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APPLICATIONS OF PLANT GROWTH PROMOTING REGULATORS AND NUTRIENTS IN THE ENZYME ACTIVITY OF SUGARCANE AND SUGAR-BIOENERGY PRODUCTION

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APPLICATIONS OF PLANT GROWTH PROMOTING REGULATORS AND NUTRIENTS IN THE ENZYME ACTIVITY OF SUGARCANE AND SUGAR-BIOENERGY PRODUCTION

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Advisor: Carlos Alexandre Costa Crusciol

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TÍTULO DA TESE: GROWTH-PROMOTING HORMONES AND NUTRIENTS APPLICATION IN SUGARCANE ENZIMATIC ACTIVITY AND SUGAR-BIOENERGY PRODUCTION

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—Carta de Issac Newton a Robert Hooke—

A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável.

—Galileu Galilei—

Uma vez eliminado o impossível, o que resta por mais improvável que seja, deve ser a verdade.

—Arthur Conan Doyle—

ABSTRACT

The production of sugarcane is of global importance due to the high technology used and the supply of sugar, ethanol and biomass for energy production. One of the biggest challenges to reach high productivity is the unfavorable edaphoclimatic conditions, significantly reducing the productivity and quality of sugarcane. The expansion of sugarcane occurred in areas where the soils are limited in nutrients, as well as the predominance of adverse climates that negatively influence the culture to explore all its genetic potential. The foliar application of nutrients and plant regulators constitute an alternative to stimulate the primary and specialized metabolism of the plant, stimulating both the photosynthetic process and the defense system in mitigating possible biotic and abiotic stresses, consequently, a greater balance of photosynthesis, thus increasing productivity of sugar cane. The primary objective of these experiments was to understand how the supply of nutrients and plant regulators via foliar spraying could stimulate photosynthesis, the activity of antioxidant enzymes, greater productivity, quality and bioenergy. In this context, fifteen experiments were carried out with foliar applications containing in (mg l^{-1}) with 17 of GA_3, 820 of IAA and 43 of zeatin (Experiments RP) and seventeen experiments with foliar applications of fertilizers whose concentration in (g ha^{-1}) was 330.0 of N, 260.0 of B, 12.0 of Cu, and 300.0 of Zn associated with plant regulators containing in (mg ha^{-1}) 25 of indolebutyric acid, 25 of gibberellic acid and 45 of kinetin (FRP experiments) conducted during four consecutive harvests (2017-2018 , 2018-2019, 2019-2020 and 2020-2021), and in three sugarcane harvest seasons [beginning (May-July), middle (July-September) and end (November-December) of the harvest]. Foliar applications of plant regulators alone or associated with fertilizers were carried out in two stages, vegetative and maturation: Chapter 1 (RV) - (I) control, without foliar application of plant regulators (Control), (II) foliar application of regulators plants in the vegetative phase of sugarcane (V), (III) foliar application of plant regulators in the vegetative and maturation phase of sugarcane (VM). Chapter 2 (FRV) - (I) control, without foliar application of fertilizers associated with plant regulators (Control), (II) foliar application with fertilizer and plant regulators in the vegetative phase of sugarcane (V), (III) foliar application with fertilizer and plant regulators in the maturation phase of sugarcane (M), (IV) foliar application with fertilizer and plant regulators in the vegetative and maturation phase of sugarcane (VM). Both experimental models with foliar applications in (V and VM) promoted positive results for the photosynthetic enzyme phosphoenolpyruvate carboxylase (PEP; EC 4.1.1.31) and only (RV) resulted in increased activity of ribulose 1,5-bisphosphate carboxylase (Rubisco: EC4.1.1.39) when applied to V, M and VM, respectively. Lower values for malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) and increased activity of the enzyme peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC:1.11.1.11) and proline concentration were observed in VM for both experimental models and superoxide dismutase (SOD; EC 1.15.1.1) in (FRV). There were increases in stalk diameter and length in (FRV) and stalk yield in both experimental models at the three harvest times in VM. The use of plant regulators

alone or in combination with fertilizers in these studies promoted increases in the activity of photosynthetic and antioxidant enzymes, productivity and bioenergy, especially when performed in two seasons, in the vegetative stages and in maturation, confirming the proposed hypothesis.

Keywords: *Saccharum spp*. Growth regulator. Sucrose metabolism. Plant protection.

RESUMO

A produção de cana-de-açúcar possui importância global devido à alta tecnologia empregada e pelo fornecimento de açúcar, etanol e de biomassa para produção de energia. Um dos maiores desafios para atingir altas produtividades são a condições edafoclimáticas desfavoráveis, reduzindo significativamente a produtividade e qualidade da cana-de-açúcar. A expansão da cana-de-açúcar ocorreu em áreas onde os solos são limitados em nutrientes, bem como predomínio de climas adversos que influenciam negativamente a cultura a explorar todo seu potencial genético. A aplicação foliar de nutrientes e reguladores vegetais perfazem uma alternativa para estimular o metabolismo primário e especializado da planta, estimulando tanto o processo fotossintético quanto o sistema de defesa na mitigação de possíveis estresses bióticos e abióticos, consequentemente, maior saldo de fotossintatos incrementando assim a produtividade da cana-de-açúcar. O objetivo primordial destes experimentos foi entender o que o fornecimento de nutrientes e reguladores vegetais via pulverização foliar poderia estimular a fotossíntese, a atividade de enzimas antioxidantes, maior produtividade, qualidade e bioenergia. Neste contexto quinze experimentos foram realizados aplicações foliares de reguladores vegetais contendo em (mg l^{-1}) 17 de GA_3, 820 de IAA e 43 de zeatina (Experimentos RP) e dezessete experimentos com aplicações foliar de fertilizantes cuja concentração em (g ha^{-1}) foi de 330.0 de N, 260.0 de B, 12.0 de Cu, e 300.0 de Zn associados a reguladores vegetais contendo em (mg ha^{-1}) 25 de ácido indolbutírico, 25 de ácido giberélico e 45 de cinetina (Experimentos FRP) conduzidos durante quatro safras consecutivas (2017- 2018, 2018-2019, 2019-2020 e 2020-2021), e em três épocas de colheita da cana-de- açúcar [início (maio-julho), meio (julho-setembro) e final (novembro-dezembro) de safra]. As aplicações foliares de reguladores vegetais isolados ou associados a fertilizantes foram realizados em dois estágios, vegetativo e de maturação: Capítulo 1 (RP) - (I) controle, sem aplicação foliar de reguladores vegetais (Controle), (II) aplicação foliar de reguladores vegetais na fase vegetativa da cana-de-açúcar (V), (III) aplicação foliar de reguladores vegetais na fase vegetativa e de maturação da cana-de-açúcar (VM). Capítulo 2 (FRP) - (I) controle, sem aplicação foliar de fertilizantes associados a reguladores vegetais (Controle), (II) aplicação foliar com fertilizante e reguladores vegetais na fase vegetativa da cana-de-açúcar (V), (III) aplicação foliar com fertilizante e reguladores vegetais na fase de maturação da cana-de-açúcar (M), (IV) aplicação foliar com fertilizante e reguladores vegetais na fase vegetativa e de maturação da cana-de-açúcar (VM). Ambos os modelos experimentais com aplicações foliares em (V e VM) promoveram resultados positivos para a enzima fotossintética fosfoenolpiruvato carboxilase (PEP; EC 4.1.1.31) e somente (RP) resultou em aumento da atividade da ribulose 1,5-bifosfato carboxilase (Rubisco: EC4.1.1.39) quando aplicadas em V, M e VM, respectivamente. Menores valores para malondialdeído (MDA) e peroxido de hidrogênio (H_2O_2) e aumento na atividade das enzimas peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), ascorbato peroxidase (APX; EC:1.11.1.11) e

concentração de prolina foram observados em VM para ambos os modelos experimentais e superoxide dismutase (SOD; EC 1.15.1.1) em (FRP). Houve incrementos de diâmetro e comprimento de colmos em (FRP) e rendimento de colmos em ambos os modelos experimentais nas três épocas de colheita em VM. A utilização dos reguladores vegetais isolados ou em associação aos fertilizantes nestes estudos promoveram incrementos na atividade das enzimas fotossintéticas e antioxidantes, de produtividade e bioenergia, principalmente quando realizados em duas épocas, nos estágios vegetativos e na maturação comprovando a hipótese proposta.

Palavras chave: *Saccharum spp*. Regulador de crescimento. Metabolismo da sacarose. Proteção de plantas.

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GENERAL INTRODUCTION

The growth of the world's population results in increased demand for food, fuel and electricity, and sugarcane can meet this demand by generating both sugar, ethanol and electricity through its products and by-products. The expansion of new areas of non-traditional sugarcane cultivation in Brazil has led crops to be exposed to adverse conditions, impacting the entire sugar and alcohol sector.

Currently, part of the areas occupied by sugarcane in Brazil are marginal areas with low soil nutrient content, high light intensity, low rainfall and altitude, being more susceptible to possible increasingly frequent and intense abiotic stresses, which which reduces productivity and in some cases, even the potential quality of sugarcane.

One of the main objectives of world agricultural production is to increase productivity, using fewer inputs and respecting environmental aspects. For this reason, a focus on stimulating the photosynthetic rate is crucial, as primary metabolism is the key to higher yields. To achieve this objective, new managements with new technologies must be adopted, with the use of plant regulators and nutrients via foliar application an alternative.

Plant regulators are artificially produced synthetic compounds with effects similar to phytohormones that are organic and naturally produced by plants, such as auxins, cytokinins, gibberellins, ethylene, abscisic acid, brassinosteroids, jasmonates and salicylates, but in modern agriculture, the exogenous application of substances with hormonal characteristics in plants are widely used and can be applied directly to plants, whether of natural or synthetic origin.

Plant regulators have essential functions from a physiological, biochemical and anatomical point of view, as growth promoters, modulate photosynthesis, stimulating the production of enzymatic and non-enzymatic antioxidants through signaling and promotion of metabolic balance. However, even at low doses, the foliar application of these substances can lead the plant to promote or inhibit physiological and morphological processes, such as stimulating growth and vegetative development in response to these changes in metabolism. Therefore, these

substances are related to biological mechanisms ranging from cell division within a tissue to stalk elongation, flowering, fruiting and maturation.

On the other side, nutrients act at various stages of the plant development process, as constituents of proteins and enzymes, as enzymatic co-factors or as part of the plant's structure. Besides, mineral nutrition plays an important role in effecting stress tolerance in plants. It induces the regulation of the osmotic potential of plant cells, providing amino acid synthesis, greater mechanical strength and cell wall structure; besides of regulating the opening and closing of stomata, enzymes activation, including those antioxidants.

Thus, it is essential to maintain a balance nutrition, keeping plants structurally integrated and physiologically active, for better tolerate biotic and abiotic adversities. Examples of some of the main nutrients acting in this context are nitrogen and magnesium, part intrinsic to chlorophylls, primordial in photosynthesis; phosphorus, providing nucleic acid energy and structure; just as potassium works in favor of cellular osmoregulation, enzyme activation and control of the opening and closing of stomata.

Likewise, nutrition attenuates the deleterious effects of stress by stimulating the defense system in relation to antioxidant enzymes, since nutrients such as nitrogen, calcium, potassium and magnesium increase the concentrations of SOD, CAT and POD, whereas zinc, copper, manganese and iron are SOD cofactors in different cell compartments.

The efficiency of nutrients and plant regulators, both in the initial and intermediate stages of the development of the sugarcane crop, is closely linked to its biostimulant action on plants, specially the exogenous application of plant regulators. Its effect on plants acts on metabolism, growth and morphogenesis, working as a chemical messenger that influences the various responses of vegetative development. Thus, the initial stages of sugarcane crop are benefited, increasing its growth rate; however, there is still a lack of information regarding the late vegetative and maturation stage, which we intend to elucidate in this research.

The objective of these studies was to evaluate the effect of foliar application of plant regulators containing GA_3, IAA and zeatin or GA_3, IAA and kinetin associated with the nutrients N, B, Cu and Zn in the vegetative and maturation stages of

sugarcane in stimulating the highest productivity and consequently the amount of biomass for energy production.

Our hypothesis is that through the exogenous use of plant regulators alone or in association with nutrients applied via the foliar route in the vegetative and maturation stages, it can stimulate the photosynthetic apparatus, promoting greater photosynthesis and, consequently, greater production of photosynthates.

The products of photosynthesis are directed both to growth in the vegetative stage and to storage in the maturation stage, in addition to stimulating greater production and activity of enzymatic and non-enzymatic antioxidants that will protect the photosynthetic apparatus against reactive oxygen species (ROS) resulting in less stress and greater stability and photosynthetic efficiency.

Chapter 1 - Foliar hormone application increases sugarcane yield and bioenergy potential by enhancing photosynthetic and antioxidant enzyme activities

Abstract

The productive potential of sugarcane has not been fully tapped, and hormone technology via foliar application could greatly benefit the production of food and renewable energy from the sugarcane production chain. In this study, we evaluated the effects of foliar hormone application at the vegetative stage (V) or vegetative and maturation stages (VM) on the photosynthetic and antioxidant enzyme activities, sucrose production, and technological and biometric parameters of early, mid-late and late harvest sugarcane. In general, foliar application increased the enzymatic, agronomic, quality and energy parameters of sugarcane. The application of hormones in V and VM increased the activities of the photosynthetic enzymes phosphoenolpyruvate carboxylase (PEPcase) and ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), decreased the contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), and increased superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and proline content. The average stalk yield over the three harvest times increased by 5.4% and 8.0% in V and VM, respectively, compared to the control. In addition, V and VM increased the sucrose concentration, theoretical recoverable sugars (TRS), and sugar productivity by averages of 2.9%, 2.6% and 9.3%, respectively, across all harvest seasons. The best results for straw, bagasse and energy production were observed in VM, with average increases of 8.0%, 7.7% and 8.0% compared with the control. Thus, phytohormone application can increase sugarcane metabolism, growth and development. Although single phytohormone application in the vegetative stage improved all sugarcane parameters, the double application of phytohormones in the vegetative and maturation stages ensured improvements in productivity and product quality.

Keywords: *Saccharum* spp.; foliar application; plant hormone; nutrient foliar, sugarcane production.

1 INTRODUCTION

The growing population will inevitably increase the global demand for food and renewable energy [1], which is driving the search for improvements in productivity per unit area and the quality of crops, especially sugarcane (*Saccharum officinarum* L.). New technologies, such as the use of phytohormones, could increase crop productivity by improving agricultural potential and mitigating abiotic stresses that restrict the genetic potential of crops and threaten global food security [2].

In the central-south region of Brazil, sugarcane is harvested from March to November. A combination of varieties with different genetic characteristics are used to ensure a continuous supply of raw material for the industry throughout the harvest period. Thus, sugarcane varieties can be classified as early, middle-late or late harvest. This study evaluates the nutritional, physiological and climatic demands of sugarcane in each harvest period. The plant's nutritional and physiological characteristics at the time of harvest have important implications for producing sugarcane of industrial quality.

Plant primary and specialized metabolism are coordinated by signaling compounds called phytohormones, which are produced at specific locations and act as chemical signals to neighboring or distal cells via the xylem and phloem to induce the synthesis of specialized proteins that stimulate photosynthesis under appropriate environmental conditions [3,4]. Plants under abiotic stress coordinate phytohormone production with biochemical, physiological, and metabolic adjustments, including the production of specific enzymatic and non-enzymatic antioxidants for plant protection, to increase productivity and plant quality [5–7]. Phytohormones influence photosynthesis directly and indirectly [8]. In photoautotrophic organisms, the interaction of light and phytohormones directly regulates chloroplast development by directly influencing pigment accumulation, organelle size and division, the

organization of the thylakoid membrane and the number of copies of the chloroplast genome [9].

Phytohormone such as auxins (AX), gibberellins (GAs) and cytokinins (CKs) modulate the photosynthetic rate under ideal conditions, whereas hormones such as abscisic acid (ABA), jasmonates (Jas), salicylic acid (SA) and ethylene regulate photosynthesis under non-ideal conditions, that is, under stress [8]. Under stress conditions, the CO_2 assimilation rate drops considerably due to stomatal, mesophilic and biochemical limitations imposed by the reduction in electron transport in the photosynthetic apparatus, photoinhibition and photooxidative stress caused by excess incident light associated with abiotic stresses [10,11]. Plant physiology research has focused on the modulation of photosynthesis by redox and hormonal signaling, particularly the action of phytohormones in the regulation of the production and elimination of reactive oxygen species (ROS) derived from photosynthesis and in photoprotection [12,13]. Exogenous phytohormones have been used to stimulate plant growth and development and to mitigate the deleterious effects of abiotic and biotic stresses [14]. The main growth-promoting phytohormones are AXs, CKs and GAs, which have promising effects on yields when applied to the leaves of sugarcane [15]. However, gaps remain in the understanding of the effects of these phytohormones in the last growth stages of sugarcane. We conjecture that applying AXs, CKs and GAs at the end of the vegetative and maturation stages could increase sugarcane productivity and quality by increasing the photosynthetic rate and stimulating the synthesis of sugars, given the effects of these phytohormones on the photosynthetic apparatus and the production of antioxidant enzymes.

Endogenous AXs play important roles in cellular metabolism, including indole-3-butyric acid (IBA), phenoxyacetic acid (PAA) and indole-3-acetic acid (IAA), which is chemically similar to tryptophan. These AXs stimulate growth and development through changes in plant gene expression patterns [16]. High AX concentrations are present in organs with active cell division, that is, the apical meristems of root cells, where it is assumed that a large number of genes are involved in IAA biosynthesis [17]. AXs have positive effects on the control of stomatal opening and closing, as high atmospheric CO_2 concentrations stimulate the photosynthetic rate and, consequently, the production of photosynthates [18–20].

CKs act on several plant tissues simultaneously and thus regulate many plant developmental processes, including growth, leaf senescence and acclimatization to environmental stresses [21]. CKs directly participate in chloroplast development and function and in the biosynthesis of chlorophyll and carotenoids. Carotenoids protect plastids against photooxidative damage [22–24]. Carotenoids are also important for PSII chlorophyll a/b binding proteins and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity [25–27] and thus have direct and indirect effects on photosynthesis. According to Yang et al., (2018), the exogenous application of 6-benzylaminopurine (BA) increases the electron transport rate (ETR), reduces the relative variable fluorescence intensity and increases the quantum yield, leading to a higher photosynthetic rate. In addition, endogenous zeatin (Zt) stimulates the activity of antioxidant enzymes and reduces malondialdehyde (MDA) content.

GAs stimulate the growth of most organs through cell elongation and help stimulate cell division [29]. Environmental signaling by light actively participates in the formation of functional chloroplasts and their survival. In the dark, chlorophyll precursors can have harmful effects when illuminated; thus, plants produce plastids as a form of adaptive mechanism for developmental control in the transition to light. In addition to stimulating photosynthesis, exogenous GA_3 alters differential gene expression in sugarcane, enabling greater tolerance to drought [30].

