The model symbiotic association between *Medicago truncatula* cv. Jemalong and *Rhizobium meliloti* strain 2011 leads to N-stressed plants when symbiotic N$_2$ fixation is the main N source for plant growth

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Abstract

A better knowledge of the nitrogen nutrition of *Medicago truncatula* at the whole plant level and its modulation by environmental factors is a crucial step to reach a complete understanding of legume nitrogen nutrition. This study was based on the symbiotic system that is the most commonly used by the research community (*M. truncatula* cv. Jemalong A17×*Rhizobium meliloti* strain 2011). Plant nitrogen nutrition was analysed in relation to carbon nutrition, under a range of nitrate concentrations in the nutrient solution and different light conditions. This study shows that this ‘model symbiotic association’ does not allow the plant to meet its nitrogen requirements, when dinitrogen fixation is the main nitrogen source for plant growth. A strong interaction between nitrogen and carbon nutrition was shown: when plant nitrogen requirements were not sustained, plant leaf area was much affected whereas photosynthesis per unit leaf area remained relatively stable. Both total nitrogen uptake and leaf area increased with increasing nitrate concentration in the nutrient solution; the magnitude of these responses varied according to the light conditions. Interestingly, the plant nitrogen nutrition level remained nearly unaffected by the light conditions. The observed nitrogen-limitation in this ‘model symbiotic association’ is an important finding for the research community. Based on practical recommendations regarding both the experimental conditions and the phenotypic traits to consider, a methodological framework was proposed to (i) help genomicists to assess plant nitrogen nutrition better, and (ii) assist in the detection of new genetic variants affected for nitrogen uptake in large-scale phenotyping studies.

Key words: Carbon, genetic variability, growth, leaf area, *Medicago truncatula*, nitrate assimilation, nitrogen, phenotyping, symbiotic dinitrogen fixation.

Introduction

Due to their ability to establish a symbiosis with *Rhizobium* bacteria in root nodules, legumes have the capacity symbiotically to fix atmospheric dinitrogen (N$_2$). As a result, nitrogen (N) nutrition can rely on both the root assimilation of soil mineral N and on the symbiotic fixation of atmospheric N$_2$. In spite of this, N nutrition can be a limiting factor of seed and protein yields of legume crops such as pea (Dorcé, 1992). In order to better understand the genetic and molecular bases involved in the N nutrition of legumes, *Medicago truncatula* has been identified as a model plant (Barker et al., 1990; Cook, 1999; Colebatch et al., 2002; Stacey et al., 2006). Based on this species, the study of natural or induced variants (non-nodulating, non-fixing, and supernodulating mutants) has provided new insights into the processes involved in the establishment of the symbiosis with *Rhizobium* and in the regulation of the nodulation (Penmetsa and Cook, 1997; Wais et al., 2000; Penmetsa et al., 2003; Mitra et al., 2004; Schnabel et al., 2005). Isolating and analysing new genetic variants, for which other physiological functions involved in N nutrition are disrupted, has
become crucial in order to extend our knowledge of the N nutrition of legumes. However, whereas large efforts have been made to introduce and analyse genetic mutations, to our knowledge, no methodology has been developed for the large-scale phenotypic characterization of the genetic diversity. A good understanding of (i) how the N nutrition of *M. truncatula*, at the whole plant level, is modulated by environmental factors and (ii) how variations in N nutrition can affect plant growth traits is of major importance in allowing us to define both the environmental conditions and the phenotypic traits that should be used to perform a relevant phenotyping. Unfortunately, whereas *M. truncatula* N nutrition has been extensively studied at the genetic and molecular levels, studies characterizing the N nutrition at the whole plant level are still very scarce (Harper and Gibson, 1984; Ewing and Robson, 1990). In particular, cv. Jemalong (genotype A17) of *M. truncatula* is widely used in legume genomics work and, when this relates to biological N$_2$ fixation, this is often with regard to a symbiotic association with *Rhizobium meliloti* strain 2011 (Barker et al., 1990; Frugoli and Harris, 2001). Paradoxically, little is known about the plant N nutrition resulting from this ‘model symbiotic system’.

Conversely, for legume crops, plant N nutrition has been relatively well analysed. For these species, soil mineral N availability is commonly identified as the main environmental factor affecting the symbiotic fixation of N$_2$ and, consequently, plant N nutrition source (i.e. the relative contributions of root assimilation of soil mineral N and symbiotic fixation of N$_2$ to overall plant N uptake) (Streeter, 1988). Quantitative studies indicated that the sensitivity to soil mineral N or the availability of symbiotic fixation of N$_2$ could differ among species or genotypes within a species (Harper and Gibson, 1984; Streeter, 1988; Ewing and Robson, 1990). Generally, plant N requirements can be sustained whatever the N nutrition source (Sagan et al., 1993; Voisin et al., 2002). Nonetheless, for some species (e.g. soybean, Crozat et al., 1994; Gan et al., 2002), plant N requirements may not be fulfilled when symbiotic N$_2$ fixation is the exclusive N source, with consequences regarding the N nutrition level (indicating whether N nutrition is optimal, sub-optimal or supra-optimal for plant growth; Gastal and Lemaire, 2002).

Plant N nutrition is known to interact closely with plant carbon (C) nutrition (Minchin et al., 1981). For instance, when faced with N-limitation, the plant strikes a balance between allocation of N to maintain SLN and therefore photosynthesis per unit leaf area, and allocation of N to maintain total leaf area and therefore light interception (Sinclair and Horie, 1989). Moreover, it is known that symbiotic N$_2$ fixation can induce higher C requirements for the plant, as compared to nitrate assimilation (Silsbury, 1977; Ryle et al., 1979; Schulze et al., 1994, 1999); as a result, the C budget at the plant level can be unfavourable when plant N nutrition relies exclusively on symbiotic N$_2$ fixation (Minchin et al., 1981). Nevertheless, interactions between plant N and C metabolisms can be species- or genotype-dependent (Bethlenfalvay et al., 1978; Ryle et al., 1979; Radin, 1983; Vos et al., 2005). Accordingly, all this information clearly indicates that a complete understanding of legume N nutrition urgently requires the integration of the large set of data collected at different levels of organization, from the gene to the whole plant considered in its environment.

