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Resistance to water deficit during the formation of sugarcane seedlings mediated by interaction with *Bacillus* sp.

Resistência ao déficit hídrico na formação de mudas de cana-de-açúcar mediada pela interação com *Bacillus* sp.

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Abstract

Sugarcane is a crop of great global importance, but its production can be greatly affected by water stress normally occurring in growing areas. Currently, several solutions have been proposed to minimize such effects, among them the use of microorganisms that improve the resistance of plants to water stress. The objective of this study was to evaluate the induction of resistance of sugarcane plants to water stress using *Bacillus* sp. For this, an experiment was performed in controlled greenhouse conditions using billets provided with twenty different varieties of sugarcane, treated or not with *Bacillus* sp. isolates, with 5 replications. The plants were grown for 45 days and, after this period, the following variables were analyzed: dry mass (from top, root and whole plant) and C, N and C/N contents. Analysis of variance, mean tests (Scott-Knott and Tukey, p≤0.05) and a hierarchical analysis was performed by cluster analysis according to the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean). The analyses were performed using the software R. The isolate of *Bacillus* sp. promoted resistance to drought for different varieties of sugarcane, and may be an alternative to mitigate the effects of water stress on some sugarcane varieties.

Additional keywords: drought; microorganism; varieties.

Resumo

A cana-de-açúcar é uma cultura de grande importância mundial, porém sua produção pode ser altamente prejudicada pelo estresse hídrico que normalmente ocorre nas áreas de cultivo. Atualmente, tem-se buscado diversas soluções para minimizar tais efeitos, dentre essas a utilização de microrganismos que melhorem a resistência das plantas ao estresse hídrico. Sendo assim, o objetivo deste trabalho foi avaliar a indução de resistência de plantas de cana-de-açúcar ao estresse hídrico por meio da utilização de *Bacillus* sp. Para isto, conduziu-se um experimento em condições controladas de casa de vegetação, com a utilização de rebolos providos de vinte diferentes variedades de cana-de-açúcar, tratados ou não com o isolado de *Bacillus* sp., com 5 repetições. Estas plantas foram cultivadas por 45 dias e, após esse período, foram analisadas as seguintes variáveis: massa seca (parte aérea, radicular e total) e teores C, N, e C/N. Foram realizados a análise de variância, os testes de média (Scott-Knott e Tukey, p≤0,05) e também uma análise hierárquica, por meio de uma análise de agrupamento, de acordo com o método UPGMA (Unweighted Pair Group Method with Arithmetic Mean). As análises foram realizadas com o uso do software R. O isolado de *Bacillus* sp. promoveu resistência à seca para diferentes variedades de cana-de-açúcar, podendo assim ser uma alternativa para atenuar os efeitos do estresse hídrico em algumas variedades de cana.

Palavras-chave adicionais: microrganismo; seca; variedades.

Introduction

The scenario of climate change on our planet, especially extreme events such as reduced rainfalls, makes food production increasingly challenging (Wallace, 2000; IPCC, 2014). There is a steady increase in the demand for food production, which, in turn, increasingly drives agriculture to marginal areas where, in many cases, water deficit is constant (Foley et al., 2011).

Water deficit causes several problems to plants because water is essential for biochemical and physiological processes that allow development and growth, limiting the normal agricultural production in diverse cultures, among them sugarcane (*Sacharum* spp.) (Taiz & Zeiger, 2004; Lisar et al., 2012; Fang & Xiong, 2015). This crop is grown in some parts of the world for the production of alcohol and sugar. Brazil has been reported as the world's largest producer of sugarcane, accounting for more than half of the sugar consumed in the world, with an estimated production of 47.34 million tons of sugar in the 2018-2019 crop (MAPA, 2016).

Modern varieties of sugarcane, even with advanced genetic improvement, have not achieved a good productivity when there is water restriction polygene, and therefore difficulty to be transmitted to new varieties (Barbosa, 2001; Reis et al., 2014; Kumar et al., 2014; Phan et al., 2016).

