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Application of a mixture of five diazotrophs on sugarcane cultivated in the south of Brazil

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ABSTRACT: Sugarcane production in Brazil for the 2019/20 harvest is estimated at approximately 615 million tons. However, in the state of Rio Grande do Sul, it is estimated at only 42.5 thousand tons. It is assumed that the use of diazotrophic bacteria can contribute to the expansion of sugarcane cultivation in the state through increased productivity; hence, this study aimed to evaluate the effects of diazotrophs on the growth of sugarcane. The study was conducted using sugarcane varieties: RB867515, RB92579, RB966928, and RB975932 that were either treated with diazotrophic bacteria or left as a control. The application of diazotrophic bacteria resulted in increased leaf area and stem number in variety RB867515 (cane plant cultivation cycle), and an increase in the relative chlorophyll content of all the varieties of sugarcane except of RB92579. Furthermore, most varieties responded positively in terms of biomass, dry matter, total nitrogen, and soluble solids. These results suggest that the application of diazotrophs assists in the growth and development of sugarcane varieties in Rio Grande do Sul.

Key words: biological nitrogen fixation; *Saccharum* spp.; total soluble solids; yield

Aplicação de um mix de bactérias diazotróficas no cultivo de cana-de-açúcar no sul do Brasil

RESUMO: A produção de cana-de-açúcar no Brasil para a safra 2019/20 é estimada em aproximadamente 615 milhões de toneladas. No entanto, no estado do Rio Grande do Sul, estima-se em apenas 42,5 mil toneladas. Contudo, acredita-se que o uso de bactérias diazotróficas possa contribuir para a expansão do cultivo da cana no estado, através do aumento da produtividade, portanto, este estudo teve como objetivo avaliar os efeitos dos diazotróficos no cultivo de cana-de-açúcar. Utilizou-se as variedades de cana-de-açúcar: RB867515, RB92579, RB966928 e RB975932, que foram tratadas com bactérias diazotróficas ou deixadas como controle. A aplicação de bactérias diazotróficas resultou em aumento da área foliar e número de colmos na variedade RB867515 (ciclo de cultivo da cana) e aumento no teor relativo de clorofila de todas as variedades de cana-de-açúcar, exceto RB92579. Além disso, a maioria das variedades respondeu positivamente em termos de biomassa, matéria seca, nitrogênio total e sólidos solúveis. Esses resultados sugerem que a aplicação de bactérias diazotróficas auxilia no crescimento e desenvolvimento de variedades de cana-de-açúcar cultivadas no Rio Grande do Sul.

Palavras-chave: fixação biológica de nitrogênio; *Saccharum* spp.; sólidos solúveis totais; crescimento

Introduction

Sugarcane production in Brazil for the 2019/20 harvest is estimated at approximately 615 million tons, with a harvested area of 8 million hectares. Rio Grande do Sul (RS) appears last in the national ranking, with a cultivated area of only 22 thousand hectares, average productivity of 45 kg ha^{-1}, and expected production in the order of 42.5 thousand tons (Conab, 2019). However, the state has a large area of aptitude for cultivation, described by Manzatto et al. (2010) in the agroclimatic zoning. In addition, sugarcane is among the seven main crops of economic value, including soybeans, rice, corn, and wheat, contributing approximately 70 million reais to the economy of Rio Grande do Sul (Silva et al., 2016).

The low productivity of sugarcane in RS is due to the use of old varieties that generally have low yield and little adaptation to environmental conditions. Therefore, studies have been developed aiming at increasing productivity, in the scope of the use of cultivars adapted to the different climate and soil conditions (Antunes et al., 2017) and utilization of plant growth-promoting diazotrophic bacteria (Oliveira et al., 2006; Schultz et al., 2014).

