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HERITABILITY OF EFFECTIVENESS IN A NITROGEN FIXING SYMBIOSIS

ABSTRACT

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Symbiotic nitrogen fixation is a process in which soil bacteria, collectively called rhizobia, invade legume plants and differentiate into a form that can convert atmospheric N\textsubscript{2} into ammonium, a form that can be used by the plant. Legumes form symbiotic interactions with rhizobia by recognizing appropriate bacteria, developing a root nodule in response to bacterial signals and supporting the metabolism needed for bacterial nitrogen fixation. The plant-rhizobia interaction is very specific and any failure during this process can lead to an unproductive relationship. Symbiotic effectiveness has been experimentally defined as the amount of fixed nitrogen that is transferred to the plant. Differences in the complex interactions between the plant and the bacteria are suggested to be the main factors responsible for the variation of N\textsubscript{2} fixation in different plant-bacteria combinations. Considerable progress has been made in the identification of microbial genes that contribute to nitrogen fixation. In contrast, there is little information about the plant genes that contribute to more effective nitrogen fixation.

This study investigated the role of plant genes in the effectiveness of symbiotic nitrogen fixation. I describe a new mass spectrometry assay that measures the contribution that N\textsubscript{2} fixation makes to the nitrogen composition of pheophytin, the Mg-free derivative of chlorophyll. This method showed that nitrogen fixation can contribute substantial $^{14}$N to pheophytin isolated
from *Medicago truncatula* in symbiosis with *Sinorhizobium meliloti*. Using the mass spectrometry assay, several *S. meliloti* strains with differential nitrogen fixation effectiveness were identified on different lines of *M. truncatula*. These strains were used to study the segregation of symbiotic effectiveness in a *M. truncatula* recombinant inbred line population derived from a cross between Jemalong A17 and DZA315.16. Quantitative trait loci (QTL) analyses identify two QTLs for pheophytin mass in linkage group 3 and several QTLs for plant length in linkage groups 7 and 8. This is the first study that looks at the plant genes involved in the effectiveness of N$_2$ fixation using a novel mass spectrometry technique.
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DEDICATION

To my parents, Maximino and Marujita for their love, support and sacrifice.
CHAPTER ONE

Background Information

The ability to form a symbiotic relationship with nitrogen fixing rhizobia is common to 88% of legume species. Legumes used as crops benefit greatly from symbiotic nitrogen fixation, with a total amount of nitrogen fixed of the order of 100-250 kg N ha$^{-1}$. This accounts for between 45 and 80% of the total nitrogen accumulated by the plants, with the remainder coming from mineral nitrogen in the soil (Jensen, 1986). Agriculturally important legumes fix 40 to 60 million metric tons (Mt) of N$_2$ annually, with another 3 to 5 million Mt fixed by legumes in natural ecosystems (Smil, 1999). This is remarkable efficiency given the small quantities of nitrogenase involved. Replacing symbiotic N fixation with fertilizer N would cost $7 to 10 billion annually, whereas even modest use of alfalfa in rotation with corn could save farmers in the U.S. $200-300 million (Peterson et al., 1991). Furthermore, fertilizer N is frequently unavailable to subsistence farmers, leaving them dependent on N$_2$ fixation by legumes or other N$_2$-fixing organisms. (Graham et al., 2003).

Symbiotic N$_2$ fixation by legume-Rhizobium associations is the result of complex interactions between the host plant and the microsymbiont. Understanding the nature of specific genetic traits that affect symbiotic N$_2$ fixation will clarify important biological events of the symbiosis and this understanding may contribute to the agronomic management of N$_2$-fixing legumes.

At present, there is no information about how many genes influence the traits that allow more N$_2$ fixation in a bacterium x host combination. In this study we looked for plant genes that might contribute to differences in the effectiveness of N$_2$ fixation using an analysis based on
quantitative genetics. The development of molecular-marker information in *Medicago truncatula* helped identify of quantitative trait loci involved in nitrogen fixation effectiveness.

**Plant genes in N₂ fixation**

The final phenotype of symbiotic N₂ fixation in the *Rhizobium*-legume association is affected by many genetically controlled traits in legumes. It seems likely that large numbers of plant genes are potentially involved in determining traits such as effectiveness of N₂ fixation. These genes might include loci involved in chemical signaling (Schultze *et al.*, 1995), root nodule formation (Fearn *et al.*, 1991; Larue *et al.*, 1992) and/or physiological functions within the mature nodule (Egli *et al.*, 1989; Gantt *et al.*, 1992; Legal *et al.*, 1989; Markwei *et al.*, 1992).

In alfalfa, Barnes *et al.* (1984) has studied the interrelationships among plant morphological, plant physiological, and *Rhizobium* effectiveness traits. They found that plants with improved N₂ fixation capabilities often do not show increased plant growth, which suggests the possibility that these plants have limited ability to assimilate the increased amounts of fixed N₂. This result led them to investigate the nodule enzymes of nitrogen and carbon assimilation in the MnNC and MnPL alfalfa populations selected for high and low nitrogenase activity, as measured by the acetylene reduction assay. Glutamine-oxoglutarate aminotransferase (GOGAT) activity and phosphoenolpyruvate carboxylase (PEPC) activity were both correlated with nitrogenase activity in the MnNC population but not in the MnPL population. Pathirana *et al.* (1992) reported the maximum expression of alfalfa root nodule PEPC in two phases. The first phase coincides with nodule emergence from roots while the second phase is associated with nodule effectiveness. Ineffective nodules have substantially less PEPC mRNA, enzyme protein and activity than do effective nodules. Similar patterns of development have been reported for
glutamine synthetase (Egli et al., 1989) and aspartate aminotransferase (Gantt et al., 1992).

Several groups have shown that many enzymes of intermediary metabolism are induced in effective nodules, often as nodule-specific isozymes, and efforts have begun to engineer even higher levels of these enzymes (Vance, 1997; Verma, 1998).

Postma et al. (1988) reported that stimulated nodulation of pea mutant nod3 was associated with an altered root and shoot morphology and that these effects were pleiotropic. In supernodulating mutants of soybean, like nts382 (Carrol et al., 1985), similar pleiotropic effects on root and shoot growth were observed, indicating that in this class of mutants probably genes are mutated which are involved in the hormonal balance of the plant. Split root and grafting experiments indicated that signaling pathways involving the entire plant are altered in these plants (Olsson et al., 1989). Analysis of Medicago truncatula mutants is in the early stages but has already revealed interesting results. Grafting experiments with the M. truncatula sunn hyper-nodulated mutant demonstrated that the sunn phenotype is determined by the genotype of the shoot, implicating a mobile signal in conditioning nodule number (Penmetsa et al., 2003). Other studies with the M. truncatula sickle mutant support the hypothesis that ethylene provides a signal for induction of infection arrest (Penmetsa et al., 1997). The development of model legume systems has allowed work to progress toward understanding the Nod factor signal transduction pathway with the identification of five genes in M. truncatula that appear to have a role in this pathway. These mutants fall into five complementation groups. The first three groups designated dmi for doesn’t make infections are dmi1 dmi2 and dmi3; the fourth and fifth complementation groups designated nodulation signaling pathway (nsp) are nsp1 (Catoira et al., 2000; Wais et al., 2000) and nsp2 (Oldroyd et al., 2003). Studies with these mutants can give us
a better understanding of the mechanisms of Nod factor perception and signal transduction in the plant.

Pea mutants that are defective in nodulation have been used to characterize the symbiosis (sym) genes of the host plant (Kneen et al., 1984; Kneen et al., 1990; Kneen et al., 1987). Most of these mutants have few or no phenotypic effects except for the abnormal nodulation. Nearly all the described nodulation mutants in pea have been demonstrated to display monogenic inheritance (Kneen et al., 1994); except for the naturally occurring sym-2 (Young, 1985). Kneen et al. (1994) suggested that nodule-specific sym genes would have evolved specialized controls appropriate to infection by rhizobia. But other sym genes, presumably existing before the symbiosis evolved, may still play a role in other tissues. This model confirms the fact that sym genes are not clustered in the host’s genome (Kneen et al., 1994; Weeden et al., 1998).

**Legume × Rhizobium interactions**

$N_2$ fixation by the *Rhizobium*-legume symbiosis is influenced by the genotype of both the bacterium and the plant. When plant cultivars and bacterial strains are tested in different combinations, no one cultivar or strain is always associated with the greatest $N_2$ fixation. This indicates that the amount of nitrogen fixed is greatly affected by the legume × *Rhizobium* interaction. Numerous attempts have been made to increase $N_2$ fixation by genetic improvement of either *Rhizobium* or plant. The major obstacle that prevents the successful introduction and use of genetically improved *Rhizobium* strains is the presence of competitive indigenous rhizobia in legume production areas. Although it is important from a basic viewpoint to understand how changing *Rhizobium* strains can increase $N_2$ fixation, there is little justification for claiming practical advantages will result from such efforts (Phillips et al., 1985b). Attempts to increase $N_2$
fixation by genetically modifying the host legume include using plant genes that prevent nodule development by all but the desired strain (Devine et al., 1984) or selecting for plants that have increased N₂ fixation by any effective rhizobia. Studies with varieties of white clover (Trifolium repens L.) that differed in their preference for strains of Rhizobium trifolii present in a mixed inoculum showed that this preference was a heritable characteristic of the legume host. It was concluded from these results that it might be possible to improve the legume-rhizobia symbiosis by breeding for host genotypes that preferentially nodulate with particularly effective strains of Rhizobium (Hardarson et al., 1979; Jones et al., 1979). It has been argued that the best method for increasing N₂ fixation involves the simultaneous selection of both plant and bacterial genotypes (Mytton et al., 1984). Although that approach may be theoretically correct, it appears pragmatically weak because the tools are not presently available to manage selected bacterial strains in agricultural soils already containing rhizobia (Phillips et al., 1985a). These problems could be avoided if plant genes, or gene combinations, could be found which conferred good phenotypic expression of nitrogen fixation over a wide range of Rhizobium genotypes.

Studies considering the question whether the Rhizobium strains or plant cultivar has the greater genetic variation for traits influencing N₂ fixation found that both plant cultivars and bacterial strains had significant impact on N₂ fixation (Mytton et al., 1977; Phillips et al., 1985b; Tan, 1981). Studies in Vicia faba (Hobbs et al., 1983; Mytton et al., 1977), Pisum sativum L. (Hobbs et al., 1983), Phaseolus vulgaris L. (Rennie et al., 1983), Trifolium incarnatum L. (Smith et al., 1984), Trifolium pratense L. (Nutman, 1984) Medicago sativa (Barnes et al., 1984; Mytton et al., 1984), and Lupinus albus L. (Robinson et al., 2000) showed that the interaction between plant and Rhizobium was responsible for a large part of the variation. These studies suggest that symbiotic N₂ fixation can be significantly greater when the most effective
Rhizobium infects the roots of a plant. However, the selection of which strains of bacteria are optimal depends strongly on plant genotype.

**How to measure effectiveness**

The increased nitrogen input from the nodule to the host plant determines the effectiveness of N\textsubscript{2} fixation. Accurate measures of biological nitrogen fixation (BNF) are important as a prerequisite in determining heritability of effectiveness in *Medicago* and pea (*Pisum sativum* L.). Several techniques have been used to measure BNF and the most suitable methods are those that can distinguish between the amounts of plant nitrogen derived from atmospheric N\textsubscript{2} fixation, distinct from the nitrogen contribution from the soil and from fertilizer applied nitrogen. The most commonly used methods are: the total nitrogen difference (TND) method, acetylene reduction assay (ARA) method, H\textsubscript{2} evolution technique, xylem-solute (or ureide production) method and methods based on the use of \textsuperscript{15}N-labeled compounds.

**The TND method** measures BNF as the difference between the total nitrogen content of plants that fix N\textsubscript{2} and those that do not derive nitrogen from fixation. This method is based on the assumption that both the N\textsubscript{2} fixing and non-fixing control plants absorb equal amounts of soil nitrogen for growth (Rennie *et al.*, 1983). The fulfillment of this assumption is therefore the greatest limitation of the TND method (Danso, 1995). However, the TND method will often give reliable estimates of N\textsubscript{2} fixed in plants grown in soils or systems in which the initial nitrogen content is low (Danso *et al.*, 1988; Patterson *et al.*, 1983; Rennie, 1984).

**The ARA technique** measures nitrogenase activity. Nitrogenase, the central enzyme involved in N\textsubscript{2} fixation, is able to catalyze the reduction of acetylene to ethylene. The assay is fast, simple, and sensitive, but it has some serious problems. Nitrogenase activity is inhibited
within 8 minutes of exposure to acetylene and reaches less than 50% of its initial activity within 30 min (Minchin et al., 1983). Any disturbance in the N₂-fixing system induces an increased resistance to the flow of oxygen into nodules which affects the rate of acetylene conversion into ethylene (Ralston et al., 1982; Sung et al., 1991). Repeated assays on the same plant material result in progressively lower estimates of nitrogenase activity (Minchin et al., 1986; Rosendahl et al., 1988). The ARA technique is a short-term assay, in contrast to the process it is intended to measure, BNF, which integrates nitrogen fixation over the life of the plant. Therefore, ARA measurements have to be extrapolated to cover several periods over which no measurements were made, a difficult correction because rates of N₂ fixation exhibit very wide diurnal (Ayanaba et al., 1977; Rainbird et al., 1983) and seasonal (Zapata et al., 1987) variations. In addition, ARA measures only the early steps of the nitrogenase reaction. Electrons able to reduce acetylene might not be able to reduce nitrogen under the same circumstances. The conversion factor linking ARA to ammonia production can vary by a factor of two or more (Hardy et al., 1968). A prior calibration (eg, by using ¹⁵N gas as a standard) needs to be done to know the correct conversion ratio to be used. Even for the same Rhizobium-plant symbiosis, the conversion factor does not always remain the same; it has been reported to vary under different environmental conditions (Witty et al., 1988).

A related technique used to measure nitrogenase activity is by monitoring H₂ evolution from nodules. Rhizobium and Bradyrhizobium strains in leguminous root nodules can contain two enzyme systems involved in H₂ metabolism. Nitrogenase is always present and simultaneously reduces N₂ to NH₃ and protons to H₂. A H₂ uptake (Hup) system that oxidizes H₂ to water is common in some, but not all symbioses. Possible advantages of H₂ oxidation in symbiotic rhizobia include protection of nitrogenase from O₂ and H₂, as well as the production of
ATP and reductant. Monitoring H₂ evolution rate from nodules in air provides only a measurement of apparent nitrogenase activity (ANA) because a proportion of the total electron flux through nitrogenase is used for N₂ reduction. By shifting the gas composition surrounding the nodules to an atmosphere lacking N₂, analysis of H₂ production provides measurements of total nitrogenase activity (TNA) (Hunt et al., 1987; Hunt et al., 1989), electron allocation coefficient of nitrogenase (Edie et al., 1983; Hunt et al., 1987) and N₂ fixation rate. Measurements of ANA, and short-term measurements of TNA, do not inhibit nitrogenase activity, therefore, measurements can be performed either continuously or intermittently, over virtually any experimental period (Hunt et al., 1993). However, there are disadvantages associated with the H₂ evolution assay. The presence of an uptake hydrogenase enzyme (HUP) in certain legume symbioses recycles some or all of the H₂ produced by nitrogenase (Evans et al., 1987; Godfroy et al., 1991). The H₂ analyzer is also very sensitive to water vapor and time-consuming to calibrate and can not be used for field studies due to H₂ oxidation by bacteria and abiotic factors that may be present in the soil (Schuler et al., 1991).

The xylem-solute technique can be used to measure BNF for those species that produce significant quantities of ureides as the product of BNF. The nitrogen fixed in the nodule is exported via the xylem stream to the shoot either as amides (predominantly asparagine and glutamine) or as the ureides, allantoin and allantoic acid (Pate et al., 1983). However, production of ureides is restricted to some tropical legumes species (Peoples et al., 1989) and cannot be used in the analysis of temperate legumes. Ureide-exporting species have low nitrate reductase activity in their roots (Atkins et al., 1980), much of the absorbed nitrate is transported to the shoot unchanged. Therefore, the composition of the nitrogen compounds in the xylem of nodulated ureide-producing plants changes from one dominated by nitrate and amino compounds
to one dominated by ureides as the plant’s dependence on N₂ fixation increases (Herridge, 1982). The xylem exudate method is simple and inexpensive, and has great potential in the screening of large germplasm collections for relative N₂ fixation ability. The most serious limitation is that only a small proportion of known N₂-fixing plants are ureide exporters (Kessel et al., 1988).

The ¹⁵N labeling methodologies can be classified in three techniques: Use of ¹⁵N₂-labelled gas, the isotope dilution method and the A-value. The common principle behind these three methods is to grow plants in soil containing a different ¹⁵N/¹⁴N ratio than the atmosphere and measuring the incorporation of atmospheric N by determining the resulting ratio of isotopes in plant material (Danso, 1995). The ¹⁵N-dilution method measures the isotopic dilution in a fixing plant growing on ¹⁵N-enriched soil due to the fixation of unlabelled atmospheric N₂, which has an almost constant ¹⁵N/¹⁴N ratio of 0.3663%. ¹⁵N-labeled fertilizer is applied to the soil to amplify differences in ¹⁵N abundance between the soil nitrogen and atmospheric N₂ (Bergersen et al., 1983; Boddey, 1987; Chalk, 1985). The accuracy of BNF measurements using the isotope dilution method depends on four aspects:

1. Fixing and reference plants should assimilate the same ratio of labeled-to-unlabelled nitrogen from the soil. This ratio can change rapidly as soon as inorganic ¹⁵N is applied. In addition, differences in nitrogen uptake pattern and rooting volume after nodulation can cause deviations in the N taken up from the soil and, consequently, the estimation of plant nitrogen derived from fixation using a reference plant can be misleading (Hamilton et al., 1992).

2. The rate of addition of nitrogen fertilizer is important because combined nitrogen has direct effects on biological N₂ fixation and plant growth, and indirect effects on soil cycling processes. It is also important that the rate of ¹⁵N application should be high enough to allow isotopic discrimination effects, either during N₂ fixation or ¹⁵N assimilation, to be ignored.
The best way to achieve these two objectives is by multiple additions of small concentrations of highly labeled fertilizer to the soil (Chalk, 1985).