Focusing on the association between cv. Jemalong (genotype A17) of *M. truncatula* and *Rhizobium meliloti* strain 2011, the purpose of the present study was, first, to determine the impact of soil mineral N on total plant N uptake, and especially on the nodulation of the roots and plant symbiotic N$_2$ fixation. By applying the concept of the critical dilution curve of N into biomass (see details in the Materials and methods, and Gastal and Lemaire, 2002, for a review), our goal was then to determine to what extent symbiotic N$_2$ fixation allowed the plant to meet its N requirements. Finally, the interrelationships between C and N nutrition were analysed: (i) the relative impacts of N deficiency on plant leaf area expansion and photosynthetic activity were assessed, and (ii) the C costs associated with, respectively, symbiotic N$_2$ fixation and soil mineral N assimilation were compared. This paper was based on experimental data collected from greenhouse experiments in which plants were grown under a range of soil mineral N availability. Nitrate was the only source of soil mineral N considered in this study as it is usually the main form of N retrieved from the soil by plants (Harper, 1994). It has been established that the light conditions used during plant culture are very different between the various laboratories using *M. truncatula* (D Moreau, unpublished survey). As light conditions are a determinant in C assimilation with possible consequences on N nutrition, the experiments involved different light conditions. In this study, our goal was to fill the gap between the knowledge collected at the genetic and molecular levels and that collected at the whole plant level in order to put ‘omics’ information in their biological context. From our findings, a phenotyping framework methodology could be proposed. Based on practical recommendations regarding both the phenotypic traits and the environmental conditions to consider, it should henceforth facilitate the detection of new genetic variants of *M. truncatula* with different abilities to take up N.

**Materials and methods**

**Plant material and experimental treatments**

Two greenhouse experiments were conducted in Dijon (France). In Experiment 1, 12 treatments were generated (Table 1). Two genotypes were cultivated: A17 (cv. Jemalong) which was
inoculated with strain 2011 of *Rhizobium meliloti*, and a non-
nodulating mutant of Jemalong (Nod−; Morandi *et al.*, 2005) which
was used as a control line unable to form nodules and thus
symbiotically to fix N2. Both genotypes were grown with six
different nutrient solutions varying in their nitrate concentration
(from 0–10.5 mM). In Experiment 1, both A17 and Nod− were
grown in given light conditions (referred to as ‘no-shade’ for
concerns of homogeneity with the treatments of Experiment 2; see
below).

Several light conditions were investigated in Experiment 2. This
experiment consisted of six treatments (Table 1): inoculated A17
was grown with two nutrient solutions contrasted for their nitrate
concentration (0.625 mM and 10.5 mM), and under three levels of
incident photosynthetically active radiations (PAR) [no-shade, low-
shade (i.e. PAR reduced by 50%) and high-shade (i.e. PAR reduced
by 70%)], artificially generated using two types of net.

Both experiments were conducted under steady-state conditions
for mineral availability and were confined to the period from
sowing to the beginning of the reproductive phase, during which
roots and nodules classically establish (Voisin *et al.*, 2003a).

Cultural conditions
Plants were grown in open 1.0 l pots containing solid, inert and
draining substrate (made up of clay and attapulgite) with a density
of 50 pots m$^{-2}$. Seed preparation and sowing were performed as
in Moreau *et al.* (2006). A17 seeds were inoculated with *R. meliloti*
at sowing (with a non-limiting amount of bacteria). In agreement with
DG Barker *et al.* (http://www.noble.org/MedicagoHandbook/pdf/
GrowingMedicagotruncatula.pdf), the nutrient solutions contained
P, K, and oligo-elements, in addition to N (Table 2). They were
provided by automatic watering of pots several times a day at
a frequency allowing the pots to drain (frequency was increased with
increasing plant growth) in order to avoid the accumulation of
ions in the substrate.

In Experiment 1, additional light was supplied with sodium
lamps (MACS 400 W; Mazda, Dijon, France) to maintain a mean
daily photoperiod higher than 13 h (Table 1). In both experiments,
air temperature (PT100 sensors; Pyro-Controˆle, Vaulx-en-Velin,
France) and incident PAR (silicium sensors; Solems, Palaiseau,
France) measurements were made at 600 s intervals and stored in
a data logger (DL2e; Delta-T Devices, Cambridge, England)
(Table 1).

Plant growth traits
Plant growth was compared among treatments at two different
levels. First, plant leaf area (LI-3100 Area Meter, Li-Cor Inc.,
Lincoln, NE, USA), plant biomass (determined after drying during
48 h at 80 °C) and plant N (Dumas procedure) were all measured at
about 1000 degree-days after sowing (for calculation of thermal
time, see below). All these measurements were performed consider-
ing five and six plants per treatment, respectively, in Experiments 1
and 2. Second, for inoculated A17 plants in Experiment 1, the level
of nodulation was evaluated at different dates, using a visual scale
(see the legend of Fig. 2) that was established from previous
knowledge on agronomic legumes (N Amarger, personal communi-
cation). This scale takes into account the intensity of nodulation, the
size of nodules and their colour which reflects their functionality
(a pink colour indicates the production of leghaemoglobin that is
present only in N2-fixing nodules).

Symbiotic N2 fixation
The intensity of the symbiotic fixation of atmospheric N2 by
inoculated A17 was estimated by measuring nitrogenase activity,
using the acetylene reduction assay (ARA) (Harty *et al.*, 1968): at
one date in the middle of Experiment 1 (at about 550 degree-days),
and at six dates in Experiment 2 (between 200 and 1000 degree-
days). As discussed by Minchin *et al.* (1983, 1986), the ARA
method has limitations when it is used in closed vessels, because
of an ‘acetylene-induced-decline’. To circumvent this problem, a stan-
dardized protocol was developed (D Moreau *et al.*, http://www.
noble.org/MedicagoHandbook/pdf/Phenetyping.pdf): in particular,
based on a short time of exposure of the below-ground part to
acetylene, it enables using ARA as a qualitative method to compare
treatments (Vessey, 1994).

