THIAGO DE ALMEIDA PAULA

BIOLOGICAL NITROGEN FIXATION IN SORGHUM: A BASIS FOR REDUCTION OF SYNTHETIC NITROGEN DEPENDENCE

Thesis submitted to the Plant Sciences Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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"They swallow God without thinking, they swallow the country without thinking. Soon they forget how to think, they let others think for them … Play them the great music of the centuries and they can't hear it". (Charles Bukowski)

ABSTRACT

PAULA, Thiago de Almeida, D.Sc., Universidade Federal de Viçosa, September, 2022. **Biological Nitrogen Fixation in sorghum: basis for reducing of synthetic nitrogen dependence.** Adviser: Leonardo Duarte Pimentel. Co-advisers: Maria Catarina Megumi Kasuya and Marliane de Cássia Soares da Silva.

The increasing population and its demands require intensifying the current agricultural model to boost productivity. Chemical fertilizers, especially nitrogen fertilizers, play a crucial role in improving agriculture in Brazil, making it a major importer. However, excessive use of chemical fertilizers raises production costs, causes environmental damage, and impacts soil microbial ecology and dynamics, which could pose long-term problems as plant-microbe interactions are considered a solution for sustainable agriculture. Hence, the agricultural model must be reassessed to reduce the demand for synthetic fertilizers and increase the use of microorganisms that enhance production sustainability. The study aimed to examine the impact of nitrogen fertilization on the diazotrophic community associated with sorghum plants and evaluate the feasibility of using pre-selected bacteria in combination with different levels of nitrogen fertilization to improve sorghum productivity and reduce chemical N fertilization dependence. The study consisted of several experiments, including: isolation of N-fixing and plant growth-promoting bacteria, selection of bacteria with biotechnological potential, evaluation of the impact of nitrogen fertilization on diazotrophic microbiota during sorghum sowing, testing the agronomic efficiency of pre-selected bacteria under greenhouse conditions, and evaluating the agronomic efficiency of pre-selected microorganisms in field conditions. The results of the study showed that different levels of nitrogen fertilization affect the composition of the diazotrophic community associated with various parts of the sorghum plant, reducing its richness, uniformity, and diversity. Inoculation with selected plant-growth promoting bacteria (*Rhizobium* sp. and *Sphingomonas* sp.) in combination with reduced levels of nitrogen fertilization significantly improved stem diameter, shoot and total dry mass, and nitrogen content of the shoot under greenhouse conditions. In field conditions, inoculation with a mixture of all tested bacteria (*Rhizobium* sp. and *Sphingomonas* sp.) showed the potential to increase forage sorghum productivity and reduce nitrogen fertilization by 25-50 %. These results indicate the potential of using these microorganisms as a biotechnological tool for sustainable agriculture. The study concluded that nitrogen fertilization impacts the diazotrophic community of sorghum plants and that the N-fixing and growth-promoting bacteria selected from the sorghum rhizosphere as seed inoculants have the potential to reduce the need for synthetic nitrogen fertilizers in sorghum production. Further research is needed to determine the optimal balance between inoculants and fertilization rates for an integrated fertilization model.

Keywords: *Sorghum*. Biological Nitrogen Fixation. *Rhizobium*. *Sphingomonas*.

RESUMO

PAULA, Thiago de Almeida, D.Sc., Universidade Federal de Viçosa, setembro de 2022. **Fixação Biológica de Nitrogênio em sorgo: bases para redução da dependência de nitrogênio sintético.** Orientador: Leonardo Duarte Pimentel. Coorientadores: Maria Catarina Megumi Kasuya e Marliane de Cássia Soares da Silva.

O aumento da população e suas demandas exigem a intensificação do atual modelo agrícola para aumentar a produtividade. Os fertilizantes químicos, especialmente os fertilizantes nitrogenados, desempenham um papel crucial na melhoria da agricultura no Brasil, tornando-o um grande importador. No entanto, o uso excessivo de fertilizantes químicos aumenta os custos de produção, causa danos ambientais e afeta a ecologia e a dinâmica microbiana do solo, o que pode representar problemas de longo prazo, pois as interações planta-micróbio são consideradas uma solução para a agricultura sustentável. Assim, o modelo agrícola deve ser reavaliado para reduzir a demanda por fertilizantes sintéticos e aumentar o uso de microrganismos que melhorem a sustentabilidade da produção. O estudo teve como objetivo examinar o impacto da adubação nitrogenada na comunidade diazotrófica associada às plantas de sorgo e avaliar a viabilidade do uso de bactérias pré-selecionadas em combinação com diferentes níveis de adubação nitrogenada para melhorar a produtividade do sorgo e reduzir a dependência da adubação nitrogenada. O estudo consistiu em vários experimentos, incluindo: isolamento de bactérias fixadoras de N e promotoras de crescimento vegetal, seleção de bactérias com potencial biotecnológico, avaliação do impacto da adubação nitrogenada na microbiota diazotrófica durante a semeadura do sorgo, teste da eficiência agronômica de pré-selecionados bactérias em condições de casa de vegetação e avaliando a eficiência agronômica de microrganismos pré-selecionados em condições de campo. Os resultados do estudo mostraram que diferentes níveis de adubação nitrogenada afetam a composição da comunidade diazotrófica associada a várias partes da planta de sorgo, reduzindo sua riqueza, uniformidade e diversidade. A inoculação com bactérias promotoras de crescimento de plantas selecionadas (*Rhizobium* sp. e *Sphingomonas* sp.) em combinação com níveis reduzidos de fertilização com nitrogênio melhorou significativamente o diâmetro do caule, parte aérea e massa seca total, e teor de nitrogênio da parte aérea em condições de casa de vegetação. Em condições de campo, a inoculação com uma mistura de todas as bactérias testadas (Rhizobium sp. e Sphingomonas sp.) mostrou potencial para aumentar a produtividade do sorgo forrageiro e reduzir a adubação nitrogenada em 25-50 %. Esses resultados indicam o potencial do uso desses microrganismos como ferramenta biotecnológica para uma agricultura sustentável. O estudo concluiu que a fertilização com nitrogênio afeta a comunidade diazotrófica das plantas de sorgo e que as bactérias fixadoras de N e promotoras do crescimento selecionadas da rizosfera do sorgo como inoculantes de sementes têm o potencial de reduzir a necessidade de fertilizantes nitrogenados sintéticos na produção de sorgo. Mais pesquisas são necessárias para determinar o equilíbrio ideal entre inoculantes e taxas de fertilização para um modelo de fertilização integrado.

Palavras-Chave: Sorgo. Fixação Biológica de Nitrogênio. *Rhizobium*. *Sphingomonas*.

SUMMARY

INTRODUCTION

By 2021, Brazil had imported 39,201,535.00 tons of fertilizers to meet its demand (ANDA, 2022; SAE, 2022). With the growing population, the demand for food, fiber, and energy is also increasing (United Nations, 2019), which results in an increased demand for fertilizers in the agricultural sector. Despite the recent increase in price and scarcity of fertilizers in the agricultural sector, the use of chemical fertilizers remains a key factor in achieving better agricultural productivity.

This reliance of Brazilian agriculture on the foreign market, coupled with the fertilizer crisis, is likely to have a negative impact on upcoming harvests, leading to lower production, food shortages in the domestic market, and a rise in prices affecting both producers and consumers. Thus, technological advancements that reduce the reliance on chemical fertilizers in crop cultivation are of utmost importance.

To address this issue, the Brazilian Federal Government launched the National Bioinputs Program in 2020 and the National Fertilizer Plan 2050 to promote sustainable agriculture development (MAPA, 2020; SAE, 2022). Thus, one potential solution to this challenge may be found in the microorganisms present in agricultural soils that form beneficial relationships with plants (Qiu et al., 2019). Microorganisms used in agriculture are considered an alternative for improving crop performance, reducing costs, and promoting sustainability (Qiu et al., 2019). However, excessive use of chemical fertilizers can negatively impact the ecology and dynamics of microbial communities in agricultural soils (Norse & Ju, 2015; Bhateria & Jain, 2016; Mareque et al., 2018), which is problematic since the plant-microbe interaction is considered an asset for crop production (Qiu et al., 2019).

A group of microorganisms that can be impacted by chemical fertilizers is diazotrophic bacteria (Silva et al., 2014; Liao et al., 2017; Wang et al., 2018), which are responsible for biological nitrogen fixation. Biological nitrogen fixation is a crucial process in agriculture because nitrogen (N) plays a vital role in various physiological processes, such as photosynthesis, respiration, growth, and cell differentiation (Nunes et al., 1993; Lobo et al., 1988; Fagundes et al., 2007). Therefore, a sustainable agriculture model must take a microbiological perspective and consider how current practices, such as chemical fertilization, affect the structure of plant-microbiota communities (Mareque et al., 2018). In order to develop new technologies, such as inoculants, which aim to reduce costs and promote environmental conservation, it is necessary to balance fertilization and introduce effective bacteria to promote plant growth in the agricultural production system.

In Brazil, for instance, the association between soybean plants and *Bradyrhizobium* sp. can fulfill all the plant's nitrogen demand through biological nitrogen fixation. However, although sorghum has an association with diazotrophic bacteria (Hara et al., 2019; Wasai-Hara et al., 2020; Barros et al., 2020), and some studies are attempting to understand the association between diazotrophic bacteria and sorghum plants and the benefits of inoculation with these bacteria, reducing nitrogen fertilization, these aspects are still not well understood. As there are no inoculants available for sorghum in Brazil, new technologies that reduce dependence on nitrogen fertilizers are crucial to minimize the risks associated with the initial investment. To achieve this goal, it is necessary to conduct studies to gain a better understanding of these associations, select bacteria that are more efficient in nitrogen fixation, and balance inoculation with fertilization, so as not to harm the biological nitrogen fixation process.

In this context, the present study aimed to understand how nitrogen fertilization affects the nitrogen-fixing microbial community associated with sorghum plants. It also aimed to evaluate the agronomic efficiency of sorghum plant inoculation with pre-selected plant growth-promoting bacteria, and determine the combination of balanced fertilization with the inoculation of effective diazotrophic bacteria. This understanding is important in reducing the dependence on synthetic nitrogen in sorghum crops and other grass species. The study stages, which range from the isolation and identification of growth-promoting bacteria associated with sorghum plants to the inoculation and validation of agronomic efficiency under field conditions, are outlined in the flowchart below (Fig. 1).

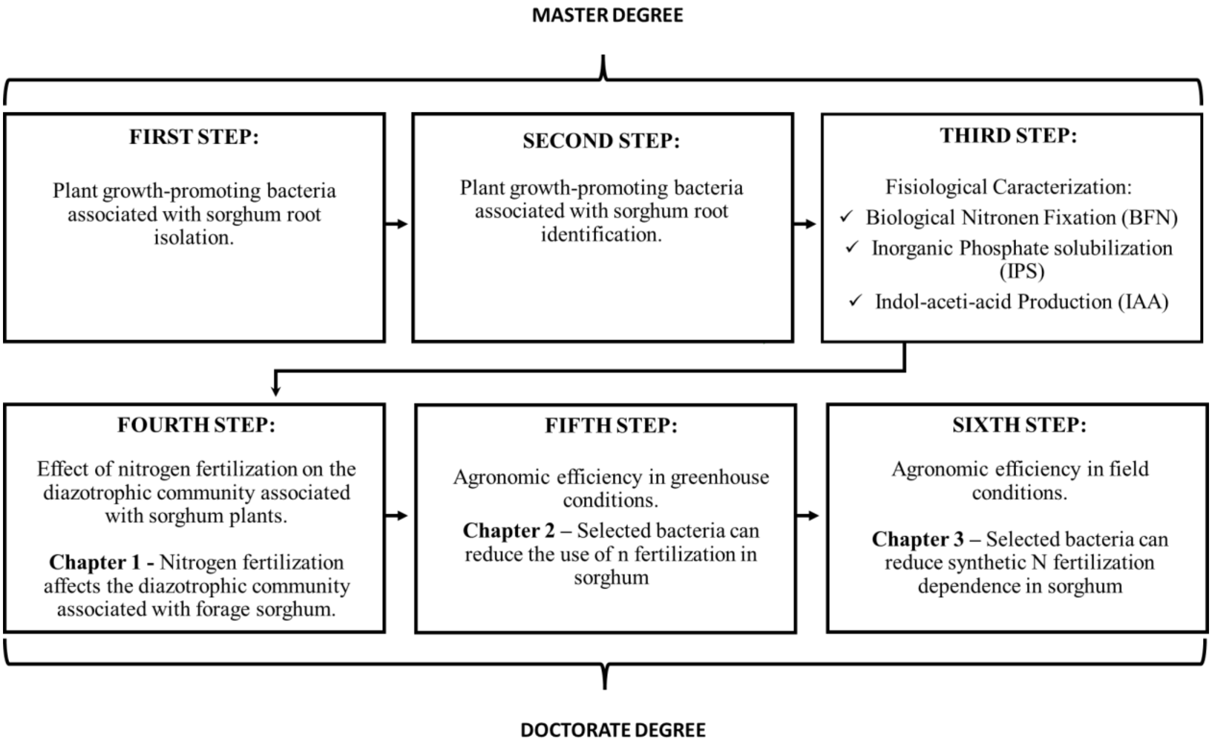

Fig. 1 – Flowchart of the study of the association between plant growth-promoting/diazotrophic bacteria and sorghum plants, including the steps to understand the impact of nitrogen fertilization on the associated diazotrophic microbiota and to determine the combination of balanced fertilization with effective diazotrophic bacteria inoculation.

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Chapter 1 - Nitrogen fertilization affects the diazotrophic community associated with forage sorghum

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RESUMO

PAULA, T. A., D.Sc., Universidade Federal de Viçosa, Setembro de 2021. **A fertilização com nitrogênio afeta a comunidade diazotrófica associada ao sorgo forrageiro.** Orientador: Leonardo Duarte Pimentel. Coorientadores: Maria Catarina Megumi Kasuya e Marliane de Cássia Soares da Silva.

Os fertilizantes nitrogenados podem exercer influência sobre a comunidade de bactérias diazotróficas associadas às diferentes plantas. Em alguns casos, o efeito pode ser tão severo que essencialmente abole a presença de fixadores de nitrogênio. Este trabalho teve como objetivo identificar o efeito de diferentes doses de nitrogênio sobre a microbiota diazotrófica associada a plantas de sorgo forrageiro durante a etapa de semeadura. Para isso, plantas de sorgo forrageiro foram cultivadas em casa de vegetação com diferentes doses de adubação nitrogenada, utilizando-se 0, 25, 50, 75 e 100% da recomendação técnica para a cultura. O DNA microbiano foi extraído da rizosfera, raízes e folhas para amplificação do gene *nif*H e análise do perfil da comunidade microbiana por DGGE. Com o aumento da dose de nitrogênio, houve aumento nas variáveis matéria seca da parte aérea, altura da planta e diâmetro do caule. Foram identificadas também diferenças na estrutura da comunidade diazotrófica que coloniza a rizosfera, raiz e folha de plantas de sorgo, sendo que as diferentes doses de N, aplicadas durante a semeadura, alteram a estrutura da comunidade diazotrófica associada a cada parte da planta, causando uma redução nos índices de riqueza, uniformidade e diversidade de Shannon. A dose de adubação nitrogenada, aplicada durante a semeadura, altera a estrutura da comunidade diazotrófica associada à rizosfera, raiz e folhas de plantas de sorgo forrageiro. Porém, mais estudos são necessários para identificar quais grupos de diazotróficos podem ser mais afetados e como isso pode interferir na fisiologia da planta.

Palavras-chave: Fixação biológica de nitrogênio, fertilizantes, meio ambiente.

ABSTRACT

PAULA, T. A., D.Sc., Universidade Federal de Viçosa, September, 2022. **Nitrogen fertilization affects the diazotrophic community associated with forage sorghum**. Adviser: Leonardo Duarte Pimentel. Co-advisers: Maria Catarina Megumi Kasuya and Marliande de Cássia Soares da Silva.

