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Effects of foliar biostimulants and two substrates (organic and conventional) on the aerial biomass and the major steviol glycosides (stevioside and rebaudioside A) of two *Stevia rebaudiana* (Bertoni) Bertoni cultivars in a pot experiment in Greece.

Abstract

Background: Steviol glycosides (SGs) from *Stevia rebaudiana* (Bertoni) Bertoni have garnered global interest due to their intense sweetness (over 300 times sweeter than sucrose) with negligible calories and potential health benefits. This study aims to address the low productivity of Greek stevia crops in terms of dry leaf yield (1235 kg/ha) despite favorable Greek agro-climatic conditions.

Aim: To investigate the agronomic reasons behind the low productivity of stevia cultivation in Greece, with final target to enhance the yield of dry leaves and major steviol glycosides (SGs) by exploring new cultivar and nutrition management strategies.

Approach: A pot experiment assessed the impact of three foliar plant biostimulants (calcite, seaweed extract, and amino acids) and two soil fertilizers (organic and conventional) on aerial biomass productivity and major steviol glycosides (stevioside and rebaudioside A) of two new stevia cultivars (cvs Olga and Ambrosia). Key growth and productivity traits such as height, covered area, leaf chlorophyll concentration, yield in dry leaves, leaf SGs concentration, and rebaudioside A/stevioside ratio were measured.

Main results: Under optimal Greek agro-climatic conditions (day length > 13 h, average temperature 23.4°C, soil moisture 41 - 43%, harvest time ≥ 110 DAT), an organic substrate enriched with N rate 1.2 g per plant from high-quality organic fertilizer (C/N < 20), along with a combination of foliar plant biostimulants (calcite 0.3%, seaweed extract 0.02%, amino acids 0.2% at low dose rates) facilitated significant improvements in dry leaf yield (87 g per plant) and major steviol glycosides concentration (17.6 g per plant), with major SGs concentration reaching 20.28% (11.09% stevioside + 9.22% rebaudioside A), and the highest rebaudioside A/stevioside ratio (0.83) in the tested stevia cultivar (cv. Olga).

Conclusion: The study suggests that the current substrate (N rate 0.88 g/plant) and cultivar (cv. SRB-128) contribute to the low productivity of stevia cultivation in Greece. It highlights the potential of suitable cultivars treated with appropriate substrate and foliar plant biostimulants to bridge the productivity gap between conventional and organic farming in the Mediterranean region. However, further research on biostimulant mechanisms, cultivar selection, and nutrition management is necessary to optimize leaf biomass, steviol glycosides concentration, and rebaudioside A/stevioside ratio across diverse local environments.

Keywords: *Stevia rebaudiana* (bertoni) bertoni steviol glycosides, rebaudioside a, stevioside, foliar biostimulants, calcite, seaweed extracts, amino acids, organic farming, conventional farming, day length, nutrition management, harvest index, reb a/stev ratio, days after transplanting (dat), biosynthesis of sgs

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Introduction

In 2011, following the EU's restrictions on tobacco cultivation and the approval of major steviol glycosides (SGs) as food additives, Mediterranean farmers embarked on testing farming strategies aimed at successfully domesticating the stevia crop. Despite these efforts, the introduction of a specific Indian cultivar (cv. SRB-128) by Greek farmers in 2012 resulted in relatively low production of dry leaves (1235 kg/ha, equivalent to 500 kg/acre), falling short of the economic viability threshold for commercial cultivation in Greece, which necessitates yields of at least 2000 kg/ha.[1] This productivity level in Greece contrasts sharply with the high yields (ranging from 3000 to 8680 kg/ha) reported in other regions globally,[2,3] highlighting the significant challenge of low productivity faced by the stevia crop in Greece.

Consequently, this study delves into the agronomic factors contributing to the low yield of dry leaves and explores the potential for enhancing productivity through the introduction of new cultivars and improved nutrition management practices involving soil fertilizers and foliar plant biostimulants.

Stevia plant –general information

Stevia rebaudiana (Bertoni) Bertoni, an herb indigenous to the mountains of the Amambay region in Paraguay and near the source of the Monday River on the border between Brazil and Paraguay, has a long history of use by native populations as a natural sweetener with purported health benefits. Renowned for its diterpene steviol glycosides (SGs) extracted from leaves and stems, stevia has gained global acclaim as a virtually non-caloric sweetener. Among the plethora of steviol glycosides (SGs) identified in stevia, only nine, including stevioside and rebaudioside A, are known for their high sweetness properties.[4,5]

The approval of major steviol glycosides (stevioside, rebaudioside A) as food additives by regulatory bodies in the USA and the European Union catalyzed a significant expansion of the stevia industry worldwide.[6,7] Stevia is among the 950 genera of the Asteraceae family, comprising over 230 species known for their intensely sweet essence.[8] It exhibits remarkable adaptability to a wide array of agro-climatic conditions, ranging from latitude 25° S to 60° N, longitude 130° W to 77° E, and altitude 500 to 1500 meters above sea level. Stevia thrives in various soil types, including sandy, sandy clay, or clay loam, with a preference for acidic soil (pH 4.5 - 5.5), and tolerates a broad temperature range (-6 to 44°C) and long photoperiods (13.0 - 15.0 hours/day).[3,9]

