Influence of *Azospirillum brasilense* on micro-propagated sugarcane seedlings

Influência do *Azospirillum brasilense* em mudas micropropagadas de cana-de-açúcar

Influencia de *Azospirillum brasilense* en plántulas micropropagadas de caña de azúcar

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Abstract

Sugarcane is an essential crop in the Brazilian economy. The adoption of techniques in the acclimatization of plants in vitro contributes to success in the field. The present work aims to evaluate the effect of *Azospirillum brasilense* on the acclimatization of sugarcane seedlings. The experiment was conducted with six treatments and twelve replications. There were two control treatments without inoculation, one with N fertilization and one without, and four with *A. brasilense* (2.0×10^8 CFU) at different doses (19.92mL + N fertilization, 9.84mL, 19.92mL, and 39.84mL). Evaluations were seedling height (SH), stem diameter (SD), number of shoots per tube (NS), number of leaves (NL), fresh aerial weight (AFM), root fresh weight (RFM), total chlorophyll (TC), foliar nitrogen (FN) and visual microscopic study of the roots. The results showed that the recommended dose of *A. brasilense* + N fertilization increased by 36% in seedling height, 29% in stem diameter, 85% in aerial fresh mass, 21% in total chlorophyll, 142% in foliar N, and also increased root hairs and the presence of microbial biofilm. Therefore, the bacterium *A. brasilense* can be used in association with nitrogen fertilizers or alone, where it obtained great benefits in the increase of biomass.

Keywords: Biofilm; Bio nutrition; Tissue culture; Rhizobacteria; *Saccharum officinarum.*

Resumo

A cana-de-açúcar é uma cultura essencial na economia brasileira. A adoção de técnicas de aclimatação das plantas in vitro contribui para o sucesso no campo. O presente trabalho visa avaliar o efeito do *Azospirillum brasilense* na

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aclimatação de mudas micropropagadas de cana-de-açúcar. A experiência foi conduzida com seis tratamentos e doze repetições. Houve dois tratamentos de controle sem inoculação, um com fertilização de N e um sem, e quatro com *A. brasilense* (2,0x10^{8} UFC) em doses diferentes (19,92mL + fertilização N, 9,84mL, 19,92mL e 39,84mL). As avaliações foram altura das plântulas (SH), diâmetro do caule (SD), número de rebentos por tubo (NS), número de folhas (NL), peso fresco aéreo (AFM), peso fresco radicular (RFM), clorofila total (TC), nitrogênio foliar (FN) e estudo microscópico visual das raízes. Os resultados mostraram que a dose recomendada de fertilização *A. brasilense* + N aumentou 36% na altura das plântulas, 29% no diâmetro do caule, 85% na massa fresca aérea, 21% na clorofila total, 142% no N foliar, e também aumentou os pêlos radiculares e observou-se a presença de biofilme microbiano. Portanto, a bactéria *A. brasilense* pode ser utilizada em associação com fertilizantes nitrogenados ou isoladamente, onde obteve grandes benefícios no aumento da biomassa.
Palavras-chave: Biofilme; Bio nutrição; Cultura de tecidos; Rhizobacteria; *Saccharum officinarum*.

Resumen
La caña de azúcar es un cultivo esencial en la economía brasileña. La adopción de técnicas de aclimatación de plantas in vitro contribuye a su éxito en el campo. El presente trabajo tiene como objetivo evaluar el efecto de *Azospirillum brasilense* en la aclimatación de plántulas micropropagadas de caña de azúcar. El experimento se realizó con seis tratamientos y doce repeticiones. Hubo dos tratamientos control sin inoculación, uno con fertilización N y otro sin, y cuatro con *A. brasilense* (2.0x10^{8} UFC) a diferentes dosis (19.92mL + fertilización N, 9.84mL, 19.92mL y 39.84mL). Las evaluaciones fueron altura de plántula (SH), diámetro de tallo (SD), número de brotes por tubo (NS), número de hojas (NL), peso aéreo fresco (AFM), peso radicular fresco (RFM), clorofila total (TC), nitrógeno foliar (FN) y estudio microscópico visual de raíces. Los resultados mostraron que la dosis recomendada de *A. brasilense* + fertilización N aumentó un 36% en la altura de la plántula, un 29% en el diámetro del tallo, un 85% en la masa fresca aérea, un 21% en la clorofila total, un 142% en el N foliar, y también aumentó los pelos radiculares y la presencia de biofilm microbiano. Por lo tanto, la bacteria *A. brasilense* puede ser utilizada en asociación con fertilizantes nitrogenados o sola, donde obtuvo grandes beneficios en el aumento de la biomasa.
Palabras clave: Biofilm; Bio nutrición; Cultivo de tejidos; Rizobacterias; *Saccharum officinarum*.

