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Symbiotic N_2 fixation by legumes growing in pots

II. Uptake of ^{15}N-labelled NO_3^-, C_2H_2 reduction and H_2 evolution by *Trifolium subterraneum* L., *Medicago truncatula* Gaertn. and *Acacia dealbata* Link.

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Summary An indirect isotopic method was developed to estimate N_2 fixation by legume grown in pots. Two pasture legumes, subterranean clover and barrel medic, and one Australian native legume, silver wattle, were grown in a N_2-depleted atmosphere of $Ar:O_2:CO_2$ (77:20:3) for a short period of time following addition of ^{15}N-labelled KNO_3 (8 mg N pot^{-1}). Uptake of fertiliser-N by these plants was compared with that of plants receiving the same amount of ^{15}N-labelled NO_3^- but grown under a normal N_2 atmosphere. Symbiotically fixed N_2 was calculated from the difference in fertiliser-N uptake by plants in the Ar and N_2 atmospheres, based on the assumption that the nitrogen requirement of the legumes grown in the former atmosphere was satisfied by an equivalent uptake of labelled $NO_3^- - N$ from the soil.

The percentage decrease in C_2H_2 reduction activity of the legumes following the addition of KNO_3 was relatively constant. The $C_2H_2:N_2$ molar ratios for subterranean clover, barrel medic and silver wattle were 5.2, 5.8 and 2.8, respectively. A large proportion (50 to 60%) of the electron flux available for N_2 fixation by the pasture legumes was used for the evolution of H_2. The $(C_2H_2 - H_2):N_2$ ratios were close to the theoretical value of 3:1. The total soil-plant system did not evolve H_2, but instead was capable of taking up exogenously supplied H_2, which was stimulated by the presence of the legumes.

Introduction

A number of isotopic techniques using ^{15}N are available for the quantitative measurement of biological N_2 fixation[14]. Indirect isotopic methods in particular are being increasingly used to measure symbiotic N_2 fixation[7,9,20,25]. The amount of N_2 fixed by legumes grown in pots and in the field has been obtained by adding ^{15}N-labelled fertiliser to the soil and determining the relative enrichment of ^{15}N in the legume and a non-fixing control plant. An important requirement of indirect isotopic methods is the selection of a non-fixing control plant. Ideally, this reference plant should be identical in every respect with the legume except for its inability to fix atmospheric N_2[6,17,18].

The C_2H_2 reduction assay provides a convenient technique for evaluating nodule activity of legumes[10]. The theoretical molar ratio of 3:1 for C_2H_2 reduced:N_2 fixed is often used to obtain quantitative estimates of N_2 fixation using C_2H_2 reduction data. However, experimentally determined C_2H_2:N_2 ratios are often significantly different from the theoretical value[3,13,21] indicating the need to calibrate the C_2H_2 reduction assay. This has usually been achieved by exposure of detached nodules to N_2 enriched in ^{15}N for short periods of time. Since significant decreases in C_2H_2 reduction activity (ARA) following removal of nodules have been observed[13,24], it is preferable to calibrate the assay with intact plants. The development of indirect isotopic methods offers the opportunity to calibrate the assay for undisturbed legumes. It has been suggested that the differences between the theoretical and experimentally determined C_2H_2:N_2 ratios are due to the evolution of H_2 by the nodules[13,22].

The objectives of this study were to estimate symbiotic N_2 fixation by two common pasture legumes, *Trifolium subterraneum* L. and *Medicago truncatula* Gaertn., and an Australian native legume, *Acacia dealbata* Link., growing in pots using an indirect isotopic method. This method was also used to calibrate the C_2H_2 reduction assay of the intact plants. In addition, H_2 evolution was measured in an attempt to explain the variations in C_2H_2:N_2 ratios between the species.

Materials and methods

Plants

Seeds of subterranean clover (*Trifolium subterraneum*), barrel medic (*Medicago truncatula*) and silver wattle (*Acacia dealbata*) were germinated on filter paper and transferred to sealed pots containing the equivalent of 200 g of oven-dry Walpeup sandy loam used in the preceding study[11]. Subterranean clover and barrel medic were inoculated with *Rhizobium trifolii* (strains WA67 + WU290) and *Rhizobium meliloti* (strain U45), respectively. Silver wattle was inoculated with a suspension of homogenized nodules collected from naturally occurring stands of this tree. An initial supplement of 2 mg N per pot as KNO_3 was required due to the low N status of the soil. A N-free nutrient solution was applied every 4 weeks at a rate of 2 ml per pot[12]. Pots were watered twice daily, and the moisture content fluctuated between 4 and 8% by weight of oven-dry soil[12]. Plants were grown in a phytotron set at a photoperiod of 12 h at 24°C with a photon flux density of 140 $\mu E\ m^{-2}\ sec^{-1}$ and a dark period of 12 h at 20°C.