3. The chemical composition of the labeled fertilizer will influence the utilization of the isotope by fixing and reference plants. Studies in the absence of plant growth showed that the concentration of $^{15}$N in the available nitrogen pool decreased more rapidly when ($^{15}$NH$_4$)$_2$SO$_4$ was added than when the same concentration of Ca ($^{15}$NO$_3$)$_2$ was added. Vallis et al. (1967) stated that NH$_4^+$ is probably better than NO$_3^-$ when the aim is to have the tracer incorporated in the internal soil nitrogen cycle whereas NO$_3^-$ is better when the objective is to minimize microbial transformations before uptake. Movement of NH$_4^+$ in soil is restricted compared to NO$_3^-$, because NH$_4^+$ is held by the net negative charge in surface soil. There is evidence that NH$_4^+$ has less of an inhibitory effect on legume–rhizobia symbioses than NO$_3^-$ (Streeter, 1988). At similar concentrations of NO$_3^-$ and NH$_4^+$, the inhibitory effects can be much more severe with NO$_3^-$ compared to NH$_4^+$ (Svenning et al., 1996; Waterer et al., 1993a, b). A few studies indicate a more inhibitory effect of NH$_4^+$ on nodulation compared to NO$_3^-$ (Guo et al., 1992).

4. The distribution of $^{15}$N should be as uniform as possible. Significant errors can arise from a bias in $^{15}$N distribution that is concurrent with a non-random distribution of microbial processes (Davidson et al., 1991) or roots sampling different parts of a heterogeneous environment.

Although isotope dilution methods have some limitations, they are widely applicable and when correctly applied can provide a powerful tool for directly measuring nitrogen fixation.
Quantitative Trait Loci (QTLs)

Quantitative trait loci (QTLs) are specific regions in the genome associated with quantitative traits. The observed variation of QTLs is due to the segregation of several to many naturally occurring polymorphic genes. It is difficult to identify these genes because the individual effects of each of these genes on phenotype are relatively small. The genes that contribute to these complex phenotypes will usually be linked only in the physiological, but not the genetic sense (Kearsey et al., 1996). Molecular marker maps can help resolve these complex characters into their contributing quantitative trait loci. Mapping QTLs has become a reality in the past 15 years, primarily because of the availability of molecular markers.

Morphological markers, protein-based markers and DNA-based markers are the three types of genetic markers used in genomic analysis. To be a genetic marker, the marker locus has to show experimentally detectable variation among the individuals in the test population. The variation can be considered at different biological levels, from the simple heritable phenotype to detection of variation at the single nucleotide. The first molecular markers used were allozymes, protein variants detected by differences in migration on starch gels in an electric field. However, allozymes have insufficient protein variation for high-resolution mapping and therefore they were rapidly replaced by DNA-based markers that became available in the mid–1980s. Molecular markers have important properties that differentiate them from morphological markers:

1. Phenotypic neutrality. Molecular markers reveal neutral sites of variation at the DNA sequence level. Alternate alleles at molecular marker loci usually cause no obvious changes in the phenotype of the organism (Jones et al., 1997; Tanksley, 1993).
2. The level of polymorphism at any given locus in natural populations is determined by many factors, including population size, selection, mutation rate, mating habit, and migration. Two of these factors, relaxed selection pressure and higher mutation rates, cause allelic variation to be higher at molecular marker loci than at morphological marker loci (Tanksley, 1993).

3. Abundance in molecular markers scattered throughout an entire genome allows the detection and characterization of QTLs affecting a quantitative inherited character (Tanksley, 1993). Today, molecular linkage maps covering the entire genome are available for quantitative-trait studies in corn (Coe et al., 1990), tomato and potato (Tanksley et al., 1992), Arabidopsis thaliana (Reiter et al., 1992), common bean (Nodari et al., 1993; Vallejos et al., 1992), rice (Wu et al., 1995; Yano et al., 1997), and Medicago truncatula (Thoquet et al., 2002).

4. Whereas most morphological markers segregate dominant-recessive alleles, most molecular markers are codominant. For loci with codominant alleles there is a one to one relationship between genotype and phenotype, where all possible genotypes can be deduced directly from the phenotype at any generation (Tanksley, 1993).

5. Epistasis is a form of interaction between nonallelic genes whereby one gene interferes with the phenotype expression of another gene. Molecular marker loci do not normally exhibit epistatic or pleiotropic effects. A virtually limitless number of segregating markers can be used in a single population for mapping QTLs through an entire genome (Kearsey et al., 1996).
Various new molecular marker techniques have been designed, resulting in an increasing number of markers for agronomically important traits that can be applied to nearly all crops. A brief description of the most common markers used in genetic mapping follows.

**Restriction Fragment Length Polymorphism (RFLP).** This technology uses restriction enzymes to cut DNA into small fragments of differing lengths. The polymorphism is identified using the same RFLP probe to compare two or more individuals. Each RFLP probe generally scores a single-marker locus, and the marker alleles are codominant, as heterozygotes and homozygotes can be distinguished. This information is highly desirable, especially for recessive traits. Conventional RFLP analysis is limited by the relatively large amount of DNA required for restriction digestion, Southern blotting and hybridization plus the requirement for radioactive isotopes and autoradiography. These factors make conventional RFLP analysis relatively slow and expensive (Kochert, 1994; Walton, 1993).

**Random Amplified Polymorphic DNAs (RAPDs).** Sequence polymorphisms are detected by using random short sequences as primers. This approach produces one to 10 fragments from a single primer PCR reaction (Winter et al., 1995) which are highly polymorphic. RAPDs will be generated if either one or two copies of an allele are present so they behave as dominant markers, indicating the presence or absence of a particular allele rather than tracking both genes at a locus. (Ragot et al., 1993) conclude that RAPDs are generally more time- and cost-effective in small studies where a modest number of individuals need to have their genotypes determined, while RFLPs are better for larger studies. Related techniques like, AP-PCR (arbitrary primed PCR) and DAF (DNA amplification fingerprinting) differ from RAPDs principally in primer length, primer to template ratio, the gel matrix used and the visualization procedure (Karp et al., 1996).
Amplified Fragment Length Polymorphism (AFLP). This method combines the use of restriction enzymes with PCR amplification of fragments. Polymorphisms are detected by the differences in length of the PCR amplified restriction fragment. The first step in the generation of AFLPs is to double-digest genomic DNA with two restriction enzymes: a rare cutter is used to create a bias towards low-copy fragments, and a frequent cutter. Next, adapters are ligated to the ends of the genomic DNA at the specific restriction sites. This DNA is then used as a template for PCR reactions. The primers are specific to the combination of adapter sequence, restriction enzyme and several selective nucleotides extensions. By changing the selective nucleotides, different subsets of fragments will be amplified. The AFLP system is technically difficult and expensive to set up, but it detects a large number of loci and reveals a great deal of polymorphism. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels (Jones et al., 1997; Liu, 1998; Vos et al., 1995).

Minisatellites, Microsatellites, and Dispersed Repetitive DNA. These techniques use hypervariable regions of the genome consisting of tandemly repeated simple sequences. These markers are called micro- or minisatellites or simple tandem repeats (STR), because their sequence organization resembles the tandem arrangement of classical satellite DNA. These sequences are dispersed in the eukaryotic genome in many copies of varying repeat-unit numbers. Minisatellites are repeat units of nine to 20 nucleotides. They can be hybridized to restricted and electrophoretically separated DNA blotted onto nylon membranes (Jeffreys et al., 1985). Microsatellites, also called simple sequence repeat (SSR) DNA, are repeat units of one to five nucleotides. The variation in size between these is based on differences in the number of repeats. Microsatellites can be hybridized to DNA in dried gels or they can be cloned, sequenced, and AFLP-detected by PCR, using oligonucleotides from the surrounding monomorphic DNA as
primers (Epplen, 1988; Litt et al., 1989). Microsatellite sites are codominant markers and therefore highly informative (Beckmann et al., 1990). However, they are expensive to establish, they have a long development time and they need specific primers (Jones et al., 1997).

Unmapped markers such as Inter-Simple Sequence Repeats (ISSRs) are generated from PCR primers that consist of a core sequence of repeated di-, tri-, tetra- or pentanucleotide motifs. The addition of a different base at the 5’- or 3’-end renders their binding sites more specific and reproducible. The sequence between two binding sites in opposite orientation within suitable distance is amplified by these primers and a loss or gain of binding sites within this region is detected as band polymorphism (Barth et al., 2002; Yang et al., 1996).

**STS, EST and CAPS.** Sequence tagged sites (STS) are short unique fragments of DNA (~300 bp). They were proposed by Olson et al. (1989) as chromosome landmarks in the human genome. An STS is a short unique fragment of DNA (~300 bp). Clones containing the same STS must overlap, so STSs are primarily used in physical mapping to join large cloned fragments. If a polymorphism can be detected using STS as probe, then anchor points between genetic and physical maps can be established (Liu, 1998). Expressed sequence tags (ESTs) are subsets of STSs derived from cDNA clones. ESTs have the advantages of representing real functional genes and are therefore more useful as genetic markers than anonymous nonfunctional sequences.

The most precise and informative molecular data on diversity are obtained from sequencing known targets in the genome. Sufficient sequence information must be known initially in order to design primer pairs that will lead to amplification of only the target sequence. The amplified product can be compared on an agarose gel to the corresponding product from another individual, but only those differences in length that result from many base pair changes
will be detected. In one of these techniques called Cleaved Amplified Polymorphic Sequence (CAPS), the amplified product is digested with a specific restriction enzyme and the products directly visualized on the agarose gel (Akopyanz et al., 1992). This simple approach is most informative when the restriction sites are mapped, rather than simply detected as RFLPs (Karp et al., 1996). In comparison with ISSRs, CAPS are more expensive to develop and apply; because specific primers for each gene have to be synthesized and expensive restriction enzymes are needed (Barth et al., 2002).

Genomic mapping data can have mistakes incorporated at any stage of the mapping process. These stages include DNA preparation, running gels and capturing gel images, scoring systems and data manipulation. A DNA marker or a single data point may give unexpected results in linkage map construction. These errors may damage the linkage map construction and the applications of the genomic information.

**Genetic map construction**

A genetic map is an abstract model of the linear arrangement of a group of genes and markers. The first step is a pairwise linkage analysis for all possible two-locus combinations. The biological foundation for linkage analysis is the homologous recombination between non-sister chromatids during meiosis. Linkage analysis for a two-locus combination is based on comparison of observed and expected frequencies of the possible genotypic classes. The number of possible genotypic classes is a function of the number of alleles at the two loci under consideration and of the reference population used. The second step is to group the marker into different linkage groups. Recombination fraction, significance level of the recombination fraction and sometimes previously established genomic information, such as number of
chromosomes, are criteria used for linkage grouping. The third step is to determine the relative position of the markers in the same linkage group. This is the key step for a high quality genetic map. Sampling, inadequate criteria and inadequate algorithms are sources of error in determining locus order. Likelihood approaches and nonparametric approaches such as bootstrap and jackknife are used for measuring the confidence of locus ordering. The last step in the genetic map construction is to estimate the multipoint recombination fractions among the adjacent loci. The multi-point map distance is based on a statistical estimate of crossover and has more in common with recombination fraction than with the physical distance. In practical genetic mapping, map distance has been estimated by converting recombination fraction to map distance using mapping functions. The most commonly used map functions are Haldane’s and Kosambi’s. The former ignores crossover interference but, when the recombination fraction is small, the map distances using Haldane’s mapping function and recombination fraction are almost equal. As the size of the fragment increases, the number of multiple-crossovers does not increase as fast as assumed in generating the Haldane function and the map distance must be adjusted to account for fewer multiple-crossovers. Kosambi’s map function considers crossover interference in a way that depends on the size of the genome segment. The interference is absent when a segment is sufficiently large. The interference increases as the segment decreases (Liu, 1998).

Once a genetic map has been constructed, QTL mapping can begin. QTL mapping tries to associate molecular markers with QTLs, and thereby to characterize these loci by determining their map locations. A large number of experimental designs and statistical methods have been developed to exploit this information. The experimental design depends on the type of line-cross population used for generating disequilibrium (e.g., RILs) and the unit of marker analysis used (e.g., single markers vs. interval mapping).
Experimental designs based on reference population: Many types of genetic reference populations can be employed for QTL mapping. Reference populations may include F2, backcross (BC), recombinant inbred lines (RIL), and double haploid (DH), open pollinated (OP) or combined related half-sibs families. RILs are the reference population used in our experiments. RILs are used when asexual reproduction is not possible. They are constructed by subjecting an F1 plant to multiple rounds of selfing or multiple generations of brother-sister mating. Producing RILs as inbred lines descended from single-seed crosses without selection to generation $\geq$F6 allows recombination and segregation to occur each generation and a rapid approach to homozygosity. Ideally, RILs are homozygous at nearly all loci, so there is very little power to detect or estimate dominance. RILs have essentially no within-line genetic variance (ignoring new mutations and small amounts of residual heterozygosity), which makes it possible to share RILs as approximately true-breeding isolates. The genetic variance between lines is considerable, as each RIL represents a different multilocus genotype (Lynch et al., 1998). Precise location of both marker loci and QTL depends upon the number of recombinations that occur between genes. Thus RILs allow high resolution mapping of QTLs because, if there are sufficient number of lines, there are likely to be gene combinations able to distinguish QTL map positions. Once the work to generate and molecularly characterize a set of RILs has been done, any character can be examined, and the previous marker information used to look for marker-trait associations. Markers in genetic reference populations have a certain expectation of segregation ratios in terms of markers (i.e, F2, 1:2:1, AA:Aa:aa; BC, 1:1, Aa:aa; RIL, 1:1, AA:aa, and DH,1:1, AA:aa). While the linkage map is constructed based on the marker data, the reference populations are simultaneously characterized for target traits (e.g., disease resistance, drought stresses, N$_2$ fixation effectiveness, etc.) (Liu, 1998).
Experimental designs based on the unit of marker analysis: Marker-trait associations can be assessed using one-, two-, or multiple-locus marker genotypes. Under a single-marker analysis, the distribution of trait values is examined separately for each marker locus. Each marker-trait association test is performed independent of information from all other markers, so that a chromosome with \( n \) markers can be subjected to \( n \) separate single-marker tests. Single-marker analysis is a good choice when the goal is only to detect a QTL linked to a marker, rather than to estimate the QTL's position and effects. Another method proposed by Lander & Botstein (1986; 1989) called interval mapping, analyzes joint frequencies of a pair of adjacent markers and a putative QTL flanked by the two markers. By using a pair of adjacent markers for analysis, it is possible to compensate for recombination between the markers and the QTL, increasing the probability of statistically detecting the QTL. Interval mapping offers a further increase in the power of QTL detection and estimates QTL effects and position more precisely. Interval analysis has been used successfully for several quantitative trait linkage studies (Doebley et al., 1991; Patterson et al., 1983; Stuber et al., 1992). Both single-marker and interval mapping approaches are biased when multiple QTLs are linked to the marker/interval being considered. Methods simultaneously using three or more marker loci attempt to reduce that bias. Composite interval mapping (CIM) is a combination of interval mapping and multiple linear regression (Zeng, 1993, 1994). CIM considers a marker interval plus a few other well-chosen single markers in each analysis, so that \( n – 1 \) tests for interval-trait associations are performed on a chromosome with \( n \) markers. One of the advantages of CIM mapping is that the markers can be used as boundary points to constrain the most likely QTL position and thus the resolution of QTL location determination can be increased greatly. Another method that considers multiple markers for analysis is called multipoint mapping. This method considers all of the linked
markers on a chromosome simultaneously, resulting in a single analysis for each chromosome (Kearsey et al., 1994).

Several factors limit the estimation of the number of genes affecting quantitative traits, such as population size, the statistical threshold for detecting putative loci, the number of molecular markers used in the analyses and the heritability of target traits. Regarding population size, a fairly large segregating population (i.e. >100 individuals) is required to detect QTLs. Although large populations are necessary to detect loci with minor effects, increasing population size above 500 plants or lines does not seem to be practical in this type of analysis (Yano et al., 1997). Another important factor that limits the detection of QTLs is the threshold employed for inferring that a QTL is statistically significant. The estimation of a significance threshold is based upon the simple observation of marker-phenotype association. It can be applied to single marker, interval, composite or multiple interval mapping approaches using any test statistic with the power to detect associations (Churchill et al., 1994). Here, one randomly shuffles the observed trait values over individuals (marker genotypes), generating a sample with the original marker information but with trait values randomly assigned over genotypes. The test statistic is then computed on this new sample, and this procedure is repeated many times, generating an empirical distribution of the test, under the hypothesis of no marker-trait associations (Lynch et al., 1998). In order to have a significance level of 5 percent it is suggested that a thousand resamplings be used. To generate a critical value of 1 percent, 10,000 or more resamplings are necessary. By keeping the marker information for each individual together, this approach nicely accounts for missing markers, differences in marker density, and any random segregation of marker alleles (Doerge et al., 1996).
Several software packages for QTL mapping are available from the public domain and can be downloaded from the Internet (Table 1). An exception is MapQTL, which is only available commercially. The different software packages have similar methods to estimate linkage and recombination fraction. For the same data set, all the packages should yield the same pairwise two-point recombination fraction matrix. The differences among these packages concern data format, computer platforms, user interface, graphic output, population types, algorithms for locus ordering and algorithms for multi-locus model building (Table 1). Statistical models that can be built in these software packages are limited and cannot handle data with complex experimental designs. Compared to commercial software, the interfaces are not very user friendly and user support is limited due to their non-commercial status. Knowledge of the location of the QTLs for a trait might open various opportunities to distinguish between the effects of linkage and pleiotropy and to improve selection efficiency. It would also be possible to look at the additive and dominance effects of individual loci and to analyze interactions between genes. This would depend on being able to identify recognizable genes or marker loci closely linked to the QTL, which could be used to tag the presence of the QTL.
Table 1. Some software packages for QTL mapping.

