Ciência Rural, Santa Maria, v.54:03, e20220628, 2024
http://doi.org/10.1590/0103-8478cr20220628
ISSNe 1678-4596
SOIL SCIENCE

Inoculation effects of *Nitrospirillum amazonense* and biofertilizer in sugarcane

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ABSTRACT: In this study, we analyzed the hypothesis that the combination of *Nitrospirillum amazonense* strain BR11145 with biological fertilizer prepared using Microgeo® and native microbiome from location of the product application results in morphological and nutritional gains for the initial development of sugarcane plants and soil chemistry. For this purpose, pre-sprouted sugarcane seedlings were grown in a greenhouse mesocosm experiment using soil amended with nitrogen/phosphorus/potassium fertilizer. The experimental treatments consisted of: 1) biological fertilizer with the addition of *N. amazonense* (100 mL ha^{-1}), 2) biological fertilizer without the addition of *N. amazonense*, 3) inoculation with *N. amazonense* at a dose of 100 mL ha^{-1} with $2x10^8$ viable cell mL^{-1}, 4) inoculation with *N. amazonense* at a dose of 200 mL ha^{-1} with $2x10^8$ viable cell mL^{-1}, and 5) control, without the addition of biological fertilizer and *N. amazonense*. The biological fertilizer was applied at dose of 300 L ha^{-1}, which was split at planting (200 L ha^{-1}) and in the post-emergence phase (100 L ha^{-1}). After 164 days of planting, it was detected an increase in leaf length +3, number of green leaves, leaf area and sulfur content in the leaves of sugarcane plants that received the biological fertilizer with the addition of *N. amazonense*. In conclusion, the combination of *N. amazonense* with biological fertilizer revealed positive effects through morphological and nutritional characteristics in sugarcane plants during their early stages of development when compared to plants grown only with the inoculation of *N. amazonense*, biological fertilizer or mineral fertilizers, with few notable positive effects on soil chemistry.
Key words: Diazotrophic bacteria, plant-bacteria interaction, soil microbiome, microbial inoculants.

Efeitos da inoculação de *Nitrospirillum amazonense* e biofertilizante em cana-de-açúcar

RESUMO: Neste estudo avaliou-se a hipótese de que a combinação de *Nitrospirillum amazonense* estirpe BR11145 com adubo biológico preparado com base em microbioma autóctone da localidade de aplicação do produto, produzido com Microgeo®, resulta em benefícios morfológicos e nutricionais para o desenvolvimento inicial de plantas de cana-de-açúcar e para a química do solo. Para tanto, mudas pré-brotadas de cana-de-açúcar foram crescidas em mesocosmos num experimento conduzido em casa-de-vegetação com solo enriquecido com fertilizante à base de nitrogênio/fósforo/potássio. Os tratamentos avaliados foram: 1) adubo biológico acrescido de *N. amazonense* (100 mL ha^{-1}), 2) adubo biológico sem o acréscimo de *N. amazonense*, 3) inoculação de *N. amazonense* na dose de 100 mL ha^{-1} ($2x10^8$ células viáveis por mL), 4) inoculação de *N. amazonense* na dose de 200 mL ha^{-1} ($2x10^8$ células viáveis por mL), e 5) testemunha, sem a adição de fertilizante biológico e *N. amazonense*. O adubo biológico foi aplicado na dose de 300 L ha^{-1} sendo esta parcelada no plantio (200 L ha^{-1}) e na fase de pós-emergência (100 L ha^{-1}). Após 164 dias do plantio constatou-se aumento no comprimento da folha +3, número de folhas verdes, área foliar e no teor de enxofre nas folhas das plantas de cana-de-açúcar que receberam o adubo biológico combinado com *N. amazonense.* Conclui-se que a combinação de *N. amazonense* com o adubo biológico produzido com Microgeo® revelou efeitos positivos por meio de caraterísticas morfológicas e nutricionais em plantas de cana-de-açúcar durante os seus estádios iniciais de desenvolvimento quando comparadas com plantas crescidas apenas com a inoculação de *N. amazonense*, fertilizante biológico ou fertilizantes minerais, com poucos efeitos positivos notáveis na química do solo.
Palavras-chave: Bactéria diazotrófica, interação planta-bactéria, microbioma do solo, inoculante microbiano.

INTRODUCTION

The massive planting of the sugarcane (*Saccharum* sp.) crop brings environmental risks that include a potential impact on tropical soil ecosystem sustainability (NAVARRETE et al., 2018). The increased need for fertilizers due to the expansion of sugarcane production is a threat to the ability of the soil to maintain its potential for self-regulation in the long term, i.e., its sustainability (SCHWAB et al., 2023). Soil management practices used in sugarcane agriculture require synthetic mineral fertilizers (nitrogen/phosphorus/potassium – NPK) and full recycling of waste products from the

Received 11.14.22 Approved 06.02.23 Returned by the author 06.27.23
CR-2022-0628.21
Editors: Leandro Souza da Silva Alessandro Dal'Col Lucio

ethanol production to sugarcane fields in the form of organic fertilizer (SICA et al., 2020). As a result of the increased economic importance of sugarcane, the requirements for large-scale production in an environmentally sustainable manner have also increased. In this sense, the utilization of biofertilizers in combination with microbiome-based technologies can become essential for sustainable crop production.

