Studies of Nodulation, Nodule Function, and Nitrogen Fixation of *Vicia faba* L. and *Pisum sativum* L.

by

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To my late Mother and my Father

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND SYMBOLS USED IN THE TEXT

Throughout this thesis, S.I. units are used. Other abbreviations follow:

AR	Acetylene reduction
HE	Hydrogen evolution
AR - HE	Apparent N_2 fixation
RE	Relative efficiency of N_2 fixation, 1 - HE/AR
Hup^+	Hydrogen uptake positive
Hup^-	Hydrogen uptake negative
e^-	Electron
ADP	Adenosine diphosphate
RuBP-case	Ribulose biphosphate carboxylase
PPFD	Photosynthetic photon flux density
Pi	Inorganic phosphate
ABA	Abscisic acid
EDTA	Ethylene diamine tetra acetate
PEP	Phosphoenol pyruvate
cv	cultivar
DAS	Days after sowing
Dwt	Dry weight
No	Number
v/v	Volume : volume
SE	Standard error
LSD	Least significant difference
NS	Not significant at $P<0.05$
*, **, ***	Analysis significant at $P<0.05$, 0.01, 0.001 respectively

SUMMARY

The aim of the research described in this thesis was to examine, in two grain legumes, faba bean (*Vicia faba* L. cv. 'Fiord') and pea (*Pisum sativum* L. cvs. 'Early Dun' and 'A 102'): (i) the use of acetylene reduction (AR) assay as an estimate of nitrogenase activity (NA) and its limitations; (ii) effective nodulation; (iii) nodule function; (iv) the accumulation and partitioning of dry matter and nitrogen between plant parts according to phenological development; and (v) the relationship between NA and the respiration of the nodulated roots. These several aspects were considered with respect to their contribution to growth and productivity, experiments being conducted under glasshouse and controlled environment conditions to minimize variation in response to particular treatments as far as possible. The studies therefore pertain more to Crop Physiology than to conventional field Agronomy.

Major limitations to the use of AR assay as an estimate of NA have been suggested, including: (i) a C_2H_2 induced decline in C_2H_4 production; (ii) effects from the use of detached, nodulated roots rather than whole plants; (iii) effects of disturbance of nodulated roots when removing the rooting medium; and (iv) variation with assay temperature. Rates of C_2H_4 production in 10% C_2H_2 in air of intact faba bean and pea plants were determined with an open system to ascertain whether they were constant or decreased during the assay period. Neither CO_2 efflux nor C_2H_4 production declined when young, vegetative plants of faba bean were assayed, but both rates declined by about 30% when plants were reproductive. In pea, exposure to C_2H_2, always induced a decline of about 30% in the rates of both C_2H_4 production and the respiration of nodulated roots.

AR rates were lower when detached, nodulated roots were used rather than intact plants. The rate decreased by about 14% during the vegetative stage and by 26% during the reproductive stage. The AR rates of plants grown in jars of 'oil dry' (calcined clay) as a rooting medium and assayed undisturbed (in a closed system) did not differ significantly from those of plants assayed after removal of the rooting medium. The removal of the rooting medium either by gentle washing with water or by gentle shaking, had no significant effect on AR rate. Assay temperatures at 12.5, 17.5, and 22.5°C influenced the specific rate of AR but not the absolute rate, with the optimum at 17.5°C. This may have been due to

differences in the degree of nodulation between plants at different temperatures. It is concluded that: (i) a C_2H_2 induced decline in the rate of C_2H_4 production, where present, results in underestimation of NA; (ii) intact plants rather than detached, nodulated roots should be used for assay; (iii) 'oil dry' is a suitable rooting medium as its removal during preparation for assays had no effect on AR; and (iv) AR assay should be conducted at the growth temperature.

Inoculated faba bean seedlings grew slowly after emergence, possibly due to: (i) the use of ineffective inoculant; and/or (ii) an inhibitory effect of the cotyledons on nodulation. Both strains of *Rhizobium leguminosarum*, TA 101 and SU 391, used to prepare the commercial peat inoculant (strain E), proved to be poor inoculants on 'Fiord' faba bean. TA 101 was more effective than SU 391, especially when plants were grown without NO_3^-. A small amount of combined N as a 'starter' (2.5 mM) was needed to promote growth and nodulation with SU 391. Strain SU 391, however, appeared to be effective for both pea cultivars, 'Early Dun' and 'A 102'. Concentrations of 2.5 mM and 5.0 mM NO_3^- suppressed NA of pea more than that of faba bean.

Removal of cotyledons from faba bean 14 days after sowing severely depressed growth, nodulation, and AR activity; but removal on day 18 had little effect on growth, increased AR activity on a nodule dry weight basis and increased nodule number. This suggests that the role of the cotyledons as a source of substrate for growth appears to be completed by day 18 at 20°C and their removal at this time removes an unknown inhibitor. It is concluded that: (i) the commercial inoculants TA 101 and SU 391 are not particularly effective on 'Fiord' faba bean; (ii) different combinations of legume and rhizobia vary in their capacity to produce nodules, fix N_2, and tolerate NO_3^-; (iii) the depression in nodule activity by applied NO_3^- is related to the level of nodule activity; (iv) cotyledons of faba bean provide both promoting and inhibiting factors for nodulation and NA.

Since plants nodulated with *R. leguminosarum* TA 101 and grown without mineral N derived all their nitrogen from N_2 fixation, rates of accumulation of N by whole plants could be estimated from the total N content. In combination with AR assay, it was possible to elucidate how the process of N_2 fixation contributed to the overall N economy, especially in relation to N translocated first from the seed to the plant during emergence and subsequently

from the plant to the seed during grain filling. At maturity the grain of faba bean contained 50% of the total dry matter and 78% of the total N: in pea the amounts were 34% and 56%. The amount of N in the vegetative parts and pods decreased by 49 % in pea and and by 51% in faba bean during the final stage of grain filling. Thus 80% of the N in the grain of faba bean was fixed during grain filling but only 20% in pea, the rest being translocated mainly from the leaves. Faba bean is evidently capable of fixing N_2 throughout most of the plant growth cycle, but the capacity for N_2 fixation declines rapidly during the grain filling period in pea. The potential of these legumes to contribute to soil organic N and thus to soil fertility is also discussed and it is concluded that such grain legume crops appear likely to sustain a legume/cereal rotation in N balance only when a large proportion of the above ground parts contribute N to the soil in addition to the root fraction.

N_2 fixation is commonly observed to decline during grain filling which may be due to a reduction in the carbon supply to the nodule. This was examined in a disbudding experiment where the C_2H_4 production rate of disbudded pea plants was found to be significantly higher than that of intact plants. In faba bean, the absolute rates of CO_2 efflux and rates of C_2H_4 production of disbudded plants did not differ from those of intact plants. This suggests that there was no significant reduction in availability of carbon to the nodule in faba bean during the reproductive stage in contrast to pea, and that faba bean is capable of fixing N_2 throughout most of the plant growth cycle.

NA and the respiration of nodulated roots of both pea and faba bean were closely linked: both varied markedly over a diurnal 12 h/12 h cycle. The respiration of nodulated roots began to increase soon after the beginning of the photoperiod, reaching a plateau after about 6 h of light and remaining stable or increasing slightly, to reach a maximum at the end of the photoperiod after which there was a rapid decline during the dark period. Nodule removal decreased root respiration to a level below the minimum shown by the nodulated roots during a diurnal cycle, and there was then no diurnal variation suggesting that such variation is attributable to nodule respiration. Respiration of the nodules accounted for 45% of that of the nodulated root, and it was estimated that respiration increased about 1.6 times during the day in both pea and faba bean. Both the increase in the respiration of nodulated roots during the day and the ratio between nodule and root respiration varied both within the

species according to the degree of plant nodulation, and between species. AR showed a diurnal variation of 2.0 times in faba bean and 2.75 times in pea. This difference in response relative to respiration was attributed to minimal diurnal variation in root respiration whereas nodule respiration, and presumably AR, was closely linked to change in the carbohydrate supply.

A prolonged light period increased the efflux of CO_2 markedly and efflux declined steadily in prolonged darkness. After a normal day of 12 h the respiration rate of the nodulated root had increased about 1.7 times that at the beginning of the photoperiod. Extension of the photoperiod beyond 12 h led to a gradual increase to 2.2 and 2.4 times at 24 h and 36 h respectively. Whereas the 12 h of a normal dark period reduced the respiration rates of nodulated roots of faba bean 0.60 times that at the end of the photoperiod, a further 12 h of darkness reduced the ratio to 0.37. In pea the figures were 0.66 and 0.36. After 36 h of continuous darkness AR rates were only 4% of those before exposure. On transfer of plants back to 12 h daily photoperiods for 50 h, AR rates were almost double those before exposure to prolonged darkness.

Since AR assay alone fails to provide information on how electron flow *in vivo* is partitioned between reduction of N_2 and reduction of protons, measurement of diurnal variation in hydrogen evolution (HE) in air and argon/O_2 in an open system, was used to estimate these rates. Diurnal variation in both rates was examined at 'low' PPFD (300 $\mu E\ m^{-2}\ s^{-1}$) and at 'high' (1300 $\mu E\ m^{-2}\ s^{-1}$) to explore whether diurnal variation could be attributed to variation in carbohydrate supply. Both HE in the presence and absence of N_2 and the respiration of nodulated roots varied diurnally, the variation being more marked when plants were exposed to 'low' PPFD. The apparent N_2 fixation (HE in Argon/O_2 - HE in air : 3) at a 'high' PPFD did not differ between day and night, indicating that the diurnal variation in NA was here due to changes in the rate of HE. At 'low' PPFD, however, there was diurnal variation in the rate of apparent N_2 fixation, which could be due to a limited availability of photosynthate. The symbiosis seems to be very inefficient since some H_2 was still produced at low PPFD.

The relationship between the supply of photosynthate and NA was tested further by detaching half the nodules from faba bean plants and examining NA of the remaining

nodules. After 5 days, AR activity of the remaining nodules had increased, so that whole plants with half the nodules removed exhibited the same activity as intact control plants. The volume of the active N_2 fixing region of the remaining nodules doubled so that, after 5 days, the nodule volume was the same on control and treated plants. Nodule weight and nodule numbers increased only slightly, however, there was a marked difference between treatments in the rate of AR per g nodule dry weight. This difference in AR rate was due mainly to a higher percentage (22.8%) of senescent nodules in control plants than in those from which half the nodules had been detached (6.3%). It is concluded that: (i) the volume of active N_2 fixing region of indeterminate nodules appears to be flexible and to increase when the available substrate per nodule increases; and (ii) expression of the rate of AR on a nodule weight basis can be misleading due to variation between nodules in the ratio of active/senescent N_2 fixing regions.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other 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.

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

Herdina

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PREFACE

Some of the data presented in this thesis has been presented to scientific meetings as indicated below:

(1) Growth, nitrogen fixation, and nitrogen accumulation in faba bean (*Vicia faba* L. cv. Fiord) and pea (*Pisum sativum* L. cv. Early Dun).
Herdina and J.H. Silsbury.
24th Aust. Soc. Plant Physiol. Meeting (1984) p. 91.

(2) Relations between phenological development of faba bean (*Vicia faba* L. cv. Fiord), nitrogenase activity (acetylene reduction assay), and root respiration.
Herdina and J.H. Silsbury.
26th Aust. Soc. Plant Physiol. Meeting (1986) p. 139.

(3) Relations between phenological development of faba bean (*Vicia faba* L. cv. Fiord), nitrogenase activity (acetylene reduction assay), and root respiration.
Herdina and J.H. Silsbury.
8th Aust. Nitrogen Fixation Conf. (1986) p. 35. (AIAS Occasional Publication No. 25).

CHAPTER 1

General Introduction

Most farming in South Australia consists of integrated cereal/livestock production based on a cereal/pasture rotation (Puckridge and French 1983). The success of this system can mainly be attributed to the use of pasture legumes for the provision of soil N for subsequent cereal crops and for high quality feed for grazing animals. It is widely accepted that annual grain legumes offer an alternative to pasture legumes in at least some agricultural districts of Australia (Farrington 1974; Kingma 1982). Lupins are used on light sandy soils in Western Australia (Gladstones 1970) and elsewhere, and peas have been a small but useful crop on loamy well drained soils in the wetter regions of the wheat belt of South Australia and Victoria for more than 80 years (Gross 1954). Several attempts have been made over the last decade to improve the range, adaptability and yield of several grain legume species available to agriculture in southern Australia (e.g., Silsbury 1975; Laurence 1979). Interest in such crops is likely to continue as there has been a decline in pasture legumes coupled with a more favourable financial return from cropping compared with livestock enterprises in recent years (Kingma 1982).

The faba bean has been proposed as one species offering considerable potential as a grain legume crop in South Australia (Laurence 1979) but little is known of its cultural requirements and even less concerning its capacity to contribute to the soil organic N through N_2 fixation. There is a general paucity of information on the extent to which grain legumes can provide the N needed by cereal crops traditionally supplied in southern Australia by pasture legumes. In rotational experiments cereal yields following grain legume crops are higher than yields under continuous cereal (Boundy 1978; White *et al.* 1978; Doyle and Herridge 1980; Hawthorne and Lewis 1980; Askin *et al.* 1982; Reeves *et al.* 1982). Grain legumes, however, may make only a small contribution to the N content of the soil in a rotational system due to the large amounts of N removed by the grain, even though the amount of N removed and the amount of N_2 fixed varies greatly between species (Gladstones and Loneragan 1975; Farrington *et al.* 1977; Pate and Flinn 1977; Russell

1980). It is crucial to the success of grain legumes as a replacement for pasture legumes in the maintenance of soil fertility, that the former is able to utilise N_2 rather than soil N.

The broad aim of this project was to study the growth, N economy and N_2 fixation of faba bean and pea as a contribution toward an understanding of the growth physiology of these important crop plants.

Acetylene reduction (AR) assay is widely used for the measurement of N_2 fixation as a cheap, simple and sensitive assay. The assay is based on the inhibition of N_2 fixation by C_2H_2 (Schöllhorn and Burris, 1966) and the consequent reduction of C_2H_2 to C_2H_4 (Dilworth 1966). Limitations of the AR assay have been described by Hardy *et al.* 1968; Bergersen 1970; Dixon 1976; Turner and Gibson 1980; and Minchin *et al.* 1983b, 1986, so clearly the assay needs to be critically examined. Minchin *et al.* (1983a) showed that there was a decline in C_2H_4 production induced by C_2H_2 which consequently underestimates the actual value. Others, Maderski and Streeter 1977, Paterson *et al.* 1983, and Layzell *et al.* 1984 have not found such a decline. Furthermore, Minchin *et al.* (1986) claimed that substantial errors occurred when a detached nodulated root system from which the rooting medium had been removed (normally by shaking) was used in the 'standard' AR assay (Hardy *et al.* 1968) performed in closed vessels. Manifestation of the response to C_2H_2 is not universal, but appears to vary with cultivar, *Rhizobium* strain and plant age. Since there is doubt in the use of the standard AR assay, a proportion of the present investigation was devoted to the development, testing and assessment of the value of AR in estimating nitrogenase activity (NA) (detail in Chapter 3.3. and Chapter 6). AR assays were conducted in both a closed and an open system. An open system was designed to obtain direct measurements of rate, rather than accumulation, of C_2H_4 or H_2 production together with the respiration rate of attached nodulated roots.

One opportunity for increasing the utility of grain legumes is to enhance their capacity for symbiotic N_2 fixation by achieving the right association between symbiont and plant cultivar. However, the effectiveness of the *Rhizobium* used as a commercial inoculant in South Australia has not been examined to date. It appears that nodulation in faba bean is comparatively late, therefore it is necessary to examine whether the delay in faba bean nodulation is due to the ineffectiveness of *Rhizobium* or possibly an inhibitor in the

cotyledons. Some authors reported that cotyledons contain a nodulation promoting factor (Thornton 1929; Schaffer and Alexander 1967; Peat *et al.* 1981b) whilst others found that they contain a nodulation inhibiting factor (Phillips 1971; Bano *et al.* 1983).

In the field, the proportion of the N of a grain legume crop which is derived from N_2 fixation rarely exceeds 60% (Herridge 1982), the remainder being taken up as NO_3^- from the soil. Recently Silsbury *et al.* (1986) concluded that N_2 fixation by subterranean clover in the field may be depressed below its potential due to the presence of soil N. In order to achieve higher rates of N_2 fixation, symbioses which are capable of maintaining N_2 fixation activity in the presence of soil NO_3^- need to be developed, and it is not known for the pea and faba bean used in southern Australia whether the NO_3^- in the soil depresses or stimulates nodulation and NA. It is commonly known that NO_3^- can either stimulate (Pate and Dart 1961; Dart and Wildon 1970; Dart 1974; Gibson 1976; Vigue *et al.* 1977) or depress nodulation according to concentration, but generally has a depressive effect on the activity of nitrogenase (McEwen 1970a, b; Oghoghorie and Pate 1971; Candlish and Clark 1975; Gibson 1976; Chen and Phillips 1977; Munns 1977; Sosulki and Buchan 1978; Hill-Cottingham and Lloyd-Jones 1980; Bauer 1981;Yousef and Sprent 1983; Silsbury and Catchpoole 1984; Silsbury *et al.* 1986). The effect of NO_3^- on nodule formation and functioning are not fully understood, although several hypotheses have been proposed concerning either the stimulation or the suppression mechanism (Pate and Dart 1961; Munns 1968; Copeland and Pate 1970; Oghoghorie and Pate 1971; Ryan *et al.* 1973; Chen and Phillips 1977; Gibson and Pagan 1977; Dazzo and Brill 1978; Wong 1977; Houwaard 1980; Malik *et al.* 1987). Little data is available on nodulation, early functioning of nodules and the effect of NO_3^- on nodulation and NA in faba bean and pea in South Australia. Therefore, it is necessary to study the nodulation, effect of cotyledon removal, effect of different strains of rhizobia, and NO_3^- levels during early growth (Chapter 4).

Nitrogen fixation is known to show diurnal variation in some legumes (Hardy *et al.* 1968; Bergersen 1970; Mague and Burris 1972; Minchin and Pate 1974; Halliday and Pate 1976; Vaughn and Jones 1976; Mahon 1977; Eckart and Raguse 1980; Rainbird *et al.* 1983), but not in others (Hardy *et al.* 1968; Fishbeck *et al.* 1973; Masterson and Murphy 1976). The occurrence of diurnal variation in NA of faba bean and pea was assessed and an

explanation for diurnal variation in N_2 fixation was sought by measurement of HE in argon (Ar) and in air at 'high' and 'low' photosynthetic photon flux density (PPFD) (Chapter 7), since AR assay fails to provide information on how electron flow *in vivo* is partitioned between reduction of N_2 and reduction of protons.

The rate of symbiotic N_2 fixation is often also observed to increase markedly around the time of flowering and to decrease rapidly during the main period of grain filling. The cause of the marked decrease in the rate of fixation is not known despite the fact that the phenomenon is widespread, having been reported for pea (*Pisum sativum*), soybean (*Glycine max*), clover (*Trifolium repens*), and cowpea (*Vigna unguiculata*) (Lawn and Brun 1974; Lawrie and Wheeler 1974; Masterson and Murphy 1976; Bethlenfalvay and Phillips 1977; Minchin and Summerfield 1978). There are several hypotheses which may explain the decline : (1) the bacteriods in the older nodules may decay or nodules may be shed; (2) a decline in photosynthesis may restrict the availability of C to the nodules; (3) fruits may have a hormonal influence on leaf senescence and on abscission which may combine with the mobilization of N from the leaves to impair photosynthetic activity; (4) N_2 fixation in the nodules may be turned off by an alternative source of N arising from the breakdown of leaf protein ; (5) fruits and nodules may compete for photosynthate. The latter hypothesis was examined by disbudding plants during the reproductive stage of both pea and faba bean (Chapter 6). Differences in the effect of disbudding and depodding on the N_2 fixing process both between species and in the same species under different conditions of growth have been recorded (Pate 1958; Roponen and Virtanen 1968; Hardy *et al.* 1968, 1971; Lovell *et al.* 1972; Mague and Burris 1972; Lawn and Brun 1974). The extent to which photosynthesis of the plant can satisfy the demand for assimilates by different metabolic 'sinks' has not been clarified in relation to changes in N_2 fixation and accumulation of photosynthates in the nodules during flowering and grain filling.

Fixed N is accumulated and distributed through the plant during grain filling. Growth, N accumulation and the distribution of dry matter and N between plant parts was studied in faba bean and pea (Chapter 5). This study is of interest for four main reasons: (i) it adds to our knowledge of the growth physiology of legume crops; (ii) it provides an opportunity to compare rates of AR at different stages of ontogeny; (iii) rates of N accumulation in the

whole plants can be related to rates of accumulation in the grain and to the mobility of N in different plant organs; and (iv) some tentative conclusions can be drawn about the capacities of faba bean and pea to provide soil organic N in a cereal/grain legume rotation.

The relationship between the supply of substrate arising from photosynthates and nodule activity of faba bean was examined by: (i) exposing the plant to prolonged darkness and prolonged light, (ii) removal of shoot and leaves, and (iii) reduction of nodule number (Chapter 8).

CHAPTER 2

Literature Review

2.1. Grain Legume Crops

There are some 10,000 species within the family Leguminosae, sub family Papilionoideae, but only about 18 of these are widely cultivated as grain legumes (Farrington 1974). The most striking feature of the grain legume crops is their high seed protein content, which is in the range of 20-30% (Laurence 1979), most of the required nitrogen being fixed symbiotically. *Vicia faba* L., the faba, field, tick, horse or broad bean, is the sixth most important pulse crop in terms of global production after soybean, dry bean (*Phaseolus*), groundnut (*Arachis*), field pea and chickpea (F.A.O. 1985).

Experiments by Laurence (1979) indicated that faba bean could be developed into a valuable leguminous grain crop in South Australia. The highest yielding lines observed by Laurence (1979) came from the Mediterranean and matured early. One was subsequently developed by the Waite Agricultural Research Institute during the 1970's and was released in 1980 as the cultivar 'Fiord'. It has proved to be well suited to South Australian conditions.

The local potential of faba bean has not been clearly established since most research on the species has been conducted in Europe where climatic conditions differ greatly from those of southern Australia. The growing season in southern Australia begins with autumn rains and the reproductive phase of the crop begins in the coldest and wettest part of winter. In northern Europe faba beans are either an autumn or a spring crop. In England they are predominantly autumn sown between August and Mid-October with spring sowing from mid-February to March (Bond and Fyfe 1962). The reproductive phase occurs in the summer between May and August (Soper 1956).

Faba beans are not well adapted to withstand dry conditions but with favourable rainfall can produce relatively high yields. Early sowing (late April to early May) appears to be very important for dryland production in the Mediterranean type climate of South Australia (Baldwin 1980; Marcellos and Constable 1986) so as to make the best use of

limited rainfall in the drier areas. Some drought tolerance is desirable to give stable yields in dry areas.

The field pea (*Pisum sativum* L.) is the principal grain legume in the medium rainfall (400-500 mm) cereal areas of Victoria, South Australia, and Western Australia where it is generally sown as a dryland field crop in rotation with cereals (Farrington 1974). Pea cultivation is favoured in areas where the weather is cool and moisture abundant in early growth (autumn or spring) but where rainfall is minimal or absent during the later stages of crop development (Pate 1977).

Faba beans and peas are usually sown at relatively high plant densities of 40 to 80 plants m^{-2}, respectively (Meadley and Milbourn 1970; Baldwin 1979, 1980; Marcellos and Constable 1986). Faba bean is especially valuable for heavy or alkaline soils, unsuited to lupins, or for areas where peas suffer from wet conditions. The cultivar 'Fiord' prefers loam to clay soils within a pH range of 6.5-8.0, generally on the wetter edge of the wheatbelt.

2.2. N_2 Fixation during Growth and Development

The pattern of N_2 fixation in many agriculturally important grain legumes is for the rate of fixation to increase from the seedling stage during early vegetative growth to reach a peak at about flowering followed by a decrease during the main period of grain filling. The cause of this decrease is not known despite the fact that the phenomenon is widespread, having been reported in pea (*Pisum sativum* L.), soybean (*Glycine max* (L.) *Merr.*), white clover (*Trifolium repens* L.), and cowpea (*Vigna unguiculata* (L.) Walp.) (Lawn and Brun 1974; Masterson and Murphy 1976; Bethlenfalvay and Phillips 1977; Minchin and Summerfield 1978). Considerable variation is found in the pattern. The peak of NA relative to flowering was found by Ham *et al.* (1976) to differ with the cultivar of soybean used. In 'Clay', the peak occurred just before the end of flowering whilst in 'Chippewa 64' it was just after the end of flowering. Maximum activity also varied considerably. Planting density also influences activity of soybean per plant (Sprent 1976). At wide spacing (7 plants m^{-2}), a peak of activity occurred at about the time of full flowering but at very high densities (200 plants m^{-2}) the peak was less marked. Other workers with soybean, however, have also

found a marked drop in nodule activity during pod filling (Harper 1974; Lawn and Brun 1974; Thibodeau and Jaworski 1975; Sprent 1976).

There are several hypotheses which may explain the decline: (i) the bacteroids in the older nodules may decay or nodules may be shed; (ii) a decline in photosynthesis may restrict the availability of C to the nodules; (iii) fruits may have a hormonal influence on leaf senescence and abscission which may combine with the mobilization of N from the leaves and impair photosynthetic activity; (iv) N_2 fixation in the nodules may be turned off by an alternative source of N arising from the breakdown of leaf protein; and (v) fruits and nodules may compete for photosynthate.

The Bacteroids in the Older Nodules may Decay or Nodules may be Shed

Decline in the transport of carbon to the nodule during the reproductive stage could result in nodule senescence. Some authors have found that senescence commenced during the early pod filling stage, causing a marked decline in symbiotic N_2 fixation (Pate 1958; Streeter 1972; Weil and Ohlrogge 1972; LaRue and Kurz 1973). Herridge and Pate (1977) showed that in cowpea, net daytime gain of C by the shoot rose to a maximum at flowering, then declined sharply due to abscission of leaves; shedding the nodules reduced fixation to zero by mid-fruiting. These results indicate that decline in the supply of carbohydrate during grain filling may induce nodule senescence.

A Decline in Photosynthesis may Restrict the Availability of C to the Nodules

Since nodules appear to use carbohydrate for N_2 fixation with similar efficiencies throughout vegetative and reproductive growth, the decline in N_2 fixation per plant commonly observed during the grain filling period may reflect a restricted availability of C to the nodules rather than an efficient use of C within them. The supply of carbohydrate available to nodules constitutes the main factor limiting symbiotic fixation of atmospheric N_2 in leguminous plants (Hardy and Havelka 1976; Lawn and Brun 1974; Bethlenfalvay and Phillips 1977). Sucrose, the major translocation product of photosynthesis must be exported from the leaf and imported by the nodule to fuel the N_2 fixation reaction (Hardy *et al.* 1980). Reduction in this supply will reduce the capacity of a nodule for N_2 fixation. A number of

workers have shown that the rates of photosynthesis of individual leaves of soybeans decline during grain filling (Boote *et al.* 1978; Mondal *et al.* 1978). Loss of RuBP-case during fruiting may be a primary event responsible for the decline in photosynthesis (Wittenbach *et al.* 1980). Other factors such as *in vivo* regulation of photosynthetic enzymes, stomatal aperture, or changing chloroplastic structure and thylakoid membrane properties may play important roles in photosynthetic rates in senescing leaves (Friederich and Huffaker 1980; Nooden 1980; Jenkins and Woolhouse 1981).

Fruits may Influence Leaves and Nodules through a Hormonal Signal

Effects of changes in hormonal balance during pod filling on NA have not been investigated thoroughly. This could be a major factor in determining the effect of flowering and fruit formation on N_2 fixation in different species, perhaps by controlling the competition for photosynthates between the different metabolic sinks of the plant. In many legumes transition from the vegetative to the reproductive phase of development is associated with a marked increase in the rate of symbiotic N_2 fixation. The work reported by Peat *et al.* (1981a) which involved the removal of reproductive parts of soybeans at different stages of their development showed that an increase in N_2 fixation rate was primarily due to the presence of flower buds. The marked effects on vegetative growth of removing the reproductive parts suggests that the mechanism involved in the promotion of N_2 fixation may be hormonal. It is not clear to what extent variation in supply and demand for C and N by different organs causes these declining leaf and nodule functions, nor whether the changes are mediated through specific adjustment in the gradients of particular translocated growth regulator (Nooden and Leopold 1978), which in turn regulate photosynthesis or the onset of senescence. A grafting experiment with soybean (Malik 1983) has demonstrated that post-anthesis decline in N_2 fixation is reversible and that changes in the shoot other than in the provision of carbohydrates may regulate nodule senescence. All known plant hormones have been implicated in either promotion or inhibition of leaf senescence (Woolhouse 1982). While a role has been suggested for some of these substances in nodule senescence, there is

little convincing evidence for a specific interaction between the nodule and rest of the plant in this way (Sutton 1983).

N_2 Fixation in the Nodules may be Turned off by an Alternative Source of N Arising from the Breakdown of Leaf Protein

Grain legume plants require large amounts of N during grain filling and although a proportion of this comes from symbiotic N_2 fixation the rate of fixation is commonly found to decline early in pod filling when the demand for N for seed formation is at a maximum. At the same time there is a decrease in N concentration of all vegetative parts of the plant consistent with the 'self-destruct' hypothesis of Sinclair and de Wit (1976), namely that the transfer of large amounts of N from the vegetative tissues in order to support seed growth leads to a reduction in photosynthetic activity, which in turn causes leaf and nodule senescence (Boon-Long *et al.* 1983). However, the increase in soluble N arising from protein breakdown in the leaf may itself turn off N_2 fixation.

Fruits and Nodules may Compete for Photosynthate

Results of some experiments are consistent with the hypothesis that fruit and nodules may compete for photosynthate during the grain filling period. Lawrie and Wheeler (1974) found that both NA and the accumulation of ^{14}C-labelled photosynthates in the nodules of pea plants in N-free culture reached maxima shortly before flowering and fruit development. During the period from flowering to fruiting, NA and the accumulation of labelled ^{14}C-photosynthate in the nodules both declined by 60% whereas the photosynthesis of the whole plant doubled. These results suggest that the nutritional demands of the reproductive process may starve the nodules of assimilates necessary to support optimum levels of NA. Blomquist and Kust (1971) and Hume and Criswell (1972) also found that only a very small amount of ^{14}C-label photosynthates had been recovered from the roots and nodules after pod filling commenced.

In contrast, Hardy *et al.* (1968; 1971) found that the AR rates of field grown soybeans did not rise substantially until flowering, with a decrease in rate occurring only at senescence. Other field work supports these findings that AR rates do not decline until grain

growth is nearly completed and the plant begins to senesce (Klucas 1974; Thibodeau and Jaworski 1975; Sloger *et al.* 1975; Duke *et al.* 1979; Nelson and Weaver 1980; Nelson *et al.* 1984; Denison and Sinclair 1985. Hardy *et al.* (1971) concluded that N_2 fixation in soybean is tailored to demand, the main period of activity being pod filling in contrast with other results (Harper 1974; Lawn and Brun 1974; Thibodeau and Jaworski 1975; Sprent 1976). The rate of NA and the duration of each phase of the seasonal profile appears to vary with the legume species, the cultivar, the *Rhizobium* strain and the environment.

2.3. Effect of Disbudding on Nitrogenase Activity

One hypothesis which may explain a decline in NA during the reproductive stage is that developing grains and nodules compete for photosynthate. This can be examined by disbudding experiments in which flower buds are removed from the plant. Some authors have reported that disbudding or depodding results in a large increase in the accumulation of photosynthate in the nodules and in a delay in plant senescence which in turn promotes nodule development and NA (Pate 1958; Roponen and Virtanen 1968; Lawn and Brun 1974). Photosynthate in the nodule is considered to decline during reproductive growth (Blomquist and Kust 1971; Hume and Criswell 1972; Lawrie and Wheeler 1974). Others have reported that disbudding prevents an increase in NA and nodule weight which normally occurs during fruit development of soybean (Hardy *et al.* 1968; Hardy *et al.* 1971; Mague and Burris 1972). It has been suggested that an increase in NA reflected the plant's demand for nitrogen (Hardy *et al.* 1968; Lovell *et al.* 1972) and/or hormonal control (Peat *et al.* 1981a). The reasons for these differences both between species and in the same species under different conditions of growth, are not known. It may be that the mechanism differs in different species. If diversion of photosynthate away from the root system to the reproductive parts is the critical factor in the reduction in NA, then disbudding or depodding will be expected to stimulate NA. If NA increases as the plant demand for N increases during the reproductive stage, disbudding or depodding will result in a fall in NA by reducing the demand for N if this is a major mechanism for the control of NA.

The extent to which photosynthesis of the plant can satisfy the demands for assimilate by different metabolic 'sinks' has not been clarified in relation to changes in NA and the accumulation of photosynthate in the nodules during the reproductive stage.

2.4. Nitrogen Redistribution during Grain Filling

The movement of N from vegetative parts and pod to grain is well documented in grain legumes (Neves *et al.* 1981; Peoples *et al.* 1983; Warembourg and Fernandez 1985) as well as in wheat (Peoples and Dalling 1978; Peoples *et al.* 1980; Dalling 1985), but the amount of N which is distributed to the grain, varies. Several workers have reported that 50-64% of the nitrogen in the grain of soybean comes from the redistribution of N from the vegetative plant parts during the pod filling period (Hanway and Weber 1971a, b; Egli *et al.* 1978). The remainder is presumed to originate from N assimilated during grain filling development and mechanisms exist for the rapid flux of fixed N_2 through vegetative structures into grains (Hanway and Weber 1971b). A similar dependence on redistributed N in the grain of cowpea and chickpea has been reported (Eaglesham *et al.* 1977; Peoples *et al.* 1983), but only a small contribution of leaf N to grain occurred in broad bean (Sprent and Bradford 1977) compared to cowpea, chickpea or soybean. Hanway and Weber (1971a, b), Zeiher *et al.* (1982) and Loberg *et al.* (1984) revealed that in soybean the leaves are more important than other sources of mobilized nutrients. Nelson and Weaver (1980) and Nelson *et al.* (1984) found that soybean plants could obtain all their N from fixation with only a minimal redistribution of leaf N during growth of the seed.

It is clear that accumulation of protein in the developing grains of legume plants may be associated with the mobilization of N from senescing vegetative organs. The extent of reliance of grain on vegetative N is obviously greater if little or no N is available from the soil and/or from N_2 fixation. If N_2 fixation is to continue into late fruiting, sufficient photosynthate must be available to meet the demands of the nodules as well as the demands of the developing grain, so abscission of leaves must be delayed and more specifically, the degradation of RuBP-case must be restricted (Peoples and Dalling 1978; Nooden 1980; Peoples *et al.* 1980). The interaction of nodules, leaves, and developing fruits in this syndrome represents one of the least understood areas of legume functioning.

2.5. Inoculation

Inoculation is the practice of increasing the number of rhizobia in that region of the soil where legume root hairs will develop to ensure the early formation of nodules with ability to fix nitrogen. Infection of leguminous plants can occur naturally but it often depends on the application of rhizobia to the seed or soil at sowing (Roughley *et al.* 1983). The presence of a compatible strain of *Rhizobium* in the soil will result in nodulation.

Legume inoculants are needed to meet two agricultural objectives. First, inoculants are often required to permit the initial establishment of a legume in soils lacking compatible rhizobia. The second is to replace an inefficient population of rhizobia with a more efficient one. It is accepted that legumes sown in soils rich in available nitrogen or well supplied with effective rhizobia do not need inoculation. It is also clear that unfavorable soil type and soil pH, extremes of temperature, and soil water, all affect rhizobial population and nodule formation adversely (Date 1970).

Inoculation of seed can be improved by pelleting, in which the inoculant is combined with lime, gypsum, or clay and applied to the seed with an adhesive. This technique has been used extensively in Australia. It has the advantages of: (i) permitting modification of the soil environment immediately adjacent to the seed; (ii) allowing application of more rhizobia to the seed; and (iii) protecting rhizobia from inhibitory substances released by the seed or combined with the seed at planting (fertiliser, pesticides etc) (Peterson and Loynachan 1981).

A liquid suspension of rhizobia can also be used as a seed inoculant although rhizobia applied in this way die more rapidly than those applied as an aqueous suspension containing peat (Burton and Curley 1965). Aqueous suspensions are usually applied directly to the soil (Schiffmann and Alper 1968; Weaver and Frederick 1974; Scudder 1975).

2.6. Root Nodules

The nodules of legumes are highly specialised structures which arise as the result of a sequence of interactions between the host plant and the invading micro-organism (Meijer 1982; Postgate 1987). Amino acid, tryptophan or homoserine (specific to pea), is released as soon as lateral roots begin to emerge and stimulate the growth of rhizobia (van Egeraat 1975). The root hair curls or branches and bacteria of the right type align on the root hair.

Lectins produced by the host are capable of binding the bacteria to the root hair surface (Law and Strijdom 1977). The *Rhizobium* which nodulates the Vicieae is always fast-growing (Sprent and Minchin 1985). The bacteria enter the host cell by the formation of an infection thread. There are some differences between infection and nodulation in the tribes Phaseoleae and Vicieae. After the infection thread has passed through the cortical cells, the cells of the inner cortex, endodermis, and pericycle begin to divide and become infected by the advancing infection thread. The planes of the division are more ordered in nodules of the Vicieae than in those of the Phaseoleae and a meristem and differentiation region can soon be distinguished (Sprent and Minchin 1985). In the Vicieae the infected cells do not divide, rhizobia are released and develop into N_2 fixing bacteroids. Nitrogenase and leghaemoglobin are produced for the reduction of N_2 to NH_3 and the transport of O_2 respectively. Nodules of the Phaseoleae have determinate growth, the vascular strands fuse at the apex forming a closed loop of the root stele. Nodules of the Vicieae have an apical meristem and indeterminate growth; the branches of the root stele enter and dichotomise within the nodule. The gaseous exchange with the soil atmosphere in the Vicieae is via intercellular spaces scattered over the entire nodule surface but in Phaseoleae gas exchange is via lenticels (Sprent 1980; Sinclair and Gaurdiaan 1981).

Nodules vary widely in their ability to fix N_2 (Wynne *et al.* 1980). Variation in effectiveness is poorly understood but several environmental factors are known to be involved (Lie 1971). Completely ineffective nodules may be found which neither contain haemoglobin nor fix N_2 and which may therefore drain a host plant of resources (Sprent 1979). Developmentally, ineffective nodules may have a blockage in the final differentiation of bacteroids (Bassett *et al.* 1977). Effectiveness and all other stages in nodule development are under the genetic control of both host and *Rhizobium* (Nutman 1969; Jordan 1974; Holl 1975).

Ineffective nodules are usually smaller than effective ones of the same species, which may be due to an alteration in level of growth regulators. Ineffective nodules of pea induced by a mutant strain of *R. leguminosarum* showed a decline in cytokinin levels earlier than effective nodules on the same cultivar (Syono *et al.* 1976; Newcomb *et al.* 1977). This

decline was correlated with a decline in mitotic activity in the nodule meristem from which most nodule cells arise and thus could partially explain the smaller size of the ineffective nodules (Newcomb 1981).

Hagedorn (1979) found that inoculation with two effective strains of *R. trifolii* in the field in southwestern Oregon significantly increased both dry weight and total N in *Trifolium subterraneum* when uninoculated sub clover plants became nodulated ineffectively. Furthermore, the increase in N_2 fixation and dry matter production through inoculation was most pronounced and consistent in soils that received phosphorus, sulphur, and molybdenum fertiliser in accordance with soil test recommendations. Thus, effective N_2 fixation depends not only on the presence of competent strains of *Rhizobium*, but also on adequate supplies of nutrients so that the plant can grow satisfactorily (Gibson 1977).

Effectiveness may also have a more straight-forward basis: for example, strains that possess an uptake hydrogenase (Hup^+) may be more effective than similar strains which lack this enzyme (Dixon and Wheeler 1986). Hup^+ may result in a conservation of energy by recovering the H_2 evolved by nitrogenase. Emerich *et al.* (1979) reported that strains having this system form ATP during H_2 oxidation and compensate partially for the ATP-dependent evolution of H_2 catalysed by the nitrogenase enzyme complex (Bulen and LeComte 1966). Thus, Hup^+ *Rhizobium* strains generally are more effective symbionts than those with Hup^- phenotypes (Albrecht *et al.* 1979; DeJong *et al.* 1982).

2.7. The Role of the Cotyledons

Cotyledons are important for the normal development of legume seedlings (Vierskov 1985), although it has been reported that it is possible to excise up to 80% of the cotyledons of soybean without affecting plant growth and development (Kester 1953; Wightman and Thimann 1980; Ndunguru and Summerfield 1975).

Results concerning the effect of cotyledons on nodulation are equivocal. Peat *et al.* 1981b; and Thomas and Hungria (unpublished) found that cotyledons of *G. max* and *P. vulgaris* contain promoting factors which could be nutritional and /or hormonal. The cotyledonary inhibitor may be abscisic acid (ABA). Isogai *et al.* (1967) crystallized ABA from fresh pea seeds where most of the compound was located in the cotyledons. ABA has

been chemically identified in lupin cotyledons (Cornforth *et al.* 1966). Bowen and Hoad (1968) demonstrated that ABA was translocated in the phloem of willow. Finally, exogenous ABA is a potent inhibitor of nodulation in peas, and appears to act by reducing cytokinin stimulated cortical cell mitosis (Phillips 1971) .

In the early stages of nodulation, ABA could act by modifying permeability (Glinka and Reinhold 1971) and hence root exudation (Markhart 1982). Although there is no direct evidence to substantiate this, treatment with ABA has been observed to retard the rate of acidification of the culture medium and to affect root turgidity in *V. faba* (Bano *et al.* 1983). Later in seedling development, when the endogenous ABA content reached a maximum in the cotyledons and root, little metabolism of exogenous radio-labelled ABA occurred and applied ABA could inhibit nodule development by inducing root dormancy (Phillipson and Coutts 1979).

ABA affects the N_2-fixing ability of the nodule by delaying bacteroid tissue formation, thereby temporarily reducing the specific activity of nitrogenase relative to the controls. At later stages of nodule development in *V. faba*, two compensatory effects were observed *viz.*: an increase in both the amount and the longevity of the nodular bacteroid tissue (Bano *et al.* 1983). These authors also found an inhibitor of root nodulation in *P. vulgaris* cotyledons. This was demonstrated by the effects of cotyledon excision on the radial location and development of nodules on the primary root. Exogenous ABA affected shoot, leaf, and root growth, delayed nodule development, and the number of nodules per plant.

There is some evidence that cotyledons supply a factor required in the bacterial-plant interaction. Thornton (1929) reported that removal of both cotyledons in lucerne severely reduced nodulation. Schaffer and Alexander (1967) extracted a substance from *P.vulgaris* cotyledons which stimulated nodulation on adventitious bean roots *in vitro*.

Phillips (1971) found an inhibition of nodulation by pea cotyledons. When one cotyledon was removed before germination, there was a highly significant increase in nodulation on lateral roots. He suggested that a cotyledonary inhibitor acts at a step between the infection process and the appearance of a macroscopic nodule. It appears that cotyledons contain a substrate for the growth of the seedling and an unknown inhibitor for nodulation and therefore for N_2 fixation.

