DIVISÃO 2 - PROCESSOS E PROPRIEDADES DO SOLO

Comissão 2.1 - Biologia do solo

INOCULATION OF SUGARCANE WITH DIAZOTROPHIC BACTERIA[1]

Nivaldo Schultz[2], Jeferson Alves da Silva[3], Jailson Silva Sousa[3], Rafael Cassador Monteiro[3], Renan Pedula Oliveira[4], Valfredo Almeida Chaves[4], Willian Pereira[5], Marinete Flores da Silva[6], José Ivo Baldani[7], Robert Michael Boddey[7], Veronica Massena Reis[7] & Segundo Urquiaga[7]

SUMMARY

The sugarcane industry, a strategic crop in Brazil, requires technological improvements in production efficiency to increase the crop energy balance. Among the various currently studied alternatives, inoculation with diazotrophic bacteria proved to be a technology with great potential. In this context, the efficiency of a mixture of bacterial inoculant was evaluated with regard to the agronomic performance and N nutrition of sugarcane. The experiment was carried out on an experimental field of Embrapa Agrobiologia, in Seropédica, Rio de Janeiro, using a randomized block, 2 × 3 factorial design (two varieties and three treatments) with four replications, totaling 24 plots. The varieties RB867515 and RB72454 were tested in treatments consisting of: inoculation with diazotrophic bacteria, N-fertilized control with 120 kg ha^{-1} N and absolute control (no inoculation and no N fertilizer). The inoculum was composed of five strains of five diazotrophic species. The yield, dry matter accumulation, total N in the shoot dry matter and the contribution of N by biological fixation were evaluated, using the natural ^{15}N abundance in non-inoculated sugarcane as reference. The bacterial inoculant increased the stalk yield of variety RB72454 similarly to fertilization with 120 kg ha^{-1} N in the harvests of plant-cane and first ratoon crops, however the contribution of biological N fixation was unchanged by

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(2) Post-graduate student at CPGA-CS, Soil Science Department, UFRRJ. BR 465, km 7. CEP 23890-000 Seropédica (RJ), Brazil. E-mail: nsufrrj@yahoo.com.br

(3) Undergraduate student in Agronomy, UFRRJ. E-mail: jefersonufrrj@yahoo.com.br, jailson_ufrrj@hotmail.com, cassadormonteiro@hotmail.com

(4) Master student at CPGA-CS, Soil Science Department, UFRRJ. E-mail: renanpedula@agronomo.eng.br, valfredo_agronomia@hotmail.com

(5) Doctoral student at CPGA-CS, Soil Science Department, UFRRJ. E-mail: willpmg@yahoo.com.br

(6) D.Sc., Embrapa-Acre. BR 364, km 14. CEP 69908-970 Acre (AC), Brazil. Fellowship DCR/CNPq E-mail: marineteflores@yahoo.com.br

(7) Researcher, Embrapa Agrobiologia. E-mail: veronica.massena@embrapa.br, segundo.urquiaga@embrapa.br, ivo.baldani@embrapa.br, robert.boddey@embrapa.br

inoculation, indicating that the benefits of the inoculant in sugarcane may have resulted from plant growth promotion.

