The effects of temperature on growth and nitrogen fixation in *Trifolium subterraneum* L. communities.

by

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To my mother and my late father.

"I climb the hill: from end to end
Of all the landscape underneath,
I find no place that does not breathe
Some gracious memory of my friend"

'In Memoriam'. Alfred, Lord Tennyson.

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ABBREVIATIONS USED IN THE TEXT

Nitrogen metabolism:

AR	C_2H_2 reduction.
HE	H_2 evolution.
AR-HE	Quantity equivalent to N_2 fixation when Hup absent.
RE	Relative efficiency of N_2 fixation, 1 - HE/AR.
Hup	H_2 uptake.
GS	Glutamine synthetase.
GOGAT	Glutamate synthase.
ADP	Adenosine diphosphate.
ATP	Adenosine triphosphate.
p	Partial pressure.
K_m	Michaelis-Menten constant.
E_A	Activation energy.

Carbon metabolism:

CER(s)	CO_2 exchange rate(s).
D	CO_2 uptake during 12 hour day.
N	CO_2 efflux during 12 hour night.
D-N	CO_2 exchange over 24 hours, growth rate.
P_N	Net photosynthesis (=D).
P_G	Gross photosynthesis.
RuBISCO	Ribulose bisphosphate carboxylase : oxygenase.
RuBP	Ribulose bisphosphate.

Other:

CEC	Controlled environment chamber.
DI	Days after inoculation.
DM	Dry matter.
LAI	Leaf area index.
-N	Nutrient solution lacking combined nitrogen.
+N	Nutrient solution containing combined nitrogen.
PPFD	Photosynthetic photon flux density.

SUMMARY

Survey of the literature showed there to be much information on the effects of temperature on nitrogen fixation. However, most of this was derived from short-term exposure of single plants, or nodulated roots, to different temperatures. Also, much of this research involved the acetylene reduction assay without consideration of associated evolution of hydrogen. It appears to have been assumed that the relationships between acetylene reduction, hydrogen evolution, and nitrogen fixation are not influenced by the environment nor do they change during ontogeny. Little information was available concerning the response of nitrogen fixation by legumes to temperature when plants are grown as swards at densities comparable with those experienced under commercial conditions. The aim of this investigation was to assess the responses to temperature of swards of Trifolium subterraneum between seedling establishment and dry matter yields regarded as economic in the field.

Swards of T.subterraneum (2,300 plants m^{-2}) were grown at temperatures of $10^{o}C$, $15^{o}C$, $20^{o}C$ and $25^{o}C$ at photosynthetic photon flux densities (PPFD) of 500 or 1000 µmol quanta $m^{-2}s^{-1}$. Growth rates were measured by infra-red analysis of CO_2 exchange, and by increment in biomass with time. Nitrogen fixation was estimated by hydrogen evolution and acetylene reduction assays, and also by increment in organic nitrogen with time.

The growth rates of nitrogen-fixing swards, of 190-475 g dry matter m^{-2}, were inversely related to temperature, and in this respect their response was very similar to that of swards assimilating combined nitrogen. However, swards fixing nitrogen grew more slowly than the latter. This was reckoned to result from the high energy costs of nitrogenase activity, and a lower growth efficiency (g carbon respired

g dry matter retained 24 h^{-1}) for nitrogen-fixing plants. The effect of temperature on the nitrogen fixation rate was similar to that on the growth rate.

Acetylene reduction accurately reflected the response of nitrogen fixation to temperature between 10°C and 20°C, but above 20°C there was a temperature-induced disruption of this relationship. The difference between acetylene reduction and hydrogen evolution is theoretically equivalent to nitrogen fixation, but in long-term experiments the development of hydrogen uptake made this difference an unreliable index of nitrogen fixation.

Hydrogen uptake occurred at high amounts of biomass (greater than 380 g dry matter m^{-2}) at 15°C and 20°C, and also at lower amounts of biomass (greater than 190 g dry matter m^{-2}) at 25°C. Appearance of hydrogen uptake was not quickened when plants were treated with hydrogen. It is suggested that hydrogen uptake developed as a normal event in the physiological ageing of nodules, which was accelerated at high temperature and served in protecting nitrogenase from damage by oxygen.

There was substantial diurnal variation in nitrogen fixation in all environments except 25°C. The data supported a model in which a diurnal change in the transpiration rate resulted in a diurnal rhythm of accumulation of nitrogen in nodules at night and release of nitrogen during the day, and this rhythm affected the nitrogen fixation rate. Nitrogen fixation was finely regulated in the short term through proton reduction.

The hydrogen evolution and acetylene reduction techniques were useful in measuring nitrogen fixation when consistently employed.

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.

Robin P.G. Gates.

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CHAPTER 1

1. General introduction.

Legumes have developed immensely useful symbiotic associations with N_2-fixing prokaryotes; useful not only to the legumes, by permitting growth in soils poor in combined nitrogen, but also to Man in that nitrogen fixed in the legume is eventually released to the soil through decomposition. This offers for agriculture an ecologically acceptable alternative to expensive nitrogenous fertilisers. Trifolium subterraneum is an annual pasture legume of great importance in southern Australia. In areas of low inherent fertility, or exhausted of nitrogen by repeated cereal cropping, soils dressed with superphosphate and seeded with T.subterraneum increase in organic nitrogen content to benefit the growth of cereals (Donald and Williams, 1954). However, the traditional view of cereal yields being immediately improved through the uptake of nitrogen fixed by a preceding legume has been questioned by Ladd et al. (1981). They suggested that legumes in rotations are more important for maintaining the soil's organic nitrogen pool than in fixing N_2 for instantaneous use by a cereal. Nevertheless, the cardinal function of legumes in fertility building in the ley farming system is irrefragable. In 1981-1982, 3,643,000 ha of rural South Australia were under legume-based pasture (S.A. Yearbook, 1983).

Variation in the amount of nitrogen fixed in the field is of concern to farmers. Factors influencing the rate of N_2 fixation are readily investigated by crop physiologists since techniques are available to quantify both the growth of legume communities and their rates of N_2 fixation. Whereas an infra-red gas analyser for measuring CO_2 exchange has been successfully wielded in the paddock (Musgrave and

Moss, 1961). The simple nitrogenase-catalysed reduction of C_2H_2 (AR) to C_2H_4 (Dilworth, 1966) has been less reliably applied there because it necessitates gross disturbance to plants growing in soil and thereby yields large errors (Goh et al. 1978). Application of the AR assay in the project to be described was facilitated through the use of plants from controlled environments, which also allowed repetition of growth conditions.

There is an extensive literature on the effects of temperature on the growth of single legumes but few data pertain to communities, and those that do so indicate notably different responses than for single plants. The main aim of the project to be described was to examine the interaction of growth and N_2 fixation as affected by growth temperature, in the range $10^{o}C$ to $30^{o}C$, in T.subterraneum communities. The approach taken embodied both diurnal and ontogenetic investigations, so recognising the critical ability of plants to adapt to temperature, just as a single leaf, for example, can show a diversity of structural and physiological strategies in adapting to shade within a community (see Hawkins, 1982). Previous studies in this area have, almost invariably, referred to single plants and AR assays were often performed on excised nodules, or severed roots, which did not accurately reflect the responses of whole plants.

C_2H_2 is not a normal physiological substrate for nitrogenase, and the influence of temperature on factors which modify the electrochemistry of nitrogenase reductions (Hageman and Burris, 1980; Wassink and Haaker, 1984) is unknown. AR has been widely applied in the field, the glasshouse and the controlled environment, based on an assumption that its relationship to N_2 fixation is constant. Hence, an additional interest of the project was to judge the competence of the AR assay in estimating N_2 fixation at different temperatures. Essential

companion measurements of H_2 evolution (HE) were made, since AR represents total nitrogenase activity while, in normal air, a portion of this activity is engaged in H^+ reduction (see Hardy, 1979).

Variation in HE offers a potential explanation for the apparent caprice of the AR assay (see Knowles, 1981). The effects of growth temperature on HE are unknown, and HE also lends a new perspective to diurnal variation in N_2 fixation. Together with the development of H_2 uptake (Hup) by Rhizobium trifolii, HE is a topic forming a pillar of discussion in this project. Diurnal variation and Hup, in particular, are both issues which have been clouded in the literature by contradictory results. Direct measurement of organic nitrogen was used as a means of calibrating the indirect HE and AR assays.

It has been borne in mind, if not frequently stated, that the legume - Rhizobium symbiosis is an enigma at the fundamental levels of the union. For example, why H_2 should be produced is a mystery. It may be that nitrogenase evolved from an hydrogenase which, in a primitive reducing atmosphere, required energy to expel H_2 (Broda and Peschek, 1980). Most recently, the view has been reiterated that the symbiosis represents an "highly regulated disease" (see Vance, 1983) since so many features of its development resemble a pathogenic infection.

CHAPTER 2

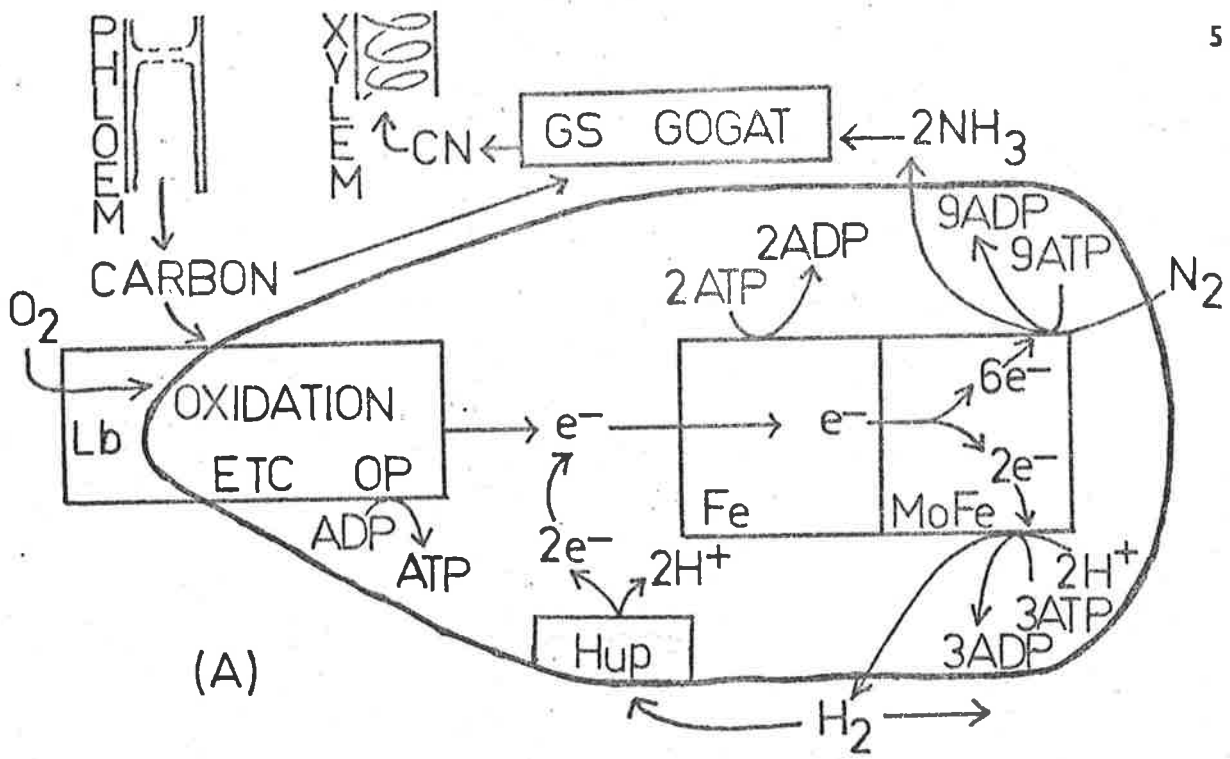
2. Literature review.

2.1.0. Symbiotic interaction.

Species of Rhizobium do not normally show nitrogenase activity in a free-living state, but in their bacteroid forms in legume nodules the prokaryotic genes coding for nitrogenase synthesis are derepressed, enabling fixation of atmosphere N_2. The host legume represses expression of the prokaryotic genes coding for enzymes of nitrogen assimilation, so that almost all of the nitrogen fixed is exported in to the host cytoplasm (O'Gara and Shanmugam, 1976a,b).

Nitrogenase consists of two subunits. Electrons are transferred from the first of these, an Fe protein, to a MoFe protein upon which substrates are reduced (Hageman and Burris, 1978). The most important reductions by nitrogenase are those of the triple-bonded substrates N_2 and C_2H_2 to NH_3 and C_2H_4, respectively, and of H^+ to H_2. These are shown in Figure 1. NH_3 is assimilated by the host-produced enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) to provide the legume with fixed nitrogen, but C_2H_4 is immediately released and can be detected to estimate nitrogenase activity since its production uses the full electron flux of nitrogenase (Dilworth, 1966; see Burns and Hardy, 1975; Boland et al. 1978).

Nitrogenase is irreversibly inactivated by O_2, yet O_2 is required as terminal electron acceptor in the respiratory electron transport chain which supports nitrogenase activity. This paradox is resolved through the functioning of leghemoglobin. Leghemoglobin, a joint production of legume and Rhizobium, exists within the membrane sacs



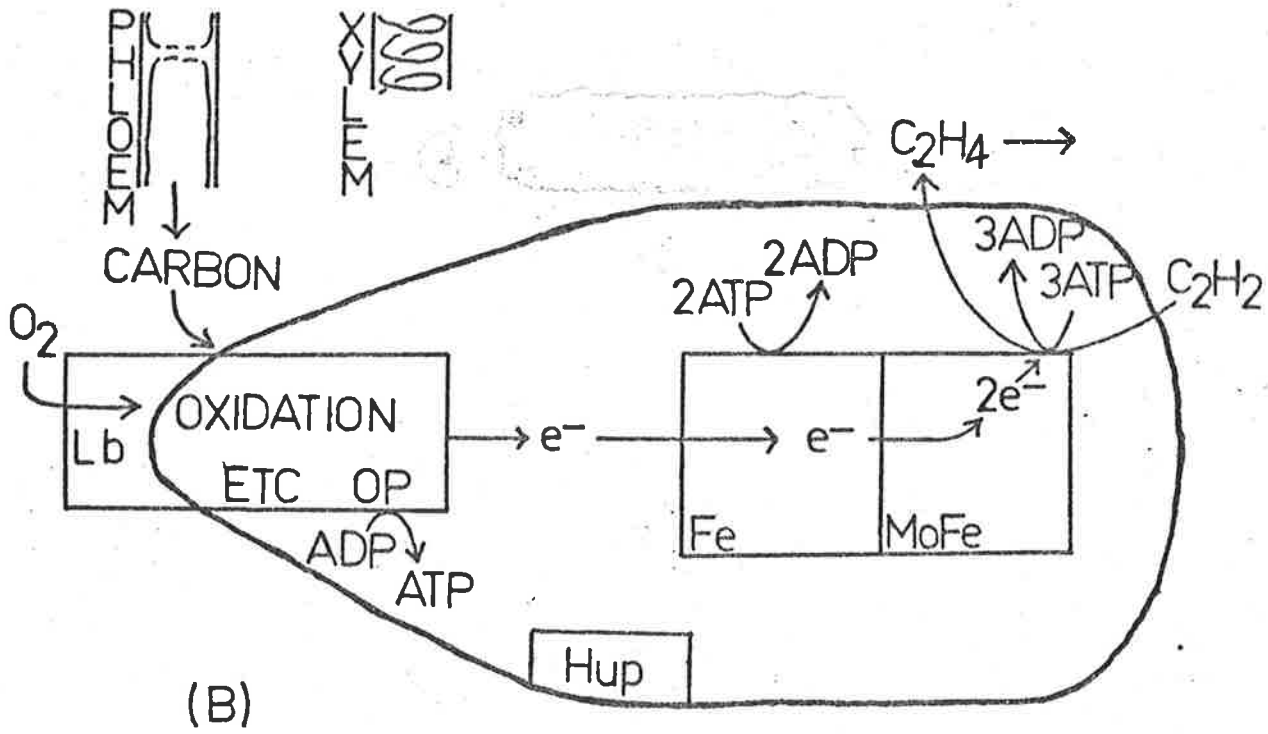


Figure 1. (A), Nitrogenase – catalysed reductions of H^+ and N_2; (B), Nitrogenase – catalysed reduction of C_2H_2. Lb, Leghemoglobin; ETC, Electron transport chain; OP, Oxidative phosphorylation; e^-, electrons; Fe, FeMo, Subunits of nitrogenase; Hup, Uptake hydrogenase; GS, Glutamine synthetase; GOGAT, Glutamate synthase.

enclosing bacteroids (Bergersen and Appleby, 1981) and supplies O_2 at a level which permits respiration but which is inocuous to nitrogenase.

The functioning of nitrogenase has an obligate requirement for ATP which binds to the Fe protein and allows electron transfer to the MoFe protein (Hageman and Burris, 1978). The ATP cost of N_2 fixation is increased by seemingly unavoidable reduction of the endogenous substrate H^+ to H_2. With a minimum of 2 ATP being hydrolysed per electron transferred, N_2 reduction and associated H^+ reduction use 28 ATP (see Evans et al. 1980). In strains of Rhizobium possessing an ATP-independent uptake hydrogenase (Hup^+ strains), some of the ATP lost in H_2 evolution (HE) may be recouped (Hyndman et al. 1953; Dixon, 1972). In the absence of Hup, the difference between rates of C_2H_2 reduction (AR) and H_2 evolution (HE) is proportional to the rate of N_2 fixation (see Hardy, 1979).

In addition to influencing Rhizobium metabolism and participating in leghemoglobin production, the host legume has an essential role in supplying photosynthetically-fixed carbon to the nodules. Fixed carbon is used mainly in respiration to provide ATP and reductant for N_2 fixation, and in the provision of molecules for the assimilation of fixed nitrogen. Fixed nitrogen, in the form of amides, amino acids, or ureides, depending on legume species, is actively secreted into the nodule vascular tissue via transfer cells rich in mitochondria, but is passively transported from the nodule in the xylem, relying on the tension created by transpiration (see Pate, 1976). The nitrogen required for distal root regions is returned via the phloem (Oghoghorie and Pate, 1972).

The question of whether CO_2 fixation limits N_2 fixation, or vice versa, has often been debated. Observations on the responses of these processes to environmental factors may help to resolve this point.

2.2.0. The effects of temperature on the growth of legumes.

In this section the effects of temperature are reviewed with respect to the fixation of CO_2 in leaves, the use of carbon in respiration, translocation of fixed carbon, and root growth. Distinctions are drawn between single plants and communities, and between tropical and temperate species.

2.2.1. CO_2 fixation and dry matter production.

In the field, the photosynthetic rate of C3 plants is determined largely by the CO_2 concentration in the leaf. Net photosynthesis (P_N) is most influenced by temperature when CO_2 conductance is high since the chemical processes of CO_2 assimilation are affected by temperature much more than those of photochemistry (see Gaastra, 1962; Berry and Bjorkman, 1980). Stomata generally open with rise in temperature to satisfy the CO_2 requirement for photosynthesis. In Pisum sativum and Vicia faba, stomatal aperture increased with temperature up to 27°C and 21°C respectively (Hofstra and Hesketh, 1969). For single leaves of N_2-fixing Trifolium repens, at a PPFD of 1150 µmol quanta $m^{-2}s^{-1}$, P_N was higher for plants from an 18°C day, 14°C night, regimen than for those from an 8°C day, 4°C night, regimen and this was attributed to differences in CO_2 conductance (Woledge and Dennis, 1982a).

Photorespiration is inextricably enmeshed with photosynthesis since both processes are catalysed by the same enzyme, ribulose bisphosphate carboxylase: oxygenase (RuBISCO), and compete for ribulose bisphosphate (RuBP). The concentrations of CO_2 and O_2 at the chloroplast dictate the proportion of fixed carbon channelled into each pathway (see Lorimer, 1981). More than 25% of fixed carbon is immediately lost in

photorespiration (Zelitch, 1975). The quantum yield, measured as moles CO_2 fixed per quantum radiant energy absorbed, falls in C3 plants with rise in temperature between 10°C and 35°C. This is due to the greater oxygenation of RuBP at higher temperatures, which some authors have claimed to be caused by the K_m of RuBISCO for CO_2 rising more rapidly with temperature than that for O_2 (Laing et al. 1974) and others by a decrease in the CO_2:O_2 solubility ratio (Hall and Keys, 1983). It has been suggested that the concentration of RuBP limits CO_2 fixation above 20°C (see Berry and Bjorkman, 1980) since RuBP is used both in photosynthesis and photorespiration, and RuBP regeneration is reduced by lower rates of photophosphorylation and NADP reduction at high temperatures. The increase in the proportion of fixed carbon wasted in photorespiration, caused by a rise in temperature, is particularly important in legumes, because the supply of fixed carbon to nodules can limit N_2 fixation (Hardy and Havelka, 1976). When photorespiration was chemically inhibited in Medicago sativa, or when P.sativum and Glycine max were grown in an atmosphere enriched with CO_2, nitrogenase activity was enhanced (Phillips et al. 1976; Hardy and Havelka, 1976; Bedmar and Olivares, 1980).

Early studies with T.subterraneum communities, both at constant and fluctuating temperatures, indicated a minor influence of temperature on growth rate (Black, 1955; Mitchell, 1956). Also, a flat temperature response curve for P_N was found in small communities of M.sativa in the range 5°C to 30°C (Murata et al., 1965). Several authors concluded that growth rates of crop stands were little affected by temperature (Morley, 1958; Stanhill, 1962; Black, 1964). More recent work with T.subterraneum communities showed that the crop growth rate was indeed affected by temperature, due to the temperature-dependence of dark respiration and the respiratory costs of maintaining accumulated biomass

(Fukai and Silsbury, 1976). The optimum growth temperature was relatively high in young communities and fell with increases in age and leaf area index, such that total dry matter production was eventually inversely related to temperature in the range 15°C to 30°C (Cocks, 1973; Fukai and Silsbury, 1976).

A brief change in temperature had little effect on P_N in T.subterraneum communities, but the relationship between P_N and temperature depended on PPFD. P_N showed a gentle increase with temperature at high PPFD, and a progressively more marked decrease with temperature as PPFD was reduced (Fukai and Silsbury, 1977b). For example, at 1150 µmol quanta $m^{-2}s^{-1}$ P_N was about 11% higher at 28°C than at 16°C, while at 690 µmol quanta $m^{-2}s^{-1}$ P_N was about 9% lower at 28°C than at 16°C.

Plants appear to adapt to their growth temperature. At high PPFD, a brief change in temperature had a larger effect on P_N in T.subterraneum and in T.repens than was evident between rates of P_N measured at different growth temperatures (Fukai and Silsbury, 1977b; Woledge and Dennis, 1982a). This suggests leaves to have adapted to temperature during growth through alterations in their photosynthetic and respiratory properties. But, whereas M.sativa was found to show no photosynthetic response to continuous diurnal variation in temperature, simulating the field (Harding and Sheehy, 1980), it, too, adapted to its long term growth temperature. M.sativa had a lower optimum temperature for P_N when grown in a cool temperature regimen than when grown in a warm temperature regimen (Pearson and Hunt, 1972).

Improved growth at low temperature has been partially explained by leaf cells being larger, so enhancing CO_2 diffusion, and by cells in vascular tissues being larger, which facilitates translocation, than at high temperature (Bula, 1972). In T.subterraneum communities, the rates

of leaf appearance and leaf death increased with temperature (Fukai and Silsbury, 1976). Milthorpe (1959) suggested that a large number of leaves expanding at high temperature increased the likelihood of a demand for minerals exceeding their supply, which would result in smaller leaves being produced. This could be important in legumes since, if N_2 fixation is affected by high temperature, nitrogen deficiency could limit leaf growth.

Ketellaper (1963) added a variety of chemicals, including vitamins and ribosides, to V.faba and P.sativum and found that at least part of the reason why plant growth was reduced above or below an optimum temperature was a temperature-induced shortage of metabolites. Amino acid interconversions, many of which require energy, are important both in leaves and nodules of legumes. The activation energies for some of the reactions involved were shown to be higher at 10°C than at 23°C in G.max, and activities of GS and asparagine synthetase were lower at 10°C (Duke et al. 1978). However, G.max is a chilling-sensitive legume and the meaning of this is explained in the next section. Legumes show metabolic adaption to their region of origin. Thus, although the curves relating P_N to temperature are of a similar shape for tropical (chilling sensitive) and temperate species, those for the tropical legumes have optima displaced towards higher temperatures and commonly at about 30°C (Ludlow and Wilson, 1971; Fukai and Silsbury, 1977b).

It is worth remarking that, when leaves have been subjected to a range of temperatures in brief experiments on photosynthesis, they have often had altered or unstable photosynthetic rates after exposure to high or low temperature (Pearson and Hunt, 1972; Woledge and Dennis, 1982a). Nolan and Smillie (1976) reported that the Hill reaction of chloroplasts was disrupted in Hordeum vulgare at 9°C and 29°C,

coincident with temperature-induced modifications to thylakoid membrane fluidity.

Many workers have grown plants in environments with diurnal changes in temperature. However, maintaining constant temperatures can simplify the interpretation of data. Moreover, at constant PPFD and with a 12h. photoperiod, the growth rates of single P.sativum and Phaseolus vulgaris plants, and of T.repens communities, were no different from those of plants grown at the same mean temperature but with a $10^{o}C$ or $20^{o}C$ diurnal change (Friend and Helson, 1976; McCree and Amthor, 1982).

2.2.2. Dark respiration.

A rapid increase in respiration with rise in temperature has been noted for a variety of legumes. This was found to have a pronounced negative effect on forage production in the field for M.sativa (Robinson and Mesengale, 1968). In T.subterraneum communities the most important factors affecting respiration were the amount of biomass present and the growth temperature (Fukai and Silsbury, 1977a). A ready explanation of this is provided through the concepts of synthesis and maintenance respiration. In short experiments with T.subterraneum and T.repens, synthesis respiration, associated with the production of new material, was independent of temperature, while maintenance respiration, associated with sustaining existing biomas, increased with rise in temperature and had a Q_{10} of about 1.8 (McCree, 1974; McCree and Silsbury, 1978). Protein turnover, and active transport to preserve ion concentrations in cells, have been described as primary maintenance processes. Both are stimulated by a rise in temperature because enzyme activity and membrane permeability are increased considerably (Penning de Vries, 1975). The maintenance respiration of roots was shown to increase more with temperature between $10^{o}C$ and $30^{o}C$ than that of shoots

(Szaniawski and Kielkiewicz, 1982), perhaps because the roots take up nutrients and maintain ionic balance with the environment for the whole plant.

There is some evidence that respiration can adapt to the growth temperature. For example, in T.repens, leaves from a cool regimen had higher respiration rates than those from a warmer regimen when examined at the same temperature, both on the basis of leaf area and dry weight (Woledge and Dennis, 1982a). Similarly, when T.subterraneum communities were compared at the same biomass and temperature, those from a cool regimen had higher respiration rates than those from a warm regimen (Fukai and Silsbury, 1977a). These findings suggest that there are some compensations for the decreased kinetic energies of reacting molecules, and lower reaction rates, expected at low temperature. In M.sativa, adaptation to low temperature was positively correlated with the activity of malate dehydrogenase, the controlling enzyme in the tricarboxylic acid cycle. The authors of this work (Duke and Doehlert, 1981) pointed out that although some enzyme activities in M.sativa appeared not to be directly affected by temperature (for example, amylase, which degrades starch ; invertase, which splits sucrose ; NADP - isocitrate dehydrogenase, which supplies carbon skeletons for NH_3 assimilation), temperature could indirectly modify enzyme activities in vivo through cryptic effects on tissue pH or the levels of allosteric inhibitors.

McCree (1974) emphasised that respiration should not be viewed as a negative process. Aside from oxidising fixed carbon, respiration also produces ATP and NADPH for synthetic reactions and carbon skeletons for the organic components of plant structure. However, a second type of dark respiration has been identified which branches from the normal electron transport chain and does not produce ATP. The effect of

temperature on this pathway is unknown. It was found to operate only when carbohydrate levels were in excess, and glycolytic activity exceeded the processing ability of the cytochrome chain and mitochondria (Azcon-Bieto et al., 1983). The energy demands for N_2 fixation and root growth in nodulated legumes have been shown to be high, and may account for the low level of this alternative-pathway respiration in nodulated legumes (Lambers et al., 1980 ; de Visser and Lambers, 1983).

Temperature in the range 10°C to 30°C affected respiration in P.sativum through the rate of degradation of the respiratory system which occurred normally at 20°C, the plant's optimum temperature for growth (Geronimo and Beevers, 1964). Physiological ageing of the material was manifested through general disorganization and lowered biochemical efficiency of mitochondria. The ageing process was simply accelerated above 20°C, and retarded below 20°C.

The review so far has largely considered species of Trifolium, Medicago, Pisum, and Vicia, which mostly grow in temperate or mediterranean regions, yet much research both in photosynthesis and in N_2 fixation relates to species of Phaseolus, Glycine, and Vigna which originate in tropical or subtropical areas. These latter species generally grow best above 20°C. As an example, when P.vulgaris was grown at 12.5°C it had disturbed chloroplast structure and a reduced rate of photosynthesis (Austin and Maclean, 1972). Such chilling-sensitive species have shown discontinuities in Arrhenius plots for mitochondrial respiration at about 10°C, indicating that phase changes occur in mitochondrial membranes. In chilling resistant species, respiration fell linearly with temperature between 25°C and 1.5°C (Lyons and Raison, 1970). Low temperature has been found to alter the molecular ordering of membrane lipids of chilling-sensitive species, such that the kinetic properties of membrane-bound enzymes are disturbed, for example, those

of succinate oxidase in the mitochondria of Vigna radiata. In chilling-resistant species the molecular ordering was shown to be stable (Raison and Chapman, 1976). This information is pertinent in that it supports the view that the productivity of pasture legumes such as T.subterraneum can be maintained at low temperatures.

2.2.3. Translocation.

Assimilate partitioning is considered in a later section, but here it can be noted that temperature affects source and sink activities but not the process of long distance assimilate movement in the phloem. Vein-loading, an active process, showed an optimum temperature at 30°C in Triticum aestivum, but the movement of carbon in the phloem was independent of temperature between 1°C and 50°C (Wardlaw, 1974). Majumder and Leopold (1967) indicated that assimilate movement out of leaves was inhibited at low temperature by the formation of callose plugs in the petiolar phloem. Plug frequency increased linearly with fall in temperature between 20°C and 5°C.

Export of fixed carbon from a leaf has been shown to increase as P_N and the sucrose level in the leaf increased, even though sink requirements were apparently unaltered (Ho, 1976). Nitrogen movement in the xylem is passively controlled by the transpiration rate, while nitrogen movement in the phloem should be subject to similar constraints as carbon movement.

2.2.4. Root growth.

The responses of roots to temperature have been less intensively examined than those of shoots. The mitotic rate of V.faba root meristem

cells was found to increase linearly with temperature between $3^{o}C$ and $25^{o}C$ (Evans and Savage, 1959), and Crawford and Huxter (1977) reported root extension to increase with temperature between $2^{o}C$ and $14^{o}C$ in P.sativum. Interestingly, the ratio of glucose to sucrose in the roots fell from about 2.7 at $14^{o}C$ to 0.4 at $2^{o}C$, but invertase had a lower K_m at the lower temperature so that the decline in reaction rate at low temperature was to some extent counterbalanced by the greater affinity of the enzyme for its substrate. This can be taken as an example of enzymic adaptation to temperature.

A rise in temperature affects water uptake by roots through the lowered viscosity of water, the increased osmotic permeability of membranes, and an increase in active solute uptake (Dalton and Gardner, 1978).

2.3.0. The effects of temperature on N_2 fixation.

The responses of N_2 fixation to temperature have been studied almost exclusively with single plants. Evidence for legume cultivar-Rhizobium strain interactions is reviewed, and the application of the AR assay to measuring the effect of temperature on N_2 fixation is considered. The effects of temperature on bacterial development and nodule respiration are also examined.

2.3.1. Responses of whole plants to temperature.

Roponen et al. (1970) have shown that N_2-fixing legumes have a narrower temperature tolerance than those assimilating combined nitrogen. They found that nodulated legumes only grew well when the

growth temperature was close to that optimal both for root growth and *Rhizobium* growth.

Early experiments on the responses of N_2 fixation to temperature in *T.subterraneum* showed a depression in the process above 20°C (Meyer and Anderson, 1959). Root temperature, rather than shoot temperature, was considered to have the main influence over N_2 fixation in *T.subterraneum* and *M.sativa* (Possingham *et al.*, 1964 ; Harding and Sheehy, 1980). Temperature affected N_2 fixation more than plant growth, since the nitrogen concentration in tissues decreased with rise in temperature (Possingham *et al.*, 1964). In *T.subterraneum*, its *R.trifolii* partner has influence over the response of N_2 fixation to temperature. For single plants of cv. Dwalganup, the optimum temperature for N_2 fixation was about 22°C, but the most effective strain of *R.trifolii* in fixing N_2 was TA1 above 22°C and NA30 below 22°C (Gibson, 1961). *T.subterraneum* can be particularly sensitive to high temperatures. A drastic decrease in N_2 fixation above 25°C was dependent not only on the *R.trifolii* strain, but also on the host cultivar (Gibson, 1963). For a range of cultivar-strain combinations, the amounts of nitrogen fixed and dry matter produced fell steadily between 18°C and 5°C. Concomitant with a reduction in temperature from 14°C to 9.5°C, the nitrogen concentration in the plants fell by as much as 0.9%. Below 18°C, the movement of nitrogen to the shoots was suggested to have been impeded by slowed transpiration (Gibson, 1963 ; Gibson, 1966). Gibson's results, based on the measurement of organic nitrogen, were largely confirmed by Roughley and Dart (1969 ; 1970) and Roughley (1970) who used the AR technique with single *T.subterraneum* plants grown similarly in unaerated nutrient agar. The latter workers inoculated their plants at the growth temperatures, however, so that temperature effects were also evident on infection and nodulation. Both processes are slowed at low temperature.

Communities of T.subterraneum, of about 230 plants m^{-2}, responded to temperature rather differently from spaced plants. The amounts of nitrogen fixed by communities were not lower at 12°C than at 22°C (Davidson et al., 1970). Growth restrictions imposed on each plant within a community, by mutual shading for example, are evidently an important facet of experimental design not only in studies of photosynthesis (Cocks, 1973) but also in those relating to N_2 fixation.

Tropical legumes generally show reduced nitrogenase activity below about 20°C. For example, a reduction in shoot temperature from 27°C to 18°C decreased the AR rate of G.max to only 39% of the rate at 27°C within 5 hours, showing there to have been a greater response of nitrogenase activity to shoot temperature than was the case in T.subterraneum (Possingham et al., 1964 ; Schweitzer and Harper, 1980). However, Possingham et al. (1964) measured N_2 fixation directly whereas Schweitzer and Harper (1980) estimated N_2 fixation using an uncalibrated AR assay in G.max.

2.3.2. Effects of temperature on different Rhizobium strains and on bacteriod development.

T.subterraneum inoculated with R.trifolii strains NA30 or CC17 began to die when transferred to 28°C, while plants inoculated with strain TA1 grew satisfactorily (Gibson, 1961 ; Gibson, 1967). Strain WU95, that currently incorporated in commercially available inoculant preparations for T.subterraneum, is similar to NA30 and CC17 in being sensitive to high temperature. Although such bacteria were found to grow well at 30°C independently, they developed abnormal symbiotic characteristics in nodules (Pankhurst and Gibson, 1973). At high temperature the bacteroid tissue was observed to break down and the low

proportion of membrane envelopes containing viable bacteroids was said to have limited N_2 fixation. It was suggested that there was not a strong effect of temperature on the N_2 fixation reaction itself.

Persistence of bacteroids has been shown to be temperature-dependent, with degeneration occurring sooner as temperature rises. At low temperature, bacteroid development and subsequent degeneration were slow. The conversion of bacterial rods into bacteroids was retarded at low temperature and suppressed at high temperature. Whereas meristematic activity in nodules was slowed at low temperture, nodules continued to grow but with a large base of degenerate cells at high temperture (Roughley, 1970 ; Pankhurst and Gibson, 1973 ; Roughley et al., 1976).

2.3.3. Responses of excised roots and nodules to temperature.

AR assays which have used excised roots or excised nodules have been found not to reflect in vivo changes in nitrogenase activity accurately because a major source of fixed carbon was absent viz. the shoot or the root (Bergersen, 1970 ; Magne and Burris, 1972 ; Murphy, 1981).

When excised roots (Dart and Day, 1971) or detached nodules (Waughman, 1977) of temperate and tropical legumes were transferred to a range of temperatures for AR assays, substantial differences between species were seen in the optimum temperatures for nitrogenase activities, although some temperature response profiles were rather flat. Tropical species, such as G.max and V.unguiculata, had maximum AR rates around 30°C or higher, while V.faba, a temperate species, performed better at lower temperatures.

Although these data revealed nitrogenase to have a broad tolerance to temperature (2°C to 40°C), the relevance of such temperature profiles

to whole plants is equivocal. Exposure of small nodules from species of Trifolium, for example, to high temperature, would have stimulated respiration and perhaps depleted the carbohydrate pool necessary to support nitrogenase activity, rather than having had a direct effect on the enzymic reaction. Data for HE were not presented, but it was stated that temperature profiles for HE followed those for AR with a suggestion of a change in the ratio HE : AR (Dart and Day, 1971).

2.3.4. Temperature and the relationship between AR and N_2 fixation.

The AR assay has been widely applied in the field, and in controlled environments, with the assumption that the relationship between AR and N_2 fixation is constant. However, there are indications in the literature that this assumption is not sound. The most lucid example of this is in the data of Munevar and Wollum (1981). Single plants of G.max inoculated with Rhizobium japonicum 587 had similar nodule mass and specific AR rates (AR expressed on the basis of nodule mass), 20 days after sowing, when grown at 28°C and 33°C. However, the amount of nitrogen fixed per plant was 50% higher at 28°C than at 33°C, indicating a higher AR : N_2 fixation ratio at 33°C than at 28°C. Fair comparisons are harder to draw from the rest of their data since there were differences in nodule mass, and AR was expressed on the basis of nodule mass while amounts of nitrogen fixed were given per plant.

Since the electron flux directed to N_2 fixation is most appropriately measured by the difference between AR and HE rates (see Burns and Hardy, 1975), apparent changes in the AR : N_2 ratios with temperature might well be rationalised if HE data were available. Notwithstanding this, there are several reports of discontinuities in Arrhenius plots of AR rate versus temperature occurring at around 20°C

for nitrogenases of legumes and free-living N_2-fixing organisms (Hardy et al., 1968 ; Waughman, 1977 ; Cralle and Heichel, 1982). The activation energy for AR was found to be about 2.5 to 4 times greater below 20°C than above 20°C.

2.3.5. Temperature and the relationship between nodule respiration and AR.

In view of the carbon cost to legumes of fixing N_2, the relationship between respiration and nitrogenase activity is of importance. In P.sativum, the amount of respiration associated with AR was independent of root temperature when subjected to brief change between 10°C and 30°C (Mahon, 1979) while both processes had a Q_{10} of about 2. Nodule respiration also increased rapidly with rise in temperature between 10°C and 30°C in P.vulgaris and G.max, but showed discontinuities in Arrhenius plots typical of chilling-sensitive legumes (Pankhurst and Sprent, 1976). Exposure to 5.5°C irreversibly depressed AR, but respiration recovered on return to 20°C. This indicates that the integrity of the N_2-fixing process was less tolerant to temperature extremes than the respiratory pathway which supported it.

In Vigna unguiculata, the efficiency of nitrogenase activity in terms of carbon respired fell sharply above 20°C, although AR was steady between 20°C and 35°C (see Sprent, 1979). This was attributed to the expression of anaerobic respiration above 20°C. Below 20°C, respiration was entirely aerobic and more ATP was produced per amount of carbon respired. In a similar vein, Minchin and Pate (1974) had earlier shown nitrogenase activity in P.sativum to be more efficient, in terms of carbon respired, at 12°C than at 18°C.

2.4.0. Growth, N_2 fixation, and PPFD.

The effects of PPFD on photosynthesis, dark respiration, and photorespiration, are reviewed, and the consequences of these effects for N_2 fixation are examined with respect to assimilate partitioning to nodules. Where necessary, photometric and radiant energy units reported in the literature have been converted to the form µmol quanta $m^{-2}s^{-1}$.

2.4.1. Effects of PPFD on photosynthesis, and evidence for adaptation to PPFD.

Before taking account of losses in respiration, about 9-11% of available light energy is used in photosynthetic CO_2 fixation (Coombs and Hall, 1982). P_N rates for swards of T.repens, M.sativa, and T.subterraneum have been found to increase curvilinearly with PPFD and to be saturated at about 1,300, 1,700, and 2,000 µmol quanta $m^{-2}s^{-1}$ respectively (Brown et al., 1966 ; Silsbury, 1981). Single leaves of many C3 plants are PPFD-saturated at about 400-650 µmol quanta $m^{-2}s^{-1}$ (Blackman, 1962) whereas, within communities, shaded leaves continue to respond to PPFD although those at the top of the canopy are PPFD-saturated. PPFD has a marked influence on the passage of CO_2 to the chloroplasts. In M.sativa and V.faba, stomatal aperture increased with PPFD to an extrapolated maximum at about 600 µmol quanta $m^{-2}s^{-1}$ (Ehrler and van Bavel, 1968), and conductance has been shown to increase curvilinearly with aperture size (Hsaio et al., 1973).

