TECHNICAL ADVANCE

Long-term non-invasive and continuous measurements of legume nodule activity

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SUMMARY

Symbiotic nitrogen fixation is a process of considerable economic, ecological and scientific interest. The central enzyme nitrogenase reduces H^+ alongside N_2, and the evolving H_2 allows a continuous and non-invasive *in vivo* measurement of nitrogenase activity. The objective of this study was to show that an elaborated set-up providing such measurements for periods as long as several weeks will produce specific insight into the nodule activity's dependence on environmental conditions and genotype features. A system was developed that allows the air-proof separation of a root/nodule and a shoot compartment. H_2 evolution in the root/nodule compartment can be monitored continuously. Nutrient solution composition, temperature, CO_2 concentration and humidity around the shoots can concomitantly be maintained and manipulated. *Medicago truncatula* plants showed vigorous growth in the system when relying on nitrogen fixation. The set-up was able to provide specific insights into nitrogen fixation. For example, nodule activity depended on the temperature in their surroundings, but not on temperature or light around shoots. Increased temperature around the nodules was able to induce higher nodule activity in darkness versus light around shoots for a period of as long as 8 h. Conditions that affected the N demand of the shoots (ammonium application, Mg or P depletion, super numeric nodules) induced consistent and complex daily rhythms in nodule activity. It was shown that long-term continuous measurements of nodule activity could be useful for revealing special features in mutants and could be of importance when synchronizing nodule harvests for complex analysis of their metabolic status.

Keywords: H_2 evolution, nitrogenase, nitrogen fixation, legumes, *Medicago truncatula*, N_2 fixation, Nodule, CO_2 concentration, technical advance.

INTRODUCTION

Biological nitrogen fixation is a fundamental part of the global N cycle. This process is the main N input in the world of living organisms and is of significant economic and ecological importance (Fowler *et al.*, 2013). Nitrogen-fixing legumes are a crucial factor in crop rotations, particularly in ecological forms of agriculture (Peoples *et al.*, 1995; Badgley *et al.*, 2007). Although free-living or root-associated bacteria can fix substantial amounts of

nitrogen, the most intensive nitrogen fixation occurs in the highly developed legume/rhizobia symbiosis (Herridge *et al.*, 2008). In this symbiosis the microsymbiont receives energy from the higher plant, especially through the provision of organic acids (Schulze *et al.*, 2002; White *et al.*, 2007). Furthermore, the higher plant harbours the bacteria in the nodules, which is a highly regulated, optimal environment (Brewin, 1991). In particular, fine regula-

tion of the oxygen pressure inside the nodule is ensured as nitrogenase is oxygen labile and its expression depends on low oxygen partial pressure (David *et al.*, 1988; Mylona *et al.*, 1995; Thumfort *et al.*, 1999). In turn, the microsymbiont provides the higher plant with reduced nitrogen in amounts that can almost fully cover the demand for it under field conditions (Larue and Patterson, 1981; Salvagiotti *et al.*, 2008).

An important tool for nitrogen fixation research is that the enzyme nitrogenase inevitably produces H_2 through the concomitant reduction of H^+ when atmospheric nitrogen is reduced (Hunt and Layzell, 1993; Witty and Minchin, 1998a,b; Marino *et al.*, 2007; Fischinger and Schulze, 2010). The gaseous H_2 evolves from the nodules and provides an – albeit indirect – non-destructive measure of the enzyme's nitrogen fixation activity. H_2 evolving from the nodules can be taken up by an airstream and measured. This approach enabled a number of milestones to be reached in the advancement of knowledge about physiological aspects of nodule nitrogen fixation. For example, it demonstrated the importance of a regulated oxygen supply to the nodules and the involvement of that process in the responses of nitrogen fixation to stress (Witty *et al.*, 1984; Layzell and Hunt, 1990; Minchin *et al.*, 1992, 1994; Kuzma *et al.*, 1993; Kaiser *et al.*, 1994; Minchin, 1997; Denison, 1998; Wei and Layzell, 2006; Naya *et al.*, 2007).

Although the measurement of H_2 evolution is non-invasive and does not disturb the nodule, continuous measurements with a high time resolution based on that approach have only been published for periods <48 h (Gordon *et al.*, 2002). Part of the reason for this is that existing flow-through H_2 analysers are highly sensitive to humidity, which means a gas flow has to be dried completely. In order to collect the H_2 evolving from nodules, the gas flow has to pass through a wet environment, at least for long-term measurements. In addition, the analysers react to oxygen pressure in the gas flow and temperature in their surroundings, both of which are difficult to maintain constant for long-term measurement. These two factors also strongly influence the nodules themselves.

A set-up is described here for measuring nodule activity non-invasively, continuously and with a high time resolution (measurements can be taken every second) over a period of several weeks (Cabeza *et al.*, 2014b). It is possible to change rapidly the composition and/or temperature of a gas flow passing the root nodule compartment and collecting the evolving H_2. The system also allows the long-term maintenance and manipulation of the shoot environment independently of the atmosphere around the roots and nodules. This study's data showed that such a set-up could provide specific insights into nitrogen fixation regulation and dynamics. For example, consistent daily rhythms in nodule activity developed when various treatments induced variations in the N-demand of the shoots.

RESULTS

Plant growth

Medicago truncatula plants A17 developed up to 12 g dry matter per plant in 8 weeks of growth in this system when depending solely on nitrogen fixation for N nutrition. The plants showed a vigorous appearance overall (Figure 1). H_2 evolution rates of up to 35 µmol h^{-1} $plant^{-1}$ [apparent nitrogenase activity (ANA)] were measured.

The influence of temperature on nodule activity

To mimic natural conditions, the standard settings of the light/temperature regime in the growth chamber was 25°C/400 µmol m^{-2} sec^{-1} during the light period (6 a.m. to 10 p.m.) and 20°C/0 µmol m^{-2} sec^{-1} during the dark period (10 p.m. to 6 a.m.). A typical time course of activity under these conditions over 4 days is shown in Figure 2(a). Nitrogen fixation showed a steep decline at the beginning of the dark period and a corresponding increase when the light period began. Nodule activity was more or less constant during darkness (8 h). When the temperature was set to a constant 25°C during several day/night cycles, the

