AN INVESTIGATION INTO THE EFFICIENCY OF NITROGEN FIXATION IN SAINFOIN (ONOBRYCHIS VICIIFOLIA SCOP.)

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ABSTRACT

Earlier reports have indicated that growth of the forage legume sainfoin (*Onobrychis viciifolia* Scop.) is limited by its capacity to fix adequate quantities of N_2.

Symbiotic N_2 fixation and development of sainfoin up to the flowering stage was studied under glasshouse conditions. Growth and development of plants that were dependent solely on fixed N_2 for their N requirements, were compared with plants supplied with abundant combined (nitrate) N. The effect of a low rate of combined N on symbiotic N_2 fixing activity and plant growth was also investigated.

From an early stage, plants dependent on symbiotic N_2 fixation had lower relative growth rates than plants supplied with combined N, indicating that the N_2 fixing system of sainfoin was not capable of providing enough N to meet the requirements of the plant, or that N_2 fixation required an energy input greater than that for the assimilation of mineral N.

The mode of N nutrition was found to influence the dry matter distribution in sainfoin to a greater extent than reported for most other legumes. Plants dependent on symbiotic N_2 fixation allocated a substantially greater proportion of dry matter to root and nodule growth and consequently had lower top:root + nodule ratios than plants provided with combined N.

Sainfoin was found to produce abundant nodules, and had a relatively high nodule weight in relation to total plant weight, compared to other legumes. Specific nodule activity, however, was found to be relatively low, and possible reasons for this are discussed.

For plants dependent on symbiotic N_2 fixation, total plant N, and hence N_2 fixation appeared to be the major factor limiting plant growth. Evidence was obtained which indicated that the N_2 fixing system of sainfoin may be relatively inefficient. The observed ratio of C_2H_2 reduced:N_2 fixed, was higher than the theoretical ratio, and appeared to be high relative to other legumes, which suggested possible wastage of energy by the N_2 fixing enzyme. The addition of a low rate of combined N had the effect of immediately reducing $N_2[C_2H_2]$ fixing activity, and the combined N appeared to substitute for, rather than supplement, symbiotic N_2 fixation, further indicating an inefficient symbiotic N_2 fixing system.

Leaf area ratio was found to be lower in sainfoin dependent on N_2 fixation than reported values for other N_2 fixing legumes; this suggests that sainfoin is less efficient at intercepting photosynthetically active

radiation. Leaf area was highly correlated with total plant N, and there was evidence that this link was via energy supply to the symbiotic N_2 fixing system. Thus leaf area may have been limiting N_2 fixation and hence total plant N.

Overall, a mutual dependence between the ability of the root nodules to fix N_2 and the ability of the leaves to supply energy was indicated. There was evidence that both of these factors may play a role in limiting the growth of sainfoin, relative to other more productive legumes, such as lucerne.

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INTRODUCTION

There is some interest in growing the forage legume sainfoin (*Onobrychis viciifolia* Scop.) in New Zealand at present. It is thought that it could play a similar role to that already played by lucerne (*Medicago sativa* L) particularly in providing forage for livestock under dry summer conditions. Sainfoin has two advantages over lucerne, in that it has non-bloating properties (e.g. Cooper *et al.* 1966) and it is not subject to some of the major pests of lucerne (e.g. Hanna *et al.* 1977a; Lance, 1980). Its major disadvantage at present, is that its yield potential appears to be substantially lower than that of lucerne in many instances (e.g. Spedding & Diekmahns, 1972). However, it is thought that sainfoin could possibly be useful as a bloat preventing crop to supplement the diet of ruminants in certain circumstances.

Problems in establishing a satisfactory stand of sainfoin and maintaining it after defoliation have been widely reported. The nitrogen fixing ability of sainfoin is reported to be insufficient to provide for the nitrogen requirements of the plant (e.g. Koter, 1965a), and nitrogen deficiency symptoms have been observed on inoculated sainfoin at an early stage in the development of the crop (Sims *et al.* 1968; Roath & Graham, 1968; Schneiter *et al.* 1969; Meyer, 1975; Smoliak & Hanna, 1975) despite plants being abundantly nodulated (Burton & Curley, 1968).

Up to the time of this project there had been little work specifically investigating nitrogen fixation and nodulation in sainfoin, and possible causes of its poor nitrogen fixing performance. The aim of this project was to investigate the nodulation and nitrogen fixation of sainfoin in relation to overall plant development, and to compare the performance of plants dependent on symbiotic nitrogen fixation with those supplied with abundant mineral nitrogen.

CHAPTER 1

REVIEW OF LITERATURE

For convenience, the review of literature is divided into three main sections, dealing with sainfoin, nitrogen fixation and the acetylene reduction technique.

1 SAINFOIN

1.1 AGRICULTURAL POTENTIAL OF SAINFOIN

1.1.1 YIELD

Comparisons of sainfoin (*Onobrychis viciifolia* Scop.) with lucerne (*Medicago sativa* L.) are probably inevitable, and provide a useful frame of reference in the evaluation of sainfoin (Hanna & Smoliak, 1968). If sainfoin is to be utilised as a hay or pasture crop, even if only under specific environmental conditions which favour its growth, it is logical to place considerable emphasis on its yield performance in comparison to lucerne, which is one of the highest yielding forage legumes over a wide range of temperate environments (Hanna & Smoliak, 1968).

Yields of sainfoin in pure stands have generally tended to be lower than those of lucerne (Hanna & Smoliak, 1968; Murray & Slinkard, 1968; N.I.A.B. 1974; Spedding & Diekmahns, 1972; Hanna *et al.* 1975; Rogers, 1976; Hanna, 1977) and red clover (*Trifolium pratense* L.) (Spedding & Diekmahns, 1972). The annual yield of sainfoin is often in the order of 20 - 30% lower than lucerne (Spedding & Diekmahns, 1972; Melton, 1973; Hanna *et al.* 1975; Rogers, 1976). Hanna & Smoliak (1968) state that even the better yielding strains of sainfoin are not likely to out-yield lucerne, where the latter is well adapted, especially where two or more cuttings can be obtained. Forage yields of sainfoin are frequently inferior to those of lucerne, where seasonal rainfall is adequate, due to poor regrowth and plant vigour (Meyer, 1975).

There are, however, a number of situations where sainfoin has out-yielded lucerne. Murray & Slinkard (1968) report dry matter yields of sainfoin (one cut type) similar to those of lucerne, in Idaho under dry summer conditions. This result was due to the early growth and relatively high first cut yields of sainfoin. Carleton *et al.* (1968b) report that, for irrigated hay, Eski sainfoin (a one cut type) yielded more than, the same as, or less than lucerne, depending on location and year. Sainfoin generally yielded more than lucerne at the first cut and less at the second, and appeared to have a comparative advantage where conditions enabled only one cut per year. Roath (1968) found that under dryland conditions, hay yields of sainfoin compared favourably with lucerne, red clover and cicer milkvetch (*Astragalus cicer* L.) except on acid soil. Smoliak & Hanna (1975) grazed subirrigated sainfoin with sheep over five years. Over the five years sainfoin yielded slightly higher than lucerne, with both yielding substantially higher

than cicer milkvetch. Kozyr (1948) reported higher hay yields from sainfoin than lucerne, and attributed this to drought endurance and resistance to pests.

Thus, although sainfoin yields are often reported to be lower than the better forage legumes such as lucerne, yields may surpass those of lucerne under particular environments to which sainfoin is better adapted. This, considered together with the desirable nutritional and non-bloating characteristics of sainfoin (Sections 1.1.2 and 1.1.3) may make it a desirable forage legume under certain circumstances.

Reports on the performance of sainfoin with companion grass species are conflicting. A companion grass can tend to increase total yields and decrease weed invasion (Bland 1971). Sainfoin is found to be competitive with grasses when planted in mixtures (Dubbs, 1968). Dubbs (1968), Roath (1968) and Hanna *et al.* (1977b) report good yields of sainfoin grown with a range of grasses, and found that sainfoin grass mixtures tend to yield higher than grass alone but in most cases, not higher than sainfoin alone. It has been found that sainfoin performs better in mixtures with bunch grasses, rather than rhizomatous grasses. The legume component tends to be reduced when the latter grasses are present, resulting in a lower quality forage (Hanna *et al.* (1975). Hanna *et al.* (1977b) measured forage yields of sainfoin/grass and lucerne/grass mixtures and found the legume component yield to be consistently higher in the lucerne/grass mixtures. Cooper (1972) found that sainfoin/grass mixtures were less productive than sainfoin/birdsfoot trefoil (*Lotus corniculatus* L.) mixtures. Sainfoin has been found to perform better in alternate rather than mixed row seedings with grass (Hanna *et al.* 1977b) or birdsfoot trefoil (Krall *et al.* 1971).

Thus, grass may be sown along with sainfoin to decrease weed invasion and increase stand longevity, but this is at the expense of decreased forage quality, and usually results in slightly decreased total yield.

To summarise, sainfoin often does not compare favourably, in terms of yield, with some of the more commonly grown forage legumes such as lucerne. However, there are certain situations, such as on dry, free draining, high pH soils (Section 1.5.1) in which the performance of sainfoin may be superior to that of other forage legumes. The potential also exists to improve yields of sainfoin by plant breeding. South Australian workers appear to have selected an acceptable cultivar within the space of two to three years (Lance, 1980). Sheehy & Harding (pers.comm.) suggest

that sainfoin yields would benefit from a selection programme aimed at increasing its specific leaf area (and hence leaf area ratio) and rate of leaf growth (section 1.4).

Sainfoin also has certain attributes, which may make it a more desirable crop, than for example lucerne, even at the expense of reduced yield. Three of these attributes are its non-bloating characteristic, its very high nutritional value (discussed in sections 1.1.2 and 1.1.3) and its resistance to some lucerne pests, such as the alfalfa weevil (*Hypera postica*) (Eslick, 1968; Hanna *et al.* 1977a) and the spotted alfalfa aphid (Lance, 1980).

1.1.2 NON BLOATING CHARACTERISTIC

Bloat is a problem of ruminant animals in which a persistent foam develops in the rumen, in amounts sufficient to prevent the animal from belching the large amounts of gas formed therein (Wright & Reid, 1974). Bloat is a particular problem in New Zealand because of the large number of ruminant animals grazing legume based pastures. Bloat occurs most frequently in New Zealand, in dairy cattle, in which it is a common disorder. Up to 90% of the herds in a particular district may experience bloat, and most animals in an individual herd may be affected (Reid, 1976). Deaths, however, are not usually high (as a result of preventative measures) with regional average death rates seldom higher than 2% (Reid, 1976). As at 1976 the annual cost of materials alone for bloat prevention could be as high as 10% of the value of a good dairy cow, or 4% of the probable annual gross income from the cow.

Soluble plant proteins have been implicated as surfactants responsible for the persistent foam that develops in the rumen of animals suffering from boat (Gutek *et al.* 1974; Jones & Mangan, 1977). McArthur & Miltimore (1964) indentified the foaming agent in lucerne as an 18-S protein. They stated that the 18-S protein was probably chloroplast lamellae, and that it was found in all legumes, but may vary qualitatively because of physical characteristics.

Three common forage legumes which cause bloat are lucerne, white clover (*Trifolium repens* L.) and red clover (McArthur & Miltimore, 1969). There are, however, some forage legumes which do not cause bloat. These include sainfoin and birdsfoot trefoil (*Lotus corniculatus* L.). Cooper *et al.* (1966) found sainfoin to have the lowest foam formation of twenty seven legume species that were evaluated for bloat potential.

Non bloating legumes such as sainfoin, have been found to contain protein precipitating substances called condensed tannins or flavolans (Reid, 1976). The soluble dietary protein of green leaves forms insoluble complexes with tannins, which are stable over the pH range 3.5 to 7.0, which includes the rumen pH range of 5.6 to 6.8 (Jones & Mangan, 1977). These complexes break up in the more acid conditions (pH 2.5) of the abomasum (Jones & Mangan, 1977). A complete absence of soluble protein has been observed in the rumen of cattle grazing pasture species that contain flavolans (Jones & Mangan, 1977). The flavolans serve a dual purpose in that they also protect dietary protein from deamination by rumen bacteria and thus have a beneficial effect on N metabolism (Jones & Mangan, 1977). Sainfoin is found to be highly palatable to herbivores, in contrast to other species containing tannins, in which they have been reported to cause reduced palatability (Jones *et al.* 1976). Sarkar *et al.* (1976) report that the flavolan composition of sainfoin is such that nutritive value is high, unlike some other flavolan containing legumes.

Reasons other than flavolan content have also been cited as being responsible for the non bloating characteristic of forages such as sainfoin. McArthur and Miltimore (1969) state that non bloating legumes, including sainfoin, were found to contain low levels of the bloat causing 18-S protein relative to bloat causing legumes.

Howarth *et al.* (1978a) found that the mesophyll cells in non bloating legumes were more resistant to mechanical rupture than those from bloat causing legumes (without cell rupture, the major foaming agents, which are intracellular, would not be released from the cells, and foam formation would not occur). Howarth *et al.* (1978a) state that the presence of flavolans, and the resistance of cells to rupture are two separate and complimentary explanations for the bloat safe nature of sainfoin.

Pectin methyl esterase (PME) activity has also been linked to stable foam formation on ruminants (Rumbaugh, 1972). Sainfoin was found to have the lowest level of PME activity (zero) of the four forage legumes tested.

Howarth *et al.* (1978b) hypothesised that colloidal sized fragments of chloroplast membranes may act as nucleation sites for bubble formation during the onset of pasture bloat. They cited evidence to support this hypothesis, and stated that sheep fed sainfoin had lower concentrations of suspended chloroplast fragments in their rumens than sheep fed other forage legumes.

1.1.3 NUTRITIONAL VALUE

Sainfoin is reported to be a very nutritious forage (Shain, 1959; Schneiter *et al.* 1969; Krall *et al.* 1971) which is highly palatable to all classes of livestock (Hanna *et al.* 1975).

Sainfoin is reported to contain higher levels of N-free extract, total digestible nutrients and phosphorus than lucerne, similar levels of ether extract, and lower levels of crude protein, crude fibre, total ash and calcium (Baker *et al.* 1952; Carleton *et al.* 1968a; Jensen *et al.* 1968; Schneiter *et al.* 1969; Smith *et al.* 1974; Ditterline & Cooper, 1975). It is found that sainfoin provides a very good balance of protein and total available energy (Carleton *et al.* 1968a; Ulyatt *et al.* 1977). Kaldy *et al.* (1979), scored sainfoin and lucerne in terms of protein quality for non ruminants. Scores were 68 and 71 respectively, compared to 100 for the ideal (whole egg) protein.

Sainfoin is reported to be similar, in terms of digestibility, to lucerne (Jensen *et al.* 1968; Chapman & Carter, 1976) and red clover (Osbourn *et al.* 1966). The leaves of sainfoin have been found to be less digestible than those of lucerne, but the stems more so (Ulyatt *et al.* 1977). In terms of animal live weight gain, sainfoin appears to be as food as, or better than, other forage legumes. The efficiency of utilisation of metabolisable energy for liveweight gain has been found to be higher for sainfoin than lucerne, white clover or subterranean clover (*Trifolium subterraneum* L.) (Ulyatt *et al.* 1977). Forage consumption, feed conversion (kg of forage consumed per kg of live weight gain) and live weight gain were similar in beef cattle fed either sainfoin or lucerne (Jensen *et al.*1968). Sainfoin fed to beef cattle is found to give superior weight gains and a better feed to beef conversion than grasses or grass/ladino clover (*Trifolium repens*) mixtures, at the expense of decreased stocking rate (Krall, 1968; Wilson, 1976). Young lambs allowed unlimited grazing have been found to make better growth on sainfoin than any other grass or legume tested (Spedding & Diekmahns, 1972). Krall *et al.* (1971) found that sainfoin produced superior daily weight gains and more beef per acre than ladino clover, or grass fertilised with N. Newman (1968) compared rates of growth of pigs fed a diet containing 3% of ground sainfoin or lucerne hay and found that average daily gains were not significantly higher with the sainfoin containing diet.

Sainfoin is reported to be very palatable, and to be superior to lucerne in this regard (Chapman & Carter, 1976). Osbourn *et al.* (1966) found the voluntary intake of sainfoin by sheep to be greater than for lucerne or red clover, and Smoliak and Hanna (1975) found that sheep preferred to graze sainfoin rather than lucerne or cicer milkvetch (*Astragalus cicer* L.).

The maximum yield of sainfoin is achieved at late bloom. Koch *et al.* (1972) found that little was lost, in terms of quality, if sainfoin was not harvested until late bloom. Lignification occurs before flowering, and the proportion of lignin remains nearly constant thereafter. The stems of sainfoin have high digestibility despite their coarse appearance (Koch *et al.* 1972), and this probably accounts for the fact that the nutritive value of sainfoin does not decline with increasing maturity to the same extent as for other forage legumes. However, changes in the chemical composition of the whole plant, as it matures, are brought about principally by changes in the leaf to stem ratio (Baker *et al.* 1952).

Sainfoin seed has been evaluated as a source of protein for monogastrics. Ditterline (1974) found it to contain 36% crude protein and to have an essential amino acid composition similar to soybean meal. Weanling pigs fed sainfoin seed as a diet supplement wasted more feed and gained less weight than pigs fed soybean meal (Ditterline, 1974; Ditterline & Cooper, 1975). Weanling rats fed diets with sainfoin seed or soybean meal as the protein sources showed similar average daily gains, feed consumption and feed conversion (Ditterline, 1974; Ditterline & Cooper, 1975). Sainfoin seed did have an advantage in that the trypsin inhibitors it contained did not increase pancreas size like those in soybean meal, which usually has to be treated to overcome this problem (Ditterline, 1974).

1.1.4 CONCLUDING COMMENTS - AGRICULTURAL POTENTIAL

If yields of sainfoin can be raised to an acceptable level it could become an attractive proposition for the dryer areas of New Zealand with suitable soils (section 1.5.1). The non bloating characteristic and high nutritional value may compensate for the higher yields which may be able to be obtained from other crops.

1.2 AGRICULTURAL HISTORY OF SAINFOIN

The genus *Onobrychis* comprises 80 to 100 species of plants native to southern Europe, northern and western Africa, and western Asia (Whyte *et al.* 1953). Sainfoin (*Onobrychis*) species have been part of native pastures in the eastern Mediterranean for as long as 6,000 years (Hely & Offer, 1972). *Onobrychis viciifolia*, originating in central and southern Europe, and temperate Asia, is the most important agricultural species. It is used in central Europe, Mediterranean countries and Great Britain as a hay and pasture plant (Whyte *et al.* 1953). Shain (1959) states that sainfoin has been used in parts of the U.S.S.R. for over 1000 years and that it was transferred to western Europe about 400 years ago. Sainfoin appears to have been first cultivated in France, the first definite record, according to Vianne, being in 1582 (Piper, 1924). Sainfoin was grown in Germany in the 17th century but not in Italy until the 19th century (Piper 1924). The spread of sainfoin over Europe led to the profitable cultivation of much dry, calcareous land (Piper, 1924).

It is thought that sainfoin came to England from the continent, particularly since the name sainfoin, meaning healthy hay, is of French origin (Bland, 1971). The writings of Jethro Tull in 1733 indicate that sainfoin must have been widespread and popular in England at this time (Spedding & Diekmahns, 1972). Sainfoin cultivation was widespread in England in the 18th, 19th and early 20th centuries (Bland, 1971; Spedding & Diekmahns, 1972) but the area grown now is almost negligible (Spedding & Diekmahns, 1972). The demise of the crop in Britain over the last 60 years has been attributed to its lack of ability to respond, as well as alternative fodder crops, to the changing requirements of British agriculture (Hutchinson, 1965). Sainfoin is still a very important crop in parts of Europe and according to Shain (1959), is more important than lucerne or red clover in many parts of the U.S.S.R.

Piper (1924) stated that sainfoin had never attained agricultural importance in the U.S.A., although often tested, and went on to say that, on suitable soils, its culture might become profitably established. The lack of interest in sainfoin in the U.S.A. has been attributed to the crop being tested under conditions (such as low pH, high rainfall and frequent irrigation, see section 1.5) to which it was not well adapted in comparison to lucerne (Eslick, 1968; Jensen & Sharp, 1968). Also, factors such as its non bloating charactertistic may have been overlooked

and its coarse appearance may have led to an impression of low palatability (Eslick, 1968).

Renewed interest in growing sainfoin in North America has resulted from the need for a dryland forage, and a substitute for lucerne on irrigated land during periods of heavy lucerne weevil infestation (Ditterline & Cooper, 1975).

The introduction of well adapted genotypes, e.g. Eski & Remont in the U.S.A., and Melrose & Nova in Canada, has also made sainfoin a more attractive proposition (Ditterline & Cooper, 1975; Hanna *et al.* 1977a; Hanna, 1980).

1.3 MORPHOLOGICAL DESCRIPTION

Sainfoin (*Onobrychis viciifolia* Scop.) is a member of the Fabaceae family.

Sainfoin is a slightly pubescent, long lived perennial herb (Whyte *et al.* 1953; Spedding & Diekmahns, 1972). From a branched crown arise numerous erect, ribbed, hollow, branched stems, which are decumbent at the base, and which may grow to about 1 metre in height (Piper, 1924; Percival, 1943; Andreev, 1963; Schneiter *et al.* 1969; Spedding & Diekmahns, 1972). Leaves are borne on long petioles, and are pinnately compound with 5 to 14 pairs of oblong leaflets and a terminal one (Percival, 1943; Spedding & Diekmahns, 1972). The number of leaflets per leaf decreases acropetally (Thomson, 1951a).

The flowers, which are rose coloured and papilionaceous, are borne on dense, erect racemes. These are carried on long, erect axillary stalks (peduncles) which enable maximum exposure for pollination (Piper, 1924; Percival, 1943; Carleton & Weisner, 1968; Spedding & Diekmahns, 1972). Flower and pod maturation begins at the base of the inflorescence and proceeds upwards (Schneiter *et al.* 1969). Inflorescences may consist of 5 to 80 flowers, and each flower has the capability of producing a single seed. A plant may have 5 to 40 stems each having 3 to 5 inflorescences (Carleton & Weisner, 1968). The flowering of sainfoin is indeterminate, but is more determinate than lucerne (Carleton & Weisner, 1968).

Seeds are borne singly in brown indehiscent pods. The pods are bilaterally symetrical and almost semi-circular in side view, with a straight ventral and a curved dorsal suture. On the sides are networks of prominent vascular ridges, often projecting from which are spines (Piper, 1924; Thomson, 1951b; Spedding & Diekmahns, 1972). The true

or milled seed is kidney shaped, with the hilum situated about the middle of the concave edge. The seed colour tends to be olive to brown, or black (Thomson, 1951b; Spedding & Diekmahns, 1972). Sainfoin seeds tend to be larger than those of other forage legumes. The weight of 1000 seeds was found to be approximately 21.5 g unmilled and 15.5 g milled (Thomson, 1951b). In comparison lucerne seed weighs approximately 2 g per 1000 seeds (Schneiter *et al.* 1969).

Sainfoin has a thick (up to 5 cm diameter) tap-root which normally extends to a depth of 1 to 2 metres, but up to 10 metres (Piper, 1924; Whyte *et al.* 1953; Andreev, 1963; Spedding & Diekmahns, 1972). The roots of sainfoin may penetrate to depths even greater than lucerne in open, dry subsoils (Percival, 1943). The root system has a few main branches and numerous fine laterals (Spedding & Diekmahns, 1972). Sainfoin is reported to have a better developed root system with twice as many laterals as lucerne (Kozyr, 1948; Kalugin, 1950; Massaudilov, 1958).

Root nodules occur mainly on the fine lateral roots, but a few also occur on the jeuvenile tap-root (Spedding & Diekmahns, 1972). The nodules are large (3 x 6 mm approximately), wedge shaped, orange-white in colour, possess a subterminal meristem (Wittmann, 1968; Spedding & Diekmahns, 1972), and tend to be formed in clusters (Schreven, 1972).

The early development of the plant is described briefly by Thomson (1938) and Percival (1943). After the kidney shaped cotyledons reach the soil surface, foliage leaves with various numbers of leaflets are produced. The first foliage leaf is usually simple, the second and third, trifoliate, and the later leaves pinnately compound. Short lateral branches are formed and the plant forms a rosette which tends to be more prostrate in the one cut or 'common' type than in the multi-cut or 'giant' type.

Sainfoin can be classified into two taxonomically indistinguishable types, a one cut or 'common' type and a multi-cut or 'giant' type, according to its growth behaviour after about the six leaf stage (Thomson, 1938).

In the one cut type, stem elongation is limited during the establishment year. Flowering usually first occurs in the second year, and occurs once a year (Spedding & Diekmahns, 1972). Stems tend to be shorter and leaflets smaller than in the multi-cut type (Spedding & Diekmahns, 1972).

The multi-cuttype is said to be shorter lived than the one cut type (Spedding & Diekmahns, 1972). Stem elongation and flowering occur on the establishment year (Thomson, 1938). After cutting, the multi-cut type again sends up flowering stems (Thomson, 1938; Spedding & Diekmahns, 1972). Stem elongation is found to take place in the upper internodes, with the first four to six remaining short. Lateral buds develop in the axils of the lower leaves, and stems either elongate, or remain short, producing a tuft of leaves at the base of the plant (Thomson, 1938). In the axils of the upper leaves, either inflorescences or branches may be produced (Thomson, 1938).

1.4 GROWTH PATTERN

Sainfoin is easy to establish. The seeds germinate readily and produce vigorous seedlings that grow rapidly (Hanna *et al.* 1977a). Seedling weight is highly correlated with seed size. However, for a given increment in seed size in sainfoin, the associated cotyledon area increase is less than for lucerne or birdsfoot trefoil (Carleton & Cooper, 1972). Sainfoin is a rapid developing legume, the seedlings of which are more aggressive than those of birdsfoot trefoil or cicer milkvetch (*Astragalus cicer* L.) in mixed culture (Smoliak & Hanna, 1977). Sainfoin seedlings may have an initial advantage over the other two legumes because of a relatively large seed and cotyledon area. It is thought that this advantage would persist throughout seedling growth (Smoliak & Hanna, 1977). Cooper and Fransen (1974) looked at energy relationships and the photosythetic contribution of the cotyledons during early seedling development. Growth was dependent on seed stored carbohydrate during the first seven days of growth (Cooper & Fransen, 1974). The store of substrate was not adequate for normal first leaf formation and expansion, which appeared to depend on cotyledonary photosynthesis. Once stored reserves have been used, photosynthesis by the cotyledon is of major importance to early seedling growth (Cooper & Fransen, 1974). The decrease in photosynthetic contribution of the cotyledons was in proportion to their decrease in area as a proportion of total leaf area, and was only 18% of the total when the seedlings were 19 days old. Smoliak *et al.* (1972) found that lucerne had a greater mean relative growth rate (RGR) and net assimilation rate (NAR) than sainfoin, indicating that lucerne seedlings grew more rapidly during the ten week period of their test. Mean LAR (LAR = leaf area ÷ total dry weight) was slightly higher for sainfoin

than lucerne, as were top and root weights. Accumulated leaf area was also higher for sainfoin than lucerne (Smoliak *et al.* 1972). Mean NAR and RGR values for cicer milkvetch were intermediate between those of sainfoin and lucerne. The important difference between sainfoin and lucerne appeared to be in terms of NAR, with the higher NAR of lucerne enabling a higher RGR despite having a lower leaf area. However differences between these species in terms of seedling top growth were found to be reflected in yields throughout the first year, with sainfoin being highest yielding, followed by lucerne and cicer milkvetch.

Sainfoin begins to grow in the spring before most other perennial legumes (Hanna *et al.* 1977a). A description of the early development has been provided in section 1.3, as has a discussion of the classification of sainfoin into one and multi-cut types according to its growth behaviour and propensity to flowering. The one cut type has a winter requirement for flowering and remains prostrate in its first year, producing one crop of herbage towards the end of the growing season (Bland, 1971; Spedding & Diekmahns, 1972). In subsequent years it grows vigorously, sending up stems and flowering once only (Bland, 1971). The large bulk of herbage produced at this time, in association with flowering, is normally cut and fed, or conserved. The regrowth, or aftermath, which is prostrate and much lower yielding, is normally grazed (Thomson, 1938; Bland, 1971).

The multi-cut type flowers on its first year and flowers two or more times per year (Thomson, 1938; Thomson, 1951a; Bland, 1971). In association with flowering it produces a sufficient bulk of erect growing foliage for cutting twice or more each year, and the aftermath of the final cut can be used for grazing (Bland, 1971). The multi-cut type is probably more appropriate where cutting and conservation is the primary object, as it is a more rapid and luxuriant grower, it flowers earlier than the one cut type, and it will produce two or more heavy crops per year (Percival, 1943).

Thomson (1951a) found that, in the seeding year, a multi-cut type of sainfoin yielded much higher than a one cut type at the first cut (cut when the multi-cut type was in full flower), but that yields for the remainder of the season were similar. In the second year, yields for the one cut type were greater under both frequent (cut monthly) and non frequent (cut when the multi-cut type flowered) defoliation regimes. The multi-cut out yielded the one cut type at the second cut (in the second year). This could have been associated with a higher proportion of leaf (compared to total top) relative to the one

cut type at the time of the first cut (Thomson, 1951a). The higher proportion of leaf could have been due to the more luxuriant growth of fresh leaves and shoots from the base of the multi-cut type at the late flowering stage, or to the fact that the multi-cut type had more leaflets per leaf, shorter internodes on the flowering stems and therefore more leaves per unit length of stem.

Cooper (1972) compared growth and development of the one cut variety Eski, with the two cut variety Remont, during their second season of growth. There was an initial period of leaf formation after which leaf area appeared to increase as a result of leaf expansion. This was followed by a period of stable leaf area index (LAI). Crop growth rate increased with increasing LAI, and was highest during the period of stable LAI. This period coincided with the time of most rapid increase in stem weight, and with the period of restoration of root carbohydrate reserves used in spring growth. The later maturing Eski had a more rapid RGR than Remont as the season progressed. The higher growth rate of Eski was related to a significantly higher LAR, and its yield advantage was thought to be primarily due to this, particularly as the amount of available radiant energy was increasing with increasing day length (Cooper, 1972).

From the stand point of getting early grazing, Remont would have an advantage, because early on its yields were greater than those of Eski. Both varieties were found to recover rapidly following spring clipping, but the regrowth of Remont was more rapid following a hay harvest or late season clipping (Cooper, 1972). No data were given for regrowth following hay harvest, but it was reported that soil moisture was a major limiting factor to later growth. The ability of a two cut variety to produce a second hay crop would probably be negated to some extent under these conditions.

Sainfoin tends to recover more slowly from defoliation than lucerne, and does not produce as much regrowth (Hanna *et al.* 1975). Sheehy & Harding (pers.comm.) compared the growth patterns of sainfoin and lucerne during a summer regrowth period of 48 days. At the end of 48 days, the amount of herbage produced by sainfoin was approximately 32% less than that produced by lucerne. The main difference was in weight of stem, with leaf weights being similar. LAI's however, were markedly different, with the final LAI of lucerne being over twice that of sainfoin. The higher LAI of lucerne was reflected in higher rates of canopy photosynthesis. The more rapid increase in LAI of lucerne

was explained partly by the difference in rate of leaf appearance. Axillary leaves accounted for the higher rate of leaf appearance in lucerne. A contributory factor to the higher rate of leaf appearance was thought to be the greater number of nodes on lucerne plants (24) compared with sainfoin (6-7). Specific leaf area (SLA = leaf area ÷ leaf dry weight) for sainfoin was less than half that for lucerne throughout the growing period. Rates of individual leaf photosynthesis per unit leaf area were similar for the two species, as were rates of dark respiration per unit leaf area and the conductance of leaves to water loss. Thus, it appears that the critical difference between the two species was one of leaf morphology. Lucerne, by using its assimilate to produce a greater leaf area instead of the thicker leaves produced by sainfoin, increased its interception of photosynthetically active radiation and hence its photosynthetic capacity.

The best management for optimum yield of sainfoin seems to be to cut when flowering is well advanced (Spedding & Diekmahns, 1972). Frequent or early defoliation can adversely affect the productivity and stand persistence of sainfoin (Thomson, 1951a; Badoux, 1965; Carleton *et al.* 1968b; Jensen & Sharp 1968; Bland, 1971; Hassell, 1971). Hassell (1971) found that pre-bloom and mid-bloom cutting reduced competitiveness and allowed invasion of weeds. Jensen and Sharp (1968) found that weeds severely invaded frequently cut plots of sainfoin, with those most frequently cut having the most weeds. Carleton *et al.* (1968b) also reported loss of stand accompanied by weed invasion on frequently cut plots. However, if defoliation is delayed to the late bud or early flowering stage, regrowth of sainfoin is satisfactory, and stand longevity is not reduced (Spedding & Diekmahns, 1972; Hanna *et al.* 1975). Hassell (1971) cut sainfoin at the prebloom, midbloom and early pod stages, and found yields to be significantly greater at the early pod stage. Carleton *et al.* (1968a) found that maximum accumulation of dry matter and crude protein in sainfoin occurred at 100% bloom, compared to 2-45% bloom for lucerne.

Thomson (1951a) found that sainfoin yielded lower under frequent (monthly) defoliation, in comparison to less frequent defoliation, such as might be encountered under a hay cutting regime. In the second season of growth, the frequent defoliation regime reduced yields by 63% (multi-cut type) and 50% (one cut type), relative to infrequent defoliation. The difference in performance of the two types can be explained largely in terms of their growth behaviour (Thomson, 1951a). The multi-cut type exhibited a much greater propensity to flower than

the one cut type, and its propensity to flowering was modified under conditions of frequent defoliation much less than that of the one cut type. The multi-cut sainfoin produced abundant flowers even under frequent defoliation (Thomson, 1951a). It was thought that the lack of flowering in the one cut type was related to its greater resistance to frequent defoliation and that it utilised relatively more of its root carbohydrate reserves in producing leaves, which yielded a return of carbohydrates. In contrast, the flowers of the multi-cut type yielded no such return, and hence root reserves were more rapidly exhausted. Also a greater tendency to flower can result in fewer leaves at low levels on the plant, and hence destruction of a high proportion of the top when cut, resulting in slow regrowth (Spedding & Diekmahns, 1972).

Under a hay cutting regime, sainfoin seems to produce over half of its total annual production at the first cut (Piper, 1924; Thomson, 1951a; Evans 1961; Jensen & Sharp, 1968). The one cut type of sainfoin tends to produce a greater proportion of its yield by the first cut than the multi-cut type, which will undergo stem elongation and flower a second time (Thomson, 1951a).

Regrowth of sainfoin after cutting is slower than lucerne, and is more adversely affected by frequency of cutting than lucerne (Carleton *et al.* 1968b). The slow regrowth of sainfoin relative to lucerne has been discussed previously in this section, in relation to the work of Sheehy & Harding (pers.comm.). This slow regrowth and sensitivity to frequent cutting may be further explained by the results of Cooper and Watson (1968), who found that total available carbohydrate in the roots of sainfoin were lower and showed less cyclic fluctuation with cutting than in lucerne. Total available carbohydrate remained at low levels until late summer or early autumn. It is hypothesised that, following the use of carbohydrate reserves in early spring growth, regrowth of sainfoin depends primarily on carbohydrates synthesised in existing leaf area (Cooper & Watson, 1968). Sheehy & Harding (pers.comm.) calculated that sainfoin, on average, translocated 9% of its photosynthate to the roots, compared to a figure of 3% for lucerne. They suggest that it is possible that the figure for lucerne could be an under estimate. However, if these figures are approximately correct, the additional photosynthate translocated to the roots of sainfoin is being used for some purpose other than building up root reserves. As discussed in section 1.3 it has been observed that sainfoin has a more highly developed root system than lucerne, and

produces a greater weight of nodule tissue. It is also possible that its symbiotic nitrogen fixing system is biochemically less efficient, and thus more energy demanding, than that of lucerne.

There appear to be some features of the morphology and growth pattern of sainfoin which contribute to its apparently poor performance in relation to the more productive forage legumes, particularly lucerne. Sainfoin has been found to have a lower SLA and LAR than lucerne, and hence a lesser ability to produce photosynthate. Also the lack of build up of carbohydrate reserves during regrowth cycles may well contribute to slow initial regrowth after defoliation. As discussed in section 1.5.2, however, the low SLA and slow rate of regrowth may be advantageous under very dry conditions.

1.5 ECOLOGICAL NICHE

The two types of sainfoin, the one cut and multi-cut types are best suited to differing environmental conditions. The one cut type, because it produces a greater proportion of its seasonal herbage production at the first cut (e.g. Thomson, 1951a), is best adapted to an environment with a short growing season or a low mid season moisture supply (Murray & Slinkard, 1968; Cooper, 1972, Carleton *et al.* 1968b). Its slow regrowth is thought to enhance its drought resistance (Koch *et al.* 1972), making it suitable for regions having very dry summers. The multi-cut type, because of its ability to produce two or more hay crops in a single year (e.g. Thomson, 1938), appears best suited to environments with a longer growing season, where more than one cut (or grazing) is desired, and where there is adequate mid season moisture to support vigorous regrowth.

1.5.1 SOILS

Sainfoin is especially adapted to dry, well drained, calcareous soils, containing at least 0.3% CaO (Whyte *et al.* 1953; Spedding & Diekmahns, 1972).

Historically the culture of sainfoin in Europe and Britain has been largely confined to chalky or other calcareous soils, particularly where these are subject to drought (Piper, 1924; Bland, 1971), and sainfoin has been found growing wild on the chalk soils of south-east England (Bland, 1971). Both low pH values, and high concentrations of Al have been found to markedly reduce the growth of sainfoin (and lucerne) in comparison to lupins (*Lupinus* species), serradella (*Ornithopus sativus*) and white clover (Rorison, 1957). Al toxicity was found

to be most important in the early seedling stage of growth. It is felt that sainfoin is excluded from certain acid grasslands mainly because of the toxic effect of Al on the soil solution (Rorison, 1965).

Traditionally, sainfoin has been grown on the dry calcareous soils of Russia and Eruope, and has shown promise as a hay and pasture crop in dry locations of USA, South America and South Africa (Whyte *et al.* 1953; Andreev, 1963; Spedding & Diekmahns, 1972; Ditterline & Cooper, 1975). Sainfoin is reported to have good persistence under dry land conditions, but may be less persistent (e.g. less than lucerne) under irrigation (Cooper *et al.* 1968a; Ditterline & Cooper, 1975; Chapman & Carter, 1976). Under dry conditions, sainfoin does respond to irrigation, but does not require it as frequently as, for example, lucerne (Hanna *et al.* 1977a). Sainfoin is found to have the poorest tolerance to flooding of a range of forage legumes, including lucerne (Heinrichs, 1970). The sensitivity of sainfoin to wet soil conditions is probably largely explained by its susceptibility to crown and root rot diseases (Ditterline & Cooper, 1975). Ditterline and Cooper (1975) cite the crown and root rot pathogenic complex as being the most limiting factor to sainfoin production. This problem has generally been associated with the fungus *Fusarium solani* (Mathre, 1968; Ditterline & Cooper, 1975; Auld *et al.* 1976). Gaudet *et al.* (1980), however, have evidence that the causal organisms may be one or more bacteria, rather than a fungal pathogen. Although crown and root rot is found under both dryland and irrigated conditions (Sears *et al.* 1975), the problem seems to be more acute under wetter conditions.

As would be expected from the previous discussion, sainfoin generally does not thrive on heavy clay soils (Spedding & Diekmahns, 1972). However, Bland (1971) points out that establishment can be quite satisfactory on clay soils provided the pH is not low. The importance of moisture level is highlighted by Carleton *et al.* (1968b) who report more frequent loss of sainfoin stands on heavy soils, and particular problems following irrigation. The fact that sainfoin appears to perform best on well drained soils is supported by Kornilov and Verteleskaia (1952) who state that sainfoin is exceptionally resistant to unfavourable environment when grown on sandy soil, with good persistence being observed in the desert climate of Karastan, U.S.S.R.

Jensen and Sharp (1968), and Chapman and Carter (1976) state that sainfoin has considerable tolerance to salinity, whereas Hanna *et al.* (1977a), state that it does not tolerate saline soils. Jensen and Sharp (1968) presented evidence indicating that sainfoin does have good salt tolerance, and the ability to persist in highly saline areas. The other authors did not substantiate their claims with evidence.

Thus, it appears that a number of soil factors, including pH, Al level, moisture conditions and texture, in combination determine whether or not sainfoin will thrive on a particular soil. It appears that adverse soil moisture conditions have their effect via the crown-root rot complex.

1.5.2 CLIMATE

Schnieter *et al.* (1969) stated that sainfoin is more drought hardy than lucerne. The deep root system of sainfoin has been cited as a reason for its drought resistance (Bland, 1971). It has also been suggested (Koch *et al.* 1972) that the slow regrowth of sainfoin, particularly the one cut type, may contribute to its drought tolerance. This idea is supported by Shain (1959) who reports lower drought tolerance from multi-cut sainfoin types. Thus it appears that one cut types of sainfoin might be more suitable for areas with very limited supplies of mid season moisture.

Sheehy *et al.* (1978) found that values for leaf area index and leaf area per unit leaf weight of sainfoin were about half those of lucerne. In addition, as the crops grew, mean daily leaf water potentials were substantially less negative, and mean daily turgor potentials were substantially higher in sainfoin than lucerne. There were no significant differences between stomatal resistances of the crops (Sheehy *et al.* 1978), indicating that leaf area is probably the critical factor. Hence the low specific leaf area (SLA) of sainfoin may be a contributing factor to its ability to tolerate drought conditions and selection for increased SLA to improve photosynthetic performance, as suggested by Sheehy & Harding (pers.comm.) may have the effect of reducing drought tolerance. Percival and McQueen (1980) found that the regrowth after cutting, of sainfoin, appeared to be more adversely affected than that of lucerne by dry conditions. It is not however clear, whether the dry conditions were the cause of the slow regrowth, or whether regrowth was just inherently slow, and would have been slow regardless of soil moisture conditions because of the

cultivar under study being a one cut type. Poor regrowth of a one cut sainfoin cultivar has been observed where seasonal rainfall was adequate (Meyer, 1975). Despite its reported draught tolerance, however, sainfoin has not been recommended for dryland areas in Montana or Western Canada where rainfall is less than 300 mm per year (Cooper *et al.* 1968b; Hanna *et al.* 1977a).

Sainfoin is reported to be winter hardy and frost resistant (Schneiter *et al.* 1969; Chapman & Carter 1976) with seedlings and mature plants being highly tolerant of autumn and spring frosts (Hanna *et al.* 1977a). It is thought, however, that sainfoin is less winter hardy than the lucerne varieties recommended for locations having severe winters (Hanna *et al.* 1977a), and Andreev (1963) states that sainfoin will stand heavy frost only if there is good snow cover. Sainfoin is found to have a wide range of winter hardiness according to area of origin (Cooper *et al.* 1968b). Introductions from Russia and Turkey (which would probably tend to be one cut types) had good and fair survival respectively, while introductions from England (which would presumably include some multi-cut types, and would tend to be of Mediterranean origin) showed poor survival. Jensen & Sharp (1968) state that, at the succulent stage, sainfoin is more tolerant to frost than lucerne, and that this should enable it to maintain later growth in the autumn, and commence growth earlier in the spring than lucerne. During the autumn, plants develop a low rosette type of growth that may remain green under snow for most of the winter (Hanna *et al.* 1977a).

Thus, the absolute frost tolerance of sainfoin appears to be lower than that of lucerne, but it is reportedly able to maintain growth under colder conditions than lucerne.

1.5.3 CONCLUDING COMMENTS - ECOLOGY OF SAINFOIN

Sainfoin appears to have some quite specific environmental requirements, particularly with regard to soil moisture and pH. Evans (1961) states that lucerne is better adapted to a wider range of environmental conditions than sainfoin. One cut sainfoin varieties appear to display better survival under dry mid season conditions, and may tend to be more winter hardy than multi-cut varieties. In New Zealand, with a relatively long growing season, and an absence of severe winter conditions, multi-cut sainfoin would likely be the more appropriate type.

1.6 NITROGEN FIXATION OF SAINFOIN

The nitrogen fixing ability of sainfoin is, in many instances, reported to be insufficient to provide for the total nitrogen requirement of the plant.

Nitrogen deficiency symptoms have been observed on inoculated sainfoin at an early stage in crop development (Burton & Curley, 1968; Sims *et al.* 1968; Roath & Graham, 1968; Meyer, 1975; Smoliak & Hanna, 1975). Cooper *et al.* (1968a), growing sainfoin with grasses and other legumes under irrigated pasture conditions, found that sainfoin recovered very slowly following defoliation, and that plants were a light green to yellowish colour characteristic of nitrogen deficiency. Schneiter *et al.* (1969) also report the development of nitrogen deficiency symptoms in sainfoin, and state that this is a very unusual condition for a legume which indicates that the strain of nitrogen fixing bacteria present is inefficient or short lived.

Nitrogen deficiency symptoms have been observed in sainfoin despite plants being abundantly nodulated (Burton & Curley, 1968). The nodules on healthy plants were observed to vary greatly in size and shape, with frequently only the small nodules containing the red pigment, leghaemoglobin, associated with nitrogen fixation. They state that this indicates a very delicate balance between the host plant and its microsymbiont, and dominance by the host. Nitrogen deficiency symptoms have been confirmed by low protein levels found on analysis of chlorotic plants (Sims *et al.* 1968). Growth responses of inoculated field grown sainfoin to added nitrogen have been observed by Sims *et al.* (1968), Jensen and Sharp (1968) and Meyer (1975). On soils containing low levels of nitrate-N, Sims *et al.* (1968) reported yield responses to up to 336 kg per hectare of added N. On a site with higher levels of nitrate-N, the effect of added fertiliser N was less pronounced. Nitrogen deficiency symptoms were manifest early in the season, and then disappeared as the activity of nitrifying bacteria increased, and as the plant root systems developed (Sims *et al.* 1968). Jensen and Sharp (1968) observed responses to nitrogen fertiliser by inoculated sainfoin in central Nevada. N at 112 kg per hectare substantially increased yields in the second year (first harvest year) of the crop. The response was diminished in the third year, and non significant in the fourth year of the crop (Jensen & Sharp, 1968). It was thought that the reduced benefits from nitrogen fertiliser in the third and fourth

years may have been related to increased nitrogen fixation by rhizobia in the more mature plants. Forage yields of sainfoin are frequently inferior to lucerne, where seasonal rainfall is adequate for two or more cuttings, because of sainfoin's poor regrowth and vigour (Meyer, 1975). Inoculated sainfoin plants typically showed N deficiency symptoms at Fargo, North Dakota, and it was thought that inferior recoverability and stand persistence could be due in part to its inability to obtain sufficient nitrogen via symbiotic fixation (Meyer, 1975). Meyer (1975) applied N, P and K to a three year old inoculated sainfoin stand. N increased yields for each increment applied, up to 448 kg per hectare. The growth and vigour was enhanced by N fertilisation but stand persistence remained poor for all but the 448 kg per hectare treatment. Fourth and fifth year yields did not compare favourably with unfertilised, inoculated lucerne (Meyer, 1975). Tap roots of plants from 0, 224 and 448 kg N per hectare treatments were examined for nodules. Large pink nodules were observed on most plants. It was thought that the *Rhizobium* inoculant was ineffective, or that the nodules were short lived (Meyer, 1975).

Babian & Karagulian (1959), on two soils, found that sainfoin responded more to added N than lucerne. Koter (1965a) grew sainfoin and red clover in pure sand and applied a range of N levels. The presence of combined N in the medium accelerated the growth rate of both species in comparison to plants reliant solely on symbiotically fixed N_2. Larger responses to N addition were obtained with sainfoin than with red clover. At the highest rate of N, the amount of herbage produced by sainfoin was increased by 112% and that of red clover by 55% compared with plants supplied with no combined N. Sainfoin not supplied with combined N also flowered later than plants supplied with N. The introduction of N into the nutrient solution reduced the nodulation of both species (Koter, 1965a). The increase in yield of nitrogen, above the quantity fixed from the atmosphere by the zero N plants was greater for sainfoin (20 - 33%) than for red clover (7 - 15%).

Koter (1965b) found that low levels of combined N stimulated nodulation and N_2 fixation in sainfoin. N_2 fixation was stimulated by 21 - 28%. High rates of combined N severely checked nodulation and nitrogen fixation, with most of the plant's nitrogen requirements being absorbed from the medium.

Major *et al.* (1979) measured $N_2[C_2H_2]$ fixing activity (N_2 fixing activity as measured by the acetylene reduction technique) in seedlings of sainfoin, lucerne and cicer milkvetch. It was found that the amount of acetylene reduced by lucerne was not as closely related to shoot dry weight as it was for sainfoin and cicer milkvetch. The relationship between nodule weight and top weight was closer in sainfoin than it was for the other two plants (Major *et al.* 1979). This may indicate a more critical relationship between plant performance and N_2 fixing activity in sainfoin than in lucerne. Sainfoin had a greater weight of nodules and a greater $N_2[C_2H_2]$ fixing activity per plant than the other two species, but a lower specific $N_2[C_2H_2]$ fixing activity per nodule weight. Sainfoin had more nodule tissue and a greater acetylene reducing activity per weight of shoot dry matter than lucerne (Major *et al.* 1979). Copley (1972) also reports higher $N_2[C_2H_2]$ fixing activity, on a per plant basis for sainfoin than lucerne. Although nodulation of sainfoin appears to be good (Karpov, 1957; Stergeeva, 1957; Burton & Curley, 1968; Major *et al.* 1979) and acetylene reducing activity appears to be adequate (Copley, 1972; Major *et al.* 1979), there is very strong evidence (Koter, 1965a; Burton & Curley, 1968; Sims *et al.* 1968; Roath & Graham, 1968; Meyer, 1975) that sainfoin is less able to provide for its own nitrogen requirements via symbiotic fixation than other forage legumes such as lucerne and red clover.

There is an apparent contradiction between the seemingly low quantity of N fixed, and relatively high $N_2[C_2H_2]$ fixing activity per weight of plant compared with, for example, lucerne. It could be that the acetylene reduction technique over-estimates N_2 fixing activity in sainfoin relative to lucerne, and that greater inefficiencies, or energy losses, perhaps in the form of H_2 evolution (section 2.2.2), are present in the nitrogen fixing system of sainfoin.

There are, however, contrasting reports which indicate that sainfoin can fix adequate amounts of N. Badoux (1965) reports that yields of sainfoin in pure stands were slightly reduced by the application of 45 and 90 kg N per hectare, and that, in the glasshouse, added nitrate decreased top and root weights, and the number and size of nodules. There are two possible explanations for this result. It could indicate that symbiotic fixation of N was adequate to meet the requirements of the plant, or that the amount of N added was sufficient to inhibit N_2 fixing activity and partially replace N_2 fixation, but not sufficient to give a growth response. Stergeeva (1957) reported that sainfoin was

found to have a greater soil enriching effect than lucerne. Crops were found to have greater yields and higher %N content following sainfoin. The greater nodule weights, and more highly developed root systems of sainfoin (section 1.3), and their subsequent decay on cultivation may contribute to this effect. An alternative explanation could be that there is leakage of N from living sainfoin plants into the soil.

The information on N_2 fixation in sainfoin can be summarised as follows:

1. Many reports of N deficiency symptoms in sainfoin stands.
2. Many reports of responses to added N.
3. A lesser number of reports comparing the response of sainfoin to added N with the response of other forage legumes, in which sainfoin is usually found to give a greater response than the better performing plants, such as lucerne or red clover.
4. Nodulation generally appears to be adequate in terms of number and weight of pink nodules. Nodule weight on a plant weight basis appears to be greater than that of lucerne.
5. From the little work that has been done, the $N_2[C_2H_2]$ fixing activity of sainfoin appears to be lower than that of lucerne on a weight of nodule basis, but higher on a per plant or weight of plant basis.

Overall, the evidence appears to suggest that sainfoin, although it produces a proportionally greater weight of nodule tissue than for example lucerne, and may have a higher $N_2[C_2H_2]$ fixing activity per weight of plant (thus using more energy), actually fixes, or utilises, less N_2 than lucerne.

Burton & Curley (1968) speculate that tannins contained in sainfoin would have an adverse effect on the symbiotic relationship between plant and bacteria. This could perhaps explain the relatively low $N_2[C_2H_2]$ fixing activity per weight of sainfoin nodule tissue.

Because of the possibly higher energy demand of the symbiotic N_2 fixing system of sainfoin and the apparently lesser ability of sainfoin to utilise photosynthetically active radiation (section 1.4) relative to lucerne, it could be that the energy relationships of sainfoin are particularly critical, and that relationships between such parameters as leaf area and nodule weight or $N_2[C_2H_2]$ fixing activity are more critical than in other legumes such as lucerne.

2 NITROGEN FIXATION

2.1 INTRODUCTION

2.1.1 THE IMPORTANCE OF BIOLOGICAL NITROGEN FIXATION

The four most abundant elements present in plant tissues are usually carbon, hydrogen, oxygen and nitrogen (Stewart, 1966). Plants, like all organisms, require nitrogen (N) for their growth and reproduction. Nitrogen is a constituent of all proteins and enzymes, many metabolic intermediates involved in synthesis and energy transfer, the photosynthetic pigment chlorophyll and the deoxyribonucleic acids which make up the genetic code (Viets, 1965; Bond, 1977).

Nitrogen is the element which most frequently limits crop growth (Quispel, 1974; Yates, 1976; Fowden, 1979). Carbon, hydrogen and oxygen are available to the plant in ample supply from atmospheric and soil sources (Stewart, 1966). The situation regarding nitrogen is somewhat different in that while there are abundant amounts (unlike other growth limiting nutrients) much of it is inaccessible to most plants (Stewart, 1966; Quispel, 1974). Fertile soils may contain as much as 6.7 tonnes of combined N per hectare, but only a few kg of this will be in plant available mineral forms (Stevenson, 1965). The atmosphere contains an abundant supply of gaseous nitrogen (N_2); it has been calculated that the air over one hectare of land contains about 78,500 tonnes of elemental N (Stevenson, 1965). However, the vast majority of plants cannot utilise atmospheric N_2, and are dependent on the relatively small quantities of mineral N present in the soil, largely in the form of nitrate and ammonium-N (Stewart, 1966).

A small minority of plants can utilise gaseous nitrogen (N_2) as a source of N (Stewart, 1966). These plants assimilate, or fix, atmospheric N_2 by reducing it to ammonia from which amino acids and amides (the basic material for synthesising proteins and other biological compounds) can be formed.

A group of agriculturally very important N_2 fixing plants are the legumes. Legumes belong to the family Fabacae which consists of 12,000 to 14,000 species (Burns & Hardy, 1975). Legumes account for almost half of the annual quantity of N fixed by biological systems (Evans & Barber, 1977).

The causative agents of N_2 fixation in the legumes are bacteria of the genus *Rhizobium*, which colonise the roots of legumes causing the

formation of nodules, within which they change to a non-reproductive form called bacteroids and fix N_2 (Yates, 1976).

Symbiotic N_2 fixation is of great importance to agriculture because it enables access to the vast reserves of N_2 in the atmosphere. Symbotic N_2 fixation by plants, such as legumes, enhances the growth and productivity of the N_2 fixers themselves, and may also make N available to successive plants or crops, via the death and decay of plant tissue or the deposition of dung and urine by grazing animals, both of which tend to increase soil N levels (Ball, 1969).