Optimal harvest timing for stevia typically precedes flowering and varies with cultivar and day length.[10] The genetic diversity within stevia has led to the development of approximately 90 new cultivars, with some exhibiting high heritability properties. The biosynthesis of SGs in stevia leaves involves intricate pathways governed by multiple genes. Traditionally, dry leaf concentrations of major SGs like stevioside and rebaudioside A range from 5 - 10% and 2 - 4%, respectively, and in reb A/stev ratio at ≤ 0.5.[5,11,12]

Cultivated stevia plants have heightened nutrient requirements compared to their wild counterparts. Adequate supply of primary and secondary macronutrients, micronutrients, and plant biostimulants (PBs) is crucial for enhancing plant growth and productivity. Calcium deficiency can impede SGs accumulation, while nitrogen positively correlates with foliage growth and SGs accumulation.[13,14] Soil fertilization with appropriate nitrogen rates and micronutrients has been demonstrated to improve yield and SGs concentration, particularly in Mediterranean regions.[3,10]

Under normal soil conditions, organic fertilizers with high organic matter content and low C/N ratios promote microbial decomposition and nutrient release.[15–17] Plant biostimulants, such as seaweed extracts and protein hydrolysates, enhance nutrient use efficiency and stress tolerance in crops.[18,19] Foliar application of calcites, seaweed extracts, and amino acids can further augment biomass yield and chlorophyll concentration in various crops, including stevia.[20]

The background problem of stevia in Greece

The primary issue affecting stevia crop productivity in Greece is the current low yield of dry leaves. Essential factors influencing stevia productivity include agro-climatic conditions, cultivar selection, and nutrition management. While Greek agro-climatic conditions generally meet optimal levels, they were not the primary focus of this study.

The current Indian cultivar (cv. SRB-128) and nutrition management using two soil N – P - K fertilizers (6 – 24 - 24 and 46 – 0 - 0 at dose rates of 100 and 140 kg/ha, respectively, translated as N rate 0.88 g/plant) result in a low yield of dry leaves (1235 kg/ha), contrasting with higher yields (3000 - 8680 kg/ha) reported in Italy. Testing new cultivars (cvs Ambrosia and Olga), along with different soil N – P - K fertilizers (mineral 15 – 6 - 12 and organic 7 – 7 - 7 at a dose rate of 150 kg N/ha, (equal to 1.2 g N per plant) and foliar plant biostimulants (calcite, seaweed extract, and amino acids), aims to identify reasons behind Greece's low productivity in Greek stevia.

The experimental design focuses on addressing key research questions:

a) Do the new cultivars and nutrition levels significantly affect plant growth and productivity traits?

b) Can these levels notably increase biometric and productivity parameters?

c) Which cultivar and nutrition level show the most promise for improving productivity in Greece?

The discussion section of the pot experiment results provides answers to these research questions.

Hypotheses for testing new cultivars and nutrition levels include:

i. The tested cultivars (cvs Ambrosia and Olga) are selections of productive parent cultivars (cv. Morita II x cv. Criolla, and cv. Morita II x cv. Eirete, respectively).[21,22]

ii. Both mineral and organic soil fertilizers with N rate 1.2 g/plant positively correlate with foliage growth, yield in dry leaves, major steviol glycosides leaf yield and concentration, and reb A/stev ratio.[3,17]

iii. Foliar plant biostimulants have shown positive effects on productivity in various leafy crops when applied alone or in combination with soil fertilizers.[23,24]

Aim of the research study

The aim of this research study was to investigate the agronomic reasons behind the low productivity of stevia cultivation in Greece, with final target the improvement of yield in dry leaves and major steviol glycosides (SGs). The main objective was to measure the effects of new cultivar and nutrition levels on agronomic characteristics of stevia, under Greek agro-climatic conditions. The results of the study may answer the posed research questions. Furthermore, our findings may recommend new farming practices to increase the stevia productivity in Greece, though the added knowledge does not refer directly to the crop management under field conditions.

Materials and methods

Experimental design and location

The pot experiment, conducted over one growth season from March to June 2016, was situated within a greenhouse at the Perrotis College of American Farm School in Thessaloniki, northern Greece (latitude 40○ 36' N, longitude 22○ 54' E, 5 m a.s.l.). The greenhouse, equipped with plastic sheet sidewalls and roofing, facilitated ample sunlight exposure and shelter while allowing for ambient temperature regulation. Thessaloniki's local climate adheres to the Mediterranean Csa classification, characterized by mild winters and springs devoid of frosts, transitioning into warm and humid summers and falls, with an annual rainfall range of 600 to 700 mm.[25]

Pots used in the experiment were filled with a soil mixture comprising clay loam and sandy loam at a ratio of 2:1 v/v, sourced from the American Farm School (AFS). This soil blend was enhanced with N – P - K fertilizers, and the plants received treatments with foliar plant biostimulants. Table 1 provides an overview of the nutrients available to the plants from both the pot soil and the foliar treatments.