Nitrogen fertilizers can affect the community of diazotrophic bacteria associated with different plants, and in some cases, the consequences can be so severe that they abolish the presence of associative nitrogen fixation. The work aimed to identify the effect of different nitrogen doses applied at sowing on the diazotrophic-associated microbiota of sorghum plants. Forage sorghum plants were grown under greenhouse conditions with different nitrogen fertilization doses, ranging from 0 to 100 % of the technical recommendation for the crop. The DNA was extracted from the rhizosphere, roots, and leaves, and the *nifH* gene was amplified for analysis by DGGE. As the nitrogen dose increased, there was an increase in plant height, stem diameter, and shoot dry matter. There were also differences in the structure of the diazotrophic community that colonized the sorghum rhizosphere, root, and leaves. The amount of nitrogen applied during sowing changes the structure of the diazotrophic community associated with each part of the plant, resulting in a reduction in richness, evenness, and the Shannon diversity index. Further studies are needed to identify which groups of diazotrophs are most affected and how this can affect the plant's physiology.

Key-words: Biological nitrogen fixation, fertilizer, environment.

INTRODUCTION

In Brazil, the use of chemical fertilizers, particularly nitrogen fertilizers, is essential for enhancing crop productivity and must increase with agriculture intensification in the coming years. However, the intensive use of chemical fertilizers can cause environmental problems and impact the ecology and dynamics of microbial communities in agricultural soils (Norse & Ju, 2015; Bhateria & Jain, 2016; Mareque et al., 2018). This is a concern since the plant-microbe association is seen as a solution for cost-effective and sustainable agriculture (Qiu et al., 2019). For instance, the association between soybean plants and the diazotrophic bacteria *Bradyrhizobium* sp. can meet all the plant's nitrogen demand through biological nitrogen fixation (BNF). However, one group of beneficial microorganisms impacted by chemical fertilizers is diazotrophic bacteria (Silva et al., 2014; Liao et al., 2017; Wang et al., 2018), which play a crucial role in colonizing the leaves, roots, and rhizosphere of plants (Gao et al., 2019).

The rhizosphere, being a dynamic habitat, hosts complex microbial communities that perform important functions such as biological nitrogen fixation, inorganic phosphate solubilization, indole-acetic acid production, and protection against plant pathogens (Singh et al., 2004; Philippot et al., 2013; Pande et al., 2017; Qiu et al., 2019). The rhizosphere microbiome can be modulated by plant cultivars and human activities, such as the use of chemical fertilizers (Igiehon & Babalola, 2018). This modulation can influence the recruitment of different microbial species, which is reflected in the environment surrounding plant tissue (Oliveira et al., 2003; Valé et al., 2005). Nitrogen fertilizers can affect the community of diazotrophic bacteria associated with various plants (Valé et al., 2005; Coelho et al., 2009; Silva et al., 2014). In some cases, the effects can be so severe that associative nitrogen fixation is essentially abolished (Bahulikar et al., 2020). This fundamental process of nitrogen fixation and its associated microbial community will become increasingly suppressed as terrestrial fertilization continues to increase (Fan et al., 2019).

With this scenario and its challenges in mind, studying the impact of chemical fertilizers on plant-microorganism associations is critical for the development of new technologies that aim to reduce production costs and promote sustainable agricultural practices. This can only be achieved through the preservation of biodiversity in the various ecosystems involved in plant production.

The aim of this work was to investigate the effect of different nitrogen doses at sowing on the diazotrophic microbiota associated with sorghum plants.

MATERIALS AND METHODS

Sowing, fertilization, and cultivation period

Forage sorghum plants were cultivated under greenhouse conditions, and the experiment followed a completely randomized design with three replications per treatment. For this, low fertility soil, classified as dystrophic red-yellow latosol, was used. The soil was stored in 3-L polyethylene bags. At the time of sowing, each treatment was added with 10 g of soil from a sorghum area cultivated during the second crop (February – March) to serve as an inoculum for microorganisms. The soil that served as a microorganism inoculum was obtained from the Experimental Field "Diogo Alves de Mello" (lat 20º 45 '14 "S, long 42º 52' 55" W and alt 648 m) belonging to the Universidade Federal de Viçosa, Viçosa, MG, Brazil. The experimental field is characterized not only by the cultivation of sorghum (*Sorghum bicolor*) crop but also by the cultivation of other summer crops such as soybeans (*Glycine max*), corn (*Zea mays*), and beans (*Phaseolus vulgaris*) during the period of the main crop (October - December).

Fertilization was carried out in a pit at a distance of 6 to 8 cm from the seed planting. Phosphate and potassium fertilization were carried out according to the technical recommendation for the crop after chemical analysis of the soil (Table 1) (Alves et al., 1999). Super simples (18 % P_2O_5) and Potassium Chloride (60 % KCl), respectively, were used.

Nitrogen fertilization with ammonium sulfate $(NH_4)_2SO_4$, was performed using 0, 25, 50, 75, and 100 % of the technical recommendation for the crop, 120 kg ha^{-1}.

The plants were grown for 45 days, so samples of rhizospheric soil (soil adhered to the roots), root, and leaf were collected. For the sampling of the rhizospheric soil, after the first root's cleaning, the soil that remained adhered to the roots was considered rhizospheric soil and collected (von der Wade et al., 2000). Evaluation of agronomic parameters such as plant height, stem diameter and shoot dry mass also were carried out.

Shoot dry matter, plant height, and stem diameter

Plant height (cm) was measured with a tape measure considering the distance from the neck to the end of the last developed leaf. Stem diameter (mm) was determined at the height of the cervix with the aid of a digital caliper.

For shoot and root dry matter determination, the collected material was washed using tap and distilled water, and dried was carried out at 60 °C in a ventilated oven for 72 hours until reaching weight constant (Hungria et al., 2010).

DNA extraction, *nifH* gene amplification and DGGE

For the analyses by DGGE, DNA was extracted from the rhizospheric soil, roots, and leaf. It was used 250 mg of sample from each part of the plant to perform the procedure. The extraction of the total DNA was performed using the Nucleo Spin Plant Kit II and NucleoSpin Soil Kit (Macherey-Nagel, GmbH & Co. KG, Germany) according to the manufacturer's instructions.

The *nifH* gene was amplified by PCR reaction using the 19F and 407R primer pairs (Coelho et al., 2009; Ueda et al., 2015). The PCR mix consisted of 20 ng total DNA, 0.2 µM of each oligonucleotide, 200 µM dNTP, 2 mM $MgCl_2$, 0.5 mg mL^{-1} bovine serum albumin (BSA) and 1.25 polymerase units. GO Taq DNA, so the total reaction volume was 50 µL. In the reaction, the following time and temperature conditions were used, and the initial annealing temperature of 58 ºC followed by a decrease of 1 ºC every two cycles until reaching 48 ºC the reaction was performed during 25 cycles (Direito & Teixeira, 2002). This step was followed by nested-PCR using the oligonucleotides 19F-GC and the primer 278R (Silva et al., 2014), under the same conditions, which yields a 260-bp fragment. Quantification and visualization of the PCR product were performed on 1.5 % agarose gel along with a 1500 bp marker.

The three replications per treatment of DNA fragments obtained by nested-PCR technique were analyzed by DGGE (Dcode System, Bio-Rad Inc., California). For the comparison, in a single DGGE, between the different parts of the plant (rhizosphere, root, and leaf) at different N doses, the three replicates of each treatment were mixed to form a composite sample for application on the gel. 20 µl of the nested-PCR products ranging from 150 to 200 ng of DNA was loaded onto an 8 % (w/v) polyacrylamide gel in 1 X Tris-acetate EDTA (TAE) buffer. The gel was prepared with a denaturing gradient ranging from 50 to 65 % (Silva et al., 2014). The gel was subjected to vertical electrophoresis at 60 V for 12 h at 60 °C, then stained for 40 min with Sybr Gold (1x) (Molecular Probes, Leiden, The Netherlands); the gel was then photographed under UV light on a Molecular Imaging System (Loccus Biotecnologic L-Pix Chemi).

Statistical Analysis

Data analysis was performed with the statistical software R (R Core Team, 2018). The data were submitted to ANOVA, followed by the Tukey test for comparison of means and t-test for the slope of the different equations ($p<0.05$). The DGGE profiles, and diversity indexes, Richness, evenness and Shannon, were analyzed and compared using BioNumerics software (Version 6.0, Applied Maths NV).

RESULTS AND DISCUSSION

Plant height, stem diameter, and shoot dry matter

With the increase in nitrogen fertilization, there was a corresponding increase in the shoot dry matter, plant height, and stem diameter of the sorghum plants (Fig. 1). This trend was observed in previous studies that examined the effect of nitrogen dose on the growth of various economically valuable crops (Uchino et al., 2013; Olugbemi & Ababyomi, 2016). This increase can be attributed to the crucial role nitrogen plays in various plant metabolic processes, including photosynthesis, respiration, growth, and cell differentiation. Nitrogen is also an essential component of key macromolecules such as enzymes, proteins, nucleic acids, chlorophyll, storage proteins, and cell walls (Nunes et al., 1993; Lobo et al., 1988; Fagundes et al., 2007).

Change in the microbial structure of the rhizosphere

The amount of nitrogen applied at the sowing of sorghum influenced the composition of the diazotrophic community associated with its rhizosphere, and the treatment with 100% nitrogen dose was the furthest from the other treatments (Fig. 2). There was also a reduction in richness and diversity (Shannon) index when 100% nitrogen dose was applied, in the rhizospheric soil, roots, and leaves (Fig. 3). This result shows that an increase in the nitrogen dose altered the recruitment of diazotrophic bacteria by sorghum plants, and intermediate doses shared a more similar structure.

Indeed, nitrogen fertilization decreases the richness and intensity of the DGGE bands of diazotrophic bacteria in the rhizosphere (Silva et al., 2014). Therefore, microbial diversity in the soil showed a robust reduction with an increase in the nitrogen applied due to a decrease in microbial biomass (Wang et al., 2018). The use of different fertilizers, especially in the long term, alters the abundance, composition, and activity of the diazotrophic community (Liao et al., 2017). For example, urea-based chemical fertilizers induce changes in the profile of the rice rhizobacteria community (Jorquera et al., 2014). With the increase in inorganic nitrogen, there is a tendency to reduce organic carbon in the soil, causing a change in the profile of the microbial community present in the environment (Wang et al., 2018). In higher doses of nitrogen fertilizers, fragments of the *nif*H gene have less diversity than those found in lower doses, indicating that certain groups present in the rhizosphere are sensitive to the introduction of nitrogen into the system (Meng et al., 2012).

Thus, a relatively low level of balanced nitrogen fertilization can be beneficial in maintaining the diversity of the diazotrophic communities present in the soil (Wang et al., 2020). Some studies have shown that fertilization with NPK (nitrogen-phosphorus-potassium) changes the diazotrophic community. However, chemical fertilization combined with organic material can improve the diversity of the diazotrophic community and the rate of N fixation (Sarkar et al., 2013; Liao, 2017).

It is worth mentioning that the profile of the diazotrophic community in the soil and rhizosphere is not only influenced by the nitrogen fertilization regime but also by factors such as the species and cultivar, plant age, season, stress, and the presence of diazotrophs in a specific environment (Coelho et al., 2009; Philippot et al., 2013; Silva et al., 2014; Sharma & Singh, 2017).

Change in the microbial structure of the root and leaf

The increase in the nitrogen dose also impacted the diazotrophic community in the roots and leaves of the sorghum plants (Fig. 4 and 5). Like in the rhizosphere, there was also a decrease in richness and diversity (Shannon) index for both the root and leaf with the increase in the nitrogen dose (Fig. 4). This is likely due to changes in the structure of the rhizosphere microbial community, the presence of diazotrophic bacteria capable of associating with other plant organs such as roots and leaves, and physiological changes caused by the change in the nitrogen dose.

As the root is usually the first point for the entry of bacteria, fertilization with N can lead to physiological changes in the tissues of this organ and, consequently, to changes in the structure of its microbial community (Carvalhais et al., 2013; Trivedi et al., 2020). So, the application of N during the planting of sorghum affects the profile of its associated diazotrophic community, and this change is pronounced at the root and must depend on the physiological state of the plant (Mareque et al., 2018). In addition, with the increase in the N availability in the environment, plants tend to exclude associations with some groups of diazotrophic bacteria since this association becomes an unnecessary energy cost. Thereby, that results in lower levels of diazotrophic bacteria in the roots, with a higher level of N essentially tending to abolish the association of roots with diazotrophic bacteria (Bahulikar et al., 2020). However, the organic components provide changes in the bacterial community associated with the corn rhizoplane, providing an increase in the number of bacterial groups, observed by the increase in the rare bands by DGGE analysis (Ferreira et al., 2009).

The changes in the rhizosphere and root tend to affect the structure of the diazotrophic community in the leaves (Gong & Xin, 2021; Chi et al., 2005). The treatment with 100% N dose showed the greatest difference compared to the others (Fig. 5), likely due to the significant impact it had on the diazotrophic structure, particularly in the rhizosphere, which serves as the primary recruitment point for microorganisms forming associations with plants. Hence, specific bacterial groups in the rhizosphere that use the root as an entry point for other tissues may not be able to establish these associations.

Change in the microbial structure of the rhizosphere, root, and leaf

Differences were observed in the structure of the diazotrophic community that colonizes the rhizosphere, root, and leaf (**Fig. 6**). Roots and leaves, being plant tissues, tend to share more microorganisms, but it was possible to observe that they shared about 40 % of their diazotrophic community with the rhizosphere (**Fig. 6**). Some studies reported that the microbiota in the roots and leaves depend partially on the community present in the soil, which explains the similarity of 40 % found in this work (Finkel et al., 2019; Tkacz et al., 2020). Thus, the rhizosphere is a richer and more diverse environment in terms of microbial structure, whereas the root and leaf tend to have more specific microorganisms associated with both organs' tissues (Trivedi et al., 2020). This difference results from the necessity of each plant added to the environmental conditions (Trivedi et al., 2020; Gong & Xin, 2021). Thus, N fertilizer, besides the characteristics and necessity of each tissue, can influence the final structure of its associated microbiota (Trivedi et al., 2020; Gong & Xin, 2021). It is also important to note that the rhizosphere, root, and leaf shared a mutual DGGE band and that it was presented practically at all doses tested (**Fig. 6**), which should represent a group that plays a chief role in the development of sorghum.

Therefore, the amount of N applied at sowing changed the structure of the diazotrophic community associated with each part of the sorghum plant (**Fig. 6**) since the community found in the 100 % N dose always differed from the other treatments. For the rhizosphere and root, the 0% N dose also differed from the intermediate doses, which can demonstrate more clearly how the application of N during the cultivation of sorghum and other grasses affects the structure of the diazotrophic community associated with plants. These results are significant because it is only possible to think of a sustainable agriculture model from a microbiological point of view based on the comprehension of how current practices, such as chemical fertilization, affect the structure of communities present in plant-microbiota associations (Mareque et al., 2018).

CONCLUSION

An increase in the nitrogen fertilizer application rate at planting results in an increase in the dry matter, height, and stem diameter of sorghum plants. However, changes are observed in the composition of the diazotrophic community in the rhizosphere, roots, and leaves of sorghum plants. The recommended 100% nitrogen fertilizer rate has the greatest impact on the diazotrophic community. Further studies are necessary to determine which diazotrophic groups are most affected and the impact on plant physiology.