2.1.2 THE BIOLOGICAL NITROGEN FIXING REACTION

The N_2 molecule is normally very unreactive, because of the very stable triple bond which links the two atoms of the structure N≡N (Postgate, 1978). Red hot magnesium or a catalyst at highly elevated temperatures and pressures, as in the Haber-Bosch process, are normally necessary to make N_2 reactive. Biological N_2 fixation, which has an energy requirement of 355 KJ per mole of NH_4^+, is approximately twice as efficient as the industrial Haber-Bosch process which has an energy requirement of approximately 680 KJ per mole of NH_3. Energy is required in both systems to overcome the activation energy for N_2 reduction and in the industrial process also for the production of hydrogen (Rawsthorne *et al.* 1980).

In biological nitrogen fixing systems the nitrogen fixing enzyme, nitrogenase, catalyses the conversion or reduction of atmospheric nitrogen (N_2) to ammonia (NH_3) a form of nitrogen which plants can assimilate (Stewart, 1966; Conn & Stumpf, 1972; Postgate, 1978) as follows:

$$N_2 + 6H^+ + 6e \xrightarrow[\text{1 atm. pressure}]{\text{normal temperatures}} 2NH_3$$

Nitrogenase renders the N_2 molecule reactive at normal temperatures and pressures and the biological reaction takes place in water, under an atmosphere of oxygen, both substances which would interfere with the two systems of chemical fixation mentioned (Postgate, 1978).

Nitrogenase consists of two non-haem, iron sulphur proteins with molecular weights of approximately 220,000 and 60,000 respectively (Yates, 1976; Postgate, 1978). The larger protein contains two molybdenum atoms (Yates, 1976).

A scheme for the action of nitrogenase is shown in fig.1. Apart from the two nitrogenase proteins, there are a number of requirements

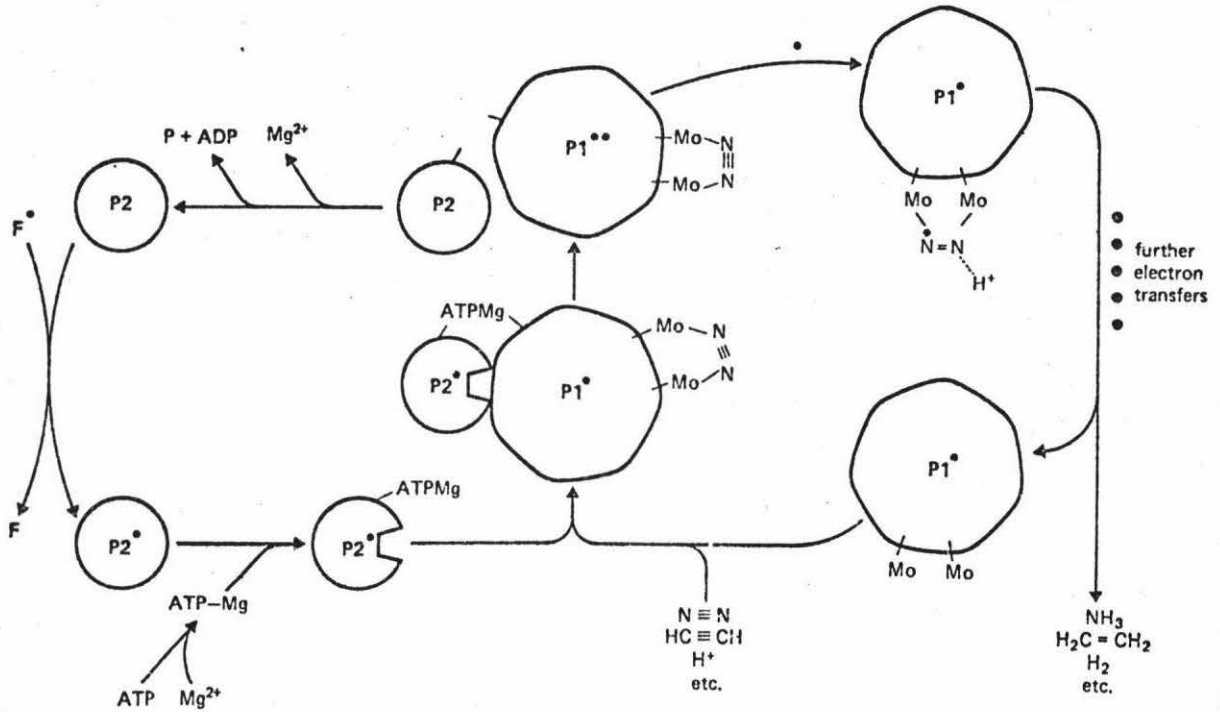


Fig 1 Scheme showing a possible mode of action of the N_2 fixing enzyme, nitrogenase (after Postgate, 1978). P1 is the larger protein containing iron and molybdenum ations and P2 is the smaller protein with iron atoms only. F is an electron donating substance (ferredoxin or flavodoxin). Black dots represent electrons.

for nitrogenase catalysis. Nitrogen fixation is a reductive process, and a reductant of low potential is required (Ljones,1974). The immediate donor of electrons to nitrogenase is thought to be ferredoxin (Ljones, 1974). The free energy for reduction of N_2 by ferredoxin is negative, but additional energy is required for nitrogenase activity. This energy is supplied by the hydrolysis of ATP to give ADP and orthophosphate (Ljones, 1974). ATP, which is present in all living tissue, is the principal molecule whereby chemical energy is mobilised for biological processes. The hydrolysis of ATP to ADP results in a net loss of chemical energy which can be used to support various biological processes (Postgate, 1978). In nitrogen fixation 12 to 15 molecules of ATP are consumed in the conversion of one molecule of N_2 to ammonia (Postgate, 1978). In physiological terms, N_2 fixation is energy expensive because the synthesis of ATP and production of reductant require energy. This energy is provided by the oxidative degradation of carbon substrates, from photosynthesis, through a series of enzymes and coenzymes (Evans & Barber, 1977). Another essential ingredient in the nitrogenase reaction is the magnesium ion, Mg^{2+}. The exact role of Mg is not understood, but ATP reacts with it to form a monomagnesium salt in order to perform its function with nitrogenase (Postgate, 1978).

Thus, nitrogen fixation requires the two nitrogenase proteins in equimolar proportions, a reducing agent (ferredoxin), ATP and Mg^{2+} (Postgate, 1978). A possible scheme of events (Postgate, 1978) is as follows (also see fig.1):

> ATP reacts with magnesium ions to produce a compound which activates the reduced form of the small protein (altering its redox potential from -280 to -400 mV). Meanwhile, the large protein with a 'spare' electron among its iron atoms, has bound a reducible substrate, normally N_2, at transition metal atoms which are probably molybdenum. The activated small protein joins the large protein carrying the substrate to form the 'nitrogenase complex,' assisted by ATP-Mg, within which an electron from the iron atoms of the small protein is transferred to the iron atoms of the large protein, prior to reaching the bound substrate. After several such electron transfer events the product of the enzyme action, normally NH_3, is released. Each electron transfer requires a new electron from the small protein, and each time a transfer takes place, a molecule of ATP is hydrolysed to ATP and pyrophosphate.

Nitrogenase has the ability to reduce several substrates in addition to N_2. One of these which is of particular interest is the reduction of acetylene (C_2H_2) to ethylene (C_2H_4), because C_2H_2 and C_2H_4 can be measured quantitatively using gas chromatography, and the reaction can be used as an assay for N_2 fixing activity.

In the absence of other substrates, the enzyme will react with water, forming gaseous H_2. In fact, this reaction actually accompanies N_2 fixation and a substantial proportion of the total reductant provided may be expended in the reduction of H^+ to H_2 (Hardy *et al.* 1975; Evans & Barber, 1977; Postgate, 1978). This ATP dependent H_2 evolution represents an inefficiency in the N_2 fixing system. Some N_2 fixing systems, including some legume symbioses, have evolved mechanisms involving an 'uptake' hydrogenase (an enzyme which takes up H_2) whereby H_2 from the nitrogenase reaction is recycled in the electron transport system (Schubert & Evans, 1976; Evans & Barber, 1977; Peters *et al.* 1977).

Nitrogenase activity can be regulated by such factors as photosynthate supply and the level of combined N. Photosynthate supply is thought to influence nitrogenase activity via the provision of energy and this is discussed in more detail in section 2.2.1. Combined N in the form of ammonia has been found to repress nitrogenase synthesis (Evans & Barber, 1977). This repression is thought to occur at the transcriptional level, and that ammonia acts indirectly by repressing the activity of glutamine synthetase in the cell (Yates, 1976). This enzyme, which catalyses the formation of glutamine from glutamic acid and ammonia, is considered to be the activator of nitrogenase synthesis (Yates, 1976).

2.1.3 NITROGEN FIXATION IN LEGUMES

Nitrogen fixation in legumes occurs in root nodules. The legume root nodule is a highly developed plant organ that possesses several characteristics that appear to be particularly conducive to symbiotic N_2 fixation (Evans & Barber, 1977). Bacteroids within the nodules are located, usually in groups of three or four, enclosed in a membrane of plant origin and surrounded by the cytoplasm of the host cell (Stewart, 1966; Evans & Barber, 1977). The mass of bacteroids enclosed in these membranes makes up about 30% of the total nodule weight. The bacterial tissue is supplied with water and carbohydrate from the plant (Pate *et al.* 1969) and with atmospheric CO_2 which serves as a substrate for carboxylation reactions (Evans & Barber, 1977).

Ammonia, the first product of nitrogenase is excreted from the bacteroids into the plant cytoplasm (cytosol) where it is converted into glutamine, asparagine and other amino acids (Pate *et al.* 1969). A group of highly specialised cells surround the xylem elements of the root and function as secretory glands in the delivery of products of N_2 fixation into the xylem elements (Pate *et al.* 1969).

The nitrogenase enzyme is very sensitive to oxygen, which affects it in two ways (Burns & Hardy, 1975): 1) by irreversible non competitive inhibition of ATP hydrolysis and substrate reduction and 2) inactivation of the enzyme, which is generally considered to be irreversible.

The mechanisms that nodulated legumes have evolved to protect nitrogenase from O_2 damage are very specialised and effective. A barrier to free diffusion of O_2, near the periphery of nodules, has been demonstrated (Evans & Barber, 1977). In addition the cytoplasm surrounding 'packets' of bacteroids contains a pigment called leghaemoglobin. Leghaemoglobin participates on a facilitated diffusion process in which O_2 from outside of the nodule is transferred to the bacteroids through oxyleghaemoglobin, in a way that continuously maintains an exceedingly low concentration of free O_2 (Evans & Barber, 1977). Leghaemoglobin thus transports oxygen, but its affinity for oxygen is so high that it delivers it to the baceteroids (which require it for metabolism) at a concentration which is harmless to their nitrogenase. Thus N_2 fixation and O_2 consumption are rendered physiologically compatable (Postgate, 1978).

2.2 FACTORS INFLUENCING NITROGEN FIXATION

Primary factors which could limit the nitrogen fixing reaction are the concentration of nitrogenase; the degree of saturation of nitrogenase by the substrates of the reaction, ATP, reduced electron donor and N_2; and the concentration of the product, ammonia (Hardy & Havelka, 1976).

Nitrogenase concentration is probably not limiting above 20°C but may possibly be limiting at lower temperatures (Hardy *et al.* 1968; Gibson, 1971; Hardy & Havelka, 1976). The low km (high affinity) of 0.02 to 0.05, of the enzyme for N_2, eliminates the partial pressure of N_2 as a practical limitation in the field.

Biological N_2 fixing systems have a high energy demand, and more energy is consumed in N_2 fixation than would be consumed if the organisms concerned were assimilating ammonia (Burris, 1977; Postgate & Hill, 1979) or nitrate (Silsbury, 1977). Ryle *et al.* (1978) found that soybean (*Glycine max* L.) nodules exhibited rates of respiration three to four times that for an equivalent weight of root. Ryle *et al.*

(1979b) working with white clover, soybean and cowpea (*Vigna unguiculata* L.) found that the rate of respiration of nodulated roots carrying out symbiotic nitrogen fixation was sometimes as much as twice that of equivalent root systems lacking nodules and taking up nitrate N. In all three legumes, those plants dependent on symbiotic fixation for their supply of nitrogen respired 11 to 13% more of their fixed carbon each day, than equivalent plants lacking nodules and supplied with abundant combined N. Higher rates of respiration in the nodulated root systems of the nitrogen fixing plants than on the root systems of the nitrate fed plants were largely responsible for this difference. The source of nitrogen was found to have little or no effect on the rate of photosynthesis or shoot respiration (Ryle *et al.* 1979b). Mahon (1977) found that the rate of root plus nodule respiration of pea (*Pisum sativum* L.) plants decreased after addition of NH_4NO_3. Similar photosynthetic rates suggested that the decreased respiration was not related to a decrease in assimilate supply. Using a method of CO_2 exchange, Silsbury (1977) found that subterranean clover plants fixing N_2 symbiotically from the air used 37.8% of the net daily CO_2 uptake for synthesis of new material over 24 hours, whereas those assimilating mineral nitrogen used 27.4% over 24 hours. Silsbury (1977) concluded that the energy requirement for symbiotic fixation was substantially greater than that required for the assimilation of mineral nitrogen from the soil. In contrast, Gibson (1966) grew subterranean clover on agar slopes and compared relative growth rates (to minimise the effect of time, and initial difference in plant size). During the early stages of nodule initiation and development there was a carbohydrate requirement which was not attributed directly to nitrogen fixation but which caused nodulated plants to have lower weights, early on, than nitrogen fed control plants (even though the relative growth rates of the control and nodulated plants may thereafter be similar, there will be a small increase in absolute dry weight difference with time). Otherwise the carbohydrate requirements for symbiotic nitrogen fixation were found to be similar or only slightly greater than requirements for the assimilation of combined N (Gibson, 1966). It was assumed in this work that a lower carbohydrate requirement for assimilation of combined N compared to the requirement for N_2 fixation would make more carbohydrate available for the growth of plants taking up combined N, and thus lead to higher relative growth rates. Minchin and Pate (1973), working with pea plants, carried out direct measurements of respiration,

and carbon and nitrogen analyses on plants dependent on symbiotic nitrogen fixation and plants supplied with nitrate N. Nodule respiration was measured on detached nodules, but when expressed as a proportion of total root respiration was similar to values obtained elsewhere (e.g. Ryle *et al.* 1979a). It was found that the energy cost in terms of mg C per mg N acquired by the plant was similar for symbiotic N_2 fixation and NO_3 assimilation.

If nitrate is reduced in the root, or in the shoot in the dark, biochemical considerations suggest that the energy cost would be similar to that of reducing N_2 to NH_3 in the root nodule (Bergersen, 1971). Ryle *et al.* (1979b) state that no such respiratory burdens were manifest in the nitrate fed legumes they examined. Alternatively nitrate may be reduced in the shoot in the light period. Ryle *et al.* (1979b) observed no sign of this in terms of a reduced rate of photosynthesis. However, Mahon (1977) did notice such a competitive effect at low light intensities. It is now thought that the entry of N into the organic form in the leaves of higher plants can occur in the chloroplast, and that ATP and reducing power generated during photosynthesis may participate directly in some of the reactions involved (Lea & Mifflin, 1974). Plants utilising only nitrate in bright light might benefit from such sources of energy if they are generated in excess of the requirements for the reduction of carbon, but no such advantage would be available to plants synthesising amino acids in root nodules (Ryle *et al.* 1979b).

Thus, a conflict exists between evidence suggesting a similar energy demand for fixation of N_2 and assimilation of nitrate, and evidence suggesting a greater energy requirement for N_2 fixation. The work of Gibson (1966) can be criticised in that it did not directly measure physiological performance and Minchin & Pate (1973) state that their work was restricted to a relatively short time interval and looked at only a single species. Some of the other work cited, particularly that of Ryle *et al.* (1979b) is not so subject to these criticisms. Ryle *et al.* (1979a) point out that in some of the published data, including that of Minchin & Pate (1973), the efficiency of N_2 fixation, in terms of CO_2 respired, is greater than the biochemical considerations would allow. Thus, there could be some doubt as to the accuracy of such data. The evidence appears to be somewhat in favour of symbiotic nitrogen fixation requiring more energy than nitrate assimilation, and it has been widely observed that legumes fed combined nitrogen often

grow more rapidly than when dependent solely on symbiotic nitrogen fixation (section 2.2.3).

There is a large ATP requirement for nitrogenase activity, with 'in vitro' measurements indicating that about twelve molecules of ATP are used per molecule of N_2 fixed (Dixon, 1975). ATP, whose production is dependent on reduced substrate and oxygen, may be a possible limiting factor in nitrogenase activity (Hardy & Havelka, 1976). The reduction of a molecule of N_2 requires six electrons to be provided by electron donors, the reduction of which is dependent also on reduced substrate (Hardy & Havelka, 1976). As well as sub-optimal concentrations of substrates, product accumulation may limit N_2 fixation. Ammonia is a repressor of nitrogenase synthesis, although not a feedback inhibitor of nitrogenase activity. Suitable carbon skeletons are necessary for ammonia incorporation prior to transfer to the aerial parts of the plant, mainly in the form of asparagine (Hardy & Havelka, 1976). A limitation in ability to translocate N to the aerial parts of the plant could be a cause of low levels of plant N.

The photosynthate available to the nodule is a secondary factor common to the above three possible primary limitations, since it provides the substrate for ATP generation, reduction of electron donors, and removal of ammonia. Factors which affect photosynthesis and photosynthate supply to N_2 fixing nodules such as light (Lie, 1971), temperature (Gibson, 1971), moisture stress (Engin & Sprent, 1972; Sprent, 1976) and nitrate (Pate, 1977) may also have a direct effect on nodulation or nitrogen fixation.

Thus, photosynthate may limit nitrogen fixing activity, and factors such as light, temperature, moisture stress and nitrate may also impose limitations, either directly or via photosynthate supply. The debate as to the relative efficiencies of symbiotic N_2 fixation and nitrate assimilation is of considerable interest, but the key fact to be remembered is that the energy required for symbiotic N_2 fixation is supplied by the sun, and does not involve the use of increasingly scarce and expensive fossil fuels, as does the chemical fixation of nitrogen by the Haber-Bosch process.

2.2.1 RELATIONSHIPS BETWEEN NITROGEN FIXATION AND CARBOHYDRATE SUPPLY

Changes in $N_2[C_2H_2]$ fixation have been found to be associated with parallel changes in root respiration rate in nodulated pea plants (Mahon, 1977), thus linking N_2 fixing activity with energy use. A link between symbiotic N_2 fixation and energy supply was suggested by the work of Allison (1935), who found that relationships between nodule bacteria and their hosts were strongly dependent on carbohydrate supply. A link between N_2 fixation and photosynthate supply is supported by the work of Ching *et al.* (1975) who found that a decrease in nitrogenase activity in the nodules of dark treated soybean plants was closely correlated with the decline in a number of energy parameters (ATP, sucrose, total adenosine phosphates and ATP/ADP ratio). Lawrie & Wheeler (1975a) observed maximum accumulation of ^{14}C assimilates in the root nodules of *Vicia faba* L. within 90 minutes of synthesis. This, together with the short term and diurnal fluctuations sometimes associated with irradiance (e.g. Ruegg & Alston, 1978) further supports the concept of a close relationship between symbiotic N_2 fixation and photosynthesis. Brun (1972) applied treatments to soybean plants designed to alter relationships between photosynthetic source and sink components of canopies. The treatments involved lighting, shading, partial leaf removal and partial pod removal. Treatments increasing the photosynthate supply increased nodule $N_2[C_2H_2]$ fixing activity, and those decreasing photosynthate supply decreased $N_2[C_2H_2]$ fixing activity. $N_2[C_2H_2]$ fixation rates of detached nodules and decapitated root systems (of soybean plants) have been found to be less than those of attached nodules and complete plants respectively (Mague & Burris, 1972; Wych & Rains, 1978) indicating the dependence of fixation rates on current translocate. Vance *et al.* (1979) observed that nodule activity of lucerne decreased sharply after harvesting, and consequent decrease in leaf area. Streeter (1973) increased photosynthetic source size by grafting a second top onto a nodulated soybean root system and increased $N_2[C_2H_2]$ fixing activity by up to 100% for a short time.

Phillips *et al.* (1976) found short term CO_2 enrichment to promote symbiotic $N_2[C_2H_2]$ fixation in pea plants by increasing total plant and root nodule development. CO_2 enrichment was found to result in an integrated growth of the entire plant, with no unique promotion of nodule growth. There was, however, a short term increase in nitrogenase activity. This could have come about because of an increase in the

level of reductant or ATP, or because an additional supply of photosynthetic products to the roots stimulated nitrogenase synthesis, perhaps by providing additional carbon skeletons enabling the removal of inhibitory levels of ammonia (Phillips *et al.* 1976). Havelka & Hardy (1976) observed a dramatic increase in $N_2[C_2H_2]$ fixation by field grown soybean plants enriched with CO_2 on open top enclosures. The increase was observed after one week of CO_2 enrichment, but in a separate experiment an increase was demonstrated after only six hours of CO_2 enrichment. The maximum rate of N_2 fixation was over three times that of the controls, and total $N_2[C_2H_2]$ fixed per enriched plant was five times that of the control plants. The increase in $N_2[C_2H_2]$ fixation was a result of both an increase in specific nodule activity and an increase in nodule weight. The short time (6 hours) for an increase of 70% in nitrogenase activity suggested that an excess of nitrogenase was present and that more complete saturation with one or more of the substrates obtained from photosynthate occurred (Havelka & Hardy, 1976). The increased $N_2[C_2H_2]$ fixation extended the exponential growth phase of the plant, and resulted in a doubling of plant dry weight and a 56% increase in total plant N at the end of the experiment. It appears that elevated pCO_2 increased net photosynthesis, thus making more photosynthate available for N_2 fixation. Sheehy *et al.* (1980a) working with lucerne and soybeans found that with younger plants (three to four weeks old) $N_2[C_2H_2]$ reducing capacity was utilised fully at low CO_2 exchange rates. However, with six weeks old lucerne, $N_2[C_2H_2]$ reduction rates were found to increase linearly with CO_2 exchange rate. With the younger plants it could have been that maximum $N_2[C_2H_2]$ reducing activity was being expressed, or, alternatively, that control of carbon partitioning was being mediated by the shoot, and that there was a potential advantage in utilising newly available carbohydrate to produce additional photosynthetic tissue (Sheehy *et al.* 1980a). The fraction of current photosynthate allocated to root nodules in older plants appeared not large enough for them to make full use of their N_2 fixation capacity (Sheehy *et al.* 1980a).

Sheehy *et al.* (1980b) found that $N_2[C_2H_2]$ fixing activity of lucerne was closely correlated with whole plant carbon exchange rate (CER) ($r = 0.75$) and leaf area ($r = 0.80$). (Sheehy *et al.* (1980b) found whole plant CER to be highly correlated with leaf area, and suggested that leaf area could consequently be used as an index for whole plant CER). Bethlenfalvay *et al.* (1978b), working with pea plants, also found N_2

fixation (estimated by measuring acetylene reduction and hydrogen evolution) to be closely related to whole plant CER. Atkins *et al.* (1978) found, similarly, that the rate of N_2 fixation and the mass of nodules per root, of *Lupinus albus* L. and cowpea, increased in parallel with increases in leaf area and rate of production of net photosynthate. Thus, a strong measure of dependence between N_2 fixing performance and photosynthetic activity is suggested. Sheehy *et al.* (1980b) found total plant N to be highly correlated to $N_2[C_2H_2]$ fixing activity ($r = 0.76$), whole plant CER ($r = 0.96$), leaf area ($r = 0.97$) and plant dry weight ($r = 0.99$). Seetin and Barnes (1977) and Duhigg *et al.* (1978), also working with lucerne, found close relationships between top weight and $N_2[C_2H_2]$ fixing activity. Sheehy *et al.* (1980b) found that the specific activity of nodules showed no relationship with plant dry weight and that average specific leaf weight was not correlated with leaf area or $N_2[C_2H_2]$ fixing activity. Thus, there were good relationships between whole plant N_2 fixation and those factors such as leaf area and whole plant CER which are related to the photosynthetic potential of the whole plant and between the total amount of N fixed and total dry weight. Poor relationships existed between individual leaf characteristics and N_2 fixing activity. Yield and growth rate of legumes are also found to be strongly related to indices of whole plant photosynthetic potential and poorly related to individual leaf morphological characteristics such as specific leaf weight or CER per unit leaf area (Kaplan & Koller, 1977; Hart *et al.* 1978).

A key factor in determining the energy available for N_2 fixation is the manner in which legumes partition their carbohydrate supplies. Minchin & Pate (1973) drew up a budget of carbon and nitrogen in the root shoot and nodules of vegetatively growing pea plants. The carbon gained photosynthetically was distributed as follows:

Top dry weight	:	26% + 15% from nodules		
Root growth	:	7%		
Root respiration:	35%			
Nodule	:	32%	growth	: 5%
			respiration:	: 12%
			returned to shoot as amino compounds:	15%

Herridge & Pate (1977) working with vegetative cowpea, estimated that 14% of the total C gained is utilised by the nodules, and 28% by the supporting roots. Nodule respiration accounted for 5% and root + nodule respiration for 19% of the total C gained by the plant. Haystead *et al.* (1979) found that in white clover 17% of the C assimilated by the plant

was lost via respiration of nodulated roots. A high proportion of the C allocated to the nodule, in the vicinity of 30 to 50%, may be returned to the plant top in the form of organic nitrogen compounds (Minchin & Pate, 1973; Herridge & Pate, 1977; Layzell *et al.* 1979). Although there are considerable differences between figures quoted for nodule and root plus nodule respiration, they serve to show that substantial amounts of carbon are utilised by nodulated N_2 fixing root systems, and by the nodules themselves.

Cassman *et al.* (1980), suggest that there are two functional equilibria operational in N_2 fixing plants, namely the partitioning of dry matter between the shoot and the root, and between the root and the nodules.

The way in which C is partitioned is found to change with mode of N nutrition, stage of plant development, and to vary between species. Atkins *et al.* (1980), working with nodulated and non-nodulated cowpea, found that greater proportions of photosynthate (37%) were translocated to the below ground parts of nodulated plants than of NO_3 fed plants (23 to 26%). Herridge & Pate (1977), and Atkins *et al.* (1978) observed the utilisation and flow of C during the growth of nodulated cowpea and *L. albus* plants. In the vegetative stage of growth a high proportion of the net C of photosynthate was allocated to the leaves, and an even higher proportion to the nodulated root. About half of the C allocated to the root was lost in respiration. In the flowering and early fruiting stage there was a tendency, especially in cowpea, for a reduced allocation of C to the roots and nodules, and a consequent increase in the shoot:root ratio. The proportion of root C utilised in respiration increased possibly because of higher consumption in the maintenance of a larger root system. This trend continued into the seed filling stage, with reduced proportions of C being allocated to leaves, roots and nodules in cowpea and leaves and nodules in *L. albus* plants. The proportion of C allocated to the roots of lupin plants remained high throughout development. The percentage of net photosynthate utilised by lupin nodules was higher than that utilised by cowpea nodules (Atkins *et al.* 1978). This was because of a higher expenditure by lupin nodules in respiration and a greater requirement for C in the transport of fixation products from *L. albus* nodules. The efficiency of C use in cowpea nodules, in terms of C usage per unit of N_2 fixed, was greater than in *L. albus* or pea (Atkins *et al.* 1978; Minchin & Pate, 1973). The export of N from cowpea nodules in the form of ureides was thought to be a major factor in the better efficiency of cowpea (Atkins *et al.* 1978), as ureides have a

lower C:N ratio than the commonly exported amide, asparagine (Rawsthorne *et al.* 1980). In addition nodules of cowpea had a higher proportion of their volume as bacteroid containing tissue than nodules of pea or *L. albus* and this may have had some bearing on apparent differences in the respiratory efficiency, and on the higher specific $N_2[C_2H_2]$ fixing activity of cowpea nodules.

The evidence points to a strong measure of dependence between nitrogen fixing performance and photosynthetic activity. There seems to be overall support for the idea that N_2 fixation is 'source' rather than 'sink' limited, at least in vegetatively growing plants. This support is provided by the direct relationships between several factors that influence the total production of photosynthate and N_2 fixation, and also by the observation that treatments increasing the supply of photosynthate, such as CO_2 enrichment, result in rapid increases in N_2 fixation. There seems to be considerable scope for plant breeders to improve the symbiotic nitrogen fixing performance of legumes by improving their photosynthetic characteristics. This could be especially true for plants which appear to have inherently poor photosynthetic characteristics, such as sainfoin, as discussed previously.

A factor which could influence the directness of relationships between symbiotic N_2 fixation and photosynthesis is the availability of stored energy for N_2 fixation.

Glycogen and poly-B-hydroxybutyric acid (PHB) have both been identified as storage compounds in bacteroids (Rawsthorne *et al.* 1980). PHB which can represent up to 50% of the dry weight of soybean nodules (Rawsthorne *et al.* 1980), may act as an insoluble carbon store which may be used to support N_2 fixation during periods of darkness when photosynthate is less readily available (Bergersen, 1970). Kretovich *et al.* (1977) suggest that PHB is an energy source for dark N_2 fixation in lupin nodules and demonstrated an inverse correlation between PHB concentration and energy demands in the nodule. They also suggest that PHB might be a source of C skeletons for ammonia assimilation. These findings have not been supported by other workers (Rawsthorne *et al.* 1980).

Pate (1976) suggests that nodules in general maintain meagre reserves of readily utilisable carbohydrate relative to their requirements for N_2 fixation. Vance *et al.* (1979) found that the decline in $N_2[C_2H_2]$ fixing activity of partially defoliated lucerne preceded the depletion of starch in roots, and concluded that starch granules in nodules and root non-structural carbohydrates were not readily available to sustain nodule

activity in lucerne. Haystead *et al.* (1979) found that white clover showed constant $N_2[C_2H_2]$ fixing activity in 21.5 hours continuous darkness, a result which was tentatively attributed to the buffering effect of carbohydrate reserve materials, probably located in the stolons. It is thought, however, that leguminous nodules often rely heavily for their growth and functioning on photosynthetic products currently translocated from leaves, or on carbohydrate mobilised from other regions of the plant (Pate, 1976).

Extracts of *Phaseolus vulgaris* L. roots have been found to be capable of fixing large amounts of CO_2 through carboxylation of phosphoenolpyruvate (Jackson & Coleman, 1959), and the presence of CO_2 in the vicinity of roots of red clover has been found to enhance nodulation and N_2 fixation (Mulder & Van Veen, 1960). In addition, Lawrie & Wheeler (1975b) have shown that suitable acceptor molecules derived from CO_2 fixation by detached nodules of *V. faba* plants can provide carbon skeletons for N_2 fixation when the carbohydrate supply is removed. They state that further work is required however, to establish the importance of the pathway in nodules attached to the plant.

There appears to be no real concensus of opinion as to the role of carbohydrate reserves or of root CO_2 fixation in supplying carbohydrate for N_2 fixation when photosynthate supply is limited. The close links between N_2 fixation and photosynthate supply discussed earlier in this section tend to support the view that carbohydrate reserves and root CO_2 fixation do not play a major role, although there are many examples (e.g. Haystead *et al.* 1979) of N_2 fixation being seemingly independent of current photosynthate supply. Plants, such as white clover, which can maintain N_2 fixation independently of current photosynthate supply appear to have an advantage in terms of ability to fix N, and this could well be translated into increased yields.

2.2.2 HYDROGEN EVOLUTION

Hydrogen (H_2) evolution is a phenomenon associated with N_2 fixation by many nodulated N_2 fixing symbionts (Schubert & Evans, 1976). The significance of this phenomenon to N fixation by legumes is discussed, as follows, by Schubert & Evans (1976). An evaluation of the magnitude of energy loss in terms of the efficiency of electron transfer to nitrogen via nitrogenase, in excised nodules, suggested that hydrogen (H_2) production may severely reduce N_2 fixation where photosynthate supply is a factor limiting fixation. In most symbionts, including soybeans, only 40 to 60% of the electron flow to nitrogenase was transferred to

nitrogen, the remainder being lost because of H_2 evolution. In situ measurements of H_2 and C_2H_2 reduction by nodulated soybeans confirmed the results obtained with excised nodules. The extent of H_2 evolution is a major factor affecting the efficiency of N_2 fixation by many agronomically important legumes, including lucerne, birdsfoot trefoil, a range of clovers, soybeans and peas (H_2 evolution appeared to represent a substantial inefficiency in 17 of the 19 legumes tested). If photosynthesis limits N_2 fixation, then conservation of the energy lost in this way would theoretically increase N_2 fixation (Schubert & Evans, 1976). If some other factor limits N_2 fixation, then reduction of H_2 evolution would presumably increase dry matter yield (Schubert & Evans, 1976).

Not all nitrogen fixing symbioses exhibit the degree of inefficiency discussed by Schubert & Evans (1976). Roelofsen and Akkermans (1979) report that most non legumes with actinomycetous nodules, and a few legumes e.g. cowpea were found to evolve very little H_2. This is thought to be due to hydrogenase activity, in taking up evolved H_2. The *Azolla - Anabaena azollae* relationship is another exhibiting little net H_2 evolution (Peters *et al.* 1977) and work by Peters *et al.* (1976) implied the presence of an uptake hydrogenase. There are found to be two separate hydrogenase enzyme systems in the bacteroids of leguminous nodules, one evolving H_2, thought to be the nitrogen fixing enzyme nitrogenase, and another, at a separate site catalysing the uptake of H_2 (Dixon, 1967; Dixon, 1978). Schubert *et al.* (1977) observed two classes of legume - *Rhizobium* combination in studies with soybeans and cowpeas. One group evolved H_2, the other did not exhibit net H_2 evolution. The latter group metabolised H_2 formed, within the nodule. The capacity to oxidise H_2 was strongly linked to strain of *Rhizobium*. Bethlenfalvay *et al.* (1979) tested five strains of *R. leguminosarum* and found two to possess hydrogenase activity. Pea plants when infected with these had significantly higher rates of N_2 fixation. Albrecht *et al.* (1979) and Ruiz-Argueso *et al.* (1979) describe strains of *Rhizobium japonicum* that form nodules on soybean roots which evolve little or no H_2. More N_2 was fixed and greater yields were produced compared with plants inoculated with strains lacking the H_2 uptake capacity (Albrecht *et al.* 1979). The H_2 uptake capacity of bacteroids showing hydrogenase activity is estimated to far exceed the capacity of nitrogenase to evolve H_2 (Ruiz-Argueso *et al.* 1979; Emerich *et al.* 1980). Plants nodulated with strains not exhibiting H_2 uptake activity had greater nodule weights (Albrecht *et al.* 1979), presumably to compensate for their less efficient N_2 fixing ability.

The probable role of the 'uptake' hydrogenase is to use H_2 as a respirable substrate, thus recouping some of the energy lost in its production (Dixon, 1978). In performing this role, a second role, that of maintaining low ρO_2 in the nodule by using H_2 as a substrate for respiratory protection of the nitrogenase, would be accomplished (Dixon, 1978).

Thus, the presence or absence of 'uptake' hydrogenase activity in N_2 fixing bacteroids can have a strong influence on N_2 fixing efficiency and legume productivity. Schubert *et al.* (1978) found that the presence of uptake hydrogenase activity increased total dry matter yield and total N_2 fixed by 24 and 31% in soybeans and by 11 and 15% in cowpea. Thus, the potential exists to substantially increase legume productivity by inoculating with strains of *Rhizobium* having the ability to take up the H_2 evolved in nitrogenase activity.

2.2.3 EFFECTS OF COMBINED N

It has been found that combined N depresses nodulation on a wide range of legumes including lucerne (Munns, 1968; Heichel & Vance, 1979), *V. faba*, *P. vulgaris*, peas (Dean & Clark, 1980) and lentils (*Lens esculenta* Moench.) (Wong, 1980). Wong (1980) found that lentils growing in 15 mM nitrate had 84% fewer, and 71% less weight of nodules than plants growing in nitrate fee solution. Observations on young seedlings suggest that root hair curling and the formation of infection threads are more susceptible to injury than the later stages of nodulation, and that nitrate and nitrite are more potent suppressors of nodulation than ammoniacal forms of N (Munns, 1968; Pate, 1977). It has been proposed that nitrate acts externally through the catalytic action of its reduction product, nitrite, in the destruction of indole acetic acid, the presumed agent of root hair curling (Tanner & Anderson, 1964). It is generally agreed that, within limits, the effects of added N on nodulation are proportional to the amount of nitrogen supplied and its frequency of application. Certain species and varieties are more tolerant than others (Allos & Bartholomew, 1959; Pate, 1977). There is evidence that small, correctly timed additions of combined N can stimulate nodulation and N_2 fixation (Pate, 1976).

Combined N, particularly nitrate, is found to substantially reduce N_2 fixing activity in legumes (Allos & Bartholomew, 1955; Allos & Bartholomew Bartholomew, 1959; Oghoghorie & Pate, 1971; Pate, 1977; Hojjati *et al.* 1978; Dean & Clark, 1980; Wong, 1980). Allos & Bartholomew (1955) working with a range of legumes including lucerne, birdsfoot trefoil,

ladino clover (*Trifolium repens* L.) soybeans and peanuts (*Arachis hypogaea*) found that N_2 fixation decreased, and combined N uptake increased with increase in quantity of available combined N. High increments of N had less influence than lower increments in increasing plant growth, but showed a greater tendency to replace fixed N_2. In no instance was N_2 fixation completely inhibited (N was applied as ammonium sulphate). Allos and Bartholomew (1959) found that a range of legumes, including lucerne, birdsfoot trefoil, sweet clover (*Melilotus* spp.), ladino clover and soybeans responded in terms of growth and N uptake to the addition of combined inorganic N. In some instances, increases in growth resulting from N fertilisation caused increases in N_2 fixation. When applied N exceeded that necessary for increased growth it tended to replace N_2 fixation (Allos & Bartholomew, 1959). Hojjati *et al.* (1978) and Barta (1979) similarly found that combined N increased plant size, and decreased N_2 fixation in red clover and big flower vetch (*Vicia grandiflora*) and birdsfoot trefoil respectively. Copeland and Pate (1969) grew white clover in sand culture with varying rates of nitrate N. Maximum dry matter was produced with a medium concentration of 140 ppm nitrate N. Higher levels of nitrate, up to 420 ppm,produced lower yields. A threefold stimulus to number and mass of leghaemoglobin pigmented nodules was observed at low levels of nitrate. Higher levels of nitrate only slightly reduced nodule member and weight below the levels encountered in control plants receiving no nitrate. Hoglund (1973) observed a bimodal response of lucerne to application of combined N, and attributed this to the tolerance of nodulation and nitrogen fixation to lower levels of nitrogen, and to the suppression of nodulation at higher levels. Bethlenfalvay *et al.* (1978a) found that ammonium N applied to peas increased growth and photosynthesis and when applied at low levels enhanced N_2[C_2H_2] fixation.

Generally, legumes grown on combined N produce higher yields and a higher proportion of soluble N. Allos and Bartholomew (1959) found that a range of legumes (see previously) exhibited an apparent capacity to supply by symbiotic fixation only about half to three-quarters of the total N which could potentially be used by the plant. Richards & Soper (1979) found that *V. faba*, nodulated with an effective rhizobial strain, and receiving no N fertiliser fixed 87.1% of their total N. A high rate of N fertiliser increased yield by 13.2%. Thus, the plants were presumably fixing about 77% of the N which could potentially be

used by the plant. Hill-Cottingham & Lloyd-Jones (1980) found that *V. faba* on a low rate of nitrate fixed over 90% of its total N, and that the total N of low nitrate plants was about 90% of that of high nitrate plants. Thus, the plants were fixing approximately 81% of their potential N requirement. Ryle *et al.* (1979a) found that the nitrogen requirement of cowpea appeared to be almost fully met by symbiotic fixation, but that N assimilation in soybeans dependent on symbiotic fixation was only about one third of the N assimilation of equivalent plants provided with abundant combined N. Semu & Hume (1979) found that symbiotic N_2 fixation can support maximum yields of soybeans in the field, but they were presumably acquiring significant amounts of mineral N from the soil.

There are two possible mechanisms by which nitrate could reduce symbiotic N_2 fixation. It can have a secondary effect by reducing photosynthate to the root nodules, or alternatively it could have a direct effect on the nitrogenase system. If nitrate is supplied in the rooting medium, some of this will be reduced in the roots, causing a substantial drain on the C reserves of the root (Pate, 1976). If combined inorganic N reaches the shoot, the photosynthesis associated assimilation of this N may lead to a considerable reduction in the total amount of translocate available to the roots. It has been found that it is nodules, and not roots, which suffer most from the shortage of translocate (Pate, 1976). Small & Leonard (1969) showed that the level of ^{14}C translocated to nodules of pea and subterranean clover plants (after exposure to $^{14}CO_2$), which were provided with 200 ppm N as $NaNO_3$ for five days, was reduced by 55 to 75% relative to N-free controls. With increasing availability of combined N to the roots, competition between nodules and roots for photosynthate changed in favour of the roots (Small & Leonard, 1969). Latimore *et al.* (1977), working with soybeans, also found combined N to decrease ^{14}C accumulation in root nodules as well as decreasing $N_2[C_2H_2]$ reducing activity. Barta (1979) found reduced amounts of photosynthate in roots of birdsfoot trefoil when supplied combined N. In field pea (*Pisum arvense* L.) levels of combined N which severely suppress symbiotic N_2 fixation reduce downward translocation of photosynthate, lower the efficiency of N_2 fixation per mass of nodules and generate a pattern of nitrate reduction in which the greater share of assimilation takes place in the shoot (Oghoghorie & Pate, 1971). Where plants are dependent solely on fixed N, N appears to be cycled to the roots via the shoot. Where

combined N is supplied the dependence of roots on cycled N will be circumvented (Pate, 1976).

The time interval between nitrate addition or removal, and significant curtailment or restoration of N_2 fixing activity is such (48 to 60 hours) as to suggest feedback control rather than any immediate effect operating for example, through nodule metabolism (Pate, 1976). The concept of the effect of nitrate on N_2 fixing activity being mediated through photosynthate supply is supported by Wong (1980) who found that added sugars alleviated the inhibitory effects of nitrate, not only by increasing the carbohydrate supply to support both N_2 fixation and nitrate reduction, but also by eliminating the accumulation of nitrate and hence lowering nitrate reductase activity in the leaves.

The more direct effect of nitrate within the nodules, however, is not precluded. Trinchant & Rigaud (1980) found that nitrite strongly inhibited nitrogenase. It appeared to bind the Mo-Fe protein of nitrogenase without any effect on the Fe protein, and gave a completely reversible inhibition. The accumulation of nitrite in the vicinity of nitrogenase, however, could presumably be attributed to a lack of energy to complete the reduction to nitrate and a lack of C skeletons. Streeter (1980) observed that carbohydrate levels did decrease in nodules of nitrate fed soybean, but that this did not appear to be a causative factor in the lack of development and activity of nodules in the presence of nitrate.

It is often desirable, in terms of yield, for legumes to be provided with N additional to what they can acquire via symbiotic fixation. However, if it is desired to maintain a maximum input of N from symbiotic fixation, the quantity and timing of inputs of combined N is critical. The quantity must be such as to supplement but not replace N_2 fixation and such as not to inhibit nodulation. Addition of fertiliser N can sometimes be useful in the initial stages of growth, before plants have developed a N_2 fixing system (e.g. Harper, 1974). The possibility also exists to supplement symbiotic fixation at other times when the symbiotic N_2 fixing system is not meeting the potential requirements for N, e.g. during the seed filling stage of soybeans.

2.2.4 LIGHT AND TEMPERATURE

Light and temperature have a very significant influence on the growth and development of plants. They influence the nitrogen fixing symbioses of legumes through the combined effect that they have on photosynthesis, and each may also have direct effects on the N_2 fixing symbiosis.

2.2.4.1 Temperature

The temperature at which a legume grows greatly affects the plant-rhizobium symbiosis (Dart & Day, 1971).

The optimum root temperature for nodulation of a range of temperate legumes has been found to be in the range 20° to 30°C (Gibson, 1971). Optimum temperatures for nodule formation are usually similar to those for nodule development and N_2 fixation; all are inhibited by extremes of heat and cold (Dart & Day, 1971). Lower than optimal root temperatures are found to retard root hair infection more than nodule initiation, nodule development or nitrogen fixation (Gibson, 1971). Gibson (1963) found that symbiotic N_2 fixation of subterranean clover was reduced at root temperatures below 22°C, and in some host-rhizobial strain combinations, was reduced at 30°C. Possingham *et al.* (1965) also observed an apparent decrease in N_2 fixation of subterranean clover plants at root temperatures of 30°C. Nodule development is influenced by temperature. It was observed by Roughly (1970) that greater amounts of bacteroid tissue were present at lower temperatures (11° and 15°C compared to 19°C) and that there was an increasing proportion of degenerate tissue at 19°C. It was proposed that the larger amounts of nodule tissue noted at lower temperatures could be a form of compensation for reduced nitrogen fixation per unit of bacteroid tissue (Roughly 1970). An increase in $N_2[C_2H_2]$ fixing activity of nodule tissue with increasing root temperature (from 5° to 25°C) has been observed in lucerne (Harding & Sheehy, 1980), which was suggested to be a result of increased enzyme activity. $N_2[C_2H_2]$ fixation and H_2 evolution rates by root nodules of several legume species, including lucerne and subterranean clover, have been found to depend on incubation temperature (Dart & Day, 1971). Maximum $N_2[C_2H_2]$ fixation rates usually occurred between 20° and 30°C. Nitrogenase has been found to function at temperatures which would limit other aspects of the N_2 fixing symbiosis (Dart & Day, 1971). Barta (1978) found 30°C temperatures to reduce $N_2[C_2H_2]$ fixing activity in lucerne and birdsfoot trefoil.

The activities of lucerne, which were much higher than those of birdsfoot trefoil, were reduced by a larger proportion. Graham (1979) found that lower day/night temperatures (25°/15°C compared to 35°/25°C and 30°/20°C) gave increased maximum rates of $N_2[C_2H_2]$ fixation in *P. vulgaris*, but that the timing of the diurnal peak of activity was delayed. Significant interactions between plant varieties and bacterial strains have been observed with respect to the optimum temperature for N_2 fixation (Gibson, 1963).

Possingham *et al.* (1965), found that high shoot temperatures had no inhibitory effect on nitrogen fixation in subterranean clover. Plant yield was, however, reduced, indicating that any effects of shoot temperature on N_2 fixation would be mediated via plant growth. However, in the lower range (5° to 20°C shoot temperatures) increased N_2 fixation has been observed with increase in shoot temperature (at root temperatures of 14° and 20°C) (Gibson, 1971). Although dry weight and N increased with increasing shoot temperatures (in plants dependent on symbiotic N_2 fixation), there was a greater dry weight response by plants supplied with mineral N, especially at the lower end of the temperature range (Gibson, 1971). This was taken to indicate that the physiological processes in the shoot associated with the assimilation of mineral N, possibly the nitrate reductase enzymes, show a greater response to increase in shoot temperature than those associated with symbiotic N_2 fixation (Gibson, 1971). No response to increased shoot temperature was found at 10°C root temperature (Gibson, 1971).

The air temperature in which the shoot and leaves are growing has been found to be the dominant factor controlling the rate of development of leaf area in lucerne (Harding & Sheehy, 1980). The supply of assimilate to nodules is important for N_2 fixation, and it is suggested that this is often a limiting factor in the rate of fixation (Havelka & Hardy, 1976). Thus, it could well be that shoot temperature influences symbiotic N_2 fixation via photosynthate supply. The influence of root temperature on N_2 fixation is probably more directly related to the biology and biochemistry of nodulation and N_2 fixation, with reduced enzyme activity at low temperatures, and increased degeneration of nodule tissue at higher temperatures.

2.2.4.2 Light

It is suggested that the main effect of light on nodulation and N_2 fixation in legumes can be attributed to the effect of light on photosynthesis and hence carbohydrate supply to the growing and functioning nodules (Masterson & Sherwood, 1969; Lie, 1971; Gibson, 1976).

Bergersen (1970) found $N_2[C_2H_2]$ fixation by nodulated soybean root systems to be related to light intensity preceeding sampling; activity of detached nodules was lower and less influenced by light. Mague & Burris (1972) found that soybean plants exhibited a diurnal cycle of $N_2[C_2H_2]$ reduction dependent on both light intensity and air temperature.

There is evidence for an additional action of light, mediated by the phytochrome system, a photoreceptor system in plants controlled by red and for red light (Lie, 1971). Root nodule formation requires the activation of certain plant root cells to divide, in particular, those cells having a double number of chromosomes. It is thought that light could have a role in this stimulation, as apparently similar results have been obtained with far red light, as with cytokinins which are believed to have a role in activating cell division (Lie, 1971). Abundant nodules have been produced in red light, and few, slow growing nodules in blue light. Far red light (high under shady conditions) has the effect of counteracting the red light effect (Lie, 1971). Sprent (1973) demonstrated the effect of shade in *Lupinus arboreus*. A linear relationship was shown between log of relative irradiance and $N_2[C_2H_2]$ fixing activity per plant. Shading principally reduced nodule number and size, with only the deepest shade reducing activity per weight of nodule tissue. Gibson (1976) states that short term changes in light intensity can affect nitrogenase activity, and Ruegg & Alston (1978) state that short term fluctuations in $N_2[C_2H_2]$ fixation activity in *Medicago truncatula* Gaertn. were mainly associated with irradiance. This plant also showed marked diurnal fluctuation in $N_2[C_2H_2]$ fixing activity. However, not all plants seem to be this sensitive to light intensity and some plants show no diurnal fluctuation in $N_2[C_2H_2]$ fixation activity (e.g. Haystead *et al.* 1979).

Minchin & Pate (1974) studied the diurnal functioning of legume root nodules. They imposed treatments of alternating light and dark at constant temperature of $18^{\circ}C$ and at alternating light/dark temperatures of $18^{\circ}/12^{\circ}C$. Nodule starch and sugar levels increased during the photoperiod and decreased in the dark. At constant temperature, N_2 fixation was slightly greater in the photoperiod, but at the alternating temperatures slightly less N_2 was fixed during the photoperiod. This was thought to be linked to more efficient use of carbohydrate at lower temperatures (Minchin & Pate, 1974). Thus, it appears that periods of darkness can be times of high nodule activity provided sufficient carbohydrate is available in, or to, the nodule. The ability of a legume to buffer its N_2 fixing system against short term

and diurnal fluctuations in light intensity appears to depend on its ability to assimilate and store sufficient carbohydrate to carry it over periods of low light intensity. The ability of legumes to store carbohydrate in the nodule or other plant organs has been discussed in section 2.2.1, as has the ability of nodules to fix CO_2.

The level of irradiance, in addition to influencing its rate of production, can also influence the partitioning of photosynthate. Williams & Phillips (1980) state that at high levels of irradiance the partitioning of recent photosynthate may favour top growth relative to N_2 fixation.

There is little that can be done to control temperature in a field situation, so such things as sowing dates need to be decided on with temperature requirements for plant growth, nodulation and N_2 fixation in mind. Light is also a seasonal factor and as such cannot be controlled in a field situation. Shading can be avoided, however, by manipulation of plant populations and spacings.

2.2.5 MOISTURE STRESS AND WATERLOGGING

2.2.5.1 Moisture stress

The effects of moisture stress and waterlogging have an adverse effect on N_2 fixation in a wide range of legumes under both laboratory and field conditions (Sprent, 1976). In the field N_2 fixation is at its highest at or near field capacity. The legume nodule requires water for maintenance of the turgidity of its tissues and for export in the xylem of the products of fixation (Pate, 1976). Stress in nodules occurs, according to Sprent (1976), when the root system cannot supply sufficient water to support these functions and compensate for moisture losses from the nodule surface. The pattern of response appears to be similar in all species. It has been found that where nodules do not fall below about 60% of maximum fresh weight, nodule activity is restored on watering within hours (Sprent, 1976). The ability of plants to recover, and the time taken for recovery are both related to the duration of the stress period (Engin & Sprent, 1973). Plants with meristematic nodules, such as white clover (Engin & Sprent, 1973) and *L. arboreus* (Sprent, 1973), can recover from damaging stress by regrowth of existing nodules (Engin & Sprent, 1973). In plants with spherical nodules, such as soybeans, severe stress causes nodule shedding, and recovery is likely to be slower, involving the formation of new nodules (Engin & Sprent, 1973; Sprent, 1976).

Wilting of the lower leaves is usually an indication that nodules are functioning at suboptimal rates, however, provided soil moisture is adequate, shoots may wilt without a noticable effect on N_2 fixing activity (Sprent, 1976). In times of moisture stress, water may be transferred from a wet zone, to nodules on a dry zone (Sprent, 1972).

When $N_2[C_2H_2]$ fixing activity is reduced by moisture stress a concommitant reduction in respiration has been observed in detached soybean nodules (Sprent, 1971). This observation along with examination of the histochemstry of respiratory enzymes in stressed nodules is a demonstration that water stress has a direct effect on bacteroids (Sprent, 1976). Pankhurst & Sprent (1975) showed that resistance to oxygen diffusion increased in soybean nodules under water stress, which led to decreased nodule respiration.

Ahmed & Quilt (1980), however, found nodulation and nitrogenase activity in the tropical forage legumes *Macroptilium atropurpureum* and *Desmodium intortum* to be less effected by moisture stress than top weight. Huang *et al.* (1975a) found that the decrease in $N_2[C_2H_2]$ fixing activity of Soybean root nodules was more closely related to decreases in photosynthesis and transpiration than decrease in dark respiration, and concluded that photosynthesis, transpiration or some direct effect on the nodules, other than that caused by respiration, were most likely to account for the inhibition of acetylene reduction in moisture stressed plants. Huang *et al.* (1975b) found that the inhibition of $N_2[C_2H_2]$ fixation caused by low water potentials and their after effects could be reproduced by depriving shoots of atmospheric CO_2, even though the soil remained at favourable water potentials. This is not conclusive evidence however, for a link between $N_2[C_2H_2]$ reduction and photosynthesis. However, it was also found that the inhibition of $N_2[C_2H_2]$ reducing activity at low water potentials could be partially reversed by exposing the shoots to high CO_2 concentrations. Finn & Brun (1980), found that CO_2 assimilation and specific nodule $N_2[C_2H_2]$ fixing activity decreased and stomatal resistance increased with increasing water stress. There was a significant negative correlation ($r = -0.71$) between specific nodule activity and stomatal resistance. Dry weights of leaves, stems plus petioles, and nodules decreased under water stress, and root dry weights increased. There was a redistribution of ^{14}C, with a greater proportion allocated to the roots and nodules at the expense of leaves. A time course study showed that carbon exchange rate (CER) decreased after the imposition of abrupt water stress, but total nodule activity was not affected (Finn & Brun, 1980). It was suggested that decreased nodule

activity under long term water stress is not caused solely by decreased CER, but also by changes in photosynthate pool sizes.

The evidence seems generally to favour the proposition that the effect of moisture stress on N_2 fixation is mediated via photosynthate supply, particularly since the key evidence for a direct effect, that of Sprent (1971) relating reduced $N_2[C_2H_2]$ fixing activity to reduced respiration, was carried out on detached nodules.