The onset of symbiotic N2 fixation was defined as the delay,
expressed in degree-days, before symbiotic N2 fixation became
effective. In Experiment 2, in which several ARA measurements
were performed, it was calculated as the mean date between the last
date at which nitrogenase activity was nil and the first date at which
nitrogenase was active. As ARA was estimated at only one date in
Experiment 1, an alternative method was used to determine the
onset of fixation. For A17 and Nod− in the six nitrate treatments,
the total amount of N in the whole plant and the below-ground

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**Table 1. Experimental treatments and environmental conditions in Experiments 1 and 2**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Genotypes</th>
<th>Light treatment</th>
<th>Nitrate concentrations in the nutrient solution (mM)</th>
<th>Daily temperature (°C)</th>
<th>Daily photoperiod (h)</th>
<th>Mean incident PAR (μmol m$^{-2} s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A17 and Nod−</td>
<td>No-shade</td>
<td>0, 0.625, 1.25, 2.5, 5, and 10.5</td>
<td>18.9±1.4</td>
<td>14.7±0.6</td>
<td>278±76</td>
</tr>
<tr>
<td>2</td>
<td>A17</td>
<td>No-shade</td>
<td>0.625 and 10.5</td>
<td>21.6±3.1</td>
<td>15.7±0.2</td>
<td>607±202</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>Low-shade</td>
<td>0.625 and 10.5</td>
<td>21.9±3.2</td>
<td>15.7±0.2</td>
<td>311±110</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>High-shade</td>
<td>0.625 and 10.5</td>
<td>22.1±3.2</td>
<td>15.7±0.2</td>
<td>171±63</td>
</tr>
</tbody>
</table>

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**Table 2. Composition of the six nutrient solutions varying for their nitrate concentration, from 0 to 10.5 mM**

The six solutions also contained iron versenate (0.050 mM) and oligo
elements (traces).

<table>
<thead>
<tr>
<th>Name of the nutrient solutions$^{a}$</th>
<th>Concentration of the elements (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>KNO3</td>
<td>0.000</td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$+4H$_2$O</td>
<td>0.000</td>
</tr>
<tr>
<td>Na$_2$NO$_3$</td>
<td>0.000</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.400</td>
</tr>
<tr>
<td>MgSO$_4$+7H$_2$O</td>
<td>2.000</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>5.000</td>
</tr>
<tr>
<td>K$_2$SO$_4$</td>
<td>1.400</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.200</td>
</tr>
</tbody>
</table>

$^{a}$ The solutions are named according to their nitrate concentration (mM).
biomass (made up of roots and nodules) were both measured at eight dates between around 200 and 1000 degree-days, considering five plants per treatment. Using these data, it was considered that the symbiotic N\textsubscript{2} fixation by A17 became effective from the date at which the amount of N in the plant became higher for A17 than for Nod\textemdash, whereas the below-ground biomass remained similar between both genotypes.

**Determination of the N nutrition level**

Plant N nutrition level was determined using the concept of the critical dilution curve (for a review see Gastal and Lemaire, 2002). It enables estimating, for a given shoot biomass (SB), the ‘critical shoot N concentration’ (\%N\textsubscript{c}), i.e. the minimal N concentration allowing a maximum biomass production. \%N\textsubscript{c} is known to decrease during the plant cycle with increasing SB, according to a unique relationship that is stable for a given species. To date, the critical dilution curve of *M. truncatula* has never been defined. However, the critical dilution curve is known to be comparable among C\textsubscript{3} species: this has been validated for agronomic species (Greenwood *et al.*, 1990) as well as for non-agronomic species (e.g. *A. thaliana*: Richard-Mollard *et al.*, 2004). The critical dilution curve of *Medicago sativa* was considered in this study. As the critical dilution curve varies according to plant density (Lemaire *et al.*, 2005, 2007), \%N\textsubscript{c} was determined in two ways: (i) for an SB lower than 100 g m\textsuperscript{-2}, \%N\textsubscript{c}=8.1×SB\textsuperscript{-0.32} (Lemaire *et al.*, 2007) and (ii) for an SB higher than 100 g m\textsuperscript{-2}, \%N\textsubscript{c}=28.9×SB\textsuperscript{-0.36} (Lemaire *et al.*, 1985).

First, the relevance to *M. truncatula* of this critical dilution curve was validated using data from inoculated A17 in the different nitrate treatments of Experiment 1, for which shoot N concentration and shoot biomass were measured at three dates between 800 and 1000 degree-days after sowing, considering five plants per treatment. Second, based on this critical dilution curve, a N nutrition index (NNI) was calculated to quantify plant N nutrition level (Gastal and Lemaire, 2002). For a given shoot biomass (SB), it was calculated as the ratio between actual shoot N concentration and \%N\textsubscript{c} (defined by the critical dilution curve). When the NNI is close to 1, it is classically interpreted that N nutrition level is optimal; a NNI lower (respectively, higher) than 1 reveals an N-deficiency (respectively, an N-excess). For each treatment, mean values of NNI and standard deviations were calculated considering the data between 800 and 1000 degree-days.

**Measurements of shoot net photosynthesis and respiration of the below-ground part**

Shoot net photosynthesis and respiration of the below-ground part were measured for different treatments at a given date (about 740 degree-days and 625 degree-days, respectively, in Experiments 1 and 2) in a growth-chamber. Measurements were performed on entire and intact plants. For measuring shoot net photosynthesis (respectively, respiration of the below-ground part), the shoot (respectively, below-ground) part of each plant was isolated in a air-tight plexiglass container. Mean air temperature was, respectively, 21 °C and 23 °C in Experiments 1 and 2. Using an infrared gas analyser (IRGA; Qubitac; Qubit Systems Inc., Kingston, Canada), shoot net photosynthesis (respectively, the respiration of the below-ground part) was estimated by measuring the rate at which the CO\textsubscript{2} concentration within the container decreased (respectively, increased) between 300 ppm and 400 ppm, with three plants per treatment. The part of the leaf area exposed to light (evaluated by combining photography and image analysis), the specific leaf N (SLN, i.e. the amount of N in the leaves per unit leaf area), and the below-ground biomass were all measured for each plant.

