UNIVERSIDADE FEDERAL DO PARANÁ

HELOÍSA DA SILVA BALDINOTTI

PHOTOSYNTHESIS AND BIOLOGICAL NITROGEN FIXATION IN COMMON BEAN
CULTIVARS WITH DIFFERENT GROWTH CYCLES

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Dissertação apresentada ao curso de Pós-Graduação em Ciência do Solo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Ciência do Solo.

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À vida.

AGRADECIMENTOS

Life on Earth depends on the sun.

(Voet and Voet, 2010, 901.)

RESUMO

A simbiose entre leguminosas e rizóbios tem sido estudada há anos devido a grande oportunidade para o desenvolvimento agronômico sustentável. Essa é baseada na troca de nutrientes de carbon C da planta por nitrogênio (N) proveniente da fixação biológica de N_2 (FBN), de forma que a planta teria um custo de C para um benefício de N. Entretanto, os supostos custos de C são compensados pela estimulação da taxa fotossintética a medida que a FBN é aumentada. A cultura de feijão comum (*Phaseolus vulgaris* L.) é conhecida por sustentar uma das menores taxas FBN entre as leguminosas graníferas cultivadas, alcançando maiores rendimentos na presença de fertilizante nitrogenado (fertilizante-N). Por isso, a compreensão dos fatores que governam os processos de fotossíntese e FBN do feijão comum pode orientar estratégias para superar a limitação de produtividade sem adição de altas doses de fertilizante-N. Este trabalho teve como objetivo estudar as relações entre a capacidade fotossintética de feijão comum com diferentes ciclos de crescimento e possíveis variações na capacidade de FBN, quando inoculada com *Rhizobium tropici* CIAT 899, ou adubada com fertilizante-N. A hipótese foi de que a fotossíntese e a FBN são processos sincronizados com a demanda de crescimento da planta e variam de acordo com o grupo de cultivares. Sementes de feijão comum dos cultivares (1) de ciclo superprecoce BRS FC104 (65 dias, da emergência à maturação fisiológica), (2) de ciclo intermediário BRS Pontal (87 dias); e (3) de ciclo longo BRS Vereda (93 dias) foram semeadas em vasos de solo, considerando os seguintes tratamentos: (i) plantas foram cultivadas com fertilizante-N, (ii) inoculadas; e, (iii) plantas não inoculadas e sem fertilizante-N. Durante o crescimento das plantas, foram medidas as taxas fotossintéticas e na colheita, foram determinadas as concentrações de ureídos, N total, açúcares solúveis e insolúveis em folhas e raízes secas sob circulação de ar forçado, bem como as atividades das enzimas Glutamina Sintetase (GS) e Glutamato Sintetase (GOGAT) em nódulos retirados frescos e congelados. O estudo indicou a inoculação de rizóbios em cultivares superprecoces é capaz de suprir as demandas de N, mesmo sem ter trazido incrementos da taxa fotossintética, de massa aérea e raízes secas em relação aos tratamentos que receberam fertilizante-N. A concentração de N nas folhas não apresentou diferença significativa em função dos tratamentos, porém a atividade enzimática de GS e GOGAT nos nódulos foi maior nas plantas inoculadas. Portanto, os custos de C do *R. tropici* CIAT 899 permitem um desenvolvimento satisfatório do feijão comum sem a necessidade de adição de fertilizante-N.

Palavras-chave: Glutamato Sintetase (GOGAT) em nódulo, Glutamina Sintetase (GS) em nódulo, inoculantes, melhoramento genético de feijão comum, simbioses radiculares, ureídos,

ABSTRACT

The symbiosis between legumes and rhizobia has been studied for years due to the great opportunity for sustainable agronomic development. It is based on the exchange of carbon (C) nutrients from the plant for nitrogen (N) derived from biological N_2 fixation (BNF), so that the plant would have a C cost for an N benefit. However, the supposed C costs are offset by the stimulation of the photosynthetic rate as the BNF increases. Common bean (*Phaseolus vulgaris* L.) is known to sustain one of the lowest BNF rates among cultivated grain legumes, achieving higher yields in the presence of nitrogenous fertilizer (N-fertilizer). Therefore, understanding the factors governing the photosynthesis and BNF processes of common bean can guide strategies to overcome productivity limitations without the addition of high doses of N-fertilizer. This study aimed to investigate the relationships between the photosynthetic capacity of common bean with different growth cycles and possible variations in BNF capacity when inoculated with *Rhizobium tropici* CIAT 899 or amended with N-fertilizer. The hypothesis was that photosynthesis and BNF are synchronized processes with the plant's growth demand and vary according to the cultivar group. Seeds of common bean cultivars (1) BRS FC104 super-early cycle (65 days from emergence to physiological maturity), (2) BRS Pontal intermediate cycle (87 days), and (3) BRS Vereda long cycle (93 days) were sown in soil pots, considering the following treatments: (i) plants were grown with N-fertilizer, (ii) inoculated with *R. tropici* 899; and (iii) non-inoculated plants without N-fertilizer. During plant growth, photosynthetic rates were measured, and at harvest, concentrations of ureides, total N, soluble and insoluble sugars in leaves and roots were determined under forced air circulation and, as well as the activities of the enzymes Glutamine Synthetase (GS) and Glutamate Synthetase (GOGAT) in freshly harvested and frozen nodules. The study indicated that rhizobia inoculation in super-early cultivars can meet N demands, even without increasing photosynthetic rate, aerial mass, and dry roots compared to treatments receiving N-fertilizer. The N concentration in the leaves did not show significant differences due to treatments, but the enzymatic activity of GS and GOGAT in nodules was higher in inoculated plants. Therefore, the C-costs of *R. tropici* CIAT 899 symbiosis allow satisfactory development of common bean, without the amendment with N-fertilizer.

Keywords: Nodule Glutamate Synthetase (GOGAT), Nodule Glutamine Synthetase (GS), inoculants, common bean genetic improvement, root symbioses, ureides.

SUMMARY

1   INTRODUCTION

Nitrogen (N) is one of the most important nutrients for the development of biological systems. In plants, it is the building block of chlorophyll, ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), amino acids, nitrogenous bases, and adenosine triphosphate (ATP) (Kusano et al., 2011; Burén & Rubio, 2018, Hessini et al., 2019). In agriculture, it is regarded as a macronutrient and limits crop production (Kuypers et al., 2018; Chen et al., 2020; Zhang et al., 2022). Plants mostly absorb N from the soil, as nitrate (NO_3^-) or ammonium (NH_4^+). N availability in the soil derives from organic N decomposition, biological N_2 fixation (BNF), in agricultural soils, by adding N-fertilizer (Marschner, 2012) and atmospheric precipitation (Xu et al., 2020). All crops demand N, but in general terms, legumes crop has a reduced need for the addition of N-fertilizer due to their association with diazotrophic bacteria, commonly known as rhizobia, which realize BNF by converting N_2 into NH_3 (Herridge et al., 2008; Kaschuk et al., 2016).

Brazilian agriculture has depended upon importation of N-fertilizer to reach better harvests, mainly due to the predominance of infertile soils in the country (Moreira et al., 1980). In 2020, 7 million of tons of N-fertilizer was imported to Brazil (Boletim Informativo, 2022), and Conab reported that Brazil imported 2.77 million of tons in january 2024. The price of N-fertilizer varies due to changes in global socio-economic scenarios, for example, as the happen during the war between Ukraine and Russia (Arndt et al., 2023). Arndt et al (2023) exposed that the developing nations are more susceptible to food insecurity and poverty due the consequences of the wars, as they affect the prices of fuels, fertilizers, and food.