Endophytic diazotrophic bacteria inhabit the interior of sugarcane plants and assist in obtaining nutrients through biological nitrogen fixation and production of phytohormones that modify root architecture, CO_2 assimilation, and leaf number (Fischer et al., 2012). They also act in other processes such as phosphate and zinc solubilization (Estrada et al., 2013), production of auxins, gibberellins, and cytokines (Lin et al., 2012) and protection against pathogens, which result in increased growth and productivity (Pedula et al., 2016; Schultz et al., 2017).

The application of bacteria presents variability from the soil, the studied variety, and the applied strains (Oliveira et al., 2006). In this study, the inoculant used contained a pool of five strains that showed a beneficial effect in sugarcane, such as increased productivity (Schultz et al., 2017); however, little is known about the cultivation of inoculated sugarcane in Rio Grande do Sul. It is assumed that the use of diazotrophic bacteria can contribute to the expansion of sugarcane cultivation in the state through increased productivity. Therefore, the purpose of the present research was to evaluate the effects of the application of a mix of diazotrophic bacteria on the growth, maturation, and productivity of four sugarcane varieties grown in the state of Rio Grande do Sul.

Materials and Methods

Site characterization and experimental area preparation

The study was carried out from November 2015 to July 2017, in a rural property in Monte Bonito, 9th district of Pelotas, located at latitude 52°22’10’’ West and 32°41’08’’ South with an of altitude of 50 m. The climate in the region is warm and temperate, classified as Cfa. The average temperature is 18 °C, and there is significant rainfall throughout the year, with an average of 1378 mm (Figure 1). The soil is characterized as Dystrophic Bruno-gray Argisol, and the experimental area was prepared by plowing, harrowing and liming the soil with 4.8 Mg ha^{-1} of dolomitic limestone (PRNT 100%) also, 210 Kg ha^{-1} of triple superphosphate and 120 kg ha^{-1} of potassium chloride were added to the soil. The fertilization was carried out according to the official fertilization, and liming recommendations adopted in the states of Rio Grande do Sul and Santa Catarina (CQFS RS/SC, 2004) and based on chemical soil analysis carried out in 0-20 cm depth sampling, with the following results: pH (H_2O) 5.5; SMP Index 5.7, 0.4 cmol dm^{-3} Al; 1.4 cmol dm^{-3} Ca; 0.7 cmol dm^{-3} Mg; 70 mg dm^{-3} K and 2.9 mg dm^{-3} of P.

Installation and conduction of the experiment

The experimental design was a randomized block design with four replications, and a 4 x 2 bifactorial scheme was used, using four sugarcane varieties (RB867515, RB92579, RB966928, and RB975932), with and without diazotrophic bacteria inoculation, which totaled eight treatments.

Sugarcane propagation was done by transplanting pre-sprouted seedlings produced at Embrapa Clima Temperado, from small stalks, previously treated by thermotherapy at 52 °C for 30 minutes and fungicide of systemic action based on pyraclostrobin, through a solution containing 50 mL of

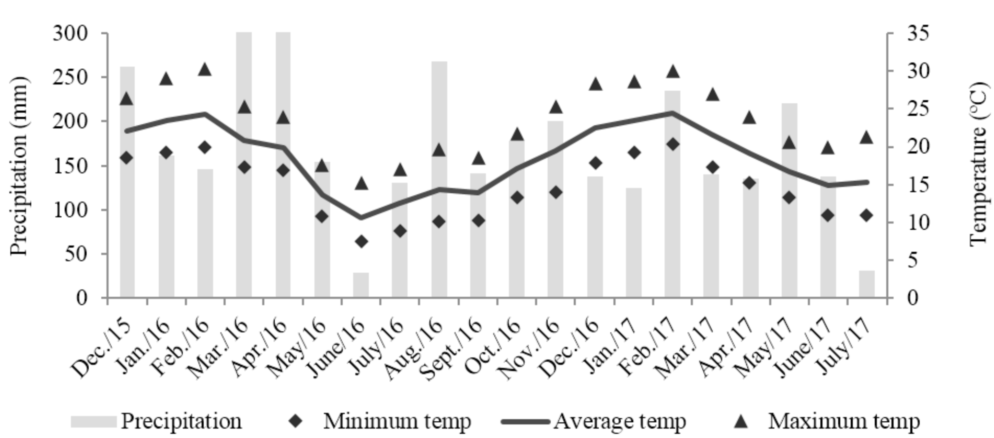

Figure 1. Precipitation data (mm) and minimum, average, and maximum temperatures (° C) from December 2015 to July 2017, provided by Embrapa Weather Station.

