

# Leaf Photosynthetic Metabolism and N<sub>2</sub> Fixation at the Flowering Stage in Three Genotypes of Cowpea [*Vigna unguiculata* (L.) Walp.]

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## Abstract

Biological nitrogen fixation (BNF) in cultivars of cowpea is not yet understood. The hypothesis proposed by this study is that lack of basic technology, including sufficient mineral nutrition, combined with periods of water shortage during the crop cycle leads to insufficient photosynthetic metabolism in the flowering stage in nodulated plants. Two experiments were conducted in northeastern Brazil, one under field conditions and another in a greenhouse at near optimal conditions. Two of the principal cultivars selected for northeastern (Mulato and Gurguéia) and one for northern Brazil (Milênio) were evaluated regarding physiological, biochemical and biometric variables between the late vegetative and early reproductive stages under mineral nutrition and BNF treatments. Gas exchanges, chlorophyll fluorescence and leaf contents of soluble sugars, amino acids, proteins and chlorophyll of inoculated plants were similar to plants fertilized with mineral nitrogen, in the three cultivars in both experiments, with emphasis on Gurguéia. Leaf nitrogen concentrations and the number and dry weight of nodules were higher in Mulato and Gurguéia compared to Milênio. Milênio and Gurguéia under BNF showed higher total dry weight compared to controls; however, the number of pods per plant was higher in inoculated plants compared to controls. In conclusion, based on these results under non-stressful conditions, cultivars selected for the northeastern region inoculated with an effective rhizobia strain and receiving a mild dose of mineral nitrogen can ensure development similar to that verified in plants fertilized only with correct nitrogen fertilization.

**Keywords:** Biological nitrogen fixation, Mineral nutrition, Photosynthesis, Semiarid

## 1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] and others beans are the main source of protein for poorer populations living in the semiarid region, cowpea is able to grow in saline and low fertility soils and tolerates irregular water supply (Pimentel and Hébert, 1999; Figueiredo et al., 2007; Valdez-Pérez et al., 2011). Furthermore, it has a strong ability to fix atmospheric nitrogen through symbiosis with rhizobia strains (Neves et al., 1982; Pelegrin et al., 2009). It is a good option for crop rotation, because it enriches the soil with nitrogen (Franzluebbers et al., 1994).

Legumes and rhizobia is an example of intensively studied biological association (Neves et al., 1982; Senanayake et al., 1987; Howard and Rees, 1996; Graham and Vance, 2000; Kaschuk et al., 2010) and its benefits to agricultural sustainability are recognized due to the process of biological nitrogen fixation (BNF). However, factors like soil acidity and high concentrations of Al toxicity often limit all stages of roots infection, nodules and nitrogen assimilation by plants (Martinez-Romero et al., 1991; Rumjanek et al., 2005). This process is also influenced by genotypic characteristics of macro and microsymbionts and is modulated by an intense

exchange of molecular signals (Hartwig, 1998; Figueiredo et al., 2007; Subramanian et al., 2008). Although drought is the main abiotic factor responsible for the reduction in BNF efficiency (Hamidou et al., 2007), in tropical areas, high soil temperature also limits the process (Simões-Araújo et al., 2008).

The degree of specificity of certain species of tropical legumes and of tropical strains of rhizobia has been previously discussed (Thies et al., 1991; Simões-Araújo et al., 2008); however, for cowpea, the ease with which this plant is nodulated by several types of rhizobia has discouraged the selection of strains for crop. From the point of view of crop, the strains in the soil responsible for nodulation are very competitive the formation of nodules, but evidence suggests they are less efficient in N<sub>2</sub> fixation (Rumjanek et al., 2005). Furthermore, studies have shown good results obtained under field conditions with cowpea in northeastern Brazil and for common bean in Africa, especially when this practice is accompanied by a dose of mineral nitrogen (Silva et al., 1993; Hungria et al., 2003; Xavier et al., 2008). On the other hand, the plant genotype seems to have more influence on the mechanism of nodulation by bacteria and plays a central role in the selection of microsymbionts (Nutman, 1969; Demezas et al., 1995).

The relation between tropical legumes, such as cowpea, an important protein source in arid regions, and BNF has not received adequate attention. Under symbiotic conditions, the cowpea shows changes in photosynthesis (Lippi et al., 1999), key enzymes activities (Figueiredo et al., 2007), gene expression (Simões-Araújo et al., 2008) and morphology (Serraj et al., 1999). These changes may be the cause of low grain production in arid regions, such as northeastern Brazil, when under BNF conditions. In contrast, due to intensive research, excellent soybean production in Brazilian territory has occurred independent of mineral N fertilization (Kaschuk et al., 2010) and the success of BNF in this legume must be attributed to strong investment in technology and specific physiological characteristics.

