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Determination of nitrogen fixation effectiveness in selected *Medicago truncatula* isolates by measuring nitrogen isotope incorporation into pheophytin

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Abstract

Effectiveness is a term used to describe the input that a bacterial nitrogen-fixing symbiosis makes to plant nitrogen metabolism. In legumes, effectiveness is considered a polymorphic trait where specific interactions between the plant and symbiotic rhizobia contribute to the success of the interaction. Evaluation of effectiveness using model legumes like *Medicago truncatula* may open new avenues for genetic studies. In previous work, an isotope dilution mass spectrometry method, which uses the effect of nitrogen fixation on the nitrogen isotope composition of chlorophyll in plants grown on ^{15}N fertilizer as a measure of effectiveness, was developed for estimating the contribution of symbiotic nitrogen fixation to plant nitrogen content. This ^{15}N-dilution assay was used to evaluate the level of nitrogen fixation effectiveness in three *Medicago truncatula* lines that have been used as parents in generating recombinant inbred lines. Three *Sinorhizobium meliloti* strains, USDA 1600, 102F51 and MK506, differ in this measure of effectiveness on three lines of *M. truncatula*: Jemalong A17, DZA315.16 and F83005.5. Plant–rhizobia combinations grown in two different conditions showed comparable differences in effectiveness.

Introduction

Many legumes have the ability to form nitrogen-fixing symbiosis with gram-negative soil bacteria known as rhizobia (Crawford et al., 2000). The symbiotic interaction involves expression of many specific bacterial and plant genes during the development of differentiated plant tissues that can accommodate the unusual metabolic needs of the symbiotic bacteria. Differences in the complex interactions between the plant and bacteria have been suggested to be the main factor responsible for the ultimate variation in N_2 fixation observed in different host-bacterial interactions (Barnes et al., 1984; Hobbs and Mahon, 1983; Mytton et al., 1984; Mytton et al., 1977; Nutman, 1984; Rennie and Kemp, 1983; Robinson et al., 2000; Smith and Knight, 1984). When a bacteria-host combination can fix nitrogen, it is said to be effective but effectiveness is really a quantitative trait that has been variously defined by measuring parameters related to the amount of nitrogen the symbiosis contributes to the plant or to the efficiency of the conversion of plant resources to fixed nitrogen (Danso, 1995). Although effectiveness is at the heart of legume productivity, there is little information about the plant genes that determine this quantitative measure of the success of the symbiosis.

In order to study the genetics of N_2 fixation effectiveness in a rhizobia–legume symbiosis, two components are crucial. The first is to have well defined genetic variants of a legume host that differ in their response to a rhizobial strain. The alfalfa relative, *M. truncatula* was chosen for this study because it has characteristics useful in studying the molecular genetics of the *Rhizobium*-legume symbiosis (Barker et al., 1990; Cook, 1999; Galibert et al., 2001). Im-

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portant characteristics include its small and diploid genome (500–600 Mbp), autogamous reproduction that leads to a high level of homozygosity; prolific seed production and rapid generation time (Cook, 1999). Populations of *M. truncatula* exhibit considerable phenotypic variation for features such as growth habit, flowering time, disease resistance and symbiotic specificity (Bonnin et al., 2001; Cook, 1999; Lie and Timmerman, 1983; Tirichine et al., 2000). Symbiotic polymorphisms in *M. truncatula* populations make a comparison of symbiosis between isolates a potentially important resource for studying the genetic basis of N_2 fixation effectiveness. Recombinant inbred lines of *M. truncatula* have been developed and partially mapped (Thoquet et al., 2002).

The second component needed to study N_2 fixation effectiveness is to have the ability to quantify the amount of N_2 fixation in different legume–rhizobia combinations. This can be complicated by the interaction of nitrogen metabolism with other parameters of plant growth (Danso, 1995). The most direct measure of nitrogen fixation's contribution to plant nutrition is to measure the amount of nitrogen actually coming from fixation using mass spectrometry but other parameters commonly used to estimate the degree of N_2 fixation have included nitrogenase activity, shoot fresh weight and nodule fresh weight. A mass spectrometry method developed in our laboratory (Kahn et al., 2002), measures the contribution that nitrogen fixation makes to the nitrogen composition of pheophytin, the Mg-free derivative of chlorophyll. Nitrogen in pheophytin is derived directly from glutamate and is therefore representative of the plant nitrogen pool composition at the time of pheophytin synthesis. Advantages of this assay are that sample preparation is rapid, it requires relatively little material and it integrates over a significant portion of the plant growth history.

