Identifying quantitative trait loci for symbiotic nitrogen fixation capacity and related traits in common bean

Lara Ramaekers · Carlos H. Galeano ·
Nayibe Garzón · Jozef Vanderleyden ·
Matthew W. Blair

Abstract Symbiotic nitrogen fixation (SNF) through association with root-nodulating rhizobia effectively contributes to improved nitrogen nutrition of leguminous plants and is an ecologically sound and low-cost strategy for improving pulse productivity. One of the main factors determining the efficiency of SNF is the plant genotype, but little is known about the plants' genetic contribution to SNF in the case of common bean. Therefore, quantitative trait loci (QTL) analysis for SNF and related growth traits was performed in a common bean recombinant inbred line (RIL) population under both greenhouse and field conditions. The RIL population was generated from the cross G2333 × G19839. Additionally, the existing genetic map of the population was improved through the mapping of 42 markers out of a set of 108 nodulation gene-based markers. All greenhouse and field experiments showed significant differences between RIL genotypes for most SNF and related growth traits. In the first greenhouse experiment, two QTL for percent nitrogen (N%) fixed were identified on linkage groups b01 and b04, explaining 21 and 20 % of the observed phenotypic variance, respectively. The QTL on linkage group b01 overlapped with a QTL detected for total N content at harvest in the field experiment ($R^2 = 14$ %). In the second greenhouse experiment, two QTL for total plant N fixed were identified on linkage groups b04 and b10 ($R^2 = 18$ and 17 %, respectively). The field experiment also yielded one QTL for the N% fixed at harvest on linkage group b04 and another for the total N fixed in the shoot at late pod-filling stage on linkage group b01. The latter two QTL identified in the field explained 19 and 21 %, respectively, of the observed phenotypic variance. DNA sequence comparison of markers closely linked to QTL identified some potential candidate genes underlying the QTL. One of these genes encodes for an auxin-responsive transcription factor which could explain differences in growth and possibly yield and N accumulation between climbing beans and bush beans. Another putative gene was identified for an AP2/ERF-domain-containing

Lara Ramaekers and Carlos H. Galeano have contributed equally to this work.

L. Ramaekers · C. H. Galeano (✉) · J. Vanderleyden
Department of Microbial and Molecular Systems,
Centre of Microbial and Plant Genetics, KU Leuven,
Kasteelpark Arenberg 20, 3001 Heverlee, Belgium
e-mail: galeanomendoza@gmail.com

N. Garzón · M. W. Blair (✉)
Universidad Nacional de Colombia, Sede Palmira,
vía Chapinero, km12, Valle de Cauca, Colombia
e-mail: mwb1@cornell.edu

M. W. Blair
Department of Plant Breeding and Genetics, Hall,
Cornell University, 242 Emerson, Ithaca, NY, USA

transcription factor underlying the QTL for the total amount of symbiotic nitrogen fixed in the field.

Keywords QTL analysis · Symbiotic nitrogen fixation · Common bean · Climbing bean · Putative genes

Abbreviations

DAP	Days after planting
LG	Linkage group
Masl	Meters above sea level
N	Nitrogen
N_{dfa}	N derived from air (N fixed)
N_{dfs}	N derived from soil
NDW	Nodule dry weight
NN	Nodule number
NR	Number
NUE	Nitrogen use efficiency
SDW	Shoot dry weight
SNF	Symbiotic nitrogen fixation
SPAD	Soil and plant analysis development
QTL	Quantitative trait loci
RIL	Recombinant inbred lines
RDW	Root dry weight
SSCP	Single strand conformation polymorphism
TDW	Total dry weight
TF	Transcription factor

Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important food legume for direct human consumption (Broughton et al. 2003). Nutritionists characterize this crop as the "poor man's meat", a nearly perfect food because of its high protein content that complements other foods like maize and rice. In Latin America and Africa, common bean is produced mostly by resource-poor farmers on small-scale, marginal farms. An environmentally friendly and low-cost strategy for improving productivity in common bean cultivation is the exploitation of symbiotic nitrogen fixation (SNF) which involves a symbiotic interaction between leguminous plants and *Rhizobium* bacteria. However, present-day commercial bean varieties are poor nitrogen fixers (Martínez-Romero 2003). A number of studies comparing several bean genotypes for SNF capacity pointed out that, in general, climbing beans tend to have higher SNF potential as well as higher

yield potential compared to bush beans (Graham and Halliday 1976; Graham and Rosas 1977; Kumarasinghe et al. 1992). Climbing common bean genotypes might therefore be promising genetic sources for improving SNF capacity in common bean cultivation.

Only a few genetic studies to detect natural variation and quantitative trait loci (QTL) for SNF and related traits in legumes have been performed so far. Most of these studies focused on QTL analyses of nodulation traits and shoot dry weight under nitrogen-fixing conditions. For soybean, Nicolas et al. (2006) used an F2 population from a cross between two Brazilian soybean cultivars and revealed two genomic regions associated with nodule number (NN) and nodule dry weight (NDW) and six epistatic interactions among non-linked QTL for shoot dry weight (SDW), NN and NDW explaining up to 15 % of the trait variation. In an F2-derived recombinant inbred line (RIL) population, Tanya et al. (2005) found various QTL for NN, NDW, SDW as well as for acetylene reduction activity, which is a direct measure of the amount of nitrogen fixed per plant. Recently, a study in pea identified nine QTL for NN, four for NDW and eight for nodule area in a RIL population derived from parents which are contrasting for root and nodule traits (Bourion et al. 2010).

In common bean, Nodari et al. (1993) were the first to report on the identification of four QTL for NN in the RIL population of a cross between BAT-93 and Jalo EEP558, with the QTL together accounting for 50 % of the phenotypic variation. This same RIL population was used to detect QTL for NN under low and high mineral nitrogen (N) fertilization (Tsai et al. 1998; Souza et al. 2000). Under low N conditions, Tsai et al. (1998) identified the same four QTL and two additional QTL. Under high N conditions, three additional regions were linked to NN. Souza et al. (2000) reported the identification of seven QTL under low N conditions and only five QTL under high N conditions, accounting for 34 and 28 % of the total phenotypic variation in NN respectively. However, it is difficult to know if the same QTL for NN were identified in the latter two studies as the genetic maps used for QTL analysis appeared to be different, with varying numbers of linkage groups. In conclusion, genetic studies on SNF in common bean and even in legumes are sparse, despite the need for genetic information on SNF in breeding. Moreover, the reported studies do not make an effort to associate detected QTL with actual SNF or yield.

The objective of this study was to identify QTL controlling SNF capacity and related traits in a bush (G19839) × climbing (G2333) bean cross. This population has previously been used in other genetic studies. A genetic map of this population has been published (Ochoa et al. 2006). Prior to QTL analysis, a set of 108 markers based on genes known to be involved in the nodulation process in model legumes (Galeano et al. 2012) were evaluated in the population. Phenotypic characterization of the parents and RIL population for SNF and related growth traits was performed under greenhouse conditions and in field conditions in tropical soils. Using these phenotypic data and the improved genetic map of the population, QTL analyses were performed for the SNF- and growth-related traits, and candidate genes were tentatively identified among the nodulation markers.

Materials and methods

Plant material and bacterial strains

A total of 83 F5:8 RIL were developed from the cross G2333 × G19839 by single-seed descent as described by Checa et al. (2006), where G2333 (climber) is a landrace from Mexico, known as 'Colorado de Teopisca', which belongs to the Mesoamerican gene pool and G19839 (bush) is a Peruvian germplasm accession from the Andean gene pool (Singh 1991).

As *Rhizobium* inoculant, *R. tropici* CIAT899 was used. The inoculum preparation was carried out following the methodology reported by Remans et al. (2007). Briefly, the strain was grown overnight in liquid tryptone-yeast extract (TY) medium at 30 °C. These liquid cultures were washed twice with 10 mM $MgSO_4$ and resuspended in 10 mM $MgSO_4$ at a density of 10^7 colony forming units (cfu) per ml. In the greenhouse experiment, this bacterial inoculum was directly applied at planting. For the field experiment, 30 ml of this inoculum was injected into sterilized humus in air-closed plastic bags (50 g humus per bag). The humus was thoroughly shaken and left at room temperature for 7 days (shaken once a day).

Molecular marker and linkage analysis

DNA was extracted from the parental lines and all RIL using proteinase K extraction as described by Mahuku (2004). DNA concentrations were measured in a Hoefer fluorometer (Dynaquant 200, Hoefer Scientific Instruments, San Francisco, CA, USA). The DNA was diluted to 5 ng/μl for further use in PCR reactions. A total of 108 gene-based markers based on nodulation genes were evaluated by single strand conformation polymorphism (SSCP) and 67 allele-specific primers were evaluated by Sequenom as reported by Galeano et al. (2009, 2012).

The polymorphic markers plus 29 new simple sequence repeat (SSR) markers (Garzon, unpublished data) were placed into the earlier genetic map for G2333 × G19839 by Ochoa et al. (2006). This was done using Mapdisto software version 1.7.5 beta (Lorieux 2012).

Experimental design, growth conditions and trait measurements in the greenhouse

The greenhouses were located at the KU Leuven (Heverlee, Belgium). Due to space limitations, a subset comprising 73 RILs with good germination capacity and both parental lines were used in the greenhouse experiments. Two experiments were carried out: the first one in the period October–November (fall) 2009 and the second one in the period June–July (summer) 2010, referred to as Oct09 and June10, respectively. Seeds of every RIL and of the parental lines were surface-sterilized according to Vlassak et al. (1998) and pre-germinated for 3 days on agar plates (10 % agar in distilled water) in the dark at 30 °C. Three seedlings were planted per one-liter pot. The seedlings were inoculated at time of planting with 100 μl of inoculum (10^6 *Rhizobium* cells) per seedling.

