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# Do *Azospirillum brasilense* application methods and doses influence the quality of *Cordia alliodora* seminal seedlings?

## Métodos de aplicação e doses de *Azospirillum brasilense* influenciam na qualidade de mudas seminais de *Cordia alliodora*?

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## Abstract

The objective of this study was to determine the best method of bacteria application and the dose of maximum technical efficiency for the growth and morphophysiological quality of *Cordia alliodora* seedlings in the northern Amazon. The variables under study were: shoot height (H), collar diameter (CD), number of leaves (NL), shoot dry mass (SDM, g plant$^{-1}$), root dry mass (RDM, g plant$^{-1}$) and total dry mass (TDM, g plant$^{-1}$), Dickson quality index, contents of chlorophyll *a*, chlorophyll *b* and total chlorophyll, leaf area (LA, m$^2$ m$^2$) and nitrogen balance index (NBI). Foliar application of *Azospirillum brasilense* at the dose of maximum technical efficiency of 0.40 ml L$^{-1}$ positively influences the increase of collar diameter in *Cordia alliodora* seedlings. Application via substrate at the dose of maximum technical efficiency of 0.41 ml L$^{-1}$ leads to larger leaf area in *Cordia alliodora* seedlings. Application of *Azospirillum brasilense* at the appropriate doses through both leaves and substrate promotes increments in all physiological parameters studied in *Cordia alliodora* seedlings at 90 days after transplantation.

**Keywords:** Freijó; Biological nitrogen-fixing bacteria; Chlorophylls; Seedling quality.

## Resumo

Objetivou-se determinar o melhor método de aplicação e a dose de máxima eficiência técnica de uma bactéria no crescimento e qualidade morfofisiológica das mudas de *Cordia alliodora* na Amazônia setentrional. As variáveis em estudo foram: altura da parte aérea (H), diâmetro de caule (DC), massa seca da parte aérea (MSPA, g plant$^{-1}$), massa seca das raízes (MSR, g plant$^{-1}$) e massa seca total (MST, g plant$^{-1}$), índice de qualidade de Dickson, teor de clorofilas *a, b* e total, índice de área foliar (AF, m$^2$ m$^2$), índice de balanço de nitrogênio (IBN). A aplicação via foliar da bactéria *Azospirillum brasilense* na dose de máxima eficiência técnica de 0,40 ml L$^{-1}$ influência de forma positiva no incremento do diâmetro de caule em mudas de *Cordia alliodora*. A aplicação via substrato na dose de máxima eficiência técnica de 0,41 ml L$^{-1}$ exibe maior aérea foliar em mudas de *Cordia alliodora*. A aplicação nas doses adequadas tanto via foliar quanto via substrato de *Azospirillum brasilense* promove incremento para todos índices fisiológicos estudados em mudas de *Cordia alliodora* aos 90 dias após o transplantio.

**Palavras-chave:** Freijó; Bactéria fixadora biológica de nitrogênio; Clorofilas; Qualidade de mudas.

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## INTRODUCTION

The increased demand for wood and its derivatives, in the sector of production of native forest seedlings and/or energy sector, requires quality seedlings for the establishment of increasingly productive forests (Smiderle et al., 2020a; Wang et al., 2019). In this context, *Cordia alliodora* Ruiz & Pavon belonging to the family Boraginaceae, popularly known as Spanish elm, component of the local flora of the state of Roraima, stands out among the potential species for implementation and establishment of reforestation in the northern region of Brazil. It is a secondary, semi-deciduous, heliophile tree species, 6-15 m tall, with an upright, cylindrical trunk with 30-50 cm in diameter (Maranho et al., 2013). *Cordia alliodora* stands out as a tree that produces wood of excellent quality and comparable to those of species already established in the international market, such as mahogany (*Swietenia macrophylla* King.) and teak (*Tectona grandis* L.F.). However, there is little information in the literature regarding the seedling production sector for *Cordia alliodora*, despite its potential for supplying timber and non-timber forest products (Maranho et al., 2013).