the product in 50 L of water, where they were immersed for 3 min (Santos et al. 2019). Small stalks were inoculated with a pool of diazotrophic bacteria containing the strains: *Gluconacetobacter diazotrophicus* strain BR11281^T = PAL-5^T; *Herbaspirillum seropedicae* BR11335 (= HRC54); *Herbaspirillum rubrisubalbicans* BR11504 (= HCC103); *Paraburkholderia tropica* BR11366^T = PPe8^T, and *Nitrospirillum amazonense* BR11145 (= CBAMc). These bacteria were previously tested and selected by Oliveira et al. (2002, 2006) and were provided by the Embrapa Agrobiology Diazotrophic Bacteria Collection - Johanna Döbereiner. Each strain was grown separately in DYGS medium (Baldani et al., 2014) at 175 rpm for 24-48 h depending on the strain and run on ground, neutralized (pH 6.0) and autoclaved sterile peat (121 °C, 20 min, 2 times) in the proportion of 70 mL of cell suspension of each strain in 250 g of peat. Peat inoculants were diluted in the proportion of 250 g / 50 L of water, and the small stalks were immersed in the solution for 30 min (Reis et al., 2009). After inoculation, they were planted in 290 cm^3 tubes containing Turfa Fértil® substrate and kept for about 30 days in a greenhouse for budding and growth of seedlings. After this stage, the seedlings were transferred to a 35% shading screen for the first phase of acclimatization to occur, then left in the open to complete acclimatization in sunlight for a total of 15 days, and finally taken to the field.

The seedlings were planted manually with the aid of a manual planter, where three rows of three meters represented each plot. Each of the rows had six plants, with a spacing of 0.5 m between plants and 1.4 m between rows, with the useful area of the plot represented by the central row. At 90 days after transplantation, plant cane fertilization was performed with 20 kg ha^{-1} of N and 30 days after the first cut, 150 Kg ha^{-1} of triple superphosphate, 135 Kg ha^{-1} of potassium chloride, and 100 Kg ha^{-1} of urea were added for ratoon cane fertilization purposes. Weed management in the experimental area was done by weeding between planting rows, and the application of selective and post-emergent Mesotrione herbicide at 400 mL ha^{-1}. The first harvest was held in September 2016 and the second harvest in July 2017.

Evaluation and statistical analysis parameters

At 120 days after transplanting cane plant cycle seedlings to the field and after the regrowth of the ratoon cane, the leaf area was estimated from the number of leaves and the leaf length and width measurements of a tiller. The values were substituted into the equation where LAI = [C x W x 0.75 x (NF + 2)]. The formula was described by Hermann & Câmara (1999) and considered the length (C) and width (L) of the +3 leaves and the number of open leaves with at least 20% green area in the plant (NF). Then, through the relationship between the leaf area and the soil area occupied by the plant, the leaf area index (LAI) was determined. The relative chlorophyll content was observed in the intermediate portion of the +3 leaves, through the chlorophyll meter ClorofiLOG, brand FALKER, and model CFL 1030. This equipment uses three light frequency ranges, allowing for a detailed analysis that can be viewed instantly or stored in the computer. Optical measurement analyzes leaf light absorption, indicating the presence of chlorophyll at SPAD (Soil Plant Analysis Development) values.

