Differential tolerance to lime-induced chlorosis of N₂-fixing common bean (*Phaseolus vulgaris* L.)

Abdelmajid Krouma[*], Tarek Slatni, and Chedly Abdelly

Laboratory of Plant Adaptation to Abiotic Stress, Centre of Biotechnology, Borj Cedria Ecopark, BP 901, Hammam Lif 2050, Tunisia, Tel. +216-79-412-848, Fax. +216-79-412-638, Email. krm_abdel@yahoo.fr

Abstract

Common bean (*Phaseolus vulgaris* L.) is one of the most important protein-rich crops in the Mediterranean regions. However, about one third of the planet's cultivated land is a calcareous soil in which iron availability is very low. Studies on the development of common bean varieties adapted to environmental constraints as existing in these regions are limited. This study was initiated to evaluate growth, nodulation and nitrogen and Fe nutrition in three N₂-fixing common bean varieties. Seedlings were inoculated with an efficient strain of *Rhizobium tropici* CIAT 899 and were grown on non-calcareous or calcareous soil. In comparison to Coco blanc and BRB17, Flamingo develop a high level of whole plant and nodule growth and maintained an adequate photosynthetic activity and symbiotic nitrogen fixation on calcareous soil. The Fe use efficiency for nodule growth and nitrogen accumulation discriminate clearly Flamingo from the other varieties.

Keywords: Common bean, genotypic variability, lime-induced chlorosis, Fe use efficiency

1. Introduction

Iron, an essential nutrient, is not readily available in the soil. Although abundant, iron is found mainly as stable, insoluble oxyhydroxide polymers that effectively limit free Fe(III) to an equilibrium concentration of approximately 10^{-17} M at neutral pH, a value far below that required for the optimal growth of microbes or plants (estimated to be 10^{-8} to 10^{-6} M, Guerinot and Yi, 1994). In calcareous soils which constitute more than 30% of the planet's cultivated land (Wallace and Lunt, 1960), the soil solution does not provide more than 10% of the plant requirements for Fe (Mortvedt, 1991). Lime-induced chlorosis affects many annual crops and perennial plants growing on calcareous soils. The total Fe content in these soils is high but the available fraction for the plant is insufficient. This is caused by the very low solubility of iron oxides at the alkaline pH conditions that are buffered by the presence of bicarbonate in these soils (Lindsay and Schwab, 1982). Ammari and Mengel (2006) indicate that Fe concentration found in calcareous soil was high enough to meet the plant's demand and prove that Fe chlorosis on such soils is not a question of the Fe availability in the soil. 50–90% of the Fe found in the soil solution was complexed by organic molecules and the percentage of organic Fe complexes was correlated with the soil pH.

Fe is known to be essential for many physiological and biochemical processes such as: photosynthesis, respiration, DNA synthesis and N₂ fixation. The legume-rhizobia symbiosis is particularly sensitive to Fe deficiency (Tang et al., 1990). The latter can limit root nodule bacteria survival and multiplication, as well as host-plant growth, nodule initiation, development and function. In particular, Fe is required for some key proteins like nitrogenase which represents ca. 10% of total protein in bacterial cells, and leghaemoglobin which represents ca. 20% of total protein in infected plant cells (Moreau et al., 1995). Bavaresco and Poni (2003) demonstrated that the reduction of biomass in susceptible plants of *Vitis vinifera* L. is related to a reduced root growth due to the presence of bicarbonate in the soil and to a lower photosynthesis rate which also depends on the reduced leaf area per plant and chlorophyll concentration encountered under iron stress conditions.

However, differences among species and genotypes in plant response to Fe deficiency have been reported (Ellsworth et al., 1997, 1998). Froehlich and Fehr (1981) demonstrated that soybean seed yield decreased by 20% per unit of visual leaf to Fe deficiency chlorosis (FeDC) when grown on calcareous soils. Common bean seed yield of 15