On March 6, 2016, following germination, a total of 160 plantlets (80 of cv. Ambrosia and 80 of cv. Olga sourced from AGROAXON S.A. nursery) with heights ranging from 8 to 10 cm were transplanted into 160 pots, each with a soil volume of 20 L per pot. Additionally, four extra plantlets (2 of cv. Ambrosia and 2 of cv. Olga) were transplanted into four guard pots to ensure experimental integrity.

The experimental design comprised 160 pots subjected to 16 different treatments, with each treatment replicated 10 times. Pot numbering was conducted according to the factors, levels, and types of treatment, as detailed in Table 2. The pots, each containing 10 replicated treatments, were organized into 10 distinct random blocks. Within each block, 16 pots were treated with one of the 16 different treatments. The arrangement of the 16 pots within each block was randomized.

Table 1 Plant nutrients available from pot soil and treatments with soil fertilizers and foliar biostimulants

	Composition			
	ppm		**g/pot or g/plant**	
Elements	**AFS Soil**	**Pot soil**	**Soil Ferilizer**	**(N –P –K)**
	(Clay loam+Sandy loam, 2:1)		**(15 –6 –12 7 –7 –7)**	
Nitrogen (-NO3) (ppm)	38.9 – 45.32	0.78 – 0.90	1.2	1.2
Phosphorous (P) (ppm)	48.1	0.96	0.48	1.2
Potassium (K) (ppm)	614 – 615.52	12.3	0.96	1.2
Magnesium (Mg) (ppm)	470 – 496.19	9.7		
Calcium (Ca) (ppm)	5027 – 7181.38	122		
Iron (Fe) (ppm)	5.15 – 6	0.1		
Zinc (Zn) (ppm)	2.77 – 3.45	0.06		
Manganese (Mn) (ppm)	14.3 – 18.08	0.32		
Copper (Cu) (ppm)	1.17 – 1.8	0.02		
Boron (B) (ppm)	0.96 – 1.21	0.001		
Calcite (HG)			0.012	
Seaweed extract (FL)			0.0008	
Amino acids (AA)			0.008	

Table 2 The basic experimental design of 160 pots numbered per factor, level and 10 times replicated treatments

Total 160 pots (No 1 – 160)			
Substrate fertilizer		**Substrate fertilizer**	
(Conventional)		**(Organic)**	
80 pots (No 1-80)		**80 pots (No 81-160)**	
Cultivar	**Cultivar**	**Cultivar**	**Cultivar**
(cv. Ambrosia)	(cv. Olga)	(cv. Ambrosia)	(cv. Olga)
40 pots (No 1 –40)	40 pots (No 41 – 80)	40 pots (No 81 – 120)	40 pots (No 121 – 160)
Foliar biostimulants	**Foliar biostimulants**	**Foliar biostimulants**	**Foliar biostimulants**
40 pots (No 1 – 40)	40 pots (No 41 – 50)	40 pots (No 81 – 90)	40 pots (No 121 – 130)
Control	**Control**	**Control**	**Control**
10 pots (No 1 – 10)	10 pots (No 41 – 50)	10 pots (No 81 – 90)	10 pots (No 121 – 130)
HG	10 pots HG	10 pots HG	10 pots HG
10 pots (No 11 – 20)	10 pots (No 51 – 60)	10 pots (No 91 – 100)	10 pots (No 131 – 140)
HG+FL	10 pots HG+FL	10 pots HG+FL	10 pots HG+FL
10 pots (No 21 – 30)	(No 61 – 70)	10 pots (No 101 – 110)	10 pots (No 141 – 150)
HG+FL+AA	10 pots HG+FL+AA	10 pots HG+FL+AA	10 pots HG+FL+AA
10 pots (No 31 – 40)	10 pots (No 71 – 80)	10 pots (No 111 – 120)	10 pots (No 151 – 160)

Cultural practices

The tested levels of cultivar, soil fertilizers, and foliar plant biostimulants were applied at specified dose rates as follows:

Cultivar: Two levels (cvs Ambrosia and Olga), resulting from the out-crossing of parents cv. Criola x cv. Morita II and cv. Morita II x cv. Eirete, respectively.

Soil fertilizers: The common substrate for all pots consisted of a soil mixture of clay loam + sandy loam at a ratio of 2:1 v/v. Fertilization included N – P - K plus trace elements Mg, B, S, Zn from two different sources, one conventional (15 – 6 - 12) and one organic (7 – 7 - 7). The N rate of 1.2 g per pot was split into 4 partial applications at 15-day intervals. Each partial application comprised 2 g per pot for conventional and 4.3 g per pot for organic.

Foliar plant biostimulants: Four levels (control[1], HG[2], HG + FL[3], HG + FL + AA[4])

[1]**Control:** Water at a dose rate of 4 mL per pot for comparison.

