DOI: http://dx.doi.org/10.1590/1678-4499.256

Endophytic bacteria affect sugarcane physiology without changing plant growth

Fernanda Castro Correia Marcos[1], Raquel de Paula Freitas Iório[2,3], Adriana Parada Dias da Silveira[3], Rafael Vasconcelos Ribeiro[1], Eduardo Caruso Machado[4], Ana Maria Magalhães de Andrade Lagôa[4]*

1. University of Campinas - Institute of Biology - Campinas (SP), Brazil.
2. Agronomic Institute - Graduate Program in Tropical and Subtropical Agriculture - Campinas (SP), Brazil.
3. Agronomic Institute - Center of R&D in Soils and Environmental Resources - Campinas (SP), Brazil.
4. Agronomic Institute - Center of R&D in Ecophysiology and Biophysics - Campinas (SP), Brazil.

ABSTRACT: The aim of this study was to evaluate if endophytic bacteria inoculants would be beneficial to the sugarcane varieties IACSP94-2094 and IACSP95-5000, promoting changes in photosynthesis and plant growth. The plants, obtained from mini stalks with one bud, were treated with two bacteria mixtures (inoculum I or II) or did not receive any inoculum (control plants). The inocula did not affect shoot and root dry matter accumulation as compared to the control condition (plants with native endophytic bacteria). However, photosynthesis and electron transport rate (ETR) increased in IACSP94-2094 treated with the inoculum II, whereas the inoculum I enhanced photosynthesis and stomatal conductance in IACSP95-5000. The inoculum II caused increase in leaf sucrose concentration of IACSP94-2094 and decrease in IACSP95-5000 leaves. Leaf nitrogen concentration was not affected by treatments, but bacteria inoculation increased nitrate reductase activity in IACSP95-5000, and the highest activity was found in plants treated with the inoculum II. We can conclude that bacteria inoculation changed sugarcane physiology, improving photosynthesis and nitrate reduction in a genotype-dependent manner, without promoting plant growth under non-limiting conditions.

Key words: *Saccharum* spp., plant-bacteria interaction, photosynthesis, nitrogen, mini stalks with one bud.

INTRODUCTION

The interaction between plants and microorganisms is quite complex and depends on organisms involved and environmental conditions, being affected by plant physiological status and nutrition (Oliveira et al. 2006; Moutia et al. 2010). The abundance and diversity of bacteria are huge under field (or non-desinfested soil/substrate) conditions, reducing or masking the effects of bacterial inoculation (Rosenblueth and Martínez-Romero 2006).

In order to assess the effects of bacterial isolates on plant physiology, most studies use plants free of microorganisms as micropropagated ones and evaluate the inoculation of only one bacterium species in plant material (Singh et al. 2011). Under such conditions, some specific changes due to plant-microorganism interaction have been revealed (Oliveira et al. 2006); however, such response may be different from those ones found in plants when more than one bacterium is present (such as in mini stalk with one buds) or when the inoculum is confronted with soil native microorganisms. For instance, the inoculation of bacterial mixtures of different species or strains (as usual in commercial inoculants) caused increases in growth and yield of tomato as compared to the single inoculation, which was justified by improvements on nitrogen (N) and phosphorus (P) nutrition (Botta et al. 2013)

Endophytic bacteria are microorganisms that live within the plant, isolated from tissues whose surface was desinfested (Hallmann et al. 1997). They are able to colonize different plant tissues, from roots to flowers (Compant et al. 2005), without causing any visible damage to plants. While the interaction between plants and endophytic bacteria has been studied taking into account plant growth promotion (Ryan et al. 2007) and plant dry matter production (Botta et al. 2013), little is known about the physiological basis of the process of improving plant growth. Sugar beet plants treated with endophytic bacteria showed higher potential →

*Corresponding author: alagoa@iac.sp.gov.br

Received: Jun. 16, 2015 – Accepted: Jul. 22, 2015

quantum efficiency of photosystem II, electron transport in the thylakoid membranes, leaf CO_2 assimilation and carbohydrate content than untreated ones (Shi et al. 2010). In general, growth promotion is usually attributed to improved plant nutrient acquisition (Barretti et al. 2008) and production of phytohormones as indoleacetic acid by bacteria (Shi et al. 2010). Biological nitrogen fixation is another advantage of the association between diazothrophic bacteria and plants and it has been shown that sugarcane varieties can get at least 40 kg $N \cdot ha^{-1}\, yr^{-1}$ through this process (Urquiaga et al. 2012). However, the underlying physiological changes related to the improved nutrition in plants treated with bacterial inoculum remain unclear.

As there is a genotypic variation when considering sugarcane yield and physiological responses to constraining environmental conditions (Landell et al. 2005a, b; Machado et al. 2009), it would be reasonable to assume that sugarcane varieties present differential ability in establishing a beneficial interaction with microorganisms. The understanding of how plants respond to bacterial inoculation and what mechanisms are stimulated is important to optimize the use of bacteria as an alternative technology to improve plant production from mini stalks and for increasing crop yield. Herein, we evaluated if mixtures of endophytic bacteria would be beneficial to two sugarcane varieties IACSP94-2094 and IACSP95-5000 with differential stalk yield (Landell et al. 2005a,b), aiming to answer the following question: does the inoculation of endophytic bacteria promote changes in sugarcane physiology and growth in a genotype-dependent manner?

