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Microorganisms for increasing sugarcane productivity: a way to complement and reduce chemical fertilization

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Abstract: This study evaluated the efficiency and agronomic feasibility of four inoculants - Azotrop, Biofree, BTP 143-20, and BTP 167-20 - in the development and productivity of sugarcane crops as a complement to chemical fertilization across five Brazilian locations from 2022 to 2023. The experimental design used was a randomized complete block design, with 14 treatments and four replicates in the experiments with Biofree, BTP167-20 and BTP143-20 and eight treatments in the experiment with Azotrop. Plant height, stalk diameter and stalk productivity variables were measured. The variance components and genetic parameters were estimated using Restricted Maximum Likelihood (REML), with the results supported by the Deviance analysis at 5% probability using the chi-square test. The Best Linear Unbiased Predictor (BLUP) was used to determine the effect of treatments on the measured traits and ranked. Results revealed strong environmental effects on plant height and stalk diameter, while productivity was more stable across environments. Inoculation with BTP167-20 represents the greatest efficiency and has agronomic feasibility for sugarcane crops, with superior performance to inoculants already on the market and capable of reducing the need for nitrogen and phosphate chemical fertilization by up to 25%, acting in a complementary way in the efficiency of use and absorption of nutrients.

Keywords: *Saccharum officinarum L.*, Plant growth-promoting rhizobacteria, Inoculant efficiency, Nutrient use efficiency, Sustainable agriculture, REML/BLUP.
Abbreviations: REML_ Restricted Maximum Likelihood; BLUP_ Best Linear Unbiased Predictor.

Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the most important crops in the world, with significant growth in recent years, mainly due to ethanol production (Ali et al., 2021). Its production chain benefits the biofuels sector, produces clean energy, sugar and renewable by-products, and generates millions of jobs (Cardoso et al., 2018). This crop has a remarkable capacity to adapt to different climatic conditions; however, its production potential depends significantly on adequate management of soil fertility, an essential factor for the metabolic processes that drive growth and biomass accumulation. However, most Brazilian soils are highly weathered, resulting in extensive areas with some level of degradation and low levels of fertility (Souza et al., 2024).

Degraded areas can be defined as the physical, chemical, and biological deterioration of soil due to anthropogenic activity, leading to a serious decline in soil volume, productivity, and fertility (Wang et al., 2023). Sugarcane is commonly grown in degraded soils, where acidity correction and mineral fertilization become essential for crop establishment, quality, and longevity, being a practice adopted to maintain and increase productivity by meeting the nutritional requirements of plants. This, in turn, results in economic costs of importing raw materials and environmental impacts associated with the extraction and use of fertilizers (Fink et al., 2016; Iqbal et al., 2020). The intensification of agriculture over time has led to a substantial increase in the demand for fertilizers, especially in acidic soils, with high retention (and low availability) of phosphorus (Roy et al., 2017), in addition to lower nitrogen availability due to reduced levels of soil organic matter (Tully et al., 2023).

With an emphasis on reducing the use of these inputs, some strategies can be employed in crop management in order to maintain acceptable productivity with less use of external resources. In this context, the use of plant growth-promoting rhizobacteria (PGPR) emerges as a promising tool to improve the efficiency of nutrient use and supply (Rocha et al., 2022). These microorganisms present several mechanisms that favor crop development, such as nitrogen fixation, greater root development, phosphate solubilization, and disease resistance (Muthukumarasamy et al., 2017). The genera *Pseudomonas*, *Azospirillum, Azotobacter, Enterobacter, Arthrobacter, Alcaligenes, Bacillus, Serratia, Burkholderia, Acinetobacter*, and *Klebsiella* are some of the best known and most used PGPR genera.

The *Bacillus* genus is composed of bacteria widely distributed in different environments, which have physiological characteristics of great biotechnological interest, including remarkable sporulation capacity (Harirchi et al., 2022). This aspect makes them extremely relevant for the development of commercial products, since spores are highly stable structures capable of remaining viable even under adverse conditions, such as lack of nutrients and hostile environments (Bahadir et al., 2018). Kruasuwan and Thamchaipenet (2016) reported 14 endophytic *Bacillus* isolates that were associated with sugarcane roots in Thailand, while Wang et al. (2019) also verified PGP potential from the use of *B. velezensis* and *B. aryabhattai*. Some strains of this bacterium make immobilized phosphorus in the soil available to plants through solubilization, which transforms insoluble inorganic forms into soluble ones, and mineralization, which converts organic phosphorus into inorganic phosphorus, facilitating its absorption by plants (Rawat et al., 2021).

In this same context, another alternative to improve nitrogen use efficiency and reduce fertilizers is to use diazotrophic bacteria, which promote biological nitrogen fixation by converting atmospheric N_2 into forms assimilable by plants (Scudeletti et al., 2023). Studies show that the response to *Azospirillum brasilense* inoculation is dependent on soil and climate conditions, being more effective in low and medium fertility soils, as well as in adverse environmental conditions (Gonçalves et al., 2020). The benefits of *Azospirillum* inoculation, which include improvements in root growth, plant vigor, and mineral nutrition, are mainly attributed to two processes: biological nitrogen fixation and phytohormone synthesis (Martins et al., 2018; Santos et al., 2019).

