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Gas exchange, growth, and antioxidant activity in sugarcane under biological nitrogen fixation

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Abstract

The aim of this study was to characterize the key physiological aspects of three sugarcane cultivars (RB92579, RB867515 and RB872552) under biological nitrogen fixation (BNF). Plants were generated in tubes containing aseptic substrates and these plants were transferred to pots containing washed sand, but watered with a mineral fertilizer, and inoculated with a mixture of five diazotrophic bacteria three times at seven-day intervals. Under BNF, all of the cultivars contained half of their total leaf nitrogen content and 50% less shoot dry mass. The leaves of plants under BNF showed approximately 65% less of the total protein content (TP). The gas-exchange control plants had twice the CO_2 assimilation rates than the BNF plants. The activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) was increased in all cultivars under BNF when compared with the control; thus, the content of hydrogen peroxide (H_2O_2) was also increased in these plants. The results of this study indicate that after acclimatization, the inoculation of young plants from tissue culture with diazotrophic bacteria could supply approximately 50% of their nitrogen requirement.

Additional key words: biomass; mineral nutrition; oxidative stress; photosynthesis; reactive oxygen species.

Introduction

Sugarcane (*Saccharum officinarum* L.) as a C_4 plant has high photosynthetic efficiency, increased rate of biomass conversion from solar energy and high efficiency of water use (Ward *et al*. 1999). In Brazil, sugarcane mobilizes approximately 100 to 200 kg of nitrogen (N) h^{-1} y^{-1}, although the level of fertilization is relatively small (Momose *et al*. 2009). Indeed, some places in Brazil have not applied nitrogen fertilizer for more than 100 years (Dong *et al*. 1994). In a recent study comparing the energy production of nine cultures, sugarcane demonstrated the third most efficient use of nitrogen, water and resources available in poor soils and trade-off materials, in which residues are reused as an energy source for the processing of the final product from culture (de Vries *et al*. 2010).

The BNF is prominently used in Brazil in the cultivation of the soybean crop, which is grown only with the fixed nitrogen from symbiosis with bacteria (Kaschuk *et al*. 2010). However, since the development of semi-solid nitrogen-free medium in the 70s, BNF is also used in the cultivation of grasses (Döbereiner 1992). Thus, different species of diazotrophic bacteria have been isolated, including endophytic bacteria, from sugarcane grown in Brazil. In fact, several authors have reported the low response to nitrogen fertilization by sugarcane in Brazil (Urquiaga *et al*. 1992). Therefore, the authors

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Abbreviations: APX – ascorbate peroxidase; BNF – biological nitrogen fixation; CAT – catalase; E – transpiration rate; g_s – stomatal conductance; H_2O_2 – hydrogen peroxide; MDA – malondialdehyde; PPFD – photosynthetic photon flux density; P_N – net photosynthetic rate; RWC – relative water content; ROS – reactive oxygen species; SOD – superoxide dismutase; SS – soluble sugars; TP – total protein.
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proposed that this low response resulted from the efficiency of BNF in this species.

The efficient selection of bacterial species that participates in the process of BNF resulted in the production of an inoculant containing five bacteria (Reis *et al*. 1999, Oliveira *et al*. 2002, 2009). Since then, several studies were conducted primarily by inoculating plants in tissue culture flasks (Oliveira *et al*. 2002). In a second stage, the development of young plants inoculated during tissue culture was tested under conditions of a growth chamber (James *et al*. 2001). A third stage tested the ability of N_2 fixation using the technique of $^{15}N_2$ injected into ten cultivars kept in concrete tanks (Urquiaga *et al*. 1992). This study demonstrated a high capacity to supply N to sugarcane through N_2 fixation. Subsequently, using $^{15}N_2$-labeling, several localities in southeastern Brazil were studied to evaluate the ability of sugarcane to fix N_2 under field conditions. Under these conditions, the BNF was between 30 and 50% (Yoneyama *et al*. 1997). None of these studies maintained the plants without the possibility of acquiring nitrogen except through BNF, due to the presence of soil, which contains residual nitrogen, or cultivation in areas in which sugarcane had previously been planted. In addition, the physiological variables were not evaluated in sugarcane plants inoculated with a bacterial mixture.

Under nitrogen deficiency, in plants, the protein content is low (Huang *et al*. 2004), shoot growth decreases, photosynthetic activity is impaired due to the lower contents of Rubisco, chlorophyll content and sucrose accumulates in leaves (Ciompi *et al*. 1996, Shangguam *et al*. 2000a, Huang *et al*. 2004). This scenario promotes increased reactive oxygen species (ROS), such as H_2O_2, leading to membrane damage in different species. To neutralize ROS, an increase in antioxidative enzyme activity is required (Huang *et al*. 2004, Jaleel *et al*. 2009, Rubio-Wilhelmi *et al*. 2011).