MATERIALS AND METHODS

Plant material and growth conditions

Sugarcane (*Saccharum* spp.) plants cvs. IACSP94-2094 and IACSP95-5000 (Landell et al. 2005a,b) were propagated by planting stalk segments containing one bud. Plants were grown under greenhouse conditions, where air temperature varied between 37.4 ± 2.8 °C (maximum) and 18.2 ± 1.5 °C (minimum) and the average air relative humidity was 73 ± 7%. Thirty-two days after germination, plantlets were transferred to 5-L pots containing a sterile mixture of sand, soil and substrate (Carolina Soil of Brazil, Vera Cruz SC, Brazil, composed of sphagnum peat, expanded vermiculite, limestone dolomite, agricultural gypsum and NPK fertilizer — traces) 1:1:1 (v/v/v) sterilized in autoclave. They were irrigated three times a week with nutrient solution with low N concentration (57 mg $N \cdot L^{-1}$). Such solution was composed by 2 mL of solution A [200 $g \cdot L^{-1}$ of $Ca(NO_3)_2$, 250 $g \cdot L^{-1}$ of $CaCl_2$, 20 $g \cdot L^{-1}$ of ConMicros Standard (commercial product) with 1.45 $g \cdot L^{-1}$ of Fe-EDTA, 0.25 $g \cdot L^{-1}$ of Cu-EDTA, 0.15 $g \cdot L^{-1}$ of Zn-EDTA, 0.36 $g \cdot L^{-1}$ of Mn-EDTA, 0.36 $g \cdot L^{-1}$ of B, 0.072 $g \cdot L^{-1}$ of Mo and 0.07 $g \cdot L^{-1}$ of Ni] and 3 mL of solution B [200 $g \cdot L^{-1}$ of KNO_3, 150 $g \cdot L^{-1}$ of KH_2PO_4, 300 $g \cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$ and 100 $g \cdot L^{-1}$ of KCl] per liter. Sugarcane tillers were removed and each plant was kept with only the main stalk. The physiological measurements and plant sampling were taken 72 days after planting (dap).

Inoculants and bacterial counting

Ten bacteria isolates from stem and roots, belonging to the Soil Microorganisms Collection held by the Instituto Agronômico (Campinas, SP), were previously selected as micropropagated sugarcane plant growth-promoting bacteria (PGPB) and chosen to prepare the inocula.

The ten selected strains were grown separately in flasks with Dygs liquid medium (Döbereiner et al. 1995) until the concentration of 10^8 $CFU \cdot mL^{-1}$. Two inocula were prepared by mixing five bacterial strains each. The composition of each inoculum, as well as the characteristics regarding indol production, the presence of *nif*H gene and plant-growth promotion are shown in Table 1.

For inoculation, small stalk segments (4 cm length) of both varieties were immersed in culture medium with inoculum (I or II) or without inoculum (control) for a period of one hour before planting. Then, they were placed in plastic cups (200 mL) with sterilized substrate. At 32 and 69 dap, an additional application of inoculum was carried out with 10 mL of inoculum (I or II) solution with a concentration of 10^8 $CFU \cdot mL^{-1}$ or sterilized culture medium (control).

The quantification of native endophytic bacteria was done in small stalk segments at planting and also in roots at the end of the experiment in both control and treated plants, following the procedure described by Döbereiner et al. (1995).

Leaf gas exchange, photochemistry and chlorophyll content

The leaf gas exchange and chlorophyll fluorescence emission were measured using an infrared gas analyzer model LI-6400 (Licor, Lincoln NE, USA), equipped with a →

Table 1. Inoculum composition, presence of *nif*H gene and bacteria ability in promoting root and shoot growth as well as producing indol substances*.

Inoculum	ID	Species	GenBank number access	*nif*H gene	Root	Shoot	Indol substances
I	IAC/BECa-088	*Burkholderia caribensis*	KJ588194	+	+	+	-
	IAC/BECa-089	*Kosakonia oryzae*	KJ588195	+	+	+	+
	IAC/BECa-090	*Pectobacterium* sp.	KJ588196	+	+	+	+
	IAC/BECa-096	*Kosakonia oryzae*	KJ588197	-	+	-	+
	IAC/BECa-101	*Enterobacter asburiae*	KJ588198	-	+	+	+
II	IAC/BECa-133	*Enterobacter radicincitans*	KJ588199	+	+	+	+
	IAC/BECa-137	*Kosakonia oryzae*	KJ588200	+	+	+	+
	IAC/BECa-140	*Kosakonia oryzae*	KJ588201	-	+	+	+
	IAC/BECa-141	*Pseudomonas fluorescens*	KJ588202	-	+	+	+
	IAC/BECa-146	*Enterobacter cloacae*	KJ588203	-	+	+	+

*(Soil Microorganisms Collection). + and - indicate promotion/presence or non-promotion/absence, respectively. ID = identification in the Genbank.

modulated fluorometer (6400-40, Licor, Lincoln NE, USA). The evaluations were taken under constant air CO_2 concentration (380 μmol·mol^{-1}), photosynthetic active radiation (Q) of 1,600 μmol·m^{-2}·s^{-1} and natural variation of air temperature and humidity. The evaluations were performed between 14h00 and 15h00 in the first fully expanded leaf with visible ligule (leaf +1) at the middle third of the leaf blade. Leaf CO_2 assimilation (P_N), stomatal conductance (g_S) and intercellular CO_2 concentration (C_i) were evaluated. The instantaneous carboxylation efficiency (k) was estimated as P_N/C_i (Machado et al. 2009). The chlorophyll fluorescence was evaluated simultaneously to leaf gas exchange, measuring the steady-state (F_S), maximum (F_M') and variable ($\Delta F = F_M' - F_S$) fluorescence emission under light conditions. As general index of photochemical activity, we calculated the apparent electron transport rate as ETR = ($Q \times \Delta F/F_M' \times 0.4 \times 0.85$), where $\Delta F/F_M'$ is the actual quantum efficiency of photosystem II (PSII), 0.4 is the distribution of electrons between photosystems I and II in C_4 plants (Edwards and Baker 1993), and the light absorption by leaves was considered 0.85 (McCormick et al. 2008).