The use of microorganisms, such as atmospheric nitrogen-fixing bacteria and plant growth-promoting bacteria, can play a relevant and strategic role to ensure high yields at low cost and with less dependence on the import of fertilizers (Hungria, 2011). In order to improve the forest seedling sector, the use of inoculation with adequate doses of *Azospirillum brasilense* has grown, mainly because it is an alternative for the efficient use of nutrients (Fukami et al., 2018; Kolachevskaya et al., 2017). These bacteria fix atmospheric $N_2$ and then make it available to plants. There are currently technological packages using plant varieties and efficient bacterial strains, which can supply more than 50% of the N needed by the plant (Hungria, 2011; Hungria et al., 2022).

In a survey of controlled trials with the *Azospirillum* bacterium in the first 20 years of pioneering field research, Okon & Labandera-Gonzalez (1994) reported that in 60 to 70% of the experiments, increases in productivity were obtained due to inoculation, with statistically drawn increases in in the order of 5% to 30%, highlighting the great importance that these inoculations can bring to the global agricultural scenario. Furthermore, in a survey of results obtained between 1994-2001 in Belgium, Uruguay, Mexico and Israel, most responses were positive, especially in relation to the increase in water and nutrient absorption (Dobbelaere et al., 2001). Smiderle et al. (2022) with research in Brazil, verified that foliar application of *Azospirillum* promoted increments in the biomass production of *Hymenaea courbaril* and in the photosynthetic parameters of its leaves, including chlorophyll content, stomatal conductance and leaf area.

Studies on foliar and soil application of *Azospirillum brasilense* in forest species native to the northern Amazon have not been reported and are needed. In this context, the present study aimed to determine the best method of application of *Azospirillum brasilense* and the dose of maximum technical efficiency for the growth and morpho-physiological quality of *Cordia alliodora* seedlings in the northern Amazon, Brazil.

## MATERIAL AND METHODS

The study was carried out in a greenhouse of Embrapa Roraima from March to June 2022. For the propagation of *Cordia alliodora* seedlings, seeds were collected at Embrapa Roraima's headquarters, located in the municipality of Boa Vista, RR, Brazil. The climate of the municipality of Boa Vista is Am type (tropical monsoon climate), with average temperatures of 27.2 °C in the hottest month and 23.3 °C in the coldest month, with an annual average of 25.4 °C. The average annual rainfall is 1808 mm, with averages of 365 mm and 26 mm for the months of highest (June) and lowest (February) precipitation, respectively.

After obtaining the seeds, they were processed and then sown in a bed, which contained sand medium. The sand substrate moisture was maintained by manual irrigation, with four daily watering.

Approximately twelve days after sowing, the seedlings began to emerge and, as soon as they homogeneously reached an approximate height of 5.0 cm, they were transplanted to polyethylene bags (15 cm x 35 cm) containing substrate consisting of soil + carbonized rice husk + organic compost (2:2:1). The average temperature within the greenhouse in the experimental period was 28 ± 3 °C and the relative humidity was 60% to 80%. Irrigation was performed manually to the field capacity.

The results of the chemical and physical analyses of the substrate (Tables 1 and 2) were obtained using the methodology described by the Official Network of Soil and Plant Tissue Analysis Laboratories of RS and SC - ROLAS (Sociedade Brasileira de Ciências do Solo, 2016).

**Table 1.** Chemical characteristics of the substrate used in the production of *Cordia alliodora* seedlings

| | pH | K | P | Ca | Mg | Al | H+Al | CEC | SB | OM | Zn | Fe | Mn | Cu | B | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | --------cmol/dm³------- | | | | | | | | dag/kg | ---------------mg/dm³------------- | | | | | |
| Subst. | 6.7 | 0.31 | 0.87 | 11.0 | 0.7 | 0.0 | 1.1 | 13.31 | 12.01 | 3.5 | 16.5 | 13.5 | 88.6 | 0.3 | 0.5 | 17.2 |