[2]**HG (Herbagreen Z20):** A natural organic mixture of calcite and zeolite containing macro- micro- and beneficial elements, applied as a water solution of 0.3% HG at a dose rate of 4 mL per pot, containing 0.012 g HG.

[3]**FL (Herbagreen FL):** A natural organic mixture of plant and seaweed extracts, applied as a water solution of 0.02% at a dose rate of 4 mL per pot, containing 0.0008 mL FL.

[4]**AA (Herbagreen A):** A plant source mixture of amino acids (43-51%), seaweed extract (5%) from algae (Ascophyllum nodosum), and water. It contains 4% organic nitrogen (N) and 21-25% carbon (C), applied as a water solution of 0.2% at a dose rate of 4 mL per pot, containing 0.008 mL AA.

The application of foliar plant biostimulants at a dose rate of 4 mL per plant was repeated 4 times at 15-day intervals.

Agronomic observations – measurements – data collection

During the growth season from March to June 2016, the experiment was meticulously monitored with daily observations on environmental conditions, weed and pest management, and plant growth characteristics. The effects of three factors and eight levels were assessed across various traits:

Biometric traits: Plant height (cm) and cover area (cm2) were measured using square frames of different dimensions, 0.5 x 0.5 m and 1 x 1 m, respectively. Leaf relative chlorophyll concentration (Spad units) was determined with a portable Chlorophyll Meter SPAD-502 Plus device, measuring absorbance of chlorophyll at specific wavelengths. Volumetric water concentration (VWC %) and soil temperature were measured using a portable WET Kit device by inserting the probe into the pot soil. Biometric traits were recorded at four growth stages (50, 70, 85, and 110 DAT), and the data were organized into four sets.

Productivity traits: Yield (g/plant) of dry leaves, were measured at harvest time (110 DAT).

Dry leaves chemical traits: Concentration (%) of stevioside (stev), rebaudioside A (reb A), and total steviol glycosides (SGs), yield of stev, reb A, total SGs, and the reb A/stev ratio were assessed. Major steviol glycosides (stev and reb A) were extracted using a new solid phase extraction (SPE) method with C18 silica sorbent cartridges under vacuum. Analysis was performed through a validated new HPLC method using a Shimadzu LC-10AD VP system coupled with UV–visible wavelength detector SPD-M10Avp Diode array. The mobile phase comprised a mixture of acetonitrile/water, and the entire run time did not exceed 25 minutes. The quantitative determination of major steviol glycosides was calculated through calibration curves. This comprehensive approach allowed for the thorough evaluation of the effects of different factors and levels on the growth, productivity, and chemical composition of stevia dry leaves. Typical chromatogram of the HPLC method is shown in Figure 1.

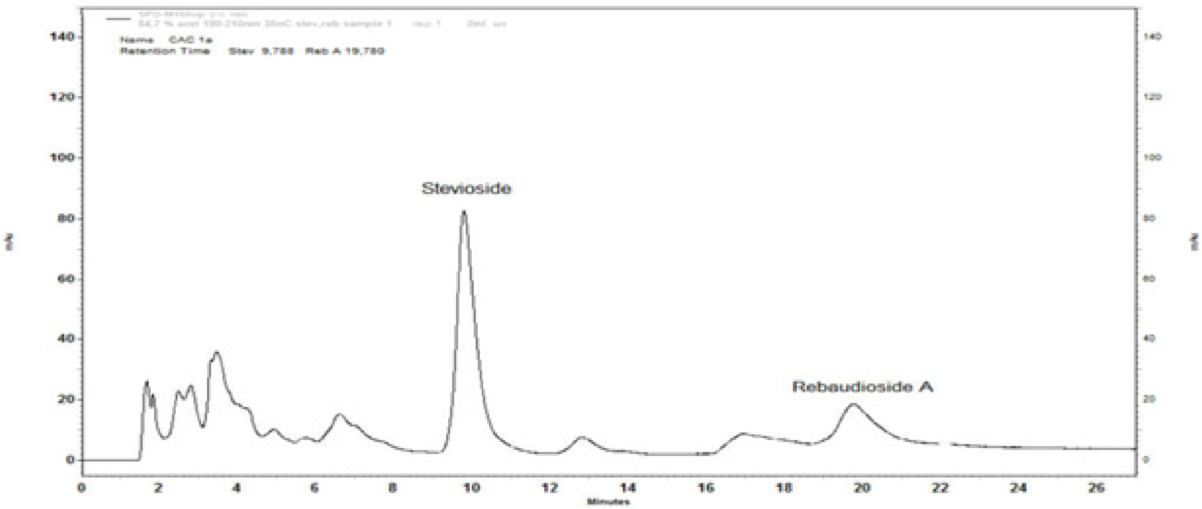

Figure 1 Representative chromatogram of major steviol glycosides by new HPLC method.

