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REGULAR ARTICLE

Hydrogen emission from nodulated soybeans [*Glycine max* (L.) Merr.] and consequences for the productivity of a subsequent maize (*Zea mays* L.) crop

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Abstract Hydrogen (H_2) is a by-product of the symbiotic nitrogen fixation (N_2 fixation) between legumes and root-nodule bacteria (rhizobia). Some rhizobial strains have an uptake hydrogenase enzyme (commonly referred to as Hup^+) that recycles H_2 within the nodules. Other rhizobia, described as Hup^-, do not have the enzyme and the H_2 produced diffuses from the nodules into the soil where it is consumed by microorganisms. The effect of this phenomenon on the soil biota and on the soil itself, and consequent stimulation of plant growth, has been demonstrated previously. Soybeans [*Glycine max* (L.) Merr.] cv. Leichhardt, inoculated with either a Hup^+ strain (CB1809) or one of two Hup^- strains (USDA442 or USDA16) of *Bradyrhizobium japonicum* and uninoculated soybeans, plus a non-legume control [capsicum (*Capsicum annuum* L.)] were grown in the field at Ayr, North Queensland, Australia. The objectives were to examine (1) relationships between N_2 fixation and H_2 emission, and (2) the influence H_2-induced changes in soil might have during the legume phase and/or on the performance of a following crop. Strains CB1809 and USDA442 were highly effective in N_2 fixation ("good" fixers); USDA16 was partly effective ("poor" fixer). The soil had a large but non-uniformly distributed naturalised population of *B. japonicum* and most uninoculated control plants formed nodules that fixed some N_2. These naturalised strains were classified as "poor fixers" of N_2 and were Hup^+. H_2 emissions from nodules were assessed for all treatments when the soybean crop was 62 days old. Other parameters of symbiotic N_2 fixation and plant productivity were measured when the crop was 62 and 96 days old and at crop maturity. Immediately after final harvest, the land was sown to a crop of maize (*Zea mays* L.) in order to determine the consequences of H_2 emission from the soybean crop on maize growth. It was estimated that soybeans inoculated with USDA442, the highly effective Hup^- strain of *B. japonicum*, fixed 117 kg shoot N/ha (or about 195 kg total N/ha if the fixed N associated with roots and nodules was taken into account), and contributed about 215,000 l H_2 gas per hectare to the ecosystem over the life of the crop. The volume of H_2 evolved from soybeans nodulated by the Hup^+ strain CB1809 was only 6% of that emitted by the USDA442 treatment, but there was no indication that soybean inoculated with USDA442 benefited from the additional H_2 input. The shoot biomass, grain yield, and amounts of N fixed (105 kg shoot N/ha,

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175 kg total N/ha) by the CB1809 treatment were little less than for USDA442 plants. Three days after the soybean crop was harvested, the plots were oversown with maize along the same row lines in which the soybeans had grown. This procedure exposed the maize roots to whatever influence soybean H_2 emission might have had on the soil and/or the soil microflora immediately surrounding soybean nodules. The evidence for a positive effect of soybean H_2 emission on maize production was equivocal. While the consistent differences between those pre-treatments that emitted H_2 and those that did not indicated a trend, only one difference (out of the 12 parameters of maize productivity that were measured) was statistically significant at $P<0.05$. The findings need substantiation by further investigation.

Keywords Crop rotation · *Glycine max* · Hydrogen · Hydrogenase · Nitrogen fixation · *Zea mays*

Introduction

The value of legumes in crop rotations has been known for millenia (Fred et al. 1932). For nearly a century after Hellriegel's (1886) discovery of legume nitrogen fixation (N_2 fixation), it was generally believed that these benefits stemmed overwhelmingly from N_2 fixation. While there still is no doubt that some of the benefits of including legumes in a cropping sequence can be attributed to improvements in soil N availability (e.g. Rochester et al. 2001), this does not always satisfactorily explain the extent of enhancement in growth and yield of following crops. For example, Chalk (1998) made comparisons of quadratic response curves for wheat (*Triticum aestivum* L.) grain yield to increasing rates of applied fertilizer N for (1) lupin (*Lupinus angustifolius* L.)–wheat, and (2) wheat–wheat sequences. In 26 experiments, he noted that yield improvements following lupin attributable to increased soil N fertility occurred in only five cases (19% of trials), whereas non-N-benefits either dominated the rotational effects, or were identified as major contributing factors to the enhanced wheat yields, on 16 occasions (62% of the trials).

Peoples and Herridge (1990) proposed a range of potential non-N-benefits that could accrue from including legumes in a crop rotation. They included improvements in soil structure and water infiltration (e.g. Rochester et al. 2001; McCallum et al. 2004), enhanced nutrient availability (e.g. Nuruzzaman et al. 2005), and the breaking of cereal disease and pest cycles (e.g. Stevenson and van Kessel 1996). However, recent research now suggests that hydrogen (H_2) gas released into the soil from nodules during N_2 fixation, and its consequent impact of soil microbial activity, might also be contributing to improvements in crop growth and yield (Dong and Layzell 2002; Dong et al. 2003; Dean et al. 2006).

It is well documented that molecular H_2 is generated by the enzyme nitrogenase as a by-product of the N_2 fixation process in legume nodules, and its production may account for about 35% of the energy consumed in the overall nitrogenase activity (e.g. reviews by Evans et al. 1988; Arp 1992; Hunt and Layzell 1993). In some legume systems, the rhizobial bacteria possess a hydrogenase uptake enzyme system (usually designated as Hup^+ symbioses) that is able to recycle almost all of the H_2 evolved and recover most of the energy that might otherwise be lost (Evans et al. 1988). The Hup^+ trait appears to be common in nodules formed by *Bradyrhizobium* spp. (representing between 64% and 77% of all host-strain combinations tested: Evans et al. 1988; Arp 1992). However, less than 25% of all *B. japonicum* strains effective on soybean [*Glycine max* (L.) Merr.] appear to be Hup^+ (Uratsu et al. 1982; Arp 1992), and the characteristic seems to be rare in legumes nodulated by strains of *Rhizobium*, *Sinorhizobium*, or *Mesorhizobium* spp. (Evans et al. 1988; Arp 1992). In those symbiotic associations that either lack the hydrogenase enzyme (Hup^-), or have low Hup activity, H_2 diffuses out of the nodules into the soil. The exposure of soil to H_2 induces a change in the activity and composition of the soil microbiology toward species capable of oxidising H_2 (McLearn and Dong 2002; Stein et al. 2005) and, within a short period after the initial exposure, most of the H_2 is rapidly consumed by the microflora within a few centimetres of the nodules and none escapes from the soil surface (Conrad and Seiler 1979; La Favre and Forcht 1983; Dong and Layzell 2001).

