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APPLICATION OF THE ACETYLENE REDUCTION TECHNIQUE FOR QUANTIFYING DINITROGEN FIXATION IN *LUPINUS ANGUSTIFOLIUS* L.

A thesis presented in fulfilment of the requirements for the Degree of Doctor of Philosophy, Faculty of Agricultural Science, University of Adelaide.

by

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SUMMARY

In southern Australia, narrow-leafed lupins (*Lupinus angustifolius* L.) are increasingly being grown as a replacement for pasture legumes in integrated farming systems based on the cereal-legume rotation. The lack of suitable techniques for measuring the amount of nitrogen fixed by lupin crops has prevented an assessment of whether the crop makes a net contribution of nitrogen to the soil in view of the large amounts of nitrogen removed in the grain at harvest.

The acetylene reduction (AR) technique was investigated as a means of quantifying symbiotic nitrogen fixation in field-grown crops of narrow-leafed lupins. The major problem addressed by the research programme was the calibration of rates of AR (A) with rates of N_2 fixed (N). Because the A/N ratio was known to vary widely between legume symbionts and possibly throughout ontogeny of a given symbiotic association, attempts were made to determine if and how any such ratio may alter during the growth cycle of the lupin plant.

The programme was initially based on the postulates that variations in experimentally-derived A/N ratios may be caused by

a) variation in nitrogenase-catalysed hydrogen (H_2) evolution, which was known to occur concomitantly with N_2 fixation in a wide range of legume symbionts *in situ* and/or

b) effects of perturbating the plant-soil system during assay procedures.

Two experiments were performed with lupin plants grown in a naturally-lit glasshouse to determine if the total electron flux through nitrogenase, as measured by AR, equated with the sum of the fluxes through H_2 evolution in air and N_2 fixation measured using $^{15}N_2$. A second aim was to investigate whether plant perturbation affected AR and/or the A/N ratio.

Measurements of H_2 evolution in whole-plant assays were confounded with the capacity of micro-organisms associated with the rooting medium to utilize H_2. In nodulated-root assays, removal of the rooting medium prior to assay enabled nitrogenase-catalysed H_2 evolution to be determined. On all occasions of measurement, nitrogenase activity determined by H_2 evolution under Ar/O_2 equated with that determined using AR, and on no occasion was exogenously-supplied H_2 taken up by nodulated roots exposed to an atmosphere of 10-15% acetylene. These results indicated that uptake hydrogenase activity did not occur in the *L. angustifolius* x *R. lupini* (strain WU 425) symbionts used during the investigations.

The glasshouse experiments demonstrated that considerable H_2 evolution occurred in unwashed, nodulated-root assays but this H_2 evolution could not account for the difference in electron flux through nitrogenase as measured by AR and $^{15}N_2$ fixation. Furthermore, similar A/N ratios were obtained irrespective of whether the assays were conducted on excised unwashed roots, or on undisturbed whole plants.

A third experiment was performed on plants grown in a growth chamber, under conditions of unlimited supply of water and nutrients (but in the absence of combined nitrogen) and controlled regimes of temperature and irradiance. AR, H_2 evolution (H) and N_2 fixation (measured by the increment in plant nitrogen) were monitored throughout the growth cycle of the plants, and it was clear that not only did the A/N and H/N ratios vary considerably throughout plant ontogeny, but they did so in a congruous manner. High values of both ratios were associated with low specific rates of N_2 fixation (sN) and *vice versa*. The relationships of the specific rates of AR (sA) and H_2 evolution (sH) to sN were curvilinear, and were linearized by plotting sA and sH against ln sN.

Similar relationships between sA and sN, and between sH and sN were obtained in a further two studies in which simultaneous measurements of AR, H_2 evolution and $^{15}N_2$ fixation were made on plants grown in consecutive years in the field. Although considerable diurnal variation occurred in sA, sH and sN no diurnal variation was evident in either the A/N or H/N ratios.

Evidence from the field experiments suggests that low A/N and H/N ratios occur at high rates of sN when the latter occurs as a possible response to an increased demand for nitrogen by the plant. Variation in environmental conditions resulted in periods of high demand for nitrogen which could not be met in the short term by an increase in nodule mass. It is postulated that, in such circumstances, the lupin plant increases the specific activity of nitrogenase with an associated change in the substrate kinetics of the enzyme in relation to the reduction of C_2H_2, H^+ and N_2.

This postulate and the reason for the low A/N ratios found during the investigations (the mean of 15 separate determinations was 2.46) is discussed in the light of very recent research published by Minchin *et al.* (1983), who have observed an C_2H_2-induced decline in nodular nitrogenase activity in a wide range of legume symbionts when a flow-through assay system was used. That an C_2H_2-induced decline does occur in the *L. angustifolius* x *R. lupini* symbiosis was confirmed by the author in a further experiment using a flow-through assay system.

The relationship observed between sH and sN is discussed in relation to published reports of the environment of the host plant affecting the relative efficiency of nitrogenase (defined as 1-[H/A]) in other legume symbionts.

Despite the occurrence of an C_2H_2-induced decline in nitrogenase activity, the results of the experiments described in this thesis indicate that the relationship between sA and sN in narrow-leafed lupins may be usefully

applied as a technique for quantifying rates of N_2 fixation over a season. However, the technique requires the routine use of ^{15}N-enriched N_2 gas for calibration purposes and the necessary laboratory facilities for measuring isotope ratios. Relatively close agreement in both the slope and intercept for regressions of sA on ln sN in two field experiments in different seasons and using different cultivars of lupins suggests that a general robust relationship for narrow-leafed lupins may apply in a variety of situations. Should this be the case, then $^{15}N_2$ assays would not be required on every occasion of assay. However, further studies of rates of N_2 fixation and AR in narrow-leafed lupins over a range of seasons and environments would be necessary before any such general relationship could be proposed.

Estimates based on integrated measurements of $^{15}N_2$ fixation of the amounts of N_2 fixed in field-grown crops of *L. angustifolius* grown in consecutive seasons, were 136 and 155 kg ha^{-1} respectively. However, 40 and 31% of the maximum nitrogen accumulated by the respective crops was derived from the soil and it is estimated that following the removal of seed at harvest only 22 and -3 kg ha^{-1} of nitrogen had been added to the soil-plant system. Furthermore, it is estimated that between 78 and 100% of the nitrogen in the lupin stubble would need to be incorporated into the soil to replace the nitrogen removed from the soil by the lupin crop.

It is concluded that narrow-leafed lupin crops growing in soils of low to moderate fertility in southern Australia, may fix sufficient nitrogen for their own requirements but cannot be expected to make a major contribution to the nitrogen status of the soil in which they grow.

STATEMENT

The investigations reported herein were carried out as part of my duties as an employee of the South Australian Department of Agriculture and as a student in the Department of Soil Science, Waite Agricultural Research Institute, University of Adelaide.

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

I consent to this thesis being made available for photocopying and loan.

Peter R. Gibson

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1. INTRODUCTION

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1. Introduction

Most of southern Australia has a Mediterranean-type climate with cool, wet winters and hot, dry summers. The principal farming system in regions receiving 250-500 mm mean annual rainfall is the integrated cereal-livestock system based on the cereal-pasture rotation. The success of this system can be mainly attributed to the use of pasture legumes (species of *Medicago* or *Trifolium*) for the provision of nitrogen for subsequent cereal crops and high quality feed for the grazing animal.

The contribution of annual pasture legumes to soil nitrogen accretion has been well documented (Donald and Williams 1954; Russell 1960; Greenland 1971; Watson *et al.* 1976; Dahmane 1978; Tuohey and Robson 1980) and there is now widespread concern that there has been a general decline in the legume component in the pasture phase of many of the currently practiced rotations. Carter *et al.* (1982) list some of the factors they consider responsible for this decline in medic and subterranean clover stands across southern Australia and these include not only the relatively recent advent of pasture aphids (species of *Therioaphis* and *Acyrthosiphon*) and sitona weevil (*Sitona humeralis* Stephens) but also factors relating to increased cropping intensity and overgrazing of pastures.

Concern for the decline in thrift of the pasture legumes, coupled to more favourable financial returns from cropping compared to livestock enterprises in recent years (Kingma 1982), has provided the impetus for continuous cropping using grain legumes, mainly lupins (*Lupinus angustifolius* L.) and field peas (*Pisum sativum* L.), to replace the traditional pasture ley in rotational systems. The total area sown to peas and lupins in Australia has more than doubled in the eight years prior to, and including, 1980/81 (Australian Bureau of Statistics, Canberra) and this

trend is likely to continue, particularly if suitable varieties can be found for the lower rainfall regions of the cereal belt.

There is, however, a paucity of information on the extent to which grain legumes can provide the quantity of fixed nitrogen removed by subsequent cereal crops and traditionally supplied by the pasture legumes. Quantitative measurements of the amounts of dinitrogen fixed by grain legumes under field conditions in southern Australia have rarely been reported because of the lack of suitable techniques. Instead, rotational experiments comparing cereal yields following grain legume crops with yields under continuous cereals have more frequently been reported, with the former system usually giving higher yields (Drover 1956; White *et al.* 1978; Boundy 1978; Schultz 1980; Hawthorne and Lewis 1980; Doyle and Herridge 1980; Reeves *et al.* 1982; Askin *et al.* 1982).

The increased cereal yields following grain legumes have, in a number of cases, been associated with higher amounts of mineral nitrogen in the soil at the time of sowing (Wells 1970; Boundy 1978; White *et al.* 1978; Schultz 1980), and with the control of cereal root diseases such as haydie, *Gaeumannomyces graminis* (White *et al.* 1978; King *et al.* 1979) or common root rot, *Cochliobolus sativus* (Moore *et al.* 1982). There appear to be different interpretations in the literature of results indicating higher amounts of mineral nitrogen in soils sown to grain legumes the previous year. The conclusion that the grain legume has made a net contribution of nitrogen to the soil is commonly stated (*e.g.* White *et al.* 1978) without direct evidence of nitrogen gain. Differences in soil mineral nitrogen may reflect lower utilization of mineralized nitrogen by the grain legume compared to a corresponding cereal crop, or to a difference in the rate of breakdown of the different residues. Recent work

by Ladd *et al.* (1981) indicates that the proportion of nitrogen in a cereal crop derived from decomposing legume residue, incorporated the previous year in the field in South Australia, is only about 10%. Their results indicate that the value of the legume may be not so much in its capacity to deliver large amounts of nitrogen immediately to subsequent cereal crops, but to maintain or possibly increase the total amount of nitrogen in the soil; thus ensuring the long-term supplies of nitrogen from the decomposition of relatively stable organic residues (Ladd 1981). This is well illustrated in one study by these workers in which increased cereal yield following medic pasture could be entirely attributed to the increased inorganic nitrogen which remained after the previous season (Ladd *et al.* 1983). Thus, in relation to nitrogen, the benefit of legumes in cereal-legume rotations in the short term may be the 'saving' of mineralized nitrogen during the legume phase by supplementation with nitrogen from dinitrogen fixation.

Evidence of a net contribution by grain legumes to the nitrogen status of the soil under a cereal-legume rotation can best be obtained by the long-term monitoring of soil total nitrogen and careful budgeting of nitrogen throughout all phases of the rotation (Greenwood 1976). Soil nitrogen accretion in systems utilizing grain legumes will depend not only on whether the legume can supply sufficient nitrogen *per se*, but also on the effect of soil cultivation on the mineralization of soil organic nitrogen and on the likelihood of nitrogen losses from leaching volatilization and denitrification.

To the author's knowledge, there is no documented evidence of an increase in total soil nitrogen following the growth of grain legumes which have been harvested for grain in southern Australia. Hawthorne and Lewis

(1980) found that cropping lupins did not consistently increase soil nitrogen relative to existing poor pastures on deep sand in the south-east of South Australia, and Schultz (1980) found no difference in organic carbon or total nitrogen after two cycles of lupin-wheat or pea-wheat rotations. Reeves *et al* (1982) compared a lupin-wheat rotation under both normal cultivation and direct drilling treatments over two cycles and at three sites in north-west Victoria and could not demonstrate increased total soil nitrogen in either treatment (Reeves, pers. comm.).

A wheat-peas treatment was included in the permanent rotation experiment at the Waite Institute in 1926 and over 50 years the soil nitrogen content declined, although the decrease was not as great as with the wheat-fallow rotation (Greenland 1971).

It appears therefore, on the limited experimental data available, that grain legumes cannot be assumed to make a net contribution to the nitrogen content of the soil in rotational systems in which they are included just because they, like the pasture legumes, fix dinitrogen. Three important factors are likely to contribute to differences in the nitrogen status of soils under rotations employing grain legumes compared to pasture legumes; *viz.* the return of nitrogen via the grazing animal, the absence of tillage in the pasture phase of the traditional pasture rotations and the large amounts of nitrogen removed in the grain of the grain legume crop. The first two aspects are beyond the scope of the investigations reported herein and only the third aspect will be considered further.

Russell (1980) makes the point that one should not generalize about the effect of grain legumes on soil nitrogen status as both the amount of nitrogen removed in the grain and the amount of nitrogen fixed can vary greatly between species. He cites the findings of Lucas *et al*. (1977) in

Michigan, U.S.A., who found that soybeans (*Glycine max* L.) and field beans (*Phaseolus vulgaris* L.) returned less carbon to the soil than wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), grass and lucerne (*Medicago sativum* L.), and depletion of soil organic matter was greater with the grain legumes. The classical study of Lyon and Bizzell (1933, 1934) also demonstrated an accumulation of soil nitrogen under clovers, lucerne and vetch (*Vicia sativa* L.) over a ten-year period, but a decrease with soybeans, peas, beans, barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.) and oats (*Avena sativa* L.).

In southern Australia, lupins and peas are predominantly grown for the harvest of grain, and it is the removal of large quantities of nitrogen in the grain that contributes most to the uncertain role that grain legumes play in the nitrogen economy of farming systems. Farrington *et al.* (1977) reported that the quantity of nitrogen found in the grain of lupins at maturity represented about 50% of the maximum accumulation of nitrogen by the whole plant and over 70% of the nitrogen present in the plant at maturity. Gladstones and Loneragan (1975) recorded that a minimum of 75% of the total nitrogen in mature lupin tops was present in the seed, and Pate and Flinn (1977) reported 61% of the total plant nitrogen in peas was present in the seed at maturity.

Thus, allowing for the removal of nitrogen in the grain, about 60% and 70% respectively of the total nitrogen in peas and lupins at the time of harvest would have had to be derived from dinitrogen fixation for there to be any net input of nitrogen to the soil. Furthermore, in the case of lupins, if it is assumed that root material contains less than 10% of the total nitrogen in a lupin plant when nearing maturity (Farrington *et al.* 1977) and that this material is the major source of organic nitrogen

returned to the soil, then the amount of nitrogen which has to be fixed is in the order of 80% of the total amount of nitrogen in the plant. This will only maintain the soil nitrogen status, and not provide sufficient nitrogen for a subsequent cereal crop.

Crucial to the success of grain legumes as a replacement for pasture legumes in the maintenance of soil fertility is therefore the extent to which the former are able to utilize dinitrogen rather than soil nitrogen. The following investigations attempted to build the necessary framework whereby quantitative data on this important subject could be cheaply and accurately derived over a range of seasons and cropping sequences.

Because no simple techniques for measuring the amount of dinitrogen fixed were readily available without major modification and testing, a substantial proportion of the investigations was necessarily devoted to the development and testing of techniques in both the glasshouse and in the field. The acetylene reduction (AR) technique was chosen as one which could most likely be adapted for routine use across a range of field sites and seasons in future studies on nitrogen fixation in field-grown lupins. The major attributes of this technique are cheapness, sensitivity and ease of application in the field, but major problems related to calibration and integration (over time) needed to be considered. Techniques employing the use of ^{15}N-enriched nitrogen fertiliser were precluded because of the high costs of ^{15}N-materials for routine experimental use and the lack of a suitable non-fixing control plant which is essential to the deployment of the technique.

The investigations described in this thesis primarily address the calibration of rates of AR (A) with rates of nitrogen fixed (N). Because the A/N ratio was known to vary widely between legume symbionts and possibly throughout ontogeny of a given symbiotic association, attempts

were made to determine if and how any such ratio may alter during the growth cycle of the lupin plant.

The programme was initially based on the postulates that variations in experimentally-derived A/N ratios may be caused by

a) variation in nitrogenase-catalysed hydrogen evolution, which was known to occur concomitantly with nitrogen fixation in a wide range of legume symbionts *in situ* and/or

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A third experiment was performed on plants grown in a growth chamber, under conditions of unlimited supply of water and nutrients (but in the absence of combined nitrogen) and controlled regimes of temperature and irradiance. AR, hydrogen evolution (H) and nitrogen fixation (measured by the increment in plant nitrogen) were monitored throughout the growth cycle of the plants to determine if, and how, the A/N and H/N ratio varies throughout plant ontogeny.

Finally, two field experiments were performed in consecutive years in commercial lupin crops and AR, hydrogen evolution and nitrogen fixation (measured using $^{15}N_2$) again were monitored throughout the growth cycle of the plants. Estimates based on integrated measurements of $^{15}N_2$ fixation of the amounts of nitrogen fixed by these field-grown crops are used as the basis for the formulation of nitrogen balances for the respective soil-plant systems.

2. LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Introduction

The literature review is divided into three sections. The first section reviews aspects of nitrogenase function with emphasis on the reduction of alternative substrates and provides the broad biochemical framework on which the acetylene reduction (AR) technique is based. An important aspect reviewed in this chapter is the involvement of nitrogenase-catalysed hydrogen evolution and its significance in the quantitative use of the AR technique. Some review of the present stage of knowledge in relation to uptake hydrogenases is also presented because of their apparent link with nitrogenase and the possible negation of hydrogen evolution measurements as a meaningful adjunct to AR assays, if this enzyme is present and functioning.

The second section reviews the methods currently available to measure nitrogen fixation in field-grown legumes and the third section covers the methodology involved in the use of the AR assay and factors relating to its deployment for field use.

The investigations reported in this thesis cover such a wide range of subject matter (*viz.* from the biochemistry of enzyme function through to agronomic evaluation of plant species in the field) that to maintain a degree of conciseness, some aspects of nitrogen fixation were not formally reviewed, although they are later developed and referred to in the text. Two such aspects (worthy of reviews in their own right) are the effects of light and temperature on nitrogenase activity and the related phenomenon of diurnal variation, and the effect of combined nitrogen on the legume-*Rhizobium* symbiosis.

2.2 Nitrogenase function and alternative substrates

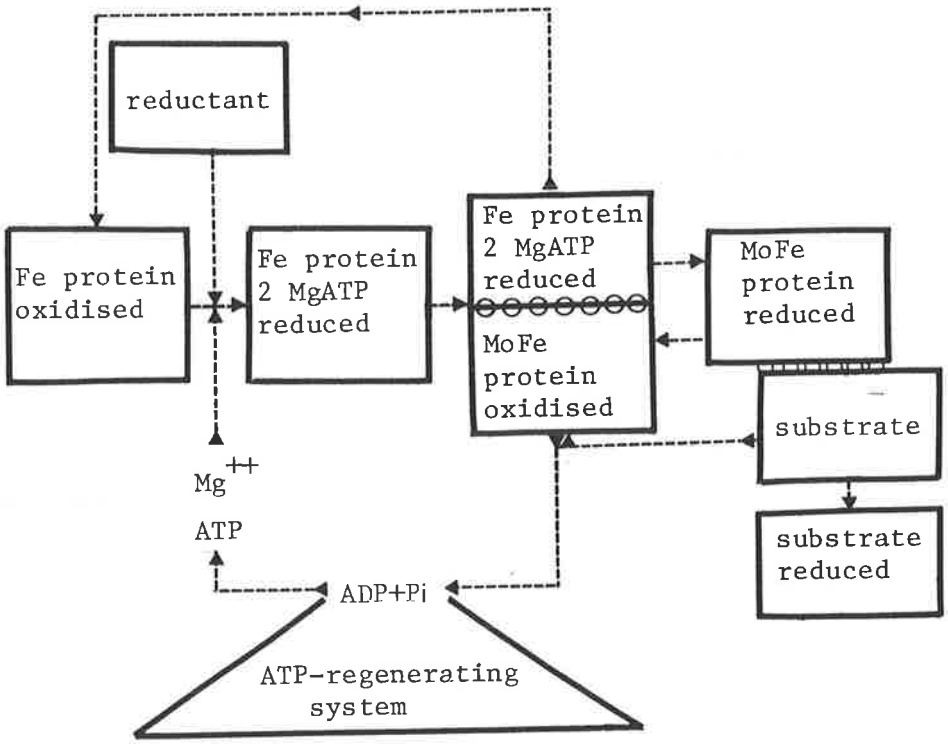
2.2.1 Enzyme structure and function

Diazotrophs, organisms which can utilize nitrogen gas (N_2) to meet their nitrogen requirements, comprise a highly diverse group which varies in complexity from primitive bacteria such as *Clostridium* through to the physiologically and genetically intricate legume root-nodule symbiosis. Despite this variability in the diazotrophs, the enzyme complex responsible for the reduction of N_2, i.e. nitrogenase, conforms to a common pattern, indicating fundamentally similar mechanisms among the wide variety of nitrogen-fixing organisms (Burns and Hardy 1975).

The term 'nitrogenase' is used hereafter to describe the functional nitrogen reducing system which is composed of two dissociating protein components. One (Fe protein) contains 4 iron and 4 acid-labile sulphur atoms; the other (MoFe protein) contains 2 molybdenum, 28 to 32 iron and approximately 28 acid-labile sulphur atoms (Mortenson and Thorneley 1979). These two proteins can now be readily separated and purified independently (Winter and Burris 1976) and this has allowed extensive fundamental studies on the interaction of the two proteins.

In addition to the two protein components, the nitrogenase system requires an effective reductant, the divalent cation (Mg^{++}) and adenosine triphosphate (ATP). The reductant donates electrons to the Fe protein, which binds MgATP, acquires a very low potential, transfers a single electron at a time to the MoFe protein which in turn binds and reduces substrates (Orme-Johnson *et al.* 1972; Eady *et al.* 1972).

All reductions by nitrogenase are two electron, or multiples of two electron, transfers and it has been suggested (Hageman and Burris 1978) that a transitory complex is formed between the two proteins with association and dissociation more than once for each reduction effected.



Path of electron transfer in the nitrogenase system

However, more recent evidence (Smith *et al.* 1981) suggests that the Fe protein may have more roles than merely that of a reductase function and may include facilitating the internal transfer of electrons within the MoFe protein and possibly the reduction and release of substrate.

The ATP requirement for N_2 fixation was initially substantiated by Hardy and D'Eustachio (1964) and Mortenson (1964) in *Clostridium* preparations following the demonstration that ferredoxin was a link between pyruvate metabolism and nitrogenase. However, the role of ATP in nitrogenase function is far from clear and the absolute requirement of nitrogenase function for ATP is difficult to rationalize on the basis of energy needs because the overall reduction of N_2 to NH_3 is thermodynamically favourable.

Still, the limiting reaction of nitrogenase is the hydrolysis of ATP to ADP and inorganic phosphate coupled with electron transfer to a reduced species (Hardy and Knight 1966). Evidence has been produced indicating that ATP participates in electron transfer between the nitrogenase proteins (Smith *et al.* 1973; Thorneley 1975) and ATP hydrolysis accompanies the transfer (Eady *et al.* 1978). Moreover, Thorneley and Eady (1977) and Thorneley and Cornish-Bowden (1977) have suggested second roles for ATP which may be either a once-only activation of nitrogenase toward the reduction of N_2 or C_2H_2 or a catalytic role in each round of electron transfer.

ADP, the product of ATP hydrolysis, is a potent inhibitor of nitrogenase activity by directly competing with ATP at the same binding sites (Moustafa and Mortenson 1967; Bui and Mortenson 1968). Recently it has been suggested that there may be three binding sites for ADP and only two for ATP (Mortenson and Upchurch 1981), and that the degree of inhibition by MgADP depends on the concentration of Mg^{++} (Davis and Kotake 1980). However, the role of ADP and/or the ADP/ATP ratio in nitrogenase control is still obscure, particularly in the aerobes. According to Haaker *et al.* (1974), Haaker and Veeger (1976, 1977) and Veeger *et al.* (1981), full inhibition of nitrogenase activity can be achieved by lowering the energized state of the cytoplasmic membrane without affecting the intracellular ADP/ATP ratios.

In addition to an ATP and divalent cation requirement, N_2 fixation requires a continuous source of low potential electrons. Evans and Phillips (1975) have devised possible electron transport systems for various N_2-fixing micro-organisms, including *Rhizobium* bacteroids. In one possible scheme, reduced nicotinamide adenine dinucleotide phosphate

($NADPH_2$) generated from various citric acid cycle intermediates and glucose-6-phosphate, reduces nitrogenase via electron carriers like ferredoxin and flavodoxin. The ATP-driven N_2 fixation reaction, which is irreversible, helps the coupling and electron passage to the low potential electron carriers.

According to Sloger (1976) ferrodoxin and flavodoxin are thought to be the electron carriers in bacteroids, although their order in the sequence has not been proven. More recently, Haaker and Veeger (1977) and Veeger *et al.* (1980) reported that flavodoxin hydroquinone is the electron donor for nitrogenase in *Azotobacter vinelandii* and *Rhizobium leguminosarum*. Furthermore, these workers demonstrated that the generation of low-potential reducing equivalents (i.e. flavodoxin hydroquinone) was induced by the electrochemical proton gradient of the cytoplasmic membrane.

2.2.2 Alternative substrates and H_2 evolution

N_2 is the natural substrate for nitrogenase and, until 1966, was the only established reducible substrate of the enzyme (Burns and Hardy 1975). The only products of N_2 reduction are two molecules of ammonia formed by the addition of 6 protons and 6 electrons to N_2, although transient forms of nitrogenase and a nitrogenase-bound dinitrogen hydride have recently been identified during catalytic cycles of nitrogenase (Thorneley *et al.* 1978).

i.e. $\quad N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$

Since 1966, a number of triple-bonded compounds have been identified as reducible substrates for nitrogenase and indeed, nitrogenase is the only isolated enzyme known to reduce the triple bond (Burns and Hardy 1975). Susceptible triple bonds include only, but not all, those which bridge N to

N, N to O, N to C, and C to C; in all known examples, at least one of these atoms is terminal or bonded to H. Six different classes of substrates can be established, based on decreasing N≡N character to increasing C≡C character (Burns and Hardy 1975), i.e. N_2, azide, N_2O, nitrites, isonitrites and alkynes.

Of these various alternative substrates so far identified, acetylene (C_2H_2) has become the most significant due to its extensive use in the AR assay for nitrogenase activity. This assay was first proposed by Hardy and Knight (1967) following independent observations by Schöllhorn and Burris (1967) and Dilworth (1966) of inhibition of N_2 fixation by C_2H_2 with extracts of *Clostridium pasteurianum*.

The reduction of C_2H_2 to C_2H_4 requires 2 electrons compared to the 6 required for the reduction of N_2 to $2NH_3$.

i.e. $C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4$

Thus the stoichiometric relationship between C_2H_2 and N_2 reduction requires 3 moles of C_2H_2 be reduced for each mole of N_2 reduced. However, early cell-free preparations of nitrogenase revealed that hydrogen gas (H_2) was released concomitant with nitrogen reduction and that ATP was required for this H_2 evolution (Bulen *et al.* 1965; Burns and Bulen 1965; Bergersen 1966; Mortenson 1966; Koch *et al.* 1967).

i.e. $2H^+ + 2e^- \rightarrow H_2$

It was also demonstrated that carbon monoxide (CO), a potent inhibitor of N_2 reduction, did not inhibit this ATP-dependent H_2 evolution. This is in contrast to the inhibition by CO of ATP-independent H_2 evolution catalysed by a classical H_2-evolving hydrogenase, such as found in *Clostridium* preparations. Furthermore, N_2 was not required for

nitrogenase turnover, nor could saturating concentrations of N_2 totally inhibit H_2 evolution.

Thus, because of the substantial electron flow to H^+ (even in the presence of saturating concentrations of N_2) and because H_2 evolution is almost completely inhibited at saturating concentrations of C_2H_2, the expected C_2H_2:N_2 (A/N) ratio is greater than 3.

Recently, mechanistic models of nitrogenase have been proposed in which a stoichiometric relationship exists between H_2 production and N_2 reduction (*e.g.* Schrauzer 1977; Chatt and Richards 1977; Yates and Walker 1980). Most studies have demonstrated a maximum electron allocation ratio between N_2 reduction and H_2 evolution of 3:1 (3 pairs of electrons are required to reduce N_2 and only one pair to reduce $2H^+$) or 1 mole of H_2 produced for every mole of N_2 reduced. For example, Yates and Walker (1980) reported always finding H_2:N_2 molar ratios greater than one in their studies with *Azotobacter chroococcum*. They suggest that there may be two separate mechanisms for H_2 evolution; one that is intrinsically associated with nitrogenase activity and a second that is determined by the stoichiometry of the N_2 reduction process.

Smith *et al.* (1981) reported from studies of N_2 reduction in *Klebsiella pneumoniae*, that they had never found less than a 30% allocation of electrons to H_2 at near neutral pH levels. However, the allocation ratio did drop to about 23% at pH values above 8.7. They proposed a mechanistic model whereby H_2 can be evolved at both intermediate and more-fully reduced enzymic states, but N_2 can only be reduced at the latter. On binding of N_2, H_2 is displaced and hence at least one H_2 molecule is generated for each N_2 molecule reduced.

However, irrespective of the mechanisms involved, it is apparent that in cell-free preparations, at least 25% of the electron flux through nitrogenase is utilized in the production of H_2 rather than the reduction of N_2. Thus the A/N ratio should have a minimum value of 4.

Of major importance in the use of the AR assay for measuring nitrogenase activity, particularly if quantitative information is required, is the question of the dependence or otherwise of the total electron flux through nitrogenase on the nature of the substrate being reduced. The experimental evidence to date is somewhat variable on this matter, which may reflect the variation in experimental systems and N_2-fixing organisms used.

Ljones (1973) reported that the electron flux through nitrogenase was independent of the substrate employed. However, Thorneley and Eady (1977) showed that the total electron flux increased in the presence of C_2H_2 compared to that under Ar/O_2 at certain component-protein ratios. The effect was temperature dependent, with the promotion of electron flux by C_2H_2 declining with temperature from 30°C to 10°C. Andersen and Shanmugam (1977) reported that electron turnover for both nitrogenase and glucose consumption by intact cultures of *Klebsiella pneumoniae* under an atmosphere of Ar/O_2 was only 75-80% of the corresponding rate under N_2. Apte *et al.* (1978), working with the blue-green algae, demonstrated conformational changes in the nitrogenase complex *in vivo* by pre-incubation under C_2H_2, and Scherer *et al.* (1980) reported up to a four-fold increase in light-induced H_2 production when C_2H_2 pre-treated cells were assayed under Ar. Although the significance of the results of pre-incubation experiments to the use of the AR assay remains uncertain, the results do indicate that different substrates may have different effects on nitrogenase conformation and function.

Of interest in the report of Scherer *et al.* (1980) is the suggestion that nitrogenase activity may be regulated by the H_2 concentration present in the cell. Thus these workers suggest that pre-incubation of algae under C_2H_2 leads to a decrease in the H_2 level in the cell to almost zero. The subsequent absence of H_2 leads to significant increases in nitrogenase activity which can be prevented by externally-added H_2.

It is important to emphasize here that conditions imposed in many *in vitro* experiments may not be relevant to the true conditions operating *in vivo*. For example, Davis and Wang (1980) reported that intact cowpea plants appeared to have an effective excess of Fe protein even under growth conditions conducive to restricted energy supply, and hence wide-ranging component ratios may not occur in this symbiosis *in vivo*.

In recent years, a number of studies have been reported in which nodules or nodulated roots of legumes have been assayed for AR and the rates compared to those of H_2 evolution under an atmosphere of Ar/O_2 or $Ar/O_2/CO_2$. Assuming nil (or equal) effect of substrate (i.e. H^+ or C_2H_2) on nitrogenase activity, the two rates should be the same because both represent total electron flux through nitrogenase. Substantially lower H_2 evolution rates compared to AR rates have been used as an indication of the presence of an active uptake hydrogenase.

Table 1 summarizes some published rates of AR and H_2 evolution under Ar/O_2 or $Ar/O_2/CO_2$ in experiments where the two phenomena have been measured under identical experimental conditions and where evidence has been supplied for the lack of uptake hydrogenase activity. The criterion for the latter was the absence of uptake of exogenously-supplied H_2 and/or evidence that H_2 evolution in air was > 25% of AR. It is evident that, in most cases, H_2 evolution rates equated reasonably well with

TABLE 1:

Summary of published comparisons of rates of AR with H_2 evolution under Ar/O_2 in legume symbionts

Symbiosis	Assay System	Assay Temp. °C	H_2 evol. under Ar/O_2 (μmol h^{-1})+	AR	Difference %	Reference
various legumes (mean of 16)	excised nodules	28	8.2	8.4	2.1	Schubert & Evans 1976
2 soybean cultivars x range R. strains (mean of 26)	nodulated roots	28	16.4	15.6	4.9	Carter *et al.* 1978
soybean x strain USDA 31 - 4 weekly occasions	excised nodules	28	12.6	12.2	3.3	Schubert *et al.* 1978
peas x 10 strains	excised nodules	28	42.9	37.8	13.5	Ruiz-Argueso *et al.* 1978
alfalfa x 9 strains	nodulated roots	28	27.6	26.3	4.7	Ruiz-Argueso *et al.* 1979b
white clover x 7 strains of *R. trifolii*	nodulated roots	28	43.1	46.0	6.2	Ruiz-Argueso *et al.* 1979b
soybean x 3 strains	nodulated roots	-	33.0	31.4	5.2	Ruiz-Argueso *et al.* 1981
peas x 128C53	nodulated roots	30	33.0	33.5	1.5	Dixon *et al.* 1981
soybean	nodulated roots	30	6.6	6.3	4.8	Dixon *et al.* 1981

+ usually per g fresh weight nodule - comparisons always in same units.

rates of AR, indicating either no effect, or equal effects of the two substrates (H^+ or C_2H_2) on nitrogenase activity in legume nodules.

The allocation of electrons to different substrates of nitrogenase (present in saturating concentrations) at the same time (*e.g.* N_2 and H^+) has been reported to depend on several factors. Silverstein and Bulen (1970) reported that the ratio of H_2 evolved to N_2 reduced was dependent on the concentration of ATP used in the assay, and Davis *et al.* (1975) demonstrated that both ATP and the protein component ratio controlled the relative ability of the enzyme to reduce different substrates. Effects of component ratio on electron allocation have also been reported by Bergersen and Turner (1973) and Thorneley and Eady (1977).

However, Hageman and Burris (1980) have reported that the electron flux through the MoFe protein is the sole determinant of the electron allocation pattern to alternative substrates and that this flux controls the kinetic properties of nitrogenase. They suggest that the concentration of ATP and reductant, and the component ratio are only important in that they affect the electron flux rather than confer changes *per se*.

Other changes which have been shown to affect the allocation ratio are pH (Andersen and Shanmugam 1977; Hageman and Burris 1980) and temperature (Thorneley and Eady 1977; Andersen and Shanmugam 1977).

2.2.3 Uptake hydrogenase

In contrast to the situation with *in vitro* or cell-free systems (clarified in the mid-sixties), convincing evidence for nitrogenase-catalysed H_2 evolution *in vivo* was not produced until the mid-seventies. This was despite the fact that H_2 evolution had been observed

from detached nodules of some legumes as early as 1957 (Hoch *et al.* 1957; Hoch *et al.* 1960; Bergersen 1963; Dixon 1968, 1972; Dart and Day 1971). At this time, it was not clear whether the hydrogen evolved by detached nodules was catalysed by nitrogenase or by a conventional hydrogenase. Reports of H_2 uptake, rather than evolution, in pea bacteroids (Dixon 1968) tended to confuse the picture.

The situation was clarified in 1976 when Schubert and Evans (1976) reported their survey of H_2 evolution from the nodules of a range of both legume and non-legume nitrogen-fixing symbionts. The survey revealed extensive *in vivo* liberation of H_2 from many of the symbionts tested. The production of H_2 was O_2-dependent and was higher in an atmosphere in which N_2 was replaced by Ar. This behaviour was consistent with ATP-dependent H_2 evolution being catalysed by nitrogenase, rather than H_2 evolution being catalysed by a classical hydrogenase.

Schubert and Evans also produced quantitative estimates of the proportion of the total electron flow to nitrogenase that was diverted to H_2 evolution. This was expressed in the term 'Relative Efficiency' (RE) defined as

$$1 - \frac{\text{rate of } H_2 \text{ evolution in air}}{\text{rate of } H_2 \text{ evolution in Ar:}O_2\text{:}CO_2 \text{ or rate of AR}}$$

In relation to the substrates H^+ and N_2 the concept of RE is essentially the same as that of allocation ratio (p. 14) but, because the rate of reduction of N_2 to NH_3 is more difficult to ascertain *in vivo*, total electron flux is assumed to be the rate of AR.

In the study of Schubert and Evans, values of RE for the legumes ranged between 0.4 and 0.6 and for the nodulated non-legumes values

approached unity. Thus it was apparent that substantial nitrogenase-catalysed H_2 evolution did occur *in vivo* in a wide range of legume symbionts and that considerable energy may be wasted by the diversion of nitrogenase activity toward H_2 evolution rather than N_2 reduction.

The fact that many leguminous plants of agricultural importance may suffer substantial energy losses from H_2 evolution during N_2 fixation, stimulated a significant research input into the relationship between H_2 metabolism and N_2 fixation in root-nodule bacteria and other aerobic N_2 fixers. The enzyme hydrogenase, responsible for H_2 metabolism, can catalyse a reversible reaction *in vitro* in the presence of suitable electron donors or acceptors. However, *in vivo*, the reaction is usually irreversible, either consuming or evolving H_2 (Adams *et al.* 1981).

To the author's knowledge, there have been no reports of H_2 evolution occurring via a classical hydrogenase in legumes *in vivo*. Lim (1978) and Bethlenfalvay and Phillips (1979) have claimed that the hydrogenase complex in pea nodules catalyses an exchange reaction, although Dixon (1967) had earlier reported little or no exchange between $^2H_2$ and $^1H_2$ by the hydrogenase from pea bacteroids. Emerich *et al.* (1980) have since been unable to demonstrate reversibility of the hydrogenase reaction *in vivo* and believe the former two reports described $^3H_2$ oxidation via the oxyhydrogen reaction rather than exchange.

The hydrogenase is known to occur in the bacteroids (McCrae *et al.* 1978), is membrane-bound, O_2 dependent, and involves cytochromes and non-heme iron proteins in the pathway of electron transport from H_2 to O_2 (Emerich *et al.* 1979, 1980).

The capacity to recycle H_2 produced by nitrogenase in legume symbionts appears to be determined predominantly by the strain of *Rhizobium*. For example, Schubert *et al.* (1977) using both soybeans and

cowpeas, formed two groups of legume-*Rhizobium* combinations based on whether or not they evolved H_2 in air. The allocation of combinations to the respective groups was predominantly dependent on rhizobial strain. Carter *et al.* (1978) found that, of 32 strains tested on 2 cultivars of soybeans, 6 exhibited no H_2 evolution in air and were shown to take up H_2 if it was exogenously supplied. Nodules formed by the remaining 25 strains had RE^s ranging from 0.41 to 0.80. Maier *et al.* (1978) have reported conclusive data that the genetic information for hydrogenase synthesis is located in the cells of *R. japonicum*. They were able to develop a suitable growth medium on which certain strains of *R. japonicum* expressed hydrogenase activity (H_2 uptake) under free-living conditions.

Despite the fact that the genetic information for hydrogenase synthesis may reside in the strain of *Rhizobium*, the environment within the nodule (itself dependent on the legume host) may have an important role in the expression and activity of hydrogenase in the bacteroids. Factors which have been shown to affect the expression of hydrogenase under free-living conditions are low concentrations of carbon substrates, decreased oxygen tension and the quantity of combined nitrogen in the medium (Maier *et al.* 1978). Hydrogenase activity is also dependent upon a pre-incubation period in the presence of H_2, indicating that H_2 may participate in the control of hydrogenase synthesis (Maier *et al.* 1978). Hence, in the legume symbiosis, the host plant may exert a major influence on hydrogenase expression and activity by regulation of one or more of the above factors.

However, there appears to be little published information on factors affecting hydrogenase expression in the intact legume symbiosis, although considerable effort is currently being directed to this aspect (A.H. Gibson, pers. comm.). One exception is a study using a tritium

exchange assay (Lim 1978) in pea root nodules reported by Bethlenfalvay and Phillips (1979). Hydrogenase activity was shown to decline with increased irradiance and to vary with plant ontogeny. The variation with plant ontogeny (a maximum in activity at the onset of flowering and decline thereafter) was associated with decreased photosynthate available to the nodule as the plants aged.

The relevance of hydrogenase activity to the overall energetics of nitrogen fixation and bacteroid metabolism is now being diligently researched. Dixon (1972) first suggested that there may be a relationship between hydrogenase and nitrogenase activity. He proposed that the oxidation of H_2 by this uptake hydrogenase served to: a) utilize excess O_2 and thereby maintain an anaerobic environment within the cell; b) prevent the build-up of H_2 evolved by nitrogenase which may otherwise inhibit nitrogenase; c) enhance the efficiency of nitrogenase by recycling the evolved H_2.

Recent research has since proved Dixon's postulates substantially correct, although the exact nature of the inter-relationships between hydrogenase and nitrogenase have yet to be elucidated. The oxidation of H_2 mediated by the H_2-uptake system has been shown to protect nitrogenase from O_2 damage (Emerich *et al.* 1979; Ruiz-Argueso *et al.* 1979a) although no respiratory protection was afforded in the case of *Azospirillum brasilense* (Pedrosa *et al.* 1982). The oxidation of H_2 by the hydrogenase has also been shown to generate ATP (Dixon 1972; Emerich *et al.* 1979; Ruiz-Argueso *et al.* 1979a).