The aim of this study was to demonstrate that cultivars selected for the Northeast inoculated with *Rhizobium* and when properly treated with mineral fertilizer and without water deficit during critical periods of the cycle, has the same performance as plants under mineral nutrition treatment. To achieve this, the study assessed key physiological responses of the two principal northeastern cultivars that have a short cycle and tolerance to weather conditions and major diseases of semiarid northeastern Brazil, in symbiosis with the most promising selected rhizobia under field and greenhouse conditions with sufficient mineral nutrition and water supply.

## 2. Materials and methods

### 2.1 Study site, plant species and growth conditions

Three genotypes of cowpea were used, provided by the Piauí State branch of the Brazilian Agricultural Research Company (*Empresa Brasileira de Pesquisa Agropecuária*, EMBRAPA). Cultivar BR17-Gurguéia is stable and well adapted to the conditions common in semiarid areas, while BR14-Mulato is sensitive to climate changes and is better adapted to environments that support high productivity (Freire-Filho et al., 2005a). Both species have cycles between 65 to 75 days and are resistant to the principal diseases of this crop. BRS-Milênio has a cycle between 70 to 75 days and was selected under the conditions of northern Brazil; i.e., it does not possess the local semiarid characteristics and has no resistance to the principal diseases.

The bacterial strain used for inoculation was BR3267 (SEMIA 6462) belonging to the genus *Bradyrhizobium*, which was isolated in the semiarid region in 1996. The main features observed for this strain are salt tolerance measured in the culture medium (Xavier et al., 1997), competitiveness with soil strains (Zilli et al., 2006) and high rates of biological atmospheric nitrogen fixation (BNF) assessed under greenhouse and field conditions (Martins et al., 2003). The inoculant dose was 200 g per 10 kg of seeds.

### 2.2 Experiment A

Field experiment was conducted at an Experimental Station of Pernambuco Research Company (*Empresa Pernambucana de Pesquisa*, IPA, Goiana, PE) from September to December 2007. For each genotype, Gurguéia, Mulato and Milênio, planting consisted of three 8.0 m rows, with 0.50 m spacing between plants and 0.60 m between rows (Cardoso et al., 2005). During the experiment, rainfall was 215 mm and the average temperature ranged 18 to 26°C. Since rainfall at the time of the experiment was low, irrigation was performed to maintain the plants well hydrated.

The supply of nutrients in Yellow Podzolic soil was achieved with an equivalent dose of K<sub>2</sub>O (40 kg ha<sup>-1</sup>) in the form of potassium chloride, P<sub>2</sub>O<sub>5</sub> (30 kg ha<sup>-1</sup>) in the form of superphosphate and N (60 kg ha<sup>-1</sup> – 30 kg at planting and 30 kg to 25 DAE) using ammonium sulfate, according to Cavalcanti et al. (1998), and was based on soil analysis. Fertilization was performed before the experiment. A randomized block design containing 2 genotypes x 2 microbiological treatments: control, no inoculation with all the nutrients and mineral nitrogen (30

kg) at 25 days after plant emergence (DAE); and inoculated at planting (biological nitrogen fixation – BNF) and application of mineral nitrogen (30 kg), at 25 DAE, with 5 replicates. The parameters analyzed 35 DAE were: gas exchange, chlorophyll fluorescence and certain parameters of leaf photosynthetic metabolism, including the contents of soluble sugars, free amino acids and proteins and yield components. The soil chemical characterization determined that phosphorus content was  $13 \text{ mg dm}^{-3}$ , pH ( $\text{H}_2\text{O}$ ) 6.2, and that potassium, aluminum, calcium and magnesium were 0.07, 0.0, 1.25 and  $0.5 \text{ cmol}_c \text{ dm}^{-3}$ , respectively. The amount of organic matter was  $2.4 \text{ g kg}^{-1}$ .

### 2.3 Experiment B

Experiment was conducted in a greenhouse from April to July 2008. The seeds were previously sterilized in a solution of 10% sodium hypochlorite for 2 min, followed by distilled water. Soaking was performed in distilled water for 2 h at  $25^\circ\text{C}$  and continuous light. After soaking, part of the seeds were inoculated. They were then planted in plastic bags with capacity of 7 kg of substrate containing a mixture of topsoil (organic), red clay and sand at a ratio of 3:1:1 and transferred to a greenhouse. This substrate was autoclaved for 1 h at  $120^\circ\text{C}$  in order to reduce the population of microorganisms. During the experiment, the average temperature ranged 19 to  $27^\circ\text{C}$ , irrigation was performed daily by maintaining the moisture in the pot at capacity. The supply of nutrients was achieved similar described above, according to Cavalcanti et al. (1998). The experiment was conducted under a randomized block design containing 3 genotypes x 2 microbiological treatments: control, no inoculation and with all the nutrients; and inoculated at planting, with mineral N fertilization (30 kg) up to 25 DAE, with 12 replicates. Parameters were assessed at 25 and 35 DAE. The parameters analyzed 35 DAE were: gas exchange, chlorophyll fluorescence and certain parameters of leaf photosynthetic metabolism, including the contents of soluble sugars, free amino acids and proteins, dry weight and number of nodules and biometric variables. The soil chemical characterization determined that phosphorus content was  $25 \text{ mg dm}^{-3}$ , pH ( $\text{H}_2\text{O}$ ) 5.5, and that potassium, aluminum, calcium and magnesium were 0.9, 0.0, 4.0 and  $1.9 \text{ cmol}_c \text{ dm}^{-3}$ , respectively. The amount of organic matter was  $3.3 \text{ g kg}^{-1}$ .