One promising alternative for sustainable sugarcane production is the use of diazotrophic bacteria. The association of diazotrophic bacterial species with sugarcane is known for their beneficial effects on promoting plant growth in different ways, especially through nitrogen-fixing and the production of phytohormones by plant-associated bacteria and siderophores, inorganic phosphate solubilization, and improving the resistance of plants to pathogens (SPAEPEN et al., 2007; SANTI et al., 2013; GÍRIO et al., 2015). Bacteria belonging to *Nitrospirillum* genus, previously belonging to the genus *Azospirillum*, described by MAGALHÃES et al. (1983) and reclassified by LIN et al. (2014), were isolated from tissues of different sugarcane varieties and selected for sugarcane bioinoculant formulation, aiming to reduce the use of nitrogen fertilizers (ANTUNES, 2019).

The use of microbes in agriculture can be an alternative to reduce the use of chemical fertilizers and generate increased productivity, in addition to mitigating the effects of soil degradation (REIS et al., 2020). Considering biological fertilization as a means of application of beneficial microbes for the crop, it is worth noting that the use of this type of fertilizer in agricultural soils benefits the soil microbiome, including the microbes inhabiting the soil that interact with the plants (FERREIRA et al., 2020). Additionally, biological fertilization with the use of biofertilizers contributes to the increase of organic matter, maintenance of pH after the crop is harvested, gradual improvement in soil fertility, availability of phosphorus and reduction of soil compaction (BELLINI et al., 2013; PINHEIRO et al., 2019).

Biofertilizers have been produced using the continuous liquid composting method in biofactories installed directly on agricultural farms. The process uses non-chlorinated water and an inoculant based on fresh cattle manure, which is later enriched with a nutritious organic component. The Microgeo® is an organic component, prepared on the basis of several organic and mineral sources (MEDEIROS et al., 2003). It is regulated through an authorization issued by the Ministry of Agriculture, Livestock and Food Supply (MAPA) for the production of biofertilizer through the continuous liquid composting process and certified by the Biodynamic Institute (IBD). The biological fertilizer produced with Microgeo® aims to re-establish the native microbiome of the location where the biofactory is located.

The present study was designed to evaluate the effect on the initial development of sugarcane plants and on the soil of the combination of *Nitrospirillum amazonense* and biological fertilizer prepared using Microgeo® and native microbiome from location of the product application. We hypothesized that the combination of *N. amazonense* with the biological fertilizer results in morphological and nutritional gains for sugarcane plants and soil chemistry.

MATERIALS AND METHODS

Growth of the N. amazonense in laboratory

The *N. amazonense* strain BR11145 was initially grown in Liquid Glucose Ivo (LGI) medium (solid), prepared according to EMBRAPA document no. 110 '*Protocolos para Preparo de Meios de Cultura da Embrapa Agrobiologia*' (Protocols for Culture Media Preparation by Embrapa Agrobiology) (DÖBEREINER et al., 1999). All the materials used to cultivate and prepare the growth curve were previously sterilized in autoclave at 121 °C for 20 minutes. The Petri dishes were incubated in a Biochemical Oxygen Demand (BOD) incubator at 30 °C for two days until the growth of colonies.

A sterile wooden stick was used to divide well-defined colonies into three 100 mL Erlenmeyer flasks containing 50 mL of liquid LGI medium each. One flask with the culture medium and no inoculation was utilized as the control (blank) to monitor any potential contamination throughout the process. These flasks with the pre-culture in the liquid medium were incubated at 30 °C under constant agitation at 120 rpm for 24 hours. To prepare the definitive culture used to prepare the growth curve, 1 mL of the pre-culture was transferred to new Erlenmeyer flasks containing 50 mL of liquid LGI medium, kept under the same conditions. The pre-culture was not inoculated in one flask, which was used as the control.

To prepare the growth curve of *N. amazonense*, ten growth time points of the culture were collected during 22 hours of incubation, beginning immediately after transfer of the pre-culture (time 0). Intervals of 4 to 6 hours were defined between the growth time points. At each point, optical density was determined in a NanoDrop spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Inc., Wilmington, DE, USA) at 640 nm, and serial dilution in 0.9% NaCl solution and plating on solid

LGI media was performed to count colony-forming units (CFUs).

Greenhouse experiment

Between August 2019 and February 2020, sugarcane was planted in a greenhouse in the Fernandópolis, *Campus* of University Brazil, located between the geographical coordinates 20°16' S, 50°17' W. A biological fertilizer prepared using Microgeo® and *N. amazonense* strain BR11145 bacteria was used in the greenhouse experiment.

