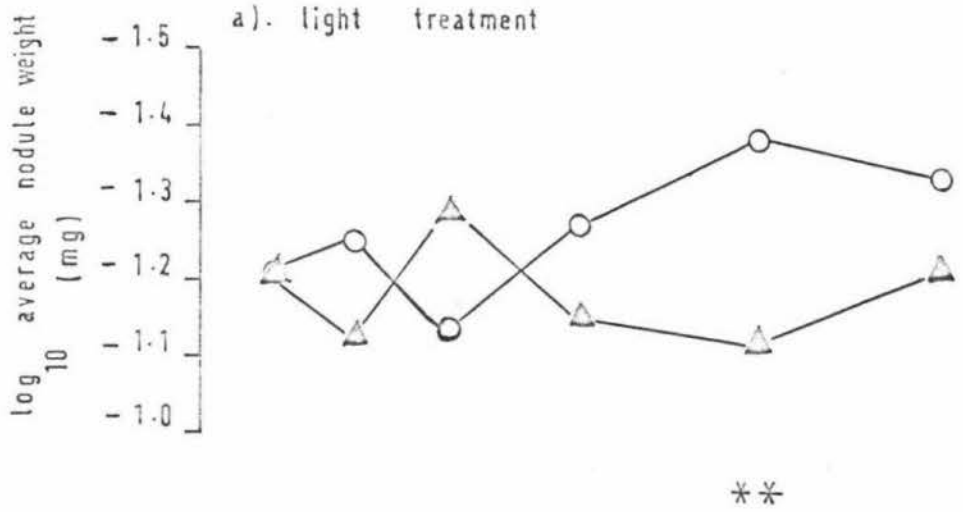


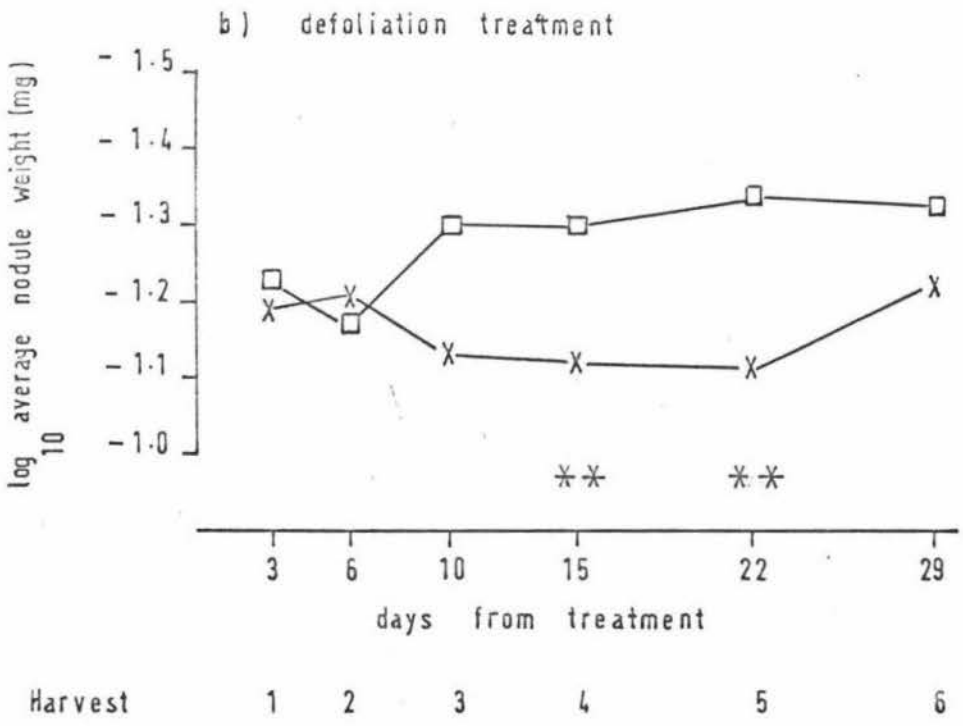
FIGURE III.2.8 AVERAGE DRY WEIGHT PER NODULE

light = ○ shade = △

uncut = □ cut = X

* = $P<0.05$ ** = $P<0.01$





Harvest	1	2	3	4	5	6

2.3 Relationship between nodulation data and root dry weight

2.3.1 Number of nodules per 100 mg root

The within-harvest analysis of variance is presented in Appendix III. 2.3.1.a; there is no L x C interaction.

Shading and defoliation had no effect on the nodule number per 100 mg root dry weight. This is clearly evident in Figure III. 2.9. That the means are not significantly different across all harvests has been confirmed by the results from the between harvest analysis of variance (using the split-split-plot design as detailed in section II. 7. 1); a summary of the results is presented in Appendix III. 2.3.1.b.

2.3.2 Nodule dry weight per 100 mg root

The within-harvest analysis of variance is presented in Appendix III. 2.3.2.b; again the L x C interaction is non-significant.

Shading caused a reduction in nodule dry weight per 100 mg root by harvest 4, although only that in harvest 5 was statistically significant ($P < 0.05$); the lower nodule dry weight per unit root weight could be the result of a faster root dry weight increase relative to that of the nodule dry weight (Figure III. 1.2.2 & Figure III. 2.6). Similarly, defoliation caused an early (less than 3 days) and persistent reduction in nodule weight per unit root weight. The differences could be due to a similar reason as that given for the 'shade' plants. The results are presented in Figure III. 2.10.

2.4 Relationship between components of nodulation data

2.4.1 Relationship between nodule dry weight and nodule number

Although the average within group regression of mean dry weight per nodule (dependant variable) and the square root nodule number per plant (independant variable) were highly significant ($P < 0.001$), results from the within group analysis of variance (Appendix III. 2.4.2) indicated that the individual regressions were significantly different from each other ($P < 0.05$).

Two interesting points emerged from these results:-

1. there was an inverse relationship between the two nodule characters, and
2. the low correlation ($r = -0.23$ to -0.55) and a non-significant

test for departure from linear regression (Snedecor and Cochran 1967 p. 455
indicated a diffuse relationship between the two variables.

FIGURE III.2.9 NUMBER OF NODULES PER 100 MG ROOT

light = ○ shade = △

uncut = □ cut = X

* = P<0·05 ** = P<0·01

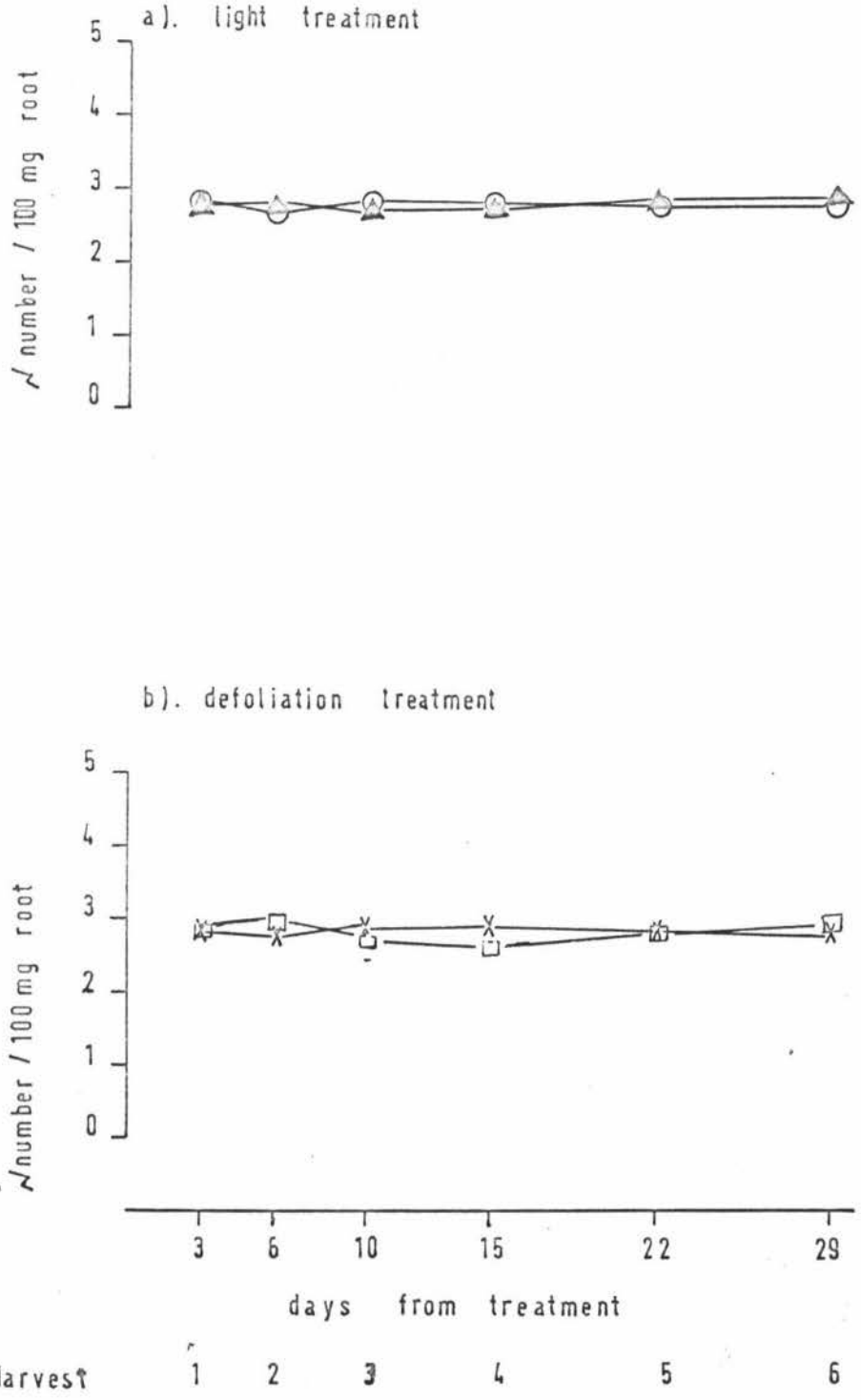
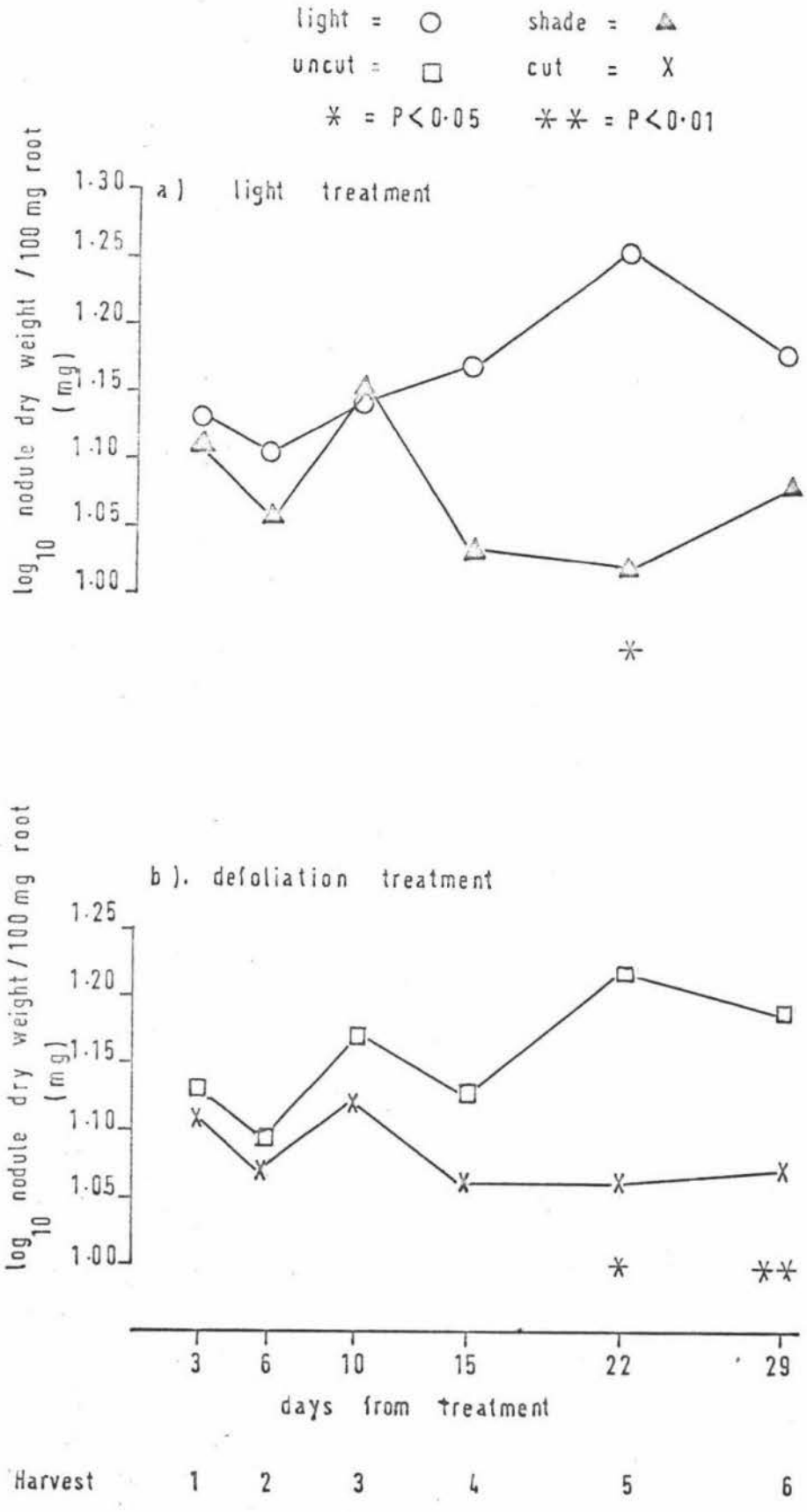


FIGURE III.2.10 NODULE DRY WEIGHT PER 100 MG ROOT



3. NITROGEN FIXATION

In this section results of plant nitrogen measurements, and the methods (as detailed under section II. 6.6.2) for and results of the evaluation of nitrogen fixation efficiency are presented.

3.1 Plant nitrogen

The results of the plant nitrogen data are presented in two sections; percentage nitrogen, and nitrogen content of the plant.

3.1.1 Percentage nitrogen

The data for percentage nitrogen (N%) of top, root and nodule were analysed using the untransformed values. The results of the within-harvest analysis of variance are presented in Appendix III. 3.1.

3.1.1.1 Light treatment

Shading caused a reduction in percentage nitrogen in the top (from harvest 3) and nodule (from harvest 2), but not in the root. The N% of the tops remained significantly lower ($P < 0.05$) in the 'shade' plants even at the last harvest, whereas differences in the nodule N% due to shading disappeared by harvest 6. The results are presented in Figure III. 3.1.

3.1.1.2 Defoliation treatment

Under defoliation, the N% of the tops was lower than that of the control (undefoliated) plants from harvest 1 to harvest 3; part of this difference could be due to the change in the proportion of leaf to stem tissue after the removal of the leaves under the defoliation treatment. Figure III. 3.2 illustrates the general trend of the effects of defoliation on the percentage nitrogen.

In both root and nodule percentage nitrogen, the significant differences in the earlier harvests due to defoliation disappeared by harvest 4 in the roots and harvest 5 in the nodules.

3.1.1.3 Light x cutting interaction

The root and nodule N% showed no L x C interaction, whereas in the top a statistically significant interaction ($P < 0.05$) was detected in harvest 4 which could be due to the recovery of the light-cut plants (Appendix III. 3.1).

3.1.2 Nitrogen content

The nitrogen content of the plant (i.e. of top, root and nodule) was calculated as the product of dry weight and percentage nitrogen. The expression 'relative rate of nitrogen assimilation' (RRNA) in mg N / mg N / day is synonymous with 'relative nitrogen assimilation rate' as described by Gibson (1965), and is used to describe the changes in nitrogen yield. (see Appendix II. 2 for details). The total nitrogen yield was also used for examining the relationship with nodule dry weights as described by Döbereiner (1966) (see section III. 3.3). Table III. 3.1 presents the transformed means of the nitrogen contents of the different plant components.

3.1.2.1 Light treatment

Shading generally caused a reduction in the nitrogen content of the different plant parts, although significant differences were detected only after harvest 2 in the top and nodule, and in harvest 6, in the root. The relative rate of nitrogen assimilation (RRNA) of the top decreased with time, whereas that of the root and nodule increased. Although shading tended to reduce the RRNA for each organ, the effect was not always statistically significant (Figure III. 3.3).

3.1.2.2 Defoliation treatment

Defoliation reduced the mean nitrogen yields of all plant components throughout the experimental period (Table III. 3.1). Defoliation increased the top RRNA (non-significantly) but decreased that of the root (also non-significantly). However, the RRNA of the nodule was significantly reduced in the 'defoliated' plants up to harvest 3, after which recovery occurred. (Figure III. 3.4).

3.1.2.3 Light x cutting interaction

There was no significant L x C interaction in the top nitrogen yield, but for the root and nodule components there were significant interactions at harvest 3 ($P < 0.05$) for the root and at harvest 1 ($P < 0.05$) for the nodule. These interactions were of minor importance (Appendix III. 3.2).