Subst.: substrate consisting of soil + carbonized rice husk + organic compost (2:2:1)

**Table 2.** Physical composition of the substrates formulated for the production of *Cordia alliodora* seedlings

| Substrate | Particle size | | |
|---|---|---|---|
| | Clay | Silt | Medium Sand |
| | -------------------------dag kg⁻¹-------------------------- | | |
| | 22.99 | 11.78 | 67.23 |

Substrate consisting of soil + carbonized rice husk + organic compost (2:2:1)

Foliar applications of *Azospirillum brasilense* were carried out using a manual sprayer with capacity for 100 mL, applying 20 mL of the solution (*Azospirillum brasilense* + water) per plant, in the afternoon period from 16:30 h, according to the doses established for each treatment. For the substrate applications of *Azospirillum brasilense*, the solution was deposited in four small depressions of 5 cm on the surface, 2 cm away from the plant collar, using an automatic pipette. The experimental design was completely randomized, in a 2 x 4 factorial scheme, corresponding to two methods of application (foliar and soil) and four doses (0.0, 0.2, 0.4 and 0.6 ml $L^{-1}$), with five replicates, each of which consisted of five seedlings (one in each container).

At 90 days after transplantation (DAT), the plants were evaluated for shoot height (H), with a graduated ruler, and collar diameter (CD), with a digital caliper. Nitrogen balance index (NBI) was also determined using a chlorophyll meter (Dualex Model). Between 9 and 11 a.m., measurements were performed on two fully expanded leaves, located in the apical third of each plant. Contents of chlorophyll *a*, chlorophyll *b* and total chlorophyll were determined using a portable chlorophyll meter (Falker model) in two fully expanded leaves of the middle region of the canopy of each plant in the experimental unit. The measurements were performed in two leaf positions (opposite sides). From the four readings, the average was obtained for both sampled leaves, using the meter itself. Leaf area (LA, $m^2$ $m^2$) was obtained with a Li-Cor LI3100C leaf area meter.

Subsequently, the plants were dried in a forced air circulation oven at 70 ºC for 72 hours until reaching constant mass, for individual determination of shoot dry mass (SDM, g $plant^{-1}$), root dry mass (RDM, g $plant^{-1}$) and total dry mass (TDM, g $plant^{-1}$), obtained by summing SDM and RDM. The Dickson quality index was determined using the formula DQI=TDM/[(H/CD) + (SDM/RDM)], according to Dickson et al. (1960). The doses of maximum technical efficiency were obtained by deriving and equaling to zero the average quadratic production functions that best fitted the data (Equation 1 and 2) (Tiesdale et al., 1993).

$$y = cx^2 + bx + a \quad (1)$$

$$\frac{dy}{dx} = 2cx+b=0 \quad (2)$$

All variables were subjected to comparison of means by Tukey test, at 5% probability level, and quantitative variables were subjected to regression analysis in order to assess the growth response of the plants as a function of time. Data analysis was performed in the statistical package Sisvar (Ferreira, 2014).

## RESULTS

The analysis of variance revealed a significant effect for the interaction between the factors doses of *Azospirillum brasilense* (D) and methods of application (A) on the variables height, number of leaves, leaf area and shoot dry mass (Table 3). However, for the single factor dose of *Azospirillum brasilense* all the variables in the present study were significantly affected (Table 3).

**Table 3.** Summary of the analysis of variance for seedling height, collar diameter (CD), number of leaves, leaf area, shoot dry mass, root dry mass, total dry mass and Dickson quality index (DQI) of *Cordia alliodora* as a function of the doses of *Azospirillum brasilense* (0, 0.2, 0.4 and 0.6 g $L^{-1}$) and foliar or soil application