<table>
<thead>
<tr>
<th>Program</th>
<th>Characteristic</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Map Manager QTX</td>
<td>Function</td>
<td>Interval Mapping, Multiple-QTL Modeling (MQM), nonparametric mapping</td>
</tr>
<tr>
<td></td>
<td>Population type</td>
<td>F2, backcross</td>
</tr>
<tr>
<td></td>
<td>Computer Platform</td>
<td>Macintosh and PC</td>
</tr>
<tr>
<td></td>
<td>Contact</td>
<td><a href="http://mapmgr.roswellpark.org/mapmgr.html">http://mapmgr.roswellpark.org/mapmgr.html</a></td>
</tr>
<tr>
<td>QTL Cartographer</td>
<td>Function</td>
<td>Single Marker, Interval and Composite Interval Mapping</td>
</tr>
<tr>
<td></td>
<td>Population type</td>
<td>F2, backcross, RIL, DH</td>
</tr>
<tr>
<td></td>
<td>Computer Platform</td>
<td>Unix, Macintosh, PC Windows</td>
</tr>
<tr>
<td></td>
<td>Contact</td>
<td><a href="http://statgen.ncsu.edu/qtlcart/cartographer.html">http://statgen.ncsu.edu/qtlcart/cartographer.html</a></td>
</tr>
<tr>
<td>MapMaker/QTL</td>
<td>Function</td>
<td>Interval Mapping, MQM</td>
</tr>
<tr>
<td></td>
<td>Population type</td>
<td>F2, backcross, RIL, DH</td>
</tr>
<tr>
<td></td>
<td>Computer platform</td>
<td>Unix/C</td>
</tr>
<tr>
<td></td>
<td>Contact</td>
<td><a href="http://www-genome.wi.mit.edu/ftp/distribution/software/">http://www-genome.wi.mit.edu/ftp/distribution/software/</a></td>
</tr>
<tr>
<td>MapQTL</td>
<td>Function</td>
<td>Interval Mapping, MQM, nonparametric mapping</td>
</tr>
<tr>
<td></td>
<td>Population Type</td>
<td>F2, backcross, RIL, DH, heterozygous F1</td>
</tr>
<tr>
<td></td>
<td>Computer platform</td>
<td>Vax, Unix, Macintosh, PC</td>
</tr>
<tr>
<td></td>
<td>Contact</td>
<td><a href="http://www.plant.dlo.nl/default.asp?section=contacts">http://www.plant.dlo.nl/default.asp?section=contacts</a></td>
</tr>
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</table>

DH: double haploid; RIL: Recombinant Inbred Line
The magnitude of the effect that different QTLs can exert on a single character is not generally the same. In many instances, a large proportion of quantitative variation can be explained by the segregation of a few major QTL. It is not uncommon to find individual QTL that can account for more than 20% of the phenotypic variation in a population and values as high as 42% have been reported for single QTL (Tanksley, 1993). Cumulative phenotypic variance attributable to the combination of all significant QTL is a measurement that determines how efficient an experiment has been in identifying the QTL responsible for a trait. Quantitative studies, where complete molecular maps have been used, have reported phenotypic variance of around 30-40% (Tanksley, 1993).

In 47 studies involving QTL analysis in maize, cereals, and brassicas including *Arabidopsis* individual studies reported that particular traits mapped in from 1 to 16 QTL with a mean of 4. From the literature it is often difficult to determine how much of the genetic variation is explained by the QTL because only the total phenotypic variance is reported. Typically, the percentage of variance explained by detected QTL for quantitative traits is around 46%, although it varies from 10-95% in individual studies (Kearsey et al., 1998). One might expect that those studies finding fewer QTL would explain less variation than those studies finding more, but this is not the case. In studies where most variation is explained just 1-6 QTL were involved (Yano et al., 1997).

There are strong limitations in QTL mapping. QTLs are hypothetical genes based on statistical inference. The genetic models on which QTL mapping is based are only approximate. The amount of genetic information contained in a reasonable size population is generally not adequate for high resolution mapping and, to make fine distinctions, the statistics of the analytical methods are not powerful enough. However, QTL analysis can be very useful in
getting to an approximate idea of how many genes influence a trait and how strong that influence is. Since there is essentially no genetic information about the heritability of effectiveness as a quantitative trait, QTL analysis might provide an opportunity to assess the parameters involved in breeding plants for increased nitrogen fixation ability.

**Work completed in this dissertation**

The work presented in this dissertation is described in four chapters. The second chapter describes the development of a mass spectrometry method for measuring $^{15}$N incorporation into pheophytin. The first part of this article uses *Chlamydomonas reinhardtii* to establish the concept of $^{15}$N incorporation into chlorophyll without the complication of unlabeled nitrogen derived from the starting tissue, such as seeds. This work was done by Michael L. Kahn and Christine L. Ford in collaboration with Forest Kaser and David McCaskill. My contribution in this article was in carrying out experiments using the technique to measure nitrogen fixation in *Medicago truncatula* plants inoculated with *Sinorhizobium meliloti* 104A14. We showed that atmospheric $^{14}$N$_2$ contributes to the *M. truncatula* pheophytin in plants growing under symbiotic conditions. This study concludes that determination of the fraction of nitrogen fixed can be estimated to less than 1% for each sample with around five repetitions per sample. Nitrogen in pheophytin is derived directly from glutamate and is therefore likely to be a representative sample of the nitrogen pool of the plant at the time of pheophytin synthesis. The assay seems to be very robust, precise, sensitive and simple. The mass spectrometry parameters were established by exploiting the expertise of Raymond E. Ketchum.

The third chapter describes the use of the mass spectrometry method to identify *S. meliloti* strains with differential performance in symbiotic nitrogen fixation in *M. truncatula*
lines Jemalong A17, DZA315.16 and F83005.5. We have identified three *S. meliloti* strains that differ in symbiotic effectiveness, USDA 1600, 102F51 and MK506. The last strain is a citrate synthase mutant of 104A14. Strains USDA 1600 and 102F51 fixed more nitrogen in Jemalong A17 than in DZA315.16. In contrast, strain MK506 fixed more nitrogen with DZA315.16 than with Jemalong A17. In the first part of the article we described the plant growth conditions and the fertilization regime used. The second part showed that the differential symbiotic performance of the three *S. meliloti* in *M. truncatula* lines did not change when conditions of plant growth and fertilization regime changed. The article also compares the different parameters used to measure nitrogen fixation such as nodule fresh weight, leaf fresh weight, nitrogenase activity and $^{15}$N incorporation into pheophytin. We showed that the $^{15}$N incorporation has the lowest variation in all experiments and is highly consistent.

The fourth chapter describes the results obtained from QTL analysis on Recombinant Inbred Lines (RILs) of *M. truncatula* derived from the cross of JemalongA17 and DZA315.16. The F7 seeds of these RILs were provided by Thierry Huguet and Jean-Marie Prosperi, INRA – SGAP Montpellier-France. Three parameters were measured: $^{15}$N incorporation in pheophytin using the mass spectrometry technique, plant length, and leaf fresh weight. A QTL for $^{15}$N incorporation was found in linkage group three in two of the three independent experiments involving *S. meliloti* USDA 1600. There is also the presence of QTLs for plant length in linkage groups 7 and 8 with high LOD score that served as an internal control for the QTL analyses.
Literature Cited


CHAPTER TWO

A mass spectrometry method for measuring $^{15}$N incorporation into pheophytin

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Abstract

The $^{15}$N content of pheophytin, the magnesium-free derivative of chlorophyll, can be measured with great accuracy and precision using positive-ion atmospheric pressure ionization electrospray mass spectroscopy following a simple solvent extraction of small amounts of plant tissue. The molecular weight of pheophytin prepared from *Chlamydomonas reinhardtii* grown in different ratios of $^{14}$N/$^{15}$N showed linear regression with the isotopic input, with a precision of 0.5-1%. Using an isotope dilution strategy, we have shown that nitrogen fixation can contribute substantial $^{14}$N to pheophytin isolated from *Medicago truncatula* plants grown in symbiosis with *Sinorhizobium meliloti*. The assay is sensitive, precise, rapid, simple and robust. These features suggest it could become an important tool for measuring the contribution of symbiotic and associative nitrogen fixation to plant metabolism.
The ability of plant-bacterial associations to convert atmospheric dinitrogen to ammonia is important in agriculture and in natural ecosystems. However, quantifying the impact of bacterial nitrogen fixation on individual plants has been problematic. The most definitive evidence for concluding that nitrogen fixation is occurring and is benefiting the plant is observing the incorporation of isotopically labeled N derived from labeled N\textsubscript{2} into plant tissue. This can be done by isotope ratio mass spectrometry (IRMS) using flash combustion and reduction of ground plant materials to generate N\textsubscript{2} (Montoya et al., 1996) or by subjecting the materials to Kjeldahl degradation, which uses strong acid to convert nitrogen compounds to ammonia (Bergersen, 1980; Danso, 1995), followed by mass spectrometry. Because these methods destroy the components of the tissue, both lose information about the nature of the nitrogen-containing compounds that are present. Surrogate methods for measuring fixation that determine the activity of nitrogenase, the key enzyme of nitrogen fixation, by measuring the reduction of the N\textsubscript{2} analog acetylene to ethylene, are rapid and inexpensive but may not give an accurate estimate of the amount of nitrogen fixed, for various reasons that have been discussed extensively (Danso, 1995; Hunt et al., 1993; Minchin et al., 1983).

In the familiar rhizobial and actinomycete symbioses, bacterial nitrogen fixation occurs in specific tissues formed after bacterial invasion. When plants infected with these bacteria are exposed to \textsuperscript{15}N\textsubscript{2}, the observation of \textsuperscript{15}N in plant tissues that do not contain nitrogen-fixing bacteria is good evidence that bacterially fixed N has been assimilated by the plant. A variant of this method use in the field is based on dilution of the \textsuperscript{15}N isotope found in the soil by N derived
from atmospheric $^\text{15}\text{N}_2$. Although the fraction of excess $^\text{15}\text{N}$ in natural soils is small, this method has been used to estimate nitrogen fixation in unperturbed situations (Danso, 1995).

We have recently become interested in the possibility that nitrogen-fixing bacteria could contribute biologically significant amounts of fixed nitrogen directly to monocots, such as, grasses (Boddey et al., 1995). It has been shown in some cases that nitrogen-fixing bacteria stimulate the growth of the associated plant, but the evidence that increased growth is due to N transfer directly from the bacteria to the plant is not compelling. One problem that has made determining the contribution of the bacteria to growth difficult is that, while it has been possible to measure bacterial nitrogen fixation associated with the grasses, there has not been a good method for determining whether the fixed nitrogen was retained in the bacteria or was incorporated into plant metabolism. In associations between nitrogen-fixing bacteria and monocots, the bacteria are thought to be within plant tissues, such as the xylem and phloem, and it is difficult to separate the organisms adequately to be sure that nitrogen in the plant fraction is actually in the plant. The amount of nitrogen thought to be fixed in these associations is only a fraction of the plant's total nitrogen requirement and is often in the range that can be accounted for by the endophytic bacterial population. This nitrogen might ultimately be available in the ecosystem, but an important biological question is whether a portion of it might be immediately accessible to the plant, like the situation in rhizobial and actinomycete symbiotic relationships, where plant growth can be entirely supported by bacterial nitrogen fixation.

We present here a possible solution to the problem of determining whether there is transfer of fixed nitrogen to the plant by showing that positive-ion atmospheric pressure ionization electrospray mass spectrometry (API-ES-MS) of a directly injected sample can be used to determine the $^{15}\text{N}$ content of plant-derived pheophytin. Pheophytin, which is derived
from chlorophyll by the acid-catalyzed removal of the central magnesium atom, contains four N atoms. Pheophytin is clearly a molecule of plant origin and finding N derived from N\textsubscript{2} in pheophytin would definitively show that the plant was receiving fixed nitrogen from the bacteria. In addition, pheophytin is abundant, is found in nearly all plants, and can be readily extracted and purified from potential contaminants before analysis by direct injection into the mass spectrometer. We chose chlorophyll as a candidate for this analysis over several other plant molecules on the basis that the others might be found in either the plant or bacteria (like an amino acid or nucleotide), that they are specific to one plant (like an alkaloid), that they were too complex (like a plant-specific protein) or that they could not be purified easily because of low initial concentration.

Earlier methods for analyzing the isotopic content of chlorophyll prepared from natural samples used extensive prepurification of the material before analysis by IRMS (Sachs et al., 2000). Our results, using pheophytin prepared by dioxane precipitation from cultures of *Chlamydomonas* labeled with ¹⁵N-ammonia and from leaves of *Medicago truncatula*, show that API-ES-MS is an excellent method for measuring incorporation of nitrogen isotopes into green photosynthetic tissue. The rapid analysis of ¹⁵N-labeled pheophytin directly injected into the API-ES-MS interface, without the need for a chromatographic separation, clearly makes this method suitable for high-throughput analysis of nitrogen fixation in legume and actinorhizal symbioses. The sensitivity and simplicity of the method may also make it suitable for using nitrogen incorporation into pheophytin to follow other aspects of plant nitrogen metabolism.
Materials and Methods

Growth and labeling of Chlamydomonas: Chlamydomonas reinhardtii strain 137+ was obtained from Dr. Charlotte Omoto at WSU and grown at 23°C in 10 ml of Sueoka high-salt medium (Harris, 1989) under fluorescent illumination. The ammonium concentration of the cultures was 10 mM and was partitioned between $^{14}\text{NH}_4\text{Cl}$ and $^{15}\text{NH}_4\text{Cl}$ as indicated. Cells were inoculated at a dilution of 1:40 and mechanically stirred. After being grown to a high density in a particular medium, cultures were diluted in the same medium, grown again, and then harvested at a mid-logarithmic phase of growth by centrifugation at 5000g for 5 min at 4°C. The cells were resuspended in deionized water and then centrifuged again, resuspended in 1 ml of methanol, and transferred to a 1.5 ml microcentrifuge tube.

Growth of Medicago truncatula: M. truncatula seeds were pretreated with concentrated sulfuric acid for 8 min and rinsed several times with water. Seeds were then soaked in sterile water for 48 h at 4°C. Seeds were rinsed 6-8 times with sterile water and then transferred to water agar plates. The plates were placed in the dark at room temperature for 24 h. Four germinated seeds were placed in Magenta boxes filled with lightweight expanded clay aggregate (Eco Enterprises, Shoreline, WA) and sand as support material. Plants were fertilized on the day of planting and at 22 and 52 days after planting (dap) with 1/4 strength Gibson’s nutrient solution (Gibson, 1980) that contained 2 mM $^{15}\text{NH}_4\text{Cl}$ or $^{14}\text{NH}_4\text{Cl}$, as indicated. This low ammonium concentration is not optimal for plant growth and there should be no inhibition of N$_2$ fixation or nodulation (Streeter, 1988). As indicated, plants were inoculated with Sinorhizobium meliloti strain 104A14 (Mcdermott et al., 1992). At 55 dap plants were harvested and leaf material was extracted as described below.
Analysis of chlorophyll (pheophytin): *Chlamydomonas* chlorophyll was extracted in a procedure similar to that of Iriyama *et al.* (1974). After 3-5 min, cells were removed by centrifugation at 10,000g for 30 s. The supernatant was transferred to a new tube, the centrifugation was repeated, and 1 ml of supernatant was taken. One seventh volume of dioxane (145 µl) and a slightly larger volume of deionized water (180 µl) were added and mixed with the methanol solution. The resulting mixture was chilled at -80ºC for at least 1 h and then the chlorophyll was pelleted in a microcentrifuge by centrifugation at 12,000 rpm at 4ºC for 15 min. The supernatant was discarded, the tubes were drained well, and the chlorophyll was resuspended in 200 µl of 1:1 methanol:acetone. In control experiments, samples from several stages of the preparation above could be stored in the dark for periods of at least two weeks without affecting the extraction or subsequent spectrum. For the *Medicago truncatula* samples, leaves were extracted in methanol for several hours, the leaves were removed, and extraction proceeded as described above, with the exception that the second dioxane extraction was omitted.

Chlorophyll was converted to pheophytin by adding 1 µl concentrated HCl to the sample. After several minutes at room temperature, the resulting preparation was centrifuged through a 0.2 µm Alltech microspin filter. The concentration of pheophytin (µg/ml) in the filtered solution was estimated by multiplying the absorbance at 654 nm by 17.2, a conversion factor determined empirically for the methanol:acetone solvent based on measurements for pheophytin in acetone and methanol (Lichtenthaler, 1987). These filtered samples were used directly for analysis on a Hewlett Packard Series 1100 series HPLC pump and autosampler interfaced to an 1100 series mass selective detector operating in electrospray, positive-ion mode. For the data of Figs. 1 and 2, and Table 1, the pump and autosampler were used to inject 1 µl of each sample directly into
the mass spectrometer, using a solvent of 10% water in acetonitrile at a flow rate of 0.3 ml/min. The spray chamber conditions were optimized at 11.0 L/min of nitrogen, a nebulizer pressure of 60 psig, a fragmentor voltage of 175 V, and a capillary voltage of 6000 V. Run time under these conditions is about 1 minute. Five replicates of each sample were analyzed to improve the statistics of the results. The molecular formula of pheophytin \( a \) is \( C_{55}H_{74}N_{4}O_{5} \), accordingly, the base peak of pheophytin \( a \) appeared at \( m/z \) 872.1 [M+H] with natural abundance isotope peaks contributed from \(^{15}\text{N},^{13}\text{C},^{2}\text{H}\) and \(^{18}\text{O}\) appearing at \( m/z \) 873.1 to 876.1. Calculation of the theoretical isotopic distribution of pheophytin \( a \) using Isotope Pattern Calculator v1.6.6 (http://www.macinsearch.com/infomac2/science/isotope-pattern-calc-166.html) gives a predicted relative abundance of the various pheophytin isotopomers \([M/(M+1)/(M+2)/(M+3)]\) of 100/64.5/21.5/4.9. Analysis of unlabeled pheophytin \( a \) (Fig 1) gave an experimental distribution of isotope peaks in excellent agreement with the theoretical values. Data were collected in scan mode, operated between \( m/z \) 850 to 900, and the mass peaks corresponding to pheophytin \( a \) were quantified. The mean mass of pheophytin \( a \) was determined by computing a weighted average of the various pheophytin peaks for each experimental sample. From these an average and standard deviation for each experimental treatment were calculated. Pheophytin \( b \) sometimes produced a similar pattern of peaks with an intensity about 10\% of the pheophytin \( a \) with a base peak at \( m/z \) 886.1. In preliminary experiments, we used commercially available chlorophyll \( a \) (Sigma, Kansas City, MO) as standard.

We have also been able to obtain high-quality data on a Waters Alliance 2690 HPLC pump and autosampler interfaced to a Waters Micromass ZQ detector operating in electrospray, positive-ion mode, although no data from this machine are included in the results presented here. The parameters used for the Waters instrument were optimized at 46 L/h cone gas flow, 284 L/h
desolvation gas flow, a capillary voltage of 3000 V, a cone voltage of 50 V, and an extractor voltage of 13.1 V. Samples were run using a solvent of 10% water in acetonitrile containing 0.2% formic acid and a solvent flow rate of 0.5 ml/min. Run time under these conditions was about 30 s.

**Results and Discussion**

Chlorophyll contains four nitrogen atoms, all derived from δ-amino levulinic acid. It is generally abundant in photosynthetic tissue and procedures for extracting chlorophyll and converting it to pheophytin are well established. For the purposes of measuring incorporation of $^{15}$N into plant tissues, chlorophyll has the disadvantage of chemical instability and the presence of three naturally occurring isotopes of Mg that complicate the interpretation of $^{15}$N enrichment. Conversion of chlorophyll to pheophytin produces a more stable molecule with a simpler mass spectrum and increases the sensitivity of the mass spectrometry measurement due to better ionization of the molecule (Airs et al., 2000).