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Table 1. Chemical soil analysis

pH	P	K	Na	Ca^{2+}	Mg^{2}	Al^{3+}	H+Al	SB	(t)	(T)	V	m
H_2O	mg / dm^3			cmol$_c$/dm^3							%	
5,40	1.41	13	-	0.24	0.08	0.00	0.66	0.32	0.32	0.98	34.7	0.00

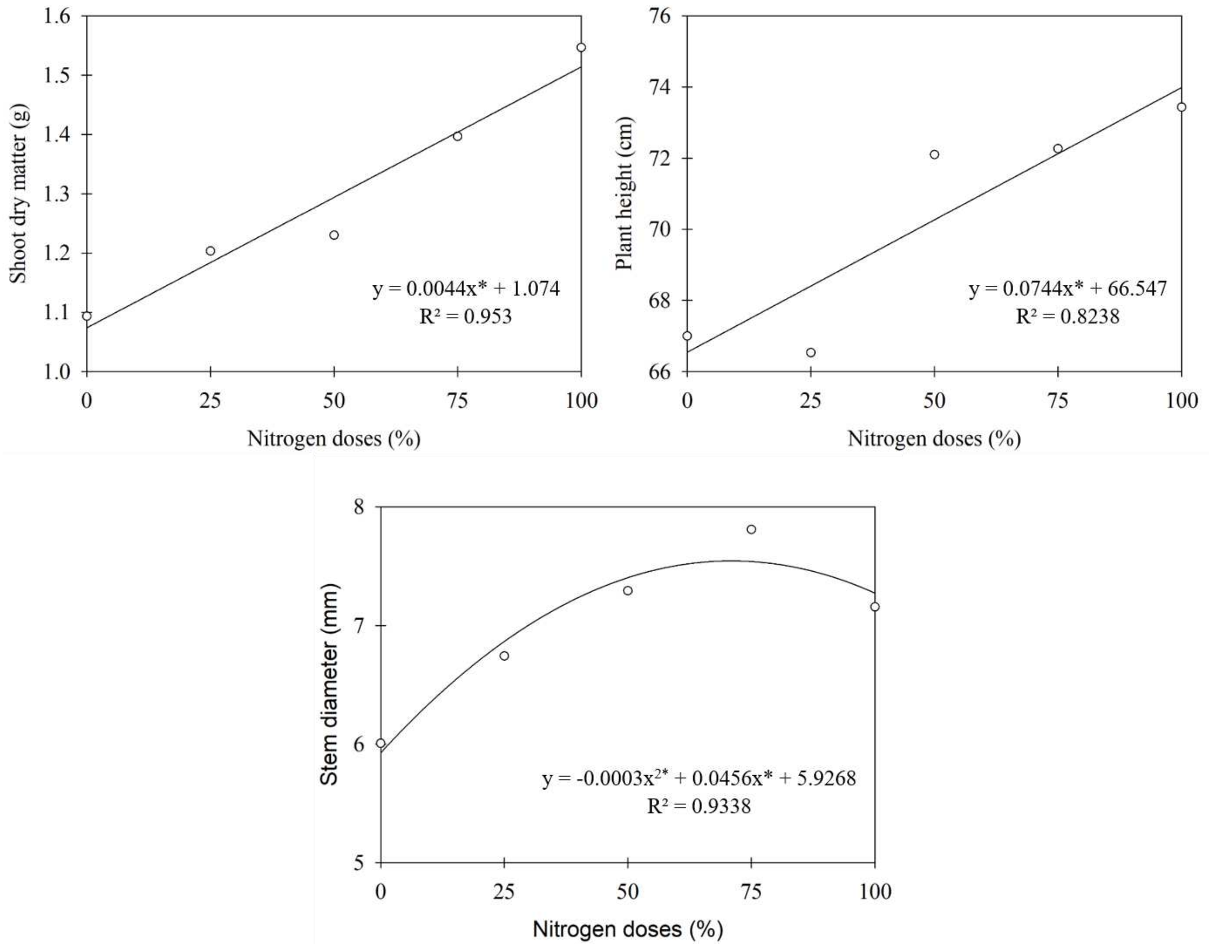

Fig. 1 - Shoot dry matter, Plant height, and stem diameter of forage sorghum plants at different doses of nitrogen fertilization (0, 25 50, 75, and 100% of the recommended dose).

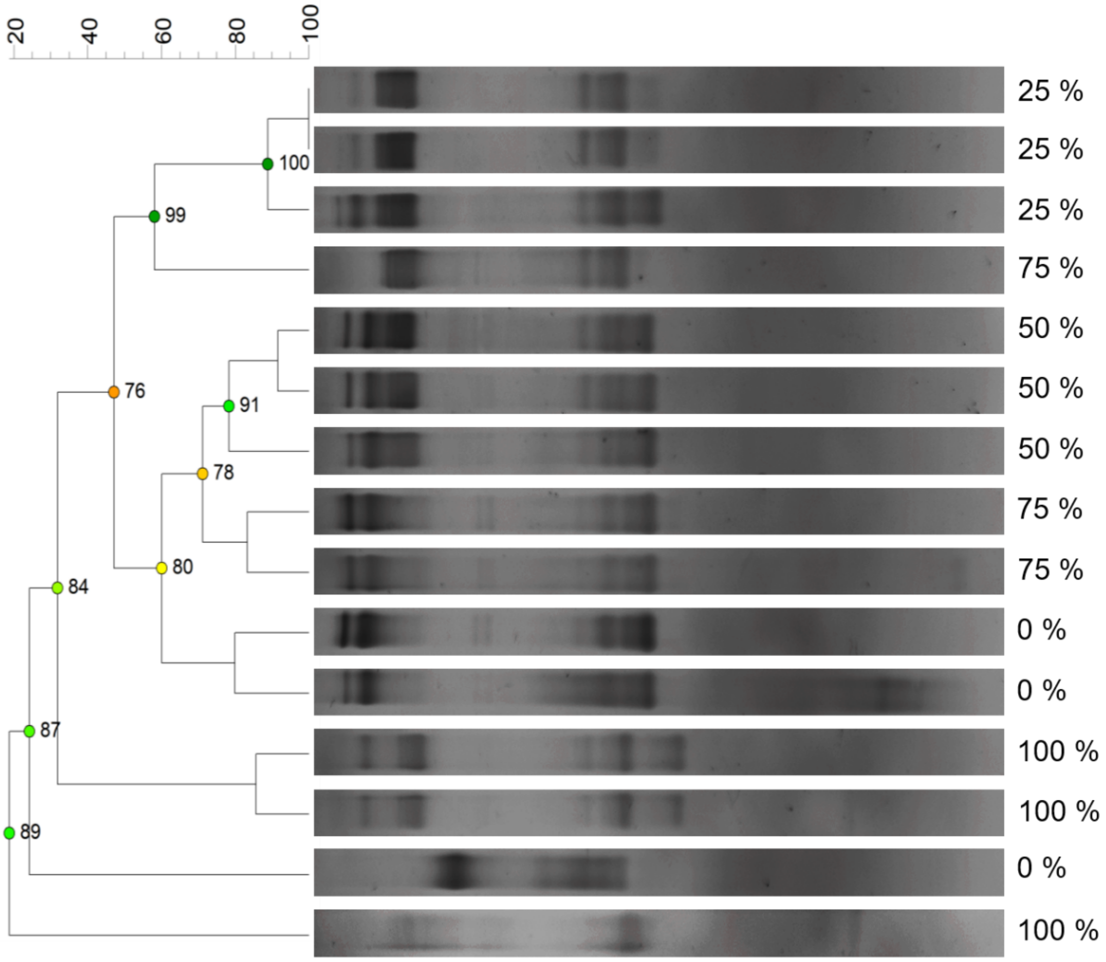

Fig 2 - Profile of the diazotrophic community associated with sorghum rhizosphere at different doses of nitrogen fertilization (0, 25 50, 75, and 100% of the recommended dose).

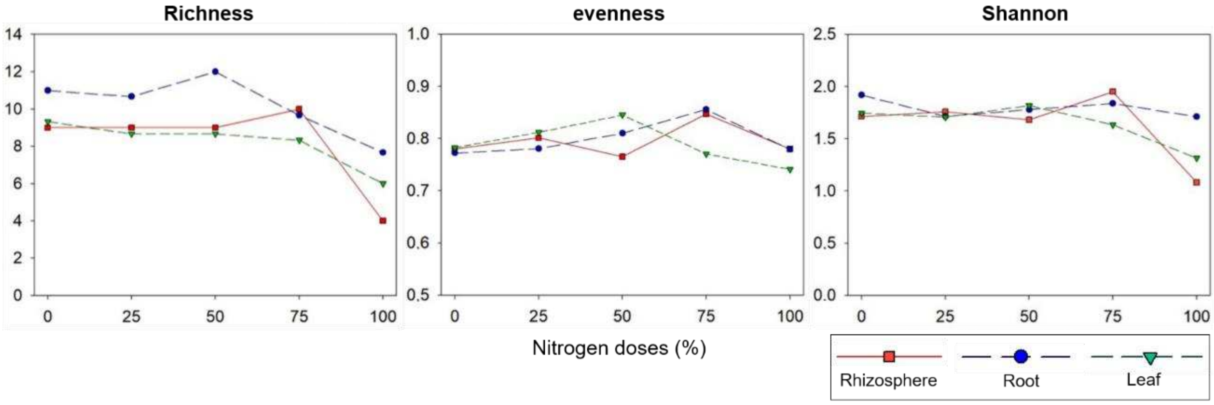

Fig. 3 - Diversity indexes (richness, evenness, and Shannon) of the diazotrophic community of sorghum rhizosphere, root, and leaf at different nitrogen fertilization (0, 25 50, 75, and 100 % of the recommended dose).

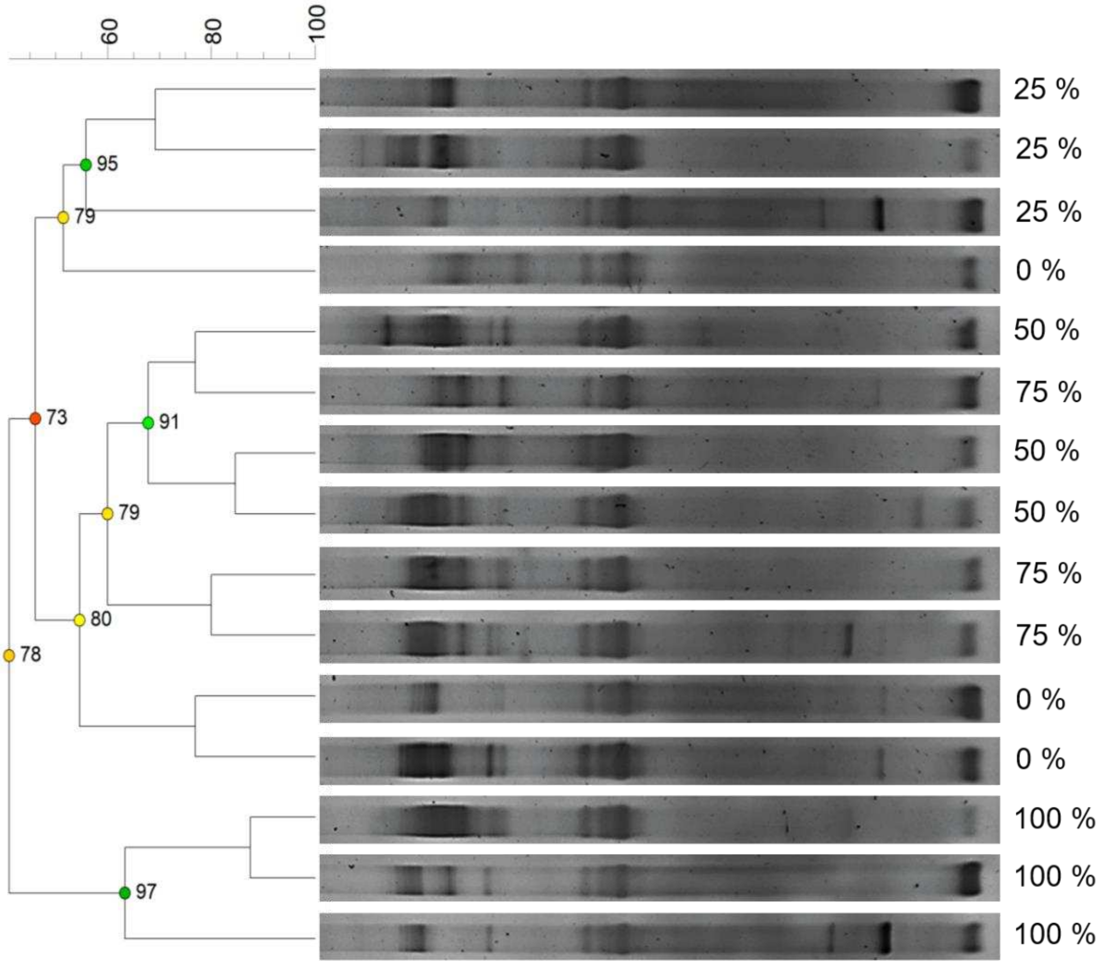

Fig. 4 - Profile of the diazotrophic community associated with sorghum root at different doses of nitrogen fertilization (0, 25 50, 75, and 100 % of the recommended dose).

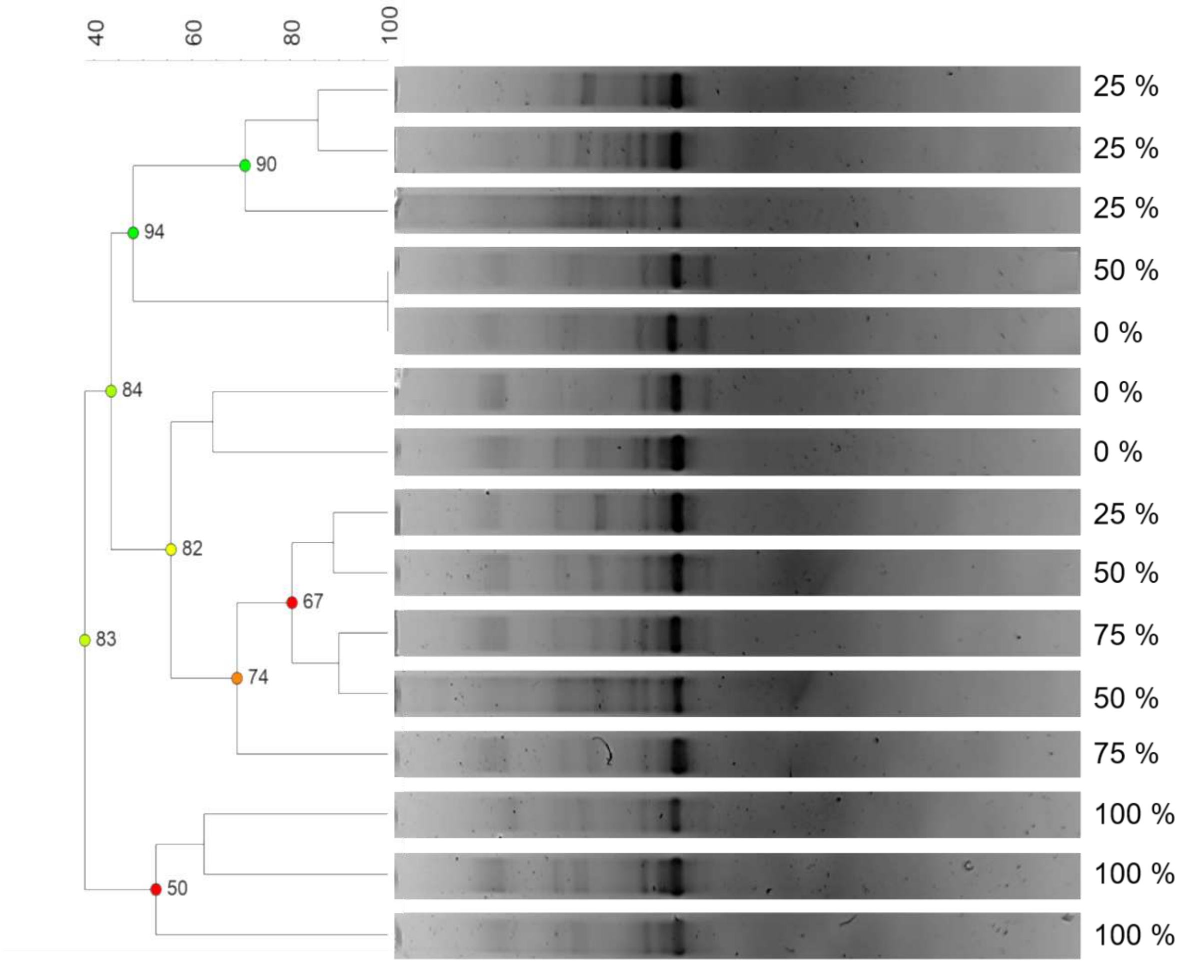

Fig. 5 - Profile of the diazotrophic community associated with sorghum leaves at different doses of nitrogen fertilization (0, 25 50, 75, and 100 % of the recommended dose).

