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# Pea nodule gradients explain C nutrition and depressed growth phenotype of hypernodulating mutants

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**Abstract** Legumes are major plants for sustainable agriculture because legumes fix atmospheric N<sub>2</sub> in symbiosis with soil bacteria. Previous investigations have attempted to increase N<sub>2</sub> fixation by creating hypernodulating mutants for several legume species. However, such genetic mutation has failed to increase symbiotic fixation because hypernodulation is usually associated with depressed plant growth. Such failure is due to our lack of knowledge on the mechanisms of carbon flow. In particular, the impact of hypernodulation on carbon uptake and partitioning between the shoot, roots and nodules has never been quantified. Here we grew two wild pea genotypes and nine hypernodulating pea mutants in hydroponics. Measurements were made at the end of the vegetative period. We counted the nodules: for pea mutants, nodule number ranged from 500 to 2,600 per plant using genetic and allele strength variation. For pea wild types, nodule number ranged from 184 to 955 per plant, using environmental manipulation. We also measured carbon nutrition traits such as leaf area, radiation use efficiency for biomass production and biomass partitioning among shoot, roots and nodules. Relationships between nodule number and traits were analysed within hypernodulating mutants. Those relationships were then compared to those obtained for wild types. Our results show that total nodule biomass increases with nodule number for hypernodulating mutants and wild types. Mean nodule size decreases with nodule number for hypernodulating mutants and wild types. For mutants, root growth was reduced from 60 % for the lower hypernodulating to 80 % for the higher hypernodulating, compared to wild types. For mutants, shoot growth was reduced from 0 % for the lower hypernodulating to 60 % for the higher hypernodulating, compared to wild types. Evidence for

decreased C uptake capacity is shown by 50 % less leaf area and 18 % less radiation use efficiency of the higher hypernodulating phenotypes. This is the first whole plant comparative study of hypernodulating mutants and wild types revealing relationships between carbon nutrition and nodule gradients. Our findings reveal some mechanisms that account for the depressed growth phenotype associated with hypernodulation in pea.

**Keywords** Hypernodulating mutants · Roots · Nodules · Symbiotic N<sub>2</sub> fixation · Carbon nutrition · Legume · *Pisum sativum* · Symbiosis

## 1 Introduction

The imminent increase in world food demand due to increasing world population and to dietary changes in emerging countries is a challenge that agriculture will face in the next decades. At the same time, the forthcoming context of nutrient and energy scarcity will bring additional constraints to agricultural production. As such, there is a need for agricultural systems that enhance sustainability while maintaining productivity. In this context, legumes have a major role to play (Siddique et al. 2012): due to their unique ability to fix atmospheric N<sub>2</sub> in symbiosis with soil bacteria, they can (a) provide protein-rich feed or food in soils poor in nitrogen (N) and (b) contribute to restore soil N fertility, at low environmental cost. Atmospheric N<sub>2</sub> is a renewable resource, in contrast to synthetic N fertilizers that potentially contribute to N pollution in ecosystems and that require large amounts of fossil energy for their synthesis (Nemecek et al. 2008). Thus, compared to the Haber–Bosch process for N fertilizer production, the energy that drives symbiotic nitrogen fixation in legumes is virtually “free” as

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it is derived from photosynthesis. Still, at the plant level, N benefits from N<sub>2</sub> fixation by symbiotic rhizobia have a carbon cost counterpart for the formation of specific symbiotic structures (root nodules) and for their N<sub>2</sub>-fixing activity. Nodules are therefore strong sinks for photoassimilates (Hacin et al. 1997), which drain carbon at the expense of other plant organs (Voisin et al. 2003a). As such, up to now, breeding crops for enhanced N<sub>2</sub> fixation has always faced the problem of balancing increasing N<sub>2</sub> fixation by increased carbon uptake or efficiency to sustain nodule requirements for carbon assimilates. Several mutants with hypernodulating phenotype have been selected as an attempt to increase symbiotic N<sub>2</sub> fixation by greatly augmenting nodule number, but have failed to substantially increase N uptake by legume plants, at least in their genetic background. Indeed, hypernodulation was associated with impairment of plant growth and productivity (Salon et al. 2001; Bathia et al. 2001; Novak 2010).

Several mutants displaying the hypernodulating phenotype were isolated in different species (Novak 2010; Reid et al. 2011 for recent reviews). Hypernodulating mutants were shown to be defective in the regulation that leads to an “autoregulation” of nodule number (AON) by the host plant (Reid et al. 2011 and Mortier et al. 2012 for reviews). In pea, three genes have been shown to control nodulation, namely NOD3 (Postma et al. 1988), SYM29 and SYM28 (Sagan and Duc 1996). On the basis of homologies with *Medicago truncatula* or *Lotus japonicus*, these three genes were identified (Krussel et al. 2002, 2011; Schnabel et al. 2011) and shown to encode peptides and receptors for developmental regulation. Many studies have reported pleiotropic effects associated with hypernodulation. Most of these effects were associated with modifications of growth and development, with depressed shoot growth, shortened internodes or fasciation of the stem and also underdeveloped roots or decreased root mass (e.g. pea: Postma et al. 1988; Duc and Messenger 1989; Sagan and Duc 1996; Salon et al. 2001; Krussel et al. 2002; Bourion et al. 2007). However, these observations are sparse because either they only described one or few mutants at the same time and/or because they did not take into account all the determinants of carbon nutrition and partitioning together. The pleiotropic effects associated with hypernodulation might be directly due to the mutation or may result from a physiological consequence of changed nodule number, structure or activity. Hence, excessive nodule number and/or nodule biomass in hypernodulating mutants was suspected to create an imbalance between shoot photosynthetic capacity and nodule requirements for carbon assimilates (Novak et al. 2009). However, the impact of hypernodulation on carbon uptake and partitioning within the plant has never been precisely quantified at the plant level.

