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Anne-Sophie Voisin, Marion Prudent, Gérard Duc, Christophe Salon. Pea nodule gradients explain N nutrition and limited symbiotic fixation in hypernodulating mutants. *Agronomy for Sustainable Development*, 2015, 35 (4), pp.0. 10.1007/s13593-015-0328-8 . hal-01353750

HAL Id: hal-01353750

<https://hal.science/hal-01353750>

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Pea nodule gradients explain N nutrition and limited symbiotic fixation in hypernodulating mutants

Anne-Sophie Voisin¹ · Marion Prudent¹ · Gérard Duc¹ · Christophe Salon¹

Accepted: 17 July 2015 / Published online: 13 August 2015
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Abstract Legumes fix atmospheric nitrogen by symbiosis with soil bacteria in root nodules. Legume yields are limited by the low capacity of N₂ fixation. Hypernodulating mutants have been selected decades ago to try to increase nodule number. However, literature data show that N fixation of hypernodulating mutants was not increased compared to parental lines. Here, we study the functional basis of limited N fixation associated to hypernodulation. We grew two wild type genotypes and nine hypernodulating mutants of pea in hydroponics in three greenhouse experiments. We measured the following traits related to N nutrition during the vegetative period: nodule number, plant N uptake, nodule-specific activity, and plant and nodule concentrations. Genetic and environmental variations induced nodule gradients. These gradients were used to set quantitative relationships between N nutrition traits and nodule number. We compared the relationships obtained for hypernodulating and for wild types. N nutrition traits were analysed together with C nutrition traits, through correlation networks. Our results show that higher nodule number of hypernodulating mutants is correlated with lower levels of nodule activity, from –25 to –60 %, by comparison to the wild type. Higher nodule number of hypernodulating mutants is also correlated with lower total N uptake by symbiotic fixation, from –0 to –60 %, by comparison to the wild type. Findings demonstrate that N nutrition is not a factor limiting growth in hypernodulating mutants, as shown by N nutrition index higher than 1, indicating N nutrition in excess. The correlations suggest that limited N₂ fixation in hypernodulating mutants arises from restricted shoot growth,

which limits the plant capacity to accumulate N. Furthermore, symbiotic efficiency decreased with increasing nodule number, down to a minimal value for hypernodulating mutants. Thus, to overcome the trade-off between N benefits from N₂ fixation and carbon nodulation costs, the hypernodulation trait should be associated with high shoot growth capacity in breeding programs.

Keywords *Pisum sativum* · Hypernodulating mutants · Nodules · Symbiotic N₂ fixation · Symbiosis · Correlation networks

Abbreviations

AON	Autoregulation of nodulation
C	Carbon
N	Nitrogen
NNI	Nitrogen nutrition index
SNU	Specific nitrogen uptake

1 Introduction

Due to their unique ability to fix atmospheric N₂ in symbiosis with soil bacteria, legumes have a major role to play in sustainable agriculture. As such, they can provide protein-rich feed or food in soils poor in nitrogen (N) at low environmental cost. However, N₂ fixation remains a limiting factor of growth and yields, as it is often impaired by biotic and abiotic factors in agricultural conditions (Corre-Hellou and Crozat 2005). This can be partly attributed to the low ability of the N₂ fixing nodules to withstand stresses. Two main reasons can account for this particularity of legume plants: first, nodule number is limited by the host plant (see below); second, legume plant response to restore its N status following a stress mainly relies

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on the enhancement of nodule biomass and on the increase of nodule number, as shown in split-root experiments in which one part of the nodulated root system was deprived of N_2 (Jeudy et al. 2010). Indeed, no short-term increase of N_2 -specific activity of the nodules still exposed to N_2 could be observed, whereas this was the case for root-specific nitrate uptake in root systems partly deprived of nitrate (Jeudy et al. 2010). Therefore, increasing symbiotic fixation through an increase of nodule number and/or biomass could be a promising perspective.

Two major pathways involved in the control of nodule number have been identified. First, an internal regulation leads to an “auto-regulation of nodule number” (AON) by the host plant, through a systemic feedback repression of nodulation by pre-existing nodules (Reid et al. 2011; Mortier et al. 2012 for reviews). Deregulated mutants with a hypernodulating phenotype were isolated in several legume species (Novak 2010; Reid et al. 2011). In the case of pea (*Pisum sativum* L.), hypernodulating mutants have been selected after mutagenesis and three genes have been shown to be involved in the regulation of 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 have been identified (Krusell et al. 2002, 2011; Schnabel et al. 2011), NOD3 being of unknown function when the two other genes encode a leucine-rich receptor like kinase.

In an attempt to greatly increase nodule number and therefore symbiotic N_2 fixation, several hypernodulating mutants have been characterised in field or greenhouse studies. The hypernodulating phenotype was never associated to a substantial increase in N uptake by the mutant plants as compared to their wild type. Instead, hypernodulation most often limited plant growth and productivity (Sagan et al. 1993; Bhatia et al. 2001; Novak 2010; Voisin et al. 2013). Hence, most studies on hypernodulating mutants have reported negative pleiotropic traits related to carbon nutrition such as depressed shoot growth, shortened internodes or fasciation of the stem, and reduction of roots development and growth (e.g., pea: Postma et al. 1988; Duc and Messenger 1989; Sagan and Duc 1996; Krusell et al. 2002; Bourion et al. 2007; soybean: Matsunami et al. 2004; Voisin et al. 2013). These pleiotropic effects might be a direct effect of the mutated gene on those carbon nutrition traits, or it may result from a physiological consequence of deregulated (and therefore excessive) nodule number (Novak et al. 2011).

The regulation of nodule development by the whole plant N status was shown to be the second major pathway involved in the control of nodule number by the host plant (Jeudy et al. 2010). When nodulation occurs in wild types, whole plant studies have shown a positive environmental correlation between nodule number and plant growth rate (Voisin et al. 2010). This was interpreted as

an adjustment to the host plant N requirements, at minimum carbon cost for nodule formation. In hypernodulating mutants, which are characterised by defective AON regulation, the loss of nodule number regulation occurs simultaneously with loss of nitrate sensitivity of nodulation (Oka-Kira and Kawaguchi 2006). This suggests that the AON pathway shares common elements with the control of nodule development by the whole plant N status (Jeudy et al. 2010). Therefore, we hypothesized that hypernodulation would first impact the relationships between nodule number and the plant N status, and that this would in turn impact carbon nutrition traits. In deregulated mutants, our hypothesis is that excessive nodule formation in hypernodulating mutants could lead to an imbalance between nodule biomass and nitrogen needs of plants. Nevertheless, only sparse information is available on the impact of hypernodulation on nitrogen nutrition in relation with carbon nutrition traits. In a few studies, hypernodulation was associated with higher N content in root tissues (Day et al. 1986; Novak et al. 2011), in the shoot (Novak et al. 2011 and references therein) or in harvested seeds (Bourion et al. 2007), but this was not related to carbon nutrition traits. In other studies, hypernodulation was associated to lower specific N_2 fixing activity of nodules (in g N_2 fixed per g nodule) and high N status as compared to wild type (Voisin et al. 2007), without full characterisation of carbon nutrition traits. As such, the actual impact of hypernodulation on N_2 fixation remains to be precisely characterised, considering both its structural and functional components, and its relationships with the plant N status and with plant carbon nutrition traits, with a special attention to carbon use for nodule formation.

