Consequences of the water deficit on water relations and symbiosis in *Vigna unguiculata* cultivars

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ABSTRACT

The study aimed at evaluating and comparing changes provoked by the water deficit on water relations and nitrogen fixation in two *Vigna unguiculata* cultivars, as well as at indicating which cultivar is more tolerant under water deficiency. The experimental design used was entirely randomized in factorial scheme, with 2 cultivars (Pitiuba and Pérola) and 2 water regimes (control and stress). The parameters evaluated were the leaf relative water content, stomatal conductance, transpiration rate, nodule number, nodule dry matter, nitrate reductase enzyme activity, ureide concentration and leghemoglobin in nodule. The stomatal conductance of the Pitiuba and Pérola cultivars under water deficit were 0.20 and 0.01 mmol $H_2O/m^2/s$, respectively. The nitrate reductase activity of the plants under stress was significantly reduced in both cultivars. The leghemoglobin in the Pitiuba and Pérola cultivars under water stress had the concentrations of 58 and 41 g/kg dry matter, respectively. The parameters investigated in this study suggest that the Pitiuba cultivar under water deficit suffers from smaller changes, when compared with Pérola cultivar.

Keywords: *Vigna unguiculata* (L.) Walp.; water deficit; stomatal conductance; nodulation; N_2 fixation

The main characteristics of the species *Vigna unguiculata* (L.) Walp. is to be highly rustic, and has greater protein content in grain. This crop is frequently used in Brazil and in the agricultural areas of world under influence of abiotic stresses. These areas frequently present small rain index and high temperature. Besides this, the soil is susceptible to salinity or to fertility loss (Lobato et al. 2008a).

The water stress provokes a decrease in the plant water content, reduction of the cellular turgor and consequently, the decrease on the cellular expansion, and changes in several physiological and biochemical processes that can affect the growth and yield (Lobato et al. 2008b).

The bacteria of the *Rhizobium* genus are intensely used as inoculant in tropical legume species as *Glycine max*, *Phaseolus vulgaris* and *Vigna unguiculata*; it reveals several positive effects, such as the increase of yield, as well as it promotes increase of the leaf area and plant dry matter (Figueiredo et al. 1999). It also maximizes the assimilation and accumulation of the protein amount in the grain. The symbiotic fixation of atmospheric N_2 occurs due the nodule formation, in which it promotes the nitrogen assimilation (Ramos et al. 2003), it is presented as an advantage in smaller expense with nitrogen fertilizer.

The nutritional supplement is fundamental to keep the biochemical balance of the plant, in which the nitrogen (N) is the mineral element most required by the plants. The nitrogen can be absorbed as ammonium (NH_4^+) and nitrate (NO_3^-), which promotes the production of metabolic as nucleotides, enzymes, amino acids and proteins (Maman et al. 1999).

Simultaneously, the water deficit can promote changes in the capacity of the nitrogen fixation in legumes, due to the smaller water absorption and consequent reduction in the water supplement in tissues, in which it might promote damage in the nitrogen compound translocation. This way, it inhibits the biochemical activity responsible by the nitrogen metabolism (Serraj et al. 1999).

The study aimed at evaluating and comparing changes provoked by the water deficit on water relations and nitrogen fixation in two *Vigna unguiculata* cultivars, as well as at indicating which cultivar is more tolerant under conditions of water deficiency.

MATERIAL AND METHODS

Growth conditions. The study was carried out in the Instituto de Ciências Agrária (ICA) of the Universidade Federal Rural da Amazônia (UFRA), Belém city, Pará state, Northern region, Brasil (01°27′N and 48°26′E) in the period of June and July of 2007. The plants remained in glasshouse environment under natural conditions day/night (air temperature minimum/maximum and relative humidity of 24.1/38.2°C and 72/89%, respectively). The photoperiod medium was of 12 h of light and photosynthesis radiation active maximum of 580 μmol/m^2/s (at 12:00 h).

Plant materials. The *Vigna unguiculata* (L.) Walp. seeds of the Pitiuba and Pérola cultivars used in this study were harvested in the 2006 season and came from Empresa Brasileira de Pesquisa Agropecuária/Meio Norte (Embrapa), estado Piauí, Brasil.