Therefore, this study measured physiological variables such as photosynthesis, nutrient contents, leaf carbon metabolism, and antioxidative enzyme activity of young plants from three sugarcane cultivars inoculated with a mix of five preselected bacteria without sources of mineral nitrogen. The main objective of this study was to evaluate the ecophysiological performance of sugarcane cultivars under BNF as a nitrogen source without interference from other sources.

Materials and methods

Plants and growth conditions: Three sugarcane cultivars (RB92579, RB867515, and RB872552) were chosen for wide cultivation in northeastern Brazil. The plants were produced in a tissue culture at the Biofactory (Centro de Tecnologias Estratégicas do Nordeste – CETENE). All cultivars were grown for 120 d and subsequently transferred to 3-L pots of washed sand in a greenhouse. A solution containing five diazotrophic bacteria strains (BR 11145, BR 11281, BR 11335, BR 11366, and BR 11504) were used for the inoculation of the plants (Oliveira *et al*. 2006). The plants under BNF received 100 mL of the bacterial solution at a concentration of 2.0 g L^{-1} in distilled water, which was applied three times at 7-d intervals. After 30-d post-inoculation, the cultivars received 200 mL of water every 2 d and 150 mL of nutrient solution (Hoagland and Arnon 1950) every 7 d. The nitrogen was removed from the nutrient solution for the plants under BNF. Throughout the experiment, the plants were grown under conditions of natural variation in air temperature (25 to 31.5ºC) and humidity (55 to 86%). The photosynthetic photon flux in the greenhouse varied from 600 to 1,400 μmol m^{-2} s^{-1}.

Relative water content (RWC): The water status of each plant was determined according to Barrs and Weatherley (1962). Leaf discs were collected at 06:00 h from four plants per treatment - for more method details *see* Souza *et al*. (2010).

Gas exchange: The rates of the net CO_2 assimilation (P_N), stomatal conductance (g_s), and transpiration (E) were measured with a portable infrared gas analyzer (*ADC, model LCi*, Hoddesdon, UK). The measurements were performed between 10:00 and 11:30 h on the younger expanded leaves.

Contents of nitrogen, phosphorus and potassium: To quantify the macronutrients, sulfur extraction was performed (Thomas *et al*. 1967), and the contents of nitrogen (Thomas *et al*. 1967), phosphorus (Murphy and Riley 1962), and potassium (da Silva 1999) per unit dry mass were determined.

Biometrics: After 93 d under BNF or mineral nutrition, plants were separated into shoots and roots, and then dried in a forced air oven at 60°C for 72 h to perform dry mass.

Biochemical analysis: The leaves were routinely collected at 15:00 h, at the period of the highest photoassimilate accumulation (Santos and Pimentel 2009). After collection, the plant tissue was immediately wrapped in aluminum foil, frozen in liquid nitrogen and stored at –80°C. The total soluble carbohydrates, soluble proteins, amino acids, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) were analyzed according to the methodologies of Dubois *et al.* (1956), Bradford (1976), Moore and Stein (1948), Cakmak and Horst (1991) and Alexieva *et al*. (2001), respectively. The cell damage was assessed by accumulation of malondialdehyde (MDA) through the TBA test, which measures MDA as an end product of lipid peroxidation (Cakmak and Horst 1991), and the

accumulation of H_2O_2 was measured spectrophotometrically, calculated using a standard curve prepared with the known concentrations.

Enzyme activities of superoxide dismutase (SOD, EC 1.15.1.1), L-ascorbate peroxidase (APX, EC 1.11.1.11), and catalase (CAT, EC 1.11.1.6) were determined. The total SOD activity was measured by its ability to inhibit the photochemical reduction of NBT at 560 nm (Giannopolitis and Reis 1977). The CAT activity was estimated by the rate of decomposition of H_2O_2 at 240 nm. The total APX activity was estimated by the reduction of H_2O_2 to water using ascorbate as a reducing agent and monitoring the decrease in absorbance at 290 nm (Nakano and Asada 1981). The total activity of SOD was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The 3 mL reaction mixture contained 75 μmol NBT, 2 μmol of riboflavin, 0.013 mol methionine, 0.1 μmol EDTA, 0.05 mol K-phosphate buffer (pH 7.8), and 5 mm^{-3} of the enzyme extract. The test tubes containing the mixture were placed in a wooden box with two fluorescent lamps at 40 W, receiving 130 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) at the surface of the reaction tube. The reaction was initiated by switching on the light and was conducted for 10 min. The light was switched off to terminate the reaction, and the absorbance at 560 nm was recorded. A nonirradiated reaction mixture that did not develop color served as the control, and its absorbance was subtracted from the sample measurements of absorbance. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction (Beauchamp and Fridovich 1971).

Statistical analysis: The experimental design was completely randomized, forming a 3 × 2 factorial (three cultivars and two ways of supplying nitrogen) with 10 plants in individual pots per treatment combination. The data were submitted to *ANOVA* tests, and the means were compared by the Student *Newman Keuls* method at a 5% probability.

Results

The relative water content of the plants under BNF was not changed as compared with the plants in mineral nitrogen (control) (P>0.05) (Table 1). However, RB92579 showed the lowest relative water content among the cultivars in both treatments.