In this context, our objectives were to provide a functional basis for the low N_2 fixation usually associated with hypernodulation in pea. Our hypothesis is that there would be a trade-off between symbiotic N_2 fixation and carbon costs induced by nodule formation. For this, we used a set of hypernodulating mutants including an allelic series of SYM29 mutants, thus providing a continuous trend in the strength of hypernodulation. In a previous study, we have used this original set of hypernodulating mutants to explain the pleiotropic depressed growth associated with hypernodulation in pea (Voisin et al. 2013). In the present study, the nodule gradients obtained among hypernodulating genotypes allowed drawing trends between nodule number and traits related to the host plant nitrogen nutrition through symbiotic N_2 fixation. Such trends obtained for isogenic hypernodulating mutants were compared to those obtained for wild types. Then, we have combined those two sets of data to analyse global interactions between carbon and nitrogen nutrition at the plant level and to identify traits reflecting symbiotic efficiency of N_2 fixation by the host plant.

2 Materials and methods

2.1 Plant material

The 2004, 2006, and 2007 experiments were conducted using pea (*P. 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-methane sulfonate (EMS) 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; Krusell 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), each differing in sequence of the *sym29* allele (Krusell et al. 2002) and varying in hypernodulating intensity, as described in Voisin et al. 2013.

2.2 Growing conditions

Plants were grown in hydroponics culture (Fig. 1) 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, in the same experiment as that described in our joint study describing the impact of hypernodulation on C nutrition (Voisin et al. 2013). All experiments spanned from germination until the appearance of the first flowers and thus lasted around 6 weeks. For each experiment, 1200 to 1320 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 without mineral nitrogen 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

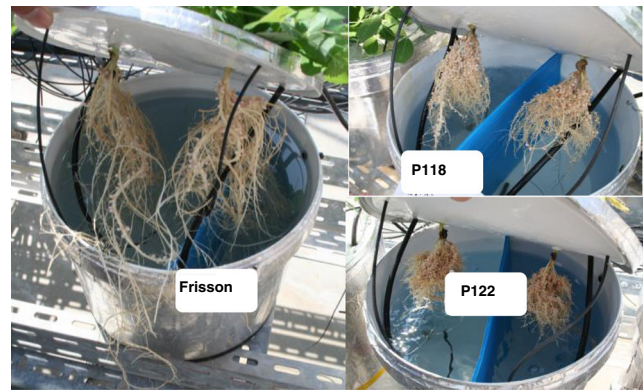


Fig. 1 Nodulated roots of the genotypes Frisson, and P118 and P122 hypernodulating mutants, as grown in hydroponics in our greenhouse experiment

by bubbling air in each pot compartment. The air flow was calibrated so that oxygenation was always close to 100 % of its maximal value (LDO sensor, Hatch Lange). Plants were inoculated with the N_2 -fixing bacteria *R. leguminosarum* bv. *Viciae* P221 (MIAE01212) with a concentration of 10^8 colony forming units per plant. This strain was obtained from “Microorganismes d’intérêt Agroenvironnemental” located at UMR Agroécologie, INRA, Dijon, France. The inoculum was prepared from cultures grown on Bergersen slopes washed with sterile water. 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

Meteorological were described in Voisin et al. (2013). 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 PAR was measured using silicium sensors (Solems, Palaiseau, France). Measurements were made at 600 s intervals, stored in a data logger (DL2e; Delta-T Devices, Cambridge, England). Temperatures were 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. 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 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⁻² day⁻¹ among years and treatments.

2.4 Harvests and measurements

Plants were harvested two to three times in 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. 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. 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. Total nitrogen concentration was determined on dried, ground tissue samples according to the Dumas procedure, using a Carlo Erba Elemental Analyzer CN2500 (Thermo Fisher Scientific) adapted with a Multi separation Column (PTFE, SS, 2 m, 6×5 mm, CE Elantech), which allows the rapid combustion of organic N in N₂ and its subsequent measurement in a chromatographic column.

2.5 Determination of the critical N dilution curve for isolated pea plants

As previously performed in the field for dense stands (Sagan et al. 1993), a critical nitrogen dilution curve was calculated here for isolated plants. It enables estimating the “critical N concentration” for a given shoot biomass, i.e., the minimal N concentration allowing a maximal biomass production. To that end, a specific experiment was conducted in the greenhouse, using non-nodulating mutants of pea (mutant P2; Sagan et al. 1993). Using this mutant unable to establish the *Rhizobia* symbiosis, it was possible to induce N deficiency by varying nitrate supply in the nutrient solution. Thus, in a specific greenhouse experiment, P2 mutants were grown in pots in a 1:1 (v/v) mixture of sterilized attapulgit and clay balls (diameter 2–6 mm), according to six N treatments with varying nitrate concentration of the nutrient solution (namely 0.625, 1.25, 5, 7.5, 10, and 14 N-NO₃⁻ mol m⁻³). Plants were harvested between germination and beginning of flowering.

For each of the nine sampling dates, the critical N concentration was identified as the shoot N concentration from which an increase in N concentration was no longer associated to an increase in shoot biomass (DW). The critical N dilution curve was defined by fitting a power curve on the nine “critical N concentration” values, as plotted vs. shoot biomass, thus defining the critical shoot N concentration (N_c) for optimal shoot growth in isolated plant.

2.6 Calculations and statistical analysis

Our analysis was conducted up to the end of the vegetative growth as nodule, and root establishment is mainly restricted to this period (Bourion et al. 2007), due to strong competition for C with the formation of reproductive organs afterwards.

Nitrogen nutrition index (NNI) In pea as in most plants, there is a dilution of nitrogen in biomass as biomass increases through the growth cycle. As such, total N concentration does not directly indicate whether N nutrition is a limiting factor of growth or not. To assess the nitrogen nutrition level relative to growth requirements, the nitrogen nutrition index (NNI) was calculated as the ratio between shoot N concentration and the “critical N concentration”, as defined above. When NNI is close to 1, it is classically interpreted that N nutrition is optimal. An NNI value lower than 1 indicates an N-deficiency while an NNI values higher than 1 indicates N uptake in excess.

