



Article

Bacillus velezensis Associated with Organomineral Fertilizer and Reduced Phosphate Doses Improves Soil Microbial—Chemical Properties and Biomass of Sugarcane

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Abstract: Appropriately using phosphorus (P) for the fertilization of sugarcane is critical to achieving high productivity because it is one of the most limiting nutrients in agricultural cropping systems. Thus, the objective of this research was to evaluate the morphological, biochemical, and yield responses of sugarcane, and the soil microbial—chemical properties, under the use of organomineral fertilizer (OF) associated or not with *Bacillus velezensis* strain UFV 3918 (B) combined with mono ammonium phosphate (MAP) doses. The experimental design used was completely randomized, consisting of eight treatments [Control (3/3 MAP); OF (without MAP); OF + 1/3 MAP; OF + 2/3 MAP; OF + 3/3 MAP; B + OF + 1/3 MAP; B + OF + 2/3 MAP; B + OF + 3/3 MAP] and four replicates. B + OF + 1/3 MAP provided increases in accumulated soil basal respiration (11.9%), carbon of microbial biomass (35.9%), fluorescein diacetate (12.7%), arylsulfatase (25.5%), and acid phosphatase (10.1%) activity compared to the control, which implied in higher shoot and total biomass. These results present a potential strategy for sugarcane fertilization, using bacteria in combination with OF to improve P nutrition and growth in sugarcane with reduced economic and environmental impact.

Keywords: *Saccharum* spp.; plant growth-promoting bacteria; soil microbial activity; soil enzyme activity; biomass production



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1. Introduction

Brazil is the world's largest producer of sugarcane, and due to the excellent yield potential, with large areas of arable and favorable edaphoclimatic conditions, Brazil has become one essential country for supplying the increased world demand for sugar and ethanol from renewable sources [1,2]. The estimated sugarcane yield in Brazil in the 2022/23 crop season is 596.1 million tons [3].

Fertilization is one of the most critical factors in achieving high productivity. It is essential to study fertilization management, mainly with phosphorus (P), one of the most limiting nutrients in agricultural cropping systems [4]. Phosphorus is indispensable for plant growth and reproduction, which does not reach the maximum productive potential without an adequate nutritional supply [5].

The importance of P consists of the fact that this nutrient participates, directly and indirectly, in processes such as cell division, photosynthesis, respiration, storage, and transfer of energy, which makes it essential for good initial formation and the development of the root system, and adequate sprouting and tillering, influencing stalk productivity, technological quality, and longevity of sugarcane field [6,7].

However, P has been the nutrient most critical in fertilization in recent years in Brazil due to its high interaction with the soil and high retention rate of its ions [8,9]. Most of the soluble phosphate fertilizers applied to the soil dissolve and remain retained in the solid

phase because the minerals of Fe and Al absorb P, and both decrease the availability of P for plant growth [10–13].

In addition to meeting the demand for P during plant development, studies have shown that some microorganisms [14] and organomineral fertilizers [9] can improve the efficiency of P use in the soil–plant system [15]. Thus, in addition to the excellent P supply favoring photosynthetic activity and increasing plant biomass, studies show that inoculation with bacteria [16,17] and the use of organomineral fertilizers [9,18] favor crop growth and productivity.

Phosphate-solubilizing bacteria release metabolites that solubilize inorganic P and mineralize organic P, transforming this nutrient into an available form for plant uptake and stimulating its development [19]. Bacteria of the genus *Bacillus* are examples of abundant microorganisms in the rhizosphere and promising as phosphate solubilizers [20].

Soil microbial communities perform essential processes during the decomposition of organic matter and nutrient solubilization, influencing the crop's productivity [21]. The activity of these communities in the soil can be evaluated to understand the mechanisms of action of bacteria in P solubilization. These evaluations can be undertaken using microbiological indicators, the activity of enzymes linked to biogeochemical cycles of nutrients, and the activity of enzymes of bacteria intracellular metabolism [22].

In this context, the objective of this research was to evaluate the morphological, biochemical, and yield responses of sugarcane until 180 days after planting and the cultivation of soil microbial–chemical properties, under the use of organomineral fertilizer associated or not with the *Bacillus velezensis* strain UFV 3918 combined with mono ammonium phosphate (MAP) doses.

We hypothesized that the association of organomineral fertilizer with *Bacillus velezensis* UFV 3918 would allow for reducing MAP doses without harming biomass production and promoting improvements in soil quality.

2. Materials and Methods

2.1. Cultivation Conditions, Experimental Design, Plant Material, and Treatments

The experiment was conducted for six months in a protected environment in the Department of Crop Production of the School of Agricultural Sciences—FCA/UNESP, located in Botucatu (22°51'01" S, 48°25'55" W, 786 m asl), Sao Paulo, Brazil. The protected environment was a wooden structure with galvanized steel arches. It had a high ceiling of 3 m and a total area of 120 m², with anti-aphid fabric (2 mm diameter) on the sides and a transparent plastic roof (150 µm diameter).

According to the Thornthwaite methodology, the region's climate is characterized as B₂rB'₃a', which describes a humid climate with a low water deficit in April, July, and August. The climate is also mesothermal and has a water potential evapotranspiration concentration of 33% in the summer. The annual average rainfall is 1428.4 mm, and the air temperature is 20.3 °C [23]. The soil used was classified as dystrophic Red Latosol [24]. The granulometric analysis showed 68.2% of sand, 25.7% of clay, and 6.1% of silt, characterizing a medium textured soil, according to the texture class grouping triangle [24].

The RB966928 cultivar was used because it is the most planted sugarcane cultivar in Brazil, accounting for 17.7% of the cultivated area in Sao Paulo [25]. In addition, this cultivar has a high development speed, medium stem density, high tillering index, high productivity, and high resistance to diseases.

The treatments were composed of three commercial products, a solid and branny P-based fertilizer (MAP) containing 60% of P soluble in neutral ammonium citrate and 12% of nitrogen (N); a solid and branny organomineral fertilizer (OF) constituted of 4.04% of organic carbon, 0.78% of hydrogen, 0.39% of N, 175 mg dm^{−3} of P, 3.5 mmol_c dm^{−3} of potassium, 50 mmol_c dm^{−3} of calcium, 15 mmol_c dm^{−3} of magnesium, 96.17 mg dm^{−3} of sulfur, 5.30 mg dm^{−3} of boron, 0.8 mg dm^{−3} of iron, 16.9 mg dm^{−3} of manganese, and 12.5 mg dm^{−3} of zinc, with a recommended dose of 500 kg ha^{−1} (11.27 g pot^{−1}); and a microbiological one containing 7 g L^{−1} of the active ingredient *Bacillus velezensis* strain

UFV 3918 (B), with a minimum of 1.0×10^8 colony forming units (CFU) mL^{-1} , with a recommended dose of 2 L ha^{-1} .

The experimental design used was completely randomized, consisting of eight treatments [Control (recommended dose of MAP—3/3 MAP); OF (without MAP); OF + 1/3 MAP; OF + 2/3 MAP; OF + 3/3 MAP; B + OF + 1/3 MAP; B + OF + 2/3 MAP; B + OF + 3/3 MAP] and four replicates.

To minimize the varietal effect on the sprouting, planting was carried out using buds with an average length of 5 cm and an average diameter of 2 cm, of the same age and good sanitary aspect. Five buds were sowing per pot, and after the sprouting stabilization, the thinning took place, keeping only one bud per pot.

Each plot was composed of 50-L pots containing 45 dm^3 of soil, corrected based on the chemical analysis according to Vitti et al. [26] and following the doses of phosphate fertilization with MAP required by the treatments (Table 1). For sugarcane, it is recommended to use 30 to 60 kg ha^{-1} N in coverage before stalk formation, with higher doses in more clayey soils [24]. Thus, the cover fertilization with urea was performed at the amount of 66.6 kg ha^{-1} (30 kg ha^{-1} N).

Table 1. Initial soil chemical analysis and doses of mineral fertilizers added at planting.

pH	O.M.	Al ³⁺	H + Al	K	Ca	Mg	SB	CEC	V%	P _{resin}	S	Cu	Fe	Mn	Zn	B
CaCl ₂	g dm ⁻³															
6.0	40.1	0.7	20.8	0.6	75.2	26.4	102.2	123.0	83.0	61.8	34.1	0.3	22.7	0.7	1.2	0.2
Planting fertilization																
Dose			N			P			K							
MAP recommended dose			36.12 kg urea ha ⁻¹ (0.813 g urea pot ⁻¹)			125 kg MAP ha ⁻¹ (2.8 g MAP pot ⁻¹)			250 kg KCl ha ⁻¹ (5.635 g KCl pot ⁻¹)							
2/3 of the MAP			46.3 kg urea ha ⁻¹ (1.04 g urea pot ⁻¹)			83.33 kg MAP ha ⁻¹ (1.878 g MAP pot ⁻¹)			250 kg KCl ha ⁻¹ (5.635 g KCl pot ⁻¹)							
1/3 of the MAP			56.47 kg urea ha ⁻¹ (1.272 g urea pot ⁻¹)			41.7 kg MAP ha ⁻¹ (0.939 g MAP pot ⁻¹)			250 kg KCl ha ⁻¹ (5.635 g KCl pot ⁻¹)							
Without MAP			66.6 kg urea ha ⁻¹ (1.5 g urea pot ⁻¹)			—			250 kg KCl ha ⁻¹ (5.635 g KCl pot ⁻¹)							

O.M (organic matter); SB (sum of bases); V% (base saturation); cationic exchange capacity (CEC).

