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Effect of light and atmospheric carbon dioxide concentration on nitrogen fixation by herbage legumes

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Summary Lucerne, red clover and white clover were grown at two atmospheric concentrations of CO_2 (300 and 1000 $\mu l\ l^{-1}$) and the effects on N_2 fixation, nodule mass/number and root/shoot dry matter production determined. Pea plants were similarly evaluated as a comparison with grain legumes. CO_2 enrichment increased N_2 fixation activity in all cases but activity/unit nodule mass was significantly increased only in the pea. The enhancement of N_2 fixation in herbage legumes by CO_2 enrichment reflected an increase in nodule mass which in turn was attributed to increased nodule number, and results show that under the experimental conditions obtaining here photosynthate supply did not limit nodule N_2 fixation in these plants though it was limiting in the case of peas.

White clover growing in a 6 and 14 hour photoperiod was studied for response of the N_2 fixing system to light. Long photoperiod (14 hour) plants assayed at constant temperature (20°C) did not show a significant response to light at the end of the dark period either in terms of fixation per plant or per unit nodule mass, in contrast with short photoperiod (6 hour) plants which showed significant responses. Short photoperiod plants compensated for reduced photosynthates by maintaining only half the root nodule mass and fixation activity of 14 hour photoperiod plants though plants in both systems supported similar rates of N_2 fixation per unit mass of nodule during the photoperiod. Comparison of N_2 fixation activities in whole and decapitated plant systems indicates the importance of shoot reserves for sustaining nitrogenase activity in white clover during short-term interruption of photosynthesis. These results support the conclusion of the CO_2 enrichment studies, that herbage legumes have the potential for supplying their nodule photosynthate requirements for sustaining optimum rates of N_2 fixation and excess carbon supply is used solely to promote further nodulation.

Nodules of short photoperiod white clover plants were less efficient in N_2 fixation in that they evolved more H_2 relative to N_2 (C_2H_2) reduced than did long photoperiod plants.

Introduction

The concept of carbohydrate supply in the plant being a primary factor in legume symbiosis was considered in early studies on N_2 fixation[1,6,7,26,27]. Factors favouring carbohydrate supply to legume roots were shown to increase nodule mass and number though measurements of N_2 fixation activity were not possible in these experiments. More recent work with both grain[10,18] and herbage legumes[16] demonstrated the beneficial effects of CO_2 enriched atmospheres on root nodule N_2 fixation activity. In apparent contradiction with earlier work Masterson and Sherwood[16] did not detect any increase in nodule

density of white clover plants subjected to increased atmospheric CO_2 concentration and similar disparities are evident in results reported for grain legumes. Test species, bacterial strain, plant physiological state and environmental conditions might be expected to influence the response of the legume nodule system to carbohydrate supply and so contribute to the contradictory literature reports. The present study was initiated to determine if energy supply represents a rate limiting step for nodule N_2 fixation in herbage legumes growing under optimum conditions. Carbohydrate supply within the plant was modified by adjusting atmospheric CO_2 concentration and photoperiod and the effects on nodule mass and nitrogen fixation determined.

Nitrogenase mediated reduction of protons appears to be an obligatory process accompanying N_2 reduction in all N_2 fixing organisms. ATP and reductant required for fixation of N_2 may be dissipated in the seemingly energy wasteful process of H_2 production. Some N_2 fixing systems possess an uptake hydrogenase capable of recycling and utilising the evolved H_2 thus conserving energy loss in the fixation system[5], and a survey of agriculturally important legumes described H_2 evolution as being a major factor affecting the efficiency of N_2 fixation by nodulated symbionts where photosynthate supply is limiting[22]. This aspect was investigated in the present study to determine if H_2 evolution from white clover nodules is influenced by carbohydrate supply within the plant.