Among the PGPR, the genus *Pseudomonas* represents a group of significant importance that has been widely studied in agriculture (Kong et al., 2016). Aguado-Santacruz et al. (2024) found that the use of the inoculant composed of *Pseudomonas fluorescens*, *A. brasilense* and *B. subtilis* in sugarcane promoted improvements in quality in terms of ºBrix and sucrose content, similar to the use of isolated chemical fertilizer. Another important group are the bacteria of the genus *Paenibacillus*, commonly isolated from the soil and associated with plant roots, including *Paenibacillus azotofixans*, initially classified as *Bacillus azotofixans* and later reclassified (Stepien et al., 2022). *P. azotofixans* performs nitrogen fixation (Aquino et al., 2021) and encodes nitrogenase with alternative cofactors of vanadium (V) and iron (Fe), in addition to the traditional molybdenum (Mo), conferring a selective advantage in environments with Mo deficiency (Grady et al., 2016).

The adoption of sustainable management in sugarcane cultivation for the production of sugar and biofuel using PGPR presents crucial benefits for the environment (Scudeletti et al., 2023), especially regarding the reduction in the use of chemical fertilizers. Thus, the objective of this study was to evaluate the efficiency and agronomic feasibility of the inoculants Azotrop (*Azospirillum brasilense*), Biofree (*Azospirillum brasilense* and *Pseudomonas fluorescens*), BTP 143-20 (*Bacillus aryabhattai*, *Bacillus circulans*, *Bacillus haynesii*) and BTP 167-20 (*Paenibacillus azotofixans, Bacillus subtilis, B. licheniformis, B. circulans*) in the development and productivity of sugarcane crops as a complement to chemical fertilization.

Results and Discussion

Meteorological data

The mean air temperature and precipitation are shown in Figure 1. In general, the mean air temperature was higher most of the time in Chapadão do Céu-GO and Chapadão do Sul-MS, ranging from 25 to 27 °C in September, October and November. The mildest temperatures occurred in Lavras-MG, throughout the sugarcane growing cycle, between 17 and 22 °C. Sugarcane has an optimal temperature of 21 °C for maximum plant growth and development (Lopes & Lima, 2015). High temperatures in Chapadão do Céu and Chapadão do Sul may have accelerated the plant's metabolism and development, increasing water demand and evapotranspiration. The volume of precipitation was higher in December, January and February in Chapadão do Céu-GO, Chapadão do Sul-MS and Lavras-MG, with a daily average between 7 and 12 mm. The averages suggest lower precipitation in May, June, July and August. Daily water consumption in most Brazilian producing regions varies between 2 and 6 mm per day, depending on the variety, stage of development and evapotranspiration demand (Oliveira et al., 2018).

Overall inoculant performance evaluation

Deviance analysis for the overall performance of inoculants (i.e., considering all products in all environments) shows a significant effect of the treatment x environment interaction (TxE) for all measured variables, at 5% probability (Table 1). There was a high environmental effect on the plant height and stalk diameter variables, due to the low heritability values found, with $H^2 = 0.03$ and $H^2 = 0.08$. Heritability is quantified to determine the proportion of phenotypic variation that genetic variation can statistically explain (Barry et al., 2023). However, it is noted that the TxE interaction is of the simple type, since the $GEIr^2$ and rge values are greater than 0.70, which shows that the treatments remained stable under environmental variation. The environmental effect was slightly smaller for the expression of stalk productivity, with a complex TxE interaction ($GEIr^2 = 0.316$ and rge = 0.484). The coefficient of variation values of the treatment ranged from 2.91 to 7.08.

This initial analysis considers the overall performance of all treatments, since not all inoculants were evaluated in all environments. The overall BLUP shows little variation among treatments for plant height (Supplementary Figure 1), where 75N+Azotrop(1.2), 75N+Azotrop(0.9), 100N/75P+Biofree(1.2), 75N/100P+Biofree(1.2), and 75N+100P+Aprinza(1.0) performed best, superior to the 100NP treatment. Based on these results, it can be inferred that the application of *A. brasilense*, alone or combined with *P. fluorescens*, is effective in stimulating sugarcane development. It was found that 75P+BTP143-20(0.2), 75P+BTP143-20(0.4), 75P+BTP143-20(0.8), 75N+BTP143-20(0.2) and 75N+BTP143-20(0.8) were the treatments with the worst performance, with results inferior to the control without fertilizers (CWF). The results corroborate the study by Scudeletti et al. (2023), which found that inoculation with *A. brasilense* is sufficient to stimulate sugarcane growth and productivity.