Substrate and pot. The substrate used for the plant growth was composed by sand and silic in the proportion of 2:1, respectively, and it was autoclaved at 120°C/atm for 40 min. The container used for the plant growing was Leonard pot with 2 l capacity and it was adapted in the Laboratório de Fisiologia Vegetal Avançada (LFVA).

Experimental design and water regimes. The experimental design used was entirely randomized in factorial scheme, with 2 cultivars (Pitiuba and Pérola) and 2 water regimes (control and stress), with 10 repetitions and 40 experimental units, in which each experimental unit was constituted by 1 plant/pot.

Inoculation and conduction of plant. Three seeds per pot were sowed and singled after germination. The seedlings were inoculated with 1 ml of *Bradyrhizobium* sp. bv. BR 3256 suspension in the concentration of 1.0 × 10^9 CFU by 3 times and at regular intervals in the 5th, 10th and 15th day after the experiment implementation. The control and stress treatments received macro- and micronutrients in the form of nutritive solution of Hoagland and Arnon (1950) without nitrogen, for the period of 30 days; the nutritive solution were changed with 2 days of interval, always at 09:00 h and the pH of the nutritive solution was adjusted to 6.0 ± 0.1 with addition of HCl or NaOH. On the 30th day after the experiment implementation, the plants of the treatment under stress were submitted to period of 5 days without nutritive solution, in which the stress was simulated in the 30th until 35th day after of the experiment start. After this period, the plants were physiologically and biochemically analyzed.

Leaf relative water content. The leaf relative water content was evaluated with leaf disks with diameter of 10 mm and it was carried out in each plant; 40 disks were removed and the calculation was done in agreement with the formula proposed by Slavick (1979):

$$LRWC = [(FM - DM)/(TM - DM)] \times 100$$

where: FM is fresh matter, TM is turgid matter evaluated after 24 h and saturation in deionized water at 4°C in dark and DM is the dry matter determined after 48 h in oven with forced air circulation at 80°C

Leaf gas exchange. The stomatal conductance and transpiration were evaluated in totally expanded leaves, under light and present in the medium third of the branch main, in which porometer of state static was used (LICOR AM-300, model 1600), with the gas changes evaluated immediately during the period between 10:00 and 12:00 h in all the plants of the experiment.

Nodulation. The nodule numbers present in the plant roots were evaluated after the nodules were removed of the plant roots and the nodule dry matter was obtained from the nodules placed in oven at 65°C during the period of 72 h.

***In vivo* nitrate reductase activity.** The extraction of the nitrate reductase enzyme (E.C. 1.6.6.1) was carried out with leaf disks until the weight of 200 mg was reached; the samples were incubated in 5 ml of extraction buffer [KH_2PO_4 at 0.1M, KNO_3 at 50mM, isopropanol at 1% (v/v) and pH 7.5] by 30 min at 30°C, and all the procedures were carried out in dark. The quantification of the enzyme activity was made according to the method of Hageman and Hucklesby (1971) with absorbance at 540 nm and using spectrophotometer (Quimis, model Q798DP).

Ureide concentration in xylem sap. The ureide concentration in xylem was carried out with cuts in the plant stem in the height of 3 cm up of the root collar, at 9:00 h, in which the xylem sap was harvested during the period of 60 min. After the xylem sap was stored at –20°C until the extraction moment. The extraction was carried out with solution contained xylem sap, ultra pure water and NaOH at 0.5M in the proportion of 1:149:50 (v/v). Subsequently this solution was incubated for 8 min at 100°C and was promoted with the thermal shock for 5 min at 2°C. The ureide-N concentration was determined at 535 nm in agreement with the colorimetric method described by Vogels and Van Der Drift (1970), in which alantoine was used as standard (Sigma chemicals).

Leghemoglobin. The leghemoglobin present in the nodule of the plant root was extracted using 200 mg of nodule dry matter, in which the fresh nodules were retired of the plant roots and immediately placed in oven with forced air circulation at 65°C for 72 h. Subsequently, the dry nodules were triturated and incubated with 10 ml of solution with extraction buffer containing KCN at 0.8mM, $K_3Fe(CN)_6$ at 0.6mM and $NaHCO_3$ at 12mM in the proportion of 3:3:4 (v/v) at 30°C by 15 min, after the homogenization it was centrifuged at 10 000 g for 20 min at 4°C. The quantification was carried out at 540 nm in agreement with Wilson and Reisenauer (1963), where human hemoglobin was used as standard.