Synthetic N-fertilizers are produced by the Harber-Bosch method, which combines N_2 and H+, under high pressure and temperatures to produce NH_3 (ammonia). This combustion demands a lot of energy, derived principally from non-renewable sources such as natural gas (Smith et al., 2020; Gao et al., 2023). Consequently, this process contributes to the greenhouse effect by emitting 1.5 t CO_2^{-eq} per one tonne of NH_3, responsible for 1,2% of this gas in the atmosphere (Bicer et al., 2016).

Studies show that plants only absorb 50% to 70% of N-fertilizers applied (Dimkpa et al., 2020). The rest is lost to the atmosphere as nitrous oxide (N_2O - this gas is more detrimental to the greenhouse effect than CO_2) or/and ammonia (Coskun et al., 2017). Excessive application of N-fertilizer to the soil can alter physico-chemical

properties, for example, stimulating the degradation of soil organic matter (Pahalvi et al., 2021) and, causing soil acidification (dim et al., 2020). When N descends the soil profile or flows on the soil surface, it contaminates underground water and contributes to the eutrophication of aquatic ecosystem (Zhang et al., 2021). Warm blood animals and humans who ingest this contaminated water are intoxicated, and may develop serious harm such as cancer (Zhang et al., 2015). Furthermore, N that is applied, and cultivated plants do not take it up is an economic loss to the farmers (Dimkpa et al., 2020). Therefore, being N an indispensable nutrient to plant physiology development, researchers are studying the best way, including the more sustainable way, to provide N for plants. The future of agriculture is the meeting between sustainability and the economy (Foyer, Nguyen, & Lam, 2019).

Legumes (Fabaceae) and rhizobia developed a symbiotic relationship during their evolution (Zhao et al. 2021). In this association, the plant provides sources of carbon (C) (e.g. dicarboxylic acid) to rhizobia housed in root nodules and these perform BNF making N available to the plant vascular system (Udvardi & Day, 1997; Poole et al., 2018). The N supplied in the symbiosis contributes to better N nutrition, increasing photosynthetic capacity, and plant growth. This exceptional relationship provides Fabaceae a positive relation to the environment and enhances soil fertility when used in intercropping or rotative systems helping provide N for the other cultures (Yang et al., 2024), reducing the requirement for N-fertilizers (Ladha et al., 2022).

Common beans (*Phaseolus vulgaris* L.) are considered a quality nutritional source for humans, they have a high content of protein, in combination with carbohydrates, fiber, and minerals (Didinger et al., 2022), playing a prominent role in combat malnutrition and ensure food security (Siddiq et al., 2022). Nevertheless, *Phaseolus vulgaris* L has a low BNF compared with other legumes, such as *Glycine max* L. (soybean; Peoples et al., 2021).

The BNF occurs in specialized organs called nodules. This process begins with a biochemical signaling (flavonoids) by the plant. Flavonoids attract *Rhizobium* spp. then starts to express the *Nod* factor which triggers nodule organogenesis (Lou et al., 2023). The root apical cells start curling and then reshaping to form infection threads, at this moment rhizobia enters the plant cell in parallel to the vegetal cortex cells that multiply to form the primordial nodule (Zhou et al., 2021). Posteriorly, bacteria come into contact with the cytosol of the host cells, which forms a periobacteriodal membrane (PM). During nodule maturation, the rhizobial cell expands and

differentiates into the endosymbiont form, the bacteroid. The PM and the bacteroid form the symbiosome, the site of the reduction of atmospheric N_2 to NH_3 (ammonia) (Poole et al., 2018; Mergaert et al., 2020).

The catalyzing of N_2 in NH_3 occurs by the metalloenzyme nitrogenase (E.C. 1.18.6.1). This reaction consumes 16 ATP per molecule of reduced N, corresponding to approximately 6 g C for each g of N fixed, it is an expensive process due to the stability of the bounds between these two atoms (Pfau et al., 2018). Since NH_3 is a gas, it reacts with H^+ protons in the bacteroid, forming NH_4^+, released in the plant cell (Marschner, 2011). Following the process, the enzyme glutamine synthetase (GS, E.C.6.3.1.2) is responsible for the incorporation of inorganic-N in an organic form, glutamine, in sequence glutamate synthase (NADH-GOGAT, E.C.1.4.1.1.4) adds the N-amide, from glutamine, to α-ketoglutarate, resulting in two glutamates (Hungria & Kaschuk, 2014; Liu et al., 2021). In tropical legumes (common beans) these amino acids are incorporated and transported as ureide to the other parts of the plant (Tegender, 2014).

BNF is, therefore, a process with great energy demand, arising from the respiratory oxidation of C sources from the plant's photosynthetic process. Some researchers consider this symbiotic relation just as drainage of a plant's carbon, not beneficial enough to the plant, considering that NO_3^- assimilation just costs 2.5 g C g^{-1} N and the N_2 fixation demands 3.3 and 6.6 g C g^{-1} N (Michin and Witty, 2005). Kaschuk et al (2009) calculate that the cost of symbioses (rhizobial and arbuscular mycorrhiza) is 4-16% of organic compounds produced in photosynthesis. Additionally, some authors (Ferguson et al., 2019; Liu, et al., 2018) corroborate that the plant controls the symbiosis by controlling the availability of C. Other studies reveal that the plant overcomes C deficiency by increasing the photosynthetic rate, which can infer a more complex relationship than just cost: benefit (Kaschuk et al., 2009).

The greater carbon dioxide assimilation (CO_2), the more the source area develops, and a greater amount of chlorophyll and photosynthetic enzymes, ATP, and inorganic phosphorus (Pi), which stimulates photosynthesis. However, the nutritional increase alone does not explain the increase in photosynthesis, as this process is also regulated by the plant's source:sink relationship (Kaschuk et al., 2010). Kaschuk et al (2010) state that one way to quantify the relationship between source:sink in plants in symbiosis with rhizobia is by comparing the changes in photosynthesis and the nutrients acquired by the symbiosis, compared to plants without symbiosis.

Photosynthesis is a metabolic process controlled by different physiological processes such as the concentration of carbohydrates in the leaves or the quantity of N available, and CO_2 concentration externally (Sigiura et al., 2019). Rogers et al (2009) affirm that legumes when in elevated concentrations of CO_2 in consortium with BNF can increase the rates of photosynthesis. However, Peoples et al (2009) estimated that only 40% of the N demanded by the common bean plant could be supplied by the BNF and the capacity varies according to the cultivar analyzed (Pacheco et al., 2020). At the same time, there is evidence that beans with different cycles or origins have variable photosynthetic rates, with a maximum rate of around 20 µmol CO_2 m^{-2} s^1 (Pimentel et al., 2011).

There are no studies with Brazilian common bean cultivars that correlate the rates of CO_2 assimilation (photosynthesis) with the activity rates of GS and GOGAT (FBN), which could confirm the phenomenon of stimulation of photosynthesis by symbiosis in common beans previously proposed for all legumes (Kaschuk et al., 2010) and confirmed with soybeans (Kaschuk et al., 2012; Kaschuk et al., 2016).

Therefore, this work aims to study the relationships between the photosynthetic capacity of *P. vulgaris* L with variable cycle growth length (cultivars of indeterminate growth with super-early, intermediate, and long cycles) in regards to its capacity to perform BNF, plant growth (dry mass) and N shoot concentration, when inoculated with *R. tropici* CIAT 899, with or without addition of N-fertilizer. The main hypothesis of this study was elucidated if photosynthesis and BNF are processes synchronized with the plant's growth demand and if it varies according to the cultivar group.

2   MATERIAL AND METHODS

2.1   EXPERIMENT SETUP

The experiment was performed in a glasshouse with no control over temperature or humidity, at the Department of Soils and Agricultural Engineering, Federal University of Paraná, Curitiba, Paraná, Brazil. Curitiba's climate is classified as humid subtropical (Cfb) in the Köppen-Geiger classification (Alvares et al., 2013), with relatively defined four seasons. The experiment started on January 2, 2023, and finished on February 13, 2023. The average temperature in the outside location during this period ranged from a maximum of 28.2°C to a minimum of 17.7°C, and the mean radiation was 1260.218 kJ m^{-2} (Inmet, 2024). The temperature in the glasshouse varies from 0.5 to 1 °C compared to the outside location.

