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DANIELE SCUDELETTI

THE SUGARCANE DEVELOPING AS A FUNCTION OF MICROORGANISM ADOPTION TO POTENTIALIZE THE PRODUCTION

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TÍTULO DA TESE: THE SUGARCANE DEVELOPING AS A FUNCTION OF MICROORGANISM ADOPTION TO POTENTIALIZE THE PRODUCTION

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To my beloved parents,

Leucina and Mauricio,

I dedicate.

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ABSTRACT

The use of microorganisms has contributed to the greater optimization of agricultural areas by increasing productivity, as they may contribute to some mechanisms related to plant growth, biological nitrogen fixation and attenuation of water stress. Thus, the objectives were divided into two studies (Chapters 1 and 2). In Chapter 1, the study aimed to evaluate the efficacy of doses and inoculation modes of *Azospirillum brasilense*, on biometric, physiological and technological parameters, as well as improvements in the absorption of nutrients and productivity of sugarcane. The study was carried out in an experimental area belonging to Raízen Mill, located in the municipality of Santa Maria da Serra - SP, in two crop seasons (2015/2016 and 2016/2017), in which the doses of the inoculant containing *Azospirillum brasilense* and the method of inoculation were evaluated. In general, the application of the dose of 15 and 20 × 10^{10} UFC ha^{-1} of the inoculant *Azospirillum brasilense*, mainly stimulated the biometric characteristics, reflected in the higher productivity of stalks, sugar and economic return. As for the mode of inoculation, further investigation of the impact of weather conditions on microbial shelf life and on the efficiency of the use of the inoculant is necessary, guaranteeing the most appropriate time and method of application to minimize potentially adverse conditions. In Chapter 2, the objective of the study was to evaluate the inoculation of *Trichoderma asperellum* in the absence or presence of drought stress in the biometric, physiological, biochemical and enzymatic parameters, and in the physico-chemical characterization of leaf sugars and the possible improvements in sugarcane conditioning. sugar in situations of drought stress. The experiment conducted in a greenhouse at College of Agricultural Science (FCA-UNESP) and the response of plants by association with *Trichoderma asperellum* under conditions of drought stress in sugarcane was evaluated. The use of this microorganism provided better results, contributing significantly to the conditioning of plants under drought stress and improving the biometric, physiological, biochemical, enzymatic parameters, as well as the physicochemical characterization of leaf sugars. In summary, both experiments found benefits from microorganisms: *Azospirillum* stimulates the growth and development of sugarcane, reflecting in increased productivity and greater economic return from sugarcane production; and *Trichoderma* improved the physiological parameters, the activation of the enzyme nitrate reductase and the antioxidant metabolism, providing better growth, development, tillering and the productivity of sugarcane, mainly under drought stress conditions.

Keywords: *Azospirillum brasilense*. *Trichoderma asperellum*. *Saccharum* spp. Productivity.

RESUMO

O uso de microrganismos tem contribuído para a maior otimização das áreas agricultáveis por meio do aumento da produtividade, pois podem contribuir para alguns mecanismos relacionados ao crescimento das plantas, fixação biológica de nitrogênio e atenuação do estresse hídrico. Assim, os objetivos foram divididos em dois estudos (Capítulos 1 e 2). No Capítulo 1, o estudo visou avaliar a eficácia de doses e os modos de inoculação do *Azospirillum brasilense*, sobre os parâmetros biométricos, fisiológicos e tecnológicos, bem como as melhorias na absorção de nutrientes e na produtividade da cana-de-açúcar. O estudo foi realizado em área experimental pertencente à Usina Raízen, localizada no município de Santa Maria da Serra - SP, em duas safras (2015/2016 e 2016/2017), nos quais foram avaliados as doses do inoculante contendo *Azospirillum brasilense* e o modo de inoculação. Em geral, a aplicação da dose de 15 e 20 × 10^{10} UFC ha^{-1} do inoculante *Azospirillum brasilense*, estimulou principalmente as características biométricas, refletiu na maior produtividade de colmos, açúcar e retorno econômico. Quanto ao modo de inoculação, é necessária uma investigação mais aprofundada do impacto das condições meteorológicas na vida de prateleira microbiana e na eficiência do uso do inoculante garantindo tempo e o método de aplicação mais apropriados para minimizar condições potencialmente adversas. No Capítulo 2, o objetivo do estudo foi avaliar a inoculação de *Trichoderma asperellum* na ausência ou presença de estresse hídrico nos parâmetros biométricos, fisiológicos, bioquímicos e enzimáticos, e na caracterização físico-química dos açúcares foliares e as possíveis melhorias no condicionamento da cana-de-açúcar em situações de estresse hídrico. O experimento foi conduzido em casa de vegetação na Faculdade de Ciências Agronômicas (FCA-UNESP) e foi avaliada a resposta das plantas inoculadas com *Trichoderma asperellum* sob condições de estresse hídrico na cana-de-açúcar. O uso deste microrganismo proporcionou melhores resultados, contribuindo significativamente para o condicionamento de plantas sob estresse hídrico e melhorando os parâmetros biométricos, fisiológicos, bioquímicos, enzimáticos, bem como a caracterização físico-química dos açúcares foliares. Em resumo, ambos os experimentos encontraram benefícios advindos dos microrganismos: o *Azospirillum* estimula o crescimento e o desenvolvimento da cana-de-açúcar, refletindo no aumento da produtividade e maior retorno econômico da produção de cana-de-açúcar; e *Trichoderma* melhorou os parâmetros fisiológicos, a ativação da enzima nitrato redutase e o metabolismo antioxidante, proporcionando melhor crescimento, desenvolvimento, perfilhamento e a produtividade da cana-de-açúcar, principalmente em condições de estresse hídrico.

Palavras-chave: *Azospirillum brasilense. Trichoderma asperellum. Saccharum* spp. produtividade

SUMMARY

GENERAL INTRODUCTION

Climate change and increasing climate variability are affecting agricultural productivity, food production, and natural resources, with impacts on food systems and rural livelihoods, including a decline in the number of farmers and steady growth of people living in urban areas (FAO, 2019). The world population today is 7.7 billion and in 2050 it will surpass 9.7 billion people. These numbers represent a 26% growth from 2020 to 2050(EMBRAPA, 2020). The increase in population, life expectancy and purchasing power, implies greater consumptions of water, energy, food and fiber. All of this leads to major changes in the way food is produced, distributed, and consumed worldwide (FAO, 2019), causing consequently new challenges with the need to increase world agricultural production due to population growth.

Brazil is an important producer and one of the main global food suppliers. Among the main crops produced in Brazil, sugarcane (*Saccharum* spp.) stands out for being one of the activities that generate more jobs in the agribusiness sector, generates income helping in the trade balance for the production of sugar, ethanol, and bioenergy, being considered one of the main alternatives within this sector by the great production potential.

Between 1975 and 2019 the production of sugarcane increased from 91.5 million tonnes to 668 million tonnes, occupying just over 1% of the national territory (EMBRAPA, 2020). According to CONAB (2020), the estimated area for sugarcane production in the 2020/2021 harvest is close to 8,605 thousand hectares, which represents a increase of 1.9% concerning the last harvested area. The estimated average productivity of stalks is approximately 77 t ha^{-1}, a relatively low value when compared to its production potential (380 t ha^{-1}) (WACLAWOVSKY et al., 2010). Increase production and plant adaptation to stress conditions is a great challenge for current agriculture since the increase in production cannot be based solely on the increase in the cultivated area, but also on the improvements in the productivity of the area consolidated agriculture (VAN REES et al., 2014; LIZANA et al., 2006). However, the production approach nowadays is different from the agricultural model adopted since the Green Revolution (increase in productivity, expansion of agricultural frontiers, intensive soil use, reduction of biodiversity, etc.). The

concern about socio-economic and environmental importance is necessary for the development of sustainable practices to ensure greater productivity.

Thus, it is necessary to use new technologies that can provide productivity improvements. The intensification of research with the use of some microorganisms has shown to be a promising technology. In this context there are potential alternatives: the use of diazotrophic bacteria, such as *Azospirillum*, are capable of carrying out biological nitrogen fixation (BNF) and can also produce growth-promoting substances in plants, consisting of a promising technology, which can benefit crop growth and productivity; the use of the fungus *Trichoderma asperellum* are also an option that acts in the pathogens defense, contributing plant development and growth nutrients and stress resistance (OLIVEIRA et al., 2006; SCHULTZ et al., 2012, 2014, MENDES, R. et al., 2011; COLEMAN-DERR et al., 2014; COMPANT, S. et al., 2010; de SANTI FERRARA et al., 2012).

Diazotrophic bacteria are bridges in the production of growth regulators, obtaining greater development of the root system, which makes the absorption of water and nutrients more efficient (SPAEPEN et al., 2007; CASSÁN et al., 2014). The promotion of plant growth occurs because these bacteria stimulate the production of phytohormones, solubilization of phosphates, zinc and can also contribute to the induction of resistance to diseases, competition with pathogens, or even induction of water stress, by providing resistance to stresses (DOBBELAERE et al., 2003; ARENCIBIA et al., 2006; SARAVANAN et al., 2007).

Several diazotrophic bacteria were found and associated with sugarcane (SOUZA et al., 2016). Various species have been described, obtained from different parts of the plant (stalk, root and leaves), such as *Gluconacetobacter diazotrophicus* (CAVALCANTE; DÖBEREINER, 1988), *Herbaspirillum* (BALDANI et al., 1996), *Azospirillum* (BALDANI et al., 1997), *Burkholderia* (REIS et al., 2004), *Microbacterium* (LIN et al., 2012) and *Raoultella* (LUO et al., 2016). The selection of strains, that is, the efficient lineage of growth promotion can increase biomass and sugar (SCHULTZ et al., 2012, 2014), it can also promote other indirect benefits, such as protection against pathogens (BLANCO et al., 2005; ARENCIBIA et al., 2006; SCHULTZ et al., 2017).

Trichoderma spp. in addition to acting as a pathogen control due to the ability to produce antibiotics and enzymes (HOYOS-CARVAJAL et al., 2009), they can produce morphological and biochemical changes in the host plant, contributing to the solubilization or sequestration of inorganic nutrients such as phosphate solubilization and other minerals making them available to the plant or even contributing to stress tolerance (HARMAN, 2000; VITERBO et al., 2002). This fungus has shown benefits in the seed germination process (ALTOMAR; TRINGOVSKA, 2011), as well as the metabolic production with activities analogous to plant hormones (HOYOS-CARVAJAL et al., 2009), which consequently activates the growth of plants, which has been observed in several cultures (KLEIFELD; CHET, 1992; OUSLEY et al., 1994; ALTOMARE et al., 1999; HARMAN, 2000; YEDIDIA et al., 2001).

The use of bacteria, and the fungus adoption (Trichoderma) has versatility and mechanism of action, used as biological control of nematodes, and as a plant stimulator, which makes it very attractive, being one of the most researched fungi in laboratories and tested in greenhouses (WIJESINGHE et al., 2011; TRUSHINA et al., 2013; FILHO et al., 2008). Some isolates can act in the phosphate solubilization and other minerals, making them available to the plant (HARMAN, 2000). Another feature is the determination of auxin synthesis, favoring root growth, and shoot growth (HOYOS-CARVAJAL et al., 2009). Among the mechanisms of action exercised can be highlighted: competition, parasitism, and predation, in addition to inducing morphological and biochemical changes in the host plant and stress tolerance (VITERBO et al., 2002; LORITO et al., 2010).

Thus, the study was divided into two chapters. The first chapter consists of the inoculation of *Azospirillum brasilense* as a growth promoter in sugarcane, where the effectiveness in its methods of inoculation and doses of inoculant during two agricultural harvests in two crops cycles were evaluated through the evaluations of biometric and technologies with the objective of possible improvements in soil nutrient absorption and productivity. In the second chapter, the topic addressed was the use of microorganisms as attenuators of water stress in sugarcane. Thus, the inoculation of *Trichoderma asperellum* was evaluated as a water stress conditioner, through biometric, physiological, biochemical, enzymatic evaluations, physicochemical characterization of leaf starches, and the possible improvements provided by bacteria under water stress conditions. This

research had as a main interest to address and clarify the effects of growth and conditioning to drought stress that microorganisms can and provide for sugarcane crop.

CHAPTER 1

Azospirillum brasilense inoculant adoption as a strategy to enhance sugarcane production and bioenergy potential[1]

Abstract

Azospirillum inoculation has gained wide prominence in the search for strategies that combine increased sustainability with enhanced agricultural productivity. Plant growth-promoting bacteria like *Azospirillum* improve resource use by plants, but proper management of *Azospirillum* inoculant application in sugarcane is key to achieving crop production potential and improving product quality and economic return for farmers. The present study evaluated a series of concentrations of *A. brasilense* inoculant (0, 5, 10, 15 and 20 × 10^{10} CFU ha^{-1}) applied on sugarcane at the setts/sprouding and tillering stages in two crop seasons at two sites under field conditions. The experiment comprised plant cane and first ratoon at Site A and first and second ratoons at Site B. No effects of inoculation on crop nutrition and technological quality of the raw material were observed. However, the effects of the inoculant on the biometric parameters and yield of sugarcane varied with the dose. The two highest *A. brasilense* doses (15 and 20 × 10^{10} CFU ha^{-1}) increased the growth variables, stalk population, sugar, trash and bagasse yields and energy production of plant cane and ratoon sugarcane. No clear pattern of effects of inoculant application time on sugarcane was observed, and the effects of inoculation were related to weather conditions rather than sugarcane stage. Overall, inoculation with *A. brasilense* promoted sugarcane productivity when applied at high inoculant doses and has the potential to maximize agronomic and economic return to the sugarcane sector.

[1] This chapter was submitted in the Biomass and Bioenergy follows the formatting rules of the same.

Keywords: *Saccharum* spp.; clean energy; diazotrophic bacteria; bacterial inoculation; sustainable production.

1.1 Introduction

Sugarcane (*Saccharum* spp.) is a globally important contributor to biomass production and atmospheric CO_2 sequestration (Macedo et al., 2008). The sugarcane production chain benefits the biofuel sector by producing ethanol, clean energy and renewable by-products and generates millions of jobs (Cardoso et al., 2018; Nass et al., 2007). However, the expansion of cultivation areas and increased fertilizer use have raised concerns about sustainability and sparked demands for technologies to improve production efficiency (Lal, 2012). In particular, technologies that sustainably increase sugarcane productivity are needed, as current yields reach only approximately 60% of the estimated potential (Marin et al., 2016).

A promising option for increasing productivity alongside economic return and environmental sustainability is biotechnological technologies such as microbial inoculants (Moraes et al., 2015). Bacteria belonging to the diazotrophic genus *Azospirillum* are used as plant growth-promoting bacteria (PGPB) for several crops worldwide (Cassán and Diaz-Zorita, 2016; Hungria et al., 2013; Moretti et al., 2020; Pankievicz et al., 2019). The benefits of *Azospirillum* inoculation, which include improved root growth, plant vigor, and mineral nutrition and induction of tolerance to abiotic and biotic stresses, have been attributed mainly to two processes: biological nitrogen fixation (BNF) and phytohormone synthesis (de-Bashan et al., 2012; Fukami et al., 2016; Kaushal and Wani, 2016; Martins et al., 2018; Santos et al., 2019). In sugarcane, diazotrophic bacteria can play a key role in enhancing crop sustainability and agronomic factors, in addition to impacting the industrial sector (Pereira et al., 2019). PGPB investigated for use with sugarcane include *Gluconacetobacter diazotrophicus* (Cavalcante and Dobereiner, 1988) and bacteria of the genera

Herbaspirillum (Bueno dos Reis Junior et al., 2000), *Burkholderia, Pantoea* and *Azospirillum.* Schultz et al. (2012) studied the effects of supplementing inoculation with five diazotrophic bacteria (*Gluconacetobacter, Herbaspirillum* spp., *Azospirillum* and *Burkholderia*) with the application of 40 kg N ha^{-1}, as diazotrophic bacteria alone cannot supply the N demand of the plant. The plant growth-promoting effects of these bacteria go far beyond BNF, as the enhanced synthesis of phytohormones and other molecules also improves nutrient uptake (Giri and Dudeja, 2013).

These studies notwithstanding, there have been few studies of the effects of *Azospirillum* on semi-perennial crops such as sugarcane (de Castro Gava et al., 2019; Schultz et al., 2014, 2012), and the appropriate doses and timing of application have not been examined (Brusamarello-Santos et al., 2017; Cuna et al., 2016). In the present study, we hypothesized that (*i*) inoculation with *A. brasilense* improves sugarcane growth and (*ii*) inoculation at the bud-sprouting or ratoon stage of sugarcane provides the best response. To test these hypotheses, we performed a field experiment in which we applied *A. brasilense* inoculant to sugarcane at different stages in two crop seasons and evaluated morphological parameters, nutrient content, yield and the technological quality of the raw material.