In this aspect, the use of diverse tools for the conviviality of plants cultivated commercially with water deficit has been studied, among them microorganisms. Studies show the viability of this biological tool in several agricultural crops, such as *Helianthus annuus* (Santos et al., 2014), *Zea mays* (Kavamura et al., 2013, Amada et al., 2015) and sugarcane (Schultz et al., 2012).

Among the most promising microbial genera to confer plants with resistance to drought, we can mention *Bacillus.* They are cosmopolitan microorganisms that can be found in varied and extreme environments, colonizing and promoting innumerable beneficial processes to plants (Zhang et al., 2014; Qin et al., 2015). For example, biological control, as presented by Araújo et al. (2002): isolates of *B. subtilis* generate toxic metabolites that compromise the movement of nematodes, inhibit the hatching of juveniles and still impair the action of root penetration of soybean plants. The genus *Bacillus* may also increase the availability of plant nutrients, such as phosphorus and nitrogen, in plants inoculated with rhizobacteria in seeds (Araújo, 2008). According to Vardharajula et al. (2011), the genus *Bacillus*, plant growth promoters, promotes induction of drought tolerance in corn plants.

Thus, this study aims to verify the induction of resistance to water deficit by seedlings of different sugarcane varieties inoculated with *Bacillus* sp.

Material and methods

The experiment was conducted in the city of Jaguariúna, SP (altitude 584 m, 22º42'20'' S and 46º59'09'' W), in the experimental area of the Embrapa Environment during 2014, in a greenhouse with moisture and temperature control.

The planting was carried out on a soil from the same locatity. It is classified as a dystrophic Red--Yellow Latosol with a clayey texture. The physical--chemical characteristics of this soil, at the 0-20 cm layer, are 77 g kg^{-1} of clay, P = 24.2 mg dm^{-3}, K^+ = 3.6 mmol dm^{-3}, $Ca2^+$ = 45.6 mmolc dm^{-3}, Mg2+ = = 22.8 mmolc dm^{-3}, and organic matter= = 38.6 g kg^{-1}, pH(H2O) = 5.1.

The microorganism tested in this study was isolated from bacteria associated with cactus from the Brazilian semi-arid region, a bacterium of the genus Bacillus (Kavamura et al., 2013). The isolate is deposited at the collection of microorganisms of the Laboratory of Environmental Microbiology (LMA) of Embrapa Environment.

The experiment was completely randomized in a 2 x 20 factorial design with five replications: with and without the addition of Bacillus sp. isolates and 20 commercial varieties of sugarcane (Table 1).

Table 1 - Commercial varieties of sugarcane used in the experiment.

IAC	RIDESA	CTC	
IAC 3396	RB 867515	CTC 02	CTC 20
IAC1099	RB 72454	CTC 04	CTC 25
IAC 5094	RB 935744	CTC 09	CTC 9001
IAC 5000	RB 825211	CTC 14	CTC 9002
	RB 855156	CTC 15	CTC 9003
		CTC 17	

Bacteria were cultured in Petri dishes containing solidified yeast extract (GY) and kept in a BOD growth chamber at 25 °C for 24 hours. Bacteria colonies were transferred to a liquid GY culture medium and incubated for 72 hours at 25 °C in an orbital shaker at 5,488 x g. After this period, the material was centrifuged at 25 ºC and 7,000 rpm for 15 min for the formation of pallets, and cell concentration was adjusted in a spectrophotometer to obtain 10^8 CFU mL^{-1} of *Bacillus* sp.