The objective of this study was to implement a mass spectrometry method for measuring nitrogen isotope incorporation into pheophytin in order to estimate the effectiveness of different strains of *Sinorhizobium meliloti* in symbiosis with three lines of *M. truncatula* (Jemalong A17, DZA315.16 and F83005.5) that have been used as parental lines for the development of plant genetic tools. In doing this we needed to refine the plant growth methods of Kahn et al. (2002) to make the assay more reproducible. The study examines the effect of bacterial strain, plant line and the interaction of these two symbionts on plant characteristics associated with nitrogen fixation.

Materials and methods

Plant material and growth conditions

M. truncatula genotypes DZA315.16 and Jemalong A17 were obtained from Douglas Cook (UC Davis) and Thierry Huguet (Laboratory Biologie Moleculaire des Relations Plantes-Microorganisms, CNRS-INRA-France), respectively. Seeds were scarified in 5 volumes of concentrated sulfuric acid for 5 to 10 min, the seeds were rinsed in distilled water five times, treated with 5% [w/v] NaOCl for 3 min, then rinsed six to eight times in sterile deionized water. Seeds were soaked in sterile water at room temperature for 4–6 h, and then placed in the dark at 4 °C for 48 h in 3 volumes of standing sterile water to synchronize germination. Vernalized seeds were rinsed eight times with sterile water at room temperature then germinated in the dark at room temperature on 1% water agar plates for 36 h. Sterile seedlings were planted into 300 mL boxes containing autoclaved (121 °C for 60 min) LECA (Lightweight Expanded Clay Aggregate, Eco Enterprises, Shoreline, WA) and sand as support material. The initial experiments were performed in open 300 mL boxes and the procedure was later adapted to smaller 50 mL conical bottom test tubes.

Plants were fertilized at planting and at 13, 20, 27, 34, 41, 48 days after planting (dap) with sterile Gibson's nutrient solution (Gibson, 1980) which contained 1 mM urea 98% ^{15}N atom (Isotec Inc., Miamisburg, OH). Plant nutrient solution was buffered with 10 mM 2-[N-Morpholino]ethanesulfonic acid (MES) to reduce acidification resulting from N_2 fixation and urea metabolism. Fertilization and watering was performed using a 10 cc sterile syringe for each treatment to avoid cross contamination. To monitor contamination and establish non-symbiotic nitrogen use, each experiment included uninoculated controls.

All experiments were carried out in a growth chamber at 25 °C under continuous light provided by a combination of supersaver cool white F40CW/SS and Gro-Lux F40/GRO/AQ/WS (Sylvania, Canada) bulbs. Plants inoculated with wild type *S. meliloti* were harvested 55 dap. Tubes or boxes that contained plants inoculated with citrate synthase mutants were placed in a 17 °C water bath to control root temperature, and were fertilized three times, at planting and at 15 and 23 dap. These plants were harvested 37 dap to avoid overgrowth by faster growing *gltA+* revertants. Symbiotic performance was evaluated by measuring

pheophytin mass as an index of ^{14}N incorporation. Additional parameters that did not compromise the measurement of pheophytin mass, such as nodule fresh weight, leaf fresh weight, and nitrogenase activity, were also evaluated.

Plants were organized in a completely randomized design with a two way treatment structure (*S. meliloti* strain and *M. truncatula* line). Analysis of variance (anova) was performed using the General Linear Model procedure of SAS version 8.02 (SAS Institute Inc., Cary, NC, U.S.A.).

