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#### Citation:

Marrou, H.; Ricaute, Jaumer; Ghanem, M.E.; Clavijo Michelangeli, J.A.; Ghaouti, L.; Rao, Idupulapati M.; Sinclair, T.R.. 2017. Is nitrogen accumulation in grain legumes responsive to growth or ontogeny? . *Physiologia Plantarum*. 162(1): 109-122

#### Publisher's DOI:

<https://doi.org/10.1111/ppl.12617>

#### Access through CIAT Research Online:

<http://hdl.handle.net/10568/88059>

#### Terms:

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## Is nitrogen accumulation in grain legumes responsive to growth or ontogeny?

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### Abstract

Nitrogen accumulation in legumes is one of the main determinants of crop yield. Although N accumulation from symbiotic nitrogen fixation or N absorption from the soil has been widely investigated, there is no clear consensus on timing of the beginning of N accumulation and the termination of N accumulation, and the physiological events that may be associated with these two events. The analyses done in this study aimed at identifying the determinant of N accumulation in two grain legumes species. Nitrogen accumulation dynamics as well as mass accumulation and development stages were recorded in the field for several genotypes of common bean (*Phaseolus Vulgaris*) and faba bean (*Vicia faba*) under different growing conditions. This study showed that during the vegetative stages N accumulation rate was correlated with mass accumulation rate. However, maximum accumulation of N did not correspond to the time of maximum mass accumulation. In fact, for both species N accumulation was found to persist into seed growth. This challenges a common hypothesis that seed growth causes a decrease in N accumulation due to a shift of photosynthate supply to support seed growth. Even more surprising was the shift of the active accumulation of N in faba bean to late in the growing season as compared to common bean. N accumulation by faba bean only was initiated at high rates very late in vegetative growth and persisted at high rates well into seed fill.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ppl.12617

## Introduction

Nitrogen (N) is widely recognized, together with water, as the most crucial resources to promote crop yield increase (Sinclair & Wit 1975; Godwin & Jones 1991; Soltani & Sinclair 2012). However, intensive use of mineral fertilizer to increase N availability for crops during the Green Revolution has resulted in concern for environmental issues such as groundwater pollution, eutrophication of aquatic ecosystems, and increase in greenhouse gas emissions (Tilman et al. 2002; IPCC 2007). As a consequence, the need for a better understanding of the physiological determinants of N capture and use by plants has been growing over the past decades. Legumes are of particular interest because they have the capacity to accumulate N in two different, interactive processes: symbiotic N<sub>2</sub> fixation from the atmosphere and N uptake from the soil.

Nitrogen accumulation in crops has been extensively studied (Ney et al. 1997; Jamieson & Semenov 2000; Lemaire et al. 2007; Cooper et al. 1976; Smith et al. 1988) using various approaches. However, studies exploring source-sink relations in cereals, for example, have generated inconsistent results to explain N uptake and allocation within plants (Martre et al. 2003; Dreccer et al. 1997; Rajcan & Tollenaar 1999). Fewer studies have been conducted with legumes, and even more confusion seems to remain due to the involvement of N accumulation from the soil and symbiotic N<sub>2</sub> fixation. Nevertheless, it is generally assumed that N accumulation is correlated with crop mass growth causing N concentration in the different organs to remain equal to or less than a target concentration (Boote & Hoogenboom 1998; Robertson et al. 2002; Martre et al. 2003; Coleman et al. 1993). However, a few studies also indicate that N accumulation in aerial mass during vegetative growth can be better related to leaf area expansion since stem may serve as a temporary storage pool to support leaf expansion (Sinclair et al. 2003; Jamieson & Semenov 2000). In addition, the nature and size of sinks for N is often related to ontogeny, and N accumulation dynamics are commonly related to developmental stages (Cooper et al. 1976; Voisin & Gastal 2015; Ney et al. 1997).

Uncertainty and confusion remains specifically about the time during the growing season of beginning of N accumulation, the termination of N accumulation, and the intervening rate of N accumulation. The timing of the beginning N accumulation and the switch from germinating seed reserve to alternative sources of N is poorly documented. Also, proposals for the termination of N accumulation range from the beginning of the seed

filling stage to some time during seed fill. To complicate matters, during seed fill N is generally translocated from leaves and stems to reproductive organs to support the growth of grain. Several authors reported that total N accumulation in above-ground dry matter plateaus around the beginning of seed growth as the high demand for carbohydrates by the seeds may result in little or no carbohydrate to support additional N accumulation (Lawlor 2002; Soltani & Sinclair 2012; Voisin et al. 2002). However, contradictory results have been reported in cowpea (*Vigna unguiculata*) and black gram (*Vigna mungo*) (Sinclair et al. 1987). In faba bean (*Vicia faba*), Herdina & Silsbury (1990) showed that half of plant N is accumulated after the beginning of seed growth, although N<sub>2</sub> fixation starts decreasing at the onset of the seed-filling phase. Their results also indicated large discrepancies in the N accumulation dynamics between faba bean and field pea (*Pisum sativum*).

Over all, few experiments have provided insight about the actual ontogenetic events associated with N accumulation in grain legumes by either N absorption from the soil or N<sub>2</sub> fixation. There are at least two reasons for this failure. First, limitation in N uptake is known to affect plant growth through a close interaction with other limiting resources, mainly water, in a complex relationship (Thomas et al. 2004; Quemada & Gabriel 2016; Devries et al. 1989). Second, differences in the dynamics of N accumulation and remobilization during grain fill have been shown both among cool-season species (Herdina & Silsbury 1990) and tropical species (Devries et al. 1989; Muchow et al. 1993). However, these species-comparative studies have all been based on the assessment of one genotype per species and did not offer results for direct comparison between cool-season and tropical species. There is no published study comparing the dynamics of N accumulation among grain legumes species that included several genotypes.

The objective of this study was to analyze the temporal dynamics of N accumulation of two grain legume species: common bean (*Phaseolus vulgaris*), a warm-season species, and faba bean, a cool-season species. These two species were selected to represent diversity in N<sub>2</sub> fixing capacity and area of production. Field experiments were done to document N dynamics through a growing season of several genotypes within each species. An important part of this objective was an attempt to correlate ontogenic events with the temporal dynamics of N accumulation, and total plant N content. The influence of high temperature and drought were considered in the common bean and faba bean experiments, respectively.

## Material and Methods

### *Experimental sites and plant material*

#### *Common bean*

Experiments on common bean were conducted at two sites in southwestern Colombia: Palmira – Valle del Cauca (PAL, 15° 09' N, 76° 51' W) and Darien – Valle del Cauca (DAR, 9° 09' N, 76° 51' W). The soil at both locations was a Mollisol but the soil at PAL was high in phosphorus and at DAR low in phosphorus. The altitude difference between the two locations resulted in environmental differences. At high altitude PAL, maximum temperature averaged 31°C, daily mean radiation was 14.2 MJ m<sup>-2</sup> d<sup>-1</sup> during the experimental time period. At low altitude DAR the maximum average temperature was 25.5°C and mean daily radiation was 18.6 MJ m<sup>-2</sup> d<sup>-1</sup>. Thus, the PAL site can be considered as a suboptimal growing situation compared to DAR due to the lower daily incoming radiation at PAL. Also, the higher temperature at PAL resulted in a shorter growing season than at DAR.

Six genotypes (CAL 96, ICA Quimbaya, DOR 364, SER 118, G 21212, Carioca) were grown. These genotypes were chosen to represent two major gene pools (Andean: CAL 96, ICA Quimbaya; and MesoAmerican: DOR 364, SER 118, G 21212, Carioca). Also, these genotypes included three widely grown growth-habits (I: CAL 96, ICA Quimbaya; II: DOR 364, SER 118 ; III: G 21212, Carioca).