One week after planting, two out of the three seedlings were randomly removed by cutting the stalk, so that only one seedling per pot remained. For each bean genotype, eight plants were used in a randomized complete block design (eight blocks) resulting in a total of 600 plants per experiment. Plants were growing in sand:vermiculite mixture (proportion of 2:1 by volume) in the absence of mineral N. One-liter pots were filled with the sand:vermiculite mixture and moistened with 250 ml N-free Snoeck medium (Snoeck 2001). During growth, a drip irrigation system was used. In terms of phenotyping, leaf chlorophyll values were measured weekly with a soil and plant analysis development (SPAD) chlorophyll

meter model 502 (Soil and Plant Analysis Development) (Monje and Bugbee 1992). Sampling of plants was performed at 42 days after planting (DAP) at the onset of flowering (R6 stage). Harvested plants were oven-dried at 60 °C for at least 2 days. Shoot and root dry weights were recorded. Shoot and root samples were ground and N concentration (%) was measured by ignition with a Variomax CN analyser (Dumas method).

Field trials and traits measured

The field trial was conducted at the CIAT research farm in Darien, Colombia (03°56′N; 76°28′W, elevation 1,537 masl) during the rainy season from September 2010 until February 2011. The average yearly temperature for this site is 20.8 °C and annual rainfall totals 1288 mm. The soil at the field site is an Udand with pH 5.4 (soil:water 1:2), 13.3 % organic matter (dicromate–sulfuric acid oxidation), 7,274.9 ppm total N, 90.6 ppm plant-available N (NH_4^+ and NO_3-) and 2.3 ppm native soil phosphorus as determined by Bray II extraction (Olsen and Sommers 1982).

A total of 88 bean genotypes were used including the two parental lines G2333 and G19839, 83 F5:8 RIL and three non-nodulating lines, BAT477-NN, DOR364-NN and Gloriabamba-NN. Non-nodulating isolines were used as non-fixing controls to measure the amount of SNF by the $\delta15N$ natural abundance method according to Shearer and Kohl (1986).

Before planting, 90 kg/ha phosphorus (as Triple Super Phosphate) and 60 kg/ha potassium (as KCl) were applied. The inoculation was carried out following the methodology reported by Remans et al. (2008). Briefly, seeds of the 88 bean lines were mixed with inoculum (30 g humus/kg seed) and distilled water (1 ml per 30 g seed). This mix was left to dry in the shade for 15 min and seeds were then manually sown at a density of 20 cm between seeds. Sowing at this density corresponds to approximately 50,000 plants/ha. Later, at 12, 20 and 28 DAP, micro-elements boron and zinc were applied by foliar spray over the developing plants (300 g/ha).

The field experiment was organized as a randomized complete block design with three replications.

Each experimental unit was a double 3-m-row plot sown with 15 seeds per row (30 plants/plot and thus 90 plants/genotype). One meter spacing between rows was used to accommodate both climbing and semi-climbing RILs.

During the experiment, a total of four samplings was taken for phenotypic trait analysis. The first two samplings were taken at 21 and 42 DAP, corresponding to the primary trifoliate V3-growth stage and early flowering R6-growth stage of the bean plants, respectively (Singh 1982). For these two samplings, three plants per plot (nine plants per bean genotype) were separately harvested by cutting the plant at the first node (starting from the soil surface) and afterwards the root was dug up, taking care that the whole root was collected.

Subsequently, nodules were separated from the root. Shoot, root and nodules of every plant were oven-dried separately at 60 °C for at least 2 days. Shoot, root and nodule dry weights were recorded. For the first sampling, nodule number was also recorded. For the second sampling, both shoot and root samples were ground and $\delta15N$ and total N concentration (%) were measured at the Stable Isotope Facility (UC Davis, California) using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK; Sharp 2005). For the third sampling at late pod-filling stage (R7/R8-growth stage; Singh 1982), shoots of three plants per plot were harvested together. The first three samples were taken from one row of the experimental unit while the second row was left intact for harvest. Shoot dry weight, $\delta15N$ and total N was measured for these pooled samples as explained earlier.

Leaf chlorophyll values of ten plants per plot were measured with a SPAD chlorophyll meter model 502 for the first three samplings. At harvest, a sample of three plants from the second row was taken, and afterwards the entire row was harvested. Plant counts per row were recorded for yield calculation. For the three-plant sample taken at harvest, the following traits were recorded: seed and pod number; seed, pod and plant dry weight; $\delta15N$ and total N of seed, pod and plant, as explained earlier.

The amount of nitrogen fixed (%N_{dfa}) was calculated using the following formula (Shearer and Kohl 1986):

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of non}-\text{fixing reference plant} - \delta^{15}\text{N of N}_2\text{fixing legume}}{\delta^{15}\text{N of non-fixing reference plant} - \text{B}} \times 100$$

where B is the $\delta15$N value from the nitrogen-fixing bean plant grown in N-free medium. This B-value was obtained by taking the average of $\delta15$N measurements of a total of 20 plants grown in the greenhouse experiment.

Data analysis

First, normal distribution of the data was verified per trait using the Shapiro–Wilk distribution test. Data analysis was done with the ANOVA Mixed Model procedure using the statistical analysis system (SAS) 9.2 combined with the statistical interface SAS Entreprise Guide 4 (SAS Institute, Cary, NC, USA).

In the analysis of variance (ANOVA), the main factor of interest was 'genotype' [73 or 83 RIL (greenhouse and field respectively) and two parental lines]. Significant differences were determined by the Tukey–Kramer post hoc test at significance level $P = 0.05$. The Kenward and Roger calculation was selected as the degrees of freedom method. Correlations for all experiments were calculated using Pearson correlation coefficients. For the greenhouse experiment, %N Root, %N Shoot, %N Plant, Total N Shoot, Total N Root and Total N Plant were all correlated. Therefore, only Total N Plant was considered.

QTL analysis was conducted with the resulting genetic map of the population and the phenotypic means for each RIL using the computer software program QTL Cartographer version 2.5 for Windows (Wang et al. 2011). QTL were identified with composite interval mapping (CIM) features. Traits for CIM analysis included a forward/backward regression with a window size of 10 cM, a walk speed of 1 cM and probability thresholds of 0.05 each for the partial F-test for both marker inclusion and exclusion. The empirical thresholds for QTL detection with the CIM method were estimated using 1,000 permutation tests as suggested by Churchill and Doerge (1994). Additive genetic effect estimates for each trait were computed by the QTL Cartographer program at the peak of the LOD profile. Identified QTL were added on the genetic map using Mapchart version 2.1 (Voorrips 2002).

Sequence comparison for identification of putative genes underlying QTL

For some QTL identified in greenhouse and/or field experiments, sequences of markers that were found to be closely linked to the QTL were available. These sequences were compared to (1) the most recent expressed sequence tag (EST) assembly of common bean (TC-gene index available at http://compbio.dfci. harvard.edu/cgi-bin/tgi/tc_ann.pl?gudb=p_vulgaris) and (2) to a collection of gene sequences in soybean (available at http://www.phytozome.org). Additionally, the most similar common bean EST, or alternatively the gene sequence of soybean, was BLASTed (http://blast.ncbi.nlm.nih.gov). These three types of comparisons made it possible to annotate the marker sequences linked to identified QTL and, in this way, to suggest putative genes underlying the identified QTL.

Results

Genetic mapping of nodulation gene-based markers

Based on SSCP and Sequenom techniques, a total of 42 markers could be mapped, comprising 16 markers (15 %) by SSCP and 26 markers (39 %) by Sequenom (Fig. 1). The final linkage map consisted of a total of 207 markers comprising 57 rapid amplification of polymorphic DNA, 106 SSRs, 1 sequence characterized amplified region, 1 isoenzyme and 42 single nucleotide polymorphisms (SNPs). In addition, the linkage map had a total map length of 1,601 cM and the average length of the linkage groups was 145 cM (Supplementary Table 1).
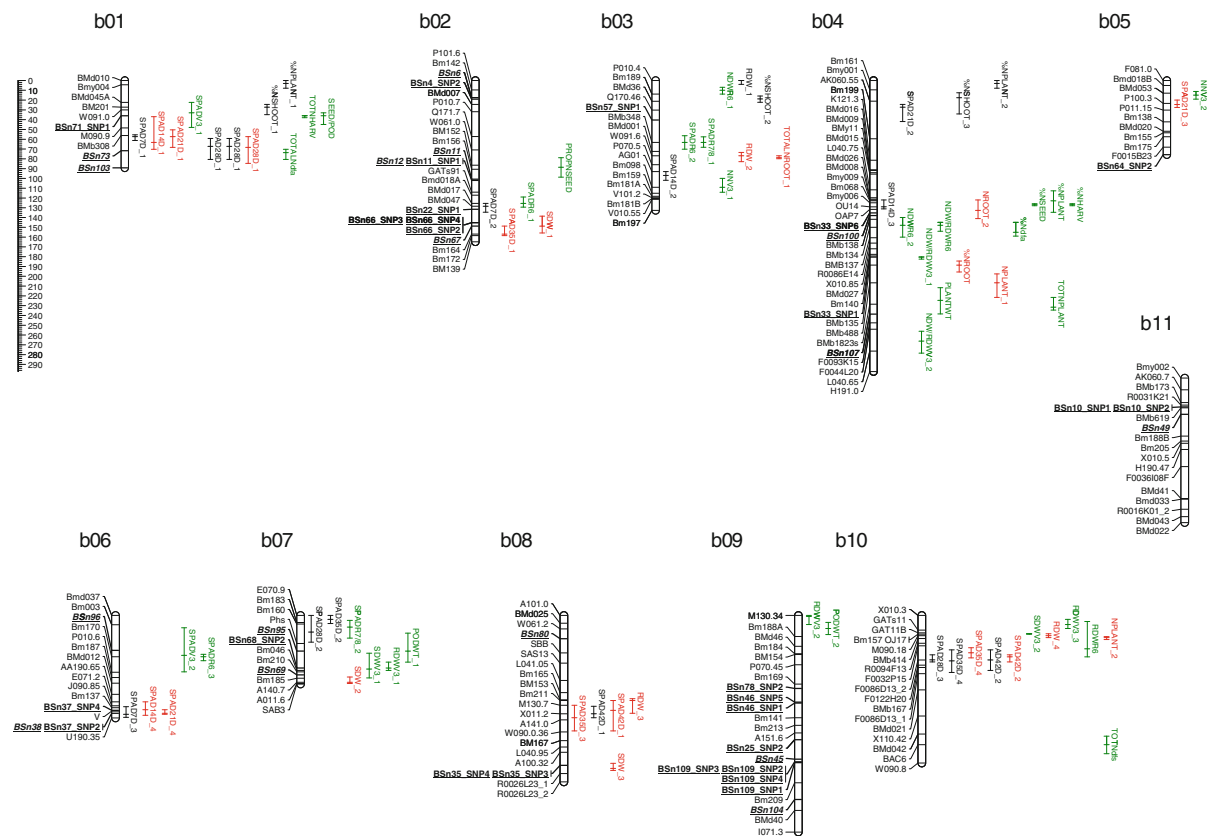
Fig. 1 Genetic map of the G2333 × G19839 common bean RIL population showing location of quantitative trait loci (QTL) for traits evaluated in both greenhouse experiments and in the field experiment. The QTL identified in the first greenhouse experiment (Oct09) are indicated in *black*, those from the second greenhouse experiment (June10) in *red* and the QTL of the field experiment in *green*. Nodulation gene-based markers that were added to the existing map are shown in *bold and underlined* (Sequenom MassARRAY iPLEX platform), and the SSCP markers are differentiated with *italics*

