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PLANT GROWTH-PROMOTING BACTERIA ASSOCIATED WITH NITROGEN FERTILIZATION IN *Eucalyptus urophylla* INCREASE GROWTH

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ABSTRACT

The objective of this study was to evaluate plant growth-promoting bacteria in eucalyptus, associated with different levels of ammonium sulfate, to know the benefits to initial plant growth, determining which nitrogen level and bacterial isolate is the most effective. Five bacterial isolates and one control without inoculation were used, besides 4 levels of ammonium sulfate, 0, 20, 40 and 60 mg dm^{-3} N, in a factorial scheme and randomized block design, set in a 20-L pot, with *Eucalyptus urophylla* clone AEC144. Height, diameter, dry matter, leaf area, Spad index, Ntotal and photosynthesis were evaluated. The isolates UESBJMR32E and UESBJNR32E promote initial growth in eucalyptus plants associated with intermediate doses of ammonium sulfate.

KEYWORDS: *Eucalyptus*; rhizobacteria; ammonium sulfate.

BACTÉRIAS PROMOTORAS DE CRESCIMENTO VEGETAL ASSOCIADAS À ADUBAÇÃO NITROGENADA EM *Eucalyptus urophylla* INCREMENTAM O CRESCIMENTO

RESUMO

Objetivou-se com esse trabalho estudar bactérias promotoras de crescimento vegetal em eucalipto, associado a diferentes níveis de sulfato de amônio, para conhecer os benefícios ao crescimento inicial das plantas, determinando qual nível de nitrogênio e isolado bacteriano é mais eficaz. Foram utilizados 5 isolados bacterianos e um controle sem inoculação, e 4 níveis de sulfato de amônio 0, 20, 40 e 60 mg dm-3 N, em esquema fatorial e delineamento em blocos casualizado, montado em vaso de 20L, com o clone AEC144 de *Eucalyptus urophylla*. Foram avaliados altura, diâmetro, massa seca, área foliar, índice Spad, Ntotal e fotossíntese. Os isolados UESBJMR32E e UESBJNR32E, promovem o crescimento inicial em plantas de eucalipto associados as doses intermediárias de sulfato de amônio.

PALAVRAS-CHAVE: *Eucalyptus;* rizobactérias; sulfato de amônio.

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1. INTRODUCTION

The sustainable and ecologically viable crop development has become increasingly important and necessary, especially with a view to reducing the use of chemical products, which are used to favor the development and control of diseases and pests, but causes major problems in the environment.

The eucalyptus planted area in Brazil is 6.97 million hectares, with 590 thousand hectares belonging to the state of Bahia (Ibá, 2020). Due to its wide use as a raw material (Gomide et al. 2010), various production techniques such as the use of plant regulators, beneficial microorganisms, different cultural treatments are tested to improve seedling initial growth, aiming to achieve a quality standard and increase yield.

Among the tools used are plant growth-promoting bacteria which, due to their mutualism relationship with the host plant, favor growth through nutrient availability (Abadi and Sepehri 2015), biological nitrogen fixation (Hungria, 2011; Klein et al. 2012), phosphate solubilization (Bolle et al. 2013), production of hormones such as auxins, gibberellins and cytokines (Cassán et al. 2009; Dartora et al. 2013; Rodrigues et al. 2014), or act indirectly through biological control of pathogens.

Nitrogen is a very important nutrient in yield, required in high quantities due to its functions as a structural component of macromolecules, enzymes, synthesis of chlorophylls, amino acids, proteins, vitamins, cytochromes, nucleic acids and hormones (Floss, 2011). In nature, lighting, photochemical reactions, biological nitrogen fixation and soil organic matter degradation supply most nitrogen to plants (Taiz and Zeiger, 2017).

Growth-promoting bacteria are quite efficient in biological nitrogen fixation (Baldani and Baldani, 2005; Butke and Leite, 2020), nutrient availability for crop, its use may represent a great strategy to reduce dependence on synthetic nitrogen fertilizers (Conceição et al. 2009). The use of these microorganisms is an important advantage, since the interaction eukaryote x prokaryote does not represent a source of environmental contamination, and can partially supply the nitrogen (N) requirements by several crops, thus reducing the use of nitrogen fertilizers, with cost reduction for the producer (Moreira et al. 2010) .

