

Germination of *Saccharum* complex genotypes in response to inoculation using selected diazotrophic bacteria before and after a heat treatment

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ABSTRACT: Breeding of new cultivars of sugarcane uses several species belonging to the *Saccharum* complex genus. In this study, a set of 20 genotypes from the *Saccharum* complex was assessed for their growth promotion during germination using a mixed inoculant containing five selected diazotrophs and their resistance to short heat treatment (52°C / 30 min). The percentage of germination was evaluated, leading to the identification of three response groups. *Saccharum officinarum* (group I) showed the highest germination percentage, succeeded by *Erianthus arundinaceus* genotypes (group II) and the other genotypes (group III). The mean germination time (MGT) varied among the genotypes and segregated the growth response into two groups. *Saccharum officinarum* only required less than 15 days to reach the MGT, whereas the other genera required more than two or three days during the initial growth process. Bacterial counting also differed between the groups, being effective for *S. officinarum* and other genotypes, improving the colonization of the plants 100–1,000 times higher than the control and heat treatments. Further comparison was made among eight genotypes, including one *Saccharum spontaneum* (US72-1319), one *S. officinarum* (IN845), and six *E. arundinaceus*. This comparison measured biomass accumulation and root parameters. The *E. arundinaceus* group demonstrated less susceptibility to the heat treatment, resulting in stimulation of the root dry mass of IJ76-381 and root parameters of this genotype, as well as IJ76-364, although not associated to the inoculation treatment. Inoculation applied to US72-1319 and IN845 enhanced only the Shoot/Root ratio.

Key words: growth promotion, plant-bacteria interaction, root architecture.

INTRODUCTION

Sugarcane crop production in Brazil has been important to its economy since its introduction, in 1515, with the establishment of the first mill, in 1532. Over 500 years, this crop has been extensively cultivated in five regions of Brazil, especially in the Southeast and Northeast regions, and has had diverse impacts on the local economy. As a result, Brazil has become the world's largest sugarcane producer (CONAB 2023). This crop plays a vital role as a primary-producing crop, with sugar accounting for approximately 80% of its production. Moreover, it holds great potential as a renewable bioenergy crop, as ethanol constitutes around 50% of this output in world agriculture (Waclawovsky et al. 2010). Energy cane can be defined as a specific cultivar of sugarcane that is selectively bred to have higher fiber levels compared to traditional sugarcane hybrids, which are primarily bred for their high sucrose content, lowering the fiber content (Matsuoka et al. 2014).

Cultivated sugarcane (*Saccharum* interspecific hybrids) is a perennial C4 grass crop belonging to the subtribe Saccharinae under the tribe Andropogoneae of the family Poaceae. The *Saccharum* genus was described by Linnaeus (1753) and comprises

35-40 species. Since this description, sugarcane has gone through an extensive and complex process of domestication and hybridization (Babu et al. 2021, Vasquez et al. 2022, Xiong et al. 2022). This genus contains six main species: the two wild ones are *Saccharum spontaneum* L. and *Saccharum robustum* (Brandes & Jesw. Ex Gressl), and the four cultivated species: *S. officinarum* L., *S. sinense* (Roxburgh), *S. barberi* (Jeswiet), and *S. edule* (Hassk) (Zhang et al. 2014). The currently cultivated sugarcane plants are highly polyploid or aneuploid hybrids derived from crossings mainly between plants of *S. officinarum* and *S. spontaneum* wild sugarcane (Fukuhara et al. 2013, Fickett et al. 2020).

It has been hypothesized that an intercrossing group named the *Saccharum* complex is involved in the origin of modern sugarcane, which displays large genomes and complex chromosomal alterations (Ling et al. 2022). This hypothetical group refers to four related genera: *Saccharum*, *Erianthus* Michx. (including E. sect. *Ripidium* or *Tripidium*), *Narenga* Bor, and *Sclerostachya* (Andersson ex Hack.) A. Camus. *Miscanthus* Andersson (sometimes accepted as *Miscanthidium* Stapf) is also closely related to this group (Amalraj and Balasundaram 2006). This concept has been widely adopted by sugarcane breeders, as plants belonging to this complex serve as the primary gene pool. Additionally, the relationships between these ancestral genera have been extensively characterized using various molecular methods (Zhu et al. 2008, Ling et al. 2022). Evolution is intricately linked to the adaptation of species to their geological origins (Wang et al. 2023).

There is a lack of information available regarding the characteristics, crossing, and evolution of the sugarcane genotypes especially for breeding purposes. Consequently, the response of these plants being grown in a typical field dedicated to energy production is not well understood. The lack of knowledge also extended to the germination process, as little research has been done on how these plants respond to common sugarcane practices such as heat treatment, the time required to produce a single plant, or the effects of bacterial inoculant on the germination velocity. Information on these topics is well-documented for *Saccharum* hybrids (Santos et al. 2017, 2019), but not for these specific genotypes.

Utilizing various genotypes derived from ancestral genera within the *Saccharum* complex sourced from the germplasm local bank (sugarcane bank of Embrapa Agrobiologia, a copy of the USDA Collection), the primary inquiry arising from the germination response of these genotypes pertains to the effects the application of an inoculant containing a blend of five diazotrophic bacteria, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Nitrospirillum amazonense*, and *Paraburkholderia tropica*. This consortium, developed by Oliveira et al. (2006), was identified as the most efficacious in stimulating biological nitrogen fixation by the sugarcane cultivars planted in three different places over two consecutive years. Subsequently, the effectiveness of this bacterial combination was assessed in a controlled greenhouse trial (Chaves et al. 2015) and field conditions (Schultz et al. 2014, 2017). The application method involved direct inoculation of the five selected strains onto the bud through a 30-min immersion process, as outlined by Reis et al. (2009) without heat treatment (HT).