Figure 1. *Medicago truncatula* plant after 8 weeks of growth in the glass tubes.
The plant depended solely on N_2 fixation for N nutrition. After a period of 3 weeks for germination and early cultivation in special growth boxes, the plant was grown for 8 weeks in a controlled environment (16 h light, 25°C; 8 h darkness, 20°C) in glass tubes with nutrient solution. The plant received an N-free but otherwise complete nutrient solution.
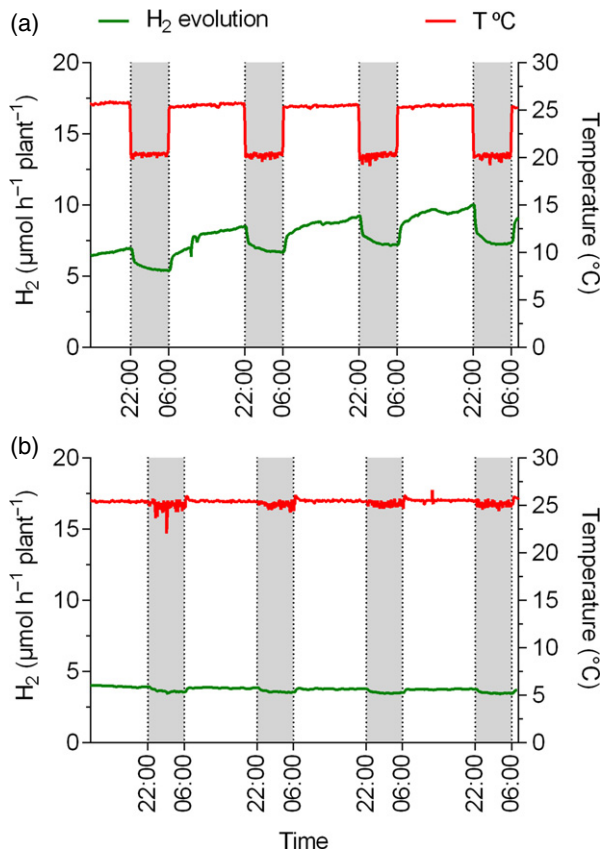
Figure 2. Time course of nodule H_2 evolution at different temperature settings during the day and night.
(a) Nodule H_2 evolution over 4 days at different temperatures during the day (25°C) and night (18°C). Data are the mean value of six replicates. Measurements were taken every 5 min.
(b) Nodule H_2 evolution over 4 days of constant temperature (25°C) during the day and night. Data are the mean value of six replicates. Measurements were taken every 5 min.

Figure 3. Time course of nodule H_2 evolution with either higher or lower temperatures during the day versus at night.
The temperature was set at 18°C during the light period of days 1 and 2 and at 25°C during the night. During days 3 and 4, the temperature during the day was 24 and 20°C at night. Data are the mean value of six replicates. Measurements were taken every 5 min.

Table 1 Relative efficiency of nitrogenase (EAC = electron allocation coefficient) of *Medicago truncatula* plants in different light and temperature conditions and at different points during the day/night cycle

Conditions around shoots	Time	Temp. °C around nodules	EAC ± SE	Replicates
Light	11 a.m.	25	0.58 ± 0.03	12
Light	11 a.m.	18	0.59 ± 0.03	10
Darkness	5 a.m.	18	0.56 ± 0.03	12
Darkness	5 a.m.	25	0.59 ± 0.03	11
Darkness	5 a.m.	30	0.58 ± 0.04	10

Total nitrogenase activity (TNA) was measured as the peak H_2 evolution during a 5-min exposure of the nodules to an Ar/O_2 (80/20; v/v) atmosphere. The EAC was calculated as EAC = 1 − ANA/TNA. The light regime was set to 16 h light and 8 h darkness (6 a.m. to 10 p.m. and 10 p.m. to 6 a.m. respectively) with different temperatures as indicated in the table. The EAC showed no significant differences among the different conditions, ANOVA test ($P < 0.05$). SE, standard error.

activity did not differ between the light and dark periods (Figure 2b). These results showed that the lower nodule activity during the dark period under the normal temperature setting was exclusively the result of the lower temperature. In fact, when the temperature during the light period was set to around 18°C and increased to 25°C during the night, the activity during the dark period was greater than during the light period throughout the 8 h darkness, and followed the development of the temperature closely (Figure 3, days 1 and 2). On day 3 (around 10 a.m.) the temperature setting was put back to normal conditions, and nodule activity again followed the temperature closely and showed the typical time course during day 4 of the measurements displayed in Figure 3. The electron allocation coefficient (EAC) was measured to ascertain possible shifts in relative efficiency of nitrogen fixation under various light and temperature conditions. At no time did the relative efficiency of nitrogenase appear to depend on
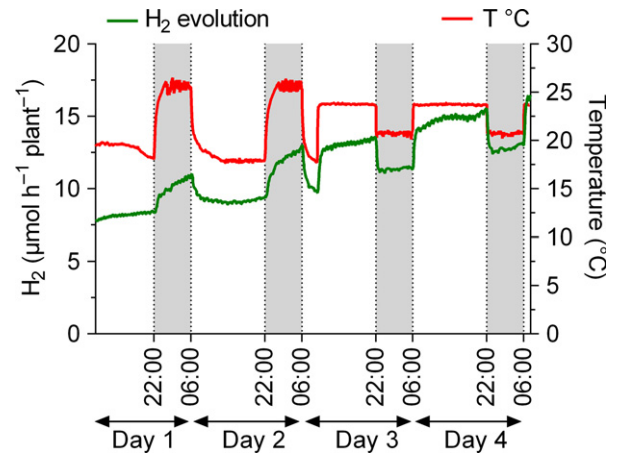
temperature or light conditions. The EAC of nitrogenase activity remained unaffected even after almost 8 h of darkness (Table 1). Figure S1 shows the time course of nodule activity over a 3-day period during which random shifts in temperature were performed. Irrespective of the length of previously relatively high or low activity, nodule activity followed the temperature changes closely. However, when the temperature was set to 36°C, a relatively sharp and continuous decline in H_2 evolution occurred, starting at approximately 33°C. The temperature around the nodules in the glass tubes was monitored in a parallel tube without a plant. A temperature sensor (Vernier

Instruments, see Methods S1) was inserted through the rubber stopper on the upper side of the tubes. The tubes were sealed with modelling clay so as to be airtight between the rubber stopper and the temperature sensor. The temperature in the tubes followed that in the growth chamber with a delay of about 9 min. The H_2 analysers react to temperature in their surroundings; therefore it was helpful to keep them in a different room from the plants to study the effects of temperature on nitrogen fixation. For the study presented here, the analysers were kept in an air-conditioned and temperature-controlled room adjacent to the climate chamber. Using the Plexiglass chamber set-up to regulate the atmosphere around the shoots separately (Figure 4 and Methods S1), it was possible to study the individual effect of the temperature around the shoots or root/nodules. For that purpose the tubes were fixed on the basic plate of the shoot compartment above the tubing connectors directly below the upper edge with tight rubber rings, so that they were completely exposed to the climate chamber temperature while the temperature around the shoots could be regulated separately through the set-up shown in Figure 4. The data revealed that in the short to medium term the effect of temperature on nodule activity was solely caused by the temperature around the roots/nodules (Figure 5). Under constant high temperature around the shoots, nodule activity closely followed a

temperature increase (24–32°C) in their surroundings (Figure 5a). When the activity at 24°C was set at 100%, nodule activity increased to 147%, or by 6% per degree. In contrast, a temperature increase around the shoots (24–31°C) had no effect on nodule activity when the temperature around the roots/nodules was kept constant (19°C; Figure 5b).