1. Introduction

Sugarcane (*Saccharum officinarum* L.), cultivated over 500 years ago, is an influential crop in the Brazilian economy, generating approximately 24 billion liters of alcohol and 33,9 million tons used in sugar production. This market continues to expand in the southeastern and northeastern states, where they exhibit productivity differences (IBGE, 2020). The northeast region is affected by climatic irregularities and lower rainfall rates than expected in essential periods for the crop, which impacts the productive potential of the plant and the quality of ethanol/sugar. For this reason, it requires better strategic planning and changes/innovations of technology in cultivation to ensure high productivity (Singh et al., 2019; Urgesa & Keyata, 2021).

Techniques to obtain high-level seedlings, like in vitro propagation from selected varieties (with genetic uniformity, high-quality phytosanitary) quality, direct influence on the percentage of survival, vigor, and growth, can contribute to production in a short time (Kodym & Leeb, 2019) creasing between 10% to 30% productivity and 30% longevity of sugarcane fields (Alina Martínez, 2020).

Process optimization in the acclimatization phase of micro-propagated seedlings is still not well established (Hartati et al., 2021). This phase involves the preparation of seedlings for the field, under the attenuation of variations of meteorological elements (solar radiation, temperature, air, and soil humidity), in a determined period (± 45 days). That depended on the variety used and gradually adapted to the natural conditions, resulting in rooted seedlings suitable for planting (Almeida Neto et al., 2020).

Fertilization is an essential step for promising crop development, where an indispensable nutrient is nitrogen (N) (Reis et al., 2020), responsible for a high input cost in grasses with the recommendation of 180 kg/ha^{-1} for 100 mg/ha^{-1} of produced stalks (Martins et al., 2020; Singh et al., 2020). Nitrogen absorption by plants depends on its slow mineralization and the other processes limiting its availability in the soil, such as lixiviation, volatilization, and denitrification, representing significant fertilizer losses for farmers. For this reason, research for new sustainable and economical alternatives independent of chemical inputs, like microorganisms that use atmospheric nitrogen fixation, is in current demand (Anas et al., 2020; Guo et al., 2020).

One of the most studied groups of microorganisms for inoculating grasses of agronomic interest is the genus Azospirillum (Galindo et al., 2022). The endophytic species, A. brasilense, can colonize plant roots, fix atmospheric $N_{2,}$ and supply N in the form of nitrite and nitrate to the plant. Also, synthesize growth-promoting substances (auxins, gibberellins, and cytokinins), increase nitrate reductase enzyme activity, induce plant resistance to abiotic and biotic stresses, and solubilize phosphates (Fukami et al., 2018; Rosa et al., 2022).

This bacterium has been reported in several crops, mainly in grasses (e.g. maize, rice, wheat), to contribute to plant growth, nutrition, and increased productivity (Boyko et al., 2011; Pham et al., 2022). In sugarcane, several works assure a positive response of Azospirillum in vegetative contribution, in seedling production, ratoon-cane, and pre-sprouted seedlings (May et al., 2021; Santos et al., 2021)

Although, there is still insufficient information about the response of micro-propagated sugarcane seedlings, especially during the acclimatization phase, with the inoculation of nitrogen-fixing bacteria like A. brasilense. Weather controls are necessary for this phase to ensure the microorganism colonization and the mutual interaction response. Consequently, research on this management practice is needed. This work aimed to evaluate the response of micro-propagated sugarcane seedlings in the acclimatization phase through the inoculation of A. brasilense species.