2.8. The Structure and Function of the Enzyme Nitrogenase

The enzyme complex which reduces nitrogen to ammonia in the bacteroid of legume root nodules is known as nitrogenase. The general structure of the nitrogenase complex is remarkably constant among different nitrogen-fixing organisms (Roughley *et al.* 1983). It is a large molecule with several subunits (Burns and Hardy 1975). There are two proteins often referred to as Fe protein or component II (molecular weight around 60 kDa) and the MoFe protein or component I (molecular weight around 200 kDa) (Burgess 1984; Orme-Johnson 1985). The two parts of the enzyme have distinct roles: for this reason Hageman and Burris (1978) suggested they be called nitrogenase reductase and nitrogenase for components I and II, respectively.

The three requirements for the overall reduction of nitrogen to ammonia are as follows. Firstly, a source of low redox potential reductant (approximately –430eV) is needed, which in a bacteroid is probably a ferredoxin (Carter *et al.* 1980). It donates electrons singly to the nitrogenase reductase. Secondly, 12-30 ATPs are necessary for the reduction of each molecule of N_2. ATP is produced by oxidative phosphorylation in bacteroids and at least some of it, in the form of its Mg complex, unites with the reduced nitrogenase reductase (Eady *et al.* 1980; Hageman and Burris 1980; Burgess 1984). Thirdly, N_2 is essential to join with nitrogenase almost certainly at its Mo-containing centre. The N_2-nitrogenase then unites with the reduced nitrogenase reductase-ATP-Mg to form the active nitrogenase complex. Electrons flow to N_2, which is reduced in steps until NH_3 is formed (Orme-Johnson *et al.* 1972; Eady *et al.* 1972; Smith *et al.* 1981; Burgess 1985).

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. The role of ATP in nitrogenase, however, is far from clear, and the absolute requirement of nitrogenase function for ATP is difficult to rationalise on the basis of energy needs because the overall reduction of N_2 to NH_3 is thermodynamically favourable.

Supplies of oxygen and of carbon skeletons are most important for the nitrogenase system to function properly in the bacteroid. Oxygen is vital for the production of ATP, but

it irreversibly inactivates the nitrogenase enzyme complex, leghaemoglobin maintains a rapid flux of oxygen but at a low concentration (Sprent 1979).

The supply of carbon skeletons originates mainly as photosynthate in the leaves but this may be supplemented by CO_2 taken up directly into nodules. The latter has been demonstrated in *V. faba* by Lawrie and Wheeler (1975) although Wheeler (1978) could not find a close relationship between nitrogen fixation and carboxylation reaction. PEP carboxylase activity could form part of a system for the removal of CO_2 produced by nodules (Wheeler 1978). Christeller *et al.* (1977) and Cookson *et al.* (1980), however, reported that CO_2 taken up by nodules is incorporated into amino acids or other reduced nitrogen compounds. Thus the possibility remains that carboxylation reactions within nodules are an integral part of nodule economy.

Carbon compounds in the nodules are used for various purposes. There are respiratory requirements for both host cells and bacteroids (Sprent 1979), the latter including generation of ATP and reductant for nitrogenase activity. It is not known which particular compounds are supplied to the bacteroids. Lawrie and Wheeler (1975) could not detect sucrose, but found that when $^{14}CO_2$ was fed to the shoots, $^{14}CO_2$ appeared in the amino acids in the nodules within 30 min. Fyson and Sprent (1982), in contrast, found large amounts of sucrose as well as various other carbohydrates in *V. faba* nodules.

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). 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, Thornely and Eady (1977) and Thorneley and Cornish-Bowden (1977) have suggested second roles for the ATP which may be either a once-only activation of nitrogenase toward the reduction of N_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). The role of ADP and/or the ADP/ATP ratio in nitrogenase control remains obscure.

Haaker *et al.* (1974), Haaker and Veeger (1977) and Veeger *et al.* (1981) reported that full inhibition of nitrogenase activity can be achieved by lowering the energised state of the cytoplasmic membrane without affecting the intracellular ADP/ATP ratios.

2.9. Substrates for Nitrogenase

Nitrogenase catalyses the reduction of a variety of substrates, most being small molecules which, like N_2, contain triple bonds (Dalton and Mortenson 1972; Burns and Hardy 1975; McKenna and Huang 1979). Alternative substrates for nitrogenase, including C_2H_2, nitriles, isonitriles, as well as evolution of H_2 from H^+ have been used in recent years to measure N_2 fixation.

The AR assay procedure proposed by Hardy and Knight (1967) involves the nitrogenase-catalysed reduction of C_2H_2 to C_2H_4 coupled with sensitive gas chromatographic analyses, and is based on the inhibition of N_2 fixation by C_2H_2 (Schöllhorn and Burris 1966) and the reduction of C_2H_2 to C_2H_4 (Dilworth 1966). Hydrogen evolution (HE) has also become a routine assay for NA and the measurement by gas chromatography, of the release of H_2 by nodules may be a reliable assay for N_2 fixation when insignificant H_2 uptake by hydrogenase occurs (Fuchsman and Hardy 1972).

The reduction of C_2H_2 to C_2H_4 requires two electrons compared with the six required for the reduction of N_2 to $2NH_3$. Thus the stoichiometric relationship between C_2H_2 and N_2 reduction theoretically requires 3 moles of C_2H_2 to be reduced for each mole of N_2 reduced. Early cell-free preparations of nitrogenase revealed, however, that hydrogen gas (H_2) was released concurrently with nitrogen reduction, and that ATP was required for this H_2 evolution (Bulen *et al.* 1965; Bergersen 1966; Koch *et al.* 1967). Furthermore, Schubert and Evans (1976) and Evans *et al.* (1980) reported that as much as 60% of the electron flow through nitrogenase *in vivo* may result in proton reduction.

Quantitative field tests have shown that the C_2H_2/N_2 conversion factor varies from 2 to 25 (Hardy *et al.* 1973). Possible reasons for such variations include: (i) a greater proportion of electrons being diverted to H_2 production when N_2 rather than C_2H_2 is the substrate; and

(ii) C_2H_2 inhibiting several metabolic processes thereby exposing nitrogenase to conformation changes in its presence (Yates 1980). Such effects must distort any simple C_2H_2/N_2 conversion ratio.

The electron flux through nitrogenase has been assumed to be independent of the substrate employed (Ljones 1973). Thorneley and Eady (1977), however, showed that the total electron flux increased in the presence of C_2H_2 compared with 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 Shanmugan (1977) reported that electron turnover for both nitrogenase and glucose consumption by intact cultures of *Klebsiella pneumoniae* under an atmosphere of argon/O_2 was only 75-80% of the corresponding rate under N_2. Apte *et al.* (1978), working with blue-green algae, demonstrated conformational changes in the nitrogenase complex *in vivo* by preincubation under C_2H_2 and Shearer *et al.* (1980) reported up to a four-fold increase in light-induced H_2 production when C_2H_2 pretreated cells were assayed under AR. Although the significance of the results of pre-incubation experiments to the use of AR assay remains uncertain, the results indicate that different substrates may have different effects on the conformation of nitrogenase and on its function.

2.10. The Importance of Hydrogen Metabolism

The evolution of H_2 during N_2 fixation by soybean nodules was reported by Hoch *et al.* (1957) and evidence that this H_2 could be recycled in nodules of *R. leguminosarum* on pea was found by Dixon (1967). During the past ten years there has been a proliferation of investigations concerning H_2 metabolism in N_2 fixing organisms. The practical goal of much of this research has been to increase the efficiency of energy utilization by N_2 fixing organisms, particularly symbioses that contribute significant quantities of fixed nitrogen to the environment.

Hydrogen Evolution during N_2 Fixation

All purified nitrogenase preparations which have been thoroughly investigated evolve about 1 mole of H_2 per mole of N_2 reduced under optimum conditions (Yates 1980). The overall reaction is formulated as follows:

$N_2 + 8H^+ + 8e^- + 16\ ATP \text{ ---> } 2NH_3 + 16ADP + 16\ P_i + H_2$

In the absence of N_2, electrons are transferred to protons and H_2 produced. When nitrogenase catalyses the reduction of N_2 to $2NH_3$ with evolution of H_2 or when the enzyme catalyses the reduction of protons to H_2 in the absence of N_2, about four moles of ATP are consumed per pair of electrons transferred (Yates 1980). The nitrogenase reaction consumes energy in the forms of ATP and reductant for nitrogenase-dependent H_2 evolution, and this accounts for about 25% of the energy consumed in the overall nitrogenase reaction.

Hydrogen Recycling

Some strains of several species of *Rhizobium* form nodules which do not evolve H_2 during N_2 fixation. This phenomenon was first explained by Dixon (1967, 1968, 1972) who demonstrated that two *R. leguminosarum* strains possessed a capacity to synthesise a system for activating H_2 and oxidizing it, thus preventing H_2 from escaping into the atmosphere.

It is now known that only a minority of strains among several species of *Rhizobium* are capable of recycling H_2 (Schubert and Evans 1976). Nodules from 11 different species of legumes and actinorrhizal species which lacked the H_2 oxidation system (Hup^-) lost an average of 32% of the nitrogenase electron flux as H_2, whereas the comparable figure for Hup^+ nodules with efficient H_2 recycling was only 3.8% (Evans *et al.* 1981). Nodules formed by several Hup^+ *Bradyrhizobium japonicum*, *R. leguminosarum* and cowpea *Rhizobium* strains recycle essentially all of the H_2 produced by the nitrogenase system (Schubert *et al.* 1977; Albrecht *et al.* 1979; Nelson and Salminen 1982).

Several potential advantages of an efficient H_2 recycling system in N_2-fixing organisms proposed by Dixon (1972) have been discussed by Eisbrenner and Evans (1983). If it is assumed that the energy supply available to the N_2 fixing process within nodules limits N_2 fixation, then the recovery by H_2 recycling of some portion of the energy expanded

in the evolution of H_2 by nitrogenase would be expected to be beneficial. Nelson and Salminen (1982), however, reported that only five of 14 strains of *R. leguminosarum* efficiently coupled H_2 oxidation to ATP synthesis. They concluded that a main factor of the uptake hydrogenase system was protection of the nitrogenase from O_2 damage, since H_2 oxidation consumed O_2.

2.11. Factors Limiting N_2 Fixation

The energy for nitrogen fixation in legumes comes from respiration by bacteroids inside specialised cells of the host plant within the root nodules (Sprent 1979). Respiration consumes both photosynthate and O_2, so either could conceivably limit nitrogenase activity. Because the enzyme nitrogenase is inactivated by even traces of oxygen (Bergersen 1968; Robson and Postgate 1980) there is a simultaneous requirement for low oxygen partial pressure inside the nodule and adequate flux of O_2 into the nodule.

Hardy and Havelka (1976) summarised the evidence that N_2 fixation may be limited by photosynthate. Short-term measurements using the AR method showed that nitrogenase activity of soybean was often higher during the day than it was at night (Hardy *et al.* 1968; Sloger *et al.* 1975). The lower rates at night were attributed to a shortage of photosynthate, but more recent work has shown that such diurnal change in nitrogenase does not occur in soybean plants at constant temperature (Schweitzer and Harper 1980; Williams *et al.* 1982). In field-grown soybean, the decline at night was small and nitrogenase activity was correlated with soil temperature rather than with photosynthetically active radiation (Denison and Sinclair 1985). These results suggest that a short-term dependence of nitrogenase activity on current photosynthesis is questionable.

Nodule anatomy plays an important role in the protection of nitrogenase from high oxygen concentration, but it also limits the supply of oxygen for respiration. To reach the bacteroids, oxygen diffuses first through the nodule cortex, then through the network of airspaces in the interior, and finally through the cytoplasm of the host cells and into the bacteroid-containing envelopes. Oxygenases on the bacteroid surfaces consume the oxygen and thus provide energy to support nitrogen fixation. Oxygen diffusion in air is faster than in cytoplasm, so the tightly packed inner cortex and the host cell cytoplasm are the only

significant barriers to oxygen (Bergersen and Appleby 1981; Dixon *et al.* 1981; Selker and Newcomb 1985).

Numerous experiments have shown that nitrogenase activity reponds to changes in the external pO_2 to which the nodules are exposed. Moderate increases in external pO_2 lead to immediate increases in nitrogenase activity in soybean (Pankhurst and Sprent 1975; Ralston and Imsande 1982; Witty *et al.* 1984; Weisz *et al.* 1985) and pea (Witty *et al.* 1984) although nitrogenase can be damaged if the increase in pO_2 is too large. Short term decreases in nitrogenase activity at lowered pO_2 have also been reported for soybean (Criswell *et al.* 1976; Wasfi and Prioul 1986). It appears that chickpea (*Cicer arietinum*) demonstrates a similar response to pO_2 (Witty *et al.* 1983).

The experimental data on manipulation of supply leads to the conclusion that nitrogenase activity is limited by both oxygen and photosynthate.

2.12. Diurnal Variation in the Activity of Legume Root Nodules

Several workers have reported that NA as assayed by the reduction of C_2H_2 varied diurnally (Hardy *et al.* 1968; Bergersen 1970). A range of species and growth conditions has now been studied and several attempts made to relate the observed fluctuation to: (i) the daily cycle of change in the environment such as temperature (Schweitzer and Harper 1980; Williams *et al.* 1982; Rainbird *et al.* 1983), vapour pressure deficit (Ayanaba and Lawson 1977),or the intensity and duration of light (Mahon 1977; Eckart and Raguse 1980); (ii) changes in the respiration of the nodulated root (Minchin and Pate 1974); (iii) the carbohydrate status of nodules (Schweitzer and Harper 1980); (iv) the accumulation and export of nitrogenous solutes by the nodules (Minchin and Pate 1974); and (v) H_2 evolution (Rainbird *et al.* 1973). Other workers have reported insignificant diurnal variation in AR (Fishbeck *et al.* 1973; Trinick *et al.* 1976; Haystead *et al.* 1979).

Acetylene reduction is directly proportional to intensity of radiation whether the nodules are of plants grown in the field (Hardy *et al.* 1968; Mague and Burris 1972), or in growth cabinets (Bergersen 1970; Minchin and Pate 1974), or even of non-legumes (Wheeler 1971).

Ayanaba and Lawson (1977) reported the effects of five environmental factors, namely soil, canopy and air temperatures, global radiation and vapour pressure deficit, on variation in the rate of AR in two cowpea and two soybean cultivars at two stages of growth in the field. The results showed two peaks in the AR rate in nodulated cowpea roots, one between 0600-1200 h and another between 1800-2400 h and two minima between 1200-1600 h and between 2400-0600 h. The two soybean cultivars did not show any definite pattern. It was concluded that vapour pressure deficit appeared the most likely factor influencing the decline in AR between 1200-1600 h.

Minchin and Pate (1974) reported that when the relative humidity in growth cabinets was decreased, C_2H_2 reduction in pea plants also decreased because of increased transpiration. A study with soybeans by Huang *et al.* (1975a), however, showed a positive correlation between transpiration and AR but in a subsequent study (1975b) they concluded that this positive correlation was fortuitous and that the observed decline in AR rate resulted from the inhibition of shoot photosynthesis.

It has been argued that the rate of N_2 fixation is primarily controlled by the availability of photosynthate to the nodules (Hardy and Havelka 1976; Pate 1977). Diurnal fluctuation in light flux density, temperature or other environmental factor(s) may affect photosynthate supply and thereby influence N_2 fixation. Experimental treatments of defoliation (Haliday and Pate 1976), daily exposure to high or low irradiance (Bethlenfalvay and Phillips 1977), and short-term changes in light (Gibson 1976) have produced changes in apparent N_2 fixation. Daily changes in temperature may affect source-sink relationships between leaves and root nodules or metabolic pools available to bacteroids.

Temperature is an important environmental variable influencing N_2 fixation rates (Kuo and Boersma 1971; Pankhurst and Sprent 1976). The response of AR by root nodules to incubation temperature differs considerably between species (Dart and Day 1971; Waughmann 1977), although the activity of temperate legume nodules was affected only slightly by decreasing temperature from 20 to 12°C (Dart and Day 1971; Gibson 1971). Masterson and Murphy (1976) reported that AR rate in white clover showed no significant fluctuation between day and night at constant temperature. Similarly in cowpea (Rainbird *et al.* 1983) NA showed a marked diurnal variation in ratio of N_2 fixed to HE when maintained

under conditions of a 12 h day at an air temperature of 30°C (800-1000 μE m^{-2} s^{-1}) and a 12 h night at an air temperature of 20°C. When the plants were maintained under the same diurnal illumination regime but at constant (day and night) air temperature (30°C), the difference was abolished and there was a relatively constant ratio of N_2 fixed to HE. N_2 fixation was largely unaffected over the temperature range from 15-35°C whereas HE increased with increasing temperature. These results imply that if the AR assay were used to estimate N_2 fixation activity, it would misrepresent the diurnal variation which was in fact due to the sensitivity of HE to temperatures and that there was no diurnal variation on N_2 fixation activity. Therefore the measurement of diurnal fluctuation in HE in air and Ar/O_2 to estimate N_2 fixation activity is better than the use of AR which fails to provide information on how electrons are partitioned between protons and N_2. Nevertheless, in almost all studies on diurnal variation of NA, AR has been measured and the electron flux has not been partitioned between N_2 and proton reduction. Haystead *et al.* (1979) concluded that there was no diurnal variation in AR for *T. repens* at constant temperature (15°C) and with 12 h of 370 μmol quanta m^{-2} s^{-1}. When *T. repens* was grown at the same PPFD and day:night temperatures of 20°C:16°C, the AR rate was higher during the day.

Minchin and Pate (1974) studied diurnal changes in pea plants in two environments, both with a 12 h, 27000 lx daylight, but one with a fluctuating temperature-humidity regime, the other with constant temperature and humidity. The fluctuating environment produced the greater fluctuation in transpiration and nodule soluble N, and resulted in more N_2 being fixed during the night than during the day, whereas the constant environment induced a more pronounced decrease in fixation at night. These results were consistent with what is already known for other cultivars of pea (Roponen *et al.* 1970) in that a fluctuating environment allowed greater growth and N_2 fixation than a constant one.

Schweitzer and Harper (1980) found AR rates of soybean to be unaffected by diurnal variation in light treatment, activity being directly related to temperature change from 18 to 27°C. They suggested that the soybean nodule was not carbon limited at night, but that fixed carbon stored during the day supported NA at night at the rate which was modified by temperature. Similarly, Eckart and Raguse (1980) found in subterranean clover that diurnal temperature fluctuations have a much greater effect on apparent N_2 fixation that diurnal light

changes. More recently Denison and Sinclair (1985) found in field-grown soybeans much of the diurnal variation in rate to be accounted for by changes in soil temperature.

Others have reported that under constant temperature, diurnal change in apparent N_2 fixation corresponds to fluctuation in light intensity for soybean (Bergersen 1970) and pea (Mahon 1977). Fishbeck *et al.* (1973) and Hardy *et al.* (1968) observed no significant change in AR by soybeans grown under 16 h photoperiods with diurnal temperature changes in growth cabinets, however, the latter reported diurnal fluctuation of AR in field grown soybean. Differences between legume species and environmental conditions appear to influence the pattern of diurnal variation of AR.

Most techniques used to study diurnal changes in N_2 fixation have been destructive (Mague and Burris 1972; Minchin and Pate 1974; Ayanaba and Lawson 1977) using excised root systems rather than intact, undisturbed plants (Hardy *et al.* 1968; Lawn and Brun 1974; Thibodeau and Jaworski 1975). AR rates of excised systems may differ from those of intact plants (Fishbeck *et al.* 1973; Wych and Rains 1978; Minchin *et al.* 1986; Hansen *et al.* 1987). Denison *et al.* (1983) reported that the size, number and placement of nodules and the extent of leaf photosynthesis of plants grown in a chamber for *in situ* measurements of AR assay in an open system for up to three weeks were similar to other plants in the field and there were no abnormal visible effects, e.g., colour, height and organ abscission.

There is evidence that C_2H_4 is a plant growth hormone (Leopold and Kriedemann 1975) and adverse effects of C_2H_4 on nodulation and N_2 fixation have been reported (Grobbelaar *et al.* 1971; Haystead *et al.* 1979). Repeated exposure of the same plants to C_2H_2 by using an open system may reduce the effect of C_2H_2/C_2H_4 on NA. Sinclair (1973) reported no serious effect on the subsequent growth and N content of white clover plants after repeated exposure. Eckart and Raguse (1980) found that repeatedly assayed *T. subterraneum* plants showed a gradually increasing AR rate. The effect of prolonged exposure to C_2H_2 on nodulated root respiration or NA may vary with the species of the host plant (Apte *et al.* 1978).

There is a considerable benefit in observing N_2 fixation rates of the same plant through time (minutes to days) as conditions change either within the plant or the environment. The potential for repeated observation becomes especially important since the high variability in

AR observed among plants (Thibodeau and Jaworski 1975) makes interpretations of a number of single-point samples difficult. A non-destructive assay allows responses to treatment of individual plants to be measured through time, thus minimizing the problem of plant-to-plant variability.

2.13. The Effect of Combined N on Nodulation, Nitrogenase Activity, and Plant Growth

The two types of N nutrition, symbiotic fixation and assimilation of combined N, have been compared in many experiments, either under controlled conditions, supplied with nutrient solutions (Harper 1974; Hill-Cottingham and Lloyd-Jones 1980; Phillips *et al.* 1981; Finke *et al.* 1982; Silsbury 1984; Davidson and Robson 1986; Silsbury *et al.* 1986) or in the field with or without fertiliser (Hanway and Weber 1971a, b; Bhango and Albritton 1972; Harper 1974). Generally the results show that plants which assimilate combined N have a dry matter yield and a total N content similar to or slightly greater than plants which fix atmospheric N_2 and yields of grain are generally higher. Few experiments lead to the opposite conclusion (Summerfield *et al.* 1977).

NO_3^- assimilation, like symbiotic N_2 fixation, requires energy to provide the ATP and reducing power to reduce NO_3^- to NH_4^+ and to incorporate the NH_4^+ into organic compounds. The energy costs to legumes using NO_3^- has been estimated in several studies (Atkins *et al.* 1978; Pate *et al.* 1979; Ryle *et al.* 1979a, b; Neves *et al.* 1981). Almost all experiments, however, have been done by comparing energy costs between non-nodulated NO_3^--fed plants and nodulated, N_2-fed plants. Pate *et al.* (1979) estimated CO_2 loss per unit N assimilated in nodulated root of white lupin as 10.2 mg C/mg N, and 8.1 mg C/mg N by non-nodulated, NO_3^--fed roots. Neves *et al.* (1981) estimated C consumption by nodulated and non-nodulated cowpea roots as 8.0 and 4.5 mg C/mg N assimilated, respectively. Atkins *et al.* (1978) reported that CO_2 losses of below-ground parts of cowpea were less in non-nodulated, NO_3^--fed plants than in nodulated, N_2-fed plants. There is other evidence that symbiotic N_2 fixation costs cowpea plants more fixed C than mineral N assimilation provided by several authors (Herridge and Pate 1977; Ryle *et al.* 1979a, b). Ryle *et al.* (1979a) also reported for soybean, cowpea and white clover that plants fixing all of their N

respire 11-13% more of their carbon than equivalent plants lacking nodules and using NO_3^-. The general conclusion from these results is that the energy costs to the legume of using N_2 gas are slightly higher than the costs of using combined N.

There have been many attempts to increase the yield of nodulated legume crops by the application of combined N. Beverley and Jarrell (1984) studied the response of cowpea to N application and reported that fertilizer increased seed yield, with side-dressing being more effective than a pre-planting application. A side-dressing at flowering was more effective than application during pod filling. They proposed that the yield increase obtained was due to decreased consumption of photosynthate by nodules following inactivation of N_2 fixation by N assimilated from the fertilizer.

The average seed yield of effectively nodulated cowpeas was 38% greater than that of non-nodulated plants when both received applied N at concentrations ranging from 60-240 ppm during one of three periods: emergence to first flower, first flower to mid pod-filling, or mid pod-filling to maturity (Dart *et al.* 1977).

Application of 200 kg N/ha as a mixture of NH_4NO_3 and $CaCO_3$ to the soil as split dressing did not affect yield of *V. faba*, but 80 kg N/ha as urea applied in four foliar sprays increased yield by 8.6% (Day *et al.* 1979). Richard and Soper (1979) reported that the yield of faba bean (*V. faba* var. *minor*) in Western Canada was not affected by N fertiliser up to 600 mg N/pot (200 mg N/kg soil) applied at the seedling stage, by 300 mg N/pot applied in four 75 mg portions, nor by single, mid-season applications of 300 mg/pot. Only the highest rate of N employed, 900 mg N/pot at sowing, significantly increased yield by 13.2%. Protein content and total N uptake into the shoots were unaffected by all N applications used.

Attempts to increase yield of faba bean by applying fertiliser N are also reported by McEwen (1970a, b). Nodulation was reduced by 50% when 125 and 375 kg N/ha were applied to the seed bed and the effect on yield was only small and non-economic. When N was applied at sowing and 11 weeks later, yield increases were generally greater. Richard and Soper (1979) reported that when nodulated with effective strains of rhizobia, faba bean obtained its N from both the soil and symbiotic fixation. This result suggests that these two

sources of N are able fully to satisfy the N demand of faba beans throughout the entire growth cycle so application of fertilizer N may not affect seed yield.

Day *et al.* (1979) found that recovery of ^{15}N in the grain of faba bean varied from 28% when applied during vegetative growth to 8% when applied in the reproductive phase and 15% in the foliar spray. This result indicates that time of application of N influences the seed yield in faba bean. It can be concluded that the timing, type and amount of N application, and environmental factors influence seed yield, and the effect may vary between the host plant and *Rhizobium* species combination.

The degree of inhibition on the N_2 fixation activity by combined N varies in different legume-*Rhizobium* combinations (Gibson 1971; Munns 1977; Evans 1982) depending on the amount of combined N applied and the time of application. Low concentrations of combined N may promote nodulation whilst high concentrations are almost always depressive. Addition of NO_3^- has been reported to diminish the formation of new nodules when applied to seedlings by inhibiting attachment of rhizobia to the root (Dazzo and Brill 1978), by inhibiting the formation of new root hairs (Pate and Dart 1961), by preventing the induction of root hair curling by bacteria (Munns 1968), by blocking the initiation of infection threads (Dazzo and Brill 1978; Malik *et al.* 1987), and by limiting the development of nodule mass (Vigue *et al.* 1977; Miller *et al.* 1982). In contrast, Jones *et al.* (1981) reported that the presence of NO_3^- and its assimilation, even at the high level of 21.4 mM, did not inhibit the development of functional nodules of soybean. Neither mass nor AR activity of nodules were reduced by high NO_3^-, but when NO_3^- was applied to actively nodulated plants, it appeared to affect nodule function both at the level of rapid, reversible effects on N_2 fixation and at the level of longer term, irreversible breakdown of tissue organisation (Dart 1977; Silsbury *et al.* 1986).

Nitrogenase activity appears to respond rapidly to the addition of NO_3^-, declining within days after application and rising when NO_3^- is withdrawn in clover (Caroll and Gresshoff 1983; Silsbury *et al.* 1986; Davidson and Robson 1986), in cowpea (Manhart and Wong 1980), and in soybean (Streeter 1985). In contrast, Masterson (1982) reported that application of N fertiliser to a mixed grass/clover sward in a field still reduced AR rate substantially four months later.

Silsbury *et al.* (1986) reported that applying 0.5 mM NO_3^- to an active symbiosis of subterranean clover suppressed NA significantly, and 3 to 5 mM stopped it completely within 7 days. This effect could be reduced by increasing the photon irradiance to the host plant thereby increasing the carbohydrate supply. It was concluded that the presence of soil mineral N in the field may depress the N_2 fixation of subterranean clover, especially in winter when the photon irradiance is relatively low.

Addition of 0.1 mM sucrose stimulated NA of soybean in the presence of NO_3^- and decreased the level of NO_2^- accumulated within the nodules (Stephens and Neyra 1983). NO_2^- is a known inhibitor of several aspects of N_2 fixation (Kennedy *et al.* 1975; Pagan *et al.* 1977; Rigaud and Puppo 1977; Trinchant and Rigaud 1980; Streeter 1982), but Gibson and Pagan (1977) and Manhart and Wong (1980) suggested that NO_3^- may not play a role in the inhibitory effect of NO_3^- on AR.

Additions of low levels of N have been reported to increase seedling growth, vigour and nodulation in many leguminous plants, i.e. soybean, alfalfa, clover, medic, vetch, cowpea, pea and faba bean (Allos and Bartholomew 1959; Richardson *et al.* 1957; Pate and Dart 1961; Dart and Wildon 1970; Oghoghorie and Pate 1971; Hill-Cottingham and Lloyd-Jones 1980; Evans 1982; Yousef and Sprent 1983), although the mechanism is not clearly understood.

Sprent and Thomas (1984) reported that N stress is more common in the Phaseoleae than in the Vicieae since the first N_2 fixed is used for nodule growth rather than being exported to the shoot system. The Vicieae can synchronise the exhaustion of seed reserves of N with the availability of fixed N so that the addition of mineral N at sowing as a 'starter' for improving growth and nodulation is not necessary.

N stress has been observed in *G. max* (Jones *et al.* 1981) and in *P. vulgaris* (Franco and Munns 1982), before nodules begin N_2 fixation. Richard and Soper (1979) reported that initiation of symbiotic fixation occurred rapidly, such that low quantities of available soil and seed N were sufficient during early growth. This explains the failure of low concentrations of N applied at seedling to have a 'starter' effect. These findings support Sprent and Thomas (1984). In contrast, N stress at early growth and the response to a little combined N at germination were also reported by others in Vicieae or other tribes which

have indeterminate nodules: i.e., in medic (Pate and Dart 1961; Dart and Wildon 1970), field pea (Oghoghorie and Pate 1971), faba bean (Hill-Cottingham and Lloyd-Jones 1980; Yousef and Sprent 1983), alfalfa (Richardson *et al.* 1957; Allos and Bartholomew 1959), cowpea (Pate and Dart 1961; Summerfield *et al.* 1977), vetch (Pate and Dart 1961) and subterranean clover (Bauma 1970; Silsbury 1984).

N stress has been observed in soybean when grown at day/night temperatures of 26/22°C (Jones *et al.* 1981). There was a rapid early growth which caused a depletion of cotyledonary reserves of N before nodules became active and thereafter the plants were unable to develop adequate leaf area to support nodule development and functioning. When grown at 22/18°C, however, nodule development was less inhibited and cotyledonary reserves of N were not depleted before the nodules became active. This result suggests that the growth conditions such as temperature may also influence the depletion of cotyledonary reserves and consequently the occurrence of N starvation.

A critical period in growth for contribution of combined sources of N is the first few weeks after plant emergence, before functional nodules are established in sufficient mass to meet the N requirements for growth (Hardy *et al.* 1971; Mahon and Child 1979). During this period the newly emerged seedling must rely upon its cotyledonary reserves and availability of combined N for absorption by roots. Since the onset of the rapid growth stage in non-inoculated seedlings is directly related to level of available N (Mahon and Child 1979; Raper *et al.* 1977), the cotyledonary reserves provide inadequate N to promote maximum developmental rates.

The potential for combined N to supplement cotyledonary reserves may be limited by inhibition of development of N_2 fixing capacity by combined N (Harper 1971; Hardy *et al.* 1980), by possible effects of temperature during seedling growth on nodule establishment and function (Graham 1979), and by effects of temperature on the rate of N utilisation required to support temperature-dependent growth rates (Raper *et al.* 1977).

It can be concluded that the synchronisation between the exhaustion of seed reserves of N and the availability of fixed N_2 vary with the host and strains of *Rhizobium*, and it is likely that the effectiveness of inoculant plays an important role.

CHAPTER 3

General Methods

3.1. Plant Culture

Plant Material

Faba bean (*Vicia faba* L. cv. Fiord), pea (*Pisum sativum* L. cvs. A102 and Early Dun) and soybean (*Glycine max* (L.) Merr. cv. Clark) were used in this study. 'Fiord' was selected during the 1970s at the Waite Agricultural Research Institute from material of Mediterranean origin. 'Early Dun' is a widely used cultivar introduced into SA from UK. 'A 102' is a cross between Derrimut and an introduced line from Turkey and has resistance to black spot. Seed was surface-sterilised by shaking with 80% ethanol for 15 s, then with 0.2% $HgCl_2$ for 3 min, followed by several changes of sterile water.

Rooting Medium

The rooting material was 'oil dry', a fritted clay normally used to absorb water from garage floors. It is chemically inert and easily removed from roots. After free drainage, the material holds about 0.31 by volume of available plant water and has an air filled porosity of 0.28 (Van Bavel *et al.* 1978). The clay thus has a large quantity of extractable water over a broad range of pressure potential with excellent gas exchange capacity. It is very suitable for growing plants under a 'pour-through' nutrient system (Silsbury 1979; Silsbury *et al.* 1984).

Pots and Arrangement

Square (15 x 15 cm) black plastic pots of 1 l capacity were used. These were steam sterilised, washed with 80% ethanol, dried, filled with steam-sterilised clay which was then wetted with deionised water. In most experiments plants were grown without inter-plant competition but when established at a plant density of one (faba bean) or two (pea) per pot and arranged in a compact block, plant densities of 44 and 88 m^{-2} respectively could be created. These correspond to densities commonly used in the field (Baldwin 1979) and represent sowing rates of approximately 200 kg ha^{-1} for both faba bean and pea. Thus,

competitive conditions above ground similar to those in the field could be established in the glasshouse.

Nutrient Solutions

Four basic nutrient solutions were used namely, half-strength Hoagland solution containing NO_3^- at 2.5, 5.0 and 7.5 mM or the same solution with NO_3^- replaced with SO_4^{2-} so that K^+ and Ca^{2+} concentrations remained the same. Nutrient solutions were prepared from stock solutions every few days using deionised water. At least 0.5 l of solution was flooded through each pot each day. Once each week pots were flushed with 1 l of deionised water to avoid the accumulation of salt. KOH was used for adjusting the pH of the solution to 7.0. The compositions of the various nutrient solutions were as follows:

	($-NO_3^-$) solution	($+NO_3^-$) solutions		
	0.0 mM	2.5 mM	5.0 mM	7.5 mM
	(mg l^{-1})	(mg l^{-1})	(mg l^{-1})	(mg l^{-1})
KNO_3	-	84.17	168.34	252.51
$Ca(NO_3)_2.4H_2O$	-	196.59	393.18	589.77
$MgSO_4.7H_2O$	246.38	246.38	246.38	246.38
KH_2PO_4	34.00	34.00	34.00	34.00
H_3BO_3	2.86	2.86	2.86	2.86
$MnCL_2.4H_2O$	1.81	1.81	1.81	1.81
$ZnSO_4.7H_2O$	0.22	0.22	0.22	0.22
$CuSO_4.5H_2O$	0.08	0.08	0.08	0.08
$Na_2MoO_4.2H_2O$	0.12	0.12	0.12	0.12
EDTA	23.82	23.82	23.82	23.82
$FeSO_4.7H_2O$	19.92	19.92	19.92	19.92
K_2SO_4	217.75	145.02	72.51	-
$CaSO_4.2H_2O$	430.00	287.00	143.50	-

Some experiments were conducted in a mist (aeroponic) or a hydroponic chamber in which the nutrient solution was changed daily or every second day. When plants were grown in nutrient solution free of mineral N, they were entirely dependent on N_2 fixation once seed reserves had been exhausted since the oil dry contained no available nitrogen.

Inoculation

Infection and nodulation with *Rhizobium leguminosarum* were usually achieved by applying an excess of commercial peat inoculant (Nodulaid, group E; Agricultural Laboratories Pty Ltd, NSW, Australia) which contained either strain TA 101 or SU 391. Seeds were normally inoculated by applying the peat inoculant as a paste at sowing, and one week after emergence as a slurry in sterile water poured onto the 'oil dry'. For soybean, cultured inoculant of *R. japonicum* CC705 was used. For study of the effectiveness of different strains of rhizobium, cultured inoculants (10 ml) were applied for each plant at emergence and 1 week later.

Controlled Environment Chambers and Glasshouses

Plants were grown in both glasshouses and in growth rooms. Temperatures inside the glasshouse were normally slightly above outside ambient during the day, but much the same during the night. Daily solar radiation inside the glasshouse measured with a 'Kipp' thermopile (Kipp and Zonen, Delft, The Netherlands) was, on average, 0.60 of that incident outside. The radiation, however, was largely diffuse, and produced a more-or-less square wave of uniform light flux density during each day even when there was no cloud. The plants thus experienced about 60% of the solar radiation and slightly higher temperature than would have been the case if grown outside. Sometimes a temperature controlled glasshouse (constant 20°C) was used with natural irradiance and daylength.

Growth rooms were set at 20°C with a 12 h day (0900 h - 2100 h) of 600-1000 $\mu E\ m^{-2}\ s^{-1}$ provided by 400 W sodium vapour 'lucolux' lamps (GTE Sylvania Canada Ltd, Drummondville, PQ, Canada). Light flux density was measured at the top of the plant canopy with a LI-Cor (LI-Cor meter LI-170, Lamda Instrument Corporation, Lincoln, NE, USA).

3.2. Estimation of Nitrogenase Activity, Measurement of Dark Respiration, and Post-Assay Procedures

Reduction of C_2H_2 to C_2H_4 (Dilworth 1966) is widely employed as an assay for estimating nitrogenase activity of nodules, decapitated intact root systems, or intact plants. Since Minchin *et al.* (1983b) claimed that AR assay in a closed system over a period can seriously underestimate the rates of acetylene reduction, an open gas system was designed in the form of a mist chamber for the simultaneous measurement of the rates of C_2H_4 production, respiration, and HE by attached, nodulated roots.

3.2.1. Closed System

Plants were removed from pots by gently washing away the oil dry with water at 20°C. This process took only a few seconds. Excess water was then blotted from the nodulated roots before incubation commenced in 1.06 l 'Agee' glass jars with screw-down metal lids each penetrated by a No 25 subaseal. Assays always started less than 10 min after plants were removed from the growth room or glasshouse. The roots were kept in darkness during assay.

Hydrogen evolution (HE)

HE assay was conducted before AR assay. Gas samples (500 µl) were taken at 10 min and at 40 min.after closure of the jars by 1 ml syringes each fitted with 0.5 mm x 16 mm needle (Terumo Pty Ltd, Melbourne, Vic., Australia) and injected into an L&D Portable Gas Chromatograph. This operated at room temperature (20°C) and was fitted with a semi-conductor sensor and light-emitting diode display (J.A.S. Instruments, Melbourne, Vic., Australia). The carrier gas was air at 50 kPa head pressure. The retention time for H_2 was 28 s. Jars were opened at the end of HE assay and flushed with air prior to commence the AR assay.

Acetylene reduction (AR)

Each jar was sealed and 10% of its volume replaced by C_2H_2 (Commonwealth Industrial Gases Ltd., NSW, Australia). Samples of 500 µl of gas were normally removed

10 min and 40 min after exposure to C_2H_2 in 1 ml syringes fitted with 0.5 mm x 25 mm needles. Samples were injected into a Varian Aerograph model 940 gas chromatograph equipped with a flame ionisation detector (Varian Instrument Division, Walnut Creek, CA, USA) and a column of 80-100 mesh Porapak R (Waters Associates Inc., Milford, MA, USA). Column, detector, and injector temperatures were 50°, 150° and 150°C, respectively. With the carrier gas (N_2) flowing at 65 ml min^{-1}, C_2H_4 eluted in 30 s. The C_2H_4 peaks were displayed on a flat-bed chart recorder ('Omniscribe', Houston, TX, USA).

Standards and calculations

Gas-tight glass syringes (S.G.E. Scientific Pty Ltd, Ringwood, Vic., Australia) were used to withdraw a known quantity of either H_2 or C_2H_4 as a standard. Standards of C_2H_4 were made up in 10% C_2H_2 in air at 20°C since it was found that there was a detectable amount of C_2H_4 in C_2H_2. Standards of H_2 were made as a range of concentrations of H_2 in air since the gas chromatograph did not give a linear response to H_2. The rate of C_2H_4 or H_2 accumulation is given by:

$\mu mol\ C_2H_4 \text{ or } H_2\ plant^{-1}\ h^{-1} = (a/b) \times (1/c) \times (d/e) \times f \times 2$

where

a = known quantities of C_2H_4 or H_2 as a standard (μl)

b = volume of standard jar (l)

c = volume of 1 mole of gas at assay temperature

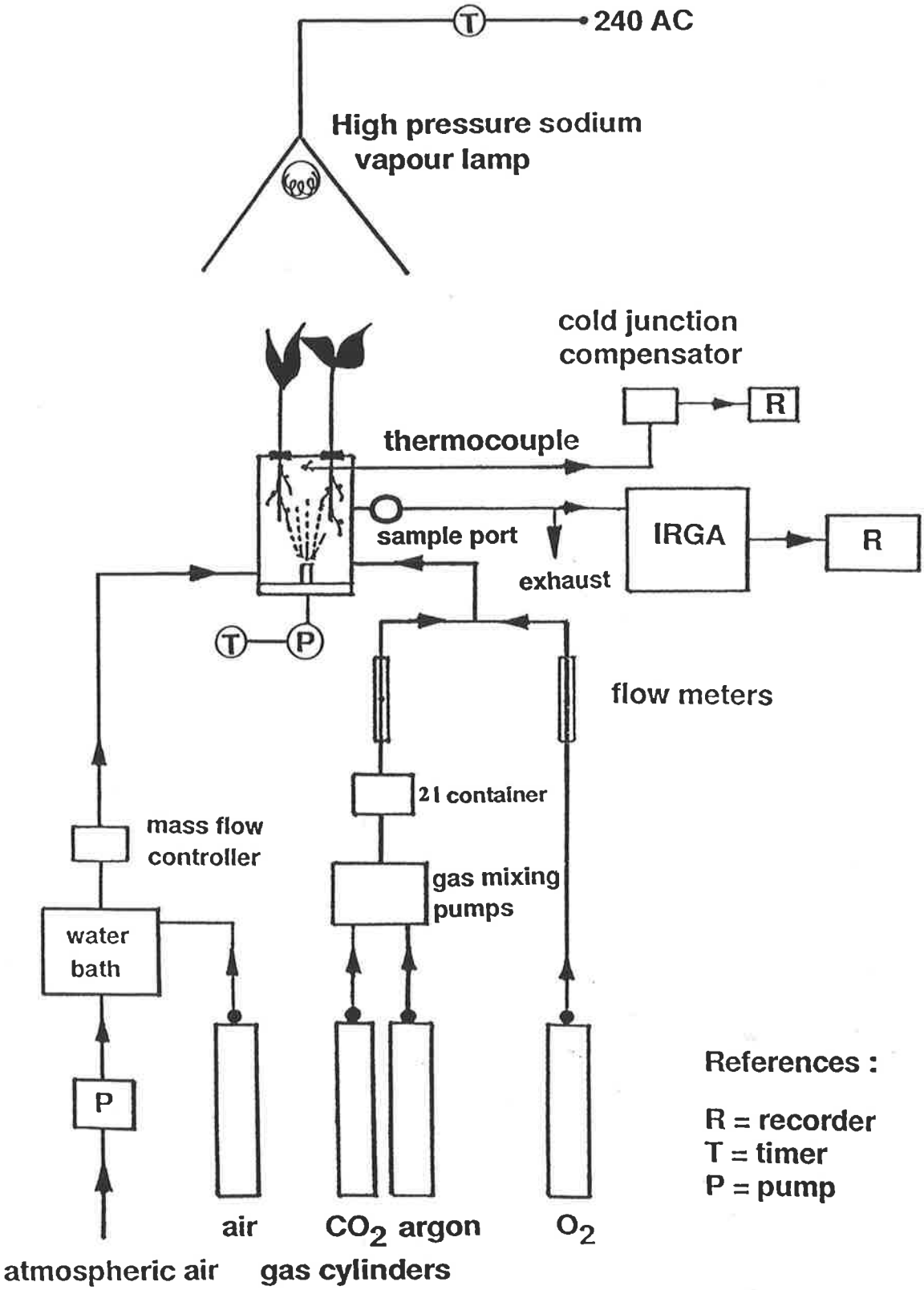
d = the difference in peak heights between two sample injections taken at 10 min and 40 min

e = the peak height of the standard

f = the volume of gas in the assay vessel containing plants, found by displacement with water (l).