1.2 Materials and Methods

1.2.1 Site description, experimental design, and sugarcane management

The field experiments were carried out at two experimental sites (Sites A and B) located in Santa Maria da Serra, São Paulo State, Brazil (48°20’ W, 22°31’ S, elevation 495 m). The evaluations included plant cane (Site A) and first ratoon (Site B) in the first year (2015/16) and first ratoon (Site A) and second ratoon (Site B) in the second year (2016/17) (Fig. 1). The climate

type is classified as Cfa, which is characterized as humid subtropical according to the Köppen classification. The mean annual temperature and rainfall are 22.3°C and 1,467 mm, respectively. The weather parameters were measured throughout the experimental period, and the water balance was calculated according to Allen (1998) (Fig. 2).

Prior to installation of the experimental areas (sugarcane planting at Site A and after sugarcane harvesting at Site B), soil samples were collected to evaluate the chemical attributes at 0.0-0.20 m depth (Table 1) according to van Raij et al. (2001). The soil was classified as Quartzipsamments (Soil Survey Staff, 2014). The sugarcane variety used was RB867515, which is characterized by erect growth, easy shedding, good sprouting capacity even in late planting under low temperatures, medium tillering, and regular stalk diameter (Chapola et al., 2010).

At both experimental sites, the experimental design was randomized blocks with a 5 × 2 factorial scheme and 4 replications. The liquid inoculant contained the diazotrophic bacterium *A. brasilense* strain AbV-5 (=CNPSo 2083). The primary factor was the inoculation dose of *A. brasilense* AbV-5: 0, 5, 10, 15 or 20 × 10^{10} CFU (colony forming units) ha^{-1}. The second factor was the inoculation application time: at sugarcane sprouding via the drench application method (plant height, 0.3-0.4 m) or at sugarcane tillering by leaf-spraying (plant height, 0.8-1.0 m). The plots, which were 9.6 m wide and 14 m long, consisted of four sugarcane rows with a double-row spacing of 1.5 x 0.9 m. Inoculation was carried out using a backpack sprayer with pressurized CO_2 and a flat fan nozzle, and the weather conditions during inoculant application are reported in Supplementary Table S1. Agronomic practices including fertilizer application and weed and pest control followed the specifications of the mill for each site (Supplementary Table S2).

1.2.2 Sugarcane plant evaluation throughout the plant cycle

For sugarcane leaf sampling, the top visible dewlap (TVD) was collected from plants in each plot at the full vegetative growth stage according to the Kuijper numbering system (Bonnett, 2013; Dillewijn, 1960). Only the middle third of the leaf blade was retained, and the leaf midrib was discarded. The samples were dried in a forced-air oven at 65°C until a constant mass was obtained. The material was then milled, and leaf nutrient concentrations (N, P, K, Ca, Mg, and S) were determined according to the method proposed by AOAC (2016).

Sugarcane development and biomass production in each plot were evaluated according to the following biometric parameters: (1) plant height (m), (2) stalk diameter (using a digital caliper), (3) number of stalks per meter (m^{-1}), (4) stalk yield (SY), (5) number of internodes and (6) average internode length (Marafon, 2012). The number of stalks m^{-1} was calculated by converting the number of stalks in the two central rows of each plot to number m^{-1}. Plant height, stalk diameter, number of internodes and average internode length were determined from the means of ten stems collected from each plot, defoliated, cut at the apical bud height, and measured using a digital scale, caliper, and ruler (m) from the soil surface up to the auricular region of +1 or TDV leaf. Then, the cleaned stalks were processed as defined in the Sucrose Content-Based Sugarcane Payment System in accordance with Consecana's semiannual updates for technological quality and according to the methodology described by Fernandes (2003).

SY was determined at sugarcane harvest by collecting the stalks in 2 m of two double rows. The collected samples were weighed using an electronic balance, and SY was calculated from the obtained weight and stalk population (Mg ha^{-1}). The sugar yield (Mg ha^{-1}) was obtained by multiplying SY (Mg ha^{-1}) by the pol (%) divided by 100. Trash yield was determined using 140 kg of trash per Mg of stalk (Hassuani et al., 2005) and 60% collection from the soil surface. Bagasse

yield was calculated using the results of fiber and stalk yield at 50% moisture. Energy production was calculated considering 1 Mg of trash has 4.96 MWh of primary energy (Hassuani et al., 2005).

1.2.3 Agronomic and economic viability

Agronomic and economic viability (AEV) was estimated considering inoculation with *A. brasilense* (presence or absence) and two doses (15 or 20 × 10^{10} CFU ha^{-1}) according to the following equation:

$$AEV(US\$\ ha^{-1}) = [(TRS\ price \times TRS\ value)] - (Y \times FR)] - (H\ cost \times Y) - (APC)$$

where Y is yield, FR is field rent, H is harvest, and APC is agronomic practices costs. AEV was calculated considering data from plant cane and ratoon (both sites), independent of the application time.

1.2.4 Statistical analyses

Data were submitted to the procedures of Shapiro-Wilk to check for normal distribution and homogeneity of variance. Analysis of variance (ANOVA) was performed using the statistical software SISVAR (Ferreira, 2014). Inoculant dose and inoculant time were considered fixed factors. If the null hypothesis was rejected, means were compared with the t test (LSD) at 10% probability.

1.3 Results

1.3.1 Nutritional status

In general, no effects of inoculation dose, inoculation time, or their interaction on nutrient concentration in TVD leaves were detected at Sites A and B in all cycles evaluated (Tables 2 and

3). The exception was the P concentration in the first ratoon at Site A and in the second ratoon at Site B, which was affected by the inoculation dose (Tables 2 and 3). In both cases, the best results were achieved with the highest inoculation dose (20 $\times 10^{10}$ CFU ha^{-1}).

1.3.2 Biometric parameters and agronomic and economic viability

At Site A, inoculation with *A. brasilense* improved plant height (Fig. 3A) and internode length (Fig. 3C) but not the number of internodes (Fig. 3B) or stalk diameter (Fig. 3D). No differences were observed related to the time of inoculation (sprouting or tillering) (Supplementary Table S3). At Site B, where *A. brasilense* inoculation was performed in successive ratoon cycles, increases in plant growth were observed at higher doses of inoculum (15 and 20 x10^{10} CFU ha^{-1}) (Figs. 3A-3D).

A. brasilense inoculation in the plant or ratoon cane cycle influenced stalk m^{-1} and SY (Figs. 3E, F). Inoculation of plant cane increased the stalk population by nearly 2 stalks m^{-1}, especially at high doses (15 or 20 × 10^{10} CFU ha^{-1}), and this increase in the number of stalks contributed to an increase in SY of nearly 20 Mg ha^{-1} compared with the non-inoculated control (Fig. 3F). With respect to timing, application of *A. brasilense* in the tillering stage resulted in a larger population and SY compared with application in the sprouting stage. Similar responses of the stalk population and SY were observed when *A. brasilense* was applied to ratoon cane; doses of 15 or 20 × 10^{10} CFU ha^{-1} increased SY to ~89.4 Mg ha^{-1} for first ratoon at Site A (control = 73.3 Mg ha^{-1}), ~88.2 Mg ha^{-1} for first ratoon at Site B (control = 71.5 Mg ha^{-1}), and ~63.2 Mg ha^{-1} for second ratoon at Site B (control = 50.0 Mg ha^{-1}) (Fig. 3F).

A. brasilense inoculation also provided promising AEV for both plant cane and ratoon. Considering all cycles evaluated, inoculation resulted in approximately 15% higher economic

return for plant cane and ratoon compared with non-inoculated areas (on average US$ 497.9 ha^{-1}) (Table 4).

1.3.3 Technological quality of raw material, products, and subproducts of sugarcane processing

There were no effects of inoculation dose and time ($p < 0.10$) on purity, fiber, reducing sugars, and total recoverable sugars (Fig. 4 and Supplementary Table S4). Although fiber was not affected by inoculation (Fig. 4A), the pattern of trash yield (an indirect parameter of fiber) was the same as that of SY. The two highest inoculant doses promoted increases in trash yield in plant cane and first ratoon (Site A) and second ratoon at Site B (Fig. 4B). In addition, inoculant application at tillering improved trash yield in plant cane at Site A and in first ratoon at Site B but reduced trash yield in first ratoon at Site A.

Bagasse yield, energy production and sugar yield followed the same pattern as SY (Figs. 5 and 6), whereas sucrose (the pol content) was not affected by *A. brasilense* (dose and time of application). On average, sugarcane inoculated with the two highest doses of *Azospirillum* had 23.5% higher bagasse yield in plant cane at Site A, 15% higher energy production in first ratoon at Site A, and 22% higher sugar yield in second ratoon at Site B compared to that of control. In addition, application at tillering increased bagasse yield, energy production and sugar yield more efficiently than application at sprouting in plant cane at Site A and in first ratoon at Site B. By contrast, in first ratoon at Site A, application at sprouting resulted in higher bagasse yield and energy production compared to application at tillering.

1.4 Discussion

This study provides a novel perspective on an alternative strategy for promoting the production of biofuel and sugar, as well as subproducts of sugarcane production, based on inoculation with *A. brasilense*. Important parameters for inoculation that were previously unavailable were determined, including the adequate dose and time of application of the inoculant. Interestingly, although the effects of *A. brasilense* inoculation have often been linked to N nutrition via BNF (Martins et al., 2020; Matoso et al., 2020a), we found that increasing the inoculant dose affected P concentrations in sugarcane leaves, regardless of the time of application. The improvements in P concentrations might be related to the synthesis of phytohormones and increased root growth, as reported previously for the Ab-V5 strain of *A. brasilense* (Rondina et al., 2020). Greater root growth enhances the uptake of water and nutrients (Pereira et al., 2019; Reis et al., 2020). In addition, studies have shown that inoculation with PGPB activates different biological mechanisms that improve the uptake of nutrients and plant growth (dos Santos et al., 2020; Matoso et al., 2020b). The differences in leaf P concentrations may also be a consequence of plant absorption of organic phosphate via microbial solubilization, which occurs through absorption by the large surface area of the root system and is essential for sugarcane cultivation (Estrada et al., 2013; Fischer et al., 2012; Magnani et al., 2013). The plants were not nutrient-limited at either site, as the leaf concentrations of N, P, K, Ca, and Mg were within the range of sugarcane sufficiency [i.e., 18-25, 1.5-3.0, 10-16, 2.0-8.0, 1.0-3.0, and 1.5-3.0 g kg^{-1}, respectively (van Raij and Cantarella, 1997)].

Previous studies of sugarcane have generated inconclusive results on the best timing of application (da Silva Viana et al., 2020; Simões et al., 2019; Umali-Garcia et al., 1980). We also observed inconsistent effects of inoculation at sprouting or tillering among plant cane, first ratoon

and second ratoon. Inconsistent results are typically attributable in part to effects of agricultural practices on sugarcane growth, such as irrigation, fertilization, genotypes, and pesticides (Cassán and Diaz-Zorita, 2016; Schultz et al., 2012; Simões et al., 2019). In the present study, adverse weather conditions (Fig. 1) at the time of application might have negatively influenced the establishment and efficiency of *A. brasilense*. Several of the effects of *A. brasilense* are dependent on conditions such as soil type, plant species, edaphoclimatic conditions and management practices (Compant et al., 2010; Ferreira et al., 2013; Moutia et al., 2010; Mus et al., 2016).

The highest inoculant doses (15 and 20 × 10^{10} CFU ha^{-1}) increased stalk diameter by 13%, possibly reflecting higher plant levels of auxins, which regulate tillering (Matoso et al., 2020b; Puente et al., 2018). Auxins synthesized by PGPB have two main effects on plants: stalk elongation and impediment of lateral bud development (apical dominance). Under high luminous intensity, auxin production decreases, which releases the inhibition of lateral buds (increasing stalks) (Cunha et al., 2020; Shi et al., 2018). Our findings emphasize the importance of adjusting the inoculant dose to avoid growth inhibition of sugarcane since application timing and diverse environmental factors affect the microorganism's efficiency on the plant (Fukami et al., 2016).

The improvement of sugarcane yield by inoculation of *A. brasilense* is probably due to a combination of BNF, phytohormone synthesis, and other secondary metabolites in plants (Duca et al., 2014; Fukami et al., 2018; Tahir et al., 2017; Santos et al., 2021). Diazotrophic bacteria such as *Azospirillum*, *Gluconacetobacter*, *Herbaspirillum* and *Burkholderia* have previously been shown to increase sugarcane growth and yields (dos Santos et al., 2020; Martins et al., 2020) as well as productivity (de Oliveira et al., 2006); their contribution to sugarcane N nutrition is estimated to be approximately 20% (Cassán and Diaz-Zorita, 2016). Growth promotion likely influences sugarcane plant height, stalk diameter, and number of stalks per meter, which in turn affect stalk yields, sugar production, bagasse yield, and energy production.

The evaluation of yield parameters (stalk, sugar, bagasse, and trash yields and energy production) over the crop seasons confirmed that inoculation with the two highest doses, 15 and 20×10^{10} CFU ha^{-1}, improved sugarcane performance. Studies have shown that inoculation with *A. brasilense* at sugarcane seeding improves production and crop response (Gírio et al., 2015; Matoso et al., 2020b; Pereira et al., 2019). However, as expected, inoculation had no effect on technological parameters (fiber, purity, reducing sugars, total recoverable sugars). The increase in sugarcane yields per hectare with inoculant application indicates that inoculation produces higher yields of sugar per hectare not by influencing synthesis pathways but by increasing stalk yield, which is beneficial for both farmers and industry.

Notably, at both sites, all variables studied were higher in the first crop season than in the second crop season. A hailstorm between the two crop seasons damaged the plants, and thus the averages of the evaluated data were lower compared with the previous harvest. However, even under these adverse conditions, the effects of the inoculant doses were the same in both crop seasons.

Inoculation of sugarcane with PGPB could be an important strategy to maximize agronomic and economic return to the sector, including farmers and mills, by increasing biomass and bioenergy production. This is particularly important in the context of recent sugarcane expansion to areas of low soil fertility (Bordonal et al., 2018), which requires improved agronomic practices to enhance ratoon cane cycles (Otto et al., 2016). We estimated favorable economic returns of inoculation of US$ 90 per hectare for plant cane and US$ 58 per hectare for ratoon cane (Table 5). Furthermore, inoculation is projected to increase sugarcane yield by an average of nearly 14 Mg ha^{-1} in each cycle; after four crop cycles, this increase would be equivalent to one ratoon cycle, thereby augmenting sugarcane longevity and reducing production costs.

In summary, our results support the feasibility of inoculation of sugarcane with *A. brasilense* to improve plant development and suggest that this strategy has outstanding potential to improve crop yields and sustainability.

1.5 Conclusions

The adoption of sustainable management in sugarcane cultivation for biofuel and sugar production has crucial environmental benefits, including the replacement of fossil fuels with biofuels and reduced fertilizer use. Inoculation of sugarcane with *Azospirillum* is a promising strategy for enhancing bioethanol and sugar production as well as agronomic and economic return and the entire sugarcane production chain. An inoculant concentration of *A. brasilense* of approximately 15 to 20 × 10^{10} CFU ha^{-1} is sufficient to stimulate plant growth and yields in the field while reducing production costs. Further investigation of the impact of weather conditions on microbial shelf life and inoculant use efficiency is needed to ensure an appropriate timing and method of application and to minimize potentially adverse conditions.

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Table 1. Chemical attributes evaluated along the experimental period at 0-0.20 m depth – site A (plant cane and first ratoon) and site B (first and second ratoon)

Crop season		pH $CaCl_2$	SOM g dm^{-3}	P_{resin} mg dm^{-3}	Al^{3+}	H+Al	K^+	Ca^{2+}	Mg^{2+}	SB	CEC	V %
						———		$mmol_c$ dm^{-3}	———			
Site A	Plant cane	5.25	13.5	10.5	0.42	14.5	6.8	18.9	11.5	37.3	51.5	72
	1st Ratoon	5.25	13.5	10.5	0.42	14.5	6.8	18.9	11.6	37.3	51.5	72
Site B	1nd Ratoon	5.45	10.5	13.0	0.43	13.5	0.3	11.3	7.3	19.0	32.5	56
	2 nd Ratoon	5.13	13.2	14.0	0.65	17.2	1.5	11.9	6.2	19.6	37.0	52

SOM – Soil Organic Matter; SB: Sum of Bases; CEC: Cation Exchange Capacity; V% Bases Saturation.

Table 3. Nutrient (N, P, K, Ca, Mg, and S) levels in the sugarcane TVD leaves as affected by inoculation rate and inoculation time of *Azospirillum brasilense* in plant cane and first ratoon in the Site A.

