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Preharvest Biostimulant–Calcium Application Enhances Blueberry Fruit Quality Through Structural and Cuticular Modifications

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Abstract

The increased demand for higher-quality, longer-lasting blueberries has led to the development of preharvest strategies to improve their structural integrity sustainably. This study analysed the effects of the foliar application of two biostimulant–calcium (Ca) combinations, using *Ecklonia maxima* extract (EM + Ca) and glycine betaine (GB + Ca), on yield, biometric, mechanical, and histological properties, as well as cuticular wax composition of blueberries. Both biostimulants increased yield per plant and fruit weight and size in 'Duke', with superior results for GB + Ca. Fruit yield increased by 80% with GB + Ca and 40% with EM + Ca. Histological analysis showed increases in cuticle thickness, epidermal cell area and thickness, and hypodermal cell area and area/perimeter ratio. This thicker, denser tissue ultimately improved blueberries' mechanical properties. Specifically, 'Draper' berries treated with GB + Ca had 36%, 15%, and 20% higher values for flesh firmness, stiffness, and deformation work, respectively, relative to the control. However, cuticular wax accumulation was more pronounced with EM + Ca for the 'Duke' cultivar, increasing by 12%. Overall, GB + Ca had the greatest impact on blueberry structural quality and may represent a promising strategy to improve postharvest quality and commercial production.

Keywords: *Vaccinium corymbosum* L.; seaweed extract; glycine betaine; calcium; fruit firmness; histology; cuticular waxes; postharvest quality

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1. Introduction

Blueberry quality at harvest is crucial for the commercial success of fruit production, as blueberries are highly perishable and susceptible to mechanical damage, water loss, microbial spoilage, and biochemical degradation during storage and distribution [1–3]. Thus, adopting preharvest strategies that increase plant productivity and improve fruit quality (e.g., size, firmness, skin integrity, and resistance to physiological disorders) is essential to extend shelf-life and ensure consumer satisfaction. The use of biostimulants has sustainably improved crop development and fruit quality under both optimal and stress conditions [4,5]. Among the biostimulants available on the market, seaweed extracts derived from *Ecklonia maxima* (EM) and osmoprotectants such as glycine betaine (GB) have shown considerable potential for various fruit crops. EM extracts contain high concentrations of bioactive compounds, such as phlorotannins, alginates, auxins, and cytokinins, that modulate plant metabolism, thereby improving photosynthetic efficiency and antioxidant defence systems [6,7]. GB is a quaternary ammonium compound that primarily acts as an osmolyte, stabilising proteins and cell membranes and promoting cell homeostasis [8]. In addition, the application of essential plant nutrients, such as calcium (Ca), positively influences cell wall structure, acts as a secondary messenger in signal transduction pathways, and is a determinant of fruit firmness and postharvest quality [9,10]. However, Ca exhibits limited phloem mobility and is predominantly transported via the xylem through transpiration-driven mass flow [9], which can result in deficiency in fruits with low transpiration rates. In blueberries specifically, Ca uptake is further constrained by declining stomatal conductance during fruit development due to cuticular wax deposition, making foliar application of soluble Ca sources at appropriate phenological stages an effective strategy to bypass these physiological limitations and enhance fruit mineral status [11]. While the combined application of biostimulants and Ca has demonstrated synergistic effects on several fruit species, the underlying mechanisms remain partially understood.

Research on blueberries has primarily focused on agronomic parameters (e.g., productivity, vegetative growth, and fruit biochemical composition) and physiological responses, such as photosynthetic efficiency and antioxidant capacity (AC). However, it is also important to examine how preharvest application of these treatments affects the fruit's microstructural properties and the composition of the cuticular barrier. Fruit cuticle is composed of a cutin polymer, impregnated and covered by epicuticular and intracuticular waxes, constituting the primary interface of the fruit and playing an important role in regulating gas exchange, controlling water loss, and acting as a physical barrier against pathogens [12,13]. In particular, epicuticular waxes are complex mixtures of very long-chain aliphatic compounds whose composition and crystalline microstructure profoundly influence fruit surface properties such as hydrophobicity, gloss, and susceptibility to cracking and microbial colonisation [12,14,15]. The histological parameters of blueberry tissues are also related to their mechanical properties, such as firmness, which is a very important factor for the quality and conservation of this fruit [16]. Therefore, understanding how the combined application of biostimulants and Ca regulates microstructural properties is essential for elucidating the physiological basis of observed improvements in fruit quality and for optimising application protocols.