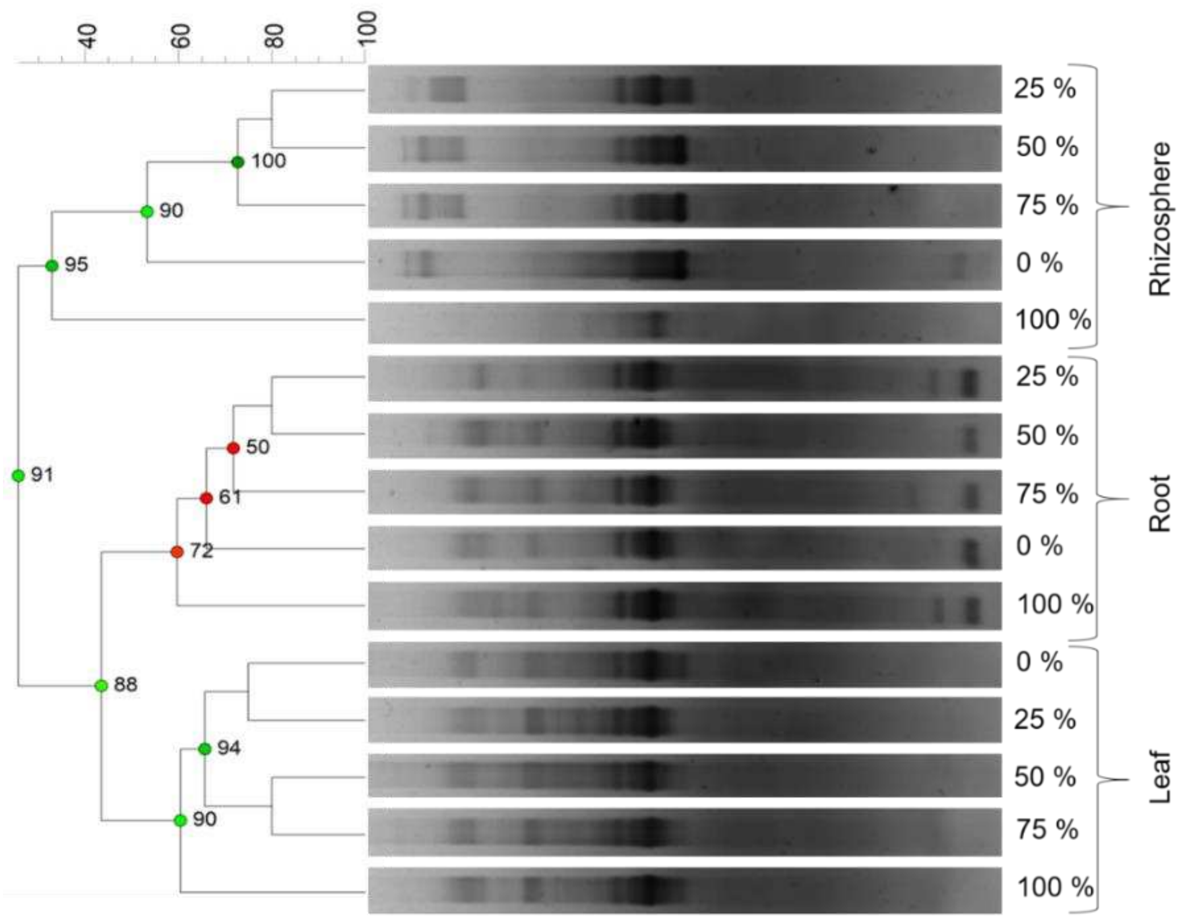

Fig. 6 - Profile of the diazotrophic community associated with sorghum rhizosphere, root, and leaves at different doses of nitrogen fertilization (0, 25 50, 75, and 100 % of the recommended dose).

Chapter 2 – Selected bacteria can reduce the use of N fertilization in sorghum

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RESUMO

PAULA, T. A., D.Sc., Universidade Federal de Viçosa, Setembro de 2022. **Bactérias selecionadas podem reduzir o uso de adubação nitrogenada em sorgo.** Orientador: Leonardo Duarte Pimentel. Coorientadores: Maria Catarina Megumi Kasuya e Marliane de Cássia Soares da Silva.

Com o aumento da população, a demanda por alimentos, fibras e energia também resultará em aumento. Assim, é esperado aumento na demanda por fertilizantes, como o nitrogênio (N), o que deve aumentar o custo de produção e causar problemas ambientais. Portanto, é necessário desenvolver tecnologias sustentáveis para reduzir o uso de fertilizantes químicos durante o cultivo. Este trabalho teve como objetivo testar a eficiência de bactérias diazotróficas na promoção do crescimento do sorgo e avaliar o potencial biotecnológico como inoculante para a cultura. Sementes de sorgo forrageiro foram inoculadas com uma das quatro bactérias (SB1 - *Burkholderia* sp; SB5 - *Rhizobium* sp .; SF1 - *Burkholderia* sp .; SF4 - *Sphingomonas* sp.) ou um MIX contendo todos os isolados testados, em solo fertilizado com diferentes doses de adubação nitrogenada, durante 60 dias, em casa de vegetação. O aumento da dose de adubação nitrogenada e a inoculação com isolados distintos (SB5 - *Rhizobium* sp., SF4 - *Sphingomonas* sp.) e o MIX aumentaram o diâmetro do caule, a massa seca da parte aérea e total, e o teor de N da parte aérea ($p < 0{,}01$). Assim, quando comparado às plantas que receberam 100% da dose recomendada de N, a inoculação com os isolados SB5 (*Rhizobium* sp.), SF4 (*Sphingomonas* sp.) ou MIX apresentou potencial de redução de 25% da dose de fertilizantes nitrogenados utilizada durante a semeadura do sorgo forrageiro ($p < 0{,}1$). Bactérias diazotróficas selecionadas e o MIX têm potencial para reduzir o uso de fertilizante químicos nitrogenados, em condições de casa de vegetação, durante o cultivo do sorgo forrageiro, proporcionando economia no uso de fertilizantes e possível preservação do meio ambiente.

Palavras-chave: Sorgo; bactérias diazotróficas, fixação de nitrogênio.

ABSTRACT

PAULA, T. A., D.Sc., Universidade Federal de Viçosa, September, 2022. **Selected bacteria can reduce the use of N fertilization in sorghum**. Adviser: Leonardo Duarte Pimentel. Co-advisers: Maria Catarina Megumi Kasuya and Marliane de Cássia Soares da Silva.

With the increasing population, the demand for food, fiber, and energy is also on the rise. As a result, there is an expected increase in the demand for fertilizers such as nitrogen (N), which would result in increased production costs and environmental problems. Hence, it is crucial to develop sustainable technologies that can reduce the use of chemical fertilizers in crop cultivation. This study aimed to test the efficiency of diazotrophic bacteria in promoting sorghum growth and evaluate their potential as inoculants for sorghum crops. Forage sorghum seeds were inoculated with one of four bacteria (SB1 - *Burkholderia* sp.; SB5 - *Rhizobium* sp.; SF1 - *Burkholderia* sp.; SF4 - *Sphingomonas* sp.) or a mix containing all tested isolates, in soil fertilized with different doses of N fertilization, during 60 days in greenhouse conditions. The increase in fertilization rates, as well as inoculation with distinct isolates and the mix, increased stem diameter, shoot and total dry mass, and shoot N content ($p<0.01$). Thus, compared to plants that received 100% of the recommended N dose ($p<0.1$), inoculation with isolates SB5 (*Rhizobium* sp.), SF4 (*Sphingomonas* sp.), or the mix showed the potential to reduce the N fertilizer dose used during sowing by 25% for forage sorghum plants. The selected diazotrophic bacteria and the mix can reduce the use of N fertilizer in forage sorghum, leading to cost savings and environmental conservation.

Key-words: Sorghum; diazotrophic bacteria, nitrogen fixation.

INTRODUCTION

Nitrogen is considered the main limiting nutrient for the growth and development of crops since this element in nature is present in a chemically stable compound (Fryzuk et al., 2000). Thus, plants cannot use it directly, requiring its transformation to a combined form to facilitate their assimilation (Santi et al., 2013). Currently, the way to supply nitrogen in an assimilable form to plants is through fertilization with synthetic fertilizers that are manufactured by the Harber-Bosch method, which takes place under high temperatures (approximately 500°C) and pressures (200-600 atm) and requires the burning of fossil fuels, which makes the process onerous and contributes directly and indirectly to the high cost of agricultural production and environmental pollution (Leigh, 1998; Lutzenberger, 2001). Therefore, it is chief the development of sustainable technologies that can reduce the use of chemical fertilizers applied to crop cultivation resulting in a reduction in the cost of production without affecting the final productivity and environmental conservation.

Sorghum (*Sorghum bicolor* L. Moench), an excellent source of food for both humans and animals and a source of bioenergy (Hara et al., 2019; Wasai-Hara et al., 2020; Barros et al., 2020), presents a high capacity to withstand environmental tensions, such as high temperatures and water deficit, being important in arid and semi-arid regions. Sorghum cultivation in Brazil, has widely been in the second crop, that is, in succession to summer crops (Coelho et al., 2002), a period when climatic conditions are unfavorable. Due to the greater risk, producers invest less, and nitrogen fertilization input is the one of most affected, which makes the performance of the crop below its potential (Dos Santos et al., 2017). Moreover, although sorghum is associated with diazotrophic bacteria (Hara et al., 2019; Wasai-Hara et al., 2020; Barros et al., 2020), there are no commercial inoculants registered for being used in sorghum in Brazil. So, for sorghum, new technologies that reduce the dependence on nitrogen fertilizers become even more important to reduce the risks linked to the initial investment.

The input of beneficial microorganisms is considered an alternative for increasing agricultural productivity, reducing costs, and achieving sustainable agriculture (Qiu et al., 2019). These microorganisms can colonize the rhizosphere, root surface, or the interior of plant tissues and act through several mechanisms, such as the ability to perform biological nitrogen fixation (BNF), inorganic phosphate solubilization (IPS), and the production of indole-acetic acid (IAA) (Kuklinsky-Sobral et al., 2004; Pande et al., 2017; Qiu et al., 2019; Zhou et al., 2020). An example is diazotrophic bacteria associated with plants of commercial value (Gruber & Galloway 2008). In Brazil, the interaction between soybean (*Glycine max*) and diazotrophic bacteria of the *Bradyrhizobium* genus can supply all the plant's N demand. Among grasses, the crops that have demonstrate relative success in the ability to make efficient associations with diazotrophic bacteria are sugarcane (Boddey et al., 1991, 1995; Taulé et al., 2012; Wei et al. al., 2014) and corn (De Salamone et al., 1996; Montañez et al., 2009; Alves et al., 2014). Boddey et al. (1991) demonstrated that some sugarcane varieties obtain about 60 to 80% of the total nitrogen of the plant through BNF. But studies involving the inoculation of diazotrophic bacteria in grass species and its efficiency in fixing N when associated with such plants are still scarce, especially with sorghum.

This work aimed to test selected diazotrophic bacteria efficient in promoting plant growth and evaluate the biotechnological potential as inoculants for sorghum crops.

MATERIALS AND METHODS

The work was carried out in the Laboratory of Sorghum, Department of Agronomy, and Laboratory of Mycorrhizal Associations (LAMIC), located in the Institute of Applied Biotechnology for Agriculture and Livestock (BIOAGRO), Department of Microbiology, both belonging to Universidade Federal de Viçosa - UFV, in Viçosa - Minas Gerais, Brazil.

Sowing, fertilization, and cultivation period

The experiment was carried out under greenhouse conditions from April to June, totalizing 60 days. The experimental design was a randomized block with three replications per treatment, following a double factorial with additional treatment (4 x 6) + 1, totalizing 25 treatments, in which four nitrogen fertilizers doses were used (0, 25, 50, and 75 % of the recommended dose), and six inoculant formulations (without inoculation, each bacterium inoculated separately, and a MIX containing all bacteria). The additional treatment corresponds to the treatment that received 100 % of the recommended N dose without bacteria inoculation. The uninoculated treatment corresponds to C- and the additional treatment corresponds to C +. For this, forage sorghum seeds and low fertility soil classified as dystrophic red-yellow latosol was used. The soil was stored in 10-liter polyethylene bags.

Forage sorghum seeds were inoculated with one of four bacteria (SB1 – *Burkholderia* sp; SB5 – *Rhizobium* sp.; SF1 – *Burkholderia* sp.; SF4 – *Sphingomonas* sp.) or a MIX containing all tested isolates capable of performing biological nitrogen fixation and previously isolated from the roots of sorghum plants. These isolates were obtained from isolation from roots of biomass and forage sorghum plants cultivated during the second crop (February – March) at the Experimental Field "Diogo Alves de Mello" (lat 20º 45 '14 "S, long 42º 52' 55" W and alt 648 m) of the Universidade Federal de Viçosa, Viçosa, MG, Brazil. The “Diogo Alves de Mello” experimental field is characterized not only by the cultivation of sorghum (*Sorghum bicolor*) but also by other summer crops such as soybeans (*Glycine max*), corn (*Zea mays*), and beans (*Phaseolus vulgaris*) during the period of the main crop (October - December).

The different isolates stored in NBY (nutrient broth yeast extract) medium (Schaad, 1998) containing glycerin (15 %) were grown in liquid medium broth potato dextrose (PD), under constant agitation of 120 rpm, until reaching the value of 2 x 10^9 CFU mL^{-1}, and to reach this value the growth curve of each tested isolate was performed. The growth curve was performed by cultivating the different isolates in liquid PD medium under constant agitation of 120 rpm and measuring the optical density (OD) at the wavelength of 520 nm, followed by the plating of an aliquot of 0.1 mL to calculate the colony-forming units (CFU), the procedure was performed every four hours until the isolates reached the stability phase.

Fertilization was carried out in a pit at a distance of 6 to 8 cm from the seed planting. Phosphate and potassium fertilization were carried out according to the technical recommendation after chemical analysis of the soil (**Table 1**) (Alves et al., 1999); super simples (18 % P2O5) and potassium chloride (60 % KCl) were used. Nitrogen fertilization was performed using ammonium sulfate in doses 0, 25, 50, 75, and 100 % of the technical recommendation, 120 kg ha^{-1} (Alves et al., 1999). For the inoculation of the isolates and the MIX was used a dosage recommended for inoculants registered for other grasses in Brazil, 100 mL for 50 kg of seed. The MIX was carried out MIX cultivating the different isolates separately and after mixing with the appropriate proportions to adapt the dosage of 100 mL for 50 kg of seed.

After sowing, the plants were grown for 60 days when they were harvested to carry out evaluations of agronomic parameters.

Plant height, stem diameter, shoot and root dry matter

Plant height (cm) was measured with a tape measure considering the distance from the neck to the end of the last developed leaf. Stem diameter (mm) was determined at the height of the cervix with the aid of a digital caliper.

For shoot and root dry matter determination, the collected material was washed using tap and distilled water, and dried was carried out at 60 °C in a ventilated oven for 72 hours until reaching weight constant (Hungria et al., 2010).