The aim of this study was to evaluate the effects of the foliar application of exogenous hormones on sugarcane, including photosynthesis, sugar and stalk yields, and the regulation of antioxidant enzymes. The central hypothesis of this study is that applying growth-promoting phytohormones such as AXs, CKs and GAs to sugarcane will increase the photosynthetic rate by stimulating the enzymes phosphoenolpyruvate carboxylase (PEPcase; EC:4.1.1.31) and Rubisco and antioxidant enzymes involved in ROS scavenging, leading to improvements in sugarcane yield, raw material quality, biometric parameters, and biomass production.

2 MATERIALS AND METHODS

2.1 Site description

The study was carried out in 15 commercial sugarcane areas in Brazil in 2017, 2019 and 2020 in the early (May-June), mid-late (July-August) and late (October-

November) harvest seasons. The field experiments were located in Pontal-SP (1), Santa Maria da Serra-SP (2), Luís Antônio-SP (3), Paulo Faria-SP (4), Mirassol-SP (5), Olímpia-SP (6), Uberaba- MG (7), Agissê-SP (8), Cruzeiro do Oeste-PR (9), Sertãozinho-SP (10 and 13), Igaraçu do Tietê-SP (11 and 14) and Pradópolis-SP (12 and 15). The details of the harvest times, geographic coordinates, cultivars, crop management, row spacing, and applications are shown in Table 1. All cultivars were managed according to recommendations for the specific environmental conditions [31,32].

The predominant climates in the experiments were Aw, with hot, rainy summers; Cfa, temperate without a dry season and with hot summers; and Cwa, temperate with hot, humid summers according to the Köppen-Geiger climate classification. The average temperatures in the early, mid-late and late seasons were 22.4°C, 21.6°C and 21.7°C, respectively. Precipitation data were obtained from meteorological stations located near each site (Table 2).

Soil classification was performed using the international system (SOIL SURVEY STAFF, 2014). The soil characteristics (0.00-0.25 and 0.25-0.50 m depths) were determined prior to the installation of the experiments according to van Raij et al. (1997). The soil data are shown in Table 3.

Table 1. Sugarcane cultivar, ratoon, line spacing and date of establishment and first and second applications and harvest in each season.

Harvest period	Sites	Latitude	Longitude	Cultivar	Ratoon	Row spacing	1st Application*	2nd Application	Harvest
Early	1	21°02'06.0"S	48°05'18.6"W	RB 85 5453	3	0.9 × 1.60	Feb, 2017	Mar, 2017	Jun, 2017
	2	22°34'18.8"S	48°13'28.6"W	RB96 6928	1	0.9 × 1.50	Feb, 2017	Apr, 2017	May, 2017
	3	21°33'03.2"S	47°49'48.4"W	RB 85 5156	2	1.5	Feb, 2017	Mar, 2017	May, 2017
	4	20°03'29.0"S	49°21'39.0"W	IAC91 1099	1	1.5	Jan, 2019	Mar, 2019	Apr, 2019
	5	20°55'48.8"S	49°31'38.6"W	CTC4	2	1.5	Jan, 2019	Mar, 2019	Apr, 2019
	6	20°40'00.2"S	49°05’23.1"W	RB85 5476	2	1.5	Jan, 2019	Mar, 2019	Apr, 2019
	7	19°45'37.1"S	47°47'13.5"W	CTC4	1	1.5	Jan, 2019	Mar, 2019	Apr, 2019
	8	22°31'34.7"S	50°59'19.4"W	RB86-7515	2	0.9 × 1.50	Jan, 2020	Apr, 2020	May, 2020
	9	23°49'36.1"S	53°20'53.5"W	RB86-7515	1	1.5	Jan, 2020	Apr, 2020	May, 2020
Mid-late	10	21°08’43.9”S	48°04’23.8"W	CTC4	3	0.9 × 1.60	Jun, 2017	Aug, 2017	Ago, 2017
	11	22°30’06.9”S	48°28’23.7”W	SP80 3280	2	0.9 × 1.50	Jul, 2017	Aug, 2017	Sep, 2017
	12	21°26’20.1”S	48°02’06.8”W	CTC4	3	1.5	Jun, 2017	Jul, 2017	Aug, 2017
Late	13	21°05’57.2”S	48°05’18.5”W	SP80-3280	2	1.5	Sep, 2017	Oct, 2017	Nov, 2017
	14	22°33’44.1”S	48°33’11.6”W	SP80-3280	1	0.9 × 1.50	Oct, 2017	Nov, 2017	Dez, 2017
	15	21°21’59.0”S	47°59’25.5”W	SP80-3280	2	1.5	Sep, 2017	Oct, 2017	Nov, 2017

*First and second applications were performed at vegetative and maturation stages of sugarcane.

Table 2. Climate classification and precipitation between planting or last ratoon, 1st to 2nd application, and 2nd application to harvest of experiments at each site (early, mid-late, and late sugarcane seasons).

Harvest period	Sites	Climate classification	Precipitation		
			From planting/last cutting to 1st application	From 1st to 2nd application	From 2nd application to harvest
Early	1	Aw	1201	208	62
	2	Cfa	1235	161	192
	3	Cwa	1148	205	81
	4	Aw	990	614	252
	5	Aw	516	199	103
	6	Aw	580	301	164
	7	Aw	1379	260	0
	8	Cfa	760	246	27
	9	Cfa	944	180	20
Mid-late	10	Aw	994	0	22
	11	Cfa	1234	0	68
	12	Aw	630	11	8
Late	13	Aw	822	79	94
	14	Cfa	1342	228	167
	15	Aw	658	52	133

Table 3. Soil classification and chemical characteristics of experiments at each site.

Harvest Period	Site	Soil classification	Depth	pH	SOM*	$P_{(resin)}$	S	Al^{+3}	$H+Al^{+3}$	K	Ca	Mg	BS	CEC	V%
				$CaCl_2$	g dm^{-3}	mg dm^{-3}			---------- $mmol_c$ dm^{-3} ----------						%
Early	1	Rhodic Hapludox	0.00-0.25	5.8	32	22	78	0.0	19	14.0	53	18	71	90	79
			0.25-0.50	5.8	19	4	100	0.0	19	7.9	22	10	32	51	63
	2	Typic Quartzipsamment	0.00-0.25	5.5	22	180	18	0.0	14	5.6	59	12	71	85	84
			0.25-0.50	5.0	12	7	30	1.0	19	2.3	28	6	34	53	64
	3	Typic Quartzipsamment	0.00-0.25	5.6	21	31	8	0.0	12	1.2	31	15	46	58	79
			0.25-0.50	5.7	9	9	5	0.0	12	0.5	13	6	19	31	61
	4	Rhodic Hapludox	0.00-0.25	5.2	22	10	9	0.0	12	0.2	32	21	53	65	82
			0.25-0.50	5.0	12	6	10	0.0	13	0.1	9	7	16	29	55
	5	Typic Eutrudult	0.00-0.25	5.5	32	17	5	3.0	17	0.5	12	7	19	36	53
			0.25-0.50	5.1	25	10	5	6.0	10	0.3	6	2	8	18	44
	6	Rhodic Eutrudox	0.00-0.25	6.6	30	26	19	1.1	14	2.3	35	7	42	56	75
			0.25-0.50	5.4	22	6	46	1.2	17	2.9	10	4	14	31	45
	7	Rhodic Eutrudult	0.00-0.25	5.7	24	18	1	0.1	17	1.2	31	9	40	57	70
			0.25-0.50	5.6	19	12	0	1.1	18	1.1	27	9	36	54	67
	8	Typic Hapludox	0.00-0.25	6.4	16	18	15	0.4	12	0.5	36	12	48	60	80
			0.25-0.50	5.7	10	7	16	0.3	13	0.4	22	7	29	42	69
	9	Typic Hapludox	0.00-0.25	5.7	8	6	0	0.9	14	1.1	8	6	14	28	50
			0.25-0.50	5.0	8	2	9	2.2	17	1.6	6	4	10	27	37
Mid-late	10	Rhodic Eutrudox	0.00-0.25	5.6	58	140	0	0.0	44	12.0	82	22	104	148	70
			0.25-0.50	5.5	42	40	10	0.0	37	8.0	53	17	70	107	65
	11	Rhodic Eutrudox	0.00-0.25	5.3	19	10	0	0.2	21	1.1	39	18	57	78	73
			0.25-0.50	5.2	20	9	0	0.3	23	1.0	41	19	60	83	72
	12	Rhodic Eutrudox	0.00-0.25	5.8	55	54	8	0.0	27	10.1	37	22	59	86	69
			0.25-0.50	5.5	42	45	2	0.0	21	7.2	23	12	35	56	63
Late	13	Rhodic Eutrudox	0.00-0.25	5.6	2	80	0	0.0	7	2.2	33	9	42	49	86
			0.25-0.50	5.6	1	20	65	0.0	14	2.1	21	7	28	42	67
	14	Rhodic Eutrudox	0.00-0.25	4.5	10	22	0	1.0	25	3.9	14	6	20	45	44
			0.25-0.50	4.6	7	10	0	2.0	20	3.5	10	5	15	35	43
	15	Rhodic Eutrudox	0.00-0.25	6.2	49	38	34	0.0	32	9.2	33	25	58	90	64
			0.25-0.50	6.0	39	35	22	0.0	29	9.2	18	16	34	63	54

* SOM, Soil Organic Matter; BS, Base Saturation; CEC, cation exchange capacity.

2.2 Experimental design and treatment applications

The plots consisted of eight rows with a length of 10 m; the inter-row spacing at each site is provided in Table 1. The experimental design consisted of three treatments of foliar hormone application in completely randomized blocks with 8 replications in the early, mid-late, and late harvest seasons. The treatments were as follows: (i) control with no application of hormones (control), (ii) foliar application of hormones at the vegetative stage of sugarcane (V), and (iii) foliar application of hormones at the vegetative and maturation stages of sugarcane (VM). Hormone application at the vegetative stage was performed 100, 75 and 60 days before harvest (DBH) in the early, mid-late and late harvest seasons, respectively; hormone application at the maturation stage was applied 30 DBH in all sugarcane harvest seasons (Figure 1).

Foliar hormone application was performed in each plot by spraying with pressurized backpack equipment (CO_2) coupled to a 2.6-m-long rod with a single tip, brass jet type 1/4KLC-9 with an average flow of 100 L ha^{-1} and a pressure of 4 kgf cm^2 or 58.0 PSI. The hormone doses were (mg l^{-1}): 17 of GA_3 activity, 820 of IAA activity and 43 of zeatin.

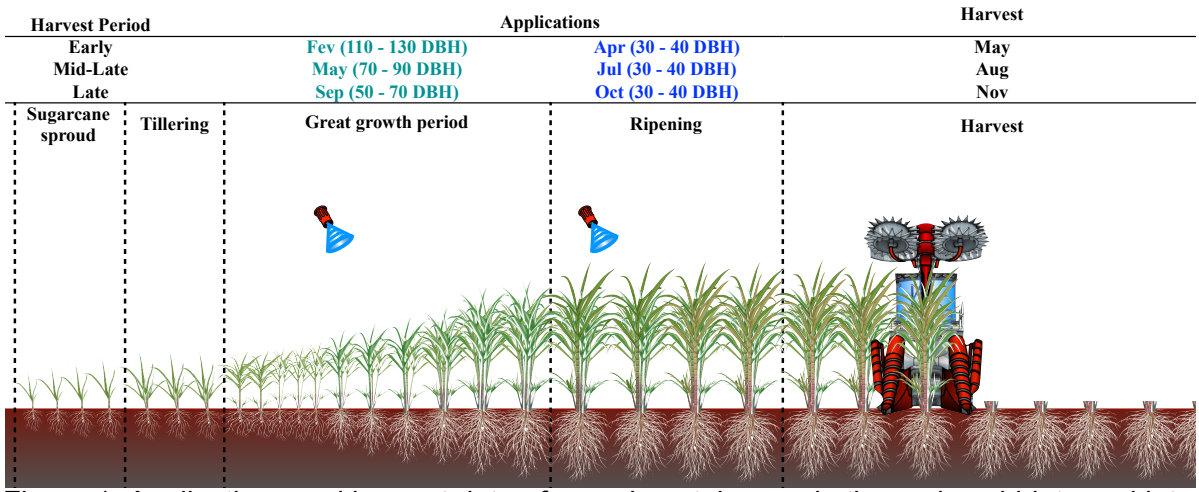


Figure 1. Applications and harvest date of experimental areas in the early, mid-late and late harvest seasons.

2.3 Photosynthetic and antioxidant enzymes

Fully expanded (+1) or top visible dewlap (TVD) leaves were collected from early-harvest canes, stored in 50-mL Falcon tubes and frozen in liquid nitrogen immediately after collection for the analysis of photosynthetic enzymes and antioxidants. The leaves were collected between 8:00 and 10:00 a.m. in all plots of site 5 (Mirassol-SP). After collection, the samples were taken to the laboratory and stored in a freezer at -80°C. Enzymatic analyses were performed only at site 5 due to the high cost, distance and difficulty of storing samples.

The procedure for the analysis of photosynthetic and antioxidant enzymes was performed by extraction of leaf. The leaves were crushed in a mortar with liquid N to obtain a fine powder, and 1 part crude extract was homogenized with 2 parts extraction buffer on ice for 20 s in an Ultra-Stirred (BIOMT: 0.5 to 250 mL, 5 mm stainless steel rod; 50-60 HZ).

The activity of PEPcase (EC:4.1.1.31) was determined by monitoring the oxidation of NADH in a spectrophotometer at 340 nm for 120 s and expressed in μmol CO_2 min^{-1} mg^{-1} protein using a molar absorptivity of 6.22 $M^{-1}cm^{-1}$ [34].

Rubisco (EC4.1.1.39) activity was determined by grinding leaves in liquid N and extraction in ice-cold Eppendorf tubes containing 0.2 M KPi (pH 7.8), 5 mM $MgCl_2$, 1 mM DTT, 1% (w/v) polyvinylpyrrolidone PM 40,000 (PVP-40), and 5 mM acid ascorbic acid (Sigma-Aldrich). The tubes were centrifuged at 14,000 g and 4°C for 30 min, and the supernatant was immediately used to measure Rubisco activity at 25°C (ASHTON et al. 1990). Rubisco was activated by incubation (35 μL of crude extract) with 450 μL of buffer containing 100 mM Bicine (pH 8.0), 25 mM KHO_3, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25 mM NADH, 80 nkat G-3-P dehydrogenase (EC1.2.1.12), 80 nkat 3-phosphoglycerate phosphokinase (EC 2.7.2.3), 80 nkat creatine phosphokinase (EC 2.7.3.2) and 0.25 mM NADH for 15 min. The oxidation of NADH was initiated by the addition of 0.5 mM RuBP (Sigma-Aldrich). The difference in absorbance at 0 and 3 min was used to calculate the activity expressed in μmol CO_2 min^{-1} mg^{-1} protein using a molar absorptivity of NADH of 6.22 $M^{-1}cm^{-1}$.

Superoxide dismutase (SOD; EC:1.15.1.1) and catalase (CAT; EC:1.11.1.6) activity were extracted according to the methodology proposed by Silva et al. (2020). Extraction was performed in buffer [200 mM KPi (pH 7.8) containing 10 mM EDTA, 20 mM ascorbic acid, 1% PVP-40 (Sigma-Aldrich) and 1 mM 1,4-dithiothreitol (DTT,

Sigma-Aldrich)]. Crude extracts for SOD and CAT activity were obtained from the supernatant after centrifugation at 12,000 g for 30 min at 4°C. The units of the photochemical activity of SOD were expressed in mg of protein [37] and obtained in an assay system consisting of 13 mM methionine (Sigma-Aldrich), 100 nM EDTA, 2 µM riboflavin (Sigma-Aldrich) and 75 µM NBT (Sigma-Aldrich) in 50 mM KPi buffer. CAT activity was assayed according to Havir and Mchale (1987) by monitoring the consumption of 250 µM hydrogen peroxide and expressed in µmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 39.4 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX; EC:1.11.1.11) activity was measured by extraction in 10 mM EDTA, 1% PVP-40, 1 mM DTT, and 200 mM KPi (pH 6.0). The extract obtained after homogenization was centrifuged for 30 min at 12,000 g and 4°C. The reaction was initiated by adding 1 mM hydrogen peroxide and 80 µM ascorbic acid to the crude extract. The decrease in absorbance at 290 nm was monitored for 120 s, and the results were expressed in µmol min^{-1} mg^{-1} protein [39,40].

Free peroxidase activity (POD; EC:1.11.1.7) was analyzed in buffered solution containing 10 mM EDTA, 1% PVP-40, and 200 mM KPi (pH 6.7) using an extract collected by centrifugation at 8,000 g for 20 min at 4°C [41].

MDA levels were determined by reacting leaf tissue extracts with thiobarbituric acid (TBA). The resulting TBA-MDA adduct was measured spectrophotometrically at 532 nm. The MDA concentration was calculated from a standard curve of 1,1,3,3-tetramethoxypropane (TPE) and expressed in nanomoles of MDA per g of fresh weight [42]. The concentration of hydrogen peroxide (H_2O_2) was determined according to the method of Alexieva et al. (2001) using a standard curve prepared with known concentrations of H_2O_2. Proline content was measured based on the methodology proposed by Bates et al. (1973).

2.4 Sugarcane measurements

The biometric parameters consisted of plant height, which was measured from the base of the stalk to the base of the TVD (+3) [44]; stalk diameter, which was measured at the third internode; and stalk yield (StY), which was determined by

harvesting the sugarcane plants in 4 m of the central two rows of each plot and extrapolating to tons of sugarcane per hectare.

Sucrose, juice purity (PUR), fiber (FIB), reducing sugars (RS) and theoretical recoverable sugars (TRS) were determined by collecting 10 sugarcane plants (Supplementary Material). Laboratory analyses were performed according to the methodology of Fernandes [45]. Sugar per hectare, i.e., sugar yield (SY), was calculated by multiplying StY by TRS and dividing by 1000.

Energy production was analyzed using the FIB and StY results at 50% humidity to determine bagasse productivity. Straw yield was calculated by multiplying StY by 140, and energy production was calculated assuming that 1 Mg of straw produces 4.96 MWh of primary energy (WALDHEIM; MONIS; VERDE LEAL, 2001)

2.5 Data analysis

The homogeneity of variances and data normality were evaluated with the F-Snedecor and Shapiro–Wilk tests, respectively. The values were then submitted to analysis of variance (ANOVA) to compare means between the treatments in each sugarcane harvest (early, mid-late and late) by the LSD test ($p < 0.10$) in SISVAR [46].

3 Results

3.1 Photosynthetic enzymes activity, oxidative stress and antioxidant enzymes activities

In early-harvest sugarcane, foliar phytohormone application increased PEPcase activity in VM (56.1 μmol CO_2 min^{-1} mg^{-1} prot) and V (52.3 μmol CO_2 min^{-1} mg^{-1} prot) compared with the control (34.3 μmol CO_2 min^{-1} mg^{-1} prot) (Figure 2A). Sugarcane Rubisco activity in V and VM was 0.68 and 0.77 μmol CO_2 min^{-1} mg^{-1} prot higher than in the control (1.55 μmol CO_2 min^{-1} mg^{-1} prot) (Figure 2B).