AR was taken as a measure of the total electron flux through nitrogenase, while HE assessed the fraction of that flux used in reducing H^+ (Hardy 1979). The quantity (AR - HE) was therefore equivalent to the electron flux allocated to N_2 reduction in normal air and used as a measure of apparent N_2 fixation (Bethlenfalvay *et al.* 1978).

Fig. 1. Diagram of an open system which employed the mist chamber for simultaneous measurement of nitrogenase activity (AR and HE) and respiration of attached nodulated root. C_2H_2 and air cylinders were used when AR was performed


T
240 AC
High pressure sodium
vapour lamp
cold junction
compensator
R
thermocouple
sample port
exhaust
IRGA
R
T
P
flow meters
2 l container
mass flow
controller
gas mixing
pumps
water
bath
P
air
CO_2 argon
O_2
atmospheric air
gas cylinders
References :
R = recorder
T = timer
P = pump

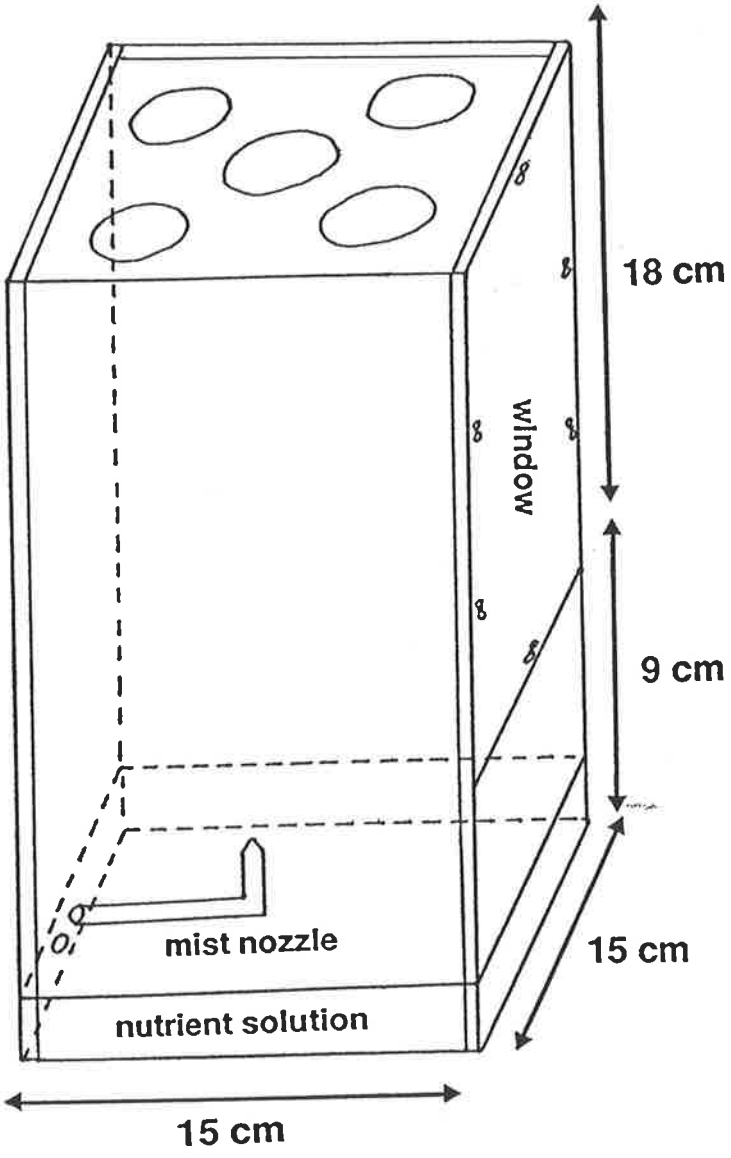
3.2.2. Open System

A flow-through gas system was designed as a mist chamber for the near-simultaneous estimation of AR, HE and respiration rate by attached, nodulated roots (Fig. 1). This technique has several advantages over the destructive batch assay method conducted as a closed system. Oxygen depletion (Hardy *et al.* 1968), CO_2 accumulation within the reaction chamber (Bethenod *et al.* 1984), water stress (Sprent 1971), ethanol accumulation (Sprent and Gallacher 1976) and decreased carbohydrate supply to the nodules (Pate 1976; Quispel 1974), all of which may affect NA, are avoided. In the mist chamber the large gas volume serves as a reservoir for sufficient quantities of O_2 and the liquid that coats the root, and the nodule surface is always saturated with both acetylene and O_2. Consequently, diffusion of these gases across the liquid boundary layer is lessened as a factor limiting rates of AR (Wych and Rains 1978).

Assay chamber

A chamber was constructed of 13 mm thick clear Plexiglass® with interior dimensions 15 cm wide, 27 cm high and 15 cm deep (Fig.2). The net capacity including internal fittings was thus 3.8 l. A stainless steel nozzle (Spraying Systems Unijet, Wheaton, IL, USA) with a full cone spray pattern was mounted in the middle of the chamber. The nozzle was fed by a pump (Flojet, Irvine, CA, USA) which circulated 350 ml of nutrient solution from the bottom of the chamber as a mist toward the top of the root system. The pump was operated by an electric timer (Omron H3BA, Tokyo, Japan) for 1 s every minute. The top section of the chamber had five holes (40 mm diameter) which allowed the whole root systems of up to 5 plants to be suspended in the chamber. An access panel was fitted on one side. Inlet and outlet ports positioned diagonally opposite each other assisted mixing of gases inside the chamber (*see* Fig. 2). The chamber was covered by black cloth during measurements to simulate darkness in the soil and to limit the growth of algae. No algal growth was ever observed.

Fig. 2. Diagram of the mist chamber designed for the simultaneous measurement of nitrogenase activity and respiration of attached nodulated roots.


18 cm
window
9 cm
mist nozzle
15 cm
nutrient solution
15 cm

Transfer of plants and measurements

Root systems of plants selected for study were carefully removed from the rooting medium by washing gently with water at 20°C. 'Prestik' sealing strip (Bostik Australia Pty Ltd, Melbourne, Vic., Australia) was used to seal stems into rubber bungs. Except where otherwise specified, shoots were exposed to a 12 h photoperiod (0900-2100 h) at a PPFD of 800-1300 $\mu E\ m^{-2}\ s^{-1}$ provided by a 'metalarc' lamp. The incident light flux density was reduced when required by optically neutral mesh screens.

Ambient temperature of the shoot was controlled at 20 ± 2°C. The air inside the chamber was controlled at 20 ± 2°C and was monitored with a copper-constantan thermocouple connected to a cold junction compensator (Rikadenki, Kogyo Co. Ltd, Tokyo, Japan) and a chart recorder. Shoot temperature was also monitored with a temperature probe.

Stability of the system

Attached nodulated roots were acclimatised in the mist chamber for 12-36 h with ambient air flowing at 2 l min^{-1} before an assay was started.

Respiration of the nodulated root

Air was drawn from the outside through a metal coil in a water bath at 20°C to control the temperature. A mass flow controller (Tylan model FC-202 with model RO14-200 readout box, Tylan Corp., Torance, CA, USA) enabled the flow rate to be varied from 1 to 3 l min^{-1}. An ADC series 225 infrared gas analyser (Analytical Development Co., Hoddesdon, UK) normally operated in differential mode, however, when measuring changes in $[CO_2]$ in the atmospheric air which was used as a reference, the absolute mode was used. Two air streams were drawn at about 250 ml min^{-1}, one for reference before entering the mass flow controller, and the other from inside the chamber for analysis. PVC tubing (Tygon, Akron, OH, USA) was used for the gas lines. The gas analyser was calibrated against dilutions of CO_2 in N_2 gas generated by two cascading gas mixing pumps (Wösthoff, Bachum, West Germany). The analyser output was fed to a chart recorder.

The CO_2 efflux rate was given by

$\mu mol\ CO_2\ plant^{-1}\ min^{-1} = a/b \times c \times d/e \times 1/f$

where

a = $[CO_2]$ of reference gas

b = peak of reference gas

c = peak of sample

d = flow rate ($l\ min^{-1}$)

e = volume of 1 mole of gas at temperature of assay

f = total number of plants.

Acetylene reduction

When AR assay was needed the outside air was replaced by that from a cylinder to ensure a steady [CO2] of the air during assay. A volume of 350 ml of C_2H_2 was injected directly into the chamber and a mixture of 10% C_2H_2 in air turned on simultaneously at a flow rate of 2 $l\ min^{-1}$. This procedure was adopted to reduce the time required to replace the air in the chamber with an air/C_2H_2 mixture so that the nodulated root was rapidly exposed to 10% C_2H_2. The gas from the chamber was sampled every 2 min over a period of 20 min. and exhausted outside the laboratory.

The rate of ethylene production is given by

$\mu moles\ C_2H_4\ plant^{-1}\ min^{-1} = a/b \times c/d \times e/f$

where

a = vol.C_2H_4 in standard jar (μl)

b = vol. of standard jar (l))

c = sample peak

d = standard peak

e = flow rate ($l\ min^{-1}$)

f = vol. 1 mole gas at assay temperature.

Hydrogen evolution

The measurement of HE in $Ar:CO_2:O_2$ and air provides information on the partitioning of the electron flow through the enzyme complex *in vivo* between N_2 and proton reduction. In the absence of hydrogenase reaction which utilises H_2, the rate of HE into $Ar:CO_2:O_2$ is regarded as a measure of the total flow of electrons to nitrogenase functioning.

Nodulated roots of attached plants were suspended in the mist chamber and allowed to stabilise in this system for 36 h with ambient air flowing at 1 l min^{-1}. Air from a cylinder was then directed through the chamber for 20 min and samples taken for $[H_2]$ every minute over a period of 5 min. A gas mixture of 79.96% Ar : 0.04% CO_2 : 20% O_2, v/v, was prepared by mixing CO_2 with argon (0.05 : 99.95) by gas mixing pumps, and then diluting with 20% O_2 (Fig. 1). The chamber was gassed with the $Ar:CO_2:O_2$ at a flow rate of 1 l min^{-1}. Samples for $[H_2]$ were taken every 3 min over a period of 15 min. HE in air and in $Ar:CO_2:O_2$ were used to estimate the rate of nitrogen fixation for each 4 h period of the day. Assuming that reduction of N_2 requires three electron pairs compared with one for H_2 production, the following relationship was used to estimate the rate of N_2 fixation (Rainbird *et al.* 1983): N_2 fixation = (HE in $Ar:CO_2:O_2$ - HE in air) / 3.

Solubility of gases

It is known that C_2H_2, C_2H_4 and H_2 are soluble in water (Weast 1980) and since the assays in the mist chamber employed these gases, it was important to determine if the nutrient solution and the spray system affected their concentration.

Attached nodulated root systems of 5-week old faba bean plants were suspended in a sealed mist chamber. 2 ml C_2H_4 were injected into the chamber and samples of gas were removed at 3 min intervals over 30 min for the estimation of $[C_2H_4]$. $[C_2H_4]$ was constant (Fig.3A).

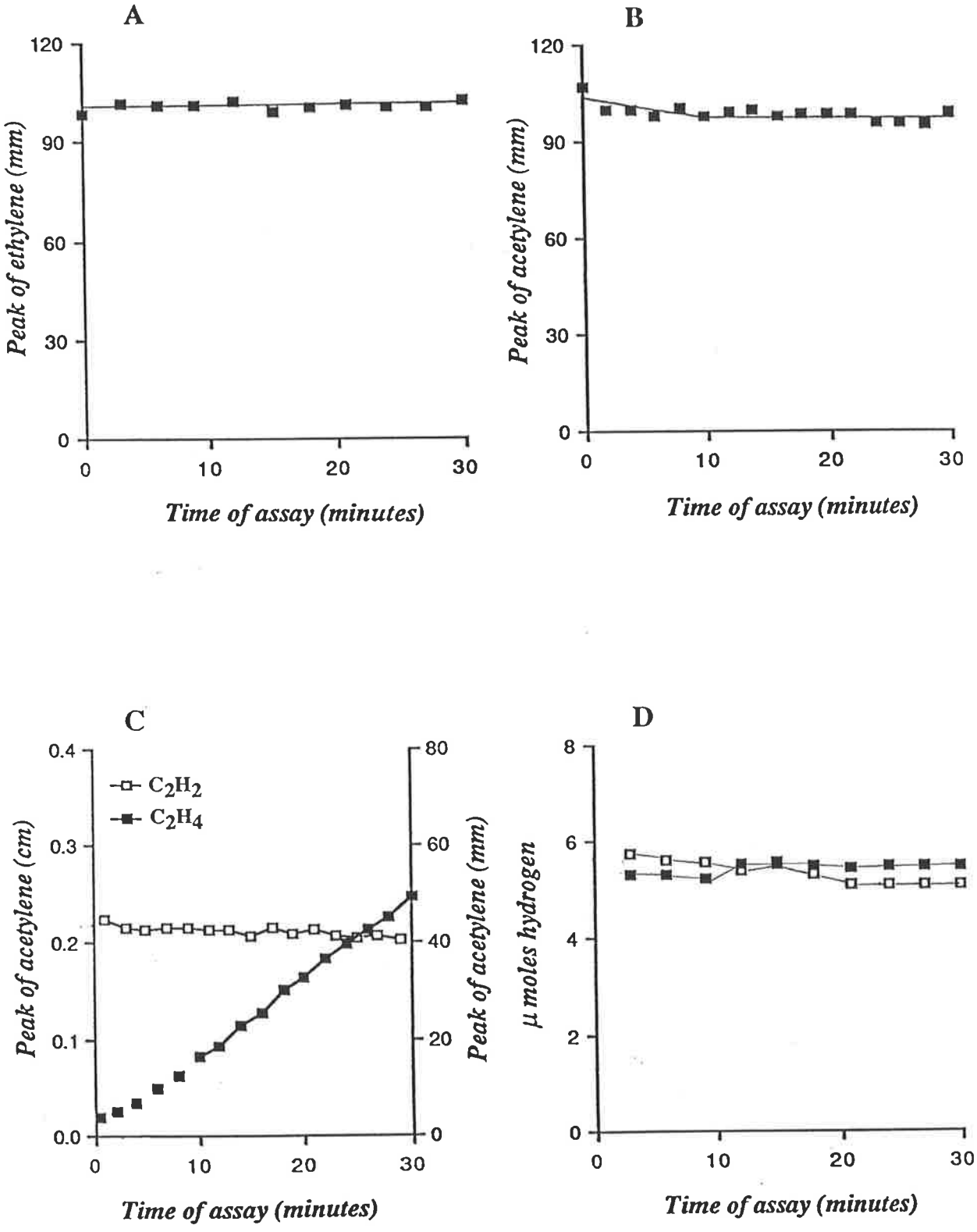
The concentration of C_2H_2 in the mist chamber was monitored over 30 min after the chamber had been sealed and 200 ml of C_2H_2 injected. $[C_2H_2]$ declined during the first 10 min by about 10% after which no further decrease was detected (Fig. 3B). The decline may have occurred in the first 2 min, probably due to the gas dissolving in water as it is more

Figs. 3 A. 2 ml C_2H_4 was injected into the closed mist chamber and [C_2H_4] monitored over a period of 30 min. Nutrient at the bottom of chamber was sprayed for 1 s on each minute.

B. 200 ml C_2H_2 was injected into the closed mist chamber and [C_2H_2] monitored over a period of 30 min. Nutrient at the bottom of chamber was sprayed for 1 s on each minute.

C. 200 ml C_2H_2 was injected into the closed mist chamber which contained attached nodulated roots of faba bean plants. The [C_2H_2] and [C_2H_4] were monitored over a period of 30 min. Nutrient at the bottom of chamber was sprayed for 1 s on each minute.

D. 700 µl H_2 was injected into the closed mist chamber with (□) and without (■) nutrient being sprayed and [H_2] monitored over a period of 30 min.


A
120
90
60
30
0
Peak of ethylene (mm)
0
10
20
30
Time of assay (minutes)
B
120
90
60
30
0
Peak of acetylene (mm)
0
10
20
30
Time of assay (minutes)
C
0.4
0.3
0.2
0.1
0.0
Peak of acetylene (cm)
80
60
40
20
0
Peak of acetylene (mm)
C2H2
C2H4
0
10
20
30
Time of assay (minutes)
D
8
6
4
2
0
μ moles hydrogen
0
10
20
30
Time of assay (minutes)

soluble in water than C_2H_4 (Weast 1980). The constant level of C_2H_2 thereafter indicates that equilibrium had been attained between the liquid and gas phases. [C_2H_2] and [C_2H_4] were measured at 2 min intervals for 30 min during incubation of nodulated roots in 10% C_2H_2. [C_2H_2] decreased as previously by about 10% (Fig.3C) and the rate of C_2H_4 accumulation was constant (r^2 = 0.999). Thus O_2 was not limiting and [C_2H_2] was saturating in the system, presumably at the site of reduction, the interior of the nodule.

When 700µl of H_2 was injected into the mist chamber both with and without the nutrient spray, [H_2] was constant for 30 min (Fig. 3D). These results show that: (i) the chamber was gas tight; and (ii) that the nutrient solution and the misting system did not influence [C_2H_2], [C_2H_4] and [H_2].

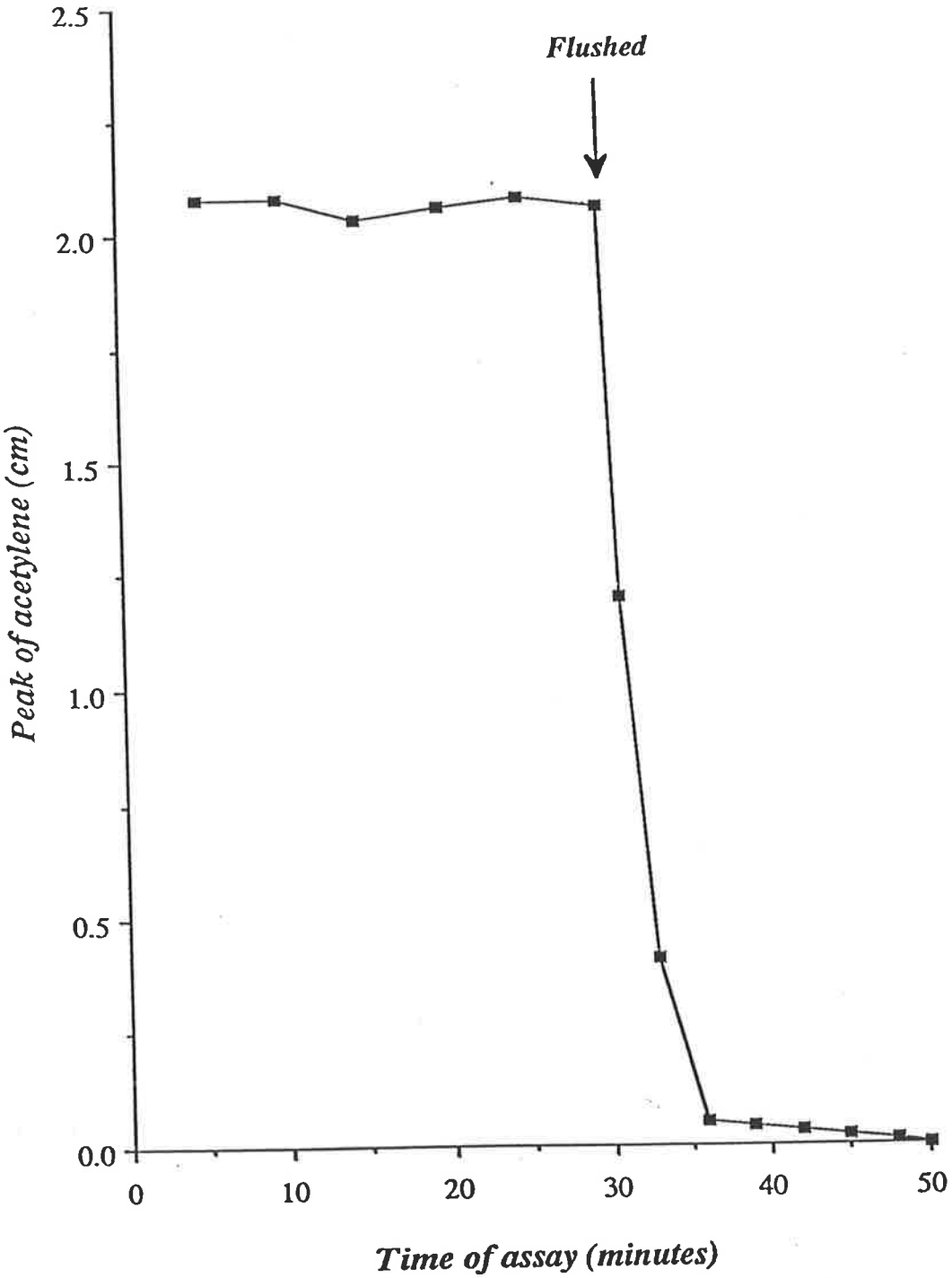
Time taken to flush C_2H_2 from the mist chamber

In some experiments AR assays were performed 5 times per day on the same plants. It was necessary to estimate the time taken to flush C_2H_2 from the mist chamber. A volume of 200 ml of C_2H_2 was injected into the chamber and [C_2H_2] monitored every 5 min for 30 min before air was flushed through at a flow rate of 2 l min^{-1}. It took about 6 min to flush out the C_2H_2 completely (Fig. 4). No C_2H_4 was evolved by nodulated roots when C_2H_2 was not added to the system.

3.2.3. Uptake Hydrogenase

Dixon (1967) found that pea bacteroids take up rather than evolve H_2. The hydrogenase responsible for H_2 metabolism can catalyse a reversible reaction *in vitro* in the presence of suitable electron donors or acceptors. *In vivo*, however, the reaction is usually irreversible, either consuming or evolving H_2 (Adams *et al.* 1981). Capacity to recycle H_2 produced by nitrogenase in legume symbionts appears to be determined predominantly by the strain of *Rhizobium* (Schubert *et al.* 1977). The possibility that the symbiosis of *V. faba* cv. Fiord and *R. leguminosarum* TA101 lacked an uptake hydrogenase needed to be examined to established the validity of the use of the difference between HE in the presence and absence of N_2 as an estimate of the rate of nitrogen fixation (Schubert and Evans 1976).

Fig. 4. Time taken to flush 200 ml C_2H_2 from the mist chamber which contained an attached nodulated root of faba bean plant with an air flow rate of 2 l per min.


Flushed
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Peak of acetylene (cm)
Time of assay (minutes)

Gas chromatography and amperometric determination were used to test for an uptake hydrogenase.

Gas chromatography method

Uptake of H_2 was measured by the disappearance of exogenous H_2 in the presence of p 0.1 C_2H_2 (Bergersen 1970; Nelson and Salminen 1982; Keyser *et al.* 1982; Bedmar *et al.* 1983). An attached nodulated root system was placed in a 1 l jar, which was then sealed and the rate of evolution of H_2 measured over 30 min. The jar was then flushed, resealed, and 180 ml of C_2H_2 injected, displaced gas being allowed to escape through a needle. C_2H_2 inhibits H_2 production by nitrogenase (Begersen 1970). 200 µl of H_2 were then injected into the assay vessel and samples taken for 35 min. Four replicates were used in these experiments (Fig. 5A).

Amperometric determination

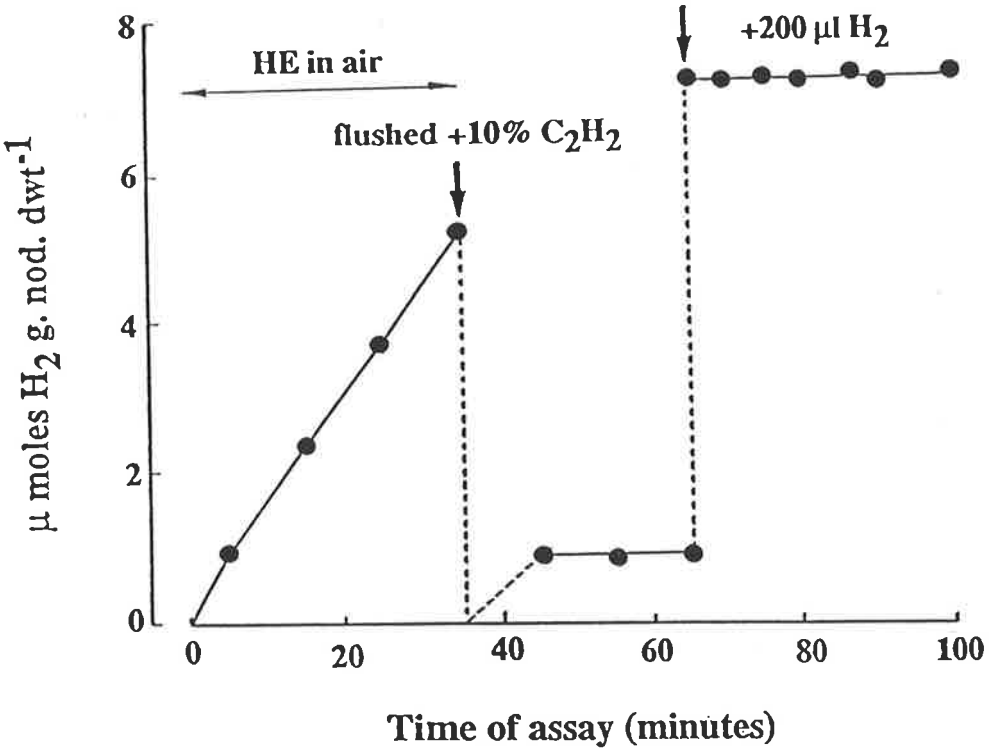
This method (Hanus *et al.* 1979) employs a YSI 5331 electrode (YSI Co., Inc., Yellows Spring, OH, USA) with the polarising voltage reversed for measurement of H_2 in aqueous solution. The probe consists of a platinum cathode and a silver wire anode, each isolated from the external environment by a Teflon® membrane permeable to O_2 but not to water or ions. Before applying the membrane, the electrode surface was immersed in saturated KCl solution and 0.01 *M* HCl. The Pt electrode was polarised at –4.5 v with respect to the Ag electrode with a current of 0.1 mA for 2-5 min. The current source was 3 flashlight cells and one 3 KΩ resistor wired in series. This generated AgCl. After the membrane was applied, the Pt electrode was polarised alternately (50 cycles min^{-1}) at +0.2 and 0.8 V with respect to the Ag electrode for 1-2 min. This was repeated each time the membrane was charged. The conditioned electrode was then attached to a polarising unit which also contained the components necessary to convert the current to a proportional voltage for display on a strip chart recorder.

The Pt electrode was polarised at 0.6 V with respect to the Ag electrode and after baseline drift stabilised (1-2 h), the apparatus was ready for use. The reaction is assumed to be:

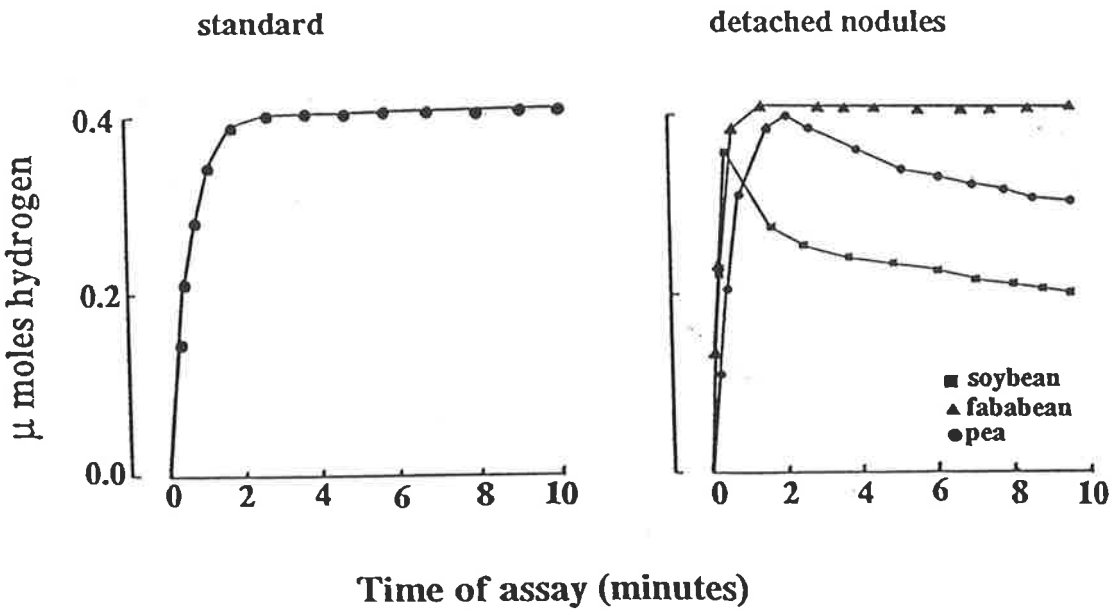
Fig. 5 A. The measurement of H_2 uptake employing the gas chromatography technique in faba bean plants.

B. The measurement of H_2 uptake employing the amperometric determination technique in 3 species of plants.

A


HE in air
flushed +10% C_2H_2
+200 µl H_2
µ moles H_2 g. nod. dwt^{-1}
0
2
4
6
8
0
20
40
60
80
100
Time of assay (minutes)

B


standard
detached nodules
µ moles hydrogen
0.0
0.2
0.4
0
2
4
6
8
10
soybean
fababean
pea
Time of assay (minutes)

$$H_2 \xrightarrow{Pt} 2\,H^+ + 2e^-$$

$$2\,AgCl + 2e^- \xrightarrow{Ag} 2\,Ag + 2\,Cl^-$$

The probe was fitted into a water jacketed cuvette (Gibson Medical Electronics, WI, USA) and detached nodules of pea, faba bean, or soybean plants introduced.

Results (Fig. 5A, B) show no H_2 uptake in faba bean symbiosis, indicating that the assumption implicit in the use of the difference between HE in the presence and absence of N_2 to estimate the rate of N_2 fixation was justified for this species. H_2 was taken up by pea and soybean nodules.

3.2.4. Post-Assay Procedures

After assay, plants were normally divided into leaf, petiole, stem, fallen (senescent) leaf, flower, pod and grain. After drying at 85°C for 24 h in a forced-draught oven, the dry weight of each portion was determined. Fractions were then ground separately to pass through a 0.5 mm sieve, and analysed when required for total organic nitrogen by Kjeldahl digestion. Samples of 250 mg were digested with an auto Kjeltab (Thompson and Capper Ltd, Runcorn, Cheshire, UK) in 4 ml conc. H_2SO_4 for 20 min at 400°C, then for a further 15 min at 350°C. Samples were allowed to cool and diluted to 75 ml with distilled water. Nitrogen was estimated by steam distillation of 5 ml samples and titration (Ballentine 1957).

3.3. Experimental Techniques

3.3.1. Introduction

The 'standard' AR assay (Hardy *et al.* 1968) is commonly performed in closed assay vessels containing detached nodulated root systems from which the rooting material has been removed. The effect of this disturbance on the rate of production of C_2H_2 is controversial. Some authors have reported an apparent decrease in NA with time e.g. *Glycine max* (Mague and Burris 1972; Wych and Rains 1978); *Acacia* sp. (Hansen *et al.* 1987); *Trifolium repens* (Minchin *et al.* 1986). Others report no response to disturbance e.g. *Lupinus* sp. (Trinick *et al.* 1976); *Phaseolus vulgaris* (Saito *et al.* 1980); *Trifolium subterraneum* (Hopmans *et al.*

1982; Gates 1984). The existence of a C_2H_2 induced decline and disturbance of the root system during extraction may limit the use of a closed system.

The 'oil-dry' used as the growth medium in the current experiments ensured little mechanical disturbance to plants during preparation for nitrogenase assay *in vitro*. The nodulated roots were saturated with water when the 'oil-dry' was removed. Hopmans *et al.* (1982) reported there to be no difference between the AR rates of washed roots of *T. subterraneum* and those assayed without previous wetting.

The effect of temperature on the C_2H_2-reducing activity of nitrogenase has been examined with N_2-fixing bacteria, legumes and non-legumes (Dart and Day 1971; Gibson 1971; Wheeler 1971). The activity of soybean nodules increased as the incubation temperature was increased from 10 to 20°C, reached a maximum at 20 to 30°C and declined above 30°C (Hardy *et al.* 1968). Other legumes (Dart and Day 1971; Gibson 1971; Waughmann 1977; Cralle and Heichel 1982) show generally similar responses to temperature but differing critical and optimum temperatures. *Alnus glutinosa* and *Hippophäe rhamnoides* nodules showed about tenfold increases in NA from 5-20°C with maximum activity in the area of 20-25°C followed by a sharp decline from 25-40°C (Wheeler 1971).

It has been reported that NA of some legumes fluctuates diurnally (Bergersen 1970; Halliday and Pate 1976; Mague and Burris 1972; Vaughn and Jones 1976; Mahon 1977). Others report that NA remains constant day and night (Hardy *et al.* 1968; Fishbeck *et al.* 1973; Masterson and Murphy 1976).

3.3.2. Effect of Root Removal, Assay Temperature, and Time of Day on AR Activity in a Closed System

It was considered necessary to determine whether the method of removal of 'oil-dry', the assay temperature, and the time of harvest had any effect on the NA of nodulated roots of faba bean.

Groups of four faba bean plants, raised in a growth room (20°C) for 4 weeks, were removed from the rooting medium by saturating with water at temperatures of 12.5, 17.5 and 22.5°C or by gently shaking off the 'oil dry'. Plants were then incubated in the morning

(1000 h) and again in the afternoon (1400 h) at the same temperatures for AR assay. The AR rates were expressed on an absolute (per plant) and on a specific (per gram nodule dry weight) basis.

Table 1. Analysis of variance for the AR activity of faba bean plants measured in a closed system: I. at different assay temperatures, II. after different methods of root extraction, and III. at different times of the day

Treatments	(A) µmol C_2H_2 plant^{-1} min^{-1}	(B) µmol C_2H_2 g nodule dwt^{-1}
I. Temperature		
12.5°C	0.13	1.85
17.5°C	0.20	2.79
22.5°C	0.18	2.17
LSD 5%	NS	0.44**
II. Method of root extraction		
washed	0.17	2.24
shaken	0.17	2.30
LSD 5%	NS	NS
III. Time		
1000 h	0.16	2.12
1400 h	0.18	2.42
LSD 5%	NS	0.30*
Interaction		
Time x Temperature x Method of root extraction	NS	NS

An analysis of variance showed there to be no significant difference ($P > 0.05$) between treatments for absolute rates but there were significant differences for time of assay

and of temperature for specific rates (Table 1). There was no significant interaction between treatments ($P > 0.05$). Plants harvested at 1400 h had a higher AR (specific rate) than those measured at 1000 h, suggesting diurnal variation in nitrogenase activity. AR assays should therefore be performed for comparative purposes at the same time each day or the assay time should represent an average of 24 hours of activity.

There was a broad optimum temperature around 17.5°C for specific activity. Since the incubation temperature influenced NA, it was decided to conduct all future assays at the growth temperature, normally 20°C. The method of root removal did not influence either the specific or the absolute rates of NA. Thus removal of the oil dry by saturating with water or by shaking gently had no significant effects ($P>0.05$) on AR, in accordance with Trinick *et al.* (1976) with *Lupinus* sp.

3.3.3. The Rate of Ethylene Production

Haystead *et al.* (1979) and Minchin *et al.* (1982) recorded a marked decrease in respiration rate in the presence of C_2H_2 when nodulated white clover roots or detached pea nodules were measured simultaneously in closed, circulating gas systems. Furthermore they also noted that the cumulative curve of C_2H_4 production was non-linear over a 30 or 60 min assay period. There was no common response in nitrogenase activity to C_2H_2, it varied with plant cultivar, *Rhizobium* strain, plant age, and the pre-assay environment (Minchin *et al.* 1982). Other users of open systems have not reported a C_2H_2 induced decline with soybean (Maderski and Streeter 1977; Patterson *et al.* 1983; Layzell *et al.* 1984). It was necessary to find out if a decline in AR rate was induced by C_2H_2 during assay of the plants used in this study.

Linearity of C_2H_4 production in a closed system was first tested by sampling for C_2H_4 accumulation every 5 min over a period of 30 min. Figure 6 shows that when plants were tested individually, each showed a near constancy in rate of C_2H_4 production or that the rate decreased slightly. Sometimes the rate increased slightly with time. Overall, rates were almost constant. However, it is argued by Minchin *et al.* (1983a) that even when there is a 50% decrease in the rate over a 60 min incubation period, a plot of the cumulative [C_2H_4]

produces a convincing linear relationship. The open system was therefore developed to obtain direct measurements of rate of C_2H_4 production..

Faba bean, pea and soybean plants were used in an early study of the open system. Changes in $[CO_2]$ and in $[C_2H_4]$ were measured 16 times over 40 minutes. $[C_2H_4]$ increased during an initial 4 min mixing period (time taken for C_2H_2 concentration in the assay vessel to rise from zero to its equilibrium value) after which it was either constant (Fig. 7A) or stabilised for about 2 min., and then declined rapidly to a new steady state (Fig. 7B). $[CO_2]$ responded in a similar manner. A C_2H_2 induced decline in the CO_2 efflux and the rate of C_2H_4 production varied with the host species (see Table 2). It was not found in soybean, but always in pea, and sometimes in faba bean. In faba bean the AR rate remained constant during assay for plants in the vegetative stage but a decline usually occurred later in ontogeny. The stage of plant development of faba bean appears to influence the manifestation of the response. Based on these results, faba bean at the vegetative stage was always used for AR assay except when N_2 fixation was monitored throughout the growth cycle. It is concluded that a closed system is satisfactory when the AR rate remains constant during assay but when AR rates decline, only the open system is satisfactory.

Fig. 6. Accumulation of C_2H_4 over a period of 30 min for faba bean plants incubated in a closed system. Assays were performed in the morning (1000 h) and in the afternoon (1400 h). Each curve is for a separate plant. Results show rates to remain constant, to increase slightly with time, or to decrease slightly with time.

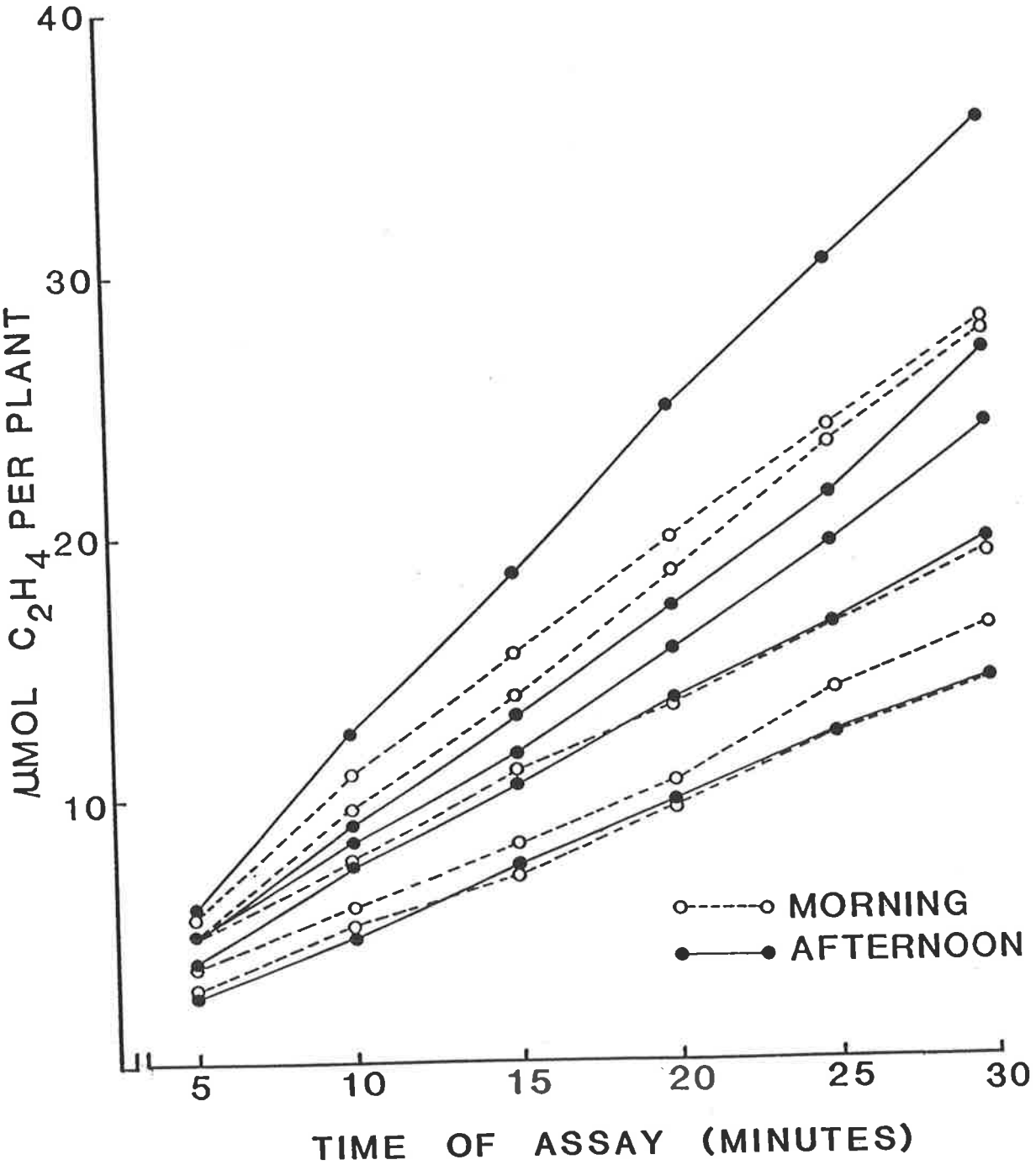
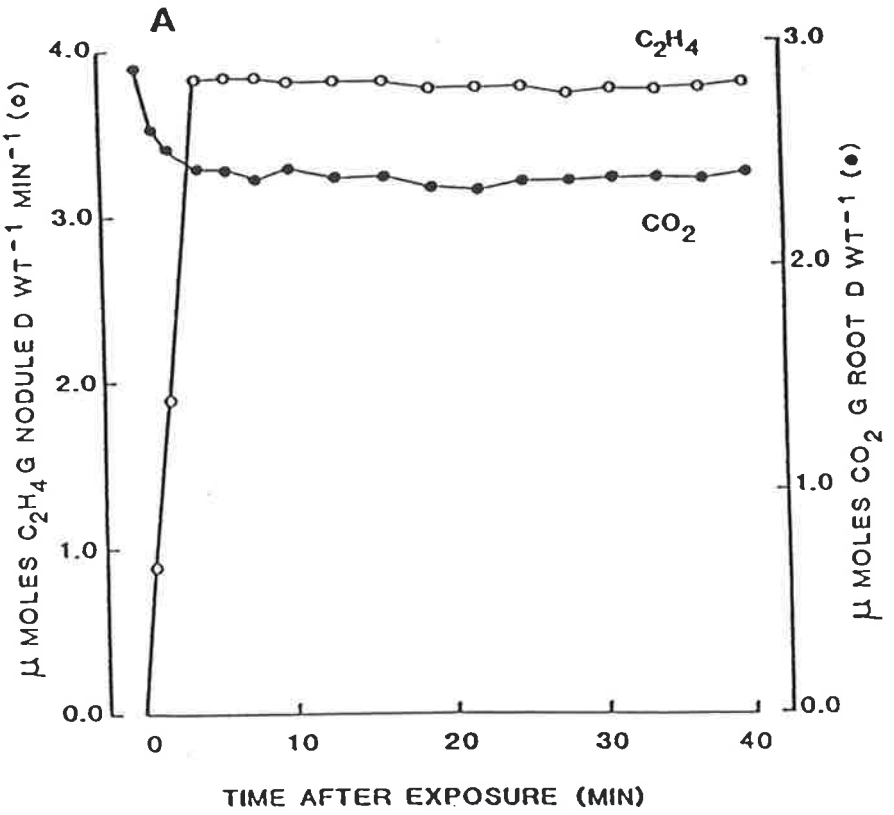

40
30
20
10
μMOL C_2H_4 PER PLANT
5
10
15
20
25
30
TIME OF ASSAY (MINUTES)
MORNING
AFTERNOON

Fig. 7. Rates of AR activity and CO_2 efflux of attached nodulated root of faba bean in an open system. Data from single replicate runs are chosen as representative of the characteristic of the constant (A) or decline (B) in both rates in over 30 measurements.