Analytical

C_2H_2 reduction assays of the legumes growing in pots were carried out in 1250-ml jars[12]. The pots were incubated for 1 h at 20°C at a partial pressure of 10 kPa of instrument grade C_2H_2. The jars were also used to measure H_2 uptake and evolution by the legumes. H_2 was determined by gas chromatography using a Packard 428 instrument fitted with a Carbosieve 5B column (100 cm) and a Porapak Q column (150 cm) in series and a constant temperature thermal conductivity detector. The column temperature was 35°C and the Ar carrier gas flow rate 7 ml min^{-1}. CO_2 and N_2 were also determined by gas chromatography using a Porapak Q column (150 cm) and a Molecular Sieve 5A column (400 cm) connected in series[2]. The column temperature was 65°C and the H_2 carrier gas flow rate 16 ml min^{-1}.

Total N in plant material was determined by steam distillation[4] following a catalysed acid digestion[16]. $NH_4^+ - N$ in the distillates was converted to N_2 under vacuum by alkaline hypobromite oxidation[23]. The m/e 28:m/e 29 ratios were determined using a magnetic sector, double collector mass spectrometer (Associated Electrical Industries, Model MS3).

Experimental

The experiment was carried out in the phytotron and consisted of 3 treatments with 6 replicate pots of barrel medic and silver wattle and 4 replicate pots of subterranean clover. Rates of symbiotic N_2 fixation and C_2H_2 reduction were measured simultaneously. Rates of C_2H_2 reduction of control pots and pots treated with 8 mg N as KNO_3 (24·75 atom % ^{15}N excess) were determined at 6, 24, 48, 72 and 96 h. In addition, pots treated with ^{15}N-labelled KNO_3 were transferred to a controlled environment chamber[11]. The legumes were maintained for 100 h in an atmosphere of $Ar:O_2:CO_2$ (77:20:3), which was circulated over a 3% CO_2 buffer solution[15] at a rate of 100 ml sec^{-1}. The concentration of CO_2 and N_2 were determined at regular intervals and the chamber was flushed with the gas mixture whenever the residual level of N_2 reached 0.1 kPa. After 100 h the complete plants were harvested and total N and isotope ratios in the plant material were determined.

In a separate experiment C_2H_2 reduction, H_2 uptake and evolution were measured for 3 replicate pots of subterranean clover, barrel medic, silver wattle and ryegrass (*Lolium perenne* L.). Ryegrass was grown under the same conditions except that the pots received KNO_3 every 2 weeks at a rate of 1 mg N pot^{-1}. Endogenous H_2 evolution was measured after 1, 2 and 3 h of incubation. H_2 uptake was determined by incubating the plants with H_2 at a partial pressure of 0.1 kPa in air. The change in H_2 concentration was measured after 1 and 2 h. In addition, plants were removed from the pots and after washing the soil from the roots, H_2 evolution was measured after 1 h incubation as described above.

Results and discussion

C_2H_2 reduction

The addition of 8 mg N caused an immediate decrease in the rate of C_2H_2 reduction by subterranean clover, barrel medic and silver wattle as shown in Fig. 1. Total ARA of the three legume species was obtained by integrating the areas under the curves. Residual activity following the addition of KNO_3 was 31%, 31% and 38% for subterranean clover, barrel medic and silver wattle, respectively. It was assumed that diurnal variation in C_2H_2 reduction was negligible as demonstrated in previous experiments under the same environmental conditions[12]. The effect of soil inorganic-N on the ARA of intact legumes is consistent with the findings reported for N_2 fixing organisms and nitrogenase[10].

N_2 fixation

The legumes in the controlled environment chamber were maintained in an N_2-depleted atmosphere ($N_2 < 0.1$ kPa) with a relatively high concentration of CO_2. Symbiotic N_2 fixation by the legumes in the chamber was considered to be negligible since the K_m for *Glycine max* under ambient conditions was 6 to 8 kPa N_2[3]. Short-term exposure of the legumes to elevated concentrations of CO_2 in the chambers (2.71 kPa) compared to the phytotron (0.05 to 0.06 kPa) was shown in preceding experiments[11] not to affect ARA. Variations in moisture content of the soils (4 to 8% of the oven-dry weight) did not significantly affect ARA and was