Materials and methods

Plant material and growing conditions

Treatment A. The effect of atmospheric CO_2 enrichment on N_2 fixation by cultivars of white clover (Blanca and S100) red clover (Hungaropoly), lucerne (Europa) and pea (Meteor) was studied under glasshouse conditions. Plants were grown in a 50:50 soil/sand mixture in 150 mm diameter pots. The mean day temperature and light intensity (14 h photoperiod) over the duration of the experiment was 25°C and 25,000 lux respectively. Plants were grown at a mean CO_2 concentration of 300 $\mu l\, l^{-1}$ for 42 days after which half of the plants (10 replicates in each treatment) were transferred to a CO_2 concentration of 1000 $\mu l\, l^{-1}$. Enrichment was maintained during the photoperiod for 6 hours over 21 days after which plants were assayed. The effect of short term enrichment was studied in white clover by maintaining 1000 $\mu l\, l^{-1}$ CO_2 in the atmosphere for 10 hours during the photoperiod. N_2 fixation was measured at intervals over a period of 4 days.

Treatment B. The effect of length of photoperiod on N_2 fixation was determined on S100 white clover plants growing in pots containing perlite under controlled environmental conditions (Conviron Model PGW-36). Plants were inoculated with an effective strain of *Rhizobium trifolii* and supplied with nutrient medium containing 30 ppm NO_3-N with plant and nodule development being stimulated by the added N. Plants were grown for 42 days at 26,000 lux (14 h photoperiod) and day/night temperatures of 20/15°C. The photoperiod was then reduced to 6 hours in half of the plants and clovers in both treatments were grown for a further 14 days.

Assays

N_2 fixation was measured by the acetylene reduction technique[25] [$N_2(C_2H_2)$]. In treatment A whole plants, including the soil-root mass, were assayed using methodology previously described[17]. Following incubation (60 min) of plants in containers containing 0.1 atm C_2H_2, 30 ml of the gas phase was subsampled to an evacuated McCartney bottle. Samples were analysed by gas chromatography. Ethylene (C_2H_4) was measured with a flame ionisation detector and a stainless steel column 1 m × 1 mm packed with Porapak N and operated at 70°C using N_2 as carrier gas.

In the photoperiod experiments (treatment B) N_2 fixation was measured both on whole plants and on nodulated roots washed free of perlite. The chamber was set at a constant temperature (20°C) for measurement of nodule activity in the dark/light period. Plants were assayed at the end of the 10 hour dark period, just prior to the photoperiod (time 0 assay) and at intervals throughout the photoperiod. For nodulated root assays, plants were decapitated just prior to assay. H_2 evolution in air was measured on nodulated root segments, washed free of perlite and which were incubated in 20 ml vacutainers for 2 hours. H_2 was determined by gas chromatography using a hot wire detector system. Gases were separated on a 3 m × 1 mm stainless steel column, packed with molecular sieve, operating at a column temperature of 70°C, with a detector temperature of 120°C and a filament current of 65 mA, using argon as carrier gas.

Following assay, plants were separated into root, shoot and nodule material, freeze dried and weighed and total nonstructural carbohydrate (TNC – sugars and starch) of legume herbage determined[23].

Results

CO_2 enrichment

The effect of short-term CO_2 enrichment on N_2 fixation in white clover is shown in Fig. 1. Results show a gradual increase in activity with time and there was no evidence of a significant response to CO_2 within hours of enrichment. In contrast long-term enrichment with CO_2 produced increases in N_2 fixation with all the species studied (Table 1). The increase in N_2 fixation/plant was associated with increased nodule mass which in turn was reflected in increased nodule number. Activity/unit nodule mass was not significantly affected by CO_2 treatment in herbage legumes though pea plants showed a significant increase in this activity.

Dry matter yields of root and shoot material were increased on CO_2 enrichment, though only in the case of red clover was there an indication of preferred root growth as indicated by the root/shoot values (Table 2). Nodule mass as a percentage of root mass was little affected by CO_2 treatment. There was a significant response to enrichment in terms of shoot total N though only in the case of the pea was a significant increase in percent N obtained, with clovers and lucerne showing a decrease in this parameter.