Factors inducing daily rhythms of nitrogen fixation

In a further experiment, the option of manipulating the nutrient solution composition was applied. Mg was left out of the nutrient solution in one treatment to induce whole-plant Mg depletion. The removal of Mg from the nutrient solution resulted in a decline in Mg concentration in shoots, roots and nodules by about 25% of the initial concentration during days 2–6 of the depletion process (Figure S2). Nodule Mg concentration was about double that in the roots and equalled the concentration in leaves. After about 4 days of Mg depletion, nodule activity showed a clear and consistent pattern during the 24 h light/dark cycles. The temperature was set to 25°C during both the light and dark periods of the experiment, which in fully nourished plants resulted in a more or less constant activity over time (Figure 2b). With growing Mg depletion, a decline in nodule activity began at around noon that continued until around 5 p.m. (Figure 6a). Subsequently, there was a clear
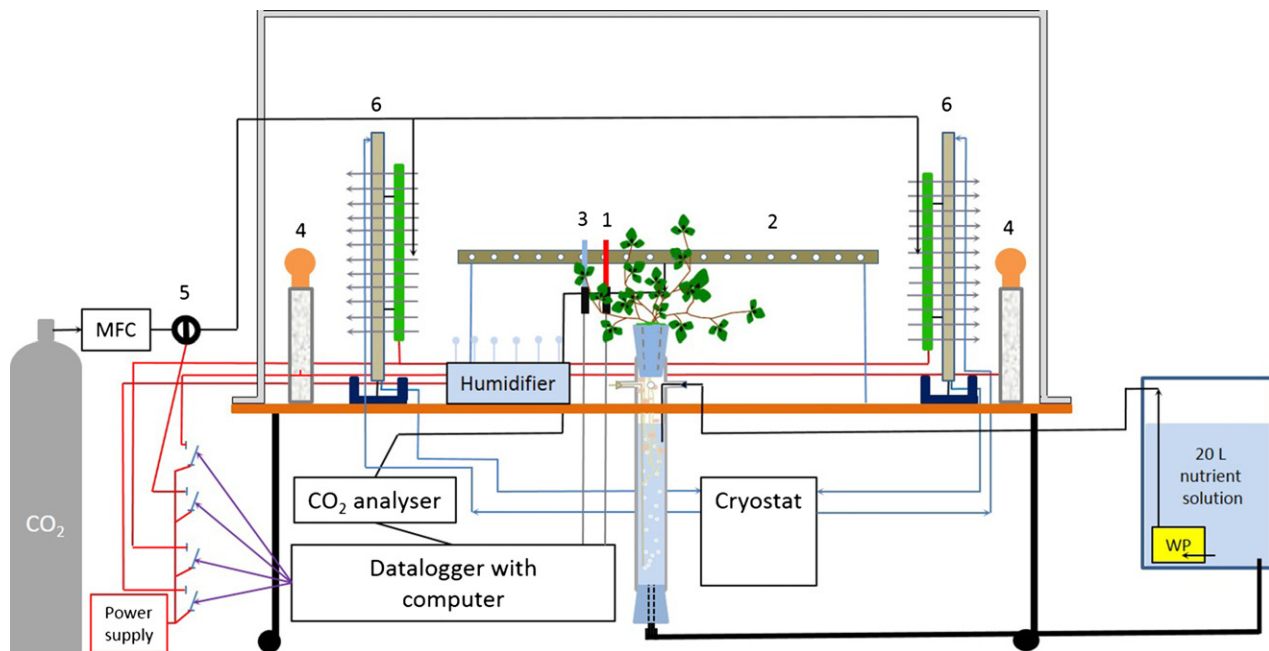

Figure 4. Experimental set-up for maintaining and manipulating environmental conditions around plant shoots in parallel to continuous measurements of root/nodule H_2 and CO_2 release.
The system allowed up to 12 plants to be enclosed. Temperature, CO_2 concentration and humidity around the shoots could be taken continuously (1, 2 and 3 respectively), regulated and rapidly altered. This was achieved by setting computer-controlled thresholds with an automatic switch on/switch off system consisting of a humidifier, two radiant heaters (4) and a valve (open/closed) (5) for CO_2 influx into the cuvette. Temperature, humidity and CO_2 concentration were lowered through two cooler systems (6) and plant shoot photosynthesis respectively. The flow rate and time of an open valve for CO_2 influx into the cuvette allowed net shoot CO_2 uptake to be measured. MFC = mass flow controller; WP = water pump. For details on the equipment used see Methods S1.
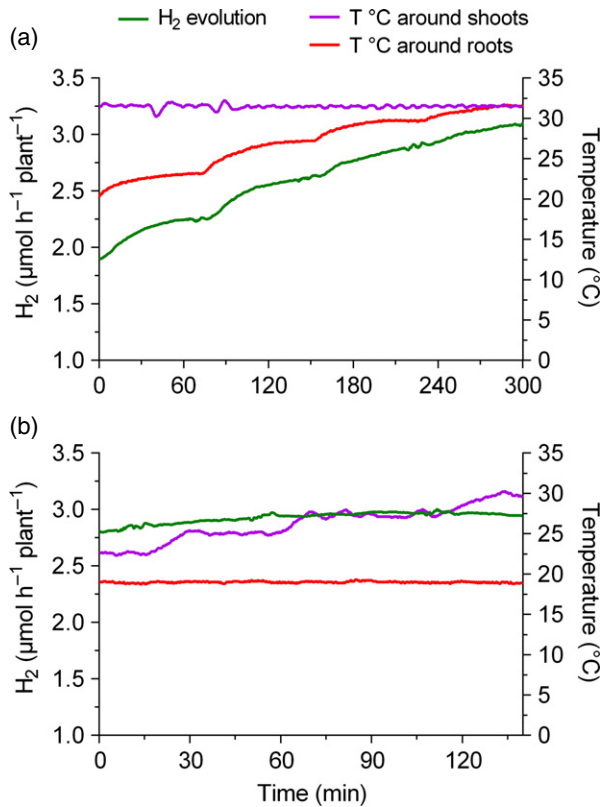
(a)

(b)

Figure 5. Time course of nodule H_2 evolution during separate increases in temperature around roots/nodules (a) or around shoots (b).
Data are means of eight replicates. Measurements were taken between 9 a.m. and 3 p.m. and data recorded every 12 sec. A closed glass tube without a plant but the addition of a constant low amount of calibration gas was used as a control.