The biological fertilizer was obtained through the Continuous Liquid Composting Process – CLC®. A CLC® Biofactory was installed in an area under direct sunlight, equipped with a 180 L plastic tank, which was filled with the following materials as a function of the tank's volume (200 L): 15% cow manure from University Brazil herd, 2.5% Microgeo® START, and completed with clean non-chlorinated water. The biological fertilizer was stirred daily with a wooden stick, and it was ready for use 15 days after installation.

A thin layer of crushed stone covered with a shade cloth was added to the perforated bottom of twenty-four 110 L plastic pots. The pots were then filled with 90 kg of soil classified as Yellow-Red Acrisol abruptic eutrophic, which had a medium sandy texture.

Upon planting on August 31, 2019, the soil in all the pots was fertilized with the equivalent of 27 kg ha^{-1} of N, 135 kg ha^{-1} of P_2O_5, and 135 kg ha^{-1} of K_2O in the form of urea, simple superphosphate, and potassium chloride, respectively, following the recommendations by RAIJ et al. (1997). The amount of fertilizer was determined by considering the area of the pot and was incorporated into the soil.

In each pot, three pre-sprouted sugarcane seedlings of the IACSP01-5503 cultivar with approximately 30 cm in height were planted, and the following treatments were evaluated:

1) biological fertilizer prepared using Microgeo® and *N. amazonense* (100 mL ha^{-1})
2) biological fertilizer prepared using Microgeo® without the addition of *N. amazonense*
3) inoculation of *N. amazonense* at a dose of 100 mL ha^{-1} with $2x10^8$ viable cells mL^{-1}
4) inoculation of *N. amazonense* at a dose of 200 mL ha^{-1} with $2x10^8$ viable cells mL^{-1}
5) without the addition of biological fertilizer and *N. amazonense* (control)

A dosage of 300 L ha^{-1} of the biological fertilizer prepared using Microgeo® was applied in doses of 100 L ha^{-1} upon planting and 200 L ha^{-1} in the post-emergence phase, according to the customary technical recommendations in the field. In each phase, a 0.5 L aliquot part of the biological fertilizer was removed from the CLC® Biofactory, and the suspended particles were eliminated.

The biological fertilizer and *N. amazonense* bacteria were applied with an automatic volumetric pipette at the base of the seedlings near the soil surface immediately after planting. The dose was divided equally among the three sugarcane plants in each pot.

The pots were distributed in an entirely randomized design inside the greenhouse. There were three replications of the treatments with *N. amazonense* bacteria dosed at 100 mL ha^{-1} and 200 mL ha^{-1}. Due to the heterogeneous nature of the inoculum (cow manure) and the importance of greater experimental representativeness of the control, six replications were set up for the other treatments. In total, there were 24 pots.

Each pot was watered by hand with 1.4 L of non-chlorinated water on alternate days during the first 30 days after planting (DAP). Between 30 and 60 DAP, 2.8 L pot^{-1} of water was applied on alternate days. After this period, 5.0 L pot^{-1} of water was applied on alternate days. This schedule was determined by the average available water capacity of the soil (0.63 mm m^{-1}) based on the model by VAN DEN BERG (2000) and the average evapotranspiration of the crop (4.0 mm day^{-1}) (LIMA et al., 2009).

The biological fertilizer inside the CLC® Biofactory was constantly stirred, and 43 days after installation of the CLC® Biofactory, it was replenished with 3 kg of Microgeo® and then homogenized.

The biological fertilizer prepared using Microgeo® was applied a second time 54 DAP via foliage at a dose of 200 L ha^{-1} with a manual sprayer in the treatment containing only biological fertilizer. Top-dressing fertilization occurred 60 DAP, applying the equivalent of 40 kg ha^{-1} of nitrogen in the form of urea in all pots. The plants developed without the occurrence of pests or diseases, and weeds were removed manually from the pots whenever necessary.

Morphological evaluations of the sugarcane plants

The experiment was completed in 164 DAP, and several parameters were evaluated. The number of tillers and the average number per pot were determined by directly counting the tillers on each plant 45, 110, and 164 DAP. Plant height was measured by a graduated ruler from the neck of the plant to the insertion of leaf +1 for all the tillers of each plant, and the average height per pot in centimeters was determined. The diameter of all the stalks of each plant was measured with a digital caliper at

about 5 cm above the soil level in the central region of the internode, and the average diameter per pot in centimeters was determined. The number of stalks and the average number of stalks per pot were calculated by directly counting the visible stalks on each plant. The length of leaf +3 of all tillers of each plant was measured by a tape measure, and the average length per pot in centimeters was determined. The width of leaf +3 was measured with a digital caliper at the median portion of all tillers of each plant, and the average width per pot in centimeters was calculated. The number of green leaves was determined by counting the fully expanded leaves with a minimum of 20% green area from leaf +1 in all tillers of each plant, and the average number per pot was then obtained. Leaf area was estimated by counting the number of green leaves and by the measurements for the leaves +3 (length and width). This estimative was based on the methodology proposed by HERMANN & CÂMARA (1999): LA = L×W×0.75×(NOL + 2), where L is the length of leaf +3; W is the width of leaf +3; 0.75 is the correction factor for the crop's leaf area; and NOL is the number of open leaves with at least 20% green area in cm^2. The fresh and dry mass of leaves in grams was determined by weighing the freshly harvested leaves packing them in paper bags, drying them in a hot air circulation oven (72 °C until reaching constant mass), and weighing on scales with a precision of 0.01 g.