The soil was obtained in the layer of 0-20 cm, in a non-cultivated area, from the Canguiri Experimental Farm in Pinhais, Paraná, Brazil, and is classified as Cambissolo in the Brazilian Soil Classification System (Santos et al., 2018), as Cambisols in the FAO system (IUSS Working Group WRB, 2022), and as Inceptisols in the Soil Taxonomy system (Soil Survey Staff, 2022). Soil chemical analyses and textural characterization were performed according to Santos et al. (2018). For the experiment, the soil was sifted through a 4mm sieve and the fertility of the native soil was adjusted by incorporating fine lime (Relative Power of Total Neutralization = 90%, 28.5% ± 0.5 CaO, 19% MgO), Triple Superphosphate (41% P_2O_5), and Potassium Chloride (50% K) fertilizers (Motta & Pauletti, 2019) as shown in Table 1. Subsequently, the was soil mixed with washed sand in a 1:1 ratio (soil : sand), and allocated to pots with a volume of 7 dm^3.

Table 1. Soil chemical and granulometric analyses

Soil attribute	Unity	Cultivated Soil
Organic matter	g/dm^{-3}	36.55
P	$mg\ dm^{-3}$	40.95
K	$cmol_c\ dm^{-3}$	0.35
Ca	$cmol_c\ dm^{-3}$	4.31
Mg	$cmol_c\ dm^{-3}$	2.88
Al	$cmol_c\ dm^{-3}$	0
H+Al	$cmol_c\ dm^{-3}$	6.7
SB	$cmol_c\ dm^{-3}$	7.54
CEC t	$cmol_c\ dm^{-3}$	7.54
CEC T	$cmol_c\ dm^{-3}$	14.24
V	%	52.95
m	%	0
S	$mg\ dm^{-3}$	4.98
B	$mg\ dm^{-3}$	0.21
Fe	$mg\ dm^{-3}$	31.07
Cu	$mg\ dm^{-3}$	0.65
Mn	$mg\ dm^{-3}$	11.2
Zn	$mg\ dm^{-3}$	2.45
Clay	$g\ kg^{-1}$	450
Silt	$g\ kg^{-1}$	150
Sand	$g\ kg^{-1}$	400
pH in $CaCl_2$		5.21

Notes: Soil P was extracted with Mehlich-1 solution. Abbreviations: CEC t = effective Cation Exchange Capacity; CEC T= Cation Exchange Capacity at pH 7.0. The organic matter was quantified by Walkley Black methodology. The H+Al was determined by calcium acetate pH 7.0. The extraction of Ca, Mg, Fe, Mn, and Cu were done by microwaves and determined by optical emission spectrometer. The soil granulometric was determined with the methodology of Santos et al (2018).

The experiment was performed under a completely randomized design in a two-factorial arrangement with six replicates per treatment. The two factors were based on common beans (*P. vulgaris* L.) genotypes and N sources supply. Common bean genotypes differed in their life cycle: (1) super-early-cycle, represented by BRS FC104, which takes 65 days from emergence to physiological maturity (Melo et al., 2019); (2) intermediary-cycle, represented by BRS Pontal, which takes 87 days from emergence to physiological maturity; and (3) long-cycle, represented by BRS Vereda, which takes 93 days from emergence to physiological maturity (de Faria et al., 2003). The treatments for N sources were: i) Control: non-inoculated and non-N-fertilized plants; ii) N-fertilized plants: application of 40 kg N ha^{-1} in the form of urea just after plant emergence, in moist soil (stage V1; de Oliveira et al., 2018), and an additional 40 kg N ha^{-1} in the form of urea in the reproductive stage (R5; Oliveira et al., 2018); and, iii) Plants inoculated with *Rhizobium tropici* CIAT 899 at sowing (a volume corresponding to 100 ml of inoculant (approximately 10^9 cells ml^{-1}) for 50 kg of seeds). Five seeds

were sown, and all five emerged, but three plants were thinned out after emergence in each pot. Thus, each experimental unit (replicate) was represented by two plants.

2.2 PHOTOSYNTHESIS AND PHOTORESPIRATION

The rates of photosynthesis and photorespiration were measured from 10:00 AM until 13:00 PM, in a chamber with 8 cm^2, was used the third fully expanded leaf (from top to bottom; Melo et al., 2020; Cruz et al., 2022), with the open gas exchange system Portable Gas Exchange Fluorescence System GFS-3000™, with leaf temperature varying from 30 °C to 32 °C, the CO_2 flow at 400 ppm, and radiation at 1500 μmol quanta m^{-2} s^{-1}.

2.3 SHOOT AND ROOT MASSES

The plants were harvested after the measurements of photosynthesis and separated into shoot and root parts. The roots were washed, nodules were detached, counted, weighted (fresh matter), frozen in liquid N, and stored in the freezer -20 °C. Shoots and roots were dried in a forced-air circulation oven at 65°C until they reached constant mass, and weighed in a semi-analytical balance (Ohaus™).

2.4 GLUTAMINE SYNTHETASE (GS; E.C. 6.3.1.2)

Enzymes were extracted from 0.5 g frozen nodules by maceration with the extracting solution described by Hungria et al. (1991). Then, the suspension was treated with a solution consisting of buffer phosphate (50 mM, pH 7.5), $MgCl_2$ (10 mM), EDTA (ethylenediaminetetraacetic acid, 5 mM), DTT (Dithiothreitol, 5 mM), PMSF (Phenylmethylsulfonyl fluoride, 0.5 mM), and PVPP (Polyvinylpyrrolidone, 0.5%). The suspensions were incubated for 10 min and then centrifuged at 6,000 g for 30 min at 4 °C. The aqueous phase was desalted through Sephadex G-25™, and 50 uL of this solution was submitted to the assay that measures the activity of GS at 30 °C using glutamate as substrate (Hungria et al., 1991). The reactions were analyzed using a light spectrophotometer (Biospectro SP-22™) with an absorbancy measurement at 505 nm. The activity was expressed in nmol γ-glutamyl min^{-1}.

2.5   NADH-GLUTAMATE SYNTHASE (GOGAT; E.C. 1.4.1.14)

For the GOGAT assay, nodules were also macerated with the extraction buffer described by Hungria et al. (1991). Then, the suspensions were incubated with a solution containing potassium phosphate buffer (200 mM, pH 7.5), mercaptoethanol (2%), 2-oxoglutarate (10 mM), NADH (1 mM), and EDTA (1 mM), in a total volume of 1 mL. Subsequently, the suspensions were stirred, incubated at 30 °C for 10 min, and centrifuged at 6,000 g for 30 min at 4 °C. The aqueous phase was desalted using Sephadex G-25™, and 50 μL of this solution was then submitted to the assay measuring the activity of GOGAT at 30 °C using L-glutamine as a substrate (Hungria et al., 1991). Samples without and with L-glutamine were read at a wavelength of 305 nm. The activity was expressed in μmol NADH min^{-1}.