Data analysis. The data were submitted to variance analysis and when significant differences occurred, they were applied to the Tukey test at 5% level of error probability; the standard errors were calculated in all evaluated points. The statistical analyses were carried out with the SAS software.

RESULTS AND DISCUSSION

Leaf relative water content

The variance analysis reveals significant differences in the leaf relative water content in both evaluated treatments. The Pitiuba cultivar presents the leaf relative water content at 92 and 63% under the control and stress treatments (Figure 1A), respectively. Besides, a reduction of 31.5% occurred in this parameter. The Pérola cultivar had the leaf relative water content reduced by 41.5% under stress; it was thus more intense compared with the Pitiuba cultivar.

The results reveal that the Pitiuba cultivar keeps higher amount of water in the tissues than Pérola. Results reported by Costa et al. (2008) working

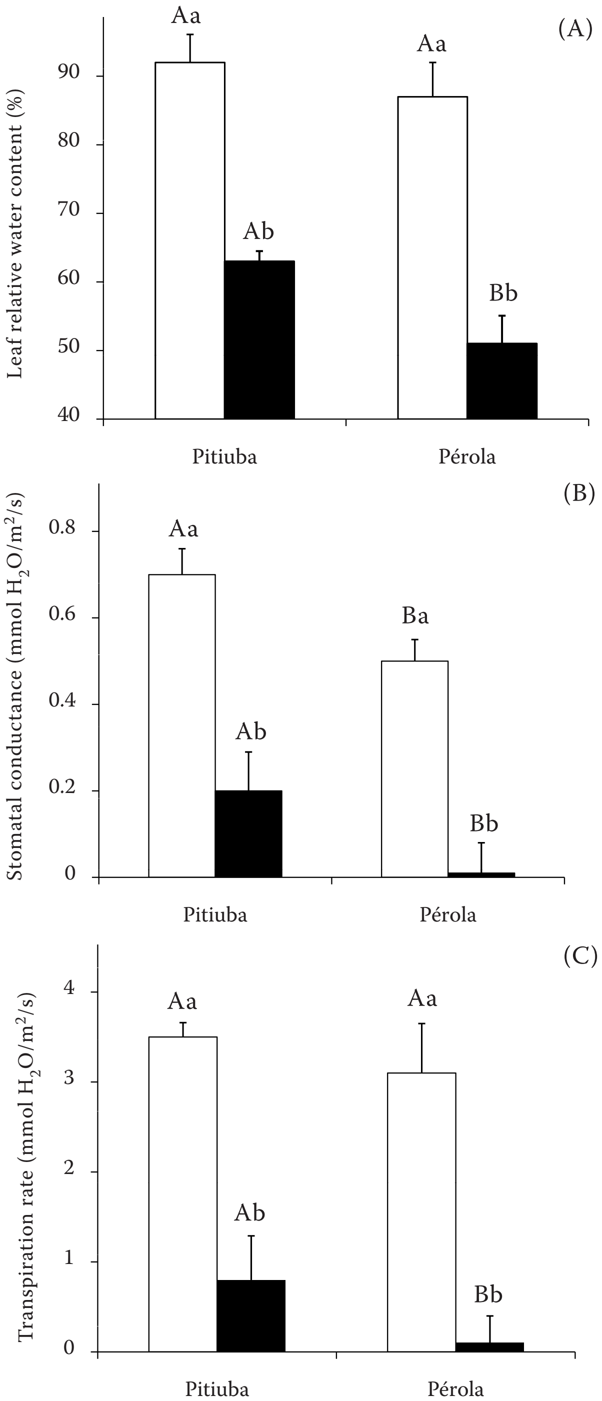

Figure 1. Leaf relative water content (A), stomatal conductance (B) and transpiration rate (C) in *Vigna unguiculata* plants cultivars Pitiuba and Pérola submitted to 5 days of water restriction

Averages followed by the same lowercase letter within the cultivars and uppercase letter within the treatments do not differ among themselves at the Tukey test at 5% probability. The bars represent the mean standard error with two *Vigna unguiculata* cultivars corroborated the higher adaptability of the Pitiuba cultivar under water deficit. The reduction shown in this parameter in both cultivars is a classic response of the deficiency/absence of water in the substrate, because during the photosynthesis process the plants need to carry out the gas changes through the stomatal opening; the plant thus loses water to the environment. Similar results were found by Lobato et al. (2008a) investigating the behavior of *Vigna unguiculata* submitted to water deficiency.