2.2.5.2 Water logging

Depression of N_2 fixation by waterlogging is thought to result largely from O_2 deficiency (Schwinghamer *et al.* 1970; Mague & Burris, 1972; Sprent, 1976). Huang *et al.* (1975a) suggested that rates of gas exchange between the nodules and the atmosphere are reduced at high soil water potentials, thus reducing $N_2[C_2H_2]$ fixation. Nodules have been found to produce increased surface area under waterlogged conditions, and nodule number, size and N_2 fixing activity have been found to decrease relative to non waterlogged controls (Sprent, 1976). Nodule initiation appears to be more tolerant to waterlogging than nodule functioning (Pate, 1976).

The sensitivity of N_2 fixation to water stress and waterlogging can no doubt affect the productivity of legumes in the field. Under irrigated conditions the opportunity would exist to optimize moisture levels for maximum crop photosynthesis and N_2 fixation, and to avoid the adverse effects of waterlogging.

3 THE ACETYLENE REDUCTION TECHNIQUE

3.1 THE ACETYLENE REDUCTION REACTION

The nitrogen fixing enzyme, nitrogenase, catalyses the conversion of the very stable N_2 molecule to ammonia, a form of N which plants can assimilate (section 2.1). Dilworth (1966) found that nitrogenase could reduce acetylene (C_2H_2) to ethylene (C_2H_4), in a reaction analogous to the reduction of N_2 to NH_3. Schollhorn & Burris (1967), and Dilworth, independently observed inhibition of N_2 fixation by C_2H_2 and Schollhorn & Burris (1967) established the competitive nature of this inhibition.

The two reactions are as follows (Bergersen, 1970):

Acetylene reduction:

$$3C_2H_2 + 6H^+ + 6e \rightarrow 3C_2H_4$$

Nitrogen fixation:

$$N_2 + 6H^+ + 6e \rightarrow 2NH_3$$

The stoichiometric equivalence of C_2H_2 to N_2 is 3:1, therefore, theoretically, if energy and reductant supply are not limiting the ratio of C_2H_2 to N_2 reduced should be 3:1.

The first reported application of acetylene reduction for the measurement of N_2 fixation was by Koch & Evans (1966) who assayed detached soybean nodules.

C_2H_2 is preferred as a substrate for the N_2 fixation assay, over other possibilities such as HCN because: 1) a relatively large amount of product is formed (the reduction of C_2H_2 to C_2H_4 requires only two electrons) 2) the detection of C_2H_4 by gas chromatography using flame ionisation detection is highly sensitive, 3) ethylene is the sole product of the reaction and 4) ethylene does not inhibit N_2 fixation (Hardy *et al.* 1968; Hardy *et al.* 1973).

The validity of the acetylene reduction assay for the measurement of N_2 fixing activity depends on the similarity between the N_2 and C_2H_2 reduction reactions. Schollhorn & Burris (1967) state that C_2H_2 is isoelectronic and isosteric with N_2 and should therefore fit into the chemisorbing site of the enzyme. They further state that the energy dependence and competitive inhibition of N_2 fixation suggest that the C_2H_2 molecule is attached to and reduced at the same enzyme site as N_2. The inhibition by C_2H_2, of ATP dependent H_2 evolution by

nitrogenase, by an amount equivalent to the formation of ethylene, establishes the electron activating reaction of nitrogenase as the source of electrons for C_2H_2 reduction (Hardy, *et al.* 1968). The similar competitive inhibitions of N_2 fixation and C_2H_2 reduction by CO provides indirect support for the role of the substrate complexing site of nitrogenase in both C_2H_2 and N_2 reduction (Hardy *et al.* 1968). Koch & Evans (1966) found that the time course of C_2H_2 production by soybean nodules was similar to a reported time course for N_2 fixation, and that conditions optimal for C_2H_2 reduction were similar to those optimal for N_2 fixation, suggesting the same enzyme system was involved in both reactions. C_2H_2 and N_2 have been found to evoke identical responses from nitrogenase (Hardy *et al.* 1968). The essential relationship between C_2H_2 reduction is supported very convincingly by studies involving cell free extracts, bacterial cultures and symbionts (Hardy *et al.* 1968). Using *Azotobacter* preparations, close similarities have been observed between the N_2 and C_2H_2 reduction reactions catalysed by nitrogenase with respect to the following (Hardy *et al.* 1968):

(a) Requirement for ATP and reductant.
(b) Linear time course for the reactions.
(c) Optimal pH.
(d) Sigmoidal relationship between rate of reaction and enzyme concentration.
(e) Competitive inhibition by CO.
(f) Relative insensitivity to presence of NH_4^+.
(g) Activation energies.
(h) Lack of activity in urea grown cells.
(i) Similar distribution of activity during fractionation of N_2 fixing extracts.
(j) Requirement for both the Mo-Fe and Fe protein fractions of nitrogenase.

Whole cell experiments were completely consistent with the results obtained 'in vitro', and also demonstrate parallel C_2H_2-N_2 relationships (Hardy *et al.* 1968).

Experiments with N_2 fixing symbionts demonstrate that parallel C_2H_2-N_2 relationships are consistantly applicable to even the most complex natural N_2 fixing systems (Hardy *et al.* 1968). Thus legumes also reduce C_2H_2 to C_2H_4 with characteristics similar to those of N_2 fixation. These include an aerobic requirement, activity only in nodules containing leghaemoglobin, absence of activity in either the root or infecting bacteria

and a similar rate of C_2H_2 or N_2 reduction per season based on electron requirement (Hardy *et al.* 1968).

The characteristics of nitrogenase activity, as exemplified by C_2H_2 reduction appear to be consistent through the entire range of organisation studied, and all systems are found to reduce C_2H_2 to a single significant product, C_2H_4 (Hardy *et al.* 1968). The C_2H_2 reduction assay is thus a sensitive, universal and specific analysis for N_2 fixing activity (Hardy *et al.* 1968) which has become widely used (Sinclair, 1973).

Despite the similarities between the N_2 fixation and C_2H_2 reduction reactions catalysed by nitrogenase, there are certain differences which should be considered.

The enzyme is thought to have approximately a 2.5 times greater affinity for N_2 than acetylene (Schollhorn & Burris, 1967), but because of the much greater solubility of C_2H_2 than N_2 (65 times greater at 1 atmosphere pressure and 25°C), the Michaelis constant is much lower for C_2H_2 than N_2 (Schollhorn & Burris, 1967; Bergersen, 1970). This factor enables C_2H_2 reduction assays to be carried out in air (containing N_2) with little (10-20% in soybean nodules) reduction in C_2H_2 reducing activity (Hardy *et al.* 1968). Algal and non legume nodule fixation rates showed no advantage of air removal, possibly, in the case of the algae, because of a high p C_2H_2 of 0.2 atmospheres (Hardy *et al.* 1973). Even at low concentrations, C_2H_2 is a powerful inhibitor of N_2 fixation (Schollhorn & Burris, 1967). In view of this fact, and the fact that complete evacuation can present problems when using large incubation containers, Sinclair (1973) performed incubations in a C_2H_2-air mixture. Trinick *et al.* (1976) found no decrease in $N_2[C_2H_2]$ fixing activity when whole plants of *Lupinus luteus* were incubated in 0.05 atm.C_2H_2 in air compared with 0.05 atm.C_2H_2 in Ar and O_2, or when nodulated root systems of *Lupinus cosentinii* were incubated in 0.1 atm. C_2H_2 in air compared with 0.1 atm.C_2H_2 in Ar and O_2. Differences were found at 0.025 and 0.02 atm.C_2H_2 to *L. luteus* and *L. cosentinii* respectively. Mahon (1977) assayed nodulated root systems of intact pea plants in 0.02 atm.C_2H_2 and found no significant difference in acetylene reduction rate when air was replaced by Ar and O_2.

In whole cell systems, the N from N_2 fixation enters the N pool of the cells and contributes to protein systems, while acetylene reduction measures the activity of the nitrogenase system only, and makes no contribution to the metabolism of the cell (Bergersen, 1970). If there was a system of feedback inhibition of nitrogenase activity by

combined forms of N, this could conceivably lead to increased rates of acetylene reduction because of depletion of N in systems being assayed for N_2 fixing activity. Hardy *et al.* (1973) state that no evidence has been found for feedback inhibition of nitrogenase activity by combined N, which is a desirable charateristic from the point of view of the acetylene reduction assay. Combined forms of N, however, do appear to control nitrogenase activity in living organisms by influencing nitrogenase synthesis (Hardy *et al.* 1973).

This is potentially of great concern with respect to the acetylene reduction assay (Hardy *et al.* 1973), and may cause rates of C_2H_2 reducing activity to increase with time in lengthy incubations. This may have been the cause of an increase in $N_2[C_2H_2]$ reducing activity observed in subterranean clover over 24 hours by Eckart & Raguse (1980).

Finally, the two substrates, N_2 and C_2H_2, have differing abilities to enter living cells through lipoprotein membranes (Bergersen, 1970).

3.2 THE RELATIONSHIP BETWEEN ACETYLENE REDUCTION AND NITROGEN FIXATION.

If the acetylene reduction technique is to be used for measurement of the absolute amount of N_2 fixed by fixing systems, the equivalence of measured acetylene reduction rates with N_2 fixation, and factors affecting this, must be considered.

Stoichiometrically the ratio of C_2H_2 to N_2 reduced is 3:1 (Bergersen, 1970). However, it has been found that the theoretical ratio often does not apply exactly in practice, and that the ratio found experimentally for a particular system is to some extent a characteristic of the test itself (Sinclair, 1973; Sinclair, 1975). Factors which may differentially affect N_2 fixation and C_2H_2 reduction are discussed in the following paragraphs.

(a) H_2 Evolution

In the acetylene reduction assay, C_2H_2 reduction replaces not only N_2 fixation, but also H_2 evolution. Because 40 to 60% of the reducing power may be lost via H_2 evolution, the ratio of C_2H_2 reduced to N_2 fixed may often be about 6:1 rather than the theoretical 3:1 (Schubert & Evans, 1976).

(b) pO_2

C_2H_2 reduction may be more sensitive to pO_2 (partial pressure of oxygen) than N_2 fixation. Bergersen (1970) found that increased pO_2 decreased the ratio of C_2H_2 reduced to N_2 fixed in soybean nodules.

It is thought that H_2 evolution might have a function in providing reductant for O_2 via hydrogenase and thereby decrease pO_2 in the vicinity of nitrogenase (Hardy *et al.* 1973). This function would cease, and nitrogenase activity may be reduced as a result, when H_2 evolution ceased resulting from the use of C_2H_2 as a substrate.

(c) N_2

When N_2 and C_2H_2 are both present they compete for electrons in the nitrogenase reaction. Even though C_2H_2 is reported to be a strong inhibitor of N_2 fixation (Schollhorn & Burris, 1967), the presence of N_2 under some circumstances, has been found to cause a small reduction in C_2H_2 reducing activity (Hardy *et al.* 1968).

(d) pC_2H_2

At levels less than required to saturate the nitrogen fixing enzyme (nitrogenase), C_2H_2 reduction is proportional to pC_2H_2 (partial pressure of acetylene), and will tend to underestimate $N_2[C_2H_2]$ fixing activity (Hardy *et al.* 1973). Hardy *et al.* (1973) state that a C_2H_2 partial pressure of 0.1 atmospheres of C_2H_2 should produce saturation of 'in vivo' nitrogenase comparable to 0.8 atmospheres of N_2. Burris (1974) states that 0.15 to 0.20 atmospheres of C_2H_2 virtually saturates the nitrogenase enzyme system, but many authors report saturation at lower values than this, and most incubations seem to be carried out at a pC_2H_2 of 0.1 atmospheres, or less.

Fishbeck *et al.* (1973), carrying out non destructive assays on soybeans, found that saturation of the nitrogenase enzyme occurred at a pC_2H_2 of 0.1 atmospheres. Trinick *et al.* (1976) found the nitrogenase of nodulated root systems of *L. consentinii* to be saturated at a pC_2H_2 of 0.1 atmospheres, and of whole *L. luteus* plants to be saturated at a pC_2H_2 of 0.05 atmospheres. Eckart & Raguse (1980), non destructively assaying subterranean clover plants growing in sand, found that C_2H_2 reduction capacity was saturated by 0.03 atmospheres of C_2H_2. It was thought that the large pore sizes of the rooting medium, together with the technique used, of forced reticulation of gases through the medium accounted for the low value. Partial pressures of C_2H_2 used in acetylene reduction assays by various workers include the following. Duhigg *et al.* (1978) with nodulated lucerne root systems, Barta (1978) with nodulated root systems of lucerne and birdsfoot trefoil, Bethlenfalvay & Phillips (1977) with nodulated pea root systems, Zaroug & Munns (1979) with nodulated root systems of *Lablab purpureus* and Major *et al.* (1979) with whole sainfoin plants used a pC_2H_2 of 0.1 atmospheres. Seetin & Barnes (1977) with nodulated lucerne root systems used a pC_2H_2 of 0.7

atmospheres. Cassman *et al.* (1980) with nodulated soybean root systems, and Harding & Sheehy (1980) with exposed root systems of intact lucerne plants used a pC_2H_2 of 0.05 atmospheres. Sinclair (1973) and Sinclair *et al.* (1978) incubated undisturbed legume plants at a pC_2H_2 of approximately 0.05 atmospheres. Hardy & Holsten (1977) suggest that acetylene should be added to give a definite final pC_2H_2 of 0.05 to 0.10 atmospheres, which will provide equivalent saturation of nitrogenase to 0.8 atmospheres of N_2.

(e) Duration of Incubation

Length of incubation time may also differentially affect N_2 fixation and C_2H_2 reduction. Lengthy incubations may lead to a depletion of combined N, which in turn may cause an increase in C_2H_2 reducing activity with time (Hardy *et al.* 1973; Eckart & Raguse, 1980). However, Sinclair (1973) over a 30 hour incubation period observed no net effect of this kind in white clover plants.

Where a plant-in-soil system is being assayed there may be an initial lag period in the production of C_2H_4 because of restriction of gaseous diffusion by the growth medium (Sinclair, 1973). Huang *et al.* (1975a), non-destructively assayed soybean plants growing in a soil/peat/perlite mixture, and observed an initial lag in C_2H_4 production of 30 to 60 minutes. Hence, non-destructive acetylene reduction assays over short time periods may underestimate N_2 fixing activity (Sinclair 1973).

(f) Diurnal variation

Diurnal variation in acetylene reducing activity has been widely observed in soybean nodules (Hardy *et al.* 1968; Bergersen, 1970; Mague & Burris, 1972). Specific C_2H_2 reducing activity (on a nodule weight basis) has been found to be maximal for samples collected over the period 12.00 noon to 8.00 p.m.. and minimal over the period 12.00 midnight to 8.00 a.m. (Hardy *et al.* 1968). The effect of light, as distinct from temperature has been demonstrated (Hardy *et al.* 1968). Sinclair (1973) demonstrated diurnal variation in the C_2H_2 reducing activity of undisturbed white clover plants, and Ruegg & Alston (1978) found that short term fluctuations in the C_2H_2 reducing activity of *Medicago truncatula* Gaertn. were associated mainly with irradiance. It was found that C_2H_2 reduction rates at 12.00 noon were 10 to 60% (average 33%) higher than mean daily rates. In contrast, Trinick *et al.* (1976) and Haystead *et al.* (1979) observed no diurnal variation in C_2H_2 reducing activity of two *Lupinus* species, and white clover respectively.

Eckart & Raguse (1980) found that C_2H_2 reducing activity in subterranean clover varied diurnally in relation to temperature, but not to light. The implication was that this species is buffered against short term fluctuations in photosynthate supply (Eckart & Raguse, 1980). Presumably the low temperatures (13°C) reduced the activity of the N_2 fixing enzyme. Gibson (1963) found N_2 fixation of this species to decrease at root temperatures below 22°C.

Thus, acetylene reduction assays carried out during the photoperiod may tend to overestimate mean daily N_2 fixing activity because of both greater photosynthate supply and higher temperatures during the photoperiod. In contrast, N_2 fixation as derived from plant N analysis integrates N_2 fixation over the entire experimental period, where plants are dependent solely on fixed N (Sinclair *et al.* 1978).

(g) Growth conditions

The day to day changes in nitrogenase activity, depending on environmental conditions, can be a problem in obtaining reliable estimates of overall N_2 fixing activity using the acetylene reduction technique (Sinclair, 1975). Moisture levels may have a small effect on the relationship between C_2H_2 reduced and N_2 fixed (Sinclair *et al.* 1978). A tendency for moisture stress to increase the ratio of C_2H_2 reduced to N_2 fixed (the latter derived from N analysis) in non destructive assays was observed. More rapid diffusion of gases through a drier rooting medium, or N loss from noduleswere thought to be possible explanations of this effect. Ahmed & Quilt (1980) found that moisture stress had little effect on $N_2[C_2H_2]$ fixing activity of the tropical legumes *Macroptilium atropurpureum* or *Desmodium intortum*. In contrast, water stress has generally been found to decrease C_2H_2 reduction by detached nodules, and in soil samples containing nodules, C_2H_2 fixing activity was usually increased by increasing moisture supply (Hardy *et al.* 1973).

(h) Species

Significant differences in the ratio of C_2H_2 reduced to N_2 fixed have been observed between species (Sinclair *et al.* 1978). Lucerne and birdsfoot trefoil had higher ratios than clovers. It was pointed out, however, that this does not imply inherently different conversion factors for lucerne or birdsfoot trefoil. The effect could possibly arise from higher day/night differentials in $N_2[C_2H_2]$ fixing activity for lucerne and birdsfoot trefoil compared to the clovers, from nodule bacteroids being more readily accessible to gas exchange during incubation, or even from exudation of N from the nodules causing an

underestimate of N_2 fixation by chemical analysis (Sinclair *et al.* 1978).

Within species and growth conditions however, the acetylene reduction assay has been found to be very closely related to N_2 fixation (Sinclair *et al.* 1978).

(i) Ethylene

The capacity of low concentrations of ethylene (C_2H_4) to inhibit nitrogenase activity may possibly affect results obtained by the acetylene reduction assay (Koch & Evans, 1966; Hardy *et al.* 1973; Goodlass & Smith, 1979). Goodlass & Smith (1979) state that the acetylene reduction technique is valid for comparative studies, but that the extent to which absolute nitrogenase activity may be reduced as a result of physiological activity of C_2H_2 and C_2H_4 merits investigation.

3.3 THE ROLE OF THE ACETYLENE REDUCTION TECHNIQUE

The three most used methods of estimating N_2 fixation are probably measurement of total nitrogen using the Kjeldahl analysis, measurement of N_2 fixation using the stable isotope ^{15}N, and measurement of nitrogenase activity using the acetylene reduction technique.

The measurement of total nitrogen using the Kjeldahl method is a destructive method and is useful for determining the total accumulated amount of N at a particular point in time.

The $^{15}N_2$ technique involves exposing the test material to N_2 enriched with the stable isotope $^{15}N_2$, after which the ^{15}N content of the material can be determined using a mass spectrometer (Stewart, 1966). From the original proportions of $^{15}N_2$ and $^{14}N_2$, total N uptake over the period of exposure of the material can be calculated. The $^{15}N_2$ technique enables measurement of N_2 fixation over a specified time period. It is a much more sensitive method for measuring N_2 fixation than the Kjeldahl-N method (Stewart, 1966). The $^{15}N_2$ technique is fundamentally the most satisfactory method for measuring N_2 fixation because it is an absolute method and not subject to the correction factors which must be applied to convert acetylene reduction to potential N_2 fixation (Burris, 1974). The disadvantages of the method are that it is destructive, expensive and it is less sensitive than the acetylene reduction method.

The acetylene reduction method is the most sensitive method of determining N_2 fixing activity, and it is also simple , inexpensive and can be readily carried out in the field, where earlier methods limited experimentation to a few samples (Burris, 1974). The acetylene

reduction technique is of particular use in making comparisons between N_2 fixing systems, comparisons between treatment effects on N_2 fixation (Bergersen, 1970), or in monitoring changes in N_2 fixing activity during the growth of a N_2 fixing organism such as a legume (Sinclair *et al.* 1978). Acetylene reduction enables short term changes in nitrogenase activity to be followed and enables nitrogenase activity to be related to growth or environmental parameters. Bergersen (1970) states that acetylene reduction is most useful where the absolute amount of N fixed is not required to be measured. It is suggested that the theoretical conversion factor between C_2H_2 reduced and N_2 fixed should not be assumed, and that for quantitative estimates of N_2 fixation, the particular acetylene reduction assay system should be calibrated against a N based method to allow for different efficiencies of nitrogenase when reducing C_2H_2 compared with N_2 (Bergersen, 1970; Sinclair, 1973; Hudd *et al.* 1980). Sinclair *et al.* (1978), state that the C_2H_2 assay alone would be unsuitable as a basis for calculating N_2 fixation in absolute terms. Eckart & Raguse (1980) support this view, and suggest that estimates of absolute N_2 fixation in the field should be obtained from some more reliable method

Within species and growth conditions, however, the acetylene reduction assay has been found to be very closely related to N_2 fixation as measured by a micro-Kjeldahl technique (Sinclair *et al.* 1978). Hudd *et al.* (1980) measured N_2 fixation of *V. faba*, grown at two rates of nitrate, using acetylene reduction and a $^{15}N_2$ technique. A constant relationship between C_2H_2 reduced and N_2 fixed was found regardless of the rate of nitrate application. Bergersen (1970) found a high correlation between C_2H_2 reducing activity of soybean nodules and total N in the tops.

Thus, it seems appropriate to use the acetylene reduction assay for comparative purposes, but where estimates of absolute rates of N_2 fixation are required, it should be calibrated against a N based method (Bergersen, 1970; Hudd *et al.* 1980).

Sinclair (1975) listed criteria for the validity of the acetylene reduction assay:

(a) There should be a close linear relationship between C_2H_4 production and N uptake.

(b) Ratios of C_2H_2 reduced : N_2 fixed should be compatable with published experimental values.

(c) There should be a logical explanation for differences between C_2H_2 reduction and N_2 fixation.

3.4 THE NON DESTRUCTIVE ACETYLENE REDUCTION ASSAY

The acetylene reduction technique which has most generally been applied to legumes, has involved the removal of the root nodules from the environment in which they were growing, and exposure either completely excised or attached to portions of root, to an atmosphere containing C_2H_2 (Sinclair, 1973). This technique is rapid and sensitive, but has several disadvantages. Collecting nodules or nodule bearing roots can be time consuming, particularly with plants bearing many small nodules and growing in compacted soil. Also, it must be accomplished quickly, since nitrogenase activity starts to decline soon after nodules or roots are severed from the plant (Hardy *et al.* 1968; Moustafa *et al.* 1969). Excised nodules have been found to have reduced nitrogenase activity compared to nodules attached to root systems or intact plants (Hardy *et al.* 1968; Bergersen, 1970; Mague & Burris, 1972; Fishbeck *et al.* 1973; Trinick *et al.* 1976; Hudd *et al.* 1980). Decreases in C_2H_2 reducing activity of 70 to 85%, about 67% and 77% have been observed in excised nodules of *Lupinus* species (Trinick *et al.* 1976), *V. faba* (Hudd *et al.* 1980) and soybeans (Mague & Burris, 1972) respectively. Hardy *et al.* (1973) recommended that assays be carried out on nodulated root systems and that comparisons with whole plants are needed. Fishbeck *et al.* (1973) and Trinick *et al.* (1976) found that decapitation of plants had little effect on acetylene reducing activity of soybeans and *Lupinus* species respectively. Mague & Burris (1972), however, found that activity in nodulated root systems of soybeans was only about 46% of that of intact plants. When nodules are removed from plants the environment of the nodules during the test bears little similarity to their environment when attached to actively growing plants (Sinclair, 1973). Bergersen (1970) pointed out that major errors are likely to result when conditions in acetylene reduction assays are not carefully matched with the conditions under which N_2 fixation is occurring. Acetylene reduction assays on detached nodules at best reflect the N_2 fixing activity at the time of harvest. As this can fluctuate sharply with environmental conditions, a quantitative estimation of N fixed would require integration of results obtained at several harvest times each day (Bergersen, 1970).

Hardy *et al.* (1968) described a technique in which cores were taken from around the tap root of decapitated soybean plants and used in acetylene reduction assays. The results from these assays were acceptable, without the removal of soil from the root system . Lie (1971)

carried out acetylene reduction assays on pea plants growing in pots containing soil. Dobereiner *et al.* (1972) also described acetylene reduction assays on plant in soil samples. Sinclair (1973) carried out acetylene reduction assays in which white clover plants growing in pots containing sand or soil were placed, intact, in an C_2H_2 air atmosphere. It was found that production of C_2H_4 began almost immediately, and continued for much longer than generally reported for experiments using detached nodules, or nodulated root systems. Sinclair (1973) evaluated C_2H_2 reduction by actively growing plants as an index of N_2 fixation, by comparing with the C_2H_2 reduction rate of nodulated root systems. It was found that rates of C_2H_2 reduction by the intact plant system was lower than for nodulated roots for the first two hours of incubation, but then higher than the nodulated root system. The lower C_2H_2 reduction of exposed roots after two hours was thought to be because of loss of nodules or other damage incurred during extraction and washing. Fishbeck *et al.* (1973) carried out the acetylene reduction assay on intact soybean plants growing in perlite or soil. For the assay they were exposed to C_2H_2 in large polyethylene containers with plexiglass lids. C_2H_2 reduction rates were found to be similar to those of detached root systems.

Sinclair (1973) states that the success of the plant-in-soil acetylene reduction assay depends on the rapid penetration of C_2H_2 through the growth medium to the root nodules and the rapid redistribution of C_2H_4 between the soil and the surrounding atmosphere. A retardation of C_2H_4 production occurred during the early stages of the plant-in-soil incubation of both Sinclair (1973) and Fishbeck *et al.* (1973). Sinclair (1973) states that the retardation was more pronounced in soil than sand. Eckart & Raguse (1980) observed no lag period when subterranean clover growing in sand was non-destructively assayed. Sinclair (1973) suggests that, because of the lag period which may occur at the start of plant-in-soil incubations, very short incubations should be avoided. Fishbeck *et al.* (1973) found that the application of the acetylene reduction tecnique to plants growing in soil was complicated by the water content of the soil. Soil water content greatly influences rates of gas diffusion, since higher moisture levels result in less available air space for gaseous diffusion. It was found that when plants were non destructively assayed, C_2H_2 reducing activity increased with increasing soil water suction, and when they were removed from the growth medium (soil), C_2H_2 reducing activity increased as the soil water suction at which they had been grown decreased

(Fishbeck *et al.* 1973). Thus, high soil moisture appeared to be favourable for the root nodules, but to restrict gaseous diffusion.

Generally, for acetylene reduction assays of excised nodules and decapitated root systems, it has been found that the period during which C_2H_4 production has been linear with respect to time has been relatively short, with declines in rate of C_2H_4 production after 60 minutes reported by Hardy *et al.* (1968) and Koch & Evans (1966), and after 30 minutes reported by Schwinghamer *et al.* (1970). Sinclair (1973) reported sustained C_2H_4 production by white clover plants growing in soil, for periods of 30 hours. Root washed whole plants showed sustained production over the same period, being slightly above the plant-in-soil or sand samples for the first five to ten hours, and slightly below thereafter (Sinclair, 1973). Fishbeck *et al.* (1973) found that soybeans undergoing 'in situ' acetylene reduction assay exhibited linear C_2H_4 production with time over at least 90 minutes, and Eckart & Raguse (1980) found that 'in situ' acetylene reduction by subterranean clover growing in sand was linear with respect to time for at least two hours.

Provided acetylene reduction assays are not too frequent, successive assays may be made on individual samples without seriously impairing their N_2 fixing activity (Sinclair, 1973). It has been found (Sinclair, 1978), that the acetylene reduction assay (one 1 hour incubation per week) had no effect on the dry matter yield or %N of a range of forage legumes.

A high correlation ($r = 0.976$) has been obtained between N_2 fixed and C_2H_2 reduced in the non destructive acetylene reduction assay (Sinclair, 1973).

Thus, the non-destructive or plant-in-soil acetylene reduction assay appears to provide a very simple, and apparently accurate, assay of N_2 fixing activity when applied to plants growing in pots containing a relatively porous growth medium. The advantages of the technique are as follows (Sinclair, 1973).

(a) It is non destructive.
(b) It eliminates root washing and the risk of nodule damage or loss.
(c) The nodules are operating under test conditions which are very similar to those under which N_2 fixation normally occurs.
(d) Acetylene reducing activity is sustained over relatively longer periods, compared to excised nodules or nodulated root systems, with the advantage that changes in N_2 fixing activity over time can be traced using a single sample (e.g. Sinclair, 1973).

CHAPTER II
EXPERIMENTAL

1 OUTLINE OF EXPERIMENTS

1.1 EXPERIMENT 1(a)

The objectives of experiment 1(a) were to observe the early development of sainfoin (*Onobrychis viciifolia* Scop.) particularly that of its N_2 fixing system, and to contrast the development of plants dependent on symbiotic fixation for their supply of nitrogen, with those provided with abundant combined nitrogen.

All plants were inoculated, and up to day 50 supplied with a low rate of combined N (N_L). At day 50, half the pots were supplied with no further combined N (treatment N_O), and half were provided with a high rate of combined N (treatment N_1). Plant growth and development was monitored up to day 50, and after day 50 comparisons between treatments were made.

1.2 EXPERIMENT 1(b)

The objectives of experiment 1(b) were as follows:

(i) To investigate the effect of a low rate of combined N on plants previously completely dependent on symbiotic fixation for their supply of N.

(ii) To investigate the ability of plants, previously supplied with abundant N to nodulate and commence symbiotic N_2 fixation, when reduced to a low rate of combined N.

Plants were treated as for experiment 1(a) up to day 80. On day 80 additional rates of nitrogen were introduced as shown in section 2.1.2.

1.3 EXPERIMENT 2

The objectives of experiment 2 were as follows:

(i) To contrast the performance of plants dependent on symbiotic N_2 fixation for their nitrogen supply with plants provided with abundant combined N.

(ii) To isolate possible factors contributing to the relatively poor performance of sainfoin when dependent upon symbiotic fixation for its nitrogen supply.

(iii) To observe any differences between inoculated and non-inoculated plants, both supplied with combined N.

Treatments were as follows:

I_1N_O : inoculated, zero nitrogen

I_1N_1 : inoculated, abundant nitrogen

I_0N_1 : not inoculated, abundant nitrogen

I_0N_0 : not inoculated, zero nitrogen

All pots were supplied with the low rate of N, as for experiment 1(a), up to day 50, when the N_1 and N_0 treatments were applied. Destructive harvests commenced on day 84, 34 days after the nitrogen treatments, above, had been imposed,and by which time their effects had presumably become established.

2 EXPERIMENTAL DESIGN

2.1 TREATMENTS

2.1.1 INOCULATION

All pots in experiment 1 and I_1 pots in experiment 2 were inoculated on days 16, 31 and 109. It was planned that the third inoculation would coincide with the re-introduction of the low rate of N in experiment 1(b) on day 81. However, contamination problems with two successive rhizobial cultures caused a delay.

Two inoculations were carried out early on in the experiments, to ensure that adequate numbers of rhizobia were present when the plants were susceptible to infection. The third inoculation was carried out, because it was thought that rhizobial populations may not have been adequately maintained in the sand culture environment with the regular flushing through of nutrient solution. It was important that adequate numbers of rhizobia be present especially in the N_1/N_L treatment of experiment 1(b), to enable renodulation.

2.1.2 RATES OF NITROGEN

The concentrations of nitrogen in the nutrient solutions were as follows (Table 1):

Table 1 Concentration of nitrogen in nutrient solutions

Treatment	Concentration of N
N_O	0 ppm
N_L	35 ppm (as NH_4NO_3 or $NaNO_3$ *)
N_1	210 ppm (as $NaNO_3$*)

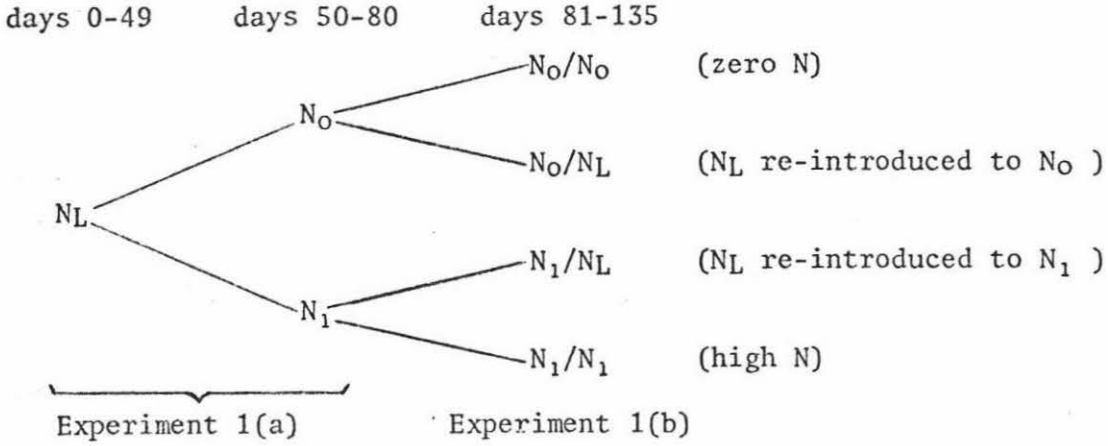
* In the early stages of the experiments, N was added as NH_4NO_3 to reduce inhibition of nodulation by nitrate which is a more potent inhibitor than ammonium N (Chapter I, Section 2.2.3.). After day 50, N was applied solely in the form of nitrate to avoid possible confusion over the differing effects nitrate and ammonium N may have had on N_2 fixing activity.

Up to day 50 the N_L rate (as NH_4NO_3) was applied to all pots. From day 50 the N_O and N_1 rates were introduced to both experiment 1 and experiment 2.

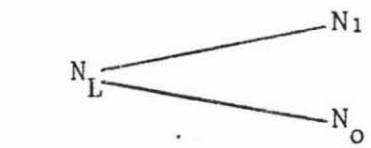
From day 81, the N_L treatment was re-introduced to half of the pots from each of the N_O and N_1 treatments (as $NaNO_3$).

The nitrogen treatments can be summarised as follows:

Nitrogen Treatments - Experiments 1(a & b)



Nitrogen Treatments - Experiment 2



2.2 STATISTICAL DESIGN

Both experiments were laid out in modified completely randomised designs.

2.2.1 EXPERIMENT 1(a & b)

The 96 pots in experiments 1(a) and 1(b) were randomly distributed over four tables A to D (24 pots each), and randomly allocated to positions within the tables, which were arranged in six rows of four. the four tables were arranged end to end, running north-south.

Tables were moved and reorientated to minimise table and block effects, and pots were moved within tables to minimise pot effects. On a weekly basis, tables were randomly reassigned to one of the four possible positions, reorientated north-south or south-north and pots on the edges of tables were exchanged with those inside them.

Zero (N_O) and high (N_1) rate of nitrogen treatments were introduced, on day 50, to two tables each. N_O was applied to tables A and D, N_1 to B and C. From this point on, re-randomisation of tables was done in such a way as to ensure that one table of each treatment was in each half of the row of four tables.

On day 81 half of the pots on each table (randomly chosen) were re-introduced to the low rate of nitrogen (N_L).

Experiments 1(a & b) were not completely randomised in that N_o and N_1 treatments were on separate tables, and then later the N_o/N_o and N_o/N_L treatments respectively, and the N_1/N_1 and N_1/N_L treatments respectively were at separate ends of tables. These constraints were placed on the randomisation to simplify and thus reduce the chance of errors in nutrient application.

2.2.2 EXPERIMENT 2

In experiment 2 there were 48 pots in each of treatments I_1N_o, I_1N_1 and I_oN_1, and 16 pots in treatment I_oN_o.

As in experiments 1(a & b) certain restraints, for practical reasons, were placed on randomisation. To minimise the risk of cross infection inoculated (I_1) and uninoculated (I_o) treatments were kept on separate tables. To facilitate nutrient application zero nitrogen (N_o) and high nitrogen (N_1) treatments were kept in separate rows within tables. Twenty-four pots (6 rows of 4) were randomly allocated to each of four 'I_1' tables and 16 (4 rows of 4) to each of four 'I_o' tables. The pots on each table were randomly allocated to positions within the table. On each 'I_1' table, N_o and N_1 treatments were randomly allocated to three rows each, and on each 'I_o' table N_o and N_1 treatments were randomly allocated to one and three rows respectively. On the 'I_o' tables, with only 16 pots, the spacing between rows was kept the same as for the 'I_1' tables. Each treatment was spread over four tables, there being eight tables altogether.

The glasshouse space allocated to this experiment was divided into quarters, and one 'I_1' and one 'I_o' table always occupied each quarter.

As for experiments 1(a & b), tables were moved and reorientated to minimise block and table effects, and pots were moved within tables to minimise pot effects. Rerandomisation was carried out on a weekly basis, as follows:

(i) Each 'I_1' and 'I_o' table was randomly allocated to a quarter, and to one of the two positions within a quarter. Tables were randomly orientated north-south or south-north.

(ii) Each pot on the end of a row was exchanged with the pot inside it to minimise edge effects.

A view of experiment 2 in the glasshouse is shown in plate 1.

Plate 1 View of experiment 2 in glasshouse on day 51.

Plate 2 Acetylene reduction technique in progress, showing incubation containers, gas chromatograph and samples awaiting analysis.

3 EXPERIMENTAL CONDITIONS

3.1 NUTRIENT APPLICATION

The nutrient solutions were as described in section 4.3.

It was intended that the frequency and quantity of nutrient application would be such as to provide the fastest growing plants with more nutrients than they actually required. Assumptions were made regarding the chemical composition of sainfoin based on Baker *et al.* (1952), Thomas *et al.* (1952), Whitehead and Jones (1969) and Smith *et al.* (1974). The information on chemical composition together with dry matter production measurements enabled calculation of the probable nutrient requirement for the various nutrient elements. Based on estimated requirements, the frequency of nutrient application was such as to provide approximately twice as much nutrient as the fastest growing plants would require.

The volume of application was generally such (350 ml) as to exceed the container capacity of the pots. Thus salt solution remaining in the pots was flushed through, or at least greatly diluted. Up to day 108 pots were flushed with 350 ml of nutrient solution on a weekly basis. From day 108 pots were flushed with 350 ml of nutrient solution twice weekly. Nutrient solutions were spread evenly onto the pots using a hand sprinkler.

The adequacy of nutrient supply was tested by carrying out analyses of herbage collected at regular intervals throughout the experiments. The levels of nutrients in the herbage were compared to levels quoted by the authors cited above. Analyses were carried out on N_1 plants only, as it was assumed that these, because they were the fastest growing, were the most likely to encounter nutrient shortage.

At the end of Experiment 2, the total amount of soluble nutrients present in the pots at the end of an interval between nutrient application was estimated. The herbage of several remaining pots was removed and their contents were immersed in 4875 ml of distilled water and stirred for two hours. The resulting solution was analysed for P, K, Mg and Ca.

Pots were periodically flushed with distilled water to ensure that no buildup of salts was occurring in the growth medium.

3.2 WATERING

3.2.1 OBJECTIVES OF WATERING

The objectives of the watering regime were as follows:

(i) To provide sufficient water for vigorous plant growth.

(ii) To ensure that water was not lost, along with nutrients, from the bottom of pots, between nutrient applications.

(iii) To avoid water logging in the bottoms of pots and allow adequate aeration of roots and nodules by not over watering.

3.2.2 WATERING REGIME

Pots were observed every day, and a few pots were check weighed every two days to ensure that the plants had an adequate supply of water. If plants were showing signs of moisture stress they were watered. If the sand in the pots appeared dry on the surface, they were check weighed. When pots fell below dry weight plus 100 g they were watered up to within 100 g of container capacity. It was found that if sufficient water was added to bring the pots right up to container capacity, significant quantities of solution were lost from the bottoms of the pots, and also air space was reduced to a low level.

As the plants became larger, and the days became longer and warmer, water use increased. From about day 100 (1st November) pots were check weighed daily and water was applied as necessary.

Larger volumes of water were applied to treatments which were using water more rapidly. Even so, the water content of the faster growing 'N_1' pots often became lower than the slower growing 'N_0' pots during the interval between waterings. However, since 90% of the water held at container capacity was easily available (at < 100 cm H_2O, see Fig.2) plants should have been able to use most of the water in the pots before suffering moisture stress. Hence, provided water in the 'N_1' pots was not allowed to become too depleted, the fact that the 'N_0' pots contained more water towards the end of intervals between waterings, should not have had a significantly unfavourable effect on the N_1 relative to the N_0 treatments.

3.2.3 ACETYLENE REDUCTION ASSAY

Care was taken to ensure that plants were not suffering from moisture stress during acetylene reduction assays. Pots were check weighed before incubation. If watering was necessary, pots were watered up to approximately 50% of container capacity. They were not watered up to container capacity, to facilitate diffusion of O_2, C_2H_2 and C_2H_4 through the sand.

3.3 INOCULATION

At each of the two initial inoculations (days 16 and 31) 12.5 ml of rhizobial suspension was trickled around the base of the stem of each plant, using a pipette. Since the cultures contained at least 1.5×10^7 bacteria per ml, there should have been at least 18.8×10^7 bacteria in the vicinity of each root system.

For the third inoculation, on day 109, rhizobial cultures (containing 1.5×10^7 bacteria per ml) were diluted 7:9 with distilled water. 100 ml of the diluted culture was sprinkled onto the surface of each 'I_1' pot. Thus each pot should have contained in excess of 117×10^7 rhizobial bacteria.

The day before each inoculation, pots were flushed with nutrient solution. For the first two inoculations, care was taken in the two weeks following the inoculation not to add water or nutrient solution in quantities sufficient to cause loss of solution and possibly rhizobia from the pots. For the third inoculation care was taken for the week following inoculation not to cause loss of solution from the pots.

3.4 GROWTH TEMPERATURES

The plants were grown in glasshouses with thermostatically controlled heating and cooling systems, at Soil Bureau, DSIR, Lower Hutt.

Glasshouse temperatures were controlled as in Table 2.

Table 2 Glasshouse temperatures (^{o}C)

Time period (days from sowing)	Air Temperatures (approx.) Day		Night	
	min	max	min	max
3 - 83	17	23	13	15
83 - 108	23	28	16	18
108 - 143	23	28	14	17

Temperatures were raised on day 83 (15 October) to simulate spring-time conditions of temperature increasing with day length. The slight change in temperatures on day 108 was because the experiments were moved to another glasshouse.

During October over-heating of the glasshouse became a problem. On very sunny days the temperature sometimes reached $30^{o}C$ (on about 10 occasions for short periods). On day 78 (mid October) the glasshouse was coated with whitewash to help control daytime temperature. This helped, but overheating was still a problem. It was felt that the

temperatures being experienced on hot days could be harmful to the plants (loss of leaflets on some lower leaves was observed on many plants) and they were moved on day 108 to a glasshouse with a more effective cooling system. This resulted in slightly better temperature control on very hot days.

4 MATERIALS

4.1 PLANTS

Onobrychis viciifolia Scop. cv. Fakir was chosen because it is an erect growing multi-cut type of sainfoin, which flowers during its first growing season, and under New Zealand conditions is relatively high yielding compared to other varieties (J.Lancashire pers. comm.). The seed, Grasslands no. AZ 1278 was harvested in 1977/78 and had a germination of 95% in July 1978. A further germination test was carried out before sowing in July 1979. Germination after six days on moist filter paper at 20°C was found to be 95%.

4.2 RHIZOBIUM

The rhizobial strain used was 3157 (NZP 5301) supplied by Applied Biochemistry Division, D.S.I.R. Cultures were made up containing at least 1.5×10^7 rhizobia per ml.

4.3 NUTRIENT SOLUTIONS

The nutrient solutions used were based on those of Small & Leonard (1969). Composition of the zero nitrogen solution was as shown in Table 3.

Table 3 Chemical composition of zero N nutrient solution

Compound, and concentration in stock solution	ml stock solution per litre nutrient solution	Concentration of element in nutrient solution (ppm)
Macroelements		
1 M $MgSO_4.7H_2O$	1.0	Mg = 24.31, S = 32.06
1 M KH_2PO_4	0.5	K = 19.55, P = 15.485
0.5 M K_2SO_4	5.0	K = 195.5, S = 80.15
0.5 M $CaCl_2$	5.0	Ca = 100.2, Cl = 177.25
Microelements		
$H_2MoO_4.H_2O$(0.09 g/litre)	1.0	Mo = 0.053
$ZnSO_4$ (0.22 g/litre)	1.0	Zn = 0.050, S = 0.025
$CuSO_4.5H_2O$(0.79 g/litre)	1.0	Cu = 0.201, S = 0.101
H_3BO_3 (2.86 g/litre)	1.0	B = 0.500
$MnSO_4.4H_2O$ (2.03 g/litre)	1.0	Mn = 0.500, S = 0.292
$Co(NO_3)_2.26H_2O$ (0.056 g/litre)	1.0	Co = 0.011
EDTA - Ferric monosodium salt (27.5 g/litre)	2.0	Fe = 8.37

Elemental composition of the zero nitrogen nutrient solution was as shown in Table 4.

Table 4 Elemental composition of zero N nutrient solution

Element	Concentration (ppm of nutrient solution)
P	15.48
K	215.05
Mg	24.31
S	112.63
Ca	100.2
Mo	0.053
Zn	0.050
Cu	0.201
B	0.500
Mn	0.500
Co	0.011
Fe	8.37
Cl	177.25

Up to day 50, N was added to all pots as NH_4NO_3 at a concentration of 35 ppm N.

When rate of N treatments were applied, from day 50, N was added in the form of $NaNO_3$.

The high low and zero rates of N used in the experiments were designated N_1, N_L and N_O and were 210, 35 and 0 ppm (15, 2.5 and 0 mM) N respectively.

4.4 GROWTH MEDIUM

4.4.1 CHOICE AND PREPARATION

Sand was used as the growth medium, to enable root systems and nodules to be easily and cleanly extracted. Because sainfoin is susceptible to crown and root rot diseases (Ditterline & Cooper, 1975), a coarse sand was chosen to avoid any problems which may have been caused by having high moisture levels and poor aeration in the pots. A sand with relatively high pH was thought to be desirable because sainfoin has historically thrived on dry calcareous soils (e.g. Piper, 1924).

A range of sands available in the Wellington region was obtained. A Wainuiomata beach sand, taken from the vicinity of the Orongorongo river mouth was chosen for use in the experiment because it was a coarse sand with reasonably uniform particle size, and relatively high pH. Before use it was thoroughly washed to remove soluble salts and fine material.

4.4.2 PROPERTIES OF SAND

The properties of the Wainuiomata sand, derived as in appendix 1, were as in Table 5.

Table 5 Properties of Wainuiomata sand

Container capacity	346 ml
Moisture content at container capacity	30.3% (by volume)
Easily available water (<100 cm H_2O)	27.2% (by volume)
Amount of easily available water at container capacity	311 ml
Air space at container capacity	7.1%
Water buffering capacity	3.06% by volume = 35 ml

The moisture release curve of the sand is shown in Fig.2, and reinforces the data in the above table, indicating that most of the water contained in the sand at container capacity was easily available and that the water buffering capacity was low.

Particle size analysis indicated that the Wainuiomata sand was a coarse sand with a quite narrow range of particle size, and very little material finer than 0.25 mm. The particle size analysis is shown in appendix 1.

4.5 POTS

Plants were grown in plastic pots of approximately 1.38 l volume. The dimensions of the pots were as follows:
height = 12 cm, top = 13.5 cm diameter, base = 10.5 cm diameter.
For drainage there were four basal holes of 12 mm diameter. Discs of fibreglass insect screen with a mesh size of 1.2x1.8 mm were placed in the bottoms of the pots to prevent loss of sand.

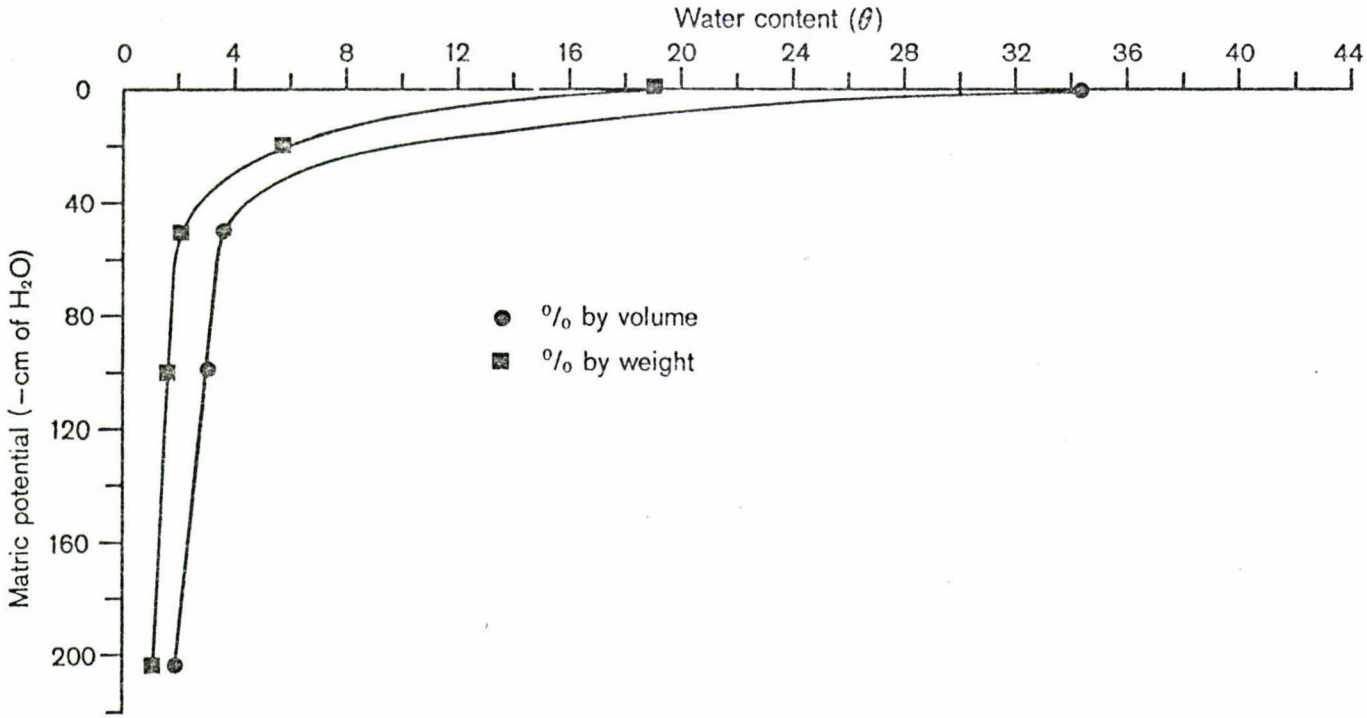


Fig 2 Moisture release curve for Wainuiomata sand. Each point is the mean of six determinations.

5 SETTING UP OF EXPERIMENTS

5.1 PACKING POTS AND SOWING

The appropriate amount of moist sand, equivalent to 1950 g of oven dry sand, was pre-weighed and stored in plastic bags. All but 265 g of the sand was packed into the pots and levelled. Two seeds were sown at each of five (experiments 1(a & b)) or two (experiment 2) uniformly spaced positions on top of the sand. The remaining 265 g of sand was then placed on top of the seeds and levelled to give a sowing depth of 1 cm (sowing depth derived from Jensen & Sharp 1968; Hanna *et al.*, 1977a).

Immediately after sowing, 100 ml water was added to the surface of each pot. The pots were then covered with heavy paper to reduce moisture loss and left at a temperature of approximately 20°C until the seedlings began to emerge. The surfaces of the pots were sprayed daily with deionised water to prevent the sand from drying out.

5.2 EMERGENCE

On day 3 after sowing, the seedlings began to emerge. The paper was removed and the pots were transferred to a glasshouse. At day 15, emergence was 93%. At this stage, thinning and transplantation, where necessary, were carried out to give the required number of plants per pot (five for experiments 1(a & b), two for experiment 2). When thinning, plants which were non-uniform, or out of position, were selectively removed.

There were three types of first true leaf. No attempt was made to select for a particular type of first true leaf. It would have been impossible to achieve uniformity in this regard without a great deal of transplanting. The significance of first true leaf type is discussed in Chapter III, 1.1.

6 DESTRUCTIVE HARVESTS

6.1 SCHEDULE OF HARVESTS

6.1.1 EXPERIMENT 1(a)

Preliminary harvests were carried out on days 10 and 24. Detailed destructive harvests were carried out twice weekly from day 30 to day 52 and once weekly from day 52 to day 80.

The pots on each of tables A to D were numbered 1 to 24. Using a table of random numbers, one pot was selected from each table at each harvest. This means that up to day 50, four N_L pots were being harvested at each harvest date. From day 50 to day 80, two pots from each of the treatments N_0 and N_1 were being harvested at each date.

6.1.2 EXPERIMENT 1(b)

Three destructive harvests were conducted, on days 121, 128 and 135. Pots for destructive harvest were chosen from each treatment using a table of random numbers. The harvest on day 121 was treated as a preliminary harvest, and only two pots per treatment were harvested. On day 128 six pots per treatment were harvested, and on day 135 all remining pots were harvested (six each from treatments N_0/N_0 and N_1/N_1, and four each from treatments N_0/N_L and N_1/N_L).

6.1.3 EXPERIMENT 2

Destructive harvests were carried out twice weekly from day 84 to day 143. Two pots from each of the treatments I_1N_0, I_1N_1 and I_0N_1 were harvested twice weekly. At the first of the twice weekly harvests, two pots (from each of the three treatments above) were chosen, one each from two randomly selected tables, by drawing numbers. At the second twice weekly harvest, two further pots from a particular treatment were chosen from the two tables containing that treatment not selected at the beginning of the week. Thus, each week one pot of each treatment was chosen from each 'quarter' of the glasshouse, or from each of the four tables over which a particular treatment was spread. I_0N_0 pots were harvested at approximately 14 day intervals, and chosen in a similar manner to that described for the other treatments.

6.2 EXTRACTION OF ROOTS

Pots were tipped upside-down on a stiff 13 cm diameter plastic disc with radial slots cut in it to allow the stems to come through. The pots were lifted off and the roots disentangled from the fibreglass gauze from the bottoms of the pots. The root systems plus sand were then

immersed in water enabling the root systems to be floated clear of the sand, and disentangled from one another.

6.3 DATA RECORDED

6.3.1 EXPERIMENT 1(a)

Data were recorded on an individual plant basis as follows:

Height of plant from base of crown, to nearest 0.5 cm.

No. of leaves

No. of leaflets on leaves

Petiole length, to nearest 0.5 cm (days 30 and 34 only)

Leaf area

Dry weight of top

Overall length of root system from bottom of crown, to nearest 0.5 cm (up to day 59)

No. of lateral roots (up to day 59)

Position of lateral roots; distance from bottom of crown, to nearest 0.5 cm (days 30 and 34)

Length of lateral roots, to nearest 0.5 cm (days 30 and 34)

Dry weight of root system

No. of nodules

No. of pink nodules (containing leghaemoglobin)

Position of nodules; position on lateral and position of lateral (days 30 to 42)

Fresh weight of nodules (started day 45)

Dry weight of nodules (started day 45)

Presence or absence of root hairs and whether or not their region of greatest abundance corresponded to the region of greatest abundance of nodules (up to day 49)

6.3.2 EXPERIMENT 1(b)

Data were recorded on a per pot basis as follows:

Top weight

Root weight

Nodule weight } All plants on days 121 and 128,
Nodule number } selected plants on day 135

6.3.3 EXPERIMENT 2

Data were recorded on an individual plant basis, as follows:

Plant height to nearest 0.5 cm

Number of leaves

Leaf area

Number of growth points

Number of stems elongated

Number of buds
Number of flowers
Top dry weight
Root dry weight
Number of nodules
Fresh weight of nodules
Dry weight of nodules

6.4 MEASUREMENT OF DATA

6.4.1 LEAF AREA

(a) Experiment 1(a)

On days 30 to 49, leaf areas of all plants were traced onto graph paper. The traced areas were cut out and weighed, enabling calculation of leaf area based on the area to weight ratio of the paper.