On the basis of largely controlled-environment studies, there appears to be substantial consequences of H_2 emission, and its uptake by soil microbes, on the soil since plants grown in such soils tend to accumulate more biomass than in equivalent soils not

exposed to H_2 (Fyson and Oaks 1990; McLearn and Dong 2002; Dong et al. 2003). One class of microorganism that apparently responds to H_2 emission is the Order Actinomycetales (Osborne 2007), but it is not known whether they have a role in the observed stimulation in plant growth. Whatever the factors involved, and provided they are persistent in the soil, H_2 evolution from nodulated legumes and its consequences for the soil environment may be of benefit to the legume during growth, and/or improve the performance of a succeeding crop (Dong et al. 2003; Dean et al. 2006). The work described here was initiated to test this hypothesis in the field.

Seeds of soybeans were inoculated with Hup^- (H_2-emitting) or Hup^+ (non-H_2-emitting) strains of *B. japonicum*. Both highly effective and poorly effective Hup^- and Hup^+ strains of rhizobia were used. The soybean crop was grown to maturity and, immediately after grain harvest, the land was sown to a crop of maize (*Zea mays* L.) to quantify what effect, if any, the H_2 treatments may have had on growth and yield. To our knowledge this is only the second report of such a field study. The previous Canadian research focused on comparing rates of H_2 uptake by soils collected adjacent to Hup^- soybean or lucerne (alfalfa, *Medicago sativa* L.) nodules, and Hup^+ soybean nodules, and examined the subsequent effects of these legume pre-treatments on grain yield by a following barley crop (*Hordeum vulgare* L.; Dean et al. 2006). The current work differs from the earlier investigation in that the experimentation was designed so that, were differences in either soybean or maize growth and grain yield to occur, it might be possible to differentiate between (1) additional fixed N_2 or N availability, and (2) the impact of H_2 emissions on soil characteristics. Our study also included comparative field assessments of the extent of nodulation, rates of H_2 emissions from nodules, and inputs of fixed N_2 by the different soybean treatments.

Materials and methods

The experimental site

The experiment, which occupied an area of approximately 1 ha, was located at the Queensland Department of Primary Industries Experiment Station at Ayr in North Queensland (19°34′S, 147°24′E). The site was on an alluvial soil of the Burdekin Delta (Gregory 1969). It has been described by McClurg (1990) as a well-structured, free-draining, dark clay loam (mapping unit Jarvisfield), and is also known as a Prairie Soil (Stace et al. 1968) or an Ustochrept (Soil Survey Staff 1975). Principal Profile Forms (Northcote 1979) are Um6.31, Um6.32 and Um6.42. When the experiment commenced, soil pH was relatively uniform down the profile (pH 6.33 in the top 10 cm to pH 6.40 at 90 cm), with a total N content of 0.07% (0–20 cm) and total carbon content of 1.13% (0–20 cm).

The experimental site had a long history of mixed cropping and dairy farming. There was, however, no record of soybean cropping although soybeans had been grown regularly on adjoining areas. At the time when the site was chosen for the experiment, fertility levels were non-uniform. In an attempt to correct this, and to lower the concentrations of soil mineral N prior to soybean cropping to maximize N_2 fixation, the whole area was subdivided into 10 blocks of equal size (each approx. 20×50 m) and sown to sorghum [*Sorghum bicolor* (L.) Moench s.lat.] in the summer of 2004–2005. The sorghum was cut twice during growth (March and May 2005) and the residues were baled and removed from the site.

The three blocks where sorghum growth was least uniform were set aside and the remaining seven blocks were used for the experiment. The site was then cultivated, fertilised [100 kg/ha superphosphate (9% P, 11% S) and 80 kg/ha potassium sulphate (41% K, 18% S)] and formed into ridges at 75-cm intervals in preparation for cropping soybeans.

The soybean crop was sown on 15 June and harvested on 10 October 2005; maize was oversown into the soybean plots on 13 October 2005 and harvested on 12 January 2006. Irrigation was provided during cropping of both soybeans and maize via plastic trickle irrigation tapes buried to a depth of 10–15 cm in the centre of each ridge. This was to supplement rainfall particularly for winter-grown soybean; rainfall at Ayr is strongly summer (December to March)-dominant (annual mean, 927 mm). Fertiliser [40 kg/ha mono-potassium phosphate (28% K, 23% S) and 500 g/ha zinc sulphate] was applied dissolved in irrigation water. Molybdenum fertiliser, 1 kg/ha Mo, as a foliar dressing was applied to the soybeans during the early vegetative stage. No nitrogen was applied to either crop.

Counting rhizobia

Most probable numbers (MPN) of rhizobia in soil samples were counted with a serial-dilution, nodulation-frequency, plant-infection test (Brockwell 1963; Brockwell et al. 1975). Prior to sowing the soybean crop, the mean population of *Bradyrhizobium* spp. at the site was estimated from a composite soil sample comprising 25 sub-samples (0–10 cm) dug from points on a grid pattern across the site. Siratro [*Macroptilium atropurpureum* (Mocino & Sesse ex DC.) Urban] was used as the test plant because seed of wild soybean (*Glycine soja* Siebold & Zucc.), a more desirable test plant for enumerating soybean rhizobia (*B. japonicum*), was not available at the time. The plant-infection test with siratro as a test plant provides an estimate of the numbers of all *Bradyrhizobium* species in a sample, whereas the test using wild soybean merely estimates the population of *B. japonicum* (Brockwell 1982).

After the maize crop, soil samples were taken from points (1) within the experimental site where no soybeans had ever been grown, (2) where only uninoculated soybeans had been grown, and (3) within the CB1809 inoculated soybean treatment (see below). MPN of rhizobia in those samples were estimated with the plant-infection test using both siratro and wild soybean as test plants. The data obtained permitted assessment of the extent to which counting of *Bradyrhizobium* spp. (test plant siratro) over-estimated the population of *B. japonicum* (test plant wild soybean).

Nitrogen-fixing effectiveness of naturalised *B. japonicum*

Twenty-four strains of *B. japonicum* were isolated from Ayr soil using Siratro and wild soybean as bait-plants (Date 1975) and tested for effectiveness of N_2 fixation on soybean cv. Leichhardt. The plants were grown from surface-sterilised, inoculated seed in 25-cm pots of washed vermiculite and river sand (1:1 by volume) that had been steam-sterilised (cf. Bergersen and Turner 1968). The pots were placed in a temperature-controlled (21°C day, 15°C night) glasshouse and watered on alternate days with dilute, N-free McKnight's (1949) seedling nutrient solution and rhizobia-free water.

There were additional inoculation treatments for comparative purposes, *viz. B. japonicum* strains CB1809 and USDA16 and an uninoculated control. CB1809 is highly effective for soybeans and is Hup^+ (and, therefore, its nodules emit little or no H_2); USDA16 is partially effective for soybeans but is Hup^- (and, therefore, forms H_2-emitting nodules). Another strain, USDA442, that was used in the field experiment and which is highly effective in soybean N_2 fixation and Hup^-, was not used in this glasshouse experiment. The plants were harvested at 42 days after sowing (DAS), the roots examined for nodulation and the shoots oven-dried at 70°C for 48 h. Shoot dry weight was used as an index of N_2 fixation (Vincent 1970; Somasegaran and Hoben 1994).