In preliminary experiments with spinach, alfalfa and oats, we found that the determination of a standard curve of pheophytin mass as a function of isotopic substitution was complicated by the unlabeled nitrogen in the seed and by a background of other molecules derived from these biochemically more complex tissues. We were able to eliminate the contaminants using the solvent extraction protocol described above so that the regions between the peaks and at m/z below 871 generally had a very low background (compare Figs. 1 and 2). The keys to obtaining a low background are the specificity of the solvent extraction for chlorophyll and the use of electrospray mass spectrometry conditions that are gentle enough to limit fragmentation of pheophytin and minimize the contribution of degradation products of
molecules of higher mass to signals within the pheophytin mass range. Attempts to use material
directly from the methanol extract had unacceptable contamination, seen as a high background at
nearby mass values that presumably contribute signals in the range of interest (Fig 2). A single
extraction with dioxane was sufficient to remove most of this contaminating material.
Fig. 1. Mass spectrum of pheophytin from *M. truncatula* grown in $^{14}$NH$_4$Cl. Unedited spectrum of dioxane-precipitated extract prepared as described in the text showing absence of significant background in regions adjacent to pheophytin. Peak abundance is reported as percent of base peak (100%) versus mass to charge ratio ($m/z$). The highest peak had a value of 470,272.
Fig. 2. Mass spectrum of methanol extract from *M. truncatula* grown in $^{14}$NH$_4$Cl.

Spectrum of the leaf material extracted directly into methanol showing high background in regions adjacent to pheophytin. This is the same sample as that analyzed in Fig. 1 after dioxane extraction and concentration. Peak abundance is reported as percent of base peak (100%) versus mass to charge ratio (m/z). The highest peak had a value of 15,758.
We used the green alga, *C. reinhardtii*, to establish the concept of $^{15}$N incorporation into chlorophyll and to develop some of the methods presented here. This allowed us to obtain a standard curve for the correlation of pheophytin mass and $^{15}$N source without the complication of unlabeled nitrogen derived from the starting tissue, such as seeds. *C. reinhardtii* can be grown in completely defined medium and, after extraction as described under Materials and methods; it gave a simple and clean spectrum. Fig. 3 shows an averaged spectrum for each of four fractional $^{15}$N substitutions. The data obtained for these cultures and others grown in media of different fractional $^{15}$N composition are summarized in Fig. 4. Over the range of 0.4-98% $^{15}$N, the pheophytin $m/z$ shifted linearly by 3.9 units, indicating that there was no major isotope effect during biosynthesis that discriminated for or against the incorporation of $^{15}$N into the pheophytin structure. Standard deviations for this data set averaged 0.025 mass units and the linear regression was very good ($r = 1.00$), indicating that both the accuracy and the precision of this technique were excellent. The sensitivity of this technique was such that material from about 3 mg of alfalfa tissue was needed to determine the mean $m/z$ of a single sample to this degree of precision. Actually analyzing samples this small is currently limited by the occasional difficulty in recovering the small amount of precipitate available from these samples. Experiments with spinach chlorophylls showed that a minimum chlorophyll concentration of around 24 mg/ml in the methanol extract was needed to reliably obtain a pellet (data not shown). With a suitable carrier it is possible that smaller quantities could be isolated and analyzed, since the sensitivity of the mass spectrometer is very high. To date, we have successfully extracted chlorophyll with dioxane from alfalfa, *M. truncatula*, oats, spinach, and sugar cane, to a degree of purity suitable for electrospray analysis.
Fig. 3. Spectra of pheophytin from *C. reinhardtii* labeled with $^{15}$N. Fractional substitutions were obtained by mixing "$^{14}$N" (99.6% $^{14}$N) and "$^{15}$N" (98% $^{15}$N) in the proportions 100:0 (A), 75:25 (B), 50:50 (C) and 0:100 (D). Pheophytin was extracted and analyzed as described in the text.
Fig. 4. Mean mass of *C. reinhardtii* pheophytin as a function of nitrogen isotope composition of the growth medium. The average mass was calculated from the relative abundance of pheophytin isotopomers at different percent substitution ratios of the input nitrogen. Error bars for each point were less than 0.04 mass units.

\[ Y = 0.039X + 0.613 \]

\[ r = 1.000 \]
The contribution of symbiotic nitrogen fixation to plant nitrogen composition was estimated by measuring pheophytin mass using a variation of an isotope dilution technique similar to those often used for this purpose (Danso, 1995).

Isotope dilution strategies are most suitable for long-term labeling of plants under nitrogen-fixing conditions, where maintaining an $^{15}\text{N}_2$-enriched atmosphere is difficult. In an isotope dilution protocol, the plants are grown in the presence of a nitrogen source enriched in $^{15}\text{N}$, such as, $^{15}\text{NH}_4$ or $^{15}\text{NO}_3$. Atmospheric $\text{N}_2$ contains 99.64% $^{14}\text{N}$ and addition of $\text{N}$ from this source due to bacterial nitrogen fixation will dilute the $^{15}\text{N}$ in the nitrogen pool. Incorporation of less $^{15}\text{N}$ into pheophytin can then be estimated by comparing the mass of pheophytin from infected and uninfected plants. Since the ratio of different isotopomers is used in the determination, the absolute efficiency of chlorophyll recovery is not very important, as long as there is sufficient signal to calculate accurate ratios.

Table 1 shows the application of this principle in an experiment with *M. truncatula* seedlings. Seeds of *M. truncatula* weigh about 4 mg, so we expected a contribution of seed $^{14}\text{N}$ to the nitrogen composition of pheophytin. Plants were grown either with 99.6% $^{14}\text{NH}_4$ or 98% $^{15}\text{NH}_4$ and, as indicated, were inoculated with *S. meliloti*. The mean mass of pheophytin in the uninoculated samples grown on $^{15}\text{NH}_4$ was about 3 mass units higher than those grown on $^{14}\text{NH}_4$, indicating that, for these small plants, about 75% of the nitrogen in pheophytin was derived from the $^{15}\text{N}$ in the medium with the balance coming from $^{14}\text{N}$ in the seed nitrogen reserves. In striking contrast, pheophytin from the inoculated sample grown on $^{15}\text{N}$ was only 0.4 mass units higher than the $^{14}\text{N}$-inoculated control. The inoculated plants were about twice the mass of the uninoculated plants and, since the concentration of N in the medium was already limiting growth, the effect of the bacteria on isotopic concentration was not due to the bacteria.
sequestering $^{15}$N and denying it to the pheophytin precursor pool. We have also shown in experiments examining the kinetics of N incorporation into pheophytin that soybean chlorophyll in mature leaves does not turn over much, if at all, at least during vegetative growth of the whole plant (A. Parra and M.L. Kahn, unpublished data).
Table 1. Atmospheric $^{14}$N contributes to the nitrogen budget of *M. truncatula* grown under symbiotic conditions.

<table>
<thead>
<tr>
<th>Nitrogen source $^a$</th>
<th>Inoculation $^b$</th>
<th>Mean $^{m/z}$± SD $^c$</th>
<th>SD/sample $^d$</th>
<th>Shoot fresh weight (mg/plant) $^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$N No</td>
<td>872.962±.091 (3)</td>
<td>0.0202</td>
<td>56.54</td>
<td></td>
</tr>
<tr>
<td>$^{14}$N Yes</td>
<td>872.786±.076 (7)</td>
<td>0.0159</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>$^{15}$N No</td>
<td>876.007±.301 (5)</td>
<td>0.0201</td>
<td>40.23</td>
<td></td>
</tr>
<tr>
<td>$^{15}$N Yes</td>
<td>873.197±.034 (5)</td>
<td>0.0131</td>
<td>78.6</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Indicates whether the fertilizer nitrogen was $^{14}$N (99.6%) or $^{15}$N (98%) ammonium chloride.

$^b$ Plants were inoculated with symbiotic bacteria at planting, as indicated.

$^c$ Average of mean $^{m/z}$ values calculated for pheophytin peaks in the $^{m/z}$ range 871-881 ± SD (number of different experimental measurements).

$^d$ Average of the SD values for the five replicate measurements made for each sample.

$^e$ Average of four plants for shoot fresh weight; ND, not determined.
The data suggest that only 10-15% of the nitrogen incorporation was from the medium. The variation between plants in each of the different treatments was also small, except for the $^{15}$N-uninoculated plants, where a single point contributed 0.16 mass units to the standard deviation value of 0.30. The standard deviation/sample based on five replicate injections of the same material was 0.02 or less, about 0.5% of the difference between fully substituted and unsubstituted chlorophyll. Some of this variation is due to instrument fluctuations and more replicates would decrease the instrument contribution to variation. Using our number of repetitions, the routine determination of the fraction of nitrogen fixed can be estimated to less than 1% for each sample and this sets an approximate limit on the sensitivity of the technique, i.e., it is likely to be necessary to have at least 1% $^{15}$N in the soil to observe a shift of the $^{15}$N content of pheophytin.

In preliminary experiments, we have not been able to demonstrate an effect of bacterial nitrogen fixation on chlorophyll from grasses. Although it has been shown that nitrogen-fixing bacteria can stimulate plant growth (Boddey et al., 1995), demonstrating directly and reproducibly that this is due to nitrogen transfer from the bacteria has been difficult. We are continuing to investigate the possibility of nitrogen transfer to grasses by endosymbiotic bacteria.

While the procedures for measuring chlorophyll mass described here are presented in the context of measuring nitrogen metabolism during symbiotic nitrogen fixation, they may be useful in any circumstance where a measurement of the composition of the nitrogen pools is to be followed isotopically and where that nitrogen would appear in chlorophyll. So, for example, seeds could be germinated to increase chlorophyll content prior to analysis. The assay seems to be very robust and it is sensitive enough to measure pheophytin in very small quantities of material. Nitrogen in pheophytin is derived directly from glutamate and is therefore likely to be
representative of the plant nitrogen pool composition at the time of pheophytin synthesis. The sensitivity, simplicity, and rapid nature of the analysis indicate that it is suitable for high-throughput analysis of nitrogen fixation in plant tissues.

Previous work that used the nitrogen isotope ratios in chlorophyll to measure nitrogen fixation in geological samples (Sachs et al., 1999) worked with much larger initial samples, purified the chlorophyll derivatives using HPLC, and then determined the isotope ratio using IRMS (Sachs et al., 2000). For samples where other materials are not present in the mass range of pheophytin, there is no question that the method described here is faster, requires less sample and is easier to interpret. The precision that we report is not yet sufficient to allow nitrogen fixation to be measured in the soil where natural abundance of $^{15}$N is only slightly above the atmospheric concentration of $^{15}$N. However, by using additional replicates and more advanced data handling techniques we extrapolate that this assay can be adapted to the field with a precision equal to those currently in use. If so, it would have many uses in plant physiology and genetics related to nitrogen acquisition from both bacterial and mineral sources.
Acknowledgements

We thank Rodney Croteau for allowing us to use his laboratory's mass spectrometer and Charlotte Omoto and Atsuka Kanazawa for assistance with *Chlamydomonas*. Parts of the work presented here were supported by grants the United States Department of Energy Biosciences Program (DE-FG03-96ER20225) and from the National Science Foundation (9816583). FK was supported by a Summer Undergraduate Fellowship from the Plant Biochemistry Research and Training Center through grant DE-FG06-94ER20160 from the U.S. Department of Energy.
References


CHAPTER THREE

Effectiveness of *Sinorhizobium meliloti* strains in symbiosis with selected isolates of *Medicago truncatula*

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ABSTRACT

Effectiveness describes the input that a bacterial nitrogen-fixing symbiosis makes to plant nitrogen metabolism. Effectiveness is considered a polymorphic trait that is greatly affected by the bacteria × host combination and, in legumes, specific interactions between the plant and symbiotic rhizobia contribute to the effectiveness of a particular combination. Evaluation of effectiveness using a model legume, Medicago truncatula, may open new avenues for genetic studies. In previous work, we developed a mass spectrometry method to estimate the contribution of symbiotic nitrogen fixation to plant nitrogen content that 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. This work explores the utility of this $^{15}$N-dilution assay in evaluating the level of nitrogen fixation effectiveness in three Medicago truncatula lines that have been used as parents in generating recombinant inbred lines. Using this assay, we have identified three Sinorhizobium meliloti strains, USDA1600, 102F51 and MK506, with differential symbiotic performance 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

Nitrogen is a major limiting nutrient for most plant species growing under most conditions. Almost all plants acquire nitrogen from the soil by transport of organic or inorganic nitrogen compounds but some can obtain sufficient nitrogen to satisfy their needs through symbiotic interactions with bacteria able to reduce atmospheric N_2. Many legumes have the ability to form nitrogen-fixing symbiosis with gram-negative soil bacteria known as rhizobia (Szczyglowski and Amyot 2003). The rhizobia–legume symbiosis involves specific expression of both bacterial and plant genes during the development of specialized 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 is able to fix nitrogen, it is said to be effective. Effectiveness 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 lead to a more effective symbiosis.

In order to study the genetics of N_2 fixation effectiveness in a rhizobia–legume symbiosis, two components are essential. The first is to have genetic variants of a legume host plant that differ in their response to rhizobial strains. If the host contribution to effectiveness is the target of study, situations where there are differences between hosts using a single strain of bacteria are ideal. 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). Important 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.

The second component needed to study N$_2$ fixation effectiveness is to have the ability to quantify the magnitude of N$_2$ fixation in different legume–rhizobia combinations. This is not always straightforward because of 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. Other parameters commonly used to estimate the degree of N$_2$ fixation include 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 a 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 determine 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. The study examines the effect of bacterial strain, plant line and the interaction of these two symbionts on plant characteristics associated with nitrogen fixation. Measurement of incorporation of $^{14}$N into pheophytin in plants grown on $^{15}$N-urea is proposed to be a good parameter for estimating N$_2$ fixation effectiveness in *M. truncatula*.

**RESULTS**

An isotope dilution strategy was used to measure the incorporation of nitrogen derived from symbiotic nitrogen fixation into pheophytin. The isotope dilution 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 so that it does not interfere with nodulation or nitrogen fixation. In preliminary experiments we used ammonium, nitrate or urea as sources of $^{15}$N. Ammonium supported relatively poor plant growth and nitrate, which is known to have strong effects on nodule formation and senescence, gave somewhat variable results (data not shown). 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). As explained in Materials and Methods, 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 have a pheophytin mass of ~872.2 Daltons whereas uninoculated plants growing in the presence of urea $^{15}$N have a pheophytin mass of ~875.50 Daltons (Tables 1 to 6). We had
previously shown using Chlamydomonas that full substitution of $^{15}$N leads to a shift of four mass units as predicted and there was not significant isotope effects in pheophytin synthesis (Kahn et al. 2002).

**Some S. meliloti strains differ in performance on Medicago truncatula lines**

Several S. meliloti isolates have been previously reported to show differences in symbiotic performance on M. truncatula lines based on plant dry weight (T. Huguet, personal communication). From these isolates, S. meliloti strains 2011, CC109, ABS7, RF22, 12, 102F65, USDA1600 and 102F51 were screened on M. truncatula lines Jemalong A17, DZA315.16 and F83005.5. Initial experiments were carried out using 2 mM Na$^{15}$NO$_3$ (99% atom) as a nitrogen source and the parameters used as indices of effectiveness were nodule number, nodule weight, leaf weight, nitrogenase activity and pheophytin mass. Significant differences were found for strain × line interaction for all parameters except for pheophytin mass (data not shown).

From these experiments, S. meliloti strains USDA1600, 2011 and 102F51 were selected for further analysis because of differences in shoot fresh weight among M. truncatula lines. S. meliloti USDA1600 was isolated from M. arborea and it is ineffective in 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). Nodule fresh weight, leaf fresh weight, nitrogenase activity and pheophytin mass were the parameters measured to evaluate symbiotic performance. For this and subsequent experiments the nitrogen source was changed to urea for reasons explained above and fertilization was done as explained in Materials and Methods. The analysis of variance for the different parameters 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 of 4 mass units. Measurements ranged from ~872.2 Daltons for non-inoculated plants to a "full substitution" of ~875.9 Daltons in uninoculated plants growing only in the presence of $^{15}$N. 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 $\times$ 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).

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 and mass 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 USDA 1600-infected plants were grown under 4 mM $^{15}$N. The 4 mM nitrogen treatment was also evaluated in uninoculated plants. However, no significant improvement in plant growth was observed (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), the bacteria were determining the difference. Based on the pheophytin results, it was determined that 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 × 102F51 interaction (Table 2).

*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 is needed to do a large number of replicates in our facilities. Analysis of variance showed that nitrogenase activity and pheophytin mass were significantly different at $P \leq 0.0001$ and $P \leq 0.05$, respectively for the line × strain interaction (data not shown). Mean separation using least square means for these two dependent variables is presented in Table 3. The effectiveness of USDA1600 was significantly different between 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%. A similar difference between lines was obtained in the previous experiment in which plants were grown in 300 ml boxes (Table 2). Nitrogenase activity was inversely correlated with the $^{15}$N incorporation measurement—high nitrogenase activity corresponded to low $^{15}$N incorporation (Table 3). These results indicated that the observed differences are robust and that the line × strain interaction plays an important role in determining the effectiveness of nitrogen fixation (Table 3).
Table 1. Analysis of variance for parameters measured on *M. truncatula* lines inoculated with *S. meliloti*  

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>NFW&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LFW&lt;sup&gt;c&lt;/sup&gt;</th>
<th>(M_{\text{pheophytin}}&lt;sup&gt;d&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>MS&lt;sup&gt;e&lt;/sup&gt;</td>
<td>df</td>
</tr>
<tr>
<td>Line (L)</td>
<td>2</td>
<td>107.0*</td>
<td>2</td>
</tr>
<tr>
<td>Strain (S)</td>
<td>5</td>
<td>641.9**</td>
<td>5</td>
</tr>
<tr>
<td>(L \times S)</td>
<td>9</td>
<td>179.9**</td>
<td>10</td>
</tr>
<tr>
<td>Mean</td>
<td>12.2</td>
<td>61.5</td>
<td>873.8</td>
</tr>
<tr>
<td>SD</td>
<td>5.81</td>
<td>25.28</td>
<td>0.45</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.76</td>
<td>0.59</td>
<td>0.89</td>
</tr>
</tbody>
</table>

<sup>a</sup> SD = Standard Deviation; \(R^2\) = Coefficient of determination  

<sup>b</sup> NFW = Nodule Fresh Weight  

<sup>c</sup> LFW = Leaf Fresh Weight  

<sup>d</sup> Incorporation of \(^{15}\text{N}\) into pheophytin is expressed as mass charge\(^{-1}\) (m \(z^{-1}\)) ratio.  