Inoculation with S. meliloti

S. meliloti strains USDA 1600 and 2011 were obtained from Thierry Huguet at INRA, France. Strain 102F51 was obtained from David Bezdicek in Crop Science at Washington State University. Several *S. meliloti* isolates have been previously found to differ in symbiotic performance on *M. truncatula* lines based on plant dry weight (T. Huguet, personal communication). From these isolates, *S. meliloti* strains USDA 1600, 2011 and 102F51 were selected for further analysis because of differences in shoot fresh weight among *M. truncatula* lines. *S. meliloti* USDA 1600 was isolated from *M. arborea* and it is ineffective with *M. sativa* (P. van Berkum, personal communication). *S. meliloti* 2011 is also of interest because of its popularity and its close relationship to the sequenced *S. meliloti* strain 1021 (Casse et al., 1979). *S. meliloti* USDA 1600 was further evaluated with *M. truncatula* lines Jemalong A17 and DZA315.16 in 50 mL plastic tubes instead of the 300 mL boxes – the smaller format was needed to do a large number of replicates in our facilities. Among the *S. meliloti* strains tested, we included a set of *S. meliloti gltA* mutant strains derived from the wild type strain Rm104A14 that had various levels of bacterial citrate synthase (CS) activity. *Medicago sativa* plants inoculated with CS mutants that had CS activities greater than about 7% of the wild type CS activity formed fully effective nodules, with nitrogenase activities comparable to the wild type; however in mutants with $\leq 3\%$ of the CS activity, fixation was significantly impaired (W. Grzemski, J. Akowski and M. L. Kahn, unpublished; Mortimer et al., 1999). To determine if this relatively sharp difference between bacterial mutants in symbiotic effectiveness might depend on the host plant, mutants with CS activity between 3% and 13% of wild type activity were tested for differential performance in nitrogen fixation on the *M. truncatula* lines. These strains were: wild type 104A14 (100% CS activity = 336.9 nmole min^{-1} mg $protein^{-1}$), MK504 (13.3% CS activity), MK506 (3.8% CS activity), and MK511 (2.8% CS activity).

Wild type *S. meliloti* strains were grown for 5 d with continuous shaking at 30 °C on yeast mannitol broth (Somerville and Kahn, 1983) and citrate synthase mutants were grown on minimal mannitol ammonia broth ($MMNH_4$) (Somerville and Kahn, 1983) supplemented with 0.25% arabinose. Cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C in a Sorvall SA600 rotor (Newton, CT), and then washed once with 0.85% NaCl under the same conditions. The pellet was resuspended in 1:3 (v/v) Gibson's nutrient solution and 1 mL of this suspension was added per box or tube.

Acetylene reduction assay

Nitrogenase activity was measured using the acetylene reduction assay (Hardy et al. 1968). Nodules were removed from rinsed roots, weighed and placed in a 10 mL glass vial. The vial was sealed with a rubber stopper and 1 mL of air was replaced with 1 mL of acetylene using a 1 cc syringe. 50-μL samples were taken 3, 6 and 9 min after acetylene addition and injected into a Shimadzu GC-8A gas chromatograph containing a Poropak N column and equipped with an SP4290 Integrator (Spectra Physics Inc., San Jose, CA). Ethylene gas standards were used for calibration.

Isotope dilution strategy

An isotope dilution strategy was used to measure the incorporation of nitrogen derived from symbiotic nitrogen fixation into pheophytin. The isotope labeling technique proposed by Vallis et al. (1967), using multiple additions of small amounts of highly labeled fertilizer, was implemented. Using this strategy is challenging because sufficient ^{15}N fertilizer needs to be present to allow ^{15}N labeling of pheophytin but, at the same time, fertilizer nitrogen must be limiting enough that it does not interfere with nodulation or nitrogen fixation (Streeter 1988). Ammonium, nitrate and urea were tested as sources of ^{15}N in preliminary experiments. Ammonium supported relatively poor plant growth with a shoot dry weight average of 12.7 mg/plant for inoculated plants 40 days after planting. Nitrate, which is known to have strong effects on nodule formation and senescence, gave inconsistent results (data not shown), perhaps by transiently interfering with nodule development and maintenance. The nitrogen source ultimately chosen was 1 mM ^{15}N-urea

(98% atom), which provides 2 mM ^{15}N. At this concentration there should be no inhibition of N_2 fixation or nodulation (Streeter, 1988).

Chlorophyll extraction and determination of nitrogen derived from fixation

All *M. truncatula* leaves present at the moment of harvest were removed from the stems and leaf fresh weight was measured before chlorophyll extraction. Leaf dry weight was not determined because drying the leaves will affect chlorophyll extraction. Preparation of samples for chlorophyll extraction, conversion to pheophytin and filtration has been described (Kahn et al., 2002). The filtered samples were directly analyzed using a Waters Alliance 2690 HPLC pump and autosampler interfaced to a Waters Micromass ZQ detector (Waters, Milford, MA) operating in electrospray, positive-ion mode. The parameters were optimized at 46 L h^{-1} cone gas flow, 284 L h^{-1} desolvation gas flow, a capillary voltage of 3000 V, cone voltage of 50 V and extractor voltage of 13.1 V. The pump and autosampler were used to inject 1 μL of each sample directly into the mass spectrometer, using a solvent of 10% v/v water in acetonitrile containing 0.2% v/v formic acid and a solvent flow rate of 0.5 mL min^{-1}. Run time under these conditions is about 30 sec.