Effect of photoperiod in white clover

Plant growth. Growth characteristics of the clover plants in the two

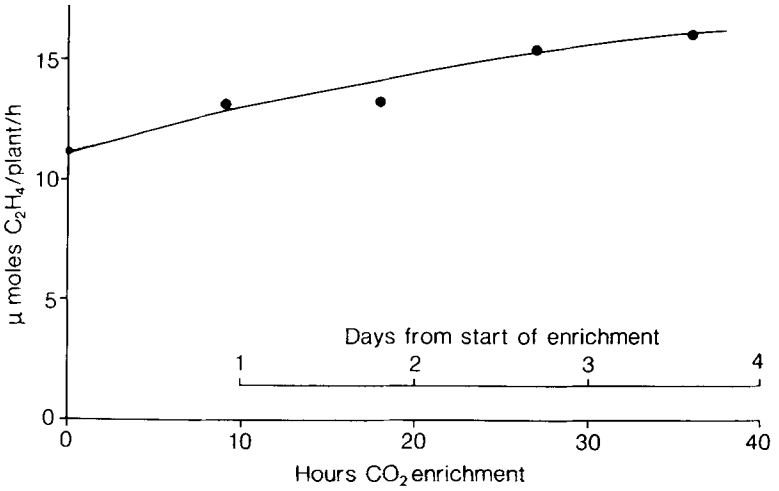

Fig. 1. Effect of short-term CO_2 enrichment ($1000\,\mu l\,l^{-1}$) on $N_2(C_2H_2)$ fixation by white clover.

Table 1. Effect of long-term CO_2 enrichment on nodulation and N_2 fixation in grain and herbage legumes

			Clovers		
	Pea	Lucerne	Red	Blanca	S100
300 μl l⁻¹ CO_2					
μ moles C_2H_4/plant/h	18.0	10.0	16.0	13.0	12.0
η moles C_2H_4/mg nodule/h	192	356	242	355	277
Nodule weight (mg)	93.7	28.0	66.1	58.0	43.3
Nodule number	77.0	ND	275	ND	150
Nodule – % root mass	13.2	3.3	9.7	14.2	13.3
1000 μl l⁻¹ CO_2					
μ moles C_2H_4/plant/h	37.0***	16.0***	24.0***	21.0***	19.0**
η moles C_2H_4/mg nodule/h	226*	344	284	232	303
Nodule weight (mg)	139.1***	46.4***	84.5***	90.5***	62.7***
Nodule number	124**	ND	344***	ND	263*
Nodule – % root mass	16.2	3.8	6.0	15.4	12.5

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. ND – Not determined.

Table 2. Effect of long-term CO_2 enrichment on dry matter production in grain and herbage legumes

			Clovers		
	Pea	Lucerne	Red	Blanca	S100
300 µl l⁻¹ CO_2					
Shoot (g/plant)	2.47	1.15	1.68	1.20	1.02
Root (g/plant)	0.62	0.81	0.61	0.35	0.28
Root/shoot	0.25	0.70	0.36	0.29	0.27
Total N (g)	0.056	0.034	0.051	0.034	0.032
N (%)	2.28	2.95	3.03	2.81	3.16
1000 µl l⁻¹ CO_2					
Shoot (g/plant)	3.87***	1.91***	2.47***	1.82***	1.72***
Root (g/plant)	0.72	1.18***	1.33***	0.50***	0.44***
Root/shoot	0.19	0.62	0.54**	0.27	0.26
Total N (g)	0.096***	0.048***	0.068***	0.047**	0.046*
N (%)	2.48*	2.52***	2.47*	2.59*	2.70**

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

photoperiods are shown in Table 3. Dry matter production of both roots and shoots in the short photoperiod plants was reduced by half as also was the root nodule mass. There was no significant difference in nodule mass as a percentage of the root mass or in per cent TNC content of the herbage, between the two light treatments.