Chemical analyses of sugarcane leaf and soil samples

Immediately after the biometric assessments, the leaves were removed and soil samples were collected for chemical analyses. For the leaf analysis of the plants in each pot, 12 open leaves with at least 20% green area were removed (four leaves from each plant). The plant materials were identified, packed in paper bags, and submitted for analysis to determine nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), and aluminum (Al), according to the methodology by BATAGLIA et al. (1983).

Soil samples were collected at a depth of 0-20 cm from the surface using a soil auger. Three soil samples were collected per pot, and combined into a composite sample that was homogenized. From the composite sample, a soil subsample was collected and identified for submission to the laboratory.

The pH of the soil was determined in a 1:2.5 soil:water suspension. Exchangeable Al, Ca, and Mg were extracted with 1 M KCl. Ca and Mg were determined by atomic absorption spectrometry, and Al was assessed by acid-base titration. P and K were extracted by ion-exchange resin. Potential acidity (Hydrogen/H + Al) was estimated by an equation based on the pH determined in the SMP buffer solution (pH SMP). Available micronutrients (Fe, Mn, Zn, and Cu) were extracted by Mehlich 1 and determined by atomic absorption spectrophotometry. B was extracted with hot water and determined by spectrophotometry with azomethine H at 420 nm. The results facilitated the calculation of other parameters, such as exchangeable bases (EB) the sum of Ca, Mg, and K; cation-exchange capacity (CEC), the sum of Ca, Mg, K, Al, and H; base saturation (V%), the ratio between EB and CEC in percentage; and Al saturation (m%), the ratio between exchangeable Al and CEC in percentage.

Statistical analysis

Firstly, Shapiro-Wilk test was used to assess the normality of data in the samples within all the three datasets. After the checking that the data followed a normal distribution for the vast majority of samples, analysis of variance was used for a statistical analysis of the data, using the F test. When there was statistical significance, the Tukey test was applied to the morphological and foliar evaluations of the sugarcane plants, and the Scott-Knott test was adopted for the soil chemical analysis, at 5% significance level for the comparison of means. The SISVAR statistical program was utilized for all the analyses. To evaluate the contribution of the combined use of *Nitrospirillum amazonense* strain 11145 with biological fertilizer prepared using Microgeo® and native microbiome from location of the product application to the total variation in morphological characteristics of sugarcane plants, chemical factors of their leaves, and chemical factors of the soil where sugarcane plants were grown, a variance partitioning BORCARD et al. (2018) was performed using the function "varpart" of the "vegan" package version 2.5.6 (OKSANEN et al. 2016) (R CORE TEAM, 2017).

RESULTS AND DISCUSSION

Viable cell count

The growth of *N. amazonense* in the liquid culture medium presented a standard growth curve with four distinct phases: lag, exponential, stationary, and death (KONEMAN et al., 2001). Figure 1 shows the curves obtained with the optical density values and the number of viable cells per mL of the culture medium over 40 hours of incubation. After 30 hours of incubation, a large decline in the number of viable

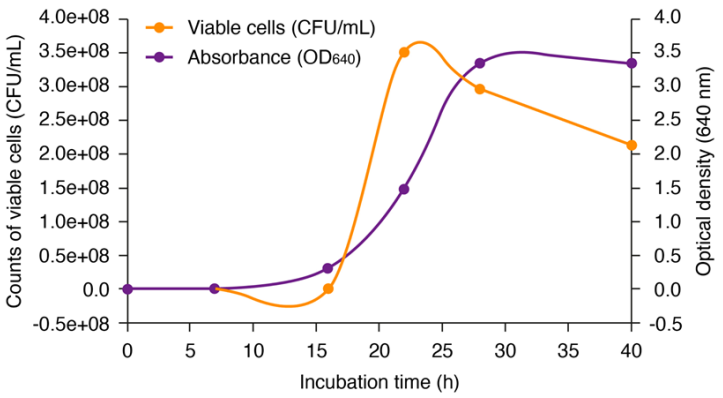

Figure 1 - Growth curve of *Nitrospirillum amazonense* in liquid LGI medium as a function of the values obtained for optical density and the number of viable cells per mL, over 40 hours of incubation at 30 °C and agitation at 180 rpm.

bacterial cells was observed. This suggested that in order to obtain a minimum concentration of $2x10^8$ viable cells per mL, found in most commercial inoculants, the inoculum should be kept under proper incubation for a period of 20 to 30 hours. The optical density evaluation is based on the fact that microbial cells scatter light; therefore, they can be detected by measuring absorbance in a spectrophotometer.