A weighted average of the pheophytin peaks was calculated and is reported as a mean mass to charge ratio, $M_{pheophytin}$. Results of incorporation of ^{15}N into pheophytin were used to calculate nitrogen derived from fixation (Ndf) using the equation:

$$\% \text{ NDF} = \frac{M_{pheophytin}\ NI\ ^{15}N - M_{pheophytin}\ sample}{M_{pheophytin}\ NI\ ^{15}N - M_{pheophytin}\ NI\ ^{14}N} \times 100,$$

where '$M_{pheophytin}$ NI ^{15}N' is the incorporation of ^{15}N in non-inoculated plants growing in ^{15}N-urea. '$M_{pheophytin}$ NI ^{14}N' is the incorporation of ^{15}N in non-inoculated plants growing only in the presence of ^{14}N urea. '$M_{pheophytin}$ sample' is the incorporation in the inoculated sample grown in ^{15}N-urea as described above.

Results

Nitrogen derived from fixation was estimated from the relative incorporation of ^{14}N and^{15}N into pheophytin of inoculated and uninoculated plants. Uninoculated plants growing only in the presence of urea ^{14}N had a pheophytin mass of ~872.2 Daltons whereas uninoculated plants growing in the presence of ^{15}N–urea had a pheophytin mass of ~875.50 Daltons (Tables 2, 3, 5 and 6). We had previously shown using *Chlamydomonas* that full substitution of ^{15}N leads to a shift of 3.9 mass units as predicted and there were no significant isotope effects in pheophytin synthesis (Kahn et al., 2002).

Table 1. Analysis of Variance for parameters measured on three *M. truncatula* lines inoculated with *S. meliloti*[a]

Source of variance[b]	NFW		LFW		$M_{pheophytin}$	
	df	MS[c]	df	MS	df	MS
Line (L)	2	107.0*	2	4910.0**	2	2.09**
Strain (S)	5	641.9**	5	8776.2**	5	23.00**
L × S	9	179.9**	10	1043.5	10	0.70**
Mean Square Error	33.791		639.316		0.210	
Mean		12.2		61.5		873.8
SD		5.81		25.28		0.45
R^2		0.76		0.59		0.89

[a]Analysis of variance for Nodule Fresh Weight (NFW), Leaf Fresh Weight (LFW) and $M_{pheophytin}$ for three *M. truncatula* lines inoculated with three *S. meliloti* strains and non-inoculated controls.
[b]Standard Deviation (SD); R^2 = Coefficient of determination.
[c]Mean squares (MS) * F ratio significant at $P \leq 0.05$. ** F ratio significant at $P \leq 0.0001$.

Evaluation of S. meliloti *strains on* Medicago truncatula *lines*

The analysis of variance for the different parameters evaluated in *S. meliloti* strains USDA1600, 102F51 and 2011 is summarized in Table 1. The standard deviation for the pheophytin mass variable is small because the range of variation of this parameter is only 4 mass units. Measurements ranged from ~872.2 Daltons for uninoculated plants to a 'full substitution' of ~875.9 Daltons in uninoculated plants growing in the presence of ^{15}N–urea. The coefficient of determination (R^2) indicates that 89% of the variability was accounted for by the pheophytin mass (Table 1). Nodule fresh weight and pheophytin mass showed significant differences between *M. truncatula* lines, *S. meliloti* strains and the line X strain interaction. Nitrogenase activity (acetylene reduction) did not differ significantly for any of the sources of variation (data not shown). Based on the ANOVA results, means for nodule fresh weight and pheophytin mass were separated by Least Square Means at $P < 0.05$ (Table 2). The separation

Table 2. Differential symbiotic performance of *S. meliloti* strains in *M. truncatula* lines

