



Regulation of N_2 fixation and NO_3^-/NH_4^+ assimilation in nodulated and N-fertilized *Phaseolus vulgaris* L. exposed to high temperature stress



Mariangela Hungria^{a,*}, Glaciela Kaschuk^b

^a Embrapa Soja, C.P. 231, 86001-970 Londrina, PR, Brazil

^b UNIPAR, Campus Umuarama, 87502-210 Umuarama, PR, Brazil

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ABSTRACT

Legumes need large amounts of N to grow satisfactorily. Under low NO_3^- availability in the soil, many legumes meet their N requirements by N_2 fixation in association with rhizobia. Both NO_3^- uptake and N_2 fixation decrease as temperature exceeds optimal growth conditions, but the mechanisms of regulation of N_2 fixation and NO_3^-/NH_4^+ assimilation under high temperature stress are not completely understood. We describe an experiment in which physiological mechanisms regulating N metabolism of common bean (*Phaseolus vulgaris* L.) are investigated in plants submitted to daily maximum temperatures of 28, 34 and 39 °C. Common bean was grown in symbiosis with each of six rhizobial strains—belonging to four different species and varying in N_2 fixation effectiveness—or fertilized with NO_3^- until flowering. Harvest measurements included the activities of shoot, stem and root NO_3^- reductase (NR), nodule glutamine synthetase (GS), NADH-dependent glutamate synthase (GOGAT), nitrogenase, phosphoenol pyruvate carboxylase (PEPcase), N-export rates by nodules and concentration of N compounds in the xylem sap. Higher temperatures inhibited N_2 fixation resulting in lower proportion of ureide-N in nodules and xylem sap of nodulated plants in relation to amide-N and α -amino-N. Higher temperatures consistently reduced the activity of NR in leaves of N-fertilized plants. Higher temperatures also decreased N exported from nodules and activities of nitrogenase, GS, GOGAT and PEPcase. The rate of decreases varied in plants with different strains. Furthermore, the activities of GS and GOGAT were more strongly affected by high temperatures than the activity of nitrogenase. There was a remarkable increase in the concentration of NH_4^+ -N and ureide-N in the nodules when GS and GOGAT activities decreased. Therefore, the results provide evidence that N_2 fixation in common bean submitted to heat stress is limited by NH_4^+ assimilation via GS-GOGAT rather than by decreased activity of nitrogenase. Rhizobial effectiveness determined the degree of down-regulation of GS-GOGAT activity in nodule tissues.

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1. Introduction

Legumes need large amounts of N to grow satisfactorily. Under low NO_3^- availability in the soil, they meet their N requirements through N_2 fixation in association with rhizobia. Both uptake of soil NO_3^- and N_2 fixation decrease as temperature exceeds optimal growth conditions, and N_2 fixation is often more sensitive to high temperatures than leaf gas exchange and plant growth (Montañez

et al., 1995; Serraj and Adu-Gyamfi, 2004). We infer that N_2 fixation may limit productivity under a global-warming scenario.

Climate projections predict increases in annual average temperatures in many countries around the world. In Brazil, where present annual average temperatures vary from 22 °C in the south to 26 °C in the north, annual average temperatures are expected to rise by about 4 °C in the next six to ten decades (Cline, 2007). More importantly, whereas annual average temperatures may seem adequate, maximum air temperatures are often much higher (Marengo and Camargo, 2008), and soil temperatures frequently exceed 40 °C in the summer, the main cropping season (Hungria and Vargas, 2000).

Legumes such as common bean (*Phaseolus vulgaris* L.) perform best when grown under temperatures ranging from 15 °C to 30 °C (van deer Maesen and Somaatmadja, 1989) and grain yield is likely to be adversely affected when temperatures at bloom stage exceed 30 °C (Wahid et al., 2007). In common bean, nodule formation and activity are inhibited by soil temperatures of 38 °C and

Abbreviations: NR, nitrate reductase; GS, nodule glutamine synthetase; GOGAT, NADH-dependent glutamate synthase; PEPcase, phosphoenol pyruvate carboxylase; PHB, poly- β -hydroxybutyrate.

* Corresponding author. Tel.: +55 4333716206; fax: +55 4333716100.

E-mail addresses: mariangela.hungria@embrapa.br, hungria@pq.cnpq.br, biotecnologia.solo@hotmail.com (M. Hungria), glaciela.kaschuk@gmail.com (G. Kaschuk).

32 °C, respectively (Hernandez-Armenta et al., 1989), but significant decreases in N₂ fixation have been observed just above 28 °C (Hungria and Franco, 1993). High soil temperatures can affect N₂ fixation by reducing the viability of rhizobia in soil (Hungria and Vargas, 2000), and in the free-living state several proteins in the Rhizobium are expressed to mitigate adverse effects of temperature stress (Gomes et al., 2012). Also, high temperatures hamper the release of *nod*-gene inducers from the host plant (Hungria and Stacey, 1997), as well as *nif*-gene expression (Michiels et al., 1994). High temperatures also inhibit nitrogenase activity (Hernandez-Armenta et al., 1989; Hungria and Franco, 1993; Montañez et al., 1995; Hungria and Vargas, 2000), but little is known about the feedback responses between nitrogenase activity and other enzymes related to N metabolism.