**Thermal time**

Thermal time (expressed in degree-days after sowing) was calculated as the sum of the mean daily effective temperatures, i.e. mean daily temperatures (\(T_m\)) minus the base temperature (\(T_b\)) (for reviews, see Bonhomme, 2000; Trudgill *et al.*, 2005):

\[
\text{Thermal time} = \int \max[0; (T_m - T_b)]dt
\]

with \(T_b\) (defined as the temperature below which plant development is thought to be nil) considered as 5 °C (Moreau *et al.*, 2006, 2007).

**Data analysis**

The impact of the nitrate concentration in the nutrient solution on plant growth was analysed in given light conditions (i.e. either in Experiment 1, for which only the light treatment ‘no-shade’ was applied, or in a given light treatment of Experiment 2). Besides, as shown in Table 1, Experiments 1 and 2 differ in terms of both photoperiod and incident PAR. Consequently, the analysis of the global impact of the light conditions (i.e. both photoperiod and incident PAR) on plant N nutrition was based on the comparison among all the light treatments (considering both experiments) for a given nitrate concentration in the nutrient solution. The analysis of the specific impact of the level of incident PAR (independent of the impact of photoperiod) on plant N nutrition pertained only to the different light treatments of Experiment 2.

Statistical analyses were performed with the GLM procedure of SAS (SAS Institute, 1987). Means were compared using the Student–Newman–Keuls test. Only differences significant at \(P < 0.05\) were considered.

**Results**

**The nitrate concentration in the nutrient solution influenced total plant N uptake differently according to the light conditions**

The amount of N in the whole plant was measured at about 1000 degree-days after sowing (Fig. 1A). For Nod\textemdash, increasing the nitrate concentration in the nutrient solution resulted in a gradual increase of the amount of N accumulated in the plant; this result was expected, as nitrate assimilation was the only source of N for Nod\textemdash. More unexpectedly, when A17 was inoculated, the amount of N accumulated by this genotype also increased in response to the nitrate concentration in the nutrient solution with a magnitude comparable to that observed for Nod\textemdash (Fig. 1A).

Inoculated A17 was analysed under different light conditions in Experiment 2, to determine how the response of the plant to the nitrate concentration in the nutrient solution was affected by C nutrition (Fig. 1B). For a given nitrate concentration in the nutrient solution, the amount of N accumulated in the plant was different between light conditions (differences could reach a factor of three). For given light conditions, increasing the nitrate concentration in the nutrient solution from 0.625 mM to
10.5 mM resulted systematically in higher amounts of N accumulated by the plant. However, it increased plant N by a factor of nine in the no-shade treatment, and only by a factor of four in the high-shade treatment. Thus, the extent of the impact of the nitrate concentration in the nutrient solution on plant N uptake was reduced as incident PAR decreased.

Nitrogen nutrition of Medicago truncatula

Nitrate concentration conversely affected symbiotic N\textsubscript{2} fixation and nitrate assimilation by A17, resulting in a complementarity between both N sources

For inoculated A17, the impact of the nitrate concentration in the nutrient solution on the level of nodulation was visually assessed during Experiment 1 (Fig. 2). On the first observations dates (200–300 degree-days), levels of

Fig. 1. Impact of the nitrate concentration in the nutrient solution on the amount of N in the plant (A, B), N nutrition index (NNI) (C, D) and plant leaf area (E, F). (A, C, E) The results of Experiment 1 in which both inoculated A17 (hatching) and Nod- (points) were grown in given light conditions (no-shade). (B, D, F) The results obtained for A17 at two different nitrate concentrations in the nutrient solution and under different light conditions: Experiment 1 (corresponding to a ‘no-shade’ treatment; hatching), and no-shade (white), low-shade (grey), and high-shade (black) treatments of Experiment 2. Data are means and vertical bars indicate +SD. Values were measured at the end of both experiments; they could not be calculated for Nod– at 0 mM of nitrate, as the probable severe N-limitation experienced by this treatment did not allow the plants to be maintained until the end of the experiment. For NNI, the dotted lines at NNI=1 indicate an optimal N nutrition level. Means were classified using a Student–Newman–Keuls test (\(P <0.05\)). (A, C, E) Mean comparisons were performed considering simultaneously both genotypes and all nitrate treatments. (B, D, F) Means were compared among light conditions at a given nitrate concentration.
nodule size, and their color which reflects their functionality (a pink color indicates the production of leghaemoglobin that is present only in N2-fixing nodules). The scale includes five scores: 0 (absence of nodules), 1 (only some white nodules of small size), 2 (both white and pink nodules of small size), 3 (mainly pink nodules of larger size), and 4 (many pink nodules of large size). Nodulation was scored for the six nitrate concentrations of Experiment 1: 0 mM (diamonds), 0.625 mM (squares), 1.25 mM (crosses), 2.5 mM (triangles), 5 mM (open circles), and 10.5 mM (closed circles). Data are means and vertical bars indicate ±SD.

**Fig. 2.** Impact of the nitrate concentration in the nutrient solution on the time-course of nodule establishment by inoculated A17. Nodulation was visually assessed, using a qualitative scale established from previous knowledge on agronomic legumes (N Amarger, personal communication). The scale takes into account the intensity of nodulation, the size of nodules, and their color which reflects their functionality (a pink color indicates the production of leghaemoglobin that is present only in N2-fixing nodules). The scale includes five scores: 0 (absence of nodules), 1 (only some white nodules of small size), 2 (both white and pink nodules of small size), 3 (mainly pink nodules of larger size), and 4 (many pink nodules of large size). Nodulation was scored for the six nitrate concentrations of Experiment 1: 0 mM (diamonds), 0.625 mM (squares), 1.25 mM (crosses), 2.5 mM (triangles), 5 mM (open circles), and 10.5 mM (closed circles). Data are means and vertical bars indicate ±SD.