Specific nitrogen uptake (SNU) Due to the absence of mineral N in the nutrient solution, plant N accumulation exclusively arises from symbiotic fixation and seed N reserves. Thus, the quantity of N₂ fixed can be calculated as total N accumulated in the plant minus seed cotyledon N decrease from sowing. To account for the efficiency of the *Rhizobial* symbiosis in fixing N₂, the specific nitrogen uptake from N₂ fixation of indeterminate nodules of pea (SNU, in mgN g nodule⁻¹ day⁻¹) was defined as the ratio between the quantity of N₂ fixed from date t_a to date t_b (N_b–N_a) and the integrated nodule dry weight (DW_{nod}) between date t_a to date t_b. SNU was calculated as follows (Voisin et al. 2007):

$$\text{SNU} = \frac{N_b - N_a}{\int_{t_a}^{t_b} DW_{\text{nod}} \cdot dt}, \text{ with } \int_{t_a}^{t_b} DW_{\text{nod}} \cdot dt = \sum_{i=a}^b (DW_{\text{nod}i} + DW_{\text{nod},i+1}) \times (t_{i+1} - t_i) / 2$$

A correlation network analysis was performed in order to visualize relationships among variables as a whole. The

network consisted of a set of nodes (measured or calculated variables) connected by a system of lines that represented the correlations (positive or negative) among variables when they were significant at the 0.001 probability level. The network layouts were generated using the Fruchterman-Reingold algorithm. The networks were constructed using Pajek graph-drawing software (<http://vlado.fmf.uni-lj.si/pub/networks/pajek/>), allowing to differentially coloring variables related to carbon fluxes (data from Voisin et al. 2013), variables related to nitrogen fluxes (from this study), and the number of nodules. Two correlation networks were drawn: the first one was drawn based on correlations among variables in the wild type genotypes, while the second one was drawn based on correlations among variables in hypernodulating mutant lines.

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

3 Results and discussion

Hypernodulation not only induces more nodules but it also alters additional plant traits. The aim of our study was thus to provide new insights into the understanding of those additional effects associated with hypernodulation, considering the main determinants of N nutrition at the plant level, in relation with C nutrition. This study is connected with a previous analysis on the determinants of C nutrition on the same data set (Voisin et al. 2013). For this, nodule number gradients were attained as described in Voisin et al. (2013) among a large set of hypernodulating mutants lines and for wild type genotypes. Relationships between nodule gradients and a set of parameters describing N nutrition were first analysed for hypernodulating mutant lines and compared to those obtained for the wild types. Our study thus provides an original analysis of the functional basis accounting for the relatively low N_2 fixation usually observed in hypernodulating mutants. Combining the analysis of N nutrition and C nutrition traits, we also propose an original analysis of the legume—Rhizobia symbiosis, through the evaluation of the trade-off between N benefits and C costs of nodulation process.

3.1 Hypernodulation negatively and gradually affected symbiotic N_2 fixation

For the Baccara genotype grown in diverse environments, total plant N uptake by symbiotic fixation was strongly

positively correlated with nodule number. Conversely, for Frisson and hypernodulating mutants, total plant N was negatively correlated with nodule number (Table 1). As such, total plant N was reduced by around -60% compared to wild type for the strongest hypernodulating phenotype. Still, in the 2009 experiment only, total plant N of the P118, P90, and P91 hypernodulating genotypes was not significantly different from that of wild type. Specific nitrogen uptake by nodules (SNU, in $\text{mgN g nodule}^{-1} \text{ day}^{-1}$) varied greatly among years and treatments for the Baccara genotype, but these variations were not strongly linked to variations in nodule number (Table 1). SNU of hypernodulating mutants was much lower than that of Frisson, even for the weakest hypernodulating phenotypes. Among hypernodulating mutants, SNU was negatively correlated with nodule number, with SNU being reduced by -25 to -60% compared to wild type (Table 1). As such, in wild type genotypes, increasing nodule number was closely related to increasing total plant N uptake, as revealed by positive correlations between nodule number and either nodule biomass (Voisin et al. 2013) or nodule-specific N uptake (SNU) (Table 1), together determining total N uptake. Conversely, in hypernodulating mutants, despite similar or higher nodule biomass than in wild type (Voisin et al. 2013), increasing nodule number was associated with decreasing levels of SNU and finally to decreased total N uptake, in accordance with the previous field observations (Voisin et al. 2007).

3.2 The “critical N dilution approach” allowed demonstrating that the limited symbiotic N_2 fixation of hypernodulating was not a factor limiting growth

Nitrogen is required for leaf expansion and for photosynthetic activity that together determine carbon uptake and biomass accumulation by the plant. Knowing this, we investigated whether decreased N uptake by N_2 fixation in hypernodulating mutants was a factor limiting plant growth. We first considered plant total N concentration that represents the ratio between N and C accumulation in the plant. For Baccara, total plant N concentration varied among years and treatments, but these variations were independent of nodule number (Table 1). Total N concentration in hypernodulating mutants was systematically equal or higher than that of wild type Frisson. Within hypernodulating mutant lines, it increased with nodule number, from 4.7% up to 6.7% .

Separating an “N demand” effect from an “N acquisition capability” is very crucial for understanding the dynamics and response of plant N uptake to different environment or genotype manipulations. Plant N demand can be evaluated through the use of the “critical N dilution” approach, which allows calculating the minimum N uptake necessary to achieve maximum biomass accumulation. For that, it was necessary to establish a “critical N dilution curve” for isolated pea plants,

Table 1 Impact of variation in nodule number on nitrogen nutrition traits, as measured at the end of the vegetative period