2. Methodology

The experiment was realized in the greenhouse of the Governador Miguel Arraes Bio factory in the Northeast Strategic Technologies Center (CETENE), located in Recife- Pernambuco, Brazil (07° 51.461' S, 035° 14.322 W), in the period from February to November 2021. During the experiment, the average climatic condition inside the greenhouse regarding average air temperature was 32 °C maximum and 21 °C minimum (± 2 °C). The sugarcane variety used was SP 79 1011, characterized by demanding soils, high yield, tolerance to drought, excellent sprouting of ratoons, and no flowering.

Micro-propagated seedlings were used in the acclimatization phase, 30 days post-planting, using tubes with commercial substrate Basaplante® (BASE AGRO, São Paulo, Brazil). The seedlings were placed under 40% shade and automatically irrigated daily in four irrigations (08:00, 11:00, 14:00, and 16:00 hours) with 4 min duration to maintain approximately 80% of the water in the soil.

A Completely Randomized Design was used, with six treatments and twelve repetitions repeated twice. There were two control treatments without inoculation, one with N fertilization – 50g/10 L of water (positive control) and another without N fertilization (negative control). And four treatments with the inoculation of *A. brasilense* (AbV5 and AbV6 strains; $2.0\text{x}10^8$ CFU/mL), commercial product AzzoFix® (Microquímica-Tradecorp®, São Paulo, Brazil) in different doses. First with recommended dose (19.92 mL) + N fertilization 50g/10 L of water, the second with half the recommended dose (9.84 mL), the third with recommended dosage (19.92 mL), and the fourth with a higher dosage (39.84 mL), all diluted in 240 mL of water. After 10mL of each treatment was suspended directly in the experimental unit, which consisted of one seedling in a tube, resulting in 144 experimental units performed two times, the methodology is shown in.

At 30 days after inoculation, the seedlings began to be monitored weekly according to their agronomic characteristics, including seedling height (SH), stalk diameter (SD), number of shoots per tube (NS), number of leaves (NL), aerial fresh mass (AFM), root fresh mass (RFM), total chlorophyll (TC), and in the nutritional area the quantification of foliar nitrogen (FN).

The SH was measured in centimeters using a ruler, from soil level to height of leaf apex (+1) (Kuijper numbering system) (Cheavegatti-Gianotto et al., 2011), and the SD, using a digital pachymeter at the base of the stalk, distanced 1cm from the substrate, both measured according to (Filho et al., 2021). The determination of the fresh mass of the aerial part (leaves + stalks) (AFM) and roots (RDM) was performed with a precision digital balance, and the results were expressed in g/plant. The TC was measured with SPAD 502 Plus Chlorophyll Meter (Konica Minolta, Tokyo, Japan). The reading was always performed at a fixed time (9:00 am), in the middle third of the leaf apex (+1), instantly measuring the chlorophyll content. Determination on the FN, the material was dried in a forced-air circulation oven at 65° C for one week; after 0.2g of the dried and ground sample of each treatment in triplicate, it was digested in sulfuric acid under heating in a digester block on a plate until ± 450°C. Then the sample was distilled in 30% sodium hydroxide and titrated in ammonium borate with 0.1N hydrochloric acid solution. The results obtained were calculated in the percentage of total protein, and then the values transformed to N according to the Kjeldahl methodology, as proposed by (Filho et al., 2021).

The middle thirds and the apex of roots inoculated with *A. brasilense* were collected 60 days after inoculation for scanning electron microscopy. The Microscopy Laboratory of CETENE, samples were fixed in 2.5% glutaraldehyde in 0.1 mol. L^{-1} sodium phosphate buffer at pH 7.2, washed with the same buffer and dehydrated in an alcoholic series. After dehydration, the samples were dried to the critical point, according to (Hakki et al., 2010). Finally, they were fixed on aluminum supports with the help of double-sided carbon tape and coated with 20 nm of gold by sputtering for ultra-structural study in Scanning Electron Microscope-SEM (FEG model-Quanta 200FEI, FEI Company, USA).