### 2.4 Measurements and analyses

#### 2.4.1 Gas exchange

Gas exchange measurement were performed at 35 DAE from 09:00 to 11:00 h using a portable infrared  $\text{CO}_2$  Gas Analyser (IRGA), ADC, model Lci (Hoddesdon, UK). Leaf gas exchange was measured on a mature, but not senescent leaf in the middle of plant with a maximum PAR of  $1080 \mu\text{mol m}^{-2} \text{ s}^{-1}$  in the leaf cuvette of the IRGA. The local PAR at the time of measurement was  $1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$  and mean temperature ranged from  $27.1$  to  $29.2^\circ\text{C}$  in experiment A and  $29.3$  to  $31.5^\circ\text{C}$  in B. The vapor pressure deficit (VDP) was approximately 2.1 kPa in experiment A and 2.8 kPa in B.

#### 2.4.2 Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was measured using an ADC fluorometer (Hoddesdon, UK). Maximal ( $F_m$ ) and basal ( $F_o$ ) fluorescence yields were measured in dark adapted (30 min) leaves, and steady-state ( $F_s$ ) and maximal ( $F_m'$ ) fluorescence yields were determined in a light-adapted state (Schreiber et al., 1994; Van Kooten and Snel, 1990). In addition, measurements were also taken under light-adapted conditions, being referred as  $F_o'$  (minimum) and  $F_m'$  (maximum). The  $F_o'$  signal was measured after PSI excitation by far-red light. The fluorescence signal under light-adapted conditions before the saturation pulse is referred as  $F_s'$ . The variable fluorescence signal under light conditions is  $\Delta F' = F_m' - F_s'$ . The following photochemical variables were calculated: maximum ( $F_v/F_m$ ) and actual ( $\Delta F'/F_m'$ ) quantum yield of primary photochemistry; non-photochemical [ $\text{NPQ} = (F_m - F_m')/F_m$ ] and photochemical [ $q_P = (F_m' - F_s')/(F_m' - F_o')$ ] quenching (Roháček, 2002).

#### 2.4.3 Leaf soluble sugars, free amino acids, proteins and total nitrogen

Leaf samples were collected in the middle of the afternoon, from 14:00 to 15:00 h. Sample leaves were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  in humidity proof containers until analysis. For free amino acid determination and Soluble carbohydrates using 50 mg of fresh leaf tissue for the preparation of the ethanol extract (Robbins and Pharr, 1988; Trethwey et al., 1998). Soluble carbohydrates were determined according to Dubois et al. (1956), using d(+)-glucose as standard. Free amino acid according to Moore & Stein (1948) using as standard a 1mM solution with the amino acids glycine, glutamic acid, phenylalanine and arginine. For extraction Leaf protein content aliquots of 100mg of fresh leaf tissue using the buffer (Armengaud et al., 2009) consisting of 20% (v/v) glycerol, 0.25% (w/v) bovine serum albumin, 1% (v/v) Triton X-100, 50 mM HEPES/KOH, pH 7.5, 10 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM EGTA and 0.25 mM dithiothreitol. Leaf protein content was determined according to Bradford (1976), using as standard bovine serum albumin. Leaf nitrogen was determined at plants reproductive stage (35 DAE) from the leaf dry mass according to Silva and Queiroz (2002). Quantification of chlorophylls was according to Lichtenthaler (1987).

#### 2.4.4 Morphological parameters, potential for nodulation and yield components

To measure the shoot (stem and leaves), the distance between ground level and decapitation was considered. Dry biomass was determined by weighing on a semi-analytical balance (DNA - Model 200) after drying at 80°C for 48 h. Leaf area was measured by a meter portable leaf area (LI-COR, model LI-3000 A). To assess the potential for nodulation, dry weight was determined and root nodule counts were performed. The yield components were evaluated by counting the number of pods per plant and number of grains per pod towards the end of crop season.

#### 2.5 Statistical analysis

The data were analyzed using the program STATISTICA 7.0. The results were submitted to variance analysis by factorial ANOVA and means were compared by Duncan's test with a significance level of 5%.

### 3. Results

#### 3.1 Experiment A

##### 3.1.1 Gas exchange and chlorophyll a fluorescence

Control plants were fertilized with mineral nitrogen twice: half when sown and at 25 DAE. Nodulated plants presented values of gas exchange and chlorophyll fluorescence similar to plants fertilized with mineral nitrogen at 35 DAE ( $p > 0.05$ ).