The chlorophyll content was measured with the chlorophyllmeter clorofiLOG (CFL1030, Falker, Porto Alegre RS, Brazil). The device provides indirect readings of chlorophyll *a*, *b* and *a* + *b* contents and data are shown as Falker Chlorophyll Index (FCI).

Carbohydrate and total free amino acids

Carbohydrate and total free amino acids were evaluated in samples of the third fully expanded leaves with visible ligule (leaf +3) collected and kept at –80 °C. Such metabolites were extracted from lyophilized samples (75 mg) with a methanol:chloroform:water solution (12:5:3, v:v:v), according to Bieleski and Turner (1966). The concentrations of soluble sugars (SS) and sucrose (Suc) were quantified according to Dubois et al. (1956) and Van Handel (1968), respectively. Starch (Sta) was quantified by the enzymatic method proposed by Amaral et al. (2007). The concentration of non-structural carbohydrates (NSC) was calculated as the sum of SS and Sta. Amino acids were determined quantitatively by using the colorimetric method of Yemm et al. (1955).

Nitrogen content and activities of nitrate reductase and glutamine synthetase

The total nitrogen concentration in leaves was determined by the Kjeldahl method and expressed in mol·kg^{-1} of dry matter (Bremner 1965). To obtain the enzymatic extract and estimate the activities of nitrate reductase (NR) and glutamine synthetase (GS), we used the procedure described by Silveira et al. (2010), with modifications. Extracts were obtained from 2 g of leaves macerated until fine powder in a mortar with liquid nitrogen and polyvinylpolypyrrolidone (PVPP). The extraction buffer (100 mM Tris-HCl buffer, pH 7.5 containing 10 mM FAD + 20 mM EDTA, 5 mM DTT + 0.5% BSA + mixture of inhibitors: 0.1 mM PMSF + leuptine 10 mM + 1 mM benzidine) was then added to the sample (2.5 mL·g^{-1} of fresh weight). The homogenate was filtered through two layers of cheesecloth and centrifuged at 4 °C for 20 min at 3,000 g. Enzyme activity was measured in the supernatant. →

The activity of nitrate reductase (NR, EC 1.7.1.1) was determined by adding 200 µL of enzymatic extract to a mixture of 500 µL of buffer (Tris-HCl 100 mM pH 7.5 + EDTA 10 mM + KNO_3 5 mM + DTT 5 mM + FAD 10 µM) and 15 µL of NADH 1 mM. The reaction was carried out in a water bath at 30 °C for 30 minutes and stopped at 100 °C for 10 minutes. Then, 750 µL of sulfanilamide (sulfanilamide 1% [w/v] + naphthylethylenediamine dihydrochloride in HCl 2.4 N) were added in the reaction mixture, and the absorbance was measured at 540 nm. The activity was expressed in $nmol \cdot h^{-1} \cdot g^{-1}$ FW.

The activity of glutamine synthetase (GS, EC 6.3.1.2) was determined by adding 100 µL of enzymatic extract to a mixture of 300 µL of buffer (Tris-HCl 50 mM pH 7.0 + $MgSO_4$ 5 mM + hydroxylamine NaOH 100 mM + Na glutamate 50 mM) and 100 µL of ATP 100 mM. The reaction was carried out in a water bath at 30 °C for 30 minutes and stopped by adding 500 µL of ferric solution ($FeCl_3$ 0.37 M + TCA 0.2 M dissolved in HCl 0.67 M). The absorbance was measured at 540 nm and a standard curve was made using γ-glutamyl hydroxymate (GGH) solutions of 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 $\mu mol \cdot mL^{-1}$. The activity was expressed in nmol $GGH \cdot h^{-1} \cdot g^{-1}$ FW.

Biometry

After 72 days of planting, leaves were counted and plant height was evaluated with a tape-measure. At this time, shoot and root dry matter were evaluated after drying samples in a forced air oven at 60 °C.

Statistical analysis

The experiment was arranged in a randomized design, testing a 3 × 2 factorial. One cause of variation was the inoculation (control; inoculum I and II) and the other was the sugarcane genotype (IACSP94-2094 and IACSP95-5000). The data were subjected to ANOVA procedure and mean values were compared by the Tukey test at 5% probability level.

RESULTS AND DISCUSSION

The counting of endophytic bacteria present in stalk segments before inoculation indicated a concentration seven times higher in IACSP95-5000 (22.0×10^5 $CFU \cdot g^{-1}$) than in IACSP94-2094 (3.3×10^5 $CFU \cdot g^{-1}$) at planting. After 72 days of planting, the number of endophytic bacteria found in roots of inoculated plants was higher than that found in control plants, which had only the native bacteria. IACSP94-2094 roots presented 66.7×10^5 $CFU \cdot g^{-1}$ when inoculated with the inoculum I and 74.8×10^5 $CFU \cdot g^{-1}$ when inoculated with the inoculum II, whereas the control plants had 16.7×10^5 $CFU \cdot g^{-1}$. Bacterial counting in IACSP95-5000 ranged from 11.3×10^5 $CFU \cdot g^{-1}$ in plants treated with the inoculum I and 5.3×10^5 $CFU \cdot g^{-1}$ in plants treated with the inoculum II to 3.2×10^5 $CFU \cdot g^{-1}$ in control plants. There was an increase on endophytic bacteria counting in the root of IACSP94-2094, even in control plants, which may be related to specific plant compounds that stimulate bacterial growth (Rosenblueth and Martínez-Romero 2006). Instead, the bacterial community was inhibited in IACSP95-5000. Such differences in colonization are likely due to the complexity of endophytes ecology, being the interactions endophytes-endophytes and endophytes-plants affected by biotic and abiotic factors. In fact, a single plant species has thousands of epiphytic and endophytic microbial species and the interactions between those microorganisms may regulate several physiological processes in the host (Andreote et al. 2014).