Table III. 3.1 **Nitrogen content**

a). Log_{10} top nitrogen content (mg)

Harvest	1(3)	2(6)	3(10)	4(15)	5(22)	6(29)
Treatment						
Light	1.4870	1.5753	1.7377	1.8711	2.1028	2.1380
Shade	1.4546	1.5198	1.5904	1.7496	1.8564	1.9962
	ns	ns	*	*	**	*
Uncut	1.7923	1.8411	1.9670	2.0709	2.2613	2.3047
Cut	1.1493	1.2540	1.3612	1.5498	1.6980	1.8295
	**	**	**	**	**	**

b). Log_{10} nitrogen content (mg) root

Harvest	1(3)	2(6)	3(10)	4(15)	5(22)	6(29)
Treatment						
Light	0.8394	0.7756	0.9239	0.8928	1.0974	1.1769
Shade	0.7790	0.7463	0.7882	0.8545	0.9830	1.0232
	ns	ns	ns	ns	ns	**
Uncut	0.9251	0.8504	1.0085	1.0213	1.2137	1.2339
Cut	0.6932	0.6714	0.7036	0.7260	0.8667	0.9663
	**	*	**	**	**	**

c). Log_{10} nodule nitrogen content (mg)

Harvest	1(3)	2(6)	3(10)	4(15)	5(22)	6(29)
Treatment						
Light	0.5897	0.4867	0.6487	0.6823	0.9895	0.9743
Shade	0.5377	0.3932	0.5247	0.4596	0.5763	0.7371
	ns	ns	*	*	**	**
Uncut	0.6760	0.5438	0.7702	0.7655	1.0240	1.0441
Cut	0.4514	0.3361	0.4031	0.3764	0.5418	0.6673
	**	**	**	**	**	**

FIGURE III.3.1 Percentage nitrogen

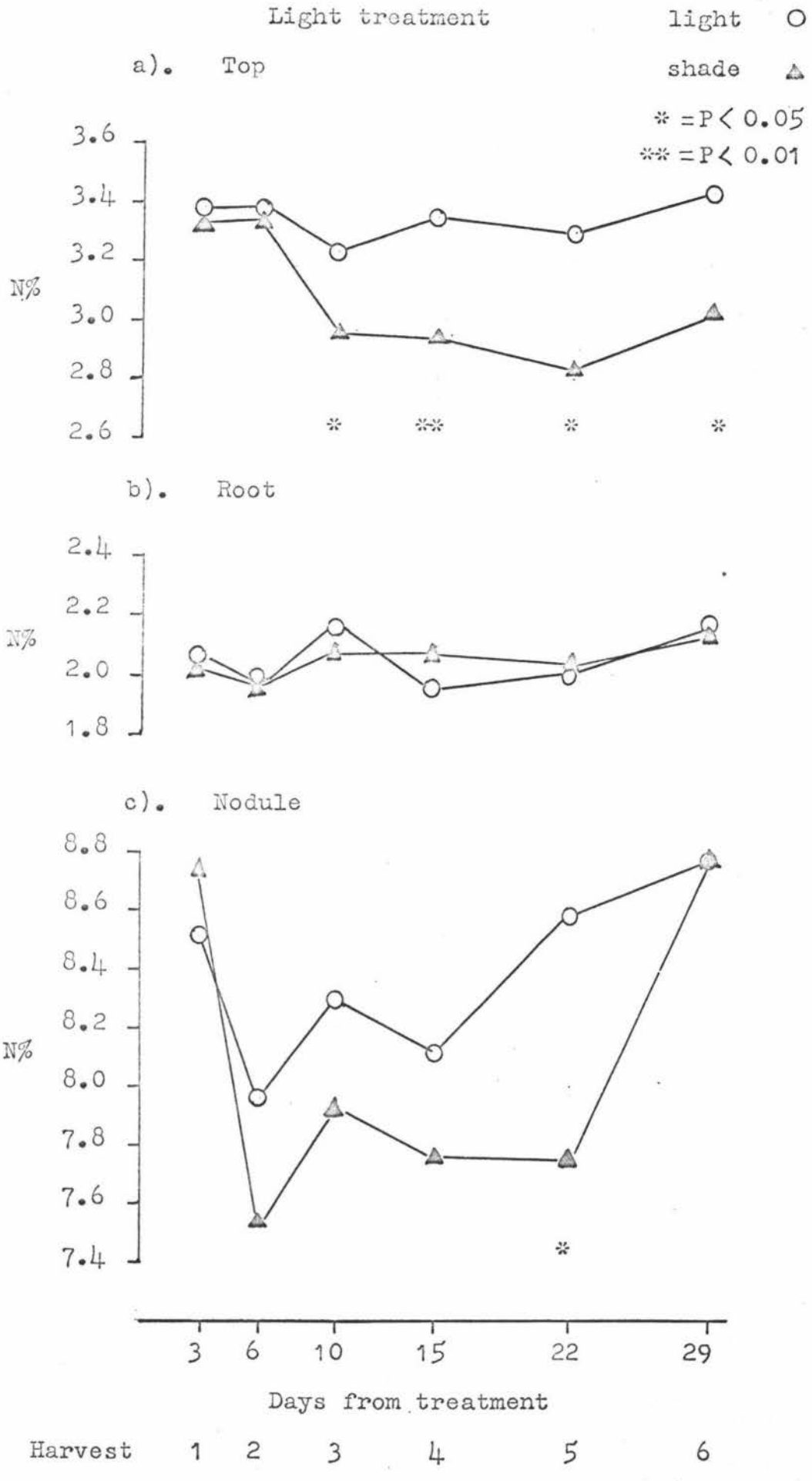


FIGURE III.3.2 Percentage nitrogen

Defoliation treatment uncut □

cut X

* = P < 0.05 ** = P < 0.01

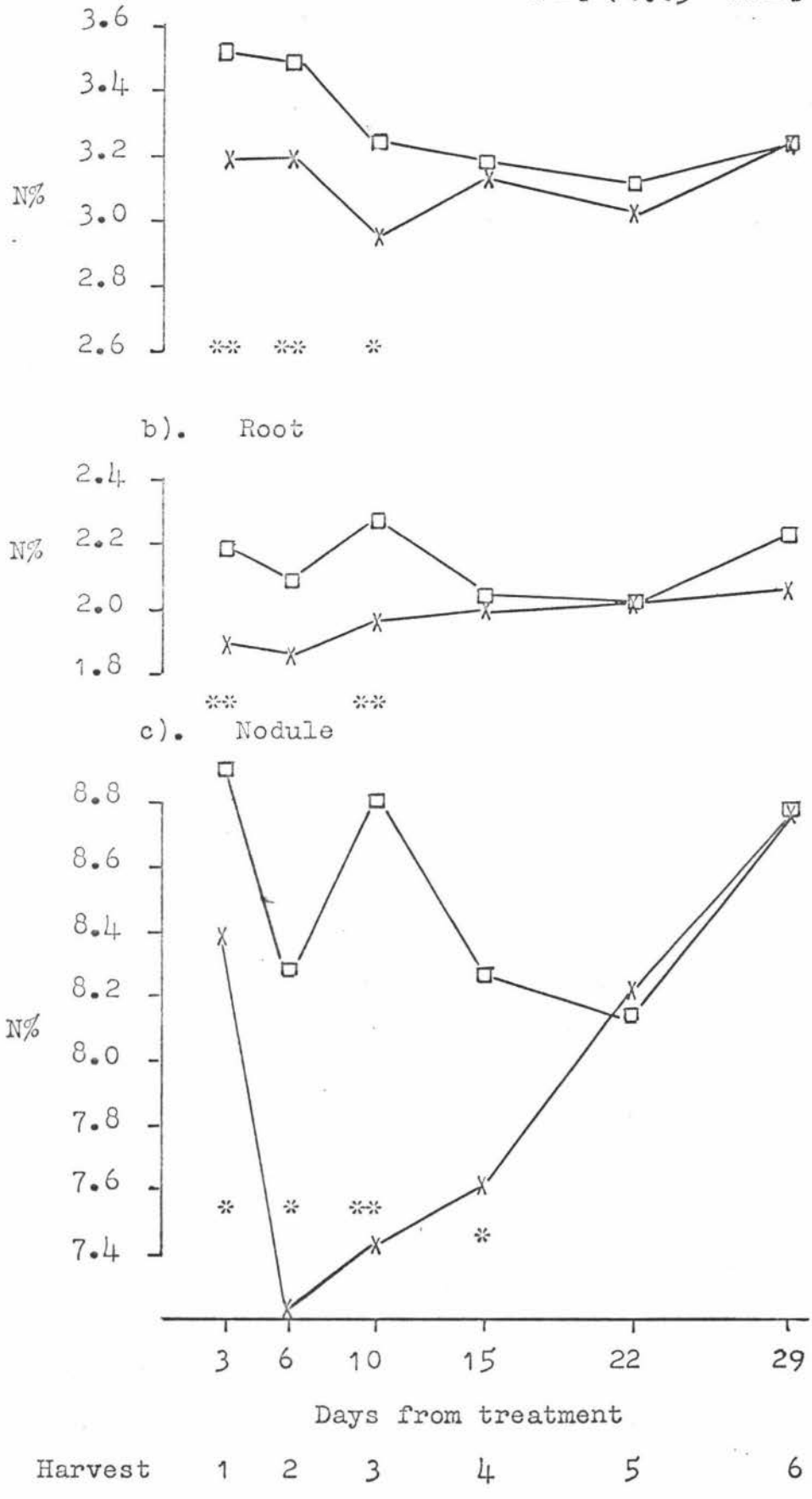


FIGURE III.3.3 PLANT NITROGEN CONTENT

(light treatment)

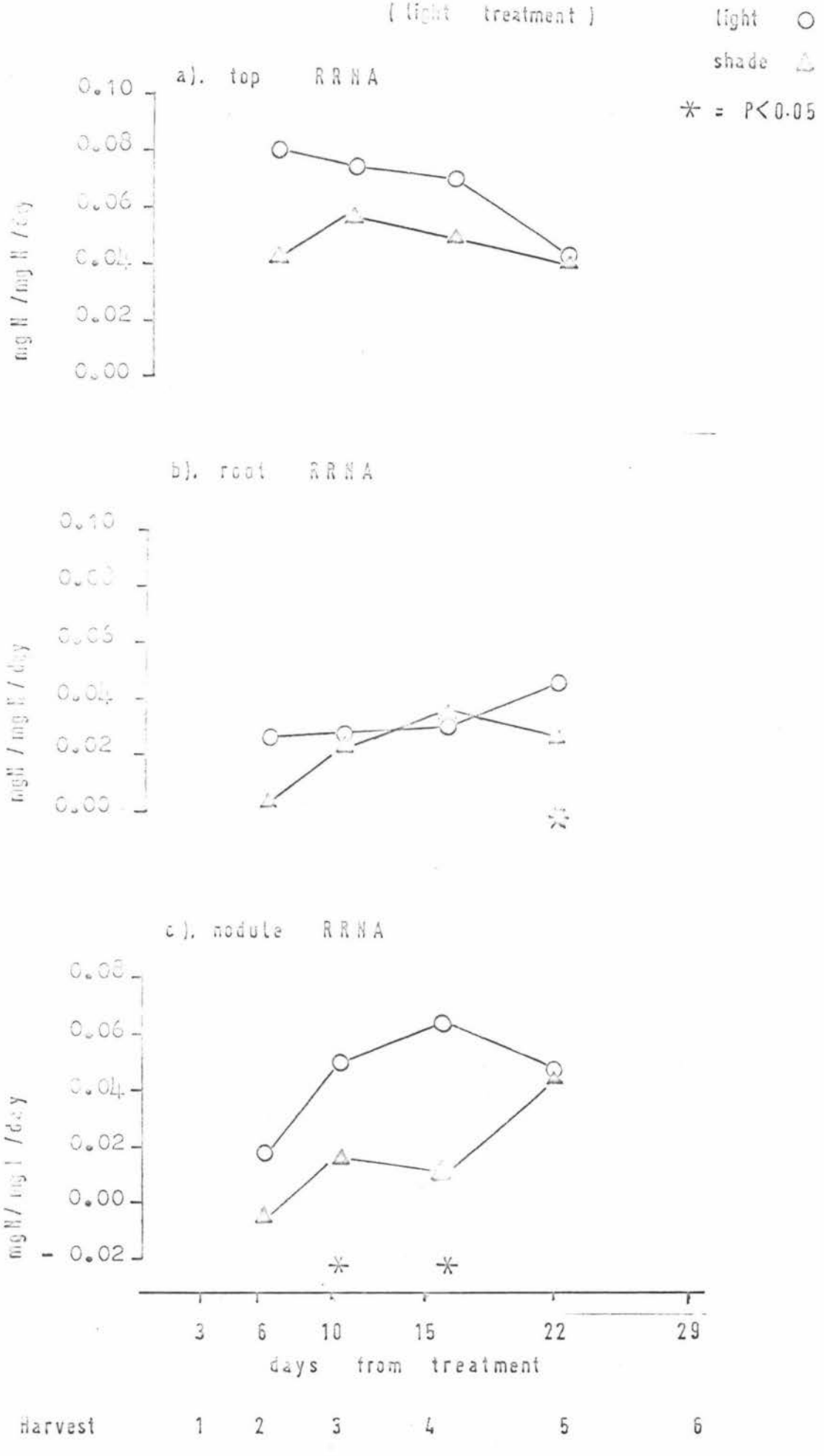
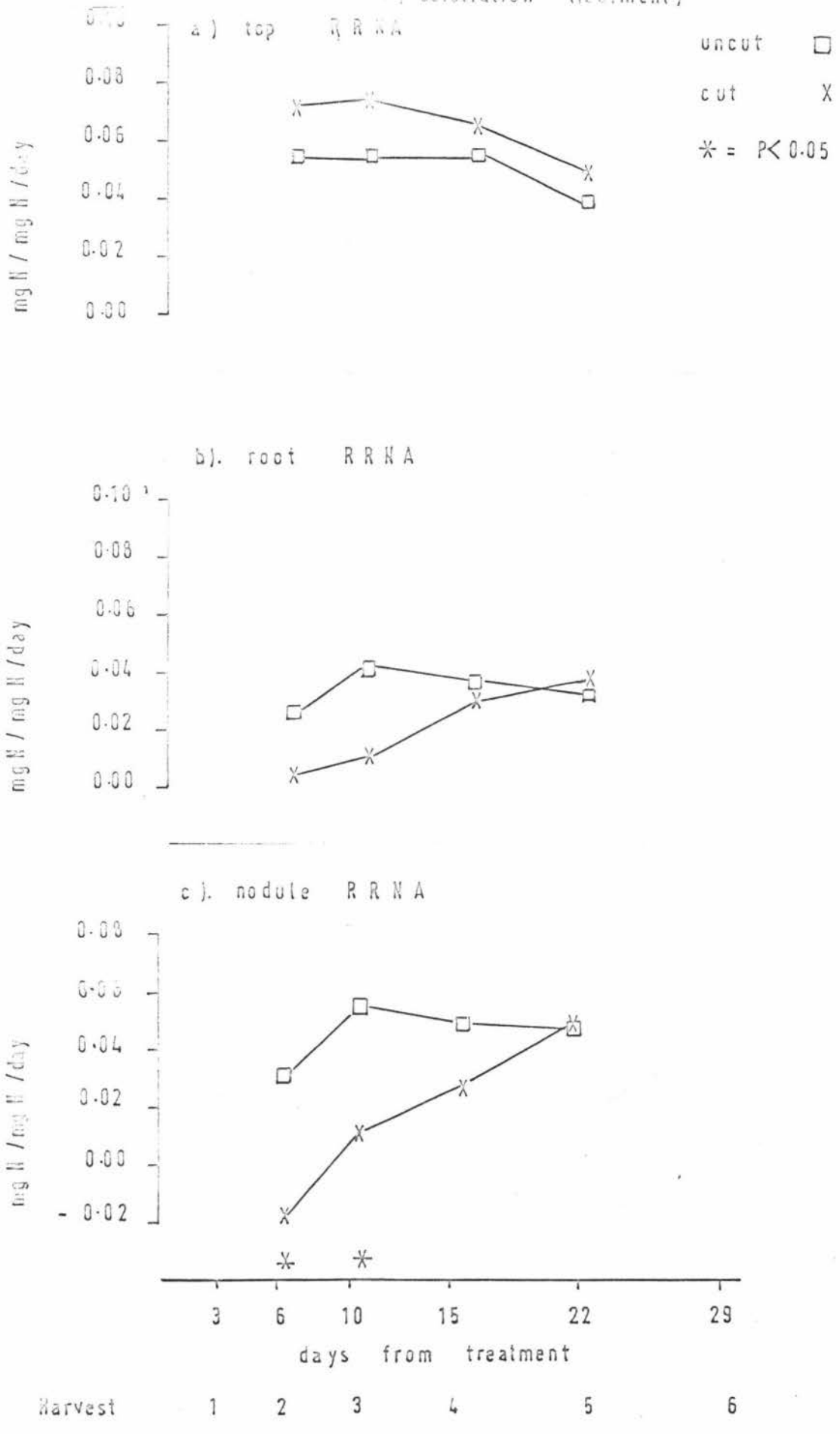


FIGURE III.3.4 PLANT NITROGEN CONTENT

(defoliation treatment)



3.2 Nitrogen fixed per gram nodule dry weight per day

The summary of within-harvest analysis of variance of this character is presented in Appendix III. 3.3. There was no L x C interaction, hence only the main effects are presented in the text.