A sustained level of PPFD during plant growth elicits adaptive responses which are distinct from those observed during brief exposure to a new level of PPFD. Adaptation to PPFD is important in plant communities because shaded leaves need not be parasitic on the plant but

can show a net uptake of CO_2. This is achieved through a lower respiration rate and a lower PPFD-compensation point than for leaves at high PPFD (see Hawkins, 1982). Although Trifolium leaves are heavily shaded during development in a community, it has been suggested that the laminae arrive at the top of the canopy, where most of the PPFD is intercepted and CO_2 is fixed, before the photosynthetic machinery has been harmed by shade (Woledge and Dennis, 1982b). When leaves of P.sativum developed at 90 µmol quanta $m^{-2}s^{-1}$, incident quanta were distributed between photosystem I and photosystem II more efficiently than in leaves produced at 320 µmol quanta $m^{-2}s^{-1}$, so that cyclic electron flow was optimised at the lower PPFD (Hodges and Barber, 1983).

PPFD affects CO_2 fixation in ways beyond the generation of ATP and NADPH for the Calvin cycle, since it leads to elevated pH, and Mg^{2+} concentration of the chloroplast and increases in the activity of several Calvin cycle enzymes located in the stroma (see Kelly and Latzko, 1982).

2.4.2. Effects of PPFD on dark respiration and photorespiration.

In T.subterraneum communities, dark respiration measured at night was a relatively constant proportion of P_N (McCree and Silsbury, 1978), and therefore increased up to saturating levels of PPFD. Whether dark respiration continues in the light is a vexed question. It is generally held that glycolysis and tricarboxylic acid cycle activity do continue, possibly at reduced rates, providing carbon skeletons for synthetic processes, while oxidative phosphorylation does not. This is reasonable since ATP and NADPH are produced by photochemical processes in the daytime (see Graham 1980).

The rates of dark respiration and photorespiration affect the pool of available carbohydrate in a legume, and perhaps N_2 fixation in consequence. The photorespiration rate in P.vulgaris was shown to increase in parallel with the rate of P_N up to saturating levels of PPFD (Catsky and Ticha, 1980) as would be expected since they both operate by RuBISCO activity.

2.4.3. Effects of PPFD on carbon partitioning and on N_2 fixation.

Bacteroid metabolism depends on a carbon supply from the host. The fixation of one mole of N_2 costs a legume at least 28 ATP (see Minchin et al., 1981) and for T.subterraneum communities this was mirrored in the expenditure of 59% more fixed carbon to produce a gramme of dry matter in N_2-fixing plants than in those assimilating combined nitrogen (Silsbury, 1977). Non-nodulated plants can reduce NO_3^- in leaves using photochemically-produced energy, a process which was found to become more rapid when the PPFD was increased (see Schrader and Thomas, 1981 ; Wild et al., 1981).

In grain legumes, from 10 to 32% of photosynthetically-fixed carbon has been found to be allocated to nodules, and is either used in respiration or is returned to the shoot carrying fixed nitrogen (see Pate and Minchin, 1980). This three-fold difference in carbon allocation raises the possibility of N_2 fixation being more influenced by photosnythesis in some species than in others. Such a scenario seems feasible, because the AR rate of M.sativa depleted of carbohydrate by a 40 hour dark treatment was subsequently limited in the light even at very high CER, whereas the AR rate of similarly treated G.max was saturated at a low CER (Sheehy et al., 1980). However, there may have been differences between species in the oxidation of leghemoglobin in

the dark, which occurred within 24 hours in P.sativum (Virtanen et al., 1955).

Translocation rate and vein loading have been found not to be directly influenced by PPFD up to about 450 μmol quanta $m^{-2}s^{-1}$ and 1300 μmol quanta $m^{-2}s^{-1}$, respectively (Vernon and Aronoff, 1952 ; Wardlaw and Marshall, 1976). Ho (1976) reported that an increase in CO_2 fixation produced proportional increases in sucrose synthesis and transport from leaves, although sink strength was not altered. It is not clear whether sink activity or the photosynthetic rate have the dominant influence on translocation of carbon. Ho's (1976) data suggested that photosynthesis dominated, but Thorne and Koller (1974) had earlier shown that CO_2 fixation by a source leaf was stimulated by sink demands of other leaves. When CER was high, the ability of sinks to absorb sucrose was suggested to limit export from source leaves (Wyse and Saftner, 1982). Partitioning of fixed carbon into starch, for storage, or sucrose which can be translocated, is controlled by sucrose phosphate synthase, the activity of which was shown to increase coordinately with sink demands for sucrose (Rufty, 1983).

In Trifolium swards, the leaf canopy is a unified source for all sinks (Harvey, 1970). Nodules are strong sinks for fixed carbon, and in T.subterraneum were strongly labelled within one hour of $^{14}CO_2$ being fed to the leaves (Small and Leonard, 1969). Nodule respiration was found to make up about 50% of total root respiration in species of Trifolium (Ryle et al., 1983). Nodule respiration and AR rate were found to increase, in parallel, with PPFD between 100 and 400 μmol quanta $m^{-2}s^{-1}$ in P.sativum (Mahon, 1977b). A temporary change in the carbon supply to nodules results in variation of the specific AR rate, that is AR per unit of nodule tissue, but after a more permanent change in carbon supply an equilibrium between the amount of nodule tissue and plant

growth was found to be restored (Streeter, 1974). When P.sativum leaves were fed $^{14}CO_2$, and ^{14}C arriving in nodules was measured for a range of growth PPFD, it was shown that translocation of fixed carbon to nodules increased dramatically with growth PPFD, and corresponded with higher AR rates (Sheikholeslam et al., 1980). Transfer of plants between PPFDs showed no effect on ^{14}C translocation within 10 hours, so PPFD was suggested to affect carbon partitioning through a long-term influence on plant development. The data of Sheikholeslam et al. (1980) allow AR-HE, which is theoretically proportional to N_2 fixation (see Hardy, 1979), to be calculated. AR-HE was 14.5 at 200 µmol quanta $m^{-2}s^{-1}$, 17.8 at 500 µmol quanta $m^{-2}s^{-1}$, and 21 at 800 µmol quanta $m^{-2}s^{-1}$ after 10 hours. Apparently, large changes in the N_2 fixation rate occurred without change in ^{14}C translocation to the nodules. These data imply that the N_2 fixation rate can be changed independently of carbon supply, perhaps through an altered HE rate.

When T.subterraneum communities were transferred to a range of PPFDs for 72 hours, at 20°C, the AR rate was a linear function of P_N. The proportion of the electron flux used in N_2 reduction, relative efficiency (RE), was about 0.7 at all PPFDs (Silsbury, 1981). Assimilate supply to nodules would seem not to have affected electron allocation to substrates. However, when P.sativum was grown at a range of PPFDs, the apparent rate of N_2 fixation, calculated from AR and HE data, increased with PPFD, but RE fell from 1 at 200 mol quanta $m^{-2}s^{-1}$ to about 0.62 at 800 mol quanta $m^{-2}s^{-1}$. These data differed from those of Silsbury (1981) in showing a correlation between RE and conditions limiting CO_2 fixation. They also showed that maximum RE did not correlate with the maximum N_2 fixation rate (Bethlenfalvay and Phillips, 1977b). These authors calculated the ratio of N_2 fixed to CO_2 fixed, and the ratio increased linearly with PPFD, perhaps indicating that the proportion, as

well as the amount, of carbon translocated to nodules was increased by raising the PPFD.

The question of whether CO_2 fixation limits N_2 fixation, or vice versa, might be inconsequential on a practical basis. An analogous question is "which wheel of a bicycle is the most important?" Both are obviously necessary for the machine to function properly. Nevertheless, Feigenbaum and Mengel (1979) produced some interesting results which suggest that a tricycle would provide a better analogy. They found that when M.sativa was grown at about 190 µmol quanta $m^{-2}s^{-1}$, the N_2 fixation rate was depressed by about the same order of magnitude as the growth rate, when compared to plants grown at about 370 µmol quanta $m^{-2}s^{1}$. Consequently, the nitrogen concentration in the plants was unaffected. Closer examination of the nitrogen fractions revealed the plants from the low PPFD to have lower protein concentrations, but higher levels of NH_4-nitrogen and amino acids, than the plants from the high PPFD. The plants from the low PPFD had a similar nitrogen constitution to plants grown with a low K^+ supply. The authors of this work concluded that photosynthesis did not limit N_2 fixation, but instead protein synthesis was limited by energy supply.

Changes in the relationship between N_2 fixation and photosynthesis are certainly apparent for whole plants or communities which pass undetected when only single-leaf photosynthesis is measured. In P.sativum, a photosynthetic rate was achieved by single leaves beyond which an increase in the amount of nitrogen fixed had no influence. For whole plants, the photosynthetic rate went on increasing as the N_2 fixation rate increased (Dejong and Phillips, 1981).

2.4.4. Effects of PPFD on young legumes.

Chlorosis is often seen in recently nodulated legumes at high PPFD. The inability of young plants to maintain carbon and nitrogen sufficiency in all their organs has been suggested to result in inhibition of CO_2 fixation and N_2 fixation at high PPFD (Orcutt and Fred, 1935 ; Williams and Phillips, 1980). If nodules become carbon-limited they are unable to fix N_2, and the consequent nitrogen deficiency in the plant reduces CO_2 conductance in the mesophyll, reduces carboxylation efficiency, and reduces the rate of leaf expansion (Ryle and Hesketh, 1969 ; Bouma, 1970 ; Bethlenfalvay et al., 1978a). Nitrogen deficiency during nodulation is less common at low PPFD.

2.4.5 Effect of PPFD on the relationship between AR and N_2 fixation.

Incidental to the main subjects discussed by Trang and Giddens (1980), it can be seen in their data that the AR rates per plant for G.max at 410 and 190 µmol quanta $m^{-2}s^{-1}$ were similar to that at 500 µmol quanta $m^{-2}s^{-1}$. However, the amounts of nitrogen fixed were 65% and 75% less at 410 and 190 µmol quanta $m^{-2}s^{-1}$, respectively, than at 500 µmol quanta $m^{-2}s^{-1}$. The data, therefore, suggest that AR was less responsive to a change in PPFD than was the N_2 fixation rate, such that the AR : N_2 ratio changed with PPFD. It has already been indicated that temperature can affect this ratio (Munevar and Wollum, 1981).

2.5.0. Ontogenetic variations in CO_2 fixation and N_2 fixation.

Information linking photosynthesis and N_2 fixation through ontogeny is fragmentary, since most studies have described responses of legumes

to fairly brief environmental changes. That which does exist pertains mainly to grain legumes.

2.5.1. Ontogenetic developments in leaves.

A leaf canopy differs from a single leaf inasmuch as its photosynthetic capacity is maintained for a longer period through the emergence of new leaves. Nevertheless, a community of plants of a common age, in which development is synchronised, can be expected to show some of the developmental changes evident in single leaves. An example of why such changes are of interest is that a decline in the CER of leaves supplying nodules with photosynthate was coincident with an increase in the RE of N_2 fixation in P.sativum (Bethlenfalvay and Phillips, 1977a).

In very young leaves of P.vulgaris, high rates of photorespiration and dark respiration were found to result in low P_N, while poor CO_2 conductance was associated with low P_N in both young and old leaves. Gross photosynthesis (P_G), the sum of P_N, photorespiration, and dark respiration, reached a plateau during the time of minima in the PPFD- and CO_2 concentration-compensation points (Catsky et al., 1976 ; Catsky and Ticha, 1980). Photorespiration and P_N rose to a maximum, and then fell during senescence, befitting both their rates being dictated by RuBISCO activity. Dark respiration, which was maximal in young and old leaves, was indicated to be subject to change in the amount of the process inhibited by light during ontogeny (Catsky et al., 1976 ; Peisker et al., 1981). These data from P.vulgaris were expressed on the basis of leaf area. Smillie (1962) found that, on the basis of the entire leaf, maximum P_N was reached before full expansion in P.sativum.

Thus, in a community, leaves could be at a stage of maximum potential for photosynthesis while submerged in the shade of the canopy.

An obvious trend with time in a community is that leaf area index (LAI) increases. The concept of an optimum LAI for Trifolium communities with respect to dry matter production (Black, 1964) appears to be no longer tenable. P_N has been shown to reach a plateau above an LAI of about 3 to 4, or even to increase steadily in diffusely-lit plants (McCree and Troughton, 1966 ; King and Evans, 1967).

During senescence, the protein and chlorophyll concentrations of leaves fall. Up to 95% of the nitrogen released from leaves during senescence was found to originate from chloroplasts (Morita, 1980). The rate of protein degradation in leaves of G.max was regulated by sink demands for nitrogen (Kato, 1980) and, in T.subterraneum, protein degradation provided nitrogen for new leaf growth when the N_2 fixation rate was low (Phillips et al., 1983). So, nitrogen released during senescence is redistributed in the plant to sustain protein synthesis in younger tissues.

The capacity of mitochondria to oxidise tricarboxylic acid intermediates and to carry out oxidative phosphorylation was found to reduce with age in P.sativum. This was suggested to be a natural feature of ontogeny, which was accelerated by increase in temperature (Geronimo and Beevers, 1964).

2.5.2. Ontogenetic variation in N_2 fixation.

In single plants of T.repens, the percentage of photosynthate translocated to nodulated roots was found to increase to a maximum at 51 days after sowing and then to fall. The amount of carbon respired by nodulated roots per unit of nitrogen fixed, a measure of efficiency,

decreased between 30 and 65 days (Ryle et al., 1979 ; Ryle et al., 1981). This provides evidence that the efficiency of nodule metabolism increases during ontogeny, and similar suggestions were made by Bethlenfalvay et al. (1978b) and Bethlenfalvay and Phillips (1979). These authors found that in P.sativum the index of the amount of the electron flux used to reduce N_2, RE, increased from about 0.7 at 32 days to 1 at 53 days, and that the capacity of Hup to recover H_2 increased with age. A proportionate increase in Hup activity with age, relative to HE, was considered to be a response to less fixed carbon being available to the nodules. Despite large drifts in AR and HE with age, RE was reported to be steady at about 0.7 in G.max and P.vulgaris (Schubert et al., 1978 ; Saito et al., 1980). Pate (1958) observed that the number of nodules on Pisum arvense declined during ontogeny, but that this decline was compensated for by increased efficiency of N_2 fixation (amount of nitrogen fixed per unit of nodule tissue) in remaining nodules.

In single plants, age effects on N_2 fixation can be confounded by change in plant growth rates, and the N_2 fixation rate per plant usually increases exponentially with time (Ryle et al., 1979). However, when T.subterraneum was grown as a community, at 20°C, with a fairly steady growth rate, there was no obvious trend in AR with time over 35 days (Silsbury, 1979).

2.5.3. Ontogenetic variation in the relationship of AR to N_2 fixation.

Data of Saito et al. (1980) reveal there to have been no effect of age on either the AR:N_2 or AR-HE:N_2 ratios in P.vulgaris, but, for the same species, Rennie and Kemp (1981) found the AR:N_2 ratio to decrease

with age. In three cultivars, Rennie and Kemp (1981) showed the AR:N_2 ratio to have been less than 1:1 late in ontogeny, compared to about 7 to 8:1 according to Saito et al. (1980). The AR:N_2 ratio also decreased with age in V.faba (Sprent and Bradford, 1977). Apparent differences between legumes in AR:N_2 ratios could be caused by variations in HE and Hup between Rhizobium strains.

2.6.0. H_2 evolution and H_2 uptake.

Many studies of N_2 fixation have reported AR data only, but N_2 fixation is more precisely estimated when HE is taken into account (see Hardy, 1979). Schubert and Evans (1976) found that, in a variety of legumes, 40 to 60% of the electron flux through nitrogenase was used in HE. HE appears to be an unavoidable consequence of nitrogenase activity in air, and has been suggested to result from nitrogenase having evolved from an hydrogenase (Broda and Peschek, 1980). Some legumes have the capability to take up H_2 via an hydrogenase (Hup) before it leaves the nodule. H_2 so recovered can be oxidised to yield ATP or electrons for further substrate reductions (Hyndman et al., 1953; Emerich et al., 1979).

Expression of the Hup characteristic was found to be controlled by the host legume species (Keyser et al., 1982), and more recently by the cultivar in P.sativum (Bedmar et al., 1983). These results make it likely that more instances of Hup will be found in the future. To date, Rhizobium strains have been classified either as Hup^+ or as Hup^-, but these labels can only be applied when a strain's phenotype has been defined with a given host legume. Notwithstanding their classification, there are suggestions in the literature that the Hup phenotype can be influenced by age and environment in P.sativum (Bethlenfalvay

et al., 1978b ; Bethlenfalvay and Phillips, 1979). Gibson et al. (1981) also reported that Hup was induced in T.subterraneum if the carbon supply to nodules was interrupted by defoliation.

Interpretations of the consequences of HE and Hup for legume growth have not achieved a consensus. Reports can be classified as those which have shown increases in N_2 fixation and growth in legumes inoculated with Hup^+ Rhizobium strains over those inoculated with Hup^- strains (Schubert et al., 1978 ; Zablotowicz et al., 1980 ; Hanus et al., 1981) and those which have shown no benefits of Hup (Gibson et al., 1981 ; Nelson, 1983 ; Rainbird et al., 1983a). Perhaps these contrary findings can be reconciled by the recent evidence that in some Hup^+ Rhizobium strains H_2 oxidation was coupled to ATP production, whereas in others it was not so coupled; also, some Hup^- strains evolved very little H_2 (Nelson and Child, 1981 ; Nelson, 1983).

The primary role of Hup may not be in saving energy. Hup may function to maintain a suitable redox environment for nitrogenase to operate in when the respiration rate is slow (Dixon, 1978) or when the leghemoglobin content of nodules is low (Dadarwal et al., 1982). Nitrogenase is an O_2-sensitive protein, and is normally protected through the function of leghemoglobin as an O_2-carrier supplying O_2 for respiration at a concentration inocuous to nitrogenase.

Gibson (see Dilworth, 1980) has calculated that the amount of energy lost from the legume in HE represented 0.43% of total photosynthate in G.max and 1.5% in T.subterraneum. It was said that such minor wastage would have a very small effect on legume growth. Dixon (1978) calculated that if all the H_2 produced by a legume with a RE of 0.5 could be oxidised via Hup, then 25% of the ATP used in nitrogenase activity could be recouped. Any benefit to N_2 fixation would depend on how the ATP was used. Hup has been found to increase the ATP pool in

bacteroids and to stimulate AR (Emerich et al., 1979 ; Dadarwal et al., 1982).

The K_m of hydrogenase for H_2 has been reported to be between 0.05 µM and 5.3 µM (Emerich et al., 1980 ; Nelson and Salminen, 1982), so the enzyme is well suited to functioning as an H_2 scavenger. Hup was said to have been induced by its substrate, H_2. Low O_2 and low carbohydrate levels were also required for its expression (Maier et al., 1978 ; Maier et al., 1979).

Much attention has been focussed on Hup, but less has centred on HE. HE has been regarded only as a wasteful process, and its response to temperature has not been examined.

2.7.0. Diurnal variation in N_2 fixation.

For the purpose of extrapolating N_2 fixation in time, it is customary to measure nitrogenase activity at intervals over 24h, and this is facilitated by the AR technique. Almost all studies to date have measured AR only, and have therefore combined the electron flux to N_2 reduction with that to H^+ reduction.

2.7.1. Important elements of technique in diurnal assays.

Several studies have used detached segments of nodulated root, so that the major source of fixed carbon for the nodules has been absent. This approach has sometimes been found to erroneously suggest diurnal variation to exist (Bergersen, 1970 ; Magee and Burris, 1972 ; Murphy, 1981).

Matching the assay temperature with the growth temperature has been recognised as important (Masterson and Murphy, 1976 ; Cralle and

Heichel, 1982). When AR assays were performed at the prevailing growth temperature on T.repens, the AR rate was lower at night than during the day, but when all AR assays were done at a standard temperature then the nighttime rate was as high as, or higher than, that of the daytime (Masterson and Murphy, 1976). It was implied that the lower night temperature resulted in the low nighttime AR rate, and that carbon supply did not limit nitrogenase activity. However, the validity of extrapolating from short assays at other-than the in vivo nighttime temperature to an entire night can be questioned. Accelerated respiration could exhaust carbon supplies if the night temperature was increased over a long period.

The regularity of assay is a salient aspect of technique. Gerson et al. (1978) showed that large fluctuations in the AR rate of Lupinus angustifolius under controlled conditions occurred within 3hours. Thus, if only two or three assays are done in 24h diurnal variation can be misrepresented. At the other extreme, if the same plants are repeatedly assayed at short intervals, results can be confounded by the effect of the AR assay in depriving plants of fixed nitrogen. The consequence of this for AR is unclear. Eckart and Raguse (1980) found that repeatedly-assayed T.subterraneum plants showed a gradually increasing AR rate. In G.max, on the other hand, there was a progressive decrease in the AR rate, and also a decline in CO_2 fixation in the day which became steeper with each day of the treatment, when plants were subjected to 20 minutes C_2H_2 per hour (Mederski and Streeter, 1977). Haystead et al. (1979) showed that nodulated root respiration was depressed by an AR assay. Apte et al. (1980) suggested that prolonged exposure to C_2H_2 induced a conformational change in nitrogenase which resulted in greater electron flow.

2.7.2. Environmental factors which influence nitrogenase diurnally.

There is an absolute dependence of nodule metabolism on fixed carbon from the host legume (see Minchin et al., 1981), but the immediacy of this may vary between species. For example, species of Lupinus which attained high AR rates at night were said to do so because they had ample carbohydrate reserves in their large nodules (Trinick et al., 1976). However, Lawrie and Wheeler (1973) found that much of the carbohydrate stored in nodules of P.sativum was unavailable to support N_2 fixation and that the process depended on a supply of newly-fixed carbon. Hence, factors which affect the size of the available carbohydrate pool in plants, such as PPFD, day length, and temperature, can be expected to have influence over the diurnal course of N_2 fixation.

While P_N for communities of T.subterraneum increased curvilinearly with increase in PPFD (Silsbury, 1981), dark respiration increased proportionally with P_N and with rise in temperature (McCree and Silsbury, 1978 ; Azcon-Bieto and Osmond, 1983). Taking extremes, under long days, high PPFD, and low temperature the carbohydrate pool available to support growth is high. In these circumstances metabolic processes are not expected to be carbon-limited at night. Contrarily, under short days, low PPFD, and high temperature, metabolic processes at night might be carbon-limited.

Carbon export from leaves has been shown to be a linear function of P_N (Servaites and Geiger, 1974 ; Ho, 1976), and carbon was found to arrive in nodules of T.subterraneum within one hour of its fixation (Small and Leonard, 1969). At night, anabolic activities must be supported by the carbon released from starch breakdown in leaves. In G.max under controlled conditions, starch accumulation in leaves slowed

and the soluble carbohydrate level increased towards the end of a 16h day while P_N fell (Uppmeyer and Koller, 1973). Starch accumulation also slowed before the end of a 14h day in Beta vulgaris leaves, and carbon export from leaves at night was at only 40% of the daytime rate (Fondy and Geiger, 1982). Chatterton and Silvius (1980) showed that when plants were transferred from long days to short days, the rate of starch accumulation in leaves, during the day, increased. It seems that there can be endogenous control of the amount of carbohydrate stored during the day for use at night, but that a diurnal change in the availability of carbohydrate to roots and nodules can exist.

Differences between species in their reliance on recently fixed carbon are highlighted by the results of experiments in which the AR rate of nodules has been measured when the CER of shoots has been increased by elevated CO_2 concentrations. In G.max, AR was not affected (Williams et al., 1982), but in P.sativum the AR rate increased rapidly (Phillips et al., 1976).

Temperature, radiation, humidity, and the ambient CO_2 concentration can all affect stomatal aperture, which controls the transpiration rate. As fixed nitrogen is transported from nodules in the xylem, diurnal variation in transpiration may be of some importance with respect to N_2 fixation. In P.sativum maintained in a 12h day of 325 µmol quanta $m^{-2}s^{-1}$ at constant 18°C and humidity, the soluble nitrogen concentration in the nodules rose at night and fell during the day because the transpiration rate at night was only 43% of that of the daytime (Minchin and Pate, 1974). However, transfer of plants between different humidities affected the transpiration rate and nitrogen export rate, but not the AR rate. In Lupinus albus, Lambers et al. (1980) found that if the transpiration rate was increased, by low ambient CO_2 and humidity, the AR rate increased too.

2.7.3. Diurnal studies of N_2 fixation in the field, the glasshouse, and the controlled environment.

An oft-reported observation has been of high rates of nitrogenase activity in the day and low rates at night, for example, in L.angustifolius in a controlled environment chamber (CEC), and in T.repens assayed at prevailing field temperature (Gerson et al., 1978 ; Carran et al., 1982). Hardy et al. (1968) attributed this pattern of variation to the diurnal light and dark change affecting assimilate supply to nodules in G.max, but a diurnal temperature change probably influenced their results too. Mague and Burris (1972) showed that this pattern of change in AR for G.max in the field was positively correlated both with PPFD and air temperature. Thus, the peak in AR at 12.00 to 16.00 hours and minimum at 04.00 hours reported by Vaughn and Jones (1976) for glasshouse-grown T.subterraneum was probably affected by a temperature fluctuation of up to 22°C. When T.subterraneum was grown in a CEC with a 12h:12h, light:dark, cycle and 750 µmol quanta $m^{-2}s^{-1}$, there was no diurnal variation in AR unless there was also a diurnal temperature change. Precisely the same responses were noted by Schweitzer and Harper (1980) for G.max in a CEC. It appears that the nodules of G.max and T.subterraneum were not carbon-limited at night, but that fixed carbon stored during the day supported nitrogenase activity at night at a rate which was modified by temperature.

Based on 4 assays done over 24 hours, Haystead et al. (1979) concluded there to have been no diurnal variation in AR for T.repens at constant 15°C and with 12h of 370 µmol quanta $m^{-2}s^{-1}$. However, 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. There was no diurnal variation in AR for plants at 740 µmol quanta $m^{-2}s^{-1}$ at 16°C:12°C. On

the basis of numbers of starch grains in stolons, the higher temperature regimen and lower PPFD were suggested to have resulted in nitrogenase being carbon-limited at night (Haystead and Sprent, 1981). Murphy (1981) reasoned similarly to explain why diurnal variation in AR occurred for T.repens grown in a 6h day, but not in a 14h day, at constant 20°C and 360 μmol quanta $m^{-2}s^{-1}$.

Ruegg and Alston (1978) grew Medicago truncatula in a glasshouse at constant temperature, and found the AR rate to fluctuate in parallel with changes in PPFD. However, in a CEC, at constant temperature and with constant PPFD, the AR rate declined well before the end of the day. There may have been an association between the fall in AR rate and a decline in CER, which was seen towards the end of the day for G.max at constant PPFD (Uppmeyer and Koller, 1973). CER and AR rate could have been linked by a temporal change in carbon partitioning (Fondy and Geiger, 1982).

There are few data for diurnal assays of HE. Lambers et al. (1980) found HE to be the same day and night in L.albus at constant 19°C, but AR to be lower at night, although they only assayed once during the day and once at night. When V.unguiculata was grown with a 12h day of 800 - 1000 μmol quanta $m^{-2}s^{-1}$ and day:night temperatures of 30°C:20°C, HE in air was found to fall at night and increase during the day, following temperature (Rainbird et al., 1983b). Despite this, there was no statistically significant variation in N_2 fixation because the total electron flux, measured by HE in Ar(80%):O_2(20%), varied similarly to HE in air.

Although Edie and Phillips (1983) did not present diurnal HE and AR data for normal days and nights, they indicated there to have been no diurnal change in RE for P.sativum with a 16h day of 550 μmol quanta $m^{-2}s^{-1}$ and day:night temperatures of 19°C:14°C. They did not refer to

T.subterraneum in the same context, but both species showed increased RE in a 20h dark period, although AR-HE decreased in the dark by over 50% in T.subterraneum. A fall in the AR rate after dark periods in excess of 12h has also been noted by others in G.max, T.repens, and T.subterraneum (Hardy et al., 1968 ; Silsbury, 1979 ; Murphy, 1981 ; Williams et al., 1982). The data of Edie and Phillips (1983) showed that the effect of the fall, presumably due to carbon limitation, on N_2 fixation could be partially offset by an increase in RE.

One of the more remarkable instances of diurnal variation is that documented by Rawsthorne et al. (1981) for Cicer arietinum. The AR and root respiration rates were highest midway through the 12h night although the night temperature was 7°C lower than that of the day. This unusual result was attributed to dark fixation of CO_2 by phosphoenolpyruvate carboxylase in the leaves and subsequent translocation of the carbon fixed to the nodules. Wheeler and Lawrie (1976) also found AR to be maximal at night in glasshouse-grown P.sativum. They suggested that this maximum was caused by the delay in transport of fixed carbon to nodules, and that the minimum in AR during the day was due to supra-optimal temperature.

The results of Hopmans et al. (1982) are unusual in that they show no diurnal variation in AR for T.subterraneum grown with a 12h day of very low PPFD, 140 µmol quanta $m^{-2}s^{-1}$ (less than 7% of full sun PPFD), and day:night temperatures of 24°C:20°C. According to Haystead and Sprent (1981), nodules are carbon-limited at night under these conditions. As all AR assays were conducted at 20°C, the daytime AR rate may have been underestimated. Fishbeck et al. (1973) also assayed at a constant temperature, 25°C, although their G.max plants were grown at 24°C:18°C, day:night. The lack of diurnal variation that they noted might not, therefore, have reflected the in vivo activity. No diurnal

variation in AR was identified for field-grown or glasshouse-grown species of Lupinus by Trinick et al. (1976), but the profile of AR which they presented for Lupinus consentinii showed some large fluctuations in activity. AR reached a minimum between 12.00 and 16.00 hours, and was at a steady high value at night.

Data from the field are often difficult to interpret. The vagaries of PPFD, temperature, and rainfall make repetition of experiments difficult, and errors are often large. Notwithstanding these drawbacks, Ayanaba and Lawson (1977) obtained a similar diurnal pattern in AR for V.unguiculata to that found by Wheeler and Lawrie (1976) for P.sativum. The AR rate was maximal early in the night and subsequently fell during darkness. A second peak in AR occurred early in the day, and a minimum in the early afternoon. They suggested that the afternoon minimum was correlated with the vapour pressure deficit.

Diurnal variation in N_2 fixation is a topic which, like Hup, is marked by a lack of consensus. It has important consequences for interpretation of instantaneous assays, and is not confined to the field and glasshouse. For example, recent data for V.faba were reported to show "enormous differences in diurnal patterns between plants grown at $10^{o}C$ and $18^{o}C$" under controlled conditions (Fyson and Sprent, 1982). Unfortunately, further detail is unavailable.

2.8.0. Combined nitrogen and N_2 fixation.

The assimilation of combined nitrogen by legumes is of interest for two reasons. Firstly, a comparison of the growth of legumes dependent on N_2 fixation with that of legumes receiving combined nitrogen can reveal responses to treatments which are specific for those fixing N_2. Secondly, nitrogenase activity of species of Trifolium has been found to

be depressed in the field by combined nitrogen in the form of waste products from grazing animals (Ledgard et al., 1982). It would be an advantage if legumes could continue fixing N_2 in the presence of combined nitrogen because they would remove less nitrogen from the soil for their own growth.

2.8.1. Use of combined nitrogen in legume growth.

Single T.subterraneum plants receiving combined nitrogen were found to assimilate almost twice as much nitrogen as those fixing N_2 at $10^{o}C$, and were also larger. The growth disadvantages of the N_2-fixing plants were greatest at low temperature (Gibson, 1963 ; Gibson, 1976).

Plants take up combined nitrogen predominantly as NO_3^-, and this can be reduced in leaves using surplus photochemically-generated reductant (see Schrader and Thomas, 1981). NO_3^- reduction was found to be independent of temperature, but stimulated by increase in PPFD (Nicholas et al., 1976 ; Wild et al., 1981). NO_3^- was shown to stimulate electron transport on the donor side of photosystem II in thylakoids, a role which is independent of that for NO_3^- as a nitrogen source (Osman et al.,1982).

Ions taken up by legumes assimilating NO_3^- were found to be mainly anions, and hydroxyl ions were excreted, whereas, N_2-fixing legumes took up mainly cations and excreted protons (van Beusichem, 1981). As the maintenance of ion fluxes is a major component of maintenace respiration (Penning de Vries, 1975), legumes fixing N_2 could have different maintenance costs from those assimilating NO_3^-.

2.8.2. The effect of combined nitrogen on N_2 fixation.

The host cell cytoplasm and organelles of T.subterraneum nodules were found to collapse in the presence of 10.7 mM NH_4NO_3 (Dart and Mercer, 1965), and bacteroids were lysed after just 3 days of treatment. Small and Leonard (1969) showed that NO_3^- assimilation by T.subterraneum led to nodules being starved of fixed carbon, but this did not occur in G.max (Streeter, 1981). Some workers have found high concentrations of NO_3^- not to depress nitrogenase activity in G.max, L.albus, and L.angustifolius (Manhart and Wong, 1980 ; Lambers et al., 1980 ; Jones et al., 1981), but it has been shown that in G.max and L.angustifolius any effect of NO_3^- on nitrogenase activity was dependent on the Rhizobium strain (Manhart and Wong, 1980 ; Evans, 1982).

Reduction of NO_3^- to NO_2^- has been suggested to be the critical step leading to inhibition of nitrogenase activity. Nitrogenase in two symbioses with NO_3^- reductase deficient mutants of P.sativum and R.japonicum, respectively, was not inhibited by NO_3^- (Feenstra et al., 1982 ; Stephens and Neyra, 1983). NO_3^- reductase has been found in the nodule cytosol (Streeter, 1982) which can explain why some authors have reported NO_3^- inhibition of nitrogenase even with NO_3^- reductase-deficient mutants of Rhizobium (Manhart and Wong, 1980).

2.9.0. The programme of investigation.

A singular theme of 'temperature' connected the entire sequence of observations on growth and N_2 fixation in T.subterraneum communities, while secondary themes appeared as the project developed. Preparatory work concerned the appropriate application of HE and AR techniques to meet the requirements of the project. This was followed by definition of

the nascent period of N_2 fixation, in order that a reference point be established from which all further treatments would be imposed.

The primary investigation of CERs and nitrogenase activities at a range of growth temperatures, and how they were affected by ontogeny, was elaborated early on by estimations of the extent of diurnal variation in N_2 fixation and then by attempts to rationalise such variation as was found. Experiments were designed to assess the effects of night length and of transpiration rate on N_2 fixation. Similarly, very low or non-existent HE from swards of high biomass, or grown at high temperature, prompted an examination of Hup. Rates of H_2 uptake were used to calculate the K_m for H_2 of an uptake hydrogenase, and some plants were grown in an atmosphere containing H_2 to see if Hup was induced by its substrate.

When residual nitrogenase activity was found in nodulated control swards receiving combined nitrogen, this made it worthwhile to examine the effect of combined nitrogen on N_2 fixation briefly. Hydroponically-grown communities were transferred to 7.5 mM NO_3^- nutrient solution and the response of N_2 fixation was measured.

Growth and N_2 fixation were also measured by increases in biomass and organic nitrogen with time, respectively, which served as yardsticks for CER, HE and AR data.

An exposition enabled the competence of the HE and AR assays in measuring N_2 fixation to be judged. Also, growth was linked to N_2 fixation through calculation of the amount of nitrogen fixed per unit of CO_2 fixed, and the daily costs of N_2 fixation were reckoned in terms of photosynthate. A crude estimate of growth efficiency, expressed in terms of CO_2 respired per unit of dry matter retained, was compared between treatments.

CHAPTER 3

3. General methods.

3.1.0. Plant culture and harvesting.

Trifolium subterraneum L. cv. 'Woogenellup' was sown at a density of 98 seeds per 210 cm^2 pot of oil-dry. Oil-dry is a fritted clay which is chemically inert and which is easily removed from roots; it drains rapidly to retain 0.31, by volume, of plant available water and an air-filled porosity of 0.28 (van Bavel et al., 1978). The oil-dry was soaked with deionised water, and seeds germinated in a naturally-lit glasshouse at 20°C. Seedlings were thinned to 49 equally-spaced plants per pot (2,300 plants m^{-2}) on day 9, when the spade leaves had expanded. For some experiments, plants were subsequently raised hydroponically in containers of aerated -N nutrient solution (see Table 1 for composition of nutrient solutions) and were inoculated by addition of Rhizobium trifolii WU95 (Agricultural Laboratories Ltd., Sefton, N.S.W.) to the solution on day 10. For plants continuing growth in pots, inoculant was poured into the oil-dry. WU95 is an effective and competitive strain of R.trifolii for 'Woogenellup' nodulation (Gibson et al., 1976). Oil-dry swards received 0.6l of -N at the growth temperature at 9 am each day and a flush of deionised water each week. -N was changed daily for hydroponic swards of 12 plants in 0.53 l jars (the same plant density as for oil-dry swards) or every 3 days for hydroponic populations of a maximum of 50 plants in 8l plastic containers. Control swards received nutrient solution containing 7.5 mM NO_3^-(+N) from 10 days after inoculation (10DI).

TABLE 1 Composition of nutrient solutions

Compound	Molarity(mM) -N	Molarity(mM) +N
KNO_3	-	2.50
$Ca(NO_3)_2.4H_2O$	-	2.50
$CaSO_4.2H_2O$	8.72	-
$MgSO_4.7H_2O$	1.00	1.00
KH_2PO_4	0.12	0.25
K_2SO_4	1.25	-
EDTA	0.06	0.06
$FeSO_4.7H_2O$	0.07	0.07
H_3BO_3	0.19	0.19
$MnCl_2.4H_2O$	0.04	0.04
$ZnSO_4.7H_2O$	3.06×10^{-3}	3.06×10^{-3}
$CuSO_4.5H_2O$	1.28×10^{-3}	1.28×10^{-3}
$Na_2MoO_4.2H_2O$	1.98×10^{-3}	1.98×10^{-3}

Plants were maintained in the 20°C glasshouse until 10DI, when N_2 fixation had begun, and were then transferred to a CEC at a constant temperature of 10°C, 15°C, 20°C, 25°C, or 30°C and with a 12h photoperiod (9am to 9 pm) of either 500 µmol quanta $m^{-2}s^{-1}$ (low PPFD) or 1000 µmol quanta $m^{-2}s^{-1}$ (high PPFD) measured at the centre of each leaf canopy (LiCOR meter LI-170, Lambda Instrument Corporation, Lincoln, Nebraska). Silsbury (1981) found that T.subterraneum communities were PPFD-saturated at about 2000 µmol quanta $m^{-2}s^{-1}$. Based on his P_N-PPFD curve, the low and high PPFD approximate to 47% and 75% of PPFD-saturation. 18.5 cm high wire-mesh sleeves were fitted to the pots to confine developing leaf canopies to a constant 210 cm^2. The PPFD was adjusted as the canopies advanced vertically inside the sleeves.

Stems of hydroponically-grown plants in 0.53l jars were sealed through jar lids with Blutac (Bostic Division, Thomastown, Victoria) and formed canopies within the confines of 53 cm^2 wire-mesh sleeves soldered to the lids. Plant growth in the 8l plastic containers was not restricted by sleeves. The aeration rate for plants in 0.53 l jars was 0.1l air min^{-1}, and in 8l containers was 1l air min^{-1}. Exposed roots were regularly sprayed with water to avoid salt accumulation.

The CEC had a maximum capacity of about 50 oil-dry swards, but the PPFD distribution from the 4 400W high pressure sodium Lucalox lamps (General Electric Co., Cleveland, Ohio) dictated that no more than 36 swards be maintained at the same time, so swards were raised sequentially in batches. Dew-point control was not possible in the CEC, but maintenance of free water surfaces and regular watering ensured that plants were not water-stressed.

Exceptions were made to this routine for an experiment in which nitrogenase assays were made over 4 day:night cycles and an experiment in which night length was varied, for which a growth room (with similar lamps) was used because large numbers of communities were required. Also, one experiment examined CER during nodulation, and swards were maintained at 20°C and 1000 µmol quanta $m^{-2}s^{-1}$ from emergence. For some experiments concerning assay procedure, and treatment of plants with H_2, plants were grown in naturally-lit glasshouses.

Plants suffering from insect attack or disease were discarded. At each temperature and PPFD, oil-dry swards were destructively assayed at intervals to give 4 or 5 ranges of biomass over which nitrogenase activity was compared. At high temperature and the lower PPFD plants grew slowly and demand on CEC space restricted the range of biomass. Swards were selected by random numbering, between 08.30 and 09.30 hours, and were removed from pots by washing the oil-dry away in water

at the growth temperature, which took about one minute. Water was blotted from the roots and nodules, and assays were started within 10 minutes of plants being taken from the CEC.