The application of the inoculants I and II did not affect the dry matter accumulation of shoots or root as compared to the control (Table 2). In addition, treated plants did not differ from the control plants in height, ranging from 37 to 39 cm for IACSP94-2094 and from 25 to 27 cm for IACSP95-5000. Regardless of genotype, inoculations did not change the number of leaves, with plants showing in average 7 ± 2 leaves.

Herein, shoot and root growth were not enhanced in treated plants (Table 2), indicating that any beneficial effect of bacterial inoculation can be hidden by species-specific interactions between bacteria and also between bacterium and plant. Native bacteria in plant tissues and the bacteria introduced by inoculation can compete for space, carbon and nutrients, a quite different condition from the application of individual bacteria species. Such competition could prevent plant growth promotion as already reported by Ögüt et al. (2005) in bean and wheat plants. On the other hand, these results could indicate that the native endophytic bacterial community was very adapted inside the plant and the plant-bacteria interaction balance was quite stable. Despite the absence of plant growth promotion (Table 2), physiological changes due to inoculation were noticed in both genotypes →

and suggest that growth promotion is a consequence of several mechanisms by which endophytic bacteria may influence plant development.

Leaf CO_2 assimilation was stimulated in inoculated plants (Figure 1a), but the underlying processes causing improved photosynthesis were different when comparing genotypes. Increased photosynthesis in IACSP94-2094 treated with the inoculum II was associated with increases in chlorophyll *a* content (Table 3) and in ETR (Figure 1c). These changes suggest an improved absorption of light energy and use in photochemistry, a key physiological process responsible for ATP and NADPH production in chloroplasts. Shi et al. (2010) also reported that bacteria inoculation caused an increase in ETR and improved photosynthesis, suggesting the presence of unknown compounds produced by bacteria that could increase ETR and chlorophyll metabolism. The presence of bacteria in leaves may also upregulate photosynthetic genes related to ferredoxin and NADPH ferredoxin (Bilgin et al. 2010). On the other hand, increased photosynthesis in IACSP95-5000 treated with the inoculum I was caused by higher stomatal aperture (Figure 1b). Stomatal regulation is affected by endophytic bacteria (Ryan et al. 2007), which may be present in stomatal cells (Compant et al. 2005). Such regulation was previously associated with compounds produced by bacteria as coronatine, with similar action to jasmonate (Brader et al. 2014). Chlorophyll *b* content was also increased in IACSP95-5000 treated with the inoculum II (Table 3). Carboxylation efficiency (*k*) was not affected by inoculation, varying around 3.84 ± 0.78 $\mu mol \cdot m^{-2} \cdot s^{-1} \cdot Pa^{-1}$ in IACSP94-2094 and 3.85 ± 0.54 $\mu mol \cdot m^{-2} \cdot s^{-1} \cdot Pa^{-1}$ in IACSP95-5000.

IACSP94-2094 treated with the inoculum II presented higher leaf sucrose content than the control plants (Figure 2a), a response associated with improved photosynthesis (Figure 1a). However, photosynthesis stimulation in IACSP95-5000 treated with the inoculum I did not increase leaf carbohydrate content (Figures 1a and 2) and plants that received the inoculum II presented large reduction in leaf sucrose content (Figure 2a). As such reduction did not result in low soluble sugars content (Figure 2b), our data indicate the presence of other sugars derived from sucrose hydrolysis. In fact, Shi et al. (2010) found higher fructose concentration in leaves of plants treated with endophytic bacteria. The inocula did not change Sta and NSC concentrations, regardless sugarcane genotype (Figures 2c, d).

There was no significant difference in leaf nitrogen concentration between treatments and the mean value was 1.64 ± 0.03 $mol \cdot kg^{-1}$. However, bacteria inoculation increased nitrate reductase activity in IACSP95-5000, with the highest activity being found in plants treated with the inoculum II (Figure 3a). Regardless of genotype, glutamine synthetase activity was not affected by inoculation (Figure 3b). There →

Table 2. Shoot and root dry matter of IACSP94-2094 and IACSP95-5000 plants treated with the inoculum I and II or untreated (control)*.

Treatment	IACSP94-2094			IACSP95-5000		
	Shoot	Root	Total	Shoot	Root	Total
Control	9.3 ± 1.1[a]	9.6 ± 2.5[a]	18.9 ± 2.6[a]	7.5 ± 0.2[a]	11.7 ± 2.0[a]	18.9 ± 2.3[a]
Inoculum I	8.4 ± 2.1[a]	10.4 ± 2.6[a]	18.4 ± 5.9[a]	7.4 ± 1.4[a]	14.5 ± 3.1[a]	20.6 ± 4.7[a]
Inoculum II	9.2 ± 1.1[a]	10.6 ± 2.0[a]	19.8 ± 2.3[a]	7.7 ± 0.9[a]	13.0 ± 1.8[a]	19.5 ± 3.5[a]

*Mean values ± standard deviation (n = 4). Similar lower case letters indicate non-significant differences between treatments by Tukey's test ($p > 0.05$).