At the time of harvesting, the number of stalks was determined, and two clumps were weighed per plot to estimate from the sugarcane weight per linear meter, the total shoot biomass production per hectare. To determine the dry mass of the sugarcane, leaf and stem samples were individually ground in a Trapp model TR 200 branch and organic waste shredder, then weighed and taken to the greenhouse, where they remained at 60ºC for about seven days and were weighed again to obtain dry weight. Total nitrogen (g) was evaluated from 2 mm solid samples of plant tissue in the Perkin-Elmer Elemental Analyzer (world standard). Moreover, in ratoon cane, as there was a better development of stalks, it was possible to observe the total soluble solids content (° Brix) at harvest. In addition, with the removal of the pointer containing the leaves, it was possible to estimate the stem yield (Mg ha^{-1}) and sugar yield (Mg brix ha^{-1}).

Statistical analysis was performed using the SAS 8.2 software, and graphical synthesis was performed in SigmaPlot 10.0. The data obtained were analyzed for normality by the Shapiro-Wilk test, homoscedasticity by the Hartley test, and the independence of the residuals was verified graphically. Subsequently, the data were subjected to analysis of variance ($p < 0.05$). In the case of statistical significance, the effects and inoculation of the varieties were compared by the Tukey test and t-test, respectively.

Results

With the application of the F-test in the analysis of variance, we identified the significance ($p < 0.05$) of the bifactorial interaction (varieties x inoculation) for all evaluation parameters in both production cycles. Thus, the isolated effects of the factors were disregarded, and the interactions were analyzed in detail (Figures 2, 3, 4, and 5).

In cane plants, the highest leaf area index (Figure 2a) was presented by the variety RB867515 in the inoculated treatment and in control, which does not differ from the RB975932 variety. In addition, this variety was the only one that had increased LAI when inoculated with diazotrophic bacteria. However, in ratoon cane (Figure 2b), differences between the varieties were observed only in the control, where the LAI was higher in the varieties RB867515 and RB92579.

The SPAD index, which reflects relative chlorophyll content, was similar in all cane plant treatments, ranging from 37.8 to 49.3 (Figure 2c) and in ratoon cane was higher in the varieties RB966928, RB867515, and RB975932 in inoculated treatments with values of 39.2, 42.76, and 51.63, respectively (Figure 2d).

The inoculated treatments presented higher dry matter content than the control in the varieties RB867515 and RB975932 in cane plant for the two years of evaluation and in RB966928 only in the cultivation of ratoon cane (Figure 3a and 3b). For nitrogen accumulation (Figure 3c and 3d), inoculation