A
C_2H_4
CO_2
μ MOLES C_2H_4 G NODULE D WT^{-1} MIN^{-1} (o)
μ MOLES CO_2 G ROOT D WT^{-1} (•)
4.0
3.0
2.0
1.0
0.0
3.0
2.0
1.0
0.0
0
10
20
30
40
TIME AFTER EXPOSURE (MIN)

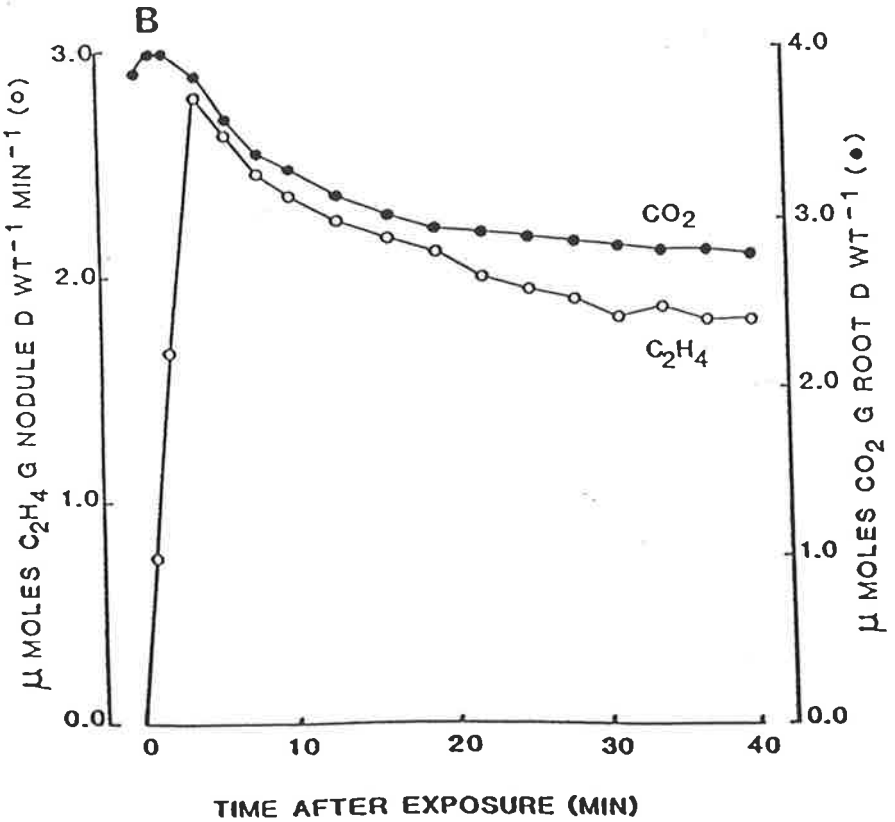

B
CO_2
C_2H_4
μ MOLES C_2H_4 G NODULE D WT^{-1} MIN^{-1} (o)
μ MOLES CO_2 G ROOT D WT^{-1} (•)
3.0
2.0
1.0
0.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
TIME AFTER EXPOSURE (MIN)

Table 2. The ratio between the minimum and the maximum rates of C_2H_4 production during AR assay of soybean (A), pea (B) and faba bean (C) plants in an open system

The maximum rates were taken at 4 min after the exposure of C_2H_2 and the minimum rates were taken at the end of assay period (40 min). Plants were assayed 7 times during a 18 day period when plants were 5-7 weeks old

No.	Time (day)	µmol C_2H_4 g nodule dwt^{-1} min^{-1} maximum	minimum	Ratio max:min
Soybean				
1	30	2.46	2.46	1
2	33	2.24	2.14	0.96
3	36	2.36	2.36	1
4	39	2.02	2.02	1
5	42	2.21	2.21	1
6	45	2.04	2.04	1
7	48	2.27	2.27	1
Pea				
1	31	6.58	3.81	0.58
2	34	7.35	4.41	0.60
3	37	9.38	6.56	0.70
4	40	7.60	5.25	0.69
5	43	7.98	5.58	0.70
6	46	6.24	4.47	0.72
7	49	5.48	3.78	0.69
Faba bean				
1	32	3.95	3.95	1
2	35	3.82	3.82	1
3	38	4.03	4.03	1
4	41	2.63	2.29	0.87
5	44	2.58	2.06	0.80
6	47	2.89	2.28	0.79
7	50	2.76	2.15	0.78

3.3.4. The Effect of Plant Disturbance on Acetylene Reduction Activity and on the Respiration of Nodulated Roots

If removal of the rooting material and the use of detached nodulated roots produce a substantial decrease in nitrogenase activity, extraction of root from the rooting medium which is normally done for a 'standard' AR assay, will produce substantial errors even when used comparatively (Minchin *et al.* 1986). It is therefore important to measure the effect of the removal of rooting material and the use of detached nodulated roots on both AR activity and respiration of nodulated roots.

Removal of the rooting medium

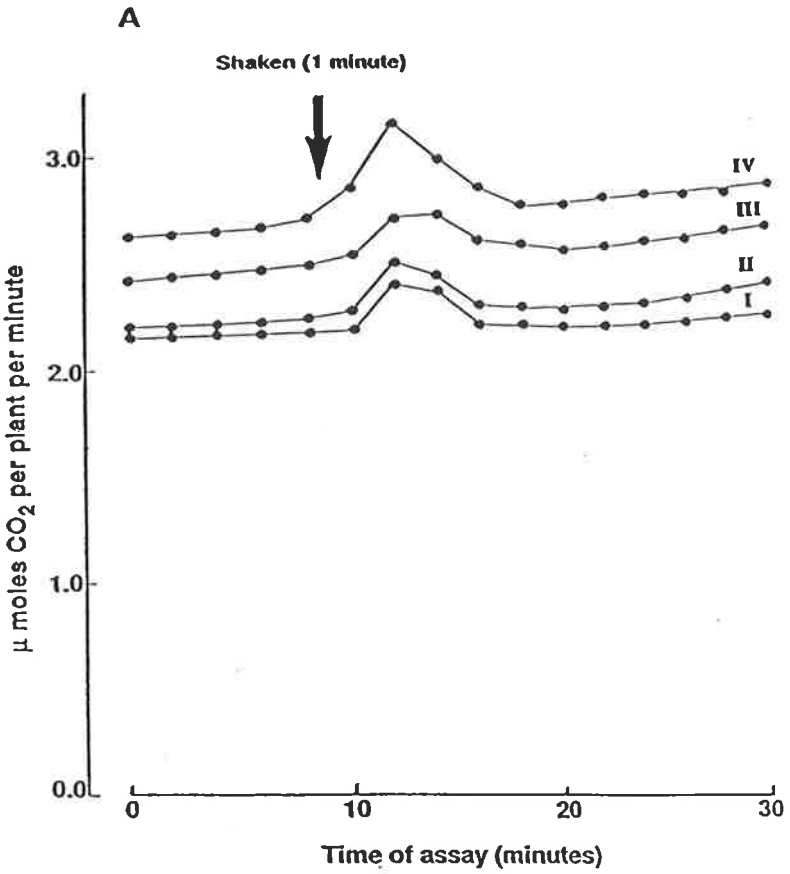
An attached nodulated root was suspended in the mist chamber and gas exchange allowed to stabilise for 4 hours before the root was shaken by means of a wire loop inserted through the outlet port to simulate the disturbance which might occur during extraction. The root system was shaken for 1 min (at 1200 h), allowed to equilibrate and shaken again 3 times at intervals of 30 min. Another root system was shaken for 1, 2, 3 and 5 min at intervals of 30 min. Results showed that in both treatments shaking increased the nodulated root respiration for 5-10 min after which it stabilised at the initial value (Fig. 8).

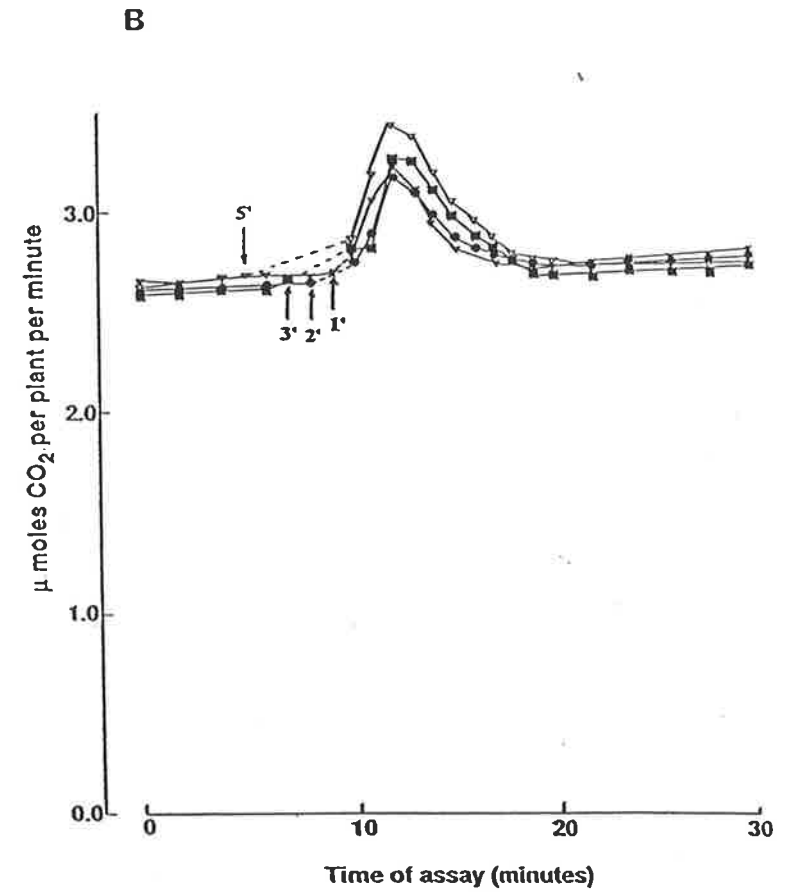
The above results suggest that mechanical disturbance of nodulated root systems has only a transitory effect on respiration. However, the treatment given was probably not as severe a disturbance as that which occurs when plants are removed from oil dry for normal AR assay in a closed system. A series of tests was therefore conducted to compare AR activity of undisturbed plants assayed *in situ* with those of plants at a comparable growth stage but disturbed in various ways. Five week old bean plants were used. Altogether three tests were made using plants of slightly different ages over a period of several days.

Test I involved growing plants in oil dry contained in the 1 l jars in which AR assay of whole plants were usually conducted. A jar with a hole in the bottom for drainage was filled with oil dry and a faba bean seedling grown in it with the shoot through a hole in the lid. Roots were irrigated daily in the usual way. After about 5 weeks of growth the jars could be sealed by pressing 'prestik' sealing strip around the stem and screwing down the lid. The drainage hole was sealed by using a rubber bung. A subaseal also inserted into the lid

Fig. 8 A. Effect of disturbance (root shaken) on the respiration rate of the nodulated root of faba bean plant. The root was shaken for 1 min (I), allowed to equilibrate and then shaken 3 times (II-IV) at intervals of 1 h. The measurements were started at 1200 h.

B. Effect of disturbance (root shaken) on the respiration rate of nodulated root of faba bean plant. The root system was shaken for 1, 2, 3, and 5 min at intervals of 30 min.


A
Shaken (1 minute)
IV
III
II
I
3.0
2.0
1.0
0.0
μ moles CO_2 per plant per minute
0
10
20
30
Time of assay (minutes)


B
5'
3'
2'
1'
3.0
2.0
1.0
0.0
μ moles CO_2 per plant per minute
0
10
20
30
Time of assay (minutes)

permitted the introduction of the necessary amount of C_2H_2 to bring the air in the jar to p 0.01 and the removal of samples of air for measurement of C_2H_4 after various periods of incubation. Oil dry has an air filled porosity of about 20% and as it was not saturated with water, it was expected that both C_2H_2 and C_2H_4 would diffuse through it very rapidly. The actual test consisted of measuring the concentration of C_2H_4 after injection of C_2H_2 every 10 min for 125 min. For comparison with the next test (II) the rate of accumulation of C_2H_4 was expressed as a first period of 20 to 65 min and a second period of 65 to 125 min.

Test II involved root disturbance by removal of the oil dry by gentle shaking. After 65 min of *in situ* measurement as test I, the oil dry was then removed. The system was re-gassed with C_2H_2 and assay continued for further 60 min. In this way the same nodulated root system could be measured undisturbed and then disturbed.

In test III nodulated roots from which the oil dry had been removed by gentle washing were assayed suspended in jars continuously for 125 min. Ten replicates were used for each treatment. The essence of the measurements was to compare the rates of C_2H_4 accumulation for the 10 to 65 and 65 to 125 min periods in test I, II, and III.

Table 3 and Figure 9 show that when an assay was conducted *in situ* in oil dry for 125 min (I) the rate of C_2H_4 production by undisturbed plants appeared constant over the first period of 20 to 65 min but then decreased over the second period. Test II shows that removal of the oil dry after an initial *in situ* rate had been measured did not result in any major shift in the rate of C_2H_4 production. Thus although in I AR declined during the second period of undisturbed assay, disturbance itself in II did not reduce AR in any noticeable way. Test III shows that when roots were suspended in jars for the full period of 125 min C_2H_4 production was maintained at a steady rate throughout.

Oil dry is a suitable rooting medium as its removal had no effect on AR activity and the C_2H_4 production of disturbed plants was constant over a 125 min period. The decline in the second period (65-125 min) of *in situ* measurements suggest that other factor(s) may be involved. The depletion of O_2 and accumulation of CO_2 during the second period may have caused the reduction in C_2H_4 production. The accumulation of C_2H_4 arround the roots may also have had an adverse effect on AR activity as reported by Grobbellar *et al.* 1971 in *P. vulgaris*.

It can be concluded that the AR rates of intact nodulated root assayed without oil dry can be considered representative and comparable with those of intact plants assayed *in situ*.

Table 3. The AR rates of undisturbed faba bean plants (grown and assayed in jars with oil dry) compared with those of disturbed plants (grown in pots and assayed in jars without oil dry)

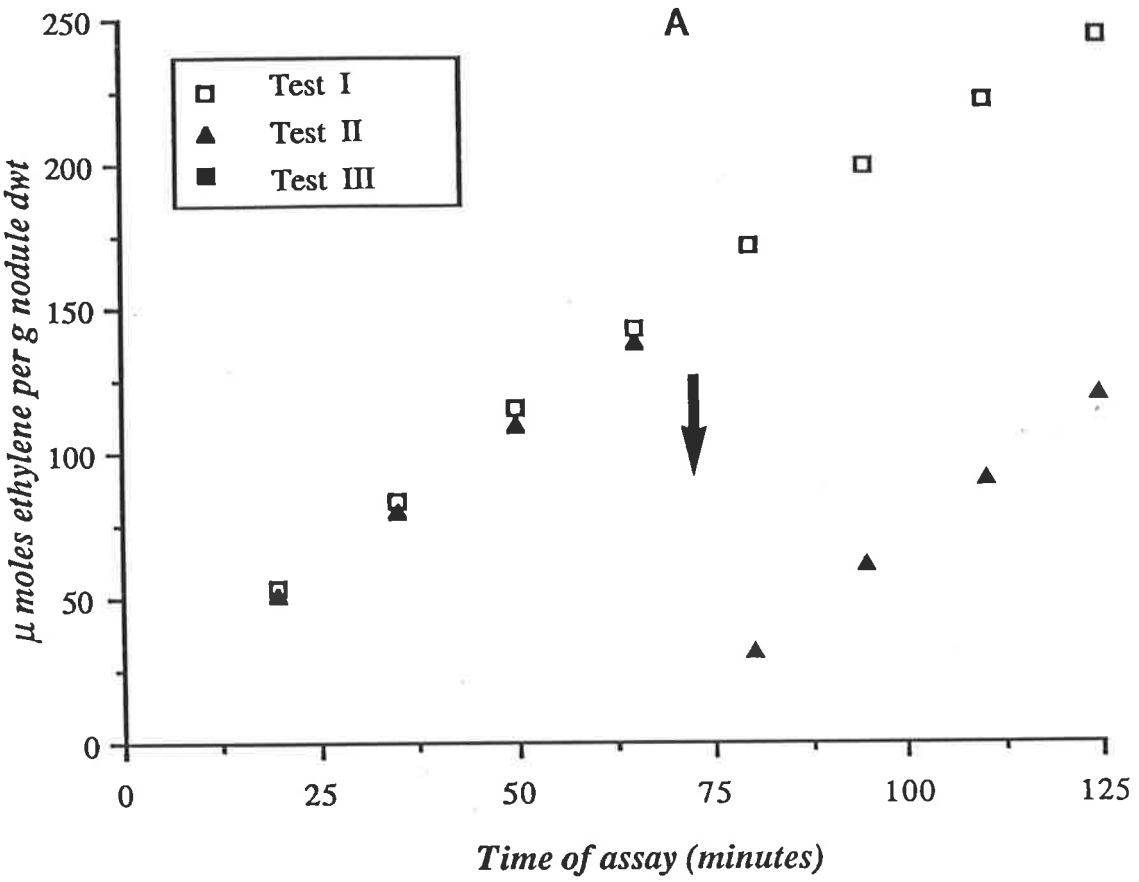
The oil dry was removed either by saturating with water (20°C) or by gentle shaking. Values are the means of 10 replicates with standard errors in brackets

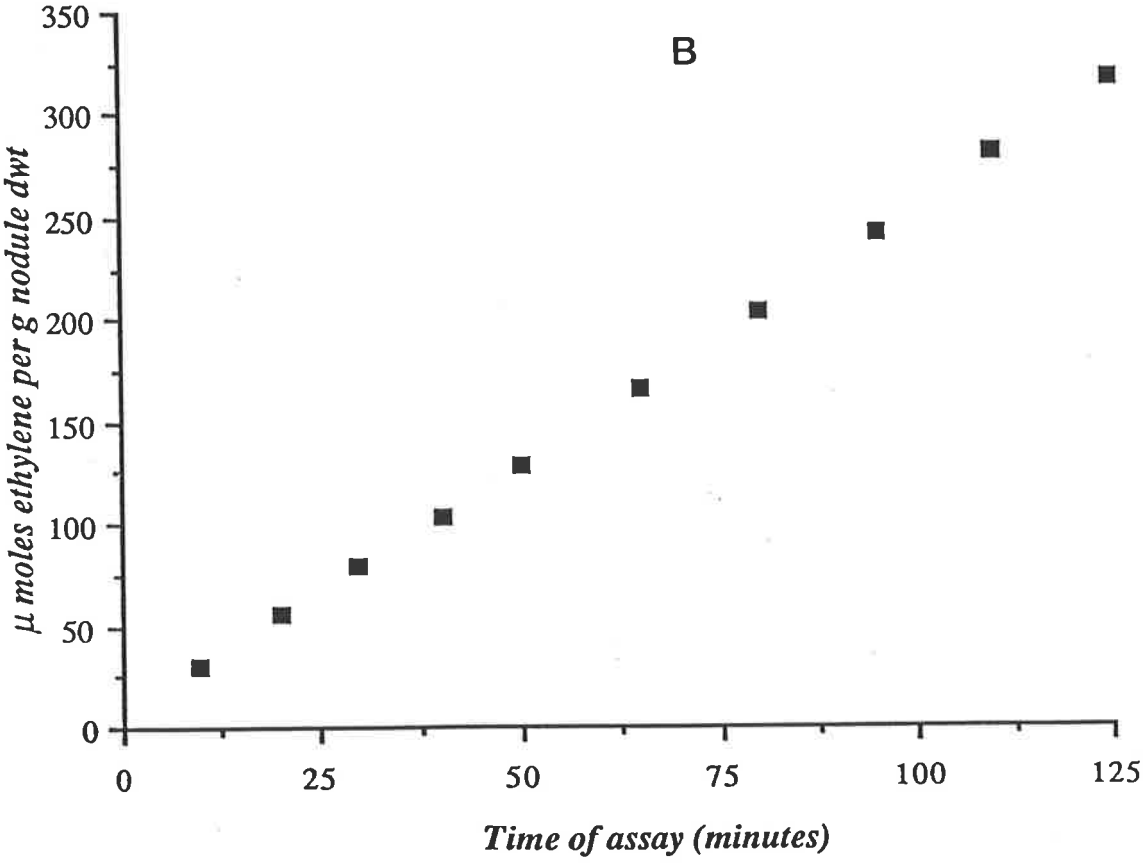
Test	Treatments	Duration of assay	AR rate*
I	Grown and assayed in oil dry :		
	(a) first period (undisturbed);	20-65 min.	2.001 (0.165)
	(b) second period (undisturbed).	65-125 min.	1.669 (0.157)
II	Grown and assayed in oil dry for the first period, oil dry removed by gentle shaking and assay re-commenced:		
	(a) first period (undisturbed);	20-65 min.	1.946 (0.189)
	(b) second period (disturbed).	15-60 min.	1.953 (0.228)
III	Grown in oil dry in pots, oil dry removed by saturating in water, and roots assayed in jars.		
	(a) first period;	10-65 min.	2.447 (0.205)
	(b) second period.	65-125 min.	2.548 (0.246)

* μ moles C_2H_4 g nodule dwt^{-1} min^{-1}.

Fig. 9 A. AR activity of: (I) undisturbed faba bean plants assayed *in situ* for 125 min; (II) undisturbed plants assayed *in situ* for 65 min and then the oil dry removed and assay continued for further 60 min. An arrow indicates the time when the oil dry was removed by gentle shaking and the system re-gassed with C_2H_2.

B. AR activity of faba bean plants disturbed by removal of the oil dry by gentle washing and assayed continuously for 125 min (Test III). Values are the means of 10 replicates.


A
Test I
Test II
Test III
250
200
150
100
50
0
0
25
50
75
100
125
µ moles ethylene per g nodule dwt
Time of assay (minutes)


B
350
300
250
200
150
100
50
0
0
25
50
75
100
125
µ moles ethylene per g nodule dwt
Time of assay (minutes)

Excision of the shoot

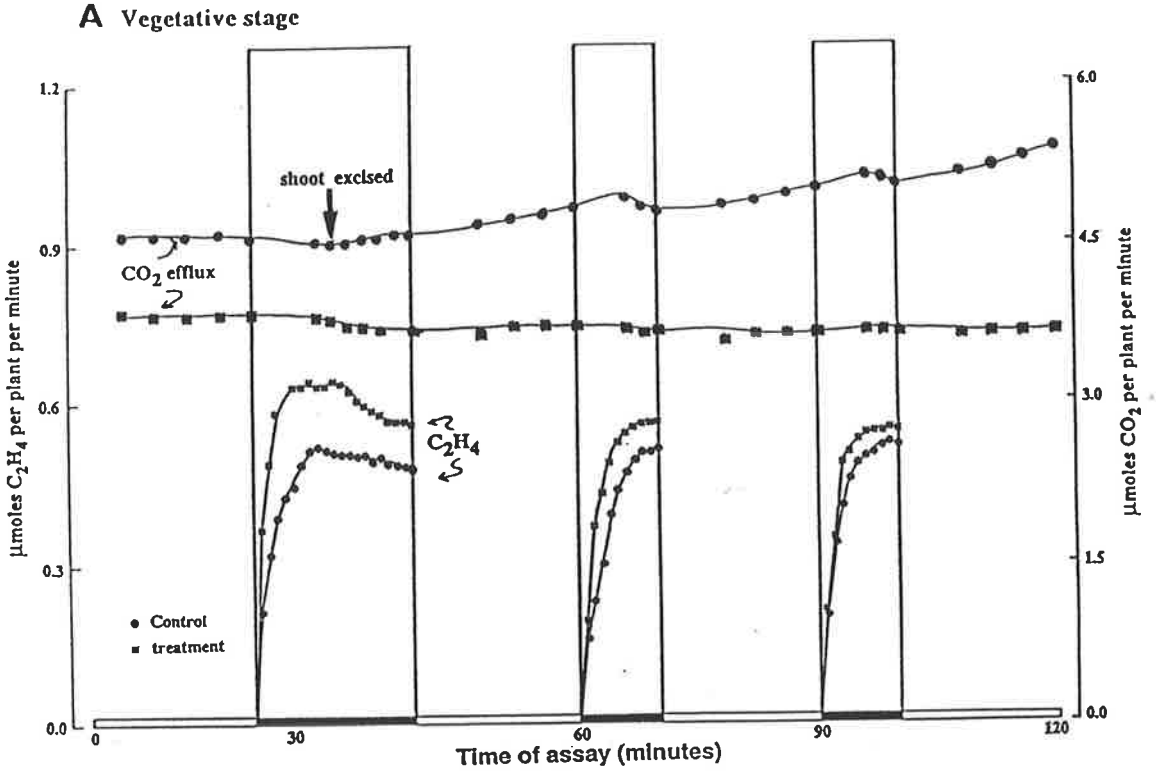
The effect of excision of the shoot on the respiration of nodulated root and on the maximum rate of C_2H_2 reduction was measured in faba bean plants during both the vegetative and reproductive stages. Four attached, nodulated root systems were allowed to stabilise in the mist chamber for 24 h before exposure to C_2H_2. The shoot system was irradiated at 800 $\mu E\ m^{-2}\ s^{-1}$. Samples were taken 10 times over a period of 10 min. AR assays were performed 10 min before and 0, 30 and 60 min after shoots were excised and rates compared with those obtained from uncut roots.

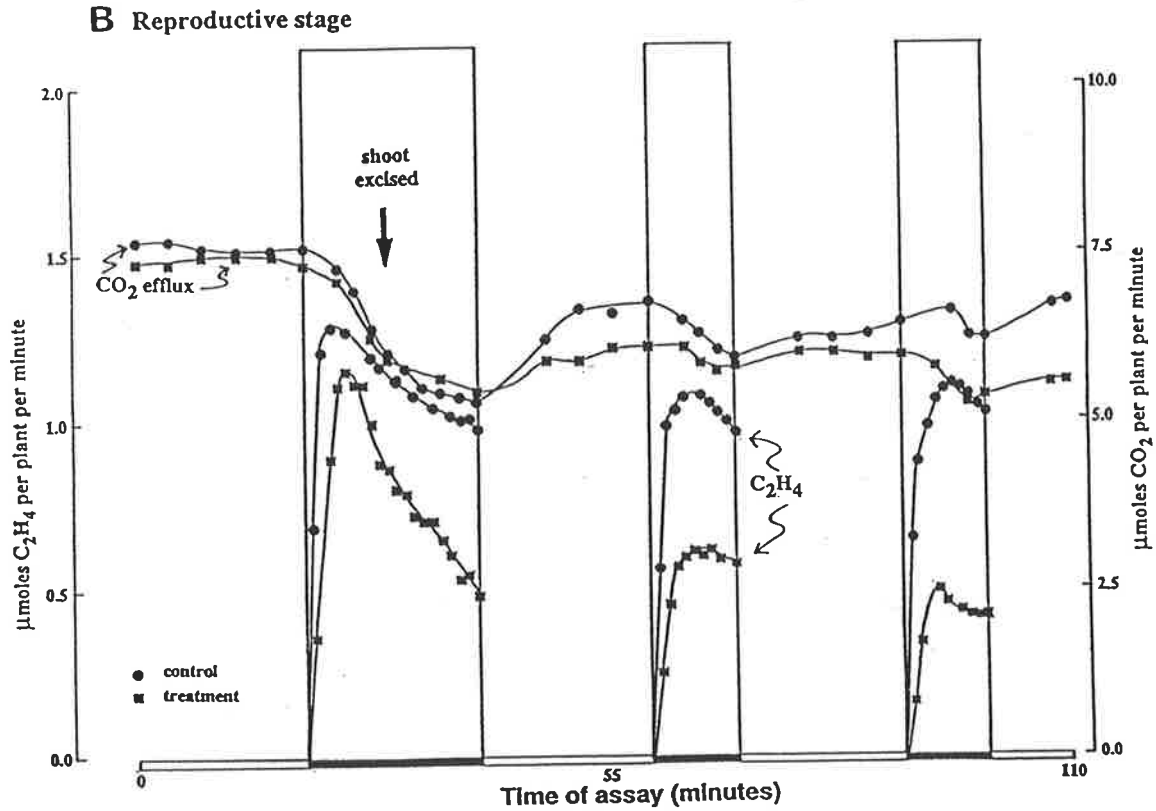
In the vegetative stage, AR rates of attached nodulated roots remained constant and CO_2 efflux increased by about 17%, but when the shoots were excised the rate declined about 14% and the CO_2 efflux decreased about 5% in 60 min (Fig. 10A). During the reproductive stage both rates declined more markedly in detached nodulated roots than in attached nodulated roots (Fig. 10B). In the former CO_2 efflux and AR rates declined about 26% and 56%, but in the later the corresponding figures were 11% and 14%.

The initial decline in the AR rate suggests that the nitrogenase activity depends on continuity of a supply of photosynthate from the shoot. These results also show that the AR assay of detached nodulated roots does not reflect *in vivo* changes in nitrogenase activity accurately because a major source of fixed carbon is absent (Bergersen 1970; Mague and Burris 1972; Murphy 1981) or carbohydrate supply to the nodule is decreased (Pate 1976; Quispel 1974). Minchin *et al.* (1986) claimed that a decrease in maximum nitrogenase activity of excised/shaken roots is a reflection of decreased O_2 supply to the bacteroids which may be a response to reduced photosynthate supply following shoot excision. Decrease in nitrogenase activity of excised shoots is not apparent, however, in the work of Fishbeck *et al.* (1973). It appears possible that the effects of shoot excision in any species may be markedly dependent on the carbohydrate status of the nodules and the extent of disturbance-induced decrease (shoot excision/shaking root) in maximum nitrogenase activity may vary with the host plant and the *Rhizobium* strain used.

It is concluded that the use of detached nodulated roots of faba bean in a closed system would produce a serious error in the AR assay and could markedly underestimate the actual rate of nitrogenase activity.

Fig. 10. The effect of shoot excision on the respiration rate of nodulated root and AR rates of faba bean plants during the vegetative stage (A) and the reproductive stage (B). The enclosed areas indicates times when AR assays were performed. The treated plants were compared with control plants.


A Vegetative stage
shoot excised
CO_2 efflux
C_2H_4
Control
treatment
µmoles C_2H_4 per plant per minute
µmoles CO_2 per plant per minute
1.2
0.9
0.6
0.3
0.0
6.0
4.5
3.0
1.5
0.0
0
30
60
90
120
Time of assay (minutes)


B Reproductive stage
shoot excised
CO_2 efflux
C_2H_4
control
treatment
µmoles C_2H_4 per plant per minute
µmoles CO_2 per plant per minute
2.0
1.5
1.0
0.5
0.0
10.0
7.5
5.0
2.5
0.0
0
55
110
Time of assay (minutes)

CHAPTER 4

Nodulation

4.1. The Effect of *Rhizobium* Strain and Nitrate Level on Nodulation, Nodule Activity, and the Growth of Pea and Faba Bean

4.1.1. Introduction

Different combinations of legumes and rhizobia vary in their capacity to produce nodules and to fix N_2. Some combinations produce long-lived nodules which fix N_2 at high rates, the highly 'effective' combinations, whilst at the other extreme ineffective nodules fix no detectable N_2 and may even be parasitic, reducing the yield of nodulated plants compared with non-nodulated plants. Between these extremes lie many degrees of effectiveness (Bergersen 1974; Sprent 1979). Strains of rhizobia which are effective on one species or cultivar may be less effective or even ineffective on another, although producing nodules readily on both. It was observed early in the present study that the commercial peat inoculant, Group E was late in nodulating 'Fiord' faba bean and inoculated plants showed N deficiency during the early stage of growth. The inoculant therefore appeared to be relatively ineffective.

It is widely accepted that the N_2 fixing activities of nodulated legumes are influenced by mineral N in at least two major ways. Firstly, nodulation may be promoted by relatively low levels of mineral N in the soil solution, high concentrations almost always being depressive. Secondly, N_2 fixation by actively growing plants is inhibited or at least suppressed by NO_3^-. It is not known how the faba bean and pea used in southern Australia respond to applied NO_3^-, whether nodulation is stimulated or depressed or whether nodule activity is reduced in the presence of NO_3^-.

The experiments reported in this chapter were designed to determine the effect of strain of *Rhizobium* , plant cultivar and NO_3^- level on nodulation, nodule activity and growth of pea and faba bean. Broadly, three areas were investigated: (i) a comparison of the relative effectiveness of strains TA 101 and SU 391 of *Rhizobium leguminosarum* on 'Fiord' faba

bean; (ii) the effect of NO_3^- level on nodulation, nodule activity and plant growth; and (iii) the effect of strain of *Rhizobium*, TA 101 on two cultivars of pea.

4.1.2. Methods

Plants were grown in a glasshouse under natural irradiance and photoperiod over the period June to August 1984 for faba bean and September to October 1984 for pea. Plants were arranged in pots in a randomised, complete block design. Faba beans were inoculated with a culture of either strain TA 101 or SU 391 of *R. leguminosarum* (Horticultural Research Station, NSW) whereas the pea cultivars 'Early Dun' and 'A102' were inoculated with strain TA 101 on commercial peat as the inoculant, group E. Faba beans were given 0.0, 2.5 and 5.0 mM NO_3^- and peas 0.0, 2.5, 5.0 and 7.5 mM NO_3^- after emergence.

Faba beans were harvested eight times, 29, 34, 38, 44, 50, 56, 63 and 70 days after sowing (DAS) with AR assays made at each harvest. Hydrogen evolution was measured only from day 44. For peas, harvests, HE and AR assays were carried out 22, 27, 32 and 44 days after sowing.

4.1.3. Results

Plant growth

In faba beans there was no significant effect of strain of Rhizobium or NO_3^- treatment on dry matter accumulation up to 50 DAS ($P>0.05$) but there was a significant interaction between strain of *Rhizobium*, NO_3^-, and time of harvest on dry matter accumulation ($P<0.01$). Figure 11 shows that after day 50, plants inoculated with SU 391 had a lower growth rate than those inoculated with TA 101 when both were grown without mineral N. A 2.5 mM solution overcame the N deficiency apparent in the SU 391 plants so that SU 391 + 2.5 mM plants did not significantly differ in yield from TA 101 + 2.5 mM plants. Overall NO_3^-, at 2.5 mM increased dry matter only slightly, 5.0 mM did so further but there was no significant difference between TA 101 and SU 391 in the presence of NO_3^-. NO_3^- and *Rhizobium* strain did not affect the growth of roots. NO_3^- increased the shoot growth of both cultivars of pea but had little effect on the growth of root (Fig. 12).

Fig. 11. Cumulative dry weight of root and shoot of faba bean grown at 3 levels of nitrate (0.0; 2.5; and 5.0 mM NO_3^-) and inoculated with either *R.leguminosarum* TA 101 or SU 391.
Strains x NO_3^- x Harvests ***, LSD 5%: 0.46

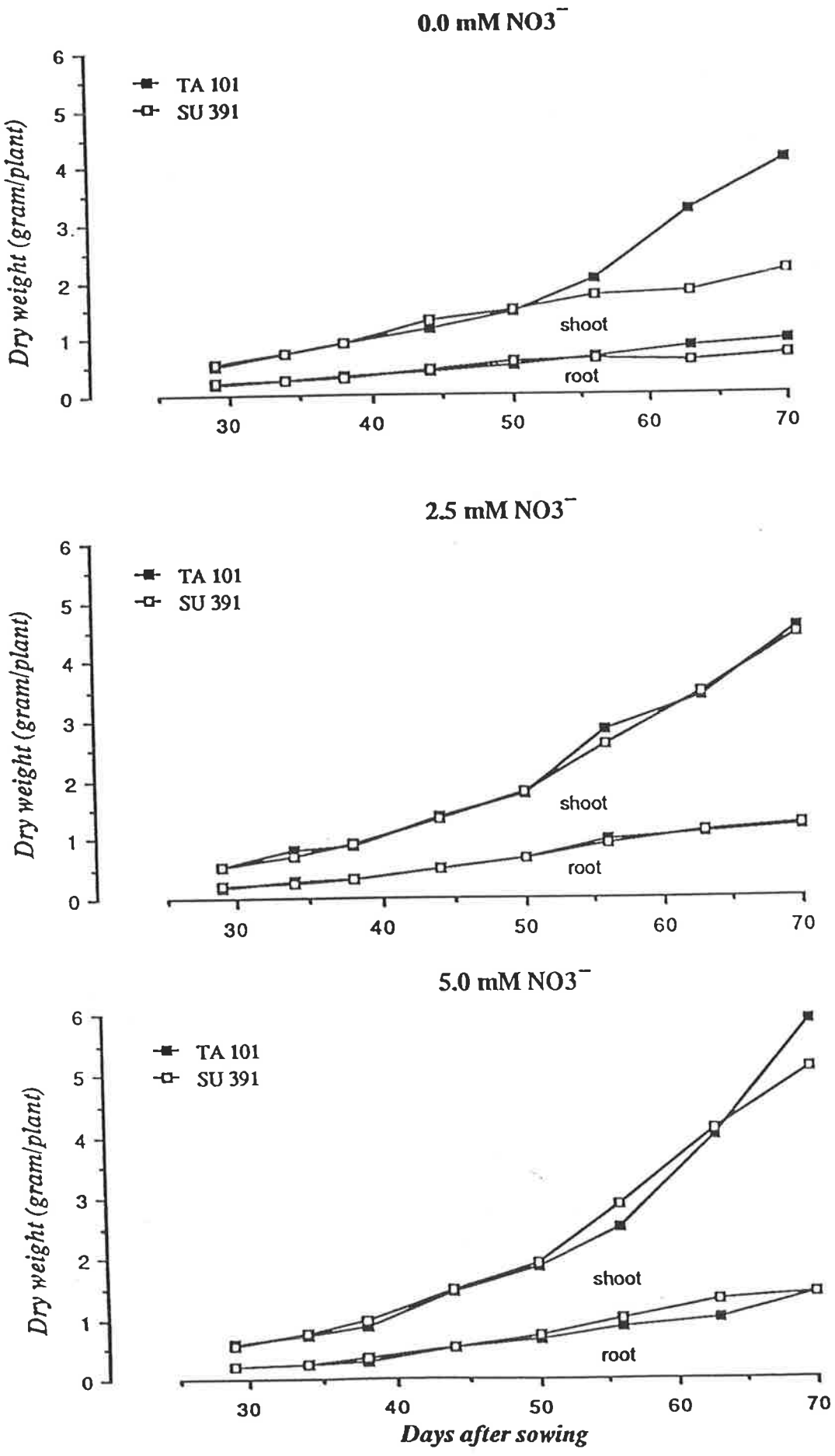

0.0 mM NO3⁻
TA 101
SU 391
Dry weight (gram/plant)
shoot
root
2.5 mM NO3⁻
TA 101
SU 391
Dry weight (gram/plant)
shoot
root
5.0 mM NO3⁻
TA 101
SU 391
Dry weight (gram/plant)
shoot
root
30
40
50
60
70
Days after sowing

Fig. 12. Cumulative dry weight of root and shoot of *P. sativum* cv A102 (A) and cv Early Dun (B) which were grown in 4 levels of NO_3^- (0.0; 2.5; 5.0; and 7.5 mM NO3). Plants were inoculated with *R. leguminosarum* TA 101 .
Genotypes x NO_3^- x Harvests: NS
Harvests x NO_3^- ***, LSD 5%: 0.27

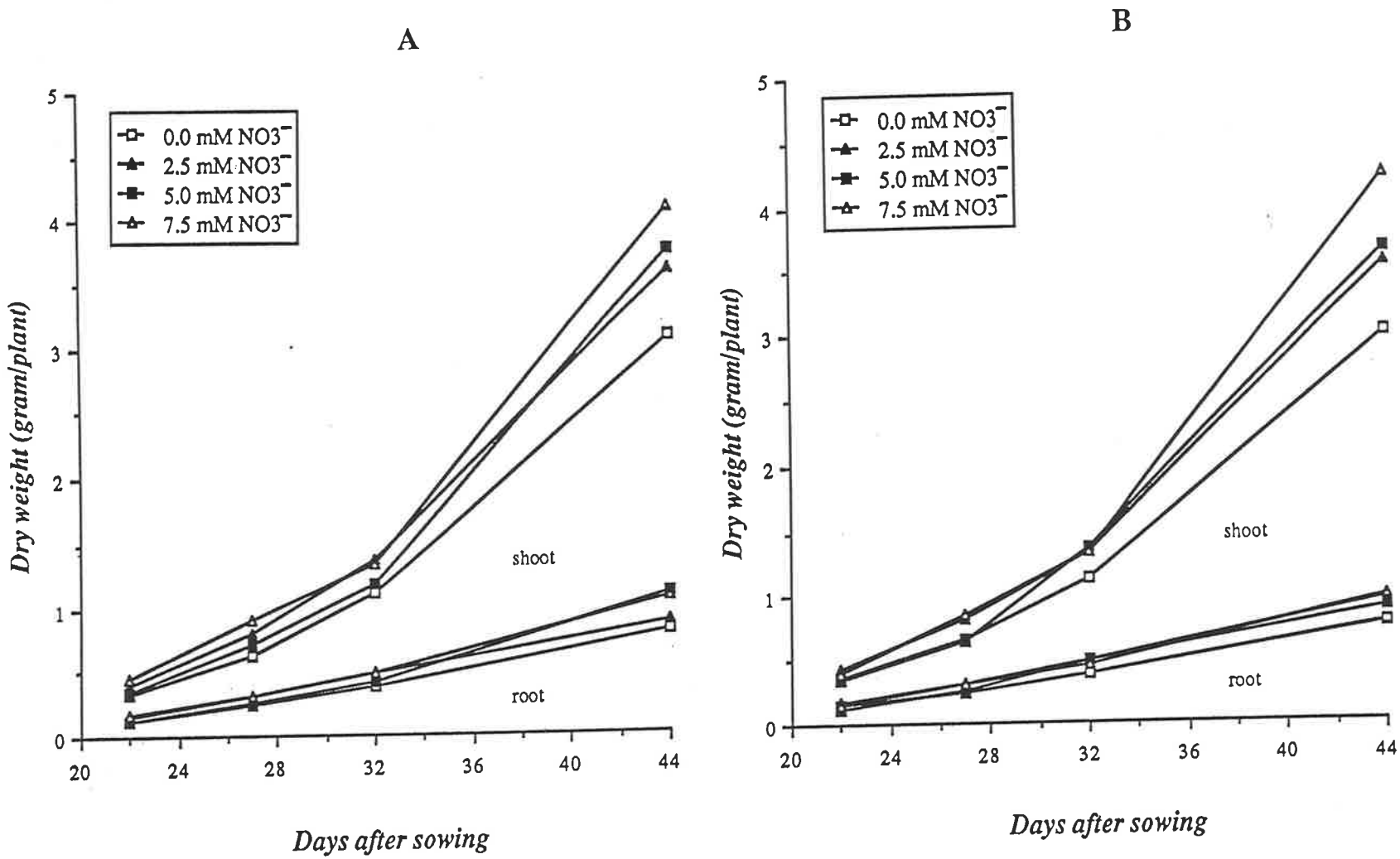

A
B
0.0 mM NO3⁻
2.5 mM NO3⁻
5.0 mM NO3⁻
7.5 mM NO3⁻
shoot
root
Dry weight (gram/plant)
Days after sowing
0
1
2
3
4
5
20
24
28
32
36
40
44

Table 4. The effect of NO_3^- level (0.0, 2.5 and 5.0 mM) and *Rhizobium* strains TA 101 and SU 391 on nitrogenase activity of faba bean

AR: µmol C_2H_4 per plant per hour

AR-HE: µmol C_2H_4 - µmol H_2 per plant per hour

	TA 101						SU 391					
	AR			AR-HE			AR			AR-HE		
NO_3^- (mM)	0.0	2.5	5.0	0.0	2.5	5.0	0.0	2.5	5.0	0.0	2.5	5.0
DAS												
29		0.32	0.29				0.37	0.02				
34	0.53	0.46	0.50				0.06	0.19	0.37			
38	0.70	0.42	0.67				0.35	0.25	0.43			
44	3.84	1.78	1.43	2.46	1.01	0.54	0.33	1.24	0.63	0.28	0.96	0.46
50	6.71	4.63	2.40	3.55	2.37	1.36	4.27	4.52	1.38	3.10	2.81	1.59
56	17.02	12.74	4.70	10.27	7.76	2.72	10.14	9.46	7.55	7.57	6.66	5.49
63	14.13	11.50	9.01	8.16	6.66	5.34	9.20	11.01	8.06	6.17	6.69	5.54
70	9.41	8.95	5.36	4.36	5.01	2.89	7.38	9.30	6.35	4.59	6.09	4.22

Source of variation	LSD 5%	
	AR	AR-HE
Strains x NO3- x Harvests	NS	NS
NO3- x Harvests	2.52**	NS
NO3-	-	1.83*
Harvests	-	1.60***

Nitrogenase activity

The AR activity of faba bean with TA 101 was first detected at about day 44, but with SU 391 there was little activity until day 50 (Table 4). AR and AR-HE were greater in TA 101 than in SU 391 when plants were grown without mineral N, but 2.5 mM NO_3^- depressed AR and AR-HE of TA 101 but not of SU 391. Nodule activity was suppressed in both symbioses by 5.0 mM NO_3^-.