The Hup status of strains of *B. japonicum* that occurred naturally in Ayr soil was not determined as part of the glasshouse trial, but nodules that formed on uninoculated plants were tested for H_2 evolution during the field experiment (described below).

Measurement of nitrogen

Soil mineral nitrogen

At the time that the soybeans were sown, multiple soil samples were collected at different locations along the length of each of the seven experimental blocks using a 10-cm diameter hand auger. A second set of soil samples was taken from each treatment replicate immediately after sowing the maize. For each plot, ten samples were collected to 20 cm depth (separated into 0–10 cm and 10–20 cm sections), and three samples from 20–90 cm (separated into 20- to 30-cm, 30- to 50-cm and 50- to 90-cm sections). Soils taken at equivalent depths from each plot were bulked, thoroughly mixed and subsampled for later analysis. The subsamples were stored at −18°C. After thawing, soil was extracted with 2 M KCl and analysed for mineral N (ammonium+nitrate) using a segmented flow analyser (Alpken, Wilsonville OR, USA) as described by Markus et al. (1985). Soil mineral N was calculated by reference to previously-measured bulk densities and expressed as a total for the 0- to 90-cm section of the profile.

Plant nitrogen

Plant material was dried (70°C for 48 h), weighed, ground and analysed for total N concentration and

^{15}N abundance using an automated N and carbon analyser (ANCA-SL) interfaced with a 20–20 stable isotope mass spectrometer (Europa Scientific, Crewe, UK). The ^{15}N natural abundance ($\delta^{15}N$) of the plant material was expressed as parts per thousand (‰) relative to atmospheric N_2 (0.3663 atom%^{15}N) using the following equation (Peoples and Herridge 1990):

$$\text{Sample } \delta^{15}N\ (‰) = 1000 \times (\text{sample atom\% } ^{15}N - 0.3663)/0.3663 \quad (1)$$

The field experiment—soybeans

Treatments

There were four treatments involving soybean cv. Leichhardt and a fifth treatment of capsicum (*Capsicum annuum* L. cv. Aries):

1. Soybean, inoculated with *B. japonicum* strain USDA442 ("good" N_2 fixation, Hup^-).
2. Soybean, inoculated with *B. japonicum* strain USDA16 ("poor" N_2 fixation, Hup^-)
3. Soybean, inoculated with *B. japonicum* strain CB1809 ("good" N_2 fixation, Hup^+)
4. Soybean, uninoculated
5. Capsicum, non-legume

The five treatments were imposed in a randomised design across the seven replicate blocks. Every treatment plot was four rows wide (75-cm spacing) and 50 m long. All plant samplings were restricted to the central two rows.

Uninoculated soybean and capsicum were included in the experiment as control treatments intended *inter alia* for use as non-N_2-fixing reference plants to allow estimates of N_2 fixation to be determined for the inoculation treatments (Peoples and Herridge 1990; Peoples et al. 2002; see below). It was found subsequently that a population of *B. japonicum* occurred naturally in the soil at the site and that many uninoculated plants became nodulated. The rhizobia responsible were "poor" N_2 fixers and assumed to be Hup^+ on the basis of field measurements of low rates of H_2 evolution from their nodules. Thus, the four soybean treatments represented two categories of N_2 fixation and two categories of H_2 emission:

N_2 fixation	"Good" fixers (highly effective)	CB1809, USDA442
	"Poor" fixers (poorly effective)	USDA16, uninoculated
H_2 emission	Emitters	USDA442, USDA16
	Non-emitters	CB1809, uninoculated

Sowing the soybean phase

Peat inocula of *B. japonicum* strains USDA442, USDA16 and CB1809 were prepared (Roughley and Vincent 1967) and applied to the seed using a commercial adhesive. The rate of inoculation was approximately 2×10^6 rhizobia per seed. The inoculated seed was sown into a moist seed bed on the day following inoculation with a two-row Gaspordo air seeder. Germination and emergence were assisted with trickle irrigation. Five-week-old capsicum seedlings were transplanted into the non-legume control plots 2 days later.

Observations on indices of soybean crop growth

At 62 days after sowing A total of 20 soybean plants was dug from each plot 62 DAS (R2 stage of physiological development; Fehr et al. 1971). Observations were made on the extent of nodulation using a subjective rating system adapted from Corbin et al. (1977) based on the number of effective (pink) nodules in the crown-root zone (regarded as the region 5 cm below the first lateral roots) and elsewhere on the root system (0–4 nodule rating scale, where 0=no nodules and 4=>10 nodules on both the crown and laterals). Shoots were retained for measurement of dry matter (DM), analysis of nitrogen (N) and quantification of symbiotic N_2 fixation (see below).

At the same time, H_2 evolution from nodules was assessed for two separate samples of nodulated roots, each comprising two root systems, collected from different points in each plot (giving, for each treatment, 14 separate measurements of 28 plants in total). The volumes of H_2 emitted (μmol/g nodule DM/h) were measured using a portable H_2 analyser similar to the one described by Dong et al. (2000) except that we utilised a H_2 sensor supplied by Qubit

Systems Inc. (Model S121, Kingston ON, Canada) interfaced with a LabPro data logger (Vernier Software, Portland OR, USA) connected to a lap-top computer; also, the system was powered by a petrol-fired generator instead of a 12-V battery. Briefly, a small pump drew ambient air at a rate of 300 ml/min through an external column filled with a magnesium perchlorate drying agent (replaced each 3 h) and then through the H_2 sensor. The sensor was calibrated by passing gases of known concentrations of H_2 through the analyser while measuring the H_2 sensor output. In order to quantify H_2 evolution by nodules, two plants were collected, the shoots removed, the root systems lightly shaken to remove soil and excess roots trimmed. Within 3–4 min of collection, the nodulated crowns were inserted into a "cuvette" constructed from the barrel of a 60-ml syringe, which was immediately connected to the inlet of the drying column so that air was drawn over the nodules prior to flowing through the H_2 sensor. The H_2 concentration in the air stream was then monitored until the output from the H_2 sensor stabilised (usually <1 min). Once the reading was recorded, the cuvette was disconnected and the sensor was re-flushed with air. When the sensor output had returned to the base calibration, the next sample of nodulated roots was introduced.

After each analysis of nodule H_2 emission, all nodules were removed from the root systems for DM determination. Estimates of the total amounts of H_2 emission (litres per hectare) from the various inoculation treatments over the soybean's life-cycle were derived by assuming that N_2 fixation, and therefore H_2 production, occurred at the same rate as that measured at 62 DAS for 24 h per day (Walsh et al. 1987) over a total period of 75 days (e.g. see information provided by Bergersen et al. 1991 to support the notion of on-going N_2 fixation during grain fill in soybean, and data presented by Herridge and Peoples 2002, which indicates that an instantaneous measure of symbiotic dependence collected at R2 can be indicative of the whole growing season).