The application of the sprouting method of *Saccharum* hybrids called pre-sprouting buds (PSB) has several benefits in terms of reducing planting costs and controlling diseases. Using a single bud (stem node) means fewer stalks are needed to obtain propagules, and plants produced in tubes are subsequently transplanted to the field (Landell et al. 2012). The bud used for propagation is heat-treated using short HT, 52°C/30 min. Although there is a long HT (50°C/2 h), it is normally used under field conditions. These HT are both used to reduce the natural population of *Leifsonia xyli* subsp. *Xyli* (Lxx). This bacterium is responsible for causing ratoon stunting disease (RSD) (Sanguino et al. 2006). Moreover, HT can also help control some fungal diseases such as whip smut caused by *Sporisorium scitamineum* (Gupta et al. 1979) and phytoplasma diseases when long HT is applied (Kumar and Pratap 2023). Alternatively, this inoculation technique could also be employed post-HT, as demonstrated by Santos et al. (2017, 2019). These five strains, besides the ability to fix nitrogen, also have the capability of secreting indole-3-acetic acid (IAA) (Chaves et al. 2015) and other phytohormones (Bottini et al. 2004), among other mechanisms that can modify plant development and nitrogen allocation in the sugarcane cultivars (Girio et al. 2015, Santos et al. 2017).

This study aimed to investigate how a diverse group of 20 genotypes from eight different species responded to a single application of bacterial inoculant containing five strains of the diazotrophic species previously selected by Oliveira et al. (2006). The experiment also included subjecting genotypes to a short HT with and without bacterial inoculation. To measure the initial growth of the plants, several parameters were assessed. This included calculating the germination percentage of the mean germination time (MGM), counting of bacterial population, and biomass accumulation of the shoots and roots. In eight of the materials, the root architecture was analyzed using the WinRHIZO Pro™ software.

METHODS

Twenty experiments were conducted in a temperature-controlled greenhouse to assess the response of 20 sugarcane genotypes (Table 1) to inoculation using a mixture of five diazotrophic bacteria, previously selected by Oliveira et al. (2006). In addition, the same group was submitted to the short HT (52°C/30 min), inoculated or not with the same mixture of diazotrophs.

Table 1. Identification of the 20 genotypes classified into the *Saccharum* complex.

Saccharum species	Name	Harvest time (DAP)
Group I (SO)		
<i>Saccharum officinarum</i>	Bourbon Sur	33
	NG27-1124	60
	Bamboo Rose	60
	IJ76-315	60
	Branca*	54
	Q44830	33
	Biscuit	60
	IN845*	45
Group II (EA)		
<i>Erianthus arundinaceus</i>	IJ76-364	120
	IJ76-358	60
	IJ76-359	60
	IJ76-381	60
	IJ76-384	60
Group III - Other genotypes		
<i>Saccharum</i>	Q45416	60
<i>Saccharum spontaneum</i>	US72-1319	120
<i>Saccharum barberi</i>	White Pararia*	25
<i>Saccharum sinense</i>	Maneria*	54
<i>Miscanthus sp.</i>	Fiji 15	60
<i>Saccharum robustum</i>	IJ76-414	45
	IM76-228*	54

NG: Papua, New Guinea; IJ: Purari, New Guinea; Q: Queensland, Australia; US: United States of America; Fiji: Fiji Republic; IN: Indonesia; IM: Mauritius Republic. The acronyms represent the countries of origin of this germplasm; *material obtained from the Collection of Rede Interuniversitária para o Desenvolvimento do Setor Sucroenergético/Universidade Federal de São Carlos. Original material from the National Center for Genetic Resources Preservation/ARS/USDA, Fort Collins, CO, USA. After quarantine, buds were propagated at the germplasm bank localized at Embrapa Tabuleiros Costeiros (Sergipe, Brazil), and transferred to Embrapa Agrobiologia (Rio de Janeiro, Brazil) for local propagation.

The experiments were carried out at Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Centro Nacional de Pesquisa em Agrobiologia (CNPAB), in Seropédica, RJ, Brazil (22°44'38"S and 43°42'28"W; altitude 26 m). The experiments were conducted throughout the year, from January to May and August to December. During this period, the average daylight duration was approximately 12-13 hours per day.

The plant material was originated from a collection of the National Center for Genetic Resources Preservation/ARS/USDA, Fort Collins, CO, United States of America. After undergoing quarantine, this plant material was micropropagated and then planted in an active germplasm bank of *Saccharum* complex at Embrapa Tabuleiros Costeiros, located at Jorge Prado Sobral, the city of Nossa Senhora das Dores, Sergipe, Brazil (10°29'27"S; 37°11'34"W). Four genotypes originated from the Collection of Rede Interuniversitária para o Desenvolvimento do Setor Sucroenergético/Universidade Federal de São Carlos (Table 1). To simplify the plant response, three groups were created:

- Group I: *Saccharum officinarum*, containing eight genotypes (SO);
- Group II: *Erianthus arundinaceus*, contains five genotypes (EA);
- Group III: other genotypes, including seven genotypes (OG).

Plants were cultivated in plastic boxes (37 × 30 cm and 14-cm high) filled with 10 kg of sand and vermiculite sterile substrate (2:1 v/v) using a vertical autoclave (two times at 121°C/30 min with 48-h intervals). The chemical analysis of the substrate was: pH (H₂O), 6.36; C (%) 0.23; exchangeable elements (in cmol_c·dm⁻³): Al³⁺, 0.06; Ca²⁺, 0.48; H+Al, 0.33; Mg²⁺, 1.59; in mg·L⁻¹, K - 21.20 and P (Mehlich) - 8.33 and N (%) = 0.02. At the budding stage, no fertilizer was applied as the propagation material contained sufficient nutrient reserve for initial growth. The pots were watered every other day to keep the soil moisture up to the required levels. Each treatment was composed of 60 buds collected from the germplasm bank after 18 months of growth. The buds were distributed in five boxes containing 12 buds (2-cm long) each. Buds were previously selected from the stalk and separated by size. Each genotype was considered one experiment, and the boxes were experimental units, arranged in five complete casualized blocks (repetitions) in an automated greenhouse with temperature and humidity control. The first trial tested the inoculation using the blend of five bacterial strains (control and inoculated buds), and the second trial tested the HT (52°C/30 min) with and without inoculation. Both trials used five replicates (box) with 12 buds each.