SNF capacity and related growth traits in the G2333 × G19839 population in the greenhouse

For the results of the first experiment (Oct09), comparison of parental means for SPAD values measured weekly during a time course of 6 weeks showed superior SPAD values of the bush bean G19839 in the first 3 weeks as compared to G2333 (Table 1). However, during the last 3 weeks of growth, which is the main period of symbiotic nitrogen fixation, the climber G2333 showed superior SPAD values. Other growth traits including shoot and root dry weight, and N content of shoot, root and plant, showed that the climbing bean parent G2333 had somewhat a superior growth rate and N fixation compared to the bush bean parent G19839.

The parameter nitrogen use efficiency (NUE), which is a measure of the amount of tissue growth per unit of N fixed by the plant, was similar for both parental bean lines. The second experiment also showed superior SPAD values of the bush bean parent G19839 in the second week (SPAD values in the first week were not determined for this experiment) as compared to G2333, while in the third week parental SPAD values were similar (Table 1). During the last 3 weeks, SPAD values of both parents increased, with the bush bean parent G19839 increasing more rapidly than the climbing bean parent G2333. In the last week, both parents showed a sudden decrease in SPAD values with a higher decrease for G2333 than G19839. Only SPAD values at 2 and 6 weeks were significantly different.

Phenotypic growth traits in this second experiment, including shoot and root dry weight, showed that

G19839 was also superior for these traits and significantly different from G2333 (Table 1). Similarly, N% in shoot and total plant and total N contents of shoot, root and plant were also significantly higher for G19839. An exception was the N% in the root which was higher, though not significantly so, for G2333. NUE, which was similar for both parental varieties in the previous experiment, was significantly higher for G19839 in this experiment.

The analysis of variance showed significant differences between RIL genotypes for almost all traits measured for both experiments (Table 1). The average trend of the SPAD values was similar to the trend observed for the parents, with an initial decrease in SPAD values followed by a gradual increase in SPAD values.

Population histograms for all traits in the G2333 × G19839 RIL population are shown in Supplementary Figure 1. All the traits and all the histograms showed normal distributions. There was no evidence of extreme kurtosis or skewing in any of the histograms which suggested that all traits measured were inherited in a quantitative manner. In almost all cases, parental means tended to be less distinct than the lowest and highest RIL, suggesting that transgressive segregation was important in the inheritance of the traits with both parents contributing positive as well as negative alleles for each trait.

Correlations among all measured traits were analyzed. Only significant correlations were listed in Supplementary Table 2. For both experiments, SPAD values from 3 weeks onwards were significantly correlated with biomass accumulation (shoot, root and total dry weight) measured at 6 weeks. This showed that from 3 weeks onwards, SPAD values were able to predict the biomass of the plant at 6 weeks. For both experiments, shoot and root dry weight strongly correlated, suggesting that plants invest biomass in both tissues simultaneously without favoring root over shoot tissue or vice versa.

In addition, the total nitrogen content of the shoot was correlated with SPAD values from 4 weeks onwards in both experiments. In the second experiment the N% in the shoot was significantly correlated with the SPAD values at 6 weeks. These last two correlations indicated the utility of the SPAD equipment as a cheap and time-saving alternative for N analysis.

SNF capacity and related growth traits in the G2333 × G19839 population in the field

SPAD values were measured at each sampling (corresponding to plant growth stages V3, R6 and R7/8). Comparisons of parental mean for SPAD values showed superior SPAD values of the climber G2333 at all samplings as compared to G19839 (Table 2), suggesting greater N uptake (through SNF and soil absorption) by the climber G2333. Average shoot dry weight of G2333 was initially (V3 stage) somewhat lower than G19839 but was higher at the R6 and R7/8 sampling stages, although the difference was only significant at the second date. For the root dry weight, the bush bean parent G19839 had higher root dry weights with a significant difference only at the V3 stage.

Generally, at the V3 stage, plants had very few nodules, while at the R6 stage the amount of nodules was abundant, suggesting that formation of nodules mainly took place between 3 and 6 weeks after planting. At the early V3 stage, G19839 had a significantly higher average nodule dry weight compared to G2333, while at flowering stage (R6) the opposite was true. When looking at nodule dry weight and thereby accounting for differences in root dry weight (NDW/RDW), the difference between G2333 and G19839 is even clearer.

Percentage of N_2 fixed (%N derived from air or %N_{dfa}) and total amounts of N fixed (total N_{dfa}) were calculated at R6 and R7/8 growth stages as well as at harvest. At R6 growth stage, the climbing bean parent G2333 had fixed about 50 % of its total N content while G19839 had only fixed approximately 30 %. However, at harvest, the opposite was observed, with 58 % fixed by the bush bean genotype G19839 and 43 % fixed by G2333. The latter difference was significant, and the same trends could be observed when comparing total N contents fixed at V3 and R6 growth stages and at harvest. The main period of SNF for the climbing bean parent G2333 appeared to be the period between flowering and pod-filling (313.6 mg/plant), while for G19839 this peak of SNF activity appeared to be between pod-filling and harvest (180 mg/plant).

Percentage and total N absorbed from soil (%N derived from soil or %N_{dfs} and total N derived from soil or N_{dfs}) were also calculated. In the beginning (up to R6), the main part (about 70 %) of the plant N was

Table 1 Parental and RIL population (G2333 × G19839) means of the greenhouse experiments (Oct09, June10)

Trait	Experiment	Parents[b]			RIL[b]		
		G2333	G19839	P_p^c	Mean	Range	P_{RIL}^c
SPAD 7 days[a]	Oct09	32.4 ± 1.4	33.1 ± 1.4	ns	34.1 ± 0.2	28.2–40.1	***
SPAD 14 days	Oct09	25.2 ± 1.1	28.7 ± 1.2	ns	27.2 ± 0.2	21.9–36.3	***
	June10	25.5 ± 1.2	32.7 ± 0.7	***	29.6 ± 0.2	22.4–34.7	***
SPAD 21 days	Oct09	23.5 ± 1.6	27.9 ± 0.4	*	24.6 ± 0.2	19.9–30.7	***
	June10	21.8 ± 0.6	22.1 ± 0.8	ns	21.8 ± 0.1	16.6–28.9	***
SPAD 28 days	Oct09	25.4 ± 1.3	28.2 ± 1.2	ns	26.1 ± 0.2	18.4–33.9	***
	June10	26.2 ± 1.0	27.2 ± 0.7	ns	25.6 ± 0.2	19.5–34.6	***
SPAD 35 days	Oct09	29.5 ± 1.2	27.4 ± 0.7	ns	28.5 ± 0.2	23.5–35.6	***
	June10	27.5 ± 0.8	29.6 ± 0.7	ns	27.1 ± 0.1	21.9–34.5	***
SPAD 42 days	Oct09	28.3 ± 1.2	25.6 ± 0.9	ns	27.5 ± 0.2	21.9–36.9	***
	June10	20.3 ± 1.7	24.5 ± 0.5	*	23.3 ± 0.2	17.1–31.6	***
SDW (g)[d]	Oct09	3.25 ± 0.37	2.32 ± 0.39	ns	3.01 ± 0.06	1.03–5.37	***
	June10	2.40 ± 0.23	3.81 ± 0.19	***	2.88 ± 0.04	1.55–4.48	***
RDW (g)[e]	Oct09	1.21 ± 0.23	1.10 ± 0.20	ns	1.43 ± 0.04	0.33–2.36	***
	June10	1.09 ± 0.17	2.05 ± 0.14	**	1.35 ± 0.02	0.84–2.30	***
%N shoot[f]	Oct09	3.71 ± 0.14	3.55 ± 0.14	ns	3.85 ± 0.02	3.22–4.40	***
	June10	2.31 ± 0.17	2.80 ± 0.06	*	2.53 ± 0.03	1.78–3.68	***
%N root[f]	Oct09	2.67 ± 0.26	2.16 ± 0.26	ns	2.66 ± 0.03	2.04–3.45	**
	June10	2.89 ± 0.13	2.51 ± 0.08	ns	2.78 ± 0.03	2.09–3.74	***
%N plant[f]	Oct09	3.41 ± 0.17	3.01 ± 0.15	*	3.49 ± 0.02	2.98–4.01	***
	June10	2.55 ± 0.12	2.72 ± 0.07	ns	2.63 ± 0.02	1.99–3.43	***
Total N shoot (mg)[f]	Oct09	121.3 ± 14.9	85.6 ± 14.9	ns	114.70 ± 2.46	35.3–188.3	***
	June10	55.9 ± 7.6	109.0 ± 7.6	**	73.5 ± 1.4	33.8–146.4	***
Total N root (mg)[f]	Oct09	33.5 ± 6.6	23.6 ± 5.8	ns	35.23 ± 0.91	8.92–62.93	***
	June10	29.6 ± 4.8	56.2 ± 6.1	*	40.0 ± 0.7	23.4–59.7	***
Total N plant (mg)[f]	Oct09	139.9 ± 22.3	109.2 ± 19.3	ns	147.38 ± 3.29	50.63–242.25	***
	June10	85.4 ± 15.4	167.4 ± 13.8	*	115.8 ± 2.2	61.4–198.0	***
NUE (g/(mg N/g DW))[g]	Oct09	0.124 ± 0.016	0.122 ± 0.017	ns	0.123 ± 0.003	0.039–0.206	***
	June10	0.129 ± 0.013	0.227 ± 0.020	*	0.171 ± 0.003	0.091–0.273	***

ns Not significant

* Significant at 0.05 probability level

** Significant at 0.01 probability level

*** Significant at 0.001 probability level

[a] SPAD values were taken weekly per plant during the time course of the experiment (6 weeks; 7–14–21–28–35–42 DAP). The remaining data were taken per plant at 42 DAP