Given the above, this study aimed to evaluate the use of plant growth-promoting bacteria in eucalyptus plants, associated with different levels of ammonium sulfate.

2. MATERIAL AND METHODS

2.1 Place of study development and material used

This study was conducted at Universidade Estadual Paulista - UNESP - FCAV, campus Jaboticabal - SP, from June to September 2017, in pot, in open field. *Eucalyptus urophylla* clone

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AEC144 seedlings with 100 days of age were used, being standardized for height, pair of leaves and collar diameter.

2.2 Bacterial isolates and fertilization levels

Five bacterial isolates and the control without inoculation were used; three isolates were from the isolation of *Eucalyptus urophylla* clone AEC144 seedlings, previously held at the State Universidade Estadual do Sudoeste da Bahia- UESB, Vitória da Conquista campus, and 2 isolates from the collection of Embrapa Agrobiologia, Seropédica-RJ. Four levels of ammonium sulfate were also used, and a 6 x 4 factorial was organized, which consisted of B1, control without inoculation; B2, isolate ZAE94- Embrapa; B3, isolate AM82- Embrapa; B4, isolate UESBJMR5E-Uesb; B5, isolate UESBJNR32E-Uesb; B6, isolate UESBJMR32E-Uesb X 0 mg dm^3 N; 20 mg dm^3 N; 40 mg dm^3 N and 60 mg dm^3 N ammonium sulfate, totaling 24 treatments, in a randomized block design with 3 replications, making 72 plots.

2.3 Isolate growth and experiment installation

Bacterial isolates were grown in liquid Dygs medium and inoculated into seedlings, according to their treatments, with 3 mL of bacterial solution placed directly in contact with the root before planting; all isolates were standardized with a population of 10^9 ufc.

Subsequently, the seedlings were planted in 20-L pots, with soil classified as clayey red eutroferric latosol. The sample presented the following chemical characteristics: pH in $CaCl_2$ 5.4; M.O 7 g dm^{-3}; P 10 mg dm^{-3}; S 16 mg dm^{-3}; Ca 17 mg dm^{-3}; Mg 4 $mmol_c$ dm^{-3}; K 0,6 $mmol_c$ dm^{-3}; Al 0 $mmol_c$ dm^{-3}, and had its fertility correction performed according to the Gonçalves (2005) and Sá et al. (2014).

The soil was sieved and homogenized with the fertilizers before planting, limestone was applied to increase base saturation to 60%, simple superphosphate was applied at 100 mg dm^{-3} P, KCl at 200 mg dm^{-3} K; this fertilizer was split, with the first dose at planting and the second at 50 days in the coverage with micronutrients. Ammonium sulfate application was performed after planting and bacterial inoculation.

The fertilizer was applied in solution, with 500 mL of water for each plant to avoid its loss. The plants were irrigated on alternate days during the first 40 days of the experiment and, subsequently, irrigation was performed daily up to 90 days, according to pot capacity (Casaroli and Lier, 2008) and plant growth.

2.4 Plant growth assessment

The evaluations started at 87 days, with the measurement of gas exchange, using an Infrared Gas Analyzer (IRGA LI-6400, LI-COR®, Nebraska/USA), and net photosynthesis was evaluated. Therefore, a leaf was used in the middle third of each plant. Readings were taken from 08.30 to 11.00 a.m. At the end of the experiment, at 90 days, the following characteristics were evaluated: Plant height (cm), taking the apex bud as a standard, measured with a graduated ruler;

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Stem diameter (mm), measured with a digital caliper; Spad index, using a CCM/200 Opti-scienses chlorophyll meter.

The evaluation was performed in three fully expanded and physiologically mature leaves, located in the middle portion of the crown. For each plant, the SPAD index was considered as resulting from the arithmetic mean of these three leaves; Leaf area, with an LI-310 LI-COR leaf area meter; Shoot dry weight, where the material was stored in paper packages and dried in an oven with forced air circulation, at 65 ºC for 72 h; weight was verified using a precision scale of 0.001 mg; N total, conducted at the Animal Nutrition Laboratory of Universidade Estadual do Sudoeste da Bahia, by the Semi-micro-Kjeldahl method, after the leaves were dried in a greenhouse and subsequently ground, according to the methodology proposed by Malavolta et al. (1997).