Statistical analysis

The study measured the effects of three variance factors (substrate fertilizer, cultivar and foliar biostimulants) on the plant growth and productivity characteristics of stevia, at four growth stages (data Set 1 –4). All data were subjected to analysis of variance (ANOVA) using JMP8 (www.jmp.com by SAS Institute Inc., Cary, NC, USA). Two-way ANOVA test estimated the significance of effects of two factors interaction pairs. One-way ANOVA test estimated the significance of the main effects of each separate factor (substrate fertilizer, cultivar and foliar biostimulants). For the effects on plant biometric and productivity characteristics and on dry leaves chemical characteristics, a two way ANOVA student's t-test (means comparisons) was carried out to compare the levels of the three factors, at probability level $\alpha = 0.05$ ($P < 0.05$). The means were separated when the ANOVA test showed significance at probability level < 0.05.[25]

Results

Two-way ANOVA t-test (means comparisons) estimated the significance of difference between the mean values of the compared levels, at probability level $\alpha = 0.05$ ($P < 0.05$). The main effect on plant productivity variables are summarized in Table 3. The main effect of each separate factor on biometric and dry leaves chemical variables are summarized in Table 4 and Table 5, respectively.

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Table 3 Means comparison in yield of dry leaves (g/plant)

Dry Leaves (g/plant)							
Substrate	**Cultivar**		**Foliar biostimulants**				**Mean**
	Ambrosia	**Olga**	**Control**	**HG**	**HG+FL**	**HG+FL+AA**	
Conventional	64.2	74.71	58.95 g	66.18 f	73.61 e	79.10 c	69.46
Organic	76.2	85.92	67.80 f	76.24 d	86.11 b	94.10 a	81.06
Mean	70.20 b	80.31 a	63.37	71.21	79.86	86.6	

Table 4 Main effects by each separate factor and level on plant growth variables at Set 4 (110 DAT)

Factors	**Levels**	**Variables**			
		Plant height (cm)	**Cover area (cm²)**	**SPAD (units)**	**VWC (%)**
Substrate Fertilizer	Conventional	93.01	571.13	44.28	42.24
	Organic	101.91	744	49.59	41.68
Cultivar	Ambrosia	92.76	583.75	44.24	43.36
	Olga	102.16	731.38	49.63	40.57
Foliar biostimulants	Control	86.25	503.5	41.74	41.33
	HG	94.68	599.25	45.48	41.8
	HG+FL	101.85	697.5	48.57	42.81
	HG+FL+AA	107.08	830	51.96	41.92

Table 5 Main effect by each separate factor and level on dry leaves concentration and yield of steviol glycosides

Factors	**Levels**	**Variables**							
		Stev %	**Reb A %**	**SGS %**	**Stev g/ plant**	**Reb A g/ plant**	**SGs g/ plant**	**Reb A/Stev Ratio**	**Dry leaves g/ plant/lot**
Substrate Fertilizer	Conventional	8.41	7.19	15.61	6.06	5.18	11.25	0.85	69.46
	Organic	7.29	6.81	14.08	6.11	5.75	11.87	0.93	81.06
Cultivar	Ambrosia	7.52	5.96	13.48	5.38	4.36	9.74	0.79	70.2
	Olga	8.19	8.04	16.2	6.8	6.57	13.37	0.98	80.32
Foliar Biostimulants	Control	5.31	4.87	10.18	3.35	3.16	6.5	0.92	63.37
	HG	6.13	5.44	11.57	4.37	3.94	8.31	0.89	71.21
	HG+FL	8.87	8.48	17.35	7.06	6.77	13.83	0.96	79.85
	HG+FL+AA	11.09	9.22	20.28	9.58	8	17.58	0.83	86.6

Results by main effects of tested variables

In optimal Greek agro-climatic conditions, various factors including soil fertilizers (conventional and organic), cultivars (Ambrosia and Olga), and foliar biostimulants (calcite, seaweed extract, amino acids) were tested for their effects on biometric, productivity, and leaf chemical traits at harvest time 110 days after transplanting (DAT).

Regarding substrate fertilizers, organic fertilizer generally led to higher biometric traits except for VWC, with the highest values in plant height (102 cm), cover area (744 cm²), and chlorophyll concentration (50 Spad units). Organic fertilizer also resulted in higher dry leaf yield (81 g/plant) compared to conventional (69 g/plant), with differences in stevia glycosides concentrations and yields.

Cultivar differences were observed as well. Cv. Olga generally exhibited higher values in plant height (102 cm), cover area (731 cm²), and leaf chlorophyll concentration (50 Spad units) compared to Ambrosia. However, cv. Ambrosia had the highest volumetric water content (VWC) at 43.3%. Cv. Olga also yielded more dry leaves (80 g/plant) compared to Ambrosia (70 g/plant), with higher concentrations of major steviol glycosides (16.20%).

Foliar plant biostimulants generally increased biometric traits across growth stages, with the combination of calcite, seaweed extract, and amino acids (HG+FL+AA) resulting in the highest values. This combination also led to the highest dry leaf yield (86 g/plant) and concentrations of stevia glycosides (SGs).