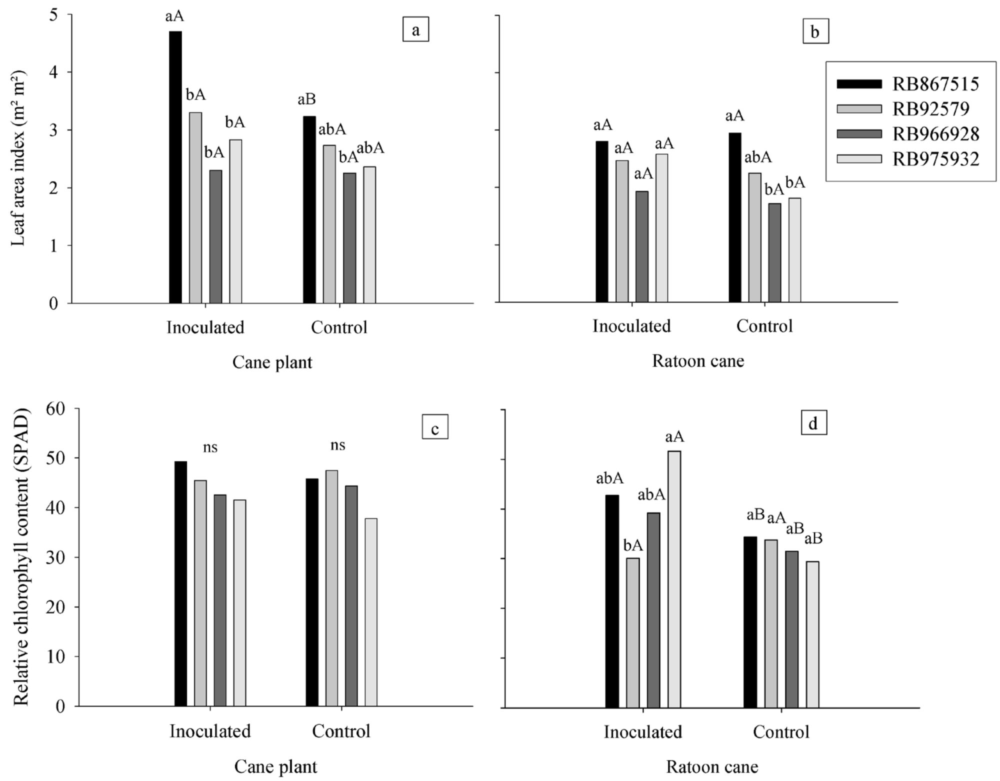

Figure 2. Leaf area (a and b) and relative chlorophyll content (c and d) of four varieties of sugarcane inoculated or not with a mixture of five strains of diazotrophic bacteria in two cultivation cycles (cane plant and ratoon cane). Non-significant (ns) and bars having the same upper and lower case letters do not differ, comparing the inoculation within each variety by the t-test and the variety within the inoculation by the Tukey test (p ≥ 0.05).

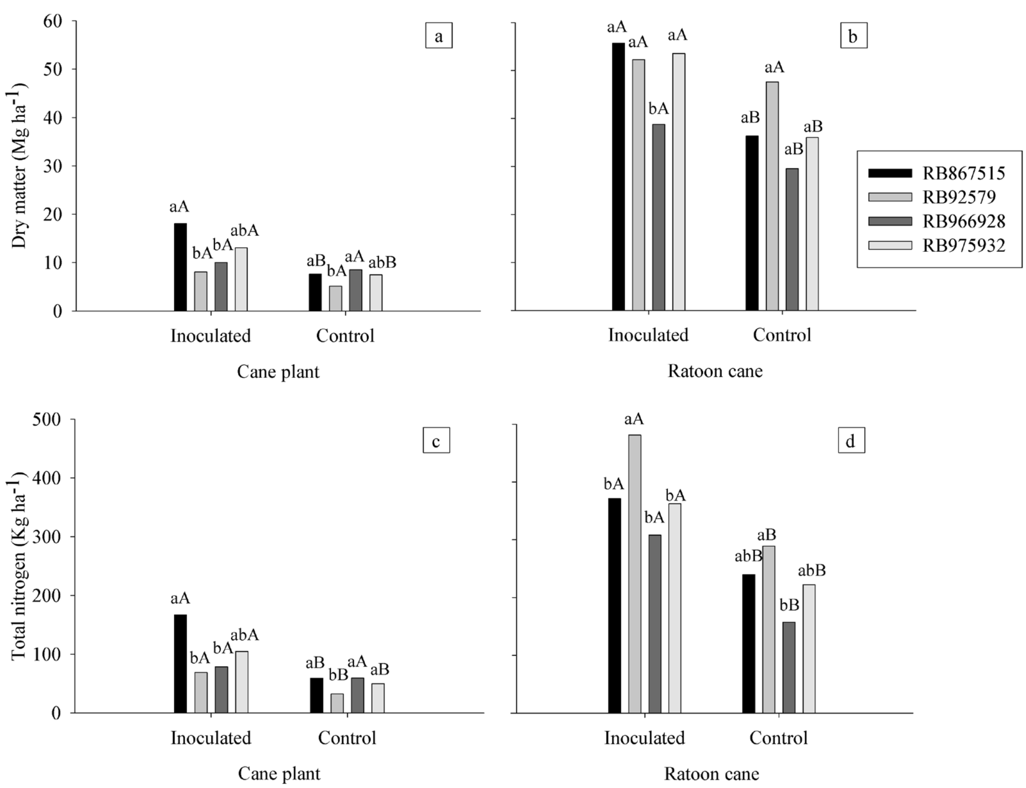

Figure 3. Dry matter (a and b) and total nitrogen (c and d) of four varieties of sugarcane inoculated or not with a mixture of five strains of diazotrophic bacteria in two cultivation cycles (cane plant and ratoon cane). Bars having the same upper and lower case letters do not differ, comparing the inoculation within each variety by the t-test and the variety within the inoculation by the Tukey test (p ≥ 0.05).