3.2.0 Assay techniques and post-assay procedures.

Reduction of C_2H_2 to C_2H_4 (Dilworth, 1966) is a widely used assay for nitrogenase activity (Hardy et al., 1973) and its application to swards similar to those used in this project has been described (Silsbury, 1981). Nevertheless, it was deemed desirable to examine certain aspects of the AR and HE assays. Results of experiments concerning technique are given in Section 3.4 and they justified adoption of the following procedure.

Plants were incubated in 1.06l 'Agee' glass jars with screw-down metal lids (New Zealand Glass Manufacturer's Company, Auckland) each penetrated by a No. 25 subaseal. The jars were held in darkness at the growth temperature using water baths.

Jar lids which supported hydroponically-grown swards were removed from culture jars and, having blotted the nodulated roots, were attached to 0.40l jars in water baths for assay. Each jar was submerged in water and checked for leaks by slight pressurisation with air, injected through a No. 25 subseal which fitted the aperture normally accommodating an aeration tube. Single hydroponic plants were incubated in 45 ml vials, for which No. 55 subseals formed tight-fitting lids.

The rate of H_2 evolution (HE) in air was estimated from the concentration of H_2 present in gas samples taken 10 minutes and 40 minutes after assay vessels were closed. 1 ml syringes fitted with 0.5 mm x 16 mm needles (Terumo Pty., Ltd., Melbourne) were used and were stored for a few minutes with the needles inserted into rubber bungs.

200 µl samples were injected into a gas chromatograph which operated at room temperature (20°C) and was fitted with a semiconductor sensor and light-emitting diode display (J.A.S. Instruments, Melbourne). The carrier gas was air, and with 50 kPa column head pressure, the retention time for H_2 was 28 s. One minute before gas sampling, both in HE and C_2H_2 reduction (AR) assays, atmospheres were mixed using a syringe since plant material offered a potential impediment to gaseous diffusion. Vessels were opened for 5 minutes at the end of HE assays to renew the air prior to commencing AR assays on the same plants.

The vessels were sealed and C_2H_2 was added to each to give p0.1 C_2H_2, and the rates of AR were calculated from the C_2H_4 concentrations in 500 µl gas samples taken at 10 minutes and 40 minutes in 1ml 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, California) and a column of 80-100 mesh Porapak R (Waters Associates Inc., Milford, Mass.). Column, detector, and injector temperatures were 50°C, 140°C, and 140°C, respectively. With the carrier gas (N_2) flowing at 65 ml min^{-1}, C_2H_4 eluted in 30 s. C_2H_4 peaks were displayed on a flat-bed chart recorder ('Omniscribe', Houston Instruments, Austin, Texas).

Using gas chromatography, H_2 uptake (Hup) was measured by the disappearance of exogenous H_2 in the presence of p 0.1 C_2H_2 as described by others (Nelson and Salminen, 1982 ; Keyser et al., 1982 ; Bedmar et al., 1983) and detailed later.

Standards of H_2 and C_2H_4 were made up in air at 20°C using gas-tight glass syringes (S.G.E. Scientific Pty. Ltd., Ringwood, Victoria). Calculations of H_2 and C_2H_4 concentrations included corrections for assay temperature. The volume of gas in an assay vessel containing plants was found by displacement with water. The difference in peak

heights (X) between 3 sample injections taken at 10 minutes and 3 taken at 40 minutes was converted to μmol H_2 or C_2H_4 $m^{-2}h^{-1}$ by reference to the peak heights (Y) given by injections from standards of known quantities of H_2 or C_2H_4 in air (Z) at 20°C. Standards were injected before and after samples were injected.

$$\mu\text{mol } C_2H_4 \text{ (or } H_2)\ m^{-2}h^{-1} = \frac{Z(\mu l)}{\text{Volume of standard jar (ml)}} \cdot \frac{1}{\text{Volume of 1 mole of gas at assay temperature}} \cdot \frac{X}{Y} \cdot \text{assay jar volume (ml)} \cdot 2 \cdot 47.62$$

47.62 was the factor converting results for communities to those per m^{-2}. The coefficients of variation for sample injections of H_2 and C_2H_4 were consistently less than 3%.

Relative efficiency (RE), the proportion of electrons apparently allocated to N_2 reduction, was given by RE = 1 - HE/AR (Schubert and Evans, 1976). AR represented the total electron flux through nitrogenase, while HE represented the amount of that flux used in reducing H^+ (see Hardy, 1979). The quantity AR - HE was, therefore, equivalent to the electron flux allocated to N_2 reduction in normal air, and was used as a measure of N_2 fixation as reported elsewhere (Bethlenfalvay et al., 1978a). The ratios of AR:N_2 and AR - HE:N_2 were calculated to examine the relationship of the indirect assays to the quantity of N_2 actually fixed. AR - HE was not expressed in moles since, conceptually, it represents a quantity similar to RE, ie. electrons allocated to N_2 reduction and this quantity was not directly measured. When H_2 uptake occurs, RE and AR - HE assume different meanings and these are discussed later.

After assays, plants were separated into roots, live shoots, and sensecent shoots. These fractions were dried in a forced-draught oven at 86°C for 24 hours, weighed, and stored. Material analysed for Kjeldahl

organic nitrogen was ground to less than 1 mm particle size in a mill and 250 mg samples were digested with an auto kjeltab (Thompson and Capper Ltd., Runcorn, Cheshire) in 4 ml conc. H_2SO_4 for 20 minutes at 400°C, then a further 15 minutes 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).

Leaf areas of young nodulated plants were measured using a planimeter (Paton Industries Pty., Ltd., Adelaide). Nodules were excised in water using a scalpel, under the low power of a Zeiss dissecting microscope.

3.3.0. Measurement of growth rates and transpiration rates.

The CERs of N_2 fixing and NO_3^--supplied swards, and also of nodulated roots in isolation, were measured by open-circuit infra-red gas analysis (Figure 2). A perspex assimilation chamber housed a sward at constant temperature. Water was pumped through a heat exchanger in the chamber from a water bath, and the temperature of the bath was lowered when a 400W 'Metalarc' lamp (G.T.E. Sylvania Canada Ltd., Drummondville, Quebec) came on above the chamber at 09.00 hours and increased the heat load. A fan circulated air in the chamber, and leaf canopy and oil-dry temperatures (which varied by about 1°C) were monitored by copper-constantan thermocouples. Air was supplied to the chamber via a rotary-vane pump, a humidifier, and a mass flow controller (Model FC202, Tylan Corporation, Torrance, California). CER over 24 h was calculated from the difference between net CO_2 influx during the day, D, and net CO_2 efflux at night, N. The difference in CO_2 concentrations of samples taken from air before and after it entered the chamber was measured by an infra-red gas analyser (Series 225, The

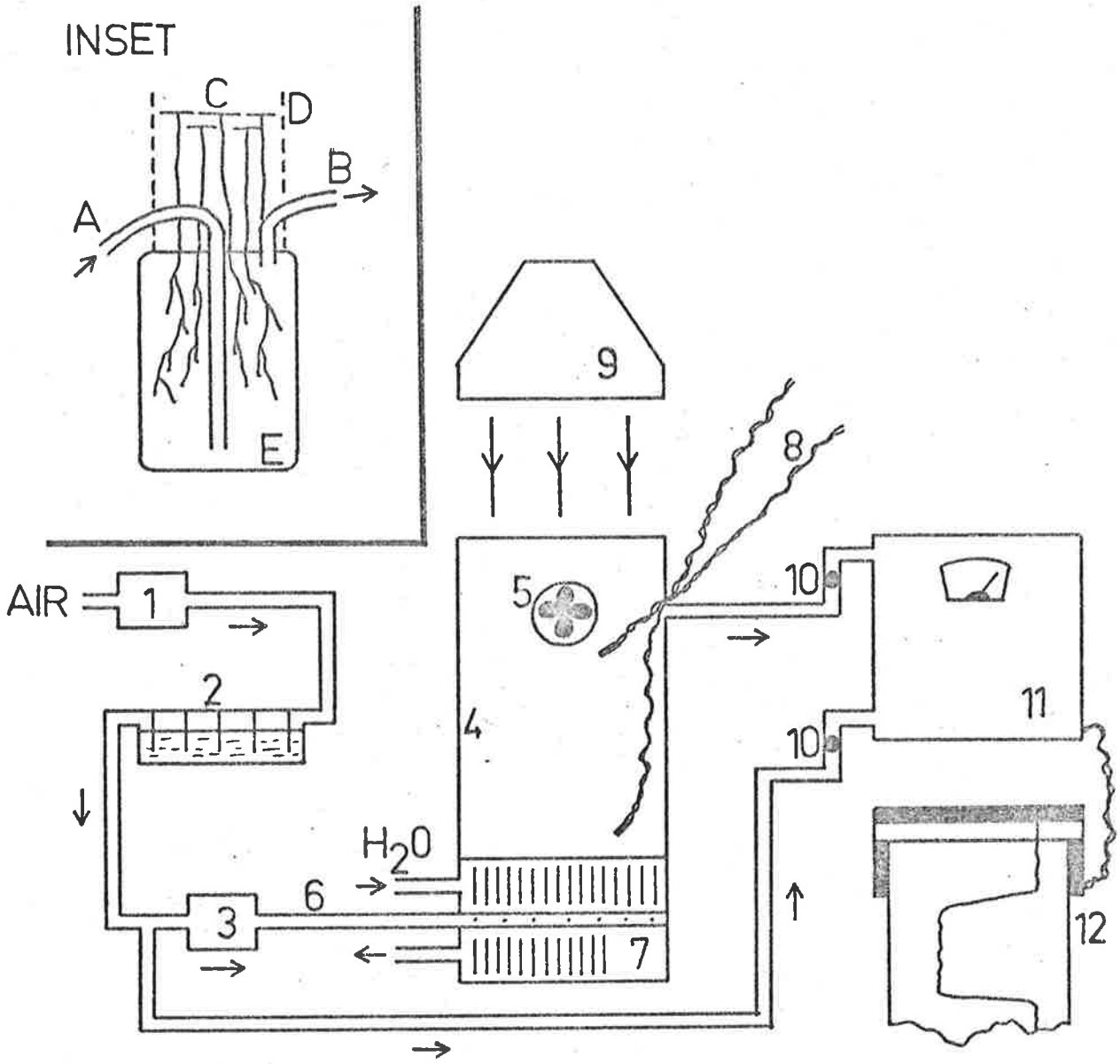


Figure 2. Front elevation of CO_2 exchange circuit. 1, air pump; 2, humidifier; 3, mass flow controller; 4, assimilation chamber; 5, fan; 6, air inlet; 7, heat-exchanger; 8, thermocouples; 9, lamp; 10, flow meters; 11, infra-red gas analyser; 12, chart recorder.

INSET. Apparatus for in vivo measurements of respiration of nodulated roots and AR. A, gas entry; B, gas exit; C, leaf canopy; D, wire mesh sleeve; E, oil-dry.

Analytical Development Co. Ltd., Hoddesdon, England) and displayed on a flat-bed chart recorder (Model 385, Linear Instruments Corporation, Irvine, California). Air was nearly saturated with water at 20°C when it entered the chamber, but further control of dew-point temperature was not possible. The analyser was calibrated each day using air of precisely-determined CO_2 concentration, and the 'chamber zero' was found when the sward was removed for irrigation with nutrient solution. The CER of a control pot of saturated oil-dry was measured over several days and was found to be negligible even when heavily inoculated with R.trifolii.

Hourly differences (A) in CO_2 concentration between 'reference' air and 'analysis' air from the assimilation chamber, were summed over the 12 h day and 12 h night and converted to D and N values in mg CO_2 m^{-2} 12 h^{-1} as follows:

$$\text{D or N} = \sum^{12h} \frac{A}{10^6} \cdot \text{air flow rate} \cdot 60 \cdot 4.4.10^3 \cdot \frac{1}{\text{Volume of 1 mole of gas at measurement temperature}} \cdot 47.62$$

The air flow rate was 15l min^{-1} for swards at 1000 µmol quanta $m^{-2}s^{-1}$ and 7l min^{-1} for those at 500 µmol quanta $m^{-2}s^{-1}$.

Growth rates were also calculated from the regressions of biomass on time, and agreed closely with those calculated from D and N data using an established conversion factor of 1 g CO_2 ≡ 0.74 g dry matter (Silsbury, 1979).

Transpiration rates of communities were estimated gravimetrically using a top-pan balance. For each environment, the mean day and night transpiration rates were calculated for 18 swards, and were corrected for evaporation from oil-dry surfaces using shaded pots containing oil-

dry only. When it was desired to generate different transpiration rates at a common level of PPFD, the irradiance was increased by carbon-filament lamps and was measured with a Kipp solarimeter (300 - 2000 nm) and millivolt meter. This is detailed later.

For convenience, fine detail pertinent to specific experiments is given immediately before them.

3.4.0. Procedural experiments.

3.4.1. Introduction.

The use of oil-dry as the growth medium ensured little mechanical disturbance to plants during preparation for nitrogenase assays *in vitro*, yet some physiological shock to the plants must occur during preparation and assay.

The nodulated roots were saturated with water when oil-dry was removed, but 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. Incubation in the presence of a saturating pC_2H_2, a non-physiological substrate for nitrogenase, effectively prevents N_2 fixation and curtails nitrogen assimilation. AR also employs the full electron flux through nitrogenase and thereby stops HE (see Knowles, 1981). It has been suggested that the binding of C_2H_2, and of N_2, to nitrogenase modifies the electron flux, possibly through a conformational alteration (Thorneley and Eady, 1977 ; Apte *et al.*, 1978). It would be surprising if disruption of the steady state, from the molecular level to that of the whole plant, did not lead to some departure from the nitrogenase activity of undisturbed plants. The wide range in reported AR:N_2 ratios (see Hardy *et al.*, 1973) may possibly be accounted for by differences in technique, especially with

respect to the handling of plants (Masterson and Murphy, 1976 ; Wheeler et al., 1978 ; Murphy, pers. comm.).

3.4.2. pC_2H_2 and AR rate.

In normal air, part of the electron flux through nitrogenase is used in reducing H^+ to H_2. When the enzyme is saturated with C_2H_2, H^+ reduction is inhibited and the full electron flux is used in reducing C_2H_2 to C_2H_4. Hence, when the AR assay is used, it is important to establish the pC_2H_2 required to saturate nitrogenase.

Groups of 3 replicates of T.subterraneum communities from a 20°C glasshouse were incubated separately in one of 4 pC_2H_2, made up by replacing appropriate volumes of the atmosphere with C_2H_2. AR was measured over a 30 minute interval.

TABLE 2 Analysis of variance of AR rates at four pC_2H_2. Means are of 3 replicates, with SE.

pC_2H_2	AR^+ µmol C_2H_4 $m^{-2}h^{-1}$	F value from AOV	LSD P0.05
0.05	1192 (36) A	6.40*	115
0.01	1103 (30) AB		
0.15	1199 (8) A		
0.20	1014 (59) B		

* Significant at P0.05

\+ Means with different postscripts are significantly different at P0.05.

AR rates are represented in Table 2. Nitrogenase was saturated by p0.05 C_2H_2, in near agreement with Eckart and Raguse (1980) who found

that pO.03 C_2H_2 saturated nitrogenase in T.subterraneum and with Crush and Tough (1981) who showed pO.06 C_2H_2 to totally inhibit HE and saturate nitrogenase in T.repens. Nitrogenase activity was apparently inhibited at pO.20 C_2H_2.

It was decided to retain pO.10 C_2H_2 as standard, because it saturated nitrogenase of T.subterraneum 'Woogenellup' and it was in line with the pC_2H_2 most frequently used for AR in previous work (Trinick et al., 1976 ; Nelson and Child, 1981 ; Silsbury, 1981).

3.4.3. Linearity of AR with time.

Minchin et al. (1982) demonstrated the existence of marked curvilinearity in a plot of the amount of C_2H_2 reduced versus time for T.repens assayed in a closed vessel. Using continuous-flow AR in an open system, these authors found that the curvilinearity was due to an C_2H_2-induced decline in the instantaneous rate of AR. After about 15 years of world-wide use of AR, the finding of an apparently fundamental flaw in the technique is undeniably dramatic. A survey of legume species indicated that the decline was not common to all symbioses (Minchin et al., 1983). AR rates in sealed and continuous-flow systems were investigated for the T.subterraneum 'Woogenellup'-R.trifolii WU95, symbiosis.

AR assays in sealed vessels were performed at 25°C, as detailed earlier. A continuous-flow system was built to enable estimation of instantaneous AR rates, and is illustrated in Figure 3. Gas mixtures of pO.10 C_2H_2 in humidified air entered via tubes to the bases of the assay vessels, and the exit gas mixtures were sampled 18 times over 60 minutes for C_2H_4 concentration. Flow rates were 0.56 l gas min^{-1} for in vitro,

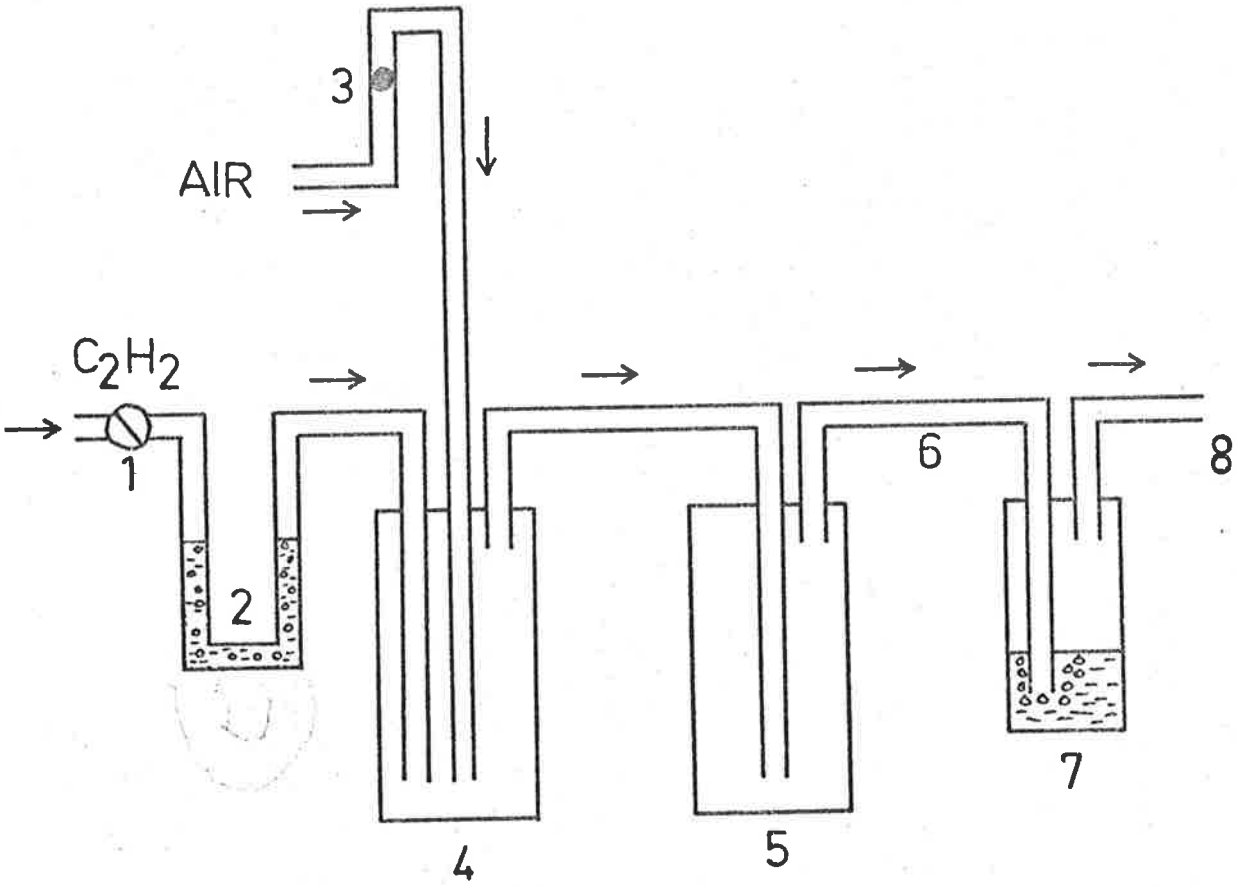


Figure 3. Continuous-flow AR apparatus. 1, needle valve; 2, bubble tube; 3, flow meter; 4, mixing jar; 5, assay jar; 6, sampling tube; 7, bubble jar; 8, waste exit.

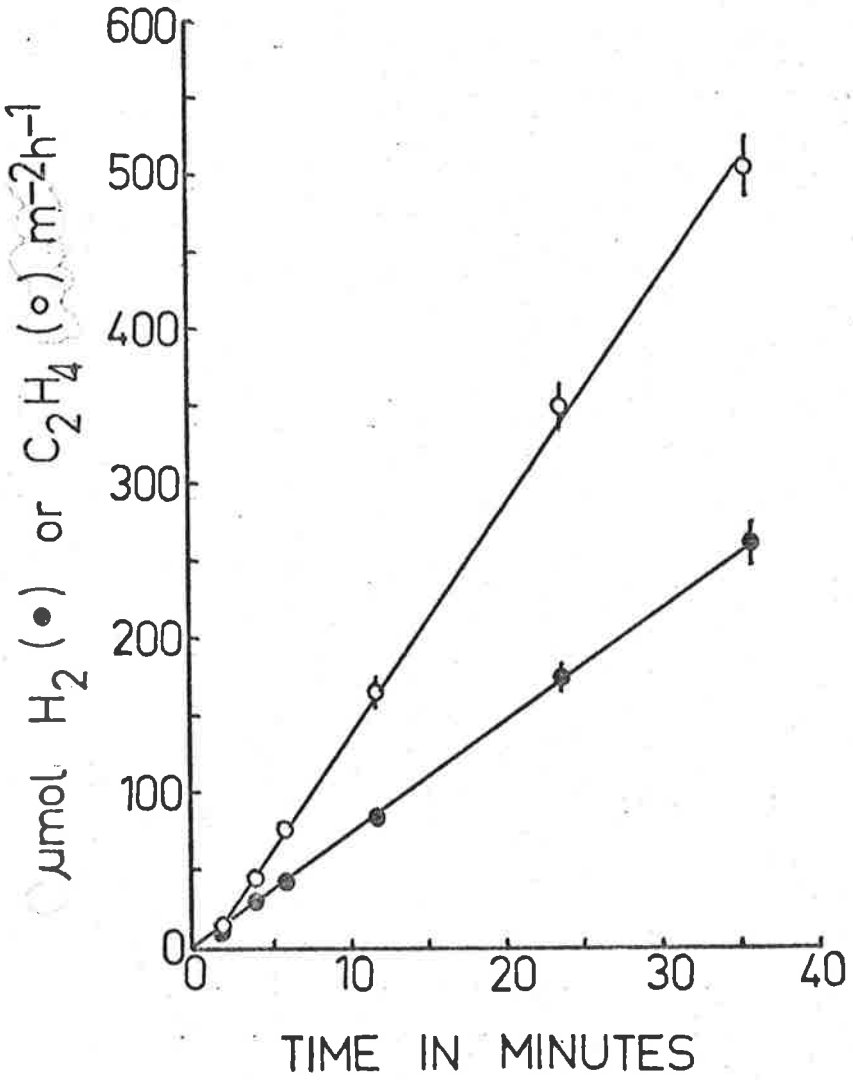


Figure 4. Time course of AR and HE in sealed vessels. 25C, high PPFD. Means, with SE, for 6 values.

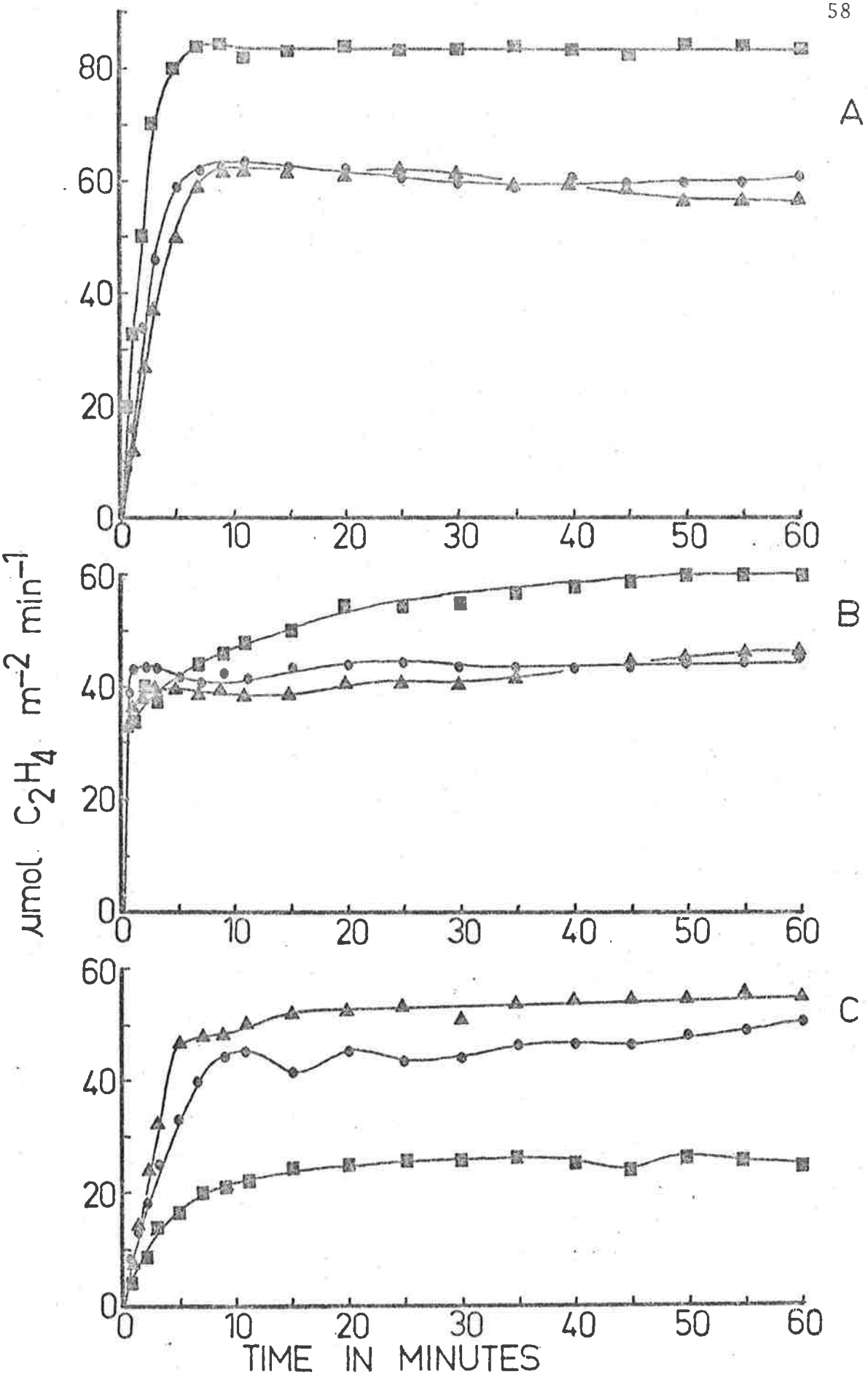


Figure 5. Instantaneous AR rates from continuous-flow assays. A, 15 C in vitro; B, 20 C in vivo; C, 25 C in vitro.

and 1.12 l gas min^{-1} for *in vivo*, assays. The instantaneous AR rate was given by:

$$\mu mol\ C_2H_4 m^{-2} min^{-1} = \frac{\text{vol } C_2H_4 \text{ in standard jar } (\mu l)}{\text{vol standard jar (ml)}} \cdot \frac{\text{sample peak}}{\text{standard peak}} \cdot \frac{1}{\text{vol 1 mole gas at assay temperature}} \cdot \text{flow rate } (ml\ min^{-1}) \cdot 47.62$$

Communities were assayed *in vivo* at 20°C during exposure to 1000 µmol quanta $m^{-2}s^{-1}$ (see Figure 1) and *in vitro* (with oil-dry removed), at 15°C and 25°C after exposure to 1000 mol quanta $m^{-2}s^{-1}$ and 500 mol quanta $m^{-2}s^{-1}$, respectively.

The AR data are shown in Figures 4 and 5. AR in closed vessels was linear with time, and there was no evidence of a decline in the instantaneous rate of AR whether measured *in vivo* or *in vitro*, at 15°C, 20°C, or 25°C, at high or low PPFD. In the first assay at 20°C *in vivo* the AR rate appeared to increase with time (Figure 5). This was probably an artifact due to the slow mixing of gases in oil-dry since, when the flow rate was increased to 1.12 min^{-1} for the other two assays at 20°C, equillibration was reached much sooner.

In the *T.subterraneum* L.'Woogenellup' - *R.trifolii* WU95 symbiosis, C_2H_2 appeared not to induce the decline in nitrogenase activity described by *Minchin et al.* (1983) for other legumes. Constancy of the instantaneous AR rate was not affected by temperature, PPFD, or the preparation of plants for assay during which the roots were washed. The AR assay was considered as a technique for making fair comparisons of nitrogenase activities between temperatures and PPFDs.

3.4.4. Gaseous losses during assays.

HE appeared to be linear with time over the assay period (see Figures 4 and 7), but since the H_2 molecule is the smallest there is, it therefore has the greatest ability to leak from assay vessels via imperfect seals. Bethlenfalvay and Phillips (1979) recognised the need to correct data for H_2 adsorption to surfaces. It was unreasonable to expect precise reproducibility of leakage and adsorption of H_2, yet H_2 losses from the assay atmosphere had to be taken into account if HE was not to be underestimated.

A batch of non-nodulated NO_3-supplied swards was raised at 20°C in a glasshouse. Harvests were made at intervals to provide 5 different levels of biomass. At each level of biomass, 2 replicate swards were incubated with each of 6 concentrations of H_2 in air, and the rate of loss of H_2 from the gas phase was measured. Assay jars were also checked for disappearance of C_2H_4.

It was expected that the amount of H_2 lost would be proportional to the amount of biomass present, but this was not found to be so. Instead, as shown in Figure 6, the amount of H_2 lost (y) was logarithmically related to the amount of H_2 present at the start of the incubation (x) according to the equation:

$$y = 14.861\ln x - 40.13$$

The leakage of C_2H_4 from assay jars was considered to be negligible.

A lack of correlation between the amount of H_2 lost and the amount of biomass present indicated that H_2 adsorption to surfaces was either not constant between harvests, or was, perhaps, confounded by variable rates of H_2 leakage. Adsorption is normally maximal when a unimolecular

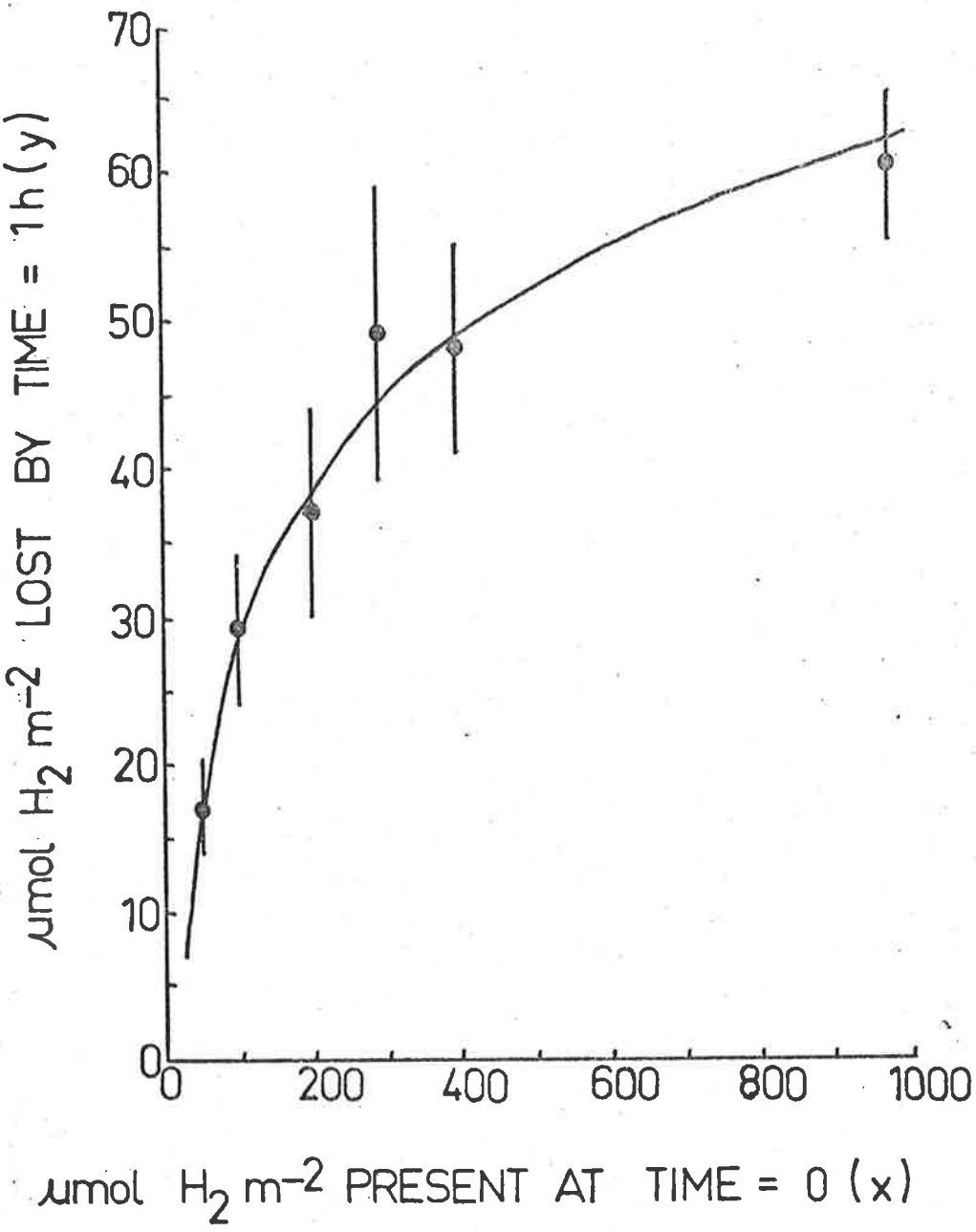


Figure 6. Loss of H_2 from the gas phase during incubation. Means, with SE, for 10 values. Curve defined by $y = 14.86 \ln x - 40.13$.

layer of adsorbent covers the adsorbate, but the relationship linking adsorbent concentration to the amount of it adsorbed is complicated by the heterogeneity of physical states of adsorbing surfaces (Rideal, 1928). In this regard, the degree of wetness of the plant material may have been important.

Above 25 µmol H_2 m^{-2} h^{-1}, the amounts of H_2 evolved from N_2-fixing plants were corrected according to Figure 6. This incurred a small error, since the amount H_2 used to calculate a loss was that accumulated in the gas phase during an assay, which was, therefore, less than the total amount of H_2 produced in the same time.

3.4.5. Assays for HE and AR on the same plants.

To reduce variation, and to make efficient use of CEC space, it was considered advantageous to do HE and AR assays sequentially on the same communities, rather than to do them separately using matched pairs of communities. It was necessary to know whether an HE assay had any effect on the subsequent AR rate.

A comparison was made, at 20°C, between the AR rates of 3 swards previously assayed for HE and 3 which had been held in darkness, in the CEC, during the HE assay and then assayed only for AR.

The AR rates are shown in Table 3. The AR rate of communities previously asayed for HE was no different from that of similar communities assayed only for AR.

It was considered permissible to do both HE and AR assays on the same communities. This maximised the number of communities which could be maintained in the CEC.

TABLE 3 Effect of doing HE and AR assays on the same plants. Means are for 3 replicates, with SE.

Treatment	AR μmol C_2H_4 $m^{-2}h^{-1}$
HE and AR assays	520 (28)
AR assay only	528 (16)

3.4.6. Effect of assay temperature on nitrogenase activity.

The temperature of water used in root washing, and the assay temperature, were found to influence nitrogenase activity in T.pratense and M.sativa (Masterson and Murphy, 1976 ; Cralle and Heichel, 1982). It was necessary to find out if nitrogenase activity of T.subterraneum was similarly affected.

Groups of 3 swards, from a 20°C glasshouse, were washed and incubated at each of 4 temperatures for AR assays.

An analysis of variance showed that the influence of a brief change in temperature on the AR rate was not significant at P0.05, but the results, presented in Table 4, indicate that the AR rate was underestimated at temperatures other than 20°C.

The results obtained here differed from those of Cralle and Heichel (1982) in that nitrogenase activity was not increased when temperature was increased above the growth temperature. However, the results were compatible with those of Masterson and Murphy (1976) in showing the optimum to be at about 20°C. The temperature of water used in root washing, and the incubation temperature during assay, may have influenced nitrogenase activity, and it was considered necessary to conduct these procedures at the growth temperature.

TABLE 4 Analysis of variance for nitrogenase activities at different assay temperatures. Means are for 3 replicates, with SE.

Temperature ^{o}C	AR µmol C_2H_4 $m^{-2}h^{-1}$	F value from AOV
10	309 (28)	2.72 NS
15	358 (47)	
20	460 (53)	
25	343 (18)	

NS Not significant at P0.05

3.4.7. Purity of C_2H_2 and nitrogenase activity.

When impure C_2H_2 was used in AR assays with T.repens, the AR rates were lower than when purified C_2H_2 was used (Tough and Crush, 1979). A similar test was performed on T.subterraneum.

The AR rates of two groups of 4 replicate communities were compared at 20^{o}C, using industrial grade C_2H_2 for one group and C_2H_2 purified by bubbling through 4M H_2SO_4 and then water for the other group.

The data in Table 5 show there to have been no obvious benefit in using purified C_2H_2.

Use of industrial grade C_2H_2 was considered as an acceptable practice for all AR assays. A typical analysis of industrial C_2H_2 was provided by C.I.G. Ltd. of Torrensville, S.A. (the suppliers of all gases used in this project): C_2H_2, 99.5% (dry basis); H_2S, < 0.02% (vol); PH_3, < 0.01% (vol); NH_3, < 0.02% (vol); CO_2, < 0.01% (vol); moisture, < 0.25% (wt); air, < 1%.

TABLE 5 The effect of C_2H_2 purity on nitrogenase activity. Means are for 4 replicates, with SE.

Type of C_2H_2	AR μmol C_2H_4 $m^{-2}h^{-1}$
Industrial	484 (37)
Purified	479 (54)

3.4.8. Time of day for nitrogenase assays.

Assays were always started between 08.30 hours and 09.30 hours, largely for reasons of convenience. The relationship between nitrogenase activity at this time and that averaged over 24 hours only became apparent well into the experimental programme, when diurnal assays had been completed.

Three swards were selected, at random, every 4 hours starting at 11.00 hours and finishing at 07.00 hours the next day, and were assayed for HE and AR. The average of the 07.00 hours and 11.00 hours AR rates was assumed to approximate the rate at 09.00 hours.

The assumed AR and AR-HE rates at 09.00 hours are compared with those averaged over 24 hours in Table 6. Exceptionally, the morning AR rate for 20°C and 500 μmol quanta $m^{-2}s^{-1}$ underestimated the average rate over 24 hours by 20%, while for other environments the average AR rate over 24 hours was underestimated by between 2 and 8.5%. Much of the diurnal variation in N_2 fixation was due to HE (details of diurnal profiles are presented in chapter 6), and morning assays ranged from underestimating the mean AR-HE rate over 24 hours by 18% to overestimating it by 16%. H_2 uptake was absent in communities used for diurnal assays.

In view of the large diurnal variations which were apparent in some environments, a morning assay was judged to have provided a reasonable standard upon which to base comparisons of nitrogenase activities between treatments. However, the use of a single assay time was an undoubted source of error. This error could only have been avoided by a frequency of assay which would have entailed the use of a very large number of pots - more than could be accommodated in the controlled environment cabinet.

TABLE 6 Estimation of nitrogenase activity over 24 hours.

Temperature, ^{o}C and PPFD	µmol C_2H_4 $m^{-2}h^{-1}$ 09.00 Estmate/ 24h Mean +	C_2H_4-HE $m^{-2}h^{-1}$ 09.00 Estimate/ 24h Mean +	Standing of estimates. C_2H_4	C_2H_4-H_2
10 Low	687/705	247/235	-3%	+5%
15 Low	378/412	274/271	-8.5%	+1%
20 Low	215/269	144/175	-20%	-18%
25 Low	620/665	408/432	-7%	-6%
10 High	619/665	297/282	-7%	+5%
15 High	677/690	304/262	-2%	+16%
20 High	786/848	405/367	-7%	+11%
25 High	831/884	473/504	-6%	-6%

\+ Different levels of biomass are represented for each environment, so that values cannot be compared between environments.

3.4.9. Watering regimen, CER, and nitrogenase acitivity.

According to some authors CER, transpiration and AR are reduced by moderate water deficits in T.subterraneum (Aparicio-Tejo et al. 1980 ;

Dejong and Phillips, 1982). The effects of withholding water on nitrogenase activity and on CER were examined.