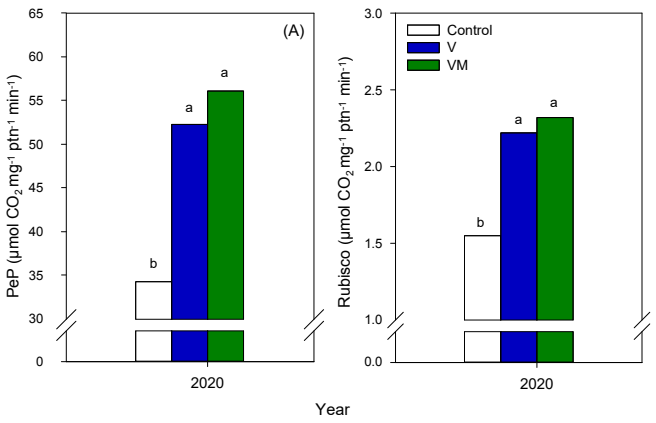


Figure 2. Activities of phosphoenolpyruvate carboxylase (PEPcase) (A) and ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) (B) in early harvest sugarcane in 2020 as a function of foliar hormone application. The treatments were as follows: control, control with no foliar application of hormone (white); V, foliar application of hormones at the vegetative stage (blue); VM, foliar application of hormone at the vegetative and maturation stages (green). Means followed by the same letter do not differ by the t test (LSD) at 10% probability.

In general, foliar hormone application decreased MDA and H_2O_2 contents (Figures 3A and 3B). H_2O_2 content decreased by 36.7% in VM compared with the control (3.85 µmol CO_2 min^{-1} mg^{-1} prot). MDA content decreased by 14.2% and 36.2% in V and VM, respectively, compared with the control.

Foliar hormone application did not affect SOD activity but did increase POD, CAT, and APX activities and proline content (Figures 3C-G). POD activity increased by 3.23-fold in VM compared with the control (0.13 µmol CO_2 min^{-1} mg^{-1} prot) (Figure 3D). CAT activity was 9.87% and 23.8% higher in V and VM than in the control (Figure 3E). APX activity increased by 18.4% and 23.5% in V and VM, reaching 0.64 µmol CO_2 min^{-1} mg^{-1} prot (Figure 3F). Finally, proline content increased by 12.5% and 16.9% in V and VM, respectively, compared with the control (0.31 µmol g^{-1} FW) (Figure 3G).

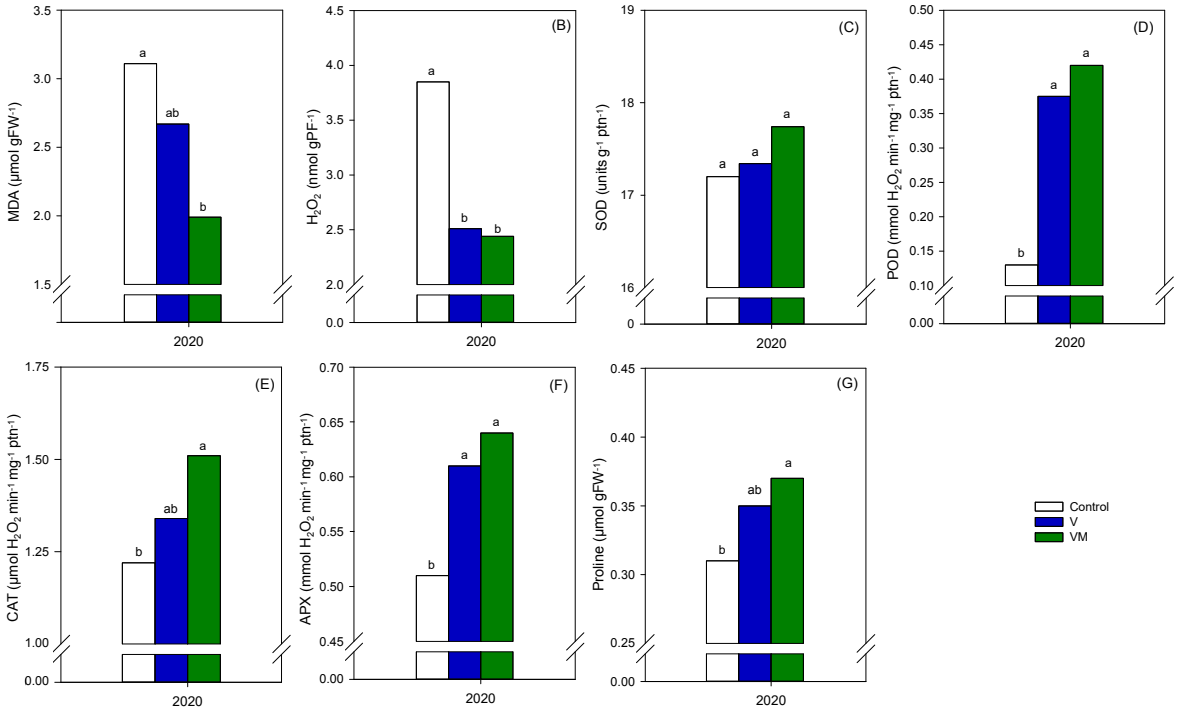


Figure 3. Malondialdehyde (MDA) content (A), hydrogen peroxide (H_2O_2) content (B), superoxide dismutase (SOD) activity (C), peroxidase (POD) activity (D), catalase activity (CAT) (E), ascorbate peroxidase (APX) activity (F) and proline content (G) in early harvest sugarcane in 2020 as a function of foliar hormone application. The treatments were as follows: control, control with no foliar application of hormone (white); V, foliar application of hormones at the vegetative stage (blue); VM, foliar application of hormone at the vegetative and maturation stages (green). Means followed by the same letter do not differ by the t test (LSD) at 10% probability.

3.2 Agronomic Parameters

Foliar hormone application did not affect plant height or stalk diameter (Figure 4A-F) but significantly ($p < 0.10$) increased sugarcane StY in all harvest seasons (Figure 4G, 4H and 4I). In early, mid-late, and late harvest sugarcane, the average StY was 127, 101, and 91 Mg ha^{-1} in V, 130, 104, and 93 Mg ha^{-1} in VM, and 118, 97, 87 Mg ha^{-1} in the control (Figures 4G, 4H and 4I). These values correspond to average increases of 7.1%, 4.8% and 4.4% in V and 9.7%, 7.7% and 6.7% in VM.

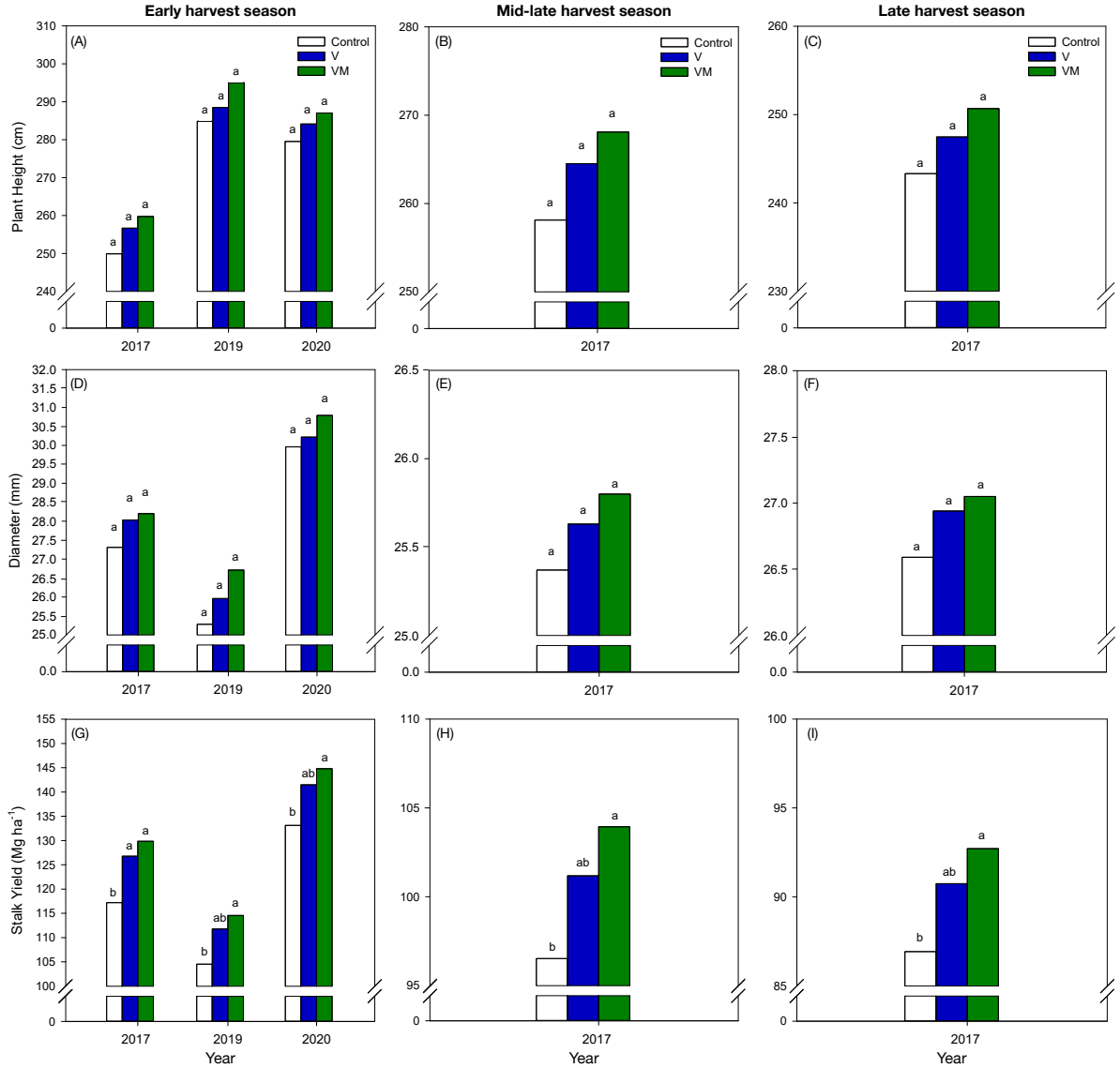


Figure 4. Plant height (A, B and C), stalk diameter (D, E and F) and stalk yield (G, H and I) of early, mid-late and late harvest sugarcane as a function of foliar hormone application. The treatments were as follows: control, control with no foliar application of hormone (white); V, foliar application of hormones at the vegetative stage (blue); VM, foliar application of hormone at the vegetative and maturation stages (green). Means followed by the same letter do not differ by the t test (LSD) at 10% probability.

3.3 Quality parameters

In general, foliar hormone application increased sucrose content and TRS in early-harvest sugarcane and sugar production in all harvest seasons ($p < 0.10$) (Figure 5 A). The highest sucrose contents were 10.9% in VM in 2019 and 12.4% in both V and VM in 2020. The largest increases in TRS occurred in early-harvest sugarcane and were 6.1% (111 kg of sugar ha^{-1}) in VM in 2019 and 5.7 and 5.8% (124 kg of sugar ha^{-1}) in V and VM, respectively, in 2020; TRS was lowest in the

control in 2019 and 2020, with values of 104 and 117 kg of sugar ha^{-1}, respectively (Figure 5D). Foliar application also increased sugar yield, which averaged 15.2 Mg ha^{-1} in V and VM (Figures 5G, 5H and 5I). Compared with the control (14.4, 15.4 and 11.9 Mg ha^{-1}), sugar yield increased by 11.0%, 7.1% and 5.1% in V and 14.6%, 10.3% and 7.7% in VM in early, mid-late and late harvest sugarcane, respectively. In early and mid-late harvest sugarcane, sugar yield was similar in V and VM, whereas in late harvest sugarcane, sugar yield was higher in VM than in V (2017).

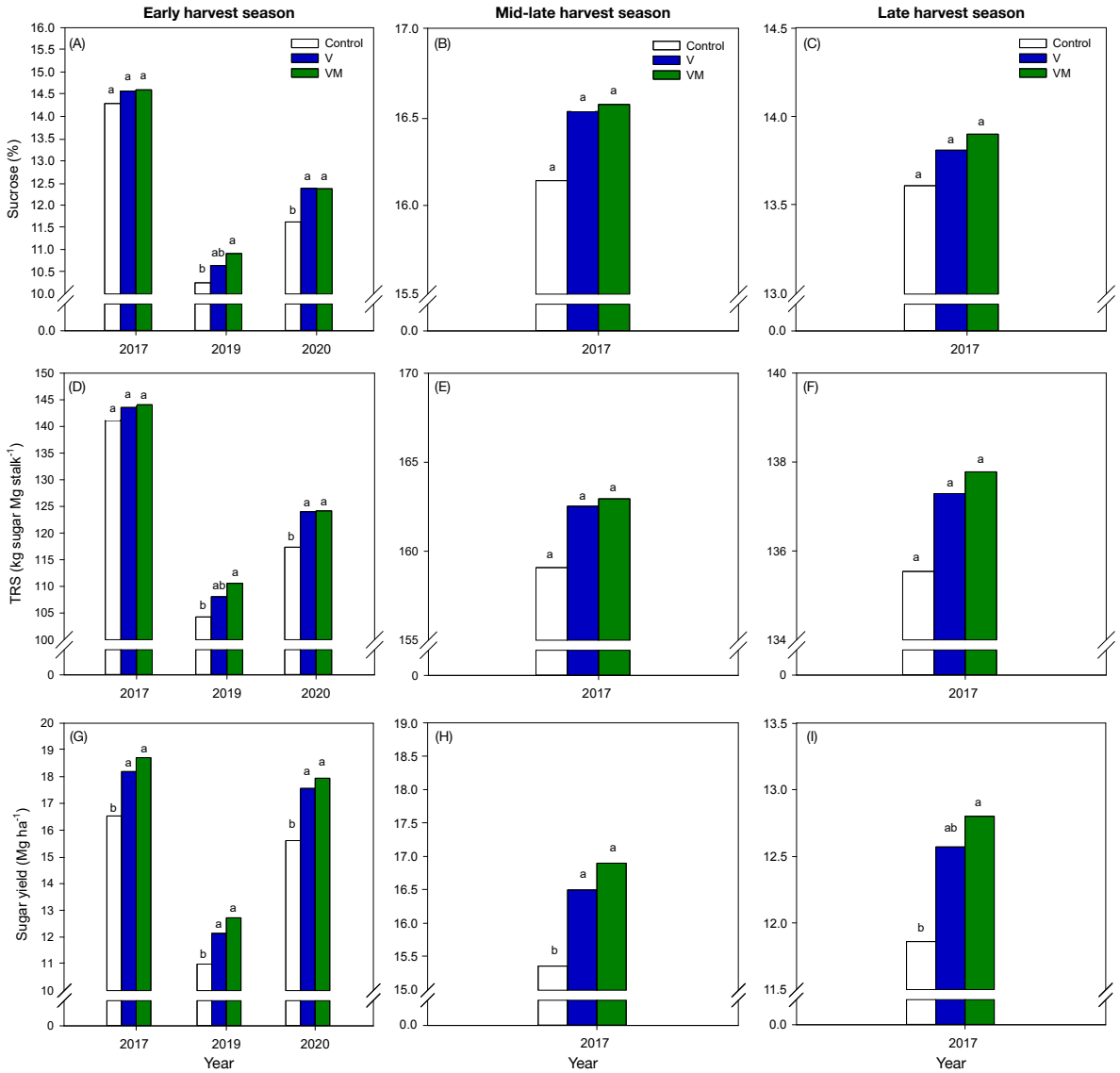


Figure 5. Sucrose (A, B and C), theoretical recoverable sugar (TRS) (D, E and F) and sugar yields (G, H and I) in early, mid-late and late harvest sugarcane as a function of foliar hormone application. The treatments were as follows: control, control with no foliar application of hormone (white); V, foliar application of hormones at the vegetative stage (blue); VM, foliar application of hormone at the vegetative and maturation stages (green). Means followed by the same letter do not differ by the t test (LSD) at 10% probability.

3.4 Energy parameters

Foliar hormone application increased straw yield significantly in early harvest sugarcane (Figure 6A) but not mid-late and late harvest sugarcane (Figures 6B and 6C), with average increases of 9.7% in VM compared with the control (16.6 Mg ha^{-1}) in early harvest sugarcane (Figure 6A). V and VM also increased bagasse production in early harvest sugarcane (Figure 6D) but not mid-late and late harvest sugarcane (Figures 6E and 6F). Compared with the control, V and VM significantly increased bagasse by averages of 6.9% and 10.2% compared with the control (6.8 Mg ha^{-1}) (Figure 6D). Finally, energy production in early harvest sugarcane was 7.1% higher in V and 9.7% higher in VM than in the control (82.1 kWh) (Figure 6G); foliar hormone application did not affect energy production in mid-late and late harvest sugarcane (Figures 6H and 6I).