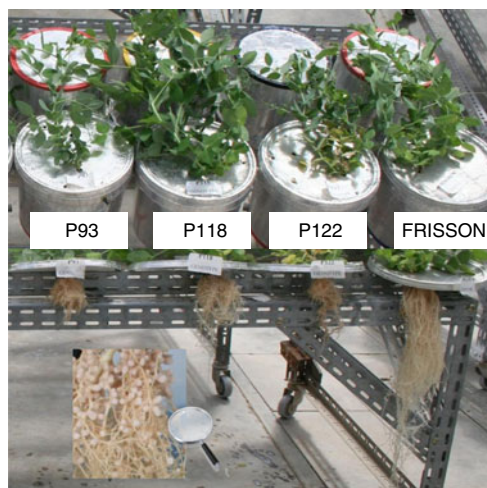
In this context, our objectives were to characterise and quantify the depressed growth phenotype usually associated with hypernodulation in pea, considering the impact of

hypernodulation on carbon nutrition at the plant level. Carbon nutrition was analysed through an ecophysiological approach taking into account carbon assimilation and biomass partitioning between the shoot, the roots and nodules. A set of mutants including an allelic series of SYM29 mutants was characterised (Fig. 1). This provided a continuous variation in the strength of hypernodulation among genotypes and therefore in nodule number. This original plant genetic material allowed us to draw trends between nodule number and plant parameters related to carbon nutrition. The trends that we obtained for isogenic hypernodulating mutants were compared to those obtained with wild types.

## 2 Materials and methods

### 2.1 Plant material

The 2004, 2006 and 2007 experiments were conducted using pea (*Pisum sativum* L.) cv Baccara. The 2008 and 2009 experiments involved cv Frisson and its hypernodulating mutants. The hypernodulating lines of Frisson were derived from ethyl methanesulfonate mutagenesis (Duc and Messenger 1989). Among them, three lines differing in the genetic basis of hypernodulation were selected, namely mutants of the SYM28, SYM29 and NOD3 genes (Sagan and Duc 1996; Postma et al. 1988; Krussel et al. 2002). Among diverse mutants of each gene, we first selected the allele displaying the weakest hypernodulating phenotype, i.e. which displayed the least depressed shoot growth compared to the parental line (i.e. P64, P118 and P121 mutants for the SYM28, SYM29 and NOD3 genes, respectively; Bourion et al. 2007). In the 2009 experiment, wild-type Frisson was grown together with an allelic series of the seven hypernodulating mutants of SYM29 (P118, P87, P90, P89, P91, P93 and P122; Table 1), each



**Fig. 1** Three SYM 29 hypernodulating mutants and wild-type Frisson in our greenhouse experiment

**Table 1** Experimental treatments aimed at modifying nodule number and biomass, through genetic or environmental manipulation. Photosynthetically active radiation (PAR) was averaged over the whole vegetative period (mean±standard deviation). Nodule number and nodule biomass were measured at the end of the vegetative period.

Statistical analysis was performed in a first group among Baccara treatments and in a second group among Frisson and hypermodulating mutants. Within each group, means followed by similar superscript letters are not significantly different at the 0.05 probability level

Treatment identification	Year	Line (mutated allele)	Light treatment	PAR (mol m <sup>-2</sup> day <sup>-1</sup> )	Nodule number (nb plant <sup>-1</sup> )	Nodule biomass (g plant <sup>-1</sup> )
Baccara <sub>2004-shaded</sub>	2004	Baccara	Shaded	15±6	184 <sup>D</sup>	0.05 <sup>C</sup>
Baccara <sub>2004</sub>	2004	Baccara	Natural light	36±14	393 <sup>C</sup>	0.16 <sup>B</sup>
Baccara <sub>2006</sub>	2006	Baccara	Natural light	25±14	803 <sup>B</sup>	0.33 <sup>A</sup>
Baccara <sub>2007</sub>	2007	Baccara	Natural light	27±13	955 <sup>A</sup>	0.28 <sup>A</sup>
Frisson <sub>2008</sub>	2008	Frisson	Natural light	11±4	532 <sup>h</sup>	0.19 <sup>e</sup>
P64 <sub>2008</sub>	2008	Frisson ( <i>sym28-1</i> )	Natural light	11±4	776 <sup>g</sup>	0.21 <sup>de</sup>
P118 <sub>2008</sub>	2008	Frisson ( <i>sym29-1</i> )	Natural light	11±4	803 <sup>fg</sup>	0.23 <sup>de</sup>
P121 <sub>2008</sub>	2008	Frisson ( <i>nod3-1</i> )	Natural light	11±4	1840 <sup>b</sup>	0.39 <sup>a</sup>
Frisson <sub>2009</sub>	2009	Frisson	Natural light	16±7	497 <sup>h</sup>	0.27 <sup>cd</sup>
P118 <sub>2009</sub>	2009	Frisson ( <i>sym29-1</i> )	Natural light	16±7	864 <sup>efg</sup>	0.39 <sup>a</sup>
P87 <sub>2009</sub>	2009	Frisson ( <i>sym29-2</i> )	Natural light	16±7	1,187 <sup>def</sup>	0.24 <sup>de</sup>
P90 <sub>2009</sub>	2009	Frisson ( <i>sym29-3</i> )	Natural light	16±7	1,230 <sup>de</sup>	0.34 <sup>ab</sup>
P89 <sub>2009</sub>	2009	Frisson ( <i>sym29-4</i> )	Natural light	16±7	1,423 <sup>cd</sup>	0.39 <sup>a</sup>
P91 <sub>2009</sub>	2009	Frisson ( <i>sym29-5</i> )	Natural light	16±7	1,630 <sup>bc</sup>	0.34 <sup>abc</sup>
P93 <sub>2009</sub>	2009	Frisson ( <i>sym29-6</i> )	Natural light	16±7	1,942 <sup>b</sup>	0.27 <sup>bcd</sup>
P122 <sub>2009</sub>	2009	Frisson ( <i>sym29-7</i> )	Natural light	16±7	2,613 <sup>a</sup>	0.37 <sup>a</sup>