Table 5 shows that in both cultivars of pea, NO_3^- had a marked effect on AR and on AR-HE, A102 always showing a higher activity ($P<0.05$). At 2.5 mM, NO_3^- did not affect AR and AR-HE up to day 32 but thereafter the activity in each symbiosis was reduced about 50%. 'A102' appeared to be more tolerant to NO_3^- at 5.0 and at 7.5 mM than 'Early Dun'.

Nodulation

In faba bean (Table 6), there was no significant effect of *Rhizobium* strain on nodule weight when NO_3^- was supplied. TA 101 appeared to produce better nodulation than SU 391 when grown without NO_3^-. In both symbioses, 5.0 mM NO_3^- depressed nodule weight and 2.5 mM did not affect nodule weight with TA 101 but increased it with SU 391. NO_3^- depressed nodulation more markedly in both cultivars of pea than in faba bean.

Table 5. The effect of NO_3^- level on nitrogenase activity of two pea cultivars, 'A102' and 'Early Dun' inoculated with *R. leguminosarum* TA 101

AR: μmol C_2H_4 per plant per hour

AR-HE: μmol C_2H_4 -μmol H_2 per plant per hour

	A102								Early Dun							
	AR				AR-HE				AR				AR-HE			
DAS	22	27	32	44	22	27	32	44	22	27	32	44	22	27	32	44
NO_3^- (mM)																
0.0	3.71	6.78	10.56	13.84	2.07	3.50	7.41	9.79	6.53	5.27	8.21	8.56	4.31	2.36	5.42	5.75
2.5	3.06	6.93	10.68	6.14	1.81	4.13	7.29	4.12	2.09	6.04	8.93	4.01	1.41	3.08	5.99	2.77
5.0	0.36	1.53	2.60	2.29	0.18	0.71	1.64	1.55	0.47	0.79	2.49	1.50	0.26	0.43	1.56	1.09
7.5	0.36	0.54	1.42	2.41	0.09	0.19	0.83	1.52	0.17	0.25	0.44	0.90	0.13	0.16	0.26	0.57

Source of variation	LSD 5% AR	AR-HE
Harvests x Genotypes x NO_3^-	NS	NS
Harvests x NO_3^-	2.20***	1.79***
Genotypes	0.90*	NS

Table 6. The effect of NO_3^- on the fresh weight of nodules of two symbioses of faba bean (A) and two cultivars of pea (B)

	Nodule fresh weight (g/plant)							
NO_3^- (mM)	0.0	2.5	5.0	7.5	0.0	2.5	5.0	7.5
DAS								
A. Faba bean								
	TA 101				SU 391			
50	0.42	0.39	0.15		0.29	0.48	0.18	
56	0.82	0.85	0.41		0.64	0.64	0.51	
63	1.59	1.55	0.94		1.36	1.58	0.95	
70	1.15	0.92	0.96		0.77	1.02	0.80	
B. Pea								
	'A102'				'Early Dun'			
22	0.17	0.10	0.04	0.04	0.14	0.05	0.03	0.01
27	0.27	0.18	0.07	0.05	0.22	0.14	0.03	0.04
32	0.43	0.43	0.19	0.09	0.36	0.33	0.15	0.03
44	1.01	0.65	0.45	0.30	0.75	0.54	0.21	0.16

Source of variation	LSD 5%	
	Faba bean	Pea
Strains/Genotypes x NO_3^- x Harvests	NS	NS
Strains/Genotypes x Harvests	NS	0.06***
NO_3^- x Harvests	NS	0.08***
Harvest	0.26***	-
NO_3^-	0.18**	-

4.1.4. Discussion

The symbiosis between *V. faba* and *R. leguminosarum* TA 101 appeared to be more effective than that between *V. faba* and SU 391, especially when plants were grown without NO_3^-. It appears also that TA 101 is more tolerant to the highest level of NO_3^- than SU 391. Strain SU 391, however, became as effective at symbiotic N_2 fixation in the presence of 2.5 mM NO_3^- as TA 101 grown without NO_3^-. This result indicates that SU 391 is less effective than TA 101 and needs a low concentration of mineral nitrogen at the seedling stage to give good nodulation and good early growth.

Stimulatory effects of combined N on nodule development and N_2 fixation have been described before (Dart and Wildon 1970; Gibson 1976; Hill-Cottingham and Lloyd-Jones 1980) though they are not yet clearly understood. They are common only in the tribe Phaseoleae (Sprent and Thomas 1984). The hypogeal species *P. sativum* and *V. faba* , both in tribe Viciae may synchronise the exhaustion of seed reserves of N with the initiation of the ability to fix N_2, and therefore do not require a dose of 'starter N', when the symbioses are effective.

SU 391 became active in N_2 fixation as measured by NA very late (day 50) and there were few nodules on the tap root which again indicates ineffectiveness. Bergersen (1974) considers extreme lateness of nodulation to be associated with a poor level of effectiveness. It has been reported that nodulation in *V. faba* begins shortly after germination when it is inoculated with effective *Rhizobium*, and fixation starts at about the time when the first true leaf (node 3) is fully expanded (Fyson and Sprent 1982). The upper parts of the tap root become almost entirely covered with nodules, except where lateral roots emerge.

The AR and AR-HE during the experiment increased up to day 56 and then decreased, which was apparently related to changes in the irradiance and temperature. At day 56, plants received 13.3 MJ m^{-2} solar radiation and the temperature was 11.2-19.3°C whereas on days 62 and 70 they received only 5.9 and 8.4 MJ m^{-2} respectively and the temperatures were 8.3-12.3 and 8.3-14.7°C (Meteorological records, Waite Agricultural Research Institute). It is known that low temperature and low irradiance can each limit N_2 fixation and increased temperature and irradiance will increase activity (Gibson 1977; Sprent *et al.* 1977; Sprent and Bradford 1977).

The *Rhizobium* strain SU 391 appeared to be effective for both cultivars of pea since nodulation and NA were both active on day 22, but pea plants appeared to be less tolerant of NO_3^- compared with faba beans. Five mM NO_3^- reduced nodule activity of faba bean by about 50% compared with the NO_3^- free plants but in pea the high concentrations of NO_3^- (5.0-7.5 mM) reduced activity by as much as 80%. Generally, SU 391 was more efficient with cv. 'A102' than with 'Early Dun'. 'A102' had a higher nodule activity and was more tolerant to higher NO_3^- than 'Early Dun'. These results agree with those of Hill-Cottingham and Lloyd-Jones (1979) and Roughley *et al.* (1983) that faba beans are relatively tolerant to NO_3^-.

NO_3^- at 2.5 mM did not depress nodule activity in either cultivar of pea up to day 32 but thereafter it did so markedly. It is difficult to explain why a low concentration of NO_3^- depressed nodule activity only at the later stage. It could be that at the early stage plants did not take up the NO_3^- and there was enough N in the seed for seedling growth and nodulation. It appears also that when an effective symbiosis has been achieved and nodule activity has started, NO_3^-, even at a low concentration, will inhibit nodule activity. In contrast, when the host plant is inoculated with a relatively inefficient *Rhizobium*, a low concentration of NO_3^- will promote nodulation, and nodule activity. This suggests that the ability of NO_3^- to switch off nodule activity is linked to the fixed N input from the nitrogenase system. This may be due to a competition for energy substrates between N_2 fixation and NO_3^- assimilation.

It can be concluded from these results that poor inoculants for faba bean caused N deficiency between emergence and the onset of N_2 fixation. Shortage of N was found to reduce vegetative growth and would subsequently also be likely to reduce yield and N content of the grain, especially when soil nitrogen is low. When soil mineral N in the field is high it could markedly reduce the potential N_2 fixation of pea plants since they are sensitive to NO_3^-.

4.2. The Effect of Removing the Cotyledons on Nodulation and Nodule Activity of Faba Bean Grown with and without NO_3^-

4.2.1. Introduction

Results presented under 4.1.3. show that nodulation and nodule activity of faba bean inoculated with the commercial peat inoculant 'Group E' are very late (day 50) so that seedling plants become stressed for N. Some authors have reported that there is an inhibitor to nodulation in the cotyledons of both *P. sativum* (Phillips 1971) and *V. faba* (Bano *et al.* 1983). Such an inhibitor could explain these results so it was decided to conduct an experiment to examine whether the delay in faba bean nodulation was due to this cause.

4.2.2. Methods

Plants were grown in a temperature controlled glasshouse at 20°C over the period July-September 1985, arranged in a randomised complete block design. Plants were inoculated with *Rhizobium leguminosarum* strain TA 101 and grown without NO_3^- ($-NO_3^-$) or inoculated and supplied with 2.5 mM NO_3^- ($+NO_3^-$). Cotyledons were removed either 14 or 18 days after sowing. Treated plants were compared with control plants when both were harvested 5, 9, 14, 18, 22, 25, 29, 33 and 37 days after sowing. AR assay was conducted at each harvest and the weights of roots, shoots, cotyledons, and nodules and numbers of nodules (white and red) determined.

4.2.3. Results

Plant growth

The dry weight of the cotyledons declined very rapidly from day 5 to day 20 after sowing (Fig. 13) and then remained constant under all treatments until at least day 37. At day 14, some 40% of the original weight of the cotyledon was still present, but by day 18 almost all reserves had been transferred to the growing seedling or had been respired.

Root and shoot growth were not increased significantly by NO_3^- supply between days 22 and 33 (Figs 14 and 15), but by day 37 the growth of $+NO_3^-$ plants was significantly

Fig. 13. Dry weight of faba bean plants except for cotyledons grown without mineral nitrogen for 37 days. Dry weight of cotyledons at each harvest is also shown.

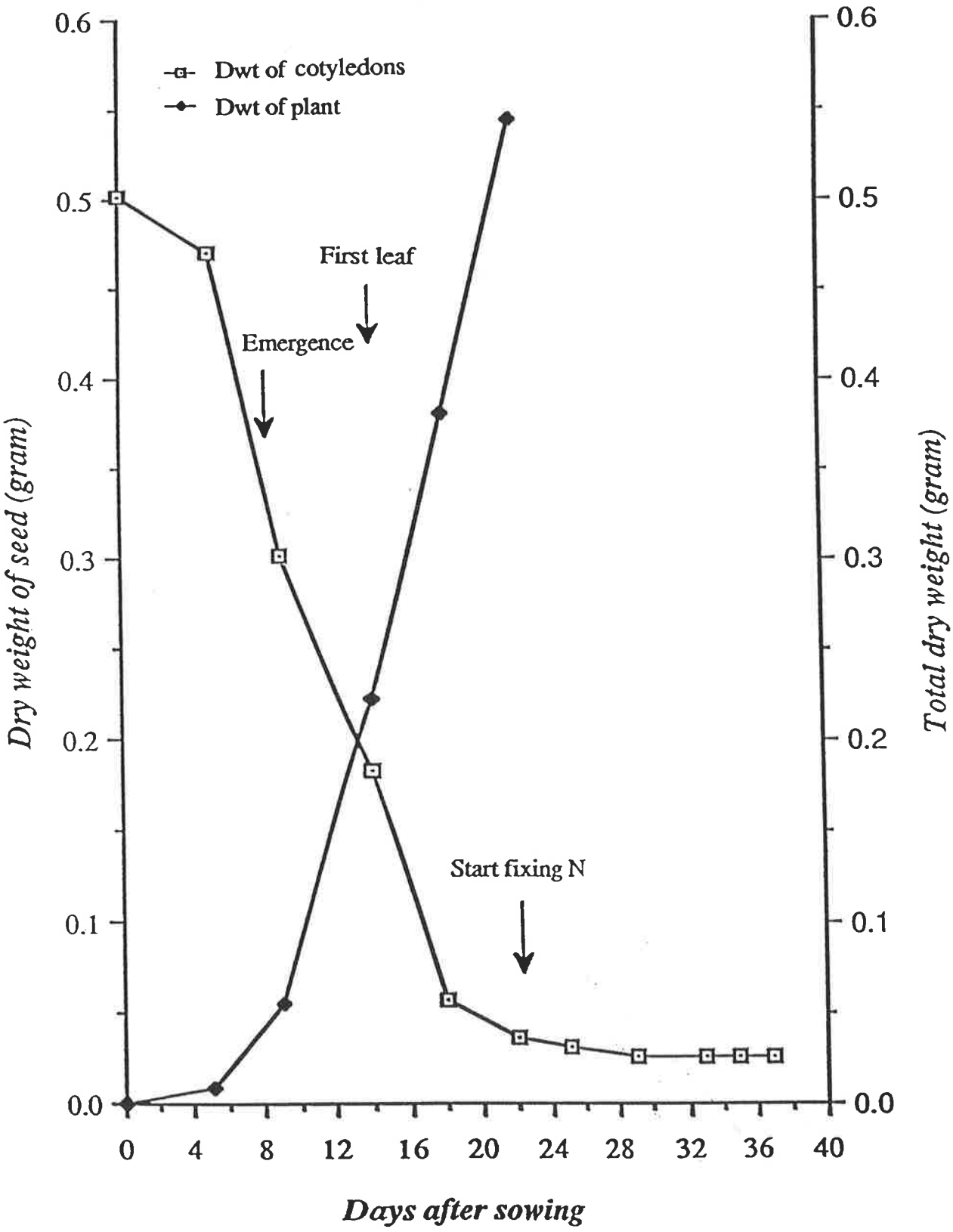

Dwt of cotyledons
Dwt of plant
First leaf
Emergence
Start fixing N
Dry weight of seed (gram)
Total dry weight (gram)
Days after sowing
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
4
8
12
16
20
24
28
32
36
40

Fig. 14. Dry weight of the roots of faba bean plants measured from day 22 to day 37 after sowing. Cotyledons were removed either on day 14 (treatment I) or on day 18 (treatment II) and supplied with either 0.0 mM NO_3^- solution throughout (Treatment I - NO_3^- or Treatment II - NO_3^-) or with 2.5 mM NO_3^- from day 14 (Treatment I + NO_3^-) or day 18 (Treatment II + NO_3^-). Control plants were supplied with 0.0 mM NO_3^- solution throughout (Control I - NO_3^-) or with 2.5 mM NO_3^- from day 14 (Control I + NO_3^-) or from day 18 (Control II + NO_3^-). Control plants were compared with treated plants.

Treatments x Harvests **, LSD 5%: 0.04.

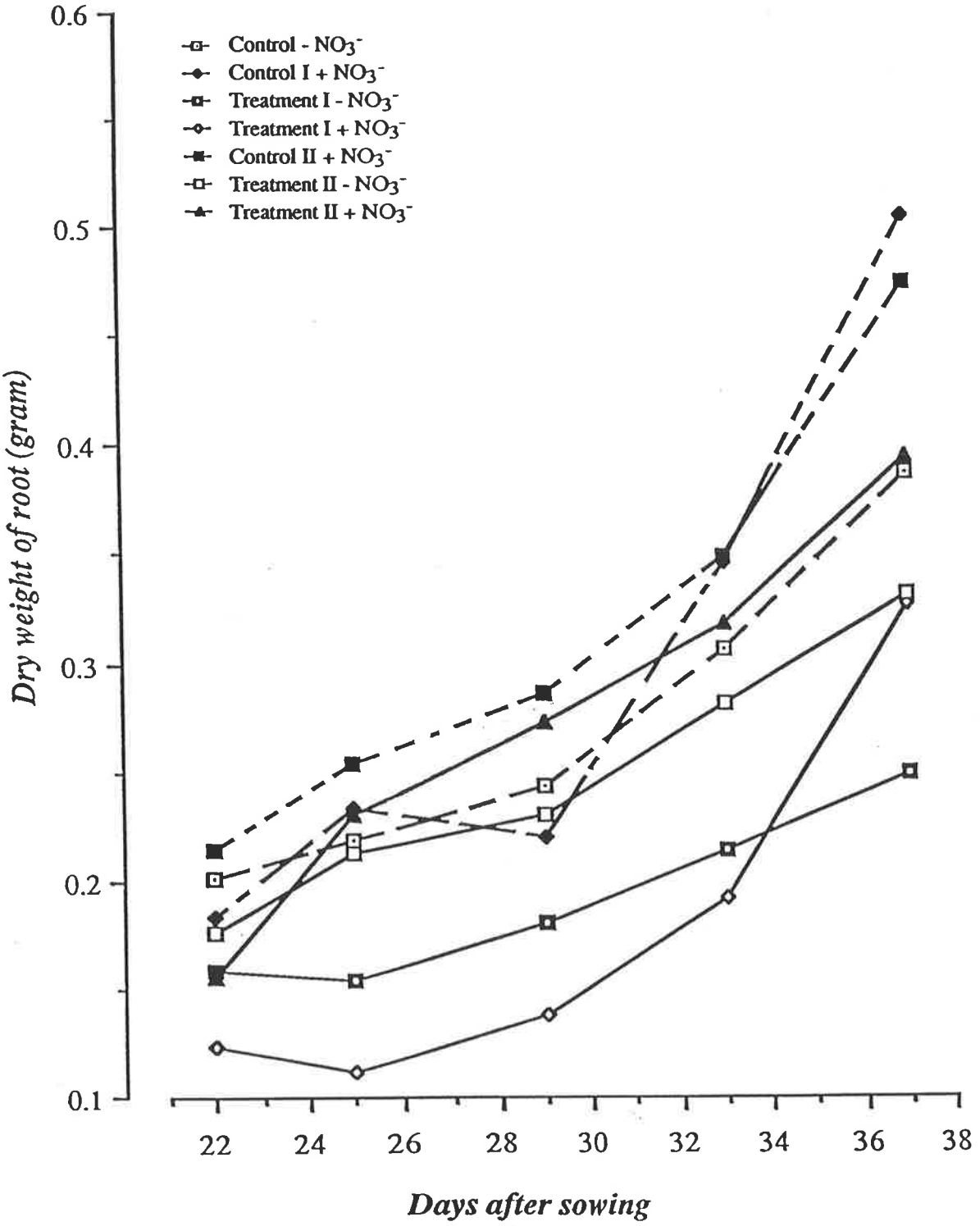
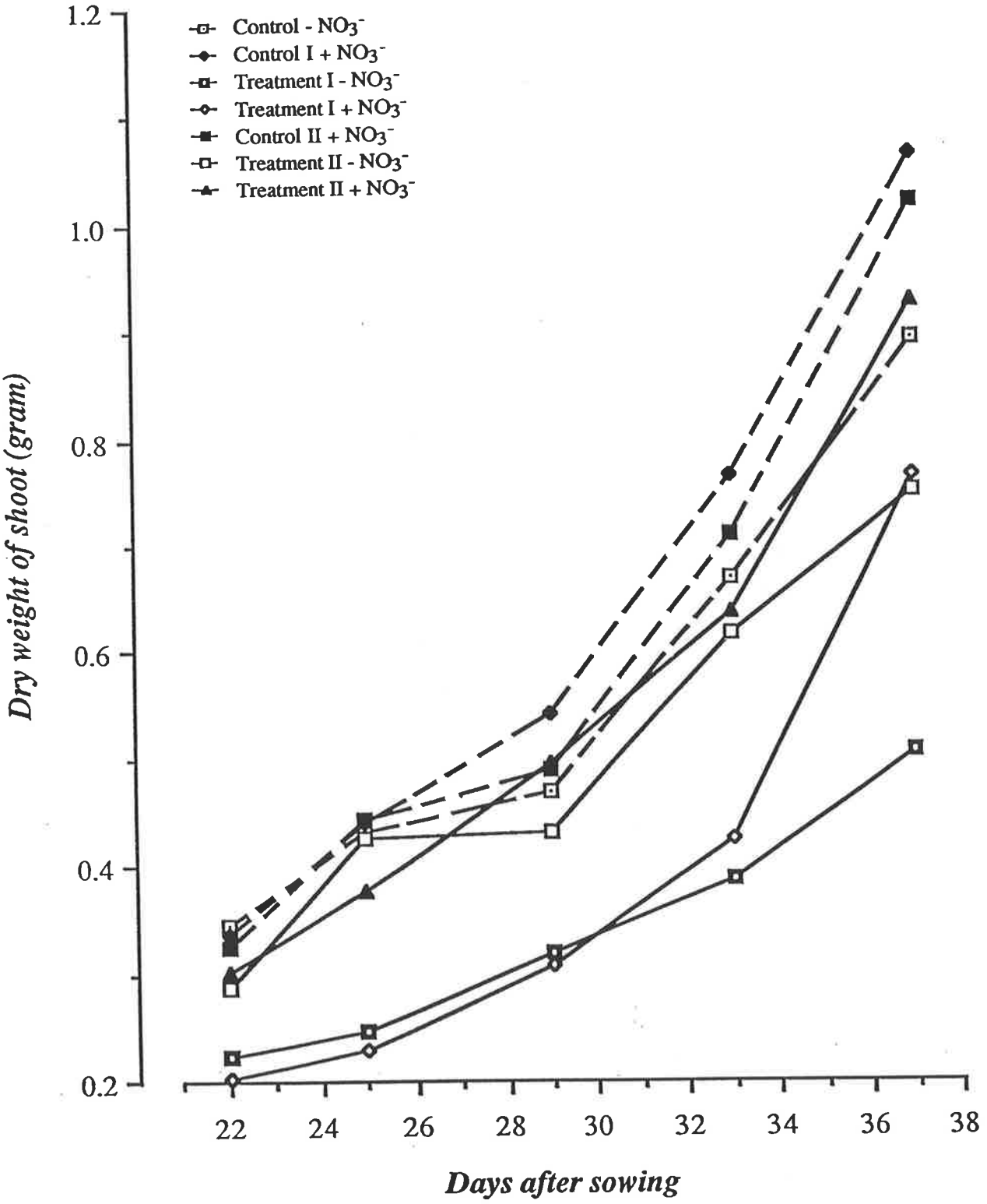

Control - NO_3^-
Control I + NO_3^-
Treatment I - NO_3^-
Treatment I + NO_3^-
Control II + NO_3^-
Treatment II - NO_3^-
Treatment II + NO_3^-
0.6
0.5
0.4
0.3
0.2
0.1
Dry weight of root (gram)
22
24
26
28
30
32
34
36
38
Days after sowing

Fig. 15. Dry weight of the shoots of faba bean plants measured from day 22 to day 37 after sowing. Cotyledons were removed either on day 14 (treatment I) or on day 18 (treatment II) and supplied with either 0.0 mM NO_3^- solution throughout (Treatment I - NO_3^-/Treatment II - NO_3^-) or with 2.5 mM NO_3^- from day 14 (Treatment I + NO_3^-) or day 18 (Treatment II + NO_3^-). Control plants were supplied with 0.0 mM NO_3^- solution throughout (Control I - NO_3^-) or with 2.5 mM NO_3^- from day 14 (Control I + NO_3^-) or from day 18 (Control II + NO_3^-). Control plants were compared with treated plants.
Treatments x Harvests **, LSD 5%: 0.08.


Control - NO_3^-
Control I + NO_3^-
Treatment I - NO_3^-
Treatment I + NO_3^-
Control II + NO_3^-
Treatment II - NO_3^-
Treatment II + NO_3^-
1.2
1.0
0.8
0.6
0.4
0.2
Dry weight of shoot (gram)
22
24
26
28
30
32
34
36
38
Days after sowing

higher than that of -NO_3^- plants. Removal of the cotyledons on day 14 significantly reduced growth, but removal on day 18 had no effect.

Nodulation

Nodulation began about day 20 in all treatments, the first count of nodules being made on day 22 (Table 7). Nodule number increased markedly between day 22 and day 37 with no significant interaction between time of harvest and treatment. An average number of nodules can therefore be calculated for comparison of treatments (Table 8). The addition of NO_3^- reduced the number of nodules by about 42% in control plants but was less severe in the excised treatments where the reduction was only about 26%. Removal of the cotyledons at day 14 significantly reduced nodule number but removal at day 18 had no effect. Close to 50% of all nodules were red under all treatments so neither cotyledon removal nor NO_3^- treatment had any apparent influence on the proportion of nodules formed which become effective.

Table 7. Numbers and dry weight of red and white nodules of faba bean plants from day 22 to day 37 after sowing

Data are the means of the treatments and were transformed to logarithms (values in brackets) for statistical analysis

Time (days)	Number of nodules/plant			Dry weight of nodules (mg/plant)		
	white nodules	red nodules	total	white nodules	red nodules	total
22	5.2 (1.7)	2.5 (1.0)	7.7 (2.7)	-	1.5 (0.7)	1.5 (1.3)
25	9.6 (2.0)	5.5 (1.5)	15.5 (3.7)	1.3 (0.7)	5.5 (1.4)	6.8 (2.4)
29	15.1 (2.4)	17.3 (2.6)	32.3 (5.5)	1.6 (0.9)	11.8 (2.2)	13.3 (3.5)
33	21.7 (2.7)	30.1 (3.2)	51.7 (7.0)	2.6 (1.0)	21.1 (2.9)	23.7 (4.7)
37	41.7 (3.4)	46.7 (3.7)	88.4 (9.0)	4.7 (1.5)	42.0 (3.6)	46.8 (6.6)
LSD 5%	(0.3)	(0.7)	(0.6)	(0.2)	(0.3)	(0.4)

As with nodule number there was not a significant interaction between treatment and time of harvest on nodule dry weight although the latter quantity increased markedly with time. The weight of nodules formed on $+NO_3^-$ plants was 0.53, 0.61, and 0.67 of $-NO_3^-$ plants for control, excised day 14 and excised day 18 treatments. Removal of the cotyledons at day 14 reduced nodule weight to about one half of control but removal at day 18 had no effect on nodule mass.

Table 8. Dry weights and numbers of red and white nodules of faba bean plants grown without NO_3^- or supplied with 2.5 mM NO_3^-

Cotyledons were removed at day 14 and day 18 after sowing. Data are the means of measurements from day 22 to day 37 and have been transformed to logarithms (values in brackets) for statistical analysis

Treatment	Number of nodules/plant			Dry weight of nodules (mg)/plant		
	white nodules	red nodules	total	white nodules	red nodules	total
Control - NO_3^-	23.5 (2.8)	27.1 (2.7)	50.6 (6.4)	3.6 (1.3)	25.0 (2.6)	28.6 (4.6)
Excised day 14						
Control I + NO_3^-	12.4 (1.9)	16.6 (2.1)	29.0 (4.6)	1.6 (0.7)	14.1 (1.9)	15.7 (3.2)
Treatment I - NO_3^-	17.7 (2.4)	18.2 (2.2)	35.9 (5.3)	2.1 (1.0)	11.7 (1.9)	13.8 (3.2)
Treatment I + NO_3^-	12.8 (2.0)	13.9 (1.9)	26.6 (4.6)	1.4 (0.7)	7.2 (1.4)	8.6 (2.5)
Excised day 18						
Control II + NO_3^-	16.2 (2.5)	20.9 (2.6)	37.1 (5.6)	2.2 (1.0)	18.3 (2.3)	20.5 (4.0)
Treatment II - NO_3^-	29.3 (2.8)	25.8 (2.9)	55.1 (6.8)	4.3 (1.3)	22.8 (2.6)	27.1 (4.6)
Treatment II + NO_3^-	19.3 (2.5)	21.7 (2.6)	41.0 (5.9)	2.8 (1.1)	15.7 (2.2)	18.5 (3.8)
LSD 5%	(0.4)	(0.3)	(0.7)	(0.3)	(0.3)	(0.5)

Acetylene reduction activity

Treatment and time of harvest did not interact with AR on a plant basis but interaction was detected when AR was calculated as a specific rate i.e. per g nodule dry weight. On a plant basis AR increased markedly from day 22 to day 44 and was depressed significantly by NO_3^- (Tables 9 and 10). Removal of the cotyledons at day 14 depressed the rate significantly in both - and + NO_3^- plants. Removal at day 18 had no significant effect on -NO_3^- plants compared with control but with late removal the addition of NO_3^- was slightly less depressive of NA than early or no removal.

Table 9. The absolute rate of AR in faba bean plants from day 22 to day 27 after sowing

Data are the means of treatments and were transformed to logarithms (values in brackets) for statistical analysis

Time (days)	AR (µmol C_2H_4 per plant per minute)
22	0.07 (0.06)
25	0.34 (0.27)
29	0.76 (0.52)
33	1.37 (0.82)
37	3.10 (1.34)
LSD 5%	(0.12)

Figure 16 shows how the specific rate of AR varied with time and treatment. Whilst overall NO_3^- depressed the rate, its effect was greater when the cotyledons were removed at day 14 rather than at day 18. Specific activity increased significantly from day 25 to day 37 after removal of the cotyledons on day 18.

Fig. 16. Specific rate of AR activity of faba bean plants measured from day 22 to day 37 after sowing. Cotyledons were removed either on day 14 (treatment I) or on day 18 (treatment II) and supplied with either 0.0 mM NO_3^- solution throughout (Treatment I - NO_3^-/Treatment II - NO_3^-) or with 2.5 mM NO_3^- from day 14 (Treatment I + NO_3^-) or day 18 (Treatment II + NO_3^-). Control plants were supplied with 0.0 mM NO_3^- solution throughout (Control I - NO_3^-) or with 2.5 mM NO_3^- from day 14 (Control I + NO_3^-) or from day 18 (Control II + NO_3^-). Control plants were compared with treated plants. The broken and solid lines indicate the control and treated plants respectively.

Treatments x Harvests **, LSD 5%: 0.26.

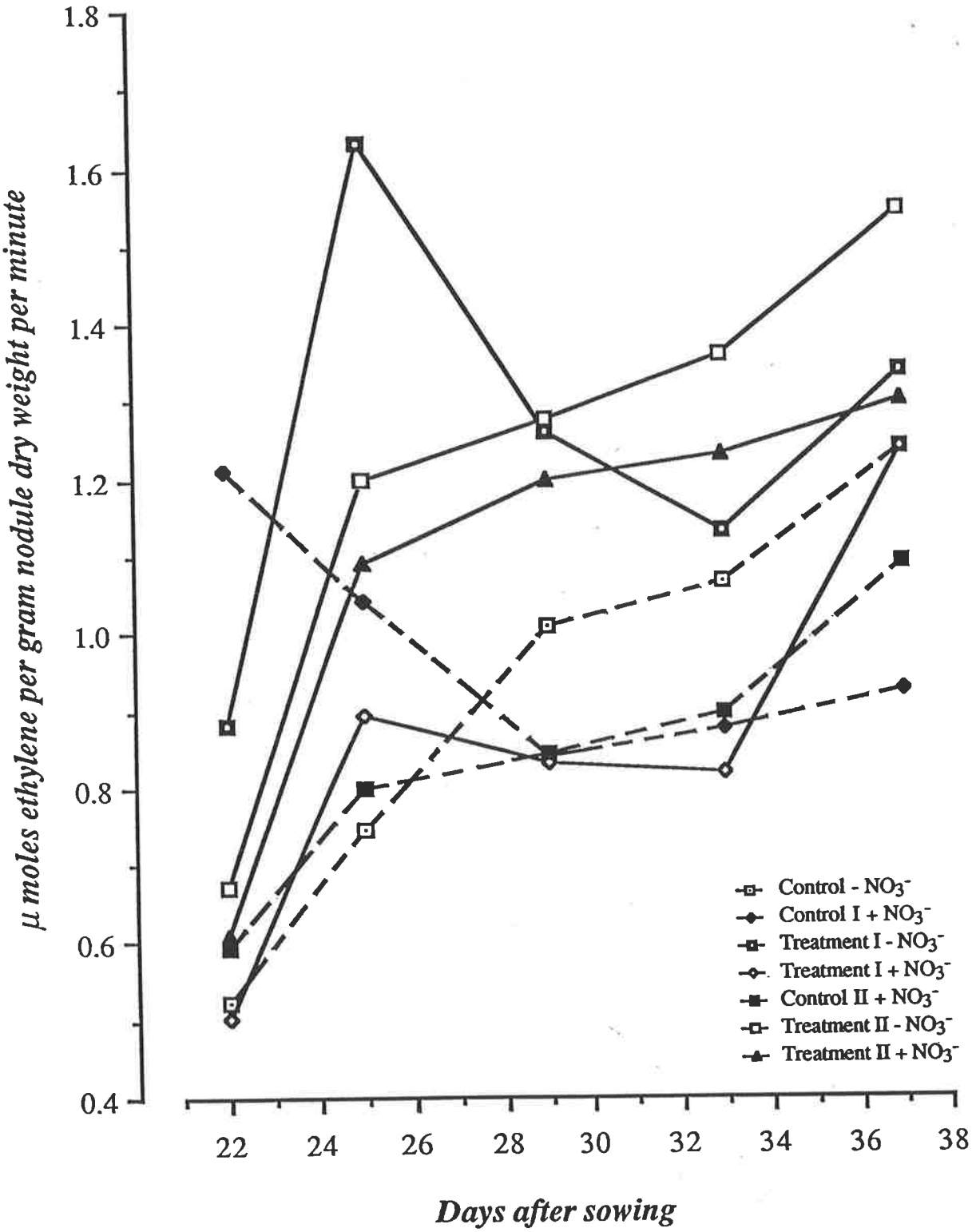

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
22
24
26
28
30
32
34
36
38
μ moles ethylene per gram nodule dry weight per minute
Days after sowing
Control - NO3-
Control I + NO3-
Treatment I - NO3-
Treatment I + NO3-
Control II + NO3-
Treatment II - NO3-
Treatment II + NO3-

Table 10. The absolute rate of AR of faba bean plants grown without mineral NO_3^- or supplied with 2.5 mM NO_3^-

Cotyledons were removed at day 14 and day 18 after sowing. Data are the means of the measurements from day 22 to day 37, and have been transformed to logarithms (values in brackets) for statistical analysis

Treatment	AR (μmol C_2H_4 per plant per hour)
Control - NO_3^-	1.7 (0.8)
Excised day 14	
- NO_3^-	0.9 (0.5)
+ NO_3^-	0.5 (0.3)
Control + NO_3^-	0.7 (0.5)
Excised day 18	
- NO_3^-	1.9 (0.9)
+ NO_3^-	1.2 (0.6)
Control + NO_3^-	1.0 (0.6)
LSD 5%	(0.1)

4.2.4. Discussion

Removal of cotyledons may remove: (i) a source of substrate for growth; (ii) a source of fixed N which may reduce nitrogenase activity; and (iii) an unknown inhibitor which affects nitrogenase directly.

The severe depression of seedling growth following cotyledon removal on day 14 with little effect of cotyledon removal 4 days later, suggests that transfer of substrate from the cotyledons was largely completed by day 18. If the mobilization of food reserves from cotyledons was positively correlated with seed dry weight (Veierskov 1985), it would be possible to say that N_2 fixation began on day 22 (see Fig.13), when cotyledonary N was running low. These results may also be interpreted as an early inhibitory effect of cotyledons on nodulation, as some workers have postulated (Phillips 1971; Bano *et al.* 1981). The severe depression in growth of faba bean seedlings which followed removal of the cotyledons 14 days after sowing emphasises the major role of the cotyledons as a source of substrate for

the developing seedling. Veierskov (1985) obtained a similar result also with *V. faba* but Ndunguru and Summerfield 1975, and Wightman and Thimann 1980 found it possible to remove 80% of the cotyledons of soybean without influencing growth and development. AR activity was also depressed by cotyledon removal on day 14, both on a per plant and a nodule dry weight basis, possibly for the same reason that removing the cotyledons removes a source of substrate. However, cotyledon removal on day 18 increased nodule activity on a nodule dry weight basis. These results suggest that removing the cotyledons on day 18 may remove either: (i) a source of organic N which may inhibit NA; or (ii) an unknown inhibitor which affects NA. This is despite the fact that most of the cotyledon had been metabolised by this time.

Removal of the cotyledons did not increase the dry weight of the nodules. Indeed, removal on day 18 had no effect but removal on day 14 reduced nodule dry weight. Phillips (1971) suggested that an inhibitor may be translocated from the cotyledons of young pea plants to their roots. When he removed one cotyledon before germination, nodulation was increased by 32% in that part of the primary phloem which had been connected to the detached cotyledon. Cotyledons were removed from faba bean plants later in the present experiment than in Phillips's experiment with pea, and he also removed only one cotyledon. It is perhaps then not surprising that nodulation as measured by nodule dry weight was not increased in this present experiment.

Removing cotyledons increased nodule number both at 14 days and grown with NO_3^-, and at 18 days grown either with or without NO_3^-. This suggests that a cotyledonary inhibitory factor is indeed involved. Removal of the cotyledons at day 14 and grown without NO_3^- resulted in depression of nodule number. This suggests that the cotyledons supply N which at this stage is also needed for nodulation. Phillips (1971) suggested that a cotyledonary inhibitor acts for a period between the infection process and the appearance of a macroscopic nodule. This result does not, however, agree with this present experiment since the nodule number of treated plants (removal of cotyledons at day 14) was less than that of control plants. It may be possible that some factor(s) in the cotyledon reduce nitrogenase activity by delaying bacteroid tissue formation instead of inhibiting the infection process (Phillips 1971) or the number of nodules per plant (Bano *et al.* 1983). Thornton (1929),

Schaffer and Alexander (1967), and Peat *et al.* (1981b) found that cotyledons of *Glycine max*, *Medicago sativa*, and *Phaseolus vulgaris* contain a nodulation promoting factor which could be nutritional or hormonal. Either way, removal of cotyledons reduced nodulation. This may explain why, when cotyledons were removed at day 14, the dry weight of the nodule, nodule number and NA were reduced because the plants may not have had sufficient nutrient for nodule growth and activity.

It can be concluded that poor nodulation of 'Fiord' faba bean appears to be due to ineffective inoculant and also to the presence of inhibitor in the cotyledons.

CHAPTER 5

Growth, Nitrogen Accumulation, Nitrogen Partitioning, and Acetylene Reduction Activity in Faba Bean and Pea

5.1. Introduction

Faba bean and pea offer considerable potential as grain legume crops in cereal rotations in South Australia (Laurence 1979). Little is known, however, of their cultural requirements and even less concerning their capacity to contribute to the soil organic nitrogen through nitrogen fixation.

Grain legumes require large amounts of N during grain filling and a proportion of this may come from symbiotic N_2 fixation. However, it is commonly found that N_2 fixation declines early in pod filling (Bethlenfalvay and Phillips 1977; Minchin and Summerfield 1978), when the demand for N for grain formation is at a maximum. There is also often a decrease (50-70%) in the concentration of N of all the vegetative parts of the plant consistent with the 'self-destruct' hypothesis of Sinclair and de Wit (1976), namely, the need to transfer large amounts of N from the vegetative tissue to support normal grain growth. Since a decrease in the amount of N in the vegetative parts accompanies grain filling, grain legumes may make only a small contribution to the N content of the soil in a rotational system. The amount of fixed N removed in the grain varies greatly between species (Farrington *et al.* 1977; Pate and Flinn 1977; Russell 1980; Piha and Munns 1987).

The experiments reported in this chapter were designed to determine how the process of N_2 fixation contributes to the overall N economy, especially in relation to N translocated first from the seed to the plant during emergence and subsequently from the plant to the seed during grain filling. The investigation covered: (i) the growth physiology of faba bean and pea; (ii) the relationship between rate of N accumulation by the plant and rate of AR at different stages in ontogeny; (iii) the distribution of N between different plant organs; and (iv) the capacity of faba bean and pea to contribute organic nitrogen to the soil in a cereal/grain legume rotation.