On days 52 to 80, the leaf area of one plant from each pot was traced as above, and the leaves of each plant were weighed separately from the stems. A mean specific leaf area was established for a particular harvest, and leaf area was calculated on the basis of leaf weight.

(b) Experiment 2

Leaf areas were measured using an electronic leaf area measuring machine. The machine gave values to 0.01 cm^2, but reliable accuracy was to the nearest 1 cm^2.

6.4.2 PLANT AND NODULE DRY WEIGHTS

Dry weights were measured after oven drying at 70°C for 48 hours.

Tops and roots from experiment 1(a) were weighed in tared containers on a Mettler H54AR balance which had an accuracy of 0.00001 g. Fresh and dried nodules from all experiments were weighed in a similar manner.

Tops and roots from experiments 1(b) and 2 were weighed on a Mettler P1210 top loading balance, to an accuracy of 0.01 g. Weighings were done in the paper bags in which they were dried. A standard weight was subtracted for the bags, which were found to have a mean weight of 2.50 g (standard error = 0.02 g). The error in bag weight was small in comparison to the weight of plant material.

6.4.3 NODULE NUMBER, CLASSIFICATION AND FRESH WEIGHT

Nodules often appeared as clusters (Plate 9). The clusters of nodules, or branched nodules arising from a single point on a root were counted as one nodule. Nodules with a clearly visible pink or red area when sectioned were classed as 'pink'. Where there was any doubt, they were classed as not pink.

Nodules were removed for weighing using a pair of fine forceps and a scalpel. They were placed in sealed plastic containers lined with moist filter paper and stored in a refrigerator until fresh weights were measured.

6.4.4. SAND IN ROOTS AND NODULES

Problems were encountered with sand in dried roots and nodules, even though careful washing had been carried out. Particular problems occurred with large branched nodules. These often had sand occluded within them which only became apparent on drying. To obtain reliable dry weights, sand was manually removed from all root systems, and from nodules collected after day 80. This material was then redried and reweighed.

7 ACETYLENE REDUCTION

7.1 ACETYLENE REDUCTION ASSAYS

7.1.1 SELECTION OF POTS FOR ACETYLENE REDUCTION ASSAY

(a) Experiment 1(b)

Two pots from each of the N_O, N_O/N_O, N_O/N_L and N_1/N_L treatments were assayed on a weekly basis from day 65 to day 135. The N_1 and N_1/N_1 treatments, which exhibited no measurable $N_2[C_2H_2]$ reducing activity, were assayed less frequently.

Up to day 80 one pot was chosen from each table using random numbers, and the pots assayed were also destructively harvested. From day 81, one pot was chosen from each half table (2 pots from each treatment), also using random numbers.

(b) Experiment 2

Four pots from the I_1N_O treatment were assayed twice weekly. The pots chosen were those chosen for destructive harvest plus one pot (randomly chosen) from each of the two 'I_1' tables from which pots for the current destructive harvest were not chosen. Two randomly chosen I_ON_O pots were routinely included as checks for background N_2 fixation, by for example, any blue-green algae on the sand.

7.1.2 INCUBATION AND GAS SAMPLING

A non-destructive acetylene reduction assay was used, similar to that of Sinclair (1973). An acetylene reduction assay in progress is shown in plate 2.

Plants were incubated in 4.5 litre, opaque, white polyethylene boxes with blue snap on lids, similar to those used by Sinclair (1973). Rubber septa were fitted in the lids to allow for sampling of gases.

To allow for any lag period before acetylene reduction became linear with time, a period of pre-incubation was included before the initial gas sampling. Two sets of gas samples were taken, the initial sampling at the end of the pre-incubation period, and a final sampling two hours later at the end of the incubation period. A pre-incubation period of approximately 1.5 hours (longer than reported lag periods associated with acetylene reduction of plant-in-soil samples) was necessary to enable C_2H_4 to reach concentrations that were reliably measurable at the first sampling time. This technique also enabled assays to be conducted over a more exactly comparable time period than would otherwise have been the case, as sometimes up to twenty minutes elapsed between the introduction of C_2H_2 to the first and last incubation containers.

Tests showed that C_2H_2 production continued in a linear fashion for at least five hours.

Intact plants in pots were placed in the containers, and lids fitted at 10.00 to 10.30a.m. 250 ml of air was removed from each of the containers by syringe and immediately replaced by 250 ml of commercial grade acetylene to give a pC_2H_2 of 0.07 to 0.08 atm. depending on the moisture content of the sand, and plant size. At 12.00 noon, and again at 2.00 p.m. three 1 ml gas samples were taken using 1 ml disposable plastic syringes. The samples were stored for the short period prior to analysis by plunging the syringe needles into a rubber bung.

Incubation temperatures were maintained at between 20° and 24°C.

7.1.3 GAS ANALYSIS

Gas samples were analysed using a Varian Aerograph series 1700 gas chromatograph fitted with a flame ionisation detector. Attached to this was an Autolab Minigrator which printed out peak areas and retention times (Plate 2). The column, which was made for these experiments, was 1 m long, made from 1/8 inch internal diameter stainless steel tubing, and packed with Porapak T 80-100 mesh. The carrier gas was N_2.

Conditions for the analysis of gas samples were as follows:

Temperature program	:	isothermal
Oven (column) temperature	:	100°C
Injector temperature	:	175°C
Detector temperature	:	155°C
Carrier gas flow rate	:	20 ml.min^{-1}

Before the samples were analysed a duplicate set of gas mixture standards, to cover the expected range of C_2H_2 and C_2H_4 concentrations in the samples, were analysed. At the end of the sample analysis one of the standards was again analysed (in duplicate). If agreement with the previous analysis was good (within 5%) no further standards were run. If agreement was poor, all standards were analysed again, and the mean peak area ratio of the beginning and end standards used in the calculation of C_2H_4 produced. Agreement was usually considerably better than 5%.

7.2 GAS MIXTURE STANDARDS

7.2.1 PREPARATION OF STANDARDS

A set of standard gas mixtures (containing air plus C_2H_2 and C_2H_4) was made up. The concentrations of C_2H_2 and C_2H_4 in the standards were such as to approximate the concentrations in the incubation containers.

The standards were made up in 100 ml conical flasks as follows:

(i) The flasks were sealed using Gallenkamp suba seals. 1.5 ml of mercury (enough to cover the neck of the flasks) was placed in each, using a syringe.

(ii) The flasks were allowed to equilibrate to atmospheric pressure by inserting a syringe needle through the suba seal.

(iii) Air, equivalent in volume to the amount of C_2H_2 + C_2H_4 to be added, was removed from the flasks using gas tight syringes.

(iv) 5 ml of C_2H_2 (commercial grade) was injected into each flask using a high precision 5 ml gas tight syringe.

(v) Various amounts of C_2H_4 (C.P. grade), were injected into the flasks using high precision gas tight syringes. The quantities of C_2H_4 were 1, 2.5, 5, 12.5, 25, 50, 125 and 250 microlitres (µℓ).

The standards were analysed prior to use to ensure that they lay on a smooth curve when peak area ratio was plotted against ethylene concentration. This was done to ensure that no major errors had occurred in making up the standards.

The set of standards made initially were used from day 65 to day 115. A new set of standards was made up, and on days 118 and 121, these were analysed alongside the old standards. The peak area ratios of the new standards were found to be lower than those of the old standards by a constant proportion (approximately 14%). A new syringe had been used to deliver the 5 ml of C_2H_2 in making up the new standards. It was found that the old syringe was delivering lower volumes than it should have been, thus causing the C_2H_4:C_2H_2 peak area ratios of the old standards to be greater than they should have been. On the basis of the comparative analyses on days 118 and 121 the peak area ratios of the old standards were reduced by a constant proportion to make them correspond to the new standards. Calculation of C_2H_4 production prior to day 118 was then done on the basis of the adjusted standard peak area ratios.

7.2.2 CALCULATION OF ETHYLENE PRODUCTION

Ethylene production (equivalent to acetylene reduction) was calculated by comparing the C_2H_4:C_2H_2 peak area ratios of the gas samples with the peak area ratios of standard gas mixtures, as follows (based on formulation by Plant Physiology Division, DSIR):

	ml C_2H_4	ml C_2H_2	Peak area ratio
Standard	x	y	A
Sample	u	z	B

Volume of C_2H_2 injected for incubation = X

Gas mixture ratio $\frac{x}{y}$ gives peak area ratio A

Gas mixture ratio $\frac{u}{z}$ gives peak area ratio B

The curve of peak area ratio against the ratio of the concentrations of C_2H_4 and C_2H_2 in the standards was found in most cases to be very slightly convex upwards through the origin. The curve was approximated by the equation -

$$A = a_o \left(\frac{x}{y}\right)^2 + a_1 \left(\frac{x}{y}\right)$$

The coefficients a_o and a_1 were fitted by least squares.

Now given an observed value, B, for the sample peak area ratio, we can write -

$$B = a_o \left(\frac{y}{z}\right)^2 + a_1 \left(\frac{y}{z}\right)$$

The appropriate solution for $\frac{y}{z}$ is L where

$$L = \frac{-a_1 + \frac{a_o}{|a_o|}\sqrt{a_1^2 + 4a_oB}}{2a_o}$$

Thus $u = Lz$

Also $u = X - z$

Eliminating z we get

$$u = \frac{LX}{1+L}$$

This gives u in ml C_2H_4 at the temperature T^oC and pressure, P atmospheres, at which C_2H_2 is measured into the incubation vessel. Using the relationship that 1 µmole of gas = 22.4/1000 ml at 0^oC and 1 atmosphere pressure, we have

$$u \text{ in } \mu\text{mole} = \frac{FLX}{1+L} = \begin{matrix}\mu\text{moles } C_2H_4 \\ \text{produced}\end{matrix} = \begin{matrix}\mu\text{moles } C_2H_2 \\ \text{reduced}\end{matrix}$$

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

8 LABORATORY ANALYSES

8.1 TOTAL N

Selected plant samples were analysed for total N using a micro Kjeldahl method as described by Metson (1972).

The samples analysed were as follows:

(i) Experiment 1(a): all pots of one harvest per week.

(ii) Experiment 1(b): all pots from the harvest of day 128, most pots from day 135.

(iii) Experiment 2: all pots from treatments I_1N_0 and I_0N_0. Every second harvest from treatments I_1N_1 and I_0N_1.

The plant samples were bulked on a per pot basis for analysis. Tops and roots (including nodules) were analysed separately.

8.2 X-RAY FLUORESCENCE ANALYSIS

Selected herbage samples from the 'N_1' treatments of experiment 2 were analysed for major and micronutrients as a check on the adequacy of nutrient supply.

8.3 ANALYSIS OF NUTRIENT FROM POTS

As outlined in section 3.1, the soluble salts contained in a number of pots, at the end of an interval between nutrient applications, were dissolved in distilled water. Analysis was carried out as follows, according to the methods of Blakemore *et al.* (1977).

(a) Phosphorus

Analysis of P was a colorimetric procedure involving reduction of a phospho-molybdate complex by ascorbic acid in a reaction catalysed by antimony, and was carried out in an automated analyser.

(b) Magnesium

Analysis of Mg was carried out using atomic absorption spectrometry.

(c) K, Ca and Na

Analysis of K, Ca and Na was carried out using flame emission spectrometry.

9 ANALYSIS OF RESULTS

Statistical analyses were carried out using the Genstat statistical package, prepared by Lawes Agricultural Trust, Rothamsted Experimental Station. Because of the high degree of variability between individual plants, statistical analyses were carried out on per pot data.

9.1 ANALYSIS OF VARIANCE

Standard treatment by harvest analyses of variance were carried out where it was desirable to compare mean values of particular variables between treatments, or to make comparisons at particular points in time. Where variability increased with time, analysis of variance was conducted on the logged variates. Differences between individual means were evaluated using standard errors of differences.

9.2 CORRELATION AND REGRESSION ANALYSIS

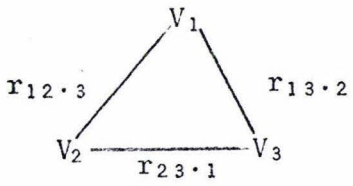
9.2.1 CORRELATIONS AND REGRESSIONS BETWEEN PAIRS OF VARIABLES

Correlations or regressions of pairs of variables against one another, where both are changing with time, contain a time component which may result in spurious values for the correlation or regression coefficients (e.g. Kendall & Stewart, 1967). It was desired to quantify relationships between sets of variables with the influence of time removed. This was done by regressing the logged (because growth was generally exponential) variables against time, and storing the residuals from the regression lines. The sets of residuals corresponding to particular variables were then correlated with one another to give a measure of the relationships between pairs of variables, which was independent of time. The sets of residuals were also regressed against one another as a check that straight line relationships did exist between sets of residuals.

9.2.2 PARTIAL CORRELATIONS

Where it was desired to make comparisons of the relationships between pairs of variables, with one another, partial correlations were calculated as described by Steel & Torrie (1960) and Nie *et al.* (1975). First order partial correlation coefficients were calculated between pairs of variables while holding the effect of a third variable constant. Second order partial correlation coefficients were calculated between pairs of variables with the effect of a third and fourth variable held constant. First and second order partial correlation coefficients were diagrammed as follows:

Diagrams of first order partial correlations

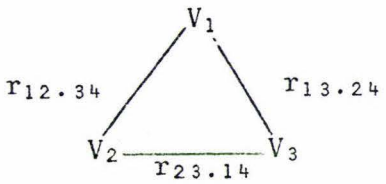


V_1, V_2 and V_3 represent variables 1, 2 and 3.

$r_{12.3}$ is the first order partial correlation coefficient (r_p) between variables 1 and 2, with the effect of variable 3 held constant.

$r_{23.1}$ and $r_{13.2}$ are, similarly, the first order partial correlation coefficients between variables 2 and 3, and 1 and 3, with the effect of variables 1 and 2 respectively, held constant.

Diagrams of second order partial correlations



(Effect of variable 4 also held constant)

As previously V_1, V_2 and V_3 represent variables 1,2 and 3.

A fourth variable being held constant for all three second order partial correlations is indicated in brackets on the righthand side of the diagram.

$r_{12.34}$ is the second order partial correlation coefficient r_{2p} between variables 1 and 2, with the effect of variables 3 and 4 held constant.

$r_{23.14}$ and $r_{13.24}$ are, similarly, second order partial correlation coefficients between variables 2 and 3, and 1 and 3 with the effects of variables 1 and 4, and 2 and 4 respectively held constant.

9.2.3 PATH COEFFICIENTS

In Figs. 33 and 34 diagrams were constructed using path coefficients as described by Nie *et al.* (1975). Arrows indicate the assumed direction of influence of one variable on another. The numbers, or path coefficients, associated with the arrows are standardised regression coefficients. Each variable was regressed on all of the variables which were assumed to have a direct influence on it. The coefficients were standardised to

correct for the different variances of the variables concerned. Arrows coming from outside the diagram, and their associated coefficients, represent external sources of variation.

9.3 RELATIVE GROWTH RATES

The relative growth rates for particular variables were calculated as by Raper *et al.* (1977), by regressing the logged variables against time. The coefficient of time is then the estimated relative growth rate, as follows:

By definition, relative growth rate, RGR = $\frac{1}{W} \cdot \frac{dw}{dt}$

If $\log W = at + b$

Then $W = e^{at + b}$

$\therefore \quad \frac{1}{W} \cdot \frac{dw}{dt} = \frac{ae^{at + b}}{e^{at + b}} = a \equiv$ coefficient of time

9.4 NET ASSIMILATION RATE

Overall net assimilation rates for particular time periods were calculated using the overall total plant relative growth rate (RGRp), and the mean leaf area ratio for the period.

i.e. Net Assimilation Rate = $\frac{RGRp}{LAR}$

CHAPTER III

RESULTS AND DISCUSSION

1 EXPERIMENT 1(a)

1.1 EARLY GROWTH AND DEVELOPMENT

Emergence began on day 3. At day 10 the first true leaf had recently unfolded, or was in the process of unfolding. It was noted that plants varied in the type of first true leaf they produced. The frequency of first true leaf type was observed over 442 plants, and was as shown in table 6.

Table 6. Frequency of first true leaf type

Type of first true leaf	Proportion of total plants
unifoliate or spade leaf	61%
trifoliate leaf	29%
bifoliate leaf	10%

Compared to the seedlings described by Thomson (1938), and most of the accessions listed by Cooper (1974), the seedlings of sainfoin var.Fakir had a substantially lower proportion of unifoliate first leaves, and a correspondingly higher proportion of trifoliate and bifoliate first leaves. This fact may have some importance because Cooper (1974) found that trifoliate first leaves had greater area than unifoliate leaves, and that seedlings with trifoliate first leaves had higher rates of net carbon exchange, and grew more rapidly during the early seedling growth period. At eight weeks of age, however, the forage yields of seedlings with unifoliate or trifoliate first leaves were found to be similar (Cooper, 1974).

Root length on day 10 was on average 8.2 cm. The root systems had an average of approximately three laterals, and approximately 50% of the root systems had root hairs.

At day 24, plant height was 8.4 cm and most plants had three true leaves, or two, with the third unfolding. Root systems were on average 15 cm in length, and had an average of 12 laterals. Root hairs were evident on most root systems. Root hairs occurred on both the lateral and tap roots, but appeared to be more abundant on the lateral roots, particularly on those closer to the surface. No nodules were in evidence at this time, despite inoculation having occurred at day 16.

1.2 GROWTH AND DEVELOPMENT, DAY 30 TO DAY 50.

At day 30, the time of the first full scale harvest, the plants were at the three (true) leaf stage. Root systems, which had reached the bottoms of the pots, had an average of 16.3 laterals, of which over 30% were at least 5 cm in length. Abundant root hairs were observed on the root systems of most plants. Root hairs tended to be most abundant on the lateral roots, particularly the upper laterals, being most apparent close to the root tips, starting about 5 mm back from the tip.

The early development of the plant (up to day 50) was similar to that described by Thomson (1938). In terms of the rate at which successive leaves unfolded and expanded, the development of sainfoin under the conditions of this experiment was similar to that of the greenhouse grown sainfoin described by Cooper (1974), and more rapid than in the sainfoin described by Thomson (1938) which was grown in the open.

1.2.1 TOTAL DRY WEIGHT

Total dry weight increased exponentially with time over the period day 30 to day 50, with the plot of $\log_e$ [total dry weight] against time appearing to be linear, and the regression of $\log_e$ [total dry weight] on time having a percentage variance accounted for ($\bar{R}^2$) of 90.3% (fig. 3, table 7). Whole plant relative growth rate (RGRp) from day 30 to day 50 was 0.0510 $g.g^{-1}.day^{-1}$ (table 7). This relative growth rate was very similar to the value of 0.0496 for the sainfoin variety Melrose, over an almost identical age period, derived from the data of Smoliak *et al.* (1972). It was also similar to relative growth rates of 0.054 and 0.047 for the field grown sainfoin varieties Eski and Remont respectively, in their first spring regrowth cycle (Cooper, 1972).

The RGRp (day 30 to day 50) of sainfoin in this experiment, of 0.051 $g.g^{-1}.day^{-1}$, was substantially less than the value of 0.073 for lucerne of similar age, derived from the data of Smoliak *et al.* (1972), and the mean value of 0.089 for 35-63 day old lucerne derived from the data of Tan & Tan (1981).

1.2.2 TOP AND ROOT WEIGHT

Top and root + nodule dry weight also showed exponential growth with the regressions of $\log_e$ [top dry weight] and $\log_e$ [root + nodule dry weight] on time, having $\bar{R}^2$ values of 87.3 and 89.7% respectively

Table 7 Relative growth rates -Experiment 1(a)

Treatment, Time period		RGP_P	RGR_T	$RGR_{R + Nod}$	RGR_R	RGR_{Nod}	$RARN_P$	$RARN_T$	$RARN_{R + Nod}$	RLAGR
		$g.g^{-1}.day^{-1}$								$(cm^2 cm^{-2} day^{-1})$
Trt N_L,										
Days	RGR	0.0510	0.0461	0.0557			0.0601	0.0608	0.0592	0.0442
30-50	S.E.	0.0035	0.0037	0.0039			0.0050	0.0060	0.0044	0.0036
	Resid.d.f.	22	22	22			13	13	13	22
	$\bar{R}^2$ (%)	90.3	87.3	89.7			91.0	87.8	92.8	86.8
Trt N_O,										
Days	RGR	0.0374	0.0343	0.0401	0.0342	0.0723	0.0314	0.0263	0.0397	0.0229
50-80	S.E.	0.0026	0.0025	0.0029	0.0030	0.0069	0.0038	0.0039	0.0041	0.0025
	Resid.d.f.	8	8	8	8	8	8	8	8	8
	$\bar{R}^2$ (%)	95.7	95.5	95.5	93.6	92.3	88.2	83.1	91.2.	90.5
Trt N_i,										
Days	RGR	0.0475	0.0548	0.0384	0.0406	-0.0197	0.0548	0.0571	0.0495	0.0433
50-80	S.E.	0.0032	0.0031	0.0044	0.0047	0.0120	0.0047	0.0049	0.0051	0.0035
	Resid.d.f.	8	8	8	8	8	8	8	8	8
	$\bar{R}^2$ (%)	96.0	97.1	89.3	89.0	15.8	93.9	93.7	91.1	94.3

$\bar{R}^2$ is the percentage variance accounted for from the regressions of the logged variables against time. The standard error of the difference between pairs of relative growth rates can be calculated using their respective standard errors (S.E.) and residual degrees of freedom.

(figs. 4 and 5, table 7). The relative growth rate of root + nodule (RGR_{R+Nod}), of 0.0557 $g.g^{-1}.day^{-1}$, was significantly greater ($P< 0.05$) than the relative growth rate of tops (RGR_T) of 0.0461 $g.g^{-1}.day^{-1}$. The mean top to root + nodule dry weight ratio over this period was 0.98. The higher RGR_{R+Nod} compared to RGR_T along with the top:root + nodule ratio of less than unity highlight the emphasis which was being placed on the 'below ground' development of the plant. The top:root + nodule dry weight ratio observed was greater than that of four week old sainfoin (mean over two years = 0.75) but less than that of six week old sainfoin (mean over two years =1.41) dependent for its N supply on symbiotic fixation (Koter, 1965a). The top : root + nodule dry weight ratio observed over the period day 30 to day 50 was substantially lower than that of 4.30 observed by Smoliak *et al.* (1972). This was perhaps a result of a higher rate of N supplied to the latter plants (the rate of N was not given). Top:root + nodule ratios of other species appeared to be higher than was that of sainfoin in this experiment over the period day 30 to day 50. Sheehy *et al.* (1980a) observed ratios of 1.51 and 1.57 respectively for four and six week old lucerne, and 1.95 for three and four week old soybeans dependent on symbiotic N_2 fixation for their supply of N. Koter (1965a) observed ratios (means over two years) of 2.35 and 2.07 respectively for 4 and 6 week old red clover.

The RGR_T (day 30 to day 50) of sainfoin in this experiment of 0.0461 $g.g^{-1}.day^{-1}$, was substantially less than the values ranging from 0.057 to 0.132 for 25 to 42 day old (approximately) field grown soybeans (Buttery & Buzzell, 1972). The above data may, however, not be strictly comparable with that of this experiment in that the plants may have had access to greater amounts of combined N.

1.2.3 LEAF AREA

Leaf area showed exponential growth over the period day 30 to day 50 with the regression of $\log_e$ [leaf area] on time having an $\bar{R}^2$ value of 86.8% (fig. 7, table 7). The relative leaf area growth rate (RLAGR) of 0.0442 (table 7) was similar to RGR_T, but significantly less than RGR_{R+Nod} ($P< 0.01$). The RLAGR of sainfoin over the period day 30 to day 50 was lower (similarly to RGR_T), than the RLAGR's of soybeans, ranging from 0.047 to 0.141, observed by Buttery & Buzzell (1972). The mean leaf area ratio (LAR = leaf area ÷ total dry weight) over the day 30 to day 50 period, of 103 cm^2/g, was lower than the mean value of 172 cm^2/g, for a similar age period, derived from the data of

Smoliak *et al.* (1972), but very similar to the values of 102 and 94 $cm^2\ g^{-1}$ for field grown Eski and Remont sainfoin respectively (Cooper, 1972).

The LAR of sainfoin (day 30 to day 50) was lower than the value of 149 $cm^2.g^{-1}$ reported by Smoliak *et al.* (1972) and the mean value of 120 cm^2/g derived from the data of Tan & Tan (1981) for lucerne. The higher LAR of sainfoin relative to lucerne reported by Smoliak *et al.* (1972) is not consistent with other reports (e.g. Sheehy & Harding, pers.comm.) which indicate that the specific leaf area and leaf area index of sainfoin is substantially lower than that of lucerne.

1.2.4 NET ASSIMILATION RATE

The net assimilation rate (NAR) of sainfoin over the period day 30 to day 50 of 0.00050 $g.cm^{-2}.day^{-1}$ was greater than the mean value for sainfoin, of 0.00028 $g.cm^{-2}.day^{-1}$, derived from the data of Smoliak *et al.* (1972), but very similar to the values of 0.00053 and 0.00050 for the sainfoin varieties Eski and Remont respectively, grown in the field (Cooper, 1972). It may be that the low value of Smoliak *et al.* (1972) is a result of their very high LAR values (discussed in section 1.2.3) compared to those in this experiment, and those of Cooper (1972), as relative growth rates were similar in all three cases.

The NAR of sainfoin over the day 30 to day 50 period was similar to the value for lucerne of 0.00051 $g.cm^{-2}.day^{-1}$ derived from the data of Smoliak *et al.* (1972), indicating that in terms of leaf area, sainfoin in this experiment had a similar ability to produce dry matter to that of lucerne. Lucerne, according to the data of Smoliak *et al.* (1972) had a higher LAR than did sainfoin in this experiment (section 1.2.3). This combined with a similar NAR would explain the higher relative growth rate of lucerne discussed in section 1.2.1. However Tan & Tan (1981) reported a higher mean NAR of 0.00076 $g.cm^{-2}.day^{-1}$ for lucerne. For soybeans, Kaplan & Koller (1977) observed a mean NAR of 0.00107 $g.cm^{-2}.day^{-1}$ with potted plants over the age period 22 to 29 days, and Buttery and Buzzell (1972) observed mean values of NAR for slightly older (approximately 25 to 42 day old) field grown soybeans ranging from 0.00048 to 0.00100 $g.cm^{-2}.day^{-1}$. Thus soybeans and perhaps lucerne have a relatively greater ability, than the sainfoin in this experiment, to produce dry matter from a given leaf area. This combined with their slightly greater LAR values (section 1.2.3) would account for their higher relative growth rates (sections 1.2.1 and 1.2.2).

1.2.5 PLANT NITROGEN

Over the period day 30 to day 50, the increase in total N appeared to be exponential, with the plot of $\log_e$ [total plant N] against time appearing to be linear and the regression of $\log_e$ [total plant N] on time having an $\bar{R}^2$ value of 91.0% (table 7). The relative accumulation rate of total plant N,

($RARN_p$) of 0.0601 $g.g^{-1}.day^{-1}$ was similar to $RGR_{R + Nod}$, but was significantly higher than the total plant relative growth rate, RGR_p ($P < 0.05$) and the relative growth rate of tops, RGR_T ($P < 0.01$).

The similarity between $RARN_p$ and $RGR_{R + nod}$ means that total plant N and root + nodule dry weight were increasing in proportion to one another, suggesting a possible link between root + nodule growth and N assimilation.

The fact that $RARN_p$ was higher than RGR_p suggests that the proportion of total plant N was increasing, which was the case (fig.11).

The mean herbage N concentration over the day 30 to day 50 period of 3.0%, was comparable to mean values of 3.09% and 2.87%, obtained by Baker *et al.* (1952), and Whitehead & Jones (1969) respectively, indicating that the plants were not suffering from severe N deficiency over that of this period.

As for RGR_p, $RARN_p$ of sainfoin appeared lower than for lucerne, with a mean $RARN_p$ for 35-63 day old lucerne of 0.085 reported by Tan & Tan (1981).

1.2.6 NODULATION

1.2.6.1 General

On day 30 (14 days after inoculation), small swellings which were thought to be future nodules were observed on 7 of the 20 plants harvested. On day 34 (18 days after inoculation) fully formed nodules were first observed, some of which appeared pink when sectioned, indicating the presence of leghaemoglobin and N_2 fixing activity. This is in accord with other observations (Greenwood, pers.comm.) that sainfoin is relatively slow to nodulate. Alternatively, there could be a delay before sainfoin becomes susceptible to infection. By day 45, 90% of plants were nodulated, and most nodules contained leghaemoglobin (fig.12). The position of nodules was recorded up to day 38. It was found that nodules occurred almost exclusively on the lateral roots, as described by Spedding & Diekmahns (1972), and tended to be more abundant on the upper laterals. The region of greatest abundance of nodules tended to coincide, in a general way, with the region of greatest abundance of root hairs, which is consistent with the belief that rhizobial infection takes place via root hairs in a large number of legume species (Dart, 1974). Nodules of sainfoin have been found to contain infection threads (Dangeard, 1926)

The presence of root hairs was observed on day 10, so presumably it was not a lack of infection sites that caused the delay in nodulation. The morphology of the nodules was similar to that described by Wittmann (1968), Spedding & Diekmahns (1972) and Schreven (1972), with nodules possessing a subterminal meristem, being branched, and giving the appearance of being formed in clusters (plate 9). The lobed, or branched nodules generally appeared to arise from a single point on the root, rather than

being several, originally separate nodules, which had merged together.

1.2.6.2 Number and weight of nodules

Total nodule number appeared to increase approximately linearly with time over the period day 30 to day 50 (fig.12). The number of pink (and presumably leghaemoglobin containing) nodules increased in a similar manner (fig.12).

Nodule weight was recorded separately from root weight starting on day 45. At day 50, nodule dry weight made up approximately 4.2% of total dry weight. This compares with proportions of 3.1 and 2.8% respectively for 28 and 42 day old lucerne and 6.2 and 6.5% for 21 and 28 day old soybeans (Sheehy *et al.* 1980a), 1.9 and 9.2% for 42 day old lucerne and cicer milkvetch respectively (Major *et al.* 1979), 3.7% for 28 day old peas (Phillips *et al.* 1976), all fed zero N nutrient solution; and 9% and 2% respectively for 35 day old soybeans fed 0 mM and 5 mM N (Cassman *et al.* 1980). Thus nodule dry weight of sainfoin fed 2.5 mM N, in this experiment at day 50, expressed as a proportion of total plant dry weight appeared to be greater than that of lucerne, similar to that of peas and less than that of soybeans and cicer milkvetch of approximately similar age.

1.3 EFFECT OF THE N_0 AND N_1 TREATMENTS

1.3.1 PLANT GROWTH

1.3.1.1 Total dry weight

Total dry weight increased exponentially with time in treatments N_0 and N_1 over the period day 50 to day 80 (fig.3). Plots of $\log_e$ [total dry weight] against time were linear, and the regressions of $\log_e$ [total dry weight] on time had $\bar{R}^2$ values of 95.7 and 96.0% for treatments N_0 and N_1 respectively (table 7).

The RGR_p in the N_1 treatment (day 50 to day 80), of 0.0475 $g.g^{-1}.day^{-1}$ (table 7) was similar to that prior to day 50 (0.0510 $g.g^{-1}.day^{-1}$) and was slightly higher than the RGR_p of 0.0439, observed in sainfoin over a similar age period by Smoliak *et al.* (1972). In the N_0 treatment RGR_p dropped significantly ($P < 0.001$) (compared to before day 50) to 0.0374 $g.g^{-1}.day^{-1}$ (table 7). As a result, total plant dry weight in the N_0 treatment fell significantly ($P < 0.05$) below that in the N_1 treatment by day 66, 16 days after the introduction of the N_0 and N_1 treatments (fig.3). Thus plants dependent on symbiotic fixation as their source of N were unable to maintain the level of growth of plants supplied with abundant combined N.

Tan and Tan (1981) found 35-63 day old lucerne, solely dependent on symbiotic N_2 fixation, to have a mean RGR_p of 0.089 $g.g^{-1}.day^{-1}$, indicating its superior growth potential.

1.3.1.2 Top and root dry weight

Top dry weight showed exponential growth with the regressions of $\log_e$ [top dry weight] on time having $\bar{R}^2$ values of 95.5 and 97.1% for treatments N_0 and N_1 respectively (table 7). After day 50 RGR_T in the N_1 treatment increased significantly ($P < 0.05$) to 0.0548 $g.g^{-1}.day^{-1}$, and that in the N_0 treatment decreased significantly ($P < 0.01$) to 0.0343 $g.g^{-1}.day^{-1}$, compared to treatment N_1 (0.0461 $g.g^{-1}day^{-1}$). Significant differences in top weight between the N_0 and N_1 treatments had developed by day 59, 9 days after introduction of the treatments (fig.4).

Root and root + nodule dry weight showed exponential growth after day 50, with regressions of $\log_e$ [root + nodule dry weight] on time having $\bar{R}^2$ values of 95.5 and 89.3% for treatments N_0 and N_1 respectively (table 7). $RGR_{R + Nod}$, in both the N_0 and N_1 treatments decreased significantly ($P < 0.001$) from 0.0557 $g.g^{-1}.day^{-1}$ before day 50 to 0.0401 and 0.0384 $g.g^{-1}.day^{-1}$ respectively (table 7). In terms of $RGR_{R + Nod}$, the two treatments N_0 and N_1 were not significantly different. In terms of RGR_R the N_1 treatment appeared higher than N_0 but the difference was not significant (table 7). By day 66 root weight in the N_0 treatment was significantly lower ($P < 0.05$) than in the N_1 treatment, but when nodule weight was

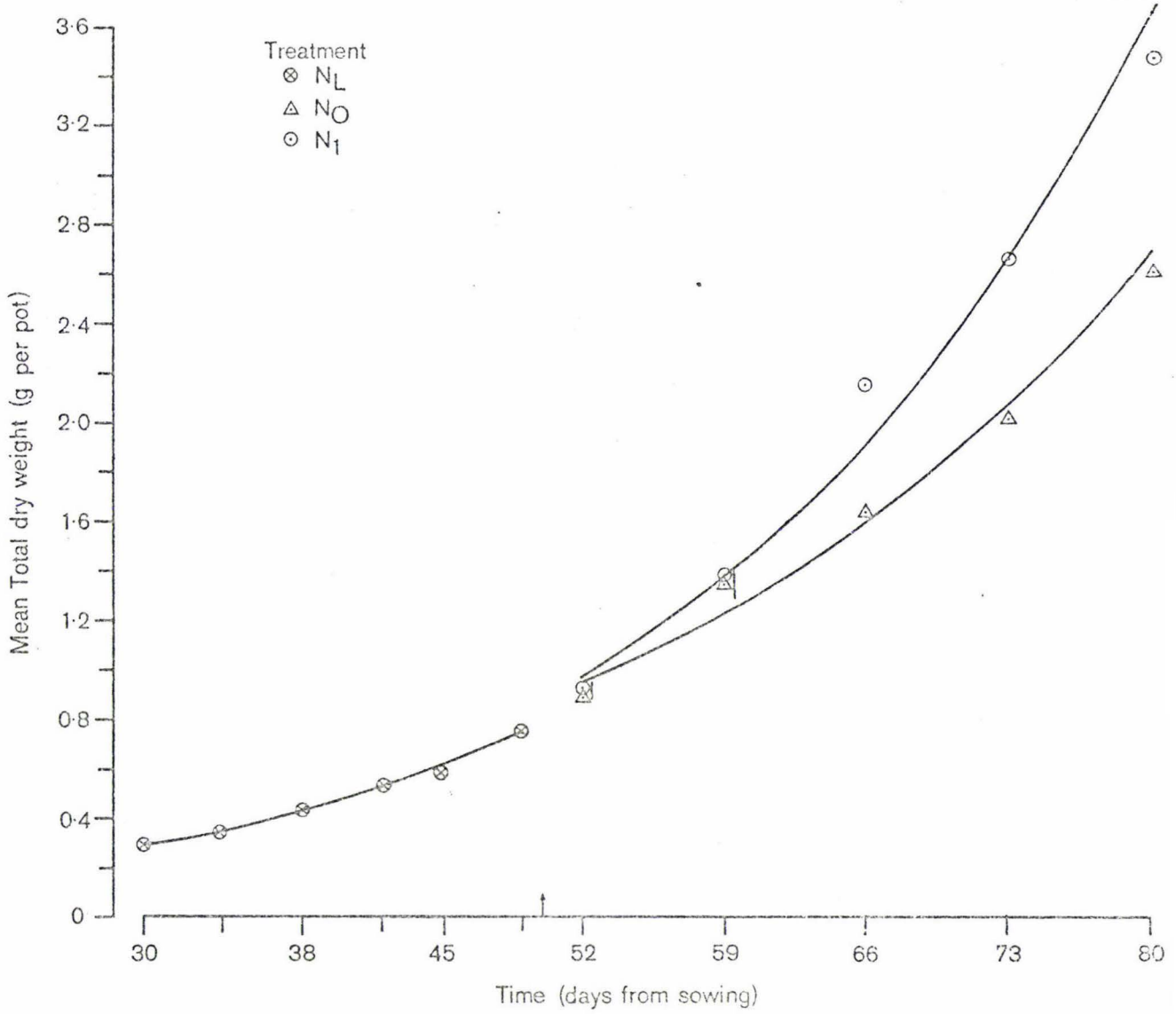


Fig 3 Total plant weight - expt.1(a). The lines drawn are relative growth rate curves. Up to day 50 a low rate of N (N_L) was applied. From day 50, zero (N_O) and high (N_1) rates of N were applied. Up to day 50 each point is the mean of four pots and after day 50 each point is the mean of two pots. Points joined by vertical bars do not differ at the 5% level of significance according to standard errors of differences.

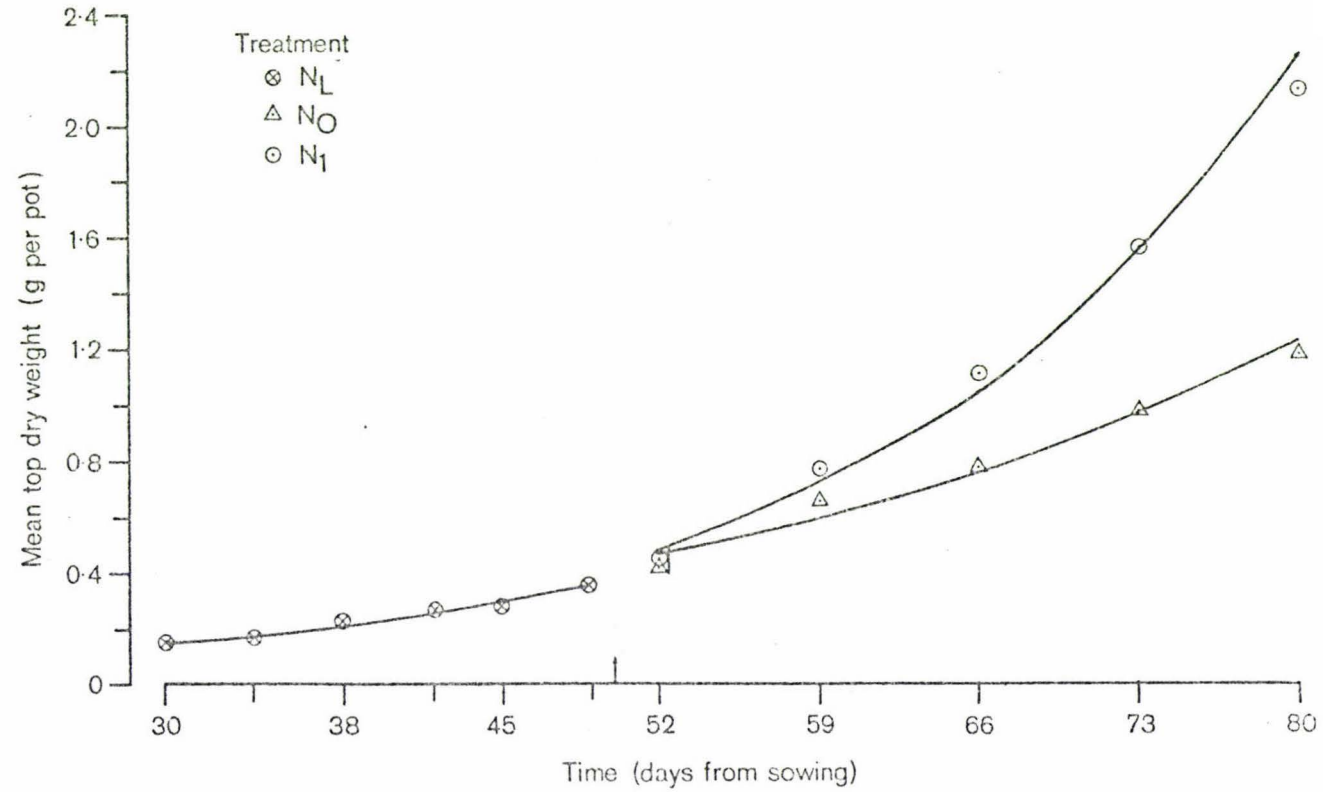


Fig 4 Top weight - expt.1(a) (details as for fig 3).

included the difference tended to disappear (fig.5).

In the N_1 treatment, RGR_T was significantly higher ($P < 0.01$ and 0.001 respectively) than RGR_R or $RGR_{R + Nod}$ (table 7). In contrast, in the N_0 treatment RGR_T was identical to RGR_R and significantly less ($P < 0.05$) than $RGR_{R + Nod}$.

Top:root + nodule dry weight ratio in the N_0 treatment (mean = 0.91) decreased slightly, and that in the N_1 treatment (mean = 1.26) increased markedly (both significant at $P < 0.05$) relative to the mean value of 0.98 in the N_L treatment, before day 50. Top:root + nodule ratio was significantly lower in treatment N_0 than in treatment N_1 from day 59, 9 days after introduction of the N_0 and N_1 treatments (fig.6).

Thus, in addition to influencing the rates of total dry matter production (discussed in section 1.3.1.1) the mode of N nutrition has been found to exert a strong influence on the pattern of dry matter distribution in sainfoin. It was found that much greater emphasis was placed on 'below ground' development, relative to over all growth, in plants dependent on symbiotic N_2 fixation, compared to plants supplied with abundant combined N. Koter (1965a & b) also observed a change in dry matter distribution of sainfoin dependent on the mode of N nutrition, with plants provided with combined N, as in this experiment, having a higher top:root + nodule dry weight ratio than plants dependent for their supply of N on symbiotic N_2 fixation. The same effect was not observed in red clover (Koter, 1965a). Cassman *et al.* (1980) suggest that two functional equilibria operate in the N_2 fixing plant, namely the partitioning of dry matter between the underground portion of the plant and the top, and between the root and the nodules. Cassman *et al.* (1980) found that at intermediate levels of P, the mode of N nutrition of soybeans had little effect on the top:root + nodule ratio. At high levels of P, when total yields were substantially higher in soybeans fed combined N, than in soybeans dependent on symbiotic N_2 fixation, the top:root + nodule ratio was only slightly higher in the soybeans fed combined N than in those dependent for their N supply on symbiotic fixation (Cassman *et al.* 1980). The data of Summerfield *et al.* (1977) showed that the top:root + nodule dry weight ratio in cowpea was smaller in nodulated plants provided with 0, 30 or 60 ppm of combined N, than in non-nodulated plants supplied with an adequate level (120 ppm) of combined N. Atkins *et al.* (1980), also working with cowpea, found that a greater proportion of net photosynthate was utilised for growth and respiration by below ground organs in nodulated plants (29%) than in plants fed 10 mM (21%) or 20 mM

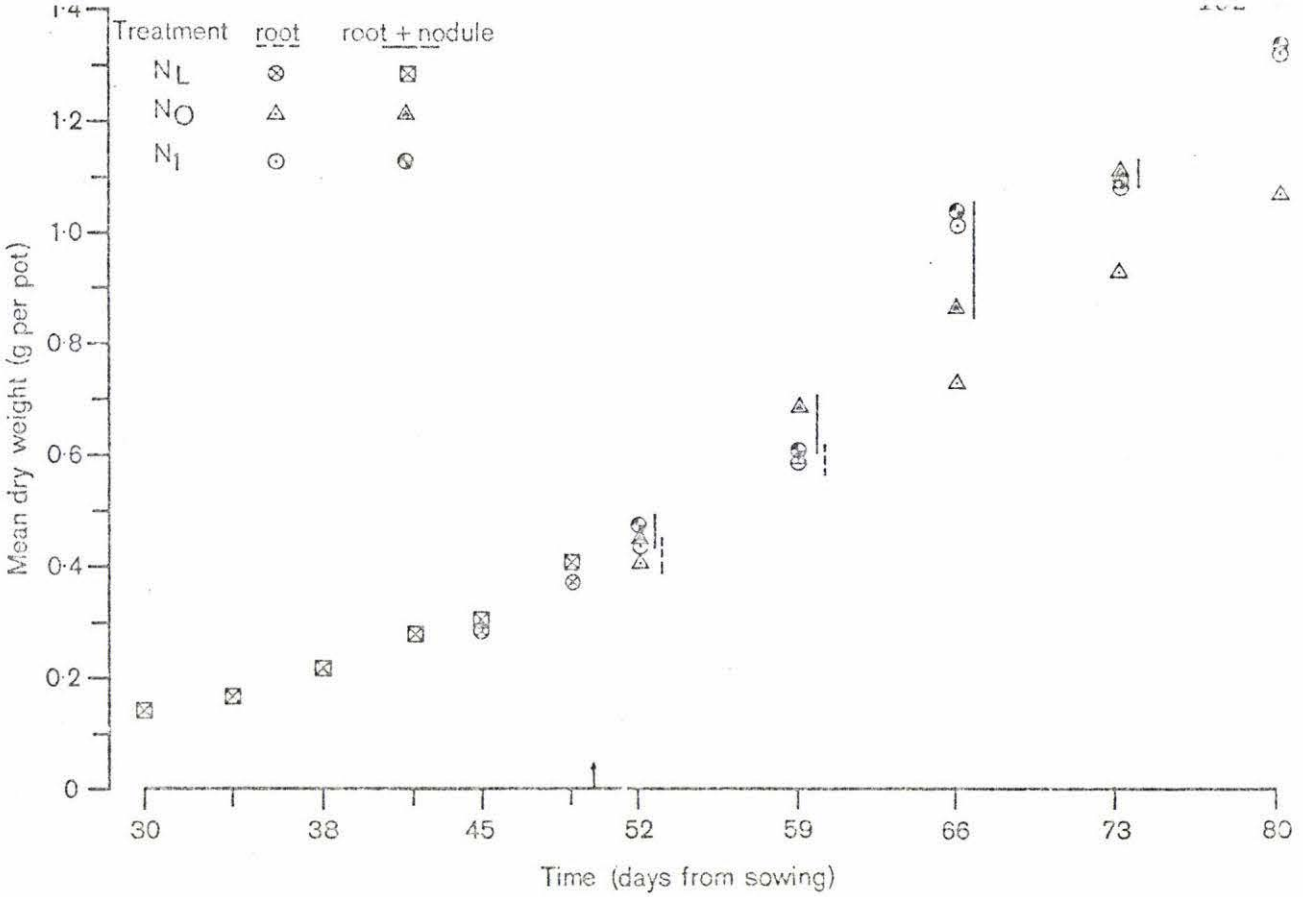


Fig 5 Root and root + nodule weight - expt.1(a). Up to day 50 a low rate of N (N_L) was applied. From day 50, zero (N_O) and high (N_1) rates of N were applied. Up to day 50 each point is the mean of four pots and after day 50 each point is the mean of two pots. Root and root + nodule weights joined by dotted and solid vertical bars respectively, do not differ at the 5% level of significance.

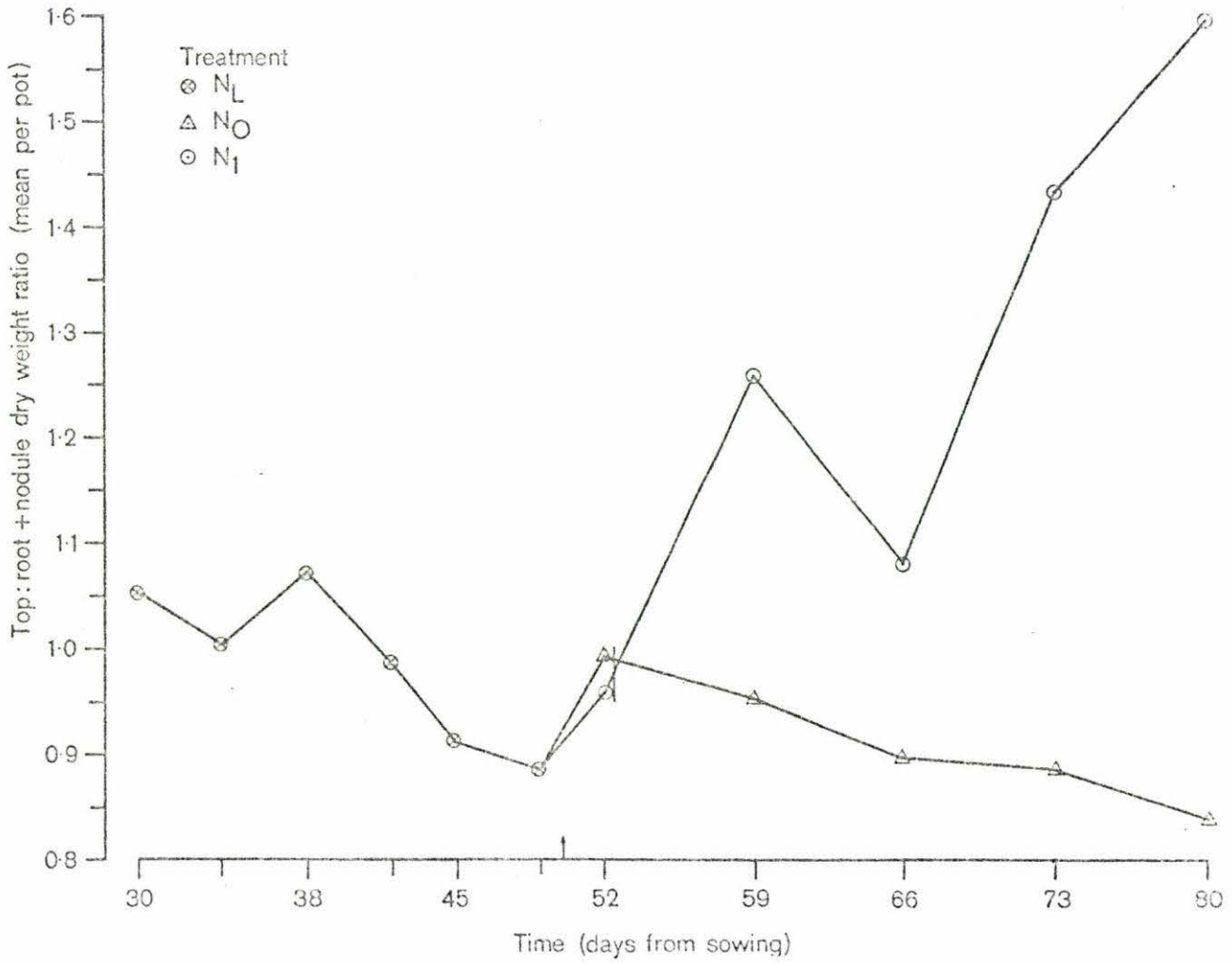


Fig 6 Top:root + nodule ratio - expt.1(a). Up to day 50 a low rate of N (N_L) was applied. From day 50, zero (N_O) and high (N_1) rates of N were applied. Up to day 50, each point is the mean of four pots and after day 50 each point is the mean of two pots. Points joined by vertical bars do not differ at the 5% level of significance according to standard errors of differences.

nitrate (18%). Barta (1979) observed a difference in photosynthate partitioning in birdsfoot trefoil, which was dependent on the mode of N nutrition. As in this experiment, the top:root + nodule ratio was observed to be higher in plants supplied with combined N than in plants dependent for their N supply on symbiotic fixation. The difference in top:root + nodule weight ratio between the N_0 and N_1 treatments was similar to the effect observed by Turner (1922) and Brouwer (1962), in which plants deficient in N accumulated relatively more dry matter in their roots than plants which were adequately supplied.

1.3.1.3 Leaf area

Leaf area showed exponential growth in treatments N_0 and N_1, with regressions of $\log_e$ [leaf area] on time giving $\bar{R}^2$ values of 90.5 and 94.3% respectively (table 7).

In the N_1 treatment the relative leaf area growth rate (RLAGR), of 0.0433 $cm^2.cm^{-2}.day^{-1}$, was virtually unchanged both in absolute terms, and relative to RGR_P (table 7), compared to that in the N_L treatment prior to day 50. In the N_1 treatment RLAGR was similar to RGR_R and RGR_R + Nod but significantly less ($P < 0.01$) than RGR_T. In the N_0 treatment, the RLAGR, of 0.0229 $cm^2.cm^{-2}.day^{-1}$, was significantly lower ($P < 0.001$) than in the N_L treatment, prior to day 50 (0.044)$cm^2.cm^{-2}.day^{-1}$) and was significantly lower ($P < 0.001$) than the relative growth rate of all components of plant dry weight measured, being lower in comparison to RGR_P than before day 50 (table 7). Leaf area in the N_0 treatment had become significantly lower than that in the N_1 treatment by day 66, 16 days after the introduction of the N_0 and N_1 treatments (fig. 7). Lower leaf number was a contributing factor to the lower leaf area in the N_0 treatment (figs. 7 & 8).

Leaf area ratio (LAR) declined with time in both the N_0 and N_1 treatments (fig.9). However, mean LAR was significantly higher ($P < 0.05$) in the N_1 treatment (mean = 109 cm^2/g) and lower ($P < 0.05$) in the N_0 treatment (mean = 93 cm^2/g) than in the N_L treatment (mean = 103 cm^2/g). LAR was significantly lower in the N_0 relative to the N_1 treatment from about day 59 (fig. 9). LAR values in the N_1 treatment were comparable to those for sainfoin reported by Cooper (1972) but less than those reported for sainfoin of similar age by Smoliak *et al.* (1972). LAR values in the N_1 treatment were comparable to values reported for lucerne of similar age (Smoliak *et al.* 1972), but less than the mean of values reported by Tan & Tan (1981) for lucerne cultivars supplied with N-free nutrient solution.