Treatment	N	P	K	Ca	Mg	S
			(g kg^{-1})			
Inoculation Rate (IR)			Plant cane – Site A			
0	19	2.2	10	8.5	1.8	1.7
5 x 10^{10}‡	19	2.2	10	8.5	1.8	1.7
10 x 10^{10}	19	2.2	10	8.5	1.8	1.7
15 x 10^{10}	19	2.3	10	8.5	1.8	1.7
20 x 10^{10}	19	2.3	10	8.6	1.9	1.7
Inoculation Time (IT)						
Budding/via Drench	19	2.2	10.5	8.4	1.9	1.7
Tillering/Leaf-spray	19	2.3	10.5	8.6	1.9	1.7
Source of variation						
IR	0.198	0.397	0.989	0.376	0.139	0.962
IT	0.675	0.154	0.576	0.223	0.551	0.902
IR × IT	0.880	0.727	0.961	0.919	0.883	0.873
			First ratoon – Site A			
Inoculation Rate (IR)						
0	18	2.0 b†	11	2.5	1.7	1.6
5 x 10^{10}‡	18	2.0 b	11	2.6	1.8	1.7
10 x 10^{10}	19	2.0 b	11	2.5	1.7	1.6
15 x 10^{10}	19	2.0 b	12	2.5	1.8	1.7
20 x 10^{10}	18	2.2 a	12	2.5	1.7	1.6
Inoculation Time (IT)						
Budding/via drench	19	2.0	12	2.5	1.8	1.7
Tillering/Leaf-spray	18	2.0	11	2.6	1.7	1.6
Source of variation	*P* > *F*					
IR	0.220	0.065	0.186	0.685	0.323	0.963
IT	0.798	0.983	0.188	0.741	0.409	0.729
IR × IT	0.246	0.195	0.924	0.992	0.486	0.154

†Means followed by different letters in the column differ statistically ($P \leq 0.10$) according to the LSD test.
‡Rate (CFU ha^{-1}) of *Azospirillum brasilense* inoculant applied to sugarcane.

Table 4. Nutrient (N, P, K, Ca, Mg, and S) levels in sugarcane TVD leaves as affected by inoculation rate and inoculation time of *Azospirillum brasilense* in first ratoon and second ratoon in the Site B.

Treatment	N	P	K	Ca	Mg	S
			(g kg^{-1})			
Inoculation Rate (IR)			First ratoon – Site B			
0	21	2.0	11	5.7	2.0	1.6
5 x 10^{10}‡	21	2.0	11	5.7	2.0	1.6
10 x 10^{10}	20	2.0	11	5.7	2.1	1.6
15 x 10^{10}	21	2.0	11	5.9	2.0	1.6
20 x 10^{10}	21	2.0	11	5.9	2.0	1.6
Inoculation Time (IT)						
Budding/via Drench	21	2.1	11	5.7 b	2.0	1.6
Tillering/Leaf-spray	21	2.0	11	5.9 a	2.0	1.6
Source of variation	*P > F*					
IR	0.427	0.347	0.618	0.525	0.831	0.969
IT	0.257	0.402	0.252	0.067	0.292	0.654
IR × IT	0.898	0.937	0.451	0.963	0.493	0.998
			Second ratoon – Site B			
Inoculation Rate (IR)						
0	19	2.2ab†	12	3.5	1.8	1.6
5 x 10^{10}‡	19	2.1b	11	3.6	1.8	1.5
10 x 10^{10}	20	2.3a	11	3.6	1.8	1.6
15 x 10^{10}	19	2.2ab	11	3.6	1.8	1.5
20 x 10^{10}	20	2.3a	12	3.3	1.7	1.5
Inoculation Time (IT)						
Budding/via drench	20	2.3	12	3.5	1.8	1.5
Tillering/Leaf-spray	19	2.2	11	3.5	1.8	1.5
Source of variation	*P > F*					
IR	0.830	0.048	0.651	0.834	0.876	0.718
IT	0.760	0.346	0.526	0.955	0.168	0.594
IR × IT	0.854	0.415	0.510	0.927	0.141	0.794

†Means followed by different letters in the column differ statistically ($P \leq 0.10$) according to the LSD test.
‡Rate (CFU ha^{-1}) of *Azospirillum brasilense* inoculant applied to sugarcane.

Table 4. The agronomic and economic viability (AEV) of *Azospirillum* inoculation (15 or 20×10^{10} CFU ha^{-1}) according to the cycle (plant or ratoon cane) in sugarcane cultivated.

Sugarcane crop cycle	AEV (US$ ha^{-1})	
	With *Azospirillum*	Without *Azospirillum*
Plant Cane	690.2	599.3
Ratoon Cane	454.6	396.5

	2015/16		2016/17	
	Sugarcane	**Application time**	**Sugarcane**	**Application time**
Site A	Plant cane	Setts Tillering	First ratoon	Budding Tillering
Site B	First ratoon	Sprouting Tillering	Second ratoon	Budding Tillering

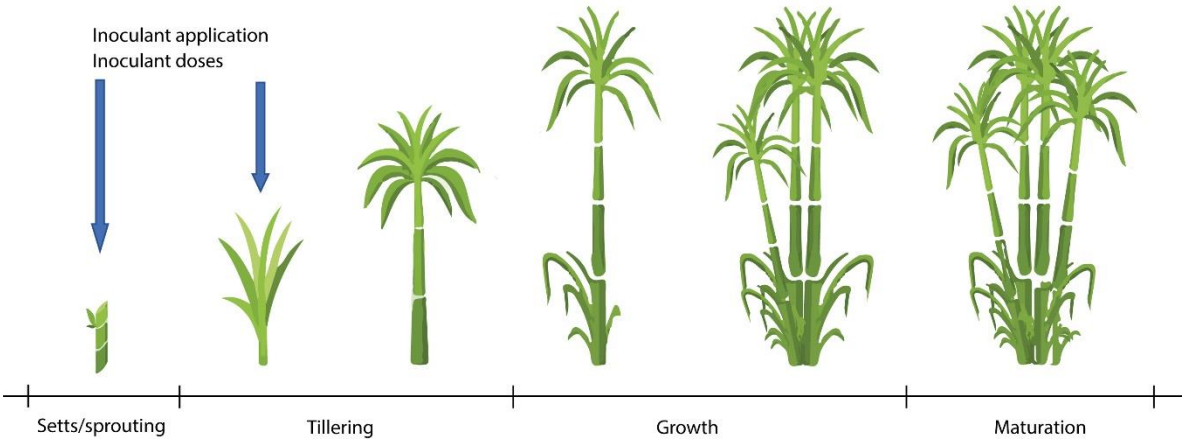

Figure 1. Schematic representation of the inoculant application timing in sugarcane in the Site A and B during the study (2015/16 and 2016/17).

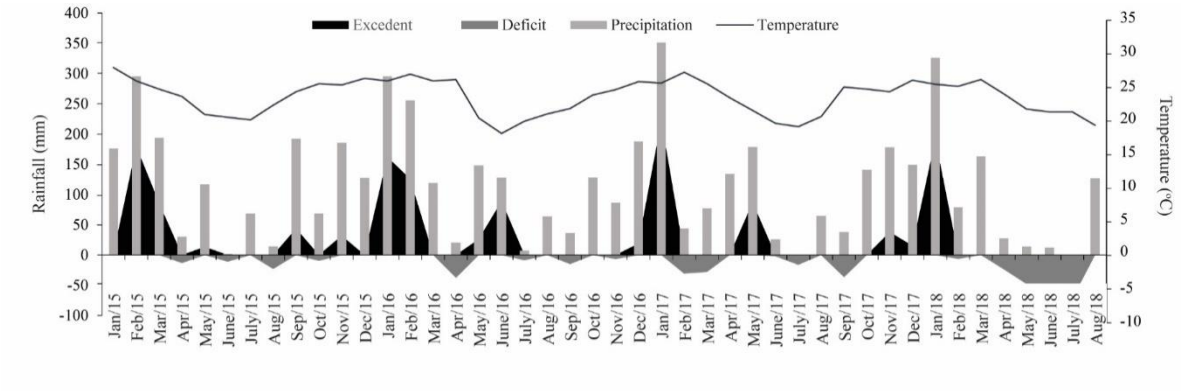

Figure 2. The water balance calculated in both areas (sites A and B) during the experimental period from June/2015 to Aug/2018.

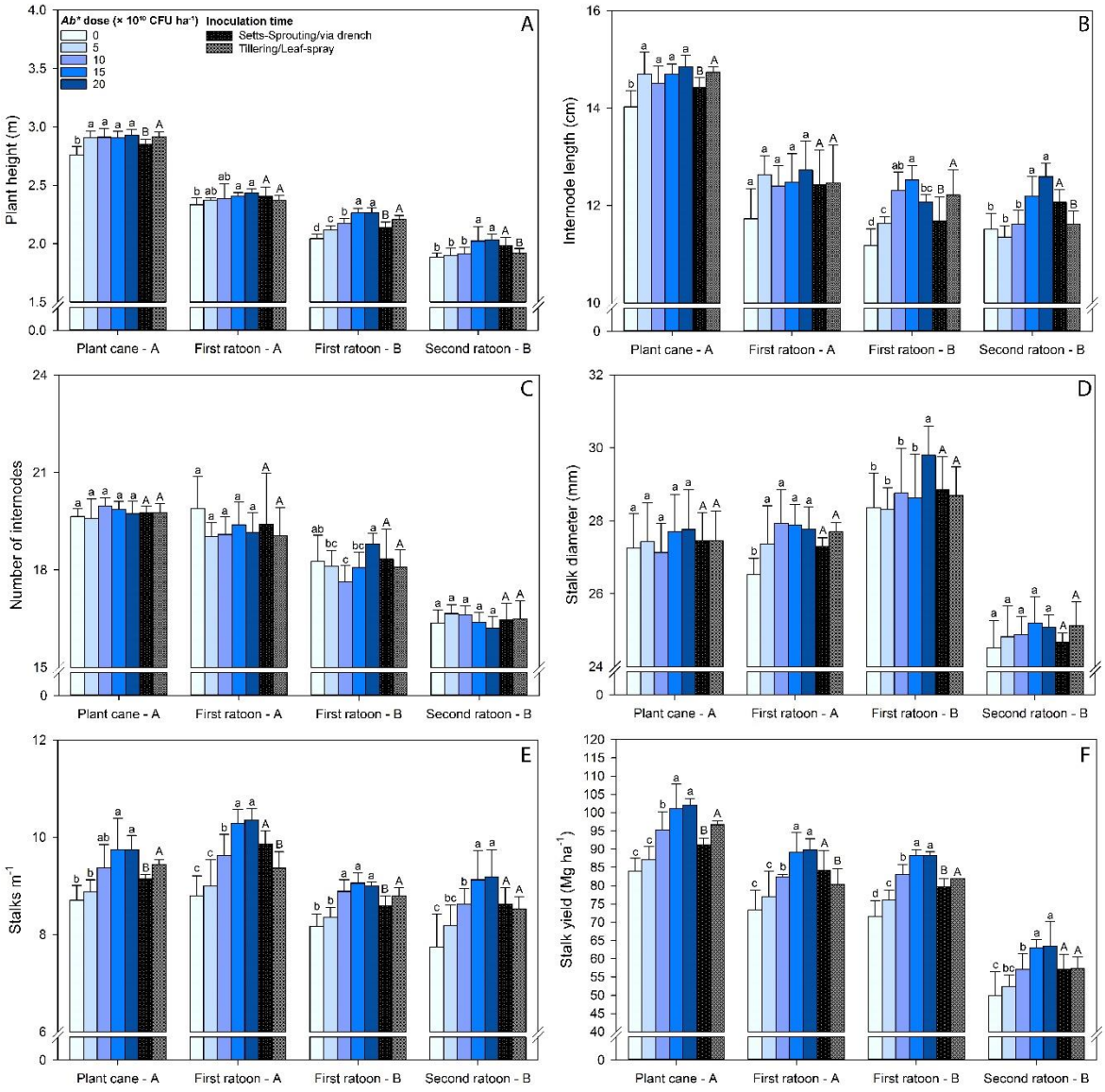

Figure 3. Biometric analysis of plant height (A), number of internodes (B), internodes length (C), stalk diameter (D), stalks m^{-1} (E) and stalk yield (F) affected by *Azospirillum brasilense* dose and inoculation time on sugarcane in Sites A and B. The methods followed by letters differ from each other by the least significant difference LSD test at $p \leq 0.10$. Lowercase letters compared the *Azospirillum* dose and uppercase letters compares the inoculation time.

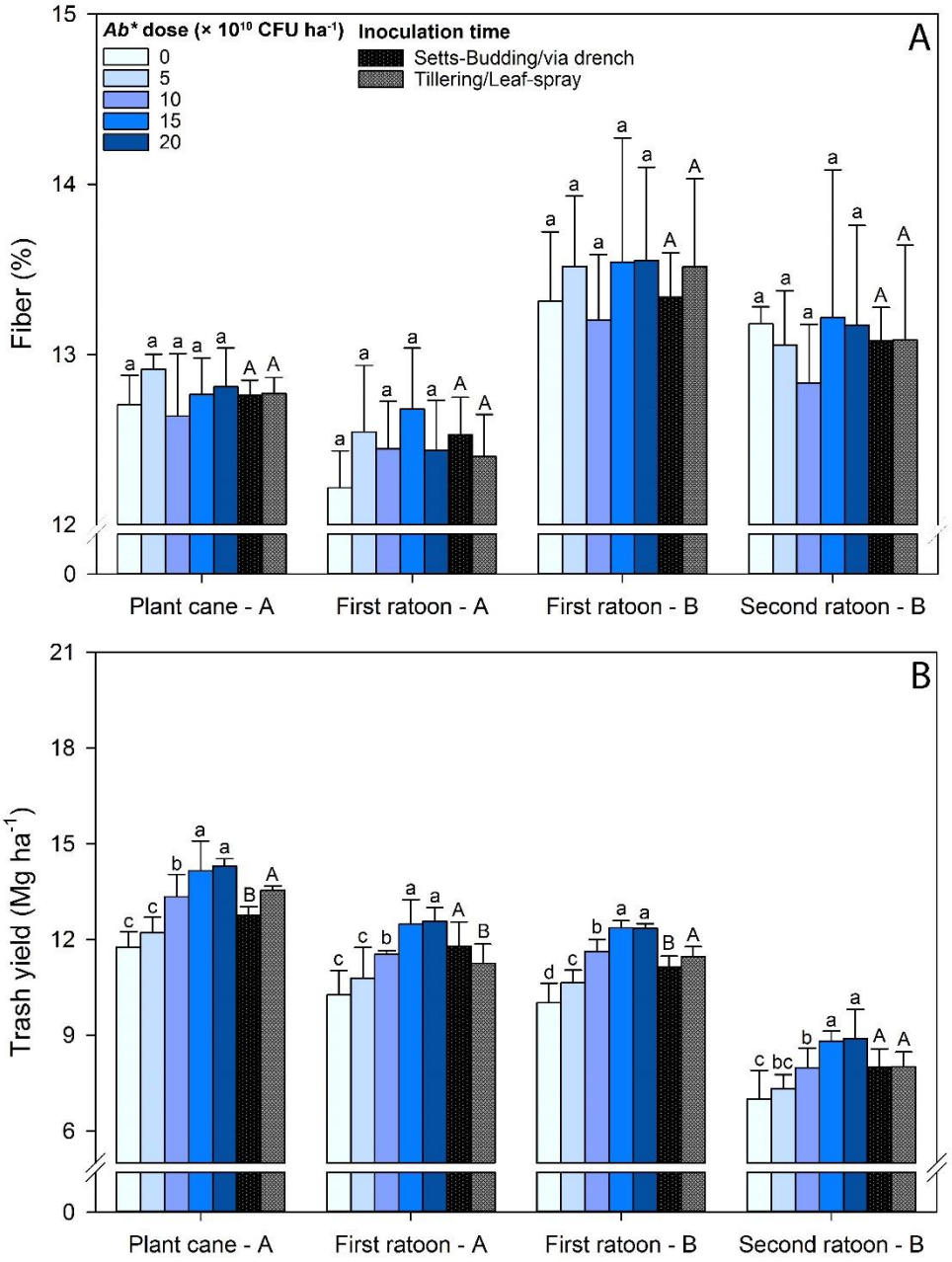

Figure 4. Fiber (A) and Trash yield (B) affected by *Azospirillum brasilense* dose and inoculation time on sugarcane in Sites A and B. The methods followed by letters differ from each other by the least significant difference LSD test at $p \leq 0.10$. Lowercase letters compared the *Azospirillum* dose and uppercase letters compares the inoculation time.