2.6   UREIDE

The method for quantification of ureide was based on Vogels and Van der Drift (1970) with adaptations from Hungria (1992) and Lescano (2020). The petioles samples were ground into 1 mm pieces. Aliquots of 30 mg grounded samples were placed in microcentrifuge tubes (with holes in the caps to prevent them from opening), amended with 1 mL of 0.5 M NaOH, and incubated in boiling water for 30 min. Following that, the extracts were centrifuged at 13,460 rpm for 10 min. The supernatant was transferred to clean tubes and the pellet was discarded. New tubes were subsequently filled with 100 μL of the extract supernatant, 650 μL of Milli-Q water, and 250 μL of 0.05 M NaOH, boiled for 8 min, cooled in ice, refilled with 250 μL of 0.65 N HCl, and boiled for another 4 min. In this step, the samples were digested, liberating glycolate into the solution. In the next step, digested extracts were amended with 250 μL of phenylhydrazine solution and 250 μL of 0.4 M phosphate buffer, and incubated at room temperature. In this step, glycolate and phenylhydrazine reacted and formed glyoxylic acid phenylhydrazone. The digests were then amended with 1.25 mL of concentrated HCl and 250 μL of ferricyanide, resulting in a reddish color of 1,5-diphenylformazan. The reactions were read at a wavelength of 520 nm using a spectrophotometer (Biospectro SP-22®), and calculated given the unit of nmol N-ureide/g DW.

2.7   TOTAL NITROGEN

A subsample of 0.1 g of ground dried leaves was subjected to the Kjeldahl method (Aguirre, 2023). The process involved digestion, converting organic-N into

NH_4^+, followed by distillation, converting NH_4^+ into NH_3. The NH_3 then reacted with boric acid, and this solution was titrated with 0.1 N sulfuric acid.

2.8   CARBOHYDRATES

Aliquots of 100 mg of dried ground-leaves or roots were incubated with 1 mL of an extraction solution (Leach and Braun, 2016), containing methanol, chloroform, and water (at a ratio of 12:5:3) in a 50 °C water bath for 30 min. Then, the suspensions were centrifuged at 13,640 g for 5 min. These steps were repeated three times. The aqueous phase was into a conical tube of 15 mL and measured the final volume and 0,6 volume of water in the conical tube, the solid phase (pellet) was kept in the initial tube. In the end, both the aqueous phase and the pellet were stored in the refrigerator.

For the quantification of soluble sugars is used the aqueous phase. The tubes were centrifugated at 6.000g and then pipet 1 mL from the top in a tube containing anthrone-sulfuric acid solution (Plummer, 1971). The anthrone-sulfuric acid solution digests carbohydrates, forming a green-colored furfural. For reference, the same procedure was repeated with known concentrations of glucose, as described in Hungria et al. (1992). The concentrations of soluble sugars were measured at a wavelength of 620 nm, the value expressed as mg of glucose/g DW.

The fraction amide was extracted from the saved pellets with adaptations from Leach and Braun (2016). Initially, the pellet was resuspended in 1 mL of $CaCl_2$ 4 M and incubated in boiling water for 5 min (Jane and Shen, 1993). Subsequently, 50 µL of the resuspended sample was mixed with 950 µL of $C_2H_3NaO_2$ 100mM and 100 µL of amyloglucosidase (~30 U) solution. The mixes were incubated in boiling water for 15 min and then cooled in a water bath at 50 °C for 3 min. In the next step, an additional 50 µL of amyloglucosidase solution was added to the mixes, which were then incubated again in a 50 °C water bath for 1 hour. The concentrations of soluble sugars in the digests were then determined as above, using the anthrone-sulfuric acid solution (Plummer, 1971; Hungria et al., 1992).

2.9   DATA ANALYZES

The data were analyzed using the Shapiro-Wilk normality test and Bartlett's homoscedasticity test at a significance level of 5%. When the residuals did not pass the homoscedasticity test, the 'box-cox' test transformed the residuals. This was applied to the data regarding the number of nodules. After meeting the necessary

prerequisites, a two-way ANOVA was performed, and the means were subjected to the Tukey test at a 5% significance level. The analyses were performed in R Studio™ using the 'ExpDes.pt' package. Anova values are presented in Supplementary Table 1.

3   RESULTS

3.1   RATES OF PHOTOSYNTHESIS AND PHOTORESPIRATION

In this experiment with common beans, the rates of photosynthesis (leaf CO_2 assimilation) ranged from 8.09 to 21.04 µmol CO_2 m^{-2} s^{-1} in the V4 phenological stage and from 8.99 to 21.61 µmol CO_2 m^{-2} s^{-1} in the R7 phenological stage. Significantly, the rates of photosynthesis were affected by the interaction between genotypes and N sources in both V4 and R7 phenological stages ($p \leq 0.05$). Notably, the super-early-cycle cultivar BRS FC104 responded very differently to N sources compared to the intermediary and long-cycle cultivars BRS Pontal and BRS Vereda.

In the V4 stage, N fertilization and *Rhizobium* inoculation stimulated the rates of photosynthesis in the intermediary and long-cycle cultivars BRS Pontal and BRS Vereda compared to the counterpart control plants, which did not receive either N-fertilizer or *Rhizobium* inoculation (Tab. 2). However, *Rhizobium* inoculation led to a decrease in photosynthesis for the super-early-cycle BRS FC104 cultivar compared to the control counterpart plants; i.e., it changed from 19.1 in control plants to 8.1 µmol CO_2 m^{-2} s^{-1} in *Rhizobium* inoculated plants. (Tab. 2). Interestingly, N fertilization also decreased photosynthesis in BRS FC104, but these changes were not statistically significant.

In the R7 stage, there was less variation in photosynthesis among the treatments. Most plants exhibited photosynthesis rates above 16 µmol µmol CO_2 m^{-2} s^{-1}. However, the *Rhizobium*-inoculated intermediary-cycle BRS Pontal had a significantly lower rate of photosynthesis than its counterpart treatments, dropping below 10 µmol CO_2 m^{-2} s^{-1} (Tab. 2).

Table 2. Photosynthesis and Photorespiration rates (µmol CO_2 m^{-2} s^{-1}) of three genotypes of common (carioca) beans FC104 (super-early-cycle BRS FC104), Pontal (intermediary-cycle BRS Pontal), and Vereda (long-cycle BRS Vereda)], subjected to three treatments: (i) Control (not fertilized with N-fertilizer, not inoculated), (ii) N-Fertilizer (treated with ca. 40 kg N ha^{-1} at emergence and ca. 40 kg N ha^{-1} at the R5 phenological stage), and (iii) *Rhizobium* (inoculated with *R. tropici* CIAT 899 at sowing), of plants grown in pots containing 7 dm^3 of soil, under greenhouse conditions.

Genotypes N Source	BRS FC104	BRS Pontal	BRS Vereda	Mean	BRS FC104	BRS Pontal	BRS Vereda	Mean
	Photosynthesis V4				Photosynthesis R7			
Control	16.70 Aa	9.77 Bb	8.09 Bb	11.52	17.19 Aa	21.62 Aa	19.57 Aa	19.46
N-Fertilizer	13.57 Ab	18.29 Aab	21.04 Aa	17.63	14.95 Ab	20.85 Aa	18.44 Aab	18.08
Rhizobium	8.14 Bb	16.88 Aa	19.25 Aa	14.76	19.07 Aa	8.99 Bb	17.44 Aa	15.17
Mean	12.80	14.98	16.13		17.07	17.15	18.48	
	Photorespiration V4				Photorespiration R7			
Control	16.93 Aa	10.77 Bb	9.16 Bb	12.29	16.96 Aa	22.22 Aa	19.13 Aa	19.44
N-Fertilizer	16.24 Ab	19.49 Aab	23.38 Aa	19.70	17.78 Aa	18.81 Aa	18.90 Aa	18.5
Rhizobium	9.44 Bb	18.77 Aa	21.86 Aa	16.69	19.75 Aa	11.20 Bb	18.72 Aa	16.56
Mean	14.20	16.34	18.13		18.16	17.41	18.92	

Following Tukey's test ($p \leq 0.05$), means of individual genotypes (in columns), at the same developmental stage (V4 or R7), followed by different uppercase letters, are significantly different among N-sources. Similarly, means of individual N-sources (in rows), at the same developmental stage (V4 or R7), followed by different lowercase letters, are significantly different among genotypes.