This research builds on previous studies investigating the effect of preharvest management practices on the 'Duke' and 'Draper' blueberry cultivars. Previous studies conducted by our group (manuscript in preparation) identified EM + Ca and GB + Ca as two effective treatments for enhancing the content of bioactive compounds, AC, photosynthetic performance, and yield in both cultivars. This study extends these findings by employing advanced analytical methods to elucidate how the two treatment approaches improve fruit physical quality through enhanced metabolic activity and resource allocation, as well as structural modifications in cellular architecture and cuticular properties that confer greater mechanical resistance and improved barrier function. The objective of this study was to (1) assess the impact of foliar application of EM + Ca and GB + Ca on blueberry yield, morphology, and texture; (2) determine the histology of berry fruit tissue quantitatively; and (3) determine the soluble cuticular wax content of fruits subjected to biostimulant–calcium treatments.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The experimental trial was conducted during 2024 in a commercial blueberry orchard located in Vilarandelo, Valpaços municipality, in the northern region of Portugal (41°40′8.38″ N, 7°19′22.81″ W; altitude 593 m a.s.l.). The investigation focused on two northern highbush blueberry cultivars, namely 'Duke' and 'Draper', grown under drip irrigation management. The plants were spaced 1.0 m within rows and 3.0 m between rows. Prior soil characterisation, performed at the UTAD soil analysis laboratory, indicated a slightly acidic pH of 5.6, medium textural class, elevated K_2O concentration (426 mg kg^{-1}), high available P_2O_5 content (105 mg kg^{-1}), and an organic matter content of 4.71%. All bushes received the same fertilisation, pruning, weed control, and pest management practices.

2.2. Experimental Design and Treatments

The experimental design comprised treatment groups incorporating $CaCl_2$ (Ca, Stopit®) at a rate of 3 L ha^{-1}: EM + Ca (*Ecklonia maxima* extract; Kelpak®, 3 L ha^{-1}) and GB + Ca (glycine betaine; Greenstim®, 3 kg ha^{-1}). An untreated water control was also included. Foliar applications were performed at BBCH 69 (petal fall), BBCH 79 (late green fruit), and BBCH 89 (before harvest) phenological stages [17] using a backpack sprayer in the morning, ensuring no rainfall within the following 24 h to allow adequate absorption. Fruits were hand-harvested from the selected blueberry bushes on 11 June ('Duke') and 20 June ('Draper').

2.3. Fruit Yield and Quality Assessment

2.3.1. Fruit Yield, Weight, and Biometric Properties

The fruits were harvested and assessed for productivity at commercial maturity. Yield per plant was calculated (kg) and presented as mean ± standard deviation (SD) (n = 9). Biometric properties were established from a random selection of 50 fruits per treatment. Individual fruit weight (g) was measured using an analytical balance (EW2200-2NM, Kern & Sohn GmbH, Balingen, Germany), and a digital calliper was used to record the following morphological fruit measurements: equatorial diameter (width, mm), polar diameter (height, mm), and calyx scar diameter (mm).

2.3.2. Texture Analysis

Multi-Parameter Texture Tests

Epidermis puncture force (EPF), epidermis rupture force (ERF), flesh firmness (FF), and external fruit firmness (EFF) were assessed using the TA.XTPlus texture analyser (Stable Micro Systems, Godalming, UK), at a velocity of 1.0 mm s^{-1}. EPF and ERF (N) were determined using a 2.0 mm diameter needle and cylindrical probes, respectively. FF and EFF (N mm^{-1}) were measured using a cylindrical probe with a diameter of 2.0 mm and a plate probe with a 7.5 cm diameter, respectively. Results are expressed as mean ± SD (n = 10).

Uniaxial Compression Test

The fruits were subjected to uniaxial compression, and three mechanical parameters were determined from the force–deformation curves (Figure 1). Stiffness (S, N mm^{-1}), calculated as the slope of the linear elastic region of the curve; deformation work (W_P, N·mm), as the area under the force–deformation curve up until tissue rupture; and force at break (F_b, N), as the maximum force recorded at the time of skin rupture, were determined using an INSTRON 5848 MicroTester (Instron, Norwood, MA, USA) with a 25 mm diameter cylindrical probe, using a constant crosshead speed of 5 mm min^{-1}. Data are reported as mean ± SD (n = 15).

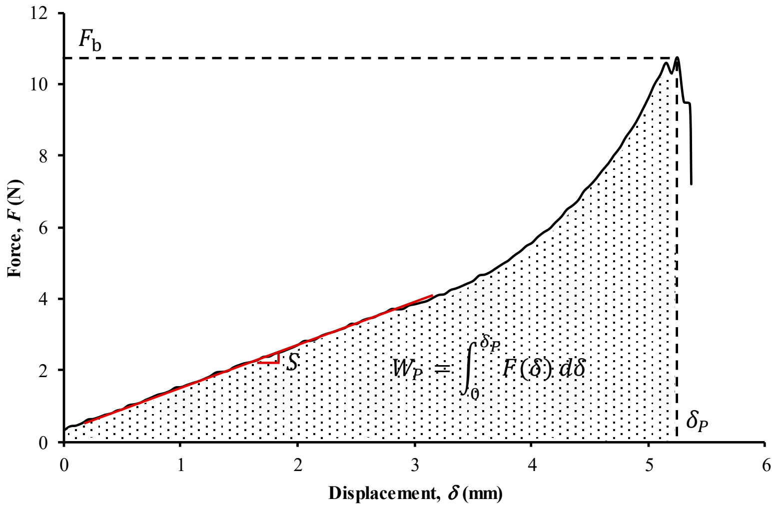

Figure 1. Typical force–displacement curve, with the schematic representation of measured parameters.

