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The efficiency of nitrogen fixation of the model legume *Medicago truncatula* (Jemalong A17) is low compared to *Medicago sativa*

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ABSTRACT

Medicago truncatula (Gaertn.) (barrel medic) serves as a model legume in plant biology. Numerous studies have addressed molecular aspects of the biology of *M. truncatula*, while comparatively little is known about the efficiency of N_2 fixation at the whole plant level. The objective of the present study was to compare the efficiency of N_2 fixation of *M. truncatula* to the genetically closely related *Medicago sativa* (L.) (alfalfa). The relative growth of both species relying exclusively on N_2 fixation versus nitrate nutrition, H_2 evolution, nitrogen assimilation, the concentration of amino acids and organic acids in nodules, and $^{15}N_2$ uptake and distribution were studied. *M. truncatula* showed much lower efficiency of N_2 fixation. Nodule-specific activity was several-fold lower when compared to *M. sativa*, partially as a result of a lower electron allocation to N_2 versus H^+. *M. truncatula* or *M. sativa* plants grown solely on N_2 fixation as a nitrogen source reached about 30% or 80% of growth, respectively, when compared to plants supplied with sufficient nitrate. Moreover, *M. truncatula* had low %N in shoots and a lower allocation of ^{15}N to shoots during 1 h $^{15}N_2$ labeling period. Amino acid concentration was about 20% higher in *M. sativa* nodules, largely as a result of more asparagine, while the organic acid concentration was about double in *M. sativa*, coinciding with a six-fold higher concentration of malate. Total soluble protein in nodules was about three times lower in *M. truncatula* and the pattern of enzyme activity in that fraction was strongly different. Sucrose cleaving enzymes displayed higher activity in *M. truncatula* nodules, while the activity of phosphoenolpyruvate carboxylase (PEPC) was much lower. It is concluded that the low efficiency of the *M. truncatula* symbiotic system is related to a low capacity of organic acid formation and limited nitrogen export from nodules.

Introduction

Barker et al. (1990) proposed *Medicago truncatula* (Gaertn.) cv. Jemalong as a model legume. Several factors favor this species for studies on molecular genetics of the legume–rhizobia symbiosis. In addition to its agronomic importance in e.g., Australia and the Mediterranean area, *M. truncatula* has a comparatively small genome (approximately 500 mbp) (Bennett and Leitch, 1995) that is now sequenced in large part (Ané et al., 2008). Moreover, the species is diploid and autogamous with a comparatively short generation time. These features favor *M. truncatula* in particular versus *Medicago sativa* (L.). However, the available genomic data reveal that genome synteny is highly conserved between the two species (Choi et al., 2004), and both are readily nodulated by *Sinorhizobium meliloti*. In studies on the biology and genomics of nitrogen fixation, the fully sequenced *S. meliloti* strain 1021 (or the closely related 2011) is most commonly used. Using this model symbiosis system, striking advances have been made in understanding the nodulation process. In fact, the genes involved in nodule signal transduction and the role of cytokinins in spontaneous nodulation have been revealed. Moreover, *M. truncatula* has also recently been used for studies on the genetic basis of tolerance to drought (Zhang et al., 2005) and salinity (Merchan et al., 2007).

By contrast, comparatively little is known on nitrogen fixation efficiency on a whole plant basis in *M. truncatula*. Several studies have shown wide variation in the agronomic performance of several *M. truncatula* lines (Parra-Colmenares and Kahn, 2005) and differences in the efficiency of combinations with different *S. meliloti* strains (Mhadhbi et al., 2005, 2009; Terpolilli et al., 2008). *M. truncatula* displays only slow growth and susceptibility to phosphorus toxicity when cultivated in nutrient solution (Tang et al., 2001, and our own observations). Recent studies have presented evidence for low symbiotic efficiency between

Abbreviations: AAT, aspartate aminotransferase; AI, alkaline invertase; ANA, apparent nitrogenase activity; CA, carbonic anhydrase; DM, dry matter; EAC, electron allocation coefficient; FW, fresh weight; MDH, malate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; SS, sucrose synthase; TNA, total nitrogenase activity

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M. truncatula cv. Jemalong (A17) and *S. meliloti* 1021 (Simsek et al., 2007; Terpolilli et al., 2008). In fact, Moreau et al. (2008) showed that nitrogen fixation cannot meet the nitrogen demand of the shoot with this combination.

The objective of the present research was to conduct a comparative study on nitrogen fixation efficiency between *M. truncatula* and *M. sativa* to reveal a possible physiological background for the apparent low efficiency in *M. truncatula*. The comparative approach was based on the use of the *S. meliloti* strain 2011. In addition, the strain 102F51 we included, which is known to be highly effective on *M. sativa*, with the objective to separate effects of the micro and macrosymbiont.

Materials and methods

Experimental design

The objective of the experiments was to compare nitrogen fixation efficiency between *Medicago truncatula* and *Medicago sativa*. For that purpose, two sets of experiments were performed. In the first experiment, plants of both species were grown for 49 days after emergence (DAE) on coarse quartz sand and depending either solely on N_2-fixation or nitrate nutrition for their nitrogen nutrition. To analyze nitrogen fixation efficiency in more detail, a second set of experiments were performed on plants grown in nutrient solution. At 62–64 DAE (subsequently 64 DAE), nitrogen fixation activity was measured through the determination of nodule H_2 evolution. Moreover, on parallel plants, $^{15}N_2$ uptake and distribution among plant organs was determined. Additional parallel plants were used for the collection of nodule material in which total protein, along with *in vitro* enzyme activities, sugars, amino acid and organic acid concentrations were measured. %N in the dry matter was determined in the organs of both species at various times during growth in nutrient solution. Nodule H_2 evolution and %N values were determined on plants in combination with two *Sinorhizobium meliloti* strains (2011 and 102F51) differing in efficiency, while the other parameters were determined on plants in combination with the fully sequenced, yet comparatively inefficient, strain 2011.