differing in sequence of the *sym29* allele (Krussel et al. 2002) and varying in hypermodulating intensity.

## 2.2 Growing conditions

Plants were grown in hydroponic culture so as to allow complete recovery of intact nodulated roots and reproducible measurement of nodule numbers and biomass (Voisin et al. 2010). The experiments were conducted in the greenhouse in years 2004, 2006, 2007, 2008 and 2009 in Dijon, in Eastern France. All experiments spanned from germination until the appearance of the first flowers and thus lasted around 6 weeks. For each experiment, 1,200 to 1,320 seeds were first weighed and calibrated in order to obtain homogeneous seedlings. Selected seeds were germinated on moist filter paper on 24 May 2004, 6 May 2006, 27 April 2007, 7 March 2008 and 15 March 2009. As soon as the radicle had reached 2–3 cm (referred to as “germination”), 400 to 440 seedlings were selected for developmental homogeneity and transferred to hydroponic culture, with two seedlings per pot. Plants were grown in 5-L pots covered with an aluminium foil both to maintain roots in the dark and to prevent excessive heating of the nutrient solution by solar radiation. The lid was bored with two holes for the seedlings to grow through. Within each pot, an inner wall separated the root systems of the two plants. Nutrient solution was supplied in

excess to each pot compartment several times a day to compensate for plant consumption and transpiration. The nutrient solution was continuously aerated by bubbling air in each pot compartment. The air flow was calibrated so that oxygenation was always close to 100 % of its maximal value when equilibrated with ambient air (LDO sensor, Hatch Lange). Plants were inoculated with *Rhizobium leguminosarum* bv *viciae* (strain P221) with a concentration of 10<sup>8</sup> rhizobia per plant. In order to allow nodulation to take place freely, nutrient solution was so deprived of mineral nitrogen. The pH of the nutrient solution was controlled and maintained between 6.5 and 7.5, which enables rhizobia to survive freely in solution and to efficiently nodulate pea plants (Amarger, personal communication). The nutrient solution was totally renewed and re-inoculated every 2 weeks.

## 2.3 Meteorological conditions

Air temperature was measured using PT100 sensors (Pyro-Contrôle, Vaulx-en-Velin, France) and temperature of nutrient solution in the pots (i.e. of the roots) with thermocouples. Incident photosynthetically active radiation (PAR) was measured using silicium sensors (Solems, Palaiseau, France). Measurements were made at 600 s intervals and stored in a data logger (DL2e; Delta-T Devices, Cambridge, England). Temperature was in the same range for all experiments, with

mean air temperature being 20.3, 19.4, 20.4, 18.4 and 19.2 °C in the 2004, 2006, 2007, 2008 and 2009 experiments, respectively. Mean temperature of the root medium (i.e. the nutrient solution) was between 1.1 and 1.9 °C higher than mean air temperature depending on the experiment. Air and root temperatures were not significantly affected by shading in the 2004 experiment. As no external light supplementation was used, the light level varied among years, depending on external solar radiation which increased when the sowing date was delayed in the growing season (Table 1). As such, the light level was similar in both experiments in which Frisson and its hypernodulating mutants were grown (2008 and 2009). This allowed comparison between years for Frisson and P118 genotypes being grown in both years. Conversely, in an aim to induce intra-genotype variation in nodule number, the Baccara genotype was grown in contrasted conditions. As such, for the Baccara genotype, the light level varied between years, both due to natural variation and to artificial shading using special nets in the 2004 experiment. As such, for Baccara, light conditions varied from 15 to 36 mol m<sup>-2</sup> day<sup>-1</sup> among years and treatments (Table 1).

#### 2.4 Harvests and measurements

Plants were harvested two to three times a week from germination until the appearance of the first flowers (i.e. until days 40–42 for Baccara and day 44 for Frisson and mutants). For each harvest, three pots per genotype were randomly selected and measurements were made on the six (Baccara plants) or five (Frisson and mutants) plants harvested. Shoot development was characterised by counting the number of nodes on main stem and the number of branches. Leaf area was measured using a LI-3100 Area Meter (LICOR Inc., Lincoln, NE, USA). Nodules were counted as soon as they were visible to the eye, i.e. when nodule meristem protruded from the root and could be unambiguously differentiated from root meristem. Nodules were detached from the roots using forceps as soon as they could be physically separated from the roots without damage. The plants were divided into several parts (seed cotyledons, leaves, stems, roots and nodules) that were weighed separately after drying at 80 °C for 48 h.