Table 1. Changes in the relative water content (RWC) in the three sugarcane cultivars inoculated with N-fixing bacteria (BNF) or under complete mineral nutrition (control). The results are given as the means ± SE, n = 3. Different *capital letters* denote the difference among the cultivars, and different *lowercase letters* denote the difference between the treatments in each cultivar (P<0.05).

Cultivars	Control	BNF
RB92579	85.80 ± 1.23^{Ba}	88.54 ± 1.64^{Ba}
RB867515	92.15 ± 1.63^{Aa}	92.44 ± 1.92^{Aa}
RB872552	90.88 ± 1.39^{Aa}	90.34 ± 1.44^{Aa}

Plants under BNF showed a lower content of nitrogen (N) in the shoots of the three cultivars as compared with the control plants (Fig. 1*A*). However, the content of phosphorus (P) and potassium (K^+) was not altered under BNF (P>0.05) (Fig. 1*B,C*), except in the cultivar RB92579, which exhibited the highest amount of P and a reduced amount of K^+ in the BNF plants (P<0.05) (Fig. 1*B,C*), when compared with control.

The lack of nitrogen in the shoots of the BNF plants reduced gas exchange in the plants as compared with the control (Fig. 2). The biggest difference was observed in the net photosynthetic (P_N), where the plants under BNF generally had half the amount of P_N as the control plants (Fig. 2*A–C*). The g_s values were similar between the treatments in all cultivars (Fig. 2*D–F*). The behavior was confirmed when the measurements were taken during a day when the light intensity varied from minimum before dawn to the maximum radiation at 14:00 h. Under these conditions, E and g_s values did not differ between treatments (data not shown). However, Fig. 3 shows the trend in the measurements taken daily at a single time (Fig. 2*A–C*).

The leaf content of soluble sugars (SS) was not changed (P>0.05), except in the cultivar RB92579, which accumulated a higher amount of sugar in the leaves under BNF (P<0.05) when compared with control. However, under BNF, all cultivars had a lower content of total protein (TP) and free amino acids (AA) as compared with the control plants (P<0.05) (Fig. 4).

Nitrogen deficiency reduced the whole plant dry mass (P<0.05) in the cultivar RB872552 (Fig. 5). In the cultivars RB92579 and RB867515, the reduction of dry mass in BNF occurred only in the shoot, which was 50% lower than the control (nitrogen supplied) (Fig. 5*A*) (P<0.05); however, the root system showed a higher mass in the plants without mineral nitrogen (Fig. 5*B*).

The reduced growth rate (Fig. 5*A*) under conditions of a tropical climate led to a high production of H_2O_2 in the plants under BNF as compared with the control plants in the three cultivars (P<0.05) (Fig. 6*D*). However, the leaf MDA content was not increased in any of the three cultivars under BNF (P>0.05) as compared with the control plants (Fig. 6*E*).

The antioxidative enzyme activity was increased in plants under BNF as compared with the control plants, and the activities of SOD and APX were increased around 50% in the three cultivars, whereas no increase in the CAT activity was observed (Fig. 6*A–C*).

Discussion

The leaf N content in plants under BNF was 50% lower than that of plants treated with mineral fertilizer (Fig. 1*A*). However, in the roots, the N content was similar, regardless of the nutritional treatment (Fig. 1*B*). This higher N content in the roots of the sugarcane is in accordance with other studies of BNF, in which the root system was the site of the highest accumulation of nitrogen, followed by the stem and leaves (Momose *et al*. 2009). The supply of nitrogen through biological fixation in association with endophytic bacteria has been confirmed in different grasses, such as *Brachiaria decumbens,* which has been shown to obtain 40% of its required N from atmospheric N_2 fixation (Boddey and Dobereiner 1995). Through $^{15}N_2$-labeling, different varieties of sugarcane were demonstrated to contain a variable amount of N_2 through symbiosis, varying between 30 and 50% of the total N present in the tissue of the shoot under field conditions, depending on the location in Brazil (Yoneyama *et al*. 1997). Under controlled conditions, $^{15}N_2$-labeling experiments showed the incorporation of higher values of N_2 between 60 to 70% (Urquiaga *et al*. 1992) in plants grown in soil.

To assess how far the BNF could maintain plant growth as compared with control nitrogen fertilization, we used washed sand. Previous studies have shown that in soils of low fertility, the growth of plants under BNF is less than that in soils with higher fertility (Oliveira *et al*. 2006). Therefore, in this study, all the nitrogen present in plants was supplied by BNF in the control treatment. In fact, the inoculation of the young plants after acclimation resulted in them showing 50% of the control N content (Fig. 1*A*), which was reflected in the dry mass of the shoots among the three cultivars (Fig. 5*A*). Another possible explanation for the growth under these conditions is that when plants are inoculated with diazotrophic bacteria, the tissue might respond to the microorganism-mediated stimulation of plant hormones, such as auxins and gibberellins, to promote growth under these conditions (Bastian *et al*. 1998).