Overall, cv. Olga, organic fertilizer, and the combination of HG+FL+AA plant biostimulants showed promising results in enhancing various traits related to plant growth, productivity and leaf chemical traits.

Discussion

This study investigated the agronomic reasons behind the low productivity of stevia cultivated under optimal Greek agro-climatic conditions. The results at harvest time (110 DAT) indicate that the tested two levels substrate fertilizers (conventional and organic), two levels cultivar (cvs Ambrosia and Olga), and three levels foliar plant biostimulants calcite (HG), seaweed extract (FL), amino acids (AA) behaved differently on biometric, productivity and leaf chemical traits. The main effects of tested variables showed that new levels of substrate fertilizers, cultivar and foliar biostimulants may significantly improve the biometric, productivity and leaf chemical characteristics of stevia. These results indicate that the current cultivar and nutrition management are responsible for the low productivity of Greek stevia. The discussion is dealing with answers to the research questions, explanation of findings, comparison with results of the cited literature, achievement of the study aim, and implications to the background problem of stevia cultivation in Greece.

Main effects of two levels substrate fertilizer

Biometric – productivity – leaf chemical traits: The tested substrate fertilizer levels, both conventional and organic, exhibited a significantly positive effect on all measured growth and productivity traits, underscoring their essential role in stevia nutrition management. However, differences were observed between the effects of the two soil fertilizer sources.

In terms of biometric traits, organic fertilizer generally resulted in higher values than conventional throughout the four vegetative stages, except for volumetric water concentration. The observed biometric results (height 93 −102 cm, cover area 571 −744 cm^2, and leaf relative chlorophyll concentration 44 −50 Spad units) are comparable to or higher than those reported in the literature (height 85 – 95 cm, cover area 25 −33 cm^2, leaf relative chlorophyll concentration 18 −36 CCI units).[3,16,27,28]

Regarding productivity traits, both fertilizer sources showed a significantly positive effect, with organic fertilizer achieving a notably higher dry leaf yield compared to conventional fertilizer. The results suggest that soil fertilizer sources at an N rate of 1.2 g/plant could potentially lead to dry leaf yields up to 80% higher than those observed with the current field substrate in Greece. Our results, with dry leaf yields (69 - 81 g/plant at harvest time 110 DAT), are comparable to or higher than dry leaf yields reported in Italy (82 - 103 g/plant at 127 DAT) by Angelini and Tavarini,[3] in Greece (53 - 60 g/plant at the first harvest) by Vasilakoglou et al,[28] and in France (11 - 21 g/plant at 70 DAT) by Barbet-Massin et al.[29] These findings align with previous research indicating the positive association between soil N – P - K fertilizers at an N rate of 1.2 g/plant and improvements in foliage growth, yield of major steviol glycosides, and the rebaudioside A/stevioside ratio. The efficacy of the N rate of 1.2 g/plant may be attributed to enhancements in various physiological processes within the plant.[16] While other organic fertilizer sources with the same N rate have shown lower performance compared to conventional sources, the favorable performance of the organic fertilizer in this study may be attributed to optimal pot soil conditions (moisture 43.1%, temperature 23.4°C) and the characteristics of the organic fertilizer, including high organic matter content (25%) and a low C/N ratio (25/7 = 3.6). This interpretation aligns with the hypothesis that organic fertilizers characterized by high organic matter content and low C/N ratios (< 20) can accelerate the rate (> 50%) of microbial decomposition of organic matter and subsequent N mineralization, resulting in the rapid release (within 3 - 6 weeks) of water-soluble N and expedited plant uptake (FAO, 2006).[15] Overall, these results underscore the importance of substrate fertilizers, particularly at an N rate of 1.2 g/plant, in improving growth and productivity traits in stevia cultivation.

In leaf chemical traits, the analysis revealed a significantly positive effect ($P < 0.0001$) from both fertilizer sources. The conventional fertilizer tended to result in higher concentrations of stev (8.4%), reb A (7.2%), and major steviol glycosides (15.6%), while the organic fertilizer led to higher yields of stev (6.1 g/plant), reb A (5.8 g/plant), total SGs (11.9 g/plant), and a higher reb A/stev ratio (0.93). The disparate performance of the two fertilizer sources could be attributed to variations in the temporal availability of nitrogen from the organic and inorganic sources. These results are consistent with previous findings suggesting that under suitable agro-climatic conditions (temperature and long day length), soil N – P - K fertilizers at an N rate of 1.2 g/plant may prolong the vegetative stage and increase the reb A concentration and reb A/stev ratio.[17] This phenomenon may be explained by the hypothesis that rebaudioside A is formed through the glucosylation of stevioside, and during prolonged vegetative periods, a portion of stevioside is converted to rebaudioside A.[11,12,30,31] Our findings regarding the dry leaf concentrations of stev (7.3 - 8.4%), reb A (6.8 - 7.2%), total major steviol glycosides (14 - 16%), and reb A/stev ratio (0.85 - 0.93) are comparable to or higher than the typical reported profile of dry leaf concentrations in steviol glycosides (stev 5 - 10%, reb A 2 - 4%, total steviol glycosides 10 - 20%, and reb A/stev ratio 0.58 - 0.85) (Gasmalla et al, 2014).[17] The tested soil N – P - K fertilizers, both conventional (15 – 6 - 12) and organic (7 – 7 – 7), applied at an N rate of 1.2 g/plant, appear to be promising alternatives to the current soil N – P – K fertilizers (6 – 24 - 24 and 46 – 0 – 0) applied at an N rate of 0.88 g/plant.