[*]*The author to whom correspondence should be sent.*

genotypes varied from 300 to 2600 kg per hectare under Fe deficiency (Zaiter and Coyne, 1992). Ksouri et al. (2007) demonstrated in grapevine that lime-induced Fe shortage is genotype dependent, the growth parameters and leaf chlorophyll content were differently affected, depending on both genotype and bicarbonate dose. For symbiotic legumes, nodule initiation was less sensitive to Fe deficiency in *Arachis hypogaea* than in *Lupinus angustifolius* (Tang et al., 1991). Some genotypes of *Lens culinaris* and *Cicer arietinum* differed in their ability to nodulate and to fix N_2 on calcareous soil. Tolerant genotypes showed a better Fe uptake efficiency and a preferential allocation of this nutrient towards the nodules (Tang et al., 1992). In hydroaeroponic culture, Krouma et al. (2006) demonstrated that iron deficiency decrease nodule initiation and development in common bean, and the relative tolerance of ARA14 genotype was linked to its high Fe use efficiency for nodule growth and nitrogen fixation.

The exploration of genotypic variability in the responses of common bean to Fe deficiency may allow identification of physiological and/or biochemical tools to screen tolerant varieties and to improve the productivity of this crop in calcareous soils. Such soils are very frequent in fertile regions of the Mediterranean basin, particularly Tunisia.

The aim of the present study was to evaluate the tolerance of some common bean varieties to Lime-induced chlorosis, and to identify some parameters involved in their differential response to Fe deficiency, which could be used in further screening program within N_2-dependent *Phaseolus vulgaris* L.

2. Material and Methods

Soil and plant material

Three common bean varieties were used, Coco blanc, Flamingo and BRB17. Seeds were disinfected with 2% hypochlorite calcium solution and sown individually in 1 kg pots filled with a fine, mixed calcareous (sampled in the region of Hammamet, Tunisia) or non-calcareous soil (sampled in the region of Soliman, Tunisia), in the glasshouse under natural light. The main soils characteristics are given in Table 1.

One ml of *Rhizobium tropici* CIAT 899 inoculum containing approximately 10^{10} cells per ml was added directly in the soil to ensure plant nodulation. This inoculation was renewed after one week and two weeks after sowing to guarantee the presence of the *Rhizobium* strain in the plantlets rhizosphere during the first stages of growth. The *Rhizobium tropici* strain was provided by the Laboratory of Legume-Microorganisms Interaction, Center of Biotechnology, Borj Cedria Technopark. Soil moisture was maintained near field capacity using tap water. At the beginning of flowering, 45 days after germination, plants

were harvested, and separated into leaves, stems, roots and nodules. Nodulated roots were soaked in 0.01 M $CaCl_2$ (Tang et al., 1990) solution and washed thoroughly and successively in 3 baths of ultra-pure water in order to avoid the contamination of roots and nodules with iron and elements from the soil.

Leaf area and chlorophyll concentration

Leaf area was measured using a Portable Area Meter, LI-COR 3000A. Chlorophyll concentration was determined according to Arnon et al. (1949). The second leaf from the plant top was used.

Table 1. Main characteristics of soils used to test sensitivity to lime.

	Non-calcareous	Calcareous
Sand (%)	77.50	62.00
Silt (%)	18.20	22.50
Clay (%)	4.00	14.00
pH	7.70	8.50
Total carbonates (%)	7.30	38.00
Active lime (%)	2.50	13.50
Organic matter (%)	1.04	0.76
C (%)	0.60	0.61
N (mg/g)	1.87	1.64
P (g/kg)	0.24	0.19
Extractible Fe (mg/kg)	33.20	8.80
K (mg/kg)	95.00	82.00

Chemical analysis

Samples of fresh material were dried at 70°C for 72 h and ground to fine powder using a grinder with agate pots. Dry nodules were previously numbered and separated into three classes, diameter < 1 mm, 1 mm < diameter < 2 mm, and diameter > 2 mm. Total nitrogen was determined by the Kjeldahl method. Quantification of active Fe was performed according to Köseoglu and Acikgöz (1995).