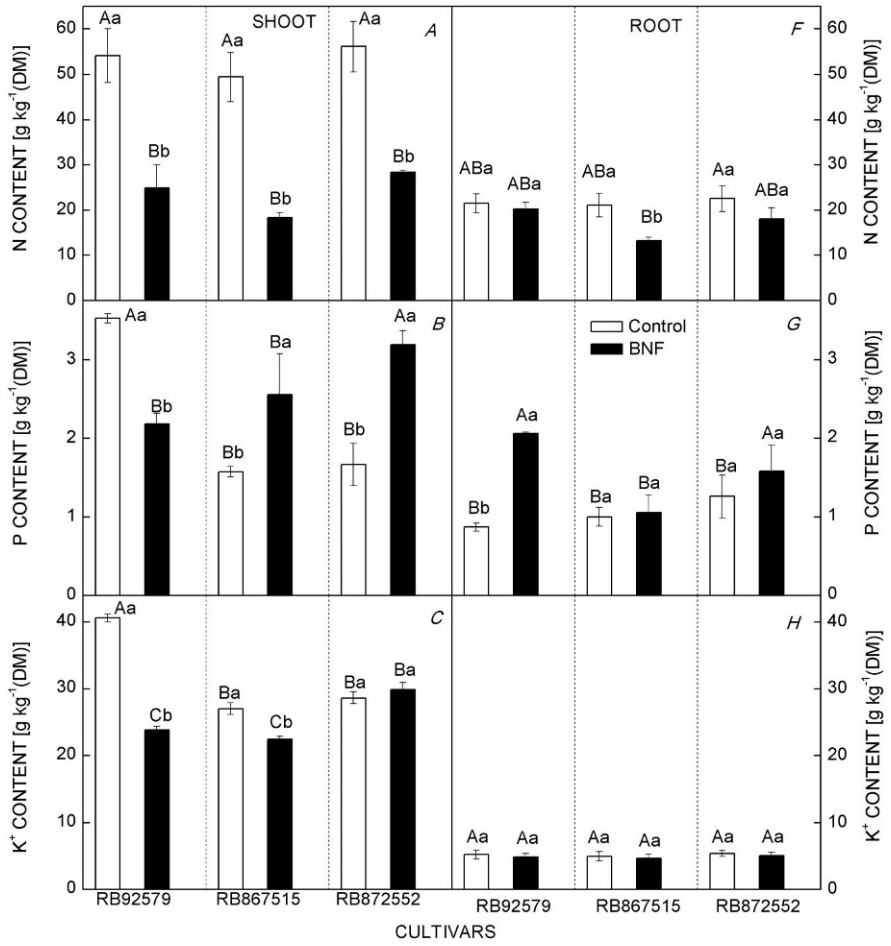

Fig. 1. The leaf (*A–C*) and root (*F–H*) content of nitrogen (N), phosphorus (P) and potassium (K) in the three sugarcane cultivars inoculated with N-fixing bacteria (BNF) or under complete mineral nutrition (control). The results are given as the means ± SE, $n = 5$. Different *capital letters* denote the difference among the cultivars and different *lowercase letters* denote difference between the treatments in each cultivar ($P<0.05$).