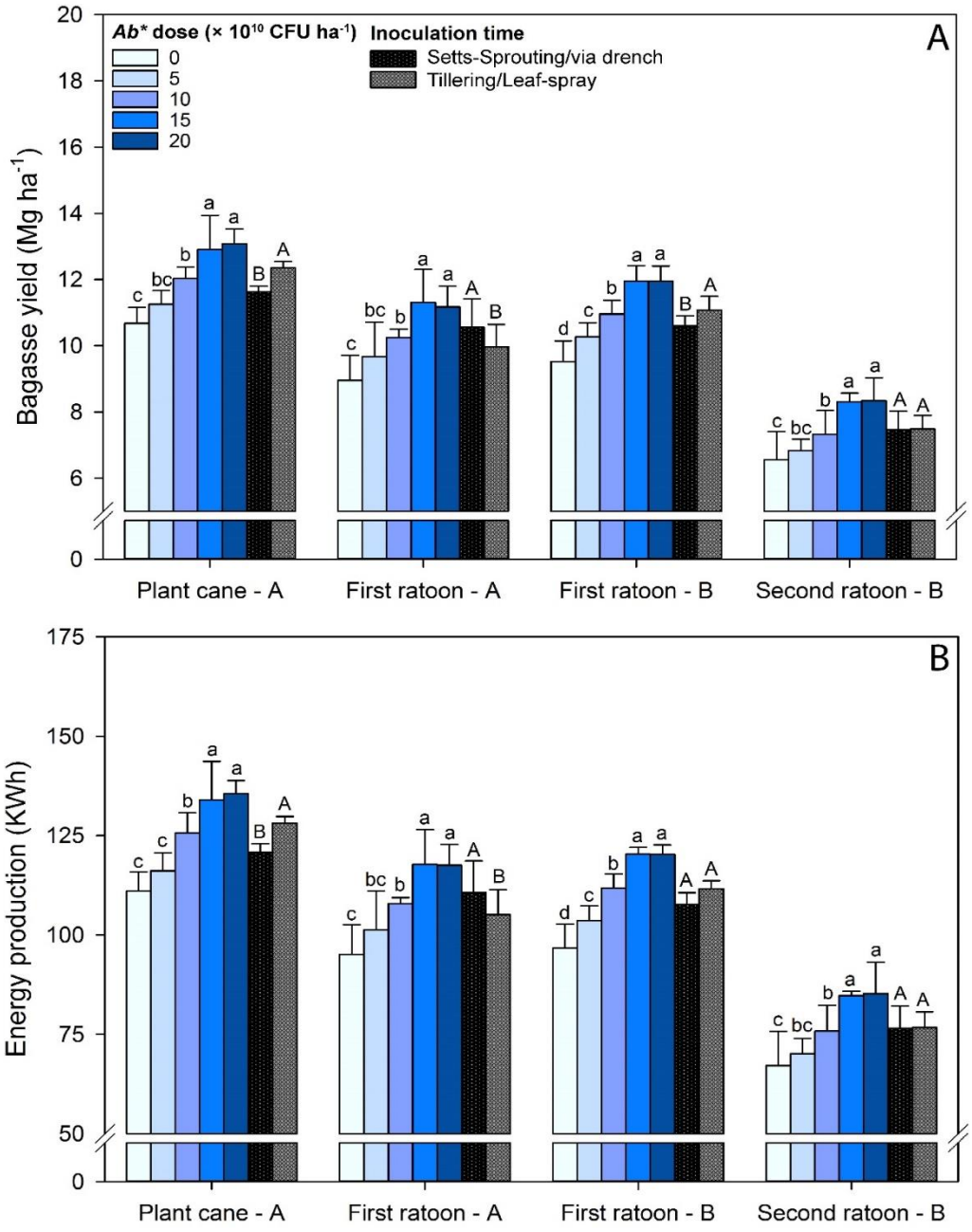

Figure 5. Bagasse yield (A) and energy production (B) affected by *Azospirillum brasilense* dose and inoculation time on sugarcane in Sites A and B. The methods followed by letters differ from each other by the least significant difference LSD test at $p \leq 0.10$. Lowercase letters compared the *Azospirillum* dose and uppercase letters compares the inoculation time.

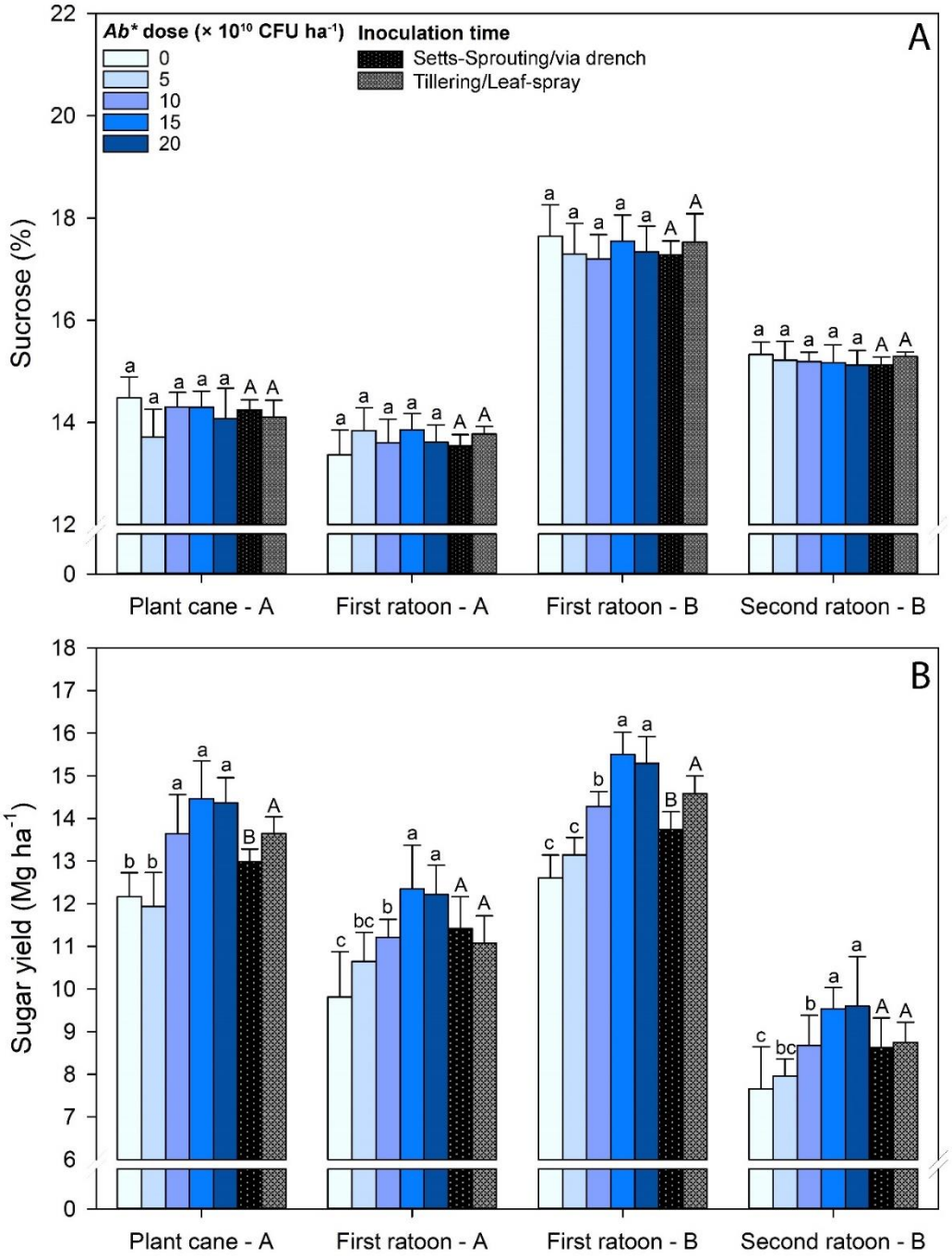

Figure 6. Sucrose (A) and sugar yield (B) affected by *Azospirillum brasilense* dose and inoculation time on sugarcane in Sites A and B. The methods followed by letters differ from each other by the least significant difference LSD test at $p \leq 0.10$. Lowercase letters compared the *Azospirillum* doses and uppercase letters compares the application time.

Supplementary Table S1. Date and weather parameters monitored during the inoculation application timing of each experimental area (site A and B).

Date	Sugarcane / Application timing	Average temperature	Relative humidity	Wind speed
		°C	%	m s^{-1}
		Site A		
06/18/2015	Plant cane / Buds	22.5	83.1	1.5
09/24/2015	Plant cane / Tillering	29.0	46.6	1.1
10/11/2016	First ratoon / Budding	22.4	74.6	4.3
10/31/2016	First ratoon / Tillering	23.6	75.7	2.8
		Site B		
11/23/2015	First ratoon / Budding	22.7	94.5	2.2
12/11/2015	First ratoon / Tillering	25.4	80.7	1.3
11/24/2016	Second ratoon/ Budding	25.7	78.1	3.3
12/14/2016	Second ratoon/ Tillering	25.4	84.9	2.5

Supplementary Table S2. Agronomic practices adopted in each experimental area during the sugarcane cultivation.

Application date	Product	Purpose	Rate	Syrupy liquid
		Plant cane – Site A		
06/18/2015	NPK (10-25-25)	Fertilizer	550 kg ha^{-1}	-
	Azoxistrobina	Fungicide	50 g ha^{-1}	200 L ha^{-1}.
	Ciproconazol	Fungicide	20 g ha^{-1}	
	Fipronil	Insecticide	31,25 g ha^{-1}	
	Clomazona	Herbicide	864 g i.a ha^{-1}	
	Diurom	Herbicide	1280 g ha^{-1}	
		First ratoon – Site A		
07/14/2016	NPK (25-00-25)	Fertilizer	480 Kg ha^{-1}	-
07/26/2016	Tebutiurom	Herbicide	639 g i.a. ha^{-1}	200 L ha^{-1}.
07/26/2016	Clomazona	Herbicide	1533,6 g i.a ha^{-1}	
		First ratoon – Site B		
01/12/2015	*Cotesia flavipes*	Biological control	7.500 parasites ha^{-1}	-
07/31/2015	NPK (25-00-25)	Fertilizer	514 Kg ha^{-1}	-
11/16/2015	NPK (25-00-25)	Fertilization	491 Kg ha^{-1}	-
11/30/2015	Clomazona	Herbicide	864 g i.a ha^{-1}	200 L ha^{-1}
11/30/2015	Diurom	Herbicide	1280 g ha^{-1}	
		Second ratoon – Site B		
10/10/2016	NPK (25-00-25)	Fertilizer	418 Kg ha^{-1}	-
10/17/2016	Tebutiurom	Herbicide	928,5 g i.a. ha^{-1}	200 L ha^{-1}
10/17/2016	Clomazona	Herbicide	1114,4 g i.a ha^{-1}	

Supplementary Table S3. ANOVA (*F probability*) experimental factors inoculation dose × inoculation time for plant height, number of internodes, internode length, stalk diameter, number of stalk m^{-1}, stalk yield of sugarcane affected by inoculation dose and inoculation time.

Treatment	Plant height	Internodes	Internode length	Stalk diameter	Stalk m^{-1}	Stalk yield
			ANOVA (*F probability)*			
			Plant cane – Site A			
Inoculation Rate (IR)	0.006	0.719	0.056	0.045	<0.001	<0.001
Inoculation Time (IT)	<0.001	0.906	0.053	0.023	<0.001	0.007
IR × IT	0.356	0.824	0.653	0.984	0.067	0.393
			First ratoon – Site A			
Inoculation Rate (IR)	0.045	0.526	0.235	0.898	<0.001	<0.001
Inoculation Time (IT)	0.672	0.162	0.879	0.345	<0.001	0.052
IR × IT	0.872	0.889	0.909	0.780	0.089	0.389
			First ratoon – Site B			
Inoculation Rate (IR)	0.026	0.026	<0.001	0.044	<0.001	<0.001
Inoculation Time (IT)	0.044	0.317	<0.001	0.535	<0.001	0.004
IR × IT	0.565	0.937	0.572	0.767	0.238	0.410
			Second ratoon – Site B			
Inoculation Rate (IR)	0.049	0.586	0.002	0.209	<0.001	<0.001
Inoculation Time (IT)	0.038	0.841	<0.001	0.130	<0.001	0.934
IR × IT	0.236	0.089	0.090	0.823	0.982	0.692

Supplementary Table S4. ANOVA (*F probability*) experimental factors inoculation dose × inoculation time for fiber, purity, reductor sugar (RS), total recoverable sugars (TRS), trash yield, sucrose, sugar yield (SY), bagasse yield (BY) and energy production (EP) of sugarcane.

Treatment	Fiber	Purity	RS	TRS	Trash yield	Sucrose	Sugar yield	Bagasse yield	Energy production
					ANOVA (*F probability)*				
					Plant cane – Site A				
Inoculation Rate (IR)	0.345	0.180	0.200	0.110	<0.001	0.2300	<0.001	<0.001	<0.001
Inoculation Time (IT)	0.882	0.492	0.529	0.441	0.007	0.4535	0.052	0.007	0.0482
IR × IT	0.296	0.549	0.506	0.199	0.393	0.7834	0.180	0.393	0.2343
					First ratoon – Site A				
Inoculation Rate (IR)	0.328	0.548	0.510	0.493	<0.001	0.5452	<0.001	<0.001	<0.001
Inoculation Time (IT)	0.269	0.348	0.425	0.233	0.042	0.5394	0.293	0.042	0.0345
IR × IT	0.857	0.729	0.724	0.850	0.389	0.8920	0.422	0.389	0.3452
					First ratoon – Site B				
Inoculation Rate (IR)	0.496	0.126	0.205	0.333	<0.001	0.3453	<0.001	<0.001	<0.001
Inoculation Time (IT)	0.250	0.692	0.725	0.679	0.004	0.5563	0.001	0.004	0.6738
IR × IT	0.936	0.556	0.626	0.856	0.410	0.7012	0.268	0.410	0.3883
					Second ratoon – Site B				
Inoculation Rate (IR)	0.976	0.914	0.914	0.914	<0.001	0.0873	<0.001	<0.001	<0.001
Inoculation Time (IT)	0.799	0.230	0.230	0.230	0.934	0.5638	0.658	0.934	0.4554
IR × IT	0.899	0.785	0.785	0.785	0.692	0.4563	0.682	0.692	0.1876

CHAPTER 2

Trichoderma asperellum inoculation as a tool for attenuating drought stress in sugarcane[2]

Abstract

Drought stress is an important concern worldwide that reduces crop yield and quality. To alleviate this problem, *Trichoderma asperellum* has been used as a plant growth promote fungi capable of inducing plant tolerance to biotic and abiotic stresses. Here, we examined the effect of *T. asperellum* inoculation on sugarcane plants above and belowground development under drought stress, and investigated the role of this fungus on inducing tolerance to drought at physiological and biochemical levels. The experiment was performed in pots under greenhouse conditions, with four treatments and four replicates. The treatments consisted of sugarcane plants inoculated or not with *T. asperellum* and grown under drought stress and adequate water availability. Drought-stressed sugarcane plants inoculated with *T. asperellum* changed the crop nutrition, and chlorophylls and carotenoids concentration, resulting in increased photosynthesis rate, stomatal conductance and water use efficiency compared to the non-inoculated plants. In addition, the antioxidant metabolism also changed, increasing the SOD and POD enzyme activities, as well as the proline concentration and sugar portioning. This cascade effects enhanced the root and stalk development, demonstrating that the *T. asperellum* inoculation is an important tool to alleviate the negative effects of drought stress in sugarcane. Future studies should be performed to elucidate if *T. asperellum* should be reapplied to the sugarcane ratoons.

Keywords: Plant growth promoting fungus, low water availability, reactive oxygen species, antioxidant metabolism.

2.1 Introduction

Drought stress is one of the major abiotic stresses worldwide affecting plant growth, development, and crop yield (Basu et al., 2016; Rosa et al., 2020). Recently, Gupta et al. (2020) reported that drought alone might cause more losses in crop yield than all plant diseases combined. Drought is defined as a stress condition in which there is a deficit of water in the

[2] This chapter was approved in the Journal Frontiers in Plant Science following the formatting rules of the same.

soil and, consequently, there is an increase in soil temperature (especially during the day), a reduction in the nutrient availability and, eventually, an increase in soil salinity (Tardieu, 2013; Tardieu et al., 2018).

Sugarcane (*Saccharum* sp.), the most important energy crop in the world, in general, is considered to be partial-drought sensitive (Marcos et al., 2018a). Grown under low water availability conditions, sugarcane often produce a significantly low stalk yield with poor technology quality, culminating in low sugar and ethanol production. Studies have shown that drought stress can induce several morphological, physiological, and metabolic responses of plants, resulting in reactive oxygen species (ROS) production, leading to increased cell damages and antioxidant enzyme inactivation (Vilela et al., 2017). In Brazil, with the expansion of sugarcane in the Midwest region, planting started to be done in a wide textural diversity and soil fertility (Marcos et al., 2018b). Soils with low fertility and, generally, sandy texture present themselves as limiting factors for the development of sugarcane (Bordonal et al., 2018). This occurs due to the low storage capacity of water and nutrients in the soil, especially in periods of high resource demand for the plant (Ribeiro et al., 2013; Marcos et al., 2018b).

As a semi-perennial crop, sugarcane experiences seasonal variations in water availability and higher temperatures (Marcos et al., 2018b), leading to loss of yield and quality of the final product, as well as a reduction in remuneration to the producer. Thus, drought stress seriously threatens the sugarcane production chain (Ribeiro et al., 2013), reducing the production of its derivatives and raising its prices in the domestic and foreign markets. Therefore, it is critically important to develop effective managements practices to alleviate the negative impacts of drought stress on sugarcane growth and development (Fahad et al., 2017). Genetic breeding has sought to obtain new cultivars more resistant to drought (Mall et al., 2020), however, it is necessary the establishment of management practices that increase the mitigation of environmental stresses.