It appears that when sainfoin alters its dry matter distribution in accordance with its mode of N nutrition, this has an influence on leaf area, which in turn will determine the ability of the plant to assimilate carbon,

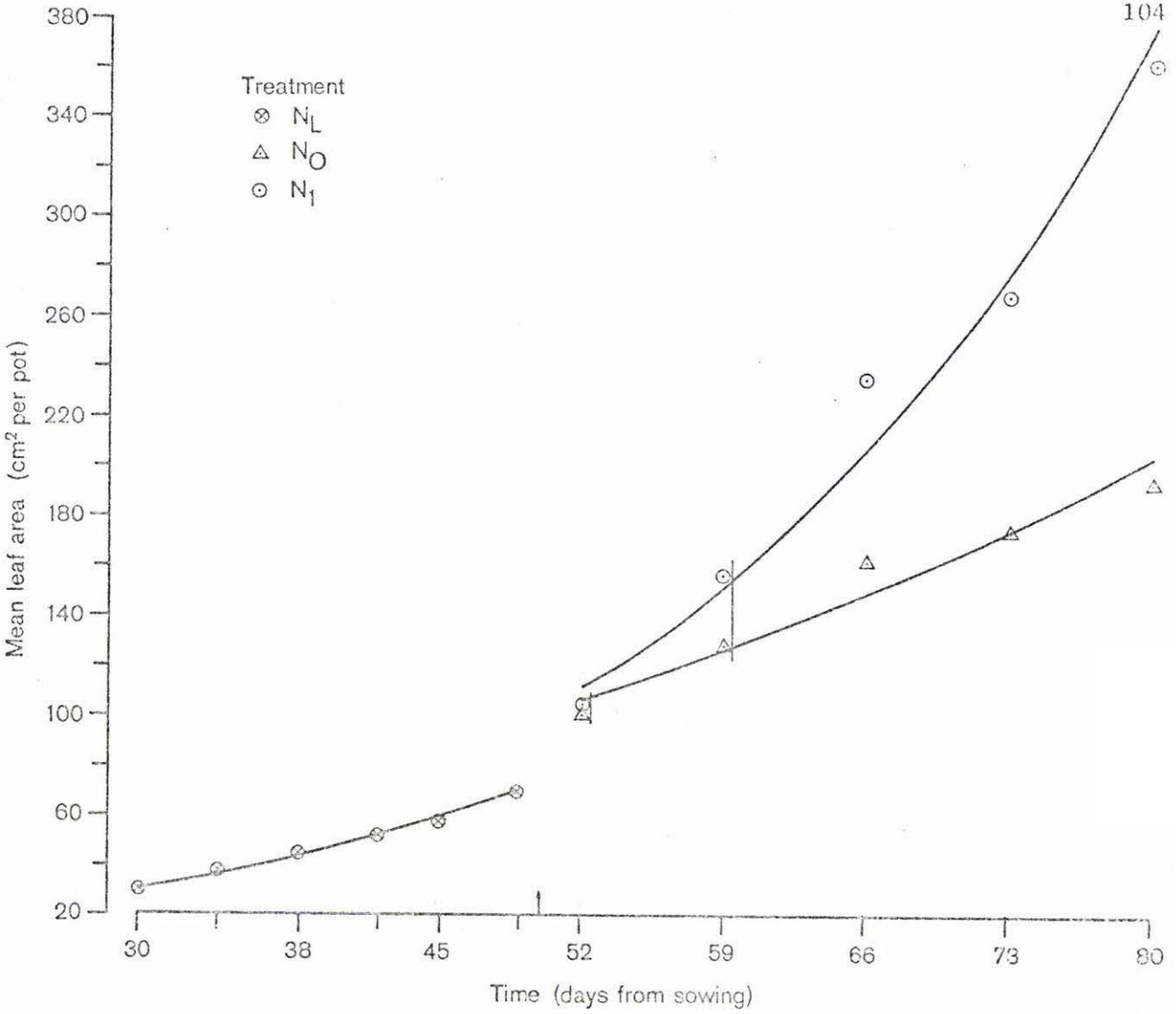


Fig 7 Leaf area - expt.1(a) (details as for fig 3).

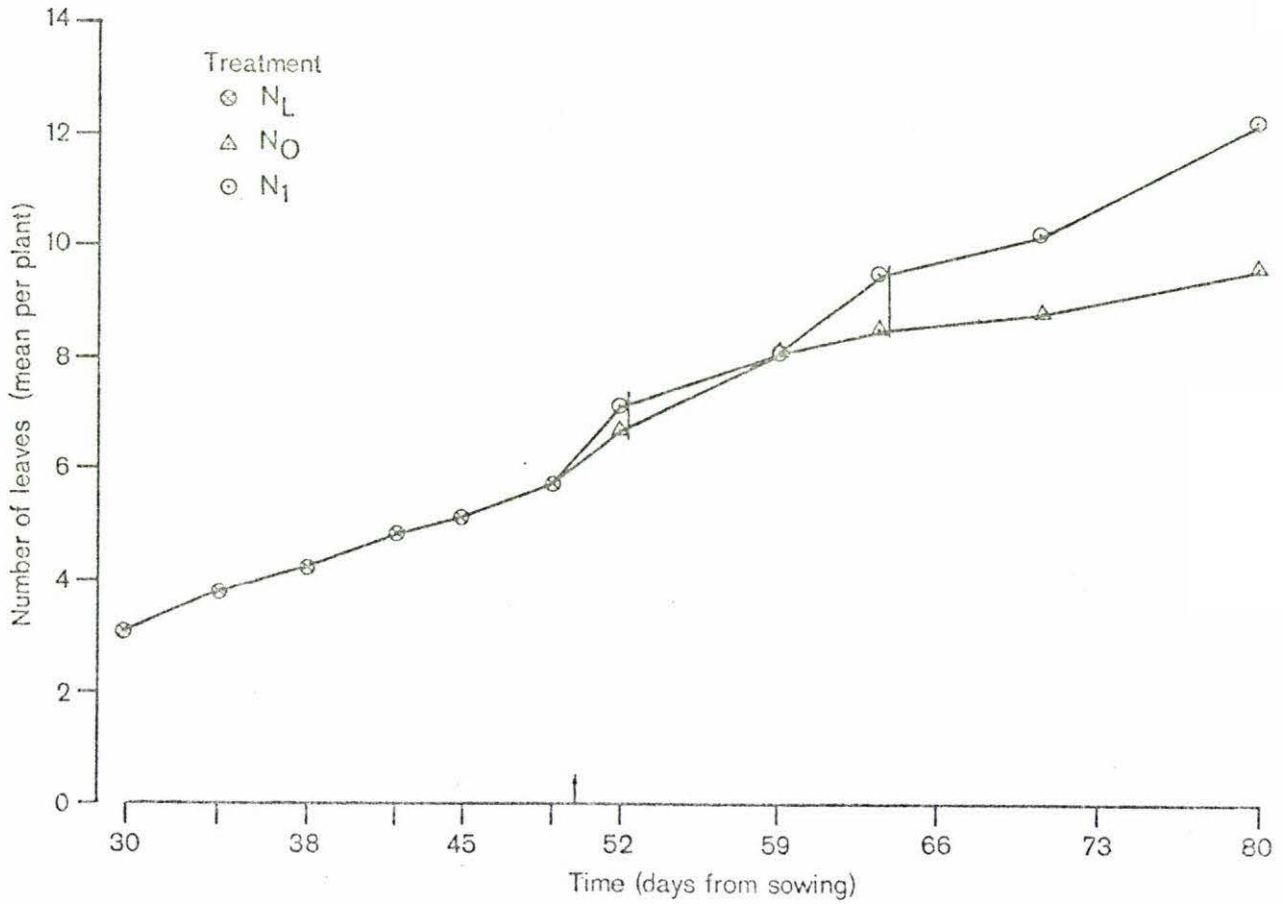


Fig 8 Number of leaves - expt.1(a) (details as for fig 6).

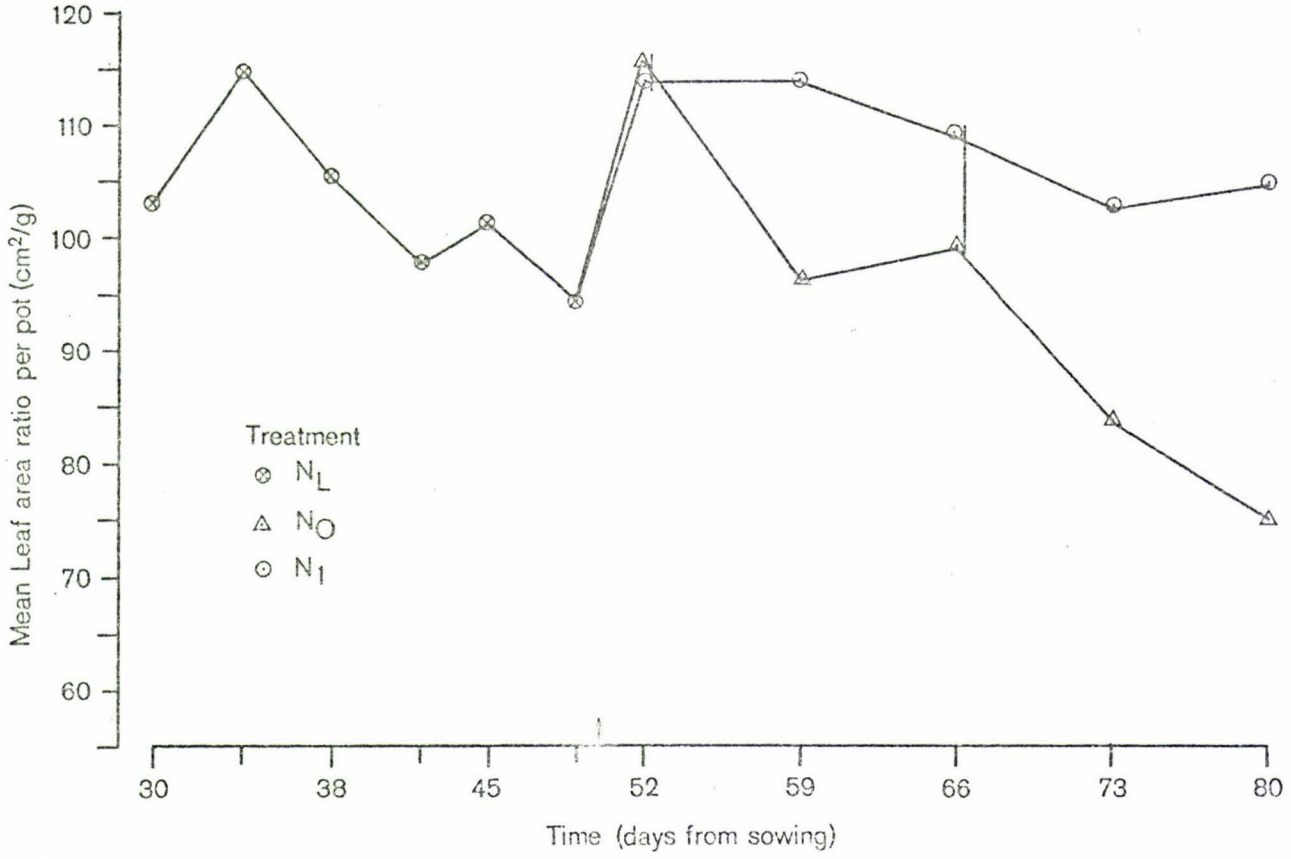


Fig 9 Leaf area ratio - expt 1(a) (details as for fig 6).

and hence grow. Sheehy *et al.* (1980b) have found leaf area to be highly correlated with whole plant carbon exchange rate in lucerne, with r = 0.94. Kaplan & Koller (1977) have found soybean growth rate to be positively correlated with leaf area growth rate, with r = 0.85. Thus a close relationship between leaf area and plant growth is indicated.

Sainfoin plants dependent on symbiotic fixation for their N supply are found to have a lower top:root nodule weight ratio, LAR and leaf area than plants provided with combined N. Thus they can be expected to have a reduced capacity to assimilate carbon, and therefore to grow more slowly than plants provided with adequate combined N.

1.3.1.4 Net assimilation rate

Net assimilation rate (NAR) appeared to decrease in both the N_1 and N_0 treatments compared with the N_L treatment (table 8).

Table 8 Net assimilation rates - Experiment 1(a)

	Treatment		
	N_L days 30-50	N_0 days 50-80	N_1 days 50-80
N.A.R. ($g.cm^{-2}.day^{-1}$)	0.00050b*	0.00039a	0.00044ab

* Figures followed by different letters differ at the 5% level of significance

It appears that a greater proportion of the acquired photosynthate (where photosynthate acquired is assumed to be proportional to leaf area, see section 1.3.1.3) was being used to produce additional dry matter in the N_1 treatment, than in the N_0 treatment, where presumably energy was being consumed in symbiotic N_2 fixation (although the difference in NAR values between treatments N_1 and N_0 was not statistically significant).

Tan and Tan (1981) found a mean NAR for 35-63 day old lucerne dependent on symbiotic N_2 fixation of 0.00076 $g.cm^{-2}.day^{-1}$, indicating that lucerne is more photosynthetically efficient on a unit leaf area basis than sainfoin.

1.3.2 PLANT NITROGEN

Over the period day 50 to day 80, the plot of $\log_e$ [total N] against time for the N_0 treatment appeared linear and the regression of $\log_e$ [total N] on time had an $\bar{R}^2$ value of 88.2%. The equivalent plot for the N_1

treatment, however, appeared slightly curved, but the regression of $\log_e$ [total N] on time, still had an $\bar{R}^2$ value of 93.9% (table 7). The increase in total N appeared approximately linear in both cases (fig.10), in contrast to the increase in total dry weight which appeared to be exponential (fig. 3, table 7). This effect was probably caused, in the N_o treatment by a decreasing percentage N from day 50, and in the N_1 treatment by a sudden increase in percentage N immediately after day 50, which then appeared to level off (fig.11).

Relative accumulation rates of N were still derived from the regression of $\log_e$ [total N] against time, even though, in treatment N_1 there was a suspicion that the log plot was not linear, as it can still be valid to fit the linear model in order to gain a single value of relative growth rate for comparative purposes (Hunt, 1978).

The relative accumulation rate of total plant N ($RARN_P$) in the N_1 treatment of 0.0548 $g.g^{-1}.day^{-1}$ was not significantly different from the value of 0.0601 $g^{-1}.day^{-1}$ in the N_L treatment (before day 50) and was identical to RGR_T. As before day 50, $RARN_P$ was higher than RGR_P (although non significantly so) and was significantly higher ($P < 0.01$) than $RGR_{R + Nod}$ (table 7). Thus, total plant N was increasing at a faster relative rate than total plant dry weight, and this is confirmed by the fact that percentage N is increasing with time (fig. 11). $RARN_T$ appeared higher than $RARN_{R + Nod}$, (table 7) probably as a result of the high RGR_T relative to $RGR_{R + Nod}$ in this treatment, but the difference was not statistically significant.

The introduction of the N_o treatment on day 50 resulted in a significant decrease ($P < 0.001$) in $RARN_P$ from 0.0601 $g.g^{-1}.day^{-1}$ to 0.0314 $g.g^{-1}.day^{-1}$. $RARN_P$ was lower (non-significantly) than RGR_P, in contrast to the situation before day 50, where $RARN_P$ was significantly higher than RGR_P (section 1.2.5, table 7). This implies that N as a proportion of total dry weight is decreasing, and this is supported by the decreasing percentage N in the N_o treatment (fig.11). $RARN_T$ was significantly lower ($P < 0.05$) than RGR_T, but $RARN_{R + Nod}$ was the same as $RGR_{R + Nod}$. As was the pattern for RGR_T and $RGR_{R + Nod}$, $RARN_T$ was significantly lower ($P < 0.01$) than $RARN_{R + Nod}$.

Tan & Tan (1981) found 35 to 63 day old lucerne, dependent on symbiotic N_2 fixation, to have a mean $RARN_P$ of 0.085 $g.g^{-1}.day^{-1}$, indicating a greater capacity for symbiotic N_2 fixation of this species.

Total plant N, and total plant percentage N were significantly lower in the N_o than in the N_1 treatment at day 59, 9 days after introduction of the N_o and N_1 treatments (figs 10 & 11).

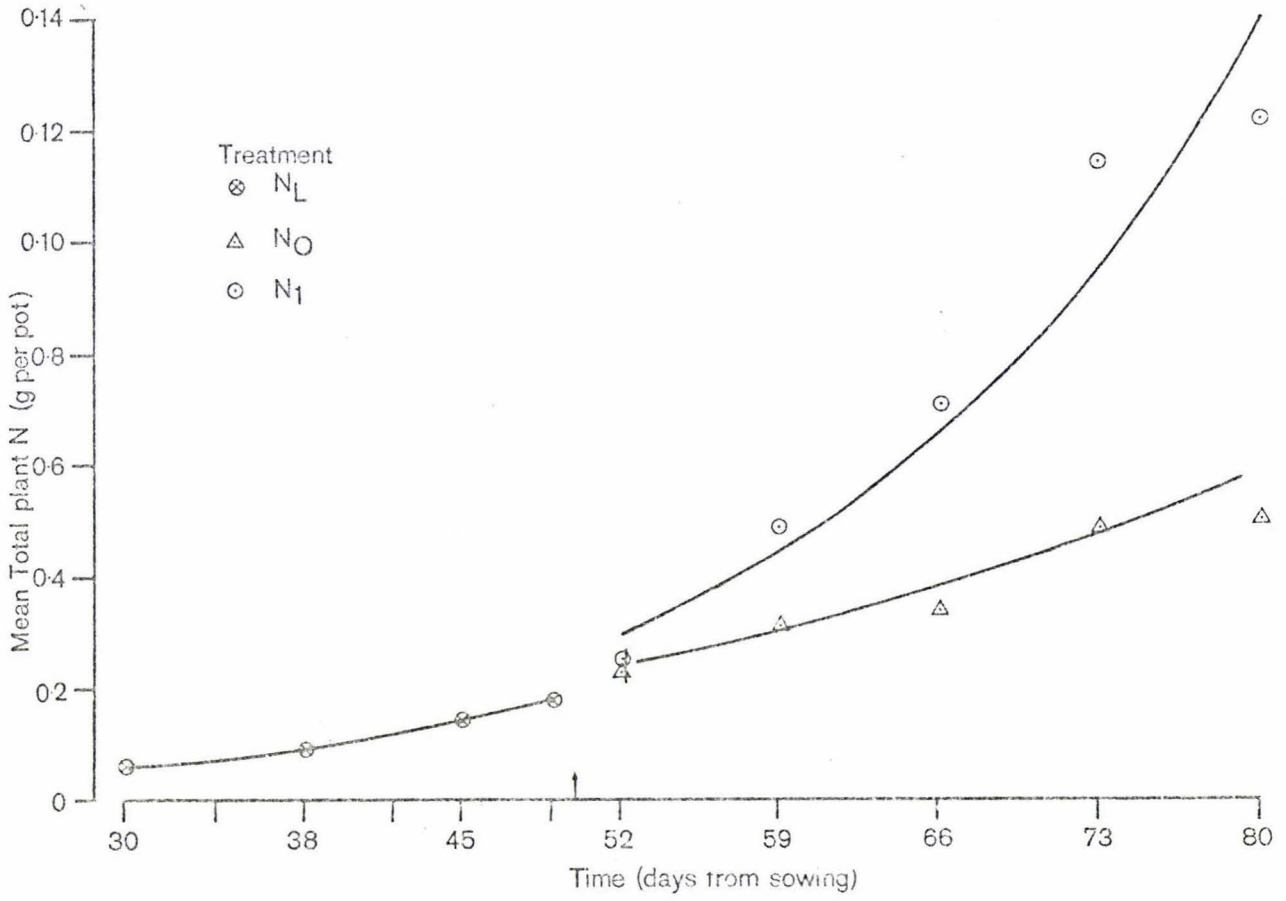


Fig 10 Total plant N - expt.1(a) (details as for fig 3).

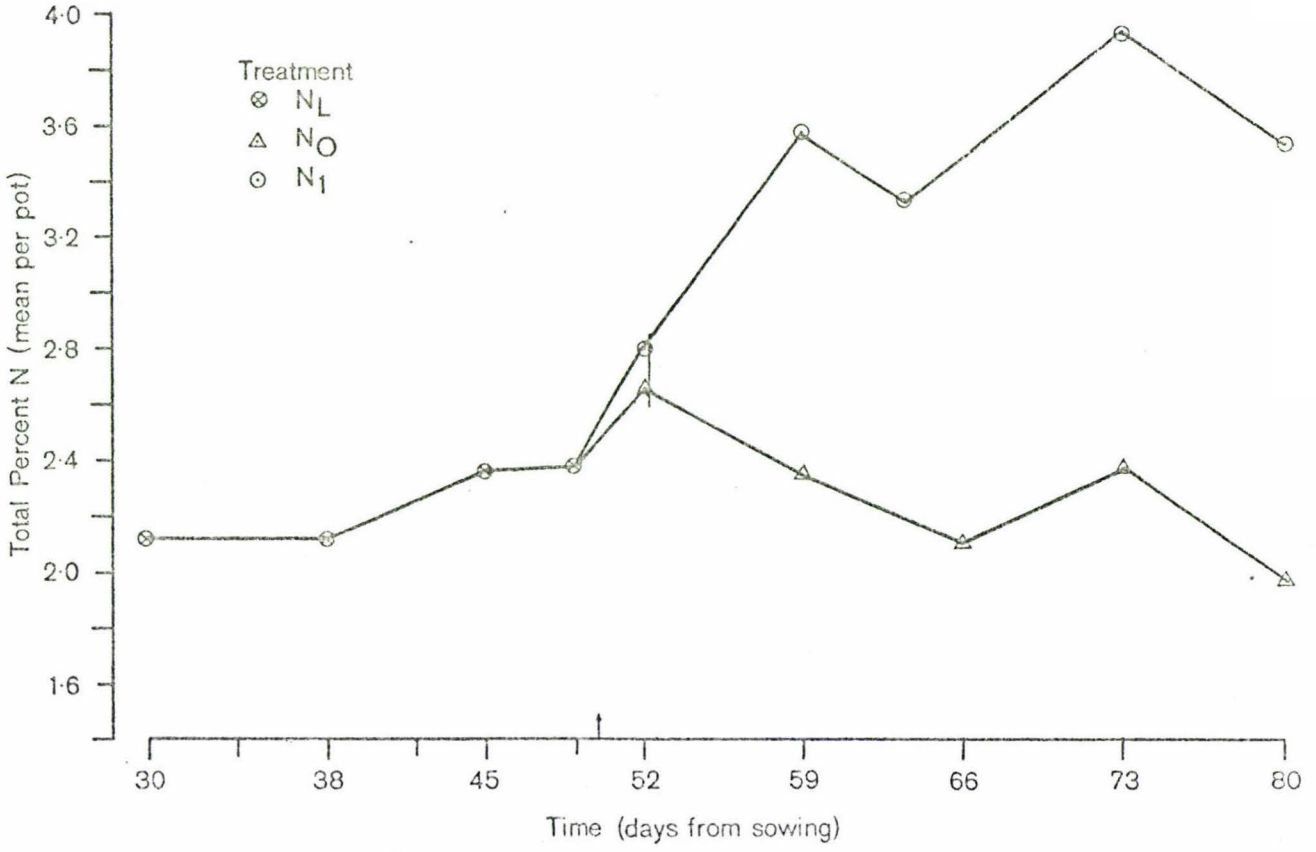


Fig 11 Total plant percent N - expt 1(a) (details as for fig 6).

$RARN_P$ in the N_0 treatment was lower (albeit non-significantly) than RGR_P, and total plant N in the N_0 treatment became significantly lower than that in the N_1 treatment earlier (day 59) than total plant dry weight in the N_0 treatment became significantly lower than that in the N_1 treatment (day 66) (figs.3 & 10). This suggests that the decline in RGR_P of the N_0 treatment may have been the result of a shortage of N. Gibson (1966) working with sub-clover, and Minchin & Pate (1973) working with peas, found that in the early stages of nodule development, energy and N utilisation by developing nodules may retard plant development.

1.3.3 NODULATION

After day 50, nodule number continued to increase in the N_0 treatment but tended to decrease in the N_1 treatment (fig.12). Nodule number tended to be very variable. This appeared to be caused mainly by variability in nodule size as nodule weight was much less variable (figs. 12 & 13). The number of pink, and presumably actively N_2 fixing nodules in the N_0 treatment was very similar to the total nodule number, whereas the number of pink nodules in the N_1 treatment had fallen to zero by day 66. Nodule number had become significantly less in the N_1 than in the N_0 treatment by day 80, but the number of pink nodules in the N_1 treatment had become significantly less than in the N_0 treatment by day 59, 9 days after the application of the N_0 and N_1 treatments (fig. 12).

Nodule dry weight was recorded separately from root weight from day 45. After introduction of the N_0 and N_1 treatments on day 50, nodule dry weight increased rapidly in the N_0 treatment, but slowly decreased in the N_1 treatment (fig.13), as nodules senesced. By day 59, 9 days after the introduction of the N_0 and N_1 treatments nodule dry weight in the N_1 treatment was significantly less than that in the N_0 treatment (fig. 13).

Nodule dry weight in the N_0 treatment was found to increase exponentially, with the regression of $\log_e$ [nodule dry weight] on time having an $\bar{R}^2$ value of 92.3% (table 7). Relative nodule growth rate (RGR_{Nod}) in the N_0 treatment was 0.0723 $g.g.^{-1}day^{-1}$, significantly higher ($P < 0.001$) than the RGR_P of 0.0374 $g.g^{-1}.day^{-1}$ and the RGRs of all other plant components (table 7). In the N_0 treatment, nodule dry weight as a proportion of total plant dry weight increased from 4.3% at day 50 to 12.4% at day 80 (estimated from relative growth rate curves).

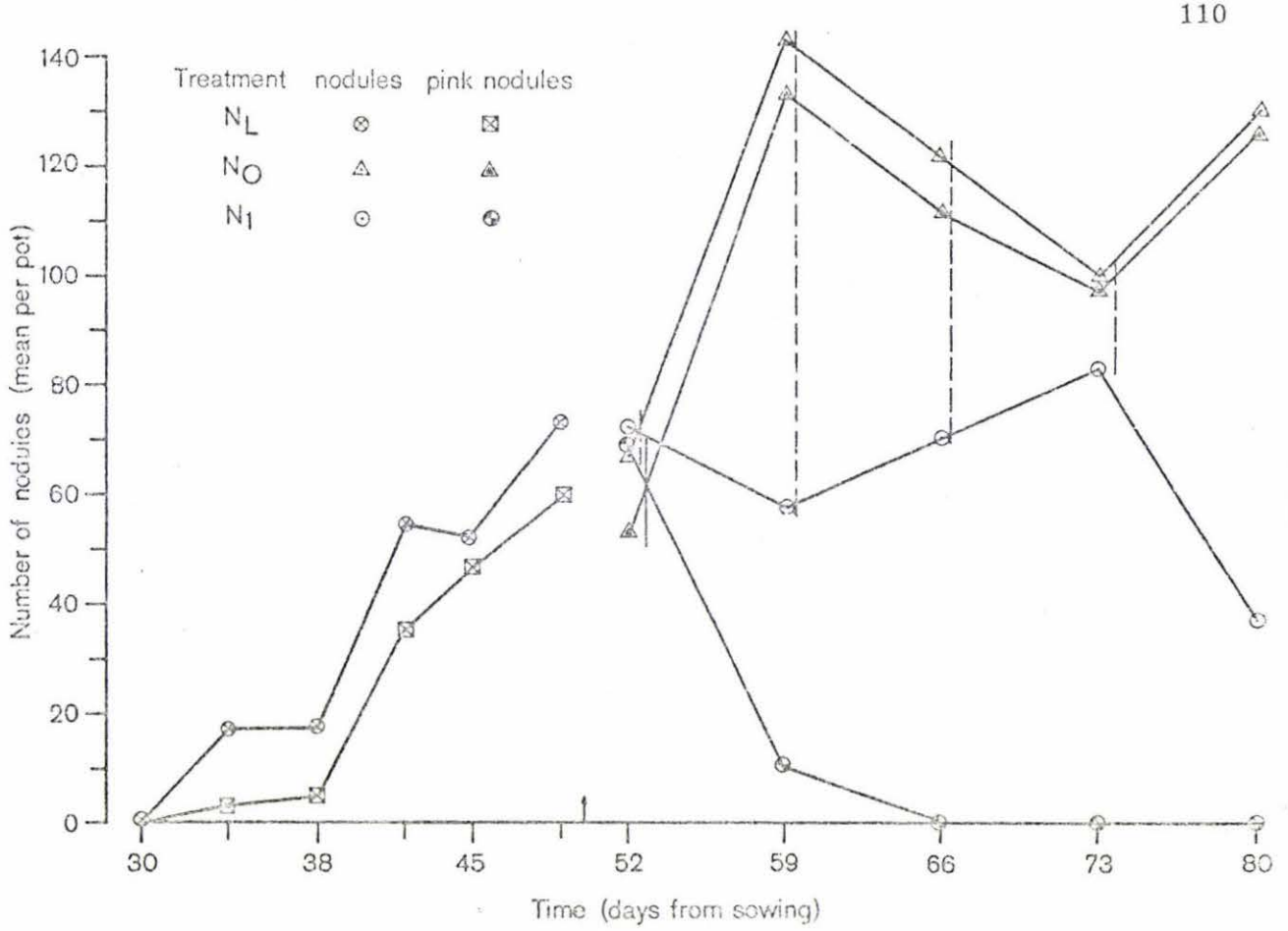


Fig 12 Number of nodules - expt.1(a). (Details as for fig 6 except that total numbers of nodules and numbers of pink nodules joined by solid and dotted vertical bars respectively, do not differ at the 5% level of significance.)

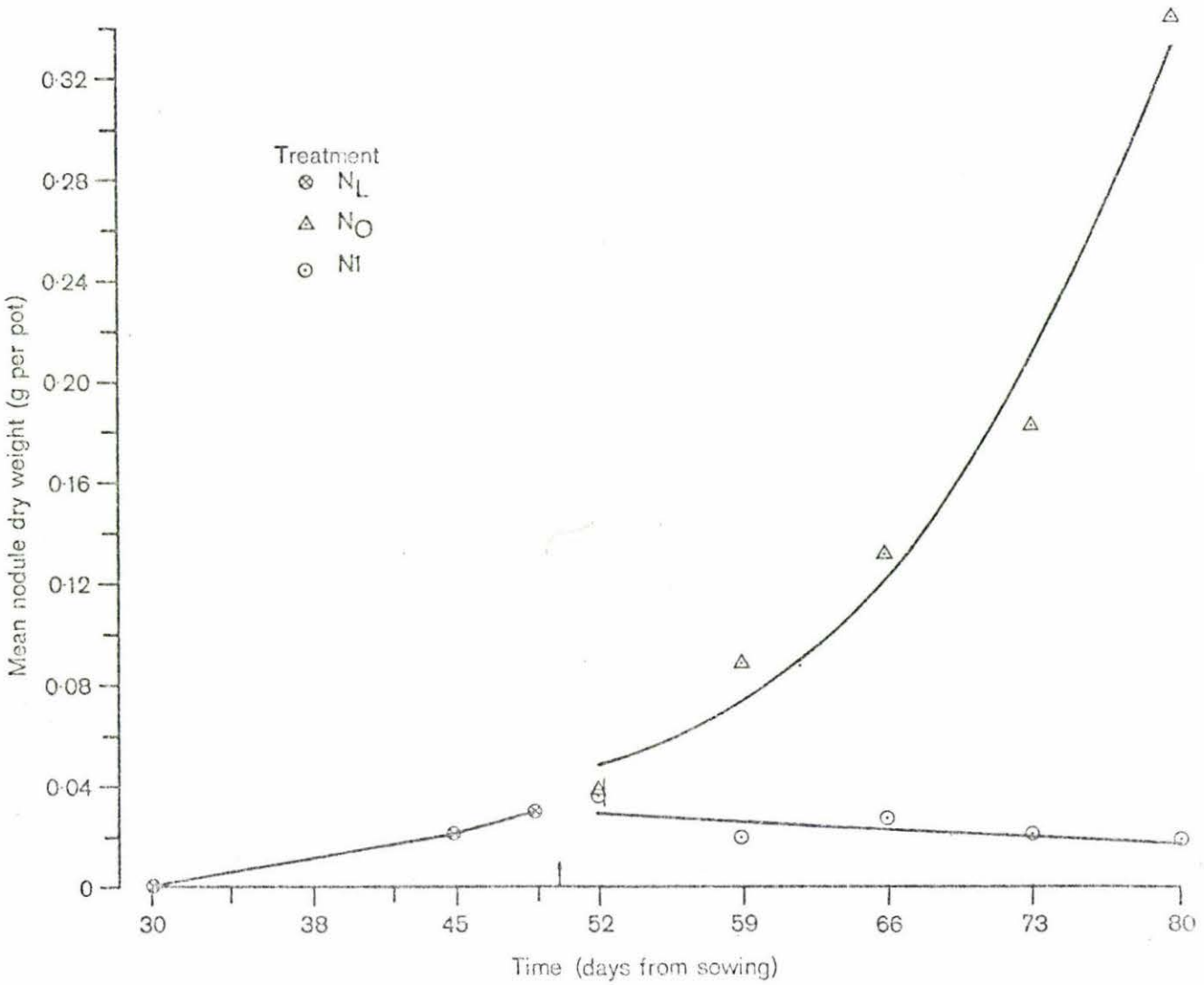


Fig 13 Weight of nodules - expt.1(a) (details as for fig 3).

Over the period day 50 to day 80, average nodule size increased approximately four fold in the N_0 treatment (based on mean values of nodule number at days 52 and 80, and values for nodule weight derived from the relative growth rate equation). Thus, the increase in nodule dry weight was a result of increases in both size and number of nodules.

The depression of nodulation in the presence of combined N (discussed in chapter I, section 2.2.3), as in the N_1 treatment, is a phenomenon which is observed in a wide range of legumes (e.g. Munns, 1968; Heichel & Vance, 1979; Dean & Clark, 1980; Wong, 1980). It is suggested that root hair curling and the formation of infection threads are more suseptible to injury than the later stages of nodulation, and that nitrate is a more potent supressor of nodulation than ammoniacal forms of N (Munns, 1968; Pate, 1977). High rates of combined N, particularly nitrate, are also found to substantially reduce fixing activity in legumes where a symbiotic N_2 fixing system is established (Allos & Bartholomew, 1955 & 1959; Copeland & Pate, 1969; Latimore *et al.* 1977; Dean & Clark, 1980; Wong, 1980). The high RGR_{Nod} relative to other plant components in the N_0 treatment was an indication of the urgency with which sainfoin dependent on symbiotic N_2 fixation was seeking to increase the capacity of its N_2 fixing system.

1.4 CONCLUDING COMMENTS - EXPERIMENT 1(a)

Sainfoin in the N_L and N_0 treatments of this experiment appeared to be well nodulated in comparison to other legumes. Despite this, however, sainfoin dependent on symbiotic N_2 fixation for its supply of N produced substantially less dry matter than when supplied with abundant combined N and exhibited a decreasing percentage N. Thus, the N_2 fixing system appeared unable to meet the N requirements of the plant. Dry matter partitioning in plants dependent on symbiotic N_2 fixation favoured the underground portion of the plant, particularly the nodules, in comparison to plants supplied with abundant combined N. As part of the decreased emphasis on top growth in plants dependent on symbiotic N_2 fixation, there was a substantially lower RLAGR which resulted in lower leaf area and presumably a decreased ability to assimilate carbon, in comparison to plants supplied with abundant combined N. This, in turn, would lead to a decreased supply of energy for nodulation and N_2 fixation.

2 EXPERIMENT 2

2.1 INTRODUCTION

Harvests were carried out twice weekly, from day 84 to day 143. At each harvest, two replicate pots from each treatment were chosen, as outlined in Chapter II, Section 6.1.3, and destructively harvested.

Acetylene reduction assays were also carried out twice weekly, on four replicate I_1N_0 pots chosen as outlined in Chapter II, Section 7.1.1. Acetylene reduction assays were also carried out on the other treatments, at regular intervals, to check that no $N_2[C_2H_2]$ fixation was occurring. Two I_0N_0 pots were included in each set of assays to check for background N_2 fixation by, for example, blue-green algae, and to check for cross infection. This was the main function of the I_0N_0 treatment and it is not discussed at any length in this chapter. From day 50, plant weight in the I_0N_0 treatment remained approximately static, with a slow change in dry matter distribution from the above to the below ground portion of the plant. The decline in the above ground portion appeared to be a result of leaf senescence.

For convenience treatments I_1N_1 and I_0N_1 are often collectively referred to as the N_1 treatments. Treatments I_1N_1 and I_0N_1 were in many respects very similar, but there were certain differences as discussed in section 2.2.5. Where treatments I_1N_1 and I_0N_1 do substantially differ, treatment I_1N_0 will be discussed in relation to treatment I_1N_1 with which it is more directly comparable than treatment I_0N_1.

As a check on the adequacy of nutrient supply, foliar samples from experiment 2 were analysed for major and micro nutrients. Levels of nutrients were generally found to be similar to, or greater than those reported in the literature (Appendix 2). Nutrient levels in pots at the end of an interval between nutrient applications were also checked and the indications were that ample nutrients remained at the end of an interval between nutrient applications (Appendix 2). The exponential growth observed over the entire experimental period is a further indication that nutrient supply was not seriously limiting.

A problem encountered in this experiment was the high degree of variability in growth and development between plants, particularly in the I_1N_0 treatment. The lower $\bar{R}^2$ values from the regressions of 'growth' variables against time in the I_1N_0 treatment compared with the N_1 treatments, was a result of this variability. A wide variety in growth form was observed within treatments, with some plants producing a rosette of leaves close to the surface of the pot, and some forming elongated

stems early on and having few leaves at a low level. A contrast in growth form is illustrated in plate 3.

2.2 THE EFFECT OF MODE OF NITROGEN NUTRITION ON PLANT GROWTH AND DEVELOPMENT

2.2.1 PLANT DEVELOPMENT

The mode of N nutrition was found to have a substantial influence on the development of sainfoin.

The number of regrowth points and overall mean plant height were significantly higher ($P < 0.05$) in the N_1 treatments, than in the I_1N_0 treatment (according to treatment by harvest analysis of variance). Stem elongation began to occur in the two N_1 treatments from day 87, but not in the I_1N_0 treatment until day 115.

The first buds appeared in the N_1 treatments on day 108 and in the I_1N_0 treatment on day 115. First flowers appeared on days 108 and 133 in the I_1N_0 and N_1 treatments respectively.

On day 106, a comparison of stage of development between treatments was made. This comparison is summarised in Table 9.

Table 9 Plant development at day 106

Treatment	Percentage of plants having: Elongated stems	Buds	Flowers
I_1N_0*	3.6	0	0
I_1N_1	71	38	18
I_0N_1	57	30	14
I_0N_0	0	0	0

* 56 plants observed in each treatment

2.2.2 PLANT WEIGHT AND TOTAL PLANT NITROGEN

For the period day 84 to day 143, total dry weight was found to increase in an exponential manner with time, with regressions of $\log_e$ [total dry weight] on time having $\bar{R}^2$ values of 79.2, 97.2 and 96.2% respectively for treatments I_1N_0, I_1N_1 and I_0N_1 (Fig.14, table 10).

The relative growth rates of plants supplied with adequate levels of combined N, 0.0318 and 0.0295 g g $^{-1}$ day $^{-1}$ for treatments I_1N_1 and I_0N_1 respectively, were significantly higher ($P \leq 0.05$) than that of 0.0250 g g $^{-1}$ day^{-1} for plants dependent on symbiotically fixed N (treatment I_1N_0) (Table 10).

Plate 3 A contrast in growth forms. Two I_1N_1 plants at day 105.

Plate 4 Top growth in experiment 2 at day 136
(left to right, treatments I_0N_0, I_1N_0, I_0N_1, I_1N_1)

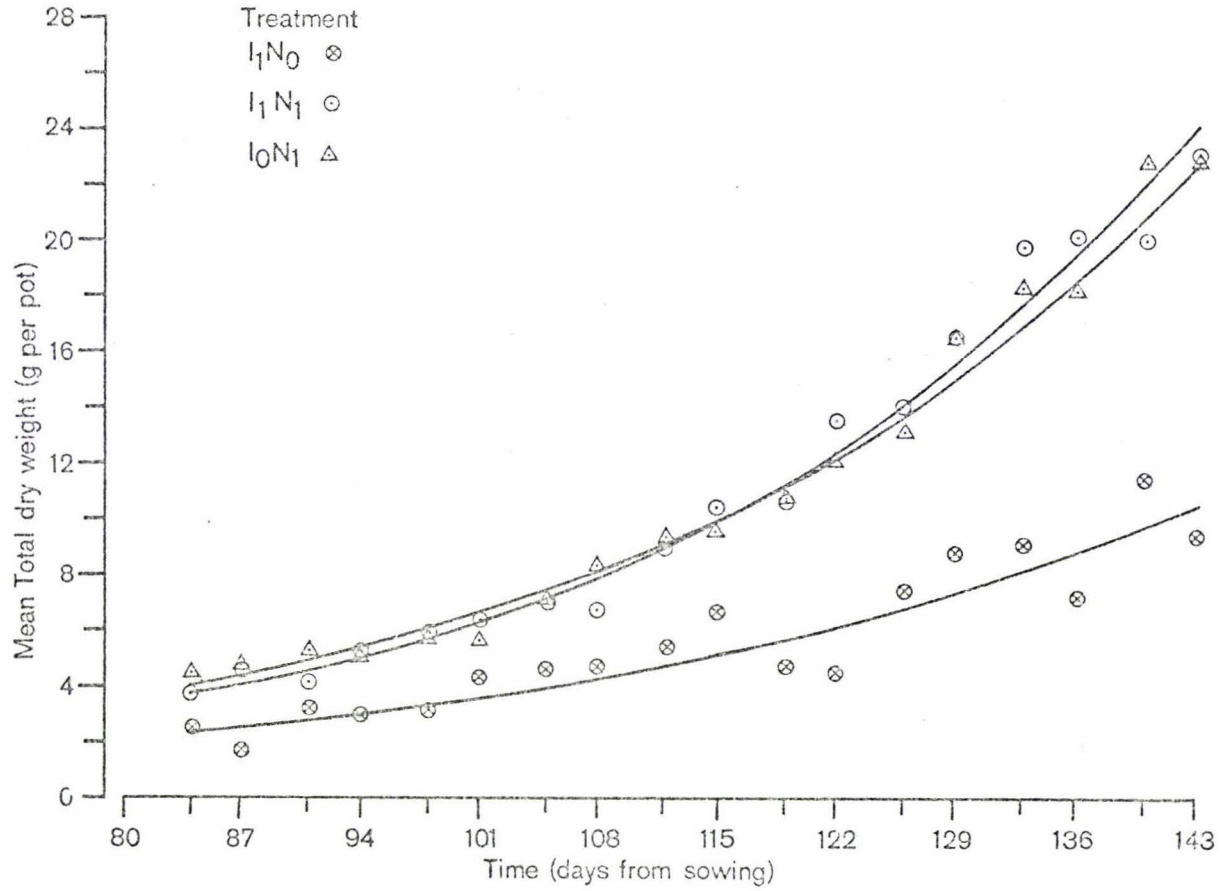


Fig 14 Total plant weight - expt 2. The lines drawn are relative growth rate curves. Each point is the mean of two pots.

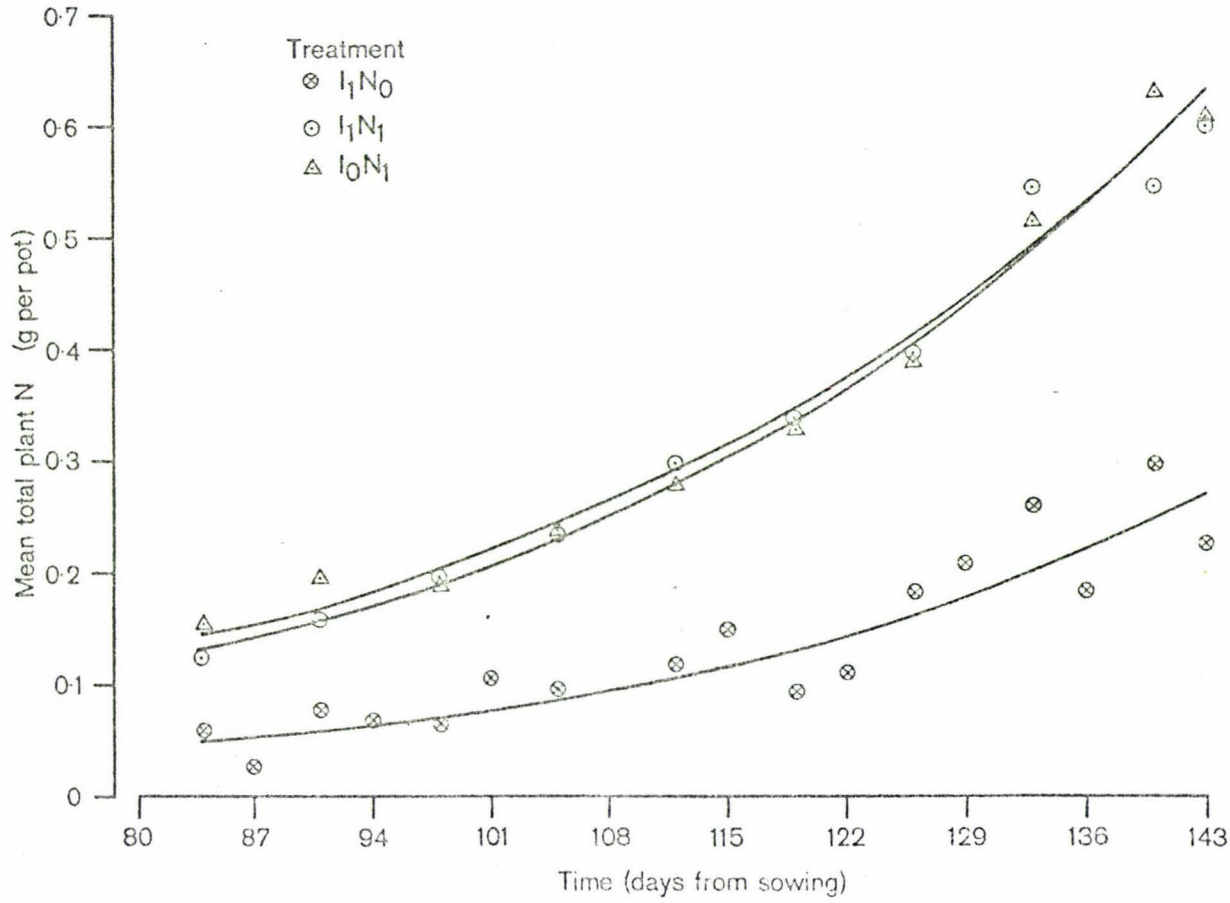


Fig 15 Total plant N - expt.2 (details as for fig 14).

Table 10 Relative growth rates - Experiment 2

	Treatment		
	I_1N_0	I_1N_1	I_0N_1
RGR_P ($g\ g^{-1}day^{-1}$)	0.0250	0.0318	0.0295
s.e. *	0.0022	0.0009	0.0010
% variance accounted for = $\bar{R}^2$	79.2	97.2	96.2
RGR_T ($g\ g^{-1}\ day^{-1}$)	0.0300	0.0346	0.0343
s.e.	0.0030	0.0012	0.0011
$\bar{R}^2$	74.4	95.9	96.6
RGR_R ($g\ g^{-1}\ day^{-1}$)	0.0178	0.0274	0.0223
s.e.	0.0018	0.0010	0.0014
$\bar{R}^2$	75.6	96.0	87.5
$RGR_{R\ +\ Nod.}$ ($g\ g^{-1}\ day^{-1}$)	0.0207		
s.e.	0.0019		
$\bar{R}^2$	77.7		
RLAGR ($cm^2\ cm^{-2}day^{-1}$)	0.0291	0.0295	0.0313
s.e.	0.0036	0.0014	0.0014
$\bar{R}^2$	66.6	92.6	93.2
RGR_{Nod} ($g\ g^{-1}day^{-1}$)	0.0386		
s.e.	0.0064		
$\bar{R}^2$	51.8		
RFA (moles $mole^{-1}\ day^{-1}$)	0.0434		
s.e.	0.0047		
$\bar{R}^2$	71.5		
$RARN_P$ ($g\ g^{-1}\ day^{-1}$)	0.0294	0.0264	0.0251
s.e.	0.0033	0.0010	0.0016
$\bar{R}^2$	69.7	97.3	93.6
$RARN_T$ ($g\ g^{-1}\ day^{-1}$)	0.0324	0.0281	0.0273
s.e.	0.0036	0.0014	0.0018
$\bar{R}^2$	71.0	95.8	93.0
$RARN_{R\ +\ Nod.}$ ($g\ g^{-1}\ day^{-1}$)	0.0254	0.0230	0.0204
s.e.	0.0032	0.0017	0.0021
$\bar{R}^2$	64.8	91.1	84.8

* s.e. = standard error

Overall mean dry weights of the N_1 treatments were significantly ($P < 0.05$) higher than that of the I_1N_0 treatment and individual harvest means were also significantly higher in the N_1 treatments than in the I_1N_0 treatment throughout the period day 84 to day 143 (according to treatment by harvest analysis of variance). At the end of the experiment, day 143, plant weights in the N_1 treatments were approximately 2.2 times higher than in the I_1N_0 treatment (Fig.14). Top dry weights were also greater in the plants provided with combined N, with top dry weight in the N_1 treatments being approximately 3.0 times higher than in the I_1N_0 treatment at day 143 (Fig.19).

Total plant N increased in an exponential manner with time (Fig.15, Table 10) with the regressions of $\log_e$ [total plant N] on time having $\bar{R}^2$ values of 69.7, 97.3 and 93.6% for treatments I_1N_0, I_1N_1 and I_0N_1 respectively.

Relative accumulation rate of total plant N (RARNp) in the I_1N_0 treatment appeared to be higher than in the N_1 treatments, but the differences were non significant. However total N was significantly higher ($P<0.05$) in the N_1 treatments than in the I_1N_0 treatment throughout the whole experimental period (day 84-143) according to treatment by harvest analysis of variance).

RARNp in the I_1N_0 treatment was higher than RGRp, and although the difference was non significant it was still reflected in an increasing percentage N (Fig.16). RARNp in both the N_1 treatments was significantly lower ($P<0.001$) than RGRp, suggesting that N as a proportion of total dry weight was decreasing. This was reflected in a decreasing percentage N in these treatments (Fig.16). The possibly lower RARNp in the N_1 treatments relative to the I_1N_0 treatment, and the decreasing total percentage N in the N_1 treatments is probably explained by the onset of stem elongation in the N_1 treatments (which appeared to result in a decreasing proportion of leaf material) from about day 87. However, the decrease in total percent N in the N_1 treatments was a result of decreasing percentage N in both roots and tops (Figs 17 and 18), and thus cannot be entirely explained by an increasing proportion of stem in the tops. However, the decline in percent N in the tops was greater than that in the roots and this combined with the fact that at the end of the experiment top weight was approximately 2.2 times greater than root weight (in the N_1 treatments) suggests that the decline in percentage N in the tops was the main contributor to the decline in percentage N of the whole plant.

At day 143 total plant N in the N_1 treatments was approximately 2.4 times greater than in the I_1N_0 treatment (Fig.15).

In summary, RGRp, total dry weight, total top weight and total plant N were all substantially (and significantly) higher in plants provided

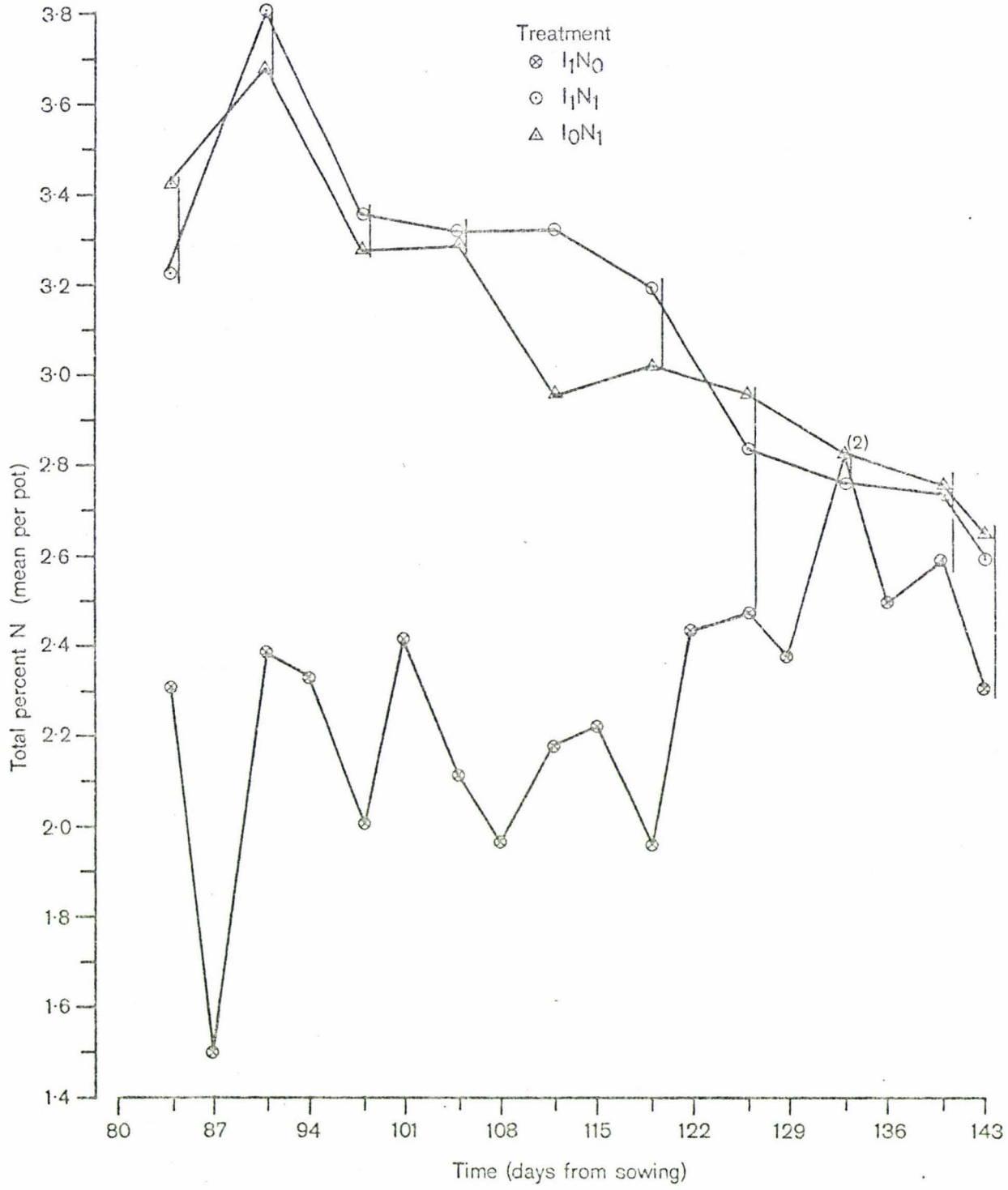


Fig 16 Total plant percent N - expt.2. Each point is the mean of two pots. Points joined by vertical bars do not differ at the 5% level of significance according to standard errors of differences.