The dataset was submitted to variance analysis (ANOVA), and the significance of the effect of inoculation with *A. brasilense* was tested by Tukey's test at a 5% probability level. Statistical analysis was performed using Sisvar® software version 5.6 for Windows (Ferreira, 2019).

3. Results

The analysis of variance revealed significant effects ($p \leq 0.05$) of the interaction between the treatment with inoculation of *A. brasilense* (AbV5 and AbV6 strains) combined with N fertilization in micro-propagated sugarcane seedlings in the acclimatization phase (Table 1). Also, it promotes influence on some important variables, like seedling height (SH) and stalk diameter (SD), resulting in increments of 36% and 29%, respectively, when compared to the negative control. This helps a positive expression of symbiotic interaction and increased survival of seedlings in the field, therefore, better productivity and profitability. These variables' development will result in sugar-alcohol derivatives and sugar manufacturing potential (Figure 1).

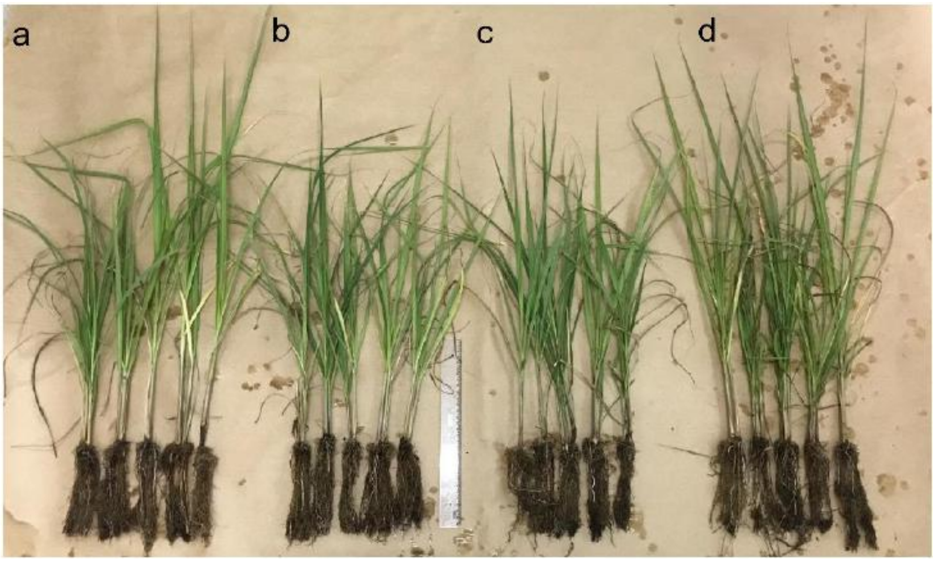
Table 1 - Agronomic development of micro-propagated seedlings[1] of sugarcane Var. SP 79 1011, under the application of all treatments (Recife-Pernambuco, Brazil).

Treatments[2]	SH[a]	SD[b]	NL[c]	NS[d]
Negative control	59.6 c	3.8 b	6.0 a	2.1 a
Positive control	74.4 b	4.2 b	6.0 a	2.1 a
***A. brasilense* + N**	80.8 a	4.9 a	6.0 a	3.1 a
***A. brasilense* dose 1**	51.0 d	3.7 b	6.0 a	2.6 a
***A. brasilense* dose 2**	58.2 c	3.8 b	6.0 a	2.5 a
***A. brasilense* dose 3**	54.9 cd	4.0 b	6.0 a	3.2 a
CV%	4.8	8.5	0.0	26.5
MG	63.1	4.1	6.0	2,6

[1]Micro-propagated seedlings 60 days after planting; [2]**Treatments: Negative control** – without inoculation and without N fertilization; **Positive control –** without inoculation and with N fertilization; ***A. brasilense* + N**– inoculation of the recommended dose of ***A. brasilense*** (19.92mL) and N fertilization; ***A. brasilense* dose 1**– lower dose (9.84mL); ***A. brasilense* dose 2**– recommended dose (19.92mL); ***A. brasilense* dose 3**– higher dose (39.84mL). [a]**SH:** Seedling height (cm); [b]**SD:** Stalk diameter (cm); [c]**NL:** Number of leaves (unit); [d]**NS:** Number of shoots per tube (unit). *Averages followed by the same letter do not differ statistically at a 5% probability level. Source: Authors.