Main effects of two levels cultivar

Biometric – productivity – leaf chemical traits: It appears that both tested cultivars, cvs Ambrosia and Olga, exhibited a significantly positive effect on all measured plant growth and productivity variables, underscoring the essential role of cultivar in stevia crop management. However, the two cultivars differed in their effects on growth and yield, with cv. Olga generally resulting in higher values than cv. Ambrosia across biometric, productivity, and leaf chemical traits.

The observed biometric results (height 92.76 - 102.16 cm, cover area 583.75 - 731.38 cm^2, and leaf relative chlorophyll concentration 44.24 - 49.63 Spad units) are comparable to or higher than those reported in the literature. For instance, studies in Italy by Angelini and Tavarini [3] and Tavarini et al.,[16] reported height values of 94 cm and 85 cm, respectively, and chlorophyll concentration of 33 mg cm^{-2} for the Italian clones NU2008 and RG, respectively. In Greece, Vasilakoglou et al.,[26] reported lower chlorophyll concentration indices (24 - 36 CCI units) for cvs Morita II and Candy-stevia. In India, Kumar et al.,[27] reported lower corresponding cover areas (490 - 855 cm^2) and chlorophyll concentrations (18 - 36 CCI units) for an unknown Indian cultivar.

Regarding productivity traits, our results in dry leaf yield (mean 70.20 - 80.31 g/plant) for cvs Ambrosia and Olga, are comparable to or higher than those reported in Italy (82 - 103 g/plant) for the Italian clone NU2008,[2] in Greece (53 - 60 g/plant) for cvs Morita II and Candy-stevia,[28] and in France (56 - 68 g/plant) for cultivars selected from cv. Criola.[29]

In leaf chemical traits, our results in dry leaf steviol glycosides concentration, yield, and reb A/stev ratio (Stev 7.52 - 8.19%, Reb A 5.96 - 8.04%, major steviol glycosides 13.48 - 16.20%, Stev 5.38 - 6.80 g/plant, Reb A 4.36 - 6.57 g/plant, major steviol glycosides 9.74 - 13.37 g/plant, and reb A/stev ratio 0.79 - 0.98) are comparable to or higher than those reported in the literature for different cultivars. Lower or comparable dry leaf concentration and yield of Stev, Reb A and major steviol glycosides (SGs), and Reb A/Stev ratio have been reported for different cultivars by various authors. For instance, Stev 7.4%, Reb A 6.0%, SGs yield 11 - 14 g/plant,[3] Stev 4.3 - 4.9%, Reb A 2.7 - 3.5%, and Reb A/Stev ratio 0.58 - 0.85 (Tavarini et al, 2015b), SGs yield 0.7 - 8.4 g/plant,[29] major steviol glycosides 10.4 - 12.6%, SGs yield 6 - 7 g/plant, Reb A/Stev ratio 0.61.[28]

The robust productivity of the tested cultivars can be primarily attributed to the high heritability of leaf yield (h^2= 62.1) and leaf steviol glycosides concentration (h^2= 76.6) inherited from their productive parents, cv. Morita II x cv. Criolla, and cv. Morita II x cv. Eirete, respectively. Additionally, the selection of the parents of cv. Olga, namely cvs Morita II and Eirette, for their high leaf concentration of reb A in intensive cultivation systems further contributes to its productivity. Our findings align with those of other authors, suggesting that stevia productivity is primarily influenced by the cultivar, with its phenotypic expression being highly dependent on agro-climatic conditions and nutrition management.[8,32,33] They demonstrate that genetically controlled productivity traits are enhanced by proper N rates and optimal agro-climatic conditions.[3] The tested cultivars (cvs Ambrosia and Olga) show promise as alternatives to the current cv. SRB-128 and might contribute to the productivity improvement of stevia in Greece.

Effects of four levels foliar biostimulants

Biometric – productivity – leaf chemical traits: The tested foliar biostimulants exhibited significantly positive effect on all measured plant growth and productivity variables, indicating their essentiality on stevia productivity. The four levels foliar plant biostimulants (Control, HG, HG+FL, HG+FL+AA) behaved differently on the plant growth and productivity variables. The positive effects on biometric, productivity and leaf chemical variables followed the order HG+FL+AA > HG+FL > HG > control.

In biometric traits, the level HG+FL+AA achieved the highest values (height 107.00 cm, cover area 830.00 cm^2, and leaf relative chlorophyll concentration 51.96 Spad units).