Digestion and determination of N content in the shoot

After dry mass determination, the samples went through the grinding process, carried out using a knife mill and a 1 mm sieve for further digestion and analysis of nutrient content. Nitrogen content was determinate by the Kjeldahl semi-micro method (Bremner and Mulvaney 1978; Tedesco et al., 1995).

Statistical analysis

Data analysis was performed with the statistical software R (R Core Team, 2018). The data were submitted to ANOVA, followed by the Tukey test for comparison of means and t-test for the slope of the different equations ($p < 0.01$). To compare the distinct isolates about the 100 % N dose, the data were subjected to analysis of variance (ANOVA) followed by the Dunnett test ($p < 0.1$).

RESULTS

For plant height, stem diameter, dry mass, and shoot N content no interaction was observed between inoculated isolates and nitrogen doses applied at sowing ($p<0.01$), so the results are shown independently. Inoculation of diazotrophic bacteria combined with nitrogen doses during maize sowing also does not present interaction for variables such as plant height, stem diameter, and dry mass accumulation (Repke et al., 2013).

Plant height and stem diameter

There was no effect of inoculation or N dose on plant height (Fig 1 a, b). For the stem diameter, there was a significant increase with both the inoculation and the N dose (**Fig. 1 c, d**), which corresponds to 16.34 % (SF4) with the inoculation of the isolates (**Fig. 1 c**) and 38.4 % with the increase in the N dose (**Fig. 1 d**).

Shoot, root, and total dry mass

The inoculation of all tested isolates and the MIX increased shoot and total dry mass accumulation compared to C- treatment, which corresponds to an increase of 44.7 % (SF4) and 40.90 % (SB5), respectively (**Fig. 2 a, e**). The increase in the N dose also increases shoot and total dry mass, which corresponds to 43 and 38.4 %, respectively (**Fig. 2 e, f**).

Root dry mass variable was the only one in which there was no significant effect of the different inoculated isolates concerning the C- treatment (**Fig. 2 c**). But there was an increase in the root dry mass with the increase in N dose (**Fig. 2 d**).

Shoot N content

The inoculation of the isolates and the MIX also results in a higher shoot N content (**Fig. 3 a**), which corresponds to 68.75 % about treatment C-. As the dose of N increases, there is also an increase in the shoot N content (**Fig. 3 b**).

25 % reduction in Nitrogen dose

For forage sorghum, whose purpose is animal feed, aiming for higher productivity, a better nutritional value, and lower production cost, it is interesting to combine the dose and isolate in which the pronounced accumulation of dry mass occurs. Therefore, it was compared the plants that received inoculation + 75 % of N dose with plants that received 100 % of the N dose (Fig 4). For the shoot and total dry mass, the treatments inoculated with the isolates SB5 (*Rhizobium*), SF4 (*Sphingomonas*), and MIX showed results equal to the treatment receiving 100 % N (**Fig. 4**), this result corresponds to an increase of 74.97 % and 70.05 % about the C-, respectively (**Fig. 4 c, e**). Regarding the shoot N content, just the treatment C- was different from the treatment C+ (Fig 4 f), which demonstrates the potential of the isolates plus the MIX tested in the present study to improve the N content in the shoot, providing a forage with better nutritional value.

Thus, when compared to plants that received 100 % of the N dose, under greenhouse conditions, the inoculation with the isolates SB5, SF4, or the MIX of bacteria can reduce by 25 % the dose of nitrogen fertilizers used during the sowing of forage sorghum plants.

DISCUSSION

Plant height and stem diameter

The inoculation of diazotrophic bacteria during the sowing of sorghum plants does not affect their growth (Andrade et al., 2019). Other studies also present no effect of increasing the N dose on the grass plants' height at 44 and 60 days after emergence (DAE) (Repke et al., 2013; Wolschik et al., 2003).

Regarding the stem diameter, the increase of 16.34 % (SF4) with the inoculation of the isolates (**Fig. 1 c**) differs from the results found by Kappes et al. (2013) and Andrade et al.

(2019), in which no difference was observed for the stem diameter of sorghum and corn plants inoculated with diazotrophic bacteria. An increase in stem diameter is interesting because it reflects less plant lodging and breakages, and it is also related to increased productivity due to the greater capacity to store photo-assimilates (Kappes, 2011).

Shoot, root, and total dry mass

In the present study, all tested isolates and the MIX increased shoot and total dry mass accumulation (**Fig. 2 a, e**). Dry mass is an important variable that is directly related to the productivity of plants (Soares et al., 2017). There is a relationship between the increase in the N dose and the accumulation of dry mass, and the inoculation with plant growth-promoting bacteria can also increase the dry mass (Montañez et al., 2012; Mut et al., 2017). For forage sorghum, whose final purpose is forage for animal feed, that result represents an important characteristic. Some studies reported that inoculation of rice plants with diazotrophic bacteria of the genus *Herbaspirillum* and *Serratia* can result in 20 to 37.6 % more accumulation of total dry mass (Gyaneshwar et al., 2001; James et al., 2002). This increase may be due to the substantial addition of N since the element is related to protein synthesis, ion absorption, photosynthesis, respiration, multiplication, and cell differentiation. Thus, plants well supplied about the demand for N tend to achieve a better dry mass accumulation (Lemaire et al., 2007; Hirel et al., 2007). Although, the increase in dry mass may be due not only to BNF but also to the ability to produce other compounds, like phytohormones, related to plant growth promotion. Bacteria of *Sphingomonas*, *Burkholderia*, and *Rhizobium* genus can also promote plant growth through phosphate solubilization, phytohormones production, and antioxidative response (Datta & Basu 2000; Zeng et al., 2017; Wang et al., 2020). All isolates tested in this work can perform biological nitrogen fixation, phosphate solubilization, and indole-acetic acid production, which may have contributed to obtaining such results.

The isolates and the MIX did not influence the root dry mass accumulation (**Fig. 2 c**).

The inoculation with *Azospirillum* did not increase the sorghum root dry mass either (Andrade et al., 2019). That may occur because the soil volume exploited was limited due to cultivation in pots, and the supply of phosphorus, which is one of the most limiting nutrients for root growth in tropical soils, was carried out according to the technical recommendations to the crop. Another factor that can influence is the genotype of plants. The inoculation with *Azospirillum* influenced the root dry mass just in some genotypes of maize plants (Pereira et al., 2015). Some studies have demonstrated a relationship between plants and bacteria, and different responses can occur depending on sorghum genotype (Boddey et al. 1991; Yoon et al., 2016).

Shoot N content

The shoot N content is considered an important variable to provide a forage with better nutritional value. Thus, the increase in N content present in the shoots may result in better zootechnical indexes due to the higher protein value of this forage. As the dose of N increases, there is an increase in the shoot N content (**Fig. 3 b**). The application of a higher level of N tended to increase the nitrogen content and uptake by sorghum (Chaudhary et al., 2018). In soils with low N availability, such as the one used in this work, the application of nitrogen fertilizer can supply this deficiency and reflect better nitrogen content in the plant (Chaudhary et al., 2018).

The inoculation of the isolates and the MIX results in higher shoot N content, which corresponds to a 68.75 % increase. That can occur due to the capacity of these isolates to perform the BNF. These genera are reported as nitrogen fixers, even when associated with non-leguminous plants (Santi et al., 2013). These bacteria can colonize both the rhizosphere and the interior of the plant, including sorghum plants (Coelho et al., 2009, 2008; Mareque et al., 2015). Bacteria of the genus *Burkholderia*, for example, are characterized by high activity of nitrogenase, being a highly effective nitrogen fixer and, when associated with rice plants, can contribute with 31 – 42 % of plant nitrogen, which should reflect in the content of N in the shoot, which may indicate their potential use for inoculation with non-leguminous plants (Baldani et al., 2000; Elliott et al., 2007; Govindarajan et al., 2007).

There are also reports of the capacity of association between bacteria of the *Rhizobium* genus with grass species, yet their presence is more associated with benefits involving other mechanisms of growth promotion (Silva et al., 2020; Osorio Filho et al., 2016). Inoculation of rice plants with *Azorhizobium* sp. RFNB31 results in an increase in the accumulation of N by the plant when compared to the treatment uninoculated (Islam et al., 2009). Another report suggests that functional N_2-fixing bradyrhizobia associated with the roots of field-grown sorghum plants have significant N_2-fixing activities in late growth stages (Hara et al., 2019).

25 % reduction in Nitrogen dose

For the shoot and total dry mass, the treatments inoculated with the isolates SB5 (*Rhizobium*), SF4 (*Sphingomonas*), and MIX showed results equal to the C+ treatment (**Fig. 4**), this result corresponds to an increase between 74.97 % and 70.05 % about the C- (**Fig. 4 c, e**), respectively. The genus *Sphingomonas* include endophytic diazotrophic bacteria that form associations with different tropical grasses (Rangjaroen et al., 2015; Oliveira et al., 2018). However, studies involving efficiency in fixing N when associated with grasses plants are still scarce. There are also reports of the capacity of association between bacteria of the genus *Rhizobium* with non-leguminous species like maize, rice, lettuce, tomato, pepper, and canola, yet, their presence is more associated with other mechanisms of growth promotion like phosphate solubilization, IAA, and siderophores production (Chabot et al., 1996; Noel et al., 1996; García-Fraile et al., 2012). Regarding the shoot N content, just the treatment C- was different from the treatment C+ (**Fig. 4 f**), which demonstrates the potential of the isolates plus the MIX tested in the present study to improve the N content in the shoot, providing a forage with better nutritional value.

Thus, compared to plants that received 100 % N dose, the isolates SB5, SF4, or the

MIX can reduce by 25 % the N dose at the sowing of forage sorghum plants under greenhouse conditions. Other studies report that the inoculation of *Azospirillum* bacteria can reduce by 25 to 50 % the N dose applied at the sowing of grass plants (Hungria et al., 2010; Piccinin et al., 2013; Fukami et al., 2016). There is also a report that sorghum plants inoculated with *Glucanocetobacter* bacteria obtained 5 to 45 % of N through BNF (Zinniel et al., 2002). For forage sorghum, a MIX containing bacteria of the genus *Burkholderia* and *Herbaspirilum*, there may be a contribution of up to 31.4 % in obtaining N via BNF, which is equivalent to 23.3 kg ha^{-1} (Santos et al., 2017). Even presenting an average equal to SB5 and SF4 isolated, maybe the MIX can confer advantages in field conditions due to functional redundancy, so even if there are unfavorable conditions for some isolates, the others can still contribute to the promotion of plant growth.

CONCLUSIONS

The isolates SB5 (*Rhizobium* sp.), SF4 (*Sphingomonas* sp.), and the mixture containing both isolates have the potential to promote growth of forage sorghum plants and reduce the need for fertilizer by up to 25% under greenhouse conditions. This demonstrates the potential for using these isolates and the mixture as biotechnology. However, further studies are required to evaluate the impact on plant physiology and agronomic performance in field conditions.

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DISCLOSURE STATEMENT

The author declares no conflict of interest.

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pH	P	K	Na	Ca^{2+}	Mg^{2}	Al^{3+}	H+Al	SB	(t)	(T)	V	m
H_2O	mg / dm^3			$cmol_c/dm^3$							%	
5,40	1,41	13	-	0,24	0,08	0,00	0,66	0,32	0,32	0,98	34,7	0,00

Table 1 – Chemical soil analysis

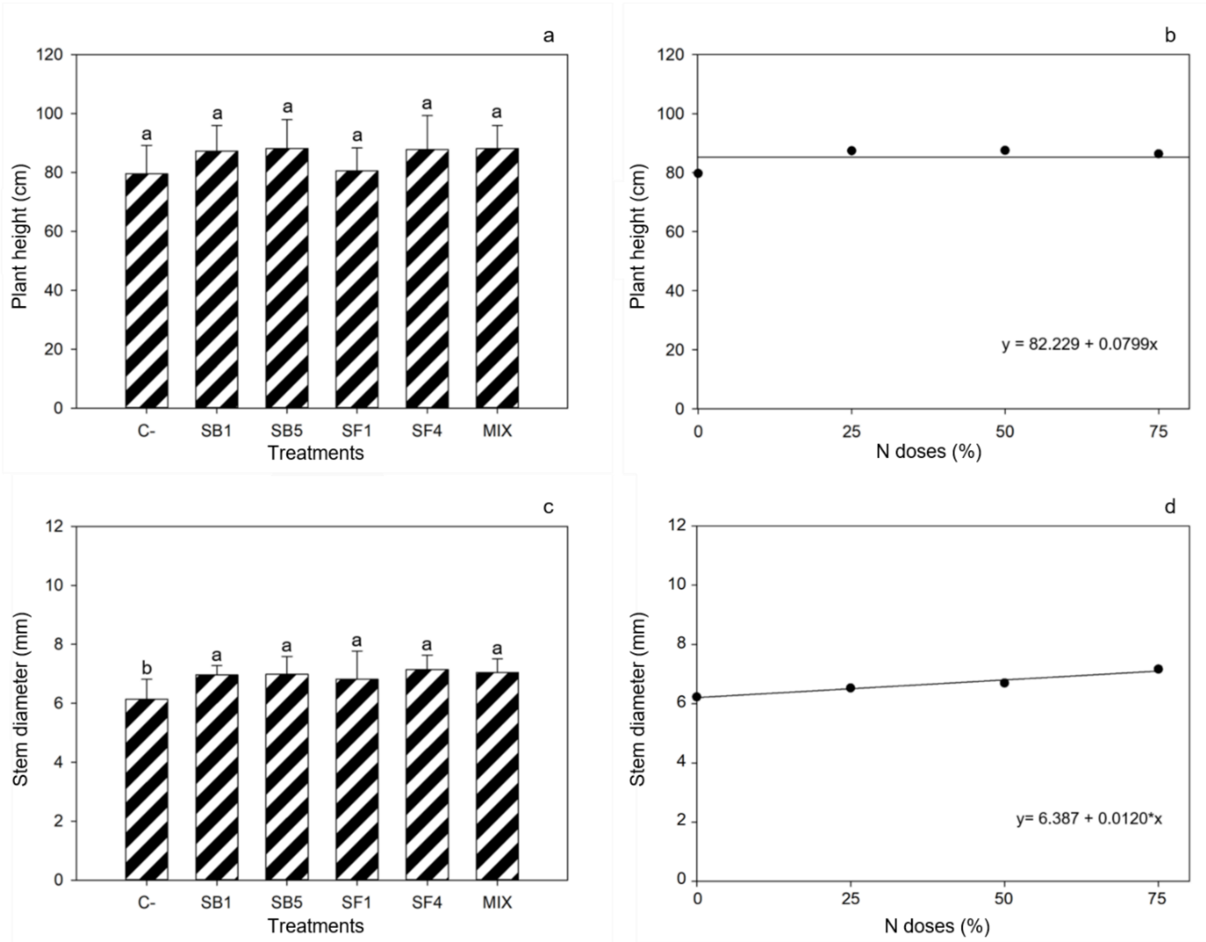

Fig. 1 - Plant height values (1 a) and stem diameter (1 c). Uninoculated (C-), plants inoculated with different bacterial isolates (SB1, SB5, SF1, and SF4) and the mixture of all isolates (MIX). Means followed by the same letter do not differ from each other by the Tukey test ($p < 0.01$). The relationship between plant height (1 b), stem diameter (1 d), and different nitrogen fertilizer doses (0, 25, 50, and 75% of the recommended dose), angular coefficients followed by * are significant by the test T ($p < 0.01$).