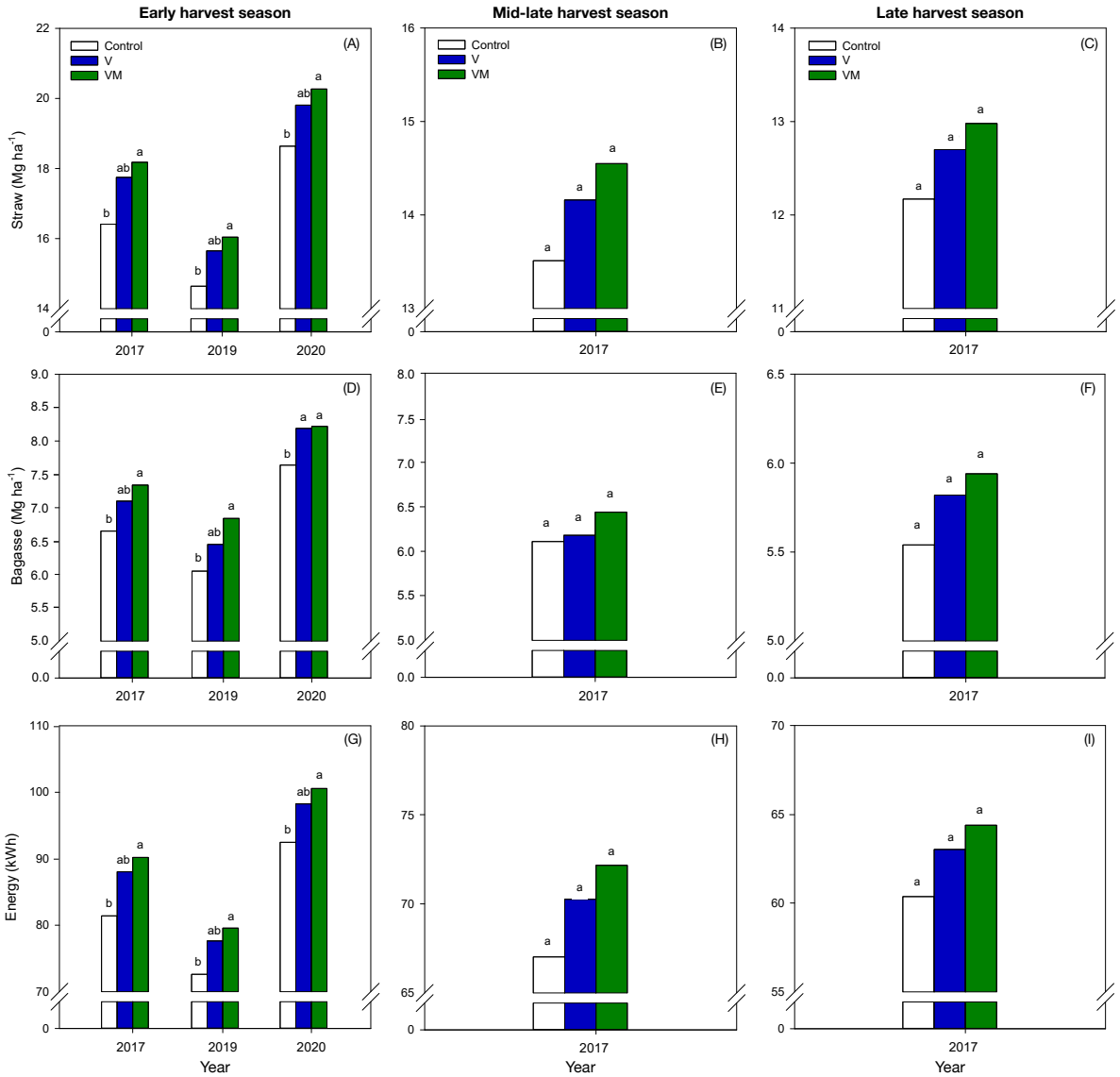


Figure 6. Straw (A, B and C), bagasse (D, E and F) and energy production (G, H and I) in early, mid-late and late harvest sugarcane as a function of foliar hormone application. The treatments were as follows: control, control with no foliar application of hormone (white); V, foliar application of hormones at the vegetative stage (blue); VM, foliar application of hormone at the vegetative and maturation stages (green). Means followed by the same letter do not differ by the t test (LSD) at 10% probability.

4 Discussion

In the present study, foliar application of phytohormones induced high sugarcane growth when performed once (vegetative growth stage) or twice (vegetative and maturation growth stages). Several studies have reported that phytohormones, e.g., ethylene, AXs, GAs, and CKs, are important metabolic engineering targets for stimulating crop development and production and improving abiotic stress tolerance in plants [3,47]. Foliar hormone application is a recent practice in agriculture around the world [48–50], and there are few studies of its effectiveness in sugarcane in different harvest seasons in tropical regions.

Here, we confirmed our hypothesis that the application of phytohormones (AX, CK and GA) during sugarcane production has positive effects on crop development, as evidenced by gains in StY. Phytohormones help plants overcome various environmental deficits, such as abiotic stresses and low availability of soil nutrients [51]. Plant hormones are a diverse class of biomolecules that promote plant acclimatization to environmental conditions by regulating development, growth, nutrient acquisition and allocation, and molecular and physiological crop responses [52,53].

In the early stages of plant growth, IAA promotes cell elongation and regulates gene expression [54,55]. Other functions of IAA include promoting adequate nutrition and controlling plant growth under stress [3,56]. Although the essential role of IAA in plants is well known [57,58], IAA-related metabolism and pathways and the interactions of IAA with nutrients and other hormones in specific crops, such as sugarcane, are still unclear, particularly under different growth conditions.

GAs act in germination, stem elongation, leaf expansion, trichome initiation and plant development [59]. GAs positively influence the photosynthetic rate, light interception, nutrient use, and the regulation of several processes throughout the plant life cycle [60]. Similar to plants treated with IAA, plants that receive GAs have low stomatal resistance, better water use efficiency and, consequently, healthy and higher crop yields [61]. GAs frequently interact with other hormones and promote crop development and pathway activation [62,63].

In summary, phytohormones are extremely important in different stages of crop growth and development, which can explain the differences in the effects of foliar hormone application once at the vegetative stage and twice at the vegetative and maturation stages of sugarcane. Single foliar hormone application at the sugarcane vegetative stage stimulated growth, i.e., more parenchymatous cells to store sucrose in the next stages. In this stage, IAA activates cell division and plant development by stimulating the growth of roots, stalks and leaves [64,65], and GAs regulate cell division and elongation, promote hypocotyl and stem growth, and increase root and leaf meristem size [66]. Our results suggest that hormone application at the vegetative stage can increase sugarcane development and yields by enhancing plant photosynthesis overall.

In the treatments with two foliar applications of phytohormones (vegetative and maturation stages), the first hormone application stimulated vegetative development and the formation of parenchymatous cells to store sucrose, and the second hormone application increased photoassimilate and sucrose production. The benefits of foliar application of hormones at two stages of sugarcane growth included increases in biometric parameters and sucrose, TRS and sugar yields. Hormones can promote shoot growth, increase xylem and decrease root growth [67,68]. Chen et al. showed that applying hormones at the maturation stage increases sucrose phosphate synthase activity in leaves and stalks while decreasing soluble acid invertase activity in stalks, thereby increasing sucrose production and accumulation.

Sucrose content and sugar production are important indicators of sugarcane quality, and sugarcane industry efforts are focused on improving sucrose content and accumulation [69,70]. Products that can be applied at the maturation stage to increase sucrose accumulation are desirable, especially hormones that enhance sugarcane yield and mitigate environmental stresses in plants. In this study, the

increases in stalk productivity and sucrose content were accompanied by gains in bagasse, straw and energy production, suggesting potential economic gains. Both timings of hormone application (V and VM) helped decrease ROS levels, as H_2O_2 and MDA contents were reduced. These decreases were the result of higher antioxidant enzyme activities (SOD, CAT, POD and APX) in phytohormone-treated sugarcane [71]. SOD, CAT, POD and APX form a complex enzymatic antioxidant system that protects the photosynthetic process from oxidative stress and prevents ROS production [72,73]. These four key cellular detoxification enzymes convert H_2O_2 to H_2O during the plant cycle [74]. Thus, foliar hormone application can promote antioxidant activity in sugarcane and mitigate unfavorable environmental conditions.

In most countries, the economy is dependent on agriculture, which relies on suitable climate conditions and fertile soil. An extensive body of research has examined the molecular mechanisms that regulate hormone synthesis, signaling, and actions; phytohormones have many functions in plant development and responses to abiotic stresses. Our results indicate that appropriate supplementation of sugarcane with phytohormones can enhance yields and quality by improving plant metabolism, regardless of harvest season.

5 Conclusion

This study found that a single hormone application was sufficient to enhance sugarcane production. Both a single application at the vegetative stage and two applications at the vegetative and maturation stages increased sugarcane growth and productivity. We found that foliar hormone application at the vegetative and maturation stages stimulated sugarcane development and enhanced photosynthetic and antioxidant metabolism. Application at both stages resulted in metabolic improvements that increased sucrose accumulation, stalk and sugar yields, and crop development and productivity.

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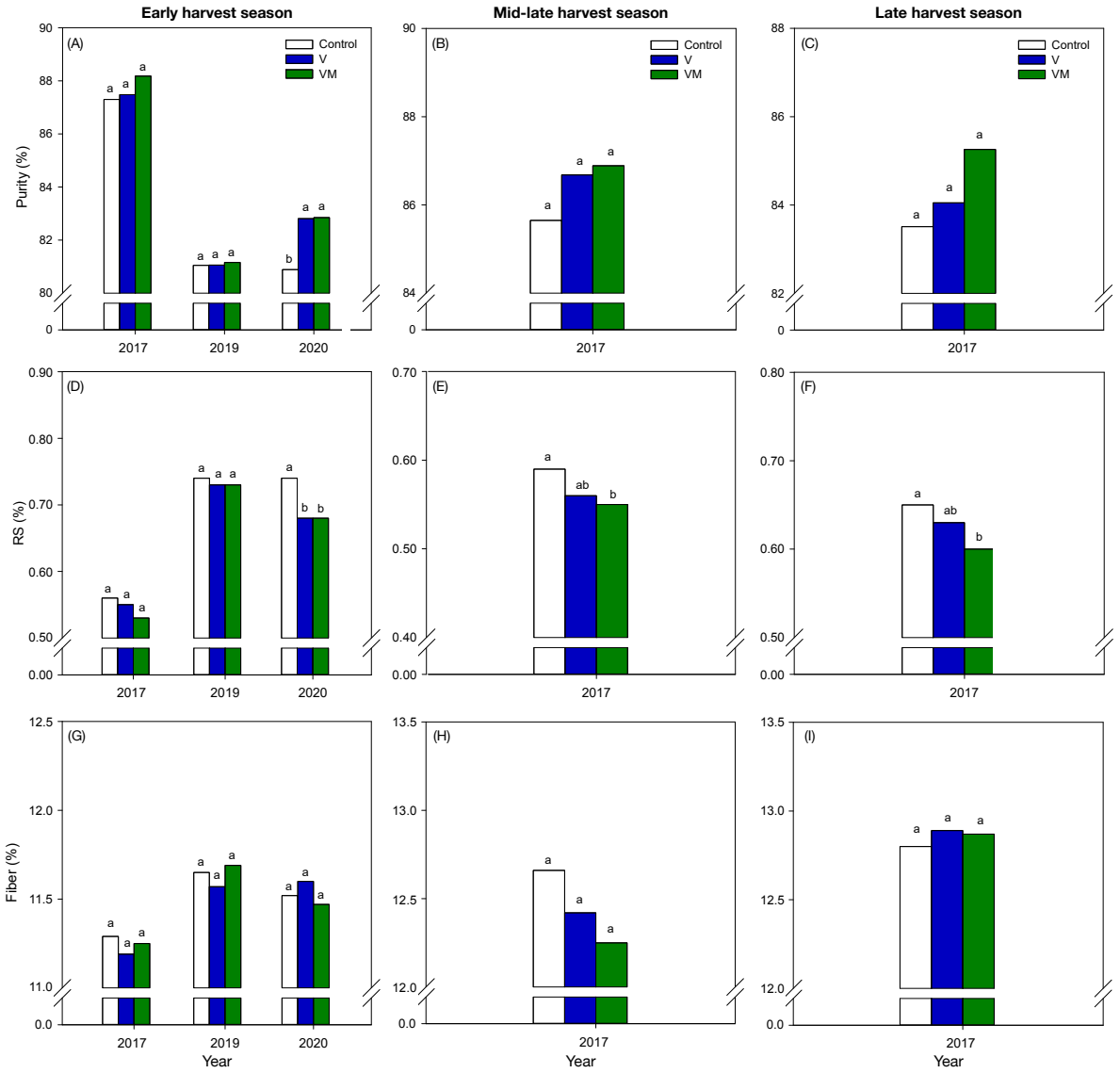
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Supplementary Material



Supplementary Figure S1. Purity (A, B and C), reducing sugar (D, E and F) and fiber (G, H and I) of early, mid-late and late harvest sugarcane as a function of foliar hormone application. The treatments were as follows: control, control with no foliar application of hormone (white); V, foliar application of hormones at the vegetative stage (blue); VM, foliar application of hormone at the vegetative and maturation stages (green). Means followed by the same letter do not differ by the t test (LSD) at 10% probability.

Chapter 2 - The frequency and phenological stage of foliar hormone-nutrient application can increase sugarcane yield and biomass and bioenergy productions

Abstract

Achieving higher productivity while reducing the expansion of cultivation is challenging. A potential strategy for increasing sugarcane yields and productivity is the foliar application of nutrients and biostimulants. This study assessed the impact of the foliar application of nutrients and a hormone-containing biostimulant on sugarcane metabolism, stalk and sugar production, and quality. Field experiments were performed at 17 sites during four growing seasons (2017-2018, 2018-2019, 2019-2020 and 2020-2021) using sugarcane with three harvest times (early, mid-late and late harvests). The treatments were as follows: (i) no foliar application of the biostimulant and nutrients (Control), (ii) foliar application at the vegetative stage of sugarcane (V), (iii) foliar application at the maturation stage of sugarcane (M), and (iv) foliar application at both the vegetative and maturation stages (VM). Foliar application did not increase rubisco activity but increased phosphoenolpyruvate carboxylase activity and the activities of the antioxidant enzymes. Peroxidase and catalase activities were highest in VM, whereas superoxide dismutase activity was highest in M. Consistent with these results, hydrogen peroxide and malondialdehyde contents were lowest in VM. In addition, the plant biometric parameters of early, mid-late and late harvest seasons were highest in VM. In summary, the foliar application of the biostimulant and nutrients efficiently promoted the growth, productivity and quality of early, mid-late and late harvest seasons. The positive effects of foliar application on the enzymatic and physiological responses of sugarcane can be achieved when applications are performed in both the vegetative and maturation stages in all harvest seasons.

Keywords: *Saccharum officinarum* L., plant growth promoters, multinutrient fertilization, phytohormones, micronutrient.

1 INTRODUCTION

Keep Sugarcane (*Saccharum officinarum* L.) is an important cultivated crop and generates more than 70% of sugar consumed globally (ZHENG et al., 2022). Sugarcane also ranks second globally among crops used to produce ethanol, and its byproducts are used in electricity production and as organic fertilizers and fodder. In Brazil, the world leader in sugarcane production (SOLTANGHEISI et al., 2021), sugarcane is cultivated in a variety of edaphoclimatic environments. Non-traditional areas of cultivation are challenging for crop management because of inadequate soil nutrition and frequent abiotic stresses, such as drought, extreme temperatures and high light inductance (ASHRAF; FOOLAD, 2007). The growing demand for food and renewable energy to support the rapidly expanding global population is forcing modern agriculture to seek alternative technologies that can increase yields while improving crop tolerance of adverse environmental conditions (FAO, 2021), such as extreme temperatures, frost, drought, excessive water and other abiotic stresses.

A key component of the sustainable management of agricultural crops is foliar fertilization, which can increase productivity and efficiency under unfavorable edaphoclimatic conditions in which the soil is inadequate for total plant requirements (FERNÁNDEZ; BROWN, 2013; HAVLIN et al., 2005). The application of nutrients and biostimulants such as phytohormones can further enhance positive interactions among crop physiology, development, and yield (MOBIN et al., 2010). Photosynthesis, which is intrinsically linked to vegetative growth and crop yield, is an important target for optimization. Inadequate conditions can decrease CO_2 assimilation due to stomatal limitations, reduced photosynthetic electron transport and photoinhibition (TAKAHASHI; BADGER, 2011), resulting in low crop productivity. Thus, crop foliar fertilization during the growth and development cycle can improve nutritional balance and increase final productivity in terms of both quantity and quality (SMOLEŃ, 2012).

Biostimulants such as phytohormones regulate plant growth and development via effects on plant metabolism (KHAN et al., 2020). Phytohormones are organic molecules produced by plants that are essential for fully functional metabolism. Among the nine known phytohormone types, five are classified as plant growth promoters: auxins (AXs), gibberellins (GAs), cytokinins (CKs), brassinosteroids (BRs), and strigolactones (SLs) (VERMA; RAVINDRAN; KUMAR, 2016). These

phytohormones are highly relevant to modern agriculture because they can increase the photosynthetic rate, which is key for improving food security, increasing renewable energy production, and promoting plant tolerance to stressful conditions (MÜLLER; MUNNÉ-BOSCH, 2021; PAUL et al., 2020; RODRIGUES et al., 2021). Previous studies have shown that endogenous hormone content is closely related to the formation and development of source-sink organs and yield parameters (DARYANTO et al., 2019). Plant hormones are also crucial for the adaptation of plants to different environments via changes in growth, differentiation, source/sink ratios and nutrient allocation (WANI et al., 2016). Finally, there are direct and complex relationships between plant hormones and cellular redox balance, which influences the response of photosynthesis to environmental stresses. Phytohormones are essential not only for the vegetative development of plants but also for controlling ROS imbalance and the plant's tolerance to different abiotic stresses (GURURANI; MOHANTA; BAE, 2015; MAYZLISH-GATI et al., 2010).

In addition to phytohormones, specific micronutrients influence photosynthesis and are important for yield regulation. These micronutrients participate in carbohydrate metabolism (B), enzymes that fix carbon (Fe and Mn), the electron transport chain (Cu), and the activation of photosynthetic enzymes belonging to the oxidase class (Zn) (CAKMAK; RÖMHELD, 1997; SANTOS et al., 2013).

Our hypothesis is that the foliar application of both biostimulants and nutrients will improve the photosynthetic rate by stimulating the activities of the enzymes phosphoenolpyruvate carboxylase (PEPcase; EC:4.1.1.31) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the plant antioxidant system. In turn, optimizing the conditions for photosynthetic function will increase the generation of photoassimilates, stalk yield and sugar accumulation. To test this hypothesis, this study evaluated the effects of the foliar application of micronutrients and plant hormones (growth promoters) to sugarcane on the regulatory processes of the photosynthetic apparatus, antioxidant enzyme activity, ROS scavenging, yield and raw material quality.

2 MATERIALS AND METHODS

2.1 Site description

Field experiments were carried out at 17 sites in 6 locations in the state of São Paulo (Figure 1) during four crop years (2017-2018, 2018-2019, 2019-2020 and 2020-2021). The sugarcane was harvested at one of three times of year: early harvest between May and June (high rainfall and high temperature), mid-late harvest between July and September (low temperature and low rainfall), and late harvest between October and December (resumption of rainfall and rising temperatures). According to the Köppen-Geiger climate classification system, the climate is Cwa, i.e., hot summers and dry winters, at sites 3 and 6; Cfa, i.e., humid, hot summers, at sites 2, 5, 7, 9, 13 and 16; and Aw, i.e., dry winters, at all other sites. The average temperature ranges over the four growing seasons were 20.8–22.3°C, 21.0–22.0°C and 21.0–22.3°C in the early, mid-late and late harvest seasons, respectively. Meteorological data were obtained from meteorological stations located near each site (Figure 2).

Soil correction and fertilization management followed the recommendations for sugarcane cultivation in Brazil (van Raij et al., 1997). The chemical characteristics of the soil were determined at depths of 0.00-0.25 and 0.25-0.50 m (VAN RAIJ et al., 2001), and the results and soil classification are shown in Table 1 (SOIL SURVEY STAFF, 2014).

All phytosanitary management of the experimental sites was carried out according to the regular standards for sugarcane production. Cultivars were selected based on the best harvest time characteristics (DAROS et al., 2010; UDOP, 2018). All data on cultivars, ages, spacing and times of treatment applications and harvest are included in Table 2.