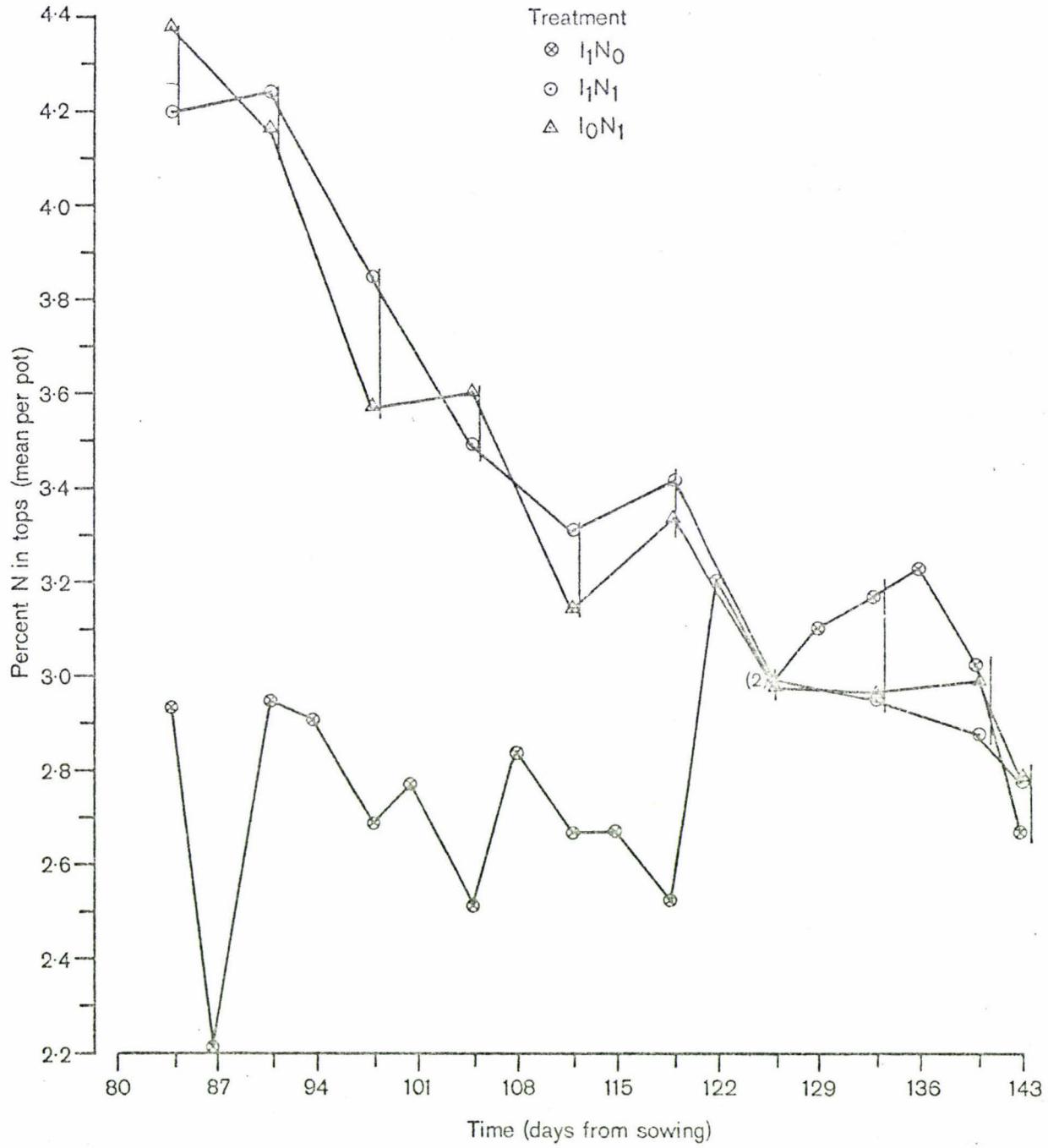


Fig 17 Percent N in top - expt.2 (details as for fig 16).

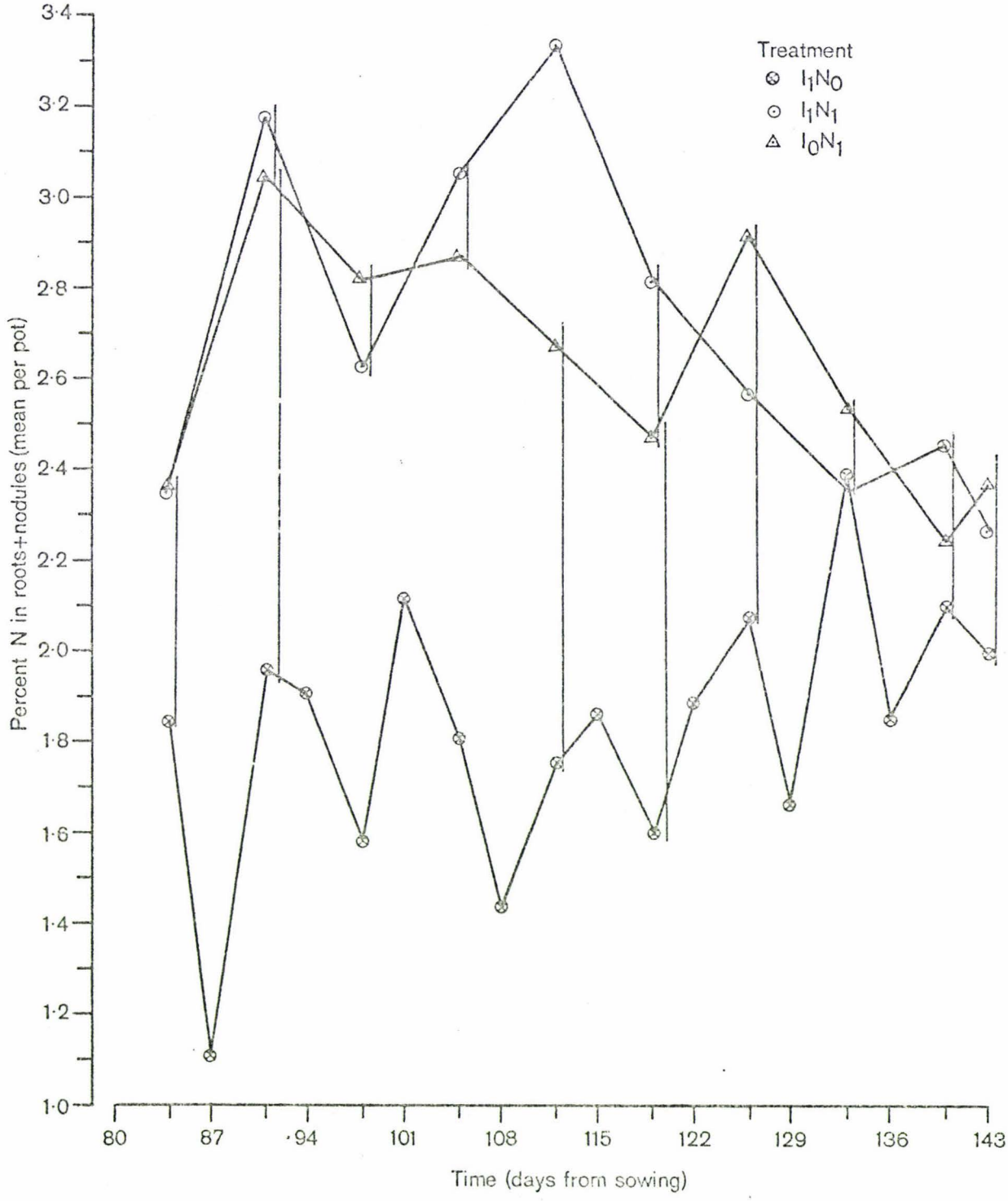


Fig 18 Percent N in root - expt. 2 (details as for fig 16).

with abundant combined N compared to plants dependent on symbiotic fixation for their supply of N. Plants dependent on symbiotic N_2 fixation produced approximately 45% of the total dry matter, 33% of the total forage (total top) and 43% of the total N of plants supplied with abundant combined (nitrate) N. Koter (1965b) found that sainfoin (at the flowering stage) solely dependent on symbiotic N_2 fixation had accumulated 50% of the total dry matter, 43% of the forage and 55% of the total N of plants supplied with high rates of combined N. Thus sainfoin in the experiment of Koter (1965b) appeared to perform relatively better than it did in this experiment. It may have been, however, that the highest rate of N supplied, in the work of Koter, was not sufficient to allow the sainfoin to grow to its full potential, especially since it was not sufficient to fully suppress nodulation as the high rate of N did in this experiment. It is difficult to compare the rates of N between the two experiments, because in the experiment of Koter (1965b) the N was added in one application.

The performance of sainfoin, in terms of its growth and N assimilation when dependent on symbiotic N_2 fixation, is compared, in Table 11, with other legumes also dependent on symbiotic fixation for their supply of N.

Table 11 N_2 fixing performance - comparison between species (percentage of total wt, top wt and total N in plants dependent on symbiotic N_2 fixation, compared to plants supplied with a high rate of combined N)

Plant (age in days)	Total dry wt	Total top wt	Total N	Reference
Sainfoin (143)	45	33	43	This experiment
Sainfoin (flowering)	45	44		Koter (1965a)
Sainfoin (flowering)	50	43	55	Koter (1965b)
Lucerne (70)	47		55	Allos & Bartholomew (1959)
Birdsfoot trefoil (70)	56		45	" "
Red clover (flowering)	61	61		Koter (1965a)
White clover	∿38	41		Ryle *et al.* (1979b)
Sweet clover (70)	30		44	Allos & Bartholomew (1959)
Ladino clover (70)	31		33	" "
Soybean (70)	48		79	" "
Soybean	∿31	∿ 32		Ryle *et.al.* (1979a)
Vicia faba			77	Richards & Soper (1979)
Vicia faba			81	Hill-Cottingham & Lloyd-Jones (1980)
Cowpea	70	73		Summerfield *et al.* (1977)
Cowpea	∿71	∿70	∿100	Ryle *et al.* (1979a)

Sainfoin dependent on N_2 fixation for its N supply appears to fix a similar proportion of N, relative to the amount accumulated by plants supplied with abundant combined N, as several of the other legumes in Table 11. In terms of N accumulation, dry matter accumulation and dry matter distribution, however, there were large differences between species. In the work of Allos & Bartholomew (1959), for example, it was found that although lucerne and birdsfoot trefoil fixed reasonably similar proportions of their own potential N requirements (Table 11), lucerne produced approximately 2.5 times as much dry matter as birdsfoot trefoil when both were dependent on symbiotically fixed N. In the work of Koter (1965a) total dry matter production of sainfoin was only slightly less than that of red clover, but the top:root + nodule ratio was much lower in sainfoin (Section 2.2.3).

Thus, the symbiotic N_2 fixing system of sainfoin appears to have a similar ability to meet the N needs of the plant, to the N_2 fixing systems of birdsfoot trefoil, lucerne, ladino clover and sweet clover. Red clover, cowpea, *V. faba* and perhaps soybeans, appeared to have a greater ability to meet their own N requirements via symbiotic fixation.

The fact that sainfoin appears to be able to fix a similar proportion of its own N requirements to lucerne, but that the latter is generally higher yielding (e.g. Spedding & Diekmahns, 1972), suggests that perhaps there is a plant factor, other than N_2 fixation, which limits the overall performance of sainfoin, including that of its N_2 fixing symbiosis.

2.2.3 PARTITIONING OF DRY MATTER AND N BETWEEN TOP AND ROOT + NODULE

As in experiment 1(a) the mode of N nutrition was found to have a substantial influence on dry matter distribution within the plant.

Top dry weight showed exponential growth in all treatments over the period day 84 to day 143, with the regressions of $\log_e$ [top dry weight] against time having $\bar{R}^2$ values of 74.4, 95.9 and 96.6% respectively for treatments I_1N_0, I_1N_1 and I_0N_1 (Fig.19, Table 10).

The relative growth rate of tops (RGR_T) in the I_1N_1 treatment, of 0.0346 g g^{-1} day^{-1}, was significantly higher ($P < 0.05$) than the value of 0.0300 g g^{-1} day^{-1} in the I_1N_0 treatment (Table 10). However, RGR_T in the I_0N_1 treatment was significantly higher than in the I_1N_0 treatment only at the 10% level. RGR_T was significantly greater ($P \leq 0.001$) than RGR_{R+Nod} ($=RGR_R$ in the N_1 treatments) in all treatments. This means that there was an increasing proportion of top weight with time in all treatments. As discussed in section 2.2.2 top weights were significantly higher in the N_1 treatments than in the I_1N_0 treatment. The effect of the treatments on top growth is illustrated in plate 4.

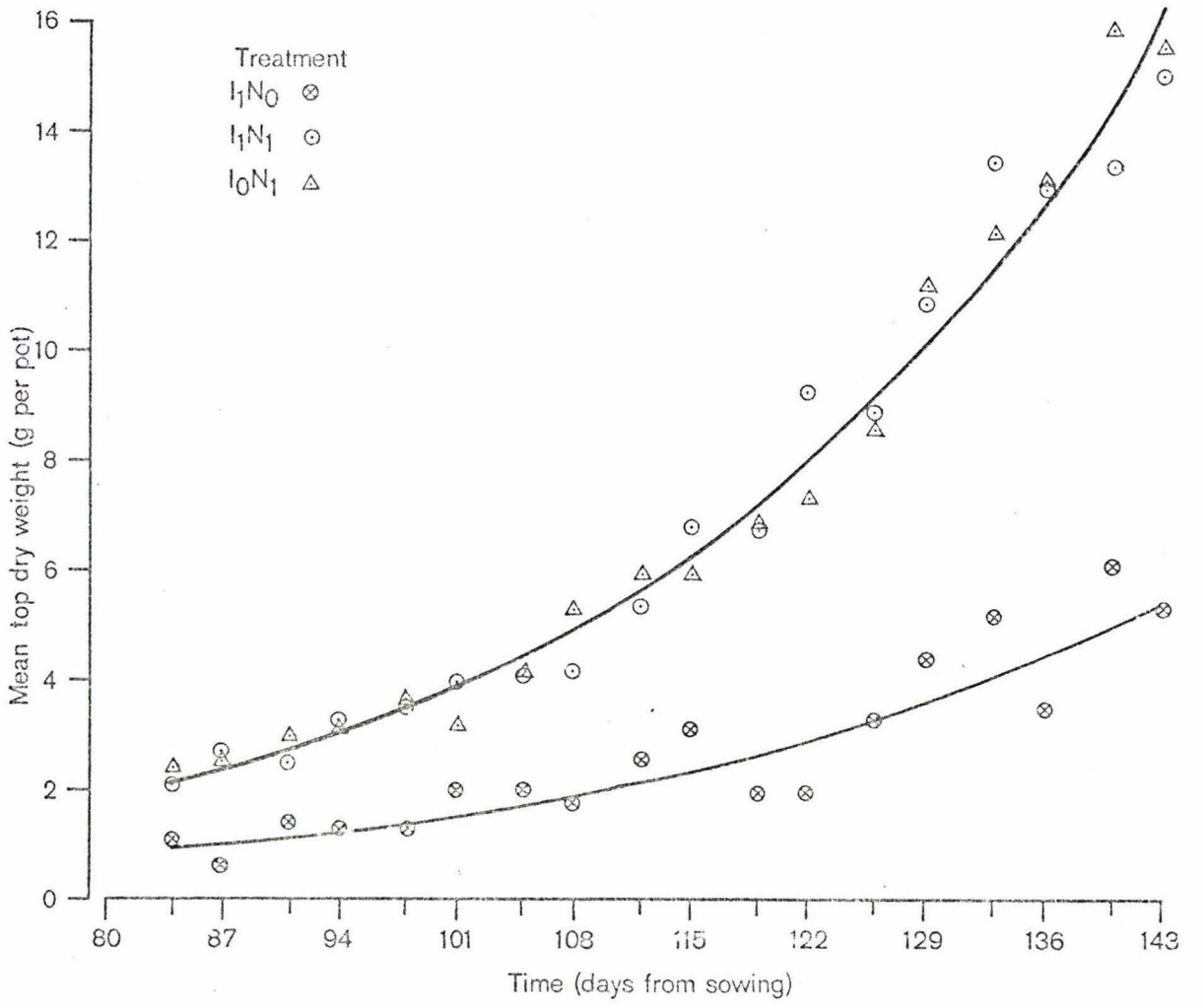


Fig 19 Top weight - expt. 2 (details as for fig 14).

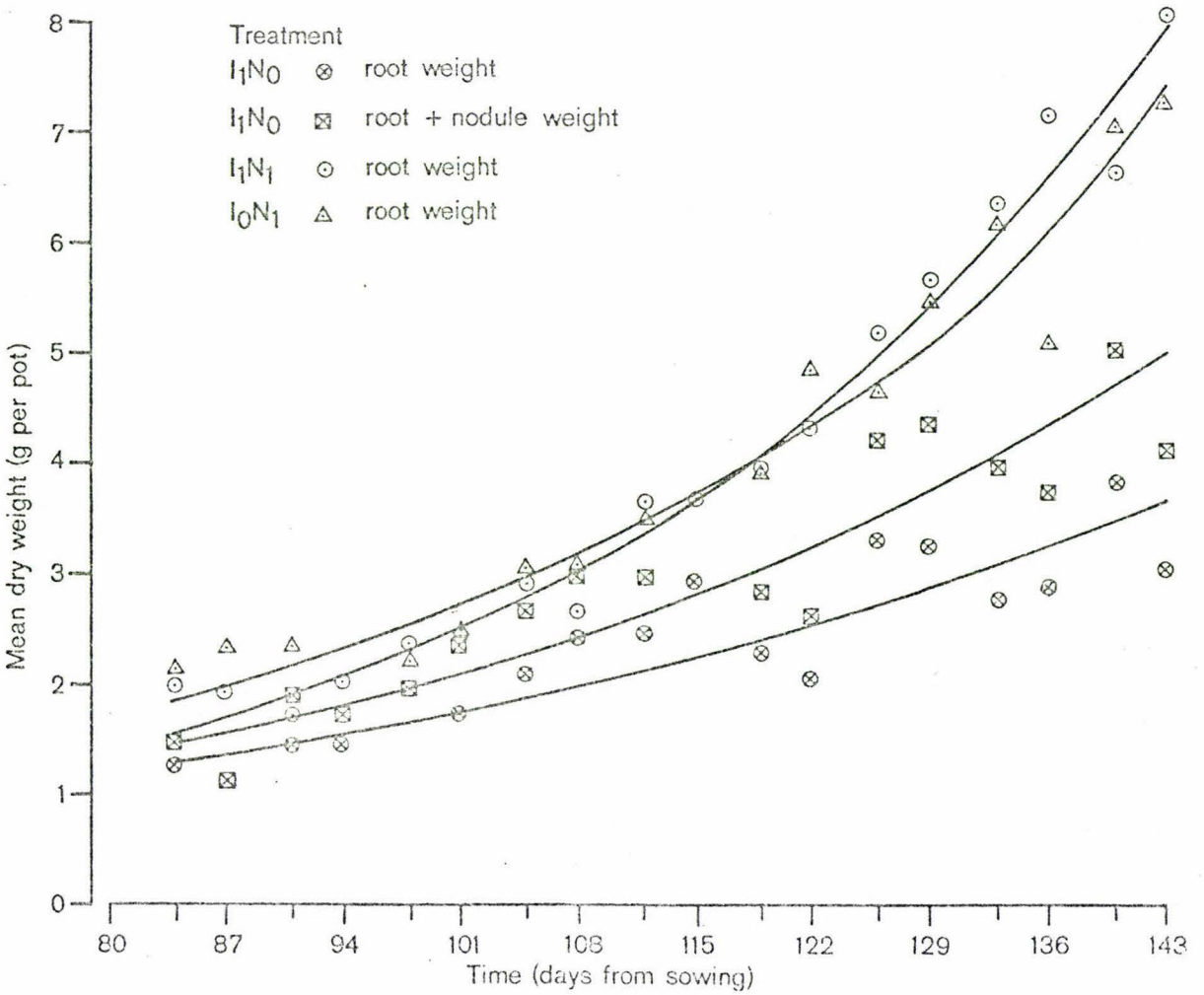


Fig 20 Root and root + nodule weight - expt. 2 (details as for fig 14).

Root dry weight also increased exponentially over the period day 84 to day 143 with regressions of $\log_e$ [root dry weight] against time having $\bar{R}^2$ values of 75.6, 96.0 and 87.5% respectively in treatments I_1N_0, I_1I_1 and I_0N_1 (Fig.20, Table 10).

The relative growth rate of roots (RGR_R) was significantly higher ($P < 0.001$) in treatment I_1N_1 (0.0274 g g^{-1} day^{-1}) than RGR_R or RGR_{R+Nod} (relative growth rate of roots+nodules) in the I_1N_0 treatment (0.0178 and 0.0207 g g^{-1} day^{-1} respectively). RGR_R in the I_0N_1 treatment was significantly less than in the I_1N_1 treatment and this is discussed in Section 2.2.5.

Root weights in the N_1 treatments were significantly greater than root or root+nodule weights in the I_1N_0 treatment (according to treatment by harvest analyses of variance) from approximately day 112. However root growth in the I_1N_0 treatment gave the appearance of being more vigorous than in the N_1 treatments, with a greater number of more healthy looking roots at the bottoms of the pots (plate 5). At day 143 root weight of the I_1N_1 treatment was approximately 2.2 times the root weight, and approximately 1.6 times the root+nodule weight in the I_1N_0 treatment (Fig.20).

The relative magnitudes of RGR_T and RGR_{R+Nod} appeared to be reasonably similar between treatments, with RGR_T being greater than RGR_{R+Nod}. This is in contrast to experiment 1(a), where in treatments N_L and N_0 RGR_T was significantly lower than RGR_{R+Nod} and in the N_1 treatment RGR_T was significantly higher than RGR_R similarly to this experiment. However top:root+nodule ratios in the N_1 treatments (mean = 1.78) were significantly higher ($P < 0.05$) than in the I_1N_0 treatment (mean = 0.882) by a factor of approximately two (Fig.21, plate 6). Top:root+nodule (root+nodule = root, in the N_1 treatments) ratio increased significantly ($P < 0.05$) with time in all treatments (according to treatment by harvest analysis of variance), which is to be expected, since RGR_T was greater than RGR_{R+Nod} in all cases. The trend towards a lower top:root+nodule weight ratio in plants dependent upon symbiotic N_2 fixation relative to those supplied with abundant combined N, first observed in experiment 1(a), has been extended to this experiment, with the difference in ratio between the treatments tending to become slightly larger with time. The relative growth rates of the 'above' and 'below ground' portions of sainfoin, relative to one another, were reasonably similar between treatments. This and the fact that the ratio of top:root+nodule weight in the N_1 treatments was higher than in the I_1N_0 treatment suggests that these differences developed when the N_2 fixing symbiosis was developing

Plate 5 A contrast in root growth
(left to right, treatments I_0N_1, I_1N_0, I_1N_0)

Plate 6 A contrast in top:root + nodule ratio at day 101
(left to right, treatments I_1N_0, I_1N_1)

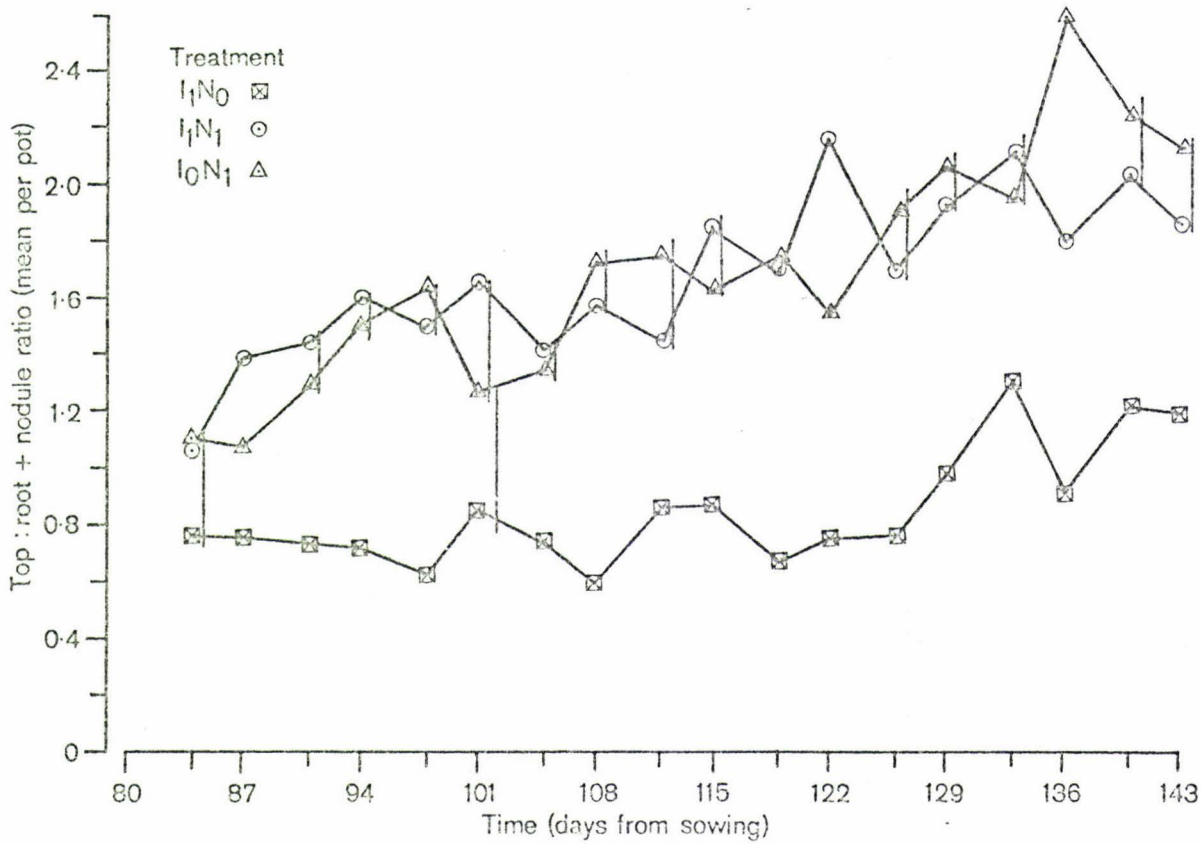


Fig 21 Top:root + nodule ratio - expt. 2 (details as for fig 16).

in the latter treatment. This is consistent with the fact that RGR_{R+Nod} was higher relative to RGR_p in treatments N_L and N_o in experiment 1(a) than in treatment I_1N_o in experiment 2.

The relative accumulation rate of N in tops and roots+nodules ($RARN_T$ and $RARN_{R+Nod}$) followed a similar pattern to that for total N (Section 2.2.2) in terms of differences between treatments (Table 10). $RARN_T$ was significantly higher ($P < 0.05$) than $RARN_{R+Nod}$ in the three treatments I_1No, I_1N_1 and IoN_1, as would be expected since RGR_T was higher than RGR_{R+Nod} in the three treatments. The increase in total N with time, especially in the N_1 treatments, was largely a result of increase in top N (Figs 22 and 23). $RARN_T$ and $RARN_{R+Nod}$ in relation to RGR_T and RGR_{R+Nod} differed between treatments. RARN was non-significantly higher than RGR in both tops and roots+nodules in the I_1N_o treatment. In the N_1 treatments RGR_T was significantly higher ($P<0.001$) than $RARN_T$, supporting the suggestion made in Section 2.2.2, that the decline overall percentage N was primarily caused by a decline in the percentage N of the tops. RGR_R in the I_1N_1 treatment was significantly higher ($P<0.01$) than $RARN_R$, but the corresponding difference was not significant in either the I_1N_o or I_oN_1 treatments (Table 10).

2.2.4 LEAF AREA

Leaf area, as for plant weight, increased in an exponential manner with time in all treatments with regressions of $\log_e$ [leaf area] on time having $\bar{R}^2$ values of 66.6, 92.9 and 93.2%, for treatments I_1N_o, I_1N_1 and I_oN_1 respectively (Fig.24, Table 10).

In absolute terms, leaf area in the N_1 treatments was generally significantly greater ($P < 0.05$) than in the I_1N_o treatment from about day 119 (see Fig. 24). At day 143, leaf area in the N_1 treatments was approximately 2.1 times greater than in the I_1N_o treatment. There was very little difference between treatments, however, in terms of relative leaf area growth rate (RLAGR) (Table 10), suggesting perhaps that reduced leaf area in the I_1N_o treatment at the end of the experiment was due to events, such as nodulation, which took place early in the development of the 'I_1N_o' plants. However the RGR_T was higher in the N_1 treatments than in the I_1N_o treatment. This, together with the similar RLAGR's suggests a difference in dry matter partitioning, within the top, between species. Relative to RGR_T in the respective treatments, RLAGR was higher in the I_1N_o treatment than in the N_1 treatments (Table 10). In the I_1N_o treatment RLAGR was very similar to

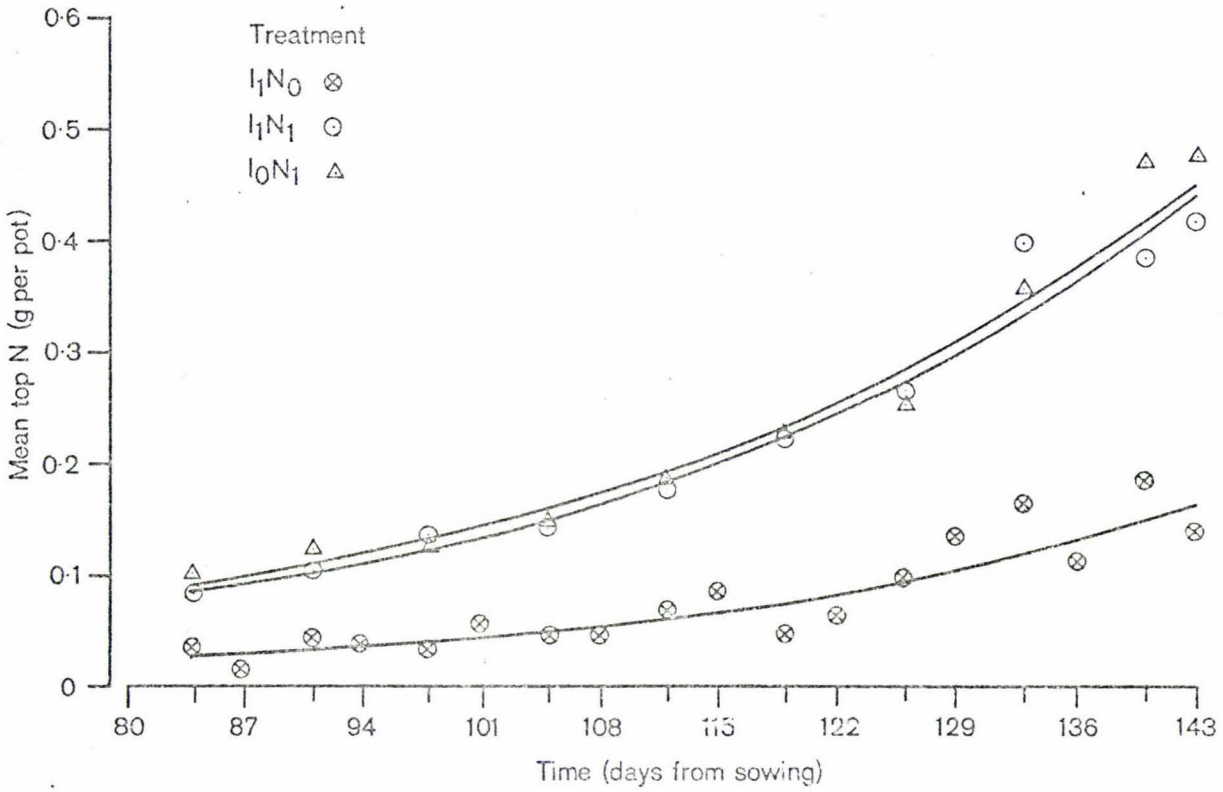


Fig 22 Top N - expt. 2 (details as for fig 14).

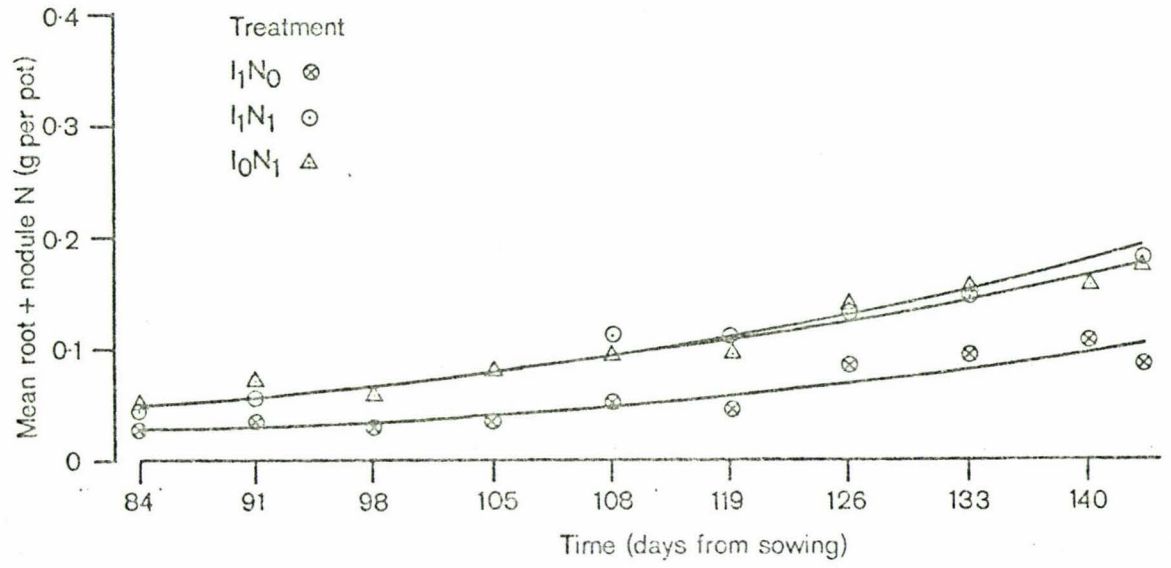


Fig 23 Root + nodule N - expt.2 (details as for fig 14).

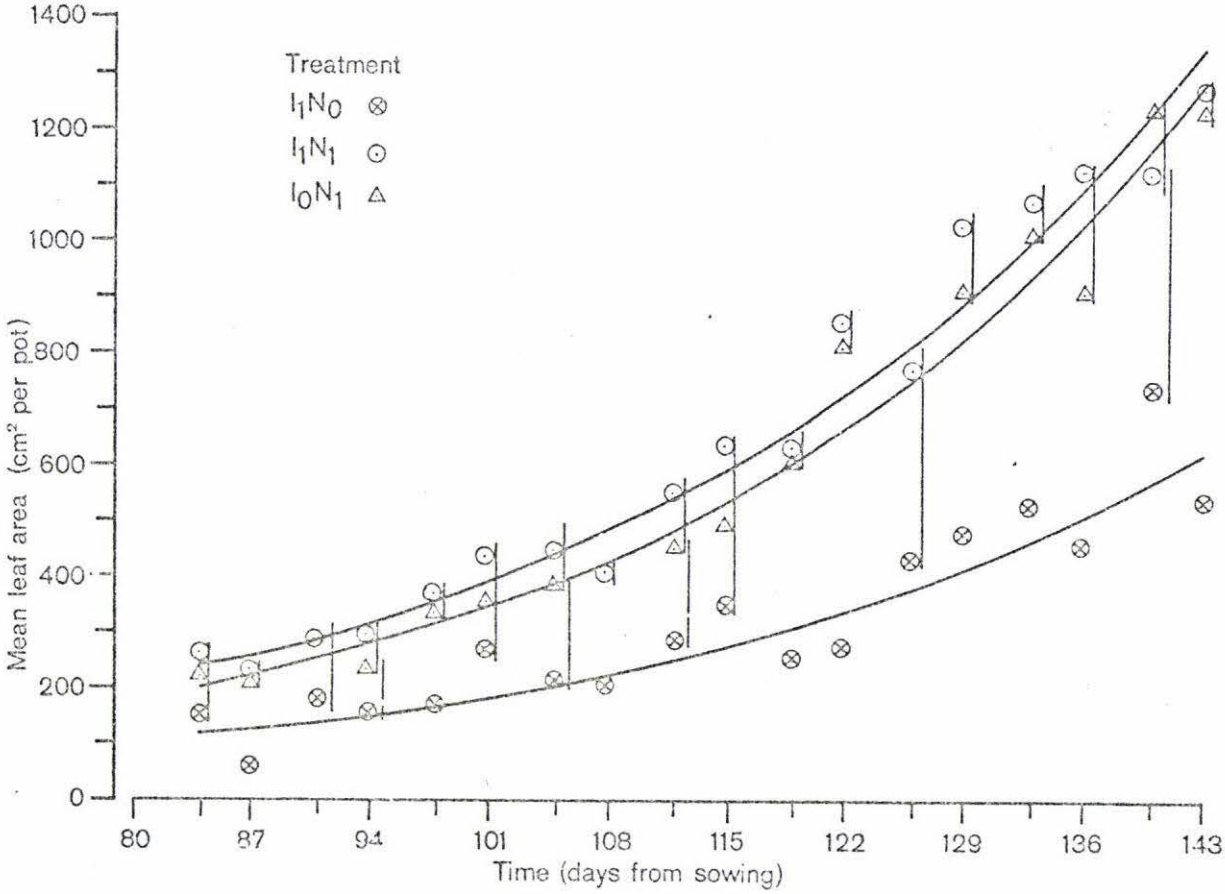


Fig 24 Leaf area - expt.2. The lines drawn are relative growth rate curves. Each point is the mean of two pots. Points joined by vertical bars do not differ at the 5% level of significance according to standard errors of differences.

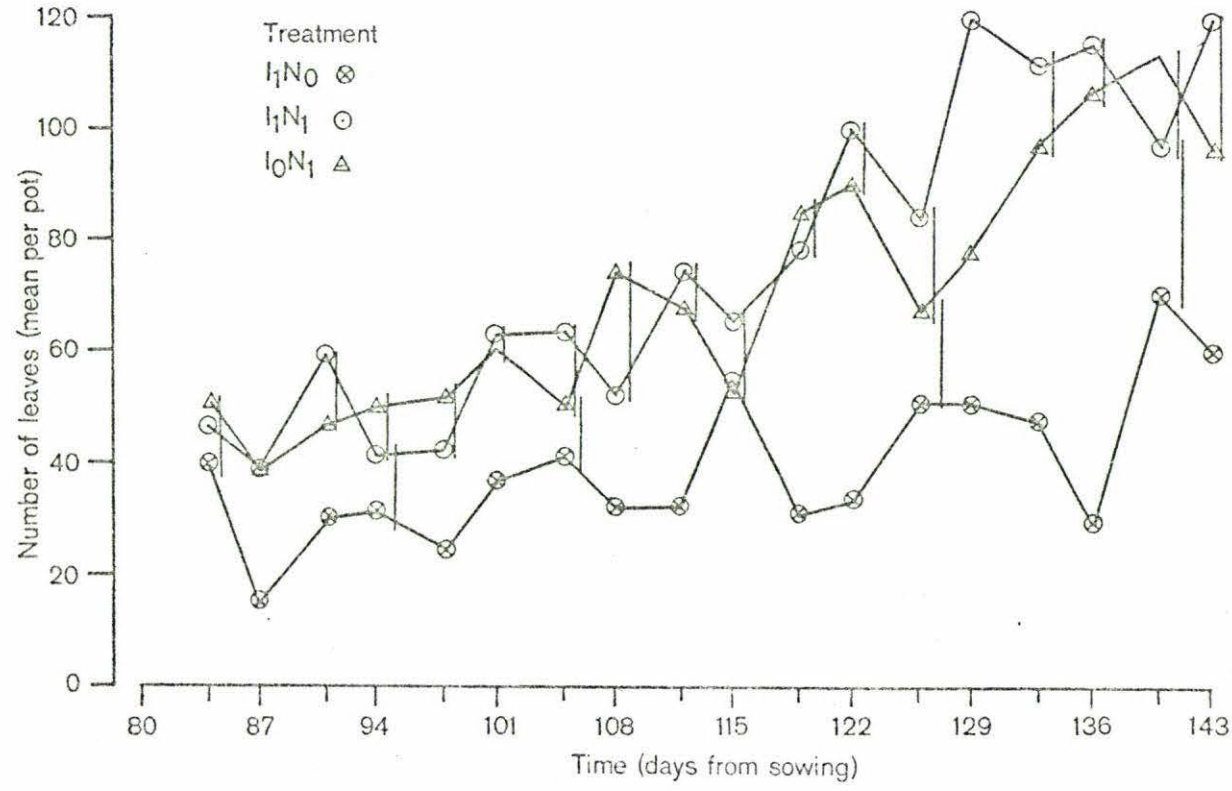


Fig 25 Number of leaves - expt.2 (details as for fig 16).

RGR_T, whereas in the I_1N_1 and I_0N_1 treatments RLAGR was significantly less ($P<0.001$ and 0.05 respectively) than RGR_T, probably reflecting an increasing proportion of stem as stem elongation and flowering took place.

RLAGR was non-significantly higher than RGR_p in treatment I_1N_0, in contrast to the situation in treatment N_0, experiment 1(a), where RLAGR was significantly lower than RGR_p. This may well be the result of the respective stages of development of the N_2 fixing symbiosis in experiments 1(a) and 2, with nodulation influencing dry matter partitioning to a greater extent early in the development of the plant. In the I_1N_0 treatment RGR_{Nod} (0.039 g g^{-1} day^{-1}) was higher than RGR_p (0.025 g g^{-1} day^{-1}), but was not relatively as high as in the N_0 treatment of experiment 1(a) (where RGR_{Nod} = 0.072 g g^{-1} day^{-1}, and RGR_p = 0.037 g g^{-1} day^{-1}). This indicates that the N_2 fixing symbiosis is utilising a lesser proportion of the resources available to the plant in experiment 2, where RGR_{Nod} is more in line with RGR_p, than in experiment 1(a) when the symbiosis was becoming established, and the plant was attempting to increase its capacity to assimilate atmospheric N_2.

There was a reasonably good relationship between leaf area and leaf number (correlation coefficients, r = 0.81, 0.89 and 0.88 for treatments I_1N_0, I_1N_1 and I_0N_1 respectively). However, data for leaf number was much more variable than that for leaf area (Figs 24 and 25), indicating that leaf size was also influencing leaf area.

Overall mean leaf area ratio (LAR = leaf area ÷ total dry weight) was significantly higher in treatment I_1N_1 than in treatment I_1N_0 (Table 12).

Table 12 Mean leaf area ratio - Experiment 2

	Treatment		
	I_1N_0	I_1N_1	I_0N_1
LAR cm^2/g	53.7 a	59.0 b	53.9 a

* Figures followed by different letters differ at the 5% level of significance (according to standard errors of differences).

This implies a greater capacity on the part of I_1N_1 plants to assimilate carbon and may explain the higher RGR_p value of this treatment relative to that of the I_1N_0 treatment.

Overall net assimilation rates for the period day 84 to day 143 were 0.00054 g cm^{-2} day^{-1} for the two N_1 treatments and 0.0047 g cm^{-2} day^{-1} for the I_1N_0 treatment. The differences in NAR between treatments were non-

significant ($P < 0.05$). This possibly lesser capacity of plants with a given leaf area to produce dry matter, in the I_1N_0 treatment, may be an indication that the symbiotic N_2 fixing system is making energy demands on the plant over and above those made by the N assimilation system of plants with access to nitrate N.

2.2.5 COMPARISON BETWEEN THE I_1N_1 AND I_0N_1 TREATMENTS

The I_1N_1 treatment was inoculated as was treatment I_1N_0, and the plants presumably formed nodules and fixed N_2 prior to day 50 when the high (N_1) rate of combined N was applied. In contrast the plants of treatment I_0N_1 were not inoculated and consequently never formed nodules or carried out symbiotic N_2 fixation. Certain differences between I_1N_1 and I_0N_1 treatments were observed.

RGR_p in the I_1N_1 treatment (0.0318 g g^{-1} day^{-1}) was significantly greater ($P < 0.05$) than in the I_0N_1 treatment (0.0295 g g^{-1} day^{-1}) (Table 10). Total dry weight was lower in the I_1N_1 treatment at day 84 and higher at day 143 than in the I_0N_1 treatment (not significantly so in either case according to treatment by harvest analysis of variance) (Fig.14). RGR_R (relative growth rate of root) was also significantly greater ($P<0.001$) in the I_1N_1 (0.0274 g g^{-1} day^{-1}) than in the I_0N_1 treatment (0.0223 g g^{-1} day^{-1}), with root weight, as for total weight, tending to be lower in the I_1N_1 treatment at day 84 and higher at day 143, than in the I_0N_1 treatment (not significantly so in either case, according to treatment by harvest analysis of variance) (Fig. 20). RGR_T, $RARN_p$ and RLAGR were not significantly different between the I_1N_1 and I_0N_1 treatments. However leaf area in the I_1N_1 treatment was higher than in the I_0N_1 treatment over the period day 84 to day 143 with a treatment by harvest analysis of variance on treatments I_1N_1 and I_0N_1 having an F value for the treatment effect significant at the 5% level. Leaf area in the I_1N_1 treatment was approximately 16% higher at day 84 and 4.5% higher at day 143 than in the I_0N_1 treatment (taken from the RLAGR equations, Fig.24). Leaf area ratio (LAR) was also higher in the I_1N_1 than in the I_0N_1 treatment, with a treatment by harvest analysis of variance on treatments I_1N_1 and I_0N_1 having an F value for the treatment effect significant at the 1% level (see also Table 12). LAR appeared to be higher at the beginning of the day 84 to day 143 period in the I_1N_1 treatment, but similar in both treatments at the end (Fig.26). Differences between treatments at individual harvests were generally not significant (according to standard errors of differences). Top:root weight ratios were not significantly different between the two treatments.

Thus, at day 84, 34 days after the introduction of the N_1 treatment, plants in the I_1N_1 treatment appeared to have a slightly lower total dry weight and root weight but were growing faster, particularly in terms of

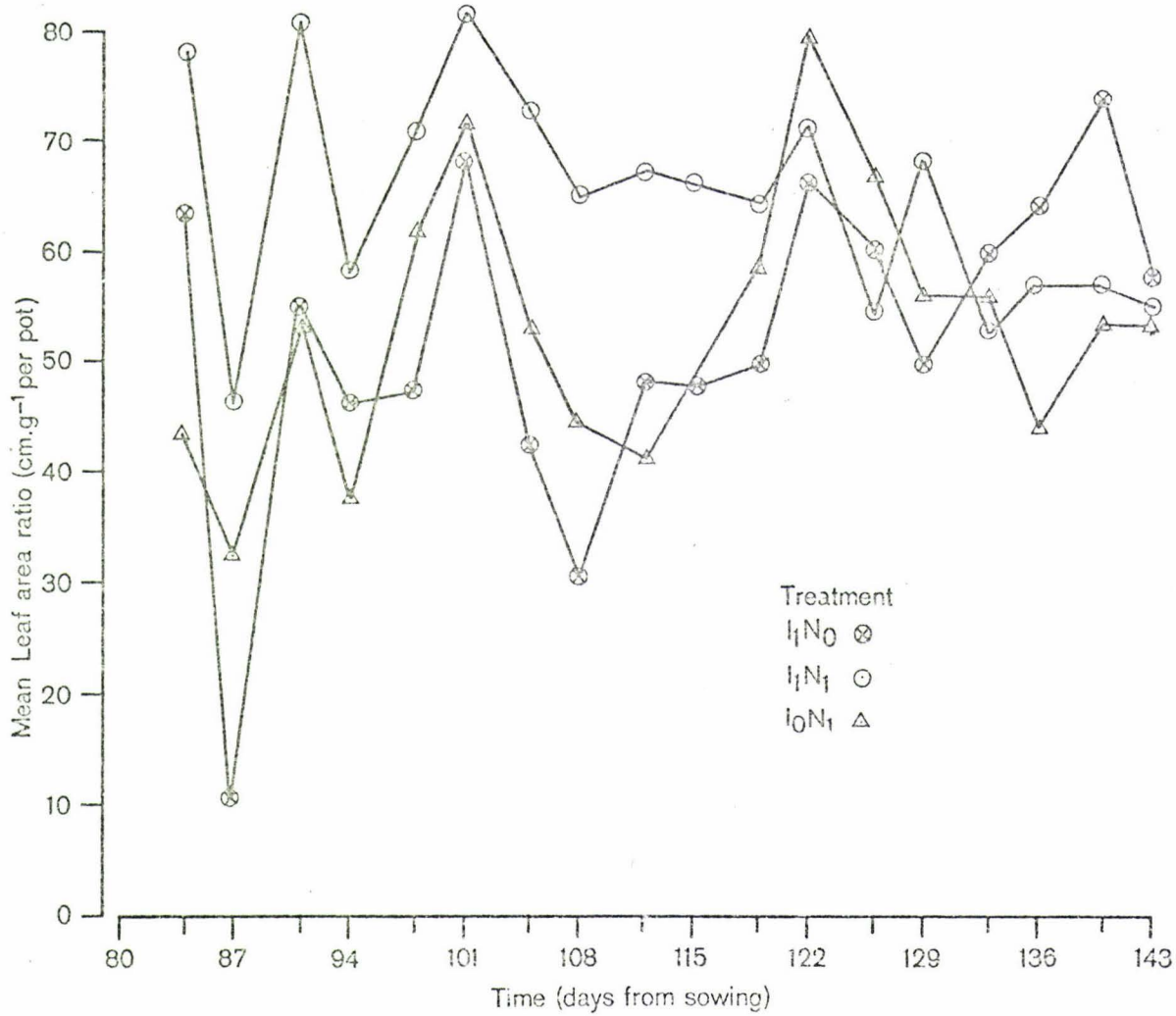


Fig 26 Leaf area ratio - expt. 2. Each point is the mean of two pots.

root weight, than in the I_0N_1 treatment. Up to day 50, a proportion of the photosynthate available to roots in the I_1N_1 treatment was presumably being channelled into the production of nodules. Thus root weight, excluding nodules, may well have been higher in the I_0N_1 treatment, but this was not measured. When the N_1 rate of nitrogen was applied, the nodules on the roots of the I_1N_1 plants would have been lost (as in experiment 1(a)). The change of emphasis to producing roots only under conditions of abundant N supply, as opposed to roots and nodules when N was limiting, appears to have resulted in an increase in RGR_R in the I_1N_1 relative to the I_0N_1 treatment. The slightly lower total plant weights in the I_1N_1 relative to the I_0N_1 treatment at day 84 may be a result of the photosynthate which was channelled into nodule production prior to day 50 in the former treatment, presumably at the expense of the rest of the plant (see Section 3.1). This effect appears to have outweighed any advantage gained from the I_1N_1 plants in terms of improved N status as a result of symbiotic fixation. It may be that the N_2 fixing symbiosis had not been established long enough for the amount of N_2 fixed to offset the energy cost of establishing the symbiosis. The higher relative growth rates in the I_1N_1 treatment, which appeared to enable it to catch up with the I_1N_0 treatment in terms of plant weight, appeared to be the result of significantly higher leaf area and, in particular, LAR. It may be that leaf area, or particularly LAR, was higher in the I_1N_1 than in the I_0N_1 treatment at day 50 (as indicated at day 84), and that this enabled a higher RGR_p thereafter. It could be argued that this was a result of improved N nutrition. The effect could possibly have been cyclical, with increased leaf area, in turn, providing additional carbohydrate for further nodulation and N_2 fixation. If the greater leaf area in the I_1N_1 relative to the I_0N_1 treatment did result from superior N nutrition alone, it could also be expected that plant dry weight would increase, relatively, in treatment I_1N_1. The reverse, in fact, appeared to happen with plant dry weight being greater in treatment I_0N_1 than I_1N_1. The effect of nodulation appeared to be a change in the growth form of the plant, with an increase in leaf area despite slightly decreased total dry weight in the I_1N_1 treatment. This latter hypothesis receives some support from the work of Heichel & Vance (1979), who found that leaf development was greater in inoculated lucerne plants than in non-inoculated controls at a range of N levels up to 50 µg/ml (50 ppm). They state that this may have reflected a limitation of growth by N. Also, however, lucerne and pea nodules have been found to produce hormones or hormone precursors in abundance, and possibly the nodules, apart from fixing molecular N_2, produce other substances that

are important in growth or nutrient utilisation (Heichel & Vance, 1979). The fact that leaf area was significantly correlated with top growth in the I_1N_1 treatment (r= 0.68) and not in the I_0N_1 treatment (r=0.26) (Table 17) may provide some evidence for additional growth regulation in treatment I_1N_1 relative to I_0N_1.

2.2.6 DISCUSSION OF GROWTH DATA

It was found that plant development and plant growth were retarded in plants dependent on symbiotic fixation for their N supply, relative to those supplied with abundant combined N. In addition the different modes of N nutrition resulted in differences in dry matter distribution as discussed in Sections 2.2.3 and 1.3.1.2.

The fact that RGR_p and possibly NAR were lower in the I_1N_0 treatment than in the N_1 treatments suggests that symbiotic fixation placed a continuing energy burden on sainfoin in addition to that imposed by nitrate assimilation and also that the energy supply for N_2 fixation may have been limiting. This is in contrast to the view of Gibson (1966), Bergersen (1971) and Minchin and Pate (1973) that similar energies are involved in the fixation of N_2 and the assimilation of nitrate, and that it is during the actual period of growth and development of nodules that the growth and development of the N_2 fixing plant is retarded. The results obtained in this experiment appear to be more in accord with the findings of Silsbury (1977), Mahon (1977), and Ryle *et al.* (1978, 1979b) which suggest that a symbiotic N_2 fixing system does make energy demands on a plant greater than those associated with the assimilation of combined N. If, however, NAR is not lower in the I_1N_0 than in the N_1 treatments, the lower RGR_p in the I_1N_0 treatment could be simply explained by an inefficient symbiotic N_2 fixing system.

2.3 NODULATION AND NITROGEN FIXATION

2.3.1 NODULATION

A typically well nodulated root system from the I_1N_0 treatment is shown in plate 7.

By day 84 there were no effective nodules on the N_1 plants, and none were found throughout the period day 84 to day 143. Thus the N_1 rate of nitrogen was such as to suppress nodulation and N_2 fixation.

Nodule number in the I_1N_0 treatment was very variable, but there did seem to be a general increase in nodule number with time (Fig.27). Re-inoculation was carried out on day 109, and this may have influenced nodule number by about day 130 (based on the re-inoculation in experiment 1(b), Fig.35).

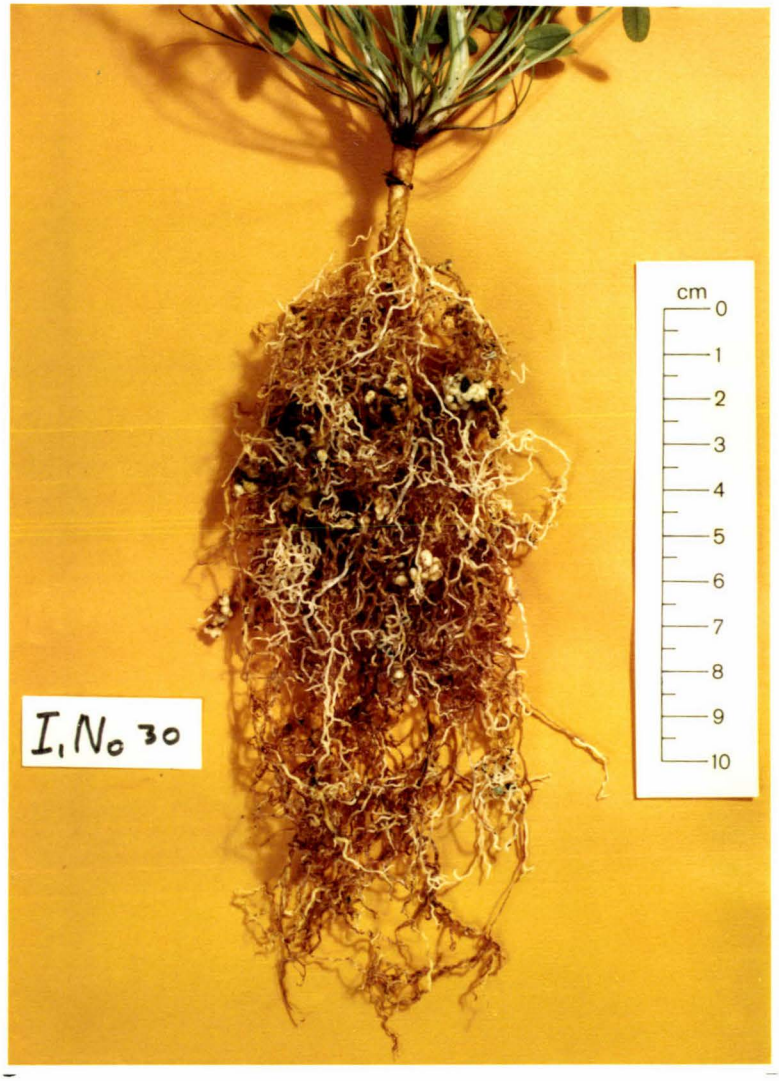


Plate 7 A typically well nodulated I_1N_0 root system at day 101.