<sup>e</sup> MS = Mean squares * F ratio significant at \(P \leq 0.05\). ** F ratio significant at \(P \leq 0.0001\).
Table 2. Differential symbiotic performance of *S. meliloti* strains in *M. truncatula* lines

<table>
<thead>
<tr>
<th>Strain</th>
<th>Line</th>
<th>NFW&lt;sup&gt;b&lt;/sup&gt; (mg plant&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>SD</th>
<th>M&lt;sub&gt;pheophytin&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (m z&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>SD</th>
<th>Ndf&lt;sup&gt;d&lt;/sup&gt; %</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA1600</td>
<td>Jemalong A17</td>
<td>14.1 cde</td>
<td>7.2</td>
<td>873.523 f</td>
<td>0.886</td>
<td>63</td>
</tr>
<tr>
<td>USDA1600</td>
<td>DZA315.16</td>
<td>8.7 def</td>
<td>6.1</td>
<td>874.207 bcde</td>
<td>1.059</td>
<td>44</td>
</tr>
<tr>
<td>USDA1600</td>
<td>F83005.5</td>
<td>15.6 bcd</td>
<td>7.0</td>
<td>874.503 b</td>
<td>0.602</td>
<td>37</td>
</tr>
<tr>
<td>USDA1600 + 4 mM&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Jemalong A17</td>
<td>22.6 bc</td>
<td>6.2</td>
<td>873.788 def</td>
<td>0.352</td>
<td>56</td>
</tr>
<tr>
<td>USDA1600 + 4 mM&lt;sup&gt;e&lt;/sup&gt;</td>
<td>DZA315.16</td>
<td>34.8 a</td>
<td>10.2</td>
<td>874.385 bcd</td>
<td>0.873</td>
<td>40</td>
</tr>
<tr>
<td>USDA1600 + 4 mM&lt;sup&gt;e&lt;/sup&gt;</td>
<td>F83005.5</td>
<td>8.2 ef</td>
<td>ND</td>
<td>875.733 a</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>102F51</td>
<td>Jemalong A17</td>
<td>23.5 b</td>
<td>6.8</td>
<td>873.385 f</td>
<td>0.302</td>
<td>67</td>
</tr>
<tr>
<td>102F51</td>
<td>DZA315.16</td>
<td>9.5 ed</td>
<td>6.5</td>
<td>874.449 bc</td>
<td>0.747</td>
<td>38</td>
</tr>
<tr>
<td>102F51</td>
<td>F83005.5</td>
<td>9.3 ed</td>
<td>4.0</td>
<td>873.871 cdef</td>
<td>0.134</td>
<td>54</td>
</tr>
<tr>
<td>2011</td>
<td>Jemalong A17</td>
<td>14.3 cde</td>
<td>3.9</td>
<td>873.615 ef</td>
<td>0.173</td>
<td>61</td>
</tr>
<tr>
<td>2011</td>
<td>DZA315.16</td>
<td>10.2 de</td>
<td>7.1</td>
<td>873.485 f</td>
<td>0.450</td>
<td>65</td>
</tr>
<tr>
<td>2011</td>
<td>F83005.5</td>
<td>17.7 bcd</td>
<td>6.4</td>
<td>873.394 f</td>
<td>0.361</td>
<td>67</td>
</tr>
<tr>
<td>NI&lt;sup&gt;f&lt;/sup&gt; 14N</td>
<td>Jemalong A17</td>
<td>0 f</td>
<td>872.192 g</td>
<td></td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>NI&lt;sup&gt;f&lt;/sup&gt; 14N</td>
<td>DZA315.16</td>
<td>0 f</td>
<td>872.205 g</td>
<td></td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td>NI&lt;sup&gt;f&lt;/sup&gt; 14N</td>
<td>F83005.5</td>
<td>0 f</td>
<td>872.214 g</td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>NI&lt;sup&gt;f&lt;/sup&gt; 15N</td>
<td>Jemalong A17</td>
<td>0 f</td>
<td>875.806 a</td>
<td></td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td>NI&lt;sup&gt;f&lt;/sup&gt; 15N</td>
<td>DZA315.16</td>
<td>0 f</td>
<td>875.811 a</td>
<td></td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>NI&lt;sup&gt;f&lt;/sup&gt; 15N</td>
<td>F83005.5</td>
<td>0 f</td>
<td>875.827 a</td>
<td></td>
<td>0.022</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Means

<sup>b</sup> NFW: Nodulation Fresh Weight

<sup>c</sup> M<sub>pheophytin</sub>: Membrane Pheophytin

<sup>d</sup> Ndf: Number of degrees of freedom

<sup>e</sup> 4 mM: 4 millimolar

<sup>f</sup> NI: Nitrogen Isotope
Means of three repetitions. Mean separation by Least Square Means. Means followed by a common letter are not significantly different for the strain × line interaction at P < 0.05.

NFW = Nodule Fresh Weight and SD = Standard Deviation.

Incorporation of $^{15}$N into pheophytin is expressed as mass charge$^{-1}$ (m z$^{-1}$) ratio. SD = standard deviation.

Ndf = Nitrogen derived from fixation.

Plants were fertilized with 4mM $^{15}$N. ND = Not determined due to plant loss.

NI = Uninoculated plants
Table 3. Differential symbiotic performance of USDA1600 in *M. truncatula* lines growing in 50 ml tubes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Line</th>
<th>Nitrogenase (nmol⁻¹ mg⁻¹ min⁻¹)</th>
<th>Mₜₚₑₜₕyτin b (m z⁻¹)</th>
<th>SD</th>
<th>SD</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA 1600</td>
<td>Jemalong A17</td>
<td>0.110 a</td>
<td>872.493 c</td>
<td>0.063</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>USDA 1600</td>
<td>DZA315.16</td>
<td>0.042 b</td>
<td>873.252 b</td>
<td>0.490</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>NI ¹⁴N</td>
<td>Jemalong A17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI ¹⁴N</td>
<td>DZA315.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI ¹⁵N</td>
<td>Jemalong A17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI ¹⁵N</td>
<td>DZA315.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Means of six repetitions. Mean separation by Least Square Means. Means followed by a common letter are not significantly different for the strain × line interaction at P < 0.05.

SD = Standard deviation.

b Incorporation of ¹⁵N into pheophytin is expressed as mass charge⁻¹ (m z⁻¹) ratio.

c Ndf = Nitrogen derived from fixation.

d NI = Uninoculated plants
Evaluation of *S. meliloti* citrate synthase mutants on parental lines of *M. truncatula*

In previous experiments, *S. meliloti* *gltA* mutant strains were derived from the wild type strain Rm104A14 that had various levels of bacterial citrate synthase (CS) activity (Grzemski et al. 2003; Mortimer et al. 1999). *Medicago sativa* plants inoculated with 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 ≤ 3% of the CS activity, fixation was significantly impaired. To see 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).

Results from the ANOVA analysis showed significant differences in line, strain and line × 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 ≤ 0.05 for parameters showing significant differences for line × strain interaction (Table 5).
Table 4. Analysis of variance for parameters measured on *M. truncatula* lines with *S. meliloti* citrate synthase mutants

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>NFW&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LFW&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Nitrogenase</th>
<th>M&lt;sub&gt;pheophytin&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>MS&lt;sup&gt;e&lt;/sup&gt;</td>
<td>df</td>
<td>MS</td>
</tr>
<tr>
<td>Line (L)</td>
<td>2</td>
<td>24.1**</td>
<td>2</td>
<td>5124.4***</td>
</tr>
<tr>
<td>Strain (S)</td>
<td>5</td>
<td>274.9***</td>
<td>5</td>
<td>4954.3***</td>
</tr>
<tr>
<td>L × S</td>
<td>10</td>
<td>3.6</td>
<td>10</td>
<td>885.1***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>6.0</th>
<th>53.3</th>
<th>0.9</th>
<th>873.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>2.02</td>
<td>13.93</td>
<td>1.08</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.90</td>
<td>0.86</td>
<td>0.78</td>
<td>0.97</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> SD = Standard Deviation; R<sup>2</sup> = Coefficient of determination.

<sup>b</sup> NFW = Nodule Fresh Weight

<sup>c</sup> LFW = Leaf Fresh Weight

<sup>d</sup> Incorporation of<sup>15</sup>N into pheophytin is expressed as mass charge<sup>-1</sup> (m z<sup>-1</sup>) ratio.

<sup>e</sup> MS = Mean Squares * F ratio significant at P ≤ 0.05, ** P ≤ 0.01 or *** P ≤ 0.001.
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 DZA315.16 except for the plants nodulated by MK511 that showed equal percentage of fixation 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 confirmed 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 × strain interaction, 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 reported in *Medicago sativa* (Grzemski et al., 2003). 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 same 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 (Table 5 and 6). High nitrogenase activity and nodule fresh weight are correlated with low pheophytin mass.
Table 5. Symbiotic performance of *S. meliloti* citrate synthase mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Line</th>
<th>LFW (^{a})</th>
<th>Nitrogenase (^{c})</th>
<th>M(_{\text{pheophytin}}^{d})</th>
<th>Ndf (^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mg plant(^{-1}))</td>
<td>SD</td>
<td>(m z(^{-1}))</td>
<td>SD</td>
</tr>
<tr>
<td>104A14</td>
<td>Jemalong A17</td>
<td>106.5 ab</td>
<td>9.33</td>
<td>0.645</td>
<td>872.612 f</td>
</tr>
<tr>
<td>104A14</td>
<td>DZA315.16</td>
<td>64.4 de</td>
<td>18.54</td>
<td>0.870</td>
<td>872.571 fg</td>
</tr>
<tr>
<td>104A14</td>
<td>F83005.5</td>
<td>69.4 cd</td>
<td>37.79</td>
<td>0.866</td>
<td>872.754 ef</td>
</tr>
<tr>
<td>MK504</td>
<td>Jemalong A17</td>
<td>71.1 cd</td>
<td>4.49</td>
<td>1.745</td>
<td>873.105 de</td>
</tr>
<tr>
<td>MK504</td>
<td>DZA315.16</td>
<td>51.9 def</td>
<td>12.19</td>
<td>1.667</td>
<td>872.842 ef</td>
</tr>
<tr>
<td>MK504</td>
<td>F83005.5</td>
<td>35.1 fg</td>
<td>8.05</td>
<td>1.907</td>
<td>873.416 d</td>
</tr>
<tr>
<td>MK506</td>
<td>Jemalong A17</td>
<td>34.5 fg</td>
<td>2.90</td>
<td>0.215</td>
<td>874.276 c</td>
</tr>
<tr>
<td>MK506</td>
<td>DZA315.16</td>
<td>43.2 efg</td>
<td>6.25</td>
<td>0.406</td>
<td>873.390 d</td>
</tr>
<tr>
<td>MK506</td>
<td>F83005.5</td>
<td>28.7 g</td>
<td>7.18</td>
<td>0.479</td>
<td>873.935 c</td>
</tr>
<tr>
<td>MK511</td>
<td>Jemalong A17</td>
<td>37.6 fg</td>
<td>4.27</td>
<td>0.025</td>
<td>874.823 b</td>
</tr>
<tr>
<td>MK511</td>
<td>DZA315.16</td>
<td>31.3 fg</td>
<td>11.63</td>
<td>0.035</td>
<td>874.906 ab</td>
</tr>
<tr>
<td>MK511</td>
<td>F83005.5</td>
<td>20.5 g</td>
<td>4.21</td>
<td>ND(^{f})</td>
<td>874.859 b</td>
</tr>
<tr>
<td>NI (^{15})N(^{g})</td>
<td>Jemalong A17</td>
<td>125.1 a</td>
<td>16.20</td>
<td></td>
<td>872.178 h</td>
</tr>
<tr>
<td>NI (^{14})N</td>
<td>DZA315.16</td>
<td>92.2 bc</td>
<td>4.63</td>
<td></td>
<td>872.209 gh</td>
</tr>
<tr>
<td>NI (^{14})N</td>
<td>F83005.5</td>
<td>34.0 fg</td>
<td>12.64</td>
<td></td>
<td>872.220 gh</td>
</tr>
<tr>
<td>NI (^{15})N</td>
<td>Jemalong A17</td>
<td>43.1 efg</td>
<td>1.54</td>
<td></td>
<td>875.173 ab</td>
</tr>
<tr>
<td>NI (^{15})N</td>
<td>DZA315.16</td>
<td>43.0 efg</td>
<td>23.46</td>
<td></td>
<td>875.143 ab</td>
</tr>
<tr>
<td>NI (^{15})N</td>
<td>F83005.5</td>
<td>28.0 g</td>
<td>11.55</td>
<td></td>
<td>875.247 a</td>
</tr>
</tbody>
</table>
a Means of three repetitions. Means separation by Least Square Means. Means followed by a common letter are not significantly different for the strain × line interaction at P < 0.05.

b LFW = Leaf Fresh Weight. SD = Standard deviation

c Nitrogenase activity is expressed in nmol-1mg-1min-1.

d Incorporation of $^{15}$N into pheophytin is expressed as mass charge-1 (m z-1) ratio. SD = Standard deviation.

e Ndf = Nitrogen derived from fixation.

f ND = no determined

g NI = Uninoculated plants
Table 6. Differential symbiotic performance of *S. meliloti* citrate synthase mutants in *M. truncatula* lines growing in 50 ml tubes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Line</th>
<th>NFW(^b) (mg plant(^{-1}))</th>
<th>SD</th>
<th>LFW(^c) (mg plant(^{-1}))</th>
<th>SD</th>
<th>Nitrogenase(^d) (nmol•mg•min(^{-1}))</th>
<th>SD</th>
<th>M(_{\text{pheophytin}})(^e) (m z(^{-1}))</th>
<th>SD</th>
<th>Ndf(^f) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>104A14</td>
<td>Jemalong A17</td>
<td>11.8 a</td>
<td>3.14</td>
<td>119.9 a</td>
<td>26.65</td>
<td>0.253 a</td>
<td>0.185</td>
<td>873.242 a 0.204</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>104A14</td>
<td>DZA315.16</td>
<td>12.5 a</td>
<td>3.44</td>
<td>89.3 b</td>
<td>22.30</td>
<td>0.417 b</td>
<td>0.196</td>
<td>873.037 a 0.249</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>MK506</td>
<td>Jemalong A17</td>
<td>6.9 b</td>
<td>1.89</td>
<td>74.4 bc</td>
<td>18.34</td>
<td>0.204 a</td>
<td>0.096</td>
<td>874.004 b 0.383</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>MK506</td>
<td>DZA315.16</td>
<td>11.8 a</td>
<td>4.78</td>
<td>87.3 b</td>
<td>23.64</td>
<td>0.445 b</td>
<td>0.146</td>
<td>873.259 a 0.299</td>
<td>70</td>
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</tr>
<tr>
<td>NI (^{14}) N</td>
<td>Jemalong A17</td>
<td>0.0 c</td>
<td>60.1 c</td>
<td>8.99</td>
<td>0.000 c</td>
<td>872.229 c</td>
<td>0.019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI (^{14}) N</td>
<td>DZA315.16</td>
<td>0.0 c</td>
<td>59.8 c</td>
<td>9.32</td>
<td>0.000 c</td>
<td>872.322 c</td>
<td>0.118</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI (^{15}) N</td>
<td>Jemalong A17</td>
<td>0.0 c</td>
<td>63.8 c</td>
<td>10.80</td>
<td>0.000 c</td>
<td>875.223 d</td>
<td>0.162</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI (^{15}) N</td>
<td>DZA315.16</td>
<td>0.0 c</td>
<td>53.3 c</td>
<td>8.95</td>
<td>0.000 c</td>
<td>875.493 d</td>
<td>0.191</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
a Means of six repetitions. Mean separation by Least Square Means. Means followed by a common letter are not significantly different for the strain × line interaction at P < 0.05.

SD = Standard deviation.

b NFW = Nodule Fresh Weight.

c LFW = Leaf Fresh Weight.

e Mean mass of pheophytin expressed as mass charge⁻¹ (m z⁻¹) ratio.

f Ndf = Nitrogen derived from fixation.

g NI = Uninoculated plants
DISCUSSION

In this study we have 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 used commonly to determine the effectiveness of a particular rhizobia strain.

In this study, measurement of $M_{\text{pheophytin}}$ was used as an index for estimating nitrogen fixation effectiveness in *M. truncatula* and it was the only parameter that detected differences in all experiments for line $\times$ strain interactions. Isotope dilution measurements have the advantage of measuring nitrogen incorporation directly and of integrating over a time period, days to weeks, which 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 $^{15}\text{N}$ 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. In order to achieve these objectives, a labeling technique proposed by Vallis et al. (1967) was followed, using multiple additions of small amounts of highly labeled fertilizer to the soil. Initial experiments revealed that many additions of Na$^{15}$NO$_3$ inhibited the symbiosis and that lowering the level of $^{15}$NO$_3$ compromised the sensitivity of the measurement (data not shown). 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 objective of this kind of study is to identify traits that correlate with effectiveness. For strains USDA1600 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 nitrogenase 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 (Grzemski et al. 2003). 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 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 (Grzemski et al. 2003).