5.2. *Methods*

Faba bean and pea were grown in a glasshouse over the period July to November 1983 and supplied daily with a nutrient solution lacking NO_3^-. *R. leguminosarum* TA101 was used as an inoculant. Ten pots of each species were removed every 7 days giving 17 harvests throughout the growth period of 144 days. The growing period in the field is normally about 180 days, May to October. AR assay, dry weights and N contents of plant parts were determined at each harvest (see Chapter 3).

5.3. *Results*

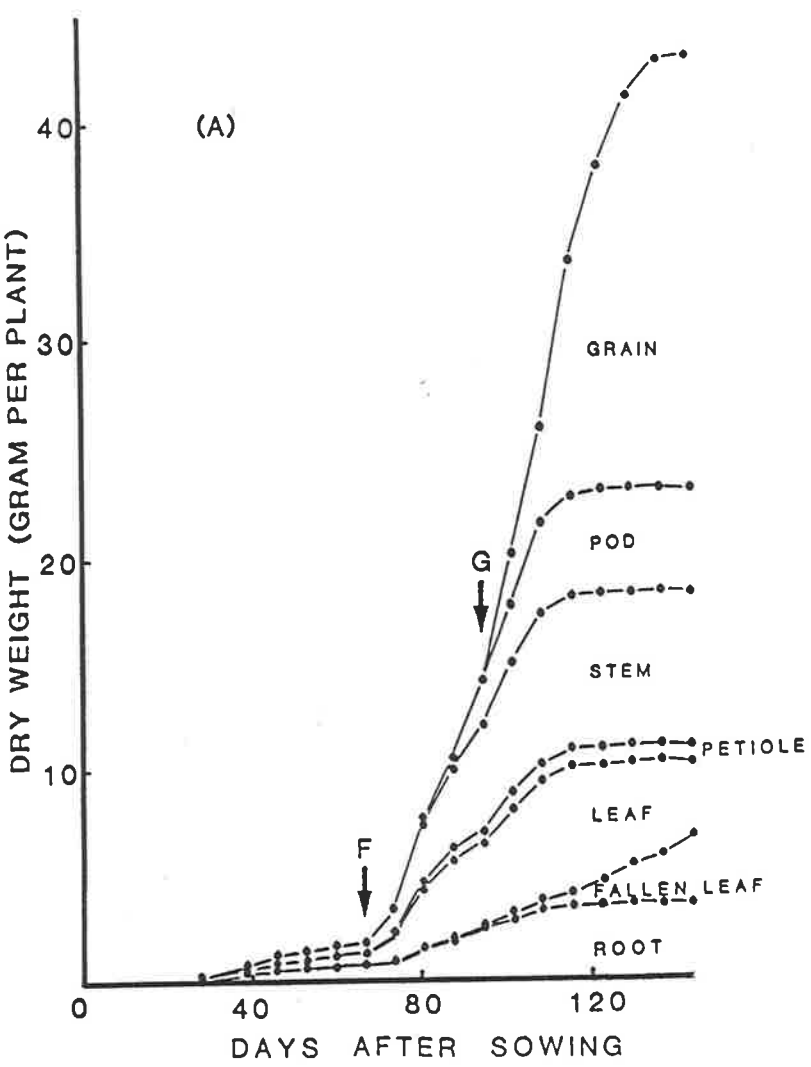
Dry matter accumulation

The growth curves of each species (Figs 17A and B) are considered to consist of three stages: (1) a period of slow growth up to the start of flowering; (ii) a period of near constant growth rate over several developmental stages including stem elongation, pod formation and grain filling; and (iii) a fairly rapid cessation of growth during the later part of grain filling. Some variation in this pattern between the two species is apparent. Flowering began at about day 60 in bean and day 40 in pea.

Bean plants appeared to suffer some N deficiency up to flowering in that they were quite chlorotic. Between days 70 and 90 the growth rate was near constant with a slight acceleration during grain filling (days 90-120). Growth ceased about day 130. The growth rate of pea increased to a maximum about the start of flowering after which a steady but slower rate held until a further decline occurred at the start of grain filling.

Root, stem, leaf and pod weight reached a maximum when grain filling began in each species at about day 116 for faba bean and day 99 for pea. After this, root, petiole, stem and pod of faba bean remained constant whilst grain weight increased and leaf weight decreased about 40%. In pea, leaf and root decreased about 25% and 40% respectively, while petiole, stem and pod remained constant. Grain accounted for 50% of the total dry weight in faba bean and 34% in pea at maturity.

Fig. 17. Cumulative dry weight as a function of time and the distribution of dry matter between plant parts for faba bean (A) and pea (B) plants grown in a glasshouse July to November 1983. F and G indicate the beginning of flowering and of grain filling respectively.


(A)
DRY WEIGHT (GRAM PER PLANT)
40
30
20
10
0
40
80
120
DAYS AFTER SOWING
F
G
GRAIN
POD
STEM
PETIOLE
LEAF
FALLEN LEAF
ROOT

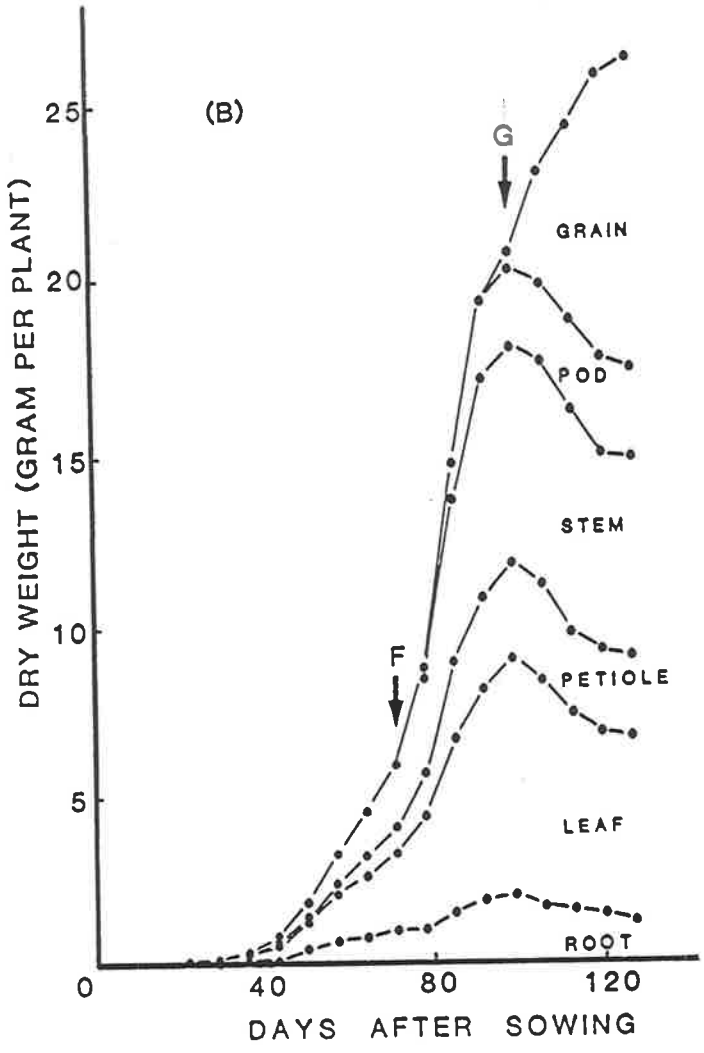
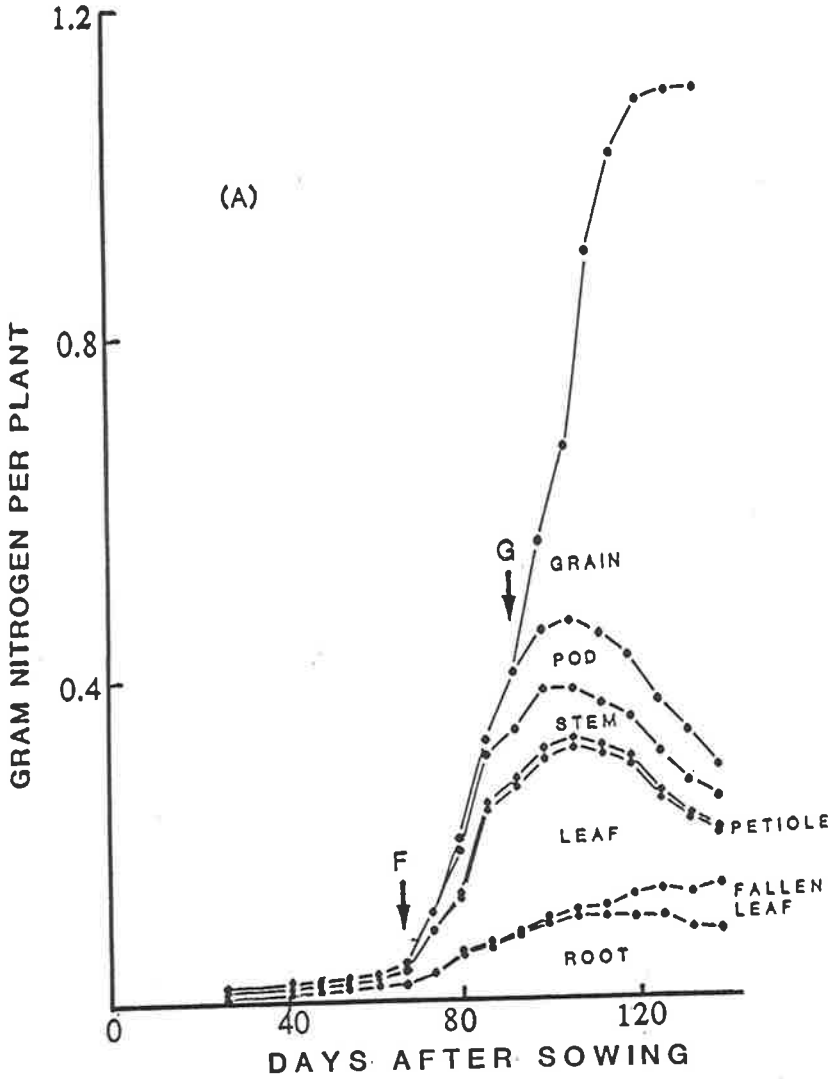
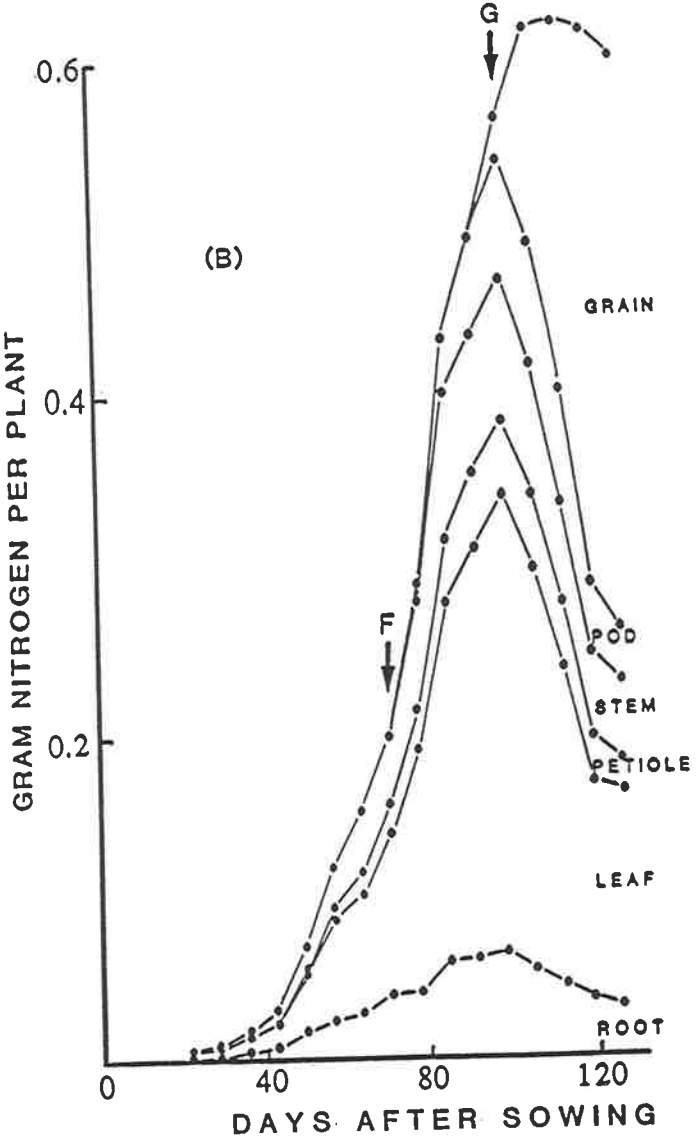

(B)
DRY WEIGHT (GRAM PER PLANT)
25
20
15
10
5
0
40
80
120
DAYS AFTER SOWING
F
G
GRAIN
POD
STEM
PETIOLE
LEAF
ROOT

Fig. 18. Cumulative amount of nitrogen as a function of time and the distribution of nitrogen between plant parts for faba bean (A) and pea (B) plants grown in a glasshouse July to November 1983. F and G indicate the beginning of flowering and of grain filling respectively.


(A)
GRAM NITROGEN PER PLANT
1.2
0.8
0.4
0
40
80
120
DAYS AFTER SOWING
G
F
GRAIN
POD
STEM
LEAF
PETIOLE
FALLEN LEAF
ROOT


(B)
GRAM NITROGEN PER PLANT
0.6
0.4
0.2
0
40
80
120
DAYS AFTER SOWING
G
F
GRAIN
POD
STEM
PETIOLE
LEAF
ROOT

Accumulation and partitioning of nitrogen

The patterns of accumulation and partitioning of N were similar to those for dry weight (Figs 18 A and B). Faba bean gained almost no N until after the beginning of flowering after which the rate of accumulation by the whole plant was near constant until the end of grain filling. The N in leaf, stem and pod reached a maximum about day 109 and then declined about 49%. At maturity, 78% of the total N was in the grain. Pea behaved similarly except that the accumulation of N started at about day 40 and only 56% of the N was in the grain at maturity.

Nitrogen content of plant parts

Percentage N in the root, leaf and stem of faba bean decreased from 4-5% just after emergence to about 2% between days 40 and 50 (Fig. 19A) with a sharp rise soon after N_2 fixation was first detected. The leaves became green very rapidly between days 50 and 60. All organs showed a maximum concentration at the beginning of grain filling but subsequently the percentage N decreased during grain filling. The percentage N of the grain remained steady during grain filling. The percentage N of the various organs of pea declined steadily during the whole growth period (Fig. 19B) and the percentage N of the grain also decreased during grain filling.

Acetylene reduction rate

The AR rates together with the cumulative amount of N in the grain are shown for each species in Fig. 20. AR activity of faba bean did not commence until about day 53, after which there was a rapid increase from the beginning of flowering to a maximum at the beginning of grain filling. Activity remained high during most of grain filling. AR activity of pea, began about day 20, increased very markedly from the beginning of flowering, reached a maximum at the beginning of grain filling and then declined.

Fig. 19. Percentage nitrogen in plant organs of faba bean and pea during ontogeny. The figures are an average for each designated organ regardless of age. A. Faba bean. B. Pea. F and G indicate the beginning of flowering and of grain filling respectively.

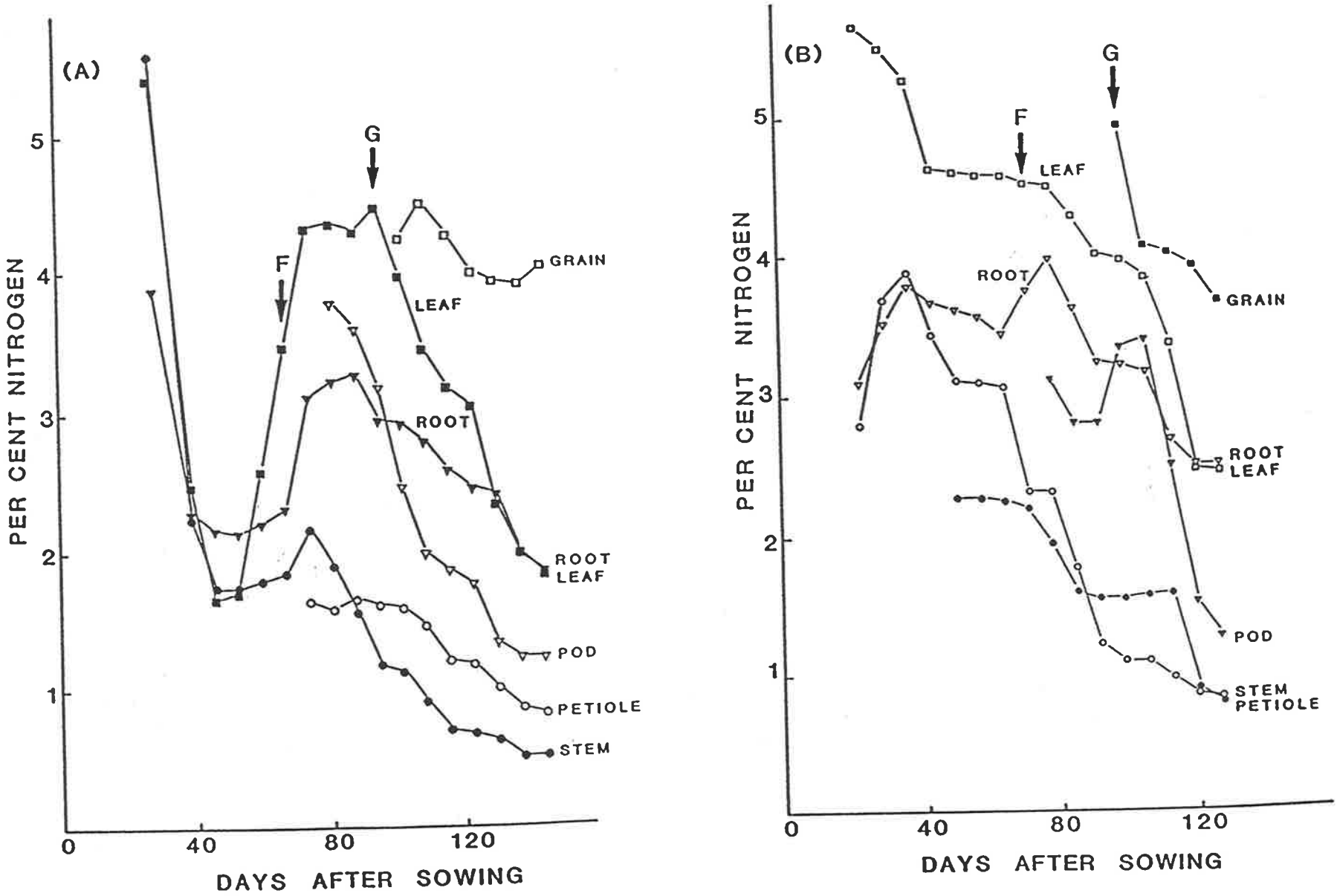
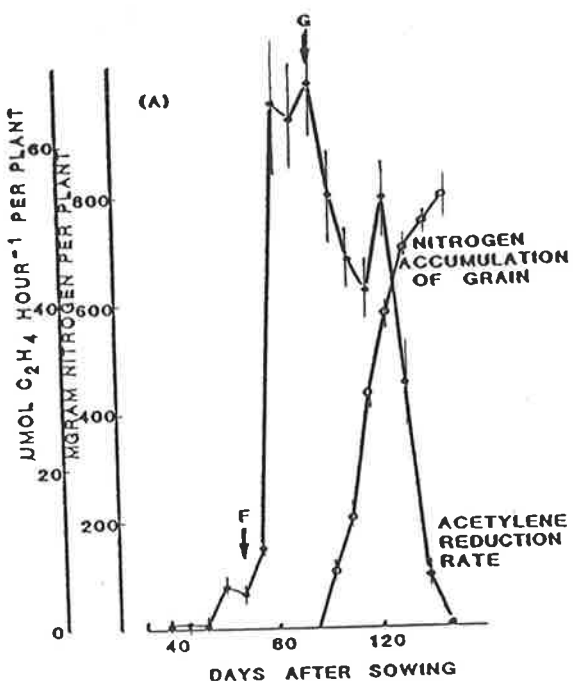
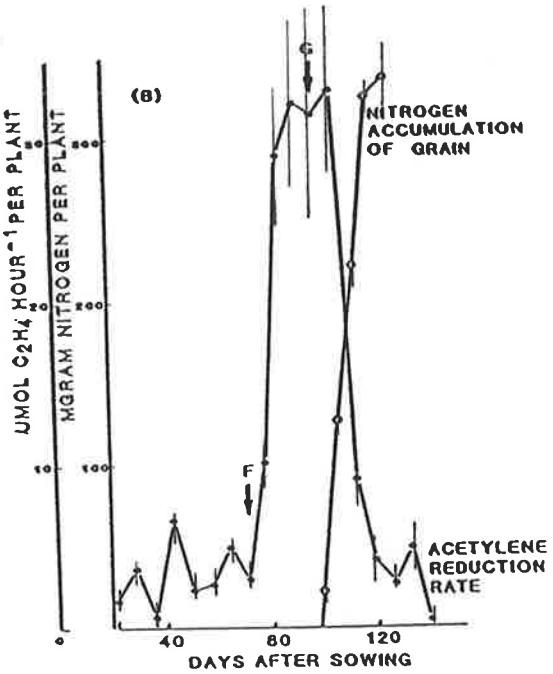
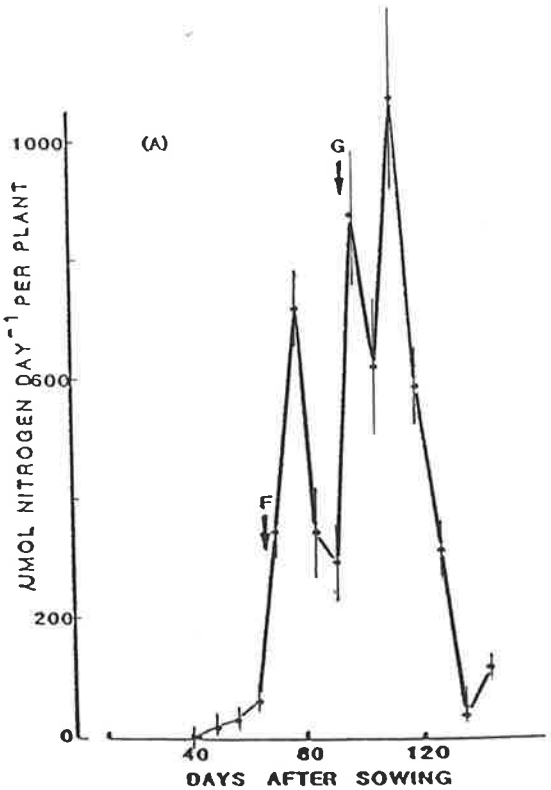

(A)
PER CENT NITROGEN
5
4
3
2
1
F
G
GRAIN
LEAF
ROOT
ROOT
LEAF
POD
PETIOLE
STEM
0
40
80
120
DAYS AFTER SOWING
(B)
PER CENT NITROGEN
5
4
3
2
1
F
G
LEAF
ROOT
GRAIN
ROOT
LEAF
POD
STEM
PETIOLE
0
40
80
120
DAYS AFTER SOWING

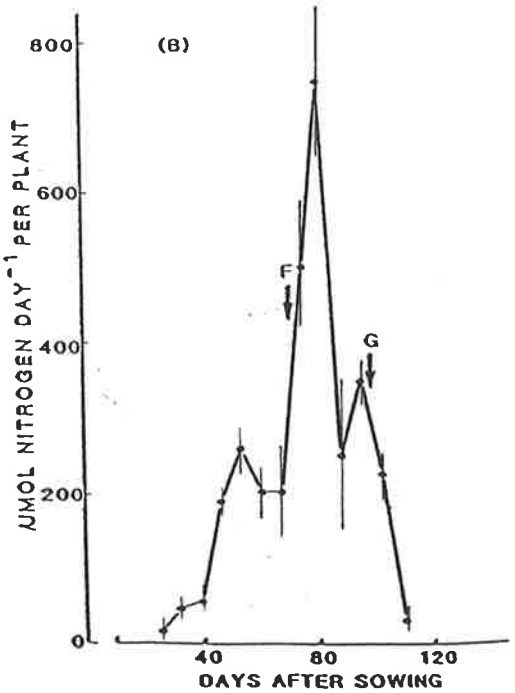
Fig. 20. Acetylene reduction rate as a function of time together with rates of accumulation of nitrogen in the grain. A. Faba bean. B. Pea. F and G indicate the beginning of flowering and of grain filling respectively. Bars indicate the standard error of the mean.

Fig. 21. Rates of accumulation of nitrogen (mg N per plant per day) determined by dry matter harvest and chemical analysis of the dry matter at each harvest. A. Faba bean. B. Pea. F and G indicate the beginning of flowering and of grain filling respectively. Bars indicate the standard error of the mean.


(A)
G
F
NITROGEN ACCUMULATION OF GRAIN
ACETYLENE REDUCTION RATE
UMOL C_2H_4 HOUR^{-1} PER PLANT
MGRAM NITROGEN PER PLANT
0
20
40
60
200
400
600
800
40
80
120
DAYS AFTER SOWING


(B)
G
F
NITROGEN ACCUMULATION OF GRAIN
ACETYLENE REDUCTION RATE
UMOL C_2H_4 HOUR^{-1} PER PLANT
MGRAM NITROGEN PER PLANT
0
10
20
100
200
300
40
80
120
DAYS AFTER SOWING


(A)
G
F
UMOL NITROGEN DAY^{-1} PER PLANT
0
200
600
1000
40
80
120
DAYS AFTER SOWING


(B)
F
G
UMOL NITROGEN DAY^{-1} PER PLANT
0
200
400
600
800
40
80
120
DAYS AFTER SOWING

Nitrogen accumulation rates

The rates of accumulation of N were estimated from the general curve of N accumulation (Fig. 18) and the time course is shown for each species in Fig. 20. For faba bean the rate ranged from 400 to 1000 μmol N day^{-1} $plant^{-1}$ between day 60 and day 120. The fluctuations are due to irregularities in the N accumulation curve (Fig. 18) which may be attributed to overestimation followed by underestimation of the dry weight at successive harvests or to variation in the environment during the growth period. A well defined peak in the rate of accumulation of N rather than a plateau is evident for pea. N accumulation rate was clearly at a maximum about day 80 just after the beginning of flowering, after which there was a sharp decline to zero coinciding with the onset of grain filling.

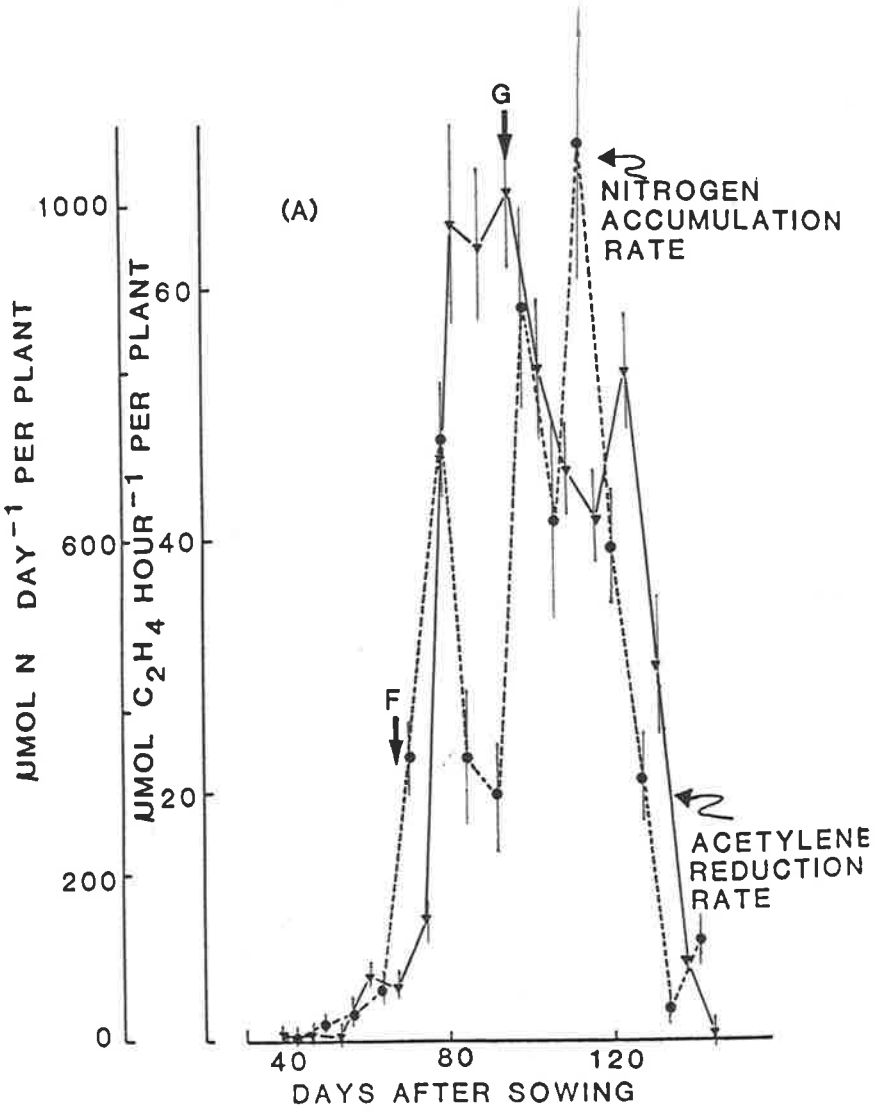
Grain filling of faba bean began where N was accumulating in the whole plant at a maximum rate but in pea almost all the N in the plant at maturity was fixed before grain growth began. About 72% of the N in the grain of faba bean was fixed during the period of grain filling whereas in pea the proportion was only about 20%, the rest being translocated, mainly from the leaves.

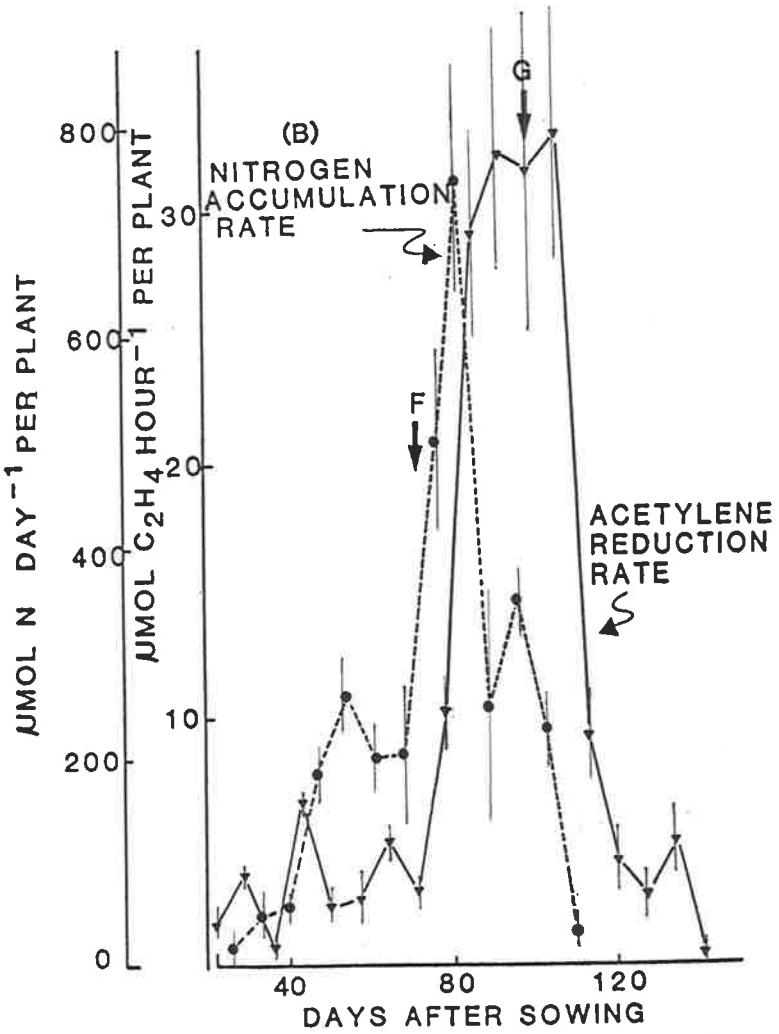
Nitrogen accumulation and acetylene reduction rates

Since the AR rate provides an estimate of the rate of N_2 fixation it is to be expected that change in nitrogenase activity as measured by AR should parallel change in rate of N accumulation as measured by increase in total N content. Fig. 22 shows that in faba bean there was good agreement between the two methods early in ontogeny as well as during maturation. In the middle stage, AR assay remained relatively high while the N accumulation rate fluctuated. Each method, however, identified an early rise, a middle period of relatively steady rate and a final decline.

There was a reasonable agreement between the two methods in pea early in ontogeny but a major difference developed after flowering. N accumulation was at a maximum rate about day 80 after which it declined very rapidly. In contrast AR activity reached a maximum rate somewhat later and continued at this level for about 40 days after which it also declined very rapidly nearly to zero at maturity.

Fig. 22. Rates of accumulation of nitrogen (mg N per plant per day) determined by dry matter harvest and chemical analysis compound with rates of nitrogenase activity as measured by acetylene reduction assay at each harvest. A. Faba bean. B. Pea. Bars indicate the standard error of the mean.


(A)
G
F
NITROGEN ACCUMULATION RATE
ACETYLENE REDUCTION RATE
μMOL N DAY⁻¹ PER PLANT
μMOL C2H4 HOUR⁻¹ PER PLANT
1000
600
200
60
40
20
0
40
80
120
DAYS AFTER SOWING


(B)
G
F
NITROGEN ACCUMULATION RATE
ACETYLENE REDUCTION RATE
μMOL N DAY⁻¹ PER PLANT
μMOL C2H4 HOUR⁻¹ PER PLANT
800
600
400
200
30
20
10
0
40
80
120
DAYS AFTER SOWING

5.4. Discussion

The patterns of accumulation of dry matter and nitrogen for faba bean and pea found in this study are very similar to those shown previously by Hanway and Weber (1971a), Shibles *et al.* (1975), and Warembourgh and Fernandez (1985) for soybean, Farrington *et al.* (1977) for lupin, and Piha and Munns (1987) for cowpea. Considering the data for all five species, some general principles concerning the growth of a grain legume crop can be proposed. It is clear that the three phase approach proposed initially for analysis of the growth of a sward of annual medic (Silsbury *et al.* 1979) also has validity in describing the growth of a community of grain legume plants. After an initial period characterised by an accelerating growth rate (stage I), plants enter a second stage during which the rate remains reasonably constant for a considerable period of time (stage II). Linear regression of dry weight on time accounts for more than 95% of the variation in the data for faba bean and 90% for that of pea. An increase in solar radiation from 8 to 22 MJ m^{-2} day^{-1} (data from meteorological station, the Waite Agricultural Research Institute) during stage II did not have a positive effect on the growth rate of either legume. Soybean and cowpea (Herridge and Pate 1977), and lupin (Farrington *et al.* 1977) have shown similar responses when grown in the field or in the glasshouse.

Another major aspect of the growth patterns of faba bean and pea is that there is no obvious change in the growth rate with change in plant ontogeny during stage II. Figure 18 shows that growth was dominated first by root, stem and leaf growth, then by pod growth and to some extent by stem growth after flowering. Finally the only investment in total dry weight arose from grain filling.

Since N_2 was the only exogenous source of nitrogen for these plants it is of interest to discern how the process of N_2 fixation contributed to the overall nitrogen economy especially in relation to nitrogen translocated first from seed to the plant during emergence and subsequently from the plant to the seed during grain filling.

The slow rate of growth of faba bean observed after emergence appears to have been due to a very slow rate of nodulation or to a relatively ineffective inoculant, *Rhizobium leguminosarum* TA 101. Little nitrogenase activity was evident until 53 days after sowing which contrasts with the 24 days observed for soybeans (Bergersen 1958), 12-14 days for

subterranean clover (Silsbury *et al.* 1984), and 20 or 40 days for faba bean grown at 18 or 10°C, respectively (Fyson and Sprent 1982). However, the large seed of faba bean is clearly able to sustain the growth of the seedling for some considerable time. The slow rate of nodulation suggests that a different inoculant might be an advantage especially in the field when the alternative N source, soil NO_3^-, might be at low concentration. Until an improved inoculant is available a 'starter' dose of nitrogenous fertiliser in the field may result in increasing growth of faba bean and ultimately improved grain yield. Pea plants nodulated early and showed no sign of N stress.

Carbon accumulated in leaves and other organs during the vegetative growth of grain legumes can subsequently be mobilised and moved elsewhere in the plant, presumably to the developing grain. Shibles *et al.* (1975), for example, recorded that substantial quantities of labile carbohydrate present in leaves, petioles and stems of soybean prior to grain development are later utilised in the growth of seed. Herridge and Pate (1977) working with cowpea found that substantial reserves in non-reproductive parts were lost in respiration or moved to the fruits. In the present species the leaves decreased appreciably in dry weight during grain filling and it is likely that this was primarily due to translocation.

The movement of nitrogen from vegetative parts and pod to grain is well documented in grain legumes (Neves *et al.* 1981; Peoples *et al.* 1983). The amount of nitrogen in the vegetative parts and pod decreased by about 50% during the final stages of growth of both faba bean and pea. Rainbird *et al.* (1983) have shown that, in soybean, glutamine and asparagine are the principal nitrogenous solutes important to grain development during pod filling. If these compounds, other amines or any nitrogenous material, are also mobilized in pea, it is clear that the movement of substantial quantities of N will result in the movement of carbon as well, since such compounds consist of about 40% C. Thus, a flux of carbon will automatically accompany a flux in N. This will explain the concomitant decrease in both the dry weight and N content of leaves in both faba bean and pea.

Relations between nitrogen accumulation, nitrogen mobilisation during grain filling and nitrogenase activity (as measured by AR assay) have been much debated, especially since the 'self-destruct' hypothesis of Sinclair and de Wit (1976). In cowpea, nitrogenase activity decreases rapidly after flowering (Peoples *et al.* 1983) but in both faba bean and pea

in this study it increased rapidly after the first flowers were observed (Fig.18). This result appears to be in contrast to that of Pate and Minchin (1980) for pea where fixation declined abruptly after flowering.

Nitrogenase activity continued for longer in faba bean than in pea, so that the former species appeared capable of fixing N_2 throughout most of the growth cyle. This result suggests that the commencement of nodulation may influence the time of nodule senescence. In faba bean, nodulation started late and the nodules senesced late, whereas pea nodulated early nodules probably senesced early. Rinno *et al.* (1973) and Richard and Soper (1979) also concluded that faba bean derived sufficient N from symbiotic fixation for grain filling because a single large application of nitrogenous fertilizer at the onset of flowering had no effect on grain filling. In contrast a similar application to peas significantly increased shoot yield. N_2 fixation in pea is suppressed during grain filling either by substrate limitation (Lawn and Brun 1974; Wilson *et al.* 1978; Pate and Minchin 1980) or by the increase in soluble N arising from protein breakdown in the leaf. The accumulation of soluble N during the grain filling may inhibit nitrogenase activity in a feed-back mechanism (see Silsbury *et al.* 1986).

CHAPTER 6

Relations Between Phenological Development, Acetylene Reduction Activity, and Root Respiration of Faba Bean and Pea

6.1. Introduction

It was observed that nodule activity of pea declined very markedly during grain filling (Chapter 5). The cause of the marked decrease in the rate of N_2 fixation is not known, despite the fact that this phenomenon is widespread, having been reported in pea (*Pisum sativum*), soybean (*Glycine max*), clover (*Trifolium repens*), and cowpea (*Vigna unguiculata*) (Lawn and Brun 1974; Masterson and Murphy 1976; Bethlenfalvay and Phillips 1977; Minchin and Summerfield 1978). There are several hypotheses which may explain the decline (see Chapter 2.2), however, only one hypothesis was examined in this study, namely that nodule and fruit may compete for photosynthate in the whole plant. This hypothesis was examined by disbudding plants of both pea and faba bean during the reproductive stage.

A supplementary objective was to determine whether the phenology of faba bean influenced the pattern of change in ethylene production during incubation in acetylene. It was found in a previous experiment that the rate of ethylene production declined during assay when beans became reproductive (see Chapter 3.3.3.).

6.2. Methods

Plants of faba bean were grown in a naturally lit glasshouse at 20±0.5°C constant temperature and with nutrient solution supplied daily. The open system was used to measure the rate of C_2H_4 production and the respiration of intact root systems over a 40 min assay period. Normal plants were compared with disbudded plants, i.e. with flower buds removed as they developed.

During vegetative growth, 6 plants were measured from 0900 h to 1700 h for 3 days starting day 25, day 27, and day 29 after sowing. During the flowering and pod filling stages, 3 plants for each treatment were measured from 0900 h to 1700 h for 5 days, on days

55 to day 59 during flowering and days 76 to day 80 during pod filling. The dry weight of shoot, root, and nodule was determined at each harvest.

Pea plants were grown in a growth room set at 20°C with a 12h day of 800 $\mu E\ m^{-2}\ s^{-1}$. Plants were disbudded as flower buds emerged. AR assay was conducted in a closed system and dry weights of plant parts were measured on 66 day old plants and compared with intact plants at the pod filling stage. 6 replicates were used for each treatment. The shoot was divided into leaf, stipule, petiole, stem, root, pod, and grain.