3.2.1 Light treatment

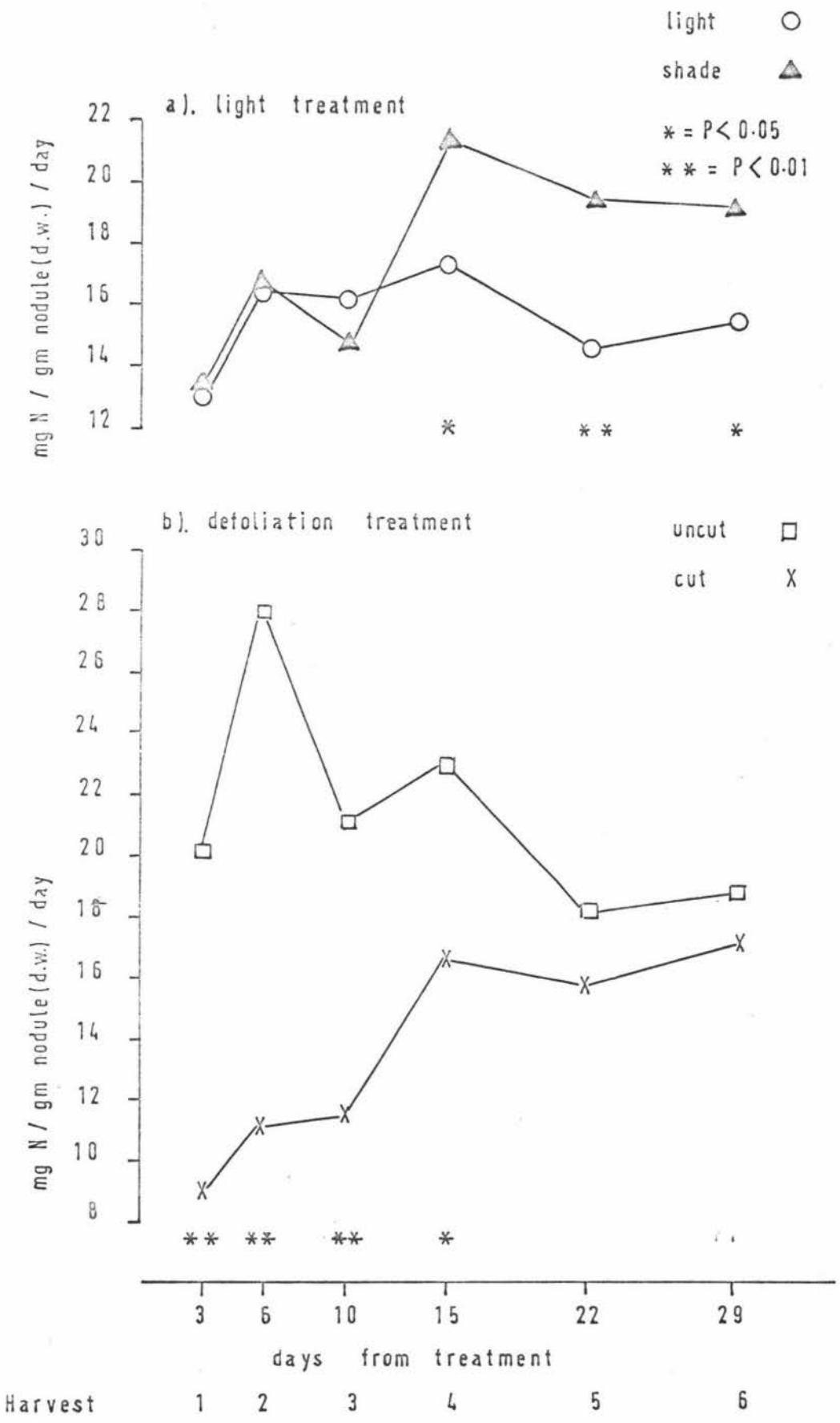
The rate of fixation became higher in the 'shade' plants by harvest 4 (Figure III. 3.5.a). This appeared to suggest, at first glance, increased efficiency due to shading; however on closer examination it was more of an artefact, since some of the nodules which had contributed towards the total plant nitrogen yield had been lost (Figure III. 2.6), and were not accounted for in the calculation.

3.2.2 Defoliation treatment

Similarly, an artefact due to the removal of the top caused the much lower fixation rate in the 'defoliated' plants for at least the first four harvests (Figure III. 3.5.b).

Where treatments actually removed part of the plant either directly e.g., defoliation or indirectly as a result of treatment e.g., loss of nodules under shading, this statistic is subject to bias.

FIGURE III.3.5 Mg nitrogen fixed per gm nodule per day
(decoded means)



3.3 Regression between plant nitrogen content and nodule dry weight

Log_{10} total plant nitrogen (dependant variable) was plotted against nodule dry weight per plant (independant variable), and a linear regression was fitted. The results are summarised in Table III. 3.2. Although the average within-group regression was highly significant ($P < 0.001$), the individual group regressions were significantly different from each other ($P < 0.001$). Low total plant nitrogen due to defoliation had contributed towards the much higher regression coefficients in the defoliated plants.

Table III. 3.2 Comparison of regressions (using general statistical program : see section II. 7.4).

a). Individual group regressions

Treatment	df	regression equation	std. err.	correlation
Light - uncut	40	Y= 1.33 + 0.0032 X	0.0002	0.90
Light - cut	40	Y= 1.14 + 0.0097 X	0.0008	0.88
Shade - uncut	40	Y= 1.57 + 0.0067 X	0.0004	0.91
Shade - cut	40	Y= 1.17 + 0.0111 X	0.0024	0.59
b) Average within group regression				
	163	Y= 1.54 + 0.0045 X	0.0003	0.74

c). Analysis of within group variance of Y

Source of variation	df	S.S.	M.S.	F
Total within group	164	11.341	0.069	
Due to average regression	1	6.277	6.277	202.04 ***
Deviation from average regression	163	5.064	0.031	
Between individual group regressions	3	1.752	0.584	28.22 ***
Deviation from individual regressions	160	3.312	0.020	

*** P 0.001

3.4 Acetylene reduction assay

The amount of ethylene (C_2H_4) reduced from acetylene (C_2H_2) per unit fresh weight (mg) of nodule is calculated as shown in Appendix II. 9. For each treatment (within each harvest) the results from seven replicates were pooled and the amount of C_2H_4 detected was plotted against time in minutes. One example is shown in Figure III. 3.6.

In order to demonstrate the linearity of the fitted regression line, the results were subjected to a test for departure from linear regression (Snedecor and Cochran 1967 p. 455). A computer program was written for the calculation (Appendix III. 3.4). Within each harvest the treatments were tested for incubation periods of 300 minutes, 240 minutes and 180 minutes. The analysis of variance for departure from linear regression is summarised and presented in Appendix III. 3.5. The results indicate that the reaction is linear for at least 180 minutes.

Furthermore it is logical to assume that at time zero there should be no detectable C_2H_4 (other than the control), hence the straight line should pass through the origin. A test for fitting the straight line through the origin (Snedecor and Cochran 1967 pp 166) was performed and the results shown in Appendix III. 3.6, which also showed the summary for the linear regressions after adjusting to fit through the origin i.e. all equations ($Y=A+bX$) were adjusted to the form $Y=bX$.

Shading caused no detectable difference in the rate of acetylene reduction, but defoliation significantly reduced the rate at harvests 1 & 2 (i.e., during the week following treatments), after which there were no significant difference between treatments. Figure III. 3.7 shows the rates of acetylene reduction expressed as μm moles C_2H_4 per mg nodule per hour.

FIGURE III.3.6 ACETYLENE REDUCTION ASSAY

(reduction of $C_2H_2 \rightarrow C_2H_4$ by nodulated white clover root , note the linear relationship between incubation time and C_2H_4 formation)

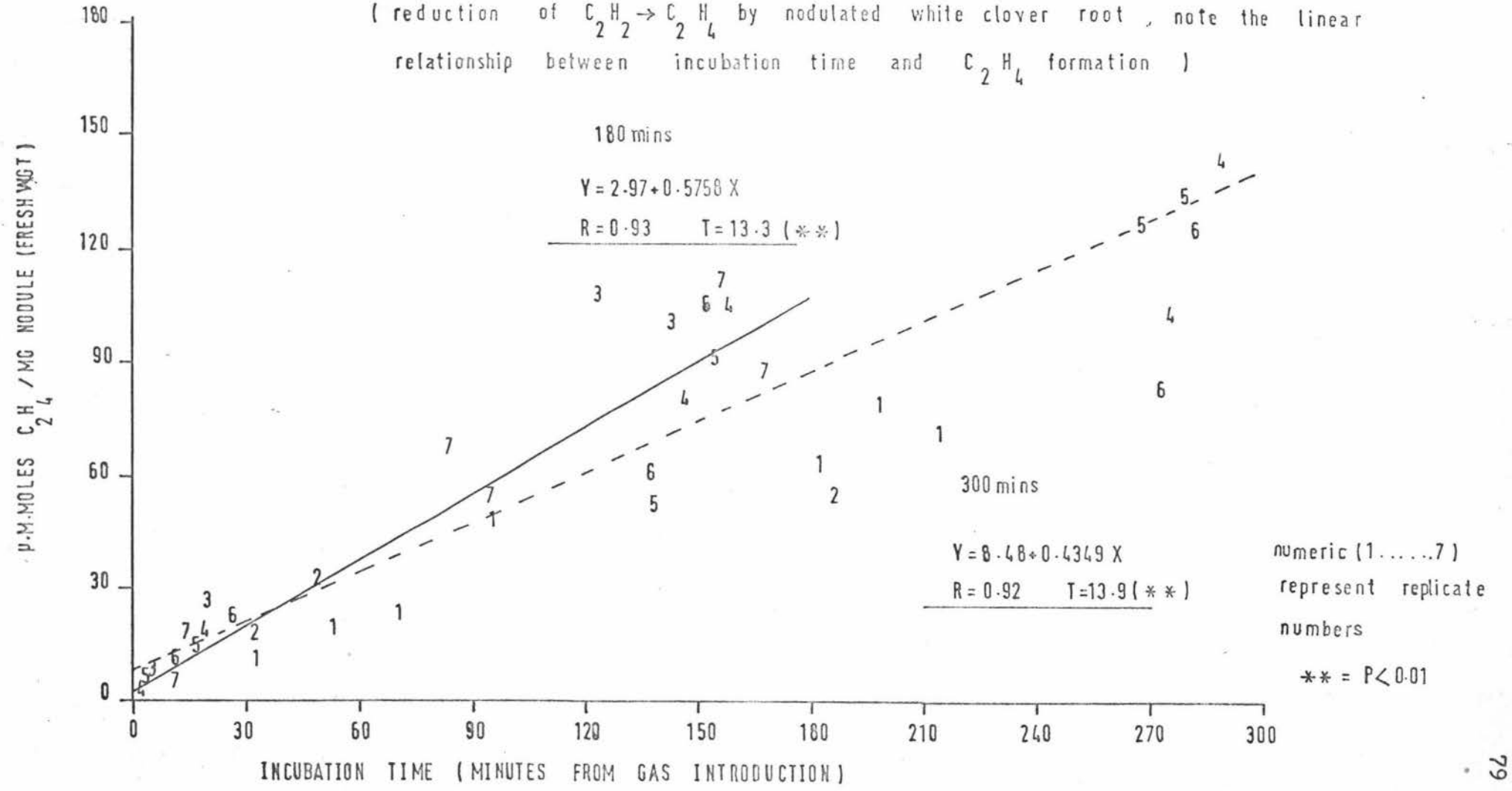
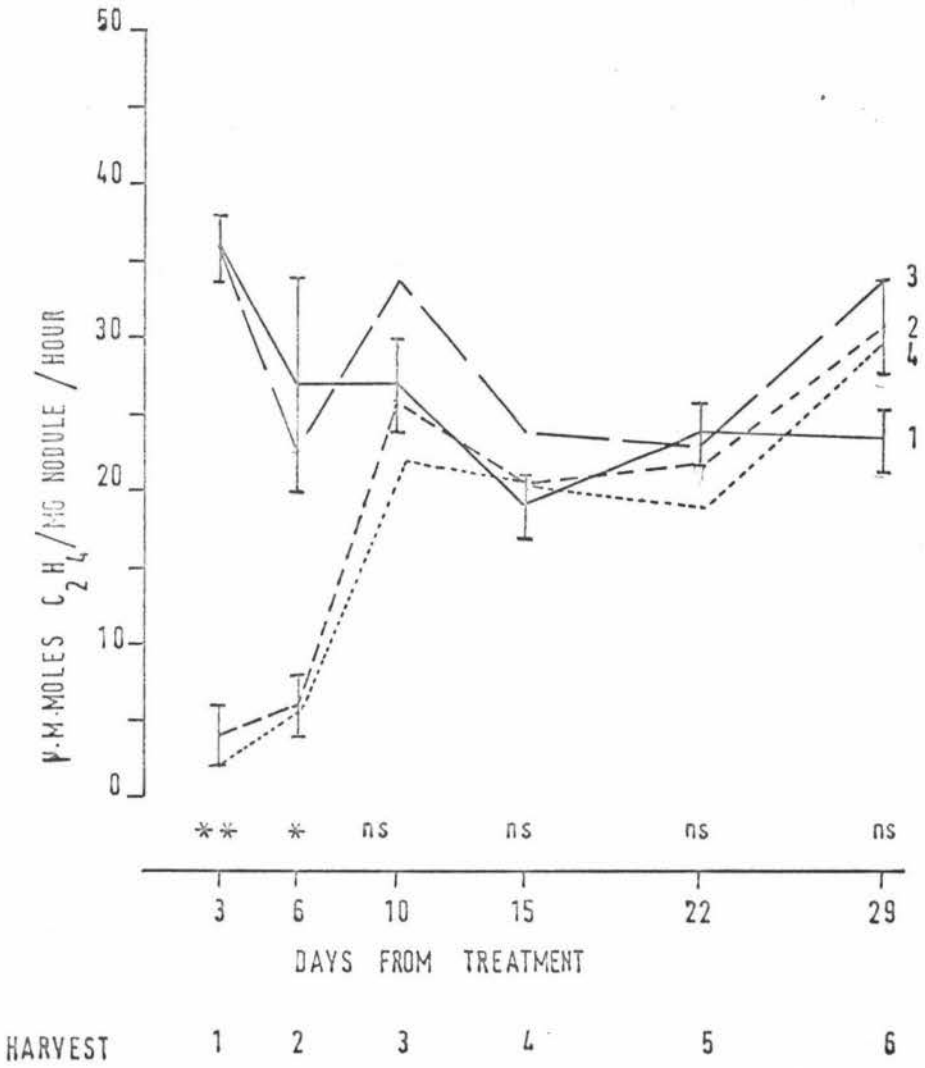


FIGURE III.3.7 Effects of shading and defoliation on the rate of $C_2H_2 \rightarrow C_2H_4$ reduction per mg nodule (fresh weight) per hour.



light - uncut ——— 1

light - cut — — — 2

shade - uncut —— —— 3

shade - cut -------- 4

results of within-harvest comparison of regression lines

ns = not significant

* = $P < 0.05$

** = $P < 0.01$

CHAPTER IV

DISCUSSION

Results of this study should be interpreted only within the conditions of the experiment and the specific host-rhizobium combination. Comparison of results from other experiments will require caution, while direct extrapolation of the results to field conditions would be unjustified. However some general trends may still be of use for future research.

Grouping the plants into comparable sizes for each replicate (section II. 4.2) resulted in significant block effects in the analyses of variance, but this tended to reduce within-block variation. Furthermore, when parameters are expressed on a per unit basis e.g. area per leaf, average weight per nodule and number of nodules per 100 mg root dry weight, this discrepancy disappears.

Temperature, moisture and the level of nutrients were satisfactory. The early iron deficiency systoms were soon corrected and thus were unlikely to have influenced the subsequent plant growth. The same could be said for the clover mite damage and the Malathion sprays (detailed in chapter II).

Lim (1963) showed that in the Trifolium species she studied, root hair infections could be limited if the rhizobial population in the rhizosphere fell below 10^4 cells per root system. Although the inoculation technique and frequency of inoculation used in this experiment ensured an adequate rhizobial population at all times (Greenwood per. comm.) most of the nodules further down the root would undoubtedly have arisen from infections by second generation rhizobia.

Pumice, probably due to its unavailability, is seldom used in detailed nodulation studies, hence to what extent it influenced nodulation (if any) is not known. The volume of the rooting medium could limit the number of nodules per pot (Nutman 1945), but from observations it seemed unlikely that for the duration of this experiment the volume of the rooting medium would have become limiting.

In this chapter, the discussion of the results falls into three main sections : 1). plant growth, 2). nodulation and 3). nitrogen fixation.

1. Plant growth

Reducing the light intensity by 42% had no significant effects on the mean leaf area per plant (Table III. 1.1.a), and although the 'shade' plants had a lower number of leaves per plant, except at harvest 5 the

differences between treatments were statistically non-significant (Table III. 1.1.b). The higher mean area per leaf in the 'shade' plants after harvest 4 (Figure III. 1.1) was partly the result of a lower mean leaf number in the 'shade' plants (Table III. 1.1.b). However, by harvest 6, a real increase in mean area per leaf under shading became evident (Appendix III. 2.4).

Reductions in dry weights of tops and roots due to shading have been reported in a number of species (Shirley 1929; Pritchell & Nelson 1951; Hiroi & Monsi 1963 and Buttrose 1968). The lower top and root dry weights of the 'shade' plants are shown in Table III. 1.2.a & b; they are probably the result of a lower relative growth rate (RGR) of these two plant components (Figure III.1.2.1). That relative growth rate is lower for 'shade' plants has been reported by Hiroi & Monsi in Helianthus annuus.

The 'shade' plants also had a lower top to root (T/R) dry weight ratio in harvest 2 (about 82% that of the control 'light' plants), which indicated that the plant's response to shading was detected within a week and that the effect was more severe in the roots than the tops. This resulted in a higher top to root ratio than the control. Similar changes in top to root ratio have been documented (Pritchell & Nelson 1951; Bula Rhykerd & Langton 1959); in addition, legume roots (viz. lucerne, red clover and birdsfoot trefoil) were shown to be more sensitive to shading than the tops (Gist & Mott 1957).

Growth factors produced by the action of light have been reported to influence the top growth in broad bean seedlings (Butler R.D. 1963); in what way and to what extent growth regulators were involved in this experiment was not known.

Defoliation can have a complicated effect on plant dry matter production, not only because it affects the relative rates of top and root growths, but also because actual loss of root tissue can occur (Crider 1955; Zykor 1968). In addition, the importance of root reserves and growth regulators has been frequently implicated in regrowth studies (among others Carlson 1966a & b; Mitchell & Denne 1967). Carlson suggested that the mobilization and utilization of reserves in white clover after defoliation might be controlled by growth regulators. That regrowth in white clover depended on reserves for short periods only, up to one week, was reported by Hoshino & Oizumi (1968).

In the 'defoliated' plants, the higher top relative growth rates, while statistically not significant, indicate that the top growth was more efficient than that of the 'undefoliated' control. This agrees

with earlier findings of Brown, Cooper & Blaser (1966) in that younger (one week or less) leaves of white clover are more efficient in terms of dry matter production and carbon dioxide uptake than older (one month) leaves.

The much lower root relative growth rates reflect a partial stoppage in root growth soon after defoliation. Cessation in root growth after defoliation has been well documented (Crider 1955; Butler et al 1959; Ennik 1966; Mitchell & Denne 1967). However root relative growth rates of both 'defoliated' and 'undefoliated' plants became comparable by day 22 (Figure III. 1.2.2).