An innovative technique increasingly studied and implemented in agriculture is the use of plant growth-promoting microorganisms (bacteria and fungi) to induce plant resistance to abiotic and biotic stresses (Zhang et al., 2016; Poveda, 2020). This approach is new and effective in increasing the plant tolerance to environmental climate challenges, which can play an important role in the development of sustainable agricultural systems (Rajesh et al., 2016). Among this group of microorganisms, *Trichoderma* spp. is a rhizospheric fungus of great agricultural and environmental importance. , which can confer numerous beneficial effects in promoting plant growth and development (Harman et al., 2004), in inducing systemic

resistance in plants (Kashyap et al., 2017; Sousa et al., 2020) and as a biocontrol agent for fungal diseases (Harman et al., 2004; Mastouri et al., 2010). There are countless species within the genus *Trichoderma*, and a highlight has been given to *T. asperellum* (Harman, 2005; Sousa et al., 2020; Zhao et al., 2020). Several authors have reported the ability of this group of microorganisms to relieve biotic and abiotic stresses of germinating plants and seeds, reducing the damage caused by reactive oxygen species (ROS) (Poveda, 2020). However, the underlying mechanisms responsible for the ROS scavenging have yet to be elucidated (Kashyap et al., 2017). While most of the work is done on crops with a short growth cycle, little information is available on the potential of *T. asperellum* to act as plant growth promoters in increasing tolerance against drought stress, especially in a semi-perennial crop as sugarcane.

Based on these arguments, our study hypothesized that inoculation with *T. asperellum* may reduce the negative effects of drought on nutrition, physiological and morphological parameters of sugarcane plants. To test these hypotheses, our study evaluated the acquisition of nutrients, the concentration of photosynthetic pigments, gas exchange parameters, antioxidant metabolism, and root growth and stalk yield in sugarcane plants submitted to drought stress under application of *T. asperellum* inoculant.

2.2 Material and methods

2.2.1 Growth conditions

This study was conducted in an experimental greenhouse with climate-controlled environment in Botucatu, São Paulo State, Brazil (22 ° 51 'S, 48 ° 26' W, elevation 815 m above sea level). Greenhouse had an internal heating/cooling air circulatory system to maintain the temperature between 22 °C and 32 °C, and 70% of relative humidity. The experiment was performed using polyethylene pots with a capacity of 38 dm^3 and 50 kg soil (soil density: 1.43 g cm^{-3}). Each pot was filled with RedYellow Latosol soil previously air-dried and then, mixed homogenously with lime (18 g 50 kg $soil^{-1}$, aiming to obtain 70% of base saturation) according to the methodology proposed by van Raij et al. (1997). The pots with soil and lime were moistened and incubated for 30 days for the lime to react with the soil. Subsequently, the soil of each pot was homogenized individually with the fertilizers [8 g urea (45% N), 8.13 g potassium chloride (KCl; 60% K_2O), 8.6 g triple superphosphate (42% P_2O_5 and 10% Ca), and 13.4 g micronutrients (2% Mn, 1% Cu, 10% Zn and 0.2% Mo)], according to the soil physicochemical analysis (Kiehl, 1979; van Raij et al., 2001; Donagema et al., 2017) (Table S1). Fertilizers application occurred in order to mimic the management practices carried out in

the cane fields by producers in Brazil. Sugarcane variety used was RB855536 (RIDESA BRASIL, Araras, Brazil), characterized as sensitive to water deficit (Hoffmann et al., 2008). The seedlings were produced by extracting a layer of reserve tissue, placing a buds per tube, filled with filter cake, substrate and sand.

2.2 Experimental design and treatments establishment

The experimental design was completely randomized block with four replicates and a 2 × 2 factorial scheme. The treatments consisted of sugarcane seedlings inoculated with water (control treatment) or with *Trichoderma asperellum* (isolates-GF332), and grown under conditions of adequate water availability (field capacity) and moderate drought stress (50% available water capacity (AWC)). The biological inoculant was a commercial conidia-based formulation of *T. asperellum* strain GF 332 (media culture: synthetic polymer polyvinylpyrrolidone (PVP), 1×10^{10} CFU g^{-1} of granules, water dispersible granules (WG), Lallemand®, Patos de Minas, MG, Brazil). The inoculations was carried out according to the technical recommendations of the manufacturers. These products are registered with the Ministry of Agriculture, Livestock, and Food Supply of Brazil. After 20 days of bud planted in the tubes, sugarcane seedlings were immersed in a solution containing inoculant with *Trichoderma asperellum* (GF332) to a concentration of 1.0 x 10^{10} CFU mL^{-1} and then, transplanted to the experimental pots. From the soil used in this experiment, the soil water retention curve was obtained at 0.00-0.15 m depth (see details in supplementary material) and the amount of water to raise the soil to field capacity was calculated. The pots with sugarcane seedlings were kept in field capacity until the seedlings reached the phenological stage of tillering and canopy development (Bonnett, 2013). During this period, two top-dressing fertilizations were carried as follows: at 30 days after transplanting (DAT) [3.9 g urea (45% N), and 4.3 g KCl (60% K_2O)] and at 115 DAT [15 g ammonium sulfate (21% N and 23% S), and 2.8 g KCl (60% K_2O)]. At beginning of the tillering and canopy development (~120 DAT), treatments with drought stress started.

The management of irrigation occurred through the use of a vacuum tensiometer, which indicated the daily irrigation up to 0.15 cm depth, considering the soil water retention curve (SWRC) (Fig. S1). The data obtained from the tensiometer were supplied in a digital spreadsheet that automatically calculated the volume of water to be applied to raise the soil's potential to field capacity (-10 kPa as 100% AWC = control treatment) according to SWRC.

Based on the SWRC, the amount of water needed to maintain moderate drought stress (50% AWC) was also determined. The tensiometer readings and irrigation occurred daily in the morning (8:00-10:00h am).

2.2.3 Sugarcane chemical and physiological parameters assessed

All nutritional, biochemical and physiological analysis occurred from sugarcane leaves [leaf +1 or Top Visible Dewlap leaf (TDV)] collected during the grand growth phenological stage (Bonnett, 2013), at ~60 days after beginning of the drought stress, collected between 9:00 h and 10:00 h am.

2.2.3.1 Sugarcane crop nutrition

The samples from leaf +1 were dried and ground in a Willey-type mill with a 1-mm screen, and subsequently, the macronutrients were determined All of the macronutrients (P, K, Ca, Mg, and S), except N, were extracted by nitroperchloric digestion and determined by atomic absorption spectrophotometry. Nitrogen was extracted through sulfuric digestion and determined by the Kjeldahl distillation method as described by AOAC (2016).

2.2.3.2 Photosynthetic pigments

To determine the photosynthetic pigments (Chl *a*, Chl *b*, and carotenoids) in the leaf tissues, five discs were cut between the edge and the leaf central vein from the fresh leaf +1. The discs were kept for 24 h in glass vials with 2 mL of N, N-dimethylformamide (DMF), as proposed by Lichtenthaler (1987). The determination of Chl *a*, Chl *b*, and carotenoids occurred through readings in absorbance at wavelengths of 664, 647, and 480 nm, respectively. The occurred according to the methodology proposed by Wellburn (1994).

2.2.3.3 Gas exchange

Sugarcane leaf gas exchange was assessed between 8:00 and 10:00 am am Leaf +1 was evaluated using a portable Infra Red Gas Analyzer (PPsystem CIRAS-3 Portable Photosynthesis System) with photon irradiance of 1000 μmol m^{-2} s^{-1}.The variables assessed were: net photosynthesis (*A*; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (*gs*; mol H2O m^{-2} s^{-1}); substomatal CO_2 concentration (*Ci*; mmol CO_2 mol^{-1} air); leaf transpiration (*E*; mmol H_2O m^{-2} s^{-1}); water use efficiency (WUE; μmol CO_2 (mmol H_2O)$^{-1}$), calculated by the *A*/*E* ratio, and carboxylation efficiency, calculated by the *A*/*C*i ratio.

2.2.3.4 Nitrate reductase activity

The in vivo nitrate reductase (NR) activity was adapted from Reis et al. (2009). Sugarcane fresh leaves (100 mg) was cut with scissors and transferred to assay tubes containing 3 mL of phosphate buffer solution pH 7.4 (50 mM L^{-1} plus 200 mM L^{-1} KNO_3). These samples were vacuum-infiltrated for 5 min to enhance the solution penetration into tissues. Afterwards, the assay tubes were incubated in a 33 °C water bath for 30 min wrapped in aluminum to prevent light penetration. The reaction was stopped by adding 1 mL of 1% sulfanilamide in 2 mol L^{-1} HCl solution plus 1 mL aliquot of 0.05% naphtylenediamine solution. The results obtained were based on the nitrite (NO_2^-) standard curve and read in absorbance in a spectrophotometer at wavelength of 540 nm. The nitrate reductase activity in the fresh leaves was expressed as nM NO_2^- h^{-1} g FW^{-1}.

2.2.3.5 Obtaining the crude extract from leaf samples for antioxidant metabolism

To obtain the crude extract (Giannopolitis and Ries, 1977), sugarcane leaves (0.3 g) were ground with a mortar and pestle under liquid nitrogen and suspended in 5.0 mL of 0.1 M potassium phosphate buffer, pH 6.8, supplemented with 200 mg of polyvinylpolypyrrolidone (PVPP). Then, the samples were centrifuged for 15 minutes at 5,000 × g for 20 min. A 2 mL aliquot of supernatant was collected and stored in a freezer at (-80ºC). From this extract, analyzes of the total soluble protein, activity of the superoxide dismutase, catalase and peroxidase were performed.

For proline determination (Bates et al., 1973), a second extract was obtained. 500 mg of the plant material (ground) was resuspended in 10.0 mL of sulfosalicylic acid (3% in deionized water). Then, the samples were centrifuged for 12 min at 4,000 × g at 4 ºC, and the supernatant was collected and stored in a freezer at -80 ºC for later determination of the proline content.

2.2.3.6 Total soluble protein content

Total soluble protein determination occurred according to the methodology proposed by Bradford (1976). A 50 µL aliquot of the crude extract was mixed with 4,950 µL of Bradford's solution and analyzed in a spectrophotometer at a wavelength of 595 nm. Bovine serum albumin (BSA) was used as a standard curve. The results and protein extract were used to determine the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and proline.

2.2.3.7 Superoxide dismutase activity (SOD; EC 1.15.1.1)

The activity of SOD was determined according to the method proposed by Giannopolitis and Ries (1977). The reaction chamber under a 15-W fluorescent lamp at 25ºC was used. A 50 μL aliquot of crude protein was mixed to 2 mL of potassium phosphate buffer solution at 50 mM and pH 7.8, plus 13 mM methionine, 75 μM NBT, 100 nM EDTA and 2 μM riboflavin in 3.0 mL. The samples were vortexed, stored in a closed chamber free of any external light, and illuminated for 20 min in the reaction chamber. After that, the samples were homogenized and read in absorbance at spectrophotometer wavelength of 560 nm, and the results are expressed as U SOD mg^{-1} protein.

2.2.3.8 Peroxidase activity (POD; EC 1.11.1.7)

The POD activity was assayed according to the methodology of Peixoto et al. (1999). A 50 μL aliquot of crude extract was added in 4.95 mL of 25 mM potassium phosphate buffer, pH 6.8, containing 20 mM Pyrogallol and 20 mM hydrogen peroxide (H_2O_2). After 1 min of incubation the reaction was stopped with 0.5 mL of H_2SO_4. The absorbance reading was performed at 420 nm. A molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used for the calculation of the POD specific activity and expressed in μKat μg $protein^{-1}$.

2.2.3.9 Determination of proline

The determination of the proline content occurred according to the method proposed by Bates et al. (1973). A 2.0 mL aliquots of the crude extract was mixed with 2.0 mL of acid ninhydrin and 2.0 mL of glacial acetic acid. Then, the mixture was heated at approximately 100 °C for 60 minutes. After the cooling, the absorbance readings were performed at 520 nm. The results were applied to the standard curve of pure proline, and the values were expressed in μmol g fresh $weight^{-1}$ (FW).

2.2.3.10 Sugar concentration

Sugar fractions (reducing sugar, sucrose, and total soluble sugar concentration) on sugarcane leaves were extracted according to the modified method of Xu et al. (2015). Sugarcane leaf ground sample (0.1 g) was extracted with 80% (v/v) ethanol. The samples were mechanically stirred at 65 ° C for 1 h, followed by centrifugation at 10,000 × g for 15 min. The sugar fractions was determined spectrophotometrically according to the methods of Somogyi-Nelson (Nelson, 1944; Somogyi, 1952). The readings were performed by a spectrophotometer at 535, 480 and 620 nm wavelength, respectively for each sugar fraction. The ethanol-insoluble

residue was used to determine the starch contents following the methodology proposed by Kuai et al. (2014) After evaporation of ethanol, 2 mL of deionized water was added into the samples, and incubated at 100 ° C for 15 min. Starch hydrolyzation occurred by addition of 9.2 M and later, 4.6M $HClO_4$ to the samples. Starch concentration was determined spectrophotometrically with anthrone reagent at 620 nm wavelength.

2.2.4 Root parameters

Prior to harvest, the root sampling occurred using a probe with 0.48 cm in diameter by 0.20 m in depth (same depth of the pots). The subsamples were washed under running water and scanned by a digitizer coupled to a computer with the WinRhizo software version 3.8-b (Regent Instruments Inc., Quebec, Canada), as described by Tennant (1975) to determine the root length. The values obtained were converted to m dm^{-3} from the pot volume and the probe. Afterwards, samples were dried in a forced air oven at 60 ° C until reached constant weight to determine the root dry weight. The values obtained were converted to g dm^{-3}.

2.2.5 Sugarcane morphological attributes

The sugarcane biometric and production parameters were assessed at harvest. The height of plants was determined by measuring with a graduated rule belonging to the soil up to the leaf +1. The stalk diameter was measured with a digital pachymeter, measuring the internode of the first third of the plant from the soil. The number of tillers occurred by the tillers count, discounting the mother plant. The leaf width was measured in the middle third of the leaf +1 with graduated rule. Stalk fresh weight was determined after the harvest (stalks without leaves), and the stalk dry weight was determined after the samples were subjected to drying in a forced air oven at 65 °C until the material was completely dried.

2.2.6 Statistical analysis

The normality and homoscedasticity of the data in all datasets were checked using the Anderson-Darling test, and homoscedasticity was analyzed with Levene's test. Subsequently, the means were subjected to analysis of individual variance (ANOVA) by the F test ($p \leq 0.05$) and, when significant, were analyzed using the Fisher's protected least significant difference (LSD) at $p \leq 0.05$.

2.3 Results

2.3.1 Crop nutrition

Changes in sugarcane crop nutrition occurred ($p < 0.01$) only for N and S concentrations (Table S2; Fig. S2). Nitrogen leaf concentration from sugarcane plants inoculated with *T. asperellum* increased by 14% when established under optimal water availability, and 22% under drought stress (Fig. S2a). However, under drought stress, N uptake by non-inoculated plants reduced 23%, and 18% in inoculated plants. Sulfur leaf concentration reduced in drought-stressed sugarcane plants, but *T. asperellum* inoculation increased the S acquisition by the plants, not differing from treatments without drought stress (Fig. S2f). The other macronutrients (P, K, Ca and Mg) were not affected by the treatments (Fig. S2b-e).

2.3.2 Photosynthetic pigments and gas exchange

Photosynthetic pigments changed according to the treatments (Table S2; Fig. 2). In no-stressed plants, leaf chlorophyll concentration (Chl *a*, *b* and total) increased ($p < 0.01$) in inoculated plants (14%, 15% and 14%, respectively) (Fig. 2a, b and c). In drought-stressed plants, *T. asperellum* inoculation also increased the chlorophylls concentration (13%, 10% and 12%, respectively for Chl *a*, *b* and total), but the values found were lower than in non-stressed plants. *Trichoderma asperellum* inoculation did not change the carotenoids concentration, but under drought stress, non-inoculated plants presented 17% less carotenoids than plants established plants under adequate water availability; whereas in inoculated plants, drought stress reduced 12% the concentration of this pigment.

Trichoderma asperellum inoculation increased ($p < 0.01$) net photosynthetic rate (*A*) by 50% in non-stressed plants, and 68% in drought-stressed plants (Table S2; Fig. 3a). Regarding the drought effects, *A* values reduced in 90% in non-stressed, and 70% in drought-stressed plants. Stomatal conductance (*gs*), internal CO_2 concentration (*Ci*) and transpiration (*E*) not changed in non-stressed plants according to the inoculation treatment, but in drought-stressed plants, *T. asperellum* increased *gs* by 12% compared with non-inoculated plants (Fig. 3b, c and d). In general, the drought stress effects in inoculated plants were lower than in non-inoculated one. Drought stress reduced 50%, 19% and 32% the *gs*, *Ci* and *E* values in non-inoculated plants and by 47%, 24% and 39% in inoculated plants, respectively.

Considering the water use efficiency (WUE; *A*/*E* ratio), *T. asperellum* inoculation increased ($p < 0.01$) these values in 42% in non-stressed plants and in 78% in drought-stressed plants (Fig. 3e). Drought stress did not change the WUE in inoculated plants; whereas reduced

23% this parameter in sugarcane plants non-inoculated. Regarding carboxylation efficiency (*A*/*Ci* ratio), drought stress reduced in 37% and 20% this parameter in non-inoculated and inoculated plants, respectively (Fig. 3f). On the other hand, when sugarcane plants were inoculated, carboxylation efficiency increased by 59% in non-stressed plants and 86% in drought-stressed plants.