The planting was performed on 12 November 2020. The mineral and organomineral fertilizers were added to the soil at the planting time. For the bacteria inoculation, a solution composed of 75 mL of water at pH 7.0 and 2 mL of the commercial product containing an isolate of *B. velezensis* UFV 3918 (10^8 CFU mL^{-1}) was applied per pot. The buds were positioned at 2 cm depth, and each bud of treatments containing bacteria received 15.4 mL of this solution. In contrast, the buds of the other treatments received 15.4 mL of water. In the end, all were covered with soil.

2.2. Morphological Variables

The sprouting assessments were performed every day from the first bud to sprouting stabilization. The final number of sprouted buds per pot was transformed into a percentage. Additionally, the sprouting speed (SS) and the sprouting speed index (SSI) were calculated according to Equations (1) and (2), respectively, proposed by Maguire [27].

The SS equation is as follows:

$$SS = \frac{[(N1 S1) + (N2 S2) + \dots + (Nn Sn)]}{(S1 + S2 + \dots + Sn)} \quad (1)$$

where SS is the sprouting speed (days), S is the number of sprouts observed in each count, and N is the number of days from planting to each count.

The SSI equation is as follows:

$$SSI = \frac{S1}{N1} + \frac{S2}{N2} + \frac{S3}{N3} + \dots + \frac{Sn}{Nn} \quad (2)$$

where SSI is the sprouting speed index, S_n is the number of sprouts observed in the “n” counts, and N_n is the number of days from planting to the “n” counts.

The evaluations of the number of tillers (NT), stalk height (SH), stalk diameter (SD), and leaf area (LA) were performed at 30, 60, 90, 120, 150, and 180 days after planting (DAP). The NT was determined by counting the tillers present in the usable area of each pot. SH was determined using a tape measure, and the measurement was taken from the ground to the +1 leaf sheath insertion. The SD was determined using a digital caliper (MeterMall, 150 mm and 0.1 mm reading, Marysville, OH, USA), and the measurements were performed in the middle third of the plant. Additionally, the LA was calculated according to Equation 3, proposed by Hermann and Câmara [28], using measurements of the width and length of the middle part of the +3 leaf.

The LA equation is as follows:

$$LA = L \times W \times 0.75 \times (N + 2) \quad (3)$$

where L is the length of +3 leaf, W is the width, 0.75 is the crop correction factor, and N is the number of open leaves with at least 20% green area.

2.3. Foliar Biochemical Variables

One week before harvest, the +1 leaves were collected and stored in liquid nitrogen immediately upon collection and were then stored in a -80°C ultra freezer (NUAIRE Inc., NU-9668GC, Plymouth, MN, USA) until analysis.

The soluble protein content was determined in 100 mg leaf tissue samples, which had been macerated in liquid nitrogen and added to a homogenization medium containing 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 200 mg of polyvinylpyrrolidone (PVPP) for extraction. The tissue homogenates were centrifuged at $5000 \times g$ at 4°C for 10 min, and then the supernatants were used as crude extracts. Then, 10 μL aliquot of crude extract was added to 5 mL of Coomassie Brilliant Blue G-250 solution. After 15 min, the readings were performed in a spectrophotometer (Shimadzu, UV-2700, Kyoto, Japan) at 595 nm. The soluble protein concentrations were calculated based on the standard curve of bovine serum albumin (BSA) at 1 mg mL^{-1} , and the results were expressed in mg g^{-1} of fresh matter (FM) [29].

The soluble sugars were determined through 20 mg of freeze-dried leaf tissue samples, using 4 mL of deionized water for the extraction. The mixture was stirred for one hour, then the samples were centrifuged at $3000 \times g$ for 15 min, and the supernatant was collected. The supernatant was centrifuged at $6000 \times g$ for 10 min, and this extract was used to determine the soluble sugars using the methodology of Dubois et al. [30]. An aliquot of 0.5 mL of the solution, 0.5 mL of 5% phenol, and 2.5 mL of sulfuric acid were placed in test tubes, followed by stirring, cooling, and color fixation in trays containing ice. The readings were performed in a spectrophotometer (Shimadzu, UV-2700, Kyoto, Japan) at 490 nm. The results were expressed in $\text{mg g}^{-1} \text{ FM}^{-1}$.

The determination of the total amino acids was performed using freeze-dried leaf tissue samples, homogenized in 0.2 M sodium citrate buffer (pH 5.0), 5% ninhydrin, 0.0002 M potassium cyanide, and 60% ethanol (v/v) for the extraction of the total amino acids. The absorbance was analyzed in a spectrophotometer (Shimadzu, UV-2700, Kyoto, Japan) at 570 nm, and the results were compared with the glycine standard curve ($0.1\text{--}1.0 \mu\text{mol mL}^{-1}$) [31]. The results were expressed in $\mu\text{mol g}^{-1} \text{ FM}^{-1}$.

The acid phosphatase activity was determined in 500 mg leaf tissue samples, which had been macerated in liquid nitrogen and added to a homogenization medium containing 0.1 mol L^{-1} sodium acetate buffer (pH 5.6) for extraction. The tissue homogenates were centrifuged at $15,000 \times g$ at 4°C for 20 min, and then the supernatants were used as crude extracts. The reaction was initiated by incubating 500 μL of enzyme extract with 200 μL of substrate [2 mmol L^{-1} of disodium p-nitrophenyl phosphate (pNPP) and 150 mmol L^{-1} of sodium acetate buffer (pH 5.6)] for five minutes at 37°C and interrupted by the addition

of 300 μL of saturated Na_2CO_3 solution. After mixing by stirring, the absorbance was determined in a spectrophotometer (Shimadzu, UV-2700, Kyoto, Japan) at 400 nm [32]. The enzyme activity was expressed in $\text{nmol pNPP min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.4. Microbiological Variables

The soil samples for the microbiological variables were collected after the sugarcane harvest and stored in a $-10\text{ }^\circ\text{C}$ freezer until analysis.

Soil basal respiration (SBR) was measured by determining the C-CO_2 released, according to Silva et al. [33], by static incubation of 50 g of soil in airtight glasses at $25\text{ }^\circ\text{C}$ (BOD). The C-CO_2 was analyzed at 3, 7, 14, and 21 days after installation in 10 mL of 1 mol L^{-1} NaOH solution, which was titrated with HCl after adding Ba(OH)_2 for the precipitation of carbonates. The released C-CO_2 was expressed as $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ dry soil}$ for 21 days and as $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ dry soil per day}$.

Carbon (CMB) and nitrogen (NMB) of the microbial biomass were determined by the irradiation-extraction method [34,35]. For carbon, K_2SO_4 0.5 mol L^{-1} was used as the extracting agent. The extracts' organic carbon (C) was estimated by oxidation with $\text{K}_2\text{Cr}_2\text{O}_7$. The difference in C concentration between irradiated and non-irradiated samples of the extracts was expressed as CMB by multiplying a conversion factor (K_c) of 0.33 [35]. The results were expressed as $\mu\text{g C g}^{-1} \text{ soil}$.

N extraction was performed with 60 mL of $0.5 \text{ mol L}^{-1} \text{ K}_2\text{SO}_4$ (pH 6.8) in a shaker with horizontal circular motion (Quimis Inc., Q225M22, Diadema, SP, Brazil) at 150 rpm for 40 min. After decantation, aliquots of 20 mL were removed and transferred to glass tubes, followed by the addition of 3 mL of concentrated H_2SO_4 and 1 g of catalyst (mixture of K_2SO_4 , CuSO_4 , and selenium powder in a ratio of 1:0.1:0.01). The digestion was carried out at $160\text{ }^\circ\text{C}$ for 2 h, then raising the temperature to $350\text{ }^\circ\text{C}$ for 1 h. The distillation was performed with 20 mL of 400 g L^{-1} NaOH and the distillate was collected in a 50 mL Falcon tube containing 10 mL of $20 \text{ g L}^{-1} \text{ H}_3\text{BO}_3$. Then, 15 mL of 0.1% bromocresol green in an alcoholic medium, 6 mL of 0.1% methyl red in an alcoholic medium, and three drops of 0.1 N sodium hydroxide were added, followed by titration with $0.0025 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$. The difference in the N concentration between the irradiated and non-irradiated samples of the extracts was expressed as NMB by multiplying a factor (K_N) of 0.54 [35]. The results were expressed in $\mu\text{g N g}^{-1} \text{ soil}$.