Statistical analysis

Analysis of variance (ANOVA), using the AV1W MSUSTAT program with orthogonal contrasts and mean comparison procedures, was performed to detect differences between treatments. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD) (P<0.05) (Slama et al., 2007).
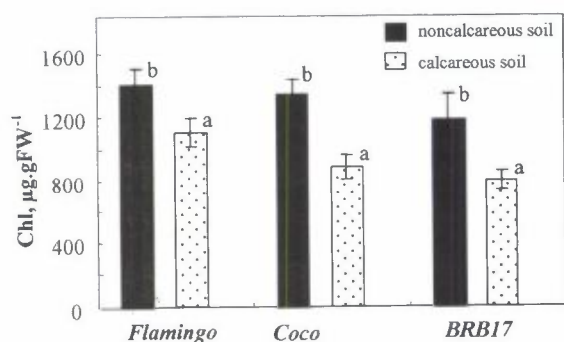
Figure 1. Chlorophyll concentration in leaves of common bean varieties cultivated in calcareous and non-calcareous soils. Vertical bars represent ± standard errors of means of 10 replicates.
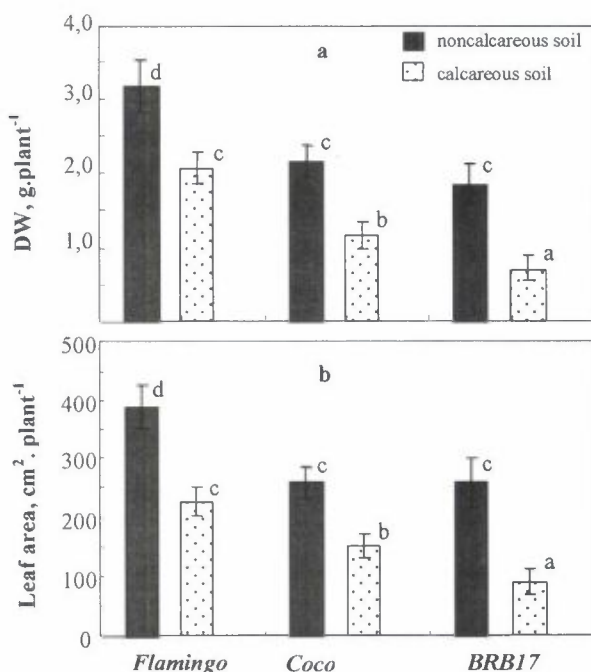

Figure 2. Host-plant growth (a) and leaf area (b) of common bean varieties cultivated in calcareous and non-calcareous soils. Vertical bars represent ± standard errors of means of 10 replicates.
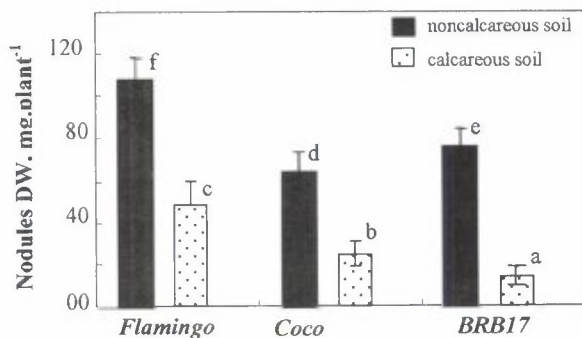

Figure 3. Nodule biomass production of common bean varieties cultivated in calcareous and non-calcareous soils. Vertical bars represent ± standard errors of means of 10 replicates.

3. Results

Plant morphology and chlorophyll content

Independently of the varieties, plants cultivated on calcareous soil expressed the symptoms of iron chlorosis on young leaves 10 days after the germination (BRB17). Moreover, the BRB17 variety expressed these symptoms faster than the other ones, Coco blanc and Flamingo, i.e. after 13 and 15 days, respectively.

All plants cultivated on calcareous soil exhibited a significant decrease of chlorophyll content as compared to those cultivated on non-calcareous one (Fig 1). This effect can explain the visual symptoms of chlorosis expressed on young leaves of plants cultivated in calcareous soil. However, Flamingo maintains the most photosynthetic leaves on calcareous soil, as compared to Coco blanc and BRB17. Chlorophyll concentration decreased by 21% in Flamingo and 37% in Coco blanc and BRB17.

Host-plant and nodule growth

The calcareous soil reduced host-plant growth as compared to the non-calcareous soil (Fig. 2a). Flamingo was the less affected by 35% of dry weight decreasing against 46 and 62%, respectively in Coco and BRB17 varieties. Calcareous soil decreased also leaf expansion (Fig. 2b). Leaf area measures show the same schema of variation as dry weight production, Flamingo remain the less affected with 41% of leaf area decreasing against 65% in BRB17.