2.5 Statistical analysis

The results were submitted to normality (Lilliefors test) and homogeneity (Bartlett test) data analyses, as recommended by Banzatto and Kronka (2013). The analysis of variance (ANOVA) was performed by the statistical software SISVAR (Ferreira, 2011) and, when the F test was significant, the means were submitted to the Scott Knott test at 5 %.

Regression analysis was performed for the quantitative parameter, and second order linear and polynomial models were tested, taking into account the significance of the parameter, biological adjustment of the data and the coefficient of determination.

3. RESULTS

Height, diameter, leaf area, shoot dry matter, SPAD index, photosynthesis and total nitrogen were significant for the interaction of factors (bacterial isolate X ammonium sulfate doses). The doses were then split within each isolate (Table 1).

Table 1: Analysis of variance in relation to the evaluations of height (H), diameter (DIA), leaf area (LA), shoot dry matter (SDM), SPAD index (SPAD), photosynthesis (PHOT) and total nitrogen (TOTN) of *Eucalyptus urophylla* clone AEC144, under inoculation of bacterial isolates and nitrogen fertilization.

SV	DOF	Mean square						
		H	DIA	LA	SDM	SPAD	PHOT	TOTN
ISOLATE	5	183.46*	0.72ns	152165.82*	610.00*	45.83*	56.19*	23.00*
DOSE	3	59.64ns	0.37ns	1830381.59*	202.57*	76.08*	34.94*	61.39*
I x D	15	119.23*	5.43*	2336025.99*	598.99*	29.37*	10.47*	7.70*
BLOCK	2	108.66	3.52	102515.84	195.22	19.47	8.22	0.46
ERROR	46	23.55	1.38	103767.54	45.78	14.7	2.68	1.16

(1) * significant effect at 5% probability, and ns = not significant;

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Evaluating the action of bacteria in the absence of nitrogen fertilization, it was observed that the isolate UESBJMR5E was superior to the others in the evaluations of height, leaf area and shoot dry matter, with values of 56.83cm; 2171.75 cm^2 and 49.88 g, respectively. For diameter and SPAD index, there was no distinction of the action of isolates. Photosynthesis and TOTN were able to differ as superior in relation to isolate UESBJNR32E, with values up to 55.39%, higher than the control without inoculation (Table 2).

Table 2: Height (H), diameter (DIA), leaf area (LA), shoot dry matter (SDM), SPAD index (SPAD), photosynthesis (PHOT) and total nitrogen (TOTN) of *Eucalyptus urophylla* clone AEC144, under inoculation of bacterial isolates.

Treatments	H	DIA	LA	SDM	SPAD	PHOT	TOTN
	cm	mm	cm^2	g		µmol Co_2 m^{-2} s^{-1}	mg kg^{-1}
Control	49.33C	7.6A	1530.66C	35.75C	32.14A	10.29D	8.44B
ZAE 94	48.16C	7.67A	1801.83B	42.75B	29.46A	12.10C	6.42C
AM 82	45.41C	7.86A	1171.41D	30.49C	29.45A	10.93D	6.47C
UESBJMR5E	56.83A	8.1A	2171.75A	49.88A	29.15A	14.40B	6.98C
UESBJNR32E	49.50C	7.52A	1320.91D	33.04C	26.22A	15.99A	9.92A
UESBJMR32E	52.25B	8.00A	1599.08C	40.87B	27.97A	13.44B	6.91C
FV (%)	9.66	15.07	20.14	17.44	16.41	12.74	14.35

* Means followed by the same letter in the column do not differ by the Scott Knott test at 5% significance.

After studying the mean bacterial isolates, the trend of the regression data was studied (Table 3), taking into account the biological phenomenon after inoculation associated with nitrogen fertilization, the select models were those with significance on the coefficients of the parameters of highest degree by t test ($p<0.05$), concomitantly with largest coefficient of determination (R2).