The plots were sown on 3 Oct 2013 at DAR and 29 May 2014 at PAL as part of a study on the effects of planting density on common bean growth and development (Ricaurte et al. 2016). Fully randomized design was used at both sites with 3 replicates at DAR and 4 replicates at PAL. Experimental units were 4-m long with 7-row plots at DAR and 8-row plots at PAL. The row spacing was 0.6 m and the results for the 25 plants m<sup>-2</sup> density are presented here. At both sites non-limiting conditions were maintained through the growth cycle by applying 60 kg P ha<sup>-1</sup> at sowing, micronutrients by foliar sprays, and irrigating to avoid water-deficit stress. No N fertilization was applied, but seeds were inoculated with *Rhizobium tropici* CIAT 899.

#### *Faba bean*

The faba bean experiment was performed at the Institut Agronomique et Vétérinaire Hassan II, in Rabat, northern Morocco. The soil at this location was a low-fertility sandy

luvisol. Four genotypes (Féverole de Fès, Aguadulce, and two breeding lines of the Agricultural Center for Agricultural Research in the Dry Areas (ICARDA): HBP/SOF/2009 and HBP/SO/06-L4301-4/09) were included in the study to represent two seed-size types (broad bean: Aguadulce, HBP/SOF/2009; small seeds: Féverole de Fès, HBP/SO/06-L4301-4/09). Also, these genotypes represented two intensities of breeding: local traditional genotypes (Féverole de Fès, Aguadulce) and newly bred lines (HBP/SOF/2009, HBP/SO/06-L4301-4/09).


Prior to the experiment, mustard (*Sinapis alba*) was grown as green manure. The field was fertilized with diamonophosphate ( $100\text{kg ha}^{-1}$ ) before sowing and ammonitrate ( $30\text{kg/ha}$ ) on 18 March 2015, around flowering date. Weeding and aphid control were performed to avoid biotic stress in both treatments.

The experiment was sown on 2 December 2014 in a split-plot design with three replicates. Experimental units were 3-m long with 4-row plots spaced at 0.6 m. The experiment included two watering treatments: Irrigated (Ir) and Non-irrigated treatment (Nlr). Irrigation treatment was the main plot and genotypes were the subplots. The Ir treatment was started on 6 March 2015, shortly before anthesis. Cumulative rainfall from sowing to beginning of irrigation was 284 mm. Since cumulative ET<sub>0</sub> was only 95mm by this time, all plots were well watered up to anthesis. Cumulative rainfall after 6 March was only 80 mm, most of which was received before the end of March, resulting in drought for the Nlr treatment. In the Ir treatment, irrigation was applied at 1 to 3 days intervals so that greater than 300 mm was applied. This irrigation was sufficient to fully meet faba bean water use from anthesis to harvest under Mediterranean conditions (Siddique et al., 2001; Maalouf et al., 2015).

### **Measurements**

Plants in both experiments were observed at least weekly to determine 50% flowering stage (R1), beginning of seed growth (BSG – the date when 50% of plants had 1-cm long pods), and physiological maturity, which was assumed equivalent to termination of seed growth.

Crop growth was monitored through destructive sampling. For common bean, sampling was performed at DAR every two weeks from 22 days after sowing (DAS) for a total of eight sampling dates, and at PAL from 14 DAS for a total of six sampling dates. At each sampling date, all plants in a 30-cm length of row from two central rows of each plot were collected (around 7 plants). The plants components were divided into leaves, stems, and if

Pods existed the podwalls and seeds were separated. The samples were dried in an oven for 15 h at 105°C. 

For faba bean, samples were also collected every two weeks, from 80 DAS to termination of seed growth in both water treatments (six sampling dates for Ir, and five sampling dates for Nlr). At each sampling date, 25-cm length was collected from each of the four rows of a plot. Samples were then separated by organ, dried in an oven at 60°C for four days, and weighed.

All dried samples were analyzed for N concentration of each organ and total N content per plant ( $NTOT_{gpp}$ ,  $g \cdot plant^{-1}$ ) at each sampling date was calculated as follows.

$$NTOT_{gpp} = \sum_{organ \in \{leaves, stems, podwalls, seeds\}} (DW_{organ} \times [N]_{organ}) \quad [1]$$

where  $DW_{organ}$  is the dry mass of organ ( $g \cdot plant^{-1}$ ), and  $[N]_{organ}$  is the N concentration in the corresponding organ ( $gN \cdot g^{-1}$ ).

Sampling date and date of occurrence of ontogenetic stages were recorded as days after sowing (DAS). These data were converted into cumulative temperature units (CTU, °C), using a three segment function to describing growth rate response to temperature as described by Soltani and Sinclair (2012). For common bean, base temperature, optimal temperature and critical temperature were set respectively to 10°C, 24°C, and 30°C (Marrou et al. 2014). For faba bean, the base temperature was set to 0°C (Boote et al. 2002), and optimal and critical temperatures were assumed to be greater than the maximum average temperature experienced during the experiment (26 °C).

### ***Ontogenetic events***

The visual observations of time of beginning of seed growth (tBSG) and time of termination of seed growth (tTSG) were not fully consistent with the estimates of tBSG and tTSG based on the harvested seed weights. Therefore, updated estimates of ontogenetic stages were computed based on a logistic regression model between seed weight and CTU. Estimates of tBSG were defined as the CTU when seed weight reached 5% the final asymptote value and of tTSG were define as the CTU when seed weight reached 95% of the asymptote value. (Comparison of these estimations of tBSG and tTSG with the visual field notations are given in Appendix A).

### ***Growth rate (GR) dynamics***

Since mass accumulation has been hypothesized as a possible driver for initiation and termination N accumulation, growth rate (GR) was calculated through the growth cycle. Estimation of GR can be calculated using two different and contrasting approaches. The classical approach consists of calculating the difference in dry matter accumulation per plant between two measurement dates, divided by the time elapsed between the two measurements. The value calculated can be considered as an estimate of the GR at a date approximately in the middle between the two measurement dates. This approach has the advantage of using directly observed values for computation, but the approach is destructive so different plants are sampled at each measurement resulting in potentially high variability.

The second approach is based on estimating GR continuously from regression of sample mass against time through the whole crop season. In these regressions, the fit was done between total dry weight per plant (DW) and CTU since CTU was a better descriptor of progress in plant development and growth than date. The following logistic function was fitted for each genotype ( $g$ ) and each site or treatment ( $t$ ).

$$DW_{g,t}(CTU) = \frac{A_{g,t}}{1+e^{\left(\frac{CTU-m_{g,t}}{s_{g,t}}\right)}} \quad [2]$$

where  $DW_{g,t}(CTU)$  is the total dry weight per plant at CTU, for genotype  $g$  and treatment  $t$ ;  $A_{g,t}$ ,  $m_{g,t}$ ,  $s_{g,t}$  are the regression parameters of the logistic function. The asymptote,  $A_{g,t}$ , was an output from the regression for common bean. However, for faba bean the last sample was taken shortly after physiological maturity so these data were used to define  $A_{g,t}$  based on maximum dry weight per plant, averaged over repetitions per treatment and genotype.

Growth rate was computed continuously as the derivative of Eq [2] (Hoffmann & Poorter 2002; Poorter & Gamier 1996; Radford 1967). By definition, maximum GR was reached when CTU equaled  $m_{g,t}$  and stopped at  $A_{v,t}/(4 * s_{v,t})$ . A caution has been noted that in these estimates of GR a limited number of repetitions may not allow a good statistical fit to the data, which could result in anomalous estimations of GR (Hunt 2003; Poorter 1989).