Biological material and plant growth

M. truncatula (Gaertn.) cv. Jemalong A17 (barrel medic) and *M. sativa* (L.) cv. Saranac (alfalfa) were studied in combination with *S. meliloti* 2011 or 102F51. Seeds of *M. sativa* were surface-sterilized with 70% ethanol for 10 min, washed in sterile water and germinated on fine quartz sand supplied with tap water to 70% of the maximum water holding capacity. *M. truncatula* seeds were soaked in concentrated H_2SO_4 for 5 min with intermittent gentle shaking. The acid was decanted and the seeds were rinsed thoroughly with sterile water five times. Subsequently, seeds were placed in 5% sodium hypochlorite for 3 min, followed by rinsing with sterile water eight times immediately after decanting the bleach. Following scarification and surface sterilization, seeds were placed in sterile water at 4 °C for 48 h. Subsequently, seeds were sown on fine quartz sand and inoculated. For inoculation, *S. meliloti* was grown in YEM to an approximate cell density of 10^7. First nodules became visible about 10 d after inoculation. Plants were maintained in a growth chamber with a 16/8 h day/night cycle at temperatures of approximately 25/18 °C and a relative humidity of about 70%. The light intensity was 360 μmol m^{-2} s^{-1} photosynthetic active radiation.

Growth experiment

In the first experiment, *M. truncatula* and *M. sativa* plants inoculated with *S. meliloti* 2011 were germinated on coarse quartz sand in PVC tubes (one plant per tube). 200 mL of a nutrient solution of the following composition (μM): pH 6.5, K_2SO_4, 700; KH_2PO_4, 15; $MgSO_4$, 500; $CaCl_2$, 800; H_3BO_3, 4; Na_2MoO_4, 0.1; $ZnSO_4$, 1; $MnCl_2$, 2; $CoCl_2$, 0.2; $CuCl_2$, 1; FeNaEDTA, 10, and 2 mM MES was supplied daily to the sand. Urea to a concentration of 0.2 mM was added for the first 10 DAE. Thereafter, the plants of the N_2 fixation treatment did not receive any external source of N, while in the nitrate treatment, $Ca(NO_3)_2$ was added to a concentration of 2 mM in the nutrient solution. Plants were harvested at 49 DAE and nodule number and shoot, root and nodule dry matter were determined.

Growth in nutrient solution

In a second experiment, plants were cultivated in a hydroponic system that allowed root/nodule gas exchange measurement and the application of a $^{15}N_2/O_2$ mixture (80/20%, v/v). Plantlets were transferred at 9 DAE from sand to nutrient solution in darkened glass cylinders (h=600 mm, inner diameter=20 mm) with rubber stoppers at both ends. The stalk base of each intact plantlet was carefully placed in a hole (diameter 5 mm) through the upper rubber stopper. The cylinder contained 250 mL of the N-free nutrient solution, including KH_2PO_4 in a concentration of 9 μM P. The nutrient solution was renewed daily at 09.00 am. Plants were held at their stem bases on the open side of the cylinder with sterilized sponge, leaving roots in the nutrient solution. Plants were inoculated with 1 mL of *S. meliloti* (102F51 or 2011) inocula (10^7 cells mL^{-1}) at transfer, and were reinoculated during the first 10 days at each solution change. The first nodules became visible five to seven days after inoculation. Urea to a concentration of 0.2 mM N was added to the nutrient solution during the initial 14 days of growth to avoid N-deficiency during nodule development. The solution was intensely aerated by an airflow of normal air of about 1 vol min^{-1} during the experiment. The pH of the nutrient solution was adjusted to 6.5. Evaporated solution was replaced by deionized water 1–3 times a day.

H_2 evolution measurements

For the H_2 evolution measurement, roots and nodules had to be enclosed in airtight gas cylinders, allowing us to apply a defined amount of air to flow through the cylinder and measure the composition of the outflowing gas. For this purpose, the hole in the upper rubber stopper was sealed with a plasticine material with high beeswax content. The beeswax gave the material a soft and pliable consistency that ensured a tight adherence to the rubber stopper and the plant stem. The material is not toxic to plants. Before sealing, stiff tubing to act as an inflow and outflow of the sealed root/nodule compartment was laid through the hole in the upper rubber stopper. The inflow tubing reached to the lower end of the glass cylinder, while the outflow was put above any nodules on the lower side of the upper rubber stopper.

The sealed root/nodule compartment was connected to an open flow gas exchange measurement system that allowed us to apply a mixture of N_2/O_2 (80/20%, v/v) to the root/nodule compartment. For the measurements, the nutrient solution level was lowered to about one-third of the gas cylinder, leaving the lower, virtually nodule-free part of the root system in the solution. An airflow of 200 mL min^{-1} (about 1.2 vol min^{-1}) was applied to the root compartment. A subsample (100 mL min^{-1}) of the outflowing gas was taken, dried (ice trap and $MgClO_4$) and passed through a H_2 analyzer (Qubit Systems, Canada). When a stable H_2 outflow from the root/nodule compartment was reached, this value was taken as apparent nitrogenase activity (ANA). Subsequently, the air composition in the inflowing