2.5. Soil Biochemical Variables

After the sugarcane harvest, the soil samples were collected and stored in a $-10\text{ }^\circ\text{C}$ freezer until analysis.

The catalytic enzyme activity (intra and extracellular) was estimated by the hydrolysis of fluorescein diacetate (FDA), as proposed by Green et al. [36]. The FDA activity was determined by adding 1 g of soil, 5 mL of potassium phosphate buffer (pH 7.6), and 0.2 mL of the substrate (fluorescein diacetate). The samples were incubated at $30\text{ }^\circ\text{C}$ for 1 h. Then, fluorescein was extracted with 25 mL of chloroform-methanol solution (2:1). The product formed was sodium fluorescein, and the samples' quantification was performed on a standard curve. The absorbance was analyzed in a spectrophotometer (ThermoFisher Scientific, BioMateTM3, Waltham, MA, USA) at 490 nm, and the results were expressed in $\mu\text{g FDA g}^{-1} \text{ dry soil h}^{-1}$.

The dehydrogenase enzyme was determined according to the method of Mersi and Schinner [37]. In a 50 mL falcon tube containing 1 g of soil, 1.5 mL of hydroxymethyl aminomethane buffer (TRIS, 1 mol L^{-1} , pH 7.5), and 2 mL of INT substrate (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium, 4.4 mmol L^{-1}) were added. Then, the samples were stirred and incubated at $40\text{ }^\circ\text{C}$ for 5 h. The product of the enzymatic activity (INTF, iodonitrophenylformazan) was extracted by adding 10 mL of ethanol-dimethylformamide solution (1:1), and the samples were kept in the dark for 20 min. A 2 mL aliquot of the supernatant was collected and centrifuged at $5000\times g$ for 10 min. The absorbance was

analyzed in a spectrophotometer (ThermoFisher Scientific, BioMate™3, Waltham, MA, USA) at 490 nm, and the results were expressed in $\mu\text{g INTF g}^{-1} \text{ soil h}^{-1}$.

The urease activity was determined according to the modified methodology of Kandeler and Gerber [38]. In a Falcon tube containing 1 g of soil, 4 mL of citrate buffer (pH 6.7) and 1 mL of 10% urea solution as substrate were added. The samples were incubated at 37 °C for 2 h. The product formed was the released N-NH₄, determined in a spectrophotometer (ThermoFisher Scientific, BioMate™3, Waltham, MA, USA) at 600 nm by the commercial Urea 500® kit (Doles Inc., Goiania, GO, Brazil). The results were expressed as $\mu\text{g N-NH}_4 \text{ g}^{-1} \text{ dry soil h}^{-1}$.

The extracellular activity of the β -glycosidase enzyme was determined according to Eivazi and Tabatabai [39]. The β -glycosidase was measured after incubating 1 g of soil with 1 mL of the substrate PNPG (p-nitrophenyl- β -D-glucopyranoside, 4.2 mmol L⁻¹) and 4 mL of modified universal buffer (MUB, pH 6.0) at 37 °C for 1 h. Then, 1 mL CaCl₂ (1 mol L⁻¹) and 4 mL NaOH (1 mol L⁻¹) were added. A 2 mL aliquot of the supernatant was collected and centrifuged at 5000 $\times$ g for 10 min. The reaction product (p-nitrophenyl—PNP) was determined in a spectrophotometer (ThermoFisher Scientific, BioMate™3, Waltham, MA, USA) at 410 nm. The results were expressed as $\mu\text{g PNP g}^{-1} \text{ dry soil h}^{-1}$.

The activity of arylsulfatase was performed in the same way as β -glycosidase by replacing the synthetic substrate with 0.05 mol L⁻¹ p-nitrophenyl-sulfate in sodium acetate buffer (pH 5.8). The results were expressed as $\mu\text{g PNP g}^{-1} \text{ dry soil h}^{-1}$.

The acid phosphatase activity was evaluated by the release of PNP from the synthetic substrate p-nitrophenyl phosphate (2.4 mmol L⁻¹), using a MUB (pH 6.0), as described by Tabatabai and Bremner [40]. In a 50 mL falcon tube, 1 g of soil, 4 mL of MUB, and 1 mL of substrate were added. The samples were incubated at 37 °C for 1 h. After, 1 mL of CaCl₂ (1 mol L⁻¹) and 4 mL of NaOH (1 mol L⁻¹) were added, followed by hand stirring. A 2 mL aliquot of the supernatant was centrifuged at 5000 $\times$ g for 10 min. The released PNP was determined in a spectrophotometer (ThermoFisher Scientific, BioMate™3, Waltham, MA, USA) at 405 nm. The results were expressed as $\mu\text{g PNP g}^{-1} \text{ dry soil h}^{-1}$.

2.6. Harvest and Yield Variables

At 180 DAP, the plants were harvested to analyze the yield. Initially, the plants were separated into leaves (leaf sheath + leaf), stalks, and roots, followed by the evaluation of SH and SD. To obtain biomass production, the plant parts were kept in a forced air circulation oven at 65 °C until they reached a constant mass and then weighed on a balance (Balmak, ELC-6/15/30, Santa Barbara d'Oeste, SP, Brazil). The shoot (leaves + stalks) (SB), root (RB), and total (shoot + root) (TB) biomass were determined in units of g pot⁻¹.

2.7. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) with the F test, with a subsequent comparison of means using the Tukey test ($p \leq 0.05$) through the statistical software AgroEstat (AgroEstat, version 2015, Jaboticabal, SP, Brazil). The biometric data were submitted to regression adjustment throughout the evaluation periods to evaluate the behavior of the treatments using Minitab software (Minitab®, version 19, State College, PA, USA).

Additionally, all of the other variables were submitted to regression adjustment considering the doses of MAP associated with organomineral fertilizer (OF, OF + 1/3 MAP, OF + 2/3 MAP, and OF + 3/3 MAP) and organomineral fertilizer + *B. velezensis* (OF, B + OF + 1/3 MAP, B + OF + 2/3 MAP, and B + OF + 3/3 MAP), through the Minitab software (Minitab®, version 19, State College, PA, USA).

3. Results

3.1. Environmental Conditions during the Experiment

During the experiment, the air temperature within the protected environment ranged from 12.5 to 32.0 °C, with an average of 21.2 °C, 22.4 °C, 23.4 °C, 21.9 °C, 21.6 °C, 21.7 °C,

19.2 °C, and 18.0 °C during planting, 1st biometric evaluation (BE), 2nd BE, 3rd BE, 4th BE, 5th BE, 6th BE, and harvest, respectively (Figure 1). The relative humidity ranged from 60.4 to 88.1% throughout the cultivation cycle, with an average of 76.5%, 76.7%, 86.4%, 69.7%, 82.9%, 66.0%, 70.9%, and 75.6% during planting, 1st BE, 2nd BE, 3rd BE, 4th BE, 5th BE, 6th BE, and harvest, respectively (Figure 1).

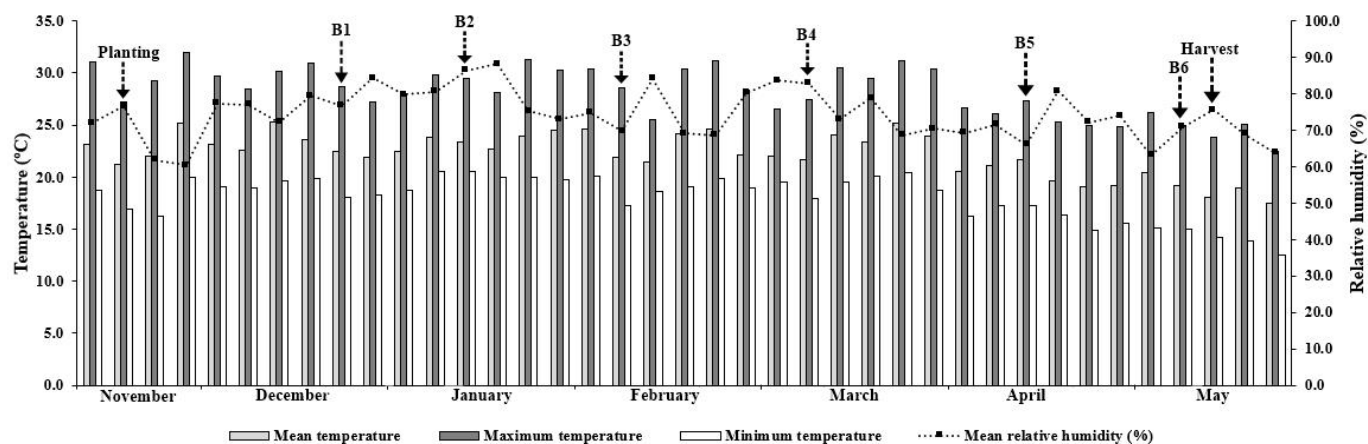


Figure 1. Mean, maximum, and minimum air temperature and average relative humidity inside the protected crop enclosure during the experimental period. B1: 1st biometric evaluation (BE); B2: 2nd BE; B3: 3rd BE; B4: 4th BE; B5: 5th BE, and B6: 6th BE.