The complementarity between nitrate assimilation and symbiotic N₂ fixation did not necessarily allow achievement of an optimal N nutrition

To assess the N nutrition level in the different treatments, the first aim was to validate, in the case of *M. truncatula*, the critical dilution curve of N into biomass previously established for *M. sativa* (see Materials and methods). For that purpose, shoot N concentration was analysed as a function of shoot biomass, for inoculated A17 in the different nitrate concentrations in Experiment 1 (Fig. 5). It has to be remembered that the critical N concentration corresponds to a N concentration at which shoot biomass does not increase when N fertilization increases, in spite of an increase of shoot N concentration (Justes *et al.*, 1994). In Fig. 5, the different nitrate treatments (from 0 mM to 10.5 mM) of one sampling date are linked, and the critical dilution curve of *M. sativa* is shown. For each of the three sampling dates, increasing the nitrate concentration in the nutrient solution increased both shoot N concentration and shoot biomass, for the data situated below the critical curve of *M. sativa*. However, when shoot N concentration was close to values defined by the critical dilution curve of *M. sativa*, increasing the nitrate concentration in the nutrient solution increased shoot N concentration but not shoot biomass. Hence, it seemed...
that the critical dilution curve of *M. truncatula* was close to that of *M. sativa*, allowing the critical dilution curve of *M. sativa* to be used in the following analysis. Our data set did not allow the first part of the critical dilution curve to be validated, i.e. for shoot biomass lower than 100 g m⁻². It was hypothesized that the dilution of N into biomass was similar between *M. truncatula* and *M. sativa*, in agreement with the known stability of the critical dilution curve among species (Greenwood *et al.*, 1990; Richard-Mollard *et al.*, 2004).

A N nutrition index (NNI) was then calculated in order to determine to what extent plant growth could be sustained by nitrate assimilation and/or N₂ fixation (see details in the Materials and methods). For Nod–, the NNI increased progressively with increasing nitrate concentration in the nutrient solution (Fig. 1C); the NNI value was close to 1 at 10.5 mM nitrate, indicating that, when nitrate assimilation was the only N source, 10.5 mM of nitrate was necessary to fulfill the plant N requirements. For A17, the NNI also increased progressively with the rise in nitrate concentration in the nutrient solution (Fig. 1C). At 5 mM and 10.5 mM nitrate, the NNI values were 0.95 and 1.10, respectively, demonstrating that N nutrition was, respectively, optimal and slightly supra-optimal for these treatments. However, when inoculated A17 was grown with nitrate concentrations lower than 5 mM, the NNI remained lower than 1 (Fig. 1C): N uptake was then insufficient to meet plant N requirements, despite the complementarity between symbiotic N₂ fixation and nitrate assimilation. Interestingly, for a given nitrate concentration in the nutrient solution, the NNI was only slightly affected by light conditions (Fig. 1D): only the high-shade treatment stood out, with NNI values higher and lower (respectively, at 0.625 mM and 10.5 mM of nitrate) than in the other light conditions.

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Plants adapted to a N-limitation primarily by modulating leaf area but tended to maintain a constant photosynthesis per unit leaf area

To establish a link between C and N nutrition, plant C assimilation was characterized by dissecting it into two
components: plant leaf area and shoot net photosynthesis per unit leaf area.

Plant leaf area was measured for each treatment at about 1000 degree-days after sowing (Fig. 1E). For both genotypes, increasing the nitrate concentration in the nutrient solution resulted in an increase of plant leaf area. For Nod–, plant leaf area rose progressively across the entire range of nitrate concentrations. For A17, raising the nitrate concentration in the nutrient solution from 0 mM to 5 mM increased leaf area; the similarity of leaf area values at 5 mM and 10.5 mM of nitrate (Fig. 1E) was in accordance with the fact that the N nutrition of A17 plants reached an optimal level by 5 mM of nitrate (Fig. 1C).

As previously indicated for plant N uptake (Fig. 1B), plant leaf area was differently affected by the nitrate concentration in the nutrient solution according to light conditions (Fig. 1F). Indeed, for given conditions of nitrate concentration in the nutrient solution, clear differences in plant leaf area were recorded among light conditions. In addition, the extent of the impact of the nitrate concentration in the nutrient solution on plant leaf area decreased as incident PAR was reduced.

Shoot net photosynthesis was measured for treatments contrasting in both N nutrition sources and N nutrition level, and grown under three different light conditions (Table 3). Data of the no-shade treatment of both Experiments 1 and 2 revealed that shoot net photosynthesis per unit leaf area was stable whatever the N treatment, under a given set of light conditions. Only for the high-shade treatment of Experiment 2 was photosynthesis per unit leaf area lower at 0.625 mM than at 10.5 mM of nitrate. SLN, which was determined for each experimental treatment (Table 3), was higher at 10.5 mM than at 0.625 mM of nitrate in both the no-shade treatment of Experiment 1 and in the high-shade treatment of Experiment 2.

\[ N_2 \text{ fixation could generate higher carbon costs as compared to nitrate assimilation} \]

To characterize better the relationships between C and N nutrition and especially to be able to formulate hypotheses explaining the sub-optimal N nutrition detected for \( N_2 \) fixing A17 plants, the respiration of the below-ground part was measured for the treatments whose shoot net photosynthesis was previously characterized. The dates of measurement (see Materials and methods) were chosen in accordance with the dates of the onset of \( N_2 \) fixation (Fig. 3) in order to be able to analyse both nodulated plants (A17 grown with 0, 0.625, and 1.25 mM of nitrate) and non-nodulated plants (A17 grown with 10.5 mM of nitrate, and Nod–).