#### 2.5 Conceptual framework and calculations

Our analysis was conducted at the end of the vegetative growth as nodule and root establishment is mainly restricted to this period due to strong competition for C with the formation of reproductive organs afterwards (Voisin et al. 2003a; Bourion et al. 2007). Functional and structural components of carbon nutrition were analysed at the plant level using the following framework:

Carbon uptake by the leaves was characterising using both leaf area and leaf efficiency for biomass production.

The efficiency of the leaves to convert solar radiation into biomass (i.e. radiation use efficiency (RUE)) was expressed in grams of total biomass per megajoules of intercepted PAR) was calculated from date  $t_a$  to date  $t_b$ , as the ratio between total plant dry weight ( $DW_{total}$ ) and cumulated intercepted PAR (as the product of daily PAR by leaf area (LA)) as follows:

$$RUE = \frac{DW_{total\ b} - DW_{total\ a}}{\sum_{i=a}^b PAR_i \cdot LA_i}$$

Carbon partitioning among plant organs was evaluated by examining biomass partitioning (a) between root and shoot compartments (using “nodulated root over total plant biomass ratio,” in grams per gram), (b) between root and nodule compartments (using “nodule over nodulated roots biomass ratio,” in grams per gram) and (c) among nodules (using mean nodule size, in milligrams per nodule). Biomass partitioning among nodules was analysed using a theory of competition for resources within a population of fruit units described by Lescourret and Génard (2003). For this, we made an analogy between nodules in the root system and fruit in that of the shoot. According to this theory, a single relationship between nodule size ( $S$ ) and nodule number ( $n$ ) can be fitted on all experimental points:  $S = S_{max}[1 - (n/n_{max})^\alpha]$ , and an optimal number of nodule value can be calculated:  $n_{opt} = n_{max} / [(a + 1)^{1/\alpha}]$ .

#### 2.6 Statistical analysis

Means were calculated on five or six plants. Analysis of variance was performed using the GLM procedure of SAS (SAS Institute 1987). Means were compared using the least significant difference test at the 0.05 probability level. Correlations were calculated using the Pearson coefficient using the R software (R Development Core Team 2008) and were considered as significant at the 0.05 probability level.

### 3 Results and discussion

The aim of our study was to provide new insights in the understanding of the pleiotropic depressed growth associated with hypernodulation, considering the main determinants of carbon nutrition at the plant level. For this, relationships between nodule number and a set of appropriate plant parameters were analysed in an enlarged and original set of hypernodulating mutants and compared to a wild-type reference. To our knowledge, this is the first time that such a whole plant comparative study between hypernodulating mutants and wild types has been conducted.



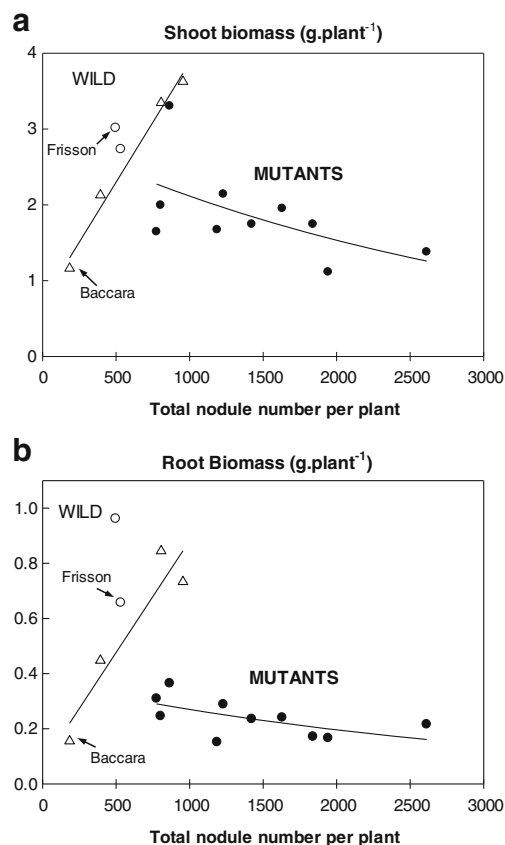
### 3.1 Our experimental setup provided plant material with continuous variation in total nodule number per plant

Variation of nodule number was attained using a series of isogenic hypermodulating mutants via allele strength variation while in the wild-type reference it was modulated by environmental manipulation. Wild-type Baccara was grown under a range of environmental conditions so as to modulate nodule number for a wild-type genotype. This induced variation in nodule number among years and treatments between 184 and 955 nodules per plant at the end of the vegetative period (Table 1). Wild-type Frisson and nine of its hypermodulating mutant lines were grown under similar conditions in two experiments. At the end of the vegetative period, the 2008 and 2009 experiments provided a range of phenotypes in a similar genetic background for the hypermodulating genotypes, with nodules ranging from 500 up to 2,600 per plant, from the weakest to the highest hypermodulating phenotype (Table 1).