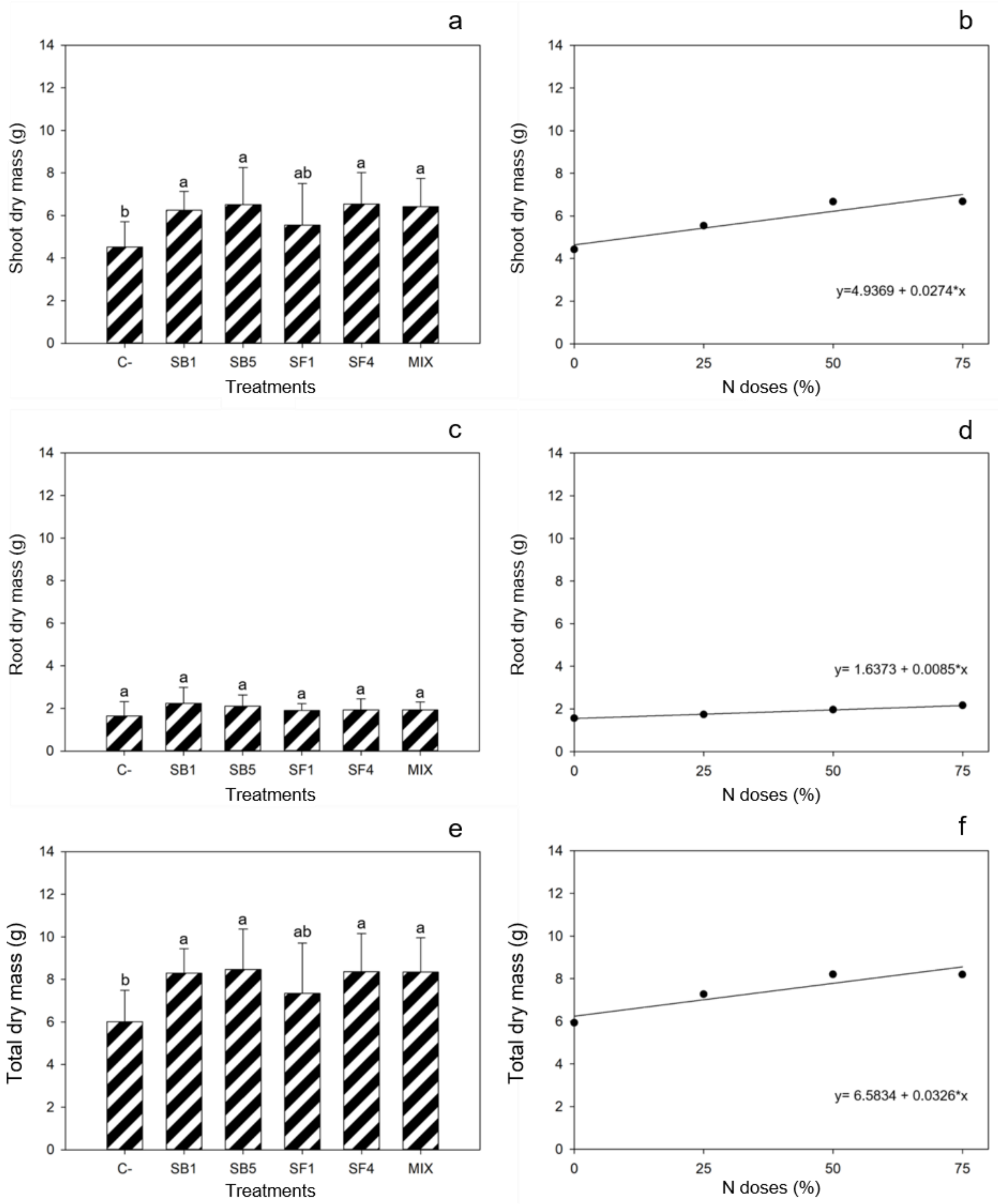

Fig. 2 - Values of the shoot (2 a), root (2 c), and total dry mass (2 e). Uninoculated (C-), plants inoculated with different bacterial isolates (SB1, SB5, SF1, and SF4) and the mixture of all isolates (MIX). Means followed by the same letter do not differ from each other by the Tukey test ($p < 0.01$). The relationship between shoot (2 b), root (2 d), and total dry mass (2 f) and different nitrogen fertilizer doses (0, 25, 50, and 75 % of the recommended dose), angular coefficients followed by * are significant by the test T ($p < 0.01$).

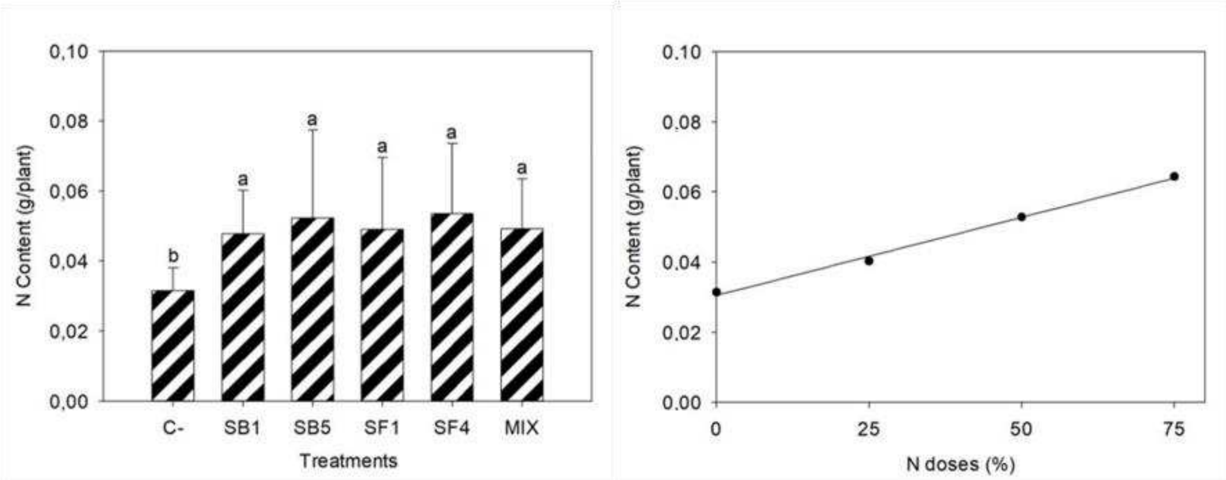

Fig. 3 – Shoot N content. Uninoculated (C-), plants inoculated with different bacterial isolates (SB1, SB5, SF1, and SF4) and the mixture of all isolates (MIX). Means followed by the same letter do not differ from each other by the Tukey test (p <0.01). The relationship between N content and different nitrogen fertilizer doses (0, 25, 50, and 75 % of the recommended dose), angular coefficients followed by * are significant by the test T (p <0.01).

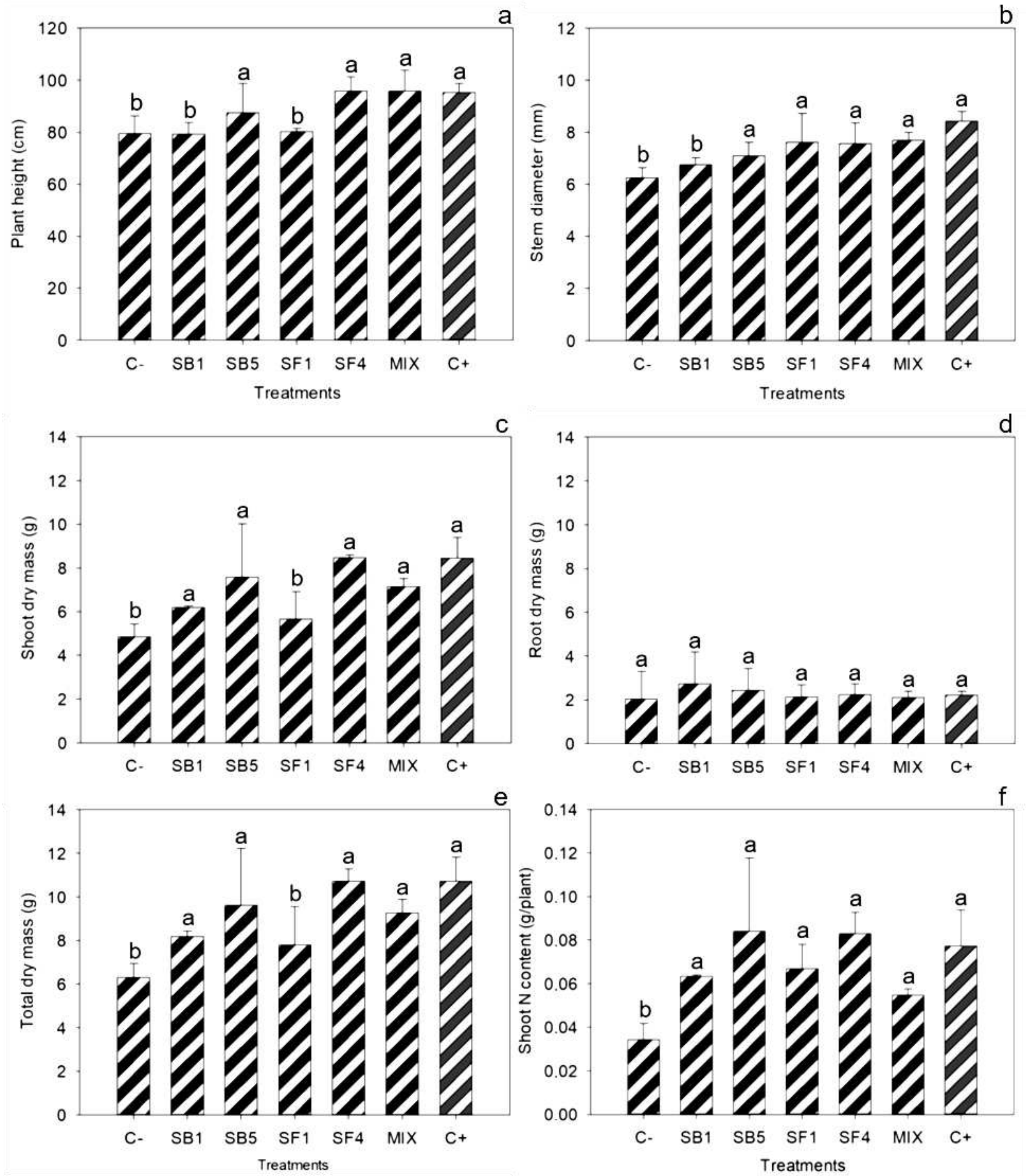

Fig. 4 – Plant height (4 a). Stem diameter (4 b), shoot dry mass (4 c), root dry mass (4 d), and total dry mass (4 e). Comparison between the control with 100 % of the dose of N and the different treatments in the dose of 75 % of N. Plants without inoculation of bacteria + dose of 100% (C+), Uninoculated + dose of 75 % of N (C-), inoculated plants with the different bacterial isolates (SB1, SB5, SF1, and SF4) and the mixture of all isolates (MIX) + dose 75 % of N. Means followed by the same letter do not differ from the Control with 100% of N by the Dunnett test ($p < 0.1$).

Chapter 3 – Selected bacteria can reduce synthetic N fertilization dependence in sorghum

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RESUMO

PAULA, T. A., D.Sc., Universidade Federal de Viçosa, Setembro de 2022. **Bactérias selecionadas podem reduzir a dependência da adubação nitrogenada sintética em sorgo.** Orientador: Leonardo Duarte Pimentel. Coorientadores: Maria Catarina Megumi Kasuya e Marliane de Cássia Soares da Silva.

Nas últimas safras, o setor agrícola brasileiro enfrentou aumento no preço e escassez de fertilizantes. Na safra 2021/2022, a quantidade de fertilizantes importados pelo mercado brasileiro foi recorde e atingiu 39 milhões de toneladas. Essa dependência pelo mercado externo é um dos fatores responsáveis pelo alto custo da produção agrícola brasileira. Neste contexto, é fundamental desenvolver novas tecnologias para reduzir a dependência do país pela importação de fertilizantes. Este trabalho teve como objetivo testar bactérias diazotróficas selecionadas e avaliar o potencial biotecnológico como inoculante para culturas de sorgo em condições de campo. Sementes de sorgo forrageiro foram inoculadas com bactérias fixadoras de nitrogênio combinadas a diferentes doses de fertilizante nitrogenado sintético, a fim de se obter uma combinação ótima de adubação biológica associada à adubação mineral. Para isso foram testados dois isolados pré-selecionados de bactérias diazotróficas (SB5 – *Rhizobium* sp.; SF4 – *Sphingomonas* sp.) e sua mistura (MIX), em solo adubado com diferentes doses de N sintético em cobertura, durante 120 dias, em condições de campo. O aumento da dose de fertilizante sintético e a inoculação com o MIX aumentam o diâmetro do caule, a massa seca da parte aérea, o teor de N da parte aérea e a produtividade da cultura do sorgo em condições de campo ($p<0,05$). Assim, quando comparadas às plantas que receberam 100 % da dose recomendada de N sintético, a inoculação com o MIX combinada à adubação mineral tem potencial para reduzir entre 25 a 50% a dose de fertilizantes nitrogenados sintéticos utilizados durante o cultivo do sorgo forrageiro para se obter a produtividade máxima da cultura ($p<0,1$).

Palavras-chaves: Fertilizantes; Nitrogênio sintético; Redução de custos.

ABSTRACT

PAULA, T. A., D.Sc., Universidade Federal de Viçosa, September, 2022. **Selected bacteria can reduce synthetic N fertilization dependence in sorghum**. Adviser: Leonardo Duarte Pimentel. Co-advisers: Maria Catarina Megumi Kasuya and Marliande de Cássia Soares da Silva.

The Brazilian agricultural sector has faced challenges in recent harvests, including an increase in the price and scarcity of fertilizers. In 2021, fertilizer imports reached a record 39 million tons, making the country heavily dependent on the foreign market and contributing to the high cost of production. In order to reduce this dependence, it is necessary to develop new technologies. The study aimed to test selected diazotrophic bacteria and evaluate their potential as an inoculant for sorghum crops in field conditions. Forage sorghum seeds were inoculated with nitrogen-fixing bacteria and combined with increasing doses of synthetic nitrogen fertilizer to find the optimal combination of biological and mineral fertilization. Two bacteria isolates (SB5 - *Rhizobium* sp. and SF4 - *Sphingomonas* sp.) and their mixture (MIX) were tested with different doses of synthetic N applied to the soil, for 120 days, in field conditions. An increase in the dose of synthetic fertilizer and inoculation with the MIX resulted in a significant ($p<0.05$) increase in stem diameter, shoot dry mass, shoot N content, and sorghum crop yield under field conditions. Compared to plants that received 100% of the recommended synthetic N dose ($p<0.1$), inoculation with the MIX combined with mineral fertilization showed the potential to reduce N fertilization by 25-50% during the cultivation of forage sorghum, leading to the maximum crop productivity.

Key-words: Fertilizers; Synthetic nitrogen; Cost reduction.

INTRODUCTION

Brazil occupies fourth place in the ranking of the largest consumers of agricultural fertilizers, but it has only 2% of global production, and more than 80% of the fertilizer used comes from imports (SAE, 2022). In 2021, the total amount of fertilizers imported by the Brazilian market was a record and reached 39,201,535.00 tons between January and December, an increase of 19.3% compared to 2020, while national production corresponded to 6,990,065 tons (ANDA, 2022) In the last crops, the Brazilian agricultural sector faced an increase in the price and scarcity of fertilizers. This crisis in the fertilizers sector should have consequences in the coming harvests, especially during the second crop. Thus, it should result in lower production and, consequently, a shortage of food for the domestic market and a rise in prices, with the producer and the final consumer impact.