2. Nodulation

The number of factors that can influence nodulation (see under review of literature) makes interpretation of the data difficult. Although shading and defoliation have been regarded as a means of reducing the supply of carbohydrate (Wilson 1942), this is not their sole effect. As mentioned earlier, growth regulators can be involved in regrowth; to what extent these endogenous growth factors influence nodulation is still not well known. Section I. 3.3.2 presents a more detailed discussion.

A number of workers have examined the effects of defoliation and/or shading on the nodulation of white clover (Wilson 1942), white clover, lucerne and Lotus uliginosus (Butler et al 1959), soyabean (Eaton 1931) and Desmodium uncinatum and Phaseolus atropurpureus (Whiteman & Lulham 1970; Whiteman 1970 a & b). In general nodulation is reduced by shading and defoliation .

In this experiment the level of defoliation was severe. A 30% difference (this percentage being based on the decoded means using the control as 100%; see Appendix IV.1 for details) in total nodule number due to defoliation was seen less than three days (Figure III. 2.2) and this difference increased to 47% by the end of the experiment.

Under shading, the difference reached 32% by harvest 6 (Figure III.2.1) Butler et al (1959), using four-months-old white clover grown in glass-sided boxes, found that reducing the light intensity by 75% was more deleterious to the number of pink nodules than was defoliation to a height of half an inch; the former treatment had 4% and the latter 46% of the original nodules still pink six days after commencement of treatment. This apparent difference in plant response to the imposed treatments was probably a reflection of their relative severity.

However, as will be discussed in more detail later, for about a week none of the defoliated plants in this present experiment had any pink nodules.

Although the effects differed in magnitude, both 'shade' and 'defoliated' plants had a lower number of nodules in all size categories as compared with their respective controls. Overall, reducing the light intensity had little effect on the number and the rate of appearance of small size nodules. Although the number of medium size nodules was lower than that of the control, the rate of appearance was similar (Figure III. 2.1.c). This was not so in the defoliated plants (Figure III. 2.2.c), in which the rate of appearance of medium size nodules was much lower than that of the 'undefoliated' control plants after the fourth harvest. In both treatments, a marked reduction in the number of large size nodules ten days after the commencement of the treatments was clearly evident. A decrease in the number of large size nodules in the control plants (Figure III. 2.3) later on during the experiment (around day 22), could indicate that some of these large, hence earlier produced nodules had reached the end of their natural 'life expectancy'. The appearance of 'green' nodules around this time in the control (light-uncut) plants as shown in Figure III. 3.5, supports this explanation. In the field, white clover nodule longevity and nitrogen fixation can be prolonged. Thus, it is possible that the picture of the relative changes in nodule number under both the 'control' and 'treated' regimes has been complicated by the presence of this 'interphase' between two nodule cycles. To what extent the age of nodules affected the changes in nodule number (in addition to effects caused by the imposed treatments) is unknown.

Bearing the above in mind, the difference in nodule number between the 'control' and 'treated' plants can be attributed to three causes at least. Firstly, there is the loss of individual nodules occurring mostly on the crown roots. Wilson (1942) gave a comprehensive description of the various stages involved in nodule decay and such empty nodule 'hulls' still attached to the crown roots could be seen frequently in this experiment (Plates 9 and 10). Secondly, losses of nodules can occur when the roots to which they are attached decay and slough off (Butler et al 1959). Thirdly, losses due to non-production of roots may occur; root growth may come to a complete stop after

severe physiological shock, as has been well documented (Crider 1955; Butler et al 1959; Mitchell & Denne 1967 and others). The very low root relative growth rates (Figures III. 1.2.1 and Figure III. 1.2.2) show that the defoliation and shading treatments in this experiment severely depressed root growth relative to the control treatment.

From the analysis of nodule number per 100 mg root dry weight (Figure III. 2.9), and from the results of root dry weights (Table III. 1.2.b), it can be seen that the differences in root dry weights on a percentage basis (as shown in Appendix IV. 1) were similar to those in nodule number, indicating that nodule losses were closely related to root losses. However, in this study it is not possible to distinguish between losses due to decay and sloughing from those of non-production. In order to appreciate the contribution of nitrogen to the soil system by nodule and root decay, a detailed study of the rates of root and nodule turnover must be conducted.

The relatively constant number of nodules per unit dry weight root tissue supports the contention of Young (1958) that in white clover the most important factor governing the total number of nodules is the amount of clover root material.

The concept that the nodule (and root) meristem is the centre of an inhibitory activity which will determine further nodulation and that the degree of this inhibition will vary according to the size of the meristem (i.e., larger nodules more inhibitory than small ones) has been discussed by Nutman (1958, 1965). The losses of large size nodules in both 'control' and 'treated' plants, possibly through different causes as discussed above have, nevertheless, similar effects on the rate of appearance of the smaller (medium and small size) nodules. This explanation of the behaviour of the medium size nodules is in agreement with the inhibition concept. The lower rate of appearance of smaller size nodules is possibly a reflection of a rapid increase in their size and in turn has contributed towards the rate of appearance of medium size nodules. This general trend of nodule number increment can also be seen in the three-dimensional graphs (Figure III. 2.4).

Nutman (1958) and later Lim (1963) both using much younger plants (seedlings), have reported a compound Mitscherlich curve type of increment in nodule number with time. Pankhurst (1970) using slightly older (eight weeks) Lotus pedunculatus and L. corniculatus also found a rhythmic trend in nodule number appearance with time. The plants used in this experiment were much older (fourteen weeks), and it was of interest to note that the stepwise increment in nodule number was

still evident.

The negative relationship between the mean nodule weight and nodule number per plant (section III. 2.4.2) agrees with previous suggestions that for a given plant size, there is a relatively constant volume of nodular tissue per plant for a specific host-rhizobium combination (Nutman 1965), and that in white clover this relationship is rather diffuse (Jones 1962).

The nodule colour scheme adopted in this experiment differs from that used by Pate (1958a), who divided nodule colour more or less according to function, i.e. white (new), pink (active) and green (senescent) nodules. It would have been too laborious to divide the nodules into both size and functional groupings, hence a slightly different but much simpler scheme was adopted (detailed in section II. 6.6.1.c).

The change in nodule colour from pink to completely green (Figure III. 2.5 and Plate 7) in less than three days after defoliation is the result of the breakdown of leghaemoglobin in the nodules into legcholeglobin (Virtanen et al 1947). More recently Roponen (1970) reported that in total darkness, pink pea nodules turned green in three days when about half of the haem was broken down. In darkness, production of photosynthate ceases, resulting eventually in the oxidation of leghaemoglobin bacteroids and changes in amino acid metabolism.

Under reduced light intensity there were signs of 'greening' at the base of some large nodules which were however still classified as 'pink' within the classification system used. Plate 8 illustrates some of these slightly green nodules in the 'shade-uncut' plants at harvest 3 (ten days after treatment).

After the initial shock of defoliation, the nodules were green for up to six days, but some of them had pink tissues growing at the apex by day 10 (Plate 8). It is significant that some of the nodules which had turned green were able to produce new active tissues (pink) again. One possible explanation is that, upon resumption of photosynthate supply, some of the younger (hence physiologically more active) nodules were able to resume cell division at the meristem. By harvest 5 (22 days) most nodules in the defoliation treatments were classified as 'pink'. The relationship between nodule colour and fixation will be discussed under the next section.

Reduction in nodule dry weight per plant as a result of shading or defoliation (Figure III. 2.6) follows a similar trend to that

described for total nodule number. Again defoliation was more deleterious to the nodule dry weight than was shading.

The low nodule RGR under shading (0.0105 - 0.0117 mg/mg/day) and defoliation (0.0010 - 0.0181 mg/mg/day)** as compared with their respective controls (0.0223 - 0.0620 mg/mg/day for uncut plants), indicates a partial reduction in nodule growth for at least two weeks after the commencement of the treatments. Again losses can be attributed to decomposition and non-production.

The losses of large nodules possibly caused the decrease in average weight per nodule (Figure III. 2.8). This effect was earlier in the 'defoliated' plants.

3. Nitrogen fixation

Shading reduced the nitrogen percentage of the tops (which did not recover during the experiment) but not the nitrogen percentage of the roots (Figure III. 3.1.). Moderate shading (27% of control) has been found to cause a reduction in the nitrogen percentage of lucerne seedings (Pritchell & Nelson 1951).

Under defoliation the initially lower N% in the tops could be due partly to change in stem to leaf ratio; leaves were known to have higher N% in Lolium perenne (Alberda Th. 1965). The difference between the 'cut' and 'uncut' plants disappeared by harvest 4 (Figure III. 3.2.a).

A similar reduction in N% in the roots under defoliation treatments (Figure III. 3.2.b) was probably the result of a protein demand for top growth. Nitrogen fixation was grossly reduced for about two weeks (Figure III. 3.5 and III. 3.7) and since the pumice has no available NO_3^- nitrogen, the protein for top growth would have to come from deamination and translocation of the root proteins or amino acids. Thus N% in the roots fell and N% for the tops was as low as compatible with top growth and nitrogen supply, until nitrogen fixation was resumed by harvest 4, whereby the N% of both the tops and roots in the 'defoliated' plants became similar to their respective 'undefoliated' controls. Davidson and Milthorpe (1965) pointed out that in cocksfoot (Dactylis glomerata) 40% of the material used for regrowth after defoliation may come from non-carbohydrate reserves.

(** figures in parenthesis are for the first 16 days after the commencement of treatments).

In the nodules both shading and defoliation caused marked reduction in N%. When nitrogen fixation is impaired, glycogen will accumulate within the nodule (Nutman 1965); this change can lead to a lower nitrogen percentage. Autoylsis of bacteroids and their subsequent decomposition could contribute towards some of their losses of nitrogenous material from the nodules. Nodule nitrogen might serve as a source of reserves for regrowth and respiration.

Wilson (1940) cautioned that one might not be able quantitatively to assay all the nitrogenous compounds by Kjeldahl digestion (e.g. nitrate). Although changes in the proportions of different amino acids have been reported by Roponent (1970), it is unlikely that differences in nodule N% could have resulted from inability of Kjeldahl digestion to break down the amino acids presented in the nodules.

Although excretion of nitrogenous compound from nodules could also lead to a lower nodule N%, there was no evidence of increases in the level of nitrogen in either the leachate or the pumice rooting medium (for all samples, the nitrogen detected was similar to that of the blank controls), thus ruling out the possibility of large scale excretions.

If the nitrogen percentage of nodules accurately indicates protein percentage, and hence the amount of bacteroidal tissue present, then nodules with lower bacteroidal content (reflected as lower N%) due to shading, were not impaired in nitrogen fixation. In other words, nitrogen fixation per unit bacteroidal tissue was greater under shading. Shade-uncut plants have slightly higher (but statistically non-significant) rates of acetylene reduction than the light-uncut controls (Figure III. 3.7) provides support for the above hypothesis.

As expected, the results of the relative rates of nitrogen assimilation (RRNA) of the three plant components followed a similar trend to that of their respective relative growth rates (compare Figures III. 1.2.1, III. 1.2.2, III. 2.6 with Figures III. 3.3, III. 3.4). Deviations in RRNA from RGR simply reflect changes in N%, thus difference between tissues (top, roots and nodules) in RRNA depends more on the difference in N% than in their respective RGR.

Total plant nitrogen yield (minus control i.e. seed nitrogen) divided by the weight of nodules and the plant's age in days has been used as a means of assessing the rate of nitrogen fixation in legumes. However this method of estimating relative fixation efficiency does not hold when losses of the components have occurred, either directly (defoliation) or indirectly (loss of nodules) as a result of the treatment. This has been emphasised in section III. 3.2.

However one interesting point appeared in the 'light' control plants (Figure III. 3.5.a) in that the rate was relatively constant (range within 13 - 17 mg nitrogen per gram nodule per day) during the experimental period. In the 'undefoliated' treatment except for harvest 2, where the rate was very high (28 mg nitrogen per gram nodule per day), the rate fell within the fairly narrow range of 18 - 23 mg nitrogen per gram nodule per day. This was comparable to that reported by Pate (1958a), who estimated that in pea nodules the rate of nitrogen fixation was approximately 30 - 100 mg nitrogen per gram nodule fresh weight per day. In the present study the nodule weights were expressed as dry weights.

The second method for the estimation of relative nitrogen fixing efficiency was based on that described by Döbereiner (1966), who plotted linear regressions between $\log_{10}$ total plant nitrogen and total nodule weight, and concluded that the regression coefficient was an index of nitrogen fixing efficiency (see section II. 6.6.2 for details). However this relationship is linear only when both plant and nodule weights are increasing in parallel (Whiteman 1970b). Under the conditions of this experiment, where part of the plant had been removed and nodules lost before sampling, the validity of the regression coefficient as an index for nitrogen fixing efficiency is questionable. As evident from results presented in section III. 3.3, the defoliated plants had apparently higher efficiencies than their controls which supports Whiteman's contention that the linear relationship holds only when there is no loss in plant and nodule weights.

The third method used in this experiment was the acetylene reduction assay. Prolonged acetylene reduction for up to eight hours by detached soyabean nodules has been reported (Sprent 1969), which appeared to depend upon the conditions of incubation. Oxygen concentration, amount for free water and the relative volumes of gas mixtures to nodular tissue were some of the factors cited. More recently Bergersen (1970) cautioned against the use of this method as a direct quantitative estimation of nitrogen fixation without carefully matching the conditions under which nitrogen fixation normally occurred. Hence results from this study should not be strictly extrapolated beyond the conditions of the assay, although one may reasonably generalise from the relative differences between treatments.

The rate of acetylene reduction was markedly reduced by defoliation (to approximately 8 - 10% of the 'undefoliated' control at day three)

and this recovered to about 20% of the control by day 6 (Figure III. 3.7). This is in general agreement with the reduction reported by Moustafa et al (1969), although in their experiment the lowest rate occurred on day 6 (on day three in this present study), this apparent variance could be due partly to the severity of treatment imposed in this experiment, or the unknown field conditions (the changes in temperature and light intensity) in Moustafa's experiment, and it could also be due to the way the rate was measured. Moustafa expressed the rate as m moles ethylene per gram root system (root plus nodule) per hour; a decrease in nodule weight per gram of root as a result of defoliation could lead to a lower reducing activity per unit weight of root tissue.

Recovery of the reducing activity by the defoliated plants was completed by day 10 and coincided with the reappearance of pink pigment in the nodule apex. It is significant that, although the nodules were still more than half green, their enzyme activity became similar to that of the control. This recovery probably resulted from the resumption of photosynthate supply from regrowth. As the proportion of this 'pink' region on nodules of the defoliated plants was much smaller than those of the control plants (wholly pink), this suggests that the apical tissue was more efficient on a per unit basis. Again if nodule percentage nitrogen reflects accurately the amount of bacteroidal tissue, then results from percentage nitrogen determination (Figure III. 3.2.c) provides further support to this suggestion. This also points to the inadequacy of using visual assessment of colour as an accurate index of nitrogen fixation. Leghaemoglobin concentration correlates well with ethylene production (Schwinghamer et al 1970) and is an index of bacteroid density (Bergersen 1961), hence a quantitative assay of leghaemoglobin could be more meaningful than a colour description.

Although shading has been reported to reduce nitrogen fixation (see under review of literature), the results of this experiment showed no immediate decrease in the acetylene reduction activity due to shading. It was probable that reducing the light intensity by 42% (0.96 m.v., see Table III. 1) was insufficient to cause a lowering of photosynthate supply to influence the actual fixation mechanism, although slight greening at the base of some nodules were observed (Plate 8). It is therefore, likely that nodulation had been affected by a reduction in photosynthate before nitrogen fixation. The absolute reduction in nitrogen fixation by the whole symbiotic system (Table III. 3.1) as a result of shading was due therefore to the reduced rate of nodule

formation (Figure III. 2.1 and III. 2.6), rather than a loss in efficiency.

The absence of any significant difference between the rates of all treatments after day 10 indicated that a common level of efficiency existed for this particular host-rhizobium combination, which supports the result estimated from the total plant nitrogen yield (i.e. method one).

The variability between replicates in reducing acetylene to ethylene is clearly evident (Figure III. 3.6). Although diurnal variations in acetylene reduction due to photosynthetic activity have been reported (Hardy et al 1968; Schwinghamer et al 1969) it is also possible that the variations are inherent differences in the symbionts, thus points to the possibility of selecting for plants with higher nitrogen fixing ability even within a relatively uniform (10% open pollination) population.

The result also points to the usefulness of the acetylene reduction assay for detecting instantaneous activity of the nitrogen fixing enzyme system which cannot be detected by the traditional long term method previously described. Besides, in this experiment, the technique also allows one to distinguish between effects on nodulation and effects on fixation per unit nodule tissue.

CONCLUSION

1. Both shading and defoliation caused root losses, the magnitude of which reflected the severity of treatment.
2. Both treatments caused marked reduction in nodule numbers in each of three size categories. The rate of medium size nodule appearance in the 'shade' plants was similar to that of the 'light' plants whereas that of the 'defoliated' plants was much lower than that of the 'undefoliated' controls. The release of inhibitory activity due to the inactivation of the larger nodules could have been responsible for the subsequent increases in the number of medium and small size nodules in all treatments.
3. Losses in nodule number due to defoliation were attributed to three major causes,
   - i. nodule decay and autolysis leaving behind empty nodule 'hulls'
   - ii. sloughing off either independently or with the root material on which they were attached; and
   - iii. non-production due to stoppage in root growth.
4. The constant number of nodules per unit weight of root tissue confirmed that the amount of root material is one of the main determinant factors of nodule number in white clover.
5. Reduction in total nodule weight per plant under shading and defoliation was probably due to the overall reduction in nodule number per plant and a trend towards a lower average weight per nodule in the 'treated' plants was probably due to losses of large size nodules.
6. The negative correlation between average weight per nodule and the number of nodules per plant was in agreement with previous findings, that for each host plant-rhizobial combination there tends to be a relatively constant volume of bacterial tissue for a given weight of plant.
7. From studies of nodule density per inch, the following points emerged:-
   - i. With time the point of maximum nodule concentration shifted from 1 - 2 inches around the crown to a region 3 - 6 inches from the crown;
   - ii. In the control (light-uncut) plants the nodule number and hence nodule weight increased in a stepwise fashion with time; and
   - iii. This stepwise increment was along the entire length of the roots bearing nodules.