| Variable | Sources of variation | | | CV (%) |
|---|---|---|---|---|
| | Doses (D) (DF = 4)[1] | Application (A) (DF = 1) | D × A interaction (DF =4) | |
| Height (cm) | 4136.4916** | 483.0250$^{ns}$ | 505.0250* | 16.68 |
| Collar diameter (mm) | 6.4282** | 0.5522$^{ns}$ | 0.3149$^{ns}$ | 6.04 |
| Number of leaves | 531.4666** | 102.4000** | 192.0000** | 11.77 |
| Leaf area $m^2m^2$ | 4972699.266** | 182790.4000$^{ns}$ | 1411844.066** | 12.86 |
| Shoot dry mass (g $plant^{-1}$) | 173.0858** | 4.1990$^{ns}$ | 18.6267** | 9.80 |
| Root dry mass (g $plant^{-1}$) | 43.9122** | 13.3402* | 4.0587$^{ns}$ | 5.84 |
| Total dry mass (g $plant^{-1}$) | 389.3891** | 32.4360* | 12.1037$^{ns}$ | 17.84 |
| Dickson Quality Index | 1.5748** | 0.8323* | 0.2374$^{ns}$ | 23.25 |

**Significant and $^{ns}$ not significant at 1% probability level (p<0.01) by the F test. DF: degrees of freedom. CV: Coefficient of variation.

According to the results obtained in the present study, the increase in the doses of *Azospirillum brasilense* through foliar application (Figure 1A) caused greater height of *Cordia alliodora* seedlings. However, to obtain the maximum height (79.97 cm) of *Cordia alliodora* seedlings, the estimated dose of maximum technical efficiency (DMTE) was 0.53 ml $L^{-1}$ with foliar application (Figure 1A). In addition, the plants exhibited a 56.23% gain in height when compared to the zero dose (control).

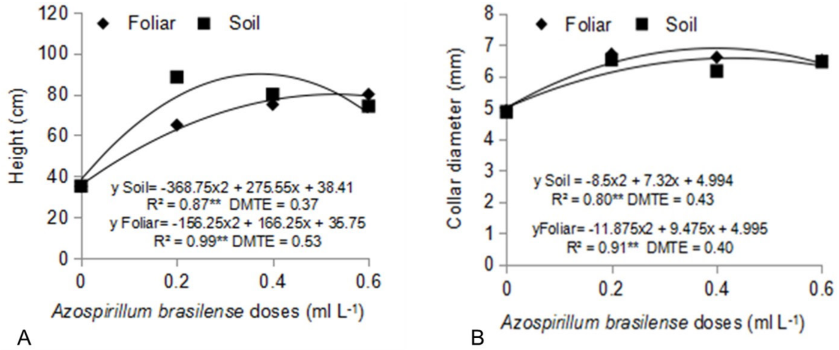


**Figure 1.** Mean values of height (A) and collar diameter (B) obtained with foliar and soil application of different doses of *Azospirillum brasilense* (0, 0.2, 0.4 and 0.6 ml $L^{-1}$) in *Cordia alliodora* seedlings at 90 days after transplanting.

However, for the number of leaves of *Cordia alliodora*, soil application of *Azospirillum brasilense* exhibited DMTE of 0.38 ml $L^{-1}$, which led to 39 leaves (Figure 2A). In turn, when foliar application was used, *Cordia alliodora* plants produced 36 leaves, with DMTE of 0.51 ml $L^{-1}$, which was sufficient to increase leaf area (LA).

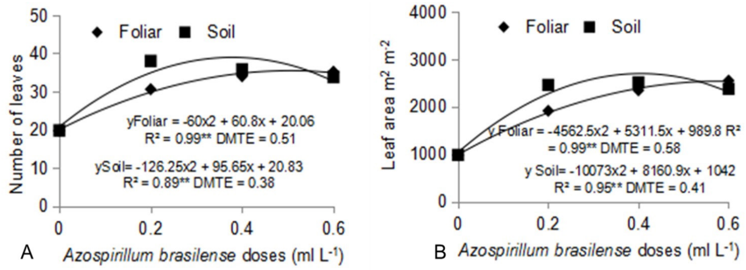


**Figure 2.** Mean values of number of leaves (A) and leaf area (B) obtained with foliar and soil application of different doses of *Azospirillum brasilense* (0, 0.2, 0.4 and 0.6 ml $L^{-1}$) in *Cordia alliodora* seedlings.