2.4. Histological Analysis

2.4.1. Sample Processing and Sectioning

Blueberry fruit tissues were prepared for light microscopy following a standard paraffin embedding protocol. Small fruit segments were placed in cassettes and immersed in FAA fixative (formalin–acetic acid–alcohol, 5:5:90 v/v) at room temperature for 24 h. Subsequently, they were transferred to 70% ethanol before dehydration through an ascending ethanol series (70%, 80%, 90%, 95%, 100%), with each step lasting 1 h and concluding with a graded immersion in 100% ethanol–xylene. Tissue clearing was then accomplished by immersion in pure xylene, rendering the tissue optically translucent and preparing it for paraffin infiltration (overnight at 56–58 °C). Transverse sections of 6 µm were then obtained using a rotary microtome (RM2255, Leica-Microsystems, Wetzlar, Germany), for a total of 3 sections cut per fruit and 5 fruit replicates per treatment. The sections were placed in a 30% ethanol bath, adhered to glass slides in a 53 °C water bath, and then stored in an oven at 30 °C for at least 48 h to ensure firm tissue adhesion. The sections were stained with 0.1% toluidine blue [18] for 10 min, rinsed under running water, and dehydrated through the ascending ethanol series described above, followed by at least 5 min in xylene, and permanently mounted using Entellan™ (Sigma-Aldrich, St. Louis, MO, USA).

2.4.2. Microscopic Imaging and Analysis

Histological sections were examined using an inverted optical microscope (Olympus IX 51; Olympus Optical Co., GmbH, Hamburg, Germany), coupled with an Olympus Colorview III digital camera and the image analysis program Cell^A (Soft Imaging System GmbH, Hamburg, Germany). Using high-magnification micrographs, the fruit skin tissues were morphometrically characterised for cuticle thickness, epidermal cell thickness and area, as well as hypodermal cell area and hypodermal area/perimeter ratio. Each fruit replicate was subjected to five transverse cross-sections, from which five independent measurements were taken per section, resulting in 25 total data points for each fruit replicate. Considering three fruit replicates per treatment, the total number of data points for each treatment was 75 (n = 75; 5 replicates × 3 sections × 5 measurements per section).

2.5. Soluble Cuticular Wax Content Determination

Soluble cuticular wax content was determined following the method described by Hamilton [19]. For each treatment, 2 fruits per treatment were submerged in 50 mL of a chloroform:methanol solution (75:25, v/v) for 2 min at 25 °C under agitation, a process repeated 10 times. The resulting solutions were transferred to individual beakers and allowed to air-dry at room temperature until complete solvent evaporation. The dried residues were gravimetrically quantified, and results expressed as micrograms of soluble cuticular waxes per gram of fresh weight (μg g^{-1} FW), with data presented as mean ± SD.

2.6. Statistical Analysis

Data were analysed using analysis of variance (ANOVA) and a post hoc Tukey's HSD test at a significance level of 5% to compare means, using version 27.0 of SPSS software (SPSS-IBM, Orchard Road, Armonk, NY, USA). This analysis included one-way and two-way ANOVAs to assess the impact of treatments and cultivars.

3. Results

3.1. Production per Plant and Biometric Properties

The results regarding the production per plant and the biometric properties of 'Duke' and 'Draper' blueberry cultivars submitted to foliar treatments are presented in Table 1. There was a significant interaction between treatment and cultivar for the weight, height, width and calyx scar diameter ($p < 0.05$), reflecting a different response by the cultivars relative to the treatments applied, and all the analysed parameters varied significantly between cultivars ($p < 0.001$). In general, 'Draper' blueberries showed higher values of yield and fruit size. The only exception was the calyx scar diameter, which was higher in 'Duke' fruits. Overall, production per plant, weight, height, width and height-to-width ratio were affected by the treatments ($0.001 < p < 0.05$).

Biostimulant–Ca sprays increased 'Duke' blueberry yield per plant compared to the control ($p < 0.001$). Specifically, plants treated with GB + Ca showed a higher yield (2.21 ± 0.31 kg $plant^{-1}$; an increase of 80%), followed by EM + Ca (1.74 ± 0.42 kg $plant^{-1}$; an increase of 41%) and control (1.23 ± 0.39 kg $plant^{-1}$). The same trend was observed for this cultivar's fruit weight, with the application of GB + Ca promoting significantly heavier fruits ($p < 0.001$), increasing weight by approximately 27% compared to the control. In addition, the application of EM + Ca significantly increased the weight of 'Duke' blueberries (+11%). A similar pattern was observed for fruit width ($p < 0.001$) after the application of EM + Ca (+5%) and GB + Ca (+9%). The latter treatment also increased fruit height by 7% ($p < 0.001$). Comparable results were verified for cv. 'Draper', whose weight, height and width of fruits increased significantly after the application of biostimulant–Ca sprays compared to the control ($p < 0.001$). Particularly, fruit weight increased by approximately 27% with the application of GB + Ca and by 11% with EM + Ca.