8. Both the traditional method for the estimation of the nitrogen fixation and the regression of total plant nitrogen content on nodule weight were found to be unsuitable for short term experiments in which part of the plant material was lost either directly or indirectly as a result of treatment. Howver, the acetylene reduction assay as an index of the nitrogen fixing enzyme activity proved to be satisfactory in detecting instantaneous changes in nitrogen fixing capabilities.

9. The conventional colour scheme adopted in this study as a visual assessment of the fixation activity proved to be inadequate. Since partly green nodules were found to be capable of reducing acetylene and therefore (presumably) of reducing nitrogen, a haemoglobin concentration assay would have been more satisfactory.

10. Using the acetylene reduction assay and conventional techniques, it was demonstrated that moderate shading reduced the amount of nitrogen fixed per plant primarily by reducing the rate of formation of nodule tissue. However, the reduction resulting from complete defoliation was only partly due to this cause, being augmented by (a) actual loss of nodules and (b) a temporary gross reduction in the fixation capability of all nodules.

Appendix II.1 Nutrient medium for rhizobial cultures

Mannitol	10.0	g per litre
KH_2PO_4	0.5	
$MgSO_4$	0.2	
NaCl	0.1	
$CaCO_3$	3.0	
Yeast extracts	5.0	
Agar	12.5	

Distilled water to make up to one litre.

Appendix II.2 Nutrient solution for the plants

$CaCl_2$	10	ml (molar solution) per litre
KCl	10	
$MgSO_4$	4	
KH_2PO_4	4	
Fe-EDTA	2	
Micro-nutrients	2	

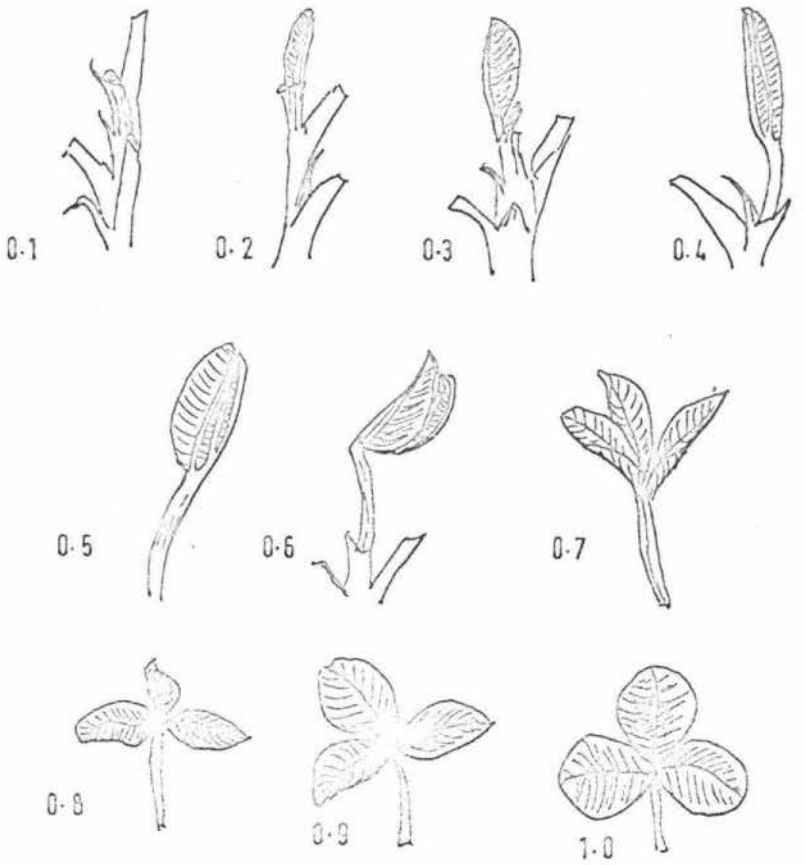
The composition of the micro-nutrients were as follows :-

H_3BO_3	2.86	g per litre
$MnCl_2$	1.18	
$ZnSO_4$	0.11	
$CuSO_4$	0.05	
$NaMoO_4$	0.025	
$CoCl_2$	0.05	

The Fe-EDTA was made up from the following procedures :-

Dissolve 5 g of NaOH in 800 ml of distilled water, add 33.2 g of EDTA (tetra-sodium salt), stir until dissolve, add 24.9 g of $FeSO_4$ and stir. Make up to one litre, and aerate the solution overnight using an aquarium airpump. The pH of the nutrient solution was 6.5 .

Appendix II.3 The ten stages of morphologic development of white clover (Trifolium repens L) leaves as described by Carlson (1966).



0.0 Leaf bud is not visible in axil of proceding leaf.

0.1 Approximately 25% of the folded leaf is visible as it emerges from its membranous stipule.

0.2 Folded leaf is completely visible but the petiole remains enclosed in its membranous stipule.

0.3 Petiole is visible and all leaflets aretightly folded.

0.4 A slight separation of individual leaflets is apparent at the midvein.

0.5 Individual leaflets are folded but are starting to separate from each other.

0.6 Leaflets are clearly separated from each other, and each leaflet is approximately 10% unfolded.

0.7 Leaflets are approximately 30% unfolded.

0.8 Leaflets are approximately 60% unfolded.

0.9 Leaflets are approximately 90% unfolded

1.0 Leaflets are 95% unfolded or slightly cupped.

Appendix II.4 Calender of work

Date	Events
15-12-69	Germination
21-12-69	Thin to three plants per pot
28-12-69	Thin to two plants per pot
29-12-69	First pretreatment harvest
5- 1-70	Thin to one plant per pot
11- 1-70	Weekly rhizobium inoculation terminates
12- 1-70	Second pretreatment harvest
	First spray of Malathion
26- 1-70	Third pretreatment harvest
2- 2-70	Spray malathion
8- 2-70	All regrouping of pots stops
9- 2-70	Fourth pretreatment harvest
	Spray Malathion
22- 2-70	Base harvest
23- 2-70	Treatment commences
26- 2-70	First harvest
1- 3-70	Second harvest
5- 3-70	Third harvest
10- 3-70	Fourth harvest
14- 3-70	Spray Malathion
17- 3-70	Fifth harvest
24- 3-70	Sixth harvest
	Experiment terminates

Appendix II.5 Leaf area ratings (from Williams, Evans & Ludwig 1964)

Rating	Area cm^2	Rating	Area cm^2	Rating	Area cm^2
1	-	9	0.794	17	5.01
2	0.158	10	1.00	18	6.31
3	0.200	11	1.26	19	7.94
4	0.251	12	1.58	20	10.00
5	0.316	13	2.00	21	12.6
6	0.398	14	2.51	22	15.8
7	0.501	15	3.16	23	20.0
8	0.631	16	3.98	24	25.1

Appendix II.6 Calculation of Relative Growth Rate (RGR) and Relative Rate of Nitrogen Assimilation (RRNA).

The following formula was used in the calculation of RGR :-

$RGR = (\log_e W_2 - \log_e W_1)/(t_2 - t_1)$ in mg/mg/day , where W_2 and W_1 were the dry weights at time t_2 and t_1 respectively.

Due to plant variations, some of the samples collected in the earlier harvests were heavier than those from the later harvests. To reduce this problem, the RGR was calculated from alternate harvests ie.,

RGR 1 (day 6½) = harvest 3 (day 10) - harvest 1 (day 3)
RGR 2 (day 10½) = harvest 4 (day 15) - harvest 2 (day 6)
RGR 3 (day 16) = harvest 5 (day 22) = harvest 3 (day 10)
RGR 4 (day 22) = harvest 6 (day 29) - harvest 4 (day 15)

The RGR was determined for each replicate and statistical significances were tested by the within-harvest analysis of variance (split-plot), using the untransformed values.

Using the same analytical method, the RRNA as described by Gibson (1965) was calculated ie.,

$RRNA = (\log_e N_2 - \log_e N_1)/(t_2 - t_1)$ in mgN/mgN/day , where N_2 and N_1 were the nitrogen yields in mg at time t_2 and t_1 respectively.

RRNA 1, 2, 3 & 4 were calculated as above. By using RRNA the initial differences in total nitrogen content between plants were taken into consideration, hence a sounder basis for comparing nitrogen fixing ability.

Appendix II.7 Summary of micro-Kjeldahl nitrogen determination (as described by Clements 1970)

Reagents :-

Digestion mixture conc. H_2SO_4 (1 litre) with K_2SO_4 (100 g) and selenium (1 g).

Sodium hydroxide 250 g per litre.

Boric acid 2% w/v H_3BO_3 with distilled water containing 2% v/v indicator mixture.

Indicator mixture 5 volumes of 0.1% ethanolic solution of bromocresol green and 1 volume of 0.1% ethanolic solution of methyl red.

Hydrochloric acid 0.01 N HCl (diluted from standard 1 N HCl Kindly supplied by Dr. C.V.Fife , Soils Dept. Massey University).

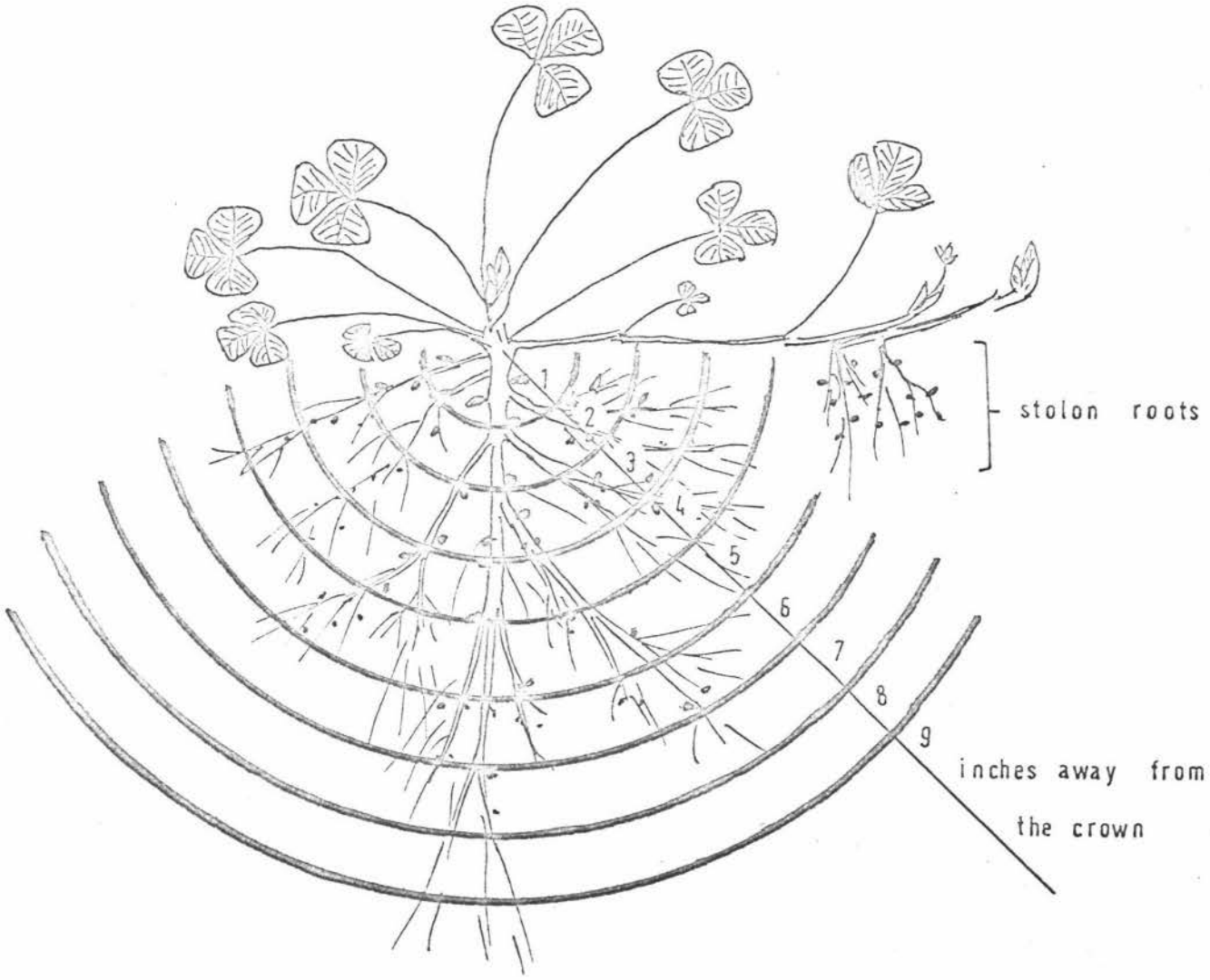
Procedures :-

1. 230 mg of the oven dried sample was accurately weighed and digested with 5 ml of conc. H_2SO_4 in a fume cupboard.
2. The digestate was diluted to 100 ml in a volumetric flask with distilled water, and a 5 ml sample was taken for distillation with 10 ml of NaOH in a Markham still.
3. The distilled sample was collected into 5 ml boric acid-indicator mixture.
4. The amount of ammonia present was determined by titration with 0.01 N HCl.
5. For 230 mg sample the percentage nitrogen (N%) would be, N% = titre (sample) - titre (control), where the titres were in ml 0.01 N HCl. For other sample weights appropriate corrections were made.

Macro-Kjeldahl nitrogen digestion

This was carried out in the animal physiological unit, Massey Uni. For each determination 5 g oven dried pumice (or 5 ml leachate) was used, in all samples the amount of nitrogen detected was similar to that of the blank controls, both before and after the treatments.

Appendix II.8 showing the system adapted for the numbering of root inch-segments



Inches 7 + 8 + 9 are grouped as 7 in the text

Appendix II.9 Acetylene reduction technique

The technique was basically similar to that used in the laboratory of Dr. W.B.Silvester (Department of Botany, Auckland University), to whom the author is indebted.

A gas chromatography unit (Varian aerograph series 1200), with a hydrogen flame ionization detector and nitrogen carrier gas was used.

a. temperatures

oven	70° C
detector	120° C
injector	40° C

b. gas sample 160 ul, using disposable syringes for normal sampling and gas tight glass syringe for the standard determinations.

c. carrier gas (nitrogen) flow rate 25 ml/min
hydrogen gas at 10 p.s.i. giving a total gas flow rate of approximately 60 ml/min.

d. filtered room air for combustion.

e. the gases were measured on range 10^{-11} and the size of the peaks regulated by attenuation.

f. column packing 'Poropak T', 120 mesh ; column length 4 feet ; column diameter $\frac{1}{8}$ inch .

Procedures

The freshly cut root and nodule segments were placed in labelled 30 ml McCartney bottles and sealed with air-tight rubber septum. These were incubated at room temperature (average 23° C). The air in the bottles was completely replaced with the following gas mixture by a vacuum device (shown in Plate 11) before incubation.

Acetylene	10%
Oxygen	20%
Argon	70%

Several precautionary measures were taken to ensure that the system was leak proof and the correct proportions of the three gases were given each time.

a. argon was used to flush the system and the vials three times after the previous gas had been removed by the vacuum pump.

b. after flushing, the argon was pumped out until the manometer reached 600 mm Hg., the vacuum pump was cut off at control A (see Plate 11).

This slight positive pressure (0.23 atmosphere) prevented any possible leakage of air into the system through the needle holes in the rubber septum.

c. acetylene and oxygen were then introduced to the appropriate levels.

d. finally argon was introduced to bring the manometer reading to 1.0 atmosphere.

e. a new rubber septum was used for each harvest.

f. all joints were sealed with silicon vacuum grease.

Generally for each replicate of a treatment, three samples were taken within the first hour, later three to four more samples were taken at approximately hourly intervals up to five hours.

On completion of the assay the volume of the gas used in the vial was determined by water displacement, and the number and weight of the nodules determined as detailed in section II.6.6 .

Water displacement method

With the root and nodule tissue in the vial the gas was displced with water from a finely graduated burette (0.1 ml) ; any air bubbles formed were removed with a needle.

Calculation of the acetylene and ethylene standards

As the hydrogen flame ionization detector is more sensitive to ethylene (C_2H_4) than acetylene (C_2H_2), a correction factor has been used to bring the value of C_2H_4 to the same molar basis as that of the C_2H_2 . The correction factor was obtained from fifty-three independent samples collected from the following ratio combinations :-

	Acetylene	Ethylene	Argon	Ratio of $\frac{C_2H_2}{C_2H_4}$
a.	100%	-	-	1 : 0
b.	-	100%	-	0 : 1
c.	10%	10%	80%	1 : 1
d.	10%	20%	70%	1 : 2
e.	20%	10%	70%	2 : 1

The average was found to be 0.828 and was taken as 0.83 which agreed with that used by Dr. W.B.Silvester.

Calculation of ethylene formation

The amount of ethylene formed from the reduction of acetylene was calculated as follows :-

Knowing the volume of acetylene in the vial (10% of the gas mixture) and the ratio of the two gases, (from their relative areas) the percentage of ethylene could be calculated.

$$\% \text{ of } C_2H_4 = \frac{E \times 0.83}{A} \times 0.1$$

where E and A were the relative areas of ethylene and acetylene minus their respective controls (as measured by the integrator), and corrected to the same molar basis (0.83 was the correction factor).

In terms of actual volume ie., ml

$$C_2H_4 \text{ (ml)} = \frac{E \times 0.83}{A} \times 0.1 \times \text{vol}$$

where vol was the volume of the gas mixture in ml, determined by the water displacement method.

$$C_2H_4 \text{ (}\mu\text{l)} = \frac{E \times 0.83}{A} \times 0.1 \times \text{vol} \times 10^3$$

where μl = micro-litre

Assuming standard temperature and pressure, 1 μ mole of gas would occupy 22.4 μl ; converting this to m.u.mole (ie. x 10^3) and expressing the result as that from 1 mg of the nodule, we would now have :-

$$\text{m.}\mu\text{.mole } C_2H_4 \text{ / mg nodule}$$

$$= \frac{E \times 0.83}{A} \times 0.1 \times \text{vol} \times 10^3 \times 22.4^{-1} \times Wt^{-1} \times 10^3$$

$$= \frac{E \times \text{vol}}{A \times wt} \times \frac{0.83}{22.4} \times 10^5$$

$$= \frac{E \times \text{vol}}{A \times wt} \times 3.7053 \times 10^3$$

where wt = weight of nodule in mg.