Stomatal conductance

In the control and stress treatments, the Pérola cultivar presents the stomatal conductance of 0.5 and 0.01 mmol $H_2O/m^2/s$, respectively, and it had a significant decrease by 98% (Figure 1B). The stomatal conductance of the Pitiuba cultivar fluctuated between 0.7 a 0.2 mmol $H_2O/m^2/s$ in the treatments that were kept in the nutritive solution (control) and under water deficiency, respectively, and it showed a decrease at 71.4%.

The water deficit simulated in this study provokes higher stomatal closing in the Pérola cultivar, however the more intense stomatal closing is related to smaller water content in the leaf of this cultivar and not the efficiency of the stomatal mechanism, because the leaf relative water content was smaller in the Pérola cultivar, when compared with the Pitiuba cultivar. Similar results were found by Vendruscolo et al. (2007) with transgenic plants of *Triticum aestivum* under water deficit.

The stomatal conductance was reduced in the Pérola and Pitiuba cultivars due to stomatal closing, because this strategy is used by the plants to decrease the water loss to the environment. Studies conducted by Liu et al. (2005) with *Solanum tuberosum* plants prove that the abscisic acid (ABA) is responsible for regulation of the stomatal closing and consequently the photosynthesis rate, in which it reveals an exponential correlation among the ABA levels in xylem and the stomatal conductance.

Transpiration

The transpiration rate was influenced by the water restriction (Figure 1C), in which it evidenced that the Pitiuba cultivar had a significant decrease by 77.1%, whereas that the Pérola cultivar had reduction by 96.8%.

The more intense decrease in the transpiration rate of the Pérola cultivar is directly linked with the stomatal closing observed at this cultivar; besides it proves the existence of smaller amount of water in the plant tissue of the Pérola cultivar, when compared with the Pitiuba cultivar.

The transpiration is controlled by the stomatal opening; under conditions of water deficiency the stomatal is closed, and the gas changes are reduced as well as the transpiration. The stomatal has a central position among the water loss pathway and the CO_2 absorption used in the photosynthesis process. Under water deficiency smaller CO_2 absorption occurs that provokes a reduction of the metabolic activity and consequently smaller growth and development of plant. Similar results on the reduction in the transpiration were found by Inamullah and Isoda (2005) investigating the effects of the water restriction in *Glycine max* and *Gossypium hirsutum* cultivars.

Nodule number

In this parameter both cultivars present smaller nodule number under conditions of water deficit (stress), compared with respective control; for Pitiuba and Pérola cultivars under water deficiency the results were 29.4 and 22.0 nodules/plant, respectively (Figure 2A), representing an increase of the control treatment compared to water stress by 120.4 and 48.6%, respectively.

The smaller nodulation of the Pérola cultivar under water deficit is due the smaller adaptability of this cultivar, when compared with the Pitiuba cultivar under water deficiency. The water deficit probably decreased the root capacity and liberated the exudate responsible for nodulation; this way it reduced the nodule number present in the plant root in water deficit conditions. Similar results were found by Costa (1999) investigating the effects of water deficit on the nodulation of *Vigna unguiculata* plants.

Nodule dry matter

The Pitiuba cultivar had 1.64 and 0.55 g/plant of nodule dry matter (DM) in the control and stress treatments, respectively. It represents a significant decrease by 66.5% after the period under stress. The values of the Pérola cultivar were 1.54 and 0.34 g/plant of nodule DM in the control and stress treatments, respectively, the reduction being 77.9% (Figure 2B).