N_2 fixation – whole plants. N_2 fixation activity of intact white clover plants growing in a 14 h photoperiod did not show a response to light, and activity at the end of the dark period (time 0 assay) was not significantly different to values measured 6 hours into the photoperiod (Fig. 2A). Whole plant fixation in the short photoperiod treatment showed a small response to light with the major effect here being an overall reduction in activity reflecting the decreased nodule mass of these plants (Table 3). However specific N_2 fixation activity showed a marked response to light in the short photoperiod plants with values approaching those of the long photoperiod plants early in the light cycle (Fig. 2B).

N_2 fixation – nodulated roots. Decapitation of long photoperiod plants at the end of the 10 h dark period reduced fixation activity/unit nodule mass 2-fold (Fig. 3) with respect to whole plant systems

Table 3. Effect of photoperiod on dry matter production, nodulation, and total nonstructural carbohydrate (% TNC in herbage) of white clover (cv. S100)

Photoperiod (h)	g/pot			Nodulation % of root mass	TNC
	Shoot	Root	Nodule		
14	1.56	0.99	0.115	10.4	7.17
6	0.82	0.37	0.047	11.3	6.82

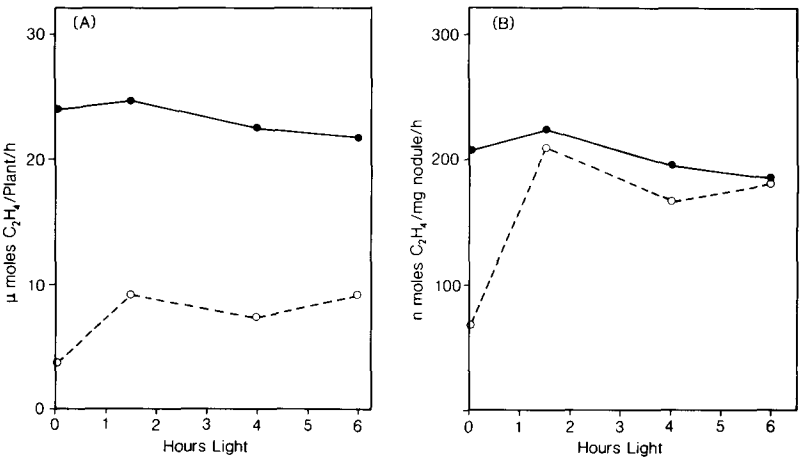

Fig. 2. Effect of length of photoperiod on whole plant $N_2(C_2H_2)$ fixation by S100 white clover growing in 14 h (•—•) and 6 h (o– –o) photoperiods. Activity per plant (A) and per unit nodule mass (B) was measured at constant temperature (20°C).

(Fig. 2B). The effect of this treatment was less when plants were decapitated 2, 4.5 and 6 h after the commencement of the light cycle with values increasing with photoperiod though still remaining short of activities of whole plant systems. These results implicate both shoot and root carbon sources as functioning in the maintenance of nodule activity in the dark period and in the early part of the photoperiod. The effects of decapitation on short photoperiod plants are even more pronounced with activities showing a slow response to light and never achieving values of comparable whole plant systems (Fig. 3).

H_2 evolution

The observed increase in fixation activity of nodulated roots as the photoperiod progressed was accompanied by an increase in H_2 evolution (Fig. 3). These results are expressed in terms of relative efficiency (R.E.) values[22] in Table 4. Plants growing in the short photoperiod had significantly lower R.E. values indicating reduced fixation efficiency relative to long photoperiod plants.

Table 4. Relative efficiency (R.E.) of nitrogen fixation by white clover (cv. S100) growing under short (6 h) and long (14 h) photoperiods. Assays at conclusion of dark period (time 0) and at intervals throughout the photoperiod

Treatment	Dark/light cycle – hours light			
	0	2.0	4.5	6.0
6 h photoperiod R.E.	0.77	0.64	0.69	0.66
14 h photoperiod R.E.	0.72	0.75**	0.80**	0.77**

**$P \leq 0.01$. Relative efficiency (R.E.) $= 1 - \frac{\text{moles } H_2 \text{ in air}}{\text{moles } C_2H_4 \text{ in air}/C_2H_2}$