At 96 days after sowing Plants were sampled from two 2-m lengths of row (R6 stage of physiological development; Fehr et al. 1971) and observations made on indices of shoot DM and shoot N and N_2 fixation.

At 117 days after sowing For each soybean plot, mature seed was harvested from a 50-m length of row (R8; Fehr et al. 1971), dried at 70°C to 12% moisture content and weighed. The capsicum plots were harvested at the same time (115 days after transplanting), but parameters of growth were not measured.

Calculations of N_2 fixation

The ^{15}N natural abundance technique (Unkovich et al. 1994; Peoples et al. 2002) was used to provide estimates of the proportion of the soybean N derived from atmospheric N_2 (%Ndfa). Such determinations provide "time-integrated" average measures of plant reliance upon N_2 fixation for growth between sowing and the time of sampling (Peoples and Herridge 1990; Peoples et al. 2002). Estimates of %Ndfa were calculated from the $\delta^{15}N$ of the soybean shoot and the non-N_2-fixing reference species capsicum (as an index of the $\delta^{15}N$ of the plant-available soil N) using the following equation (after Peoples and Herridge 1990):

$$\%\text{Ndfa} = 100 \times \left(\delta^{15}\text{N capsicum} - \delta^{15}\text{N soybean}\right) \Big/ \left(\delta^{15}\text{N capsicum} - B\right) \quad (2)$$

where B represents the $\delta^{15}N$ of soybean shoots fully dependent upon N_2 fixation for growth.

There is evidence to indicate that the shoot B value for a particular legume species can be influenced by the strain of rhizobia forming the symbiosis (Peoples et al. 2002). The B value (−1.30‰) had already been determined for the shoots of soybean inoculated with *B. japonicum* strain CB1809 and grown in N-free pot culture (i.e. under conditions where the nitrogenous products of growth were derived entirely from atmospheric N_2; Bergersen et al. 1989). Although no such data were available for soybean nodulated by strains USDA442 or USDA16 or by the strains of *B. japonicum* naturalised in Ayr soil, a previous study (using the same growth conditions) had been undertaken with eight *B. japonicum* strains of different origin that varied in effectiveness and Hup status. Analysis of the shoots from this study indicated a range of $\delta^{15}N$ from −1.7‰ to −1.0‰, with a mean of −1.40‰ (M. B. Peoples, unpublished data). This mean value was used as B when calculating %Ndfa for the USDA442, USDA16, and uninoculated treatments.

The mean $\delta^{15}N$ of the capsicum reference determined using equation (1) was +8.9‰ and +8.0‰ at

the 62 DAS and 96 DAS samplings, respectively. These values were substantially greater than the +2.0‰ generally considered as the lowest reference $\delta^{15}N$ required to provide reliable measures of N_2 fixation (Unkovich et al. 1994). They were almost identical to the $\delta^{15}N$ values for individual non-nodulated soybean plants recovered from the uninoculated plots (+8.4‰) and for sorghum plants harvested from the trial site a few months earlier (+8.6‰ and +8.8‰ in March and May 2005, respectively).

The amounts of N_2 fixed between sowing and R2 or R6 (Fehr et al. 1971) were calculated from determinations of soybean %Ndfa, shoot DM and N content (%N) at the 62 DAS and 96 DAS samplings, respectively, as follows (after Peoples et al. 2002):

$$\text{Soybean shoot N} = \%\text{N}/100 \times (\text{soybean shoot DM}) \quad (3)$$

$$\text{Amount shoot N fixed} = \%\text{Ndfa}/100 \times (\text{soybean shoot N}) \quad (4)$$

The field experiment—maize

Treatments

Immediately following final harvest, the soybean trash was removed from one-half of every plot. Capsicum trash was fully removed. Within 3 days, all plots were sown with maize with a Max Emerge John Deere vacuum planter along the same row lines in which the soybeans had grown. This procedure was adopted to ensure that the roots of the maize would be intimately exposed to whatever influence H_2 emission might have had on the soil and/or the soil microflora immediately surrounding soybean nodules. Thus, there were five maize pre-treatments, identical to the soybean treatments, that similarly represented two categories of H_2 emission and two categories of N_2 fixation:

H_2 emission	Emitters	USDA442, USDA16
	Non-emitters	CB1809, uninoculated, (non-legume=capsicum)
N_2 fixation	"Good" fixers	CB1809, USDA442
	"Poor" fixers	USDA16, uninoculated

Observations on indices of maize crop growth

The maize crop was harvested at 91 DAS at the "soft-dough" stage of grain development. Ten plants were cut from each plot and the cobs were separated from the shoots. All plant material was oven-dried at 70°C to constant weight, sub-sampled and analysed for total N content and for $\delta^{15}N$ as described above. The N data for cobs are for whole cobs, not merely for the grain.

Treatment of the data

Two data sets for "harvest index" were derived from the maize data, *viz*. a DM harvest index (HI_{DM}) and a N harvest index (HI_N):

$$HI_{DM} = 100 \times \text{DM of cobs}/(\text{crop DM}) \quad (5)$$

$$HI_N = 100 \times \text{cob N}/(\text{crop N}) \quad (6)$$

Untransformed data relating to indices of plant production, N uptake and N_2 fixation were subjected to analysis of variance. For the data from soybean, where differences between treatments were clearcut, least significant differences were calculated at $P=0.05$. For the data from maize, where only trends may have been apparent, confidence limits were calculated to $P=0.20$.

In order to compare the size of populations of *Bradyrhizobium* detected with different test species, $\log_{10}$ transformation was applied to MPN data. Fiducial limits were +(MPN×3.8) and −(MPN×0.26) (Grassia and Brockwell 1978); that is, a difference of 1.16 $\log_{10}$ units represents a significant difference ($P<0.05$) between two MPN values.

Results

Populations of rhizobia in Ayr soil

Total populations

Total soil populations of *Bradyrhizobium* spp. in uninoculated plots before soybean cropping (2.30×10^4per g) were little different from populations detected after maize cropping (1.46×10^4 rhizobia per g; Table 1). Growth of inoculated soybeans led to

Table 1 Comparison of the most probable numbers (MPN) of rhizobia in Ayr soil (MPN per gram, 0–10 cm) estimated with plant-infection tests using either (1) Siratro (to detect total *Bradyrhizobium* spp.) or (2) wild soybean (to detect *B. japonicum*) as test plants

Source of sample	Test plant	
	Siratro	Wild soybean
Before soybeans		
Composite sample across experimental area	2.30×10^4 ab[a]	–
After maize		
Block 1, treatment 1[a], capsicum (uninoculated)	2.34×10^4 ab	2.29×10^3 bc
Block 1, treatment 2[a], uninoculated soybean	0.91×10^4 abc	0.58×10^3 c
Block 1, treatment 3, inoculated (CB1809) soybean	9.33×10^4 a	–

[a] Values that share the same letter are not significantly different from one another (P=0.05).

a sixfold increase in total numbers of *Bradyrhizobium* in the soil. After cropping, numbers of *B. japonicum* in uninoculated plots were significantly fewer than numbers of *Bradyrhizobium* spp. (1.15×10^3 *B. japonicum* per g soil; 1.46×10^4 total *Bradyrhizobium* spp. per g soil).