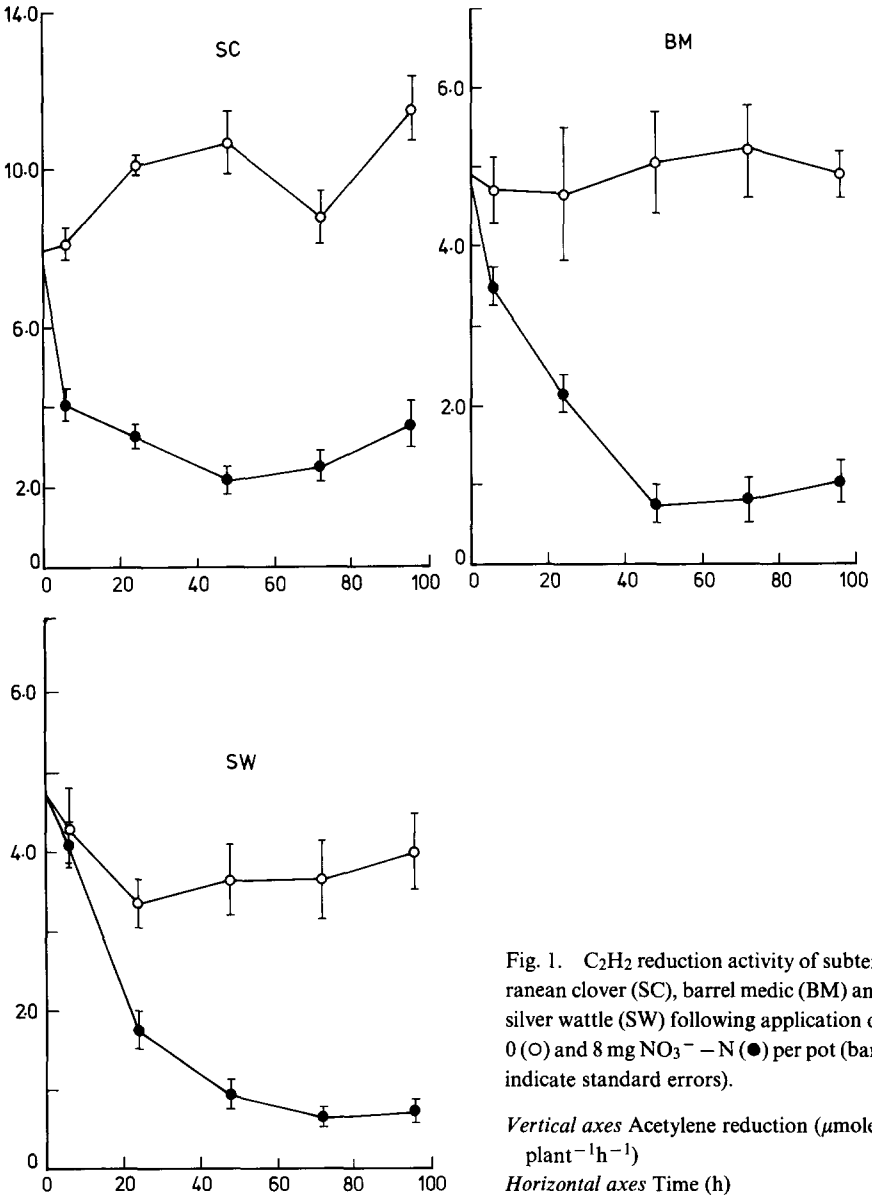

Fig. 1. C_2H_2 reduction activity of subterranean clover (SC), barrel medic (BM) and silver wattle (SW) following application of 0 (○) and 8 mg $NO_3^- - N$ (●) per pot (bars indicate standard errors).

Vertical axes Acetylene reduction (μmoles plant^{-1}h^{-1})
Horizontal axes Time (h)

optimum over this range[12]. It was considered that the legumes in the Ar atmosphere were identical to those under ambient atmospheric conditions in the phytotron except for the inability to fix atmospheric N_2.