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Table 1. Influence of applied treatments (T) on production per plant and biometric properties in 'Duke' and 'Draper' blueberry cultivars (Cv).

Treatment	Production Plant^{-1} (kg)		Weight (g)		Height (mm)		Width (mm)		Ø Calyx Scar (mm)		Width/Height	
	'Duke'	'Draper'	'Duke'	'Draper'	'Duke'	'Draper'	'Duke'	'Draper'	'Duke'	'Draper'	'Duke'	'Draper'
Control	1.23 ± 0.39 ^{a}	4.06 ± 1.60 ^{A}	1.42 ± 0.31 ^{a}	2.29 ± 0.36 ^{A}	10.62 ± 0.83 ^{a}	12.95 ± 0.63 ^{A}	14.92 ± 1.06 ^{a}	17.71 ± 1.07 ^{A}	7.38 ± 0.75 ^{a}	6.97 ± 0.50 ^{A}	1.41 ± 0.08 ^{a}	1.37 ± 0.06 ^{A}
EM + Ca	1.74 ± 0.42 ^{b}	5.13 ± 1.50 ^{A}	1.57 ± 0.26 ^{b}	2.62 ± 0.38 ^{B}	10.91 ± 0.66 ^{a}	13.28 ± 0.62 ^{B}	15.61 ± 0.78 ^{b}	18.51 ± 1.03 ^{B}	7.22 ± 0.65 ^{a}	7.23 ± 0.64 ^{A}	1.43 ± 0.07 ^{a}	1.39 ± 0.06 ^{A}
GB + Ca	2.21 ± 0.31 ^{c}	5.01 ± 1.17 ^{A}	1.81 ± 0.27 ^{c}	2.64 ± 0.38 ^{B}	11.38 ± 0.65 ^{b}	13.27 ± 0.62 ^{B}	16.31 ± 0.83 ^{c}	18.44 ± 1.03 ^{B}	7.42 ± 0.70 ^{a}	7.09 ± 0.41 ^{A}	1.43 ± 0.06 ^{a}	1.39 ± 0.07 ^{A}
p (T)	<0.05		<0.001		<0.001		<0.001		>0.05		<0.05	
p (Cv)	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
p (T × Cv)	>0.05		<0.05		<0.05		<0.05		<0.05		>0.05	

Values are means ± SD (n = 9 for production per plant; n = 50 for biometric properties). Lowercase letters indicate significant differences ($p < 0.05$) between 'Duke' treatments, and uppercase letters indicate significant differences ($p < 0.05$) between 'Draper' treatments by Tukey's test.

3.2. Texture

3.2.1. Epidermis Puncture and Rupture Force, Flesh Firmness, and External Fruit Firmness

Blueberry texture was affected by the foliar application of the treatments and by the cultivars (Figure 2). The treatment × cultivar interaction was statistically significant ($p < 0.01$) for FF, and EPF, ERF, and FF showed significant differences among treatments ($p < 0.001$). In addition, cultivar significantly affected the EPF and EFF ($0.001 < p < 0.01$). For cv. 'Draper', blueberries sprayed with GB + Ca showed a 14% lower EPF ($p < 0.05$) and a 36% higher FF ($p < 0.01$) compared to the control. On the other hand, EM + Ca resulted in a 10% reduction in the ERF ($p < 0.001$).