Treatment identification	Nodule number (nb plant ⁻¹)	Total plant N (mg N plant ⁻¹)	Specific nitrogen uptake (SNU) (mg N g nodule ⁻¹ day ⁻¹)	Total N concentration (%)	Nodule N concentration (%)	Nitrogen nutrition index (NNI)
Baccara _{2004-shaded}	184 ^D	47 ^C	49 ^B	3.5 ^B	6.94 ^B	0.69 ^B
Baccara ₂₀₀₄	393 ^C	94 ^B	45 ^B	3.4 ^B	6.74 ^B	0.68 ^B
Baccara ₂₀₀₆	803 ^B	110 ^B	45 ^B	2.4 ^C	5.28 ^C	0.49 ^C
Baccara ₂₀₀₇	955 ^A	183 ^A	77 ^A	3.9 ^A	7.56 ^A	0.85 ^A
Correlation with nodule number		0.89	0.47	ns	ns	ns
Frisson ₂₀₀₈	532 ^h	168 ^{bc}	89 ^a	4.7 ^e	8.07 ^{bcde}	1.03 ^{de}
P64 ₂₀₀₈	776 ^g	103 ^e	42 ^{cdef}	4.7 ^e	7.61 ^{ef}	0.93 ^e
P118 ₂₀₀₈	803 ^{fg}	138 ^{cde}	49 ^{cd}	5.6 ^d	8.40 ^{abc}	1.12 ^{cd}
P121 ₂₀₀₈	1840 ^b	140 ^{cde}	34 ^f	6.1 ^{bc}	7.98 ^{cde}	1.20 ^{bc}
Frisson ₂₀₀₉	497 ^h	187 ^{ab}	83 ^a	4.5 ^e	8.05 ^{bcde}	0.96 ^e
P118 ₂₀₀₉	864 ^{efg}	224 ^a	63 ^b	5.5 ^d	8.20 ^{abcd}	1.17 ^{bc}
P87 ₂₀₀₉	1187 ^{def}	122 ^{de}	48 ^{cde}	5.9 ^{bcd}	8.39 ^{abc}	1.16 ^{bc}
P90 ₂₀₀₉	1230 ^{de}	166 ^{bc}	51 ^c	6.0 ^{cd}	8.47 ^{ab}	1.19 ^{bc}
P89 ₂₀₀₉	1423 ^{cd}	135 ^{cde}	39 ^{def}	5.9 ^{cd}	7.59 ^f	1.15 ^{cd}
P91 ₂₀₀₉	1630 ^{bc}	160 ^{bcd}	49 ^{cd}	6.3 ^{abc}	8.50 ^a	1.28 ^{ab}
P93 ₂₀₀₉	1942 ^b	104 ^e	37 ^{ef}	6.7 ^a	7.65 ^{ef}	1.35 ^a
P122 ₂₀₀₉	2613 ^a	126 ^{cde}	32 ^f	6.4 ^{ab}	7.79 ^{def}	1.27 ^{ab}
Correlation with nodule number (hypermodulating mutants only)		-0.31 (ns)	-0.66 (-0.56)	+0.77 (±0.68)	ns (ns)	+0.66 (±0.57)

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 “nitrogen nutrition trait” and nodule number is shown. For the second group, the coefficient shown in brackets is the correlation coefficient considering hypermodulating mutants only. “ns” indicates non significant correlation at the 0.05 probability level

which had never been done. The critical nitrogen dilution curve obtained here defines the critical shoot N concentration (N_c) for optimal shoot growth (DW) in isolated plant as follows: $N_c = 4.756 DW^{-0.088}$ ($R^2 = 0.93$). The “dilution coefficient” of -0.088 is very close to curves already calculated for other isolated C3 plants, within the range of -0.08 to -0.10 (Lemaire et al. 2007). As for other C3 crops (Lemaire et al. 2007), the dilution coefficient obtained in isolated plants is higher than calculated for dense crops (-0.32 for pea; Ney et al. 1997). This indicates that in isolated plants, plant N concentration declines more slowly with plant mass compared to dense crop. Indeed, N dilution in biomass was shown to be accelerated in situation of competition for light, as N is re-directed within the plant towards illuminated leaves (Lemaire et al. 2005).

The “critical N dilution approach” thus allowed evaluating the N uptake level relative to growth requirements, using the Nitrogen Nutrition Index (NNI, Table 1). For Baccara, NNI increased with nodule number, from 0.49 up to 0.85, while NNI was close to 1 for the Frisson genotype. This indicated deficient N nutrition for Baccara and optimal N nutrition for Frisson. In contrast, NNI was systematically equal or higher than 1 for hypermodulating mutant lines, indicating N nutrition in excess.

To further analyse the relationships between symbiotic fixation activity and its ability to sustain growth requirements, SNU values were plotted as a function of NNI (Fig. 2a). In wild types, SNU was positively and linearly related to NNI, with NNI values increasing up to one. For wild type genotypes, this suggests that nodule N₂ fixing activity was a limiting factor of growth. In contrast, for hypermodulating mutants, low SNU values were associated to NNI values above 1. It was postulated as a general rule that in situations where access to N substrate is not limiting, N uptake by the plant is determined by N demand, as determined by plant growth rate (Devienne-Barret et al. 2000). As such, in hypermodulating mutants, the high levels of NNI indicate that the low N₂ fixing activity of hypermodulating mutant tended to saturate the host plant sink capacity. Indeed, on the one hand, the N uptake capacity of hypermodulating mutants was higher than that of wild types, due to their high nodule biomass arising from deregulated nodulation. On the other hand, plant N requirements for growth were lower in hypermodulating mutants than in wild types; indeed, a limited shoot and root growth were previously observed in hypermodulating mutants and interpreted as a result from C competition with excessive nodule growth (Voisin et al. 2013). At the physiological level, a