[b] Values represent means of eight plants ± mean standard error

[c] Effects of genotype were tested and significance levels obtained are indicated in the columns P_p and P_{RIL} (SAS, *t* test for independent samples or ANOVA for parental comparison and populational comparison respectively)

[d] Shoot dry weight (SDW)

[e] Root dry weight (RDW)

[f] %N and total N content of shoot, root and total plant

[g] Nitrogen use efficiency (NUE)

Table 2 Parental and RIL population (G2333 × G19839) means of the field experiment at the CIAT research farm in Darien, Colombia

Sampling stage	Trait[a]	Parents			RIL		
		G2333	G19839	P_p^b	Mean	Range	P_{RIL}^b
SPAD	SPAD V3	35.7 ± 0.7	36.7 ± 1.0	ns	36.2 ± 0.2	30.9–42.1	****
	SPAD R6	41.4 ± 0.9	36.9 ± 0.9	**	36.7 ± 0.2	29.1–43.9	****
	SPAD R7/8	45.2 ± 3.7	39.7 ± 0.9	ns	43.8 ± 0.3	29.7–51.3	****
SDW (g)[c]	SDW V3	1.05 ± 0.09	1.24 ± 0.12	ns	1.04 ± 0.01	0.57–1.60	****
	SDW R6	7.36 ± 0.72	5.38 ± 0.74	*	6.93 ± 0.12	4.34–11.39	****
	SDW R7/8	22.78 ± 4.12	16.15 ± 2.24	ns	25.80 ± 1.68	13.69–166.52	*
RDW (g)[d]	RDW V3	0.25 ± 0.03	0.41 ± 0.04	****	0.31 ± 0.01	0.16–0.48	****
	RDW R6	0.72 ± 0.06	0.99 ± 0.16	ns	0.76 ± 0.01	0.50–1.19	****
NDW (mg)[e]	NDW V3	2.8 ± 0.6	8.8 ± 3.0	*	2.6 ± 0.1	0.1–8.6	****
	NDW R6	143.5 ± 26.6	58.1 ± 20.2	**	95.1 ± 3.2	11.5–326.6	****
NDW/RDW (mg/ 100 mg root)	NDW/RDW V3	11.9 ± 2.8	18.7 ± 8.1	ns	7.55 ± 0.29	0.12–22.70	****
	NDW/RDW R6	193.1 ± 31.0	49.9 ± 11.6	***	121.4 ± 3.7	25.6–344.4	****
NN[f]	NN V3	18 ± 2	25 ± 8	ns	14 ± 1	1–39	****
$\%N_{dfa}$	$\%N_{dfa}$ shoot R6	27.90 ± 5.83	29.15 ± 2.40	ns	23.22 ± 0.77	0.00–56.64	ns
	$\%N_{dfa}$ shoot R7/8	49.53 ± 15.89	28.65 ± 13.49	ns	45.95 ± 1.26	0.00–83.06	ns
	$\%N_{dfa}$ HARVEST	43.40 ± 4.59	57.71 ± 2.53	*	43.24 ± 0.84	9.55–77.68	*
Total N_{dfa}(mg)	Total N_{dfa} shoot R6	51.6 ± 2.8	52.3 ± 18.7	ns	49.3 ± 2.1	0.0–180.5	**
	Total N_{dfa} shoot R7/8	365.2 ± 163.2	135.4 ± 60.7	ns	296.5 ± 13.8	0.0–1268.6	ns
	Total N_{dfa} HARVEST	133.5 ± 58.0	315.6 ± 195.5	ns	261.6 ± 10.2	9.8–856.2	ns
$\%N_{dfs}^g$	$\%N_{dfs}$ shoot R6	72.10 ± 5.80	70.85 ± 2.40	ns	76.70 ± 0.77	43.36–100.00	ns
	$\%N_{dfs}$ shoot R7/8	50.47 ± 15.89	71.35 ± 13.49	ns	54.05 ± 1.26	16.94–100.00	ns
	$\%N_{dfs}$ HARVEST	56.60 ± 4.59	42.30 ± 2.53	*	56.76 ± 0.84	22.32–90.45	*
Total N_{dfs}(mg)	Total N_{dfs} shoot R6	148.1 ± 38.6	119.5 ± 28.1	ns	169.4 ± 5.3	17.0–475.8	ns
	Total N_{dfs} shoot R7/8	441.0 ± 246.0	389.6 ± 114.2	ns	411.2 ± 21.6	20.9–1959.6	ns
	Total N_{dfs} HARVEST	171.1 ± 63.0	204.7 ± 110.5	ns	379.9 ± 17.8	19.3–1341.1	*
NUE (g/(mg N/g DW))[h]	NUE R6	0.301 ± 0.094	0.173 ± 0.016	ns	0.248 ± 0.008	0.073–1.573	ns
	NUE R7/8	0.798 ± 0.278	0.529 ± 0.096	ns	0.931 ± 0.034	0.195–3.074	ns
	NUE HARVEST	0.338 ± 0.210	0.365 ± 0.262	ns	0.493 ± 0.020	0.032–1.467	****
SEED HARVEST[i]	SEED NR	64.3 ± 24.2	19.6 ± 9.7	ns	51.0 ± 1.8	19.0–84.9	****
	SEED NR/POD	5.7 ± 0.9	3.1 ± 0.3	**	5.0 ± 0.1	3.1–7.6	****
	DRY SEED WEIGHT (g)	20.88 ± 10.37	9.93 ± 5.34	ns	17.36 ± 0.62	5.43–33.33	****
	%N SEED (mg N/100 mg seed)	3.14 ± 0.11	3.77 ± 0.33	ns	3.56 ± 0.05	0.50–6.35	ns
	N SEED (mg)	319.0 ± 184.2	404.8 ± 231.3	ns	597.9 ± 24.5	64.4–2191.5	**
	PROP N SEED (%)	93.48 ± 0.09	79.52 ± 1.53	***	87.79 ± 0.33	68.47–95.79	****
POD HARVEST	POD NR	12.7 ± 6.5	5.8 ± 2.4	ns	10.0 ± 0.3	4.6–16.3	***
	DRY POD WEIGHT (g)	5.33 ± 2.72	3.69 ± 2.01	ns	5.01 ± 0.18	1.48–9.37	****
	%N POD (mg N/100 mg pod)	0.67 ± 0.14	0.81 ± 0.08	ns	0.72 ± 0.02	0.17–1.88	****
	N POD (mg)	12.4 ± 5.8	40.9 ± 29.5	ns	36.0 ± 1.5	2.6–127.0	**
PLANT	DRY PLANT WEIGHT (g)	2.57 ± 1.29	4.37 ± 2.06	ns	3.93 ± 0.15	1.24–8.27	****

Table 2 continued

Sampling stage	Trait[a]	Parents			RIL		
		G2333	G19839	P_p^b	Mean	Range	P_{RIL}^b
HARVEST	%N PLANT (mg N/100 mg plant)	0.96 ± 0.27	1.38 ± 0.07	ns	1.16 ± 0.03	0.45–2.00	****
	N PLANT (mg)	39.8 ± 30.3	29.9 ± 13.9	ns	47.1 ± 2.2	2.9–173.7	***
TOTAL	YIELD (kg/ha)	$2,169 \pm 654$	614 ± 204	*	$1,512 \pm 38$	535–3,082	****
HARVEST	%N TOTAL (mg N/100 mg total)	3.14 ± 0.11	3.77 ± 0.33	ns	3.56 ± 0.05	0.50–6.35	ns
	N TOTAL[a] (mg)	304.6 ± 119.3	520.3 ± 306.0	ns	644.2 ± 26.3	36.2–2,366.3	*

ns Not significant

* Significant at 0.10 probability level

** Significant at 0.05 probability level

*** Significant at 0.01 probability level

**** Significant at 0.001 probability level

[a] Data taken at V3 and R6 stage were taken per plant with nine replicate plants, except for N data at R6 stage. All other data, including the latter, were taken from three replicate groups obtained by pooling the nine replicate plants in three groups corresponding to the three field replicates. Therefore, values represent means of nine or three replicates $\pm$ mean standard error

[b] Genotype effects were tested and significance levels are indicated in the columns P_p and P_{RIL} (SAS, t test for independent samples for parental comparison and ANOVA for populational comparison)

[c] Shoot dry weight (SDW)

[d] Root dry weight (RDW)

[e] Nodule dry weight (NDW)

[f] Nodule number (NN)

[g] %N and total N fixed (N derived from air or N_{dfa}), %N and total N absorbed from soil (N derived from soil or N_{dfs})

[h] Nitrogen use efficiency (NUE) for several samplings corresponding to V3, R6, R7/8 bean growth stages and harvest

[i] Additional parameters measured exclusively at harvest were pod number (POD NR), seed number (SEED NR), seed number per pod (SEED NR/POD), dry weight and total N content of seed, pod and plant, yield and the proportion of total N located in the seed (PROP N SEED)

taken up from the soil rather than fixed from atmospheric N. From R6 to R7/8 growth stages, the climbing bean parent G2333 started fixing considerable amounts of N (up to 50 % at R7/8) while G19839 kept relying mainly on N absorption from the soil (70 %).