2.3.3 Enzymatic activity and proline concentration in leaves

Sugarcane plants inoculated with *T. asperellum* increased ($p < 0.01$) the nitrate reductase (NR) activity in both non-stressed and drought-stressed plants by 91% and 114%, respectively, and the drought stress most negatively affected the NR activity in non-inoculated plants (47%) than inoculated one (40%) (Table S2; Fig. 4a). Conversely, antioxidant enzymatic activity and proline concentration reduced ($p < 0.01$) when sugarcane plants were inoculated, in both water availability conditions. Drought stress in non-inoculated plants increased the activity of superoxide dismutase (SOD) (16%), peroxidase (POD) (19%) and proline concentration (63%), whereas in inoculated plants, the increase occurred by 11.5% , 20% and 63% (Fig. 4b, c and d). On the other hand, *T. asperellum* inoculation decreased the same parameters by 19% for SOD, 24% for POD and 40% for proline in non-stressed plants, and by 26%, 22% and 38%, respectively, in sugarcane plants under drought stress.

2.3.4 Leaf sugar fractions

Reducing sugar concentration increased ($p < 0.01$) in drought-stressed plants, but sugarcane inoculated with *T. asperellum* reduced this value by 38% compared with non-inoculated plants (Table S2; Fig. 5a). Increases ($p < 0.01$) occurred for sucrose (non-stressed = 26%; drought-stressed = 65%), total sugar (non-stressed = 12%; drought-stressed = 18%) and starch (non-stressed = 33%; drought-stressed = 15%) concentrations (Fig. 5b, c and d). Notably, drought stress reduced sucrose (non-inoculated = 42%; inoculated = 24%), total sugar (non-inoculated = 15%; inoculated = 10%), and starch (non-inoculated = 32%; inoculated = 41%) concentrations compared with non-stressed plants; however for sucrose and total sugar, drought stress effects were stronger in non-inoculated plants.

2.3.5 Root and morphological attributes

All biometric and yield parameters were improved ($p < 0.01$) by the treatments, except stalk diameter (Table S2; Fig. 6). *Trichoderma asperellum* inoculation in sugarcane plants increased plant height (non-stressed = 8.0%; drought-stressed = 7.6%) and in 8.2% the leaf

width in drought-stressed plants (Fig. 6a and c). Drought stress also reduced plant height (non-inoculated = 9.5%; inoculated = 9.8%), and by 11% the leaf width in non-inoculated plants. In addition, *T. asperellum* inoculation increased the number of tillers plant^{-1} (12%) in non-stressed plants (Fig. 6d). As result these parameters combination, stalk fresh and dry weight also improved (Fig. 6e and f). Inoculated plants increased both fresh (non-stressed = 9.5%; drought-stressed = 28%) and dry (non-stressed = 14%; drought-stressed = 41%) weight of stalks. On the other hand, drought stress reduced stalk fresh weight (non-inoculated = 30%; inoculated = 18%), and stalk dry weight (non-inoculated = 39%; inoculated = 25%) compared with non-stressed plants.

Interestingly, *T. asperellum* inoculation not only improved the root length, but also enhanced the root dry weight production (Table S2; Fig. 7). In inoculated plants, increases ($p < 0.01$) occurred by root length (non-stressed = 42%; drought-stressed = 16%) and root dry weight (non-stressed = 21%; drought-stressed = 24%) (Fig. 7a and b). Conversely, drought stress reduced the root length (non-inoculated = 20%; inoculated = 35%), and root dry weight (non-inoculated = 16%; inoculated = 15%).

2.4 Discussion

Our understanding *Trichoderma* spp. impacts on sugarcane nutrient uptake, plant physiology and yield are limited, especially under environmental stresses conditions. Previous studies have demonstrated that drought stress is one of the major environmental stress factors that causes biochemical alterations in plants, reduces plant growth, and decreases plant yield (Basu et al., 2016; Gupta et al., 2020). Therefore, our findings can help in understanding the effects of *T. asperellum* inoculation on sugarcane crop alleviating the drought stress effects in photosynthetic and antioxidant metabolism and their reflexes on stalk yield. Our results showed that the *T. asperellum* inoculation not only improved sugarcane nutrition and agronomic parameters, but also its physiological metabolism and yield (Figs. 2-7). These findings are consistent with previous studies that reported *Trichoderma* spp. can enhance yield and quality of tomato (Khoshmanzar et al., 2020), wheat (Zhang et al., 2016), rice (Doni et al., 2019; Sousa et al., 2020), and maize (Fu et al., 2017; Estévez-Geffriaud et al., 2020) under abiotic stresses.

Generally, water deficiency reduces the nutrient uptake by the roots and transport from root to shoot (Ghassemi et al., 2018). However, the use of some microorganisms can assist in the acquisition of nutrients from the soil even under water restrictions (Khoshmanzar et al.,

2020). Inoculation with *T. asperellum* presented higher N and S uptake in both non-stressed and drought-stressed plants (Fig. S2). The increase in N and S absorption can occur directly through the production of hydrolases (e.g., proteases and chitinases), which are responsible for degrading rhizospheric proteins and chitin and increasing the N and S uptake (Khoshmanzar et al., 2020); as well as indirectly, assisting in the supply of simple degradation compounds to heterotrophic nitrogen fixing microorganisms (Shoresh et al., 2010; Doni et al., 2019). In addition to the improvement in the nutrient acquisition from the soil solution, the N use efficiency seems to have been increased, given the greater activity of NR in inoculated plants (Fig. 4a). Several evidences have shown that *Trichoderma* spp. are directly involved in the N metabolism, mediated by the activation of NR in plants (Sherameti et al., 2005). The amount of nutrients applied to the plants was the same, so the increase in the nutrition of sugarcane plants, especially N, may be related to the increase in the efficiency of the use of this element. Nitrate reductase is the first enzyme in the nitrate assimilation pathway and probably is a determining factor in the efficiency of this process. Notwithstanding, *Trichoderma* spp. are widely recognized as phytohormones synthesizers (e.g., auxin) and hormone-like compounds called harzianolide and swollenin, substances that help along with auxin, in the expansion of cell wall (Rajesh et al., 2016; Sousa et al., 2020). These compounds enhances not only the shoot dry weight, but also the root and rootlets development (Sousa et al., 2020) involved in water and nutrients absorption, as reported in our study (Figs. S2 and 7). Therefore, sugarcane inoculated with *T. asperellum* performed better in terms of water and nutrients absorption by the roots compared to the non-inoculated plants, resulting in higher plant height and stalk production (Fig. 6).

The benefits of *T. asperellum* inoculation in sugarcane plants are notorious, even under drought stress. The photosynthetic machinery of plants inoculated with this fungus has been improved, starting with the increase in chlorophyll concentrations (Fig. 2). Chlorophylls concentration is widely used as an important indicator of abiotic tolerance in crops (Chutipaijit et al., 2011). As previously reported, *T. asperellum* changed the concentration of N, a nutrient directly involved in the composition of chlorophyll (Lawson and Flexas, 2020). In addition, plants exposed to drought stress usually show pronounced chlorosis due to rapid chlorophyll degradation (Zhang et al., 2016) leading to overall plant growth retardation. Our results showed that drought-induced stress significantly decreased chlorophylls concentration, but these values were reversed back to a level close to non-inoculated sugarcane plants grown under available water. In fact, we discovered that the chlorophylls concentration increased in sugarcane plants

inoculated with *T. asperellum* whether or not subjected to drought stress. Our findings reinforce the important role of this microorganism in regaining the vigour of plants established in stressful environments.

As a result, *T. asperellum* also promoted benefits in photosynthesis rate (*A*), stomatal conductance (*gs*), water use efficiency (WUE) and carboxylation efficiency (Fig. 3). Our results are in agreement with the findings of Bae et al., (2009) and Sousa et al. (2020) who reported that plants inoculated with *Trichoderma* spp. reduced the drop on *A* and *gs* values, in addition to increasing the photosynthetic area of sugarcane leaves (Fig. 6c). The increase in photosynthetic efficiency can also be seen in the increase in the levels of sugars and starch in sugarcane leaves (Fig. 5). Although *E* and *Ci* values did not change by inoculation, sugarcane plants photosynthetically active and with stomatal conduction control showed higher WUE and carboxylation efficiency, since a higher rate of photosynthesis occurred for each unit of water that was evaporated by the stomata and CO_2 that was carboxylated in the biochemical phase of photosynthesis. Stomatal conductivity can be altered by inoculation with *T. asperellum*, since this microorganism acts as an abscisic acid producer (Poveda, 2020). Abscisic acid is an important phytohormone related to the modulation of stomatal opening-closure movement (Zargar et al., 2017). These results are very relevant to sugarcane fields, as it is a semi-perennial crop, which is harvested in a cycle between 1-1.5 years and is subject to numerous periods of environmental stresses such as higher temperatures and water scarcity throughout its cycle (Marcos et al., 2018a). *Trichoderma asperellum* inoculation promoted an increase in the efficiency of gas exchange parameters, hence the higher stalk and roots weight in these plants was obtained (Figs. 6 and 7). These results revealed that the phytohormones and other similar compounds produced by this fungus are not the only mechanism to enhance root and stalk weight, that is, the relationship between water absorption to the losses from stomata, as well as the increase in photosynthesis in inoculated than non-inoculated plants are also involved in enhancement in sugarcane below and aboveground development.

The reduction in chlorophyll degradation and the increase in the photosynthetic process may also be related to the increase in antioxidant systemic resistance in sugarcane plants inoculated with *T. asperellum* (Fig. 4). The complex interaction between fungus and host may inhibit the production and accumulation of reactive oxygen species (ROS) by increasing the activity of enzymes and other antioxidant compounds in plant tissue (Brotman et al., 2013; Fu et al., 2017). The overproduction of ROS in plant tissues is considered a biochemical change responsible for damages to macromolecules and plants cellular structures under drought stress

Zargar et al., 2017); on the other hand, ROS production can also indicate signalling pathways (Foyer, 2018; Farooq et al., 2019). Under environmental stress conditions for example, ROS production can function as sensors that trigger repair mechanisms, assisting the plant in several processes that are linked to defence metabolism or cell growth (Foyer et al., 2017; Foyer, 2018). Although we did not evaluate ROS concentrations directly in our study, the antioxidant enzyme activity and, especially, the proline content indicated that in this case, the ROS produced in non-inoculated plus drought-stressed plants acted as causes of cell damage. To alleviate the ROS damages, plants produce the wide range of antioxidant defence mechanisms to detoxify free radicals accumulation in their cells and protect them from oxidation (Zhang et al., 2016). The findings from our study demonstrated that drought stress induced plants to produce higher levels of SOD and POD activities, besides proline concentration than the non-stressed plants, but *T. asperellum* reduced these values, probably due to the lower stress level in inoculated plants. Our results suggested that SOD and POD enzymes played a central protective role in the ROS scavenging in sugarcane plants treated *with T. asperellum*. In addition, drought stress increased proline biosynthesis, which enhances protein turnover (Khan et al., 2010). The proline compound is an important nitrogen source produced and used by plants to recover from abiotic stresses and restore their growth (Trotel et al., 1996). (Karuppiah et al., (2019) studying Co-cultivation of *T. asperellum* and *Bacillus amyloliquefaciens* in wheat growth stated that this fungus induced the production of secondary metabolites, including proline, and increased the activity of antioxidant enzymes, both involved in increasing plant resistance against abiotic factors. In addition, another mechanism that may be involved in increasing resistance against drought stress is the participation of *Trichoderma* spp. in the regulation of resistance genes (Harman et al., 2004; Sousa et al., 2020). In particular *T. asperellum* is known as a modulator of defence genes expression during interaction with their host, regulating the pathway of jasmonic acid and salicylic acid (Segarra et al., 2007), both involved in increasing plant responses against environmental stresses (Segarra et al., 2007; Mastouri 476 et al., 2010; Sousa et al., 2020).

Interestingly, in drought-stressed plants, the reducing sugar increased, but *T. asperellum* inoculation decreased this parameter (Fig. 5a). The opposite occurred for sucrose, total sugars and starch concentration (Fig. 5b-d). This occurred due to under challenging environmental conditions, more complex carbohydrates (e.g., sucrose and starch) are generally remobilized to provide energy and carbon at times when photosynthesis may be potentially limited (Thalmann and Santelia, 2017), which results in an increase in reducing sugar concentrations (Fig. 5).

Carbohydrates portioning support plant growth under stress, acting as osmoprotectants and signalling molecules, mitigating along with antioxidant metabolism, the negative effect of the stress (Krasensky and Jonak, 2012; Thalmann and Santelia, 2017).

2.5 Conclusions

In summary, sugarcane inoculated with *T. asperellum*, altered the physiological parameters such as photosynthetic pigments, photosynthesis rate, stomatal conductance, water use efficiency and carboxylation efficiency, activation of nitrate reductase enzyme, and antioxidant metabolism. This cascade effect culminated in sugarcane plants growth, tillering and stalk yield even under drought stress. It is a significant result for sugarcane fields that constantly experience periods of water scarcity during the crop cycle. Inoculation is a feasible management tool, with promising and low-cost results that can increase the useful life of the sugarcane field and the farmer's rentability. Our study evaluated the impact of inoculation on sugarcane seedlings, however, future studies should be carried out under field conditions to verify the viability and the need for the reinoculation of *T. asperellum* on sugarcane ratoons.

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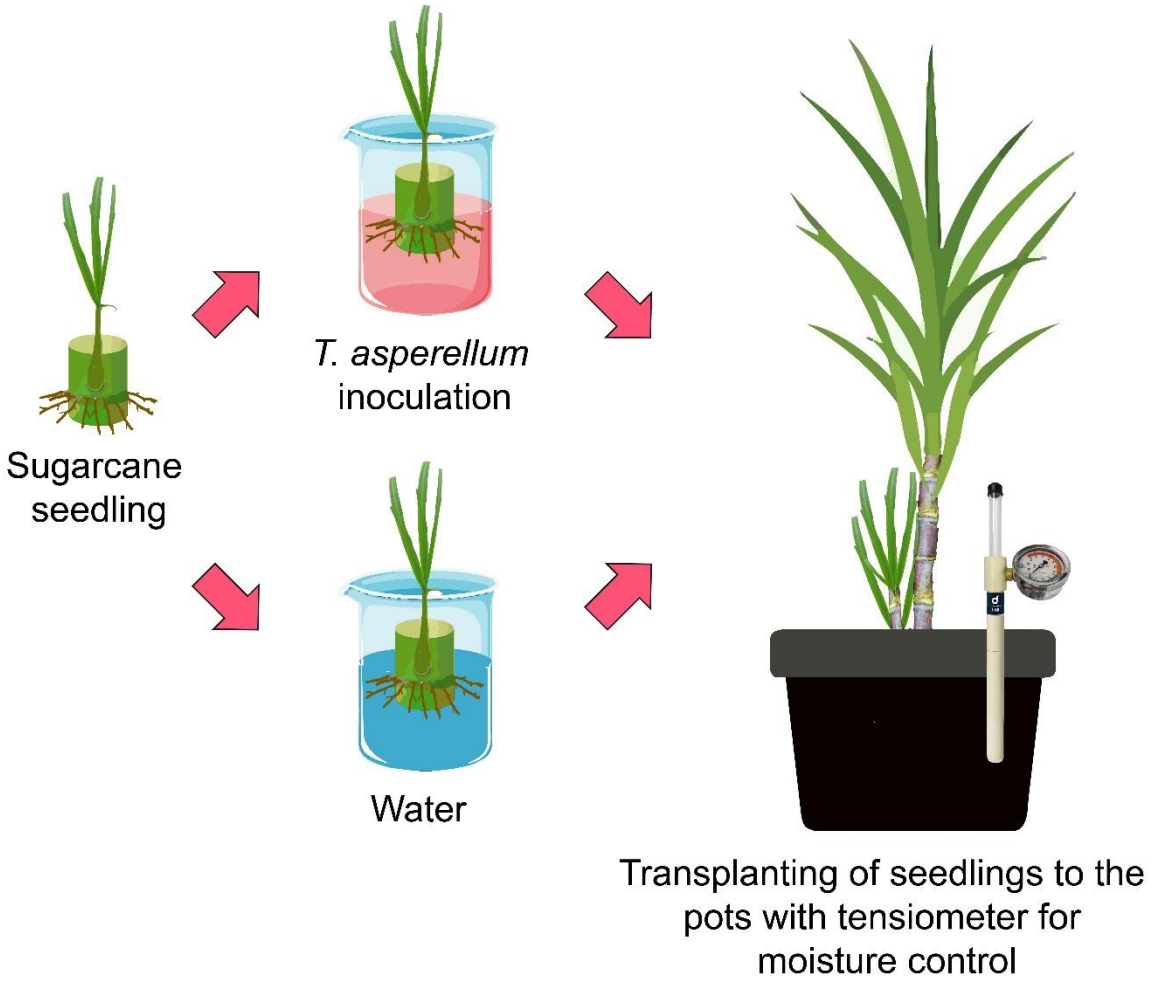

Figure. 1. Schematic figure representing the experiment setup.