6.3. Results

6.3.1. Dry weights of plant parts

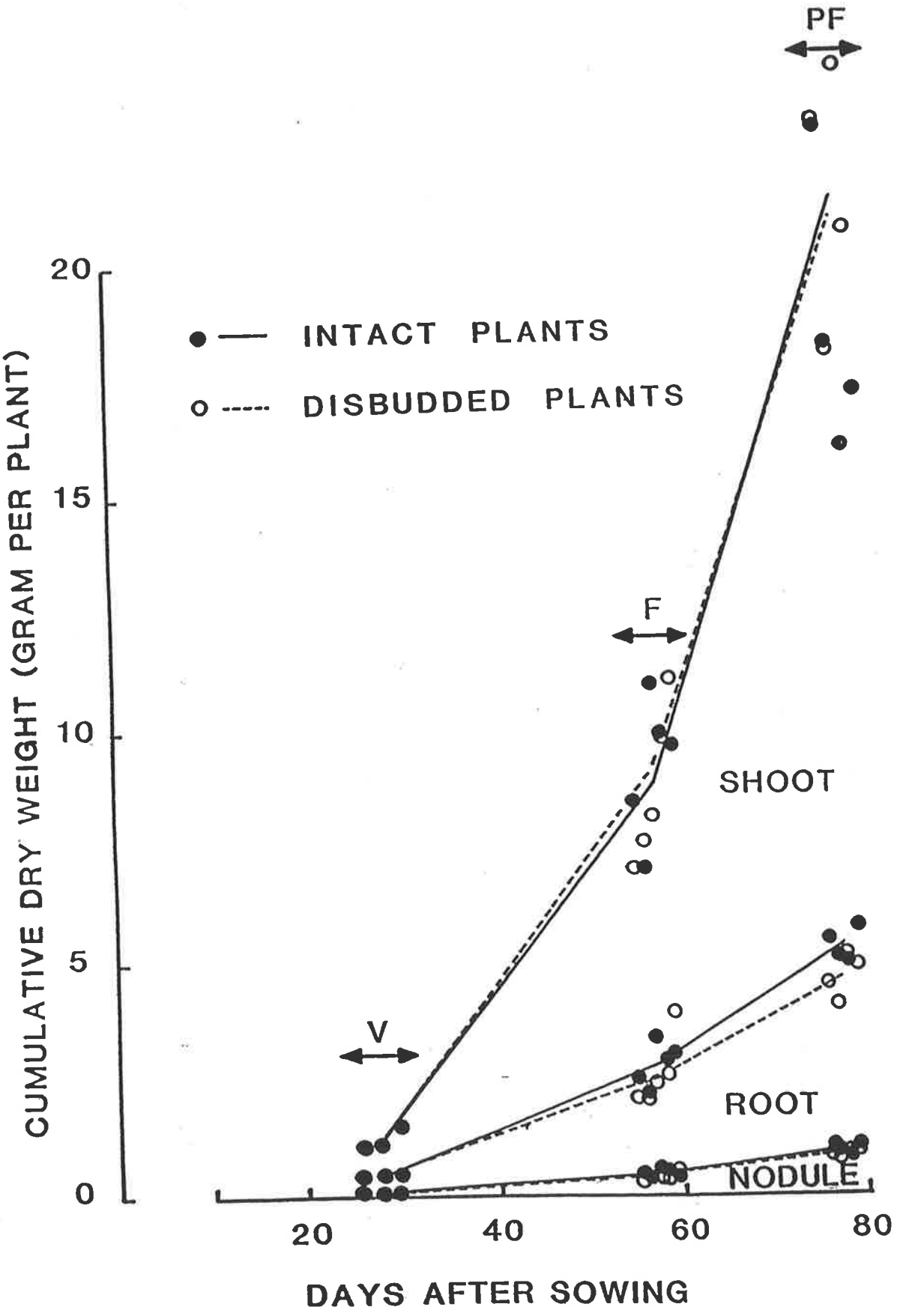
The dry weight of shoot, root, and nodule of both intact plants and disbudded plants of faba bean each increased significantly from the vegetative, to the flowering, and pod filling stages (Fig 23) the increase in shoot weight being greater than that in root and nodule. There were, however, no significant differences in the weights of shoot, root or nodule of intact plants compared with disbudded plants. Disbudding influenced the partitioning of dry matter between plant parts of pea (Table 11).

Table 11. The dry weights of intact plants of 66 day old pea plants compared with those of disbudded plants

Values represent the mean and standard error (in brackets) for 6 measurements

Treatment	Dry weight (g/plant)							
	Root	Shoot	Stem	Stipula	Leaf	Petiole	Grain	Pod
Intact	2.3	27.4	3.4	1.8	3.1	2.3	11.4	5.3
	(0.5)	(4.6)	(0.6)	(0.5)	(0.6)	(0.5)	(0.7)	(0.4)
Disbudded	3.9	25.1	8.1	5.2	5.9	5.1	-	-
	(0.8)	(0.9)	(1.2)	(0.6)	(0.7)	(0.6)	-	-
	NS	NS	*	*	*	*		

Fig. 23. Cumulative dry weight of nodules, roots, and shoots of faba bean plants, which were harvested during the vegetative (V), flowering (F), and pod-filling (PF) periods. Whole plants are compared with disbudded plants.


PF
20
INTACT PLANTS
DISBUDDED PLANTS
15
F
10
SHOOT
5
V
ROOT
NODULE
0
20
40
60
80
CUMULATIVE DRY WEIGHT (GRAM PER PLANT)
DAYS AFTER SOWING

The dry weights of the whole shoot of intact pea plants including grain and pod did not differ from those of disbudded plants. Root weights were also similar for the two treatments. However, disbudding influenced the distribution of dry matter within the shoot in that the dry weights of stem, stipule, leaf, and petiole of disbudded plants were significantly higher than those of intact plants.

Table 12. CO_2 efflux and C_2H_4 production rates of nodulated roots of faba bean plants measured at 3 different stages

Intact plants are compared with disbudded plants

Values represent the mean with standard error in brackets for 15-18 measurements taken 4 minutes after the introduction of C_2H_2 into the air stream (the initial rate)

Stage	Treatment	µmol.CO_2 per g root dwt per min	µmol.C_2H_4 per g nodule dwt per min
Vegetative	Intact	3.15 (0.19)	3.83 (0.60)
Flowering	Intact	2.97 (0.24)	2.55 (0.34)
	Disbudded	3.24 (0.22)	2.53 (0.34)
Pod filling	Intact	3.65 (0.19)	2.54 (0.43)
	Disbudded	3.67 (0.26)	2.25 (0.36)
LSD 5%		NS	NS

6.3.2. The effect of disbudding on CO_2 efflux and rate of C_2H_4 production

Neither the rate of CO_2 production nor the rate of C_2H_4 production of beans was significantly affected by disbudding: neither did these rates change significantly with development (Table 12). The AR activity of disbudded pea plants was significantly higher than that of intact plants, 39.7 (SE=11.5) for intact and 94.7 (SE=15.8) µ mol C_2H_4 per g nodule dwt per min for disbudded plants.

Table 13. Test for the linearity of C_2H_4 production during assay (y=a-bt) Ethylene production rates of nodulated roots of faba bean plants measured at 3 growth stages

Intact plants were compared with disbudded plants

Logarithmic transformation was used for statistical analysis and each value is the mean of 15-18 measurements

Stage	Treatment	AR rate (ln y = a-bt)	SE (b)
Vegetative	Intact	1.299	
Flowering	Intact	0.867 - 0.011 t	0.0013
	Disbudded	0.867 - 0.011 t	0.0013
Pod filling	Intact	1.738 - 0.010 t	0.0018
	Disbudded	2.053 - 0.010 t	0.0018
LSD 5%		NS	

6.3.3. Changes in rates of CO_2 efflux and C_2H_4 production by faba bean during AR assay

The CO_2 efflux and rate of production of C_2H_4 rate were constant with time during assay (Fig. 7A) during vegetative growth but during the reproductive period both decreased with time during assay by about 20% for CO_2 and 30% for C_2H_4 (Fig. 7B). Intact and disbudded plants behaved similarly (Table 13). In pea exposure to C_2H_2 always induced a decline of about 30% in rates of both C_2H_4 production and the respiration of nodulated roots. This decline was similar to the decline exihibited by faba bean during the reproductive stage.

6.3.4. Comparison of the rates of acetylene reduction in a closed system with an open system

The integral of the rate of C_2H_4 production over a 4 to 40 min period in the open system was used to estimate the rate of C_2H_4 production which would be obtained in a closed system in which C_2H_4 accumulated and this value compared with an initial rate taken 4 min after the introduction of C_2H_2. The maximum rate at 4 minutes is taken to represent the rate of nitrogenase activity as would be determined by N_2 uptake (see Minchin *et al.* 1983b). The results showed (Table 14) that the C_2H_4 production rate measured by a closed system would not under estimate nitrogenase activity when the plants were vegetative but during the reproductive stage when C_2H_2 reduction decreased with time, a 36 min assay period in a closed jar would underestimate nitrogenase by about 17%.

Table 14. Acetylene reduction rates of a closed system (integral of C_2H_4 production over a 4 to 40 min period) were compared with the open system (initial rate) of faba bean plants which were measured at 3 growth stages

Intact plants are compared with disbudded plants

Stage	Treatment	Acetylene reduction rates (μ moles C_2H_4 per g nodule dwt per min.)		Ratio
		closed system	open system	closed:open
Vegetative	Intact	3.88	3.88	1
Flowering	Intact	2.09	2.55	0.82
	Disbudded	2.07	2.53	0.82
Pod-filling	Intact	2.13	2.54	0.84
	Disbudded	1.89	2.25	0.84

6.4. Discussion

Disbudding did not alter the growth rate either of shoot, root, or nodules of either pea or faba bean but disbudded pea plants had higher leaf and stem weights than intact plants.

This could be due to disbudding causing a build-up of photosynthate along the entire translocation pathway and increasing specific leaf weight through the accumulation of starch. The whole plant may thus store carbohydrate, perhaps leading to new growth from buds at the nodes. Similar results were found by Wittenbach (1982) with soybean.

Disbudding increased AR activity of pea but not of faba bean. These results suggest that there was no significant reduction in the availability of carbon to the nodule in faba bean during the reproductive stage but that there may have been in pea. It is widely accepted that the supply of carbohydrate available to nodules constitutes the main factor limiting symbiotic fixation (Lawn and Brun 1974; Hardy and Havelka 1976; Bethlenfalvay and Phillips 1977). Sucrose, the major translocation product of photosynthesis, must be exported from the leaf and imported by the nodule to fuel the N_2 fixation reaction (Hardy *et al.* 1980). Reduction in this supply will reduce the capacity for N_2 fixation (Hardy and Havelka 1976). Several workers have shown that the rates of photosynthesis of individual leaves of soybeans decline during grain filling (Boote *et al.* 1978; Mondal *et al.* 1978). Loss of RuBP-case during fruiting may be responsible for the decline in photosynthesis (Wittenbach *et al.* 1980). Other factors such as *in vivo* regulation of photosynthetic enzymes, stomatal aperture, or changing chloroplastic structure and thylakoid membrane properties may play important roles in photosynthetic rates in senescing leaves (Friederich and Huffaker 1980; Nooden 1980; Jenkins and Woolhouse 1981).

Lawrie and Wheeler (1974) found that both NA and the accumulation of ^{14}C-labelled photosynthates in the nodules of pea plants in N-free culture reached maxima shortly before flowering and fruit development. During the period from flowering to fruiting, NA and the accumulation of labelled ^{14}C-photosynthates in the nodules declined by 60 %, whereas the photosynthesis of the plant doubled. These results suggest that the nutritional demands of the reproductive process may starve the nodules of the assimilates necessary to support optimal levels of NA. Disbudding of pea plants appear to result in an increase in the accumulation of photosynthate in the nodule and in a delay in plant senescence which in turn promotes nodule activity.

The effect of disbudding faba bean appears to be different from disbudding pea. In the former, senescence did not begin until grain growth was nearly completed and disbudding

may not have altered the carbohydrate supply. A similar response has been found by others with soybean (Hardy *et al.* 1968; Hardy *et al.* 1971; Klucas 1974; Thibodeau and Jaworski 1975; Sloger *et al.* 1975; Duke *et al.* 1979; Nelson and Weaver 1980; Nelson *et al.* 1984; Denison and Sinclair 1985). Decline in both AR rate and the rate of CO_2 efflux (20-30%) were much less than the 70 % reported by Minchin *et al.* 1983b. A suggested cause of the decline is that ammonia production ceases during assay (Minchin *et al.* 1983b). For lupin, an asparagine producer, the key intermediary step of phosphoenol pyruvate carboxylation was apparently reduced by about 50% when ammonia production was curtailed by exposing nodules to C_2H_2 or an atmosphere of helium (Laing *et al.* 1979). Then again, pyruvate kinase activity may be inhibited by higher ATP/ADP ratios resulting from the absence of ammonia assimilation (Peterson and Evans 1978). An unidentified product reported by Sprent (1969), may inhibit C_2H_4 reduction or alternatively low CO_2 concentration in the root atmosphere may decrease available malate and thus slow down nitrogenase activity by energy deprivation (Bethenod *et al.* 1984), since malate is a substrate of bacteroid respiration (de Vries *et al.* 1980). Lastly C_2H_2 may increase the diffusion resistance of the nodules resulting in decreased oxygen flux from the atmosphere to the bacteroids (Minchin *et al.* 1983a; and Witty *et al.* 1984).

Closed systems may be useful for comparative purpose if the percentage decrease in ethylene production is the same under all treatments. The greater curvature of the C_2H_4 cumulative curve at higher temperature (Dart and Day 1971) and, the variation in C_2H_2/N_2 ratios with different levels of water stress (Engin and Sprent 1973) suggest that this may not be valid. Underestimation of nitrogenase activity due to an C_2H_2 induced decline in C_2H_4 production in a closed system, may account for variation in the estimated rates of nitrogen fixation between species, and for variation within a species at different times. The error, however, would be proportional to the magnitute of the decline observed.

CHAPTER 7

Diurnal Variation in Nitrogenase Activity of Faba Bean and Pea

7.1. Introduction

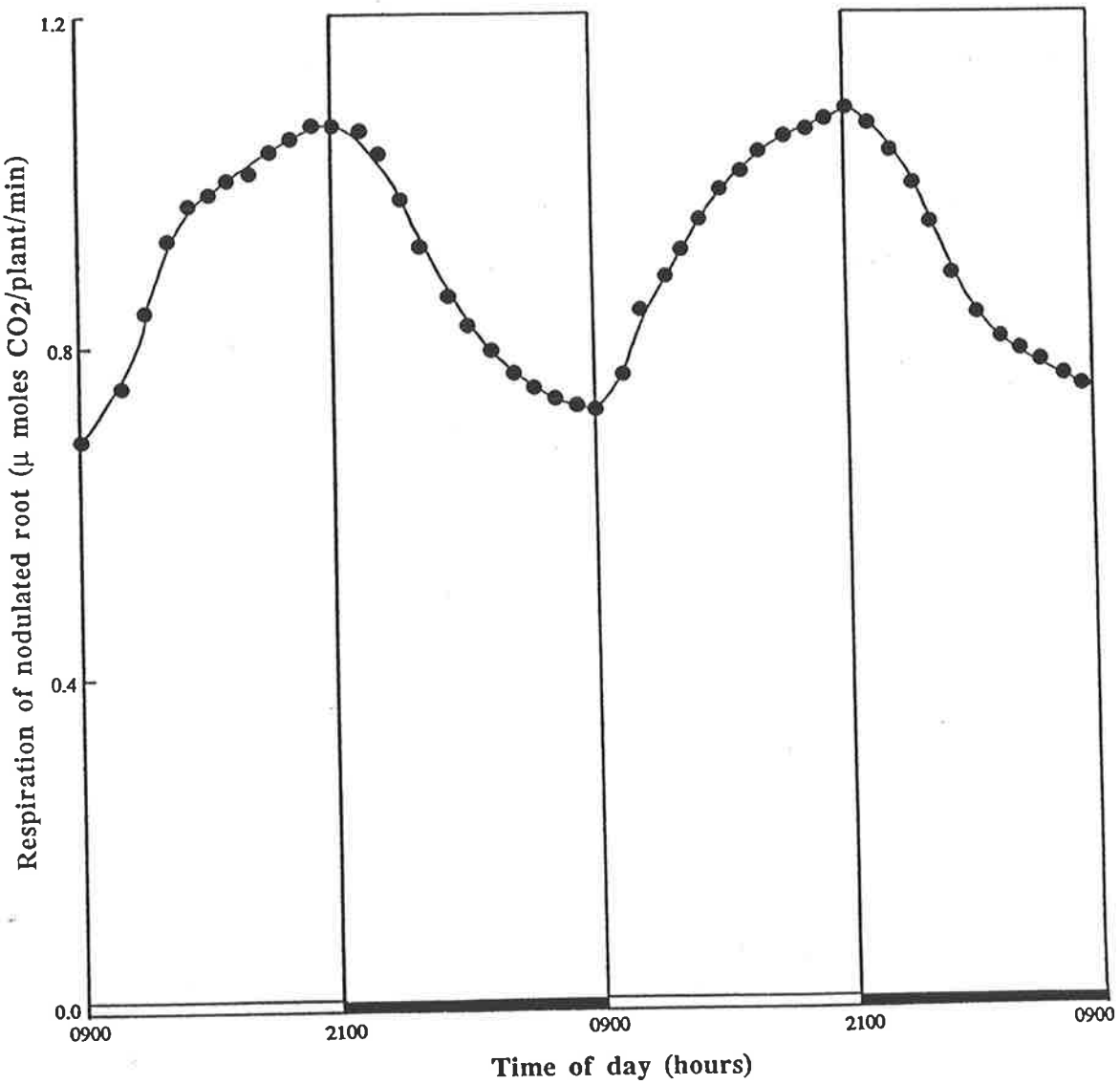
Nitrogenase has been reported by some authors to vary in its rate of activity over a 24 h period in certain legumes, for example in soybean (Bergersen 1970), in pea (Minchin and Pate 1974), and in subclover (Eckart and Raguse 1980). However, Hardy *et al.* (1968) and Fishbeck *et al.* (1973) found little variation in soybean and Materson and Murphy (1976) found none in white clover. Both pea and faba bean appear not to have been studied in this respect and the experiments reported in this chapter were designed to remedy this. The respiration of nodulated roots was first measured as a guide to the level of nodule activity as it is relatively easy to monitor dark respiration continuously. Several observers have found that there is a close linear relationship between CO_2 and C_2H_4 efflux rates (Minchin and Pate 1974; Haystead *et al.* 1979; Silsbury 1979; Minchin *et al.* 1983a).

7.2. Diurnal Variation in the Respiration of Nodulated Roots over a 12 h/12 h Cycle

Plants were grown in a controlled environment chamber set at constant temperature of 20°C with a 12 h day of 600-800 $\mu E\ m^{-2}\ s^{-1}$ for 6 to 9 weeks (see Chapter 3.1.). Root systems were extracted by gentle washing with water at 20°C and suspended in the mist chamber (*see* Fig. 2). Shoots were exposed to a 12 h photoperiod of 600 $\mu E\ m^{-2}\ s^{-1}$. Respiration of the nodulated roots was monitored over 48 h at a gas flow rate of 2 to 3 l per min.

Respiration of nodulated roots of both species varied markedly over a diurnal 12h/12h cycle (Fig. 24). CO_2 efflux began to increase soon after the beginning of the photoperiod, reached a plateau after about 6 h of light, remained stable or increased slightly over the next 6 h, and finally reached a maximum at the end of the photoperiod. There was a rapid decline during the dark period. From minimum values at the beginning (0900 h) and maximum values at the end (2100 h) of a photoperiod, it was estimated that respiration increased about 1.6 times during the day in both pea (1.64, SE = 0.07) and faba bean (1.58, SE = 0.07).

Fig. 24. Diurnal variation in respiration of nodulated roots of 8 week old faba bean plants. Data from a single replicate run was chosen as representative of the pattern of diurnal variation in the respiration of nodulated roots of faba bean and pea in over 60 measurements. The enclosed areas represent dark periods.


1.2
0.8
0.4
0.0
Respiration of nodulated root (μ moles CO2/plant/min)
0900
2100
0900
2100
0900
Time of day (hours)

The respiration rate of nodulated roots of faba bean was allowed to reach a maximum (1700 h), the chamber opened and the nodules detached rapidly, the chamber resealed and the respiration of the remaining roots measured. Root respiration could be thus separated from root plus nodule respiration. Measurements were repeated 12 times. The nodules accounted for 45% (SE = 1.5) of the respiration. The specific activity of the nodule was 7.6 (SE=0.6) and that of the root 1.1 (SE=0.1) μ moles CO_2 per g root dry weight per min. It was also found that after nodules had been detached at the beginning of the photoperiod (0900 h), at 1700 h, or after prolonged darkness, or when seedlings had not yet produced any nodules (early vegetative stage), the respiration of the root showed no diurnal variation (Fig. 25 A-D).

7.3. Diurnal Variation in Acetylene Reduction over a 12 h/12 h Cycle

Some measurements were made of AR at regular intervals over 24 h to explore whether rates also fluctuated diurnally in parallel to the respiration of nodulated roots. Measurements of AR of faba bean at the vegetative stage were made at 3 h intervals during a day (0900, 1200, 1500, 1800 and 2100 h) and at 0900 h the next morning (*see* Chapter 3.2.2.). Respiration was monitored continuously for 48 h.

Respiration of nodulated roots and C_2H_4 production rates of both faba bean and pea were closely linked: both varied markedly over a diurnal 12 h/12 h cycle. In contrast to a change of about 1.6 fold in the respiration of nodulated roots, AR showed a diurnal variation of 2.0 fold in faba bean and 2.75 fold in pea (Fig. 26).

7.4. Apparent N_2 fixation, Respiration, and Hydrogen Evolution (HE)

Since AR assay fails to provide information on how electron flow *in vivo* is partitioned between reduction of N_2 and reduction of protons, measurement of diurnal variation in HE, in air and in Ar/O_2 in an open system was used to estimate rates of N_2 fixation and proton reduction. Since it had been found that the symbiosis of *V. faba* cv. 'Fiord' and *R. leguminosarum* TA101 lacked an uptake hydrogenase (Hup^-) (Chapter 3.2.3.), it is justifiable to estimate N_2 fixation from the difference between HE in the presence and absence of N_2. Plants were exposed to 'low' (300 μE m^{-2} s^{-1}) and 'high' (1300 μE m^{-2} s^{-1}) PPFD to explore

Fig. 25. The respiration of nodulated root of faba bean when nodules:

(A) have not been established (early vegetative period)

(B) were detached at 1700 h

(C) were detached at the beginning of the photoperiod (0900 h)

(D) were detached after 36 h in prolonged darkness.

Arrows indicate times when nodules were detached and the enclosed areas represent darkperiods.

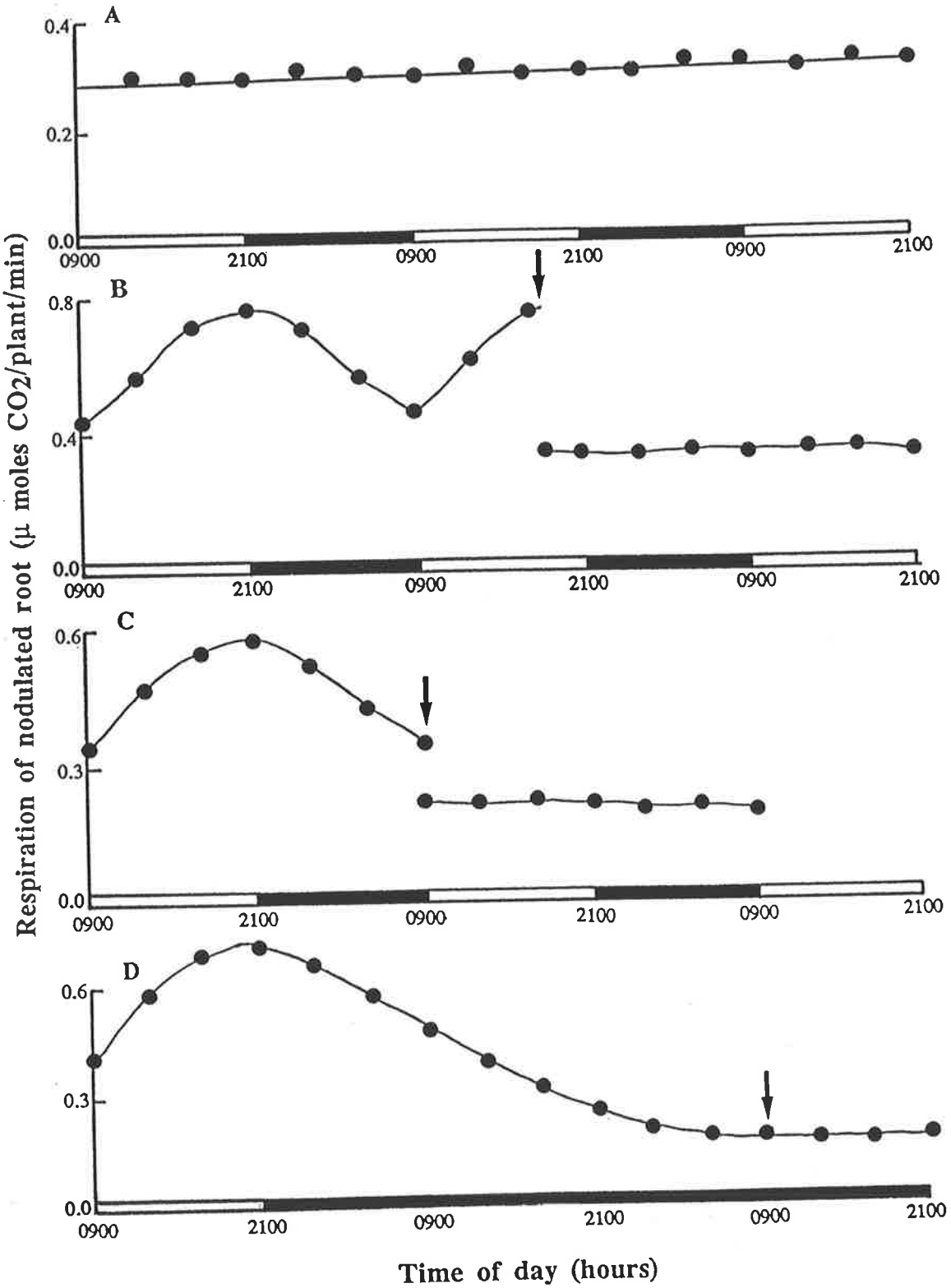
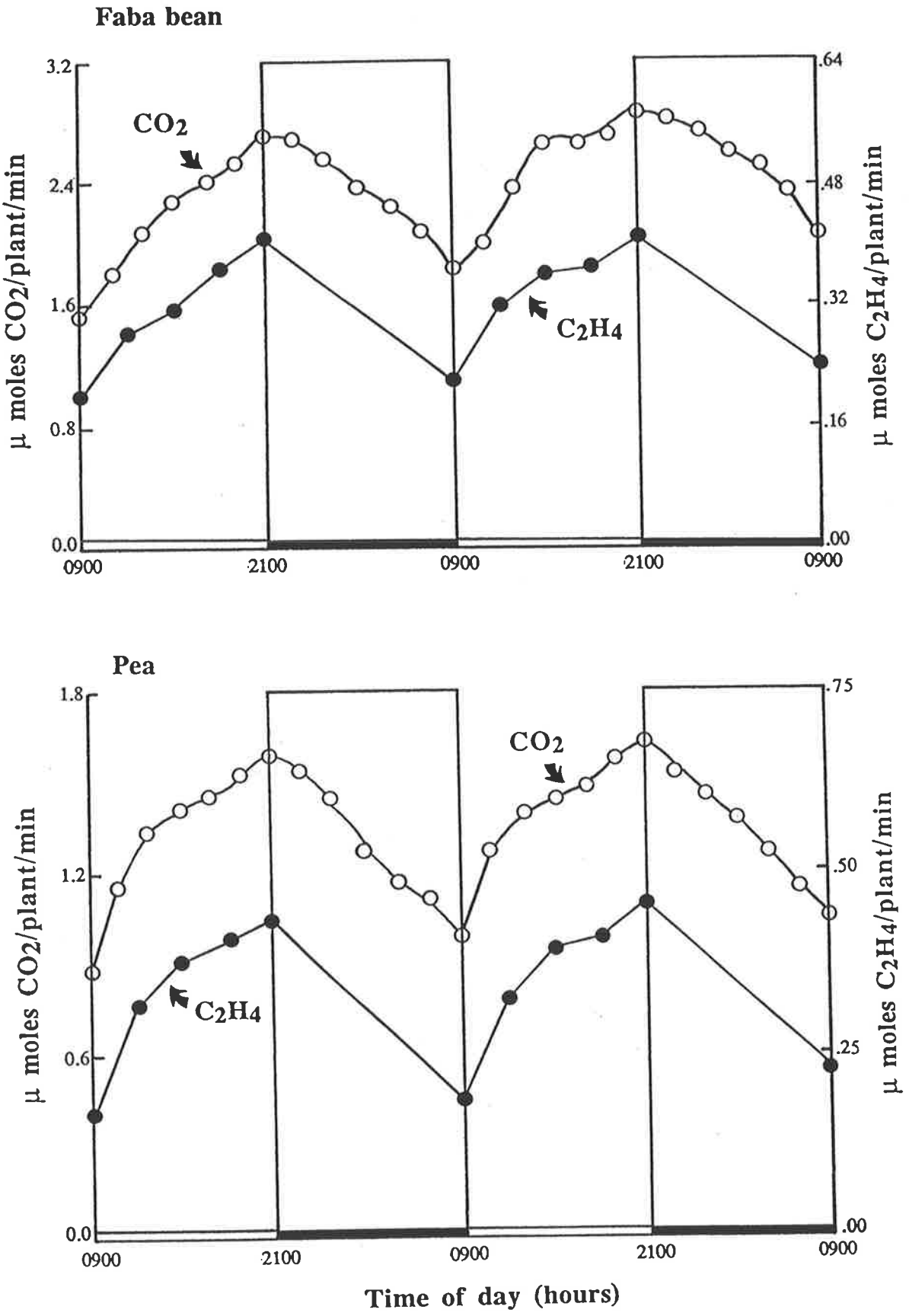

A
B
C
D
0.4
0.2
0.0
0.8
0.6
0.3
0900
2100
Respiration of nodulated root (μ moles CO2/plant/min)
Time of day (hours)

Fig. 26. Diurnal variation in the respiration of nodulated roots and AR rates of faba bean and pea plants during the vegetative stage. Nodulated roots were placed in the mist chamber and allowed to acclimatize for 36 h before exposure to a continuous gas stream containing 10% C_2H_2 in air. The enclosed areas represent dark periods.


Faba bean
3.2
2.4
1.6
0.8
0.0
.64
.48
.32
.16
.00
CO2
C2H4
μ moles CO2/plant/min
μ moles C2H4/plant/min
0900
2100
0900
2100
0900
Pea
1.8
1.2
0.6
0.0
.75
.50
.25
.00
CO2
C2H4
μ moles CO2/plant/min
μ moles C2H4/plant/min
0900
2100
0900
2100
0900
Time of day (hours)

whether the diurnal variation in respiration and in NA was influenced by the carbohydrate supply.

Respiration and the evolution of H_2 of nodulated roots were followed in air and in Ar/O_2 in the mist chamber for 48 h, measurements being made at 4 h intervals during the day (0900, 1300, 1700 and 2100 h) and at 0900 h the next morning (*see* Chapter 3.2.2.). HE in air was measured for 5 min and samples were taken every minute. HE in Ar/O_2 was measured for only 15 min since it was found that the H_2 and CO_2 effluxes were constant after the 12 min mixing period representing the time taken for the gas mixture $Ar:O_2:CO_2$ to rise to its equilibrium concentration of CO_2 (Fig. 27).

Table 15. Percentage of H_2 evolved in 'high' and 'low' PPFD
Values are the means of 5 measurements with standard errors in brackets

Time (h)	Percentage of total electron flux to H_2	
	'Low' PPFD	'High' PPFD
Day 1		
0900	26	31
1300	32	36
1700	30	34
2100	35	35
Day 2		
0900	24	31
1300	27	37
1700	31	35
2100	30	36
0900	23	30
Mean (SE)	28.7 (1.3)	33.9 (0.9)

Fig. 27. Rates of H_2 production and the respiration of intact nodulated roots of faba bean plants over a 40 min period. Roots were exposed at time zero to a continuous gas stream containing 79.96% argon : 20% O_2 : 0.04% CO_2.

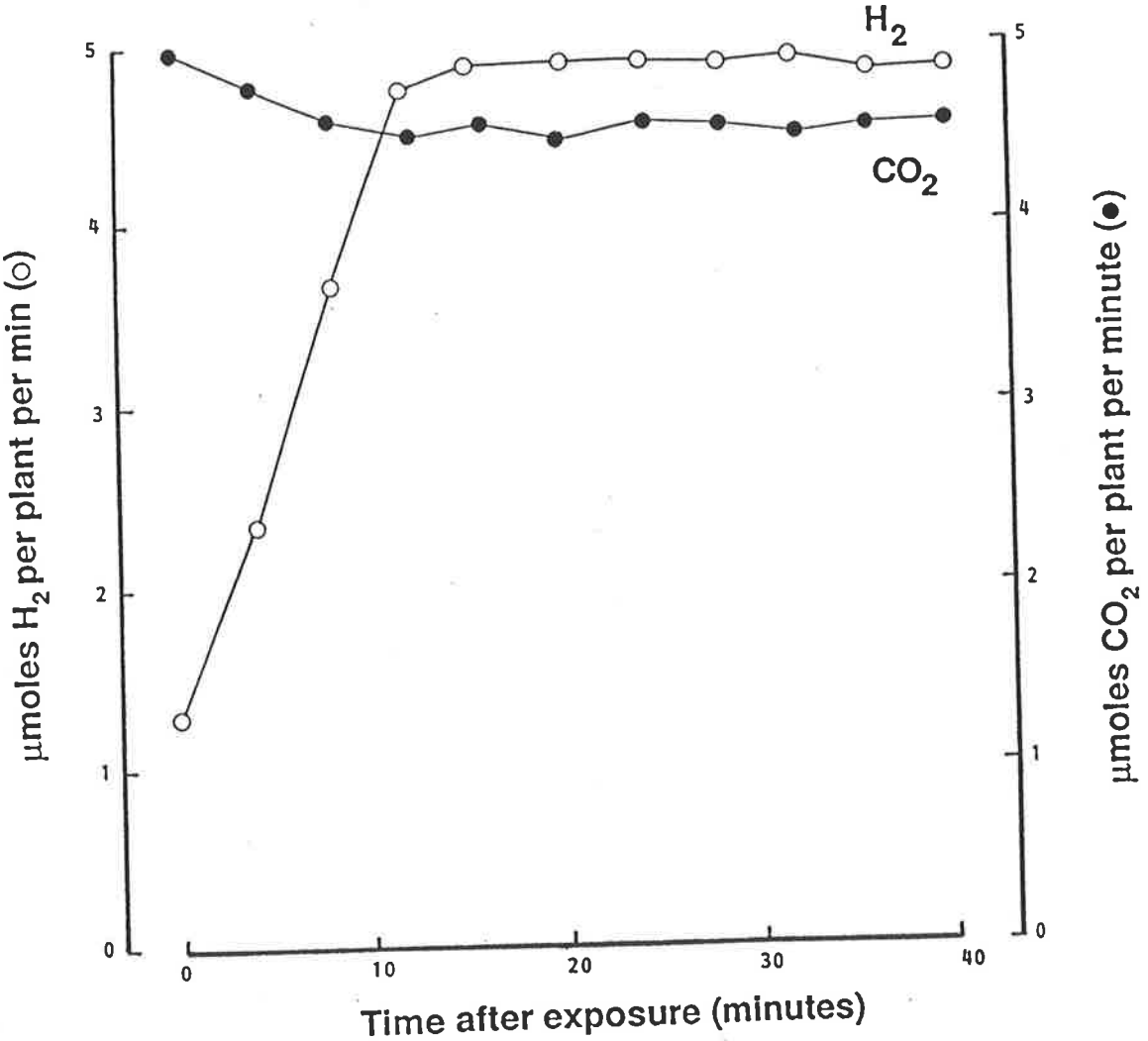
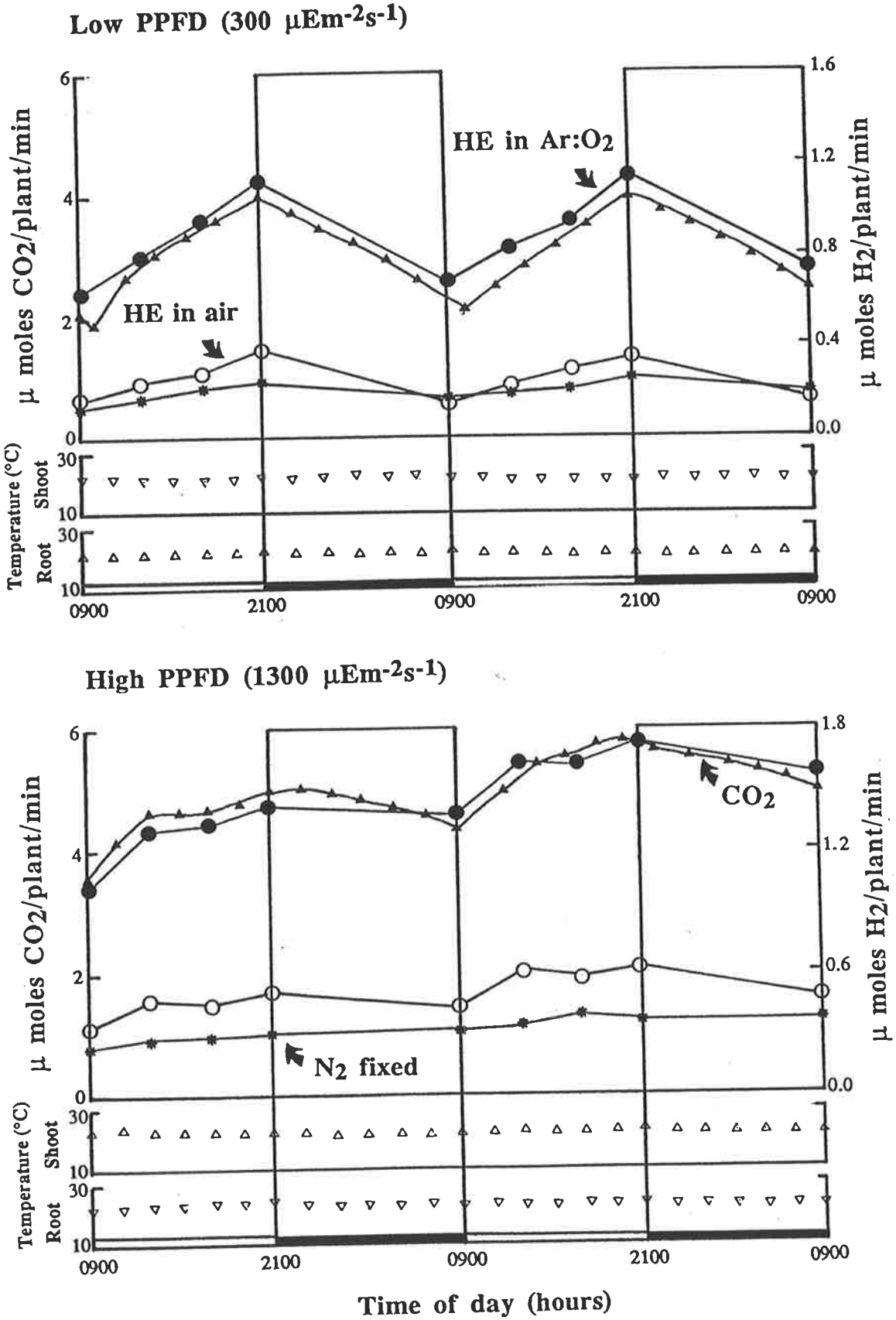

H2
CO2
µmoles H2 per plant per min (○)
µmoles CO2 per plant per minute (●)
Time after exposure (minutes)
0
1
2
3
4
5
10
20
30
40

Fig. 28. Diurnal variation in: (i) the rate of HE into a flowing steam of air (○) or into a mixture of 79.96% argon : 20% O_2 : 0.04% CO_2 (v/v) (●), (ii) the estimation N_2 fixation (✱), and (iii) the CO_2 efflux by attached nodulated roots of faba bean plants (▲). Shoots were exposed to a 'low' (300 μE m^{-2} s^{-1}) and 'high' (1300 μE m^{-2} s^{-1}) PPFD. Shoot and root temperatures and [CO_2] were also monitored. The enclosed areas represent dark periods.


Low PPFD (300 μEm-2s-1)
μ moles CO2/plant/min
μ moles H2/plant/min
HE in Ar:O2
HE in air
Temperature (°C)
Shoot
Root
0900
2100
0900
2100
0900
High PPFD (1300 μEm-2s-1)
CO2
N2 fixed
Time of day (hours)

The evolution of hydrogen in both the presence and absence of N_2 and the respiration of the nodulated root varied diurnally, the variation being more marked when plants were exposed to 'low' PPFD (Fig. 28). HE in air and in argon/O_2 during a day of 'low' PPFD increased by about 2.3 and 1.9 times respectively. In 'high' PPFD the figures were 1.5 times. Both rates declined during the dark period. The apparent N_2 fixation at 'high' PPFD did not differ between day and night, but there was diurnal variation in the rate of estimated N_2 fixation at a 'low' PPFD. In 'high' PPFD, 33.9% of the total electron flux was to H_2 and at 'low', 28.7% (Table 15).

7.5. Discussion

The respiration of nodulated root of faba bean and pea appears to be very sensitive to the carbohydrate supply since an increase in respiration can be detected very soon after the beginning of each photoperiod and a decline occurs during the dark period. In contrast to this result, Haystead *et al.* 1979 found that white clover maintained at 15° C in a 12h-80-W m^{-2} photoperiod showed no significant light-dependent diurnal variation. It is likely that pea and faba bean have little carbohydrate reserve in the nodules compared to white clover.

Since no fluctuation was found after the nodules had been detached, diurnal variation appears to be due mainly to variation in nodule activity. In this study, it was estimated that respiration increased about 1.6 times during the day under constant 20°C temperature both day and night in both faba bean and pea. Similarly, Rainbird *et al.* (1983) found that the CO_2 efflux by attached nodules of cowpea showed a marked variation, with rates of respiration during the 30°C photoperiod being double those during most of the 20°C night period.

Nodule respiration was 45% of total respiration of the nodulated root. Thus about half the respiration of nodulated roots is attributable to growth and maintenance processes of the root and the rest to three nodular processes: (i) assimilation of ammonia, (ii) nitrogenase activity, and (iii) the growth and maintenance of nodules. The very intense activity in the nodules is reflected in the differences in specific activities between nodule and root since the specific activity of nodule respiration was 7 times higher than specific root respiration. Both the increase in the respiration of nodulated roots during the day and the ratio between nodule and root respiration varied from plant to plant in this study according to the degree of

nodulation. However, the proportion between nodule and root respiration appears also to vary with the species. For example, Minchin *et al.* 1983a found approximately 80% of the total root respiration of white clover to be associated with N_2 fixation and ammonia assimilation and less than 50% of the respiration of nodulated root of pea associated with N_2 incorporation. Ryle *et al.* (1983) also found that about 50% of root respiration of *Trifolium sp.* was nodule respiration.

AR activity and the respiration of nodulated root were closely linked, however, the fluctuation of the respiration of nodulated root was less marked compared to AR activities. This difference in response relative to respiration was attributed to minimal diurnal variation in root respiration whereas nodule respiration, and presumably AR activity was closely linked to change in the carbohydrate supply.

Marked diurnal variation in the rate of C_2H_4 production makes the use of AR assay of limited value for comparative purposes. Furthermore, AR measurement can overestimate actual N_2 fixation if the percentage of the electron flux to proton reduction is high. Schubert and Evans (1976) and Evans *et al.* (1980) reported that as much as 60% of the electron flow through nitrogenase *in vivo* may lead to proton reduction. The evolution of hydrogen has to be taken into account when AR assay is used to estimate of N_2 fixation. The difference between HE in air and HE in argon/O_2 appears to be a better way of estimating N_2 fixation than is AR - HE in air as it avoids effects of the C_2H_2 induced decline in C_2H_4 production during AR assay. Although both HE in air and HE in argon/O_2 were closely linked with the respiration of the nodulated root, apparent N_2 fixation showed slight diurnal variation at 'low' light and almost none at 'high'. Variation at 'low' light may be due to substrate limitation since the supply of carbohydrate available to nodules constitutes the main factor limiting N_2 fixation (Hardy and Havelka 1976; Bethlenfalvay and Phillips 1977). The fluctuation in the respiration of nodulated root in 'high' PPFD may also be due to the loss of H_2 since the apparent N_2 fixation is relatively constant during day and night. However, the symbiosis appears to be inefficient since at 'low' PPFD, 28.7% of the total electron flux through nitrogenase was used for proton reduction. The relative efficiency (RE) at the beginning of the photoperiod was higher than that at the end of the photoperiod under each light flux density and RE was lower in a 'high' PPFD, which suggests that more H_2 is being

produced when the substrate available is high. The plants were growing in 'high' PPFD during a 48 h period of measurements since the all the activities: respiration of nodulated root, HE, and N_2 fixation were consistently higher in the second photoperiod than in the first.