Specific respiration was calculated as the amount of C respired g\(^{-1}\) of below-ground biomass (Table 4). In the light conditions used, specific respiration was systematically higher for nodulated than for non-nodulated roots. For A17, compared to non-nodulated roots (10.5 mM nitrate), the specific respiration of the nodulated roots was increased by 13% and 25% (respectively, for 0 mM and 1.25 mM of nitrate) in the no-shade treatment of Experiment 1, and by 30% and 38% (respectively, for the
Table 3. Shoot net photosynthesis for different treatments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Genotype</th>
<th>Light treatment</th>
<th>Nitrate concentration in the nutrient solution (mM)</th>
<th>Shoot net photosynthesis (μg C s⁻¹ cm⁻² of leaf)ᵃ</th>
<th>SLN (mg N cm⁻² of leaf)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nod–</td>
<td>No-shade</td>
<td>0</td>
<td>0.0137 a</td>
<td>0.153 b</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>0</td>
<td>0.0129 a</td>
<td>0.160 b</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>1.25</td>
<td>0.0116 a</td>
<td>0.178 b</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>10.5</td>
<td>0.0144 a</td>
<td>0.270 a</td>
</tr>
<tr>
<td>2</td>
<td>A17</td>
<td>No-shade</td>
<td>0.625</td>
<td>0.0188 a</td>
<td>0.271 a</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>10.5</td>
<td>0.0226 a</td>
<td>0.314 a</td>
</tr>
<tr>
<td>2</td>
<td>A17</td>
<td>High-shade</td>
<td>0.625</td>
<td>0.0077 b</td>
<td>0.157 b</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>High-shade</td>
<td>10.5</td>
<td>0.0093 a</td>
<td>0.223 a</td>
</tr>
</tbody>
</table>

ᵃ Means were classified using a Student–Newman–Keuls test (P <0.05): for Experiment 1 or for a given light treatment in Experiment 2, identical letters indicate no significant differences between nitrate treatments.

Table 4. Respiration of the below-ground parts for different treatments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Genotype</th>
<th>Light treatment</th>
<th>Nitrate concentration in the nutrient solution (mM)</th>
<th>Nodulation of the rootsc</th>
<th>Specific respiration (μg C s⁻¹ g⁻¹ of below-ground biomass)</th>
<th>Percentage of C arising from shoot net photosynthesis which is allocated to the respiration of the below-ground part c,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nod–</td>
<td>No-shade</td>
<td>0</td>
<td>Non-nodulated</td>
<td>0.278 c</td>
<td>26%</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>0</td>
<td>Nodulated</td>
<td>0.470 ab</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>1.25</td>
<td>Nodulated</td>
<td>0.520 a</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>10.5</td>
<td>Non-nodulated</td>
<td>0.415 b</td>
<td>13%</td>
</tr>
<tr>
<td>2</td>
<td>A17</td>
<td>No-shade</td>
<td>0.625</td>
<td>Nodulated</td>
<td>0.402 a</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>No-shade</td>
<td>10.5</td>
<td>Non-nodulated</td>
<td>0.291 b</td>
<td>9%</td>
</tr>
<tr>
<td>2</td>
<td>A17</td>
<td>High-shade</td>
<td>0.625</td>
<td>Nodulated</td>
<td>0.555 a</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>A17</td>
<td>High-shade</td>
<td>10.5</td>
<td>Non-nodulated</td>
<td>0.428 b</td>
<td>11%</td>
</tr>
</tbody>
</table>

c Nodulation was determined for each treatment at the date of measurement of respiration. Roots were considered as non-nodulated when the onset of the symbiotic fixation of N₂ occurred after the date of measurement, and reciprocally.

ᵇ Means were classified using a Student–Newman–Keuls test (P <0.05): for Experiment 1 or for a given light treatment in Experiment 2, identical letters indicate no significant differences among nitrate treatments.

ᶜ Percentage of C arising from shoot net photosynthesis which is allocated to the respiration of the below-ground part. A mean plant was considered for each treatment by taking into account both the plants used for the measurements of the photosynthesis and the respiration of the below-ground part. The calculations were made for a period of 24 h, assuming shoot photosynthesis and the respiration of the below-ground part to be constant, respectively, during a photoperiod of 15 h and during 24 h. Due to this hypothesis, it is likely that the calculated percentages have no physiological meaning; however, they enabled comparisons among N treatments to be made regardless of light conditions.

Discussion

The analysis presented here is, to our knowledge, the first of its kind, investigating at a whole plant level the N nutrition of *M. truncatula* in relation to C nutrition. Focused on the association between *M. truncatula* cv. Jemalong (genotype A17) and *R. meliloti* strain 2011, it has allowed the specificities of the 'model symbiotic system' considered in most genomics works (Barker et al., 1990; Frugoli and Harris, 2001) to be characterized in terms of the relationships between soil nitrate concentration, plant N nutrition, and plant growth traits. From our findings, workable methods can be proposed in order to help the *M. truncatula* research community, both for better handling plant N nutrition and for assisting in the detection of new genetic variants with contrasting N nutrition in large-scale screening.

high-shade and no-shade treatments with 0.625 mM of nitrate) in Experiment 2.

To be able to compare the respiration of the below-ground parts among treatments that were grown under different light conditions (and that therefore experienced different levels of photosynthesis), below-ground respiration was expressed as a percentage of the C assimilated by shoot net photosynthesis (Table 4). Interestingly, whatever the light conditions, a larger proportion of the C assimilated by shoot net photosynthesis was respired by the below-ground part for nodulated plants (on average 24%) than for non-nodulated plants (on average 11%) (Table 4). For Nod–, the proportion of C respired by the roots was high (26%) even though the roots were not nodulated. Thus for this treatment, a severe disruption of plant metabolism seemed to have strongly affected C economy, presumably due to an extreme N-limitation.
The commonly used association between line A17 of M. truncatula and strain 2011 of R. meliloti can lead to N-stressed plants; consequences for better handling plant N nutrition

Our data supported the existence of a complementarity between symbiotic N₂ fixation and nitrate assimilation. While this has been commonly observed in many legumes, it had never before been demonstrated for M. truncatula. Interestingly, in spite of this, the N uptake of M. truncatula plants was not able to match plant N requirements when symbiotic N₂ fixation was the only or the main source of plant N (Fig. 1C). This suggested that symbiotic N₂ fixation was unable to support full plant growth. This result disagrees with observations on M. sativa (Lemaire et al., 1992) and pea (Sagan et al., 1993; Voisin et al., 2002) but agrees with those of Harper and Gibson (1984), assuming that the total reliance of M. truncatula on N₂ fixation could lead to a sub-optimal N nutrition. Thus, because of this sub-optimal N nutrition, it appears that the symbiotic system considered as a model by the research community shows specificities in terms of N nutrition. It cannot be determined whether the observed sub-optimal N nutrition is general for M. truncatula species or specific to the association between the symbionts of M. truncatula and R. meliloti studied (Mhabdi et al., 2005).