Sugarcane seedlings were inoculated with a suspension of *Bacillus* sp. by applying a bacterial solution over them. The application during the period of seedling formation occurred on the day of planting of buds (0 days) and 30 days after planting (30 days). After 30 days, the plants were inoculated weekly until transplanting to a definitive vessel. The seedlings were formed in tubes containing a substrate based on carbonized Pinus husk from the billets, containing one sugarcane bud per tube, according to the method of multiplication of pre-budded seedlings (Landell et al., 2012). The plants were kept without irrigation until reaching the wilting point for a period of 24 days, after which irrigation was performed up to 70% of field capacity, and the test was monitored for another 20 days.

At 45 days after the transplantation of seedlings to pots, the plants were removed, and shoots were separated from roots. Shoot dry matter (SDM), root dry matter (RDM) and total dry matter (TDM) were determined after drying the plant material in a forced-air ventilation oven at 65 ºC until constant mass. Leaf samples and culms of sugarcane seedlings were also used for the determination of total carbon and nitrogen contents (%) by dry combustion in a C and N elemental analyzer (TruSpec CN LECO®).

Analysis of variance (ANOVA) and Scott-Knott mean tests ($p \leq 0.05$) were used to evaluate the means obtained in the different treatments for the variables SDM, RDM, TDM, N and C/N. The variable C was evaluated by Tukey test ($p \leq 0.05$). Statistical analyses were performed using the software R. To guarantee assumptions of homogeneity and normality, the variables RDM and TDM were transformed by square root, the variable C/N was transformed by logarithm, and the C variable was transformed by its square within a generalized model with heteroscedasticity between varieties (R, Core Team, 2016).

We also performed a hierarchical analysis of data obtained for all studied variables considering data without transformation by cluster analysis according to UPGMA, generating a dendrogram considering the Euclidean distance in the matrix of dissimilarity for all variables. For such analysis, the software R (R Core Team, 2016) was used.

Results

By the analysis of variance (ANOVA) of the results obtained in this study it was possible to observe that the following variables showed a significant interaction among the factors treatments and varieties: root dry matter (RDM), total dry matter (TDM) ($p<0.000$), shoot dry matter (SDM) ($p<0.001$), C/N ratio ($p<0.000$) and C content ($p<0.004$). The nitrogen variable (N) presented a significant main effect only for the factor variety ($p<0.000$). There were differences reported by mean tests in relation to the results obtained for the interaction between the factors analyzed, except for some varieties (Table 4).

Regarding the variable SDM, the results of interaction among factors presented statistical differences between them. The most expressive results were observed with the presence and absence of the bacterial isolate, respectively, for the varieties RB 855156 (5.3569 g plant^{-1}) and IAC 5000 (4.799 g plant^{-1}) (Table 2). The varieties CTC 20, IAC 3396, RB 855156 and RB 925211 presented the highest values for SDM when subjected to exposure to bacteria in relation to the control with water. CTC 9003 was the only one that presented the highest significant increase in the value of aerial part in the absence of bacteria.

According to the results observed for the variable RDM, it can be understood that, in the presence of the isolate, the variety RB 855156 (1.4074 g plant^{-1}) presented a better result; in the absence of the isolate, the variety IAC 1099 stood out (1.3407 g plant^{-1}) (Table 2). There was a greater increase in roots for the varieties CTC 15, CTC 9001 and RB 855156 in the presence of bacteria. However, without the presence of the isolate, some varieties presented high root dry matter values, especially the variety IAC 1099.

The best results were observed for TDM with the presence and absence of the tested microorganism, respectively, for the varieties RB 855156 (2.8656 g plant^{-1}) and CTC 5000 (2.58 g plant^{-1}) (Table 2). In the presence of bacteria, there was an increase in the total dry matter of CTC 17, CTC 20, CTC 9003, RB 855156 and RB 925211, while the varieties IAC 1099 and IAC 3396 presented a decrease in total dry matter with the application of bacteria.

The C/N ratio presented the highest values, which were obtained in the presence and absence of the bacteria, respectively, for the varieties IAC 1099 (3.8025 g plant^{-1}) and RB 925211 (3.7295 g plant^{-1}). There was an increase in the C/N ratio in the varieties IAC 1099 and IAC 5000 when exposed to isolates of *Bacillus* sp., and a decrease in the varieties CTC 15 and RB 855156 (Table 3).