In relation to Dixon's second postulate that, by decreasing the concentration of H_2 in the cell, hydrogenase eliminates the inhibition of nitrogenase by H_2, Walker and Yates (1978) have showed that (at least in

A. chroococcum) the H_2 dispersed too rapidly to inhibit N_2 reduction. In blue-green alga, *Anabaena cylindrica* (Bothe *et al.* 1977); and *A. chroococcum* (Walker and Yates 1978), H_2 oxidation provided both ATP and reductant for nitrogenase but it is still uncertain as to whether H_2 serves as a reductant for nitrogenase in legume bacteroids (Emerich *et al.* 1979).

Possession of the uptake hydrogenase has been reported to increase plant yields (nitrogen yield and dry matter in whole plants). For example, soybeans inoculated with *Rhizobium* strains that synthesized the hydrogenase system, fixed significantly more nitrogen and produced greater dry-matter yields than strains lacking hydrogen-uptake capacity (Schubert *et al.* 1978; Albrecht *et al.* 1979; Zablotowicz *et al.* 1980; Hanus *et al.* 1981). Similar results have been obtained with cowpeas (Schubert *et al.* 1978) and very recently with mungbean, *Vigna radiata* (Pahwa and Dogra 1981).

It should be noted here that in the above reports, comparisons were always made between *Rhizobium* strain-host combinations that possessed the H_2-uptake system and either the H_2-evolving system or H_2-uptake negative mutants. It is possible that the observed benefits were due to other unidentified differences (even in the case of the mutants unless the latter have been proved to be strictly isogenic except for hydrogenase).

2.2.4 Host factors and hydrogen dynamics

Environmental effects on H_2 loss from nodules may be due to effects on either the allocation of reductant to H^+ or N_2 by nitrogenase or to uptake hydrogenase activity, or both. Distinction between the two

processes is not readily apparent when only H_2 evolution is measured without specific assays for hydrogenase activity. This is particularly the case in some symbionts where the hydrogenase activity may be insufficient to recycle all the H_2 produced by the nitrogenase system under normal aerobic conditions. Thus Ruiz-Argueso *et al.* (1978), by eliminating nitrogenase-catalyzed H_2 production in peas by deactivating the nitrogenase with high O_2 concentrations, were able to demonstrate H_2 uptake in some symbiont combinations which actually evolve H_2 under ambient conditions. Hydrogenase activity in nodules which evolve H_2 in air has also been observed in nodules of red clover (*Trifolium pratense*) and vetch (*Vicia sativa*) nodulated by native *Rhizobium* species in the field (Evans *et al.* 1978).

The most extensive published studies of environmental effects on hydrogen dynamics in a legume symbiosis have been reported by Bethlenfalvay and co-workers using *Pisum sativum* L. cv. Alaska, inoculated with *R. leguminosarum*, strain 128C53 (Bethlenfalvay and Phillips 1977a, b, 1978, 1979; Bethlenfalvay *et al.* 1978a, b, c). Considering these reports as a whole, one has to conclude that, although environmental factors (such as form and quantity of combined nitrogen, light intensity, plant ontogeny and source-sink manipulations) are shown to affect H_2 loss and/or RE, the results are not always consistent (Table 2). This is not unexpected because of the problem of differentiating between a change in electron allocation between substrates and uptake hydrogenase activity.

In one study, Bethlenfalvay and Phillips (1979) attempted to separate the effects of ontogeny and irradiance on nitrogenase and hydrogenase activities. Hydrogenase activity was measured by tritium exchange (Lim

TABLE 2:

The effect of environmental factors

on the apparent RE in peas

Treatment Variable	Treatment Range	RE Range	Nature of Response	Reference
1) light intensity	PAR† 200-800 $\mu E\ s^{-1}\ m^{-2}$	1.0 - 0.62	directly inverse	Bethlenfalvay and Phillips 1977a
2) light intensity	PAR 400 & 700 $\mu E\ s^{-1}\ m^{-2}$	0.93 & 0.91+ respectively	nil	Bethlenfalvay and Phillips 1978
3) combined N 16 mM NH_4^+	-	0.93+		
or 16 mM NO_3^-	-	0.97+	nil	Bethlenfalvay and Phillips 1978
or nil	-	0.91+		
4) combined N (NH_4^+)	0-16 mM	0.47 to 0.78	bimodal max. at 2-4 mM	Bethlenfalvay *et al.* 1978b
5) plant ontogeny	onset flowering to senescence	0.4 to 0.7	linear increase to maximum at pod filling	Bethlenfalvay and Phillips 1977b
6) source-sink manipulations x plant ontogeny	control depodded defoliated	0.52++ - 0.99 0.65 - 0.98 0.75 - 1.00	decapitation (nil) ontogeny* (linear increase to near maturity)	Bethlenfalvay *et al.* 1978a

† Photo-synthetically active radiation.

\+ Calculated from H_2 evolution and AR rates reported.

++ Sampled 7 days prior to the other two treatments.

* Control bimodal with a minimum during mid-vegetative stage.

1978) which, as the authors point out, measures activation of H_2 rather than allowing quantitative estimates of H_2 produced by nitrogenase which is recovered by hydrogenase in the system. Their results indicated that effects of increased light intensity on RE (Table 2 Ref. 1) were probably due to both a shift in electron allocation by nitrogenase from N_2 to H^+ reduction and to higher levels of uptake hydrogenase activity at lower irradiance. Furthermore, ontogenetic studies indicated that the rate of decline differed as the nodules matured, probably resulting in increased rates of H_2 recapture in older plants.

The question of whether the electron allocation pattern by nitrogenase or hydrogenase expression and activity is related to photosynthate availability to the nodule remains uncertain from the above studies. The coincidence of the increase in RE with declining photosynthetic activity in the leaves and with the development of reproductive structures (Bethlenfalvay and Phillips 1977b) suggests a causal relation between the change in RE and changes in source-sink activity. However, in a subsequent experiment (Bethlenfalvay *et al.* 1978a) in which the source-sink activity was varied by either depodding or defoliating the plants, the RE was unaffected. As suggested by the authors, an alternative cause for an increase in RE as the host plant matures may be changes in hormonal factors within the host plant, perhaps triggered by flowering, which affect hydrogenase or nitrogenase functions in the bacteroids.

Other more recent reports tend to support the view, nevertheless, that photosynthate supply to the nodules is implicated in the control of RE. Lambers *et al.* (1980) found that the RE in *Lupinus albus* was lower at night than during the day and Murphy (1981) reported higher RE in white clover plants grown under longer photoperiod (14 h) conditions than in plants grown under conditions of short photoperiod (6 h).

The above two reports suggest that carbon depletion may reduce the RE which contrasts to recent evidence gained by Gibson *et al.* (1981), in which factors likely to reduce photosynthate supply to the nodules of subterranean clover (i.e. lowered light intensity, addition of mineral nitrogen or defoliation of the plant) resulted in an elevated RE. As pointed out by these workers, closer definition of the physiological processes involved is required before the observed effects of host manipulation on both H_2 production or uptake can be rationalized.

2.3 Methods of measuring N_2 fixation in the field

2.3.1 Nitrogen balance

Estimates of N_2 fixed by the symbiotic association of a legume and *Rhizobium* derived from nitrogen balance studies of the overall plant-soil-system are extremely unreliable and difficult to obtain. Not only are there other factors contributing to the input of nitrogen such as asymbiotic N_2 fixation and precipitation, but there are potentially major losses through denitrification, volatilization, leaching and erosion, all of which must be measured. In addition, the pool of organic nitrogen in the soil usually far exceeds the quantities of N_2 fixed and any losses that occur through the above processes, thus rendering balance studies insensitive and subject to site variability.

2.3.2 Nodule number, mass and leghemoglobin content

Although a correlation can usually be established between nodule number, nodule mass or leghemoglobin content of the nodule and the rate of N_2 fixed at any given time, the relationship usually does not remain constant over time, i.e. either diurnally or throughout plant ontogeny (Mague and Burris 1972; Trinick *et al.* 1976; Farrington *et al.* 1977). Therefore, with the possible exception of leghemoglobin concentration, this class of measurement is not recommended (Criswell *et al.* 1976). In the case of leghemoglobin content, only approximate estimates of N_2 fixation are possible and the presence or absence of this compound in the nodule is not always a true indication of nitrogenase activity (Criswell *et al.* 1976).

2.3.3 Total plant nitrogen

Total plant nitrogen can be readily determined by the kjeldahl or dumas analytical methods (Bremner 1965) and may be suitable in laboratory studies when exposures are made for a substantial time and when the percentage change in plant nitrogen is reasonably large (Burris 1974). However, this method is severely limited in field studies because it does not differentiate between symbiotic N_2 fixation and mineral nitrogen derived from the soil. The method can be modified to include control (non-fixing) plants which allow an estimate to be made of the mineral nitrogen contribution of the soil.

This technique has been widely used in field studies and is based on the assumptions that the control plant takes up the same amount of soil nitrogen as does the legume under investigation and that the nitrogen in the control plant is derived solely from soil nitrogen. The type of control plants used have varied from uninoculated legumes of the same variety as the inoculated legume (Vest 1971; Bezdicek *et al.* 1978), inoculated, but with ineffective bacteria (Nutman 1976), non-nodulating isolines (Weber 1966; Harper 1974; Ham *et al.* 1975; Bhangoo and Albritton 1976), or a non-legume species (Wagner 1954; Bell and Nutman 1971; Brockwell 1971; Cocks 1980).

The use of this technique can be criticised because the control and N_2-fixing plant may not, and in some cases have been shown not to, take up equal quantities of soil mineral nitrogen (Harper 1974; Williams *et al.* 1977; Rennie *et al.* 1978; Deibert *et al.* 1979; Amarger *et al.* 1979).

2.3.4 ^{15}N-labelled methods (indirect)

The use of ^{15}N-enriched nitrogen fertilizer has overcome some of the problems associated with the 'difference' techniques outlined in 2.3.3 above. Besides being in the order of 100 times as sensitive as kjeldahl methods (Burris and Wilson 1957; Hardy and Holsten 1977) the assumption that control and N_2-fixing plants take up the same amount of nitrogen from the soil is not required.

Two variants of the indirect isotope method have been used; both require similar measurements but are based on different assumptions. In one variant, ^{15}N-labelled fertilizer is applied to both the N_2-fixing legume and the control in small quantities and it is assumed that both treatments have access to similar pools of soil nitrogen (McAuliffe *et al.* 1958; Vallis *et al.* 1967; Legg and Sloger 1975; Haystead and Lowe 1977; Vallis *et al.* 1977; Goh *et al.* 1978; Edmeades and Goh 1979; Heichel *et al.* 1981). The second variant ("A" value) is independent of the total nitrogen taken up by either the control or N_2-fixing legume. In this case, an amount of fertilizer nitrogen can be applied to the control crop so that it can be grown with an adequate supply of nitrogen while the amount of nitrogen applied to the legume crop may be kept to a minimum to prevent inhibition of N_2 fixation (Fried and Broeshart 1975, 1981; Fried and Middelboe 1977; Heichel *et al.* 1981).

The general equation for isotope dilution is

$$X_2 = (C_1/C_2 - 1)\ X_1\ (M_2/M_1)$$

where X_1 = weight of added labelled compound

X_2 = weight of the compound in the unknown sample

C_1 = enrichment of original labelled compound

(atoms-% ^{15}N excess)

C_2 = enrichment of compound recovered from mixture (atoms-% ^{15}N excess)

M_2/M_1 = correction for molecular weight change of compound as its isotopic concentration changes upon dilution.

The A-value concept as applied in the second variant of the isotope dilution method is derived from the assumption that a plant having two sources of a nutrient will absorb this nutrient from these sources in direct proportion to the amount available (Fried and Dean 1953). Thus the amount of nutrient available in the soil (A) can be represented by:

$$A = B(1 - y)/y$$

where B is the amount of nutrient in the standard, and y is the proportion of the total nutrient in the plant derived from the fertilizer.

The amount of fixed nitrogen (N) contained in a legume crop can be calculated from the relationship:

$$N = 1 - \frac{\text{atom \% } ^{15}N \text{ excess in legume crop}}{\text{atom \% } ^{15}N \text{ excess in control crop}} \times \text{total N in legume crop}$$

Although the success of this method is independent of the rate of application of fertilizer, the control plant must still take up fertilizer and soil nitrogen in the same proportion as the legume.

This requirement may be a major problem with the use of the indirect ^{15}N-labelled methods and the success of this technique is very dependent on the appropriate choice of a non-fixing control plant (Rennie *et al.* 1978; Witty 1983). In field experiments, application of ^{15}N-enriched fertilizer may result in uneven labelling of the pool of nitrogen available to the plants at different depths of soil. Differences between the legume plant and non-fixing control plant in relation to the density of root growth at various depths of soil may result in the latter plant taking up

fertilizer and soil nitrogen in a different proportion to that of the former. An extreme case, but one which may commonly occur in relation to the measurement of N_2 fixation in lupins, is where the applied ^{15}N-enriched fertilizer may be rapidly leached through sandy surface soil horizons and be unavailable to a more shallow-rooting, non-fixing control plant. Some tropical legumes, e.g. pigeon pea (*Cajanus cajan*) are further examples of particular legumes with patterns of root growth that may not be easily matched by the root growth of any non-fixing plant (Rennie *et al.* 1978).

It should be noted here that, not only must the legume and non-fixing control plant have similar rooting patterns, but also similar nitrogen-uptake profiles. Witty (1983) has shown that, although the legume and control plant may take up the same quantity of nitrogen from the soil, any difference in the time at which the uptake occurs may introduce large errors. These errors may be caused by changing (usually declining) soil ^{15}N-enrichment with time and hence a decline in the ^{15}N-enrichment of nitrogen available to the plant.

Other assumptions are also implied in these indirect methods, such as the constancy of the A-value for a given soil, and these have been addressed by Hauck and Bremner (1976). Nevertheless, evidence is accumulating that indirect methods of measuring N_2 fixation using ^{15}N-labelled fertilizer or ^{15}N-labelled organic residues (Ladd 1981) may give reliable estimates of N_2 fixation where suitable control plants can be used (Ruschel *et al.* 1979, 1982; Deibert *et al.* 1979; Rennie *et al.* 1978, 1982; Heichel *et al.* 1981; Wagner and Zapata 1982; Witty 1983). However, studies comparing the technique with a direct, independent method

are still lacking, although some experiments comparing $^{15}N_2$ reduction, AR, nitrogen difference and ^{15}N-isotopic dilution were in progress in 1978 (Rennie *et al.* 1978).

2.3.5 Natural abundance methods

A modification of the indirect ^{15}N-method is to use the difference in natural abundance in ^{15}N which may occur between fixing and non-fixing plants (Delwiche and Steyn 1970; Rennie *et al.* 1976, 1978; Amarger *et al.* 1979; Kohl and Shearer 1980; Shearer *et al.* 1980). This eliminates the need to apply ^{15}N-enriched (or depleted) fertilizer which may be expensive in large-scale field experiments and which may give rise to errors associated with uneven distribution of fertilizer with depth as well as changes in rates of mineralization of soil organic matter and/or immobilization of added and perhaps mineralized nitrogen (Hauck and Bremner 1976).

However, a number of problems are evident in the use of this method and more studies are required before wider adoption could be recommended. The major assumption in the use of the technique is the same as for all the indirect ^{15}N methods of estimating N_2 fixation, i.e. equal exploitation of soil by the roots of the N_2-fixing and control plants such that the mineral nitrogen taken up by both plants is of the same ^{15}N-enrichment. That this may not be always the case, at levels of natural abundance, is highlighted by studies which indicate that not only is there significant variability between soil types in soil ^{15}N-natural abundance (Cheng 1964; Shearer *et al.* 1978; Broadbent *et al.* 1980) but also spatial variability exists within a particular field (Broadbent *et al.* 1980) and variation in

^{15}N-natural abundance occurs with depth of soil (Delwiche and Steyn 1970; Steele and Wilson 1981). Although it is considered that careful selection of sites may overcome spatial variability (i.e. if topographical discontinuities are avoided - Karamanos *et al.* 1981), variation in ^{15}N-natural abundance with depth of soil cannot so easily be overcome.

A further problem is the uneven distribution of ^{14}N and ^{15}N in the plant (Moore and Craswell 1976; Shearer *et al.* 1980) making it imperative that the whole plant (including roots) is sampled.

Another potential source of error may be introduced if significant isotope discrimination occurs during the N_2 fixation process *per se*. There is some evidence that this may occur and give rise to major errors in natural abundance methods (Rennie *et al.* 1978). However, isotope discrimination is of neglible significance when ^{15}N-enriched fertilizer techniques are employed.

Despite the various potential sources of errors that may be associated with the use of the indirect ^{15}N techniques, their major advantage is that seasonal estimates of N_2 fixation can be made without having to integrate numerous short-term measurements taken throughout the season, as required for the $^{15}N_2$-reduction and AR techniques described below.

2.3.6 $^{15}N_2$ reduction

Measurement of the uptake of ^{15}N-enriched N_2 is generally accepted as the most reliable method for measuring biological nitrogen fixation (Burris 1974; Hauck and Bremner 1976). The method is both direct and sensitive but, because it is an almost instantaneous method, requires integration of numerous measurements if seasonal estimates of N_2 fixation are required. Another major problem is that the nodules or root system of

the plant being investigated must be confined within some sort of enclosure so that the ^{15}N-enrichment of the gas phase can be controlled and measured. This requires removal of the plant from the soil in field studies and errors associated with plant perturbation may be introduced.

The only assumption implied in this technique is that nitrogenase does not discriminate between ^{14}N and ^{15}N. Hoering and Ford (1960) and Delwiche and Steyn (1970) reported that very little, if any, discrimination occurs in N_2 fixation by *Azotobacter vinelandii* that would be of practical significance to the use of the technique. Although isotope discrimination may be significant in natural abundance methods (Rennie *et al.* 1978) it is unlikely to introduce serious error when higher enrichments of $^{15}N_2$ are used (Bergersen 1980).

The methodology involved in using ^{15}N-enriched N_2 to measure N_2 fixation has been described in several reviews (Burris 1972, 1974; Hauck and Bremner 1976; Criswell *et al.* 1976; Bergersen 1980) and will not be elaborated on here. The use of suitable enclosures, gas phases and sampling techniques are further discussed in conjunction with the AR assay.

2.3.7 Nitrogenous solute analysis

Certain leguminous species have been shown to export fixed nitrogen as ureides with the abundance of these compounds in the xylem sap depending on the symbiotic dependence of the plant (McClure and Israel 1979; McClure *et al.* 1980; Pate *et al.* 1980). The technique has been modified for use in the field with soybean by analysing tissue extracts (rather than xylem solute, which is often difficult to obtain in field-grown plants) for the relative abundance of ureides and nitrate (Herridge 1982a,b).

Insufficient testing of this technique has been carried out to date but it is worthy of consideration if investigating seasonal patterns of N_2 fixation in ureide-producing plants. However, lupins do not produce significant amounts of ureides (Pate *et al.* 1980).

2.4 The acetylene reduction (AR) assay

This section reviews the methodology involved in the application of the AR assay to nodulated roots and whole leguminous plants, with particular emphasis on techniques applicable to field use. Because the basis of the assay is the measurement of the rate of production of a gas (C_2H_4) some type of enclosure is required to enable changes in gas concentration to be measured. The type of enclosure used with legumes is primarily dependent on the aims of the experiment and the degree of perturbation of the plant-soil system deemed necessary or desirable to fulfil these aims. Various assay systems have been used, varying in complexity from the use of isolated bacteroids to the use of whole plants growing *in situ* in the field.

Factors which need to be considered when using the AR assay are the gas phases involved, *i.e.* partial pressures of O_2, N_2, CO_2, C_2H_2, and environmental factors such as temperature, and water regimes maintained within the system. Each of these factors will be considered in relation to the AR assay and the degree to which the assay reflects the true rates of AR *in situ*. It is important to stress here, that close matching of the environmental conditions of the assay system with those of the *in situ* system being measured may not be imperative, if only comparative rates of nitrogenase activity between treatments are required, but may become critical if absolute rates of nitrogenase activity are sought.

2.4.1 Plant mutilation

Plant assays usually fall into one of three categories based on the degree of plant mutilation, *i.e.* detached nodules, nodulated roots (plant top removed) or intact, whole plants (usually measured *in situ* but

sometimes removed from the growth medium). All studies where comparisons have been reported have shown that excised, nodulated roots yield (commonly two-fold) higher rates of AR than excised nodules, e.g. soybean (Bergersen 1970; Mague and Burris 1972; Fishbeck *et al.* 1973; Huang *et al.* 1975a), lupins (Trinick *et al.* 1976) and birdsfoot trefoil (Ranga Rao 1977).

The reasons for the decline in nitrogenase activity with nodule detachment have been assumed to be lowered carbohydrate supply (as discussed below for decapitated plants) and physical damage to important membranes and other barriers within the nodule which may allow leakage of carbohydrate or the entry of excessive O_2. In lupins, for example, nodules have a large proportion of their surface embedded in the root and detached nodules often have pink tissue directly exposed to the atmosphere (Trinick *et al.* 1976). However, recently Ralston and Imsande (1982) reported that the nitrogenase activity of detached soybean nodules could be restored to the original activity prior to detachment by increasing the partial pressure of O_2 (pO_2) in the assay vessel from 0.2 to 0.5 atm. Furthermore, maximum nitrogenase activity of attached nodules was achieved in the presence of 0.2 to 0.3 atm O_2. These workers suggest that the entry of O_2 is actually impaired upon detachment from the nodules.

Decapitation of the plant ultimately decreases nitrogenase activity of the legume nodule through the annexation of photosynthate from the leaves and depletion of reserve substances. However, the extent to which maximum rates of nitrogenase activity can be maintained in the short term, without current translocated photosynthate from the tops, is uncertain. Pate (1962) and Small and Leonard (1969) demonstrated with experiments using ^{14}C that photosynthate was translocated very rapidly from the shoot to the nodules of a number of species. Lawrie and Wheeler (1973) suggested

that maintenance of optimum rates of N_2 fixation in peas required an adequate supply of recently manufactured photosynthate to the nodules.

Huang *et al.* (1975a), using apparatus that allowed measurement of AR *in situ* simultaneously with net photosynthesis and transpiration of the shoot, reported that the time lag between detopping soybeans and the first detectable effects on AR varied between 30 and 60 minutes. These workers also concluded after further experiments (Huang *et al.* 1975b) that root reserves supporting AR were low in soybean and that nitrogenase activity depended mainly on current photosynthesis. This was later challenged by Mederski and Streeter (1977) who reported considerable (> 50% maximum rate) nitrogenase activity in the absence of current photosynthesis in soybean. However the latter workers were able to demonstrate an almost immediate (after few minutes) decline in AR following detopping, when they used continuous, automated AR assays on intact plants *in situ*.

Wych and Rains (1978) compared rates of AR in intact plants assayed in a mist chamber with those of excised roots assayed both in the mist chamber and in glass jars. Immediately after the shoots were removed the AR of the excised roots declined by 24% and remained constant for one hour before dropping to only 21% of the rate of the intact control for a further 2.5 h. The AR of excised roots assayed in the jars responded in a similar manner after decapitation, although the decline in AR during the first hour was more pronounced than for the excised roots assayed in the mist chamber.

Two other studies with soybean, in which the AR by nodulated roots has been directly compared with that of whole plants, yielded conflicting results. Mague and Burris (1972) reported a 50% mean drop in activity following decapitation but Fishbeck *et al.* (1973) reported no difference. However, in the latter study the whole plant (i.e. tops and roots) and

decapitated treatments were assayed in the dark at a pC_2H_2 of 0.025. It is possible that removing the plant tops from the light may have eliminated any difference in AR between the two treatments. In addition, the assays were performed at a sub-saturating concentration of C_2H_2 (as determined by these same workers for plants *in situ*). Hence any effect of the removal of the plants from the rooting medium on concentration of C_2H_2 required to achieve saturation of nitrogenase may have affected the comparisons.

However, Trinick *et al.* (1976) with lupins and Saito *et al.* (1980) with beans, also reported no significant difference in AR rates in intact plants (tops not exposed to C_2H_2 in some cases) and nodulated-root (decapitated) systems. Minchin and Pate (1974) reported a significant decrease in AR within 2 h of decapitation in peas with a further decrease after 4 h.

With respect to pasture legumes, Moustafa *et al.* (1969) reported that AR in white clover began to decrease 30 minutes after removal of plant tops and Halliday and Pate (1976) reported an 80% decline in AR rates within 6 h of defoliation. AR in lucerne decreased by 88% (Vance *et al.* 1979) and 70% (Groat and Vance 1981) within 24 h following harvest of top growth (70-80% shoot removal).

Irrespective of plant species, it appears that removal of the plant top may have an adverse effect on AR within 24 h in most cases and within 1 h of decapitation in many cases. As the amount of carbohydrate reserves sustaining nitrogenase activity may affect how quickly AR of the excised root system declines and, as the former may vary with experimental conditions, it is impossible to generalize on the effect of decapitation on nitrogenase activity in the short term. However, it would seem imperative

that no effect of decapitation on nitrogenase activity be demonstrated under the conditions of each experiment before assays of excised nodulated roots can be taken as reflecting absolute rates of AR *in situ*.

2.4.2 Effects of oxygen and water

The enigmatic role of O_2 in the process of N_2 fixation is well documented. The nitrogenase enzyme complex is extremely sensitive to O_2; bacteroids are deactivated within 15 minutes by exposure to ambient O_2 (Bergersen and Turner 1968). Nevertheless, O_2 is essential for the supply of ATP by oxidative phosphorylation, which in turn supplies the reducing power required for nitrogenase function (Bergersen 1960). As noted by Mague and Burris (1972) and Criswell *et al.* (1977), reports in the literature of the effect of pO_2 on nitrogenase activity are conflicting. For example, Bergersen (1962) demonstrated that the optimum rate of uptake of ^{15}N-enriched N_2 in soybeans occurred at about 0.5 atm O_2 and Koch and Evans (1966) observed saturation of nitrogenase activity between 0.2 and 0.4 atm O_2. Bergersen (1970) and Mague and Burris (1972) reported maximum rates of AR at a pO_2 between 0.7 and 0.9 atm. Trinick *et al.* (1976) with lupins and Masterson and Murphy (1976) with white clover, obtained maximum rates of AR at a pO_2 of 0.2 atm. In the former case, AR declined rapidly from a pO_2 of 0.4 atm to almost zero at a pO_2 of 1 atm, but in the latter case no inhibition of AR was observed up to a pO_2 of 0.4 atm, the highest concentration tested.

With the exception of the study by Trinick *et al.* (1972), in which whole plants of one variety of lupins tested displayed an AR response to

pO_2 similar to that of nodulated-root systems, none of the above studies were performed on whole plants. That plant mutilation may have a decisive impact on the response of nitrogenase to rhizosphere O_2 concentration is well illustrated in experiments performed with two varieties of soybean by Criswell *et al.* (1977). They found that the response in AR to pO_2 by excised nodulated roots differed in a number of important aspects from that of whole plants. Not only was the response more variable in the former, but AR was always stimulated by supra-ambient concentrations of O_2. In contrast, it was not possible to increase AR by whole plants above that obtained with the ambient concentration of O_2. Furthermore, exposures to low pO_2 did not decrease AR activity as much in intact plants as in excised roots. As stressed by these workers, maintenance of plant integrity proved essential for evaluating the effects of pO_2 on nitrogenase activity in soybeans.

In relation to the use of the AR assay and O_2 status, Bergersen (1970) suggested that, for the calibration of the relationship between AR and N_2 fixation, the C_2H_2 concentration should be adjusted to give equimolar concentrations of C_2H_2 and N_2 in solution. This would then eliminate differential effects of pO_2 upon the separate assays and the C_2H_2:N_2 ratio should remain constant over a wider range of pO_2. This obviously may not be so important with intact-plant assay systems where the AR response to pO_2 may be negligible within the normal range of O_2 concentrations likely to be encountered (Criswell *et al.* 1977), or where minimal disturbance is made to the gas composition of the rooting medium (as in some *in situ* assay systems).

A second important aspect of O_2 concentration in relation to the AR assay is the maintenance of the required (usually ambient) concentration of

O_2 in the assay vessel during the AR assay. Nodules have a greater requirement for O_2 than a similar mass of root tissue. Allison *et al.* (1940) and Bond (1941) reported similar requirements for O_2 by nodules and roots but more recently Layzell *et al.* (1979) reported that nodules of lupins respired 73% of the total CO_2 respired during a 30 minute assay of nodulated roots and 63% on the basis of equal fresh weight of roots and nodules. Ryle *et al.* (1978) found that soybean and cowpea nodules respired at 3-4 times that of an equal mass of root during intense periods of N_2 fixation.

Thus the size of the enclosure must be sufficient to prevent serious depletion of O_2 concentration during the assay. Mague and Burris (1972) suggested that samples of detached nodules should occupy no more than 20% (v/v) of the incubation vessel for a half-hour assay, although this may well be conservative. Sprent (1969) reported linear rates of AR in 910 mg samples of soybean nodules for up to 2 h in 7 ml assay vessels. This would be equivalent to the nodules occupying well over 20% of the assay volume on the basis of a half-hour assay. Obviously the rate of respiration of the enclosed tissue (and therefore the rate of O_2 depletion) will depend on the activity of the nodules which in turn will depend on plant species and experimental conditions.

As indicated by Gibson (1977), the effects of high O_2 concentrations on nitrogenase activity are of little more than academic interest as the O_2 concentrations of agricultural habitats will be lower (rather than higher) than that of the atmosphere. The two principal environmental factors associated with lowered O_2 concentrations are poor soil structure and waterlogging. Smith and Dowdell (1974) reported that concentrations of

O_2 in a heavy clay soil at 15 cm depth remained below 10% (v/v) throughout the winter and spring, but there followed a gradual rise in O_2 concentration to near-atmospheric levels as the soils dried out. However, the O_2 concentration in a sandy loam declined below 12% only for a short period (approximately 1 week) and was greater than 15% for most of the growth period. Russell (1973) quotes some typical figures for the composition of the soil atmosphere in surface soils of north-western Europe in which the O_2 content was usually over 20% and rarely below 15%. For field evaluation of nitrogenase activity, assay techniques such as described by Balandreau *et al.* (1978) should best mimic the actual concentrations of O_2 to which the plant is exposed *in situ*.

Effects of water

Experiments reported by Sprent (1969) and Sprent and Gallacher (1976) clearly demonstrated decreased AR in soybean nodules caused by a film of water on the nodules. Van Straten and Schmidt (1975) maintained that a water layer *per se* had no effect on AR as long as the nodule surface was undamaged, i.e. not detached from the root, but Sprent and Gallacher (1976) and Trinick *et al.* (1976) also found decreased AR in excised, roots with nodules intact. Minchin and Pate (1975) found that waterlogging decreased the production of nodule tissue and decreased AR in peas, and Pate (1976) reported that optimum rates of AR occurred when sand-cultured peas were watered on alternate days with free drainage at all times. Mague and Burris (1972) reported that soybean nodules, blotted dry after soaking in water for 10 minutes, displayed a decrease in AR but a brief washing and drying had no effect. Trinick *et al.* (1976) reported similar results with lupin nodules on both intact and excised-root systems. The latter workers also observed a significant decrease in AR activity in field-grown

lupins after excessive rain. Gibson (1980) has observed a 90% decline in AR activity in lupin nodules following washing, due to temperature effects and the blockage of aeration pathways in the nodules.

It should not be inferred here that the presence of water on the nodule will automatically decrease AR activity. Wych and Rains (1978) demonstrated that substantial AR in soybeans could be maintained in an aeroponic system. The continual coating of the nodule surface with a mist saturated with both C_2H_2 and O_2 seemed to ensure adequate diffusion of these gases across the liquid-boundary layer. The fact that a stagnant liquid layer on the nodule surface may impose an O_2 diffusion barrier of sufficient effectiveness to decrease nitrogenase activity, indicates that diffusion of O_2 from the nodule surface to the bacteroids within the nodule is probably through a gas phase. A liquid barrier on the nodule surface would be unlikely to cause such dramatic effects on AR if movement of O_2 within the nodule already had to diffuse through a liquid.

Sprent (1972) and Bergersen and Goodchild (1973) have deduced from anatomical studies, that continuous intercellular spaces exist between the nodule surface and centre. The recent calculations of Sinclair and Goudriaan (1981) further highlight the importance of nodule structure in O_2 assimilation. By solving differential equations for the various gradients and concentrations of O_2 in the nodule they calculated that intercellular gas spaces must exist in nodules for adequate O_2 distribution. An interesting conundrum is their hypothesis that a continuous shell of water only 45 μm thick around the bacteroid-containing cells in the inner cortex of the nodule would be required to decrease the concentration of O_2 to a level required for normal nitrogenase functioning within the bacteroids (Tjepkema and Yocum 1974).

2.4.3 CO_2 in the rhizosphere

There appears to be a paucity of information in the recent literature related to possible effects on N_2 fixation of CO_2 concentration in the rhizosphere. Early reports are variable; e.g. Stolwijk and Thimann (1957) reported that the growth of roots of some legumes (*Pisum, Vicia* and *Phaseolus*) was completely inhibited if the rooting medium was aerated with 6.5% CO_2 in air. Peas were inhibited at concentrations as low as 1.5%, although a small stimulation of root growth occurred at 5% CO_2. Grable and Danielson (1965) found that CO_2 concentrations below 29% had little influence on soybean growth. Mulder and van Veen (1960) reported that both N_2 fixation and growth were considerably decreased in red clover, peas and beans grown in a CO_2-free culture solution. With red clover the effect of CO_2 on N_2 fixation was dependent on the pH of the nutrient solution.

To the author's knowledge, the only report of the CO_2 concentration in the rhizosphere affecting the specific nitrogenase activity of legume nodules is that of Bergersen (1971) in which he states that there was increased uptake of $^{15}N_2$ in two experiments with detached soybean nodules when CO_2 was added to treatments containing low pO_2 concentrations. There was no effect in treatments containing 0.3 pO_2 levels or higher, presumably due to increased nodule respiration removing any CO_2 limitation.

Recent evidence of possible links between CO_2 fixation *in vivo*, phosphoenolpyruvate (PEP) carboxylase and nitrogenase activity *in vitro* during nodule development (Christeller *et al.* 1977) may be important in relating nitrogenase activity to the CO_2 concentration in the rhizosphere. However, the above workers have shown that PEP carboxylase

saturates *in vitro* at very low concentrations of CO_2 and that the quantities of CO_2 normally evolved via nodule respiration would be sufficient to saturate the enzyme.

During the AR assay, which is usually performed in a vessel in which 10% v/v of the air has been replaced by C_2H_2, CO_2 may increase to relatively high concentrations during the course of the assay, depending of course on the volume of the assay vessel. For example, 1 g of fresh lupin nodules assayed for half an hour in a 7 ml serum bottle (Sprent 1969) would raise the CO_2 concentration to approximately 7.5% at 20°C if the respiration rate was 43.5 μmoles CO_2 h^{-1} per g fresh weight of nodules [as reported by Layzell *et al.* (1979)].

In practice, the CO_2 concentration of the soil is many times greater than that of air and is usually in the range of 0.1 to 1.5%, although values as high as 10% have been recorded (Russell 1973). Soil factors that affect gaseous diffusion, such as soil structure and water content, together with the presence of actively respiring plant roots and microflora, will determine the CO_2 concentration at any soil depth at any time. The practice of including CO_2 at concentrations approximating atmospheric in flushing mixtures used for short AR assays (e.g. Schubert and Evans 1976) would seem unnecessary and probably poorly reflects the true CO_2 concentration *in situ* in many cases.

2.4.4 C_2H_2 concentration

The rate of AR is proportional to pC_2H_2 at concentrations less than required to saturate the enzyme and, for most experimental purposes, a pC_2H_2 should be selected to saturate nitrogenase to the same degree that it is saturated by ambient N_2 (Hardy *et al.* 1973). The reported Km (Michaelis constant) for N_2 in nodulated legume roots varies between

0.04 and 0.08 atm (Hardy *et al.* 1973; Bergersen 1970). pN_2 concentrations between 0.2 and 0.3 atm closely approach, but do not saturate, nitrogenase (Burris 1974; Bergersen 1980) and saturation can occur at a pN_2 greater than that of ambient air (Bergersen 1970).

The Km (C_2H_2) for nitrogenase in legume nodules has been reported to vary between 0.007 and 0.3 (Koch and Evans 1966; Hardy *et al.* 1968; Davis 1980) and is dependent on whether or not N_2 is present in the gas phase during the assay (Davis 1980). According to Hardy *et al.* (1973), a pC_2H_2 of 0.1 atm should produce saturation comparable to a pN_2 of 0.78 atm (i.e. ambient air) *in vivo*, and this C_2H_2 concentration has since been adopted in most reported uses of the AR assay. Where the effect of pC_2H_2 on AR has been checked, nitrogenase saturation has occurred at a pC_2H_2 within the range of 0.05 to 0.2 atm. For example, in excised nodules or excised nodulated roots, saturation has occurred at 0.1 atm C_2H_2 in lupins (Trinick *et al.* 1976); 0.05 atm (Koch and Evans 1966) to 0.2 atm C_2H_2 (Mague and Burris 1972) in soybean and 0.1 atm in subterranean clover (Vaughn and Jones 1976).

Mague and Burris (1972) also reported an apparent inhibition of AR by high concentrations of C_2H_2 (>0.2 atm) but suggested this may have been due to diminished pO_2 rather than toxicity of the C_2H_2. However, Vaughn and Jones (1976) also observed inhibition of nitrogenase activity at a pC_2H_2 of 0.5 atm in subterranean clover.

When intact plants have been assayed *in situ* a similar, but variable range in pC_2H_2 required to achieve maximum AR has been reported; e.g. pC_2H_2 of 0.035 (Mederski and Streeter 1977) and 0.10 (Fishbeck *et al.* 1973) in soybeans using continuous gas recycling and stationary gas phases

respectively; 0.05 pC_2H_2 in beans (Westermann and Kolar 1978); 0.05 pC_2H_2 in lupins removed from the soil (Trinick *et al.* 1976) and 0.03 pC_2H_2 in subterranean clover (Eckart and Raguse 1980).

There appears to be little evidence of inhibition of nitrogenase activity in intact plants at pC_2H_2 concentrations greater than that required to achieve saturation. The lack of evidence results mainly from a lack of investigation by research workers rather than from a demonstrable absence of inhibition. One exception was reported by Fishbeck *et al.* (1973) where a slight decline in AR occurred between 0.1 and 0.2 pC_2H_2.

2.4.5 Depression of nitrogenase activity by C_2H_2

Prolonged, continuous exposure of the root system of intact plants to air/C_2H_2 mixtures may depress nitrogenase activity (Mederski and Streeter 1977). However, the opposite effect (stimulation of AR) was observed by Eckart and Raguse (1980) in subterranean clover plants exposed to repeated AR assays over a 24-h period.

There is no evidence for feedback inhibition of nitrogenase activity by combined forms of nitrogen in cell-free systems (Hardy *et al.* 1973) but combined nitrogen may indirectly control nitrogenase activity in living organisms. The absence of formed NH_3 in the presence of saturating levels of C_2H_2 may stimulate increased synthesis of nitrogenase (Shanmugam and Valentine 1975). Moreover, in some micro-organisms, long periods of incubation with C_2H_2 may lead to a multifold enhancement of AR (David and Fay 1977). Long-term assays have therefore not been recommended [Hardy *et al.* (1973)].

The use of low concentrations (sub-saturation) of C_2H_2 (LaRue and Kurz 1973; Mahon 1977; Mahon and Salminen 1980), rapid pulsing (20 min

C_2H_2/air - 40 min flush cycle - Mederski and Streeter 1977) or short multiple assays on the same plants at one or two weekly intervals (Sinclair *et al.* 1978; Ruegg and Alston 1978) are methods that have been employed either to minimize effects of prolonged exposure of plant material to C_2H_2 and C_2H_4 or to make maximum use of experimental material.

2.4.6 Replacement of N_2

Of practical significance to the use of the AR assay, particularly in large and/or *in situ* systems, is whether the N_2 in the assay vessel needs to be replaced by an inert gas. Early data of Hardy *et al.* (1968) with soybean nodules indicated that failure to replace the N_2 with Ar decreased AR between 10% and 20%.

Davis and Wang (1980), reported studies of *in vivo* nitrogenase kinetics in which N_2 was only a weak inhibitor of AR at high concentrations of C_2H_2 but was a potent inhibitor at low concentrations. This effect has also been demonstrated in two species of lupins (Trinick *et al.* 1976) where substantial differences in AR were detected between assays conducted in Ar/O_2 and in air, with a pC_2H_2 of 0.02 to 0.025 atm. However, at concentrations of C_2H_2 sufficient to saturate nitrogenase in the two species, no effect of N_2 replacement on AR was detected. Mague and Burris (1972) working with soybean, and Mahon (1977) working with peas, also found no decrease in AR if the air was not replaced by Ar/O_2 mixtures.

2.4.7 Enclosures

As alluded to at the start of this section, the type of enclosure suitable for the AR assay depends on the aims of experimenter and the type of plant material (*e.g.* nodules, or whole plants) being assayed.

Provided attention is paid to the factors discussed above, there are few restrictions in the type of enclosure that can be used.

One important factor that may affect the type of enclosure chosen is the maintenance of the desired temperature. Because nitrogenase activity in legume nodules is sensitive to temperature and the response varies between varieties as well as between species (Dart and Day 1971; Gibson 1971; Waughman 1977), care must be exercised in maintaining a constant temperature during the assay. Furthermore, the assay temperature should be the same as that to which the nodules *in situ* are exposed, if the AR assay is to reflect nitrogenase activity *in situ*.