Other the host-plant growth, nodule biomass decreased markedly in plants cultivated on calcareous soil (Fig. 3). This decrease was less pronounced in Flamingo variety (–55%), than Coco blanc (–64%) and BRB17 (–83%). However, the numbering of the nodules shows that on calcareous soil nodule initiation was inhibited. In fact, the total number of nodules decreased significantly in calcareous soil as compared to the non-calcareous one (–22% in Flamingo versus –38%, and –42% in Coco blanc and BRB17, respectively) (number in Fig. 4).

Moreover, Fig. 4 shows that the proportion of big nodules (diameter of dry nodules >2 mm) decline from 22% in non-calcareous soil to 12% in calcareous soil in Flamingo variety. In the other varieties, this class of nodule disappears in calcareous soil. The proportion of modest nodules (1 mm < diameter of dry nodules < 2 mm) was maintained in Flamingo (52% of the total number in the two soils) and decline in Coco blanc from 70% in non-calcareous soil to 52% in calcareous soil and in BRB17 from 59% in non-calcareous soil to 39% in calcareous soil. In contrast to these variations, calcareous soil increased the proportion of small, no functional, nodules (diameter of dry nodules < 1 mm), particularly in BRB17 in which this class of nodule represent 66% of the total nodule number

Table 2. Fe concentrations in common bean tissues cultivated on two Tunisian soils.

Fe ($\mu g.g^{-1}$ DW)		Leaves	Roots	Nodules
Flamingo	Calcareous soil	312.70 ± 28.70	563.40 ± 41.60	$1,195.20 \pm 121.30$
	Non-calcareous soil	392.50 ± 18.82	590.10 ± 56.55	$1,370.60 \pm 135.20$
Coco	Calcareous soil	286.80 ± 24.69	527.30 ± 42.16	971.00 ± 142.10
	Non-calcareous soil	335.80 ± 34.22	576.00 ± 83.20	$1,114.00 \pm 111.30$
BRB17	Calcareous soil	273.20 ± 9.07	482.00 ± 62.37	974.50 ± 117.60
	Non-calcareous soil	355.90 ± 29.76	567.00 ± 70.11	$1,195.30 \pm 152.30$

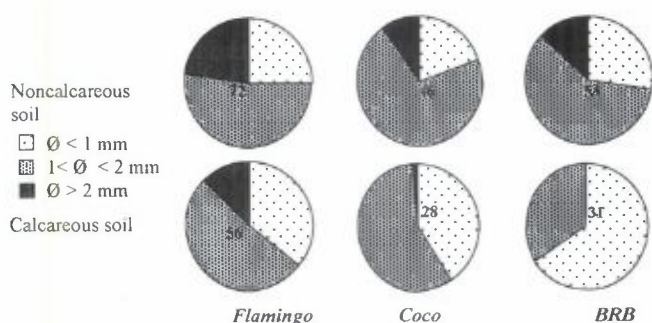Figure 4. Effect of lime-induced iron deficiency on the small (diameter of dry nodules < 1 mm), modest (1 mm < diameter of dry nodules < 2 mm) and big (diameter of dry nodules > 2 mm) nodules distribution. Total nodule number given as numbers in histograms, means of 10 replicates.
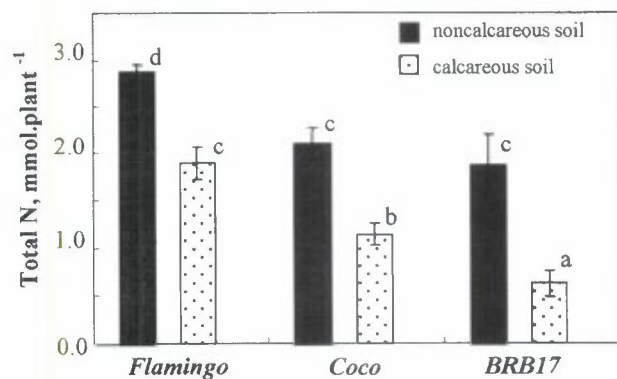

Figure 5. Effect of lime-induced iron deficiency on nitrogen accumulation (mmol.plant^{-1}). Vertical bars represent ± standard errors of means of 10 replicates.