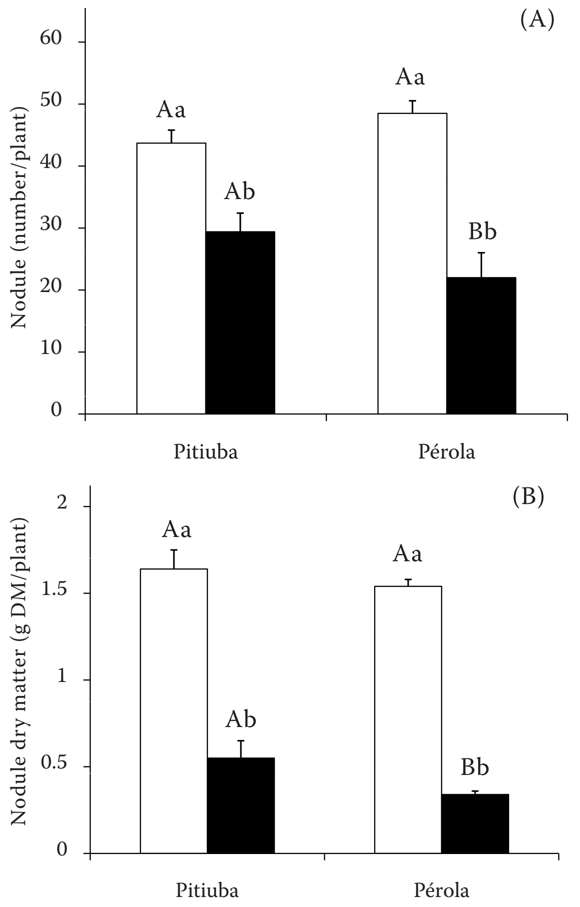

Figure 2. Nodule number (A) and nodule dry matter (B) in *Vigna unguiculata* plants cultivars Pitiuba and Pérola submitted to 5 days of water restriction

Averages followed by the same lowercase letter within the cultivars and uppercase letter within the treatments do not differ among themselves at the Tukey test at 5% probability. The bars represent the mean standard error

The results on the nodule dry matter demonstrate smaller accumulation of photo assimilates and consequently smaller dry matter of Pérola, caused by more intense reduction than in the Pitiuba cultivar.

Nitrate reductase activity

The Pérola cultivar presents the nitrate reductase activity by 1.158 and 0.045 μmol NO_2^-/g/h in the control and stress treatments, respectively (Figure 3A), compared to the results of the Pitiuba cultivar, 0.771 and 0.285 μmol NO_2^-/g/h in the control and stress treatments, respectively. The results reveal a significant reduction in the nitrate reductase activity, with the reductions of 63 and 96.1% in the Pitiuba and Pérola cultivars, respectively; they suggest that the Pitiuba cultivar is more tolerant to water restriction than Pérola. This decrease is caused by dependence of the nitrate reductase enzyme activity on the water flux assimilated by