The dry matter yields of the non-fixing plants were significantly higher than those of fixing plants for subterranean clover and silver wattle (Table 1). However, the N contents of the fixing and non-fixing plants were not significantly different. The ^{15}N-enrichments were consistently lower for the fixing plants but

Table 1. Dry matter yield, plant N, N content and ^{15}N-enrichment of subterranean clover, barrel medic and silver wattle maintained under ambient and N_2-depleted atmospheres for 100 h

Species	Treatment*	Dry matter yield (g)	Plant N (%)	Plant N content (mg)	Atom % ^{15}N excess**
Subterranean clover	Non-fixing	3.473	2.86	99	1.549
	Fixing	2.798	3.11	87	1.264
		(0.566)†	(0.14)	(21)	(0.358)
Barrel medic	Non-fixing	5.132	2.62	134	1.013
	Fixing	4.744	2.70	128	0.923
		(0.520)	(0.16)	(14)	(0.125)
Silver wattle	Non-fixing	1.843	2.92	53	2.302
	Fixing	1.562	3.14	48	1.802
		(0.312)	(0.28)	(9)	(0.401)

* Non-fixing, N_2-depleted atmosphere; fixing, ambient atmosphere.

** Applied 8 mg N per pot as KNO_3 at 24.75 atom % ^{15}N excess.

† Data in parentheses are least significant differences at $P \leqslant 0.05$.

differences between fixing and non-fixing plants were only significant for silver wattle, which also had the lowest N content (Table 1). The uptake of fertiliser-N by the fixing plants was significantly less than that for the non-fixing plants (Table 2). It was assumed that the N requirements of the legumes that were suddenly deprived of atmospheric N_2 were met by an equivalent uptake of labelled NO_3^- from the soil. This assumption is based on the inverse linear relationship between fertiliser-N uptake and symbiotic N_2 fixation reported for a number of legume species[1,19]. The amount of N_2 fixed was therefore estimated as the difference in fertiliser-N uptake between the fixing and non-fixing legumes (Table 2).

A comparison of the estimated N_2 fixed with the total C_2H_2 reduced over 100 h gave molar C_2H_2:N_2 ratios for subterranean clover, barrel medic and silver wattle of 5.2, 5.8 and 2.8, respectively (Table 2). The C_2H_2:N_2 ratios for the pasture legumes were considerably higher than the theoretical value of 3:1. The ratios found for subterranean clover (5.2) using this indirect isotopic method compares quite favourably with the ratio (5.1 ± 0.4) derived from direct exposure of this legume to N_2 of low ^{15}N enrichment[11]. Therefore, the decrease in ARA due to fertiliser-N addition did not alter the C_2H_2:N_2 ratio. This result lends support to the indirect isotopic technique adopted in this study, which is a yield

Table 2. C_2H_2 reduced, fertiliser-N taken up, estimated N_2 fixed and molar ratios of C_2H_2 reduced:N_2 fixed over 100 h for subterranean clover, barrel medic and silver wattle

Species	Treatment*	C_2H_2 reduced (μmoles)	Fertiliser-N uptake** (mg)	Estimated N_2 fixed (μmoles)	Molar ratio C_2H_2:N_2
Subterranean clover	Non-fixing	Nil	6.07	Nil	
	Fixing	308	4.43	59	5.2
			(0.19)†		
Barrel medic	Non-fixing	Nil	5.49	Nil	
	Fixing	151	4.76	26	5.8
			(0.53)		
Silver wattle	Non-fixing	Nil	4.90	Nil	
	Fixing	144	3.45	52	2.8
			(0.76)		

* Non-fixing, N_2-depleted atmosphere; fixing, ambient atmosphere.

** (N uptake by plant) $\times \frac{(\text{atom \% } ^{15}\text{N excess in plant})}{(\text{atom \% } ^{15}\text{N excess in fertiliser})}$

† Data in parentheses are least significant differences at $P \leqslant 0.05$.

dependent, N-difference method for estimating symbiotic N_2 fixation. It is an alternative to the yield independent ^{15}N-dilution technique[17] and the yield dependent 'A' value method[7]. The classical N-difference method measures symbiotically fixed N_2 as the difference between the total N contents of fixing and non-fixing plants, with the N content of the former being greater than the latter. The technique adopted in this study differs from the classical approach in that N_2 fixation is determined in a short-term experiment as the difference in fertiliser-N uptake between fixing and non-fixing plants, with more fertiliser-N being taken up by the non-fixing plant.

An important advantage of the indirect isotopic technique used in this study is the use of the same legume as the non-fixing control plant. This overcomes the problem of non-uniformity in morphology, physiology and biochemistry between the fixing and non-fixing plants associated with the use of a non-nodulated isoline of the legume or a grass as the non-fixing control plant[7,9,18,20,25].

H_2 uptake and evolution

It has been suggested that H_2 evolution by nodules decreases the efficiency of the legume to fix N_2[22], and recent findings have indicated that H_2 evolution by

nodules resulted in C_2H_2:N_2 ratios greater than the theoretical value of 3:1[8,21]. The possibility that H_2 evolution by subterranean clover, barrel medic and silver wattle could explain the differences in the experimentally determined C_2H_2:N_2 ratios was therefore investigated.