Nitrogen-fixing effectiveness of naturalised rhizobia in Ayr soil

Of the 24 naturalised strains of *Bradyrhizobium* specific for soybean that were trapped from uninoculated plots using wild soybean as the trap-plant, 16 were ineffective in N_2 fixation with soybean cv. Leichhardt. Only two of the other eight strains that fixed N were significantly better in shoot DM production than uninoculated controls but they were significantly inferior to inoculum strains *B. japonicum* CB1809 and USDA16. Shoot DM (mg per plant): CB1809=1,148>USDA16=895> mean of 2 N_2-fixing naturalised strains=685>uninoculated control=534 (P<0.05).

Subsequently, in dealing with parameters of soybean N_2 fixation due to various inoculation treatments, USDA16 and the naturalised strains (uninoculated plots) were classed as "poor" N_2 fixers and USDA442 and CB1809 as "good" N_2 fixers.

General observations on crop growth

Germination and establishment of soybeans was relatively uniform across blocks and treatments (mean rate of establishment of about 320,000 plants per hectare, at 62 DAS). On the other hand, it was evident that the distribution of naturalised *B. japonicum* was non-uniform across the site. Although most uninoculated soybean plants nodulated, there were some non-nodulated plants in uninoculated plots. Visual differences between the uninoculated treatment and the other treatments were discernible as a paler shade of green at 62 DAS, but not at 92 DAS or afterwards. It was assumed that the strains of *Bradyrhizobium* spp. naturalised in Ayr soil were slower in forming nodules and commencing N fixation than the inoculant strains of *B. japonicum*.

By the standards for soybeans grown at higher latitudes, the yield of the crop at Ayr was relatively low (mean 1.51 t/ha). However, harvest index (seed yield/crop biomass) averaged 35%, which is consistent with the performance of short-season soybean cultivars grown at wide row-spacing at lower latitudes during the short days of winter and early spring as in the current experiment (cf. Lawn and Byth 1974; Mayers et al. 1991).

The establishment of maize was about 69,000 plants per hectare. Although germination and establishment was uniform across blocks and treatments, it was less uniform than for soybeans along the row, because soybean stumps and root systems made for a non-uniform seed bed for the maize. Accordingly, it was deemed prudent to present maize production data on both per-plant and per-area bases.

There were some differences in soil mineral N before soybean cropping (range 14–54 kg N/ha for individual blocks, mean 35 kg N/ha to 90 cm), but levels were below values that might be expected to impact significantly on soybean N_2-fixing potential (Herridge et al. 1990; Peoples and Herridge 1990). There were no differences in soil mineral N when maize was sown after soybean harvest; totals were

<20 kg N/ha to 90 cm across all treatments. There was no effect of soybean trash removal on the subsequent growth of maize (data not given). This may have been associated with the brief turn-around between the two crops which allowed insufficient time for substantial decomposition and mineralisation of N from soybean residues.

The soybean crop

Hydrogen emission from soybean nodules Soybean nodules from the two Hup^- treatments emitted large volumes of molecular H_2, equivalent to 119.5 and 72.3 l/ha per hour for USDA442 and USDA16, respectively. Nodules from the Hup^+ treatments emitted considerably less: 7.7 and 14.4 l/ha per hour for CB1809 and uninoculated, respectively (Table 2). H_2 emission for USDA442 was significantly greater than for USDA16 and both were many times greater than for CB1809 and uninoculated ($P<0.05$). Total H_2 release into the soil from the USDA442 treatment was calculated to exceed 215,000 l per hectare over the life of the soybean crop. The estimate of H_2 evolution from USDA16 nodules was nearly two-thirds of this figure, while CB1809 and the uninoculated treatments were around only 6% and 13% of USDA442, respectively (Table 2).

Soybean crop productivity At the time that H_2 emission measurements were undertaken 62 DAS during the early reproductive phase, there were significant ($P<0.05$) differences between each inoculation treatment in extent of nodulation (measured as nodule score) with strains USDA442 and CB1809 being best and strain USDA16 and uninoculated being poorest (Table 3). At this time, indices of growth and N uptake for the uninoculated treatment were significantly inferior to the other three treatments. Measures of shoot $\delta^{15}N$ for the various soybean treatments (range from +3.54 to +4.78‰ for the USDA442 and uninoculated treatments, respectively) were all measurably lower than detected in the shoots of the non-fixing capsicum reference (+8.9‰). The strain treatments designated "good" fixers of N_2, USDA442 and CB1809, were calculated to have derived 50–52% of their N requirements between sowing and 62 DAS from N_2 fixation, representing 104–106 mg shoot N fixed per plant (equivalent to 30–35 kg shoot N/ha), which was significantly more that the designated "poor" fixers,

Table 2 Hydrogen emission from nodules of Leichhardt soybeans inoculated with strains of *Bradyrhizobium* spp. that combine differing characteristics of N_2-fixing effectiveness ("good" fixers; "poor" fixers) and Hup status (Hup^-, H_2-emitting; Hup^+, non-H_2-emitting), and estimates of total H_2 emitted by the crop

Inoculum strain(s) effectiveness; Hup status[a]	H_2 emission (µmol/g nodule DM/h)	Nodule mass (kg/h)	Crop H_2 emission	
			l/ha per hour	l/ha per crop[b]
Individual inoculation treatments (each value is the mean for 28 plants)				
CB1809 ("good" N_2 fixer; Hup^+)	3.6 b[c]	73.2 a	7.7 c	13,860
USDA442 ("good" N_2 fixer; Hup^-)	70.3 a	77.5 a	119.5 a	215,082
USDA16 ("poor" N_2 fixer; Hup^-)	57.9 a	61.5 a	72.3 b	130,176
Uninoculated ("poor" N_2 fixer; Hup^+)[a]	23.1 b	26.6 b	14.4 c	25,974
Data pooled according to N_2-fixing effectiveness of inoculation treatments (each value is the mean for 56 plants)				
"Good" N_2 fixers (CB1809 + USDA442)	36.9 a	75.4 a	63.6 a	114,480
"Poor" N_2 fixers (USDA16 + uninoculated)	40.5 a	44.1 b	43.4 a	78,084
Data pooled according to H_2 emission of inoculation treatments (each value is the mean for 56 plants)				
Hup^-; H_2 emitting (USDA442 + USDA16)	64.1 a	69.5 a	95.9 a	172,638
Hup^+ ; non-H_2 emitting (CB1809 + uninoculated)	13.3 b	49.9 a	11.1 b	19,926

Measurement of H_2 evolution from nodules undertaken 62 days after sowing (R2 stage of physiological development; physiological stage of soybean development according to Fehr et al. 1971)

[a] The soil in all four treatments contained strains of *Bradyrhizobium*, presumed to be Hup^+, that occurred naturally in Ayr soil.