An effective symbiosis requires a coordinated expression of genes of both the bacterium and the plant (Neves and Hungria, 1987; Gonnet and Díaz, 2000; Vance and Lamb, 2001). In legume-rhizobia symbioses without environmental constraints, atmospheric N₂ is reduced by nitrogenase (EC 1.18.6.1) to NH₄⁺ in the bacteroids; the NH₄⁺ is quickly exported to the cytosol of plant cells, where it is assimilated via the glutamine synthetase (GS; EC 6.3.1.2)–NADPH-glutamate synthetase (GOGAT; EC 1.4.1.14) pathway to form glutamine and glutamate, respectively. These N-compounds are incorporated into aspartate and asparagine, and further exported as ureides-N or amides-N, depending on the plant species. In the case of predominance of ureide-N-transporter nodules—such as common bean—N-compounds are metabolized into allantoin and allantoic acid via a purine pathway and transported through xylem to shoots. The C skeletons required for N assimilation originate from the oxidation of malate to oxaloacetate, and from the activity of phosphoenolpyruvate carboxylase (PEPcase; E.C. 4.1.1.31), which returns some of the respired CO₂ back to the nodules (Neves and Hungria, 1987; Gonnet and Díaz, 2000; Vance and Lamb, 2001). If not all consumed, the C skeletons supplied to nodules are stored as poly-β-hydroxybutyrate (PHB) in the bacteroids (Bergersen et al., 1991). Maintenance of symbiotic N₂ fixation under environmental stress is likely to be dependent on the perfect match of the plant variety and the rhizobial strain nodulating it (Talbi et al., 2012, this study). When plants rely mainly on NO₃⁻ for N uptake, nitrate reductase (NR; EC 1.7.99.4) performs the first steps of NO₃⁻ assimilation. These steps are coordinated depending on the N demands of the plant, photosynthate availability and environmental conditions.

This study aimed at understanding the regulation of N metabolism, including enzymatic activities, the accumulation of N-compounds and transport through xylem in common-bean plants inoculated with one of six different *Rhizobium* spp. strains, or fertilized with nitrate, and submitted to daily maximum temperatures of 28 °C, 34 °C or 39 °C. We provide evidences that rhizobial effectiveness may influence the activity of plant enzymes in the nodules, which allows plants to tolerate moderate high temperature stress.

2. Materials and methods

2.1. Plant and bacterial material

The experiment was performed with common bean (*P. vulgaris* L.) cultivar Negro Argel (a small, black-seeded cultivar of indeterminate growth habit, semi-climbing, type III, Mesoamerican origin). Six rhizobial strains were used as inocula, and, based on the analysis of the 16S rRNA gene (data not shown), were classified as: *Rhizobium tropici* strains BR 814 and BR 817 (originally isolated from *Leucaena* sp. in Brazil) and CIAT 899^T (isolated from common-bean nodules in Colombia); *Rhizobium leucaenae* CFN 299^T (isolated from common-bean nodules in Brazil); *Rhizobium leguminosarum* bv. phaseoli CNPAF 126 (isolated from common-bean nodule in Brazil);

and *Bradyrhizobium elkanii* BR 6010 (originally isolated from *Lonchocarpus* sp. in Brazil). In a previous study, the strains varied in N₂-fixing efficiency under high-temperature conditions such that, CIAT 899, BR 817 CNPAF 126 and BR 6010 were more tolerant than CFN 299 and BR 814 (Hungria et al., 1993).

2.2. Experimental setup

Common-bean plants of cultivar Negro Argel were grown in polystyrene pots, each containing 2 kg of sand, in growth chambers with controlled light and temperature conditions (Eaglesham et al., 1983). Plants were inoculated with *Rhizobium* strains under three temperature regimes: 28 °C, 34 °C or 39 °C for 8 h/day, and 23 °C for the rest of the day and night. To assure that enzymatic responses were not influenced by suboptimal growth due to N limitation under symbiotic conditions, we set a control with non-inoculated plants fertilized with KNO₃. Measurements indicated that temperatures around the root system oscillated within 1 °C of the air temperatures. Chamber lamps supplied 800 μmol photons m⁻² s⁻¹ for a daily 12-h photoperiod. Relative humidity in the chamber ranged from 60% to 80%. Water deficit was prevented by keeping water in the pot saucers.

Temperatures were not replicated because of the availability of just three controlled-environment chambers; pots were randomized in seven blocks inside each chamber.

For inoculation, rhizobia were grown in yeast-mannitol medium for five days at 28 °C under agitation (Vincent, 1970). Common-bean seeds were surface sterilized (Vincent, 1970) and treated with 1 mL of rhizobial suspension (approximately 10⁹ cells mL⁻¹) per 15 seeds and incubated for 1 h. At sowing, pots were covered with approximately 2.5 cm of styrofoam beds to reduce surface evaporation and the risk of airborne contamination. Five days after emergence (5 DAE) plants were thinned to two per pot.

All plants received 250 mL of N-free nutrient solution twice a week (Eaglesham et al., 1983). Non-inoculated controls received 50 mg of N as KNO₃ at the first week and afterwards 75 mg of N per week until totaling 350 mg of N by the fifth week, and no nodules were observed in these plants. Two harvests were performed, the first at early vegetative growth stage, at 18 days after emergence (DAE), and the second at full flowering, at 38 DAE.

At harvest, four blocks were used for evaluation of nitrogenase activity, H₂ evolution and xylem-sap sampling, as described below. In the other three blocks, one plant of each treatment in each block was taken to determine nodule PEPcase activity in vivo, and NR activity in leaves, stems and roots. Nodules from the other plants were quickly picked off, frozen in liquid nitrogen and kept at -80 °C until analyses of GS and GOGAT activities and protein content. For dry weight, total N and ureide-N in tissues, four replicates were used. At harvest, shoots (leaves and stems), roots and nodules were detached from the plant, dried to constant weight at 75 °C and weighed.