A similar ranking of treatments was observed for stalk diameter, with Biofree inoculation showing relatively better performance than Aprinza under the same levels of complementary chemical fertilization, resulting in stalk diameters close to 22 mm (Supplementary Figure 2). 75N+Azotrop also demonstrated good performance, being the fifth treatment with the highest average. Plants with higher stalk diameter tend to allocate a greater amount of nutrients, mainly phosphorus, which is related to greater root growth, water and nutrient absorption and, consequently, final productivity (Reis et al., 2020). All treatments with BTP143-20 resulted in stalk diameter below 21 mm, regardless of the dose used, being similar to CWF and CF/75P. Intermediate performance was attributed to BTP167-20, with stalk diameter between 21 and 22 mm, with similarity to 75N+Aprinza(1.0) and CF/75N.

In contrast, the BLUP for stalk productivity revealed marked differences among treatments, with BTP167-20 standing out (100-110 t ha^{-1}; Supplementary Figure 3). This inoculant outperformed Biofree, Aprinza, Azotrop, and 100% fertilizer controls, indicating greater effectiveness of the *Bacillus* strains present. Desalegn et al. (2023) reported that sugarcane productivity is a complex trait, and achieving high yields depends on the appropriate combination of variety, nitrogen input, and the use of bioregulators and growth promoters. Despite not promoting taller plants or thicker stalks, BTP167-20 led to higher productivity. Almeida et al. (2024) concluded that the use of inoculants based on *Bacillus licheniformis* and *Bacillus subtilis* increased the survival of sugarcane plants, along with benefits for growth and final productivity. In contrast, BTP143-20, Azotrop with 75% N, and several controls showed lower productivity (85 to 100 t ha^{-1}).

Inoculant performance stratified by environments

The effect of the treatments was significant for all variables in Andirá-PR (Table 2). The percentage of contribution of the treatments to the results obtained was similar among the variables, with 75.10%, 72.40 and 77.40 for plant height, stalk diameter and stalk productivity. These parameters indicate little influence of the environment on the performance of the treatments, which was reflected in h^2mg and accuracy values above 0.90, that is, there is the possibility of selecting promising treatments. The coefficient of variation values were low, ranging from 4.72 to 4.98 for CVg and 2.69 to 2.97 for CVr.

In that context, the greatest effectiveness in promoting sugarcane growth was observed with 75P+BTP167-20(1.2), which reached a plant height of approximately 2.4 m (Supplementary Figure 4). The treatment 75N+Azotrop(1.2) ranked third, outperforming CF/100 and Aprinza. These results indicate that the potential for reducing chemical fertilization trough microbial inoculants is greater when higher doses are applied. Among the lower doses, only 75N+BTP167-20(0.4) and 75P+BTP167-20(0.4) exceeded the overall average, while the others were similar or inferior to treatments without inoculation. For stalk diameter (Supplementary Figure 5), BTP167-20 treatments showed superior performance (24-26 mm), with 75N+BTP167-20(1.2), 75P+BTP167-20(1.2), and 75N+BTP167-20(0.8) outperforming CF/100.

These findings are consistent with Tolera et al. (2024), who reported a strong causal relationship between stalk length and diameter with stalk and sugar yield. Aroh et al. (2020) also noted that productive sugarcane plants typically range from 2.0 to 4.5 m in height, with the 2.0-4.0 m interval providing the best balance between biomass and sugar content.

The other inoculants tested showed greater variability in their performance, with 75P+Aprinza(1.0), 75N+Aprinza(1.0), 75N+Azotrop(1.2) and 75N+Azotrop(0.9) ranked as superior to the overall average, which showed similarity with CF/75P. The remaining treatments were inferior to the overall average, with stalk diameter values between 22 and 24 mm, with the exception of CWF, which obtained performance below 22 mm. The BLUP for stalk productivity listed inoculation with BTP167-20 at doses of 1.2 and 0.8 mL ha^{-1}, together with 75N/100P+Biofree(1.2), as the best performing treatments (Supplementary Figure 6). It was also found that 75P+BTP167-20(0.4) and 75N+BTP-20(0.4) were the only treatments with doses lower than 0.8 mL ha^{-1} above the general average. Aquino et al. (2021) reported that inoculation with *Paenibacillus azotofixans*, present in the BTP167-20 inoculant, promoted the greatest accumulation of nitrogen and dry matter in corn and sorghum plants, which corroborates the potential presented by this microorganism in the study.

Deviance analysis revealed a significant effect of treatments on all traits measured in Chapadão do Céu-GO (Table 3). Similar to what was observed for Andirá-PR, the parameters show that the environmental effect had a low influence on the performance of treatments, with percentages of 11.6, 11.9 and 23.4 for plant height, stalk diameter and stalk productivity, respectively. This resulted in high values of heritability and selection accuracy. However, there was greater variability between treatments for plant height and stalk diameter in this environment, with CVg of 25.6 and 19.3%.