The AR rates of communities from a 15°C glasshouse were compared between those watered 2 hours before assay and those not watered for 26 hours. Similarly, the CER of communities was measured when water was withheld for one day.

The AR rates presented in Table 7 show that nitrogenase activity was not influenced by the daily watering regimen. When watering was interrupted for one day, an effect on CER was only apparent at 25°C, at which temperature D (CO_2 uptake in the photoperiod) began to decline about 30 hours after watering.

TABLE 7 The effect of watering on the AR rate. Means are for the number of replicates indicated, with SE.

Plant Material	AR µmol C_2H_4 $m^{-2}h^{-1}$	
	Watered 2h before assay	Unwatered for 26h
Young swards (5 reps)	907 (58)	840 (39)
Mature swards (3 reps)	326 (49)	330 (21)

The rooting medium, oil-dry, held an amount of available water sufficient to maintain normal nitrogenase activity and CER over the 24 hour interval between application. In view of the lesser variability in AR data from unwatered plants, communities were not watered before assays subsequently conducted between 08.30 hours and 09.30 hours.

The water holding capacity of the oil-dri was 45%.

3.4.10. Residual effect of an AR assay on HE.

The use of communities grown hydroponically was seen as a technique which permitted frequent non-destructive AR assays on the same plants, and was potentially well suited to a study of diurnal variation. Imsande and Ralston (1981) reported repeated AR assays on single legumes grown hydroponically but considered HE and diurnal variation as "complicating factors", and ignored them. AR uses the full electron flux through nitrogenase, inhibiting HE (see Hardy, 1979), and it was necessary to find out for how long the inhibition lasted.

Small swards were grown hydroponically in 0.53l jars of aerated - N nutrient solution (see Table 1). The canopy area was 53 cm^2, with a plant density of 2,300 plants m^{-2} - the same as for larger swards grown in oil-dry. Three communities were maintained at 15°C and 1000 µmol quanta $m^{-2}s^{-1}$ in the assimilation chamber shown in Figure 2. After 2 days in the chamber, the communities were subjected to an AR assay, then returned to the chamber. HE and CER were measured over the next 12 hours.

HE was completely inhibited by p0.1 C_2H_2, and was first detectable 2 hours after the AR assay. However, the HE rate did not recover to the pre-AR assay rate for 8 hours. Canopy CER was unaffected by exposure of the roots to C_2H_2. CER of roots was not measured.

Because C_2H_2 inhibited HE, and a residual effect lasted for about 8 hours, when hydroponically-grown swards were used for repeated diurnal assays the same community was not used more frequently than once in 12 hours. However, in view of the disturbance to nodule metabolism, repeated assays were soon abandoned in favour of destructive assays. The hydroponic method was reintroduced to assess the response of nitrogenase to combined nitrogen, for which AR assays were less frequent.

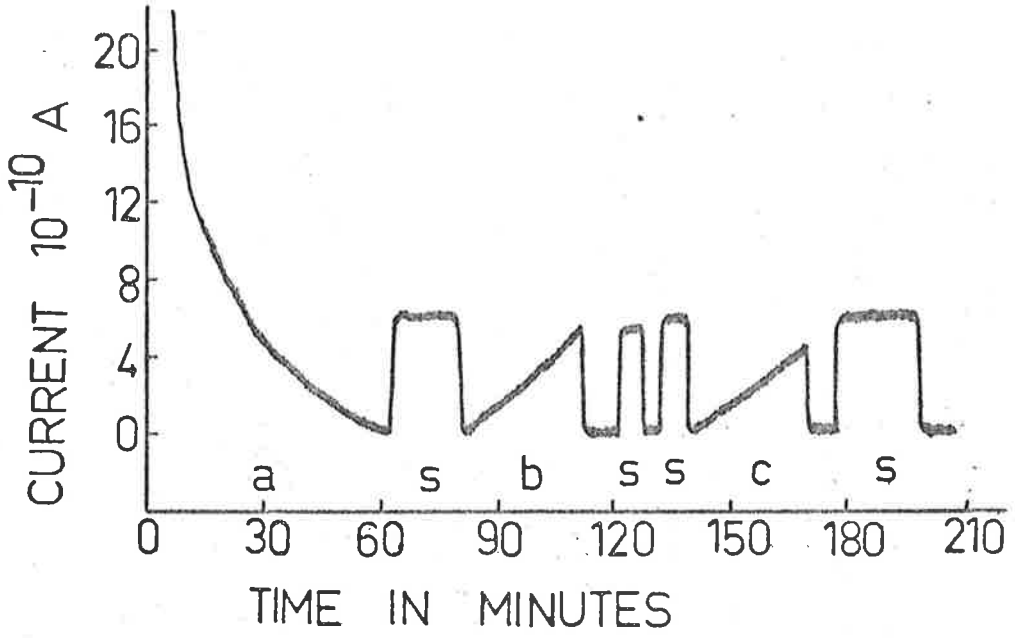
The observations on CER were in agreement with those made for plants assayed for AR *in vivo* at 20°C using a continuous-flow system (see 3.4.3, but CER is not mentioned in the context of that experiment), and suggest that cessation of nitrogen assimilation under AR had no immediate consequences for the rate of photosynthesis.

3.4.11. Amperometric H_2 measurement and linearity of HE with time.

Amperometric H_2 detection has been described as a method for measuring HE over a few minutes, in miniature curvettes, from nodule extracts or excised nodules (Schubert and Evans, 1976 ; Sweet *et al.*, 1980) and was said to suffer from a fall in sensitivity of at least $17\%h^{-1}$ (Peterson and Burris, 1978). The advantage of the method is that it provides a continuous response to H_2, and does not rely on discrete gas samples. The suitability of the method to measuring HE from whole plants was investigated.

Factorial combinations of preconditioning times (using a voltage oscillator), sensitisation (in pure H_2), and general handling procedures for a YSI 5331 electrode (YSI Co. Inc., Yellow Springs, Ohio) resulted in a method of preparation which yielded the sensitivity and stability required to measure HE over about 30 minutes from excised roots of swards in 0.285l vessels. It was impractical to use whole plants, because doing so necessitated using larger assay vessels and thereby reduced the H_2 concentration to a level not reliably detected. Since routine H_2 measurements were subsequently made using gas chromatography, the details of circuitry, preconditioning, sensitisation and handling are omitted, but are available from the author on request.

The method provided a continuous and stable response to H_2. A facsimile of the recorder trace for HE from two swards is shown in



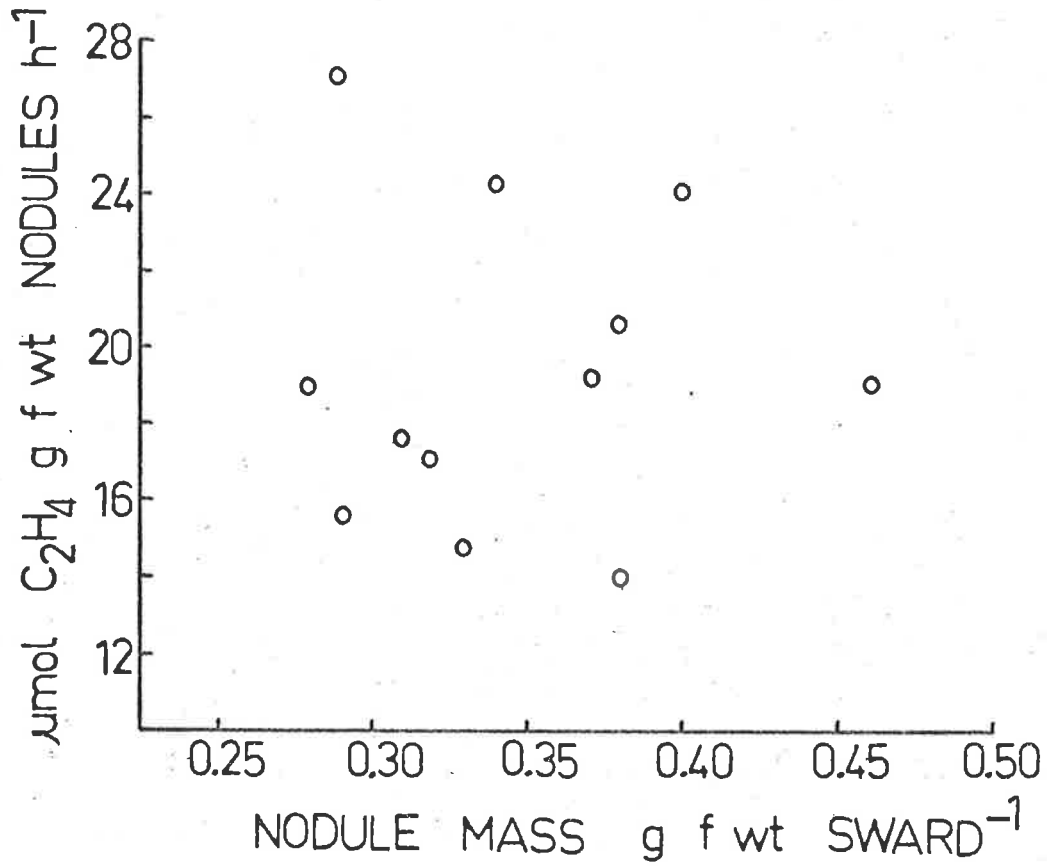


Figure 7,(Top). Facsimile of recorder trace for amperometric measurement of H_2. a, period of stabilisation; s, standards of 200 µl H_2; b,c, HE from roots. Assay chamber volume was 0.285l.

Figure 8,(Bottom). Relationship between specific AR rate and nodule mass of swards from 10C and high PPFD.

Figure 7, and demonstrates that HE was linear with time during assay. This was confirmed by gas chromatography (Figure 4).

The amperometric technique was extremely time-consuming in preparation, and was not suited to assays requiring replication. The procedure has been described as being "based as much on folklore as precise scientific principles" (Dr. W.J. Sweet, pers. comm.) since the fine physical chemistry of the electrode surfaces is not understood. Amperometric H_2 measurement was not suited to the requirements of this project.

3.4.12. The expression of nitrogenase activity.

Nitrogenase activity is sometimes stated specifically in terms of nodule weight, for example as µmol C_2H_4 gramme fresh weight nodules. This may be appropriate to brief experiments in which the nodule mass remains constant, since a rapid change in the N_2 fixation rate is manifested through immediate change in specific activity. Treatments imposed over a longer period of growth can result in altered nodule mass (Streeter, 1974). The suitability of specific activity as an expression of nitrogenase activity by a community was investigated.

Twelve communities were grown hydroponically at $10^{o}C$ and 1000 µmol quanta $m^{-2}s^{-1}$, and their AR rates were measured at 11.00 hours. All nodules were excised from the roots, and the fresh weight of nodules found for each community.

Data shown in Figure 8 reveal that the potential specific AR rate of a given nodule mass covered a wide range. The AR rate for a community (the mean AR rate, with SE, for all 12 was 1257 (94) µmol C_2H_4 $m^{-2}h^{-1}$) could be achieved by a large or a small nodule mass with a low or a high specific activity, respectively.

In view of the variation in nodule mass between swards with similar total nitrogenase activities, the most serviceable expression of nitrogenase activity was taken to be that based on canopy area (μmol product $m^{-2}h^{-1}$) as suggested by Silsbury (1981).

CHAPTER 4

4. Community growth.

4.1.0. Effect of temperature on community growth.

4.1.1. Results.

The growth of N_2-fixing and of NO_3^--supplied (control) swards was examined at 4 temperatures and two levels of PPFD. The control communities were originally nodulated, but were given 7.5 mM NO_3^- from the day after N_2 fixation commenced. Their nitrogen nutrition quickly became independent of nodules, which ceased to function except those at 10°C which were found to have 30-40% of the normal N_2 fixation rate. The data presented for plant growth are for CER, total dry matter production (DM), a crude measurement of growth efficiency, and root to shoot ratios.

The method of calculating growth rate from CER is compared with the more traditional approach of mesuring the increase in biomass between harvests in Table 8. An assumption was made in the calculations based on CER that the percentage carbon of the N_2-fixing and the NO_3^--supplied legumes was the same, as has been done elsewhere (Israel and Jackson, 1982). The conversion factor of 1g CO_2 ≡ 0.74g DM was originally calculated for N_2-fixing communities at 20°C (Silsbury, 1979). Plots of DM versus time fitted straight lines very well for all environments (r ranged between 0.96 and 1), but although growth rates measured by CER were relatively constant from one day to the next, they showed a tendency to decrease over several weeks in some instances. This tendency was most noticeable at 25°C. It was mainly attributable to the respiratory cost of maintaining an increasing biomass (Figures 9 to 12).

TABLE 8 Comparison of growth rates of communities measured by CO_2 exchange and dry matter accumulation.

Temperature, PPFD, N source	D-N^{+} g $CO_2 m^{-2} 24h^{-1}$	D-N . 0.74^{++} g $DM m^{-2} 24h^{-1}$	a^{+++} g $DM m^{-2} 24h^{-1}$	$\frac{D-N}{a}$
10°C,low PPFD,N_2	14.52	10.76	11.24	0.96
10°C,low PPFD,NO_3^-	16.19	12.00	12.95	0.93
15°C,low PPFD,N_2	13.43	9.95	10.67	0.93
15°C,low PPFD,NO_3^-	14.67	10.86	ND	ND
20°C,low PPFD,N_2	11.71	8.67	8.43	1.03
20°C,low PPFD,NO_3^-	13.62	10.10	10.90	0.93
25°C,low PPFD,N_2	9.05	6.71	6.71	1.00
25°C,low PPFD,NO_3^-	11.76	8.71	ND	ND
10°C,high PPFD,N_2	23.95	17.71	19.33	0.92
10°C,high PPFD,NO_3^-	28.38	21.00	21.38	0.98
15°C,high PPFD,N_2	23.24	17.19	17.52	0.98
15°C,high PPFD,NO_3^-	28.48	21.10	20.57	1.03
20°C,high PPFD,N_2	21.91	16.19	15.48	1.05
20°C,high PPFD,NO_3^-	24.86	18.38	18.19	1.01
25°C,high PPFD,N_2	16.19	12.00	12.62	0.95
25°C,high PPFD,NO_3^-	20.10	14.86	14.95	0.99

ND Not Determined

\+ D was the net assimilation of CO_2 during the day, N was the efflux of CO_2 at night, each over 12 hours. D-N is the mean for closed canopies over the same range of biomass as used to calculate a, either 95-475 gm^{-2} (20 values) or 95-570 gm^{-2} (25 values). Values of D-N for 10°C, high PPFD in the range 95-190 gm^{-2} were omitted because the canopies were not closed.

\++ 1g $CO_2 \equiv$ 0.74 g dry matter (see text).

\+++ a is the linear regression coefficient for dry matter versus time. Based on 7-11 points representing single communities for NO_3^- plants, and on 8-10 points each an average for 3 communities for N_2 plants.

However, a rough assumption was made that growth rates were constant. To facilitate comparison, average growth rates were calculated from CER and DM data over similar ranges of biomass. The growth rates estimated by CER ranged from 93% to 105% of those found from the linear regression of DM on time (Table 8), showing that CER reliably represented the crop growth rate. Thus, it was sound to interpret the CERs of swards measured in the assimilation chamber as representing the growth rates of similar swards in the growth cabinet which were used for N_2 fixation assays.

Reference to Table 9 shows growth rates to have increased substantially at 1000 µmol quanta $m^{-2}s^{-1}$ (high PPFD) over those at 500 µmol quanta $m^{-2}s^{-1}$ (low PPFD) at all temperatures, as has been found for other legume communities (Brown et al., 1966). An increase in PPFD provides more energy for CO_2 fixation, as well as increasing stomatal aperture and, therefore, CO_2 conductance. In a community it is the shaded leaves which respond most to increase in PPFD, while those not shaded may be PPFD-saturated (Blackman, 1962). The data on nitrogenase activities in Table 9 are referred to in chapter 5.

The effects of temperature on CER for N_2-fixing and NO_3-supplied communities are shown for discrete intervals of the amount of biomass in Figures 9 to 12. At each PPFD the CER responses were very similar for N_2-fixing and NO_3^--supplied plants, but there was a difference between PPFDs with respect to ontogeny. At high PPFD the pattern of temperature response with increase in biomass was similar to that found earlier for dry matter accumulation by non-nodulated communities (Fukai and Silsbury, 1976) in that the optimum temperature was high, at 20°C, in early growth and then shifted, through 15°C ultimately to 10°C at high biomass (Figures 9 and 11). However, Figures 10 and 12 show that the growth rate was highest at 10°C throughout the period of measurement for swards grown at low PPFD.

TABLE 9 Effect of PPFD on growth rate and nitrogenase activities at four temperatures.

Variable	Temperature, °C	Low PPFD		High PPFD		F value+++
D-N+						
g $CO_2 m^{-2} 24h^{-1}$	10	14.67	(1.37)	23.95	(1.26)	26.49**
	15	13.67	(1.55)	24.62	(1.82)	24.84**
	20	11.67	(1.11)	22.00	(1.31)	32.78**
	25	8.71	(1.30)	16.00	(3.21)	11.42**
AR++						
µmol $C_2H_4 m^{-2} h^{-1}$	10	432	(17)	660	(31)	6.37**
	15	486	(25)	605	(36)	2.75**
	20	293	(27)	533	(22)	7.00**
	25	537	(19)	842	(37)	7.38**
HE++						
µmol $H_2 m^{-2} h^{-1}$	10	254	(16)	403	(29)	4.54**
	15	236	(39)	330	(48)	1.52NS
	20	88	(21)	211	(26)	3.65**
	25	126	(22)	98	(28)	0.80NS
AR-HE++						
C_2H_4-$H_2 m^{-2} h^{-1}$	10	178	(14)	257	(19)	3.32**
	15	250	(21)	275	(21)	0.83NS
	20	205	(18)	319	(19)	4.36**
	25	410	(27)	744	(45)	6.32**
N_2 fixation						
µmol $N_2 m^{-2} h^{-1}$	10	538		924		24.04**
	15	597		904		5.13*
	20	408		714		30.01**
	25	279		529		11.84**

NS - Not Significant

* Significant for P0.05 to P0.01. ** Significant at P<0.01

\+ 190-475 gm^{-2} range, mean for 15 values, with SE.

++ 95-475 gm^{-2} range, mean for 24 values, with SE.

+++ Linear regression lines for nitrogen fixed versus time were compared as described in Snedecor and Cochran (1967). All other data were compared using the t test.

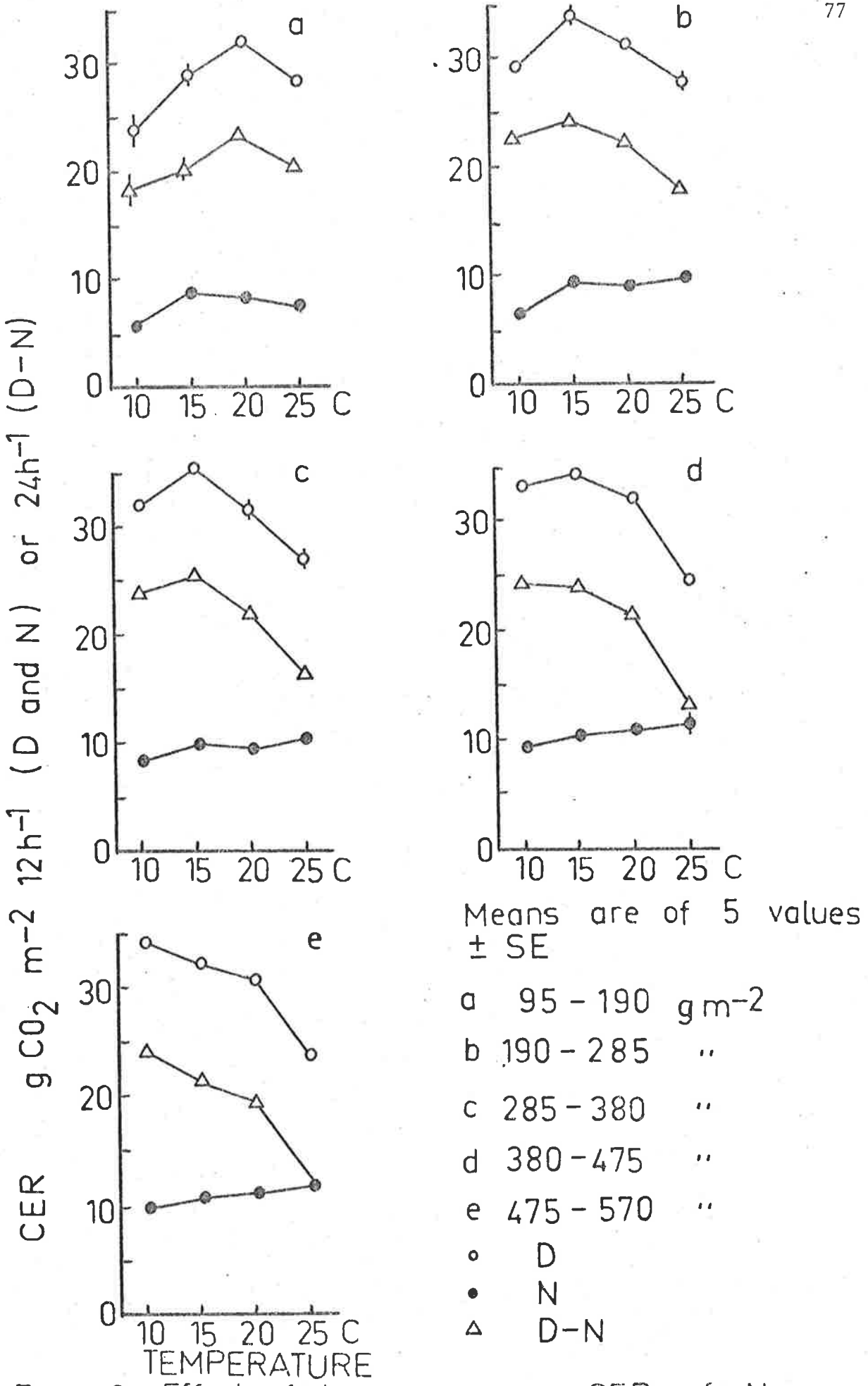
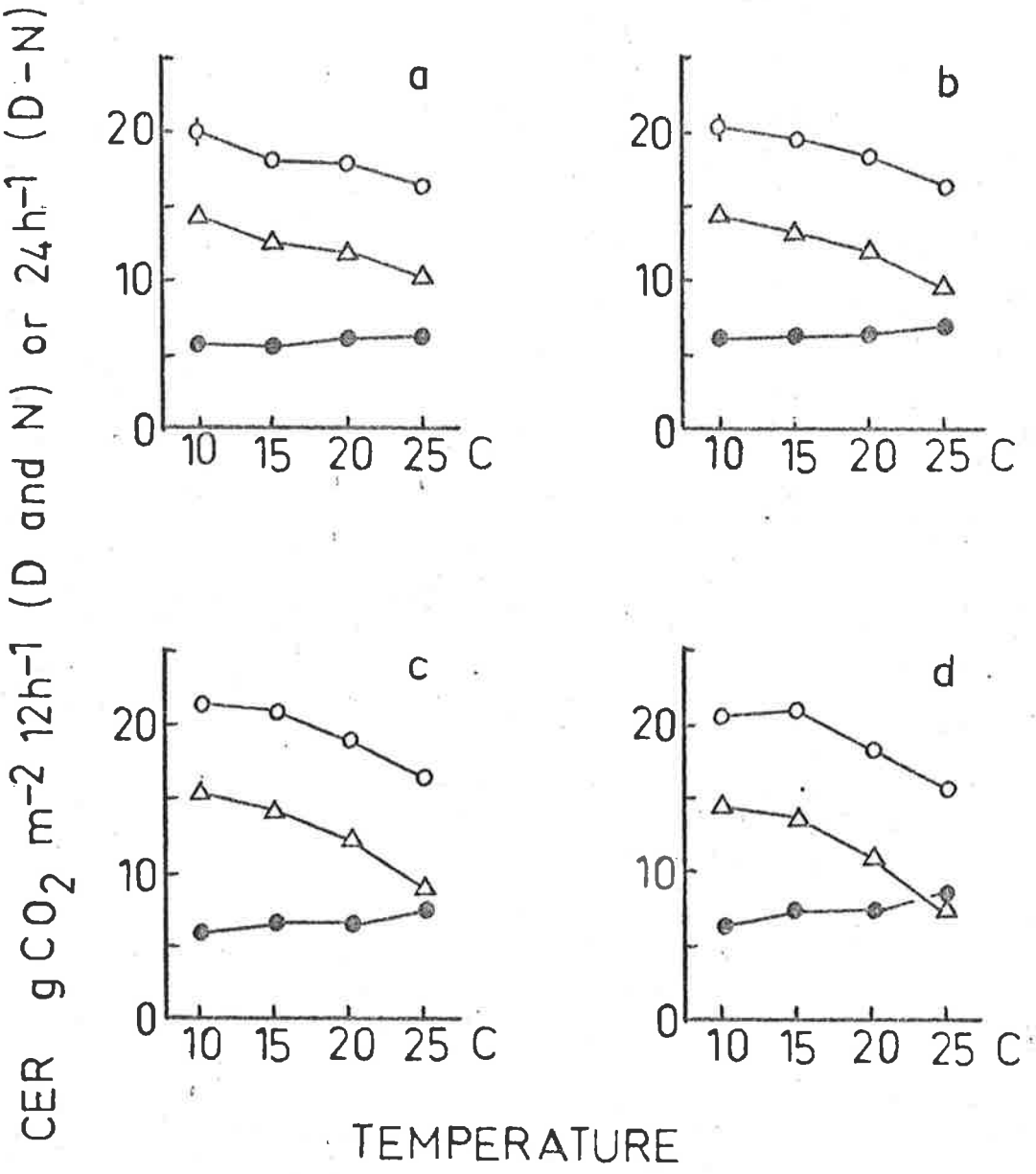
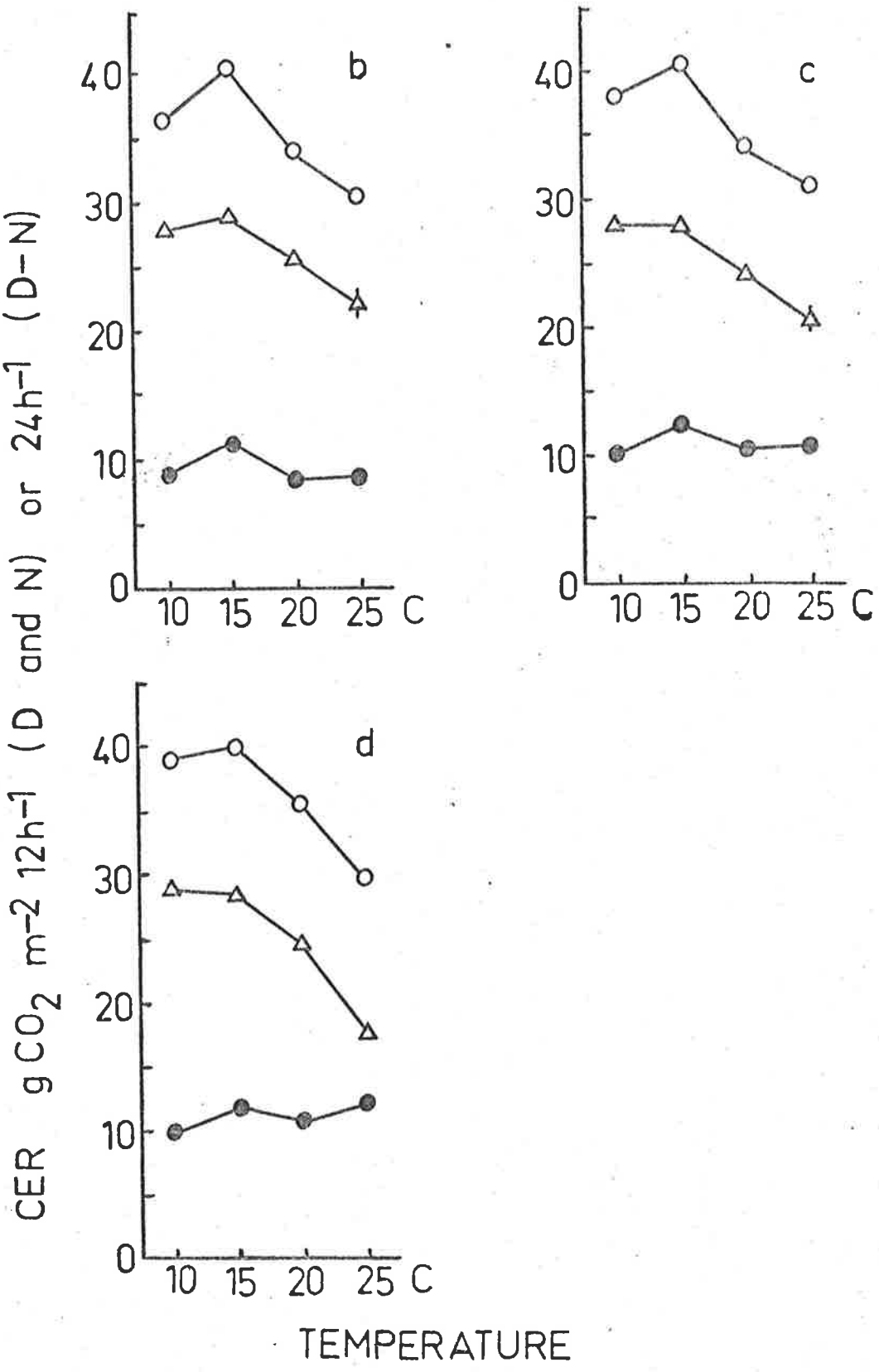


Figure 9. Effect of temperature on CER of N_2-fixing plants at high PPFD.



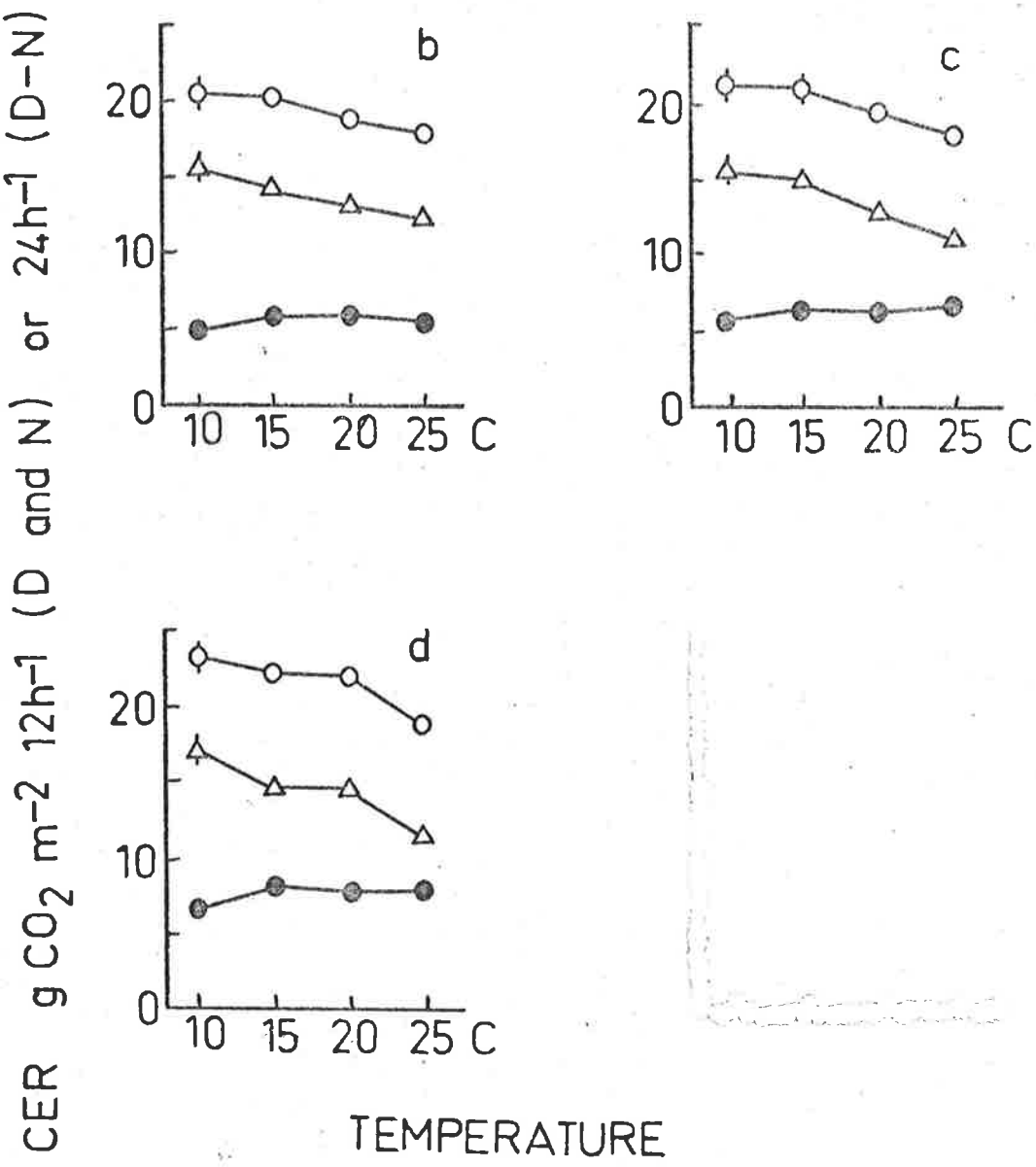
Symbols as for figure 9

Figure 10. Effect of temperature on CER of N_2-fixing plants at low PPFD.



Symbols as for figure 9

Figure 11. Effect of temperature on CER of NO_3^--supplied plants at high PPFD.



Symbols as for figure 9

Figure 12. Effect of temperature on CER of NO_3^- supplied plants at low PPFD.

The response of growth rate to temperature over a range of biomass common to both PPFDs, and nitrogen treatments, has been subjected to an analysis of variance in Table 10.

TABLE 10 Analysis of variance of the response of growth rate to temperature. 190-475 g m^{-2} biomass range. Means for 15 values, with SE.

Temperature, ^{o}C	D-N, g CO_2 $m^{-2}24h^{-1}$.			
	Low PPFD		High PPFD	
	N_2	NO_3^-	N_2	NO_3^-
10	14.67 (1.37)A	16.19 (2.41)A	23.95 (1.26)A	28.38 (1.69)A
15	13.67 (1.55)B	14.67 (1.09)B	24.62 (1.82)A	28.48 (1.09)A
20	11.67 (1.11)C	13.62 (1.33)C	22.00 (1.31)B	24.86 (1.64)B
25	8.71 (1.30)D	11.76 (1.51)D	16.00 (3.21)C	20.10 (3.31)C
F value, AOV	113.21**	37.12**	104.78**	106.0**
LSD P0.05	0.70	0.87	1.08	1.10

** Significant at P0.01

Figures within columns followed by different letters are significantly different at P0.05

At low PPFD, growth rates were highest at $10^{o}C$ and were reduced with rise in temperature while, at high PPFD, the most favourable temperature for growth broadened to $10^{o}C$ to $15^{o}C$, but growth rate was reduced with rise in temperature above $15^{o}C$. Both N_2-fixing and NO_3^--supplied plants responded similarly. Figures 9 to 12 show that growth rates were reduced at the higher temperatures through a larger proportion of the CO_2 fixed during the day being lost in respiration at night and, presumably, also during the day. For all communities above

380 gm^{-2} DM, the growth rate was inversely related to temperature between 10°C and 25°C.

Tables 8 and 10 show that the growth rates of NO_3^--supplied communities were greater than those of N_2-fixing communities, and this is substantiated statistically in Table 11.

TABLE 11 Comparison of growth rates between N_2-fixing and NO_3^--supplied communities. 190-475 gm^{-2} biomass range. Means for 15 values, with SE.

Temperature, °C, and PPFD.	D-N, g $CO_2 m^{-2} 24h^{-1}$				F value from t test.
	N_2		NO_3^-		
10,low	14.67	(1.37)	16.19	(2.41)	24.13**
10,high	23.95	(1.26)	28.38	(1.69)	4.10**
15,low	13.67	(1.55)	14.67	(1.09)	14.53**
15,high	24.62	(1.82)	28.48	(1.09)	4.06**
20,low	11.67	(1.11)	13.62	(1.33)	10.49**
20,high	22.00	(1.31)	24.86	(1.64)	8.69**
25,low	8.71	(1.30)	11.76	(1.51)	6.84**
25,high	16.00	(3.21)	20.10	(3.31)	11.62**

** Significant at P0.01

The N_2-fixing communities grew more slowly, by an average of 15%, than those assimilating NO_3^- under all conditions. The growth rates of N_2-fixing plants ranged from 74% to 93% of those of NO_3^--supplied plants, with no obvious trend with temperature or PPFD in evidence, except that N_2-fixing plants were most disadvantaged at 25°C for both levels of PPFD. Figures 9 to 12 indicate the NO_3^--supplied plants to

have had greater values for D and, in most cases, similar values of N, in comparison with N_2-fixing plants.

The comparison between nitrogen treatments is developed in Table 12 through a crude estimate of growth efficiency, g C respired g DM retained 24 h^{-1}. This calculation was made with the simplification that daytime respiration was taken to be the same as nighttime respiration. It also ignored photorespiration, and did not resolve respiration into synthesis and maintenance components.

TABLE 12 Crude estimates of growth efficiency calculated from total respiration, and dry matter retained, over 24 hours. Biomass range 190-475 gm^{-2}, means for 15 values, measured on the same plants.

Temperature, ^{o}C	g C respired g DM retained $24h^{-1}$			
	Low PPFD		High PPFD	
	N_2	NO_3^-	N_2	NO_3^-
10	0.30	0.26	0.25	0.25
15	0.36	0.33	0.30	0.30
20	0.42	0.36	0.33	0.29
25	0.62	0.41	0.49	0.38

Notwithstanding the limitations of this index, it did reveal effects of temperature, PPFD and nitrogen source. According to Table 12, more carbon was respired in association with the production of a unit of dry matter by N_2-fixing plants than NO_3^--supplied plants, at all temperatures for low PPFD. At high PPFD, the index was similar at $10^{o}C$, and also at $15^{o}C$, for plants fixing N_2 and assimilating NO_3^-. Above $15^{o}C$, a larger proportion of carbon was respired by plants fixing N_2, and this quantity rose most rapidly with increase in temperature for N_2-fixing plants at both PPFDs. Finally, growth was always more efficient,

in terms of the proportion of carbon respired, at high PPFD than at low PPFD.

The root : shoot ratios of N_2-fixing swards are presented in Table 13.

TABLE 13 Effects of temperature and PPFD on root:shoot ratios of N_2-fixing communities. Biomass range 190-475 gm^{-2}. Means, and SE, for 18 values (3 harvests, each of 6 replicates, corresponding to 3 ranges of biomass common to all environments).

PPFD	Temperature				F value	LSD
	10°C	15°C	20°C	25°C	of AOV+	P0.05
Low	0.35 (0.02)A	0.28 (0.01)B	0.26 (0.01)B	0.26 (0.01)B	8.97**	0.04
High	0.43 (0.02)A	0.35 (0.02)B	0.30 (0.01)B	0.31 (0.01)B	12.03**	0.05
F value of t test	3.00**	2.88**	2.04*	2.94**		

* Significant for P0.05 to P0.01
** Significant for P<0.01
\+ Figures within rows followed by different letters are significantly different at P0.05

While this ratio undoubtedly results from the interaction of many variables, its simple representation of the proportion of carbohydrate that is translocated to below ground parts is sound enough (Brouwer, 1962). In this regard it may be a useful number in indicating the availability of carbohydrate to nodules. The effect of temperature was the same for both PPFDs, the root : shoot ratio being markedly increased at 10°C. The ratio increased with PPFD at all temperatures.

Some N_2-fixing communities were transfered to 30°C and high PPFD, but their leaf canopies did not close and leaves present at the time of transfer began to senesce. New leaves formed but they were small, and represented a few pale green islands in a sea of dying tissue. The stunted canopies appeared to be very nitrogen deficient. Nitrogen for new leaf production seems to have come almost exclusively from older leaves, since only the roots showed a net nitrogen gain and growth, that is, increase in biomass. The linear regression coefficients for organic nitrogen plotted against time for roots (28.6mg nitrogen m^{-2} $24h^{-1}$) and for senescent shoots (33.3 mg nitrogen m^{-2} $24h^{-1}$) when summed almost equalled the value for whole plants (62.9 mg nitrogen m^{-2} $24h^{-1}$). The growth rate for N_2-fixing swards at 30°C fell from 13.19 to 8.52 g CO_2 m^{-2} $24h^{-1}$ over 11 days, contrasting with a comparatively stable growth rate for communities assimilating NO_3^- of 21.48 (0.29) g CO_2 m^{-2} $24h^{-1}$ over 16 days. NO_3^--supplied communities were dark green, large-leaved and tall, appearing not to suffer from nitrogen deficiency.

Between 10°C and 25°C, N_2-fixing swards showed no marked symptoms of a nitrogen limitation on growth, but those given NO_3^- invariably had larger leaves.