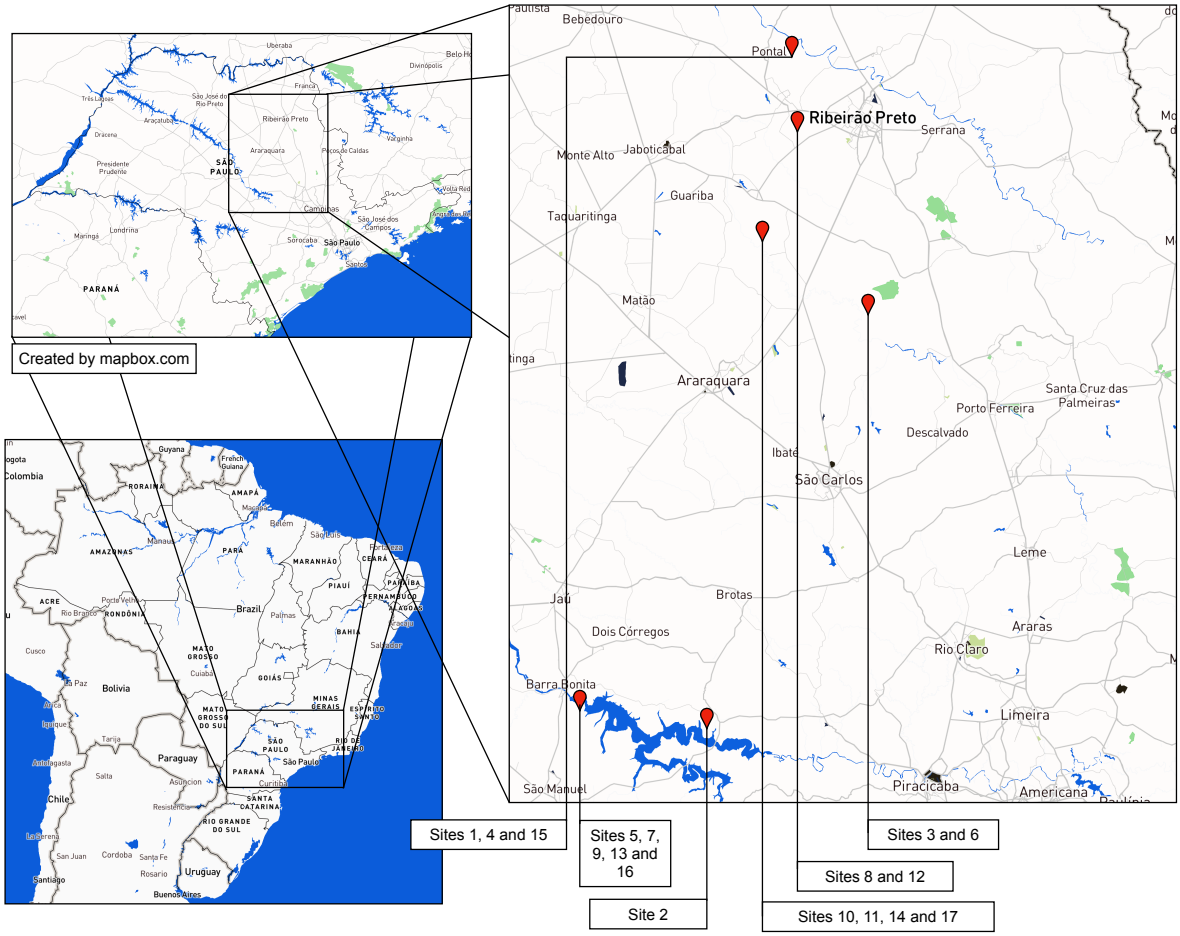


Figure 1. Experimental sites. The early harvest season trials were located at Pontal-SP (Site 1 – 21°02'06.0"S 48°05'18.6"W, Site 4 – 20°57'49.8"S 48°08'41.7"W), Santa Maria da Serra-SP (Site 2 – 22°34'18.8"S 48°13'28.6"W), Luis Antônio-SP (Sites 3 and 6 – 21°33'03.2"S 47°49'48.4"W), and Igaraçú do Tiête-SP (Sites 5 and 7 – 22°33'39.63"S 48°28'39.05"W). The mid-late harvest season trials were located at Sertãozinho-SP (Site 8 – 21°08'43.95"S 48°04'23.74"W), Igaraçú do Tiête-SP (Site 9 – 22°30'06.93"S 48°28'23.74"W), and Pradópolis-SP (Sites 10 and 11 – 21°26'20.1"S 48°02'06.8"W). The late harvest season trials were located at Sertãozinho-SP (Site 12 – 21°05'57.19"S 48°05'18.50"W), Igaraçú do Tiête-SP (Site 13 – 22°33'44.06"S 48°33'11.58"W, Site 16 – 22°33'24.32"S 48°30'59.23"W), Pradópolis-SP (Site 14 – 21°21'59.01"S 47°59'25.53"W, Site 17 – 21°22'24.06"S 48°02'52.75"W) and Pontal-SP (Site 15 – 21°03'40.00"S 48°03'53.40"W).

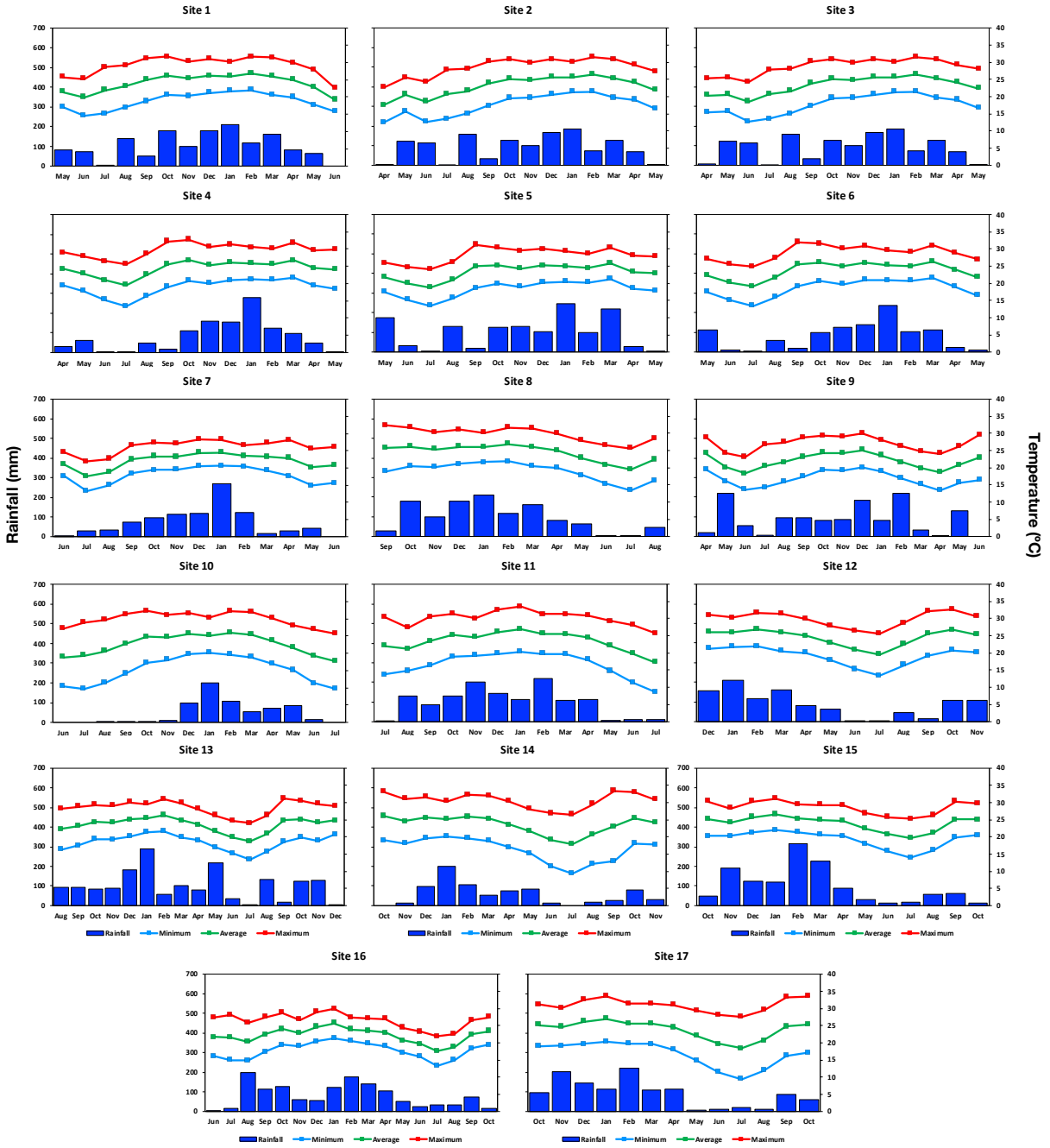


Figure 2. Rainfall (mm) and maximum and minimum temperature averages at all sites during the early, mid-late and late harvest periods. Early harvest in 2017: Pontal-SP (Site 1), Santa Maria da Serra (Site 2), and Luis Antônio (Site 3); in 2018: Pontal-SP (Site 4), Igaraçú do Tiête-SP (Site 5), and Luís Antônio-SP (Site 6); in 2020: Igaraçú do Tiête-SP (Site 7). Mid-late harvest in 2017: Sertãozinho-SP (Site 8), Igaraçú do tTiête-SP (Site 9), and Pradópolis-SP (Site 10); in 2019: Pradópolis-SP (Site 11). Late harvest in 2017: Sertãozinho-SP (Site 12), Igaraçú do Tiête-SP (Site 13), and Pradópolis-SP (Site 14); in 2019: Pontal-SP (Site 15), Igaraçú do Tiête-SP (Site 16), and Pradópolis-SP (Site 17).

Table 1. Soil classification and chemical characteristics at each site.

Harvest period	Site*	Soil classification	Depth m	pH $CaCl_2$	M.O. g dm^{-3}	$P_{(resin)}$ mg dm^{-3}	S	Al^{+3}	$H+Al^{+3}$	K	Ca	Mg	SB	CTC	V% %
								$mmol_c$ dm^{-3}							
Early harvest season	1	Rhodic Hapludox	0.00-0.25	5.7	31	23	77	0.0	19	14.0	53	18	71	90	79
			0.25-0.50	5.7	20	3	101	0.0	19	7.9	22	10	32	51	63
	2	Typic Quartzipsamment	0.00-0.25	5.4	24	184	17	0.0	14	5.6	59	12	71	85	84
			0.25-0.50	5.2	13	6	34	1.0	19	2.3	28	6	34	53	64
	3	Typic Quartzipsamment	0.00-0.25	5.4	20	30	10	0.0	12	1.2	31	15	46	58	79
			0.25-0.50	5.5	10	8	3	0.0	12	0.5	13	6	19	31	61
	4	Rhodic Hapludox	0.00-0.25	4.8	13	11	0	0.9	33	1.6	26	12	38	71	54
			0.25-0.50	4.9	12	5	60	1.4	29	0.6	13	6	19	48	40
	5	Typic Quartzipsamment	0.00-0.25	4.2	8	6	0	3.1	25	0.6	5	2	7	32	22
			0.25-0.50	4.6	8	5	0	1.1	24	0.6	8	3	11	35	31
	6	Typic Quartzipsamment	0.00-0.25	5.4	20	30	10	0.0	12	1.2	31	15	46	58	79
			0.25-0.50	5.5	10	8	3	0.0	12	0.5	13	6	19	31	61
	7	Typic Quartzipsamment	0.00-0.25	4.2	8	6	0	3.1	25	0.6	5	2	7	32	22
			0.25-0.50	4.6	8	5	0	1.1	24	0.6	8	3	11	35	31
Mid-late harvest season	8	Rhodic Eutrudox	0.00-025	5.5	58	145	0	0.0	44	12	82	22	116	160	73
			0.25-0.50	5.5	41	40	0	0.0	37	8.0	53	17	78	115	68
	9	Rhodic Eutrudox	0.00-025	5.3	19	10	0	0.2	21	1.0	39	18	58	79	73
			0.25-0.50	5.2	20	9	0	0.3	23	1.0	41	19	61	84	73
	10	Rhodic Eutrudox	0.00-0.25	5.8	55	54	8	0.0	27	10	37	22	69	96	72
			0.25-0.50	5.5	42	45	2	0.0	21	7.0	23	12	42	63	67
	11	Rhodic Eutrudox	0.00-0.25	5.8	55	54	8	0.0	27	10	37	22	69	96	72
			0.25-0.50	5.5	42	45	2	0.0	21	7.0	23	12	42	63	67†

†To be continued...

Harvest period	Site*	Soil	Depth	pH	M.O.	$P_{(resin)}$	S	Al^{+3}	$H+Al^{+3}$	K	Ca	Mg	SB	CTC	V%
Late harvest season	12	Rhodic Eutrudox	0.00-0.25	5.6	1.8	80	0	0.0	7	2.0	33	9	44	51	86
			0.25-0.50	5.6	1.6	20	65	0.0	14	2.0	21	7	30	44	68
	13	Rhodic Eutrudox	0.00-0.25	4.5	10	22	0	1.0	25	3.0	14	6	23	48	48
			0.25-0.50	4.6	7.1	9.7	0	2.0	20	3.0	10	5	18	38	47
	14	Rhodic Eutrudox	0.00-0.25	6.2	49	38	34	0.0	32	9.0	33	25	67	99	68
			0.25-0.50	6.0	39	35	22	0.0	29	8.0	18	16	42	71	59
	15	Rhodic Hapludox	0.00-0.25	4.4	13	10	1	1.0	31	2.0	28	14	42	73	58
			0.25-0.50	4.7	11	6	58	1.3	30	1.0	12	10	22	52	42
	16	Rhodic Eutrudox	0.00-0.25	5.3	19	10	0	0.2	21	1.0	39	18	58	79	73
			0.25-0.50	5.2	20	9	0	0.3	23	1.0	41	19	61	84	73
	17	Rhodic Eutrudox	0.00-0.25	6.0	47	40	35	0.4	30	12	32	28	72	102	71
			0.25-0.50	5.9	37	33	28	0.0	25	9.0	15	20	44	69	64

*Sites: Early harvest season 2017: Pontal-SP (Site 1), Santa Maria da Serra (Site 2), and Luis Antônio (Site 3); 2018: Pontal-SP (Site 4), Igaraçú do Tiête-SP (Site 5), and Luís Antônio-SP (Site 6); 2020: Igaraçú do Tiête-SP (Site 7). Mid-late harvest season 2017: Sertãozinho-SP (Site 8), Igaraçú do Tiête-SP (Site 9), and Pradópolis-SP (Site 10); 2019: Pradópolis-SP (Site 11). Late harvest season 2017: Sertãozinho-SP (Site 12), Igaraçú do Tiête-SP (Site 13), and Pradópolis-SP (Site 14); 2019: Pontal-SP (Site 15), Igaraçú do Tiête-SP (Site 16) and Pradópolis-SP (Site 17).

Table 2. Sugarcane cultivar, ratoon, row spacing and dates of establishment, first and second applications and harvest.

Season	Sites	Cultivar	Ratoon	Row spacing	1st Application†	2nd Application	Harvest
Early harvest	1	RB 85 5453	3	0.9 × 1.60	February 2017	April 2017	June 2017
	2	RB96 6928	1	0.9 × 1.50	February 2017	April 2017	May 2017
	3	RB 85 5156	2	1.5	February 2017	April 2017	May 2017
	4	RB85-5536	3	0.9 × 1.60	January 2018	March 2018	May 2018
	5	RB96-6928	1	0.9 × 1.50	February 2018	April 2018	May 2018
	6	RB 85 5156	3	1.5	February 2018	April 2018	May 2018
	7	RB-96-6928	2	0.9 × 1.50	February 2020	April 2020	June 2020
Mid-late harvest	8	CTC4	3	0.9 × 1.60	June 2017	August 2017	August 2017
	9	SP80 3280	2	0.9 × 1.50	July 2017	August 2017	September 2017
	10	CTC4	3	1.5	June 2017	July 2017	August 2017
	11	CTC4	5	1.5	May 2019	June 2019	July 2019
Late harvest	12	SP80-3280	2	1.5	September 2017	October 2017	November 2017
	13	SP80-3280	1	0.9 × 1.50	October 2017	November 2017	December 2017
	14	SP80-3280	2	1.5	September 2017	October 2017	November 2017
	15	CTC4	3	1.5	September 2019	October 2019	November 2019
	16	SP80-3280	1	0.9 × 1.50	August 2019	September 2019	October 2019
	17	SP80-3280	5	1.5	August 2019	October 2019	November 2019

†The first application was performed at the vegetative stage, and the second application was performed at the maturation stage.

2.2 Experimental design and treatment application

The experiments were established in a completely randomized block design with 8 replications. The plots consisted of eight rows with a length of 10 m; the row spacing at each site is given in Table 2. The treatments consisted of the foliar application of nutrients with a phytohormone-containing biostimulant in the vegetative stage, maturation stage, or both stages of sugarcane: (i) no foliar application (Control), (ii) foliar application of the biostimulant and nutrients at the vegetative stage of sugarcane (V), (iii) foliar application of the biostimulant and nutrients at the maturation stage of sugarcane (M), and (v) foliar application of the biostimulant and nutrients at both the vegetative and maturation stages (VM). In VM, two applications were performed; thus, plants in this treatment received twice the doses applied in V or M. For the early, mid-late and late harvest seasons, foliar application at the vegetative stage was performed an average of 100, 75 and 60 days before harvest (DBH), respectively; foliar application at the maturation stage was performed 30 DBH regardless of harvest time (Figure 3).

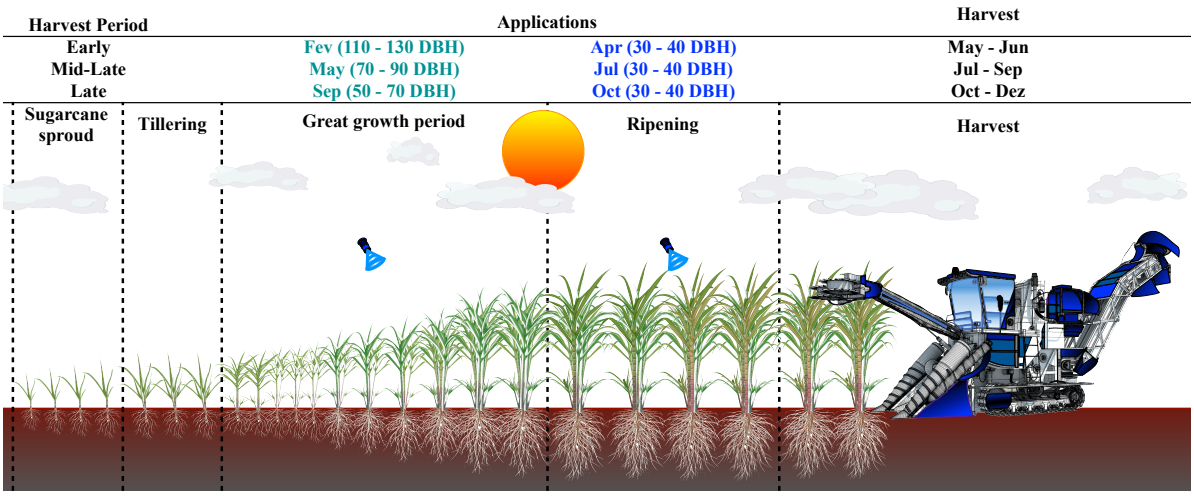


Figure 3. Dates of foliar applications and harvest at the experimental sites according to the early, mid-late and late harvests.