[b] Estimate derived by assuming that N_2 fixation (and H_2 emission) occurred during 24 h each day for 75 days; data not subjected to analysis of variance.

[c] In any one column, values with a common letter are not significantly different ($P>0.05$).

Table 3 Effect of inoculation treatment on extent of nodulation (0–4 nodule rating scale), nodule dry matter (DM), shoot DM, shoot nitrogen (N), and fixed N in shoots (Ndfa[a]) for soybean cv. Leichhardt 62 days after sowing (R2 stage of physiological development[b])

Inoculum	Nodule score (0–4)	Nodule DM (mg/plant)	Shoot DM (g/plant)	Shoot N (%)	Shoot N (mg/plant)	Ndfaa (%)	Fixed N in shoots (mg/plant)
Individual inoculation treatments							
CB1809 ("good" fixer; Hup^+)	1.78 b[c]	262 a	6.33 a	3.26 a	207 a	50 a	104 a
USDA442 ("good" fixer; Hup^-)	1.96 a	258 a	6.54 a	3.11 a	204 a	52 a	106 a
USDA16 ("poor" fixer; Hup^-)	1.60 c	204 a	6.02 a	2.98 a	179 a	43 ab	77 b
Uninoculated ("poor" fixer; Hup^+)[d]	0.83 d	68 b	4.53 b	2.61 b	119 b	34 b	40 b
Data pooled according to N_2-fixing effectiveness of inoculation treatments							
"Good" N_2 fixers (CB1809 and USDA442)	1.87 a	257.7 a	6.43 a	3.19 a	206 a	51 a	105 a
"Poor" N_2 fixers (USDA16 and uninoculated[d])	1.21 b	137.9 b	5.28 b	2.80 b	149 b	39 a	58 b
Data pooled according to Hup status of inoculation treatments							
Hup^-; H_2 emitting (USDA442 and USDA16)	1.78 a	227.0 a	6.28 a	3.05 a	192 a	47 a	91 a
Hup^+; non-H_2 emitting (CB1809 and uninoculated[d])	1.30 b	168.6 a	5.43 b	2.94 a	163 a	42 a	72 a

[a] Ndfa=shoot N derived from atmospheric N_2

[b] Physiological stage of soybean development according to Fehr et al. (1971)

[c] In any one column of each set, values with a common letter are not significantly different ($P>0.05$).

[d] The soil in all four treatments contained Hup^+, "poor" N_2-fixing strains of *B. japonicum* that occurred naturally in Ayr soil.

uninoculated and USDA16 treatments (%Ndfa values of 34–43%, 40 and 77 mg shoot N fixed/plant, respectively; Table 3).

There was no evidence that H_2 emission had any influence on soybean productivity at this time. The significant differences ($P<0.05$) between Hup^- and Hup^+, in terms of nodule score and shoot DM (Table 3), were attributable entirely to the poor performance of the uninoculated treatment at this stage of crop development.

At around the time of peak biomass 96 DAS, shoot $\delta^{15}N$ had declined to between −0.37 and +0.74‰ (for CB1809 and USDA16, respectively) for the inoculated soybean, and to +2.77‰ for the uninoculated treatment (cf +8.0‰ for the capsicum) indicating substantial N_2 fixation had occurred since the previous sampling 62 DAS. Integrated seasonal estimates of %Ndfa for USDA442 and CB1809 treatments were 79–83% and both had fixed >100 kg shoot N/ha (Table 4). When the data were pooled according to the N_2-fixing effectiveness of the treatments, the "good" fixers were consistently superior ($P<0.05$) to the "poor" fixers in indices of shoot growth, N accumulation and N_2 fixation (Table 4). In contrast, there was little or no difference between Hup^- and Hup^+ treatments when the data were pooled according to the Hup status of inoculation treatments. There was no difference in grain yield between any soybean treatments (Table 4).

The maize crop

It was part of our hypothesis that the substantial emissions of H_2 from the nodules of soybeans inoculated with the Hup^- strains of *B. japonicum*, USDA442 and USDA16, would have beneficial residual effects on the growth of a subsequent crop of maize. This was not borne out by the data (Table 5, DM data; Table 6, N data). Of the 12 indices of maize growth and productivity (not all independent variables) that were measured, there was only one, cob DM per ha, in which the combined H_2-emitting pre-treatments were superior ($P<0.05$) to the combined non-H_2-emitting pre-treatments (Table 5). However, there were trends in the data that suggested a possible benefit of H_2 emission. In every one of those 12 indices of maize growth, H_2-emitting pre-treatments were greater than non-H_2-emitting pre-treatments

Table 4 Effect of inoculation treatment of Leichhardt soybeans on crop biomass [shoot dry matter (DM)], shoot nitrogen (N), and fixed N in shoots (Ndfa, shoot N derived from atmospheric N_2) 96 days after sowing (R6 stage of physiological development; physiological stage of soybean development according to Fehr et al. 1971), and on seed yield 117 days after sowing (R8, physiological stage of soybean development according to Fehr et al. 1971)

Inoculum	Shoot DM (t/ha)	Shoot N (%)	Shoot N (kg/ha)	Ndfa (%)	Fixed N in shoots (kg/ha)	Seed yield (t/ha)
Individual inoculation treatments						
CB1809 ("good" fixer; Hup^+)	4.44 a	3.20 a	135 ab	83 a	105 ab	1.57 a
USDA442 ("good" fixer; Hup^-)	4.72 a	3.02 a	144 a	79 a	117 a	1.54 a
USDA16 ("poor" fixer; Hup^-)	4.30 a	2.86 a	116 bc	70 a	75 b	1.45 a
Uninoculated ("poor" fixer; Hup^+)[a]	3.88 a	2.80 a	105 c	49 b	41 c	1.48 a
Data pooled according to N_2-fixing effectiveness of inoculation treatments						
"Good" N_2 fixers (CB1809 and USDA442)	4.58 a	3.11 a	139 a	81 a	111 a	1.55 a
"Poor" N_2 fixers (USDA16 and uninoculated[a])	4.09 b	2.83 b	111 b	59 b	58 b	1.47 a
Data pooled according to Hup status of inoculation treatments						
Hup^-; H_2 emitting (USDA442 and USDA16)	4.51 a	2.94 a	130 a	74 a	96 a	1.49 a
Hup^+; non-H_2 emitting (CB1809 and uninoculated[a])	4.16 a	3.00 a	120 a	66 a	73 a	1.53 a

In any one column of each data set, values with a common letter are not significantly different ($P>0.05$).