### ***N accumulation temporal dynamic***

Similar to total dry weight per plant, the temporal dynamic of total plant N content was represented using a logistic model fitted between the total N content (NTOT, g plant<sup>-1</sup>) and



time from sowing, expressed in CTU. As given in Eq [3],  $NTOT_{g,t}(CTU)$  is the total N content in the plants ( $\text{g plant}^{-1}$ ) for genotype  $g$ , in treatment or site  $t$  at CTU, and  $A_{N,g,t}$ ,  $m_{N,g,t}$ ,  $s_{N,g,t}$  are the parameters of the logistic function for each variety and site.

$$NTOT_{g,t}(CTU) = \frac{A_{N,g,t}}{1 + e^{\left(\frac{CTU - m_{N,g,t}}{s_{N,g,t}}\right)}} \quad [3]$$

For faba bean as for common bean,  $A_{N,g,t}$  was set equal to the maximum N content (averaged over repetitions) observed for each variety and treatment. For common bean, data from the final harvest were discarded to fit the logistic adjustment since dry leaves were not collected at that date.

The logistic model (Eq 3) was first adjusted without any effect on the parameters of the logistic function (simple model), then with treatment effect and, eventually, with a genotype effect within each site or treatment, on each parameter of the logistic equation (complete model). The CTU for the beginning of nitrogen accumulation (tBNA) and termination of nitrogen accumulation (tTNA) were estimated for each variety and treatment or site as the CTU when total nitrogen content reached respectively 5% and 95% of the asymptote for each genotype and each treatment.

### **Statistical tools and methods**

All nonlinear adjustments were performed with the R software (<https://cran.r-project.org/>) using the `nls` and `gnls` function of package `nlme`. Comparison of the dynamics of N accumulation between sites, genotypes, and growing conditions, was made using nested model comparison based on the Akaike criterion (AIC). The estimated values of parameters for logistic models of total dry mass and total N accumulation are presented in Appendix B. Effect of sites, growing conditions and genotypes on total mass or N accumulation at final harvest was tested by Analysis of Variance models (ANOVA) using `lm` and `aov` functions in R software. Genotype was considered as a nested effect within the Site or Irrigation treatment effect to comply with the experimental design. Mean comparisons were done using Tukey's HSD test.

## Results

### *Ontogeny*

Mass accumulation data were used to estimate seed development stage as described previously. The tBSG estimated from the logistic regression were always estimated to occur after visual flowering by an average of 125 CTU in common bean and 245 CTU in faba bean (Table 1). The relative root mean square error between estimated ontogenetic stages based on seed growth and visually observed ontogenetic stages was only 7% (see Appendix A). Thus estimations of tBSG and tTSG based on logistic regression on seed growth were considered as reliable. Under stresses of high temperature (common bean) or water-deficit (faba bean) conditions, crop cycle was significantly shortened, with tBSG and tTSG occurring earlier.

### *Mass accumulation*

In the case of common bean, total accumulated mass was approximately equal ( $p$ -value= 0.07) between the PAL site ( $12.2 \text{ g plant}^{-1}$ ) and the DAR site ( $14.5 \text{ g plant}^{-1}$ ) with similar values among genotypes within each site ( $p$ -value=0.13). The entire dynamic of mass accumulation modeled by Eq [2] was found to differ between sites when comparing models using AIC (Fig. 1). Mass accumulation did not differ among genotypes in DAR, while under non-optimal conditions at the PAL site, significant differences in the dynamic of mass accumulation were detected among genotypes.

In the case of faba bean, total mass at final harvest was reduced significantly ( $p$ -value = 0.0018) under Nlr ( $13.1 \text{ g plant}^{-1}$ ) as compared to Ir ( $42.7 \text{ g plant}^{-1}$ ). However, no significant differences in final dry weight were found among genotypes within the same watering treatment ( $p$ -value=0.67) (Fig. 1). For each site, model comparison using AIC showed that all genotypes grown at the same site could be modeled with a common set of parameters in Eq [3].

Growth rate (GR) was calculated both by using the difference approach and the derivative approach. For common bean at PAL, GR was very similar between the two approaches (RMSE between the two approaches ranged between  $0.0039$  and  $0.0068 \text{ g plant}^{-1} \text{ CTU}^{-1}$ ) (Fig. 2). This similarity in estimating GR by the two approaches was not found at DAR for common bean and for faba bean in both watering treatments. The difference approach

for calculating GR was found inappropriate as it resulted in unrealistic values with time for some genotypes.

The GR as calculated from the derivative of the mass accumulation regression followed a symmetrical dynamic: GR increased until a maximum and then declined until maturity. For common bean, maximum GR was approximately  $0.05 \text{ g plant}^{-1} \text{ CTU}^{-1}$  at the two sites, however, the time when this maximum was reached differed (Fig 2). Maximum GR was achieved around flowering at DAR site. At the PAL site, where development was accelerated due to high temperature and low radiation likely limiting photosynthesis, maximum GR was not reached until between flowering and the beginning of seed growth.

In the case of faba bean (Fig. 3), maximum GR of the Nlr treatment was much lower (approximately  $0.015 \text{ g plant}^{-1} \text{ CTU}^{-1}$ ) than of the Ir treatment (approximately  $0.055 \text{ g plant}^{-1} \text{ CTU}^{-1}$ ). For Ir faba bean, maximum GR was achieved during the seed filling phase. In contrast, GR did not increase after flowering for Nlr faba bean.

### ***Nitrogen accumulation***

N accumulation could be successfully modeled in all cases using Eq [3], regression parameters were significantly different between sites for common bean, and between watering treatments for faba bean, based on the comparison of AIC of nested models (Fig. 4). In particular, NTOT at the final harvest was affected in different ways by climate and crop management. For common bean, there was no significant difference between the two experimental sites ( $p$ -value=0.18). For faba bean, NTOT in the Ir treatment ( $1.32 \text{ gN plant}^{-1}$  on average) was significantly greater than in the Nlr treatment ( $0.40 \text{ gN plant}^{-1}$ ) ( $p$ -value=0.003).

Nitrogen accumulation under optimal growth conditions was initiated at different times in the growth cycle depending on the species. For common bean, N accumulation started (tBNA) at 69 to 179 CTU at PAL and 248 to 322 CTU at DAR, which was soon after germination in both cases (Table 1). In contrast, tBNA for faba bean was delayed to an average of 1026 CTU, or only about 200 CTU before flowering (Table 1). Comparisons of the estimates of tBNA from optimal conditions with non-optimal conditions also showed significant differences. There was a delay in tBNA for both stress conditions of higher-temperature for common bean at the PAL site and of Nlr treatment for faba bean (Table 1).

Termination of N accumulation (tTNA) was highly correlated with tBSG in common bean ( $R^2=0.63$ ), and with tTSG in faba bean ( $R^2=0.87$ ). However, the CTU for these stages were offset with tTNA occurring by an average of 222 CTU after tBSG in common bean and 525 CTU after tBSG in faba bean. Based on ANOVA analysis, tTNA was found to be significantly earlier when plants were stressed ( $p\text{-value}<0.001$  for common bean and  $p\text{-value}=0.025$  for faba bean) with tTNA occurring shortly before tTSG by 64 CTU in common bean and by 80 CTU in faba bean.

No significant differences in final NTOT were found among genotypes within the same site or treatment, neither in common bean ( $p\text{-value}=0.37$ ), nor in faba bean ( $p\text{-value}=0.62$ ). Yet, common bean and faba bean genotypes used in these experiments were selected to cover a wide range of morphologies, seed size, and gene pools. Comparing N accumulation dynamics, significant differences were found among genotypes only for common bean at the PAL site and for Ir faba bean. Thus, genetic variability may exist in the dynamic of N accumulation, but it was detected only when differences among genotypes were large compared to the variability in measurements.