Table 3. Chlorophyll readings (FCI) in leaves of IACSP94-2094 and IACSP95-5000 treated with the inoculum I, II or untreated (control)*.

Genotype	Treatment	Chl *a*	Chl *b*	Chl *a*+*b*
IACSP94-2094	Control	34.3 ± 1.3 [Ab]	13.6 ± 1.9 [Ba]	51.2 ± 0.6 [Aa]
	Inoculum I	34.3 ± 1.9 [Ab]	13.5 ± 0.7 [Ba]	52.1 ± 3.0 [Aa]
	Inoculum II	37.6 ± 1.8 [Aa]	13.8 ± 0.7 [Ba]	52.8 ± 3.9 [Aa]
IACSP95-5000	Control	36.3 ± 0.6 [Aa]	16.9 ± 0.9 [Ab]	54.5 ± 2.6 [Aa]
	Inoculum I	36.7 ± 0.7 [Aa]	15.8 ± 1.2 [Ab]	55.5 ± 0.8 [Aa]
	Inoculum II	35.6 ± 1.8 [Aa]	20.1 ± 1.1 [Aa]	52.2 ± 0.9 [Aa]

*Mean values ± standard deviation (n = 3). Different capital letters indicate statistical differences between genotypes in a given treatment, whereas lower case letters indicate statistical differences between treatments in a given genotype by Tukey's test ($p < 0.05$).

was no difference in total free amino acids among treatments and the mean values were around 2.32 ± 0.16 mg·g^{-1} in IACSP94-2094 and 2.58 ± 0.32 mg·g^{-1} in IACSP95-5000.

Decreases in leaf sucrose content may be related to plant-bacteria interactions (Fuentes-Ramírez et al. 1999) in IACSP95-5000, which also showed higher nitrate reductase activity (Figure 3a). According to Donato et al. (2004), bacteria can affect the N metabolism through nitrate reductase activity, increasing the intake of nitrate and then leaf nitrogen content. As the activities of glutamine synthetase (unaffected) and nitrate reductase (increased) were differently affected by inoculation, we may argue that the bacterial treatment improved the absorption and translocation of nitrate to the leaves. Such assumption is based on leaf nitrogen concentration, which was →

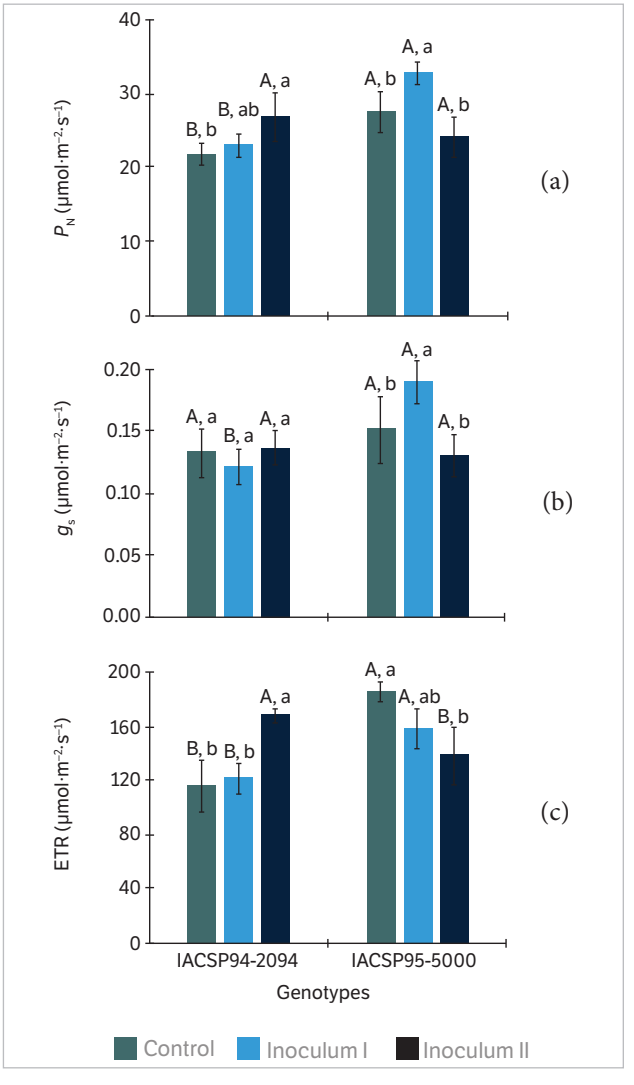

Figure 1. Leaf CO_2 assimilation (P_N, in a), stomatal conductance (g_S, in b) and apparent electron transport rate (ETR, in c) in IACSP94-2094 and IACSP95-5000 treated with the inoculum I, II or untreated (control). Mean values ± standard deviation (n = 3). Different capital letters indicate statistical differences between genotypes in a given treatment, whereas lower case letters indicate differences between treatments in a given genotype by Tukey's test ($p < 0.05$).