##### 3.1.2 Leaf soluble sugar, amino acid, protein and chlorophyll contents

Regarding the variables for leaf photosynthetic metabolism assessed at 35 DAE, leaf contents for soluble sugars (SS), amino acids (FA), total proteins (TP) and chlorophyll were similar ( $p > 0.05$ ) between treatments (Table 1). SS and TP presented differences between cultivars ( $p < 0.05$ ), with Gurguéia presenting the highest values.

##### 3.1.3 Yield components

In analysis of yield components, the number of pods per plant (NPP) is indicative of the potential yield of a cultivar. Plants under biological nitrogen fixation (BNF) produced more pods per plant (NPP) compared to controls ( $p < 0.05$ ), and did not differ from controls regarding the number of seeds per pod (NSP).

#### 3.2 Experiment B

##### 3.2.1 Gas exchange and chlorophyll a fluorescence

The variation in nitrogen source did not result in changes in gas exchange (Fig. 2) or chlorophyll fluorescence (data not shown) at 35 DAE for plants grown in pots with 7 kg of substrate in the greenhouse regarding the three cultivars.

##### 3.2.2 Leaf soluble sugar, amino acid, protein and chlorophyll contents

Leaf SS and TP contents differed among cultivars in two different periods of evaluation at 25 and 35 DAE, in preflowering and flowering ( $p < 0.05$ ). At flowering, the Gurguéia cultivar presented the highest contents of SS and TP in control conditions and under BNF (Table 3). Leaf FA content was only different between cultivars at 25 DAE; however, the contents of photosynthetic pigments were the same for both assessments and treatments among cultivars ( $p > 0.05$ ).

##### 3.2.3 Leaf nitrogen content

Assessment of the amount of leaf nitrogen in the three cultivars verified that the Gurguéia cultivar under BNF presented the greatest accumulation of this element in dry weight compared to Milênio and Mulato ( $p < 0.05$ ) and between the values obtained for control plants (Table 4). The number and biomass of root nodules presented by Gurguéia and Mulato were the highest values when under BNF ( $p < 0.05$ ) at 35 DAE, during the flowering period (Table 4), indicating good efficiency of the rhizobia.

##### 3.2.4 Dry mass and specific leaf area

Plant development in general showed no marked difference between treatments or among cultivars for the parameters of biomass of the roots, stems and leaves and specific leaf area among treatments (Table 5), except for Milênio and Gurguéia, which presented greater dry weight compared to controls.

##### 3.2.5 Yield components

Plants under biological nitrogen fixation (BNF) produced the same number of pods per plant (NPP) and number of seeds per pod (NSP) compared to controls ( $p > 0.05$ ). Except Milênio for NPP, that produced more under mineral nutrition (data not shown).

#### 4. Discussion

The treatments followed in this study, mineral nutrition (control) or biological nitrogen fixation (BNF) with mineral nitrogen supply at preflowering to 25 days after emergence (DAE), helped to clarify the hypothesis that lack of technology, nutrition mineral base and sufficient water supply are the main obstacles to improving the performance of selected cultivars in numerous crops in northeastern Brazil under BNF. Previous studies have shown that a dose of nitrogen at planting or coverage benefits the development of bean plants (Hungria et al., 2003; Xavier et al., 2008); hence the absence of the conventional treatment in this study, involving BNF alone, without any source of mineral nitrogen. In this study, seeding in pots with only sand as substrate was also avoided, since previous studies have shown that the bean is strongly hindered by this substrate without organic matter (Melo et al., 2005); moreover, this condition can easily lead to water deficit or high temperature in nodulated roots, which is harmful (Hamidou et al., 2007; Simões-Araújo et al., 2008).