According to the results for the variable C, it is possible to understand that, in the presence of the isolate, the variety IAC (2085.278 g plant^{-1}) presented the best results, and in its absence, the variety IAC 5000 stood out among the others (1,163.426 g plant^{-1}) (Table 3). Carbon contents increased in the presence of bacteria in the varieties IAC 1099 and IAC 5000. The varieties CTC 14, RB 855156 and RB 925211 presented a decrease in the presence of the isolate.

For the N variable, it was verified just effect significant for variety factor. The varieties CTC 15, CTC, CTC 20, CTC 9001, CTC 9002, CTC 9003, IAC 5094 and RB 7515 showed better results for the factor variety, and were statistically different from several other varieties (Table 4).

According to the analysis of the dendrogram (Figure 1), the results obtained in this study presented differences between them as for studied variables. The results obtained between the interaction of factors (treatments and varieties) were separated into two different groups. The first group included the following combinations: IAC 1099 in the presence in the isolate and CTC 14 and RB 925211 in its absence. The other combinations were described within a single group (Figure 1).

Table 2 – Dry matter of the aerial part (SDM), dry matter of the roots (RDM), and dry matter of the whole plant (TDM) of sugarcane's varieties treated or not with *Bacillus* sp.

Variety	SDM		RDM		TDM	
	Bacillus	without	*Bacillus*	Without	*Bacillus*	without
CTC 02	3.3669 cA	3.8339 bA	1.0601 cA	1.0592 cA	2.1414 dA	2.2426 cA
CTC 04	2.6979 dA	2.5032 eA	1.1027 cA	1.0705 cA	2.0175 dA	1.9336 dA
CTC 14	3.5535 cA	3.7092 cA	0.9086 dA	0.9186 dA	2.1144 dA	2.1748 cA
CTC 15	4.2736 bA	4.3318 bA	1.0848 cA	0.9288 dB	2.4089 cA	2.3335 bA
CTC 17	3.8236 cA	3.3567 cA	1.0692 cA	0.9871 dA	2.2719 cA	2.1008 cB
CTC 20	4.0534 bA	3.5309 cB	1.0560 cA	1.0349 cA	2.2995 cA	2.1557 cB
CTC 25	4.2145 bA	4.0157 bA	1.1299 cA	1.1378 bA	2.3601 cA	2.3425 bA
CTC 09	4.2835 bA	4.4437 aA	1.0290 dA	1.1051 cA	2.3229 cA	2.4343 bA
CTC 9001	3.5907 cA	3.4394 cA	1.1397 cA	1.0006 cB	2.2888 cA	2.1671 cA
CTC 9002	3.0408 dA	3.0906 dA	1.0141 dA	0.9726 dA	2.1435 dA	2.1017 cA
CTC 9003	3.5154 cB	4.0927 bA	0.9724 dA	1.0185 cA	2.1376 dB	2.3185 bA
IAC 1099	4.0250 bA	4.2739 bA	1.1919 bA	1.3407 aB	2.3616 cB	2.5320 aA
IAC 3396	5.2735 aA	4.5703 aB	1.3569 aA	1.3251 aA	2.7379 aA	2.5584 aB
IAC 5000	4.4838 aA	4.7990 aA	1.2542 bA	1.2189 bA	2.5322 bA	2.5800 aA
IAC 5094	3.3857 cA	3.3790 cA	0.9934 dA	1.0323 cA	2.0922 dA	2.1302 cA
RB 72454	3.0911 dA	3.1294 dA	0.9801 dA	0.9125 dA	2.0244 dA	1.9889 dA
RB 7515	3.8471 cA	3.6835 cA	1.1179 cA	1.1476 bA	2.2660 cA	2.2529 cA
RB 855156	5.3569 aA	4.5389 aB	1.4074 aA	0.9320 dB	2.8656 aA	2.4012 bB
RB 925211	3.8513 cA	3.1012 dB	0.9534 dA	0.9178 dA	2.2184 cA	1.9967 dB
RB 935744	4.5857 bA	4.2731 bA	1.0860 cA	1.0909 cA	2.4973 bA	2.4138 bA

Means followed by different lowercase and capital letters in the column and line, respectively, differ significantly by Scott-Knott test (p≤0.05).