4.1.2. Discussion.

N_2 fixation by legumes has sometimes been studied as an isolated topic, either in the field (Carran et al., 1982) or under controlled conditions (Fyson and Sprent, 1982). Responses of N_2 fixation to environmental variables have then been interpreted to have occurred through the mediation of the host plant, although quantitative evidence for such mediation was not given. Fundamentally, there must be two sides to a discussion of symbiosis. With reference to measured attributes of

the growth of swards under the various treatment conditions, discussion can encompass, on an empiric basis, a reasonable theorem that the N_2 fixation rate is influenced by the host requirement for nitrogen in growth.

The finding that the growth rates of N_2-fixing communities were highest at low temperature extended the similar observation made for non-nodulated communities (Fukai and Silsbury, 1976). The results were consistent with the view that T.subterraneum underwent metabolic adaptation to low temperature. This species is not chilling-sensitive and so the enzymes associated with such processes as respiration and nitrogen assimilation are not expected to have undergone kinetic disturbance at low temperature, neither was there any bleaching of leaves nor inhibition of photosynthesis which have been observed in chilling-sensitive tropical legumes (Austin and Mclean, 1972 ; Raison and Chapman, 1976 ; Duke et al., 1978). The thermal adaptation of respiratory enzymes has been identified as important in growth at low temeperature (Duke and Doehlert, 1981), and the normal reduction in a reaction rate seen when the temperature is reduced was found to be compensated for by an enzyme having a lower K_m for its substrate (Crawford and Huxter, 1977). Through greater oxygenation of RuBP, the quantum yield for C3 plants decreases with increase in temperature above $10^{o}C$ (see Berry and Bjorkman, 1980) and this is one of the reasons why low temperature was optimal for community growth. Low photorespiration rates contributed to the high values of D at low temperature.

For communities, the effect of temperature on growth rate was found to be realised via dark respiration (Fukai and Silsbury, 1977) since the maintenance component of dark respiration had a Q_{10} of about 1.8 (McCree and Silsbury, 1978). Hence, the growth rates were highest at low temperature (Table 10) largely because the carbon costs of maintaining

biomass were least. The change in the optimum temperature for growth at 1000 μmol quanta $m^{-2}s^{-1}$ (high PPFD) resulted mostly from parallel reductions in D and D-N (Figure 9). As biomass accumulated, the benefit of stimulation to photosynthetic CO_2 fixation by high temperature was outweighed by the carbon loss of maintenance respiration. Old leaves began to senesce when they were shaded by new leaves, and they did so faster at high temperature. Senescence is a natural event both for respiration and photosynthesis processes (Geronimo and Beevers, 1964 ; Catsky et al., 1976) which is hastened by high temperature, and senescent respiration contributed increasingly to the total measured respiration rate with time. At $10^{o}C$ there was virtually no senescent leaf material, even in the highest range of biomass, but at $25^{o}C$ senescent leaves comprised about 20% of the shoot dry weight in the 475-570 g m^{-2} range of biomass.

Surprisingly, D appeared to increase with time at $10^{o}C$ and $15^{o}C$ for both PPFDs. The rise in D between 95 and 190 g m^{-2} at high PPFD and $10^{o}C$ was due to the leaf canopies not closing until 190 g m^{-2} (Figure 9), but the other increases were probably due to an artifact of the growth technique. At low temperature shaded leaves stayed green for longer than at high temperature, and were probably able to use radiation reflected from the walls of the assimilation chamber to make a contribution to the net CO_2 fixation rate. The four sides of the mesh sleeve (which each had a transmittance of about 55%) admitted reflected radiation into the canopy, and this would have gained in importance as the amount of leaf tissue in the lower canopy layers increased. McCree and Troughton (1966) found that canopy CER increased similarly with time for T.repens in diffuse radiation. Also, the canopy geometry changed with time. As the canopy became more crowded by the production of new leaves, many of them remained inclined upwards during the photoperiod. Rhythmic vertical leaf

movement, at the beginning and end of the photoperiod, was observed to always coincide with a higher CER, and this may have resulted from improved penetration of light into the community.

Leaf canopies closed earlier at the low PPFD, possibly because leaves were larger in area, which is a common response to low PPFD (see Hawkins, 1982). The low temperature optimum for growth was also established sooner than at high PPFD (Figure 10). N_2 was fixed very inefficiently early on, as discussed in section 4.3.0. While the nodules probably supplied sufficient nitrogen for growth at low PPFD, when plants were transferred to high PPFD at 10DI the combined effects of a large demand for nitrogen (because CER was initially high) and the slowing of nodule development on transfer to low temperature shown by Roughley (1970), probably resulted in the plants being unable to maintain nitrogen sufficiency in the leaves. Consequently, at high PPFD early growth was favoured at a higher temperature. This occurred in G.max (Williams and Phillips, 1980) so that for several weeks of early growth CO_2 fixation and AR rates were lower at 1500 µmol quanta $m^{-2}s^{-1}$ than at 700 µmol quanta $m^{-2}s^{-1}$. Thus, in young plants at high PPFD and low temperature, nitrogen deficiency probably slowed community development.

The temperature optimum for D-N, averaged over the range 190-475 g DM m^{-2} narrowed to 10°C at low PPFD (Table 10). One cause of this, the absence of an ontogenetic change in the optimum temperature for growth at low PPFD and the presence of a change at high PPFD, has already been discussed. Another factor contributing to the narrowed temperature optimum was that the respiration rate gained in importance with respect to determining the growth rate at the low PPFD. For similar biomasses, P_G was smaller at the low PPFD but the amount of respiration for maintenance should have been similar at both PPFDs. Thus, the proportion

of P_G, and of the carbohydrate pool, used in maintenance was higher at low PPFD than at high PPFD and, due to the responsiveness of respiration to temperature, increase in temperature had a more marked effect on CER at low PPFD than at high PPFD.

The extremely poor and declining growth rate of N_2-fixing communities at 30°C, and the continuity of growth of those assimilating NO_3^- showed that the high temperature specifically inhibited N_2 fixation, and confirmed the high temperature-sensitivity of R.trifolii WU95 in symbiosis (Pankhurst and Gibson, 1973).

The carbon costs for synthesis of dry matter were shown by Silsbury (1977) to be higher for N_2-fixing plants than for non-nodulated plants. The crude index of growth efficiency calculated in Table 12 did not seperate synthesis respiration from that for maintenance, being the total g carbon respired per g DM retained 24 h^{-1}, but the results for low PPFD, and 20°C and 25°C at high PPFD, were in agreement with those of Silsbury (1977). The data were calculated for a common level of biomass, and the increase in the index with temperature reflected the response of maintenance respiration to temperature. The synthesis coefficient was found to be independent of temperature (McCree and Silsbury, 1978). The difference in growth efficiency between nitrogen sources probably arose out of the respiratory burden of nodules borne by N_2- fixing plants. Nodule respiration has been shown to accelerate rapidly with rise in temperature (Pankhurst and Sprent, 1976 ; Mahon, 1979), and in T.repens and T. pratense nodule respiration accounted for about 50% of nodulated root respiration (Ryle et al., 1983). N_2-fixing legumes were found to partition a larger fraction of fixed carbon into sucrose, and less into starch, than legumes assimilating NO_3^- (Huber and Israel, 1982) and this was probably to meet the larger carbohydrate requirement for respiration in plants fixing N_2 since de Visser and

Lambers (1983) have found the ATP cost for root growth of N_2-fixing legumes to be higher than for those assimilating NO_3^-.

The growth efficiency data suggested that the respiratory burden of the nodules was more important when the growth rate was low. The differences between high and low PPFD in growth efficiency were quite large for N_2-fixing plants, but small for those receiving NO_3^-. N_2 fixation continually draws on the carbohydrate pool, whereas NO_3^- reduction can be achieved virtually 'free' in the daytime using photochemically generated energy (Schrader and Thomas, 1981). The high PPFD resulted in there being a greater pool of carbohydrate in the plants available for growth, and the nodules detracted from this by a fraction which had less consequence for other community growth processes than at low PPFD. The differences in g C respired g DM retained $24h^{-1}$ between PPFDs were largely attributable to the maintenance component of the total respiration measured. At low PPFD, where P_G was low, the cost of maintaining a unit of tissue became more important, since it would have been the same as at high PPFD but used up a larger proportion of P_G.

The responses of the root : shoot ratio to temperature and PPFD were as commonly seen in other plants (Brouwer, 1962), and are discussed later with respect to N_2 fixation rates.

The data in Tables 8, 11, and 12, uphold the view that the carbon costs of N_2 fixation and growth in N_2-fixing plants result in lower growth rates for plants obtaining nitrogen symbiotically than for those assimilating combined nitrogen (Gibson, 1976 ; de Visser and Lambers, 1983). Compromise is a precept for symbiosis, such that mutual advantages are obtained. The cost of the compromise was evidently manifested in terms of growth rate. However, as the legume-Rhizobium symbiosis probably evolved in response to nitrogen deficiency, a

relatively low growth rate for a plant fixing N_2 may be of small consequence in an environment where competition with other plants is secondary to growth itself.

Between 10°C and 25°, at both PPFDs, the responses of growth rates to temperature, and the patterns of change in response with increase in biomass, were very similar for N_2-fixing and NO_3^--supplied communities. The supply of nitrogen from nodules must have been adequate to meet the requirements for growth of the N_2-fixing plants. Only at 30°C did the growth of N_2-fixing plants appear to be nitrogen-limited. In the range 10°C to 25°C, the marked difference between N_2-fixing and NO_3^--supplied plants did not relate to their growth responses to temperature, but to a growth response to the source of nitrogen. The NO_3^--supplied plants grew faster. The data suggest that the growth of N_2-fixing plants between 10°C and 25°C was not limited by nitrogen, but by the carbon cost of fixing N_2. It was, therefore, worthwhile to calculate how much of the net photosynthate was used in fixing N_2, and this is reported in the next section.

4.2.0. Amount of photosynthate used to support nitrogenase activity.

4.2.1. Methods.

The principles upon which calculations were based have been outlined by Gibson, in Dilworth (1980), but the assumptions made in calculating the cost of nitrogenase activity differed slightly from those made by Gibson.

For each environment net photosynthesis, P_N, was converted to dry matter using the factor of 0.74 (Silsbury, 1979) and it was assumed that all dry matter was equivalent to glucose. The total amount of

nitrogenase activity over 24 hours was calculated from diurnal profiles of AR, which are presented in chapter 6. The ATP cost of reducing one mole of C_2H_2 was assumed to be 7ATP, based on the ATP cost of transferring two electrons to a substrate shown in Evans et al. (1980). The complete oxidation of one mole of glucose was taken to yield 38 ATP. Hence, the proportion of net photosynthate used in AR during 24 hours was arrived at. A correction factor of 4 was used because, as discussed in chapter 5, AR assays in sealed vessels underestimated nitrogenase activity by 4 times.

The average RE over 24 hours was arrived at from the data on diurnal variation (given in chapter 6) and was used to calculate the proportions of net photosynthate used in HE and in N_2 reduction.

4.2.2. Results.

The proportion of net photosynthate used in support of nitrogenase activity is shown in Table 14. This proportion increased with temperature at high PPFD, but the trend was not repeated at low PPFD although the proportion was again highest at 25°C. In the absence of a confirmed effect of either temperature or PPFD on the costs of nitrogenase activities, average costs were calculated. Mahon (1979) showed that temperature and PPFD did not affect the carbon cost of N_2 fixation in P.sativum. 5.36% of net photosynthate was lost in HE, and 5.76% was used to support N_2 reduction (Table 14).

4.2.3. Discussion.

As nitrogenase activity was reckoned to use about 11% of the net photosynthate in 24 hours, this may go some way towards accounting for

TABLE 14 Daily costs of nitrogenase activities, in terms of net photosynthate (glucose equivalents). The table assumes that CO_2 fixed during the day was as glucose, and that 1g $CO_2 \equiv 0.74$ g glucose.

Temperature, °C and PPFD	AR mmol C_2H_4 $m^{-2}24h^{-1}$	ATP used in AR µmol $m^{-2}24h^{-1}$	g glucose used in AR $24h^{-1}$	P_N g CO_2m^{-2} per $12h^{-1}$	Glucose g m^{-2} per $12h^{-1}$	RE $\bar{x}$ $24h^{-1}$	% Photosynthate used for: HE	N_2	Total
10 low	16.91	119.0	0.56	21.29	15.76	0.35	2.34	1.26	3.60
15 low	9.90	70.0	0.33	19.33	14.29	0.64	0.83	1.47	2.30
20 low	6.43	45.0	0.21	18.10	13.38	0.65	0.56	1.04	1.60
25 low	15.95	112.0	0.53	16.10	11.91	0.65	1.57	2.93	4.50
10 high	15.95	112.0	0.53	32.43	28.76	0.42	1.04	0.76	1.80
15 high	16.57	116.0	0.55	35.43	26.24	0.37	1.32	0.78	2.10
20 high	20.38	143.0	0.68	31.57	23.38	0.45	1.59	1.31	2.90
25 high	21.19	149.0	0.70	27.95	20.67	0.57	1.46	1.94	3.40
						$\bar{x}$	1.34	1.44	2.78
						$\bar{x} \times 4^+$	5.36	5.76	11.12

+ The conversion factor of 4 was used because assays in sealed vessels underestimated nitrogenase activities by 4 times, compared to continuous flow assays (discussed in chapter 5).

why the growth rates of N_2-fixing communities were lower, by an average of about 15%, than those of communities receiving NO_3^- (Table 11). The figure of 11% is in near agreement with values of 9% and 12% found for the amount of net photosynthate used in nodule respiration during vegetative growth of L.albus and P.sativum, respectively (Minchin and Pate, 1974 ; Pate and Herridge, 1978) but is substantially greater than that of 5% found for V.unguiculata (Herridge and Pate, 1977). The data are not strictly comparable because the figure of 11% represents only photosynthate used in support of nitrogenase operation and not total nodule respiration. Nodule respiration also supports the in vivo cost of assimilating fixed nitrogen via GS-GOGAT which requires further ATP and NADH (see Miflin, 1980), and the cost of maintaining nodule tissues. The communities which received NO_3^- were probably able to reduce NO_3^- virtually 'free' in the daytime, using photochemically-produced reductant (Schrader and Thomas, 1981), and they did not incur a large energy cost for HE.

The use of 5.36% of net photosynthate in reducing H^+ highlights the expense to the swards of what is generally held to be an unavoidable and wasteful process. This theme is taken up in chapters 5 and 6, where it is proposed that during the course of evolution legumes have gained some utility from HE.

4.3.0. Community growth during the nascence of N_2 fixation.

4.3.1. Introduction.

Between the time of germination and the onset of N_2 fixation, the T.subterraneum swards used in this project could only draw on reserves of nitrogen in the seed to support growth, as no combined nitrogen was

supplied. There are several references in the literature to legumes being nitrogen deficient in early growth, and it was decided to explore this in detail. In particular, it was necessary to find out how soon after inoculation N_2 fixation commenced, so that combined nitrogen could be applied to controls from the day that N_2 fixation began. This would ensure a fair comparison between the growth of NO_3^--supplied and N_2-fixing communities. It would also allow communities to be transferred from the nodulation temperature, of 20°C, to different growth temperatures after a known period of N_2 fixation.

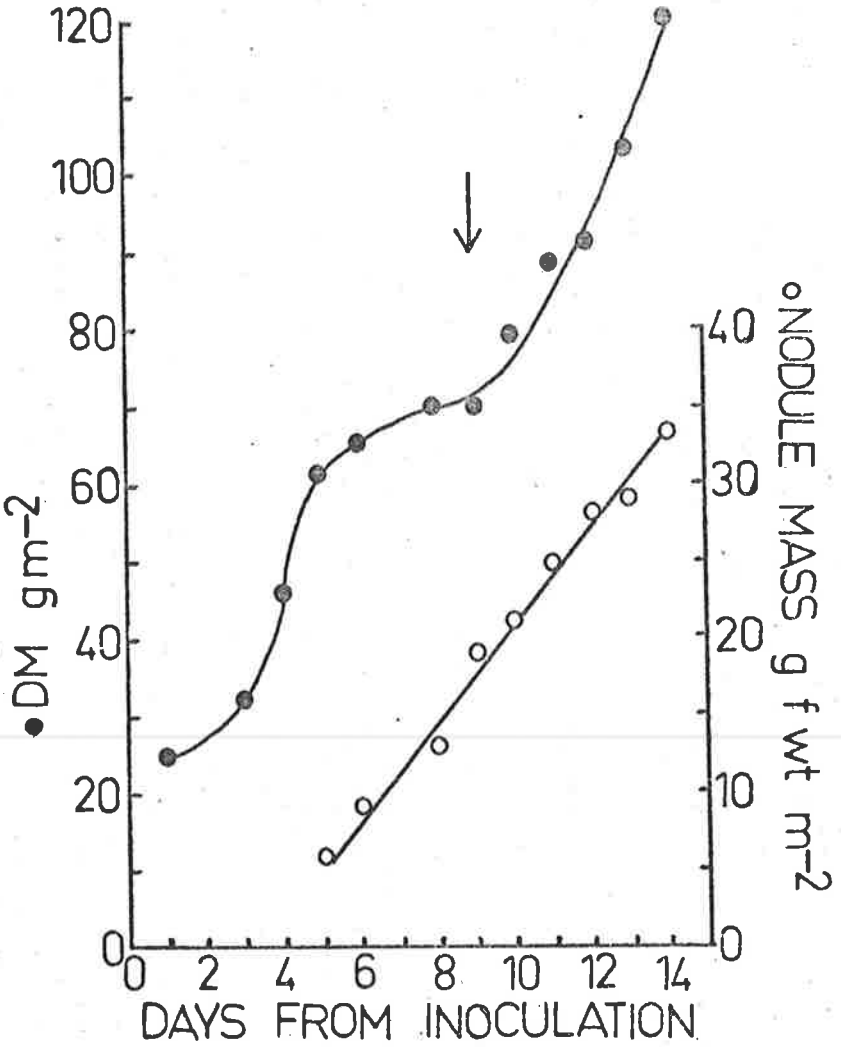
4.3.2. Methods.

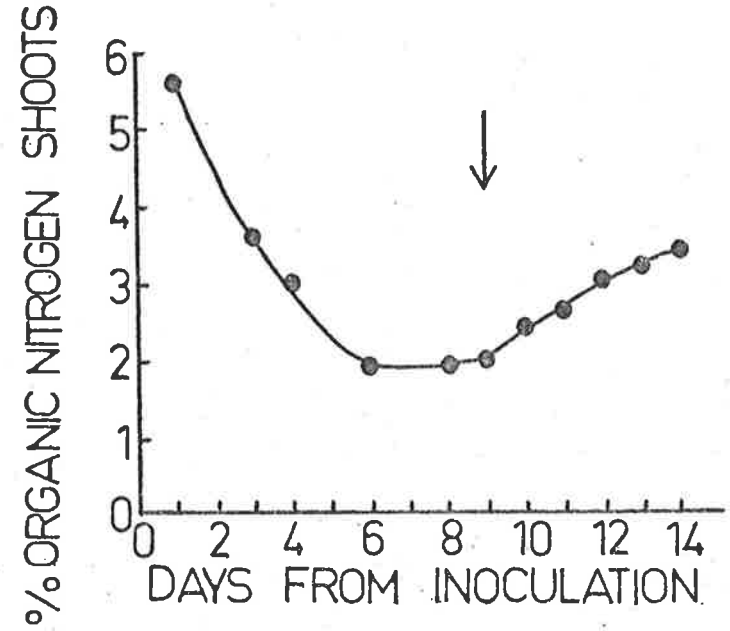
Communities were grown at 20°C and 1000 µmol quanta $m^{-2}s^{-1}$ (high PPFD) from emergence. They were inoculated and given - N nutrient solution as described in chapter 3, except for one community which was not inoculated and received 7.5 mM NO_3^- (+N) from the day on which the inoculated plants started to receive - N.

While CER was measured during nodulation for some communities, others were subjected to in vitro HE and AR assays to determine when N_2 fixation began, as described in chapter 3. Leaf areas were measured with a planimeter, so that the specific photosynthetic rate could be calculated. Growth of whole communities and of nodules was measured by increase in dry matter and fresh weight, respectively. The organic nitrogen concentration in shoots was found by Kjeldahl digestion and titration.

4.3.3. Results.

HE and AR data for the early stages of N_2 fixation are shown in Table 15. N_2 fixation began 9 days after inoculation (9DI) and was





ARROWS SHOW WHEN N_2 FIXATION BEGAN

Figure 13, (Top). Increase in community DM and in nodule mass during nodulation at 20C and high PPFD.

Figure 14, (Bottom). Change in nitrogen concentration of shoots during nodulation at 20C and high PPFD.

initially very inefficient for, despite a high rate of nitrogenase activity, most of the energy available to reduce N_2 was used to reduce H^+.

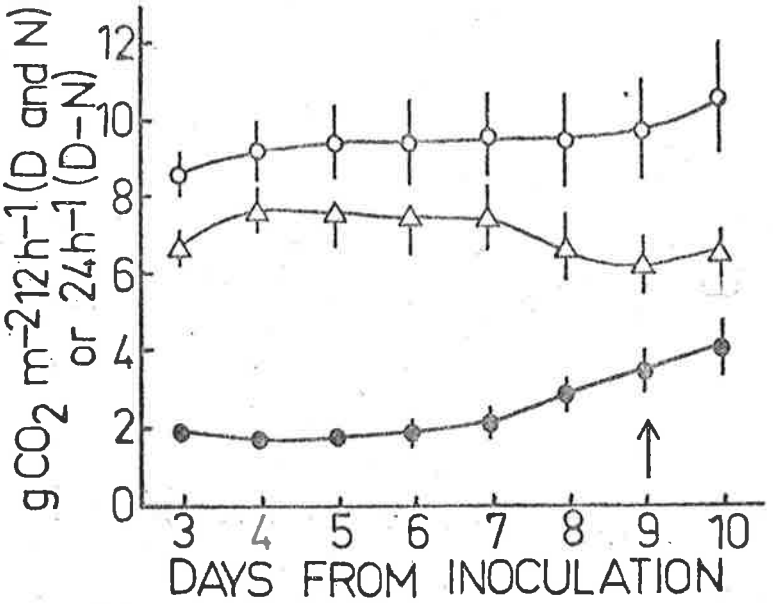
Prior to 9DI the communities experienced a period of nitrogen deprivation. The consequence of this for community growth is shown in Figure 13, where it can be seen that dry matter accumulation slowed markedly before nodules began to fix N_2. Nodule mass increased linearly with time from the first day that nodules were large enough for excision, 5DI. Change in the organic nitrogen concentration in the

TABLE 15 The nascent period of nitrogenase activity at 20°C and high PPFD. Means of 4 replicates, with SE.

Days from sowing	AR	HE	AR-HE	RE
	μmol C_2H_4 $m^{-2}h^{-1}$	μmol H_2 $m^{-2}h^{-1}$	$m^{-2}h^{-1}$	
7	-	-	-	-
9	540 (20)	487 (8)	53 (17)	0.10 (0.03)
11	450 (15)	346 (22)	104 (36)	0.23 (0.07)
13	700 (32)	564 (17)	136 (32)	0.19 (0.04)
14	890 (38)	550 (8)	340 (32)	0.38 (0.02)
15	691 (68)	439 (21)	251 (47)	0.36 (0.03)

shoots is shown in Figure 14. Nitrogen was rapidly diluted as new leaves appeared, stabilising at less than 2% during the time when the growth rate had slowed considerably. Before N_2 fixation began, leaves were pale green, and the specific rate of photosynthesis fell from 27.7 g CO_2 m^{-2} leaf area 12 h^{-1} at 2DI to 1.26 g CO_2 m^{-2} leaf area 12 h^{-1} at 9DI.

In Figure 15, CER data are presented for the period covering nodulation. D, the daytime uptake of CO_2 for the whole sward, changed



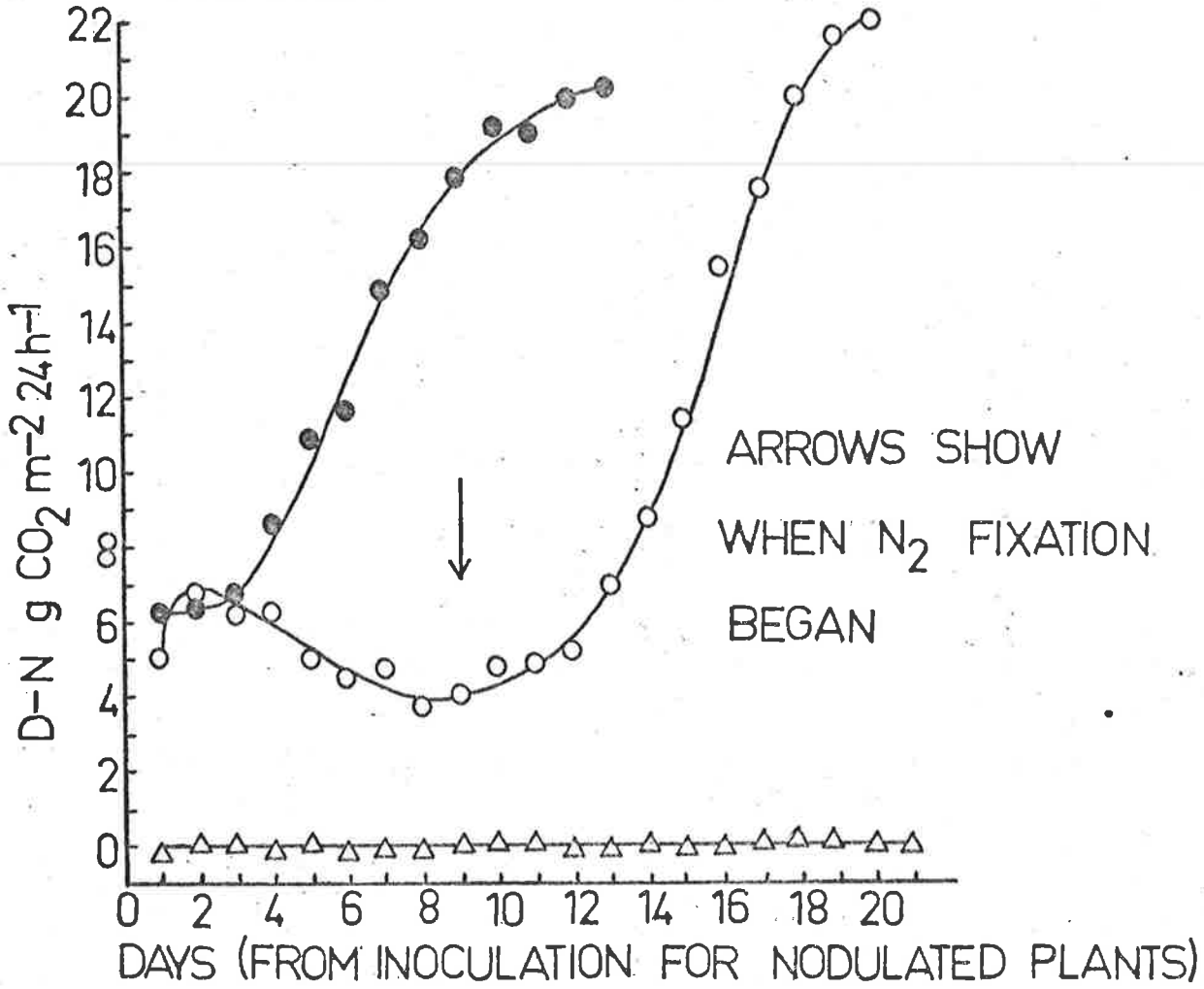


Figure 15, (Top). CER during nodulation at 20C and high PPFD ∘ D, • N, △D–N. Means, with SE, for 5 values.

Figure 16,(Bottom). Growth rates of nodulated (∘) and non-nodulated (•) plants at 20C and high PPFD. CER for control pot of inoculated oil– dry △.

very little between 3 and 10DI, but N, the nighttime efflux of CO_2, began to increase at about 7DI, by which time the nodules were growing rapidly (Figure 13). Thus, the growth rate measured over 24 hours, D-N, was decreasing before N_2 fixation began.

The combined effects of nodulation and nitrogen deficiency on inoculated plants are evident in Figure 16. The growth rate of a nodulated community is compared with that of a non-nodulated community receiving combined nitrogen. There was a lengthy pause in the growth of N_2-fixing plants before growth accelerated when N_2-fixation began at 9DI, but the growth of non-nodulated plants was unabated until they approached canopy closure, which occurred 8 days sooner than for nodulated plants of the same age.

4.3.4. Discussion.

Synthesis respiration during nodulation was probably small, while maintenance respiration was probably steady because little new biomass was being made. Hence, the increase in respiration before N_2 fixation began (Figure 15) can be attributed to nodule growth. Nodule growth continued apparently independent of the hesitation in community growth (Figure 13), suggesting that assimilates were preferentially directed to the nodules. The nodules constituted 3.5% of sward fresh weight by the time N_2 fixation started.

Once N_2 was being fixed, the growth of the nodulated communities accelerated more rapidly than that of non-nodulated plants (Figure 16). This may have been due to the sudden influx of nitrogen to existing leaves and their improved photosynthetic viability, noted visually in the greening of previously pale tissues.

Continued growth without N_2 fixation during nodulation brought the nitrogen concentration in shoots down to a level which almost qualified them as senescent (Figure 14). Peat *et al*. (1981) suggested that nitrogenase activity in *G.max* seedlings was inhibited by substances emanating from the cotyledons, a device which permitted increase in leaf area to occur without additional below-ground competition for carbohydrate. A similar mechanism may operate in *T.subterraneum*.

The results are generally in agreement with those of Williams *et al*. (1981) which showed that the growth of *G.max* during nodulation was limited by nitrogen. The growth responses of communities transferred to different environments at 10DI, when N_2 fixation had just begun, might have reflected in part the capacity of communities to recover from nitrogen deficiency during nodulation. As reported in chapter 5, the RE of N_2 fixation was low in early growth after transfer to $10^{o}C$ and $15^{o}C$ at high PPFD. This is suggested to have been due to the period of low RE seen at $20^{o}C$ (Table 15) being extended by slowed nodule development on transfer to low temperature.

T.subterraneum would probably benefit from an application of 'starter' nitrogen during early growth, but this should be applied at a low level because combined nitrogen inhibits root hair infection, by *Rhizobium*, and N_2 fixation.

CHAPTER 5

5. Relations between N_2 Fixation and temperature.

5.1.0. Introduction.

Survey of the literature had revealed that the ways in which temperature influenced N_2-fixation by mature legume communities were largely unknown. Previous work on T.subterraneum in this area had been with single plants grown in agar (Gibson, 1961 ; Gibson, 1963). In particular, the responses of HE to temperature had not been examined, and the role of Hup was unclear. Cocks (1973) and Fukai and Silsbury (1976) had shown the effects of temperature on the growth of legume communities to be very different from those of single plants. Roughley et al (1976) had shown temperature to have a marked influence on the physiological ageing of nodules, and the consequence of this for N_2 fixation during community growth had not been examined.

The experiments reported in this chapter were designed to determine and quantify some of the specific interactions between temperature and the growth and N_2 fixation of T.subterraneum swards. Broadly, four areas were investigated. These were:

(i) the relations between nitrogen accumulation, the AR assay, and HE;

(ii) the existence of Hup;

(iii) the induction of Hup, and calculation of the K_m of Hup for H_2;

(iv) the interaction of temperature with inhibition of N_2 fixation by combined nitrogen.

5.2.0. Methods.

Communities were grown at two levels of PPFD, 1000 and 500 µmol quanta $m^{-2}s^{-1}$, which are referred to as high and low PPFD, respectively. They correspond to about 75% and 47% of PPFD-saturation of P_N for T.subterraneum communities at 20°C (Silsbury, 1981).

Growth temperatures were maintained constant at 10°C, 15°C, 20°C, or 25°C. A few swards were maintained at 30°C, but this temperature was soon abandoned because the plants grew very poorly. 10°C to 25°C covers the range of mean soil temperatures experienced by pasture legumes in South Australia between June and November, which is the period of rapid vegetative growth (Waite Agricultural Research Institute Biennial Report, The University of Adelaide, 1980-1981). The dry matter yields obtained in the growth cabinet were as high as 570 g m^{-2}.

Rates of AR and HE were determined for 6 communities, by destructive harvest, at intervals throughout the growth period and changes in these attributes were assessed in relation to the amount of biomass present rather than with respect to time. The CER measurements, reported in chapter 4, enabled the amount of biomass present at a given time to be forecasted with reasonable accuracy.

Determination of the amount of organic nitrogen present at each harvest enabled the construction of curves for nitrogen accumulation as a function of time. These were always linear, so the regression of organic nitrogen on time allowed an 'absolute' rate of N_2 fixation to be calculated for each of the 8 environments used.

When the RE value indicated that Hup was operating, the existence of Hup was tested for in two ways. In one method, a quantity of H_2 (usually 500 µl, about 21 µmol) was added to plants in the assay vessels after HE and AR assays, in the presence of p0.1 C_2H_2 to inhibit

HE but not Hup, and the rate of disappearance of H_2 was measured by gas chromatography as described by Nelson and Salminen (1982). The effect of Hup on nitrogenase activity was examined by comparing the rates of AR before and after H_2 was added. Alternatively, the HE rate in an Ar (80%) : O_2 (20%) atmosphere was compared to the AR rate. Both assays have been reported to estimate the total electron flux when Hup is absent (Schubert and Evans, 1976). If Hup is present, the HE rate is less than the AR rate. This technique was only used with plants which had been found to evolve no H_2 and to which no AR assay had been applied, because C_2H_2 inhibits HE.

The induction of Hup, or acceleration of its appearance, was studied by growing plants hydroponically in a glasshouse at 25^oC and supplying them with 2% H_2 in the aeration stream. Except for the gas inlet and outlet, the 8l containers used in this experiment were made airtight. Nutrient solution was renewed every day, as were the airtight seals between the top and bottom of the container. Control plants received no H_2. The aeration rate was 1l min^{-1}.

Using a batch of swards that were known to be showing Hup, two replicates were incubated in the presence of p0.1 C_2H_2 with each of 5 concentrations of added H_2. The initial rates of H_2 uptake were measured over the first 10 minutes of incubation, and a correction for H_2 adsorption and leakage was made, according to Figure 6, so that the true rates of uptake, V, were obtained. The slope of the line relating V to V/S, where S was the concentration of H_2, was equal to the K_m of Hup for H_2 (Hofstee, 1952).

Some control swards, receiving NO_3^-, were subjected to HE and AR assays, at 20^oC and 10^oC. Nitrogenase activity was found at 10^oC, and this was investigated further using 4 hydroponically-grown communities which were transferred from -N nutrient solution to +N solution (7.5 mM

NO_3^-) at 10°C so that the effect of combined nitrogen on HE and AR could be observed by repeated assays. Four control communities were maintained in -N solution during the course of the experiment and were also assayed for HE and AR. Nutrient solutions were renewed daily, and the aeration rate was 0.11 min^{-1}.

5.3.0. Results.

5.3.1. Relations between nitrogen accumulation, the AR assay, and HE.

The AR, HE, and N_2 fixation rates (calculated from organic nitrogen accumulation), are given in Table 16. Means for each variable have been compared between temperatures for a range of biomass common to all temperatures. The N_2 fixation rate was the yardstick to which the AR and HE data were compared. The N_2 fixation rates were similar at 10°C and 15°C, and decreased markedly above 15°, thus showing the same trend as was observed for the growth rate (Table 10). Clearly, the N_2 fixation rate was, in the long term, a function of the effects of temperature on community growth.

Between 10°C and 20°C, the effects of temperature on N_2 fixation can be seen to have been paralleled in those on AR, but at 25°C N_2 fixation was depressed whereas AR was stimulated. Surprisingly, AR-HE appears not to have been a reliable index with which to have measured the response of N_2 fixation to temperature, as the differences in AR-HE between temperatures did not mirror those in N_2 fixation at either PPFD (Table 16). The inadequacy of AR-HE arose out of an effect of temperature on HE. This can be seen in Figures 17 and 18. In comparison with the other temperatures, HE at 10°C was relatively stable throughout

TABLE 16 Analysis of variance for the effects of temperature on nitrogenase activities. 95-475 g m^{-2} biomass range. Means of 24 values, with SE.

Temperature	AR µmol $C_2H_4m^{-2}h^{-1}$		HE µmol $H_2m^{-2}h^{-1}$		AR-HE		RE		N_2 fixation µmol $N_2m^{-2}h^{-1}$	
	High++	Low++	High	Low	High	Low	High	Low	High	Low
10°C	660A	432A	403A	254A	257A	178A	0.40A	0.41A	924A	538A
	(31)	(17)	(29)	(19)	(19)	(14)	(0.03)	(0.03)		
15°C	605AB	486AC	330A	236A	275A	250B	0.51A	0.56B	904AB	597A
	(36)	(25)	(48)	(39)	(21)	(21)	(0.05)	(0.06)		
20°C	533B	293B	211B	88B	319A	205AB	0.62B	0.77C	714B	408B
	(22)	(27)	(26)	(21)	(19)	(18)	(0.04)	(0.05)		
25°C	842C	537C	98C	126B	744B	410C	0.88C	0.76C	529C	279C
	(37)	(19)	(28)	(22)	(45)	(27)	(0.03)	(0.04)		
F Value for AOV	17.14**	22.63**	15.77**	9.93**	66.24**	25.20**	28.62**	13.94**	+	+
LSD P0.05	89.53	61.91	94.76	72.86	80.00	58.10	0.11	0.13		

** Significant at P0.01

\+ Linear regression lines, of organic nitrogen on time, were compared as described in pp. 135-141, and pp. 432-436, of Snedecor and Cochran (1967).

Figures followed by different letters within columns are significantly different at P0.05.

++ PPFD

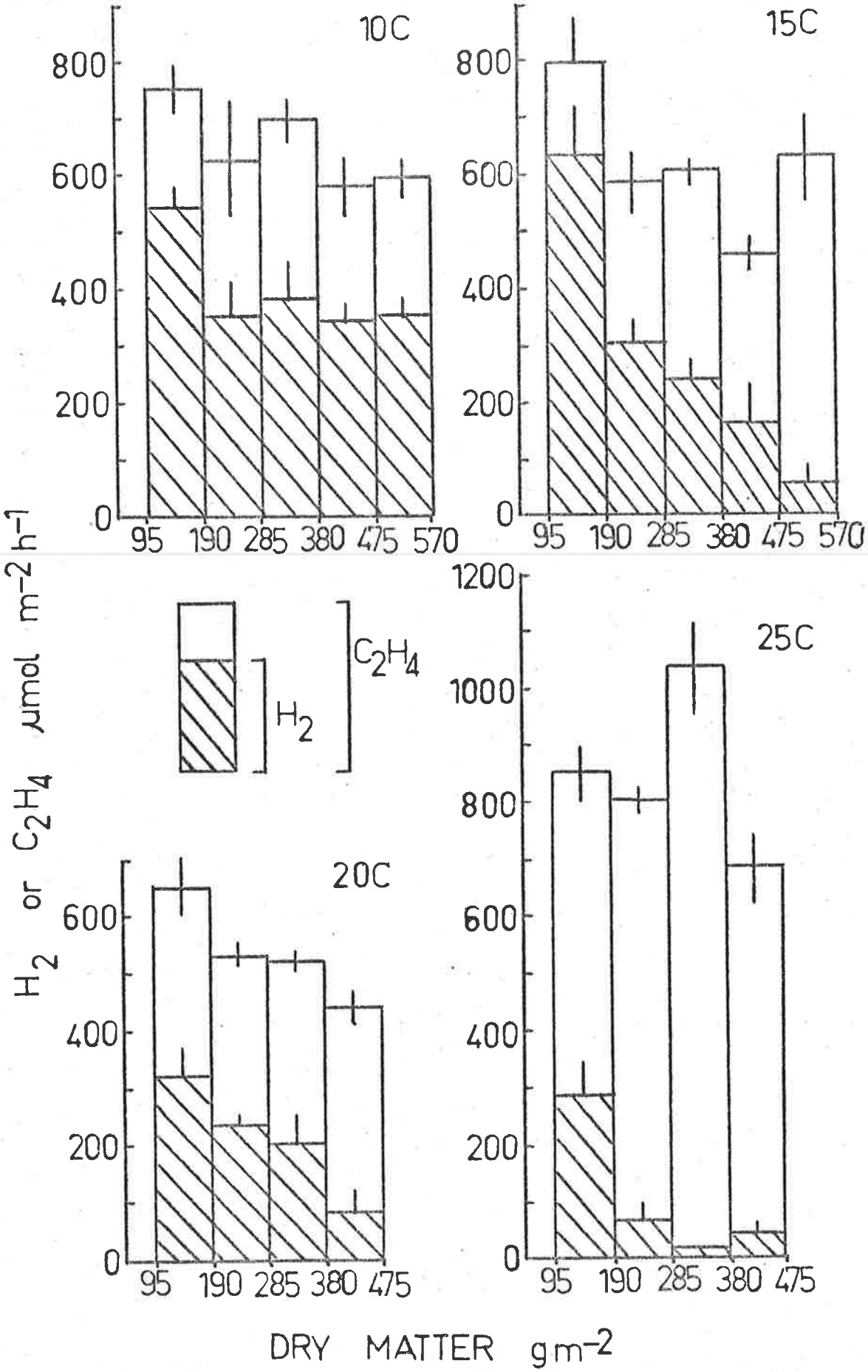


Figure 17. Ontogenetic patterns in HE and AR at high PPFD. Means, with SE, for 6 values. Only one half of error bar shown for H_2.