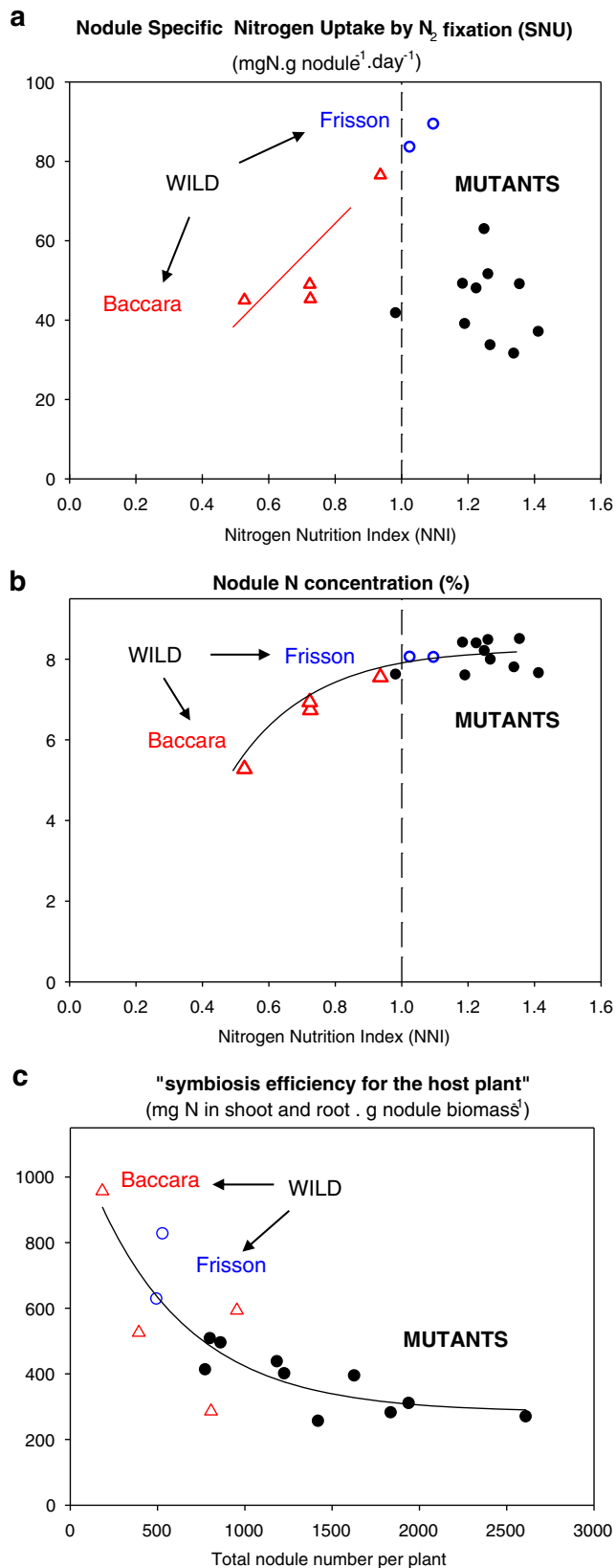


Fig. 2 Traits reflecting N nutrition as related to the N nutrition level (a and b) or to nodule number (2c) for wild type Baccara (red triangle), wild type Frisson (blue circles), and hypernodulating mutants (black circles). Each point represents the means calculated on five or six plants. Nodule-specific nitrogen uptake by N₂ fixation (SNU) (a) and nodule N concentration (b) as plotted vs. NNI (Nitrogen Nutrition Index, see Material and methods for calculation). In (a), a linear regression was performed on experimental points corresponding to Baccara plants; in (b), a single exponential-like relationship rising to a maximum was fitted to all experimental points ($Y = -15.5 + 23.8 (1 - \exp(-4.2 X))$; $R^2 = 0.88$). "Symbiosis efficiency for the host plant" (c), as indicated by the relationship between N accumulated in the host plant (as the sum of N in shoot plus N in roots, without nodules) and total nodule number per plant. A single exponential like relationship decreasing to a lower level was fitted to all experimental points ($Y = 283 + 876 \exp(-0.0018 X)$, $R^2 = 85$)

and Layzell 1997; Fischinger et al. 2006). This regulation involves for example amides in temperate legumes. We therefore make the hypothesis that low SNU values in hypernodulating mutants were the result of a down-regulation of N₂ fixation associated with high levels of plant N concentration. And we conclude that the limited N₂ fixation in hypernodulating mutants is limited by the limited capacity of their shoots to store N, due to limited shoot growth.

As the nodules are the organs in which N₂ fixation occurs, nodule N concentration was also considered (Table 1). In wild types, nodule N concentration varied between 5.28 and 7.56 %, while nodule N concentration was around 8 % for the whole set of hypernodulating genotypes (Table 1). Nodule N concentration was then plotted as a function of NNI (Fig. 2b). For all genotypes, N concentration increased with NNI values, up to an 8 % plateau for NNI values equal or higher than 1. As such, considering functional nodules of similar age for all genotypes and experimental situations, high levels of nodule N concentration presumably indicate a saturation of the N₂ fixing enzymatic machinery, as inferred by excess N nutrition. Interestingly, unlike for SNU, the relationship between nodule N concentration and NNI was valid for all genotypes. And unlike plant N concentration, nodule N concentration saturated to a single level for supra optimal levels of NNI. As such, it appears that nodule N concentration can be considered as a convenient indicator of the capacity of nodules to sustain plant N requirements for growth. Indeed, in our study, the relationship proved here to be valid across the large set of genotypes that we used, and its absolute value directly indicates the level of N nutrition, without the need of a reference for optimal N concentration (unlike plant N concentration).

3.3 A global view of relationships between C and N nutrition traits evidences shoot growth as an important trait driving N uptake both in wild types and hypernodulating mutants

systemic repression of N₂ fixation by N-rich phloem downstream metabolites has been shown in numerous studies (Neo

In order to globally visualise how nodule number differentially affects interactions between C and N nutrition in