Figure 6. Relationship between nodule growth (mg DW.plant^{-1}) and quantities of iron allotted to these organs ($\mu g.plant^{-1}$) (a) and between total nitrogen accumulated (mmol.plant^{-1}) and quantities of iron allotted to nodules ($\mu g.plant^{-1}$) (b). Vertical and horizontal bars represent ± standard errors of means of 10 replicates.

(Fig. 4). These results confirm the inhibition of nodule development in calcareous soil.

Nitrogen and iron content

According to varieties and soils, N concentration was the highest in nodules, root and shoot organs show comparable concentrations of nitrogen (not shown).
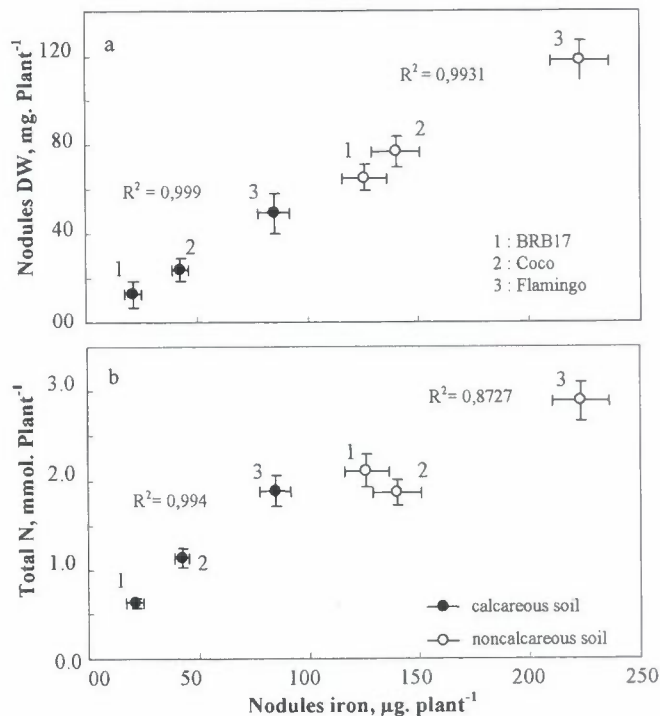
The quantities of nitrogen accumulated during the experiment were estimated from the N accumulation in all organs (Fig. 5). Results in Fig. 5 show that the quantities of total N were the highest in Flamingo independently of the soil. However, calcareous soil decreased significantly N accumulation as compared to non-calcareous one. This decrease was less important in Flamingo (–34%) than Coco blanc (–46%) and BRB17 (–67%).

The quantification of active Fe (Table 2) demonstrates that nodules are the organs with the highest Fe concentrations, leaves were the organs with the lowest Fe concentrations. As compared to non-calcareous soil, all tissues of plants cultivated on calcareous soil show lower
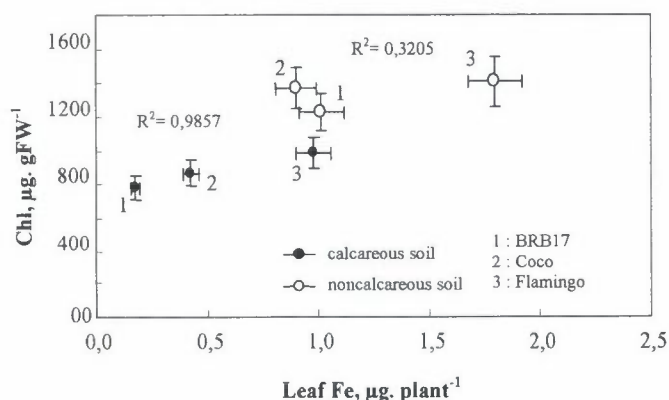
Figure 7. Relationship between chlorophyll concentration (μg.gFW^{-1}) and quantities of iron allotted to leaves (μg.plant^{-1}). Vertical and horizontal bars represent $\pm$ standard errors of means of 10 replicates.

Fe concentrations. However, the calculation of the quantities of Fe allocated to nodules demonstrates that Flamingo allocate more Fe to these symbiotic organs than the other two varieties (Fig. 6a, b). Fig. 6a shows a positive linear regression of nodule mass as a function of nodules Fe content suggesting that growth of these organs is highly dependent on their Fe content. Furthermore, Fig. 6b shows a closely relationship between total nitrogen accumulation in plant and Fe content of nodules. Symbiotic nitrogen fixation (SNF) seems to be the origin of the N differences between varieties cultivated on calcareous soil.