3.2. Morphological Analysis

Generally, % sprouting was not uniform; the control and B + OF + 1/3 MAP provided the highest values (Figure 2a). The single-use of OF showed that sprouting increased as the MAP doses increased. In contrast, the sprouting decreased as the MAP doses increased with B + OF. It suggests that B + OF was more efficient under lower doses of MAP, showing P solubilization potential because the soil had high P content (Table 1). OF + maximum dose of MAP provided the highest %sprouting (0.99*), but B + OF + MAP doses had no regression adjustment (Figure 2a).

There was no difference between the treatments for SS, which demonstrates that the use of *Bacillus* and OF did not affect the average number of days required for sprouting (Figure 2b). Additionally, 3/3 MAP showed the highest SSI, followed by OF + 3/3 MAP, evidencing a higher average number of sprouted buds per day (Figure 2c). There was a tendency for SSI to increase with the application of OF and increasing doses of MAP (0.83*), while there was a tendency for SSI to decrease with inoculation of B + OF and increasing doses of MAP (0.70*).

In general, there was an increase in the NT from 30 DAP to 120 DAP in the control; OF; OF + 1/3 MAP, 2/3 MAP, and 3/3 MAP; and OF + B + 2/3 MAP, followed by a sharp decrease in values. This may have occurred due to the reduction in mean temperature from the end of March (Figure 1), which negatively affects tillering, as well as by competition and spatial restriction because the stalk elongated and its diameter increased. Whereas B + OF + 1/3 MAP and 3/3 MAP showed lower NT at 30 DAP, followed by increases in NT until 120 DAP and stabilization from this point (Figure 3a).

The control showed the highest SH throughout the evaluation period. Still, the association of B + OF + doses of MAP enabled the average SH to be similar to the control at 180 DAP (Figure 3b).

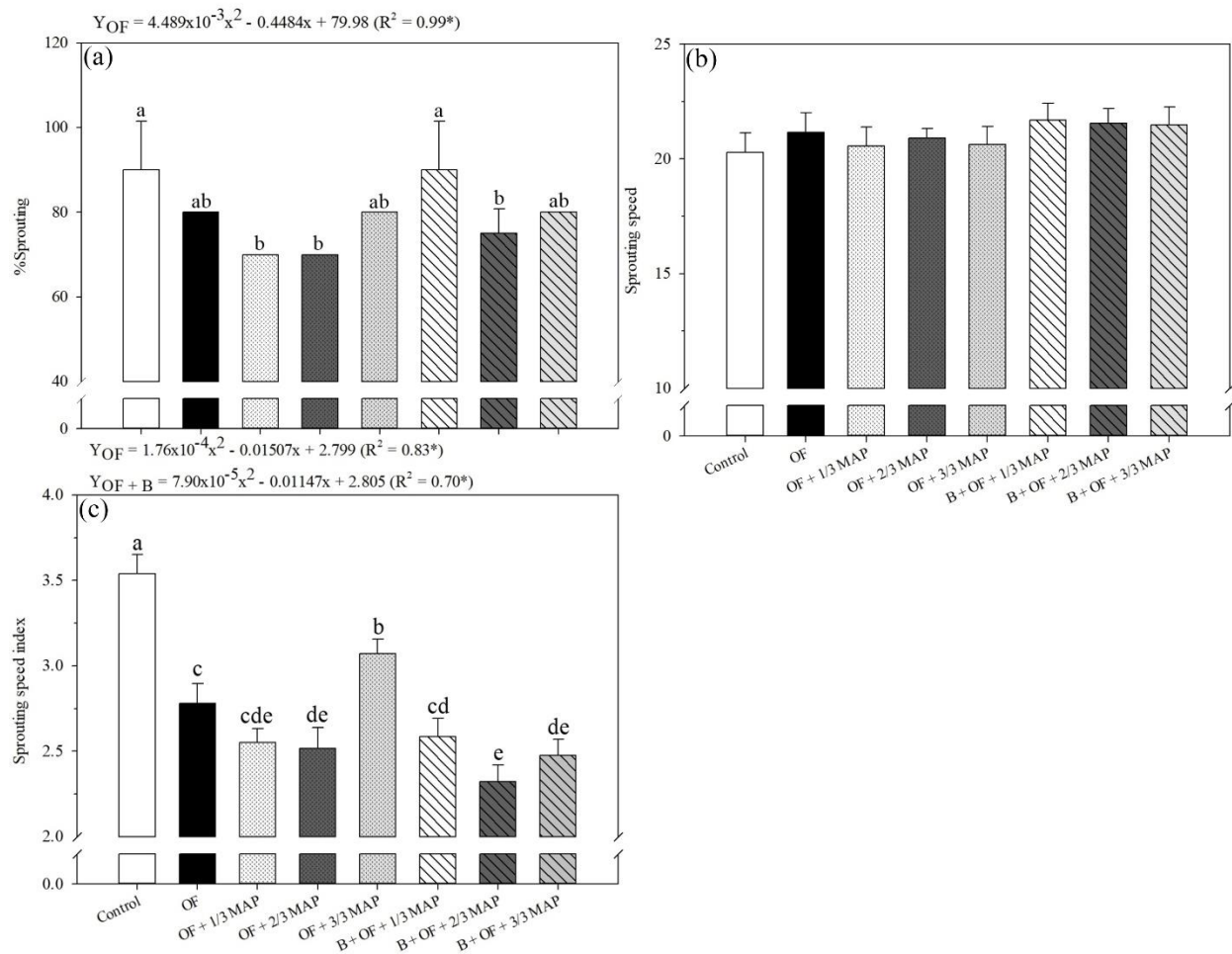


Figure 2. %Sprouting (a), sprouting speed (b), and sprouting speed index (c) of sugarcane seedlings under the association of organomineral fertilizer (OF), *B. velezensis* (B), and doses of mono ammonium phosphate (MAP). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. The error bars express the standard deviation of the mean ($n = 4$). The regression equations and R^2 above the figures include the MAP doses associated with organomineral fertilizer (OF) and with organomineral fertilizer + *B. velezensis* (OF + B) at the 5% significance level (*).

The behavior of the SD curves was very similar between the treatments (Figure 3c). There was an increase in SD until 150 DAP, followed by a decrease in this variable caused by the end of the tillering phase and beginning of the grand growth phase of the crop, known by the elongation of the stalks and internode spaces and the possible reduction in its diameters.

The LA curves were also similar between the treatments, with increases in LA until 180 DAP without stabilization. Sugarcane grand growth phase of development is marked by the rapid rise in leaf development from the 6th to the 7th month [41,42], favoring the high production of photo-assimilates and the beginning of sucrose accumulation, which explains the highest LA verified at 180 DAP (Figure 3d). The association of B + OF + doses of MAP provided the largest leaf areas.

The equations referring to NT, SH, SD, and LA for each treatment are shown in Table S1.