Morphological analyses of the sugarcane plants

The results of the morphological characteristics of the sugarcane plants grown in the different experimental treatments are presented in table 1. The data pointed to significant differences in the length of leaf + 3, the number of green leaves, and leaf area in the plants grown in the soil treated with the biological fertilizer with *N. amazonense*.

Table 1 - Morphological analyses of sugarcane plants grown in soil containing biological fertilizer with Microgeo® and addition of *Nitrospirillum amazonense.*

	----------Experimental treatments----------					
Morphological characteristics	Biological fertilizer	*Nitropirillum amazonense* (200 mL ha^{-1})	*Nitrospirillum amazonense* (100 mL ha^{-1})	Biological fertilizer with addition of *N. amazonense*	Control	Statistics (ANOVA)
Tillers 15/10	$6.3^{\delta}\pm1.2^{\dagger}$	6.7±0.7	6.3±0.5	5.6±0.8	6.0±1.3	
Tillers 17/12	6.3±0.9	6.6±0.7	7.4±1.3	6.7±0.6	6.8±0.9	
Tillers 10/02	3.7±0.5	4.2±0.7	4.4±1.1	4.7±0.7	4.2±0.5	
Plant height (cm)	95.6±8.9	103.9±6.4	97.1±8.3	92.9±11.9	93.7±9.8	
Stalk diameter (cm)	1.843±0.01	1.839±0.01	1.831±0.01	1.828±0.02	1.840±0.01	
Stalk number	3.2±0.8	3.2±0.7	3.9±0.8	3.5±0.6	3.4±0.3	
Length of leaf +3 (cm)	135.8ab±5.7	136.7ab±5.2	127.7b±12.1	133.7ab±4.2	138.8a±6.1	P<0.042
Leaf width (cm)	1.93±0.03	1.99±0.06	1.93±0.01	1.97±0.03	1.98±0.07	
No. of green leaves	3.1b±1.0	4.9ab±1.0	4.8ab±1.5	5.5a±1.4	$4.4^{ab}\pm1.2$	P<0.006
Leaf area (cm^2)	1012.5b±245.1	1401.6a±270.6	1266.3ab±281.5	1473.5a±234.1	1326.4ab±214.9	P<0.004
Fresh mass of leaves (g)	1474.6±245.2	1584.7±387.7	1573.60±286.8	1955.2±598.5	1748.4±174.0	
Dry mass of leaves (g)	472.2±57.1	552.3±43.5	472.5±66.9	508.7±159.8	543.2±46.5	

$^{\delta}$Average for the replicates soil.

†Standard deviation of the average of the replicates soil.

Values with the same letters were not significantly different (P<0.05) – within the same line – based on upon a Tukey's HSD test.

It is not common to evaluate the leaf length of sugarcane in isolation in response to different factors. However, this variable is adopted in formulas which determine the plant's leaf area (CARVALHO et al., 2008). Accordingly, the changes promoted by the application of biological fertilizer with *N. amazonense* on the length of the leaves influenced leaf area. The number of leaves that accumulate in a plant is a parameter of plant development that is also associated with the expansion of leaf area, which, in turn, is related to the interception of solar radiation, photosynthesis, biomass accumulation, and evapotranspiration (DELLAI et al., 2005; STRECK et al., 2005). According to LUCCHESI (1987), an increase in leaf area induces an increase in the plant capacity to absorb solar energy and carry out photosynthesis. Therefore, this criterion can be used to indicate productivity.

Chemical characteristics of the leaves

Table 2 demonstrates the results of the chemical analyses of the leaves of the sugarcane plants grown in the different treatments. The results revealed a higher S content in the leaves of sugarcane plants grown in soil containing the biological fertilizer with *N. amazonense* (1.5±0.6 g kg^{-1}) and inoculation of *N. amazonense* (100 mL ha^{-1}) (1.9±0.3 g kg^{-1}) compared to the other treatments (mean of the other treatments: 1.1±0.3 g kg^{-1}). S is crucial as a nutrient and as a plant defense mechanism against pests and diseases (EBLOEM et al., 2015; NWACHUKWU et al., 2012). It plays an essential role in plant metabolism as it is a component of amino acids (cystine, cysteine, methionine, and taurine), coenzymes (thiamine and biotin), and polysaccharide esters. S also makes up ferredoxin, a molecule responsible for electron transport in photosynthesis (COLEMAN, 1966; CRAWFORD et al., 2000); in nitrogen fixation (SANTOS et al., 2019); and in the reduction of oxidized compounds, such as nitrate (MARTINS et al., 2020). S also influences chlorophyll content, which increases in plants treated with sulfate fertilization (TISDALE, 1977).