N allocation to organs was found to respond significantly to growing conditions only during the seed-filling phase (data not shown). Comparison of the proportion of N content located in leaves, stem, and grain at a given date during seed filling showed that under non-optimal conditions (PAL site and Nir treatment), a higher proportion of N content was located in the seeds, while leaves would contain a lower proportion of N compared to plants grown under optimal conditions (DAR site and Ir treatment). It appeared that, non-optimal conditions accelerated the translocation of N from leaves to seeds, while N dynamic in stems remains unaffected. No difference was found in the allocation of N between leaves and stems before flowering, neither between sites or treatments, nor genotype, for both species.

## Discussion

The objective of this study was to compare the temporal dynamics of N accumulation by several genotypes of two grain legumes. The approach was to examine experimental results using a logistic function to analyze accumulated plant mass and accumulated N through the cropping season. The results of the regression showed the functions (Eq 2 & 3) were appropriate in representing the change in mass and N with CTU (Fig. 1).

Analysis of the results expressed by the logistic equation allowed insight into the beginning and termination of N accumulation in these grain legumes. This study clearly showed that the timing of N accumulation in common bean and faba bean was quite different. While tBNA in common bean occurred shortly after germination and tTNA occurred on an average of 222 CTU after tBSG, in faba bean tBNA was not initiated until nearly flowering and sustained N accumulation well into seed fill (525 CTU after tBSG). These results for faba bean are consistent with the results of Herdina and Silsbury (1990) with glasshouse grown plants in which N accumulation began at flowering and more than half of the accumulation occurred during seed fill. The apparently unique delayed N accumulation pattern of in faba bean seems especially deserving of further physiological investigation to understand its unexpected ability to sustain N accumulation in competition with seed growth.

During vegetative development it has been hypothesized by various authors (Sinclair et al. 2003; Lawlor 2002; Lemaire et al. 2007; Jamieson & Semenov 2000) that N accumulation is sink driven and closely related to leaf expansion and to mass accumulation. The data from these experiments were used to examine this relationship by plotting NTOT ( $\text{gN plant}^{-1}$ ) and GR ( $\text{g CTU}^{-1} \text{plant}^{-1}$ ) against each other. However, before doing this analysis an initial consideration is that since NTOT experimentally includes plant mass in its determination, there may be a linkage between NTOT and GR, which is consistent with the mathematical linkage between GR and NTOT (derivative of [Eq 2] and [Eq 3])



In all experimental situations, the same parametric equations could be fitted for GR and NTOT although parameters varied across species, genotypes, and growing conditions. Sharp acceleration of NTOT increase versus GR appears when parameters  $s_{Ng,t}$  and  $m_{Ng,t}$  (Eq 3) are respectively lower than  $s_{g,t}$  and  $m_{g,t}$ , (Eq 2). That is, N accumulation dynamic is much delayed and NTOT starts increasing steadily only after maximum GR. These results indicate that there is no threshold in GR that triggers the beginning of N accumulation, unless dry matter is accumulating at a very slow rate due to unfavorable conditions.

The differences in GR and NTOT increase resulted in different patterns when GR and NTOT were plotted against each other (Fig 6 and 7). Generally, during the stages before flowering there was approximately a linear relationship between increase in GR and NTOT until reaching maximum GR. Following maximum GR, NTOT continued to increase while GR

decreased to zero. In the case of common bean, the relation between NTOT and GR was approximately linear in the two halves of the cycle.

In the case of Nlr faba bean in which the crop was exposed to quite severe stress (P-PET<250 mm), the relation between GR and NTOT followed a somewhat different pattern for the other cases (Fig 7). First, there appeared to be a threshold in GR of about 0.015 g plant<sup>-1</sup> CTU<sup>-1</sup> before NTOT increase was initiated. NTOT remained nearly constant at the beginning of the cycle, then N accumulation showed a sharp acceleration when GR became maximum (0.01 g plant<sup>-1</sup> CTU<sup>-1</sup> to 0.02 g plant<sup>-1</sup> CTU<sup>-1</sup>, depending on genotype).

### Concluding remarks

It is often suggested that N accumulation by crops is demand driven, and thus directly linked to dry matter accumulation (Hammer et al. 2010; Imsande & Touraine 1994; Bertheloot et al. 2011). Thus, N accumulation from the environment – either from the soil or from the atmosphere for grain legumes – is anticipated to be closely tied to sink size or sink growth rate. While previous studies, e.g. Lemaire et al (2007), showed N accumulation and mass accumulation were correlated, in this study the comparison of accumulation was pushed one step further by calculating and comparing accumulation rates. These results provided strong evidence in both the cool-season and  warm-season legume that while initially N accumulation is closely tied to mass accumulation, N accumulation continued well into the reproductive cycle. The large fraction of N accumulation during seed fill by faba bean is especially contradictory to the widely accepted hypothesis that photosynthate competition stops N accumulation soon after seed filling has started (Amir & Sinclair 1991; Hammer et al. 2010). Previous experimental studies on non-legume crops also support this result: Ruiter & Brooking (1996) showed that in barley, substantial amounts of N can be acquired by crops during seed filling phase. Our findings bring additio evidence about the variability that exists in N accumulation in legumes, both during vegetative and reproductive phases and their representation in mechanistic crop models.

### Acknowledgments

The authors would like to thank the CGIAR CRP Grain Legumes for funding this study. The authors are grateful to Ms S. Ben Sadoun, master student from Montpellier SupAgro and to

the technical staff from IAV Hassan II for their valuable support in acquiring field data on faba bean. Contribution of CIAT and University of Florida in acquiring and analyzing data on bean is greatly acknowledged.

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Table 1. Dates of flowering (tR1, observed in field) beginning of seed growth (tBSG), termination of seed growth (tTSG), beginning of nitrogen accumulation (tBNA), and termination of N accumulation (tTNA), expressed in CTU of faba bean and common bean genotypes growing under different treatments ( I = irrigated, NI = non-irrigated) and at different sites (DAR = Darien, PAL = Palmira). Calculations of tBSG, tTSG, tBNA, and tTNA are based on logistic regression of biomass or total nitrogen accumulation against thermal time.

Species	Treatment	Genotype	tR1	tBSG	tTSG	tBNA	tTNA
Faba bean	I	Fev de fes	1183	1550	2159	1000	2202
		Aguadulce	1305	1518	2075	999	2119
		HBP/SOF/2009	1183	1503	2373	1180	2291
		HBP/SO/06-L4301-4/09	1183	1330	2353	924	2222
	NI	Fev de fes	1183	1366	1954	766	1975
		Aguadulce	1267	1558	1839	1319	1630
		HBP/SOF/2009	1267	1492	1897	1237	1730
		HBP/SO/06-L4301-4/09	1183	1396	1906	1181	1756
Common bean	DAR	CAL 96	511	663	1079	105	863
		Carioca	578	746	914	179	968
		DOR 364	562	653	1094	69	1000
		G 21212	530	706	846	78	909
		ICA Quimbaya	500	750	862	70	918
		SER 118	535	554	1109	159	763
	PAL	CAL 96	428	528	714	248	751
		Carioca	483	605	837	264	822
		DOR 364	486	686	809	299	805
		G 21212	453	556	796	261	821
		ICA Quimbaya	422	497	961	323	718
		SER 118	455	546	914	322	817

## Appendices

### Appendix A : determination of *tBSG* and *tTSG*. Quality of estimations

A non linear regression model was fitted between observed seed dry weight and time of observation in cumulated thermal unit (CTU) (equation A.1).

$$DW_{seed_{g,t}}(CTU) = \frac{A_{DWS,g,t}}{1 + e^{\left(-\frac{CTU - m_{DWS,g,t}}{s_{DWS,g,t}}\right)}} \quad [A.1]$$

where  $DW_{seed_{g,t}}(CTU)$  is the seed dry weight for a genotype  $g$ , in treatment or site  $t$ , at CTU.  $A_{DWS,g,t}$ ,  $m_{DWS,g,t}$ ,  $s_{DWS,g,t}$  are the parameters of the logistic function for each genotype and site. Parameter  $m_{DWS,g,t}$  represents the date (in CTU) when seed growth rate (SGR) is maximum and this date is noted as  $tSGRX$ .  $tBSG$  was calculated as the date, in CTU when  $DW_{seed_{g,t,r}} = 0.05 \times A_{DWS,g,t}$  and  $tTSG$  as the date when  $DW_{seed_{g,t,r}} = 0.95 \times A_{DWS,g,t}$ . Equation 2 was applied to each genotype and each treatment or site for both species.