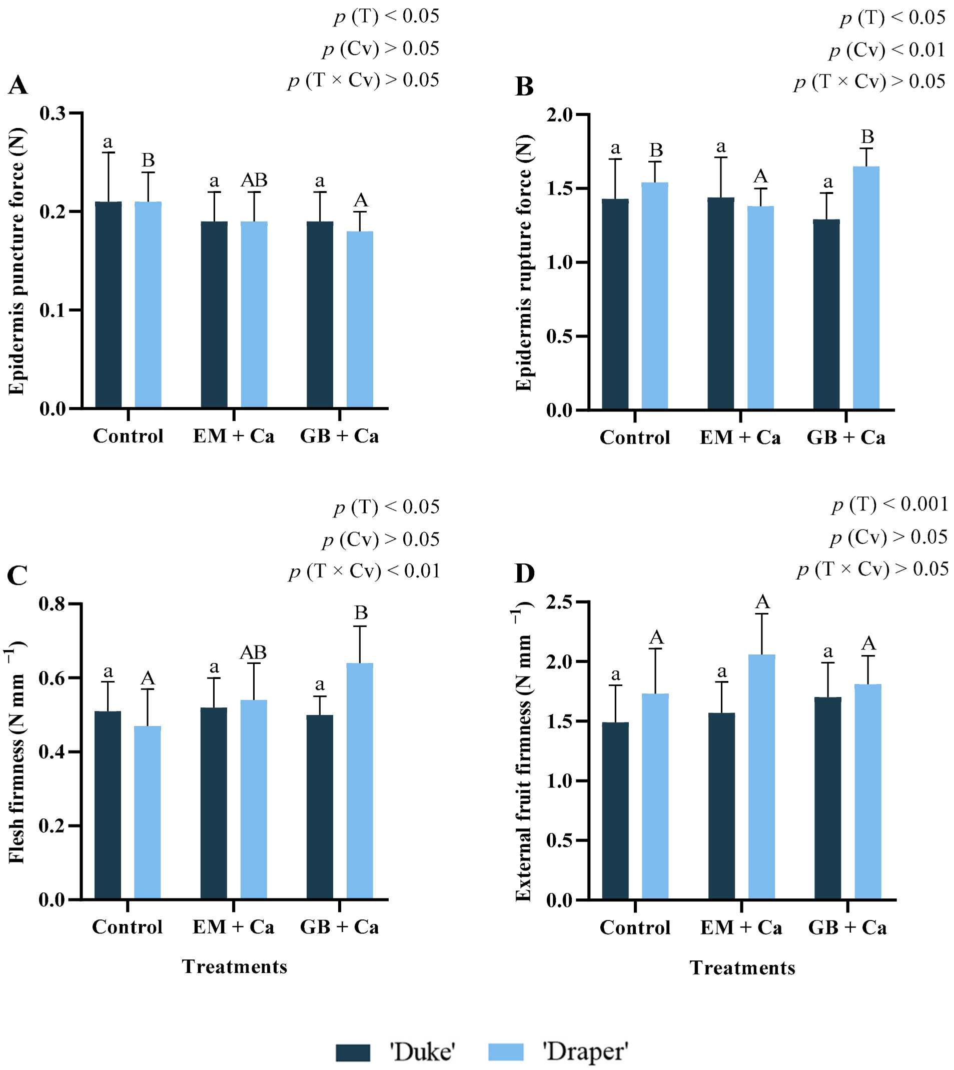

Figure 2. Treatment (T) effects on fruit texture parameters of 'Duke' and 'Draper' blueberry cultivars (Cv) using probes P2N (**A**), P2 (**B**,**C**), and P75 (**D**). Means ± SD ($n = 10$); different lowercase and uppercase letters indicate significant differences among treatments for 'Duke' and 'Draper', respectively (Tukey's test, $p < 0.05$).

3.2.2. Stiffness, Deformation Work, and Force at Break

Regarding stiffness (S), deformation work (W_P) and force at break (F_b), the interaction between treatment and cultivar was not significant ($p > 0.05$), indicating that treatment effects were consistent among cultivars. However, fruits treated with both formulations showed higher average S in both cultivars, with GB + Ca showing significant differences compared to the control ($p < 0.05$), with values approximately 20% higher for cv. 'Duke' and 15% for cv. 'Draper' (Table 2). In addition, the latter treatment increased the W_P of 'Draper' blueberries by 20% ($p < 0.05$). In contrast, F_b was not significantly affected by treatments ($p > 0.05$). All texture parameters were significantly affected by cultivar ($p < 0.001$), with 'Draper' fruits exhibiting higher S, W_P, and greater F_b than 'Duke', regardless of treatment.

Table 2. Effect of biostimulant treatments on stiffness (S), deformation work (W_P), and force at break (F_b) of blueberry fruits from 'Duke' and 'Draper' cultivars.

Treatment	S (N mm^{-1})		W_P (N·mm)		F_b (N)	
	'Duke'	'Draper'	'Duke'	'Draper'	'Duke'	'Draper'
Control	1.31 ± 0.18 ^{a}	1.66 ± 0.20 ^{A}	23.40 ± 5.88 ^{a}	32.71 ± 6.75 ^{A}	10.70 ± 2.15 ^{a}	12.48 ± 2.65 ^{A}
EM + Ca	1.46 ± 0.18 ^{ab}	1.71 ± 0.20 ^{AB}	23.98 ± 4.63 ^{a}	36.41 ± 4.24 ^{AB}	11.59 ± 1.90 ^{a}	13.87 ± 1.98 ^{A}
GB + Ca	1.57 ± 0.22 ^{b}	1.91 ± 0.33 ^{B}	26.35 ± 5.56 ^{a}	39.20 ± 6.16 ^{B}	11.69 ± 2.33 ^{a}	13.58 ± 2.99 ^{A}
p (T)	<0.001		<0.01		>0.05	
p (Cv)	<0.001		<0.001		<0.001	
p (T × Cv)	>0.05		>0.05		>0.05	

Values are means ± SD (n = 15). Lowercase letters indicate significant differences ($p < 0.05$) between 'Duke' treatments, and uppercase letters indicate significant differences ($p < 0.05$) between 'Draper' treatments by Tukey's test.

3.3. Histological Parameters

The interaction of treatment and cultivar was significant for cuticle thickness ($p < 0.05$). While this parameter was not significantly affected by biostimulant treatments in cv. 'Duke' ($p > 0.05$), 'Draper' fruits treated with GB + Ca showed greater cuticle thickness compared to the control ($p < 0.001$), with an increase of 17% (Table 3). Overall, cv. 'Duke' had a statistically thicker cuticle than cv. 'Draper' ($p < 0.001$).