Index terms: ***Saccharum*** **sp, inoculant, biological nitrogen fixation.**

RESUMO: *INOCULAÇÃO DE BACTÉRIAS DIAZOTRÓFICAS NA CULTURA DE CANA-DE-AÇÚCAR*

O aumento da eficiência produtiva da cana-de-açúcar com tecnologias que possibilitem aumentar seu balanço energético é uma necessidade do setor sucroenergético, por se tratar de uma cultura estratégica para o país. Entre as diversas alternativas atualmente estudadas, o inoculante com bactérias diazotróficas vem se evidenciando uma tecnologia com elevado potencial. Diante desse contexto, este estudo teve como objetivo avaliar a eficiência do inoculante bacteriano no desempenho agronômico e na nutrição nitrogenada da cana-de-açúcar. O ensaio foi realizado no campo experimental da Embrapa Agrobiologia, em Seropédica, RJ. O delineamento experimental foi em bloco ao acaso, em esquema fatorial 2 × 3 (duas variedades e três tratamentos), com quatro repetições, totalizando 24 parcelas. As variedades utilizadas foram a RB867515 e RB72454. Os tratamentos foram: inoculação com bactérias diazotróficas, controle nitrogenado com 120 kg ha^{-1} de N e controle absoluto (sem inoculação e sem N). O inoculante foi composto por cinco estirpes de cinco espécies de bactérias diazotróficas. As variáveis avaliadas foram a produtividade de colmos, o acúmulo de matéria seca e N total da parte aérea das plantas e a contribuição da fixação biológica de N (FBN), utilizando como referência a abundância natural de ^{15}N da própria cana-de-açúcar não inoculada. O inoculante bacteriano aumentou a produtividade de colmos na variedade RB72454 de maneira similar à adubação com 120 kg ha^{-1} de N, nas colheitas de cana-planta e primeira soqueira; no entanto, não houve modificação na contribuição da FBN advinda da inoculação, dando evidências de que os benefícios do inoculante para a cana-de-açúcar podem ser provenientes do efeito da promoção de crescimento de plantas.

Termos de indexação: Saccharum *sp., inoculante, fixação biológica de nitrogênio.*

INTRODUCTION

In recent years, sugarcane was one of the fastest-growing crops in Brazil, reaching new frontiers, decisively influencing the economic, social and cultural development in the regions where new sugar mills were built. According to the Brazilian Institute of Geography and Statistics (IBGE, 2012) in 2012, sugarcane was planted on 9.7 million hectares in Brazil, with an average yield of 71.7 Mg ha^{-1} and a total production of 661 million tons.

While monocultures such as sugarcane bring progress and development to the expanding agricultural frontiers, they also impose a burden of environmental impacts, in the case of sugarcane especially the emission of greenhouse gases, particularly of CO_2, CH_4 and N_2O, resulting from the use of N fertilizers mainly applied to the soil surface after harvest (Soares et al., 2009; Denmead et al., 2010). In view of this scenario it is crucial to invest in research on alternatives that would ensure a competitive and sustainable development of the sugarcane industry.

Among the various possibilities of increasing the crop yield and use efficiency of available soil N and applied fertilizer, the diazotrophic-based inoculant or inoculant of diazotrophic bacteria could be a strategic alternative for the sugar-energy industry, for being a low-cost technique without environmental impacts (Reis et al., 2009). Field studies showed the efficiency of the inoculant in increasing stalk and dry matter yield of different sugarcane varieties (Oliveira et al., 2003; Silva et al., 2009; Schultz et al., 2012). The benefits of inoculation with diazotrophic bacteria for the crop are not yet fully understood (Oliveira et al., 2009), however there are reports that the plant-bacteria association may increase the contribution of atmospheric N_2 by biological fixation in plants, according to the cultivar and environmental conditions (Oliveira et al., 2003; Baldani et al., 2009). The application of these bacteria also induces other growth-promoting processes, e.g., the production of phytohormones, especially of auxins (Videira et al., 2012); phosphate solubilization (Singh et al., 2007; Shukla et al., 2008), solubilization of zinc compounds (Saravanan et al., 2007), increase in soil organic C content and increased N contents and retention of essential nutrients in the rhizosphere (Yadav et al., 2009). Due to all these mechanisms, these bacteria are known as plant growth-promoting and can improve nutrient absorption and even the biological control of plant pathogens (Spaepen et al., 2007)

The purpose of this study was to evaluate the response of two sugarcane varieties to the inoculation of five growth-promoting diazotrophic bacteria strains in terms of agronomic performance and the contribution of biological N fixation over three consecutive years.