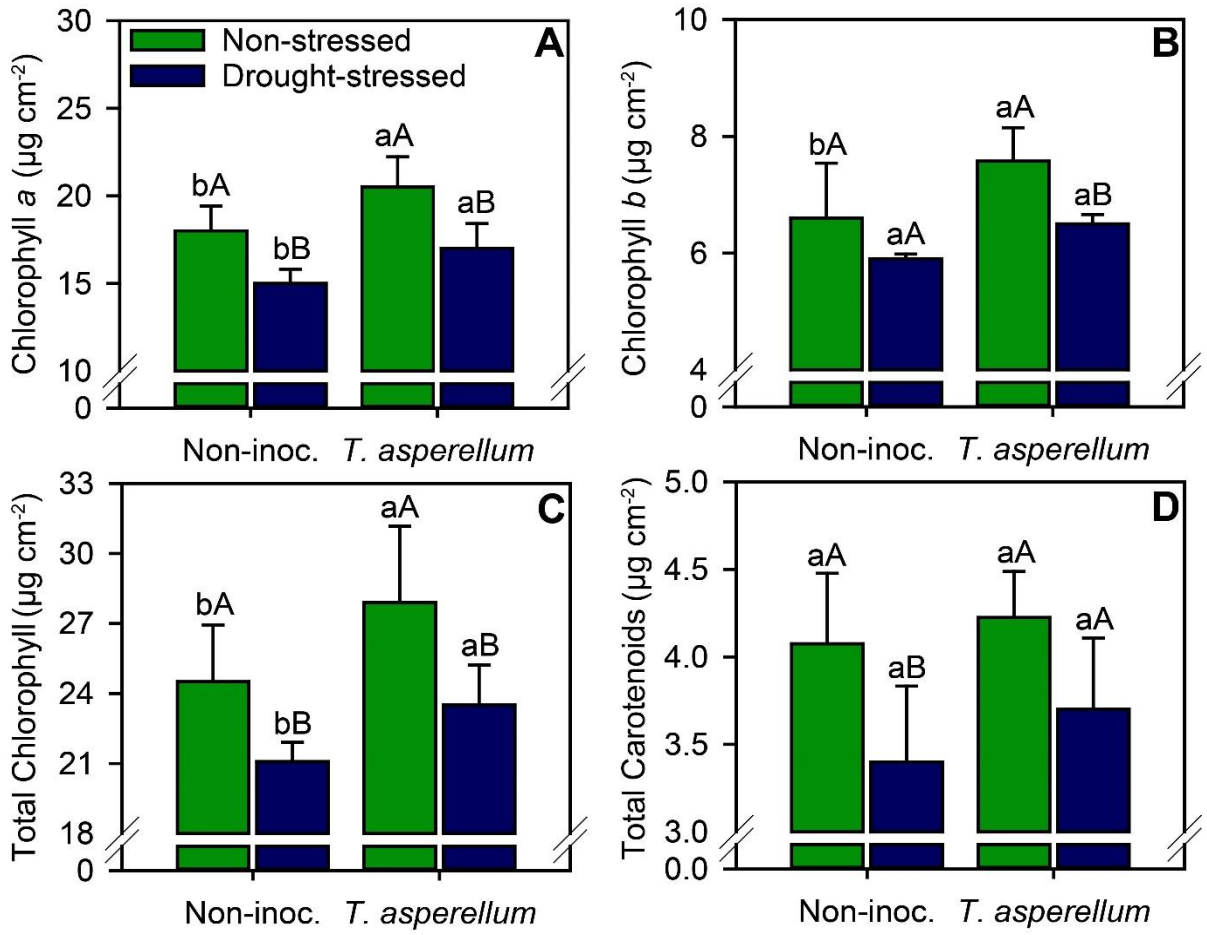

Figure. 2. Chlorophyll *a* (a), chlorophyll *b* (b), total chlorophyll (c) and total carotenoids (d) concentration in sugarcane leaves according to the treatments. Different lower-case indicate significant differences between presence or absence of moderate drought stress, and different capital letters indicate significant differences between presence or absence of inoculation with *T. asperellum* by Fisher's protected LSD test at $p \leq 0.05$. Error bars express the standard error of the mean (n = 4).

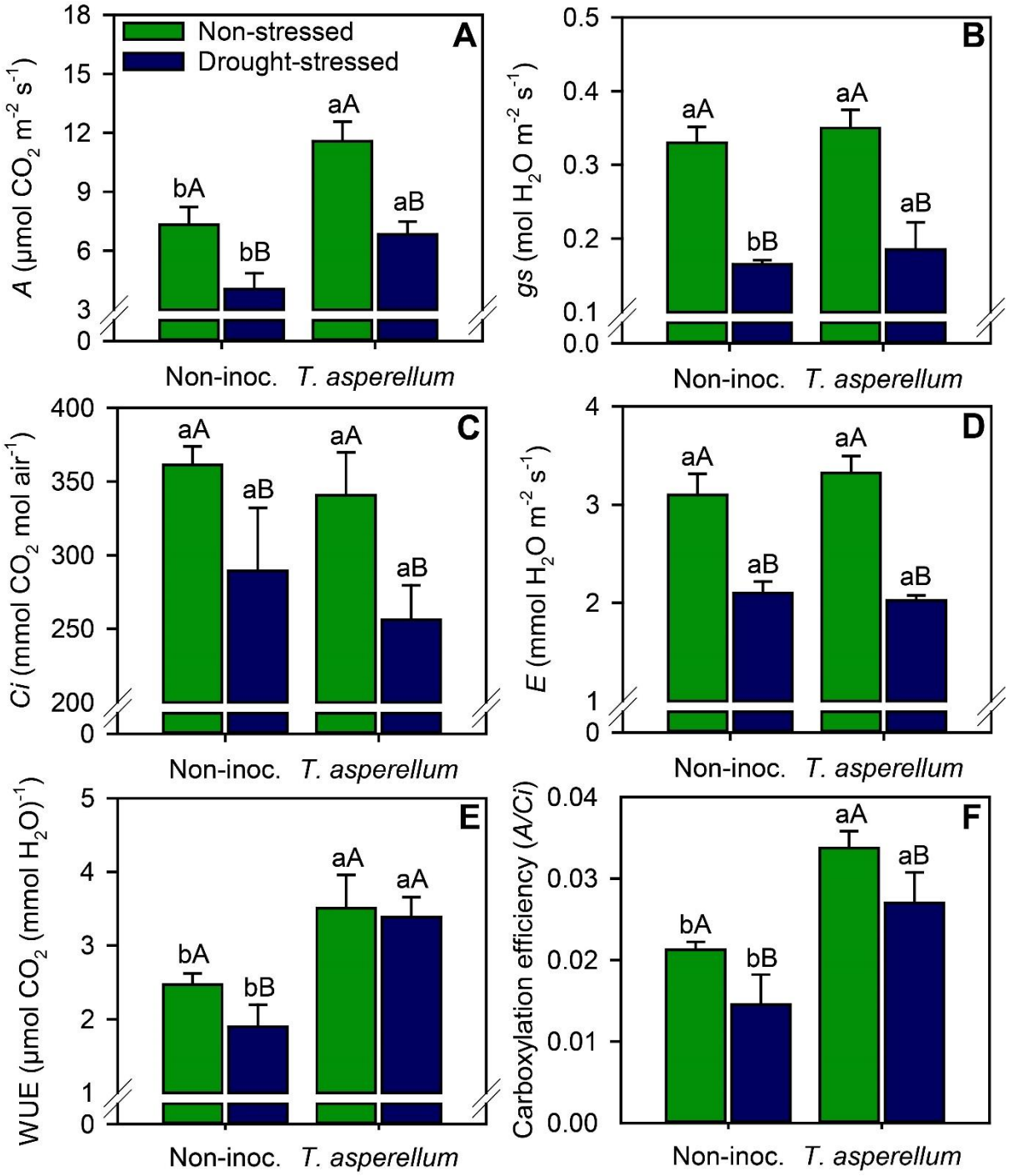

Figure. 3. Net photosynthesis rate (a), stomatal conductance (b), substomatal CO_2 concentration (c), leaf transpiration (d), water use efficiency (e), and carboxylation efficiency (f) measured in sugarcane leaves according to the treatments. Different lower-case indicate significant differences between presence or absence of moderate drought stress, and different capital letters indicate significant differences between presence or absence of inoculation with *T. asperellum* by Fisher's protected LSD test at $p \leq 0.05$. Error bars express the standard error of the mean (n = 4).

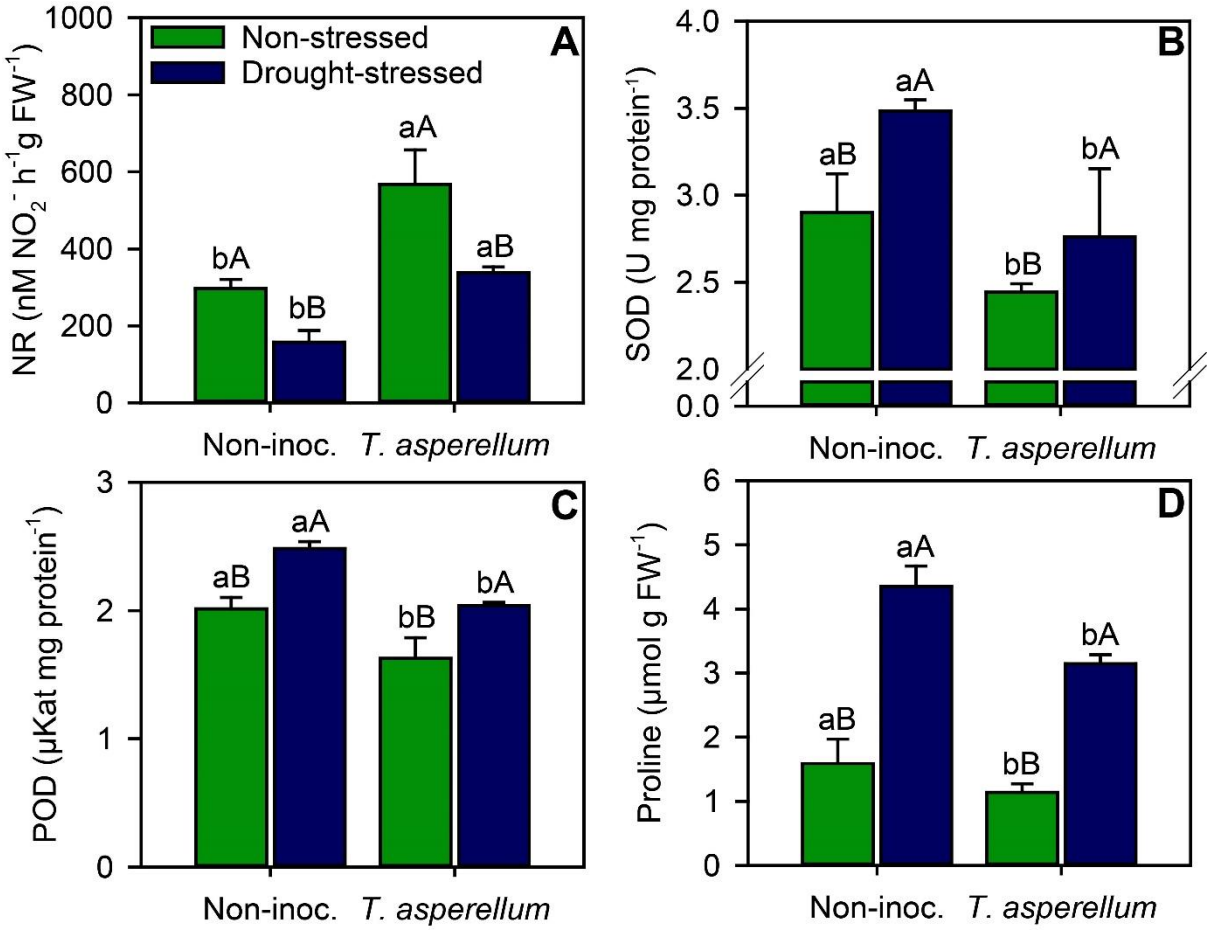

Figure. 4. Activities of nitrate reductase (a), superoxide dismutase (b) and peroxidase (c), and proline concentration (d) measured in sugarcane leaves according to the treatments. Different lower-case indicate significant differences between presence or absence of moderate drought stress, and different capital letters indicate significant differences between presence or absence of inoculation with *T. asperellum* by Fisher's protected LSD test at $p \leq 0.05$. Error bars express the standard error of the mean (n = 4).

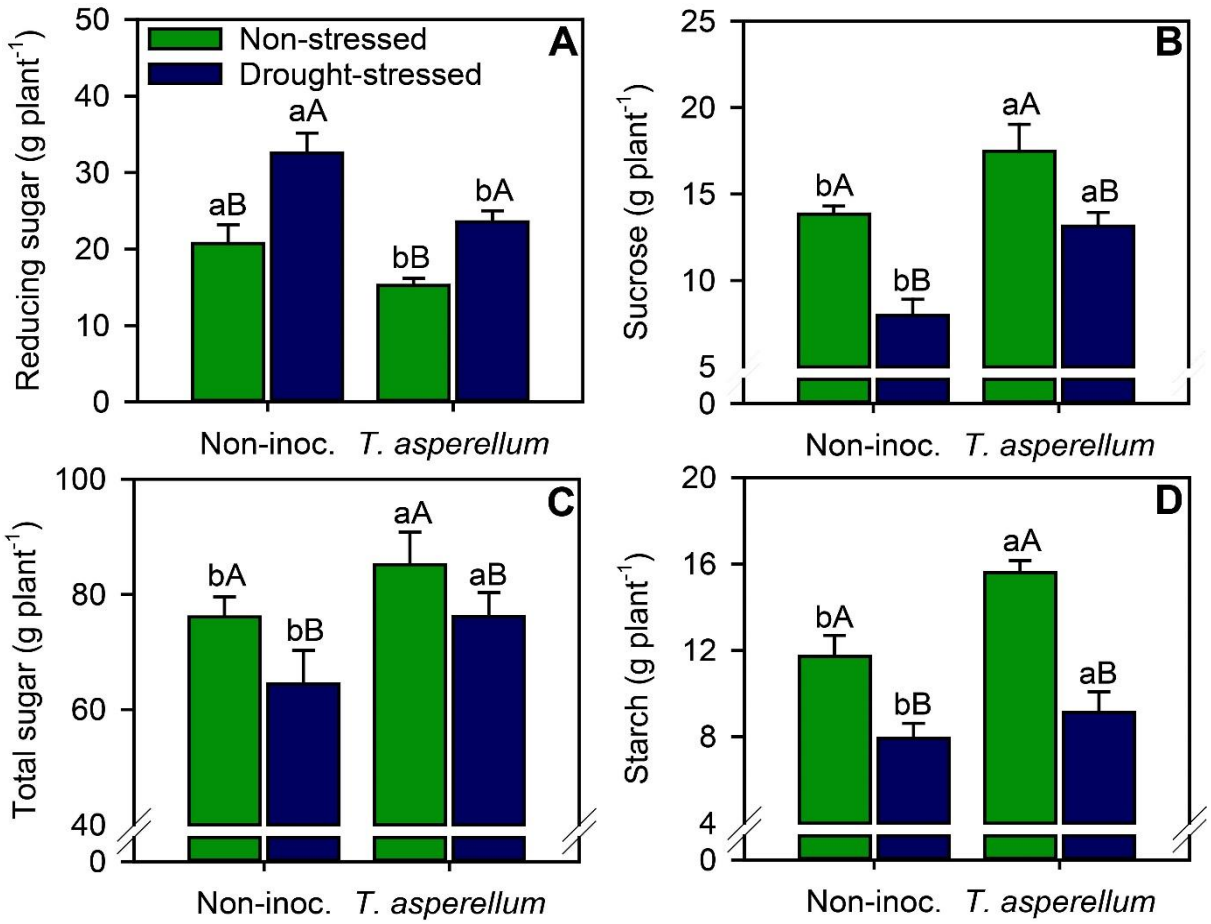

Figure. 5. Reducing sugar (a), sucrose (b), total sugars (c), and starch (d) concentrations in sugarcane leaves according to the treatments. Different lower-case indicate significant differences between presence or absence of moderate drought stress, and different capital letters indicate significant differences between presence or absence of inoculation with *T. asperellum* by Fisher's protected LSD test at $p \leq 0.05$. Error bars express the standard error of the mean (n = 4).