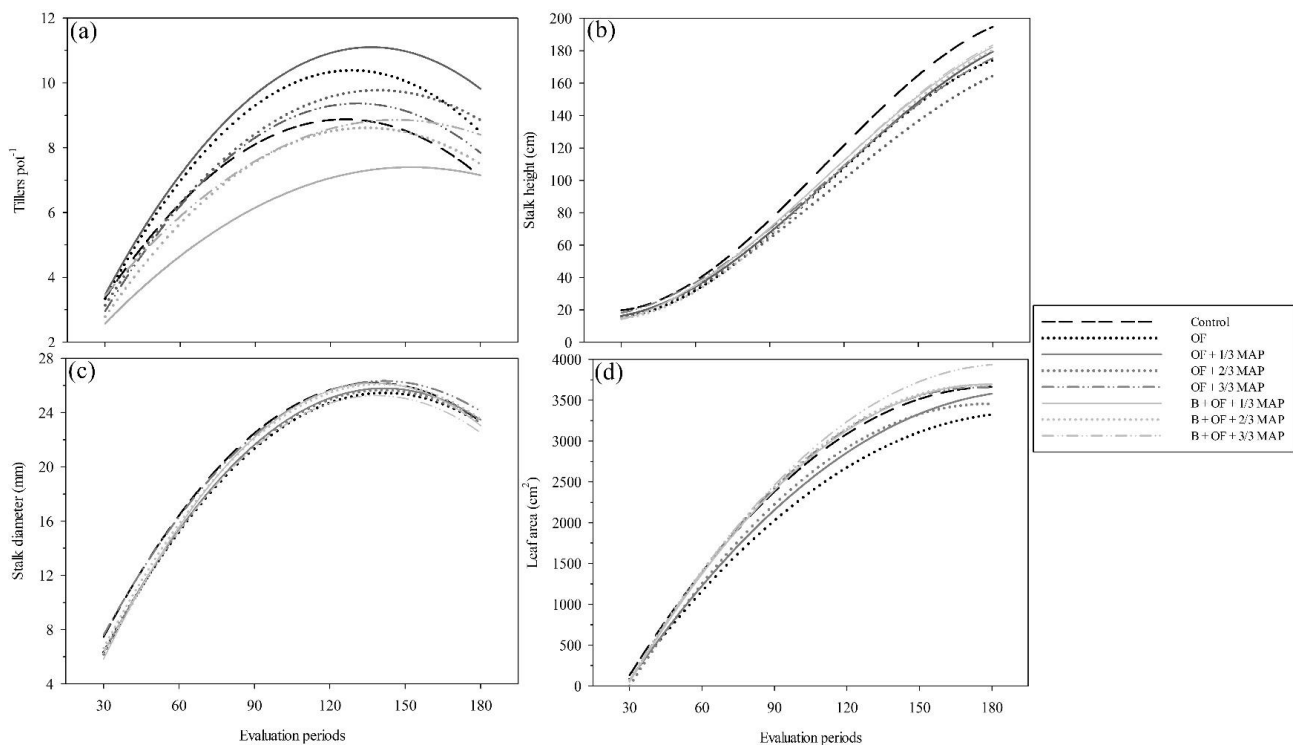


Figure 3. Regression curves along the evaluation periods (30, 60, 90, 120, 150, and 180 DAP) for tillers pot^{-1} (a), stalk height (b), stalk diameter (c), and leaf area (d) of sugarcane under the association of organomineral fertilizer (OF), *B. velezensis* (B), and doses of mono ammonium phosphate (MAP).

3.3. Foliar Biochemical Variables

There was no difference between the control and the different doses of MAP associated with OF or B + OF for the protein content (Figure 4a).

The total sugar (TS) content when OF or B + OF were combined increased as the doses of MAP increased. OF + 1/3 or 2/3 MAP and B + OF + 1/3 MAP or 2/3 MAP showed TS similar to the control, while B + OF + 3/3 MAP promoted an increase of 8.6% in this content compared to the control (Figure 4b). B + OF + maximum MAP dose provided the highest TS (0.82*), but OF + MAP doses had no regression adjustment.

The highest total amino acid (TA) contents were observed in the treatments with B + OF. B + OF + 1/3 MAP or 2/3 MAP and B + OF + 3/3 MAP provided increases of 16.2% and 31.2% in TA content, respectively, compared to the control (Figure 4c). OF + MAP doses promoted increases of TA up to 66% of the MAP dose, followed by a decrease in this content with 100% of MAP (0.77*), and B + OF + MAP doses increased the TA as the MAP doses were increased (0.84*).

Regarding the foliar acid phosphatase (AP) activity, B + OF + 1/3 or 2/3 MAP showed the highest enzyme activities, with an average increase of 9.4% compared to the control (Figure 4d). Both OF + MAP doses (0.97*) and B + OF + MAP doses (0.83*) provided increases in AP activity up to 66% of the MAP dose, followed by a decrease in enzyme activity under the use of higher phosphate doses.

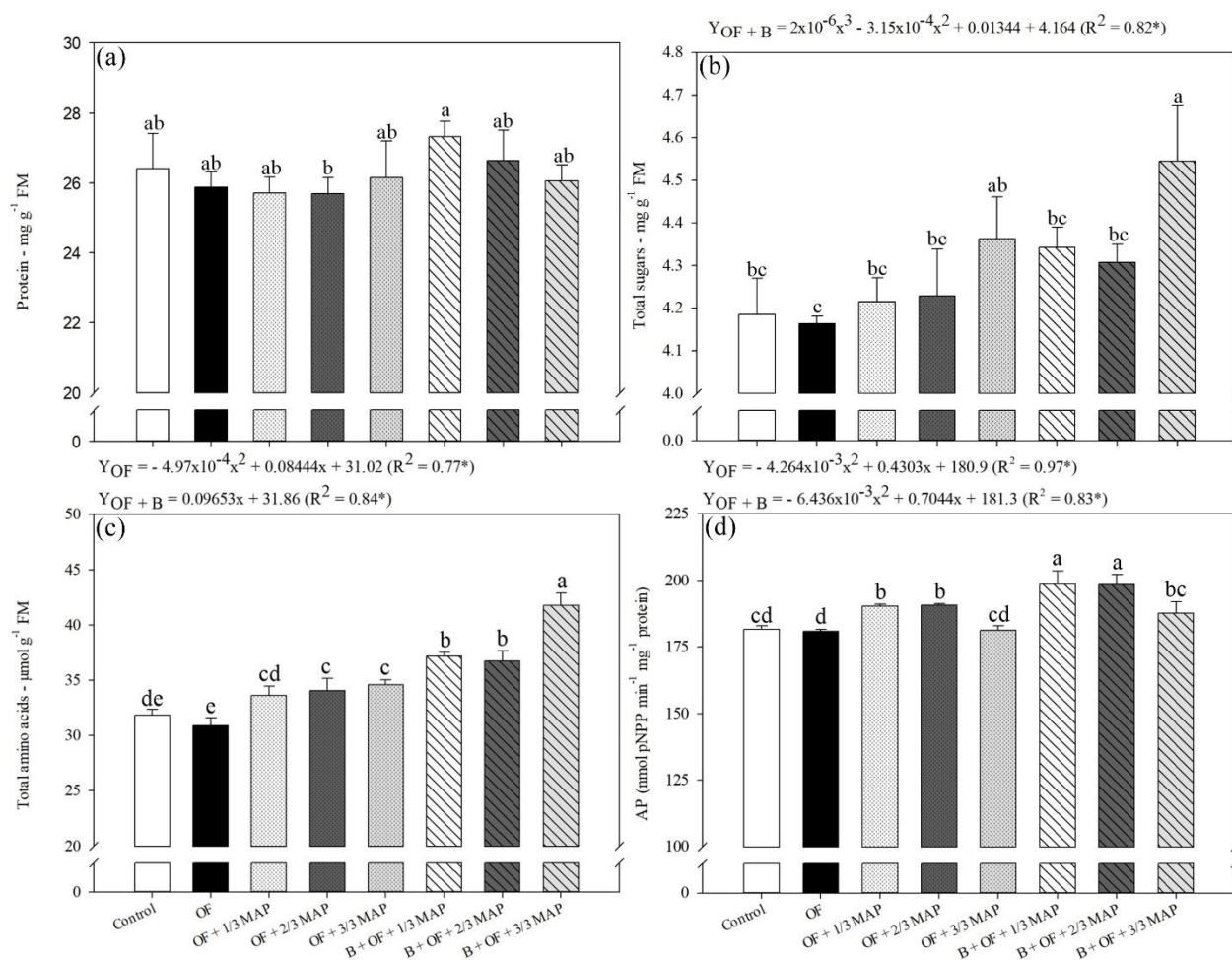


Figure 4. Protein content (a), total soluble sugars (b), total amino acids in the leaves (c), and leaf acid phosphatase (AP) activity (d) of sugarcane under the association of organomineral fertilizer (OF), *B. velezensis* (B), and doses of mono ammonium phosphate (MAP). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. The error bars express the standard deviation of the mean ($n = 4$). The regression equations and R^2 above the figures include the MAP doses associated with organomineral fertilizer (OF) and with organomineral fertilizer + *B. velezensis* (OF + B) at the 5% significance level (*).

3.4. Microbiological Variables

The highest accumulated SBR in 21 days was verified with B + OF + 1/3, 2/3, and 3/3 MAP, which provided increases of 11.9%, 45%, and 49.2%, respectively, compared to the control (Figure 5a). Both OF + MAP doses (0.93*) and B + OF + MAP doses (0.94*) provided increases in SBR up to 100% of the MAP dose, with higher values of SBR verified under the use of B + OF in relation to a single OF.

Considering the SBR per day, OF + 1/3 MAP and OF + 2/3 MAP were similar to each other and equivalent to the control. B + OF + 2/3 MAP and B + OF + 3/3 MAP were similar but showed an average increase of 47.1% in SBR compared to the control (Figure 5b). Both OF + MAP doses (0.91*) and B + OF + MAP doses (0.93*) increased the SBR up to 100% of the MAP dose, but B + OF promoted higher values of SBR per day compared to single OF.

Regarding CMB, OF + all the doses of MAP were similar to the control, while the association of *B. velezensis* and OF increased the CMB. B + OF + 1/3, 2/3, and 3/3 MAP provided increases of 35.9%, 27.1%, and 48.9%, respectively, in CMB compared to the control (Figure 5c). The optimal dose of B + OF + MAP doses for CMB was 33% of MAP (0.85*) since it provided a CMB similar to 100% of MAP, while OF + MAP doses did not adjust.