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 $^{14}\text{N}/^{15}\text{N}$ 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 but we believe that the pheophytin assay is simple enough to allow us to investigate questions that have previously been outside the scope of a reasonable research effort.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

*M. truncatula* genotypes Jemalong A17 and DZA315.16 were obtained from Douglas Cook (UC Davis) and Thierry Huguet (INRA, France), respectively. Seeds were scarified in 5 volumes of concentrated sulfuric acid for 5 to 10 min. Acid was decanted and the seeds were rinsed in distilled water five times. Seeds were then treated with commercial bleach (approximately 5% [w/v] NaOCl) for 3 min, and 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. After this period, seeds were rinsed eight times with sterile water at room temperature. Treated seeds were germinated in the dark at room temperature on 1% water agar plates for 36 hours. Sterile seedlings were planted in-300 ml boxes containing LECA (Lightweight Expanded Clay
Aggregate, Eco Enterprises, Shoreline, WA) and sand as support material. The initial characterization experiment was performed in open 300 ml boxes and the procedure was adapted to carry out the experiments in a smaller scale in 50 ml conical bottom test tubes. Plants were fertilized at planting and at 13, 20, 27, 34, 41, 48 days after planting (dap) with Gibson’s nutrient solution (Gibson 1980) that contained 1 mM Urea 98% 15N atom (Isotec Inc., Miamisburg, OH). Plant nutrient solution was buffered with 10 mM 2-[N-Morpholino]ethanesulfonic acid (MES) to reduce acidification resulting from N₂ fixation and urea metabolism. Plants inoculated with wild type S. meliloti were harvested 55 dap. Plants inoculated with citrate synthase mutants were grown in water baths at 17°C, and fertilized at planting, 15 and 23 dap. These plants were harvested 37 dap to avoid contamination by faster-growing gltA+ revertants. 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 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 of S. meliloti**

S. meliloti strains USDA 1600, 2011, CC109, ABS7, RF22 and 12 were obtained from Thierry Huguet, Laboratory. Biologie Moleculaire des Relations Plantes-Microorganisms, at CNRS-INRA-France. Strain 102F65 and 102F51 were obtained from David Bezdicek in Crop Science at Washington State University. S. meliloti citrate synthase mutants, MK504, MK506 and MK511 were isolated from S. meliloti 104A14 by Wojciech Grzemski in our laboratory.
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 (MMNH₄) supplemented with 0.25% arabinose (Somerville and Kahn 1983). Cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall SA-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**

Nitrogen fixation was measured with acetylene reduction assays as described previously (Hardy et al. 1968). In short, roots of *M. truncatula* plants were rinsed in water to remove sand. Nodules were then picked from the roots, weighed and placed in a 10 ml glass serum vial. The vial was sealed with a rubber stopper and 1 ml of air was removed and replaced with 1 ml of acetylene using a 1 cc syringe. Acetylene was generated by adding calcium carbide (Sigma, St. Louis, MO) to water and trapping the gas formed. 50-µl samples were taken at 3, 6 and 9 min after acetylene addition using a 250-µl syringe (Hamilton Co., Reno, NV) and injected into a GC-8A gas chromatograph (Shimadzu, Columbia, MD) containing a Poropak N column and equipped with an SP4290 Integrator (Spectra Physics Inc., San Jose, CA). Ethylene gas standards were injected into the gas chromatograph as controls. Acetylene reduction rates were calculated using the following equation:

$$\frac{[(\text{Slope} \times A_c) + \text{Intercept}] 	imes (V/V_{\text{inj}})}{M \times T}.$$  

Slope and Intercept are calculated from a linear regression generated with the ethylene controls. $A_c$ is the area under the experimental ethylene peak, $V$ is the volume of the reaction vial (10,000
µl), \( V_{\text{inj}} \) is the volume of experimental injection (50 µl), \( M \) is the mass of experimental nodules in milligrams, and \( T \) is the time in minutes of exposure to acetylene. Acetylene reduction rates were expressed as nmole mg\(^{-1}\) min\(^{-1}\).

**Chlorophyll extraction and determination of nitrogen derived from fixation**

Chlorophyll was extracted in a procedure similar to that of Iriyama et al. (1974). All the leaves present in the plant at the moment of harvest were removed and placed in a 10-ml plastic tube. Approximately 1.2 ml of methanol was added and the leaves were placed at 4°C for one or two days to extract the chlorophyll. The resultant methanol solution was transferred to a new tube and centrifuged in a Brinkman microcentrifuge (Westbury, NY) for 30 s at 14,000 rpm to remove plant debris. One ml of the methanol supernatant was mixed with one-seventh volume of dioxane (145 µl) and a slightly larger volume of deionized water (180 µl). The resulting mixture was placed at -20°C overnight. The chlorophyll was pelleted by centrifugation in a Jouan MR22 centrifuge (Winchester, VA) at 12,000 rpm at 4°C for 15 min. The supernatant was discarded, the tubes were drained well and the chlorophyll pellet was resuspended in 200 µl of 1:1 methanol:acetone. Chlorophyll was converted to pheophytin by adding 1 µl 25 % v/v HCl to the sample. The resulting mixture was centrifuged through a 0.2 µm microspin filter (Alltech, Deerfield, IL). The filtered samples were directly analyzed on 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 seconds.

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

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

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

**ACKNOWLEDGEMENTS**

We thank Thierry Huguet (CNRS – INRA Toulouse, France) for providing us with $S.\ meliloti$ strains and $M.\ truncatula$ seeds of Jemalong and DZA315.16. We also thank the greenhouse team of the Institute of Biochemistry, Sue Vogtman and Julianna Gothard for their assistance in making available seeds of $M.\ truncatula$, Gerhard Munske for his assistance in the mass spectrometer. Finally, we thank Taryn Urion, Joseph Powers and David Kahn for help in the completion of the experiments presented in this study. This work was supported by the United States Department of Agriculture.
LITERATURE CITED


CHAPTER FOUR

Approaching the genetic basis of *Medicago truncatula* contribution to symbiotic effectiveness

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ABSTRACT

During the evolution of nitrogen fixation, the major benefit to the host plant is the opportunity to acquire nitrogen efficiently from the bacteria. The efficiency with which the fixed nitrogen is transferred to the plant is a factor that depends mainly on the legume – rhizobia combination. Two *Sinorhizobium meliloti* strains with different degrees of symbiotic performance on parental lines Jemalong A17 and DZA315.16 were evaluated on a recombinant inbred line (RIL) population generated by crossing these two lines. Segregation of N₂ fixation effectiveness was evaluated by measuring parameters such as shoot fresh weight, plant length and pheophytin mass. It has been showed previously that this last parameter is an excellent tool to measure the contribution of N₂ fixation to the nitrogen composition of chlorophyll. Two QTLs were identified for pheophytin mass in linkage group three. One QTL had a logarithm of odds (LOD) of 4.0 and was located around 3.4 cM, the second QTL identified in a different experiment lies around 14.6 cM and has an LOD of 7.6. One of these QTLs co-maps with a QTL for leaf fresh weight. Several QTLs for plant length were also identified in linkage groups seven and eight. They are considered part of the non-symbiotic phenotypic difference between parental lines Jemalong A17 and DZA315.16.
INTRODUCTION

Success in symbiotic nitrogen fixation is determined by coordinated expression of plant and bacterial genes to produce a root nodule environment in which bacteria reduce atmospheric N\textsubscript{2} to NH\textsubscript{4}\textsuperscript{+}, a process that is fueled by carbon-rich nutrients from the plant (Verma, 1998). The plant host can participate in symbiotic development in several ways, including recognition (Downie and Walker, 1999), invasion and nodule development (Schultze and Kondorosi, 1995), physiological functions within the mature nodule (Vance, 1997) and possibly through the suppression of host defense responses required for compatibility. While considerable progress has been made in understanding the way in which microbial genes and gene products contribute to nitrogen fixation, less progress has been made in understanding which plant genes contribute to symbiosis and how these genes act. In a variety of legume species, many mutations have been identified that block some aspect of nodulation and nitrogen fixation (Vance, 1997; Udvardi, 2002). However, only a few of these genes have been identified and there is little information about plant genes that lead directly to a more effective symbiosis.

Productivity of the symbiosis is often referred to as effectiveness, which is usually defined by measuring parameters related to the amount of nitrogen that symbiosis contributes to the plant (Danso, 1995). Correlation of plant genes with effectiveness is difficult due to the complexity of nitrogen fixation as a quantitative trait and the difficulty of implementing some assays of nitrogen fixation on a large enough scale to carry out quantitative genetics. A new mass spectrometry technique has been developed in our laboratory that measures the contribution that nitrogen fixation makes to the nitrogen composition of pheophytin, the Mg-free derivative of chlorophyll (Kahn et al., 2002). Pheophytin contains four nitrogen atoms. When plants are grown in \textsuperscript{15}N-urea, these nitrogen atoms can be derived either from fertilizer \textsuperscript{15}N or from \textsuperscript{14}N available
to the plant from nitrogen fixation. Dilution of the $^{15}$N by $^{14}$N is correlated with the amount of nitrogen coming from fixation. Operationally, this measurement is an index of the effectiveness of nitrogen fixation because nitrogen in pheophytin is derived directly from glutamate and therefore represents the plant nitrogen pool at the time of pheophytin synthesis. This method is an excellent tool for quantitative genetic studies because it measures nitrogen effectiveness with reasonable precision and it is simple enough that it can be used to evaluate the hundreds of samples needed for a genetic analysis of effectiveness as a quantitative trait.

Quantitative trait loci (QTL) mapping consists of identifying (through linked markers) the individual genetic factors influencing the value of a quantitative trait. To carry out a QTL analysis of effectiveness, we chose an alfalfa relative, Medicago truncatula, because it has a number of characteristics required for studying the molecular genetics of the Rhizobium-legume symbiosis (Barker et al., 1990; Cook, 1999; Galibert et al., 2001). Key attributes of M. truncatula include: diploidy and autogamous fertilization, a small genome (500-600 Mbp/1C) (Blondon et al., 1994), a rapid reproductive cycle, a high level of biodiversity, and a number of available cultivars. In addition, a set of more than 170,000 M. truncatula expressed sequence tags (ESTs) generated world-wide have been assembled in a database available to the public at http://www.medicago.org/MtDB (Lamblin et al., 2003). Seeds of M. truncatula are small enough that large numbers can be screened for symbiotic traits in limited space.

Recombinant inbred line populations of M. truncatula appropriate for a QTL study have recently been described (Thoquet et al., 2002). The advantages of using recombinant inbred lines (RILs) to study QTLs is discussed by Austin et al (1996). RILs undergo multiple cycles of meiosis before homozygosity is reached. After several generations, most loci are homozygous
and a given RIL will represent one of a large number of combinations of the original parental genes.

Jemalong A17 and DZA315.16 were originally chosen for genetic analysis based on their phenotypic and molecular properties. The two lines differ in pod shape, leaf pigmentation pattern, and other observable phenotypes. They have very similar DNA content (1.16 pg/2C). About 32% of PCR-amplified fragment bands are polymorphic in comparisons between Jemalong 6 and DZA315.16 (Thoquet et al., 2002). In this study and for creation of the RILs, Jemalong A17, which is considered as having an identical genotype as Jemalong 6 (Thoquet et al., 2002), was used. Single seeds from Jemalong A17 and from DZA315, an Algerian natural population, were selfed at least twice in order to increase homozygosity of the *M. truncatula* line because, in some natural populations, *M. truncatula* flowers occasionally cross-pollinate (<1%) (Bonnin et al., 2001).

In previous experiments, *S. meliloti* strains USDA 1600 and a citrate synthase mutant MK506 showed differential symbiotic performance on *M. truncatula* parental lines Jemalong A17 and DZA315.16. When Jemalong A17 was inoculated with USDA 1600, 63% of pheophytin nitrogen was from symbiotic nitrogen fixation, while DZA315.16 received only 44% from the bacteria. Conversely, when inoculated with MK506, Jemalong A17 obtained 30% from fixation and DZA315.16 received 60% (article in preparation). These two *S. meliloti* strains were used to evaluate how nitrogen fixation effectiveness segregated in a *M. truncatula* population of 93 RILs generated from the cross between Jemalong A17 and DZA315.16. This RIL population was generated by T. Huguet in collaboration with Jean-Marie Prosperi (SGAP-INRA, France) (Thoquet et al. 2002).
In this study, we identified several loci in the RILs that correlate with the variability of pheophytin mass, leaf fresh weight and plant length. This study represents, to our knowledge, the first approach to study the effectiveness of N\textsubscript{2} fixation in *Medicago truncatula* using QTL analysis.
RESULTS

Experimental strategy for RILs tested with *S. meliloti* USDA 1600

After obtaining seeds of each RIL from Dr. Prosperi, increasing the number of seeds available for the 93 RILs became a limiting step. Due to the low germination percentage for some of the lines and to assure the evaluation of all the 93 RILs in the growth space available to us, we initially decided for experiment one to include seven independently grown sets of plants per RIL and evaluate all the RILs in three separate sets as explained in Materials and Methods. The three sets of RILs were grown with overlapping generations but all plants from one RIL were handled at the same time. Several sets of parental and uninoculated controls were present to overlap the entire growth period. Robust plantlets were chosen after sowing in order to decrease the heterogeneity caused by differences in the early stages of plant development. In experiments two and three only 81 and 84 RILs were evaluated, respectively. Among the group of RILs not evaluated in experiments two and three were lines 35, 38, 49, 51, 96 and 108 that we could not obtained enough seeds in our facilities and additional seeds of those lines obtained from Dr. Prosperi did not germinate.

Phenotypic variation and QTL analysis for USDA 1600

A summary of the analysis of variance for line × strain interaction for experiments testing USDA 1600 is presented in Tables 1 and 2. The means for leaf weight, plant length and pheophytin mass for the RILs were significantly different for line × strain interaction in all experiments except for set three of experiment one. In experiment two, the difference among means for pheophytin mass was statistically significant only at the 5% level. In this experiment, the symbiotic performance of USDA 1600 on parental lines of *M. truncatula* differed from the
results obtained in the other two experiments (Fig. 2). In this case, Jemalong A17 had a $^{15}\text{N}$ incorporation value higher than DZA315.16, indicating more $\text{N}_2$ fixation in DZA315.16. No QTLs were identified for pheophytin mass (Table 3) in experiment two. Values for the coefficient of determination ($R^2$) for all the traits indicated that there was some degree of variability within repetitions. For the traits followed in these experiments, the coefficient of variation (CV) for pheophytin mass had the lowest values in all the experiments, indicating a higher degree of precision for comparison using this index (Tables 1 and 2).

A histogram showing the distribution of the phenotypic variation in the RILs is presented for each trait on Figures 1, 2 and 3. The distributions for pheophytin mass and leaf fresh weight showed wide ranges of variation from experiment to experiment. In contrast, the phenotypic distributions for plant length were similar among experiments with a range from 9 cm to 37 cm. The mean values for the parental lines in each of the traits measured are indicated for all experiments in Figures 1, 2 and 3. Mean separation for all traits by Least Squares Means at 5% level indicated no difference among the parental lines for any of the traits in any of the experiments except for plant length in experiment one which was significantly different between parental lines in all the three sets (data not shown). *M. truncatula* parental lines showed a similar trend among experiments for leaf fresh weight values, these being higher for DZA315.16 compared to Jemalong A17. In contrast, plant length values were higher for Jemalong A17 compared to DZA315.16 (Fig 1, 2, 3). Pheophytin mass values for parental lines in experiments one and three, showed higher values for DZA315.16 than for Jemalong A17.
Table 1. Summary of the analysis of variance for RILs inoculated with *S. meliloti* USDA 1600 in experiment 1 evaluated in three sets

<table>
<thead>
<tr>
<th>Set No.</th>
<th>Trait</th>
<th>Mean</th>
<th>F Value</th>
<th>DF 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DF 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2d&lt;/sup&gt;</th>
<th>CV&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leaf Weight</td>
<td>401.37</td>
<td>4.84 ***</td>
<td>32</td>
<td>173</td>
<td>97.29</td>
<td>0.47</td>
<td>24.24</td>
</tr>
<tr>
<td></td>
<td>Plant Length</td>
<td>25.77</td>
<td>12.54 ***</td>
<td>32</td>
<td>173</td>
<td>4.41</td>
<td>0.70</td>
<td>17.13</td>
</tr>
<tr>
<td></td>
<td>Pheophytin Mass</td>
<td>872.75</td>
<td>3.59 ***</td>
<td>32</td>
<td>173</td>
<td>0.18</td>
<td>0.40</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>Leaf Weight</td>
<td>206.41</td>
<td>5.70 ***</td>
<td>47</td>
<td>224</td>
<td>63.15</td>
<td>0.54</td>
<td>30.59</td>
</tr>
<tr>
<td></td>
<td>Plant Length</td>
<td>22.50</td>
<td>13.16 ***</td>
<td>47</td>
<td>224</td>
<td>4.60</td>
<td>0.73</td>
<td>20.46</td>
</tr>
<tr>
<td></td>
<td>Pheophytin Mass</td>
<td>873.19</td>
<td>6.00 ***</td>
<td>47</td>
<td>224</td>
<td>0.33</td>
<td>0.56</td>
<td>0.04</td>
</tr>
<tr>
<td>3</td>
<td>Leaf Weight</td>
<td>283.69</td>
<td>6.23 ***</td>
<td>19</td>
<td>112</td>
<td>61.17</td>
<td>0.51</td>
<td>21.56</td>
</tr>
<tr>
<td></td>
<td>Plant Length</td>
<td>19.75</td>
<td>9.54 ***</td>
<td>19</td>
<td>112</td>
<td>4.32</td>
<td>0.62</td>
<td>21.86</td>
</tr>
<tr>
<td></td>
<td>Pheophytin Mass</td>
<td>872.81</td>
<td>1.65</td>
<td>19</td>
<td>112</td>
<td>0.27</td>
<td>0.22</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*** F test is significant at 0.01% level.

<sup>a</sup> DF 1 Degrees of Freedom (treatments – 1)

<sup>b</sup> DF 2 Degrees of Freedom (number of observations - 1) – (treatments – 1)

<sup>c</sup> SD Standard Deviation

<sup>d</sup> R<sup>2</sup> Coefficient of determination.