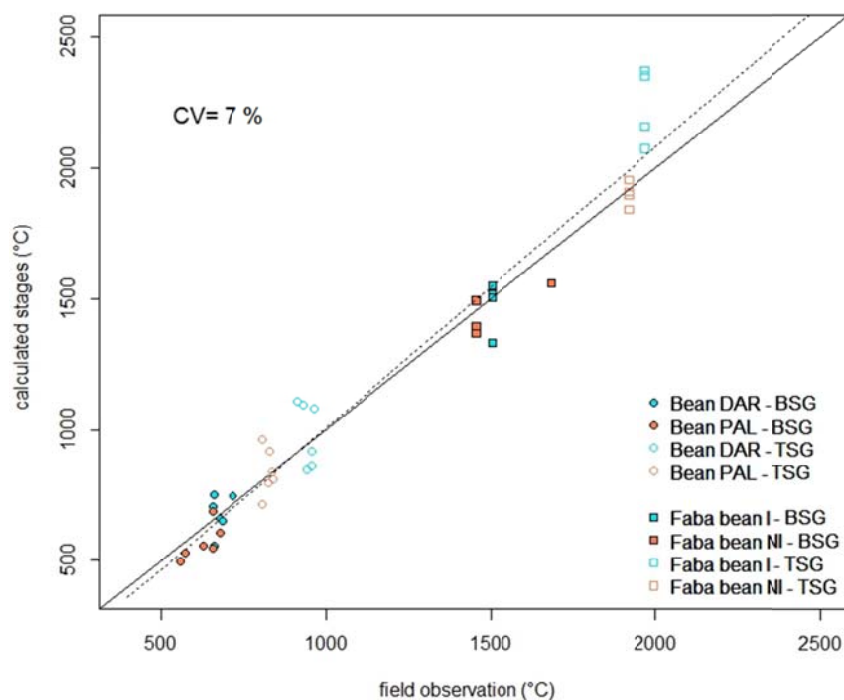


Fig A.: estimated versus observed dates of BSG and TSG for bean in PAL and DAR sites, as well as for faba bean in I and NI treatments. Solid line represents the graph bisector. Dotted line represents the prediction of linear regression between estimated and observed values. Coefficient of variation (CV) of the error of the estimation is equal to 7%.

**Appendix B : adjusted coefficients for dynamic of DW and NTOT.**

Faba bean	DW=f(CTU)			NTOT=f(CTU)			TGR=f(NTOT)						
	A	m	s	mN	AN	sN	A	An	s	E	P	max(NTOT)	max(GR)
Fev de fes	47.11	1631.35	227.04	1597.65	1.40	207.43	47.1	1.40	227.04	1.16	0.91	0.757	0.052
Aguadulce	28.09	1540.44	169.07	1558.94	0.89	190.19	28.1	0.89	169.07	0.90	1.12	0.423	0.042
HBP/SOF/2009	53.70	1708.08	254.46	1728.97	1.79	200.52	53.7	1.79	254.46	0.92	0.79	0.847	0.053
HBP/SO/06-L4301-4/09	41.75	1604.39	265.82	1567.50	1.24	227.42	41.7	1.24	265.82	1.15	0.86	0.670	0.039
Fev de fes	11.26	1235.76	313.78	1368.49	0.37	199.13	11.3	0.37	313.78	0.66	0.63	0.127	0.009
Aguadulce	14.25	1336.93	223.19	1470.89	0.41	45.99	14.3	0.41	223.19	0.55	0.21	0.021	0.016
HBP/SOF/2009	11.00	1330.24	266.38	1480.86	0.34	82.60	11.0	0.34	266.38	0.57	0.31	0.047	0.010
HBP/SO/06-L4301-4/09	16.03	1374.15	258.94	1465.97	0.49	96.18	16.0	0.49	258.94	0.70	0.37	0.137	0.015

Bean	DW=f(CTU)			NTOT=f(CTU)			TGR=f(NTOT)						
	A	m	s	mN	AN	sN	A	An	s	E	P	max(NTOT)	max(GR)
CAL 96	14.76	529.83	105.57	483.85	0.42	128.76	14.8	0.42	105.57	1.55	1.22	0.246	0.035
Carioca	18.28	634.45	101.70	573.43	0.50	134.13	18.3	0.50	101.70	1.82	1.32	0.307	0.045
DOR 364	16.77	585.41	78.74	534.32	0.49	158.14	16.8	0.49	78.74	1.91	2.01	0.287	0.053
G 21212	19.64	610.84	108.02	493.81	0.47	141.12	19.6	0.47	108.02	2.95	1.31	0.325	0.045
ICA Quimbaya	19.60	587.76	102.28	493.93	0.47	143.86	19.6	0.47	102.28	2.50	1.41	0.308	0.048
SER 118	15.51	525.86	83.87	461.07	0.40	102.52	15.5	0.40	83.87	2.17	1.22	0.260	0.046
CAL 96	9.25	526.56	67.07	499.66	0.28	85.42	9.2	0.28	67.07	1.49	1.27	0.164	0.034
Carioca	12.22	567.08	78.57	543.24	0.38	94.76	12.2	0.38	78.57	1.35	1.21	0.216	0.039
DOR 364	15.13	603.88	71.48	551.97	0.40	85.87	15.1	0.40	71.48	2.07	1.20	0.258	0.053
G 21212	13.26	579.94	75.33	541.18	0.42	95.10	13.3	0.42	75.33	1.67	1.26	0.250	0.044
ICA Quimbaya	13.92	543.95	70.52	520.40	0.39	66.99	13.9	0.39	70.52	1.40	0.95	0.227	0.049
SER 118	15.28	635.82	85.82	569.53	0.36	84.11	15.3	0.36	85.82	2.16	0.98	0.249	0.045

Table B: adjusted coefficients for dynamic of DW and NTOT against CTU and for the dynamic of TGR as a function of NTOT. Max(NTOT) and max(TGR) are the coordinate of the curve maximum.