A second consideration is the type of material used to construct assay vessels because absorption and subsequent release of C_2H_4 by the vessel may occur. This has been shown to be important in the repeated use of rubber closures (Kavanagh and Postgate 1970). Types of assay vessels and systems employed for the AR assay have been thoroughly discussed in several reviews (*e.g.* Criswell *et al.* 1976; Gibson 1980) and the subject requires no further elaboration here.

3. MATERIALS AND METHODS

3. Materials and Methods

This chapter describes the methodology utilized in all experiments undertaken. Where methods varied from those stated here, the variations are described in the "Methods" sections relating to specific experiments.

3.1 Plant culture

Pots for use in the glasshouse or growth room were constructed from PVC (poly [vinyl chloride]) pipe of 100 mm internal diameter and were 290 mm in length. An end cap, sealed with silicon sealant[+] and drilled with small holes to allow drainage, formed the base of the pot. The rooting medium consisted of siliceous sand (3 kg dry weight) washed with acid and deionized water and sieved to between 0.5 and 1.0 mm particle size. The pots, including sand, were sterilized prior to sowing by exposure to heat (95^{o}C for 48 hours) in a forced-draught oven; thereafter no further microbiological control was exercised.

Lupin (*Lupinus angustifolius*) seeds, graded to even size, were surface sterilized using $HgCl_2$ (Vincent 1970), washed ten times in sterile water and allowed to stand in sterilized water at 50^{o}C for 30 minutes prior to inoculating with commercial peat inoculum (Nodulaid G - *Rhizobium lupini* strain WU 425) mixed with sterile gum arabic. The seeds were then sown, one per pot, at a depth of 3 cm in the moist sand and sterilized polypropylene beads added to form a 1 cm layer on the sand surface to minimize water loss from evaporation.

To obtain uniform plants, sufficient pots were prepared to allow between 15 and 20% of the pots to be culled prior to the imposition of treatments. Once the plants had emerged, the pots were flushed daily with

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[+] Silicone 781 - Dow Corning Australia Pty. Ltd., 21 Tattersall Road, Blacktown, N.S.W.

deionized water and on alternate days with nutrient solution. The nutrient solution contained no nitrogen and was adjusted to pH 6.5 with HCl (Appendix 1, p. 159).

3.2 Plant analyses

3.2.1 Nitrogen content

Separate nitrogen determinations were always made on tops and roots. Roots were washed with water and both tops and roots dried to constant weight at 85°C. Total nitrogen of the tops was determined using a micro kjeldahl method (Appendix 2, p. 160). The method was modified to include nitrate and organic nitro-compounds in the case of field samples. The extent of contamination of the roots by sand or soil was determined by difference from measurements of ash content of the material and the 'true' ash content of the plant, assumed to be 8%.

3.2.2 Nitrogen-15 (^{15}N) determination

Duplicate aliquots of distillate, prepared during kjeldahl analysis but with no indicator added, were acidified to between pH 3 and 4 with sulphuric acid and evaporated to dryness in an oven at 85°C. Mass 29:28 ratios were determined on an AEI mass spectrometer (Kelley *et al.* 1980). Samples whose ^{15}N enrichments (atoms-%^{15}N excess) were below 0.01 were analysed with a Micromass 602C mass spectrometer, utilizing a rapid gas sample preparation system (Ross and Martin 1970). Although ^{15}N enrichments could be resolved at lower values than this on the AEI, the analytical error was considered prohibitive. The natural abundance of ^{15}N of plant material (tops and/or roots), treated in identical manner (both experimentally and analytically) except for exposure to a ^{15}N-

enriched rhizosphere, was used as the standard in all calculations of atoms-%^{15}N excess of samples.

To determine the atoms-%^{15}N excess of the N_2 to which the nodulated roots were exposed during assays, duplicate 1 ml gas samples were taken in disposable syringes, and the needles sealed with rubber stoppers. The gas was injected into the AEI mass spectrometer using apparatus described by Burris (1974). Gas samples were stored in the syringes for usually no more than 24 h prior to analysis. Exceptions were necessary however, particularly during diurnal studies in the field. The syringes, with needles inserted in rubber stoppers, were sealed in jars filled with degassed, deionized water to minimize contamination from N_2 in the air during storage of the syringes. The atoms-%^{15}N excess of the gas samples were obtained by measuring the peak heights of the three masses 28, 29 and 30 (Bergersen 1980).

3.3 Soil analyses

3.3.1 Total nitrogen

Total nitrogen in soil was determined using the micro kjeldahl procedure described for plant nitrogen but without modifications to include nitrate or organic nitro compounds. The soil including course organic matter was previously dried to constant weight and ground to pass through a 0.5 mm sieve.

3.3.2 Mineral nitrogen

Ten g of soil was extracted with 100 ml of 2N potassium chloride within 24 h of sampling and stored at 4^{o}C until analysed. Ammonium and nitrate in the soil extracts were determined by the automated distillation method of Keay and Menage (1970).

3.3.3 Water content

Water content was determined on soil concurrently subsampled for mineral nitrogen analysis. Weighed fresh samples were dried to constant weight and the weight of water expressed as the percentage of the dry weight of soil at a temperature of 105°C.

3.3.4 pH

The pH of the soil was determined on a suspension of soil in distilled water (1:5 w/v) following mixing (30 minutes) and standing (1 h).

3.3.5 Organic carbon

The organic carbon content of the soil was determined by the method of Walkley and Black (1934).

3.4 Gas assays

3.4.1 Enclosures

Three basic enclosure systems were employed during the course of these investigations.

System 1 - Nodulated root assay (hereafter referred to as NRA)

NRA is defined as the incubation of one or more entire nodulated-root systems washed or shaken free of soil and severed from the plant tops. In all cases, incubation commenced within 3 minutes of removing the plant from its growth environment.

Two types of enclosures were used with this system

a. Syringe enclosures were used in some experiments prior to 1981. They were either single '50 ml' centre-lock disposable syringes which were graduated to 60 ml, or double syringes (*i.e.* two of the above syringes joined end to end with the tip assembly of one

removed to allow access of plunger throughout) sealed with a No. 9 Suba-Seal at the needle-lock end. The double syringe was used when the volume of the nodulated roots exceeded 20% of the single syringe volume. Maximum working volume of the double syringe enclosure was 150 ml.

This system was adopted because it was inexpensive, allowed rapid flushing of the enclosure with different gas mixtures, and allowed easy adjustment to atmospheric pressure (by movement of the plunger) following the addition of gas (*e.g.* C_2H_2) or following removal of a gas sample through the Suba-Seal. Flushing was accomplished by connecting the flushing-gas supply to a needle and inserting the latter through the Suba-Seal. Gas was allowed to escape by holding the plunger slightly ajar at the other end.

b. Glass jar enclosures (Figure 1a) were used for most work on the NRA system, eventually replacing the syringe enclosures entirely. Elimination of all N_2 from the syringe enclosures proved unreliable as some air frequently entered before the plunger could be firmly re-inserted into the syringe following flushing. This was of little consequence except in experiments where complete elimination of N_2 was required; i.e. where comparative assays were made of H_2 evolution under Ar/O_2 and AR. A second reason for adopting the jar enclosure was its more robust flushing features due to the incorporation of quick-fitting and precise gas connections, and its larger volume (270 ml).

System 2 - Whole plant assay in PVC pot (Plate 1) (hereafter referred to as WPA)

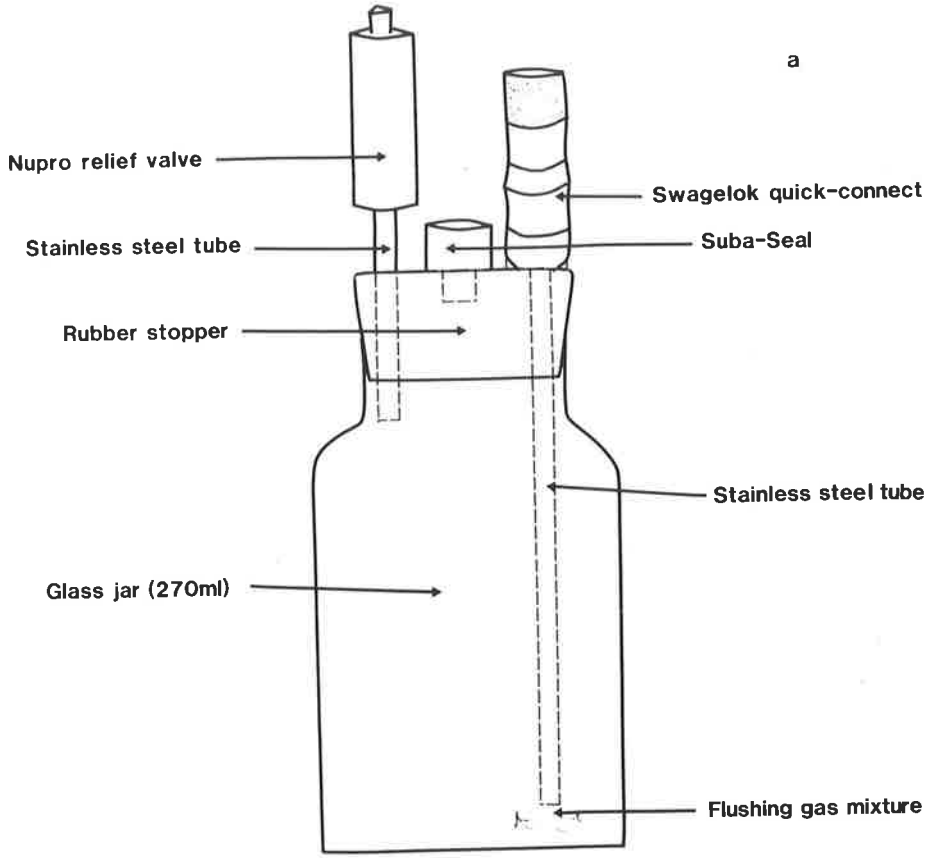
Figure 1

a) Glass jar enclosure for NRA system

b) Gas flow diagram for the WFA System

A. - Gas proportioner model 7351T with 603 and 604 gas tubes and glass floats (Matheson Gas Products, East Rutherford, New Jersey, U.S.A.)

B. - Iwaki air pump, model AP-115 (Iwaki Co. Ltd. Japan)


a
Nupro relief valve
Swagelok quick-connect
Stainless steel tube
Suba-Seal
Rubber stopper
Stainless steel tube
Glass jar (270ml)
Flushing gas mixture

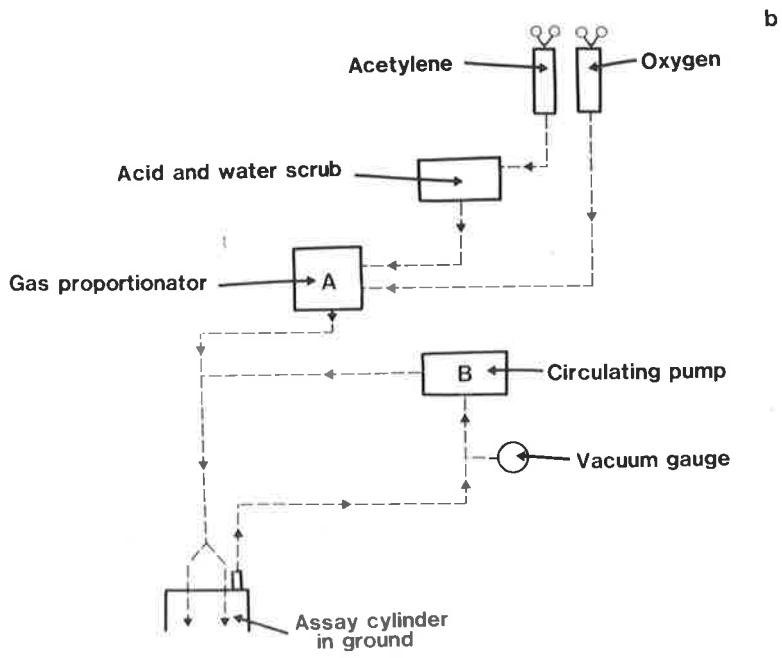
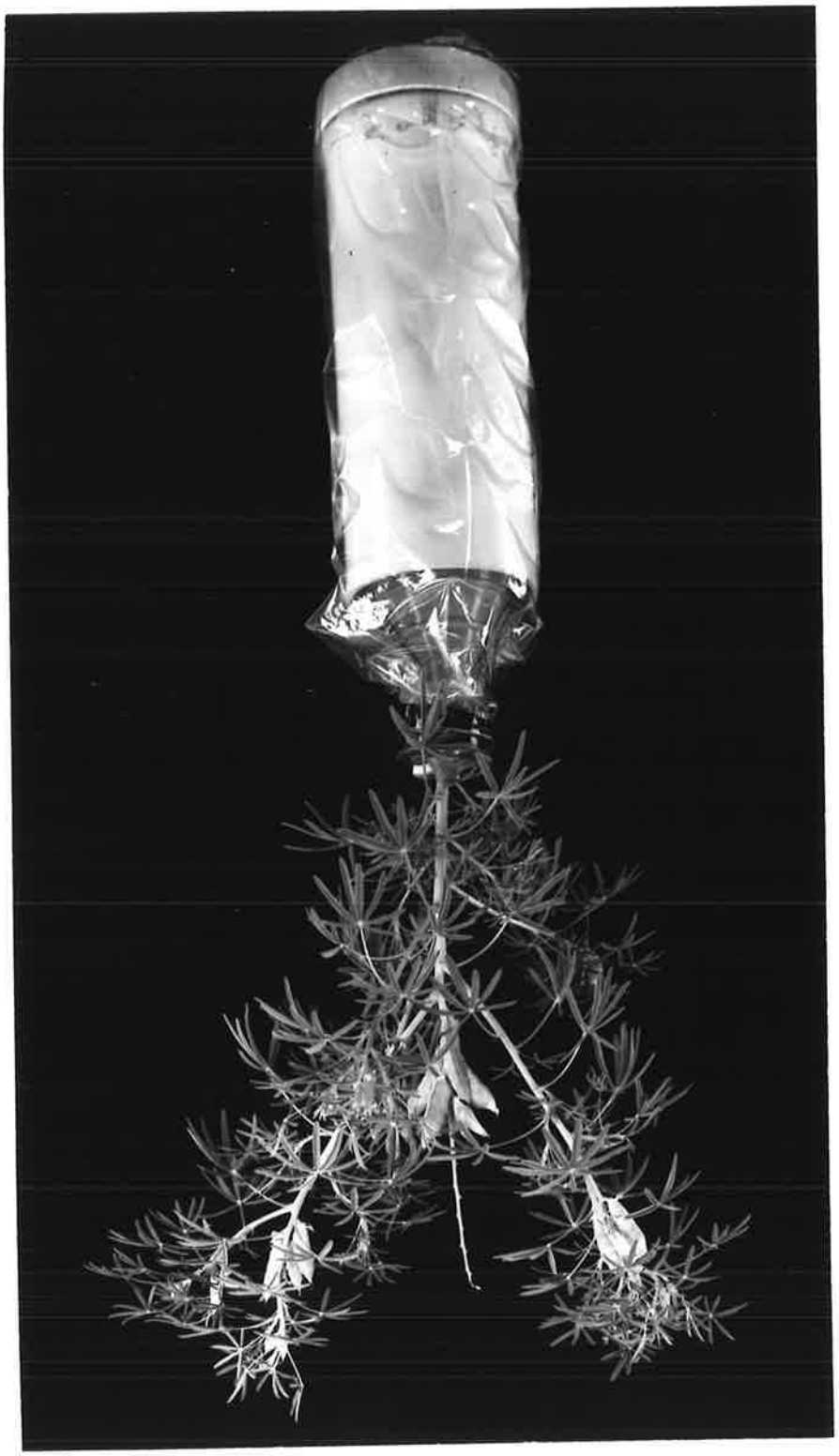

b
Acetylene
Oxygen
Acid and water scrub
Gas proportionator
A
B
Circulating pump
Vacuum gauge
Assay cylinder
in ground

Plate 1

Lupin plant being assayed using the WPA system (description p. 59).



WPA is defined as the incubation of the entire unperturbated soil-plant-root system with the top of the plant intact and exposed to its normal growth environment. This system was achieved by placing the undisturbed PVC pot into a Saran+ bag (205 x 450 mm) and wrapping the mouth of the bag around a split rubber stopper through which the stem of the plant had been placed. Modelling clay was used to seal between the plant stem and the rubber stopper, PVC tape tied with wire maintained the seal and a plastic funnel supported the plant.

Evacuation and gassing were achieved through a hypodermic needle inserted through a Suba-Seal fixed in the rubber stopper. The volume of gas occupied by the bag once it had been tied around the plant stem and evacuated to a fully collapsed state around the PVC pot was approximately 1.2 l at a sand water content of 10%, and the maximum volume obtained when the bag was fully expanded was approximately 2.5 l. These volumes varied depending on the exact method of wrapping the mouth of the bag around the split rubber stopper.

System 3 - Whole plant *in situ* field assay (Figure 2)

(hereafter referred to as the WFA system)

WFA is defined as the incubation of the nodulated zone of an intact, unperturbated soil-plant-root system in the field with the top of the plant intact and exposed to its normal growth environment. This system was achieved immediately prior to the assay by inserting a 15 cm diameter stainless-steel cylinder over the plant in the field and into a PVC tube located in the soil to a depth of approximately 20 cm. The PVC tube was used to isolate single-plant root systems. The

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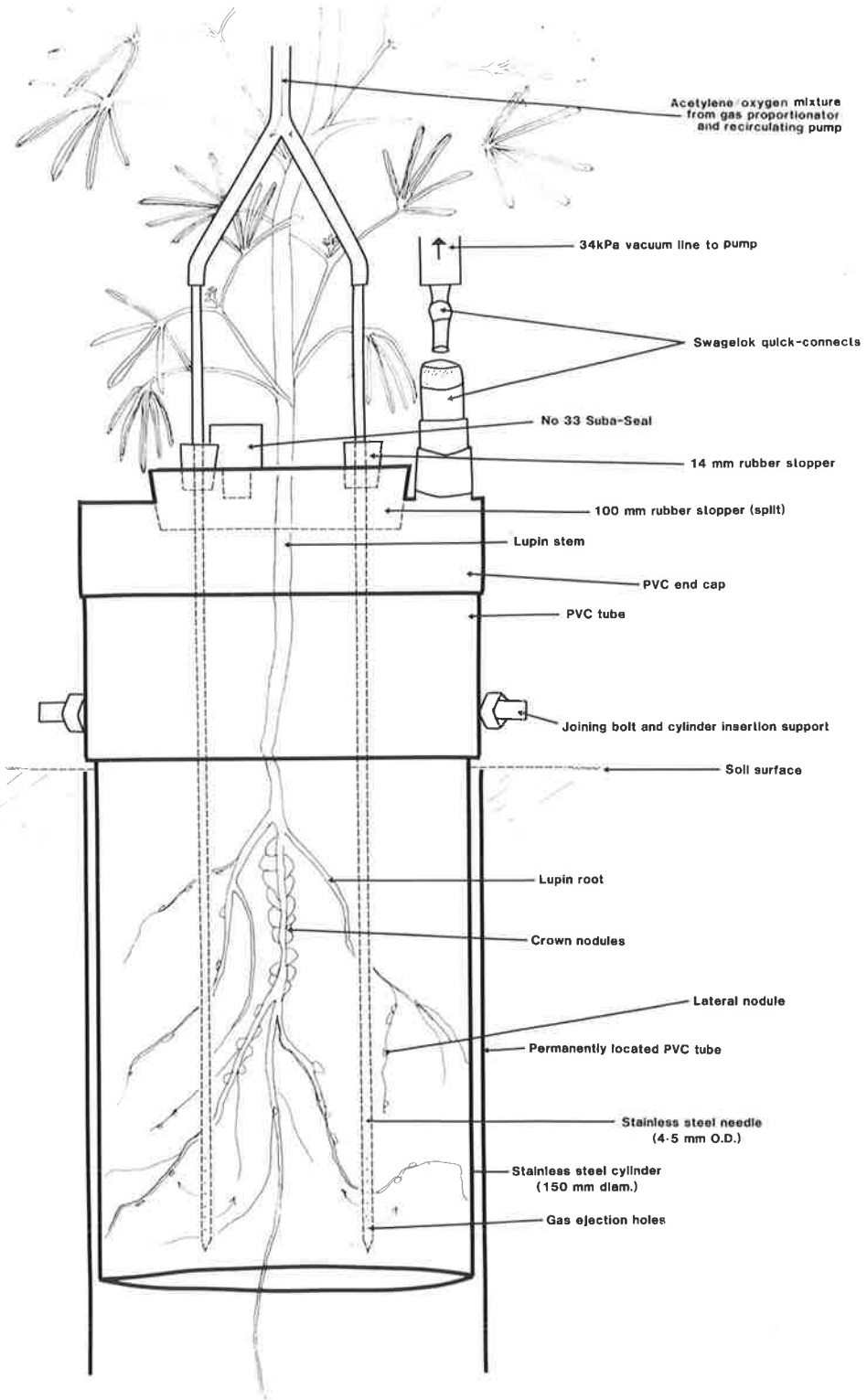
+ Plain Barrier Bag (B101.B/seal) - W.R. Grace Aust. Ltd., 1126 Sydney Road, Fawkner, Victoria (original size 205 x 865 mm).

Figure 2

WFA *in situ* field assay system (description p. 59)


Acetylene/oxygen mixture from gas proportionator and recirculating pump
34kPa vacuum line to pump
Swagelok quick-connects
No 33 Suba-Seal
14 mm rubber stopper
100 mm rubber stopper (split)
Lupin stem
PVC end cap
PVC tube
Joining bolt and cylinder insertion support
Soil surface
Lupin root
Crown nodules
Lateral nodule
Permanently located PVC tube
Stainless steel needle (4·5 mm O.D.)
Stainless steel cylinder (150 mm diam.)
Gas ejection holes

stem of the plant was inserted into a hole in a large split rubber stopper which was then pressed tightly into a PVC end cap fixed to the cylinder. Three additional holes drilled in the rubber stopper accommodated a No. 33 Suba-Seal for gas sampling and two small stoppers which sealed around stainless-steel needles (4.5 mm O.D.) feeding a mixture of C_2H_2 and O_2 to the base of the cylinder. A 'quick-connect' in the PVC end cap allowed rapid connection to the vacuum line of a circulating pump.

3.4.2 Gas phases

All gases were obtained from CIG+ and, except for C_2H_2, were graded high purity.

C_2H_2 was scrubbed through concentrated sulphuric acid and water before use. The compositions of the special gas mixtures employed for the experiments were H_2/helium (He) (51:49, v/v), Ar/O_2 (4:1, v/v) and for gas chromatographic standards, H_2 137 $\pm$ 7 vpm in air; H_2 2060 $\pm$ 40 vpm in N_2 and C_2H_4 65.3 $\pm$ 1.3 vpm in N_2.

Establishing the required gas phases within the enclosures was accomplished as follows:-

a. AR assays

1) NRA system A mixture of C_2H_2/O_2 (4:1, v/v) was injected by syringe to give the required concentration of C_2H_2 in the enclosure.

When the jar enclosure was used YV/(V-Y) ml of gas were first withdrawn to maintain the pressure within the jar at 1 atm (where

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+ Commonwealth Industrial Gases Ltd., Jervois Street, Torrensville S.A.

Y = volume of gas at 1 atm pressure injected into the jar of volume V).

2) WPA system A gas proportioner was used to meter a mixture of C_2H_2/O_2 (4:1, v/v) directly into the plastic bag.

3) WFA system The basic requirement for the gas phase was that the pC_2H_2 should not exceed 0.4 atm (refer section 4.3 p. 72) and that the entire nodulated zone be exposed to a saturating concentration of C_2H_2 (in respect of nitrogenase) for the duration of the assay. A mixture of C_2H_2/O_2 (4:1, v/v) was fed by the gas proportioner at a flow rate of 5-10 l min^{-1}. The pressure line from the circulating pump connected directly to the injection tubes inserted within the soil core and the flow rate of the feeding mixture was adjusted at the proportioner to maintain a pressure of 34 kPa in the vacuum line from the assay cylinder (Figure 1b).

Experience with the system in sandy soils of various water contents revealed that the combination of 'feeding in' the C_2H_2/O_2 mixture, together with rapid circulation of the gas atmosphere of the cylinder enabled a pC_2H_2 of between 0.3 and 0.4 atm to be reached within 1 minute of commencing gas feeding and circulating.

It should not be presumed that all the C_2H_2 injected into the soil is circulated; a large proportion escapes through the bottom of the cylinder, and conversely, air from below the cylinder enters the confines of the cylinder and is circulated.

After 1 minute the gas supply and pump were turned off, injection needles removed and holes stoppered, and vacuum line disconnected. Internal standard gas (propane - refer 3.4.3

p. 66) was then injected into the cylinder and the procedure repeated at another site.

All equipment was mounted aboard a four-wheel drive vehicle, allowing rapid mobility between sampling sites. This procedure allowed 10 cylinders to be 'gassed up' within 1 h, with approximately 5 minutes lag time between each site.

b.- H_2 evolution assays

H_2 evolution was measured simultaneously with AR assays on plants matched for size, vigour and growth stage. Where $^{15}N_2$ fixation was not simultaneously measured, the H_2 evolution measurements were made prior to, and on the same plant, as the AR measurements.

Depending on the particular aims of the experiment, H_2 evolution was measured either in an atmosphere of air or of Ar/O_2 (4:1, v/v). In the latter case (performed only in the NRA system), the jar enclosure was first flushed for 1 minute with the Ar/O_2 mixture.

c. H_2 uptake assays

Unless otherwise specified, the H_2 concentration within the enclosure was 0.5 mM prior to commencement of the assays. Tests showed that this concentration saturated the uptake 'process'.

d. $^{15}N_2$ assays

$^{15}N_2$ assays were performed in conjunction with the H_2 evolution assays with the pN_2 varying between 0.20 and 0.25 atm to

conserve $^{15}N_2$. This pN_2 was assumed to give close to maximum rates of N_2 fixation (Burris 1974; Bergersen 1970, 1980). Preliminary experiments showed that significant inhibition of H_2 evolution by N_2 occurred only at a pN_2 greater than 0.1 atm; *i.e.* H_2 evolution rates during these assays were equal to those in air.

In the NRA system, the required pN_2 and pO_2 were obtained prior to assay by first flushing the jar with Ar/O_2 mixture, withdrawing gas from the jar by syringe and injecting between 4 and 15 ml of $O_2/^{15}N_2$ mixture and up to 50 ml of air. An example of the calculations undertaken to ascertain the necessary volumes of gas injected or withdrawn during this procedure is given in Appendix 3, p. 164.

Preparation of ^{15}N-enriched N_2

^{15}N-enriched N_2 was generated prior to use in experiments by treatment of approximately 95% atoms-% ^{15}N-enriched ammonium sulphate[+] with alkaline hypobromite solution in a modified Rittenberg apparatus (Rittenberg *et al.* 1939). The N_2 generated was stored in evacuated 1 litre flasks after it was passed through a liquid N_2 trap, and brought to 1 atm pressure with distilled water using a displacement fluid reservoir similar to that described by Bergersen (1980). The 1 litre storage flask, together with the displacement fluid reservoir, were mounted in a wooden box which allowed safe transport and use under field conditions. Prior to the use of the ^{15}N-enriched N_2, O_2 was added to give a mixture of N_2/O_2 (4:1, v/v).

+ British Oxygen Company Ltd., Deer Park Road, London SW193UF UK.

In the WPA system, complete replacement of N_2 with Ar was impossible, although a pN_2 within the bag of approximately 0.2 atm could be achieved by first flushing the bag with Ar/O_2 for 2 minutes and then fully expanding the bag with Ar/O_2, waiting 2 minutes for gas equilibration and then evacuating until full collapse of the bag around the PVC pot. This procedure was routinely repeated 4 to 5 times before assay.

A particular problem of the $^{15}N_2$ assay with the WPA system was the possible lag in uptake of N_2 of uniform ^{15}N enrichment. This lag may be important in comparisons of WPA and NRA systems under certain conditions. To minimize this lag and to facilitate the uniform mixing of $^{15}N_2$ throughout the enclosure, a large PVC hand pump was used to circulate the gas rapidly throughout the bag after the injection of the ^{15}N-enriched N_2.

Six ml of 95 atoms-% ^{15}N was injected at the start of the assay, giving a final ^{15}N enrichment of the gas phase of approximately 2 atoms-% ^{15}N. Termination of all non-field $^{15}N_2$ assays was performed by immersing the nodulated roots in liquid N_2. Termination of field $^{15}N_2$ assays was performed using a microwave oven operating at maximum power for 1 minute. Tests showed that the nitrogenase in approximately 5 g (dry weight) of nodulated lupin roots was deactivated within 30 seconds and usually within 20 seconds of exposure in the microwave oven. A correction of 5 seconds per gram dry weight of root was applied when calculating the rate of N_2 fixation. The more usual sulphuric acid deactivation procedure was not used because of the difficulty of estimating the contribution of

nitrogen from contaminating soil particles in the extracts. In the WPA assays, both roots and tops were analysed for ^{15}N enrichment.

3.4.3 Internal standards

The WPA and WFA systems required an internal standard for C_2H_4, primarily because of the uncertain bag volume in the former system and the need to estimate the rate of C_2H_4 loss through the open bottom of the steel assay cylinder in the latter system. Of the number of gases that have been used as internal standards for C_2H_4 (*e.g.* methane, propylene and propane (C_3H_8), Knowles 1980) C_3H_8 was chosen because it was most compatible with the existing gas chromatographic systems employed and because it was more readily available commercially. The use of C_2H_2 as an internal standard for C_2H_4 was considered inaccurate in whole-plant systems containing a considerable amount of water in the soil, because of the much greater solubility of C_2H_2 in the water. It was found by experience that the use of C_3H_8 as an internal standard for C_2H_4 proved reliable (Chapter 4.1, p. 69) and simplified sampling procedures, particularly when using storage containers for samples where the dilution of sample was not known.

He was used as an internal standard for H_2 in all assays where H_2 evolution or uptake was measured. In assays where H_2 was introduced into the enclosure, and in the formulation of standards for gas chromatographic analysis, a commercially prepared mixture of He and H_2 was used.

The requirement that the amount of internal standard injected (*i.e.* He or C_3H_8) into the assay chamber be accurately known was ensured by the use of a precision, glass, gas-tight syringe[+] and computations based

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[+] Hamilton & Co. Inc., Whittier, California, U.S.A.

on precise measurements of injected gas temperature and pressure. The method of computing the rate of gas production (*e.g.* C_2H_4 or H_2) has been recently described by Knowles (1980). Results of preliminary experiments to check the validity of the internal standards used, are given in Chapter 4.1, p. 69, together with descriptions of sampling procedures and performance of the three assay systems.

3.4.4 Control treatments

In all experiments in which AR and/or H_2 evolution were measured, routine control treatments were included.

a) Non-symbiotic AR was checked by running the assays without plants. In the case of the WPA system, the nodulated roots were removed and the rooting medium assayed. In the WFA system, plants were detopped and assayed 2 to 3 weeks later or the assay was performed in soil containing no nodulated lupin roots.

b) Endogenous ethylene production was routinely checked by analysing gas samples from the H_2 evolution assays for ethylene. In the WFA system, this was achieved by incubating plants without the prior flush of C_2H_2/O_2 mixture.

3.4.5 Gas chromatographic analysis

A Pye Unicam[+] Series 204 Gas Chromatograph equipped with dual inlet system, flame ionization detector (FID), thermal conductivity detector (TCD), and automatic flow switching unit (FSU) was used for the routine measurement of H_2, He, C_2H_2, C_2H_4 and C_3H_8. The FSU, and the FID and TCD outputs were interfaced to a Pye Unicam DP101 Computing

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[+] Pye Unicam Ltd., Cambridge, England.

Integrator modified to include programmed detector and column-switching capability. Recorder facilities allowed peak heights to be measured in addition to the automatic calculation of peak areas computed by the integrator.

The use of the FSU incorporating the pressure balancing technique (Deans 1965) eliminated problems associated with incorporating rotary values inside the oven. Molecular Sieve columns, used for the separation of H_2 from He or air, require regular reconditioning by heating to temperatures above the maximum allowed for most valve units.

Two isolated gas separation systems were employed to separate

a) the hydrocarbons C_2H_4, C_2H_2 and C_3H_8 (FID) and

b) the low molecular weight gases H_2 and He (TCD).

The system will allow simultaneous measurement of all gases from one injection using a column splitter but in practice it was found more convenient to isolate the two systems and use two consecutive sample injections. More rigid control of column pressures could be maintained and larger samples of gas reached each detector; this was important when maximum capability for H_2 detection was required.

The length of column required to separate H_2 and He (*c.* 6m of Molecular Sieve 5A) necessitated the removal of O_2 from the sample to overcome impractically long retention times for O_2 and this was achieved by the use of a stripper column and a column by-passing technique. This system also allowed maximum TCD filament current to be used without oxidation of the filament.

Column configurations and full descriptions of operating sequences are given in Appendix 4, p. 165.

The volume of sample gas routinely injected into the gas chromatograph was 1 ml for TCD detection and 250 µl for FID detection.

4. PRELIMINARY EXPERIMENTS IN METHODOLOGY

4. Preliminary Experiments in Methodology

4.1 Use of internal standards

Gas phases containing more than 250 vpm of H_2 could be routinely measured with a coefficient of variation (CV) of less than 3%. Using He as an internal standard for H_2 decreased this CV to approximately 1% by eliminating the dependence of the determination on the absolute quantity of H_2 sampled and on injection technique. Similarly, increased precision in gas measurement was achieved by using propane (C_3H_8) as an internal standard for C_2H_4.

In the use of the two internal standards, it was assumed that the diffusion coefficients of both H_2 and C_2H_4 were the same as those of the respective internal standard gases. This assumption was shown to be valid over the time intervals used for the various incubation systems used. Figure 3a shows that there was a slightly greater loss of H_2 compared to He from a 50 ml disposable syringe (as used in the NRA syringe system) over a period of 96 h. The difference in concentration of the two gases was significant ($p<0.05$) after 48 h, assuming a CV in gas analysis of 3%. As the maximum incubation period in the NRA syringe or NRA jar system was less than 0.5 h, it is clear that the use of He as an internal standard for H_2 under these conditions was justified. However, where samples have been stored in plastic disposable syringes for periods greater than 48 h, a correction for differential rates of gas loss would be required.

Further tests, for which results are not shown, indicated that gas samples containing He and H_2 could be stored for at least one week in evacuated blood sampling (EBS) tubes[+] without detecting any difference in

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[+] Vacutainer or Venoject - Becton, Dickinson and Co., Rutherford, N.J., U.S.A. and Jintan Terumo, Tokyo, Japan, respectively. Unsterilized tubes must be used as frequently high concentrations of H_2 could be detected in sterilized tubes.

Figure 3

Preliminary experimental investigations

a) Comparative losses of He and H_2 from the NRA syringe system at 20^oC.

He- ○ , H_2- □

b) Change in concentration of He and H_2 and in the He/H_2 ratio with time following the injection of standard He/H_2 mixture into the WPA system containing sterilized sand at 20^oC.

He- ○ H_2- □ He/H_2- ▽

c) Change in the concentration of C_3H_8 and C_2H_4 and in the C_3H_8/C_2H_4 ratio with time following the injection of equal quantities of both gases into the WPA system at 20^oC.

C_3H_8- ▲ C_2H_4- ● C_3H_8/C_2H_4- ○

d) As in c) but using the WFA system in sandy soil at Coonalpyn. Air temperature 17^oC, soil temperature (10 cm depth) 14^oC, soil water content 4%.

e) H_2 evolution and AR of Unicrop lupins using the NRA system. Each point represents the mean of 10 replicate assays. Vertical bars represent 95% confidence intervals.

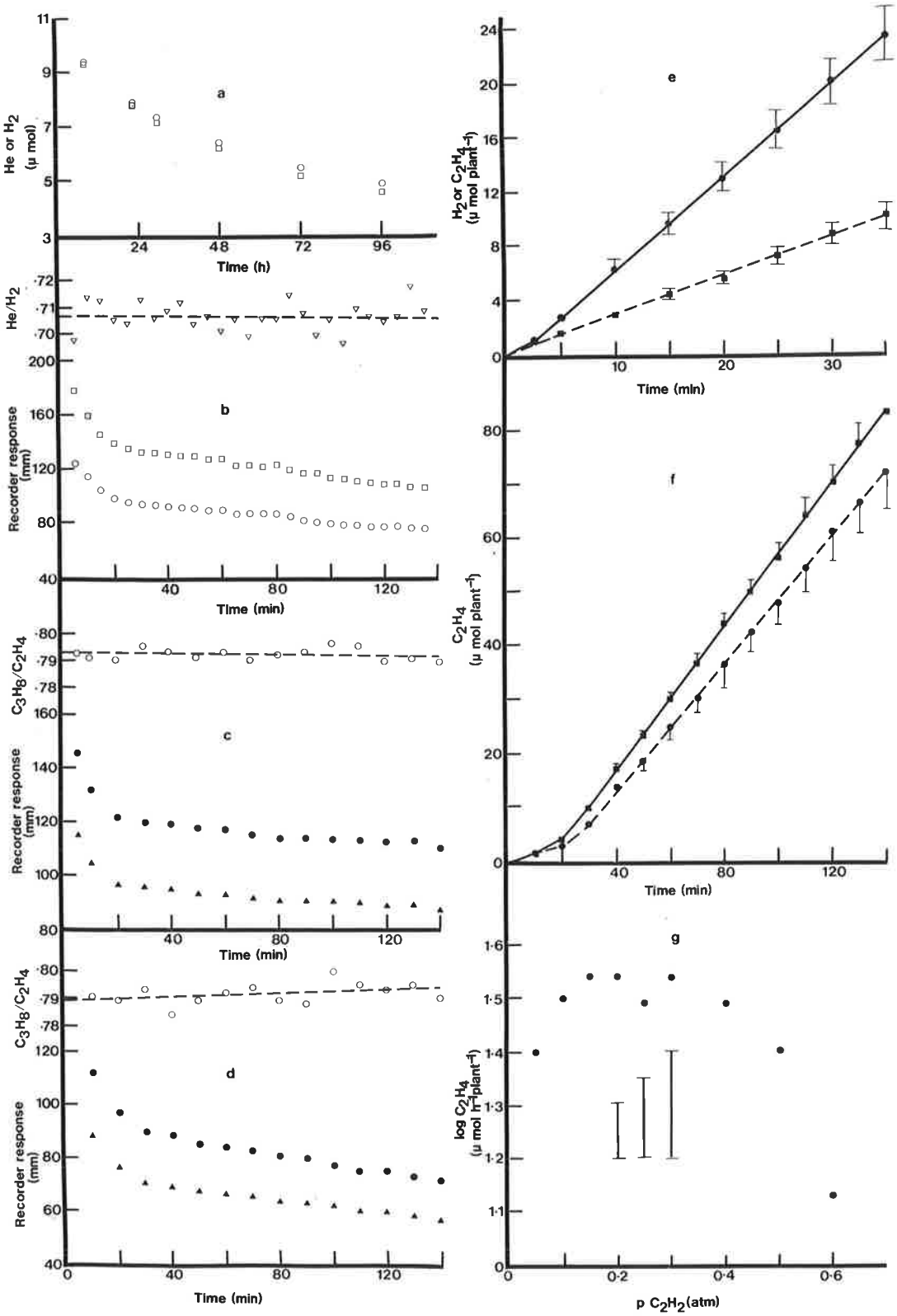
H_2 evolution ■-■ C_2H_4 production ●-●

f) AR of Marri lupins using the WPA and WFA system. Vertical bars represent 95% confidence intervals; for clarity only half the interval is shown.

WPA ■-■ WFA ●-●

g) Relationship between AR and pC_2H_2 in nodulated roots of field -grown Marri lupins. Each point represents the mean of 5 replicates and vertical bars represent the L.S.D. between means at 5%, 1% and 0.1%respectively.

Gas phases were 0.2 atm O_2, the indicated pC_2H_2 and the balance Ar to 1 atm.


a
He or H_2 (μ mol)
Time (h)
He/H_2
b
Recorder response (mm)
Time (min)
C_3H_8/C_2H_4
c
Recorder response (mm)
Time (min)
C_3H_8/C_2H_4
d
Recorder response (mm)
Time (min)
e
H_2 or C_2H_4 (μ mol plant^{-1})
Time (min)
f
C_2H_4 (μ mol plant^{-1})
Time (min)
g
log C_2H_4 (μ mol h^{-1}plant^{-1})
p C_2H_2(atm)

the rate of loss of He and H_2. All gas samples taken during the course of the following investigations were stored in EBS tubes and analysed within 72 h (usually within 24 h) of sampling.

The leakage of H_2 from the WPA system was greater than for either of the NRA systems but no significant change in the He/H_2 ratio could be detected within 2 h of injecting a mixture of the two gases into the bag (Figure 3b). Thus H_2 uptake can be accurately monitored for at least 2 h in this assay system using He as an internal standard for H_2. It is also obvious that it would be futile to attempt to measure H_2 evolution or uptake accurately in the WPA system without the use of an internal standard, even if saran bags are used and tight sealing around the plant stems is achieved.

As expected, the rate of loss of both C_2H_4 and C_3H_8 from the open WFA system was greater than from the WPA system (*cf.* Figures 3c and 3d) but no significant ($p>0.05$) change in the C_3H_8/C_2H_4 ratio was detected over a period of 140 minutes in either system. There was some indication of a slight increase in C_3H_8/C_2H_4 ratio occurring in some WFA-system tests performed when soil temperature was high and soil water content low, but never significantly so ($p>0.05$) over a 2-h period in which at least 10 measurements were taken.

No loss of C_2H_4 or C_3H_8 could be detected when soil was taken from Coonalpyn or from experimental pots in the glasshouse and incubated with these two gases in a gas-tight jar. Hence the loss of C_2H_4 and C_3H_8 in the WPA and WFA systems was assumed to only be due to the escape of these gases from the assay enclosures and not to the utilization of the gases by micro-flora in the soil.