Foliar fertilization consisted of (g ha^{-1}) 330.0 N, 260.0 B, 12.0 Cu, and 300.0 Zn and a hormone complex comprising (mg ha^{-1}) 25 indole butyric acid, 25 gibberellic acid and 45 kinetin. The foliar application was performed between the fourth and fifth rows of each plot using pressurized equipment (CO_2) with a single 1/4KLC-9 brass jet nozzle

with an average flow rate of 100 L ha^{-1}. To achieve this spray volume, a working pressure of 4 kgf cm^2 (equivalent to 58.0 PSI) was used. The equipment had a 2.6-m-long rod to overcome the height of the sugarcane and promote a homogeneous and effective application.

2.3 Sugarcane measurements

2.3.1 Leaf photosynthetic and antioxidant enzyme activities and antioxidant and nutrient contents

Seven days after foliar application, photosynthetic and antioxidant enzyme activity was assessed in fully expanded leaves (+3) or the TVD (top visible dewlap). In VM, the assessment was performed 7 days after the second application. Leaves were collected from each plot at site 7 (Igaraçú do Tietê) between 8:00 and 10:00 am, immediately frozen in liquid nitrogen, and stored in 50-ml Falcon tubes at -80°C.

To analyze photosynthetic and antioxidant enzyme activity, extracts were prepared by grinding the leaf tissue to a fine powder in a mortar with liquid nitrogen. The enzymatic assays were carried out using crude extracts prepared by homogenizing 1 part frozen sample with 2 parts extraction buffer on ice for 20 seconds in an stirred ultrafiltration cell (BIOMT: 0.5 to 250 mL, 5-mm stainless steel rod; 50-60 Hz).

To determine phosphoenolpyruvate carboxylase activity (PEPcase; EC:4.1.1.31), leaves pulverized in liquid nitrogen were extracted with extraction buffer containing 0.2 M KPi (pH 8.0), 10 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT and 1% (w/v) PVP-40 in ice-cold Eppendorf tubes. The enzymatic extract was obtained after centrifugation at 14000xg and 4°C. Oxaloacetate is reduced to malate in the presence of malate dehydrogenase (MDH: EC 1.1.1.37) and β-nicotinamide adenine dinucleotide in its reduced form (NADH). Accordingly, 50 µL of crude extract was added to 940 µL of medium containing 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 33 nkat MDH (one katal is the amount of enzyme required to convert one mole of substrate to one mole of product per second), 5 mM glucose-6-phosphate, 0.2 mM NADH, and 5 mM phosphoenolpyruvate (PEP) and incubated for 15 min. The oxidation of NADH was monitored in a spectrophotometer at 340 nm for 120 s, and the total specific

enzyme activity was expressed as μmol CO_2 min^{-1} mg^{-1} protein using a molar absorptivity of NADH of 6.22 M^{-1} cm^{-1} (DEGL'INNOCENTI et al., 2003).

To assay ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco: EC4.1.1.39) activity, leaves pulverized in liquid nitrogen were extracted with buffer containing 0.2 M KPi (pH 7.8), 5 mM $MgCl_2$, 1 mM DTT, 1% (w/v) PVP-40, and 5 mM ascorbic acid (Sigma-Aldrich) in ice-cold Eppendorf tubes. After centrifugation at 14,000 g and 4°C for 30 min, the supernatant was immediately used to measure Rubisco activity at 25°C following the methodology of Ashton et al. (1990) with minor modifications. The Rubisco extracts were initially activated by incubating 35 μL of crude extract with 450 μL of buffer containing 100 mM Bicin (pH 8.0), 25 mM potassium bicarbonate ($KHNO_3$), 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25 mM NADH, 80 nkat G-3-P dehydrogenase (EC1.2.1.12), 80 nkat 3-phosphoglyceric phosphokinase (EC 2.7.2.3), 80 nkat creatine phosphokinase (EC 2.7.3.2) and 0.25 mM NADH for 15 min. NADH oxidation was initiated by the addition of 0.5 mM RuBP (Sigma-Aldrich), and the reaction was followed for 3 min. The activity was calculated from the difference in absorbance readings at 0 and 3 min and expressed in μmol CO_2 min^{-1} mg^{-1} protein using a molar absorptivity of NADH of 6.22 M^{-1} cm^{-1}.

All antioxidant enzymes, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were extracted from leaf tissue that had been ground to a fine powder in a mortar with liquid nitrogen. One part pulverized frozen tissue was mixed with 2 parts extraction medium and homogenized on ice for 20 sec in an Ultra-Stirred (BIOMT: 0.5 to 250 mL, 5-mm stainless steel rod; 50-60 Hz). The crude extract was utilized for enzyme assays according to the methodology of Silva et al. (2020). MDA extracted from leaf tissue was reacted with thiobarbituric acid (TBA), and the absorbance of the resulting TBA-MDA adduct was measured in a spectrophotometer at 532 nm. The concentration of MDA was calculated from a standard curve of 1,1,3,3-tetramethoxypropane (TPE) and expressed in nmol MDA g^{-1} fresh weight (FW). The H_2O_2 concentration was determined according to the method of Alexieva et al. (2001). The amount of H_2O_2 was calculated using a standard curve prepared with known concentrations of H_2O_2.

To measure the activities of superoxide dismutase (SOD; EC:1.15.1.1) and catalase (CAT; EC:1.11.1.6), the extraction buffer contained 200 mM KPi (pH 7.8), 10 mM EDTA, 20 mM ascorbic acid, 1% polyvinylpyrrolidone MW 40,000 (PVP-40; Sigma-Aldrich) and 1 mM 1,4-dithiothreitol (DTT; Sigma-Aldrich). The supernatant

obtained after centrifugation at 12,000 xg and 4°C for 30 min was used as the crude extract for both SOD and CAT activity assays. SOD activity was determined photochemically using an assay system consisting of methionine (13 mM; Sigma-Aldrich), EDTA (100 nM), riboflavin (2 μM; Sigma-Aldrich), and NBT (75 μM; Sigma-Aldrich) in 50 mM KPi buffer. The results were expressed in units (U) of SOD per mg of protein as described by Giannopolitis and Ries (1977). CAT (CAT; EC:1.11.1.6) activity was determined by monitoring the consumption of H_2O_2 (250 μM) and expressed in μmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 39.4 mM^{-1} cm^{-1} (HAVIR; MCHALE, 1987).

Free peroxidase activity (POD; EC:1.11.1.7) was analyzed using the supernatant obtained after centrifugation at 8,000 xg and 4°C for 20 min. The assays were performed in buffered solution containing 200 mM KPi (pH 6.7), 10 mM EDTA, and 1% PVP (40). The activity of free POD was measured colorimetrically by the formation of the 4-aminoantipyrine phenol complex and expressed in μmol H_2O_2 min^{-1} mg^{-1} protein using a molar absorptivity of 6.8 M^{-1} cm^{-1} according to the method of Allain et al. (1974).

Ascorbate peroxidase (APX; EC:1.11.1.11) activity was extracted in 200 mM KPi (pH 6.0), 10 mM EDTA, 1% (PVP-40), and 1 mM DTT. After homogenization and centrifugation at 12,000 xg and 4°C for 30 min, the supernatant was used for activity assays. The reaction was initiated by adding H_2O_2 (1 mM) and ascorbic acid (80 μM), and the decrease in absorbance at 290 nm was monitored for 120 s. The activity was expressed in μmol min^{-1} mg^{-1} protein (KOSHIBA, 1993; NAKANO; ASADA, 1981). Proline accumulation was measured according to the methodology proposed by Bates Waldren and Teare (1973).

In all harvest seasons, leaf nutrient content was evaluated by collecting 20 TVD+1 leaves (DILLEWIJN, 1960; MALAVOLTA; VITTI; OLIVEIRA, 1997). However, the results were inconsistent and therefore are not discussed.

2.3.2 Sugarcane biometric evaluations

Sugarcane development was evaluated by determining the biometric parameters of 20 stalks randomly collected from each side at the ripening phenological stage prior to harvest. Plant height (m) was measured as the distance from the ground to the

auricular region of the +3 leaf, and stalk diameter was measured using a digital caliper. In each plot, two planting rows with a length of 2 m each were defined as the useful area. Stalk mass was evaluated at the ripening phenological stage prior to harvest. The mass of stalks in the useful area of the plot was extrapolated to obtain the stalk yield (StY) in Mg ha^{-1}.

2.3.3 Sugarcane quality

Sucrose (%), juice purity (%), fiber (%), reducing sugars (RS; %), theoretical recoverable sugar (TRS; kg sugar Mg^{-1} stalk) and sugar yield (SY; Mg ha^{-1}) were measured by collecting 10 random stalks in the two central rows of the plot (FERNANDES, 2011). SY was obtained by multiplying StY by TRS and dividing by 1000.

2.3.4 Sugarcane biomass and energy production

Fiber and StY at 50% moisture were used to calculate bagasse productivity. Straw production was assumed to be 140 kg from 1 Mg of stalks, and energy production was calculated assuming that 1 Mg of straw and 60% soil surface coverage (HASSUANI, 2005) produce 4.96 MWh of primary energy (WALDHEIM; MONIS; VERDE LEAL, 2001).

3 Data analysis

For all data, homogeneity of variances and normality were assessed with the F-Snedecor and Shapiro–Wilk tests, respectively. Data were submitted to analysis of variance (ANOVA), and means were compared between foliar fertilizer treatments in each sugarcane harvest season (early, mid-late and late) by the least significant difference (LSD) test at 10% using SISVAR (FERREIRA, 2014).

4 Results

4.1 Photosynthetic and antioxidant enzyme activity

PEPcase activity was significantly higher in M and VM than in V and the control (Figure 4A), while Rubisco activity did not differ significantly between the treatments (Figure 4B).

The contents of MDA and H_2O_2 were lowest in MV: 4.80 μmol g^{-1} FW (Figure 5A) and 5.88 nmol g^{-1} FW (Figure 5B), respectively. These contents were 32.3% and 31.4% lower than those in the control, respectively. SOD, POD and CAT activities and proline content were highest in M and VM. Compared with the control (10.9 units mg^{-1} prot.), SOD activity was 56.7% higher in M and 52.8% higher in VM (Figure 5C). POD activity was 74.2% and 103.2% higher in M and VM than in the control (0.077 μmol min^{-1} mg^{-1} prot.), respectively (Figure 5D). CAT activity was 15.2% and 45.9% higher in M and VM than in the control (0.79 μmol min^{-1} mg^{-1} Prot.), respectively (Figure 5E). In addition, APX activity was higher in V and M (Figure 5F); proline content was highest in VM and was 30.2% higher than in the control (0.39 μmol g^{-1} FW) (Figure 5G).

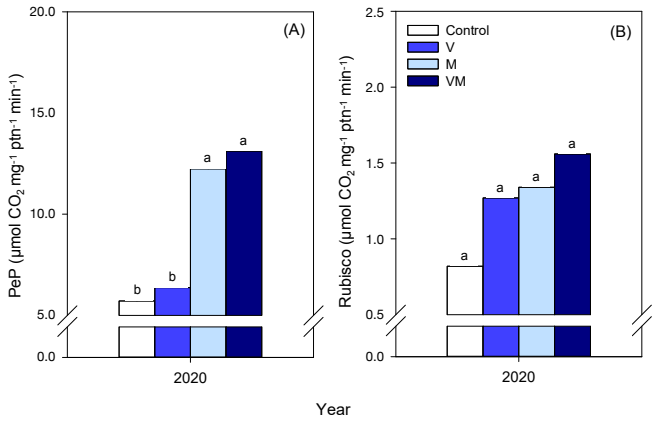


Figure 4. Phosphoenolpyruvate carboxylase (PEPcase) (A) and ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) (B) activities in the treatments with different timings of foliar biostimulant and nutrient applications in early harvest seasons. Control: no foliar application of biostimulant or nutrients; V: foliar application of biostimulant and nutrients at the sugarcane vegetative stage; M: foliar application of biostimulant and nutrients at the sugarcane maturation

stage; VM: foliar applications of biostimulant and nutrients at both the vegetative and maturation stages. Bars with the same letter do not differ by the t test (LSD) at 10% probability.

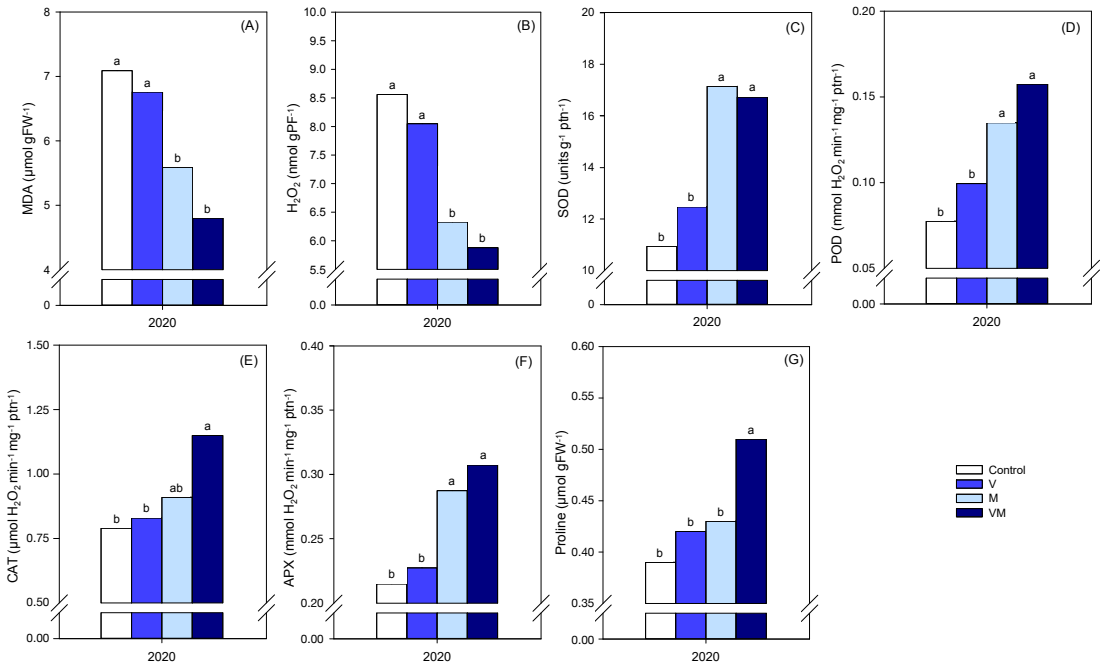


Figure 5. Malondialdehyde (MDA) content (A), hydrogen peroxide (H_2O_2) content (B), superoxide dismutase (SOD) activity (C), peroxidase (POD) activity (D), catalase (CAT) activity (E), ascorbate peroxidase (APX) activity (F) and proline content (G) in the treatments with different timings of foliar biostimulant and nutrient applications in early harvest seasons. Control: no foliar application of biostimulant or nutrients; V: foliar application of biostimulant and nutrients at the sugarcane vegetative stage; M: foliar application of biostimulant and nutrients at the sugarcane maturation stage; VM: foliar applications of biostimulant and nutrients at both the vegetative and maturation stages. Bars with the same letter do not differ by the t test (LSD) at 10% probability.

4.2 Sugarcane productivity parameters

Plant height was greatest in VM and increased by 5.7%, 4.0% and 5.4% in early, mid-late and late harvest seasons, respectively, compared to the control (2.47, 2.63 and 2.50 m, respectively) (Figures 6A, 6B and 6C). Stalk diameter was also greatest in VM and was approximately 3.8%, 1.7% and 2.1% greater than in the control (on average 27.5, 25.9 and 25.8 mm) in early, mid-late and late harvest sugarcane, respectively (6D, 6E and 6F). StY increased by 11.9%, 7.5% and 9.7%, on average, in VM compared to the control, where early, mid-late and late harvest sugarcane produced 105, 92 and 86 Mg stalk ha^{-1}, respectively (Figures 6G, 6H and 6I).

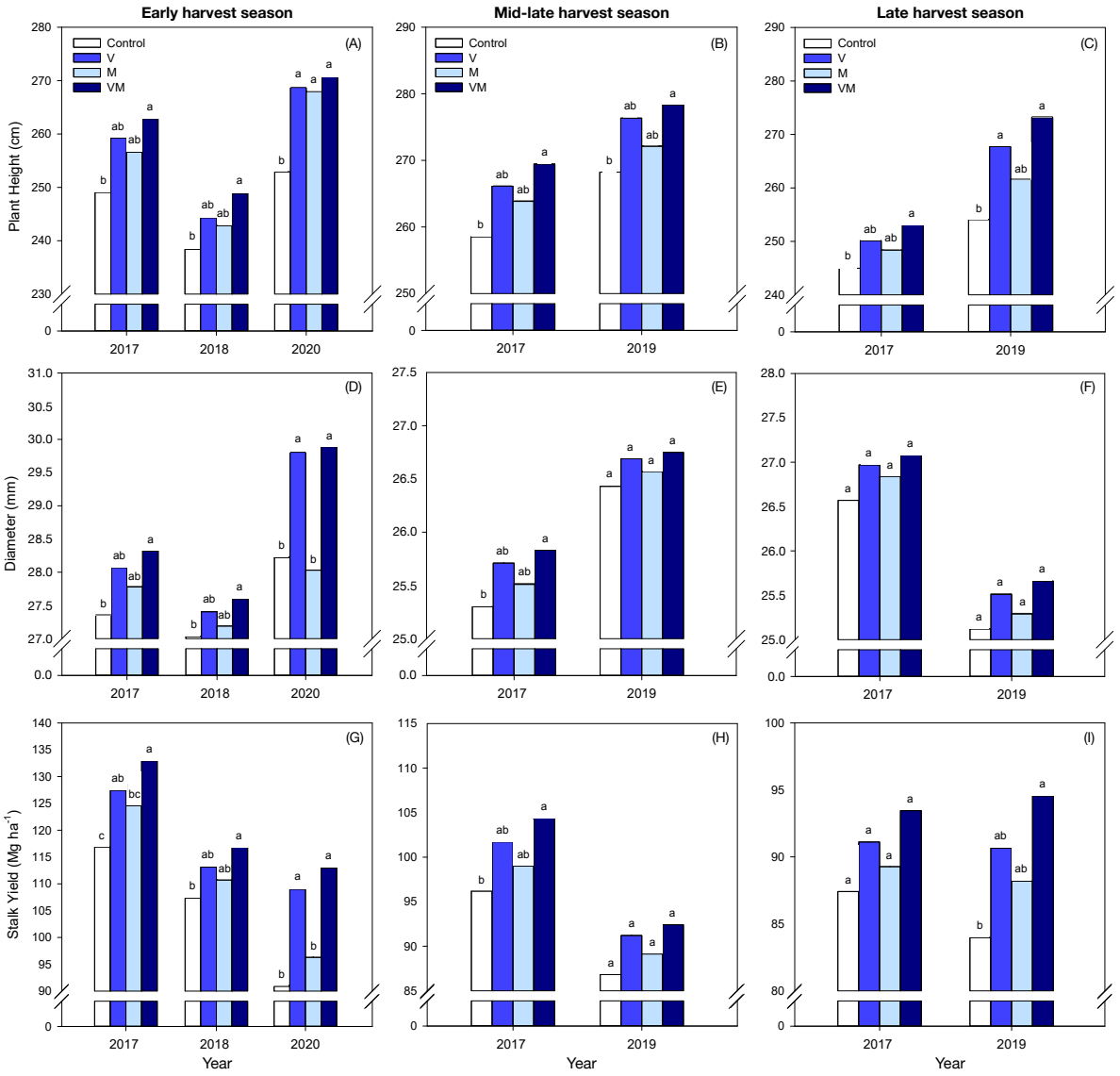


Figure 6. Sugarcane plant height (A, B and C), stalk diameter (D, E and F) and stalk yield (G, H and I) in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons. Control: no foliar application of biostimulant or nutrients; V: foliar application of biostimulant and nutrients at the sugarcane vegetative stage; M: foliar application of biostimulant and nutrients at the sugarcane maturation stage; VM: foliar applications of biostimulant and nutrients at both the vegetative and maturation stages. Bars with the same letter do not differ by the t test (LSD) at 10% probability.