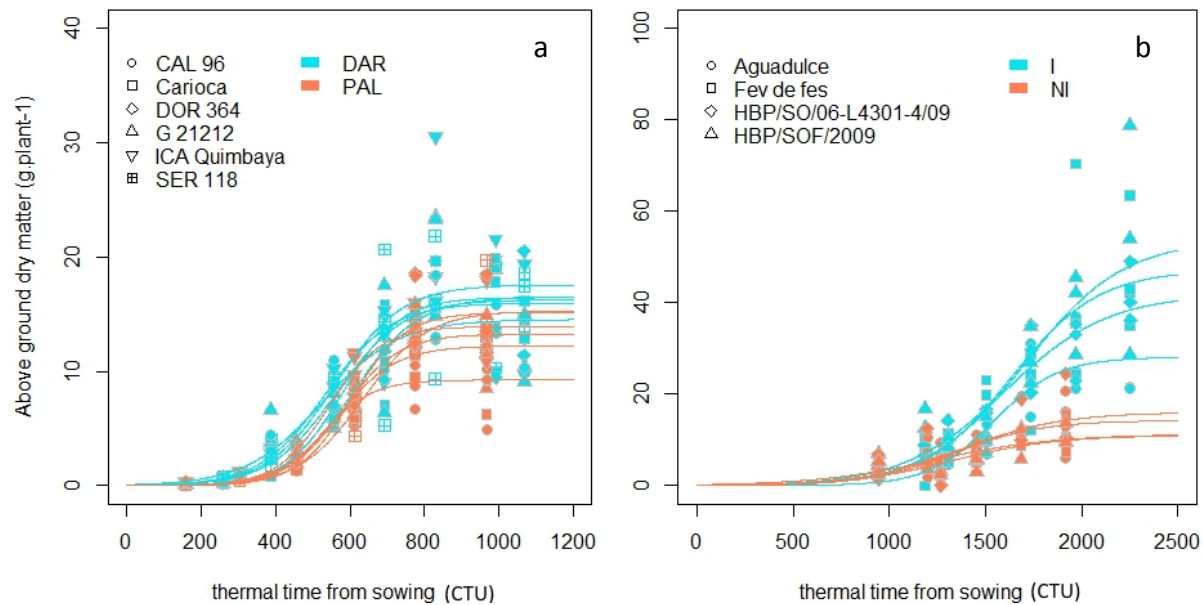


Figure 1. Observed (closed symbols) dry weight per plant ( $\text{g plant}^{-1}$ ) and predicted values of dry weight (solid lines) using the logistic model (Eqn 2) from sowing (CTU) for common bean (a) and faba bean (b) growing under in different sites (DAR = Darien, PAL = Palmira) and under different treatments (I = irrigated, NI = non-irrigated).

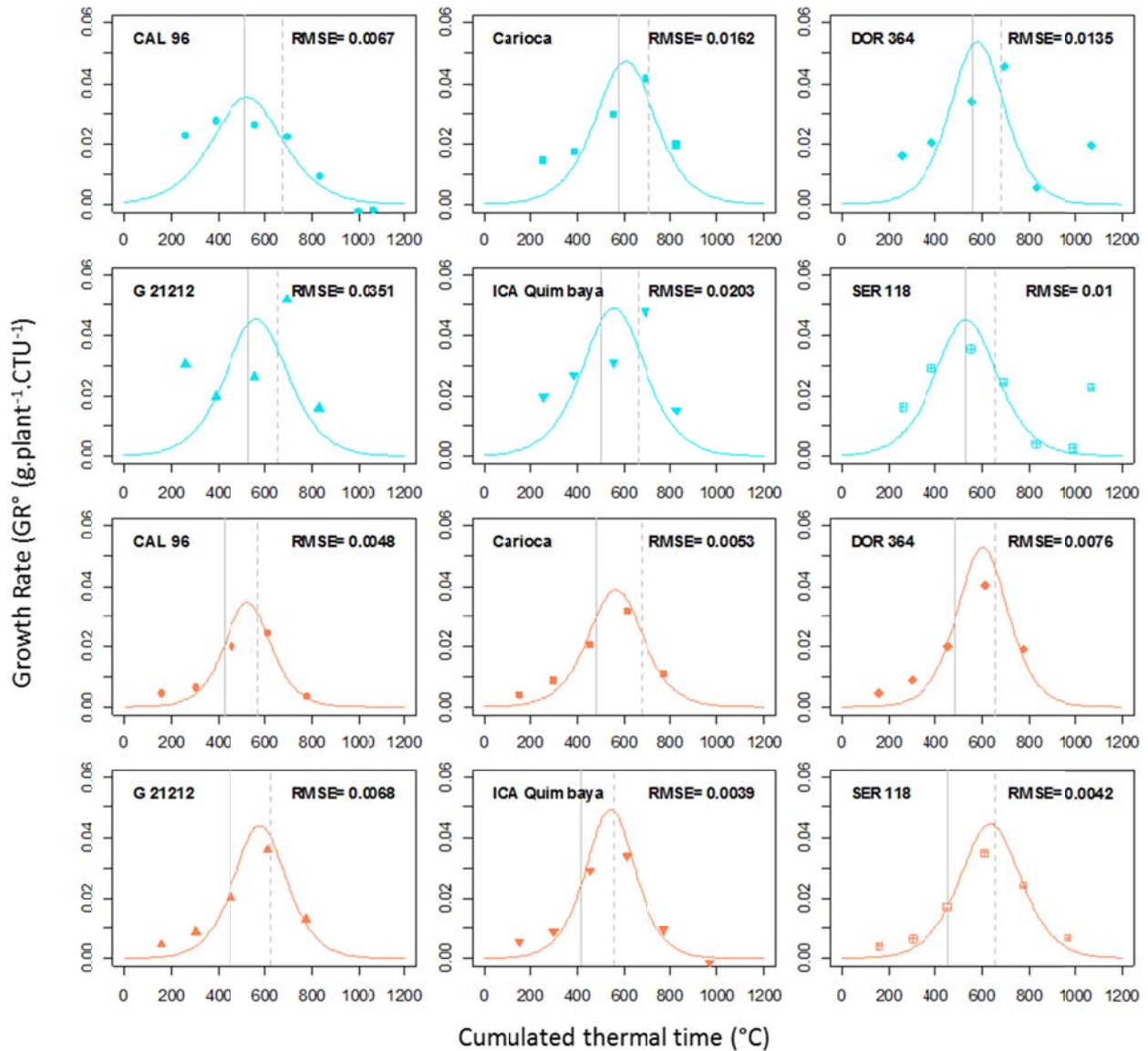


Figure 2. Growth rate (GR) as a function of CTU for common bean genotypes, as estimated by the difference approach (closed symbols) and the derivative approach (solid line) in DAR site (turquoise) and in PAL site (orange). The solid vertical line represents flowering while the dashed vertical line represents beginning of seed growth.