MATERIAL AND METHODS

The experiment was carried out on an experimental field of Embrapa Agrobiologia, at point 7 km of the highway BR 465, municipality of Seropédica, State of Rio de Janeiro (22º 44' 38'' S; 43º 42' 28'' W; 26 m asl), from September 2007 to October 2010. The climate is Aw, according to the Köppen classification, with hot dry summers and wet winters, and a mean annual temperature of 22.7 ºC.

The soil of the experimental area was classified as Albaquult, according to Santos et al. (2006). The experiment was arranged in a randomized block, 2 × 3 factorial design (two sugarcane varieties and three treatments) with four replications, totaling 24 plots. The varieties RB867515 and RB72454 (commercial *Saccharum* sp. hybrids) were evaluated. The treatments consisted of: inoculation with a mixture of five strains of diazotrophic bacteria, N-fertilized control with fertilization of 120 kg ha^{-1} N and an absolute control (no inoculation and no N fertilization).

The plots consisted of five 5-m rows, spaced 1.1 m apart, with a total area of 27.5 m^2 per plot. The plant-cane and first and second ratoon crops were assessed. In the plant-cane crop (September 2007 to October 2008), the cumulative rainfall was 1,177 mm; in the first and second ratoon crops, harvested 12 months after the respective previous cutting, the accumulated rainfall was 1,598 and 1230 mm, respectively (Figure 1).

Prior to soil tillage for planting, the soil chemical properties were analyzed in the layers 0-20 and 20 - 40 cm (Table 1).

Soil tillage consisted of plowing, harrowing and liming (2 Mg ha^{-1} lime incorporated by harrowing), and planting after 40 days. Fertilizer was applied at planting in the furrows, at rates of: 100 kg ha^{-1} P_2O_5 in the form of single superphosphate, 100 kg ha^{-1} K_2O in the form of potash, 50 kg ha^{-1} fritted trace elements (FTE BR12), and 0.4 kg ha^{-1} sodium molybdate. In the ratoon crops, fertilization consisted of K_2O, FTE BR12 and sidedressed sodium molybdate, at the same doses as at planting.

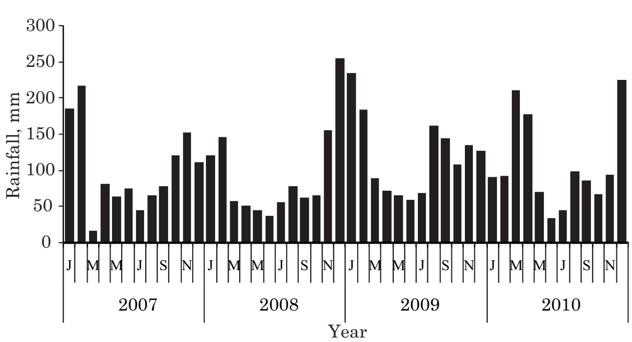

Figure 1. Monthly rainfall recorded in the region of Seropédica, RJ, between 2007 and 2010.

In the plant-cane crop, N fertilization in the N-fertilized control treatment was divided in two, applying 60 kg ha^{-1} N at the bottom of the furrow and 60 kg ha^{-1} N sidedressed 60 days after planting, using urea. In the ratoon crops, N fertilization (control with 120 kg ha^{-1} N) was applied as a single dose, approximately 30 days after the previous harvest.

The seedlings for planting (cuttings with three buds) were originated from a trial planted from micropropagated plantlets of two varieties, laboratory-inoculated by the method of Reis et al. (1999), reinoculated in a greenhouse (nursery) and reinoculated again in the field at planting, according to the method described by Schultz et al. (2012).