4.2 Lag Phase

Following the injection of C_2H_2, a lag phase was always observed prior to the establishment of a linear rate of C_2H_4 production. This lag phase was approximately 2.5 minutes in the NRA system (Figure 3e); up to 20 minutes in the WPA system (Figure 3f); and up to 30 minutes on occasions in the WFA system. A lag phase in H_2 evolution in the NRA system was rarely observed (Figure 3e).

Gas sampling during experiments was always performed during the linear phase of C_2H_4 production. This was ensured by sampling after either 10 or 15 minutes and again after 20 or 30 minutes in NRA systems, and after 1 h and 2 h in all whole plant assays.

Despite 4.5 g dry weight of nodulated root mass being assayed in the NRA jar system (root volume approximately 0.5 x volume of jar), a linear rate of C_2H_4 and H_2 production was still observed (Figure 3e) for at least 30 minutes. However, in all studies where root volume was judged to be greater than 30% of the assay vessel volume, samples were taken after 10 and 20 minutes to ensure that depleted O_2 levels did not decrease nitrogenase activity during the course of the assay.

4.3 C_2H_2 Concentration

Trinick *et al.* (1976) reported that maximum AR occurred at 0.1 atm C_2H_2 with *L. cosentinii* and at about 0.05 atm with *L. luteus*, with no decrease in activity up to 0.2 atm (the maximum pC_2H_2 used). Halliday (1975) reported maximum AR in *L. angustifolius* at about 0.15 atm C_2H_2 with a subsequent decline in activity with increasing pC_2H_2 up to 0.4 atm (the maximum pC_2H_2 used). However, statistical significance ($p<0.05$) could not be demonstrated between any of the pC_2H_2 treatments imposed (0.05 - 0.40 atm) in the latter case.

A similar response in AR with pC_2H_2 to that reported by Halliday (1975) was found in these investigations with nodulated roots of field-grown Marri lupins (Figure 3g). Saturation of nitrogenase with C_2H_2 occurred at about 0.15 atm. However, no significant difference ($p>0.05$) in AR could be demonstrated between assays conducted at 0.05 and 0.4 atm. Significant depression in AR was observed at concentrations of C_2H_2 greater than 0.4 atm (Figure 3g).

The effect of pC_2H_2 on H_2 evolution was not checked at this time. It was assumed to be negligible at 0.1 atm C_2H_2. This was not the case however, as demonstrated later in Experiment 1, p. 79.

4.4 Diurnal variation

Trinick *et al.* (1976) reported no marked diurnal fluctuation in AR in nodulated roots of field or glasshouse-grown plants of *L. luteus*, *L. angustifolius* or *L. cosentinii* when the assays were conducted at constant temperature. Halliday (1975) recorded marked fluctuations (approximately 50% variation in amplitude) in AR in field-grown *L. angustifolius* when assays were conducted at the temperature of the soil at 5 cm depth; although these fluctuations did not always parallel variations in soil temperature.

To determine if diurnal variation in nitrogenase activity did occur in narrow-leafed lupins and if some account of such variation would be necessary in later field studies, AR was monitored using Unicrop lupins in the WPA system in a naturally-lit glasshouse. The growth stage of the plants was the onset of flowering. The maximum air temperature was 21°C and the temperature declined to a minimum of 7°C during the night. Measurements taken over a 24-h period showed a 45% drop in AR from the maximum at noon to the minimum at 4.00 a.m. the following morning.

4.5 Root washing

The literature on the effect of washing nodulated-root systems prior to assay on nitrogenase activity (reviewed in 2.4.2, p. 44) indicated a range from no effect to significant decreases in nitrogenase activity, depending on length of immersion of nodules in water and the degree to which they were dried prior to assay. Trinick *et al.* (1976) demonstrated that washing nodulated lupin roots for 30 seconds and blotting them dry did not impair their activity.

During the initial stage of these investigations there appeared to be considerable advantage in removing contaminating sand or soil from the nodulated roots prior to N_2 fixation assays because immediate deactivation of nitrogenase activity was required at the termination of the assay. Lupin roots frequently acquire significant quantities of contaminating soil which adheres to the root surface aided by mucilagenous excretions from the root. Removal of soil is more difficult once deactivation of nitrogenase has been achieved by rapid freezing or by the addition of concentrated acid.

An experiment using ten, 10 week-old Unicrop plants, revealed no significant ($p>0.05$) effect on AR of a 30 second immersion of nodulated roots in water and subsequent drying with blotting paper; in accordance with the report of Trinick *et al.* (1976).

4.6 Repeated assays

In glasshouse and growth-chamber studies, large savings in space and labour can be achieved if the same plants can be used more than once (*i.e.* by using non-destructive assays such as in the WPA system). In anticipation of undertaking ontogenetic studies with a possible minimum time interval between assays of about 1 week, an experiment was performed

to check whether AR assays repeated at weekly intervals affected AR and/or growth of the plant.

Five replicate plants were assayed once per week for 8 weeks using the WPA system and the AR rates compared to those of 5 plants taken from the same population of plants but assayed once only. No detectable differences in rates of AR or plant dry weight were observed on any occasion of assay between those plants previously assayed and those that were not. It was concluded that weekly assays of 2 h duration had no harmful effect on the lupin plant that could be readily detected.

4.7 Variability and statistical analysis

The coefficient of variation (CV) in measurements of AR and H_2 evolution made on 10 single plants varied between 7% and 12% for plants grown under supposedly uniform conditions in either the glasshouse or growth room. With field-grown plants, the CV commonly ranged between 10% and 15%. The variability in H_2 evolution measurements was usually, but not always, less than the variability in AR measurements made on the same plants. There appeared to be less variability in whole-plant assays than in nodulated-root assays but the difference was neither large nor consistent.

The CV of N_2 fixation measurements (using $^{15}N_2$ assays) was frequently higher (range 10-20%) than either that of AR or H_2 evolution assays. This may have reflected the greater number of variables measured in computing the rates of N_2 fixation, rather than intrinsic variability in the N_2 reduction process *per se*.

Variation in measurements of all three parameters (AR, H_2 evolution and N_2 fixation) were decreased by about 15% if expressed on the basis of nodule dry weight rather than on an individual plant basis.

Although the CV can be lowered by increasing the number of plants assayed in each enclosure, the required gas volume becomes prohibitive in relation to ease of gas replacement and the cost of routine use of ^{15}N-enriched N_2. Thus 10-12 single-plant assays matched across treatments were usually performed in the experiments described hereafter, except very early in plant ontogeny, when up to 5 root systems could be assayed in one enclosure and replicated 10-12 times.

Graphical presentation of results of experiments has been attempted whenever possible in the following text, with 95% confidence intervals indicated. Because the standard deviation in most parameters measured during these investigations varied directly with the mean, treatment comparisons required stabilization of variance, particularly if pooled over time. Logarithmic transformations were therefore performed on the data where required, although values in figures and tables were derived from untransformed data.

5. EXPERIMENTAL

5. Experimental

5.1 General introduction

A major problem addressed in the investigations was the calibration of AR rates against rates of N_2 fixation. This problem was obviously a most serious handicap to the adoption of the AR technique as a quantitative assay for N_2 fixation studies in field-grown lupins. It was initially postulated that a) H_2 evolution would have to be measured, in addition to AR, for accurate calibration of the assay throughout the growth cycle of the plant, and b) perturbation of the plant during assay procedures may be important because of its effect on the total electron flux or the electron allocation by nitrogenase to the two substrates viz. H^+ and N_2.

It was anticipated that, although AR rates would be measured *in situ* in the field, the measurement of H_2 evolution may prove difficult without perturbating the plant-soil system. Thus, the success of the assay procedures would depend on whether the electron allocation to N_2 or H^+ was affected by plant perturbation.

The experimental programme was also designed to check for the presence of a functional uptake hydrogenase as the presence of such an enzyme system would render H_2 evolution measurements useless. In addition, problems in integration of short-term assays due to diurnal and seasonal variation in nitrogenase activity were considered.

5.2 Glasshouse experiment 1

5.2.1 Aims

The experiment had two main aims. Firstly, to discover whether the total electron flux through nitrogenase, as measured by AR, equates with

the sum of the fluxes through H_2 evolution and N_2 fixation. If this was so it would enable rates of N_2 fixation to be calculated from measurements of rates of AR and H_2 evolution in air.

The second aim was to investigate whether plant perturbation (removal of tops and/or root disturbance) affects AR and/or the ratio of AR to N_2 fixation (hereafter designated the A/N ratio).

5.2.2 Methods

Six-week old Unicrop lupins in the vegetative stage of growth were stratified into 10 replicated blocks on the basis of size for the following treatments:-

	Assay system	Gas treatment
a)	NRA	$^{15}N_2$
b)	NRA	C_2H_2 (10%)
c)	WPA	$^{15}N_2$
d)	WPA	C_2H_2 (10%)
e)	WPA - tops removed prior to assay	C_2H_2 (10%)

Assays were performed between 11.00 a.m. and 1.00 p.m. at 1 minute staggered intervals in a glasshouse maintained at 21°C. Average irradiance for the experimental period was 1060 $\mu E\ m^{-2}\ s^{-1}$ and the water content of the sand prior to assays was 9.3% $\pm$ 1%. Flushing of the WPA system to decrease the pN_2 commenced about 20 minutes before the start of the assays. The flushing procedures (both NRA and WPA systems) were applied to all treatments irrespective of whether $^{15}N_2$ was later injected. Plants used in the NRA treatments were removed from their pots, the root systems were washed with water for 30 seconds (21°C) and excess water removed with blotting paper, and the plants were decapitated.

Removal of the tops in Treatment e was performed at the same time as the corresponding NRA treatments. All NRA assays were performed using the disposible syringe system.

5.2.3 Results and discussion

The presence of some H_2 evolution in Treatment b (NRA + 10% C_2H_2) indicates incomplete saturation of nitrogenase with C_2H_2 (Table 3). Only a trace of H_2 could be detected in Treatments d and e (WPA + 10% C_2H_2) and this was assumed (at the time) to be due to saturation of nitrogenase by C_2H_2 in these treatments.

The effect of the assay system was dramatic, with over 250% less AR recorded in the NRA treatments compared to in the WPA treatments. This was surprising in view of the results of Trinick *et al.* (1976), who reported no detrimental effect of decapitation and removal of roots from the soil on AR in lupins. However, the insignificant ($p>0.05$) effect of decapitation on AR in the WPA treatments shows clearly that the difference in AR between assay systems was not due to decapitation of the plants *per se*.

The difference in rates of N_2 fixation between the two assay systems was even greater than the difference in AR, such that the (A + Ha)/N ratio was significantly ($p<0.05$) lower in the WPA system (Table 3). Ha represents H_2 evolution in the presence of C_2H_2. The ratio (A + Ha)/N represents the ratio of the total flux of electrons allocated to the reduction of C_2H_2 plus the evolution of H_2 in the presence of 10% C_2H_2 compared to the allocation of electrons to N_2 in the absence of C_2H_2. This ratio is equivalent to the A/N ratio when H_2 evolution is completely suppressed in the presence of C_2H_2.

As anticipated, a substantial proportion of nitrogenase activity in air was diverted to the evolution of H_2, giving rise to RE^s, defined here as 1 - [H/(A + Ha)], of 0.54 and 0.50 for the NRA and WPA systems

Table 3

Effect of assay system on AR, H_2 evolution and N_2 fixation in Unicrop lupins (Experiment 1)

(μmol h^{-1} $plant^{-1}$ $\pm$ SEM†)

Assay System	NRA		WPA		
Treatment	a	b	c	d	e
	($^{15}N_2$)	(10% C_2H_2)	($^{15}N_2$)	(10% C_2H_2) decapitated	
				−	+
A		9.5 (± 0.2)		25.1 (± 0.5)	23.8 (± 0.5)
H	5.2 (± 0.1)		12.6 (± 0.3)		
Ha		1.9 (± 0.1)		trace	trace
N	2.2 (± 0.1)		7.7 (± 0.2)		
(A + Ha)/N	5.3 (± 0.2)		3.3 (± 0.1)		
RE	0.54		0.50		

† = standard error of mean
A = AR
H = H_2 evolution in air
Ha = H_2 evolution in C_2H_2
N = N_2 fixation
RE defined as 1 − [H/(A + Ha)]

respectively. These values are within the range reported by Schubert and Evans (1976) for the various legume symbionts they tested. With the occurrence of such relatively high rates of H_2 evolution, it was unlikely (although not impossible) that an uptake hydrogenase was functioning during the experiment.

The insignificant ($p>0.05$) difference in RE between the two systems agrees with the finding of Schubert and Evans (1976) that perturbation of the plant has no effect on RE in the short term.

On the assumption that the total electron flux through nitrogenase is equal to the sum of the fluxes to H_2 evolution (in air) and N_2 fixation, and that the total electron flux is unaltered by the presence of C_2H_2 and is therefore equal to the sum of the fluxes to C_2H_2 and H_2 evolution in the presence of C_2H_2, the amount of N_2 fixed can be estimated by the following formula.

$$\text{Estimate of } N_2 \text{ fixed} = \left[C_2H_2 \text{ reduced} + H_2 \text{ evolved in } C_2H_2 - H_2 \text{ evolved in air} \right] / 3$$

Substituting the values for NRA treatments recorded in Table 3 gives

an estimate of N_2 fixed $= (9.5 + 1.9 - 5.2)/3$

$= 2.1 \ \mu mol \ h^{-1} \ plant^{-1}$

The measured amount of N_2 fixed during the same period, based on the rate of uptake of ^{15}N-enriched N_2, was 2.2 μ mol h^{-1} $plant^{-1}$ (Table 3).

The agreement between the estimated value and measured value of N_2 fixed in the NRA system appears to support the assumptions on which the estimated value was based as outlined above. However, this was not the case in the WPA system where the calculated estimate of N_2 fixation was 4.2 μ mol h^{-1} $plant^{-1}$ compared to that measured directly of 7.7 μ mol h^{-1} $plant^{-1}$ (Table 3).

Further small experiments were performed to investigate the reasons for the loss of AR in the NRA system. Factors investigated were detopping

(as in Experiment 1), different methods of flushing with Ar/O_2 and washing the root system. These experiments are not documented here, but loss of activity seemed on most occasions to be associated with the presence of water on the nodulated-root system. In all subsequent experiments, the root systems were not washed. It is noteworthy that Gibson (1980), subsequent to this experiment, reported a 90% decline in AR in lupin nodules where roots had been washed.

A phenomenon which also became evident in further assays of H_2 evolution performed on the same population of plants used in Experiment 1 was that the concentration of H_2 in the WPA system decreased as the assays were performed on more mature plants. From early flowering onwards, only an occasional trace of H_2 evolution could be detected in the WPA system and furthermore, H_2 that was exogenously supplied was rapidly taken up. However, no uptake of H_2 was ever recorded in the NRA system.

Two reasons were advanced to account for the occurrence of H_2 uptake in the WPA system but not in the NRA system.

1) A functional uptake hydrogenase, induced in the lupin-*Rhizobium* symbiosis during the latter stages of growth (flowering onwards), was somehow deactivated by perturbation of the root system. Tests had shown detopping of the plants to have no immediate effect on the uptake of H_2 in the WPA system.

2) The uptake of hydrogen was due to consumption of H_2 by microbial or enzyme activity associated with the rooting medium. The presence of the growing lupin plant may provide a source of substrate for the build-up of micro-flora in the rhizosphere. Furthermore, the increasing nodule mass per volume of soil,

together with a possible increase in specific H_2 evolution during plant ontogeny, may induce a rapid build-up of a population of microbes able to utilize H_2.

Although it was difficult to postulate a mechanism whereby 1) would operate, the occurrence of such a phenomenon would yield the measurement of H_2 evolution in an excised nodulated-root system of little relevance to the measurement of N_2 fixation in whole plants, particularly if 'recycling' of the H_2 by the uptake hydrogenase resulted in increased N_2 fixation (see Chapter 2.2.3, p. 23). If hypothesis 2) was correct, then the measurement of H_2 in the NRA system, devoid of soil contaminants, should have reflected the true evolution of H_2 by nitrogenase. However, the measurement of H_2 in the WPA system, if it could be detected at all would have underestimated the electron flux to H^+ on most occasions throughout plant ontogeny.

A problem related to 2) is that in the NRA system, there is the possibility that some H_2 may be taken up by micro-flora on the soil particles still attached to the nodulated roots. The abolition of root-washing prior to assays would most probably exacerbate the problem. Furthermore, if hypothesis 2) was correct, the H_2 evolution rates measured in the WPA system in Experiment 1, may have been underestimated. The absence of H_2 in Treatment d (WPA + C_2H_2) may not have been due to saturation of nitrogenase by C_2H_2 as previously assumed, but rather to the uptake by soil micro-flora of most of the H_2 evolved.

Because of the uncertainty regarding possible uptake of H_2 in the WPA system and the possible effects of root-washing procedures on the A/N ratio in the NRA system in Experiment 1, a second experiment was conducted.

5.3 Glasshouse experiment 2

5.3.1 Methods

Ten-week old Unicrop lupins in the reproductive growth stage (the inflorescence had emerged on the first order lateral stem) were assayed.

There were 6 treatments stratified into 8 replicate blocks *viz.*

	Assay system	Gas treatment
a)	NRA	$^{15}N_2$
b)	NRA	C_2H_2 (15%)
c)	NRA	flushed with Ar/O_2
d)	WPA	$^{15}N_2$
e)	WPA	C_2H_2 (*c.* 15%) + H_2 (*c.* 0.5mM)
f)	NRA with nodules removed.	C_2H_2 (15%) + H_2 (0.5 mM).

The following alterations from the treatments in Experiment 1 were made for Experiment 2:-

1) A treatment in which the N_2 was replaced with Ar was included to check for uptake hydrogenase activity in the NRA system (Treatment c).

2) H_2 was added to the WPA + C_2H_2 treatment to measure any H_2 uptake over a period of 2.5 h (Treatment e).

3) Roots were not washed and, in the AR treatments, the pC_2H_2 was increased to 0.15 atm to ensure complete inhibition of nitrogenase-catalysed H_2 evolution.

4) A treatment was included in which all the nodule tissue was removed and the roots (less nodules) exposed to exogenously-supplied H_2 and C_2H_2, to measure any H_2 uptake by micro-flora adhering to the roots (Treatment f).

5) The NRA-jar system was used instead of the NRA syringe system to ensure complete replacement of N_2 in the Ar/O_2 treatment.

The experiment was conducted in the same manner as Experiment 1 except that the roots were not washed in the NRA system prior to the assays. Average irradiance for the experimental period was 1150 $\mu E\ m^{-2}\ s^{-1}$ and the water content of the sand was 8.7% $\pm$ 1%.

5.3.2 Results and discussion

No significant ($p>0.05$) differences in AR were evident between the NRA and WPA systems. There was also no significant ($p>0.05$) difference between the rates of AR and H_2 evolution under Ar/O_2 in the NRA system (Table 4). Furthermore, neither the rates of N_2 fixation, nor the A/N ratio were significantly ($p>0.05$) affected by the assay system. This is in contrast to the results of Experiment 1, where large differences occurred between NRA and WPA treatments in rates of AR and N_2 fixation.

It is concluded that washing the nodulated-root system prior to performing the assays in Experiment 1 resulted in the low rates of AR and N_2 fixation in that experiment. This was probably caused by restriction of O_2 supply to the bacteroids by a surface film of water. In cases tested earlier where the roots were washed and blotted dry and low rates of AR were recorded, moisture may have penetrated the nodule. Alternatively, physical changes may have taken place due to altered water relations in the nodule.

It is not clear why the A/N ratio should vary under such circumstances. One can postulate biochemically-induced changes in the substrate kinetics of nitrogenase with respect to C_2H_2 and N_2 caused by

Table 4

Effect of assay system on AR, H_2 evolution and N_2 fixation in Unicrop lupins (Experiment 2)

(μmol h^{-1} $plant^{-1}$ $\pm$ SEM)

Assay system	NRA			WPA		
Treatment	a	b	c	d	e	f
	$^{15}N_2$	+ C_2H_2 (15%)	Ar/O_2	$^{15}N_2$	C_2H_2 + H_2 (15%) (0.5mM)	No nodules (C_2H_2 + H_2) (15%) (0.05mM)
A	-	44.6 (± 1.2)	-	-	40.2 (± 1.1)	0
H_2 evolution†	27.6 (± 0.7)		47.3 (± 1.1)	0	- 367 (± 6.1)	- 0.5 (± 0.2)
N	16.8 (± 0.6)	-	-	14.1 (± 0.9)	-	-
A/N	2.7			2.9		
RE	0.38					

† Previously designated H in Treatments a and d, and Ha in Treatment b of Experiment 1 (Refer Table 3, p. 80).

restricted O_2 supply, or differential effects of restricted gas exchange caused by lowered porosity of the nodule. In the latter case, the greater solubility of C_2H_2 in water compared to N_2 may have resulted in N_2 becoming more limiting than C_2H_2 as the substrate for nitrogenase; giving rise to an elevated A/N ratio.

There was a significant ($p<0.01$) difference between Experiments 1 and 2 in the A/N ratio produced by the WPA treatments *i.e.* 3.3 and 2.9 respectively. The difference would be greater if some H_2 evolution occurred in the WPA + C_2H_2 treatment (Treatment e) of Experiment 1 and was masked by H_2 uptake within the system. Assuming incomplete saturation of nitrogenase by C_2H_2 occurred to the same degree in the WPA system in Experiment 1 as it did in the NRA system in Experiment 1, total electron flux in the former would be equivalent to a rate of AR of 30.1 μ mol C_2H_4 h^{-1} $plant^{-1}$ and the A/N ratio would be *c.* 3.9.

Because the major difference between the two experiments in respect of the WPA treatments was the growth stage of the plants, it seemed likely that the A/N ratio may have been dependent on the growth stage of the plant. However, the plants were grown in a naturally-lit glasshouse which controlled only the maximum air temperature and not the variation in irradiance and temperature. Hence the environmental conditions prior to the two experiments were not identical and may have affected the A/N ratios measured during the experiments. The question of whether the A/N ratio varies with plant ontogeny, when plants are grown in a constant environment is addressed in Experiment 3.

In both assay systems in Experiment 2, the experimentally-derived A/N ratios were less than the stoichiometric equivalent value of 3 and, in the case of the NRA system (A-H)/N was 1.01. Thus, on the assumptions that

a) the reduction of one mole of N_2 was equivalent to the reduction of 3 moles of C_2H_2 and b) total nitrogenase activity was represented by the reduction of C_2H_2 and unaltered by the nature of the substrate, then the estimated amount of N_2 fixed based on the formula

$$N = (A-H)/3$$

was 5.7 μ mol h^{-1} $plant^{-1}$ compared to the amount measured directly of 16.8 μ mol h^{-1} $plant^{-1}$.

On theoretical grounds, these results are difficult to explain unless the substrate C_2H_2 actually decreased the total electron flux through nitrogenase.

In contrast to these results, Saito *et al.* (1980) reported that N_2 fixation in beans was over-estimated by the AR technique, even when H_2 evolution was taken into account.

The apparent equality in electron flux to C_2H_2 and the sum of that to H^+ and N_2 in the NRA system in Experiment 1 may have been a direct consequence of washing the roots which depressed N_2 fixation to a greater extent than AR. This is further considered in the general discussion section 6.5 (p. 139).

The insignificant difference ($p>0.05$) in rates of H_2 evolution under Ar/O_2 and AR in the NRA system (Table 4) indicates that no uptake hydrogenase was functioning during the experiment; otherwise significantly lower rates of H_2 evolution (relative to AR) would have occurred. In contrast, a massive uptake of exogenously supplied H_2 was observed in the WPA system to which C_2H_2 and H_2 had been added (Treatment e, Table 4 and Figure 4a), but only a low rate of uptake was recorded in the NRA system without the nodules (Treatment f, Table 4). The latter measurement had a very high coefficient of variation which was due partly to the

Figure 4

Time Course of H_2 Uptake in Whole Plant Assays

a) Time course of H_2 uptake in the WPA system (Treatment e, Expt. 2).

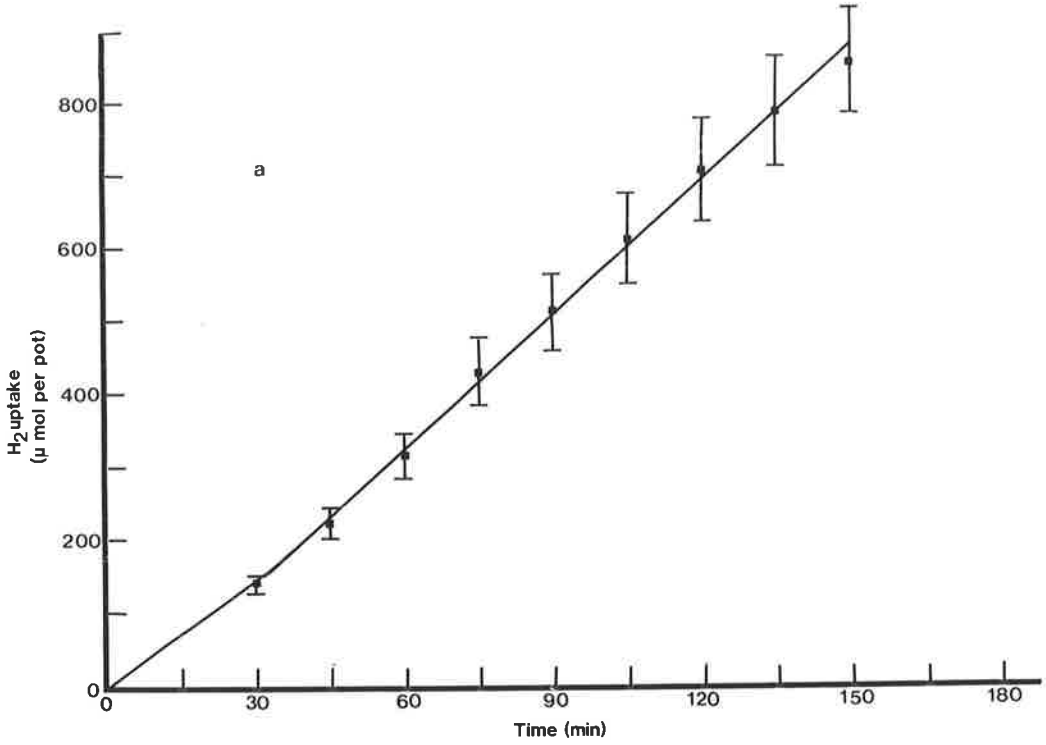
Gas phase was C_2H_2 (0.15 atm), H_2 (0.5 mM) and He (0.5 mM). Points represent the mean of 8 replicates and vertical bars represent 95% confidence intervals.

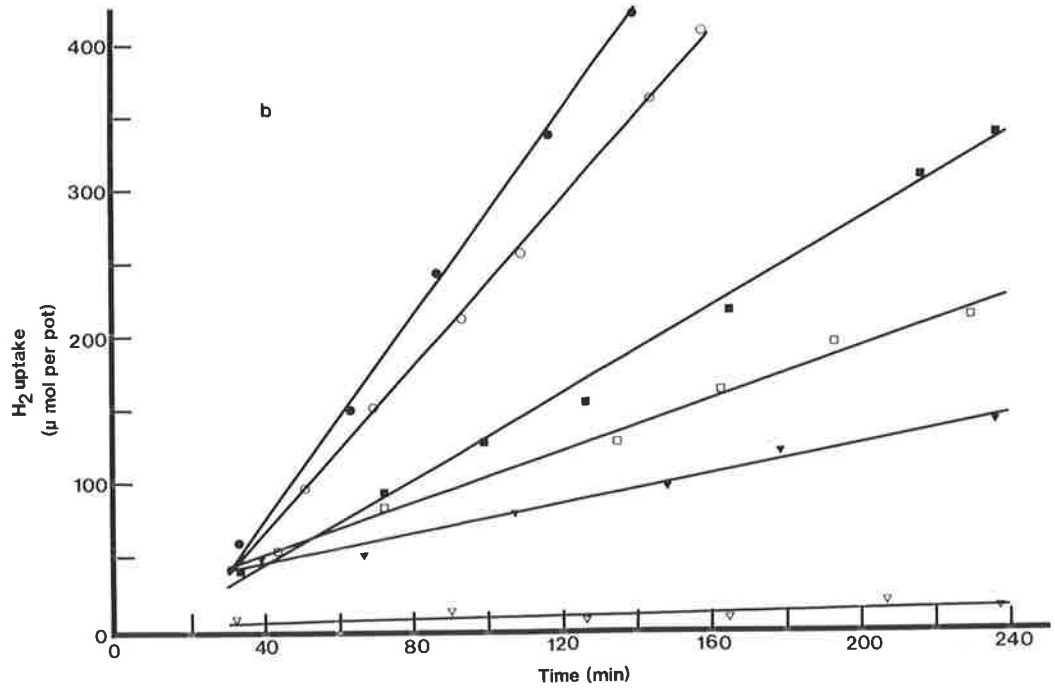
b) Time course of H_2 uptake in the WPA system as a function of time interval between plant decapitation and assay. All assays were performed on the same pot.

Interval between decapitation and assay (days)

0 ○—○ 7 ■—■ 14 □—□
17 ▼—▼ 21 ▽—▽

- after the assay on day 21, the pot exposed to 1 mM H_2 for 14 days and re-assayed ●—●


a
800
600
400
200
0
H_2 uptake
(μ mol per pot)
0
30
60
90
120
150
180
Time (min)


b
400
300
200
100
0
H_2 uptake
(μ mol per pot)
0
40
80
120
160
200
240
Time (min)

variation in H_2 uptake between replicate samples and partly to analytical error associated with the determination of small absolute differences in H_2 concentration against a high background concentration.

Nevertheless, the estimate of H_2 uptake by the latter treatment appears reasonable when the mass of rooting medium is considered and the estimate compared to the uptake of H_2 in the WPA system. The dry weight of the sand material adhering to the roots in Treatment f was estimated by ashing the roots to be about 2 g. On the basis that the uptake of H_2 is directly proportional to the mass of sand present, the uptake of H_2 in the WPA treatment, which contained 3 kg dry weight of sand, can be estimated as

$$3000 \times 0.5 / 2 = 750 \ \mu \text{ mol } h^{-1} \text{ pot}^{-1}$$

However, this assumes uniform uptake of H_2 for the whole sand mass which is likely to over-estimate the uptake of H_2 because the numbers of microflora are likely to be higher in close proximity to the roots. Further tests showed that the uptake of H_2 by nodule-free roots which were not washed prior to assay was usually between 0 and 2% of the hydrogen evolved with the nodules intact. This was considered to be within the range of experimental error for the measurement of H_2 evolution and therefore ignored in further investigations.

Although the direct relevance of the uptake of H_2 by soil microflora to these investigations was only in the area of technique (*i.e.* an awareness that nitrogenase-catalysed H_2 evolution cannot be automatically measured *in situ*), it was intriguing to discover what role the lupin plant played in inducing the H_2-uptake capability in the soil. During the course of the remaining investigations, a number of experiments were performed to assess this, both in the glasshouse and field, and some of the investigations are briefly described below.

Figure 4b shows the time course of H_2 uptake repeated at increasing time intervals from decapitation of the lupin plant in the WPA system. Nitrogenase activity as measured by AR had declined to zero after one week, but H_2-uptake capacity remained for up to 3 weeks following decapitation. Furthermore, if after 3 weeks the same pot (sand and roots intact) was then enclosed for two weeks in a plastic bag containing 1 mM H_2, the original capacity to take up H_2 was regained.

Other experiments showed that pots containing uninoculated lupin plants and watered daily with nutrient solution containing 20 mM nitrogen (to prevent nodulation) had developed no nodules and no capacity to take up H_2 by mid-flowering. However, H_2 uptake capacity was again evident after exposing the rooting medium to 1 mM H_2 for 2 weeks. Pots treated identically to the above but lacking any plants behaved in a similar fashion (although the capacity to take up H_2 was lower), indicating that the continual supply of H_2 by the nitrogen-fixing lupin plant was the main reason that the rooting medium developed H_2-uptake capacity.

It may be that the lupin plant assists in maintaining or building up the populations of micro-flora responsible for the H_2 uptake by the supply of substrate and nutrients in exudates or in the decomposition of dead root material. In addition, known hydrogen oxidising bacteria are facultative autotrophs, which can grow on either organic substrates or H_2 plus CO_2 (Aragno and Schlegel 1981). The presence of active root tissue would ensure not only the presence of organic residue but also a continual supply of CO_2.

Conrad and Seiler (1981) observed that H_2 decomposition in soil was due not only to the activity of viable micro-organisms but also soil enzymes. However, although these workers showed that the decomposition of

atmospheric H_2 at very low concentrations in the soil was probably due to the activity of abiontic soil enzymes, higher concentrations of H_2 induced H_2 uptake by resident flora of hydrogen bacteria (Conrad and Seiler 1979a).

It is interesting to note here that the same workers (Conrad and Seiler 1979b) have been able to measure the release of H_2 *in situ* in the field over a range of legumes, indicating that soil micro-organisms may not always act as a complete sink for H_2 evolution catalysed by nitrogenase.

During the field experiments to be described later in this thesis (Chapter 5.5, p. 100), uptake of H_2 was similar to that found in the glasshouse experiments, both in relation to plant ontogeny (*i.e.* from flowering to maturity) and absolute rates per unit mass of soil. It was therefore concluded that true rates of nitrogenase-catalysed H_2 evolution could be reliably measured only in the NRA system. Recent reports (*e.g.* Crush and Tough 1981) of H_2 evolution being measured in whole soil cores containing white clover plants must be regarded with caution as there is no indication that some H_2 uptake did not occur via resident soil micro-flora.

5.4 Growth-chamber experiment (Experiment 3)

5.4.1 Aims

The results from Experiments 1 and 2 indicated that the A/N ratio in lupins may not be constant, and it was postulated that the ratio may vary with either plant ontogeny *per se* or as a function of environmental conditions influencing the growth or energetic status of the lupin plant. In the latter case, the growth rate of the plant or the size of

carbohydrate reserves available for the N_2 fixation process may in some way affect the A/N ratio.

Experiment 3 was therefore designed to examine if and how the A/N and H/N ratios varied throughout plant ontogeny under conditions of unlimited supply of water and nutrients (except N) and controlled regimes of temperature and irradiance. Furthermore, whole-plant AR assays were again compared with excised-root AR assays to check for possible interaction between assay treatment and plant ontogeny on AR.

5.4.2 Methods

Narrow-leafed lupins (cv. Illyarrie) were sown in 120 pots. After 14 days, 90 pots with uniform plants were selected and grown to maturity in a growth room using a 12 h photoperiod (*c.* 550 μE m^{-2} s^{-1} at the top of the plants) and 18°/12°C, 60%/90% relative humidity day/night regimes.

Assays were performed on 10 randomly selected replicate pots per treatment on nine occasions throughout plant ontogeny.

Treatments : A. WPA system

B. NRA system

The 10 replicate plants used in the WPA treatment were returned to the main population and re-randomized after each occasion of assay. Pots were not watered in the morning of the day of assay. Limited resources did not allow the use of ^{15}N-labelled N_2 in this experiment, and N_2 fixation was measured by the increment in total plant nitrogen. The nutrient solution and the sand contained no nitrogen. Nodule tissue was also separated from the roots after each nodulated-root assay and the dry weight of nodules was determined. This allowed the three parameters, N_2 fixation, AR and H_2 evolution, to be determined on a nodule dry weight basis.

5.4.3 Results

There was no significant ($p > 0.05$) difference between the rates of AR in the NRA and WPA systems on any sampling occasion in this experiment (Appendix 5, p. 167). Therefore, all rates of AR given in the following results are those obtained using the NRA system.

The onset of flowering occurred on day 45 (*i.e.* 45 days from sowing) and coincided with the commencement of a rapid increase in the dry weight of the tops and nodules (Figures 5a and 5b). The growth rate of the whole plant reached a maximum at about day 80 (Figure 5c). The nitrogen content of the tops and roots varied in a similar manner to plant dry weight (Figure 6a), although the concentration of nitrogen in both tops and roots progressively declined throughout plant ontogeny (Figure 6b). Noteworthy was the coincidence in maximum growth rate of the plant (Figure 5c), maximum nodule growth rate (Figure 6c), maximum AR and H_2 evolution per plant (Figure 6d), and the commencement of a rapid increase in RE, defined as 1-H/A (Figure 6d). Furthermore, the RE increased while the growth rates declined.

The mean rates of AR and H_2 evolution (calculated from consecutive sampling occasions assuming a linear rate of change in these parameters for each interval) are compared to similar calculations of N_2 increment or decrement in Figure 7a. Despite increasing mean rates of AR and H_2 evolution, the rate of nitrogen accumulation, and therefore N_2 fixation, remained surprisingly constant on a per plant basis during the experiment.

An important feature of the results was the progressive decline throughout the experiment in rates of all three nitrogenase-related parameters (AR, H_2 evolution and N_2 fixation) when expressed on a nodule dry weight basis; hereafter referred to as sA, sH and sN respectively. Hence under the non-limiting conditions of water, light and

Figure 5

Dry weight and growth rate of Illyarrie lupins in the growth chamber

a) Dry weight of Illyarrie lupins plotted against time.

Tops ●—● Roots ■—■

b) Nodules

Points represent the mean of 10 replicate determinations and vertical bars represent 95% confidence intervals; for clarity small intervals are not shown.

c) Growth rate plotted against time

Values are calculated assuming a constant rate of change in dry weight between sampling occasions.

Tops ●—● Roots ■—■ Total ▲—▲

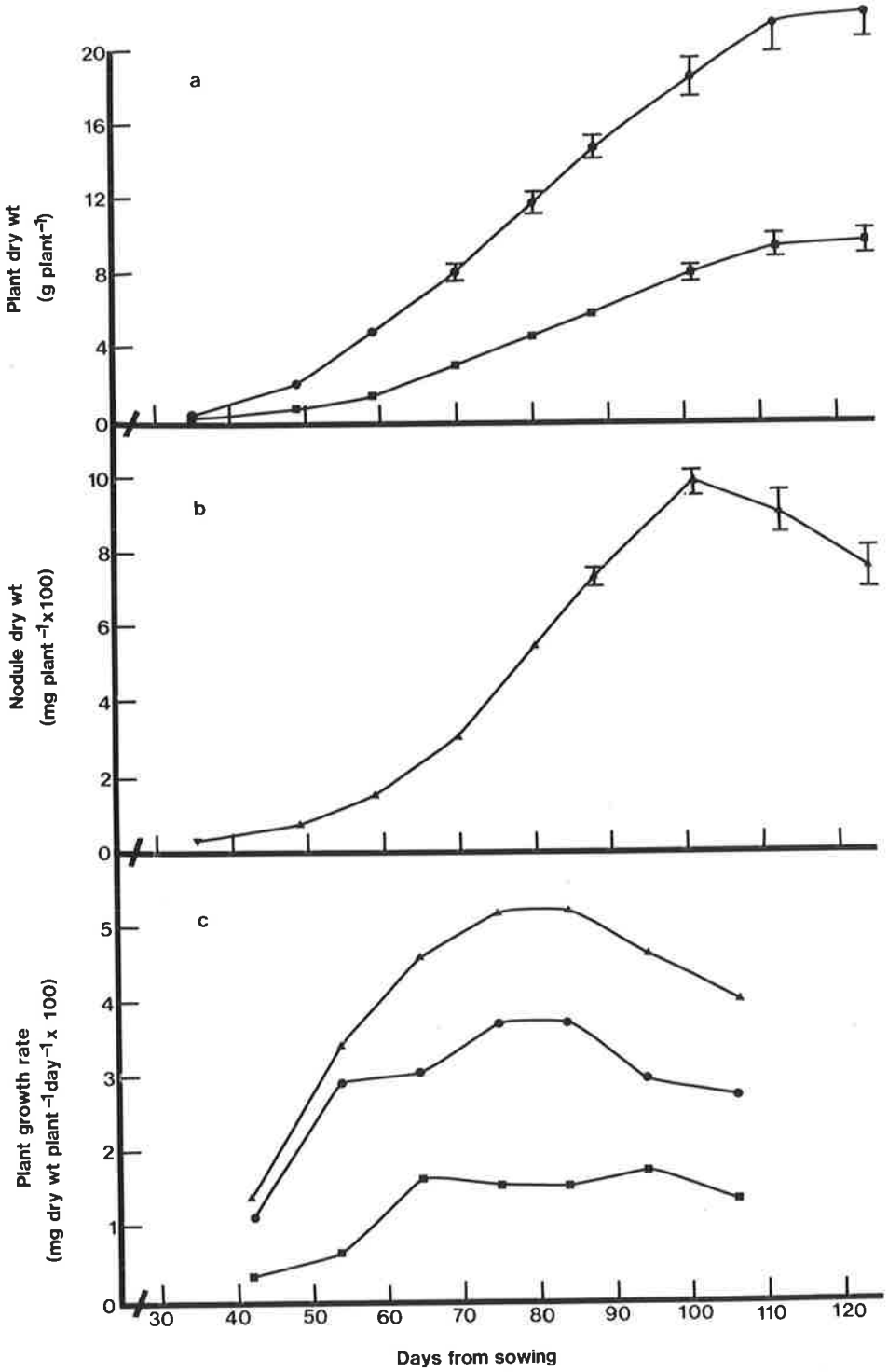

a
b
c
Plant dry wt
(g plant⁻¹)
Nodule dry wt
(mg plant⁻¹x100)
Plant growth rate
(mg dry wt plant⁻¹day⁻¹x 100)
Days from sowing
0
4
8
12
16
20
2
6
10
1
3
5
30
40
50
60
70
80
90
100
110
120

Figure 6

Nitrogen content, concentration, nodule growth rate, AR, H_2 evolution and RE during plant ontogeny in the growth chamber

a) and b) Nitrogen content (a) and nitrogen concentration (b) of Illyarrie lupins plotted against time. Points represent the mean of 10 replicate determinations and vertical bars represent 95% confidence intervals; for clarity small intervals are now shown.