increase in activity until the light was switched off (10 p.m.). The Mg-depleted plants showed a steep decline in activity unrelated to temperature when the light was switched off at 10 p.m. A strong recovery of nodule activity occurred after about 2–3 h of darkness, at around 1 a.m. The intensity of the recovery became stronger when the light was switched on at 6 a.m. and resulted in peak activity at midday. Thereafter the activity rhythm was consistently repeated over several days, but with increasing intensity. It has been reported that a corresponding P-depletion process is different in that the P concentration in nodules is maintained for longer into the P-depletion process than it is in leaves (Cabeza *et al.*, 2014b). Nevertheless, a rhythm in nodule activity appeared during the depletion process. Whereas Mg-depleted plants consistently developed the described rhythm in nodule activity after 3–4 days of the depletion process, this occurred later during P depletion. Nevertheless, the pattern of the rhythm was consistent with that of Mg-depleted plants and is shown as an example in Figure 6(b). The rhythm resembled that of Mg-depleted

plants, except that decline and recovery during the night was less strong. A corresponding rhythm in activity could be induced by applying low concentrations of ammonium to the nutrient solution (Figure 6c). Figure 7 shows the development in nodule activity for a 5-day period after the application of a high amount of ammonium, and the recovery after the nutrient solution was replaced with an ammonium-free nutrient solution (indicated in Figure 7). The complete removal of the nutrient solution (including that in the tubes) resulted in a short disturbance to measurements. Nodule activity showed a strong overall decline during the exposure to high concentrations of ammonium (Figure 7). Nevertheless, the typical 24-h rhythm was still recognisable. The rhythm disappeared within 1 day when the plants were supplied with ammonium-free nutrient solution and per plant activity recovered over time. Finally, older plants with the supernodulating feature developed a corresponding nodule activity rhythm under undisturbed conditions supplied with full nutrient solution (Figure 6d).

DISCUSSION

The gas exchange measurement system

Continuous long-term and non-invasive measurements of nodule H_2 evolution provided information about the process of nitrogen fixation and specific features of genetically manipulated plants (mutants) with respect to their nodule activity. Prerequisites for this kind of measurement are a precise, constant but also flexible mixture of a basic flow and a highly efficient multi-step way of removing humidity after the gas flow has passed the root/nodule compartment. Furthermore, a means of renewing and/or manipulating the nutrient solution (for example, the addition of NH_4^+) without opening or interfering with the sealed root/nodule compartment was very helpful since it avoided disturbance and readjustment of the 'measurement equilibrium' in the gas flow. Any direct interference with the root/nodule compartment required several hours at least before a new stable H_2 evolution measurement could be taken. A continues day to day increase in nodule activity per plant occurred in our experiments. However, the pattern depending on the temperature setting and also the induced daily rhythm showed no dependence on plant age and plant activity (see for example Figure 6), with the exception that the not treatment-induced activity rhythm occurring in Mt$_{sunn}$ plants usually developed in plants older than at least 4 weeks. The measurement with the described set-up necessitates the use of a bacteria strain that does not form an H_2-uptake hydrogenase (hup$^-$). Other than the efficient strain used in this study, three other widely used strains for studying the *M. truncatula* symbiosis (Sm1021, Sm2011 and WSM419) do not form an H_2-uptake hydrogenase (hup$^-$). In the authors' experience, there is no detectable evolution of H_2 from nodules formed by hup$^+$ strains.
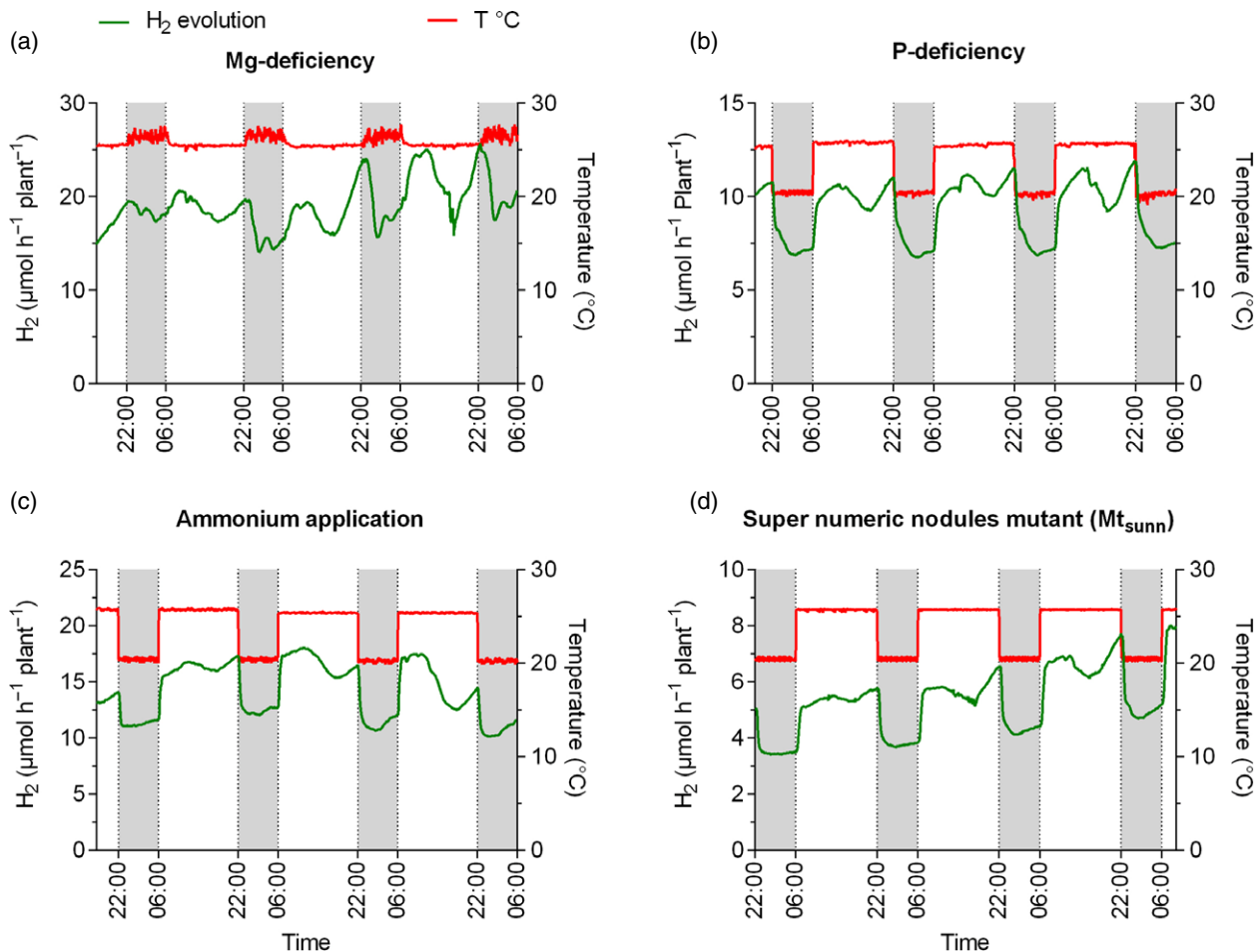
Figure 6. Daily rhythm of nodule H_2 evolution during whole-plant Mg or P depletion, exposure to low concentrations of ammonium or plants with unregulated nodulation.