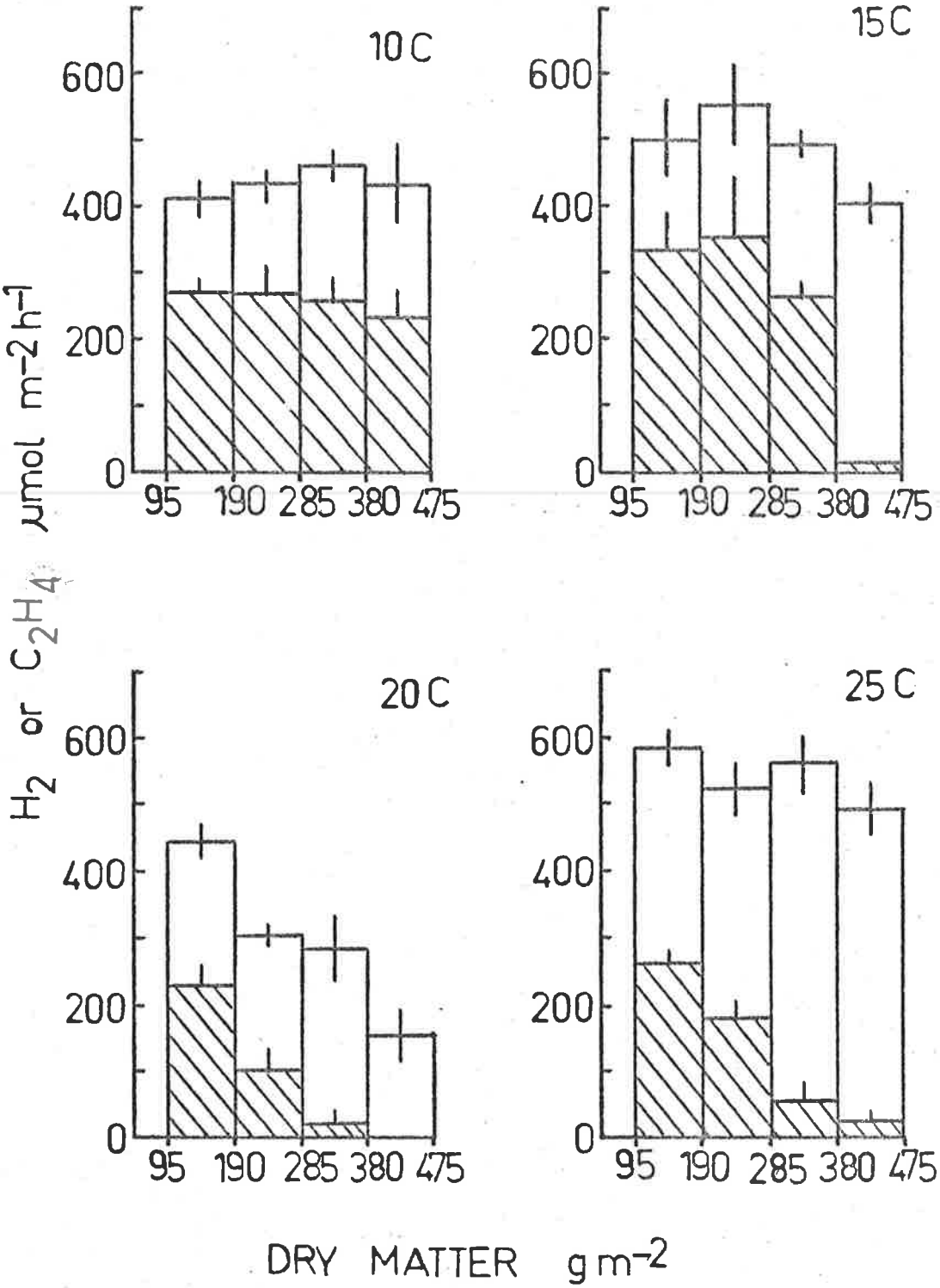


Figure 18. Ontogenetic patterns in HE and AR at low PPFD. Symbols as for figure 17.

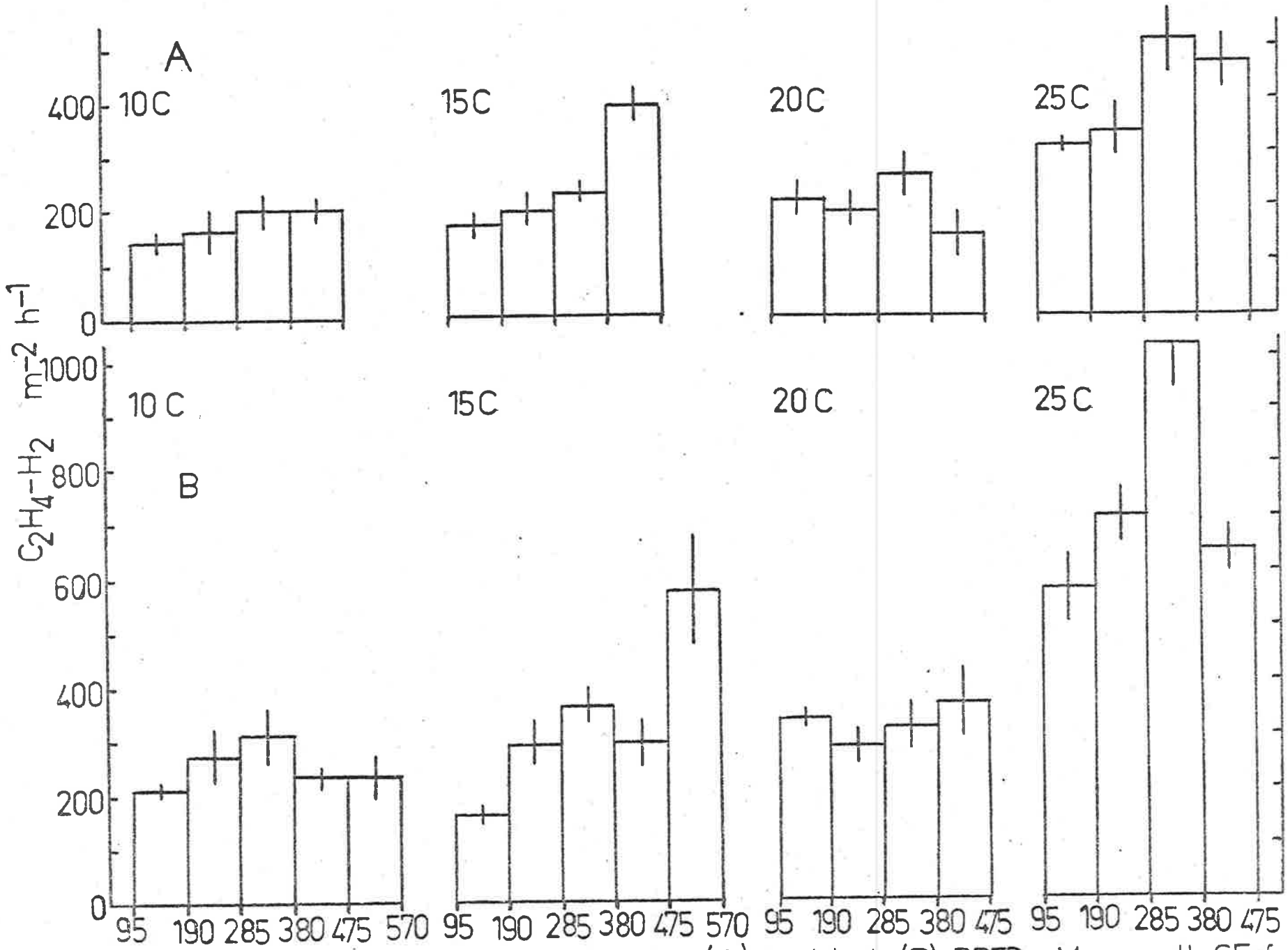


Figure 10 Ontogenetic patterns in AR-HF at low (A) and high (B) PPFD. Means, with SE, for 6 values.

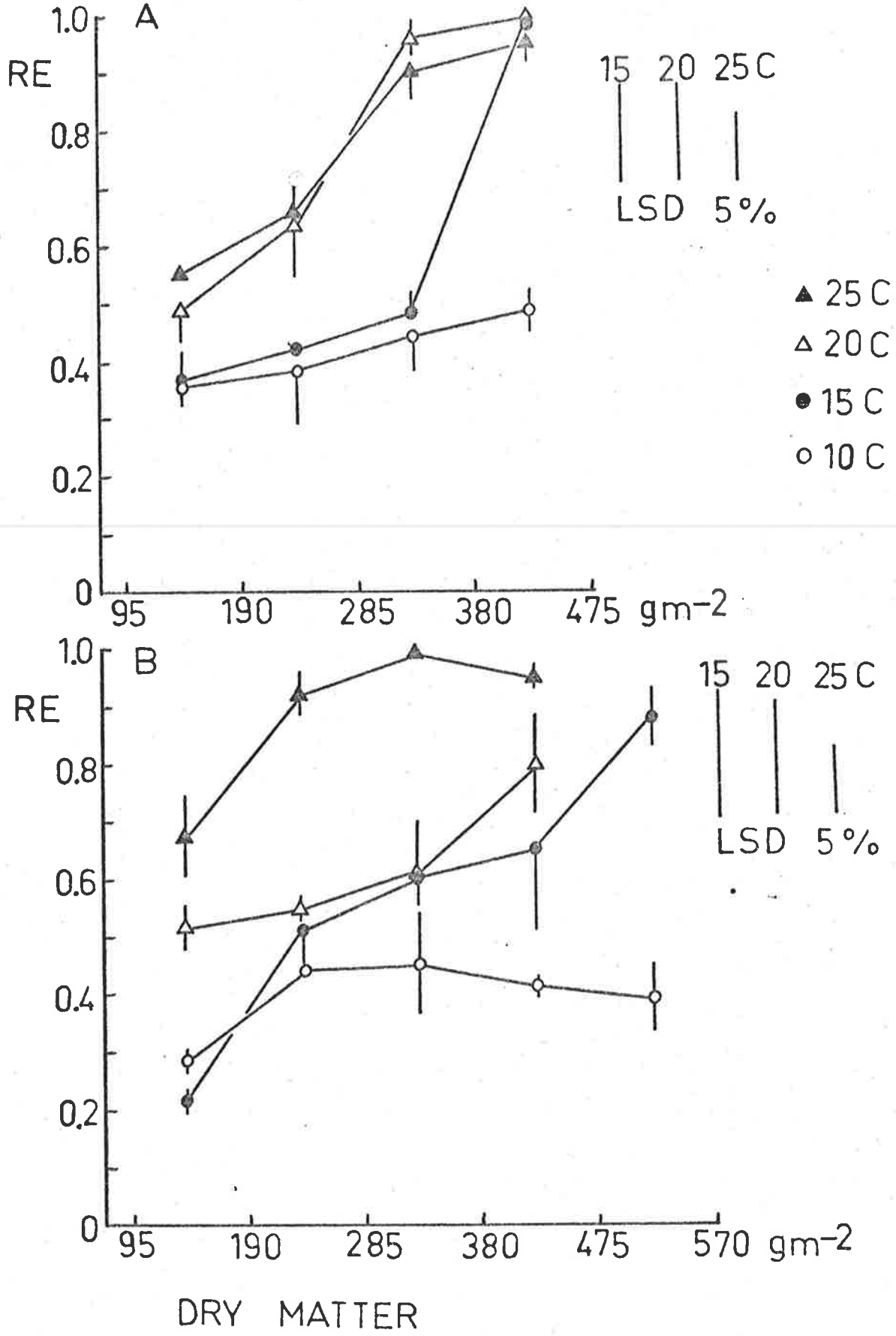


Figure 20. Ontogenetic change in RE as affected by temperature at low (A) and high (B) PPFD.

community growth, as was AR. As a result, at 10°C AR-HE showed little variation with increase in biomass (Figure 19; Appendix I). This was the expected response for communities which had an essentially steady growth rate. However, at 15°C, 20°C, and 25°C, there was a pronounced decline in HE with increase in biomass at both PPFDs (Figures 17 and 18). At 15°C and 25°C this decline was not accompanied by a proportionate decrease in AR, so that AR-HE and RE both increased greatly with age (Figure 19 ; Figure 20 ; Appendices II and III). RE also increased with age at 20°C (Figure 20) but AR-HE did not because AR decreased during ontogeny (Figures 17, 18 and 19).

The N_2 fixation rate at low PPFD showed a slightly different response to temperature from that at high PPFD in that 15°C appeared to be an optimum temperature for N_2 fixation, although not a statistically-significant one (Table 16). In Table 17 this is shown to have resulted in biomass produced at 15°C and low PPFD having the highest organic nitrogen concentration of any treatment, because 15°C was not an optimum temperature for the growth rate at low PPFD (Table 10). The nitrogen concentrations of roots and of live shoots were similarly and adversely affected by temperature above 15°C at low PPFD, and above 10°C at high PPFD (Table 17).

The effects of PPFD on community growth, AR, HE and N_2 fixation, were presented earlier in Table 9. The N_2 fixation rate was increased at the high PPFD over that at the low PPFD at all temperatures, in concord with similar increases in the growth rate (D-N). AR adequately indicated the increases in N_2 fixation at all temperatures, but AR-HE failed to do so at 15°C. The spurious result for 15°C might be partially explained through the difference in N_2 fixation rates between PPFDs being of a lower statistical significance at 15°C than at the other temperatures.

TABLE 17 Organic Nitrogen concentrations of plant fractions

Temperature, °C	Roots		Shoots		Senescent shoots		Total	
	High+	Low+	High	Low	High	Low	High	Low
10	3.74 (0.06)++	3.48 (0.05)	3.65 (0.14)	3.71 (0.07)	1.33	ND	3.67 (0.10)	3.65 (0.05)
15	3.43 (0.07)	3.86 (0.04)	3.51 (0.08)	4.07 (0.05)	1.33	1.69	3.49 (0.07)	4.01 (0.04)
20	3.23 (0.07)	3.37 (0.04)	3.39 (0.08)	3.68 (0.05)	1.34	1.29	3.33 (0.06)	3.53 (0.04)
25	3.10 (0.08)	3.00 (0.07)	3.23 (0.07)	3.17 (0.06)	1.10	1.29	3.15 (0.05)	3.05 (0.05)

ND Not determined

\+ PPFD

++ Means followed by standard errors are for 9 to 11 values.

From the AR and N_2 fixation data given in Table 16, it is clear that there was some disturbance to the relationship between AR and N_2 fixation above 20°C, such that the two quantities diverged with respect to their response to temperature. This is also evident from Figures 17 and 18. The ratios of C_2H_4 : N_2 and C_2H_4-HE : N_2 have been calculated for a range of biomass common to each temperature, and are presented in Table 18. In theory, the C_2H_2 : N_2 ratio should be larger than 3:1 if HE occurs. For example, if 25% of the electron flux was used in HE (RE 0.75) and 8 electrons passed through nitrogenase, 2 electrons would be used in reducing $2H^+$ to H_2 and 6 electrons would be used in reducing N_2 to $2NH_3$. In an AR assay, all 8 electrons would be used in reducing 4 C_2H_2 molecules to 4 C_2H_4 molecules. In this instance, the C_2H_2 : N_2 ratio would be 4 : 1. The C_2H_4 : N_2 ratios in Table 18 were all well below the theoretical ratio, but were nevertheless fairly steady between 10°C and 20°C, ranging from 0.67 : 1 to 0.81 : 1. However, above 20°C there was a sharp increase in the ratio. The C_2H_4 - HE : N_2 ratio increased between 10°C and 20°C, due to the ontogenetic decrease in HE mentioned earlier, and increased abruptly at 25°C.

Bethlenfalvay and Phillips (1977b) set great store by the ratio of moles of N_2 fixed to moles CO_2 fixed, as a means of expressing the efficiency of N_2 fixation in terms of photosynthesis. Since the AR : N_2 ratio was affected by temperature (Table 18), and AR varied diurnally (chapter 6), directly measured N_2 fixation rates were used to calculate the N_2 : CO_2 ratios in Table 19. The ratio showed no obvious response to PPFD, but varied with temperature similarly with the nitrogen concentration of whole communities.

The very low rates of HE, at high biomass for 15°C and 20°C, and at 25°C (Figures 17 and 18) indicated that Hup was operating. Results of the investigation of Hup are given in the next section.

TABLE 18 Relationship of AR, and of AR-HE, to N_2 fixation. Means with SE are for 24 values in the biomass range 95-475 g m^{-2}, except for 30°C which is a mean and SE for 19 values.

	TEMPERATURE								
	10°C		15°C		20°C		25°C	30°C	
Quantity	High+	Low+	High	Low	High	Low	High	Low	High
µmol $N_2 m^{-2} h^{-1}$	924	538	904	597	714	408	529	279	44
µmol $C_2H_4 m^{-2} h^{-1}$	660(31)	432(17)	605(36)	486(22)	533(25)	293(27)	842(37)	537(19)	95(15)
C_2H_4 : N_2	0.71:1	0.80:1	0.67:1	0.81:1	0.75:1	0.72:1	1.59:1	1.93:1	2.17
C_2H_4-$H_2 m^{-2} h^{-1}$	257(19)	178(14)	275(21)	250(21)	319(19)	205(18)	744(45)	410(27)	-
C_2H_4-HE : N_2	0.28:1	0.33:1	0.30:1	0.42:1	0.45:1	0.50:1	1.41:1	1.47:1	-

+ PPFD

TABLE 19 Ratios of N_2 fixed to CO_2 fixed.

Quantity	TEMPERATURE 10°C High+	10°C Low+	15°C High	15°C Low	20°C High	20°C Low	25°C High	25°C Low
µmol $N_2 m^{-2} h^{-1}$	924	538	904	597	714	408	529	279
mmol $CO_2 m^{-2} h^{-1}$++	60.5	39.1	65.8	38.5	60.4	34.8	50.2	30.3
µmol N_2 µmol CO_2 x 10^{-3}	15.3	13.8	13.8	15.5	11.8	11.7	10.5	9.2

\+ PPFD

++ Calculated from D

5.3.2. The existence of Hup.

The increases in RE shown in Figure 20 suggested that Hup had developed at high biomass at 15°C, and much sooner at 25°C, with 20°C being somewhat intermediate. Visual inspection of the nodules at each temperature indicated that the physiological ageing of nodules was accelerated by rise in temperature. At 25°C, the pink colour which characterises the presence of leghemoglobin, became progressively less intense with age, whereas at 10°C the nodules were dark pink even in the highest range of biomass. Some nodules at 25°C were pale green, indicating that leghemoglobin had been oxidised (Virtanen et al., 1955).

When H_2 was added to communities with a high RE, after an AR assay, but in the presence of C_2H_2 to inhibit HE, the H_2 was rapidly taken up. Also, when the rate of HE in Ar (80%) : O_2 (20%) was compared with the rate of AR, the two rates were similar for young communities at 20°C but, for communities of high biomass at 20°C, the HE rate was much lower than the AR rate (Table 20). Thus, Hup was concluded to have been operating and to have developed during ontogeny.

TABLE 20 HE in Ar (80%):O_2(20%) and AR rate for young and old communities at 20°C. Means, with SE, for 4 values.

Age of Communities	HE Ar(80%):O_2(20%) µmol H_2 $m^{-2}h^{-1}$	AR µmol C_2H_4 $m^{-2}h^{-1}$
Young	499 (92)	480 (80)
Old	292 (54)	660 (40)

Hup was never detected below an RE of 0.85, but was not always found to occur at REs greater than this.

When H_2 was added to plants with Hup during the course of an AR assay, there was no effect on the AR rate. AR continued at the same rate as before the H_2 addition. This differs from others' results (Emerich et al., 1979 ; Dadarwal et al., 1982) which showed AR to be stimulated. H_2 oxidation may, therefore, not have been linked to ATP production (Nelson and Salminen, 1982) and may have served some purpose other than energy conservation.

5.3.3. Induction of Hup, and calculation of the K_m of Hup for H_2.

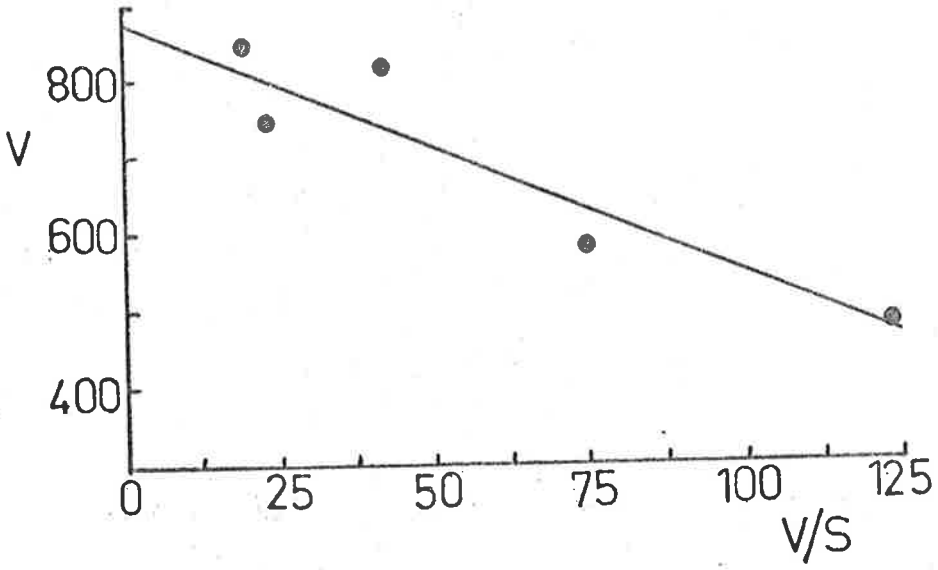
Maier et al. (1978) reported that development of Hup was dependent on preincubation with H_2 in R.japonicum, whereas Ruiz-Argueso et al. (1981) reported that H_2 was not required to induce Hup.

The result of an experiment in which it was attempted to induce Hup in T.subterraneum, by supplying plants with 2% H_2, is shown in Figure 22. Although there was clearly an effect of H_2 on RE, in no instance was Hup detected. This was in accordance with the finding of Nelson and Child (1981) that a high RE was not necessarily an indication of Hup. The observed increase in RE in the presence of H_2 is interpreted as a secondary effect of H_2 being, rather paradoxically, an inhibitor of N_2 fixation (Scherer et al., 1980) since both the growth rate and AR-HE were depressed by the H_2 treatment.

The K_m of Hup for H_2 was calculated from the rates of H_2 uptake (V) at a range of H_2 concentrations (S) using a Hofstee (1952) plot which is shown in Figure 21. The fitted line is defined by:

$$y = -3.57x + 887 \quad (r = 0.94)$$

where $y = V$, and $x = V/S$, $K_m = 3.57\ \mu M$



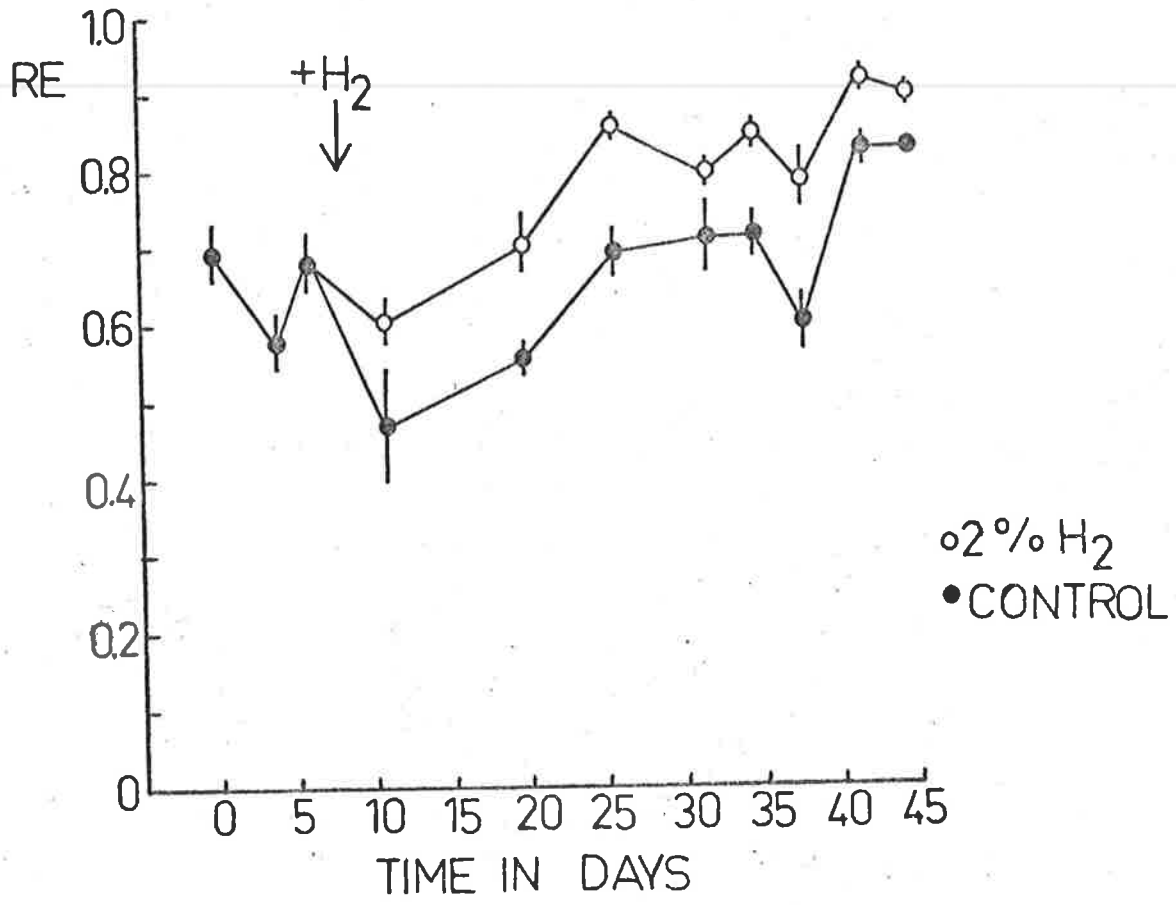


Figure 21, (Top). Hofstee plot for H_2 uptake. V is μmol H_2 taken up h^{-1} (from initial rate), S is μmol H_2 present at start. Slope, −3.57μM, is K_m. Means are for 2 values.

Figure 22, (Bottom). Effect of H_2 on RE at 25C. Means, with SE, for 4 values.

5.3.4. Interaction of temperature with inhibition of N_2 fixation by combined nitrogen.

The AR and AR-HE rates for swards which received combined nitrogen are shown in Table 21.

TABLE 21 Nitrogenase activities of communities, at 10°C and 20°C, which received 7.5 mM NO_3^-.

Temperature, °C, and PPFD	Biomass g m^{-2}	AR µmol $C_2H_4 m^{-2} h^{-1}$	AR-HE $m^{-2} h^{-1}$
20, low	96	43	32
	171	29	24
	175	+	+
	190	+	+
20, high	300	20	10
	350	5	5
	438	+	+
	534	+	+
	574	+	+
10, high	186	399	187
	283	319	191
	338	150	110
	427	155	129
	645	64	51

\+ Very low activities.

Nitrogenase activity of communities at 20°C was reduced to very low or negligible levels by combined nitrogen, both at high and low PPFD.

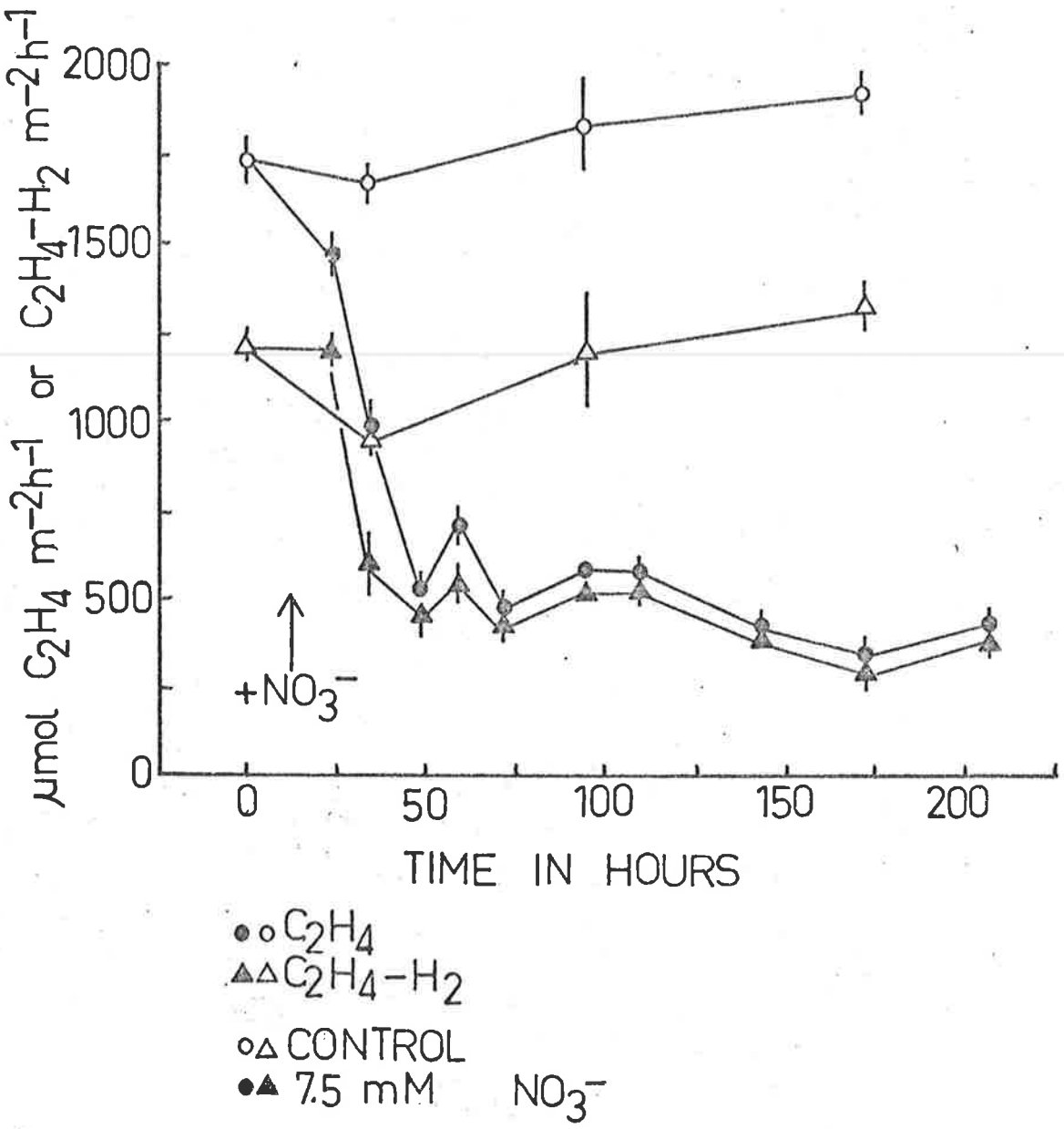


Figure 23. Effect of combined nitrogen on AR and AR-HE at 10C and high PPFD. Means, with SE, for 4 values.

The nodules of these plants were observed to be mostly white or pale green, and were often loosely attached to the roots. However, at $10^{o}C$, combined nitrogen had a much weaker inhibitory effect on nitrogenase activity and many of the nodules showed the pink colour which denotes presence of leghemoglobin. The nodules were also more firmly attached to the roots at $10^{o}C$.

The time course of inhibition of nitrogenase activity by combined nitrogen at $10^{o}C$ is shown in Figure 23. It can be seen that AR and AR-HE were depressed within 24 hours of the hydroponically-grown communities being transferred to +N (7.5 mM NO_3^-) nutrient solution. AR-HE, theoretically proportional to the N_2 fixation rate, then stabilized to about 30-40% of the AR-HE rate of the controls over the next week.

5.4.0. Discussion.

5.4.1. N_2 fixation and temperature.

The primary aim of this project was to assess the effect of growth temperature on N_2 fixation in T.subterraneum swards. However, because treatments were extended over a long period of vegetative growth, the results permit comment on the utility of the AR technique in quantifying environmental effects on N_2 fixation, and also on the ontogenetic development and role of Hup.

An appraisal of the competence of the HE and AR assays to estimate N_2 fixation is critical to this project, and also to much of the literature reporting the use of 'uncalibrated' AR assays. The AR assay has been widely used because of its simplicity and, as a consequence of the enthusiasm to answer questions that were previously unanswerable by other techniques, it has often been applied with scant regard to its

limitations (see Knowles, 1981). As a result, there is a view expressed in sections of the literature that the AR assay is unreliable. This view is likely to become more widely accepted in the light of recent evidence that, in some species, the AR assay is not linear with time (Minchin et al., 1983). However, it is considered that the present studies have shown the AR assay to have been useful for comparative experiments when it was consistently applied.

The effect of PPFD on N_2 fixation was adequately reflected by AR at all temperatures (Table 9), as was the effect of temperature on N_2 fixation between $10^{o}C$ and $20^{o}C$ (Table 16). Above $20^{o}C$, AR did not parallel N_2 fixation. While assay technique has been recognised as a factor which affects the AR rate, as a result of mechanical disruption to the whole plant (Masterson and Murphy, 1976 ; Wheeler et al., 1978), there has been no reference to any effect of temperature impinging on the validity of AR in estimating N_2 fixation rates of whole plants. Clearly, at $25^{o}C$ and $30^{o}C$ there was a temperature-induced disruption of the stoichiometric relationship between AR and N_2 fixation (Table 16). A similar temperature effect on AR can be calculated from the data presented by Munevar and Wollum (1981). It is likely that, above $20^{o}C$, there was an effect of temperature on nitrogenase kinetics which favoured reduction of C_2H_2 but not of N_2.

There is evidence in the literature that the kinetics of AR are in some way affected by temperature, since E_A (the activation energy) for AR by nitrogenase was shown to change sharply at just above $20^{o}C$ (Waughman, 1977 ; Cralle and Heichel, 1982). However, it should not be assumed that a similar change occurs for N_2 reduction because, although C_2H_2 and N_2 reductions draw on the same electron reservoir (the MoFe protein of nitrogenase), they do so at different sites on the enzyme

(Rivera-Ortiz and Burris, 1975). Thus, it is feasible that, at about 20°C, nitrogenase underwent a conformational change which differentially affected the binding of C_2H_2 and N_2. If this was true, then their relative stimulations to the electron flux caused through binding would likewise have been differentially affected (Thorneley and Eady, 1977). A second explanation centres on the capacity of the MoFe protein to accumulate the 6 electrons required to reduce N_2. The capacity for this might have been adversely affected by high temperature, the capacity to hold 2 electrons being unaffected. Hageman and Burris (1980) provided evidence that the electron flux through nitrogenase in A.vinelandii could be decreased by alterations in the MgATP concentration or in the ratio of the nitrogenase subunits one to another, and that this decreased the allocation of electrons to N_2 while allocation to C_2H_2, or H^+, was increased. At low electron flux the K_m of nitrogenase for C_2H_2 decreased while that for N_2 was relatively unchanged. Ribulose bisphosphate carboxylase : oxygenase, also a bifunctional enzyme, was found to undergo similar changes in affinity for its substrates, CO_2 and O_2, if the temperature was varied (see Berry and Bjorkman, 1980). Seemingly, then, a high temperature effect on the subunit ratio - perhaps through their relative rates of turnover - could have affected the relationship of C_2H_2 and N_2 reductions to each other through an alteration in electron flux.

The ratios of C_2H_4 produced to N_2 fixed were similar to others found for G.max, V.faba, M.truncatula, and P.vulgaris (Mague and Burris, 1972 ; Sprent and Bradford, 1977 ; Ruegg and Alston, 1978 ; Rennie and Kemp, 1981) in being much lower than the theoretical ratio of greater than 3 : 1 (Table 17). The ratios were increased from those given in Table 17 by 4 times when continuous-flow AR assays were performed (Figure 5) rather than assays in sealed vessels. It is, therefore,

postulated that the low ratios were an artifact of technique, but one which was constant and clearly did not affect the utility of the AR assay in making the comparisons required of it (Table 16). The AR assay depends on diffusion of gas into and out of nodules, and this was probably expedited in the continuous-flow assays by lowered boundary layer resistances to diffusion and removal of water films. Gaseous diffusion through water is far slower than through air.

It was interesting to find interactions of age and temperature with HE and Hup. The data in Figures 20 and 22 suggest that RE gradually increased during ontogeny. This resulted from parallel decreases in AR and HE even when Hup was not operating (Figures 17 and 18). Plasticity in RE, which hints at the capability for electron allocation to substrates to be altered, has not always been observed (Schubert et al., 1977 ; Saito et al., 1980 ; Silsbury, 1981).

Hup in R.trifolii has only been alluded to in the past, for example, strain HFRO 4 was said to have "evolved no H_2" (Haystead and Sprent, 1981). There is a report of R.trifolii DT6 having Hup activity, measured by tritium exchange (O'Gara and Hynes, 1981). Development of Hup was probably brought about through the physiological ageing of the nodules, a process which was accelerated by increase in temperature. The amount of leghemoglobin in nodules has been shown to decrease during ontogeny in P.sativum, (LaRue and Child, 1979), and visual inspection of the colour of nodules suggested it to have done so in T.subterraneum also, especially at 25^{o}C. Since Hup did not lead to stimulation of AR, it is suggested to have maintained an appropriate redox environment for the O_2-labile nitrogenase. This would have offset the diminishing capability of leghemoglobin to fulfil this function, in that H_2 oxidation removed molecular O_2 from the site of N_2 fixation. This role was first postulated by Dixon (1972) but has been neglected in the wake

of purported increases in yield arising from an energy-saving activity of Hup. When the leghemoglobin concentration of nodules was reduced during physiological ageing, Hup probably resulted in prolongation of the useful life of the nodules.

Operation of Hup explains the failure of AR-HE to mirror the effect of temperature on N_2 fixation, since the rate of H^+ reduction was masked by immediate H_2 uptake. Thus, it cannot be ruled out that the rate of H^+ reduction was very high at high 'apparent' RE, and that the true efficiency of N_2 fixation was low. Such a state of affairs would have offered the greatest O_2-protection to nitrogenase.

It may have been a consequence of the growth technique that Hup did not arise when plants were treated with a low concentration of H_2 (Figure 22). The plants used in this experiment were cultured hydroponically, and new nodules arose on young roots throughout the period of growth. Thus, it may have been unnecessary to prolong the utility of older nodules. Contrarily, in pot culture, nodules were most numerous on the oldest root tissue, and few young nodules were present on old plants.

It could be argued that the H_2 uptake recorded was not due to R.trifolii, but rather to H_2-oxidising bacteria associated with the plants and exterior to the nodules. A variety of bacteria and abiontic enzymes were said to take up H_2 in soil (Conrad and Seiler, 1981). However, after 37 days of growth in an environment supplemented with H_2 (Figure 22), no H_2 uptake was found to be associated with any plants and it, therefore, seems unlikely that such bacteria caused problems with other assays. When contaminating microorganisms have been found to take up H_2 evolved by legumes in soil, for example, from Cajanus cajan, T.subterraneum, and L.angustifolius (La Favre and Focht, 1983 ; Hopmans et al., 1983 ; Gibson, 1984), removal of the soil also removed the

contaminants. In this project all assays were conducted on roots carefully washed free from the inert growth medium. Also, the K_m of Hup for H_2 of 3.57 µM is in near agreement with that found for Hup in Rhizobium leguminosarum of 4 to 5.3 µM (Ruiz-Argueso et al., 1978 ; Nelson and Salminen, 1982). The K_m of H_2 - using bacteria common in soil was reported to be 100 to 1000 times less than this (Conrad and Seiler, 1981).

A reasonable judgement of the competence of HE and AR techniques to assess environmental influences on N_2 fixation is a qualified 'adequate'. The qualifications are that AR-HE, which was expected to be closely related to the N_2 fixation rate, did not estimate N_2 fixation when Hup was present because H^+ reduction could not be measured, and also that a change in the relationship of AR to N_2 fixation occurred above 20°C. The latter point is of major importance in the field, and glasshouse, where AR is often conducted in an environment with a large diurnal temperature fluctuation. In the long term, AR data accurately represented the response of N_2 fixation to temperature between 10°C and 20°C. In the absence of Hup, for example at 10°C and in all of the diurnal experiments reported in chapter 6, AR-HE was an accurate measure of N_2 fixation. The results of Saito et al. (1980) can be used to calculate the relationship between AR-HE and $^{15}N_2$ fixed for P.vulgaris. The ratio was steady over a wide range of rates of activity. Also, for L.angustifolius, the ratio of AR to N_2 fixation was diurnally constant although $^{15}N_2$ fixation varied diurnally (Gibson and Alston, 1984).