<sup>e</sup> Coefficient of variation (CV).
Table 2. Summary of the analysis of variance for RILs inoculated with *S. meliloti* USDA 1600 in experiments two and three

<table>
<thead>
<tr>
<th>Exp</th>
<th>Trait</th>
<th>Mean</th>
<th>F Value</th>
<th>DF 1</th>
<th>DF 2</th>
<th>SD</th>
<th>R²</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf Weight</td>
<td>290.6</td>
<td>2.84 ***</td>
<td>82</td>
<td>158</td>
<td>63.57</td>
<td>0.60</td>
<td>21.87</td>
</tr>
<tr>
<td>2</td>
<td>Plant Length</td>
<td>20.88</td>
<td>7.38 ***</td>
<td>82</td>
<td>158</td>
<td>4.40</td>
<td>0.79</td>
<td>21.10</td>
</tr>
<tr>
<td></td>
<td>Pheophytin Mass</td>
<td>872.91</td>
<td>1.40 *</td>
<td>82</td>
<td>158</td>
<td>0.34</td>
<td>0.42</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Leaf Weight</td>
<td>237.27</td>
<td>2.68 ***</td>
<td>85</td>
<td>167</td>
<td>64.52</td>
<td>0.58</td>
<td>27.19</td>
</tr>
<tr>
<td>3</td>
<td>Plant Length</td>
<td>22.98</td>
<td>7.75 ***</td>
<td>85</td>
<td>167</td>
<td>4.41</td>
<td>0.80</td>
<td>19.21</td>
</tr>
<tr>
<td></td>
<td>Pheophytin Mass</td>
<td>873.46</td>
<td>2.39 ***</td>
<td>85</td>
<td>167</td>
<td>0.47</td>
<td>0.55</td>
<td>0.05</td>
</tr>
</tbody>
</table>

***, * F test is significant at 0.01% and 5% levels, respectively

a DF 1 Degrees of Freedom (treatments – 1)

b DF 2 Degrees of Freedom (number of observations - 1) – (treatments – 1)

c SD Standard Deviation

d R² Coefficient of determination.

e Coefficient of variation (CV).
Fig 1. Trait distribution from the cross Jemalong A17 x DZA315.16 inoculated with

*S. meliloti* USDA 1600 - Experiment 1

Mean values for the recombinant inbred lines (RILs, open arrows) as well as the parental lines, Jemalong A17 (J) and DZA315.16 (D) (black arrows).
Fig 2. Trait distribution from the cross Jemalong A17 x DZA315.16 inoculated with

*S. meliloti* USDA 1600 - Experiment 2

Mean values for the recombinant inbred lines (RILs, open arrows) as well as the parental lines, Jemalong A17 (J) and DZA315.16 (D) (black arrows).
Fig 3. Trait distribution from the cross Jemalong A17 x DZA315.16 inoculated with

* S. meliloti USDA 1600 - Experiment 3 *

Mean values for the recombinant inbred lines (RILs, open arrows) as well as the parental lines, Jemalong A17 (J) and DZA315.16 (D) (black arrows).
A summary of the results from QTL analyses is presented in Table 3. These analyses were performed using composite interval mapping (CIM) analysis carried out with QTL Cartographer version 1.17 (Basten et al., 1994; Basten et al., 2000) as explained in Materials and Methods. QTL mapping was performed to determine the QTL(s) that are involved in the effectiveness of nitrogen fixation. QTL analyses for experiments one and three revealed the presence of a QTL for pheophytin mass on linkage group 3. In experiment one, the QTL found at 3.4 cM has an LOD = 4.0 and 17.6% of the variability is explained by the QTL. In experiment three, the QTL was located at 14.6 cM with an LOD = 7.6 and explained 21.4% of the variability. A LOD score of 4.0 means that there is a 1 in 10,000 chance of the association occurring by chance. The additive effect of this major QTL was negative in both experiments, which indicates that substituting a DZA315.16 allele for a Jemalong A17 allele would be predicted to cause a 0.18 point decrease in pheophytin mass in experiment one and 0.26 point decrease in pheophytin mass in experiment three. A closer look at the alleles present in the RILs in the QTL region of 3.4 and 14.6 cM on linkage group 3 revealed that 75/92 (81%) of the RILs in experiment one and 64/81 (79%) of the RILs in experiment three carried a DZA315.16 allele, much higher than would be expected if allele assortment was random. This exceptional distribution of parental alleles in the RILs might have an effect on the QTLs detected (Fig 4). Thoquet et al. (2002) reported a linear gradient of distortion on linkage group 3 ranging from Mendelian equality of male (DZA315.16) and female (Jemalong A17) markers to a 5-fold frequency in favor of male alleles in an F2 population from the cross Jemalong x DZA315.16. The standard deviation of the distribution of the range of effectiveness measurements for a single line was about 0.2 mass units in experiment one and 0.3 mass units for experiment three. Figure 5 shows the graphical output from QTL Cartographer depicting the location of the QTL detected.
in experiments one and three, which are separated by 11.2 cM. The threshold levels estimated with 1,000 permutations are indicated for each experiment. The estimation of a significance threshold is based upon the observation of marker-phenotype association. The trait values are randomly shuffle over individuals (marker genotypes), generating a sample with the original marker information but with trait values randomly assigned over genotypes. A significant linkage of the marker and a QTL with 5% probability indicates that there is statistical evidence that this linkage would be expected to occur 0.05 times in a genome.

There are only 13 lines that are genetically mosaic through the regions in linkage group three where QTLs related to pheophytin mass were detected. In experiment one, only one line of these 13 lines are present under the peak for distribution of effectiveness (Figure 4). In experiment three, none of these 13 lines is present under the highest peak for distribution of effectiveness (Figure 4). This suggests that there are other genes that will interact to detect these QTLs for pheophytin mass in linkage group 3. Both experiments contained all these 13 representative lines, except for two lines that were missing in experiment two. The identification of a QTL in the initial region of chromosome 3 in two different experiments indicates the presence of one to several QTLs that affect pheophytin mass (Figure 5).
Table 3. Map positions and effect of QTLs detected in *M. truncatula* RILs inoculated with *S. meliloti* USDA 1600

<table>
<thead>
<tr>
<th>Exp</th>
<th>Trait</th>
<th>Number QTLs</th>
<th>Linkage Group</th>
<th>Nearest Marker</th>
<th>(cM) (^a)</th>
<th>LOD</th>
<th>(r^2) (^b)</th>
<th>(a) (^c)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Pheophytin</td>
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<td>3</td>
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<tr>
<td></td>
<td>Mass</td>
<td>1</td>
<td>7</td>
<td>PE23</td>
<td>157.5</td>
<td>3.3</td>
<td>15.7</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Length</td>
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<td>7</td>
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<td>10.2</td>
<td>22.5</td>
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<tr>
<td></td>
<td></td>
<td>1</td>
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<td></td>
<td></td>
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<td>7</td>
<td>PE23</td>
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<td></td>
<td></td>
<td></td>
<td>MTIC113</td>
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<td>6.4</td>
<td>-2.06</td>
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<td>21.4</td>
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<td>8</td>
<td>enod20</td>
<td>11.1</td>
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<td>12.1</td>
<td>0.15</td>
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<td></td>
<td>Length</td>
<td>2</td>
<td>7</td>
<td>VR</td>
<td>103.2</td>
<td>8.7</td>
<td>38.3</td>
<td>-4.43</td>
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<td></td>
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<td>46.0</td>
<td>-4.93</td>
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<td>22.6</td>
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<td></td>
<td></td>
<td></td>
<td>MTIC113</td>
<td>131.7</td>
<td>5.3</td>
<td>25.1</td>
<td>-3.61</td>
</tr>
</tbody>
</table>

Leaf Weight

Leaf Weight
a Expressed in Kosambi cM from north of the linkage group.

b Percentage of variance explained by the QTL at the test site conditioned on the background markers and any explanatory variables.

c The additive effect, $a$, is the effect of replacing one DZA315.16 allele with one Jemalong A17 allele. By convention, the effect of the DZA315.16 allele relative to the Jemalong A17 allele at each locus then represents the sign of the allelic effect (positive or negative effect QTL).
Fig 4. Distribution of effectiveness in interaction of USDA 1600 with Jemalong A17 x DZA315.16 RILs

![Graph showing distribution of effectiveness](image-url)

Legend:
- LG3J Exp 1
- LG3D Exp 1
- LG3D Exp 3
- LG3J Exp 3

Number of RILs vs Mass of Pheophytin - 872
Fig 5. LOD-score plots showing a putative QTL associated with pheophytin mass located in linkage group 3
Several QTLs related to plant length were identified in linkage groups 7 and 8. A QTL for plant length found at 153.3 cM in linkage group 7 in experiment one was also found in experiment three. Analysis of experiments one and two also revealed a common QTL located at 85.5 cM in linkage group 8. A QTL that lies at 131.7 cM in linkage group 8 was also found in experiments two and three. The allelic effect of the QTLs for plant length was negative in all experiments, indicating that replacement of the Jemalong A17 allele with the DZA315.16 allele would lead to decreased plant length. Only one QTL, detected in experiment two, showed a positive allelic effect. This QTL is in linkage group 1, lies at 139.2 cM and it was detected only in experiment two (Table 3). The consistent detection of QTLs for plant length in linkage groups 7 and 8 served as an internal control for the QTL analyses.

Only one QTL was identified for leaf fresh weight, in experiment three. This QTL was located at 14.6 cM in linkage group 3, had an LOD of 4.1 and explained 19.6% of the variability. This QTL co-located with the QTL detected for pheophytin mass. Phenotypic correlations among the traits are presented in Table 4. Only experiments two and three were included in the correlation analysis since the experimental design allowed all the RILs to be evaluated at the same time. The strongest correlation was found between leaf fresh weight and pheophytin mass. Plants with high pheophytin mass (low ¹⁴N fixation) were always correlated with low leaf weight. On the other hand, plant length was not correlated with leaf fresh weight and was not affected by the amount of ¹⁵N incorporation.
Table 4. Phenotypic correlations among traits for experiments two and three\(^{a}\).

<table>
<thead>
<tr>
<th></th>
<th>Plant Length</th>
<th>Leaf Weight</th>
<th>Pheophytin Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Length</td>
<td>1</td>
<td>0.21 **</td>
<td>-0.16 *</td>
</tr>
<tr>
<td>Leaf Weight</td>
<td>0.39 ***</td>
<td>1</td>
<td>-0.57 ***</td>
</tr>
<tr>
<td>Pheophytin Mass</td>
<td>-0.32 ***</td>
<td>-0.69 ***</td>
<td>1</td>
</tr>
</tbody>
</table>

\(***\), \(**\) and \(*\) Significantly different at the 0.1\%, 1\% and 5\% level, respectively.

\(^{a}\) Values above the diagonal correspond to trait correlations in experiment 2. Values below the diagonal correspond to trait correlations in experiment 3.
Experimental strategy for RILs tested with *S. meliloti* MK506

*S. meliloti* MK506 was included in this study because it also produced differences in effectiveness between the *M. truncatula* parental lines. Measurements of pheophytin mass showed that *S. meliloti* MK506 was more effective with parental line DZA315.16 than with Jemalong A17 (Figure 6). This superiority of DZA315.16 was different than the situation observed with *S. meliloti* USDA 1600, where Jemalong A17 was more effective. The experimental design to test strain MK506 on the population of RILs had some constraints. Plants were harvested at 37 dap because, after this time, revertants or pseudorevertants of the citrate synthase mutation in MK506 can become numerous enough to affect the results. When these sets of experiments were planned, the number of seeds for some of the RILs was insufficient to do the experiment a second time. For this reason, experiment one tested 92 RILs but experiment two tested only 76 RILs. In addition, *S. meliloti* MK506 did not nodulate 64% of the plants in experiment two. For this reason, results from experiment two will not be presented. Experiments testing the survival of *S. meliloti* MK506 during the preparation for inoculation showed that bacteria did not grow very well in the final suspension solution of 3:1 v/v Gibson’s nutrient solution.

In order to assure that experiment one contain all the lines, seven repetitions per line were considered and the RILs were evaluated in separate sets as explained in Materials and Methods.

Phenotypic variation and QTL analysis for *S. meliloti* MK506

A summary of the ANOVA of *S. meliloti* MK506 on 92 RILs is presented in Table 5. The RILs were evaluated in three separate sets. There were significant differences for line × strain interaction among the means at the 0.01% level for all of the sets. The coefficient of
determination \((R^2)\) for the pheophytin mass was higher compared to experiments with USDA 1600. This indicated that a higher proportion of the variability for this trait was accounted for by the RILs. The coefficient of variation \((CV)\) for all traits had similar values to those obtained with USDA 1600. Mass of pheophytin had the lowest CV values, giving a good index of the reliability of the experiment.

Phenotypic distributions for pheophytin mass, leaf fresh weight and plant length are presented in Figure 6. There were high values for pheophytin mass in all the RILs compared to the ones observed with USDA 1600. There were no significant differences in pheophytin mass between parental lines. However, the highest value was from Jemalong A17, consistent with previous results. Mean separation by Least Square Means at 5% level showed significant differences in plant length between parental lines (data not shown). Jemalong A17 showed higher values for plant length and leaf fresh weight than DZA315.16. However, these values were low, compared to the range of values obtained with USDA 1600. The highest value obtained for leaf weight was 140 mg plant\(^{-1}\) compared to 550 mg plant\(^{-1}\) obtained with USDA 1600. The phenotypic distributions showed that \(S. meliloti\) MK506 fixed less nitrogen than USDA 1600.
Table 5. Summary of the analysis of variance for RILs inoculated with *S. meliloti* MK506

**Experiment evaluated in three sets**

<table>
<thead>
<tr>
<th>Set No.</th>
<th>Trait</th>
<th>Mean</th>
<th>F value</th>
<th>DF 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DF 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2d&lt;/sup&gt;</th>
<th>CV&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf Weight</td>
<td>104.92</td>
<td>12.62 ***</td>
<td>37</td>
<td>214</td>
<td>19.6</td>
<td>0.69</td>
<td>18.72</td>
</tr>
<tr>
<td>1</td>
<td>Plant Length</td>
<td>9.17</td>
<td>16.69 ***</td>
<td>37</td>
<td>214</td>
<td>1.8</td>
<td>0.74</td>
<td>19.44</td>
</tr>
<tr>
<td></td>
<td>Pheophytin Mass</td>
<td>874.44</td>
<td>25.56 ***</td>
<td>37</td>
<td>214</td>
<td>0.4</td>
<td>0.82</td>
<td>0.04</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Leaf Weight</td>
<td>85.84</td>
<td>8.77 ***</td>
<td>36</td>
<td>193</td>
<td>21.0</td>
<td>0.62</td>
<td>24.44</td>
</tr>
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<td>2</td>
<td>Plant Length</td>
<td>7.68</td>
<td>18.14 ***</td>
<td>36</td>
<td>193</td>
<td>1.8</td>
<td>0.77</td>
<td>23.88</td>
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<td>14.82 ***</td>
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<td>193</td>
<td>0.4</td>
<td>0.73</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Leaf Weight</td>
<td>89.81</td>
<td>4.22 ***</td>
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<td>204</td>
<td>22.2</td>
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<td>3</td>
<td>Plant Length</td>
<td>6.88</td>
<td>17.35 ***</td>
<td>40</td>
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<td>1.3</td>
<td>0.77</td>
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<td></td>
<td>Pheophytin Mass</td>
<td>874.46</td>
<td>12.52 ***</td>
<td>40</td>
<td>204</td>
<td>0.5</td>
<td>0.71</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*** F test is significant at 0.01% level.

<sup>a</sup> DF 1 Degrees of Freedom (treatments – 1)

<sup>b</sup> DF 2 Degrees of Freedom (number of observations - 1) – (treatments – 1)

<sup>c</sup> SD Standard Deviation

<sup>d</sup> R<sup>2</sup> Coefficient of determination.

<sup>e</sup> Coefficient of variation (CV).
Fig 6. Trait distribution from the cross Jemalong A17 x DZA315.16 inoculated with 

*S. meliloti* MK506

Mean values for the recombinant inbred lines (RILs, open arrows) as well as the parental lines, Jemalong A17 (J) and DZA315.16 (D) (black arrows).
Results of the QTL analysis of the 92 RILs evaluated with *S. meliloti* MK506 are summarized in Table 6. No QTLs were detected for pheophytin mass and only one QTL was detected for leaf fresh weight. For plant length, 5 QTLs were identified in linkage groups 1, 7 and 8. They were also identified in experiments with USDA 1600. The QTL in linkage group 7 lies at 151.3 cM and it has an LOD of 3.3. This particular QTL was also detected in two previous experiments with USDA 1600 with LODs of 10.2 and 8.5, respectively.

Three QTLs were identified in linkage group 8. Two of them lie at 85.5 and 118.1 cM and account for 34.8 and 39% of the variability explained by the QTL, respectively. The LOD score plot for this particular region of the chromosome showed a broad peak (data not shown). More RILs need to be evaluated to narrow the location of this QTL(s). The allelic effect for all QTLs identified for plant length was negative indicating that the Jemalong A17 allele causes a decrease in plant length.
Table 6. Map positions and effect of QTLs detected in *M. truncatula* RILs inoculated with *S. meliloti* MK506

<table>
<thead>
<tr>
<th>Trait</th>
<th>Number QTLs</th>
<th>Linkage group</th>
<th>Nearest marker</th>
<th>(cM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LOD</th>
<th>r&lt;sup&gt;b&lt;/sup&gt;</th>
<th>a&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>38</td>
<td>3.2</td>
<td>15.4</td>
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<sup>a</sup> Expressed in Kosambi cM from north of the linkage group.

<sup>b</sup> Percentage of variance explained by the QTL at the test site conditioned on the background markers and any explanatory variables.

<sup>c</sup> The additive effect, *a*, is the effect of replacing one DZA315.16 allele with one Jemalong A17 allele. By convention, the effect of the DZA315.16 allele relative to the Jemalong A17 allele at each locus then represents the sign of the allelic effect (positive or negative effect QTL).
DISCUSSION

In order to do quantitative genetics of a phenotype, the trait must be measurable with reasonable precision and accuracy. The mass spectrometry method for measuring $^{15}$N incorporation into pheophytin is potentially an excellent tool for measuring the contribution of rhizobial strains to nitrogen fixation (Kahn et al., 2002). The standard deviation of 5 replicate injections in the mass spectrometer is around 0.02 mass units. Because material preparation requires only a solvent extraction and precipitation of chlorophyll followed by acidification and removal of precipitates, the assay was predicted to be practical for measuring the hundreds of plants needed in each experiment for a genetic analysis. This study used a subset of 81 to 93 RILs from a population that has recently been expanded to 196 RILs. This subset was sufficient to give a preliminary view of the determinism of a given trait, though it has limitations in assigning precise map positions. For future work, the phenotyping data should include all 196 RILs and by that time, the genetic mapping information of these RILs should be completed.

Quality of genotyping is a key factor in quantitative genetic studies. The genetic map used here is a more developed version of the information presented by Thoquet et al, (2002) and will be presented elsewhere. It includes both anonymous and informative markers. Among the anonymous markers there are 195 amplified fragment length polymorphism (AFLP) and 11 inter-simple sequence repeats (ISSRs). AFLP markers are good for creating a core genetic map but they cannot be related to any sequence or gene, and reading an AFLP gel is not a simple task. There are 287 informative markers that originate from the sequence of a gene or a genome sequence typed as a microsatellite or as cleaved amplified polymorphic sequences (CAPS). In this case, data are usually clear and the chance of making reading errors is low but still exists.
S. meliloti strains USDA 1600 and citrate synthase mutant MK506 were chosen for these studies because, in preliminary experiments, they showed significant differences in symbiotic performance on M. truncatula parental lines Jemalong A17 and DZA315.16. S. meliloti USDA 1600 fixes more nitrogen with Jemalong A17 than with DZA315.16 but MK506 has the complementary tropism, fixing more nitrogen with DZA315.16 than with Jemalong A17. Phenotypic distributions of the RILs showed more extreme values than were seen in either parental line (Figures 1, 2 and 3). This situation might be explained by parental lines being fixed for sets of alleles having opposite effects. For example, one parental line might be fixed for +1 genes at locus 1 and -1 genes at locus 2 and the other parental line might be fixed for -1 genes at locus 1 and +1 genes at locus 2 (Lynch and Walsh, 1998). This type of explanation is the one supported by several QTL studies in Arabidopsis (Loudet et al., 2003), barley (Mickelson et al., 2003), rice (Li et al., 1995), soybean (Mansur et al., 1993), sunflower (Rachid et al., 2002), tomato (deVicente and Tanksley, 1993). However, parental line means for pheophytin mass were not significantly different in any of the three experiments in which S. meliloti USDA 1600 was evaluated, indicating a lack or reproducibility of the preliminary data and variation from experiment to experiment that was not as apparent among replicate plants within an experiment.