However, it is chief to develop new technologies to reduce the country's dependence on the foreign fertilizer market. In this sense, the Brazilian federal government, in 2020 and 2022, launched the National Bioinputs Program and the National Fertilizer Plan 2050 to promote the sustainable development of Brazilian agriculture (MAPA, 2020; SAE, 2022). Thus, the use of bacteria that inhabit agricultural soils and form beneficial interactions with plants is considered a solution to this bottleneck and an alternative to increasing production, reducing costs and achieving a sustainable agriculture model (Qiu et al., 2019). These microorganisms, called Plant Growth Promoters Bacteria (PGPB), can colonize the rhizosphere, roots, and leaves of plants and act by various mechanisms such as the biological nitrogen fixation, among others (Gao et al., 2019; Qiu et al., 2019; Zhou et al., 2020; Cubillos-Hinojosa et al., 2021; Goyal et al., 2021). Due to these characteristics, the agricultural interest in these microorganisms has increased, mainly due to the high cost of the current model and external pressures for environmental conservation. Among the plant growth-promoting bacteria, an essential group is the diazotrophic bacteria capable of performing BNF. But studies about the association of diazotrophic bacteria and its efficiency in fixing N when associated with sorghum are still scarce.

In the current context, especially during second crop cultivation in Brazil (February – March), the sorghum, an excellent source of food for both humans and animals and a source of bioenergy, appears as an alternative for planting, especially in regions with arid and semi-arid climates (Coelho et al., 2002; de Morais Cardoso et al., 2017; Adiamo et al., 2018; Ratnavathi, 2019; Velmurugan et al., 2020). However, sorghum cultivation has been growing in several Brazilian regions, with an increase of 20.8% in the cultivated area and in the production of 43.2% higher than the previous crop during the 2021/2022 harvest (CONAB, 2022). Moreover, although sorghum is associated with diazotrophic bacteria (Wasai-Hara et al., 2020; Barros et al., 2020), there are no commercial inoculants registered for being used in sorghum in Brazil. For sorghum, new technologies that reduce the dependence on nitrogen fertilizers become even more chief to reduce the risks linked to the initial investment.

This work aimed to test selected diazotrophic bacteria and evaluated the biotechnological potential as inoculants for sorghum crops under field conditions.

MATERIALS AND METHODS

The work was carried out in partnership between the Laboratory of Sorghum, Department of Agronomy, and Laboratory of Mycorrhizal Associations (LAMIC), located in the Institute of Applied Biotechnology for Agriculture and Livestock (BIOAGRO), Department of Microbiology, both belonging to Universidade Federal de Viçosa - UFV, in Viçosa - Minas Gerais, Brazil.

Sowing, fertilization, and cultivation period

The experiment was executed under field conditions from November to March, 120 days totalizing. The experimental design was a randomized block with three replications per treatment, following a subdivided parcel (5 x 4) in which five top-dressing nitrogen fertilizers doses were used (0, 25, 50, 75, and 100 % of the recommended dose), and four inoculant formulations (without inoculation, each bacterium inoculated separately, and a MIX containing all bacteria). Each plot consisted of four lines of five meters with a spacing between lines of 0.78 meters.

Forage sorghum seeds were inoculated with one of two bacteria (SB5 – *Rhizobium* sp.; SF4 – *Sphingomonas* sp.) or a MIX containing all tested isolates capable of performing biological nitrogen fixation. These isolates were obtained by isolation from roots of sorghum plants cultivated during the second crop, and their potential use as an inoculant was previously tested under greenhouse conditions.

The different isolates stored in NBY (nutrient broth yeast extract) medium (Schaad, 1998) containing glycerin (15 %) were grown in liquid medium broth potato dextrose (PD) under constant agitation of 120 rpm, until reaching the value of 2 x 10^9 CFU mL^{-1}, and to reach this value the growth curve of each tested isolate was performed. The growth curve was performed by cultivating the different isolates in a liquid PD medium under constant agitation of 120 rpm and measuring the optical density (OD) at the wavelength of 520 nm, followed by the plating of an aliquot of 0.1 mL to calculate the colony-forming units (CFU), the procedure was performed every four hours until the isolates reached the stability phase.

Fertilization was carried out in a planting furrow at a distance of 6 to 8 cm from the seed planting. Phosphate, potassium, and planting nitrogen fertilization were carried out according to the technical recommendation after chemical analysis of the soil (**Table 1**) (Alves et al., 1999). Top-dressing nitrogen fertilization was performed using urea following the technical recommendation (Alves et al., 1999), where top-dress fertilization was carried out 30 days after planting and performed using 0, 25, 50, 75, and 100% of the recommended dose, 160 kg ha^{-1}. The inoculation of the isolates and the MIX was performed using a dosage recommended for inoculants registered for other grasses in Brazil, 100 mL for 50 kg of seed. The MIX was carried out MIX cultivating the different isolates separately and after mixing with the appropriate proportions to adapt the dosage of 100 mL for 50 kg of seed.

After sowing, the plants were cultivated for 120 days when they were harvested to carry out evaluations of agronomic parameters. Three evaluations of agronomic parameters such as plant height, stem diameter, and dry mass accumulation were carried out before harvest and final evaluation of productivity, totaling four evaluations.

Plant height, stem diameter, shoot and root dry matter

Plant height (cm) was measured with a tape measure considering the distance from the neck to the end of the last developed leaf. Stem diameter (mm) was determined at the height of the cervix using a digital caliper.

For shoot and root dry matter determination, the collected material was washed using tap and distilled water and dried at 60 °C in a ventilated oven for 72 hours until reaching a weight constant (Hungria et al., 2010).

Digestion and determination of N content in the shoot

After dry mass determination, the samples were ground using a knife mill and a 1 mm sieve for further digestion and analysis of nutrient content. Nitrogen content was determined by the Kjeldahl semi-micro method (Bremner and Mulvaney 1978; Tedesco et al., 1995).

Productivity

After determining the fresh and dry matter per plot, sorghum' s productivity per hectare was estimated. For the evaluation of the final productivity, the two central lines of each parcel were considered as useful area of evaluation. For evaluation, the plants were collected in one linear meter within the five-meter planting line with a spacing of 0.78 meters between lines for sorghum, totaling 2.8 m² of useful area.

Statistical analysis

Data analysis was performed using the statistical software R (R Core Team, 2018). The data were submitted to ANOVA, followed by Tukey test for the comparison of means and the t-test for the slope of the different equations ($p < 0.05$). To compare the distinct isolates about the 100 % N dose, the data were subjected to analysis of variance (ANOVA) followed by the Dunnett test ($p < 0.1$).

RESULTS AND DISCUSSION

For plant height, stem diameter, dry mass, shoot N content, and final productivity, no interaction was observed between inoculated isolates and nitrogen doses applied at sowing ($p<0.05$), the results are shown independently.

Plant height, stem diameter and shoot dry mass

Regarding the plant height, the inoculation of the isolates or the MIX did not result in plant growth (Fig. 1). This result corroborates with the results found by Andrade 2019, in which no difference was observed for the plant height of sorghum inoculated with diazotrophic bacteria during the sowing.

The inoculation with the MIX and the increase in top-dressing N fertilization results in stem diameter increase (Fig 1c), that differs from others results in which no difference was observed for the stem diameter of sorghum and corn plants inoculated with diazotrophic bacteria (Andrade et al., 2019, Reznick et al., 2019). Other studies report an increase of up to 37 % in stem diameter due to inoculation with plant growth-promoting bacteria (Marques et al., 2020). That increase in stem diameter, by the MIX inoculation, correspond to 4,59 % about control, what can result in greater productivity, less plant lodging and breakages (Kappes, 2011).

Inoculation with the MIX and the increase in top-dressing N fertilization also increase shoot dry mass accumulation in 13,44 % and 27,17 %, respectively (**Fig. 1 e, f**). Dry mass is directly related to the productivity of plants, and there is a relationship between the increase in the N dose and the accumulation of dry mass (Soares et al., 2017). Therefore, the inoculation with diazotrophic bacteria can also increase dry mass since these can supply N to the plant, and it occurs because N is related to protein synthesis, ion absorption, photosynthesis, respiration, multiplication, and cell differentiation (References). Thus, plants well N-supplied tend to achieve a better dry mass accumulation (Lemaire et al., 2007; Hirel et al., 2007).

Productivity

Regarding productivity, both the inoculation with MIX and the increase in the top-dressing N dose resulted in an increase in final productivity, whether in terms of fresh or dry matter, which is related to the increase in dry mass accumulation per plant shown earlier (**Fig. 1 e, f**). The results obtained corroborate with the positive responses related to the accumulation of dry mass by the plants due to the PGPB inoculation and N doses application (Araújo et al., 2004; Gomes et al., 2007). Thus, inoculation with the MIX would increase N uptake by sorghum plants, which would explain the higher productivity. The plant growth process depends on N for protein synthesis, ion absorption, photosynthesis, respiration, cell multiplication, and differentiation, providing abundant green vegetation with increased foliage and rapid growth (Okumura et al., 2011). These results also demonstrate the importance of adequate fertilization during the crop to obtain better productivity rates.

The increase resulting from the MIX inoculation corresponds to 14,00 % and 13,44 % concerning the uninoculated control of fresh and dry mass, respectively, which can result in economic gains for the grower (**Fig. 2 a, c**). Studies involving inoculation with nitrogen-fixing bacteria report an increase of 11.7 to 89% in total accumulation of biomass and until 36.7% in the total accumulation of a dry mass of non-legume plants can occur (Reference). Inoculants containing diazotrophic bacteria are available for some crops, and 5 % to 30 % increases in yields have already been reported (Vessey, 2003; Karthikeyan et al., 2007; Bhattacharyya and Jha, 2012). However, there are still no efficient commercial inoculants for the sorghum crop. In this way, a commercial inoculant to promote plant growth can reduce the need for nitrogen fertilization carried out through chemical fertilizers in sorghum planting and, consequently, production costs, which can readjust the fertilization system reducing production costs and improving agricultural sustainability indicators. Thus, for new technologies development, such as inoculants, it is necessary to combine balanced fertilization with selection and the introduction of effective bacteria to promote plant growth in the agricultural production system.

Shoot N content

For the shoot N content, both the inoculation with the MIX and the top-dressing N dose increased the N content (**Fig. 3**). The shoot N content is important to provide a forage with better nutritional value. Thus, a greater N content may result in better zootechnical indexes due to the higher protein value.

The inoculation of the MIX results in higher shoot N content which corresponds to a 13,63 % increase per plant and 13, 64 % per ha. That can occur due to the capacity of these isolates to perform the BNF. In non-leguminous, such rice and maize, associative nitrogen fixation can supply until 25 % of total nitrogen requirements (Montanez et al., 2012). Bacteria of the genus *Burkholderia* are characterized by high activity of nitrogenase and can contribute with 31– 42 % of non-leguminous plant nitrogen, which should reflect in the content of N in the shoot (Baldani et al., 2000; Elliott et al., 2007; Govindarajan et al., 2007). For forage sorghum, a MIX containing bacteria of the genus *Burkholderia* and *Herbaspirilum*, can obtain an amount of N about 31.4 % by the FBN process (Santos et al., 2017).

There are also reports of the association between bacteria of the *Rhizobium* genus with grass species, and there is evidence that functional N_2-fixing bradyrhizobia is associated with the roots of field-grown sorghum plants have significant N_2-fixing activities in late growth stages (Hara et al., 2019).

Reduction in Nitrogen dose

Regarding the possible reduction and savings in the use of nitrogen fertilizers during sorghum cultivation under field conditions, the MIX inoculation has a more prominent effect when combined with intermediate doses of cover fertilization (**Fig. 4 a, b**). When combined with the 50 % recommended dose, both the productivity and the N content of the treatment inoculated with MIX are equal to the treatment C+ and differ from the treatment C-, which can demonstrate the possible effect of inoculation (**Fig 4 a, b**). From the 75% dose, there is no effect of inoculation with MIX since there is no significant difference between the different treatments (**Fig 4 c, d**). However, further experiments are needed to determine the combination between the MIX and the optimal fertilization rate since, in the present work, it was not possible to estimate the maximum within the equation generated by the regression with the different top-dressing N fertilization. Other studies report that the inoculation of *Azospirillum* bacteria can reduce by 25 to 50 % the N dose applied at the sowing of grass plants, what corroborates with the present work (Hungria et al., 2010; Piccinin et al., 2013; Fukami et al., 2016).

The "Diogo Alves de Mello" experimental field is characterized not only by the cultivation of sorghum (*Sorghum bicolor*) but also by other summer crops such as soybeans (*Glycine max*), corn (*Zea mays*), and beans (*Phaseolus vulgaris*) during the period of the main crop (October - December). In this way, the soil of the experimental field can be characterized by having fertility built up over time, so fertilization with 75% of the recommended dose for the crop would already be enough to meet the plant's needs, which would explain the effect of inoculation at intermediate doses such as 50% of the recommended dose. With the increase in the N availability in the environment, plants tend to exclude associations with some groups of diazotrophic bacteria since this association becomes an unnecessary energy cost, then with a higher level of N essentially tending to reduce the association of the plant with diazotrophic bacteria (Bahulikar et al., 2020).

CONCLUSION

Inoculation with a mixture containing all tested isolates of *Rhizobium* sp. and *Sphingomonas* sp. (MIX) increased the productivity of forage sorghum, promoted plant growth, and reduced the need for fertilizer by 25 to 50% under field conditions. These results suggest a need to reconsider the fertilizer recommendation model, taking into account the synergistic effect between biological fertilizer and mineral/synthetic fertilizer, especially with regards to nitrogen. While the results demonstrate the potential of using the mixture as a biotechnology, further experiments are necessary to determine the optimal combination of the mixture and fertilizer rate.

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DISCLOSURE STATEMENT

The author declares no conflict of interest.

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Table 1 – Chemical soil analysis

pH	P	K	Na	Ca^{2+}	Mg^{2}	Al^{3+}	H+Al	SB	(t)	(T)	V	m
H_2O	mg / dm^3			$cmol_c/dm^3$							%	
5.80	12.4	49	-	3.10	0.82	0.00	3.90	4.05	4.05	7.95	50.09	0.00

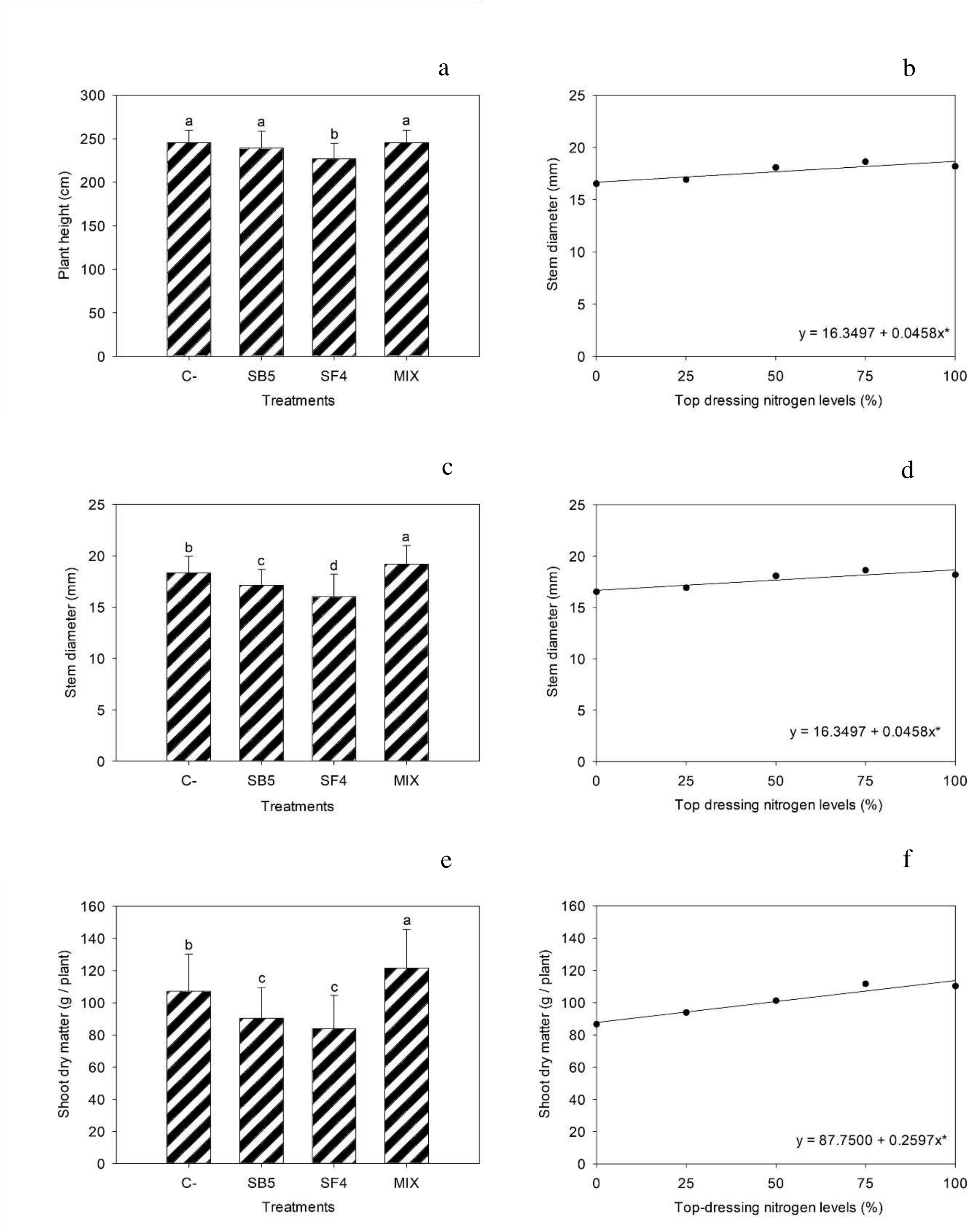

Fig. 1 - Plant height values (2 a) stem diameter (2 c) and dry matter (2 e). Uninoculated treatment (C-), plants inoculated with different bacterial isolates (SB5 and SF4) and the mixture of the two isolates (MIX). Means followed by the same letter do not differ from each other by the Tukey test ($p < 0.05$). The relationship between plant height (b), stem diameter (d), and dry mass (f) and different top-dressing N doses (0, 25, 50, 75 and 100 % of the recommended dose), angular coefficients followed by * are significant by the test T ($p < 0.05$).