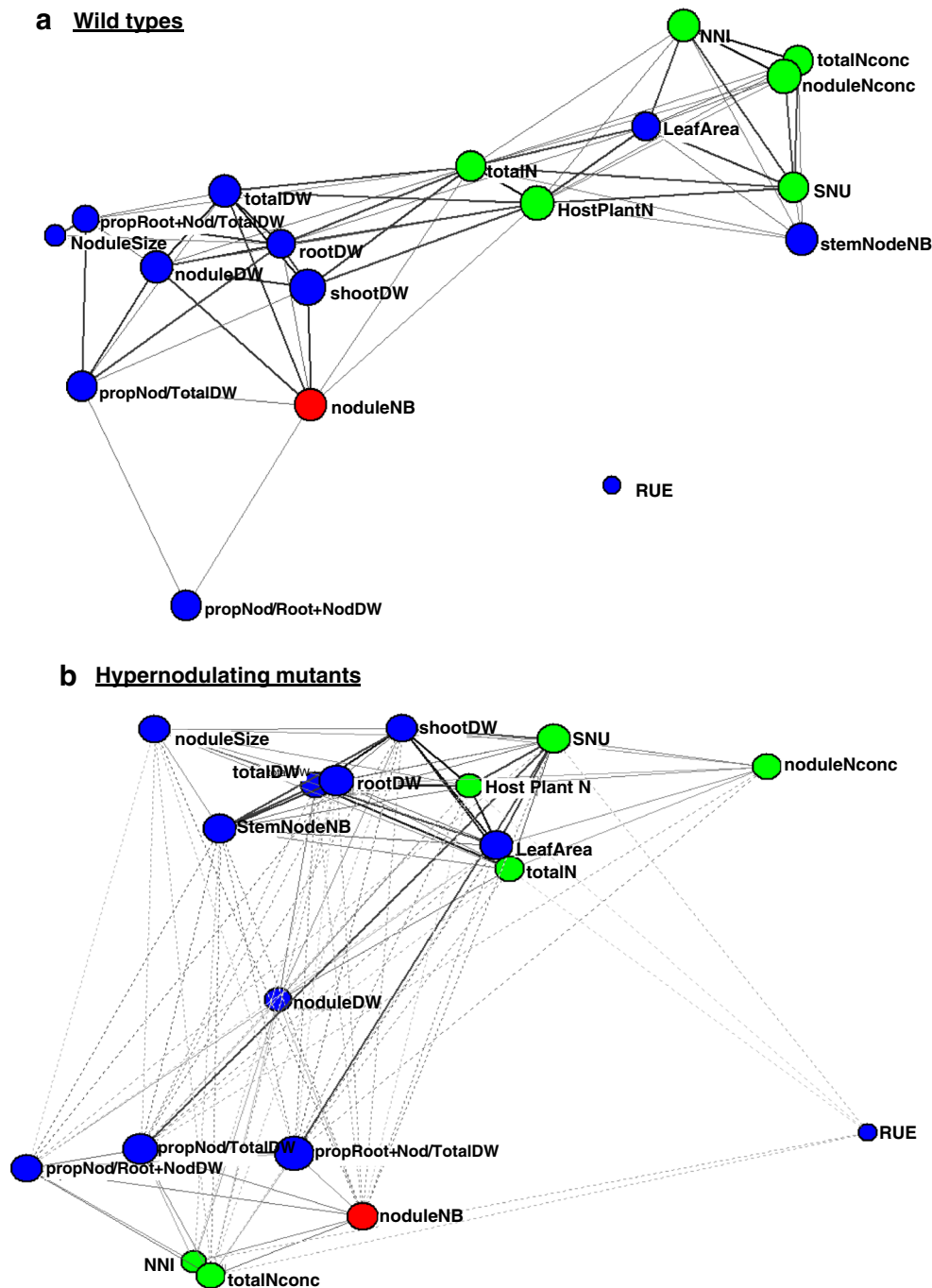


Fig. 3 Correlation networks among variables in wild type genotypes and in hypernodulating mutants. The first network (a) was drawn using data from wild type genotypes Frisson and Baccara and the second one using data from the seven hypernodulating mutant lines (b). Eleven variables related to carbon fluxes are presented as blue nodes: radiation use efficiency (RUE), leaf area (LeafArea), number of nodes on the main stem (StemNodeNB), mean nodule biomass (NoduleSize), dry weights of shoots (shootDW), roots (rootDW), nodules (noduleDW), and whole plant (totalDW), proportion of nodulated root biomass within the whole plant (propRoot+Nod/TotalDW), proportion of nodule biomass within the nodulated roots (propNod/Root+NodDW), proportion of nodule biomass within the total plant (propNod/TotalDW). Six variables related

to nitrogen fluxes are presented as green nodes: the total quantity of nitrogen contained in the plant (totalN), the total quantity of nitrogen contained in the plant minus the nitrogen contained in the nodules (HostPlantN), the nitrogen concentration in the plant (totalNconc), the nitrogen concentration in the nodules (noduleNconc), the specific nitrogen uptake from N₂ fixation (SNU), and the nitrogen nutrition index (NNI). The nodule number (noduleNB) is presented as a red node. The lines represent the correlation between two variables, when the associated probability was significant at the 0.001 probability level. A dotted line means that the correlation is negative. The bigger the node, the higher the number of connections at this node

hypernodulating mutants vs. in wild types, correlation networks were compared for wild type genotypes (Fig. 3) and for hypernodulating mutants (Fig. 3b). The correlation networks comprised all variables used to characterise N nutrition in this study and those used to characterise C nutrition in the related study on the same data set (Voisin et al. 2013).

For the wild type genotypes, all correlations among traits were positive (Fig. 3a and Table 2). Nodule number was thus positively correlated both to growth traits and to N nutrition traits. Especially, nodule number was closely positively related (i) both to shoot and root growth and (ii) to traits reflecting total N uptake by the plant (“total N” and “Host Plant N”, Fig. 3a). We have shown here so-called “environmental correlations” among traits, as they were obtained using environmental variations. Most of these relationships have also been identified as “genetic correlations” in a quantitative genetics analysis based on a large set of genotypes of pea (Bourion et al. 2010), which has demonstrated numerous colocalizations of quantitative traits loci and positive correlations between nodule number, root growth, and N uptake. This suggests the possibility of breeding new pea cultivars with increased root system size and sustained nodule number, thus improving N nutrition.

For hypernodulating mutants, relationships among variables related to C and N nutrition were strongly modified, as compared to wild types (Fig. 3b and Table 2). In particular, unlike for wild types, correlations between nodule number and shoot and root growth or total N uptake were negative. Nodule number was positively correlated only to variables reflecting either (i) nodule biomass or its biomass proportion within the host plant or (ii) the N nutrition level (plant N concentration or NNI).

Novak (2010) has recently suggested that the large increase in nodule number in hypernodulating mutants causes an imbalance between the demand of the nodulated root for carbon and the photosynthetic capacity of the host plant to sustain nodule growth and activity. Likewise, we have previously hypothesised that low nodule SNU of hypernodulating mutants could be accounted for by C competition with excessive nodule growth (Voisin et al. 2007). We have shown here that low SNU values were associated with high levels of N concentration, presumably linked to a saturation of the N_2 fixing enzymatic machinery. Interestingly, our study therefore demonstrates that nodule-specific N uptake (SNU) of hypernodulating mutants is not limited by a restriction of carbon and energy supply from photosynthesis, but SNU is rather limited by the low capacity of the shoot to store N ($NNI > 1$), due to limited shoot growth in hypernodulating mutants. In support of this hypothesis, attempts to increase nodule-specific activity through genetic modifications of the C machinery in alfalfa symbionts resulted in increased N concentration in the host plant but failed to increase total biomass and N accumulation (Tikhonovich and Provorov 2007 and 2011).

As such, in order to increase total N uptake in the shoot, the hypernodulating trait should be associated with high shoot growth capacity, in accordance with promising performance of backcrosses of hypernodulating mutants into forage varieties (Novak et al. 2009). This hypothesis is reinforced by the global view provided by correlation networks among the set of variables characterizing C and N nutrition that we used here (Fig. 3). Hence, both in wild types and hypernodulating mutants, all variables reflecting N accumulation by the host plant appeared to be closely related to variables reflecting leaf development and expansion, which therefore here appear as the key traits closely linked to N nutrition that should be genetically improved.