### 3.2 Hypermodulation results in major shifts of assimilates from roots to nodules

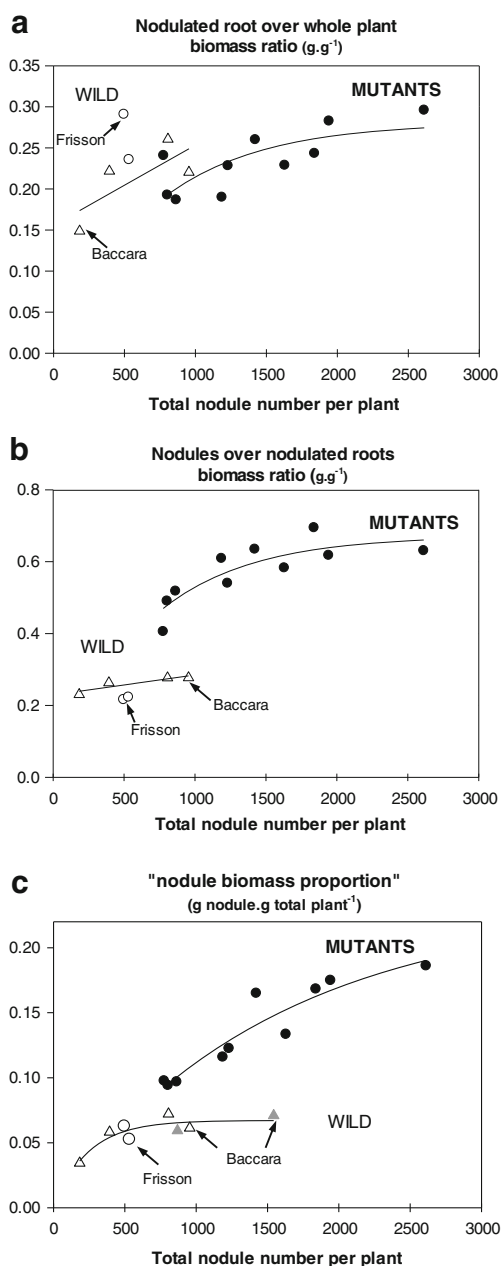
So as to characterise the impact of hypermodulation on plant growth, relationships between nodule numbers and plant growth parameters were compared between hypermodulating mutants and wild types. In wild-type Baccara, nodule, root and shoot biomass were positively correlated with nodule number (Table 1 and Fig. 2). In contrast, trends were opposite in hypermodulating mutants, as nodule number was negatively correlated with shoot and root biomass. Total nodule biomass of hypermodulating mutants was similar or higher than that of the wild type. And among hypermodulating mutants, the correlation between nodule number and nodule biomass was weak ( $r=0.31$ ,  $p=0.03$ ). Among mutants, shoot biomass progressively decreased with increasing nodule number, from levels similar to wild type down to a 60 % reduction compared to wild type (Fig. 2a). Root biomass was far more affected by hypermodulation, being reduced by 60 to 80 % compared to wild type, from the weakest to the strongest hypermodulating phenotype (Fig. 2b).

Carbon partitioning between plant organs was analysed using the proportion of below-ground biomass (root and nodules) relative to total plant biomass (“below-ground proportion”; Fig. 3a) and the proportion of nodule biomass relative to nodulated root biomass (“nodule proportion in roots”; Fig. 3b). These proportions increased with nodule number, but there were two distinct trends, in the one hand for wild types and in the other hand for hypermodulating mutants. The range of below-ground proportion was similar for hypermodulating and wild-type genotypes (Fig. 3a). In contrast, nodule proportion in roots of hypermodulating mutants increased from 40 up to 70 %, being far higher than for wild-type Frisson (20 %) and Baccara (around 25 %; Fig. 3b).



**Fig. 2** Shoot and root biomass as related to nodule number at the end of the vegetative period. Shoot (a) and root (b) as plotted vs. total nodule number per plant for wild-type Baccara (white triangle), wild-type Frisson (white circles) and hypermodulating mutants (black circles). In each graph, each point represents the means calculated on five or six plants. A linear regression was performed on experimental points corresponding to Baccara plants while the most appropriate trend was drawn on those corresponding to hypermodulating mutants

Thus, using the environmentally induced variation of nodule number in wild-type Baccara, we showed a positive correlation between nodule number and either total nodule biomass (Table 1 and Fig. 3c), root biomass or shoot biomass (Fig. 2). For this wild-type genotype, this correlation reflected the beneficial impact of the symbiotic association to the host plant growth. This beneficial impact could be accounted for by the positive correlation between the nodule number and plant growth rate during nodulation that we evidenced in a previous whole plant study (Voisin et al. 2010). This adjustment was interpreted as an adjustment of nodule number to plant N requirements for growth and was shown to be linked to the AON by the host plant (Voisin et al. 2010; Jeudy et al. 2010). A natural genetic variability for nodule number has been reported in several legumes, but there were very few studies on the quantitative genetics analysis of this trait. Especially, a quantitative trait loci (QTL) analysis in pea showed that several quantitative trait loci for root, nodule and shoot biomass traits map in similar locations (Bourion et al. 2010).