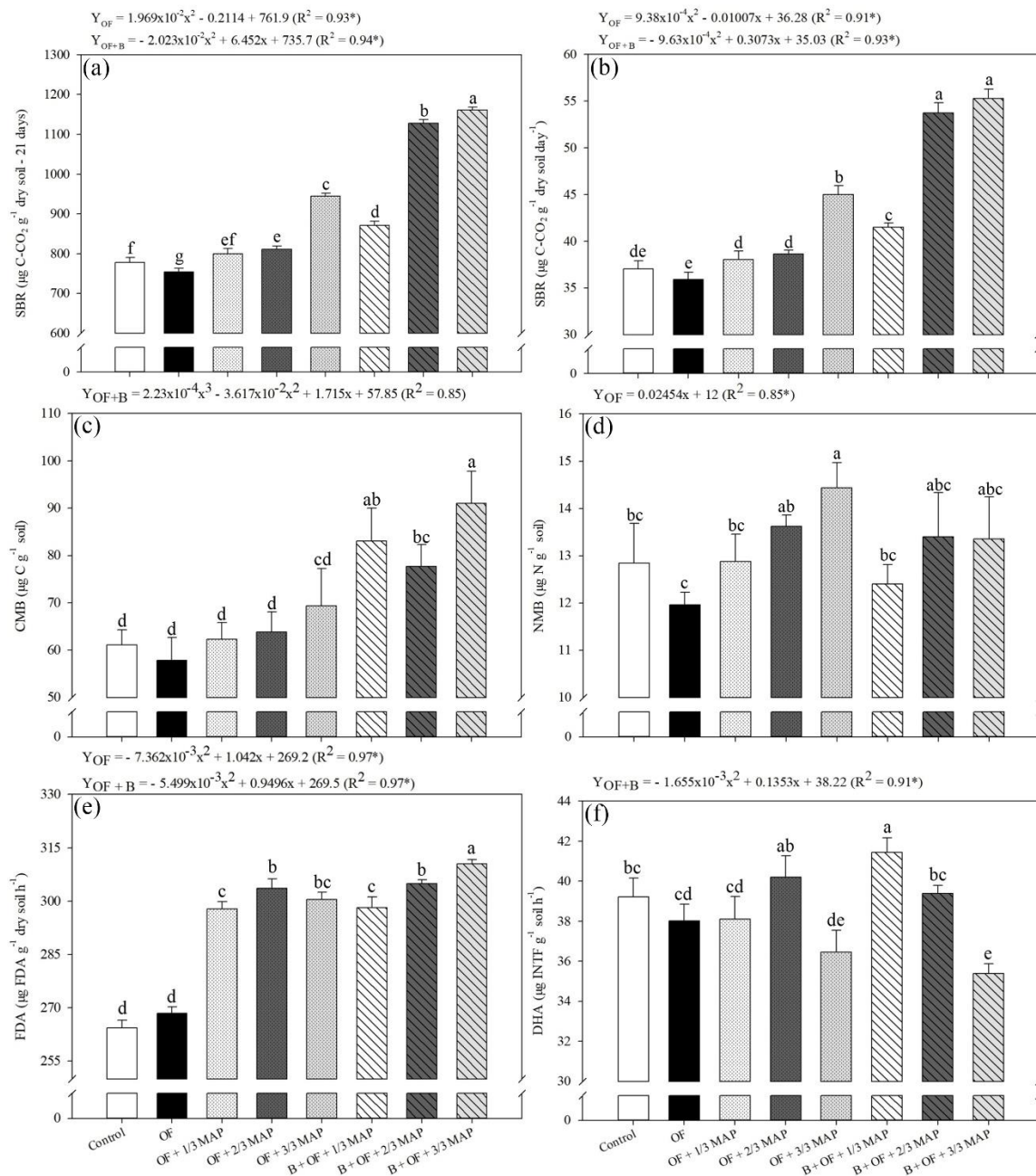


Figure 5. Soil basal respiration (SBR) accumulated in 21 days and per day (a,b); carbon (CMB) and nitrogen of the microbial biomass (NMB) (c,d); hydrolysis of fluorescein diacetate (FDA) (e); and dehydrogenase activity (f) in the soil of sugarcane cultivation under the association of organomineral fertilizer (OF), *B. velezensis* (B), and doses of mono ammonium phosphate (MAP). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. The error bars express the standard deviation of the mean (n = 4). The regression equations and R² above the figures include the MAP doses associated with organomineral fertilizer (OF) and with organomineral fertilizer + *B. velezensis* (OF + B) at the 5% significance level (*).

OF + 1/3 MAP and OF + 2/3 MAP were similar to the control for NMB, while OF + 3/3 MAP provided an increase of 12.4% in NMB compared to the control (0.85*). On the other hand, B + OF + 1/3, 2/3, or 3/3 MAP were equivalent to each other and similar to the control (Figure 5d).

Regarding FDA, OF and B + OF associated with 1/3 and 2/3 of MAP were equivalent to each other and provided average increases of 12.7% and 15.1%, respectively, compared

to the control, while B + OF + 3/3 MAP promoted an increase of 17.5% in FDA compared to the control (Figure 5e). OF + MAP doses (0.97*) provided a rise of FDA up to 66% of the MAP dose, followed by a tendency of decrease with 100% of MAP, while there was an increase in FDA with B + OF up to 100% of the MAP dose (0.97*).

Contrary to that observed for SBR, increasing P doses associated with OF or B + OF reduced the DHA activity. B + OF + 1/3 MAP provided the highest DHA activity, representing an increase of 5.7% compared to the control (Figure 5f). The optimal dose of B + OF + MAP doses for DHA was 33% of MAP (0.91*) because it provided the highest enzymatic activity, while OF + MAP doses did not adjust.

3.5. Soil Biochemical Variables

The urease activity verified with B + OF + 1/3 MAP was similar to the control. B + OF + 2/3 and 3/3 MAP provided increases of 4.2% and 5.2% in enzyme activity, respectively, compared to the control (Figure 6a). B + OF + MAP doses (0.89*) increased the urease activity as MAP doses increased, while OF + MAP doses did not adjust.

All the MAP doses associated with OF or B + OF promoted B-glucosidase activity similar to the control (Figure 6b), but there was no adjustment for B-glucosidase data with the use of OF + MAP doses or B + OF + MAP doses.

In general, OF provided increases in arylsulfatase and AP activity as MAP doses increased. At the same time, there was a tendency to decrease these enzymes' activity as MAP doses increased under B + OF (Figure 6c,d). B + OF + 1/3 MAP provided 25.5% and 10.1% increases in arylsulfatase and AP activity, respectively, compared to the control (Figure 6c,d).

OF + MAP doses (0.92*) increase the arylsulfatase activity until 66% of the MAP dose, followed by a tendency to decrease with 100% of MAP. B + OF + MAP doses (0.84*) promoted an increase in this enzymatic activity until 33% of the MAP dose, followed by decreases with higher doses of MAP (Figure 6c). OF + MAP doses increase AP activity as the MAP dose increased (0.82*), while B + OF + MAP doses (0.87*) provided increases in AP activity up to 33% of the MAP dose, followed by decreases in the enzymatic activity under the use of higher MAP doses (Figure 6d).