The buds underwent a short HT at 52°C/30 min, as described by Sanguino et al. (2006), and used to decrease the phytopathogenic microorganisms and the general microbiota associated with the setts (Reis et al. 1994). Subsequently, all buds of the two trials were immersed for 3 min in a 0.1% solution of the fungicide Methyl N-(2-[[1-(4-chlorophenyl)-1H-pyrazol-3-yl] oxymethyl] phenyl) N-methoxycarbamate (Piraclostrobin 250 g·L⁻¹, Comet®) to prevent fungal diseases.

Inoculant procedure

The inoculant used was prepared using five diazotrophic strains all isolated from sugarcane and deposited at the Diazotrophic Bacteria Collection of Embrapa Agrobiologia – CRB Johanna Döbereiner (BR label). The diazotrophs used were *G. diazotrophicus* strain BR11281^T = PAL-5^T (Cavalcante and Döbereiner 1988, Gillis et al. 1989, Yamada et al. 1997); *H. seropedicae* BR11335 = HRC54 (Baldani et al. 1986); *H. rubrisubalbicans* BR11504 = HCC103 (Baldani et al. 1996); *Paraburkholderia tropica* BR11366^T = PPe 8^T (Reis et al. 2004, Oren and Garrity 2015); and *N. amazonense* BR11145 = CBAMc (Magalhães et al. 1983, Lin et al. 2014).

The bacterial inoculant was prepared using a single colony of each strain cultivated in 5 mL of DYGS medium, as described by Baldani et al. (2014), and grown in a rotary shaker at 170 rpm for 24 h at 30°C until reaching optical density (O.D.₅₆₀) = 1 (Santos et al. 2019). This pre-inoculum was used in an Erlenmeyer of 125 mL containing 50 mL of the same medium and grew for 24 h at 170 rpm in a rotary shaker at 30°C until it reached the cell quantity of 10⁹ cells·mL⁻¹ (O.D.₅₆₀ = 1). For the inoculation procedure, the bacterial suspension (50 mL) was mixed with 170 mg of sterilized and neutralized peat (121°C, 20 min two times with one day between autoclavation) and used as a vehicle to maintain cell numbers. The inoculant was kept at 20–22°C in a controlled chamber for at least one week before use in the experiments.

The five strains were mixed up by using 10 g of the individual peat inoculum, totaling 50 g in the mixture, and diluted to 1/100 (w/v) using water. The control consisted of sterilized peat diluted in water without bacterial addition. After the chemical and HT (when applied), buds were immersed for 30 min in the inoculum solution (inoculated treatment) and water (control). Bacterial numbers present in the inoculant were measured by plating using three different solid media: LGI-P (for BR11281 and BR11366), JNFb (for BR11335 and BR11504), LGI (for BR11145), as described by Baldani et al (2014). Bacterial counting of the inoculant reached 10⁸ to 10⁹ UFC·mL⁻¹.

Bacterial counting

The colonization of the buds was measured five or six days after inoculation using the most probable number technique, as described by Baldani et al. (2014). Two plants (roots and part of the germinated bud) of each treatment were washed in tap water to remove excess of the substrate and macerated with a blender using 10 g of fresh tissue diluted in 90 mL of saline solution (0.8% NaCl). Aliquots (100 µL) of each dilution (up to 10⁻⁸) were inoculated into vials containing 5 mL of three different

N-free semi-solid media: JNFb (malate, final pH 5.8), LGI (crystal sugar, final pH 6), and LGI-P (100 g·L⁻¹ crystal sugar, final pH 5.7), as described by Baldani et al. (2014). The population size was estimated by the most probable number (MPN) using McCrady's table with three replicates (Baldani et al. 2014). Numbers were transformed in Log (10)·g⁻¹ fresh mass.

Plant evaluations

During the experiments, sprouting was evaluated daily, and, with the values recorded, the MGT was calculated as per the formula provided by Ellis and Roberts (1981) (Eq. 1).

$$\text{Mean germination time (MGT)} = \Sigma(n \times d)/N \quad (1)$$

where: n: number of setts germinated on each day; d: number of days from the beginning of the test; N: total number of setts germinated at the termination of the experiment.

After this period, the percentage of germination was measured. All the settings in each treatment were uprooted and cleaned, and the shoot and root from spent setts at different times depending on the growth of each genotype. First, the aerial tissue of the plant, the roots were not used for the scanning, was dried in a hot-air oven at 60°C till constant weight, and the its dry weight was recorded and expressed in milligrams per plant. The shoot-root ratio was also calculated by dividing shoot dry weight with root dry weight.

One *S. officinarum* IN845, one *S. spontaneum* US72-1319, and six genotypes of *E. arundinaceus* (Table 1) were also used for the measurement of root architecture using the methodology developed by Bauhus and Messier (1999) with the application of the WinRHIZO Pro™ software (Regent Instruments, QC, Quebec, Canada). The roots and minisetts were washed and immersed in 50% ethanol for storage and to study the root architecture. For measuring, the roots were separated from the sett, and the software coupled with an Epson Expression 11000XL LA2400 image scanner was used. Roots were laid out in an acrylic container (20-cm wide and 30-cm long) with approximately 1 cm of water layer and placed on the scanner. Root length (TL, cm), number of tips, forks, and crossings, and length of fine roots (0 < L < 0.5 cm) were selected from the data obtained by the software after scanning the roots. After analysis, the roots were dried at 60°C to obtain a constant weight and root dry weight.

Statistical analysis

Plant measurements were taken in five replications. Excel program of MS Office 13 version was used for the calculation of mean, percentage, and the MGT, and to plot both line and bar graphs. The experiments were analyzed by each genotype individually in a randomized complete block. The data were subjected to the analysis of variance (ANOVA) using the F-test, at a 5% probability for setting growth, biomass accumulation, and root analysis, and performed. When significant, the mean values of treatments were compared by Scott-Knott's test, also at 5% probability¹, on RStudio (RStudio Team, 2015), version 1.0.136.

Bacterial population using the MPN technique using the McCrady table is already an estimation of the bacterial population, considered a statistical method. No further analysis was performed on these data.