The five diazotrophic strains of the sugarcane inoculant were *Gluconacetobacter diazotrophicus* (Pal5T-BR11281), *Amazon Azospirilum* (Cbamc-BR11145), *Herbaspirilum seropedicae* (HRC54-BR11335), *Herbaspirilum rubrisubalbicans* (HCC103-BR11504), and *Bulkholderia tropica* (PPe8T-BR 11366). These five strains were previously tested and selected by Oliveira et al. (2003, 2006), in inoculation studies using the method described by Reis et al. (1999). To obtain the inoculum, the bacteria (with initials BR) were obtained from the collection of diazotrophic bacteria at Embrapa Agrobiologia and grown in liquid DYGS culture medium (Silva et al., 2012). Subsequently, 75 mL of DYGS culture medium with a population of 10^9 cells mL^{-1} of each strain was mixed in 175 mL of carboxymethyl cellulose polymer and starch (60 and 40 %, respectively). This polymer is a patented formulation (no. PI0506338-8, Instituto Nacional de Propriedade Intelectual) at a ratio of 60/40 carboxymethyl cellulose/starch. Before adding the bacterial suspension, 175 mL of the polymer was transferred to polypropylene bags with a thickness of approximately 0.05 mm and sealed. The bags were autoclaved at 120 °C for 20 min and the bacterial suspension (75 mL) with a population of 10^9 cells mL^{-1} was added immediately. Thus each package (polypropylene bag) contained a volume of 250 mL of a single strain and the total inoculum dose composed of the five strains was 1.25 L.

The inoculum suspension for immersion of the cuttings at planting and for the inoculation of the ratoon plants (reinoculation) was prepared by diluting the mixture of the five strains (five packages = 1.25 L) in 100 L of clean water (12.5 mL of product per L of pure water).

For inoculation, cuttings with three pre-selected and standard size buds for planting were packed in raffia bags according to the number required per plant row (15 buds per meter), immersed for 30 min in the

Table 1. Soil chemical properties in the experimental area

Layer	pH(H_2O)	C	N	Ca^{2+}	Mg^{2+}	Al^{3+}	V	P	K
cm		g kg^{-1}		cmol$_c$ dm^{-3}			%	mg dm^{-3}	
0-20	5.4	4.8	0.4	0.7	0.2	0.3	24	19	12
20-40	5.1	3.0	0.3	0.8	0.3	0.6	29	5	7

Analyses carried out according to the Laboratory Manual for soil, water, plant nutrition, animal nutrition and food (Nogueira & Souza, 2005).

inoculum suspension, in 200 L containers. After immersion, the seedlings were dried in the shade for 30 min and planted immediately. In the ratoon plants, the inoculant suspension dilution equal to that used for planting (12.5 mL of inoculant per L suspension) was applied at the plant base three days after harvest with a backpack sprayer, applying 11 mL per meter of furrow.

Agronomic evaluations were performed to determine the stalk yield, dry matter yield and total N in the plant shoots (stalks, straw and flag-leaves), in the three harvests. After weighing the stalks, straw and flag-leaves, sub-samples were taken from each fraction and dried to constant weight in a convection oven at 65 °C. Based on the fresh weight determined in the field and on the dry matter percentage, the fresh stalk yield and total shoot dry matter per hectare were estimated. Then, the subsamples were first ground in a Willey mill (2 mm) and then finely ground similarly as described by Arnold & Schepers (2004). Nitrogen was determined according to the semi-micro-Kjeldahl method (Nogueira & Souza, 2005) and the natural abundance of ^{15}N analyzed only in the flag leaf sub-samples, which, according to Boddey et al. (2001), is the isotopic label of the entire plant. After analyzing the N content in the flag leaf, the weights were determined for analysis of the delta value (d^{15}N), dividing four by the total N content in percentage, according to the spectrum of action indicated by the mass spectrometer (Finnigan MAT, Bremen, Germany) of the stable isotope laboratory of John M Day of Embrapa Agrobiologia (Ramos et al., 2001).

The uniformity of isotopic labeling with ^{15}N of the soil available N for plants, in time and depth, is one of the prerequisites for the technique of isotopic ^{15}N dilution to assess BNF, provided that the control plants have roots and similar N-uptake as the plant under study (inoculated sugarcane) (Unkovich et al., 2008). Therefore, to assess whether the inoculant affected the BNF process in sugarcane, an uninoculated control of the proper sugarcane was used as reference.