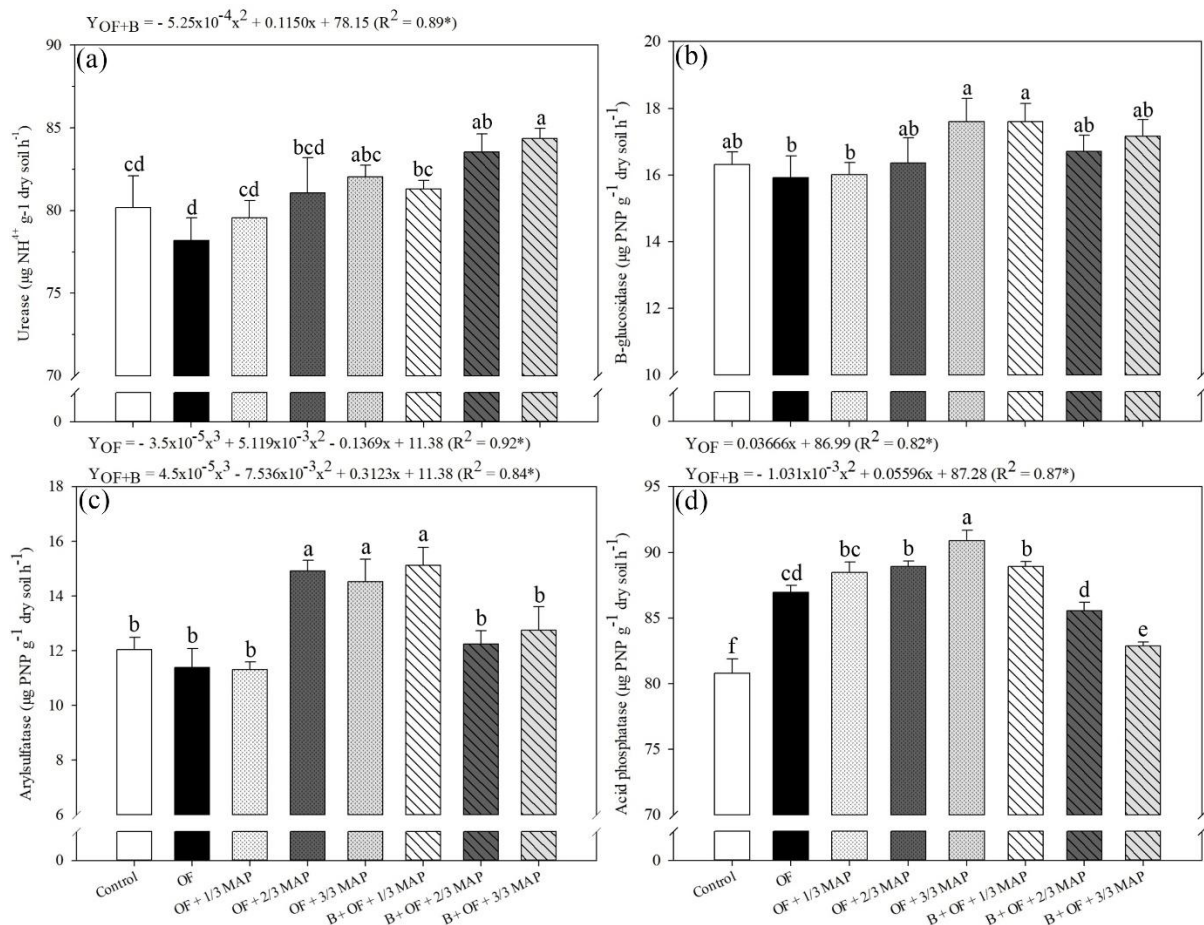


Figure 6. Urease (a), B-glycosidase (b), arylsulfatase (c), and acid phosphatase activity (d) of the sugarcane cultivation soil under the association of organomineral fertilizer (OF), *B. velezensis* (B), and doses of mono ammonium phosphate (MAP). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. The error bars express the standard deviation of the mean ($n = 4$). The regression equations and R^2 above the figures include the MAP doses associated with organomineral fertilizer (OF) and with organomineral fertilizer + *B. velezensis* (OF + B) at the 5% significance level (*).

3.6. Harvest and Yield Variables

Regardless of the MAP dose used, all of the treatments associated with B + OF were similar to the control for SH (Figure 7a), and there was no difference between the treatments for SD (Figure 7b). The OF + MAP doses and B + OF + MAP doses showed no adjustment for SH and SD.

The highest SB was verified with B + OF + 1/3 MAP, representing an increase of 3.05% compared to the control (Figure 7c). OF + MAP doses (0.81^*) increase SB up to 100% of MAP dose, while B + OF + MAP doses (0.71^*) promoted increases of SB until 100% of MAP dose, but the optimal dose was 33% of MAP, as it showed similar result to 100% of MAP.

On the other hand, the B + OF + MAP doses were similar to the control for RB, while OF + 2/3 MAP and OF + 3/3 MAP provided an average increase of 5.9% in RB compared to the control (Figure 7d). OF + MAP doses (0.94^*) increased RB up to 66% of MAP dose, followed by a tendency of decrease with 100% of MAP, while B + OF + MAP doses (0.81^*) promoted increases of RB up to 33% of MAP dose, followed by decreases with higher amounts of MAP.

As verified for SB, B + OF + 1/3 MAP also promoted the highest TB, representing a slight increase of 2.73% compared to the control (Figure 7e). OF + MAP doses (0.96^*) increased TB as the MAP doses increased, while B + OF + MAP doses (0.97^*) provided

increases in TB until 33% of MAP, followed by decreases in TB as the MAP doses increased. This decrease was caused by the low RB verified with B + OF + 2/3 and 3/3 MAP.

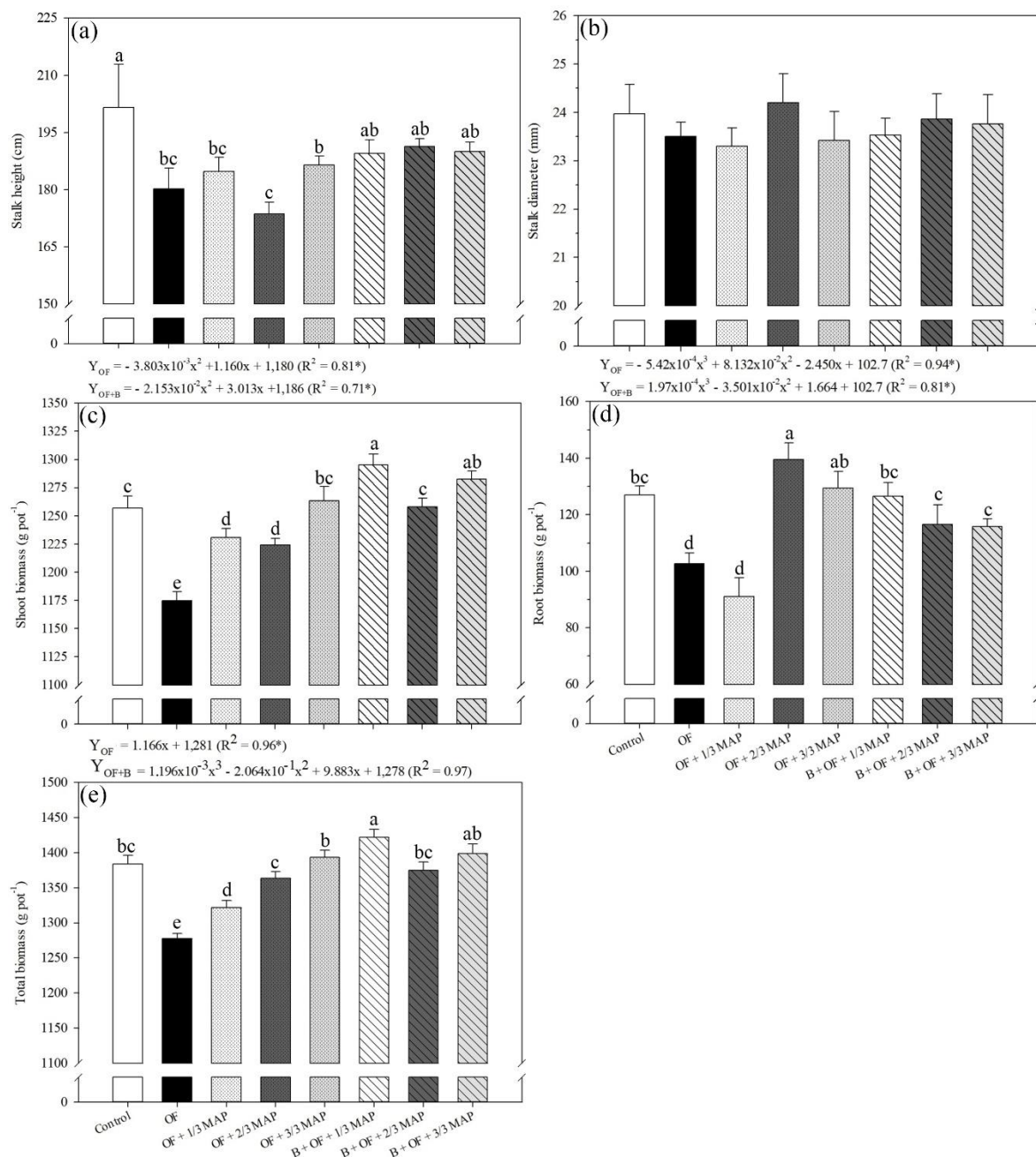


Figure 7. Stalk height (a), stalk diameter (b), shoot biomass (c), root biomass (d), and total biomass (e) of sugarcane under the association of organomineral fertilizer (OF), *B. velezensis* (B), and doses of mono ammonium phosphate (MAP). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. The error bars express the standard deviation of the mean ($n = 4$). The regression equations and R^2 above the figures include the MAP doses associated with organomineral fertilizer (OF) and with organomineral fertilizer + *B. velezensis* (OF + B) at the 5% significance level (*).

4. Discussion

One sustainable strategy for increasing P availability is using phosphate-solubilizing bacteria (PSB) [43]. PSB are microorganisms capable of solubilizing and mineralizing inorganic and organic P sources. Several soils and rhizosphere bacteria display these abilities

and thus can promote plant growth [44,45]. The combined use of PSB and organomineral fertilizer may be a successful alternative for P fertilization and plant growth since organomineral fertilizers have been used in sugarcane in eco-friendly management to achieve greater productivity and profitability [9,18].