Analysis of variance (ANOVA) was used to evaluate least significant differences ($p \leq 0.05$) as described before (La Fravre and Eaglesham, 1986). First, data were analyzed considering two-way analyses, having plants (inoculated and N-fertilized) and temperature as the main factors. Since the interactions between factors were not significant in all parameters, results were reanalyzed considering one-way ANOVA. Linear relationships between variables were tested with the Pearson correlation coefficient.

2.3. Enzyme activities

2.3.1. Nitrogenase activity, H₂ evolution and relative efficiency of nitrogenase

Nitrogenase activity was estimated by the acetylene reduction assay (ARA) in a continuous flow assay, as described before (LaRue

et al., 1975). Measurements were evaluated after 3 min of incubation per plant. Ethylene was analyzed in a Perkin-Elmer 3920 B gas chromatograph, equipped with a H₂ flame-ionization detector, operated at 110 °C, with N₂ as the carrier gas and a flow rate of 100 mL min⁻¹. Hydrogen evolution was also evaluated in the same samples, also in a Perkin-Elmer chromatograph, but equipped with a thermal conductivity detector. Relative efficiency (RE) of electrons used by nitrogenase was estimated as described by Schubert and Evans (1976), according to the equation:

$$RE = \left[\frac{1 - H_2(\text{air})}{C_2H_2} \right]$$

2.3.2. Glutamine synthetase (GS)

The GS was extracted by grinding about 0.5 g nodules with a pestle in a mortar using the extraction buffer described by Kendall et al. (1986). After centrifugation at 20,000 × g for 30 min at 4 °C, the supernatants were desalted in Sephadex G-25 columns with a solution containing 2-amino-2(hydroxymethyl)-1,3 propanediol (Tris)-HCl pH 7.8, 10 mM MgSO₄, 10% ethanediol and 5 mM dithiothreitol. Samples of 50 μL of nodule extract were assayed for transferase (Cullimore and Sims, 1980) and semi-biosynthetic hydroxylamine (Cullimore and Lea, 1982) at 4 °C.

2.3.3. NADH-dependent glutamate synthase (GOGAT)

GOGAT was determined as described before (Hungria et al., 1991), slightly modified. The enzyme was extracted by grinding about 0.5 g nodules with a pestle in a mortar using extraction buffer A (50 mM potassium phosphate pH 7.5, 5 mM dithiothreitol, 1% (v/v) 2-mercaptoethanol, 10% ethanediol) and 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethyl sulphonyl fluoride and 0.05% triton X-100. The homogenate was filtered through Miracloth and centrifuged at 20,000 × g for 30 min. The supernatant was desalted in Sephadex G-25 columns, previously equilibrated with buffer A. The enzyme assays of 50 μL of nodule extract were performed with a reaction mixture containing, in a total volume of 1 mL: 100 mM potassium phosphate pH 7.5; 1% mercaptoethanol; 1 mM 2-oxoglutarate; 0.1 mM NADH; and 0.1 mM EDTA. Reactions were evaluated by reading absorbance at 340 nm and 30 °C before and after the addition of 5 mM L-glutamine.

2.3.4. Nitrate reductase (NR)

NR activity was evaluated according to Andrews et al. (1984) on leaf discs of approximately 4 mm² (weighing 0.2–0.3 g), stems (0.3–0.4 g) and roots (0.5–0.7 g).

2.3.5. Phosphoenol pyruvate carboxylase (PEPcase)

PEPcase activity of nodules *in vivo* was determined according to Maxwell et al. (1984), with minor modifications. After extraction in a 45 °C waterbath, 1 mL of 0.1 M HCl was added to each vial. Samples were left in a sterile hood overnight or until dry. Ten milliliters of scintillation liquid were added to each vial and acid-stable radioactivity values were determined by liquid scintillation counting (LSC). Fixation of CO₂ was calculated from the associated radioactivity of root tissue.

2.4. Transport of N compounds in the xylem sap

Plants were decapitated below the cotyledonary node; the cut ends were rinsed with distilled water and dried with tissue paper. Bleeding sap was collected for 10 min in calibrated microcapillaries, so that the exudation rate could be estimated. Sap was stored at -20 °C until analysis. Determinations of ureide-N, α-amino-N, ammonium-N, amide-N, nitrate-N and total-N in the xylem sap were made on aliquots of 5 μL of sap, according to Boddey et al. (1987).

2.5. N compounds in tissues

Total plant-N values at 38 DAE (shoots, roots and nodules) were determined using 100-mg samples of dry, ground (35 mesh) tissue. Samples were digested in 6 mL of concentrated H₂SO₄ with one tablet of tecator catalyst (Tecator, Sweden), containing 1.5 g K₂SO₄ and 0.0075 g Se, for 2.5 h after clearing. Total N was then analyzed colorimetrically in an autoanalyser (Scientific Analytical Cartridge AC 200, Scientific Instruments Corporation, Pleasantville, NY).

Ureides-N were extracted from dried plant material (shoots, roots and nodules) with 0.1 M phosphate buffer pH 7.0 containing 50% ethanol at 80 °C for 5 min. Extracts were then filtered through four layers of cheesecloth, centrifuged, and 50-μL aliquots were used to analyze ureide-N, as described before (Hungria et al., 2006).

Ammonia was extracted from 100 mg of frozen nodules with 750 μL of 0.1 M HCl. Nodule extracts were placed in Eppendorf tubes and centrifuged for 10 min, and supernatants were transferred to clean tubes. To remove proteins, 50 μL aliquots of supernatant were added to 25 μL of Na-tungstate (10%, v/v) and 25 μL of 1 N H₂SO₄ tubes and centrifuged for 10 min. Supernatants were then transferred to clean tubes and ammonia analyzed according to Boddey et al. (1987).