A computer program was written to calculate this .

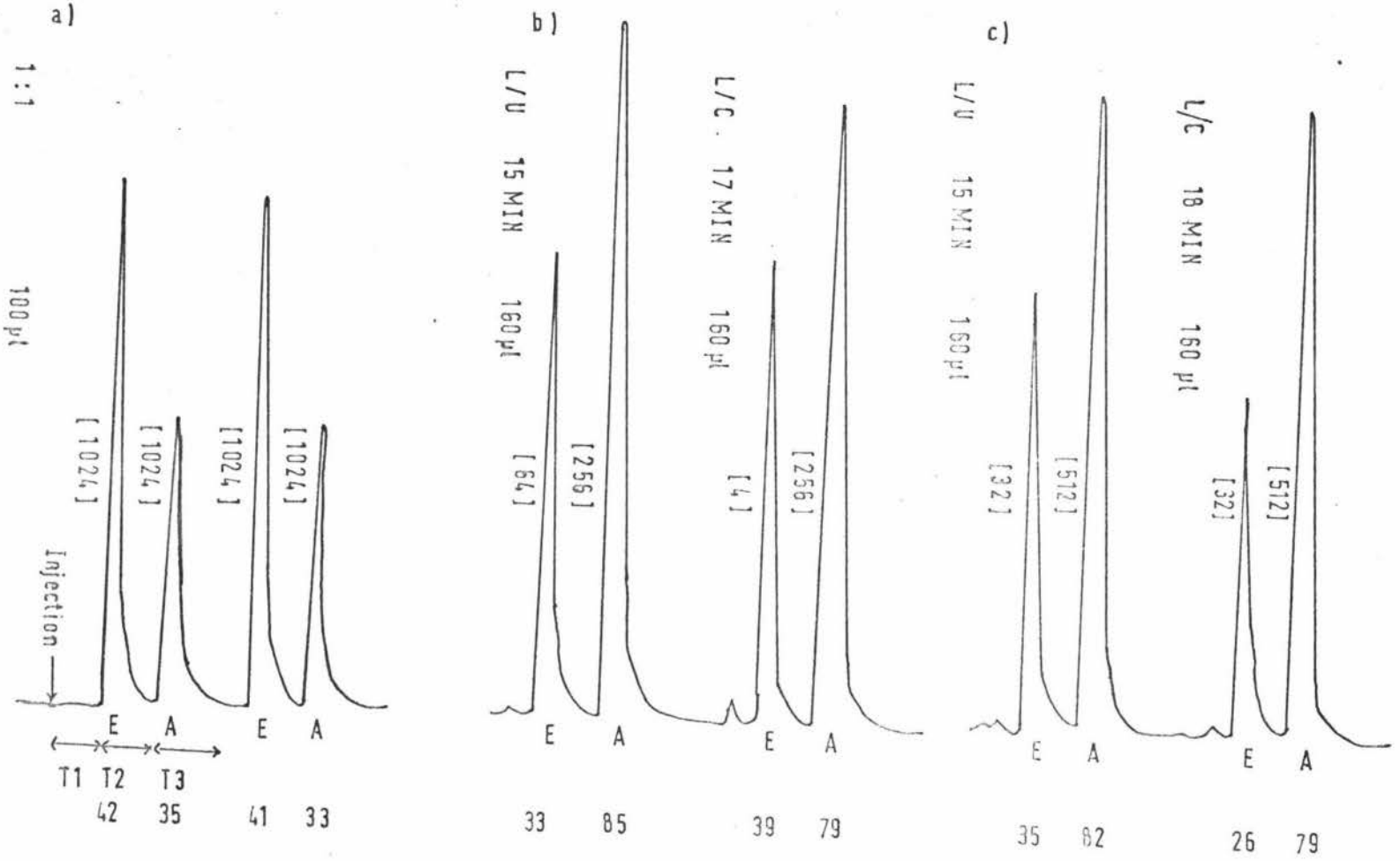


DIAGRAM 4 Typical chromatograms of :-

a). ratio standand of 10% acetylene to 10% ethylene

b). light - uncut (L/U) & light - cut (L/C) at harvest 1 (3 days from defoliation)

c). L/U & L/C at harvest 3 (10 days from defoliation)

```
C
      PROGRAM FOR CALCULATING ACETYLENE REDUCTION                    CHU
C
      DIMENSION X(100),Y(100),IRAP(100)
      IF(SENSE SWITCH  9)2,2
    2 IHOLD=0
      N=0
    1 READ 100,IREP,IHAR,ITRE,T,ET,ER,AT,AR,CON,VOL,WGT
  100 FORMAT(3I2,4X,8F7.1)
    3 IF(IHOLD)18,10,18
   18 IF(IHOLD-IHAR)21,16,21
   10 PRINT 101,ITRE
  101 FORMAT(13H1TREATMENT    ,I1)
   14 PRINT 102,IHAR
  102 FORMAT(13H HARVEST NO  ,I1/)
   16 Z=ET*ER*.83/((AT*AR)-CON)*VOL*100000./(WGT*22.4)
      N=N+1
      IRAP(N)=IREP
      X(N)=T
      Y(N)=Z
      IHOLD=IHAR
      IF(SENSE SWITCH 9)13,1
   21 SUMX=0
      SUMY=0
      SUMXX=0
      SUMYY=0
      SUMXY=0
      DO50L=1,N
      SUMX=SUMX+X(L)
      SUMY=SUMY+Y(L)
      SUMXX=SUMXX+(X(L)*X(L))
      SUMYY=SUMYY+(Y(L)*Y(L))
   50 SUMXY=SUMXY+(Y(L)*X(L))
      FN=N
      XMEAN=SUMX/FN
      YMEAN=SUMY/FN
      SXX=SUMXX-(SUMX*SUMX)/FN
      SYY=SUMYY-(SUMY*SUMY)/FN
      SXY=SUMXY-(SUMX*SUMY)/FN
      B=SUMXY/SUMXX
      R=SXY/SQRTF(SXX*SYY)
      SSDV=SYY-(SXY*SXY)/SXX
      ASDFR=SSDV/(FN-2.)
      SSDFR=SQRTF(ASDFR)
      SSDRC=SSDFR/SQRTF(SXX)
      RMSYX=(SUMYY-((SUMXY*SUMXY)/SUMXX))/(FN-1.)
      TTT=(YMEAN-(B*XMEAN))/SQRTF(RMSYX*((1./FN)+((XMEAN*XMEAN)/SXX)))
      TT=B/SSDRC
      DF=FN-2.
      PRINT 106,B,B,R,SSDRC,TT,DF,TTT
  106 FORMAT(1H ,10HREGRESSION,2X,9HCOEFF B =,F12.4/1H ,3HY =,F12.4,1X,1
     1HX/1H ,11HCORRELATION,1X,9HCOEFF R =,F12.4/1H ,6HSAMPLE,1X,3HSTD,1
     2X,3HDEV,1X,2HOF,1X,3HTHE,1X,3HREG,1X,7HCOEFF =,F12.4/1H ,3HTT=,F12
     3.4,2X,4HDF =,I4,2X,10HTEST FOR T,2X,5HTTT =,F12.4)

      IHOLD=0
      N=0
      GOTO3
   13 CALL EXIT
      END
```

APPENDIX II.10

```
C
C     SPLIT PLOT ANALYSIS OF VARIANCE          CHU   5-11-70
C
      DIMENSION X(2,2,7),T(2,2),TL(2),TC(2),F(2,7),B(7),TITLE(5),TLM(2),
     1TCM(2),TM(2,2),IH(6)
   50 READ 20,TITLE
      READ 101,(IH(N),N=1,6)
      L=0
      N=0
   51 DO 21 I=1,2
      DO 21 J=1,2
      TM(I,J)=0
   21 T(I,J)=0
      DO 22 I=1,2
      TLM(I)=0
   22 TL(I)=0
      DO 23 J=1,2
      TCM(J)=0
   23 TC(J)=0
      DO 24 K=1,7
   24 B(K)=0
      DO 25 I=1,2
      DO 25 K=1,7
   25 F(I,K)=0
      DO 26 I=1,2
      DO26 J=1,2
      DO 26 K=1,7
   26 X(I,J,K)=0
      SUM=0
      GTSS=0
      BK=0
      FL=0
      FC=0
      FMP=0
      S=0
      READ 1,(((X(I,J,K),K=1,7),J=1,2),I=1,2)
      DO 2 I=1,2
      DO 2 J=1,2
      DO 2 K=1,7
      SUM=SUM+X(I,J,K)
    2 GTSS=GTSS+X(I,J,K)*X(I,J,K)
      DO 3 I=1,2
      DO3 J=1,2
      DO 3 K=1,7
    3 T(I,J)=T(I,J)+X(I,J,K)
      DO 4 I=1,2
      DO 4 K=1,7
      DO 4 J=1,2
    4 F(I,K)=F(I,K)+X(I,J,K)
      DO 5 K=1,7
      DO 5 I =1,2
    5 B(K)=B(K)+F(I,K)
      DO 6 I=1,2
      DO 6 J=1,2
    6 TL(I)=TL(I)+T(I,J)
      DO 7 J=1,2
      DO 7 I=1,2
    7 TC(J)=TC(J)+T(I,J)
      CF=(SUM*SUM)/28.
```

```
      TOTAL=GTSS-CF
      DO 8 K=1,7
    8 BK=BK+(B(K)*B(K))
      BK=(BK/4.)-CF
      DO 9 I=1,2
    9 FL=FL+(TL(I)*TL(I))
      FL=(FL/14.)-CF
      DO 10 J=1,2
   10 FC=FC+(TC(J)*TC(J))
      FC=(FC/14.)-CF
      DO 11 I=1,2
      DO 11 K=1,7
   11 FMP=FMP+(F(I,K)*F(I,K))
      FMP=(FMP/2.)-CF
      ERRA=FMP-FL-BK
      DO 12 I=1,2
      DO 12 J=1,2
   12 S=S+(T(I,J)*T(I,J))
      S=(S/7.)-CF
      FI=S-FL-FC
      ERRB=TOTAL-FMP-FC-FI
      SM1=BK/6.
      SM2=FL
      SM3=ERRA/6.
      SM4=FC
      SM5=FI
      SM6=ERRB/12.
      FCAL1=SM1/SM3
      FCAL2=SM2/SM3
      FCAL4=SM4/SM6
      FCAL5=SM5/SM6
      SE1=SQRTF(SM3/14.)
      SE2=SQRTF(SM6/14.)
      SE12R=SQRTF(SM6/7.)
      DO 30 I=1,2
   30 TLM(I)=TL(I)/14.
      DO 31 J=1,2
   31 TCM(J)=TC(J)/14.
      DO 32 I=1,2
      DO 32 J=1,2
   32 TM(I,J)=T(I,J)/7.
      D15=SE1*2.447
      D11=SE1*3.707
      D25=SE2*2.179
      D21=SE2*3.055
      D35=SE12R*2.179
      D31=SE12R*3.055
      AV=SUM/28.
      CV=((SQRTF(SM6))/AV)*100.
      N=N+1
      PRINT 33,TITLE,IH(N)
      PRINT 34
      PRINT 35
      PRINT 34
      PRINT 36,BK,SM1,FCAL1
      PRINT 37,FL,SM2,FCAL2
      PRINT 38,ERRA,SM3
      PRINT 34
      PRINT 39,FC,SM4,FCAL4
      PRINT 40,FI,SM5,FCAL5
```

```
      PRINT 41,ERRB,SM6
      PRINT 34
      PRINT 42,TOTAL
      PRINT 34
      PRINT 52,CV
      PRINT 34
      PRINT 43
      PRINT 44,(TLM(I),I=1,2)
      PRINT 45,SE1,D15,D11
      PRINT 43
      PRINT 46,(TCM(J),J=1,2)
      PRINT 45,SE2,D25,D21
      PRINT 43
      PRINT 47,((TM(I,J),I=1,2),J=1,2)
      PRINT 45,SE12R,D35,D31
      PRINT 43
      L=L+1
      IF(L-6)51,49,49
   49 GOTO 50

      FORMAT STATEMENTS

    1 FORMAT(6X,7F9.0)
   20 FORMAT(5A4)
   33 FORMAT(1H ,20HANALYSIS OF VARIANCE,2X,5A4,2X,7HHARVEST,1X,I2)
   34 FORMAT(1H ,70H----------------------------------------------------------
     1----------)
   35 FORMAT(1H ,6HSOURCE,1X,3HD F,4X,4HS.S.,12X,4HM.S.,10X,5HF CAL,5X,5
     1HF REQ,5X,6HRESULT)
   36 FORMAT(1H ,3HREP,6X,1H6,2F16.4,F10.4,1X,11H4.28(8.47) )
   37 FORMAT(1H ,5HLIGHT,4X,1H1,2F16.4,F10.4,1X,11H5.99(13.74))
   38 FORMAT(1H ,7HERROR 1,2X,1H6,2F16.4)
   39 FORMAT(1H ,3HCUT,6X,1H1,2F16.4,F10.4,1X,10H4.75(9.33))
   40 FORMAT(1H ,3HC*L,6X,1H1,2F16.4,F10.4,1X,10H4.75(9.33))
   41 FORMAT(1H ,7HERROR 2,1X,2H12,2F16.4)
   42 FORMAT(1H ,5HTOTAL,3X,2H27,F16.4)
   43 FORMAT(1H ,/)
   44 FORMAT(1H ,15HMEANS FOR LIGHT,2F16.4)
   45 FORMAT(1H ,5HS.E.=,F10.4,1X,5HD.05=,F10.4,1X,5HD.01=,F10.4)
   46 FORMAT(1H ,15HMEANS FOR CUT  ,2F16.4)
   47 FORMAT(1H ,21HMEANS FOR LIGHT X CUT,4F16.4)
   52 FORMAT(1H ,5HC.V.=,F10.2)
  101 FORMAT(6I2)
      END
```

Appendix III.1.1.a. Total leaf area per plant

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest		1	2	3	4	5	6
Source	DF	M.S.	M.S.	M.S.	M.S.	M.S.	M.S.
Rep	6	0.0278	0.0445	0.0582	0.0984	0.0616	0.1504
Light	1	0.0091 ns	0.0090 ns	0.0052 ns	0.0003 ns	0.0292 ns	0.0022 ns
Error 1	6	0.0188	0.0159	0.0145	0.0062	0.0206	0.0224
Cut	1	4.9865 **	2.4533 **	2.0936 **	1.5559 **	1.2960 **	1.1294 **
L x C	1	0.0039 ns	0.0560 ns	0.0232 ns	0.0215 ns	0.0018 ns	0.0003 ns
Error 2	12	0.0059	0.0129	0.0119	0.0143	0.0092	0.0297
C.V. %		19	37	29	32	25	49

Appendix III.1.1.b. Total leaf number per plant

Summary of within-harvest analysis of variance sqrt (X x 100)

Harvest		1	2	3	4	5	6
Source	DF	M.S.	M.S.	M.S.	M.S.	M.S.	M.S.
Rep	6	114.8	207.0	337.9	726.9	693.4	1705.1
Light	1	5.5 ns	4.4 ns	0.4 ns	9.4 ns	1774.0 *	662.3 ns
Error 1	6	53.8	48.5	83.1	63.6	183.4	277.2
Cut	1	8584.3 **	5502.3 **	6734.6 **	6695.0 **	7957.3 **	6937.2 **
L x C	1	8.7 ns	136.0 ns	0.1 ns	111.5 ns	438.6 *	230.0 ns
Error 2	12	83.3	42.1	81.0	157.7	59.0	287.9
C.V. %		17	11	13	15	8	16

Appendix III.1.1.c Average leaf size

Summary of within-harvest analysis of variance untransformed

Harvest		1	2	3	4	5	6
Source	DF	M.S.	M.S.	M.S.	M.S.	M.S.	M.S.
Rep	6	0.97	0.23	0.46	0.34	0.57	0.07
Light	1	0.31 ns	0.38 ns	0.71 ns	0.48 ns	6.80 *	6.64 **
Error 1	6	0.38	0.43	0.36	0.62	0.58	0.09
Cut	1	37.88 **	21.24 **	22.96 **	18.19 **	16.78 **	25.38 **
L x C	1	0.14 ns	0.16 ns	4.07 **	0.23 ns	5.36 **	6.35 *
Error 2	12	0.48	0.46	0.31	0.36	0.44	0.68
C.V. %		18	16	11	13	12	14

Appendix III.1.2.a Top dry weight

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest / Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0469	0.0848	0.0699	0.0705	0.0608	0.1758
Light	1	0.0054 ns	0.0129 ns	0.0828 ns	0.0313 ns	0.2310 *	0.0553 ns
Error 1	6	0.0098	0.0169	0.0154	0.0153	0.0194	0.0213
Cut	1	2.4480 **	2.1015 **	2.2070 **	1.8258 **	2.1074 **	1.5638 **
L x C	1	0.0032 ns	0.0704 *	0.0808 *	0.0000 ns	0.0002 ns	0.0011 ns
Error 2	12	0.0061	0.0147	0.0129	0.0142	0.0088	0.0312
C.V.%		20	32	29	32	24	50

Appendix III.1.2.b Root dry weight

Summary of within-harvest analysis of variance $\log_{10}$(X x 100)

Harvest / Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0293	0.0459	0.0642	0.0637	0.0543	0.0754
Light	1	0.0188 ns	0.0049 ns	0.1004 ns	0.0279 ns	0.1379 **	0.1374 **
Error 1	6	0.0242	0.0115	0.0215	0.0067	0.0093	0.0095
Cut	1	0.2008 **	0.1181 ns	0.4132 **	0.5650 **	0.7726 **	0.4334 **
L x C	1	0.0317 ns	0.0048 ns	0.0878 *	0.0462 ns	0.0366 *	0.0002 ns
Error 2	12	0.0123	0.0285	0.0115	0.0157	0.0064	0.0252
C.V.%		29	48	28	33	20	44