When looking at total N contents (sum of total N_{dfs} and total N_{dfa}), the main period of N accumulation by G2333 was between flowering and late pod-filling (292.9 mg/plant). From late pod-filling till harvest, this genotype had a net loss of N (−501.6 mg/plant) possibly due to decreased N accumulation during the later stages of plant development. G19839 also had a significant N accumulation from flowering till late pod-filling (353.2 mg/plant) but continued to accumulate N from late pod-filling up to harvest (very small net N loss of 4.7 mg/plant despite leaf fall).

Many additional traits were measured at harvest. Only parental differences that were significant will be discussed further. The seed number per pod was significantly higher for G2333 with approximately six seeds per pod as compared to G19839 with an average of three seeds per pod. An important trait which partly determines the nutritional value (in protein content) of bean seeds was the proportion of N that is translocated to the seed by the plant. For this trait, G2333 was able to translocate about 93 % of its total N to the seed while G19839 only translocated 80 %. Finally, the average yield of G2333 was significantly higher than G19839, at more than 2 t/ha for the climbing bean parent compared to 600 kg/ha for the bush bean parent.

Population averages for NUE at the growth stage R7/8 and at harvest were also higher for many of the

RILs than for both parental means, indicating that most RILs performed better than both parents for this trait. The population mean for %N fixed (%N$_{dfa}$) was somewhat lower than for both parents at all sampling stages, suggesting that many RILs were fixing less N than either parent. Similar to the greenhouse experiments, all histograms showed normal distributions without extreme kurtosis or skewing, suggesting that all traits measured were inherited in a quantitative manner (data not shown). For each trait, parental means tended to be less distinct than the lowest and highest RIL (also observable when looking at the population ranges), suggesting that transgressive segregation was important in the inheritance of the traits with both parents contributing positive as well as negative alleles for each trait.

In correlation tests (Supplementary Table 3), the parameter for yield was inversely related to the climbing capacity scale (Checa and Blair 2008). Given this, there was an inverse relation between yield and climbing capacity which indicated that yield increased with increasing climbing ability. Yield was also correlated with total N fixed at the R7/8 growth stage and at harvest, suggesting that SNF actually contributed significantly to yield. Yield was likewise positively correlated with total N absorbed from the soil at the earlier R6 stage. The correlation of yield with SPAD values at R6 and R7/8 growth stages confirmed the important role of the plants' N status in the final yield.

Climbing capacity also showed a negative correlation with total plant N content and NUE at harvest. In other words, climbing beans tended to have higher total N contents and higher NUE than bush beans. Another intriguing correlation was that between SPAD values measured at flowering (R6) and NUE at harvest, indicating that higher chlorophyll contents at earlier growth stages coincided with a more efficient use of N. Total N contents of seeds, pods and plants at harvest were all positively correlated with total N absorbed from the soil at flowering (R6) and late pod-filling stage (R8), but not with total N fixed. These correlations indicated that N absorption from the soil, as opposed to N fixation from the air, was the most important factor determining final N contents.

QTL for SNF capacity and related growth traits

In the greenhouse experiments, 20 and 23 QTL were identified for the first and second experiments,

respectively. QTL were found for all measured traits with some QTL overlapping (Table 3). For leaf chlorophyll content (SPAD values), a QTL was found five times on linkage group b01 (at 7, 14, 21 and 28 days). This QTL was linked to two microsatellite markers and can be considered to be a major QTL as it is able to explain up to 33 and 35.5 % of the total phenotypic variance (R^2) of SPAD values at 14 and 28 days, respectively, in the second experiment. Another important QTL was found on linkage group b06 which was linked to the nodulation gene-based marker BSn38.

Additionally, overlapping QTL were found on linkage group b10 during the last 3 weeks of the experiment, which was the main period of SNF, indicating that this QTL could have a function in chlorophyll accumulation through the process of nitrogen fixation. This QTL was linked to microsatellite markers and explained from 12 to 23 % of the total phenotypic variance (R^2). G19839 provided the positive allele for this QTL. For the other QTL identified for SPAD values, both G19839 and G2333 provided an equal amount of positive alleles for higher SPAD values. QTL for shoot dry weight were found only in the second experiment, with three QTL identified. One of these QTL on linkage group b02 overlapped with a QTL found for SPAD values at 35 DAP in the second experiment and explained over 23 % of the total phenotypic variance.

Several QTL were found for root dry weight in both experiments. One of these QTL on linkage group b08 (RDW_3) overlapped with a QTL for SPAD values at 42 DAP (SPAD42D_1) which was found in both experiments. The bush bean parent G19839 provided the positive allele for this QTL. Another three and two QTL were found for N% in shoot and plant, respectively, but only in the first experiment. The QTL found on linkage group b01 for these traits overlapped (%NSHOOT_1 and %NPLANT_1) and the climbing bean parent G2333 contributed the positive allele which explained about 20 % of the total phenotypic variance. No QTL were detected for total N shoot content. Another group of QTL were identified for total N content of root and plants, but only in the second experiment. The QTL found for total N content of the plant explained 17–18 % of the total phenotypic variance, in both cases with G19839 providing the positive alleles for high total N fixed by the plant.

Table 3 Quantitative trait loci (QTL) analysis for G2333 × G19839 RIL population in the greenhouse experiments (Oct09, June10). QTL showing overlap are highlighted in the same colour

Trait	Experiment	QTL name	LG[*]	Position (cM)	Closest marker	Region	Source	Additivity[***]	Significance[**] R²(%)	TR²(%)	LR
SPAD 7 days	Oct09	SPAD7D_1	1	57.29	BMb308	55.48-61.29	G19839	-0.9430	12.38	52.24	14.8
	Oct09	SPAD7D_2	2	128.36	BMd047	125.54-134.59	G19839	-1.3073	16.16	50.38	19.8
	Oct09	SPAD7D_3	6	98.34	BSn37_SNP2	90.78-101.34	G2333	1.0055	14.26	52.16	17.2
SPAD 14 days	June10	SPAD14D_1	1	63.29	BMb308	36.97-70.29	G19839	-1.6877	32.72	62.67	28.6
	Oct09	SPAD14D_2	3	97.03	AG01	93.03-102.03	G2333	1.1703	17.77	54.44	15.6
	Oct09	SPAD14D_3	4	129.14	OU14	122.03-131.14	G19839	-1.2725	21.89	47.12	24.9
	June10	SPAD14D_4	6	93.78	Bm137	85.97-99.34	G2333	1.1858	14.82	52.44	18.3
SPAD 21 days	June10	SPAD21D_1	1	57.29	BMb308	50.48-68.29	G19839	-1.2471	20.33	62.53	26.8
	Oct09	SPAD21D_2	4	27.23	BM201	24.37-41.97	G19839	-1.0009	15.30	57.95	21.7
	June10	SPAD21D_3	5	24.47	Bm199	20.03-27.47	G19839	-1.2604	15.22	61.51	21.0
	June10	SPAD21D_4	6	97.34	BSn37_SNP2	93.78-98.34	G2333	0.9505	11.49	60.07	17.8
SPAD 28 days	Oct09	SPAD28D_1	1	67.29	BMb308	59.29-80.66	G19839	-1.1864	16.93	56.04	17.1
	June10	SPAD28D_1	1	68.29	BMb308	57.29-84.66	G19839	-1.6408	35.53	63.21	30.3
	Oct09	SPAD28D_2	7	16.60	Bm160	0.01-26.6	G2333	1.1921	16.85	55.08	16.5
	Oct09	SPAD28D_3	10	44.23	F0032P15	39.23-46.31	G19839	-1.0837	11.75	50.69	15.3
SPAD 35 days	June10	SPAD35D_1	2	156.93	Bm164	148.82-158.42	G2333	0.9856	16.84	54.35	22.4
	Oct09	SPAD35D_2	7	4.01	E070.9	0.01-8.09	G2333	1.0017	13.70	52.19	16.7
	June10	SPAD35D_3	8	101.77	X011.2	89.36-114.88	G2333	0.9602	18.49	58.53	22.5
	June10	SPAD35D_4	10	37.23	F0032P15	32.23-42.23	G19839	-0.9379	16.60	59.48	16.1
	Oct09	SPAD35D_4	10	45.31	F0086D13_2	34.23-56.66	G19839	-1.3818	22.83	51.93	26.3
SPAD 42 days	Oct09	SPAD42D_1	8	97.77	X011.2	90.36-101.77	G2333	1.3057	16.29	52.25	21.1
	June10	SPAD42D_1	8	94.36	M130.7	84.66-114.88	G2333	1.6583	25.75	61.03	31.3
	Oct09	SPAD42D_2	10	44.31	F0086D13_2	34.23-54.66	G19839	-1.3182	18.18	52.57	23.0
	June10	SPAD42D_2	10	41.23	F0032P15	39.23-46.31	G19839	-1.1903	12.14	58.58	14.3
SDW (g)	June10	SDW_1	2	148.82	BSn67	138.59-155.82	G2333	0.3029	23.44	60.10	32.6
	June10	SDW_2	7	66.51	A140.7	61.21-67.73	G19839	-0.2426	14.29	59.81	21.9
	June10	SDW_3	8	152.12	CR3_3	147.14-154.12	G19839	-0.2018	11.66	62.54	15.8
RDW (g)	Oct09	RDW_1	3	0.01	P010.4	0.01-4.01	G2333	0.1766	14.48	40.12	15.6
	June10	RDW_2	3	76.94	W091.6	73.46-82.94	G2333	0.1128	13.76	50.58	17.7
	June10	RDW_3	8	84.66	Bm211	82.84-97.36	G19839	-0.1188	16.09	50.93	19.6
	June10	RDW_4	10	20.08	M090.18	18.28-22.08	G19839	-0.1587	19.26	50.57	23.7
%N shoot	Oct09	%NSHOOT_1	1	27.23	BM201	24.37-35.01	G2333	0.1678	20.06	52.92	24.5
	Oct09	%NSHOOT_2	3	18.91	Bm189	15.91-22.34	G19839	-0.1179	16.31	56.87	17.6
	Oct09	%NSHOOT_3	4	17.61	Bmy001	12.61-34.21	G19839	-0.1208	17.08	56.09	18.4
%N root	June10	%NROOT	4	188.56	Bmb135	184.39-195.56	G19839	-0.1603	19.03	45.42	20.6
%N plant	Oct09	%NPLANT_1	1	34.01	W091.0	23.37-37.97	G2333	0.1453	21.01	56.93	25.5
	Oct09	%NPLANT_2	4	3.01	Bm161	0.01-8.01	G19839	-0.1166	20.30	40.59	16.8
total N root (mg)	June10	NROOT_1	3	78.94	W091.6	76.94-79.94	G2333	3.3289	14.69	47.63	14.4
	June10	NROOT_2	4	132.68	OAP7	122.03-141.00	G19839	-3.8788	20.87	46.96	21.2
total N plant (mg)	June10	NPLANT_1	4	206.61	BMb488	197.56-221.61	G19839	-12.0407	18.12	44.31	17.5
	June10	NPLANT_2	10	22.08	M090.18	21.08-24.02	G19839	-13.3903	16.99	50.99	20.4