3.4 Towards the identification of traits reflecting the trade-off between N benefits and C cost of nodulation: from symbiosis to parasitism

In hypernodulating mutants, excessive nodule number and biomass were associated with lower shoot and/or root growth (Fig. 3b). A “symbiosis efficiency for the host plant” of the legume-Rhizobia symbiosis was calculated here as the ratio between “N benefits” for the host plant relative to “C investment in nodule biomass” (Fig. 2c). The N benefits for the host plant were quantified using the amount of nitrogen provided to the host plant (as total N minus nodule N). Nodule C cost for the host plant was indicated by total nodule biomass, making the hypothesis that C concentration was similar among genotypes. This “symbiosis efficiency for the host plant” was plotted as a function of nodule number (Fig. 2c). All genotypes (wild types and hypernodulating mutants) followed a single trend, with “symbiosis efficiency for the host plant” decreasing from 1000 mg N g nodule⁻¹ for the lowest nodule numbers down to a 200 mg N g nodule⁻¹ lower limit for the highest numbers. The relative “efficiency” of the legume-Rhizobia symbiosis was shown here to decrease with nodule number, according to the same relationship for wild types and hypernodulating mutants. This reflects the unavoidable increase of nodule carbon cost with nodule number and biomass. Yet, in wild types, this nodule efficiency decreasing with nodule number was associated with positive effects on biomass and N accumulation in the host plant (Fig. 3a), while in hypernodulating mutants, those effects were negative (Fig. 3b). From an ecological point of view, when the symbiotic density increases, increasing host plant productivity reflects a mutualistic interaction while a decreasing host plant productivity indicates a parasitic behavior (Neuhauser and Fargione 2004). As such, hypernodulation could appear to turn the legume-Rhizobia interaction from symbiosis to parasitism. In wild type plants, thanks to the internal host plant control of nodule (therefore symbiont) number, the plant-Rhizobia interaction can remain mutualistic (Morgan et al. 2005). Conversely, in hypernodulating mutants in which the

Table 2 Correlation matrix between N nutrition and C nutrition traits for wild types and hypermodulating mutants

Wild types

	nodeNB	nodeDW	totalDW	shootDW	rootDW	totalN	totalNcnc	NNI	nodeNBcnc	LeafArea	stemNodeNB	SNU	RUE	propNod/TotalDW	propRoot+Nod/TotalDW	propNod/Root+NodDW	HostPlantN	NoduleSize
nodeNB	1.00	7.3E+10	9.9E+11	1.2E+11	1.6E+05	6.0E+05	6.1E+01	9.9E+01	4.3E+01	3.8E+01	5.2E+01	2.3E+01	4.2E+02	2.6E+05	1.7E+02	2.8E+04	2.0E+04	6.3E+01
nodeDW	0.84	1.00	2.0E+14	1.7E+11	1.4E+12	2.3E+05	5.1E+01	9.1E+01	3.0E+01	8.0E+02	1.3E+01	5.3E+01	3.9E+01	2.9E+12	5.2E+07	1.7E+03	1.3E+04	1.1E+02
totalDW	0.86	0.92	1.00	0.0E+00	4.7E+09	8.3E+01	4.0E+01	4.0E+01	9.7E+01	5.3E+02	6.7E+02	1.7E+02	3.2E+01	9.5E+06	4.2E+05	4.3E+08	4.5E+08	5.5E+02
shootDW	0.88	0.87	0.99	1.00	5.2E+10	1.1E+08	8.5E+01	4.3E+01	9.5E+01	1.3E+02	1.3E+01	1.2E+02	2.7E+01	1.5E+04	1.3E+03	4.1E+02	6.7E+08	1.9E+01
rootDW	0.67	0.89	0.91	0.84	1.00	5.0E+08	5.5E+01	2.3E+01	8.1E+01	1.6E+04	2.6E+02	2.6E+02	6.0E+01	4.3E+07	1.1E+11	5.7E+01	4.3E+07	2.6E+04
totalN	0.63	0.66	0.81	0.80	0.78	1.00	3.8E+06	8.1E+06	7.5E+04	3.8E+08	2.9E+04	1.0E+07	3.5E+01	1.0E+02	5.2E+04	9.2E+01	6.0E+00	7.1E+02
totalNcnc	-0.09	-0.12	0.04	0.03	0.11	0.60	1.00	0.0E+00	0.0E+00	5.7E+07	1.7E+04	5.8E+08	5.8E+01	3.1E+01	5.8E+01	1.5E+02	4.2E+05	6.0E+01
NNI	0.00	-0.02	0.15	0.14	0.21	0.68	0.99	1.00	0.0E+00	1.6E+08	6.1E+05	7.5E+10	4.3E+01	5.0E+01	3.3E+01	1.6E+02	1.3E+06	4.7E+01
nodeNBcnc	-0.13	-0.18	-0.01	-0.01	0.04	0.55	0.96	0.95	1.00	2.6E+05	1.8E+03	1.1E+08	3.6E+01	3.9E+01	1.3E+03	8.5E+03	2.4E+04	7.3E+01
LeafArea	0.15	0.30	0.47	0.42	0.60	0.79	0.74	0.80	0.65	0.71	1.00	1.1E+04	4.0E+01	2.4E+01	4.3E+03	9.6E+02	2.3E+04	4.1E+02
stemNodeNB	0.11	0.26	0.32	0.26	0.47	0.58	0.60	0.63	0.51	0.71	1.00	1.8E+01	1.8E+01	1.00	9.5E+01	8.7E+01	3.5E+01	2.1E+02
SNU	0.21	0.11	0.41	0.43	0.38	0.77	0.78	0.84	0.76	0.80	0.57	1.00	1.00	1.00	1.00	1.00	6.9E+09	7.4E+01
RUE	0.35	0.15	0.17	0.19	0.09	0.17	0.10	0.14	0.04	0.16	0.15	0.24	1.00	1.00	1.00	1.00	3.5E+01	2.1E+02
propNod/TotalDW	0.66	0.89	0.68	0.60	0.75	0.43	-0.18	-0.12	-0.26	0.15	0.21	-0.16	-0.01	1.00	3.7E+09	1.4E+04	3.1E+02	1.6E+03
propRoot+Nod/TotalDW	0.00	0.74	0.64	0.53	0.88	0.56	0.10	0.17	0.02	0.53	0.47	0.16	-0.08	0.82	1.00	7.9E+01	1.5E+03	3.2E+07
propNod/Root+NodDW	0.58	0.00	0.32	0.35	0.10	0.02	-0.41	-0.41	-0.45	-0.44	-0.29	-0.47	0.03	0.61	0.05	1.00	8.6E+01	5.3E+01
HostPlantN	0.60	0.60	0.78	0.78	0.75	1.00	0.64	0.72	0.59	0.80	0.59	0.81	0.17	0.37	0.52	-0.03	1.00	1.0E+01
NoduleSize	-0.09	0.43	0.33	0.23	0.59	0.31	0.09	0.13	0.06	0.41	0.35	0.06	-0.39	0.52	0.75	-0.11	0.29	1.00