Table 3 - Carbon-nitrogen proportion (C/N) and carbon content (C) of sugarcane's varieties seedlings treated or not with *Bacillus* sp.

Variety	C/N		C[1]	
	Bacillus	without	*Bacillus*	without
CTC 02	3.5181 bA	3.6042 bA	1818.38 bcdA	1848.350 cdefA
CTC 04	3.5116 bA	3.5355 bA	1763.374 bcdA	1771.520 cdeA
CTC 14	3.5065 bB	3.6574 aA	1858.190 dB	1895.910 efA
CTC 15	3.5283 bA	3.4927cA	1791.612 cdA	1780.586 cdeA
CTC 17	3.5224 bA	3.5156 cA	1865.822 dA	1854.156 defA
CTC 20	3.4812 bA	3.3878 dA	1817.992 cdA	1625.242 abcdA
CTC 25	3.3808 bA	3.4133 dA	1269.386 aA	1384.860 abA
CTC 09	3.6340 bA	3.5651 bA	1992.154 dA	1821.574 bcdefA
CTC 9001	3.4867 bA	3.4566 cA	1780.974 abcdA	1726.920 abcdefA
CTC 9002	3.5380 bA	3.5531 bA	1973.972 dA	1837.670 cdefA
CTC 9003	3.5785 bA	3.6073 bA	1970.906 dA	2160.628 fA
IAC 1099	3.8025 aA	3.5934 bB	2085.278 dA	1711.724 abcdefB
IAC 3396	3.5427 bA	3.5461 bA	1335.148 aA	1439.984 abcA
IAC 5000	3.4865 bA	3.3780 dB	1289.198 abcA	1163.426 aB
IAC 5094	3.5537 bA	3.5161 cA	1900.998 dA	1965.632 efA
RB 72454	3.5947 bA	3.5786 bA	1834.514 dA	1855.506 defA
RB 7515	3.5660 bA	3.5001 cA	1865.354 dA	1867.456 defA
RB 855156	3.4825 bB	3.6366 aA	1475.460 abcdB	1830.274 abcdefA
RB 925211	3.5466 bB	3.7295 aA	1579.096 abcdB	1777.652 abcdefA
RB 935744	3.5191 bA	3.4978 bA	1479.270 abA	1765.914 cdeA

Means followed by different lowercase and capital letters in the column and line, respectively, differ significantly by Scott-Knott test (p≤0.05).

Table 4 – N content in different varieties of sugarcane seedlings.

Variety	N (g kg^{-1})	Variety	N (g kg^{-1})
CTC 02	12.2 b	CTC 9003	12.5 a
CTC 04	12.4 b	IAC 1099	10.8 d
CTC 14	12.1 b	IAC 3396	10.7 d
CTC 15	12.7 a	IAC 5000	11.2 cd
CTC 17	12.8 a	IAC 5094	12.8 a
CTC 20	13.4 a	RB 72454	11.9 b
CTC 25	12.2 b	RB 7515	12.7 a
CTC 09	11.9 b	RB 855156	11.4 c
CTC 9001	13.0 a	RB 925211	17.5 c
CTC 9002	12.6 a	RB 935744	12.1 b

Means followed by different lowercase letters in the column differ significantly by Tukey test (p≤0.05).