These aspects are demonstrated in the BLUP for plant height (Supplementary Figure 7), where the presence of two distinct treatment groups was observed, with CWF, CF/75N and 75N+Aprinza(1.0) being the only intermediates, with values between 2.5 and 3.0 m. Thus, all treatments with BTP167-20 and BTP143-20 were below the overall average of the experiment, with performance close to or slightly higher than 2.0 m, grouped with CF/75P and 75P+Aprinza(1.0). In contrast, superiority was attributed to all treatments with Azotrop and Biofree, regardless of the dose used. In this scenario, 75N+Azotrop(0.9), 75N/100P and 75N+Azotrop(1.2) were the treatments with the best performance, close to 4.0 m.

A similar behavior was observed for stalk diameter (Supplementary Figure 8), with two distinct treatment groups. Inoculations with BTP167-20 and BTP143-20 were ineffective, showing values around 20 mm – lower than the control without fertilization or inoculation. In contrast, all Biofree treatments exceeded the overall average, surpassing 30 mm and matching the performance of 100N/75P, 75N+Azotrop(0.9), 75N/100P+Aprinza(1.0), 75N+Azotrop(1.2), and 100N/75P+Aprinza. However, greater plant height and stalk diameter did not necessarily reflect higher stalk productivity (Supplementary Figure 9). According to the BLUP, BTP167-20 inoculation led to the best productive performance across all doses and fertilizer combinations, possibly due to enhanced nutrient uptake by *Bacillus* species, as reported by Iqbal et al. (2020) in cotton. For BTP143-20 and Biofree, only 75N+BTP143-20(0.4) and 75N/100P+Biofree(1.2) outperformed the overall average.

In the Chapadão do Sul-MS environment, the effect of the treatments was significant only for the variables plant height and stalk productivity (Table 4). Unlike the previous ones, this environment had a greater effect on the performance of the treatments, with percentages of 58.5 and 50% for plant height and stalk productivity, respectively. This resulted in lower selection accuracy values than previously verified, which suggests that the selection of promising treatments will be more challenging compared to locations with a lower percentage of environmental contribution. The CVg values were 2.61 and 10.3, which shows greater variation of the treatments for productivity.

Plant height ranged from 2.0 to 2.2 m (Supplementary Figure 10), with most BTP167-20 treatments outperforming the CF/75P control, except 75P+BTP167-20(0.2) and 75N+BTP167-20(0.4). Only 75N+BTP143-20(0.4) stood out among BTP143-20 treatments, while Biofree was mostly below average, except for 75N/100P+Biofree(1.2). The BLUP for stalk productivity (Supplementary Figure 11) confirmed the superiority of BTP167-20 - particularly 75P+BTP167-20(0.8) and 75N+BTP167-20(0.2) - with an overall average of ~90 t ha^{-1}. Biofree and Azotrop showed intermediate performance, but BTP143-20 remained the least effective across all doses.

In the Lavras-MG environment, Deviance shows that the treatment effect was significant only for plant height and stalk productivity (Table 5). The treatment effect acted to a greater extent when compared to the environment effect on plant height, with a value of 69%, resulting in high selection accuracy. The coefficient of variation shows little variability among the performance of the treatments (CVg=4.61). On the other hand, the environment acted to a greater extent on the expression of stalk productivity (64.2%), which consequently reduces the selection accuracy.

The ranking for plant height shows the treatments CF/100 and CF/75N as having the best performance (Supplementary Figure 12). Similar results were observed for the inoculation with 75P+BTP143-20(1.2), 75N+BTP143-20(0.4), 75N+Aprinza(1.0) and 75P+Aprinza(1.0). However, they were not sufficient to reduce nitrogen fertilization in this case. The treatments 75N+BTP143-20(1.2), 75P+BTP143-20(0.4), 75P+BTP143-20(0.8) and 75P+BTP143-20(0.2) were below the general average. Regarding stalk productivity (Supplementary Figure 13), BLUP ranked the treatments 75P+Aprinza(1.0) and 75P+BTP143-20(0.8) as the best performing, being higher than 140 t ha^{-1} and surpassing the performance of the controls CF/75N and CF/100. Although Tena et al. (2016) reported a moderate positive correlation between plant height and productivity, this was not confirmed here, as BTP143-20 achieved higher productivity even with lower height—comparable to the commercial inoculant in Lavras-MG.

The effect of treatments was significant for all variables in Piracicaba-SP, where a high environmental influence on the expression of plant height (76.6%) was observed, while there was a higher percentage of influence of treatments on the variables stalk diameter and stalk productivity, with values of 74.2 and 92.7%, respectively. These parameters resulted in accuracy values of 0.74, 0.96 and 0.99, reflecting the heritability values that were measured (0.234, 0.742 and 0.927). The coefficient of variation for the treatment was 5.08, 8.85 and 12.1 for plant height, stalk diameter and stalk productivity, respectively.

Table 1. Restricted maximum likelihood (REML) test and genetic parameters for the general evaluation of the performance of the inoculants tested in sugarcane for the variables plant height (cm), stalk diameter (mm) and stalk productivity (t ha^{-1}).