Our findings provide insights into the soil microbial–chemical properties and growth in the first six months of development, by which *B. velezensis* associated with organomineral fertilizer improves soil and foliar biochemical properties and promote plant development. The organomineral fertilizer (OF) increased %sprouting and SSL, which was previously reported in grains [46,47]. The OF used has a high content of nutrients, especially P, that is indispensable for cell division, influencing the sprouting and initial development of the crop.

Although P is involved in protein production [6], there was no difference in the protein content between the increasing P doses; in addition, the use of OF associated or not with *Bacillus* did not promote increases in this parameter. This nutrient is also involved in sugar metabolism, and the association of *B. velezensis*, OF, and lower P doses provided similar results to the control for the total sugars, showing that the reduced applied P dose did not affect this parameter. Thus, the transformation of sugars can typically occur because under the adequate concentration of cytosolic phosphate (Pi), the triose phosphate from the chloroplast is exported to the cytosol via a Pi transporter, and sucrose is synthesized; what does not happen when the cytosolic Pi concentration is low, because triose phosphate is retained within the chloroplast and starch is synthesized [42].

The non-application of P reduced the levels of total amino acids, while inoculation with *B. velezensis* + OF increased these levels. Osmolytes, such as sugars and amino acids, help sustain the water level and regulate cellular metabolism and functionality [48]. The current research showed that the increase in amino acid concentration was significant in inoculated plants. This augmentation indicates that *B. velezensis* plays a role in plant defense against environmental stresses in addition to their roles in metabolism [49,50].

Low levels of available P are reflected in plant enzymatic activity. Plants respond to P deficiency with less efficient P usage and higher acid phosphatase (AP) activity in leaves, stalks, and roots [51]. When associated with OF or OF + B, increasing MAP doses caused reductions in leaf AP activity. Phosphatases are associated with P remobilization in plants. Therefore, the increased activity of these enzymes has been linked to low cellular levels of inorganic P [51,52]. However, our results showed that bacteria inoculation increased the activity of this enzyme compared to the control, even under higher P doses, which suggests the potential of phosphate solubilization of the UFV 3918 strain.

The OF granted nutrients to the soil, contributing to the addition of organic matter and the supply of nutrients to the plants [53]. In addition, soil organic matter can provide benefits such as improving habitat for soil microfauna and consequently enhanced microbial activity due to producing soil organic carbon during the organic matter mineralization process [54,55], which explains the increased SBR, CMB, and activity of FDA.

The inoculation of *B. velezensis* improved soil organic carbon by improving biological and enzymatic activities, with a higher increase in the higher dose of P. Soil basal respiration is determined by the bacterial population and represents the soil life [56], and soil enzyme activity is controlled by microbial population [57]. The application of agro-industrial organic wastes with *Azospirillum* has already increased microbial activity [58]. Our findings agree with previous studies that suggested the enhancement of microbial activity in soil by organic amendments [59,60].

Soil enzymes are the primary mediators of soil biological processes, playing an essential role in organic matter degradation, mineralization, nutrient recycling, and transformation, contributing to maintaining soil quality and ecosystem functionality [61]. For years FDA hydrolysis has been widely used as an indicator of overall microbial activity since lipase, protease, and esterase are involved in this reaction [62,63].

CMB is considered a key indicator of soil microbial activities, and its measurement provides a direct estimate of the relation between soil microbial activities and nutrient cycling [64–66]. Similarly, Egamberdieva et al. [67] reported increased FDA activity in

soil with applying the organic fertilizer biochar compared to the control without biochar. Furthermore, our results agree with previous studies that reported increased FDA and CMB activity under bacterial inoculation [60,68].

Phosphorus and nitrogen are nutrients that act synergistically. Urease participates in the N cycle, and the increase in its activity in the soil helps supply nutrients to plants and then promotes plant growth [69]. The highest urease activity was verified under B + OF + 2/3 and 3/3 MAP, and Duan et al. [70] had already reported an increase in this enzyme when inoculation with *B. amyloliquefaciens* was performed. In addition, NMB is considered an essential biological indicator for soil management, and the use of OF associated with increasing P doses probably improves organic residues and exudates in the soil, stimulating increases in this indicator [71].

Many bacterial groups can secrete a significant amount of β -glucosidase in soil [72], but the UFV 3918 strain of *B. velezensis* did not change this enzyme's activity. In general, β -glucosidase activity is positively correlated with CMB [73]; therefore, the use of OF and increasing P doses provided a slight increase in β -glucosidase activity, similar to that observed for CMB. Conversely, B + OF + 1/3 MAP increased arylsulfatase activity compared to the control, and the combination of OF + 2/3 or 3/3 MAP also increased this enzyme's activity. Some studies showed that applying organic substances positively affected arylsulfatase activity [74].

As observed with B + OF + 1/3 MAP, several studies have also reported an increase in DHA and AP activities with *Bacillus* inoculation [60,68]. However, there was a reduction in the activity of these two enzymes when B + OF were combined with higher doses of MAP. Mineral fertilizer, when associated with any organic compound, can be more efficient mainly by reducing P absorption in Al and Fe minerals [75]; in addition, one of the processes by which bacteria can make P available is through phosphatases, which suggests that the increase in substrate (MAP) availability led to a reduction in AP activity because there was already enough P. Interestingly, the same behavior was verified for the activity of the DHA enzyme, even though it is an exclusively intracellular enzyme, i.e., linked to viable cells and, therefore, to microbiological activity [76].

Finally, *B. velezensis* inoculation improved leaf area and shoot and total biomass production, in addition to providing stalk height and root biomass similar to the control, even under lower doses of phosphate. As already mentioned, an organic compound improves the mineral fertilizer efficiency through the reduction of P adsorption on oxides and hydroxides of Fe and Al [75]; in addition, the organic matter can help maintain its moisture near the placement of fertilizers for longer, which favors the diffusion of P from the soil [77], and increases the amount of P that reaches the roots, improving plant development.

Although the single use of OF + phosphate doses has provided a more significant number of tillers, the inoculation of *Bacillus* supplied greater stalk weight. Remarkably, the OF acted as a source of P, other macro and micronutrients, and carbon for the inoculated PSB, which may have favored the phosphate solubilization mechanisms. This is very attractive for phosphate fertilizers due to the low P use efficiency in highly weathered tropical soils, with a high capacity for P fixation [78].

Increases in leaf area facilitate sunlight absorption, favoring photosynthetic activity and, consequently, carbohydrate accumulation, which results in greater biomass, whereas increased root biomass facilitates nutrient uptake. Some studies also demonstrated that organomineral application, as well as PSB inoculation, increased sugarcane yield [18,79]. Some *Bacillus* strains are known to act as plant growth-promoting rhizobacteria either through the solubilization of minerals or the production of metabolites such as siderophores and phytohormones [80]. The improvements verified in the biomass may also have been due to metabolites and phytohormones produced by the PSB inoculated [81].

All of these demonstrate that the synergism between *B. velezensis* and organomineral allowed the enhancement of microbiological and enzymatic activities under low doses of

phosphate; thus, the consequent greater availability of nutrients probably increased soil nutrient capacity and therefore favored crop growth.

5. Conclusions

We reported how *Bacillus velezensis* associated with organomineral fertilizer and reduced P doses enhanced the soil microbial–chemical properties and sugarcane biomass. The inoculation of the strain UFV 3918 in the sugarcane buds allowed the equalization of plant height with the control (100% MAP) under low doses of MAP and provided an increase in leaf area, which may have enhanced the interception of solar radiation and contributed for successful biomass production. There were increases in amino acid content and leaf acid phosphatase activity, reflecting the regulation of cellular metabolism and the potential of phosphorus solubilization. In addition, we also observed that *B. velezensis* + organomineral fertilizer + 1/3 MAP increased soil basal respiration, carbon of the microbial biomass, FDA, DHA, arylsulfatase, and acid phosphatase activity, and all of these played nutrient recycling and then favored the plant growth, since the greater shoot and total biomass were verified in this condition, at the same time as it contributed to maintaining soil quality and ecosystem functionality. These results have important implications for the ecosystem and sustainability since P is one of the most limiting nutrients in the agricultural cropping system, especially under tropical conditions; in addition, it is a finite nutrient and irreplaceable, and sugarcane fields are dependent on high doses of P, mainly to its longevity, which evidences that this is a viable strategy to optimize the use of P in the sugarcane crop.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12112701/s1>, Table S1: Regression equations of tillers pot^{-1} , stalk height, stalk diameter, and leaf area of sugarcane under the association of organomineral fertilizer, *B. velezensis*, and doses of mono ammonium phosphate (MAP) doses along the evaluation periods (30, 60, 90, 120, 150, and 180 DAP).

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Conflicts of Interest: The authors declare that the research was conducted in collaboration with Vittia Group (São Joaquim da Barra, SP, Brazil), which provided product and technical support, and is interested in the performance of the product at a biological level.

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