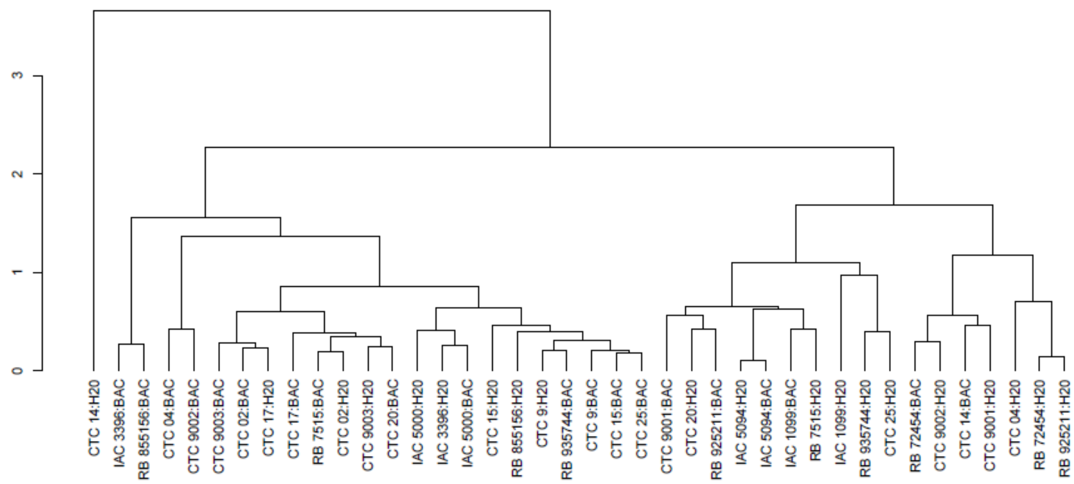

Figure 1 - Dendogram elaborated from the averages of the characteristics of sugarcane's varieties.

Discussion

The low availability of water to plants may limit their development. Thus, the use of microorganisms may provide a better resistance by plants to water stress, becoming a good alternative for the management of sugarcane crops. Morphological changes due to water deficit may be easy to verify in plants, such as decrease in height, leaf size and yield. However, evaluations may be more complex, such as increased root volume and decrease in fresh and dry matter of various parts of the plant (Torrecillas et al., 1996).

In general, among the 20 evaluated varieties, the one that presented the best behavior in face of water stress, when compared to treatments with the absence of bacteria for the majority of the variables evaluated, was the variety RB 855156. In a study by Torres et al. (2012), this variety showed a reduction in stomatal conductance after the application of herbicides, indicating that, under stress situations, this variety may have a specific protection mechanism.

The increase in shoot dry matter (SDM) in the varieties CTC 20, IAC 3396, RB 855156 and RB 925211, in the presence of bacteria, can be due to a better relation of these varieties with the isolate applied, denoting a possible higher efficiency by plants in association with the isolate for the use of water for the production of dry matter. In a study conducted by Silveira et al. (2004), there was an increase in the growth of cucumber plants inoculated with isolates of *Bacillus amyloliquefaciens* and *Enterobacter cloacae.* According to Araújo & Hungria (1999), the inoculation of soybean seeds with *Bacillus* increased the amount of nodules by 59% at the V3 stage, increased nodules occupation by *Bradyrhizobium* strains by 76% at R2, and increased grain yield by 24% when compared to the control without inoculation of *Bacillus.*

The presence of bacteria also provided an increase in root dry matter in some of the studied varieties (CTC 15, CTC 9001 and RB 855156). According to Govindarajan et al. (2006), there was an increase in sugarcane biomass (20%) with the inoculation of *Burkholderia* spp. The authors Mirza et al. (2001), testing bacteria of the genus *Enterobacter* in sugarcane plants micropropagated *in vitro*, reported an increase in root dry matter. The highest increase in root growth may provide the plant with a greater nutrient uptake and consequently a greater resistance to abiotic factors such as water deficit. The varieties CTC 17, CTC 20, CTC 9003, RB 855156 and RB 925211 showed the highest plant total dry matter. According to studies conducted by Ashraf et al. (2011), rhizobacteria promote an increase in root and shoot dry matter. According to Cohen et al. (2008), rhizobacteria can modify the hormonal signaling of plants, changing stomatal opening, improving water use and promoting a greater production of total dry matter.