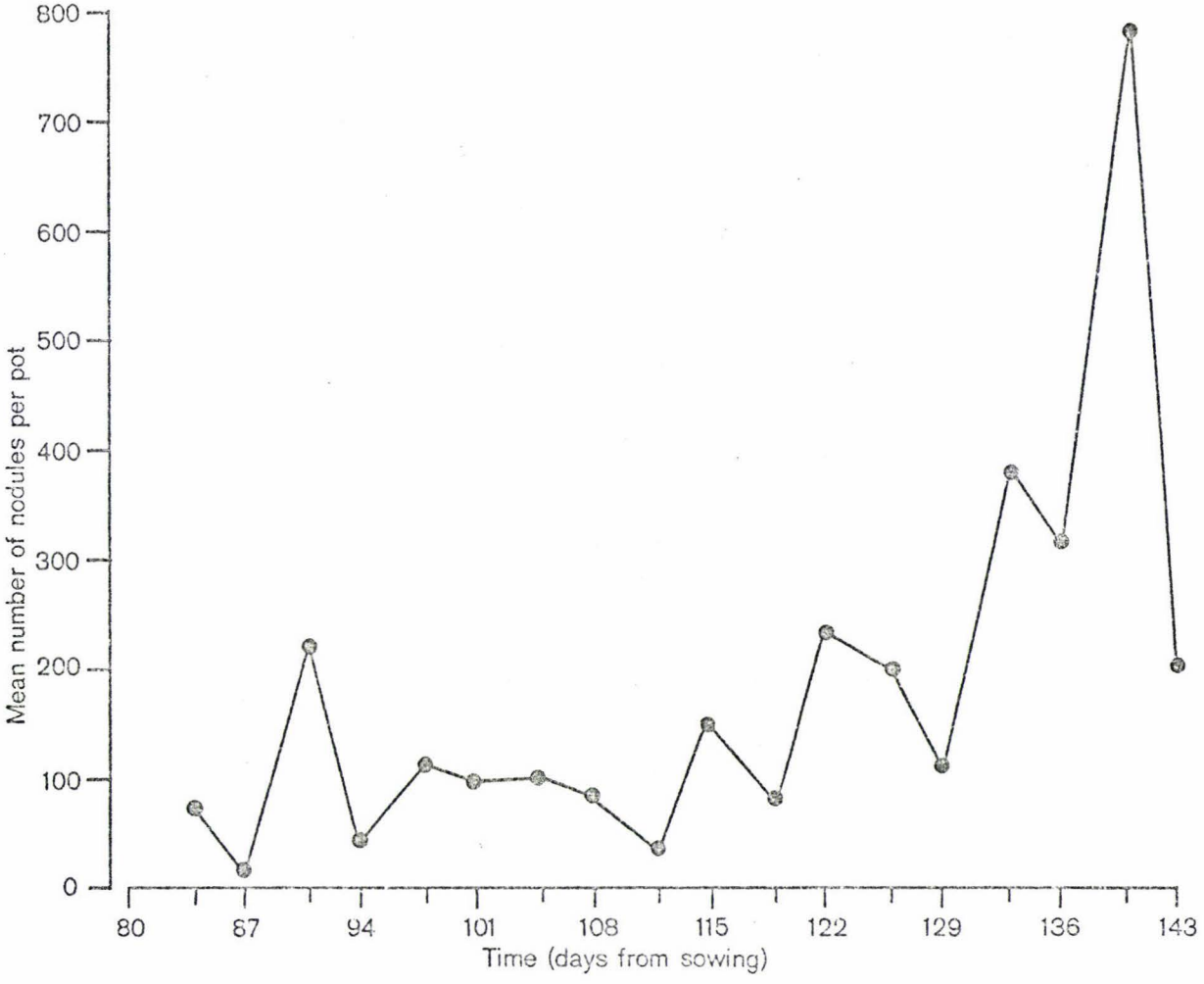


Fig 27 Number of nodules - expt.2. Each point is the mean of two pots.

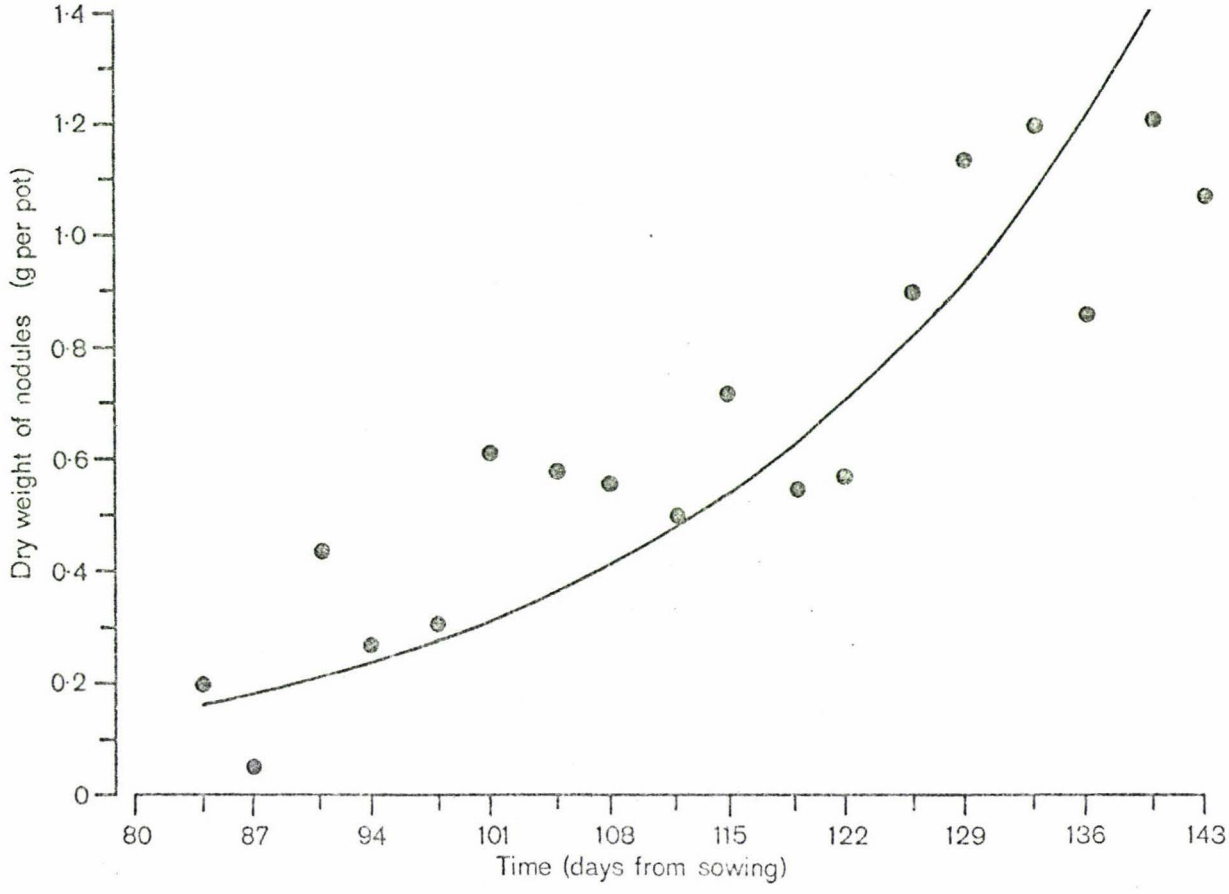


Fig 28 Weight of nodules - expt.2 (details as for Fig 14).

In some plants, for no apparent reason, nodulation did not occur. There were a number of pots where one plant was found to have abundant nodules and the other none at all (plate 8). Thus there were extremes where plants were well nodulated, or possessed no nodules, but there was also a whole range in between. This observed variability in the ability of sainfoin plants to nodulate is seen in the variability which exists in nodule numbers and nodule weights per pot (Figs 27 & 28), and is manifest in the low $\bar{R}^2$ value of the regression of $\log_e$ (nodule dry weight) on time, of 51.8% (Table 10). This variability in ability to form nodules is very likely the cause of the variability which is seen in the growth and N accumulation of sainfoin dependent on symbiotic N_2 fixation, compared to sainfoin supplied with combined N (Figs 14 & 15. Table 10). The fact that this variability exists between individual plants in their ability to nodulate suggests that a plant host factor is involved in determining the relative success or failure of the N_2 fixing symbiosis.

Nodule weight appeared to be less variable over time than nodule number (Figs 27 & 28). The increase in nodule weight over time appeared to be exponential. The plot of $\log_e$ [nodule dry weight] against time was linear in nature even though, because of the variability in the data, $\bar{R}^2$ for the regression of $\log_e$ [nodule dry weight] on time was only 51.8% (Table 10). The relative nodule growth rate (RGR_{Nod}) of 0.0386 g g^{-1} day^{-1} was significantly higher ($P<0.01$) than RGR_p (0.0250 g g^{-1} day^{-1}) over the period day 84 to day 143, but was not relatively as high as in the N_0 treatment of experiment 1(a). As discussed in Section 2.2.3, the reason for this may be that the N_2 fixing symbiosis was to a greater extent at equilibrium with the rest of the plant and utilising a lower proportion of the photosynthate available to the plant than at the earlier stage when the symbiosis was still becoming established and the plant was attempting to increase its capacity to assimilate atmospheric N_2. Over the period day 84 to day 143, nodule dry weight as a proportion of total dry weight did not show a significant increase, and had a mean value of 11.0%. Koter (1965b) found sainfoin to have a lower proportion of its dry weight as nodule tissue (than in this experiment), with nodule tissue making up 1.9 and 7.8 percent of total dry weight at 28 days and at the flowering stage respectively, when totally dependent on symbiotic fixation for its supply of N. Much of the data in the literature, relating nodule weight to total dry weight, refers to younger plants than those in this experiment, but comparisons with this data indicate that sainfoin in this series of experiments had higher proportions of nodule weight than lucerne throughout development (see Sections 1.2.6.2 and 1.3.3).

Plate 8 Well and non nodulated plants from the same I_1N_0 pot, day 85.

Average nodule size increased with time and became relatively large in some pots, but others maintained a low average nodule size (Fig.29). Overall there was not a significant trend with time (the F ratio for the effect of time from the treatment by harvest analysis of variance of nodule size was non-significant), and average nodule size varied greatly between individual pots (Fig.29).

The nodules formed on the roots of sainfoin in this experiment were similar to those described by Wittman (1968), Spedding and Diekmahns (1972) and Schreven (1972), being branched and giving the appearance of being formed in clusters (see also Section 1.2.6.1). Plate 9 illustrates a range of the shapes and sizes of nodules found in this experiment. Some of the larger nodules when sectioned were very dark in colour, but pink or red close to the periphery of the nodule, indicating perhaps that the older central tissue was no longer fixing N_2 (plate 10). If large nodules do contain a zone of inactive tissue this may affect specific nodule activity ($N_2[C_2H_2]$ fixing activity on a nodule dry weight basis) as discussed in Section 2.3.2. It may be that gaseous diffusion into these nodules is restricted, as discussed by Trinick *et al.* (1976) in relation to *Lupinus* nodules.

2.3.2 NITROGEN FIXING ACTIVITY

The increase in $N_2[C_2H_2]$ fixing activity (N_2 fixing activity as measured by the acetylene reduction technique), shown in Fig.30, appeared to be exponential in nature, with the plot of $\log_e$[C_2H_4 production] against time being linear and the regression of $\log_e$ [C_2H_4 production] against time having an $\bar{R}^2$ value of 71.5% (Table 10).

No N_2 $[C_2H_2]$ fixing activity was detected in the treatments other than I_1N_0, and no background activity was detected in the I_0N_0 pots used as blanks.

The relative increase in $N_2[C_2H_2]$ fixing activity (RFA) (a concept also used by Hoglund, 1973) was 0.0434 moles.mole^{-1} day^{-1}, which was non-significantly higher than RGR_{Nod}, but significantly higher than RGR_p ($P<0.001$) and $RARN_p$ ($P<0.01$). This indicates that $N_2[C_2H_2]$ fixing activity is increasing with time in proportion to nodule dry weight, but at a proportionately greater rate than total dry weight or total plant N. This suggests that the shortage of N for plant growth (in plants dependent on symbiotic N_2 fixation) may decrease with plant age. A reasonably close linear relationship was observed between $N_2[C_2H_2]$ fixing activity and nodule dry weight, with $r = 0.71$. The relationship between $N_2[C_2H_2]$ fixing activity and nodule number was relatively weaker with $r = 0.50$, which is consistent with the findings of Chen & Thornton (1940), that the volume of N_2 fixing tissue was a more important criteria in assessing nodule function, than nodule number.

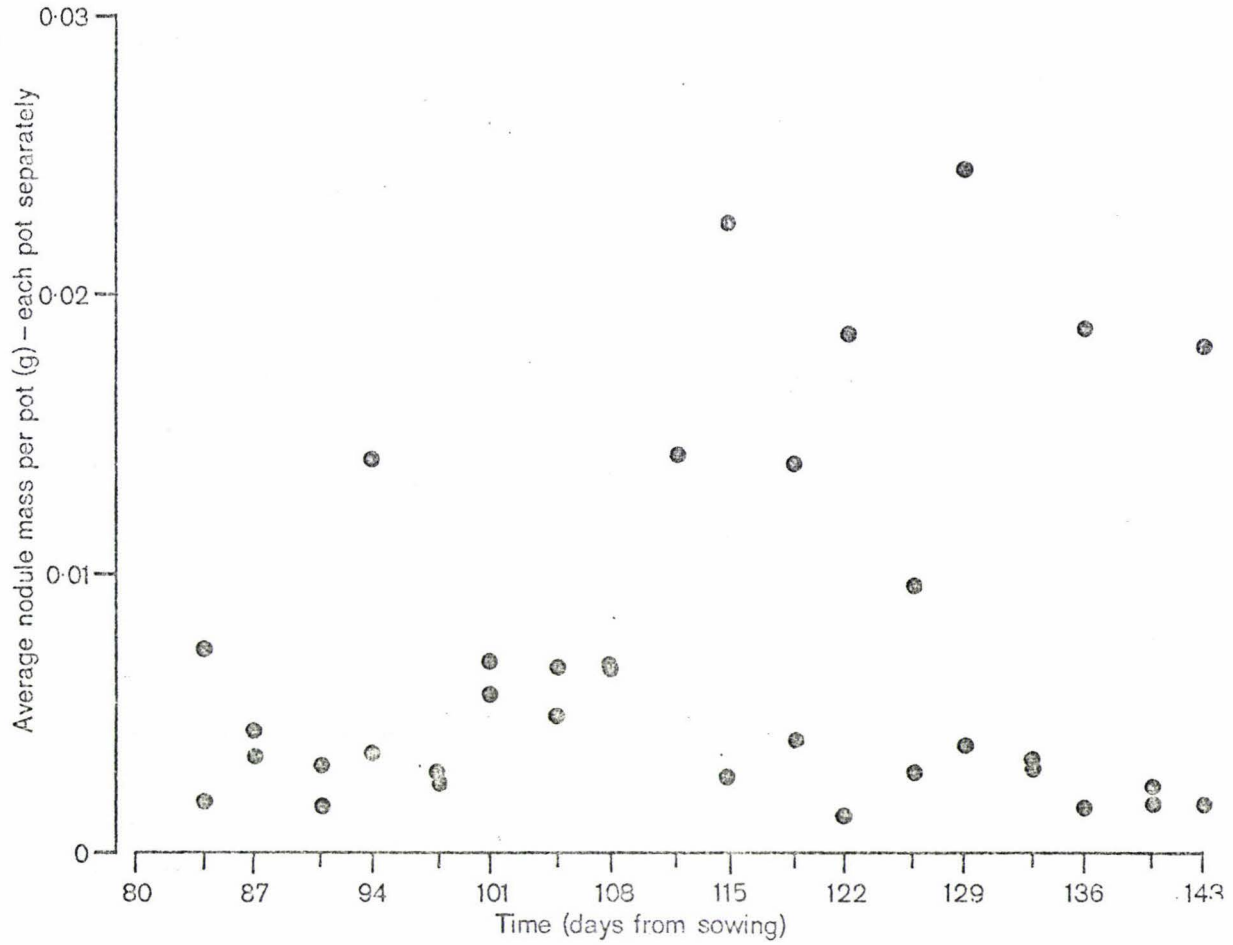


Fig 29 Nodule size - expt.2. Each point is the mean of one pot.

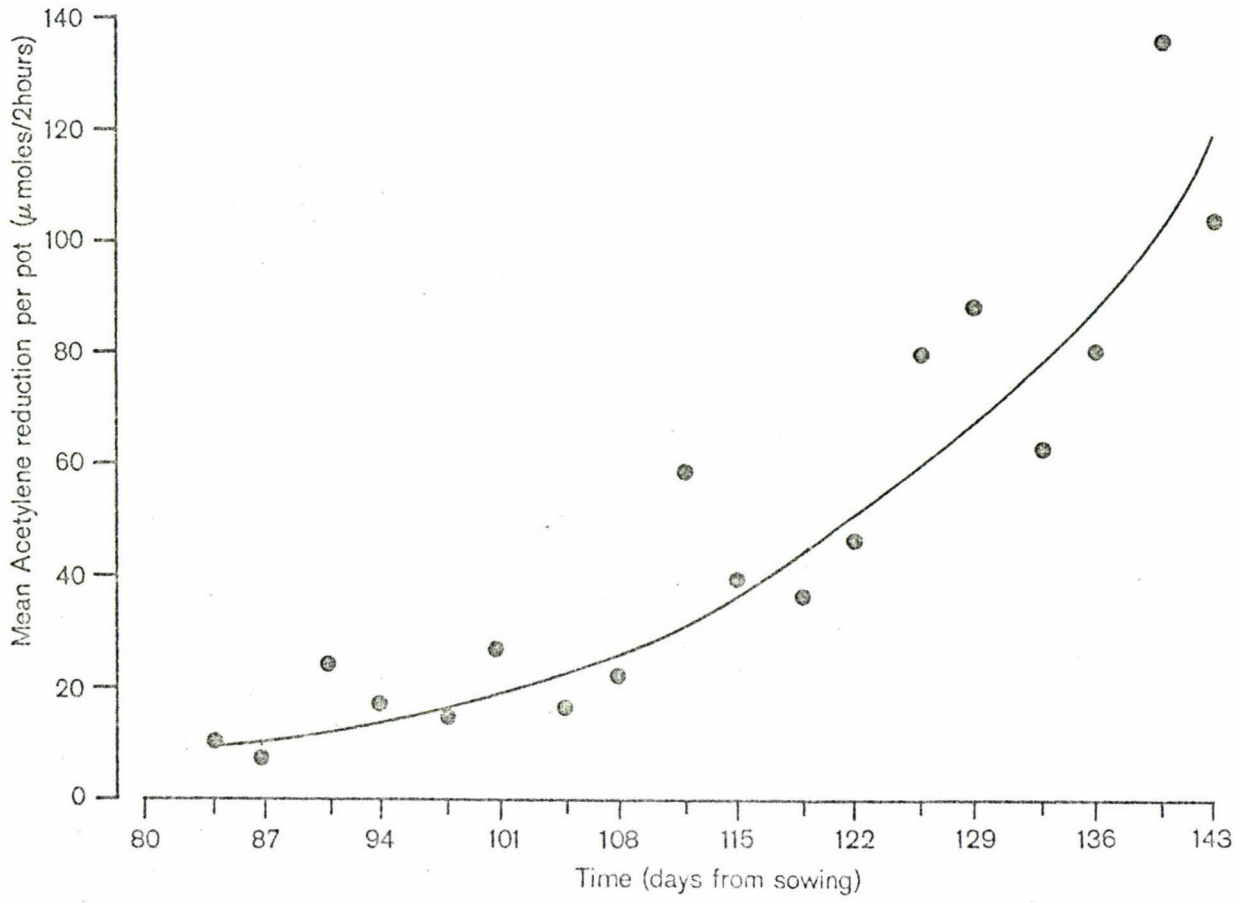


Fig 30 $N_2[C_2H_2]$ fixing activity per pot - expt.2 (details as for fig 14).

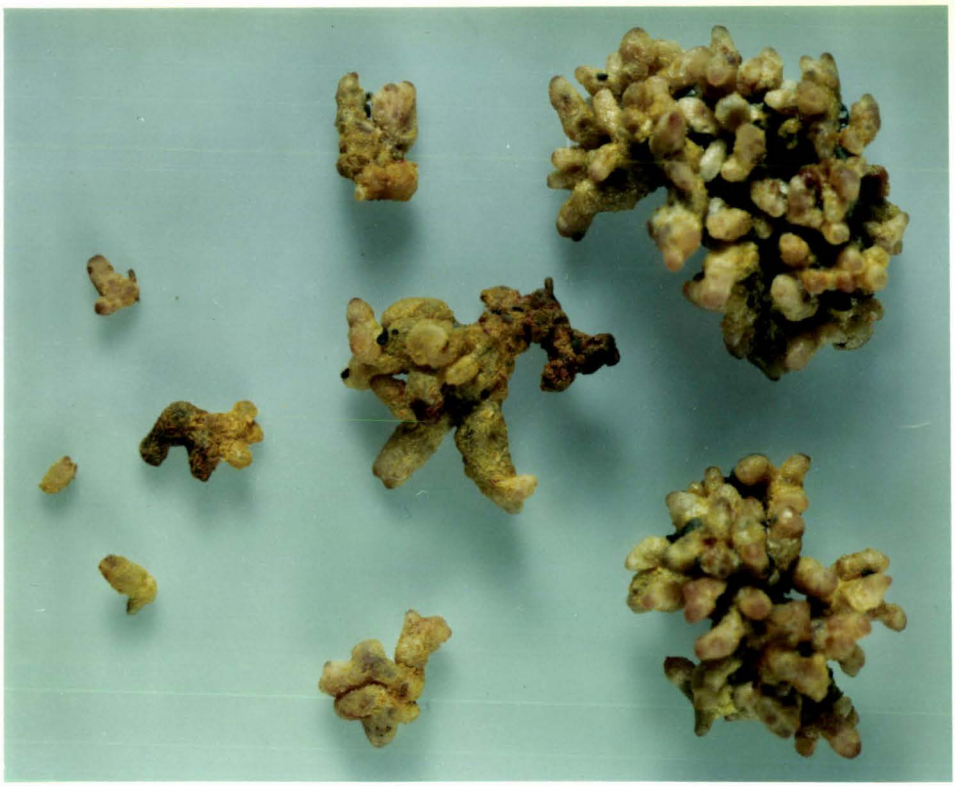


Plate 9 A range of nodule sizes and shapes (collected day 139). (Approximately 2.5 times magnification)

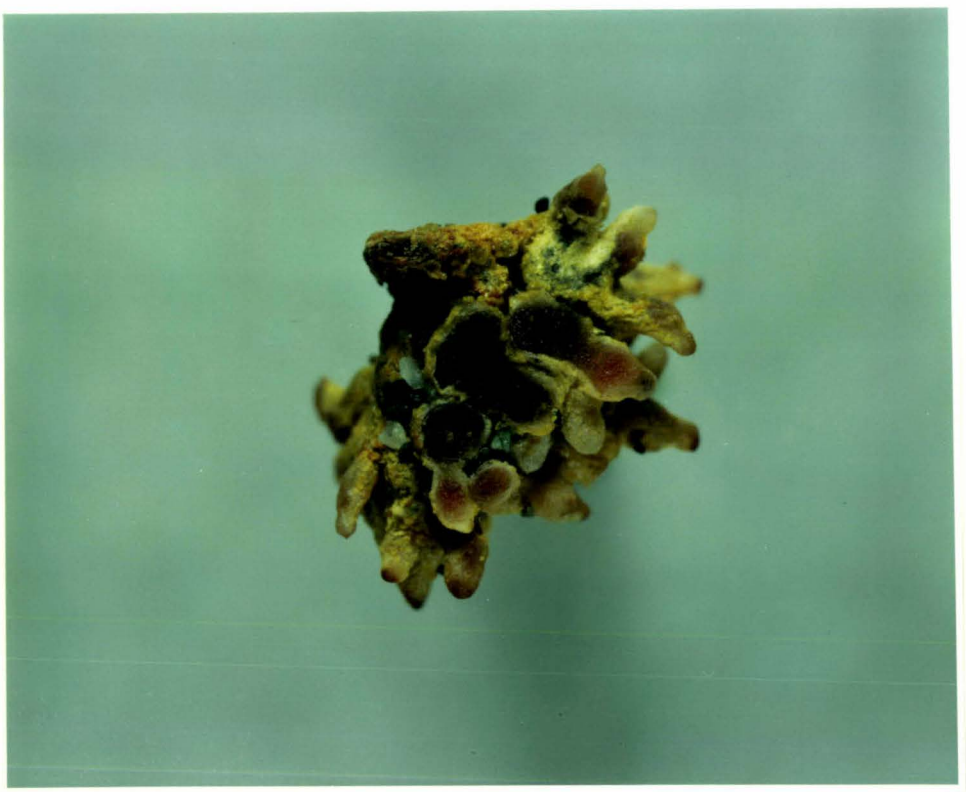


Plate 10 A large branched nodule, sectioned to show dark coloured central tissue, and pale red tissue towards its extremities (collected day 122).

Specific nodule activity ($N_2[C_2H_2]$ fixing activing ÷ nodule dry weight) increased significantly with time (the F ratio for the effect of time from the single treatment, by harvest, analysis of variance was significant at the 5% level), (Fig.31). The overall mean specific nodule activity was 73 µmoles C_2H_4 produced (or C_2H_2 reduced) per 2 hours per g of nodule dry weight. Specific nodule activity varied widely between pots however, and no doubt this was one of the reasons why the correlation between $N_2[C_2H_2]$ fixing activity and nodule weight was not higher than it was. $N_2[C_2H_2]$ fixing activity per total dry weight of plant also increased significantly with time (the F ratio for the effect of time from the single treatment, by harvest, analysis of variance was significant at the 0.1% level) (Fig.32). The mean $N_2[C_2H_2]$ fixing activity on a plant weight basis was 7.91 µmoles C_2H_4 produced per g of total plant dry weight per two hours.

The mean specific nodule activity and $N_2[C_2H_2]$ fixing activity per unit total plant weight of sainfoin in this experiment is compared with values obtained by other workers for sainfoin and a range of other legumes, in Table 13. In all of the examples quoted, the plants were supplied with zero or very low rates of combined N.

Table 13 $N_2[C_2H_2]$ fixing activity - comparison between species

Plant	Age	µmoles C_2H_4 nodule dwt.$^{-1}$ 2 hrs^{-1}	µmoles C_2H_4 plant dwt.$^{-1}$ 2 hrs^{-1}	Reference
Sainfoin	84-143 days	73	7.91	This experiment
Sainfoin	70 days	27.4	1.71	Hanna *et al.* (1978)
Lucerne	49 days	47.0	0.64	" " "
Lucerne	>28 days	216	6.47	Sheehy *et al.* (1980b)
Lucerne	>49 days	68.4		Harding & Sheehy (1980)
Lucerne	49 days	116.5		" " (1980)
Lucerne	vegetative		7.52	Barta (1978)
Birdsfoot trefoil	vegetative		1.43	" "
Lucerne	flowering		6.58	" "
Birdsfoot trefoil	flowering		2.21	" "
Cicer milk-vetch	49 days	51.4	3.46	Hanna *et al.* (1978)
Subterranean clover	30 days	333		Eckart & Raguse (1980)
Soybeans	35 days	217	18.5	Cassman *et al.* (1980)
Peas	28 days	228	16.9	Phillips *et al.* (1976)
Peas	30 days	144	4.81	Bethlenfalvay & Phillips (1977)

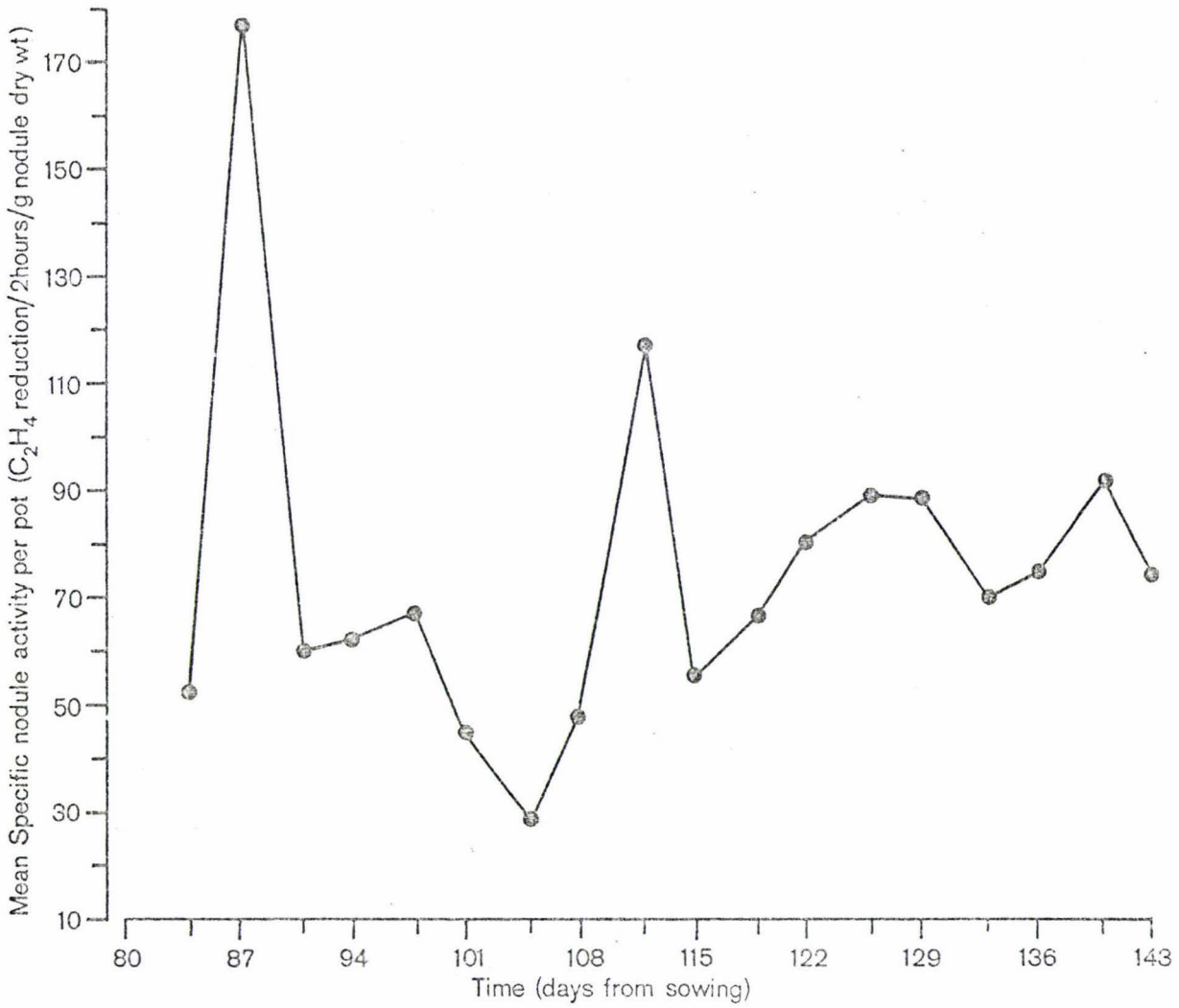


Fig 31 Specific nodule activity - expt.2. Each point is the mean of two pots.

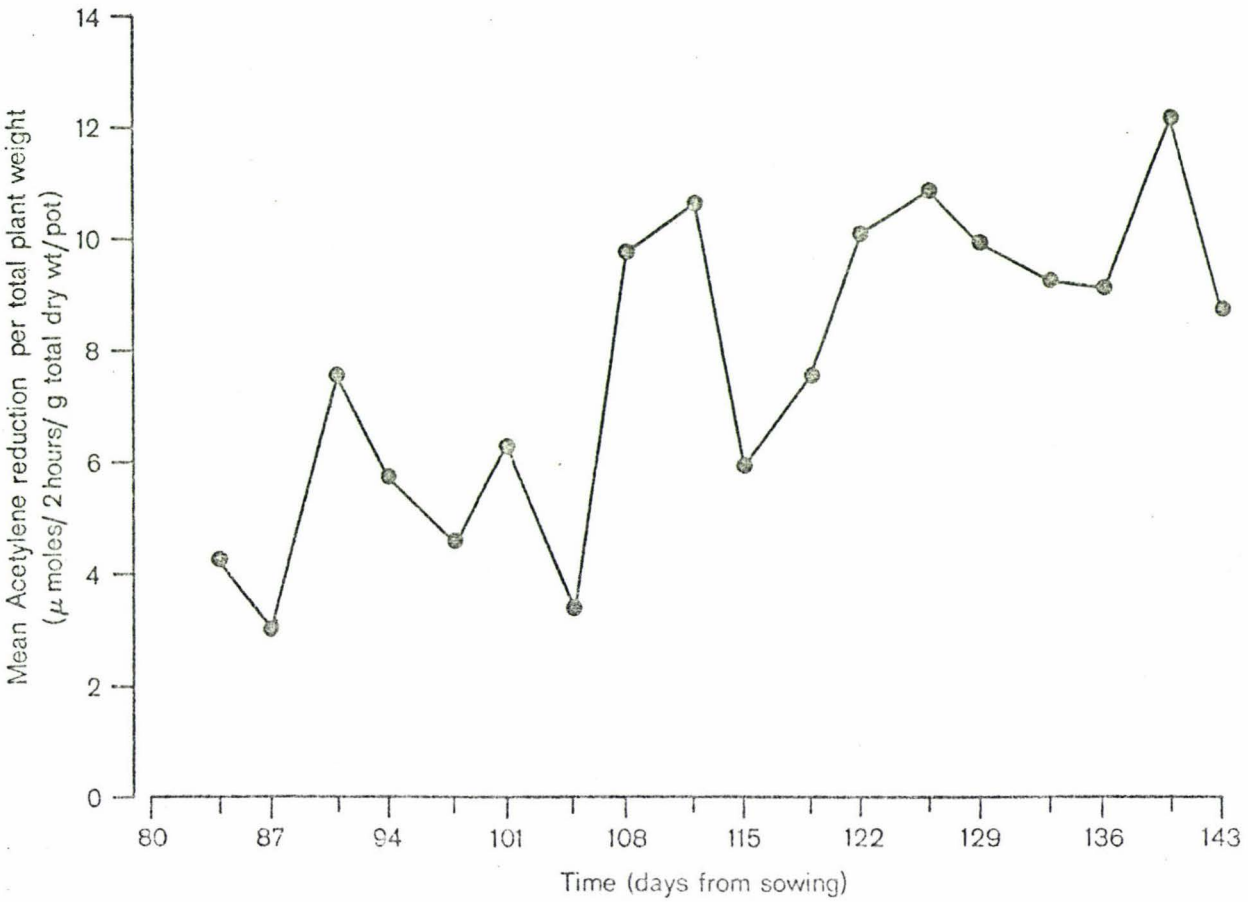


Fig 32 N_2[C_2H_2] fixing activity per unit plant weight - expt.2. Each point is the mean of two pots

Sainfoin appears to have a lower specific nodule activity than most of the other legumes quoted. In the experiments of Hanna *et al.* (1978) the specific nodule activity of sainfoin was lower than that of the other two legumes tested. The specific nodule activity of sainfoin obtained by Hanna *et al.* (1978) was substantially lower than that obtained in this experiment, and that obtained for lucerne was lower than that obtained by other workers. The mean specific nodule activity of sainfoin in this experiment was similar to, or substantially lower than for lucerne, (apart from the value of Hanna *et al.* (1978)) and appeared to be substantially lower than for subterranean clover, soybeans and peas. In terms of $N_2[C_2H_2]$ fixing activity on a total plant dry weight basis, however, sainfoin compared more favourably with other legumes. In the experiments of Hanna *et al.* (1978) sainfoin was found to have a higher $N_2[C_2H_2]$ fixing activity on a total plant weight basis than lucerne, but was lower than cicer milkvetch. The mean $N_2[C_2H_2]$ fixing activity per unit plant dry weight in this experiment appeared to be greater than that of birdsfoot trefoil, similar to that of lucerne (apart from the value of Hanna *et al.* (1978)) and less than that of soybeans and possibly peas. Sainfoin appears to have a high proportion of its dry weight as nodule tissue compared to other legumes (Section 2.3.1). This appears to enable it to have a $N_2[C_2H_2]$ fixing activity on a total dry weight basis comparable with lucerne, which has a relatively low proportion of nodule weight compared to other legumes, despite its apparently lower specific nodule activity. Soybeans and peas appear to have a proportion of nodule weight approaching that of sainfoin (Section 2.3.1), and hence appear to have a higher $N_2[C_2H_2]$ fixing activity on a total plant dry weight basis. Although useful, the above comparisons should be treated with some caution, as they are made on plants of various ages growing under different sets of encironmental conditions.

It was frequently observed that the larger branched nodules of sainfoin contained a zone of very dark, almost black, coloured tissue in the centre, with pale red towards the extremities (Plate 10). If the dark coloured tissue was not actively fixing N_2, this would tend to reduce the $N_2[C_2H_2]$ fixing activity on a nodule dry weight basis and may be a reason for the low $N_2[C_2H_2]$ fixing activity per unit nodule dry weight. A non-significant correlation of -0.12 was observed between mean nodule size per pot and mean specific nodule activity per pot. Thus there was no clear cut decrease in specific nodule activity with nodule size.

2.3.3 MOLAR RATIO OF C_2H_2 REDUCED : N_2 FIXED

The molar ratio of C_2H_2 reduced : N_2 fixed over the period day 84 to day 143 was calculated. The quantity of N_2 fixed over the period was derived from the $RARN_p$ (relative accumulation rate of total plant N) curve, by subtracting the quantity of N at day 84 from that at day 143, and converting moles of N to moles of N_2 . The quantity of C_2H_2 reduced was estimated by integrating the RFA (relative increase in $N_2[C_2H_2]$ fixing activity) curve over the period day 84 to day 143.

The calculated molar ratio of C_2H_2 reduced to N_2 fixed was 3.9:1. The theoretical molar ratio (chapter I, Section 3.2) is 3:1. Two possible reasons for a ratio which is higher than 3:1 are diurnal variation in $N_2[C_2H_2]$ fixing activity, and hydrogen evolution by the N_2 fixing enzyme, nitrogenase (Chapter I, Section 3.2). Diurnal variation in $N_2[C_2H_2]$ fixing activity has been observed in soybean (Hardy *et al.* 1968; Bergersen, 1970; Mague & Burris, 1972), white clover (Sinclair, 1973) and *Medicago truncatula* Gaertn. (Ruegg & Alston, 1978). Diurnal variation in $N_2[C_2H_2]$ fixing activity, where activity was higher during the photoperiod, would result in the measurement of $N_2[C_2H_2]$ fixing activity over the period 12.00 noon to 2.00 p.m., as in this experiment, over estimating average daily N_2 fixing activity. However, Trinick *et al.* (1976), and Haystead *et al.* (1979) observed no diurnal fluctuation in the $N_2[C_2H_2]$ reducing activity of two *Lupinus* species, and white clover respectively. Eckart & Raguse (1980) found that $N_2[C_2H_2]$ fixing activity in subterranean clover varied diurnally in relation to temperature but not to light. It is thought that low night time temperatures may reduce enzyme activity. In this experiment, night time temperatures were lower than daytime temperatures, perhaps increasing the likelihood of diurnal variation in N_2 fixing activity. It was not possible to accurately determine the extent of diurnal variation in $N_2[C_2H_2]$ fixing activity in this experiment. Maximal $N_2[C_2H_2]$ fixing activity is reported to occur around midday (Hardy *et al.* 1973). However, in this experiment acetylene incubations were carried out at a constant room temperature, regardless of the glasshouse temperature. This may have tended to reduce the influence of diurnal variation, as room temperature was usually cooler than glasshouse temperature. Most authors do not record the time at which acetylene incubations are carried out, but presumably they are during the photoperiod and could tend to over-estimate overall $N_2[C_2H_2]$ fixation as discussed for this experiment.

The other possible reason for a discrepancy between the measured and theoretical C_2H_2:N_2 ratios, is that when the symbiotic N_2 fixing system is reducing N_2, a significant proportion of the electron flow to the N_2

fixing enzyme, nitrogenase, may be lost through hydrogen evolution (Schubert & Evans, 1976; Chapter I, Section 2.2.2). However, in the acetylene reduction assay, reduction of C_2H_2 replaces not only the reduction of N_2, but also the evolution of H_2 (Chapter I, Section 3.2). Thus, H_2 evolution would have the effect of increasing the ratio of C_2H_2 reduction:N_2 fixation. It was not possible to make a choice between these two alternative reasons for the C_2H_2:N_2 ratio not being closer to 3:1 because the extent of diurnal variation in $N_2[C_2H_2]$ fixing activity was not known, and H_2 evolution was not measured.

Sinclair *et al.* (1978) calculated ratios of C_2H_2 reduced:N_2 fixed for a number of clovers, lucerne and *Lotus pedunculatus*. The ratios calculated for the clovers were less than 3:1, lucerne was 3.02:1 and *Lotus pedunculatus* was 3.88:1, similar to that calculated for sainfoin in this experiment. The plants were all incubated with C_2H_2 for 2 hours, starting 2.5 hours after the beginning of the photoperiod (Sinclair *et al.* 1978).

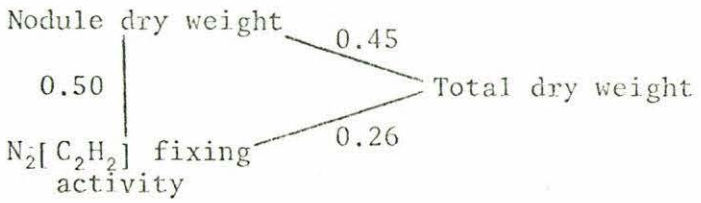
The fact that the calculated C_2H_2:N_2 ratio for sainfoin in this experiment and that of *Lotus pedunculatus* calculated by Sinclair *et al.* (1978) were similarly high, indicates that the N_2 fixing systems of the two species are similarly inefficient. This may be a result of their exhibiting H_2 evolution or a relatively high degree of diurnal variation in $N_2[C_2H_2]$ fixing activity, or a combination of both, as discussed earlier in this section. The expression of hydrogenase (H_2 uptake) activity is found to be primarily under the control of the rhizobial strain (Dixon, 1978), which suggests the possibility of improving N_2 fixing efficiency by appropriate choice of rhizobial strain. The host can, however, exert a modifying influence (Dixon, 1978). Evidence for a plant host influence on nodulation was discussed in Section 2.3.1. One host plant factor which sainfoin and *Lotus* spp. have in common is that both contain high levels of tannins (Chapter I, Section 1.1.2). It has been suggested (Burton & Curley, 1968) that tannins may have an adverse effect on the symbiotic N_2 fixing system. This may account for the apparently low C_2H_2:N_2 ratio discussed above. There appears to be very little published data giving C_2H_2:N_2 ratios, which makes comparisons between species difficult.

2.4 RELATIONSHIPS BETWEEN NITROGEN FIXATION, PLANT GROWTH, AND LEAF AREA.

In this section the relationships between pairs of variables is discussed, and an attempt is made using partial correlations to assess the relative importance of some of these relationships. In all cases correlations have had the time component removed as discussed in chapter II, section 9.2.1. An explanation of the first and second order partial correlation diagrams is given in chapter II, section 9.2.2.

2.4.1 THE INFLUENCE OF NITROGEN FIXATION ON PLANT GROWTH

Total plant dry weight and top dry weight were significantly correlated with both nodule dry weight ($r = 0.69$ in both cases) and $N_2[C_2H_2]$ fixing activity ($r = 0.62$ and 0.68 respectively) but were less well correlated with nodule number ($r = 0.39$ and 0.46 respectively) (table 14). This suggests that nodule weight and $N_2[C_2H_2]$ fixing activity have a greater influence on plant weight than nodule number. Partial correlation coefficients, (r_p) involving nodule dry weight, $N_2[C_2H_2]$ fixing activity and total dry weight, where correlation coefficients were calculated for pairs of variables with the effect of a third variable held constant (see chapter II, section 9.2.2) indicate that nodule dry weight is more closely related to total dry weight than $N_2[C_2H_2]$ fixing activity, as follows:



The correlations of plant weight with nodule weight and $N_2[C_2H_2]$ fixing activity were similar to those of Major *et al.* (1979), who observed correlation coefficients of 0.71, 0.60 and 0.65 between top dry weight and nodule dry weight in sainfoin, lucerne and cicer milkvetch respectively. Poorer relationships were observed between top dry weight and $N_2[C_2H_2]$ fixing activity, with correlation coefficients of 0.44, 0.18 and 0.45 being observed for sainfoin, lucerne and cicer milkvetch respectively (Major *et al.* 1979). Duhigg *et al.* (1978), working with lucerne, observed correlations of similar magnitude to those in this experiment, with correlation coefficients of 0.73 and 0.68 between top weight and nodule score, and top weight and $N_2[C_2H_2]$ fixing activity, respectively.

Table 14 Correlations between plant growth variables and indices of N_2 fixing capacity

Plant growth variable	Index of N_2 fixing capacity: Nodule dry weight	Nodule number	Acetylene reduction
Total dry weight	0.69***	0.39*	0.62***
Top dry weight	0.69***	0.46**	0.68***
Root dry weight	0.38*	0.07	0.31
Leaf area	0.79***	0.60***	0.74***
Total N	0.83***	0.58***	0.77***
Top N	0.75***	0.53**	0.75***
Root+nodule N	0.88***	0.62***	0.75***
Plant height	0.38*	0.44**	0.53**
Number of growth points	0.47**	0.24	0.34

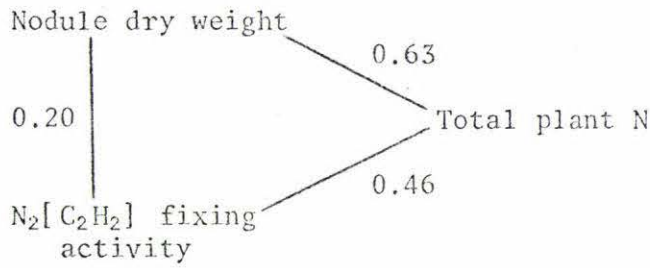
*,**,*** indicate correlations significant at the 5%, 1% and 0.1% levels respectively

Table 15 Correlations between plant growth variables and total N

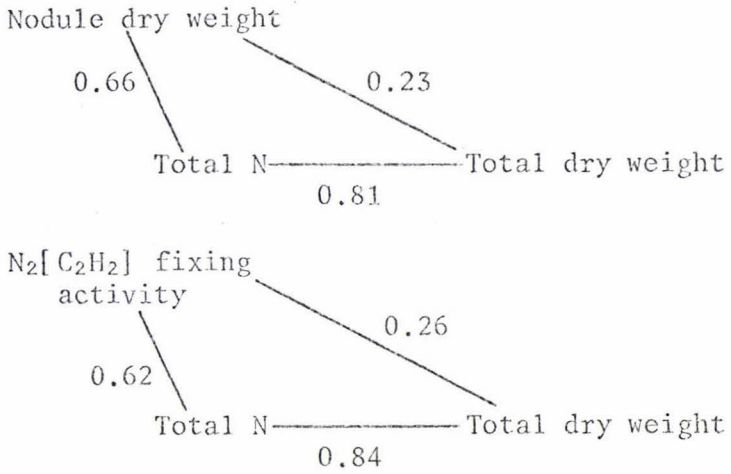
Plant growth variable	Treatment I_1No	I_1N_1	IoN_1
Total dry weight	0.90 ***	0.78 ***	0.90 ***
Top dry weight	0.95 ***	0.82 ***	0.67 ***
Root dry weight	0.50 **	0.37	0.88 ***
Root+nodule dry weight	0.72 ***		
Top:root+nodule ratio	0.69 ***	0.23	-0.14
Leaf area	0.94 ***	0.64 **	0.61 **
Leaf area ratio	0.74 ***	0.40	-0.44 *
Plant height	0.66 ***	-0.17	0.21
Number of growth points	0.62 ***	0.47 *	0.26

*,**, *** indicate correlations significant at the 5%, 1% and 0.1% levels respectively

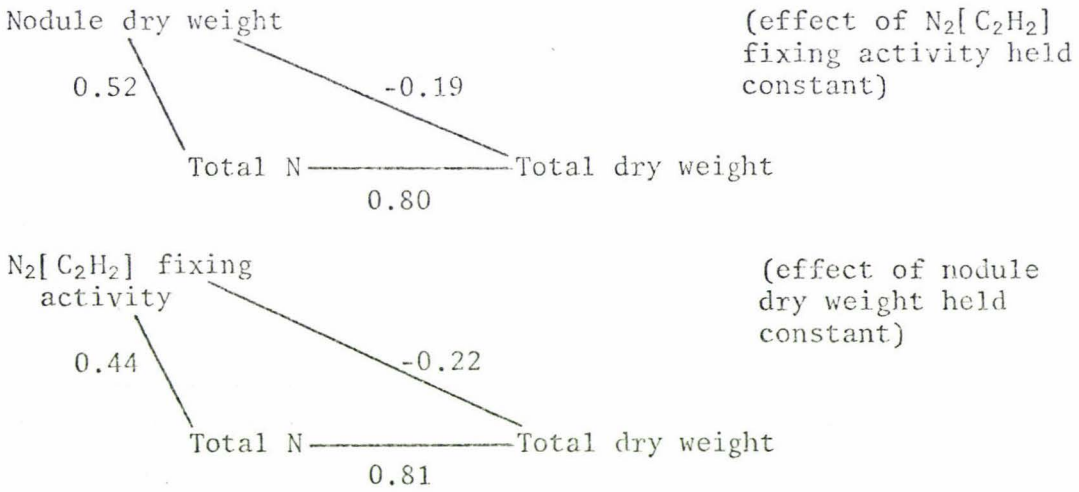
Relationships between plant N, and nodule weight or $N_2[C_2H_2]$ fixing activity appeared to be closer than between plant dry weight, and nodule weight or $N_2[C_2H_2]$ fixing activity (table 14). Total plant N, top N and root N were all significantly correlated with nodule dry weight ($r = 0.83$, 0.75 and 0.88 respectively) and $N_2[C_2H_2]$ fixing activity ($r = 0.77$, 0.75 and 0.75 respectively), but as for plant dry weight, were less well correlated with nodule number ($r = 0.58$, 0.53 and 0.62 respectively). The correlation between total plant N and $N_2[C_2H_2]$ fixing activity was similar to that of 0.76 observed by Sheehy *et al.* (1980b) working with lucerne. Duhigg *et al.* (1978), also working with lucerne, obtained correlation coefficients of 0.68 and 0.69 between top N and nodule score, and top N and $N_2[C_2H_2]$ fixing activity respectively. Partial correlation coefficients involving nodule dry weight, $N_2[C_2H_2]$ fixing activity and total plant N, showed total plant N to be more closely related to nodule dry weight than to $N_2[C_2H_2]$ fixing activity, as follows:



In the I_1N_0 treatment, there was a very close relationship between total plant N and total dry weight with $r = 0.90$ (table 15). In comparison, Sheehy *et al.* (1980b) observed an equivalent correlation of 0.99 for lucerne. Total N was also closely correlated with top dry weight ($r = 0.95$), but to a lesser extent with root + nodule dry weight ($r = 0.72$). Treatments I_1N_1 and I_0N_1, where plants were provided with combined N, tended to display slightly weaker relationships between plant N content and plant weight, having correlation coefficients between total plant N and total plant dry weight of 0.78 and 0.90 for treatments I_1N_1 and I_0N_1 respectively (table 15). Thus, it appears that total plant N plays an important role in influencing total plant weight, particularly in the I_1N_0 treatment. Partial correlation coefficients involving nodule dry weight or $N_2[C_2H_2]$ fixing activity, total plant N and total plant dry weight indicate that both nodule dry weight and $N_2[C_2H_2]$ fixing activity influence total dry weight via total N, as follows:



Two sets of second order partial correlation coefficients (r_{2p}) were calculated to enable the effects of both nodule weight and $N_2[C_2H_2]$ fixing activity to be removed (see chapter II, section 9.2.2) as follows:



The effect observed with first order correlations was enhanced, with the direct link between total dry weight and nodule dry weight or $N_2[C_2H_2]$ fixing activity assuming even less importance. This confirms that nodule weight and $N_2[C_2H_2]$ fixing activity in sainfoin solely dependent on symbiotic fixation for its N supply are linked to total dry weight via total N.

In the I_1N_0 treatment, there were significant correlations between total N and such characteristics as number of growth points ($r = 0.62$) and plant height ($r = 0.66$), whereas in the N_1 treatments these relationships were poorer or non-significant (table 15). This indicates that the development of the 'I_1N_0' plants was more strongly influenced by the level of plant N than that of the 'N_1' plants, suggesting that in the I_1N_0 treatment the level of plant N was limiting plant development.

The relationships between percent N in the plants and the various growth variables, were generally weaker than for the absolute quantities of N (table 16). However, these relationships, similarly to those for quantity of plant N, were stronger in the I_1N_0 than in the N_1 treatments, indicating the critical nature of N levels in the 'I_1N_0' plants.

Thus, it was found that dry weight and development of sainfoin in this experiment were closely linked to the indices of N_2 fixing activity, nodule dry weight and $N_2[C_2H_2]$ fixing activity. These indices of symbiotic N_2 fixing activity appeared to influence plant dry weight via total plant N.

2.4.2 THE ROLE OF LEAF AREA

Sheehy *et al.* (1980b) found whole plant carbon exchange rate to be highly correlated with leaf area (r = 0.94) in lucerne, and stated that, consequently, leaf area can be used as an index for whole plant carbon exchange rate.