Source: The author (2024).

Following the patterns of photosynthesis, photorespiration was influenced by the interaction of cultivars and N sources. For instance, in the V4 stage, the long-cycle BRS Vereda exhibited higher rates of photorespiration when treated with N-fertilizer or inoculated with *Rhizobium* than its control plants, which did not receive N-fertilizer or *Rhizobium* (Tab. 2). Conversely, although not statistically significant, the super-early-cycle BRS FC104 inoculated with *Rhizobium* showed lower rates of photorespiration than its control plants (Tab. 2).

Similar to photosynthesis, photorespiration in the R7 stage showed minimal variation. The only exception was the intermediary-cycle BRS Pontal inoculated with *Rhizobium*, which showed lower photorespiration than its counterpart plants, whether fertilized with N or not (Tab. 2).

3.2 SHOOT, ROOT AND NODULE DRY WEIGHTS

The shoot dry weight in the V4 stage was influenced by the interaction between genotypes and N sources (Tab. 3). The super-early cycle cultivar BRS FC104 accumulated similar shoot dry weights regardless of N sources ($p \leq 0.05$). In contrast, the intermediary-cycle cultivar BRS Pontal and the long-cycle BRS Vereda significantly increase the dry weights when receiving N-fertilizer compared to control plants, but they were not affected by *Rhizobium* inoculation (Tab. 3).

Following the trends of shoot dry weight, root dry weight in the V4 stage was also influenced by the interaction between genotypes and N sources. Repeating the patterns of shoot dry weight, the super-early cycle cultivar BRS FC104 accumulated similar root dry weights regardless of N sources ($p \leq 0.05$). Similarly, root dry weights of the intermediary-cycle BRS Pontal were higher when fertilized with N than the control plants (Tab. 3). Interestingly, there were no differences between *Rhizobium*-inoculated and control plants in terms of root dry weights ($p \leq 0.05$).

Treatments that exhibited greater growth in the V4 stage also demonstrated increased size in the R7 stage (Tab. 3). Consequently, the intermediary-cycle BRS Pontal, when fertilized with N and inoculated with *Rhizobium*, displayed higher aerial and root dry weights compared to control plants (Tab. 3). However, the super-early BRS FC104 was not influenced by N sources, resulting in similar root dry weights for control, N-fertilized, and *Rhizobium*-inoculated plants (Tab. 3).

Nodules were counted, and for statistical analyses, it was necessary to transform the data using the Box-Cox method due to the abnormality in the residuals.

Table 3. Shoot (g plant⁻¹), roots (g plant⁻¹), and nodule (g plant⁻¹) dry weights, and nodules numbers of three genotypes of common (carioca) beans (FC104, Pontal, and Vereda), subjected to three treatments: Control, N-Fertilizer, and *Rhizobium*, of plants grown in pots containing 7 dm³ of soil, under greenhouse conditions.

Genotypes Source of N	BRS FC104	BRS Pontal	BRS Vereda	Mean	BRS FC104	BRS Pontal	BRS Vereda	Mean
	Shoot dry weight V4				Shoot dry weight R7			
Control	3.71 Aa	2.44 Bb	2.94 Bb	3.03	7.85 Ba	8.23 Ba	7.35 Ba	7.81
Fertilizer	3.66 Ab	11.45 Aa	4.19 Ab	6.43	11.82 Aa	10.57 Aab	10.25 Ab	10.88
Rhizobium	3.85 Aa	2.96 Bb	3.72 Aa	3.51	8.57 Bb	11.38 Aa	10.23 Aa	10.06
Mean	3.74	5.62	3.61		9.42	10.06	9.28	
	Roots dry weight V4				Roots dry weight R7			
Control	2.60 Aa	1.94 Bab	1.45 Bb	2	2.92 Aab	4 Ba	1.92 Bb	2.94
Fertilizer	1.35 Bc	6.21 Aa	2.43 Ab	3.33	3.49 Ab	6.42 Aa	3.47 Ab	4.46
Rhizobium	2.30 Aa	2.10 Ba	1.45 Ba	1.95	2.85 Ab	5.50 Aa	3.67 Ab	4.07
Mean	2.08	3.41	1.78		3.09	5.31	3.02	
	Total dry weight V4				Total dry weight R7			
Control	6.31 Aa	4.37 Bb	4.38 Bb	5.02	10.77 Bb	16.93 Ba	9.28 Bb	12.33
Fertilizer	5.48 Ab	21.22 Aa	6.63 Ab	11.11	15.32 Ab	21.59 Aa	13.72 Ab	16.88
Rhizobium	6.15 Aa	5.05 Ba	6.02 Aa	5.74	11.42 Bc	21.41 Aa	13.90 Ab	15.58
Mean	5.98	10.21	5.68		12.50	19.98	12.3	
	Nodules dry weight V4				Nodules dry weight R7			
Control	0.07 Aa	0.06 Ba	0.04 Aa	0.05	ns	ns	ns	0.15 B
Fertilizer	0.02 Ab	0.32 Aa	0.04 Ab	0.13	ns	ns	ns	0.24 A
Rhizobium	0.07 Aa	0.12 Ba	0.08 Aa	0.09	ns	ns	ns	0.22 A
Mean	0.05	0.16	0.05		0.21 AB	0.23 A	0.16 B	
	Nodules number V4				Nodules number R7			
Control	13.41 Aa	13.73 Ba	10.60 Ba	12.58	ns	ns	ns	104.88 B
Fertilizer	11.68 Ab	23.04 Aa	11.88 ABb	15.53	ns	ns	ns	157.55 A
Rhizobium	15.63 Aa	17.30 ABa	17.90 Aa	19.64	ns	ns	ns	120.22 AB
Mean	13.57	18.02	13.46		131.94 A	135.83 A	114.88 A	

According to the Tukey's test (p ≤ 0.05), means of individual genotypes (in columns), at the same developmental stage (V4 or R7), followed by different uppercase letters, are significantly different among N-sources. Similarly, means of individual N-sources (in rows), at the same developmental stage (V4 or R7), followed by different lowercase letters, are significantly different among genotypes.

Source: The author (2024).

There were differences in the number of nodules in both V4 and R7 stages. When considering the average of cultivars and N sources together, plants formed a total of 81.2 nodules per plant in the V4 stage, and 127.5 nodules per plant in the R7 stage (Tab. 3).

However, there were differences in nodule weight ($p \leq 0.05$). In the V4 stage, plants of the intermediary-cycle BRS Pontal receiving N-fertilizer exhibited a higher nodule weight than any other treatment (Tab. 3). Subsequently, in the R7 stage, BRS Pontal continued to have the heaviest nodules. However, at this time, its nodule weight was only significantly higher than control plants of the super-early BRS FC104 and the long-cycle BRS Vereda. There was no statistical difference with the treatments involving its own BRS Pontal control and all the other cultivars receiving N or inoculated with *Rhizobium* (Tab. 3).

3.3 NODULE ENZYMATIC ACTIVITY

The activities of nodules GS and GOGAT were highly dynamic, influenced by the plant genotype, the N source treatment, and particularly the plant phenological stage.

The activity of GS in the nodules formed in the V4 stage of all three cultivars was consistently higher in Rhizobium-inoculated plants than in their counterpart N-fertilized or control plants (Tab. 4). Within the inoculated and N-fertilized plants, the activity of GS in the nodules of the intermediary-cycle BRS Pontal was higher than in the super-early BRS FC104 (Tab. 4). Nodules of control plants had the lowest rates of GS activity in the V4 stage (Tab. 4).