Under field conditions plants of all three cultivars exhibited similar behavior regarding gas exchange and photochemical efficiency for both treatments with BNF and not (Fig. 1). These values measured between 09:00 and 11:00 h for healthy plants without stress were similar when compared to studies with bean plants (Lippi et al., 1999; Ribeiro et al., 2004; Santos et al., 2006). Despite the smaller amount of mineral nitrogen in the leaves BNF treatment showed similar photoprotective capacity of the control treatment, which is confirmed by the content of photosynthetic pigments (Table 1) (Schreiber et al., 1994) and resulting in photochemical efficiency (Fig. 1). Under BNF, a critical time for ensuring the productivity of the bean is the preflowering period, since this is when the highest rates of CO<sub>2</sub> assimilation occur (Pimentel et al., 1999), providing a higher percentage of fertilization and, therefore, a higher number of pods per plant (Lippi et al., 1999; Pimentel and Hébert, 1999). When the nodules are active, a large amount of photosynthates are diverted to the roots (Minchin et al., 1980). The costs for N<sub>2</sub> fixation amount to approximately 25% of all the carbon fixed in photosynthesis per day, against 4-13% in nonsymbiotic plants grown at an optimum nutrient supply (Lambers et al., 2008). Due to the rapid photosynthetic peak at preflowering, most of the bean cultivars do not produce enough sugars during preflowering to fill the pods. Thus, the plants cannot sustain the high activity of the nodules and ensure high grain yields per plant, because their senescence begins quickly (Lippi et al., 1999). The situation is different for soybeans, which maintain high photosynthetic activity for a longer period in the early reproductive stage (Kaschuk et al., 2010), maintaining a high rate of sugar transport, protein synthesis and leaf chlorophyll content. This feature, a short period of peak photosynthesis in the preflowering phase, a common physiological behavior of bean, is perhaps the main reason that the culture responds to a small amount of mineral nitrogen while under BNF (Tsai et al., 1993; Hungary et al., 2003; Xavier et al., 2008; Pelegrin et al., 2009). Another determining factor for the low efficiency of BNF in bean is the occurrence of water stress (Figueiredo et al., 2007; Hamidou et al., 2007), even in *Vigna*, which is more tolerant of water shortage than *Phaseolus* (Pimentel and Hébert, 1999). This is especially true if these stressful conditions occur between vegetative and early reproductive stages, when the production of new nodules is stabilizing in cowpea (Senanayake et al., 1987), leading to low efficiency of BNF during the reproductive stage when a high amount of nitrogen is required by the leaves in legumes (Neves et al., 1982; Kaschuk et al., 2010).

Plants of the three cultivars presented high leaf contents for SS, TP and chlorophyll in both treatments under field conditions, while Gurguéia accumulated the highest value for soluble sugars (Table 1, 2). One of the first symptoms of nitrogen deficiency in the leaves is chlorophyll degradation, which causes a reduction in photosynthetic activity and TP content, characterizing the onset of leaf senescence. This early flowering response (35 DAE) of the Gurguéia cultivar supports the development of nodulated plants (Kaschuk et al., 2010), delaying leaf senescence, while maintaining high photosynthetic activity for longer (Lippi et al., 1999), thus preventing photosynthate deficiency in the nodules during the pod-filling stage, which consumes a large amount of nitrogen in the leaves.

Without the major abiotic stresses present in the Brazilian northeast, drought and nutrient deficiency, plants of three cultivars presented values of yield components, number of pods per plant (NPP) and number of seed per pod (NSP), as would be expected for each growth habit and under rainfed conditions (Freire-Filho et al., 2005b). The three cultivars under BNF presented higher NPP values compared to controls of 42%, 27% and 20% for Mulato, Gurguéia and Milênio, respectively, and the same mean number of grains per pod ( $p > 0.05$ ). Neves et al. (1982) reported increased NPP and NSP production in cowpea inoculated with a supply of nitrogen mineral, although the source mineral did not contribute significantly to the increase in production.

The experiment in an open greenhouse was designed to evaluate the cultivars under the same conditions of water availability and nutrition, without competition among plants, and measure the number of nodules and dry weight. Under these conditions, the plants showed maximum rates of gas exchange in an open environment at 35 DAE,

early flowering, between 20 and 25  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , with no significant differences between cultivars and treatments (Fig. 2B). Maintaining a high photosynthetic rate is critical to the efficiency of BNF (Kaschuk et al., 2010). The onset of leaf senescence in cowpea plants varies among cultivars (Lippi et al., 1999), leading the anticipation of the photosynthetic peak in cultivars with early senescence. Thus, during a breeding program aimed at improving BNF, a characteristic that should be considered is the onset of leaf senescence.

During pod filling, leaf nitrogen metabolism is high in nodulated legumes (Kaschuk et al., 2010), thus high leaf nitrogen content at the onset of pod filling is another desirable feature among cowpea cultivars. The leaf nitrogen content at 35 DAE was significantly higher only in Milênio under BNF compared to controls ( $p < 0.05$ ). However, when comparing between cultivars, those selected for the northeastern region under low-tech conditions (Mulato and Gurguéia) presented higher values ( $P < 0.05$ ) than cultivars suitable for the northern region (Milênio) in control conditions and under BNF. Which may be indicative of high nodule activity, supported by high production of SS in northeastern cultivars, and corresponds to higher TP content in the leaves of these cultivars on the same day (Table 3).

Nodules number and dry weight at 35 DAE (experiment B) were also measured. The Gurguéia and Mulato cultivars showed the highest values for both parameters, especially for dry weight, compared to Milênio (Table 4). This provides strong evidence that explains the high leaf nitrogen content (Table 4) and SS and TP (Table 3) remained very similar between treatments probably due to the effectiveness of BNF occurring in nodulated plants. In fact, Gurguéia under BNF presented a higher total dry weight compared to controls (Table 5); which could be the result of an efficient supply of nitrogen to the shoot from the root nodules (Table 4).