RESULTS AND DISCUSSION

Evaluation of the 20 genotypes

As observed in Table 1, 10 of the 20 genotypes were harvested 60 days after planting (DAP), but two took 120 days to reach a similar biomass accumulation reached by the others, the IJ76-364 and US72-1319. On the other side, White Pararia

¹ De Mendiburu, F. (2016). A statistical analysis tool for agricultural research. Dissertation, National Engineering University Lima-Peru.

and Q44830 harvested 25 and 33 DAP, respectively. Comparisons were made inside the genotype growth curve, not between them, as these plants differ in germination and biomass accumulation.

The germination percentage above 80% was observed in only seven untreated buds of the group I and inoculation did not improve it (Fig. 1a). Differences were observed between the group's genotypes, in *E. arundinaceus* (group II) the germination was reduced by 22%, and the other genotypes (group III) had an intermediary level. Buds subjected to a short HT showed an opposite pattern: the group II improved this percentage, and the other ones reduced by 25% in the group I and 37% in the group III, compared to group I (Fig. 1a). In both cases, inoculation did not play a role in this index.

When evaluating the MGT, the group I showed a shorter period, 16 days, regardless the bud treatment (Fig 1b). Conversely, the other two groups required more than two days to reach the MGT, and this index was again not modified by the two treatments applied (inoculation using five diazotrophs and HT).

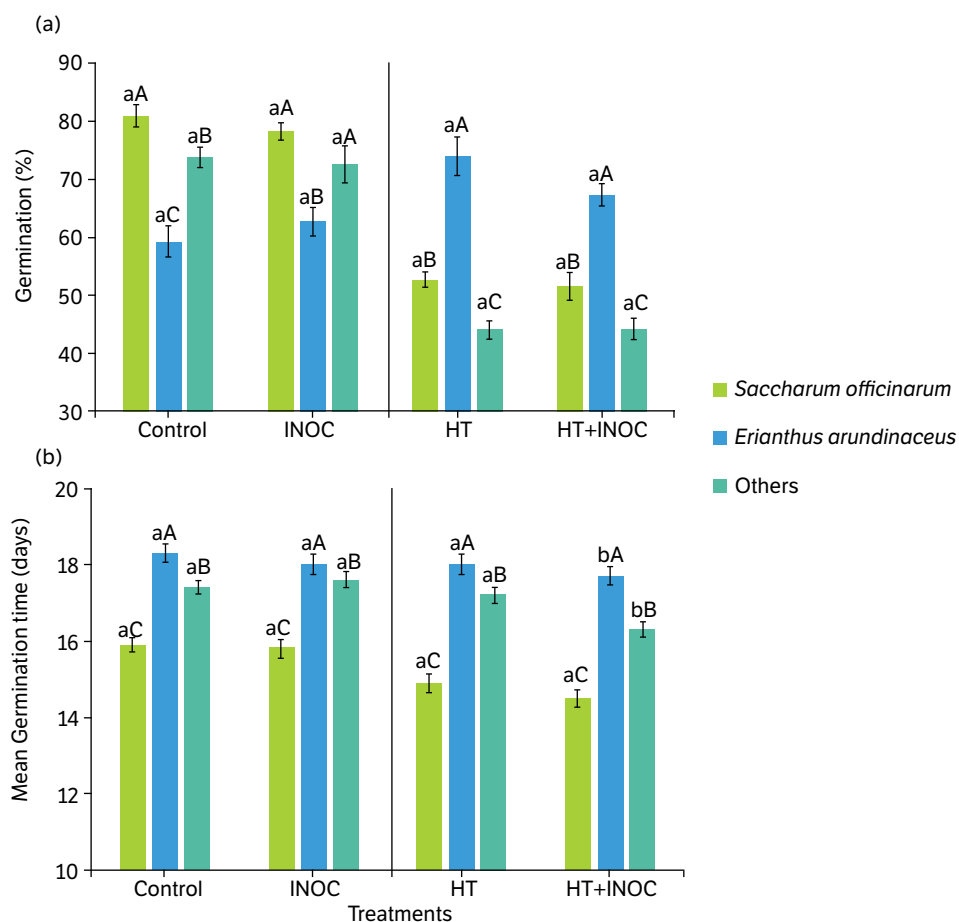


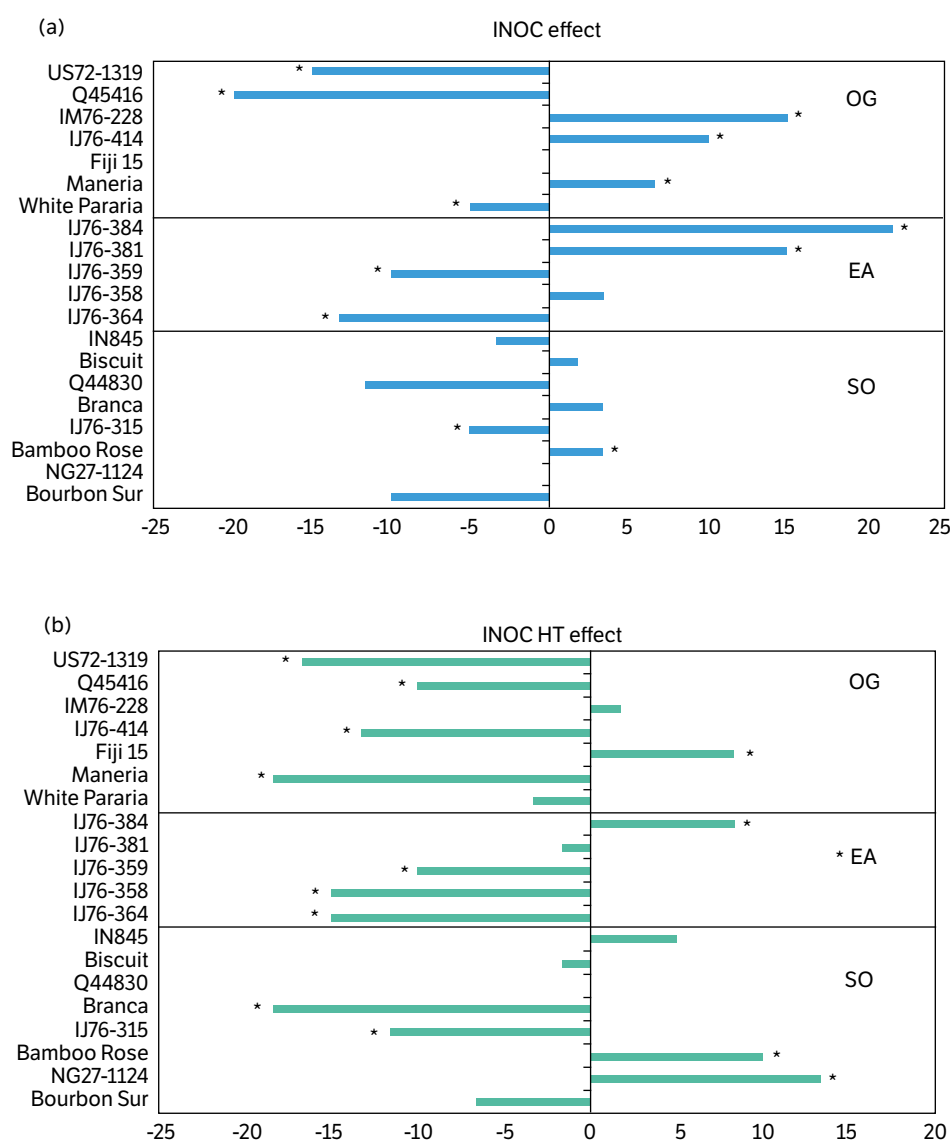
Figure 1. Evaluation of plant sprouting. (a) Germination and (b) mean germination time of three groups of *Saccharum* complex genotypes submitted to inoculation using five diazotrophs (INOC) and a short heat treatment (HT). *Saccharum officinarum* ($n = 8$); *Erianthus arundinaceus* ($n = 5$) and other genotypes ($n = 7$) were evaluated during the first 30 days of growth. Lowercase letters compare inoculation inside each treatment and capital letters compare between groups at $p < 0.05$.