In this experiment, in treatment I_1N_0, leaf area was highly correlated with total dry weight, with r = 0.88 (table 17). In treatments I_1N_1 and I_0N_1, the equivalent correlation coefficients were 0.64 and 0.42 respectively. The correlations between leaf area and top dry weight were also higher in the I_1N_0 treatment (r = 0.92) than in the I_1N_1 and I_0N_1 treatments (r = 0.68 and 0.26 respectively). This suggests that leaf area, and hence capacity to produce photosynthate, was more closely linked to total plant dry weight of sainfoin when it was dependent for its N supply on symbiotic fixation. Close relationships between leaf area and the growth of legumes have also been observed elsewhere. Foutz *et al.* (1976), observed a correlation of 0.90 between yield (top weight) and leaf area in field planted lucerne, and Hart *et al.* (1978) observed correlations between yield and estimated leaf area of 0.87 and 0.91 in field grown lucerne.

Close relationships were also found between leaf area and nodule dry weight, and leaf area and $N_2[C_2H_2]$ fixing activity, with r = 0.79 and 0.74 respectively (table 14). Partial correlation coefficients involving nodule dry weight, $N_2[C_2H_2]$ fixing activity and leaf area indicate that leaf area was slightly more closely linked to nodule dry weight than to $N_2[C_2H_2]$ fixing activity as follows:

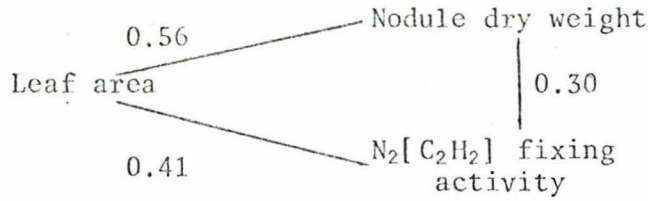


Table 16 Correlations between plant growth variables and total %N

Plant growth variable	Treatment I_1N_0	I_1N_1	I_0N_1
Total dry weight	0.46 **	-0.38	0.02
Top dry weight	0.63 ***	-0.16	-0.24
Root dry weight	-0.02	-0.51 *	0.31
Root+nodule dry weight	0.23		
Top:root+nodule ratio	0.80 ***	0.19	0.22
Leaf area	0.73 ***	-0.08	0.19
Leaf area ratio	0.71 ***	0.28	-0.52 *
Plant height	0.60 ***	-0.51 *	0.36
Number of growth points	0.43 *	0.31	-0.23

*, **, *** indicate correlations significant at the 5%, 1% and 0.1% levels respectively

Table 17 Correlations of leaf area with total and top dry weight

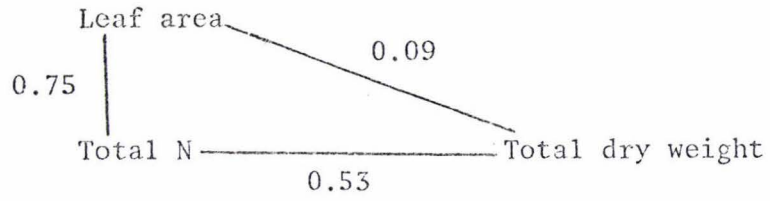
	Treatment I_1N_0	I_1N_1	I_0N_1
Total dry weight	0.86 ***	0.64 ***	0.42 *
Top dry weight	0.92 ***	0.68 ***	0.26

*,**, *** indicate correlations significant at the 5%, 1% and 0.1% levels respectively

Sheehy *et al.* (1980b) working with lucerne observed a correlation of 0.80 between $N_2[C_2H_2]$ fixing activity and leaf area, which was similar to that observed in this experiment.

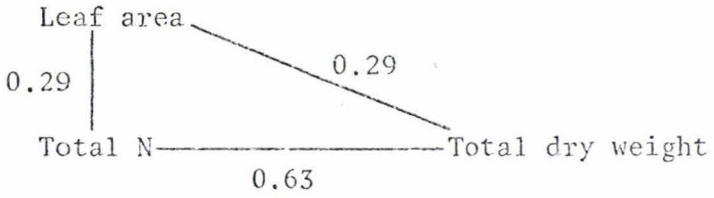
The relationship between total plant N and leaf area in the I_1N_0 treatment was particularly close, with r = 0.94, and appeared to be closer than the equivalent relationships in the I_1N_1 and I_0N_1 treatments, where correlation coefficients were 0.64 and 0.61 respectively (table 15). Thus, it appears that leaf area is more closely linked with total N, as it was with total dry weight, where plants were dependent on symbiotic fixation for their supply of N. Sheehy *et al.* (1980b) observed a correlation of 0.97 between total plant N and leaf area, similar to that observed in this experiment. The relationship between LAR and total N was also closer in the I_1N_0 treatment than in the N_1 treatments. Correlations between LAR and total N were 0.74, 0.40 and -0.44 in treatments I_1N_0, I_1N_1 and I_0N_1 respectively (table 15). Similarly, in the I_1N_0 treatment, top:root + nodule ratio was significantly correlated with total N (r = 0.69), whereas in the N_1 treatments, the equivalent correlations were non-significant (table 15). Total percent N was also highly correlated with leaf area, LAR and top:root + nodule ratio in the I_1N_0 treatment (the last of the three correlations being higher than with total N), whereas in the N_1 treatments the equivalent correlations were either non-significant or negative (table 16). Thus, in sainfoin dependent on symbiotic N_2 fixation, both total N and the proportion of N in the plant are closely linked to leaf area, and dry matter partitioning as expressed in LAR and top:root + nodule ratio.

Close relationships were found between total plant N, and total dry weight (section 2.4.1) on the one hand, and leaf area on the other. This, and particularly the fact that these relationships appear to be closer in plants dependent on symbiotic fixation for their N supply than in plants supplied with abundant combined N, suggest two possible hypotheses. The first is that N content is critical to the growth of sainfoin dependent on symbiotic N_2 fixation, and that the supply of carbon (or energy) from leaf photosynthesis has a critical influence on N_2 fixation and hence total N. An alternative hypothesis is that plant N, and therefore N_2 fixation, was limiting the production of both total dry weight and leaf area. Partial correlations involving leaf area, total N and total dry weight for the I_1N_0 treatment, indicate that leaf area is linked to total dry weight via total N and that total N is limiting plant growth rather than photosynthate supply directly as follows:

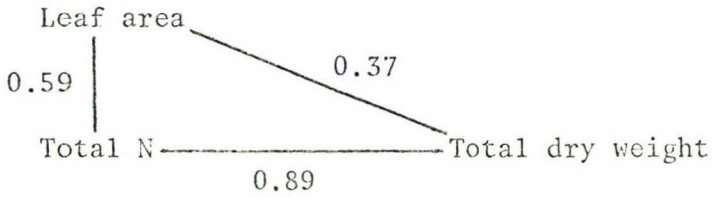


The partial correlations tend to support the first hypothesis, above, rather than the latter, because if the limited supply of N was not in any way linked to energy supply, it could be expected that the link between total N and total dry weight would be at least as strong as that between total N and leaf area, as total plant weight is probably a better indicator of N utilisation, or demand for N, than leaf area. Similar sets of partial correlations were calculated for the two N_1 treatments, as follows:

Treatment I_1N_1

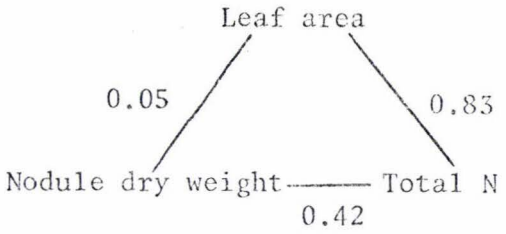


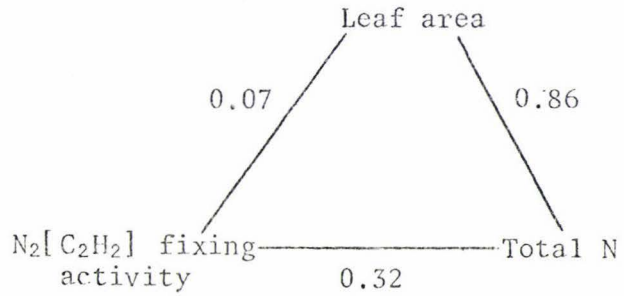
Treatment I_0N_1



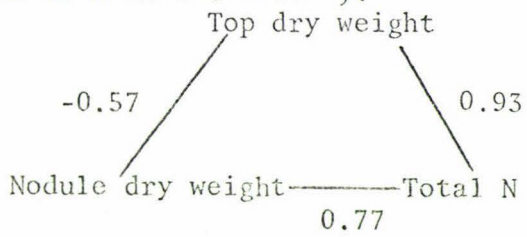
These partial correlations showed a stronger direct link between leaf area and total dry weight and a weaker direct link between leaf area and total N in sainfoin supplied with combined N, compared with sainfoin dependent on symbiotic N_2 fixation, in which the leaf area - total N - total dry weight pathway dominated. Also, the link between total N and total dry weight became stronger relative to that between leaf area and total N (compared to treatment I_1N_0) which is consistent with a decreased influence (via the provision of energy) of leaf area on N accumulation. However, energy is still required for the assimilation of combined N. In the N_1 treatments it is likely that growth is limited by factors other than N, such as environmental conditions or the physiological capacity of the plant to assimilate carbon.

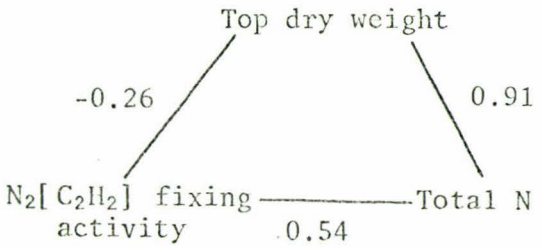
Partial correlations were calculated involving leaf area, nodule dry weight or $N_2[C_2H_2]$ fixing activity, and total plant N, as follows:





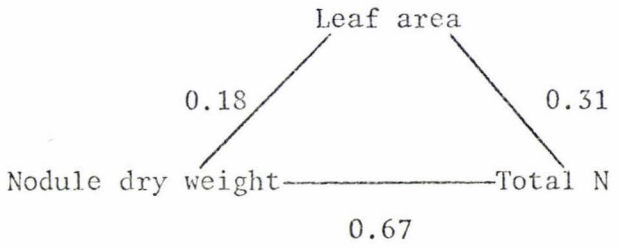
Superficially, this could suggest a weak direct link between leaf area and nodule weight or $N_2[C_2H_2]$ fixing activity, and that leaf area influences nodule weight via demand for N. It was thought that top weight would better represent a demand for N, so sets of partial correlation coefficients were calculated involving top dry weight, nodule dry weight or $N_2[C_2H_2]$ fixing activity, and total N, as follows. (It could be argued, however, that leaf area is a good indicator of accumulated N because of the high concentration of N in the leaves):



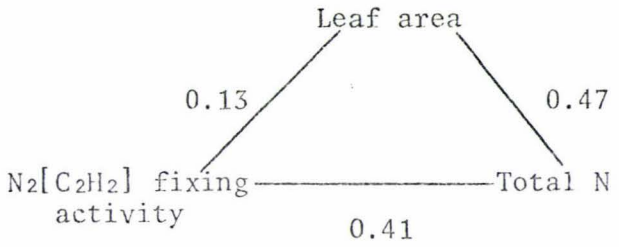


The partial correlations between top weight and nodule weight or $N_2[C_2H_2]$ fixing activity became negative, and those between total N and top weight became relatively higher when top dry weight was included instead of leaf area. This suggests that the relationship between leaf area and nodule dry weight involves something other than simply a demand for N for leaf growth and that top weight does better represent demand for N than leaf area. Indeed from the biological stand point, leaf area must almost certainly have a link to total N via N_2 fixing activity (as estimated by

nodule dry weight or $N_2[C_2H_2]$ fixing activity), given that the link between leaf area and total dry weight appears to be largely indirect, via total N, as discussed previously. A set of second order partial correlation coefficients was calculated including leaf area, nodule dry weight or $N_2[C_2H_2]$ fixing activity, and total N, with the effect of top dry weight (and hopefully its demand for N) removed, as follows:



(Effect of top dry weight also held constant)



(Effect of top dry weight also held constant)

This had the effect of relatively increasing the partial correlations between leaf area and nodule dry weight or $N_2[C_2H_2]$ fixing activity and decreasing those between leaf area and total N, further indicating that the relationship between leaf area and total N involves more than merely a demand for N by the leaves.

The seemingly poor link between leaf area and total N via nodule dry weight or $N_2[C_2H_2]$ fixing activity could result from the fact that both nodule weight and $N_2[C_2H_2]$ fixing activity are merely indices of symbiotic N_2 fixing activity. Variability in specific nodule activity may reduce the accuracy of nodule weight as an index of N_2 fixing activity,and the acetylene reduction technique as used in this experiment estimates rather than measures total N_2 fixing activity. As indices of N_2 fixing activity, however, nodule weight and $N_2[C_2H_2]$ fixing activity were consistent with one another, being interchangeable with similar results (as in this section and section 2.4.1) and having a correlation with one another of 0.71. The second order partial correlation coefficient between nodule dry weight and $N_2[C_2H_2]$ fixing activity, where the effects of both total plant N and total dry weight were held constant was, however, low ($r_{2_p} = 0.15$) suggesting a weak direct link between the two. In contrast the other measurements seem inherently more reliable, with total plant N providing a reasonably accurate

integration of N accumulation, and hence N_2 fixation (in the I_1N_o treatment) over time, and leaf area reportedly (Sheehy *et al.* 1980b) being a good index of total plant carbon exchange rate.

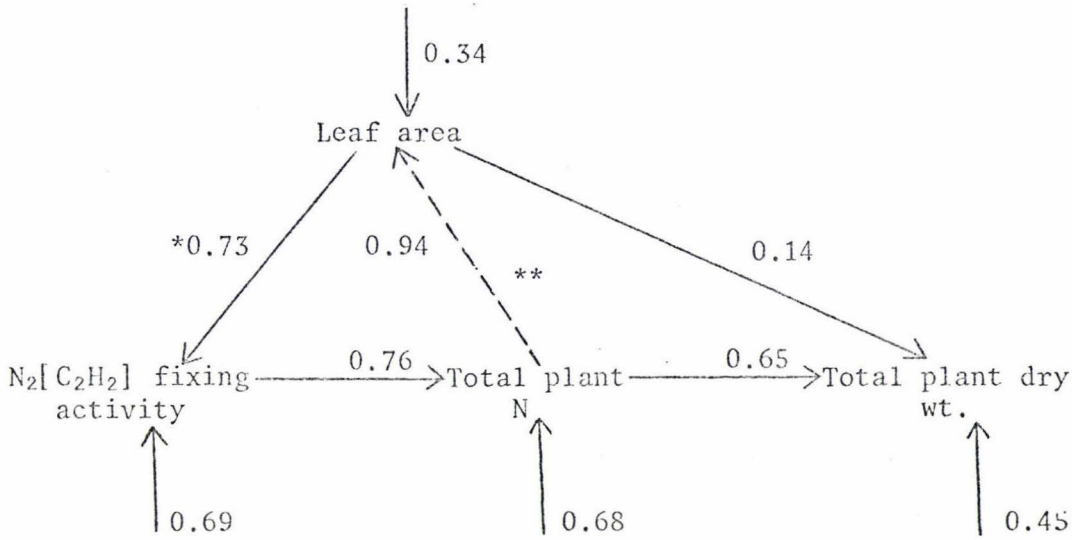
Thus, in sainfoin dependent on symbiotic N_2 fixation, leaf area appeared to be linked to total plant weight via total N rather than directly via carbohydrate supply. Although partial correlations between leaf area and nodule weight or $N_2[C_2H_2]$ fixing activity were low, there was evidence that the relationship between leaf area and total N was not merely one of demand for N by the leaves, indicating that there must be a link between leaf area and total N via N_2 fixation. Total N, in turn, may limit the production of leaf area and total dry weight.

2.4.3 SUGGESTED LINKS BETWEEN LEAF AREA, NITROGEN FIXATION, TOTAL PLANT NITROGEN AND TOTAL PLANT WEIGHT

In sainfoin dependent on a symbiotically fixed N (I_1N_o treatment), total plant N appears to be a key factor controlling plant growth. This is to be expected in plants whose growth was restricted by a shortage of N (section 2.2.2). N_2 fixing activity, as measured by nodule dry weight or $N_2[C_2H_2]$ fixing activity, appeared to be linked to total dry weight via total plant N (section 2.4.1). Leaf area also appeared to be linked to total dry weight via total plant N (section 2.4.2), in contrast to the situation in the N_1 treatments where there was a substantial direct link between leaf area and total plant weight (section 2.4.2). Assuming that leaf area is acting mainly as an energy source rather than as a sink for N, as discussed in section 2.4.2, the link between leaf area and total N must be via symbiotic nitrogen fixation, since I_1N_o plants were solely dependent on symbiotic N_2 fixation for their supply of N. Evidence was found for a direct link between leaf area and N_2 fixing activity (as measured by nodule dry weight or $N_2[C_2H_2]$ fixing activity) despite the low partial correlations between leaf area and nodule dry weight or $N_2[C_2H_2]$ fixing activity (section 2.4.2). Two possible schemes of relationships, consistent with the correlations and partial correlations discussed in sections 2.4.1 and 2.4.2, were envisaged (figs. 33 and 34). Path coefficients were calculated as described in chapter II, section 9.2.3 and these are included in the schemes.

In the first scheme (fig.33) $N_2[C_2H_2]$ fixing activity was included rather than nodule dry weight, as it was thought that $N_2[C_2H_2]$ fixing activity may be a better index of current N_2 fixing activity.

Fig.33 Simplified path diagram for sainfoin dependent on symbiotic N_2 fixation.



* Path coefficients were calculated as explained in chapter II, section 9.2.3.

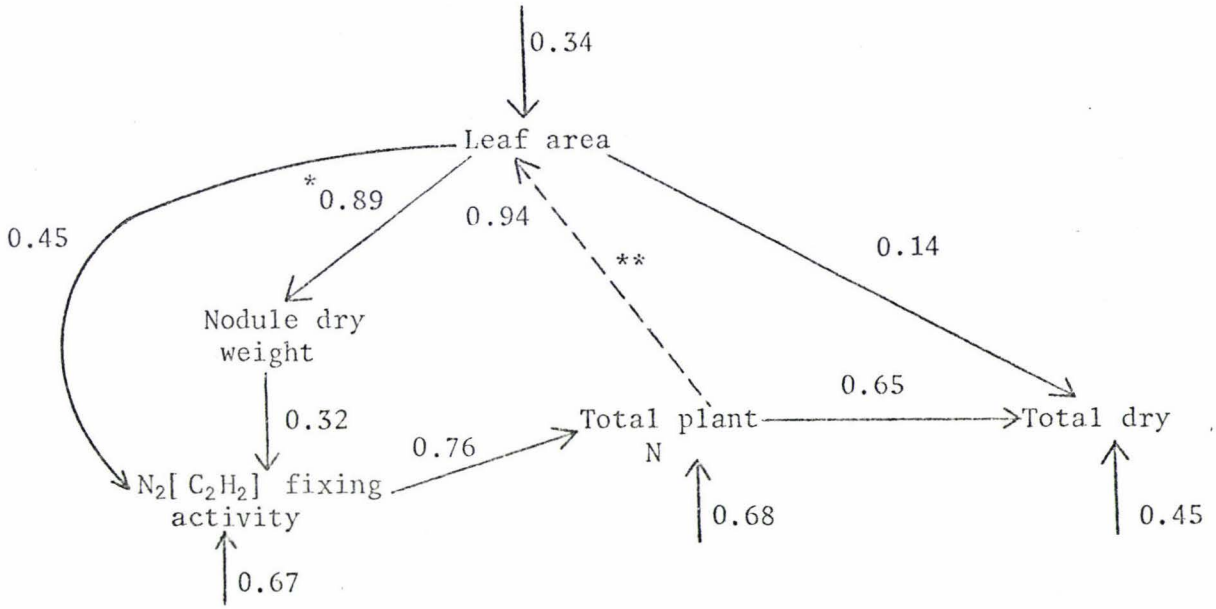
** The dotted arrow indicates the uncertain nature of the direct link between total plant N and leaf area.

The relative similarity of the path coefficients between leaf area and $N_2[C_2H_2]$ fixing activity, $N_2[C_2H_2]$ fixing activity and total plant N, and total plant N and total plant dry weight is perhaps an indication of the similar importance of each of these links and of the importance of this pathway. The weakness of the direct link between leaf area and total dry weight is again apparent.

The relative magnitudes of the path coefficients between leaf area and $N_2[C_2H_2]$ fixing activity, $N_2[C_2H_2]$ fixing activity and total plant N, and total plant N and leaf area differ somewhat from the relative magnitudes of the equivalent partial correlations (section 2.4.2). This appears to be a result of the different procedures for calculating partial correlation and path coefficients.

A second more complete scheme, including both nodule dry weight and $N_2[C_2H_2]$ fixing activity, was envisaged (fig.34).

Fig.34 Path diagram for sainfoin dependent on symbiotic N_2 fixation.



* and ** see fig.33.

This scheme enables a comparison of the relative influences of leaf area directly, and nodule weight on $N_2[C_2H_2]$ fixing activity. It appears that availability of energy has slightly more influence on $N_2[C_2H_2]$ fixing activity than nodule dry weight. A direct link between nodule weight and plant N was not drawn because nodule weight, without N_2 fixing activity, cannot influence total N.

In this experiment it has been found that total plant N is more closely linked to leaf area, and possibly total plant dry weight, in sainfoin dependent on symbiotic N_2 fixation than in sainfoin supplied with abundant combined N. The two hypotheses, suggested in section 2.4.2, to explain the poor N_2 fixing performance of sainfoin in this experiment are related to these findings. The first of these, that leaf area limits total plant N by limiting the energy available for N_2 fixation is supported by the work of Sheehy & Harding (pers.comm.), who compared the growth patterns of sainfoin and lucerne over a summer regrowth period of 48 days. Sainfoin was found to produce approximately 32% less herbage than lucerne. Leaf area indices of the two crops were markedly different, with that of lucerne being over two times greater than that of sainfoin. This higher leaf area index of lucerne was reflected in higher rates of canopy photosynthesis. Total leaf weights were similar in the two plants, but specific leaf area in sainfoin was less than half of that in lucerne. Lucerne, because of its greater

leaf area, appeared to have a greater capacity to intercept photosynthetically active radiation and assimilate carbon (Sheehy *et al.* (1980b) found a very close relationship between leaf area and total carbon exchange rate in lucerne) than sainfoin, and this was thought to result in the better performance of lucerne and its symbiotic N_2 fixing system, compared to sainfoin. Alternatively, however, it could be suggested that the better performance of lucerne resulted, at least in part, directly from a more efficient N_2 fixing system.

Thus, sainfoin is a plant which has a relatively low leaf area in comparison to its total dry weight. In a situation where it is dependent on symbiotic N_2 fixation, N_2 fixing activity may well be limited by the relatively poor (compared with lucerne) ability of the plant to assimilate carbon and provide energy for the process. Thus total plant N would be limited, which in turn would limit total dry weight and further leaf area production. The weak direct link between leaf area and total dry weight suggests that N is limiting growth rather than carbohydrate supply directly. The schemes diagrammed in figs.33 and 34 are in accord with findings indicating that symbiotic N_2 fixation is linked with energy use (e.g. Mahon, 1977) and that increases or decreases in photosynthate supply increase or decrease symbiotic N_2 fixation (Brun, 1972; Streeter, 1973; Havelka & Hardy, 1976; Phillips *et al.* 1976; Sheehy *et al.* 1980a; Vance, 1979), as discussed in chapter I, section 2.2.1.

An alternative explanation for the poor performance of sainfoin dependent on symbiotic N_2 fixation could be that it has a relatively inefficient N_2 fixing system. Evidence for this view was provided by experiment 1(b) (section 3.1) and by the fact that the molar ratio of C_2H_2 fixed:N_2 reduced, for sainfoin, is higher than the theoretical ratio of 3.0 (section 2.3.3).

The findings of this experiment suggest that both of the above explanations i.e. a relatively poor capacity to assimilate carbon and a relatively inefficient symbiotic N_2 fixing system, may contribute to the poor performance of sainfoin dependent on symbiotic N_2 fixation. A balanced interdependence between the photosynthate supplying function of the leaves and the nitrogen supplying function of the roots, as proposed by Raper *et al.* (1977) for soybeans and cotton, is indicated.

3 EXPERIMENT 1(b)

At day 80, treatment N_0 from experiment 1(a) was divided into treatments N_0 and N_L (denoted N_0/N_0 and N_0/N_L respectively), and treatment N_1 was divided into treatments N_L and N_1 (denoted N_1/N_L and N_1/N_1 respectively), as described in chapter II, section 2.1.2. $N_2[C_2H_2]$ fixing activity was monitored regularly throughout the period day 80 to day 135 (fig.35), and destructive harvests were carried out on days 121, 128 and 135.

3.1 TREATMENTS N_0/N_0 AND N_0/N_L

The effect of a low rate of combined N on symbiotic N_2 fixation and growth of sainfoin, previously dependent solely on symbiotic fixation for its N supply, was investigated by making comparisons between treatments N_0/N_0 and N_0/N_L.

The introduction of a low rate of nitrate N (2.5 mM or 35 ppm) in treatment N_0/N_L resulted in an immediate decrease in the level of $N_2[C_2H_2]$ fixing activity in plants previously dependent solely on symbiotic N_2 fixation for their N supply (fig.35). $N_2[C_2H_2]$ fixing activity in the N_0/N_L treatment appeared to be lower than that in N_0/N_0 treatment throughout the period day 80 to day 135. Treatment by harvest analysis of variance (appendix 3) indicated a significant treatment effect (F was significant at the 0.1% level) and that the overall mean $N_2[C_2H_2]$ fixing activity in the N_0/N_0 treatment was significantly higher ($P < 0.05$) than that in the N_0/N_L treatment. At individual assay times, treatment means were usually not significantly different. This reduction in $N_2[C_2H_2]$ fixing activity occurred, even though there was a great deal of potential for increased growth in plants dependent on symbiotic N_2 fixation in comparison to plants supplied with adequate combined N (i.e. treatment N_1/N_1 plants) (table 18).

Table 18 Total dry weight (g per pot) - Treatment by harvest means

Treatment	Day			Overall means
	121	128	135	
N_0/N_0	6.36a*	8.16a	9.75a	8.58a
N_0/N_L	6.88a	8.64a	10.70a	9.03a
N_1/N_L	10.08b	12.30b	15.39b	12.96b
N_1/N_1	10.04b	13.89c	18.79c	15.44c

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

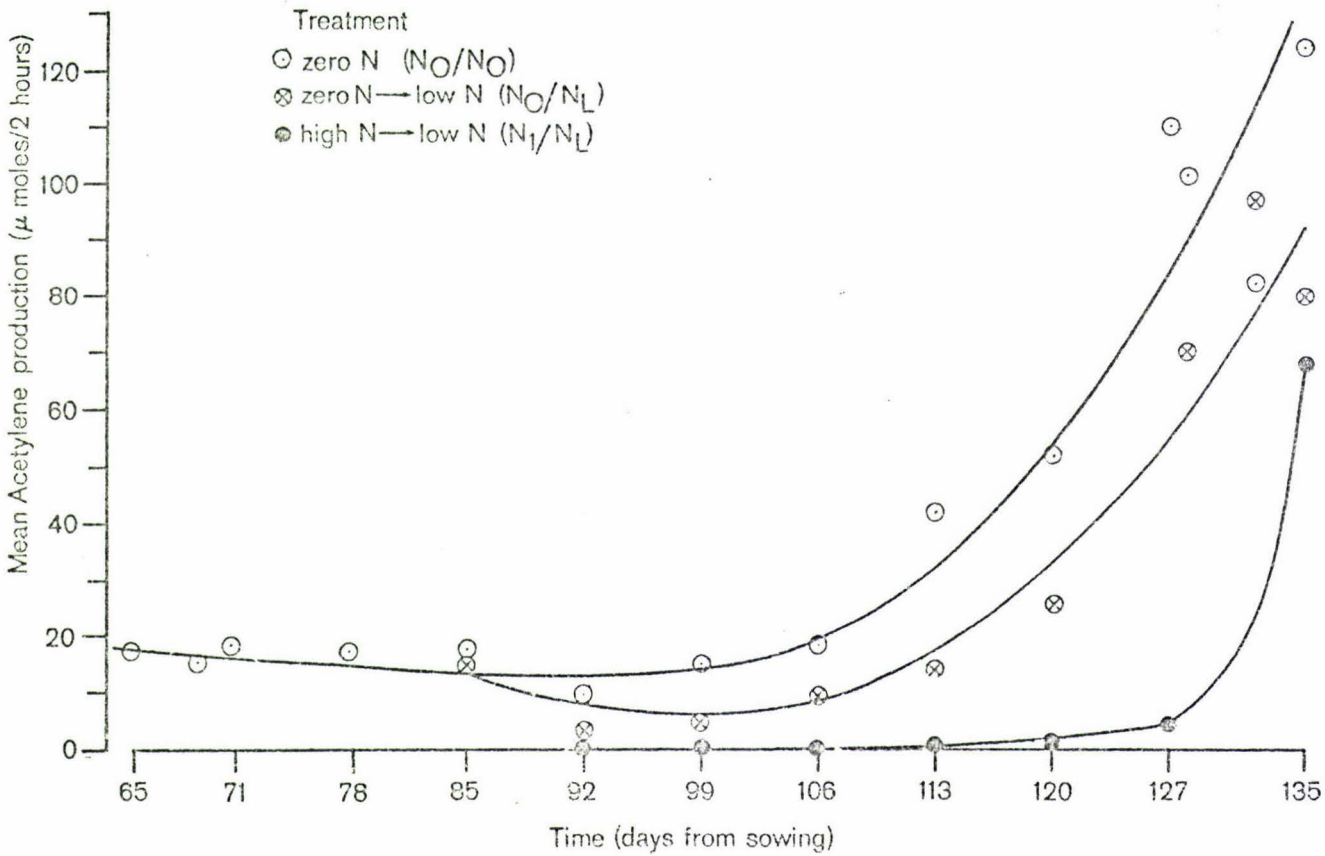


Fig 35 $N_2[C_2H_2]$ fixing activity per pot - expt.1(b). Each point is the mean of two pots. The curves drawn to show the trends were not mathematically fitted.

Mean nodule numbers per pot are given in table 19. Over the three harvests, nodule number in treatments N_O/N_O and N_O/N_L were not significantly different. However, overall, and at each of the three destructive harvests, nodule weight in the N_O/N_O treatment was significantly higher than in the N_O/N_L treatment (table 20). Nodule weight in both treatments increased quite rapidly over the final two weeks of the experiment. Nodule dry weight as a proportion of total dry weight, and as a proportion of root dry weight, was significantly higher in the N_O/N_O treatment than in the N_O/N_L treatment (table 21). $N_2[C_2H_2]$ fixing activity on a nodule dry weight basis was not significantly different between treatments (table 21) and consequently $N_2[C_2H_2]$ fixing activity per unit plant dry weight was significantly higher in the N_O/N_O treatment than in the N_O/N_L treatment (table 22). The ratios involving nodule dry weight were calculated for day 128 because the most complete nodule weight data was collected at this harvest (chapter II, sections 6.1.2, 6.3.2)

Table 19 Nodule number (per pot) - Treatment by harvest means.

Treatment	Day			Overall means
	121	128	135	
N_O/N_O	89a*	216c	229bc	203c
N_O/N_L	58a	272d	170b	202c
N_1/N_L	0a	132b	258c	152b
N_1/N_1	0a	0a	0a	0a

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Table 20 Nodule dry weight (g per pot) - Treatment by harvest means

Treatment	Day			Overall means
	121	128	135	
N_O/N_O	1.010c*	1.112c	1.363d	1.205d
N_O/N_L	0.472b	0.855b	0.808c	0.775c
N_1/N_L	0 a	0.048a	0.373b	0.148b
N_1/N_1	0 a	0 a	0 a	0 a

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Table 21 Proportion of nodule weight and specific nodule activity

Ratios, means at day 128	Treatment			
	N_O/N_O	N_O/N_L	N_1/N_L	N_1/N_1
Nodule dry wt: Total dry wt.	0.134d*	0.099c	0.004b	0a
Nodule dry wt: Root dry wt.	0.352d	0.263c	0.010b	0a
Acetylene reduction: Nodule dry wt.	92.3 b	89.1b	81.4b	0a

* Figures followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Table 22 $N_2[C_2H_2]$ fixing activity (μM C_2H_2 per 2 hours) per unit plant dry weight (g).

Treatment	Day			Overall means
	121	128	135	
N_O/N_O	8.16c*	12.62d	10.56c	11.10d
N_O/N_L	3.69b	8.39c	8.26c	7.57c
N_1/N_L	0a	0.32b	4.56b	1.68b
N_1/N_1	0a	0a	0a	0a

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Although total plant dry weight in the N_O/N_L treatment was consistently higher than in the N_O/N_O treatment at all three harvests, and in terms of overall mean, the differences were not statistically significant (table 18). Total dry weight in the N_O/N_O and N_O/N_L treatments was significantly lower than in the N_1/N_1 and N_1/N_L treatments.

The introduction of a low rate of combined N in the N_O/N_L treatment did appear to influence dry matter distribution in comparison to the N_O/N_O treatment. Overall mean top dry weight was significantly higher in the N_O/N_L than in the N_O/N_O treatment (table 23), and overall mean root + nodule dry weight in the N_O/N_L treatment was non-significantly lower than in the N_O/N_O treatment (table 24). Consequently the top:root + nodule dry weight ratio in the N_O/N_L treatment was significantly greater than in the N_O/N_O treatment (table 25).

Table 23 Top dry weight (g per pot) - Treatment by harvest means

Treatment	Day			Overall means
	121	128	135	
N_o/N_o	2.96a*	3.91a	4.88a	4.19a
N_o/N_L	3.40a	4.49a	5.78b	4.74b
N_1/N_L	5.68b	7.39b	8.85c	7.59c
N_1/N_1	6.30b	9.47c	12.85d	10.46d

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Table 24 Root + Nodule dry weight (g per pot) - Treatment by harvest means

Treatment	Day			Overall means
	121	128	135	
N_o/N_o	3.40a*	4.24a	4.87a	4.39a
N_o/N_L	3.48a	4.15a	4.92a	4.30a
N_1/N_L	4.41b	4.91b	6.54b	5.37c
N_1/N_1	3.75ab	4.43ab	5.95b	4.98b

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Table 25 Top:root + nodule ratio - Means at day 128

	Treatment			
	N_o/N_o	N_o/N_L	N_1/N_L	N_1/N_1
Ratio of top dry weight: root + nodule dry weight	0.95a*	1.10b	1.42c	2.10d

* Figures followed by different letters differ at the 5% level of significance (according to standard errors of differences)

Total plant N levels did not differ significantly between the N_o/N_o and N_o/N_L treatments (table 26), and neither did top or root N (N analyses were carried out on day 128 and 135 harvests only). Total percentage N also did not differ significantly between the N_o/N_o and N_o/N_L treatments (table 27), and this was true also for percentage N in the tops and roots + nodules.

The reduction in $N_2[C_2H_2]$ fixing activity, decrease in nodule weights and lack of significant response in terms of total plant dry weight or total N on addition of a low rate of N in the N_o/N_L treatment, suggests that

the nitrate N supplied was tending to replace rather than supplement symbiotic N_2 fixation in sainfoin. The main effect of the N_o/N_L treatment appeared to be on dry matter distribution, with added combined N resulting in higher top weights, reduced nodule weights, similar root + nodule weights and an increased top:root + nodule ratio. It could be suggested that the higher top weights, and thus presumably higher leaf areas (leaf areas were not measured, but were found to be reasonably highly correlated with top weight in experiment 2, see table 17) in the N_o/N_L treatment would benefit N_2 fixation relative to that in the N_o/N_o treatment. However, no such effect was apparent up to day 135, 55 days after the introduction of the N_o/N_L treatment. Perhaps the time period was not long enough for the probably improved photosynthetic capacity of the plant to have an influence on the N_2 fixing system.

Table 26 Total plant N (g per pot) - Treatment by harvest means

Treatment	Day		Overall means
	128	135	
N_o/N_o	0.183a*	0.216a	0.199a
N_o/N_L	0.190a	0.218a	0.202a
N_1/N_L	0.227b	0.253a	0.237b
N_1/N_1	0.413c	0.491b	0.444c

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Table 27 Total % N - Treatment by harvest means

Treatment	Day		Overall means
	128	135	
N_o/N_o	2.232b*	2.203b	2.217b
N_o/N_L	2.221b	2.040b	2.146b
N_1/N_L	1.853a	1.642a	1.754a
N_1/N_1	2.976c	2.660c	2.834c

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

The response of sainfoin, particularly that of its symbiotic N_2 fixing system to a low rate of combined N is similar to that observed in birdsfoot trefoil (Allos & Bartholomew, 1959). It was found that even at the lowest rate (80 mg per pot during 10 weeks growth), application of combined N resulted in a decrease in N_2 fixation. The birdsfoot trefoil, like sainfoin in this experiment, had ample potential for increased growth, with plants supplied with a high rate of combined N producing

approximately 1.8 times as much dry weight and 2.1 times as much N as plants solely dependent on symbiotic N_2 fixation for their N supply (Allos & Bartholomew, 1959).

Combined N, particularly in the form of nitrate, has been found to reduce N_2 fixing activity in a wide range of legumes, at levels ranging from similar to that in the N_L treatment of this experiment, to considerably higher than this (Allos & Bartholomew, 1955; Oghoghorie & Pate, 1971; Pate, 1977; Hojjati *et al.* 1978; Dean & Clark, 1980; Wong, 1980). However, it has also been found that additions of N (ranging from levels similar to the N_L level in this experiment to levels which would probably be sufficient to totally suppress symbiotic N_2 fixation in sainfoin) can stimulate N_2 fixation in legumes (Allos & Bartholomew, 1955; Allos & Bartholomew, 1959; Copeland & Pate, 1969; Hoglund, 1973; Pate, 1976; Bethlenfalvay *et al.* 1978a). The benefit of combined N to a N_2 fixing symbiosis is thought to be indirect, coming via the general stimulus which added N may give to plant growth (Pate, 1976).

Thus, sainfoin appears to behave differently to most other legumes, in that $N_2[C_2H_2]$ fixing activity was decreased by even a low rate of combined N, similarly to the effect on birdsfoot trefoil observed by Allos & Bartholomew (1959).

This response to combined N tends to support the hypothesis (section 2.4.2) that the symbiotic N_2 fixing system of sainfoin is in some way inefficient and that when combined N is supplied it is advantageous to the plant to substitute the combined N for symbiotically fixed N. Both sainfoin and birdsfoot trefoil are non bloating legumes containing tannins. It could be that the relatively high levels of tannins which they contain may have an adverse effect on the symbiotic N_2 fixing relationship, as speculated by Burton & Curley (1968).

Alternatively it could be argued that, because of its relatively low LAR, it is advantageous for sainfoin to channel energy into increasing its leaf area. The fact that combined N was applied may have enabled plants in the N_O/N_L treatment to channel carbon, normally consumed in the N_2 fixing process, into production of leaves, with no decline in N status.

3.2 TREATMENTS N_1/N_1 AND N_1/N_L

The introduction of the N_1/N_L treatment, in which plants formerly supplied with a high rate of combined N (as nitrate) were introduced to a low rate, resulted in nodulation and the initiation of $N_2[C_2H_2]$ fixing activity (table 19, fig.35). Nodulation, and the initiation of $N_2[C_2H_2]$ fixation in the N_1/N_L treatment may have been delayed by the delay in re-inoculation discussed in chapter II, section 2.1.1. In any event, evidence of nodulation and $N_2[C_2H_2]$ fixing activity was not observed until day 127, 18 days after re-inoculation. This interval between re-inoculation and nodulation was similar to that observed between initial inoculation and nodulation (chapter III, 1.2.6.1). Once nodulation had occurred, nodule numbers increased very rapidly, and within a short time the level of $N_2[C_2H_2]$ fixing activity was approaching that of the N_0/N_L treatment (fig.35). As part of the treatment by harvest analysis of variance conducted on data from the destructive harvests on days 121, 128 and 135 was an analysis of variance of acetylene reduction data for the pots destructively harvested on those days (table 28). At the time of the final destructive harvest, $N_2[C_2H_2]$ fixing activity in the N_1/N_L treatment was not significantly lower than that in the N_0/N_L treatment.

Table 28 $N_2[C_2H_2]$ fixing activity (μM C_2H_2 per 2 hours) - Treatment by harvest means

Treatment	Day			Overall means
	121	128	135	
N_0/N_0	51.6b*	105.4c	103.3c	96.8d
N_0/N_L	25.4ab	72.0b	88.6bc	69.8c
N_1/N_L	0.0a	4.0a	68.1b	24.9b
N_1/N_1	0.0a	0.0a	0.0a	0.0a

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Nodule number increased very rapidly in the N_1/N_L treatment during the two weeks over which destructive harvests were conducted, to reach a level similar to that in the N_0/N_0 and N_0/N_L treatments by day 135 (table 19). No nodules were found in the N_1/N_1 treatment. At day 135, nodule weight in the N_1/N_L treatment was still significantly lower ($P < 0.05$) than in treatment N_1/N_L (table 20).

The total dry weight in the N_1/N_L treatment was significantly lower than that in the N_1/N_1 treatment after day 121 (table 18), indicating that the N_L rate of N was insufficient to sustain maximum growth of sainfoin

under the conditions of this experiment. Total dry weight in both the N_1/N_1 and N_1/N_L treatments was significantly higher than on the N_O/N_O and N_O/N_L treatments.

The reduction in level of N supply in the N_1/N_L relative to the N_1/N_1 treatment, as well as reducing total yield, also influenced dry matter distribution. In the N_1/N_L treatment top dry weight was significantly lower than in the N_1/N_1 treatment (table 23), but root + nodule weight (in terms of overall mean) was significantly higher than in the N_1/N_1 treatment (table 24). Consequently top:root + nodule weight ratio was significantly higher in the N_1/N_1 than in the N_1/N_L treatment (table 25). Top dry weight in both the N_1/N_1 and N_1/N_L treatments were significantly higher than in the N_O/N_O or N_O/N_L treatments (table 23), as were overall mean root + nodule weights (root + nodule weight = root weight in the N_1/N_1 treatment) (table 24). At two of the three individual harvests, however, root weight in the N_1/N_1 treatment was not significantly greater than root + nodule weight in the N_O/N_O and N_O/N_L treatments. Comparative top growth of the four treatments is shown in plate 11.

The total plant N level in the N_1/N_L treatment was significantly and substantially, lower than that in the N_1/N_1 treatment, and was significantly higher than in the N_O/N_L and N_O/N_O treatments (table 26). The level of top N in the four treatments followed a pattern very similar to that of total plant N (table 29). Levels of root N in treatment N_1/N_L were significantly lower than in treatment N_1/N_1 and similar to those in treatments N_O/N_O and N_O/N_L (table 30).

Table 29 Top N (g per pot) - treatment by harvest means

Treatment	day		Overall means
	128	135	
N_O/N_O	0.101a*	0.120a	0.112a
N_O/N_L	0.107a	0.128a	0.116a
N_1/N_L	0.148b	0.155a	0.151b
N_1/N_1	0.284c	0.349b	0.315c

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Plate 11 Top growth in experiment 1(b) at day 122 (left to right treatments N_O/N_O, N_O/N_L, N_1/N_L, N_1/N_1).

Table 30 Root N (g per pot) - Treatment by harvest means

Treatment	Day		Overall means
	128	135	
N_O/N_O	0.082a*	0.096a	0.089a
N_O/N_L	0.083a	0.091a	0.086a
N_1/N_L	0.079a	0.097a	0.086a
N_1/N_1	0.129b	0.141b	0.136b

* Figures (within harvests) followed by different letters differ at the 5% level of significance (according to standard errors of differences).

Treatment by harvest, and treatment means for total percent N are given in table 27. Percent N in the N_1/N_L treatment was significantly lower than in all other treatments, and percent N in the N_1/N_1 treatment was significantly higher than in all other treatments. Percent N in both tops and roots + nodules followed patterns similar to that for total percent N.

The reduction of the rate of combined N application, from a rate which should have been fully adequate to meet the requirements of the plant, to a low rate insufficient to sustain maximum growth of sainfoin, resulted in decreased plant dry weight, decreased total plant N and the initiation of nodulation and symbiotic N_2 fixation. There was also a marked change in dry matter distribution, with the top:root + nodule dry weight ratio decreasing in the N_1/N_L relative to the N_1/N_1 treatment. The decrease in total plant N in treatment N_1/N_L relative to N_1/N_1 was proportionally greater than the decrease in total dry weight in treatment N_1/N_L relative to N_1/N_1. This, and the fact that total percentage N was lower in the N_1/N_L treatment than in any other treatment indicates that it was a shortage of N that resulted in the change in dry matter distribution and the initiation of nodulation and $N_2[C_2H_2]$ fixation in the N_1/N_L treatment. The influence of mode of N nutrition on the dry matter distribution of sainfoin has been discussed in relation to other legumes in chapter III, 1.3.1.2. A reduced top:root dry weight ratio in sainfoin dependent on symbiotic N_2 fixation compared with sainfoin supplied with high rates of combined N has also been observed by Koter (1965a & b). Similar effects have been observed in birdsfoot trefoil (Barta, 1979) and cowpea (Summerfield *et al.* 1977; Atkins *et al.* 1980). In other legumes, however, such as red clover (Koter, 1965a) and soybeans (Cassman *et al.* 1980), the mode of N nutrition was found to have a relatively small effect on dry matter distribution. The effect of a reduction in the supply of N to sainfoin

in this experiment appeared to have a similar effect to that of the withdrawal of the supply of nitrate to nitrate fed *Lupinus albus*, which resulted in substantial changes in assimilate partitioning between top and root (Pate *et al.* 1979).

3.3 CONCLUDING COMMENTS - EXPERIMENT 1(b)

In section 2.4.2 it was hypothesised that the growth of sainfoin, dependent on symbiotic fixation for its supply of N, could be limited directly by an inefficient N_2 fixing system, or indirectly, by a poor photosynthetic capacity which limits energy availability for symbiotic N_2 fixation. Experiment 1(b) tends to support the former hypothesis although it by no means rules out the latter, as discussed in section 3.1. When experiment 1(b) is viewed in conjunction with experiments 1(a) and 2, it seems likely that a combination of an inefficient symbiotic N_2 fixing system and a poor photosynthetic capacity limit the growth and development of sainfoin dependent on symbiotic N_2 fixation.

4 CONCLUSIONS - EXPERIMENT 1 (A & B), EXPERIMENT 2

(a) The symbiotic N_2 fixing system of sainfoin in these experiments was found to be capable of fixing only approximately 43% of the amount of N which could potentially be used by the plant. This is a lower proportion than for many other legumes.

(b) Sainfoin was found to form abundant nodules, and nodule dry weight as a proportion of total dry weight was equal to or greater than that reported for most other legumes.

(c) The specific activity of sainfoin nodules was found to be lower than that reported for most other legumes. It was observed that the central region of some large branched nodules was very dark red (almost black) in colour with the more normal lighter red or pink coloured tissue towards their extremities. It may well be that this dark coloured tissue was not actively fixing N_2, and that where a lot of large nodules were present, a significant proportion of nodule tissue was not actively fixing N_2.

(d) The growth of sainfoin entirely dependent on N from symbiotic fixation was closely linked to indices of its capacity to fix atmospheric N_2, namely nodule weight, $N_2[C_2H_2]$ fixing activity and particularly total plant N. Indices of the N_2 fixing capacity of sainfoin, particularly total plant N, were also closely linked to leaf area. The quantity of N_2 being fixed appeared to be limiting plant growth, and leaf area appeared to be limiting symbiotic N_2 fixation. There appeared to be a close mutual dependence between the ability of the root nodules to provide N, and the leaves to provide energy.

(e) Evidence was found suggesting that the symbiotic N_2 fixing system of sainfoin is inefficient compared with the better performed forage legumes such as lucerne and the clovers. Sainfoin was found to have a ratio of C_2H_2 reduced:N_2 fixed higher than reported ratios for lucerne and clovers, but similar to *Lotus pedunculatus*. Causes of this relatively high ratio in sainfoin could be net evolution of H_2 by nitrogenase or diurnal fluctuation in N_2 fixing activity. A low rate of combined N was found to depress rather than stimulate $N_2[C_2H_2]$ fixing activity in sainfoin (similarly to *Lotus corniculatus*) and

appeared to replace rather than supplement symbiotic N fixation. This further suggests that the symbiotic N_2 fixing system of sainfoin is in some way inefficient.

A common factor between sainfoin and *Lotus* species is their relatively high tannin content. This perhaps contributes to inefficiencies in their symbiotic N_2 fixing systems.

(f) Sainfoin in these experiments tended to have lower values for LAR and top:root+nodule dry weight ratio than those reported for other N_2 fixing legumes. Thus, the ability of sainfoin to intercept photosynthetically active radiation, as expressed in leaf area, appears to be less than for some other legumes.

(g) The mode of N nutrition influenced the dry matter distribution between top and root+nodule of sainfoin in these experiments more than reported for most other legumes. Plants dependent on symbiotic N_2 fixation for their supply of N substantially increased the proportion of dry matter allocated to root+nodule compared to plants supplied with abundant combined N.

(h) In sainfoin dependent on symbiotically fixed N, there was a substantial amount of variability in total dry weight and the variables thought to influence it, namely leaf area, nodule dry weight, $N_2[C_2H_2]$ fixing activity and total N. Thus, it should be possible, via plant breeding, to improve the N_2 fixing and overall performance of sainfoin.

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APPENDIX 1. PROPERTIES OF SAND

(a) Container Capacity

1950 g dry weight of sand, occupying 1140 cm^3, was packed into four pots. The container capacity, i.e. the amount of water held against gravity by the sand in the pots after saturation, was measured as follows:

(i) Pots were saturated by immersing in water overnight.

(ii) They were then covered with polythene sheets to prevent evaporative loss of water, and allowed to drain for 24 hours.

(iii) The amount of water retained by the sand in the pots was then determined by oven drying the sand from each pot to constant weight at 105°C.

(iv) Container capacity, and moisture content at container capacity were calculated:

Container capacity = 346 ml, S_x = 2

Moisture content at container capacity = 17.8%, S_x = 0.1 (by weight)

= 30.3%, S_x = 0.2 (by volume)

(b) Bulk Density (ρ_b)

Pot bulk density = 1.71 g. cm^{-3}

ρ_b for the determination of the moisture release curve and other properties was 1.80 $g.cm^{-3}$, S_x = 0.04.

(c) Particle Density (ρ_p)

ρ_p = 2.73 $g.cm^{-3}$ (Determined by the methods of Gradwell, 1971)

(d) Total porosity (Calculated from ρ_b and ρ_p)

Total porosity in pots = 37.4% by volume

Calculated total porosity of sand packed for determination of moisture release curve = 34.07% by volume (S_x = 1.34).

The discrepancy is caused by the slight difference in bulk densities.

(e) Air Space at Container Capacity

Air space in the pots at container capacity was 37.4-30.3 = 7.1% by volume. This represents a volume of approximately 80 ml. When pots were watered to 100 ml short of container capacity, air space was 16% (approx.) by volume.

(f) Moisture Release Curve

The moisture release curve (see Fig.2) was determined using the methods of Gradwell (1971).

(g) Water Buffering Capacity

The water buffering capacity of the sand was very low, at 3.06% by volume or 1.71% by weight (Fig.2). This means that plants could rapidly begin to suffer from moisture stress when easily available water became exhausted.

(h) Particle Size Analysis

Particle size analysis was carried out by dry sieving through a stack of sieves on a shaker. The particle size distribution of the sand was as follows:

Particle Size Distribution of Wainuiomata Sand

International classification	USDA classification	Size (mm)	% by weight (air dry)
Gravel	Gravel	> 2	9.9
Coarse sand	Very coarse sand	1-2	27.1
	Coarse sand	0.5-1	37.9
	Medium sand	0.25-0.5	24.1
Fine sand	Fine sand	0.1-0.25	1.0
	Very fine sand	0.06-0.1	trace
	Silt and clay	< 0.06	trace

APPENDIX 2. CHEMICAL ANALYSIS OF HERBAGE AND POT EXTRACT

The nutrient levels in sainfoin herbage did not change substantially over the period during which experiment 2 was harvested Mean values of herbage nutrient levels are given in table 2.1 along with mean values derived from analyses quoted by Baker *et al.* (1952), Thomas *et al.* (1952), Whitehead & Jones (1969), and Smith *et al.* (1974). The nutrient levels observed in sainfoin in this experiment were similar, or slightly higher in some instances than levels found by other workers. The levels of P in this experiment were at the low end of the range values reported in the literature. There were, however, no obvious P deficiency symptoms in the plants, and the levels of P remaining in solution at the end of an interval between nutrient applications appeared to be adequate (see table 2.2).

The levels of some of the macro nutrients extracted from pots at the end of an interval between nutrient applications is given in table 2.2. At the end of the interval, levels of all nutrients appeared to be adequate and levels of some nutrients were higher than in the original nutrient solution, indicating that they were accumulating. The levels were not excessively high, although they would undoubtedly have been higher at the beginning of an interval between nutrient applications.

Table 2.1 Chemical composition of herbage

Element	Expt.2 (N_1 treatments)	Mean of literature values	Range of mean literature values
K (%)	2.56	1.62	1.08 - 2.1
Mg (%)	0.37	0.32	0.17 - 0.54
Ca (%)	0.78	1.06	0.9 - 1.48
P (%)	0.22	0.25	0.22 - 0.28
S (%)	0.36	0.26	0.24 - 0.28
Cl (%)	1.15	0.24	0.10 - 0.38
Si (%)	0.11		
Al (%)	0.03	0.0102	0.0102
Na (%)	0.62	0.03	0.01 - 0.05
Mn (ppm)	29.5	47.2	27.5 - 62
Fe (ppm)	299.8	127	93 - 158
Cu (ppm)	29.3	6.3	5 - 7
Zn (ppm)	45.8	27	26 - 28

Table 2.2 Chemical analysis of pot extract

Element	Treatment	mg contained in pot	Concentration at container capacity (ppm in solution)
P	I_1N_0 (S.E.) †	2.8 (0.8)	8.1 (2.4)
	N_1* (S.E.)	3.0 (0.6)	8.8 (1.8)
	I_0N_0 (S.E.)	-	-
K	I_1N_0 (S.E.)	110 (20)	317 (57)
	N_1 (S.E.)	86 (13)	248 (38)
	I_0N_0 (S.E.)	93 (4)	269 (11)
Mg	I_1N_0 (S.E.)	7.37 (1.13)	21.3 (3.3)
	N_1 (S.E.)	5.22 (0.92)	15.06 (2.7)
	I_0N_0 (S.E.)	6.93 (0.42)	20.05 (1.2)
Ca	I_1N_0 (S.E.)	52.6 (6.1)	151 (17)
	N_1 (S.E.)	80.3 (15.9)	232 (46)
	I_0N_0 (S.E.)	58.8 (1.9)	170 (6)

* N_1 represents both treatments I_1N_1 and I_0N_1

† S.E. = standard error.

APPENDIX 3 ANALYSIS OF VARIANCE OF ACETYLENE REDUCING ACTIVITY - EXPT 1(b)

SOURCE OF VARIATION	DF	SS	SS%	MS	VR*
Treatment	1	3170.2	4.69	3170.2	11.290
Day no.	9	55884.1	82.68	6209.3	22.114
Treatment.day no.	9	2920.1	4.32	324.5	1.156
Residual	20	5615.7	8.31	280.8	
Total	39	67590.0	100.00	1733.1	
Grand total	39	67590.0	100.00		
Grand mean		48.1			
Total number of observations		40			

* Variance ratio ≡ F ratio