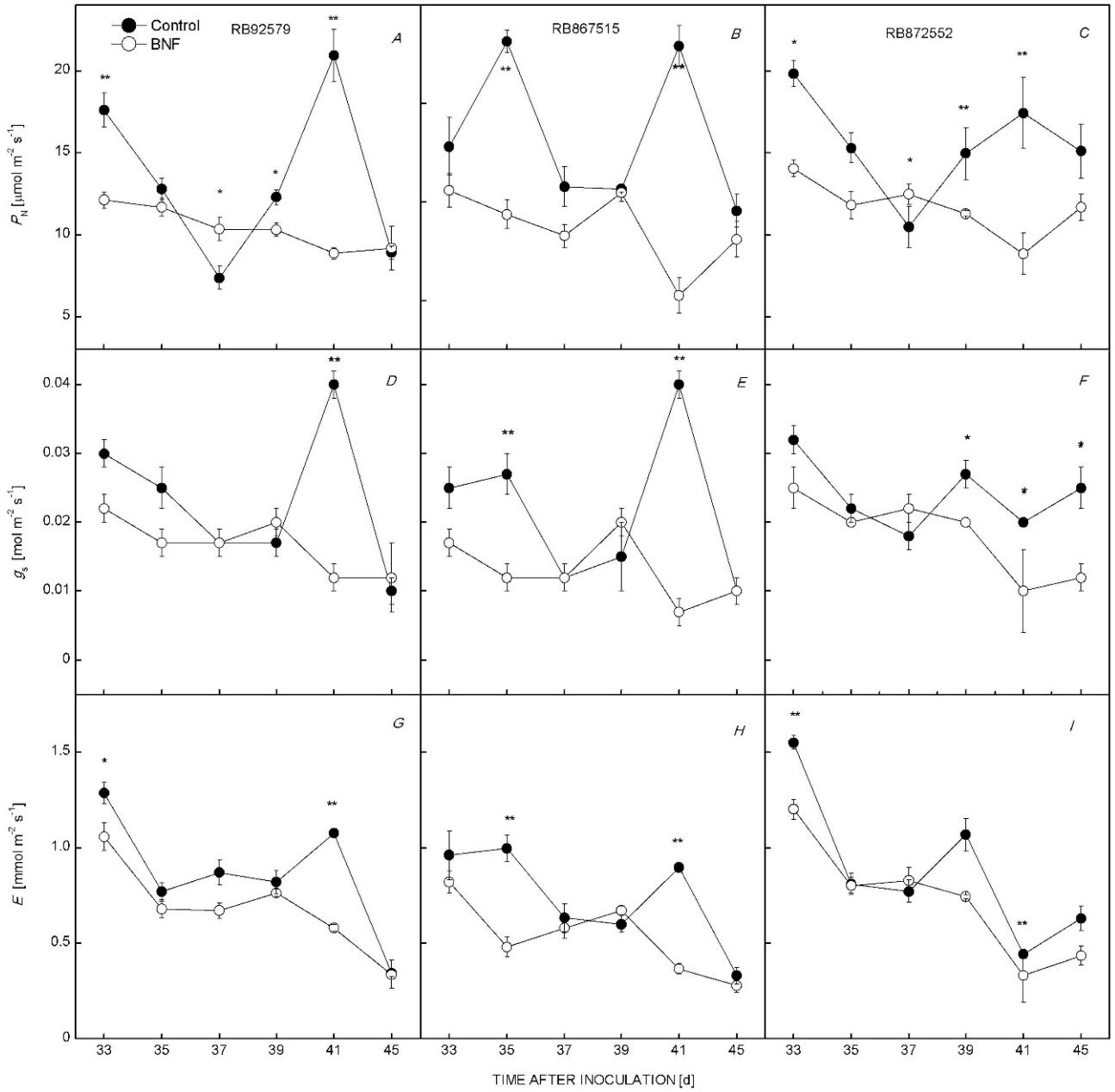

Fig. 2. Gas exchange in the three sugarcane cultivars inoculated with N-fixing bacteria (BNF) or under complete mineral nutrition (control). *A–C*: net photosynthetic rate (P_N), *D–F*: stomatal conductance (g_s), *G–I*: transpiration rate (E). The measurements were performed between 10:00 and 11:30 h on the younger expanded leaves. During this time the photosynthetic photon flux density (PPFD) was 1,200; 1,200; 800, 800; 1,000; and 600 µmol m^{-2} s^{-1}, the temperature was 29, 32, 30.4, 31.1, 31, and 27.1 °C and a humidity of 78, 66, 65, 54, 69, and 78%, each day of measurement respectively. The results are means ± SE, n = 5, * values significantly different at P<0.05 or ** at P<0.01.

P_N was reduced in the plants under BNF (Fig. 2*A–C*) in the three cultivars at a similar intensity. However, the g_s values were not reduced to that extent as due to photosynthetic metabolism, which showed a pattern typical of plants under N deficiency in the shoot (Fig. 1*A*). This result was due to the low content of total proteins and free amino acids in plants under BNF (Fig. 4*B*,*C*). Rice, another grass under N deficiency in the shoot, showed a decreased rate of photosynthetic activity of photosystem II and decrease in the total protein, including Rubisco content (Huang *et al*. 2004). If g_s does not sharply limit the rate of P_N, the reduction might be due to biochemical limitations such as in sunflower, wheat, and rice plants (Ciompi *et al*. 1996, Shangguam *et al*. 2000a, Huang *et al*. 2004), in which both the photochemistry and carboxylation were affected in plants