[a] Linkage group

[b] R^2: proportion of phenotypic variance explained by QTL at test site; TR^2: total R^2 for the QTL and the background marker; LR: likelihood ratio test statistic for H_0, H_1, where H_0 is the hypothesis of no QTL effect at the test position and H_1 is the hypothesis of a QTL effect at the test position

[c] Additivity: effects of substituting a single allele from one parent to another. Positive values indicate that allelic contribution is from G2333 and negative from G19839

In the field experiment, a larger number of QTL were identified (Table 4). At flowering, a QTL for SPAD overlapped with a QTL for SPAD in the first greenhouse experiment. Another QTL found on linkage group b07 for SPAD values at late pod-filling stage coincided with QTL for SPAD values at 28 days and 35 days in the first greenhouse experiment. In the field experiment, two QTL for SPAD values were found twice: (1) the QTL on linkage group b06 was

found for SPAD values at both V3 and R6 stages and (2) the QTL on linkage group b03 was found for SPAD values at both R6 and R7/8 growth stages.

Finally, for root biomass at flowering stage (R6), a QTL was detected on linkage group b10 and overlapped with a QTL for root dry weight at the R6 growth stage in the second greenhouse experiment. For shoot dry weight at the early V3 stage, two QTL were found on linkage groups b07 and b10 with G2333

Table 4 Quantitative trait loci (QTL) analysis for G2333 × G19839 RIL population grown in a field trial at the CIAT research farm in Darien, Colombia

Sampling stage	Trait	QTL name	LG[a]	Position	Closest marker	Region	Source	Additivity	Significance[b] R²	TR²	LR
SPAD	SPAD V3	SPADV3_1	1	33.01	W091.0	22.37-47.97	G2333	-1.1124	21.69	69.94	39.2
		SPADV3_2	6	39.73	BSn96	12.28-56.13	G19839	1.5673	43.54	69.50	65.5
	SPAD R6	*SPADR6_1*	*2*	*125.54*	*BMd017*	*119.13-129.36*	*G19839*	*1.4515*	*19.56*	*57.11*	*30.6*
		SPADR6_2	3	63.18	BMb348	56.51-70.18	G19839	0.9948	11.89	57.59	19.3
		SPADR6_3	6	41.13	Bm170	38.73-45.13	G19839	0.7916	10.39	57.09	17.3
	SPAD R7/8	SPADR7/8_1	3	63.18	BMb348	57.51-68.18	G19839	1.3758	13.17	44.18	16.7
		SPADR7/8_2	*7*	*11.60*	*Bm160*	*5.01-22.6*	*G19839*	*1.4537*	*14.89*	*46.02*	*16.5*
SDW	SDW V3	SDWV3_1	7	53.33	BSn69	37.52-62.21	G2333	-0.0940	27.14	60.38	40.1
		SDWV3_2	10	18.28	GAT11B	18.28-18.61	G2333	-0.0736	11.88	59.75	19.3
RDW (g)	RDW V3	RDWV3_1	7	52.33	BSn69	46.52-54.75	G2333	-0.0191	11.07	53.94	17.1
		RDWV3_2	9	1.01	M130.34	0.01-9.01	G19839	0.0188	11.35	54.41	16.9
		RDWV3_3	10	9.01	X010.3	4.01-13.01	G2333	-0.0197	12.36	58.29	15.6
	RDW R6	RDWR6	10	33.23	F0032P15	6.01-41.23	G2333	-0.0759	25.69	56.14	25.9
NDW (mg)	NDW R6	NDWR6_1	3	10.01	P010.4	7.01-13.91	G2333	-20.4678	12.97	43.15	13.5
		NDWR6_2	4	147.85	BMb138	140.00-159.98	G2333	-24.7119	19.07	44.69	21.2
NDW/RDW (mg/100mg root)	NDW/RDW V3	NDW/RDWV3_1	4	180.39	BSn33_SNP1	180.20-182.39	G2333	-1.7108	12.34	49.23	16.1
		NDW/RDWV3_2	4	266.09	F0044L20	256.09-278.53	G2333	-3.0617	32.34	60.85	26.2
	NDW/RDW R6	NDW/RDWR6	4	147.85	BMb138	144.85-153.98	G2333	-21.1542	12.36	47.56	14.9
NN	NN V3	NNV3_1	3	109.11	Bm098	100.03-114.11	G2333	-3.5107	17.25	54.04	17.3
		NNV3_2	5	15.03	BMd053	11.22-19.03	G2333	-4.0545	16.72	49.23	16.1
%N$_{dfa}$	%N$_{dfa}$ HARVEST	%Ndfa	4	154.98	BMb134	144.85-158.98	G2333	-3.6165	18.79	38.55	16.4
total N$_{dfa}$ (mg)	total N$_{dfa}$ shoot R7/8	TOTNdfa	1	73.66	BSn73	70.29-80.66	G19839	60.8124	21.38	43.75	19.1
total N$_{dfs}$ (mg)	total N$_{dfs}$ shoot R6	TOTNdfs	10	128.49	BAC6	120.00-137.49	G2333	-23.1028	23.52	39.41	26.9
SEED HARVEST	SEED NR/POD	SEED/POD	1	35.97	BSn71_SNP1	33.01-44.97	G19839	0.3519	17.67	45.39	21.3
	%N SEED (mg N/100 mg seed)	%NSEED	4	126.91	Bmy006	125.91-127.91	G2333	-0.2327	16.60	32.09	15.2
	PROP N SEED (%)	PROPNSEED	2	88.60	BM152	78.93-98.70	G19839	1.8121	20.67	55.17	26.1
POD HARVEST	DRY POD WEIGHT (g)	PODWT_1	7	35.52	BSn95	17.6-46.52	G2333	-0.9733	26.21	39.95	26.0
		PODWT_2	9	13.01	M130.34	7.01-19.01	G2333	-0.8640	22.41	53.17	17.1
PLANT HARVEST	DRY PLANT WEIGHT(g)	PLANTWT	4	224.61	BMb488	211.61-238.49	G2333	-0.9127	38.24	53.61	31.1
	%N PLANT (mg N/100 mg plant)	%NPLANT	4	122.85	Bm068	112.66-134.68	G2333	-0.1689	27.83	48.33	35.8
	N PLANT (mg)	TOTNPLANT	4	231.49	Bm068	221.61-234.49	G2333	-9.8328	16.17	41.46	15.0
TOTAL HARVEST	%N TOTAL (mg N/100 mg total)	%NHARV	4	126.91	Bmy006	125.91-127.91	G2333	-0.2327	16.60	32.09	15.2
	N TOTAL[1] (mg)	TOTNHARV	1	35.97	BSn71_SNP1	35.97-37.97	G19839	130.6433	14.13	36.62	15.0

QTL showing overlap for this experiment are highlighted in the same colour. QTL underlined and in italic are the QTL that overlap with QTL for the same trait found in the greenhouse experiment

[a] Linkage group

[b] R^2: proportion of variance explained by QTL at test site; TR^2: total R^2 for the QTL and the background marker; LR: likelihood ratio test statistic for H_0, H_1, where H_0 is the hypothesis of no QTL effect at the test position and H_1 is the hypothesis of a QTL effect at the test position

[c] TOTAL N: Sum of N contents of seed, pod and plant at harvest

providing the positive allele for high shoot dry weight. The QTL on linkage group b07 was found to be linked to the nodulation gene-based marker BSn69.

Nodulation parameters (NDW, NDW/RDW and NN) were also associated with some QTL including one QTL for NDW/RDW at the V3 growth stage which was most closely linked to the nodulation gene-based marker BSn33_SNP1. A QTL found for nodule and root characteristics, NDW/RDW, at the R6 growth stage overlapped with another QTL for %N fixed at harvest. The climbing bean parent G2333 provided the positive allele in both cases. For total N amounts fixed at late pod-filling stage (R7/8), a QTL on linkage group b01 was found to be closely linked to another nodulation gene-based BSn73.

QTL found for the seed number per pod and for the total N accumulated at harvest overlapped with QTL for SPAD values at V3 stage on linkage group b01. For

the seed, plant and total %N accumulation, the same QTL was detected on linkage group b04. The climbing bean parent G2333 provided the positive allele for this QTL. For the proportion of N translocated to the seed (%), one major QTL was detected on linkage group b02 that accounted for 20 % of the total observed variance.