4. Discussion

In the present study, the cultivation of common bean in a calcareous soil induce leaf chlorosis characteristic of iron deficiency. Chlorophyll concentration was also decreased in this soil as compared to non-calcareous one. In fact, iron is indispensable element for chlorophyll and carotenoids biosynthesis (Terry and Zayed, 1995; Morales et al., 1990; Thoiron et al., 1997), the photosynthesis (Rutherford, 1985; Malkin, 1987; Sandmann, 1985) as well as the metabolism of plastidial proteins (Spence et al., 1991) and therefore, the induced iron chlorosis and the decrease of chlorophyll concentration observed in this study can be explained by a drastic decrease of iron availability for these organs. In addition, many other studies explain the decrease of chlorophyll concentration in chlorotic leaves by the increase of the chlorophyllase activity, enzyme responsible for the chlorophyll degradation, under iron deficiency (Almela et al., 1983; Rodriguez et al., 1987; Fernandez-Lopezes et al., 1991). Nevertheless, Fig. 7 shows that in calcareous soil a high positive correlation exist between chlorophyll concentration and leaf iron content ($R^2 = 0.98$). The tolerant variety Flamingo allocates more iron to these

organs and produces more chlorophyll. These results are in accordance with those of Ksouri et al. (2007) on grapevine which demonstrates that lime-induced Fe shortage is genotype dependent, the growth parameters and leaf chlorophyll content were differently affected, depending on both genotype and bicarbonate dose.

Host-plant and nodule growth as well as nodule number were also significantly decreased in calcareous soil. Flamingo variety maintains an important performance of plant growth and nodulation as compared to the other varieties Coco blanc and BRB17. This genotypic variability was found hydroaeroponically in common bean subjected to iron deficiency (Krouma et al., 2003; 2006). Zaiter and Coyne (1992) demonstrated that common bean seed yield of 15 genotypes varied from 300 to 2600 kg per hectare under Fe deficiency and confirms the differences among species and genotypes in plant response to Fe deficiency. Furthermore, our results show that lime-induced iron deficiency reduced significantly nodule initiation and growth. Such results are in agreement with those of Tang et al. (1990) (in lupine), Rai et al. (1984) (in lentil) and Krouma et al. (2006) (in common bean). These authors demonstrated that Fe deficiency reduced nodule biomass and number. O'Hara et al. (1988) observed in peanut the same number of nodules initiated in plants subjected to iron deficiency and plants sprayed with iron, and suggest that the first stages of nodule development are more sensitive to iron deficiency than nodule initiation.

The major result in this work is the significant difference in the symbiosis tolerance to iron deficiency between Flamingo and the other two varieties, Coco blanc and BRB17. Such a genotypic variation in response of symbiotic common bean to Fe deficiency confirms previous intraspecific variations observed in green bean (Hemantaranjan and Garg, 1986), chickpea (Rai et al., 1982), lentil (Rai et al., 1984), peanut (O'Hara et al., 1988) and lupine (Tang et al., 1990).

The quantification of accumulated nitrogen demonstrated that this parameter was significantly reduced in calcareous soil as compared to non-calcareous one (Fig. 5). The performance of Flamingo was also maintained and this variety accumulates almost two times and three times more nitrogen than Coco blanc and BRB17, respectively. Krouma et al. (2006) demonstrated hydroponically the existence of a closely relationship between nodule growth and nitrogen fixation. In the current study, Flamingo develops more nodule biomass (Fig. 3), size and number (Fig. 4) than the other two varieties and therefore fixes more nitrogen. We suggest that the high symbiotic performance of Flamingo is the origin of this high nitrogen accumulation as compared to Coco blanc and BRB17. In fact, Slatni et al. (2008) demonstrated that Flamingo possess a higher capacity of symbiotic nitrogen fixation than Coco blanc cultivated hydroaeroponically in an N-free medium.

Table 3. Fe use efficiency for nodule growth (FeUE DW), [mg nod DW/ nod Fe-concentration (μg Fe. mg DW^{-1})] and for nitrogen accumulation (FeUE NA), [Total N (mmol.$plant^{-1}$)/ nod Fe-concentration (μg Fe. mg DW^{-1})] in two different soils.