2.6. Electron microscope studies

Excised nodules from the first harvest were maintained in fixative [3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.8] at 4 °C for 4 h. After four buffer rinses, the tissue was post-fixed in 2% (w/v) osmium tetroxide for 1 h at 4 °C, then rinsed twice with the buffer, two times with distilled water, dehydrated using an ethanol series and embedded in Spurr's epoxy resin. Thin sections were cut with a diamond knife, post-stained with 4% uranyl acetate and viewed with a Zeiss 10 transmission electron microscope.

3. Results

3.1. Enzymes activities

Nitrogenase activity (ARA) and the relative efficiency of nitrogenase (RE) indicated that the most efficient rhizobial strains were *R. tropici* BR 814 and *R. leguminosarum* bv. phaseoli CNPAF 126, and the least efficient was *R. tropici* BR 817 (Table 1). This was observed both at the early vegetative stage (18 day after emergence, DAE) and at full flowering (38 DAE). Considering the average for all of the strains at 18 DAE, ARA at 28 °C (9.1 μmol C₂H₄ plant⁻¹ h⁻¹) decreased by 22% when temperature rose from 28 °C to 34 °C, with a further decrease of 64% when the temperature rose to 39 °C (Table 1). Similar results were observed at 38 DAE.

The assimilation of N-NO₃⁻ in N-fertilized common-bean plants occurred mostly in the leaves; NR activity was much higher in leaves than in stems of roots, both at 18 and 38 DAE (Fig. 1). At 18 DAE, NR activity in leaves and stems was affected only by the highest temperature of 39 °C, but at 38 DAE the activities decreased at both 34 °C and 39 °C (Fig. 1).

High temperatures negatively affected the activity of all enzymes related to N metabolism at full flowering (Table 2). GS and NADH-GOGAT are produced and regulated by plant metabolism, but the experiment revealed differences in GS and NADH-GOGAT activities probably triggered by differences on the strain-specific activities (Table 2). At 28 °C, strains BR 814, BR 6010 and CNPAF 126 achieved rates of NADH-GOGAT activity one and half times higher than CIAT 899 and CFN 299, and six to seven times higher than BR 817. Similar trends existed for GS activity (Table 2). On average, GS activity at 28 °C (62.8 μmol γ-glutamyl mg⁻¹ protein min⁻¹) decreased 31% when

Table 1

Temperature effects on nitrogenase activity and relative efficiency in nodules of *Phaseolus vulgaris* L. cv. Negro Argel associated with six different rhizobial strains. Plants were grown under controlled-environment conditions and harvested at early vegetative growth (18 days after emergence, DAE) and full flowering (38 DAE).

Strain	T (°C)	Nitrogenase activity (ARA, $\mu\text{mol plant}^{-1} \text{h}^{-1}$)		Relative efficiency RE = $[1 - \text{H}_2(\text{air})]/\text{C}_2\text{H}_2$	
		18 DAE	38 DAE	18 DAE	38 DAE
<i>R. tropici</i> BR 814	28	13.2 a	46.6 a	0.92 a	0.94 a
	34	9.0 bcd	36.2 abc	0.90 ab	0.91 ab
	39	2.4 gh	9.2 hij	0.67 efg	0.67 ef
<i>R. tropici</i> BR 817	28	6.9 def	13.9 fghij	0.80 bcd	0.83 abcd
	34	1.8 gh	10.7 ghij	0.76 cde	0.76 cde
	39	1.1 gh	2.35 j	0.60 g	0.75 cde
<i>B. elkanii</i> BR 6010	28	2.2 g	28.3 bcd	0.76 cde	0.87 abc
	34	2.2 g	23.6 def	0.80 abcd	0.83 abcd
	39	1.0 h	12.9 fghij	0.74 def	0.74 cde
<i>R. tropici</i> CIAT 899	28	7.7 cde	25.7 cde	0.80 bcd	0.86 abc
	34	7.5 cde	15.8 efg	0.86 abc	0.78 cde
	39	2.6 gh	4.6 ij	0.59 g	0.57 f
<i>R. leucaena</i> CFN 299	28	11.1 abc	19.8 defgh	0.86 abc	0.85 abc
	34	10.8 abc	22.1 defg	0.86 abc	0.84 abc
	39	3.3 f	10.8 ghij	0.64 fg	0.71 de
<i>R. leguminosarum</i> bv. phaseoli CNPAF 126	28	13.7 a	40.2 ab	0.90 ab	0.92 ab
	34	11.8 ab	29.8 bcd	0.86 abc	0.88 abc
	39	4.8 efg	12.9 fghij	0.70 defg	0.80 bcd
LSD 5%		3.8	11.7	0.11	0.13

Values represent the means of four replicates ($n=4$) and when followed by the same letter, in the same column, do not show statistical difference by Tukey's test ($p \leq 0.05$).

temperature rose from to 34°C, with a further decrease of 52% at 39°C. For the NADH-GOGAT, activity at 28°C ($174.5 \mu\text{mol NADH mg}^{-1} \text{protein min}^{-1}$) decreased 31% when temperature rose to 34°C and decreased a further 76% when temperature rose to 39°C (Table 2).