Epidermal cell thickness and area were influenced by both treatment and cultivar ($p < 0.001$), with a significant treatment × cultivar interaction for thickness ($p < 0.01$). Fruits from cv. 'Duke' showed higher epidermal cell thickness and area than cv. 'Draper' ($p < 0.001$). GB + Ca treatments significantly increased epidermal cell thickness in both cultivars by 28–42% compared with the control ($p < 0.001$). Additionally, the use of EM + Ca increased epidermal cell thickness of 'Duke' blueberries by 35% ($p < 0.001$). A similar trend was observed for epidermal cell area. Fruits subjected to foliar treatments had significantly higher cell area (+32 to 45%) compared to the control ($p < 0.001$). It was also noted that GB + Ca increased cell area compared to EM + Ca in 'Draper' blueberries.

The different foliar treatments had a significant effect on hypodermal cell area and the area/perimeter ratio, which also varied significantly among cultivars ($p < 0.001$). Fruits from bushes sprayed with EM + Ca and GB + Ca presented higher hypodermal cell area (+43 to 72%) and hypodermal area/perimeter ratio (+18 to 33%) ($p < 0.001$).

Table 3. Influence of applied treatments (T) on histological parameters in 'Duke' and 'Draper' blueberry cultivars (Cv).

Treatment	Cuticle Thickness (µm)		Epidermal Cell Thickness (µm)		Epidermal Cell Area (μm^2)		Hypodermal Cell Area (μm^2)		Hypodermal Cell Area/Perimeter Ratio (µm)	
	'Duke'	**'Draper'**	**'Duke'**	**'Draper'**	**'Duke'**	**'Draper'**	**'Duke'**	**'Draper'**	**'Duke'**	**'Draper'**
Control	3.42 ± 1.11 ^{a}	2.44 ± 0.72 ^{A}	14.91 ± 3.49 ^{a}	13.06 ± 2.92 ^{A}	465.02 ± 146.16 ^{a}	353.94 ± 120.24 ^{A}	1107.06 ± 407.77 ^{a}	1175.18 ± 574.67 ^{A}	8.44 ± 1.73 ^{a}	8.35 ± 2.28 ^{A}
EM + Ca	3.62 ± 1.06 ^{a}	2.45 ± 0.75 ^{A}	20.10 ± 6.94 ^{b}	14.34 ± 4.64 ^{A}	657.30 ± 176.97 ^{b}	466.10 ± 208.02 ^{B}	1791.80 ± 500.46 ^{b}	1680.04 ± 781.03 ^{B}	10.62 ± 1.70 ^{b}	9.86 ± 2.61 ^{B}
GB + Ca	3.33 ± 1.55 ^{a}	2.85 ± 0.66 ^{B}	21.24 ± 5.58 ^{b}	16.70 ± 3.56 ^{B}	691.12 ± 293.47 ^{b}	585.26 ± 156.29 ^{C}	1902.33 ± 533.58 ^{b}	1732.86 ± 601.31 ^{B}	11.23 ± 1.60 ^{b}	10.12 ± 2.04 ^{B}
p (T)	>0.05		<0.001		<0.001		<0.001		<0.001	
p (Cv)	<0.001		<0.001		<0.001		>0.05		<0.001	
p (T × Cv)	<0.05		<0.01		>0.05		>0.05		>0.05	

Values are means ± SD ($n = 75$). Lowercase letters indicate significant differences ($p < 0.05$) between 'Duke' treatments, and uppercase letters indicate significant differences ($p < 0.05$) between 'Draper' treatments by Tukey's test.

3.4. Soluble Cuticular Wax Content

Fruit cuticular wax content was significantly influenced by the foliar application of the treatments, cultivars and the interaction between both factors ($p < 0.001$) (Figure 3). 'Duke' blueberries showed higher cuticular wax concentration than 'Draper'. Regarding preharvest foliar treatments, 'Duke' fruits sprayed with EM + Ca showed cuticular wax content approximately 12% and 44% higher than those of the control and those treated with GB + Ca, respectively ($p < 0.001$). In turn, GB + Ca showed a statistically lower soluble cuticular wax content than the control (−22%).

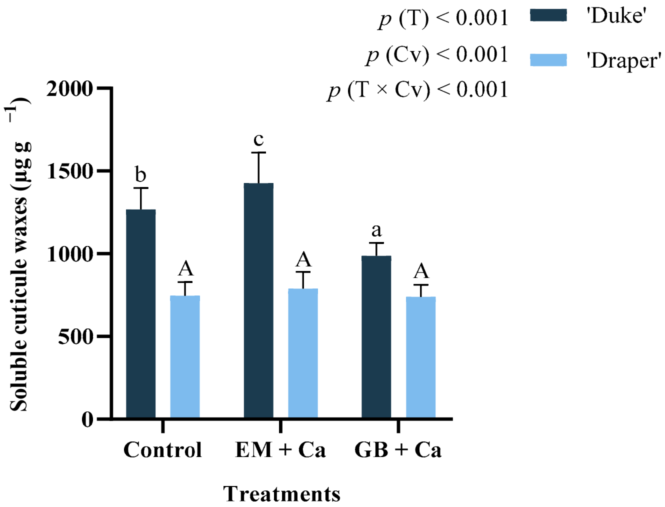

Figure 3. Soluble cuticular waxes content of 'Duke' and 'Draper' blueberry cultivars (Cv) as affected by spray treatments (T). Data are presented as means ± SD ($n = 10$). Different lowercase and uppercase letters indicate significant differences among treatments for 'Duke' and 'Draper', respectively, according to Tukey's test ($p < 0.05$).