Overall, the data agree with previous findings that N_2 fixation in T.subterraneum is depressed above 20°C (Meyer and Anderson, 1959 ; Possingham et al., 1964) but disagree with the suggestion that the optimum temperature for N_2 fixation is at about 22°C (Gibson, 1961). The results give deeper insight into an earlier conclusion that, for low

density communities of T.subterraneum (230 plants m^{-2}, cf. 2,300 plants m^{-2} in this project) "dry matter yield, N_2 fixation ... were not lower at 12°C than at 22°C" (Davidson et al., 1970). Clearly, both quantities were far greater at low temperature. It is also obvious that AR rates of trimmed roots during brief exposure to temperature changes bear little relevance to the nitrogenase activity of communities, since such brief experiments showed an optimum temperature of 30°C for T.subterraneum (Dart and Day, 1971).

5.4.2. N_2 fixation in relation to community growth.

N_2-fixation should be viewed as being subject to interactions between the host and the endosymbiont. This approach is believed to be more useful than that of considering the specific activities of nodules in isolation. The legume - Rhizobium symbiosis probably evolved to improve plant growth in nitrogen-deficient soils, giving some plants an advantage over other plant groups, and so it is of interest to see how the legume, in its entirety, has achieved this adaptation. Coordination of the growth rate and the N_2 fixation rate are of particular importance.

Tables 9, 10 and 16 show that the growth and N_2 fixation rates responded in reasonable harmony to the growth temperature, and to PPFD, although N_2 fixation was evidently more sensitive to temperature increase as the nitrogen concentrations of roots and shoots were decreased above 15°C at both PPFDs (Table 17). As reported in chapter 4, the rates of growth of N_2-fixing and NO_3^--supplied communities responded almost identically to the changes in temperature, and N_2-fixing plants showed no signs of a nitrogen limitation to growth. It is, therefore, concluded that, between 10°C and 15°C, entering into symbiosis has not

incurred any growth disadvantages for T.subterraneum with respect to their ability to obtain nitrogen. As was shown earlier, however, a growth disadvantage was related to the carbon cost of fixing N_2 (chapter 4).

Total dry matter plotted against time gave straight lines for all environments (r ranged between 0.96 and 1). Notwithstanding this, growth rates measured by CER showed a tendency to decrease during several weeks of growth in some environments. This could be ascribed to the respiratory cost of maintaining biomass ; it was most noticeable at 25^{o}C (Figures 9 and 10). Thus, the general decline in AR with increase in biomass (Figures 17 and 18) can be explained by a gradual decrease in the amount of fixed carbon available to nodules. As AR-HE was generally quite steady until Hup developed, a decrease in total nitrogenase activity was offset by a lower rate of H^+ reduction so that the N_2 fixation rate was maintained.

At an elementary level, the root : shoot ratio lends support to the view that N_2 fixation was not restricted by carbon supply. The proportion of carbon allocated to the roots increased with PPFD, and at low temperature (Table 13), which were both conditions under which the growth rate and, therefore, demand for nitrogen were elevated.

The N_2 : CO_2 ratio tended to decrease with increasing temperature (Table 19) and, because nodule respiration in species of Trifolium has been shown to constitute about 50% of total root respiration (Ryle et al., 1983), it is possible that this reflected a decrease in the amount of N_2 fixed per unit of carbon respired by nodules. In some legumes there appears to be a marked temperature effect on this measure of the efficiency of N_2 fixation, due to an alteration in the proportions of carbon oxidised in nodules via aerobic and anaerobic pathways (Minchin and Pate, 1974 ; see Sprent 1981). However, the fact

that the N_2 : CO_2 ratio was not affected by PPFD (Table 19) is worth enlarging on with reference to the work of Sheikholeslam et al. (1980). These authors found that increasing the growth PPFD for P.sativum exponentially increased the amount of fixed ^{14}C that was translocated to nodules, and also the proportion of the total ^{14}C fixed that was translocated to them. At 200, 500, and 800 µmol quanta $m^{-2}s^{-1}$, the amounts of ^{14}C in nodules were 0.38, 1.82, and 4.68 ng $plant^{-1}$ which were 3.7, 8.3 and 18.3% of ^{14}C translocated, respectively. Increases in ^{14}C translocated to nodules produced similar increases in AR rates. Thus it is likely that the increase in the N_2 : CO_2 ratio observed with increase in PPFD in P.sativum (Bethlenfalvay and Phillips, 1977b) was in accord with an increase in the proportion of fixed carbon partitioned to nodules. At the level of the whole community, the data in Table 19 suggest that the amount of N_2 fixed per unit of fixed carbon arriving at the interface of the symbiosis was similar at low and high PPFD. If this was true, then these communities probably differed from single plants of P.sativum in that the amount of fixed carbon sent to nodules increased in parallel with increase in the growth PPFD, such that the proportion sent was constant.

The optimum for N_2 fixation at 15°C and low PPFD appears to be an anomaly (Table 16), and one which has unexplained precedents in Vicia atropurpurea and Medicago truncatula (Pate, 1961). For these two species, grown singly, the percentage of nitrogen in the plants was highest at 21°C, but the total amount of nitrogen fixed - and, hence, the plant growth rate also - was lowest at 21°C. The optimum temperatures for legume growth and for N_2 fixation did not coincide.

Host legumes and their Rhizobium partners have different optimum temperatures for growth, so that there is an area of compromise for the growth of symbioses which is confined to a narrower range than that of

non-nodulated legumes. The growth of R.trifolii NA30, which was temperature-sensitive in symbiosis (as is WU95, the strain used in this project) was favoured in culture at 30°C over that at 22°C (Pankhurst and Gibson, 1973). Conversely, Table 10 shows quite clearly that community growth of T.subterraneum was optimal at low temperatures. R.trifolii is an invasive organism which uses carbon substrates from the host for its own metabolism. The faster growth of non-nodulated plants indicated that the carbon costs of supporting nodules reduced plant growth in N_2-fixing plants (chapter 4). The essence of symbiosis is mutual benefit through close interaction, and since nodules of Trifolium were found to be large sinks for carbon (Ryle et al., 1983) which must have competed for carbon with other sinks in the legume (Lawn and Brun, 1974), it can be argued that there were two components to the flux of carbon entering nodules.

The first component is already well established, being solely a property of the host, such that the amount of carbon translocated to nodules increased with CER and irrespective of sink demands, perhaps controlled by the sucrose level in the leaf (Ho, 1976 ; Sheikholeslam et al., 1980). The second component is postulated to have been the capability of the nodules to attract carbon. This idea has not been widely considered, but is a reasonable one since nodules produce cytokinins which can interact with host-produced hormones to effect hormone-directed transport of metabolites (Phillips, 1969 ; Phillips and Torrey, 1970). When the CER was low, at low PPFD, and a lesser proportion of carbon fixed was allocated to the roots (Tables 9 and 13), this would have emphasised the second, more 'active', component. At low PPFD probably only a small amount of fixed carbon was allocated to the nodules by the host, having the effect of making the amount 'attracted' by the nodules relatively more important than at high PPFD. As the

growth of R.trifolii was more favoured by high temperature than that of the host, the combination of the nodules 'attraction' of carbon (greater at high temperature) and the 'allocation' of carbon to the nodules by the legume (greater at low temperature) resulted in an optimum for N_2 fixation at the intermediate temperature of 15°C at low PPFD. This optimum was probably absent at high PPFD because the amount of carbon 'attracted' by the nodules would have been swamped by the 'allocation' of carbon to them by the legume. As community growth was carbon-limited at low PPFD (photosynthesis was about 47% saturated according to Silsbury, 1981) the extra nitrogen fixed resulted in a higher nitrogen concentration in the tissues and not in a higher growth rate. Comparisons of N_2 fixation and AR-HE data between PPFDs at 10°C, 20°C, and 25°C show a positive correlation between legume CER and N_2 fixation, but the temperature optimum for N_2 fixation at low PPFD resulted in the difference between N_2 fixation rates between PPFDs having a lower statistical significance at 15°C than at the other temperatures (Table 9). This, and the flexibility in the amount of nitrogen required per unit biomass (Table 17) signify that the growth rate was not simply dictated by the N_2 fixation rate between 10°C and 25°C and that the nodules had a degree of autonomy with respect to their rate of N_2 fixation. It may not be a precept for successful symbiosis that the participating organisms renounce all claims to independence.

5.4.3. Temperature and the effect of combined nitrogen on N_2 fixation.

N_2 fixation was eventually completely inhibited in the presence of combined nitrogen at 20°C, but at 10°C N_2 fixation continued at about 30% of the rate of plants growing in the absence of combined nitrogen (Table 21 ; Figure 23). This indicates that there was an interaction of

temperature with the effect of combined nitrogen on N_2 fixation. It can be speculated that temperature either interfered with the reduction of NO_3^- to NO_2^- in the nodule, or that nodules at 10°C were deprived of carbon by the combined nitrogen treatment to a lesser extent than were those at 20°C, because P_N was higher at 10°C, and the root : shoot ratios showed that a larger proportion of carbon was allocated to the roots. The conversion of NO_3^- to NO_2^-, and carbon deprivation of nodules, have both been suggested as mechanisms of inhibition of N_2 fixation by combined nitrogen (Small and Leonard, 1969 ; Streeter, 1982).

This information is important with respect to T.subterraneum growing in the field, because the sward is most productive at low temperature and, therefore, has a large requirement for nitrogen. Field-grown legumes often encounter low levels of combined nitrogen, and if they could continue to fix N_2 for their own growth under such conditions, then soil nitrogen would be spared.

CHAPTER 6

6. Diurnal variation in N_2 fixation.

6.1.0. Introduction.

The usefulness of the HE and AR assays lies in their simplicity, as they are readily applied to whole plants and provide results quickly. Because they measure the instantaneous rates operating during the assay period, it must be established how representative the assays are of the day's activity if it is required to extrapolate to approximate values for a whole day, or longer. This can be done by assaying frequently over 24 hours.

The literature indicates that nitrogenase activity (AR) in species of Trifolium varies if there is a diurnal temperature fluctuation, or if a combination of high temperature and low PPFD leads to exhaustion of carbohydrate reserves at night (Eckart and Raguse, 1980 ; Haystead and Sprent, 1981). However, diurnal variation in HE has received very little attention.

The experiments reported in this chapter were designed to characterise the diurnal profiles of HE, AR, and AR-HE, in T.subterraneum communities for each of the growth environments described in chapters 4 and 5. Three areas were investigated:

(i) nitrogenase activities and nodulated root respiration during a normal 12 hour day and 12 hour night;

(ii) variation in nitrogenase activities induced by change in night length;

(iii) variation in nitrogenase activities induced by change in the rate of transpiration.

6.2.0. Nitrogenase activities and nodulated root respiration during a normal 12 hour day and 12 night.

6.2.1. Methods.

Initially, communities were grown hydroponically as a space-saving device and to allow repetition of HE and AR assays on the same plants. In the hydroponic technique, detailed in chapter 3, communities were maintained at constant temperature and 1000 µmol quanta $m^{-2}s^{-1}$ and assays were performed every 4 hours between 11.00 hours and 23.00 hours. Each community was not assayed more frequently than once in 12 hours due to the inhibitory effect of C_2H_2 on HE (see chapter 3). Because of the inhibition of HE, the hydroponic technique was abandoned in favour of destructive assays on communities grown in oil-dry.

Destructive assays were performed on communities grown at $10^{o}C$, $15^{o}C$, $20^{o}C$, or $25^{o}C$, both at 500 µmol quanta $m^{-2}s^{-1}$ (low PPFD) and 1000 µmol quanta $m^{-2}s^{-1}$ (high PPFD) with a 12 hour day, 12 hour night, cycle. Using random number tables, three replicate swards were taken from the growth cabinet every 4 hours, starting at 11.00 hours and finishing at 07.00 hours the next day. HE and AR assays were conducted as described in chapter 3. Over the 24 hours preceding the HE and AR assays, the day and night transpiration rates were estimated gravimetrically for the same communities, as detailed in chapter 3. In each diurnal experiment, the 3 communities assayed at 11.00 hours were not watered before assay but all other communities were watered at 09.00 hours. As stated earlier, there was no effect of watering on the absolute rate of AR, but there was less variation between AR rates of communities not watered immediately before assay.

The low REs of communities used in diurnal studies indicated that Hup was not operating. When the RE exceeded 0.7, H_2 was added to

communities to check for Hup (as described in chapter 5). The small amounts of H_2 which disappeared from the gas phase were consistent with losses through leakage and adsorption (see chapter 3).

In one experiment the diurnal assays were extended to cover 92 hours. The 72 communities used during the course of this experiment were sampled from a population of 80 maintained at 20°C in a growth room. Because of the large number of communities used, the distribution of PPFD over the experimental material was less homogeneous than in other experiments, ranging from 900 to 1100 µmol quanta $m^{-2}s^{-1}$.

Nodulated root respiration of a community growing in an assimilation chamber at 15°C and 1000 µmol quanta $m^{-2}s^{-1}$ was measured continuously over 12 days and nights. The community was grown in oil-dry in the same type of vessel as used for growing plants hydroponically, shown in Figure 2, so that the CER of roots could be monitored by infra-red gas analysis. Air was delivered at the base of the vessel at 1l min^{-1} and was sampled when it left via the exit port at the top of the vessel, having circulated amongst roots and oil-dry. The community was given about 0.2l -N nutrient solution, which displaced all air from the jar, every second day, drained, and returned to the chamber.

6.2.2. Results.

The results of HE and AR assays conducted between 11.00 hours and 23.00 hours on hydroponically-grown swards at 10°C and high PPFD are presented in Table 22.

The outcome of variations in HE and AR was that AR-HE was highest early in the day and early in the night, with most of the electron flux through nitrogenase being used to reduce H^+ during the middle of the day. On the basis of these results, a series of diurnal investigations was made using the larger communities grown in oil-dry.

TABLE 22 Diurnal variation in nitrogenase activities of communities grown hydroponically at 10°C, high PPFD, and repeatedly assayed. Means, with SE, for 3 values.

Time of assays, Hours	AR µmol C_2H_4 $m^{-2}h^{-1}$	HE µmol H_2 $m^{-2}h^{-1}$	AR-HE C_2H_4-H_2 $m^{-2}h^{-1}$	RE
11.00	1618 (91)	777 (83)	841 (43)	0.52 (0.03)
15.00	1225 (108)	926 (49)	301 (121)	0.23 (0.08)
19.00	1202 (125)	1022 (115)	180 (9)	0.15 (0.01)
23.00	1508 (68)	660 (26)	851 (77)	0.56 (0.03)

Measurements of transpiration, made on the larger communities before they were assayed, are shown in Table 23.

TABLE 23 Transpiration rates of closed canopies. Each figure is the mean for 18 values.

Quantity	Temperature, and PPFD ----10°C---		----15°C---		----20°C--		----25°C--	
	High	Low	High	Low	High	Low	High	Low
g H_2O $m^{-2}h^{-1}$ during day	243	152	281	205	343	271	367	319
g H_2O $m^{-2}h^{-1}$ during night	+	+	14	9.5	29	29	86	71
Day/night	+	+	20	22	12	9.5	4.3	4.3

\+ Too low to be measured gravimetrically.

The transpiration rate was increased by increases in PPFD and in temperature, in accordance with the known effects of PPFD and

temperature on stomatal aperture (Ehrler and van Bavel, 1968 ; Hofstra and Hesketh, 1969). The ratio of the daytime : nighttime transpiration rates increased considerably with decrease in temperature. Because nitrogen is transported from nodules in the xylem, the effect of temperature on this ratio achieves importance in the later discussion of diurnal variation in nitrogen availability.

The diurnal profiles for HE and AR are shown in Figures 24 and 25, and the data given in these figures were used to derive the diurnal profiles of AR-HE shown in Figure 26. There were pronounced diurnal variations in HE, AR and AR-HE. The importance of measuring HE in analysing diurnal variation in N_2 fixation is readily apparent from Figures 24, 25 and 26. For example, at $10^{o}C$ and low PPFD, and $15^{o}C$ and high PPFD, there was no statistically significant variation in AR (the results of statistical tests are given in appendices V to VIII). However, AR-HE, which was directly proportional to N_2 fixation because Hup was absent, showed highly significant diurnal variation (Figure 26 ; Appendix VII). There was no statistically significant variation in AR-HE at $25^{o}C$, but in other environments the diurnal changes in AR-HE were largely due to the common pattern of an increase in HE during the day and a decrease at night, but also to the pattern in AR which showed a trough during the day, a peak late in the day or early in the night and a subsequent decline at night (Figures 24 and 25 ; Appendices V and VI). This pattern in AR is seen clearly for all temperatures at high PPFD, and also for $15^{o}C$ at low PPFD.

HE has been regarded as a wasteful process, serving no apparent purpose, so that an increase in the proportion of electrons used in H^{+} reduction has been interpreted as a decrease in efficiency according to the term RE, coined by Schubert and Evans (1976). In Appendix VIII it is shown that in all environments below $25^{o}C$, RE was at its minimum at

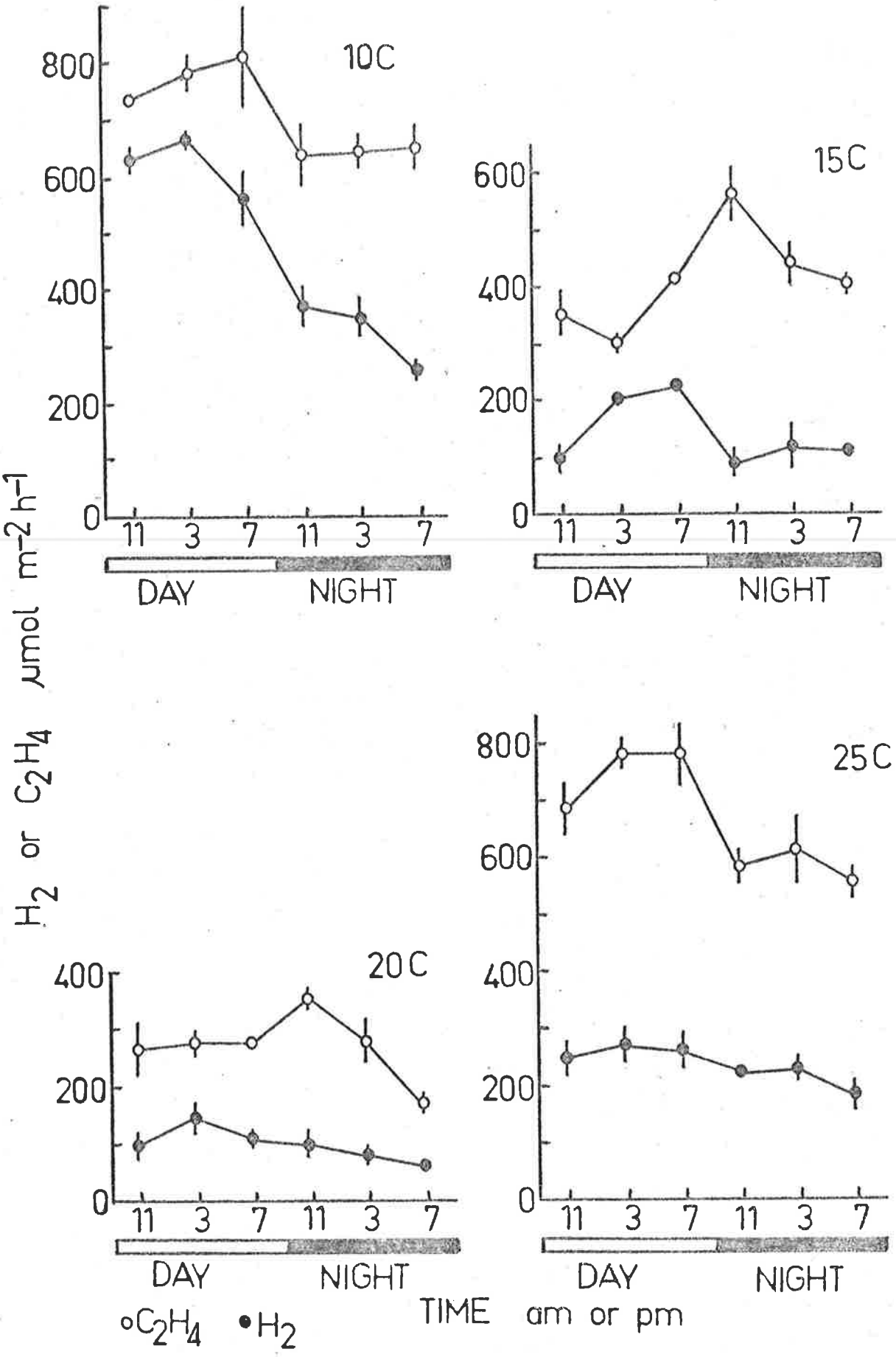


Figure 24. Diurnal variations in HE and AR at low PPFD Means, with SE, for 3 values.

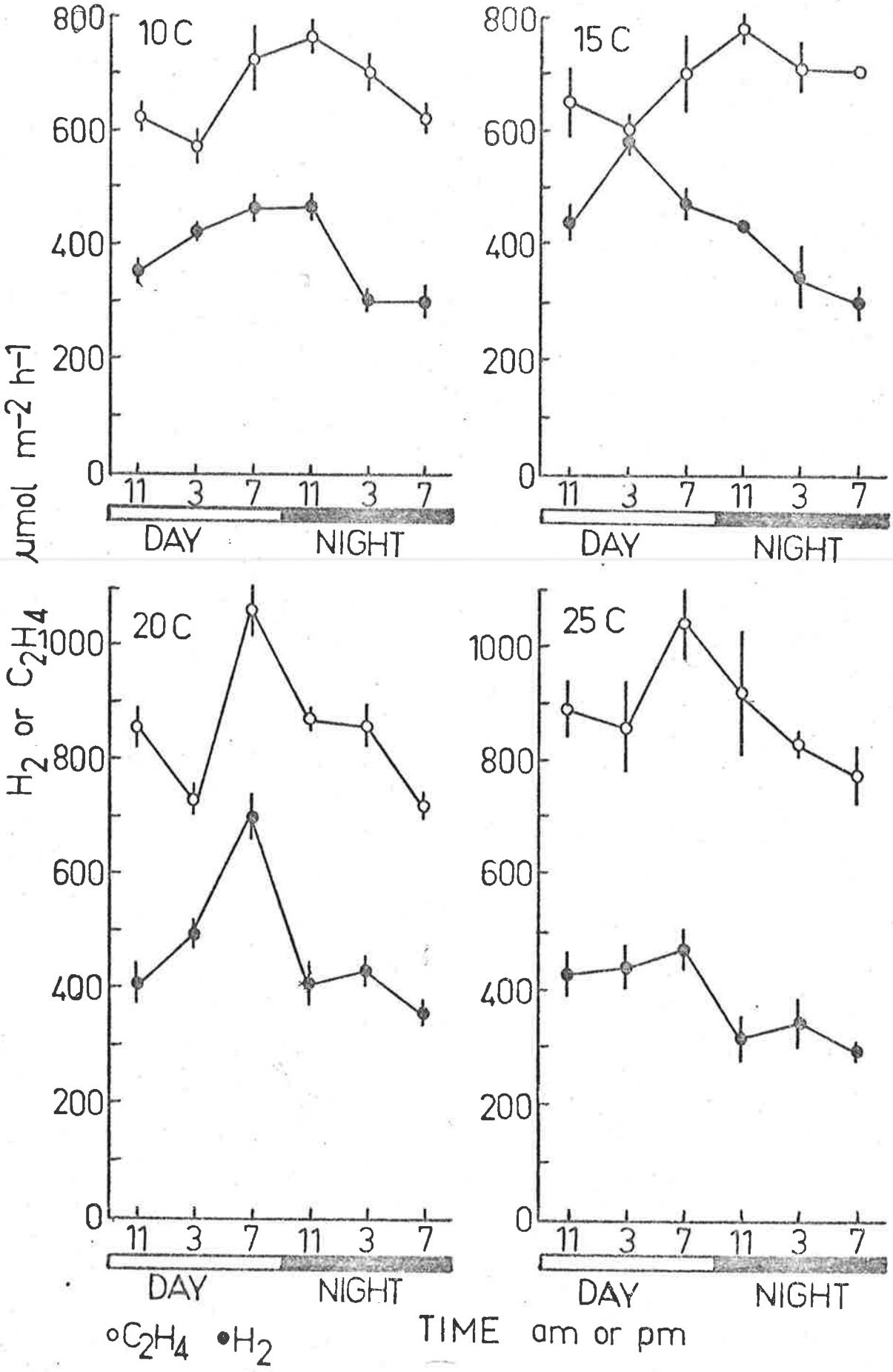


Figure 25. Diurnal variations in HE and AR at high PPFD Means, with SE, for 3 values except 20C for 12 values.

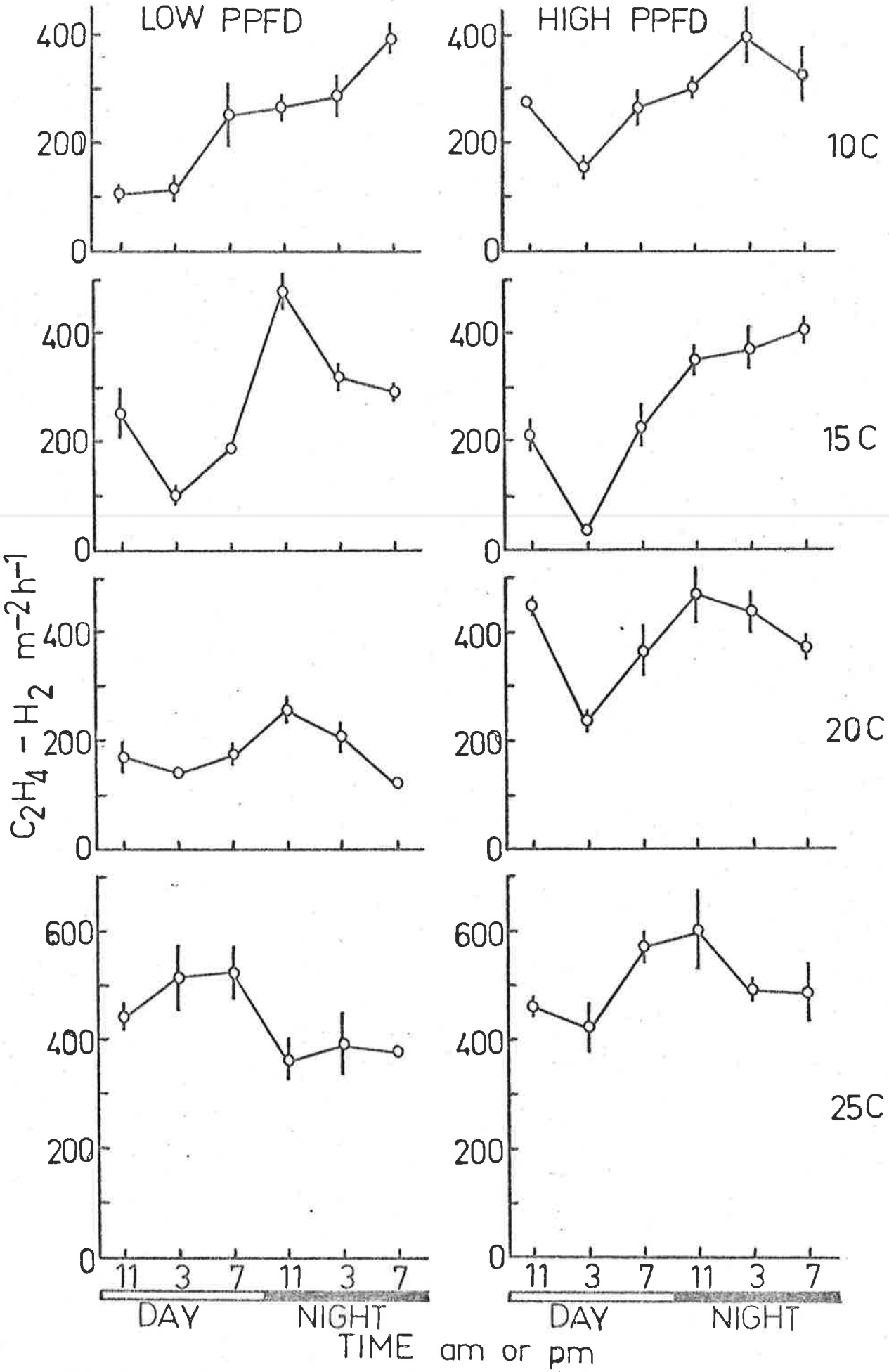


Figure 26. Diurnal variation in AR-HE, (from figures 24 and 25

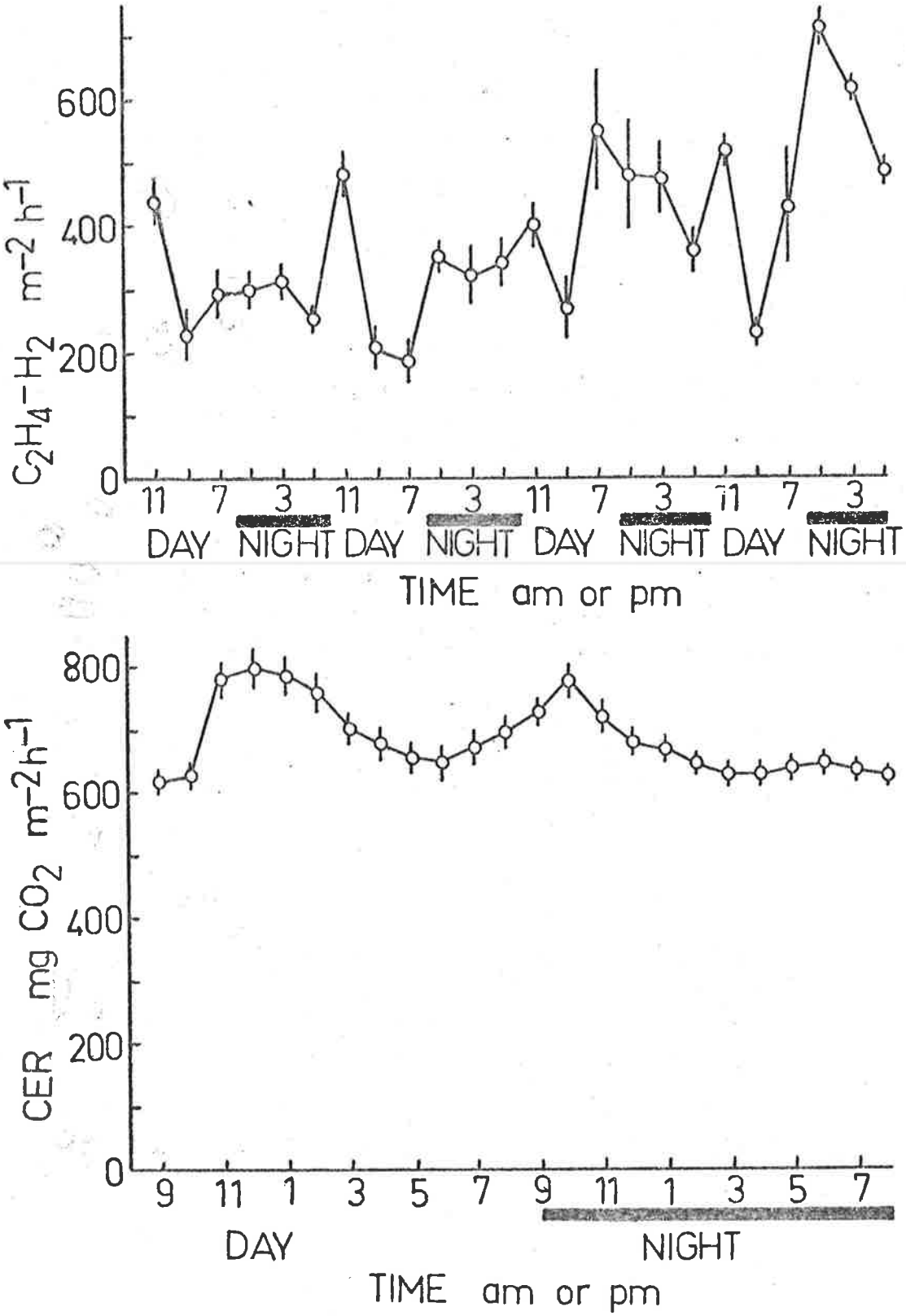


Figure 27, (Top). Diurnal variation in AR-HE at 20C and high PPFD over 92 h. Means, with SE, for 3 values.

Figure 28, (Bottom). Diurnal variation in nodulated root respiration at 15C and high PPFD. Means, with SE, for 12 values

15.00 hours and was highest at night. RE was extremely flexible.

Where diurnal variation in N_2 fixation is discussed in the literature, it is typically concluded that N_2 fixation is highest during the day and lowest at night. The diurnal profiles for AR-HE for environments in which variation was statistically significant (10^oC, 15^oC and 20^oC) are, therefore, unusual in showing N_2 fixation to have been low during the day and high at night (Figure 26).

The pattern of variation in AR-HE over four day-and-night cycles at 20^oC is shown in Figure 27. It can be seen that the daytime decrease in AR-HE noted in previous experiments was a repeatable event, occurring at about the same time in every 24 hour cycle. For each 24 hour cycle the nighttime AR-HE rate was higher than the daytime rate. For some assays shown in Figure 27 the errors were larger than usual, and this was probably attributable to the poorer homogeneity of PPFD for this experiment, which was referred to earlier.

A diurnal profile for respiration of nodulated roots, at 15^oC, is shown in Figure 28. The pattern of variation was interesting in that it showed a parallelism with the diurnal variation in AR (Figures 24 and 25).

6.3.0. Variation in nitrogenase activities induced by change in night length.

6.3.1. Introduction.

The pattern of diurnal variation in N_2 fixation (AR-HE) shown in Figure 26 was unlikely to have been caused by carbohydrate shortage in the nodules, because the minimum in AR-HE occurred in the middle of the day when newly fixed carbon has been shown to be rapidly transported to

nodules in T.subterraneum (Small and Leonard, 1969). Also, temperature was constant and could not have influenced N_2 fixation diurnally.

The strong effect of temperature on the ratio of daytime : nighttime transpiration rates (Table 23) indicated that a diurnal rhythm in nitrogen export from nodules might have been more pronounced at low temperature than at high temperature. If nitrogen export was related to the diurnal rhythm in AR-HE, this might explain why there was no statistically significant variation in AR-HE at 25°C. At low temperature, when the transpiration rate at night was very low, little nitrogen could have left the nodules at night.

Little attention has been paid to diurnal variation in transpiration in studies of N_2 fixation. Although Minchin and Pate (1974) found it to result in substantial nitrogen accumulation in nodules at night, they concluded that "there is no evidence, as yet, that the rate at which fixation products are cleared from the nodule has any influence on the current rate of fixation."

The experiment reported in this section was designed to examine the effects of nitrogen accumulation in nodules at night, and its release into the xylem during the day, on daytime nitrogenase activities.

6.3.2. Methods.

A batch of swards was grown at 15°C and 500 µmol quanta $m^{-2}s^{-1}$ with a normal 12 hour day, 12 hour night. When the leaf canopies had closed, half of the communities were transferred to an 8 hour day : 16 hour night cycle, and the other half to a 16 hour day : 8 hour night cycle, both at 15°C. The PPFD during the photoperiod was adjusted to about 800 µmol quanta $m^{-2}s^{-1}$ and 330 µmol quanta $m^{-2}s^{-1}$, respectively, so that the CER over 24 hours was similar for each environment. By making the growth

rates similar, it was hoped that the demands for fixed nitrogen would also be similar for each environment.

After 24 hours in the new environments, the transpiration rates were measured. After 48 hours, assays were begun, 3 replicate communities being taken from each treatment about every 3 hours.

It was expected that after a 16 hour night, more nitrogen would accumulate in nodules, and be delivered into the xylem in the daytime, than after an 8 hour night.

6.3.3. Results.

The higher PPFD of the 8 hour day produced a higher daytime transpiration rate than for the 16 hour day, but this probably served to magnify the treatment effects intended viz. differences in the amounts of nitrogen cleared from nodules.

The results of the experiment have been averaged over 24 hours and are presented in Table 24. AR and AR-HE did not differ between treatments, so the attempt to produce similar growth and N_2 fixation rates appears to have been successful. However, there were very marked differences in HE and RE between treatments. HE was lower, and hence RE higher, in the 16 hour night treatment. The diurnal profiles for each treatment are shown in Figure 29. It can be seen that HE, averaged over 24 hours, was lower in the 16 hour night treatment because in this environment most of the nitrogen was fixed in darkness when HE was lowest. There was a large depression in AR-HE in the daytime of the 16 hour night treatment, but almost no diurnal variation in AR-HE in the 8 hour night treatment.

The treatment effect on N_2 fixation would have been overlooked if HE had not been measured, since AR was not affected (Table 24).

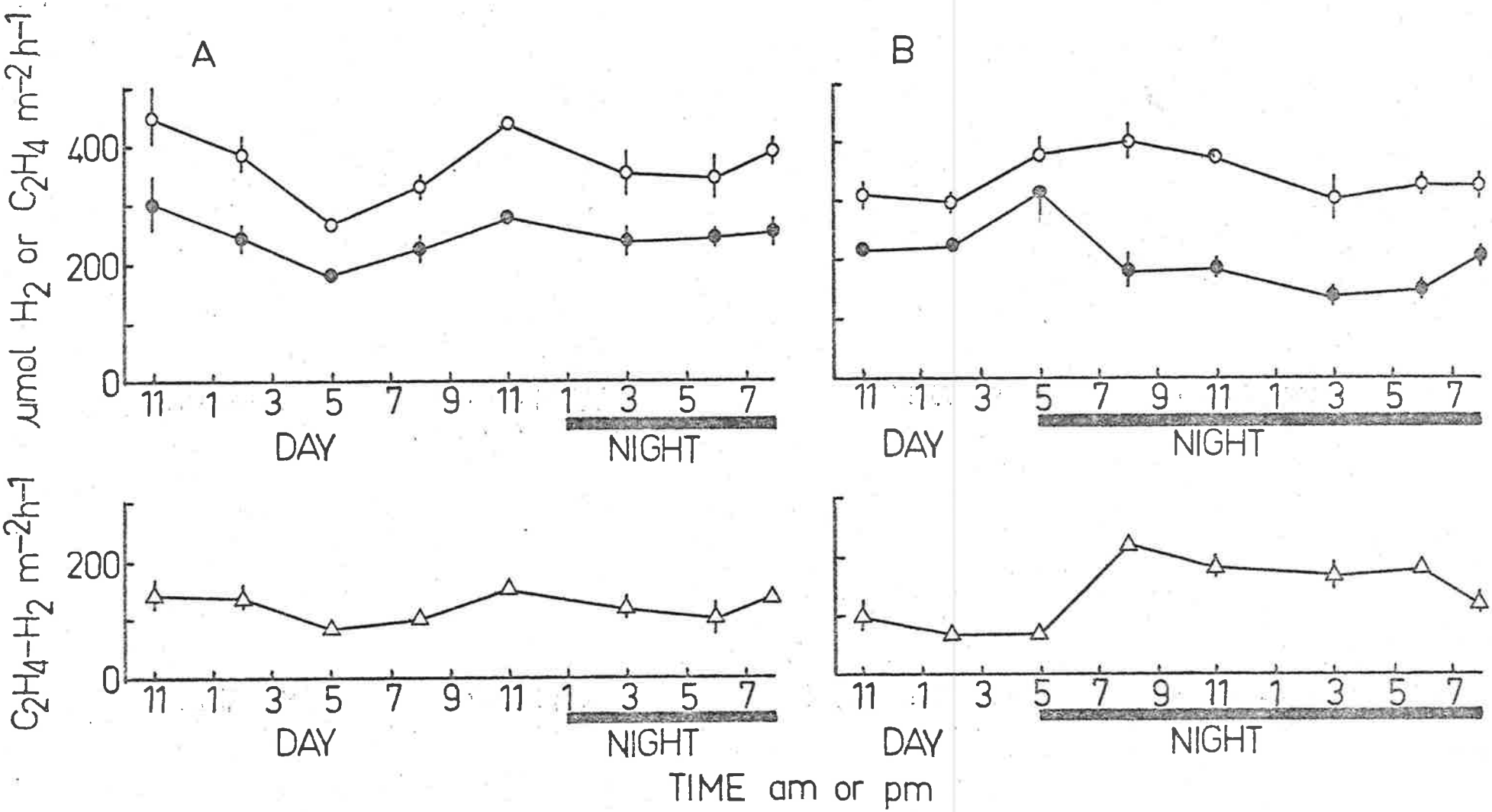


Figure 29. Diurnal patterns of HE (•), AR (o), and AR-HE (Δ) at 15C. 8h night (A) or 16h night (B). PPFD was set so that growth rates were the same for A and B. Means, with SE, for 3 values.

TABLE 24 Effect of night length on nitrogenase activities. Each mean, with SE, is for 24 values made up from 3 replicates x 8 harvests every 3 hours for 24 h.