Height is shown in Figure 1A. The means of isolate UESBJMR32E presented a quadratic behavior, with a maximum point of 61.37 cm at the level of 26.20 mg dm^{-3} N, and the means of isolate UESBJMR5E showed a linearly increasing behavior with 62.66 cm height at the level of 60 mg dm^{-3} N.

Figure 1B demonstrates the quadratic behavior for the native isolate UESBJMR32E and the increasing linear behavior of the native isolate UESBJMR5E for collar diameter. The means for isolate UESBJMR32E have a maximum point of 9.73 cm at a dose of 27.42 mg dm^{-3} N. The means for isolate UESBJMR5RE have a maximum value of 9.52 cm at a dose of 60 mg dm^{-3} N This demonstrates, in both parameters, the efficiency of the native isolate UESBJMR32E in the presence of a lower nitrogen level, yet activating plant growth.

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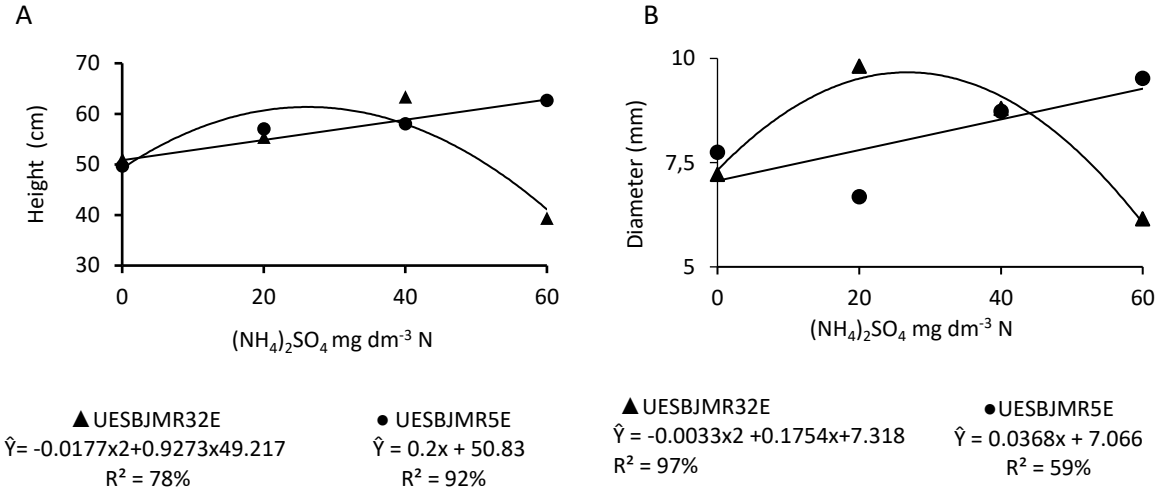
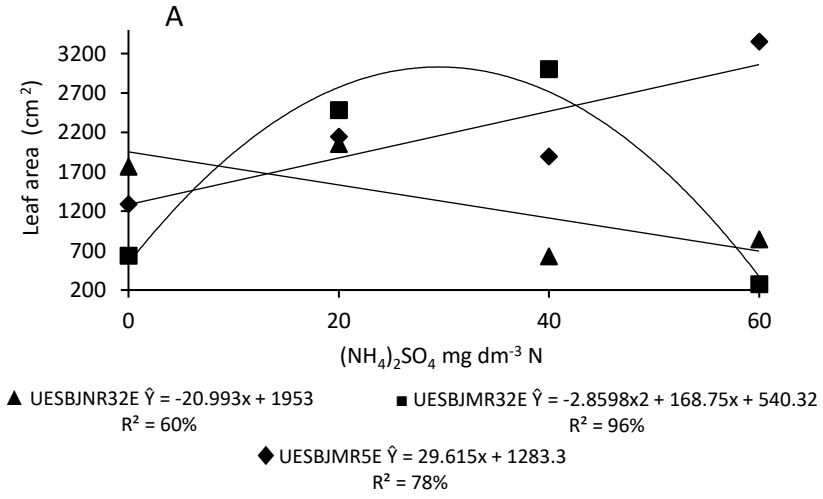


Figure 1: Height (A) and diameter (B) of *Eucalyptus urophylla* clone AEC144, inoculated with bacterial isolates UESBJMR32E and UESBJMR5E, associated with different ammonium sulfate levels.