Strain	Line	Means[a]				
		NFW		$M_{pheophytin}$		Ndf[b]
		(mg plant^{-1})	SD	(m/z)	SD	%
USDA 1600	Jemalong A17	14.1 bc	7.2	873.523 f	0.886	63
USDA 1600	DZA315.16	8.7 c	6.1	874.207 bcde	1.059	44
USDA 1600	F83005.5	15.6 bc	7.0	874.503 b	0.602	37
USDA 1600 + 4 mM[c]	Jemalong A17	22.6 b	6.2	873.788 def	0.352	56
USDA 1600 + 4 mM	DZA315.16	34.8 a	10.2	874.385 bcd	0.873	40
USDA 1600 + 4 mM	F83005.5	8.2 c	ND	875.733 a		ND
102F51	Jemalong A17	23.5 b	6.8	873.385 f	0.302	67
102F51	DZA315.16	9.5 c	6.5	874.449 bc	0.747	38
102F51	F83005.5	9.3 c	4.0	873.871 cdef	0.134	54
2011	Jemalong A17	14.3 bc	3.9	873.615 ef	0.173	61
2011	DZA315.16	10.2 c	7.1	873.485 f	0.450	65
2011	F83005.5	17.7 bc	6.4	873.394 f	0.361	67
NI ^{14}N	Jemalong A17	0		872.192 g	0.039	
NI ^{14}N	DZA315.16	0		872.205 g	0.030	
NI ^{14}N	F83005.5	0		872.214 g	0.05	
NI ^{15}N	Jemalong A17	0		875.806 a	0.053	
NI ^{15}N	DZA315.16	0		875.811 a	0.065	
NI ^{15}N	F83005.5	0		875.827 a	0.022	

[a]Mean separation by Least Square Means for Nodule Fresh Weight (NFW) and $M_{pheophytin}$ (expressed as mass charge (m/z) ratio) for three *M. truncatula* lines inoculated with three *S. meliloti* strains and two non-inoculated (NI) controls. Numbers represent the means of three repetitions. Means followed by a common letter are not significantly different for the strain × line interaction at $P < 0.05$. SD = Standard Deviation.
[b]Nitrogen derived from fixation (Ndf).
[c]Plants fertilized with 4 mM ^{15}N. ND = Not determined due to plant loss.

of means for nodule fresh weight did not include the uninoculated treatments.

Ideally, the limited nitrogen available from urea should not interfere with the need to obtain nitrogen from symbiosis. To determine if increasing the nitrogen concentration affected nitrogen fixation andmass of pheophytin, *M. truncatula* inoculated with *S. meliloti* USDA 1600 was grown in either 2 mM or 4 mM of ^{15}N. The difference among means for Jemalong A17 and DZA315.16 in pheophytin mass observed in USDA 1600-infected plants growing under 2 mM ^{15}N disappeared when the plants were grown under 4 mM ^{15}N (Table 2). In contrast, differences among *M. truncatula* lines in nodule fresh weight were seen when plants infected with USDA 1600 were grown in the presence of 4 mM ^{15}N. The 4 mM nitrogen treatment was also evaluated in uninoculated plants. No significant improvement in plant growth was observed, suggesting that N availability was not limiting growth (data not shown).

There were significant differences in pheophytin mass between Jemalong A17 and DZA315.16 when inoculated with *S. meliloti* strains USDA 1600 and 102F51 (Table 2). Since these differences between lines of *M. truncatula* were not seen when the plants were nodulated by strain 2011 or several other bacterial strains (Table 2 and data not shown), we conclude that the bacteria were responsible for the difference. Based on the pheophytin results, Jemalong A17 inoculated with USDA 1600 derived 63% of its nitrogen from fixation (Nitrogen derived from fixation, Ndf) compared to 44% for DZA315.16 and 37% for F83005.5. A similar difference in symbiotic performance was detected with *S. meliloti* 102F51, where Ndf was 67% with Jemalong A17, 38% with DZA315.16 and 54% with F83005.5. On the other hand, nodule fresh weight was significantly different only for the Jemalong A17 X 102F51 interaction (Table 2).

Analysis of variance for *S. meliloti* USDA 1600 tested on *M. truncatula* lines Jemalong A17 and

DZA315.16 growing in 50 mL tubes showed that nitrogenase activity and pheophytin mass were significantly different ($P \leq 0.0001$ and $P \leq 0.05$, respectively) for the line X strain interaction (data not shown). Mean separation using least square means for these two dependent variables is presented in Table 3. Means separation for nitrogenase activity did not include the uninoculated treatments. The effectiveness of USDA 1600 was also significantly different on Jemalong A17 and DZA315.16 when plants were grown in 50 mL tubes. Jemalong A17 received 95% of nitrogen from fixation compared to DZA315.16, which received only 69%. Nitrogenase activity was inversely correlated with the ^{15}N incorporation measurement – high nitrogenase activity corresponded to low ^{15}N incorporation as expected (Table 3). These results indicated that the observed differences are robust and that the line X strain interaction plays an important role in the effectiveness of nitrogen fixation (Table 3).