Data germination percentage of each genotype, the inoculation effect also differed inside the three groups, improving higher positive promotion in group III and group II and a low positive effect of group I (Fig. 2a). After applying the HT, the positive responses reduced in HT buds as expected after this treatment. Growth response related to the inoculation treatment was lower and, in this case, group I differed from the other two, improving this index, especially in two genotypes, NG27-1124 and Bamboo Rose (Fig. 2b).

Bacterial counting was improved by the inoculation, in two of the three groups, SO and other genotypes, but similarly in the *Erianthis* group (Fig. 3). The group II presented a natural population less impacted by the HT, and inoculation was

less effective compared to the other two groups of genotypes (Fig. 3b). In these group I and group III, the inoculation improved the bacterial counting by 100–1,000 times higher the control or HT buds. Population size improved even in the untreated or HT buds evaluated five days after planting of the group I, especially the counts using N-free semi-solid JNFB media (malate as a carbon source, final pH 5.8) and LGI-P (crystal sugar 100 g·L⁻¹, pH 5.7).

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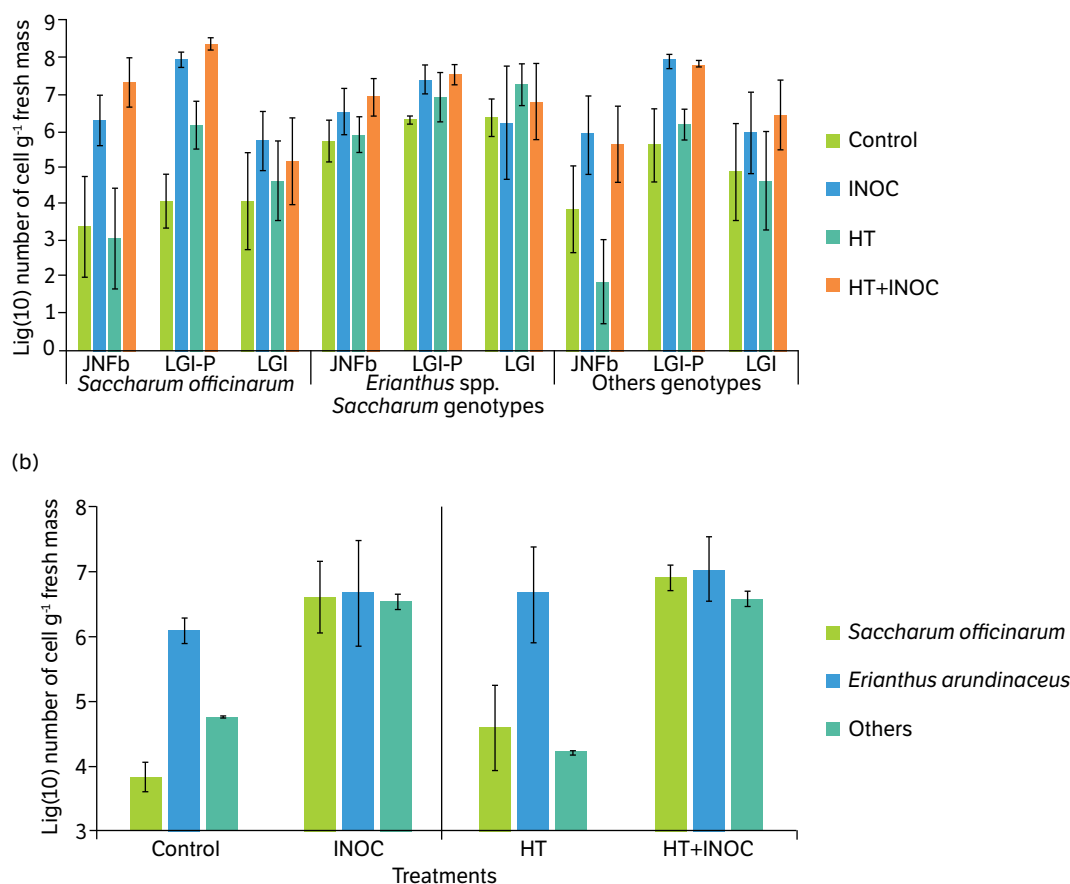


SO: *Saccharum officinarum* (group I); EA: *Erianthus arundinaceus* (group II); OG: other genotypes (group III).