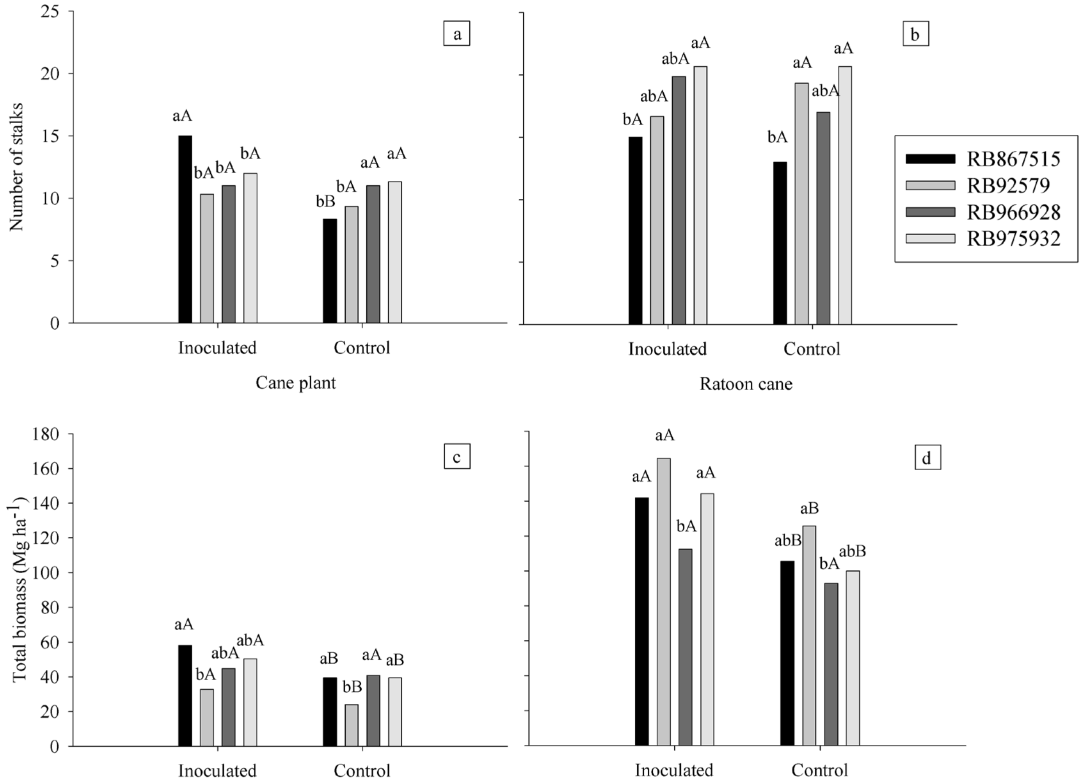

Figure 4. Number of stalks (a and b) and total biomass (c and d) of four varieties of sugarcane inoculated or not with a mixture of five strains of diazotrophic bacteria in two cultivation cycles (cane plant and ratoon cane). Bars having the same upper and lower case letters do not differ, comparing the inoculation within each variety by the t-test and the variety within the inoculation by the Tukey test ($p \geq 0.05$).

promoted growth for all varieties in the two cultivation cycles except for RB966928 in the cane plant cycle.

The number of stalks, as seen in Figure 4 (a and b), was slightly influenced by the inoculation of diazotrophic bacteria and the only variety that showed increase in this parameter was the RB867515 in the cane plant cycle, where the number of stalks was higher than the other varieties. However, in ratoon cane, the highest results were presented by the varieties RB92579 and RB975932, which did not differ from RB966928. In contrast, inoculation provided an increase in the total biomass (Figure 4c and 4d) of the varieties RB867515, RB92579, and RB975932 in both cane plant and ratoon cane.