4. Discussion

The foliar application of EM and GB, in combination with Ca, simultaneously increased blueberry crop productivity and fruit size, with different responses observed between the two cultivars studied. The increase in yield per plant after GB + Ca treatment applications to 'Duke' blueberries suggests that GB may have improved photosynthetic efficiency and plant water status, leading to increased photoassimilate accumulation and partitioning to reproductive organs. This osmoregulator protects cells by stabilising membranes and enzymes, thereby promoting the efficiency of the photosynthetic apparatus [20,21]. These results are consistent with those of Lopes et al. [22], who reported significant increases in blueberry production after the foliar application of EM and GB over two consecutive growing seasons.

Additionally, the increase in blueberry weight and dimensions in both cultivars after biostimulant application may be associated with greater cell division and expansion during development. EM is rich in compounds with hormonal activity (e.g., cytokinins, auxins, polyamines, and betaines), which stimulate cell division, tissue growth, and nutrient uptake [23,24]. According to Lopes et al. [22], the increase in the biometric properties of blueberries after EM application may be related to increased concentrations of plant hormones, through the activation of different biosynthetic pathways and network interactions that modulate hormonal pathways, as well as to the role of polysaccharides. The significant interaction between treatment × cultivar observed for fruit weight, height, width, and calyx scar diameter indicates that the magnitude of the response to biostimulants is genotype dependent. Overall, cv. 'Duke' showed more gradual and distinct responses to treatments, whereas 'Draper' responded more uniformly to both biostimulants across most biometric parameters. The stability of the observed width/height ratio indicates that, despite the increase in fruit dimensions, the general morphology and characteristic shape of the blueberries of each cultivar were preserved.

Regarding the texture and mechanical properties, differences were observed between cultivars, with more pronounced effects in cv. 'Draper'. The increase in FF compared to the control observed for this cultivar after GB + Ca application suggests that this treatment simultaneously promotes the reinforcement of the epidermal integrity and the internal structure of the fruit. In addition, this treatment improved fruit stiffness and deformation work without significantly altering breaking force, suggesting preferential action on the superficial layers of the fruit (i.e., cuticle, epidermis, and hypodermis), promoting cell turgidity and/or cell wall reinforcement. Ca plays an important role in fruit texture as it regulates cell metabolism and division, promotes osmoregulation, stabilises cell wall pectin by forming cross-links between polygalacturonic acid chains, and delays the action of hydrolytic enzymes that degrade the cell wall, such as polygalacturonase and pectin methylesterase [9,25–27]. These mechanisms are independent of each other and, together, explain the observed increase in stiffness, consistent with more turgid and compact hypodermal cells, as confirmed by the increase in the area/perimeter ratio of these cells. Similarly, Lopes et al. [22] reported that EM and GB increased the firmness of 'Duke' and 'Draper' blueberries, attributing this effect to the presence of polysaccharides and plant hormones in algae, in addition to improving the absorption and use of nutrients, with beneficial effects on the structure and composition of cell walls, as well as to the osmotic regulation and maintenance of cell turgidity by the GB. In addition, the significant increase in the dimensions of epidermal and hypodermal cells following biostimulant application may indicate structural remodelling that directly affects fruit firmness. The inclusion of Ca in both formulations may have contributed to cell wall strengthening, which may explain the increase in cell dimensions observed, particularly with GB + Ca. In fact, it was reported that GB application to apples and jujubes delayed the activity of enzymes responsible for cell wall degradation (e.g., pectinases, cellulase, and β-glucosidases), leading to the maintenance of a higher proportion of insoluble pectin and greater tissue firmness [28,29]. This process results in thicker, more intact cell walls, leading to increased epidermal thickness and cell volume. Furthermore, GB acts as an osmoprotectant in the stabilisation of membranes and proteins, maintaining membrane potential and energy metabolism [28,30,31], favouring a higher state of cell turgidity and controlled cell expansion, increasing the cell area without wall collapse. This hypothesis was corroborated after the application of GB in cherry and apple, where an increase in the cuticle and epidermis thickness was observed, as well as the diameter and area of these cells; in cherry, GB + $CaCl_2$ was also associated with a higher expression of expansin 1, a protein that loosens the cell wall and allows controlled cell expansion [31,32].