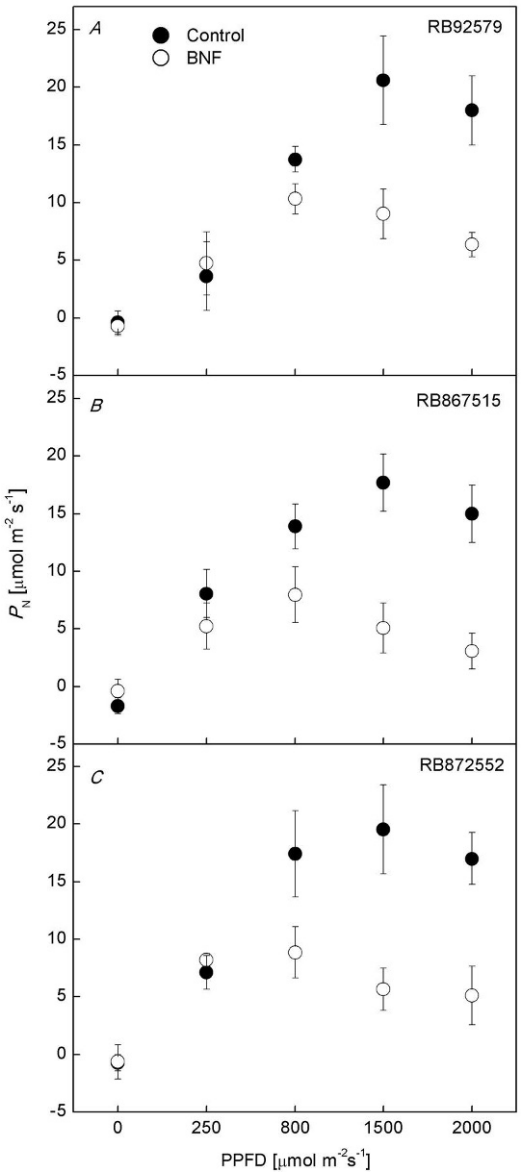

Fig. 3. Changes in net photosynthetic rate (P_N) and photosynthetic photon flux density (PPFD) under natural conditions in the three sugarcane cultivars inoculated with N-fixing bacteria (BNF) or under complete mineral nutrition (control). The results are given as the means ± SE, n = 3. The curve was fitted to the change in the air temperature during a 05:30 to 14:00 h period at 21 to 30ºC and 55 to 89% humidity.

under low N. However, this was not observed in wheat plants, which when well hydrated, show decreased g_s and P_N values due to the reduced N availability (Shangguam *et al*. 2000b); however, the plants under drought showed no difference in the gas-exchange values, independent of the leaf N content. In the present work, the sugarcane plant under BNF showed a reduced P_N (approximately 50%) (Fig. 3) as compared with the control under high PPFD, which was probably due to biochemical limitations (Fig. 4*B*,*C*), especially the extreme reduction of total protein in the leaf. Under N deficiency, the reduction of leaf amino acids and proteins was accompanied by a reduction of photosynthetic pigments (data not shown), which was perhaps the main reason for the lack of response to increasing PPFD (Fig. 3).

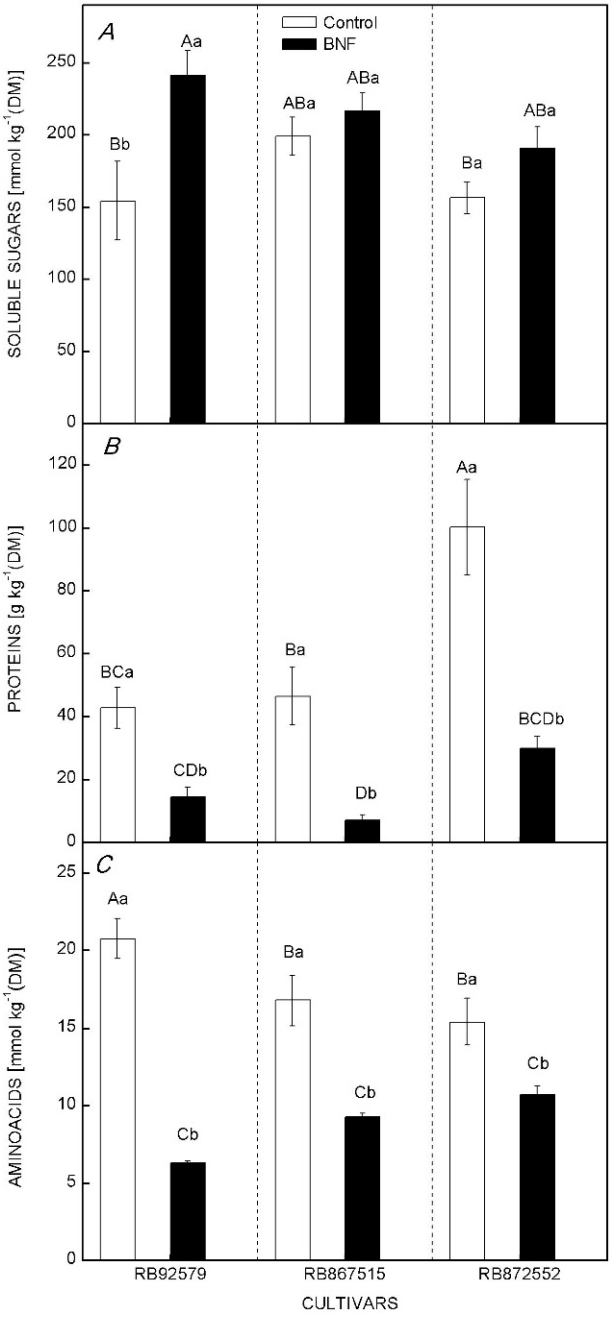

Fig. 4. Changes in the amount of leaf soluble sugars (*A*), total proteins (*B*) and free amino acids (*C*) in the three sugarcane cultivars inoculated with N-fixing bacteria (BNF) or under complete mineral nutrition (control). The results are given as the means ± SE, n = 5. Different *capital letters* denote the difference among the cultivars, and different *lowercase letters* denote the difference between the treatments in each cultivar (P<0.05).