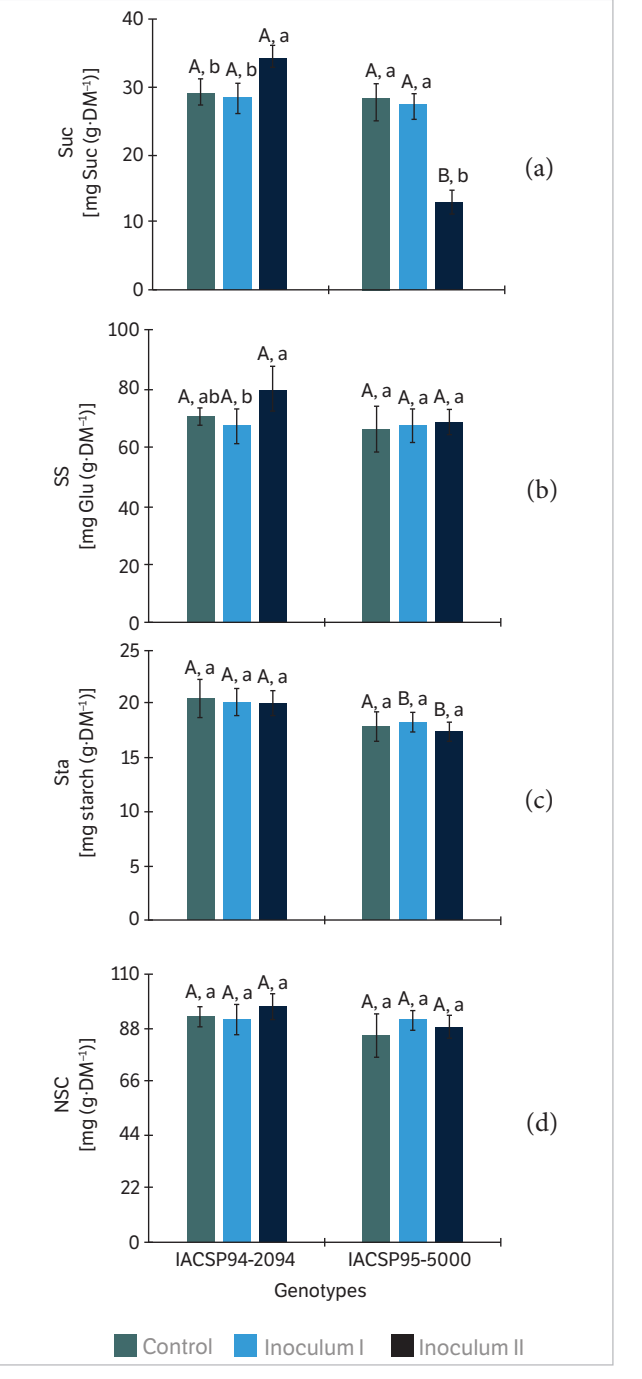

Figure 2. Leaf carbohydrate concentrations in IACSP94-2094 and IACSP95-5000 treated with the inoculum I, II or untreated (control): sucrose (a); soluble sugars (b); starch (c); and total non-structural carbohydrates (d). Mean values ± standard deviation (n = 3). Different capital letters indicate statistical differences between genotypes in a given treatment, whereas lower case letters indicate differences between treatments in a given genotype by Tukey's test ($p < 0.05$).

not changed by inoculation. Regarding the enzymatic activity, one may consider that *in vitro* essays do not necessary reflect the *in vivo* activity of enzymes as temperature, and substrate concentrations would be not the same found *in planta*. Then, the activities of nitrate reductase and glutamine synthetase reported herein would be an indicative that the inocula have changed the sugarcane N metabolism.

Is bacteria inoculation advantageous to sugarcane as there are metabolic costs without promotion of plant growth? Plants were under non-limiting conditions in this study and we should take into account that any advantage associated to inoculation may occur under stress condition (Vargas et al. 2014). For instance,

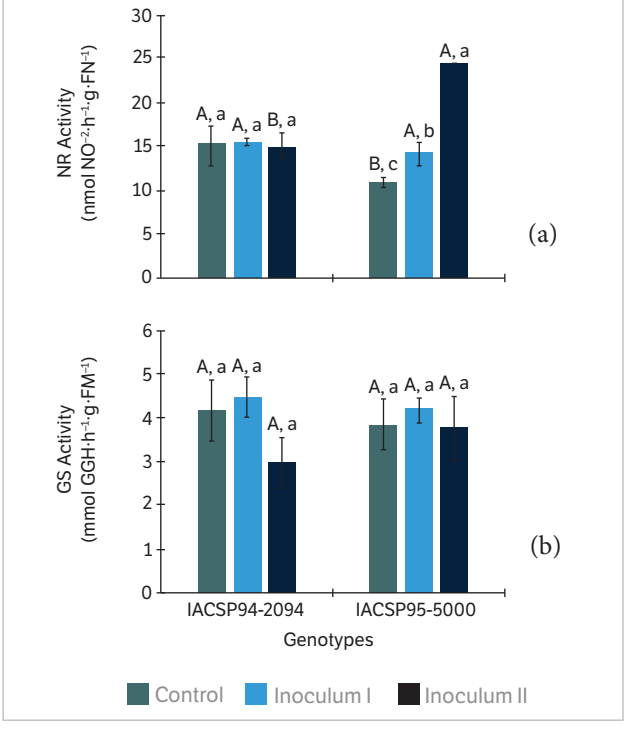

Figure 3. Activities of nitrate reductase (a) and glutamine synthetase (b) in leaves of IACSP94-2094 and IACSP95-5000 treated with the inoculum I, II or untreated (control). Mean values ± standard deviation (n = 3). Different capital letters indicate statistical differences between genotypes in a given treatment, whereas lower case letters indicate differences between treatments in a given genotype by Tukey's test ($p < 0.05$). GGH = glutamyl hydroxymate.

increases in carbohydrate status of IACSP94-2094 may benefit plant metabolism under constraining conditions, maintaining energy and carbon supply and then plant homeostasis. The increases in stomatal conductance of IACSP95-5000 due to inoculation (Figure 1b) may be a positive change under short-term water deficit, favoring CO_2 supplying to photosynthesis. Accordingly, positive effects of bacteria inoculation in plant nutrition were observed only in low fertility soils (Oliveira et al. 2006). Time after inoculation is another aspect to be considered when the interaction between plants and bacteria is studied. Chauhan et al. (2013) reported positive effects of bacteria inoculation in sugarcane plants grown under field conditions after six months, with plants showing improvements in chlorophyll content, N content and yield. On the other hand, studies have shown that the most pronounced effects could occur at the beginning of growth after inoculation.