These findings contrast with the observations of Masterson and Murphy (1976), Haystead and Marriot (1978), and Haystead *et al.* (1979) for white clover, Rainbird *et al.* (1983) for cowpea, Eckart and Raguse (1980) for subterranean clover, and Schweitzer and Harper (1980) for soybean. These investigators did not find light-dependent diurnal changes in nitrogenase activity and they claimed diurnal variation to be a direct response to temperature rather than to change in irradiance. Temperature was fully controlled in the present study but fluctuation in NA and in the respiration of nodulated roots were found. These findings are similar to those of Minchin and Pate (1974) that a constant temperature (18°C) induced more marked fluctuation in AR activity of pea plants than fluctuating temperature 18°C/12°C during day/night. Others have also reported that under constant temperature, diurnal changes in apparent N_2 fixation correspond to fluctuation in the light intensity for soybean (Bergersen 1970) and for pea (Mahon 1977).

A major strength of the present results is that the whole plants were used in an undisturbed system. There is considerable benefit in observing the same plants continuously as was done by others (Thibodeau and Jaworski 1975). The technique eliminates much of the variability arising between plants and from shoot excision when detached roots only are used.

CHAPTER 8

Nodule Function

8.1. Introduction

The aim of this study was to examine the relationship between the supply of substrate (photosynthate) and nodule activity. Several worker have shown that increase in the supply of assimilate from increased photosynthesis enhances nodule activity whereas shading or defoliation reduces it, e.g. in soybean (Lawn and Brun 1974; Peet and Kramer 1980; Bayne *et al.* 1984), in white clover (Haystead *et al.* 1979; Gordon *et al.* 1987), and in cowpea (Garg and Swaraj 1984). The reduction may be rapid or relatively slow. Haystead *et al.* 1979 found that in white clover there was no change in the rate of AR during the first 24 h after the light flux density in the photoperiod was decreased. The relation between the photosynthetic source/sink ratio to nodule activity appears to differ between species. The investigation examined the effects on nodule activity of: (i) prolonged darkness and prolonged light; (ii) excision of the shoot and defoliation; and (iii) reduction of nodule number.

8.2. The Effects of Prolonged Darkness and Prolonged Light on Nodule Activity

8.2.1. Methods

Faba bean and pea were grown in a growth room at constant 20°C with a 12 h photoperiod of 600 - 800 μE m^{-2} s^{-1} for 6 to 9 weeks. Plants were transfered to the mist chamber with shoots exposed to the same irradiance, and acclimatised for 36 h before exposure to either prolonged darkness or prolonged photoperiod for a further 36 h. Root respiration was monitored throughout and measurements were usually repeated 4 times.

The effect of prolonged darkness on AR activity was studied using 9-week old faba bean. Plants were harvested and AR rates (closed system) measured at the beginning and end of the photoperiod (0900 h, 2100 h), after prolonged darkness for 36 h, and after

transfer back to a normal daily photoperiod of 12 h for 50 h. Dry weight of root, shoot, and nodule was determined at each harvest.

8.2.2. Results

The respiration of nodulated roots of both pea and faba bean increased markedly when the light period was extended for 36 h. Whereas in a 'normal' 12 h day/12 h night the respiration rate of the nodulated faba bean roots increased 1.6 times from the beginning to the end of the photoperiod, further exposure to light increased the ratio to 2.1 and 2.3 at 24 and 36 h, respectively. In pea the figures were 1.7, 2.2, and 2.4 times after 12, 24, and 36 h of continuous light. Respiration declined to similar rates during a subsequent dark period of 12 h following normal (12 h) or prolonged (24, 36 h) exposure.

Respiration was also measured after 12, 24, and 36 h of extended darkness. After 12 h of a 'normal' dark period the respiration rate of nodulated roots of faba bean had fallen to 0.6 times that at the end of photoperiod, after an additional 12 h to 0.4, and after a further 12 h to 0.3. In pea the figures were 0.7, 0.4, and 0.3 times for 12, 24, and 36 h of darkness.

The effects of 36 h of prolonged darkness on AR activity and on the dry matter of roots, shoots, and nodules are shown in Table 16. AR declined to 0.04 of the rate prior to exposure. When plants were transferred back into 12 h daily photoperiods for 50 h, AR recovered rapidly and reached a peak rate almost double that of plants before exposure to prolonged darkness.

The dry weight of root, shoot, and nodule did not change significanlly between the beginning of a photoperiod and the end, and weight was not measurably reduced by prolonged darkness for 36 h. However, there was a significant increase in the dry weights of plant parts after transfer from prolonged darkness to a normal photoperiod of 12 h.

Table 16. Acetylene reduction rates and the dry weight of plant parts of faba beans were measured at the beginning of photoperiod (0900 h), at the end of photoperiod (2100 h), after prolonged darkness for 36 h, and after the plants were transferred back into daily photoperiods of 12 h for 50 h

Four replicates were used, and AR assays were conducted in a closed system

Values are the means and standard errors, in brackets, from 4 measurements

Time	µmol C_2H_4 per g nodule dwt per h	Dry weight (g)		
		Root	Shoot	Nodules
0900 h	58.1 (2.3)	8.5 (0.5)	20.5 (1.3)	1.3 (0.1)
2100 h	99.6 (1.9)	7.6 (0.5)	20.3 (0.8)	1.3 (0.1)
<u>After prolonged darkness for 36 h</u>				
0900 h	3.7 (0.1)	8.6 (0.4)	19.7 (0.3)	1.3 (0.1)
<u>Returned to daily photoperiod for 50 h</u>				
2100 h	146.4 (2.9)	9.2 (0.5)	29.0 (2.7)	1.4 (0.1)

8.3. The Effect of Shoot Excision and of Defoliation on Nodule Activity of Faba Bean

8.3.1. Methods

The effects of complete removal of the shoot and of defoliation (leaf removal) were studied using 6-9 week old faba bean plants acclimatised to the mist chamber as described previously. Respiration was monitored continuously and AR activity measured 1, 4, 16, and 28 h after treatments were imposed. Either the entire shoot was removed by cutting just below the first leaf or individual leaves (usually 10-13 per plant) were removed leaving the stem system in contact with the root system. Treatments were imposed at 1700 h when nitrogen fixation and root respiration were expected to be close to the daily maximum value.

8.3.2. Results

Both excision of the shoot and removal of the leaves influenced the respiration of the nodulated root markedly (Table 17). Just 1 h after the shoot was cut the respiration of the nodulated root had begun to decrease so that after 28 h the rate was only about one quarter of the initial value. Response to defoliation was similar except that the decrease over the first hour may have been more marked. Shoot excision also decreased the AR activity markedly. After 1 h AR activity had fallen to 0.66 of the initial rate, and was almost zero after 28 h.

Table 17. The effect of shoot excision and defoliation on the respiration and AR activity of nodulated root of 6-9 week old faba bean plants

Respiration was monitored continuously and AR activity measured 1,4, 16, and 28 h after treatment commenced

Treatments	Time after treatment (h)				
	0	1	4	16	28
Respiration					
Shoot excision	1.00	0.90	0.63	0.31	0.27
Defoliated	1.00	0.83	0.61	0.29	0.26
AR assay					
Shoot excision	1.00	0.66	0.26	0.11	0.04

8.4. The Effect of Reducing the Number of Nodules on Nodule Activity

8.4.1. Methods

Faba bean plants were prepared for culture in a hydroponic system by careful removal of oil dry after 5 weeks of growth in a growth room at a PPFD of 800 $\mu E\ m^{-2}\ s^{-1}$ set at constant temperature of 20°C. The hydroponic system consisted of two polyethylene tanks each of 20 l capacity coupled by a polyethylene tube at the base (see Fig. 29). Into each tank 18 plants were suspended using rubber stoppers inserted in holes drilled in a black plexiglass top. The root systems were thus suspended in nutrient solution and the shoots exposed to

light. Nutrient solution in a 55 l reservoir was pumped to and from the tanks on 5 min cycles which were controlled by liquid level switches, so that the root systems were regularly flooded with nutrient solution and kept well oxygenated. The nutrient solution was changed every other day.

After allowing 48 h for acclimatisation, nine replicate plants were removed for AR assay and for determination of nodule number, nodule weight, volume of the active and senescent regions of the nodules, and plant dry weight. Half of the nodules on the remaining 18 plants were removed as rapidly as possible leaving approximately the same volume of root sustaining half the original nodule population. Nine of these de-nodulated plants were then assayed for AR and harvested. Nine plants were left in the nutrient solution for 5 days after which they were also harvested. Control plants with a full complement of nodules were also left for 5 days for harvest and AR assay.

The volume of the active nodule and senescent region of the nodules was estimated from samples of 20 fresh nodules from each plant sectioned longitudinally. The length (l) and width (2 r) of the active (pink) and senescent (green) N_2 fixing regions were measured with a dissecting microscope: volume was calculated as $\pi r^2 l$. The volumes of the active and senescent regions of the nodules were measured at day 0 and day 5 after nodule detachment and compared with those of control plants.

8.4.2. Results

Plant dry weight

Control and treated plants did not differ significantly in root and shoot weight at day 0 or at day 5 ($P>0.05$) and there was no increase of root weight in 5 days. Dry weight of shoot, however, increased significantly with time (5 days), control plants doing a little better than treated (Table 18). There was also a significant interaction between time of harvest and treatment with respect to dry weight.

Fig. 29. Experimental device for hydroponic culture of faba bean. (a) Lamp. (b) Black plexiglass lid. (c) Polyethylene container (20 l). (d) Rubber bung. (e) P.V.C. tubing. (f) Liquid level switches. (g) Reservoir (55 l). (h) Pump. (i) Nutrient solution.

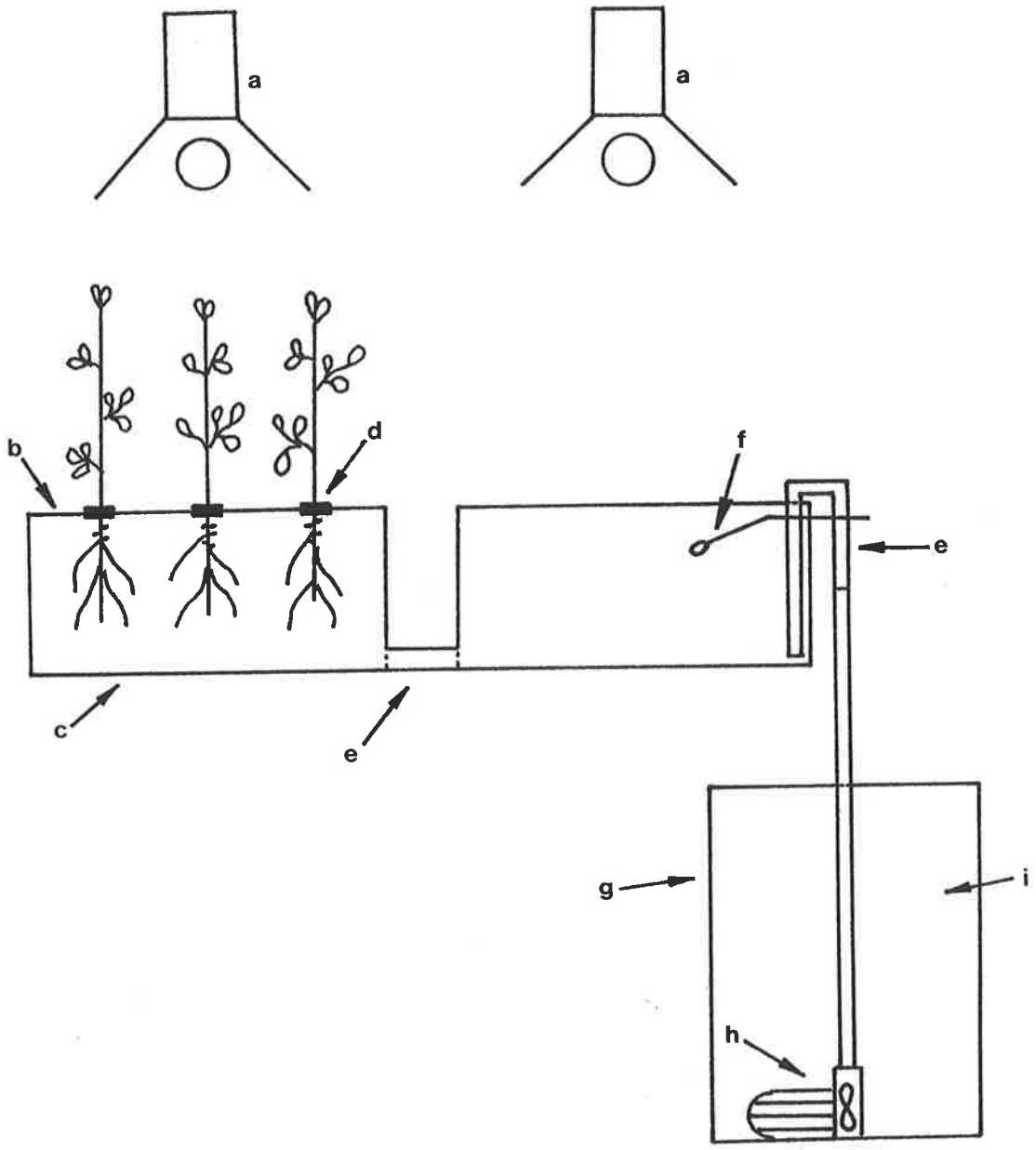

a
a
b
d
f
e
c
e
g
i
h

Table 18. Effect of nodule detachment on dry weight of shoots of faba bean plants

Plants were measured before and 5 days after half of the nodules were detached

All values, with standard errors in brackets, are means of 9 measurements

Time (day)	Dry weight of shoot (gram/plant) Control	Treatment
0	2.60 (0.13)	2.80 (0.15)
5	3.66 (0.24)	3.33 (0.22)
LSD 5%	0.34*	

Table 19. Effect of nodule detachment on nodule dry weight and nodule number

Plants were measured before and 5 days after half of the nodules were detached

Source of variation	Dry weight of nodule (gram/plant)	Nodule number per plant
Time		
Day 0	0.20	226
Day 5	0.25	268
LSD 5%	0.03**	41*
Control	0.28	321
Treatment	0.17	172
LSD 5%	0.03***	41***

Nodulation

Time of harvest and treatment had significant effects on nodule weight and nodule number but the interaction between harvest and treatment was not significant (Table 19). Table 19 shows that nodule weight and nodule number increased significantly from day 0 to day 5 and that, as expected, control plants had more nodules than treated. However, the important measurement is the response of treated and control plants to time. Neither control nor treated plants showed an increase in nodule number with time but whereas nodule size also remained constant in the control plants, the nodules left on the treated plants increased in size by 24% between day 0 and day 5 (Table 20).

Table 20. Effect of nodule detachment on nodule size of faba bean plants. Plants were measured before and 5 days after half of the nodules were detached

All values, with standard errors in brackets are means of 9 measurements

Time (day)	Nodule size (mg/nodule)	
	Control	Treatment
0	0.91 (0.05)	0.85 (0.02)
5	0.87 (0.03)	1.05 (0.03)
LSD 5%	0.09***	

The volume of the active N_2 fixing region of the nodules of the treated plants increased significantly but that of the nodules of the controls remained constant (Table 21). The volume of the senescent N_2 fixing region increased in both control and treated plants, but the increase was much greater in the control than in the treated plants so that after 5 days the senescent region of the control plants was almost twice the size of that of the treated plants. The total volume of the active N_2 fixing region when expressed per plant, however, was the same in the control plants as in the treated.

Table 21. Effect of nodule detachment on the volume of active and senescent N_2 fixing regions of faba bean plants

Plants were measured before, and 5 days after half of the nodules were detached
All values with standard errors (in brackets) of means of 9 measurements

Treatment	Time (day)	A	B	C	D
Control	0	2.37 (0.03)	0.16 (0.01)	0.70 (0.06)	6.39 (0.65)
	5	2.51 (0.04)	0.74 (0.03)	0.86 (0.13)	22.78 (1.72)
Treatment	5	5.32 (0.19)	0.35 (0.03)	0.98 (0.07)	6.27 (1.19)
LSD 5%		0.77***	0.12***	0.20*	3.75***

A : Volume of active N_2 fixing region per nodule (mm^3).

B : Volume of senescent N_2 fixing region per nodule (mm^3).

C : Volume of active N_2 fixing region per plant (cm^3).

D : Senescent nodules as percentage of total volume of nodules.

Acetylene reduction activity

Nitrogenase activity was calculated on a plant basis, on a nodule dry weight basis, and per mm^3 of the active N_2 fixing region. There was a highly significant interaction between time of harvest and treatment with respect to AR activity ($P<0.01$). Table 22 shows that, as expected, removal of half of the nodules at day 0 halved the AR activity per plant. Specific activity, however, remained the same. The treated plants (nodules removed) showed the same total activity as the controls (full nodule complement) after 5 days.

Table 22. Effect of nodule detachment on the specific and absolute rates of AR activity of faba bean plants

Plants were measured before and 5 days after half of the nodules were detached

All values, with standard errors in brackets are means of 9 measurements

Treatment	Time (day)	A	B	C
Control	0	24.16 (1.96)	92.81 (5.83)	2.14 (0.19)
	5	24.13 (1.83)	79.59 (2.78)	1.68 (0.06)
Treatment	0	12.84 (0.91)	97.22 (3.56)	-
	5	24.71 (0.61)	126.56 (2.25)	1.57 (0.11)
LSD 5%		4.42***	11.52***	0.36**

A : μ moles ethylene per plant per hour

B : μ moles ethylene per gram nodule dry weight per hour

C : μ moles ethylene per mm^3 volume of active N_2 fixing region per minute.

8.5. Discussion

Nodule activity in faba bean and pea appears to be very sensitive to the current carbohydrate supply when this is varied by change in day length, removal of the shoot or defoliation. The nodule is not well buffered against even short - term changes of 30 min or so and the path length from source to sink appears to be short. This contrasts with the work of Haystead *et al.* (1979) who showed that mature plants of white clover have the ability to reduce C_2H_4 at an undiminished rate in darkness for over 20 h and at reduced irradiance for 24 h. It was suggested that this is a result of the buffering effect of carbohydrate reserve materials which is located in the stolons (Hoshino *et al.* 1963) and a reflection of the sink strength of the nodule in N_2 fixing clover. Furthermore Gordon *et al.* 1987 found that the levels of sucrose and starch in the nodule at the end of the photoperiod were sufficient to maintain N_2 fixation for 8-9 h of the 12 h dark period. In faba bean nodules there appears to

be very little reserve material to sustain activity once the supply from the leaves is removed or diminished.

The effect of prolonged darkness for 36 h on faba bean appears to be reversible since there was a recovery 2 days after a return to normal conditions. Also it was observed that after the prolonged darkness for 36 h, nodules did not turn green. Exposure to carbohydrate stress therefore has no major apparent effect on the 'potential' activity of the nitrogenase enzyme.

The indeterminate nodule of faba bean appears to be able to increase its specific activity almost two-fold in response to treatment which increases the demand made of each nodule for fixed N. This response was not immediate and was consequent on a relatively slow increase in the active N_2 fixing region of the nodule. It thus appears to differ from the 'normal' fluctuation which occurs diurnally and perhaps in response to light flux density (Chapter 7).

N_2 fixation activity appears to be influence more by the availability of substrate rather than by nodule numbers per plant. Expression of the rate of AR on the basis of nodule weight can be misleading due to variation between nodules in the ratio of active/ senecsent N_2 fixation regions. It is suggested that a whole plant may be better for comparative purposes but where specific activity is required, active volume of N_2 fixing region may be a better basis than nodule dry weight.

CHAPTER 9

General Discussion

A variety of aspects of the growth physiology and N_2 fixation of faba bean and pea has been investigated in the studies reported in this thesis. Pot experiments and laboratory studies have been employed rather than the more traditional field experiment. The objective has been to explore the principles of growth of whole plants and the functioning of their nodules rather than to detail empirical responses of nodule and plant to environmental factors during one or more growth seasons in the field. Nevertheless it is considered that the results obtained under controlled conditions are applicable to the field and will lead to improvements in the productivity of these and other grain legume crops.

Considerable attention has been given to the acetylene reduction assay for estimating relative rates of N_2 fixation in faba bean and pea. Some of the common problems in use of the AR assay may be avoided by: (i) using 'oil dry' as the growth medium, (ii) conducting assay at the growth temperature, (iii) using intact plants, and (iv) conducting assays in an open rather than a closed system.

Oil dry is a particularly suitable rooting medium for growing plants for the study of nodulation and N_2 fixation. It holds a large volume of water at high potential and is always well aerated even after flushing with nutrient solution. Its greatest advantage is perhaps that nodulated roots may be easily extracted from it with no apparent disturbance of nitrogenase activity. Similar results were found for nodulated roots of soybean (*Glycine max*) which were removed from perlite (Fishbeck *et al.* 1973) and beans (*Phaseolus vulgaris*) removed from vermiculite (Saito *et al.* 1980). However, the AR activity of nodulated roots removed from Newberg sandy loam were four times those of nodulated plants *in situ* (Fishbeck *et al.* 1973). This was probably due to very slow diffusion rates of both C_2H_2 and C_2H_4 through the soil. The high air-filled porosity of oil dry apparently allows for rapid gas diffusion. In contrast to these results, Minchin *et al.* (1986) claimed that removal of vermiculite from nodulated roots of soybean and white clover plants reduced the AR rate. It was considered that the decline is due to a reduction in oxygen flux to the bacteroids caused by an increase in

the oxygen diffusion resistance of the nodule. Clearly, the nature of the rooting medium and its removal, as is commonly done for AR assay has to be evaluated for the particular legume under test.

Intact plants rather than detached nodules or detached roots of faba bean should be used for AR assay and assays conducted at the growth temperature, as has been found for other species (Mague and Burris 1972; Wych and Rains 1978; Cralle and Heichel 1982; Hansen 1987). It appears that in any species the effects of detaching the root system will depend on the carbohydrate status of the nodules. For example, the lack of effect of detachment on the AR of lupin found by Trinick *et al.* (1976) could be due to the presence of 'reserves' of metabolizable carbohydrate in the nodules. The AR rates of faba bean were found in this study to be very sensitive to detachment. The nodules of faba bean appear to have very little in the way of 'reserve' carbohydrate and to be dependent for their continued functioning on a supply of assimilate from the shoot and presumably from the chloroplast. The rate decreased by about 14% after excising the shoot during the vegetative stage and by 26% during the reproductive stage. It is essential that whole plants be measured, as was also reported by Denison *et al.* (1983) for soybean.

The closed system is satisfactory when the AR rates remains constant during assay but when AR rates decline only the open system is satisfactory. C_2H_4 production rate measured by a closed system does not underestimate nitrogenase when faba bean plants are vegetative but during the reproductive stage or in pea during all stages, a 36 min assay period in a closed jar would underestimate nitrogenase by about 17%. A closed system may not produce a good measurement as other workers reported that the percentage decrease in nitrogenase appears to differ for different treatments. For example, Dart and Day (1971) reported a greater curvature of the C_2H_4 cumulative curve at higher temperature, and Engin and Sprent (1973) reported variation in C_2H_4/N_2 ratio with different levels of water stress. Underestimation of nitrogenase activity due to a C_2H_2 induced decline in C_2H_4 production in a closed system, may account for variation within a species at different times. The error would be proportional to the magnitude of the decline observed. The cause of the decline in AR rate during assay remains to be determined. Witty *et al.* (1984) indicated that the decline in nitrogenase activity in the presence of C_2H_2 was due to an increase in diffusion resistance

of O_2 in the nodule. However, it is difficult to propose a mechanism for the occurrence of the C_2H_2 induced decline and to link it to the permeability to O_2 of membranes within the nodule.

Marked diurnal variation in nodule activity in both pea and faba bean can also make the use of the AR assay of limited value for comparative purposes if the assay is done at one time, is not representative of the day's activity and not coupled with short-term assays carried out over the growing season. Halliday (1975) recorded that the amplitude of diurnal fluctuation in AR in lupin varied during plant ontogeny and that the amplitude tended to decrease late in plant ontogeny. Soil temperature in the field will also be a major factor in generating diurnal variation in nitrogenase activity which could change the the variation in the relative efficiencies of N_2 and proton reduction by nitrogenase. Rainbird *et al.* (1983) claimed that unlike HE, the rate of N_2 fixation estimated from the difference in H_2 production in argon/O_2 and in air was relatively insensitive to a temperature change from 15°C to 38°C. These results also suggest that measurement in the field by AR assay alone could lead to an overestimate of the ratio between C_2H_2 and N_2. Temperature during measurements in this study was constant but fluctuations in apparent N_2 fixation by faba bean in 'low' PPFD still occurred. The activity of temperate legume nodules is known to be affected only slightly by a decrease in temperature from 20 to 12°C (Dart and Day 1971; Waughmann 1977). Minchin and Pate (1974) reported that the N_2 fixation rate was related to a diurnal rhythm in the clearance of reduced nitrogen from the nodules and that amino acids accumulated in nodules at night when the transpiration was low. However, it would seem that amino acids which accumulate in the nodule of faba bean at night do not affect the N_2 fixation rate since plants at 'high' PPFD and with presumably high rates of amino acid accumulation did not show diurnal fluctuation in the apparent N_2 fixation. Fixed nitrogen is actively transported from the site of fixation and assimilation, via transfer cells, to the vascular spaces (Pate 1976) so that N_2 fixation and nitrogen accumulation are spatially separated. Also, electron transfer to nitrogenase is controlled by the electrical component of the proton motive force across the peribacteroid membrane, which is unaffected by external NH_3 (Laane *et al.* 1980). Irradiance, rather than temperature, and consequent changes in the flux of carbon into the nodule appears to be the cause of fluctuation in both nitrogenase

activity and the respiration of nodulated root of faba bean. Insufficient carbon was stored during the day to support nitrogenase activity in the dark. These results are similar to those of Sheikholeslam *et al.* (1980) and Mahon (1977) who showed that AR increased when the amount of carbon entering the nodule increased and when nodule respiration rate increased. The pattern of fluctuation in the respiration of the nodulated root appears to be due to carbon supply and the decline during the dark period was caused by less carbon being translocated from the leaves. It appears that the nodule responds to this variation in supply but not the root. The root is probably well buffered against short-term changes and the growth and maintenance of the root may be less sensitive to variation in carbohydrate supply than the processes in the nodule associated with N_2 fixation. Trinick *et al.* (1976) found no diurnal variation in lupin when the assay temperature was constant and no effect of decapitating the plant, indicating that lupin nodules may contain sufficient carbohydrate reserves to maintain the same level of nitrogenase activity during the course of the assay, in contrast to faba bean in the present study.

The existence of marked diurnal variation in AR and HE rates indicates that the time of assay needs to be standardised when estimating nitrogenase activity. It is concluded that HE in air has to be measured, together with either measurement of AR or HE in argon/O_2 to estimate N_2 fixation. The choice between these two methods will depend on establishing the limitations to their measurement. This estimation is valid if the symbiosis lacks an uptake H_2. If Hup^+ is present, AR provides a more reliable measurement than AR-HE for comparing rates of N_2 fixation. A measurement by AR assay alone can underestimate N_2 fixation, since the percentage of total electron flux which is diverted to proton reduction can be as much as 60% (Schubert and Evans 1976; Evans *et al.* 1980). In this study it was found that about 30% of the electron flux through nitrogenase was directed to proton reduction in both 'low' and 'high' PPFD. HE has been regarded as a wasteful process and the mechanism of this process is poorly understood.

If the repeated exposure of the same plants to C_2H_2 in an open system reduces the effect of C_2H_2/C_2H_4 on NA, as reported by Grobbelaar *et al.* 1971, the measurement of HE in argon/O_2 and in air will be a better measurement for estimating N_2 fixation than AR-HE. There was no effect of C_2H_2/C_2H_4 on NA in this study since nodule activity of both pea and

faba bean increased during measurement. There is a considerable benefit in observing N_2 fixation rates of the same plant through time as conditions change either within the plants or the environment.

NA and the respiration of the nodulated root of both pea and faba bean were found to be closely linked, both varied markedly over a diurnal 12/12 h cycle. Since no fluctuation in the respiration of the nodulated root was found after the nodule had been detached, diurnal variation appears to be due mainly to variation in nodule activity. N_2 fixation in faba bean is very sensitive to the current carbohydrate supply when this is varied by change in light flux density, by alteration of the day length, by shoot removal, and by defoliation. The faba bean nodule is not well buffered against even short-term changes of 30 min or so. These findings contrast with those of Haystead *et al.* (1979) who showed that mature plants of white clover have the ability to reduce C_2H_4 at an undiminished rate in darkness for over 20 h and at reduced irradiance for 24 h. In faba bean there appears to be very little reserve material to sustain activity once the nodules supply from the leaves is removed or diminished. About half of the dark respiration of the nodulated root is associated with respiration of the nodules and thus with N_2 fixation. Variation in dark respiration is thus a good and convenient measure of N_2 fixation.

It appears that the relative efficiency in faba bean is higher under 'low' PPFD than under 'high' PPFD. This result is similar to the work reported by Edie and Phillips (1983) in which RE in pea increased under 20 h of darkness and declined with increasing irradiance following re-illumination.

Expression of the rate of acetylene reduction on the basis of nodule weight can be misleading due to variation between nodules in the ratio of active/senescent N_2 fixing regions of faba bean. It is suggested that a whole plant basis is best for comparative purposes but where specific activity is required, the volume of the active nitrogen fixing region may be a better basis than nodule dry weight.

Different rhizobia vary widely in their ability to form symbioses and to fix N_2 but this variation in effectiveness is poorly understood. The inoculants, *Rhizobium leguminosarum* TA 101 and SU 391 for faba bean and pea appear not to be fully effective, to be sensitive to nitrate, and may have a short operating life. The strains of *R. leguminosarum* available for

faba bean and pea need to be improved to increase both the dry weight and total N of the host, as has been achieved for subterranean clover (Hagedorn 1979). Effectiveness is known to be under the genetic control of both host and *Rhizobium* but environmental factors can also be involved. Effectiveness may also have a straight forward basis: for example, strains that possess an uptake hydrogenase (Hup^+) may be more effective than similar strains which lack this enzyme since Hup^+ may result in a conservation of energy by recovering the H_2 evolved by nitrogenase (Dixon and Wheeler 1986). Strains having this system form ATP during H_2 oxidation and compensate partially for the ATP-dependent evolution of H_2 catalysed by the nitrogenase enzyme complex (Bulen and LeComte 1966). Thus, Hup^+ *Rhizobium* strains generally are more effective symbionts than those with Hup^- phenotypes (Albrecht *et al.* 1979; DeJong *et al.* 1982).

Strain TA 101 appears to be effective for both 'Early Dun' and 'A102' pea, since nodulation was established relatively early at about 20 days after sowing compared to 53 days for faba bean. The establishment of nodulation in faba bean appears to be influenced by temperature, but this is not so in pea. Faba bean grown at a constant 20°C started to nodulate early, about day 20 compared with 53 days at a lower temperature (10°C). Sowing in the field is usually in May when the average soil temperature at night is about 9°C. Fyson and Sprent (1982) also reported that nodulation of faba bean was influenced by temperature.

Inoculated faba bean seedlings grew slowly after emergence and commonly showed N deficiency. Little nitrogenase activity was evident until 53 days after sowing, the relatively large seed being able to sustain growth for some considerable time. A small amount of combined N as a 'starter' was needed to promote growth and nodulation with the inoculant used in this study. This may also apply in the field when the soil NO_3^- is at a low concentration. Strain TA 101 appeared to be more effective than SU 391 especially when faba beans were grown without nitrate. When an effective inoculant is provided, faba bean is known to have the ability to synchronise the exhaustion of seed reserves of N with the ability to fix N_2 (Sprent and Thomas 1984), so that the addition of mineral N as a 'starter' is not necessary. The slow start of nodulation in 'Fiord' faba bean could also be due to the presence of an unknown inhibitor in the cotyledons, since cotyledon removal on day 18 increased AR activity and increased nodule number per plant. This postulated inhibitor may

be abscisic acid (Phillips 1971). Removing half of the cotyledons from faba bean seedlings to remove the inhibitor of nodulation is a potential means of promoting nodulation but removal of both cotyledons would depress growth as they function as a source of substrate.

There have been many attempts to increase the yield of faba bean crop by the application of combined N, but it appears that the effect on yield is only small and non-economic (Day *et al.* 1979; Richard and Soper 1979; McEwen 1970a, b). These results suggest that, when faba bean is nodulated with an effective strain of rhizobia, it is able to satisfy the N demand in the plant from the soil and through symbiotic fixation during the entire growth cycle.

Nodule senescence in pea plants started at the beginning of grain filling, however, which could markedly disrupt the N supply of N for grain production. If the life span of the symbiosis was prolonged, until the end of grain filling, it may have improved plant N content substantially, both in the grain and the rest of the plant.

Another limitation of the pea symbiosis used in this study is that it was sensitive to nitrate. A concentration of 2.5 mM suppressed NA markedly, so the presence of soil N in the field may depress N_2 fixation below the potential, as has been reported by Silsbury *et al.* (1986) for subterranean clover. The depression in nodule activity by applied NO_3^- is related to the level of nodule activity. In faba bean, at the early vegetative stage, NO_3^- did not suppress NA and even promoted nodulation. In pea, however, at the early vegetative stage applied NO_3^- suppressed NA markedly when the activity of the nodule was already relatively high.

The patterns of accumulation of dry matter and nitrogen for faba bean and pea in this study are very similar to those shown previously by Hanway and Weber (1971a, b) for soybean, and Farrington *et al.* (1977) for lupin. Considering the data for all four species, some general principles concerning the growth of a grain legume can be proposed. It is clear that the three phases proposed initially for analysis of the growth of a sward of annual medic (Silsbury *et al.* 1979) also has validity in describing the growth of a community of grain legume plants. An initial period characterised by an accelerating growth rate (stage I) is followed by a second stage (II) during which the rate remains reasonably constant for a considerable period of time. During this stage the growth rate may be independent of short-

term trends in temperature and irradiance as has been shown in soybean and cowpea (Herridge and Pate 1977), sub-clover (Silsbury and Fukai 1977), and lupin (Farrington *et al.* 1977). There is no obvious change in growth rate with change in plant ontogeny during stage II in both faba bean and pea plants. Growth was dominated first by root, stem, and leaf growth, then after flowering, by pod growth and to some extent by stem growth. Finally the only investment in total dry weight arose from grain filling. Applying the source/sink concept, it appears to be immaterial to the crop growth rate which sink is being presented, each sink is filled at approximately the same rate independently of the irradiance and temperature and, within a limited range, the size of the source (leaf area). This applies to the accumulation of both dry matter and nitrogen. The rate of accumulation of nitrogen by faba bean and pea appears to be independent of the nature of the source, N_2 fixation or remobilized N and that of the sink, leaf, stem, or grain.

At maturity the vegetative parts and pod of faba bean and pea had relatively similar N contents, the figures were 0.24 and 0.26 g N/plant, respectively. Furthermore, the amounts of N redistributed from the vegetative parts and pod of faba bean and pea to the grain were also similar, the figures were 0.23 and 0.27 g N/plant, respectively. The difference was only in the N fixed during the grain filling. In faba bean, 0.58 g N in the grain was from N_2 fixation and in pea it was only 0.07 g N. This difference was mainly due to the decline of N_2 fixation in pea during grain filling. These results are consistent with results obtained for other legumes e.g. soybean (Mague and Burris 1972), lupin (Trinick *et al.* 1976; Farrington *et al.* 1977), and pea (Hobbs and Mahon 1982) in that the maximum rates of the N_2 fixation occurred at the beginning of grain filling and declined during most of the grain filling.

Decline in nitrogenase activity during grain filling in pea may be due to reduction in the carbon supply to the nodule. NA of disbudded plants was found to be significantly higher than that of intact plants. Transport of carbon to the nodule during the reproductive stage may decline and this process may result in nodule senescence and a marked decline in symbiotic N_2 fixation. Sucrose, the major translocation product of photosynthesis must be exported from the leaf and imported by the nodule to fuel the N_2 fixation reaction (Hardy *et al.* 1980). Several workers have shown that the rates of photosynthesis of individual leaves of soybeans decline during grain filling (Boote *et al.* 1978; Mondal *et al.* 1978). Loss of

RuBP-case during fruiting may be a primary event responsible for the decline in photosynthesis (Wittenbach *et al.* 1980) and consequent decline in N_2 fixation. Other factors such as *in vivo* regulation of photosynthetic enzymes, stomatal aperture, or changing chloroplastic structure and thylakoid membrane properties may play important roles in determining photosynthetic rates in senescing leaves (Friederich and Huffaker 1980; Nooden 1980; Jenkins and Woolhouse 1981). When the carbohydrate supply of pea plants was decreased by leaving them in prolonged darkness, it was observed that the nodules turned green, which indicates that the nodules were becoming senescent. It may also be possible that during the period from flowering to grain filling of pea the photosynthate of the whole plant doubles as found by Lawrie and Wheeler (1974) but nitrogenase activity and the accumulation of photosynthate in the nodule may both decline due to the competition for assimilate between the development of grain and nodule activity.

Faba bean clearly differed from pea in its response to disbudding and in the way in which N_2 fixation changed during ontogeny. Disbudding did not reduce AR activity in faba bean and the plant retained a capacity for N_2 fixation throughout most of the grain filling period. This suggests that there was no significant reduction in availability of carbon to the nodule. The decrease in N concentration of all the vegetative parts of the plant is consistent with the 'self-destruction' hypothesis of Sinclair and de Wit (1976), namely the need to transfer large amounts of N from the vegetative tissues in order to support normal seed growth. Although this transfer occurrs mainly from the leaf it may not lead to a significant reduction in the photosynthetic activity of faba bean since the nodules were found to be relatively active during the grain filling stage. Sufficient photosynthate thus appears to be available in faba bean to meet the demands of the nodule as well as the demands of the developing grain. Hormones could be a major factor in controlling the competition for photosynthates between the different metabolic sinks of the faba bean plant. It is not clear to what extent variation in supply and demand for C and N by different organs caused these declining leaf and nodule functions or whether the changes are mediated through specific adjustment in the gradients of particular translocated growth regulators (Nooden and Leopold 1978), which in turn regulate photosynthesis or the onset of senescence. A grafting experiment with soybean (Malik1983) has demonstrated that the post-anthesis decline in N_2

fixation is reversible and that changes in the shoot other than in the provision of carbohydrates may regulate nodule senescence. All known plant hormones have been implicated in either promotion or inhibition of leaf senescence (Woolhouse 1982) and some of these have been suggested to play a role in nodule senescence (Sutton 1983).

When plants were grown without mineral N, the entire root system was retrieved and inter-plant competition was comparable with that which would occur in the field. If dry matter, grain yield and nitrogen are apportioned for the plant in the field in the same manner as occurred in the glass house, a crop of faba bean or pea will contain only 6-8% of its nitrogen in the root at maturity. Nearly 80% of the N will be in the grain in faba bean and 60% in pea. In an average year, one hectare of faba bean can be expected to accumulate, by symbiotic N_2 fixation in the absence of any NO_3^- supplied from the soil, 90 kg of N in the grain, 30 kg in the stubble and only 12 kg in the root system. The corresponding figures for pea are 90, 60 and 10 kg. Thus if the only path for symbiotically derived N from air (N_2) to the soil organic nitrogen fraction is via the legume root system, an above average legume crop would not supply sufficient N for a subsequent average wheat crop even if all the organic nitrogen became available. Under normal farm practice the amount of nitrogen transferred from cereal straw to soil is probably very small so almost all of the N in the cereal straw must be classed as a debit in a balance equation. The proportion of N in the legume straw and/or grain likely to be returned to the soil after grazing is probably of the order of 25%.

The conclusion appears inevitable that a grain legume crop such as faba bean or pea can only make a satisfactory contribution to soil organic nitrogen if a substantial portion of the N in stubble or grain is returned to the soil either by the grazing animal or by direct incorporation. Cereal/grain legume rotations appear only to be sustainable in N balance when a large proportion of the above ground parts contribute N to the soil along with the root fraction.

Perhaps the major finding was that the nodules of faba bean are very responsive to the carbohydrate supply from the host plant and/or the demand for N by the host. Indeterminate nodules can be induced to increase their specific activity almost two-fold in response to removal of half the nodules. This response was not immediate and was consequent on a

relatively slow increase in the active N_2 fixing region of the nodule. It thus differs from the normal fluctuation which occurs diurnally and in response to change in light flux density. The questions which then arise are: (i) how many nodules does a plant need?, and (ii) how is a 'normal' supply of carbohydrate distributed by the host among its population of dependent nodules to satisfy its (the host) demand for reduced N?

The major conclusions drawn from this work are as follows:

1. The assay commonly used to estimate the activity of nitrogenase, acetylene reduction, has known limitations which must be re-defined for the particular legume under study.
2. A C_2H_2 induced decline in the rate of C_2H_4 production during AR assay was dependent on the plant species and on the stage of development. It always occurred in pea but in faba bean the AR rate remained constant during a 40 min assay for plants in the vegetative stage and declined when plants were in the reproductive stage.
3. When a C_2H_2 induced decline in C_2H_4 production did occur, AR assay (40 min) in a closed system compared with an open one, underestimated the activity of the nodules by about 17%.
4. The commercial inoculants TA 101 and SU 391 normally use to inoculate pea and faba bean in Australia are not particularly effective on faba bean cv 'Fiord'.
5. Redistribution of N from the vegetative parts of a grain legume to the seed during grain filling may be linked to the capacity of the plant to continue to fix N_2 during this period.
6. Diurnal variation in the respiration of nodulated roots was closely linked to NA as measured by AR assay and was due mainly to variation in the activity of the nodule rather than to that of the root. Nodule activity was very sensitive to experimental treatments such as defoliation, shoot excision, exposure to high/low PPFD, and exposure to prolonged light and darkness, all of which produce change in the current supply of photosynthate.
7. Diurnal variation of the respiration of nodulated roots and the total electron flux to NA of faba bean as measured by AR assay and by HE in Ar/O_2 was attributable to changes in HE rather than to fluctuation in N_2 fixation. N_2 fixation fluctuated at 'low' PPFD but not

at 'high' due to presumed substrate limitation. In both cases about 30% of the total electron flux was to H_2.

8. The volume of the active N_2 fixing region of the indeterminate nodules of faba bean appears to be flexible and can increase in response to a treatment (removal of half the nodules on a plant) which increases the substrate available to a nodule or increases the need for the nodule to increase its fixation of N.
9. Expression of the rate of acetylene reduction on the basis of nodule weight can be misleading due to variation between nodules in the ratio of active/senescent N_2 fixing regions. It is suggested that a whole plant basis is best for comparative purposes but where specific activity is required, the active volume of the N_2 fixing region may be a better basis than nodule dry weight.

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