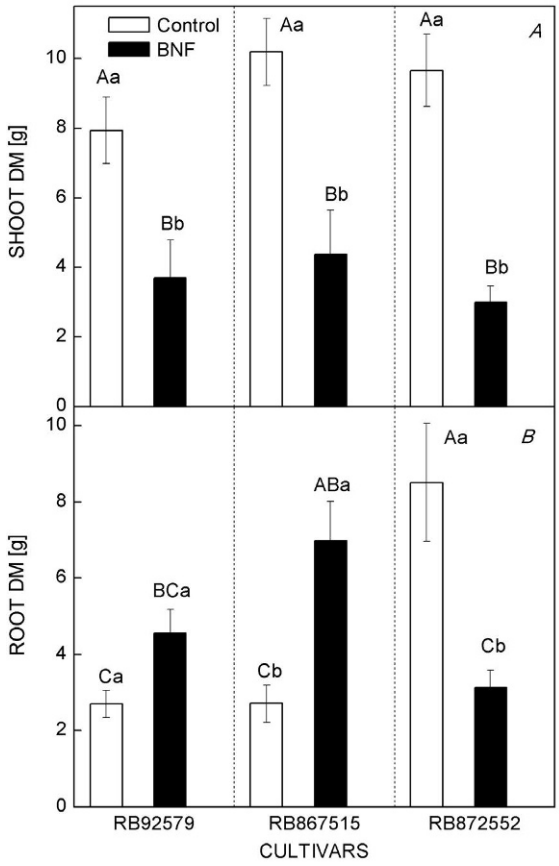

Fig. 5. Dry mass of the shoots (*A*) and roots (*B*) of three sugarcane cultivars inoculated with nitrogen-fixing bacteria (BNF) or under complete mineral nutrition (control). The results are given as the means ± SE, $n = 5$. Different *capital letters* denote the difference among the cultivars and different *lowercase letters* denote the difference between the treatments in each cultivar ($P<0.05$).

Plants under BNF, due to the low N leaf content (Fig. 1*A*), exhibited low total protein and free amino acids (Fig. 4*B*,*C*) when compared with the control, which resulted in a 50% reduction of the P_N and dry mass of the shoots (Fig. 5*A*). This reduced growth, although the PPFD and RWC (Table 1) were both adequate, might have been responsible for the increased production of H_2O_2 in the leaves (Fig. 6*D*). H_2O_2 is the product of SOD activity, which doubled when compared with the control plants (Fig. 6*A*) in response to low N content. Beyond the low N deficiency (Huang *et al*. 2004, Pompelli *et al*. 2010b, Rubio-Wilhelmi *et al*. 2011), several environmental stresses increase ROS, such as drought (Pompelli *et al*. 2010a, Arcoverde *et al*. 2011), and multiple stresses that are common in tropical environment, such as high temperature and drought (Jaleel *et al*. 2009). This increase in ROS leads to metabolic disorder, damage, and cellular senescence in different species.

In plants under abiotic stress, photosynthate partitioning tends to be reduced and SS are accumulated in the leaf (Fig. 4*A*) (Santos and Pimentel 2009, Arcoverde *et al*. 2011). This accumulation of sugars has changed the rate of assimilation in plants (Wingler *et al*. 2006), further reducing the activity of photosynthetic machinery through feedback that leads to downregulation (Paul and Pellny 2003), which will inevitably result in the increased production of ROS (Huang *et al*. 2004, Arcoverde *et al*. 2011), causing damage to membranes and producing MDA (Fig. 4*E*) (Pompelli *et al*. 2010a,b; Arcoverde *et al*. 2011). N deficiency is among the several environmental factors that can reduce the activity of carboxylation and therefore increase the production of ROS (Huang *et al*. 2004, Pompelli *et al*. 2010b, Rubio-Wilhelmi *et al*. 2011). The three sugarcane cultivars showed a marked increase in the production of H_2O_2 (Fig. 6*D*) in plants under BNF, which also exhibited half the leaf N content (Fig. 1*A*) and less than half of the total protein (Fig. 4*B*) as compared with the control plants. ROS are divided into two main classes: nonfree radicals (H_2O_2) and free radicals (O_2, $OH^{\cdot}$, $OH_2^{\cdot}$). Both forms can cause changes in DNA, membranes, lipids, and chlorophyll, among others (Jaleel *et al*. 2009). Besides the deleterious role, ROS can act as signaling molecules during biotic and abiotic stresses, even without the presence of a stressor (Vranová *et al*. 2002). After the sharp increase in SOD activity, the dismutation of free radicals in H_2O_2 (Fig. 6*A*) occurred in all three cultivars and APX activity (Fig. 6*C*) was increased, which metabolizes H_2O_2 to H_2O in higher plants (Shigeoka *et al*. 2002). The high activity of antioxidation enzymes might be essential under stress conditions to maintain the integrity of the plant tissue and thus increase tolerance to the stressor; however, in this respect, some cultivars differed, especially RB92579, which showed high SOD activity and H_2O_2 and MDA content (Fig. 6*A*,*D*,*E*).