Figure 2. Inoculation (INOC) effect of the applied mixture of diazotrophs on (a) the germination of 20 *Saccharum* genotypes and (b) after the heat treatment (HT). Effect measured using germination percentage on the inoculated buds minus the control. Horizontal lines divided the genotypes into three groups.

This data associated with the germination effect (Fig. 2) can in part explain the lower growth response in the group II, in which the natural population was not reduced by the HT, reducing the inoculation effect in this group. The opposite occurred with group I, in which the germination percentage was lower after the HT (Fig. 1b) and inoculation improved it (Fig. 3b), depending on the genotype evaluated without influencing the MGT (Fig. 1b).

Comparing two genotypes belonging to group I (IN845) and group III (US72-1319), the dry mass accumulation of *S. officinarum* IN845 and *S. spontaneum* US72-1319 were evaluated at different periods, at 45 days for IN845 and 120 days for US72-1319 (Fig. 4). It took IN845 only 45 days to reach the maximum value of 253 mg·plant⁻¹ (Fig. 4a), whereas US72-1319 took three times longer to reach a similar value of 242 mg·plant⁻¹ in the INOC treatment (Fig. 4c). The inoculation using five different diazotrophic strains did not affect the shoot dry mass, but reduced the root dry mass of IN845 (Fig. 4a) and US72-1319 after the HT (Fig. 4c). The shoot/root ratio, however, responded positively to INOC in both genotypes and after HT only in the US72-1319 (Figs. 4b and 4d).

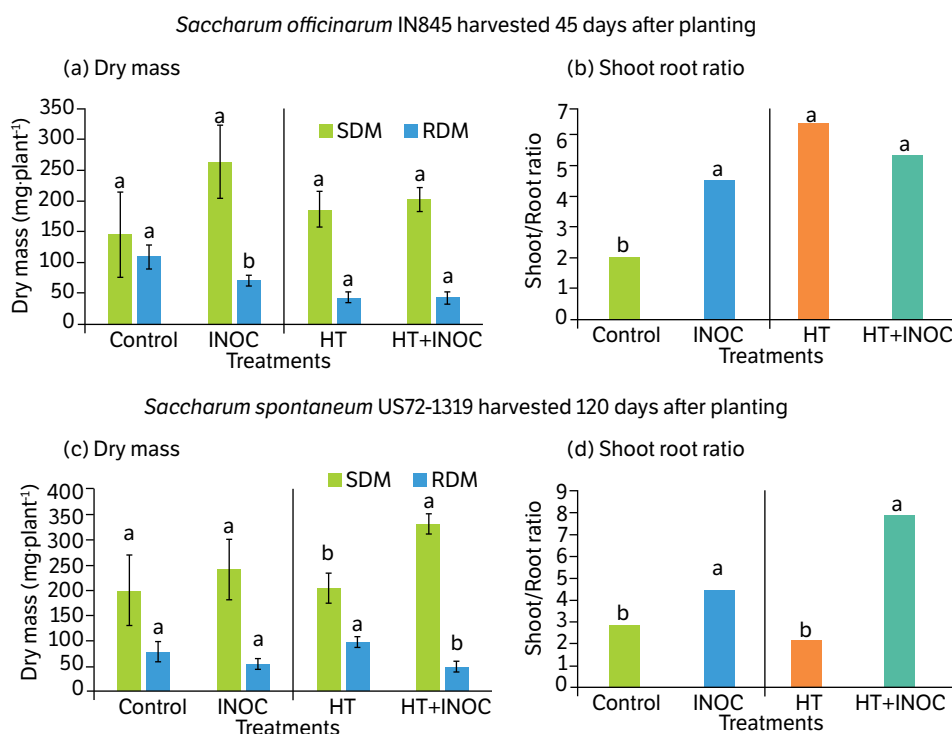


HT: heat treatment.

Figure 3. Bacterial counting using N-free semisolid media JNFb (malate), LGI-P (100 g·L⁻¹ crystal sugar) and LGI (5 g·L⁻¹ crystal sugar) of three groups of *Saccharum* complex genotypes: *S. officinarum* (n = 8); *Erianthus arundinaceus* (n = 5) and other genotypes (n = 7) evaluated five or six days after inoculation (INOC) using most probable number (MPN) technique.

Energy canes refer to crops capable of generating biomass at a low-cost production and possessing high fiber content. Notably the *E. arundinaceus* species and *S. spontaneum* exhibited these desirable characteristics. This study solely employed data derived from plants cultivated in a low fertility substrate without any supplementary fertilizer addition. Biomass production in these plants primarily relies on bud reserve and some minimal amount of nutrients present in the substrate (K and P mainly). All measurements were made within a relatively short period, specifically a maximum of 120 days. Due to variations in their origins and the absence of breeding for enhanced performance, significant differences were observed in germination and growth rates. For instance, some plants achieved an 82% germination rate, while others only reached 44%

after the HT or HT + INOC treatments (Fig. 1a). Furthermore, the MGT also varies from around 15 days of *S. officinarum* genotypes to 18 days of *E. arundinaceus* group (Fig. 1b). The setts represent a unique plant physiological stage characterized by a dormant metabolic status that is reorganized during the germination process (Singh et al. 2015). This initial process of growth involves the regulation of several plant hormones and enzymatic activity and under field conditions starts from seven to 14 days and is usually completed in about 30–45 days.



SDM: shoot dry mass; RDM: root dry mass.

Figure 4. Plant evaluation: (a, c) Dry mass accumulation and (b, d) shoot/root ratio of (a, b) *Saccharum officinarum* IN845 and (c, d) *S. spontaneum* US72-1319 and submitted to inoculation (INOC) using five strains of diazotrophs and a short heat treatment (HT). Each bar represents the mean of five replicates, and the error bars are the standard error of the mean. Lowercase letters differ at $p < 0.05$ ($n = 5$).