Tops ●—● Roots ■—■

c) Nodule growth rate plotted against time.

d) Rates of AR (●—●), H_2 evolution (■—■) and RE (✱—✱) plotted against time.

$RE = 1 - H/A$

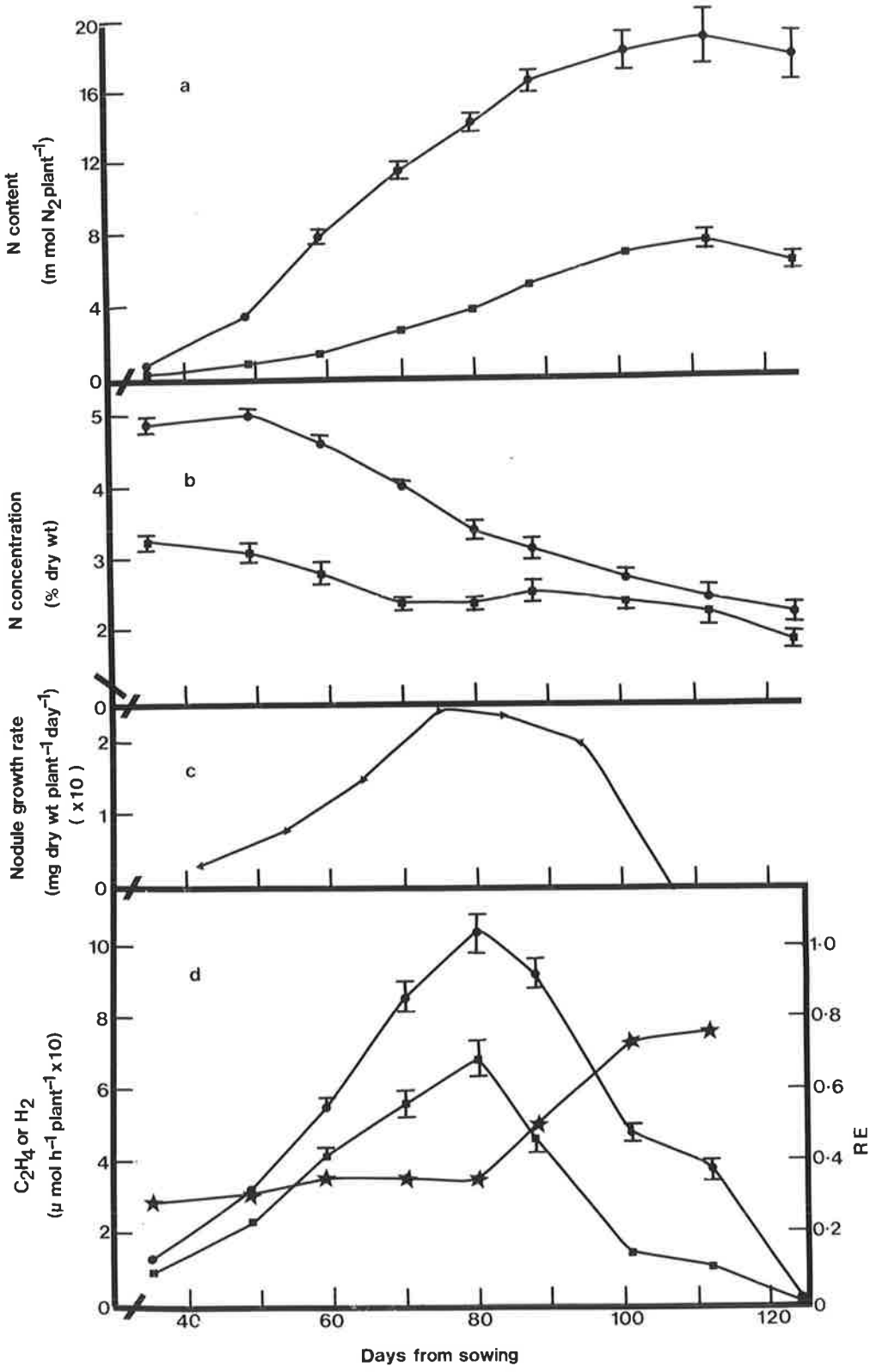
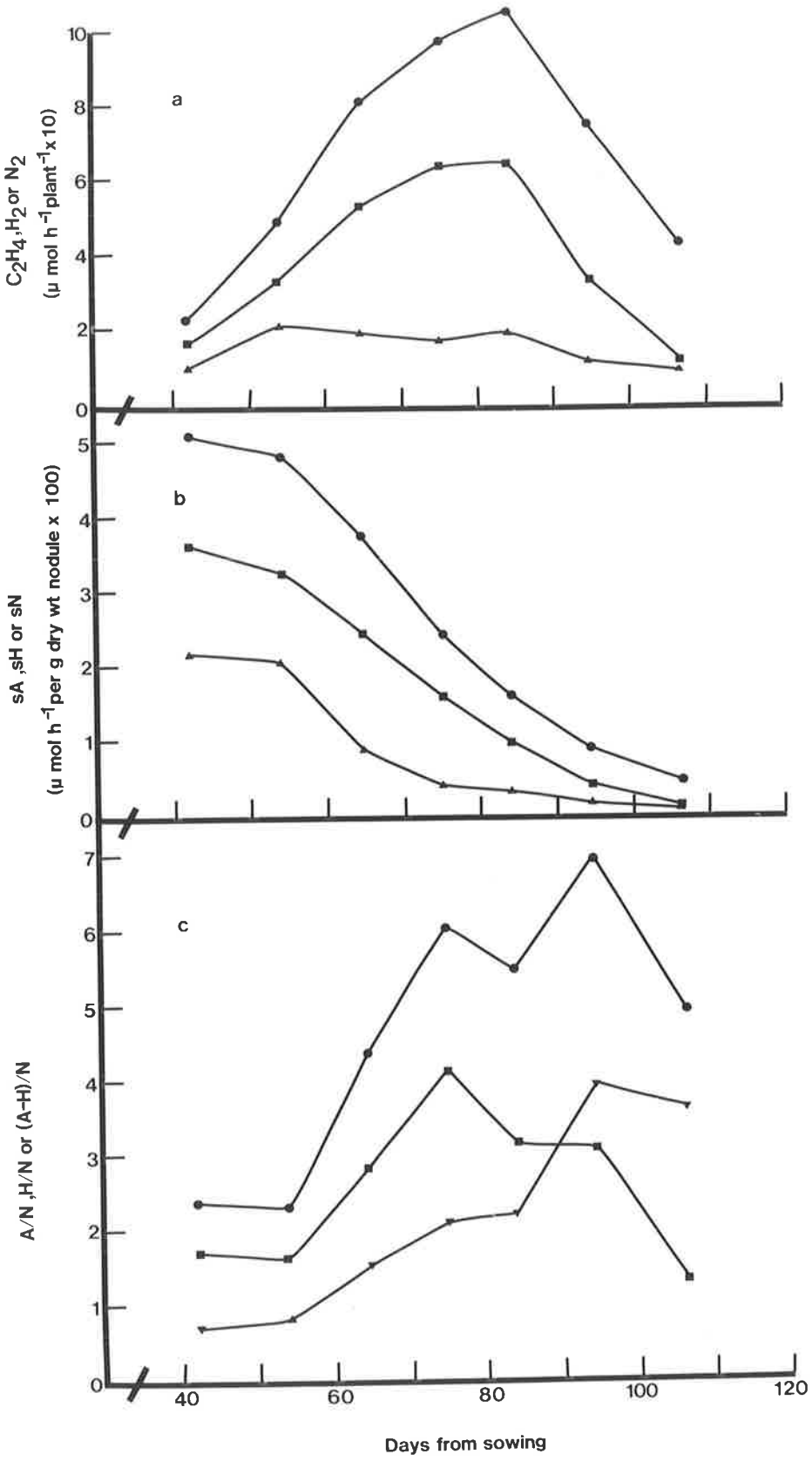

a
b
c
d
N content
(m mol N_2 plant^{-1})
N concentration
(% dry wt)
Nodule growth rate
(mg dry wt plant^{-1} day^{-1})
(x10)
C_2H_4 or H_2
(μ mol h^{-1} plant^{-1} x10)
RE
Days from sowing

Figure 7

Rates of AR, H_2 evolution and N_2 fixation and variation in A/N, H/N and (A-H)/N ratios during plant ontogeny in the growth chamber

a) and b) Mean rates per plant (a), and mean specific rates (b) of AR (●—●), H_2 evolution (■—■) and N_2 fixation (▲—▲) in Illyarrie lupins plotted against time. Values are calculated assuming a constant rate of change in each parameter between consecutive sampling occasions on which 10 replicate assays were performed.

b) A/N (●—●), H/N (■—■) and (A-H)/N (▼—▼) plotted against time. Ratios are calculated from the mean values depicted in a). Diurnal variation in AR and H_2 evolution is not taken into account and any such variation is assumed to be constant over time (refer text, p. 98).


a
b
c
C_2H_4, H_2 or N_2
(μ mol h^{-1} $plant^{-1}$ x 10)
sA, sH or sN
(μ mol h^{-1} per g dry wt nodule x 100)
A/N, H/N or (A-H)/N
Days from sowing
0
1
2
3
4
5
6
7
8
10
40
60
80
100
120

nutrients (except N) which this sand-cultured lupin crop encountered, changes in nitrogenase activity per plant, reflected increased nodule mass rather than increased activity per unit nodule mass up until maximum rates of activity were obtained.

It is significant that the experimentally-derived A/N and (A-H)/N ratios generally increased throughout plant ontogeny; from 2.4 to a maximum of 6.9 in the case of the A/N ratio (Figure 7c). The H/N ratio peaked at about 75 days and then declined. There was a significant ($p < 0.001$) direct linear relationship between sH and sA (Figure 8a) and furthermore, the extrapolation of the regression line produced a significant ($p < 0.05$) positive intercept on the abscissa.

However, the most important finding of this experiment was that high A/N and H/N ratios and high values of RE appeared to be associated with low values of sN and *vice versa*. The relationships of sA and sH to sN were curvilinear (Figure 8b) and were linearized by plotting sA and sH against ln sN (Figure 8c). The regressions of sA and sH on ln sN were not only significant ($p < 0.001$) but had significantly ($p < 0.01$) different slopes.

It is important to note here that diurnal variation in AR and H_2 evolution was not examined in this experiment. Rates of AR and H_2 evolution measured mid-way through the photo-period would probably over-estimate the mean daily rate, but this is not certain. Furthermore, although there appeared to be congruous relationships between AR and N_2 fixation, the interpretation of these relationships is confounded by the possibility that the degree of any diurnal variation which may have occurred in AR and H_2 evolution was variable over time. However, the magnitude of the change in A/N and H/N ratios during plant ontogeny precludes variation in the degree of diurnal variation in AR and H_2 evolution as being the only or even the major reason for the variable A/N

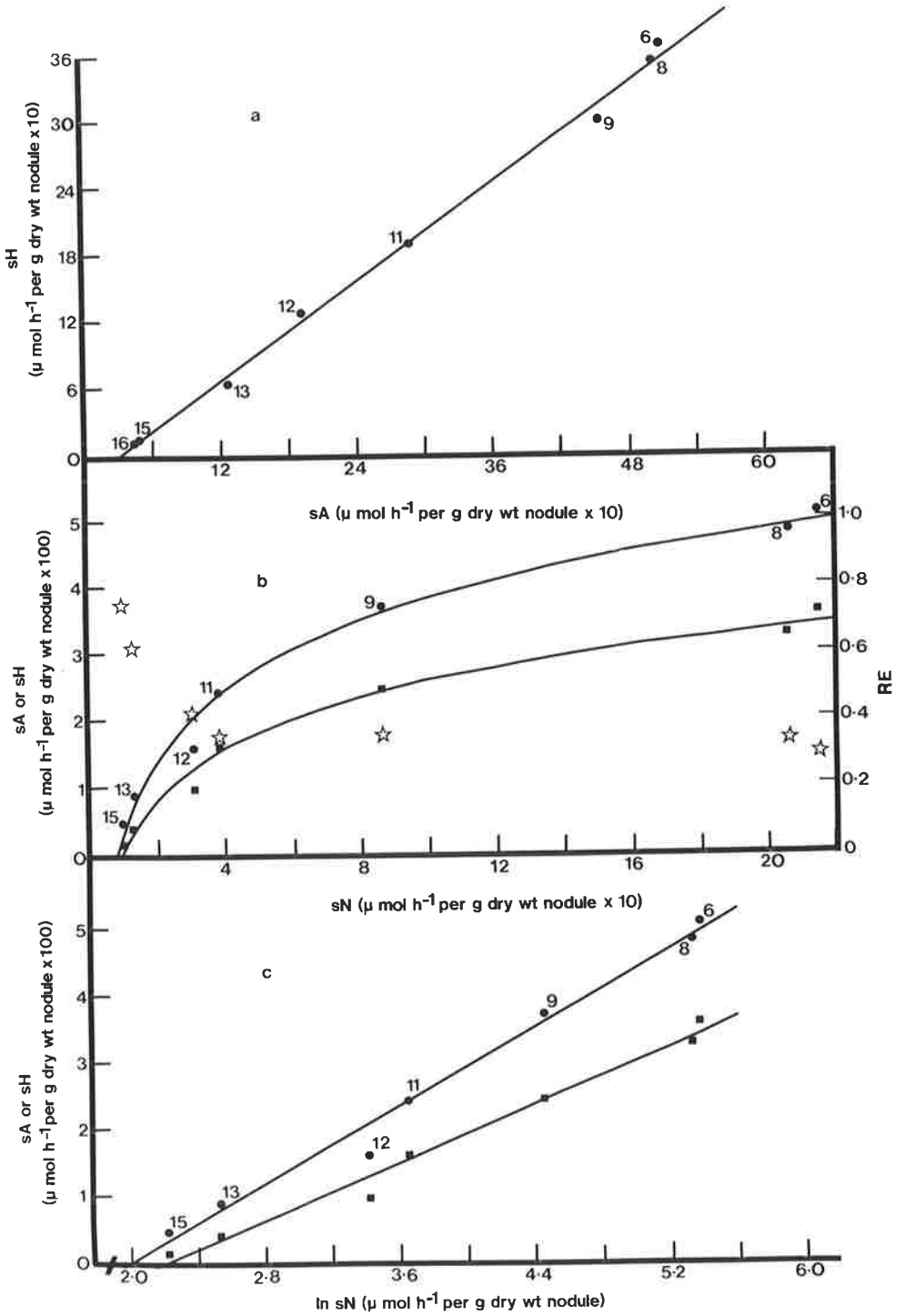
Figure 8

Relationships between sH and sA, and between sA, sH and sN in the growth-chamber experiment

a) Relationship between the mean specific rates of H_2 evolution (sH) and mean specific rates of AR (sA) in Illyarrie lupins. Points represent the mean of 10 pairs of replicate assays and point-scripts on the graph indicate the number of weeks from sowing when the assays were performed.

b) Relationships between mean sA (●—●), mean sH (■—■), RE (✡—✡) and mean sN.

c) Relationships between mean sA (●—●), mean sH (■—■) and the natural logarithm of mean sN (ln sN).


a
sH
(μ mol h-1 per g dry wt nodule x 10)
36
30
24
18
12
6
0
6
8
9
11
12
13
15
16
12
24
36
48
60
sA (μ mol h-1 per g dry wt nodule x 10)
b
sA or sH
(μ mol h-1 per g dry wt nodule x 100)
5
4
3
2
1
0
RE
1·0
0·8
0·6
0·4
0·2
0
4
8
12
16
20
sN (μ mol h-1 per g dry wt nodule x 10)
c
sA or sH
(μ mol h-1 per g dry wt nodule x 100)
2·0
2·8
3·6
4·4
5·2
6·0
ln sN (μ mol h-1 per g dry wt nodule)

and H/N ratios. Furthermore, the congruous relationships between sA and sN and between sH and sN obtained in this experiment were also obtained in subsequent field experiments (Section 5.5, p. 114) where the calculations were based on simultaneous measurements of AR, H_2 evolution and the uptake of ^{15}N-enriched N_2. More detailed examination of, and possible reasons for, these observed relationships and their potential application in the measurement of N_2 fixation will be discussed in the context of the results of the following field experiments.

5.5 Field experiments [Experiments 4 (1980) and 5 (1981)]

5.5.1 Aims

The parameters measured in the growth-chamber experiment were also measured in two consecutive years (1980 and 1981) in commercial crops of narrow-leafed lupins (cvv. Marri and Illyarrie respectively). The field experiments provided data accumulated under more adverse, but more realistic, growth conditions than that encountered in the growth chamber.

An important and ultimate aim of the field experimentation was the derivation of estimates of N_2 fixation for commercial lupin crops and the assessment of their likely contribution to the nitrogen status of the soil within a commercial farming system. Because of the likely inverse relationship between soil mineral nitrogen status and the proportion of the nitrogen in the plants derived from N_2 fixation, the sites were chosen to represent the higher end of the soil fertility scale for commercial lupin production. This would enable verification of the relationships between sA, sH and sN observed in the growth-chamber experiment with plants grown in the presence of moderate amounts of combined N in the soil.

The results from 1980 using the NRA system showed that, either significant quantities (higher than expected) of the nitrogen accumulated in the lupin plant were derived from soil mineral nitrogen or else the NRA

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system was substantially under-estimating the true quantity of N_2 being fixed *in situ*.

Therefore, in 1981, the WFA system was first utilised to investigate whether plant perturbation was affecting nitrogenase activity in plants grown in the field, particularly under conditions of water stress. Although it was clear that little difference could be demonstrated in AR and N_2 fixation between the NRA and WPA systems in the glasshouse and growth chamber (provided the roots were not washed prior to assay), it does not follow that the same would apply to field-grown plants. For example, one important difference is that plants grown in the growth chamber were adequately supplied with water whereas plants grown in the field may frequently encounter periods of water stress.

Measurements of mineral nitrogen concentrations in the soil were also made during 1981 and measurements of diurnal variation in AR, H_2 evolution and N_2 fixation were made on three occasions during plant ontogeny.

5.5.2 Methods

The two field sites were on the property of D.R. Klitscher located at Field, Hundred of Strawbridge, County Buccleuch, 25 kilometres south-east of the township of Coonalpyn, South Australia. The mean annual rainfall is 480 mm. The property is representative of most commercial areas of lupins in South Australia, and the farmer is renowned for consistently producing high-yielding, weed-free crops. The podzolic soil consists of a siliceous, grey, sandy, A1 horizon and a bleached white A2 horizon overlying a yellow-brown clay at a depth of between 45 and 100 cm. Limestone was sometimes encountered in the B horizon when soil samples were collected.

The 1980 experimental site had been sown to lupins in the previous year and the site used in 1981 had been under grassy pasture for at least the previous five years, although the paddock did contain a legume component in some of these years.

Values of organic carbon, total nitrogen and pH of the 1981 experimental site are given in Table 5.

In both years, the crops were sown with about 100 kg ha^{-1} of superphosphate using minimum tillage techniques and relying on simazine (2-chloro-4,6-bisethylamino-1,3,5-triazine) for weed control which was almost 100% effective. The experimental sites were selected after plant emergence, and the major selection criterion was uniform establishment of plants in a uniform soil.

Other management practices carried out during the growth of the two crops when required were the application of manganese sulphate for the prevention of split seed (Perry and Gartrell 1976), and DDT (dichloro diphenyl trichloroethane) and Metasystox (2-(ethylthio)ethyl diethyl phosphorothionate) for the control of heliothis (*Heliothis punctiger*) and cowpea aphid (*Aphis craccivora*) respectively.

Table 5

Organic carbon, total nitrogen and pH of the soil

(1981 experimental site)

SOIL HORIZON	DEPTH (cm)	ORGANIC C (%)	TOTAL N (%)	pH
A1	0-15	1.49 (± 0.12)	0.05 (± 0.004)	6.13 (± 0.02)
A2	15-50	0.39 (± 0.02)	0.02 (± 0.0006)	6.69 (± 0.05)
B1	50-100	0.40 (± 0.01)	0.03 (± 0.002)	6.59 (± 0.11)

(Values in parenthesis represent ± the SEM of 30 random samples from the experimental site).

PLATE 2

A. Nodulated roots of 14-week old Illyarrie lupin plants at Coonalpyn in 1981.

B. *In situ* field assay (WFA system).

C. Gas cylinders mounted aboard a 4-wheel drive vehicle.

D. and E. Soil profiles at Coonalpyn showing the grey sandy A1 horizon over a bleached white sandy A2 horizon (D). Limestone outcrops were sometimes encountered close to the soil surface (E).

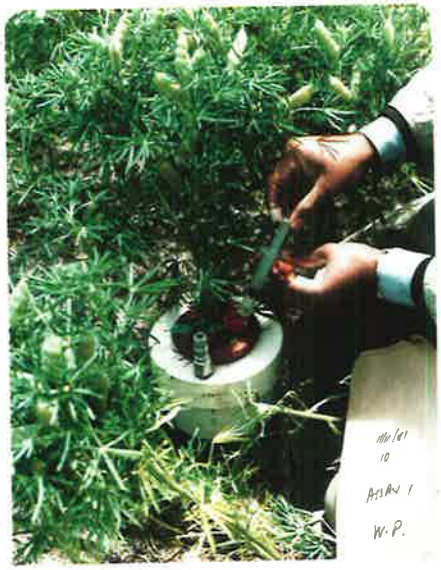
F. Soil sampling using a hydraulic ram mounted on a 4-wheel drive vehicle.

G. View of field site early in 1981 showing the incubation cylinders inserted.

H. Field site in September 1981 when the lupin plants were 14 weeks old.

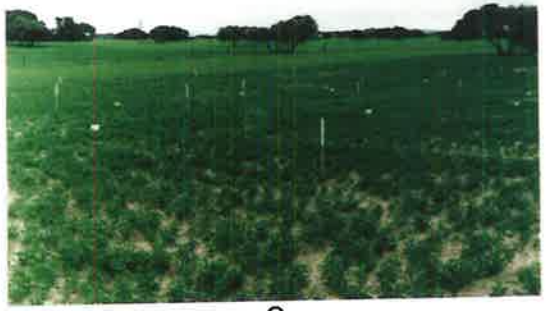
I. Leaf drop late in the season, a characteristic habit of narrow-leafed lupins in southern Australia.

A



B

C



G

H

D

E

F

I

The experimental sites were subdivided into 12 blocks (each 10 m x 10 m) in 1980 and 10 blocks of similar size in 1981. A central roadway provided immediate access to each block. Each block was further divided into 10 sub-blocks (5 m x 2 m) and on each sampling occasion measurements were made on plants in one sub-block selected at random from each block.

In the NRA system, one plant (although occasionally more at the very early stages of growth) was selected at random from the appropriate sub-plot for measurement of AR, and another plant, closely matched in size and maturity, was chosen for the measurement of H_2 evolution and N_2 fixation.

In 1981, immediately after the site was selected, two PVC tubes per sub-block were centred over individual lupin plants and inserted into the soil to a depth of 250 mm, leaving the top level with the soil surface. The PVC tubes confined most of the nodulated-root zone of the single plants within the tube and ensured that the whole-plant assays which were performed on these plants estimated nitrogenase activity for the confined plant only. The careful excavation of plants for NRA treatments, and gentle shaking to remove soil from the roots ensured a single and mostly entire nodulated-root. Comparisons of WPA and NRA systems for AR were therefore valid on a single plant basis.

To check that the presence of the PVC tube had no effect on the growth and development of the lupin plant in comparison with unconfined plants, comparative measurements of plant dry weight and AR using the NRA system were performed pre-flowering, at the first occasion of measurement after the onset of flowering and late pod-fill. Also on these occasions, two extra NRA-system treatments were included to check for hydrogen uptake.

These treatments were a) the addition of 0.5 mM H_2 plus C_2H_2 and b) flushing with Ar/O_2.

Plant dry weight and nitrogen content were measured on the individual plants used for AR and H_2 evolution assays. On all occasions, N_2 fixation was determined by measuring the rate of uptake of ^{15}N-enriched N_2.

Fallen plant parts were collected using fibreglass mesh laid on the ground under plants to be sampled on the next occasion. Results of plant dry weight and nitrogen content have been adjusted to account for fallen debris.

Soil water content and mineral nitrogen concentrations (NH_4^+ and NO_3^-) were monitored by core-sampling down to a depth of 1 m using a hydraulic ram mounted on a vehicle, and sampling the A1, A2 and B1 horizons.

Meteorological instruments were located at the farmhouse about 1 km from the experimental sites and daily records of air temperature, rainfall and class A pan evaporation were kept by the farmer.

On occasions of assays, irradiance and soil temperature at a depth of 10 cm were monitored on a continuous basis.

Irradiance was measured at crop height by a LI-COR+ quantum sensor, measuring photo-synthetically active radiation (PAR) in the 400 to 700 nm waveband, and automatically integrated over time by a LI-COR (LI550B) printing integrator.

On three occasions (early vegetative, mid-flowering and pod-filling stages of growth) in 1981, diurnal variation in AR, H_2 evolution and N_2 fixation was monitored using the NRA system. AR was also monitored using

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+ LI-COR Inc./LI-COR Ltd., 4421 Superior Street, P.O. Box 4425, Lincoln Nebraska, 68504, U.S.A.

the WFA system. Measurements were made at 6 h intervals over a period of 24 h. Plants to be sampled at night were marked with reflecting tape during the day because of the difficulty of matching the plants at night.

5.5.3 Results

Preliminary findings

Three facets of the results are addressed prior to presentation of the main findings. Firstly, the comparative studies of AR and dry weight of tops of plants growing in the PVC tubes and those growing without, revealed no demonstrable difference in either parameter (Appendix 6, p. 168). Secondly, as in the growth-chamber experiment, AR in the whole-plant assays (in this case the WFA system) was never significantly ($p>0.05$) different from AR of excised, nodulated roots (NRA system) (Appendix 7, p. 169). Thirdly, on all occasions on which measurements were made, no uptake of exogenously-supplied H_2 occurred in the NRA system and no significant ($p>0.05$) difference could be demonstrated between rates of H_2 evolution in Ar/O_2 and rates of AR (Appendix 6, p. 168).

Therefore, only the results of the NRA treatments are presented in the following figures. Furthermore, the measurements of H_2 evolution presented represent total nitrogenase-catalysed H_2 evolution in the absence of a functional uptake hydrogenase. This is considered further in the general discussion (Section 6.3, p. 127).

Plant growth and nitrogenase activity

In 1980, the dry weight of plant tops increased greatly following the onset of flowering at week 13 (Figure 9a), although the growth rate of nodule mass and roots declined at this time (Figure 9a and 10a). The change in the growth rate of the tops was associated with increased availability of soil water (Figure 9b) caused by heavy rains (Figure 9d).

Figure 9

Dry weight of Marri lupins and meteorological data during 1980 at Coonalpyn

a) Dry weight plotted against time. Points represent the means of 12 replicate assays and vertical bars represent 95% confidence intervals; for clarity only half intervals may be shown or small intervals omitted. F denotes the onset of flowering.

Tops ●—● Roots ■—■ Nodules ▲—▲

b) Soil water content plotted against time. Points represent the mean of 36 samples (3 samples in each of 12 blocks).

A1 horizon ●—● A2 horizon ■—■ B1 horizon ▲—▲

c) Mean weekly maximum (●—●) and minimum (■—■) temperatures during the growing season.

d) Weekly rainfall (—) and evaporation (----) during the growing season.

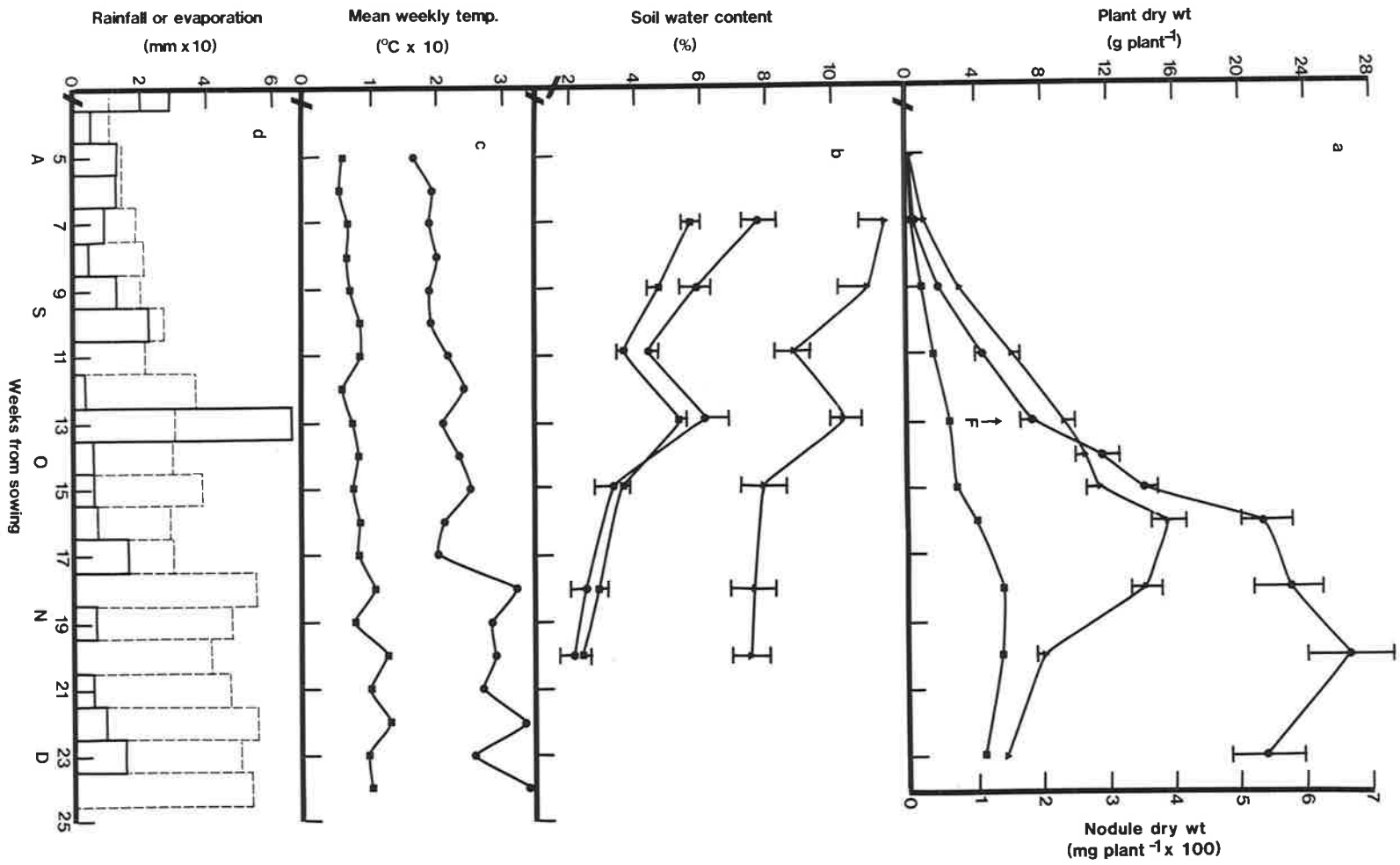

Plant dry wt
(g plant^{-1})
Soil water content
(%)
Mean weekly temp.
(°C x 10)
Rainfall or evaporation
(mm x 10)
Nodule dry wt
(mg plant $^{-1}$ x 100)
a
b
c
d
F
Weeks from sowing
A
S
O
N
D

Figure 10

Growth rate and rates of AR, H_2 evolution and N_2 fixation during 1980 at Coonalpyn

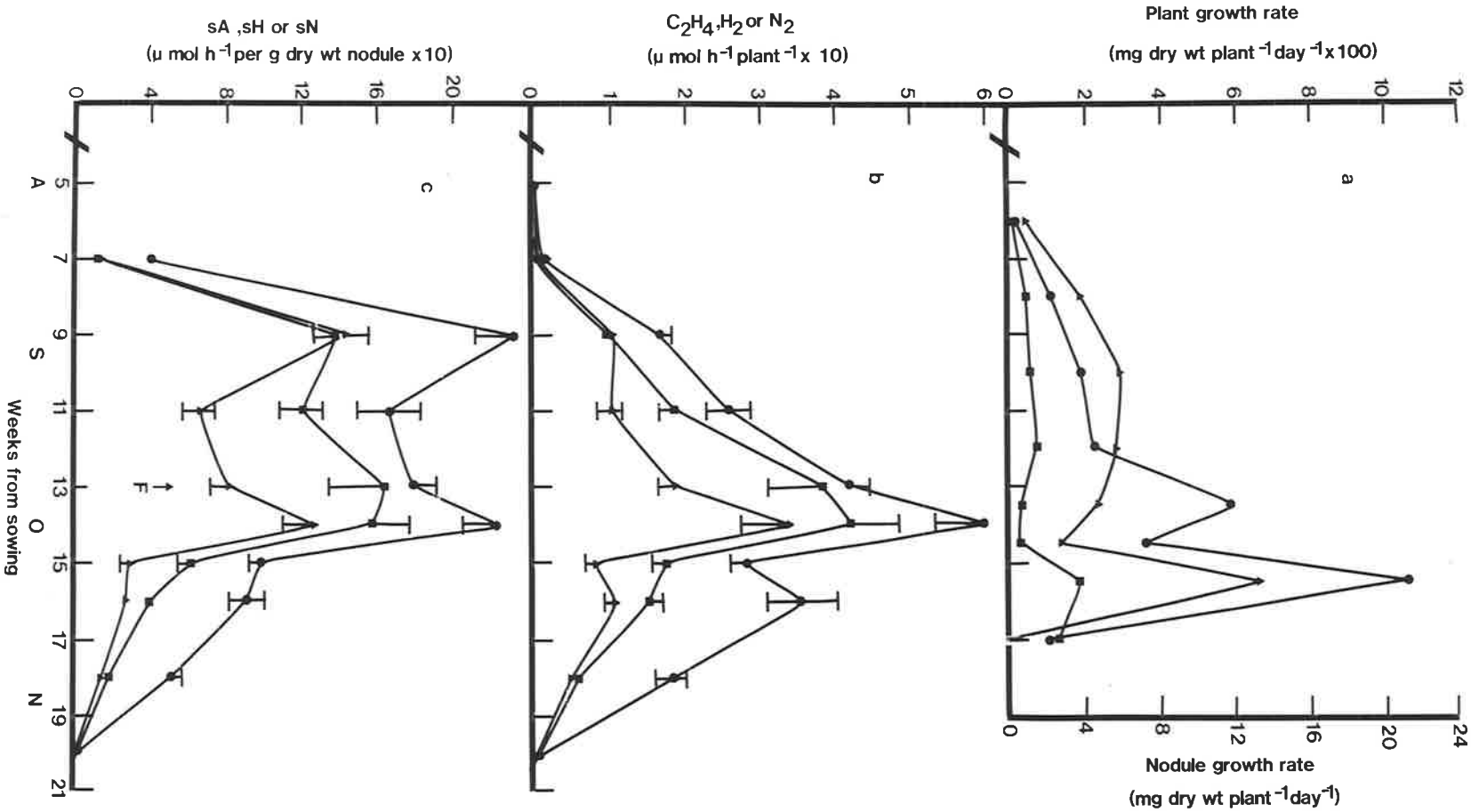
a) Growth rate plotted against time. Points represent the mean increment per unit time between consecutive sampling occasions on which 12 measurements of dry weight were recorded (assuming a constant rate of change in dry weight between sampling occasions).

Tops ●—● Roots ■—■ Nodules ▲—▲

b) and c) Rates of AR (●—●), H_2 evolution (■—■) and N_2 fixation (▲—▲) per plant (b) and specific rates (c) plotted against time.

Points represent the means of 12 replicate assays and vertical bars represent 95% confidence intervals; for clarity only half the interval may be shown or small intervals omitted.

F denotes the onset of flowering.


Plant growth rate
(mg dry wt plant $^{-1}$ day $^{-1}$ x 100)
a
Nodule growth rate
(mg dry wt plant $^{-1}$ day $^{-1}$)
C_2H_4, H_2 or N_2
(μ mol h $^{-1}$ plant $^{-1}$ x 10)
b
sA, sH or sN
(μ mol h $^{-1}$ per g dry wt nodule x 10)
c
F
A
S
O
N
Weeks from sowing

Air temperature *per se* does not appear to have been a causative factor (Figure 9c). Also at this time, the rates of AR and N_2 fixation per plant increased greatly (Figure 10b) and, because of the corresponding time-lag before the growth rate of the nodules increased, sA and sN also greatly increased (Figure 10c).

The resulting patterns in sA, sH and sN therefore contrast to the almost linear decline in these parameters throughout plant ontogeny in the growth-chamber experiment. It is clear that, under certain conditions in the field, the nodules can increase sA, sH and sN despite the overall trend for increased growth of nodules to lower these parameters.

The rapid increase in growth rate of tops, roots and nodule mass between weeks 15 and 16 (Figure 10a), together with a reversal in the declining rates of AR and N_2 fixation per plant (Figure 10b) cannot be accounted for directly by environmental factors such as temperature and soil water content, and the cause is not readily apparent.

Environmental conditions during the onset of flowering in 1981 differed from those in 1980, in that the decline in soil water content, that commenced prior to flowering in each year, continued unabated until week 19 (Figure 11b). This resulted in a more synchronous pattern of change in dry weight of plant tops, nodules and roots (Figure 11a), although a situation similar to 1980 again occurred (but for less duration) where nodule growth rate began to decline while the growth rate of the tops continued to increase (Week 13, Figure 12a).

In 1981, the profiles of AR, H_2 evolution and N_2 fixation per plant were similar to those for 1980 (Figure 12b) with maximum rates occurring one to two weeks following the onset of flowering. However, no reversal in the declining rates of these parameters occurred later in the season in 1981, although sA, sH and sN did increase during weeks 11-15

Figure 11

Dry weight of Illyarrie lupins and meteorological data during 1981 at Coonalpyn

a) Dry weight plotted against time. Points represent the mean of 10 replicate assays and vertical bars represent 95% confidence intervals; for clarity only half intervals may be shown or small intervals omitted.

Tops ●—● Roots ■—■ Nodules ▲—▲

F denotes the onset of flowering.

b) Soil water content plotted against time. Points represent the mean of 30 samples (3 x 10 blocks).

A1 horizon ●—● A2 horizon ■—■ B1 horizon ▲—▲

c) Mean weekly maximum (●—●) and minimum (■—■) temperatures during the growing season.

d) Weekly rainfall (—) and evaporation (-----) during the growing season.

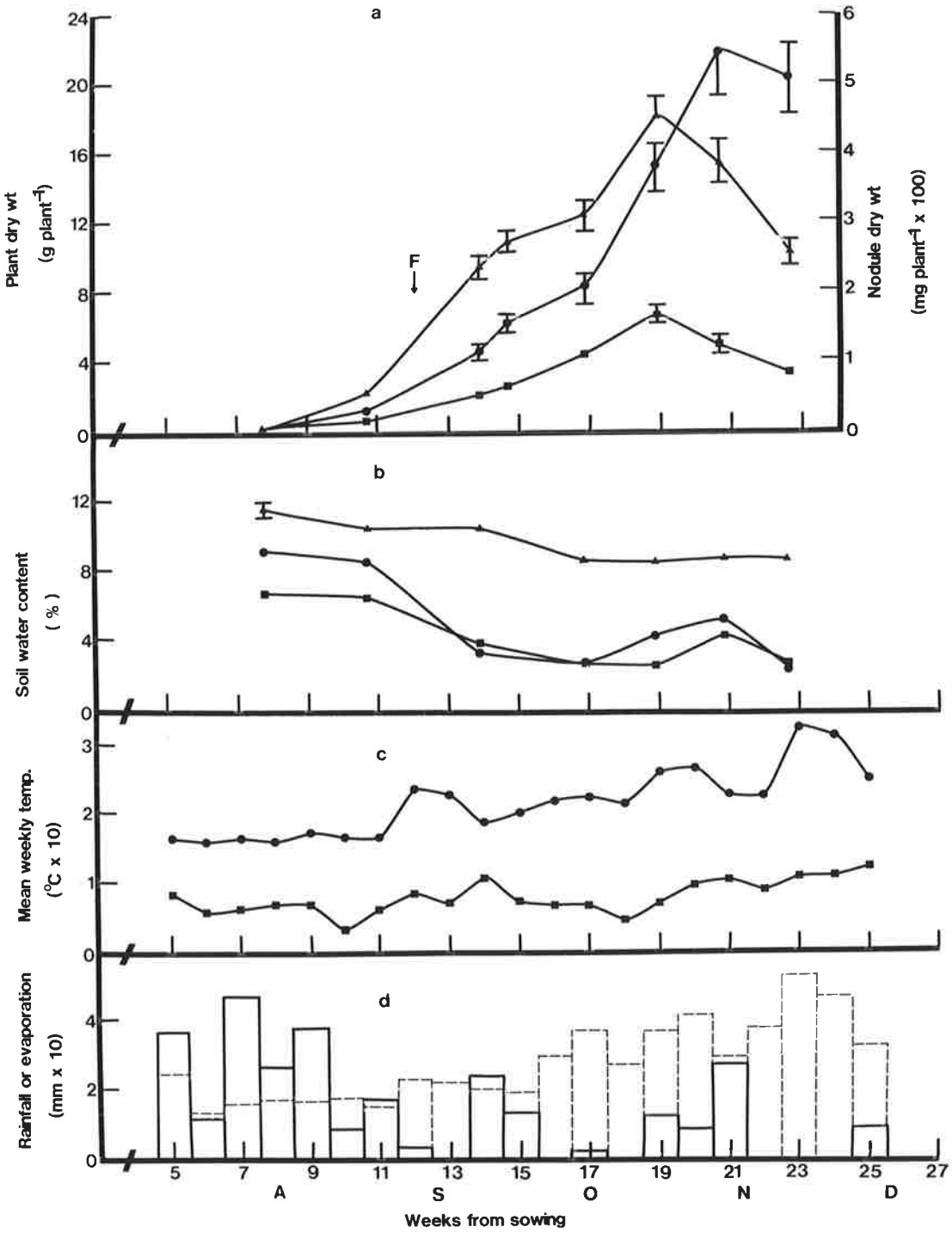

a
Plant dry wt
(g plant⁻¹)
Nodule dry wt
(mg plant⁻¹ x 100)
F
b
Soil water content
(%)
c
Mean weekly temp.
(°C x 10)
d
Rainfall or evaporation
(mm x 10)
5
7
9
11
13
15
17
19
21
23
25
27
A
S
O
N
D
Weeks from sowing

Figure 12

Growth rate and rates of AR, H_2 evolution and N_2 fixation during 1981 at Coonalpyn

a) Growth rate plotted against time. Points represent the mean increment per unit time between consecutive sampling occasions on which 10 measurements of dry weight were recorded (assuming a constant rate of change in dry weight between sampling occasions).