Evaluation of S. meliloti *citrate synthase mutants on parental lines of* M. truncatula

Results from the ANOVA analysis showed significant differences in line, strain and line X strain interaction for leaf fresh weight and pheophytin mass (Table 4). Nitrogenase activity showed significant differences only between *S. meliloti* strains. Based on these results, means were separated by the least square means at $P \leq 0.05$ for parameters showing significant differences for line X strain interaction (Table 5).

Results from the calculation of pheophytin nitrogen derived from fixation showed that, among CS mutants, MK504 had the highest level of nitrogen fixation followed by MK506 and MK511. Among the *M. truncatula* lines, fixation was greater in DZA315.16 than in Jemalong A17 and F83005.5 except for the plants nodulated by MK511 that showed equally low Ndf for Jemalong A17 and F83005.5. Mean separation by the least square means showed that the greatest difference in symbiotic performance was for MK506 inoculated on *M. truncatula* lines Jemalong A17 and DZA315.16. This result was supported by differences in leaf fresh weight between citrate synthase mutants MK504, MK506 and MK511 compared to the wild type strain 104A14, indicating some decrease in plant biomass due to reduced nitrogen fixation by the mutants (Table 5). Although nitrogenase activity did not show significant differences between line X strain interactions, the values are listed in Table 5 to illustrate that CS activity level of *S. meliloti* in symbiosis with *M. truncatula* showed the same trend as previously found in *Medicago sativa* (W. Grzemski, J. Akowski and M. L. Kahn, unpublished). CS mutant MK511 with 3% of normal CS activity formed nodules with greatly reduced nitrogenase activity.

S. meliloti MK506 was also tested to determine whether growth in 50 mL plastic tubes showed a similar difference in Ndf between plant hosts. Changing the format had the potential to affect fertilizer and watering conditions, which are critical to plant growth and labeling of the seedlings. The protocol for fertilization and watering followed in previous experiments was adapted and the results were similar to those for plants grown in boxes (Table 6). Results from the mass of pheophytin determined 70% of Ndf in DZA315.16 compared to 41% for Jemalong A17 when inoculated with MK506. There was a difference of 29% between the lines, the same difference observed in the previous experiment (Tables 5 and 6). High nitrogenase activity and nodule fresh weight are correlated with low pheophytin mass.

Discussion

In this study we surveyed various strains of *S. meliloti* to determine which of these strains differ in effectiveness on three standard *M. truncatula* lines, as measured by an isotope dilution assay. Two strains were identified that are more effective on Jemalong A17 than on DZA315.16 and a third that has the opposite discrimination. It was not particularly surprising to find strains that differ on the two hosts. A high level of polymorphism in nodulation and fixation was reported by Snyman and Strijdom (1980) in a study of the symbiotic characteristics of lines and cultivars of *M. truncatula* inoculated with *S. meliloti* strains of diverse origins. A similar study in seven natural Tunisian *M. truncatula* populations inoculated with *S. meliloti* and *S. medicae* strains showed a significant symbiotic polymorphism with the higher effectiveness for wild lines when compared to Jemalong, but often having the lowest efficient use of mineral nitrogen (Talbi et al., 2001). These studies evaluated *M. truncatula* lines and their associations with rhizobia strains in terms of nodulation, nitrogenase activity and plant biomass. These parameters are commonly used to estimate the effectiveness of rhizobia strains.

In this study, measurement of $M_{pheophytin}$ was used as an index for estimating nitrogen fixation effectiveness in *M. truncatula* and it was the only parameter

Table 3. Differential symbiotic performance of USDA 1600 in *M. truncatula* lines growing in 50 mL tubes

Strain	Line	Means[a]				
		Nitrogenase		$M_{pheophytin}$		Ndf[b]
		($nmol^{-1}$ mg^{-1} min^{-1})	SD	(m/z)	SD	%
USDA 1600	Jemalong A17	0.110 a	0.055	872.493 c	0.063	95
USDA 1600	DZA315.16	0.042 b	0.035	873.252 b	0.490	69
NI ^{14}N	Jemalong A17			872.320 c	0.132	
NI ^{14}N	DZA315.16			872.215 c	0.020	
NI ^{15}N	Jemalong A17			875.697 a	0.045	
NI ^{15}N	DZA315.16			875.593 a	0.100	

[a]Mean separation by Least Square Means for nitrogenase activity and $M_{pheophytin}$ (expressed as mass charge (m/z) ratio) for two *M. truncatula* lines inoculated with *S. meliloti* USDA1600 and two non-inoculated (NI) controls. Numbers are the means of six repetitions. Means followed by a common letter are not significantly different for the strain × line interaction at $P < 0.05$. Standard deviation (SD).
[b]Nitrogen derived from fixation (Ndf).