For some QTL identified in greenhouse and/or field experiments, sequences of markers that were found to be closely linked to the QTL were available. These sequences were compared to the most recent EST assembly of common bean and to a collection of gene sequences in soybean. Additionally, the most similar common bean EST, or alternatively the gene sequence of soybean, was subjected to a BLASTn analysis. This made it possible to annotate the marker sequences linked to some QTL, and the results are shown in Supplementary Table 4.

Discussion

The results of the greenhouse experiments varied for parental and RIL genotypes, with contrasting results in terms of parental N fixation in the first and second experiments. This could have been due to photoperiod differences in the greenhouse trials (fall vs. summer). In terms of N_2 fixation, G2333 performed better in the field experiment with higher yields but G19839 accumulated more N both from soil and through SNF, but only by the end of the experiment.

This variability in SNF responses can be considered to be quite typical for SNF and related traits and is mainly caused by a large environmental impact on SNF performance (Bliss 1993; Peoples et al. 1995). Environmental factors that affect SNF performance have been observed to include abiotic and biotic stresses such as P deficiency, drought, pests and diseases (Herridge and Rose 2000) as well as physiological parameters such as growth habit. High mineral N availability and endogenous non-fixing rhizobial populations can be contributing factors to environmental variability. These environmental factors can obscure the true SNF capacity of a legume crop and make it very difficult to obtain stable results over time and location.

Despite the variation in phenotypic results between greenhouse and field, some consistent QTL were identified in all three experiments or in at least two out of the three experiments. A first interesting QTL for leaf chlorophyll content (SPAD) at early plant growth stages was detected in both greenhouse experiments on linkage group b01 (see Fig. 1) with an overlap of QTL underlying SPAD values at the early V3 stage in the field experiment. There was also an overlap with another QTL for the total N fixed in the shoots at late pod-filling stage (R7/8). A second interesting QTL for SPAD values at later growth stages was found in both greenhouse experiments on linkage group b10. However, this QTL was not identified under field conditions. On the same linkage group, a QTL for root dry weight at flowering was found in the second greenhouse experiment as well as in the field experiment. On linkage group b07, a QTL for SPAD values at 4–6 weeks in the greenhouse coincided with a QTL for SPAD values at late pod-filling stage in the field.

Various QTL for %N fixed and total N fixed were identified. In the first greenhouse experiment, two QTL were identified for N% fixed on linkage groups b01 and b04. The QTL on linkage group b01 overlapped with a QTL detected for total N content at harvest in the field experiment. In the second greenhouse experiment, two QTL for total plant N content were identified on the same linkage groups but these did not show any overlap with other QTL for amounts of fixed N. Finally, the field experiment also yielded some QTL for SNF-related traits, including one on linkage group b04 for the N% fixed at harvest and one on linkage group b01 for the total N fixed in the shoot at late pod-filling stage. These QTL, however, did not show any overlap with previously identified QTL from the greenhouse experiments. Variability between greenhouse and field results have also been observed for studies of phosphorus uptake in common bean (Miklas et al. 2006) and therefore was not surprising for the more complex biological interactions of *Rhizobia* and common bean.

In terms of QTL detection accuracy, the present study was developed with a small mapping population (<100 individuals). Therefore, these results have to be interpreted with special caution because of the "Beavis effect" (Xu 2003). For this reason, further analysis, based on our preliminary results, must be done. Specifically, using the linked markers to QTL reported here, QTL confirmation and population size expansion (Lander and Kruglyak 1995) approaches will be useful to validate the loci involved in SNF capacity.

Previous genetic studies have been performed using the G2333 × G19839 common bean population. The first one was an inheritance study and QTL analysis of climbing ability (Checa et al. 2006; Checa and Blair 2008). In two other studies, QTL for adventitious rooting (Ochoa et al. 2006) and phytate and phosphorus content of seed (Blair et al. 2009) were described. QTL for SPAD values at V3 and R6 growth stages identified in the field experiment overlapped with QTL identified in the field for adventitious root dry weight on linkage group b06 (Ochoa et al. 2006). Another QTL for RDW at flowering (R6) in the field coincided with one of the QTL underlying specific root length measured in the greenhouse (Ochoa et al. 2006). Several QTL identified in our study overlapped with QTL identified for climbing bean growth traits in the study by Checa and Blair (2008). Of the overlap, the most interesting would be on linkage group b07, where a QTL for SPAD values measured at 28 days overlapped with one of the QTL found for climbing ability by Checa and Blair (2008). These results might strengthen the hypothesis that climbing ability is genetically linked to nitrogen fixation capacity, which would explain why many climbing beans are good at N_2 fixation as reported by previous authors (Graham and Halliday 1976; Graham and Rosas 1977; Kumarasinghe et al. 1992).

A number of studies to detect natural variation and QTL for SNF and related traits in legumes have been published (Nodari et al. 1993; Tsai et al. 1998; Souza et al. 2000; Tanya et al. 2005; Nicolas et al. 2006; Santos et al. 2006; Bourion et al. 2010). Only two of these studies used common bean for identifying QTL for nodule number. In the greenhouse study by Tsai et al. (1998), a total of four QTL for high and low level of mineral N fertilization were identified on linkage groups b01, b03, b04 and b07. In the present study, two QTL for nodule number were found on linkage group b03 and b05, explaining each about 17 % of the total observed phenotypic variance and with G2333 providing the positive allele for high nodule number.

When comparing these QTL for nodule dry weight, an overlap of QTL was evident on linkage group b03 with one QTL from Tsai et al. (1998). The QTL for NDW at R6 stage appeared to coincide with a QTL for NN under both high and low mineral N fertilization levels. Another QTL for NN at the V3 growth stage on linkage group b03 overlapped with a QTL for NN under low mineral N fertilization from Tsai et al.

(1998). Finally, a third QTL for NDW at R6 stage overlapped with a QTL for NN under high mineral N fertilization on linkage group b04 from that previous study.

A second study by the same research group (Souza et al. 2000) using the same experimental population identified a total of eight and five QTL for NN under low and high mineral N fertilization, respectively. When comparing the present study with the results obtained by Souza et al. (2000) it seems that the QTL identified in this study for NN at V3 stage on linkage group b03 again overlapped with a QTL for NN under low mineral N fertilization. However, it was difficult to exactly compare the results of this study with results from Souza et al. (2000) as different populations were used.

Analysis of N% and total N contents of all plant parts at harvest, including seed, pod and plant parts, allowed us to calculate the proportion of N that was translocated to the seed. This trait, together with the absolute N% in the seed, is important for the seed protein content. Absolute seed N% was somewhat lower for the seed of the climbing bean parent G2333 than for the bush bean parent G19839. For both seed N% and translocation of N to the seed, QTL were found including one on linkage group b04 for seed N% and one for proportion of N translocated to the seed on linkage group b02. These QTL were both linked to microsatellite markers which could be used for marker-assisted selection of these traits.

Some QTL that were identified in this study were linked to the nodulation gene-based markers that were mapped on the existing map of the G2333 × G19839 population. For these QTL and for some other QTL linked to markers with known sequences, it was possible to compare sequences with the most recent common bean EST assembly and with a soybean gene collection in order to annotate tentatively the sequences linked to the QTL identified in this study.

First, a QTL was identified for early SPAD values on linkage group b06 for the greenhouse experiments, more specifically at 7 days in the first greenhouse experiment and for SPAD at 21 days for the second greenhouse experiment, which was closely associated with the gene *ASTRAY* (BSn37_SNP2) which is a known bZIP transcription factor with a function in the regulation of nodulation (Nishimura et al. 2002). Legumes control nodulation by sensing environmental cues where *ASTRAY* can play a roll.

For SPAD at 7 days, another QTL on linkage group b01 was also linked to a bZIP transcription factor. We expected transcriptional control to be important in determining SNF-related traits as a whole, since these are responsive to complex signal perception and a cascade of gene responses (Yokota and Hayashi 2011).

Another interesting candidate gene—QTL association was identified for a QTL for shoot dry weight in the second greenhouse experiment, on linkage group b02, which was linked to the gene-based marker BSn67. This sequence contains a conserved region of an auxin-responsive transcription factor ARF4 of *Arabidopsis thaliana*. The parents of the population G2333 and G19839 might have different auxin levels, resulting in differing biomass accumulation. As the positive allele for this QTL originated from the climbing bean parent G2333, this variety might be the one with higher expression levels of the transcription factor or higher actual levels of auxin under conditions of symbiotic nitrogen fixation.

Both shoot and root dry weight at V3 growth stage in the field experiment showed overlapping QTL on linkage group b07 that in turn were linked to the marker BSn69. This sequence contains a WRKY-DNA binding domain and an associated plant zinc cluster domain. The WRKY proteins are a superfamily of transcription factors with up to 100 representatives in *Arabidopsis* (Eulgem et al. 2000). Family members appear to be involved in the regulation of various plant physiological programs. At this stage, however, there is no detailed information available yet on the exact function of each transcription factor in this process or possible associations with N_2 fixation.

Another candidate gene of interest was based on the marker BSn33_SNP1 underlying the QTL for NDW/RDW at the V3 growth stage. This sequence is homologous to LYK3 originally identified in *Medicago truncatula* and is known to be one of the LysM domain receptor kinases in a two-receptor model that regulates rhizobial infection in this model legume together with LYK4 (Limpens et al. 2003). The positive allele for high NDW/RDW was provided by the climbing bean parent G2333 for this QTL.

In addition, the QTL for total N fixed at late pod-filling stage (R7/8) on linkage group b01 was shown to be linked to the marker BSn73 (Supplementary Table 4) which showed high homology to an AP2/ERF-domain transcription factor. This family of transcription factors integrates jasmonic acid and ethylene signals in expression regulation of several plant defense or abiotic stress-related genes (Nakano et al. 2006). Additionally, in *M. truncatula*, these transcription factors have found to be involved in both activation and repression of nodulation factor-elicited gene transcription in root hairs at the beginning of the infection process by *Rhizobium* (Andriankaja et al. 2007). These findings make BSn73 a promising putative gene for marker-assisted selection or evaluation.