*Vigna unguiculata* showed results that under non-stressful conditions in an open environment with varying temperature and light intensity throughout the day, confirms our hypothesis that when cowpea is nodulated by efficient rhizobia and with a small dose of mineral nitrogen at the end of the vegetative stage, it can present very similar development to plants fertilized with mineral nitrogen. However, since a strong difference was not observed between treatments in favor of BNF, when plants are grown under limited conditions of mineral nutrition and with the occurrence of short periods of drought during the cycle, the productivity of these nodulated plants is significantly decreased. These factors, drought and nutritional deficiency, commonly occur in cultivated cowpea in most states in northeastern Brazil (Freire-Filho et al., 2005a).

The present findings suggest that this physiological and biochemical scenario characterizes the efficiency of biological nitrogen fixation for most cowpea crops, however, are often produced without technology by small farmers in northeastern Brazil, what decrease the efficiency of BNF. Furthermore, these factors must be taken into account in breeding programs seeking to enhance physiological characteristics, such as a high photosynthetic rate under growing near optimal conditions.

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Table 1. Leaf contents of soluble sugars, free amino acids, chlorophyll and soluble protein in *Vigna unguiculata* conducted under two treatments, control (with levels of nitrogen at planting and 25 days after emergence (DAE)) and inoculated at planting (Biological Nitrogen Fixation – BNF) (with mineral nitrogen at 25 DAE) under field conditions at 35 DAE

Cultivar	Soluble sugars (mmol kg <sup>-1</sup> DW)		Amino acids (mmol kg <sup>-1</sup> DW)		Proteins (g.kg <sup>-1</sup> DW)		Chlorophyll (mmol.kg <sup>-1</sup> DW)					
	Control	BNF	Control	BNF	Control	BNF	Control			BNF		
							Cl <sub>a</sub>	Cl <sub>b</sub>	Carotenoids	Cl <sub>a</sub>	Cl <sub>b</sub>	Carotenoids
Mulato	1207BCa	1112BCa	319Aa	277Aa	230Ba	184Ba	7.42Aa	2.26Aa	1.73Aa	6.62Aa	1.92Aa	1.60Aa
Milênio	1121BCa	969Ca	340Aa	286Aa	259Ba	264Ba	7.03Aa	2.29Aa	1.70Aa	6.67Aa	1.96Aa	1.38Aa
Gurguéia	1685Aa	1364ABa	337Aa	350Aa	373Aa	415Aa	8.43Aa	2.42Aa	1.85Aa	6.29Aa	1.20Aa	1.36Aa

Different capital letters denote statistical differences ( $p < 0.05$ ) among cultivars within each parameter, different small letters indicate statistical differences between treatments within each cultivar,  $n = 4$ .

Table 2. Components of productivity of the plants of *Vigna unguiculata* conducted under two treatments, control (with levels of nitrogen at planting and 25 days after emergence (DAE)) and inoculated at planting (biological nitrogen fixation – BNF) (with mineral nitrogen at 25 DAE) under field conditions. Number of pods per plant – NPP and number of seeds per plant – NSP

Cultivar	Control	BNF	Control	BNF
	NPP		NSP	
Mulato	07Cb	17Ba	08Aa	10Aa
Milênio	11Bb	19ABa	09Aa	07Aa
Gurguéia	14Ab	21Aa	14Aa	11Aa

Different capital letters denote statistical differences ( $p < 0.05$ ) among cultivars within each parameter, different small letters indicate statistical differences between treatments within each cultivar,  $n = 4$ .

Table 3. Leaf contents of soluble sugars, free amino acids, chlorophyll and soluble protein in *Vigna unguiculata* conducted under two treatments, control (with levels of nitrogen at planting and 25 days after emergence (DAE)) and inoculated at planting (biological nitrogen fixation – BNF) (with mineral nitrogen at 25 DAE), under greenhouse conditions at 35 DAE

Cultivar	Soluble sugars (mmol kg <sup>-1</sup> DW)		Amino acids (mmol kg <sup>-1</sup> DW)		Proteins (g.kg <sup>-1</sup> DW)		Chlorophyll (mmol.kg <sup>-1</sup> DW)					
	Control	BNF	Control	BNF	Control	BNF	Control			BNF		
							Cl <sub>a</sub>	Cl <sub>b</sub>	Carotenoids	Cl <sub>a</sub>	Cl <sub>b</sub>	Carotenoids
<b>25 DAE</b>												
Mulato	970.66Ba	1126.36Ba	393.89Aa	320.61ABa	308.99Ca	481.42Ba	9.61Aa	3.09Aa	1.69Aa	13.85Aa	4.72Aa	2.34Aa
Milênio	1172.30Ba	1540.31Aa	254.55Ba	269.03ABa	628.00Aa	757.08Aa	11.03Aa	3.66Aa	1.90Aa	10.10Aa	3.17Aa	1.86Aa
Gurguéia	1088.47Ba	1151.62Ba	121.29Ca	203.23BCa	278.15Ca	275.41Ca	7.81Aa	2.59Aa	1.39Aa	12.04Aa	4.05Aa	2.06Aa
<b>35 DAE</b>												
Mulato	1064.82ABa	1126.13ABa	121.97Aa	130.43Aa	119.15Ba	134.18Ba	8.25Aa	2.70Aa	1.51Aa	8.58Aa	2.77Aa	1.67Aa
Milênio	750.75Ca	848.35BCa	151.68Aa	161.19Aa	87.80Ba	112.26Ba	5.79Aa	1.91Aa	1.14Aa	4.91Aa	1.62Aa	0.99Aa
Gurguéia	1090.12ABa	1204.50Aa	153.49Aa	146.24Aa	243.68Aa	236.24Aa	9.42Aa	3.08Aa	1.87Aa	9.30Aa	3.07Aa	1.57Aa