Model	Plant height	Stalk diameter	Stalk productivity
Genotype	6.89^{-1}	2.31^{-1}	1.89^{-9*}
Genotype x Environment	2.89^{-133*}	3.32^{-87*}	3.72^{-34*}
Parameters	PH	SD	PROD
Phenotypic Variance	0.157	9.41	142
Heritability	0.0265	0.0767	0.346
$GEIr^2$	0.816	0.676	0.316
h^2mg	0.134	0.342	0.812
Accuracy	0.366	0.585	0.901
rge	0.838	0.732	0.484
CVg	2.91	3.97	7.08
CVr	7.11	7.13	7
CVratio	0.41	0.56	1.01

$GEIr^2$: coefficient of determination of treatment-environment interaction; rge: correlation coefficient of treatment-environment interaction; CVg: treatment coefficient of variation; CVr: environmental coefficient of variation; CVratio: ratio between CVg and CVr; *: significant at 5% by the χ^2 test.

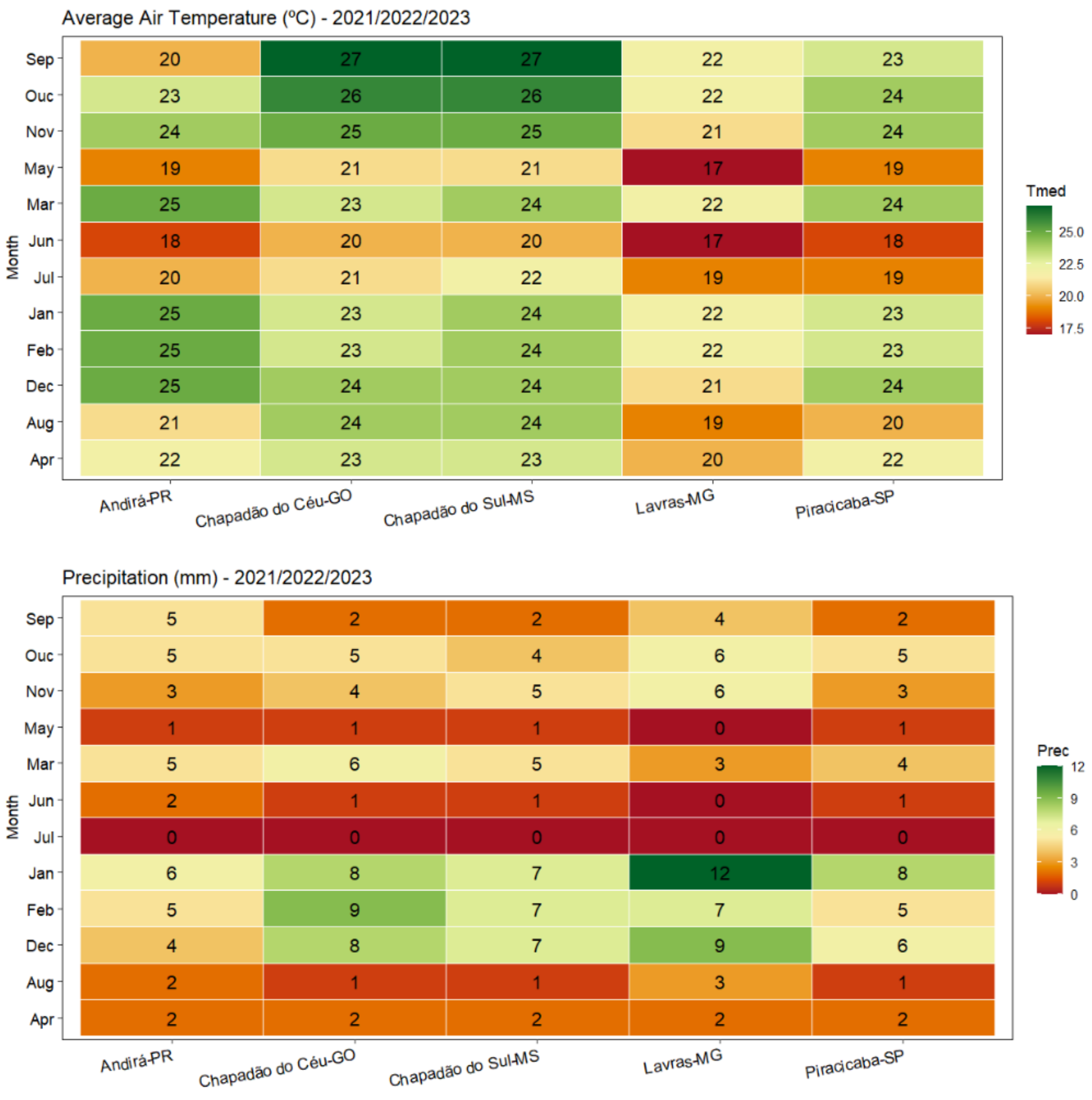

Fig 1. Satellite meteorological data of mean air temperature (Tmed, ºC) and average daily precipitation (Prec, mm) for the period of development of the experiments with Sugarcane.