[a] The soil in all four treatments contained Hup^+, "poor" N_2-fixing strains of *B. japonicum* that occurred naturally in Ayr soil.

(mean increase 15.4%). The confidence levels for the differences were $P<0.20$ in 7 of 12 instances. Pre-treatments of "good" N_2-fixers, USDA442 and CB1809, were better than pre-treatments of "poor" fixers, USDA16 and uninoculated, in 10 of 12 indices (mean increase 10.9%) and the confidence levels for the differences were $P<0.20$ in 4 of 12 cases (Tables 5 and 6). It was observed that cob $\delta^{15}N$ (+6.51‰) for the non-legume, non-N_2-fixing capsicum pre-treatment was significantly greater ($P<0.05$) than cob $\delta^{15}N$ (+5.57‰; pooled data) for N_2-fixing soybean pre-treatments (data not given); this suggests some uptake of soybean N by the maize.

It is emphasised that these data are equivocal and that they constitute inadequate evidence to confirm our hypothesis that H_2 emissions from soybeans inoculated with the Hup^- strains of rhizobia can benefit subsequent crops.

Discussion

Naturalised rhizobia in Ayr soil

Population Before the experiment commenced, the most probable number (MPN) of *Bradyrhizobium* spp. (including soybean rhizobia—*B. japonicum*) in Ayr soil was counted using Siratro as a test plant because seed of wild soybean, a superior test plant for counting *B. japonicum*, was not available. It was found subsequently (Table 1) that enumeration with Siratro over-estimated *B. japonicum* numbers by a factor of about ten. It has been assumed, therefore, that the population of naturalised *B. japonicum* at the site at the start of the experiment was 2,300 per gram of soil (0–10 cm). This is equivalent to approximately 3.5×10^{12} rhizobia per hectare (0–10 cm; cf. Brockwell and Bottomley 1995). It compares with a population of 1.0×10^{12} *B. japonicum* introduced into the soil by sowing inoculated soybean seed. The source of the naturalised rhizobia is a matter for conjecture, but it is probable that they derived from *Bradyrhizobium* spp. associated with indigenous legumes (cf. Cowdry 1954) rather than from contaminant rhizobia from adjoining areas where soybeans, inoculated with commercial inoculant containing *B. japonicum* strain CB1809 (Bullard et al. 2005), had been grown.

Effectiveness of N_2 fixation and classification of H_2 emission Individual strains of *B. japonicum* occurring naturally in Ayr soil were poorly effective in association with Leichhardt soybean and most were ineffective. However, it does not follow from this that uninoculated soybeans grown at Ayr would be unable to fix N_2. There is a large body of evidence (e.g. Nicol and Thornton 1941; Vincent and Waters 1953;

Table 5 Effect of pre-treatment (inoculation of a previous soybean crop) on growth of maize (crop biomass; weight of cobs; "harvest index"=weight of cobs/crop biomass × 100)

Soybean pre-treatment	Biomass DM (t/ha)	Cob DM (t/ha)	Biomass DM (g/plant)	Cob DM (g/plant)	"Harvest index" (%)
Individual inoculation pre-treatments[a]					
CB1809 (Hup^+; "good" fixer)	5.35	1.63	80.6	24.9	30.1
USDA442 (Hup^-; "good" fixer)	5.87	1.98	89.7	30.1	33.5
USDA16 (Hup^-; "poor" fixer)	6.14	1.79	94.6	27.4	28.7
Uninoculated (Hup^+; "poor" fixer)	5.35	1.24	76.3	18.2	23.0
Non-legume (Capsicum)	5.67	1.59	71.2	20.6	27.7
Data pooled according to H_2 emission of pre-treatments (non-legume pre-treatment excluded)					
Hup^- (H_2 emission)	6.01	1.89	92.2	28.7	31.2
Hup^+ (No H_2 emission)	5.35	1.43	78.5	21.5	26.5
Confidence[b]	P=0.093	P=0.046	P=0.106	P=0.090	P=0.197
Data pooled according to H_2 emission of pre-treatments (non-legume pre-treatment included)					
H_2 emission	6.01	1.89	92.2	28.7	31.2
No H_2 emission	5.46	1.49	76.0	21.2	26.9
Confidence[b]	P=0.162	P=0.057	P=0.062	P=0.059	P=0.188
Data pooled according to effectiveness of N_2 fixation of pre-treatments (non-legume pre-treatment excluded)					
"Good" N_2 fixers	5.61	1.80	85.2	27.5	31.8
"Poor" N_2 fixers	5.74	1.52	85.4	22.8	25.9
Confidence[b]	P>0.200	P>0.200	P>0.200	P>0.200	P=0.095

Pre-treatments comprise soybeans inoculated with strains of *Bradyrhizobium* spp. that combine differing characteristics of N_2-fixing effectiveness ("good" fixers; "poor" fixers) and Hup status (Hup^-, H_2-emitting; Hup^+, non-H_2-emitting) plus a non-legume (capsicum) control

[a] No significant differences (P=0.05) between treatments.

[b] Confidence levels calculated to P=0.200

Robinson 1969; Masterson and Sherwood 1974; Singleton and Stockinger 1983; Yates et al. 2005) to show that, when exposed to a mixture of strains of varying effectiveness, a legume will invariably nodulate with the more effective component(s) of the mixture. We assumed from this that the uninoculated soybean treatment at Ayr would fix some N_2 due to preferential nodule formation by the more effective of

Table 6 Effect of pre-treatment (soybeans nodulated by Hup^- and Hup^+ strains of *Bradyrhizobium japonicum*) on the nitrogen content and "harvest index"=cob N/crop N × 100) of a maize crop grown at Ayr for 91 days from October 2005 to January 2006

Soybean pre-treatment	Cob N (%)	Cob N (kg/ha)	Trash N (%)	Trash N (kg/ha)	Crop N (%)	Crop N (kg/ha)	HI for N (%)
Data pooled according to H_2 emission of pre-treatments (non-legume pre-treatment excluded)							
H_2 emission	1.105	20.5	0.651	26.6	0.786	47.2	43.0
No H_2 emission	1.097	15.8	0.633	24.4	0.754	40.7	39.3
Confidence[a]	P>0.200	P=0.063	P>0.200	P>0.200	P>0.200	P=0.178	P>0.200
Data pooled according to H_2 emission of pre-treatments (non-legume pre-treatment included)							
H_2 emission	1.105	20.5	0.651	26.6	0.786	47.2	43.0
No H_2 emission	1.111	16.5	0.628	24.6	0.755	41.1	38.3
Confidence[a]	P>0.200	P=0.101	P>0.200	P>0.200	P>0.200	P=0.050	P>0.200
Data pooled according to effectiveness of N_2 fixation of pre-treatments (non-legume pre-treatment excluded)							
"Good" N_2 fixers	1.117	19.9	0.684	25.6	0.814	45.6	42.9
"Poor" N_2 fixers	1.085	16.4	0.600	25.4	0.726	41.9	38.4
Confidence[a]	P>0.200	P=0.187	P=0.170	P>0.200	P=0.127	P>0.200	P>0.200