The present study demonstrated that the inoculation of plants from tissue culture could support approximately 50% of the growth of sugarcane, under the conditions presented here, which could be a promising result considering the high cost of N fertilizer. A lower dose of N in plants inoculated under field conditions might generate increased revenues for the producer. In addition, the use of few amounts of N fertilizer in the planting of sugarcane could add even greater ecological value to biofuels and other products, such as sugar processed from sugarcane. The next step is to evaluate the physiological parameters and the yield of different sugarcane cultivars inoculated with bacterial mixture under field conditions with and without the mineral N dose associated with inoculation.

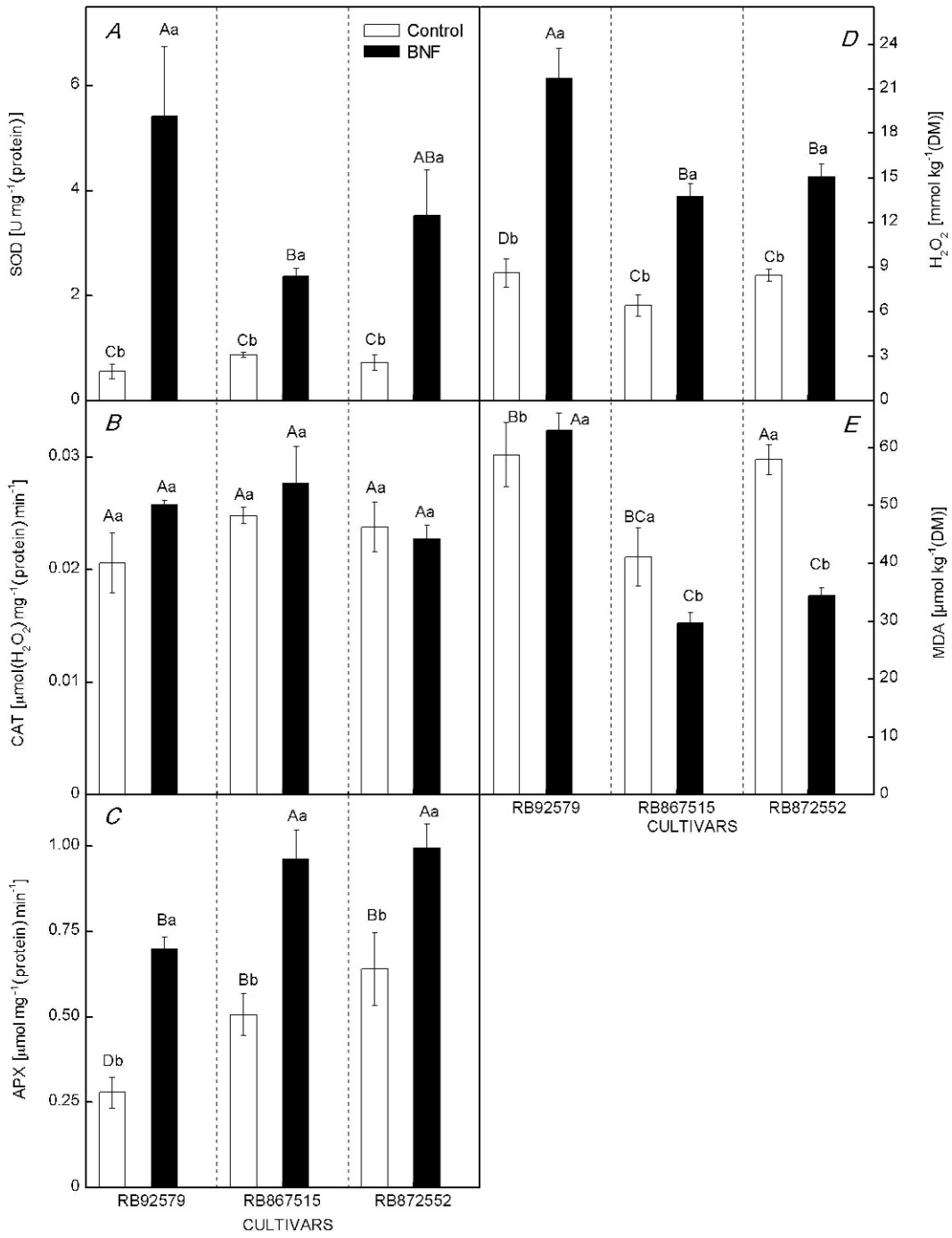

Fig. 6. Changes in the activity of superoxide dismutase (SOD) (*A*), catalase (CAT) (*B*) and L-ascorbate peroxidase (APX) (*C*). Changes in the content of H_2O_2 (*D*) and malondialdehyde (MDA) (*E*) in the three sugarcane cultivars inoculated with N-fixing bacteria (BNF) or under complete mineral nutrition (control). The results are given as the means ± SE, $n = 5$. Different *capital letters* denote the difference among the cultivars and different *lowercase letters* denote the difference between the treatments in each cultivar ($P<0.05$).

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