For SDM production, DMTE was equal to 0.54 ml $L^{-1}$ with foliar application and to 0.39 ml $L^{-1}$ with soil application, leading to biomass values of 12.76 g $plant^{-1}$ and 13.25 g $plant^{-1}$, respectively (Figure 3A). In addition, for RDM, the DMTE was 0.47 ml $L^{-1}$ with soil application and 0.41 ml $L^{-1}$ with foliar application, resulting in biomass values greater than 7.40 g $plant^{-1}$ for both application methods (Figure 3B).

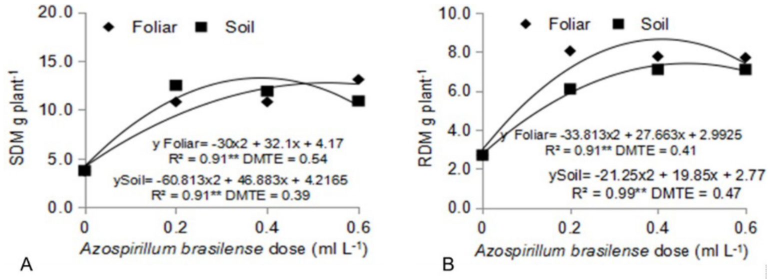


**Figure 3.** Mean values of shoot dry mass (SDM, A) and root dry mass (RDM, B) obtained with foliar and soil application of different doses of *Azospirillum brasilense* (0, 0.2, 0.4 and 0.6 ml $L^{-1}$) in *Cordia alliodora* seedlings.

Regarding the total dry mass production of *Cordia alliodora* plants (Figure 4A), there were gradual increments up to the DMTE of 0.47 ml $L^{-1}$ of the inoculant *Azospirillum brasilense* for both application methods. Thus, the supply of *Azospirillum brasilense* in adequate quantity is expected to ensure, together with micronutrients, the maintenance of the main metabolic processes that promote higher quality of these native forest seedlings.

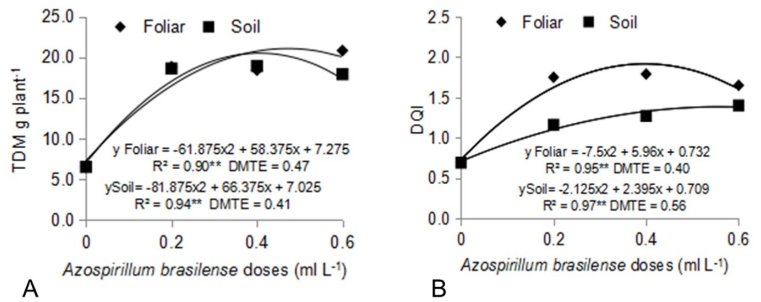


**Figure 4.** Mean values of total dry mass (TDM, A) and Dickson quality index (DQI, B) obtained with foliar and soil application of different doses of *Azospirillum brasilense* (0, 0.2, 0.4 and 0.6 ml $L^{-1}$) in *Cordia alliodora* seedlings.

According to the results, the highest DQI values obtained were 1.92 with DMTE of 0.40 ml $L^{-1}$ of *Azospirillum brasilense* in foliar application and 1.38 with DMTE of 0.56 ml $L^{-1}$ of *Azospirillum brasilense* in soil application, and both values were within the ideal range (Figure 4B).

It is worth noting that the analysis of variance for chlorophyll *a* (CHL a), chlorophyll *b* (CHL b), total chlorophyll (CHL Total) and N balance index (NBI) revealed significant effect for the interaction between the factors doses of *Azospirillum brasilense* (D) and application methods (A).