Data were analyzed for normality and homogeneity of distribution by means of the Lilliefors and Barttlet & Cochran tests, respectively. The results were subjected to analysis of variance with the F test and means compared by the (LSD) t-test at the 10 % level.

RESULTS AND DISCUSSION

In the plant-cane and first ratoon crops, inoculation promoted increases in stalk yield in the same order of magnitude as the N-fertilized control in variety RB72454, with higher yields in the inoculated and N-fertilized treatments than in the absolute control (Table 2). In the second ratoon crop, the response in stalk yield of variety RB72454 to inoculation was higher than that of the absolute control, but nevertheless lower than of the N-fertilized control. The response in total shoot dry matter of variety RB72454 accompanied that of stalk yield in the plant-cane and second ratoon cycle. In the first ratoon crop of variety RB72454, the treatments did not differ in total shoot dry matter. The accumulation of total N in variety RB72454 followed the pattern of stalk yield only in the plant-cane cycle, with significant increases by inoculation and in the N-fertilized control compared to the absolute control. In the first ratoon crop of variety RB72454, no difference was observed between treatments for total N accumulation and in the second ratoon crop, the stalk yield in the N-fertilized control was superior to that of the absolute control and inoculation treatment.

Evaluating the stalk yield of variety RB72454 in all three harvests, a high response potential to inoculation was observed in the plant-cane and first ratoon crops, followed by a decline in the second ratoon, which responded to inoculation, but less than to N-fertilization. The reduction in inoculation efficiency with successive harvests may be associated with N depletion in the soil, because some studies showed that inoculation has no influence on BNF in sugarcane (Schultz et al., 2012) and the yield increases after inoculation may be associated with the growth-promoting effects on the plant root system (Sevilla et al., 2001; Saravanan et al., 2007; Videira et al., 2012). In this case, if the benefit of inoculation is related to the increased use efficiency of plant-available soil N and the soil stock of available N decreases as the crops are being harvested, the efficiency of inoculation may decline over the years. Schultz et al. (2012) evaluated the efficiency of bacterial inoculants for sugarcane based on the ^{15}N isotope dilution of soil- available N and total N accumulated in plants. These authors found that inoculation did not influence BNF in sugarcane and concluded that the yield increase observed in variety RB867515 in a Fluventic Eutrudept

Table 2. Agronomic evaluation of sugarcane grown on a Albaquult in three treatments: an absolute control, inoculation with diazotrophic bacteria and a nitrogen-fertilized control in three consecutive growing seasons, on an experimental field of Embrapa Agrobiologia, in Seropédica, Rio de Janeiro

Variety	Stalk yield			Total shoot dry matter			Total shoot N		
	Control	Inoculated	120 N	Control	Inoculated	120 N	Control	Inoculated	120 N
	Mg ha⁻¹						kg ha⁻¹		
	Cane plant (growing season 2007/2008)								
RB72454	83.3 b	101.8 a	102.9 a	37.5 b	43.7 a	43.0 ab	120.3 b	163.3 a	161.0 a
RB867515	97.1	108.3	98.3	43.4	46.5	46.4	131.3	139.5	148.2
CV (%)		11.6			10.3			15.5	
	First ratoon (growing season 2008/2009)								
RB72454	86.3 b	100.9 a	99.4 a	47.1	50.4	48.4	116.2	125.4	133.6
RB867515	104.8	111.3	110.3	49.4 b	57.0 a	53.4 ab	116.2	128.2	128.6
CV (%)		9.1			7.7			14.5	
	Second ratoon (growing season 2009/2010)								
RB72454	34.8 c	45.9 b	72.4 a	23.7 c	29.1 b	45.7 a	57.0 b	66.2 b	124.6 a
RB867515	52.5 b	62.2 b	85.5 a	33.4 b	36.6 b	50.4 a	60.8 b	71.9 b	130.4 a
CV (%)		15.1			11.9			16.1	
Sum of three harvests	458.8	530.4	568.8	234.5	263.3	287.3	601.8	694.5	826.4
Increment of the treatments compared to the control (%)		13.5	19.3		10.9	18.4		13.3	27.2

Mean of four replications. Values followed by different letters in rows do not differ by the LSD t test at 10 % significance. The sum of three harvests and percentage values of increments of the treatments compared to the control were not subjected to statistical analysis.

in the region of Goytacazes may have resulted from growth promotion of the plant root system.