Finally, the marker BMd017, related to a DNA-J protein, was associated with a QTL for SPAD at flowering in the field experiment on linkage group b02. This gene, known as a Heat Shock Protein 40 (Hsp40), is from a family of heat-shock proteins that contain a 70 amino-acid consensus sequence known as the J domain (Schlesinger 1990). Hsp40 plays a role in regulating ATPase activity with the expression of heat-shock proteins increasing upon exposure to elevated temperatures or other stress. Heat-shock proteins also function as molecular chaperones binding substrate proteins that are in unstable, non-native structural states during protein folding (Boston et al. 1996). Parental bean varieties G2333 and G19839 might have a difference in expression of a certain heat shock protein which functions as molecular chaperone in the correct folding of Rubisco leading to a difference in chloroplast accumulation in the leaves.

In order to confirm candidate gene—QTL associations, one of the strategies could be to look at variation in expression levels of these genes (e.g. by qRT-PCR) in the population. Another strategy that can be used to further confirm and fine map some of the most interesting QTL identified in our study is through the approach of heterogeneous inbred-family analysis which is a rapid method for developing near-isogenic lines that differ only at the QTL of interest (Tuinstra et al. 1997). This method is particularly suitable in the G2333 × G19839 RIL population, as the degree of heterozygosity is substantial. For some QTL, including the QTL for N % in the seed and the proportion of N translocated to the seed (determining the nutritional value of the seed), it would also be worthwhile to further study their consistency through repeated QTL analysis and/or through association studies in natural populations. If consistent, the markers linked to these QTL could be used for marker-assisted selection in breeding efforts to improve the nutritional value of bean seeds.

References

Andriankaja A, Boisson-Derniera A, Francesa L, Sauviaca L, Jauneaub A, Barkera DG, de Carvalho-Niebela F (2007) AP2-ERF transcription factors mediate Nod factor—dependent Mt ENOD11 activation in root hairs via a novel cis-regulatory motif. Plant Cell 19:2866–2885

Blair MW, Sandoval TA, Caldas GV, Beebe SE, Paez MI (2009) QTL analysis of seed phosphorus and seed phytate content in a recombinant inbred line population of common bean. Crop Sci 49:237–246

Bliss FA (1993) Breeding common bean for improved biological nitrogen fixation. Plant Soil 152:71–79

Boston RS, Viitanen PV, Vierling E (1996) Molecular chaperones and protein folding in plants. Plant Mol Biol 32(1–2):191–222

Bourion V, Rizvi SM, Fournier S, de Larambergue H, Galmiche F, Marget P, Duc G, Burstin J (2010) Genetic dissection of nitrogen nutrition in pea through a QTL approach of root, nodule, and shoot variability. Theor Appl Genet 121:71–86

Broughton WJ, Hernández G, Blair MW, Beebe S, Gepts P, Vanderleyden J (2003) Beans (*Phaseolus* spp)—model food legumes. Plant Soil 252:55–128

Checa OE, Blair MW (2008) Mapping QTL for climbing ability and component traits in common bean (*Phaseolus vulgaris* L.). Mol Breed 22:201–215

Checa O, Ceballos H, Blair MW (2006) Generation means analysis of climbing ability in common bean (*Phaseolus vulgaris* L.). J Hered 97(5):456–465

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci 5:199–206

Galeano CH, Fernández AC, Gómez M, Blair MW (2009) Single strand conformation polymorphism based SNP and Indel markers for genetic mapping and synteny analysis of common bean (*Phaseolus vulgaris* L.). BMC Genomics 10:629

Galeano C, Cortes A, Fernandez A, Soler A, Franco-Herrera N, Makunde G, Vanderleyden J, Blair M (2012) Gene-based single nucleotide polymorphism markers for genetic and association mapping in common bean. BMC Genet 13:48

Graham PH, Halliday J (1976) Inoculation and nitrogen fixation in the genus *Phaseolus*. In: Vincent JM, Whitney AS, Bose J (eds) Exploiting the legume-*Rhizobium* symbiosis in tropical agriculture. Univ Hawaii Misc. Publ, Hawaii, pp 313–334

Graham PH, Rosas JC (1977) Growth and development of indeterminate bush and climbing cultivars of *Phaseolus vulgaris* L inoculated with *Rhizobium*. J Agric Sci 88:503–508

Herridge D, Rose I (2000) Breeding for enhanced nitrogen fixation in crop legumes. Field Crops Res 65:229–248

Kumarasinghe KS, Danso SKA, Zapata F (1992) Field evaluation of N_2 fixation and N partitioning in climbing bean (*Phaseolus vulgaris*) using 15N. Biol Fert Soils 13(3):142–146

Lander E, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11:241–247

Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R (2003) LysM domain receptor kinases regulating rhizobial Nod-factor induced infection. Science 302:630–633

Lorieux M (2012) MapDisto: fast and efficient computation of genetic linkage maps. Mol Breed. doi:10.1007/s11032-012-9706-y

Mahuku GS (2004) A simple extraction method suitable for PCR-based analysis of plant, fungal and bacterial DNA. Plant Mol Biol Rep 22(1):71–81

Martínez-Romero E (2003) Diversity of *Rhizobium-Phaseolus* vulgaris symbiosis: overview and perspectives. Plant Soil 252:11–23

Miklas PN, Kelly JD, Beebe SE, Blair MW (2006) Common bean breeding for resistance against biotic and abiotic stresses: from classical to MAS breeding. Euphytica 147:105–131

Monje OA, Bugbee B (1992) Inherent limitations of nondestructive chlorophyll meters: a comparison of two types of meters. Hort Sci 27:69–71

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the *ERF* gene family in *Arabidopsis* and rice. Plant Physiol 140:411–432

Nicolas MF, Hungria M, Arias CAA (2006) Identification of quantitative trait loci controlling nodulation and shoot mass in progenies from two Brazilian soybean cultivars. Field Crops Res 95:355–366

Nishimura R, Ohmori M, Fujita H, Kawaguchi M (2002) A *Lotus* basic leucine zipper protein with a RING-finger motif negatively regulates the developmental program of nodulation. Proc Natl Acad Sci USA 99:15206–15210

Nodari RO, Tsai SM, Guzman P, Gilbertson RL, Gepts P (1993) Toward an integrated linkage map of common bean. III. Mapping genetic factors controlling host-bacteria interactions. Genetics 134:341–350

Ochoa IE, Blair MW, Lynch JP (2006) QTL analysis of adventitious root formation in common bean under contrasting phosphorus availability. Crop Sci 46:1609–1621

Olsen SR, Sommers LE (1982) Phosphorus. In: Methods of soil analysis, part 2. Chemical and microbiological properties, ASA-SSSA, Madison, WI, USA, pp 403–430

Peoples MB, Herridge DF, Ladha JK (1995) Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? Plant Soil 174:3–28

Remans R, Croonenborghs A, Gutierrez R, Michiels J, Vanderleyden J (2007) Effects of plant growth-promoting rhizobacteria on nodulation of *Phaseolus vulgaris* L. are

dependent on plant P nutrition. Eur J Plant Pathol 119:341–351

Remans R, Ramaekers L, Schelkens S, Hernandez G, Garcia A, Reyes J, Mendez N, Toscano V, Mulling M, Galvez L, Vanderleyden J (2008) Effect of *Rhizobium–Azospirillum* coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris* L. genotypes cultivated across different environments in Cuba. Plant Soil 312:25–37

Santos MA, Nicolas MF, Hungria M (2006) Identification of QTL associated with the symbiosis of *Bradyrhizobium japonicum, B. elkanii* and soybean. Pesq Agropec Bras 41(1):67–75

Schlesinger MJ (1990) Heat shock proteins. J Biol Chem 265:12111–12114

Sharp Z (2005) Principles of stable isotope geochemistry. Prentice Hall, Upper Saddle River, NJ

Shearer G, Kohl DH (1986) N2-fixation in field settings: estimations based on natural 15N abundance. Aust J Plant Phys 13:699–756

Singh SP (1982) A key for identification of different growth habits of *Phaseolus vulgaris* L. Annu Rep Bean Improv Coop 25:92–94

Singh SP (1991) Breeding for seed yield. In: van Schoonhoven A, Voysest O (eds) Common bean: research for crop improvement. C.A.B, International, Wallingford/CIAT, Cali, pp 383–443

Snoeck C (2001) Host specificity determinants of *Rhizobium* sp. BR816 for early signalling in symbiotic interactions. PhD dissertation, Catholic University Leuven

Souza AA, Boscariol RL, Moon DH, Camargo LEA, Tsai SM (2000) Effects of *Phaseolus vulgaris* QTL in controlling host-bacteria interactions under two levels of nitrogen fertilization. Gen Mol Biol 23(1):155–161

Tanya P, Srinives P, Toojinda T, Vanavichit A, Lee S (2005) Identification of SSR markers associated with N2-fixation components in soybean. Korean J Genet 27(4):351–359

Tsai SM, Nodari RO, Moon DH, Camargo LEA, Vencovsky R, Gepts P (1998) QTL mapping for nodule number and common bacterial blight in *Phaseolus vulgaris* L. Plant Soil 204:135–145

Tuinstra MR, Ejeta G, Goldsbrough PB (1997) Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci. Theor Appl Genet 95:1005–1011

Vlassak KM, Luyten E, Verreth C, Van Rhijn P, Bisseling T, Vanderleyden J (1998) The *Rhizobium* sp. BR816 *nodO* gene can function as a determinant for nodulation of *Leucaena leucocephala, Phaseolus vulgaris* and *Trifolium repens* by a diversity of *Rhizobium* spp. Mol Plant Microbe Interact 5:383–392

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78

Wang S, Basten CJ, Zeng Z-B (2011) Windows QTL cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

Xu S (2003) Theoretical basis of the Beavis effect. Genetics 165:2259–2268

Yokota K, Hayashi M (2011) Function and evolution of nodulation genes in legumes. Cell Mol Life Sci 68:1341–1351