The time course of H_2 evolution showed 3 day/night cycles of: (a) plants beginning 3 days after complete removal of Mg from the solution; (b) plants beginning 8 days after complete removal of P from the solution; (c) plants beginning 2 days after provision of 0.25 mM ammonium; and (d) 6-week-old supernodulating Mt_{sunn} plants. Ammonium was added as $(NH_4)_2SO_4$, the additional sulphate was equalized in the control by K_2SO_4. The amount of sulphate removed with the Mg $(MgSO_4)$ was replenished by K_2SO_4 in the control. The temperature was set to a constant 25°C for (a) (day and night), while the setting for (b), (c) and (d) was 25°C/20°C during the day and night respectively. Data are the mean value of six replicates. Measurements were taken every 5 min, with data recorded from different experiments with plants of different ages.

A further set-up that allowed separate and long-term changes of the atmosphere around shoots also enabled the direct or long-term shoot-mediated influences on nodule activity to be studied (Figure 4). The possibility of studying the effect on nodule activity of elevated CO_2 concentrations around the shoots might be a particularly useful application of the system described (Lepinay *et al.*, 2012; Guo *et al.*, 2013, 2014).

Nodule activity depends on the surrounding temperature

There are various reports that nodule activity depends on the temperature around shoots or, in a broader sense, on the growth rate of the shoots (Schweitzer and Harper, 1980; Walsh and Layzell, 1986; Kessler *et al.*, 1990). However, these were point measurements of changes that might take effect mid-term, which means in the context of days. No immediate effect on nodule activity was found when the temperature around shoots was elevated, even though the temperature rise should have had an immediate effect on plant growth rate (Criddle *et al.*, 1997). In contrast, rising temperatures around the nodules increased the nodule activity significantly when the temperature around the shoots was kept constant. It was also possible to show that during the dark period of the light/dark regime, nodule activity continued unabatedly during the 8 h of darkness. In fact greater activity than during the light period could be induced by higher temperatures around the nodules. Random switches in temperature were directly and immediately followed by nodule activity, irrespective of prior activity. Taken together, these results
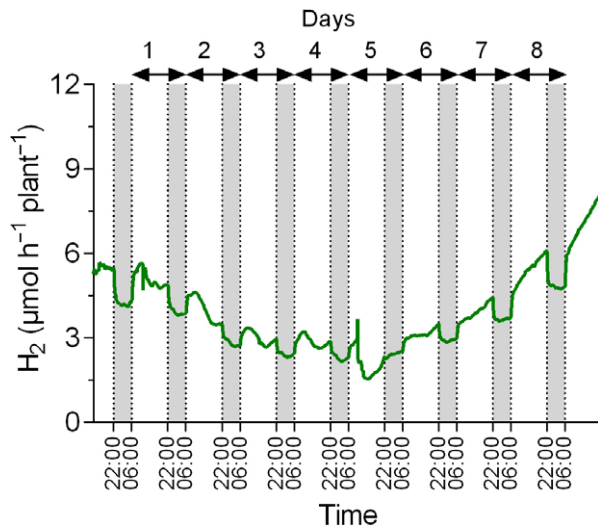
Figure 7. Daily rhythm of nodule H_2 evolution of plants after providing high concentrations of ammonium (5 mM) (day 1) and during recovery of nodule activity after subsequent ammonium removal (day 5).
The day/night conditions in this experiment were set as follows: 16 h light (400 µmol m^{-2} sec^{-1}, 25°C), 8 h darkness (20°C). Ammonium was added as $(NH_4)_2SO_4$, the additional sulphate was equalized in the control by K_2SO_4. Data are the mean of six replicates. The need to remove ammonium completely from the nutrient solution resulted in a disturbance of the system during day 5.

showed that during undisturbed conditions for the nodules, the specific activity of nodules was not regulated (restricted) in the short term either by a down-regulatory impact from shoots or limitation of a necessary supply (assimilates). Rather it appeared that during undisturbed growth the supply of NH_4^+ to the higher plant through nodules was, within limits, flexible and dependent on the temperature around the nodules. The strong and direct temperature dependence of nodule activity indicated that the amount of nitrogenase protein in conjunction with the temperature determines the specific activity of an individual nodule. This interpretation was further supported by the fact that at around 33°C a steep decline in nodule activity occurred. Instead of some resources becoming depleted (high activity at 30°C can be maintained for a whole dark period), temperatures above approximately 33°C appeared to have a detrimental effect on the conformation of nitrogenase. Although this set-up allowed numerous conditions for the nodules to be varied, no single treatment was found to induce higher specific nodule activity in the short term other than the temperature around the nodules. In contrast, under stress conditions or when alternative N sources are available, the plant can efficiently downregulate nodule activity (Denison, 1998; Kato *et al.*, 2010; Esfahani *et al.*, 2014a,b). It is proposed that increasing shoot demand is largely met by increasing the number of nodules (Voisin *et al.*, 2010), while the existing nodules work

on the temperature-dependent upper limit set by the amount of nitrogenase protein. This is consistent with the fact that an elevated CO_2 concentration around shoots results in a concerted increase in shoot biomass and nodule number, but not in short-term increased specific nodule activity (Vance and Heichel, 1991; Rogers *et al.*, 2009; Cabeza *et al.*, 2014c). In the symbiosis, the higher plant forces (punishes or sanctions) (Kiers *et al.*, 2003; Oono *et al.*, 2009, 2011) the microsymbiont to produce as much nitrogenase as possible or what is appropriate for the amount of assimilates consumed.