4.3 Quality parameters

The LSD test showed that sucrose and TRS did not differ significantly between the treatments for any of the sugarcane harvests (Figures 7A-F).

SY was lowest in the control, with average values of 15.1, 14.0 and 12.5 Mg ha^{-1} for early, mid-late and late harvest sugarcane, respectively. SY was highest in VM

regardless of harvest season; the average gains compared with the control were 14.2%, 10.2% and 10.3% for early, mid-late, and late harvest sugarcane, respectively (Figures 7G, 7H and 7I). SY decreased in the treatments in the order VM>V>M>Control.

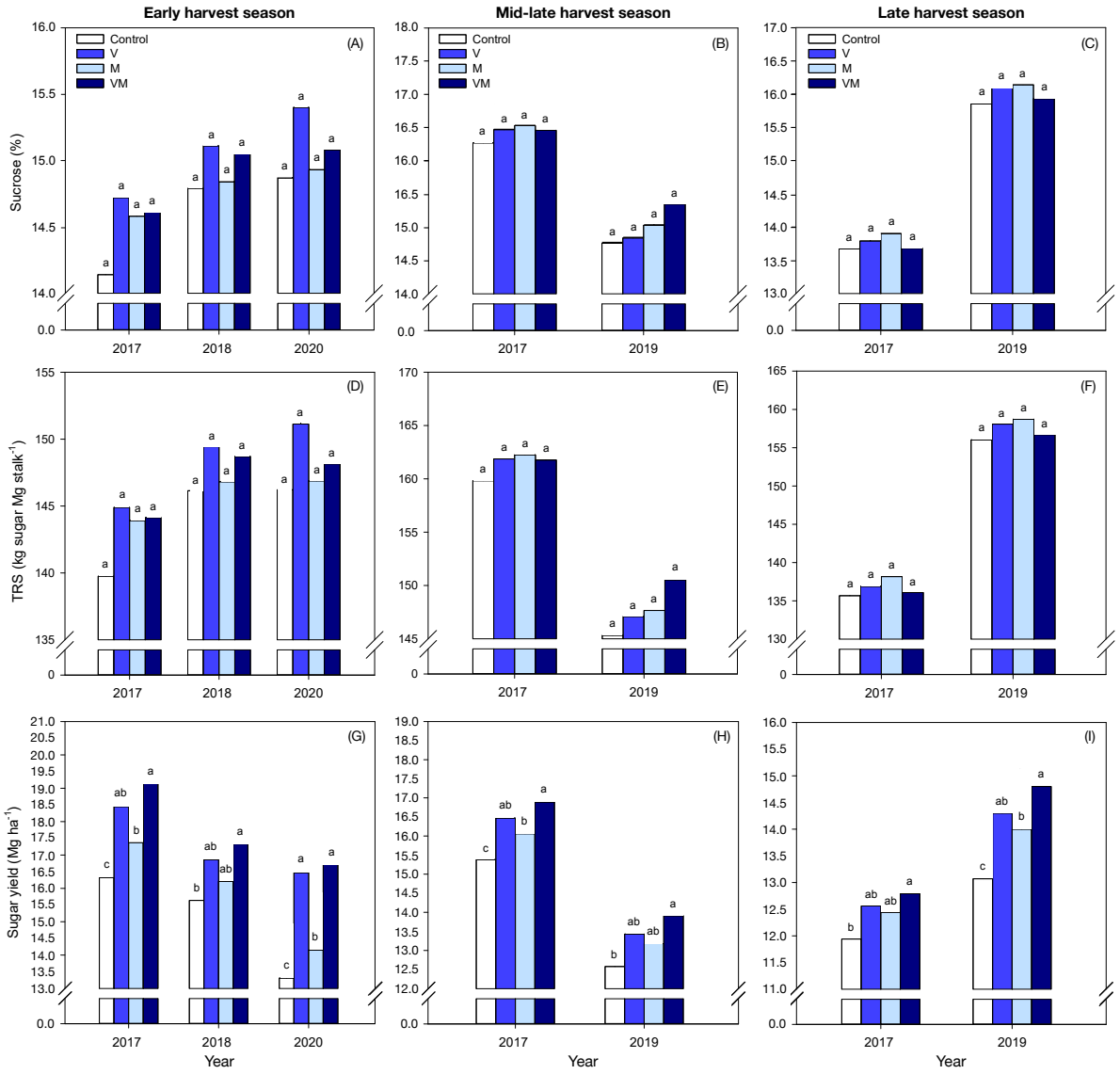


Figure 7. Sucrose (A, B and C), theoretical recoverable sugar (TRS) (D, E and F) and sugar yield (G, H and I) in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons. Control: no foliar application of biostimulant or nutrients; V: foliar application of biostimulant and nutrients at the sugarcane vegetative stage; M: foliar application of biostimulant and nutrients at the sugarcane maturation stage; VM: foliar applications of biostimulant and nutrients at both the vegetative and maturation stages. Bars with the same letter do not differ by the t test (LSD) at 10% probability.

4.4 Biomass parameters and estimate energy production

Straw production was highest in VM in all growing seasons. Compared with the control (14.7, 12.8 and 12.0 Mg ha^{-1}), straw production increased by an average of 11.9%, 7.5% and 9.7% in early, mid-late and late harvest sugarcane in VM, respectively (Figures 8A, 8B and 8C). However, there were no differences between treatments in 2018 and 201 in early and mid-late harvest seasons.

Bagasse production was highest in VM in all harvests. Compared with the control (6.2, 5.6 and 5.6 Mg ha^{-1}), bagasse production increased by an average of 10% and 9% in early and late harvest sugarcane in V and VM, respectively (Figures 8D, 8E and 8F).

Energy production was also highest in VM in all sugarcane harvests. The average energy production in VM was 81.7, 68.3 and 65.3 kWh for early, mid-late and late harvest sugarcane, respectively, which represented increases compared with the control of 11.9%, 7.5% and 9.7% (Figures 8G, 8H and 8I). However, potential energy production was similar between treatments in 2018 and 2019 in the early and mid-late harvest seasons, respectively.

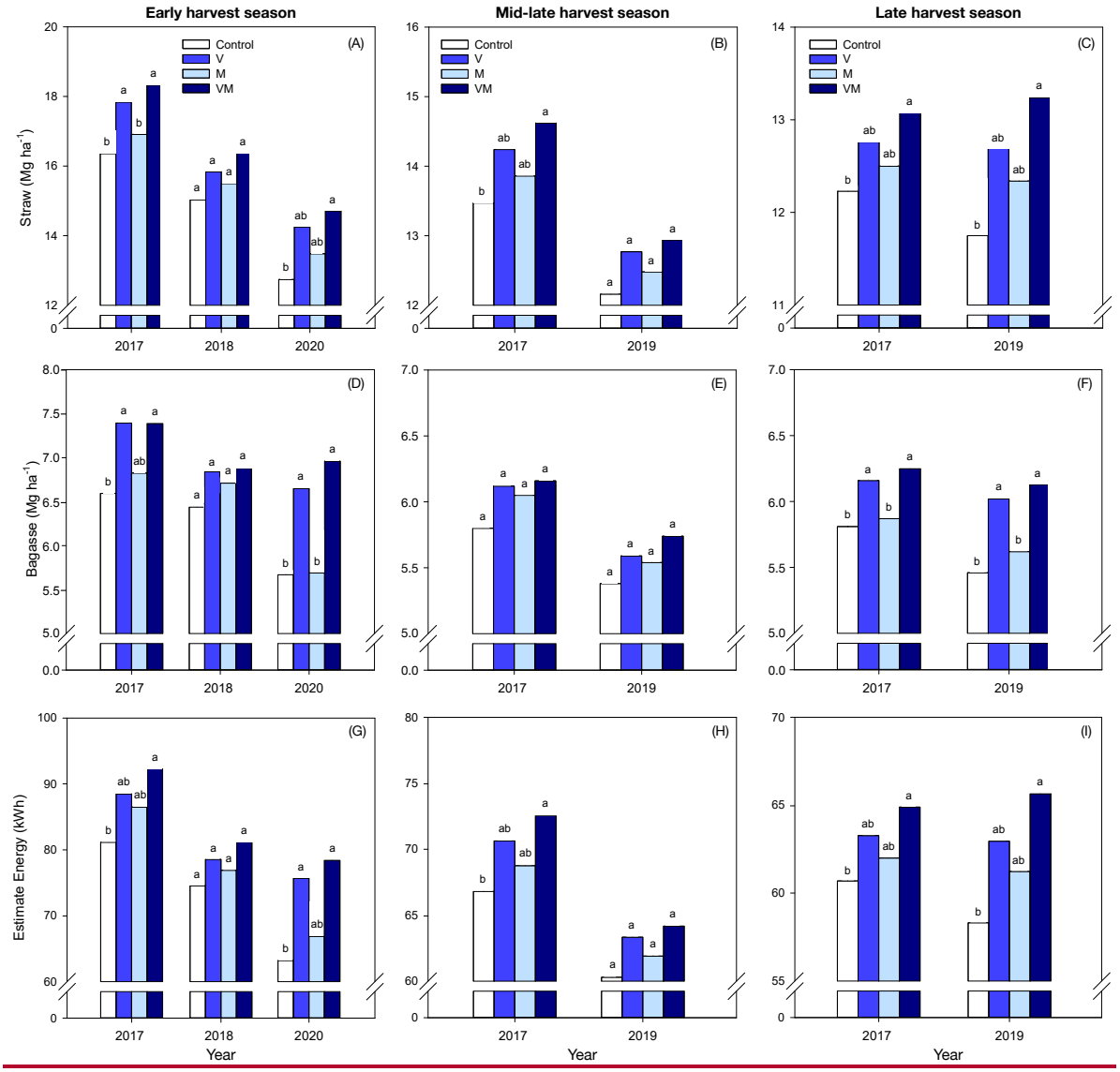


Figure 8. Straw (A, B and C), bagasse (D, E and F) and energy production (G, H and I) in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons. Control: no foliar application of biostimulant or nutrients; V: foliar application of biostimulant and nutrients at the sugarcane vegetative stage; M: foliar application of biostimulant and nutrients at the sugarcane maturation stage; VM: foliar applications of biostimulant and nutrients at both the vegetative and maturation stages. Bars with the same letter do not differ by the t test (LSD) at 10% probability.

5 Discussion

In this study, the foliar application of nutrients and the biostimulant at the vegetative and maturation stages of sugarcane increased the activity of carboxylation enzymes. Compared with the control, foliar application increased PEPcase activity by 1.1- to 2.3-fold. These increases in PEPcase activity were associated with higher sugarcane yield

as well as PEPcase is important in photosynthesis in C4 plants by its regulatory function and CAM pathways of photosynthesis such as actively participate in CO_2 fixation, pH regulation and stomatal opening (COUSINS et al., 2007). Foliar application with hormones such as the GAs promote photosynthesis both directly and indirectly, similar to CKs (MÜLLER; MUNNÉ-BOSCH, 2021; YUAN; XU, 2001). GAs also interact with other hormonal signaling pathways, such as AXs and BRs (CHAIWANON et al., 2016; NEMHAUSER; HONG; CHORY, 2006). The exogenous application of GAs has been shown to stimulate sugarcane growth and increase biomass (MOORE; BUREN, 1978). The foliar application of the phytohormone-containing biostimulant probably promoted the expression of genes encoding enzymes involved in GA biosynthesis, thereby increasing GA content and the effects of GAs, such as aerial growth and xylem lignification (ISRAELSSON et al., 2003).

In addition, AXs play an indirect role in photosynthesis by regulating chloroplast development, stomatal formation, and leaf innervation (SALAZAR-IRIBE; DE-LA-PEÑA, 2020). An increase in AX levels due to exogenous application or overexpression can reduce the number of stomata (QI; TORII, 2018) and induce mutations that cause loss of AX biosynthesis or excessive stomatal agglomeration (BALCEROWICZ et al., 2014; ZHANG et al., 2014), which will reduce leaf vein density, leaf hydraulic conductance and CO_2 assimilation (MCADAM et al., 2017). Thus, the GAs and AXs phytohormones activated SOD, CAT, POD and APX that have the potential of the activation of these enzymes mitigates abiotic stress (FAROOQ et al., 2019; GILL; TUTEJA, 2010).. The main function of SOD, CAT, POD and APX activation by hormones is to eliminate ROS through the dismutation of free radicals to prevent cellular damage since ROS directly influence photosynthesis and are produced when the redox state is disturbed by abiotic stress (BASU et al., 2016; GONG et al., 2020)

The foliar application of nutrients and the biostimulant in M and VM increased SOD, CAT and POD activity by more than 50%. This stimulation of antioxidant activity can induce long-lasting increases in metabolic activity, as evidenced by the increases in sugarcane growth and productivity. On average, the foliar application of nutrients and the biostimulant reduced H_2O_2 content by 31.4% and MDA content by 32.3%, indicating that this strategy holds promise for effectively increasing crop productivity and quality (LÓPEZ-MILLÁN; ELLIS; GRUSAK, 2005; RODRIGUES et al., 2021; WU

et al., 2018). Additionally, biostimulants containing GAs increase SOD and CAT activities in sugarcane, indicating better resistance to environmental stress (TRIPATHI; CHANDRA; PRAKASH, 2019). The stimulation of antioxidant enzyme activity by GAs reduces MDA and ROS levels (ABDEL-KADER, 2001; LI et al., 2010).

Plant height and stalk yield were greater when sugarcane received foliar application at the vegetative and maturation stage in early, mid-late and late harvest seasons. These two parameters are important biometric indicator because sucrose is stored in the stalks; taller plants have more and larger parenchyma cells for sugar storage (SPAUNHORST; TODD; HALE, 2019). Sugarcane height is the result of internode elongation, which is controlled by genetic (LI et al., 2012; QI et al., 2011), environmental (MAZZELLA; BERTERO; CASAL, 2000; PERALTA et al., 2002) and hormonal interactions (BUCK-SORLIN; KNIEMEYER; KURTH, 2005; LIU; YANG; LI, 2016). The process of internode elongation in sugarcane is mainly controlled by AXs and GAs (HILLMAN, 2011; SANTNER; CALDERON-VILLALOBOS; ESTELLE, 2009). Stimulating cell elongation and division by using exogenous growth regulators is an excellent strategy to achieve high yields (HEDDEN; SPONSEL, 2015; RADEMACHER, 2000). The addition of N and Zn via the nutrient complex associated with the biostimulant supports this process by promoting the synthesis of tryptophan, a precursor of AXs (MOGHADAM; ZAHEDI; ASHKIANI, 2013; WANG et al., 2019).

Contrarily to biometric parameters, sucrose and TRS were not influenced by foliar application. Foliar application did not interfere in the quality of the material from sugarcane due to increase of stalk yield rather than an effect on sugar synthesis pathways. However, the increases of plant height and stalk yield indirectly are linked to byproducts. On average, straw, bagasse and energy were highest in VM, with increases in bagasse production of 10.3%, 6.4% and 9.9% and straw and energy production of 11.9%, 7.5% and 9.7% in early, mid-late and late harvest seasons, respectively, compared with the control. Straw and bagasse can be used as a fuel in the cogeneration production of mills; thus, potential energy production is a byproduct from straw and bagasse.

Foliar application at the vegetative and maturation stages increased StY and SY, suggesting that nutrient fertilizers and biostimulants can be advantageous for sugarcane production. Due to higher rainfall and temperatures and a greater number of days before harvest, productivity parameters such as stalk diameter, plant height

and StY were highest in early harvest sugarcane, resulting in greater sugar yield and biomass parameters. In summary, the supplementary addition of nutrients and hormones at the vegetative and maturation stages of sugarcane is of fundamental importance because conventional fertilizer and foliar biostimulant management practices do not consider the stage of greatest sucrose production. To guarantee the success of sugarcane production, adequate management must consider the sugarcane stage.

6 Conclusion

The foliar application of the biostimulant and nutrients effectively increased sugarcane stalk yield and quality in early, mid-late and late harvest seasons. In addition, foliar application at the vegetative and maturation stages of sugarcane enhanced enzyme activity and biometric parameters. The foliar application of biostimulants combined with nutrients is a key management practice because biostimulants are precursors of plant hormone production and nutrients are involved in plant metabolism of its processes. The application of biostimulants and nutrients is an important alternative strategy to improve sugarcane antioxidant activity and growth, including taller and thicker stalks, resulting in greater stalk and sugar yields in all sugarcane harvest seasons.