There were positive significant correlations ($p \leq 0.005$) between nitrogenase activity (ARA, $\mu\text{mol C}_2\text{H}_4 \text{plant}^{-1} \text{h}^{-1}$) and GS ($\mu\text{mol } \gamma\text{-glutamyl mg}^{-1} \text{protein min}^{-1}$) ($r=0.92$), and between ARA and NADH-GOGAT ($\mu\text{mol NADH mg}^{-1} \text{protein min}^{-1}$) ($r=0.85$).

Tolerance of high temperatures was also correlated with nodule capacity to maintain high PEPcase activity, and, for plants growing at 34°C and 39°C, a significant positive correlation

occurred between nitrogenase (ARA) and PEPcase ($\mu\text{mol CO}_2 \text{g}^{-1} \text{protein min}^{-1}$) activities ($r=0.85$) was shown. Generally, high temperatures significantly decreased PEPcase activity, except in the cases of inoculation with strains BR 814, BR 6010 and CFN 299 when the temperature was increased from 28°C to 34°C (Table 2). In the case of BR 817 and CIAT 899, increases in temperature consistently decreased PEPcase activity by approximately half of its initial value (Table 2).

For all treatments, there were not only significant decreases in nodule total-protein content (Table 2), but also enzyme activities per gram of protein decreased remarkably (data not shown). An important point to mention is that concentrations of N-NH_4^+ in

Table 2

Temperature effects on nodule N metabolism of *Phaseolus vulgaris* L. cv. Negro Argel associated with six different rhizobial strains. Plants were grown under controlled-environment conditions and harvested at full flowering (38 days after emergence).

Strain	T (°C)	PEPcase ($\mu\text{mol CO}_2 \text{g}^{-1} \text{DW h}^{-1}$)	GS transferase ($\mu\text{mol } \alpha\text{-glutamyl g}^{-1} \text{FW min}^{-1}$)	NADH-GOGAT ($\mu\text{mol NADH g}^{-1} \text{FW min}^{-1}$)	Total protein ($\text{mg g}^{-1} \text{FW nodule}$)	N-NH ₄ ⁺ ($\mu\text{g g}^{-1} \text{FW nodule}$)	Ureide-N ($\mu\text{g g}^{-1} \text{DW nodule}$)
<i>R. tropici</i> BR 814	28	55.6 b	86.8 A	238.8 a	15.8 a	1.8 f	2001.5 bcd
	34	49.6 bc	67.9 Bc	149.7 b	13.8 abc	2.0 f	1139.0 Ef
	39	31.3 de	12.5 Hij	38.6 de	8.9 d	15.3 b	2234.5 B
<i>R. tropici</i> BR 817	28	20.3 fgh	14.4 Hi	33.6 de	9.6 bcd	3.4 def	605.0 Fg
	34	8.0 ij	8.2 lj	20.2 de	7.8 d	2.8 ef	487.8 G
	39	4.7 j	1.4 J	2.7 e	3.7 e	16.0 b	1111.1 Ef
<i>B. elkanii</i> BR 6010	28	33.2 de	84.2 a	215.8 a	16.0 a	3.3 def	2381.8 Ab
	34	23.0 efg	61.5 cd	167.4 b	12.5 ab	3.9 def	1418.2 De
	39	11.6 hij	49.5 def	49.5 d	9.2 c	18.6 b	2587.5 Ab
<i>R. tropici</i> CIAT 899	28	31.0 def	58.5 cde	165.7 b	14.1 ab	5.6 cde	2046.8 Bc
	34	16.3 ghij	30.5 g	106.8 c	12.6 abcd	5.3 cdef	1440.5 De
	39	6.6 j	23.5 gh	23.5 de	7.9 de	22.3 a	2182.0 B
<i>R. leucaena</i> CFN 299	28	48.7 bc	54.2 def	167.4 b	11.9 abcd	6.6 cd	1506.2 cde
	34	40.4 cd	44.3 f	142.3 bc	10.9 bcd	6.7 cd	988.2 Efg
	39	11.4 hij	20.5 gh	20.5 de	9.2 cd	25.0 a	2398.0 Ab
<i>R. leguminosarum</i> bv. phaseoli CNPAF 126	28	66.8 a	78.8 ab	249.6 a	15.8 a	6.5 cd	2307.5 B
	34	37.5 d	48.0 ef	130.0 bc	12.0 abcd	8.6 c	1122.5 Ef
	39	18.0 ghi	18.3 hi	33.4 de	9.5 bcd	22.9 a	2935.0 A
LSD 5%		10.7	12.0	41.2	4.8	3.6	600.0

Values represent the means of four replicates ($n=4$) and when followed by the same letter, in the same column, do not show statistical difference by Tukey's test ($p \leq 0.05$).

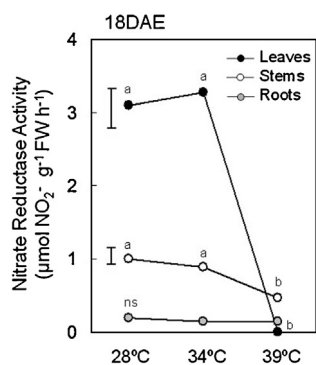


Fig. 1. Nitrate reductase activity of common-bean plants receiving 75 mg of N per week as KNO_3 at 18 and 38 days after emergence (DAE), grown at 28, 34 and 39 °C. Data represent the means of four replicates ($n=4$) and when followed by the same letter, for each plant tissue, do not show statistical difference (Tukey's test; $p \leq 0.05$).

the nodules increased by 76% when temperature rose from 34 °C to 39 °C, but no significant changes were observed with the increase from 28 °C to 34 °C (Table 2). With the increase from 28 °C to 34 °C, nodule ureide-N across different strains decreased by 20–40%, but, as temperature increased to 39 °C, nodule ureide-N returned to their initial values (Table 2). As a matter of fact, the same plants with higher activities of GS, NADH-GOGAT and PEPcase (BR 814, BR 6010 and CNPAF 126) accumulated more ureides-N in the nodules than those with lower activities (e.g. BR 817).