Table 4. Analysis of Variance for parameters measured on *M. truncatula* lines with *S. meliloti* citrate synthase mutants[a]

Source of variation[b]	NFW		LFW		Nitrogenase		$M_{pheophytin}$	
	df	MS[c]	df	MS	df	MS	df	MS
Line (L)	2	24.1**	2	5124.4***	2	0.13	2	0.26**
Strain (S)	5	274.9***	5	4954.3***	5	4.18***	5	13.08***
L × S	10	3.6	10	885.1***	10	0.11	10	0.12*
Mean Square Error	4.093		194.013		0.123		0.050	
Mean	6.0		53.3		0.9		873.6	
SD	2.02		13.93		1.08		0.22	
R^2	0.90		0.86		0.78		0.97	

[a]Analysis of variance for Nodule Fresh Weight (NFW), Leaf Fresh Weight (LFW), Nitrogenase activity and $M_{pheophytin}$ for three *M. truncatula* lines inoculated with four *S. meliloti* strains and two non-inoculated (NI) controls.
[b]Standard Deviation (SD); R^2 = Coefficient of determination.
[c]Mean Squares (MS) * F ratio significant at $P \leq 0.05$, ** $P \leq 0.01$ or *** $P \leq 0.001$.

that detected differences in all experiments for line X strain interactions. Isotope dilution measurements have the advantage of measuring nitrogen incorporation directly and of integrating over a time period – days to weeks – that is relevant to crop productivity. The accuracy of biological nitrogen fixation measurements using isotope dilution methods depends on several factors: the chemical composition of the labeled fertilizer, uniform distribution of ^{15}N and the rate of addition of nitrogen fertilizer (Chalk, 1985). It is important that nitrogen fixation is not stimulated or inhibited by changes in the concentrations of soil nitrogen coming from fertilization. It is also important that the rate of ^{15}N fertilizer application be high enough to allow isotope discrimination effects during N_2 fixation, ^{15}N uptake and metabolism to be ignored. For the experiments presented in this study, 2 mM ^{15}N from ^{15}N-urea 99 atom % was used. Development of a standard protocol was essential to measuring a relatively reproducible incorporation of ^{15}N into pheophytin.

One ultimate objective of this kind of study is to identify traits that correlate with effectiveness. For strains USDA 1600 and 102F51, it is unknown which bacterial genes might discriminate between the plant genes in Jemalong A17 and DZA315.16. On the other hand, differences in performance between *M. truncatula* lines interacting with *S. meliloti gltA* mutants was clearly related in some way to the level of CS in these bacteria. Alfalfa plants inoculated with CS mutants with activities greater than about 7% of the wild type activity formed fully effective nodules with nitroge-

Table 5. Symbiotic performance of *S. meliloti* citrate synthase mutants

Strain	Line	Means[a]					
		LFW		Nitrogenase[b]	$M_{pheophytin}$		Ndf[c]
		(mg plant^{-1})	SD		(m/z)	SD	%
104A14	Jemalong A17	106.5 ab	9.33	0.645	872.612 f	0.017	86
104A14	DZA315.16	64.4 de	18.54	0.870	872.571 fg	0.075	88
104A14	F83005.5	69.4 cd	37.79	0.866	872.754 ef	0.546	82
MK504	Jemalong A17	71.1 cd	4.49	1.745	873.105 de	0.156	69
MK504	DZA315.16	51.9 def	12.19	1.667	872.842 ef	0.117	78
MK504	F83005.5	35.1 fg	8.05	1.907	873.416 d	0.177	60
MK506	Jemalong A17	34.5 fg	2.90	0.215	874.276 c	0.232	30
MK506	DZA315.16	43.2 efg	6.25	0.406	873.390 d	0.215	60
MK506	F83005.5	28.7 g	7.18	0.479	873.935 c	0.517	43
MK511	Jemalong A17	37.6 fg	4.27	0.025	874.823 b	0.127	12
MK511	DZA315.16	31.3 fg	11.63	0.035	874.906 ab	0.329	8
MK511	F83005.5	20.5 g	4.21	ND[d]	874.859 b	0.077	13
NI ^{14}N	Jemalong A17	125.1 a	16.20		872.178 h	0.042	
NI ^{14}N	DZA315.16	92.2 bc	4.63		872.209 gh	0.050	
NI ^{14}N	F83005.5	34.0 fg	12.64		872.220 gh	0.032	
NI ^{15}N	Jemalong A17	43.1 efg	1.54		875.173 ab	0.028	
NI ^{15}N	DZA315.16	43.0 efg	23.46		875.143 ab	0.046	
NI ^{15}N	F83005.5	28.0 g	11.55		875.247 a	0.141	