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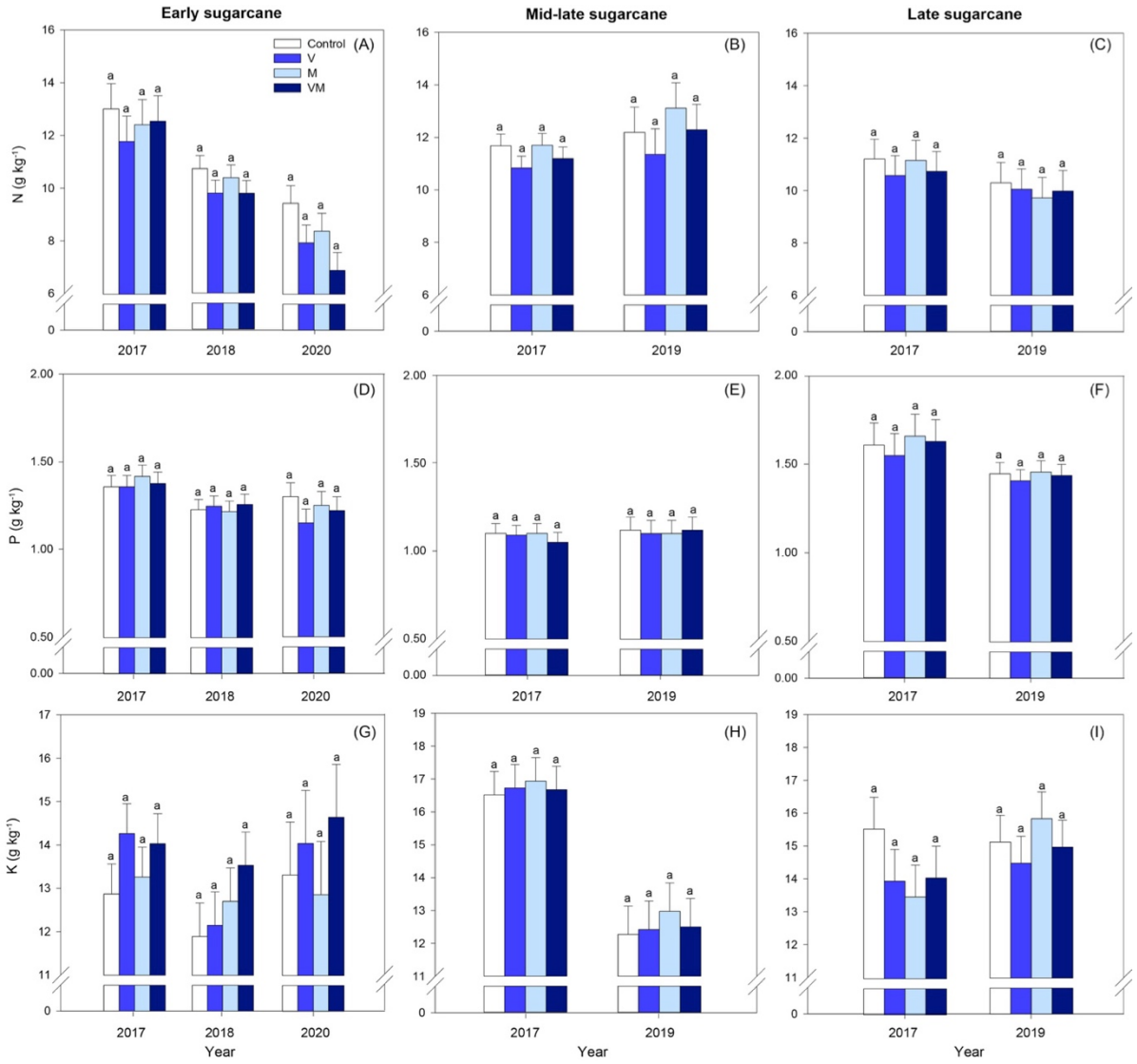
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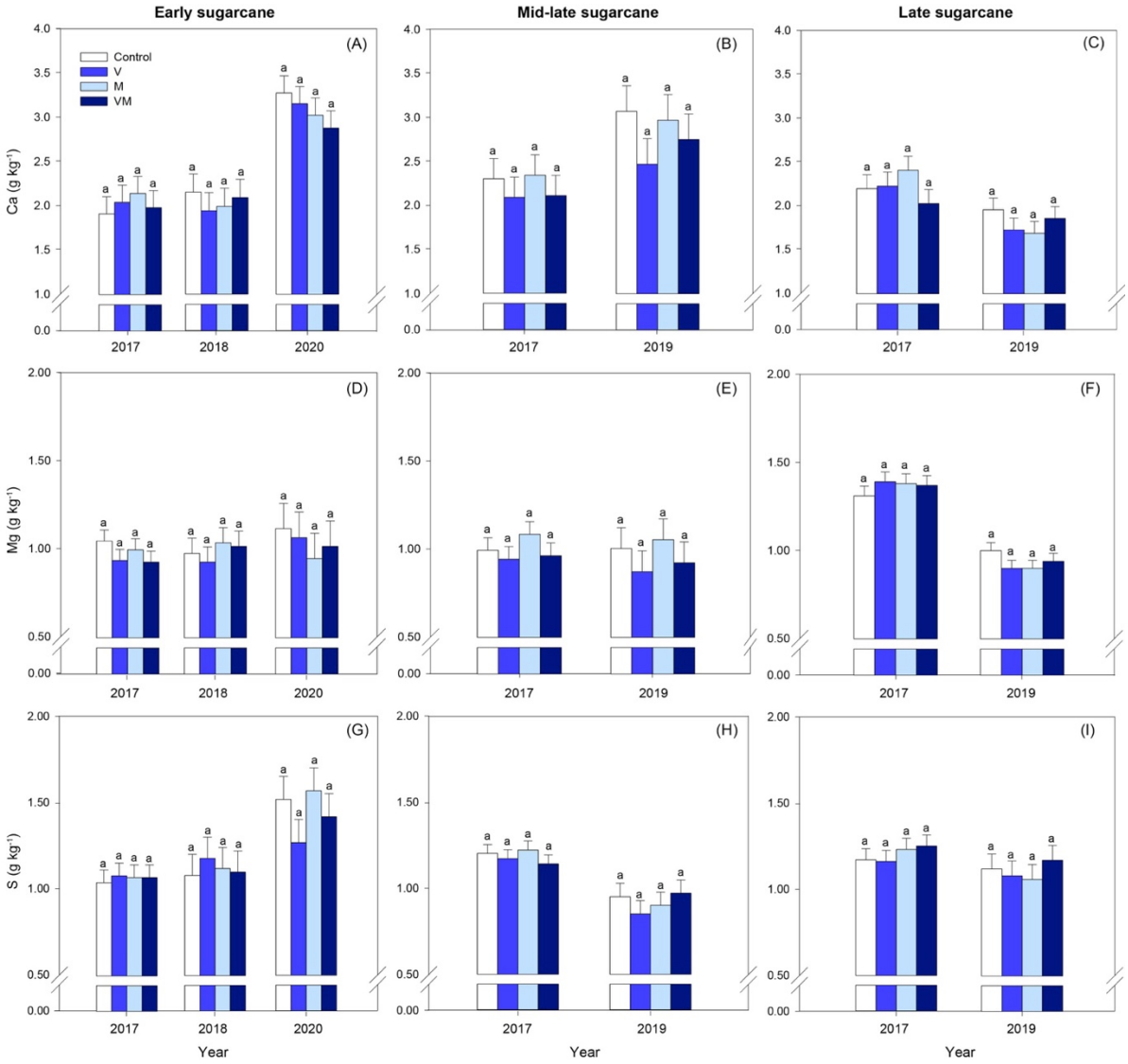
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Supplementary Material

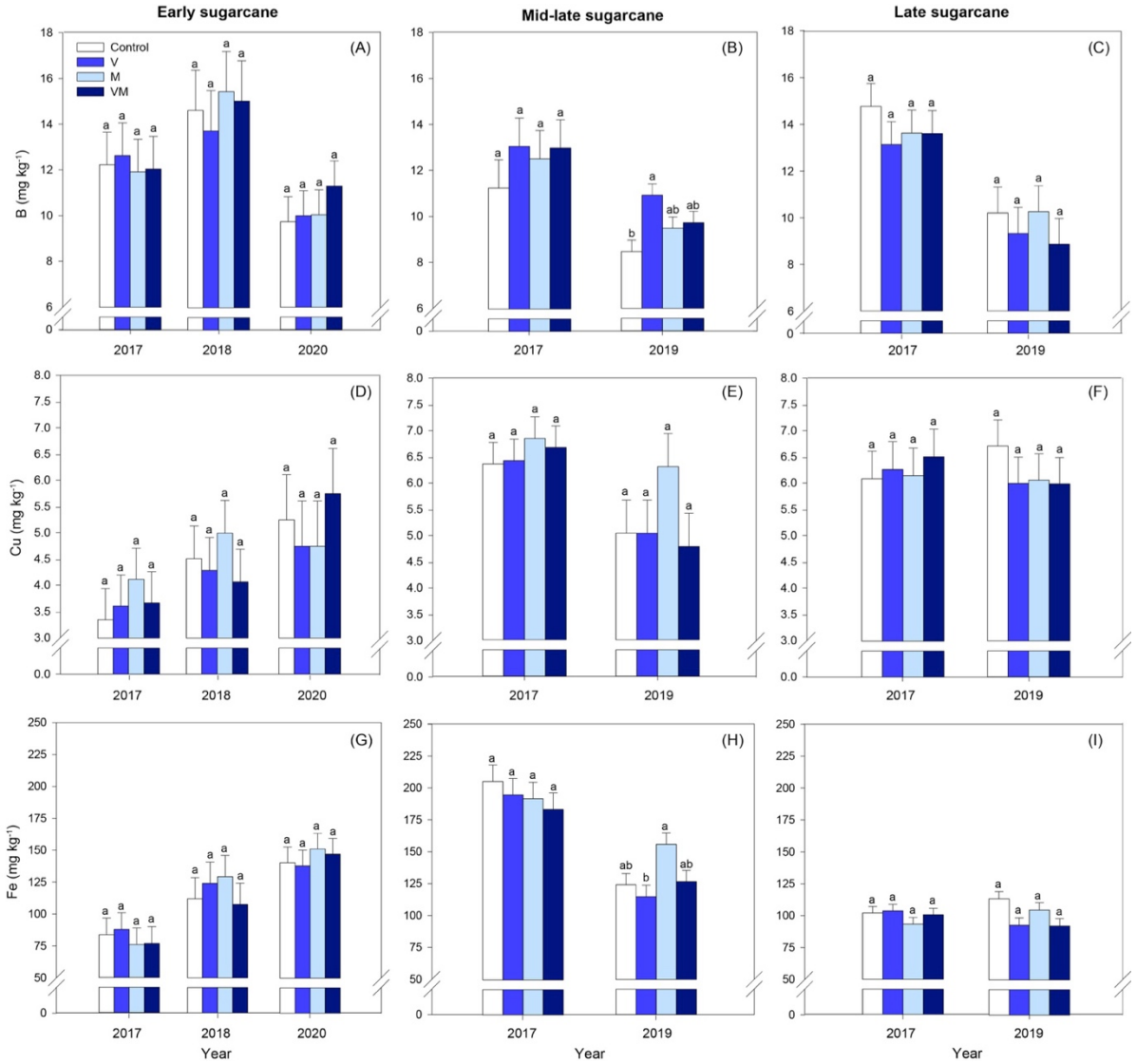
Supplemental material is compounded by five complementary figures.



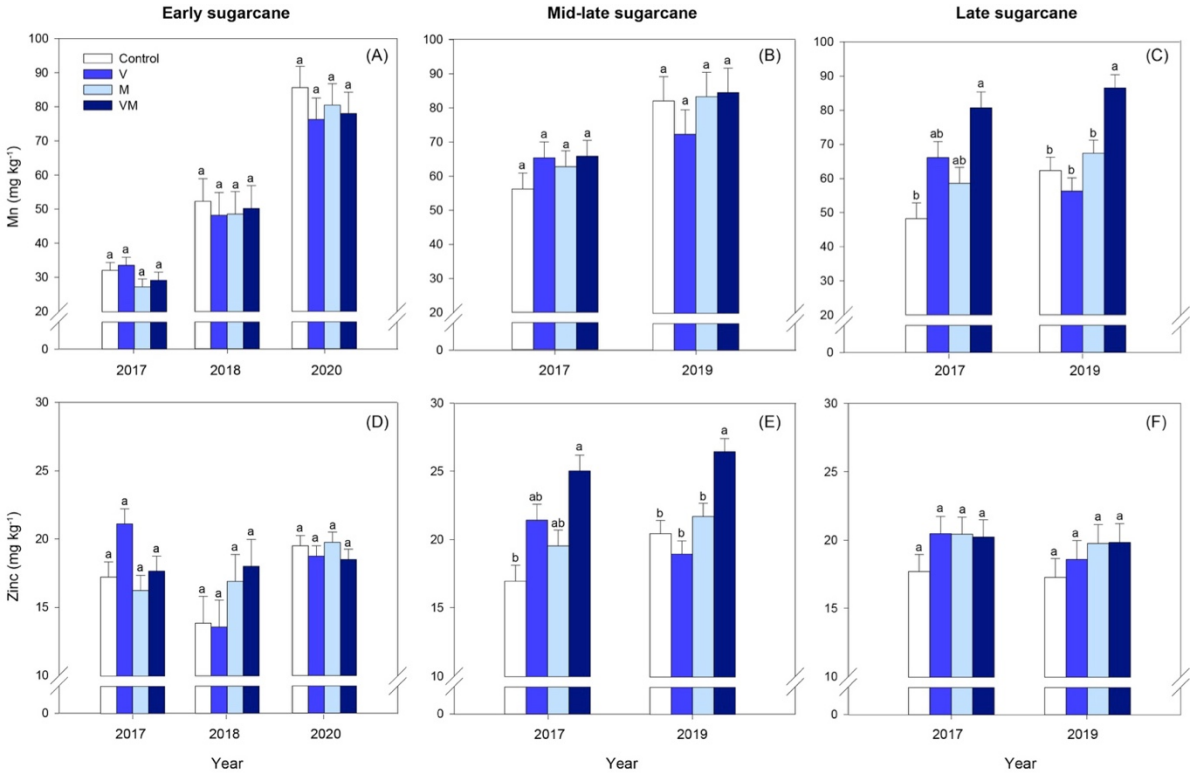
Supplementary Figure S1. Leaf nutrient contents in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons. Range of leaf nutrient contents considered suitable as reference (RAIJ et al., 1997) (g kg): N: 18-25 (A, B and C), P: 1.5-3.0 (D, E and F), and K: 10-16 (G, H and I). Bars with the same letter do not differ by the t test (LSD) at 10% probability.



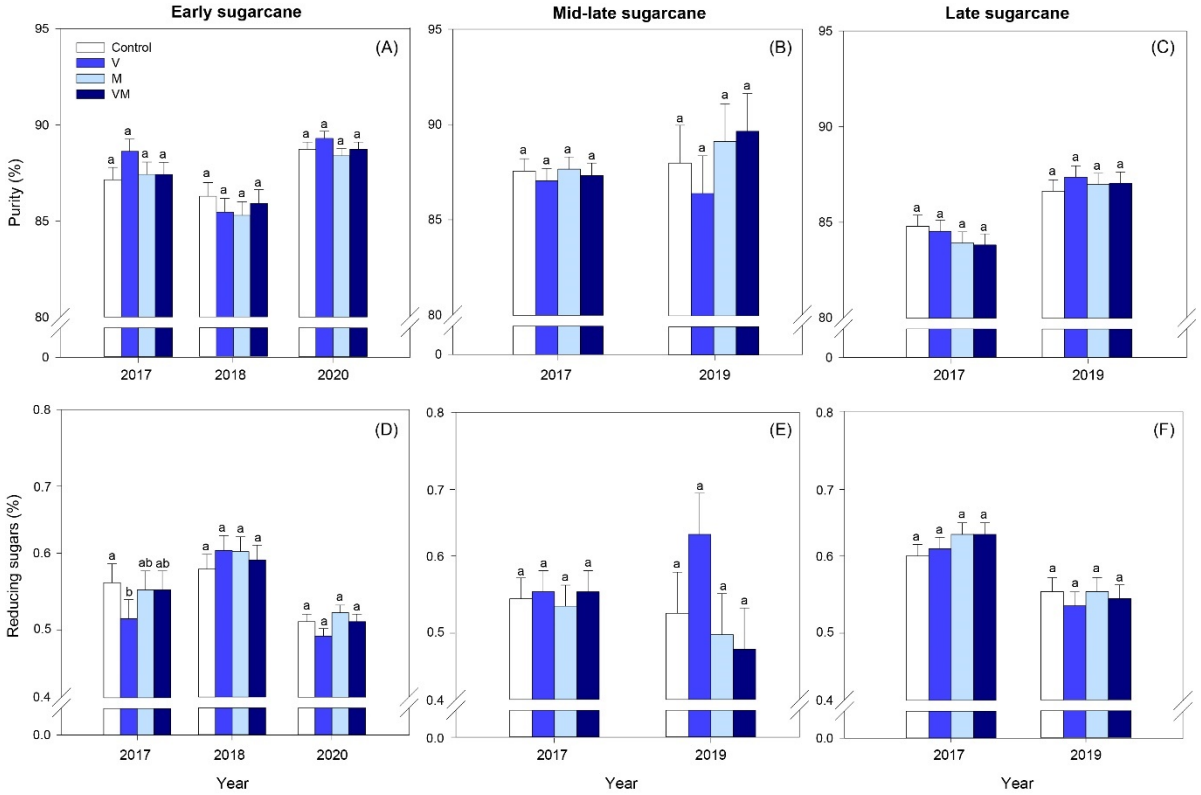
Supplementary Figure S2. Leaf nutrient contents in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons. The treatments are described in Figure 4. Range of leaf nutrient contents considered suitable as reference (RAIJ et al., 1997) (g kg): Ca: 2.0 -8.0 (A, B and C), Mg: 1.0-3.0 (D, E and F), and S: 1.5-3.0 (G, H and I). Bars with the same letter do not differ by the t test (LSD) at 10% probability.



Supplementary Figure S3. Leaf nutrient contents in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons. The treatments are described in Figure 4. Range of leaf nutrient contents considered suitable as reference (RAIJ et al., 1997) (mg kg): B: 10-30 (A, B and C), Cu: 6-15 (D, E and F), and Fe: 40-250 (G, H and I). Bars with the same letter do not differ by the t test (LSD) at 10% probability.



Supplementary Figure S4. Leaf nutrient contents in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons The treatments are described in Figure 4. Range of leaf nutrient contents considered suitable as reference (RAIJ et al., 1997) (mg kg): Mn: 25-250 (A, B and C) and Zn: 10-50 (D, E and F). Bars with the same letter do not differ by the t test (LSD) at 10% probability.



Supplementary Figure S5. Purity (A, B and C) and reducing sugars (D, E and F) in the treatments with different timings of foliar biostimulant and nutrient applications in early, mid-late and late harvest seasons The treatments are described in Figure 4. Bars with the same letter do not differ by the t test (LSD) at 10% probability.

FINAL CONSIDERATIONS

This study investigated, during four sugarcane harvests in 32 different locations, the effects of the use of plant growth regulators alone or in combination with nutrients applied via foliar application, in the vegetative and maturation stages in three different sugarcane harvest times and the effect provided on enzymatic, biometric, qualitative and bioenergetic parameters.

Through the application of the respective products, there was an increase in the activity of the photosynthetic enzyme phosphoenolpyruvate carboxylase (PEP; EC 4.1.1.31) and ribulose 1,5-bisphosphate carboxylase (Rubisco: EC4.1.1.39), consequently greater production of photosynthate, which may this being directed to both growth and storage in parenchyma cells.

There was a reduction in the activity of the enzymes malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) due to the increase in the activity of the antioxidant enzymes superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC:1.11.1.11) and proline concentration.

There were increases in the biometric parameters of diameter, length of stalks and productivity of stalks in both protocols at the three harvest times, as well as a significant increase in energy production.

Briefly, experimental models in their respective locations stimulated enzymatic activity, productivity and consequently biomass for energy production, with the best results when performed in two seasons, in the vegetative and in maturation stages, proving the proposed hypothesis.

Relevant questions about the effectiveness of the plant regulators GA_3, IAA, IBA, zeatin and kinetin and the nutrients N, B, Cu and Zn, throughout the complete cycle of sugarcane, deserve further investigation.