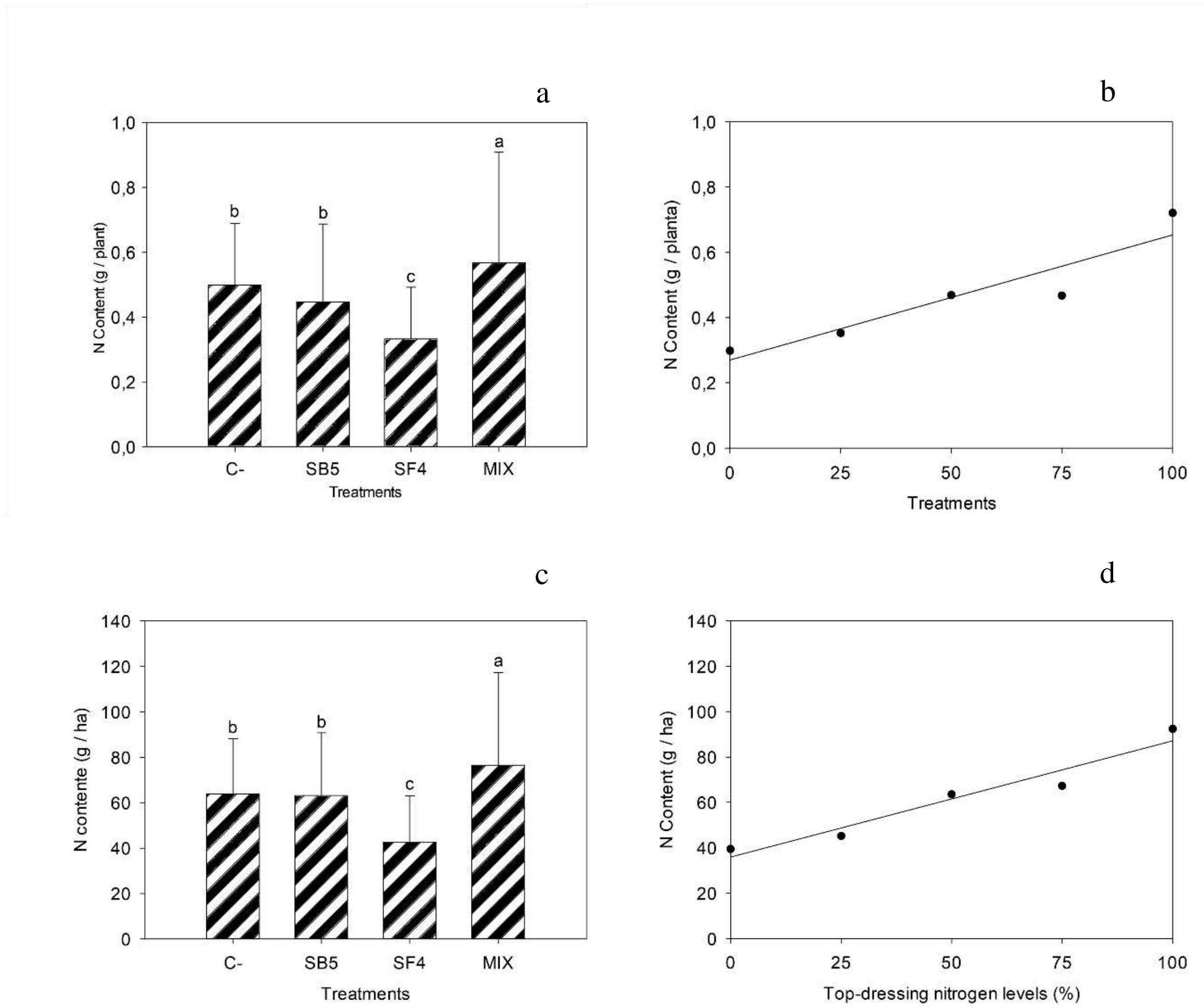

Fig. 2 – Shoot N content. Uninoculated treatment (C-), plants inoculated with different bacterial isolates (SB5 and SF4) and the mixture of the two isolates (MIX). Means followed by the same letter do not differ from each other by the Tukey test (p <0.05). The relationship between shoot N content and different top-dressing N doses (0, 25, 50, 75 and 100 % of the recommended dose), angular coefficients followed by * are significant by the test T (p <0.05).

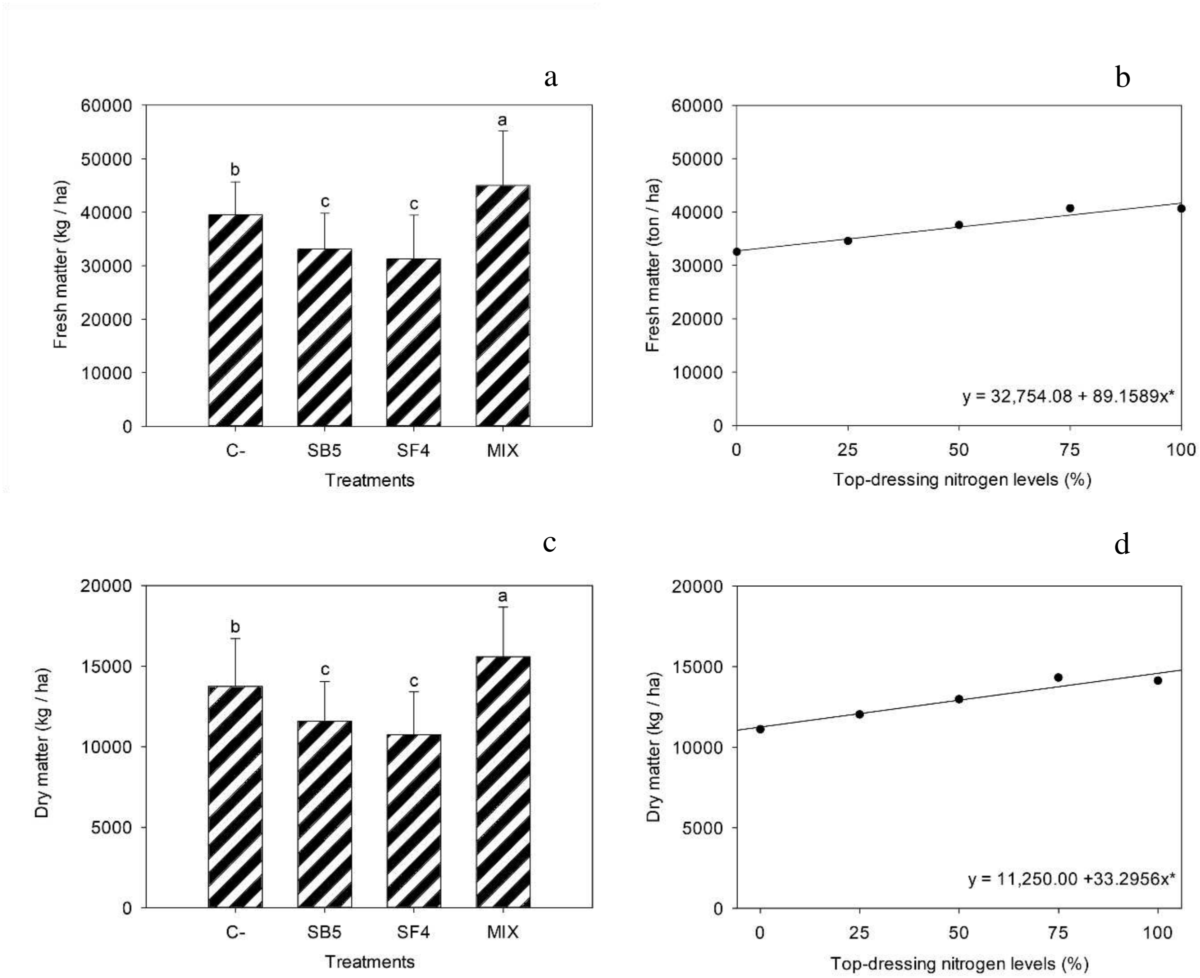

Fig. 3 - Values of the productivity. Fresh matter (3 a) and dry matter (3 c) (Kg ha^{-1}). Uninoculated treatment (C-), plants inoculated with different bacterial isolates (SB5 and SF4) and the mixture of the two isolates (MIX). Means followed by the same letter do not differ from each other by the Tukey test (p <0.05). The relationship between productivity, fresh matter and dry matter, and different top-dressing N doses (0, 25, 50, 75 and 100 % of the recommended dose), angular coefficients followed by * are significant by the test T (p <0.05).

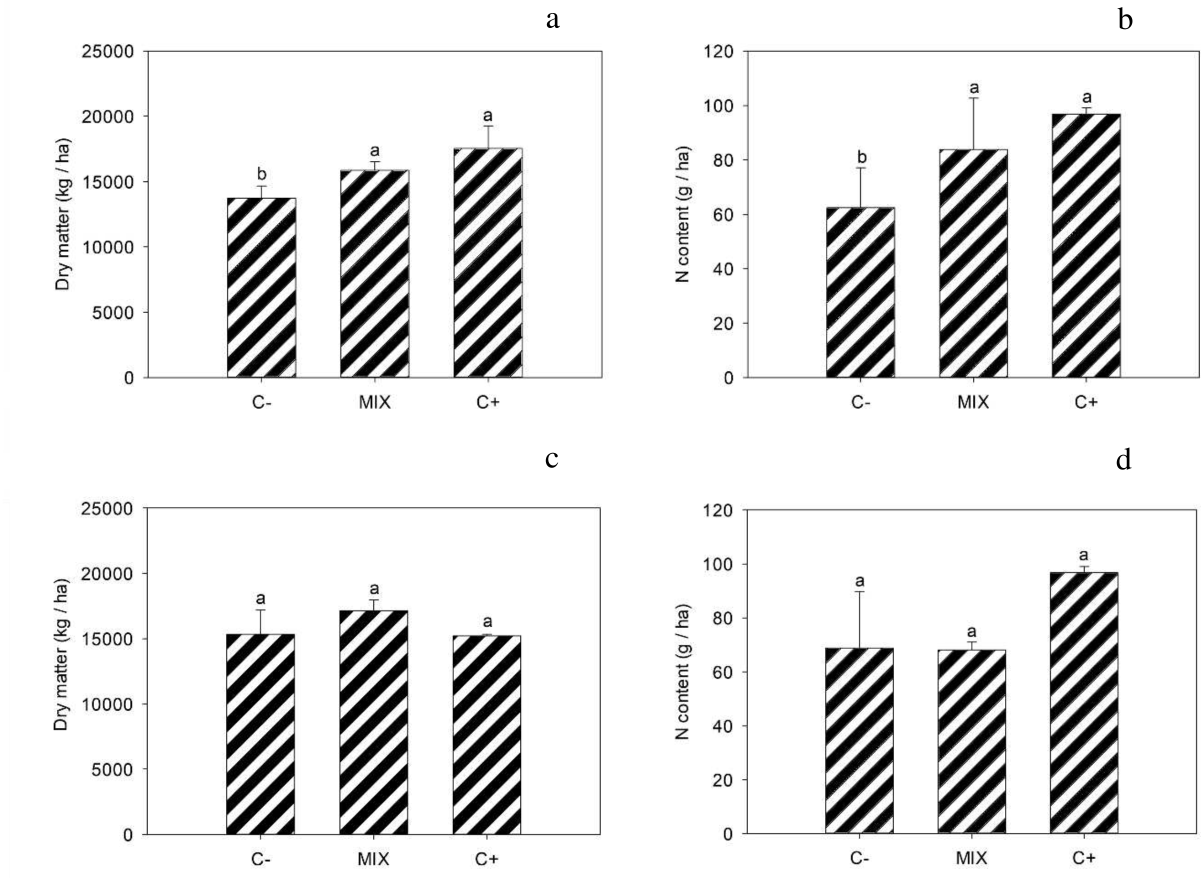

Fig. 4 – Dry matter and shoot and content. Comparison between the control with 100 % of the dose of N and the MIX treatments in the doses of 50 % (4 a, b) and 75 % (4 c, d) of top-dressing N. Plants without inoculation Uninoculated + dose of 50 % of N (C-), the mixture of all isolates (MIX) + dose 50 % of N and of bacteria + dose of 100 % uninoculated (C+). Plants uninoculated + dose of 75 % of N (C-), the mixture of all isolates (MIX) + dose 75 % of N and of bacteria + dose of 100 % uninoculated (C+). Means followed by the same letter do not differ from the Control with 100% of N by the Dunnett test (p <0.01).

FINAL CONSIDERATIONS

In the coming years, must be an increase in the use of fertilizers in Brazil, mainly nitrogenous ones, to meet the intensification of agriculture, which will increase production costs. For sorghum crop, it won't be different since its crop rising over the years, mainly due to the interest in its potential for the bioenergy sector. Thus, because the fertilize crise in the last crops, Brazil faced an increase in the price and scarcity of the agriculture input, which must result in the growing interest about inoculants containing nitrogen-fixing bacteria. This interest must be stimulated by the Brazilian Federal government, which, through the national plan for bio inputs, aims to encourage and develop research for new technologies for the agricultural sector.

Like other grasses, the present work proved that sorghum has potential to form associations with nitrogen-fixing bacteria. With the evolution of the studies, it is expected to identify the microorganisms with greater efficiency in carrying out the BNF in association with the culture, as well as to determine the optimal conditions for the interaction between sorghum and diazotrophic bacteria, in order to maximize the yield of these interactions in terms of agricultural productivity, both through biological nitrogen fixation and promotion of plant growth. In this way, it is possibility of reduce the use of nitrogen fertilizers during its cultivation, which can result in economic, environmental and social benefits. It also seeks to reduce the risks and the initial investment linked to sorghum crops in the off-season, encouraging its production and boosting the agricultural and bioenergy sector.

Therefore, it is necessary to identify and solve the bottlenecks that still exist in the research for a new fertilization model resulting from an integrated strategy combining chemical with biological fertilization.