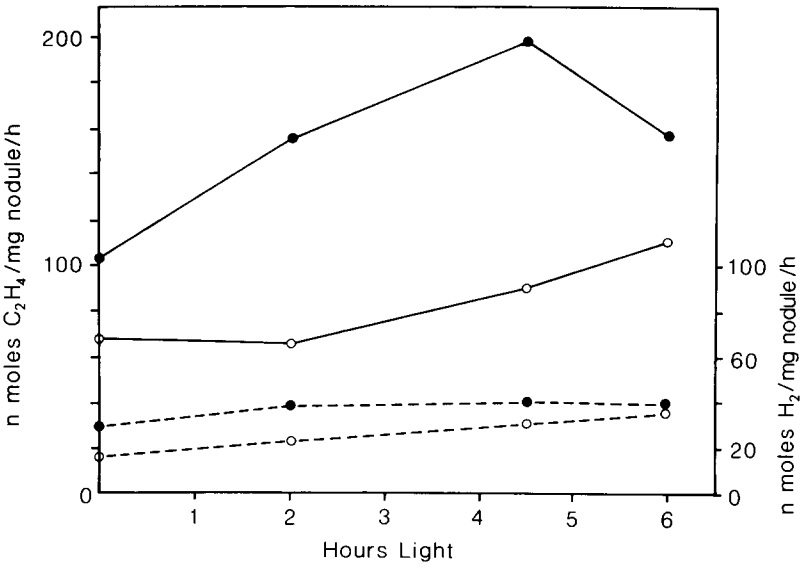

Fig. 3. Effect of length of photoperiod on $N_2(C_2H_2)$ fixation and H_2 evolution by decapitated roots of S100 white clover. $N_2(C_2H_2)$ fixation in 14 h (•—•) and 6 h (○—○) photoperiod plants and H_2 evolution by 14 h (•– –•) and 6 h (○– –○) photoperiod plants was determined at constant temperature (20°C).

Discussion

Results show that the response of herbage legumes to CO_2 is dependent on whether the plants were exposed to short or long-term enrichment. A short-term response, when mass of nodule N_2 fixing tissue would not have increased significantly, would point to nitrogenase activity being limited by photosynthate supply. The absence of a short-term response in clover fixation activity to CO_2 enrichment has also been reported by others[12]. Such a response has been obtained with pea plants where 200% increase in nodule activity after 6 h enrichment with CO_2 was reported[18]. Others varied photosynthate supply

in the soybean plant by shading, defoliation, provision of supplemental light and depodding[14] or by CO_2 enrichment[10] and concluded that photosynthate supply is a major factor limiting N_2 fixation in this system. Results reported here for peas in which plants showed a significant increase in nodule activity following long-term CO_2 enrich ment are in agreement with this conclusion. However, herbage legumes appear to behave differently in that even long-term enrichment with CO_2 did not result in a significant increase in nodule fixation activity. Augmented photosynthate supply was used solely to increase root nodule mass which resulted from increased nodulation with individual nodule mass not being significantly affected. True photosynthate limitation of nodule activity is reflected in specific activity values[24] Although nodule activity/unit nodule mass values expressed in the *in vivo* assay for nitrogenase used here are not specific activities in the strict sense they do demonstrate that under optimum conditions nodulation rather than nodule fixation activity is limited by photosynthate supply in herbage legumes. That differences may exist between grain and herbage legumes in their response to CO_2 is not surprising in view of the fact that the former exhibit a shorter, more intense period of N_2 fixation, and expend more energy in reproductive growth than the latter.