As for agricultural production, S is one of the 16 essential elements, and it is fundamental for the maximum yield and quality of crops, preventing chlorosis of the leaves of sugarcane plants (VITTI, 1989). S deficiencies are often confused with N deficiencies. The symptoms of S deficiency are delayed plant growth and general yellowing of the leaves. In less severe cases of S deficiency, visual symptoms may not be apparent, but both crop yield and quality will be affected (CECCOTTI, 1996; JESCHKE & DIEDRICK, 2010).

Table 2 - Chemical characteristics of the leaves of sugarcane plants grown in soil containing biological fertilizer with Microgeo® and addition of *Nitrospirillum amazonense*.

	----------Experimental treatments----------					
Chemical characteristis of the leaves	Biological fertilizer	*Nitropirillum amazonense* (200 mL ha^{-1})	*Nitrospirillum amazonense* (100 mL ha^{-1})	Biological fertilizer with addition of *N. amazonense*	Control	Statistics (ANOVA)
N (g kg^{-1})	9.0[δ]±1.8[†]	8.1±2.1	8.2±0.9	9.3±1.7	7.4±3.5	
P (g kg^{-1})	0.7±0.1	0.6±1.1	0.7±1.8	0.7±1.1	0.8±1.2	
K (g kg^{-1})	10.8±2.3	10.1±1.9	9.6±2.1	9.7±2.1	10.4±2.0	
Ca (g kg^{-1})	2.9±0.3	3.0±0.25	2.8±0.6	3.1±0.7	2.9±0.6	
Mg (g kg^{-1})	1.5±0.1	1.5±0.1	1.4±0.2	1.6±0.2	1.6±0.1	
S (g kg^{-1})	1.2b±0.3	1.1b±0.3	1.9a±0.3	1.5a±0.6	1.1b±0.3	P<0.067
B (mg kg^{-1})	26.4±22.5	11.6±4.2	34.1±35.9	16.3±13.6	15.5±6.2	
Cu (mg kg^{-1})	15.6±5.5	17.0±9.5	11.7±2.9	16.4±6.9	14.1±4.7	
Fe (mg kg^{-1})	145.9±55.8	141.5±19.0	124.2±10.2	133.9±22.59	155.4±32.9	
Mn (mg kg^{-1})	84.8±19.4	90.0±28.0	76.2±9.8	82.8±13.0	82.4±9.4	
Zn (mg kg^{-1})	46.2±19.6	42.8±11.5	36.5±3.8	41.8±9.0	39.2±10.2	
Na (mg kg^{-1})	57.0±45.2	40.7±5.8	38.5±6.6	46.7±11.0	45.3±23.3	
Al (mg kg^{-1})	125.7±66.4	120.7±43.4	114.3±19.8	92.9±27.5	124.8±48.2	

[δ]Average for the replicates soil.

[†]Standard deviation of the average of the replicates soil.

Values with the same letters were not significantly different (P<0.05) – within the same line – based on upon a Scott-Knott test.

Emphasis is given to N on the issue of sugarcane nutrition and fertilization, but the plants utilization of this nutrient may be reduced if S becomes limited, demonstrating a synergistic effect between N and S (SALVAGIOTTI et al., 2009; DE BONA et al., 2011). For BOLOGNA-CAMPBELL (2013), an increase in N supply implies a greater utilization of S by plants. Although no statistical difference was found for the mean N content of the leaves, this chemical factor presented higher values in the plants grown in the soil treated with biological fertilizer with *N. amazonense* (9.3±1.7 g kg^{-1}), in the order of 23.5%, 15.3%, 14%, and 3.9%, compared to the plants grown in the control and in the treatments containing a dose of 200 mL ha^{-1} of *N. amazonense*, a dose of 100 mL ha^{-1} of *N. amazonense*, and biological fertilizer, respectively. As evidenced by increasing tendencies in comparison to the other treatments, these results corroborated the statistical results for the foliar S content and demonstrate the positive effect of the combination of *N. amazonense* with biological fertilizer for a greater utilization of S by the plants.

Soil chemical factors

The results of the chemical analyses of the soil used to cultivate sugarcane plants in different treatments are shown in table 3. The data revealed decrease in the soil pH. The lowest value was found in the treatment with *N. amazonense* (200 mL ha^{-1}) (5.07±0.1), compared to the other treatments (mean of the other treatments: 5.15±0.04). According to HARTEMINK & BARROW (2023), soil acidity and soil alkalinity in relation to plant growth has been well-studied. The soil pH is often used as an indicator of the chemical fertility of the soil, and it is believed that most major and minor plant nutrients are best available around a slightly acid pH.