The trend of data for leaf area is shown in Figure 2A. The isolate UESBJMR5E showed an increasing linear behavior with a value of 3060.20 cm^2 at the level of 60 mg dm^{-3} N; the isolate UESBJNR32E showed a decreasing linear behavior with a value of 1954.76 cm^2 at the level 0 mg dm^{-3} N, and the means of isolate UESBJMR32E showed a quadratic behavior with a maximum point of 3029.76 cm^2 at the level of 29.50 mg dm^{-3} N.



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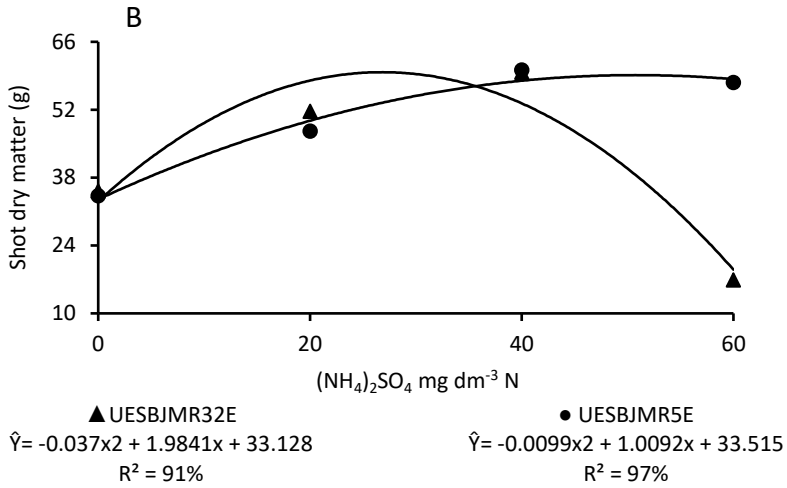
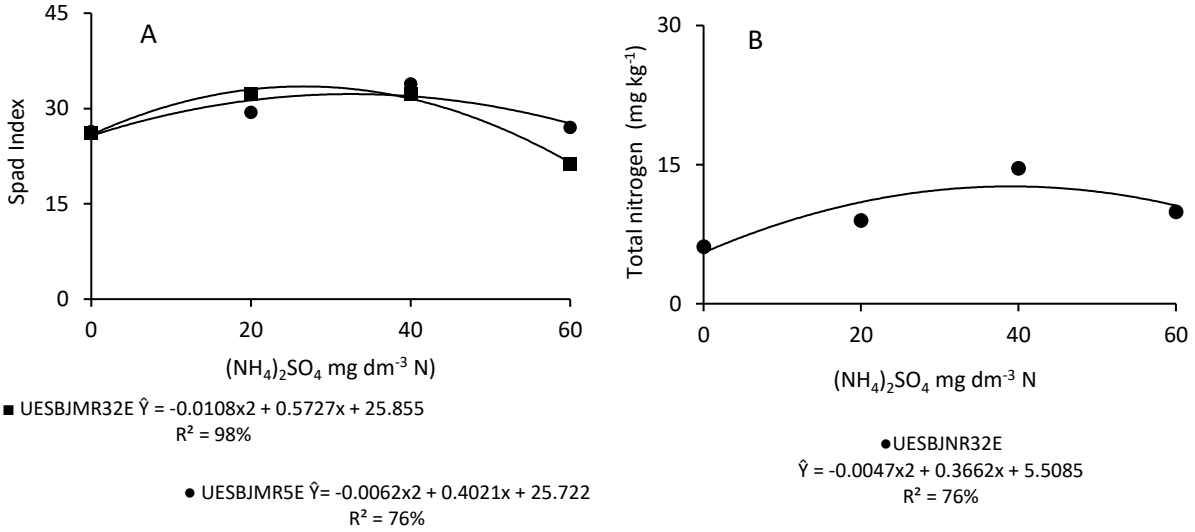


Figure 2: Leaf area (A) inoculated with isolates UESBJNR32E, UESBJMR32E and UESBJMR5E; shoot dry matter (B) inoculated with isolates UESBJMR32E and UESBJMR5E, associated with different ammonium sulfate levels.