**Fig. 3** Biomass partitioning between plant organs as related to nodule number at the end of the vegetative period. Nodulated root over whole plant biomass ratio (**a**), nodule over nodulated root biomass ratio (**b**) and nodule over total plant biomass ratio (**c**) as plotted vs. total nodule number per plant for wild-type Baccara (white triangle), wild-type Frisson (white circles) and hypernodulating mutants (black circles). In each graph, each point represents the means calculated on five or six plants. In **a** and **b**, a linear regression was performed on experimental points corresponding to Baccara plants while the most appropriate trend was drawn on those corresponding to hypernodulating mutants. In **b**, an exponential curve rising to a maximum was performed on experimental points corresponding to Baccara plants from this study to which two experimental situations were added (grey circles) from Voisin et al. (2010)

This suggests the possibility of breeding new pea cultivars with increased root system size, sustained nodule number and improved shoot growth. In hypernodulating mutants however,

increased nodule number was detrimental both to shoot and root growth compared to wild type, in accordance with many previous observations (Novak 2010). This indicates the nodule overload for the host plant growth, for those genotypes for which the autoregulation of nodulation is disrupted. More importantly, using the continuous variation in the nodulation strength provided by the allelic series of SYM29 mutants, our study reveals here that nodule excess was far more detrimental to root growth than to shoot growth: indeed, root growth depression was high and similar for all hypernodulating phenotypes while shoot growth decrease was more progressive with increasing nodule number (Fig. 2). Excess nodule formation thus appears to mainly occur at the expense of root growth in hypernodulating mutants. This is in accordance with studies conducted on wild types that show that nodule formation mainly occurs at the expense of the roots (Schulze et al. 1999; Voisin et al. 2003a), but this was amplified in hypernodulating mutants.

### 3.3 Hypernodulation limited shoot growth and carbon uptake capacity

For wild-type Baccara, nodule number was strongly positively correlated with total plant biomass, whereas the correlation was negative for hypernodulating mutants (Table 2). The effects of hypernodulation on carbon uptake capacity were analysed considering both leaf area and radiation use efficiency for biomass production. For Baccara, leaf area was positively correlated with nodule number, but the trend was opposite for hypernodulating mutants, with the leaf area of the strongest hypernodulating phenotype being decreased by 50 % compared to wild type (Table 2). In addition, increasing nodule number in hypernodulating mutants was associated to decreasing number of nodes on main stem, while for wild-type Baccara, the total number of nodes on main stem was remarkably stable whatever the nodule number (Table 2).

Our quantitative study thus originally showed that the negative impact of hypernodulation on shoot growth increased with the strength of the hypernodulating phenotype. We cannot distinguish between the possibility that shoot growth depression is directly associated with the mutation, independently of the symbiotic status, or is a consequence of increased competition for carbon by the excess nodules. A direct effect of the mutation independent of nodulation may arise from modification of phytohormone balance or transport due to disruption of the autoregulation of nodule number (Reid et al. 2011 for review), and this hormonal modification is likely to interfere with the regulation of shoot development. This hypothesis could be supported by the negative correlation that we found here between the nodule number and the total number of nodes on the main stem in hypernodulating mutants.

Photosynthetic efficiency for biomass production was evaluated using RUE (Table 2). For all genotypes, RUE varied

**Table 2** Impact of variation in nodule number on carbon nutrition traits, as measured at the end of the vegetative period. Statistical analysis was performed in a first group among Baccara treatments and in a second group among Frisson and hypermodulating mutants. Within each group, means followed by similar superscript letters are

not significantly different at the 0.05 probability level. Among each group, the Pearson correlation coefficient between each carbon nutrition trait and nodule number is shown. For the second group, the coefficient shown in brackets is the correlation coefficient considering hypermodulating mutants only

Treatment identification	Total plant biomass (g plant <sup>-1</sup> )	Leaf area (cm <sup>2</sup> plant <sup>-1</sup> )	Number of nodes on main stem (plant <sup>-1</sup> )	Radiation use efficiency (RUE) (g biomass MJ <sup>-1</sup> )
Baccara <sub>2004-shaded</sub>	1.36 <sup>C</sup>	125 <sup>D</sup>	14.7 <sup>A</sup>	1.87 <sup>B</sup>
Baccara <sub>2004</sub>	2.74 <sup>B</sup>	180 <sup>C</sup>	14.9 <sup>A</sup>	1.36 <sup>C</sup>
Baccara <sub>2006</sub>	4.52 <sup>A</sup>	233 <sup>B</sup>	14.9 <sup>A</sup>	1.91 <sup>AB</sup>
Baccara <sub>2007</sub>	4.64 <sup>A</sup>	322 <sup>A</sup>	15.2 <sup>A</sup>	1.97 <sup>A</sup>
Correlation with nodule number	0.93	0.87	ns	0.45
Frisson <sub>2008</sub>	3.58 <sup>a</sup>	699 <sup>a</sup>	16.2 <sup>ab</sup>	2.10 <sup>a</sup>
P64 <sub>2008</sub>	2.17 <sup>bc</sup>	417 <sup>b</sup>	16.5 <sup>ab</sup>	1.91 <sup>b</sup>
P118 <sub>2008</sub>	2.48 <sup>bc</sup>	473 <sup>bcd</sup>	15.8 <sup>bc</sup>	1.91 <sup>b</sup>
P121 <sub>2008</sub>	2.31 <sup>bc</sup>	425 <sup>bcd</sup>	14.5 <sup>cd</sup>	1.93 <sup>b</sup>
Frisson <sub>2009</sub>	4.24 <sup>a</sup>	681 <sup>a</sup>	16.2 <sup>ab</sup>	1.55 <sup>c</sup>
P118 <sub>2009</sub>	4.06 <sup>a</sup>	733 <sup>a</sup>	17.4 <sup>a</sup>	1.36 <sup>f</sup>
P87 <sub>2009</sub>	2.06 <sup>cd</sup>	446 <sup>bc</sup>	13.8 <sup>de</sup>	1.26 <sup>g</sup>
P90 <sub>2009</sub>	2.77 <sup>b</sup>	526 <sup>b</sup>	15.2 <sup>bc</sup>	1.42 <sup>ef</sup>
P89 <sub>2009</sub>	2.37 <sup>bc</sup>	349 <sup>cde</sup>	14.8 <sup>cd</sup>	1.51 <sup>cd</sup>
P91 <sub>2009</sub>	2.53 <sup>bc</sup>	440 <sup>bc</sup>	14.8 <sup>cd</sup>	1.40 <sup>ef</sup>
P93 <sub>2009</sub>	1.55 <sup>d</sup>	260 <sup>e</sup>	13.7 <sup>de</sup>	1.43 <sup>def</sup>
P122 <sub>2009</sub>	1.96 <sup>cd</sup>	312 <sup>de</sup>	13.0 <sup>e</sup>	1.47 <sup>cd</sup>
Correlation with nodule number	-0.57 (-0.42)	-0.62 (-0.51)	-0.64 (-0.60)	-0.31 (ns)