The application of a mixture of five diazotrophic strains normally improves the sugarcane hybrids to germinate, and grow faster (Chaves et al. 2015, Gírio et al. 2015, Santos et al. 2019) was a feature not observed in these rustic materials. Several factors can contribute to the poor germination of the buds, such as low temperatures (not applicable in this study), level of reducing sugars, activity of acid invertase, and accumulation of indole acetic acid (IAA) and total phenols present in these rustic materials. These parameters are typically associated with temperatures below the optimal range (Jain et al. 2007). This pool of bacterial strains used in these experiments produces a diverse group of plant growth regulators, including auxins (Cassán et al. 2014), and gibberellins (Bastián et al. 1999, Bottini et al. 2004). Previous research has shown that co-cultivation with other bacteria can enhance the production of these hormones by the bacteria (Cacciari et al. 1989) and was also employed in this study. Additionally, studies have reported that inoculation can modify the gene expression related to phytohormones, thereby altering the root response (Silva et al. 2020).

This variability mentioned in the statement is anticipated, as the HT used to enhance the quality of sugarcane buds typically resulted in reduced germination and growth in most of the genotypes tested (Fig. 1), as well as in the commercial hybrids used in sugarcane fields all over (Fernandes Júnior et al. 2010). However, this negative impact was not observed in the genotypes of EA species that were tested (Fig. 1). These findings demonstrate once again the ability of this genus to thrive even under unfavorable conditions that typically harm the buds of the *S. officinarum* genus (Fig. 1). The inoculation treatment, that somehow could improve these two measurements, germination and mean germination period, was not effective or even reduced them in some groups.

Bacterial counting using three different media showed that the inoculation was effective, especially for the counting using JNFB and LGI-P media (Fig. 2a). Evaluating the three groups of genotypes, the EA group presented the highest bacterial numbers in the uninoculated plants, even after the HT (Fig. 2b). Group I and group II showed a bacterial population in the inoculated plants improved by 100–1,000 times more than the respective controls. One possibility can be attributed to the lower effect of HT, which also reduces the natural population of different bacteria including *Leifsonia xyli* subsp. *xyli* (Copersucar 1989) and phytoplasma disease (Kumar and Pratap 2023). Another possibility can be attributed to a different natural microbiome as observed by Teheran-Sierra et al. (2021). Differences in the core microbiome could be also associated with bacteria adapted to a broader range of carbon sources, pH, or even osmotolerance as most of the detected bacteria were counted using the LGI-P medium, which is made with 10% crystal sugar (Fig. 2a), differing of the microbiome present in the other two groups. It can explain, in part, the differences observed in the group II in germination and tolerance to the HT. It is important to note that the experiments were performed using a sterile substrate, and the bacteria counted originated them the bud or the inoculant.

The genus *Erianthus* is closely related to the *Saccharum* and *Miscanthus* genera. However, there are ongoing confusion and controversy regarding the taxonomy of these plants (Vasquez et al. 2022, Xiong et al. 2022). Nevertheless, certain *Erianthus* species have the potential to provide high biomass yields, particularly in challenging environments, making them ideal candidates for future bioenergy production systems. Additionally, this genus can serve as a genetic resource for improving sugarcane's ability to withstand adverse conditions (Amalraj et al. 2008). This performance can be seen by the robustness of this material, which enables it to overcome issues that typically hinder the germination of *Saccharum* hybrids like the HT (Fig. 1).

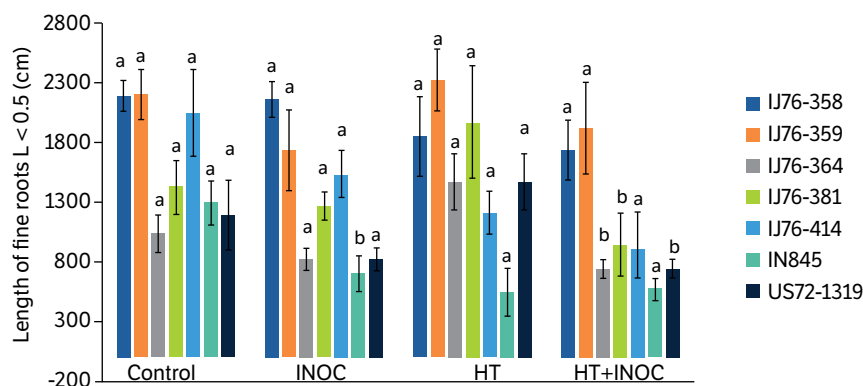
The inoculation effect, measured after 27 days of planting and using the germination percentage, showed that buds vary between positive and negative effects not related to a group of genotype species (Fig 3a). It is important to note that these buds were cut from a germplasm bank 10 months after planting and fully fertilized, an ideal state for maximum vigor for bud germination. The inoculation effect of the HT buds changed the pattern of plant response, reducing the negative percentage of germination with higher positive increments in group II and group III, being less effective for group I (Fig 3b).

The growth response was not observed in the root development and was commonly found by several authors using the same mixture of five strains tested in this study in sugarcane hybrids (Chaves et al. 2015, Santos et al. 2019) and other plants such as wheat, maize, and other cereals (Cassán et al. 2014, Carril et al. 2021). Growth response was observed in the shoot dry mass accumulation of US72-1319 (Fig. 4c) and shoot / root ratio of both genotypes IN845 and US72-1319 (Figs. 4b and 4d).

No previous studies have been reported on the growth response of the specific genotypes tested concerning inoculation using diazotrophs associated or not with HT. During this initial stage, buds contain varying amounts of carbohydrates used to produce organs, using the reserves contained in the parenchyma cells of the sett (Jain et al. 2009, Marafon 2012). Adventitious roots emerge from the culm root zone and are responsible for water uptake during bud sprouting and plant support until the permanent roots develop. The roots of the genotypes evaluated in this study were less sensitive to inoculation, measured by the length of fine roots. In this case, root length was not modified or even reduced by the inoculation (Fig. 5). Compared to hybrids used in sugarcane fields and studied previously, root biomass is positively modified by the inoculation (Girio et al. 2015, Santos et al. 2019). However, aerial stimulation observed in one genotype, the US72-1319 (Fig. 4), may be attributed to their natural growth pattern, in which they prioritize the production of aerial plant tissue over root development.

Evaluating the biomass accumulation of the *Erianthus* genotypes, inoculation did not modify the shoot or root dry mass, but HT reduced the shoot dry mass, but not the root (Fig. 6). Only genotype IJ76-381 improved the shoot dry mass in plants, not HT. So, in this genus, bacterial counting was not effective, and in the other two groups, inoculation response was null.