In addition, the evaluation of the stalk diameter x seedling height indicates that the plant will have greater or lesser success in acclimatization/rustification, consequently, a higher capacity to survive in the field after final transplanting. No significant results were observed for the variables, the number of leaves (NL) and several shoots per tube (NS), for all treatments (Table 1).

Figure 1 - Development of micro-propagated sugarcane seedlings submitted to all treatments, 60 days after inoculation. **(a)** negative control, without inoculation and without N fertilization; **(b)** lower dose, half the recommended dose (9.84mL) of *A. brasilense*; **(c)** recommended dose (19.92mL) of *A. brasilense*; and, **(d)** higher dose (39.84mL) of *A. brasilense.*



Source: Authors.

For fresh aerial mass (AFM), Table 2, the treatment *A. brasilense* dose 3 (higher dose 39,84mL), showed results equivalent to the treatments with N fertilizer, performed fortnightly. In comparison, the treatments *A. brasilense* + N and the positive control showed an increase of 85% of AFM compared to the negative control.

For fresh root mass (RFM), treatments *A. brasilense* dose 1, dose 2, and dose 3 expressed significantly promising results, according to the dosage of *A. brasilense*, with a more significant increase from the highest dose following the lowest dose, respectively. However, no significant effects were observed in the treatments *A. brasilense* + N, negative control, and positive control (Table 2).

The highest total chlorophyll (TC) production was observed in the treatment *A. brasilense* + N, and the others had similar responses to the negative and positive controls (Table 3). It is important to emphasize that the treatment *A. brasilense* dose 3 had a similar response to the positive control, in which only one application of the biological product (*A. brasilense*) was made, and the chemical (N fertilization) was reapplied every 15 days (Table 3).

Table 2 - The aerial and fresh root mass of micro-propagated seedlings[1] of sugarcane Var. SP 79 1011, under the application of all treatments (Recife-Pernambuco, Brazil).

Treatments[2]	AFM[a]	RFM[b]
Negative control	7.9 b	4.8 cd
Positive control	13.4 a	4.8 cd
***A. brasilense* + N**	14.6 a	4.4 d
***A. brasilense* dose 1**	6.6 b	6.2 bc
***A. brasilense* dose 2**	6.0 b	7.0 b
***A. brasilense* dose 3**	11.6 a	9.0 a
CV%	19.8	13.1
MG	10,0	6,0

[1]Micro-propagated seedlings 60 days after planting; [2]**Treatments: Negative control**– without inoculation and without N fertilization; **Positive control–** without inoculation and with N fertilization; ***A. brasilense* + N**– inoculation of the recommended dose of *A. brasilense* (19.92mL) and N fertilization; ***A. brasilense* dose 1**– lower dose (9.84mL); ***A. brasilense* dose 2**– recommended dose (19.92mL); ***A. brasilense* dose 3**– higher dose (39,84mL). [a]**AFM:** Aerial fresh mass (g); [b]**RFM:** Root fresh mass (g). *Averages followed by the same letter do not differ statistically at a 5% probability level. Source: Authors.

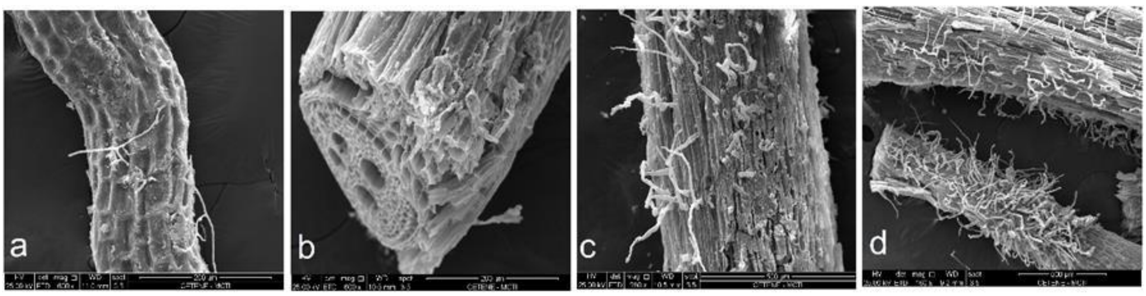
Table 3 - Total chlorophyll and foliar N of micro-propagated seedlings[1] of sugarcane Var. SP 79 1011, after application of all treatments (Recife-Pernambuco, Brazil).