3.2. Transport of N compounds in the xylem sap

Both N-fertilized and nodulated plants transported less N in the xylem sap when grown under higher temperatures, particularly at 39 °C (Fig. 2). Nodulated plants associated with strain BR 817 always transported less N in the xylem sap than the others. Rises in temperature from 28 to 34 °C decreased the transport of N compounds in the xylem sap by 14% and 24% in nodulated and N-fertilized plants, respectively. Further increases in temperature, from 34 to 39 °C, resulted in decreases of 55% in nodulated and 44% in N-fertilized plants (Fig. 2).

As expected, when N-fertilized and nodulated plants were compared, N-fertilized plants always transported more N-NO_3^- , whereas nodulated plants always transported more ureide-N in the xylem sap (Fig. 3). However, nodulated plants grown at higher temperature (39 °C) had a significantly smaller proportion of ureide-N

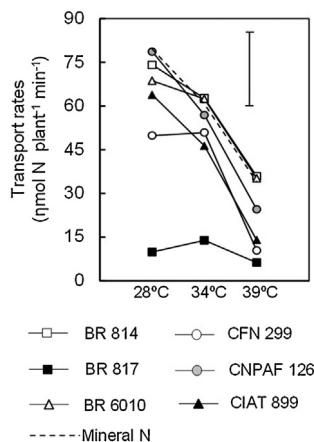


Fig. 2. Transport rates of N compounds in the xylem sap of nodulated (with strains BR 814, BR 817, BR 6010, CFN 299, CNPAF 126 and CIAT 899) or N-fertilized (75 mg of N as KNO_3 per week) common-bean plants grown at 28, 34 and 39 °C. Data represent the means of four replicates ($n=4$) and the bar indicates LSD at $p \leq 0.05$ (Tukey's test).

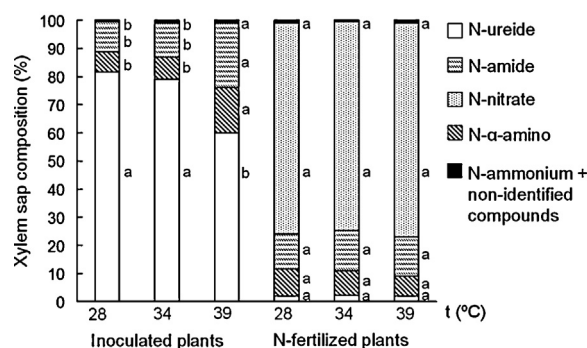


Fig. 3. Xylem-sap composition of nodulated (with strains BR 814, BR 817, BR 6010, CFN 299, CNPAF 126 and CIAT 899) or N-fertilized (75 mg of N as KNO_3 per week) common bean grown at 28, 34 and 39 °C. Evaluation performed at 38 days after emergence. Data represent the means of 24 (nodulated; $n=24$) and four (N-fertilized; $n=4$) replicates, and when followed by the same letter, within each group of N-compound, do not show statistical differences (Tukey's test, $p \leq 0.05$). A prior analysis revealed that there were no significant differences in the patterns among different strains.

in relation to amide-N and α -amino-N than their counterpart plants grown at lower temperatures (28 °C and 34 °C) (Fig. 3).

3.3. Plant growth, N accumulation in tissues and electron microscope observations

Rhizobial strain and temperature affected nodule biomass, which in turn, was closely related to shoot biomass and total plant N (Table 3). At 18 DAE and 28 °C, nodulated plants associated with rhizobial strains CIAT 899, CFN 299 and CNPAF 126 accumulated similar shoot dry weights and total N values as plants fertilized with KNO_3 , whereas plants associated with BR 814, BR 817 and BR 6010 showed poorer performance. Except for plants associated with CIAT 899 and CFN 299 and mineral N, shoot dry weights at this stage (18 DAE) were hardly affected by 39 °C. However, at the later stage (38 DAE), all plants suffered with both high temperatures, and shoot dry weights were consistently lower at 34 °C and 39 °C than at 28 °C (Table 3). Both in early and later stages, plants accumulate less total N, and total ureide-N in their tissues when submitted to high temperatures. On average, ureide-N values in the shoots were reduced by 33% from 28 °C to 34 °C, and by 75% from 34 °C to 39 °C (Table 3).

Our main objective with the microscopy was to verify if structures of carbon storage—PHB—would be decreased or depleted at high temperatures. However, for all strains, nodules were able to accumulate PHB in the bacteroids regardless of temperature regime.

4. Discussion

Down-regulation of N metabolism in common bean submitted to high temperatures was evidenced in the two routes of N acquisition: N_2 fixation and subsequent NH_4^+ assimilation as well as NO_3^- reduction. Relevant literature attributes down-regulation of N_2 fixation to limitation in carbon availability or due to N-feedback (e.g. Vance and Lamb, 2001). Along with other carbohydrate related enzymes, e.g. sucrose synthase (Silvente et al., 2003), PEPcase activity collaborates with N_2 fixation by supplying energy for nitrogenase activity and C-skeletons for ureide biosynthesis (Neves and Hungria, 1987). In this study, activities of both PEPcase and nitrogenase decreased as temperatures increased (Table 2), but these results did not indicate that N_2 fixation was limited by carbon availability at high temperatures. As a matter of fact, microscope studies revealed that nodules growing at either 28 or 39 °C accumulated large amounts of PHB (data not shown), demonstrating that,

Table 3

Temperature effects on growth and N accumulation of *Phaseolus vulgaris* L. cv. Negro Argel associated with six different rhizobial strains or receiving N-fertilizer (75 mg of N as KNO₃ per week). Plants grown under controlled-environment conditions and harvested at early vegetative growth (18 days after emergence, DAE) and full flowering (38 DAE).