However, total soluble solids content (ºBrix) and sugar yield estimated in Mg ha^{-1} were influenced by the inoculation of diazotrophic endophytic bacteria in all varieties (Figs 5a and 5c). For stem yield (Figure 5b), only RB867515 responded to inoculation. Among the varieties, RB966928 presented the highest ºBrix, regardless of the inoculation treatment, and the stalk productivity was higher in the inoculated variety RB867515 and the control RB92579, resulting in a higher sugar yield also in these two varieties.

Discussion

The study of the leaf area in sugarcane varieties allows the correlation with their productive potential, either in growth rates, stem production, dry mass, or amount of sugar. The leaf is the structure responsible for producing most of the carbohydrates essential for plant growth and development through photosynthesis; therefore, crop yield will be higher depending on how fast the plant reaches maximum leaf area and how long the leaf area remains active. Thus, inoculation with diazotrophic bacteria plays an important role due to biological nitrogen fixation, which acts on leaf elongation of plants and, consequently, on the increase of photosynthetic rate (Schultz et al., 2017).

The optimal leaf area index is not necessarily the highest index recorded, but the one in which photosynthetically active leaves are kept slightly above the compensation point. According to Machado et al. (1982), for sugarcane, the LAI close to 4.0 is sufficient to intercept 95% of incident solar radiation, however, in the present study, values from 1.7 to 4.7 were recorded, as seen in Figures 2a and 2b, which is common to occur due to differences between the sugarcane varieties (Santos, 2018).

Chlorophyll content is related to higher dry mass production, and it is proportional to how much nitrogen was absorbed by the plant (Martuscello et al., 2009). Chlorophyll content can be observed in the inoculated plants of the varieties RB867515, RB966928, and RB975932 having values superior to the control without inoculation in the ratoon cane cycle (Figure 2d), which were proportional to the increase in dry mass and nitrogen in these same treatments. (Figure 3b and 3d). However, an increase in N content was observed in