Table 2. Restricted maximum likelihood (REML) test and genetic parameters to evaluate the performance of inoculants tested in sugarcane in Andirá-PR.

Model	Plant height	Stalk diameter	Stalk productivity
Genotype	6.29^{-22*}	5.32^{-20*}	8.67^{-24*}
Parameters	Plant height	Stalk diameter	Stalk productivity
GenVar	0.010	1.330	26.600
Gen (%)	75.100	72.400	77.400
ResVar	0.004	0.506	7.760
Res (%)	24.900	27.600	22.600
PhenVar	0.014	1.830	34.300
H^2	0.751	0.724	0.774
h^2mg	0.923	0.913	0.932
Accuracy	0.961	0.955	0.965
CVg	4.720	4.800	4.980
CVr	2.720	2.970	2.690
CVratio	1.740	1.620	1.850

H^2: heritability; h^2mg: heritability of the treatment mean; CVg: treatment coefficient of variation; CVr: environmental coefficient of variation; CVratio: ratio between CVg and CVr. *: significant at 5% by the χ^2 test.

Table 3. Restricted maximum likelihood (REML) test and genetic parameters to evaluate the performance of inoculants tested in sugarcane in Chapadão do Sul-MS.

Model	Plant height	Stalk diameter	Stalk productivity
Genotype	7.70^{-46*}	3.96^{-45*}	1.17^{-28*}
Parameters	Plant height	Stalk diameter	Stalk productivity
GenVar	0.521	25.000	92.000
Gen (%)	88.400	88.100	76.600
ResVar	0.068	3.370	28.100
Res (%)	11.600	11.900	23.400
PhenVar	0.589	28.300	120.000
H^2	0.884	0.881	0.766
h^2mg	0.968	0.967	0.929
Accuracy	0.984	0.984	0.964
CVg	25.600	19.300	10.100
CVr	9.260	7.080	5.560
CVratio	2.770	2.720	1.810

H^2: heritability; h^2mg: heritability of the treatment mean; CVg: treatment coefficient of variation; CVr: environmental coefficient of variation; CVratio: ratio between CVg and CVr. *: significant at 5% by the χ^2 test.

Table 4. Restricted maximum likelihood (REML) test and genetic parameters to evaluate the performance of inoculants tested in sugarcane in Chapadão do Céu-GO.

Model	Plant height	Stalk diameter	Stalk productivity
Genotype	2.36^{-8*}	1	1.59^{-11*}
Parameters	Plant height	Stalk diameter	Stalk productivity
GenVar	0.003	-	89.900
Gen (%)	41.500	-	50.000
ResVar	0.004	-	89.800
Res (%)	58.500	-	50.000
PhenVar	0.007	-	180.000
H^2	0.415	-	0.500
h^2mg	0.739	-	0.800
Accuracy	0.860	-	0.895
CVg	2.610	-	10.300
CVr	3.090	-	10.300
CVratio	0.842	-	1.000

H^2: heritability; h^2mg: heritability of the treatment mean; CVg: treatment coefficient of variation; CVr: environmental coefficient of variation; CVratio: ratio between CVg and CVr. *: significant at 5% by the χ^2 test.

Table 5. Restricted maximum likelihood (REML) test and genetic parameters to evaluate the performance of inoculants tested in sugarcane in Lavras-MG.

Model	Plant height	Stalk diameter	Stalk productivity
Genotype	2.43^{-8*}	0.338	0.005^{*}
Parameters	Plant height	Stalk diameter	Stalk productivity
GenVar	0.009	-	108.000
Gen (%)	69.000	-	35.800
ResVar	0.004	-	193.000
Res (%)	31.000	-	64.200
PhenVar	0.014	-	301.000
H^2	0.690	-	0.358
h^2mg	0.899	-	0.691
Accuracy	0.948	-	0.831
CVg	4.610	-	8.140
CVr	3.090	-	10.900
CVratio	1.490	-	0.748

H^2: heritability; h^2mg: heritability of the treatment mean; CVg: treatment coefficient of variation; CVr: environmental coefficient of variation; CVratio: ratio between CVg and CVr. *: significant at 5% by the χ^2 test.

Table 6. Restricted maximum likelihood (REML) test and genetic parameters to evaluate the performance of inoculants tested in sugarcane in Piracicaba-SP.