Treatments[2]	TC[a]	FN[b]
Negative control	29.2 b	34.13 e
Positive control	27.6 b	62.58 b
***A. brasilense* + N**	35.4 a	82.55 a
***A. brasilense* dose 1**	29.8 b	39.81 d
***A. brasilense* dose 2**	26.8 b	45.55 c
***A. brasilense* dose 3**	28.0 b	62.58 b
CV%	11.0	2.0
MG	29.5	47.26

[1]Micro-propagated seedlings 60 days after planting; [2]**Treatments: Negative control**– without inoculation and N fertilization; **Positive control–** without inoculation and with N fertilization; ***A. brasilense* + N**– inoculation of the recommended dose of *A. brasilense* (19,92mL) and N fertilization; ***A. brasilense* dose 1**– lower dose (9,84mL); ***A. brasilense* dose 2**– recommended dose (19,92mL); ***A. brasilense* dose 3**– higher dose (39,84mL). [a]**TC:** Total chlorophyll (SPAD); [b]**FN:** Foliar N (%). *Averages followed by the same letter do not differ statistically at a 5% probability level. Source: Authors.

The bacteria *A brasilienses* increased the amount of N and chlorophyll (Table 3) and biomass (Table 2). This proves that bacteria alone or associated with fertilizers can be used. Sugarcane seedlings treated with *A. brasilense* increased RFM, and the presence of root hairs was observed by high-resolution-SEM imaging (Figure 2). Adventitious roots provide a more expansive zone of absorption, allowing the uptake of essential nutrients through solutes into the plant, demonstrating how part of the N was absorbed to participate in the synthesis of chlorophyll and reflected in the total chlorophyll content. The more significant number of secondary roots may have helped the uptake of water and nutrients and helped the plant develop better.

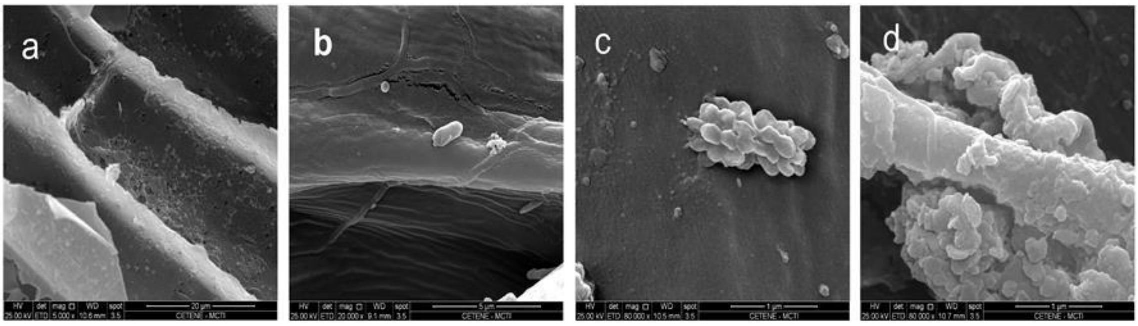
Figure 2 - Incidence of adventitious roots of micro-propagated sugarcane seedlings 60 days after application of treatments. (a) negative and (b)positive control, showing few secondary roots. (c) lower and (d) recommended dose of *A. brasilense*, offering a larger contact surface due to the high presence of root hairs.



Source: Authors.

These results may be related to the uptake of nutrients such as N (Table 3) and the formation of microbial biofilm found in the images of this work (Figure 3). The biofilm production may have helped retain water and nutrients in the rhizosphere region, demonstrating that in the treatments with more adventitious roots (Figure 2 d), the plant grew more, as demonstrated in (Table 2).