Different capital letters denote statistical differences ( $p < 0.05$ ) among cultivars within each parameter, different small letters indicate statistical differences between treatments within each cultivar,  $n = 4$ .

Table 4. Leaf nitrogen content in *Vigna unguiculata*, number and dry weight (DW) (mg) root nodules conducted under two treatments, control (with levels of nitrogen at planting and 25 days after emergence (DAE)) and inoculated at planting (biological nitrogen fixation – BNF) (with mineral nitrogen at 25 DAE), under greenhouse conditions at 35 DAE

Cultivars	Parameters					
	Leaf nitrogen (%)		Number nodules		DW nodules	
	Control	BNF	Control	BNF	Control	BNF
Mulato	3.23Ba	3.58Ba	32Bb	65Ba	44Ab	906.25Aa
Milênio	2.91Cb	3.66Ca	15Bb	60Ba	22Bb	270.48Ba
Gurguéia	3.75Aa	3.81Aa	56Ab	85Aa	115Ab	823.10Aa

Different capital letters denote statistical differences ( $p < 0.05$ ) among cultivars within each parameter, different small letters indicate statistical differences between treatments within each cultivar,  $n = 4$ .

Table 5. Changes in total dry weight and specific leaf area in *Vigna unguiculata* conducted under two treatments, control (with levels of nitrogen at planting and 25 days after emergence (DAE)) and inoculated at planting (biological nitrogen fixation – BNF) (with mineral nitrogen at 25 DAE), under greenhouse conditions at 25 and 35 DAE

Cultivar	Total dry weight (g)		Specific leaf area (cm <sup>2</sup> .g <sup>-1</sup> )	
	Control	BNF	Control	BNF
<b>25 DAE</b>				
Mulato	6.19Db	7.81BCa	398.29Aa	413.77Aa
Milênio	8.33ABa	9.40Aa	432.68Aa	526.96Aa
Gurguéia	6.91CDa	7.91BCa	474.81Aa	404.29Aa
<b>35 DAE</b>				
Mulato	21.25BCa	25.16ABa	312.38ABa	289.66Ba
Milênio	12.89Db	16.73CDa	282.82Ba	300.06ABa
Gurguéia	23.06Bb	30.31Aa	349.01Aa	341.05Aa

Different capital letters denote statistical differences ( $p < 0.05$ ) among cultivars within each parameter, different small letters indicate statistical differences between treatments within each cultivar,  $n = 4$ .