[a]Means separation by Least Square Means for Leaf Fresh Weight (LFW) and $M_{pheophytin}$ (expressed as mass charge (m/z) ratio) for three *M. truncatula* lines inoculated with four *S. meliloti* strains and two non-inoculated (NI) controls. Numbers are the means of three repetitions. Means followed by a common letter are not significantly different for the strain × line interaction at $P < 0.05$. Standard deviation (SD).
[b]Nitrogenase activity is expressed in nmol (mg min)$^{-1}$.
[c]Nitrogen derived from fixation (Ndf).
[d]No determined (ND).

Table 6. Differential symbiotic performance of *S. meliloti* citrate synthase mutants in *M. truncatula* lines growing in 50 mL tubes

Strain	Line	Means[a]								
		NFW		LFW		Nitrogenase		$M_{pheophytin}$		Ndf[b]
		(mg plant^{-1})	SD	(mg plant^{-1})	SD	nmol (mgmin)$^{-1}$	SD	(m/z)	SD	%
104A14	Jemalong A17	11.8 a	3.14	119.9 a	26.65	0.253 b	0.185	873.242 a	0.204	66
104A14	DZA315.16	12.5 a	3.44	89.3 b	22.30	0.421 a	0.196	873.037 a	0.249	77
MK506	Jemalong A17	6.8 b	1.89	74.4 bc	18.34	0.204 b	0.096	874.004 b	0.383	41
MK506	DZA315.16	11.8 a	4.78	87.3 b	23.64	0.445 a	0.146	873.259 a	0.299	70
NI ^{14}N	Jemalong A17			60.1 c	8.99			872.229 c	0.019	
NI ^{14}N	DZA315.16			59.8 c	9.32			872.322 c	0.118	
NI ^{15}N	Jemalong A17			63.8 c	10.80			875.223 d	0.162	
NI ^{15}N	DZA315.16			53.3 c	8.95			875.493 d	0.191	

[a]Mean separation by Least Square Means for Nodule Fresh Weight (NFW), Leaf Fresh Weight (LFW), nitrogenase activity and $M_{pheophytin}$ (expressed as mass charge (m/z) ratio) for two *M. truncatula* lines inoculated with two *S. meliloti* strains and non-inoculated (NI) controls. Numbers are the means of twelve repetitions. Means followed by a common letter are not significantly different for the strain × line interaction at $P < 0.05$. Standard deviation (SD).
[b]Nitrogen derived from fixation (Ndf).

nase activities comparable to the wild type. Mutants with 3–4% of wild-type CS activity had about 20% of nitrogenase activity and low plant weight (W. Grzemski, J. Akowski and M. L. Kahn, unpublished). The same trend was observed in *M. truncatula* lines inoculated with *S. meliloti* CS mutants (Table 5). Although

the wild type Rm104A14 was equally effective on all hosts, the effect of lowering CS activity was more pronounced with Jemalong A17 than with DZA315.16. Further study of the interaction of CS mutants with different *M. truncatula* lines could be valuable because it may be possible to link changes in effectiveness to changes in the tricarboxylic acid (TCA) cycle metabolism or cellular polysaccharides (Mortimer et al., 1999). For example, alfalfa plants inoculated with temperature-sensitive CS mutants showed that continued CS activity was essential for nodule maintenance (W. Grzemski, J. Akowski and M. L. Kahn, unpublished).

The identification of three *S. meliloti* strains that vary in effectiveness among Jemalong A17, DZA315.16 and F83005.5 establishes the possibility of using these strains to investigate the contribution of the plant genes to effectiveness. Using the $^{14}N/^{15}N$ composition of pheophytin method we are attempting to measure effectiveness in a set of recombinant inbred lines derived from a cross between Jemalong A17 and DZA315.16 (Thoquet et al., 2002). This analysis will require determination of the mass of pheophytin for a very large number of samples and the pheophytin assay is simple enough to allow us to investigate questions that have previously been outside the scope of a reasonable research effort.

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