Moving to plants in the R7 stage, the activity of GS in the nodules of Rhizobium-inoculated intermediary-cycle BRS Pontal remained higher than its counterpart receiving N-fertilizer (Tab. 4). However, the differences between Rhizobium-inoculated and N-fertilized plants in the super-early BRS FC104 and long-cycle BRS Vereda (Tab. 4) were no longer significant. Remarkably, in the R7 stage, nodules of the super-early BRS FC104 and the intermediary-cycle BRS Pontal, formed from indigenous rhizobia strains (not inoculated), had similar rates of GS activity to the nodules of their *Rhizobium*-inoculated counterparts.

Table 4. Glutamine synthetase (GS; nmol γ-glutamyl min⁻¹) and Glutamate synthase (GOGAT; µmol NADH min⁻¹) nodule activity of three genotypes of common (carioca) beans [FC104 (super-early-cycle BRS FC104), Pontal (intermediary-cycle BRS Pontal), and Vereda (long-cycle BRS Vereda)], subjected to three treatments: (i) Control (not fertilized with N-fertilizer, not inoculated), (ii) N-Fertilizer (treated with ca. 40 kg N ha⁻¹ at emergence and ca. 40 kg N ha⁻¹ at the R5 phenological stage), and (iii) *Rhizobium* (inoculated with *R. tropici* CIAT 899 at sowing), of plants grown in pots containing 7 dm³ of soil, under greenhouse conditions. Nodules were harvestedimmediately after haversting shoots and frozen at -4 ℃ until biochemical analyses.

Genotypes N-sources	BRS FC104	BRS Pontal	BRS Vereda	Mean
GS V4				
Control	ns	ns	ns	2.51 B
Fertilizer	ns	ns	ns	2.70 B
Rhizobium	ns	ns	ns	5.02 A
Mean	3.47 A	3.86 A	2.90 B	
GOGAT V4				
Control	5.31 Aa	5.74 Aa	5.36 Aa	5.47
Fertilizer	5.02 Aa	3.58 Bb	2.68 Bb	3.76
Rhizobium	6.06 Aa	6.61 Aa	5.3 Aa	5.99
Mean	5.46	5.31	4.44	

Genotypes N-sources	BRS FC104	BRS Pontal	BRS Vereda	Mean
GS R7				
Control	ns	ns	ns	3.31 A
Fertilizer	ns	ns	ns	3.55 A
Rhizobium	ns	ns	ns	3.37 A
Mean	3.28 A	3.49 A	3.47 A	
GOGAT R7				
Control	7.30 Aa	5.07 Bb	3.41 Bc	5.26
Fertilizer	4.43 Bb	4.20 Bb	5.80 Aa	4.81
Rhizobium	6.46 Ab	7.95 Aa	5.88 Ab	6.76
Mean	6.06	5.74	5.03	

According to the Tukey's test (p ≤ 0.05), means of individual genotypes (in columns), at the same developmental stage (V4 or R7), followed by different uppercase letters, are significantly different among N-sources. Similarly, means of individual N-sources (in rows), at the same developmental stage (V4 or R7), followed by different lowercase letters, are significantly different among genotypes.

Source: The author (2024).

In comparison to GS at a similar stage, GOGAT exhibited a somewhat different pattern of activity. For instance, in the V4 stage, nodules of both control plants (formed nodules from indigenous rhizobia) and *Rhizobium*-inoculated plants showed comparably high rates of GOGAT activity (Tab. 4). Interestingly, the GOGAT activity in the nodules of the intermediary-cycle BRS Pontal and long-cycle BRS Vereda was significantly inhibited by N-fertilizer (Tab. 4), while the activity in the nodules of super-early BRS FC104 was not affected (Tab. 4).

In the R7 stage, the patterns of nodule GOGAT activity diverged from those of GS. Unlike GS, the activity of GOGAT in the nodules of long- and long-cycle cultivars, forming nodules with indigenous rhizobia, was significantly lower than those formed with *Rhizobium* inoculant in the same cultivars. However, similar to GS, the activity of GOGAT was inhibited by the application of N, affecting the nodule GOGAT activity of super-early BRS FC104 and intermediary-cycle BRS Pontal.

3.4 TOTAL NITROGEN AND UREIDES

None of the factors had a significant impact on the total N concentration in the leaves of common beans in this experiment (Tab. 5). The average N concentration in the leaves of common beans was 3,44 %.

In the V4 stage, the concentration of ureides in the super-early BRS FC104 and long-cycle BRS Vereda were similar across all three N source treatments. Conversely, intermediary-cycle BRS Pontal plants inoculated with *Rhizobium* exhibited a higher N ureide concentration (34.93 nmol N-ureide g^{-1} DW) compared to the control (18.98 nmol N-ureide g^{-1} DW) and N-fertilized plants (9.26 nmol N-ureide g^{-1} DW) (Tab. 5).

In the R7 stage, irrespective of the N source treatment, the intermediary-cycle BRS Pontal consistently exhibited the highest shoot ureide concentrations, while the super-early BRS FC104 cultivar and the long-cycle BRS Vereda had the lowest concentrations (Tab. 5). The application of N-fertilizer hindered the accumulation of shoot ureides in the long-cycle BRS Vereda, but it had no significant impact on the other cultivars. *Rhizobium* inoculation did not enhance shoot ureide concentrations compared to control plants (Tab. 5).

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Table 5. Total Nitrogen (%) and Ureide (nmol N-ureide g^{-1} DW) in plant (leaves and petioles, respectively) of three genotypes of common (carioca) beans [FC104 (super-early-cycle BRS FC104), Pontal (intermediary-cycle BRS Pontal), and Vereda (long-cycle BRS Vereda)], subjected to three treatments: (i) Control (not fertilized with N-fertilizer, not inoculated), (ii) N-Fertilizer (treated with ca. 40 kg N ha^{-1} at emergence and ca. 40 kg N ha^{-1} at the R5 phenological stage), and (iii) *Rhizobium* (inoculated with *R. tropici* CIAT 899 at sowing), of plants grown in pots containing 7 dm³ of soil, under greenhouse conditions.

Genotypes N-sources	BRS FC104	BRS Pontal	BRS Vereda	Mean
	Total N V4			
Control	ns	ns	ns	3.31 A
Fertilizer	ns	ns	ns	3.59 A
Rhizobium	ns	ns	ns	3.47 A
Mean	3.3 A	3.6 A	3.47 A	
	Ureide V4			
Control	16.43 Aa	9.26 Ba	24.30 Aa	16.66
Fertilizer	20.87 Aa	18.98 Bb	11.96 ABab	17.27
Rhizobium	22.05 Ab	34.93 Aa	15.92 Ab	24.3
Mean	19.78	21.06	17.39	

Genotypes N-sources	BRS FC104	BRS Pontal	BRS Vereda	Mean
	Total N R7			
Control	ns	ns	ns	3.31 A
Fertilizer	ns	ns	ns	3.55 A
Rhizobium	ns	ns	ns	3.37 A
Mean	3.28 A	3.49 A	3.47 A	
	Ureide R7			
Control	18.80 Ab	37.98 Aa	26.11 Ab	27.63
Fertilizer	9.50 Bb	36.67 Aa	5.57 Bb	17.25
Rhizobium	18.03 Ab	38.51 Aa	19.21 Ab	25.25
Mean	15.77	37.72	16.96	

According to the Tukey's test (p ≤ 0.05), means of individual genotypes (in columns), at the same developmental stage (V4 or R7), followed by different uppercase letters, are significantly different among N-sources. Similarly, means of individual N-sources (in rows), at the same developmental stage (V4 or R7), followed by different lowercase letters, are significantly different among genotypes.

Source: The author (2024).

3.5   CARBOHYDRATES

Starch and soluble sugars in the roots and leaves were affected by treatments. Regarding the leaf carbohydrates, plants of the long-cycle BRS Vereda fertilized with N accumulated more starch (Tab. 6) and soluble sugars (Tab. 6) than their counterpart plants inoculated with *Rhizobium* in both V4 and R7 stages (Tab. 6). Conversely, this same treatment accumulated less carbohydrates in the roots (Tab. 6).