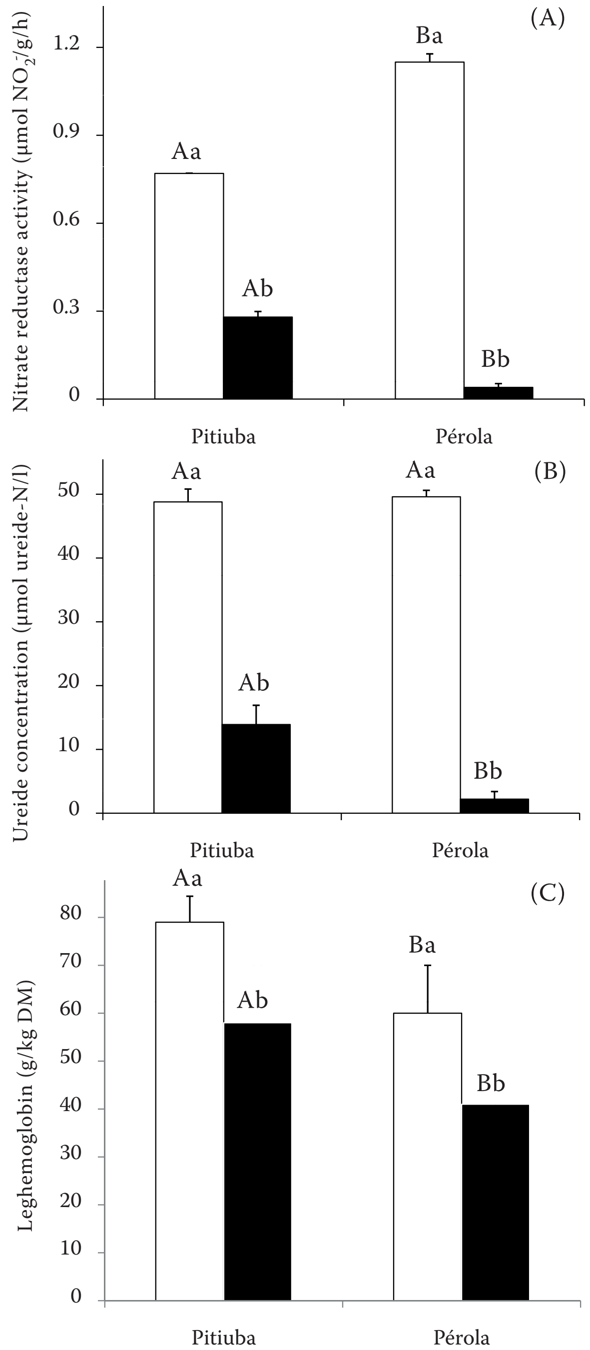

Figure 3. Nitrate reductase activity (A), ureide concentration (B) and leghemoglobin (C) in *Vigna unguiculata* plants cultivars Pitiuba and Pérola submitted to 5 days of water restriction

Averages followed by the same lowercase letter within the cultivars and uppercase letter within the treatments do not differ among themselves at the Tukey test at 5% probability. The bars represent the mean standard error the plant (Foyer et al. 1998). Studies carried out by Silveira et al. (2001) with 2 species of the *Phaseolus* genus reveal that despite belonging to the same genus these species are different in relation the amount and place (leaf and/or nodule) of higher activity of the nitrate reductase.

The nitrate reductase is an efficient indicator physiologic, because it is the first enzyme involved in the nitrogen metabolism, moreover, this enzyme shows a quick response under inadequate conditions for the plant; it is also extremely sensitive to the biotic and abiotic changes (Lobato et al. 2008b). Similar results on the reduction in the nitrate reductase activity were shown by Debouba et al. (2006) studying the consequences of the salt stress in *Lycorpesicon esculentum* plants, it corroborated the sensibility of this enzyme under conditions of abiotic stress as well.

Ureide concentration

The ureide concentration in the xylem was significantly reduced in both cultivars, when compared with the control treatment. The Pitiuba cultivar showed 48.8 and 13.9 μmol ureide-N/l in the control and stress, respectively, representing a decrease by 71.5% (Figure 3B). The values for the Pérola cultivar were 49.6 and 2.2 μmol ureide-N/l in the control and under water deficit, respectively, revealing a reduction by 95.6%. The smaller reduction in this parameter at the Pitiuba cultivar presents its higher adaptability compared with the Pérola cultivar.

The ureide concentration in the xylem also reveals higher sensibility of the Pérola cultivar to water stress. Lower ureide concentrations were also reported by Figueiredo et al. (1999) investigating the effects of water deficit in *Vigna unguiculata* plants inoculated with *Bradyrhizhobium* spp. The ureide is a compound resulting from the N_2 fixation in the root nodule of the tropical leguminous and it has the capacity to store and transport the nitrogen (Hansen et al. 1993).

Leghemoglobin in nodule

The Pitiuba and Pérola cultivars in the control treatment had the leghemoglobin concentrations of 79 and 60 g/kg DM, respectively; the stress treatment gave the concentrations of 58 and 41 g/kg DM in the Pitiuba and Pérola cultivars, respectively (Figure 3C). Significant reductions in the leghemoglobin concentrations were thus shown in the Pitiuba and Pérola cultivars, namely 26.6 and 31.7%, respectively. Thus, the Pitiuba cultivar has higher concentration of leghemoglobin in nodule than Pérola.

The leghemoglobin level in the nodule demonstrates that the Pérola cultivar under water deficiency has smaller leghemoglobin amount, when compared with the Pitiuba cultivar under the same water conditions; it indicates higher ability of the Pitiuba cultivar of to carry out the adjustment of the biochemical metabolism under conditions of low water potential and thus maintain satisfactory amount of leghemoglobin in the nodule. The leghemoglobin function is to keep the adequate supplement of oxygen (O_2) available to the nitrogenase enzyme activity, because in supra and sub-optimal concentrations of O_2 inhibition of the activity of this enzyme occurs, resulting in smaller nitrogen assimilation.

The evaluation and comparison of the parameters investigated in this study suggested that the Pitiuba cultivar under water deficit suffers from smaller changes in water relations and nitrogen fixation, when compared with Pérola cultivar; it indicates the suitability of the Pitiuba cultivar for regions with limited water supply to the plant.

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