Variable	8h. night	16h. night	t value
$\mu mol\ C_2H_4^{AR}\ m^{-2}h^{-1}$	368 (15)	337 (11)	1.71 NS
$\mu mol\ H_2^{HE}\ m^{-2}h^{-1}$	244 (10)	199 (12)	2.89**
$C_2H_4\text{-}H_2^{AR-HE}\ m^{-2}h^{-1}$	123 (17)	139 (12)	1.07 NS
RE	0.33 (0.01)	0.41 (0.03)	2.35*

* Significant at P0.05
** Significant at p0.01
NS Not Significant

A surprising result of this experiment was the relatively low AR rate in the 8 hour day of high PPFD in comparison with the AR rate of the 16 hour day of low PPFD (Figure 29). It was expected that AR would increase in parallel with daytime CER (Silsbury, 1981) but the higher rate of CO_2 fixation at high PPFD did not bolster AR in the daytime.

6.4.0. Variation in nitrogenase activities induced by change in the rate of transpiration.

6.4.1. Introduction.

The aim of the experiment reported in this section was to vary the rate at which a given amount of nitrogen, accumulated in the nodules at night, was cleared from the nodules during the day. The chosen means of accomplishing this was by alteration to the transpiration rate.

6.4.2. Methods.

Swards were grown at 15°C and 500 µmol quanta $m^{-2}s^{-1}$ in a normal 12 hour day : 12 hour night cycle until the leaf canopies had closed. The swards were then divided into 3 populations of 12, each of triple rows of 4 across the growth cabinet. Because of the difficulty in changing the CO_2 concentration of the air in the cabinet, or the dew point temperature of the air, differences in transpiration between populations were generated by increasing the irradiance. Carbon-filament lamps, with a high output of infra-red radiation, were hung from the cabinet ceiling to supplement the HID sodium lamps. Irradiance (300-2000 nm) was measured using a Kipp solarimeter, and PPFD was measured using a LiCOR meter.

Transpiration rates were measured 3 days before it was desired to run the experiment, to check on the effect of the treatments. Three markedly different transpiration rates were generated for the low (260-335 Wm^{-2}), medium (335-445 Wm^{-2}), and high (480-595 Wm^{-2}) irradiance treatments. These were 307, 386 and 491 g H_2O m^{-2} h^{-1}, respectively. The PPFD was similar for all treatments, at about 775 µmol quanta m^{-2} s^{-1}. It was hoped that growth rates during the photoperiod would also be similar.

The carbon-filament lamps were turned on at 09.00 hours on the day of the experiment, and 2 replicate communities were taken from each treatment at 09.30 hours, and every 2 hours thereafter, for HE and AR assays.

6.4.3. Results.

The profiles of HE, AR and AR-HE during the photoperiod are shown in Figure 30, and the data averaged over the 10 hours of measurement are given in Table 25.

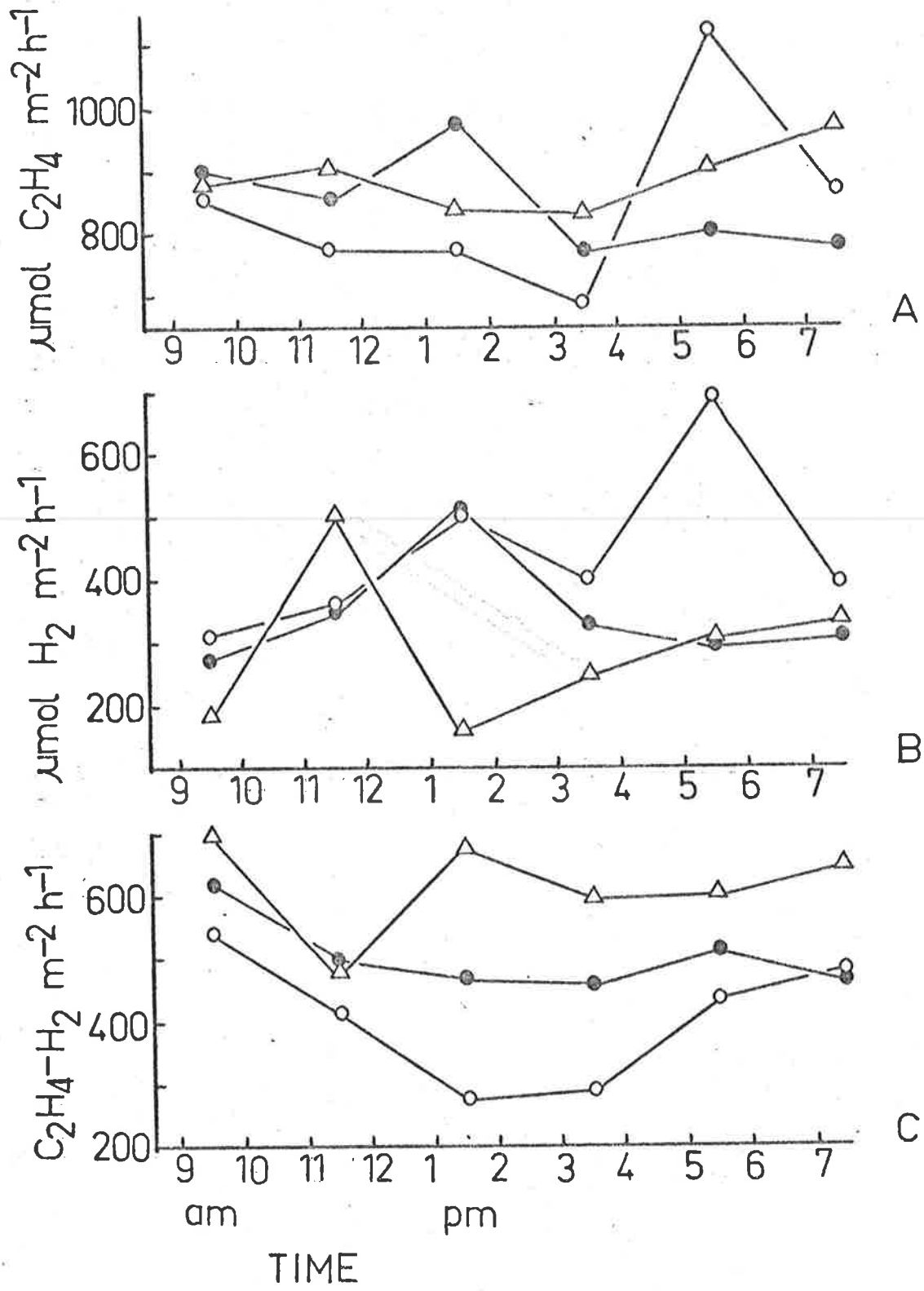


Figure 30. Effect of transpiration rate (○ low ● medium △ high) on AR (A), HE (B), and AR–HE (C) in daytime at 15C. Points are means for 2 values.

TABLE 25 Analysis of variance for the effects of transpiration rate on nitrogenase activities during the photoperiod. Each mean, with SE, is for 12 values made up of 2 replicates x 6 two-hourly harvests.

Variable	Transpiration rate (g H_2O $m^{-2}h^{-1}$) Low (307)	Medium (386)	High (491)	F value AOV	LSD P0.05
AR µmol C_2H_4 $m^{-2}h^{-1}$	848 (60)	848 (36)	891 (30)	0.32 NS	
HE µmol H_2 $m^{-2}h^{-1}$	443A (49)	344AB (30)	288B (39)	3.81*	116
AR - HE C_2H_4-H_2 $m^{-2}h^{-1}$	405C (32)	504B (22)	603A (34)	10.82**	87
RE	0.49B (0.03)	0.60A (0.02)	0.68A (0.04)	9.15**	0.09

* Significant at P0.05
** Significant at P0.01
NS Not significant

Although the experiment was conducted at an air temperature of 15°C, the extra heat load on the leaves from the carbon-filament lamps might have wrought changes in other processes as well as in transpiration. The most important of these would have been in the rate of respiration, since this could have affected the carbon supply to nodules. However, the AR rate was not affected by the treatments, and AR-HE (proportional to N_2 fixation) was highest in the high irradiance/high transpiration rate treatment. Hence, carbon availability seems not to have limited nodule activity during the experiment (Table 25).

HE decreased with increase in the transpiration rate during the photoperiod, so that AR-HE and RE increased (Table 25). Figure 30 shows

that the rate of clearance of accumulated nitrogen from the nodules affected the extent and duration of the daytime depression in AR-HE noted earlier. Although AR-HE over 10 hours was greatest for the 'high' treatment (Table 25), AR-HE was still depressed but it recovered much sooner than in the other treatments (Figure 30). The faster was the rate of transpiration, the earlier in the day HE reached a peak and declined. The data indicate that the treatment effects on the rate of N_2 fixation were realised through altered rates of H^+ reduction.

6.5.0. Discussion.

From the general observation of there being a trough in AR-HE during the daytime and a subsequent recovery to a high value at night, (Figure 26) it was hypothesised that the N_2 fixation rate was related to diurnal rhythm in the clearance of nitrogen from the nodules. Two assumptions were made during the course of experiments. The first was that nitrogen accumulated in nodules at night, when the transpiration rate was low, which has been documented for P.sativum by Minchin and Pate (1974). The second assumption was that nitrogen accumulation in nodules did not directly affect the N_2 fixation rate, within the nodules. Fixed nitrogen is actively transported from the sites of fixation and assimilation, via transfer cells, to the vascular spaces (see Pate, 1976) so that N_2 fixation and nitrogen accumulation are spatially separated. Also, electron transfer to nitrogenase was shown to be controlled by the electrical component of the proton motive force across the peribacteroid membrane, which was unaffected by external NH_3 (Laane et al., 1980). Thus, the second assumption was sound.

One factor which might affect the N_2 fixation rate is the capacity for fixed nitrogen to be assimilated. In Helianthus annus grown at

constant temperature, there was a rhythmic change in the GS activity of roots. Peaks in activity occurred at the end of the day and at the end of the night, and there was a trough in activity during the day. This pattern was suggested to be caused by allosteric effects of ATP and ADP on GS (Knight and Weissman, 1982), and resembles that found for AR-HE. As well as inhibiting GS, ADP was found to promote electron allocation to H^+ in nitrogenase (Wassink and Haaker, 1984). So, a decrease in the N_2 fixation rate during the day might have been caused by a reduced ability to assimilate NH_3. However, where statistically significant diurnal variation in AR-HE occurred, AR-HE was highest at night whereas GS activity was shown to fall rapidly at night both in P.sativum nodules and H.annus roots (Skrdleta et al., 1980 ; Knight and Weissman, 1982). It, therefore, seems unlikely that the changes seen in N_2 fixation rate were caused by variation in GS activity.

Diurnal variation in AR probably represented a gross response to changes in carbon allocation to the nodules. AR was shown to increase when the amount of carbon entering nodules increased (Sheikholeslam et al., 1980) and when nodule respiration rate increased (Mahon, 1977a). The diurnal pattern in root respiration (Figure 28) was probably due to variation in nodule respiration because in Trifolium species nodule respiration was found to make up about 50% of root respiration (Ryle et al., 1983). Veeger et al. (1981) showed that production of flavodoxin hydroquinone, the supposed electron donor for N_2 fixation, was driven by the proton motive force generated by respiration across the cytoplasmic membrane. Respiration occurs to support other processes as well as N_2 fixation, and is assumed to vary as a consequence of change in the flux of carbon into the nodule. Thus, the rate of total electron flux through nitrogenase (AR) might have been passively set. If the pattern of variation in total root respiration is attributed to carbon supply, then

the nighttime decline was probably caused by less carbon being translocated from the leaves in darkness, as seen previously (Fondy and Geiger, 1982). The two peaks separated by a trough (Figure 28) may have been caused by higher CO_2 fixation rates at the beginning and end of the day, which resulted from the circadian leaf movements of T.subterraneum.

If the AR rate was controlled primarily by respiration, rather than the legume's demand for fixed nitrogen, then a new role for HE can be proposed : HE was a regulatory mechanism by which the N_2 fixation rate and the host requirement for fixed nitrogen was coordinated. This mechanism did not necessitate changes in carbon partitioning or in respiratory processes, but operated at the site of N_2 fixation itself. HE has formerly been regarded as an entirely useless and wasteful process, in the company of photorespiration (Zelitch, 1975) and alternative pathway respiration (de Visser and Lambers, 1983). These three processes may be so categorised because they share the property of being poorly understood. Diurnal variation in the transpiration rate probably caused a strong diurnal rhythm in the amount of nitrogen in circulation in the plants. This rhythm was damped at 25^{o}C because the ratio of daytime : nighttime transpiration rates was low (Table 23), but at temperatures below 25^{o}C it might have resulted in the need for N_2 fixation to be regulated diurnally.

A model is presented in Figure 31 which has been assembled from the literature and which portrays the link between host and endosymbiont. Fixed nitrogen is excreted from bacteroids and assimilated via GS-GOGAT in the host cells (Boland et al., 1978). The products are then transported to the vascular spaces (see Pate, 1976) where they accumulate at night when the transpiration rate is low (Minchin and Pate, 1974). The transpiration rate increases during the day and carries the backlog of fixation products to the transpiring leaves, increasing

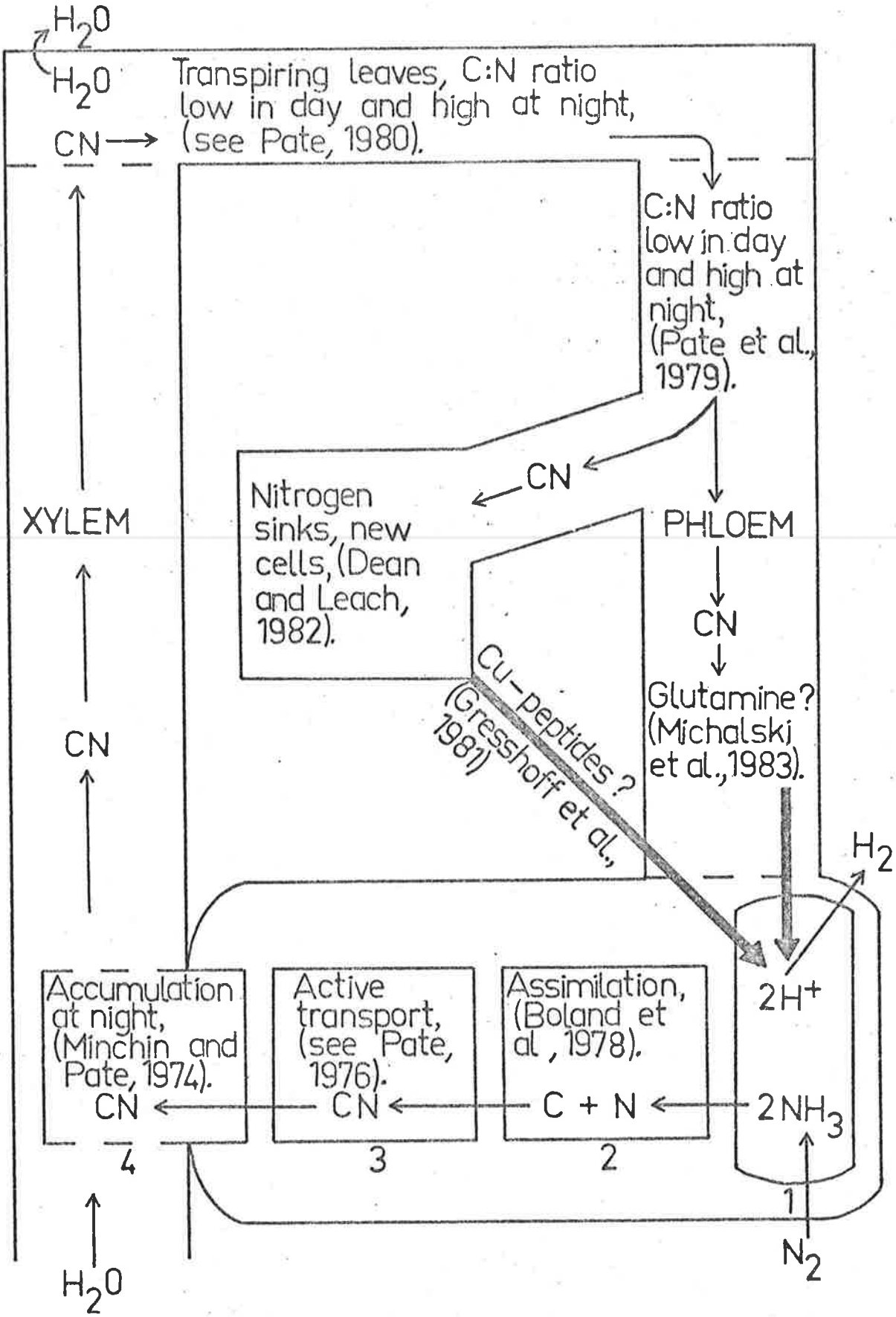


Figure 31. The pathways of nitrogen movement in a N_2-fixing legume. 1, bacteroid; 2, host cell; 3, transfer cell; 4, vascular spaces.

their nitrogen content considerably. Pate (1980) reported a daytime increase of 20% over the nitrogen content of leaves sampled at night. This results in a lower C : N ratio for the phloem sap departing leaves during the day than at night (Pate et al., 1979). Thus, diurnal variation in transport of fixed nitrogen from the nodules results in a diurnal rhythm in the C : N ratio of phloem sap arriving at all plant parts, including the nodules.

It is the rhythmic surge in the amount of nitrogen which is suggested to have resulted in the daytime decrease which characterised profiles of AR-HE (Figure 26). Increase in AR-HE at night may have been an attempt to increase nitrogen availability when there was a dearth of nitrogen in circulation. This was ineffectual because although more N_2 was fixed, the products were not exported until the following day. The low daytime : nighttime transpiration ratio at 25°C (Table 22) and the lack of statistically significant variation in AR-HE at 25°C (Appendix VII) suggest that, at this temperature, the nighttime transpiration rate was sufficiently high to avoid a large nocturnal nitrogen accumulation. Nitrogen export at night could have been maintained at 25°C by an increase in the concentration of nitrogen in the xylem sap, which was reported at high temperature in V.unguiculata although there was still diurnal variation in the amount of nitrogen transported (Rainbird et al., 1983).

Results from the experiments in which night length and transpiration rate were varied can be accommodated by the scheme outlined above. A large amount of nitrogen accumulated in the nodules during the 16 hour night and resulted in marked daytime depression of AR-HE, while a small amount of accumulated nitrogen from the 8 hour night caused no depression (Figure 29), because the 16 hour night resulted in a large diurnal amplitude in the amount of nitrogen in

circulation. The 8 hour night probably resulted in greater constancy in the pool of available nitrogen in the plants. The daytime AR rate of the 8 hour day of high PPFD was lower than expected. Increased rates of daytime starch accumulation have been found in leaves of several species when transferred from long to short photoperiods (Chatterton and Silvius, 1980) so it is likely that, after 48 hours of treatment, the communities had become entrained to the long night and they stored extra carbon in the daytime to support nitrogenase activity in darkness. AR declined very little during the 16 hour night (Figure 29) and this disagreed with the findings of Murphy (1981) for T.repens, and also with diurnal profiles of AR for normal 12 hour day : 12 hour night cycles (Figures 24 and 25). This further suggests that, after 48 hours of treatment, communities had altered the temporal allocation of carbon to nodules. However, the higher RE of the 16 hour night treatment (Table 24) was in agreement with previous work on T.subterraneum (Edie and Phillips, 1983).

When the transpiration rate was varied by supplementary infra-red radiation, the rate at which accumulated nitrogen entered into circulation affected the extent of the daytime depression in AR-HE (Figure 30). As the transpiration rate increased, so the severity of the AR-HE depression decreased, indicating that the period over which an elevated level of nitrogen was circulating in the plants had important consequences for the daytime rate of N_2 fixation. The results of this experiment (Table 25) agreed with other work which showed that brief changes in transpiration rate did not affect the AR rate (Minchin and Pate, 1974 ; Huang et al., 1975) but disagreed with that of Lambers et al. (1980) which showed AR to be affected by transpiration.

In experiments 6.3.0 and 6.4.0, the proposed effect of the release of nitrogen from the nodules during the day on the N_2 fixation rate was

via H^+ reduction (Tables 24 and 25). Two mechanisms to explain this effect are evident in the literature and are included in Figure 31. One mechanism concerns the nature of nitrogenous compounds arriving in the nodules. Michalski et al. (1983) found that glutamine induced rapid, and reversible, adenylylation of the Fe protein of nitrogenase in a photosynthetic diazotroph. As the adenylylation modified electron flow, it could also alter electron allocation to substrates (Hageman and Burris, 1980). Since this method of regulation has evolved in one N_2-fixing organism, it is quite likely to be present in others too. Extensive interconversion of nitrogenous compounds occurs in leaves (see Miflin, 1980), so a diurnal change in the type of nitrogenous compounds departing leaves and arriving in nodules might have directly affected electron allocation in nitrogenase.

A second mechanism involves the direct interaction of 'nitrogen sinks' with nitrogenase. New cells in meristems have been shown to have large requirements for nitrogen in the synthesis of nucleic acids and proteins (Dean and Leech, 1982). Meristems might have responded to the C : N ratio of phloem sap by communicating to the nodules that there was a shortage of nitrogen at night and a surfeit of nitrogen during the day. Emissary molecules from the meristems might have been copper-containing peptides, which have been isolated from a variety of legume cells including T.repens. These were shown to influence H^+ and C_2H_2 reductions through effects on oxidative phosphorylation and the ATP : ADP ratio in Rhizobium (Reporter, 1978 ; Reporter et al., 1980 ; Gresshoff et al., 1981).

Both mechanisms provide for the N_2 fixation rate to be regulated according to the status of the pool of available nitrogen in the plant, and hence the integration of the N_2 fixation rate with the use of nitrogen in growth.

Similar diurnal variations to those reported in the present study, with minimum nitrogenase activity (AR) during the day, have been found for L.cosentinii and P.sativum in the glasshouse (Trinick et al., 1976 ; Wheeler and Lawrie, 1976) but were, respectively, not discussed and attributed to change in temperature. The emergence of H^+ reduction as a mechanism to determine the rate of N_2 fixation can account for the lack of diurnal variation in N_2 fixation concluded from studies with T.subterraneum in which only the AR assay was used (Haystead et al., 1979 ; Eckart and Raguse, 1980 ; Hopmans et al., 1982).

Typical of the ambiguity of the literature is the finding that the AR rate of detached P.sativum nodules increased with temperature up to 26°C (Waughman, 1977) whereas, for nodulated roots of the same species, the AR rate was near its diurnal minimum at 26°C in the glasshouse during the daytime (Wheeler and Lawrie, 1976). As the level of complexity is increased from that of the isolated organ, the nodule, to that of the complete community, so the daily profile of N_2 fixation would seem to be influenced by more than diurnal fluctuation in a single variable. The involvement of PEP carboxylase activity in the leaves of C.arietinum with nighttime maxima in AR and root respiration provided a striking example of the need to consider plants in their entirety (Rawsthorne et al., 1981).

H^+ reduction has been identified as being instrumental in the regulation of diurnal variation in N_2 fixation under controlled conditions. The consequences of diurnal variation for the use of HE and AR assays in comparative experiments were presented in chapter 3. The results of this investigation have furthered understanding of how the physiology of the host legume is enmeshed with that of the endosymbiont.

CHAPTER 7

7. General discussion.

The small swards used in this investigation were intended to simulate the growth of T.subterraneum in the field. However, there is an important factor which operates in the field which was omitted from these studies viz. associate species. The effect of grasses in a mixed sward on N_2 fixation was not examined. It is to be expected that grasses would reduce the PPFD by shading the legume.

In all aspects examined, the growth of T.subterraneum appeared to be well adapted to a cool environment. Both the growth rate and the N_2 fixation rate were high at $10^{o}C$ to $15^{o}C$, and decreased above $15^{o}C$. The diurnal studies showed that long nights, characteristic of winter, did not decrease the amount of nitrogen fixed because sufficient carbon was stored during the day to support nitrogenase activity in the dark. In the range of biomass 190-475 g DM m^{-2} the growth rates of swards fixing N_2 and of swards assimilating combined nitrogen were affected very similarly by temperature between $10^{o}C$ and $25^{o}C$. However, one of the characteristics of the legume - Rhizobium symbiosis which became evident early on was a period of nitrogen deficiency between emergence and the onset of N_2 fixation. Shortage of nitrogen and the energy burden of nodulation, were found to result in inhibition of growth. Nitrogen deficiency may not have an effect on growth when legumes are establishing early in the season because the deficiency seems to be exacerbated at high PPFD. Also, combined nitrogen in the soil may alleviate the problem. For vegetative swards in the biomass range 95 to 475 g DM m^{-2}, the N_2 fixation rate was depressed more by increase in temperature than was the growth rate, and the lower nitrogen

concentration of plants grown at high temperature showed that there was a degree of flexibility in the amount of nitrogen required to permit the growth potential of the swards to be realised.

The high energy cost of fixing N_2 and reducing H^+ appeared to be responsible for N_2-fixing swards growing slightly slower than those assimilating combined nitrogen. This is interpreted as the forfeit which T.subterraneum has incurred by entering into symbiosis. The enzyme nitrogenase is thought to have developed from an hydrogenase which originally required energy to expel protons from the cell into a primitive reducing atmosphere, and the legume - Rhizobium symbiosis itself is thought to have become established to allow growth in nitrogen-deficient soils. As nitrogenase evolved independently of the legume, it need not follow that every feature of the legume - Rhizobium symbiosis should serve a beneficial purpose in the association. It can be argued that, with respect to Rhizobium which requires fixed carbon from the legume, the host property of photorespiration is very wasteful. Chloroplasts are thought to have evolved from photosynthetic bacteria which entered into symbiosis with land plants (Whatley and Whatley, 1981), so perhaps photorespiration fulfilled a useful function in the prokaryotes. So, it should not be expected that nodules operate with perfect efficiency. Symbiosis is a compromise. Nevertheless, the data presented in this study indicate that H^+ reduction, putatively viewed as a purposeless use of energy, has been enlisted to serve in the symbiotic interest.

If the rate of total nitrogenase activity responds passively to the respiration rate, H^+ reduction offers an intrinsic means of regulating the N_2 fixation rate. H^+ reduction is a mechanism of short term regulation, partly independent of the carbon supply to nodules. The results of this study uphold a model in which transpiration imposes a

diurnal rhythm on the status of the pool of available nitrogen and elicits regulation of the N_2 fixation rate by alterations in electron allocation to N_2 and to H^+. Also, it is proposed that oxidation of H_2 by Hup functions to remove molecular O_2 from the bacteroids when lèghemoglobin levels are low and so to protect nitrogenase from damage by O_2.

There are several reports in the literature of N_2 fixation being carbon-limited. The observed plasticity in RE, which was apparently controlled, and the optimum for N_2 fixation at 15°C and low PPFD whereas 10°C was optimal for sward growth at low PPFD, indicate that carbon availability did not limit N_2 fixation in T.subterraneum. At low temperature, in particular, the data indicate that there was ample carbon available to support synthetic activities of nodulated roots, because the rate of maintenance respiration was low. On the basis of empirical evidence, it is suggested that the use by nodules of readily available fixed carbon limited the growth of the host legume. It is of interest to recall here the work of Feigenbaum and Mengel (1979) which showed that, when the energy status of M.sativa was low, the synthetic processes of the host legume were energy-limited, but N_2 fixation was not. Hence, the increases seen in N_2 fixation when legumes have been grown in high levels of CO_2 (Hardy and Havelka, 1976) are interpreted as a response of N_2 fixation to the greater overall growth of the plants. The question can be posed: "Does an increase in CO_2 fixation primarily supply more energy to 'enable' N_2 to be fixed, or does it create a 'demand' for more N_2 to be fixed?" If N_2 fixation is not carbon-limited, then an increase in CO_2 fixation (at high levels of CO_2) will alleviate the carbon limitation on legume growth and result in a need for more N_2 to be fixed. While the dependence of N_2 fixation on carbohydrate is obligate at a fundamental level, the variation noted in H^+ reduction

showed that when carbon allocation to nodules was reduced, the efficiency of N_2 reduction could be increased . There are examples in the literature of potential for variation in other measures of symbiotic efficiency, for example, in the number of nodules which must be maintained by P.arvense and by T.subterraneum to achieve a given rate of N_2 fixation (Pate, 1958 ; Silsbury, 1981).

The information pertaining to the AR assay reported in this study is of considerable practical importance. AR was linear with time and this indicates that the low C_2H_4 : N_2 ratios found were not due to an C_2H_2-induced decline in nitrogenase activity (Minchin et al., 1983) but rather were a consequence of the handling of plants, as suggested by others (Masterson and Murphy, 1976), or of the properties of gaseous exchange in sealed assay vessels. The effect of temperature found on the C_2H_4 : N_2 ratio is especially relevant to AR work in which there are diurnal or seasonal drifts in temperature. A disturbance to nitrogenase kinetics above 20°C limits meaningful use of the AR assay in many studies in the field and glasshouse. For long-term studies, in which Hup can develop as a normal feature of physiological ageing, AR provides a more reliable index by which to compare rates of N_2 fixation than AR-HE. However, for short-term studies in which Hup is absent, AR-HE can be interpreted as being directly equivalent to the N_2 fixation rate. The diurnal variation revealed in AR-HE shows that the assay time must be standardised, and the diurnal profile must be known, if the HE and AR assays are to be used to make comparative measurements of N_2 fixation. Overall, it is concluded that the HE and AR assays are apt methods by which to estimate N_2 fixation when their limitations have been established.

While it can be claimed that research with whole plants is more representative of growth in the field than that in which organs or

enzymes are examined isolated from their normal environment, questions are posed which may best be answered by further study at these more fundamental levels of organisation. However, this is a more useful progression than that in which a response is first characterised for an excised nodule, for example, and is then found to have no relevance to the whole plant. A 'signalling' mechanism by which nitrogenase is regulated diurnally, and the conditions arising during ontogeny which lead to development of Hup, might be defined by research at the molecular level. The finding of a temperature effect on depression of nitrogenase activity by combined nitrogen warrants further investigation. The data infer that in the winter, when temperatures are low, T.subterraneum can continue to fix N_2 in the presence of soil nitrogen. Later in the season, when soil temperatures are higher and nitrogenase is more susceptible to inhibition, much of the soil nitrogen may have been leached by rain from the upper layers where most nodules of T. subterraneum are to be found. Thus, exploration of the carbon and nitrogen economies of legumes which fix N_2 and assimilate combined nitrogen at the same time, would be pertinent to the growth of legumes in the field.

Appendix I Analysis of variance for nitrogenase activities at 10°C. Means, with SE, for 6 values.

	------ AR ------		-------- HE --------		---- AR - HE ----		--------- RE ---------	
Biomass Range, g	µmol $C_2H_4 m^{-2} h^{-1}$ High+	Low+	µmol $H_2 m^{-2} h^{-1}$ High	Low	High	Low	High	Low
95-190	748(34)	411(25)	540(36)A	265(19)	208(11)	146(15)	0.28(0.02)	0.35(0.03)
190-285	624(102)	430(22)	348(60)B	264(40)	276(45)	165(39)	0.44(0.01)	0.38(0.09)
285-380	693(33)	458(20)	383(65)B	257(31)	309(56)	200(28)	0.45(0.09)	0.44(0.06)
380-475	576(46)	430(59)	341(33)B	229(40)	235(19)	202(23)	0.41(0.02)	0.48(0.04)
475-570	588(29)		351(32)B		237(44)		0.39(0.06)	
F Value for AOV	1.71NS	0.30NS	3.17*	0.26NS	1.04NS	0.99NS	1.93NS	0.81NS
LSD P0.05			138					

NS Not Significant

* Significant at P0.05

Figures within columns followed by different letters are significantly different at P0.05.

\+ PPFD

Appendix II Analysis of variance for nitrogenase activities at 15°C. Means, with SE, for 6 values.

	------ AR ------		-------- HE --------		---- AR - HE ----		--------- RE ---------	
Biomass Range, g	µmol $C_2H_4 m^{-2} h^{-1}$ High+	Low+	µmol $H_2 m^{-2} h^{-1}$ High	Low	High	Low	High	Low
95-190	791(83)A	506(57)	634(82)A	330(55)A	158(13)C	176(18)B	0.21(0.02)C	0.37(0.05)B
190-285	584(54)B	548(65)	291(53)B	351(94)A	293(40)BC	197(31)B	0.51(0.07)B	0.42(0.11)B
285-380	598(17)B	492(16)	237(27)B	258(22)A	361(29)B	235(19)B	0.60(0.05)B	0.48(0.04)B
380-475	447(32)B	399(32)	157(71)BC	6(4)B	290(40)BC	392(34)A	0.70(0.14)A	0.98(0.01)A
475-570	626(76)A		55(27)C		571(99)A		0.88(0.05)A	
F Value for AOV	4.46*	1.82NS	14.95**	8.06**	8.20**	13.47**	10.68**	20.47**
LSD P0.05	170		165	165	154	79	0.22	0.17

NS Not Significant

* Significant at P0.05

** Significant at P0.01

Figures within columns followed by different letters are significantly different at P0.05.

\+ PPFD

Appendix III Analysis of variance for nitrogenase activities at 20°C. Means, with SE, for 6 values.

	------ AR ------		-------- HE --------		---- AR - HE ----		--------- RE ---------	
Biomass Range, g	µmol $C_2H_4 m^{-2} h^{-1}$ High+	Low+	µmol $H_2 m^{-2} h^{-1}$ High	Low	High	Low	High	Low
95-190	650(54)A	439(25)A	323(48)A	224(31)A	327(15)	214(28)	0.52(0.04)B	0.49(0.06)B
190-285	526(20)B	303(14)B	236(15)A	107(28)B	279(28)	196(31)	0.55(0.02)B	0.64(0.09)B
285-380	517(14)B	278(51)B	203(46)A	18(16)C	314(41)	260(39)	0.61(0.09)AB	0.96(0.03)A
380-475	439(26)B	151(38)C	82(40)B	2(2)C	357(57)	149(37)	0.80(0.09)A	0.99(0.01)A
F Value of AOV	7.20**	11.50**	6.0**	20.57**	0.71NS	1.84NS	3.31*	17.14**
LSD p0.05	96	102	120	66			0.203	0.17

NS Not Significant
* Significant at P0.05
** Significant at P0.01
Figures within columns followed by different letters are significantly different at P0.05.
\+ PPFD

Appendix IV Analysis of variance for nitrogenase activities at 25°C. Means, with SE, for 6 values.

	------ AR ------		-------- HE --------		---- AR - HE ----		--------- RE ----------	
Biomass Range, g	µmol $C_2H_4 m^{-2}h^{-1}$ High+	Low+	µmol $H_2 m^{-2}h^{-1}$ High	Low	High	Low	High	Low
95-190	852(43)B	577(23)	280(63)A	260(17)A	572(60)B	317(9)B	0.67(0.07)B	0.55(0.01)B
190-285	794(19)BC	518(41)	61(29)B	173(22)B	740(46)B	345(51)B	0.92(0.04)A	0.65(0.05)B
285-380	1034(81)A	559(41)	10(8)B	50(23)C	1024(80)A	508(58)A	0.99(0.01)A	0.90(0.05)A
380-475	682(58)C	492(41)	41(17)B	20(11)C	641(42)B	472(48)A	0.95(0.02)A	0.95(0.03)A
F Value of AOV	7.10**	1.03NS	11.87**	34.03**	11.32**	4.24*	13.42**	24.18**
LSD P0.05	163		106	56	174	134	0.12	0.12

NS Not Significant
* Significant at P0.05
** Significant at P0.01
Figures within columns followed by different letters are significantly different at P0.05.
\+ PPFD

Appendix V Analysis of variance for diurnal variation in AR, µmol C_2H_4 $m^{-2}h^{-1}$. Means, with SE, for 3 values, except at 20°C/high, 12 values.

Time, hours	Temperature/PPFD 10°C/Low	10°C/High	15°C/Low	15°C/High	20°C/Low	20°C/High	25°C/Low	25°C/High
11.00	730(12)	619(25)BC	350(42)BC	650(62)	260(49)BC	854(35)B	685(53)AB	887(52)
15.00	773(30)	571(31)C	301(16)C	601(32)	276(19)AC	727(26)C	780(27)A	858(82)
19.00	808(93)	722(58)AB	413(6)B	702(65)	276(12)AC	1061(47)A	782(56)A	1038(60)
23.00	636(54)	760(34)A	563(47)A	777(29)	351(18)A	869(22)B	579(32)B	917(111)
03.00	638(33)	698(34)AB	442(38)B	709(46)	277(41)AC	860(40)B	612(60)B	829(22)
07.00	644(40)	620(26)BC	405(13)B	704(2)	170(13)B	719(21)C	553(31)B	775(48)
F Value	2.25NS	3.94*	8.23**	1.76NS	3.95*	13.92**	4.96*	1.72NS
LSD P0.05		113	96		90	94	139	

* Significant at P0.05

** Significant at P0.01

NS Not significant

Figures within columns followed by different letters are significantly different at P0.05

Appendix VI Analysis of variance for diurnal variation in HE, µmol H_2 $m^{-2}h^{-1}$. Means, with SE, for 3 values, except at 20°C/high, 12 values.

Time, hours	Temperature/PPFD 10°C/Low	10°C/High	15°C/Low	15°C/High	20°C/Low	20°C/High	25°C/Low	25°C/High
11.00	624(22)AB	349(23)B	97(25)A	442(32)B	89(25)	408(36)BC	244(30)	426(38)AC
15.00	659(15)A	422(13)A	200(12)B	579(19)A	137(29)	495(27)B	268(31)	441(37)AC
19.00	556(52)B	461(25)A	226(5)B	475(26)B	107(12)	698(42)A	261(35)	468(35)A
23.00	371(36)C	463(23)A	90(24)A	434(3)BC	94(24)	410(40)BC	220(6)	314(39)B
03.00	352(35)CD	302(22)B	123(39)A	345(55)CD	77(14)	428(24)BC	224(18)	341(38)BC
07.00	257(17)D	297(29)B	111(11)A	304(25)D	54(5)	354(21)C	179(23)	290(18)B
F Value	25.94**	11.04**	6.51**	10.01**	1.97NS	14.26**	1.62NS	4.52**
LSD P0.05	100	71	69	95		91		108

* Significant at P0.05

** Significant at P0.01

NS Not significant

Figures within columns followed by different letters are significantly different at P0.05

Appendix VII Analysis of variance for diurnal variation in AR-HE, C_2H_4-H_2 $m^{-2}h^{-1}$. Means, with SE, for 3 values, except at 20°C/high, 12 values.

Time, hours	Temperature/PPFD 10°C/Low	10°C/High	15°C/Low	15°C/High	20°C/Low	20°C/High	25°C/Low	25°C/High
11.00	106(10)C	270(6)B	254(46)BC	208(31)B	171(28)AC	446(17)A	441(23)	460(16)
15.00	114(27)C	149(20)C	101(13)A	31(19)C	139(10)AC	232(18)B	511(58)	417(50)
19.00	253(57)B	260(35)B	187(4)B	227(41)B	169(20)AC	363(48)A	521(50)	570(33)
23.00	265(27)B	297(13)AB	473(34)D	343(28)A	257(24)B	460(48)A	358(37)	603(75)
03.00	286(40)AB	396(53)A	319(24)C	364(40)A	200(28)BC	430(39)A	389(62)	488(19)
07.00	387(33)A	322(52)AB	293(13)C	400(23)A	116(11)A	364(24)A	375(10)	485(57)
F Value	9.35**	5.41**	22.65**	18.91**	5.22**	5.84**	2.60NS	2.23NS
LSD P0.05	109	108	82	97	66	98		

** Significant at P0.01

NS Not significant

Figures within columns followed by different letters are significantly different at P0.05

Appendix VIII Analysis of variance for diurnal variation in RE. Means, with SE, for 3 values, except at 20°C/high, 12 values.

Time, hours	Temperature/PPFD 10°C/Low	10°C/High	15°C/Low	15°C/High	20°C/Low	20°C/High	25°C/Low	25°C/High
11.00	0.15(0.02)D	0.44(0.02)BC	0.72(0.08)A	0.32(0.02)D	0.66(0.04)	0.53(0.03)A	0.65(0.02)	0.52(0.02)BD
15.00	0.15(0.03)D	0.26(0.02)D	0.34(0.04)B	0.05(0.03)E	0.51(0.07)	0.32(0.02)B	0.65(0.05)	0.49(0.02)D
19.00	0.31(0.04)C	0.36(0.02)CD	0.45(0.01)B	0.32(0.03)D	0.61(0.05)	0.33(0.04)B	0.67(0.04)	0.55(0.02)BCD
23.00	0.42(0.03)B	0.39(0.01)C	0.85(0.03)A	0.44(0.02)C	0.73(0.06)	0.52(0.05)A	0.61(0.03)	0.66(0.01)A
03.00	0.44(0.05)B	0.56(0.05)A	0.73(0.07)A	0.51(0.06)B	0.72(0.01)	0.49(0.03)A	0.66(0.02)	0.59(0.04)AB
07.00	0.60(0.03)A	0.52(0.06)AB	0.73(0.03)A	0.57(0.03)A	0.68(0.03)	0.51(0.03)A	0.68(0.02)	0.62(0.03)AC
F Value	27.01**	9.61**	14.89**	27.18**	2.98NS	8.22**	0.52NS	7.20**
LSD P0.05	0.11	0.11	0.16	0.04		0.10		0.07

** Significant at P0.01

NS Not significant

Figures within columns followed by different letters are significantly different at P0.05

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