On the other hand, plants inoculated with *Rhizobium* or nodulated with indigenous rhizobia that did not receive N accumulated less carbohydrates in the leaves in both V4 and R7 stages (Tab. 6). In the V4 stage, plants of the super-early BRS FC104 and the intermediary-cycle BRS Pontal accumulated more starch in the roots when they were inoculated with *Rhizobium* (Tab. 6). The same cultivars but forming nodules with indigenous rhizobia or receiving N-fertilizer had much lower starch concentration. A similar pattern was observed in the R7 stage (Tab. 6).

Table 6. Soluble and insoluble sugars (mg of glucose g^{-1} DW) in leaves of three genotypes of common (carioca) beans [FC104 (super-early-cycle BRS FC104), Pontal (intermediary-cycle BRS Pontal), and Vereda (long-cycle BRS Vereda)], subjected to three treatments: (i) Control (not fertilized with N-fertilizer, not inoculated), (ii) N-Fertilizer (treated with ca. 40 kg N ha^{-1} at emergence and ca. 40 kg N ha^{-1} at the R5 phenological stage), and (iii) *Rhizobium* (inoculated with *R. tropici* CIAT 899 at sowing), of plants grown in pots containing 7 dm³ of soil, under greenhouse conditions.

Genotypes N-source	BRS FC104	BRS Pontal	BRS Vereda	Mean	BRS FC104	BRS Pontal	BRS Vereda	Mean
	Glucose leaves V4				Glucose leaves R7			
Control	18.13 Ca	22.51 Ca	20.57 Ba	20,40	18.13 Ca	32.73 Ca	31.89 Ba	27.58
Fertilizer	85.27 Ab	110.60 Aa	55.13 Ac	83.66	85.27 Ab	112.39 Aa	78.26 Ab	91.97
Rhizobium	48.73 Bb	74.56 Ba	28.91 Ab	50.73	48.73 Bb	67.57 Bab	42.71 Bb	53
Mean	119.64	69.22	34.87		50.71	70.89	50.95	
	Starch leaves V4				Starch leaves R7			
Control	4.45 Ab	6.84 Aa	2.69 Cc	4.66	3.29 Bb	6.88 Aa	3.69 Bb	4.62
Fertilizer	3.86 Ab	3.72 Bb	9.61 Aa	5.73	2.01 Bb	3.30 Bb	7.33 Aa	4.21
Rhizobium	4.48 Ab	4.18 Bb	6.49 Ba	5.05	4.68 Aa	4.09 Ba	4.79 Aa	4.52
Mean	4.26	4.91	6.26		3.33	4.75	5.27	
	Glucose Roots V4				Glucose Roots R7			
Control	10.75 Cb	17.09 Bb	75.15 Aa	34.33	29.52 Bb	38.01 Bb	80.85 Aa	49.46
Fertilizer	109.45 Aa	82.86 Ab	27.18 Bc	73.16	100.02 Aa	78.52 Aa	40.36 Bb	72.96
Rhizobium	84.73 Ba	79.29 Aa	77.09 Aa	80.37	110.96 Aa	83.61 Ab	70.36 Ab	88.31
Mean	68.31	59.75	59.81		80.16	66.71	63.86	
	Starch roots V4				Starch roots R7			
Control	2.11 Ba	2.15 Ba	1.05 ABb	1.77	2.18 Ba	1.71 Ba	1.54 Aa	1.81
Fertilizer	1.51 Ba	0.80 Ca	1.40 Aa	1.24	1.47 Ba	0.39 Cb	1.39 ABa	1.08
Rhizobium	5.56 Aa	5.48 Aa	0.58 Bb	3.87	4.78 Ab	6.61 Aa	0.61 Bc	4
Mean	3.06	2.81	1.01		2.81	2.90	1.18	

Following Tukey's test (p ≤ 0.05), means of individual genotypes (in columns), at the same developmental stage (V4 or R7), followed by different uppercase letters, are significantly different among N-sources. Similarly, means of individual N-sources (in rows), at the same developmental stage (V4 or R7), followed by different lowercase letters, are significantly different among genotypes.

Source: The author (2024).

4   DISCUSSION

4.1   BNF IS LIMITED BY RHIZOBIUM INOCULATION, NOT BY PHOTOSYNTHESIS

The association between common beans and rhizobia certainly shows one of the most adaptable metabolisms among all legume symbioses. To date, this work was based on an experiment with three contrasting common bean genotypes and three contrasting N sources for the plants. Most of the variables were affected by the interaction of genotypes and N sources (Sup. Tab. 1), meaning that plants were consistently adjusting their metabolism to support their own C requirements. This implies that photosynthetic capacity and growth of different genotypes do not limit rhizobial nodule activity and biological N fixation, but *Rhizobium* inoculation with efficient strains does. Leaf photosynthesis is influenced by both N supply and C sink strength (Kaschuk et al., 2009; Baslam et al., 2021). Increased N supply raises the concentration of photosynthetic enzymes, particularly Rubisco, leading to enhanced leaf photosynthesis (Liu et al., 2020). The C sink strength provides feedback on enzymatic activity in the chloroplast (Ahouvi et al., 2022). C sink strength can be elevated through faster plant growth and pod formation (Tabatabaiepour et al., 2023) or metabolism, where nodule activity, consuming approximately 6 C per N fixed (Michin and Witty, 2005), should be considered. In the V4 stage, photosynthesis of BRS FC104 was stimulated by the C sink strength resulting from faster growth, utilizing N from its cotyledons (Tab. 2). In contrast, intermediate (BRS Pontal) and long-cycle (BRS Vereda) plants stimulated photosynthesis through both N supply (N fertilization) and C sink strength (increased nodule activity via *Rhizobium* inoculation). The impact of C sink strength was supported by data from *Rhizobium*-inoculated plants, which accumulated more starch in the roots (Tab. 6) and exhibited nodules with significantly higher enzymatic activity compared to plants forming nodules with indigenous rhizobia (Tab. 4).

Therefore, as far as CO_2 assimilation and C sink strength were concerned, it was evident that all three genotypes increased photosynthetic rates and distributed sufficient carbohydrates for growth and nodule activity (Tab. 2, 4, 6), in accordance with intrinsic mechanisms of photosynthesis regulation. Since there was no C limitation for nodule formation, all treatments exhibited a large number of nodules. However, nodules formed by indigenous rhizobia showed much less activity of GS enzymes compared to nodules formed by *Rhizobium* inoculated plants (Tab. 4), which could

suggest that common beans N fixation is limited by *Rhizobium* inoculation, but not by photosynthesis.

The common bean is a promiscuous host for rhizobia and can form an abundant nodule mass regardless of Rhizobium inoculation (this study, Kaschuk et al. 2006a, 2006b). Furthermore, indigenous rhizobia are highly diverse and competitive (Moura et al., 2020). Indigenous strains could serve to improve plant performance by other mechanisms of plant growth promotion (Moura et al., 2020), ordinarily they do not contribute to symbiotic BNF. Therefore, if the crop is not inoculated, nodules will be formed with indigenous strains, which appear to be less efficient than the inoculant (Shamseldin & Velázquez, 2020; Mendoza-Suárez et al., 2021; Mwenda et al., 2023). In this study, nodule activity of GS in the V4 stage was significantly higher in *Rhizobium*-inoculated plants than in the control (Tab. 4), but there were no significant differences in the other variables. On the other hand, in a field experiment developed by Hungria, Campo, & Mendes (2003) demonstrated that plants subjected to inoculation with *Rhizobium* (e.g., with strains H12, H20, PRF81, and CIAT899) accumulate similar shoot dry weights, N contents, and yields as N-fertilized plants fully satisfying the N requirements of the plants. In that regard, corroborating with previous studies, the results of this study suggest that common beans inoculated with *Rhizobium* have more stability in plant growth. Furthermore, it contributes to environmental safety since N derived from the symbiosis does not leach like the N from fertilizers does (Palmero et al., 2022).