A total of five QTLs were identified among the three experiments testing USDA 1600 on the M. truncatula RILs. Three of these QTLs were identified only in one of the three experiments. One of these QTLs identified in experiment one is located around 157.5 cM in linkage group 7, and the other two QTLs identified in experiment three are located around 8.7 and 11.1 in linkage groups 1 and 8, respectively. These QTLs are considered an artifact since they were not detected in the other experiments. However, the QTLs detected in linkage group 3 for pheophytin mass around 3.4 cM (in experiment one) and around 14.6 cM (in experiment
three) are more likely to be real because they were identified in two different experiments and the LOD score plots overlap indicating the presence of a common region (Figure 5).

The additive effect of the two QTLs identified in linkage group three is negative, indicating that Jemalong A17 allele causes a decrease in pheophytin mass (Table 3). These results agree with the phenotypic distributions for pheophytin mass showing that the contribution of symbiotic nitrogen was higher for Jemalong A17. However, the allelic contribution at the particular loci in linkage group three indicates that DZA315.16 allele should be present to have good fixation (Figure 5). This is interesting since, DZA315.16 inoculated with S. meliloti USDA 1600 was associated with the opposite phenotype: high $^{15}$N incorporation and low $N_2$ fixation.

If there is a QTL near the end of linkage group 3, what does it mean that different map positions were found in experiments one and three? In carrying out the analysis, the LOD scores in these two experiments were elevated in two overlapping peaks, rather than as sharp, distinct assignments (Figure 5). This is consistent with the idea that the assigned map positions are the same within experimental error. In addition, the distribution of parental alleles at this end of chromosome 3 indicates that there is some departure from the predicted segregation behavior in this region. Examination of the alleles present in the RILs revealed that most RILs have a block of parental genes in this region—there are only 13 RILs where the allele at 3.4 is not from the same parent as the allele at 14.6. Thoquet et al. (2002) reported that there were regions where there were distortions in segregation that favored male alleles in the F2 population of M. truncatula. These distortions could suggest the presence of specific gene(s) interfering with meiosis or of linkage of traits that are most useful when present in the parental combination. The small number of RILs that actually can be used to locate traits in this region of linkage group 3
means that the precision of the mapping will be even less robust than would be predicted for the size of the population used.

All experiments testing the RILs with USDA 1600 evaluated most of the representative lines that contributed to the detection of a QTL in linkage group three. It is unclear why QTLs for pheophytin mass were not detected in experiment two. Possible explanations include environmental or inoculation factors. Although the two sets of RILs were grown at the same time and in the same growth chamber, there might be environmental differences within the growth chamber that influence the result. Differences in seed germination or inoculation might also have been important. We have encountered the same variability of results previously with the parental lines. However, a QTL can have a significant effect in all environments even in the presence of very significant genotype × environment interactions. Likewise, low power of detection can result in a QTL being detected in only some of the replicates of an experiment, even when its effects are identical across environments (Lynch and Walsh, 1998).

All experiments detected QTLs for plant length in linkage groups seven and eight. These QTLs are probably related to the non-symbiotic phenotype of the parents since Jemalong A17 and DZA315.16 have different shapes, with Jemalong A17 being taller than DZA315.16. One surprising result in our analyses was the predicted negative effect on plant length when a DZA315.16 allele would be replacing a Jemalong A17 allele (Table 3). This type of situation was reported by deVicente et al. (1993) as a transgressive segregation in a study with an F2 population of tomato (*Lycopersicum* L.). In their study, 36% of the QTLs had alleles that were associated with effects on the character that were contrary to the parental-line differences, e.g. alleles correlated with short plants contributed by parents from the taller line. An alternative explanation for this result is that there are epistatic interactions between different chromosomal
regions that lead to non-linear, and perhaps negative, effects (Lynch and Walsh, 1998). However, this option was not tested in our analysis.

There were no QTLs for pheophytin mass detected in the experiment with *S. meliloti* MK506. In addition to the obvious possibility that a single locus doesn't contribute enough to performance to generate a substantial LOD score, there are several possible reasons for this result. Nitrogen fixation performance of the citrate synthase mutant MK506 was lower than in previous experiments where the parental lines were tested (Chapter 3). It is possible that the detection of QTLs involved in nitrogen fixation effectiveness is very sensitive to the amount of $^{15}$N been absorbed by the plant. Another reason might be that the level of bacterial citrate synthase interacts in a direct or indirect manner with alleles linked to effectiveness affecting its expression in most of the RILs and therefore affecting the detection of the QTL(s).

Genetic analysis is a key factor in the reliability of quantitative genetic studies. If there are several QTLs affecting a particular trait, some will have a greater effect than others. QTL detection is biased toward genes with relatively large phenotypic effects and the methods used may underestimate the number of these. In order to rule out this possibility it would be important to test the 196 RILs population with more *S. meliloti* strains to determine if the QTL in linkage group three is found consistently or if there are more QTLs in other parts of the *M. truncatula* genome that can contribute to the effectiveness of nitrogen fixation.

The two markers that were linked to the QTLs for $^{15}$N incorporation detected in experiments one and two were MTIC 87 and MTIC128, respectively. MTIC markers are microsatellites identified within EST sequences. No function is known for these particular markers and it is not possible at this point to make any conclusions about candidate genes in this region. In order to do that, a larger number of RILs would need to be sampled and a genetic
analysis more focused on this region of linkage group 3 carried out, especially in lines that were isogenic except for variation in this region. Because effectiveness in rhizobia-legume interactions is often dependent on the matching between the symbionts, the question of whether this candidate locus would affect effectiveness with other strains would also need further experiments with additional *S. meliloti* strains to determine if the region has similar effects on a variety of symbioses and contains candidate genes for improving the effectiveness of N$_2$ fixation through selective breeding.

**CONCLUSIONS**

The results presented in this study indicate that the heritability of effectiveness, as measured by an isotope dilution assay, may be influenced by a small number of loci in *M. truncatula*. A region of linkage group 3 was tentatively identified as contributing to nitrogen fixation but, in order to confirm the presence of QTLs in linkage group 3, the experimental design needs to be expanded to include more *S. meliloti* strains and a larger population of RILs generated from the cross Jemalong A17 and DZA315.16. Additional genetic manipulations with the lines such as the generation of isogenic lines would allow us to solidify the conclusion that we have found a locus involved in effectiveness and establish the contribution of parental alleles. The potential materials for these isogenic lines are the RILs heterozygotes at the region where the QTL was located.

The data presented here shows that despite our attempts to reproduce growth conditions as closely as possible, considerable experiment to experiment variation exists. But within each experiment, it appears that the mass spectrometry assay provides a useful index of a parameter
directly associated with nitrogen fixation and that sufficient samples can be analyzed to carry out genetic analysis of sufficient numbers of plants to yield informative data.

MATERIALS AND METHODS

Plant Material and Growth Conditions

F7 seeds of 93 Medicago truncatula recombinant inbred lines (RILs) derived from the cross Jemalong 6 x DZA315.16 were provided by Dr. Jean-Marie Prosperi, INRA – SGAP Montpellier-France. For this study, we used F8 seeds obtained from the last generation of single-seed descent for the 93 lines.

Seed germination was performed as described in chapter three. Seeds were placed in sterile water at 4°C for four days to synchronize germination. During this period, water was replaced daily to help subsequent germination. Seedlings 1-2 cm in length were chosen for planting. Plants were grown in open 50 ml plastic tubes filled with lightweight expanded clay aggregate (LECA, Eco Enterprises, Shoreline, WA) and sand as support material. Two seedlings were planted per tube and, after 12 days, the smaller plant was removed leaving only one plant per tube in all the lines tested. Plants were fertilized with Gibson’s plant nutrient solution (Gibson, 1980), buffered with 10 mM 2-[N-Morpholino] ethanesulfonic acid (MES) to prevent acidification from both N₂ fixation and urea metabolism. The plant nutrient solution also contained 1 mM [¹⁵N]-urea (98 atom%, Isotec, Miamisburg, IL) that provides 2 mM ¹⁵N. Plant nutrient solution containing 1 mM [¹⁴N]-urea was used for control treatments, as indicated. Plants inoculated with S. meliloti USDA 1600 were fertilized on the day of planting and at 12, 18, 25, 32, 40 and 47 days after planting (dap); plants inoculated with S. meliloti strain 104A14 or MK506 were fertilized on the day of planting and at 15 and 23 dap. Plants were harvested at
55 days (USDA 1600) or 37 days (MK506) after planting. Seed increase of the 93 lines was carried out in a greenhouse under natural light supplemented with high pressure sodium-2000 watt lighting to give a photoperiod of 14 h light, 10 h dark. Temperature was controlled to give 25°C d/20°C night. Seeds were collected seven months after planting. Symbiosis experiments were carried out in a growth chamber in which plants were grown at 25°C under continuous light provided by a mixture of supersaver cool white F40CW/SS and Gro-Lux F40/GRO/AQ/WS bulbs (Sylvania, Canada).

The following treatments were present in all experiments: 93 RILs and as controls *M. truncatula* parental lines Jemalong A17 and DZA315.16 inoculated and not inoculated. Uninoculated *M. truncatula* parental lines were grown in the presence of either 1 mM [15N]urea or 1 mM [14N] urea. All other treatments received 1 mM [15N]urea.

**Statistical Design**

In experiment one using *S. meliloti* USDA 1600, the 93 RILs were evaluated in three separate sets, 31 RILs in the first one, 46 RILs in the second one and 18 RILs in the third set. Each set was organized in a completely randomized design with one-way treatment (RIL) structure and seven repetitions per treatment and the parental and uninoculated controls. For experiments two and three with *S. meliloti* USDA 1600, 81 and 84 RILs were evaluated, respectively. Plants were organized in a completely randomized design with one-way treatment structure, three replicate plants per treatment and harvested 55 dap. These experiments were run in parallel in order to assess consistency of the experimental results.
For experiment one evaluating *S. meliloti* MK506, 92 RILs were evaluated in three separate sets of 30 RILs, 29 RILs and 33 RILs respectively. Each set included seven repetitions per treatment and the parental and uninoculated controls.

**Growth and Inoculation of *S. meliloti***

*Sinorhizobium meliloti* strains used in this study were USDA 1600, from the collection of Thierry Huguet at CNRS-INRA, France, and *S. meliloti* MK506, a citrate synthase mutant of Rm104A14 isolated by Wojciech Grzemski in collaboration with Mike Kahn. *S. meliloti* USDA 1600 was grown for 5 days at 30°C on yeast mannitol broth and citrate synthase mutant MK506 was grown on minimal mannitol ammonia (MMNH₄) supplemented with 0.25% (w/v) arabinose. Cells were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall SA-rotor (Newtown, CT). Cells were then washed once with 0.85% (w/v) NaCl and the centrifugation was repeated. The pellet was resuspended in 3:1 (v/v) Gibson’s nutrient solution and 1 ml of this final solution was added per plant.

**Measured Traits**

Leaf fresh weight, plant length and incorporation of $^{15}\text{N}$ into pheophytin were measured in all experiments. Plants were cut 1 cm below the base of the cotyledon. Leaves attached to the plant at harvest were removed and leaf fresh weight was determined. Plant length was determined from the base of the cotyledon. Incorporation of $^{15}\text{N}$ into pheophytin was determined following the chlorophyll extraction protocol described in Kahn et al. (2002). Analysis of the [$^{15}\text{N}$]pheophytin was performed on 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 containing 90% (v/v) acetonitrile and 0.2% (v/v) formic acid. Solvent flow rate was 0.5 ml m\(^{-1}\). Run time under these conditions was about 30 s per injection.

**Statistical Analysis and QTL Mapping**

Analysis of variance (ANOVA) was performed using the General Linear Model procedure of SAS version 8.0 (SAS, 2001). Genetic marker information for the 93 RILs and their parents, Jemalong A17 and DZA315.16, was generated by Thierry Huguet at CNRS-INRA, France. This genetic map contains 495 markers comprising 195 amplified fragment length polymorphisms (AFLPs), 11 inter-simple sequence repeats (ISSRs) and 287 markers that include random amplified polymorphic DNA (RAPDs), cleaved amplified polymorphic sequence (CAPs) and microsatellites identified within expressed sequence tags (ESTs). The resulting *M. truncatula* genetic map spans 1081 cM. Map Manager QT was used to make linkage groups. All QTL analyses were performed using QTL Cartographer v 1.17 (Basten et al., 1994; Basten et al., 2000). The chromosomal location of QTLs was resolved by composite interval mapping (CIM). This model integrates two parameters for CIM: the number of markers that control the genetic background (\(n_m=7\)) and a window size (\(w=10\)) that will block out a region of the genome on either site of the markers flanking the test site (Basten et al., 1994). The inclusion of background markers makes the analysis more sensitive to the presence of QTLs in the target interval. The log of the odds (LOD) significance threshold was estimated from several permutation test analyses, as suggested by Churchill and Doerge (Churchill and Doerge, 1994). One thousand
permutations of phenotypic data were analyzed for each trait and the maximum threshold obtained (overall error level, 5%) was used for all traits.

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CHAPTER FIVE

Conclusions and Future Directions

In this study we established a method that measures the effectiveness of N$_2$ fixation by determining the $^{14}$N/$^{15}$N ratio in pheophytin. This method was used successfully to screen for *S. meliloti* strains that differ in effectiveness when grown with several different lines of *Medicago truncatula*. Two of these lines that have a large number of phenotypic and genotypic differences, Jemalong A17 and DZA315.16, were chosen by Thierry Huguet, in collaboration with Jean-Marie Prosperi (SGAP-INRA, France), to generate a population of 196 recombinant inbred lines (RILs). The availability and characteristics of these RILs plus the symbiotic polymorphism of the parental lines potentially made them a good population to study the quantitative genetics of N$_2$ fixation effectiveness. QTL analyses of three different experiments carried out with *S. meliloti* USDA1600 identified five QTLs for incorporation of $^{15}$N into pheophytin. Two of these were located in linkage group three and one of them co-maps with a QTL for leaf weight. Several QTLs for plant length were identified in linkage groups seven and eight and we hypothesize that these are related to a non-symbiotic phenotype of the parents.

As future directions for this project, it would be important to evaluate N$_2$ fixation effectiveness of other *S. meliloti* strains using the population of 196 RILs generated from the cross of Jemalong A17 and DZA315.16. There are three main categories of *S. meliloti* strains that need to be screened. The first category contains *S. meliloti* strains that have different effectiveness when grown with parental lines Jemalong A17 and DZA315.16. *S. meliloti* strain 102F51 was identified in our previous screenings as a strain that fixed more nitrogen in association with Jemalong A17 than with DZA315.16. Screening more strains is likely to identify others that differ in symbiotic performance on the parental lines. These strains would be
likely to differ in performance on the RILs as the set of genes affecting performance in the parents is reassorted in the progeny lines. An important question to be answered here is how QTLs identified for other bacterial strains correlate with each other. If effectiveness with many bacterial strains is similarly influenced by the same QTLs, this suggests that the QTLs would be useful in plant cultivar improvement. If, on the other hand, each strain interacts with a different set of QTLs, using these QTLs for breeding is not likely to be useful in a field situation.

While we took USDA1600 and MK506 from the set of strains that discriminate between parents, it would be very interesting to examine *S. meliloti* strains that do not discriminate in N₂ fixation between the *M. truncatula* parental lines. This would address the issue of whether gene reassortment in crosses between parents with similar effectiveness can generate progeny that differ in effectiveness. If so, this would suggest that the parental lines contain genes that both enhance and decrease effectiveness and that diversity in the field would be generated by segregating these traits. *S. meliloti* strain 1021 would be a logical first choice for the bacterial strain for this experiment, since its genome was recently sequenced. In addition, the Kahn laboratory is now generating a set of genetic tools for this strain that would allow bacterial genes to be manipulated to test for specific interactions with plant genotypes. With the use of hybridization array chips constructed in other laboratories for both the bacteria and the plant, it would be possible to study how gene expression might differ between highly effective and less effective lines.

A third category of experiments would involve further investigation of the citrate synthase mutants isolated from *S. meliloti* 104A14. Clearly, the problems with the second MK506 experiment suggest that further adjustment of the experimental protocol might be needed to obtain reproducible results. But the observation that differences in symbiotic effectiveness
can be revealed by using mutants with altered levels of citrate synthase might allow a correlation between specific plant traits with a known bacterial gene. For this analysis, microarrays of *M. truncatula* would be evaluated to determine what differences in gene expression correlate with the use of the different citrate synthase mutants. *S. meliloti* mutants with different levels of citrate synthase activities are available in our laboratory to be tested.

It is also important to determine if the measurement of incorporation of $^{15}$N into pheophytin is really evaluating symbiotic performance. While the only way for $^{14}$N to enter in contact with the plant growing in 50-ml tube is via nitrogen fixation, the parameter we actually measure is the ratio of $^{14}$N to $^{15}$N. If some RILs differ in how they metabolize $^{15}$N-urea, this could lead to differences in the ratio. Various possible interactions between urea metabolism and nitrogen fixation could be hypothesized but initially, it would be useful to test the RILs without inoculation under low (2 mM) and moderate (10 mM) nitrogen concentrations and to use different nitrogen sources labeled with $^{15}$N such as urea, NaNO$_3$ and NH$_4$Cl. QTL analysis of these experiments would detect the QTLs involved in nitrogen metabolism and it could be established whether they correlate with those identified under symbiotic conditions.

None of the parameters used to measure N$_2$ fixation was consistently correlated with pheophytin mass. While we think that, by its nature, a parameter that directly reflects nitrogen fixation is likely to be the best indicator of effectiveness, it would be useful to correlate this parameter with key enzymes previously hypothesized to be involved in symbiotic nitrogen metabolism such as glutamate synthase (GOGAT), aspartate amino transferase (AAT) and phosphoenolpyruvate carboxylase (PEPC).

If the experiments described above identify one or more QTL that are consistently linked to N$_2$ fixation effectiveness, the next step would be to establish more directly the importance of
particular alleles in effectiveness. There are three F6 lines heterozygous in the region of linkage
group three where the QTL for N₂ fixation effectiveness was located. If the trait under
consideration is heterozygous in these lines, further inbreeding can be used to generate RILs that
share a substantial fraction of their other genes but have one or the other allele of the QTL. These
almost isogenic lines would allow the hypothetical effectiveness locus to be tested without the
complication of other genetic variation, as in the experiments already performed. If lines with the
different alleles differ in effectiveness, crosses between them could be used to determine the
dominant/recessive relationship of the alleles. This would potentially open the way for
transgenic approaches to identify the genes themselves.

Although *M. truncatula* is a model plant, results identifying a gene for effectiveness
would likely have immediate use in breeding other legumes. Recent work indicates a relatively
high degree of microsynteny among legumes, so homologues to loci involved in N₂ fixation
effectiveness in *M. truncatula* could possibly be identified in other legumes and their importance
in these other plants could be determined.