Two main hypotheses can be formulated to account for this important feature. First, it can be hypothesized that plants grown either in the absence or with small amounts of nitrate have undergone a N-limitation period due to the poor synchronization between the time when the seed N reserves have been depleted and when symbiotic N₂ fixation is sufficient to meet the plant N requirements (Mahon and Child, 1979; Williams and Phillips, 1980; Sprent and Thomas, 1984; Atkins et al., 1989). This is mainly due to the supplementary energy requirement for legume plants to synthesize the specific organs hosting symbiosis (nodules), thus leading to a delay before N uptake through symbiotic N₂ fixation becomes effective. This hypothesis seems plausible for M. truncatula because this type of N-limitation is more common for plants with an epigeal germination that preferentially directly assimilates to the shoot (and thus not to nodule formation) (Sprent and Thomas, 1984), and with small C and N seed reserves (Atkins et al., 1989). In our study, as for most legumes (Streeter, 1988), the onset of symbiotic N₂ fixation was delayed with increasing the nitrate concentration in the nutrient solution. Nevertheless, even in the absence of nitrate in the nutrient solution, the onset of symbiotic N₂ fixation occurred tardily in plant cycle (432 degree-days after sowing). This means that, during the first half of its vegetative cycle, plant N nutrition only relied on the seed N reserves that are very low [around 0.30 mg; calculated from seed N concentration (Djemel et al., 2005) and seed biomass measured in our experiments], compared to other species that do not encounter such an N-limitation (e.g. around 130 mg of N for pea seeds; calculated from Atta et al., 2004). When the nitrate concentration in the nutrient solution was low (0 mM and 0.625 mM), nodulation began early in the plant cycle (some nodules were established from 200 degree-days), but nodules started to increase in size and to turn pink (the pink colour indicates the production of leghaemoglobin that is present only in N₂-fixing nodules) only from 400–500 degree-days after sowing (it corresponds to score 3 in Fig. 2). Altogether, these indications seemed to confirm the hypothesis of poor synchronization between heterotrophy and autotrophy for N. Second, a higher C cost related to symbiotic N₂ fixation could be assumed to be partly responsible for the sub-optimal N nutrition experienced by N₂-fixing plants. Indeed, in agreement with observations on several legumes (Ryle et al., 1978, 1979; Pate et al., 1979; Schulze et al., 1994; Voisin et al., 2003b), the specific respiration of the below-ground part of M. truncatula tended to be higher for nodulated than for non-nodulated roots. Additional experiments (especially including C labelling; Voisin et al., 2003b) would be required to validate this hypothesis. Still, it can be suggested that both a C and a N-limitation during the early growth phase and possible high energy requirements of nodules (as compared to roots) could have resulted in a spiral in which C and N alternatively and progressively limited the growth of the plants whose symbiotic N₂ fixation was the main source of plant N.

The fact that N₂-fixing plants systematically displayed a sub-optimal N nutrition is a significant result for the M. truncatula research community working on genomics related to N₂ symbiotic fixation. Henceforth, it is crucial that functional genomics studies take this feature into account. The present study provides a reference frame that will be essential for better handling plant N nutrition in experiments. First, to date, little information is available regarding the nutrient solutions that should be used to grow M. truncatula plants particularly regarding the choice of the nitrate concentration. In this context, the present paper provides both (i) a range of nutrient solutions that can be used to cultivate M. truncatula (Table 2) and (ii) criteria for choosing the more suitable nitrate concentration according to the objectives of the experiments (at least for the combination between the line A17 of M. truncatula and the strain 2011 of R. meliloti). Indeed, based on our results, it can be recommended that a minimum of 5 mM of nitrate should be supplied to make sure N-limitation is avoided. A nil or close to nil (i.e. 0.625 mM) nitrate concentration should be used when the goal is to grow plants with symbiotic N₂ fixation as the exclusive (or main) N source (but this will still result in a N limitation). A nitrate concentration at about 5 (or 10.5) mM is recommended in order to cultivate plants with nitrate assimilation as the main (or almost exclusive)
N source. Therefore, for the purpose of phenotyping studies, such low and high conditions of nitrate concentration in the nutrient solution should enable the capacity of genotypes, respectively, to fix N\textsubscript{2} symbiotically and to assimilate nitrates to be evaluated.

Second, gene expression is known to be affected by plant N nutrition (Higashi et al., 2006; Girin et al., 2007). Unfortunately, the N nutrition level and source for plants used in functional genomics analyses are generally poorly characterized. The present paper demonstrated that the N nutrition index (NNI), which is classically applied for agronomic species (Gastal and Lemaire, 2002), based on the critical dilution curve of N into the biomass of M.\textit{sativa} (Lemaire et al., 1985, 2007), was appropriate to quantify the N nutrition level of M.\textit{truncatula} plants; this index should be useful for further functional genomics studies. Similar results were obtained by validating the use of the NNI in the case of the model plant \textit{Arabidopsis thaliana} (whose critical dilution curve of N into biomass was demonstrated to be similar to that of C\textsubscript{3} species; Richard-Mollard et al., 2004). Concomitantly, our analysis identified a robust linear relationship between the onset of symbiotic N\textsubscript{2} fixation and nitrate concentration in the nutrient solution that remained unaffected by light conditions (Fig. 3). Henceforth, using this relationship, the onset of N\textsubscript{2} fixation can be estimated for any experiment, knowing the nitrate concentration in the nutrient solution and the mean daily temperature (for the calculation of thermal time). So, in the scope of functional genomics studies, the comparison of both (i) the date at which plant tissue collection is performed for gene expression analyses and (ii) the date at which symbiotic N\textsubscript{2} fixation is estimated to begin (estimation from Fig. 3) is a simple way to determine the N nutrition source of the plants to be analysed; if tissue collection is performed before the calculated onset of N\textsubscript{2} fixation, nitrate assimilation is the only N source for the plants; whereas if tissue collection is performed after the calculated onset of N\textsubscript{2} fixation, plant N nutrition relies on both N sources. This approach could be of practical interest, both (i) as a decision tool for better scheduling tissue collection according to plant N nutrition source, and (ii) for checking \textit{a posteriori} if data produced in different experiments have been obtained using plants with similar N nutrition sources.