In productivity traits, the same level HG+FL+AA recorded also the highest value in dry leaves yield (86.60 g/plant).

In leaf chemical traits, the same level HG+FL+AA recorded the highest values in dry leaves concentration in Stev (11.09%), Reb A (9.22%), major steviol glycosides (20.28%) and in dry leaves yield in Stev (9.58 g/plant), Reb A (8.00 g/plant) and major steviol glycosides (17.58 g/plant). The higher values obtained by the HG+FL+AA level may be attributed to the amino acids component, which favors the increase of leaf steviol glycosides concentration.[14] The control level recorded a higher value in the dry leaves Reb A/Stev ratio (0.92) compared to the HG+FL+AA treatment (0.83). This suggests that the reb A/stev ratio is primarily influenced by the substrate fertilizer and cultivar, with the foliar plant biostimulants potentially enhancing the effects of these factors.

Our results are not directly comparable, due to lack of similar references. Pal et al (2013) reported lower dry leaves yield (17 g $plant^{-1}$) for stevia plants treated with foliar calcium nitrate ($Ca(NO_3)_2$) in India. Our findings are in agreement that under optimal agro-climatic conditions, proper N rate and cultivar, the foliar plant biostimulants may improve the biometric, productivity and leaf chemical traits of stevia.[8,17] They indicate that, under optimal conditions, the foliar plant biostimulants may increase the effectiveness of substrate fertilizer and cultivar on yield of leaves and on yield and concentration of steviol glycosides. The positive effects of foliar biostimulants on plant growth and productivity of other leafy crops have been confirmed by several studies.[23,34]

The activity of foliar plant biostimulants, including calcite, seaweed extracts, and amino acids, can be elucidated through their metabolic roles, which suggest several mechanisms:

i. Calcite acts as a signaling compound for enzyme activity, leading to increased chlorophyll concentration, plant growth, and productivity of leafy crops (Pilon-Smits et al, 2009).

ii. Seaweed extracts enhance metabolic processes such as photosynthesis, carbon and nitrogen assimilation, chlorophyll concentration, and regulation of phytohormones, thereby improving plant growth and productivity (Craigie, 2011).[35,36]

iii. Amino acids serve as carriers of organic nitrogen and signaling compounds for enzymatic and molecular processes like nitrogen assimilation, protein synthesis, glycolysis, and accumulation of macro- and micronutrients in leaves.[37-39]

The effectiveness of foliar nutrients is attributed to their fast penetration into inner leaf tissues either through diffusion through open leaf stomata or through ‘polar pores’ of the leaf cuticle.[40] These mechanisms highlight the potential of foliar plant biostimulants to enhance plant growth and productivity by facilitating key metabolic processes and nutrient uptake in leaves.

The HG+FL+AA treatment appears to be the most promising level for enhancing crop productivity. While previous studies have highlighted the benefits of plant biostimulants in conventional farming systems, their efficacy in organic systems has not been extensively documented. This study demonstrates that foliar biostimulants may indeed improve the productivity of stevia cultivated in organic systems, expanding our understanding of their potential in organic agriculture.

Conclusion

This study delved into the agronomic factors contributing to the low productivity of Greek stevia, exploring the effects of new levels of three productivity factors—cultivar, substrate fertilizers, and foliar plant biostimulants—under optimal Greek agro-climatic conditions. Results revealed that each factor significantly influenced stevia traits such as dry leaves and steviol glycosides yield, leaf SGs concentration, and reb A/stev ratio. The new levels of cultivar, substrate fertilizers, and foliar biostimulants demonstrated potential to achieve higher dry leaves yield compared to the current practices, suggesting that the current cultivar and substrate fertilizer could be key agronomic factors behind the low productivity.

Among the tested levels, the most promising were the organic substrate, cv. Olga, and the foliar biostimulants combination of calcite (HG), seaweed extract (FL), and amino acids (AA). The positive effects of foliar biostimulants followed the order: HG+FL+AA > HG+FL > HG > control. The most favorable treatment was the organic substrate (N rate 1.2 g/plant) combined with cv. Olga and foliar biostimulant HG+FL+AA.

These findings underscore that, under Greek agro-climatic conditions conducive to stevia growth, a suitable cultivar with strong genetic traits, when treated with appropriate organic substrate fertilizer and foliar biostimulants, may enhance stevia productivity. The study fulfills its aim by suggesting practical farming approaches to address the productivity issue of Greek stevia, shedding light on various aspects of stevia growth and productivity.

However, while the pot experiment contributes valuable insights, it is limited in its ability to simulate field conditions. Further research is warranted to elucidate the mechanisms of action of biostimulants and plant responses to productivity enhancements in leaves and major SGs. Additionally, ongoing studies on cultivar selection and optimal nutrition dose rates will aid in striking the right balance between leaf biomass, SGs concentration, and reb A/stev ratio across diverse local environments.

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Conflicts of interest

Authors declare that there is no conflict of interest.

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