Model	Plant height	Stalk diameter	Stalk productivity
Genotype	0.001^{*}	2.59^{-26*}	5.61^{-58*}
Parameters	Plant height	Stalk diameter	Stalk productivity
GenVar	0.009	3.620	135.000
Gen (%)	23.400	74.200	92.700
ResVar	0.029	1.260	10.600
Res (%)	76.600	25.800	7.270
PhenVar	0.038	4.880	146.000
H^2	0.234	0.742	0.927
h^2mg	0.550	0.920	0.981
Accuracy	0.742	0.959	0.990
CVg	5.080	8.850	12.100
CVr	9.190	5.220	3.390
CVratio	0.553	1.700	3.570

H^2: heritability; h^2mg: heritability of the treatment mean; CVg: treatment coefficient of variation; CVr: environmental coefficient of variation; CVratio: ratio between CVg and CVr. *: significant at 5% by the χ^2 test.

Different from what was observed in the previous environments, inoculation with Biofree promoted the best plant height results (close to 2.0 m) for 75N/100P+Biofree(1.2) and 100N/75P+Biofree(1.2), while CWF had the lowest average (close to 1.6 m) (Supplementary Figure 14). Treatments with BTP143-20 and some controls showed heights below 1.8 m. Stalk diameter ranged from 17 to 24 mm (Supplementary Figure 15), with BTP143-20 performing worst (18–20 mm), Azotrop intermediate (20–22 mm), and BTP167-20 as a transitional group. Biofree outperformed Aprinza at doses between 0.6 and 1.2 mL ha^{-1}, except for 75N+BTP167-20(1.2), which only exceeded 100NP. Productivity rankings mirrored these trends (Supplementary Figure 16), with Biofree leading (higher than 110 t ha^{-1}), followed by BTP167-20 (100–110 t ha^{-1}). Aprinza and BTP143-20 showed variable performance depending on dose, with some treatments near 95 t ha^{-1} and others dropping below 80 t ha^{-1}. Most other treatments matched controls, yielding 80–90 t ha^{-1}.
These findings indicate that the Biofree inoculant, based on *Bacillus* strains, effectively enhances vegetative growth and productivity in Piracicaba-SP, likely by improving nutrient uptake and stress resilience, while the inconsistent results with Aprinza and BTP143-20 emphasize the critical role of inoculant strain and dosage. The poor performance of the unfertilized control (CWF) highlights the need for fertilization and/or inoculation to achieve productive growth.

Material and Methods

Multi-environment trials experimental design

The experiments were conducted in 2022 and 2023. They took place in the cities of Andirá-PR (23°02'45"S 50°14'00"W, altitude of 479 m), Chapadão do Céu-GO (18°35'88.1"S and 52°85'39.8"W, altitude of 826 m), Chapadão do Sul-MS (18°47'05.6''S and 52°52'53.5''W, altitude of 840 m), Lavras-MG (21º12'39.5''S 045º03'34.9"W, altitude of 922 m) and Piracicaba-SP (22º39´50.91"S and 47º38´53.80"W, altitude of 527 m). Supplementary Figure 17 shows the location of each municipality where the experiments were conducted. These multi-environment trials sought to evaluate the efficiency of four inoculants (two commercial and two pre-commercial) in sugarcane crops. All information on climate, soil type, sowing and harvesting dates are described in Supplementary Table 1. The experimental design used was a randomized complete block design, with 14 treatments and four replicates in the experiments with Biofree, BTP167-20 and BTP143-20 and eight treatments and four replicates in the experiment with Azotrop. The experimental units consisted of a total area of 75.0 m^2 and a useful area of 24.0 m^2, with rows spaced 1 m apart and a planting density of 10,000 sections per hectare, in order to obtain 90,000 final sections per hectare. The sugarcane genotypes CTC 9003, IAC SP95 5094, CTC 04, IACSP95-3028 and CTC 04 were used in Andirá, Chapadão do Céu, Chapadão do Sul, Lavras and Piracicaba, respectively.

Description of inoculants and treatments

In all treatments, except for the absolute control without fertilization, 100 kg ha^{-1} of K_2O and the insecticide and fungicide Muneo® (Alpha-Cypermethrin 150 g per L + Fipronil 225 g per L + Pyraclostrobin 125 g L^{-1}) were applied at a dose of 1.0 L ha^{-1} via planting furrow.