ns nonsignificant correlation at the 0.05 probability level

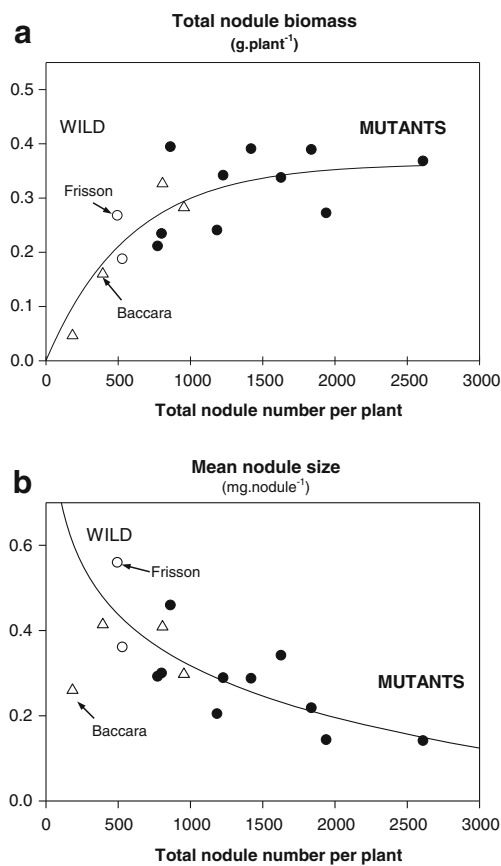
among years and treatments, without correlation with nodule number. Noticeably, RUE was 2 to 18 % lower for hypermodulating mutants than for wild types. Thus, we showed that photosynthetic efficiency for biomass production (RUE) was negatively affected by hypermodulation. Lower RUE values in hypermodulating mutants could be induced by the higher nodule biomass proportion in nodulated roots (Fig. 3b). Indeed, higher respiratory carbon costs were shown to be associated to nodule formation and activity, as compared to roots (Warembourg and Roumet 1989; Voisin et al. 2003b; Andrews et al. 2009). Thus, our detailed quantitative analysis showed that hypermodulation was associated with a concomitant limitation of leaf area and photosynthetic efficiency, and this resulted in a decrease in the C uptake capacity with increasing nodule numbers in hypermodulating mutants.

### 3.4 Competition for carbon resources in hypermodulating mutants induced a decrease of nodule size with nodule number

Variation in nodule number induced variation in total nodule biomass per plant and mean nodule size (Fig. 4).

Considering our whole range of plant material, nodule biomass increased with nodule number up to a maximal value, according to a single positive two-phase relationship. This relationship included both wild types and hypermodulating genotypes (Fig. 4a). As a result, nodule size decreased with nodule number, according to a single decreasing relationship that was also valid for all genotypes (Fig. 4b). And nodule size of hypermodulating mutants decreased with nodule number, from 0.5 down to 0.1 mg nodule<sup>-1</sup>. Novak (2010) has recently suggested that the large increase in nodule number causes an imbalance between the nodulated root's demand for carbon and the photosynthetic capacity of the host plant to sustain nodule growth and activity. Here we showed that mean nodule size decreased with nodule number in hypermodulating mutants (Fig. 4b). Nodule underdevelopment is an accompanying feature of hypermodulation (Day et al. 1987; Novak et al. 2009; Novak 2010). We originally suggest here that nodules compete for carbon resources within the root system. Our data can be related to the theory of competition for resources within a population of fruit units described by Lescourret and Génard (2003). For this, we made an analogy between nodules in