Other crops were already tested as for the use of bacteria to improve plant behavior in order to obtain better yields under conditions adverse to plant development. For example, Nowak et al. (1999) studied the effects of bacteria on potato transplanting. The authors verified that plants inoculated with bacteria behaved better in years with low rainfalls, but this did not occur in years with severe drought or heavy rains. Productivity was higher for plants inoculated with bacteria. The authors also found a significant interaction between potato varieties and bacteria regarding plant growth. Therefore, bacteria have to be tested in different varieties, because the relation can be changed and the isolate may not present a positive effect on the plant.

Thus, the use of microorganisms to reduce the deleterious effects of water stress becomes a viable alternative for management. In a study conducted by Beltrano & Ronco (2008), which verified the effects of *Glomus claroideum* on tolerance to water deficit by wheat plants, the authors found that root colonization by *G. claroideum* would probably be an appropriate management measure aiming to reduce the negative impacts of water stress on wheat. Porcel & Ruiz--Rozano (2004) also reported a positive effect of mycorrhizal fungi on water stress in soybeans.

The increase in shoot and root dry matter of some sugarcane varieties inoculated with bacteria are tolerance responses of the bacteria to water deficit. According to McCree & Fernández (1989), the best responses to water deficiency are reduction in leaf area, closure of stomata, acceleration of senescence and abscission of leaves because water is indispensable to cell growth and a fundamental component for the conservation of turgescence. According to a study by Ball et al. (1994), the greatest development of roots occurred at soil layers where water was more available, and the propagation of roots in field was more compromised by water deficit than by leaf development.

In addition to the effects on growth and productivity, plants subjected to water stress may decrease the production of enzymes and nutrients (Pereira et al., 2015).

As can be seen in Table 3 and Figure 1, which show the analysis of the variable C/N, we observed that, in general, there were no discrepant results in the absence and presence of bacteria. Such results can be explained by the fact that, on average, the C/N ratio in straws of sugarcane, according to Ramos et al. (2016), is a variable that can be influenced by many environmental factors, but that in general organic materials have very marked characteristics in relation to this variable since they are adjusted by the structure and the type of the organic materials constituent of vegetal tissues. Thus, the results show that in the presence of bacteria, there is an average increase of 0.02% in the variable C/N. We also found an increase of 0.96% in the presence of the microorganism for the variable C (Tables 3 and 4).

It is known that the fraction of carbon in the C/N ratio comprises organic fractions of different materials (Pitombo et al., 2015). The C fraction of this ratio describes the amount of organic compounds in this relation, and thus a better absorption of nutrients and CO_2 may increase or decrease the percentages of this element simply due to the production of more tissues by plants. The N fraction of the C/N ratio plays an important role in the basic functions of plant physiology, being essential for almost all functions (Ramos et al., 2016).

In the three variables of this group of analyses (C/N ratio, C and N content), the variety IAC 5094 presented good results in the absence of the microorganism among the other varieties studied, a fact that can be evidenced by the rusticity of the variety against adversities and the likely low compatibility with the isolate studied (Tables 3 and 4).

It can be described that there is a profitable relation of the use of *Bacillus* sp. isolates. However, changes in this group of analyses (C/N ratio, C content and N content) were inexpressive between inoculated and non-inoculated plants. However small that increase may be with the use of isolates, it is satisfactory when taking into account productivity and gain for thousands of hectares of sugarcane.

Conclusions

Bacillus sp. isolates promoted an improvement in the variables evaluated for the sugarcane varieties CTC 20, IAC 3396, RB 855156 and RB 925211, promoting resistance to drought during the formation of seedlings.

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