Tops ●—● Roots ■—■ Nodules ▲—▲

b) and c) Rates of AR (●—●), H_2 evolution (■—■) and N_2 fixation (▲—▲) per plant (b) and specific rates (c) plotted against time. Points represent the means of 10 replicate assays and vertical bars represent 95% confidence intervals; for clarity only half the interval may be shown or small intervals omitted. F denotes the onset of flowering.

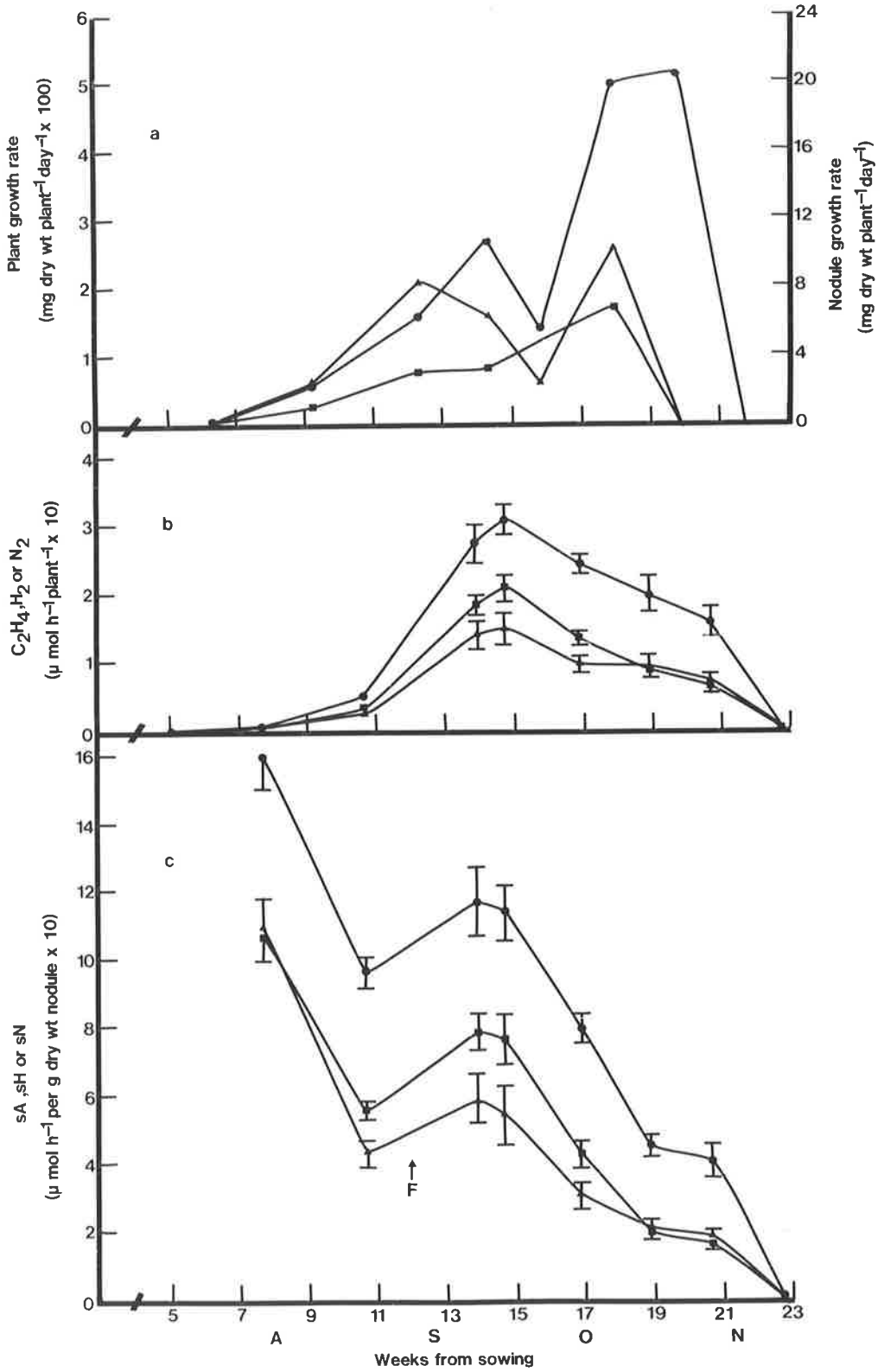

a
Plant growth rate
(mg dry wt plant^{-1} day^{-1} x 100)
Nodule growth rate
(mg dry wt plant^{-1} day^{-1})
b
C_2H_4, H_2 or N_2
(μ mol h^{-1} plant^{-1} x 10)
c
sA, sH or sN
(μ mol h^{-1} per g dry wt nodule x 10)
F
A
S
O
N
Weeks from sowing

Figure 13

Variation in A/N, H/N and (A-H)/N ratios and RE during 1980 at Coonalpyn

a) and b) A/N (●—●), H/N (■—■) and (A-H)/N (▲—▲) ratios (a) and RE (b) plotted against time. F denotes the onset of flowering.

c) RE plotted against sN.

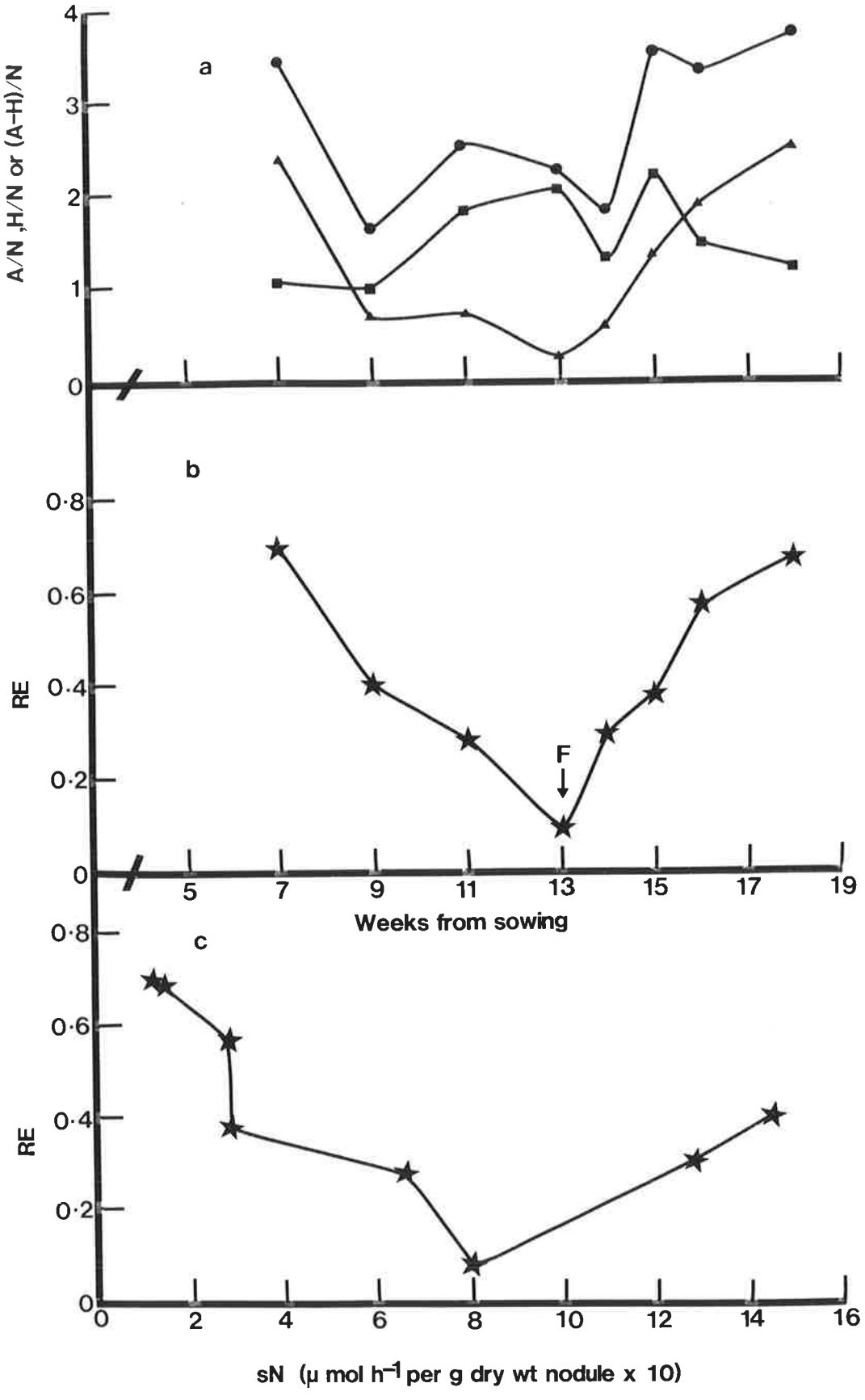
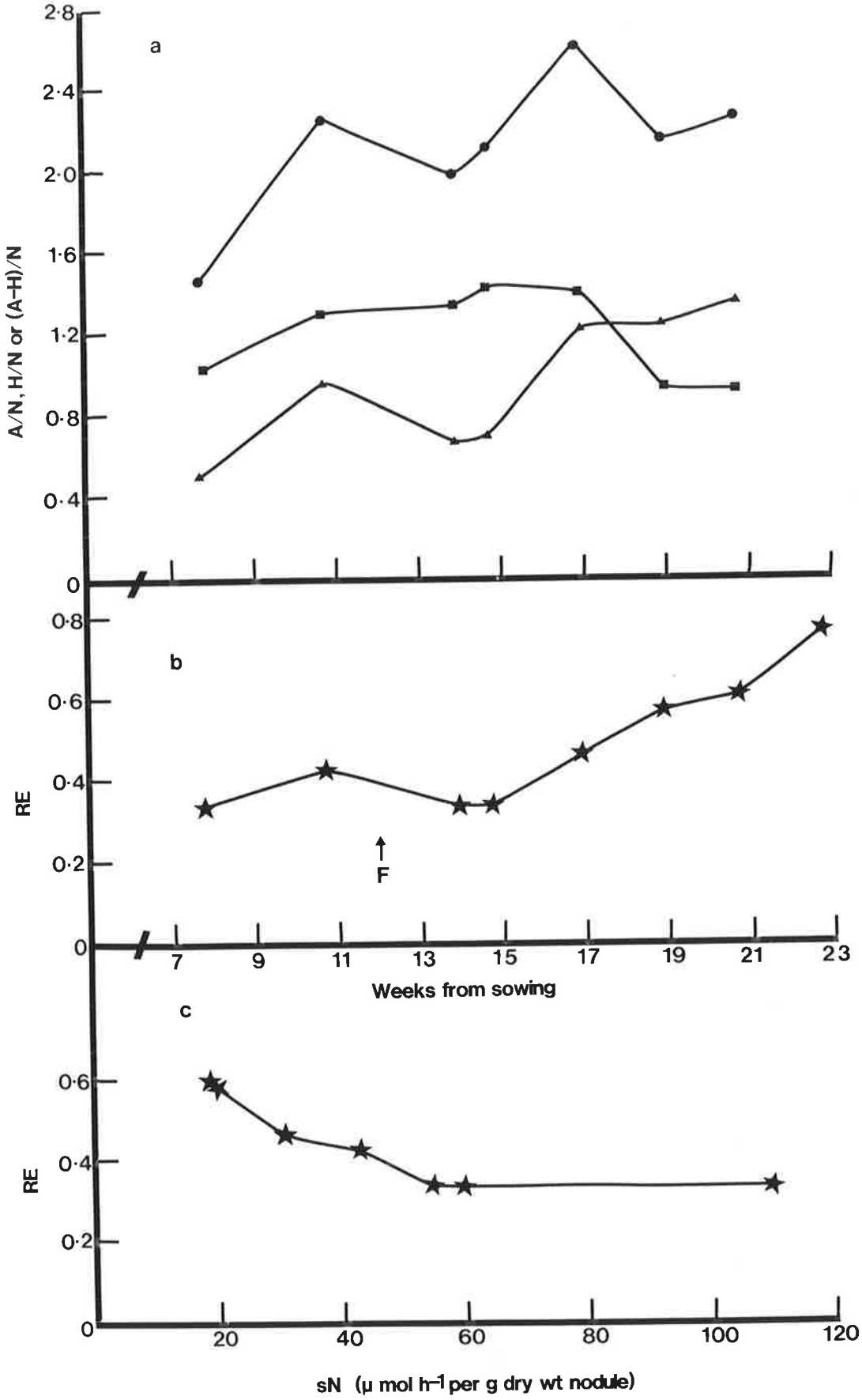

a
A/N ,H/N or (A–H)/N
4
3
2
1
0
b
RE
0·8
0·6
0·4
0·2
0
F
5
7
9
11
13
15
17
19
Weeks from sowing
c
RE
0·8
0·6
0·4
0·2
0
0
2
4
6
8
10
12
14
16
sN (μ mol h⁻¹ per g dry wt nodule x 10)

Figure 14

Variation in A/N, H/N and (A-H)/N ratios and RE during 1981 at Coonalpyn

a) and b) A/N (●—●), H/N (■—■) and (A-H)/N (▲—▲) ratios (a) and RE (b) plotted against time. F denotes the onset of flowering.

c) RE plotted against sN.


a
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
A/N, H/N or (A–H)/N
b
0·8
0·6
0·4
0·2
0
RE
F
7
9
11
13
15
17
19
21
23
Weeks from sowing
c
0·6
0·4
0·2
0
RE
20
40
60
80
100
120
sN (μ mol h⁻¹ per g dry wt nodule)

following the initial rise and decline after the onset of nodulation (Figure 12c).

In 1980, the A/N, H/N and (A-H)/N ratios varied during the season (Figure 13a). The RE (defined as 1 - H/A) declined until around the onset of flowering and then increased until plant maturity (Figure 13b). There appeared to be a general decline in RE with increasing sN at low values of the latter with a tendency to level out (or increase) at higher values of sN (Figure 13c).

In 1981, the above parameters varied in similar fashion with plant ontogeny to those in 1980, although the amplitudes of the fluctuations in A/N and H/N were noticeably less (Figure 14a). In addition, no initial decline in RE prior to flowering was recorded in 1981 (Figure 14b), although the increase in RE during the latter part of the season was a common feature in both years. In 1981, the RE declined with an initial increase in sN and tended to level out at higher values of sN (Figure 14c).

Relationships between sA, sH and sN

The data displayed in Figures 15 and 16 for the two consecutive seasons (1980 and 1981) support the findings of the growth-chamber experiment, in that a linear relationship between H_2 evolution and AR (Figures 15a and 16a) and a curvilinear relationship between both sA and sN, and sH and sN (Figures 15b and 16b) can clearly be demonstrated.

As in the growth-chamber experiment, extrapolation of the regression of H_2 evolution on AR produced a significant ($p<0.05$) positive intercept in the abscissa in both years and the relationships of sA and sH to sN were linearized by plotting sA and sH against ln sN (Figures 15c and 16c). Although the difference in slope of the regression lines of sA and sH on

Figure 15

Relationships between sH and sA, and between sA, sH and sN during 1980 at Coonalpyn

a) Relationship between sH and sA. Points represent the mean of 12 replicate pairs of assays, and point-scripts on the graph indicate the number of weeks from sowing when the assays were performed. Vertical and horizontal bars represent 95% confidence intervals; for clarity only half intervals may be shown or small intervals omitted.

b) and c) Relationships between sA (●—●), sH (■—■) and sN (b) and between sA, sH and ln sN (c).

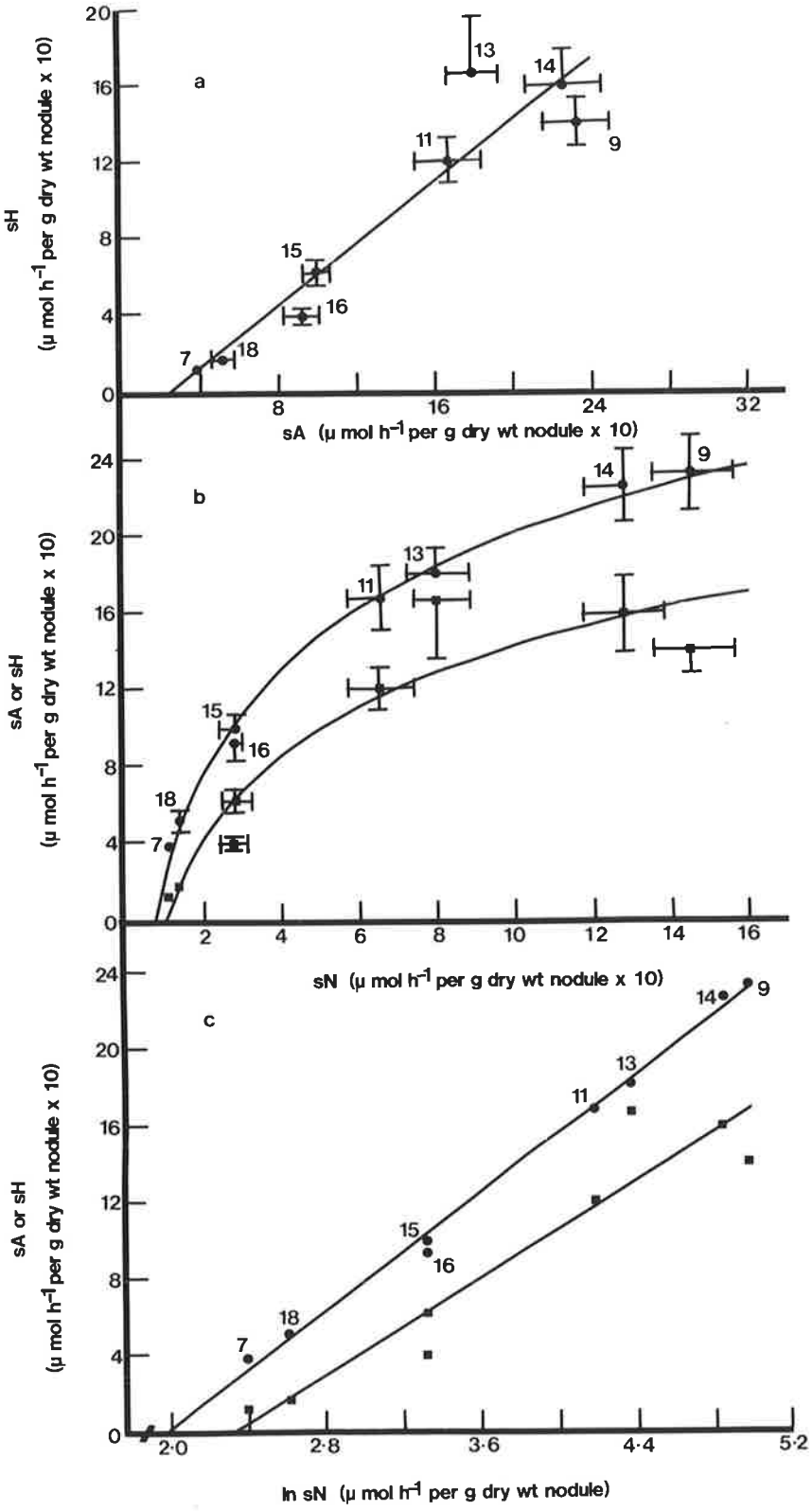

a
sH
(μ mol h⁻¹ per g dry wt nodule x 10)
sA (μ mol h⁻¹ per g dry wt nodule x 10)
b
sA or sH
(μ mol h⁻¹ per g dry wt nodule x 10)
sN (μ mol h⁻¹ per g dry wt nodule x 10)
c
sA or sH
(μ mol h⁻¹ per g dry wt nodule x 10)
ln sN (μ mol h⁻¹ per g dry wt nodule)

Figure 16

Relationships between sH and sA, and between sA, sH and sN during 1981 at Coonalpyn

a) Relationship between sH and sA. Points represent the mean of 10 replicate pairs of assays, and point-scripts on the graph indicate the number of weeks from sowing when the assays were performed. Vertical and horizontal bars represent 95% confidence intervals; for clarity only half intervals may be shown or small intervals omitted.

b) and c) Relationships between sA (●—●), sH (■—■) and sN (b) and between sA, sH and ln sN (c).

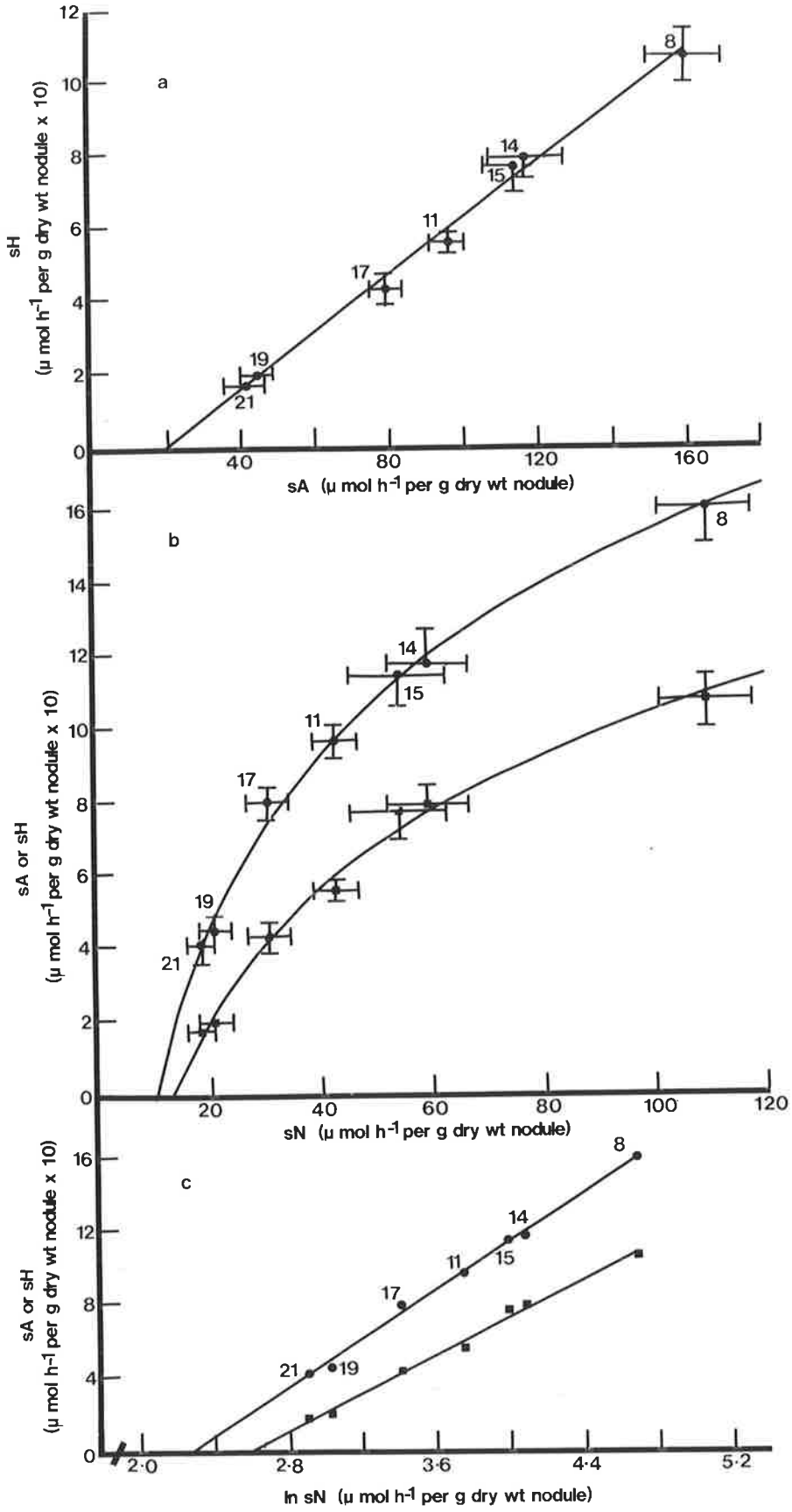

a
sH
(μ mol h-1 per g dry wt nodule x 10)
sA (μ mol h-1 per g dry wt nodule)
b
sA or sH
(μ mol h-1 per g dry wt nodule x 10)
sN (μ mol h-1 per g dry wt nodule)
c
sA or sH
(μ mol h-1 per g dry wt nodule x 10)
ln sN (μ mol h-1 per g dry wt nodule)
8
14
15
11
17
19
21

Figure 17

Diurnal variation in AR, H_2 evolution and N_2 fixation, and meteorological parameters during 1981 at Coonalpyn

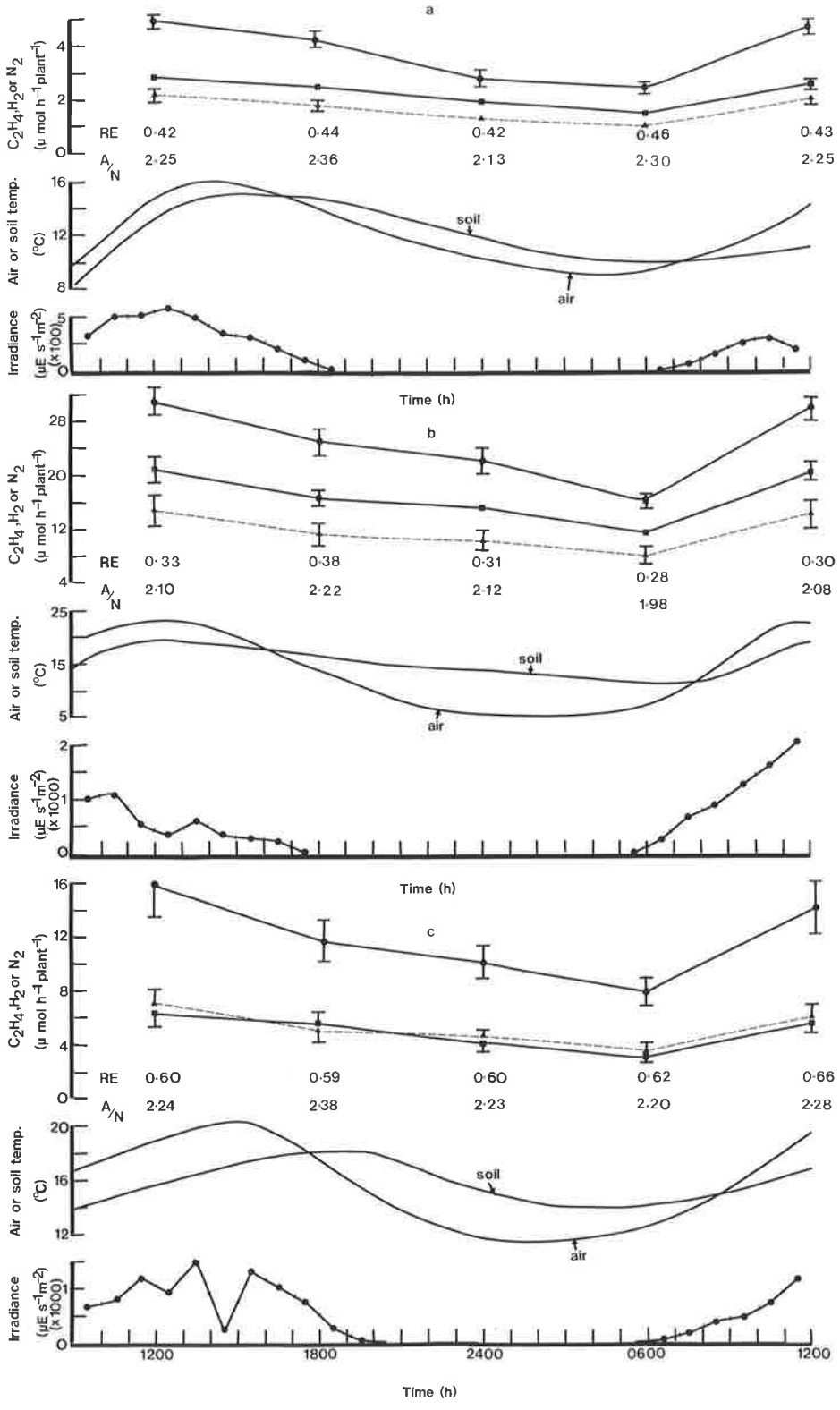
Diurnal variation in rates of AR (●—●), H_2 evolution (■—■) and N_2 fixation (▲—▲), air and soil temperature (as indicated) and irradiance on 3 occasions.

a) Sept. 2 and Sept. 3 (week 11)

b) Sept. 30 and Oct. 1 (week 15)

c) Nov. 11 and Nov. 12 (week 21)

Points depicting AR, H_2 evolution and N_2 fixation data represent the means of 10 replicate assays and vertical bars represent 95% confidence intervals. For clarity only half the interval may be shown or small intervals omitted. RE and the A/N ratio are shown for each sampling occasion.


a
C_2H_4, H_2 or N_2 (μ mol h^{-1} $plant^{-1}$)
RE 0·42 0·44 0·42 0·46 0·43
A_N 2·25 2·36 2·13 2·30 2·25
Air or soil temp. (°C)
soil
air
Irradiance (μE s^{-1} m^{-2}) (x100)
Time (h)
b
C_2H_4, H_2 or N_2 (μ mol h^{-1} $plant^{-1}$)
RE 0·33 0·38 0·31 0·28 0·30
A_N 2·10 2·22 2·12 1·98 2·08
Air or soil temp. (°C)
soil
air
Irradiance (μE s^{-1} m^{-2}) (x1000)
Time (h)
c
C_2H_4, H_2 or N_2 (μ mol h^{-1} $plant^{-1}$)
RE 0·60 0·59 0·60 0·62 0·66
A_N 2·24 2·38 2·23 2·20 2·28
Air or soil temp. (°C)
soil
air
Irradiance (μE s^{-1} m^{-2}) (x1000)
1200 1800 2400 0600 1200
Time (h)

ln sN in 1980 did not reach significance ($p>0.05$), it did so ($p<0.001$) in 1981.

Diurnal variation

On all three occasions on which diurnal measurements were taken, rates of AR, H_2 evolution and N_2 fixation declined at almost a linear rate from the mid-day sampling until the 0600 h sampling the following morning, and rose again to their original activities by mid-day (Figures 17a, 17b & 17c). Although there were some differences in absolute values and amplitude of variation in both soil and air temperatures on the three occasions, the diurnal variations in rates of AR, H_2 evolution and N_2 fixation were similar. Integration of the areas under the N_2-fixation curves in Figures 17a, 17b and 17c revealed that the mean daily rate of N_2 fixation was over-estimated by 25, 24 and 26% (average 25%) respectively if the mean value of the two consecutive mid-day values was used in each case.

A significant finding from the studies of diurnal variation was the lack of diurnal variation in both RE and A/N ratios (Figures 18a, b and c). No significant differences ($p>0.05$) in either the RE or A/N ratios could be demonstrated between sampling occasions within each 24-h period.

Partitioning of nitrogen sources

In 1980, the concentration of nitrogen in both tops and roots declined over most of the season (Figure 18a) but total plant nitrogen content increased until week 20 and then declined (Figure 18b).

Figure 18c depicts total plant nitrogen and cumulative estimates of plant nitrogen derived from the atmosphere and based on measurements of $^{15}N_2$ fixation. The bottom curve assumes that the rates of N_2 fixation measured at mid-day over-estimated the mean daily rate by 25%.

The proportion of plant nitrogen derived from N_2 fixation increased steadily up until week 14 when maximum rates of N_2 fixation were recorded, and at this stage the proportion was *c.* 73%. Thereafter it declined until at week 20, when maximum plant nitrogen was recorded, the proportion was only 61%.

Figures 19a, 19b and 19c, depict similar data for the 1981 season when similar trends occurred. In this case, the maximum percentage of total plant nitrogen derived from N_2 fixation occurred at week 17 (83%) and declined to 64% before total plant nitrogen declined (week 21). The author considers that the value of 83% of plant nitrogen derived from N_2 fixation is an over-estimation because it is obvious from Figure 18c that, either the estimate of the amount of N_2 fixed between weeks 15 and 17 is too high, or the nitrogen content in the plant at week 17 is undervalued. Otherwise, between weeks 15 and 17, more N_2 was fixed than total nitrogen accumulated in the plant, which is impossible unless a major loss of nitrogen was incurred by the plant. Although losses of nitrogen have been recorded in narrow-leafed lupins and other plant species during the growing season, the losses always occurred much later in the season, *i.e.* between flowering and maturity. This is further discussed in Section 6.9.2, p. 151. No apparent pre-flowering losses of nitrogen were recorded during the 1980 experiment.

Concentrations of mineral nitrogen in the soil

Relatively high concentrations of mineral nitrogen in the soil were recorded throughout the 1981 growing season (Figure 19d). The greater portion of this nitrogen was in the form of NH_4^+-N rather than NO_3^--N and occurred predominantly in the A1 horizon of the soil (Figure 20a).

The fluctuations in NH_4^+-N paralleled the changes in water content of the soil (Figures 20a and 20b). Peaks in both NH_4^+-N and NO_3^--N in the soil also followed periods of elevated temperatures during October and November (Figures 19a and 19c).

Seed yields

Considerable floral abortion due to hot dry conditions occurred in 1980 resulting in low seed yield. This low seed yield, and hence low amount of nitrogen accumulated in the grain of the lupin plant in 1980, has important ramifications for the nitrogen balance in the soil-plant system in this year. This is discussed in more detail in Section 6.9.2, p. 151. Seed yields for 1980 and 1981 were 1.32 and 2.56 t ha^{-1} respectively.

Figure 18

Nitrogen concentration, content and accumulated amounts fixed in Marri lupins during 1980 at Coonalpyn

a) and b) Nitrogen concentration (a) and nitrogen content (b) plotted against time. Points represent the mean of 12 replicate determinations and vertical bars represent 95% confidence intervals; for clarity only half intervals may be shown or small intervals omitted.

Tops ●—● Roots ■—■

c) Comparison of total plant nitrogen determined by kjeldahl analysis (●—●) with cumulative estimates of N_2 fixation (based on ^{15}N measurements) assuming no diurnal variation (■—■), or that the mid-day estimates of N_2 fixation over-estimated the mean daily rate by 25% (▲—▲). F denotes the onset of flowering.

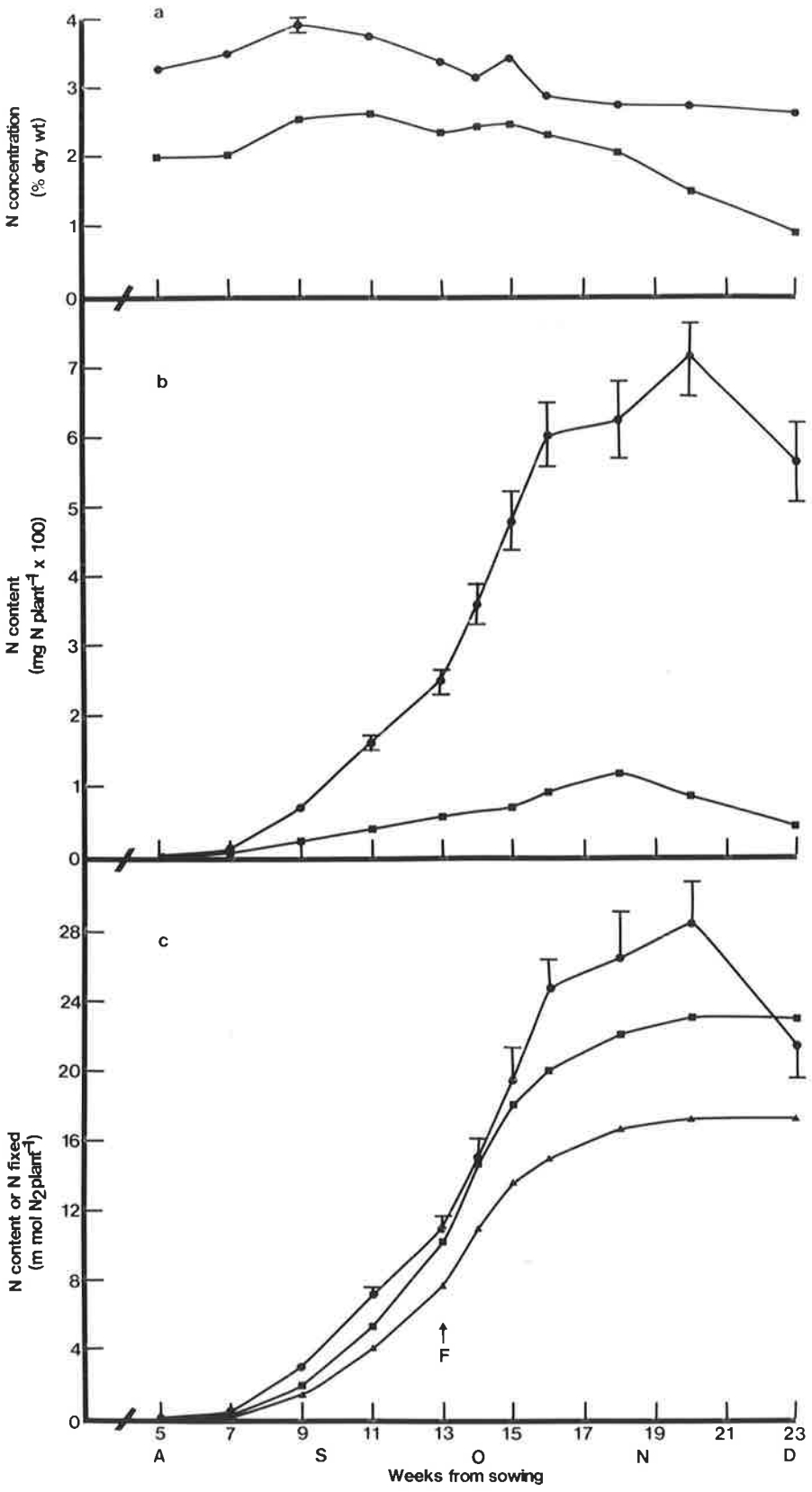

a
b
c
N concentration
(% dry wt)
N content
(mg N plant^{-1} x 100)
N content or N fixed
(m mol N2plant^{-1})
F
5
7
9
11
13
15
17
19
21
23
A
S
O
N
D
Weeks from sowing

Figure 19

Nitrogen concentration, content and accumulated amounts fixed in Illyarrie lupins during 1981 at Coonalpyn

a) and b) Nitrogen concentration (a) and nitrogen content (b) plotted against time. Points represent the mean of 12 replicate determinations and vertical bars represent 95% confidence intervals; for clarity only half intervals may be shown or small intervals omitted.

Tops ●—● Roots ■—■

c) Comparison of total plant nitrogen determined by kjeldahl analysis (●—●) with cumulative estimates of N_2 fixation (based on ^{15}N measurements) assuming no diurnal variation (■—■), or that the mid-day estimates of N_2 fixation over-estimated the mean daily rate by 25% (▲—▲).

d) Soil mineral nitrogen plotted against time. Points represent the mean of 30 (3 x 10 blocks) samples.

A1 horizon ●—● A2 horizon ■—■ B1 horizon ▲—▲

F denotes the onset of flowering.