Our results show that blueberries treated with GB + Ca had larger epidermal and hypodermal cells, suggesting a synergistic effect between these two compounds on cell expansion and integrity, which may contribute to a more cohesive tissue structure. In addition, the increase in the area/perimeter ratio of hypodermal cells indicates a more compact and rounded cell morphology. This set of morphological adaptations may be beneficial by promoting a more uniform distribution of stresses along the cell wall, which can improve resistance to compression and deformation [33], consistent with the higher stiffness and strain energy observed after the application of GB + Ca, particularly in 'Draper' blueberries. In turn, the effect of EM may be related to the plant hormones present in the extracts [6] which, depending on the dose applied and the ripening stage at which the fruit is treated, can influence the number and size of fruit cells, or redirect growth toward other organs, ultimately affecting overall fruit cell dimensions [34–36]. The significant interaction between treatment and cultivar for cuticle and epidermal cell thickness indicates that the two cultivars respond differently to biostimulant application, reflecting their intrinsic differences. Specifically, cv. 'Draper' showed a more pronounced differentiation between EM + Ca and GB + Ca.

The significant difference in soluble cuticular wax content between the two cultivars studied is consistent with variability in cuticle composition reported for blueberry genotypes, associated with differences in bloom intensity [37]. According to our results, cv. 'Duke' has a higher concentration of waxes, which may partially explain the greater average cuticle thickness of this cultivar's berries. The increased concentration of waxes after the application of EM + Ca in 'Duke' blueberries may be related to a synergistic effect between the two compounds. Although seaweed extracts contain relatively low ABA concentrations, they are rich in cytokinins, which can induce endogenous ABA biosynthesis in plants [23,38], thereby increasing the total concentrations of waxes, β-diketones, and triterpenes [37]. The preharvest application of Ca can, in turn, increase wax content and preserve its structural integrity during storage. This essential nutrient is associated with the regulation of lipid metabolism and wax biosynthesis pathways, namely through the modulation of transcription factors SHINE/WIN1 and MYB96, which activate genes encoding fatty acid elongases and wax transporters, and modulate the expression of genes involved in the conversion of fatty acids into primary alkanes and alcohols [39]. According to these authors, increased terpenoid levels can enhance the fruit's AC and stress resistance by sequestering reactive oxygen species and improving water retention. Featuring a higher concentration of waxes, blueberries treated with EM + Ca may exhibit less water and mass loss, as well as greater preservation of tissue turgidity and firmness during storage [37,40,41]. Wax removal in grapes, for example, triggers the expression of genes responsible for cell wall degradation, such as pectinesterase, leading to cell breakdown [42]. In addition, fruits with higher wax content tend to have less water and pathogen ingress, reducing membrane and organelle disorganisation and slowing the senescence processes typical of storage [15,40,43]. On the other hand, the increase in the concentration of cuticular waxes in 'Duke' blueberries after the first applications of EM + Ca may have limited the absorption of these compounds in later stages of fruit development, since the development of cuticular waxes leads to a reduction in stomatal conductance as fruit ripening progresses [44,45], and this hypothesis may explain less significant effects on other parameters after the application of this treatment. Furthermore, the reduction in the concentration of waxes in cv. 'Duke' after the application of GB + Ca may be related to the reallocation of carbon resources to cell expansion processes, evidenced by the increase in the dimensions of epidermal and hypodermal cells. The osmoprotective effect of GB, associated with the mitigation of abiotic stresses, may also have led to reduced induction of wax biosynthesis [12,46].

5. Conclusions

The combined application of biostimulants and Ca improved fruit production and morphological performance relative to control, with GB + Ca emerging as the most effective strategy, particularly in terms of yield per plant in the 'Duke' cultivar. Both GB + Ca and EM + Ca increased fruit weight and biometric parameters across both cultivars, although GB + Ca was the best treatment to improve fruit weight and height. Fruit width was similarly enhanced by both treatments. Regarding textural properties, GB + Ca reduced epidermal puncture force while simultaneously increasing flesh firmness, fruit stiffness, and deformation work, suggesting a differential effect on the mechanical behaviour of skin and flesh. At the histological level, GB + Ca was the most effective in increasing epidermal cell thickness and area, while both GB + Ca and EM + Ca promoted hypodermal cell enlargement, as reflected by increased cell area and area/perimeter ratio. Our results also suggest that GB and EM have different effects on cuticular wax biosynthesis or deposition in blueberry fruit, as EM + Ca increased soluble cuticular wax concentration compared to the control, while GB + Ca reduced it. This research endorses the integration of biostimulants into preharvest blueberry production practices as a promising strategy to improve fruit quality, structural integrity, and postharvest performance. Future research should include comprehensive economic assessments, such as benefit–cost analysis and return on investment calculations, as well as postharvest storage trials to evaluate shelf-life extension, quality retention, and marketability under commercial conditions.

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Abbreviations

The following abbreviations are used in this manuscript:

a.s.l	Above sea level
ABA	Abscisic acid
AC	Antioxidant capacity
ANOVA	Analysis of variance
BBCH	Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie
Ca	Calcium
EFF	External fruit firmness
EM	*Ecklonia maxima*
EM + Ca	*Ecklonia maxima* combined with calcium
EPF	Epidermis puncture force
ERF	Epidermis rupture force
FAA	Formalin–acetic acid–alcohol
F_b	Force at break
FF	Flesh firmness
FW	Fresh weight
GB	Glycine betaine
GB + Ca	Glycine betaine combined with calcium
HSD	Honestly significant difference
S	Stiffness
SD	Standard deviation
W_P	Deformation work

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