Appendix III.1.3. Top to root ratio

Summary of within-harvest analysis of variance untransformed

Harvest / Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.3695	4.4238	0.9478	2.0046	0.9527	1.8665
Light	1	0.0041 ns	5.0066 ns	0.0869 ns	1.1972 ns	0.6883 ns	2.3143 ns
Error 1	6	0.2612	5.3279	0.8587	3.3898	0.6193	1.7465
Cut	1	54.4887 **	114.2512 **	78.0891 **	67.9226 **	55.4696 **	60.0064 **
L x C	1	0.4128 ns	8.2297 ns	0.0357 ns	11.3793 ns	6.1946 *	3.9151 ns
Error 2	12	0.2558	7.2601	0.7032	3.3345	0.8577	1.4712
C.V.%		16	63	18	31	15	19

Appendix III.1.4.a Relative growth rate of tops (mg/mg/day)

Summary of within-harvest analysis of variance untransformed

Period ‡		1	2	3	4
Source	DF	M.S.	M.S.	M.S.	M.S.
Rep	6	0.0024	0.0010	0.0002	0.0012
Light	1	0.0050 ns	0.0002 ns	0.0013 ns	0.0000 ns
Error 1	6	0.0027	0.0028	0.0019	0.0007
Cut	1	0.0006 ns	0.0006 ns	0.0000 ns	0.0002 ns
L x C	1	0.0128 *	0.0053 ns	0.0032 *	0.0000 ns
Error 2	12	0.0019	0.0012	0.0006	0.0019

‡ (Periods 1, 2, 3 & 4 are 6½, 10½, 16 and 22 days from treatment respectively for details see Appendix II.6)

Appendix III.1.4. b Relative growth rate of roots (mg/mg/day)

Summary of within-harvest analysis of variance untransformed

Period		1	2	3	4
Source	DF	M.S.	M.S.	M.S.	M.S.
Rep	6	0.0014	0.0011	0.0006	0.0001
Light	1	0.0035 ns	0.0006 ns	0.0001 ns	0.0011 ns
Error 1	6	0.0028	0.0016	0.0011	0.0003
Cut	1	0.0040 ns	0.0108 *	0.0020 ns	0.0002 ns
L x C	1	0.0244 **	0.0013 ns	0.0003 ns	0.0010 ns
Error 2	12	0.0021	0.0015	0.0006	0.0012

Appendix III.2.1.a Total number of nodules per plant

Summary of within-harvest analysis of variance Sqrt (X x 100)

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	1429	988	2214	3140	4062	6236
Light	1	908 ns	216 ns	14842 **	1403 ns	5763 ns	12635 ns
Error 1	6	507	502	2009	1221	2117	4337
Cut	1	5901 **	7519 **	5320 *	10510 *	36566 **	33535 *
L x C	1	611 ns	332 ns	291 ns	29 ns	457 ns	4679 ns
Error 2	12	542	794	965	1540	1135	5995
C.V.%		14	19	18	23	16	35

Appendix III.2.1.b Nodule number over 3 mm per plant

Summary of within-harvest analysis of variance Sqrt (X x 100)

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	258	570	571	481	471	967
Light	1	145 ns	29 ns	370 *	1996 ns	7612 **	1373 *
Error 1	6	174	341	61	566	177	126
Cut	1	337 *	281 ns	2695 **	5454 **	5929 **	1150 *
L x C	1	94 ns	153 ns	995 *	5 ns	21 ns	845 ns
Error 2	12	57	96	72	158	227	193
C.V.%		12	17	11	19	22	29

Appendix III.2.1.c Nodule number between 1 - 3 mm per plant

Summary of within-harvest analysis of variance Sqrt (X x 100)

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	864	393	1382	977	2427	5862
Light	1	70 ns	166 ns	695 ns	911 ns	3718 ns	1530 ns
Error 1	6	138	185	501	859	2664	1537
Cut	1	1319 *	1282 ns	4063 **	1546 ns	21924 **	35607 **
L x C	1	465 ns	90 ns	1328 ns	405 ns	474 ns	895 ns
Error 2	12	221	595	430	866	1092	2740
C.V.%		17	30	23	29	24	31

Appendix III.2.1.d Nodule number under 1 mm per plant

Summary of within-harvest analysis of variance Sqrt (X x 100)

Harvest / Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	1003	1094	1528	2334	2556	2589
Light	1	725 ns	27 ns	6589 *	0.2 ns	24 ns	1116 ns
Error 1	6	513	592	620	658	652	2583
Cut	1	5803 *	8694 **	6296 *	4787 ns	10814 **	14181 *
L x C	1	356 ns	404 ns	1302 ns	736 ns	83 ns	627 ns
Error 2	12	759	622	945	1126	622	2874
C.V.%		24	22	27	28	18	38

Appendix III.2.2.1 Nodule dry weight per plant (mg)

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest / Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0346	0.0707	0.0880	0.0624	0.0384	0.1287
Light	1	0.0300 ns	0.0337 ns	0.0768 ns	0.2921 *	0.9446 **	0.3890 **
Error 1	6	0.0261	0.0263	0.0129	0.0320	0.0219	0.0094
Cut	1	0.2691 **	0.1497 **	0.6023 **	0.8706 **	1.6701 **	0.9794 **
L x C	1	0.0482 *	0.0080 ns	0.0530 ns	0.0042 ns	0.0010 ns	0.0045 ns
Error 2	12	0.0072	0.0149	0.0140	0.0249	0.0127	0.0177
C.V.%		22	32	31	44	30	36

Appendix III.2.2.2 Relative Growth Rate of nodule dry weight

Summary of within-harvest analysis of variance untransformed

Period / Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.
Rep	6	0.0044	0.00147	0.0005	0.0011
Light	1	0.0011 ns	0.0083 ns	0.0177 *	0.0001 ns
Error 1	6	0.0036	0.0015	0.0018	0.0005
Cut	1	0.0071 ns	0.0194 **	0.0097 *	0.0000 ns
L x C	1	0.0219 **	0.0015 ns	0.0025 ns	0.0000 ns
Error 2	12	0.0020	0.0018	0.0011	0.0015

Appendix III.2.3.1 a Nodule number per 100 mg root dry weight

Summary of within-harvest analysis of variance Sqrt (X x 100)

Harvest		1	2	3	4	5	6
Source	DF	M.S.	M.S.	M.S.	M.S.	M.S.	M.S.
Rep	6	15.9	24.8	22.5	30.6	27.7	39.5
Light	1	0.4 ns	12.0 ns	11.4 ns	2.4 ns	11.4 ns	28.8 ns
Error 1	6	13.4	8.7	24.6	20.8	30.6	25.7
Cut	1	1.9 ns	24.7 ns	9.1 ns	92.4 ns	2.3 ns	31.1 ns
L x C	1	2.8 ns	2.6 ns	1.0 ns	51.9 ns	24.7 ns	0.5 ns
Error 2	12	27.9	56.9	23.6	35.9	15.8	29.0
C.V.%		19	27	18	21	14	19

Appendix III.2.3.1. b Nodule number per 100 mg root dry weight

Analysed with computer program (ANMA, from Applied Maths Dept.) as Split-split-plot design with light as the main effect, cutting as the sub-effect and harvest as the sub-sub-effect. Data transformed into square root x 100 for the analysis of variance.

Source of variation	DF	S.S.	M.S.	F.ratio	
Replicate	6	2.69	0.45	4.29	*
Light	1	0.07	0.07	0.07	ns
Error 1	6	0.63	0.10		
Cut	1	0.04	0.04	0.07	ns
Cut x Light	1	0.08	0.08	0.13	ns
Error 2	12	7.40	0.62		
Harvest	5	0.13	0.03	0.11	ns
Harvest x Light	5	0.59	0.12	0.49	ns
Harvest x Cut	5	1.58	0.32	1.30	ns
Har x Cut x Light	5	0.76	0.15	0.62	ns
Error 3	120	29.10	0.24		
Total	167	43.08			

Appendix III.2.3.2. a Average dry weight per nodule (mg)

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0441	0.0556	0.0727	0.0271	0.0574	0.0630
Light	1	0.0002 ns	0.0927 ns	0.1757 ns	0.1146 ns	0.5516 **	0.0817 ns
Error 1	6	0.0232	0.0374	0.0365	0.0288	0.0227	0.0642
Cut	1	0.0117 ns	0.0106 ns	0.2103 ns	0.2250 **	0.2560 **	0.0966 ns
L x C	1	0.0067 ns	0.0020 ns	0.0216 ns	0.0000 ns	0.0054 ns	0.1396 ns
Error 2	12	0.0304	0.0299	0.0648	0.0210	0.0266	0.1159
C.V.%		49	49	80	40	46	191

Appendix III.2.3.2. b Nodule dry weight (mg) per 100 mg root dry weight

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0142	0.0118	0.0076	0.0096	0.0095	0.0139
Light	1	0.0014 ns	0.0130 ns	0.0015 ns	0.1400 ns	0.3615 *	0.0645 ns
Error 1	6	0.0127	0.0147	0.0081	0.0291	0.0099	0.0047
Cut	1	0.0050 ns	0.0019 ns	0.0178 ns	0.0329 ns	0.1712 *	0.1100 **
L x C	1	0.0016 ns	0.0003 ns	0.0043 ns	0.0793 ns	0.0513 ns	0.0073 ns
Error 2	12	0.0165	0.0183	0.0086	0.0185	0.0109	0.0197
C.V.%		34	37	24	37	27	38

Appendix III.2.4. PERCENTAGE FREQUENCY OF LEAF AREA RATING

(Ratings based on the standards of Williams et al (1964))

	Ratings	12	13	14	15	16	17	18	19	20	21	22	23
Harvest	Treat-ment	(percentage to total)											
1	L/U	12	2	5	9	9	20	16	13	9	2	2	0
	L/C	41	1	14	16	19	7	0	1	0	0	0	0
	S/U	14	4	4	9	14	21	18	12	4	1	0	0
	S/C	47	2	9	16	11	10	3	0	0	0	0	0
2	L/U	18	2	8	5	10	20	23	10	8	2	0	0
	L/C	28	2	13	16	19	17	4	1	0	0	0	0
	S/U	10	0	4	7	14	28	20	10	6	2	0	0
	S/C	24	2	8	11	21	23	9	2	1	0	0	0
3	L/U	12	0	1	3	10	15	19	18	15	8	1	0
	L/C	20	0	5	16	20	21	14	2	1	0	0	0
	S/U	9	0	2	5	9	25	28	13	8	1	0	0
	S/C	16	0	1	6	16	42	11	5	2	0	0	0
4	L/U	11	1	3	6	13	24	23	10	5	3	1	0
	L/C	14	3	8	13	27	24	8	1	0	0	0	0
	S/U	9	0	5	6	10	26	22	14	14	6	3	0
	S/C	14	0	8	12	32	27	6	1	0	0	0	0
5	L/U	11	1	2	8	13	23	25	11	5	1	0	0
	L/C	14	0	1	7	23	27	21	6	1	0	0	0
	S/U	10	0	1	2	10	14	20	16	14	11	2	0
	S/C	11	0	2	9	20	29	18	9	1	0	0	0
6	L/U	18	0	1	7	11	19	14	14	9	5	1	0
	L/C	17	1	1	5	15	25	17	11	5	3	0	0
	S/U	12	1	1	2	6	9	14	16	15	15	7	1
	S/C	15	1	1	6	15	28	20	8	5	1	0	0

Note in harvest 6 S/U plants had 60% of their leaves between ratings 18 (6.31 CM^2) and 21 (12.60 CM^2), whereas L/U plants had 58% of their leaves between ratings 16(3.98 CM^2) and 19 (7.94 CM^2). For details on rating areas see section II.6.3 .

L/U:light-uncut ; L/C:light-cut ; S/U:shade-uncut ; S/C:shade-cut

Appendix III. 2.5 Relationship between nodule dry weight and nodule number

Dependent variable = $\log_{10}$ nodule dry weight (mg)
Independent variable = square root nodule number

a. Individual group regression

Treatment	regression equation	std err	correla-tion	DF
Light - uncut	Y = 1.30 + 0.030 X	0.004	0.73	40
Light - cut	Y = 1.23 + 0.025 X	0.007	0.51	40
Shade - uncut	Y = 1.29 + 0.026 X	0.004	0.67	40
Shade - cut	Y = 1.11 + 0.024 X	0.008	0.42	40

b. Average within group regression

Y = 1.21 + 0.027 X 0.003 0.62 163

c. Analysis of within group variance of Y

Source of variation	DF	S.S.	M.S.	F ratio	
Total within group	164	8.3465	0.0509		
Due to aver. reg.	1	3.2747	3.2747	105.24	***
Dev. from aver. reg.	163	5.0718	0.0311		
Betw. indi. grp. reg.	3	0.0248	0.0083	0.26	ns
Dev. from indi regs.	160	5.0470	0.0315		

The results from this and Appendix III.2.6 were analysed with Professor Mumford's general statistic computer programs (STATIC), see section II.7.3 .

Appendix III.2.6 Relationship between average dry weight per nodule and nodule number

Dependent variable = average dry weight per nodule (mg)
Independent variable = square root nodule number

a. Individual group regression

Treatment	regression equation	std.err.	correlation	DF
Light-uncut	Y = 0.312 - 0.004 X	0.003	- 0.23	40
Light-cut	Y = 0.437 - 0.014 X	0.003	- 0.55	40
Shade-uncut	Y = 0.616 - 0.021 X	0.007	- 0.44	40
Shade-cut	Y = 0.300 - 0.010 X	0.003	- 0.52	40

b. Average within group regression

Y = 0.399 - 0.011 X	0.002	- 0.37	163

c. Analysis of within group variance of Y

Source of variation	DF	S.S.	M.S.	F ratio	
Total within group	164	3.9305	0.0240		
Due to aver. reg.	1	0.5508	0.5508	26.56	***
Dev. from aver. reg.	163	3.3796	0.0207		
Betw. indi. group reg.	3	0.2224	0.0741	3.75	*
Dev. from indi. regs.	160	3.1573	0.0197		

Appendix III.3.1. a Nitrogen percentage of tops

Summary of witnin-harvest analysis of variance untransformed

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0732	0.1168	0.2941	0.2224	0.1705	0.1870
Light	1	0.0240 ns	0.0660 ns	0.4995 *	1.0921 **	1.4950 *	1.1441 *
Error 1	6	0.0663	0.0287	0.0510	0.0091	0.1213	0.0284
Cut	1	0.8786 **	0.6603 **	0.6180 *	0.0234 ns	0.0670 ns	0.0001 ns
L x C	1	0.0024 ns	0.0011 ns	0.0603 ns	0.2040 *	0.0200 ns	0.0984 ns
Error 2	12	0.0812	0.0454	0.0966	0.0325	0.0321	0.0372
C.V.%		8	6	10	6	6	6

Appendix III.3.1. b Nitrogen percentage of roots

Summary of within-harvest analysis of variance untransformed

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0413	0.0825	0.0465	0.0348	0.0707	0.1424
Light	1	0.0146 ns	0.0015 ns	0.0585 ns	0.0937 ns	0.0116 ns	0.0009 ns
Error 1	6	0.0341	0.0233	0.0261	0.0438	0.0700	0.0375
Cut	1	0.6062 **	0.3634 ns	0.6665 **	0.0137 ns	0.0004 ns	0.2340 ns
L x C	1	0.0005 ns	0.0004 ns	0.0104 ns	0.0497 ns	0.0948 ns	0.0869 ns
Error 2	12	0.0482	0.0922	0.0274	0.0310	0.0481	0.0600
C.V.%		11	15	8	9	11	11

Appendix III.3.1. c Nitrogen percentage of nodules

Summary of within-harvest analysis of variance untransformed

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.6446	1.348	0.4231	1.4245	0.7464	0.8666
Light	1	0.4757 ns	1.2771 ns	0.8505 ns	0.7990 ns	4.5280 *	0.0000 ns
Error 1	6	0.6067	0.3514	0.3319	0.1655	0.6879	0.7297
Cut	1	1.9610 *	7.6546 *	13.0562 **	2.9639 *	0.0531 ns	0.0030 ns
L x C	1	0.4400 ns	2.0412 ns	0.0028 ns	1.1645 ns	0.6481	0.0902 ns
Error 2	12	0.3837	0.9047	0.3430	0.4271	0.3565	0.5353
C.V.%		7	12	7	8	7	8

Appendix III.3.2. a Nitrogen yield of tops

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest		1	2	3	4	5	6
Source	DF	M.S.	M.S.	M.S.	M.S.	M.S.	M.S.
Rep	6	0.0537	0.0755	0.0893	0.0924	0.0733	0.1503
Light	1	0.0073 ns	0.0215 ns	0.1518 *	0.1034 *	0.4251 **	0.1409 *
Error 1	6	0.0103	0.0165	0.0196	0.0158	0.0259	0.0193
Cut	1	2.8938 **	2.4132 **	2.5689 **	1.9010 **	2.2216 **	1.5810 **
L x C	1	0.0058 ns	0.0731 ns	0.0585 ns	0.0071 ns	0.0002 ns	0.0063 ns
Error 2	12	0.0070	0.0169	0.0128	0.0163	0.0091	0.0189
C.V.%		21	35	30	34	25	48

Appendix III.3.2. b Nitrogen yield of roots

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest		1	2	3	4	5	6
Source	DF	M.S.	M.S.	M.S.	M.S.	M.S.	M.S.
Rep	6	0.0307	0.0535	0.0777	0.0730	0.0702	0.0995
Light	1	0.0255 ns	0.0060 ns	0.1289 ns	0.0103 ns	0.0915 ns	0.1654 **
Error 1	6	0.0240	0.0134	0.0223	0.0064	0.0251	0.0081
Cut	1	0.3763 **	0.2241 *	0.6507 **	0.6106 **	0.8430 **	0.5012 **
L x C	1	0.0308 ns	0.0046 ns	0.1015 *	0.0666 ns	0.0060 ns	0.0002 ns
Error 2	12	0.0132	0.0429	0.0181	0.0205	0.0075	0.0374
C.V.%		30	62	37	39	22	56