No response to inoculation and N-fertilized control was observed in variety RB867515 in the plant-cane cycle for the evaluated variables (stalks, dry matter and total N). In the first ratoon crop, inoculation caused a significant increase in dry matter production compared to the absolute control, but did not differ from the N-fertilized control. In the second ratoon crop, variety RB867515 showed no response to inoculation, but was responsive to N fertilization in stalk yield, dry matter and total N. The lack of response of this variety (RB867515) to treatments with inoculation and N fertilization in the plant-cane crop may be characteristic of the variety, since it produces high yields on N-poor soils (Silva et al., 2009; Schultz et al., 2012). In the first ratoon crop, due to the reduction in available soil N after the plant-cane harvest, there was no response in the variety to the treatments with inoculation and N fertilization in the accumulation of total dry matter production, which was however not reflected in significant increases in stalk yield and in total N, stocks, probably due to the high use efficiency of available soil N of this variety. In the second ratoon crop of variety RB867515, our results confirmed the findings for RB72454, discussed above, i.e., the exhaustion of the available soil N and consequently the response in variety RB867515 only in the treatment with 120 kg ha^{-1} N fertilization, since the inoculum did not affect BNF, as indicated by the delta values ($\delta^{15}N$) in the sugarcane leaves (Table 3).

The sum of the values of the three harvests showed that inoculation induced an increase in stalk yield of 13.5 %, in dry matter of 10.9 % and total N accumulation of 13.3 %. The results obtained in this study corroborate several studies in the literature. Govindarajan et al. (2006) evaluated the inoculation of *Burkholderia vietnamiensis,* strain MG43, in the varieties Co 86032 and Co 86027 of micropropagated sugarcane after different evaluation periods up to 12 months after planting, and found that inoculation with strain MG43 promoted increases of 20 and 19 % in dry matter production of Co 86032 and Co 86027, respectively. Testing the same inoculum as in this study in a medium texture Hapludult, Silva et al. (2009) found that stalk yield increased by 50 Mg ha^{-1} in variety RB72454 and 30 Mg ha^{-1} in variety RB867515, 11 months after planting, compared to the absolute control; these increases were similar to those recorded in the control fertilized with 120 kg ha^{-1} N. Similar results to those mentioned above were reported by Schultz et al. (2012) in studies carried out for two years in a Fluventic Eutrudept in the region of Campos dos Goytacazes, Rio de Janeiro, for variety RB867515.

Table 3. Delta (δ15) in the sugarcane flag-leaf inoculated with diazotrophic bacteria and in the uninoculated control grown for three consecutive harvests on an Albaquult on an experimental field of Embrapa Agrobiologia, Rio de Janeiro

Treatment	RB72454			RB867515		
	Cane plant	1ª ratoon	2ª ratoon	Cane plant	1ª ratoon	2ª ratoon
	$\delta^{15}N$ in flag leaves of sugarcane (‰)					
Control	3.5	3.1	2.4	2.8	2.6	2.2
Inoculated	3.4	2.9	2.1	3.4	3.1	2.3
CV (%)	12.4	21.1	33.5	12.4	21.1	33.5

Means of four replications. CV: coefficient of variation. The absence of letters means no difference between treatments. LSD (t test) significant at 10 %.

The results obtained to date and reported in the literature show that diazotrophic inoculation in sugarcane increased the crop yield potential, but with great variability in the responses and influence of soil-climatic factors involved in the production process. These results indicate a possible reduction of N fertilization of sugarcane, translating into savings for the sugarcane industry and into environmental preservation, for being an alternative product with lower cost and lower environmental risk than N fertilizers.