4.2   CULTIVAR CYCLES AND FIXATION PERFORMANCE

This study involved three cultivars with varying cycle lengths, anticipating different responses to N sources. Interestingly, the cultivars adjusted their N metabolism to maintain similar N concentrations in the leaves, likely through different mechanisms of N acquisition simultaneously (Tab. 5). However, the intermediate-cycle BRS Pontal stood out as the cultivar that accumulated more ureides (Tab. 5), indicating a reliance on BNF (Kebede, 2021). More importantly, it was evident that this cultivar had the ability to sustain ureide translocation at the R7 stage, even in the presence of N-fertilizer (Tab. 5), highlighting a desirable attribute in common bean BNF breeding.

In fact, farmers, anticipating that common beans may fail to acquire N through rhizobial symbiosis, often apply N-fertilizers as both starter and top dressing in the cropping cycle. These applications of N are even recommended in official manuals for

crop fertilization (e.g., Pauletti and Motta, 2019). However, these N doses do not fully satisfy the crop's N demands and may even hinder nodulation and BNF (Vargas, Mendes & Hungria, 2000; Reinprecht et al., 2020). In this study, the application of N-fertilizer resulted in a decrease in petiole ureide concentration (Tab. 5) and GS-GOGAT nodule activities (Tab. 4) of the cultivars BRS FC104 and BRS Vereda, probably because they were relying on soil N instead of symbiotic BNF.

Decreases in BNF due to soil N uptake are often attributed to the N-feedback regulation of BNF (Salvagiotti et al., 2008; Pannecoucque et al., 2021), which may occur due to accumulation of nitrogenous compounds in the phloem sap, downregulating nitrogenase activity (Bacanamwo & Harper, 1996). The regulation of common bean BNF by soil N availability remains to be elucidated (Kawaka et al., 2018; Jiang et al., 2020; de Sousa et al., 2020). Thus, it is promising when a cultivar like BRS Pontal can sustain BNF despite the circumstantial availability of N in the soil. For example, further research should investigate whether the BRS Pontal cultivar can mitigate biochemical N feedback regulation of BNF (Fischinger et al., 2006), or if it is merely a coincidence due to its increased root and shoot formation in the early stages of development (Tab. 3).

Common bean is one of the crops with the lowest rates of BNF (Peoples et al., 2009), despite the breeding efforts to improve it. Some former literature already demonstrated that there was potential genetic material for supporting higher efficiency in BNF (Bliss, 1993), but still little has been advanced in that matter. For example, it has been shown that common bean varieties with a longer cycle may exhibit greater efficiency in fixing N compared to varieties with early cycles (Ruschel et al., 1982). Additionally, it has been suggested that common beans of type III may have better BNF performance than plants of types I and II (Handarson et al., 1993). In this study, the super-early BRS FC 104 and the long-cycle BRS Vereda showed the less likely results to have high efficiency of BNF, but they somewhat compensated with other mechanisms of N acquisition from the soil, probably from the degradation of soil organic matter (dos Santos Sousa et al., 2022).

On the other hand, the cultivar BRS FC104 represents an opportunity to enhance intercropping with non-legume crops. It has positive agronomic characteristics, including a grain yield of 2332 kg ha^{-1} during the rainy season, resistance to common mosaic virus and other pathogens, indicating potential resistance to phytosanitary problems (Melo et al., 2019). Notably, legume with a super-

early cycle stands out because it improves land utilization and harvest timing, reduces both biotic and abiotic stresses, and increases light use efficiency (Bedoussac & Justes, 2010). Legumes are often associated with the capacity to improve soil fertility through their symbiotic relationships with microorganisms (Duchene, Vian & Celette, 2017). Therefore, even if the crop does not reach our expectations of BNF, legume crops, such as the three cultivars of this study, recycle the N, preventing it to be wasted to the atmosphere.

5   CONCLUSIONS

The symbiosis of *Phaseolus vulgaris* L and *Rhizobium tropici* CIAT 899 it's harmonious for both of the organisms involved in this study. The experiments demonstrated that the photosynthesis could supply the vegetal physiology and prokaryotic carbon sink in all genotypes. The presence of *Rhizobium tropici* CIAT 899 and N-fertilizer stimulate the rates of photosynthesis, in BRS Pontal and Vereda (V4 and R7). The enzymatic activity in nodules of inoculated plants was higher than in the other treatments. Consequentially, the N in inoculated plants was supplied by symbioses. The sink of *Rhizobium tropici* CIAT 899 improved plant physiology development.

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Supplementary Table 1. The *p*-values of F test, obtained after the two-way variance analyses, considering the experiment that involved two factors: 1) three common bean (*P. vulgaris*) cultivars (BRS FC104, BRS Pontal, and BRS Vereda), and 2) three N sources (Control: not inoculated, not fertilized; N-fertilized; and inoculated with *Rhizobium tropici* CIAT 899).

Analyses	*p*- value		
	N source	Genotype	Genotype x source
Photosynthesis V4	< 0.0001	< 0.01	< 0.0001
Photosynthesis R7	< 0.05	< 0.1	< 0.0001
Photorespiration V4	< 0.0001	< 0.01	< 0.0001
Photorespiration R7	ns	ns	< 0.001
Shoot dry weight V4	< 0.0001	< 0.0001	< 0.0001
Shoot dry weight R7	< 0.0001	< 0.05	< 0.0001
Root dry weight V4	< 0.0001	< 0.0001	< 0.0001
Root dry weight R7	< 0.0001	< 0.0001	< 0.05
Total dry weight V4	< 0.0001	< 0.0001	< 0.0001
Total dry weight R7	< 0.0001	< 0.0001	< 0.0001
Starch Leaves V4	<0.05	< 0.0001	< 0.0001
Starch Leaves R7	ns	< 0.0001	< 0.0001
Starch Roots V4	< 0.0001	< 0.0001	< 0.0001
Starch Roots R7	< 0.0001	< 0.0001	< 0.0001
Glucose Leaves V4	< 0.0001	< 0.0001	< 0.0001
Glucose Leaves R7	< 0.0001	<0.01	< 0.01
Glucose Roots V4	< 0.0001	ns	< 0.0001
Glucose Roots R7	< 0.0001	< 0.05	< 0.0001
GOGAT V4	< 0.0001	<0.01	<0.05
GOGAT R7	< 0.0001	< 0.001	< 0.0001
GS V4	< 0.0001	< 0.0001	ns
GS R7	< 0.0001	<0.01	< 0.0001
Nodule weight V4	< 0.001	< 0.0001	< 0.0001
Nodule weight R7	< 0.001	<0.05	ns
Nodule numbers V4	< 0.05	<0.05	< 0.05
Nodule numbers R7	< 0.05	ns	ns
N% V4	ns	ns	ns
N% R7	ns	ns	ns
Ureide V4	< 0.001	ns	< 0.0001
Ureide R7	< 0.0001	< 0.0001	< 0.01

Legend: p value at < 0.0001= 99.9999% of chance in reject H0, at < 0.001 = 99.999% of chance in reject H0, at <0.01 = 99% of chance in reject H0, at <0.05 = 95% of chance in reject H0 and ns= non-significant.

Notes: 1. The experiment was performed under completely randomized design, with 12 replications. Six replications were harvested at V4 (four expanded leaves) and R7 (flowering) stages. 2. Before running, data for checked for the premisses of ANOVA. Only the data on nodule number had transformed by BoxCox to stabilize variance.