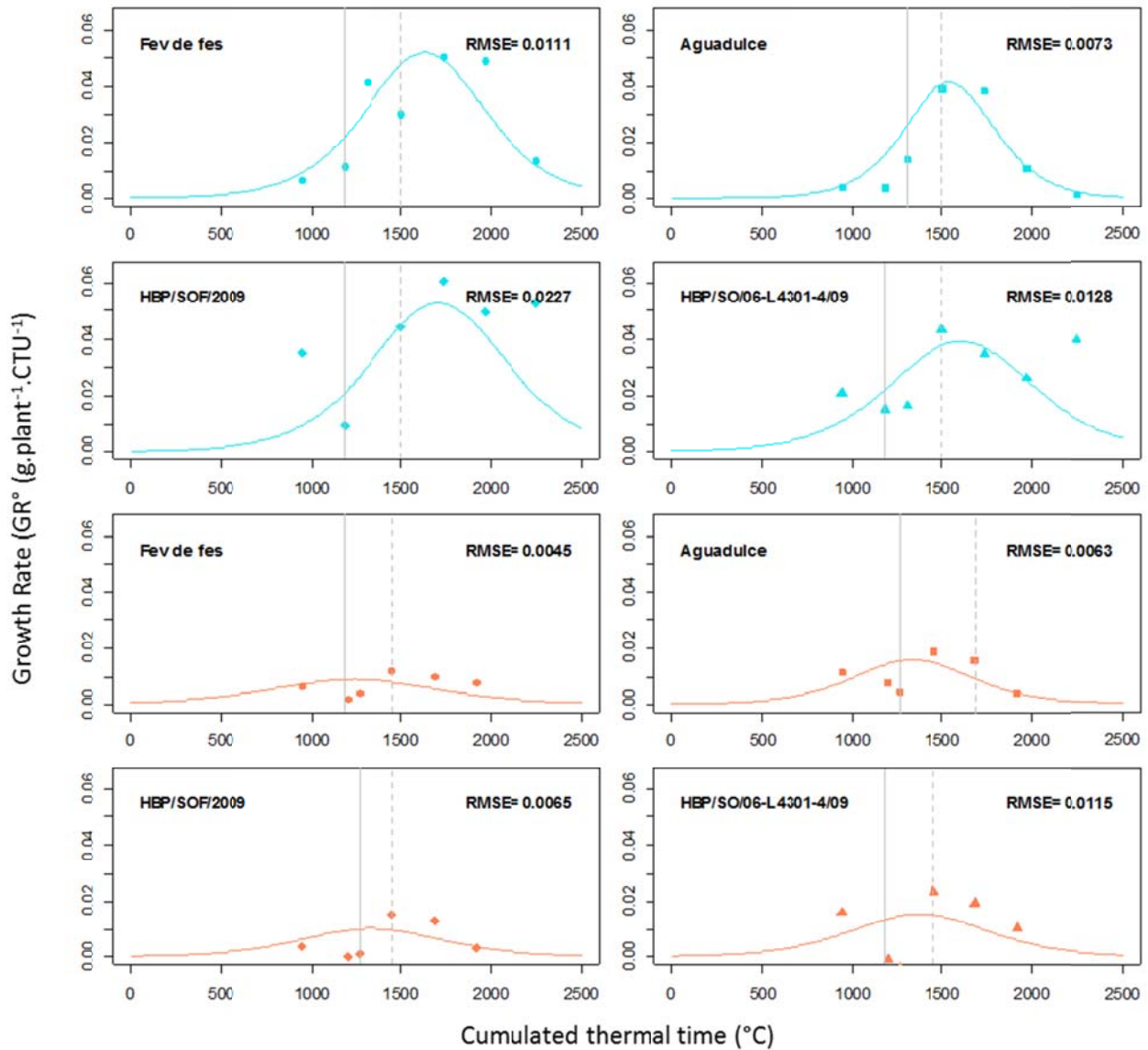


Figure 3. Growth rate (GR) as a function of CTU for faba bean genotypes, as estimated by the difference approach (closed symbols) and the derivative approach (solid line) for irrigated (turquoise) and non-irrigated conditions (orange). The solid vertical line represents flowering while the dashed vertical line represents beginning of seed growth. The solid vertical line stands for flowering time while the dashed vertical line shows beginning of seed growth.



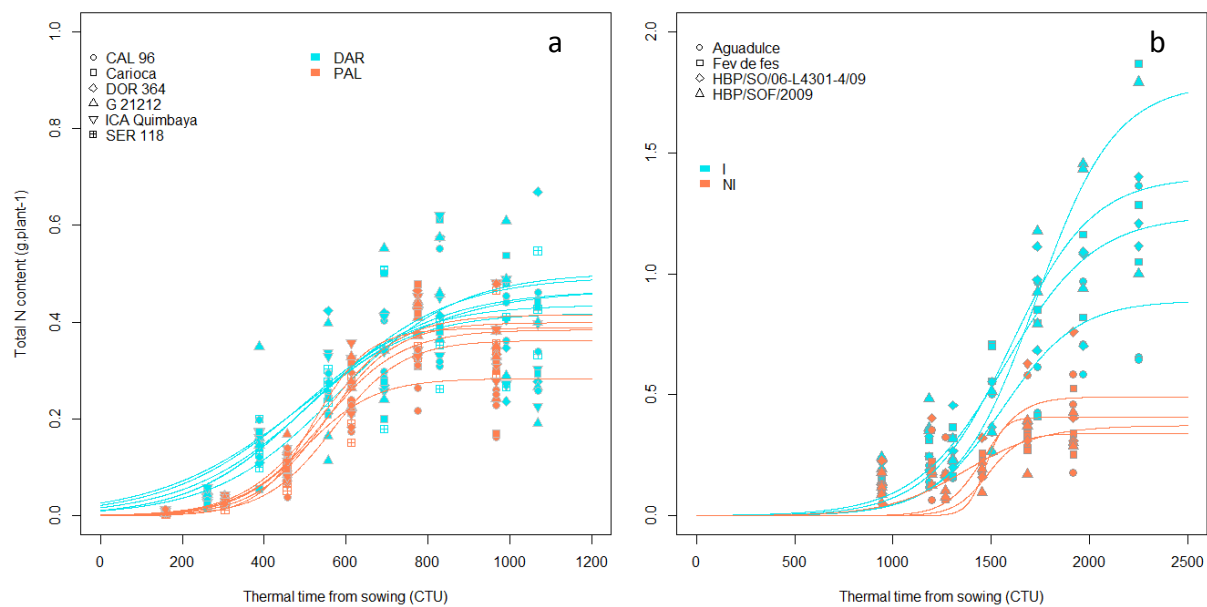


Figure 4. Observed (closed symbols) nitrogen accumulation (g plant<sup>-1</sup>) and predicted values of N accumulation (solid lines) using the logistic model (Eqn 3) as a function of CTU for (a) common bean grown at DAR and PAL and (b) faba bean grown under Ir and NIr water treatments.

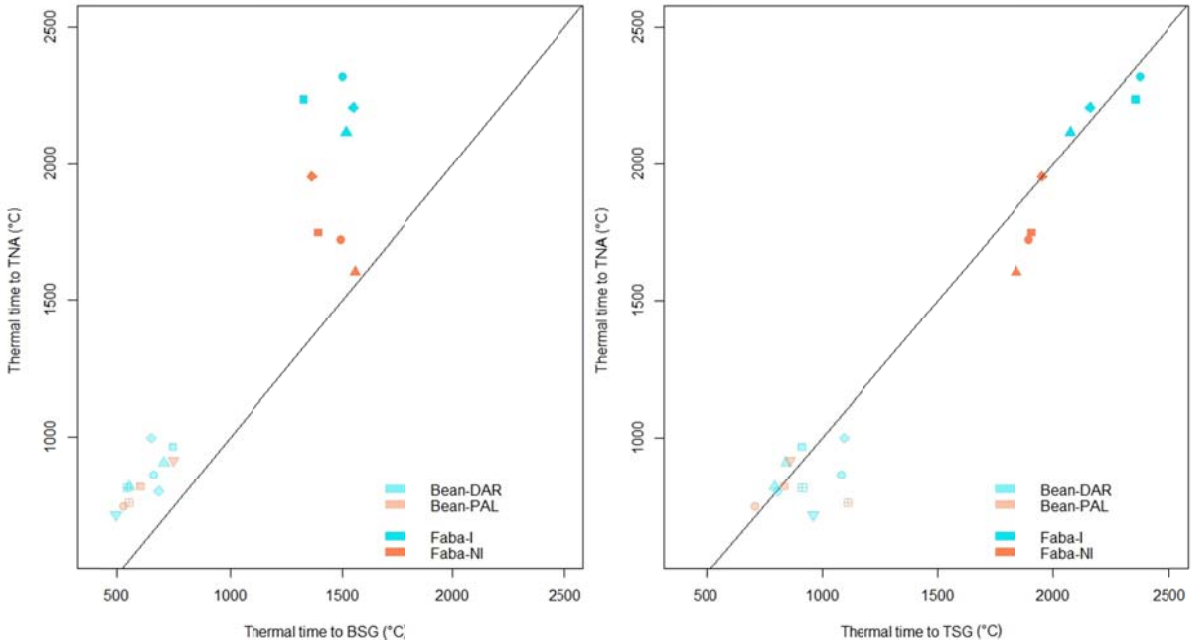


Figure 5: Plot of CTU at termination of N accumulation (tTNA) versus (a) CTU at beginning of seed growth (tBSG) and (b) CTU at termination of seed growth (tTSG). The solid line represents the 1:1 correspondence.