For chlorophyll *a* in the leaves of *Cordia alliodora*, foliar application with the tested doses of *Azospirillum brasilense* (0.2, 0.4 and 0.6 ml $L^{-1}$) led to higher values when compared to soil application. As presented in Table 4, *Cordia alliodora* seedlings under foliar application of *Azospirillum brasilense* at the dose of 0.4 ml $L^{-1}$ showed a 16.4% gain when compared to the control for CHL a, while for soil application of *Azospirillum brasilense* at the dose of 0.4 ml $L^{-1}$, the gain was 5.5% when compared to the zero dose.

**Table 4.** Mean values of chlorophyll *a* (CHL a, ěg/mL), chlorophyll *b* (CHL b, ěg/mL), total chlorophyll (CHL Total, ěg/mL) and N balance index (NBI), determined in leaves of *Cordia alliodora* under different doses and methods of application of *Azospirillum brasilense*

| Dose (ml $L^{-1}$) | CHL a, µg/mL | | CHL b, µg/mL | |
|---|---|---|---|---|
| | Foliar | Soil | Foliar | Soil |
| 0 | 35.21 Ab | 35.01 Ab | 8.08 Ab | 8.06 Ab |
| 0.2 | 39.93 Aa | 36.13 Bab | 10.98 Aa | 9.96 Aa |
| 0.4 | 40.98 Aa | 36.93 Ba | 10.90 Aa | 9.93 Aa |
| 0.6 | 36.98 Aab | 36.96 Ba | 10.99 Aa | 9.98 Aa |
| CV% | 9.03 | 10.3 | 8.99 | 8.66 |
| | CHL Total, µg/mL | | NBI | |
| | Foliar | Soil | Foliar | Soil |
| 0 | 43.29Ab | 43.07Ab | 25.82 Ab | 25.02Ab |
| 0.2 | 50.91Aa | 46.09Ba | 34.31 Aa | 31.02Ba |
| 0.4 | 51.88Aa | 46.86Ba | 35.03 Aa | 30.78Ba |
| 0.6 | 47.97Aab | 46.94Ba | 34.53Aa | 28.87Bab |
| CV% | 10.23 | 9.76 | 10.34 | 10.43 |

[1] Uppercase letters (A,B) compare the means for the variables between the methods of application of *Azospirillum brasilense*. Lowercase letters (a,b) compare the means for the variables between the doses of the *Azospirillum brasilense*, by Tukey test at 5% probability level.

It is worth pointing out that *Cordia alliodora* plants that received different doses of *Azospirillum brasilense* in both application methods showed higher mean values of chlorophyll *a*, chlorophyll *b* and total chlorophyll when compared to the control (Table 4), since the inoculation with *Azospirillum brasilense* bacteria favors the biosynthesis of chlorophyll molecules.

## DISCUSSION

For the height of *Cordia alliodora* seedlings with soil application of *Azospirillum brasilense*, DMTE was 0.37 ml $L^{-1}$, leading to an average height of 89.88 cm (Figure 1A), a 61.06% gain compared to the zero dose. It is extremely important to evaluate the practicality and benefits regarding the best method of application and doses of *Azospirillum brasilense* to be used, since both methods used in this study led to satisfactory results in plant height.

Smiderle et al. (2022), investigating the effect of doses of *Ascophyllum nodosum* on the growth of *Hymenaea courbaril*, established DMTE of 0.54 ml $L^{-1}$ for the highest gain in plant height with foliar application.

In general, the collar diameter (6.57 mm) of *Cordia alliodora* seedlings with soil application exhibited a DMTE of 0.43 ml $L^{-1}$ of *Azospirillum brasilense* (Figure 1B), while for foliar application the DMTE was 0.40 ml $L^{-1}$, with an average diameter of 6.89 mm (Figure 1B), an average increment of 28.8% in collar diameter when compared to the control treatment. Similar results were obtained by Smiderle & Souza (2022) in *Hymenaea coubaril* seedlings with doses of *Ascophyllum nodosum* seaweed extract and Stimulate®.