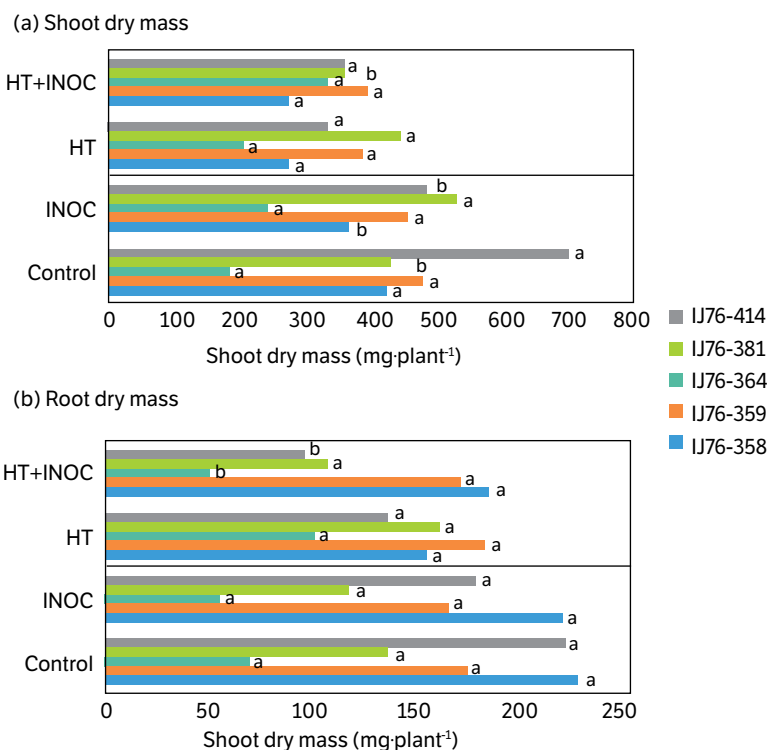
Another point to be considered is the potential competition between inoculation and the process of germination through the utilization of bud carbohydrate reserves, especially related to glucose and sucrose content that is used by the strains applied (Reis et al. 1994, 2004), and act as important signaling molecules that affect germination, growth, and development (Rolland et al. 2006).



INOC: inoculation; HT: heat treatment.

Figure 5. Length of fine roots ($L < 0.5$) of seven genotypes belonging to the *Saccharum* complex, being four *Erianthus arundinaceus* (IJ genotypes), one *S. officinarum* IN845 and one *S. spontaneum* (US72-1319). Each bar represents the mean of five replicates, and error bars represent the standard error of the mean. Lowercase letters differ at $p < 0.05$.

Different species and genotypes will differ in response to the treatments being applied. This study serves as an initial investigation into the genotypes being used. Efforts to understand how bacterial inoculation enhances the growth of selected plants have been made, demonstrating the potential for utilizing different genera to produce biomass for energy production (Cursi et al. 2022) and associating this growth to treatments that are normally used to control diseases in sugarcane and the possibility to improve growth upon the use of diazotrophic bacteria as an inoculant. The selected two genera out of the eight evaluated mark the beginning of potential new materials that could be tested in detail to enhance bacterial response, one being *S. spontaneum* US72-1319 (Fig. 2d), as well as a single genotype of *E. arundinaceus*, the IJ76-381 (Fig. 6).



INOC: inoculation; HT: heat treatment.

Figure 6. Dry mass accumulation of sugarcane genotypes. (a) Shoot and (b) root dry mass of five genotypes belonging to the *Erianthus arundinaceus* species. Each bar represents the mean of five replicates. Lowercase letters differ at $p < 0.05$.

Another point is related to bacterial concentration. This study also highlights knowledge regarding the inoculant used. While the concentration developed for commercial sugarcane hybrids (10^8 – 10^9 cell·mL⁻¹) was employed, this parameter has never been tested for this germplasm. However, the existing diversity of microorganisms in these plants and how they interact with the new microbiota added remain unknown.

Furthermore, this study represents the first trial to compare germination and HT of plants. More studies based on these plants could prove to be valuable tools in selecting an optimal plant-bacteria interaction to enhance the initial establishment of new breeding genotypes. This is especially important in soil with severe fertility limitations and regions with limited opportunities for biomass production for energy supply.

CONCLUSION

In this study, 20 different genotypes originating from different collections were planted in the same soil and transferred to Embrapa Agrobiologia for further evaluation. Each genotype exhibited a unique combination of these factors and responded differently to the treatments applied. As a result, the 20 materials belonging to the *Saccharum* complex were divided into three groups based on their growth response: group 1, consisted of *S. officinarum*; group 2, included *E. arundinaceus* and *S. spontaneum*; and group 3, consisted of *Saccharum* spp., *S. barberi*, *S. sinense*, *S. robustum*, and *Miscanthus* spp. The HT used to control several diseases had an inhibitory effect on germination in groups 1 and 3, but not in group 2.

It is well known that inoculation response is genotype-dependent and has an optimal dose response, which could be a limiting factor in this study. Interestingly, the genotypes in the *E. arundinaceus* group and two specific genotypes, *S. officinarum* IN485, and one *S. spontaneum* US72-1319, showed a positive response in the shoot biomass production at the expense of root development in response to the treatments of applied. This contrasts with the response observed in commercial hybrids and raises questions about the hormonal balance and interaction of these plants with the HT and INOC associated or not as studied. Understanding how these plants interact with different diazotrophic bacteria used as inoculants can also improve the growth response of these rustic plant genotypes that can be used as an energy crop under challenging soil conditions.

CONFLICT OF INTEREST

Nothing to declare.

AUTHORS' CONTRIBUTION

Data curation: Reis, V. M.; **Formal analysis:** Guimarães, N. F., Alves, G. C., Lorenzetti, I. J. S.; **Methodology:** Reis, V. M.; **Project administration:** Reis, V. M.; **Resources:** Reis, V. M.; **Supervision:** Reis, V. M.; **Writing – original draft:** Reis, V. M. and Alves, G. C., **Final approval:** Reis, V. M.

DATA AVAILABILITY STATEMENT

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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