		FeUE DW	FeUE NA
Flamingo	Calcareous soil	28.27 ± 1.88	1.09 ± 0.08
	Non-calcareous soil	57.13 ± 2.80	1.52 ± 0.11
Coco	Calcareous soil	13.54 ± 0.99	0.64 ± 0.03
	Non-calcareous soil	33.50 ± 1.99	1.09 ± 0.08
BRB17	Calcareous soil	7.94 ± 0.75	0.38 ± 0.04
	Non-calcareous soil	42.30 ± 2.55	1.03 ± 0.13

The most causal differences between the contrasting varieties reside at the level of iron allocation to nodules which was twice as high in Flamingo as in Coco blanc and four times as high in Flamingo as in BRB17 (Fig. 6a, b). This may be the major cause of the 2 times and 4 times higher nodule growth in Flamingo as compared with Coco blanc and BRB17, respectively, and higher tolerance of Flamingo to lime-induced iron deficiency. In fact, our results suggest that under Fe deficiency, nodule development depends closely on their Fe supply. Flamingo, which allocated more Fe to its nodules, expressed the highest growth of these organs and fix more nitrogen. An adaptation similar to that of Flamingo has been observed in such species as lentil and chickpea on calcareous soil (Tang et al., 1992). In fact, Fig 6a shows a closely positive relationship between nodule growth and their iron content. Furthermore, the same relationship was observed between total nitrogen and nodule iron content (Fig. 6b). Our results suggest that, in a first time, the tolerance of Flamingo to lime-induced iron deficiency is linked to its capacity to allocate more iron to its leaves to maintain their photosynthetic activity and to its nodules to maintain their growth and SNF. Some physiological responses can be implied in Fe mobilisation such as rhizosphere acidification and Fe^{3+}-reduction activity. In a previous study (Krouma et al., 2003), we demonstrated that some tolerant varieties of common bean are able to acidify their external medium and induce significant Fe^{3+}-reduction activity.

In order to investigate the other parameters implied in the performance of Flamingo variety, we calculated the Fe use efficiency for nodule growth (FeUE DW) and nitrogen accumulation (FeUE NA) (Table 3). The first parameter is defined as the ratio of nodule biomass (mg DW) to nodule Fe-concentration (μg Fe (mg $DW)^{-1}$). This ratio is considered as a more reliable estimate of nutrient use efficiency because it takes into account the nutrient concentration together with plant (or organ) growth (Vadez and Drevon, 2001; Krouma and Abdelly, 2003). The second parameter is defined as the ratio of assimilated nitrogen (mmol.$plant^{-1}$) to nodule Fe-concentration (μg Fe (mg $DW)^{-1}$) (Krouma and Abdelly, 2003). Obtained results

(Table 3) show that FeUE DW decreased in calcareous soil as compared to non-calcareous one in all varieties. This parameter was decreased by 51% in Flamingo, 60% in Coco blanc and 81% in BRB17. Nevertheless, Flamingo maintains a high efficiency in calcareous soil as compared to the other varieties. FeUE DW was 2 times more important in Flamingo than Coco blanc and 3 times more important in Flamingo than BRB17 cultivated in calcareous soil. The FeUE NA was also decreased in all varieties cultivated in calcareous soil as compared to non-calcareous one. This decline was estimated to 28%, 41% and 63% respectively in Flamingo, Coco blanc and BRB17. Nevertheless, Flamingo remains the most efficient variety. FeUE NA was 1.7 and 2.8 times more important in Flamingo than Coco blanc and BRB17, respectively.

It appears clearly that these parameters discriminate the three common bean varieties and Flamingo appeared as the most efficient variety. This finding gives as a new explanation of Flamingo performance in a limiting iron availability condition like calcareous soil. In fact, our results suggest that the tolerance of Flamingo was linked to two responses: firstly a better ability to allocate iron to shoots to maintain photosynthetic activity and to nodules to maintain their growth and nitrogen fixation; and secondly, to its efficiency of iron use. Flamingo showed a higher Fe use efficiency for nodule growth and nitrogen accumulation. These parameters seem to be a useful test for tolerant legumes screening.

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