Inducible rhythms in nitrogenase activity over the day/night cycle

Various conditions that in a broader sense have the plants' decreasing need for nitrogen fixation in common (high or low NH_4^+ supply, emerging P and Mg deficiency, too many nodules) induced a daily rhythm in nodule activity rather than a steady decline. This led to significantly decreased activity per day. In the experiment with a low ammonium supply, a reduction of about 13% in the amount of fixed nitrogen per plant and day occurred through the rhythm in activity. P deficiency induced declining activity after several hours of light during the light period. A recovery started after around 4 h, usually at 5 p.m. in the afternoon in this day/night regime. During darkness the recovery continued after a short decline. Four- to 5-week-old plants of the supernodulating mutant developed a daily rhythm in activity without the induction of any treatment. The principle pattern resembled that of P deficiency: after a decline starting at around noon, a recovery occurred at around 5 p.m. It has been shown that the rhythm intensifies greatly in Mt$_{sunn}$ plants under P deficiency (Cabeza *et al.*, 2014c). The pattern of the Mt$_{sunn}$ activity rhythm is an example that specific features of mutants affected in nitrogen fixation could be revealed through the use of this long-term measurement set-up. Ammonium application and emerging Mg deficiency resulted in the same rhythm during the light period, but a stronger second pattern during the dark period. In both cases a strong decline in activity occurred during the early hours of darkness, however after several hours a steep recovery commenced. These patterns during the dark period might be related to assimilate allocation to nodules being affected (Cakmak *et al.*, 1994). However, the recovery during darkness indicated that these periods of possibly insufficient assimilate supply reflected a disturbed allocation rather than an overall restriction in assimilate availability on the whole-plant level. It is noteworthy that Mg appeared to be of central importance for nitrogen fixation, both on a systemic and local level. This was reflected in the fact that the nodules had a high Mg concentration (Figure S2). The concentration was significantly higher when compared with roots and was about as high as in leaves. Comparatively little information is known about the

importance of Mg for nitrogen fixation (Ohara *et al.*, 1988). The long-term measurements during Mg depletion revealed that total nitrogen fixation was already affected by latent Mg deficiency. The strong daily rhythm in activity developed after 3–4 days of Mg depletion. There were no visible Mg-deficiency symptoms in shoots during the whole Mg-depletion period (10 days). Mg is known to be easily reallocated via the phloem (Steucek and Koontz, 1970). Consequently, the nodules had a very high, specific Mg demand that was among the primary targets of developing Mg deficiency. The observation of an emerging 24-h pattern in nodule activity under Mg depletion necessitated the described set-up for long-term measurements.

The occurrence of a decline in the afternoon might be the result of strong nitrogen fixation in the morning and a downregulatory impact of the shoots as a consequence of N satiety. An overwhelming amount of circumstantial evidence pointed to the fact that a downregulatory impact on nodules under stress conditions results from a shoot-borne factor (Schulze, 2004). It has been shown that N travels quickly from leaves to nodules (Fischinger *et al.*, 2006) and in particular the amount of asparagine increases in the phloem under treatments that reduce nitrogen fixation (Sulieman *et al.*, 2010, 2014). Nevertheless the actual mechanism by which nodule activity is affected by this shoot-borne factor remains to be shown. In fact, the high time-resolution measurements allow the precise timing of studies that have the objective of isolating this factor and characterising the underlying mechanism of its impact on nodules. From this study's results, in particular the 'afternoon decline' appears to be promising as it is a common feature under the impact of each treatment studied. We have no explanation for the fact that the feature retained most was a recovery of activity at around 5 p.m. This might indicate that processes related to the circadian clock are involved. An alternative explanation might be that the rhythm is the result of a relatively coarse regulatory mechanism induced by a substance that is formed in excess by the shoots when an unnecessary high N supply occurs and is used up during a 4- to 5-h period. These aspects warrant further research.

The appearance of a daily rhythm of nodule activity under stress conditions makes it necessary, in some cases, to re-evaluate point measurements of nitrogen fixation, for example on excised nodules or whole plants through H_2 evolution, $^{15}N_2$ application or measurements of acetylene reduction, and to consider the timing of such measurements carefully. For example, a measurement taken for up to 1 h between 11 a.m. and noon when compared with a measurement between 4 p.m. and 5 p.m. under P deficiency would have resulted in about a 25% difference or variability as a consequence of a daily activity rhythm. The fact that comparatively small amounts of ammonium already induced the activity rhythm described makes it

appear possible that under conditions of growth in soil, where a certain amount of mineral nitrogen is always available, a daily rhythm in activity is the commonly found time course of nodule activity.

In conclusion, the described set-up for growth and continuous, non-invasive gas exchange measurements of legume nodules allowed the unravelling of physiological aspects that were elusive to point measurements. As an example, the strong direct, short-term dependence of nodule activity on temperature in their surroundings could be shown, but not on light or temperature around shoots. Furthermore, specific daily rhythms in nitrogen fixation occurred when experimental treatments were imposed that induced relative N satiety in the shoots. A mutant that formed an unusually large number of nodules showed specific features of the daily rhythm in nitrogen fixation. The set-up therefore has the potential to characterise specific features of nitrogen fixation in mutants that are affected in their nodule functioning. The high time resolution of the measurements also allows the precise timing of 'omics' studies on the regulatory impacts on nodule functioning (Cabeza *et al.*, 2014a,b).

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seeds of *Medicago truncatula* (Gaertn.) cv. Jemalong A17 were submerged in H_2SO_4 (96%) for 5 min for chemical scarification, sterilised with 5% (v/v) sodium hypochlorite for 5 min and rinsed several times with deionised water. The seeds were subsequently kept at 4°C for 12 h in darkness, submerged in tap water. The next step was a 2- to 4-day slight shaking of the submerged seeds at 25°C and in continuous light. When the seed had developed a primary root about 20 mm long, 20 plantlets were transferred to small growth boxes (170 × 125 × 50 mm) filled with a 1/10 diluted and intensely aerated nutrient solution (see below for the composition of the solution). The seedlings were fixed through small x-shaped cuts in tape on the upper side of the growth boxes. The plants were grown for 2 weeks in these boxes in a growth chamber with a 16 h/8 h light/dark cycle at 25°C/20°C respectively. The nutrient solution level in the boxes was maintained by adding an appropriate amount of nutrient solution every other day. Light intensity at plant height was approximately 400 μmol m^{-2} sec^{-1}. Immediately after transfer to the growth boxes, the seedlings were inoculated with 1 ml/box of a stationary *Sinorhizobium meliloti* (Sm) (102F51) YEM culture, with an approximate cell density of 10^9 ml^{-1}. The Sm strain induced good nodulation, with the first nodules visible to the naked eye after about 7–10 days. The strain shows more efficient nitrogen fixation than the model strain 1021 (Sulieman and Schulze, 2010) and does not form an H_2-uptake hydrogenase (Blumenthal *et al.*, 1997).

Glass tubes for growth and gas exchange measurements

The plants formed primary roots about 30–50 mm long and two leaves during 2 weeks of growth in the small 1 L container. At that stage the plants were transferred to glass tubes (length 800 mm, internal diameter 20 mm) specifically designed for gas exchange measurements (Figure 8). The tubes were closed at the top by a

Figure 8. Glass tubes functioning as a root/nodule compartment that can be sealed airtight and individually aerated and supplied with a circling nutrient solution flow.