The means of isolates UESBJMR32E and UESBJMR5E and dry matter showed a quadratic behavior (Figure 2B): for the first isolate, the maximum point was 59.73g at the level 26.84 mg dm^{-3} N and, for the second isolate, the point was obtained at 59.24g at the level 50.96 mg dm^{-3} N, with the superiority of isolate UESBJMR32E.

The SPAD index (Figure 3A) presented a quadratic data behavior after inoculation with UESBJMR5E and UESBJMR32E bacteria, with values of 32.42 mg dm^{-3} N and index of 32.23 and 26.51 mg dm^{-3} N, with index of 33.44, respectively.



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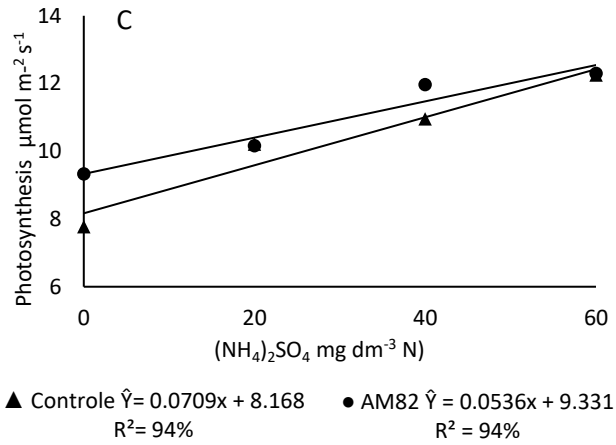

Figure 3: SPAD index (A) inoculated with isolates UESBJMR5E and UESBJMR32E; total nitrogen (B) inoculated with isolate UESBJNR32E, and photosynthesis (C) inoculated with bacteria AM82 and with control treatment, associated with different ammonium sulfate levels.

The trend of data for TOTN showed a quadratic response to isolate UESBJNR32E (Figure 3B), a maximum point of 12.78 mg kg^{-1} N at the level of 39.75 mg dm^{-3} N, respectively.

Photosynthesis values adjusted to the growing linear model for the control treatment and bacteria AM82, with maximum values of 12.30 and 12.25 µmol Co_2 m^{-2} s^{-1}, respectively (Figure 3C).

4. DISCUSSION

The isolates UESBJMR5E, UESBJN32R and UESBJMR32E, which promoted growth in eucalyptus plants, were previously characterized as auxin producers (66.63; 65.13 and 64 µg mL^{-1} IAA), were responsive to biological nitrogen fixation, and assimilated morphologically to bacteria of the genus *Azospirillum*, being characterized as plant growth promoters.

Isawa et al. (2010) and Bao et al., (2013) used *Azospirillium* strains as liquid inoculants in field and pot, and observed shoot growth and positive response to auxin production and biological nitrogen fixation.

Therefore, the response obtained for height and diameter is explained by the combination of factors: nitrogen provides the synthesis of RNA and DNA, which favors the production of new tissues, and plant growth-promoting bacteria have the ability to produce hormones, especially auxins, gibberellins and cytokines, and auxin is responsible for cell elongation and differentiation, leading to stem cell elongation in plants, being synthesized mainly in stem apical meristems (Taiz and Zeiger, 2017).

The results obtained for plant height and diameter underscore the need for an initial nitrogen supply for the plant to develop, begin to release exudates and, consequently, the bacteria start symbiosis, then resulting in a positive response.

Leaf area expansion can be explained as a response to plant growth caused by bacteria and their cytokine production. According to Montoaldo (2016), cytokinins are synthesized in roots and young tissues, as well as by microorganisms associated with plants. According to Taiz and Zeiger (2017), many bacteria produce the types of cytokine: trans-zeatin, cis-zeatin and their tissue

division-inducing ribosides; all of these structures present nitrogen in their composition, which again highlights the benefit of this association with bacteria and their nutrient availability to the plant, resulting in the conduction of various metabolic pathways.