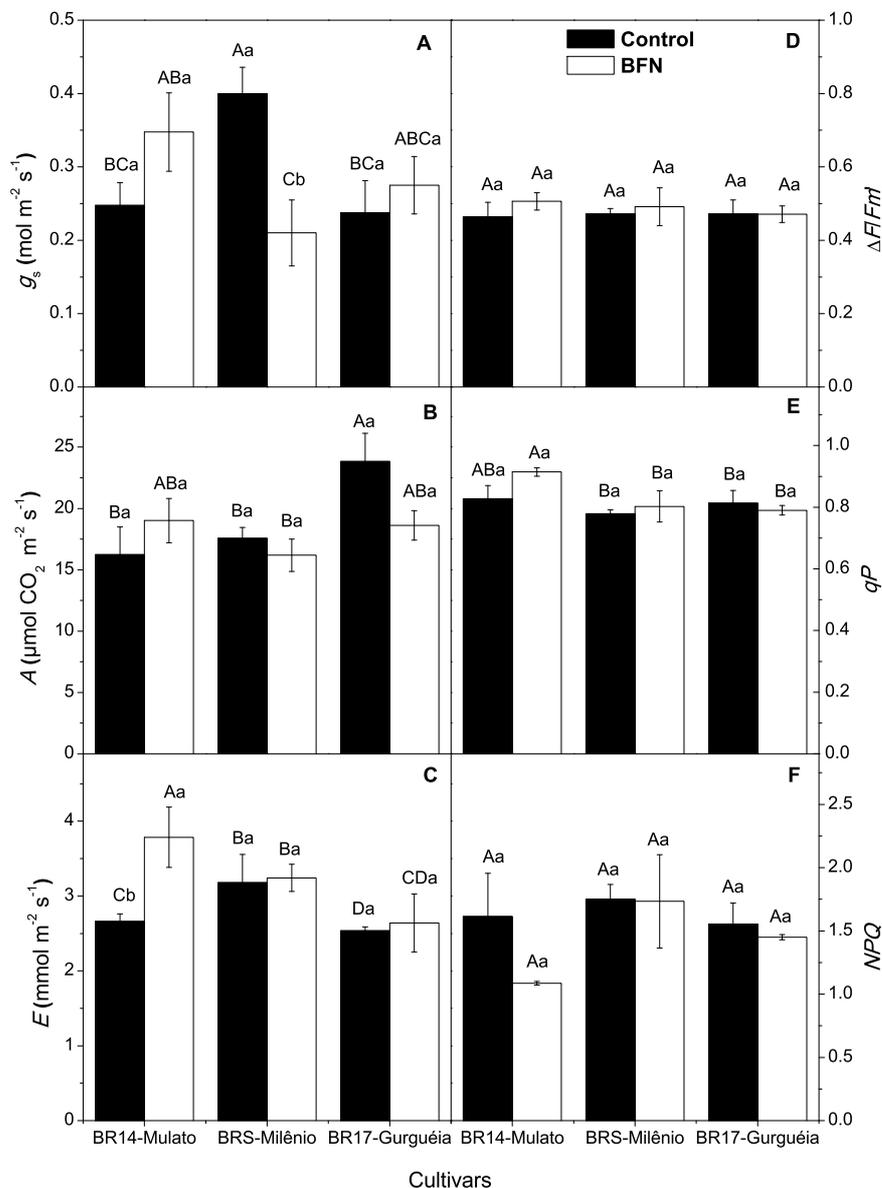


Figure 1. (A) Stomatal conductance ( $g_s$ ), (B)  $\text{CO}_2$  assimilation ( $A$ ), (C) transpiration ( $E$ ), (D) PSII effective quantum yield ( $\Delta F/F_m'$ ), (E) photochemical quenching ( $qP$ ) and (F) non-photochemical quenching ( $NPQ$ ) in plants of *Vigna unguiculata* under field conditions at 35 DAE (9:00 - 11:00 h). The plants were conducted under two treatments, control (with levels of nitrogen at planting and cover 25 DAE) and inoculated (BNF – biological nitrogen fixation) (with a dose of nitrogen 25 DAE). Different capital letters denote statistical differences ( $p < 0.05$ ) among cultivars within each parameter, different small letters indicate statistical differences between treatments within each cultivars,  $n = 4 \pm \text{SE}$

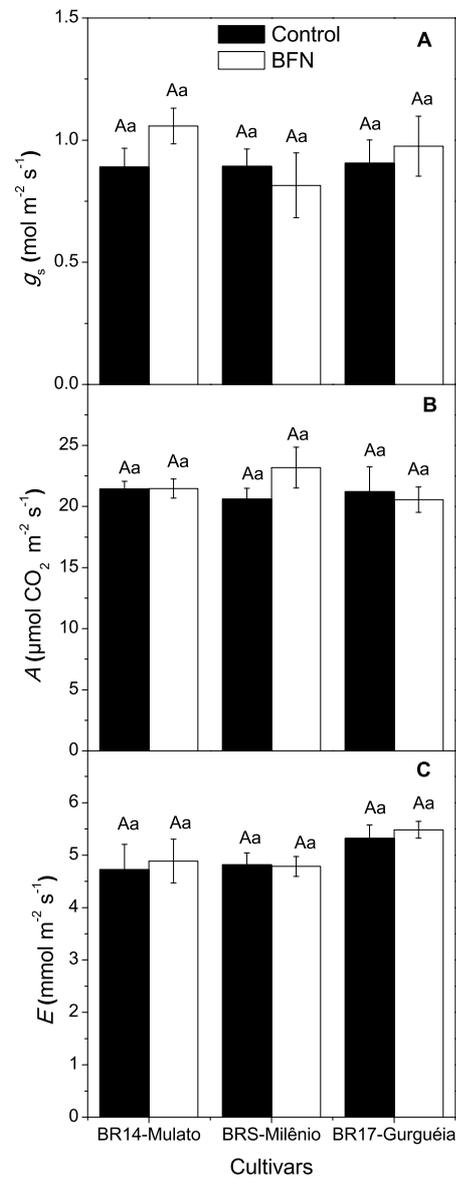


Figure 2. (A) Stomatal conductance ( $g_s$ ), (B) CO<sub>2</sub> assimilation ( $A$ ) and (C) transpiration ( $E$ ) in plants of *Vigna unguiculata* under greenhouse conditions at 35 DAE (9:00 - 11:00 h). The plants were conducted under two treatments, control (with levels of nitrogen at planting and cover 25 DAE) and inoculated (BNF – biological nitrogen fixation) (with a dose of nitrogen 25 DAE). Different capital letters denote statistical differences ( $p < 0.05$ ) among cultivars within each parameter, different small letters indicate statistical differences between treatments within each cultivar,  $n = 4 \pm \text{SE}$