Masterson and Sherwood[16] found increased N_2 fixation on CO_2 enrichment reflected increased nodule specific activity without total nodule mass being affected, in contradiction to results reported here. Plant growth vigor appears to have been less in the former experiment perhaps as a result of lower light intensities, and light limitation can reduce specific activity of nodule fixation as has been demonstrated in the photoperiod experiments reported here. CO_2 enrichment increases the efficiency of photosynthesis largely by reducing photorespiration[10], so the effect of this treatment on plants growing under irradiances limiting nodule activity may be to increase the supply of photosynthate to carbon depleted nodules thus enhancing nitrogenase activity. Where photosynthate supply is not limiting for enzyme activity effects of excess photosynthate may be evident in increased nodule mass only.

Results of the photoperiod experiments are in agreement with the conclusion that under conditions otherwise optimum for forage legume growth, nodule N_2 fixation activity is not limited by photosynthate supply. There was no evidence of a light induced diurnal variation in N_2 fixation activity of white clover growing in the 14/10 h light/dark cycle in agreement with results reported by others[11]. However additional evidence suggests a much closer coupling between short-term rates of photosynthesis and nitrogenase activity in white clover[21].

These conflicting results may be partly explained in terms of plant physiological response to varying experimental conditions, *e.g.* results obtained here demonstrate the greater dependence of short photoperiod plants on concurrent photosynthetic activity compared with non-stressed plants. Reduction of the photoperiod to 6 h resulted in a marked light induced diurnal effect both in terms of activity/plant and activity/unit nodule mass. Plants in this treatment showed a response to light with activity/unit nodule mass being restored to the level of long photoperiod plants within 2 h of commencement of the light cycle. Determination of root nodule weight as a percentage of total root mass shows that white clover growing under different photoperiods maintains a nodule mass which is in balance with overall plant growth.

White clover has been shown to exhibit diurnal variations in N_2 fixation activity resulting from fluctuations in temperature[3,9,17]. Although it is difficult to extrapolate to field conditions results obtained here suggest that where light is not limiting *i.e.* well grazed swards in summer, it is likely that observed diurnal variation in N_2 fixation arises more from fluctuations in temperature than light.

Plants growing in a short photoperiod were less efficient in N_2 fixation in that they evolved more H_2 to C_2H_2 reduced than did long photoperiod plants. H_2 evolution can be influenced by variation in electron donation for proton reduction by nitrogenase[13] or by the activity of an uptake hydrogenase[22]. Both of these mechanisms have been implicated as functioning in peas where net H_2 evolution was shown to increase with increasing growth irradiance[2]. No evidence has been obtained for the presence of an uptake hydrogenase activity in *R. trifolii* strains[20] or for its induction in photosynthate deprived nodules as has been tested in these experiments. The highest relative efficiency value reported here was 0.80 although Crush and Tough[4] reported values ranging from 0.52 to 1.0 in white clover. These authors were successful in measuring H_2 production by white clover in the field through Masterson (pers. comm.) failed to detect H_2 evolution from white clover pastures. Results obtained with pigeon peas growing in large soil columns demonstrated that despite profuse liberation of H_2 *in vitro* from nodules lacking uptake hydrogenase, H_2 gas was not detected in any of the soil columns and these workers concluded that H_2 was not lost from the soil – plant ecosystem but was conserved by H_2-oxidising bacteria[15]. Factors affecting soil H_2 uptake have been studied by Popelier *et al.*[19]. Measurements of H_2 evolution in the experiments reported here were obtained for plants growing in soilless culture and which had been washed free of perlite prior to assay.

The presence of H_2 metabolising bacteria in soil makes field measurements of H_2 evolution by legumes difficult and complicates interpretation of the date.

R.E. values of 0.6–1.0 have been reported for subterranean clover growing in soil-less culture, with the higher value being transitory and associated with defoliated plants[8]. The results were interpreted as indicating the presence of an uptake hydrogenase in photosynthate-deprived nodules of subterranean clover, an activity which appears to be absent in comparable white clover nodules. Measured values of H_2 uptake will vary depending on legume species, microsymbiont strain or plant growth stage[2]. A further complication is that exceptionally high R.E. values obtained with herbage legume nodules tend to be associated with low nodule activity so that it is possible for errors to accrue when measuring H_2/C_2H_4 at very low gas concentrations.

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