[a] Confidence levels calculated to P=0.200

the *B. japonicum* strains in Ayr soil. The assumption was borne out by the data in Tables 3 and 4 that justified the categorisation of the uninoculated soybean treatment as a "poor" fixer. Nodules of uninoculated soybean emitted relatively little H_2 (Table 2) and so the treatment was classified as Hup^+. Thus, the four soybean inoculation pre-treatments (of the maize crop) comprised two highly effective strains ("good" fixers), one Hup^- and one Hup^+, and two poorly effective treatments ("poor" fixers), one Hup^- and one Hup^+. It was hoped that this combination of pre-treatments would make it possible to identify any direct influence on soybean growth, and to distinguish between beneficial effects on the growth of maize of (1) residual N from the soybean crop, and (2) the consequences of H_2 emission from soybean nodules on the soil and its micoflora.

Overview of soybean inputs

The soybean crop and its various inoculation treatments made at least two forms of input to the ecosystem, *viz*. the products of symbiotic N_2 fixation and the consequences of H_2 emission. Soybeans inoculated with effective strains of *B. japonicum* ("good" fixers) exhibited similar shoot growth, grain yield, nodulation and N_2 fixation (105–117 kg/ha fixed N in shoots; Table 4), regardless of Hup status. While the soybeans nodulated by less effective strains ("poor" fixers) did not differ greatly in shoot biomass or grain yield, they contributed correspondingly less (41–75 kg/ha fixed N in the shoots). Much of the N in the shoots was removed in the seed harvest (estimated to be 80–90 kg N/ha, data not shown) and the remaining residues probably contributed little N directly to the following maize crop. The soybean root systems represented another potential source of legume N for the maize. Recent data suggest that 30–50% of the total N in a crop legume can be associated with nodulated roots and rhizodeposition of N during growth (Rochester et al. 1998; Khan et al. 2002; McNeill and Fillery 2008). If it is assumed that the proportion of below-ground N originating from atmospheric N_2 (i.e. %Ndfa) was similar to the proportion measured in the shoots, then below-ground N would represent an additional 70–80 kg fixed N/ha for the "good" fixers, and an additional 25–50 kg fixed N/ha for the "poor" fixers. If those estimates of N fixed below ground were added to the determinations of fixed N in shoots presented in Table 4, total inputs of N via N_2 fixation for CB1809 and USDA442 would be in the order of 175 and 195 kg N/ha, respectively.

The nodule weights for the "good fixers" (Table 3), and the rate of H_2 evolution from USDA442 nodules (Table 2) observed in the present study were both similar to those reported for other soybean field experiments (Herridge et al. 1990; Peoples and Herridge 1990; Hunt and Layzell 1993; Dong and Layzell 2001). Unlike acetylene reduction assays, the process of monitoring H_2 emissions from nodules does not directly inhibit nitrogenase (Hunt and Layzell 1993), but it is likely that excision of shoots and shaking of nodulated roots to remove surplus soil prior to gas exchange measurements resulted in some reductions of nitrogenase activity similar to those described by Minchin et al. (1986). In our field experiment, such disturbance was unavoidable if we were to obtain measures of rates of H_2 emission. Notwithstanding this potential deficiency in our assay procedures, as well as the wide assumptions inherent in calculations, our derived estimate of the amount of H_2 released into the soil over the life of soybeans inoculated with USDA442 (215,000 l per ha—Table 2) was similar to the theoretical determinations made by Dong and Layzell (2001) for a Hup^- soybean symbiosis fixing 200 kg N/ha (250,000 l per hectare). There was no evidence from our work that H_2 emissions influenced soybean growth.

It is known from previous investigations (e.g. Conrad and Seiler 1979; La Favre and Forcht 1983) that this H_2 does not percolate uniformly through the soil, but is rapidly taken up in the immediate vicinity of the nodules. The wide row spacing (75 cm) in our field experiment at Ayr meant that the considerable H_2 emissions absorbed into the soil environment were highly concentrated. It seems inevitable that concentrations of such a highly reactive element would lead to changes in the microbial, chemical and physical characteristics of the soil. Provided such changes were not transient, it could be expected that they would impact on the growth of a subsequent crop (e.g. McLearn and Dong 2002; Stein et al. 2005; Dean et al. 2006). An objective of our work was to test this hypothesis by growing a crop of maize, sown into the soybean row lines, immediately following final harvest of the soybean crop.

Overview of the maize crop

Unlike a recent Canadian field study where Hup^- legume associations were demonstrated to improve significantly the yield of a following crop of barley (Dean et al. 2006), there was little conclusive evidence of a positive effect of H_2 emissions during a legume phase on the performance of maize. While the consistent differences between those pre-treatments that emitted H_2 and those that did not indicated a trend, only one difference was statistically significant at $P<0.05$. There were several possible reasons for the situation. First, our endeavours to create a field site that was uniformly fertile by growing a pre-experiment crop of sorghum were only partly successful. The result was "noisy" data. Second, substantial N_2 was fixed during the soybean phase. Although there was no statistical evidence that residual soybean N contributed to maize growth, it may have had some confounding effect on the results relating to the effects of H_2 on soil characteristics and on the growth of the following maize crop. Third, the environmental conditions during maize cropping in our experiment were substantially different from those that obtained during the growth of barley in the Canadian work. At Ayr, day and night temperatures were higher and day length was shorter. Fourth, the carbon metabolism of maize (a C4 species) is different from that of barley (a C3 plant) and may have influenced the end results.

Conclusions

It should be possible to demonstrate more clearly the effect of H_2 emissions on plant growth by dispensing with the legume component of the experimental system and, consequently, any confounding effect of N input to the system. Instead, H_2 emission from legume nodules formed by Hup^- rhizobia might be simulated by supplying H_2 gas directly from an artificial source. However, this current study suggested that H_2 emission from soybean nodules formed by USDA442 (a Hup^- strain that is a "good" N_2 fixer) may have released 215,000 l/ha into soil over the life of a soybean crop. Logistically it would be difficult if not impossible to deliver this quantity of H_2 through gas lines embedded in fallow soil.

Irrespective of these considerations, this investigation has confirmed that the nodules of an appropriately inoculated soybean crop emit substantial quantities of molecular H_2. Our observations suggest that H_2 evolved by a nodulated Hup^- soybean crop, through its impact on the soil environment, may possibly be beneficial to the growth of a following crop of maize. However, the findings are equivocal and need substantiation by further investigation.

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