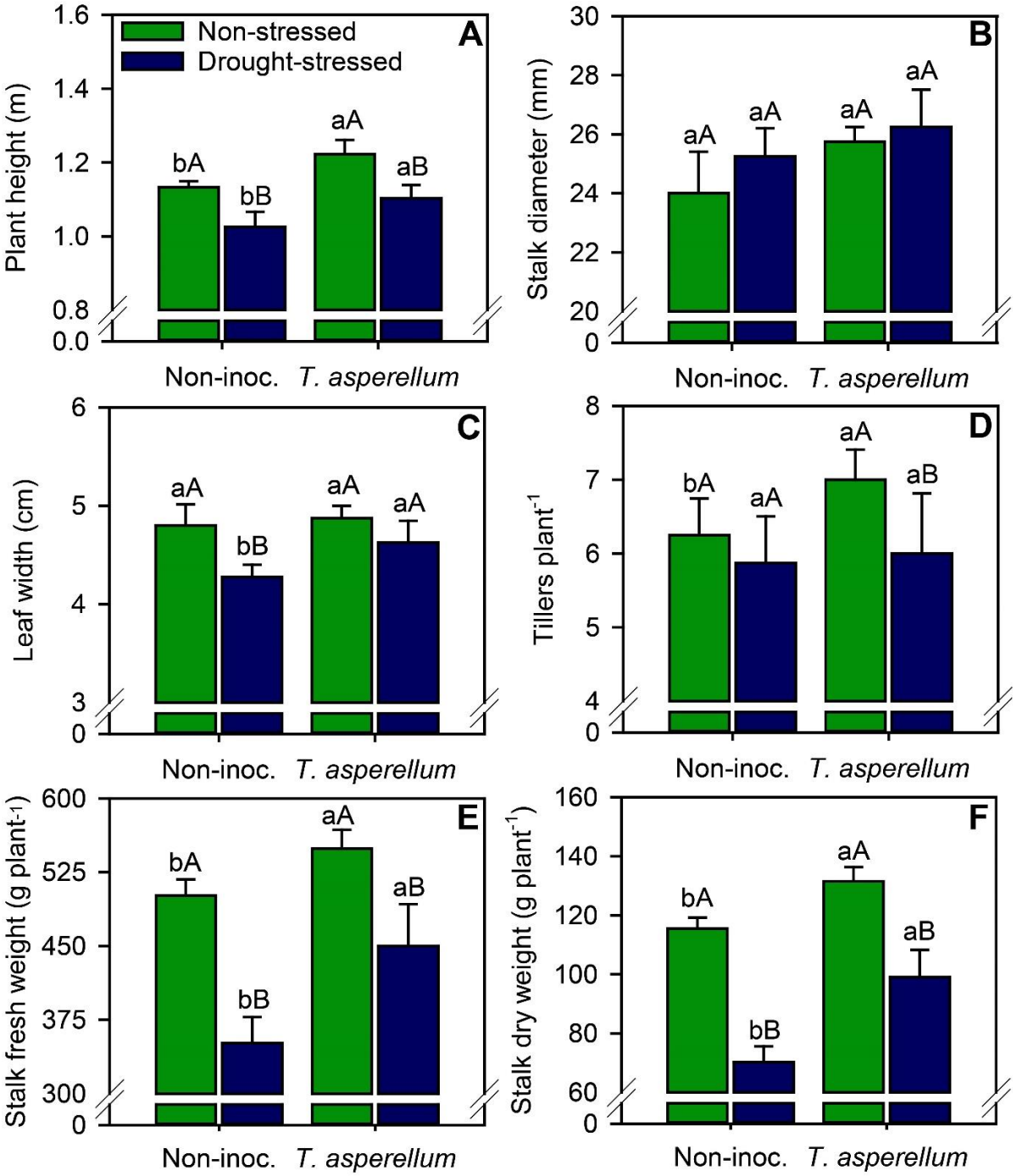

Figure. 6. Plant height (a), stalk diameter (b), leaf width (c), tillers plant^{-1} (d), stalk fresh weight (e), stalk dry weight (f) of sugarcane plants according to the treatments. Different lower-case indicate significant differences between presence or absence of moderate drought stress, and different capital letters indicate significant differences between presence or absence of inoculation with *T. asperellum* by Fisher's protected LSD test at $p \leq 0.05$. Error bars express the standard error of the mean (n = 4).

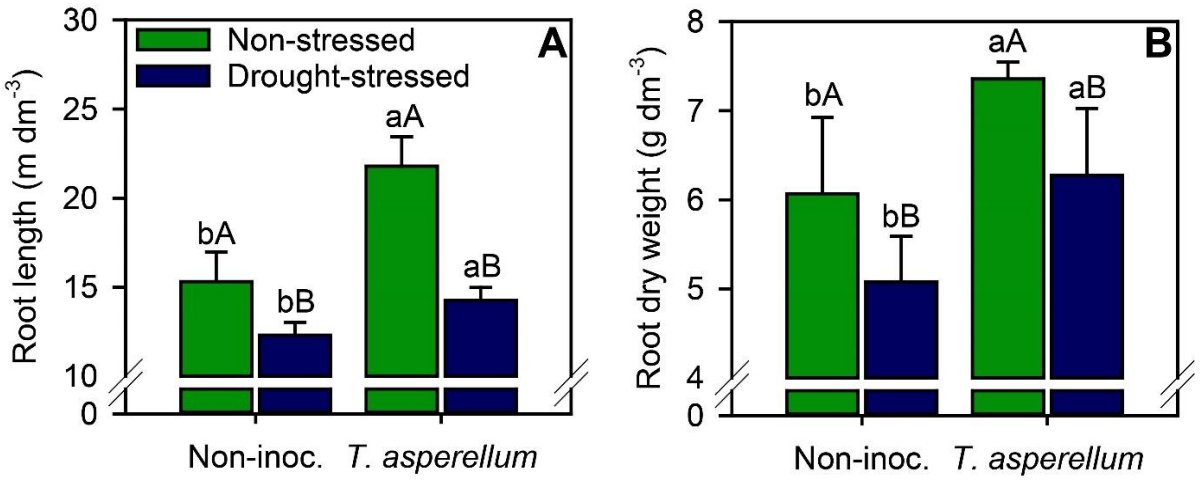

Figure. 7. Root length (a) and root dry weight (b) of sugarcane plants according to the treatments. Different lower-case indicate significant differences between presence or absence of moderate drought stress, and different capital letters indicate significant differences between presence or absence of inoculation with *T. asperellum* by Fisher's protected LSD test at $p \leq 0.05$. Error bars express the standard error of the mean (n = 4).

Table S1. Physical-chemical analysis of the soil before the beginning of the experiment.

Sand	Clay	Silt	Soil texture	Soil density
	---------- (g kg^{-1}) ----------			(g cm^{-3})
810	141	49	Sandy	1.46

pH ($CaCl_2$)	SOM (g dm^{-3})	P_{resin} (mg dm^{-3})	K^+	Ca^{2+}	Mg^{2+}	SB	CTC	BS
			---------- ($mmol_c$ dm^{-3}) ----------					
4.1	5	2	0.31	4	1	6	22	25

S (g dm^{-3})	Al^{3+}	H+Al	Fe	Cu	Mn	Zn	B
	($mmol_c$ dm^{-3})		---------- (mg dm^{-3}) ----------				
9	12	17	88	1.1	9.2	1.2	0.7

Table S2. Statistical parameters of ANOVA test (p≤0.05) for all sugarcane variables studied.

Treatments	ANOVA (*F probability)*					
			Crop nutrition			
	N	P	K	Ca	Mg	S
Inoculation (I)	0.0213	0.1070	0.8642	0.4264	0.1833	0.0322
Drought Stress (DS)	<0.001	0.5263	0.1062	0.6119	0.0760	0.1523
I X DS	0.0462	0.6330	0.7586	0.5247	0.3104	0.0425
			Photosynthetic pigments			
	Chl *a*	Chl *b*	Total chlorophyll	Total carotenoids		
Inoculation (I)	0.0016	<0.001	<0.001	0.0698		
Drought Stress (DS)	0.0022	<0.001	<0.001	0.0075		
I X DS	0.0123	0.0495	0.0144	0.0500		
			Gas exchange			
	A	*gs*	*Ci*	*E*	WUE	*A*/*Ci*
Inoculation (I)	<0.001	0.0021	0.220	0.0783	<0.001	<0.001
Drought Stress (DS)	<0.001	<0.001	<0.001	<0.001	0.0032	<0.001
I X DS	0.0037	0.0427	0.0509	0.0602	0.0352	0.0185
			Sugars concentration			
	Reducing Sugar	Sucrose	Total sugar	Starch		
Inoculation (I)	<0.001	<0.001	0.0012	<0.001		
Drought Stress (DS)	<0.001	<0.001	0.0012	<0.001		
I X DS	0.0045	0.0074	0.0372	<0.001		
			Enzymes activity			
	NR	SOD	POD	Proline		
Inoculation (I)	<0.001	<0.001	<0.001	<0.001		
Drought Stress (DS)	<0.001	<0.001	<0.001	<0.001		
I X DS	0.0487	0.0237	0.0492	<0.001		
			Root development			
	Root length	Root dry weight				
Inoculation (I)	<0.001	0.0020				
Drought Stress (DS)	<0.001	0.0066				
I X DS	0.0010	0.0015				
			Biometric parameters			
	Plant height	Stalk diameter	Leaf width	Tillers plant^{-1}	Stalk fresh weight	Stalk dry weight
Inoculation (I)	<0.001	0.0047	0.0320	0.0126	<0.001	<0.001
Drought Stress (DS)	<0.001	0.0090	0.0041	0.0126	<0.001	<0.001
I X DS	0.0325	0.0412	0.0320	0.0404	0.0135	0.0076

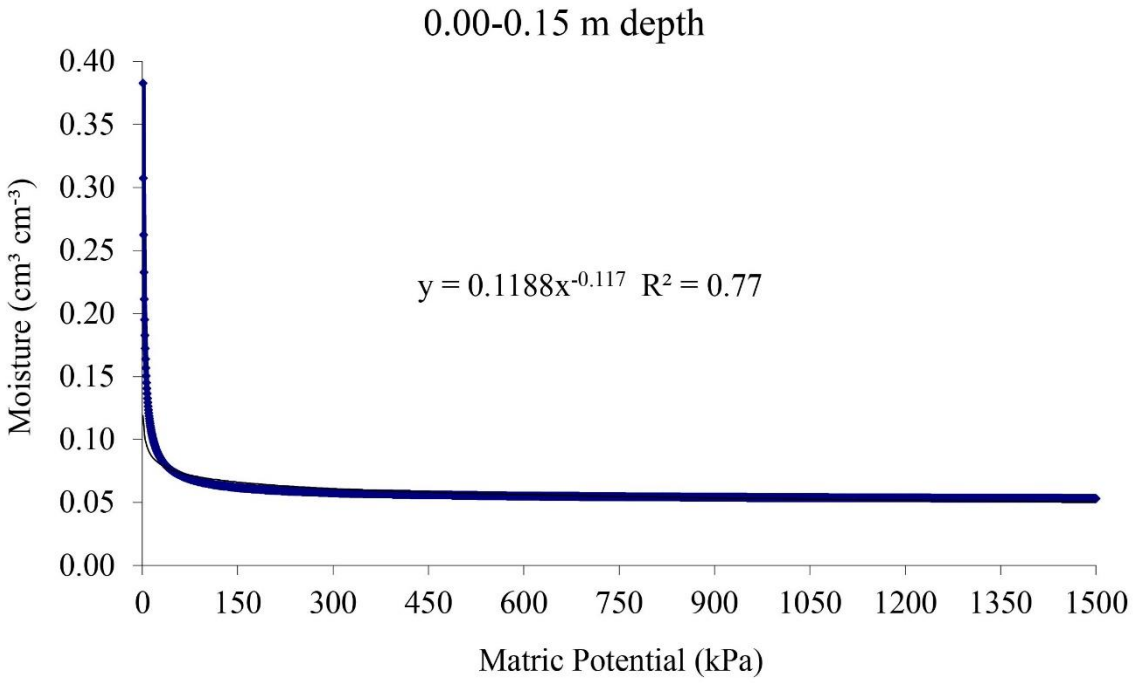

Figure. S1. Soil water retention curve from soil used in the present study.

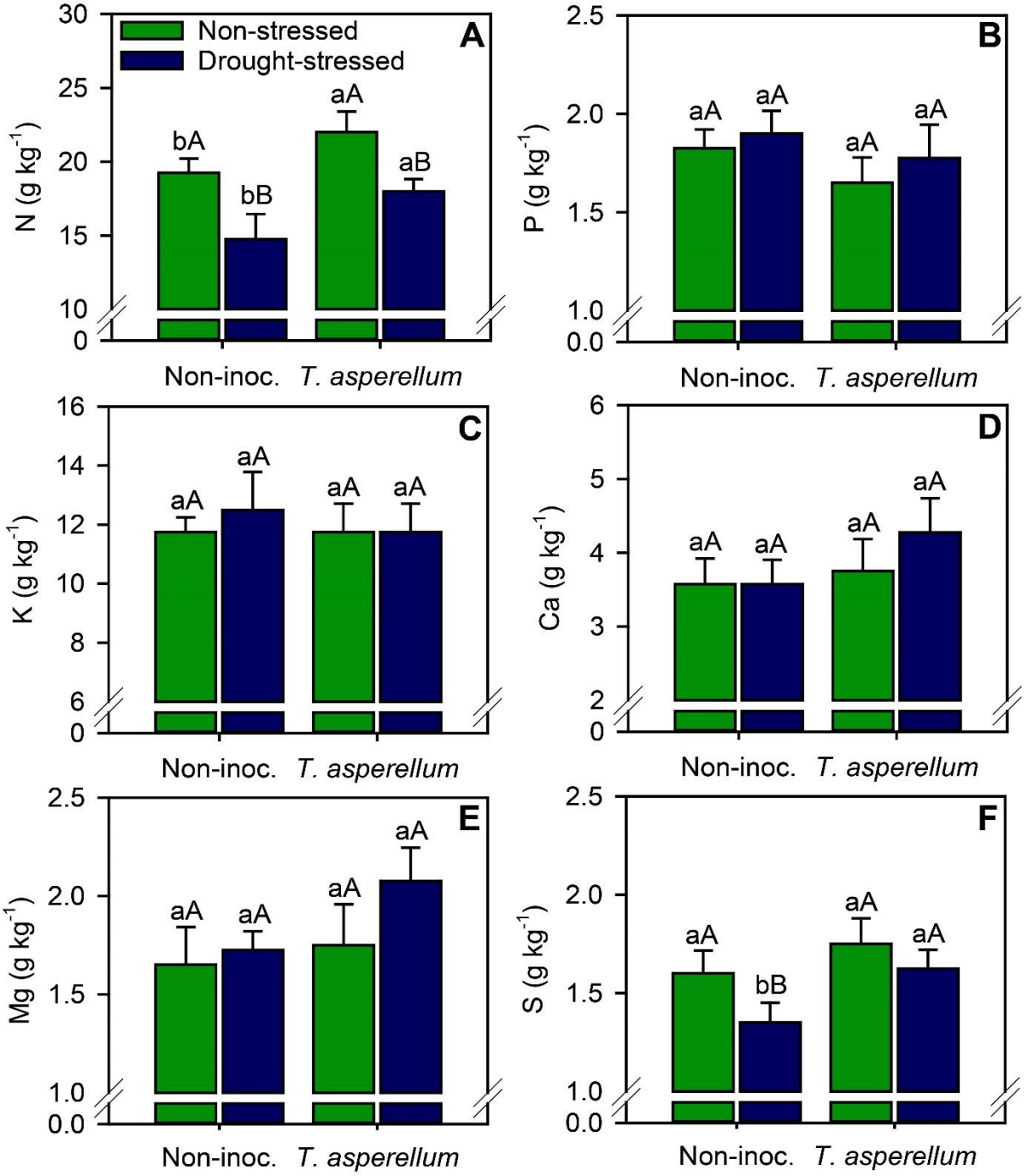

Figure. S2. Concentration of nitrogen (a), phosphorus (b), potassium (c), magnesium (d), calcium (e), and sulfur (f) in sugarcane leaves according to the treatments. Different lower-case indicate significant differences between presence or absence of moderate drought stress, and different capital letters indicate significant differences between presence or absence of inoculation with *T. asperellum* by Fisher's protected LSD test at $p \leq 0.05$. Error bars express the standard error of the mean (n = 4).

GENERAL CONSIDERATIONS

The inoculation of microorganisms in sugarcane has the purpose of enhancing productivity, by increasing the production of photoassimilates, improving the metabolism of the plant mainly in situations of water stress, consequently favoring the growth and development of the plants, contributing to the profitability for the producer.

The use of doses 15 and 20 × 10^{10} CFU ha^{-1} of the inoculant containing bacteria *Azospirillum brasilense* provided better crop development, tillering and thus higher stalk production, sugar, trash and bagasse yields and energy production of sugarcane. However, there was no pattern of results from effect of the inoculant application time. Therefore, the use of this microorganism in high rates provides greater sugarcane yields, as well as being an alternative to maximize the agronomic and economic return to the sugarcane sector; and the application of the inoculant is not related to the stage of the sugarcane, but to the climatic conditions.

Trichoderma asperellum also showed positive effects when inoculated on sugarcane plants under water stress. These findings were observed on greater crop nutrition, concentration of chlorophylls and carotenoids by increases in the rate of photosynthesis, stomatal conductance and water use efficiency on inoculated sugarcane compared to uninoculated plants. In addition, the antioxidant metabolism was also affected, increasing the activities of the enzymes SOD, POD, proline and the portioning of sugars; these effects result in the potentiation of root and stalk development of sugarcane, which *Trichoderma asperellum* inoculantion is an important tool to reduce the negative effects of drought stress in sugarcane.

For these aspects, consolidating the use of microorganisms in agriculture is an innovative alternative, and its benefits are greater plant growth, stress tolerance (biotic and abiotic), as well as increases in productivity. In addition,application of microorganisms inoculants in plants favors the environment for being an important sustainable strategy economically viable.

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