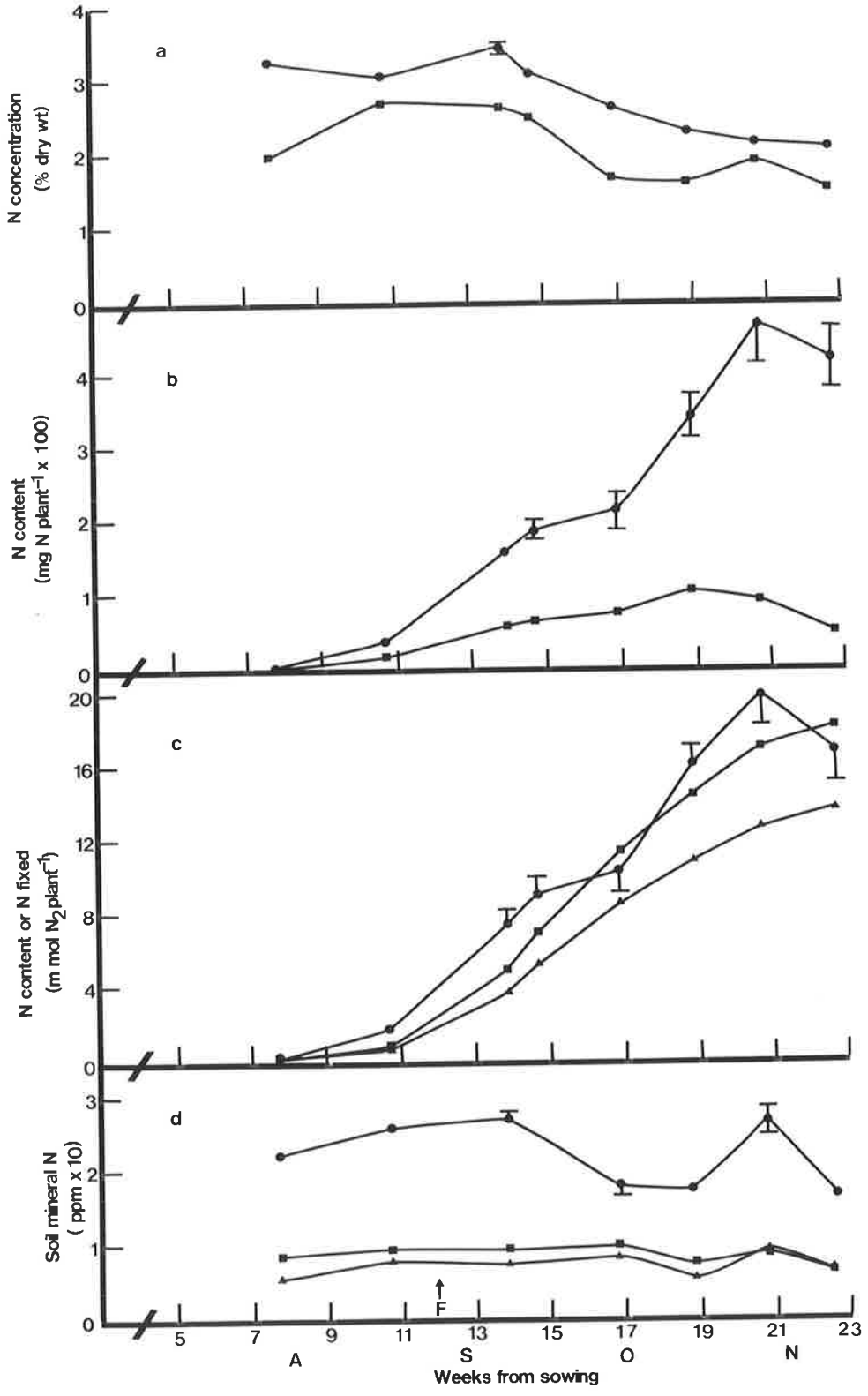

a
N concentration
(% dry wt)
b
N content
(mg N plant^{-1} x 100)
c
N content or N fixed
(m mol N_2 plant^{-1})
d
Soil mineral N
(ppm x 10)
F
5
7
9
11
13
15
17
19
21
23
A
S
O
N
Weeks from sowing

Figure 20

Soil mineral nitrogen and meteorological data during 1981 at Coonalpyn

a) Soil mineral nitrogen plotted against time. Points represent the means of 30 (3 x 10 blocks) samples. Vertical columns represent 95% confidence intervals. For clarity only half intervals may be shown or small intervals omitted.

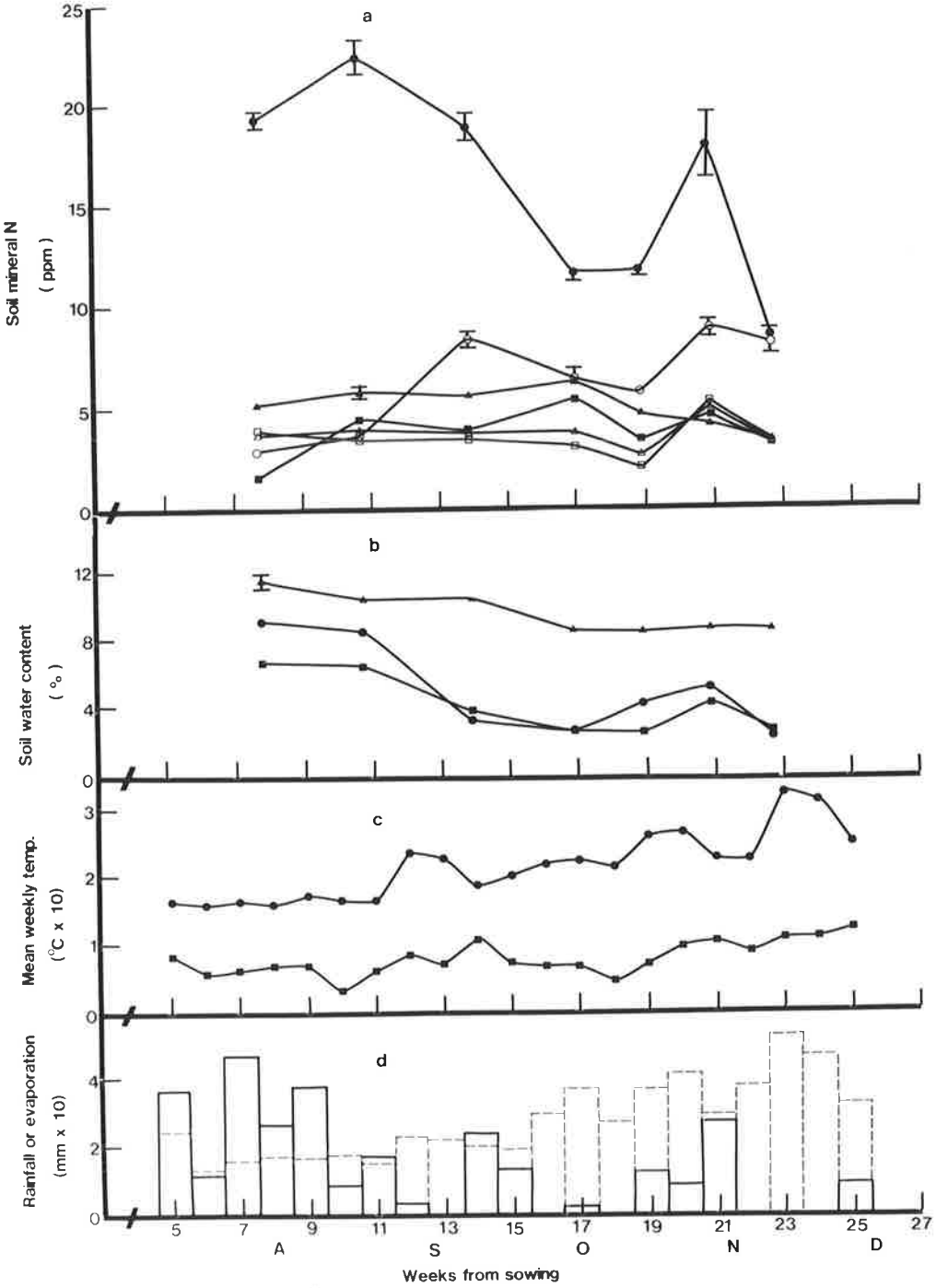
	A1 horizon	A2 horizon	B1 horizon
NH_4-N	●—●	■—■	▲—▲
NO_3-N	○—○	□—□	△—△

b) Soil water content plotted against time.

A1 horizon ●—● A2 horizon ■—■ B1 horizon ▲—▲

c) Mean weekly maximum (●—●) and minimum (■—■) temperatures during the growing season.

d) Weekly rainfall (—) and evaporation (-----) during the growing season.


a
Soil mineral N
(ppm)
25
20
15
10
5
0
b
Soil water content
(%)
12
8
4
0
c
Mean weekly temp.
(°C x 10)
3
2
1
0
d
Rainfall or evaporation
(mm x 10)
4
2
0
5
7
9
11
13
15
17
19
21
23
25
27
A
S
O
N
D
Weeks from sowing

6. DISCUSSION

6. DISCUSSION

6.1 Seasonal profiles of nitrogenase activity

Rates of AR have been monitored throughout the growth cycle of most agriculturally-important grain legume crops with maximum rates per plant and per nodule weight usually occurring shortly after flowering or during early pod-fill (Hardy *et al.* 1971, 1973; Mague and Burris 1972; Hobbs and Mahon 1982).

Trinick *et al.* (1976), Farrington *et al.* (1977) and Herridge (1983) have monitored rates of AR throughout the life cycle of narrow-leafed lupins grown in the field. In the first case, the crop was irrigated and the rate of AR per plant remained relatively constant until shortly before the death of the plants. In the latter two cases (under rain-fed conditions), the rates of AR per plant showed a distinct peak after the onset of flowering and then a decrease during the pod-filling stage, coincident with water stress. These latter profiles were similar to those recorded in the two years of the field experiments reported in this thesis. Furthermore, the environmental conditions encountered during the field experiment of Farrington *et al.* (1977) were similar to those at Coonalpyn (Greenwood *et al.* 1975) and the range in absolute rates of AR recorded at the two locations was also similar.

A decline in sA with plant ontogeny, similar to that which occurred in the growth-chamber experiment, occurred in the irrigated crop of Trinick *et al.* (1976), and distinct peaks in activity, as occurred in the present field experiments, were also reported by Farrington *et al.* (1977).

It is clear that the specific nitrogenase activity of narrow-leafed lupins can be greatly affected by environmental factors influencing the whole plant at any time during plant ontogeny and that seasonal profiles of nitrogenase activity may vary accordingly.

The profile for rates of H_2 evolution per plant throughout ontogeny was similar to that for rates of AR in both the growth-chamber and field experiments. Similar results have been reported for *Pisum sativum* (Bethlenfalvay and Phillips 1977b) and for *Phaseolus vulgaris* (Bethlenfalvay and Phillips 1977c), although in the latter two studies, maximum rates of H_2 evolution preceded those of AR by approximately one week.

6.2 Diurnal variation in nitrogenase activity

The integration of the results of short-term assays over the growing season remains as a major obstacle to using such assays for computing the amounts of N_2 fixed by field-grown legumes during a season. Although fortnightly assays (with weekly assays during periods of rapid change in nitrogenase activity) may describe the general response curve for nitrogenase activity over time, considerable error may still be introduced by using measurements made on only one occasion in any given day because of diurnal variation in nitrogenase activity.

During these investigations, the mid-day value for the rate of N_2 fixation over-estimated the mean daily rate by approximately 25% on each of three occasions of measurement in 1981. However, it is likely that this estimate of 25% would have varied considerably in many 24-h periods during plant ontogeny. In a similar environment in Western Australia, Halliday (1975) recorded a similar amplitude in diurnal fluctuations of AR in *L. angustifolius* in five experiments although the amplitude of the fluctuations in AR tended to decrease late in the season. Herridge (1983) calculated that the rate of AR at 1300 h was approximately 38% above the mean daily rate in his experiments with field-grown *L. angustifolius*. However, the difference between minimum and maximum soil temperatures was

greater in Herridge's environment than in either that of the author or of Halliday.

The effect of temperature on nitrogenase activity in *L. angustifolius* is similar to that of most other temperate legumes with a broad optimum for activity between 17°C and 30°C (Halliday 1975). Soil temperature *per se*, if outside this range, will be a major factor in generating diurnal variation in nitrogenase activity. In Halliday's studies, the fluctuations in AR generally paralleled the changes in soil temperature, although it was clear that this was not the case on several occasions.

Halliday concluded that the direct effect of temperature on the nodules was not the sole determinant of diurnal variation in nitrogenase activity in lupins. Trinick *et al.* (1976) found no diurnal variation in narrow-leafed lupins if the assay temperature was constant, and this result is consistent with the absence of any short-term effect of decapitating the plant, recorded both in their studies and in the present investigations. The assumption implicit here is that the lupin nodules contain sufficient carbohydrate reserves to maintain the same level of nitrogenase activity during the course of the assays.

Other factors which may alter the supply of carbohydrate to the nodule such as irradiance, have been implicated in causing diurnal variation in legume nodules. Minchin and Pate (1974) reported higher rates of AR during the night period in *Pisum sativum* despite lower temperatures during this period. These workers point out that this is possible provided the temperature of the night period is optimal for efficient use of carbohydrate, and that sufficient carbohydrate has been built up in the nodule by the end of the previous photoperiod.

In reviewing the subject of diurnal functioning of nodules, Minchin *et al.* (1981) state: "clearly, differences in plant morphology and phenology and variations in light intensity, air and soil temperatures, humidity and plant water status are important features in determining the diurnal pattern of N_2 fixation".

In contrast to the numerous reports in the literature of diurnal variation in AR in the legume symbiosis, direct measurements of N_2 fixation at given intervals throughout a 24-h period are lacking. In the field experiments reported in this thesis, both H_2 evolution and N_2 fixation exhibited a diurnal pattern, but there was no significant ($p>0.05$) change in the A/N or H/N ratios within the 24-h periods.

These results are in contrast to those of Rainbird *et al.* (1983) who found that the diurnal fluctuation in nitrogenase activity (as measured by AR) in *Vigna unguiculata* was almost entirely due to changes in the rate of H_2 evolution. The rate of N_2 fixation was not significantly different between the day and night despite a 10°C change in temperature. The lack of diurnal variation in A/N and H/N ratios in the present investigations is discussed in more detail below (p. 139).

6.3 Hydrogenase activity

The observed changes in RE in lupins in both the growth-chamber and field experiments cannot be attributed to variable expression of uptake hydrogenase activity during plant ontogeny, as recorded by Bethlenfalvay and Phillips (1979) for *Pisum sativum*. On no occasion during any experiment undertaken in the present study was there H_2 uptake that could be attributed to anything but micro-flora in the soil around the plant roots. If an uptake hydrogenase was functioning, uptake of exogenously-

supplied H_2 would have been observed in the presence of 0.15 atm C_2H_2 when nitrogenase-catalysed H_2 evolution was inhibited.

Although C_2H_2 has been reported to inhibit hydrogenase activity in free-living N_2-fixing bacteria (Smith *et al.* 1976), it did not inhibit uptake hydrogenase activity by the *P. sativum* x *R. leguminosarum* symbiosis in the experiments of Bethlenfalvay and Phillips (1979) and has not inhibited H_2 uptake in a range of other legume symbionts (A.H. Gibson, pers. comm.). It is unlikely therefore, that the lack of H_2 uptake in the presence of C_2H_2 was due to inhibition by C_2H_2 of an otherwise functional hydrogenase.

Further evidence for the absence of an uptake hydrogenase was the consistent finding of equal rates of H_2 evolution under Ar/O_2 and AR on all occasions of measurement during the present investigations (Schubert and Evans 1976; Ruiz-Argueso *et al.* 1978, 1979b).

To the author's knowledge, there are no published reports of an uptake hydrogenase functioning in any *L. angustifolius* x *R. lupini* symbiosis. However, A.H. Gibson (pers. comm.) has isolated two H_2 uptake (Hup^+) strains from *R. lupini* and *L. arboreus* which gave RE values (defined as 1-H/A) greater than 0.90 at 4 weeks from sowing when screened with 5 *L. angustifolius* cultivars. There is evidence (A.H. Gibson, pers. comm.) that in Hup^- symbioses, RE is affected by the *Rhizobium* strain, in addition to the changes with plant ontogeny that have been observed during the present investigations. Possible reasons for changes in RE in a single symbiosis, and the implications for the measurement of N_2 fixation are further discussed below (p. 140).

6.4 The relationships between sA, sH and sN

A significant feature of the results from both the growth-chamber and the field experiments was the occurrence of demonstrable relationships

between sA and sN, and sH and sN. The A/N ratio has been reported to vary during ontogeny of a given symbiotic association (*e.g.* Mague and Burris 1972; Sprent and Bradford 1977) but, to the author's knowledge, no rationale has been proposed and no functional relationship has been demonstrated between changes in the A/N ratio in a particular legume symbiosis and any physiological processes occurring in the intact nodule or host plant. In contrast, the kinetics of substrate reduction by nitrogenase in cell-free extracts has been shown to depend on several factors such as ATP concentration, pH, temperature and the ratio of the component proteins of the enzyme (Silverstein and Bulen 1970; Davis *et al.* 1975; Thorneley and Eady 1977; Hageman and Burris 1980).

TABLE 6

Regression parameters for sA and sH on ln sN

Experiment	Growth chamber		Field 1980		Field 1981	
Cultivar	Illyarrie		Marri		Illyarrie	
Regression	sA	sH	sA	sH	sA	sH
a	-298.5	-242.6	-154.6	-145.0	-154.8	-137.3
b	147.5	108.4	77.2	62.3	67.1	52.5
r^2	0.99	0.98	0.99	0.91	0.99	0.99
h	$p < 0.01$		$p > 0.05$		$p < 0.001$	

a and b are the intercept and slope respectively for the general regression equation $y = a + b (\ln x)$. r is the correlation coefficient for the two variables ln x and y, and h is the statistical significance of the difference in b for the two regressions sA on ln sN, and sH on ln sN.

Table 6 summarises the regression parameters for sA and sH on ln sN for the three sets of data plotted in Figures 8c, 15c and 16c (pp. 99, 115 and 116 respectively). All relationships between sA and ln sN, and sH and ln sN were linear and significant ($p<0.001$).

The relatively close agreement in both the slope and intercept for the respective regressions of sA and sH on ln sN in the two sets of field data may be coincidental, although it could reflect a general robust relationship for narrow-leafed lupins that may apply in a variety of field situations. The regression parameters derived from the growth-chamber data cannot be strictly compared with those derived from the field data because direct and simultaneous measurements of N_2 fixation using $^{15}N_2$ were made only in the field experiments.

There is a paucity of data in which AR in legumes has been directly calibrated with the uptake of ^{15}N-enriched N_2. Exceptions are studies by Stewart *et al.* (1967) and Mague and Burris (1972) of soybean; Hudd *et al.* (1980) of field bean (*Vicia faba*) and Saito *et al.* (1980) of common bean (*Phaseolus vulgaris*). There is no indication of any relationship between the A/N ratio and sN in these studies. In the latter two studies, where AR and N_2 fixation measurements were made on the same population of plants throughout plant ontogeny, there was little change in the A/N ratios despite a general decline in values of sN throughout the experiments.

To the author's knowledge, no similar studies of lupins have been published. However, the author's calculations on unpublished data of D.F. Herridge (pers. comm.) has provided further evidence of the relationship between sA and sN in narrow-leafed lupins. This data was obtained from a glasshouse study of *L. angustifolius* cv. Unicrop. AR assays were performed on the plants using a NRA system and N_2 fixation was estimated from the increment in plant nitrogen. The plants received N-free nutrient solution and were grown in N-free sand culture. The regression of sA on ln

sN was significant ($p<0.05$, $r^2 = 0.83$) and the A/N ratio increased and sN declined throughout plant ontogeny. Similar changes in the A/N ratio and sN with plant ontogeny were evident in the growth-chamber experiment reported in this thesis (Figure 8b, p. 99).

6.5 Reasons for variable A/N ratios

The question arises as to why there should be relationships between sA and sN, and sH and sN in these investigations. It could be argued, using the data from the growth-chamber experiment, that the A/N and H/N ratios varied as a function of plant or nodule age because sA, sH and sN all progressively declined with time. However, as emphasised during presentation of the results, there were periods during the field experiments (particularly in 1980) when sA, sH and sN all increased substantially despite increased nodule age and mass. Yet the relationships between sA and sN, and sH and sN were maintained. Furthermore, the increases in specific activities occurred during periods when accelerated growth rate of the plant tops foreshadowed accelerated growth rate of the nodule mass.

Hence it can be postulated that the capacity of the plants to grow rapidly generates a demand for nitrogen which, in the short term before the growth of nodule tissue can increase sufficiently, can be provided only by increased specific nitrogenase activity. Silsbury (1981) showed that the rate of N_2 fixation by a subterranean clover sward is closely related to the growth rate of the sward. He confirmed the now widely recognised relationship between assimilate supply and N_2 fixation in whole plants (*e.g.* Hardy and Havelka 1975; Brun 1976; Bethlenfalvay and Phillips 1977a) and suggested that the specific rate of N_2 fixation by the nodules of a plant may be determined by the demand for nitrogen by the plant.

In the growth-chamber experiment, uniform conditions for growth occurred throughout the experiment and growth of the nodules closely paralleled that of the whole plant. This resulted in any demand for increased nitrogen by the plant being met by increased nodule mass or mobilization of nitrogen in the plant rather than increased nitrogenase activity per unit nodule mass. In the field experiments, variation in environmental conditions resulted in periods of high demand for nitrogen which could not be met by a sufficiently high growth rate of nodule mass in the short term. Hence it is postulated that, in such circumstances, the plant increases the specific activity of its nodules in response to the increased demand for nitrogen for growth processes.

It is important to indicate here that specific nitrogenase activity has hitherto been defined as the activity of nitrogenase per unit dry weight of nodule and not the activity per unit of enzyme present. Because the minimum interval of time between the occasions of field sampling in these investigations was a week (diurnal studies excluded), one is unable to apportion changes in specific activity to variation in activity of the same amount of enzyme or to changes in the absolute amounts of enzyme present per unit dry weight of nodule. Regulation of nitrogenase by changes in enzyme concentration has been shown to occur by repression of nitrogenase synthesis or by degradation of pre-existing enzyme (Eady 1981). A second and more rapid method of regulation is the modulation of potential activity when nitrogenase is derepressed. Factors which have been implicated in this form of regulation are the MgATP/ADP ratio, the maintenance of O_2 and membrane potential and interconversion forms of nitrogenase with different activity (Eady 1981). Possible mechanisms whereby the plant may regulate the specific activity of the nitrogenase complex is discussed further on p. 137.

While this report was being prepared, some very pertinent research was published by Minchin *et al.* (1983). They observed that nitrogenase activity in attached nodulated roots of a wide range of legumes declined rapidly in the presence of C_2H_2 with a concurrent decrease in respiration. Results for white clover are shown in Figure 21a. The decrease began within a few minutes of exposure to C_2H_2 and continued for 30 to 60 minutes before a new steady state was attained. A similar decline in H_2 evolution also occurred when the N_2 was replaced with Ar (Figure 21b).

In nearly all cases in which the AR technique has been used (which amounts to considerable research input world-wide), including the cases reported in the present investigations, the rate of production of C_2H_4 has been calculated from the cumulative rate of increase in concentration of C_2H_4. According to Minchin *et al.* (1983), this technique may mask instantaneous changes in rates of AR. The fact that the peak in nitrogenase activity probably occurs during the lag phase in a conventional assay would further tend to mask any such phenomenon in the normally-used system. Minchin *et al.* (1983) used a flow-through gas system in which the rate of production of C_2H_4 (or H_2) is measured directly rather than by accumulation. $^{15}N_2$ assays indicate that it is the maximum, rather than the mean or final rate of AR which best represents the pre-assay rate of nitrogenase activity.

L. angustifolius was not included in the survey undertaken by Minchin *et al.* (1983) but a decline in nitrogenase activity was induced by C_2H_2 and Ar/O_2 in *L. albus* (white lupin). The author has since confirmed that an C_2H_2-induced decline does occur in *L. angustifolius* cv. Marri, inoculated with *R. lupini* WU 425 (Figure 22).

Figure 21

Rates of CO_2 efflux, AR and H_2 evolution in white clover according to Minchin *et al.* (1983)

a) Rates of respiratory CO_2 efflux (■—■) and AR (●—●).

b) Rates of respiratory CO_2 efflux (■—■) and H_2 evolution (▲—▲).

Assays conducted on attached roots of white clover (*Trifolium repens* L. cv. Blanca) nodulated by *Rhizobium* strain RCR 221.

Roots were exposed at time zero to continuous gas streams containing either a) 10% C_2H_2, 21% O_2, 68.9% N_2, 0.1% CO_2 or b) 78.9% Ar, 21% O_2, 0.1% CO_2.

Figure reproduced from Minchin *et al.* (1983).

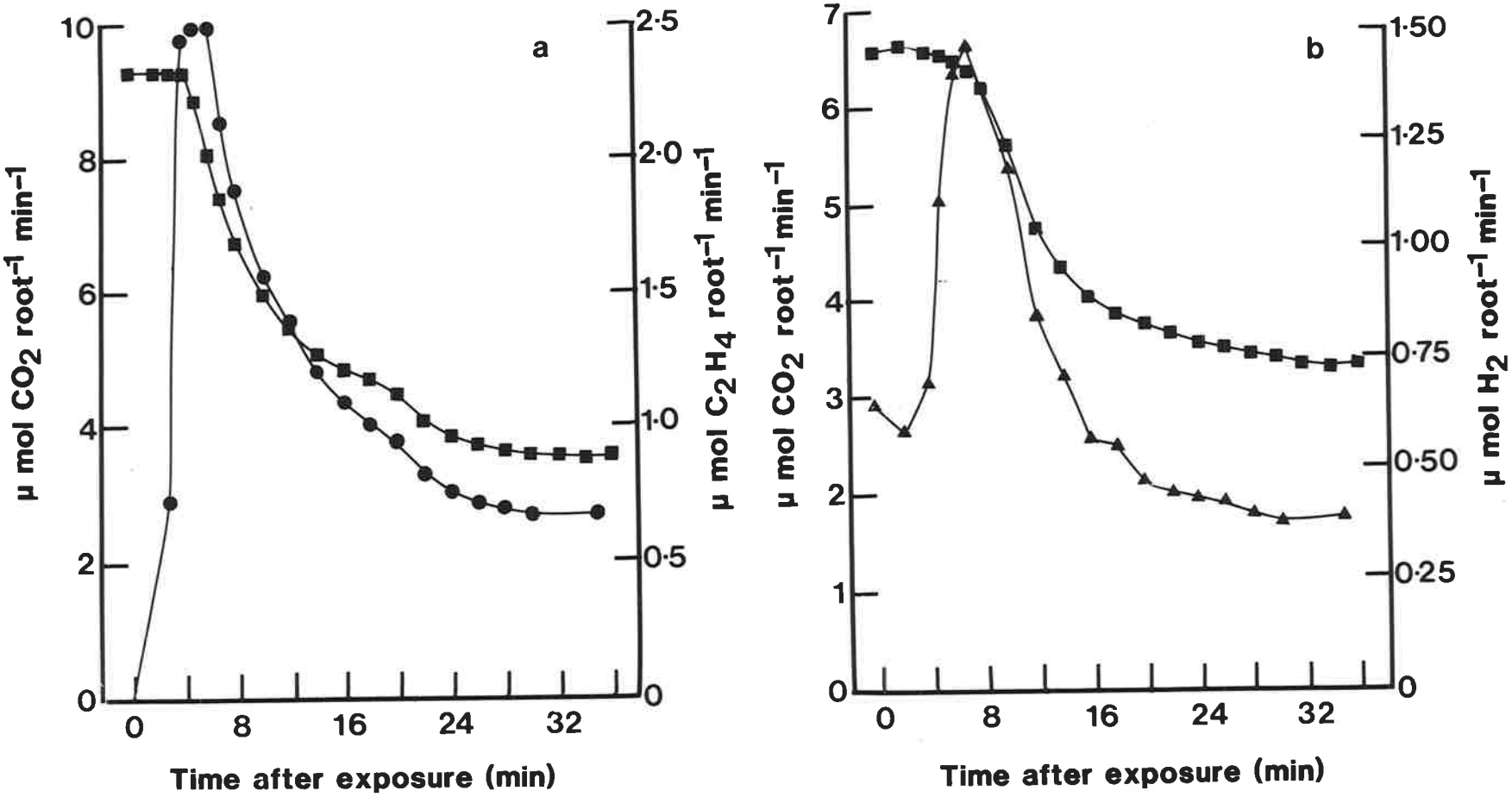

a
b
μ mol CO_2 root^{-1} min^{-1}
μ mol C_2H_4 root^{-1} min^{-1}
μ mol H_2 root^{-1} min^{-1}
Time after exposure (min)
Time after exposure (min)

Figure 22

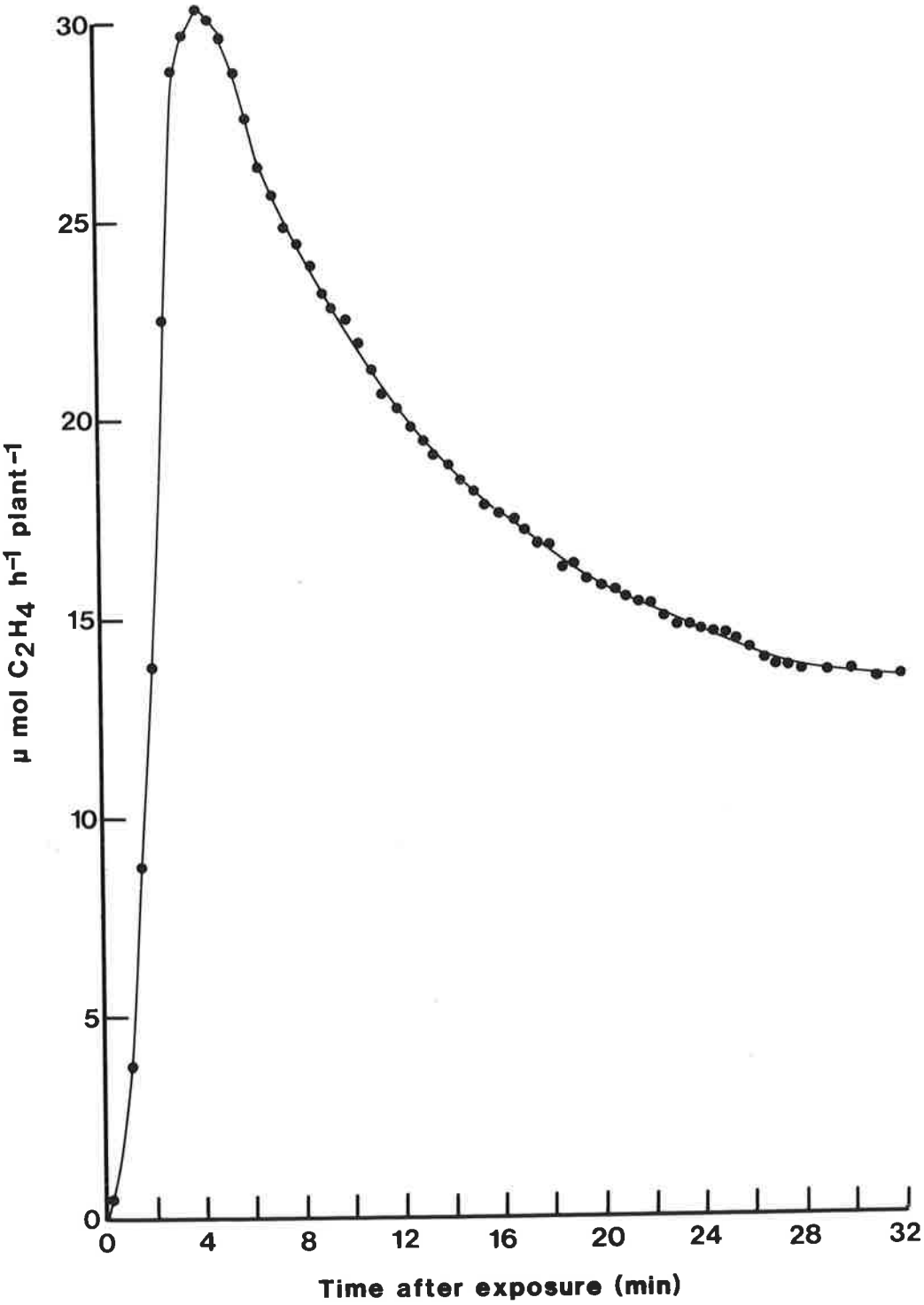
C_2H_2-induced decline in AR by Marri lupins using a flow-through assay system

Marri lupins, inoculated with *Rhizobium lupini* strain WU 425 were grown in sterilized soil in a naturally-lit glasshouse. The plants were detopped prior to the onset of flowering when they were six weeks old. Soil was shaken from the nodulated roots and the latter assayed.

Assays were performed using the jar system described on p. 56, modified to allow a continuous stream of gas to pass through the jar and then through a sampling tube prior to venting.

The gas stream comprised 15% C_2H_2, 20% O_2 and 65% N_2 fed by a gas proportioner at a flow rate of 380 ml min^{-1}. Samples of effluent gas were taken at 30-s intervals using 1 ml plastic disposible syringes, and the C_2H_4 concentration subsequently determined by gas chromatography.

The assays were repeated on five separate occasions with similar results. Points on the graph represent AR by nodulated roots from 5 lupin plants.


30
25
20
15
10
5
0
μ mol C2H4 h-1 plant-1
0
4
8
12
16
20
24
28
32
Time after exposure (min)

The phenomenon explains the low A/N ratios obtained during these investigations and provides an explanation for the relationships observed between the A/N ratio and sN. The average value of A/N obtained from measurements made on 15 separate occasions of assay (each replicated at least 10 times and including simultaneous measurements of AR and $^{15}N_2$ reduction) during the present investigations was 2.46 and the average RE (defined as 1-H/A and measured on 17 occasions) was 0.47. On the assumption that the electron flux allocated to $^{15}N_2$ reduction equals the flux to AR less the flux to H_2 evolution (in air), the A/N ratio should have averaged 6.38. Obviously, the above stoichiometry did not apply in these experiments and a decrease in nitrogenase activity in the presence of C_2H_2 is the likely cause.

That the rates of H_2 evolution under Ar/O_2 were equal to the rates of AR in the present investigations is of particular interest because the decline in nitrogenase activity observed by Minchin *et al.* (1983) in the presence of C_2H_2 also occurred in the presence of Ar/O_2 in their experiments. These workers suggest that the causal factor for the decline in nitrogenase activity is probably associated with the cessation of ammonia production as this is a feature common to both assay situations. However, one cannot rule out the possibility that some modification of the nitrogenase enzyme complex occurs when N_2 is not a substrate being reduced, resulting in altered substrate kinetics.

Very recent unpublished results of M.I. Minguez, J.F. Witty, F.R. Minchin and J.E. Sheehy (pers. comm.) indicate that an increase in resistance to the diffusion of O_2 in the nodule is associated with the decline in nitrogenase activity in the presence of C_2H_2 or Ar/O_2.

These workers postulate that those symbioses which exhibit an C_2H_2-induced decline in nitrogenase activity may protect their nitrogenase from inactivation by O_2 by increasing the diffusive resistance of certain membranes to O_2. Otherwise, under conditions of a constant rate of O_2 diffusion into the nodule, the sudden decline in nitrogenase activity, caused by the introduction of C_2H_2, would result in an intolerable increase in the internal O_2 concentration. The same workers have also found that, by pre-exposing white clover nodules to various O_2 concentrations for 45 minutes, the magnitude of the acetylene-induced decline in nitrogenase activity in this symbiosis depended upon the O_2 concentration to which the nodules had adjusted. It was greatest for nodules adjusted to 21% O_2 and least for those adjusted to 80% O_2.

On the basis of existing published knowledge of nitrogenase function and ammonia assimilation by a legume plant, it is difficult to propose a mechanism for the occurrence of the C_2H_2-induced decline and any link to the permeability to O_2 of membranes within the nodule. Based on the principle of supply and demand one would expect the absence of ammonia to increase nitrogenase activity rather than cause a decline. However, studies with crude extracts and purified components of nitrogenase have provided little evidence for feedback control of nitrogenase by ammonia or any of its assimilation products (Eady 1981).

Because the C_2H_2-induced decline occurs rapidly (within 3-4 minutes from the introduction of C_2H_2), the mode of nitrogenase control cannot be by changes in the intracellular enzyme concentrations but rather by modulation of potential nitrogenase activity. In intact bacteroids, potential nitrogenase activity may be regulated by the supply of ATP and the ADP/ATP ratio (Appleby *et al.* 1974; Laane *et al.* 1978), by the

concentration of O_2 (Laane *et al.* 1978) or by the supply of reducing equivalents (Laane *et al.* 1979). These modes of regulation may interact in a complex manner *viz.* at low rates of O_2 supply, nitrogenase activity may be limited by ATP; at saturating concentrations of ATP, generation of reducing equivalents may become limiting; and at very high rates of O_2 supply, dissolved O_2 may be present and protective mechanisms result in a switch-off of nitrogenase activity (Laane *et al.* 1978; Eady 1981).

The excretion of fixed ammonia from the bacteroids is driven by respiration which maintains the cytoplasmic membrane in an energized state (Laane *et al.* 1980). A sudden increase in internal O_2 concentration resulting from a decrease in respiration and caused by the absence of ammonia to be excreted across the cytoplasmic membrane, may cause a partial switch-off of nitrogenase activity. Alternatively, the excretion of ammonia across the cytoplasmic membrane may be linked to the latter's diffusive resistance to O_2 with a low concentration gradient across the membrane increasing the resistance of the membrane to the diffusion of O_2. In this case, the C_2H_2-induced decline may result from a decrease in internal concentration of O_2 and thereby a decrease in ATP concentration or a decrease in reducing equivalents for nitrogenase activity.

However, the mechanism whereby the lack of ammonia production, if indeed this is the causal factor, is linked to the modulation of nitrogenase activity remains to be determined.

Nevertheless, the phenomenon *per se* of an C_2H_2-induced decline and an associated change in diffusive resistance of certain membranes to O_2, as suggested by M.I. Minguez and co-workers, provides a plausible explanation for the relationship between the A/N ratio and sN observed in the experiments reported in this thesis. It can be postulated that, in

circumstances in which the sN of lupin nodules is high, the nodules adjust to a low O_2 concentration maintained by the high rate of respiration required to drive nitrogenase at this level of activity. Under these conditions, the diffusive resistance of nodule membranes to O_2 would be low and the introduction of C_2H_2 at this time may result in a large proportional decline in nitrogenase activity caused by a rapid increase in the resistance of certain membranes to the diffusion of O_2 or by other mechanisms such as discussed above. This postulate provides for a greater proportional decline in nitrogenase activity at high values of sN which is manifested in low A/N ratios.

However, the absence of any diurnal variation in the A/N ratio reported here suggests that the absolute value of sN is not the sole determinant of the magnitude the C_2H_2-induced decline. Diurnal changes in nitrogenase activity are relatively slow compared to the changes in activity brought about by the introduction of C_2H_2, and diurnal changes in the resistance to O_2 of membranes within the nodule may occur in parallel with diurnal variation in nitrogenase activity.

The absence of an C_2H_2-induced decline in nodulated roots, which had been washed prior to assay, may account for the equality in electron flux to C_2H_2 and the sum of that to H^+ and N_2 observed in the NRA system in Experiment 1 (p. 81). It can be postulated, that under circumstances where diffusion of O_2 to the bacteroids is already impeded by a film of water on the surface of the nodule, internal changes in permeability of membranes associated with the inhibition of ammonia production by C_2H_2 would be of little consequence. Hence it is feasible that no C_2H_2-induced decline in nitrogenase activity occurred in Experiment 1 when the nodulated roots were washed prior to assay, and therefore the theoretical stoichiometry (as noted above) was observed.

6.6 Reasons for variable H/N ratios and RE

The data from the experiments reported in this thesis clearly show that the partitioning of electrons by nitrogenase between N_2 fixation and H_2 evolution in *L. angustifolius* can vary considerably. Furthermore, evidence has been produced that the H/N ratio varies as an inverse function of sN.

Most recent studies which have examined H_2 evolution (in air) from legume symbionts have compared these rates with rates of AR and/or rates of H_2 evolution in Ar/O_2 and not with direct measurements of N_2 fixation. The term RE has been defined as (1-H/A) or (1-H/R), where R represents H_2 evolution in Ar/O_2, and this term has been equated with (1 - the electron allocation to H_2). It has been assumed in these cases that rates of AR, or H_2 evolution in Ar/O_2 represent the total electron flux through nitrogenase in air and that this flux remains unaltered by the treatments applied. It is important to emphasize here that the occurrence of an C_2H_2-induced (or Ar/O_2-induced) decline as reported by Minchin *et al.* (1983) would obviously lower the RE as defined above. Variation in the size of this decline would cause a corresponding variation in RE. It was noted in the review (p. 26) that no consistent relationships could be demonstrated between changes in RE and factors affecting the symbioses through the host plant and the reason for this may be the occurrence of an C_2H_2-induced decline in many symbioses.

Caution needs now to be exercised in interpreting much of the data published in relation to RE. A very recent example is work reported by Edie and Phillips (1983) in which the RE in *Pisum sativum* increased under conditions conducive to low carbohydrate supply (*i.e.* maintaining the plants for 20 h in the dark) and declined with increasing irradiance following reillumination. However, Minchin *et al.* (1983) reported that

P. sativum, inoculated with the same strain of *R. leguminosarum* (Strain 3740), exhibited a decline in nitrogenase activity induced by C_2H_2 or Ar/O_2. It can be shown that, if the decrease in the A/N ratio is greater than the decrease in the H/N ratio then the RE would appear to decrease despite the fact that the electron flow to N_2 fixation increases at the expense of H_2 evolution.

This point is illustrated in the data on RE presented in Figures 6d, 13c and 14c on pp. 96, 112 and 113 respectively. RE^s appear to increase rapidly with low and declining values of sN despite the curvilinear relationship between sH and sN (Figures 8b, 15b and 16b, pp. 99, 115 and 116 respectively) showing H_2 evolution increasing in relation to N_2 fixation at decreasing values of the latter.

Few studies have been reported where both H_2 evolution and $^{15}N_2$ incorporation have been measured simultaneously, as in the experiments reported in this thesis. Two exceptions are experiments reported by Saito *et al.* (1980) with *Phaseolus vulgaris* and Minchin *et al.* (1983) with *Pisum sativum*. In the former case, calculations based on the reported data revealed no obvious relationship between the H/N ratio (or the electron allocation coefficient to H_2 [*i.e.* H/(3 N + H)]) and sN. However, these workers reported high A/N ratios (7.3 - 8.3) and RE^s between 0.65 and 0.68. No mention is made in the paper as to the presence or otherwise of a functional uptake hydrogenase. It is therefore not clear whether the H_2 evolution data represent the true allocation of electrons by nitrogenase to H_2 evolution.

The data of Minchin *et al.* (1983) indicate that the allocation coefficient to H_2 (and therefore the H/N ratio) in *P. sativum* was inversely related to the irradiance supplied to the plants in the 48-h period prior to assays. On the assumption that sN was a direct function of

irradiance, these data are in accord with the inverse relationship between the H/N ratio and sN recorded in the present investigations.

Recent *in vitro* studies of nitrogenase by Hageman and Burris (1980) showed that decreasing concentrations of MgATP favoured H_2 evolution relative to N_2 fixation. Furthermore, unpublished experiments by H. Wassink and H. Haaker (pers. comm.), using intact *R. leguminosarum* bacteroids, demonstrated the same result.