The highest S content was detected in the control soil (18.8±3.8 mg dm^{-3}), and the lowest content of this element was observed in the treatment with *N. amazonense* (100 mL ha^{-1}) (7.7±1.5 mg dm^{-3}). This is possibly due to the greater availability of S for the plants through a process mediated by the microorganisms present in the soil and to the best availability of this element to the plants under lower

Table 3 - Chemical factors of soil cultivated with sugarcane in experimental treatments containing biological fertilizer with Microgeo® and addition of *Nitrospirillum amazonense*.

	----------Experimental treatments----------					
Soil chemical factors	Biological fertilizer	*Nitropirillum amazonense* (200 mL ha^{-1})	*Nitrospirillum amazonense* (100 mL ha^{-1})	Biological fertilizer with addition of *N. amazonense*	Control	Statistics (ANOVA)
P (mg dm^{-3})	13.8[δ]±7.2[†]	11.3±1.5	8.3±1.5	18.0±11.8	13.3±3.8	
OM (g dm^{-3})	14.5±0.8	15.0±1.0	14.3±1.5	14.7±0.5	14.0±0.8	
pH ($CaCl_2$)	5.1ab±0.05	5.07b±0.1	5.1ab±0.0	5.2a±0.06	5.2ab±0.05	P < 0.045
K (mmolc dm^{-3})	2.3±0.4	2.3±0.3	2.2±0.2	2.2±0.6	2.1±0.2	
Ca (mmolc dm^{-3})	22.0±1.7	23.0±1.0	23.0±2.0	25.0±6.0	24.5±1.6	
Mg (mmolc dm^{-3})	11.0±1.3	10.7±0.6	12.7±0.6	12.2±1.6	13.7±2.9	
Na (mmolc dm^{-3})	1.25±0.2	1.27±0.1	1.47±0.2	1.25±0.3	1.33±0.2	
H+Al (%)	23.7±0.8	23.7±1.1	22.7±0.6	23.3±1.0	23.2±1.3	
CTC (mmolc dm^{-3})	60.1±2.8	60.9±2.8	62.0±3.0	64.0±7.8	65.0±4.1	
EB (mmolc dm^{-3})	36.4±2.5	37.3±1.8	39.4±2.6	40.6±7.1	41.9±4.5	
V (%)	60.5±1.8	61.0±1.0	63.7±1.5	63.3±3.1	64.0±3.4	
S (mg dm^{-3})	12.7ab±7.2	15.0ab±3.6	7.7b±1.5	12.8ab±3.9	18.8a±3.8	P < 0.047
B (mg dm^{-3})	0.73±0.1	0.75±0.2	0.77±0.2	0.84±0.2	0.85±0.1	
Cu (mg dm^{-3})	0.73±0.1	0.63±0.1	0.60±0.1	0.70±0.08	0.65±0.05	
Fe (mg dm^{-3})	37.2±7.5	38.3±4.1	38.0±3.5	35.0±4.3	35.3±4.1	
Mn (mg dm^{-3})	11.7±1.9	11.2±0.9	12.0±0.9	11.3±1.5	10.9±1.0	
Zn (mg dm^{-3})	1.5±0.3	1.3±0.2	1.0±0.2	1.8±0.7	1.5±0.3	

[δ]Average for the replicates soil.

[†]Standard deviation of the average of the replicates soil.

Values with the same letters were not significantly different (P < 0.05) – within the same line – based on upon a Tukey's test.

soil pH. The levels of this element in the leaves of the sugarcane plants grown in the soil containing biological fertilizer with *N. amazonense* and inoculation of *N. amazonense* were higher than in the control (Table 2).

Although, there was no statistical difference, the average P content of the soil in the treatment with biological fertilizer with *N. amazonense* (18.0±11.8 mg dm^{-3}) was higher in comparison to the other treatments (biological fertilizer, and *N. amazonense* (200 mL ha^{-1}), *N. amazonense* (100 mL ha^{-1}), and control: 13.8 g dm^{-3}, 11.3 g dm^{-3}, 8.3 g dm^{-3}, 13.3 g dm^{-3}, respectively). The same findings were unveiled for the Zn content in soil. This trend indicates positive effect among the biological fertilizer, the diazotrophic *N. amazonense* bacteria, and this highly relevant macro- and micronutrient in agriculture.

As with nitrogen fertilization, the responses of the soil chemical factors to inoculation, and possibly to its combination with biological fertilization, are dependent on the sugarcane variety (LOPES et al., 2019) and may be more notable in soils with medium or low fertility (OLIVEIRA et al., 2006). Accordingly, future studies should consider different sugarcane cultivars and soil fertility status.

Variance partitioning

The variance partition showed the relative contribution of the combination of *N. amazonense* with biological fertilizer produced using Microgeo® and native microbiome from location of the product application in the total variation of morphological characteristics of sugarcane plants during their early stages of development under controlled conditions, chemical factors of their leaves, and chemical factors of the soil where these sugarcane plants were grown (Figure 2). In a particular way, combined use of *N. amazonense* and biological fertilizer influenced 24.51% in the variance of the morphological characteristics of sugarcane plants analyzed in this study. The chemical characteristics of the leaves of sugarcane plants were influenced of the order of 17.17% by this combination. In turn, soil chemical factors were influenced 12.03%.