After the plants invest in growth and development, they tend to allocate a large amount of energy to the number of leaves and leaf area expansion, in order to maximize light capture and energy production (Praet et al., 2020)

Regarding leaf area (LA), the DMTE was 0.41 ml $L^{-1}$ for soil application of *Azospirillum brasilense* and 0.58 ml $L^{-1}$ for foliar application, with estimated LA of 2695 and 2536 $m^2m^2$, respectively. According to Smiderle et al. (2022), in tropical environments, the optimum LA is between 2000 and 6000 $m^2m^2$ for most native forest species of Roraima.

This suggests that the response of *Cordia alliodora* seedlings to the application of *Azospirillum brasilense* is due not only to the fixed $N_2$, but also to the higher efficiency of absorption of the mineral nitrogen (N) available in the substrate (Table 1). In addition, substances based on phytohormones, such as auxins and cytokinins, can be released by *Azospirillum brasilense* and later improve the efficiency of N use by plants, consequently leading to greater gain in biomass production (Smiderle et al., 2022; Souza et al., 2020). This was observed in the present study with the variables shoot dry mass (SDM) and root dry mass (RDM) of *Cordia alliodora* for both application methods.

According to Souza et al. (2020), the Dickson quality index (DQI) is a good indicator of seedling quality, as it considers for its calculation the sturdiness quotient and the balance of biomass distribution among the organs, both considered important parameters for reliable recommendation of seedling quality. For Souza et al. (2018) and Smiderle et al. (2020a), the value considered ideal for DQI in native forest seedlings of Roraima is approximately 1.00.

In turn, the zero dose led to DQI of 0.67, which is below the value considered ideal by the aforementioned authors.

Foliar application of *Azospirillum brasilense* at the tested doses had a positive effect on *Cordia alliodora* seedlings, as can be verified in the N balance index (NBI), which was higher for the doses applied than for the control (Table 4), in order to meet the demand for the synthesis of photo-assimilates, amino acids and proteins, promoting higher concentrations of chlorophylls, a determinant physiological parameter for plant growth.

Among the mineral nutrients, N has been considered one of the main limiting factors for photosynthesis, since it is part of the main constituents of the photosynthetic system, such as chlorophylls and proteins, including the RuBisCO enzyme (catalyst of the photosynthetic reduction of $CO_2$) (Katiyar et al., 2021).

It is known that, for photosynthesis, higher plants depend on the absorption of light and significant presence of chlorophylls *a* and *b* and carotenoids in the leaves to direct the metabolism of carbohydrates in chloroplast and cytosol through the chemical forms ATP and NADPH (Menegatti et al., 2022). Chlorophylls are related to the photosynthetic efficiency of plants and, consequently, to their growth and adaptability to different cultivation conditions (Smiderle et al., 2020b; Leal et al., 2020).

In this context, it can be inferred that the use of bacteria for biological nitrogen fixation in *Cordia alliodora* seedlings was efficient in terms of both biomass production and plant nutrition, with significant increments in their physiological and morphological quality, since *Azospirillum brasilense* bacteria have been associated with several mechanisms, and in addition to being asymbiotic nitrogen fixers, they are also considered plant growth-promoting rhizobacteria (Galindo et al., 2022; Shukla et al., 2019).

## CONCLUSIONS

Foliar application of *Azospirillum brasilense* at the dose of maximum technical efficiency of 0.40 ml $L^{-1}$ promotes greater increase in collar diameter in *Cordia alliodora* seedlings.

Soil application at the dose of maximum technical efficiency of 0.41 ml $L^{-1}$ leads to larger leaf area in *Cordia alliodora* seedlings.

The dose of 0.40 ml $L^{-1}$ with foliar application of *Azospirillum brasilense* is recommended to obtain *Cordia alliodora* seedlings with better quality and sturdiness and shorter time of permanence in the nursery.

Application of *Azospirillum brasilense* at the appropriate doses through both leaves and soil promotes increments in all physiological parameters studied in *Cordia alliodora* seedlings at 90 days after transplantation.

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