CONCLUSION

Our data demonstrate that bacterial mixtures affect sugarcane physiology, improving photosynthesis and nitrate reduction in a genotype-dependent manner. However, such physiological changes are not associated with biomass production in sugarcane plantlets, obtained from mini stalks with one bud, already colonized by native endophytic bacteria and grown under non-limiting conditions.

ACKNOWLEDGEMENTS

We acknowledge the financial support of the São Paulo Research Foundation (FAPESP, Brazil), through the Bioen Program (Proc. 2008/57495-3 and 2008/56147-1). The authors received scholarship (FCCM) and fellowships (ECM, RVR) from the National Council for Scientific and Technological Development (CNPq, Brazil). The authors are also grateful to Dr. Matheus A. Cipriano for the critical reading and suggestions.

REFERENCES

Amaral, L. I. V., Gaspar, M., Costa, P. M. F., Aidar, M.P.M. and Buckeridge, M. S. (2007). Novo método enzimático rápido e sensível de extração e dosagem de amido em materiais vegetais. Hoehnea, 34, 425-431. http://dx.doi.org/10.1590/S2236-89062007000400001.

Andreote, F. D., Gumiere, T. and Durrer, A. (2014). Exploring interactions of plant microbiomes. Scientia Agricola, 71, 528-539. http://dx.doi.org/10.1590/0103-9016-2014-0195.

Barretti, P. B., Souza, R. M., Pozza, A. A. A., Pozza, E. A., Carvalho, J. G. and Souza, J. T. (2008). Aumento da eficiência nutricional

de tomateiros inoculados com bactérias endofíticas promotoras de crescimento. Revista Brasileira de Ciência do Solo, 32, 1541-1548. http://dx.doi.org/10.1590/S0100-06832008000400018.

Bieleski, R. L. and Turner, A. (1966). Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Analytical Biochemistry, 17, 278-293. http://dx.doi.org/10.1016/0003-2697(66)90206-5.

Bilgin, D. D., Zavala, J. A., Zhu, J., Clough, S. J., Ort, D. R. and DeLucia, E. H. (2010). Biotic stress globally downregulates photosynthesis genes. Plant, Cell and Environment, 33, 1597-1613. http://dx.doi.org/10.1111/j.1365-3040.2010.02167.x.

Botta, A. N., Santacecilia, A., Ercole, C., Cacchio, P. and Del Gallo, M. (2013). *In vitro* and *in vivo* inoculation of four endophytic bacteria on *Lycopersicon esculentum*. New Biotechnology, 30, 666-674. http://dx.doi.org /10.1016/j.nbt.2013.01.001.

Brader, G., Compant, S., Mitter, B., Trognitz, F. and Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. Current Opinion in Biotechnology, 27, 30-37. http://dx.doi.org/10.1016/j.copbio.2013.09.012.

Bremner, J. M. (1965). Total nitrogen. In C. A. Black (Ed.), Methods of soil analysis: Chemical and microbiological properties (p. 1149-1178). Madison: American Society of Agronomy.

Chauhan, H., Bagyaraj, D. J. and Sharma, A. (2013). Plant growth-promoting bacterial endophytes from sugarcane and their potential in promoting growth of the host under field conditions. Experimental Agriculture, 49, 43-52. http://dx.doi.org/10.1017/S0014479712001019.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C. and Barka, E. A. (2005). Endophytic colonization of *Vitis vinifera* L. by Plant Growth-Promoting Bacterium *Burkholderia* sp. Strain PsJN. Applied and Environmental Microbiology, 71, 1685-1693. http://dx.doi.org/10.1128/AEM.71.4.1685-1693.2005.

Döbereiner, J., Baldani, J. I. and Baldani, V.L.D. (1995). Como isolar e identificar bactérias diazotróficas de plantas não leguminosas. Brasília: EMBRAPA.

Donato, V. M. T. S., Andrade, A. G., Souza, E. S., França, J. G. E. and Maciel, G. A. (2004). Atividade enzimática em variedades de cana-de-açúcar cultivadas *in vitro* sob diferentes níveis de nitrogênio. Pesquisa Agropecuária Brasileira, 39, 1087-1093. http://dx.doi.org/10.1590/S0100-204X2004001100006.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith. F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28, 350-356. http://dx.doi.org/10.1021/ac60111a017.

Edwards G. E. and Baker, N. R. (1993). Can CO_2 assimilation in maize leaves be predicted accurately from chlorophyll fluorescence analysis? Photosynthesis Research, 37, 89-102. http://dx.doi.org/10.1007/BF02187468.

Fuentes-Ramírez, L. E., Caballero-Mellado, J., Sepúlveda, J. and Martínez-Romero, E. (1999). Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. FEMS Microbiology Ecology, 29, 117-128. http://dx.doi.org/10.1111/j.1574-6941.1999.tb00603.x.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F. and Kloepper, J.W. (1997). Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology, 43, 895-914. http://dx.doi.org/895-914, 10.1139/m97-131.