In general, the SPAD index grows linearly with the use of nitrogen fertilization, since it quantifies the intensity of the green color of the leaves that correlates with the nitrogen content in the tissue. In this study, the maximum response was 32.42 mg dm^{-3} N, which is equivalent to the other characteristics that provided optimal values at intermediate levels of 25 to 40 mg dm^{-3} N.

The values were expected since, for eucalyptus in soil with organic matter (OM) of 7 g dm^{-3} equal to the experiment, the ideal dose of N is 60kg ha^{-1} (Gonçalves, 2005); if the maximum point were at higher levels, it would probably cause excess nitrogen responses, altering the transport of produced carbohydrates, interfering with plant development.

The response to nitrogen accumulation in the plant by bacteria UESBJNR32E is due to the fact that its positive response to biological nitrogen fixation is also similar to the genus *Herbaspirillum* spp., which is able to colonize specific niches within plant tissues (James et al. 2002; RoncatoMaccari et al. 2003), and can transfer nitrogen compounds more efficiently to plants, not suffering limitations in carbon-rich sources (Gyaneshwar et al. 2002; Reddy et al. 2002).

Studies conducted with different eucalyptus species, *Eucalyptus globulus, Eucalyptus regnans* and *Eucalyptus nitens* have recorded greater N uptake when it is preferentially absorbed as NH^{4+} (Warren and Adams, 2007; Pfautsch et al. 2009). This response occurs once, according to ; Hachiya et al. (2012), absorption in the form of N-NH_4^+ requires less energy, it eliminates the reduction phases, which are required when N-NO_3^- absorption occurs and, in ammoniacal forms, nitrogen already enters the carbon skeletons; thus, the type of fertilizer used responds to the effect of nitrogen content in this study.

However, the response to N- NH_4^+ absorption should be at lower doses, as observed in the study with the isolate UESBJNR32E, since ammonium has high rhizosphere acidifying power, which interferes with the acquisition of some nutrients, and must be immediately metabolized due to its toxic effect when accumulated in plants (Prado, 2008). Furthermore, rhizosphere acidification can cause a reduction in the beneficial bacterial population that needs optimal conditions, as well as the plant for its survival.

Photosynthesis values at the highest nitrogen level were expected, since photosynthesis is governed by the action of the enzyme Rubisco, which represents 50% of the total soluble proteins present in the leaf (Prado, 2008). Photosynthesis is known to be determined, among other factors, by the amount of tissue nitrogen (Mendes et al. 2013). Therefore, the greater the amount of nitrogen available to the plant, the greater will be the production of enzymes and proteins that will assist in this and several other plant processes.

Bacterial isolates UESBJNR32E, UESBJMR32E and UESBJMR5E showed the best results in all characteristics. These are isolates like the genera *Herbaspirillum* spp. and *Burkholderia spp.*, respectively which, according to Baldani et al. (2002), are endophytic bacteria.

In an endophytic and protected environment, bacteria develop better, due to their lower competition with other isolates and greater efficiency in the transfer of compounds essential to plant development (Dobbelaere et al. 2003). As reported by LATA et al. (2018), endophytic

microorganisms facilitate plant growth by increasing nutrient uptake, which leads to changes in root morphology, changes in nitrogen storage and metabolism, increased water use efficiency and decreased environmental stress.

Another very important factor that may have optimized the results is that these bacterial isolates are native, previously isolated from *Eucalyptus urophylla* clone AEC 144, the same species and clone used in this study, thus favoring the mutualism process and the specificity of the bacteria with the plant.

According to Moreira et al. (2013); Coelho et al. (2007) the ability of bacteria to colonize tissues is mainly linked to the interaction of plant genotype and microorganism releasing exudates, which may be related to the natural coexistence of the host plant/bacteria or to the metabolic sharing between host plants and bacteria (Monteiro et al. 2012).

The response of plants to growth-promoting bacteria depends on intrinsic factors to plants in the environment. In this study, native bacteria positively affect plants when associated with nitrogen fertilization, and the expressive early growth of plants is due to different functions of isolates, including auxin production and biological nitrogen fixation.

5. CONCLUSIONS

Native bacteria in Eucalyptus, UESBJMR32E; UESBJNR32E and UESBJMR5E, promote plant growth when associated with 25 to 40 mg dm^{-3} N as ammonium sulfate.

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