The glass tubes (length 800 mm, internal diameter 20 mm) had three connectors for air inflow and outflow and nutrient solution inflow about 20 mm below the upper end of the tubes (left). The nutrient solution flowed out of the tube, driven by gravity, through a connector in the lower rubber stopper back to a 20 L nutrient solution container. The tubes were darkened at both ends to avoid algal growth. When roots/nodules were grown in the tube, the rest of the tube was darkened by a double layer of white linen (centre), which allowed occasional checks to ensure the airstream was bubbling properly and the roots and nodules were healthy (right). The plants were fixed in a conical central bore in the upper side rubber stopper by foam during normal growth and by beeswax-rich, easily formable modelling clay that is not toxic for plants. The material allowed air-proof sealing of the root compartment during measurements. The tubes contained a total of 150–250 ml nutrient solution, depending on the volume of the roots and nodules.

rubber stopper with a centric bore (internal diameter 8 mm). The plants were inserted through that bore and fixed on the lower part of the stem by a 30 mm piece of sponge. The sponge also guaranteed lightproof conditions for the roots. The bottom part of the glass tubes was closed by a rubber stopper with a connector led through a central bore. Tubing connected the bottom part of six glass tubes to a 20 L nutrient solution container (Figure 9). A pump in the container drove a nutrient solution flow of about 10 ml min^{-1} into the upper side of each individual glass tube. Each individual glass tube contained about 150–250 ml nutrient solution (depending on the size of the roots), which were then turned every 15–25 min. Through the force of gravity, the connection of the bottom part of the tubes to the nutrient solution container guaranteed that the level in the glass tubes depended on the height of the container's position, and losses through plant transpiration could be adjusted by the addition of nutrient solution to the container, thereby not interfering with ongoing measurements in the root/nodule compartment (Figure 9). All tubing and connections were darkened by black tape to prevent algal growth. The system constituted a quasi flow-through nutrient solution supply that allowed an undisturbed gas flow-through the glass tubes for collecting evolving H_2 and CO_2. The nutrient solution

contained macronutrients: K_2SO_4, 0.7 mM; $MgSO_4$, 0.5 mM; $CaCl_2$, 0.8 mM; and micronutrients: H_3BO_3, 4.0 μM; Na_2MoO_4, 0.1 μM; $ZnSO_4$, 1.0 μM; $MnCl_2$, 2.0 μM; $CoCl_2$, 0.2 μM; $CuCl_2$, 1.0 μM; FeN-aEDTA (ferric monosodium salt of ethylenediaminetetraacetic acid), 30 μM. The pH was adjusted to 6.4 through KOH and buffered with 0.25 mM 2-(N-morpholino)ethane-sulfonic acid (MES). Phosphorus (P) was added daily as KH_2PO_4 to a concentration of 5 μM P. The nutrient solution was changed every week. During this procedure the pump in the container was switched off and the backflow from the glass tubes to the container was blocked. This meant that ongoing measurements in the root/nodule compartment were not affected. The procedure to replace the nutrient solution in the container took about 10 min, and then the nutrient solution turnover system could be put back to normal functioning. If necessary up to 250 ml nutrient solution in the glass tubes could also be replaced through an outlet in the backflow tubing to the container. For the first week after the transfer of the plants to the glass tubes, the nutrient solution was adjusted once to a 0.5 mM NH_4^+ concentration by the addition of NH_4SO_4. Low concentrations of ammonium support nodule formation in *M. truncatula* (Fei and Vessey, 2009). In the following weeks of growth in the glass tubes, the plants depended solely on N_2 fixation for N nutrition. Overall the set-up allowed manipulation of the nutrient supply to the plants during ongoing measurements of nodule activity, for example through the addition of nitrate (Cabeza *et al.*, 2014a) or ammonium (within this paper) or the removal of P (Cabeza *et al.*, 2014b) or Mg (within this paper).