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\textbf{Plant leaf area is a valuable criterion for easily identifying putative differences in N nutrition between genotypes in a first step of a large-scale screening}

Plant leaf area was highly responsive to an N-limitation (Fig. 1E). Conversely, photosynthesis per unit leaf area was only slightly affected by N nutrition (Table 3). This finding indicated two main features. First, the higher SLN observed for A17 grown with 10.5 mM nitrate (Experiment 1) did not result in a higher photosynthetic activity per unit leaf area. This is in accordance with the supra-optimal N nutrition recorded for A17 grown at 10.5 mM nitrate (NNI values higher than 1 indicate that the amounts of N accumulated were higher than those required for maximal biomass production). Second, the strong N-limitation observed for the other N treatments (i.e. A17 at 0 mM and 1.25 mM and Nod- at 0 mM; Fig. 1C) did not affect photosynthesis per unit leaf area, despite a lower SLN. These results suggest the existence of a ‘SLN threshold’ above which photosynthesis per unit leaf area is unaffected by SLN, in accordance with Sinclair and Horie (1989). They also suggest that, when encountering strong N-limitation, plants tend to maintain SLN above this threshold in order to keep constant photosynthesis per unit leaf area. Altogether, these data indicate that, when N nutrition is sub-optimal, plants reduce total leaf area to maintain SLN and associated photosynthesis per unit leaf area. Authors hypothesized that this strategy of optimization of C assimilation could be general for dicot species, as opposed to Gramineae for which leaf area tends to be maintained at the detriment of SLN and therefore the photosynthetic capacity per unit leaf area (Radin, 1983; Vos et al., 2005).

These findings provide insights into the phenotypic traits that should be considered in large-scale screening for diagnosing phenotypic differences between genotypes in their capacities to take up N. In particular, as plants adapted to N-limitation primarily by modulating leaf area expansion, plant leaf area observed at a given sampling date and in given environmental conditions could be a convenient indicator for performing a first selection of the genotypes according to their capacities to take up N. Plant leaf area is particularly appropriate for phenotyping a large number of plants as it can be measured non-destructively by combining photography and image analysis (Granier et al., 2006; Walter et al., 2007). Importantly, it is recommended for measurements to be performed later in the plant cycle (as performed in our study) to maximize the phenotypic differences between genotypes. Nevertheless, as plant leaf area strongly interacts with environmental factors other than N nutrition (e.g. light), complementary measurements are required to determine whether the differences of plant leaf area originate from differences in N nutrition.

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\textbf{The N nutrition index (NNI) is a robust criterion for quantifying the intrinsic capacities of a genotype to take up N in given conditions of nitrate concentration in the nutrient solution}

At a given nitrate concentration in the nutrient solution, differences in the light conditions substantially affected N accumulation (Fig. 1B) and plant leaf area (Fig. 1F). By contrast, the N nutrition level of inoculated A17
(estimated by the NNI) was almost independent of the light conditions and was primarily determined by the nitrate concentration in the nutrient solution (Fig. 1D). This finding is probably due to the co-ordinated regulation between the C and N assimilation pathways (Foyer et al., 1998; Silveira et al., 2001): when N was limiting, C assimilation may have been adjusted to N uptake (by modulating plant leaf area); reciprocally, when N was not limiting for plant growth, N uptake may have been adjusted to C assimilation. It seems therefore that, when studying the phenotypic variability associated with N nutrition within a range of genotypes, estimating the NNI should allow the intrinsic properties of the studied genotypes (Tardieu, 2003) to take up N in a given condition of nitrate concentration in the nutrient solution to be evaluated. Hence, in order to determine if differences in plant leaf area between genotypes effectively originate from differences in N nutrition in phenotyping studies, a second step should consist of estimating the NNI for the genotypes selected on their leaf area value, by measuring their shoot biomass and their shoot N content. Accordingly, screening the plants by performing observations only on their shoot part (i.e. without focusing on their below-ground part whose characterization is generally more complex) could be sufficient, at least in a first step, for assessing their N nutrition.

Phenotyping plants with an incident PAR lower than 300 μmol m−2 s−1 is likely to mask phenotypic differences between genotypes with different capacities to take up N

A strong interaction between plant N nutrition and C nutrition was recorded at different levels. For instance, increasing the nitrate concentration in the nutrient solution for inoculated A17 plants stimulated both plant N uptake and leaf area expansion, but the extent of the impact of nitrate supply was substantially reduced as incident PAR decreased. Besides, the NNI was found to be nearly independent of light conditions, being primarily determined by the nitrate concentration in the nutrient solution but slight discrepancies were observed for the high-shade treatment. All these results have direct implications when diagnosing phenotypic differences between genotypes in their capacities to take up N in large-scale screening studies. From our work, it is recommended that situations of low incident PAR, under which plant N uptake can be affected, should be avoided: phenotyping plants with an incident PAR lower than 300 μmol m−2 s−1 is likely to mask phenotypic differences between genotypes with different capacities to acquire N. This is of key importance as M. truncatula is commonly grown with an incident PAR lower than this threshold (DG Barker et al., http://www.noble.org/MedicagoHandbook/pdf/GrowingMedicagotruncatula.pdf).

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