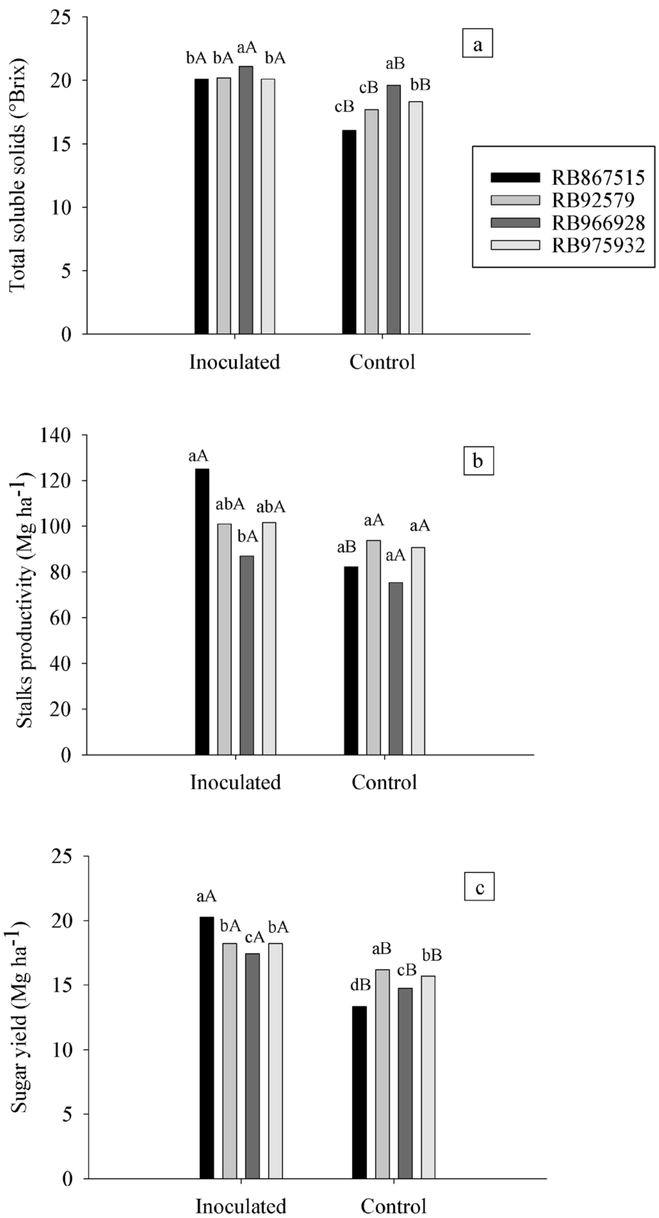

Figure 5. Total soluble solids (a), stalks productivity (b), and sugar yield (c) of four varieties of sugarcane inoculated or not with a mixture of five strains of diazotrophic bacteria in the ratoon cane. Bars having the same upper and lower case letters do not differ, comparing the inoculation within each variety by the t-test and the variety within the inoculation by the Tukey test ($p \geq 0.05$).

RB92579, although there were no differences in chlorophyll compared to control. This increase was also observed by other authors who used the same mixture of diazotrophic bacteria in the variety RB92579 (Pedula et al., 2016).

The emission of stalks in plants of the Poaceae family is called tillering, which begins around 40 days after planting and can last up to 120 days. Tillering is a physiological process of continuous underground branching of the compressed nodal joints to the primary shoot, which provides the cultivation with the number of stalks necessary for a good production (Diola & Santos, 2010).

In general, all treatments in ratoon cane showed better performance in stem formation and biomass production (Figure 4), probably due to the time tillering and development occurred in each of the growing years. The highest sugarcane production in Rio Grande do Sul occurs in the first month of the year, mainly due to the optimum temperature and light conditions, considering that it is a plant with C4 metabolism, highly efficient in the conversion of light energy into energy in chemistry through the photosynthetic process (Kluge et al., 2015). The monthly data of minimum, average and maximum temperatures, as well as precipitation, during the conduction of the experiment, in the 2015/2016 and 2016/2017 crops (Figure 1) confirm the occurrence of high temperatures, and this explains the observed performance in ratoon cane. However, in the cane plant, this period coincided with the tillering phase, thus delaying the growth and development of stalks and, consequently, the production of biomass.

In addition, genetic factors and auxin production also influence tillering; therefore, inoculation with bacteria widely recognized as inducing growth-promoting substances (Cassán et al., 2014) combined with intrinsic factors of the varieties, are closely linked to stem emission and production of sugarcane biomass.

Several factors such as genetic, climatic, and crop management factors, can affect sugarcane productivity and technological quality (Thomé et al., 2018). Bacterial inoculation promoted increases in ° Brix as well as the anticipation of sugarcane plant maturation, including known late-medium materials, RB867515 and RB92579, which in the control treatment were not ripe for harvesting. The variety RB966928 did not respond to inoculation, presenting high and similar values in both treatments, because it is a characteristic of this variety to have an early maturation behavior.

These results are quite promising, especially considering that the RB867515, RB92579, and RB966928 genotypes are among the most cultivated genotypes in Brazil. Nevertheless, further studies on sugarcane quality are needed to confirm whether these increases in total soluble solids with inoculation of diazotrophic endophytic bacteria are relative to sucrose content. Maximum accumulation of sucrose only occurs when the plant encounters some restriction in its growth (Podesta, 2015), which would also happen in uninoculated treatments; therefore, the anticipation of sugarcane maturation shows that knowledge about the potential of diazotrophic bacteria on sugarcane is still limited.

Conclusion

The application of a mixture of five diazotrophs assists in the growth and development of sugarcane varieties grown in Rio Grande do Sul, resulting in increased productivity, dry mass, nitrogen accumulation, and early maturation, which is promising for the expansion of the crop cultivation in the state.

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