The preponderant influence of the combined use of *N. amazonense* and biological fertilizer on morphological and nutritional characteristics of the sugarcane plants in comparison with its lesser influence on soil chemical characteristics may be associated with the direct benefits to the plants assured by biological, metabolic and chemical properties of the combination of microbial (native microbiome with addition of diazotrophic bacteria), organic and mineral components. Increase in soil microbial biomass and activity, expected by use of the combination tested in this study, can essentially acts as a decomposition agent for residues added to the soil and also works as a compartment that rapidly

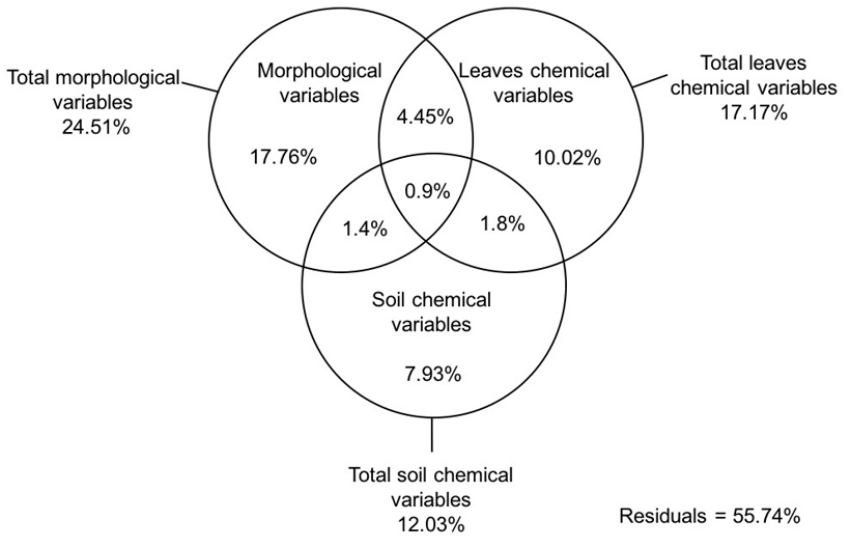

Figure 2 - Influence of the combined use of *Nitrospirillum amazonense* strain 11145 with biological fertilizer prepared using Microgeo® and native microbiome from location of the product application on morphological characteristics of sugarcane plants, chemical factors of their leaves, and chemical factors of the soil where sugarcane plants were grown.

releases nutrients to plants through the process of mineralization of residues and death of organisms (SILVA et al., 2010), representing a labile source of nutrients to crops (DICK et al., 2009). In addition, the association of diazotrophic bacterial species with sugarcane is known for their beneficial effects on promoting plant growth (SPAEPEN et al., 2007).

Despite the few notable positive effects of the treatments on soil chemistry, decrease in soil pH under application of *N. amazonense* (200 mL ha^{-1}), and the presumed greater availability of S for the plants through a process mediated by the microorganisms may also have contributed to the positive effects on the sugarcane plants.

CONCLUSION

Based on the evaluation of the early stages of sugarcane development under controlled conditions, this study identified a positive effect of the application of biological fertilizer, prepared using Microgeo® and the native microbiome from the location where the product was applied, combined with *N. amazonense* on the length of leaf +3, the number of green leaves, leaf area, and the S content in the leaves of sugarcane plants. Thus, our hypothesis was supported based on gains in the morphological and nutritional characteristics of sugarcane plants grown under the combination of *N. amazonense* with the biological fertilizer. These gains were observed in the early stages of plant development, with few notable positive effects on soil chemistry.

ACKNOWLEDGEMENTS

We wish to thank Matheus Henrique Doria Siqueira and Mateus Leonardo Welika dos Santos from Universidade Brasil for their technical support during the greenhouse experiment. This work was financially supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil (23038.006927/2014-35 / Premium 116/2017), Conselho Nacional de Desenvolvimento Científico (CNPq), Brazil (Research A. A. Navarrete Productivity Grant 309746/2021-3) and Microgeo biotecnologia agrícola - Microbiol Indústria e Comércio Ltda.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

AAN, GHV, SPVF, MSCA-K and RCB conceived and designed the experiment. RCB, GHV and AAN performed the experiment; RCB, GHV and AAN carried out the lab analyses. GHV and AAN supervised and coordinated the experiment. RCB, GHV, LSV and AAN performed statistical analyses of experimental data. AAN, RCB and GHV prepared the draft of the manuscript. AAN, ESL, SPVF, MSCA-K, JHPA-P and ACS revised and edited the manuscript. AAN and MSCA-K acquired resources and funding. All authors critically revised the manuscript and approved of the final version.

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