Figure 3 - Scanning electron microscopy-SEM of micro-propagated sugarcane seedlings after 60 days under the applications of treatments. (a) negative control, (b) detail of a bacterial cell isolated from *A. brasilense*, (c) lower dose of *A. brasilense*, (d) recommended dose of *A. brasilense* + N fertilization, showing the presence of colonization.



Source: Authors.

The biofilm produced by the bacteria (Figure 3 d) may have helped in nutrient and water retention, increasing biomass values in the plant and development (Figure 1d). They modified the root morphology with root length, surface area, and root volume increases. Root volume increased by 40% and 47% (irrigated and water deficiency conditions, respectively) compared to plants without inoculation. In both situations, the highlight is mainly for inoculants Az1 and Az2, which improved the development of the root system (irrigated and stressed), reflecting in higher root dry mass (washed).

4. Discussion

The treatment with inoculation of *A. brasilense* (AbV5 and AbV6 strains) combined with N fertilization in micro-propagated sugarcane seedlings in the acclimatization phase, promotes influence on some important variables, like seedling height (SH) and stalk diameter (SD). These variables are essential morphological parameters when required to determine the quality of seedlings after implantation in the field. This characteristic estimates the balance of growth of the plants (Galindo et al., 2021; Garcia et al., 2021).

The increase in leaf and fresh root mass is correlated with dry mass, removing the humidity factor; according to (Santos et al., 2022), this increase can be associated with the high colonization of bacteria, which helps access photo-assimilates and nutrient uptake for the plants. In maize plants, more excellent vegetative development was observed in the presence of *A. brasilense,* which contributes to the availability of nutrients to allow plants to assimilate them and accumulate representative rates of fresh mass (Galindo et al., 2019).

It was possible to observe that in all the treatments with *A. brasilense* inoculation, improved N assimilation was expressed in the leaves of the seedlings evaluated. A significant accumulation of N was observed in treating *A. brasilense* + N and decreased with the dose of bacteria. Some researchers justify the behavior of N accumulation in leaves due to the availability of N in the soil because this nutrient participates directly in cell division and expansion and the photosynthetic process (Marques et al., 2021; Santos et al., 2022).

Another effect of N absorbed by plants has increased the apical meristem activity, showing an increased growth rate (Raffi & Charyulu, 2021). Other research with the application of *A. brasilense* in corn showed modifications in the morphology of the roots, increasing the number of lateral roots and favoring the absorption of nutrients (Galindo et al., 2021), as was found in the present work.

Inoculation with plant growth-promoting bacteria stimulates the production of hormones that promote the development of secondary roots and a higher number of root hairs (Galindo et al., 2021). The increase in plant development of sugarcane seedlings with the AbV5 and AbV6 strains of *A. brasilense* may be related to the increase in the root system favoring secondary roots (Pagnussat et al., 2020). The overlay provides comfort to abiotic conditions as physical protection, better water absorption, and essential solutes for the plant (Ramirez-Mata et al., 2018).

Determination of biofilm formation is an easy tool to detect *Azospirillum*-Plant interactions (Ramirez-Mata et al., 2018). Biofilms can provide an ecological advantage for surviving environmental disturbances, as they create a protective niche against other microorganisms found in the soil (Tugarova et al., 2017). In addition, researchers (Marques et al., 2021) showed inoculation by *A. brasilense* on maize plants under irrigated and water deficiency conditions. The root architecture of plants with Az1 isolate is possibly related to a higher photosynthetic rate. These results corroborate those found by (Galindo et al., 2021), which showed a 16% increase in root dry mass in maize with *A. brasilense* inoculation.

Our work was significant in showing bacteria's importance in increasing this vital crop's biomass, especially in the acclimatization phase of micro-propagated seedlings, reaching the in 10 days less.

5. Conclusion

The *Azospirillum brasilense* bacterium can be used with nitrogen fertilizers or alone, where it has dramatically increased biomass in sugarcane seedlings.

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