Phosphate and nitrogen fertilization were applied according to each treatment, using simple superphosphate (20% P_2O_5) and urea (45% N) as sources, respectively, applied in the planting furrow. The nitrogen and phosphate chemical fertilizer doses for all treatments were: CF/100 = 30 kg N ha^{-1} + 125 kg P_2O_5 ha^{-1}; CF/100N = 30 kg N ha^{-1} at planting + 30 kg N ha^{-1} at topdressing; 100NP = 30 kg N ha^{-1} at planting + 100 kg P_2O_5 ha^{-1} at planting + 30 kg N ha^{-1} at topdressing; 100N/75P = 30 kg N ha^{-1} at planting + 75 kg P_2O_5 ha^{-1} at planting + 30 kg N ha^{-1} at topdressing; 75N/100P = 22.5 kg N ha^{-1} at planting + 100 kg P_2O_5 at planting + 22.5 kg N ha^{-1} at topdressing; CF/75N = 22.5 kg N ha^{-1} at planting + 22.5 kg N ha^{-1} at topdressing; CF/75P = 93.6 kg P_2O_5 ha^{-1} at planting. All information on the treatments used is described in supplementary Table 2.
The Azotrop product is a commercial inoculant composed of the bacteria *Azospirillum brasilense* (isolates Ab-V5 and Ab-V6), formulated with a concentration of $2x10^{11}$ CFU (Colony Forming Units) per liter. The Biofree product consists of a commercial inoculant composed of the bacteria *Azospirillum brasilense* (isolate Ab-V6) and *Pseudomonas fluorescens* (isolate CCTB03), with a concentration of $1x10^{11}$ CFU L^{-1}. The BTP167-20 inoculant is a pre-commercial product composed of the bacteria *Paenibacillus azotofixans* (CCTB10) + *Bacillus subtilis* (CCTB04) + *Bacillus licheniformis* (CCTB07) + *Bacillus circulans* (CCTB15), with a concentration of $8x10^8$ CFU L^{-1}. The pre-commercial product BTP143-20 is composed of the bacteria *Bacillus aryabhattai* (CBMAI1120) + *Bacillus circulans* (CCT0026) + *Bacillus haynesii* (CCT7926), with a guaranteed concentration of $9.9x10^{10}$ UFC L^{-1}. The product Aprinza was used as the standard and consists of a commercial inoculant composed of the bacteria *Nitrospirillum amazonense* (BR11145), with a concentration of $1x10^9$ UFC mL^{-1}. All inoculants have a liquid formulation and are guaranteed to be free of contaminants. Applications were made in the furrow at planting and via foliar application at 40 DAE, with a CO_2-pressurized backpack sprayer, 40 psi pressure, fan-type spray tip (XR110:02) and a spray volume of 200 L ha^{-1}. The inoculants Azotrop, Biofree and BTP167-20 were evaluated in Andirá, Chapadão do Céu, Chapadão do Sul and Piracicaba, while the inoculant BTP143-20 was evaluated in Chapadão do Céu, Chapadão do Sul, Lavras and Piracicaba.

Measured variables
The plant height and stalk diameter variables were measured in 20 sequenced stalks in the useful area of each experimental unit. At 60 days after emergence (DAE), stalk diameter assessments were performed using a digital caliper. Plant height measurements were performed at 180 DAE, using a graduated ruler. Stalk productivity was measured at the end of the crop cycle, from the harvest of the two central rows, with the mass determined on a precision scale. To aid in understanding the results obtained, meteorological data on mean air temperature (ºC) and precipitation (mm) were obtained with the aid of the NASA Power platform (NASA Power, 2023).

Statistical analysis

The data obtained were subjected to analysis and removal of outliers. Subsequently, the assumptions of the statistical model were verified, based on the tests of normality of errors, homogeneity of variances and independence of errors, using the Shapiro-Wilk, Bartlett and Durbin-Watson tests, respectively. With the assumptions met, the variance components and genetic parameters were estimated using Restricted Maximum Likelihood (REML), with the results supported by the Deviance analysis at 5% probability using the chi-square test ($\chi 2$). The Best Linear Unbiased Predictor (BLUP) was used to determine the effect of treatments on the measured traits and, thus, perform the ranking, based on the following model: $y_{ijk} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + \gamma_{jk} + \varepsilon_{ijk}$, where y_{ijk} is the response variable observed in the kth block of the ith genotype of the jth environment; μ is the overall mean; α_i is the effect of the ith genotype; τ_j is the effect of the jth environment; $(\alpha\tau)_{ij}$ is the effect of the interaction between the ith genotype and the ith environment; γ_{jk} is the effect of the kth block within the jth environment; and ε_{ijk} is the random error. Statistical analyses were performed with the metan package version 1.18.0 (Olivoto & Lúcio, 2020), using R software (R Core Team, 2023).

Conclusion

The inoculation of sugarcane with BTP167-20 allowed the achievement of the highest stalk productivity in the environments of Andirá-PR, Chapadão do Céu-GO and Chapadão do Sul-MS. There is superiority between inoculation with Biofree and the standard commercial inoculant in the environment of Piracicaba-SP, with similar performance mainly in complementing phosphate fertilization. The inoculation process with BTP143-20 presented stalk productivity similar to the standard commercial inoculant in Lavras-MG. Inoculation with BTP167-20 represents the greatest efficiency and has agronomic feasibility for sugarcane crops, with superior performance to inoculants already on the market and capable of reducing the need for nitrogen and phosphate chemical fertilization by up to 25%, acting in a complementary way in the efficiency of use and absorption of nutrients.

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Author`s contributions
The authors contributed to this study as follows: Bandeira WJA, data processing, literature review, statistical analysis and manuscript writing; Carvalho IR, data processing and statistical analysis; Meireles LG, Watanabe LFM, Santos LM, conducting the experiment, collecting experimental data and final revision of the paper.

Conflicts of Interest
The authors declare no conflict of interest.

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