Strain/N	T (°C)	Nodule dry weight (mg plant ⁻¹)		Shoot DW (g plant ⁻¹)		Total plant N ^a (mg plant ⁻¹)		Shoot Ureide-N (mg plant ⁻¹)
		18 DAE	38 DAE	18 DAE	38 DAE	18 DAE	38 DAE	38 DAE
<i>R. tropici</i> BR 814	28	96.8 cd	291.0 a	0.5 d	6.2 ab	19.7 d	219.5 a	15.4 A
	34	89.0 cde	213.8 ab	0.3 de	5.1 abcd	14.3 de	173.7 ab	11.1 Bc
	39	41.2 bcd	123.8 bcd	0.2 de	3.4 efg	9.2 efg	78.4 ef	3.5 Ef
<i>R. tropici</i> BR 817	28	50.0 defg	73.5 d	0.3 de	2.8 gh	11.4 def	66.1 f	1.8 Fgh
	34	42.8 efg	75.8 d	0.3 de	2.7 gh	9.0 efg	58.9 f	1.4 Fgh
	39	29.5 fg	98.8 cd	0.3 de	1.7 h	7.5 efg	35.9 f	0.5 H
<i>B. elkanii</i> BR 6010	28	31.0 fg	181.5 bc	0.2 de	5.4 abcd	8.5 efg	204.9 ab	12.8 B
	34	16.0 g	129.5 bcd	0.3 de	5.3 abcd	9.2 efg	204.2 ab	11.6 B
	39	17.0 g	130.0 bcd	0.3 de	3.1 fg	7.6 efg	93.2 cdef	2.9 Efg
<i>R. tropici</i> CIAT 899	28	123.8 bc	119.8 bcd	1.3 a	5.8 abc	46.3 ab	199.6 ab	10.7 Bc
	34	84.8 cde	165.0 bcd	1.0 abc	4.3 def	35.5 c	151.6 abcd	4.1 E
	39	79.8 def	126.8 bcd	0.4 de	3.0 gh	13.4 de	73.7 ef	1.2 Gh
<i>R. leucaena</i> CFN 299	28	208.8 a	190.0 bc	1.3 a	4.5 cde	51.9 a	142.2 bcde	8.1 D
	34	163.5 ab	290.0 a	1.2 ab	5.0 bcd	41.8 bc	157.8 abc	4.8 E
	39	67.8 defg	213.2 ab	0.3 de	2.8 gh	13.2 de	79.4 def	1.2 Gh
<i>R. leguminosarum</i> bv. phaseoli CNPAF 126	28	137.0 abc	211.0 ab	1.3 a	6.3 ab	52.8 a	221.7 a	16.0 A
	34	151.0 b	170.2 bc	0.8 c	5.0 bcd	32.9 c	171.9 ab	10.5 C
	39	61.2 defg	199.0 ab	0.3 de	3.1 fgh	12.8 de	74.6 ef	1.6 Fgh
Mineral N	28			1.3 a	6.4 a	46.7 ab	218.5 a	
	34			1.0 ab	5.1 abcd	34.5 c	166.8 ab	
	39			0.4 de	2.9 gh	10.1 defg	80.3 def	
LSD 5%		53.3	94.3	0.2	1.3	9.8	72.6	2.1

Values represent the means of four replicates ($n = 4$) and when followed by the same letter, in the same column, do not show statistical difference by Tukey's test ($p \leq 0.05$).

^a N in shoot + roots + nodules.

despite heat stress, there were abundant supplies of C to the nodules. Indeed, other studies have demonstrated that symbiotic N₂ fixation is not limited by carbon availability due to sink stimulation of photosynthesis (Kaschuk et al., 2009, 2010, 2012).

The hypothesis of N-feedback regulation of N₂ fixation claims that surplus nitrogenous organic compounds translocated via phloem from the shoot into nodules regulate N₂ fixation in the legume symbiosis (Parsons and Sunley, 2001; King and Purcell, 2005). The more N in the phloem sap entering the nodule, the lower the N₂ fixation. For example, decreases in nitrogenase activity of defoliated white clover (*Trifolium repens* L.) and white lupin (*Lupinus albus* L.) were accompanied by increases in the concentration of amino acids in the vascular tissue (Hartwig and Trommler, 2001). Decreases in leaf transpiration and xylem translocation from roots to shoots caused by drought resulted in accumulation of ureide-N in the nodules and decreased N₂ fixation (Serraj et al., 1999; Ramos et al., 2005; Sinclair et al., 2007). In our study, decreases in nitrogenase activity (ARA) (Table 1) were related to increases in ureide-N in the nodules (Table 2) when temperature rose from 34 °C to 39 °C. In this case, N₂ fixation is likely not associated with decreased leaf transpiration because high temperature without water deprivation promotes stomatal opening and increased leaf transpiration (Reynolds-Henn et al., 2010). Yet, when the temperature rose from 28 °C to 34 °C, both Ureide-N content and ARA activity decreased. Thus, it is not clear whether N-feedback regulation, related to the synthesis and accumulation of N-amino acids and ureide-N in the phloem sap and the nodules (Parsons and Sunley, 2001; King and Purcell, 2005), explains decline of symbiotic N₂ fixation under high temperature stress. As NH₄⁺ is the first stable product of nitrogenase activity (Neves and Hungria, 1987), it would be expected that increases in the concentration of NH₄⁺ would result in a much stronger negative feedback on N₂ fixation.