**Fig. 4** Total nodule biomass and nodule size at the end of the vegetative period. Nodule biomass (**a**) and nodule size (in milligrams dry weight per nodule) (**b**) as plotted vs. total nodule number per plant for wild-type Baccara (white triangle), wild-type Frisson (white circles) and hypernodulating mutants (black circles). In each graph, each point represents the means calculated on five or six plants. In **a**, a single exponential like relationship rising to a maximum was fitted on all experimental points ( $Y=0.3638(1-\exp(-0.0017 X))$ ;  $R^2=0.89$ ). In **b**, according to Lescourret and Génard (2003), a single relationship between nodule size ( $S$ ) and nodule number ( $n$ ) was fitted on all experimental points:  $S = S_{\max}[1 - (n/n_{\max})^\alpha]$ , with  $S_{\max}=10$ ,  $n_{\max}=6,000$ ,  $\alpha=0.018$  and  $R^2=0.80$  (see below) and with an optimal nodule number value  $n_{\text{opt}} = n_{\max}/[(\alpha + 1)^{1/\alpha}] = 2227$ . Calculations were made on points representing single plants (not shown)

the root system and fruit in that of the shoot. In accordance with this theory, among wild type and hypernodulating mutants, there was (a) a decrease in nodule size with increasing nodule numbers and (b) an increase in total nodule mass in the “population” as the number of nodules increases, until a maximal value (Fig. 4a). Following this theory, an optimal number of nodules for maximal total nodule biomass can be calculated (Fig. 4b). Thus, according to Lescourret and Génard (2003), a single relationship between the nodule size ( $S$ ) and nodule number ( $n$ ) was fitted on all experimental points:  $S = S_{\max}[1 - (n/n_{\max})^\alpha]$ , with  $S_{\max}=10$ ,  $n_{\max}=6,000$ ,  $\alpha=0.018$  and  $R^2=0.80$ . The optimal number of nodule was  $n_{\text{opt}} = n_{\max}/[(\alpha + 1)^{1/\alpha}] = 2,227$ . Interestingly, for wild-type genotypes, nodule numbers were always lower

than this optimum. For hypernodulating mutants, only the strongest hypernodulating phenotypes were above the optimum. Following Lescourret and Génard (2003), two hypotheses can be evoked to account for nodule numbers below the optimum: (a) either C resources are always large enough to allow the increase of the metapopulation (i.e. nodule number here) or (b) the metapopulation can act on the availability of resources. In our situation, the first hypothesis may account for wild-type behaviour, with nodule number being limited through a combination of the autoregulation of nodulation and systemic feedback by plant N status (Mortier et al. 2012; Ruffel et al. 2008). The second hypothesis most probably applies to hypernodulating mutants; this is suggested by a shift of carbon assimilates from roots to nodules, being drained by the high sink strength of numerous nodules (Hacin et al. 1997).

### 3.5 Towards the identification of indicators reflecting excess C cost of nodulation in hypernodulating mutants

Overall, our results provide a quantification of the negative impact of hypernodulation on plant carbon uptake and biomass partitioning within the plant. The negative impact of hypernodulation can be associated to carbon costs associated to excess nodule formation. We calculated an indicator of the relative carbon costs of nodule formation for the host plant using “nodule biomass proportion” (as the biomass proportion of nodules relative to total biomass). For both Baccara and Frisson wild types, nodule biomass proportion was around 6 % and increased only slightly with nodule number (from 4 to 7 %, Fig. 3c). For hypernodulating mutants, nodule biomass proportion was always higher than the wild type, increasing from 10 up to 20 %. An accurate evaluation of the total C costs associated with nodule formation and activity would need to include respiratory costs (Warembourg and Roumet 1989; Voisin et al. 2003b). However, nodule biomass is likely to be an appropriate indicator of the extra C cost associated to  $N_2$  fixation compared to nitrate assimilation as (a) C respiratory costs associated with  $N_2$  fixation and nitrate uptake activities are similar (Andrews et al. 2009) and (b) C respiratory costs associated with nodule synthesis and turnover are proportional to nodule biomass. Our study shows here that nodule biomass proportion strongly discriminates between hypernodulating mutants and wild types, as it was twice to three times higher in hypernodulating mutants compared to wild type. As such, nodule biomass proportion could be considered as an appropriate indicator of nodule overload for the host plant.

## 4 Conclusion

We provide here a functional basis to account for the depressed growth phenotype associated with hypernodulation in pea. Our experimental study was based on an integrated

functional analysis of traits related to C nutrition at the whole plant level using isogenic hypernodulating mutants in pea. This is the first time that this allelic series of SYM 29 mutants was characterised, and the use of this genetic material as a continuum to establish trends between nodule number and growth parameter is also innovative. We showed that excess nodule formation in hypernodulating mutants mainly resulted in a shift of assimilate from roots to nodules. Within nodules, increased competition for carbon also negatively affected individual nodule size. At the plant level, shoot growth limitation could be accounted for either by the extra-carbon costs induced by nodule formation and/or by a direct effect of the mutation on shoot development. This whole plant study will provide guidance for adequate traits to breed together with hypernodulation. Hence, integrative studies of the same kind have already proved to be efficient for identifying robust QTLs in quantitative genetic analysis (Moreau et al. 2012). For further studies, it should be reminded that the cost of hypernodulation not only impinges the C budget. With its negative effect on root growth, hypernodulation is likely to reduce the access to major resources like water and mineral nutrients in the field. In addition, due to the complex relationships that exist between carbon nutrition and nitrogen nutrition in legumes, an integrative approach describing the impact of hypernodulation of nitrogen nutrition traits N and their interaction with carbon nutrition (Moreau et al. 2012) would be useful to identify the most adequate traits to select and combine by breeders.

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