The natural abundance of $\delta^{15}N$ in the flag leaves of uninoculated (control) and inoculated sugarcane did not differ from each other at harvest (Table 3). The similarity between the $\delta^{15}N$ values in the flag leaves of inoculated sugarcane and uninoculated control evidenced that the inoculant did not affect the BNF process in the two tested varieties. However, in the three harvests, the inoculated variety RB72454 obtained higher increases in stalk yield, dry matter accumulation and total N than the absolute control while the inoculated variety RB867515 showed an increase in dry matter production over the absolute control in the first ratoon crop. These results led to the conclusion that inoculation provided other benefits in sugarcane, which may be associated with the action of plant hormones synthesized by diazotrophic bacteria, phosphate and Zn solubilization, N retention and other essential nutrients in the rhizosphere. Sevilla et al. (2001), comparing a strain of *Gluconacetobacter* with a nitrogenase negative (nif) mutant concluded that other factors, aside from BNF, may be responsible for the benefits resulting from inoculation. Several studies show that diazotrophic bacteria may act in promoting plant growth, mainly by changing the morphology of the root system, thus positively influencing the development and productivity of several crops of economic interest (Bashan et al., 2004; Somers et al., 2004). Videira et al. (2012) assessed the diversity of 204 diazotrophic isolates from roots and stems of fresh elephant grass (*Pennisetum purpureum* Schum) and found that 97 % of these isolates produced indole compounds and 22 % were able to solubilize phosphate. Yadav et al. (2009) evaluated the effect of *Gluconacetobacter diazotrophicus* and *Trichoderma viride* on the soil and yield of sugarcane in India and found that there was an increase in soil organic C, increase in N and retention of essential nutrients in the rhizosphere due to the increase in the microbial population in the soil-rhizosphere-root interface. In a study of Muñoz-Rojas & Caballero-Mellado (2003) with micropropagated sugarcane (variety MEX 57-473) inoculated with *Gluconacetobacter diazotrophicus,* strain $PAL5^T$, and grown in sterile vermiculite showed increases in the dry weight of roots and shoots and greater accumulation of total N 35 and 75 days after planting, although the concentrations of total N were lower than in the control treatment, indicating the plant growth-promoting effect. Roesch et al. (2005) assessed the effect of inoculation with diazotrophic bacteria of the genus *Azospirillum* on wheat and stated increases in the size and number of root hairs in inoculated plants and increased contents of total N in plant roots and shoots, proving the influence of phytohormones produced by the inoculated strain on the physiology of this cereal. Schultz et al. (2012) evaluated the agronomic efficiency of two sugarcane varieties, RB72454 and RB867515, inoculated with diazotrophic bacteria and fertilized with 120 kg ha^{-1} N and verified that in RB867515, the response to inoculant application increased the delta values $\delta^{15}N$ in the flag-leaf tissue, leading to the inference that the inoculated sugarcane root system was modified and improved soil exploration in the deeper layers, where the delta values of isotope $\delta^{15}N$ are higher. Saubidet et al. (2002) evaluated the effect of inoculation with *Azospirillum brasiliense* in wheat and found that inoculated plants were more efficient in uptake and use of soil available N.

CONCLUSIONS

1. The yield increase of sugarcane variety RB72454 in the plant-cane and first ratoon crops after inoculation with diazotrophic bacteria was comparable to that induced by fertilization with 120 kg ha^{-1} N.

2. In variety RB72454, the efficiency of inoculation declined in the second ratoon crop in relation to plant-cane and first ratoon crops.

3. In the plant-cane crop, variety RB867515 did not respond to inoculation and fertilization treatments with 120 kg ha^{-1} N.

4. Inoculation with diazotrophic bacteria did not change the natural contribution of the process of biological N fixation in sugarcane.

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