Furthermore, following expectations of N metabolism in nodulated versus N-fertilized plants (Hungria and Neves, 1987a,b;

Kaschuk et al., 2010), Fig. 3 shows that nodulated plants accumulated more ureide-N than any other type of N-compound in the sap when compared with N-fertilized plants, regardless of temperature. However, the proportion of ureide-N significantly decreased at the temperature of 39 °C, showing that decreased N₂ fixation (Table 1) also resulted in lower ureide-N concentration in the xylem sap. Our data do not allow inferences on whether high temperatures could have down-regulated ureide biosynthesis (Tajima et al., 2004; Alamillo et al., 2010). In fact, in the route from N₂ to ureide-N, several enzymes potentially regulate N metabolism in legume nodules exposed to environmental stresses (Neves and Hungria, 1987; Vance and Lamb, 2001; Christophe et al., 2011). But, since NH₄⁺ increased in nodules as temperature rose (Table 2), we hypothesize that N₂ fixation under high temperatures is likely limited by the assimilation of its first product: NH₄⁺.

Interestingly, the results evidenced a crucial role for NADH-GOGAT and GS activities (due to its role on NH₄⁺ assimilation) in the regulation of nodule N metabolism of common bean under high temperatures (Table 3). Both GS and NADH-GOGAT have been proposed as indicators of more efficient N₂-fixing legume plants (Hungria et al., 1991; Gonnet and Díaz, 2000), but their activities are not perfectly coupled under drought stress (Figueiredo et al., 2001). In this study, NADH-GOGAT activity decreased faster than GS, and even faster than nitrogenase. Greater sensitivity of nodule NADH-GOGAT activity was also demonstrated in cowpea (*Vigna unguiculata* L.) due to drought (Figueiredo et al., 1999, 2001) and in common bean due to salt stress (Khadri et al., 2001). Accordingly, the experiment provided evidence that lower activity of GS (Table 3) resulted in increases in N-NH₄⁺ concentrations in the nodule (Table 2). It is important to mention that, under normal conditions, maintenance of higher rates of GS activity in nodules has been attributed to increases in N-NH₄⁺ produced by N₂ fixation at the post-transcriptional level (Suganuma et al., 1999) but,

under high temperature conditions, increases on N-NH_4^+ were actually associated with lower GS activities (Fig. 3). In other studies, decreases of GS activity in cowpea due to drought were related to increases in the activity of glutamate dehydrogenase (GHD, E.C. 1.4.1.2) (Figueiredo et al., 1999, 2001), which is not considered to have a synthetic role in ammonia assimilation in the nodules due to its high K_m for ammonia and its inefficient competition in the presence of GS (Boland et al., 1978; Groat and Vance, 1981); however, it might assume importance under drought conditions.

Our results indicate that N_2 fixation with common beans is negatively affected in several ways under high-temperature stress. The most sensitive enzymes are regulated by plant genetic strength (i.e. GS and NADH-GOGAT; Table 2) rather than rhizobial strength (nitrogenase; Table 1). However, an important finding of our study was that plant enzyme activities were differently stimulated depending on the rhizobial effectiveness. Similarly, Talbi et al. (2012) found that common bean nodules produced by a *Rhizobium etli* strain having a ccb_3 -oxidase expressed more noduline sucrose synthase (Silvente et al., 2003) than nodules induced by a wild type *R. etli* strain. Considering that common beans may simultaneously establish symbiosis with a diverse number of strains in the field (Hungria et al., 1993; Hungria and Vargas, 2000), breeding programs for improving yields under stressful conditions may only target N metabolism if considering the best combination of both rhizobial and plant genotypes.

Since N_2 fixation is severely compromised by high temperatures, one may suppose that applying N-fertilizer would compensate restrictions of N supply. Indeed, common-beans seedlings that do not meet their N requirements from seed reserves have to rely on NR activity to obtain N before the onset of nodule N_2 fixation occurs (Hungria and Franco, 1993) and thus, NR activity should overcome N restriction under high temperatures. However, the results evidenced that NR activity in leaves and stems were significantly decreased by temperatures above 34°C at 18 DAE, and at 38 DAE decreases were already observed with 34°C (Fig. 1). Actually, although NR activity of some crops may endure up to 50°C or 60°C (Chopra, 1983), decreases in NR of legumes due to temperatures above 30°C are expected (Christophe et al., 2011). High temperatures affect photosynthetic systems, photorespiration and photosynthesis rates and, therefore, the C metabolism in the leaves (Wahid et al., 2007), which certainly brings negative consequences to NR activity. Furthermore, plants relying on NO_3^- show leaf senescence several days earlier than their counterparts relying sole in symbiotic N_2 fixation (Kaschuk et al., 2010), probably affecting yields.

Finally, it should be mentioned that, under field conditions, high temperatures are usually accompanied by drought. There are other morphological and anatomical changes in plant physiology that may contribute to mitigate the negative effects of high temperatures, such as maintenance of membrane stability, production and accumulation of antioxidants and compatible solutes, removal of reactive oxygen species, and activation of chaperone and protein kinases cascades (Wahid et al., 2007). However, our results highlight another strategy, showing that targeting the improvement of responses of N metabolism in legumes to high temperatures should also consider the activities of GS and NADH-GOGAT along with the screening of the best fit between rhizobial strain and plant genotype.

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