Hypermodulating mutants

	nodeNB	nodeDW	totalDW	shootDW	rootDW	totalN	totalNcnc	NNI	nodeNBcnc	LeafArea	stemNodeNB	SNU	RUE	propNod/TotalDW	propRoot+Nod/TotalDW	propNod/Root+NodDW	HostPlantN	NoduleSize
nodeNB	1.00	3.1E+02	2.7E+03	7.2E+04	5.9E+04	1.3E+01	6.4E+08	1.6E+05	1.7E+01	1.5E+04	4.0E+06	2.1E+05	2.1E+01	6.7E+07	1.2E+04	5.8E+07	4.1E+02	8.0E+08
nodeDW	0.31	1.00	2.0E+05	2.7E+04	7.8E+02	7.6E+08	3.9E+02	1.1E+02	5.4E+01	9.8E+03	3.9E+01	9.1E+01	2.4E+01	2.9E+02	3.6E+01	2.9E+04	8.4E+06	8.5E+02
totalDW	-0.42	0.56	1.00	0.0E+00	7.3E+10	0.0E+00	5.4E+02	6.9E+01	1.6E+03	0.0E+00	1.9E+10	3.6E+09	2.1E+01	5.2E+05	5.6E+06	7.1E+02	0.0E+00	2.5E+06
shootDW	-0.46	0.49	0.99	1.00	1.0E+08	0.0E+00	3.6E+02	6.0E+01	5.3E+04	0.0E+00	1.2E+10	8.6E+11	1.9E+01	4.2E+06	1.3E+07	5.1E+02	0.0E+00	2.4E+06
rootDW	-0.47	0.25	0.74	0.71	1.00	2.3E+06	3.6E+04	3.3E+02	3.1E+01	2.7E+07	8.6E+13	2.2E+05	8.5E+01	7.3E+05	4.1E+02	1.1E+08	5.5E+07	9.1E+05
totalN	-0.22	0.68	0.94	0.93	0.61	1.00	7.4E+01	6.6E+02	2.9E+04	2.2E+16	4.2E+06	2.1E+08	4.4E+02	4.1E+03	1.4E+04	6.6E+01	0.0E+00	1.3E+04
totalNcnc	0.68	0.29	-0.27	-0.30	-0.48	0.05	1.00	0.0E+00	6.5E+01	3.2E+02	1.2E+05	2.1E+01	8.0E+03	4.8E+05	1.3E+02	2.8E+07	1.0E+00	1.0E+02
NNI	0.57	0.36	-0.06	-0.08	-0.30	0.26	0.96	1.00	3.6E+01	5.7E+01	6.7E+03	8.4E+01	4.1E+03	9.4E+03	2.2E+01	7.2E+05	1.1E+01	1.2E+01
nodeNBcnc	-0.20	0.09	0.44	0.47	0.15	0.49	0.07	0.13	1.00	2.0E+04	1.2E+01	3.9E+05	9.7E+02	3.6E+04	1.9E+06	6.0E+01	2.2E+04	3.2E+01
LeafArea	-0.51	0.36	0.93	0.95	0.65	0.87	-0.30	-0.08	0.50	1.00	1.5E+09	5.0E+12	2.4E+01	8.6E+08	7.9E+10	2.8E+02	0.0E+00	1.6E+05
stemNodeNB	-0.60	0.12	0.76	0.76	0.81	0.60	-0.58	0.22	0.55	0.80	1.00	2.0E+07	2.9E+01	3.8E+08	3.7E+05	3.2E+07	4.2E+07	9.4E+04
SNU	-0.56	-0.02	0.72	0.77	0.56	0.70	-0.18	0.03	0.55	0.80	0.66	1.00	7.6E+03	1.0E+10	1.0E+10	1.8E+04	7.7E+11	1.1E+03
RUE	-0.18	-0.17	0.72	-0.19	0.63	-0.29	-0.37	-0.40	-0.24	-0.17	0.15	-0.37	1.00	8.6E+01	3.1E+01	1.6E+01	4.4E+02	9.5E+01
propNod/TotalDW	0.64	0.31	-0.54	-0.60	-0.53	-0.40	0.54	0.36	-0.48	-0.67	-0.69	-0.76	0.02	1.00	0.0E+00	2.4E+09	3.5E+04	9.1E+03
propRoot+Nod/TotalDW	0.52	0.13	-0.59	-0.67	-0.51	0.35	0.35	0.18	-0.62	-0.74	-0.55	-0.76	0.15	1.00	1.00	6.3E+03	9.8E+06	1.5E+02
propNod/Root+NodDW	0.64	0.49	-0.26	-0.28	-0.70	-0.06	0.65	0.53	-0.08	-0.31	-0.65	-0.50	-0.20	0.73	0.38	1.00	2.8E+01	3.9E+02
HostPlantN	-0.29	0.58	0.95	0.95	0.64	0.99	0.00	0.23	0.50	0.90	0.65	0.77	-0.29	-0.49	-0.58	-0.15	1.00	6.6E+05
NoduleSize	-0.67	0.25	0.61	0.61	0.52	-0.52	-0.36	-0.23	0.14	0.57	0.45	0.45	-0.01	-0.37	-0.34	-0.29	0.53	1.00

Pearson correlation values are presented in the lower part of the matrix and the associated probability levels in the upper part. Correlation values: red (resp blue) numbers are positive (resp negative) values that are significant at the 0.001 probability level. Black values are not significant at the 0.001 probability level. Trait identifiers are the same as in Fig. 3

host plant control on nodule number is disrupted, the interaction turns into parasitism.

4 Conclusion

Our study was originally based on an integrated functional analysis of traits related to C and N nutrition at the whole plant level. Integrative studies of the same kind have already proved efficient to identify robust QTLs in quantitative genetic analysis. Here, we provide for the first time characterisation of an enlarged set of hypernodulating mutants in pea, though a combined analysis of carbon and nitrogen nutrition, using correlation networks. Our analysis thus identified the links between plants variables and identified those that would require special attention to breed so as to increase N uptake in hypernodulating mutants and thus to improve the whole plant performance. Due to the complex relationships that exist between C and N metabolism in legumes, a modeling approach describing N uptake by symbiotic fixation together not only with the shoot C uptake and N accumulation capacities but also with the developmental patterns of the nodulated roots would be useful to identify the most adequate traits for the breeders to select and combine.

Acknowledgments We thank Drs Richard Thompson and Sergio Ochart for carefully reading the manuscript and correcting the English. Our grateful thanks are due to Vincent Durey, Patrick Mathey, Christian Jeudy, Anne-Lise Santoni, and Sylvie Girodet for their technical assistance and to the greenhouse staff for managing the experiments. We also thank Stéphanie Lacombe, Envel Kerdaffrec, Yann Corderoc'h, Arnaud Germond, Farruk Azeem, and Justine Sourisseau for their help during the experiments. This work was partly founded by INRA, UNIP, and Burgundy Region.

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