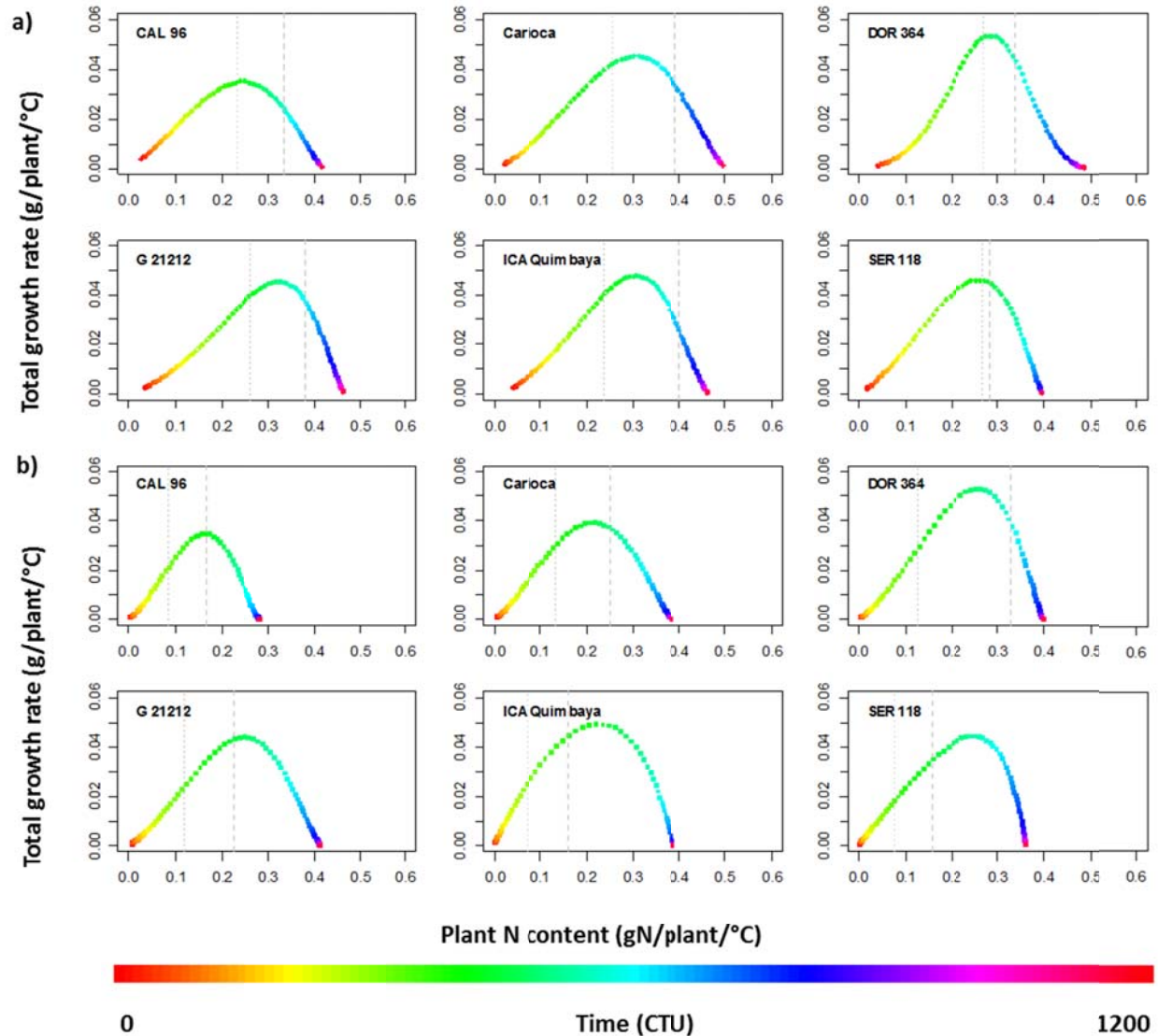


Figure 6. Growth rate (GR) vs. N accumulation rate (NAR) for common bean at (a) DAR site and (b) PAL site. These rates are based on derivatives of the logistic models fitted to observed total dry weights and total N content. Point colors refer to time from sowing: red-yellow points correspond to beginning of cycle while blue-purple points correspond to the end of the cycle. The dotted vertical line is flowering while the dashed vertical line is beginning of seed growth.

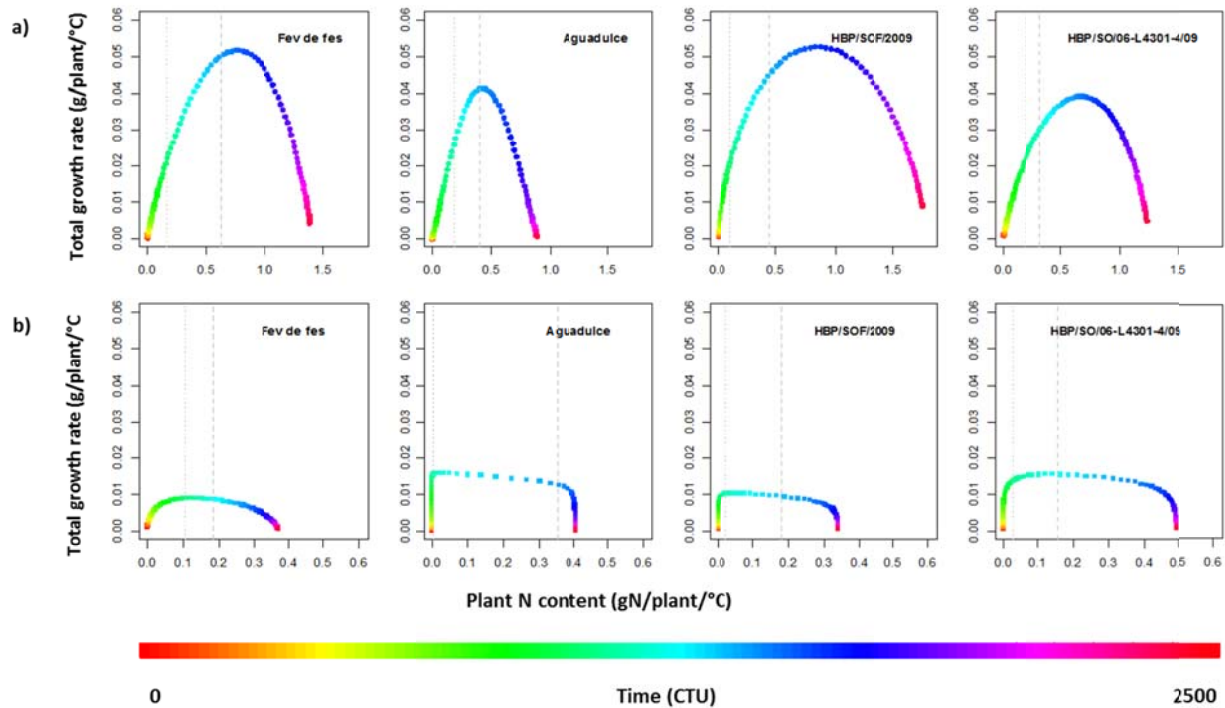


Figure 7. Growth rate (GR) vs. N accumulation rate (NAR) for faba bean for (a) irrigated and (b) non-irrigated conditions. These rates are based on derivatives of the logistic models fitted to observed total dry weights and total N content. Point colors refer to time from sowing: red-yellow points correspond to beginning of cycle while blue-purple points correspond to the end of the cycle. The dotted vertical line is flowering while the dashed vertical line is beginning of seed growth.