Landell, M. G. A., Campana, M. P., Figueiredo, P., Vasconcelos, A. C. M., Xavier, M. A., Bidoia, M. A. P., Prado, H., Silva, M. A., Dinardo-Miranda, L. L., Santos, A. S., Perecin, D., Rossetto, R., Silva, D. N., Martins, A. L. M., Gallo, P. B., Kantack, R. A. D., Cavichioli, J. C., Veiga, A. A. Filho., Anjos, I. A., Azania, C. A. M., Rossini Pinto, L. and Souza, S. A. C. D. (2005a). Variedades de cana-de-açúcar para o Centro-Sul do Brasil: 16ª liberação do programa Cana IAC (1959-2007). Campinas: Instituto Agronômico.

Landell, M. G. A., Campana, M. P., Figueiredo, P., de Vasconcelos, A. C. M., Xavier, M. A., Bidoia, M. A. P., Prado, H., Silva, M. A., Dinardo-Miranda, L. L., Santos, A. S., Perecin, D., Rossetto, R., Silva, D. N., Martins, A. L. M., Gallo, P. B., Kantack, R. A. D., Cavichioli, J. C., Veiga, A. A., Filho., Anjos, I. A., Azania, C. A. M., Rossini Pinto, L. and Souza, S. A. C. D. (2005b). Variedades de cana-de-açúcar para o Centro-Sul do Brasil: 15ª liberação do programa Cana IAC (1959-2005). Campinas: Instituto Agronômico.

Machado, R. S., Ribeiro, R. V., Marchiori, P. E. R., Machado, D. F. S. P., Machado, E. C. and Landell, M. G. A. (2009). Respostas biométricas e fisiológicas ao déficit hídrico em cana-de-açúcar em diferentes fases fenológicas. Pesquisa Agropecuária Brasileira, 44, 1575-1582. http://dx.doi.org/10.1590/S0100-204X2009001200003.

McCormick, A. J., Cramer, M. D. and Watt, D. A. (2008). Changes in photosynthetic rates and gene expression of leaves during a source-sink perturbation in sugarcane. Annals of Botany, 101, 89-102. http://dx.doi.org/10.1093/aob/mcm258.

Moutia, J. F. Y., Saumtally, S., Spaepen, S. and Vanderleyden, J. (2010). Plant growth promotion by *Azospirillum* sp. in sugarcane is influenced by genotype and drought stress. Plant and Soil, 337, 233-242. http://dx.doi.org/10.1007/s11104-010-0519-7.

Ögüt, M., Akdag, C., Düzdemir, O. and Sakin, M. A. (2005). Single and double inoculation with *Azospirillum*/*Trichoderma*: the effects on dry bean and wheat. Biology and Fertility of Soils, 41, 262-272. http://dx.doi.org/10.1007/s00374-004-0818-3.

Oliveira, A. L. M., Canuto, E. L., Urquiaga, S., Reis, V. M. and Baldani, J. I. (2006). Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria. Plant and Soil, 284, 23-32. http://dx.doi.org/10.1007/s11104-006-0025-0.

Rosenblueth, M. and Martínez-Romero, E. (2006). Bacterial endophytes and their interaction with hosts. Molecular Plant-Microbe Interactions, 19, 827-837. http://dx.doi.org/10.1094/MPMI-19-0827.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J. and Dowling, D. N. (2007). Bacterial endophytes: recent developments and applications. FEMS Microbiology Letters, 278, 1-9. http://dx.doi.org/10.1111/j.1574-6968.2007.00918.x.

Shi, Y., Lou, K. and Li, C. (2010). Growth and photosynthetic efficiency promotion of sugar beet (*Beta vulgaris* L.) by endophytic bacteria. Photosynthesis Research, 105, 5-13. http://dx.doi.org/10.1007/s11120-010-9547-7.

Silveira, J. A. G., Martins, M. O., Lima, A. B. and Ferreira-Silva, S. (2010). Redução de nitrato e assimilação da amônia em sistemas vegetais: mensuração de atividade enzimática e metabólitos. In M. V. B. Figueiredo, Biotecnologia aplicada à agricultura: textos de apoio e protocolos (p. 93-124). Brasília: EMBRAPA.

Singh, M. K., Singh, D. P., Mesapogu, S., Babu, B. K. and Bontemps, C. (2011). Concomitant colonization of nifH positive endophytic *Burkholderia* sp. in rice (*Oryza sativa* L.) promotes plant growth. World Journal of Microbiology and Biotechnology, 27, 2023-2031. http://dx.doi.org/10.1007/s11274-011-0664-z.

Urquiaga, S., Xavier, R. P., Morais, R. F., Batista, R. B., Schultz, N., Leite, J. M., Sá, J. M., Barbosa, K. P., Resende, A. S., Alves, B. J. R. and Boddey, R. M. (2012). Evidence from field nitrogen balance and ^{15}N natural abundance data for the contribution of biological N_2 fixation to Brazilian sugarcane varieties. Plant and Soil, 356, 5-21. http://dx.doi.org/10.1007/s11104-011-1016-3.

Van Handel, E. (1968). Direct microdetermination of sucrose. Analytical Biochemistry, 22, 280-283. http://dx.doi.org/doi:10.1016/0003-2697(68)90317-5.

Vargas, L., Brígida, A. B. S., Mota Filho., J. P., Carvalho, T. G., Rojas, C. A., Vaneechoutte, D., Van Bel, M., Farrinelli, L., Ferreira, P. C. G., Vandepoele, K. and Hemerly, A. S. (2014). Drought tolerance conferred to sugarcane by association with *Gluconacetobacter diazotrophicus*: a transcriptomic view of hormone pathways. PLoS One, 9, e114744. http://dx.doi.org/10.1371/journal.pone.0114744.

Yemm, E. W., Cocking, E. C. and Ricketts, R. E. (1955). The determination of amino-acids with ninhydrin. Analyst, 80, 209-214. http://dx.doi.org/10.1039/AN9558000209.