Root/nodule gas exchange measurement

The short-term measurement of nodule H_2 and CO_2 evolution, including the determination of apparent nitrogenase activity (ANA), total nitrogenase activity (TNA), the calculation of the electron allocation coefficient (EAC) and of N_2 fixation and the validation of the values by $^{15}N_2$ application, is described in Fischinger and Schulze (2010). For a continuous, long–term measurement of H_2 evolution, the set-up was extended by an efficient three-step air drying system for the gas flow out of the root/nodule compartment (Figure 9). The H_2 analysers (Quibit Systems, Canada, http://www.qubitbiology.com/environmental/q-s121-h2-gas-analyze) respond to humidity and a totally dry gas flow is a prerequisite for the measurements. The nutrient solution in each glass tube was individually aerated with a gas flow of 200 ml min^{-1} (N_2/O_2, 80/20 v/v) (Figure 9). A first condensation step after bubbling through the nutrient solution in the glass tubes was achieved by passing the gas flow consecutively through two snake-type reflux condensers, using the inner 'snake' part for the gas stream, while the surrounding outer compartment was continuously supplied with water at 0°C (Figure 9 and Methods S1). The condensing water from the gas flow was collected in a reservoir in the lower part of the reflux condenser. This first drying step removed the bulk of the humidity. Subsequently, the gas flow was bubbled through 10 ml of concentrated H_2SO_4, which took up about 1 ml of water per week. Eventually the gas flow passed through 200 × 20 mm transparent plastic tubes containing dry silica gel. This measure removed any remaining humidity in the gas flow. Exhaustion of the capacity of H_2SO_4 to remove the remaining water was indicated by a colour loss of the silica gel. Both H_2SO_4 and the silica gel were renewed weekly. It was ensured that the discolouring of the silica gel never reached the last 50 mm of the plastic tubes. The H_2 analysers used did not show significant shifts in their zero points during the experimental periods. The data for H_2 and CO_2 evolution could be taken with a resolution of up to 1 sec, collected by a LabPro data logger (Vernier Software and Technology, LLC, USA) and stored in a computer using the LABVIEW software
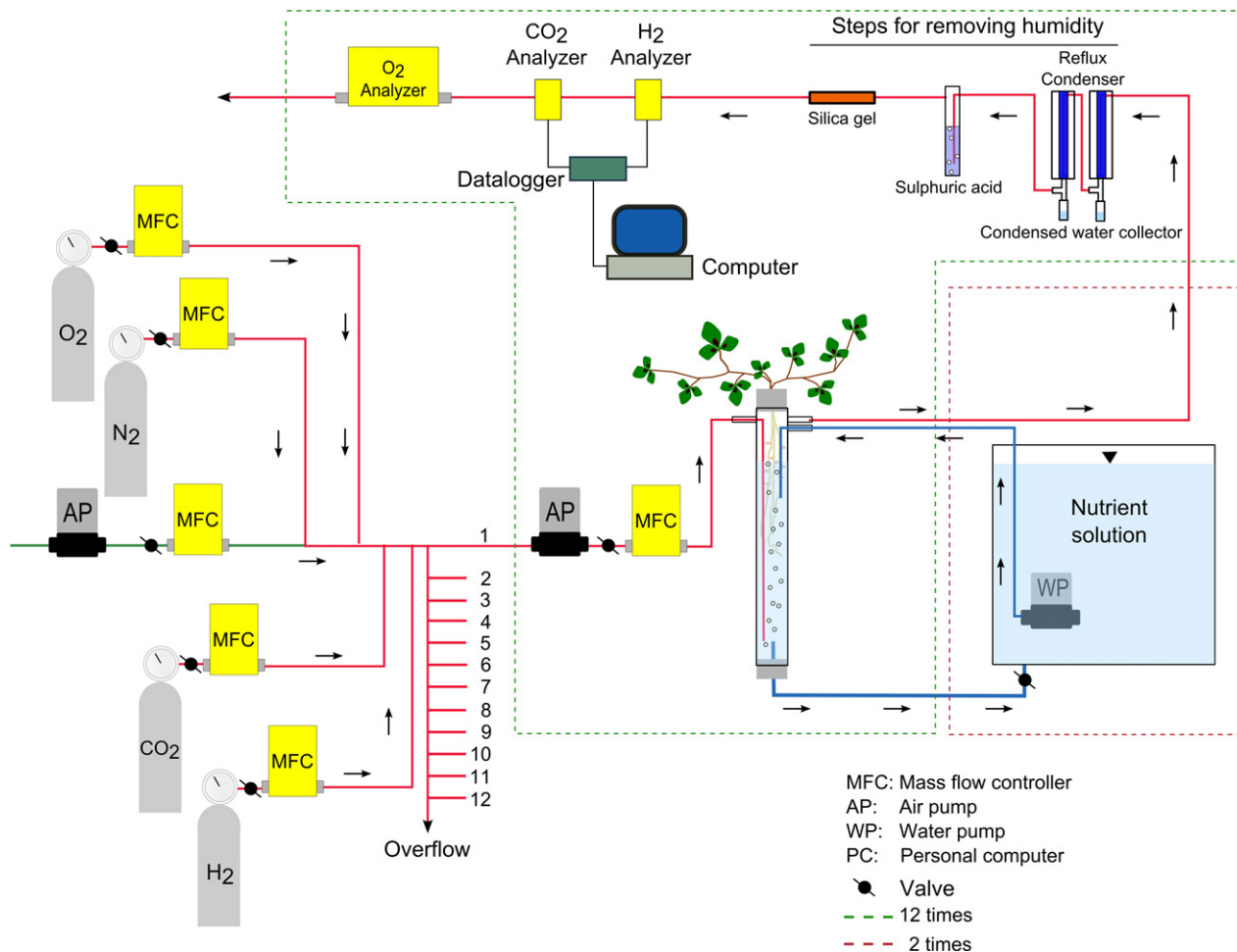
Figure 9. Experimental set-up for long-term, continuous and non-invasive measurement of nodule H_2 evolution.
A gas blending system or alternatively a pump providing outside air supplied an overflow of a basic gas flow of rapidly adjustable composition. Pumps took a constant flow (MFC = mass flow controller) from the basic flow and directed it into the lower part of the glass tubes containing the root/nodules. After bubbling through the nutrient solution in the glass tube, the flow was dried and directed to analysers for H_2, CO_2 and O_2. A data-collecting system took measurements of the concentration of the individual gas components. The glass tubes, nutrient solution container and nutrient solution tubing system were lightproof and covered by various materials (not shown in the figure). There were 12 sets of components enclosed by the green dotted line, and two sets of components enclosed by the red dotted line, allowing two treatments for nutrient solution composition and 12 individual H_2 evolution measurements.

(Vernier Software and Technology, LLC) (Methods S1). For the long-term experiments described here [Mg depletion, P depletion, ammonium addition and measurement of activity of nodules of Mt$_{sunn}$ (super numeric nodules)] data were taken every 5 min. The system was calibrated by bypassing the root/nodule compartment and adding increasing amounts of calibration gas that contained 1000 μmol mol^{-1} H_2 into the basic flow (Figure 9). The nutrient solution did not dissolve measurable amounts of H_2 and did not release measurable amounts of H_2 during increases in temperature within the range used in this study (Appendix S1 and Figure S3).

Maintenance and manipulation of the atmosphere around the shoots

Figure 4 shows a Plexiglass cuvette in which a set of 12 plant shoots could be enclosed (see also Methods S1). The material does not absorb photosynthetic active light. Temperature (10–40°C), CO_2

concentration (CO_2 compensation point to 1%) and humidity (30–60%) could be constantly regulated within the cuvette. The set-up is an extension of the system described in Schulze and Merbach (2008). The temperature was constantly measured inside the cuvette at plant shoot height and the data collected by a computer. The amount of heat provided by greenhouse warming was removed by two coolers supplied with cooled water from a cryostat. The coolers were originally designed for cars (Methods S1). The coolers were equipped with four computer fans that push air through the coolers' rows of ribs against the inside of the small wall of the cuvette. The whole set-up was placed in an accessible temperature-regulated growth chamber. The temperature in the growth chamber should typically be set slightly above the desired temperature inside the cuvette, particularly to avoid water vapour condensing on the inside of the cuvette wall. Two radiant heaters were placed behind the coolers. The power supply of the radiant heaters was connected to an automatic on/off switch system. When the temperature inside the cuvette fell below a pre-set lower limit,

the radiant heaters were switched on until an upper threshold was reached. Temperatures from 10 to 40°C could be set with 1° over- and undershooting. Humidity and CO_2 concentration were regulated using the same principle. Humidity was supplied by a commercial humidifier. A stream of CO_2 was directed behind the fans and the CO_2 concentration was measured continuously at plant height inside the cuvette. An open/close valve for the CO_2 flow was regulated in the same way as the radiant heaters. CO_2 concentrations could be set with over- and undershooting of 20 ppm. During dark periods the cuvette was flushed with outside air.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Time course of nodule H_2 and root/nodule CO_2 evolution during random shifts of temperature over a 3-day period.

Figure S2. Mg concentration in leaves, roots and nodules during whole-plant Mg depletion of *M. truncatula* plants.

Figure S3. Effect of changes in temperature on the concentration of H_2 in a calibration gas flow.

Appendix S1. Rough calculation on a possible artefact in nodule H_2 evolution measurements through dissolution of H_2 in the nutrient solution.

Methods S1. List of used material and equipment for the set-up shown in Figures 4 and 9.

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