Possible reasons can be advanced for H_2 evolution being favoured in preference to N_2 fixation when specific nitrogenase activity is low. In circumstances where the demand for nitrogen by the host plant is low, increased H_2 evolution may act as a 'relief valve', enabling nitrogenase to remain functional while reducing less nitrogen. Alternatively, when the requirement for nitrogen by the plant is high the efficiency of N_2 fixation is increased and less energy is used through the evolution of H_2.

Although a possible explanation for the relationship between sA and sN observed in the present investigations can be found in the C_2H_2-induced decline in nitrogenase activity, the relationship between sH and sN cannot be explained on the same basis. In an atmosphere of air, N_2 fixation proceeds simultaneously with H_2 evolution and the NH_3 formed is assimilated. It is difficult to account for the variable H/N ratios observed during the experiments unless some change occurs in the substrate kinetics of nitrogenase predisposing the enzyme more to the reduction of N_2 and less to H_2 evolution during periods of high N_2 fixation.

One can only speculate as to the mechanism whereby such changes could occur, but as indicated earlier in this discussion, considerable evidence now exists from *in vitro* experiments that conformational changes in

nitrogenase do occur in response to several factors such as ATP concentration, pH, temperature and the ratio of component proteins of the enzyme (Silverstein and Bullen 1970; Davis *et al.* 1975; Thorneley and Eady 1977; Hageman and Burris 1980). Recent unpublished work by H. Wassink and H. Haaker (pers. comm.) with *R. leguminosarum* bacteroids suggests that ADP may be the controlling factor influencing the electron allocation by nitrogenase to H^+ and N_2 *in vivo*. Furthermore, these same workers have shown that it is not the absolute flux of electrons through nitrogenase *per se* which influences the electron allocation to H^+ but rather the method whereby this flux is regulated.

In previous dicussion (p. 137) it was noted that nitrogenase activity in intact bacteroids could be limited by ATP and other factors such as the supply of reducing equivalents at saturating concentrations of ATP. According to H. Wassink and H. Haaker, only a decrease in ATP concentration (increase in the ADP/ATP ratio) influences electron allocation to H^+ and not other known regulators such as the supply of reducing equivalents. If this is so then it may explain why no diurnal variation in the H/N ratio was observed during these investigations. In lupins, diurnal variation appears to be predominantly determined by temperature, as discussed in 6.2 (p. 125) and the large reserves of carbohydrate stored in lupin nodules probably ensures an adequate supply of ATP during dark periods normally encountered in southern Australia. This is supported by the absence in the short term of any affect on nitrogenase activity by decapitation of the plant and by the lack of diurnal variation in nitrogenase activity when AR assays were performed at constant temperature (Trinick *et al.* 1976).

Thus although sN may decline during the dark period the concentration of ADP in the nodule may not alter significantly and hence the H/N ratio remains constant.

6.7 Stoichiometry of nitrogenase

The question arises as to whether variation in nitrogenase-catalysed H_2 evolution can account for variable A/N ratios when AR is measured in the absence of, or prior to, an C_2H_2-induced decline in nitrogenase activity. The data of Minchin *et al.* (1973) suggests that this is the case in white clover. Further detailed experiments in which simultaneous measurements of H_2 evolution, $^{15}N_2$ fixation and AR prior to the C_2H_2-induced decline would be required to validate such a stoichiometric relationship in narrow-leafed lupins.

The data from these investigations with lupins suggest that variation in nitrogenase-catalysed H_2 evolution cannot account for variable A/N ratios when AR is measured by the accumulation of C_2H_4 using the techniques described in this thesis. This is illustrated as follows.

The relationship between sA and sN, and sH and sN are described by the equations:

$$sA = a_1 + b_1 \ln sN \qquad (1)$$

$$sH = a_2 + b_2 \ln sN \qquad (2)$$

Provided $b_1 = b_2$ as demonstrated in Table 6, p. 129 in two sets of data ($p < 0.01$ and $p < 0.001$), then it is evident that (sA-sH) varies as a linear function of ln sN rather than sN.

Equations (1) and (2) can also be rearranged to show that sH is a linear function of sA as depicted in Figures 8a, 15a and 16a on pp. 99, 115 and 116 respectively.

i.e. rearranging equation (1)

$$\ln sN = (sA-a_1)/b_1$$

and substituting in equation (2)

$$sH = a_2 + b_2/b_1 \ (sA-a_1)$$

$$i.e.\ sH = (a_2 - a_1 b_2/b_1) + (b_2/b_1)\ sA \qquad (3)$$

It is interesting to determine the nature of the relationship between sH and sA should the latter be measured in the absence, or prior to, an C_2H_2-induced decline in nitrogenase activity and equate to the sum of electron flux to N_2 and H^+ in air.

If, under these conditions sA is represented by sAmax, then

$$sAmax = 3\ sN + sH \qquad (4)$$

Equation (2) can be rearranged to give

$$sN = e^{[(sH-a_2)/b_2]}$$

and substituting for sN in (4) gives

$$sAmax = sH + 3e^{[(sH-a_2)/b_2]} \qquad (5)$$

Equation (5) indicates that, under the assumptions noted above, sAmax is not linear in sH, but is dependent on the summation of a linear component (sH) and an exponential component $3e^{[(sH-a_2)/b_2]}$.

In practical terms, the linear component represents the change in sH which results directly from a change in nitrogenase activity. The exponential component represents the change in sH which results from the change in the allocation of electrons between H_2 evolution and N_2 reduction; the electron allocation varying also with nitrogenase activity.

Figure 23 illustrates equation (5) using mean values of a_2 and b_2 derived from the field experiments. It is clear that if the electron allocation to H_2 varies as a function of sN as described above (Equation 2) and variation in the A/N ratio is due to variation in sH, then rates of H_2 evolution cannot be linearly related to rates of AR as reported by some workers (Saito *et al.* 1980; Silsbury 1981) for other symbioses. The failure to account for an C_2H_2-induced decline in nitrogenase activity may have resulted in the linear relationships between

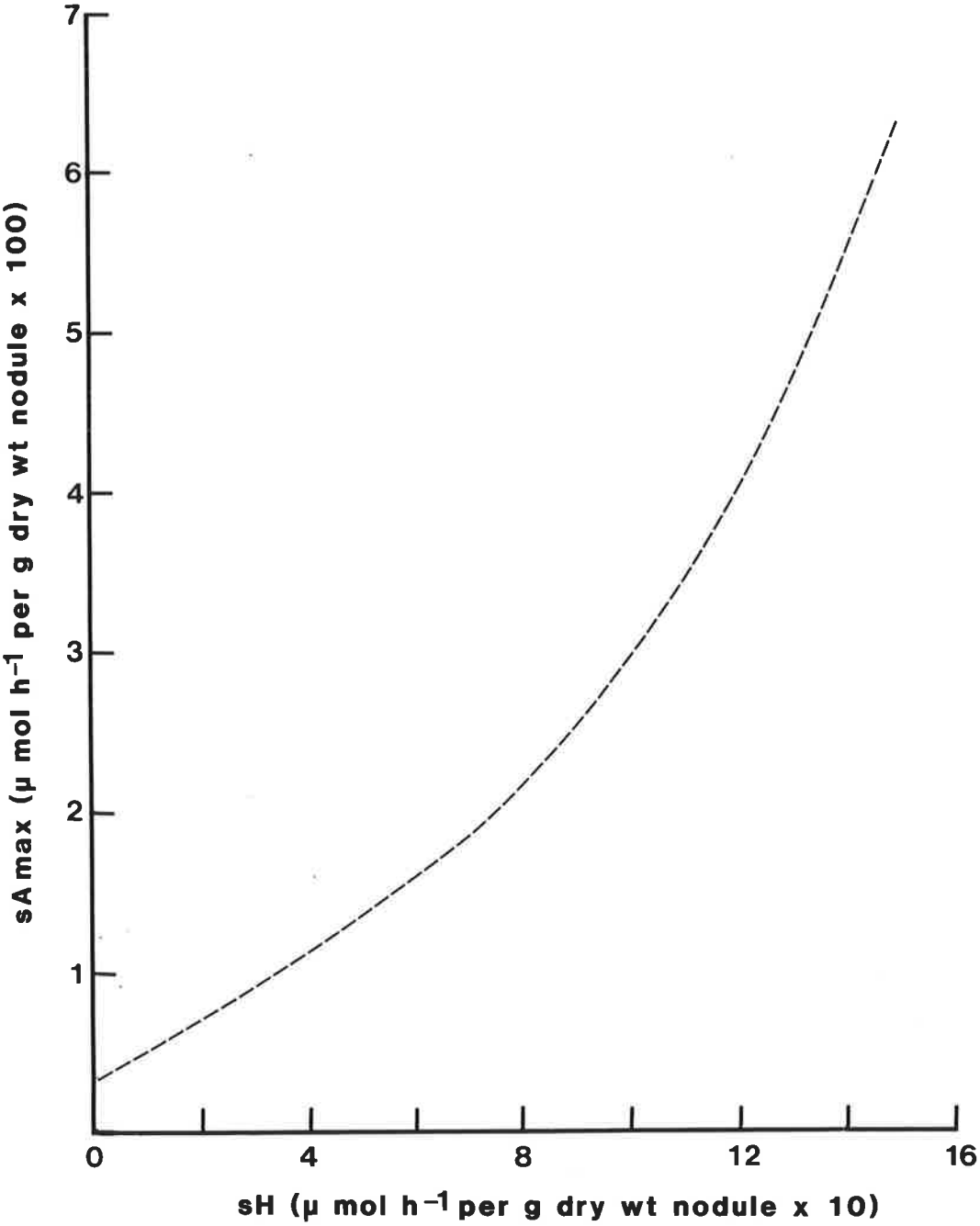
Figure 23

Relationship between sAmax and sH as described by the equation:

$$sAmax = sH + 3e^{[(sH-a_2)/b_2]}$$

$a_2 = -141.2$

$b_2 = 57.4$


7
6
5
4
3
2
1
0
4
8
12
16
sA max (μ mol h⁻¹ per g dry wt nodule x 100)
sH (μ mol h⁻¹ per g dry wt nodule x 10)

H_2 evolution and AR recorded by these workers and also in the experiments described in this thesis.

6.8 Application of variable A/N ratios

Data presented by Minchin *et al.* (1983) suggests that measurements of maximum rates of AR obtained using a flow-through assay system, and of rates of H_2 evolution in air, may reasonably predict rates of N_2 fixation in legume symbioses exhibiting an C_2H_2-induced decline in nitrogenase activity. However, a major drawback in the implementation of the flow-through technique in field studies in which quantitative measurements of N_2 fixation are required, may be the number of samples necessary to ensure that the maximum rate of AR is detected. Between 5 and 10 samples of gas may be required for each nodulated-root system being assayed. The number of samples required may be lower than this if the maximum rate of AR could be shown to occur after the same interval of time on all occasions of assay. Detailed investigations would be required to define the environmental conditions and the physiological status of the plant under which this may occur. Furthermore, separate measurements of H_2 evolution in air would be required and more elaborate equipment and procedures would be necessary to perform the flow-through technique in the field.

The experiments described in this thesis indicate that the relationship between sA and sN in narrow-leafed lupins may be usefully applied as a technique for quantifying rates of N_2 fixation over a season. The technique may be less arduous in the field than the flow-through technique but would require the routine use of ^{15}N-enriched N_2 gas for calibration purposes and the necessary laboratory facilities for measuring ^{15}N enrichments in air and plant tissue and the measurement of nodule weights.

As was noted earlier in the discussion (p. 130), the relatively close agreement in both the slope and intercept for the regressions of sA on ln sN in the two sets of field data suggests that a general robust relationship for narrow-leafed lupins may apply in a variety of field situations. Should this be the case, then the number of $^{15}N_2$ assays required for calibration purposes may well be smaller than used here, with the obvious savings in labour and expense. However, further studies of rates of N_2 fixation and AR in narrow-leafed lupins over a range of seasons and environments would be necessary before any such general relationship could be proposed.

6.9 Field estimates of N_2 fixation

The concentrations of mineral nitrogen in the A_1 horizon of the soil in the 1981 field experiment were relatively high for sandy soils. However, the concentrations were not unexpected in view of the immediate past history of the site. The nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*, are sensitive to acid conditions (Allison 1973) which probably explains the predominance of the NH_4^+ form of nitrogen (Figure 20a, p. 123). Although soil nitrogen was not measured in 1980, the fact that a lupin crop rather than a cereal crop had been grown the previous year may have resulted in some 'saving' of soil mineral nitrogen in that year. It is suggested therefore, that the lupins in both 1980 and 1981 had access to higher concentrations of mineral nitrogen in the soil during growth than the average commercial lupin crop grown on sandy soils in rotation with cereal crops and that a higher proportion of nitrogen in the former crops was derived from the soil.

However, the exact relationship between the presence of mineral nitrogen in the rooting medium and N_2 fixation in *L. angustifolius* is

uncertain. Gibson (1976) reported a stimulation of N_2 fixation in glasshouse-grown plants of *L. angustifolius* by early supplementation with 7mM KNO_3 for 14 days after sowing. Nitrogen assimilation by these plants was comparable to that of inoculated plants supplied with 7mM KNO_3 throughout the experiment (36 days). This indicates that early supplementation of nitrogen was more important than the continuous availability of mineral nitrogen. Unpublished results of P. Subramanian (pers. comm.) using hydroponic cultures, indicate that NH_4^+ nitrogen may have adverse effects, and low concentrations of NO_3^- nitrogen stimulatory effects, on N_2 fixation in *L. angustifolius*. If this is so then N_2 fixation would probably have been adversely affected in the 1980 and 1981 lupin crops because the NH_4^+ form of nitrogen was present in greater concentration in the soil, at least during the 1981 field experiment reported in this thesis.

Measurements of total nitrogen accumulation and N_2 fixation in both the 1980 and 1981 lupin crops (Figures 18c and 19c; pp. 121 and 122) showed that between 31 and 40% of the maximum nitrogen accumulated by the crops was derived from the soil and between 60 and 69% was derived from N_2 fixation.

Most reported estimates of N_2 fixation in field-grown grain legume crops have been confined to soybean (*Glycine max*). Estimates range between 15 and 160 kg ha^{-1} of nitrogen per annum, depending on the fertility and locality of the soil in which the crops were grown and on the method of determining the amounts of N_2 fixed (LaRue and Patterson 1981). There are few published estimates of N_2 fixation in other grain legume crops, particularly in crops grown in a Mediterranean-type environment characteristic of southern Australia. Indeed, LaRue and

Patterson (1981) concluded, that apart from good estimates of N_2 fixation for soybean grown in representative locations in experimental plots, there is not a single legume crop for which valid estimates of the N_2 fixed in agriculture are available.

Gladstones and Loneragan (1975) and Farrington *et al.* (1977) reported that 155 and 220 kg ha^{-1} of nitrogen accumulated in the tops and tops plus roots respectively of narrow-leafed lupins grown in light sandy soils of low fertility in Western Australia. These amounts of nitrogen are very similar to the amounts accumulated in the field experiments reported here (refer Table 7, p. 152). However, it is impossible to determine how much of the accumulated nitrogen was fixed in the former experiments. In the experiment of Gladstones and Loneragan (1975), 125 kg ha^{-1} of calcium ammonium nitrate was applied immediately before sowing the crop. The crop described by Farrington *et al.* (1977) was grown on a grey sand overlying a kaolinitic clay and received no nitrogenous fertilizer. The concentration of mineral nitrogen in the soil at sowing and at harvest was low (mean 2.7 ppm) and varied little between date or depth (to 60 cm) of sampling (Farrington, pers. comm.). It seems likely, that in Farrington's experiment, the proportion of the nitrogen accumulated in the lupin plants that was derived from N_2 fixation would have been greater than the proportions recorded in the present investigations.

Herridge (1983) reported that 263 and 124 kg ha^{-1} of nitrogen were accumulated in two crops of narrow-leafed lupins grown in sandy soils of the Pilliga Scrub, N.S.W. Based on measurements of AR (calibrated to rates of N_2 fixation using a 2.2 conversion factor determined from glasshouse experiments) and a tissue solute technique, Herridge estimated that 95% (250 kg $ha^{-1}N$) and 83% (103 kg $ha^{-1}N$) respectively of the accumulated nitrogen in the plants was fixed. It is interesting to note here, that the

extrapolation of a mean A/N conversion factor derived from glasshouse studies to the field is open to question in view of the variable A/N ratios reported here and their dependence on specific rates of N_2 fixation. Nevertheless, the value of A/N of 2.2 used by Herridge is close to the mean value of 2.46 obtained in the two field experiments at Coonalpyn.

The results from the field investigations described in this thesis, together with those from other workers referred to above, indicate that approximately 150 kg ha^{-1} of nitrogen may be fixed by a good crop of narrow-leafed lupins grown in soils of moderate fertility in southern Australia and possibly up to 250 kg ha^{-1} by crops grown in sandy soils of very low fertility.

6.9. Nitrogen balances for field crops

In 1980 and 1981 the lupin plants lost 25% and 16% respectively of their total nitrogen between the occasion of maximum recorded nitrogen content and maturity (harvest). Similar losses of nitrogen have been recorded by other workers. Gladstones and Loneragan (1975) reported a loss of plant nitrogen of 20% between flowering and maturity, and Farrington *et al.* (1977) recorded a 33% loss.

The loss of nitrogen from annual plants between flowering and maturity has been frequently reported. The phenomenon has recently been reviewed by Wetselaar and Farquhar (1980) who concluded, that while the nature of the losses is largely unknown, there is little evidence for much loss due to the translocation of nitrogen to the roots and soil. Net losses from tops may result from the cumulative effects of a large number of small losses, some of which increase with the nitrogen content of the plant or with senescence. These workers further concluded that leaching of mobile nitrogenous compounds from the tops and direct loss of nitrogen in gaseous form from the tops may be important mechanisms of loss.

TABLE 7:

Nitrogen balances for lupin crops in 1980 and 1981

		1980		1981	
		kg ha^{-1}			
a)	Maximum plant N recorded	225		226	
b)	N from N_2 fixation	136		155	
c)	N from soil	89		71	
d)	N in plant at harvest	169		191	
e)	N removed in seed	58	(0.34†)	123	(0.64)
f)	N in tops (not seed) at harvest	100		49	
g)	N in roots at harvest	11		19	
h)	N in plant at harvest (not seed)	111		68	
i)	Net input of N into soil-plant system	22		- 3	

† Values in parenthesis refer to the fraction of N in the plants at harvest.

Table 7 shows the nitrogen balances for the lupin crops in 1980 and 1981. Assuming that the nitrogen lost from the plants prior to maturity (Table 7, line a - line d) is lost to the soil-plant system, then 89 and 71 kg ha^{-1} of nitrogen was derived from the soil for 1980 and 1981 respectively (Table 7, line c). The amount of nitrogen removed in the seed in 1980 was substantially less than that removed in 1981 because of the lower seed yield in 1980 (Table 7, line e). The amount of nitrogen remaining in the plant following the harvest of seed was 111 and 68 kg ha^{-1} respectively (Table 7, line h) of which only 11 and 19 kg ha^{-1} remained in the roots (Table 7, line g).

The net input of nitrogen into the soil-plant system resulting from N_2 fixation can be calculated as the difference between the amount of nitrogen remaining in the plant following the harvest of seed and that derived from the soil (Table 7, line h - line c), *i.e.* 22 and -3 kg ha^{-1} for the years 1980 and 1981 respectively. However, for this amount of nitrogen to be available for recycling in subsequent crops, the tops would need to be incorporated into the soil.

Only an amount of nitrogen equivalent to between 12 and 27% of the nitrogen removed by the lupin plant from the soil remained in the soil as root material (Table 7, line g/line c x 100). The measured amounts of nitrogen returned to the soil in root material are probably under-estimated because of the difficulty of extracting all root material late in the season and the continual process of root and nodule decay throughout the season. Nevertheless, a substantial percentage (78 - 100% based on measured values) of the nitrogen in the tops (less seed) would need to be incorporated into the soil to replace the nitrogen removed from the soil by the lupin plant [Table 7, (line c - line g)/line f x 100].

Ladd *et al.* (1981) reported that between 88 and 93% of the ^{15}N of unground ^{15}N-labelled medic material (*Medicago littoralis*) was recovered in wheat plus soil at three sites in South Australia. The tissue was mixed with the topsoil and allowed to decompose for about 8 months prior to sowing the wheat crop. These high recoveries were recorded despite differences in the texture and organic matter contents of the soils, and differences in the rainfall and extent of leaching of the $^{15}NO_3$- nitrogen formed from the decomposing medic residues. Further studies (Ladd 1981) showed, that in this Mediterranean-type environment, decomposition rates of leaf and stem tissue of medic were similar to that of *L. angustifolius*.

It appears from the limited evidence available, that the loss of nitrogen from incorporated legume tissue is low in this environment and it is postulated that a high proportion of the nitrogen in lupin top growth would be retained within the soil providing the tops were incorporated. Obviously, the extent to which the lupin top growth is incorporated in the soil will depend on the type of stubble management adopted. Tillage practices, and soil and wind erosion will have a major bearing on the extent to which the nitrogen in lupin stubbles is returned to the soil.

Grazing of the stubble by livestock, a common practice in this environment, may result in accelerated volatilization of nitrogen. Watson and Lapins (1969) found that up to 54% of the nitrogen applied as urine to ryegrass in pots was volatilized within 54 days, with greatest losses occurring during the summer months. Gillard (1967) reported that 80% of the nitrogen was lost from faeces which were dried in the sun. Furthermore, the uneven distribution of excreta by grazing animals within a paddock may result in nitrogen from exreta being lost from a large proportion of the surface soil (Hilder 1964).

Burning of stubble may result in the loss of nitrogen as gas (Clarke and Russell 1977), although any nitrogen that is returned to the soil may be less immobilized because of a lowered C:N ratio of the returned material (Vlek *et al.* 1981).

The nitrogen balance figures reported here cast doubt on the ability of a lupin crop grown in this environment to fix sufficient nitrogen to replace that which it removes from the soil. In 1980, when relatively low amounts of nitrogen were removed in the seed, 78% of the nitrogen in the tops would need to have been returned to the soil, and this does not account for subsequent losses of nitrogen from the soil by processes such as denitrification. In 1981, the total amount of nitrogen in the lupin tops would need to have been incorporated into the soil to replace the nitrogen removed from the soil by the same crop.

As has been already stressed earlier in this discussion, if the lupin crop is grown in soils containing very low concentrations of mineral nitrogen, the amount of nitrogen removed from the soil may be less and the nitrogen balance may be more favourable.

The amount of nitrogen removed in the grain of a wheat crop is approximately 25 kg ha^{-1} per tonne of grain (Schultz and French 1978). For a lupin crop to supply the nitrogen for a subsequent wheat crop in a lupin-cereal rotation, it would need to return at least 50 kg ha^{-1} (for a 2 t crop) to the soil, in addition to replacing the amount which itself removed. The data reported in this thesis show clearly that, in the two field experiments undertaken, 50 kg ha^{-1} of nitrogen would not have been added to the soil as a consequence of growing the lupin crops. This assumes however, that the large loss of nitrogen (about 45 kg ha^{-1}) from the lupin crop immediately prior to maturity is lost to the soil-plant system. Concerted research effort should be directed towards discovering

the mechanism(s) of this nitrogen loss because of the substantial impact which the loss has on the nitrogen budgets of cereal-legume rotations.

7. CONCLUSIONS AND RECOMMENDATIONS

The following conclusions and recommendations can be drawn from the investigations on narrow-leaved lupins reported in this thesis.

1. Contrary to the report of Trinick *et al.* (1976), washing and blotting dry the root system may greatly lower AR and N_2 fixation by this species.
2. There is no evidence for a functional H_2-uptake hydrogenase in the *L. angustifolius* x *R. lupini* (strain WU 425) symbiosis.
3. Uptake of H_2 by the rooting medium prevents the use of *in situ* assay systems for the measurement of nitrogenase-catalysed H_2 evolution.
4. The ratio of rates of AR (based on measurements of rates of accumulation of C_2H_4) to rates of N_2 fixed varies as a function of the specific rate of N_2 fixed.
5. The A/N ratio is consistently less than that predicted by the theoretical stoichiometric equation; moles C_2H_2 reduced = (moles N_2 fixed)/3 + moles H_2 evolved in air.
6. An C_2H_2-induced decline in nitrogenase activity is evident in the *L. angustifolius* x *R. lupini* symbiosis when a flow-through assay system is used. This phenomenon probably accounts for the low A/N ratios obtained using closed assay systems and variation in the extent of the decline may account for the relationship obtained between the A/N ratio and the specific rate of N_2 fixation.
7. The ratio of H_2 evolved to N_2 fixed (H/N) varies as a function of the specific rate of N_2 fixation (sN), such that N_2 fixation is favoured above that of H_2 evolution when sN is high.
8. Although considerable diurnal variation occurs in AR, H_2 evolution and N_2 fixation in field-grown lupins, no such variation occurs in the A/N or

H/N ratios. However, the occurrence of diurnal variation in N_2 fixation, together with the short-term nature of assays of AR, H_2 evolution and $^{15}N_2$ fixation, remain as serious handicaps to the easy and cheap deployment of these techniques for assessing the amount of N_2 fixed throughout a growing season.

9. The pursuit of a non-nodulating isoline of *L. angustifolius*, in the overall quest for suitable non-fixing control plants for the application of ^{15}N-dilution techniques for measuring N_2 fixation in this species, is warranted. However, a major problem to be surmounted would be that of rapid leaching of ^{15}N-enriched fertilizer through the sandy soils on which most commercial crops in southern Australia are grown.

10. Narrow-leafed lupin crops, growing in soils of low to moderate fertility in southern Australia and reaped for grain, may fix sufficient nitrogen for their own seed requirements, but cannot be expected to make a major contribution of nitrogen to the soil in which they grow. Therefore it is concluded that lupins cannot adequately replace pasture leys as providers of nitrogen for subsequent cereal crops although their deployment in continuous cropping systems would obviously be beneficial.

APPENDIX 1

Nutrient solution formulation

4 mM	$CaSO_4$
2 mM	K_2HPO_4
2 mM	$MgSO_4$
1 mM	K_2CO_3
46 μM	H_3BO_3
9 μM	$MnCl_2.4H_2O$
0.76 μM	$ZnSO_4.7H_2O$
0.32 μM	$CuSO_4.5H_2O$
0.11 μM	$H_2MoO_4.H_2O$
15 μM	$FeC_6H_5O_7.5H_2O$
4.2 nM	$CoCl_2$

adjusted to pH 6.5 with HCl.

APPENDIX 2

Kjeldahl methods

2.1 General method for total nitrogen (does not include all nitrogen in nitrate and organic nitro-compounds)

Reagents

1. 10N NaOH Solution
2. 2% Boric Acid Solution
3. Indicator (methyl red - methylene blue mixture)

   Solution A - 0.2% methyl red in 95% ethanol

   Solution B - 0.2% methylene blue in 95% ethanol

   Mix in the ratio of 2 volumes of A to 1 volume of B.
4. Boric Acid + Indicator Solution

   Add 10 ml of mixed indicator to 1 litre of 2% H_3BO_3.
5. 0.01N $KH(IO_3)_2$ (1 ml = 0.14 mg N)
6. K_2SO_4/Se Catalyst

   Use 'Keltabs' Auto - (1.5 g K_2SO_4/0.0075 g Se)

Method

A. Plant material

   1. Weigh 0.25 g of ground plant material (oven dry 85°C for 24 h) into a 100 ml kjeldahl flask or tube.
   2. Add 1 'Keltab' and 2 anti-bumping granules (or glass beads).
   3. Add 4 ml of conc. H_2SO_4 (auto-dispenser).

B. Soil

   1. Weigh 5 g of air-dry soil (passed through a 2 mm screen) into 100 ml kjeldahl flask.
   2. Add 2 'Keltabs' and 2 glass beads (or anti-bumping granules).
   3. Add 10 ml of conc. H_2SO_4 (auto-dispenser).

APPENDIX 2 (Cont.)

Digestion procedure for A or B

(i) Digest samples on 'Moloney' burners (or digestion block for tubes). Heat gently at first, then boil the digests for 30 minutes after clearing. (Clearing time is about 25 minutes.)

(ii) Allow the digests to cool and dilute with distilled H_2O, to 50 ml or any suitable volume (75 ml for tubes).

Distillation procedure for A or B

(i) Immerse the tip of the condenser into 5 ml of boric acid + indicator solution.

(ii) Take an aliquot of 5 or 10 ml in a distillation apparatus, and follow this with 10 ml of NaOH solution and distil.

(iii) When about 10 ml has distilled over, lower the flask containing the indicator solution and let a further 15 ml distil over. Remove the flask, ready for titration.

(iv) Remove the distillation flask and rinse thoroughly before beginning the next sample.

(v) Titrate the distillate with 0.01N $KH(IO_3)$ to a lilac end point. Use a 5 ml or 10 ml auto-burette (1 division = 0.02 ml).

2.2 Kjeldahl method modified to include nitrate and organic nitro-compounds

Reagents

1. Salicyclic acid-sulphuric acid mixture

The mixture is made up in the ratio of 1 g of salicyclic acid to 30 ml of conc. H_2SO_4.

APPENDIX 2 (Cont.)

2. Sodium Thiosulphate ($Na_2S_2O_3.5H_2O$)

0.5 g per digest. Use powder crystals passed through a 20 mesh screen.

Method

A. Plant material

1. Weight 0.25 g of ground plant material (oven dry 85°C for 24 h) into a 100 ml kjeldahl flask or test tube.
2. Add 1 'Keltab' and 2 glass beads (or anti-bumping granules).
3. Add 6 ml of the salicyclic acid-H_2SO_4 mixture and allow to stand for several hours.
4. Add 0.5 g of sodium thiosulphate through a funnel with a long stem. Mix thoroughly and allow to stand for at least 1 h.

B. Soil

1. Weigh 5 g air dry soil (passed through a 2 mm sieve) into a 100 ml kjeldahl flask.
2. Add 2 'Keltabs', 2 glass beads (or anti-bumping granules).
3. Add 15 ml of the salicyclic acid-H_2SO_4 mixture and allow to stand for at least 1 h.
4. Add 0.5 g of sodium thiosulphate through a funnel with a long stem. Mix thoroughly and allow to stand for at least 1 h.

Digestion procedure for A or B

(i) Digest samples on Moloney burners (a digestion block for tubes). Heat gently at first until the frothing has ceased. Then boil the digests for 30 minutes after clearing (clearing time is about 25 minutes).

(ii) Allow digests to cool and dilute with distilled H_2O to 50 ml or any convenient volume (75 ml for tubes).

Distillation procedure for A or B

Distillation as for 2.1.

APPENDIX 2 (Cont.)

2.3 Distillation method for ^{15}N samples

1. Immerse the tip of the condenser into 5 ml of H_3BO_3 solution.
2. Take an aliquot of 10 ml of the sample solution in the distillation apparatus and follow this with 10 ml of NaOH solution and distil.
3. When between 30 and 40 ml has distilled over remove the flask and make up to 50 ml with distilled H_2O in a volumetric flask.
4. Pipette 20 or 25 ml from the volumetric flask into a clean beaker; add 3 drops of indicator and titrate with 0.01N $KH(IO_3)_2$ solution to a lilac end point. Use a 5 ml auto-burette (1 division = 0.01 ml). Discard this solution following titration.
5. Acidify the remaining 25 or 30 ml of distillate with 1N H_2SO_4 to between pH 3 and 4 (use pH meter) and evaporate to dryness in an oven at 85°C overnight.
6. Dissolve the residue in 1 to 2 ml of distilled H_2O and transfer the solution to a glass screw-topped tube (Trident 2-dram vial). Evaporate to dryness in an oven at 85°C.

APPENDIX 3

Calculations for $^{15}N_2$ gas phases

The following calculations are presented as an example of the procedures adopted for all NRA system assays. Calculations were performed on estimated parameters prior to the assay.

- Jar volume = 270 ml
- Estimated free volume of assay chamber after addition of nodulated roots = 210 ml
- Estimated dry weight of roots = 5 g at 1.5% N.

Therefore estimated amount of N in roots = 75 mg

- The rate of N_2 fixation was assumed to be 15 μ mol h^{-1} $plant^{-1}$

Therefore the percentage of N in the roots which has been fixed at end of a half hour assay is approximately (15 x 0.5 x 28)/(10 x 75)% = 0.28%

- The minimum enrichment of the roots was set at 0.05 atoms-% ^{15}N excess, therefore the assay gas mixture should contain N_2 at about (0.05 x 100)/0.28 = 17.9 atoms-% ^{15}N excess.
- To achieve this enrichment, N_2 of 95 atoms-% ^{15}N would need to be diluted about 95/17.9 = 5.3 times.
- The desired N_2 of the gas phase was 0.2 atm. Therefore 210 x 0.2 = 42 ml of the assay volume should be N_2. Because the assay chamber is first flushed with a gas mixture of Ar/O_2 (80:20 v/v), about (42/5.3) = 8 ml of 95 atoms-% $^{15}N_2$ should be mixed with about 34 ml of $^{14}N_2$ or (34 x 100)/78 = 43.5 ml of air.
- Amount of gas to be injected into assay chamber is about (8 x 5)/4 ml of 95 atoms-% $^{15}N_2/O_2$ mixture plus 43.5 ml of air = 53.5 ml.

  This will require (53.5 x 210)/(210 - 53.5) = 72 ml of gas to be first withdrawn from the assay chamber to maintain the pressure at 1 atm.

APPENDIX 4

GC Operating Parameters

Carrier gas - N_2
Oven temp. - Isothermal
Injector temp. - 65°C
Detector temp. - 125°C

Numerous operating combinations were possible (2 listed below) with column flow rates and oven temperatures adjusted depending on the sensitivity required for H_2 detection and the time afforded per analysis (> sensitivity of TCD to H_2 with lower carrier flow rates).

Temperatures : Oven 65°C,

	System A	System B (rapid analysis)
Carrier gas (TCD) N_2	30 ml min^{-1}	100 ml min^{-1}
Carrier gas (FID) N_2	40 ml min^{-1}	90 ml min^{-1}
Pressure Gauge E	165 kPa	275 kPa
Pressure Gauge C	180 kPa	385 kPa
Pressure Gauge G	25 kPa	275 kPa

Gas retention and detector switching times

	Time (s)	Time (s)
Inject sample at I	0	0
Inject sample at J	5	5
Solenoid Valve (L) on	20	5
Solenoid Valve (L) off	40	15
C_2H_4	50	30
C_2H_2	85	52
FID - TCD	110	70
He	160	88
H_2	190	101
TCD - FID	210	120
C_3H_8	230	142
O_2 if required (Solenoid L - activated and low filament current)	290	155

Figure 24

Column Configurations for Gas Chromatograph -

Pye Unicam Series 204

System code

A. N_2 carrier gas input (500 kPa)

B. Pressure regulator 2

C. Pressure gauge

D. Mass flow controller

E. Pressure gauge

F. Pressure regulator 1

G. Pressure gauge

H. TCD sample injection port

I. FID sample injection port

J. TCD stripper column (1 m x 2 mm ID, Molecular Sieve 5A, 80-100 mesh)

K. FID Column (2 m x 2 mm ID, Porapak N, 80-100 mesh)

L. Solenoid valve

M. TCD main column (5.5 m x 4 mm ID, Molecular Sieve 5A, 80-100 mesh)

N. Fine metering valve (Nupro 'SA' series, orifice diam. 0.79 mm)

O. TCD detector

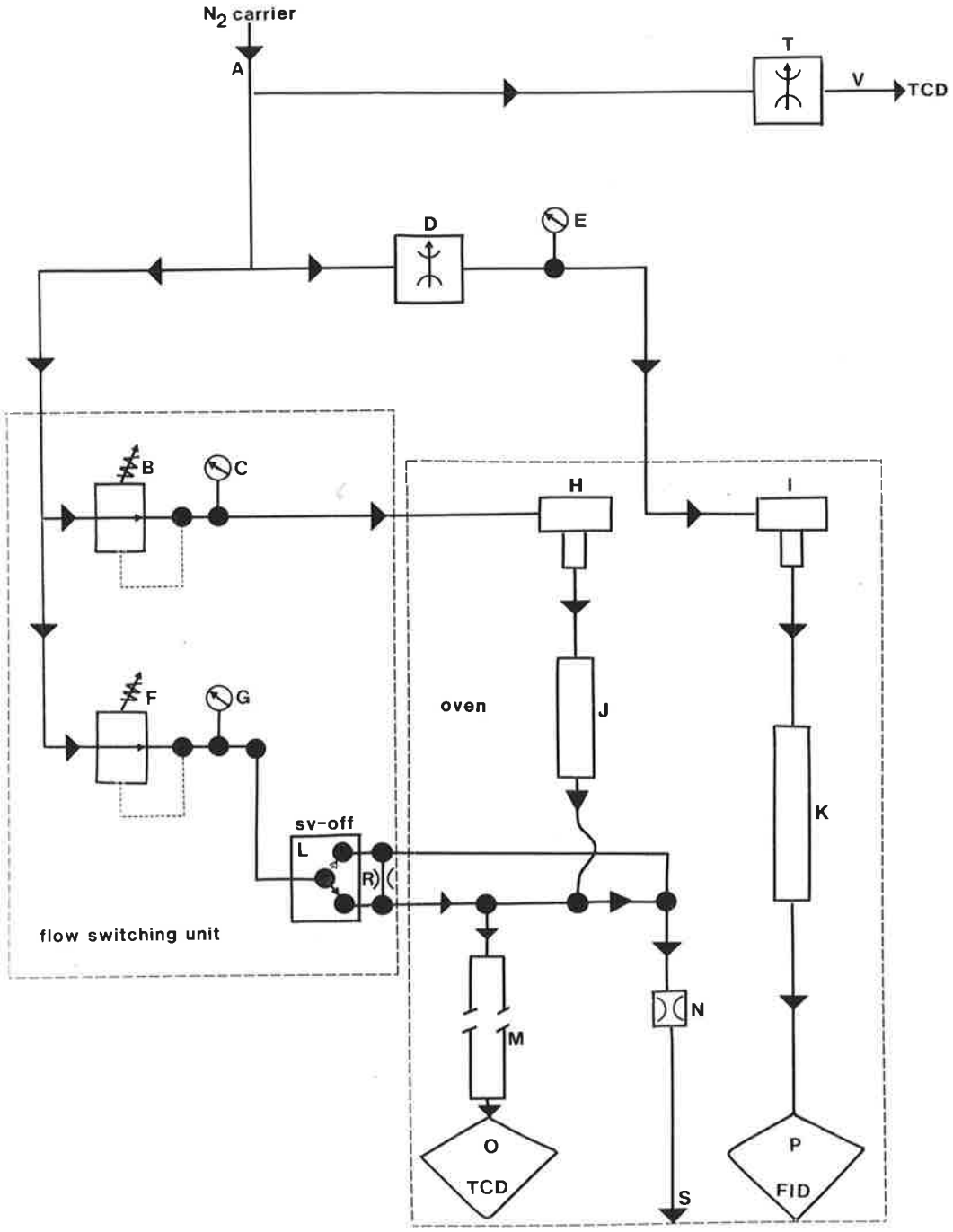
P. FID detector

Q. Restrictor (4-6 ml min^{-1} N_2 at 138 kPa)

R. Vent

S. Mass flow controller (100 ml min^{-1})

T. Dummy column to 2nd side TCD


N_2 carrier
A
T
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H
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oven
J
K
sv-off
L
R
flow switching unit
M
N
O
TCD
P
FID
S

APPENDIX 5

Rates of AR in the Growth Chamber (Experiment 3)

Days from Sowing		NRA System	WPA System
		(μ mol h^{-1} $plant^{-1}$)*	
35		12.2	13.5
49		31.9	33.2
59		54.8	53.2
70		86.0	81.3
80		104.0	113.1
88		91.7	90.7
101		47.7	51.4
112		38.1	35.3
124		2.1	2.4
	mean	52.1	52.7

* AR values are the means of 10 replicate assays. There is no significant difference ($p > 0.05$) between means within occasions of assay.

APPENDIX 6

AR, H_2 evolution in Ar/O_2, and dry weight of tops of lupins growing within and without PVC tubes at Coonalpyn in 1981.

		Date of Sampling (Weeks from Sowing)		
		2/9 (11)	24/9 (14)	11/11 (21)
Plant dry weight ($g\ plant^{-1}$)	Inside Tube	1.06 (a)*	5.13 (b)	19.95 (c)
	Outside Tube	1.10 (a)	4.47 (b)	21.80 (c)
AR† ($\mu mol\ h^{-1}\ plant^{-1}$)	Inside Tube	4.8 (d)	30.5 (e)	13.6 (f)
	Outside Tube	4.9 (d)	27.4 (e)	15.7 (f)
H_2 evolution in Ar/O_2† ($\mu mol\ h^{-1}\ plant^{-1}$)	Outside Tube	5.1 (d)	28.7 (e)	14.9 (f)

† Assays performed using the NRA jar system. Values in table are the means of 10 replicate assays.

* Like letters in parenthesis indicate treatment means are not significantly different ($p > 0.05$).

APPENDIX 7

AR in lupin plants at Coonalpyn in 1981 using the NRA and WFA assay systems

Date of Sampling	Weeks from Sowing†	NRA System	WFA System
		(μmol h^{-1} $plant^{-1}$)*	
12/8	8	0.2	0.2
2/9	11	4.9	4.1
24/9	14	27.4	29.3
30/9	15	31.0	32.7
15/10	17	24.3	22.9
29/10	19	19.8	19.1
11/11	21	15.7	16.6
25/11	23	1.0	0.9
	Mean	15.5	15.7

* AR values are the means of 10 replicate assays. There is no significant difference ($P > 0.05$) between means within occasions of assay.

† Rounded to the nearest week.

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