Appendix III.3.2. c Nitrogen yield of nodules

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest		1	2	3	4	5	6
Source	DF	M.S.	M.S.	M.S.	M.S.	M.S.	M.S.
Rep	6	0.0398	0.0933	0.0955	0.0766	0.0375	0.1536
Light	1	0.0193 ns	0.0614 ns	0.1080 *	0.3474 *	1.1950 **	0.3940 **
Error 1	6	0.0270	0.0334	0.0103	0.0331	0.0321	0.0073
Cut	1	0.3534 **	0.3021 **	0.9436 **	1.0599 **	1.6282 **	0.9942 **
L x C	1	0.0335 *	0.0001 ns	0.0523 ns	0.0165 ns	0.0002 ns	0.0072 ns
Error 2	12	0.0066	0.0194	0.0165	0.0252	0.0081	0.0201
C.V.%		21	38	35	44	23	39

Appendix III.3.3 Nitrogen fixed per mg nodule dry weight per day (mg N/ mg nodule/ day)

Summary of within-harvest analysis of variance $\log_{10}$ (X x 100)

Harvest Source	DF	1 M.S.	2 M.S.	3 M.S.	4 M.S.	5 M.S.	6 M.S.
Rep	6	0.0165	0.0059	0.0074	0.0108	0.0073	0.0058
Light	1	0.0034 ns	0.0001 ns	0.0110 ns	0.0569 *	0.1125 **	0.0520 *
Error 1	6	0.0067	0.0117	0.0039	0.0065	0.0081	0.0060
Cut	1	0.8395 **	0.9295 **	0.4815 **	0.1377 *	0.0185 ns	0.0406 ns
L x C	1	0.0089 ns	0.0173 ns	0.0005 ns	0.0003 ns	0.0023 ns	0.0001 ns
Error 2	12	0.0088	0.0064	0.0033	0.0218	0.0081	0.0113
C.V.%		24	20	14	40	23	28

APPENDIX III.3.4

```
PROG FOR CALCULATING CURVILINEAR REGRESSION FOR C2H2 REDUCTION CHU

DIMENSION X1(100),X2(100),Y(100)
IHOLD=0
N=0
 1 READ 2,IHAR,ITRE,X,Z
19 IF(IHOLD)16,17,16
16 IF(IHOLD-IHAR)4,3,4
17 PRINT 18,ITRE,IHAR
 3 IHOLD=IHAR
N=N+1
XX=X*X
X1(N)=X
X2(N)=XX
Y(N)=Z
IF(SENSE SWITCH 9)999,1
 4 SX1=0
SX2=0
SY=0
SXX1=0
SXX2=0
SXY1=0
SXY2=0
SX1X2=0
SYY=0
DO 5 L=1,168
SX1=SX1+X1(L)
SX2=SX2+X2(L)
SY=SY+Y(L)
SXX1=SXX1+(X1(L)*X1(L))
SXX2=SXX2+(X2(L)*X2(L))
SYY=SYY+(Y(L)*Y(L))
SXY1=SXY1+(X1(L)*Y(L))
SXY2=SXY2+(X2(L)*Y(L))
SX1X2=SX1X2+(X1(L)*X2(L))
 5 CONTINUE
FN=N
YBAR=SY/FN
X1BAR=SX1/FN
X2BAR=SX2/FN
SSXX1=SXX1-((SX1*SX1)/FN)
SSXX2=SXX2-((SX2*SX2)/FN)
SSXSX=SX1X2-((SX1*SX2)/FN)
SSX1Y=SXY1-((SX1*SY)/FN)
SSX2Y=SXY2-((SX2*SY)/FN)
SSYY=SYY-((SY*SY)/FN)
D=(SSXX1*SSXX2)-(SSXSX*SSXSX)
B1=((SSXX2*SSX1Y)-(SSXSX*SSX2Y))/D
B2=((SSXX1*SSX2Y)-(SSXSX*SSX1Y))/D
A=YBAR-(B1*X1BAR)-(B2*X2BAR)
PRINT 9 ,A,B1,B2

TEST FOR SIGNIFICANCE OF DEPARTURE FROM LINEAR REGRESSION

SS1=SSYY-((SSX1Y*SSX1Y)/SSXX1)
SS2=SSYY-((B1*SSX1Y)+(B2*SSX2Y))
SS3=SS1-SS2
```

```
      DF1=FN-2.
      DF2=FN-3.
      SM1=SS1/DF1
      SM2=SS2/DF2
      SM3=SS3
      F1=SM3/SM1
      F2=SM3/SM2
      PRINT 20
      PRINT 10
      PRINT 11
      PRINT 12,DF1,SS1,SM1,F1
      PRINT 13,DF2,SS2,SM2,F2
      PRINT 14,SS3,SM3
      PRINT 11
      PRINT 20

      THIS CALCULATES THE STD ERRORS

      C11=SSXX2/D
      C12=-SSXSX/D
      C22=SSXX1/D
      B11=(C11*SSX1Y)+(C12*SSX2Y)
      B22=(C12*SSX1Y)+(C22*SSX2Y)
      SB1=SQRTF(SM2)*SQRTF(C11)
      SB2=SQRTF(SM2)*SQRTF(C22)
      T1=B1/SB1
      T2=B2/SB2
      PRINT 15,B11,B22,SB1,SB2,T1,T2
      PRINT 20
    2 FORMAT(2X,2I2,2F10.0)
    6 FORMAT(1H ,21X,4HTIME,1X,4HMINS,1X,9HC2H4 PROD,1X,8HC2H4 EST,2X,8H
     1C2H4 DEV)
    9 FORMAT(1H ,10HEQUATION =,F10.2,3H + ,F12.4,3H X ,3H + ,F12.4,3H X2
     1)
   10 FORMAT(1H ,19HSOURCE OF VARIATION,1X,6H  D F ,15HSUMS OF SQUARES,1
     1X,12HMEAN SQUARES,8X,6HF.CAL.)
   11 FORMAT(1H ,68H----------------------------------------------------
     1----------------)
   12 FORMAT(1H ,20HDEV FROM LINEAR REG ,F6.0,F16.1,F16.2,F10.4)
   13 FORMAT(1H ,20HDEV FROM CURVED REG ,F6.0,F16.1,F16.2,F10.4)
   14 FORMAT(1H ,20HREDUCTION IN SUMS SQ,4X,2H1.,F16.1,F16.2)
   15 FORMAT(1H ,3HB1=,F16.4,2X,3HB2=,F16.4,5X,4HSB1=,F10.4,2X,4HSB2=,F1
     10.4,5X,3HT1=,F10.4,2X,3HT2=,F10.4)
   18 FORMAT(1H ,9HTREATMENT,I2,1X,7HHARVEST,I2)
   20 FORMAT(1H ,/)
  999 CALL EXIT
      END
```

Appendix III.3.5 Summary of analysis of variance for test for departure from linear regression

Treatment	Plant(min)	Harvest 1	2	3	4	5	6
Light-uncut	300	ns	*	ns	ns	*	**
	240	ns	ns	**	ns	*	*
	180	ns	ns	**	ns	ns	ns
Light-cut	300	ns	ns	ns	ns	ns	ns
	240	ns	ns	ns	ns	ns	ns
	180	ns	ns	*	ns	ns	ns
Shade-uncut	300	ns	ns	ns	**	ns	ns
	240	**	*	ns	*	ns	*
	180	ns	ns	ns	ns	ns	ns
Shade-cut	300	ns	ns	ns	ns	*	**
	240	ns	ns	ns	ns	*	*
	180	ns	*	ns	ns	ns	*

(Program and details in Appendix A ; ns = not significant ; * = P < 0.05 ; ** = P < 0.01)

Appendix III.3.6 Summary for linear regression coefficients for sectional reduction assay (adjusted to the form Y = bX)

	1	2	Harvest 3	4	5	6
Light-uncut						
d.f.	26	29	28	34	33	46
reg.coeff.	0.600	0.453	0.449	0.319	0.401	0.392
s.e.	0.043	0.118	0.051	0.036	0.046	0.033
significance	**	**	**	**	**	**
T.origin	ns	ns	ns	ns	ns	ns
Light-cut						
d.f.	27	24	26	34	34	43
reg.coeff.	0.069	0.093	0.440	0.344	0.365	0.512
s.e.	0.027	0.031	0.120	0.044	0.041	0.060
significance	**	**	**	**	**	**
T.origin	ns	ns	ns	ns	ns	ns
Shade-uncut						
d.f.	25	23	26	34	33	43
reg.coeff.	0.599	0.374	0.565	0.404	0.388	0.563
s.e.	0.069	0.091	0.061	0.040	0.026	0.063
significance	**	**	**	**	**	**
T.origin	ns	ns	ns	ns	ns	ns
Shade-cut						
d.f.	24	23	27	32	34	42
reg.coeff.	0.029	0.090	0.370	0.349	0.311	0.504
s.e.	0.010	0.050	0.099	0.062	0.031	0.102
significance	**	ns	**	**	**	**
T.origin	ns	ns	ns	ns	ns	ns

(Details in Appendix A; d.f.=degrees of freedom; reg.coeff.= regression coefficient ; s.e.= standard error ; significance= t.test for linear regression; T.origin = t.test for fitting the regression through the origin ; ns = not significant ; ** = P 0.01)

Appendix IV.1 Decoded means and percentage difference in root dry weights and total nodule number

Root dry weights (mg)

Harvest	1	2	3	4	5	6
Light	337	305	381	401	629	707
Shade	300	287	297	347	456	512
% to control	89	94	76	87	72	72
	ns	ns	ns	ns	**	**
Uncut	386	343	450	517	785	801
Cut	262	255	257	269	366	452
% to control	68	74	57	52	47	56
	**	ns	**	**	**	**

Total nodule number

Harvest	1	2	3	4	5	6
Light	278	221	382	324	500	584
Shade	241	238	223	275	381	397
% to control	87	108	58	85	76	68
	ns	ns	*	ns	ns	ns
Uncut	308	282	346	370	603	650
Cut	214	182	252	236	300	345
% to control	70	65	73	64	50	53
	**	**	*	*	**	*

(Controls were the Light and Uncut plants respectively)

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Plate 1

General view of the glasshouse, note shades in the background.

Plate 2

General view of the glasshouse.

Plate 3

Light meter

Plate 4

Close-up view of pot

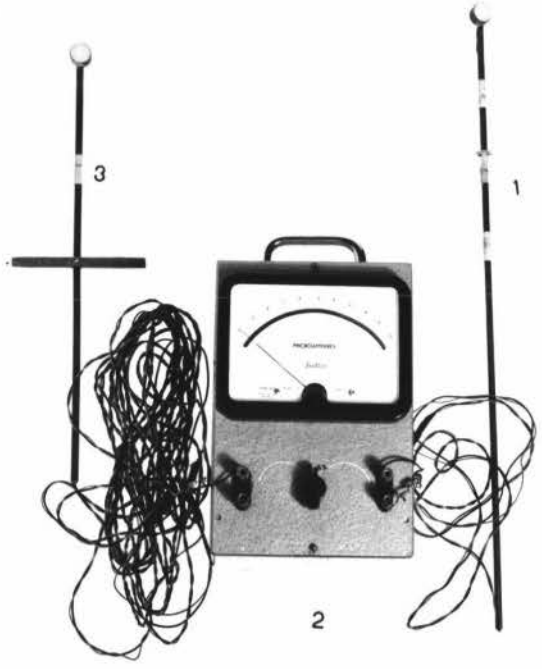

3
1
2

Plate 5

Difference between 'undefoliated' and 'defoliated' plants.

Plate 6

Amount of regrowth on 'defoliated' plants at harvest 1 (day 3).


KA 2
KA 3 LK


KA 4 LK

Plate 7

'Green' and 'pink' nodules as at harvest 1 (day3).

Plate 8

'Green' and 'pink' nodules as at harvest 3 (day 10)

1 = light-uncut (control)
2 = light-cut
3 = shade-uncut
4 = shade-cut

Note the half green, half pink nodules in treatments 2, 3 and 4.


CM.

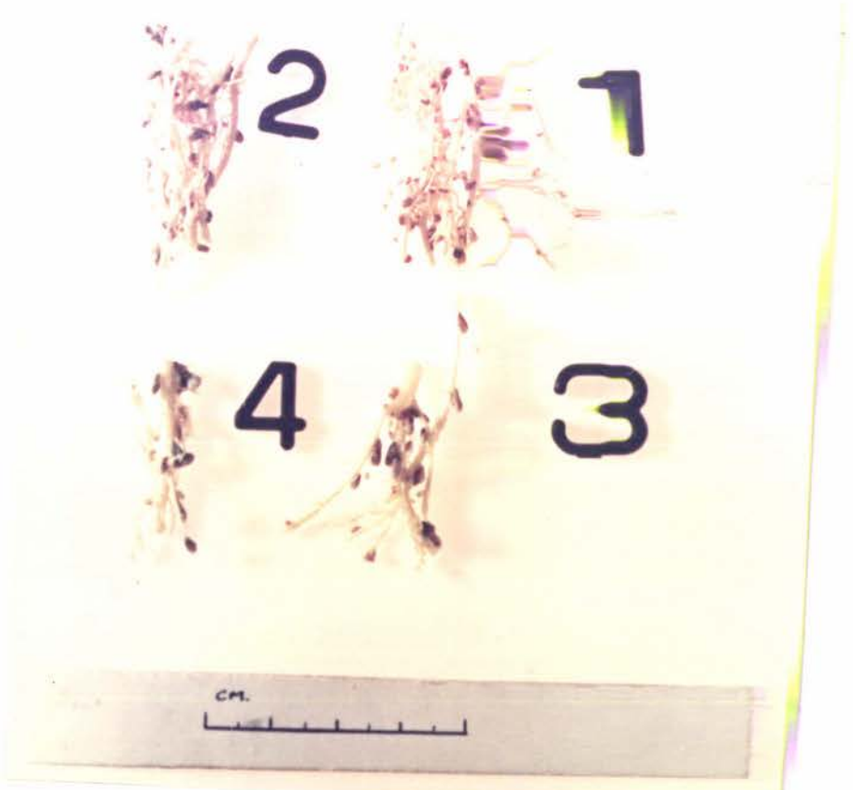

2
1
4
3
CM.

Plate 9

First inch-segment of root showing nodules at various stages of sample taken from 'defoliated' plants on harvest 3 (day 10).

Note: nodule 'hulls'
brown nodules with split apex
pink nodules

Plate 10

Lateral roots from 4th inch-segment of 'defoliated' plants showing nodules at various stages of development.

Note: nodule 'hull'
slightly pink apex
small pink nodule


CM.

Plate 11

The vacuum devise used in the experiment.

A = control to vacuum pump

B = gas inlets

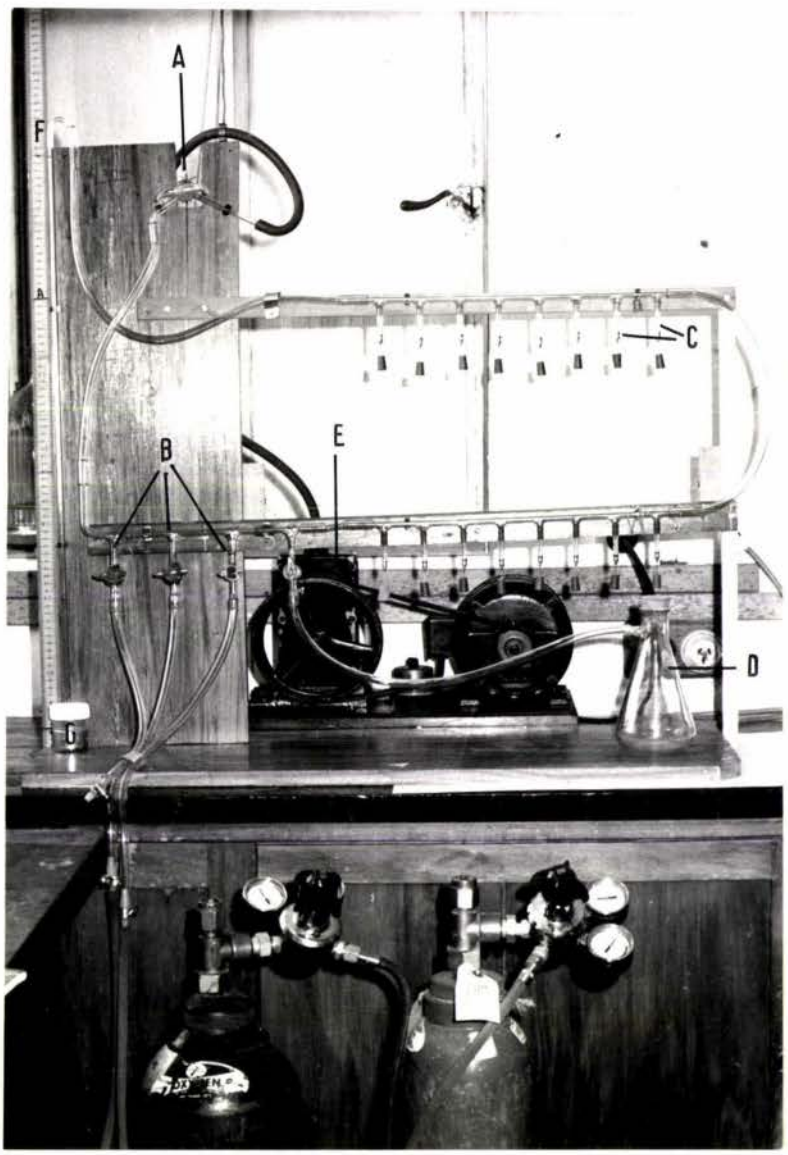
C = outlet into vial

D = gas reservoir

E = vacuum pump

F = manometer

G = Hg reservoir


A
F
C
B
E
D
G