The legumes growing in pots did not evolve H_2 at measurable rates, but instead the plant-soil system was able to absorb exogenously supplied H_2 at high rates compared with the ARA of the plants (Table 3). H_2 uptake by the legume-soil system was 3 to 5 times greater than for the ryegrass-soil system. H_2 uptake appears to be related to the ARA of the legumes as indicated by the relatively constant rates of H_2 uptake per μmole of C_2H_2 reduced, viz. 2.5, 2.2 and 2.2 for subterranean clover, barrel medic and silver wattle, respectively. These findings are consistent with increased H_2 turnover in soils enriched with organic matter derived from legumes[5].

H_2 evolution was measured for legumes with bare root systems but not for ryegrass (Table 3). N_2 and H_2 compete for the same electron flux available to the nitrogenase enzyme[10]. Although H_2 is a competitive inhibitor of N_2 fixation, C_2H_2 reduction is not affected by H_2 and therefore is a measure of the total electron flux[22], provided substrate pressures of C_2H_2 are above saturation. The

Table 3. Effect of removal of plants from soil on the rates of C_2H_2 reduction, H_2 evolution and H_2 uptake by subterranean clover, barrel medic, silver wattle and ryegrass in the presence of atmospheric N_2

		Rate (μmoles plant^{-1} h^{-1})**		
Species	Treatment*	C_2H_2 reduction	H_2 evolution	H_2 uptake
Subterranean clover	Intact plants in soil	5.3	N.D.	13.6
Barrel medic		9.1	N.D.	20.1
Silver wattle		5.5	N.D.	11.9
Ryegrass		N.D.	N.D.	3.8
Subterranean clover	Soil removed	11.6	4.6	
Barrel medic		4.7	2.2	
Silver wattle		5.4	0.1	
Ryegrass		N.D.	N.D.	

* Three replicates per treatment for each species.

** N.D., below detection limit.

relative efficiency (RE) of N_2 fixation by the legumes was calculated using the formula:

$$RE = 1 - \frac{\text{Rate of } H_2 \text{ evolution}}{\text{Rate of } C_2H_2 \text{ reduction}}$$

The relative efficiencies for subterranean clover (0.60), barrel medic (0.53) and silver wattle (0.98) indicate that the Australian native legume is very efficient in utilising the energy available for substrate reduction by nitrogenase for the fixation of atmospheric N_2. In contrast, the pasture legumes used a significant proportion of the electron flux for the evolution of H_2 which constitutes an energy loss to the legume.

Rates of symbiotic N_2 fixation were estimated from the ARA of the isolated legumes (Table 3) using the C_2H_2:N_2 molar ratios (Table 2). Corrected ratios were then calculated taking into account the rates of H_2 evolution by the legumes:

$$\text{Corrected ratio} = \frac{\text{Rate of } C_2H_2 \text{ reduction} - \text{Rate of } H_2 \text{ evolution}}{\text{Rate of } N_2 \text{ fixation}}$$

These ratios were close to the theoretical value of 3:1 for all three legume species (Table 4). These results clearly demonstrate that the C_2H_2 reduction assay measures total nitrogenase activity which is the sum of N_2 reduction and H_2 evolution in terms of energy cost to the plant. The variation in C_2H_2:N_2 ratios, which were experimentally determined using the indirect isotopic technique, mainly reflect the differences in H_2 evolution between the legume species. Changes in H_2 evolution during the growth of legumes would be reflected in the C_2H_2:N_2 molar ratios. This is illustrated by the C_2H_2:N_2 ratios calculated for

Table 4. Ratios for C_2H_2 reduction and N_2 fixation corrected for H_2 evolution by subterranean clover, barrel medic and silver wattle

Species	C_2H_2 reduction (μmoles plant^{-1}h^{-1})	C_2H_2:N_2* experimental ratio	N_2 fixation** (μmoles plant^{-1}h^{-1})	H_2 evolution (μmoles plant^{-1}h^{-1})	C_2H_2:N_2 corrected ratio
Subterranean clover	11.6	5.2	2.3	4.6	3.0
Barrel medic	4.7	5.8	0.8	2.2	3.1
Silver wattle	5.4	2.8	1.9	0.1	2.8

* Data from Table 2.

** Calculated from the rate of C_2H_2 reduction and the experimentally determined C_2H_2:N_2 ratios.

subterranean clover (2.9) and barrel medic (3.3) during early vegetative growth using the classical N-difference method[12].

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